Chapter 7

General Discussion and Future Work

7.1. Comparison of the Properties of the Glycosidase Isolated from Apple with Glycosidases from other Sources

β-D-Glycosidase has been known as a hydrolase for over 170 years (Esen, 1993), and there has been much research regarding its commercial usage, physiochemical properties, and kinetic characteristics. β-D-Glycosidases have been found in many different sources, predominantly microbial and plant tissues, and each possesses different features. The glycosidase from apple seeds studied in this thesis has not previously been studied before in terms of kinetic properties, though Yu *et al.* (2007) has used SDS-PAGE to look at the molecular weight and stability of the apple enzyme. The following relates the properties identified in the present work to literature reports of glucosidases from other sources.

Enzymes from different sources may possess different characteristics including their physiochemical properties, molecular weight, kinetic characteristics, etc., even though they may catalyse the same reaction.

7.1.1. Physiochemical Characteristics of β-D-Glycosidases from Different Sources

The physiochemical characteristics of β-D-glycosidase from different sources vary but have some things in common. Most of them operate at an optimum pH between 4-6, and an optimum temperature range of 37-70°C. Besides apple seed β-D-glycosidase, the enzymes from most plant sources have optimum temperatures below 60°C while enzymes from *Aspergillus wentii* and China white jade snail, which are of bacterial and animal sources respectively, have an optimum temperature higher than 60°C. Apple seed β-D-glycosidase can work at higher temperatures than those from other plant sources. Optimum reaction conditions are listed in table 7-1.

Table 7-1 Physiochemical characteristics of β-D-glycosidase from different sources

Sources (with *botanic name*)	Source type	Optimum pH	Optimum Temp.(°C)	Reference
Almond (*Prunus dulcis*)	Plant seeds	5.5	50	Present study
Apple seeds (*Malus domestica*)	Plant seeds	5.5	65	Present study
Alfalfa (*Medicago sativa L.)*	Plant seeds	5.0	55	(Robinson, 1996)
Almond (*Prunus dulcis*)	Plant seeds	5.0	37	(www.sigma-aldrich.com)
Almond (*Prunus dulcis*)	Plant seeds	4.8	50	(Ducret *et al.*, 2006)
Soy bean (*Glycine max*)	Plant seedlings	6.0	30	(Hsieh & Graham, 2001)
Corn Stover (*Zea mays L*.)	Plant waste	4.8	37	(Han & Chen, 2008)
Grapes (*Vitis L*.)	Plant fruit	5.0	45	(Lecas *et al.*, 1991)
Papaya (*Cirica papaya L*.)	Plant fruit	5.0	50	(Hartmann-Schreier & Schreier, 1986)
Sicilian blood orange (*Citrus sinensis* L.Osbeck)	Plant fruit	4.5	60	(Barbagallo *et al.*, 2007)
Strawberry (*Fragaria* × *ananassa* cv. Elsanta)	Plant fruit	4	60	(Orruño *et al.*, 2001)
Aspergillus wentii	Fungi	4.5-5.5	60-65	(Srivastava *et al.*, 1984)
Aspergillus oryzae	Fungi	5.0	60	(Zhang *et al.*, 2007)
Aspergillus niger	Fungi	4.5	60	(Yan *et al.*, 1998)
Stachybotroys sp.	Fungi	5	50	(Amouri & Gargouri, 2006)
China white jade snail (*Achatina fulica*)	Animal tissue	5.6	70	(Luan *et al.*, 2006)
Human livers and intestine	Human tissue	5-6	37	(Daniels *et al.*, 1981; Hays *et al.*, 1996)

The temperature and pH for enzyme stability and for reaction optimum are different concepts. The conditions for enzyme stability are the features of the enzyme molecule itself, while the conditions for optimizing enzyme reactions are affected by the enzyme, the reaction, and the environment together. As a result, these two conditions may be different. For example, the β-D-glycosidases from the fungi *Aspergillus oryzae* had optimum conditions at pH 5.0 and 60ºC (Zhang *et al.*, 2007), while optimum stability conditions were at less than 60ºC and under pH 4-5. For commercial usage, the optimum conditions are always a determined priority, while for enzyme extraction and purification, the stability conditions become more important because the principle aim of enzyme extraction and purification is to obtain the enzyme in as high a yield as possible consistent with the retention of maximal catalytic activity (Price & Steven, 2002).

In this study, β-D-glycosidases from raw materials were used and tested as crude extracts without purification, and the optimum stability conditions for the pure enzyme have not been determined. This approach is acceptable since the results show that the enzyme was very stable under the optimum reaction conditions (3.4.4. & 4.4.2.). On the other hand, another consideration was that the enzyme assay including the kinetic study had been assumed to be carried out under optimum reaction conditions.

