

Starch-based Bioethanol Process Innovation

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Submitted in accordance with the requirements for the degree of

Doctor of Philosophy

The University of Leeds

Faculty of Biological Sciences

School of Biology

November, 2013

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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“..... but over all those endowed with knowledge is the All-Knowing (Allah)”

Qur'an 12:76

Acknowledgements

First and foremost I must express my profound gratitude to Almighty Allah for all His favours; Alhamdu liLlah, Masha Allah Laa quwwata illa biLlah.

I must commend my supervisor Professor Jurgen Denecke who has being very supportive and inspirational during the course of my research work. Thank you for guiding me through and making this research a success. I also appreciate the assistance and conducive environment provided by my laboratory colleagues, David Gershlick, Jing An and Carine De Marcos Louisa. Special thanks go to all those that have taught me from cradle up to this stage.

To my parents; My Lord (Allah)! Bestow on them Your mercy as they did bring me up when I was young, Amin. Thank you for your efforts and sacrifices in giving me good education. To my wife and daughter, I love and cherish you and thank you for all the patience and perseverance. I wish to acknowledge the support and prayers of my relatives, friends and well-wishers as space will not permit me to mention them individually.

The last and not the least is to acknowledge the Petroleum technology development fund (PTDF), Nigeria for providing the sponsorship required for my PhD research.

Abstract

Starch liquefaction and saccharification are key processing steps in the bioethanol industry. The rate-limiting α -amylase plays an important role due to its endo-glycosidic activity. Work carried out in this thesis on barley α -amylase focused on ER retention to boost recombinant protein expression *in planta* and purification of the protein to facilitate a cascade refinery approach allowing other high value proteins to be co-produced together with starch for bioethanol production. Results obtained generated evidence for context-dependence of the ER retention motif HDEL, the existence of an HDEL-independent ER retention mechanism and quantitative data showing toxicity or detrimental effects of HDEL overdose. Results also revealed an effect of peptide tags on N-linked glycosylation as well as evidence that expression levels and systems can strongly affect glycosylation of proteins in the secretory pathway. Furthermore, α -amylase endo-glycosidic action on long glucan chains was shown not to be rate limiting in starch saccharification but the presence of short oligomers and their susceptibility to hydrolysis by fungal amyloglucosidase must be considered next. Interestingly, starch saccharification using acid hydrolysis was more efficient compared to enzyme catalysed hydrolysis. In order to optimise the saccharification process further, the research centred on exploring enzymes with raw-starch digesting properties at low temperature and increased specificity for low molecular weight oligosaccharides. Therefore, an alpha amylase-like gene was identified in ripening plantain (*Musa acuminata* \times *balbisiana*) using degenerate primers. The gene encodes a putative protein product with close homology to chloroplast α -amylases from *Ricinus communis*, and *Arabidopsis thaliana* (AtAMY3).

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Abbreviations

ADP	Adenosine diphosphate
Amy	α -amylase
AGPase	ADP-glucose pyrophosphorylase
ATP	Adenosine triphosphate
BE	Branching enzyme
Bp	Base pair
BiP	Binding protein
C	Carbon
CAL	Calreticulin
CBM	Carbohydrate binding module
DE	Dextrose equivalent
DBE	Debranching enzyme
DPE	Disproportionating enzyme
EC	Enzyme commission
EDDDHDEL	Glu-asp-asp-asp-his-asp-leu-glu-leu
EDTA	Ethylenediamine tetra-acetic acid
ER	Endoplasmic reticulum
ERD	ER-defective
GBSS	Granule bound starch synthase
Glc	Glucose
GLT	Glucose transporter
GWD	Glucan water dikinase
GUS	Glucoronidase
HDEL	His-asp-glu-leu
ISA	Isoamylase
kDa	Kilo-dalton
KDEL	Lys-asp-glu-leu
LDA	Limit dextrinase
LPG	Liquid petrol gas

LPVC	Late pre-vacuolar compartment
MEX	Maltose exporter
P	Phosphate
PGI	Phosphoglucoisomerase
PGM	Phosphoglucomutase
PM	Plasma membrane
PVC	Pre-vacuolar compartment
PWD	Phospho glucan water dikinase
SBD	Starch binding domain
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SS	Starch synthase
SP	Starch phosphorylase
SRP	Signal recognition particle
TGN	Trans-Golgi network

Chapter 1

Introduction

Life on earth has always depended on energy which facilitates all human activities. These include the heating of homes and larger buildings, cooking, transport but also refining raw materials and ultimately generation of electricity for the growing number of electric devices (Volk, 2009). Currently, this is mainly supplied by fossil fuels. Coal has been used to heat homes and in part facilitated the industrial revolution by powering steam engines (Hubbert, 1949; Suranovic, 2013). Nowadays the majority of coal is used in large power stations to generate electricity (Monticello and Finnerty, 1985). Petroleum based products have emerged more recently, but are now dominating the energy sector due to the ease with which they can be mined, shipped, stored and refined (Hall, 2008). Diesel, kerosene, petrol and liquid petrol gas (LPG) are mostly used in the transport sector (Yuan et al., 2013), whilst a significant number of purified side fractions in the refinery process are essential feedstock for petro-chemistry, for instance ethylene, which forms the basis for polyethylene and polyvinyl chloride manufacturing (Sujith and Unnikrishnan, 2005; Yateem et al., 2011).

Liquid fuels have a high energy density and can be stored on small scale, making them ideal for cars and airplanes, for which there is an increasing demand (Agarwal, 2007). Another side-fraction of the petroleum distillation is natural gas, mostly composed of methane, which cannot be compressed to liquid and is less suitable for transportation. However natural gas finds its use in domestic heating and cooking, supported by national gas grids. Natural gas is also used in power stations to generate electricity, albeit at much lower scale compared to coal powered stations (Akhgari and Kamalan, 2013).

Although coal deposits on earth are vast and not likely to run out soon, petroleum reserves have been speculated to be depleted by 2050 (Saxena et al., 2009). Moreover, combustion of coal releases the highest amount of carbon dioxide compared to all other fuels and is considered to be one of the causes for climate change (Gomez et al., 2008; Escobar et al., 2009; Saxena et al., 2009). Taking all arguments together, the use of fossil fuel in general is therefore considered unsustainable (Demirbas, 2007; Koh and Ghazoul, 2008; Demirbas, 2011). Moreover, the supply and price of the petroleum based fuels is fluctuating due to political instability (McLaren, 2005; Gomez et al., 2008; Philp et al., 2013); therefore, diverse sources of energy are required in order to maintain energy security (Vanholme et al., 2013).

In order to solve the problems of non-sustainable fossil energy, alternatives must be considered that should satisfy today's demand without compromising the needs of the future (Taylor, 2008; Philp et al., 2013). The chosen alternatives should lead to low level of pollution and be fully renewable in the long term (Amigun et al., 2008; Sanchez and Cardona, 2008). Currently, renewable energy sources include wind, solar, water, tidal and wave power, as well as the so-called biofuels (Goncalves da Silva, 2010).

The use of wind and solar energies (in voltaic cells and electricity generation) is restricted to instant consumption due to lack of long term storage (Baker, 1991; Sloomweg and Kling, 2003; Mekhilef et al., 2013; Qin et al., 2013). As a result, these two types of renewable energy are restricted to local use and cannot satisfy the full spectrum of energy requiring activities (Baker, 1991; Mekhilef et al., 2013). Water is the biggest form of renewable energy, but it is limited in amount and most of the suitable sites have already been exploited in hydro-electric stations to produce electricity (Paish, 2002; dos Santos et al., 2006; Lenzen, 2010). Due to geological considerations it is difficult to construct more

dams, therefore this source has a poor potential for growth (King et al., 2000; Andre, 2012).

Biofuels which are referred to as fuels produced from various crop plants and other biomass are also considered. Biofuels are attractive alternatives, because they can be produced, stored and deployed on demand (Saxena et al., 2009; Yuan et al., 2013). The production and use of biofuels has also led to the "fuel versus food" debate which will be discussed in a later section. Despite the attractive properties of biofuels other sources of energy are required to satisfy demand and maintain sustainability (Harvey and Pilgrim, 2011).

In the introduction to this section, the indispensable role of energy was discussed. Currently, the bulk of energy is provided by fossil fuels which are unsustainable and polluting the environment. One of the alternative sources of renewable energy with great potential is biofuels (Goncalves da Silva, 2010). This introduction will focus on the different types of biofuels, the necessary feedstock and the specific production strategies involved. This will set the stage for the specific research aims of this thesis to improve starch-based bioethanol production strategies.

1.1 Biofuels

Biomass is a general term that refers to any living matter; it includes plants, algae, micro-organisms and animals. They contain compounds of carbon, oxygen, nitrogen and sulphur, comprising significant amounts of free energy in the form of chemical bonds (Lora and Andrade, 2009; Saxena et al., 2009). These can be released on breaking the intra and inter molecular forces to generate heat, which can be converted to mechanical work or electricity. Biomass can also be used to produce transport fuel if it is transformed into a liquid form. Both food and non-food biomass can be used to produce fuels commonly referred to as

biofuels (Lora and Andrade, 2009). They can either be solid, gas or in liquid form; which influences the manner in which they can be deployed.

Solid biofuel is exemplified by the burning of wood for domestic and industrial use (Jensen et al., 2004; Demirbas et al., 2009; Vamvuka, 2011). Methane, carbon dioxide, monoxide, and hydrogen produced from microbes are referred to as biogas (Li et al., 2013; Serrano-Lotina and Daza, 2013). It also includes gas that is produced from chemical cracking of wood referred to as wood gas. Although gas can be used for domestic purposes; it is not flexible as transport fuels because large and heavy tanks are required for storage. Liquid fuels are more attractive because they have high energy densities and can be stored in light-weight tanks (Muffler and Ulber, 2008).

Biofuel production and usage should tackle or address problems of environmental concerns. These include sustainability, climate change, and biodegradability among others. It should also address air pollution, sequestration of carbon, national security, economy and farm economy (Balat and Balat, 2009). Man is adventurous in nature, and the production and use of biofuels dates back to time immemorial. In 1900, Dr Rudolf Diesel's engine was fuelled with peanut oil but was later abandoned due to the discovery of petrol-diesel. Sir Rudolf also predicted the use of vegetable oils in the future and that they will be as important as petroleum. Historically, in the 1930's and 1940's vegetable oils were used in place of diesel (Demirbas, 2007).

In the past, the acceptance of biofuels increased due to their potential benefits to the environment. Several countries introduced policies on biofuels production; leading to a gradual increase in biofuel production worldwide. More recently, the public acceptance decreased again due to the public concerns that gave rise to 'food versus fuel' debate, which will be discussed in the following sections (Tan et al., 2008; Philp et al., 2013). Some of the factors affecting the production of biofuels include

the feedstock usage, availability as well as inefficient production strategies (Piccolo and Bezzo, 2009). The provision of subsidies by different governments may have stimulated investment into the biofuel sector but also caused the implementation of inefficient processing of feedstock. Another problem affecting the biofuel industry is the high cost of enzymes that are required for large scale feedstock processing which make the production expensive (Sticklen, 2006; Eijnsink et al., 2008; Gressel, 2008; Sticklen, 2010). In the following sections, the different forms of biofuels and the feedstock used in the production will be discussed.

1.1.1 Biofuel Production Strategies

Biofuels can be produced using any of the strategic types of feedstock which include simple sugars, polysaccharides and lipids (Demirbas, 2007; Harvey and Pilgrim, 2011). Four generations of biofuels are being considered in the literature however this classification is based on the different stages of development of biofuel production (Gressel, 2008). In the following sections, the four generations of biofuel will be discussed.

First generation

First generation biofuels are defined as those utilising food crops as feed stock. These include ethanol produced through fermentation from either simple sugars derived from crops such as sugar cane and sugar beet, or starch crops such as corn, wheat and barley (Table 1.1). They also include biodiesel produced through transesterification of plant oils such as rapeseed, sunflower, oil palm and animal fats (Festel, 2008; Gressel, 2008).

The use of food crops for biofuels was suggested to be unsustainable and led to the “food versus fuel debate” (Harvey and Pilgrim, 2011). This is mainly due to a sharp rise in prices of food most especially cereals and oils in 2008 which was erroneously blamed on biofuels. Sugar cane is

grown in Brazil and used as a feedstock for bioethanol production and less than 1% of the total arable land as shown in Figure 1.1 is used for the plantation. The land use for ethanol is low compared to other uses and the total available land; therefore this does not affect land available for food and feed (Chauhan et al., 2011; Gauder et al., 2011). Corn is used in the US for bioethanol, but it is an energy inefficient crop because of low harvest index therefore it does not give enough evidence to make general deductions (Lal, 2005; Koh and Ghazoul, 2008). These two examples illustrate that there is no meaningful competition between fuel and food crops, and that the high food prices in 2008 must have originated from other economic practices, such as speculation (Harvey and Pilgrim, 2011). As a result of the public fear on the impact of biofuels on food; non-food crops were considered to be the sole strategies for biofuel production (Kendall and Yuan, 2013). However the first generation fuels remain a viable alternative because of high yield, and ease of processing as will be discussed below.

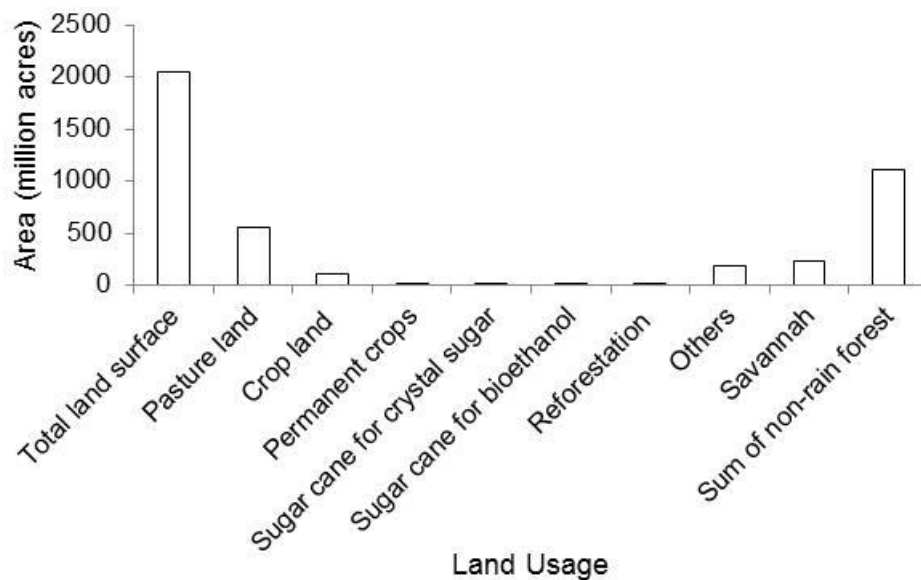


Figure 1.1. The Brazil arable land showing the land usage for different purposes. It includes; pasture land (cattle), crops (such as beans, corn, etc), and permanent crops (fruit trees), and sugar cane for crystal sugar, sugar cane for bioethanol, reforestation (pine, eucalyptus, etc), others (include urban centers, lakes, etc), savannah, occupy 9: sum of non-rain forest. Source: Latin Business Chronicle, May 18, 2007

Second generation

The strategy is based on the idea of using the whole plant to produce fuel; this will ensure hundred percent efficient use of the energy crop therefore will provide a higher harvest index. However, it is hard to convert the entire crop to fuel. The second generation of biofuel are produced from non-food crops; bioethanol and biobutanol from lignocelluloses parts of plants such as straw, wood; and biodiesel production from non-food crops such as *Pongamia pinnata*, and *Jatropha curcas* (Gressel, 2008; Demirbas, 2011). The non-food biomass mostly considered for bioethanol production is lignocellulose that includes trees such as poplar, eucalyptus, miscanthus, switch grass and other grasses among others. Another form of the biomass is agricultural wastes; this includes rice, wheat, and corn straws; and bagasse among others (Lal, 2005; Lora and Andrade, 2009; Sarkar et al., 2012). At the moment, the wastes are only used for domestic purposes such as animal feeds, and domestic fuels among others (Lal, 2005; Sarkar et al., 2012).

Table 1.1 Sources of biofuels

Bioethanol	Wheat, maize, potato, sugar cane, sugar beet, poplar tree, eucalyptus, miscanthus, switch grass
Biodiesel	Oil palm, rapeseed, soybean, peanut safflower, <i>Jatropha curcas</i>

The table shows the different sources of biofuels. Bioethanol can be produced from starch crops such as wheat corn and potatoes; and sugar crops such sugar beet and sugar cane. Biodiesel is produced from oil crops such as oil palm, rapeseed, soybean, peanut and safflower

The lignocellulosic biomass contains three major components: lignin, cellulose and hemicelluloses as fractions, and are not directly amenable to hydrolysis (Carroll and Somerville, 2009). Currently, there are no efficient conversion technologies to break the plant cell wall because of its lignin component that makes it resistant to hydrolysis (Himmel et al., 2007; Sanchez and Cardona, 2008; Carroll and Somerville, 2009; Abhary et al., 2011). In spite of the fact that significant research efforts

have been put into devising strategies for efficient conversion of lignocellulose to sugar, the technology is far from established. For instance, some researchers use antisense technology to modify lignin composition of alfalfa (Chen and Dixon, 2007; Sticklen, 2010) however, the modification did not lead to a significant improvement in the conversion process. This and other efforts have not solved the problem and much is required to be done; which makes it look unrealistic in the near future (Sticklen, 2006; Gressel, 2008; Sticklen, 2010).

Invariably, the production of bioethanol from lignocellulose requires the disassembly of the plant cell walls (Himmel et al., 2007; Arantes and Saddler, 2010) as pre-treatment prior to hydrolysis to fermentable sugar. The effect of this is that it increases the accessibility of enzymes to the complex polysaccharides (Gomez et al., 2008). Pre-treatments can be performed using acid and alkali at high temperature. The acidic pre-treatment leads to the hydrolysis of hemicelluloses with no effect on cellulose and lignin; the alkali also disrupts the lignocellulose structure (Gray et al., 2006; Himmel et al., 2007; Carroll and Somerville, 2009). In summary to produce bioethanol lignocellulosic biomass is mechanically and chemically pre-treated prior to enzymatic hydrolysis which adds to the production costs (Piccolo and Bezzo, 2009).

The pre-treatment may lead to undesirable effects such as the generation of substances such as furfurals and it must be considered that enzymatic hydrolysis is costly. These compounds in turn have negative effects on yeast therefore reduce the fermentable sugar yields and also decrease the efficiency of fermentation (Gray et al., 2006). Another problem often overlooked is the fact that only 30 to 40% of lignocellulose leads to fermentable sugar. The presence of non-fermentable sugars such as pentoses limits productivity of the approach (Gray et al., 2006; Carroll and Somerville, 2009). Current research focuses on the development of pentose-fermenting microorganisms but these studies are still in their infancy. The current technologies only

function on a low scale with unacceptable yield and involve high production costs. The net effect of the above makes the production of bioethanol from lignocelluloses much more expensive than from sugar or starch crops (Sims et al., 2010).

Although lignocellulose crop can yield an average annual production of 50-100 tonnes per hectare in the case of eucalyptus (Henry, 2010); it may be more efficient if the biomass is used directly for combustion to generate electricity. The biomass can also provide the raw material for paper production which has a higher market value than its energy content (Jensen et al., 2004; Talebnia and Taherzadeh, 2012; Stephen et al., 2013).

Biofuel production from non-food biomass has been claimed not to compete with food crops. This is because it is claimed that the lignocellulose will be grown on marginal lands; however, the yield of crops on those areas may be poor due to low quality of the land (Gressel, 2008; Stephen et al., 2013). Moreover, another concern is that of harvest from remote areas such as mountains is the lack of adequate infrastructure and cost for transportation. This means that in order to obtain a sufficient amount of the lignocellulose, lands that are currently used for food and feeds will have to be explored therefore leading to competition for agricultural land (Gressel, 2008; Escobar et al., 2009; Sims et al., 2010). Also, lignocellulose crops typically occupy land for many years, reducing flexibility of land use. Moreover, due to lack of crop rotation, the long term sustainability of this approach has not been evaluated (Sims et al., 2010).

Third and fourth generation

The term third generation and fourth generation or advanced biofuels is misleading because the two generations represent the same strategy (Fiorese et al., 2013). The third generation is defined as the processing of algal biomass for biofuel production. It includes the production of

hydrogen and electricity using the biomass from algae (Beer et al., 2009; Demirbas, 2010). The fourth generation refers to the metabolic engineering of algae for producing biofuels from oxygenic photosynthetic microorganisms (Beer et al., 2009; Zeng et al., 2011). Algal farming has been considered as source of oil which will be converted to diesel. However due to the low yield of oil from algae, the production requires drastic scaling up to generate biodiesel at meaningful scale (Gressel, 2008; Beer et al., 2009; Demirbas, 2010; Zeng et al., 2011). In order to enhance the yield, some of the options include use of land, or dams, or ponds, or rivers or oceans for algal farming. Even when algae are grown on commercial scale, only some areas will provide sufficient solar energy needed by the cells. The problem of stability and contamination of the algal cultures is perhaps the most significant limiting factor preventing its commercialisation in the near future (Gressel, 2008). Therefore, production of biodiesel from algae is not currently implemented.

The production of hydrogen is a chemical process that involves catalytic breakdown of living matter to obtain hydrogen as a side fraction. In practice hydrogen can be produced through a number of processes, including electrolysis of water, thermocatalytic reformation of hydrogen-rich organic compounds, and biological processes (Levin et al., 2004; Beer et al., 2009). The scale of hydrogen production is low because there is only very limited amount of hydrogen that can be released from biomass or from living organisms (Levin et al., 2004). Currently, low success has been recorded on this front and appears to be unrealistic compared to the liquid fuels biodiesel and bioethanol. The following sections of this thesis will focus on oil crop and biodiesel production.

1.1.2 Oil crops and biodiesel

Biodiesel is popular because it can be used directly in cars without modifications. In principle, it can be produced from any oil crops such as oil palm, rapeseed, soybean, and safflower (Bergmann et al., 2013;

Bezergianni and Dimitriadis, 2013). The yield of oil crops (see Figure 1.2) is low when compared to that of carbohydrate crop such as sugar cane of an average of 84 or 148 t/ha/y depending on harvest (Waclawovsky et al., 2010). Figure 1.2 shows the average yield of oil crops which has reached its optimum at the moment, and will be highly difficult to improve (Gressel, 2008). The best production is oil palm which is an annual crop with an average yield of 3.74 tonnes per hectare hence low productivity per surface of agricultural land (Sumathi et al., 2008; Atabani et al., 2013; Prasertsit et al., 2013). In addition oil palm takes a number of years (5-6) to establish and occupies the land for 15-20 years therefore does not allow flexibility of land use because of lack of crop rotation. Moreover, the market value of food oil from oil palm is higher than that of the combustion oil (Shuit et al., 2009) therefore; it should be used as food rather than fuel. For these reasons, biodiesel production is less viable and other alternative fuels have to be explored.

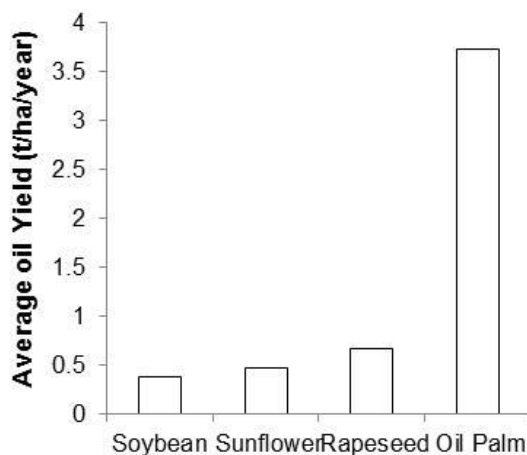


Figure 1.2. Yield from terrestrial oil crops illustrating the annual yield of different oil crops that can be used for biodiesel production. The oil yield is in tonnes per hectare of land per crop. Soybean, sun flower, rapeseed and oil palm have yields of 0.38, 0.48, 0.67 and 3.74 tonnes per hectare per year.

1.1.3 Carbohydrate crops and bioethanol

The term carbohydrate refers to a compound of carbon, hydrogen and oxygen. Based on chain length carbohydrates can be divided into three types. Firstly, simple sugars which include monosaccharides (such as glucose and fructose), disaccharides (such as sucrose and maltose) and polyols (sugar alcohols such as sorbitol and maltodextrin) (Cummings and Stephen, 2007). The second group is the oligosaccharides which are short chain carbohydrates consisting of three to nine sugars, it includes α - and non- α -glucans such as raffinose. The last group, the polysaccharides consists of polymers that include starch such as amylose and amylopectin; and non-starch polysaccharides such as inulin (a fructose polymer), cellulose, hemicelluloses (arabinoxylans), and pectin (Cummings and Stephen, 2007).

Carbohydrates represent the largest amount of fixed carbon on earth; it is present in almost all crops. Crops used starch as a store of energy with the exception of oil crops. The starch and sugar crops such as wheat, maize, sugar cane and beet; and lignocellulose biomass such as eucalyptus, miscanthus and switch grass are referred to as carbohydrate crops (Muffler and Ulber, 2008). The simple carbohydrate in sugar crops can be directly converted to ethanol by fermentation; however the polysaccharides in the starch and non-starch crops must be hydrolysed to sugars before being processed further to ethanol. Compared to the conversion of lignocellulose, it is relatively simple to hydrolyse starch; the principle of which will be discussed in the remainder of this introduction (Gray et al., 2006; Chuck-Hernandez et al., 2009; Arapoglou et al., 2010).

1.1.4 Conclusion

In summary, the concept of biofuels, its requirements and production have been introduced and the different forms of biofuels and feedstock

have also been considered. Currently, only first generation biofuel production strategies are successful and the current state of development only allows first generation bioethanol to be considered for large scale production (Sanchez and Cardona, 2008; Goncalves da Silva, 2010). This is because the exploitation of lignocellulose is unrealistic due to lack of technology to efficiently convert the biomass to sugars (Sticklen, 2006; Carroll and Somerville, 2009; Sims et al., 2010). Similarly, the production of biodiesel from oil crops is not viable due to poor yield and productivity (Basiron, 2007). The starch crops are more productive than the oil crops although corn is a bad example there are other crops with high harvest index and yield that have not yet been exploited systematically. This thesis is mainly concerned about bioethanol production therefore the remainder of this introduction will focus on bioethanol, and the use of starch crops as feedstock.

1.2 Bioethanol

Bioethanol is a liquid fuel that is currently produced from sugar and starch crops as feedstock. The sugar crops are processed by crushing, pressing and hot water extraction while starch crops such as corn processing is initiated through milling and saccharification. The saccharification is accomplished by cooking and enzymatic hydrolysis to yield fermentable sugar after milling (Leiper et al., 2006; Chuck-Hernandez et al., 2009; Arapoglou et al., 2010). This is followed by fermentation of the sugars, predominantly by the yeast *Saccharomyces cerevisiae*, and finally distillation to 96% alcohol. The ethanol has a range of applications (Calvert, 1997; Burrell, 2003), including its popularity in the beverage and food industry, but also in hospitals, to manufacture detergents, as solvents and other industrial applications, and to provide energy (Gray et al., 2006; Mussatto et al., 2010).

Bioethanol is a fuel that is not only easy to produce; it is also conveniently stored in small volumes and can be used on demand. In

rural areas it can be used for combustion (heat, cooking) and as transport fuel when needed (Gray et al., 2006; Bruni et al., 2010). In contrast to the deleterious effect of petroleum spillage and gas explosion to the environment, the spillage of ethanol does not lead to environmental disasters. These features make it a very important renewable and promising alternative to petrol and petrol based products. For instance, ethanol can be converted to ethylene by dehydration yielding a highly desired feedstock for the plastic industry that could replace fossil fuel based plastics (Haro et al., 2013; Zhu et al., 2013).

Table 1.2. Global bioethanol production (billion litres)

Country	2005	2006	2008	2009
USA	15.0	18.3	34.1	40.1
Brazil	15.0	17.5	24.5	24.9
China	1.00	1.00	1.90	2.05
France	0.15	0.25	1.00	1.25
India	0.30	0.30	0.25	0.35
Others	1.55	1.65	0.63	6.63
Total	33.0	39.0	65.4	74.0

The Table shows the major bioethanol producing countries, and the amount of this fuel produced by each country for a period of four years. It indicates an increase in the amount of bioethanol produced which were 33 and 74 billion litres in 2005 and 2009 respectively. This represents more than double the initial amount.

Bioethanol has indeed become popular across the globe, and rapid growth in the acceptance of bioethanol were recorded as illustrated in Table 1.2 (Sorda et al., 2010). It rose from 33 billion litres in 2005 to 74.0 billion litres in 2009 as shown in Table 1.2. The production was expected to reach 100 billion litres by 2015 (Balat and Balat, 2009; Mussatto et al., 2010). High rate of bioethanol consumption has been reported in the US, Brazil and Europe following policies that encourage the use of biofuels. The trend shown may continue due to the perceived benefits of the fuels in terms lower environmental pollution among others (Balat and Balat, 2009; Mussatto et al., 2010; Sorda et al., 2010). An issue of concern

remains that of sustainability and competition with food production (Saxena et al., 2009).

Some of the limiting factors in the production of bioethanol from starchy crops are the requirements for enzymes that are used in liquefaction and saccharification. These enzymes are expensive therefore make the process not cost effective for large scale operation (Smith, 2008). As a result there is need for development of more efficient and economically viable strategies of ethanol production from starch. Some of the strategies include the production of cheaper enzymes, generation of transgenics that have high expression of the key starch degradation enzymes (Eijsink et al., 2008; Lopez-Casado et al., 2008; Smith, 2008; Taylor et al., 2008). If an efficient process technology is developed, it will enable the starch conversion to be more cost effective. The following sections introduce two main crops used as feedstock for bioethanol production and explore high yielding starch crops that have yet to be considered for the future.

1.2.1 Sugar cane as feedstock

Brazil initiated the bioethanol from biomass specifically the sugar cane since 1975 after the oil crisis. The crop is basically made up of lignocellulose, water and approximately 20% sucrose, a disaccharide that is composed of glucose and fructose (Lora and Andrade, 2009). The production of ethanol from sugar crops such as sugar cane involves fermentation of the sugar and subsequent distillation to obtain pure alcohol (Liang et al., 2008; Patrick et al., 2013).

One of the factors that affect this industrial process is low percentage of alcohol in the aqueous broth. This is as a result of pressing and several steps of washing the fibre with hot water that dilutes the sugar to a low concentration. Therefore, alcohol yields are less than 10% in the fermented broth (Leiper et al., 2006), consequently, it is not cost effective

to separate alcohol from water under these conditions. The recovery of alcohol from the sugar is currently not optimised and the cost of distillation is still high due to the amount of material and infrastructure used in the process (Rossell et al., 2005; Basso et al., 2008; Son et al., 2009). However, in Brazil part of the cost is covered by using bagasse, the lignocellulosic by-product of sugar cane processing, as renewable fuel for distillation, therefore increasing the effective harvest index of the crop (Waclawovsky et al., 2010; Dias et al., 2013). Moreover, by combining bioethanol production with crystallisation of sugars for the food industry, part of the heat energy from the bagasse can be used to drive this energetically demanding processing step. Finally, any surplus energy remaining is currently used by the refineries to power steam generators to generate electricity for retail (Dias et al., 2013), allowing sugar cane processing plants to generate three products simultaneously and with high economic sustainability (Waclawovsky et al., 2010). The strategy is a typical example of the generation of various products from a single raw material and can be compared with generation of different fractions from petroleum (Hubbert, 1949).

Despite the promising properties of sugar cane, it occupies the land for longer periods of six years thereby making the land non-flexible for other uses, and only five harvests are made (Waclawovsky et al., 2010). These factors make the overall process less attractive, nevertheless there is a lot of land available for expansion as Brazil currently only uses an insignificant proportion for bio-ethanol production (see Figure 1.1). In view of the above, other carbohydrate crops that occupy land for shorter period and have higher harvest index and yield are considered for bioethanol production (Semencenko et al., 2013; Zhang et al., 2013).

1.2.2 Maize as feedstock

In contrast to sugar cane, maize is an annual crop that is amenable to crop rotation, a feature that increases market flexibility and long term sustainability. However, edible corn represents only a small proportion of the overall crop, and has a significant value as food and animal feed (Chuck-Hernandez et al., 2009; Lynch et al., 2012). An attractive feature of starch crops such as corn is that higher ethanol amount can be produced because the starch can be concentrated by sedimentation to ensure higher yield which is not the case with sugar crops (Jeon et al., 2010; Semencenko et al., 2013). The use of maize as a feedstock for industrial processes dates back to conversion of the starch within its seeds to the very popular corn syrup produced in the US since 1957. The processing of corn starch to bioethanol is a more recent technology that was simply derived from its original use. Two methods of conversion are used; dry and wet milling of the starch (Chuck-Hernandez et al., 2009; Piccolo and Bezzo, 2009). The dry milling is simple with ethanol as the sole product; in contrast the wet milling is a complicated process but yields other valuable side fractions such as protein, oil (corn oil) among others (Taylor et al., 2006; Piccolo and Bezzo, 2009).

Despite the success stories in the conversion of corn starch to sugar and subsequent fermentation to ethanol, maize is an energy inefficient crop. This is because only the seeds are used while the other part of the plant such as cob that contain biomass which cannot be easily converted to sugar is not exploited (Waclawovsky et al., 2010). It was based on this inefficiency of maize that the speculation was made on the negative impact of biofuel crops on food that led to the high prices of food in 2008 (Pimentel and Patzek, 2005; Lynch et al., 2012). However maize should not be used as a model to discredit the potentials of biofuel production from food crops thus other potential starch crops that can be used as feedstock for bioethanol production will be discussed in the next section.

1.2.3 A case for starch as feedstock

Starch is one of the most important and complex biomolecules of high significance in the world due to its diverse roles in life. It is synthesised and stored by higher plants in granules in tissues such as leaves, seeds, tubers, roots and stems (Buleon et al., 1998; Kossmann and Lloyd, 2000; Jeon et al., 2010; Halford et al., 2011). In comparison to the sugar crops, more starches are stored by mass in starchy crops due to the compact nature of starch than the sugars present in sugar crops (Goncalves da Silva, 2010; Halford et al., 2011). This high density of starch makes it an attractive feedstock for bioethanol production. Plants store carbohydrate as starch because it has low osmotic property and is chemically inert compared to sucrose or other sugars (Godin et al., 2013). It is also easy to mobilise in vivo and broken down to sugars to provide energy to the plant. This can be compared with the storage of blood glucose as glycogen in animals which is rapidly mobilised for energy on demand (Kossmann and Lloyd, 2000; Cummings and Stephen, 2007; Halford et al., 2011).

Starch is stored in photosynthetic tissues during transitory metabolism to provide a source of energy during the night. It is also stored in non-photosynthetic tissues for longer term storage (Graf and Smith, 2011). Starch is found in almost all classes of crops; tubers (such as potato, cassava and yam); cereals (rice, maize, sorghum, wheat, barley, oat), and roots (such as sago). It is also found in fruits such as banana, plantains, tomato, apple, pear among others. Starch from cereal endosperms represents most of the world's supply of starch (Abd-Aziz, 2002; Hannah and James, 2008; Keeling and Myers, 2010).

Starch is used as food and feed as livestock feeds. It finds a variety of industrial applications including, beverages, brewery, pharmaceuticals, food, and paper. Others include use as adhesives (thickeners and gelling agents), and sweeteners and syrups. Starch can be converted to

biodegradable plastics (Nigam and Singh, 1995). It serves as a substrate for acetone and butanol production; it is hydrolysed to maltose and dextrose that are fermented to lactic acid. Finally, starch is also used in the production of ethanol through fermentation of sugars. However, not every starch crop is suitable for sustainable alcohol production (Buchholz and Seibel, 2008).

Although conversion of starch to fermentable sugar is an additional step, suggesting a more costly production process compared to sugar crops there is specific advantage to the use of starch. One of these is the ability to sediment the starch prior to conversion to sugars. This means, that high concentration of sugar syrup can be produced, leading to a higher percentage of alcohol and cheaper refinery by distillation (Gryta, 2001; Leiper et al., 2006; Basso et al., 2008; Son et al., 2009). In addition, the average yield of some starch crops such as potato, sweet potato and cassava is higher than that of sugar produced by sugar cane and sugar beet (Clough, 1994; Lynch et al., 2012). Most high yielding starch crops are annual crops, amenable to crop rotation which promotes sustainability and flexibility to satisfy changing market demands (Clough, 1994; Goncalves da Silva, 2010; Lynch et al., 2012). Furthermore, the sugar crops such as sugar cane takes approximately thirteen months to grow while starch crops like potato only require a maximum of five months thus offering flexibility (Waclawovsky et al., 2010). This makes the starch crops more attractive than sugar crops as the land can be used for other purposes after harvest (Waclawovsky et al., 2010; Lynch et al., 2012). In the temperate region of the world more land is available for the growth of starch crops such as potato than that available for the growth of sugar crops in the tropics (Harvey and Pilgrim, 2011).

The popularity of starch and its use in various industries which is affected by the properties of the polymer therefore has led to several research efforts at improving starch. The properties that are targets for improvements include composition and digestibility of the polymer. In

addition there are also efforts to enhance the process of starch conversion to sugar, the aim of which is to ensure maximum yield of alcohol (Slattery et al., 2000; Burrell, 2003; Sonnewald and Kossmann, 2013).

1.2.4 Exploring potatoes

Key advantages of potato are the short growth period, the high harvest index, and the ease of harvesting, transportation and storage as well as existing wet-milling procedures to refine starch industrially. The starch can be used as food and to generate fuel, offering flexibility because such food crop will serve different purposes (Clough, 1994; Alvani et al., 2011; Blahovec and Lahodova, 2013). These properties make potato an attractive but yet unexplored feedstock for bioethanol production, glucose syrup, and pharmaceuticals (Ryffel, 2010; Alvani et al., 2011). This implies potato can be compared to petroleum that is used to generate a range of products which include petrol, diesel, kerosene, asphalt and petrochemicals. Also, potato is a good model system for genetic engineering because it has a storage organ that can be manipulated quite easily (Kossmann and Lloyd, 2000).

The host laboratory has started to develop a cascade-refinery strategy to use potatoes for the production of hydrolases and other industrial enzymes, with starch based alcohol as side fraction (Jing An and Denecke, unpublished). The strategy of using potato for protein production or enzyme and its starch for bioethanol will ensure efficient use of resources. This is because if implemented, land can be used for the rest of the season to grow other crops. Furthermore, crop rotation practices will enhance the long term sustainability of the strategy (Festel, 2008; Gressel, 2008). In order to appreciate the limiting factors in starch conversion, I will introduce the reader to the structure of starch and its biosynthesis.

1.3 Starch biosynthesis

Starch has been described as the most abundant storage carbohydrate in plants. In order to use starch as a feedstock for bioethanol, there is a need to understand starch, its composition and structure. The reason for this is because the form of starch affects its digestion to simple sugars and consequent conversion to ethanol. The following chapter will focus on the molecular architecture of starch.

1.3.1 Starch structure

Starch is a macromolecule that consists of two glucose polymers, amylose and amylopectin as its major components. Amylose is a linear polymer of glucose residues that are linked mostly by α -1,4 glycosidic bonds with very low branching (of α -1,6 glycosidic bonds) of about 0.1% (Denyer et al., 2001). Amylopectin is a high molecular weight compound of about 10^7 - 10^9 that is highly branched and consists of glucose residues linked through both α -1,4 glycosidic bonds and α -1,6 glycosidic bonds as shown in Figure 1.3 (Kossmann and Lloyd, 2000; Zeeman et al., 2010). Typically between 5-6% of the bonds represent branch points.

The two main components of starch have different chemical and physical properties. For instance, amylose is insoluble in water while amylopectin is water soluble. Hence, the form and characteristics of starch is a function of its components. Both amylose and amylopectin are responsible for the semi-crystalline nature of starch (Copeland et al., 2009), typically found as spheres or ovoid of 0.5 – 100 μ m in diameter. The granule size and relative proportions of amylose and amylopectin is highly variable and depends on the crop species (Denyer et al., 2001; Zeeman et al., 2010; Santelia et al., 2011). In a typical starch granule, approximately 20-30% of the starch molecule is made up of amylose while 70-80% is amylopectin (Kossmann and Lloyd, 2000; Keeling and Myers, 2010). Though starch generally contains both types, starch

granules have been observed that are composed of amylopectin only (Buleon et al., 1998).

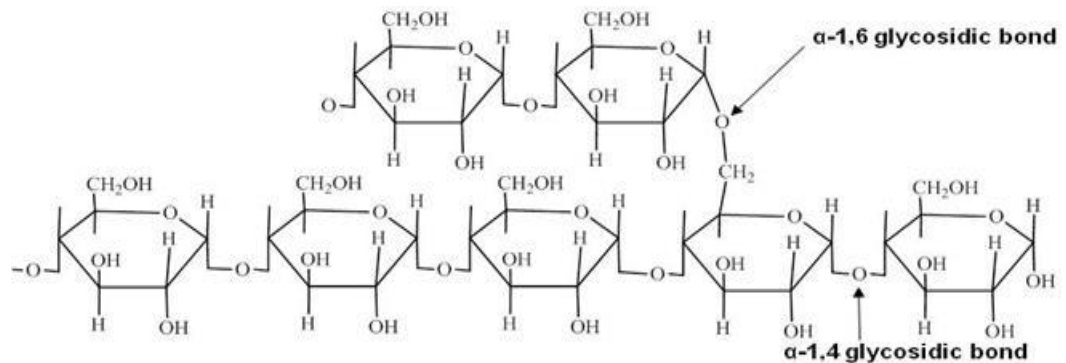


Figure 1.3. The Structure of starch showing the repeating units of glucose linked by α -1,4-glycosidic bonds and α -1,6-glycosidic bond at branch points.

The amylose to amylopectin ratio of starch strongly affects its physical characteristics. Starches subjected to heating in water form thick pastes, the long linear amylose chains are mainly responsible for the texture and viscosity of cooked starches (Zhang et al., 2005). Starch with an amylose composition of 20-30% forms a turbid paste and rapidly aggregates due to crystallization of amylose. In contrast, starch that is free from amylose gelatinises with ease forming clear liquid pastes of lower viscosity. Furthermore, starch digestibility by enzymes is also affected by its physical and chemical properties; they include granule size and shape, source, the ratio of amylose to amylopectin. The molecular interaction between the components, chain length, crystallinity and the availability of complexes of amylose-lipid can all be important factors (Zhang et al., 2005; Fuentes-Zaragoza et al., 2010; Alsaffar, 2011). It has been postulated that starch with high amount of amylopectin is hydrolysed more easily by the amylases compared to amylose-rich starch (Denyer et al., 2001; Soares et al., 2011).

1.3.2 Biosynthesis of starch

The energy from sunlight is converted into chemical energy by the Calvin cycle during the process of photosynthesis. CO₂ is first reduced to glucose which then forms the feedstock to synthesise polysaccharides, lipids, nucleic acids and proteins. Plants do not store glucose because it is a chemically and osmotically active compound in contrast to starch, and it is not efficient for the long term storage of large amounts of energy. Starch is a preferred form of storage because it is osmotically inert and exhibits a higher energy density when condensed into starch granules. In plants, it is used as an energy buffer in a variety of conditions (Smith et al., 2005; Munoz et al., 2006; Fettke et al., 2009).

Starch is synthesised and stored temporarily in the chloroplast of photosynthetic tissues such as leaves of plants and is referred to as transitory starch. Because starch cannot be transported from the leaves to the storage tissues, it is hydrolysed to maltose and glucose that are further converted to sucrose for long distance transport from leaves to the root, tuber, and stems (Smith et al., 2003; Smith et al., 2005; Orzechowski, 2008). Sucrose is a non-reducing, low viscous, more neutral and chemically less interactive disaccharide which make it a medium of choice for sugar transportation in plants (Winter and Huber, 2000; Halford et al., 2011; Stitt and Zeeman, 2012). On translocation, sucrose is converted through a series of biochemical reactions back to starch that is stored in seeds, fruits, stems and tubers, often referred to as reserve starch. In the non-photosynthetic tissues the starch is stored in amyloplast, a plastid belonging to the same family as chloroplasts but devoid of pigments.

The biosynthesis of starch is not a straightforward process and involves several structural modifications that include elongation, branching, debranching. These are then followed by the final assembly of the components until the complete molecule is produced, therefore the

structure of a starch granule depends on both synthesis and degradation (Mukerjea et al., 2002; Mukerjea and Robyt, 2005b, a; Mukerjea et al., 2009). For simplicity and to aid in understanding the synthesis, the process will be described in separate steps.

Initiation of Starch Synthesis

The process is initiated by the synthesis of ADP-glucose which is the first committed step in the pathway. (Buleon et al., 1998; Denyer et al., 2001). As shown in Figure 1.4, phosphoglucose isomerase (PGI) converts fructose 6-phosphate to glucose 6-phosphate, which is further converted to glucose 1-phosphate by the action of phosphoglucomutase (PGM). Other hexose phosphates can also be converted to glucose-1-phosphate by PGM (Smith, 2012). The enzyme ADP-glucose pyrophosphorylase (AGPase) located in the cytosol catalyses the conversion of glucose-1-phosphate into ADP-glucose in the presence of ATP (Figure 1.4) which serves as a donor of the adenosine di-phosphate. In contrast AGPase is located in the amyloplast in the storage organs of some starch. The ADP-glucose produced is transferred into the amyloplast of the endosperm of cereal crops. This implies that the synthesis of ADP-glucose can occur in the cytosol or directly in the amyloplast (Tetlow, 2006; Streb et al., 2009; Zeeman et al., 2010).

ADP-glucose pyrophosphorylase (AGPase) activity is rate-limiting in starch biosynthesis and is subject to regulations by different metabolites. In the plastid, AGPase is activated by low concentrations of 3-phosphoglycerate and inhibited as the concentration of 3-phosphoglycerate, inorganic orthophosphate and pyrophosphate increases. In contrast, cereal endosperm starch synthesis is controlled by the availability of ADP-glucose that is synthesised by the cytosolic AGPase which has less sensitivity to the allosteric regulation of the metabolites described above (Tetlow et al., 2004; Ihemere et al., 2006; Orzechowski, 2008). In some plants, AGPase has been reported to be controlled by light and sugar levels. Consequently, the synthesis of ADP-

glucose or otherwise is a major determinant of the pathway of starch synthesis (Stark et al., 1992; Orzechowski, 2008).

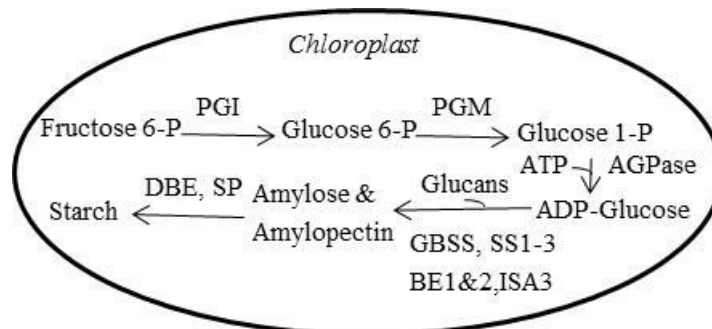


Figure 1.4. The pathway of starch biosynthesis, the process begins with the conversion of fructose-6-phosphate to glucose 6-phosphate in a reaction catalysed by phosphoglucosomerase (PGI). Glucose 6-P is converted to Glucose 1-P by phosphoglucomutase (PGM). It accepts ADP from ATP in a reaction catalysed by ADP-glucose pyrophosphorylase (AGPase) to produce ADP-Glucose. In the presence of glucan donor, a series of synthases (GBSS and SS) assemble the products to amylose and amylopectin respectively. At this stage, branching enzymes and isoamylases are involved in the creation of branched glucans. Debranching enzymes (DEB) and starch phosphorylase are involved in the final modification of the glucan to produce starch.

Elongation of Starch Structure

The ADP-glucose produced is subsequently transferred onto pre-existing glucan chains by the action of starch synthase, ADP-glucose: [1-4]- α -D-glucan 4- α -D-glucosyltransferase (Beck and Ziegler, 1989; Kossmann and Lloyd, 2000; Keeling and Myers, 2010). This prompts the question regarding the first synthesis of the acceptor molecule. Is a starch precursor available from the haploid ovary cells, or can it be de novo synthesised? Starch synthases transfer glucosyl-residues from ADP-glucose to the reducing end of an acceptor molecule, an α -1,4-linked glucan chain leading to amylose and amylopectin synthesis (Zeeman et al., 2010).

In higher plants, five gene classes encode the starch synthases; Granule-bound starch synthase (GBSS) binds tightly to the starch granule during amylose synthesis. (Orzechowski, 2008; Zeeman et al., 2010). In contrast to GBSS, starch synthases I, II, III, and IV (SSI, SSII, SSIII and SSIV) are soluble isoforms of the protein that are responsible for the generation of amylopectin chains. It has been shown that in amylopectin synthesis, there is preferential elongation of short, medium, and long chains by SSI, SSII and SSIII classes respectively (Tomlinson and Denyer, 2003). Among the starch synthases, only GBSS has been reported to have principal role in amylose synthesis, this is evident from the fact that mutants lacking amylose lack GBSS activity. One of the obvious distinctions of GBSS from other isoforms of starch synthase is localisation; some are present in the plastid while others are found in the cytosol (Denyer et al., 2001; Emes et al., 2003).

In 2010, BASF patented an 'amylopectin' potato approved for growth in the EU. Inactivation of the GBSS gene results in modification of starch phosphorylation, and prevents amylose synthesis. This affects starch degradation and lead to reduction in taste characteristics of the transgenic potato (tuber) starch (Lorberth et al., 1998), therefore altering starch composition usually lowers the yield. The use of biotechnology to improve the starch is not yet possible due to lack of understanding of the main factors that affect starch properties.

Branching in Starch

A very important structural feature of starch that affects its characteristics is the presence of branches (Kossmann and Lloyd, 2000). The branching in starch is created by the coordinated action of branching enzymes (BE, α -1,4-glucan: α -1,4-glucan-6-glucosyltransferase EC 2.4.1.18). The BE cuts α -1,4-glycosidic bond on an existing α -1,4-glucan chain (Keeling and Myers, 2010; Zeeman et al., 2010). The cleaved segment of six or more glucose units is transferred to C6 position of a glucosyl residue of the same or another glucan chain. The BEs are classified into two

classes, class I and II (also referred to as B and A respectively). Class B enzymes transfer longer chains than the A class. Thus, class A enzymes preferentially act on amylopectin while the B enzymes act on amylose (Buleon et al., 1998; Slattery et al., 2000; Tomlinson and Denyer, 2003).

Debranching enzymes (DBEs) modulate starch synthesis by cleaving the α -1-6-branch points. In plants, two types of DBEs have been reported; the isoamylase (ISA, EC 3.2.1.68) and pullulanase or limit dextrinase (LDA, EC 3.2.1.41) (Hussain et al., 2003; Tetlow et al., 2004). The isoamylase (ISA) has three classes; ISA1, ISA2, and ISA3 with ISA1 and ISA2 having critical roles in amylopectin synthesis. The limit-dextrinases and ISA3 may also have roles in starch degradation. ISA1 has preferential activity on glucans with long external chains while LDA and ISA3 act more on glucans with short external chains. ISA2 is reported to be inactive but may play modulatory roles on ISA1 (Hussain et al., 2003; Tetlow et al., 2004; Zeeman et al., 2010).

The reversible transfer of glucose units from glucose-1-phosphate (Glc1P) to the non-reducing end of α -1,4-linked glucan chains is catalysed by the starch phosphorylase (SP; EC 2.4.1.1). The SP present in the plastid has higher affinity for amylopectin than amylose. Experimental evidence has indicated the probable role of plastidic SP (Pho1 or L-form) in starch synthesis. However, more explanation is needed to fully elucidate the roles of the SP in starch metabolism in higher plants (Tetlow et al., 2004; Orzechowski, 2008).

In summary, despite a relatively simple structure consisting of just two glycosidic bonds, a large number of enzymes of the biosynthetic pathway have been discovered and full understanding of the process of starch synthesis is far from established. There are suggestions that the enzymes of starch biosynthesis interact as multi-enzyme complexes (Tetlow, 2006; Hennen-Bierwagen et al., 2008). This may be due to the complexity of starch synthesis and the roles played by the various

enzymes. However the reason for the association may be to make the process of synthesis efficient. This means it is difficult to study and understand the pathway of starch biosynthesis by reductionist approaches, whether they be biochemical or genetic in nature.

1.4 Starch degradation

Starch degradation is the process of the breakdown of starch to liberate simple sugars for metabolism. It takes place *in vivo* (in the cell) to mobilise energy during the night or during fruit ripening or seed germination. It can also occur extracellularly, usually when starch is decomposed by another organism, i.e. bacteria or fungi that depend on plant starch for their own survival (Smith et al., 2003; Orzechowski, 2008). In this section, the *in vivo* degradation referring to the breakdown of transitory and reserve starch will be discussed first

1.4.1 Degradation of transitory starch

Initiation of Starch Degradation

Transitory starch degradation occurs in the chloroplast, as introduced earlier. The circadian rhythm is crucial to mobilise starch in order to provide energy for the tissues during the night. Starch degradation can be divided into initiation of degradation and subsequent digestion into maltose, and glucose (Smith et al., 2003; Orzechowski, 2008). The process of hydrolysis of transitory is illustrated in Figure 1.5.

In this section, the discussion will focus on transitory starch degradation in the leaves. In the chloroplast; glucan water dikinase (GWD) and phosphoglucan water dikinase (PWD) phosphorylate starch prior to degradation. The phosphorylation is evident by the presence of phosphates at C-6 and C-3 position of amylopectin (Buleon et al., 1998). The enzymes GWD and PWD catalyse starch phosphorylation in the presence of ATP (Ritte et al., 2004; Zeeman et al., 2007b). The

mechanism of phosphorylation is as follows; ATP is the donor of phosphate group that is first transferred to a conserved histidine residue on the dikinases (GWD and PWD). The phosphate group is subsequently transferred to a glucose unit of the amylopectin (Fettke et al., 2009). The phosphorylation of starch molecule leads to disruption of its structural organization (Blennow et al., 2002; Santelia et al., 2011), therefore making it more amenable to the action of isoamylase and beta amylase (Buleon et al., 1998; Zhang et al., 2005).

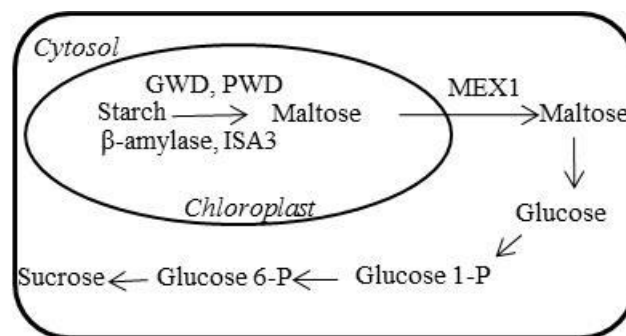


Figure 1.5. Transitory starch degradation in the chloroplast. Starch is attacked by the glucan water dikinase (GWD), phosphoglucan water dikinase (PWD), isoamylase and beta-amylase to maltose which is transported by the maltose transporter, MEX1 from the chloroplasts to cytosol, maltase hydrolyse it to glucose which is converted to glucose 1-P and further to glucose 6-P and finally to sucrose.

In a mutagenic experiment, it was reported that inactivation of GWD leads to more than 7-fold accumulation of starch compared to the wild type plant (Yu et al., 2001). In another report, mutants with defects in GWD were observed to accumulate excessive amounts of starch as opposed to 7-fold more (Ritte et al., 2004). A PWD identified in the leaves of *Arabidopsis* phosphorylates the C3 position of amylopectin in contrast to GWD that phosphorylates C6 and C3. Because transgenic plants with low expression of PWD showed starch accumulation this may imply a reduction in hydrolysis (Kotting et al., 2005). In *Arabidopsis*, an isoform that was identified and designated as AtGWD3 (or PWD) preferentially phosphorylates glucose at C3 position which indicates that

it may complement the activity of AtGWD1 (Baunsgaard et al., 2005; Kotting et al., 2005).

Debranching of starch

Starch degradation in leaves is not only restricted to hydrolytic and phosphorolytic processes that were described in the previous section, it also involves the dis-assembly of the polymer which takes place in the form of debranching. The debranching enzyme Isoamylase (ISA3) acts on the 1,6-glycosidic linkages in starch whereas β -amylase acts on 1,4-glycosidic bonds in the linear chains releasing maltose from the non-reducing ends of the glucan chains (Orzechowski, 2008; Andriotis et al., 2010) to release maltose. The disaccharide is exported to the cytosol by a transporter known as MEX1; and is subsequently converted to sucrose in steps (Zeeman et al., 2004). Indeed maltose was found to be the major sugar exported from the plastid to the cytosol at night when degradation of transitory starch occurs. A transglucosidase (disproportionating enzyme; DPE, EC 2.4.1.25) converts maltose to glucose. The glucose moieties are phosphorylated to glucose 1-phosphate by a cytosolic glucan phosphorylase, PHS2 (Weise et al., 2004). A glucose transporter referred to as GLT exports glucose (Weber et al., 2000; Andriotis et al., 2010). Moreover, GWD and DPE2 (transglucosidase) are highly significant in the control of starch breakdown. Further evidence has shown DPE deficient species to have a high accumulation of maltose (Chia et al., 2004).

Note on glycogen metabolism in animals

In contrast to plants, animals store glucose in the form of glycogen, it also consists of α -1,4 glycosidic bonds and α -1,6 at the branch points. Compared to starch, glycogen has a higher degree of branching that occurs every 8 to 12 residues and it is more compact than starch. When animals feed on starch, glucose is converted to glycogen and stored in liver and muscle. However, glycogen is more abundant in the liver, and the granules are associated with enzymes of glycogen synthesis and

degradation, it is hydrolysed and transferred to tissues when energy is needed (Nelson and Cox, 2005). Muscle glycogen is used during exercises therefore it may be stored longer than the liver equivalent. Glycogen only has one reducing end with branch points of non-reducing, thus on hydrolysis by the phosphorylase glucose is released from the non-reducing ends until four glucose residues are left. The complete hydrolysis of glycogen involves two additional enzymes, the debranching enzyme and phosphoglucomutase thus glycogenolysis is less complex compared to amylase catalysed starch hydrolysis. Therefore, glycogen in the hepatocyte is the equivalent of transitory starch in plant leaves (Nelson and Cox, 2005).

1.4.2 Degradation of reserve starch

In addition to transitory starch which undergoes continuous synthesis, branching, debranching and hydrolysis to maltose, plant cells also accumulate starch for long term storage. Degradation of this type of starch does not occur in a circadian manner. Starch will be synthesized over a long period of time, usually not in the tissue in which CO₂ fixation took place, but after long distance traffic of sucrose from the photosynthetic tissue towards a storage organ. This can either be seeds or fruits but also stems, roots or tubers. In this chapter, the degradation of reserve starch will be discussed using two examples, i.e. seeds of cereals and fruits containing significant levels of starch, to illustrate the complexity of the process.

Starch degradation in seeds

In germinating cereal endosperm, a secreted form of α -amylase converts starch to linear and branched glucans (Beck and Ziegler, 1989; Kotting et al., 2010). The oligosaccharides are hydrolysed further by the actions of enzymes; limit dextrinase attacks the α -1,6 linkages and while β -amylase hydrolyse the linear oligosaccharides from the ends. Maltose and glucose are released; and are exported to the scutellum. The maltose is

further broken down to glucose by α -glucosidase (Lao et al., 1999; Weise et al., 2004; Lu and Sharkey, 2006) while the glucose is directly converted to glucose 1-phosphate and subsequently to glucose 6-phosphate. In a series of further reactions the synthesis of sucrose needed for embryonic growth, development and long distance energy transport to the growing plant is achieved (Smith et al., 2004; Smith et al., 2005).

Disruption of starch by phosphorylation

In addition to the initial hydrolysis of starch by α -amylase, a minor pathway can occur via phosphorylation. Glucan water dikinase (GWD) and phosphoglucan water dikinase (PWD) phosphorylate starch prior to degradation (Ritte et al., 2004; Zeeman et al., 2007b). The mechanism of phosphorylation has been described in the previous section and leads to disruption of structural organization of starch, making it more amenable to the activities of the hydrolases (Blennow et al., 2002; Santelia et al., 2011).

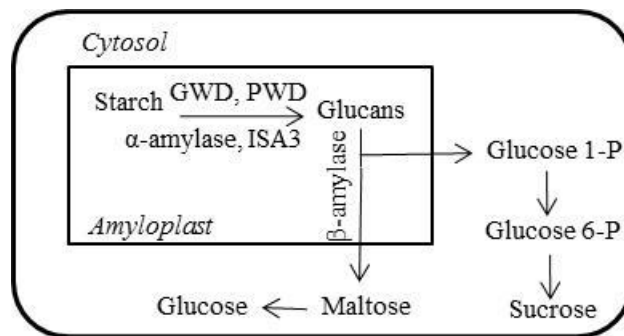


Figure 1.6. Degradation of reserve starch illustrating the pathway of starch degradation in the amyloplast. Starch is acted upon by alpha amylase which initiates the degradation, and glucans are generated. In a minor pathway, the starch is phosphorylated by GWD and PWD and is acted by ISA3. These glucans can be converted to sucrose and glucose. The combined cleavage by isoamylase and beta-amylase releases maltose which is transported by the maltose transporter, MEX1 to the cytosol, where it is further converted to glucose. Glucose 1-phosphate can also be released and is further converted to glucose 6-P and finally to sucrose.

Debranching

The debranching enzyme Isoamylase (ISA3) cleaves the α -1,6-glycosidic bonds in starch to release linear sugar chains. Then β -amylase acts on 1,4-glycosidic bonds from the non-reducing ends of the linear glucan chains to release maltose (Orzechowski, 2008; Andriotis et al., 2010). The disaccharide maltose is exported to the cytosol by a transporter known as MEX1; where is further converted to glucose (Zeeman et al., 2004). In addition the action of β -amylase may also release glucose. The glucose moieties are phosphorylated to glucose 1-phosphate by a cytosolic glucan phosphorylase, PHS2 (Weise et al., 2004). On the other hand, the glucose 1-phosphate is converted to glucose 6-phosphate which is finally converted to sucrose. A glucose transporter referred to as GLT exports glucose (Weber et al., 2000; Andriotis et al., 2010).

It was observed that starch accumulation and turnover also occurs in the embryo in cells undergoing cell division and differentiation. This indicates that starch is a temporary energy reserve in dividing cells or in the early differentiated cells, thus confirming the accumulation of starch in the embryo, endosperm and the testa (Andriotis et al., 2010).

Ripening in fruits

Fruits also contain a large amount of reserve starch and ripening is an essential developmental process in fruits where the starch is digested. Based on their ripening patterns, fruits are classified into two classes. Climacteric and non-climacteric fruits; the former are capable of ripening when detached from parent plants. The members of the latter group are not capable of ripening independent of their parents (Giovannoni, 2001; Prasanna et al., 2007). The process of ripening is characterised by some biochemical and physiological changes that affect the texture, taste and nutritional attributes of fruits. The cell walls contain complex polysaccharides such as pectin, cellulose, hemicelluloses and starch (Prabha and Bhagyalakshmi, 1998; Prinsi et al., 2011). These complex

compounds undergo series of enzymatic modifications to give the fruits the desired softness and sweetness. It is a highly complicated process that converts macromolecules into simpler ones. During ripening of fruits; there is an observed decrease in the amount of starch that is as a result of increased activity of hydrolases (Hill and Aprees, 1994; Prasanna et al., 2007).

Crops such as banana and plantains in the unripe stage may consist of 70% or more starch. As the crop ripens, the starch is gradually hydrolysed and converted to sugars. A fully ripened banana may contain 2% or less starch while plantain may contain 10% starch (Junior et al., 2006; Xu et al., 2007; Fioravante Bernardes Silva et al., 2008). Moreover, there is an accumulation of sugars predominantly sucrose which may reach 16% or more of the fresh weight of the crop. This biochemical process of complete conversion of starch to sugars is facilitated by different hydrolases (Zhang et al., 2005; Shiga et al., 2011). α -amylase hydrolyses starch to release shorter chain sugars and maltose while β -amylase releases maltose. Maltose may be converted to sucrose through the glucose 1-phosphate pathway. This is also supported further by the fact that only trace amounts of maltose are found in ripening fruit (Hill and Aprees, 1994; Prabha and Bhagyalakshmi, 1998; Fioravante Bernardes Silva et al., 2008). α -amylase and β -amylase cannot act on the α -1,6 branching in amylopectin, hence α -glucosidase or glucoamylase may be required to cleave this bond. Thus, it has been postulated that more than one pathway may be involved in this conversion process (Sarikaya et al., 2000; Prasanna et al., 2007; Derde et al., 2012).

In conclusion, a better understanding of starch degradation in vivo will assist us in process design involving starch degradation. This is because the digestion of starch to fermentable sugar is a major step in the industrial conversion of starch to bioethanol. The full elucidation of the pathway will enhance the production and yield of sugar for bioethanol

production. In the following sections, the extracellular degradation of starch will be discussed.

1.4.3 Microbial Starch Degradation

Starch degradation in nature does not only occur in the organisms that synthesize and utilise starch as part of their normal energy physiology. Starch can also be used by pathogenic or saprophytic microorganisms that take advantage of this rich source of sugars. Because essentially this process takes place outside the cells that will acquire the released sugars, the mechanisms of starch catabolism show some fundamental differences compared to the previous examples given. First of all, hydrolysis of starch by micro-organisms is achieved without phosphorylation, and generally depends on the use of secreted extracellular enzymes. Saprophytic starch degradation can occur under a multitude of conditions, including low or high pH, and a vast range of temperatures.

In bacteria and fungi, thermostable α -amylases have been found that hydrolyse starch at high temperatures. In particular α -amylases from the *Bacillus* species are thermo stable, a feature that has led to the use of these microbial enzymes in industrial processing of starch (Prakash and Jaiswal, 2010). The thermal stability has been selected due to the extreme environmental conditions faced by microbes such as high temperature in compost. Glucoamylases in microbes cleave the 1,4 glycosidic bonds at the non-reducing end of starch to release glucose (Marin-Navarro and Polaina, 2011). From the biotechnological point of view, the heat stability is an attractive because the starch is liquefied at high temperatures and enzymes that can withstand these conditions are needed.

Microbes have over time evolved enzymes that have the ability to hydrolyse raw crystalline starch (Saha and Zeikus, 1989; Gupta et al.,

2003; Sivaramakrishnan et al., 2006). Microbial breakdown of starch is catalysed by secreted amylases that have starch binding domains (SBDs). The SBDs facilitate the interaction between starch and the amylases by disrupting the starch structure which exposes starch to the active site of the amylase (Warren, 1996; Rodriguez-Sanoja et al., 2005). This feature has yet to be explored industrially but could be of high economic importance in processes where heat-labile components such as vitamins and nutrients demand processing at lower temperatures.

1.5 Industrial starch processing

Industrial processing of substances is a highly complex activity that involves conversion of feedstock to specific high value products. Starch is a raw material for various important products in different industries such as food and beverage, but also the biofuel sector. The strategy in use currently to convert starch to sugars is a combination of acid and heat-induced hydrolysis, and immobilized enzyme columns. Alcohol is currently produced from corn and some cereals, both of which are carbohydrate crops that contain starch which is the raw material in saccharification (Chen and Zhang, 2012; Duvernay et al., 2013). Since these crops are also high quality food sources, there are justified public concerns about the viability of this approach because corn and other cereals exhibit a low harvest index and occupy significant land surface with limited bio-ethanol yield (Gressel, 2008).

The starch transformation involves three basic steps, these include gelatinisation, liquefaction and saccharification (Satyanarayana et al., 2004). The process is currently highly expensive and high technology-based. It is also associated with the cost of downstream processing (Satyanarayana et al., 2004).

1.5.1 Gelatinisation and hydration of starch

Unlike starch digestion in nature, industrial processes cannot be partial and time-consuming; they need to occur fast and with minimal losses. Starch in its crystalline form cannot be easily digested by enzymes because they cannot penetrate the densely packed granule, justifying the general approach from beer brewers to hydrate and gelatinise the starch at high temperatures (Schuster et al., 2000; Benmoussa et al., 2006). Gelatinisation is defined as the phase transition starch undergoes on heating in the presence of water at high temperature giving rise to a water-soluble highly viscous gel. It begins with the diffusion of water molecule into the granule, hydration of the starch leading to the swelling of the granule. The structural order of the molecule is lost by the heat induced uncoiling and dissociation of the crystalline helices (Haralampu, 2000; Zhang et al., 2005).

The kinetics of starch gelatinisation and its temperature-dependence depends strongly on the ratio of amylose to amylopectin. The temperature ranges from 60°C to 80°C depending on the botanical source and properties of the starch. Banana starch gelatinises between 67 to 75°C however green (unripe) banana starch requires higher temperature of about 80°C to gelatinise (Zhang et al., 2005; Soares et al., 2011). It implies that the stage of ripening affects gelatinisation. This is because unripe banana has a higher concentration of starch some of which may be resistant or have low digestibility. Potato starches exhibit low gelatinisation temperature at 61°C while wheat and corn starches have temperatures of 73 and 77°C respectively as gelatinisation temperatures (Zhang et al., 2005; Carmona-Garcia et al., 2009). Therefore, an industrial saccharification process must include a robust gelatinisation step as pre-treatment to enable routine processing of starch from different feedstock.

1.5.2 Liquefaction

Liquefaction of starch is defined as the breaking down of the molecule into shorter chain units such as dextrin (Buchholz and Seibel, 2008; Dzedzoave et al., 2010). The gel-like properties are lost as the viscosity decreases, hence the term liquefaction. This is industrially achieved by acid catalysed hydrolysis of starch at high temperature and pressure (Kim et al., 2008b). It can also be achieved by enzymatic liquefaction using heat-stable α -amylase. The enzyme attacks the intact starch polymer at internal position cleaving the α -1,4 bonds to yield short glycans that can be digested further by other enzymes (Beck and Ziegler, 1989; Irving et al., 1999). Either method leads to a strong decrease in the viscosity of the starch solution thereby accelerating subsequent enzymatic digestion. It also creates a larger number of exposed non-reducing ends of the starch molecule hence providing substrates for other enzymes which exhibit an exo-glycosidic hydrolysis activity (Abd-Aziz, 2002; Buchholz and Seibel, 2008).

In the industrial starch conversion, gelatinisation and liquefaction are often combined; because high temperature is used for gelatinisation therefore liquefying enzymes are required that are stable under this condition thus the use of heat stable amylases has become the norm. Liquefaction of starch can also be achieved using acid hydrolysis; however the process is totally random therefore it is hard to control the pattern of products (Soni et al., 2003; Buchholz and Seibel, 2008). Also, both process-strategies depend on the concentration of starch, and results obtained with 10% starch solution cannot be extrapolated to a 30% starch solution. Efficient gelatinisation and liquefaction requires a great deal of optimisation.

1.5.3 Saccharification

Saccharification is defined as the breaking down of short sugars into fermentable sugars. In this case the term “fermentable” is defined for the ability of the yeast *Saccharomyces cerevisiae* to metabolise simple sugars, including glucose, maltose and maltotriose. For the food and drink industry, saccharification may also be defined as the process that leads specifically to glucose only (Carr et al., 1982; Satyanarayana et al., 2004). The extent of starch saccharification is quantitatively determined by the dextrose equivalence (DE) values and is defined as the ratio between glucose that is released or recovered from starch hydrolysis and the theoretical amount of glucose present in the starch sample (Schuster et al., 2000). The DE value of 100 means complete saccharification to glucose. Lower DE values may still contain mostly fermentable sugars if maltose and maltotriose represent the majority in the hydrolysate.

The efficiency of saccharification depends on a number of factors. These include the size and surface characteristics of starch and the molecular weight of the polymers. It has been postulated that starch with high amount of amylopectin is hydrolysed more easily by the amylases compared to amylose rich starch (Denyer et al., 2001; Soares et al., 2011). In contrast glycogen which is the storage form of carbohydrate in animals does not require gelatinisation which may be due to the high branching of the molecule.

Some α -amylases in addition to producing glucans can also hydrolyse starch to release mainly maltose which is also a fermentable sugar (Derde et al., 2012). However, β -amylase is the principal enzyme that releases maltose from non-reducing ends of starch (do Nascimento et al., 2006; Lin et al., 2008). Glucoamylase cleaves the α -1,4 and α -1,6 glycosidic bond in starch or glucans generated by the α -amylase reaction to release glucose. This implied neither of the isolated enzymes can lead

to complete digestion of starch (Pazur and Ando, 1959; Tatsumi et al., 2007), therefore, a combination of at least two of the enzymes, a liquefying and saccharifying enzyme, is required. Generally, starch is liquefied by α -amylase then followed by saccharification with glucoamylase. These strategies are used in the industry most especially for glucose production (Buchholz and Seibel, 2008).

1.5.4 Retrogradation of starch

Retrogradation is defined as the reversal of complex starch structures into native as well as non-native forms when subject to low temperature after gelatinisation. On heating starch in excess of water, the granules swell thereby rendering the amylose and amylopectin soluble. On cooling the solution, the amylose component forms a gel while aggregation of the amylopectin occurs forming a turbid solution of high viscosity. This phenomenon is referred to as retrogradation (Zhang et al., 2005; Alvani et al., 2011) and this property of starch has been used in wall-paper glue and other applications.

The structural organisation of the starch molecule is disrupted on heating, though renaturation can occur on cooling (Chung et al., 2006; Sajilata et al., 2006). However, the amylose components of starch re-associate by hydrogen bond to form double and often triple helical structures and these undergo transformations to form crystalline structures that are not native to starch (Wu and Sarko, 1978; Miles et al., 1985; Haralampu, 2000). Moreover, much higher temperatures are required for subsequent gelatinisation of the retrograded starch compared to the initial gelatinisation. The high energy required for subsequent gelatinisation can be attributed to compactness of glucose units forming higher inter-molecular hydrogen forces (Jane and Robyt, 1984; Haralampu, 2000)

The desirability of the above depends on the purpose of starch conversion; retrogradation is considered an attractive feature in thickening or when starch-based glues are employed. In the bakery for instance, stale bread is a form of retrograded starch (Karim et al., 2000). Nutritionally, a retrograded starch is often indigestible in the human diet because the α -amylases cannot hydrolyse the molecule; this can be exemplified with high starch food such as beans (Behall and Howe, 1996). Industrially, retrogradation is used in the development of resistant starch. The concept is used in the production of cereal foods such as corn flakes (Yue and Waring, 1998). When considering retrograded starch, estimates of energy content on carbohydrate-rich foods can be exaggerated as they do not always take into account digestibility.

Retrogradation is an undesirable feature when the starch is to be quantitatively digested to fermentable sugars as it will lead to reduced yields. For this reason industrial starch saccharification does not involve cooling the solution between the gelatinisation and liquefaction. The gel is kept between 65 to 90°C at all times so that retrogradation does not occur (Betancur and Chel, 1997). The temperature of the starch solution is only lowered when the heat stable α -amylase or moderately heat stable fungal glucoamylase is to be added for the hydrolysis (Aggarwal et al., 2001).

1.5.5 Raw starch digestion

Raw starch digestibility is a topic that is generating interests recently which may be due to increase in the roles starch play in the industries (Robertson et al., 2006). The digestibility of raw starch is affected by many factors, such as amylose to amylopectin ratio, presence of minor components such as phosphate and lipids, source, granule size, crystallinity, strength of molecular interaction between starch components, and amylose chain length. For instance, raw and uncooked banana starch is mostly resistant to hydrolysis so that when eaten by

humans it is extruded and passed in the faeces (Zhang et al., 2005; Cummings and Stephen, 2007).

Previous research has mainly focused on the identification of thermal stable α -amylases with little efforts on identifying amylase with raw starch digestion properties that can hydrolyse resistant starch (Muralikrishna and Nirmala, 2005). The process of hydrolysis starts with adsorption of the enzyme onto the starch granule; hence, it is necessary to make the starch susceptible to amylase activity (Sarikaya et al., 2000; Kim et al., 2008b).

In the previous and current section, the complex process of starch synthesis and degradation in nature and in the industry has been explained. The processes involve a variety of enzyme catalysed reactions and therefore in the next section, these enzymes, their nature, and mode of actions will be discussed in more detail. Understanding the properties of these proteins will assist in the design of process technology for starch hydrolysis.

1.6 Amylolytic Enzymes

Hydrolases also referred to as amylases are enzymes used to digest starch. Most of the starch hydrolases used in hydrolysis are of microbial origin whilst the beer brewing industries still adhere to the traditional use of malted barley containing endogenous plant hydrolases (Nigam and Singh, 1995; Horvathova et al., 2000; Kirk et al., 2002). Phosphorolysis and hydrolysis are the two forms of reactions enzymes used in starch degradation (Asatsuma et al., 2005). However, the discussion here will focus on the hydrolytic pathway. The enzymes involved in starch hydrolysis are classified into four: endoamylases, exoamylases, debranching enzymes, and transferases (Henrissat, 1991; van der Maarel et al., 2002).

1.6.1 Classifications

The amylases occupy a large share about one-third of the global enzyme market. This is associated with the various applications of the enzymes. The enzymes have found their usefulness due to the role they play in starch conversion. Amylases are widely used in food and beverage industries, breweries and the biofuel sector (Kirk et al., 2002).

The hydrolases differ in their sequence of amino acids that make up the primary structure. This implies that the secondary and tertiary structures of the enzymes are also variable (Henrissat, 1991; Henrissat et al., 2001; Stam et al., 2006). Similarly, the mechanism with which the reactions are catalysed is also different (McCarter and Withers, 1994). The endoamylase such as α -amylase hydrolyse α -1,4 glycosidic bonds in internal positions of amylose and amylopectin structures. It is also referred to as liquefying enzymes, this is because it cannot cleave the α -1,6 glycosidic bonds however, digests starch to liberate shorter glucans (Sarıkaya et al., 2000; Derde et al., 2012).

β -amylase and glucoamylase are referred to as exo-amylases; β -amylase cleaves the α -1,4-glycosidic bonds at the external part of the starch molecule (Hehre et al., 1979; Lao et al., 1999). The glucoamylase is capable of hydrolysing both α -1,4- and α -1,6-glycosidic bonds from the external positions of the starch molecule (Kim and Robyt, 1999; Sauer et al., 2000). It is referred to as a saccharifying enzyme because it is able to hydrolyse the dextrans to simple sugars such as glucose. Debranching enzymes, ISA and pullulanase hydrolyse the α -1,6 glycosidic bonds while the transferase cuts an α -1,4 glycosidic bond (of a donor) and transfers part of the donor to an acceptor molecule (Hussain et al., 2003; Bierhals et al., 2004).

1.6.2 Mechanisms of action by Hydrolases

The mechanisms of action of glycoside hydrolases have been studied due to their applications in starch hydrolysis (Hehre et al., 1979; McCarter and Withers, 1994). Besides elucidating their complex mode of action, research has also revealed information that is required for improvement of enzyme action (Reilly, 1999; Richardson et al., 2002; Lopez-Casado et al., 2008). In order for enzyme engineering to be successful, it is required that the amino acids that act at the active site of the protein are known. Also other residues that affect the structure and stability of the protein are also fully described (Richardson et al., 2002; Bessler et al., 2003; Cherry and Fidantsef, 2003; Johannes and Zhao, 2006). This will enable manipulation of the enzymes to increase their activity and also confer on them additional desirable features (Eijsink et al., 2004; Eijsink et al., 2008; Kelly et al., 2009).

Two modes of hydrolysis by the amylases can be distinguished; the first is retention of configuration this implies the α -configuration in starch is retained by the product of the enzyme action. In the second mechanism, the α -configuration of starch is inverted therefore the hydrolytic product has a β -configuration. In the retention mechanism, an amino acid serves as both a general acid and base while a second amino acid acts as a nucleophile and leaving group (McCarter and Withers, 1994). However, in enzymes with inversion mode of action, an amino acid serves as a general acid and another one acts as a base. Alpha amylase uses the retention mechanism while the inversion action is employed by the beta amylase (McCarter and Withers, 1994). In β -amylase from soybean Glu186 and Glu380 have been described as the general acid and base respectively (Kang et al., 2004). Because the hydrolases have significant industrial uses, understanding the mechanism of the enzyme action is important for the full exploration of these proteins and also to increase the efficiency of hydrolytic process.

1.6.3 Alpha-amylase

Alpha-amylase (1,4- α -D-glucan-4-glucanohydrolase, EC 3.2.1.1) is a member of the glucosylhydrolase class-13 that are folded into three domains A, B and C (Kuriki and Imanaka, 1999; Kumari et al., 2010). The enzyme α -amylase is found in microbes (bacteria and fungi), plants and the archaea. Thus various groups have reported the purification and characterization of α -amylase from germinating seeds of plants such as soybean (Kumari et al., 2010); malted finger millet (Nirmala and Muralikrishna, 2003); banana (Junior et al., 2006); apple (Wegrzyn et al., 2000; Stanley et al., 2002); *Bacillus amyloquefaciens* (Demirkan et al., 2005).

In plants, α -amylase may be produced and secreted by the aleurone cells (in rice) or scutellum (in maize and sorghum) or both into the starch endosperm (Ranki and Sopanen, 1984; Warner and Knutson, 1991; do Nascimento et al., 2006). Alpha amylase hydrolyses the internal α -1,4 glycosidic bonds of complex carbohydrates (Figure 1.7) such as starch leading to the generation of soluble glucans that are subsequently hydrolysed by debranching enzymes and β -amylase (Smith et al., 2005; Yu et al., 2005; Kumari et al., 2010). The name alpha refers to the configuration at carbon one of the reducing unit of the oligosaccharides generated by the action of the amylase. The degradation of starch by α -amylase has industrial uses in glucose syrups, bakery, brewing, pharmaceuticals, and detergents, treatment of sewage and livestock feeds (Chao and Serpe, 2010).

Physico-chemical factors may affect the stability and activity of α -amylases. Research has revealed that the α -amylases vary due to high and low pI form which are found on chromosomes 6 and 1 respectively (Mitsui and Itoh, 1997). Barley AMY1 and AMY2 are low and high pI of 4.9 and 5.9 forms respectively. Hence the pH optima of the α -amylases range from 4.5-5.5 (Tibbot et al., 2002; Robert et al., 2003). Temperature

or thermal stability is an important property of proteins as it affects enzyme activities. The optimum temperature of these amylases ranges between 40-55°C however in nature seed germinate at much lower temperatures therefore thermal stability is not important (Prakash and Jaiswal, 2010). Temperatures above 60°C may lead to inactivation of these proteins however, the brewing variety of barley α -amylase has been selected by the brewers for heat stability and can withstand temperature of 65°C which is not natural (Prakash and Jaiswal, 2010). The α -amylases in bacteria and plants require calcium for their stability, and activity (Tanaka and Hoshino, 2002, 2003).

Post-translational modifications such as glycosylation which is the addition of glycans (sugars) affect protein activity, stability and functions. (de Barros et al., 2009; Motyan et al., 2011). O-glycosylation involves glycan addition at hydroxyl groups of serine and threonine residues. While N-glycosylation takes place on asparagine residues of the sequence Asn-X-Ser/Thr. The addition of the N-glycans to proteins occurs in the endoplasmic reticulum. This is found in a subset of the secreted proteins including α -amylases, and the biological role of the glycosylation is not always clear (Vitale and Denecke, 1999; Motyan et al., 2011).

Several types of classification have been described for the plant α -amylases in cereals that are based on the tissues they are found (Huang et al., 1992; Mitsui and Itoh, 1997). However, a broader classification for plant α -amylases based on their cellular localisation has grouped the enzymes into three distinct families (Janecek, 2002; Stanley et al., 2002). Family one α -amylase is those that contain a signal peptide that targets the proteins to endoplasmic reticulum. The second family is the cytosolic α -amylases that are not known to contain any targeting peptide (Janecek, 2002; Stanley et al., 2002; Stanley et al., 2005). Family three α -amylases are chloroplast proteins that have transit peptide; and in addition to the enzyme (amylase) domain they contain an unknown

domain. This group of proteins are twice the sizes of both families one and two α -amylases (Stanley et al., 2002; Stanley et al., 2005).

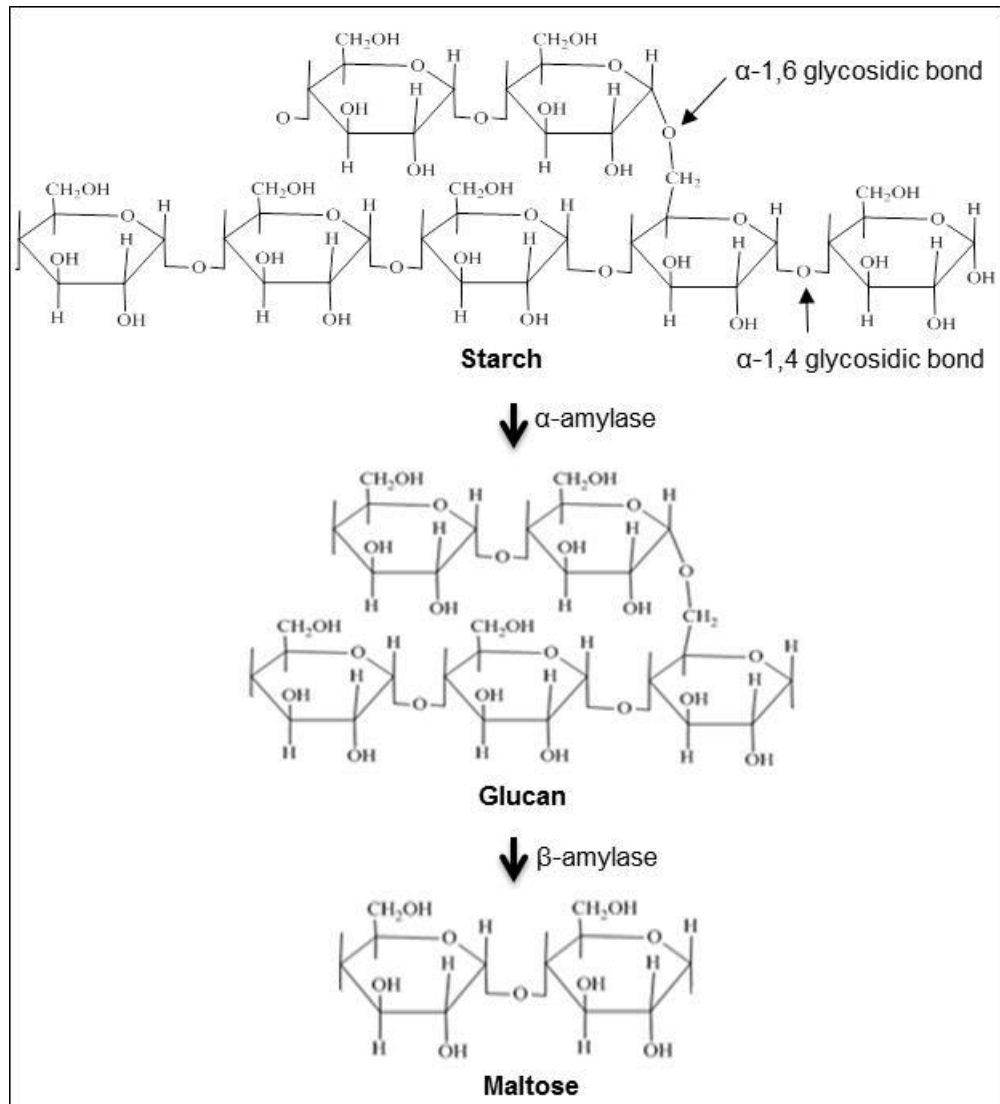


Figure 1.7. Action of α - and β -amylases on starch; the Figure illustrates the action of the two amylases on starch. The first enzyme α -amylase cleaves on the α -1,4 glycosidic bonds in starch to generate soluble glucan. The α -configuration is retained by the product. The glucans are hydrolysed further by a second amylase. β -amylase hydrolyses the glucans to generate maltose with the β -configuration.

The enzymes of the α -amylase family share some common features. These include their ability to cleave α -glycosidic bond, retaining of the α -configuration of the products (see Figure 1.7). The members also have up to seven sequence conserved regions, and similar catalytic

machinery (Janecek, 2002). The α -amylases possess the TIM barrel fold within the catalytic domain. Three amino acids are conserved in α -amylases; Asp206, Glu230 and Asp287. These two aspartate residues and one glutamate are located at the catalytic centre. An aspartate residue is located close to the end of the β -4 strand. The glutamic acid is situated near the end of strand β -5 while the second aspartic acid is close to the end of the β -7 strand (Horvathova et al., 2000; Stanley et al., 2005). In terms of the roles of the amino acid triads in chemical catalysis; the first aspartate is a nucleophile. The glutamate serves as a proton donor while the aspartate at strand β -7 stabilises the transition (Svensson, 1994; Horvathova et al., 2000).

High activity of α -amylase has been reported during seed germination. This indicates its role in starch mobilization in germinating seeds where starch reserves are used for energy (Irving et al., 1999; Zeeman et al., 2010). The synthesized α -amylase in the aleurone and scutellum is secreted into the endosperm to degrade starch. Thus, α -amylase plays a principal role in starch hydrolysis in the endosperm during germination (James et al., 2009; Kumari et al., 2010). Although α -amylase plays an important role in storage starch hydrolysis in the endosperm, it may not be involved in transitory starch hydrolysis in the chloroplasts of leaves. An *Arabidopsis* mutant (designated as *sex4* mutant) with low α -amylase activity showed normal trend of starch metabolism compared to the wild type. It is also evident that mutation in *AMY3* that is present in the chloroplast does not change the rate of starch hydrolysis in leaves (Yu et al., 2005; Zeeman et al., 2007b; Zeeman et al., 2007a). Thus alpha-amylase may be less significant in transitory starch breakdown.

In summary, α -amylase has been described as the only enzyme capable of hydrolysing crystalline starch granule (Beck and Ziegler, 1989). Evidence for this was established in a research on starch degradation in squash (Irving et al., 1999). In addition to the type of α -amylases discussed above, maltogenic α -amylases also exist, for instance

Lactobacillus plantarium α -amylase. This group of enzyme hydrolyse starch to produce short chain oligosaccharides such as maltose, maltotriose, maltoheptaose among others. The maltose is further hydrolysed to glucose and lactate (Giraud and Cuny, 1997). Therefore, the significant role played by the α -amylase in starch hydrolysis is an enzyme with endo-glycosidic activities.

1.6.4 Beta-amylase

Beta-amylase (α -1,4-glucan maltohydrolase, EC 3.2.1.2), a member of class 14 of glycosylhydrolase catalyses the hydrolysis of α -1,4 glycosidic bonds from the non-reducing ends of starch to release maltose (see Figure 1.7). It generates products that have β -configuration at the carbon one, thus the name of the amylase (Taylor and Robbins, 1993; Lin et al., 2008). Unlike α -amylase, beta amylase does not act on intact starch but it acts on the oligosaccharide chains that are released due to the action of endo-amylase. Hence, this amylase does not associate directly with starch granules. Instead it acts on glucans such as maltosaccharides which are its preferred substrate (Hehre et al., 1979; MacGregor et al., 1999; Hara et al., 2009). β -amylase hydrolyses maltoligosaccharide to β -maltose and glucose (Figure 1.7). Thus it is a major enzyme in the industrial production of maltose from starch (Shiraishi et al., 1987; Lu and Sharkey, 2006).

β -amylase has been characterised from microbes and higher plants. Unripe fruits such as banana, plantain, mango and apple contain large amount of starch that is converted to soluble sugars during ripening. This conversion is catalysed by several enzymes (Wang et al., 1995; Lao et al., 1999; do Nascimento et al., 2006). Since β -amylase cannot act on intact starch, α -amylase first hydrolyses the starch to soluble glucans. β -amylase then acts on the released glucans from the non-reducing end to liberate maltose. High activity of β -amylase and significant amount of

maltose has been reported from ripening fruits (Shiraishi et al., 1987; do Nascimento et al., 2006).

Although β -amylase cannot effectively digest starch without the previous action of α -amylase to create high numbers of non-reducing ends, research has repeatedly implicated β -amylase to be one of the key enzymes of starch hydrolysis. Maltose is a major product of starch hydrolysis during the night. Its presence is an implication that starch degradation occurs through hydrolytic pathway rather than phosphorolysis (Scheidig et al., 2002; Asatsuma et al., 2005). Unlike the α -amylases, members of the β -amylase family have two glutamic acid residues at its catalytic (active) centre. The β -amylase has a structure similar but with a difference to the TIM-barrel found in α -amylase which may be due to the two glutamate residues at its active site (Hehre et al., 1979; Kang et al., 2004). The structural difference implies that mechanism of catalysis employed by the β -amylase is different from that of the α -amylase.

The physico-chemical characteristics of β -amylases affect their chemical reactivity. The isoelectric point of these proteins is 5.1 with an optimum pH of 5.5. Similarly, the members of β -amylases exhibit different thermal stabilities (Eglinton et al., 1998; Daba et al., 2012). Most of the enzymes are not stable and inactive at temperature above 60°C however some microbial β -amylases are stable at temperature of 80°C. An example of this is a thermostable β -amylase from *Clostridium thermosulphurogenes* (Shen et al., 1988).

1.6.5 Glucoamylase

The hydrolytic degradation of starch cannot be completed by attacking α -1,4 glycosidic bonds only. Cleavage of the α -1,6 branch points is facilitated by glucoamylase (1,4- α -D-glucan-4-glucohydrolase, EC 3.2.1.3), a member of family 15 of glycosylhydrolase that is also involve

in the hydrolysis of starch. This enzyme is often referred to as amyloglucosidase (Shenoy et al., 1985; Coutinho and Reilly, 1997). It cleaves both the α -1,4 and α -1,6 glycosidic bonds (see Figure 1.8) from the non-reducing end of starch to release maltose and glucose of the β -configuration. Due to this property, glucoamylase is required for the complete hydrolysis of starch to glucose. It is an important industrial enzyme in the production of glucose syrup from starch and alcohol production (Saha and Zeikus, 1989).

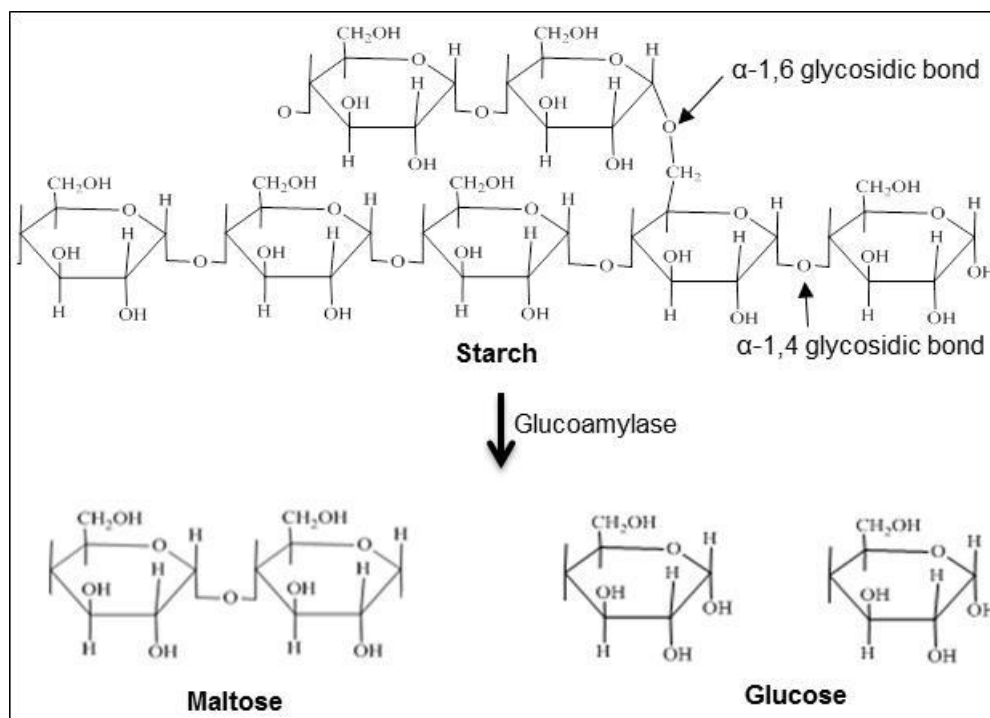


Figure 1.8. Action of glucoamylase on starch, it shows the effect of glucoamylase on starch. The enzyme is a major and important enzyme in starch processing, and it hydrolyses both the α -1,4 and α -1,6 glycosidic bonds of starch to generate maltose and glucose.

In the literature, two different glucoamylases have been described. Glucoamylase is a multi-domain protein with an active domain connected by a linker region to a starch binding domain. Of the two enzymes, glucoamylase I has a domain for reversible binding to starch that is separate from its active site. It is also larger in size than the glucoamylase II (Kim and Robyt, 1999; Kim et al., 2008a). The domain is referred to as a starch binding domain present in glycoside hydrolases.

As the name suggest, it is a region that establishes good binding between the enzyme and its substrate, starch. The interaction that is facilitated by the domain aids in adsorption of the glucoamylase by starch (Reilly, 1999; Hostinova et al., 2003).

Some conserved amino acids are found in glucoamylases, two glutamate residues at the catalytic centre. The presence of these amino acids is highly significant in the terms of the reactivity of the enzymes (Marín-Navarro and Polaina, 2011). The glucoamylases catalyse the hydrolysis of starch by an inverting mechanism. In the reaction, a proton is transferred from an acid catalyst to glycosidic oxygen. Nucleophilic attack of the water molecule occurs by a base catalyst. Glu179 serves as the general acid while Glu400 is the base. Glucoamylase can hydrolyse polysaccharides such as starch and oligosaccharides (malto-oligosaccharides) (Saha and Zeikus, 1989; Sauer et al., 2000; Sevcik et al., 2006). However, polysaccharides are the preferred substrate of this enzyme as their hydrolysis proceeds faster. In addition to these substrates, glucoamylase has the ability to hydrolyse raw starch by adsorption onto the crystalline granules (Coutinho and Reilly, 1997; Kim and Robyt, 1999).

The molecular weight of glucoamylase ranges between 48 to 112 kDa however some glucoamylases may have much higher mass. Post translational modification is a process that is significant in adding specific chemical or structural features to proteins (Saha and Zeikus, 1989; Marín-Navarro and Polaina, 2010). Glucoamylases are often glycosylated; both O and N glycosylation occur in this class of proteins, this means that they are secreted proteins. The enzyme glucoamylase is a glycoprotein composed of twenty sugars such as glucose, mannose, galactose and glucosamines. The sugars are linked to serine or threonine residues in the polypeptide, and the significance of the sugar moieties is to stabilise the enzymes. The glucoamylase are mostly from

fungi and are usually not heat-stable (Saha and Zeikus, 1989; Sauer et al., 2000).

1.6.6 Microbial and Fungal enzymes

A vast majority of industrial enzymes have been discovered from prokaryotes such as bacteria and eukaryotes such as fungi therefore the popularity of protein production from microbes is very high. Microbes are cosmopolitan and occupy a large variety of ecological niches including extreme temperatures consequently; they produce a vast variety of enzymes which enable them to survive in various environments. They can grow on a wide range of substrates that include carbohydrates such as starch, cellulose and sugars; proteins and lipids (Warren, 1996).

Among the enzymes that have been produced using microbial and fungal systems are heat-stable lipases and proteases use in washing powder, amylases such as α -amylase and glucoamylase (Shenoy et al., 1985; Saha and Zeikus, 1989; Sivaramakrishnan et al., 2006; Erjavec et al., 2012). Examples include heat stable α -amylase from *B. subtilis* (Kuranova et al., 1966), and *B. amyloquefaciens* (Demirkan et al., 2005) and glucoamylase from *Aspergillus niger* (Shenoy et al., 1985; Marín-Navarro and Polaina, 2010) and *Aspergillus oryzae* (Hata et al., 1991). In the starch processing industries such as bakeries, breweries, dairy and the beverage industries the hydrolases from bacteria and fungi are widely used (Shahani et al., 1976; Sharma and Satyanarayana, 2013). The microbial enzymes have gained popularity and are successful due to their desirable features such as heat and acid stability (Sharma and Satyanarayana, 2013).

In addition to the microbes being sources of valuable enzymes, they also serve as factories for the production of proteins. They possess systems which allow simple manipulations, thus they are widely accepted and used for protein production in small scale. Recombinant proteins can be

produced and harvested with relative ease from organism such as *E. coli* (Anne et al., 2012; Martinez et al., 2012). This has enabled its routine use in the laboratory for protein production. Despite the successes of the microbial system, there are still challenges.

The main problem of using the microbial system to produce proteins is the yield which may be sufficient for food and beverage industries. For the biofuels production to be meaningful on a global scale much larger quantities of enzymes are required which surpass the limit of contained fermentors (Rengby et al., 2004; Schumann and Ferreira, 2004; Rabhi-Essafi et al., 2007). The microorganisms lack the machinery for solubilisation of the recombinant proteins therefore the recombinant proteins often accumulate in inclusion bodies and are non-functional (Sorensen and Mortensen, 2005b). The bacteria also lack the machinery required for post-translational modifications of eukaryotic proteins such as folding, glycosylation (Kusnadi et al., 1997; Rabhi-Essafi et al., 2007). For these reasons, more efficient systems are required that provide the additional benefit of correct folding, and quality control of products as well as the necessary production scale.

1.6.6 The potential of Carbohydrate binding modules

Starch hydrolysis in nature does not only occur under extreme conditions such as high temperature in hot compost heaps. Microorganisms also degrade starch in the soil at low to very low temperatures, therefore the microbes have to devise means of dealing with crystalline starch. A characteristic feature of some members of glycoside hydrolases is the presence of carbohydrate binding module (CBM). It was originally found in enzymes involved in cellulose conversion. Thus, the domains were referred to as cellulose binding domains (Machovic and Janecek, 2006b, a; Shoseyov et al., 2006). Later, research has shown that enzymes involved in carbohydrate digestion all possess this domain. The proteins may be involved in hydrolysis or other reactions. Carbohydrate binding

modules have been classified into several groups. There are over 60 families of the CBM, with families 20, 21, 25, 26, 34, 41, 45, 48, 53 and 58 being SBDs. The domain may be found in alpha-, beta- or gluco-amylases, referred to as starch binding module (SBM or SBD) (Rodriguez-Sanoja et al., 2005; Shoseyov et al., 2006; Janecek et al., 2011). It is also present in glucan water dikinase, and glucosyltransferase. Some α -, β -, and gluco-amylases belong to family 20 of the CBM. α -amylase and glucan water dikinase also belong to CBM family 45 (Mikkelsen et al., 2005; Christiansen et al., 2009b; Christiansen et al., 2009a; Glaring et al., 2011) .

A CBM is a chain of amino acids found in enzymes of carbohydrate digestion. The number of amino acids ranges from 30 to 200 forming a single, double or triple domain. Starch binding domain may be situated at the amino or carboxyl terminus of the protein (Shoseyov et al., 2006; Chou et al., 2010). It is separate from the active site of the enzyme indicating that it may support or confer an additional function on the enzyme (Feller et al., 2011; Glaring et al., 2011).

The significance of CBM or SBDs in hydrolases has been a subject of research. It is evident that they perform functions different from that of the other domains that are found in the protein. They do not have enzymatic roles but aid in binding of the enzyme to its substrate, which is carbohydrate or starch (Juge et al., 2006; Glaring et al., 2011). Therefore, the CBM enable efficient binding between protein and starch thus facilitating an effective catalysis and subsequent conversion of the substrate to product. It may possess a disruptive function when the domain enzyme binds to its substrate, and may also mediate the adsorption of enzymes to their substrate. This was shown in an experiment where the fusion of glucoamylase to SBD was revealed to lead to an increase in enzyme activity and more efficient saccharification (Juge et al., 2006; Nielsen et al., 2009).

In conclusion, the CBM containing hydrolases may have great potential in the beverage and drink industry (Janecek et al., 2011). These include processes such as the clearance of juice at low temperatures to make crystal clear apple juice without affecting the stability of vitamins.

1.7 Plant Biotechnology

Classical breeding has been the way of improving crops both in terms of agronomic traits, such as growth rate, growth period, pest resistance, abiotic stress tolerance, development and overall productivity. Breeding has remained a strategic practice over decades where characteristics were modified or new ones often introduced (Taylor et al., 2008). However with the advancement in DNA technology, it is possible to transfer a specific foreign gene into a host cell, based on a defined hypothesis. Gene transfer methods such as *Agrobacterium* mediated transformation, electroporation of protoplasts, use of viral vectors and particle bombardment are now used routinely. The technology allows modification or even introduction of specific traits that would be hard to obtain by accident (Caplan et al., 1983). For instance the improvement of nutritional quality of food such as the development of golden rice is one typical example. This involved the introduction of two genes from *Narcissus pseudonarcissus*; phytoene synthase (*psy*) and lycopene β -cyclase (*β -lcy*) into rice, to facilitate biosynthesis of β -carotene, a provitamin A, in the rice seeds which normally do not produce meaningful quantities of this metabolite (Potrykus, 2001; Beyer et al., 2002). However, whilst the introduction of specific genes is easy, and can be exploited to produce vitamins or high value proteins in plants, it is much harder to modify stress resistance, plant-pathogen interactions and overall plant development and growth. Most of the success stories are therefore derived from strategies that involved the production of a single gene product, or perhaps two at most.

Another viable approach offered by biotechnology is protein evolution, a wide area of research where lots of enzymes of industrial, medicinal, agricultural importance are modified (Kusnadi et al., 1997; Rupp, 2013). Using these systems, properties such as thermal stability, pH optimum, reaction and produce specificities of enzymes are modified (Bornscheuer and Pohl, 2001; Cherry and Fidantsef, 2003; Khersonsky et al., 2006; Kiss et al., 2009). Specifically, α -amylase has been engineered for better enzyme performance and specificities (Richardson et al., 2002; Bessler et al., 2003; Kelly et al., 2009).

One of the key advantages of field-grown crops over contained fermentation tanks is the low impact of advanced technology and the high yields. It is the latter that could be explored to generate cheaper sources of hydrolases to render biofuel production economically viable.

1.7.1. Recombinant proteins in plants

Plant genetic engineering provided a new frontier in the area of protein production. Using the knowledge of biotechnology, proteins are being produced in plants, these include several antibodies against human diseases (Ma et al., 1998; Rigano et al., 2009a), and the viability of the system for vaccine is now being explored (Marusic et al., 2009; Rigano et al., 2009a; Gartland et al., 2013).

Different plants species have been considered for protein production; nowadays, it is possible to produce almost all types of proteins in plants. For instance, tobacco is one of the most widely used crops for recombinant protein production. This is due to its robust expression system; yield and gene transfer technique is fully established. Tissue culture is easy and plant regeneration is robust (Kapila et al., 1997; Ma et al., 2003; Vitale and Pedrazzini, 2005).

Enzymes have not only being discovered from or produced in microbes and fungi but also from higher eukaryotes such as plants (Kandra, 2003). Over a long time, proteins of industrial importance have been produced from plants. In addition to this, plants can also serve as a factory to produce proteins. This is in addition to the large amounts of enzymes that have been discovered and purified from plants (Vitale and Pedrazzini, 2005; Lynd et al., 2008). For instance, the barley malt has for a long time served as a source of amylases that is being used in brewing (Muralikrishna and Nirmala, 2005). Some other proteins have been heterologously expressed and produced in large quantities in crops. Production of proteins in transgenic plants allows significant yield of the recombinant products (Ma et al., 1998; Ma et al., 2003). This is because the efficiency of transcription and translation machinery can be increased. For instance, inclusion of an intron increases transcription in cereals. Also, depending on the type of expression required; regulated or constitutive promoters may be used (Caplan et al., 1983; Taylor et al., 2008).

Expression systems

Heterologous expression of proteins can be achieved in transient to test functionality of constructs. In this system protoplasts are being used to express different constructs, DNA transfer technique such as electroporation is used to introduce the DNA into the cells. The cells are incubated for a short time between 12 to 24 hours (Hadlington and Denecke, 1994). Then expression of the desired protein is determined using enzymatic assays. This system has lots of advantages that include reproducibility, safety, generate similar and comparable information as transgenic (Hadlington and Denecke, 1994). Another form of transient expression system that may offer higher yield of protein product is the infiltration of leaves epidermal cells. This has the advantage of longer expression period of between 48 to 72 hours (Caplan et al., 1983; Kapila et al., 1997). If the construct of interest is processed or transported, it may be more representative of the transgenic plants. The most important

asset of transient expression is that it is fast and can save time prior to the commitment to generate stable transgenics (Hadlington and Denecke, 1994).

Plant hosts for recombinant protein production

The selection of plants for expressing proteins particularly those used in biofuel production is not straightforward because a crop that can give an optimum amount of the desired protein is required (McLaren, 2005; Taylor et al., 2008). Secondly, or even more important is the amenability of the crop plant to transformation. For instance crops such as maize, corn, wheat, barley, tobacco and potato are considered easily transformable. In the context of biofuel, potato is highly desirable due to its high starch content. Therefore, selecting the appropriate plants is key to the success of protein engineering (Barrell et al., 2013). The next issue of high significance relating to the expressed protein is determination of the actual activity of the recombinant enzyme. This is because the measurement is not often performed with the actual substrate of the enzyme (Taylor et al., 2008).

1.7.2 What is the appropriate compartment for the production of recombinant proteins?

A major consideration in the production of recombinant proteins in plants is the cell organelle to target and store the proteins. These include the vacuole, apoplast, plastid (chloroplasts), mitochondria and the ER which together with Golgi, TGN make up the plant secretory pathway. Proteins are directed to the sub-cellular organelles using some specific targeting signals such as transit peptide for chloroplast (Marusic et al., 2009). Secretion of soluble proteins occurs by default thus does not require any signals (Denecke et al., 1990). This implies that recombinant proteins such as hydrolases and antibodies produced in transgenic plants can be secreted from the cells into the apoplast (Denecke et al., 1990; Vitale and Pedrazzini, 2005). However vacuolar sorting of proteins is signal

mediated by receptors (De Marcos Lousa et al., 2012). The ER retention of proteins is mediated by signals such as the HDEL (Munro and Pelham, 1987; Denecke et al., 1992).

The vacuole occupies the larger part of the cell but is less explored due to its lytic properties. The chloroplast has several advantages that include uniform expression rates, lack of gene silencing, post-translational modifications, such as oligomerization and disulphide bond formation, and effective protein accumulation due to high copy number and increased stability (Rigano et al., 2009b). However, the plastid targeting offer only options in terms of the post translational modification when compared to the ER due to its low hydrolytic ability and plasticity (Ma et al., 2003; Vitale and Pedrazzini, 2005; Rigano et al., 2009a). In order to target proteins to the secretory pathway, signal peptide is included at the N-terminus of the protein. The secretory pathway has been suggested to offer a better environment for protein production (Ma et al., 2003). An overview of the secretory pathway will be discussed in the next section.

1.7.3 The plant secretory pathway

In the eukaryotic cells, the endomembrane system of the secretory pathway is highly significant for the transport of molecules such as proteins, polysaccharides and lipids. The transport of proteins has been described to occur in a vectorial fashion beginning at the ER, followed by the Golgi apparatus and final cellular destinations such as the lysosomes (Palade, 1975).