7.1.2. Difference in Kinetic Properties of β-D-Glycosidases from Different Sources

Km and Vmax are very important parameters of an enzyme and an enzyme catalysed reaction, which can be used to not only explain how enzymes work and predict how enzymes behave, but also to compare enzyme activities under the same reaction conditions and using the same substrate. The ratio Vmax/Km is even better for comparison. Km, Vmax, and Vmax/Km were defined in chapter 4. The different kinetic properties of β-D-glycosidases from different sources are listed in table 7-2.

Table 7-2 Kinetic parameters of β-D-glycosidase from different sources

Sources (with *botanic name*)	Substrate	Km (mM)	Vmax(U/ml or mgx10^{-6})	Vmax/Km X10^{-7}	Reference
Almond (*Prunus dulcis*)	p-NP-Glc	3.32	13.54x10^{6}	4.08x10^{7}	Present study
Apple seeds (*Malus domestica*)	p-NP-Glc	5.48	15.60x10^{6}	2.85x10^{7}	Present study
Soy bean (*Glycine max*)	Isoflavone-glucoside	5.4-33 x10^{-5}	Kcat(s^{-1})=3.07-4.45	Kcat/Km($M^{-1}s^{-1}$) =1.3-8.1x10^{4}	(Hsieh & Graham, 2001)
Corn Stover (*Zea mays L.*)	p-NP-Glc	2.3	18.6x10^{6}		(Han & Chen, 2008)
	Cellobiose	4.6	36.2x10^{6}		
	Salicin	9.3	21.5x10^{6}		
Grapes (*Vitis L.*)	p-NP-Glc	1.81	43.9	242.93	(Lecas *et al.*, 1991)
Papaya (*Cirica papaya L.*)	p-NP-Glc	0.11	9.51	864.82	(Hartmann-Schreier & Schreier, 1987)
Sicilian blood orange (*Citrus sinensis* L. Osbeck)	p-NP-Glc	0.267	210x10^{6}		(Barbagallo *et al.*, 2007)
	Anthocyanins	210	3.3x10^{3}		
Vanilla (*Vanilla planifolia*)	p-NP-Glc	3.3	11.5x10^{6}	3.5x10^{7}	(Dignum *et al.*, 2004)
Strawberry (*Fragaria* × *ananassa* cv.Elsanta)	p-NP-Glc	18.5	474	256.22	(Orruño *et al.*, 2001)
Aspergillus wentii	p-NP-Glu	1.6	7.6x10^{6}		(Srivastava *et al.*, 1984)
	Cellobiose	8.0	21.3x10^{6}		
Aspergillus oryzae	Piceid	0.74	323nkat mg^{-1}		(Zhang *et al.*, 2007)
Aspergillus niger	p-NP-Glc	2.2	10.2x10^{6}		(Yan *et al.*, 1998)
	cellobiose	15.4	464x10^{6}		
Stachybotroys sp.	p-NP-Glu	0.27	78x10^{6}		(Amouri & Gargouri, 2006)
	Cellobiose	2.22	59.4x10^{6}		
	Salicin	37.14	2x10^{6}		
China white jade snail (*Achatina fulica*)	p-NP-Glc	0.224	0.203		(Hu *et al.*, 2007)
Human livers	Q-4'	27x10^{-3}	0.35-1.38x10^{6}		(Day *et al.*, 1998)
	G-7	13x10^{-3}	1.03-1.34x10^{6}		
Human small intestine	Q-4'	37x10^{-3}	1.06-1.51x10^{6}		
	G-7	14x10^{-3}	1.50-2.91x10^{6}		

p-NP-Glc: para-nitrophenyl-β-D-glucopyranoside; p-NP-Glu: para-nitrophenyl-β-D-glucoside; Q-4': quercetin-4'-glucoside; G-7: genistein-7-glucoside=genistin

In the present study, whole almond and apple seed have been extracted and tested as crude extracts by using p-NP-Glc as an artificial substrate. Results for whole almond extract showed that the kinetic parameters were Km=3.32mM, Vmax=13.54U/ml protein, Vmax/Km=4.08; for apple seed extract Km=5.48mM, Vmax=15.60U/ml protein, Vmax/Km=2.85. Compared with almond, which is a well-known enzyme source of β-D-glucosidase, the enzyme activity of apple seed extracts is less strong. However, apple seeds are a very cheap source of food waste while almond is a relatively expensive edible nut.

Table 7-2 details various kinetic properties of glycosidases from different sources. The enzyme activity in apple seed extracts is significantly stronger, with the Vmax/Km values 10^5-fold greater than grapes, papaya and strawberry, and with similar levels for the vanilla, corn stover (essentially the waste plant material left after harvest of the maize cobs), and sicilian blood orange enzymes. The enzyme in apple seed extracts may have similar activity to that from *Aspergillus*, but much lower activity than that in the enzyme from human tissues.