Two transport routes can be distinguished; biosynthetic or anterograde and endocytic or retrograde pathways. The former is used to describe the route from the endoplasmic reticulum to the plasma membrane or the vacuole which are regarded as the later part of the secretory pathway. The latter deals with recycling or movement of molecules from the

plasma membrane to the organelles of the secretory pathway (Vitale and Denecke, 1999). This constant recycling of proteins and receptors from the PM back to internal organelle balances the anterograde pathway. The intermediate organelles include the Golgi apparatus, trans-golgi network (TGN), the pre-vacuolar compartments (PVC) or endosomes and the late pre-vacuolar compartment (LPVC) mediate trafficking steps between the ER, the vacuoles and the plasma membrane (Foresti and Denecke, 2008; Foresti et al., 2010; De Marcos Lousa et al., 2012). Most of the transport between the different organelles of the secretory pathway occur via protein coated membranes vesicles (Hadlington and Denecke, 2000; Bonifacino and Glick, 2004).

Entry into the Secretory pathway

The synthesis of nuclear proteins can either occur in the cytosol and catalysed by the free ribosome. Alternatively it may be catalysed by membrane-bound ribosomes on the rough ER (Blobel and Dobberstein, 1975b, a). This is a major route through which proteins enter the secretory pathway where newly synthesised proteins are translocated across the ER membrane (Dobberstein and Blobel, 1977). Soluble proteins containing a signal peptide (SP) at the N-terminus follow this route. In the cytosol, the SP is recognised by the signal recognition particle (SRP) (Dobberstein and Blobel, 1977; Walter and Blobel, 1981a, b; Walter et al., 1981). The binding of SRP to the protein triggers conformational changes that stops or slow down translation (Walter and Blobel, 1981a). A complex is formed; mRNA-ribosome-nascent chain-SRP and is directed to the SRP receptor (SR) on the membrane. As a result the SRP is released followed by the resumption of protein synthesis. Translocation of the nascent chain across an aqueous channel in pore complex of the ER membrane occurs (Kalies et al., 1994).

The fate of the protein after translocation depends on its structural composition. A protein that possesses a transmembrane domain in its

coding region act as a 'stop transfer' and is integrated into the ER membrane by lateral diffusion out of the core complex (Walter and Blobel, 1981a). However, integration into the membrane may also occur via a post-translational mechanism. It may be through an SRP dependent or independent manner. This pathway is used by short secreted proteins or tail-anchored proteins that are synthesised in the cytosol before being targeted to the ER. In addition to protein synthesis, the ER is responsible for folding, and assembly of newly synthesised proteins. It is also the site of post translational modification of proteins such as glycosylation (Vitale and Denecke, 1999), controls calcium levels in the cell and is responsible for the biosynthesis of phospholipids (Hanton et al., 2006).

The ER-Golgi interface

After successful translocation and chaperone-assisted protein folding; proteins can be exported from the endoplasmic reticulum to the Golgi apparatus. These two organelles can be regarded as the early secretory pathway; trafficking between these organelles occurs bi-directionally, from the ER to the Golgi or the biosynthetic route, and also recycling from the Golgi back to the ER, the retrograde route. The significance of this is to create a balance between the two organelles, whilst maintaining a sustainable flow of newly synthesized proteins to reach distal locations of the secretory pathway (Sparkes et al., 2009). The movement has been described to begin at the ER proceeds via the Golgi apparatus to either lysosomes or the plasma membrane (Palade, 1975).

Various routes have been reported to mediate the transport to lysosomes; they include coated vesicles (clathrin coated vesicles, CCV), mannose-6-phosphate (M6P) receptors in mammals (Pearse, 1975; Kaplan et al., 1977; Pearse and Bretscher, 1981), and vacuolar sorting receptors (VSRs) in plants (De Marcos Lousa et al., 2012). Some other models propose that movement of proteins to PM do not require some specific signals (Stevens et al., 1986; Munro and Pelham, 1987; Valls et

al., 1987; Wieland et al., 1987). However, it was later revealed that soluble protein secretion in plants occurs via the default pathway (Denecke et al., 1990). In contrast, sorting signals may be required for membrane proteins to exit the ER (Hanton et al., 2006).

Protein retention in the ER

The concept of retention was described as a result of work on three soluble reticuloplasmins that were discovered to contain the same tetrapeptide (KDEL) sequence at the C-terminus (Haas and Wabl, 1983; Munro and Pelham, 1986; Sorger and Pelham, 1987). Subsequently, it was discovered via deletion and transplantation studies that this motif is responsible for retention of proteins in the ER (Munro and Pelham, 1987). Related sequences, such as HDEL (yeast), RDEL and KEEL were discovered and also considered to be retention motifs (Denecke et al., 1992).

Although the term retention and retention signals is used frequently, it does not describe the exact manner in which ER-resident proteins accumulate in the ER. ER resident proteins such as the chaperone BiP must freely diffuse in the lumen in order to interact with intermediates of protein folding. Exactly this behaviour was established experimentally in *Xenopus* oocytes, which revealed that the presence of the tetrapeptide KDEL did not affect the rate of diffusion of BiP in the ER lumen (Ceriotti and Colman, 1988). First evidence for a dynamic recycling mechanism originated from experiments with the lysosomal protein cathepsin D (Pelham, 1988). When the sequence KDEL was fused to the C-terminus of cathepsin D, the hybrid protein accumulated in the ER, but continued to receive glycan modifications typical of the Golgi stack (Waheed et al., 1981; Pohlmann et al., 1982; Kornfeld and Kornfeld, 1985; Vonfigura and Hasilik, 1986). It was therefore suggested that a receptor binds to the KDEL-sequence in the Golgi and mediates retrieval back to the ER (Pelham, 1988).

Identification of receptors that interact with the retention signals

Receptors that recognise the tetrapeptide, KDEL and related signals were originally identified using a genetic screen in yeast. HDEL and KDEL functionalities were initially tested using a chicken lysozyme and yeast invertase as cargo molecules respectively, by fusing the tetrapeptides to the C-terminus of the cargo. It was revealed that the retention motifs cannot be interchanged for their respective binding machinery. Because of the homology between the KDEL and HDEL it was then suggested that *S. cerevisiae* may possess an identical ER retention system with a similar receptor molecule that offers different ligand binding specificity (Lewis et al., 1990).

In order to test the hypothesis, secretion / retention experiments were performed using the yeast invertase. Chimeric genes were put under the transcriptional control of strong or weak promoters, the strong promoter led to the secretion of the invertase-HDEL fusion suggesting that the saturation of the system in yeast (Pelham et al., 1988). The assay used in the research was not based on quantitative measurement of enzyme activity in the medium and cells of the culture. Most of the enzymes were in the space between the cell membrane and cell wall therefore secretion assays were performed on washed cells extracted with or without detergents referred to as extracellular and intracellular activity respectively. The invertase that remains in the culture medium was ignored (Pelham et al., 1988).

Stable integration and low expression of the recombinant invertase fusions in yeast genome within a SUC2 deletion mutant lacking endogenous invertase led to a pronounced phenotype. (Emr et al., 1983). However significant enzyme amount were contained in the periplasm of the unmodified invertase and KDEL-tagged invertase that allowed growth on sucrose. In contrast, the enzyme tagged with HDEL was retained in the ER making growth on sucrose almost impossible.

This was landmark to the identification of the ER Retention Defective (ERD) mutants based on selection on sucrose containing medium. The ERD retention induces the leakage of HDEL tagged invertase to the periplasm thus hydrolysing sucrose to fructose and glucose which is transported into the cells to be used as a carbon source (Pelham et al., 1988). ERD1 and 2 were identified using complementation analysis on yeast mutant screen and were tested by immunoblotting of BiP secreted from a colony onto the nitrocellulose filter that was used to test the secretion of ER residents (Hardwick et al., 1990; Semenza et al., 1990).

HDEL independent retention system

In addition to the HDEL-dependent retention system described above, other retention mechanisms have been described. Many ER residents have acidic C-termini, calreticulin is a typical example. A 53-55 kDa protein is analogous to muscle calsequestrin which is a major calcium storage protein present in the ER (Denecke et al., 1995). It was previously shown that the formation of calcium chelate by the reticuloplasmins play a major role in protein retention in the ER (Booth and Koch, 1989). However, the ER accumulation of proteins may be a combination of retention and retrieval (Pimpl and Denecke, 2000).

The role of the secretory pathway in recombinant protein production

A protein should be targeted to an environment that ensures maximum yield, and accumulation. This may also particularly affect folding, assembly and other post-translational modifications of proteins. The secretory pathway has been shown to be more suitable environment for efficient production of immunoglobulins than the cytosol (Zimmermann et al., 1998; Schillberg et al., 1999). This is evident by the fact that high accumulations of antibodies were reported in the secretory pathway compared to the cytosol. The above behaviour may also apply to other proteins besides antibodies however, this depends on the specific properties of the protein (De Jaeger et al., 1999; Schouten et al., 2002). Proper targeting of a protein has a significant effect on its biological

function and activity. It has been shown to lead to increased protein accumulation and stability.

The advantage of the endoplasmic reticulum is due to its oxidizing properties, abundance of molecular chaperones and little number of proteases. The above features are suggested to aid protein folding and assembly. The specific interaction of antibodies targeted to the secretory pathway with molecular chaperone (BIP) has also been reported (Nuttall et al., 2002; Ma et al., 2003). Glycosylation which is required for the function of some proteins only takes place in the endomembrane system (Ma et al., 2003).

Recombinant protein expression has been shown to improve if the product is retained in the ER using the signal H/KDEL tetrapeptide. A two to ten-fold accumulation in comparison to the non-tagged protein has been reported (Conrad and Fiedler, 1998; Schillberg et al., 2002). Another advantage of this system is that the retained protein may contain high-mannose this is because they are not modified in the Golgi apparatus. Taken together the secretory pathway and in particular the endoplasmic reticulum could be considered as storage compartments for recombinant hydrolases and other valuable industrial enzymes.

1.8 Unpublished data in the host laboratory in support of the work

As part of a long term project on new bio-fuel production strategies, the host-laboratory has experimented with transgenic potatoes to harness the enormous productivity of this crop per surface of land and cultivation time (see section 1.2.4). The strategy involved the cost-effective production of recombinant α -amylase in potato tubers to satisfy the demand for the required quantities of recombinant enzymes to facilitate large scale starch conversion to fermentable sugar in meaningful quantities on a global scale. It was discovered that segregation of recombinant hydrolases from the starch in amyloplasts was efficient enough to prevent self-digestion during growth. This is because the protein is separated from the starch by three membranes; two membranes of the amyloplast and one of the secretory pathway.

To take these findings forward, the team established a saccharification procedure that includes a combination of mild acid hydrolysis and enzymatic hydrolysis, improving the overall yield of the process and reducing processing costs. It was discovered that HCl was found to be a good mineral acid for the hydrolysis in contrast to sulphuric acid which was shown to decompose the sugars under similar conditions. The strategy allowed the use of diluted HCl in a pre-treatment that mediates sufficient starch gelatinisation and liquefaction to enable subsequent enzymatic hydrolysis at lower temperature. Under these conditions, transgenic potato tubers producing recombinant barley α -amylase produced yields that permitted detection of an additional protein band in Coomassie stained gels. Moreover, the amount of α -amylase stored in the transgenic tubers was capable of treating 100-fold higher levels of starch than what was contained in the tuber itself, when used in the combined protocol. In conclusion, the strategy reduced the cost for recombinant enzymes, guaranteed sufficient yield to treat economically

meaningful quantities of potato starch, and paved the way for a production strategy that yields multiple products from a single feedstock. Transgenic potatoes could thus be used to produce recombinant enzymes for industrial purposes, starch for bio-ethanol production, soluble proteins to generate yeast growth nutrients, cell wall pulp for paper production and yeast extract for retail.

Due to the success of the strategy, and the continuous resistance of the public to accept the growth of transgenic crops in fields, the team explored strategies to increase productivity, so that the transgenic plants yield sufficient added value to self-fund the additional cost required for growth in containment. In addition to the hydrolases the tubers may also serve as a platform for production of higher value proteins, such as antibodies, vaccines and serum proteins. In this manner, the bio-ethanol may merely become a side fraction of the overall process, yet becoming a more economically viable approach that may be successful without subsidies. The apoplast and endoplasmic reticulum were found to be the most successful storage compartments for barley α -amylase, in contrast to tuber vacuoles which were found to be just as lytic as vacuoles in vegetative tissues such as roots, stems and leaves (J. An and J. Denecke, unpublished). In addition, barley α -amylase was produced to 100-fold higher levels compared to heat stable *Bacillus amyloliquefaciens* α -amylase. This implies that the apoplast and ER can be exploited for the production of recombinant proteins, but it depends strongly on the properties of the individual proteins to be expressed.

Interestingly, a comparison of secreted α -amylase with ER-retained α -amylase-HDEL revealed that the ER lumen is probably more suitable for protein storage compared to the apoplast, but that HDEL-overdose can lead to developmental defects. The ER retention system was found to be saturated upon producing large amount of amylase-HDEL (Phillipson et al., 2001) thereby leading to auxin-deficient phenotypes (J.An and J. Denecke, unpublished) that may be due to excessive secretion of the

auxin-binding protein, a KDEL protein that is thought to inhibit endocytosis from the plasma membrane when it leaks out to the apoplast (Robert et al., 2010). As a result, the distribution of recombinant protein levels in the population of transgenic plants was clearly shifted towards higher α -amylase-HDEL levels, but appeared to be truncated at the high production end, suggesting that the majority of overproducers did not regenerate. To overcome this problem, the team finally attempted to use the acidic C-terminus of calreticulin, a protein suggested to contain HDEL-independent ER retention signals (Pagny et al., 2000), perhaps due to the calcium chelating ability of an acidic stretch of 40 amino acids near the C-terminus. In addition the presence of peptides that increase the distance between the consensus glycosylation site and the stop codon has also been suggested to increase the efficiency of glycosylation (Nilsson and von Heijne, 2000) therefore amy-cal may have been glycosylated. Interestingly, recombinant protein activity yields were much lower than those obtained for α -amylase or α -amylase-HDEL in transgenic crops, which was in contrast to observations in electroporated protoplasts where activities of α -amylase or α -amylase-HDEL were more comparable to those of the calreticulin fusions, regardless of the presence of the HDEL retention signal.

1.9 Working hypothesis and aims of the project

The current work was mainly concerned with the continuation of the ongoing bio-fuel project, specifically focussing on the limiting factors that affect the entire process. The project can be subdivided into different work packages, each based on specific working hypotheses.

Exploring factors that affect glycosylation: It was hypothesised that the low activity of amy-cal fusions in transgenic plants (section 1.7) may have been due to glycosylation of a consensus site near the C-terminus of the barley α -amylase that was used as model protein in these studies. However, further research was required to test this hypothesis, because earlier research showed that in electroporated protoplasts, barley α -amylase is not glycosylated (Leborgne-Castel et al., 1999). The aim of the initial aspect of the project was to test whether the amylase-fused to HDEL or calreticulin can indeed be glycosylated and if C-terminal extensions influence the degree of glycosylation (Nilsson and von Heijne, 2000). This required detection by antibodies in order to test the molecular weight on denaturing gels. Therefore an antibody against the barley α -amylase was generated, and different fusion proteins were modified to eliminate the consensus site for N-linked glycosylation. These tools were used in plant expression systems to score potential glycosylation and its effect on the specific enzyme activity.

Affinity purification of recombinant α -amylase: A cascade refinery process was designed based on the initial success of recombinant barley α -amylase production in potatoes; it implies that the potatoes will be used as a model to produce a range of high value proteins. These proteins will be purified and different side fractions from the potatoes will be used as feedstock for other purposes. However, one of the problems of producing soluble recombinant proteins is purifying the protein of interest from a mixture of proteins. Therefore the barley α -amylase was tagged with a histidine octapeptide that enables the purification of the

amylase using a nickel column; if this works as a proof-of-concept this technology can be used to purify other high value proteins (Marusic et al., 2007), but also permits purification of a second high value protein from the flow through, for instance a recombinant antibody using a protein A affinity column.

Optimising combined acid and enzymatic starch hydrolysis: Even though the preliminary results on the combination of mild acid pre-treatments and subsequent enzyme hydrolysis were promising (J. An, J. Denecke, unpublished), further optimisation was necessary. In this work package, different acid hydrolysis regimes were compared, as well as a combination of liquefying and saccharifying enzymes. This is to establish an efficient protocol that ensures maximum yield of fermentable sugars with minimum loss to non-fermentable degradation products.

α -amylases with raw starch digesting properties: Previous research on the liquefying enzyme α -amylases has been mainly targeted towards heat stable fungal and bacterial enzymes; however plant enzymes have been much less explored. In view of the fact that some plant α -amylases may have attractive features such as raw starch digestion ability therefore cloning a plant amylase was attempted from ripening plantains, which contain a large quantity of resistant starch, yet undergo a very fast ripening process leading to effective conversion of starch to soluble sugar molecules.

Chapter 2

2 Engineering of amylases

2.1 Introduction

Starch is a molecule that is made of glucose linked by α -1,4, and α -1,6-glycosidic bonds. As explained in the introduction to this thesis, starch serves as food and feedstock for various high value products including bioethanol (Kossmann and Lloyd, 2000; Copeland et al., 2009). Therefore, the process of starch conversion to fermentable sugars is of interest in order to optimise biofuel yield and to minimise production cost. The conversion of starch involves three processes; gelatinisation, liquefaction and saccharification (Betancur and Chel, 1997; Copeland et al., 2009). Gelatinisation is performed using high temperature in the presence of water, while liquefaction and saccharification can be performed by the use of mineral acids or enzymes (Betancur and Chel, 1997; Soni et al., 2003; Satyanarayana et al., 2004).

Acid hydrolysis is non-discriminating; it acts on both α -1,4, and α -1,6 bonds of starch but leads to the formation of salts after neutralisation. It can also cause chemical decomposition of sugars, thus reducing the overall yield (Betancur and Chel, 1997; Kossmann and Lloyd, 2000; Soni et al., 2003; Satyanarayana et al., 2004; Copeland et al., 2009). In the enzymatic starch conversion, gelatinisation is combined with liquefaction therefore heat stable enzymes are required to deal with high gelatinisation temperature. In contrast to the acid hydrolysis, no salts are formed during enzymatic hydrolysis and decomposition of sugars is less likely (Betancur and Chel, 1997; Buchholz and Seibel, 2008).

The gelatinised starch solution is liquefied and rendered amenable to further hydrolysis by the use of liquefying enzyme, α -amylase (Beck and

Ziegler, 1989; Buchholz and Seibel, 2008). This hydrolase has the ability to cleave the α -1,4 glycosidic bonds of starch in internal position. This ability of the enzyme increases the number of non-reducing ends and releases dextrans that are substrates for β -amylase and glucoamylase to release fermentable maltose and glucose respectively. The liquefying action of α -amylase reduces viscosity and prevents retrogradation of the starch (see section 1.4.4) (Schuster et al., 2000; Abd-Aziz, 2002). However, non-fermentable products which are sugars longer than maltotriose and branched sugars that cannot be converted to alcohol are also released (Pazur and Ando, 1959). Further optimisation is thus needed to improve overall efficiency of bioethanol production from starch.

Previous work in the host laboratory established a starch conversion process that involves the combination of mild acid hydrolysis and enzymatic hydrolysis (see section 1.7). The starch is initially pre-treated with dilute HCl at a temperature of 126°C. The solution is neutralised and cooled to lower temperature for the enzyme action and then treated with α -amylase. The preliminary results indicate that the HCl pre-treatment is sufficient to mediate starch gelatinisation and liquefaction to enable subsequent enzymatic hydrolysis at lower temperature thus, reducing the amount of enzyme required (J. An and J. Denecke, unpublished). Therefore, improving the overall yield of the process and reducing processing costs lies within reach. However, in order to produce bioethanol at industrial scale, large quantities of enzymes are required at low expense to make the overall process cost effective but this cannot be provided by the microbial sources due to scale and high cost of the enzymes. Therefore, a cheaper source of enzyme is required (Eijsink et al., 2008).

Plant can serve as a factory for in-expensive and large scale production of recombinant proteins (Ma et al., 2003; Rigano et al., 2009a), thus production of α -amylase required for bioethanol production was

considered in transgenic plants (Vitale and Pedrazzini, 2005; Lynd et al., 2008). The barley α -amylase is a secreted protein with signal peptide for translocation across the ER membrane. In the cytosol, the SP of this soluble protein is recognised by signal recognition particle (SRP) and is cleaved by the signal peptide peptidase (SPP) (Walter and Blobel, 1981a; Lyko et al., 1995; Weihofen et al., 2002). Previous work in the host laboratory has used the α -amylase to study protein properties such as activity, secretion / retention by fusing the amylase to the ER retention signal HDEL (see section 1.7), and its related sequences (Phillipson et al., 2001). This has been experimentally achieved using transient expression in electroporated protoplasts as well as infiltration of leaves. Through this system, the secretion of the barley α -amylase was tested; retention of the amylase-HDEL has also been established (Crofts et al., 1999).

Preliminary work in the host laboratory attempted the use of potatoes as a model to produce recombinant barley α -amylase (see section 1.7, J. An and J. Denecke, unpublished). The recombinant barley α -amylase activity produced was more than 100-fold higher compared to heat stable *Bacillus amyloliquefeciens* α -amylase. It was also revealed that amylase extract from a single transgenic potato producing recombinant barley α -amylase can digest starch contained in hundred wild type potatoes (J. An and J. Denecke, unpublished). Based on the properties of the secreted α -amylase with ER-retained α -amylase-HDEL, the ER lumen was suggested to be more suitable for protein storage compared to the apoplast, however high HDEL levels can lead to developmental defects. The ER retention system was found to be saturated upon producing large amount of HDEL proteins (Crofts et al., 1999). As a result amylase-fused to the acidic C-terminus of calreticulin was then tested in transgenic plants. Preliminary results showed that recombinant protein activity yields were much lower than those obtained for α -amylase or α -amylase-HDEL, which may be due to protein glycosylation (J. An and J. Denecke, unpublished). However, in electroporated protoplasts

comparable activities of α -amylase or α -amylase-HDEL or those of the calreticulin fusions, regardless of the presence of the HDEL retention signals were obtained. Further, research was required to study these differences and to test if glycosylation of α -amylase could be induced by generating fusion proteins and if this depends on the expression system. These questions were addressed in this chapter together with a comparison of a variety of ER retention signals and an affinity tag for routine purification of the recombinant α -amylase.

2.2 Results

Generation of antibody against the barley α -amylase

In order to determine the molecular weight and the pattern of glycosylation of barley α -amylases expressed in plant cells, an antibody against the barley amylase was required that does not cross-react with endogenous potato or tobacco α -amylases. The process of the antibody generation was not standard due to some un-expected challenges therefore; the individual steps in the generation of the antibody will be presented and discussed.

Establishing an expression protocol for amylase fused to GST

In order to purify the barley α -amylase from the mixture of cell proteins using affinity purification, the protein was fused to the 26kDa glutathione S-transferase (GST) (Stofkohahn et al., 1992; Liu et al., 2006; Abhary et al., 2011). This would enable the purification of the recombinant protein using a GST column. A T7, inducible promoter was used to avoid the problem of protein toxicity on the *E. coli* cells whilst establishing clones and large scale cultures (Grunberg-Manago, 1999; Lopez et al., 1999). A pilot experiment involved a comparison of two different *E. coli* strains (Trabbi-Carlson et al., 2004); BL21 Star™(DE3)pLysS One shot and BL21-Gold™(DE3)pLysS are the two strains chosen (Carr et al., 1991; Miroux and Walker, 1996; Feng et al., 2002); because they enable high expression of recombinant proteins from pUC plasmids with very high transformation efficiency and significant protein yield (Kido et al., 1996; Lopez et al., 1999). The strains also limit background expression of un-induced cells to very low level (Grunberg-Manago, 1999; Lopez et al., 1999). They also reduce protein degradation due to the inactivation of expression of genes that increase the degradation rate (Rabhi-Essafi et al., 2007; Borja et al., 2012; Waegeman et al., 2013).

After gene induction, cell extracts and all pellets were boiled in sample buffer and were subject to gel electrophoresis. Figure 2.1 shows the

pattern of recombinant proteins obtained with GST and α -amylase fused to GST. An additional protein of the expected molecular weight (70 kDa) was observed in the induced samples after 3 hours of induction or more. This band was absent in the GST control which showed a lower molecular weight GST band instead. It was also observed that longer induction time may be unnecessary because significant recombinant protein levels were obtained after only 3 hours. Comparable levels of expression were obtained for both star and gold strains of the *E. coli* cells. The results imply that any of the two may be used for further expression and subsequent purification.

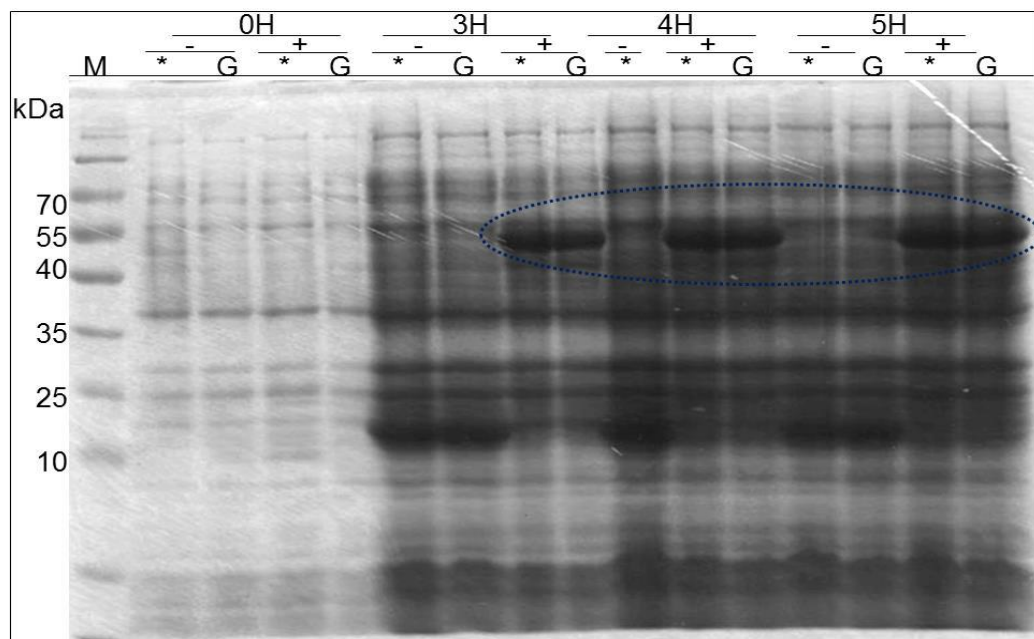


Figure 2.1. Expression of the barley alpha amylase in *E. coli*, M is the marker, * and G denotes the BL21 Star™ and Gold™ (DE3)pLysS *E. coli* strains, respectively. While – and + represents GST only and GST-fused amylase respectively. 0, 3, 4 and 5H represents hours after induction. The circled region shows the expressed amylase of the expected molecular weight of 70 kDa. The GST only of 25 kDa is also visible.

Recombinant protein solubilisation

Extraction using different buffers

To be able to purify the protein from numerous cell proteins using the GST-tag as affinity bait on GST columns, induced cell pellets were

extracted to obtain soluble proteins. The cells were pelleted and re-suspended in buffers of different compositions followed by cell disruption using sonication. Equal quantities of supernatant (s) and cell pellets (p) were boiled in sample buffer and loaded on a gel. Figure 2.2 shows that the protein of interest (70 kDa) was only detectable in the insoluble fraction (p), but could not be observed in the soluble portion (s). This indicates that soluble GST fused amylase was not solubilised under these extraction conditions.

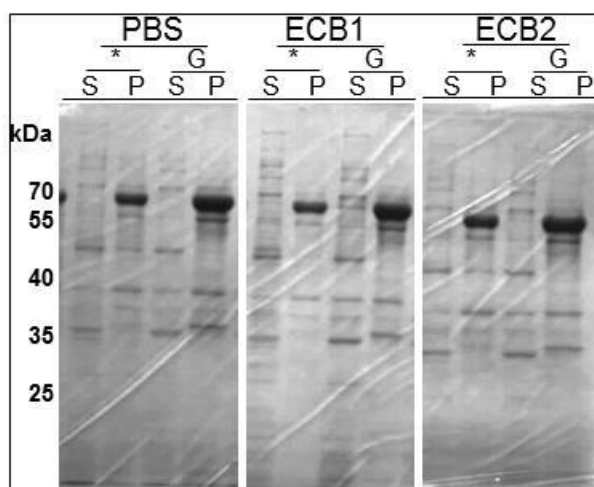


Figure 2.2. Extraction of recombinant protein, S and P are supernatant and pellet, respectively. * and G represents the BL21 Star™ and Gold™ (DE3)pLysS *E. coli* strains, respectively. PBS = Phosphate buffered saline (NaCl, Na₂HPO₄·2H₂O, KH₂PO₄, pH 7.4), ECB1 = E. coli buffer 1(50mM Tris pH 8.8, 2mM EDTA pH 8.00), 2) Buffer 2 (50mM Tris pH 8.8, 2mM EDTA pH 8.00, 150mM NaCl), were used to extract the recombinant proteins.

In order to extract soluble proteins, the experiment was repeated using the same expression and induction protocols. However, to enhance protein solubility, two different growth temperatures of 28 and 37°C were used this is because it has been suggested that expression at lower temperatures increases solubilisation efficiency. To test if the protein can be solubilised by buffers of different strengths, phosphate buffered saline (PBS), phaseolin buffers and bug buster (Merck®) were used. The PBS is judged to be mild compared to phaseolin buffer because it contains little amount of salt and phosphates. The phaseolin buffer has higher extraction abilities compared to PBS buffer due to the higher amounts of

salt and is detergent-rich. It is composed of tris, salt, EDTA, a detergent triton and a denaturant β -mercaptoethanol that cleaves disulphide bonds in proteins, and the buffer has been shown to solubilise GST-fusion proteins (Frangioni and Neel, 1993; Tao et al., 2010). Bug buster (Merck®), a buffer that has been described to be efficient in releasing soluble proteins. In addition to detergents and denaturants, it also contains nucleases that degrade DNA and RNA. Higher sonication amplitude of 50% was used to disrupt the cells, cell extracts and pellets were boiled in sample buffer and subject to gel electrophoresis. Figure 1.3 shows that the desired GST-fusion protein was still partitioned to the insoluble fraction with very little or even no soluble fusion protein extracted.

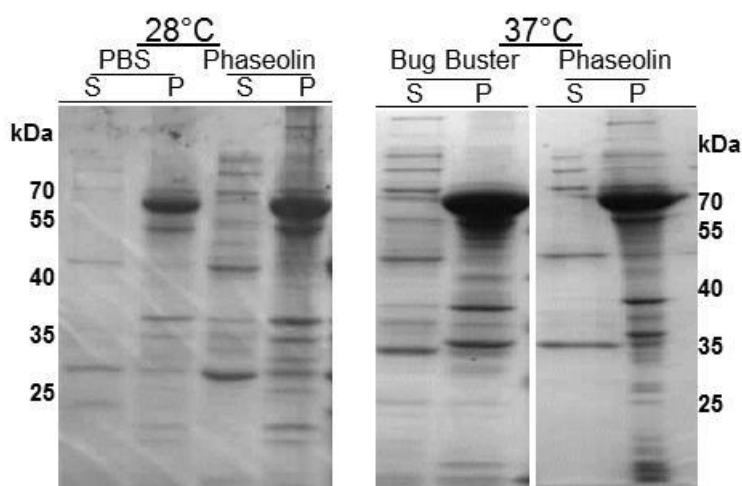


Figure 2.3. Extraction of recombinant protein 2, S and P are supernatant and pellet, respectively. The expression was performed using the BL21 Gold™ (DE3)pLysS *E. coli* strain at 28 and 37°C respectively. The proteins were extracted in bug buster and phaseolin buffers.

Comparable amount of protein was detected with both the bug buster and phaseolin (see Figure 2.3). This suggested that the difference in detergents did not influence the extraction of soluble protein. Similarly, comparing the two different growth conditions of 28 and 37°C revealed that comparable recombinant protein levels were achieved at both temperatures (Figure 2.3). But solubilisation of the protein was not affected by the growth of the bacteria at the different temperature

condition. Therefore, to yet extract the soluble protein a further modification to the expression condition was decided.

Changing the expression condition

Having failed to extract the soluble protein using two different temperatures of 28°C and 37°C, and buffers of different strengths; a different approach was required. In order to solubilise the protein, the expression was performed at lower temperatures of 10, and 16°C this is because protein aggregation has been suggested to reduce at low temperatures. This is because lower temperature usually means lower expression levels. Also, different media LB and TB were used for the growth of the bacteria, and low concentration of the inducer IPTG of 1 mM was used. After induction, centrifugation, sonication; the supernatant and the pellet were loaded on a SDS-PAGE gel. Figure 1.4 shows the recombinant proteins obtained using the different media and growth conditions. But the lower temperatures did not offer enrichment to the amount of protein compared to what was obtained at higher temperatures. However, comparable amount of protein was obtained with expression at 10 and 16°C (Figure 2.4, panel A), this is also the case with the two media used however soluble GST-fused amylase could not be obtained.

Auto-induction

In order to increase the solubility of the recombinant protein, an auto-induction experiment was performed in addition to the different conditions used. This is because auto-induction has been suggested to promote yield and solubility of recombinant protein (Grabski et al., 2005). After induction, the supernatant and pellet were boiled in sample buffer and subject to gel electrophoresis. Figure 2.4 panel A shows the protein pattern observed with the auto-inductions with insignificant amount of soluble proteins obtained (Figure 2.4, panel A). This implied that the auto-induction condition was not able to render majority of the protein

soluble. To increase the amount of soluble compared to insoluble proteins, the protocol should be improved.

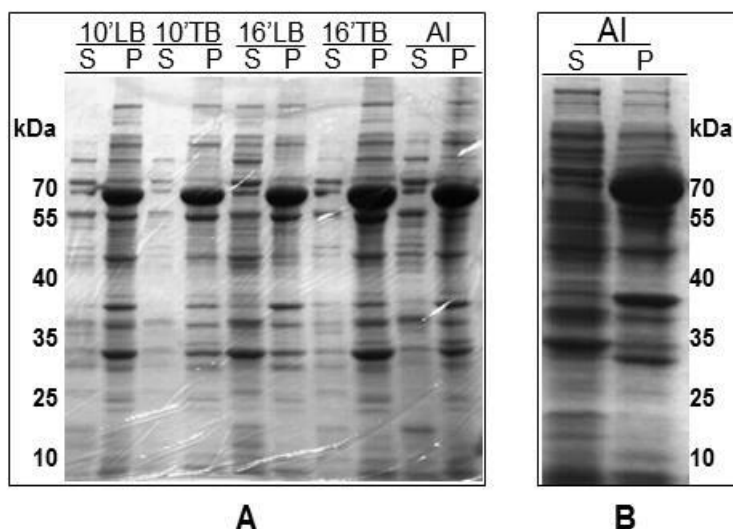


Figure 2.4. Expression using different media and temperature. Panel A shows expression using LB and TB media at different temperature. S and P are supernatant and pellet, respectively. AI represents the auto-induction protocol. The expression was performed using the BL21 Gold™(DE3)pLysS *E. coli* strain. The proteins were extracted in phaseolin buffer. Panel B shows the repeat of auto-induction experiment.

In order to increase the protein solubility by enriching the amount of the product, the auto-induction experiment was repeated in larger volume of cultures to increase the yield. Figure 2.4 (panel B) shows the soluble and insoluble fractions that were obtained after extraction and cell lysis however the solubility could still not be enhanced.

From the results presented above, it can be concluded that the recombinant product could not be solubilised using a variety of different approaches attempted, including different growth conditions and buffer types. Hence, it was decided to use the insoluble property of the fusion protein by eliminating soluble proteins in the S-fraction and by identifying a way of purifying the protein of interest from the insoluble portion.

Purifying the barley amylase

In order to purify the desired recombinant protein from the insoluble fraction, it was decided to separate the GST-fusion on a denaturing protein gel. It was first necessary to quantify the amount of recombinant protein. After induction and growth, the cultures were spun, the supernatant recovered and pellet was re-suspended in phaseolin, and mixed with sample buffer mix and boiled at 100°C and were subject to gel electrophoresis. Figure 2.5 shows the recombinant protein against BSA standard and the protein was estimated as 4µg/µl.

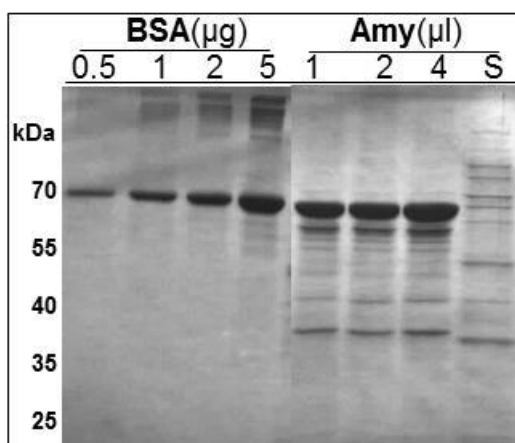


Figure 2.5. Quantification of recombinant protein, S is the supernatant. The protein was quantified against Bovine serum albumin (BSA). The amount of BSA in microgram is 0.5, 1, 2, and 5; while the amount of the amylase is in micro-litres of 1, 2, and 4 of the amylase. It was deduced that 1µl = 4 µg.

Preparative gel

In order to purify the insoluble recombinant proteins from gel, 250 µg of proteins which is the amount needed of the antigen for individual injection for the immunisation procedure was prepared. A 10% preparative SDS-PAGE gel was made and the proteins were loaded and ran slowly. The gel was stained with coomassie brilliant blue for one hour and de-stained overnight; Figure 2.6 shows a preparative gel indicating the separation of the proteins. The band of interest was thinly cut out with razor blade and transferred into a microfuge tube and weighed. This procedure was repeated eight times because four injections are required

for two rabbits each. The purified protein was sent to Eurogentec for the antibody generation programme.

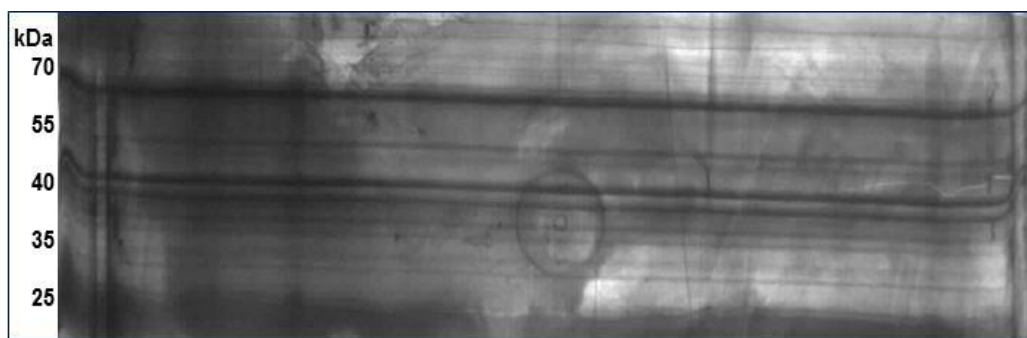


Figure 2.6. Preparative gel showing the expressed GST-fused barley amylase loaded on a 10% preparative gel. Good separations of the different *E. coli* proteins and the 70 kDa desired protein of interest were obtained.

Characterisation of the antibodies

Antisera against the barley α -amylase were received from the company; include the pre-immune, small and large bleeds. In order to characterise the antibodies generated, the first step was to compare the barley α -amylase GST fusion with GST alone as control. The two constructs were expressed in *E. coli*, and the samples were used to probe the different antisera. Figure 2.7 shows the western blot of the different antisera from two different rabbits. The antibody from rabbit 1 shown in Figure 2.7 panel A was able to specifically detect the recombinant barley α -amylase but does not cross-react with *E. coli* proteins or GST. Therefore, the antibody exhibits good specificity to the barley α -amylase. The detection ability of the antibody is acceptable particularly for the second (large) bleed. On the other hand, the pre-immune bleed did not detect anything as expected and was also free from background affinities. However, the antisera from the second rabbit as shown in Figure 2.7 panel B were deemed inferior because background affinities to *E. coli* proteins were detected even in the pre-immune serum, and specific interaction with the recombinant GST-fusion was unconvincing. This implies that the quality of the antisera from the second rabbit was very poor.

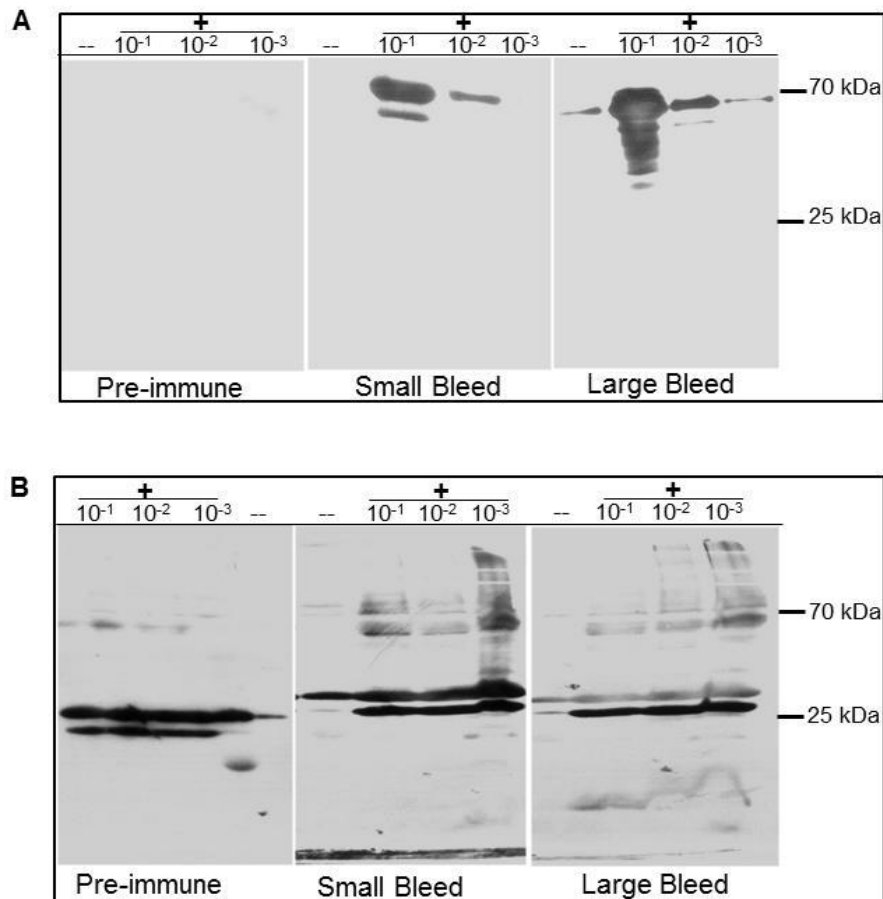


Figure 2.7. Characterisation of the anti-amylase, three antisera; pre-immune, small and large bleeds from two different rabbits were obtained. Panel A and B shows antisera from rabbit 1 and 2 respectively. – is the GST alone as control. + is the barley α -amylase GST fusion. Three dilutions of the recombinants proteins used are; 10, 100, and 1000 folds

According to the result in Figure 2.7 (panel A), affinity to *E. coli* proteins was not observed however, a different specificity may be exhibited with plant proteins. This is because plant proteins may exhibit different properties therefore it was necessary to determine the antibody specificity using plant extracts. Previous work in the host laboratory has generated transgenic potatoes over-expressing the barley α -amylase (J. An, unpublished). Therefore, I decided to work with potato tuber extracts from the wild type and barley α -amylase overproducers as reagents. The small, large and final bleeds were diluted a thousand and five thousand folds. Figure 1.8 shows a western blot of the barley amylase detected using the antibody. The three antisera were able to recognise or detect the antigen which is amylase protein. No barley α -amylase was detected

in the wild type potato that does not contain the recombinant α -amylase (Figure 2.8). Also, there is a decrease in the signal detected as the amylase concentration decreases. Stronger detection efficiency was observed with the large bleed compared with the small bleeds. However, no significant increase in detection was obtained with the final bleed of the antibody. On the other hand, background signals were observed in all cases which may likely be from plant proteins. The affinities to the background proteins increase from small bleed to being highest in the final bleed of the antibody. Similarly, dilution of the antisera reduces the background as shown in the Figure; the more diluted the antisera the lower the background and vice versa. As a result, it was decided that routine uses of the antibody will explore diluted antisera.

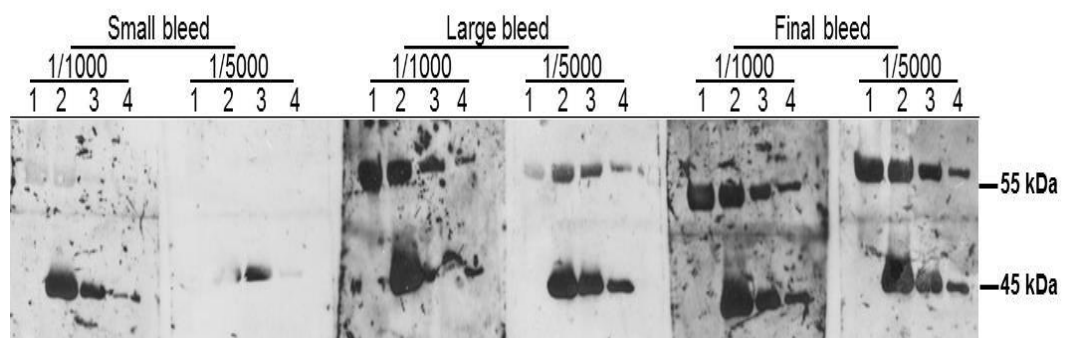


Figure 2.8. Characterisation of the anti-amylase 2, bleeds from the rabbit 1 (A) above were used for the second characterisation. The antisera are small, large and final bleeds. 1: wild type potato, 2, 3, and 4 are extracts of transgenic potatoes producing α -amylase and are undiluted, 3 and 10 fold diluted respectively. Two dilutions of the antisera were used; 1/1000 and 1/5000 respectively.

Effect of amino acid substitution on amylase properties

Having produced and characterised the barley α -amylase antibody, as was discussed in the aims section the next step was to investigate the effects of glycosylation on α -amylase. In the next section, results on the transient expression of the standard barley α -amylase and its different derivatives will be discussed.

The unpublished work in the host laboratory has suggested that the low activity obtained with the recombinant α -amylase fused to calreticulin may have been the effect of protein glycosylation. In order to test this hypothesis, a glycan mutant of the α -amylase was generated by site-directed mutagenesis. This involved a point mutation on the barley α -amylase where a single amino acid substitution that is asparagine residue was mutated to serine (N372-S372). Plasmids encoding either wild type α -amylase or the glycan mutant were then expressed transiently in the protoplast. The activities of the proteins produced 24 hours after electroporation were then measured in both the culture medium and the cell. Figure 2.9 shows the amylase activity in the medium, cells, and total activity; and secretion index which is a ratio of medium to cell activities. Although lower amylase activities were obtained with the extract of wild type enzyme compared to the mutant, comparable secretion indices were observed with the two constructs. Variability often exists due to differences in the plasmid DNA preparation, therefore it could not be concluded that increased protein synthesis was causally related to the point-mutation of the glycosylation site.

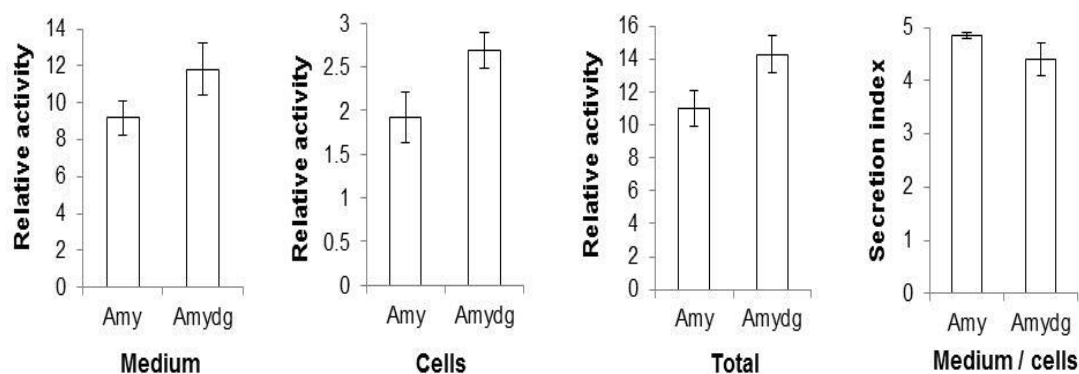


Figure 2.9. Comparison of amylase and its delta glycan mutant, the properties of the two proteins are shown. The delta-glycan form has a point mutation where an amino acid asparagine is replaced by serine (N372-S-372). It shows the amylase activities in the medium, cells, and total. Comparable secretion index was obtained with the two amylases. Error bars indicate standard deviation.

Expression of barley amylase & its mutant fused to GUS In planta

To facilitate a fair comparison between the wild type α -amylase and its glycan mutant in a quantitative manner, a cytosolic β -glucuronidase (GUS) expression construct was used as internal standard on the same plasmid to normalise transfection efficiency. The two constructs encoding either wild type α -amylase and / or its delta glycan derivative were therefore sub-cloned into a novel GUS reference plasmid (TR2-GUS-3'OCS-polylinker-3'NOS) developed by the host laboratory (D. Gershlick and J. Denecke, unpublished). The two recombinants were tested *in planta* by transient expression in the protoplasts. GUS activity was used to normalise the transfection efficiency of the two constructs. In practice, equivalent GUS activity was used to establish if there is any effect on the amylase activity and secretion. A comparable amylase to GUS ratio was obtained between the amylase and its delta glycan as shown in Figure 2.10, panel A.

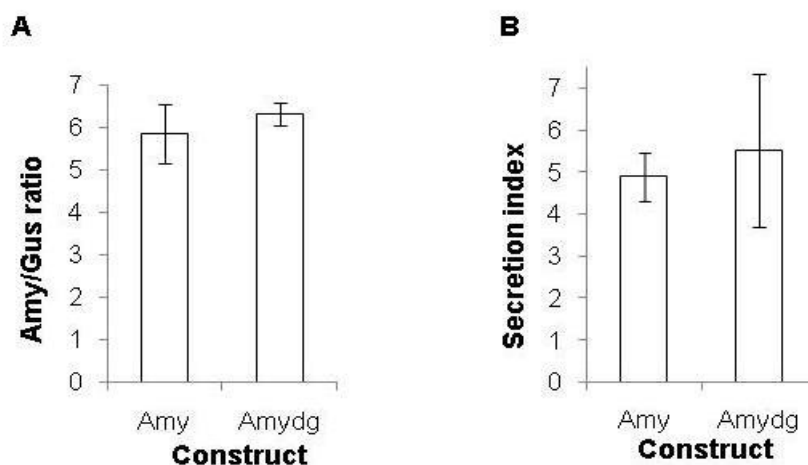


Figure 2.10. The secretion of GUS normalised amylases. Panel A shows the amylase to gus ratio of the α -amylase and its delta glycan mutant, comparable amylase to gus ratio was obtained. Panel B shows the secretion index of the two amylases indicating a comparable secretion of the two proteins. Error bars indicate standard deviation.

The secretion index (Figure 2.10, panel B) was comparable in both cases as seen before (Fig. 2.9). This means that the single amino acid substitution had no effect on the general properties of the barley α -

amylase. And since it was shown before that transient expression of wild type α -amylase was not accompanied with glycosylation, the point mutation is likely to be silent in this construct (Leborgne-Castel et al., 1999). But this does not rule out effects on glycosylation in other α -amylase fusion proteins (see below).

Effect of histidine tagging on amylase activity

Purification of the amylase from cells may not be practical due to difficulty in obtaining the pure protein as was discussed in section 1.8. In order to purify the α -amylase in a cascade refinery process leading to multiple products, an affinity purification protocol is the preferred option. Hence eight histidine residues were engineered before the stop codon using polymerase chain reaction. Binding of the engineered α -amylase via the histidine to a nickel column may lead to effective purification without loss of enzyme activity (Marusic et al., 2007). Moreover, presence of peptides that increase the distance between the consensus glycosylation site and the stop codon has been suggested to increase the efficiency of glycosylation (Nilsson and von Heijne, 2000). A delta glycan derivative of the histidine-tagged α -amylase was therefore generated to test this hypothesis. The two constructs were then sub-cloned into the β -glucuronidase (GUS) reference plasmid for comparative purposes.

In order to determine the functionality of the constructs and also the effect of the tagging on the α -amylase properties, the histidine-tagged α -amylase and its delta glycan derivative alongside positive controls were tested by transient expression in tobacco protoplasts. As shown in Figure 2.11 significantly lower amylase to gus ratios were obtained with the histidine-tagged amylases compared to the standard barley α -amylase regardless of the presence of a functional consensus site for N-linked glycosylation. This means that the addition of the eight histidine residues had a negative effect on the production of the α -amylase, either in terms of biosynthesis or specific activity.

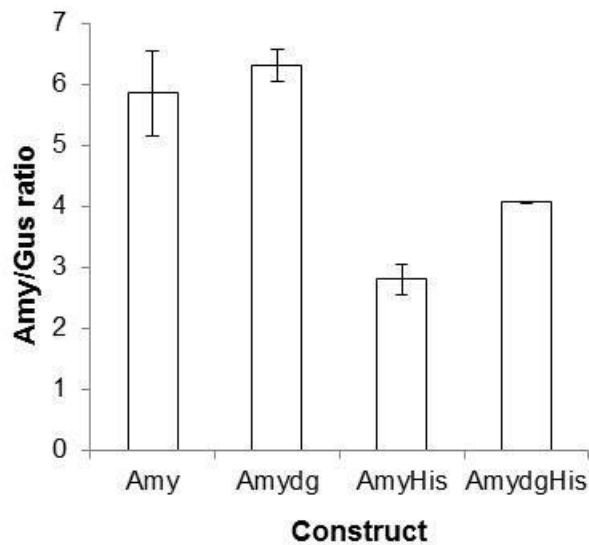


Figure 2.11. The effect of tagging on protein activity, the amylase to gus ratio of amylase, amylase delta glycan, amylase-his and amylase delta glycan his are shown. Comparable amylase to gus ratio was obtained with amylase and its delta glycan mutant however lower but significant ratio were obtained with the his-tagged amylase. Error bars indicate standard deviation.

The amylase secretion was also determined for the constructs and the result is shown in Figure 2.12. Even though lower amylase to gus ratio was obtained with the his-tagged amylase compared to the standard α -amylase (Fig 2.11), yet very high secretion index was observed with the amylase-His compared to the amylase control (Figure 2.12). This was not caused by higher levels in the medium but rather due to the very low intracellular amylase activity measured for the histidine tagged protein, leading to the very high secretion index (Fig 2.12).

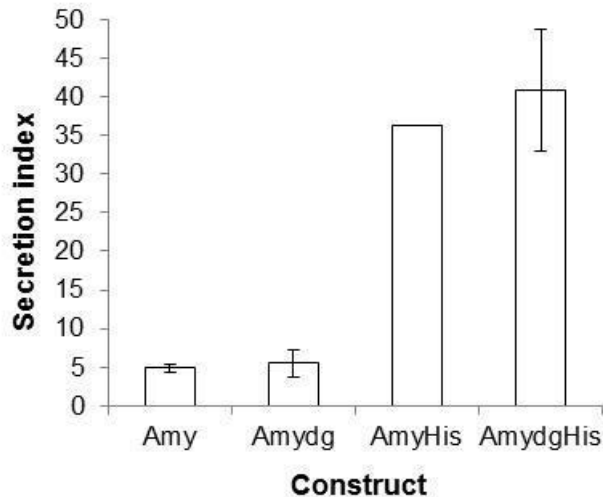


Figure 2.12. The effect of histidine tagging on amylase secretion. The secretion index of amylase, amylase delta glycan, amylase-his and amylase delta glycan his are shown. Approximately more than eight-fold factor in secretion index was obtained between the histidine tagged and non-tagged amylases. Error bars indicate standard deviation.

The positively charged histidine residues may bind to negatively charged compounds such as the phospholipids in the cell membrane, thus affecting the protein activity or possibly extractability. Since the histidine tagged α -amylase may remain bound to the cell membrane; therefore strong detergents may be required to the amylase from such bonds. In order to test this hypothesis, a 0.1 or 1% triton was used to extract the protein. Amylase activity that is comparable to what was initially measured was detected. This may imply that detergents do not compromise amylase activity. However, it did not explain the high secretion index.

Properties of amylase fused to the ER retention signal HDEL

The fact that some signal sequences such as HDEL affect protein (amylase) secretion has been established. However, it was unclear why the presence of a retention signal should cause specific shifts in the population of expression patterns in transgenic plants. In order to test if HDEL-mediated saturation of the retention machinery can cause inhibition of recombinant protein expression, further experiments were needed to allow better quantification. Two different forms of short α -

amylase fusions carrying retention signals were tested; one form is amylase fused to the tetrapeptide, histidine, aspartate, glutamate and leucine (HDEL) described earlier (Phillipson et al., 2001). The second form was amylase fused to octapeptide, EDDDHDEL which has glutamate and three aspartate residues in addition to the HDEL described earlier (kindly provided by S. Hanton). The aim of this fusion was to better mimic the biological context of the HDEL peptide by copying the acidic C-terminus of an ER chaperone BiP.

To test if the presence of glycan affects the properties of the HDEL and EDDDHDEL fusion proteins, the glycan mutants of the two amylases were generated by point-mutation (N372-S372) at the N-linked glycosylation site. The wild type amylases and mutants were then sub-cloned into the GUS reference plasmid vector to permit accurate control of transfection efficiency. In order to determine the effect of the different retention signals, the new recombinant plasmids alongside positive control, the barley α -amylase were then tested in transient expression in protoplasts. The total amylase to gus ratio was determined, and is shown in Figure 2.13. The data obtained indicate a significant reduction in the yield of α -amylase fusion carrying a retention signal. The result may imply that the retention signal affects either the synthesis rate or the activity of the α -amylase. This effect was not shown before but correlates well with the observed changes in expression population in the transgenic plants, indicating a loss of over-producers from the population (J. An and J. Denecke, unpublished).

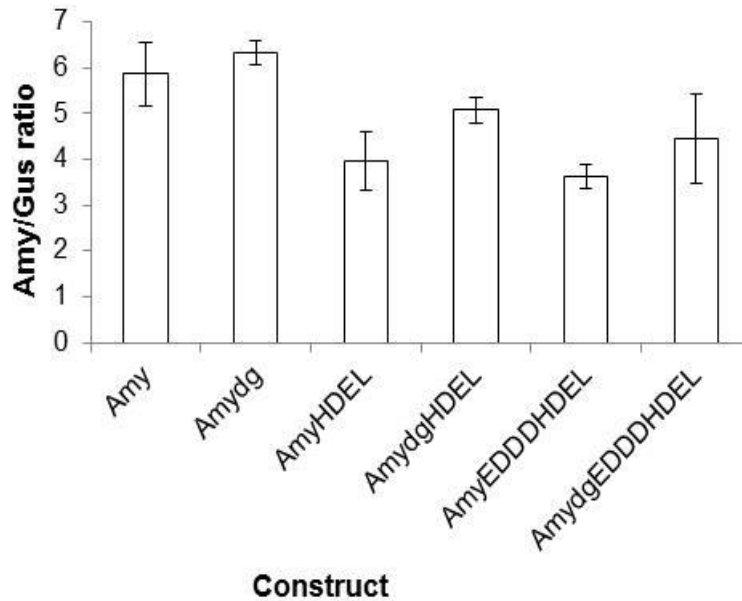


Figure 2.13. The amylase to gus ratio of shorter fusions. Different amylase fusions; amylase-HDEL, amylase-EDDDHDEL and their delta glycan derivatives were compared for protein activity. Comparable amylase to gus ratio was obtained with all the construct. Slightly lower activities were obtained with amylase-HDEL and the EDDDHDEL. Error bars indicate standard deviation.

In order to determine the retention efficiency of the different α -amylases, the secretion index of the amylases was determined. As shown in Figure 2.14, panel A amylase-HDEL and the EDDDHDEL derivative exhibit a strongly reduced secretion index compared to the standard secreted barley α -amylase. The secretion index of the amylase fusions are not background but they are measurable values as shown in Figure 2.14, panel B. It is also observed that the slightly longer fusion shows a further reduction in secretion compared to the tetrapeptide fusion alone, but this is a marginal difference. This may indicate that the EDDDHDEL peptide displays the HDEL signal better in its natural context.

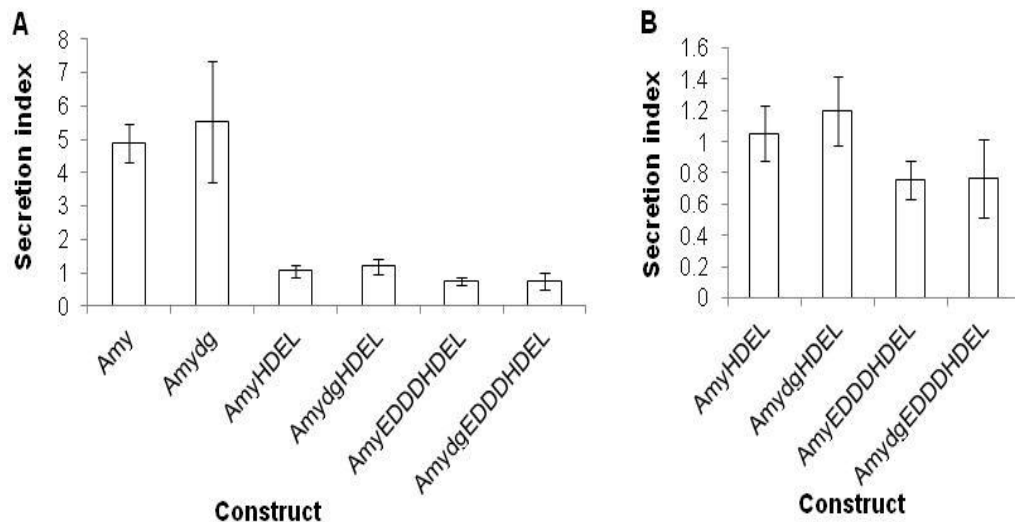


Figure 2.14. The secretion of short amylase fusions. Panel A shows secretion index of amylase and its fusions. Panel B shows secretion index of amy-HDEL compared to the amylase-EDDDHDEL fusion. High but comparable secretion index was obtained with amylase and its delta glycan. Very low secretions were obtained with the fusion constructs compared to the control. Error bars indicate standard deviation.

Testing the glycosylation of amylase fusions in leaf cells after *Agrobacterium* infiltration

Results so far indicate that HDEL tagging as well as histidine tagging reduced α -amylase expression relative to the internal marker GUS present on the same plasmid. To test if this effect may be due to glycosylation, samples were tested by western blotting. However since low amount of recombinant proteins is produced in protoplast transfection, the recombinant α -amylase fusions were below the detection limit of the western blot procedure.

To increase recombinant protein levels in plants, naked DNA transfer in protoplast was replaced by *Agrobacterium*-mediated gene transfer in infiltrated leaf sections, as the method results in transformation of the majority of cells in an infiltrated region. For this purpose, the new recombinant expression constructs yielding amydg, amyHis, amyHisDg, amyHDEL Dg, amyEDDDHDEL and amyEDDDHDELDg were subcloned in plant expression vectors and transformed in *Agrobacterium*. Amy and amyHDEL were already available (Phillipson et al., 2001).

Tobacco plants were infiltrated with the amylase constructs and incubated for 72 hours. The total amylase activity was determined in all leaf extracts and found to be much higher compared to the protoplast expression experiment as shown in Table 2.1.

Table 2.1 Comparison of amylase activity

Constructs	Protoplast	Infiltration
Amy	25.87	16933
Amydg	24.67	9833
AmyHis	3.93	1660
AmydgHis	3.47	6367
AmyHDEL	16.35	9207
AmydgHDEL	23.00	16327
AmyEDDDHDEL	7.90	12967
AmydgEDDDHEL	7.35	9753

In order to compare the different recombinant proteins directly, equal amylase activities from the infiltrated samples were loaded on a denaturing protein gel for western blot analysis. Figure 2.15 shows the different α -amylases detected using the anti-barley amylase antiserum. Interestingly, the presence of a glycan was only noticeable by the double band detected with the α -amylase fused to eight histidines (Fig 2.15, lane 4). The upper band was absent in the His-tagged fusion carrying the point-mutation in the N-glycosylation consensus site (Fig. 2.15, lane 5). The barley α -amylase and other short fusions such as amylase fused to HDEL and EDDDHDEL did not show evidence for glycosylation. It is possible that eight histidine at the C-terminus cause sufficient delay in protein synthesis to give the glycosylation machinery the opportunity to act on the nascent chain (Nilsson and von Heijne, 2000). But it is not the length per-se but rather the nature of the amino acid that causes the delay.

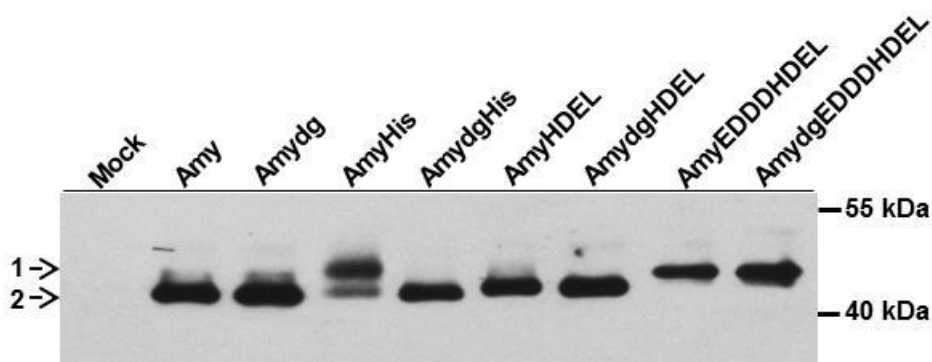


Figure 2.15. Effect of short fusions amylose glycosylation. The western blot shows properties of amylose and its short fusions. – is the negative control, are amylose and its delta glycan; Amylose-His and its delta glycan, and amylose-HDEL and its delta glycan, and amylose-EDDDHDEL and its delta glycan form. The arrows represent the different protein bands, the upper band (1) indicate the glycosylated high mannose protein while the lower band (2) is non-glycosylated protein with lower molecular weight.

Glycosylation of his-tagged amylose and amylose HDEL in stable transformation

Although α -amylose and short amylose fusions such as amylose HDEL are not glycosylated in protoplasts or infiltrated leaf epidermis cells, it cannot be ruled out that glycosylation may yet take place in transgenic plants. In order to test the glycosylation of amyHDEL and the effect on protein activities, the construct alongside a positive control amy-his which is glycosylated in transient expression (Fig. 2.15) were used to generate stable transgenic plants via leaf disc method (Deroles and Gardner, 1988; vanderGraaff and Hooykaas, 1996). After, successful regeneration of plants, leaves from the transgenics was used to extract proteins. Amylose activity was determined and significant activities were obtained. Equal enzymatic activities were loaded on the gel, Figure 2.16 shows a western blot of amy-his and amyHDEL with their respective delta glycan derivatives. It was evident from the Figure that the amylose tagged with histidine (lane 2) and amylose fused to HDEL (lane 4) were glycosylated in the transgenic plants. This means that the glycosylation machinery is affected by the expression condition because as shown in Figure 2.15, amy-HDEL was not glycosylated in a transient leaf

infiltration assay. Both lanes 2 and 4 showed double bands, the upper of which represents the glycosylated band as it is absent on lanes 3 and 5 displaying the glycan mutants. The glycosylation of the amylase-his was more pronounced in the transgenic than in transient this is evident by the increase in the intensity of the higher molecular weight high mannose protein band compared to the non-glycan band when compared to the pattern shown in Figure 2.15. Since significant enzyme activity was obtained with the plants compared to the delta glycan form this may imply that the glycosylation did not affect the stability and functionality of the protein.

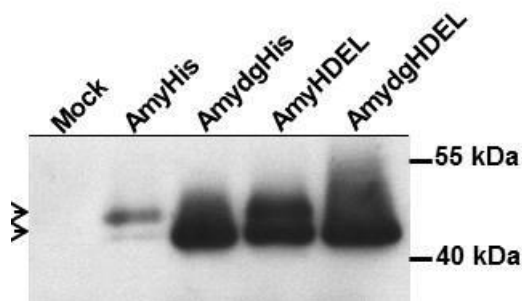
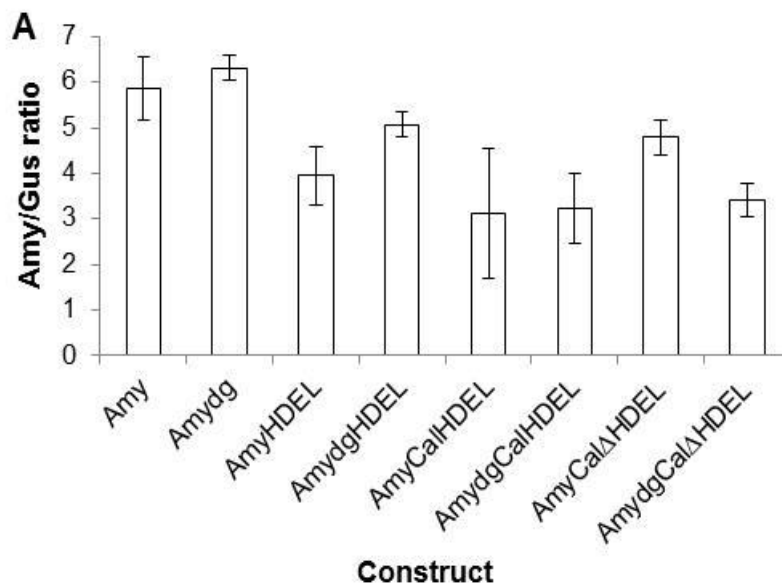


Figure 2.16. Stably expressed amylases; the western blot shows the properties of short amylase fusions. The samples tested are mock (negative control), amylase-his and its delta glycan; and amylase-HDEL and its delta glycan. The arrows represent the different protein bands, the upper band indicate the glycosylated high mannose protein while the lower band is non-glycosylated protein with lower molecular weight.

The effect of calreticulin fusion on α -amylase properties

Having established the effect of short peptide fusions on amylase properties that includes activity and secretion, it was necessary to determine the effect of larger protein fusions that were earlier shown to cause more dramatic reduction in α -amylase yield in transgenic plants (J. An and J. Denecke, unpublished). Therefore, the two fusions of α -amylase with the acidic C-terminus calreticulin (Fig. 2.17, panel B) with and without HDEL were chosen and their glycan mutants were generated by replacing asparagine with serine at the consensus N-glycosylation site (N372-S372) for comparison. The constructs were then sub-cloned in the GUS reference plasmid for quantification. The constructs alongside controls which are α -amylase and amylase-HDEL

were tested in electroporated tobacco protoplasts. The GUS activity was measured and normalised to establish the yield of the various fusions relative to the internal standard. Figure 2.17(A) shows the amylase to gus ratio of the different constructs. Significantly lower amylase to gus ratios were obtained with the calreticulin fusion constructs compared to the α -amylase and amylase-HDEL. Interestingly, the calreticulin fusion lacking the HDEL signal did not show increased amy / Gus ratios but continued to yield lower levels compared to α -amylase alone. This suggests that for these longer fusions, the peptide itself rather than the HDEL sequence was responsible for the reduction in amylase activity.



B SKAAPADSDAEEDDDADDDSDDADDKSESKDDEA

Figure 2.17. The protein properties of the amylase-calreticulin.. Panel A shows the amylase to gus ratio of standard amylase, amylase-HDEL, amylase-calreticulin, with and without HDEL, and their delta glycan derivatives. Comparable amylase to gus ratio was obtained with the constructs. The panel B shows the calreticulin protein sequence. Error bars indicate standard deviation.

In order to determine the effect of the calreticulin fusions on α -amylase retention, the secretion index was determined. Figure 2.18 shows the secretion indices of the calreticulin fusions compared to the amylase and amylase-HDEL. Generally, lower secretion index is exhibited by the HDEL compared to the secreted amylase (Fig. 2.18). However, the

secretion index of the amylase-calreticulin with HDEL was much lower than that of the amylase and amylase-HDEL (Figure 2.18), and even lower than EDDDHDEL (see Fig 2.14). On the other hand, the amylase fused to calreticulin without the HDEL signal showed comparable but higher secretion index than the amy-HDEL (Figure 2.18), but more than three-fold lower than the secreted α -amylase. This is interesting and may explain the peculiar role of the acidic calreticulin C-terminus in ER retention. The result may explain the fact that calreticulin exhibits a HDEL independent retention property as was suggested earlier (Pagny et al., 2000). These observations are independent of the presence of an N-linked glycan consensus site (Figure 2.18). However, the glycosylation status of the calreticulin fusion will have to be tested directly.

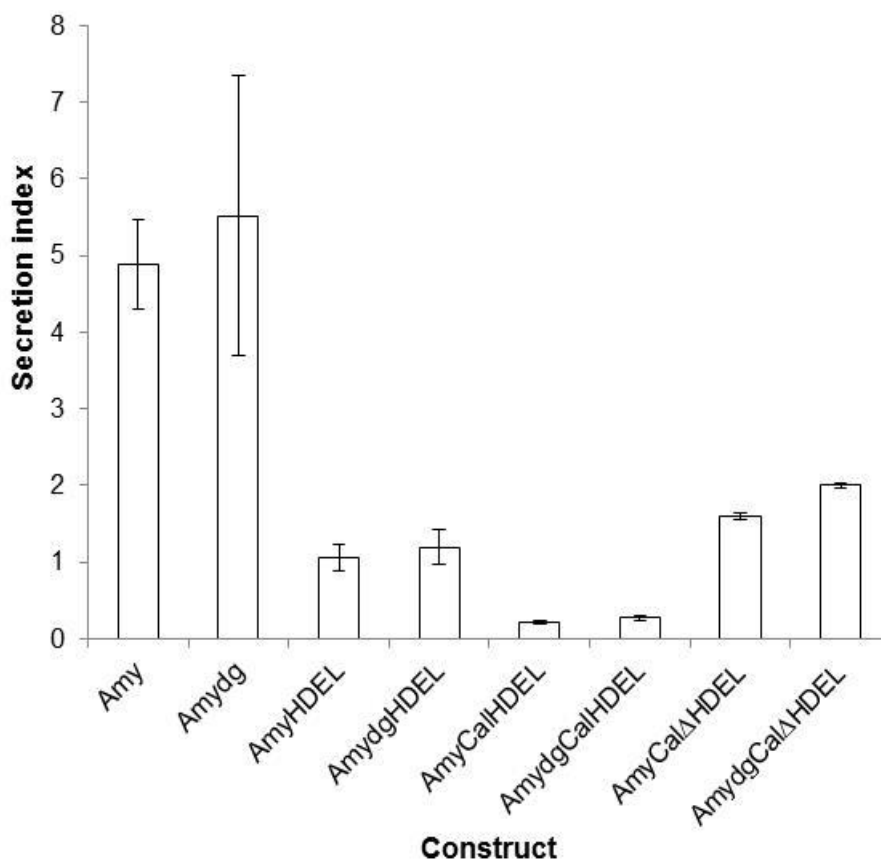


Figure 2.18. The retention of amylase fused to calreticulin, the secretion index of amylase, amylase-HDEL and amylase-calreticulin, with and without HDEL, and their delta glycan derivatives are shown. Significantly lower secretion index were obtained with the different fusions compared to the standard secreted amylase. Error bars indicate standard deviations.

Effect of calreticulin fusion on amylase glycosylation

Previous work in the host laboratory has shown that recombinant protein activity yields of α -amylase calreticulin fusions were much lower than those obtained for α -amylase or α -amylase-HDEL in transgenic crops, which was in contrast to observations in electroporated protoplasts where activities of α -amylase or α -amylase-HDEL were more comparable to those of the calreticulin fusions (Figure 2.17). Therefore, it was necessary to test the glycosylation status of the calreticulin fusions. In order to investigate the influence of the two larger protein fusions on α -amylase glycosylation, electroporated samples were tested by western blotting. However since low amount of proteins is produced in protoplast transfection, the recombinant α -amylase fusions were below the detection limit of the western blot procedure.

To increase recombinant protein levels in plants, naked DNA transfer in protoplast was replaced by *Agrobacterium*-mediated gene transfer in infiltrated leaf section, as the method results in transformation of the majority of cells in an infiltrated region. The constructs were sub-cloned into *Agrobacterium* vector for leaf-infiltration experiments. Amylase activities were measured; Table 2.2 is a comparison of the total activities in electroporated cells and infiltrated leaves, although this is random but explains the difference in the recovered amylase activities from the two expressions.

Table 2.2 Comparison of amylase activities

Constructs	Protoplast	Infiltration
Amy	25.87	16933
Amydg	24.67	9833
AmyCalHDEL	9.35	5800
AmydgCalHDEL	8.95	7107
AmyCal Δ HDEL	23.27	10893
AmydgCal Δ HDEL	13.57	5893

After measurement of amylase activities, equal activities of extracts from infiltrated samples were loaded on a denaturing protein gel for western blot analysis. Figure 2.19 is a western blot that shows the characteristic

pattern revealed by the different amylases. As shown in Figure 2.19, the presence of a glycan is clearly noticeable by the higher molecular weight band detected with the amylases fused to calreticulin with or without HDEL (Fig. 2.19; lanes 3 and 5) compared to the glycan mutant indicating that they are glycosylated. The upper band was absent in the calreticulin fusions with the point mutations in the N-glycosylation site (lane 4 and 6). However, the amylase fused to calreticulin lacking the HDEL signal (lane 4) showed the presence of four bands (1, 2, 3 and 4) compared to the amylase-calreticulin with HDEL (lane 6) which showed only the two higher bands. The bands marked 1 in lane 4 represents the glycan because it is absent in the glycan mutant (lane 5), while the band number 2 represents the non-glycan band because it is present in both the glycan and its mutant, the two upper bands (1 and 2) correspond to the pattern showed by the calreticulin-HDEL construct. The two lower bands marked number 3 and 4 are absent in the HDEL derivative of the calreticulin fusion thus are likely to be degradation products caused by escape of the protein from ER-Golgi since the construct lacks the ER retention signal HDEL. The HDEL derivative exhibits a higher mannose protein band (1) and a non-glycan band (2) which are bigger than the non-HDEL calreticulin due to the presence of additional four amino acids. The HDEL stops degradation in a post-ER compartment which makes the bands 3 and 4 of the lane 4 to disappear.

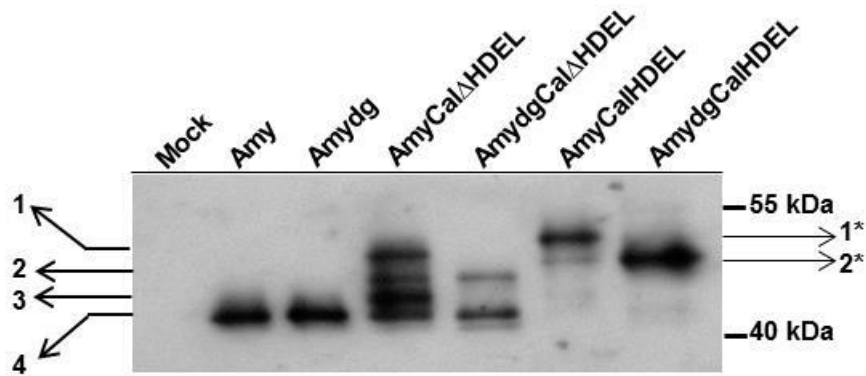


Figure 2.19. The glycosylation pattern of amylose fused to calreticulin. – is the negative control, the proteins tested include; amylose, amylose calreticulin-delta HDEL, and amylose calreticulin-HDEL and their glycan mutants respectively. The arrows indicate the different protein bands; the first and third arrows show the glycosylated high-mannose protein while the second and fourth arrows show the non-glycosylated protein. On the calreticulin HDEL derivative, the arrows 1* and 2* represent glycan and non-glycan bands since arrow no. 2 is present in the delta glycan derivative.

2.3 Discussion

Antibody generation

A major challenge facing recombinant protein production is optimum yield of the proteins (Sorensen and Mortensen, 2005a). The main variable is the nature of the recombinant protein itself. Expression of the recombinant proteins in *E. coli* may also be connected with the codon usage or the growth condition as well as the specific nature of the fusion to add as affinity tag (Trabbic-Carlson et al., 2004). Results presented here suggest that production of the GST-fused amylase in *E. coli* led to well defined high expression levels (Stofkohahn et al., 1992; Liu et al., 2006; Abhary et al., 2011). As shown in Figures 2.1 to 2.5, the yields of the recombinant α -amylase-GST fusion were high and not limiting factors. Although, tagging of proteins to GST has been suggested to decrease the formation of inclusion bodies (Kusnadi et al., 1997; Rabhi-Essafi et al., 2007; Deceglie et al., 2012), this form of tagging did not seem to enhance the protein solubility in *E. coli* in the case of α -amylase GST fusion. The results agree with previous report on purification of GST fusion microtubule associated protein (MAP 2) (Stofkohahn et al., 1992).

Reducing expression levels by lowering the concentration of the inducer IPTG to 1 mM (Deceglie et al., 2012) failed to improve solubilisation. Other conditions such as temperature of 10, 16, 28 and 37°C were explored for growth to yield lower expression levels but this also failed to yield soluble GST fusion. Lowering temperatures between 15 to 28°C have been reported to improve protein solubility; this is because usually overexpression causes aggregation. However, at temperatures below 10°C the growth of the bacteria may be hampered (Song et al., 2012). None of these attempts provided evidence for the presence of small quantities of soluble GST-amylase fusions that could be purified by affinity.

To yet enable the solubilisation of the amylases, different buffers were used. Detergents concentrations were varied from low to high to improve the extraction abilities of the buffers. Protein solubilisation was not achieved using phosphate-buffered saline. This is in agreement with what was reported previously on purification of a 180 kDa GST-fused RNA polymerase (Deceglie et al., 2012), although each protein has its peculiar properties. The more drastic extraction procedure using lysozyme treatment prior to extraction with the detergent-rich phaseolin buffer was also unsuccessful. The protein fusion remained firmly associated with the insoluble pellet (Figure 2.3).

All the different conditions attempted to solubilise the amylase GST failed; therefore the insolubility was used as a purification method instead. Using harsh extraction conditions, large amounts of contaminating proteins were removed as they were solubilised and retaining the insoluble fraction consequently led to a strong enrichment. The final step involved purifying the protein on SDS PAGE (Laemmli, 1970; Wilm et al., 1996), this gave very sharp well defined band of the insoluble GST-fusion protein which could be cut directly from the gel after coomassie staining. However, limitation of this strategy is risk of having multiple proteins of the same size although it was decided to accept this risk. The then obtained antigen yielded acceptable antiserum when tested in control plants and transgenic plants (Fig. 2.8). Therefore, the antibody can be used as a tool in blotting to recognise barley α -amylase and different amylase fusion proteins.

Epitope tagging of α -amylase changes the protein properties

The barley α -amylase is a naturally secreted protein which accumulates in the apoplast (Phillipson et al., 2001). However in order to purify the protein using affinity columns, it was tagged with eight histidine residues (Marusic et al., 2007). Lower protein activities were observed for the histidine tagged protein compared to the standard amylase (Figure 2.11). The fusion of the amylase to the histidine octapeptide may have also

affected the protein targeting (see Figure 2.12). The amylase tagged with histidine compared to the standard amylase and other short fusions such as EDDDHDEL exhibited lower activities in electroporated protoplasts as well as in infiltrated cells (see Table 2.1). The decrease in activity or protein yield may be attributed to be the effects of the eight positively charged histidine residues.

The secretion index of the amylase tagged with the histidine octapeptide was observed to be about 8-fold higher compared to the standard barley α -amylase (Figure 2.12). The behaviour of the histidine tagged α -amylase may indicate that there is possibility of faster secretion of the fusion protein which may be driven by the presence of the eight histidines. A second explanation for high secretion may be due to poor extraction of the protein from the cell as a result of histidine-binding to the phospholipids of the ER membrane, thus yielding lower cellular levels after extraction. Further research is required to understand the difference in secretion caused by the histidine octapeptide.

The glycosylation status of the amylase with and without the histidines revealed that α -amylase fused to histidine was glycosylated in transiently expressing leaf epidermis cells, while the un-tagged barley α -amylase was not (Figure 2.15). The glycosylation status was also revealed in stable transgenic plants (Figure 2.16). However the signal of the high molecular weight mannose band was found to be stronger in stable transgenic plants (Fig. 2.16) compared to the intensity in transiently expressed sample (Fig. 2.15). The glycosylation may be due to the fact that the histidine tagging slows down the translation rate prolonging the time that the nascent polypeptide remains associated with the translocation pore. This may also explain the overall yield loss. Histidine is a rare amino acid and the tRNA for its translation may not be abundant, if this hypothesis is correct it would slow down the translation as well as the co-translational translocation (Kane, 1995). The glycosylation of the amy-his may also be due to the long stretch of amino

acids that can delay the processing thereby enabling the glycosylation machinery to take effect (Kusnadi et al., 1997).

Evidence of HDEL-independent ER retention

The tetrapeptide HDEL has been described to lead to protein retention in plant cells (Denecke et al., 1992) thereby hampering the secretion of proteins, an observation that was originally shown by Munro and colleagues for KDEL in mammals (Munro and Pelham, 1987). The ability of HDEL to lead to retention of proteins in the ER was confirmed in this work (Figure 2.14), but interestingly a fusion of α -amylase to EDDDHDEL which is four amino acids longer than the HDEL was observed to be more efficiently retained (Figure 2.14, panel B). The higher retention of the EDDDHDEL derivative compared to the HDEL may be due to a better exposure of the HDEL retention signal.

Besides retention of proteins in the ER mediated by the signal HDEL, other form of retention may exist that are mediated by different mechanisms. Calreticulin is a protein that is retained in the ER and is characterised by a long acidic C-terminus (Fig. 2.17, panel B) preceding the HDEL sequence (Crofts et al., 1999). An amylase-HDEL calreticulin fusion containing 34 amino acids of the acidic C-terminus exhibited much higher retention (Fig. 2.18) than the HDEL and EDDDHDEL derivatives (Fig. 2.14). This may also be attributed to better exposure of the HDEL retention signal. However, the equivalent calreticulin fusion lacking the HDEL sequence also exhibited a significant cell retention compared to secreted α -amylase (Figure 2.18). The result implies that the calreticulin possess a HDEL-independent retention property. The ability of the calreticulin fusions to be retained independent of HDEL may be due to the calcium chelating ability of an acidic stretch of 40 amino acids near the C-terminus (Nilsson and von Heijne, 2000; Pagny et al., 2000). However, the presence of the HDEL was certainly significant as observed by the lack of proteolytic processing caused by the tetrapeptide (Fig. 2.19). The calreticulin fusions lacking the HDEL signal

exhibited protein degradation (Fig. 2.19 lanes 4 and 5) while the HDEL equivalent (Fig. 2.19, lanes 6 and 7) did not, thus signifying the strong role of the HDEL signal. This means that although HDEL-independent ER retention may assist in the process, the HDEL signal still plays a major role in calreticulin targeting confirming (Crofts et al., 1999) and refuting (Pagny et al., 2000) which stated that HDEL plays a minor role.

Glycosylation status does not affect protein properties

From the results obtained, it was observed that the substitution of asparagine at the consensus N-glycosylation site to serine (Gavel and von Heijne, 1990) did not alter overall α -amylase properties (see Figures 2.10, 2.11, and 2.12). This may not be surprising because a closely related α -amylase (NCBI accession no. CAX51373) from barley has N-S substitution (Radchuk et al., 2009). This implies that the substitution of asparagine with serine had no detectable effect on α -amylase activity and secretion. However, the effect on glycosylation was clearly demonstrated by comparing His tagged α -amylase with and without the mutation (Figures 2.15, and 2.16).

The α -amylase fused to the EDDDHDEL octapeptide as well as the HDEL derivative were not glycosylated in transient expressing leaf epidermal cells (Figure 2.15). Since the EDDDHDEL derivative has the same number of eight additional amino acids as the amylase histidine octapeptide fusion; glycosylation of the latter (Figure 2.15) must be specifically due to the properties of the histidine octapeptide. One possibility is a limitation of tRNAs for histidine which may slow down translation and translocation.

In contrast to untagged α -amylase, amy-HDEL and amy-EDDDHDEL fusions, the α -amylase fused to the acidic C-terminus of calreticulin was revealed to be glycosylated in infiltrated cells (Figure 2.19). Notably the size of the additional polypeptide at the amylase C-terminus is significantly larger. The glycosylation status was independent of the

presence of HDEL. The presence of peptides that increase the distance between the consensus glycosylation site and the stop codon has been suggested to increase the efficiency of glycosylation (Gavel and von Heijne, 1990; Nilsson and von Heijne, 2000). If this is the case, the long stretch of amino acids at the C-terminus of the protein may be responsible for the glycosylation.

The effect of expression system on protein properties

Transfection in the electroporated protoplasts involves naked DNA transfer into cells; there can be high level of expression due to the high number of plasmid copies which affects the yield and properties of the recombinant protein (Hadlington and Denecke, 1994). In infiltration of leaf cells which is *Agrobacterium* mediated, several copies of the genes are transferred which may also lead to high level of expression in the cells, however; some of the genes may be subsequently silenced at later stages (Caplan et al., 1983; Kapila et al., 1997). The consequent effect of the above is that it reduces the expression levels compared to the electroporated protoplasts (Bottanelli et al., 2012).

In this study, low quantity of proteins was obtained with the electroporated protoplasts that were below the detection limit of α -amylase antibody. In contrast, the yield of protein (α -amylase) activity was clearly demonstrated to be much higher using infiltration in leaf epidermal cells compared to electroporated protoplasts (see Tables 2.1 and 2.2). The observation was noticeable in all the amylase constructs tested, including the standard barley α -amylase, short fusions such as amyHis, amyHDEL and EDDDHDEL derivatives (Table 2.1) as well as the calreticulin fusions (Table 2.2). It is likely that the main reason for this discrepancy is the low percentage of transfected cells in protoplasts compared to that of infiltrated leaves. Therefore, individual cells in the leaves may have lower expression compared to the high expression in electroporated protoplasts but the number of transformed cells is so

much higher than the overall yield surpasses that of an electroporated protoplast suspension.

The expression system does not only affect the protein yield but also its properties such as glycosylation (see Figures 2.15 and 2.16). Whilst amylase-HDEL was not glycosylated in infiltrated leaves (Fig 2.15), the fusion protein was observed to be glycosylated in stable transgenics (Fig. 2.16). It is possible that stable regeneration removes a great deal of overexpressing lines as they may either be silenced or toxic so that they do not form normal plants (Kusnadi et al., 1997). In a transgenic plant 100% of the cells are transgenic, but the expression in each individual cell may be low enough to permit N-linked glycosylation.

Chapter 3

3 Starch saccharification

3.1 Introduction

Starch is one of the most abundant polysaccharides on earth, a glucose polymer containing only two types of covalent bonds to link the sugar monomers. It is the primary storage of photosynthetic free energy in all plants (Buleon et al., 1998; Stitt et al., 2010). Starch is also an attractive feedstock for bioethanol production because it is water insoluble and can be sedimented at low cost. It is a high energy molecule compared to simple sugar dissolved in water, the latter cannot be concentrated further without evaporating water (Denyer et al., 2001; Halford et al., 2011). Starch is easy to mobilise because it contains only α -1,4 and α -1,6 glycosidic bonds (Gerard et al., 2001; Buchholz and Seibel, 2008; Copeland et al., 2009). Industrial starch processing for biofuel production involves three basic steps; gelatinisation, liquefaction and saccharification (Duvernay et al., 2013).

Starch gelatinisation which is a process of hydrating the molecule in water is performed at high temperature depending on the starch properties. The aim of this is to disrupt the semi-crystalline starch structure, therefore making the glycosidic bonds accessible and susceptible to enzymatic action (Schuster et al., 2000; Duvernay et al., 2013). The liquefaction is the process of converting the gel to liquid and that involves reducing the viscosity of the starch molecule by producing shorter glucan chain that can be digested further. This is either achieved in the presence of inorganic acids or by treatment with α -amylase which has an endo-glycosidic hydrolase activity (Aggarwal et al., 2001; Chen and Zhang, 2012). Gelatinisation and liquefaction are often performed together in the industry at temperatures of 65°C and above using

thermostable enzymes. Either in combination with acid hydrolysis or not, and often using immobilised enzyme columns to boost thermo-tolerance and long-term stability of the enzyme (Betancur and Chel, 1997; Zhang et al., 2005; Buchholz and Seibel, 2008).

Research in the host laboratory has revealed that starch pre-treatment with acids at high temperature is a simple but effective strategy to increase the rate of starch hydrolysis both concerning α -1,4 and α -1,4 glycosidic bonds (Betancur and Chel, 1997; Kim et al., 2008b). The physico-chemical conditions chosen for starch hydrolysis affect protein stability and function therefore the optimum pH and temperature for enzyme action are used (Buchholz and Seibel, 2008). The solution is then cooled because addition of enzyme to the hot solution directly may result in the destruction of the protein. Since the solution is cooled to a low temperature for enzyme action retrogradation of the starch takes place leading to the formation of double and triple helices of amylose (Haralampu, 2000; Sajilata et al., 2006; Alvani et al., 2011). These helices cannot be found in nature and are highly resistant to heat-induced gelatinisation and enzymatic hydrolysis (Wu and Sarko, 1978; Miles et al., 1985). The solution is also neutralised by the addition of alkali setting the pH to neutral condition (Chen and Zhang, 2012). This is then subject to the action of the liquefying enzyme which act on the starch to generate linear and branched oligosaccharides (Carr et al., 1982; Beck and Ziegler, 1989). The subsequent saccharification takes place when the products obtained after liquefaction are subject to the action of the saccharifying enzyme, glucoamylase which cleaves both α -1,4 and α -1,6 glycosidic bond. The enzyme digests the glucans or dextrin from their non-reducing ends to release simple fermentable sugars such as maltose and glucose (Pazur and Ando, 1959; Carr et al., 1982).

Besides starch conversion using the enzymatic liquefaction and saccharification, the process is often performed exclusively based on

acid hydrolysis. The starch is treated at high temperatures above 100°C with mineral acids (Betancur and Chel, 1997). The advantage of acid is that it replaces two enzymes which has both α -1,4 and α -1,6 activity however; the acid catalysed hydrolysis is a totally random process that cannot be controlled. It also leads to generation of waste in the form of salts that are difficult to dispose (Soni et al., 2003; Duvernay et al., 2013).

High amounts of inorganic acids may lead to the destruction of the starch building blocks themselves; this may affect the integrity and quality of the products. The use of acid also generates hydroxyl-methylfurfurals that have been implicated to inhibit growth of yeast and also reduce fermentation efficiency (Tasic et al., 2009; Duvernay et al., 2013). In contrast, enzymatic hydrolysis of starch has lots of advantages. These include better and purer yields, more stable products. It also ensures a greater control and specificity of the reaction. In addition to the above advantages, the use of enzyme is simpler and more environmentally friendly way of sugar generation. Obtaining cheap enzymes is a key-aim to render the overall alcohol production process from starch economically viable and more sustainable (Satyanarayana et al., 2004; Buchholz and Seibel, 2008). However, when high starch concentrations above 10% are treated viscosity of the gelatinised starch is very high without liquefaction.

As was explained in the aims section of this thesis, initial work in the host laboratory has found that hydrolysis of concentrated starch suspension (30% weight / volume) can be performed by combination of high temperature using autoclaving with acid hydrolysis. This leads to gelatinisation and partial liquefaction, allowing the process to be completed by enzymes (Betancur and Chel, 1997). The strategy lowers the amount of enzyme needed in the overall process. Sulphuric acid was discovered to lead to the decomposition of starch molecule, however

hydrochloric acid was found to be efficient in starch hydrolysis at very low concentrations (J. An and J. Denecke, unpublished).

Here, it is shown that after two standard autoclave steps using low HCl levels, the degree of retrogradation is very low and glucoamylase saccharified a 30% starch solution to a DE value of 60%, hence complete conversion of starch to glucose was not obtained. A combination of the liquefying and saccharifying enzymes did not yield more glucose compared to the glucoamylase alone. A parallel approach that uses a combination of high temperature with acid was attempted, five repetitive autoclaving were used, a higher yield of glucose as evident from the DE value was obtained. The amount of the fermentable sugars was found to increase after each autoclave step. The pattern of sugars obtained on the hydrolysis was analysed using chromatography, glucose showed the highest peak. In addition two other fermentable sugars; maltose and maltotriose were released however non-fermentable saccharides were also observed. It can be concluded that though an incomplete starch conversion to glucose was realised, a 90% conversion may be obtained by using the standard autoclaving in combination with acid hydrolysis. Moreover, and the remaining 10% of the starch can be digested further with enzymes.

3.2 Results

Glucose standards

The dextrose equivalence (DE) value measures the amount of glucose produce from a given amount of starch therefore; it is used in assessing the quality of starch saccharification. There is no consensus reference for determining the DE value, researchers use different references (Schuster et al., 2000). In this project, it was assumed that 1g of starch liberates 1 g of glucose on saccharification. To determine the amount of glucose released on hydrolysis of starch a reference standard was required. This was to be used for quantification of glucose amounts in unknown samples. In order to have standards of known concentrations, glucose solutions of 0.1 – 10 mg/ml were prepared. The standard glucose oxidase-peroxidase method of estimating glucose amounts was used which is based on the oxidation of glucose in the presence of oxygen to gluconic acid and hydrogen peroxide. The peroxide in the presence 4-aminopyrine and phenol produces a quinoneimine which is indicated by the pink colour that is measured colorimetrically as absorbance. The optical densities were used to generate a standard curve. Figure 3.1 showed a glucose standard curve, the slope of the curve was used to derive a formula for estimating the amount of glucose in test samples based on the absorbance recorded. The curve in Figure 3.1 shows a progressive increase in the optical densities (absorbance) of the glucose standards. It revealed a nearly linear relationship between the absorbance and glucose concentrations for the range of concentration tested, suggesting that the reagents were not limiting under these conditions.

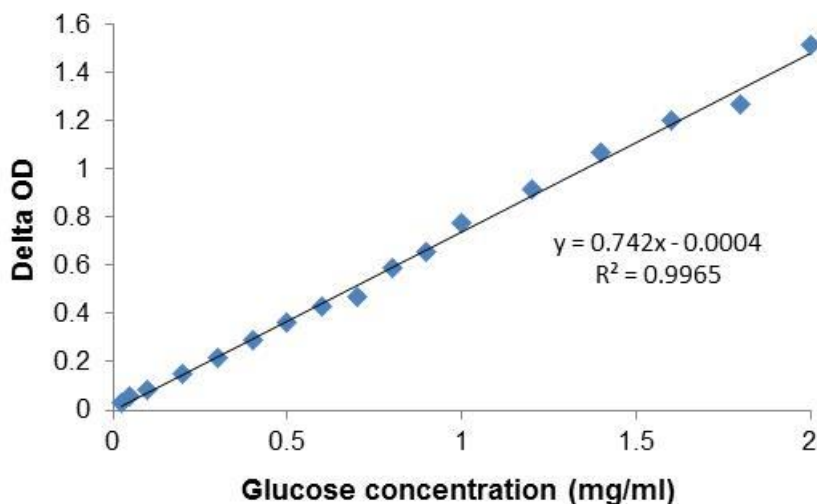


Figure 3.1. Glucose standard curve, the absorbance (optical density, OD) of glucose solutions was plotted against the mg/ml glucose used. The slope was used to derive a formula which gives the relationship between the optical density and concentration.

Glucoamylase catalysed hydrolysis of starch

Glucoamylase preparation

Aspergillus niger glucoamylase was obtained from Sigma, but the enzyme is mixed with glucose as a stabiliser. In order to use the enzyme for analytical saccharification, a purification step was required to remove the glucose therefore; gel filtration was chosen as a method of choice. Several fractions were collected and the glucose concentrations of the fractions were determined, the glucoamylase activities were also assayed. Figure 3.2 shows two curves of the glucose and glucoamylase concentrations of the gel filtrates however tailing was observed which may be as result of the sample addition. Fractions which contain no glucose but have significant enzyme concentrations (Fig 3.2, rectangle region) were considered to be suitable for analytical enzymatic digestion of hydrolysed starch.

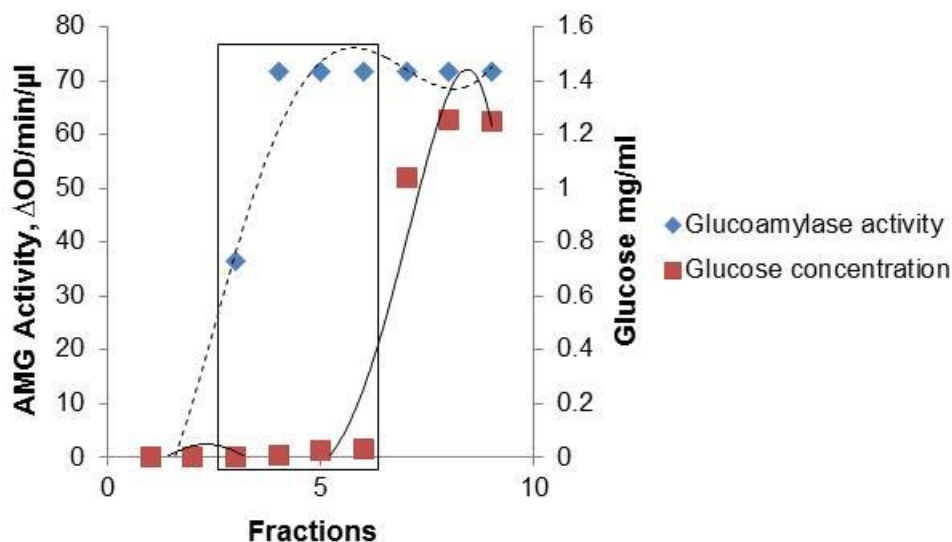


Figure 3.2. Properties of the glucoamylase gel filtrate. On the primary axis is the amyloglucosidase activity ($\Delta OD/min/\mu l$) while on the secondary axis is the glucose concentrations (mg/l). The Figure also illustrates the peak of the enzyme and glucose concentrations; four fractions exhibit significant enzyme activity with very little glucose contamination. The rectangle represents the region containing gel-filtrated glucoamylase fractions that were pooled and use for saccharification.

Saccharification

In order to attempt analytical saccharification a 30% starch solution was prepared; this is a more realistic concentration for industrial production of sugars than using a 1 or 0.1% starch as is typical for laboratory simulation (Pazur and Ando, 1959). Hydrochloric acid was added to a final concentration of 10mM, and an acid pre-treatment was carried out to enhance starch liquefaction and digestion. In order to mimic the industrial starch hydrolysis, the solution was autoclaved at 126°C for 20 minutes to liquefy the starch however; the solution became turbid after cooling to 55°C. The turbidity may indicate retrogradation of starch. As explained in the introduction, this aggregation decreases the efficiency of enzyme treatment (Wu and Sarko, 1978; Miles et al., 1985) therefore a second autoclave cycle was considered to enable full liquefaction. At the end of the cycles, the liquefied solution became significantly less turbid after cooling to 55°C. Next an equal concentration of 10mM sodium hydroxide was added to neutralise the acid. This is a pre-requisite prior

to enzyme treatment otherwise the structure of the enzyme protein can be disrupted by the acidic condition. If this happens, the enzyme may become non-functional and hence cannot catalyse the reaction.

The glucoamylase was added rapidly after autoclaving and neutralisation and incubated for a total of 8 hours with samples taken at different time intervals. Figure 3.3 shows the DE values of the saccharified solution at different time points. A progressive increase in the DE value was observed therefore increase in the amount of the glucose liberated as a function of time. The DE values obtained were below 70% this implies incomplete saccharification which may be due to some invisible retrogradation of starch that makes it impossible for the enzyme to penetrate the molecules. In order to obtain a higher degree of saccharification, improvements to the protocol became necessary; therefore one of the options considered was the use of a liquefying enzyme α -amylase after acid hydrolysis.

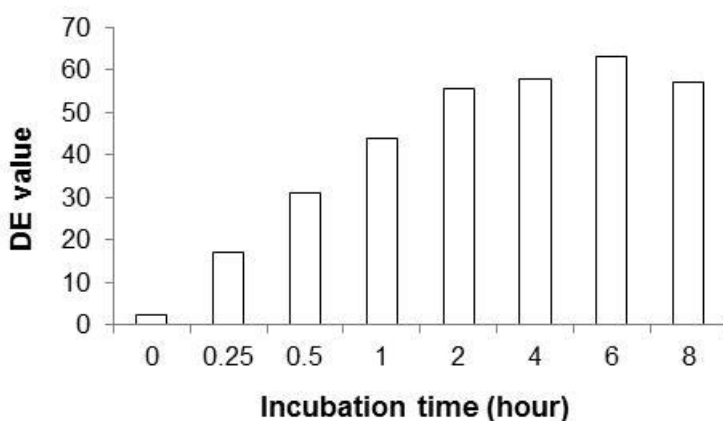


Figure 3.3. Dextrose equivalent of Glucoamylase treatment, the hydrolysed starch solution was initially treated with 10mM HCl. The starch was digested for a total of 8 hours. The DE value indicates the degree to which the starch is converted to glucose. It shows a progressive increase in the dextrose equivalent values.

Starch hydrolysis using glucoamylase and α -amylases from *Aspergillus oryzae*

A combination of two enzymes, α - and gluco-amylases was used to hydrolyse the starch and minimise retrogradation using the liquefying

ability of the α -amylase. A commercially available α -amylase from *Aspergillus oryzae* was obtained. A 30% starch solution was prepared and hydrochloric acid and the pre-treatment was identical to the previous experiment. The solution was cooled to 55°C which is an optimum temperature for the enzymes and neutralised, turbidity was not observed. The amyloglucosidase and α -amylase were added, and incubated at 55°C for 8 hours. The amount of glucose liberated by the combined action of the two enzymes was determined using a standard assay and sugar concentration was quantified using the glucose standard curve described above. Figure 3.4 shows the dextrose equivalence values of the different time points.

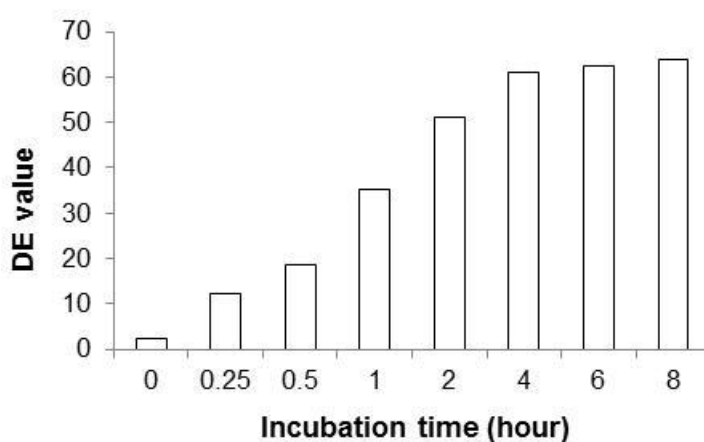


Figure 3.4. Dextrose equivalence of combined treatment with amyloglucosidase and α -amylase from *Aspergillus oryzae*. The acid hydrolysed starch solution was subject to the action of amyloglucosidase and α -amylase for a total of 8 hours. The DE values obtained showed a progressive increase in the amount of glucose released.

It was observed as shown in the Figure 3.4 that on addition of amyloglucosidase and α -amylase, glucose was released at a comparable rate as in the presence of glucoamylase alone (Figure 3.3). This implies that liquefying enzymes were not needed. It is possible that glucoamylase cannot cleave short fermentable sugars such as maltose and maltotriose which are not detected by the glucose assay.

Starch hydrolysis using glucoamylase and α -amylases from *Bacillus amyloquefeciens*

In the previous section the combination of glucoamylase with α -amylase from *A. oryzae* did not yield a complete saccharification to glucose. Here different amylase which is a heat stable α -amylase from *B. amyloquefeciens* will be used. The concentration of the starch solution and pre-treatments were as described in the previous section. The amyloglucosidase and α -amylase were added and incubated at 55°C because the glucoamylase cannot withstand high temperatures. Figure 3.5 shows the dextrose equivalence at the different time points.

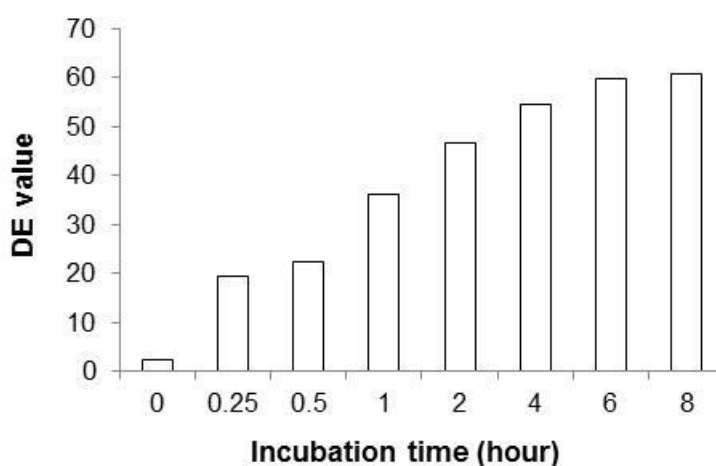


Figure 3.5. Dextrose equivalent of combined treatment with amyloglucosidase and α -amylase from *B. amyloquefeciens*. The hydrolysed starch solution was digested using amyloglucosidase and α -amylase respectively for a total of 8 hours. A progressive increase in the amount of glucose released was obtained.

The amount of glucose obtained was found to progressively increase with time (Figure 3.5), and comparable to the DE profiles shown in Figures 3.3 and 3.4. This implies a more efficient process is required to achieve a complete or near complete starch conversion to glucose.

Figure 3.6 is a combination of the dextrose equivalence of the three treatments that is saccharification using glucoamylase alone and combinations of two α -amylases from *A. oryzae* and *B.*

amyloquefeciens. From 15 minutes to 2 hours of starch digestion, a difference was observed with the three treatments (Figure 3.6), however, no significance difference was obtained afterwards. The common pattern between the different treatments was that they are all far from 100%.