7.1.3. Sources of β-D-Glucosidases

It can be seen from table 7-1, that the sources of β-D-glucosidases are predominately plant and microbial. Compared to other sources, apple seeds have significant advantages to be a new enzyme source of β-D-glucosidases, which are:

- the presence of enzyme in high activities. The enzyme activity in apple seeds is at the same levels as that of almond, which is a commercial β-D-glucosidase enzyme source.
- stability. β-D-Glucosidases from apple seeds can work at high temperatures and maintain enzyme activity at 4°C for at least 12 weeks, so it is a stable source that can be easily used, for example, in the present study. It can be used easily in a bakery, without being worried that it will lose enzyme activity.
- cost. Apple seeds are food waste of processes such as juice production, while most other plant sources, like strawberry, almond, papaya, are normally more valuable and not giving rise to waste. While no calculations have been made

(it would require detailed commercial knowledge of the economies of apple processing), it can be assumed that apple seeds might be a cheap source of the enzyme, especially if it were used in a food context. The value of apple seeds might be greatly enhanced by being used as an enzyme source.

So, using apple seeds as an enzyme source to make soy flour biscuits is a good combination, which might not only promote the health value of soy isoflavone, providing a feasible enhancement for the health benefits of phytoestrogen in the diet, but also enhancing the value of a food waste – apple seeds.

In order to test the health effects of dietary compounds it is necessary to perform human studies. However, it is difficult to select appropriate control foods. The use of the apple seed enzyme to generate two identical foods that vary only in their content of isoflavone glycoside and isoflavone aglycone allows human studies with appropriate controls. How might such a study be carried out?

7.2. Possible Human Intervention Study

7.2.1. Subjects and Diet

As in all human studies, selection of subjects is an important issue. Subjects should not be taking any prescription drugs or other medicine. Isoflavone-rich food (especially soy, soy-derived food, kudzu, bean sprouts, chick pea, runner bean, peanuts, and other legumes) should be avoided for at least 3 days prior to the experiment, and during the study. No alcohol should be consumed and strenuous exercise avoided during the experimental days.

7.2.2. Study Design

The study should be divided into 2 parts. In each part, the subject should have a "wash-out" by taking a low isoflavone diet for at least 3 days, before consuming the designated food (either un-hydrolysed soy biscuits, food A or hydrolysed soy flour

biscuits, food B) and then collecting urine samples hourly for 48hr. There should be at least a 1 week interval between the two parts of the experiment. High isoflavone content diets should be avoid during the whole experiment period.

7.2.3. Urine Sample Collection

A suggested flow diagram of the possible experimental approach is shown in figure 7-1. On the first day of experiment, the subjects would get up early in the morning, void the bladder and immediately drink 200ml water. An hour later the subjects would void the bladder again to obtain the baseline sample. Then 120g of biscuit from 6.3.3 (Food A or Food B) should be consumed with 200ml water. Urine samples would be collected every hour and a further 200ml water would be consumed immediately in order to produce enough urine for analysis. When the 15hr urine sample is collected the water drunk should return to normal. Urine produced between 15hr and 24hr should be collected as the 24hr sample. The same should happen for 36hr and 48hr samples. This proposed method is modified from the version described by Rothwell (2005).

The urine samples collected should be measured for volume after cooling down and then 50ml of urine sample would be transferred to a vial containing 0.05g ascorbic acid. The urine samples could then either be stored in a refrigerator or analysed immediately.

Low isoflavone content lunch, dinner, and snacks would be consumed during the experimental period and the subjects should record all food and drink consumed during the experimental days.