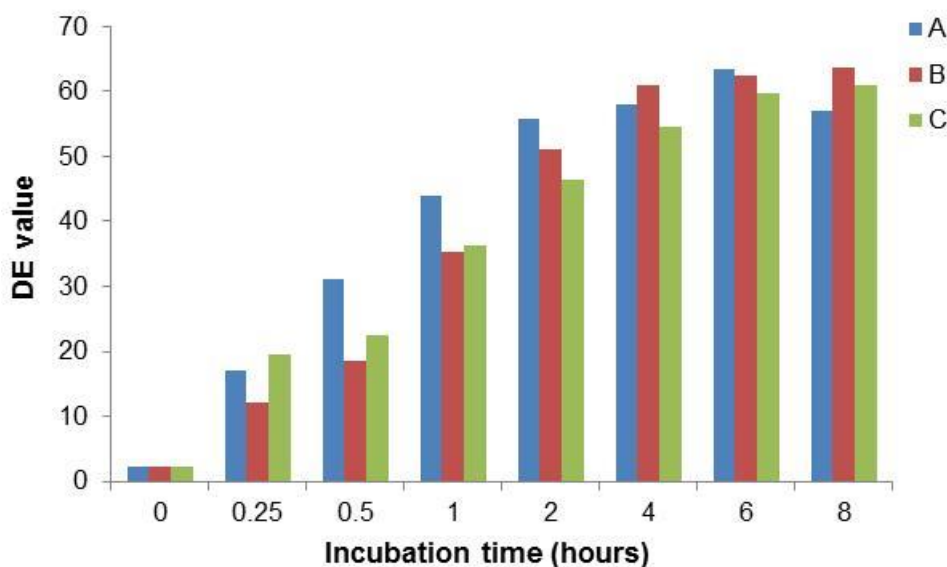


Figure 3.6. Dextrose equivalent of the combined treatment with amyloglucosidase and α -amylases from *A. oryzae* and *B. amyloquefeciens*. The dextrose equivalents of the three different treatments were compared indicating the amount of glucose liberated at the end of each treatment for 8 hours. Comparable amount of glucose was obtained in all the cases with no significant difference.

In conclusion, within the remit of this research complete hydrolysis of 30% could not be achieved using the pre-treatment and saccharifying enzyme. Similar results were obtained using pre-treatment and a combination of saccharifying and liquefying enzyme. As was discussed in the previous section, the reason for the inability of the enzymes to catalyse complete or near complete conversion of starch to sugars may be due to retrogradation. In the next section a different form of treatment using high temperatures with acid to liquefy and digest starch will be reported.

Effect of temperature and acid on starch hydrolysis

A pre-treatment at 126°C in the presence of hydrochloric acid to a final concentration of 10mM is well below the concentration that is often used in the industry but was considered for sustainability and environmental concerns. The fact that a double cycle of autoclaving resulted in much lower formation of turbidity suggested that prolonging in the time of acid hydrolysis may be a simple and cheap strategy. To test how much can be accomplished by acid hydrolysis only, five cycles of autoclaving at 126°C was used to monitor progressive saccharification of the starch. Samples were taken after each cycle and the glucose concentration was quantified and used to determine the dextrose equivalence (DE). Figure 3.7 shows a chart of the DE values obtained at end of each cycle of the five repetitive autoclaving. As shown in the Fig. 3.7, little amount of glucose was produced at the end of the cycle this implies that the second cycle was necessary. The rapid increase in the DE value means that acid hydrolysis is not linear over time but follows a complex kinetic curve. Moreover, with each progressive cycle, much more glucose was released than in the first autoclaving reaction. As shown in Figure 3.7, at the end of the repetitive autoclaving, a DE value of 70% was obtained which signifies that 30% of the starch was not digested to glucose. The DE value was significant but the saccharification was still incomplete after the five cycle of acid hydrolysis with 10mM HCl at 126°C.

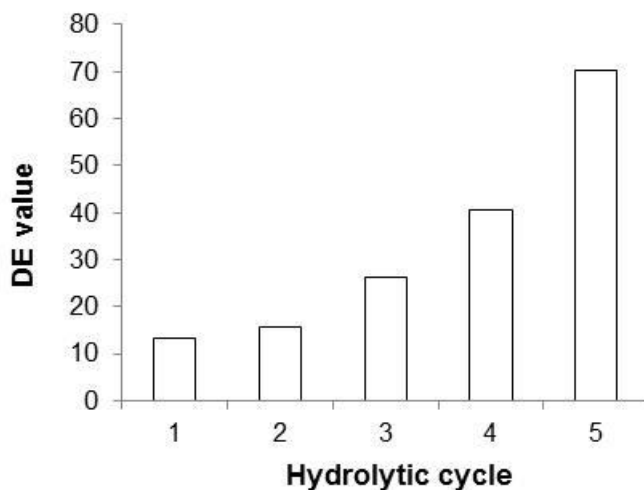


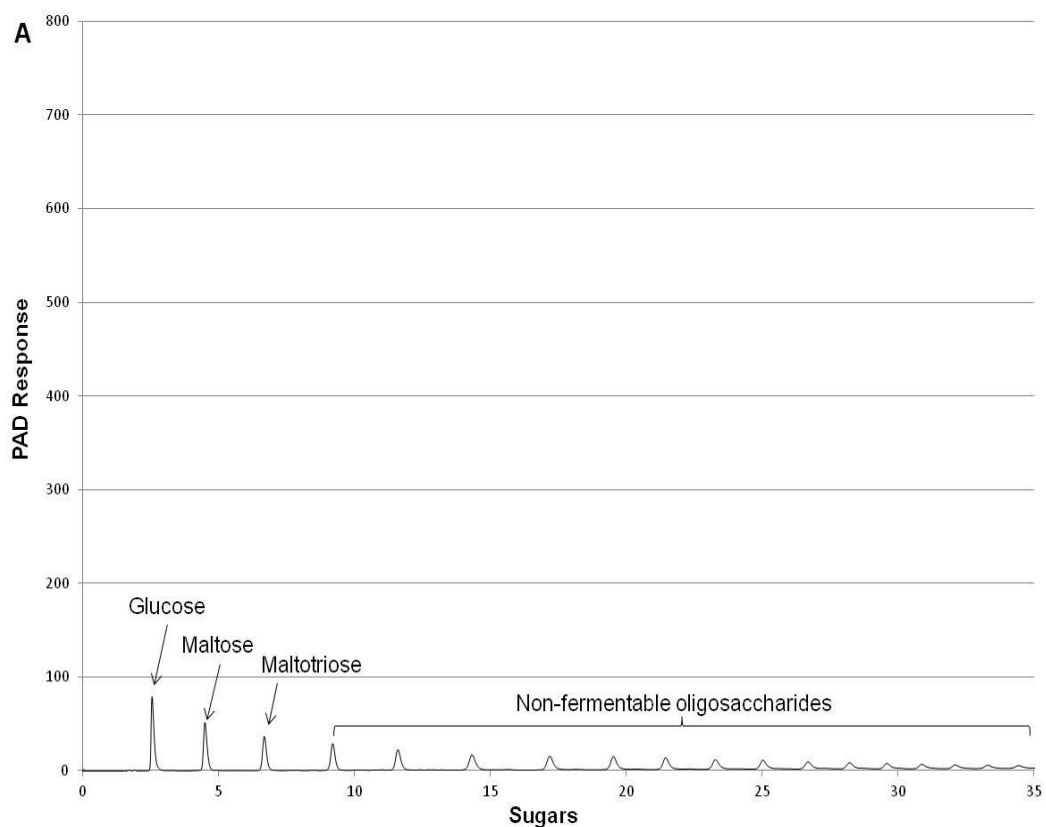
Figure 3.7. Dextrose equivalents of the hydrolytic products, 30% starch solution was hydrolysed using high temperature and mild acid treatment. 1st, 2nd, 3rd, 4th and 5th represent autoclave cycle that was used to mimic the high temperature liquefaction of starch. A significant increase in the DE value was obtained after the 5th cycle compared to the 1st cycle indicating an increase in the amount of glucose obtained.

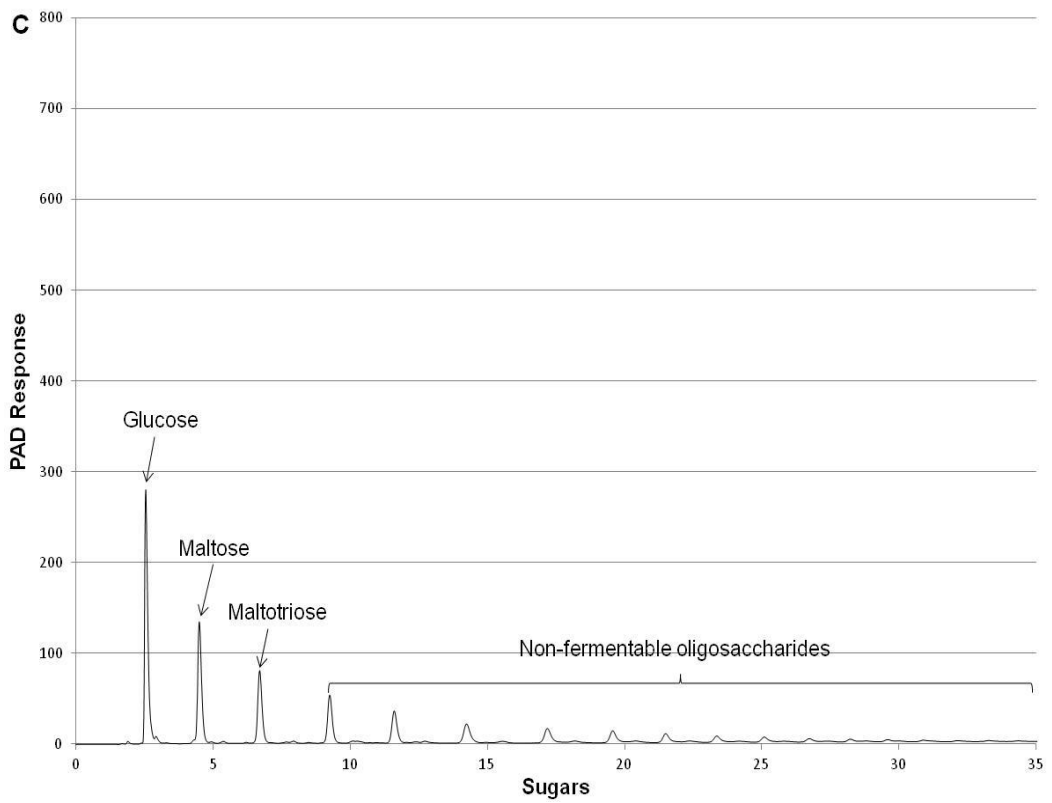
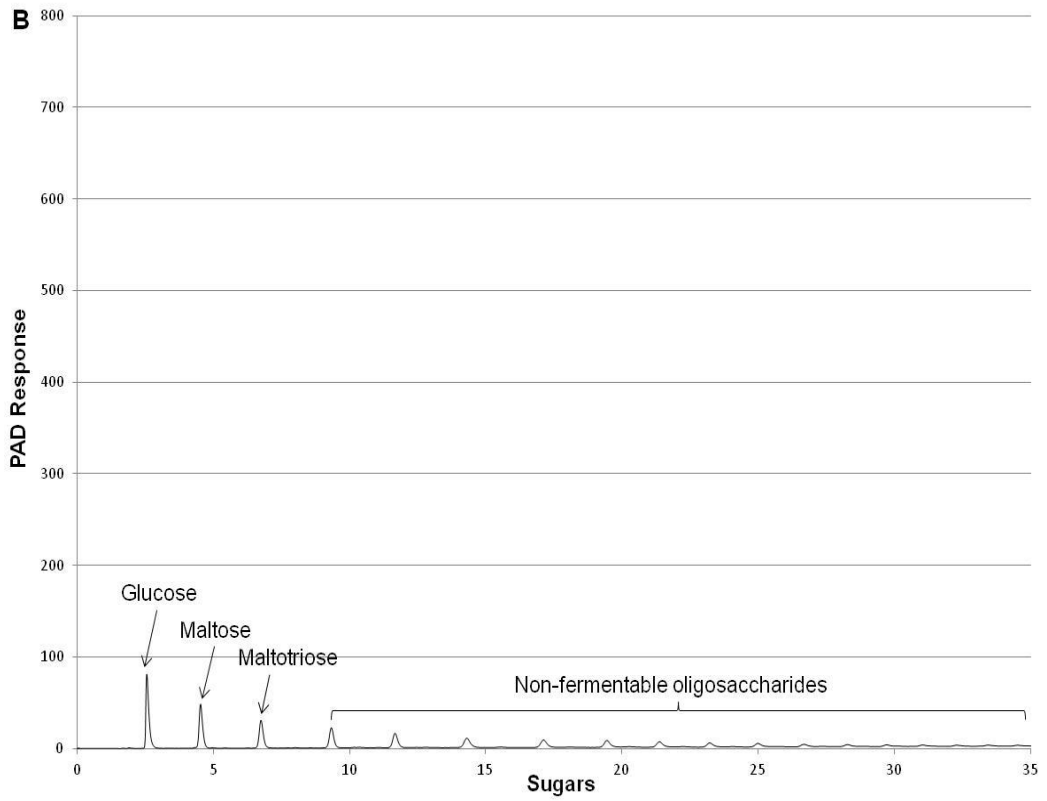
HPAE-PAD to reveal the sugars

The DE value can only report on the glucose obtained on hydrolysis of starch, in order to determine other sugars that are released from the digestion of the 30% starch at each cycle of the five repetitive autoclaving, column chromatography was considered as a method of choice. The samples were then subject to high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) to reveal the saccharides. Figure 3.8 shows chromatogram peaks indicating the different sugars and other larger chain sugars obtained on hydrolysis. As shown in Figure 3.8 (panel A) at the end of the first autoclave cycle, glucose, maltose, maltotriose, maltotetraose, maltopentose, maltohexose, maltoheptaose, and other longer sugars were released. Based on the pattern revealed by the peaks (Fig. 3.8, panel A), the amount of glucose released was higher than the other sugars. The system set-up uses a pre-column that filters and excludes longer chains therefore feed only the population of smaller chains on the real column. Moreover, among the sugars obtained in Figure 3.8, only three glucose, maltose and maltotriose are fermentable while the rest are

non-fermentable that cannot be readily converted to simpler sugars and subsequently ethanol.

The chromatograms indicated that the amount of glucose increase slightly after the second cycle (Fig 3.7, panel B) compared to the first cycle (3.7, panel A) this trend was also observed for the two sugars maltose and maltotriose. This may imply that some of these sugars are gradually being converted to smaller sugars. After the third cycle, the glucose, maltose and maltotriose peaks tripled (Figure 3.8, panel C) compared to the first and second cycles (Fig 3.8, panels A and B). In contrast, a slight increase in the longer chain sugars was shown. This means that the starch hydrolysis is progressing similarly some of the non-fermentable sugars are being converted to shorter saccharides.





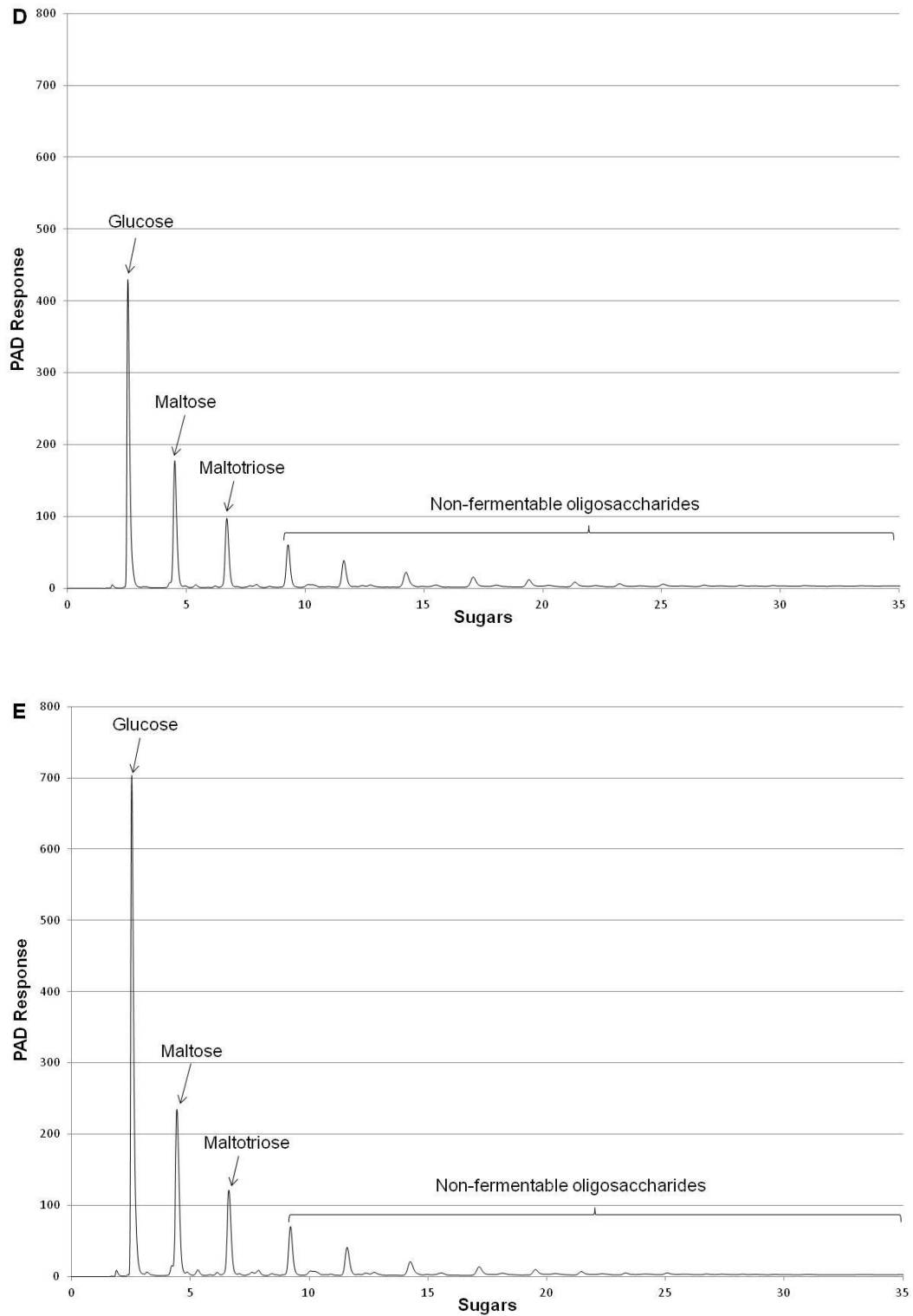


Figure 3.8. Glucose chromatogram showing the different sugars obtained on hydrolysis of a 30% starch solution. Panels A, B, C, D and E show the sugar profile of the products of the 1st, 2nd, 3rd, 4th and 5th hydrolytic cycles respectively. The first, 2nd, and 3rd peaks represents glucose, maltose, and maltotriose, while each peak show an increase in the chain length of the sugars with the 5th peak being maltopentose. Also the first three peaks are those of the fermentable sugars while the others are the saccharides that cannot be readily converted to ethanol.

In comparison to the third cycle, the fourth showed a further increase in the amount of glucose that was realised (Fig. 3.8, panel D), while the other sugars maltose and maltotriose do not increase at the same ratio. At this stage the amount of the molecules of higher than three sugars lengths does not increase. The sum of all the signals for the fermentable sugars increases in the last cycle of hydrolysis as shown in Figure 3.8, panel E. The three fermentable sugars, glucose, maltose and maltotriose increase with more than eight-fold compared to the initial peak (Fig. 3.8, panel A). This means that the repetitive hydrolysis was efficient in the conversion of starch to simple sugars. The results obtained with the chromatogram (Fig. 3.8) are in agreement with the DE values shown in Figure 3.7. It also shows that the DE value underestimates the percentage starch conversion to fermentable sugars as the glucose assay does not report on maltose and maltotriose. In conclusion, prolonging the time of acid hydrolysis is a simple and cost effective pre-treatment that strongly diminishes the need for expensive recombinant hydrolases.

3.3 Discussion

Glucose concentration and optical density reveal a linear relationship

Starch is a complex macromolecule that is made up of sugars, specifically glucose monomers and on digestion glucose is released or liberated as one of the products. Therefore, saccharification is a process of industrial and commercial importance for the production of glucose (Nigam and Singh, 1995; Buchholz and Seibel, 2008). In order to be able to quantify the amount of glucose released on starch saccharification, a relationship between the optical density (absorbance) and glucose concentration was derived using the standard curve (Fig. 3.1). The relationship between the absorbance and concentration was found to be linear. This implied that as sugar concentration increases, absorbance increases and vice versa and the standard curve can be used for quantification of unknown samples within the range of glucose concentration used to generate the graph.

Glucoamylase does not saccharify starch completely to glucose

Glucoamylase or amyloglucosidase is an enzyme that is used in industrial glucose production. It is an exo-amylase that acts on the non-reducing ends of starch polymer to release glucose (Pazur and Ando, 1959) (Figure. 3.3) but in order to function efficiently crystalline starch must be gelatinised using high temperature and liquefied using mild acidic conditions (Schuster et al., 2000). The uptake of water helps in breaking the bonds holding the molecules together (Schuster et al., 2000). Hence gelatinisation of starch provides the condition needed for a key step in enzymatic saccharification which is the adsorption of enzyme onto the granule (Aggarwal et al., 2001; Kim et al., 2008b). Acid hydrolysis reduces the viscosity and increases the number of non-reducing ends that are substrates for the glucoamylase (Betancur and Chel, 1997). Treatments of starch using amyloglucosidase yielded less than 70% glucose after 8 hours (Figure 3.3) of hydrolysis compared to

the total amount of starch however the reaction reaches peak after 4-5 hours and no further increase is expected. Though, the results are encouraging but higher saccharification efficiency was expected. Lower saccharification efficiency of about 13% starch conversion after 2.5 hours of glucoamylase treatment has been previously reported (Chen and Zhang, 2012). Retrogradation reduces the efficiency of saccharification because the starch forms double and triple helices that make the polymer inaccessible for enzyme action (Miles et al., 1985; Wu et al., 2006). The substrate specificity of the glucoamylase also affects the yield of glucose because short oligomers may be formed in the process of liquefaction that cannot be recognised by the enzyme (Shenoy et al., 1985).

In order to obtain a complete conversion of starch to glucose, the use of α -amylase in combination with glucoamylase was attempted. Alpha amylase catalyse the digestion of starch to dextrin (liquefaction) (Irving et al., 1999) while the glucoamylase mediates conversion of dextrin to glucose (Pazur and Ando, 1959; Schuster et al., 2000). The use of an endo-glucosidase was meant to test if retrogradation of long oligosaccharides was the limiting factor (Haralampu, 2000). The combination of amyloglucosidase with an α -amylase from *A. oryzae* yielded glucose levels (Figure 3.4) that were comparable to that obtained with glucoamylase alone (Fig. 3.3). The result is surprising, it was expected that the combination should increase the efficiency of hydrolysis. This implied that long-chain amylose retrogradation is probably not the cause for partial conversion to glucose.

The experiment was repeated with α -amylase from *B. amyloliquefeciens*, the obtained amount of glucose was lower than 70% (Fig. 3.5) and comparable to that of hydrolysis of starch with amyloglucosidase alone (Fig. 3.3), and a combination of glucoamylase with α -amylase from *A. oryzae* (Fig. 3.4). Therefore, the use of a different α -amylase did not improve the process. In summary, as was shown in Figure 3.6 the use of

glucoamylase or a combination of glucoamylase and α -amylase yielded comparable amount of glucose of 60%. It is possible that fermentable maltose and maltotriose are poor substrates for glucoamylase.

Acid catalysed hydrolysis of starch yielded higher amount of glucose compared to the enzyme catalysed reactions

To test if acid hydrolysis can be used alone to obtain good yields of fermentable sugars, five consecutive autoclaving steps were performed to measure progress of glucose release. The strategy used for the hydrolysis of starch was effective due to a progressive increase in the DE values which represents the amount of glucose release after each cycle. Comparing the first autoclave cycle to the last, the DE value showed five-fold increase. Therefore, the approach was able to liberate simple sugars that may be converted further to ethanol. Moreover, the dextrose equivalence was more than 70% yet this does not signify a complete or near complete saccharification. The obvious challenge in the hydrolytic process is obtaining an optimum yield of the desired product. In order to obtain a complete conversion of starch to glucose, higher concentration of acid is required. In a project where 1M and 2M HCl at 98°C were used to digest a lower percentage of starch for 8 hours; 80 and 94% dextrose recovery respectively was obtained (Tasic et al., 2009).

The chromatogram further revealed the type of sugars released and that the glucose release was not linear. After the initial saccharification, glucose, maltose and maltotriose which are fermentable sugars were recovered. In addition non-fermentable carbohydrates which are sugars of longer chain length were also obtained. With each further digestion cycle, an increase in the amount of the three fermentable sugars was observed. After the last cycle of hydrolysis, a more than eight fold increase in the amount of glucose compared to the first was obtained as revealed by the chromatogram. It is encouraging that at this stage, the amount of the non-fermentable sugar was very low compared to the

fermentable oligo-saccharides. Some other products that are too big to pass through the column might have bound to the pre-column.

In conclusion, the DE value underestimates the true conversion of starch to fermentable sugars as it does not report on the recovery of maltose and maltotriose. In addition, prolonged acid hydrolysis is likely to be a very cost-effective, yet efficient approach for starch saccharification. Remaining non-fermentable sugars are so low in abundance and polymer size that the risk for retrogradation is low. Furthermore, those non-fermentable sugars may be converted to fermentable sugars during the long fermentation process, if a transgenic yeast with the appropriate amylolytic enzyme is utilised. In nature, some plants express amylases that act at low temperatures to digest starch, these forms of enzymes can be explored so that starch saccharification can be performed at relatively low temperatures and with more efficiency.

Chapter 4

4 Scouting for new starch hydrolase with unique properties

4.1 Introduction

Starch is physiologically an attractive store of energy due to its compact nature and osmotically neutral properties. Besides oils and sugars; starch has thus been envisaged as a feedstock for bioethanol production though this has been underexplored with the exception of corn which is an energy inefficient crop (Slattery et al., 2000; Gray et al., 2006; Balat and Balat, 2009; Mussatto et al., 2010). However, many crop plants produce much larger quantities of starch per surface area and time have yet to be considered. Structurally, it is a macromolecule that consists of two glucose polymers which have different degree of branching, amylose and amylopectin (Buleon et al., 1998; Kossmann and Lloyd, 2000; Denyer et al., 2001; Keeling and Myers, 2010; Santelia et al., 2011).

Moreover, starch can also be classified into transitory starch, the type synthesized by higher plants in leaves during the day and degraded at night; and the so-called storage starch which represents reserves that are stored in seeds and tubers or other vegetative tissues (fruits and stems). Starch is found in almost all classes of crops. Tubers such as potato, cassava and yam have particularly high levels; cereals such as rice, maize, wheat, and fruits such as banana, plantain, apple, and pear also contain significant levels of this polymer (Nigam and Singh, 1995; Junior et al., 2006; Smith, 2008; Zeeman et al., 2010).

The hydrolysis of starch takes place in chloroplasts or amyloplasts and multiple enzymes are involved in this process (Lao et al., 1999; Tetlow et al., 2004; Smith et al., 2005; Zeeman et al., 2007a; Fettke et al., 2009;

Andriotis et al., 2010). These can be classified into hydrolases; endoamylases, exoamylases, debranching enzymes, and transferases. The endoamylase α -amylase hydrolyses α -1,4 glycosidic bonds in internal positions of amylose and amylopectin structures leading to the generation of smaller water soluble glucans (Smith et al., 2005; Yu et al., 2005; Kumari et al., 2010). Exoamylases such as β - and gluco-amylases cleave both α -1,4 and α -1,6 glycosidic bonds at the external part of starch molecule. Debranching enzymes, example isoamylase and pullulanase hydrolyse the α -1,6 glycosidic bonds while the transferase cut α -1,4 glycosidic bond of a donor and transfer part it to an acceptor molecule (van der Maarel et al., 2002). Of all these enzymes, α -amylase is considered as a key enzyme for industrial starch hydrolysis due to its endo-amylase activity (Beck and Ziegler, 1989; Asatsuma et al., 2005).

Alpha amylase (1,4- α -D-glucan-4-glucanohydrolase, EC 3.2.1.1) is a member of the glucosylhydrolase class-13 (Kuriki and Imanaka, 1999; Kumari et al., 2010). The protein is folded into three domains A, B and C. The enzyme is found in humans, animals, microbes, plants and the archaea (Tibbot et al., 2002; Robert et al., 2003). In plants such as barley, α -amylase may be produced and secreted by the aleurone cells or scutellum or both of seeds (Ranki and Sapanen, 1984; do Nascimento et al., 2006; Jeon et al., 2010). The synthesized protein is secreted into the endosperm of seeds. High activity of α -amylase has been reported during seed germination, indicating its role in starch mobilization in germinating seeds where starch reserves are used for energy (Kumari et al., 2010; Zeeman et al., 2010).

Alpha amylase is a multi-gene family of proteins; earlier classification was based on cereal α -amylases (Mitsui and Itoh, 1997; Janecek, 2002). However, a broader classification of plant α -amylases grouped the genes into three families. Family one α -amylase is found in cereals and seeds of dicot plants. They are secreted proteins with a signal peptide for entry into the endoplasmic reticulum. This family of enzyme has also been

described to be involved in the degradation of extracellular starch and can be found in microbes. The presence of this type of protein in seeds of higher plants may be due to the need of the enzyme to translocate across membranes to the specialised starch tissues such as the endosperm in cereals (Stanley et al., 2002; Stanley et al., 2005). The second family of alpha amylase consists of proteins that localise to the cytoplasm due to the absence of any characterised targeting peptide. It is found in leaves of monocots and dicot plants as well as gymnosperms. This group of enzymes degrade cytosolic α -glucan or heteroglycan (Stanley et al., 2002; Stanley et al., 2005). Family three α -amylases have a chloroplast transit peptide with a large N-terminal domain in addition to the α -amylase domain (Stanley et al., 2002; Stanley et al., 2005).

The semi-crystalline nature of starch necessitates that enzymes of starch degradation have some features for effective and strong binding to the starch molecule. This can be a specific substrate binding site in the catalytic domain of the enzyme or a carbohydrate binding module (CBM) or starch binding domain (SBD) (Rodriguez-Sanoja et al., 2005; Machovic and Janecek, 2006b; Chou et al., 2010). The CBMs are structural motifs that facilitate effective binding of the enzyme with the substrate (starch) leading to hydrolysis (Machovic and Janecek, 2006a; Glaring et al., 2011; Janecek et al., 2011). However, in the industry starch is gelatinised at high temperature such as 70°C, thus previous research efforts were mostly focused on identifying heat stable alpha amylase (Azad et al., 2009; Prakash and Jaiswal, 2010).

Other properties such as raw starch digesting abilities and specificity for low molecular weight oligosaccharides have not been explored extensively (Iefuji et al., 1996; Ueda et al., 2008). Microbes are able to breakdown starch at low temperatures indicating the presence of enzymes that digest crystalline starch (Nigam and Singh, 1995; Gupta et al., 2003). Similarly, ripening fruits are able to hydrolyse starch to soluble sugars at ambient temperatures; hence hydrolases may be expressed

that utilise crystalline starch as their substrate (Prasanna et al., 2007; Prinsi et al., 2011; Shiga et al., 2011). Thus, such plants may be considered as good models for the discovery of α -amylase with unique properties that may assist in low temperature starch degradation in industrial bioethanol production.

Plantain is a climacteric fruit that consists of high amount of crystalline and resistant starch when unripe. However, ripening take place in eight stages during which the starch is progressively converted into soluble sugars (Zhang et al., 2005; Prasanna et al., 2007; Soares et al., 2011) . This conversion is thought to be mediated by different hydrolases. One of these enzymes is α -amylase which has an endo-glycosidic activity to attach an intact starch granule (Hill and Aprees, 1994; Prabha and Bhagyalakshmi, 1998; Prakash and Jaiswal, 2010). Here it is shown that ripening plantain express a family three type of α -amylase. The protein consists of two distinct domains; an N-terminal domain of uncharacterised function and a C-terminal catalytic (α -amylase) domain.

4.2 Results

Extraction of plantain alpha amylase

In order to identify novel α -amylases from plantains, ripening plantain extracts were tested for endo-amylase activity using blocked para-nitrophenyl maltoheptaoside, a substrate that mimics starch. In the presence of excess glucoamylase paranitrophenyl is released after cleavage by α -amylase.

It was first necessary to establish a protein extraction protocol for water soluble proteins and test if enzyme activity can be measured so that subsequent fractions from chromatography runs can be tested for the presence of the desired hydrolase activity. This yielded significant alpha-amylase activity obtained from plantain extracts, permitting further purification techniques to be developed. It was observed that the α -amylase activity in plantains was highly dependent on the stage of ripening. Plantain ripening is a long process from a green plantain to an almost liquid fruit. Generally, higher activities and protein concentration were observed at later stages of ripening. However, analysis of ripe plantains still revealed enormous variation in enzyme activity that could not be correlated to a specific property. Therefore, a biochemical search for protein was hampered by the uncertainty of the yield in the starting material. Figure 4.1 shows the variability in α -amylase activity of different plantains. In addition, different extraction buffers were tested to compare the recovery of α -amylase activity from ripening plantains. The extract obtained using the standard low-pH alpha-amylase extraction buffer showed a greater enzyme activity compared to the pH neutral HEPES buffer (data not shown).

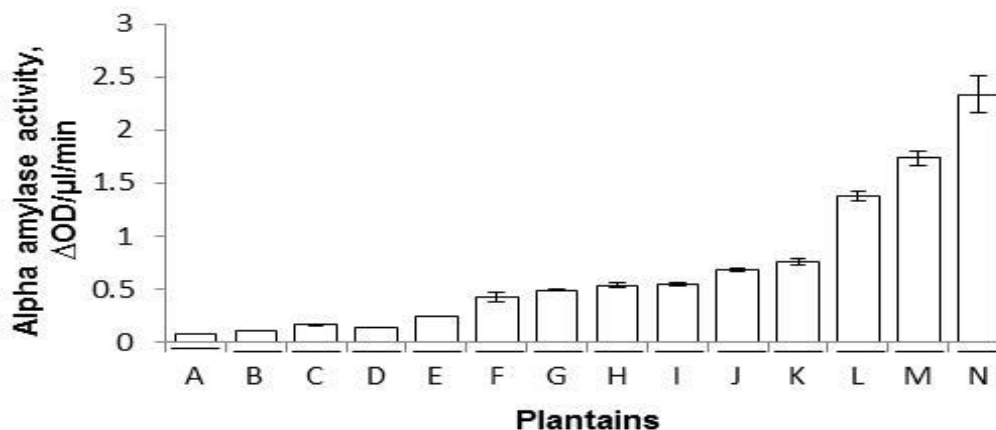


Figure 4.1. Plantains α -amylase activity profile. Soluble proteins were extracted from different ripened commercially available plantains that were fully ripened. Error bars indicate repetitions of the assays for individual plantains.

Ammonium sulphate fractionation of alpha amylases leads to strong enrichment of the desired activity

To enrich the extract for α -amylase and to separate proteins from soluble cell wall polysaccharides, proteins were subject to ammonium sulphate fractionation. The tests revealed that maximum α -amylase activity was recovered when precipitation was carried out at 30% ammonium sulphate on ice (Figure 4.2, panel A). Further increase in ammonium sulphate did not increase the recovered activity. This is encouraging as it could be used as a first step in protein purification prior to ion-exchange chromatography. Since most proteins precipitate at higher ammonium sulphate concentration, a 30% precipitate leads to a strong enrichment of the desired protein in a single step, and at the same time the volume is strongly reduced.

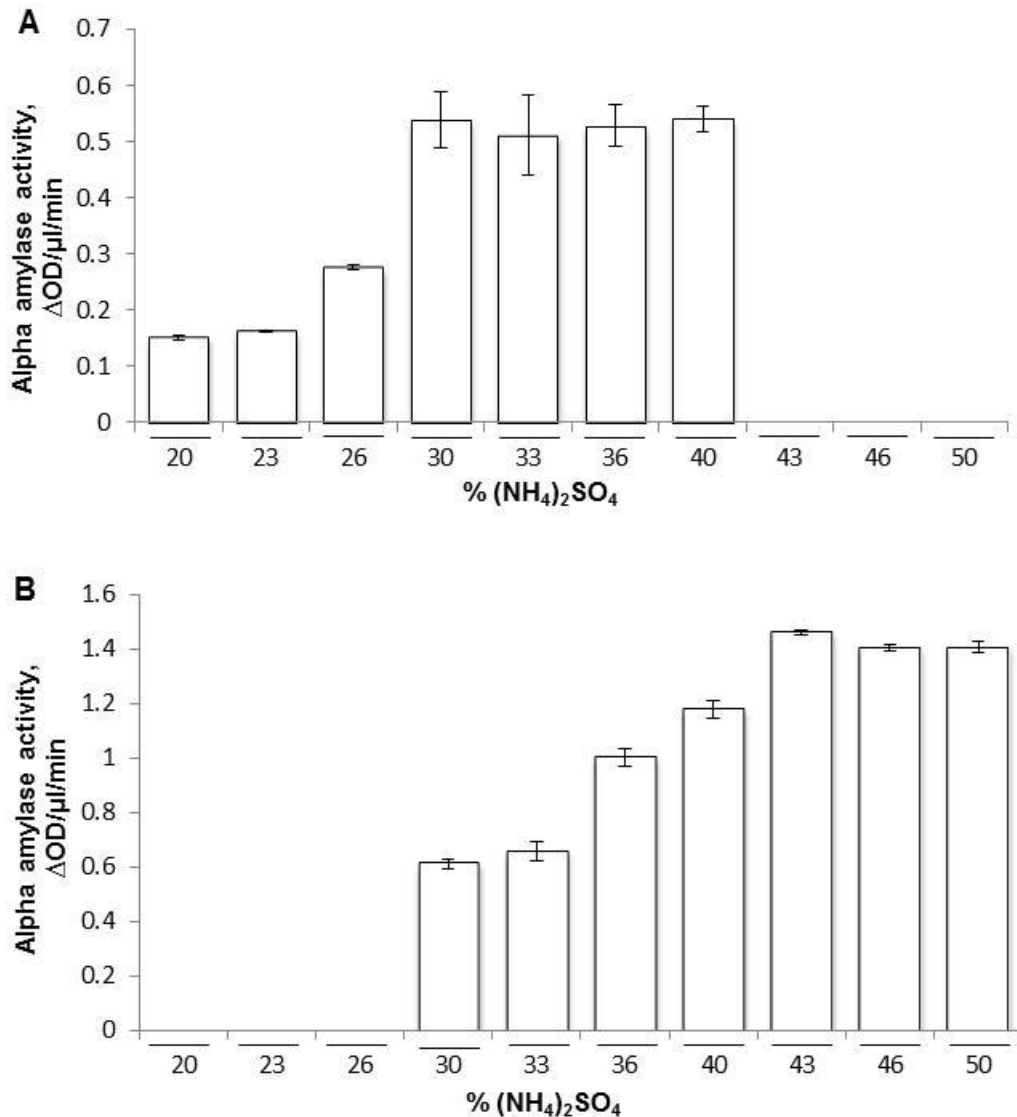


Figure 4.2. Enzyme activity and precipitation, the pattern of enzyme activity on fractionation of amylase at different percentages of ammonium sulphate are shown. Panel A shows the α -amylase activity obtained on fractionation of plantain α -amylase while panel B shows the behaviour of barley α -amylase on precipitation. Error bars indicate standard deviations.

In order to compare ammonium sulphate precipitation pattern of plantain α -amylase and barley α -amylase, ammonium sulphate fractionation was performed with a potato extract from transgenic potato tubers expressing 1000-fold higher recombinant barley α -amylase. Figure 4.2 (panel B) shows repetitions of the test revealed that maximum α -amylase activity was recovered at 43% ammonium sulphate fractionation on ice. Increase in ammonium sulphate concentration above 43% did not lead to increase

in the recovered enzyme activity. Therefore, a significant difference was observed in the physicochemical properties of the two enzymes, which are unlikely due to minor sequence differences in the catalytic domain of the enzyme.

Dialysis of the fractionated fractions

Having established that the activity in plantain extracts could be recovered after 30% ammonium sulphate precipitation, remaining salt in the pellet was removed by subsequent dialysis of the re-suspended protein pellet. This way protein stability and subsequent separation by ion-exchange chromatography could be improved. Therefore, the ammonium sulphate fraction was subject to dialysis against distilled water overnight. Alpha amylase activity and protein concentration were measured in the starting material, the re-suspended precipitate before and after dialysis. Good recovery and enrichment of enzyme activity after dialysis was observed, which is highly encouraging. Table 4.1 shows that the procedure yielded an approximately 20-fold enrichment of the enzyme relative to the total proteins. On the other hand, there was a significant loss of protein during dialysis, which could be due to the overall low protein concentration of the plantain extracts, as ripening plantains mostly contain polysaccharides. This could lead to non-specific loss of proteins binding to the dialysis membrane.

Table 4.1 Enrichment of amylase activity (Δ OD/ μ l/min)

	Blank	Starting	Precipitate	Dialysed
OD	0.111	0.412	1.6665	1.241
Delta OD		0.301	1.5555	1.13
α -Amylase activity		0.250833	1.29625	0.941667
Protein concentration		0.131519	0.064612	0.025547
Specific activity		1.907204	20.0622	36.86042

Purification of α -amylase by ion-exchange chromatography

The next step was to test binding to an ion exchange column. First experiments revealed that at neutral pH, the measured α -amylase activity remained bound to Q-sepharose, suggesting that the enzyme is negatively charged under these conditions. Subsequent elution with a salt gradient revealed conflicting results. In one experiment, a defined α -amylase activity peak was eluted whilst in subsequent repetitions the protein bound to the column but failed to elute. At low pH (5.5), the α -amylase failed to bind to either Q-sepharose or S-sepharose, suggesting that it was not charged. Several attempts were made to purify the enzyme using the ion-exchange chromatography but did not yield the desired result.

In order to test the binding of plantain alpha amylase to Q-sepharose; a sepharose binding assay was used where plantain extract with good α -amylase activity was mixed with the sepharose slurry and spun for few hours. It was centrifuged and α -amylase activity of the supernatant was measured. As shown in Table 4.2, significant enzyme activity was recovered in the supernatant. This indicates that plantain α -amylase does not bind un-specifically to sepharose and thus earlier observed loss of activity on the column may be due to low protein concentration and unspecific protein binding. Higher protein concentrations should be used to overcome this problem, but this was not possible due to large quantities of glutinous pectins.

Table 4.2 Sepharose binding experiment (Δ OD/ μ l/min)

	Blank	Supernatant
OD	0.099	0.375
Delta OD		0.276
α -Amylase activity		0.23
Protein concentration		0.014599
Specific activity		15.75443

Cloning strategy of plantain cDNA coding for amylases

Sequence conservation and design of degenerate primers

Due to difficulties in purifying the α -amylase from plantains, a different approach was envisaged based on sequence homology between α -amylases in the plant kingdom. Gene sequences of α -amylases from plantains have not been described in the literature but the high degree of sequence conservation between α -amylases from different plant species provided a cloning strategy based on degenerate primers. Cloning of plantain c-DNA encoding α -amylases was therefore attempted, although this technique does not guarantee enzymatic activity. Figure 4.3 shows an alignment of secreted α -amylases from seven plant species, displaying various regions of high sequence homology suitable for the design of degenerate primers.

A

<i>H. vulgare</i>	MANKHLSLS - - LFLVLLGLSASLASGQVLFQGFNWSWKHNGGWYNFLMGKVDDIAAAGITHVWLPPASQSVAEQGYMPG
<i>V. mungoculata</i>	---MDSFSRLSIFCLFISLLPLFSSPALLFQGFNWSWKHNGGWYNFLMGKVDDIAAAGITHVWLPPASQSVAEQGYMPG
<i>M. acuminata</i>MFLLLFLVILNLAQSQILFQGFNWSWRQQGGWYNFLKDKVSDIANAGVTHVWLPPSHSVGVQGYMPG
<i>G. max</i>	---MDTLSQLSLLCLCLSFPLFASPALLFQGFNWSWKHNGGWYNFLMGKVDDIAAAGITHVWLPPASQSVAEQGYMPG
<i>I. nil</i>	---MNLFQFLSCFCLACIFVPAIGS-TLLFQGFNWSWKHNGGWYNFLMGKVDDIAAAGITHVWLPPSHSVGVQGYMPG
<i>P. vulgaris</i>	---MHSFSRLSIFCLFISLSPFLSSSALLFQGFNWSWKHNGGWYNFLMGKVDDIAAAGITHVWLPPASQSVAEQGYMPG
<i>S. bicolor</i>	MAKHSAAACSLVLLVLLCLGSQLVQSQVLFQGFNWSWKHNGGWYNFLMGKVDDIAAAGITHVWLPPSHSVGVQGYMPG
<i>H. vulgare</i>	RLYDL DASKYGNKAQLKSLIGALHGKGVKAIADIVINHRTAEHKDGRGIYCFEGGTPDARLDWGPHEICRDDRPIADGT
<i>V. mungoculata</i>	RLYDL DASKYGSKNELKSLIAAFHEKGIKCLADIVINHRTAEKRDGRGIYCFEGGTPDRDWDGPFICRDTTAYSDGT
<i>M. acuminata</i>	RLYDLGASKYGNQDELKALIGAFHDKGVKCVADIVINHRCADKQDGRGIWCFEGGTDARLDWGPHEICRDTTQYSDGT
<i>G. max</i>	RLYDL DASKYGTQDLKSLIAAFHDKGIKCLADIVINHRTAEKRDGRGIYCFEGGTPDARLDWGPHEICRDTTQYSDGT
<i>I. nil</i>	RLYDL DASKYGNKQQLQALVAALHDKGIAVADIVINHRCADYKDSRGIYCFEGGTPDARLDWGPHEICRDTTQYSDGT
<i>P. vulgaris</i>	RLYDL DASKYGSDELKSLIAAFHEKGIKCLADIVINHRTAEKRDGRGIYCFEGGTPDARLDWGPHEICRDTTQYSDGT
<i>S. bicolor</i>	RLYDL DASKYGTHAELKSLIAAFHAKGVQCVADVIVINHRCADYKDSRGIYCFEGGTPDRDLWGPHEICRDTTQYSDGT
<i>H. vulgare</i>	GNPDTGADFGAAPDIDHLNLRVQKELVEWLNWLKADIGFDGWRDFFAKGYSAADVAKIYIDRSEPSFAVAEIWTSLAYGGD
<i>V. mungoculata</i>	GNNDSGEGYDAAPDIDHLNPQVQRELSEWMNWLKTEIGFDGWRDFVVKGYAPSIKIIYMEQTKPDFAVGEKWDSSISYGGD
<i>M. acuminata</i>	GNLDTGEGFAAPDIDHLNTPVQHELDLWLNWLKTDIGFDGWRDFFAKGYSSIAKIIYVEQTPNFVVAEIWSSLAYRND
<i>G. max</i>	GNLDSGEPYDPAPDIDHLNPQVQRELSEWMNWLKTEIGFDGWRDFVVKGYAPSIKIIYMEQTRPDFAVGEKWDSSISYGGD
<i>I. nil</i>	GNADTGMDFGGAPDIDHLNPRVQKELSEWMNWLKSEIGFDGWRDFVVRGYAASLTKIYMENTSPEFAVGEFWDSSMAYGSD
<i>P. vulgaris</i>	GNNDSGEGYDAAPDIDHLNPQVQRELSEWMNWLKTEVGFDFGWRDFVVKGYAPSIKIIYMEQTRPDFAVGEKWDPLSY-EN
<i>S. bicolor</i>	GHRDTGADFGAAPDIDHLNPRVQELSGWLNWLKSDLGFDGWRDFFAKGYSAADVAKIYVDNTAPTFFVAEIWSSLHYDGN
<i>H. vulgare</i>	GKPNLNQDQHRQELVNWVDKVGKGPATTFDFTTKGILNVAVEGELWRLRGTGDKAPGMIWGWPAKAVTFVDNHD TGSTQ
<i>V. mungoculata</i>	GKPNYNQDQHRQELVNWVESAG--GAI TAFDFTTKGILQAAVQGGELWRLIDPNGKPPGMIWVKPENAVTFVDNHD TGSTQ
<i>M. acuminata</i>	GKPTVDQNGNRQGLVNWVQVQV--GPVTAFDFTTKGILQAAVEGELWRMRDPQKAPGMMGWVPEKAVTFVDNHD TGSTQ
<i>G. max</i>	---DNYDGHGALVNWVESAG--GAI TAFDFTTKGILQAAVQGGELWRLKDSNGKPSGMIWVKPENAVTFVDNHD TGSTQ
<i>I. nil</i>	GKPDYNQDNHRNELSQVQVQV--GAVTAFDFTTKGILQAAVLDLWRLKDPNGKPPGLIGISPKNAVTFVDNHD TGSTQ
<i>P. vulgaris</i>	GKPTYNQDQHRQELVNWVESAG--GAI TAFDFTTKGILQAAVQGGELWRLIDPNGKPSGMIWVKPENAVTFVDNHD TGSTQ
<i>S. bicolor</i>	GEPSNNQDADROELVNWVAQVQV--GPAAFDFTTKGVLQAAVQGGELWRMKDNGKAPGMIWGLPEKAVTFVDNHD TGSTQ
<i>H. vulgare</i>	HMWPFPSDRVMQGYAYILTHPGTPIFYDHFWDWGLKEEIDRLVSVRTRHGIHNEKSLQIIEADADLYLAEIDG...KV
<i>V. mungoculata</i>	RLWPFPSDKVMQGYAYILTHPGTPIFYDHFWDWGLKEQIAKLSSIRLRNGINEKSTVKIMASEGDLYVAKIDN...KI
<i>M. acuminata</i>	KLWPFPSDKVMQGYAYILTHPGVPSIFYDHMFDWGLKEKIRLAKTRTRNRHSGSSLNIIASDADLYMAMIDG...KI
<i>G. max</i>	RIWPFPSDKVMQGYAYILTHPGTPIFYDHFWDWGLKEQIAKLSSIRVKGHINEKSSVNIILAAEADLCKCKDRQDLFED
<i>I. nil</i>	SMWPFPSDKVMQGYAYILTHPGVPCVYDHFWDWGLKEEISKLTSIRSRNGISETSNVEILAAADADLYVAKIDD...KV
<i>P. vulgaris</i>	RLWPFPSDKVMQGYAYILTHPGTPIFYDHFWDWGLKEQIAKLSSIRVRNGINEKSTVEILAAEADLYVAKIDN...KI
<i>S. bicolor</i>	NSWPFPSDKVMQGYAYILTHPGTPIFYDHFWDWGLKEEISALSAYRSRNGIHPGSKLNIILAADGDLVAKIDD...KV
<i>H. vulgare</i>	IVKLG.....PRYDVGNIIPGGFKVAAHGNDYAVWEKI.....
<i>V. mungoculata</i>	MVKIG.....PKMDLGNLIPSNLHVATSGQDYAVWE.....
<i>M. acuminata</i>	LTKLG.....SRYDVGNIIPSNFHVASGNDYCVWEKR.....
<i>G. max</i>	RAKDGPKPYSPKFPKCYLWPRLCRVGVT--QFITLQ.....
<i>I. nil</i>	IMKIG.....SKYDVGNIIPPNFNLVTSQDYAVWEKKI.....
<i>P. vulgaris</i>	MVKIG.....PKMDLGNLIPSNFHVATSGQDYAVW.....
<i>S. bicolor</i>	IVKIG.....SRYDVGNIIPSDFHAVAAGNNDYCVWEKSGLRVPAGRHH

B

AmyS1: 5' TTY CAR GGN TTY AAY TGG GA 3'
 AmyAS1: 5' ATR TAN GCR TAN CCY TGC AT 3'

Figure 4.3. Sequence conservation of plant α -amylases. Panel A shows the alignment of amino acid sequences of α -amylases from seven plant species. Abbreviations, Hv= *Hordeum vulgare*, Vm= *Vigna mungo*, Ms= *Musa specie*, Gm= *Glycine max*, In= *Ipomea nil*, Pv= *Phaseolis vulgaris*, Sb= *Sorghum bicolour*. The alignment shows no conservation in the signal peptide, but some areas with good sequence conservation were observed. The boxes indicate the region used in the design of degenerate primer and arrows indicate the orientation. Panel B shows the sequence of the two oligonucleotide primers, AmyS1 is the sense and amyAS1 is the antisense that were used for PCR amplification of the plantain cDNA.

Optimisation of mRNA extraction from starch tissues

In order to amplify cDNA with degenerate primers (Figure 4.3 and 4.4), high quality mRNA preparations are needed for efficient cDNA synthesis and subsequent PCR reactions with high number of cycles typical for degenerate primers. The first attempts using the standard protocols yielded low amounts of RNA of poor quality (Table 4.3), which may be attributed to the high amount of polysaccharides and polyphenols in the plantain fruit. Polysaccharide, polyphenols, and protein contamination affects the amount and quality of RNA, which can be deduced from A_{260}/A_{230} ratios for polysaccharides and A_{260}/A_{280} ratios for proteins. For this reason, alternative protocols were tested (see Table 4.3) in order to reduce contamination by polysaccharides, polyphenols and proteins. The method of Asif et al. (2000) resulted in the best yield and quality RNA from the plantains. Concentrations up to 2.76 $\mu\text{g}/\mu\text{l}$ (Table 4.3) were suitable for subsequent cDNA synthesis reactions.

Table 4.3 Properties of RNA extracts

Methods	Absorbance	Absorbance	Concentration
	260/230	260/280	(ng/ μ l)
Birnbom, 1988	0.280	1.34	109.8
Suzuki et al., 2004	0.890	1.39	303.7
Suzuki et al., 2004	0.923	1.41	217.0
Asif et al., 2000	1.613	2.16	365.0
Asif et al., 2000	2.000	2.02	2760.0

Cloning with degenerate primers reveals a novel α -amylase sequence not reported before

To identify α -amylases from plantains, complementary DNA was synthesised with reverse transcriptase from 5.5 μ g of high quality plantain RNA using oligo (dT)₁₈ as primer. Dilutions of the synthesized cDNA were amplified using a pair of degenerate primers amyS1 and AS1 (Figure 4.3B) designed based on conserved domain homology between alpha amylases from different plants species (Figure 4.3). Forty five cycles of amplifications were used to ensure efficient amplification. A PCR amplification product of approximately 900 base pairs (Figure 4.4B) was obtained consistent with the known distance of the two conserved regions in other α -amylases (Figure 4.3). The result was encouraging and could indicate that ripening plantains contain mRNA that encodes alpha amylase, a hypothesis that could be tested by sequencing the PCR product.

In order to sequence the amplified product, the PCR product was first gel purified (Figure 4.4C), eluted in TE, tested on gel again (Figure 4.4D) and sequenced with the two degenerate primers from both ends (Figure 4.4A). Good quality sequences were obtained with the sense primer (N-terminus) and antisense primer (C-terminus). The sequences obtained were informative to predict possible open reading frames. The

sequences from the two termini did not overlap because the reactions were short. This could be due to the use of degenerate primers as sequencing primers using standard commercial routine as well as insufficient template after gel-purification.

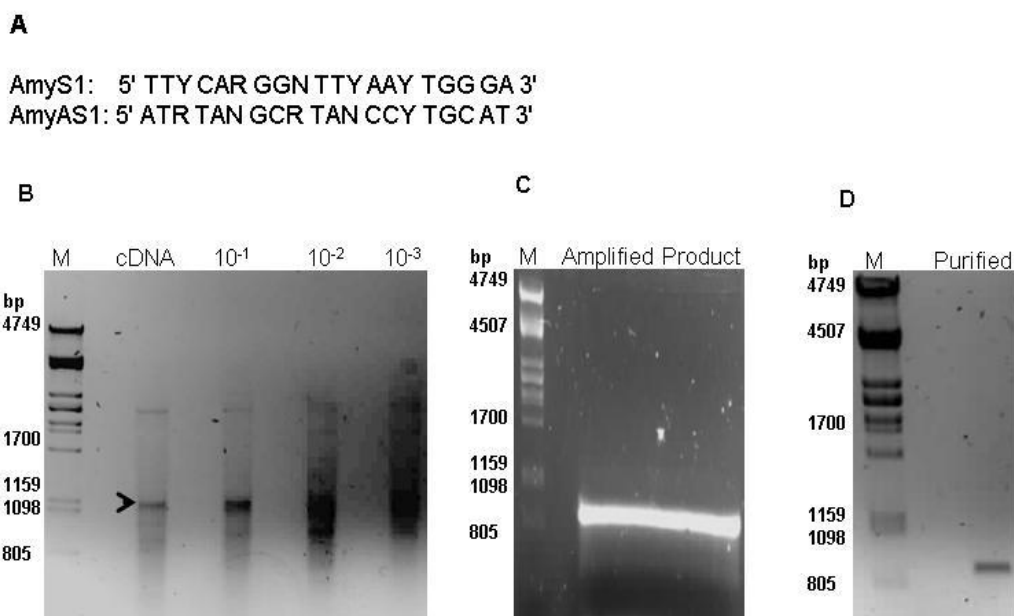


Figure 4.4. PCR amplification of plantain cDNA. Panel A shows degenerate primers designed using the conserved domain between α -amylases (Supplemental Figure 1) represented using IUPAC codes; AmyS1 is the sense primer and AmyAS1 is the antisense primer. Panel B shows the PCR amplified product using AmyS1 and AmyAS1 and different dilutions of template, arrow shows the expected product. Panel C is preparative gel showing the amplified product and panel D shows the gel purified DNA eluted in TE suitable for sequencing.

To determine the missing sequences, specific primers (PlanS1 and PlanAS1) were designed based on the sequences obtained with degenerate primers. The gel purified product (Figure 4.4D) was sequenced again and the sequences were identified with good overlap. The resulting nucleotide sequence contig is shown in Figure 4.5. The sequences were used to predict the open reading frame and the deduced amino acid sequence was determined using the Bio-edit programme.

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GAATTGTCTTCATTAGGTTTACAGTCATCTGGCTACCACCACCCACTGAGTCTGTATCTCCTGAAGGGTA
CATGCCAAAGGATTTATACAATTTAAATTCAGATATGGAAGCTTGGAGAGCTGAAGGATCTTGTAATA
GTTTCCATGAAGTGGGCATTAAGTTCTTGGTGATGCTGTCTTAAACCATCGATGTGCACATTACCAGAAT
AAGAATGGAATTTGGAACGTTTTCCGGAGGCCGATTGAATTTGGGATGATCGTGCTATTGTTGCTGATGATCC
TCACTTCCAGGGAAGGGGTAACAAAAGCAGTGGAGATAATTTTCATGCTGCCCCGAATATGACCCTCTC
AAGATTTTGTGAGGAGGGACCTGAAAGAGTGGCTCTGCTGGTTGAGAAAAGAAGTTGGATATGATGGATGG
AGATTAGATTTTGTCCGTGGGTTTTGGGGTGGTTATGTAAAAGACTACATGGAAGCAACTGAACCATATTT
TGCTGTGGGCGAGTATTGGGACTCATTAAGTTATACATATGGTGACATGGACCATAATCAAGATGCTCATA
GACAAAGGATTGTTGATTGGATAAATGCTACAAATGGAAGTGCAGGTGCCTTCGATGTACCACAAAAGGA
ATCCTCCACAGTGTCTGTGAAAAATGTGAGTATTGGCGATTGTCTGATCAAAATGGGAAACCTCCTGGAGT
AGTAGGCTGGTGGGCATCTCGTGCCGTTACTTTTTATAGAGAATCATGAC

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Figure 4.5. Nucleic acid sequence of the initial product. The sequence obtained from the amplified PCR product (Figure 4D) is shown. The sequence was from reactions using both degenerate and sense primers. Both the N-terminal and C-terminal sequence overlapped with no missing sequence in between.

Identification of Chloroplast α -amylase from plantains

In order to establish the type of protein encoded by the PCR amplification product, the open reading frames were subject to a BLAST search at NCBI. The amplified sequence showed the highest homology with α -amylase from *Ricinus communis* (Figure 4.6, panel A). Similar high homologies were observed for α -amylase of *Vitis vinifera*, *Malus domestica* and *Arabidopsis thaliana*. Interestingly, the gene sequences with the highest homology to the amplified sequence do not encode secreted α -amylases but instead encode a new type of ill-defined type 3 chloroplast α -amylase. These proteins are much larger than the type of α -amylase shown in Figure 4.3 and contain a large domain in between the chloroplast transit peptide and the α -amylase homology domain (Figure 4.6, panel B). In contrast, only low sequence homologies to secreted Hvamy2 and Atamy1; and cytosolic Atamy2 and Mdamy8 (Figures 4.6, panels A and B) were observed. In conclusion, the obtained PCR product (Figure 4.4) of the amplified cDNA is closer related to the type 3 α -amylases than any other including the barley clone used in the host laboratory.

A

<i>Plantainamy</i>	ELSSLGFTVIWLPPPTESVSP EGYMPKDLN LN - SRYGSL EELKDLVNSFHEVGIKVLGD	
<i>RcamyUD</i>	EISSLGFTVIWLPPPTESVSP EGYMPKDLN LN - SRYGSI DELKDLVKS LHRVGLKVLGD	
<i>VvamyUD</i>	ELSSLGFTV VWLPPPTASVSP EGYMPTDLYN LN - SRYGSS DELKVLVKS FHEVGVKVLGD	
<i>Atamy3</i>	ELASLGFTVLWLPPPTESVSP EGYMPKDLN LN - SRYGTI DELKDI VRKFHKVGIKVLGD	3
<i>Mdamy10</i>	ELSSLGFTVIWFPPPTDSVSP QGYMPRDLYNMN - SRYGNMDELKEI VKTIFHDAGLKVLGD	
<i>Hvamy2</i>	DI AAAGITHVWLPPASQSV AEQGYMPGRLYDL DASKYGNKAQLKSL I GALHGKGVKAIAD	
<i>Atamy1</i>	DI ANAGITHLWLPPPSQSV APEGYLP GKLYDLNS SKYGS EAELKSL I KALNQGKIKALAD	1
<i>Atamy2</i>	DI AKSGFTSAWLPPPSQSL APEGYLP QDLYSLN - SAYGSEHLKSL L RKMKQYKVRAMAD	
<i>Mdamy8</i>	DI GRSGFTSAWLPPATHSFA PEGYLPQDIYSLN - SKYGS ENLLTSL LHKMKQHKVRAMAD	2
<i>Plantainamy</i>	AVLNHRCAHYQNKNGI WNVFGGR - - - - LNWDDR - AIVADDPHFQGRGNKSS GDNFHAAP	
<i>RcamyUD</i>	AVLNHRCAHFQNGVWNI FGGR - - - - LNWDDR - AIVADDPHFQGRGSKSS GDNFHAAP	
<i>VvamyUD</i>	VVLNHRCAQYQNNGI WNI FGGR - - - - LNWDDR - AIVADDPHFQGRGNKSS GDNFHAAP	
<i>Atamy3</i>	AVLNHRCAHFKNQNGV WNLFGGR - - - - LNWDDR - AVVADDPHFQGRGNKSS GDNFHAAP	3
<i>Mdamy10</i>	AVLNHRCAEYQNNGV WNI FGGR - - - - LNWDER - AVVADDPHFQGRGNKSS GDSFHAAP	
<i>Hvamy2</i>	I VINHRTAEHKDGRGI YCIFE GGT P DARLDWGP HMICRDDR PYADG I GNPDIGADFGAAP	
<i>Atamy1</i>	I VINHRTAERKDDKCGYCY FEGGT SDDRLDWDP SFVCRND - PKFPG I GNLDIGDFDGAP	1
<i>Atamy2</i>	I VINHRVGTIRGHGGMYNRYDGI S - - - - LPWDEH - AVT S CT - - - GGLGNRSTIGDNFNGVP	
<i>Mdamy8</i>	I VINHPVGTIRGHGGKYNRYDGI S - - - - LSWDER - AAT S CT - - - GGLGNPSTIGDNFHGVP	2
<i>Plantainamy</i>	NIDHSQDFVRRDLKEWLCWLRKEVGYD GWRLDFVRFWGGYVKDYME AIEPYFAVGEYWD	
<i>RcamyUD</i>	NIDHSQDFVRQDLKEWLCWLRDEIGYNGWRLDFVRFWGGYVKDYME AIEPYFAVGEYWD	
<i>VvamyUD</i>	NIDHSQDFVREDIKEWLCWLRKEIGYD GWRLDFVRFWGGYVKDYMDASEPYFAVGEYWD	
<i>Atamy3</i>	NIDHSQDFVRKDIKEWLCWMMEEVGYD GWRLDFVRFWGGYVKDYMDASKPYFAVGEYWD	3
<i>Mdamy10</i>	NIDHSQDFVRKDIREWLCWLRDDIGYD GWRLDFVRFWGGYVKDYMDASEPYFAVGEYWD	
<i>Hvamy2</i>	DIDHLNLRVQKELVEWLNWLKADIGFDGWRFDFAKGYSADVAKIYIDRSEPSFAVAE IWT	
<i>Atamy1</i>	DIDHLNPRVQKELSEWMNWLKTEIGFHWRFDFYVRGYASSITKLYVQNTSPDFAVGEKWD	1
<i>Atamy2</i>	NVDHTQHFRKDIIGWLRWLRNTVGFQDFRDFARGYSANYVKEYIGAAKPLFSVGECCWD	
<i>Mdamy8</i>	NIDHSQLFVRKDIIGWLRWLRNNVGFQDFRDFARGYS AKYVKEYIEGAKPIFSVGEYWD	2
<i>Plantainamy</i>	SLSYTY - GDMHNQDAHRQRIVDWINATNG - - TAGAFDVTITKGI LHSALKECEYWRLS DQ	
<i>RcamyUD</i>	SLSYTY - GEMDHNQDAHRQRIIDWINATNG - - TAGAFDVTITKGI LHSALDRCEYWRLS DQ	
<i>VvamyUD</i>	SLSYTY - GEMDHNQDAHRQRIIDWINATNG - - AAGAFDVTITKGI LHSALGRCEYWRLS DQ	
<i>Atamy3</i>	SLSYTY - GEMDYNQDAHRQRIVDWINATSG - - ATGAFDVTITKGI LH TALQKCEYWRLS DP	3
<i>Mdamy10</i>	SLSYTY - GEMDHNQDAHRQRIVDWINATNG - - TCGAFDVTITKGI LHAALERCEYWRLS DE	
<i>Hvamy2</i>	SLAYGGDGKPNLNQDQHRQELVNWVDKVGKGPATTFDFTITKGI LNVAVEG - ELWRLRGT	
<i>Atamy1</i>	DMKYGGDGKLDYDQNEHRSGLKQWIEEAGG - GVLTAFDFTITKGI LQSAVKG - ELWRLKDS	1
<i>Atamy2</i>	SCNYNG - HGLDYNQDSHRQRIISWIDATGQ - - ISAAFDFTITKGI LQEA VKG - QYWRLCD A	
<i>Mdamy8</i>	SCNYNG - HGLDYIQDSHRQPIVNWINGTIGQ - - LSTAFDFTITKGI LQEA VKG - QLWRLRDP	2
<i>Plantainamy</i>	NGKPPGVVGWWASRAVIF IENHD	
<i>RcamyUD</i>	KGKPPGVVGWWPSRAVIF IENHD	
<i>VvamyUD</i>	KRKPPGVVGWWPSRAVIF IENHD	
<i>Atamy3</i>	KGKPPGVVGWWPSRAVIF IENHD	3
<i>Mdamy10</i>	KGKPPGVVGWWPSRAVIF IENHD	
<i>Hvamy2</i>	DGKAPGMIGWWPAKAVI FVDNHD	
<i>Atamy1</i>	QGKPPGMIGIMP GNAVIF IENHD	1
<i>Atamy2</i>	QGKPPGVVGWWPSRAVIF IENHD	
<i>Mdamy8</i>	QGKPPGVVGWWPSRSVIF IENHD	2

B

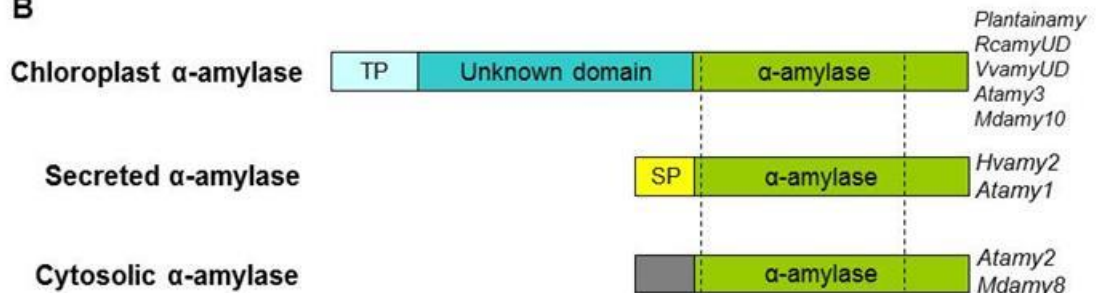


Figure 4.6. Comparison of putative plantain α -amylase with known α -amylases. Panel A shows the clustalw alignment of amino acid sequence of partial plantain amylase clone with α -amylases from the three families (1, 2 and 3). Family 1: *Hvamy2* (*H. vulgare*) and *Atamy1* (*Arabidopsis*); family 2: *Atamy2* and *Mdamy8* (apple); and family 3: *RcamyUD* (*R. communis*), *VvamyUD* (*V. vinifera*), *Atamy3* and *Mdamy10*. Panel B shows a pictorial representation of amylases from the three domains, TP=transit peptide, SP=signal peptide, the dotted lines represents the region of homology of the amylases which is also the region of the amplified sequence.

Rapid amplification of cDNA ends (3'RACE)

In order to obtain the C-terminal end of the plantain α -amylase clone, a rapid amplification of the cDNA end (3'RACE) was performed. A specific forward primer (Plans1) and a 3'RACE (reverse) primer (Figure 3.7A) were used to amplify the total plantain cDNA. A PCR amplified product of the expected size (approximately 1500bp) was obtained (4.7B), gel purified and tested on a gel (C). In order to establish further the validity of the gel purified product, a semi nested PCR amplification was performed using PlanS1 as forward and an internal reverse primers, PlanAS4 (Figure 4.7C) on the dilution series of the product shown in panel C. Products of the expected sizes (> 700bp) were obtained with the dilution series of the template (D). This again establishes validity of the initial RACE product. The 3'RACE product (panel B and C) was sequenced with specific sense primers, PlanS1, PlanS2, PlanS4 and PlanEND. Good sequences were obtained that read beyond the stop codon and confirmed that a clone corresponding to an α -amylase was obtained.

A

PlanS1: 5' GGTTTTACAGTCATCTGGCT 3'
 3'RACE: TTTTTTTTTTTTTTTTTTTTTTTT(GAC)(GATC)
 PlanAS4: 5' CAGCCTACTACTCCAGGAGG3'

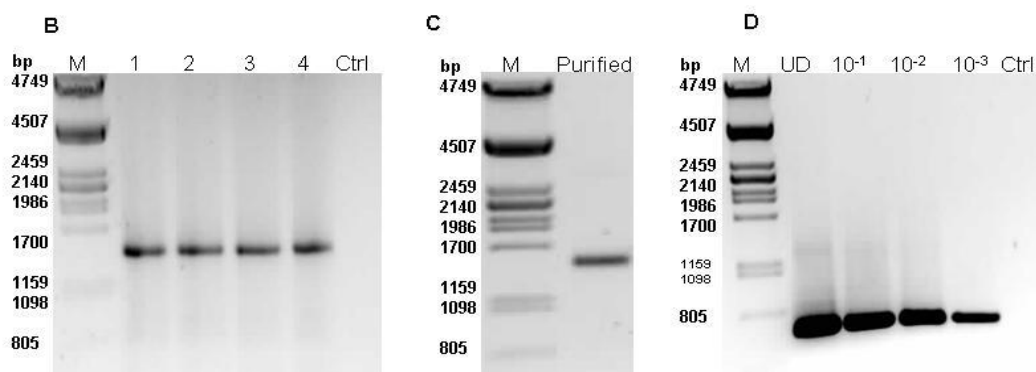


Figure 4.7. The products of 3' amplification of rapid cDNA ends, the amplified products of C-terminal end of the plantain α -amylase are shown. Panel A shows the primers used for PCR. Panel B shows the amplified product of PlanS1 and a 3'RACE primer, 1, 2 3 and 4 are all replica of the cDNA amplified product. The pooled and gel purified product is shown in panel C. Panel D illustrates the product of nested PCR amplified of dilution of purified product shown in panel B. UD represents undiluted, 10^{-1} , 10^{-2} and 10^{-3} represent the other dilution series.

The unknown domain in family 3 amylase is conserved

In order to obtain experimental evidence showing that the amplified plantain sequence encodes a type 3 α -amylase, it was necessary to identify conserved region in the N-terminal unknown domain of this class for the design of degenerate primers which will be used for PCR amplification. Protein sequences corresponding to the 500 amino acids from six different type 3 α -amylases were aligned using clustalW2 at EBI.

Figure 4.8 reveals the degree of conservation in the N-terminal unknown domain of family 3 proteins. The domain starts after the transit peptide and ends with a linker region (GTGSG). The linker connects the unknown domain to the rest part of the protein which is the amylase domain. As illustrated in Figure 4.8 significant conservation between the different proteins in the additional domain was observed. However there are some regions in the domain that are not conserved across the group. This is not uncommon as it is often the case in large family of proteins.

Consequently, several degenerate primers were designed using areas with high sequence conservation (Figure 4.8).

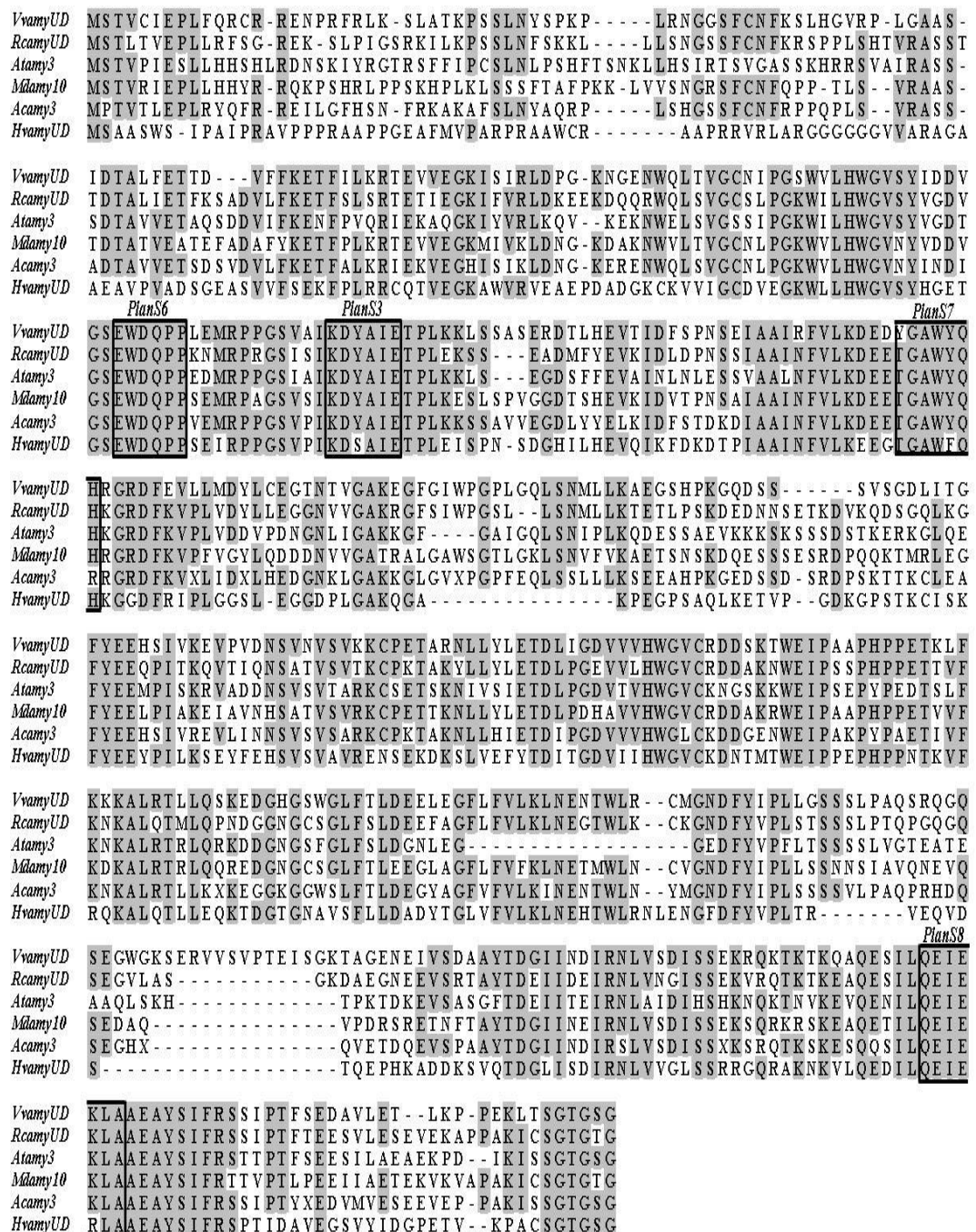


Figure 4.8. Conservation of the unknown domain in family three α -amylases, an alignment of the some of the family three α -amylases is shown. Plans3, 6, 7 and 8 are annotated degenerate primers that were used for amplification of the plantain cDNA to obtain the large or used as sequencing primers. Areas in grey indicate amino acids that are conserved in the family three proteins.

Evidence that ripening plantains express a type 3 α -amylase

In order to maximise the chance for success, the most N-terminal degenerate primer, PlanS6 (Fig 4.9, panel A) was used in conjunction with a specific antisense primer, PlanAS4 designed from the amplified sequence (Figures 4.5). This would allow further verification by nested PCR using more distal degenerate primers (PlanS3, S7, and S8, Fig. 4.8) or more proximal antisense primers. If the hypothesis is correct, it should be possible to amplify a fragment coding for a partial unknown domain fused to an amylase catalytic domain with an overall size predicted from the available sequence homology. A PCR amplified product of 1.9 kilo base pairs was obtained as shown in Figure 4.9 panel B, a size expected for a type 3 amylase with the chosen primer pair.

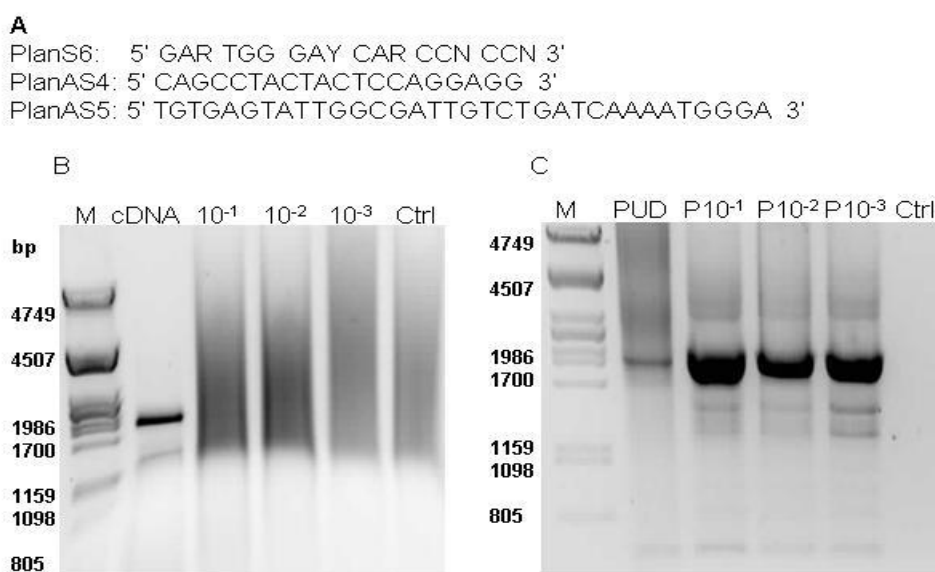


Figure 4.9. PCR amplified product of PlanS6 and AS4 showing the PCR amplified products using specific primers. Panel A shows the sequence of primers that were used for PCR. Panel B shows cDNA series amplified using PlanS6 and AS4. Panel C shows products of Semi-Nested PCR dilution of the cDNA amplified (4B) using PlanS6 and AS5.

To validate this, a semi nested PCR was necessary; the products from the PlanS6 and AS4 reaction (Fig. 4.9B) were diluted and amplified using PlanS6 as sense primer and a more internal antisense primer AS5. PCR amplified products of the expected size (1.8 Kb) were obtained (Figure

4.9, panel C). The products were gel purified and tested on a gel. A nested PCR was again performed on the purified product (data not shown) using the primer pair PlanS3 (Fig. 4.8) and PlanAS5. The gel purified product was then sequenced using the primer pair, PlanS6 and AS5. Sequences with good reads were obtained, however a small region between the unknown domain and amylase domain was missing.

In order to identify the missing sequence, two specific primers PlanUDS and Upas1 were designed and used to amplify the unsequenced central region of the new clone. Figure 4.10 shows a PCR amplification of the small missing part, which was gel purified and sequenced. The obtained sequences had 100% homology with the overlapping region from the first PCR product (Figures 4.4 and 4.5) and encoded an α -amylase catalytic domain in frame with an N-terminal open reading frame that was clearly related to the unknown domain of type 3 amylases. Therefore, the partial unknown domain clone together with the full amylase domain was cloned.

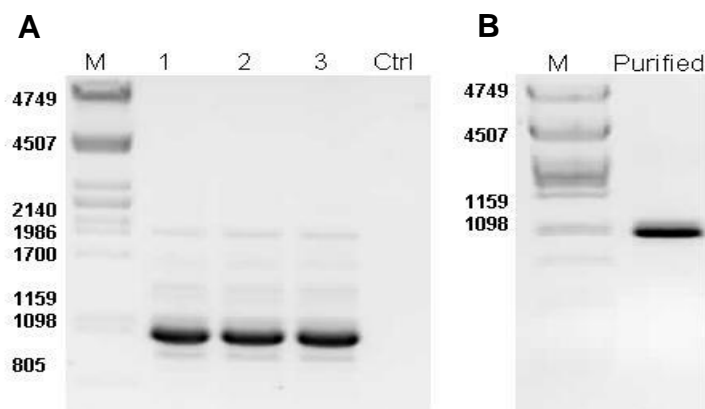


Figure 4.10. PCR amplified product of PlanUDS and Upas1. Panel A shows a cDNA series amplified using PlanS6 and AS4. Panel B shows the gel purified product obtained from panel A.

Assembly of the plantain amylase sequence

To assemble and annotate the α -amylase sequence, the nucleotide sequence obtained from various PCR and sequencing reactions shown in Figures 4.4/5; 4.7; 4.9 and 4.10 were assembled together. Good overlaps were obtained between the different sequence contigs that

were assembled. The nucleotide sequence of the partial plantain clone is shown in Figure 4.11A. The sequence excludes part of the protein that is yet to be cloned but extends at the C-terminus up to the stop codon. Similarly the amino acid sequence of the putative plantain α -amylase is shown in Figure 4.11B. This sequence shows the full amylase domain at the C-terminus but it does not include the missing region in the unknown domain and transit peptide (see Fig. 4.6).

A

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CCTTTGAAGAGATTATCATCCCAATCCGAAAGGCAGGCACCTTCACGAACTGCAGATCGAATTTGACAGCAACACCCCAATT
GCAGCAATTCATTTTGTTTTAAAGGAAGAAGAAAACAGGAGCATGGTTTCAGCACAAAGGGAAGGGATTTTCAGAATATCCTTT
ACAGACTATTTTGAAGTGGCCAAACAGTGTAGCTGGAAATCAAGGCTTGAGCATATGGCCAGGGGGTTTTGACCAGATATCT
AGCCTGCTGCTGAAAGCTGAAGAGTCTACTTCCAAAAAGAAGACCCTGATGATGAAGATGGAAATGTTGTGAAGCAGAAT
AGATGCATTGCTCCAATTTACAAAGAATTTCTATCTTAAAAGAAGAGTTTGTTCACCAACCATATGACAGTCTCTGTGAGG
AGTAGTGATAAGACAGATAAGAACATTGTGCAATTTGACACAGATCTGCCTGGTGTGTCGTAATTCATTGGGGTGTCTGT
AAGGACGATGGGAGAAAATGGTAATCCCATCTACCCACATCCACCAGCAACCAAAATCTTTCGGCATAAGGCTCTTCAA
ACTTTACTACAGCCAAAACCAGATGGCCTTGGTAGCTGGGGATTATTTTTGGTGGATCAAGGAACCTCAGGTGTGGTTTTT
GTGCTCAAACCTAATGAATACACATGGTTGAATAACAATGGAACCTGATTTTTTCATCCCAATGGAAAGTGTGAGTAGCACA
ACTGCTGAAATGGTAGTGATGATATTGTACATGAACAGCAGGACAGGTACAGAGATTGATGATGTGAGAAACAGTCTATCA
CCAAATAATTCATACCCACTGCAGAATAAATCACTGGAAGCAAGTGATCCCCAAAACATCAATAGTTTACCATGAAGCCC
CAAGTCTCTGAGGAGCTCATAGAAGCTGTTGCATATACTGATGAAATTAACAAGGAGATAAGACATTTAGTAACTGATATA
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GCTTATAGTATCTTCAGGATCTCCATCCAGTTTTGTGGAGTTGGCTTCTGACACTGAGCTGCTGAAGCCTGCTGTAAAA
TTGCTTTCAGGAACAGGCTCTGGGTATGAAATCTGTGTCAAGGATTTAACTGGGAATCTCACAATCGGGAAGATGGTAT
TCCGAGCTTAGTGACAAGGCCAAGGAATGTCTTCATTAGGTTTTACAGTCATCTGGCTACCACCACCCTGAGTCTGTA
TCTCCTGAAGGGTACATGCCAAAGGATTTATACAATTTAAATTCAGATATGGAAGCTTGGAAAGCTGGAAGGATCTTGTA
AATAGTTTCCATGAAGTGGGCATTAAGGTTCTTGGTGTGCTGCTTAAACCATCGATGTGCACATTACCAGAATAAGAAT
GGAATTTGGAACGTTTTTCGGAGGCCGATGAAATGGGATGATCGTGCTATTGTTGCTGATGATCCTCACTTCCAGGGAAGG
GGTAACAAAAGCAGTGGAGATAATTTTCATGCTGCCCCGAATATTGACCACTCTCAAGATTTTGTGAGGAGGACCTGAAA
GAGTGGCTCTGCTGGTTGAGAAAAGAAGTTGGATATGATGGATGGAGATTAGATTTTGTCCGTGGGTTTTGGGGTGGTTAT
GTAAAAGACTACATGGAAGCAACTGAACCATAATTTGCTGTGGCGAGTATTGGGACTCATTAAAGTTATACATATGGTGAC
ATGGACCATAATCAAGATGCTCATAGACAAAGGATTGTTGATTGGATAAATGCTACAAATGGAACGCAGGTGCCTTCGAT
GTCACCACAAAAGGAATCCTCCACAGTGTCTGGAAAAATGTGAGTATTGGCGATTGTCTGATCAAAATGGGAAACCTCCT
GGAGTAGTAGGCTGGTGGGCATCTCGTGCCGTTACTTTTATAGAGAATCATGACACTGGCTCCACTCAGGGTCATTGGCGA
TTCCCATCTGGAAAGGAGATGCAAGGATATGCATATATCTTAACGCATCCTGGCACACCAGCAGTCTTCTATGACCACATT
TTCTCTCACTACCAGCAAGAGATATCTAGATTGATCTCGGTGAGAAATGAAAACAAGATTCACTGCAGGAGCACGGTGAAG
ATCGTCAAGGCAGAGAGGGACGCTCTATGCGGCTGAGATCGACGAAAAGTTAGCAGTCAAAATGGGCCAGGACATTATGAG
CCACCAGATGGTCTACAAAATGGGTTGTAGCAGCTGAAGGAAGAGATTATAAAGTGTGGGAAACATCATGA

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B PLKRLSSQSERQALHELQIEFDSNTPIAAIHVFLKEEETGAWFQHKGRDFRISFTD
 YFEVANSVAGNQGLSIWPGGFDQISSLLLKAEEESTSKKEDPDDEDGNVVKQNRICIA
 PIYKEFPILKEEFVFNHMTVSVRSSDKTDKNIVQFDTDLPGDVVIHWGVCKDDGRK
 WVIPSTPHPPATKIFRHKALQTLQPKPDGLGSWGLFLVDQGTSGVVFVLKLNEYT
 WLNNGTDFFIPIGSVSSTTAEIGSDDIVHEQQAGTEIHDVRNSLSPNNSYPLQNK
 SLEASDPQNINSLPMKPQGPEELIEAVAYTDEI I KEIRHLVTDISSEK GKRAKSKE
 AQENILQEIEKLAAEAYSIFRISIPGFVELASDTELLKPAVKLSS **GTGSGYEILCQ**
GFNWESHKSRWYSELSDKAKELSSLGFTVIWLPPEPTESVSPEGYMPKDLYNLNSRY
GSLEELKDLVNSFHEVGIVLGDVNLHRCAHYQNKNGIWNVFGGRLNWDDRAIVA
DDPHFQGRGNKSSGDNFHAAPNIDHSQDFVRRDLKEWLCWLRKEVGYDGRWLDVFR
GFWGGYVKDYMEATEPYFAVGEYWDLSLSYTYGDMDNQDAHRQRIVDWINATNGTA
GAFDVTTKGILHSALEKCEYWRLSDQNGKPPGVVGGWASRAVTFIENHDTGSTQGH
WRFP SGKEMQGYAYILTHPGTPAVFYDHF SHYQQEISRLISVRNENKIHRSTVK
IVKAERDVYAAEIDGKLAVKIGPGHYEPPDGPTKWVVAEGRDYKVVETS

Figure 4.11. Full sequence of the plantain α -amylase. Panel A shows the nucleotide sequence up to the stop codon. Panel B shows the deduced amino acid from the longest open reading frame translated from the nucleic acid sequence in panel A. The amino acids in red (GTGSG) indicate the linker between the unknown and amylase (catalytic) domains of the protein. The underlined region indicate the catalytic domain

Phylogenetic relationship between plant α -amylases

To fully verify the type of α -amylase cloned from the plantains (Figure 4.11), α -amylase sequence from the three different families were retrieved from the database. The amino acid sequence of the partial plantain clone was aligned with α -amylases from families one, two and three respectively. The result of the alignment is illustrated in Figure 4.12 below. The plantain α -amylase again showed closer homologies to α -amylases from *V. vinifera* and *R. communis* which belong to the family three α -amylase. In contrast lower sequence homologies were shown by the plantain α -amylase to α -amylases from families two and three respectively (Figure 4.12). This is in agreement with the result of the earlier comparison (Figure 4.6) done with the short putative amylase. This strongly suggests that the cloned α -amylase from plantain is of the family three type. In addition, some information on the missing part of the sequence was obtained. The alignment indicated that the missing sequence at the N-terminus of the plantain amylase is approximately 150 amino acids in length (Figure 4.12). Thus, a rapid amplification at the 5' (or N) terminal end has to be performed to identify the missing part.

In order to further establish and confirm the relationship between the partial plantain α -amylase clone identified (Figure 4.12) and α -amylases from different plant species; rather than the complete plantain sequence, the sequences of the amylase domain alone and that of α -amylases from the three different families were aligned together using the clustalW2. The alignment was used to generate a phylogenetic tree using the MEGA5 programme. Figure 4.13 shows a dendrogram describing the three families of α -amylase found in plants. The secreted alpha amylase from barley and other plant species contains a signal peptide for the translocation to endoplasmic reticulum and the α -amylase domain (Figures 4.6 (B) and 4.13). The cytosolic proteins (family two) have no targeting peptide. The third family is the chloroplast non-secreted α -amylase that contains an unusual domain in between the transit peptide and the α -amylase domain (Figures, 4.6(B) and 4.13). The phylogenetic analysis also revealed that the plantain α -amylase is of the chloroplast type which is in agreement with the results of alignments shown in Figures 4.6, and 4.12.

```

Plantainamy
VvamyUD
Rcamy
Atamy3
Hvamy2
Atamy2
MSTWCIIEPLFQRCRRENPF--RFLKSLATKPSSSLN---YSPKPLR-NGGSFCNFKS---LHGVRPLGA
MSTLTVEPLLRFSGREKS--LPIGSRKILKPSSSLN---FSKLLKLSNGGSFCNFKRSPPLSHTVIR--AS
MSTWPIESLLHSHLRDNSKIYRGRTRSFIFCSLNLPSHFTSNKLLHSIRTSVGAASSKHRRSVAIR--A
-----

Plantainamy
VvamyUD
Rcamy
Atamy3
Hvamy2
Atamy2
ASIDTALFET---TDVFFKETFILKRTETVEVVEGKISIRLD-PGKNGENWQLTVGCSLIPGWSVLLHWGVSYID
STTDALIEETFKSADVLFKETFSLSRTEETIEGKIFVRLDKEEKDQQRWQLSVGCSLIPGKWLILHWGVSYVG
SSSDTAVVETAQSDVDFKFNFPVQRIEKAGGKIYVRLKQVKEK--NWELSVGSSIPGKWLILHWGVSYVG
-----

Plantainamy
VvamyUD
Rcamy
Atamy3
Hvamy2
Atamy2
DVGSEWDQPPLEMRPPGSVAIKDYAIETPLKLLSSASERDTHLVEVTDLDFSPNSEIAAIRFVLKDEEYGAW
DVGSEWDQPPKNMRPPGSIKDYAIETPLKKS--SEADMFYEVKIDLDPNSSIAAIRFVLKDEETGAW
DTGSEWDQPPLEMRPPGSIKDYAIETPLKLL--SEGDSFFEVAIINLNLLESSVAALNFVLKDEETGAW
-----

Plantainamy
VvamyUD
Rcamy
Atamy3
Hvamy2
Atamy2
FQHKGRDFRISFTDY-FEVANSVAGNQLSIVPQGFQDQISSLLKAEESTSKKEDPDEEDGNVVKQNRCTI
YQHRGRDFEVLMDYLCSEGTNVGAKEGFGIWPQGLGQLSNMLLKAEKSHPKGQDSSVSGDL---I
YQHKGRDFKVPVLDYLLLEGNVVGAKRGSFIWPQGL--LSNMLLKTEETLPSKDEENNSETKDVVKDQSGQL
YQHKGRDFKVPVLDVDPDNGNLTGAKKGF---GALGQLSNIPLKQDESSAEVKKSKSSSDSTKERKGL
-----

Plantainamy
VvamyUD
Rcamy
Atamy3
Hvamy2
Atamy2
APIYKEFPILKEEFVPHMIVSVSRSSDKTDKNIVQFDTDLPGDQVIVHWGVCKDDGKRWVIPSTPHPPATK
TGFYEEHSIVKEVPVDNSVNSVKKCPETARNLLYLETDLIGDQVIVHWGVCRDDSKTWEIPAAPHPPETK
KGFYEEQPIKQVTLQNSATVSVTKCPKTAQYLLYLETDLPGDQVIVHWGVCRDDAKNWEIPSPHPPETK
QEFYEEPIKRVADDNSVSVTARKCSETSKNLISETDLPGDQVIVHWGVCKDDGSKKWEIPSEPPEDTS
-----

Plantainamy
VvamyUD
Rcamy
Atamy3
Hvamy2
Atamy2
IFRHKALQTLQPKPDGLGSWGLFLLVDGTSVGVFVFLKLNNEYTLNNGTDFFIPIIGSVSSTAEIGSDD
LFFKALQTLQSKEDGHSWGLFLLDELEEGFLFVFLKLNENTWLRGMNDFFVPLIGLSSSLPAGSRQG-
VFNKALQTLQPNDDGGNCSGLFSLDEFEAGFLFVFLKLNENTWLRCKGNDFFVPLISTSSSLPTGPGQG-
LFFKALQTLQPKDDGNGSGLFSLDGNLEG-----GEDFYVPLITSSSSSLVGT EAT-
-----

Plantainamy
VvamyUD
Rcamy
Hvamy2
Atamy2
IVHEQQAGTEIHDVRNLSLSPNNSYPLQNKSLSEADP--QINSLPMKPGQPEELIEAVAYTDEIIEKIRH
-----QSEGWVKSERVVSVPTETISGKTAGENEIVSDAAYTDGIIINDIRN
-----QSEGLVAS-----GKDAEGNEVSRATYTDIIEIRN
-----EAAQLS-----KHTPKTKNEVSASGETEIRN
-----MANKHLSLFL
-----MGYYNNVDFECN-
-----

Plantainamy
VvamyUD
Rcamy
Atamy3
Hvamy2
Atamy2
LVTDISSEKGRKAKSKEAQNILQEIEKLAEEAYSIFRISIPGFVELASDTEL--LKPAVKLSGSGTGSGF
LVSDISSEKQRKTKTKQAQESILQEIEKLAEEAYSIFRSSIPTFSEDAVLET--LKPPEKLSGSGTGSGF
LVNGISSEKVRKTKTKQAQESILQEIEKLAEEAYSIFRSSIPTFSEVLESEVEKAPPAKICSGTGTGFH
LAIDIHSHKNQKTNVKEQENILQEIEKLAEEAYSIFRSTPTTFSEESILAEAE--KPDIKISGSGTGSGF
VLLGLSAS-----DQTDIGRIVIR-----LASE
-----DGR
-----

Plantainamy
VvamyUD
Rcamy
Hvamy2
Atamy2
EILCQGFNWESHKSGR-WYSELSDKAKELSSLGFTVIVLWLPPTESVSPPEGYMPKDLNLYNL-SRYGSLEEL
EILCQGFNWESNKSGR-WYSELSSKVAELSSLGFTVIVLWLPPTASVSPPEGYMPKDLNLYNL-SRYGSDEL
EILCQGFNWESNKSGR-WHMELEKAAEISSLGFTVIVLWLPPTESVSPPEGYMPKDLNLYNL-SRYGSDLE
EILCQGFNWESHKSGR-WYLELEKADELASLGFTVIVLWLPPTESVSPPEGYMPKDLNLYNL-SRYGTIDEL
QVLCQGFNWESWKHNGGWYVFLMGKVDIILAAAGITHLWLPASQSVLAEGYMPGRLYDLDAKSAKQAL
EVLQAGYNWESHKYD--WWRNLDGKVPDIAKSGFTSAWLPPSSQSLAEPGRIVLQDLYSLN-SAYGSEHLL
-----

Plantainamy
VvamyUD
Rcamy
Atamy3
Hvamy2
Atamy2
KDLVNSFHEVGKVLGDVAVLNHRCACHYQNKNGIWNVFGGR----LNWDDRAIVADDP-HFQGRGNKSSG
KDLVKSFHEVGKVLGDVAVLNHRCACHYQNGQIWNVFGGR----LNWDDRAIVADDP-HFQGRGNKSSG
KDLVKSLSHRVGLKVLGDVAVLNHRCACHYQNGQVWNVFGGR----LNWDDRAIVADDP-HFQGRGNKSSG
KDTVRKFKHKGKVLGDVAVLNHRCACHYKNGQVWNVFGGR----LNWDDRAIVADDP-HFQGRGNKSSG
KSLIIGALHGKGVKVAIADIIVNHRRAEHKGRGIVYICIFEGGTPDARLDWGPDMICRDRPPIADGTPNDTG
KSLLRKMKQYKVRAMADIVNHRVGTTRGHGGMYNRYDGIS--LPWDEHVAVTCCT--GGLGNRSTG
-----

Plantainamy
VvamyUD
Rcamy
Atamy3
Hvamy2
Atamy2
DNFHAAPNIDHSQDFVRRDLKEWLCWLRKEVGYDQWRLDFVIRGFWGGYVKDYMEATEPYFAVGEYWDLSL
DNFHAAPNIDHSQDFVREDIKEWLCWLRKEIGYDQWRLDFVIRGFWGGYVKDYMDASEPYFAVGEYWDLSL
DNFHAAPNIDHSQDFVRRDLKEWLCWLRDEIGYNGWRLDFVIRGFWGGYVKDYMEATEPYFAVGEYWDLSL
DNFHAAPNIDHSQDFVRKDIKEWLCWMMEEVGYDQWRLDFVIRGFWGGYVKDYMDASKPYFAVGEYWDLSL
DNFHAAPNIDHNLNRVQKDIWGLWNLWADIGFDGQWRFDFAKGYSADVAKIYIDRSEPSFAVAEIWTSLSL
DNFNGVPNVDHTQHFRVKDIIVLRLWLRNLTGFGQDFRDFARGYSANYVKEYIIGAALPLFSVGECDWCSN
-----

Plantainamy
VvamyUD
Rcamy
Atamy3
Hvamy2
Atamy2
YT-YGDMDNQDAHRQRIVDWINATN--GTAGAFDVTTKGILHLSALEKCEYWRLLSDQNGKPPGVVGGWAS
YT-YGEMDNQDAHRQRIDWINATN--GAAGAFDVTTKGILHLSALGRCEYWRLLSDQKRPKPPGVVGGWPS
YT-YGEMDNQDAHRQRIVDWINATS--GTAGAFDVTTKGILHLSALGRCEYWRLLSDQKRPKPPGVVGGWPS
YGDGKPNLNQDQHRQELVNWVDKVGKGPATTFDFTTKGILNVAVEG-ELWRLRGTGDKAPGMIGWPPA
YN-GHGLDYNQDSSHRQRIISWIDATG-QISAADFVTTKGILQEAVKG-QYWRLLCDAQKPPGMVGGWPS
-----

Plantainamy
VvamyUD
Rcamy
Atamy3
Hvamy2
Atamy2
RAVTFIENHDTGSTQGHWRFPSPGKEMQGYAYILTHPGTPAVFYDHF--SHYRSEIASLISLRNRNEIH
RAVTFIENHDTGSTQGHWRFPNGKEMQGYAYILTHPGTPAVFYDHF--SHYRSEIASLISLRNRNEIH
RAVTFIENHDTGSTQGHWRFPNGKEMQGYAYILTHPGTPAVFYDHF--SHYRSEIASLISLRNRNEIH
KAVTFVNDHDTGSTQHMWPFPSDRVMQGYAYILTHPGTPCIFDYDFD--WGLKEEIAALLSVRTRHGIH
RAVTFVLDNHDGSTQAHWPFPSHHVMEGYAYILTHPGTPCIFDYDFD--WGLKEEIAALLSVRTRHGIH
-----

Plantainamy
VvamyUD
Rcamy
Atamy3
Hvamy2
Atamy2
CRSTVVKIIVKAERDVYAAEIDGKLAVKIGPGHYEPPDGPWKVVAAEGRDYKVVWETS
CRSTIQITMAERDVYAAIDEKVAAMKIGPGYIEPPKGGQRTWTLALEGKDYKIWETS
CRSSVKITKAERDVYAAIDEKVAAMKIGPGHYEPPSGKN-WSMALIEGKDYKVVETS
CRSEVNIIDKSERDVYAAIDEKVAAMKIGPGHYEPPNGSNNWSVAEGRDYKVVETS
NESKLRITAEADLYLAEIDGKVIKLGPRVDVGNLIPGGFKVAAHGNDYAVVEKI
SRSTVRVLAKEASNLVYAAIVGKELICMKLGDGSGWCPSS--GRDWTLATSGHRYAVWHK-
-----

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Figure 4.12. An alignment of the partial (full length) plantain clone and α -amylases from the three families. Protein sequences were obtained using publicly available data (<http://www.ncbi.nlm.nih.gov/>), aligned using the BLOSUM62 algorithm with the ClustalW alignment tool (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Family I is represented by *Hvamy2*, family II represented by *Atamy2* and family three represented by *VvamyUD*, *RcamyUD*, and *Atamy3*.

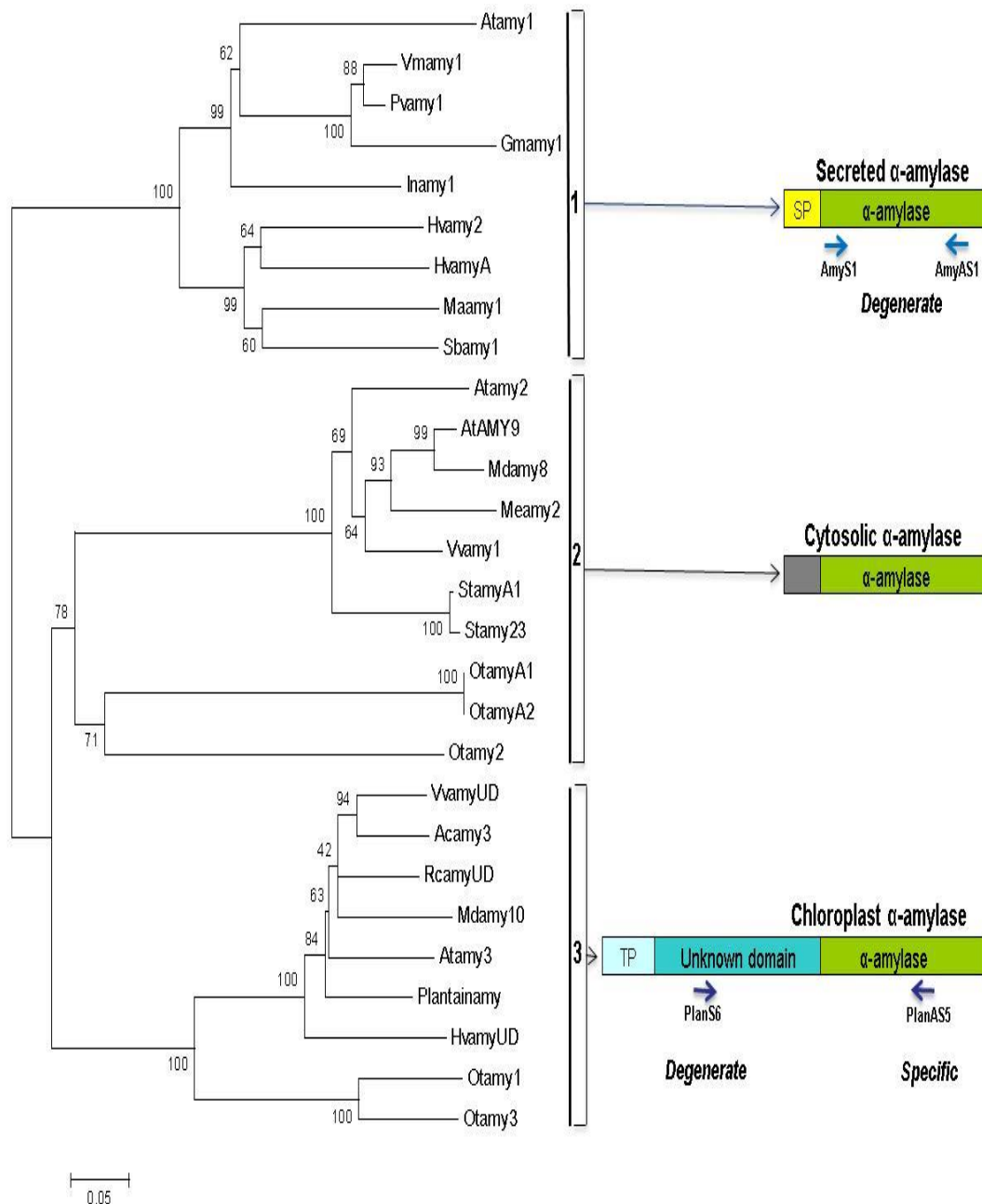


Figure 4.13. Phylogenetic tree of plant α -amylases illustrating an evolutionary relationship between plant α -amylases using only the catalytic domain for comparison. Protein sequences were obtained using publicly available data (<http://www.ncbi.nlm.nih.gov/>), aligned using the BLOSUM62 algorithm with the ClustalW alignment tool (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The aligned sequences were assembled into a phylogenetic tree using the boot-strapped neighbor-joining algorithm (Saitou and Nei, 1987) and the Jones, Taylor, and Thornton amino acid substitution model (Jones et al., 1992) in MEGA 5.05 with 1000 trials (<http://www.megasoftware.net/>) (Tamura et al., 2011). Bootstrap values are indicated as percentages of the 1000 trials at their respective node. Abbreviations: Hv (*H. vulgare*), In (*I. nil*), Pv (*P. vulgaris*), Gm (*G. max*), Vm (*V. mungoculata*), Sb (*S. bicolor*), Md (*M. domestica*), Ma (*M. acuminata*), Me (*M. esculanta*), At (*A. thaliana*), Vv

(*V. vinifera*), St (*S. tuberosum*), Ot (*O. taurii*), Ac (*A. chinensis*), Rc and (*R. communis*). 1, 2 and 3 represent the three α -amylase families. SP represents signal peptide, TP represents transit peptide. All the three families share an amylase domain but differ in their N-terminus. PlanS6 and PlanA5 are degenerate and specific primes respectively used to clone the partial plantain clone with the unknown and catalytic domains.

4.3 Discussion

Biochemical strategy

Variance in α -amylase expression

Significant α -amylase activity was measured in different plantains, with higher activities measured from fully ripened plantains. High activity of α -amylase which is one of the principal enzymes in starch degradation has been reported in ripening fruits (Junior et al., 2006; Prasanna et al., 2007; Fioravante et al., 2008; Goncalves Peroni et al., 2008; Prinsi et al., 2011). Higher α -amylase activity in plantains at later stages of ripening may suggest the role of the enzyme in starch hydrolysis during ripening (Shiga et al., 2011; Soares et al., 2011). Hence, ripening plantains may be good source of α -amylase. However, there was a strong variation in the specific activity from plantain to plantain. This means that routine purification is not trivial and always requires pilot tests to choose the best plantains. This may also have repercussions regarding the molecular approach because variation in mRNA populations could be equally high. Future work will have to address this variability for both approaches.

The biochemical strategy involves extraction of α -amylase from the ripening plantains followed by a variety of purification steps aimed at increasing the specific activity. Ammonium sulphate fractionation was identified as the first step, leading to a strong increase in the specific activity when combined with dialysis. The dialysed material was then subject to ion-exchange chromatography, but problems arose due to low protein levels and loss of enzyme activity in the eluate.

Ammonium sulphate fractionation

In contrast to plantain α -amylase that showed maximum recovery of activity at 30% $(\text{NH}_4)_2\text{SO}_4$, barley α -amylase showed different precipitation pattern with saturation at 43% $(\text{NH}_4)_2\text{SO}_4$ concentration.

The pattern showed by plantain α -amylase is in close agreement to the precipitation of α -amylase *Eisenia foetida* of 35% $(\text{NH}_4)_2\text{SO}_4$ (Ueda et al., 2008). Ammonium sulphate fractionations of α -amylases have been achieved at 35-65% concentration for α -amylase from cowpea, millet, soybean, safflower and azuki bean with a high recovery of enzyme activity has also been reported (Bastos et al., 1994; Mar et al., 2003; Kumari et al., 2010). These differences can be attributed to lots of factors, among which are the size of the proteins, stability at high salt concentration and pI (Ashraf et al., 2008; Azad et al., 2009; Lin et al., 2009). Since most proteins need more than 30% ammonium sulphate to precipitate, fractionation of α -amylase at this concentration leads to higher specific activity thus enrichment of enzyme activity.

Unfortunately, subsequent purifications of the dialysed extract by ion exchange chromatography were inconsistent and irreproducible. First experiments revealed that at neutral pH, the measured α -amylase activity remained bound to Q-sepharose, suggesting that the enzyme is negatively charged under these conditions. Subsequent elution with a salt gradient revealed conflicting results. In one experiment, a defined α -amylase activity peak was eluted whilst in subsequent repetitions the protein bound to the column but failed to elute. At low pH (5.5), the α -amylase failed to bind to either Q-sepharose or S-sepharose, suggesting that it was not charged.

The main problem with the purification strategy may arise from the fact that plantain extracts exhibit extremely low protein concentrations, even when high tissue to buffer ratios was used. This may explain loss of material by unspecific binding to dialysis membranes and ion-exchange columns. Another factor is the high viscosity of the extract due to cell wall polymers that may interfere. These include high molecular weight polysaccharides that may bind to the column, and interfere with the binding of the protein. Most of these would be lost during the initial ammonium sulphate precipitation, but further steps may have to be taken

to lower the viscosity of the re-suspended pellet prior to ion exchange chromatography.

Molecular strategy

In parallel to the biochemical approach, a gene cloning strategy based on degenerate primers corresponding to conserved regions in plant α -amylase catalytic region was attempted. The strategy was based on the assumption that conserved domains in known plant α -amylases are also present in plantain α -amylases. The approach has led to the discovery of a type 3 class of non-secreted α -amylases in plantains. However, the approach has also been faced by specific challenges that require further work.

Optimisation of RNA extraction from ripening plantain

In order to obtain good quality cDNA for PCR amplification, a RNA extraction protocol needed to be established for the plantain. Conventional RNA extraction strategies from tissues use several chemicals and steps to remove contaminating substances such as proteins, polysaccharides, lipids (Birnboim, 1988; Suzuki et al., 2004). However, extraction of RNA from plantains was not straightforward due to the high abundance of polyphenols and polysaccharides such as starch and pectin. The polysaccharides may possess similar physicochemical features to RNA; and they can also co-precipitate thus contaminating the RNA. Initial methods tested led to low yield and low quality RNA partly due to the reason mentioned earlier (Birnboim, 1988; Asif et al., 2000; Suzuki et al., 2004). Perhaps, a vigorous protocol that has many additional steps compared to earlier RNA extraction method was used. Example of such step is the use of 0.1 volume of ethanol and 1/30 volumes of 3M Na acetate that kept RNA in solution and precipitates the polysaccharide. An addition of Na acetate to a final concentration of 0.3M and 3 volumes of ethanol precipitates the RNA. The use of 20mM EDTA and 2-mercaptoethanol removes the polyphenols (Asif et al., 2000). Consequently the $A_{260/230}$ ratio measured

indicated that there was very low or no contamination by polysaccharides and polyphenols. In the same vein the $_{260/280}$ ratio also showed that there was no protein contamination in the RNA (Asif et al., 2000). This was further evident by the RNA yield which was exceptional. The method of Asif and colleagues used ensured minimum levels of contaminants that do not interfere with the integrity and quality of the RNA. The RNA was then used for the synthesis of complementary DNA.

Plantains express a family three amylase

The identification and cloning of complete α -amylases from plantains was based on sequence homology between the proteins in the database. Degenerate primers were designed based on conserved domain homology between the different secreted plant α -amylases. Interestingly, the amino acid sequences deduced from the longest open reading frame showed high similarity to non-secreted and chloroplastic α -amylases from plant species such as *R. communis* AMY10, *M. domestica* and AMY3 of *A. thaliana*. The homology to this class of enzyme was far greater than the similarity to secreted α -amylases from barley and other plant species. This suggests that mRNA levels of secreted amylases are either very low or absent in ripening plantain. This is interesting because the recently published genome sequence of the related plant banana revealed a secreted type of α -amylase but no plastid type amylase (D'Hont et al., 2012).

To fully obtain the catalytic domain which is at the C-terminus of the protein, a 3'RACE was performed successfully. The sequences obtained from initial amplifications and that from RACE were all assembled. An alignment of the partial full length clone of plantain amylase was performed with α -amylases from the three families. The result indicated a closer homology to family three α -amylases than those from families one and two. From this, it is evident that the plantain clone identified is of family type 3 of α -amylases. The alignment also enables us to identify the missing part of the sequence. This was surprising because the

degenerate primers used for the amplification were designed based on conservation of the secreted α -amylases.

Ripening plantains express a plastid type amylase

In order to establish the presence of this unusual type of α -amylases in plantains, degenerate primers were designed in the unknown domain region and some specific primers in the catalytic region of the protein. PCR amplification of the plantain cDNA with these primers and sequencing confirmed that the identified cDNA encoded a plastid-type 3 amylase. It may be possible that ripening plantains expresses the plastid amylase as the predominant starch hydrolase.

Bioinformatics and phylogenetic studies categorised plant α -amylases into three families, secreted, cytosolic and a chloroplast types. The longest open reading frame revealed that plantain α -amylase is of the family three type (Stanley et al., 2002; Junior et al., 2006). This amylase has been reported to catalyse hydrolysis of diurnal and storage starches in the plastid (Stanley et al., 2002). The three families have differences in their N-terminal domain however there is good structural similarity in their C-terminus (catalytic) domain. This homology may indicate that they share a common ancestral origin (Stanley et al., 2005). However, there are diverse opinions and arguments on conserved regions in plant α -amylases (Janecek, 2002).

Chapter 5

5 Functional analysis of the plantain amylase

5.1 Introduction

Alpha amylase is the most popular among the starch hydrolase because it has been described as the only enzyme capable of digesting intact starch molecule at endogenous glycosidic bonds (Irving et al., 1999), and it is found in different organisms. However, previous research efforts have been mainly focused on the identification and characterisation of heat stable microbial α -amylase with little done on plant amylases. Heat stable bacterial and fungal α -amylases are more popular in the industries due to the high temperatures that are used for starch gelatinisation (Gupta et al., 2003; Demirkan et al., 2005). In contrast to the microbial amylases, plants α -amylases with raw starch digestion abilities have being poorly studied and explored (Janecek et al., 2011).

Plants α -amylases are classified into three families. The α -amylases show very high homology in the catalytic domain of the protein that is composed of more than four hundred amino acids. However, there exist structural differences in the other regions of the proteins (Janecek, 2002). The family one amylase which are secreted have signal peptides, family two are cytosolic protein and do not have any targeting peptide (Stanley et al., 2005). The α -amylases of family three have transit peptides for translocation to the chloroplast at the amino terminus. In addition, the chloroplast amylases also have additional domain in between the transit peptide and the amylase domain referred to as unknown domain because its actual function is yet to be fully established (Stanley et al., 2002; Yu et al., 2005).

In living plant tissues, starch degradation often takes place at low temperatures therefore; enzymes of starch degradation possess some additional features for effective and tight binding to the substrate molecule. This can be a specific substrate binding site in the catalytic domain of the enzyme or a carbohydrate binding module (CBM) or starch binding domain (SBD) (Machovic and Janecek, 2006b). Family three α -amylases possess an unknown domain that has been recently described to be a carbohydrate binding module specifically referred to as starch binding domain (SBD) (Glaring et al., 2011). This domain has been suggested to assist in binding of the enzyme to the raw starch in plants. The class of α -amylase with starch binding domain may offer the opportunity of starch processing without prior gelatinisation. The SBD can also be used as an affinity tag or for targeting in planta in starch engineering (Janecek et al., 2011).

The gene encoding a putative plantain protein identified in chapter four of this thesis is a member of the family three type of α -amylases (Stanley et al., 2005; Yu et al., 2005). This was based on the sequence homology shown between the plantain amylase and the family three proteins (Figures 3.6, 3.12 and 3.13). The general description and function of the plant amylases in the literature is based on the roles in α -amylases play in transitory starch degradation in leaves or germination in seeds of cereals (Zeeman et al., 2004; Delatte et al., 2006; Lu and Sharkey, 2006; Andriotis et al., 2010). Little is known about the plastid type of α -amylase family as regards their functions and the roles they perform in plants. Therefore, to understand the function of the family three amylases there is need to characterise the plantain amylase which is a member of the group. This will also provide knowledge on the peculiar properties of this group of proteins and their functions

Here it is shown that the catalytic domain of the amylase does not exhibit protein activity if expressed as an independent domain without the SBD in the cytosol. To test if the SBD is needed for enzyme activity, the full

length Arabidopsis Amy3, a homologue of the plantain amylase was cloned and tested in electroporated protoplasts. The full length protein did not exhibit amylase activity, therefore; the results may suggest that the maltoheptaoside used in the assay system may not be a good substrate for the type three α -amylases.

5.2 Results

Generation of antibody against the plantain α -amylase

In order to confirm the presence of a type 3 α -amylases in plantain and determine its molecular weight, an antibody against the α -amylase was required. The process of the antibody generation was hampered by a number of un-expected challenges therefore; the individual steps in the generation of the antibody will be presented and discussed. However, similar challenges were encountered as those in the production of antibody against barley α -amylase described in the chapter 2 of results section of this thesis. Therefore, only some peculiar differences will be described.

Establishing an expression protocol for amylase fused to GST

The expression was performed as described before for the GST-fused barley α -amylase in result chapter 1. In order to purify the barley α -amylase from the mixture of cell proteins using affinity purification, the protein was fused to the 26kDa glutathione S-transferase (GST) (Stofkohahn et al., 1992; Liu et al., 2006; Abhary et al., 2011). This would enable the purification of the recombinant protein using a GST column. After gene induction, cell extracts and all pellets were boiled in sample buffer and were subject to gel electrophoresis. Figure 5.1 shows the pattern of recombinant proteins obtained with GST and α -amylase fused to GST. An additional protein of the expected molecular weight (70 kDa) was observed in the induced samples after 3 hours of induction or more. This band was absent in the GST control which showed a lower molecular weight GST band instead. It was also observed that longer induction time may be unnecessary because significant recombinant protein levels were obtained after only 3 hours. Comparable levels of expression were obtained for both star and gold strains of the *E. coli* cells (Figure 5.1).

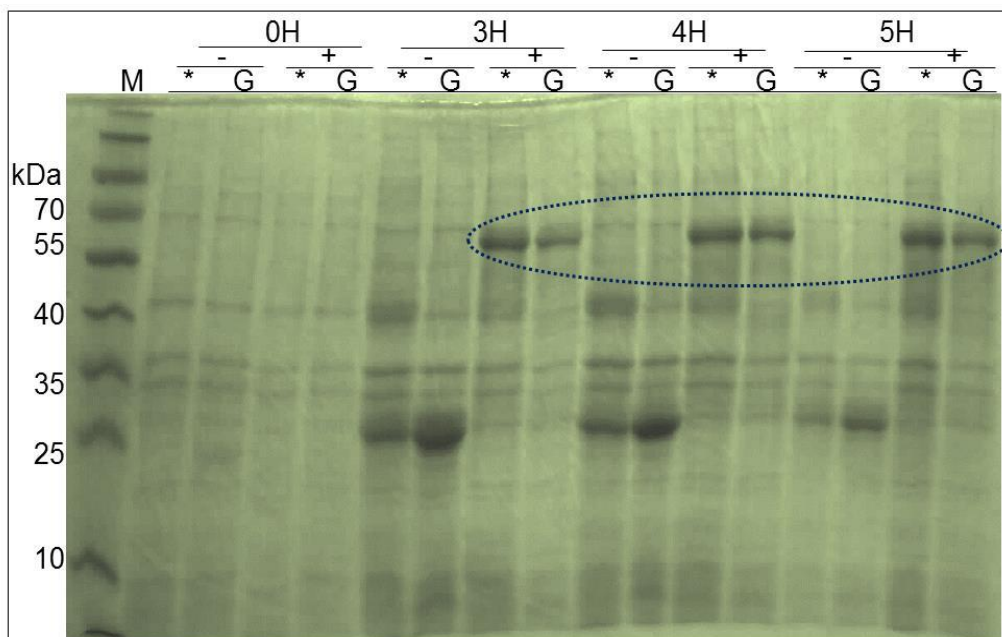


Figure 5.1. Expression of the plantain alpha amylase in *E. coli*. M is the marker, * and G denotes the BL21 Star™ and Gold™ (DE3)pLysS *E. coli* strains, respectively. While – and + represents GST only and GST-fused amylase respectively. 0, 3, 4 and 5H represents hours after induction. The cycled region shows the expressed amylase of the expected molecular weight of 70 kDa. The GST only of 26 kDa is also visible.

Recombinant protein solubilisation

Extraction using different buffers

To be able to purify the protein from numerous cell proteins using the GST-tag as affinity bait on GST columns, induced cell pellets were extracted to obtain soluble proteins. The cells were pelleted but re-suspended in buffers of different compositions followed by cell disruption using sonication. Equal quantities of supernatant (S) and cell pellets (P) were boiled in sample buffer and loaded on a gel. Figure 5.2 shows that the protein of interest (70 kDa) was only detectable in the insoluble fraction (P) but could not be observed in the soluble portion (S). This indicates that soluble GST fused amylase was solubilised under these extraction conditions.

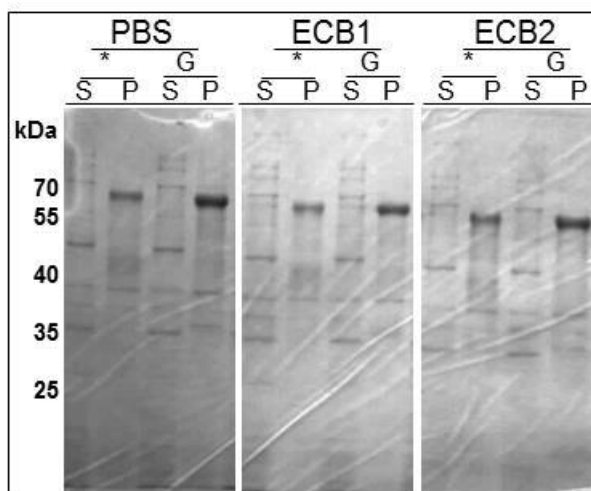


Figure 5.2. Extraction of recombinant protein. S and P are supernatant and pellet, respectively. * and G denotes the BL21 Star™ and Gold™ (DE3)pLysS *E. coli* strains, respectively. PBS (phosphate buffered saline), ECB1 and 2 (*E. coli* buffer 1 and 2) are the buffers used to extract the recombinant protein.

In order to extract soluble proteins, the experiment was repeated using the same expression and induction protocols. However, to enhance protein solubility, two different growth temperatures of 28 and 37°C were used this is because it has been suggested that expression at lower temperatures increase solubilisation efficiency. To test if the protein can be solubilised by buffers of different strengths, phosphate buffered saline (PBS), phaseolin buffers and bug buster (Merck®) were used (see section 2.2). Higher sonication amplitude of 50% was used to disrupt the cells, cell extracts and pellets were boiled in sample buffer and subject to gel electrophoresis. Figure 5.3 shows that the desired GST-fusion protein was still partitioned to the insoluble fraction with very little or even no soluble fusion protein extracted. Comparable amount of protein was detected with both the bug buster and phaseolin (Figure 5.3). This indicated that the strength of the buffers did not influence the extraction of soluble protein. Similarly, comparing the two different growth conditions of 28 and 37°C revealed that higher recombinant protein levels were achieved at the lower temperature as shown in Figure 5.3 (panel A and B).

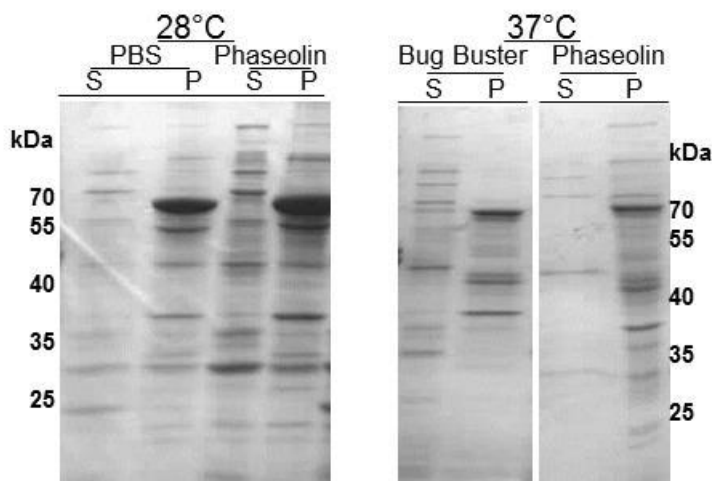


Figure 5.3. Extraction of recombinant protein in high strength buffers. S and P are supernatant and pellet, respectively. The expression was performed using the BL21 Gold™ (DE3)pLysS *E. coli* strains at 28°C and 37°C respectively. The proteins were extracted in PBS (phosphate buffered saline), bug buster and phaseolin buffers.

Changing the expression condition

Having failed to extract the soluble protein using two different temperatures of 28°C and 37°C, and buffers of different strengths; a different approach was required. In order to solubilise the protein, the expression was performed at lower temperatures of 10, and 16 °C that this is because protein aggregation has been suggested to reduce at low temperatures. This is because lower temperature usually means lower expression levels. Also, different media LB and TB were used for the growth of the bacteria, and low concentration of the inducer IPTG of 1 mM was used. After induction, centrifugation, sonication; the supernatant and the pellet were loaded on a SDS-PAGE gel. Figure 5.4 shows the recombinant proteins obtained using the different media and growth conditions. But the lower temperatures did not offer enrichment to the amount of protein compared to what was obtained at higher temperatures. However, comparable amount of protein was obtained with expression at 10 and 16°C (Figure 5.4, panel A), this is also the case with the two media used however soluble GST-fused amylase could not be obtained.

Auto-induction

In order to increase the solubility of the recombinant protein as was explained in section 2.2, an auto-induction experiment was performed in addition to the different conditions used. After induction, the supernatant and pellet were boiled in sample buffer and subject to gel electrophoresis. Figure 5.4 panel A the protein pattern observed with the auto-inductions with insignificant amount of soluble proteins obtained (Figure 5.4, panel A). This implied that the auto-induction condition was not able to render majority of the protein soluble. To increase the amount of soluble compared to insoluble proteins, the protocol should be improved. In order to increase the protein solubility by enriching the amount of the product, the auto-induction experiment was repeated in larger volume of cultures to increase the yield. Figure 5.4 (panel B) shows the soluble and insoluble fractions that were obtained after extraction and cell lysis however the solubility could still not be enhanced.

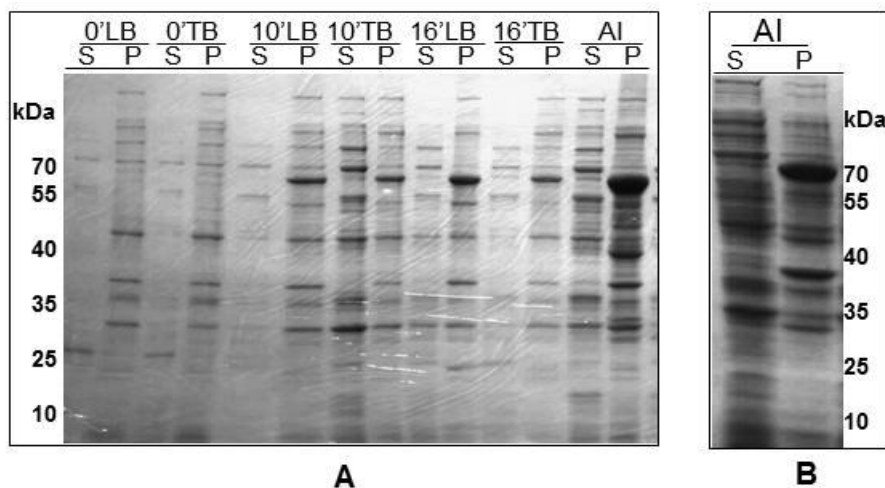


Figure 5.4. Protein expression using different media and lower temperatures. Panel A shows the recombinant products obtained using LB and TB media as well as different temperatures for the growth of the culture. S and P are supernatant and pellet, respectively. AI represents the auto-induction protocol. The expression was made using the BL21 Gold™ (DE3)pLysS *E. coli* strains. The proteins were extracted in phaseolin buffer. Panel B shows a repeat of auto-induction.

From the results presented above, it can be concluded that the recombinant product could not be solubilised using a variety of different approaches attempted, including different growth conditions and buffer types. Therefore, a way of purifying the protein of interest from the insoluble portion should be used.

Purifying the insoluble protein

In order to purify the desired recombinant protein from the insoluble fraction, the protein, it was decided to separate the GST-fusion on a denaturing protein gel. It was first necessary to quantify the amount of recombinant protein. After induction and growth, the cultures were spun, the supernatant recovered and pellet was re-suspended in phaseolin, and mixed with sample buffer mix and boiled at 100°C and were subject to gel electrophoresis. Figure 5.5 shows the recombinant protein against BSA standard and the protein was estimated as 0.5 µg/µl.

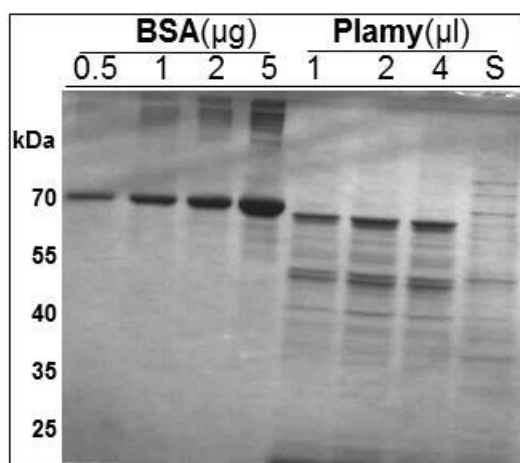


Figure 5.5. Quantification of recombinant protein, bovine serum albumin (BSA) was used as a reference standard to estimate the recombinant protein. The amount of BSA in microgram of 0.5, 1, 2, and 5; while the Plamy is the plantain amylase fused to GST. The amount is in micro-litres of 1, 2, and 4. S indicates the supernatant.

Final expression of the plantain amylase and purification

In order to enrich the amount of the recombinant protein, the expression was repeated using a different condition. The pre-culture was incubated at 37°C while the main culture was grown at 28°C this is to have a better

expression. The induction protocol was as described above except that the culture was grown for four hours after induction. This is to increase the yield of the recombinant proteins since the expression at a lower temperature of 28°C was initially observed to be better than that at 37°C (see Figure 5.3). To effectively disrupt the cell five consecutive cycles of sonication at 40% amplitude were performed. The pellet was diluted by re-suspending it in sample buffer of different amount as shown in Figure 5.6. The protein was quantified against BSA standard as shown in Figure 5.6 and the protein was estimated to be 2 µg/µl.

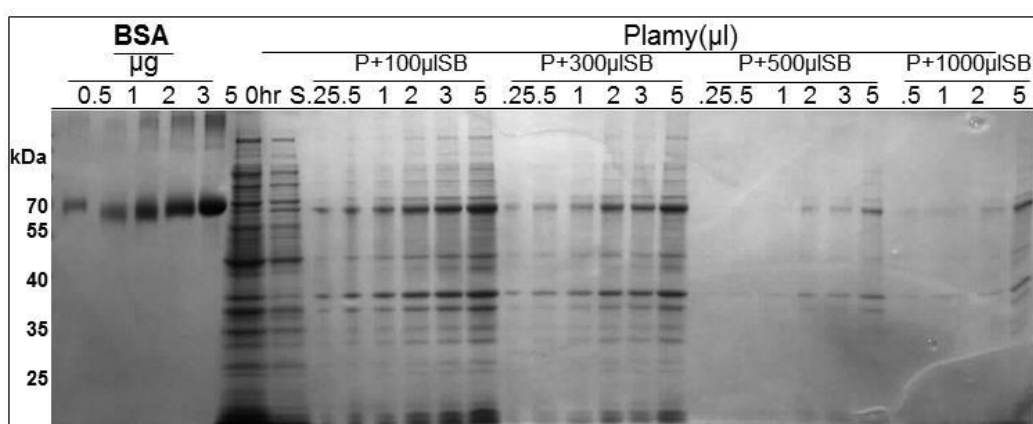


Figure 5.6. Quantification of recombinant protein 2, the expression of GST-fused amylase was repeated at 28°C, S is the supernatant. The protein was again quantified against Bovine serum albumin (BSA). The amount of BSA in microgram of 0.5, 1, 2, and 5; while the Plamy is the plantain amylase fused to GST. The pellet was extracted using different dilutions of sample buffer.

Preparative gel

In order to purify the insoluble recombinant proteins from gel, 250 µg of proteins which is the amount needed of the antigen for individual injection for the immunisation procedure was prepared. A 10% preparative SDS-PAGE gel was made and the proteins were loaded and ran slowly. The gel was stained with coomassie brilliant blue for one hour and de-stained overnight. Figure 5.7 shows a preparative protein gel. The protein of interest can be detected as shown in the Figure 5.7. This band of interest of was thinly cut out with razor blade and transferred into a microfuge tube and weighed. This procedure was repeated eight times

because four injections are required for two rabbits each. The purified protein was sent to Eurogentec for the antibody generation programme.

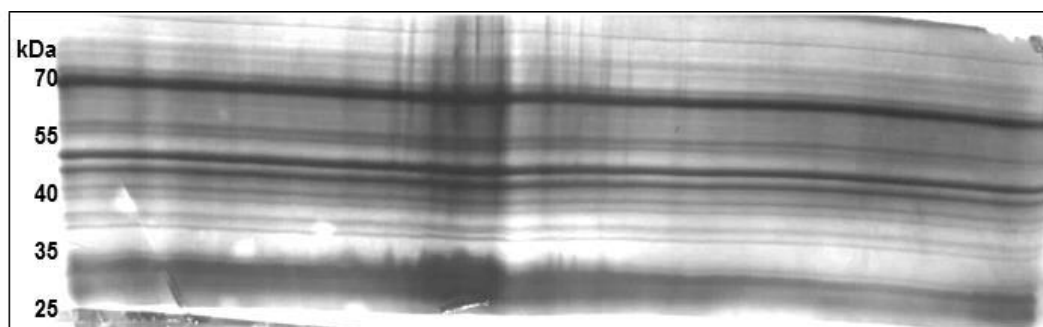


Figure 5.7. Preparative gel, the Figure shows the expressed GST-fused amylase loaded on 10% preparative gel. The proteins were resolved and separation that enabled the cutting of the protein of interest from gel with a razor blade was obtained.

Characterisation of antibodies

Antisera against the plantain α -amylase were received from the company. These include the pre-immune, small and large bleeds. In order to characterise the antibodies generated, determine their purity, titre and specificity, the GST fused α -amylases from plantains was expressed in *E. coli*, and the samples were used to probe the different antisera. Figure 5.8 shows the western blot of the different antisera from two different rabbits. The antibody from rabbit 1 shown in Figure 5.8 panel A was able to specifically detect the recombinant α -amylase from plantain. But the detection ability was very low, and some background contaminants were also observed. This is the case for both the small and large bleeds, and it may imply that the quality of the antibody is very low. On the other hand, the pre-immune bleed did not detect anything as expected and free from background contamination. However, the antisera from the second rabbit as evident from Figure 5.8 panel B could weakly detect even the highest concentration of the protein. That means the quality of the antisera from the second rabbit was very low. All the three bleeds of the antisera did not give any background bands.

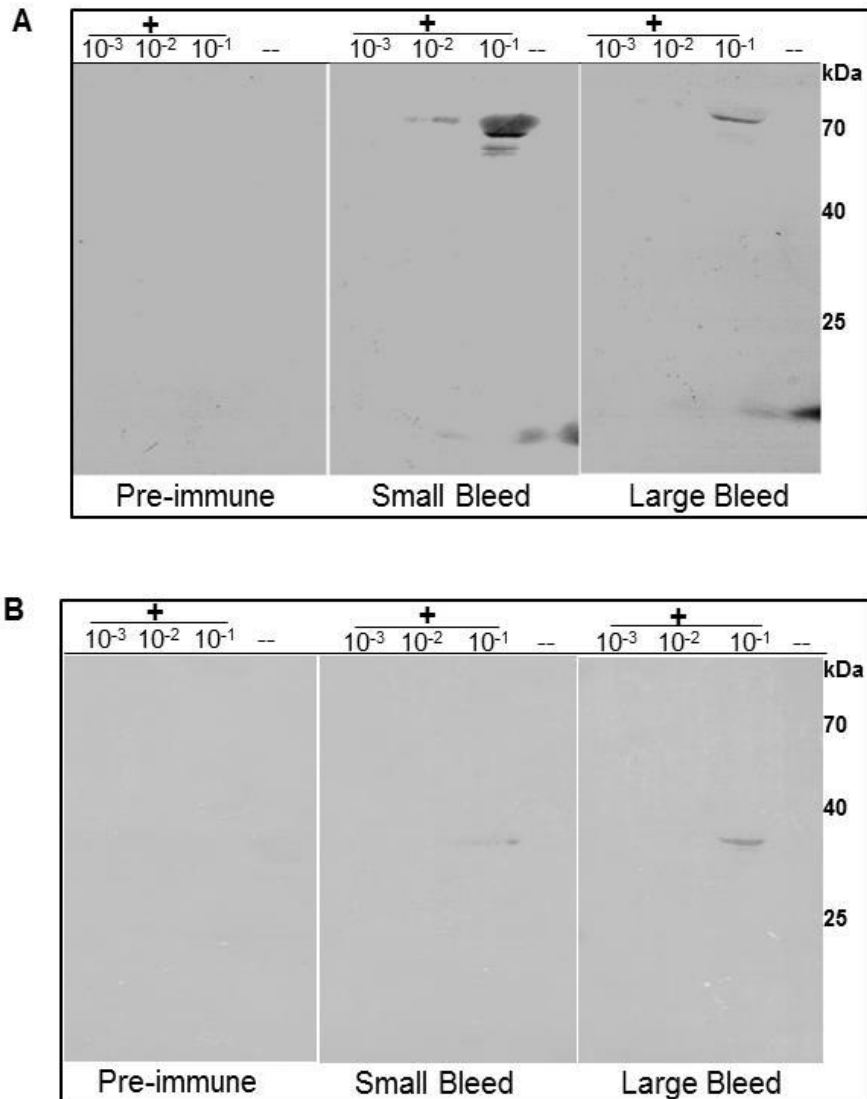


Figure 5.8. Characterisation of the anti-plantain amylase, the three bleeds; pre-immune, small and large bleeds were analysed for quality. Panel A shows antisera from rabbit one while panel B shows bleeds from a second rabbit. The proteins are the expressed GST-fused plantain amylase. Three dilutions of the recombinants proteins were used; 10, 100, and 1000 folds.

It was not possible to detect endogenous plantain α -amylase in plantain extracts with the highest activity as measured in Figure 4.1 (Chapter 4) of this thesis. Due to time constraints, a repeat of the antibody generation was not possible.

Expression of plantain amylose In planta

In the absence of specific antibodies, the remainder of this work was devoted to characterising the biological role of type 3 α -amylase identified from plantains. In order to characterise the plantain α -amylase, it was initially necessary to determine the enzyme activity of the clone in the cytosol, therefore the catalytic domain of the protein (see Fig. 4.11, panel B) was cloned into a small pUC vector under the transcriptional control of the 35S promoter. It was then expressed transiently in the protoplast alongside a positive control which is a construct of the barley α -amylase. The activity of the protein in the protoplast was then measured in both the medium and cell. The total amylase activity was determined and is shown in Figure 5.9. Even though very weak or no α -amylase activities were detected for the plantain amylase in comparison to the control (Figure 5.9); the protoplasts were competent for transfection as evident from the significant activity exhibited by the positive control.

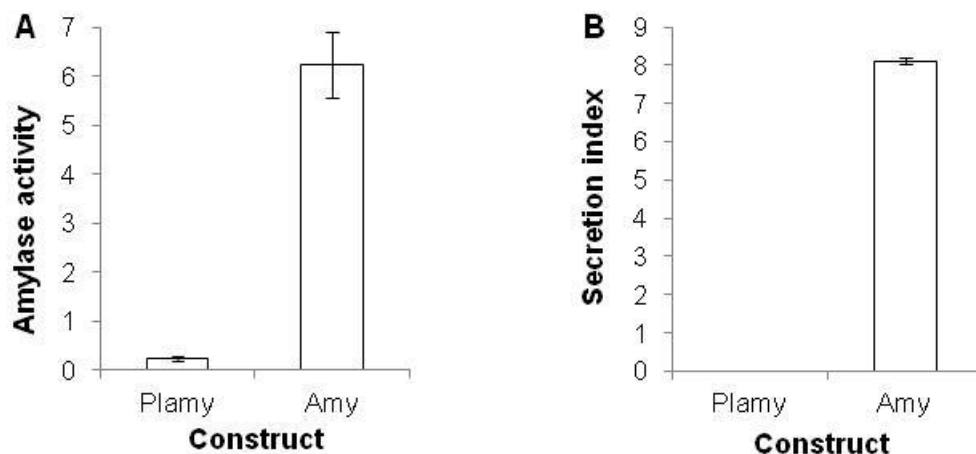


Figure 5.9. Activity of the plantain α -amylase in cells, the properties of the plantain α -amylase (plamy) and the barley α -amylase (amy) which is a positive control for the experiment are shown. Panel A shows the total amylase activity while panel B shows the secretion index which is the ratio of the medium to cell activity.

Measurement of the activity in the culture medium permitted calculation of the secretion index (Fig. 5.9, panel B). As expected significant

secretion was obtained for the standard barley α -amylase whilst no activity in the medium was observed for the sample electroporated with the plasmid encoding the catalytic domain of the plantain protein in the cytosol. Based on the results obtained above (Figure 5.9), it was hypothesised that it is possible that the plantain amylase and the family three protein activity is dependent on the unknown domain or that the cytosol is an environment that does not support the amylase activity. However the full length plantain amylase is yet to be cloned, therefore, cloning of a homologue of the protein from Arabidopsis whose sequence is available in the database (Yu et al., 2005) was considered.

Expression of plantain amylase, Atamy3 In planta

In order to test the hypothesis on whether the unknown domain of α -amylase of the family three has any role in the protein activity, specific primers were designed based on the gene sequences available on the public database (Yu et al., 2005). The Arabidopsis cDNA was PCR amplified using the primers and the amplicon was cloned into a pUC vector under the control of the 35S promoter. The cloned Atamy3, the plantain α -amylase alongside, the barley α -amylase as a positive control were then tested in plant expression vectors via transient expression in tobacco protoplasts. The activity of the protein in the protoplast was then measured in both the medium and cell, and the total activity was determined. As shown in Figure 5.10, amylase activity was not detected neither from the plantain amylase nor the Atamy3 but activity was detectable for the positive control. This is because negative absorbances should be interpreted as zero activity.

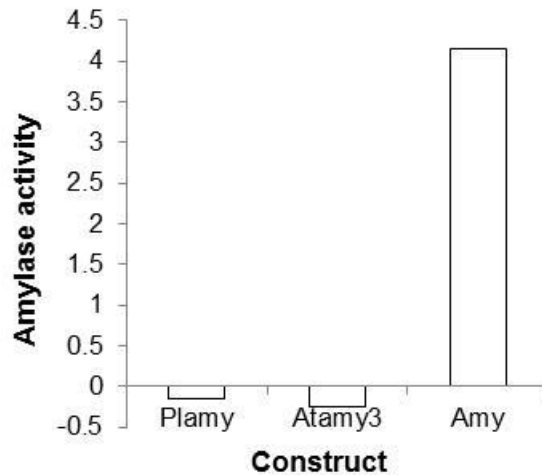


Figure 5.10. Activity of the plantain α -amylase and full length Atamy3. The total amylase activity of the plantain amylase (plamy), Arabidopsis amylase 3 (Atamy3) and the barley amylase (amy) are shown. However negative activities were obtained with the plantain amylase and Atamy3.

Fluorescently tagged Atamy3 starch binding domain

Enzyme activity was not obtained with the family three α -amylases either as a short protein with the amylase domain expressed in the cytosol or the full length protein. This seems to correspond to earlier claim regarding Atamy3 knockout plants which had no phenotype (Yu et al., 2005). To study the behaviour of this group of proteins further, it was necessary to characterise the unknown domain. Therefore, primers were used to PCR amplify the complete SBD of Atamy3 and cloned under the transcriptional control of the 35S promoter with a fluorescent protein tagged at its C-terminus replacing the catalytic domain. Hence a fusion protein of the unknown domain and YFP was produced. It was tested by transfection in tobacco protoplast alongside a positive control which is Rab7, and was expressed for 24 hours. The cells were then visualised under the microscope for fluorescence. No proteins were seen to have labelled the chloroplast or the entire cells with the unknown domain fused to the YFP. In order to determine if the construct was functional, the cells were loaded on a gel and detected using anti-YFP antibody. Figure 5.11 shows an image of the western blot for the functionality of the YFP tagged unknown domain construct. The protein was detectable with the anti-YFP antibody which implies that the recombinant proteins

were produced as also evident by the positive control which is Rab7 fused to YFP. It was not possible to test the subcellular localisation, nor to test if overexpressed SBDs without the catalytic domain had a dominant-negative effect on starch degradation.

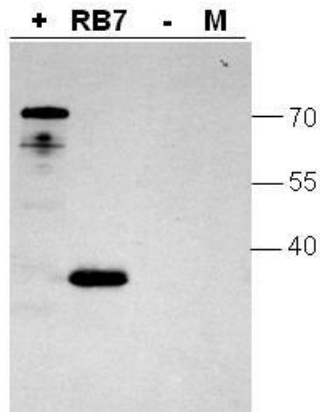


Figure 5.11. YFP fused unknown domain of Atamy3, the western blot shows the unknown domain of the Atamy3 fused to YFP. + is the unknown domain fused to YFP, RB7 is Rab7, - is the negative control and M is the marker lane. The arrow indicate band of the expected molecular weight of approximately 75 kDa. The figure shows that the experiment worked and that the proteins were expressed.

5.3 Discussion

Antibody generation

The production of antibody against the plantain α -amylase was similar to that of the barley amylase described in chapter 1 of the results section and the same protocols were explored. The discussion will not be repeated but some specific observations as relates the plantain GST-fusion will be discussed. This because each protein has its peculiar characteristics and the main variable in expression is the nature of the recombinant protein itself. Expression of the recombinant proteins in *E. coli* may also be connected with the codon usage or the growth condition as well as the specific nature of the fusion to add as affinity tag (Trabbic-Carlson et al., 2004). Results presented here suggest that production of the GST-fused amylase in *E. coli* led to well defined high expression levels (Stofkohahn et al., 1992; Liu et al., 2006; Abhary et al., 2011). As shown in Figures 4.1 to 4.5, the yields of the recombinant α -amylase-GST fusion were high and not limiting factors. Although, tagging of proteins to GST has been suggested to decrease the formation of inclusion bodies (Kusnadi et al., 1997; Rabhi-Essafi et al., 2007; Deceglie et al., 2012). The tagging did not seem to enhance the protein solubility in *E. coli* in the case of the plantain α -amylase GST fusion similar to results obtained with the barley α -amylase GST fusion (see chapter 2.2, Figures. 2.1-2.5)

Further, changing the expression conditions such as lower IPTG concentration, temperatures, buffers did not increase the protein solubility similar to results obtained with the barley protein (see section 2.2). None of these attempts provided evidence for the presence of small quantities of soluble GST-amylase fusions that could be purified by affinity. To yet enable the solubilisation of the amylases, different buffers were used. Protein solubilisation was not achieved using phosphate-buffered saline. The more drastic extraction procedure using lysozyme treatment prior to extraction with the detergent-rich phaseolin buffer was

also unsuccessful. The protein fusion remained firmly associated with the insoluble pellet (Figure 5.3).

All the different conditions attempted to solubilise the amylase GST failed; therefore the insolubility was used as a purification method instead. Using harsh extraction conditions, large amounts of the contaminating proteins were removed as they were solubilised and retaining the insoluble fraction consequently led to a strong enrichment. The final step involved purifying the protein on SDS PAGE (Laemmli, 1970; Wilm et al., 1996), this gave very sharp well defined band of the insoluble GST-fusion protein which could be cut directly from the gel after coomassie staining. However, limitation of this strategy is risk of having multiple proteins of the same size although it was decided to accept this risk. The obtained antigen did not yield acceptable antiserum when tested (Fig. 5.8). Therefore, the antibody cannot be used as a tool in blotting to recognise plantain α -amylase

The cytosolic plantain α -amylase and full length Atamy3 do not exhibit enzyme activity using the standard amylase assay

The catalytic domain of the plantain α -amylase was tested for expression and functionally in protoplasts. The undetectable activity of the catalytic domain of plantain α -amylase can be explained by the fact that the unknown domain may be a pre-requisite for the protein function. This may be possible if the domain is responsible to facilitate the effective binding of the enzyme with its substrate before catalysis can occur. Therefore, in order to test this hypothesis the full length protein from a Atamy3, a homologue of the plantain α -amylase was cloned and tested in electroporated protoplasts (see the following section). Perhaps, a reasonable inference may be drawn on the behaviour of this type of unusual chloroplast α -amylase (Yu et al., 2005). Similarly, the full length Atamy3 tested in electroporated cells did not yield enzyme activity.

The results revealed that the catalytic domains of family amylase as well as the full length protein do not exhibit enzymatic activity using the maltoheptaoside assay. This lack of enzyme activity by the plantain amylase and Atamy3 may imply that the unknown domain has no effect on the amylase activity. However, a significant enzyme activity was detected with the catalytic (C-terminal) domain as well as full length Atamy3 which is a homologue to the plantain amylase (Yu et al., 2005). It cannot be ruled out either that the amylase substrate in the kit may be an inappropriate substrate for the enzyme. If the amylase (family 3) is unable to recognise maltoheptaosides, the loss of activity could be explained. In the work carried out by Yu and colleagues, the amylase activity was detected based on the ability of Atamy3 to digest soluble starch as substrate. The soluble starch is structurally similar to the endogenous substrate of the enzyme in plant tissues (Yu et al., 2005); unlike the maltoheptaosides that was used for the assay and which is readily hydrolysed by the secreted barley enzyme. Therefore, this may also support the hypothesis that this type of amylase only act on starch and not shorter glucans such as the maltoheptaosides.

Another hypothesis is that the cytosol may not be a convenient environment and does not support the amylase activity for the family 3 proteins. This may be true because the proteins are localised to the chloroplasts and previous research has revealed that Atamy3 released both linear and branched malto-oligosaccharides from starch into the chloroplast (Yu et al., 2005; Streb et al., 2012).

The SBD and localisation of family three α -amylases

The N-terminal sequence of family three α -amylases contains a large starch binding domain (SBD) which is absent from the secreted α -amylases from barley and other different plant species. There is no obvious information available on the possible biological function of this domain. Previous research work has described the functionality of the unknown domain of the family three amylases in terms of binding to

starch. Some plant hydrolases have been reported to contain domains or modules that bind carbohydrates such as cellulose or starch (Tibbot et al., 2002; Rodriguez-Sanoja et al., 2005). These domains have been proposed to complement the activity of α -amylases, possibly by helping to establish tighter interactions between the substrate and the enzyme. In family three α -amylases, the unknown domain has been suggested to be a carbohydrate binding module (Glaring et al., 2011). It also has been suggested that the binding of the full length protein may involve the presence of secondary binding sites in the amylase domain (Glaring et al., 2011).

In this thesis, the functionality of the SBD was analysed (Figure 4.11), it was shown that the N-terminal domain (48 kDa) of family three proteins fused to YFP revealed a protein of 75 kDa. This means that the fusion protein was expressed as predicted but it was not fluorescent. The reason for this behaviour is likely to be that the unknown region which is suggested to be the starch binding domain initially associates tightly with the starch molecule before the enzyme through the amylase domain can digest the starch. It was previously shown using a fluorescently tagged unknown domain of Atamy3 that the full length protein exhibits both in vitro and in vivo binding to starch (Glaring et al., 2011). It is possible that the fluorescence of the fusion protein was below the detection limit. Further work will reveal how chloroplast-targeting of SBD-YFP occurs, and if such a molecule can act as a dominant-negative mutant that affects transitory starch metabolism.

Chapter 6

General Discussion

My PhD work was mainly concerned with the continuation of the biofuel project, specifically focussing on the limiting factors that affect the entire process. This involves answering key questions that relates to the properties of the α -amylase and its key role in mediating starch liquefaction. A major line of research involved optimising the starch hydrolysis process to ensure maximum yield of the desired products. Amylases are required that can act on starch to produce mainly simple and fermentable sugars. These sugars include the single, double or triple chain glucose referred to as glucose, maltose and maltotriose. It is of no doubt, that availability of such proteins will increase the efficiency of industrial starch saccharification. This is because it will reduce industrial waste, loss and will add value to the products. In view of the above, the use of barley α -amylase was investigated within the combined acid hydrolysis and enzymatic processing of starch. In addition, a new α -amylase with different properties was identified and characterised from plantains.

6.1 Epitope tagging as a means of affinity purification of proteins: effects of histidine octapeptide on protein property

A cascade refinery process was designed based on the initial success of recombinant barley α -amylase production in potatoes; it implies that the potatoes will be used as a model to produce a range of high value proteins. The barley α -amylase was tagged with a histidine octapeptide that enables the purification of the amylase using a nickel column (Marusic et al., 2007), but also permits purification of a second high value protein from the flow through.

Lower amount of α -amylase was produced with the histidine octapeptide fusion compared to the untagged amylase in the protoplast. In contrast to the barley amylase, the histidine fusion exhibited faster secretion which may be due to the properties of histidine. However, a reasonable expression of the histidine fusion protein was obtained in leaves compared to other α -amylase fusions. Though amylase tagged with eight histidines was glycosylated in transiently expressing leaf epidermal cells, the glycosylation was observed to be enhanced in stable transgenics which may be due to the transformation efficiency.

In the work of Marusic and colleagues, a HIV Nef (negative regulatory factor) protein was tagged with hexapeptide histidine; using transient expression in electroporated protoplasts the protein was revealed to accumulate in the cytosol. The recombinant protein was then produced in transgenic plants; the Nef protein and its truncated mutants of 25 and 27 kDa tagged with six histidines were affinity purified from a transgenic plant in a one-step using a nickel column (Marusic et al., 2007). The Nef protein hexahistidine fusion was revealed to be unstable in the secretory pathway; however the instability could be because of the protein properties and not the six histidines.

The result obtained in this thesis revealed the α -amylase octapeptide histidine fusion to be stable in both infiltrated leaf cells and the stable transgenic plants expressing the protein. This is because no degradation product was detected with the antibody against α -amylase (Figures 2.15 and 2.16). Therefore, the result implies that the synthesis of the fusion protein, rather than the stability could explain the lower yield of amylase activity that was detected in electroporated protoplasts.

6.2 Evidence that fusion tags affect protein properties and presence of HDEL-independent retention mechanisms

In addition to the amylase fused to the octapeptide histidine, other α -amylase fusions were tested. The fusion of HDEL was revealed to decrease

the yield of amylase, this is because amylase HDEL fusions were observed to exhibit lower yield of the protein using the amylase to GUS ratio compared to the secreted barley α -amylase (Fig 2.13). The decrease in the amylase activity was also exhibited by the amylase fused to the acidic C-terminus of calreticulin with or without the HDEL (Fig 2.17). The amyEDDDHDEL and amylaseHDEL fused to calreticulin exhibited lower amylase activity compared to HDEL. However the calreticulin lacking HDEL and amyHis also exhibited lower activity compared to the secreted protein. Therefore, the different fusions may slow down the rate of protein synthesis as a result of delay in translation and translocation or are toxic to the protein. The result confirms previous work that reported a low activity and lethal effect of HDEL and calreticulin fusion amylases on transgenic plants (J. An and J. Denecke, unpublished).

In this thesis the effects of peptides tags on ER retention was clearly demonstrated. It was shown in addition to HDEL, the amylase fused to EDDDHDEL exhibited more efficient retention compared HDEL fusion alone (Fig. 1.14B). Therefore, the result confirms previous reports on HDEL mediated retention in plants (Denecke et al., 1992) or KDEL-mediated in mammals (Munro and Pelham, 1987). Moreover, the results strongly suggest that a better exposure of the HDEL signal may be responsible for the observed effect. Amylase calreticulin fusion with HDEL was retained more efficiently (Fig. 2.18) compared to the HDEL and EDDDHDEL fusions. The calreticulin is an ER retained protein with a long acidic C-terminus preceding the HDEL sequence (Crofts et al., 1999). The ability of the calreticulin to increase the efficiency of retention may also be due to a better exposure of the HDEL signal. However, the significant retention of calreticulin fusion lacking the HDEL signal (Fig. 2.18) strongly confirms the existence of HDEL-independent retention mechanisms. The calreticulin-mediated retention may be related to the calcium chelating property of the acidic amino acids near the C-terminus of the peptide (Pagny et al., 2000; Nilsson and von Heijne, 2000). Although, the HDEL-independent retention

may be efficient it does not rule out the significant role played by HDEL signal in retention.

6.3 Glycosylation of α -amylases is not only affected by length of the amino acids

N-linked glycosylation is a type of modification that occurs in the lumen of the endoplasmic reticulum; it is mainly the addition of sugars to the amide group of some secreted proteins such as the barley α -amylase as they pass the secretory pathway. The glycosylation is suggested to increase stability and affects the overall properties of the protein. The initial aspect of the project tested whether the amylase-fused to HDEL or calreticulin can indeed be glycosylated and if C-terminal extensions influence the degree of glycosylation (Nilsson and von Heijne, 2000). In chapter 2.2 of this thesis, the effect of a single amino acid substitution at the consensus glycosylation site was described. It was revealed that the protein properties of the α -amylase were not affected by the change of asparagine to serine (N372-S372). The result was similar irrespective of small fusions such as AmyHDEL, EDDDHDEL and AmyHis or large fusions such as amylase fused to calreticulin with or without the HDEL compared to the standard barley α -amylase. Although the glycosylation status could not be verified using electroporated protoplasts, however, the evidence from the delta glycan mutant indicates that lack of glycosylation had no detectable effect on protein yield, stability, secretion or retention.

In this thesis, it was clearly shown that long C-terminal fusions promote glycosylation as was observed with the calreticulin fusions (Fig 2.19). The result is a confirmation of previous suggestion that glycosylation efficiency depends on the distance of the glycosylation site to the C-terminus of the protein (Nilsson and von Heijne, 2000). Moreover, the glycosylation does not only depend on the length of the fusion but also on the nature of the C-terminal fusions. Both amyHis and amyEDDDHDEL have eight additional amino acids however, only the former exhibited glycosylation in leaves while

the latter did not (Fig. 2.15). The glycosylation of the amy-his, amyHDEL and amycal with and without the HDEL signals indicates that the proteins were translocated into the ER lumen.

The efficiency and pattern of protein glycosylation is of high interest particularly in the pharmaceutical industry. For the recombinant product to be accepted as per regulatory concerns it is required to be identical to the wild-type protein because the glycosylation pattern affects the function of the protein (Ma et al., 2003).

6.4 Effect of expression systems on protein properties

Evaluation of recombinant protein expression involves either transient transfection or stable transformation. Transient expression is a system of rapidly verifying the expression and functionality of constructs. Two forms of transient expression are routinely used; these include the electroporated protoplast system (Hadlington and Denecke, 1994) and *Agrobacterium*-mediated infiltration of leaf epidermal cells (Kapila et al., 1997). The electroporated protoplasts offers the benefit of producing little amount of the recombinant protein for analysis prior to stable transformation for the production of transgenics. It also provides the opportunity to study protein localisation and compartments for the accumulation of recombinant proteins (Marusic et al., 2007). Though the electroporated protoplasts offer some amount of proteins for routine work, yet as was shown in this thesis the limitation is on the yield of adequate amount of proteins compared to *Agrobacterium* infiltration which offers a higher yield of amylase activity (Tables 2.1 and 2.2). Therefore, in a situation where a higher yield of recombinant proteins is required then electroporated protoplasts may not be the answer. In contrast to the transient expression strategy, stable transformation to produce transgenic plants expressing proteins is a more robust technique due to higher yield of recombinant products but it takes longer.

One drawback of the electroporated protoplasts system is that it may not guarantee the post-translational modifications due to overexpression or the short expression time for example amylase is not glycosylated in protoplast but it was revealed to be glycosylated in plants (Sutter et al., 2012). However, the *Agrobacterium* based infiltration offers the benefit of producing the desired modifications such as glycosylation. This thesis clearly demonstrated the glycosylation of amyHis and amyCalreticulin fusions in infiltrated leaf epidermal cells. It was also revealed that stable transformation in transgenic plants promoted the efficiency of glycosylation compared to the transient expression using *Agrobacterium*-mediated infiltration of leaf epidermal cells. This is because the glycosylation of the amy tagged with histidine octapeptide was more pronounced in stable transgenics. Similarly, amylase-HDEL which was not glycosylated in infiltrated leaf cells (Fig. 2.15) exhibited glycosylation in transgenic plants (Fig. 2.16). The result confirms glycosylation of amyHDEL in BY2 suspension cells (Sutter et al., 2012)

6.5 Optimisation of starch hydrolysis process

Although the preliminary results on the combination of mild acid pre-treatments and subsequent enzyme hydrolysis were promising (J. An, and J. Denecke, unpublished), further optimisation was necessary. In this work package, different acid hydrolysis regimes were compared, as well as a combination of liquefying and saccharifying enzymes. This was to establish an efficient protocol that ensures maximum yield of fermentable sugars with minimum loss to non-fermentable degradation products.

In principle, starch can be hydrolysed to glucose, maltose, maltotriose, maltopentose, maltohexose and other longer glucan chains (Nigam and Singh, 1995). The sugars glucose, maltose, and maltotriose can be fermented to ethanol and distilled. In contrast, sugars with chain length higher than maltotriose cannot be easily converted to alcohol. For this reason, the non-fermentable oligosaccharides can be re-cycled further and subject to hydrolysis. During the process of starch conversion to alcohol, in

addition to ethanol some broths are recovered. In practice 70% of the starch is converted to glucose this means that the remaining 30% forms part of the broth. This implies that in order to gain more fermentable sugars from the starch, the broth can be recycled and passed through the liquefaction and saccharification process. This increases the amount of sugars that are recovered and subsequently converted to alcohol. Therefore, using this system it may be possible to achieve a close to 100% conversion and recovery from the hydrolysis process.

In the acid hydrolysis of starch, the release of glucose was found not to be linear which may be due to a complex sequence of events that positively or negatively influence each other. For instance, the reaction gives rise to higher numbers of non-reducing ends that can be subject to further hydrolytic action of the acid in the process. It is also likely that some oligomers may be released that cannot be further digested by the acid or perhaps at a much slower rate than other oligomers. However, in enzyme catalysed liquefaction and saccharification, the α -1,4 endo-glycosidic action of the liquefying enzyme gives rise to dextrans.

Furthermore, the dextrans are saccharified by the exo-amylase action of the glucoamylase which has the ability to cleave α -1,4 and α -1,6 glycosidic bonds from the non-reducing ends. Therefore, glucose as well as maltose and maltotriose are released, and because the initial gelatinisation and liquefaction also involved acid hydrolysis, it is likely that some oligomers may be liberated in the process that cannot be rapidly hydrolysed by the glucoamylase. Consequently, these oligosaccharides together with the longer chain sugars make up the non-fermentable fractions. Therefore, amylases with specificity for shorter chain sugars such as maltase (an α -glucosidase) which has the glycosidic ability to digest maltose to glucose may be required and would be an excellent starting point for future research.

6.6 Exploring new enzymes for biotechnology

In this thesis an α -amylase-like gene that encodes a chloroplast type protein was cloned from the plantains. The catalytic domain of the plantain α -amylase and full length Atamy3 were cloned under the transcriptional control of the 35S promoter and transiently expressed in tobacco protoplasts, however, the two constructs did not yield amylase activity using the maltoheptaoside substrate. The maltoheptaoside may be too short for the family three amylases that may only exhibit specificity for longer chains such as starch, therefore the specificities of the enzyme may also be a limiting factor. Previous work of Atamy3 using soluble starch as substrate has reported enzyme activity (Yu et al., 2005). This is not surprising since the substrate used is similar to crystalline starch, the endogenous substrate of the enzyme. In-gel assay can be used to study the endoglycosidic activity of the family three proteins but this is beyond the scope of this thesis. Another assay system that can be used may be incubation of the enzyme with soluble starch and analysis of the products afterwards using chromatography. Therefore, it may be likely that the maltoheptaoside assay was not the appropriate method to assay the family three α -amylases even though it work well with the barley amylase used in the host laboratory. The localisation of the plantain amylase and Atamy3 could not be studied due to expression problems.

Moreover, it is noteworthy that contrasting views have been suggested on the role of α -amylase in transitory starch degradation at night. Although α -amylase plays an important role in storage starch hydrolysis in the endosperm, it may not be involved in transitory starch hydrolysis in the chloroplasts of leaves. In transgenic plants, the loss of plastid-resident Amy3 does not seem to affect transitory starch degradation therefore may imply that it is not involved in the process (Yu et al., 2005; Zeeman et al., 2007b). However, interpreting such data has to be done with caution because the observation may be affected by the growth conditions. A second hypothesis may be that other enzymes of starch degradation compensates for the lack

of the α -amylase (Yu et al., 2005; Streb et al., 2012). It has also been suggested that family three amylases may be involved in the breakdown of plastid bound starch in storage tissues and leaves of plants (Stanley et al., 2002; Stanley et al., 2005; Yu et al., 2005). Recently, this class of amylase has been suggested to contain a starch binding domain which belong to the family 45 of carbohydrate binding module (Glaring et al., 2011). The SBD has been proposed to complement the activity of α -amylases, possibly by helping to establish tighter interactions between the substrate and the enzyme (Glaring et al., 2011).

Localisation of family three α -amylases to the chloroplast

The starch binding domain of Atamy3 was fused to YFP, but the localisation could not be confirmed because the fluorescence of the fusion protein was below the detection limit. Fractionation of the chloroplast for the cell components may reveal the localisation of the SBD but this is beyond the scope of this thesis. However, previous work has shown the localisation of the Atamy3 to the chloroplast (Yu et al., 2005; Glaring et al., 2011). The localisation of α -amylase to the plastid of plantains may indicate that the enzyme and starch are synthesized in the same cells but at different times in contrast to barley and other cereal crops where the hydrolases are not only synthesised at a different time but produced by a separate tissue, the aleurone layer from which they are secreted and transported to the starchy endosperm (Ranki and Sopanen, 1984; Kitajima et al., 2009; Jeon et al., 2010). This physical segregation may be difficult in large ripening fruits such as plantains, banana, pears, apple, where progressive conversion of starch to soluble sugars is required in the entire storage tissues.

Comparison of Atamy3 like proteins to glucan, water dikinase (GWD)

Starch degradation is a very important process in energy metabolism in higher plants. It is catalysed by a variety of enzymes; one of them is glucan water dikinase (GWD) (Smith et al., 2005; Zeeman et al., 2007b; Zeeman et al., 2007a). The protein is localised to the chloroplast and has been shown to catalyse starch phosphorylation. There is conservation in the unknown

domain found in GWD to that present in family three alpha amylases (Baunsgaard et al., 2005; Mikkelsen et al., 2005; Glaring et al., 2007). This may not be unconnected with the fact that the two different enzymes act on starch. Furthermore, the similar structural feature may explain the role of this additional domain.

Amylases with unusual characteristics

The discovery of new proteins with unusual feature is not uncommon; several amylases with composite proteins have been identified. Recently an α -amylase with no known homologue was cloned from drainage of an acid mine (Delavat et al., 2012). Some researchers reported isolation of α -amylase genes from *Lactobacillus plantarum* A6 and *L. amylovorus* with unusual characteristics. *L. plantarum* gene encodes a protein of 913 amino acids with N-terminus that is homologous to α -amylase from *B. subtilis* while the C-terminus shows an unusual feature. The amylase gene from *L. amylovorus* encodes α -amylase that consists of 954 amino acids; the protein shared very close homology to the *L. plantarum* α -amylase. The C-terminal domain was suggested to be a carbohydrate binding domain (Giraud and Cuny, 1997). Similarly, an amylase of 2056 amino acids was cloned from *L. plantarum* L137; the N-terminus has the catalytic domain of both an α -amylase and pullulanase. The protein showed α -amylase and pullulanase activities; it hydrolyses starch to maltotriose and maltotetraose (Kim et al., 2008a).

6.7 General conclusion and outlook

It is possible to tag proteins for affinity purification, though the codon hypothesis on the rare tRNA for histidine has to be tested. More than one protein should be produced and the cascade refinery process of producing multiple recombinant products in potatoes should be explored. In order to validate the effect of glycosylation on amylases, stable transgenic potatoes expressing the delta glycan mutants of the calreticulin fusions can be produced. Although acid hydrolysis has been successful, the presence of degradation products such as furfurals may be considered. Furthermore, the biological function of the plantain α -amylase should be explored this is because the sweet taste of ripened plantains indicate that maltose and maltotriose may be released. The search for alternative enzymes should consider amylases that can digest short glucan chains (ten glucose and below). In addition to plants, compost heaps should be considered for isolation of amylases and their action on starch can be compared using chromatography. This may enable the identification of enzymes that can produce higher amount of fermentable sugars compared to what is currently explored.

Chapter 7

Methods

7.1 Molecular biology

All DNA manipulations were performed using the established standard protocols, all buffers, reagents and media used were prepared as described by (Sambrook et al., 1989). Chemicals and enzymes were either purchased from Sigma, NEB, Megazyme among others

7.1.1 PCR

Several PCR were carried out in the course of this work, and three DNA polymerases from different manufacturers were used. *Pfu* DNA polymerase (Promega)®, KOD Hot start DNA polymerase (Novagen)®, and the Q5 High fidelity DNA polymerase (NEB)® were used according to the manufacturer's instructions. The PCR reactions were set up for a 50 µl volume of sense and antisense primers, polymerase, dNTPs, Mg, DNA template and water. Annealing and elongation times were calculated depending on the primers and the expected product size respectively.

7.1.2 Restriction digests

For preparative digests, 10-20 µg of plasmid DNA was added in Eppendorf tube, and then the respective compatible buffer for the enzymes was added. The restriction enzyme(s) was then added and the volume made up to 50µl with TE and the mix was incubated at 37°C. The progress of the digest was usually checked at 0 mins (before enzyme addition), 20 and 40 min after the enzyme was added. After, the full digestion of the plasmid, the subsequent procedures depended on the use of the DNA. For vector preparation, the DNA was subject to dephosphorylation, phenol-chloroform clean-up, DNA

precipitation and finally re-suspension while for fragments preparation, the next step was isolation on agarose gel.

7.1.3 Dephosphorylation

To prevent self-ligation of a vector cut with a restriction enzyme, the open ends are dephosphorylated with calf intestine alkaline phosphatase (CIP). To the DNA from the preparative digests, 40 µl of TE, 5 µl of 10X CIP buffer and 5 µl CIP were added and incubated at 37°C.

7.1.4 Clean-up

To remove the enzymes and buffers used in DNA manipulations, 50 µl of phenol was added to the 50 µl of the “dirty” DNA, mixed and centrifuged at room temperature. The aqueous phase was recovered and 100 µl chloroform was added, mixed and centrifuged again. The aqueous phase was again recovered and the DNA was precipitated with a 10% 5 M NaClO₄ and 110% isopropanol and centrifugation. The supernatant was discarded and the pellet was dried using vacuum pump. The pellet was re-suspended in 50 µl of TE and tested on a DNA gel.

7.1.5 Isolation of fragments

The digested plasmid DNA was loaded on a preparative agarose gel which depended on the size of the expected fragment. It was then ran slowly at 50 V for 3 h or more to ensure good separation of the DNA. The gel was visualised using the trans illuminator and fragment of interest was then cut out with a razor blade and the DNA was purified using the Quigene® DNA extraction kit according to manufacturer’s instructions.

7.1.6 Ligation

Three eppendorf tubes were labelled 1, 2 and 3; equal amount of vector such as 1 µl was added to each of the three tubes. 1 or 2 µl of the fragment depending on the abundance and quality of the DNA was added to tube

number 3, 4 μ l of 5X ligation buffer was added to each tube. 1 μ l of DNA ligase (Invitrogen)[®] according to manufacturer's instruction was added to tubes labelled 2 and 3. Then finally the volume in each tube was made up to 20 μ l with TE, mixed and spun down. The tubes were either incubated at room temperature for 1-2 h or at 4°C overnight.

7.1.7 Competent cells

Solutions and their compositions

1 litre of 2xYT medium: 16 g bacto tryptone, 10 g bacto yeast extract and 5 g NaCl. The solution was made up to 1 litre with water and the pH was adjusted to 7.0 using NaOH and autoclave.

TFBI (200ml): 30 mM $\text{KC}_2\text{H}_3\text{O}_2$ (0.589 g), 100 mM RbCl (2.418 g), 10mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.294g), 50 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1.979 g), 15% v/v glycerol (30 ml), and was adjusted to pH 5.8 using 0.2 M CH_3COOH , and filter sterilised. The solution was stored at +4°C.

TFBII (200ml): 10 mM MOPS 0.419 g, 10 mM RbCl 0.242g, 75 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.205g, 15% v/v glycerol 30 ml and was adjusted to pH 6.6 using 5 M KOH, and filter sterilise. The solution was stored at +4°C.

The MC1061 *E. coli* cells were streaked on LB. A single colony was selected and used to inoculate 3 ml 2xYT in a new 50 ml Falcon tube and was incubated at 37°C with shaking. At O.D.₅₅₀ = 0.300 (slightly turbid), the pre-culture was poured into 200 ml of 2xYT that was pre-warmed to 37°C and was incubated at 37°C. At O.D.₅₅₀ = 0.480 the culture was transferred into four 50ml sterile Falcon tubes and were placed on ice 5 min and all further manipulations were carried out on ice. The tubes were spun down at 3K in a swing-out rotor at 4°C for 20 min and then the supernatant was discarded. The cells were re-suspended in a total of 80ml of ice cold TFBI and placed on ice for 5 minutes. The tubes were spun as before and the cells were re-suspended in 8 ml of TFBII and left on ice for 15 minutes. Using pre-chilled pipette tips in the cold room, 100 μ l aliquot were added into pre-chilled Eppendorfs (sitting on ice), and were frozen in a dry ice and stored at -80°C.

LB medium contained 10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10g/L NaCl. 15 g/L agar was added for solid medium. The medium was sterilised by autoclaving.

7.1.8 *E. coli* transformation

5 µl of each ligation mixture was added to 100 µl of competent cells, mixed and incubated for 15 min on ice (minimum), this was followed by a heat shock at 37°C for 3 min during which the *E. coli* cells take up the re-ligated plasmid. 1 ml of LB medium then added and the cells were incubated for a further 15 minutes at 37°C. The LB medium with the transformed competent cells was then poured onto Ampicillin containing LB-agar plates, dried and was incubated overnight at 37°C. The colonies which appeared originated from single transformed cells, and were resistant to the antibiotics due to the presence of the plasmid.

7.1.9 Agrobacterium transformation

1µl of mini-prep was added to 10 0µl of thawed competent cells on ice, and the tube was frozen in snow of the fridge and then incubated at 37°C for 4 minutes. 1 ml of LB medium was then added to the cells. The suspensions were then transferred to 15 ml falcon tubes and incubated overnight at 28°C with shaking. The medium and transformed cells were then poured onto a LB plate containing three antibiotics selection: streptomycin (300 µg/ml), streptomycin (100 µg/ml) and rifampicin (100 µg/ml), they were dried and incubated at 28°C for two days.

7.1.10 DNA preparations

Small scale plasmid DNA preparation (dirty mini-prep)

E. coli competent cells were transformed with the respective DNA as described above. Single colonies were selected and used to inoculate 3 ml cultures with LB medium containing 150 µg/ml ampicillin. The inocula were grown at 37°C overnight with shaking. 1.5ml of the culture was then

transferred into a microfuge tube and spun at room temperature and maximum speed for 1 min. The supernatant was then discarded and the pellet was re-suspended in 150 µl of TES buffer (composed of 10 mM Tris-HCl pH 8.0, 5 mM EDTA, 250 mM sucrose, and filter sterilised). 20 µl of lysozyme solution (10 mg/ml) was added to each tube; it was mixed by pipetting up and down thrice and incubated for 5 min at room temperature. 300 µl of distilled water was added quickly to the mix and incubated at 73°C for 15 min. The tubes were then spun at maximum speed in a microfuge for 15 min, the supernatant was transferred into a new tube. 10% 5 M NaClO₄ and 110% isopropanol were added to the solution to precipitate the DNA. The tubes were then spun at maximum speed and room temperature for 15mins. The supernatant was discarded and the pellet was dried, the pellet was then re-suspended in 50 µl TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). A qualitative digests using restriction enzymes were used to check the constructs. The positive clones were streaked on an ampicillin plate and incubated overnight at 37°C.

Wizard prep

A single colony from the streaked plate above was used to inoculate a 8-10 ml LB culture and was incubated at 37°C shaking overnight. The DNA from the bacterial culture was purified using Promega Wizard® SV kit according to the manufacturer's instructions.

Large scale plasmid DNA preparation (maxi-prep)

3 ml pre-cultures of LB medium containing 150 µg/ml ampicillin were inoculated from fresh single colony and grown for 3 h. Slightly turbid pre-cultures were then used to inoculate 500 ml LB medium cultures, and were then incubated at 37°C shaking for 24 h. The cultures were transferred into 500 ml bucket and spun down with a swing out rotor at 4°C and 3699 rpm for 1 h. The supernatant was discarded carefully and the pellets were re-suspended in 8ml of ice-cold TE 50/1 by vortexing at intervals (50 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). The suspension was then transferred into pre-chilled SS34 tubes. 2.5 ml of fresh made lysozyme (10 mg/ml) solution

was added to each tube, and the tubes were turned upside down to mix. The suspensions were then incubated on ice for 5 min, 2 ml of 0.5M EDTA pH 8.0 was then added to each tube and was mixed as above. RNase solution (50 µl of ribonuclease A (20 mg/ml), 150 µl of 10% triton and 800 µl of TE 50/1) was then added to the tubes, and then turned gently to mix and incubated for 30 min on ice. The tubes were spun for 1hr using sorvall SS34 rotor at 18000 rpm and 4°C. The clear supernatant was then transferred to new 50 ml falcon tube, 20 ml of equilibrated phenol (pH 8.0, 8-hydroxyquinoline) was then added to each tube and shaken vigorously for 1 min. The tubes were then spun for 25 min at 3699 rpm. The aqueous phase was then gently transferred to a fresh falcon tube, 20 ml of chloroform was then added to each tube, and shaken vigorously followed by centrifugation at 3699 rpm for 10 min. The aqueous phase was then transferred into 30 ml corex tube; volumes were adjusted with TE 50/1. 10% volume of the liquid (approx. 1 ml) of 5 M NaClO₄ and 8ml of isopropanol were then added to each tube and mixed. The tubes were then spun using HB-6 rotor at 4°C and 10,000 rpm for 15 min. The supernatant was discarded and the pellet was dried using vacuum pump for 3 h. The pellet was finally re-suspended in 500 µl of TE, and the solution was transferred to a new microfuge tube. The plasmid DNA was tested on gel for DNA quality and RNAs contamination.

7.1.11 Sequencing of constructs

The DNA wizard prep was sent to Source Biosciences, Nottingham, UK for sequencing.

7.1.12 Generation of recombinant plasmids

Several plasmids were generated for this thesis using techniques such as PCR, quick change and sub-cloning of various fragments. Table 7.1 shows the list of oligonucleotide primers used for generating the different constructs. Table 7.2, shows a description of the various pUC plasmids and Table 7.3 shows the list of the plant vectors that were used in this thesis.

Table 7.1 The list of primers used to generate the different constructs

Name	Sequence
BamAMY	AGGCTCTGGATCCGAAATTCTGTGTCAAGGATTT
AMYSal	GATATGCCGTCGACTCATGATGTTTCCCACACTTT
Nco1Amy	AGGCTCTGCCATGGAAATTCTGTGTCAAGGATTT
AmyBam	GATATGCCGGATCCTCATGATGTTTCCCACACTTT
AtCla1AMY	GATCCTCTCTATCGATGTCCACTGTTCCCATTGAG
AtAMYBam	TGGGAACCGGATCCTTAAGATGTTTCCCACACCTT
Nco1Amy3full	CTCTCTCTCGCCATGGCCACTGTTCCCATTGAGTCTCTTCTCCACCA
Atamy3Nhe	GGCATAATATGCTAGCTCCCGAGCCGGTTCCTGAGG
AtNco1Amy	ACCGGCTCGGCCATGGAGATATTATGCCAAGGTTTCAAC
Atamy3As	CCAGCAGCTCCACTAGTTGC
AtAMYBam	AAGGTGTGGGAAACATCTTAAGGATCCGGTTCCCA
AtAMYXba	CTACAAGGTGTGGGAAACATCTTAATCTAGAGGTTCCCAAT
HvCla1AMY	AGCTTGGCATCGATGCAAGTCCTCTTTCAGGGCTTC
HvAMYSal	TCTGCTTCGGTCGACCTAGATCTTCTCCCATACGGC
HvS	GCTTGGCCGGATCCCAAGTCCTCTTTCAGGGCTTC
Glycamy	ACCCGGCACGGGATACACTCTGAGAGCAAGCTGCAAATC
GlyamyAS	GATTTGCAGCTTGCTCTCAGAGTGTATCCCGTGCCGGGT
PUCOF	CCACACAACATACGAGCCG

Table 7.2 Description of pUC plasmids

Construct (pIKA)	Description	Mode of generation
1	35S-Maxbamy(COOH-terminus)-3'NOS	PCR
2	GST-Maxbamy(COOH-terminus)	PCR
3	35-Atamy3(COOH-terminus)-3'NOS	PCR
4	GST-HvAmy	PCR
5	35-Atamy3(Nco1)-3NOS	PCR
6	35S-Maxbamy(UD)-YFP-3'NOS	PCR
7	35S-Atamy3-3'NOS	PCR
8	35S-Hvamy(Δ G)-3NOS	PCR
9	TR2-GUS-3OCS-35S-Amy-3NOS	Sub-cloned
10	TR2-GUS-3OCS-35S-Amy(Δ G)-3NOS	Sub-cloned
11	TR2-GUS-3OCS-35S-AmyHis-3NOS	Sub-cloned
12	TR2-GUS-3OCS-35S-Amy(Δ G)His-3NOS	Sub-cloned
13	TR2-GUS-3OCS-35S-AmyCal-3NOS	Sub-cloned
14	TR2-GUS-3OCS-35S-AmyCalHDEL-3NOS	Sub-cloned
15	TR2-GUS-3OCS-35S-Amy(Δ G)Cal-3NOS	Sub-cloned
16	TR2-GUS-3OCS-35S-Amy(Δ G)CalHDEL-3NOS	Sub-cloned
17	35S-Amy(Δ G)HDEL-3NOS	PCR
18	35S-Amy(Δ G)EDDDHDEL-3NOS	PCR
19	TR2-GUS-3OCS-35S-Amy(Δ G)HDEL-3NOS	Sub-cloned
20	TR2-GUS-3OCS-35S-Amy(Δ G)EDDDHDEL-3NOS	Sub-cloned
21	TR2-GUS-3OCS-35S-AmyHDEL-3NOS	Sub-cloned

Table 7.3 List of plant vectors and descriptions

Name	Description
pTAmy	α -amylase
pTIKA2	α -amylase Δ -glycan
pTIKA3	α -amylase-His ₆
pTIKA4	α -amylase Δ -glycan-His ₆
pTAmyHDEL	α -amylase-HDEL
pTIKA5	α -amylase Δ -glycan HDEL
pTKA8	α -amylase-EDDDHDEL
pTIKA9	α -amylase Δ -glycan EDDDHDEL
pTAmyCal Δ HDEL	α -amylase-calreticulin
pTIKA6	α -amylase Δ -glycan calreticulin
pTAmyCalHDEL	α -amylase HDEL calreticulin
pTIKA7	α -amylase Δ -glycan HDEL cal

7.2 Tissue Culture

7.2.1 Generation of plant material

Nicotiana tabacum seeds were surfaced sterilised and grown on Murashige and Skoog medium (Murashige and Skoog, 1962) and 2% (w/v) sucrose at 22°C in a controlled room at 16-h day length and light irradiance of 200 mE/m²/second. The plants were used to generate protoplasts for transient expression.

Transient expression in protoplasts

7.2.2 Preparation of protoplasts

All solutions were sterilized by filtration through 0.2 μ m filter with a syringe in a laminar flow bench. Leaves were cut gently on the lower surface every 1-2 mm (without cutting through the whole surface). This was done by balancing the scalpel so that a fraction of its own weight exerts the pressure on the leaf surface. The mid nerve was removed and the two halves of the leaf were

transferred to a Petri dish containing 7 ml digestion mix 1X (TEX buffer (B5 salts, 500 mg/l MES, 750 mg/l CaCl₂ (2 H₂O), 250 mg/l NH₄NO₃, 0.4 M sucrose (13.7%), pH 5.7 (with KOH) and enzyme mix (2 % Macerozyme R10; 4% Cellulase R10)., with the cut side facing downwards. The plates were incubated in the dark overnight, and 30 min before use, and gently shaken to release protoplasts from the cuticula. The digestion mix was filtered through a 100 µm nylon filter and the filter. The protoplasts were then centrifuged in Falcon tubes (50 ml) for 15 min at 100 g and room temperature in a swing-out rotor. A long Pasteur pipette connected to a peristaltic pump which can pump up to 1 litre per minute and the Pasteur pipette was inserted through the floating cell layer and was used to underlying medium were removed until the band of living protoplasts reaches the bottom. 25 ml of electroporation buffer (0.4 M Sucrose (13.7%), 2.4 g/l HEPES, 6 g/l KCl, 600 mg/l CaCl₂, pH 7.2 (with KOH)) was added and spun again at 80 g for 10 mins. The underlying solution was removed as described above and the procedure was repeated twice. At the end the protoplasts were resuspended in an appropriate volume in order to obtain 2-5 x10⁶ protoplasts/ml. This solution was used for the electroporation.

7.2.3 Electroporation procedure

500 µl of protoplasts was pipetted gently into a disposable 1ml plastic cuvette. Plasmid DNA was diluted in 100 µl of electroporation buffer and was mixed by gentle shaking. The cells were incubated for 5 minutes. The electroporation was performed with the following conditions: 910 µF, 130 V. The electrodes were rinsed in distilled sterilized water to remove cell debris and DNA, and dipped in 99 % ethanol, then briefly flamed and was cooled down in electroporation buffer. The electroporated cells were incubated (without shaking) for 15-30 min to allow the cells to recover. The cuvette was then rinsed twice with 1 ml of TEX buffer and the obtained cell suspension is incubated in small Petri dishes in the dark during an appropriate time period (24 h).

7.2.4 Harvesting of the cells and culture medium

After incubation, the cell suspension was recovered in a small Falcon tube (15 ml). For the intracellular proteins: 1ml of the cell suspension was diluted 10 fold with 250 mM NaCl and was centrifuged for 3 minutes at 200g. Refined pasteur pipette was used to remove the suspension, a further 5 minutes centrifugation was performed, the remaining supernatant was completely removed with a peristaltic pump. The cell pellet was immediately placed on ice and was then extracted with the appropriate buffer.

To recover secreted proteins such as α -amylase; 500 μ l of the cell suspension was place on ice in an Eppendorf tube. The suspension was then sonicated later and centrifuged to obtain the total sample. The was recovered resuspended and pellet resuspended in amylase buffer.

7.2.5 Tobacco leaf infiltration

Solutions and compositions

MGL medium: yeast extract (2.5 g/L), tryptone (5 g/L), NaCl (5 g/L), mannitol (5g/L), monosodium glutamate (1.16 g/L), KH_2PO_4 (0.25 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 g/L), biotion (1 g/L) and pH 7.0

Infiltration buffer: 50 mM MES (pH 5.6), 2 mM Na_3PO_4 , 0.5% glucose, 100 μ M Acetosyringone.

The constructs of interests were cloned into the plant vector, and was used to inoculate a culture using MGL medium and was grown at 28°C overnight with shaking. 1 ml of the cells suspension was centrifuged at 5000 rpm (2200 g) for 5 min in a microcentrifuge at room temperature. The pellet was re-suspended in 1 ml of the infiltration buffer and centrifuge again. This procedure was repeated twice to remove the remaining MGL. The cell was re-suspended in 1 ml of infiltration buffer and diluted five fold, the OD_{600} was measured. The cell suspension was diluted to OD_{600} of 0.1 which is the OD required for the injection. Using a yellow tip, small hole was created in the leaves and the Agrobacterium suspension was injected into the leaf by press the nozzle of a 1 ml syringe (no needle) against the lower (abaxial)

epidermis of tobacco leaf, the small hole was covered with the nozzle and held using a gloved finger to the other side of the leaf and injected slowly. The infiltrated area turns dark and is marked with black pen. The plants were incubated under normal growing conditions for 2-3 days. The infiltrated area was excised and proteins were extracted and analysed as appropriate.

7.2.5 Generation of Transgenic plants

In a laminar flow hood, tobacco leaves were cut into 0.5 – 1 cm² squares void, the mid-rib and the primary nerves were avoided without damaging the surface of the squares. The leaf squares were put 10-20 to float upside up on 10 ml of A10 in a Petri dish. The 10ml was infected with 100 µl of Agrobacteria overnight culture (in MGL medium) and was grown at 28°C and 2-3 days in the dark. The leaves were transferred to a fresh dish with 10 ml of A10 and were incubated for 15 minutes, and swirled gently thrice to allow bacteria to come off the plant cells. The liquid was sucked away with disposable sterile plastic pipette and replaced by 10ml of A10. The plates were incubated for ten minutes and swirled gently as before. The third wash was repeated A10 supplemented with 500 µg/ml cefotaxime (a bacteriostaticum). The leaves were transferred to solid A11 medium and were pressed very gently to the surface to allow good contact. Incubated for 7 days, the leaves were then transferred to fresh A10 plants. After a week, the leaves were transferred to fresh plates and incubated for a week; the leaves were again transferred to fresh plates. The calli appeared, and were transferred to A12 medium; and placed so that good contact with the medium were established to ensure proper selection as well as nutrition. This incubation was for two weeks, the calli were transferred to fresh plates for another two weeks. The calli (5 mm) were placed in small jars containing A13 (A12 but no NAA) and incubate for a further 2 weeks, this was repeated twice or thrice. The shoots were cut with a sharp scalpel and place on MS2 medium without pushing the stem too deep in the agar. At this point, leaves of the respective shoots were cut and proteins were extracted and used for analysis.

	A10	A11	A12	A13	
800ml					
B5	+	+	+	+	2.56
g					
NH ₄ NO ₃ (250 mg/l)	+	+	+	+	200
mg					
MES (500 mg/l)	+	+	+	+	400
mg					
Glucose (2%)	+	+	+	+	16 g
Agar (0.75%)	-	+	+	+	6 g
Adenine (40 mg/l)	-	+	+	+	32mg
pH 5.7 (KOH)					

Added prior to use of medium:-

	A10	A11	A12	A13
Stock Conc				
BAP	1 µg/ml	1 µg/ml	1 µg/ml	1µg/ml
(1mg/ml)				
NAA	0.1 µg/ml	0.1 µg/ml	0.1 µg/ml	-
(1mg/ml)				
Cefotaxime	-	500 µg/ml	200 µg/ml	200 µg/ml
(200mg/ml)				
Selective pressure				
Kanamycin	-	100 µg/ml	100 µg/ml	100µg/ml
(100mg/ml)				
or				
Phosphinothricine	-	5 µg/ml	5 µg/ml	2 µg/ml
BAP = 6-benzylamino purine				
NAA = α-naphthalene acetic acid				

7.3 Enzyme assays

7.3.1 α -amylase assays

The medium and cell fractions from the harvested transiently expressed protoplasts were analysed for amylase activity using the procedure described on the Megazyme kit. To measure the total amylase activity, 500 μ l of the protoplasts was transferred into 500 μ l of amylase extraction buffer (50 mM malic acid, 50 mM NaCl, 2 μ M CaCl₂, 0.02% sodium azide and 0.02% BSA) and sonicated for 5 sec at 50 or 60% amplitude and was followed by centrifugation at 4°C and 14,000 g for 15 min. The medium samples were 2-fold diluted with amylase extraction buffer. The cells were resuspended by adding 950 μ l amylase buffer, sonicated for 5 sec at 50 or 60% amplitude and was followed by centrifugation at 4°C and 14,000 g for 15 min. The supernatant was transferred into fresh microfuge tube and used for the assay. The α -amylase substrate contained blocked P-nitrophenyl maltoheptaosides (54.5 mg), glucoamylase (100 U, pH 5.2), α -glucosidase (100U, pH 5.2) and dissolved in 10 ml of distilled water according to manufacturer's instructions.

The assay was performed as followed: 30 μ l of the substrate was added to 30 μ l of sample, and the mix was incubated at 45°C; the reaction was stopped by adding 150 μ l of 1% (w/v) Tris pH 11. 200 μ l of the solution was then added into the well of microtitre plate and the optical density was read at 405 nm. OD values of the mock electroporations (negative controls) were used to correct the absorbances. Values greater than O.D of 1 were considered out of scale; and the respective samples were diluted. The amylase activity was calculated as the change in optical density divided by the volume of extract used (μ l) and incubation times and multiplied by 1000 to convert to ml. The assays were repeated either twice or thrice and the average was computed. The secretion index (S.I) was calculated as the extracellular divided by intracellular amylase activity.

7.3.2 Gus assays

500 µl of the protoplast was transferred into a microfuge containing 500 µl of GUS extraction buffer (50 mM (P) Sodium buffer pH7.0, 10 mM Na₂EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100 and 10 mM β-mercapto-ethanol). The solution was sonicated for 5 sec at 60% amplitude, vortex for 5 sec and was followed by centrifugation at 4°C and 14,000 g for 15 min. The clear supernatant was transferred into fresh microfuge tube and used for the assay. The assay was set as followed; 80 µl of stop buffer (2.5 M 2-amino-2methyl 1,3-propanediol) was added to the zero stop tube, 100 µl of reaction buffer (50 mM (P) Sodium buffer pH 7.0, 0.1% Triton, 2 mM PNPG, 10 mM β-mercapto-ethanol (added prior to use)) was added to both the zero stop and the test tubes. 100 µl of each sample was then added to respective tube, and incubated at 37°C for 2h. The reaction was stopped by adding 80 µl of the stop solution to the test sample. 250 µl of the solution was then added to each well of microtitre plate and the optical density was read at 405 nm. The OD values of the zero stops were used to correct the absorbance for each sample. OD above 2.0 was considered out of scale, assays were initially repeated for shorter time or samples were diluted. Later, an equation was used to normalise the values.

7.4 Protein Gels and immunoblots

Solutions

Sample buffer: 0.1% bromophenol blue, 5 mM EDTA, 200 mM Tris pH 8.8, and 1 M sucrose

Sample buffer mix: 900 µl sample buffer, 300 µl 10% SDS, 18 µl 1 M DTT

Stacking gel: 5% protogel (30% acrylamide, 0.8% bisacrylamide), 15% sucrose, 66 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.2% v/v N,N,N',N'-tetramethylethylenediamine, 0.033% w/v ammonium sulphate.

Separating gel: 12% protogel (30% acrylamide, 0.8% bisacrylamide), 420 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.055% v/v N,N,N',N'-tetramethylethylenediamine, 0.033% w/v ammonium sulphate.

5X running buffer: 30 g/l Tris-HCl, 144 g/l glycine, 5 g/l SDS

7.4.1 SDS-PAGE: the gel assembly

The gel cassette was assembled, the separating gel was prepared and poured, 1ml of separation mix was then added and the gel was allowed to set for 1-1.5h. The separation mix was discarded and the gel was washed with water and dried. The stacking gel was prepared as described above and the mix was poured into the cassette, the comb was inserted. The gel was then allowed to set for 1.5-2 h, the gel was mounted onto the electrophoretic platform. 1X running buffer was added and the comb was removed, the wells were washed, all bubbles were removed. The protein samples boiled in sample buffer mix at 95°C and marker were then loaded. The gel was run initially at low current which was subsequently increased until all the proteins were separated.

7.4.2 Ponceau staining

The membrane was at times stained in ponceau solution which contained 0.1% Ponceau S, and 5% acetic acid,

7.4.3 Western blotting

The proteins were initially separated on SDS-page and then transferred onto nitrocellulose membrane using a home-made device. Electroblotting was performed for 2 h at a current 500 mA in buffer (3 g/l Tris-HCl, 14.4 g/l glycine and 10% methanol.

The membrane was then washed several times with 1X PBS and 0.5% tween 20 (10X PBS: 87 g/l NaCl, 22.5 g/l, Na₂PO₄-2H₂O, 2 g/l KH₂PO₄, pH 7.4). The membrane was then transferred into blocking solution (5% skimmed milk in PBS-T) for 1h with shaking. The membrane was then washed several times with PBS-T and then PBS. The primary antibody was diluted 1:5000 in PBS, 0.02% sodium azide and BSA. The membrane was then incubated in the primary antibody at 4°C overnight. The antibody was

removed and membrane was washed several times with PBS-T. The secondary antibody (anti-rabbit IgG) was diluted in blocking solution 1:15,000. It was then added to the membrane and incubated for 1 hour shaking. The antibody was discarded and the membrane was washed several times with PBS. Immuno-detection were usually performed with the enhanced chemo-luminescence (ECL) system. ECL solutions 1 (1 ml Tris-HCl pH 8.5, 100 μ l 250 mM luminol, 44 μ l p-coumaric acid and 8.85 ml dH₂O) and 2 (6 μ l 30% H₂O₂, 1ml IM Tris-HCl pH 8.5, 9 ml dH₂O) were prepared. The membrane was then incubated in the two solutions for few minutes, and the films were exposed for 1min or more (when the signal was weak) and the film was then developed.

7.5 Recombinant protein expression and purification

7.5.1 Expression

The barley and plantain amylase were each PCR amplified and cloned into a GST plasmid (pGEX-4T-1); and two recombinant plasmids pKA2 and pKA4 respectively were made. *E. coli* competent cells (star and gold strains) were transformed with the two constructs and GST only and grown overnight at 37°C. Single colonies were used to inoculate LB medium liquid cultures and incubated at 37°C overnight. The O.D of the overnight cultures were measured, a pre-culture using LB medium was inoculated at OD of 0.1 and was grown 37°C till OD of 0.6. When the OD was 0.6, an aliquot of the culture was taken before induction. 1 M IPTG was added to a final concentration of 1mM or lower to induce the gene, the culture was grown for five hours; 600 μ l aliquot was transferred into a microfuge tube at 3, 4 and 5 hours respectively. The sample was spun at maximum, and the pellet was frozen at -80°C. Sample buffer mix was made, and the pellets were resuspended throughly in 200 μ l of the buffer and boiled at 95°C for 5 min. A 10% SDS-PAGE was made and the samples were loaded and ran at low voltage. The gel was stained in coomasie blue for 1 hour and de-stained overnight in a solution of 10% acetic acid and 25% methanol and 35% water. The gel image was taken.

7.5.2 Solubilisation

The expressions were performed as described above. The pellets were resuspended in three buffers; 1) Buffer 1 (50 mM Tris pH 8.8, 2 mM EDTA pH 8.00), 2) Buffer 2 (50 mM Tris pH 8.8, 2 mM EDTA pH 8.00, 150 mM NaCl), and 3). Phosphate buffered saline, PBS (NaCl, Na₂HPO₄·2H₂O, KH₂PO₄, pH 7.4). The samples were then sonicated for two cycles of 30 seconds and 40% amplitude each, followed by centrifugation at maximum speed and +4°C for 15mins. The supernatants were transferred to a new microfuge tube, and the pellets were resuspended in 500µl of respective buffer. Equal volume of the samples were mixed with sample buffer mix and loaded on a 10% SDS-PAGE gel. The gel was stained and de-stained as described above.

The solubilisation experiment was repeated as described above, cultures were incubated at 28°C and induced for 3hrs. The pellets were re-suspended in Bug buster and phaseolin buffer (Tris pH 8.0, NaCl, EDTA, 10% triton and β-mercapto-ethanol). The samples resuspended in phaseolin buffer were sonicated for three cycles at 50% amplitude for 15 secs. The bug buster resuspended samples were incubated at room temperature for 30 mins. The samples were spun at maximum for 10mins. The supernatant recovered into a new tube and pellet re-suspended in 250 µl of the respective buffer. 42 µl of 6X sample buffer mix added to each sample and 10µl of each was loaded on a 10% gel and ran at 10 mA, and 20 mA later. The gel was stained and de-stained as described above.

Solubilisation experiment repeated again, cultures were incubated at 28°C and induced for 3 hrs. The pellets were resuspended in PBS and phaseolin buffers. Three cycles of sonication at 40% amplitude and 30 secs were performed. All other treatments were as above.

The solubilisation experiments was again set, cultures were grown using two different media, LB and TB. The cultures were incubated at different temperatures; 0, 10, 16, and 37°C (for auto-induction). For temperatures of

10 and 16 °C, the procedure was as described above. The auto-induction was performed as followed; a single colony was inoculated in 2 ml of LB-1D and was grown all day at 37°C. 5 µl of the pre-culture was inoculated in 10ml LB-5052 was grown overnight and whole day (22 hrs). The cultures were pellets and the pellets were resuspended in PBS (+lysozyme) and phaseolin buffer. This was followed by sonication as described above. The samples were loaded on a 10% protein gel and treated as described above.

The auto-induction experiment was repeated and the samples were re-suspended in phaseolin buffer.

7.5.3 Protein quantification

The cultures (pre- and induced cultures) were grown at 37°C, the expression was induced with 1 mM IPTG and cultures were incubated for three hours after induction. The pellets were resuspended in PBS (+lysozyme) and 2X phaseolin buffer and sonicated for 1min at 40% amplitude (3-4 cycles); followed by centrifugation at maximum. Supernatants were transferred into fresh tubes; pellets were resuspended in 1X phaseolin buffer. The samples were then mixed with 6X sample buffer. The expressed pIKA2 and pIKA4 of 1,2, and 4 µl were loaded against BSA standard (0.5, 1, 2, and 5 µg) on a 10% gel and the protein amount were quantified. 1 µl of the expressed pIKA4 was equal to 4 µg of BSA.

The expression of pIKA2 was repeated at 28°C and induced for 5 h. Due to the pattern of the protein obtained, the expression was repeated using the same condition of expression and the culture was induced for 5hrs. The pellets were sonicated five times at 40% amplitude for 1min; the proteins were loaded on gel and quantified again BSA standard.

7.5.4 Purification

The proteins were not solubilised, so could only be purified from SDS-PAGE. A preparative 10% gel with large well was made; 200-250 µg of proteins was loaded on each gel. The gel was ran at low voltage and was stained with

coomassie blue for 1 h and de-stained overnight. A microfuge tube was weighed and recorded as a reference tube, another tube was weighed and the protein band was cut using a sterile blade. The cut gel slice was transferred into the tube and weighed again. This was repeated eight times for each recombinant protein. The purified protein bands were sent to Eurogentec for antibody production.

7.5.5 Antibody production: immunisation schedule

Two rabbits were used for each antigens; pIKA2 (plantain amylase), and pIKA4 (barley amylase). The amount of protein injected is 200 µg of protein per rabbit per injection. Four injections are performed for each antigen in two hosts. Four bleeds are obtained: pre-immune bleed (before immunisation), then first immunisation. The first boost was performed and small bleed was taken, then second boost is made, large bleeding was performed. The third (last) boost was performed and final bleed was made.

7.5.6 Characterisation of Antisera

The antisera were diluted 5000 fold (1 in 5000) and the diluents were used for immunodetection. Samples from GST fused proteins expressed in *E. coli* were loaded at different dilutions on protein gels. The gels were stained with ponceau, washed and the different antisera were used to detect the respective amylases.

Further characterisation of small, large and final bleeds of antisera against the barley amylase was performed with the extract from the transgenic potatoes. Three dilutions of the antisera were used: 1:1000, 1:3000 and 1:5000.

7.6 Analytical Techniques

7.6.1 Glucose assay

The 48 ml GOPOD reagent buffer, pH 7.4 containing 0.22 M of *p*-hydroxybenzoic acid, and 0.4% w/v sodium azide was diluted to 1L with distilled water according to manufacturer's instructions. The GOPOD reagent enzymes (Glucose oxidase plus peroxidase and 4-aminoantipyrine) was dissolved in 20 ml of water and transferred to the diluted buffer solution according to the manufacturer's instructions.

Glucose stock of 10 mg/ml was prepared and dilutions to concentrations of 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.2, 1.4, 1.6, 1.8, 2.0 mg/ml were made.

The glucose assays was performed as followed; 10 μ l of test sample or blank (water) was added to 300 μ l of the GOPOD reagent mix, the solution was mixed and incubated at 45°C for 20 min. The absorbance was read at 510 nm against the reagent blank. The absorbances were used to construct a glucose standard curve in Microsoft excel.

7.6.2 Gel filtration

A column packed with Sephadex G-25 was prepared in water and about 25ml was poured into a column; 200 mM sodium acetate (pH 4.5) was used to wash the column several times. 2.5 ml of glucoamylase was added onto the column in a step wise manner and the protein was eluted with 200mM sodium acetate. Fractions of 500 μ l were collected and analysed for glucoamylase activity and glucose concentration.

7.6.3 Glucoamylase assay

The glucoamylase substrate contained blocked P-nitrophenyl- β -maltoside (4mM), plus thermostable β -glucosidase (5 U/ml) was dissolved in 10 ml of

distilled water according to manufacturer's instructions. The assay was carried out using the procedure described on the Megazyme kit. To measure the total glucoamylase activity: 30 µl of the substrate was added to 30 µl of sample, and the mix was incubated at 45 °C; the reaction was stopped by adding 150 µl of 2% (w/v) Tris pH 8.5. 200 µl of the solution was then added into the well of microtitre plate and the optical density was read at 405nm. OD values of the mock (negative controls) were used to correct the absorbances. Values greater than O.D of 1 were considered out of scale; and the respective samples were diluted. The glucoamylase activity was calculated as the change in optical density divided by the volume of extract used (µl) and incubation times and multiplied by 1000 to convert to ml. The assays were repeated either twice or thrice and the average was computed.

7.6.4 Starch saccharification

A 30% starch solution was prepared as described above, the solution was autoclaved twice. The solution was neutralised by adding 4 M NaOH to a final concentration of 10 mM. The hydrolysed solution solution was cooled to temperature of 55°C and 500 µl of the gel filtrated glucoamylase was added and mixed. The slurry was incubated in the water bath at 55C, samples were taken after 15 min, 30 min, 1, 2, 4, 6 and 8 h. Each time the sample is diluted 300 fold and the glucose amount was determined using the glucose oxidase-peroxidase method as described above. The glucose concentration was deduced using the glucose standard curve.

7.6.5 Combined Saccharification and liquefaction

The starch was gelatinised as described above, glucoamylase was then added to the hydrolysed solution as was described above, 2.5 and 5ml of α-amylase was then added to the solution and incubated at 55°C. All other treatments were as described above.

7.6.6 Acid hydrolysis of starch

A 30% starch solution was made; 30 g of starch dissolved in 70 g (ml) of distilled water, concentrated HCl was added to a final concentration of 10mM. The solution was autoclaved in 5 cycles, at the end of each cycle an aliquot of the liquefied solution was taken, diluted and glucose was measured as absorbance. The glucose concentration was deduced using the standard curve.

7.6.7 HPAE-PAD Chromatography

200 mM sodium hydroxide and sodium acetate were prepared and filtered using the vacuum pump to remove all insoluble particles. The hydrolysate samples from the five repetitive autoclaving were diluted to a concentration of 0.1 mM each. The solutions above were used as effluents in the chromatography according to manufacturer's instructions (Dionex)®.

7.7 Soluble protein extraction from plantains

7.7.1 Protein Extraction

Plantains were obtained from Leeds City Market and were kept at room temperature until extraction. Water soluble proteins were extracted from the plantain as followed; the plantain was peeled, the pulp was cut and measured. Alpha amylase extraction buffer in the ratio of 1:2 was added to the pulp and ground using a domestic blender. The homogenate was spun at maximum using the refrigerated centrifuge, the supernatant was recovered and pellet discarded. Alpha amylase activity was measured. Plantains were obtained from Leeds City Market and were kept at room temperature until extraction. Water soluble proteins were extracted from the plantain.

7.7.2 Ammonium sulphate precipitation and dialysis

The protein was precipitated by fractionated ammonium sulphate ((NH₄)₂SO₄) precipitation at 30%. Ammonium sulphate fractionations were performed repeatedly for plantain and barley extracts. Dialysis membrane CelluSep® was used according to the manufacturer's instruction. The ammonium sulphate fractions were dialysed against distilled water overnight at 4°C. The alpha amylase activity was measured using the Ceralpha Method from Megazyme according to manufacturer's instructions (McCleary and Sheehan, 1987; Sheehan and McCleary, 1988). Total protein concentrations were measured using the Bio-rad reagent (Bradford, 1976).

7.7.3 Column purification

Alpha-amylase purification was attempted using the GE Healthcare HiTrap column for anion exchange chromatography (Q-IEX) and cation exchange chromatography (SP-IEX) with Hepes buffer at pH 7.5 and elution by application of a salt gradient (0-1 M). A further purification of alpha-amylase using Q-HiTrap column was performed with alpha amylase buffer at pH 5.5 instead. The binding of alpha amylase to sepharose was tested in sepharose binding assay; 200 µl of sepharose slurry (initially washed with HEPES buffer) was added to 600 µl of HEPES buffer and 200µl of dialysed amylase extract were mixed and used.

7.8 Protocols used to identify the plantain α-amylase

7.8.1 RNA extraction

RNA extraction was attempted using modified methods (Birnboim, 1988; Suzuki et al., 2004); in brief 1 gram of plantains was ground in liquid nitrogen. 9 mls of NTES solution (1% SDS, 0.1 M NaCl, 0.01 M Tris-HCl and 1 mM EDTA in DEPC-water) was added to 6 ml of 50/50 phenol chloroform and mixed. The plantain powder was added to the mix and centrifuged in Sorvall HB6 at 8000rpm. The supernatant was collected and transferred into

a new corex tube, 800 µl of 2 M sodium acetate, and 15 ml of ice cold ethanol were added and centrifuged. The pellet was re-suspended in DEPC-water. 500µl of 4M lithium chloride was added and the solution was aliquot, vortex and incubated on ice for 3 hours. The solution was centrifuge at maximum speed and the pellet was dissolved in 400 µl of DEPC-water, 40 µl of 2 M sodium acetate and 800 µl of ethanol were then added to the pellet; the RNA was stored at -80°C till further analysis. The solution was centrifuged at maximum speed, the supernatant was removed, and the pellet was drained and re-suspended in 50 µl of water. The absorbance (A_{260}/A_{230} and A_{260}/A_{280}) and RNA concentration were measured using the Nanodrop spectrophotometer. Lots of modifications to the method above to optimise the amount and quality of RNA obtained were carried out repeatedly; these include changing the ratio of NTES solution to phenol-chloroform, the changing of ratio of phenol to chloroform (from 1:1 to 1:2).

RNA was then extracted from approximately 1 g of plantain as described (Asif et al., 2000). Plantain was sliced and deep frozen in liquid nitrogen; 10 mL of preheated (65°C) extraction buffer (100 mM Tris-Cl pH 8.2, 1.4 M NaCl, 20 mM EDTA (pH 8), 2% CTAB; 10 µl of 2-mercaptoethanol) was added to the tissue and homogenized. The homogenate was transferred to a clean 30 ml centrifuge tube and incubated at 65°C for 1 h, with gentle vortexing every 15 min. The tube was cooled to room temperature and 10 ml of chloroform (CHCl₃: isoamylalcohol, 24:1) was added. The tube was shaken vigorously until the two phases formed an emulsion. The tube was centrifuged at 12000 g for 15 min at room temperature. The aqueous phase was collected and re-extracted with an equal volume of chloroform. The tube was centrifuged again. The aqueous phase was collected and 10 M LiCl was added to a final concentration of 3 M, the RNA was allowed to precipitate at 4°C overnight. RNA was recovered by centrifugation at 17000 g at 4°C for 20 minutes. The pellet was dissolved in DEPC treated water and extracted sequentially once with phenol, phenol: chloroform (1:1), and chloroform. The aqueous phase was collected and 1/30 volume of 3 M Na acetate pH 5.2 and 0.1 volume of 100% ethanol were added to it, mixed well and kept on

ice for 30 minutes. The tube was centrifuged in a microfuge at 4°C for 25 min. A white jelly-like pellet consisting mostly of polysaccharides was obtained and discarded. To the clear supernatant 3 M Na-acetate pH 5.2 to a final concentration of 0.3 M and 3 volumes of 100% ethanol were added. The RNA was precipitated at -80°C overnight. RNA was recovered by centrifugation in a microfuge at 4°C for 20 min. The pellet was washed with equal volume of 70% EtOH. It was vacuum dried and re-suspended in 50 µl of DEPC treated water. The absorbance (A_{260}/A_{230} and A_{260}/A_{280}) and RNA concentration were measured using the Nanodrop spectrophotometer.

7.8.2 Design of degenerate primers

Alpha-amylase sequences of different plants were retrieved from the NCBI website, the sequences were aligned using the ClustalW2 multiple alignment programme using the following alpha amylase sequences from; *Hordeum vulgare* (AAA98790.1), *Sorghum bicolor* (XP_002460332.1), *Musa acuminata* (AAN01149.1), *Vigna mungo* (CAA51734.1), *Phaseolis bulgaris* (BAA33879.1), *Glycine max* (ACU18643.1), *Ipomoea nil* (BAC02435.1). Primers were designed based on areas of homology that showed conservation between the sequences. A pair of degenerate primers was designed; amyS1 and amyAS1 for forward and reverse primers respectively.

7.8.3 PCR amplification of cDNA

The cDNA was synthesized from 5 µg RNA with oligo (dT)₁₈ primer using the Fermentas first strand cDNA synthesis kit according to manufacturer's instruction. To terminate the reaction the reaction mix was boiled for 3 min. The cDNA was diluted into 10, 100, and 1000-fold. PCR amplifications were carried out using the degenerate primers amyS1 and amyAS1 with the cDNA as the template using both the concentrated stocks and the diluents. The PCR conditions were as followed; a total of 45 cycles, hot started for 5 minutes paused at 3min and 5µl of *PFU* DNA polymerase (Stratagene) added, denatured at 94°C for 15s , annealed at 50°C and extended at 72°C. 1% gel was run to visualise the PCR amplified product, a preparative gel

was used to extract the DNA. The PCR products of the desired sizes were gel purified using the protocol as instructed by the Qiagen kit manufacturer. The gel purified PCR amplified product was sequenced with the two primers, AmyS1 and AmyAS1. Bioinformatics tools that include Bioedit, BLAST (NCBI), ClustalW (EBI) were used to analyse the obtained sequences.

7.8.4 Rapid amplification of cDNA ends

Two specific primers were designed for RACE from the sequence obtained from the PCR amplified product using AmyS1 and AS1. The cDNA was then amplified using PlanS1 and PlanAS1, PCR conditions (for RACE) used were as followed; 40 cycles of annealing with a 5 min initial denaturation at 94°C (hot start for 3 min). Annealed at 60°C for 1 minute, extended at 72°C for 2 min, and denatured at 94°C for 20s. PCR products were resolved on 0.6% gel. A PCR product of 720bp was obtained and extracted from the gel using the Qiagen kit according to the manufacturer's instructions. DNA was finally eluted in TE buffer; the eluted DNA was run on 1% gel to check the efficiency of purification. The gel purified PCR amplified product was sequenced with the primers, PlanS1 and PlanAS1.

7.8.5 3'RACE

In order to obtain the sequence of the 3'end of the gene, the plantain cDNA was PCR amplified using a specific sense primer PlanS1 and a 3' RACE primer as the antisense primer. The conditions used are 40 cycles of 95°C for 2 min (initial denaturation), 95°C for 25s, 45°C for 15s and 70°C for 20s. PCR product was resolved and gel purified on a 1% gel.

Nested -3'RACE

The gel purified product was diluted 10, 100 and 1000 fold, the diluents were PCR amplified using a more internal sense primer, PlanS4 (5'CCACCACCCACTGAGTCTGT 3') and 3'RACE primer in the antisense direction. The conditions used are 25 cycles of 95°C for 2 min (initial denaturation), 95°C for 25s, 45°C for 15s and 70°C for 20s. PCR product

was resolved and gel purified on a 1% gel. The product was sequenced with PlanS4 and a more internal specific primer (sense), PlanS2.

7.8.6 PCR amplification of big plantain clone

In order to clone the big amylase clone from plantain, the cDNA was PCR amplified using a degenerate sense primer PlanS6 (and a specific antisense primer, AS4. The conditions used are 45 cycles of 95°C for 2 minutes (initial denaturation), 95°C for 25 seconds, 50°C for 15 seconds and 70°C for 40 seconds. PCR products were resolved on a 0.8% gel.

Semi nested-PCR; the PCR amplified product was diluted 100, 1000 and the diluents were PCR amplified using PlanS6 and a more internal antisense primer, AS5. The conditions used are 40 cycles of 95°C for 2 minutes (initial denaturation), 95°C for 25 seconds, 60°C for 15 seconds and 70°C for 40 seconds. PCR products were resolved on a 1.2% gel. The above was repeated for 30 cycles, and was gel purified on a 0.8% gel. The 1.9kb gel purified product was sequenced with the two primers PlanS6 and AS5.

Table 7.4 List of primers used for cloning the plantain α -amylase

S/ N	Name	Sequence	Direction
1	AmyS1	5' TTY CAR GGN TTY AAY TGG GA 3'	Sense
2	AmyAS1	5' ATR TAN GCR TAN CCY TGC AT 3'	Antisense
3	AmyS2	5' GAY TGG GGN CCN CAY ATG AT 3'	Sense
4	AmyAS2	5' GGC CAC CAG CCC ATC AT 3'	Antisense
5	PlanS1	5' GGTTTTACAGTCATCTGGCT 3'	Sense
6	PlanAS	5' AACGGCACGAGATGCCACCA 3'	Antisense
7	PlanS2	GCTACAAATGGAAGTGCAGG	Sense
8	PlanS3	5' AAR GAY TAY GCN ATH GAR AC 3'	Antisense
9	PlanS4	5' CCACCACCCACTGAGTCTGT 3'	Sense
10	PlanAS4	5' CAGCCTACTACTCCAGGAGG3'	Antisense
11	PlanAS5	TGTGAGTATTGGCGATTGTCTGATCAAAATGGG	Antisense
12	PlanS6	5' GAR TGG GAY CAR CCN CCN 3'	Sense
16	PlanS9	ATTGTTGCTGATGATCCTCA	Sense
17	PlanS10	TATGATGGATGGAGATTAGA	Sense
18	PlanS11	AGTATTGGGACTCATTAAGT	Sense
19	PlanUDs	TATCTAGCCTGCTGCTGAAAGC	Sense
20	Upas1	ATTGTATAAATCCTTTGGCAT	Antisense
22	PlanEnd	ACAAGATTCACTGCAGGAGC	Sense
23	3'RACE	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT(GAC)(GATC)	Antisense
24	Planrace1	TTGTGCTGAAACCATGCTCC	Antisense
25	Planrace2	CAGTTCGTGAAGTGCCTGCC	Antisense
26	Atgprimer	NNN-ATG-N	Sense
26	Planrace3	ATTGCAGCAATTCATTTTGT	Sense
27	Planrace5	ACAAAATGAATTGCTGCAAT	Antisense

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