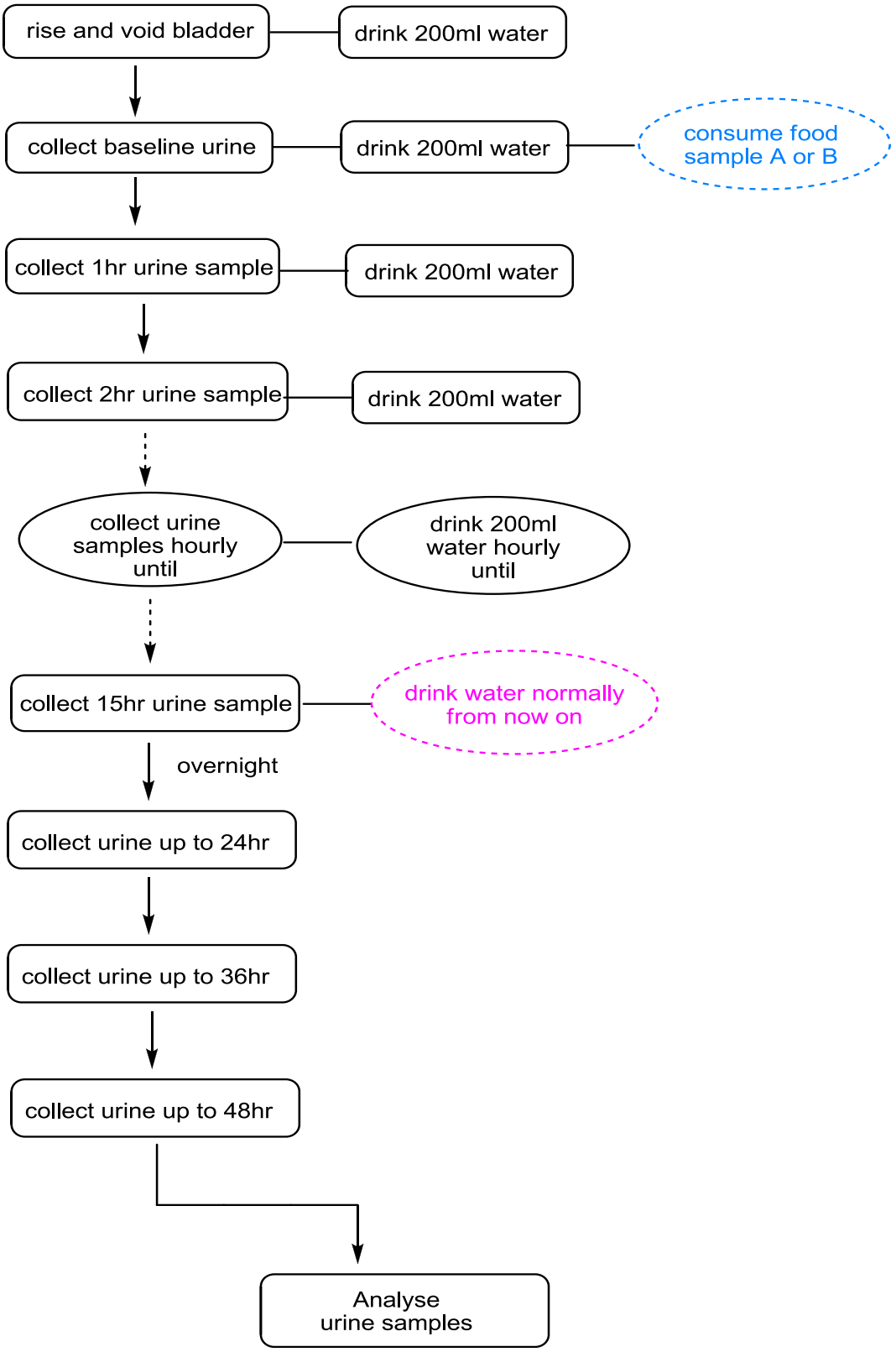

Figure 7-1 Flow diagram of a possible human study

7.2.4. Conditions for Urine Sample Analysis

Ethyl acetate is an organic solvent with very weak polarity, and can be used to extract components with high hydrophobicity. After being treated with enzyme β-glucuronidase and sulfatase, flavonoids would exist in urine as aglycone forms, which are much more hydrophobic than conjugated forms. Preliminary experiments (data not shown) of recoveries showed that the efficiencies for extracting daidzein, genistein, and equol from urine with ethyl acetate were 94.36 ± 2.3%, 95.74 ± 2.3%, and 86.58 ± 3.8% respectively, which were quite good compared with some other reports (Xu *et al.*, 1994; Zhang *et al.*, 1999; Hendrich, 2002; Wang *et al.*, 2006). The use of ethyl acetate would make it easy to separate solvent phase and water phase after extraction, so centrifugation would be unnecessary.

The method of enzymatic hydrolysis has been described by DuPont *et al.* (2002). The particular enzymes were selected because (a) they are known to be efficient in de-conjugating glucuronides and sulfates so excessive concentrations are not required, (b) they work optimally at pH 7 so acidification is not needed, whereas cellulose, for example, can also carry out this reaction but its optimum pH is around 5, and (c) the enzymes are inexpensive and relatively pure.

Urine analysis is much less used than plasma analysis in human intervention studies, because the pharmacokinetic information obtained from urine is less direct than that from plasma. However, urine metabolite concentrations are considered to represent the dose absorbed, and follow those of the plasma at any given time (Hollman, 2001). The cumulative measurement of excreted flavonoid allows bioavailability to be determined (Scalbert & Williamson, 2000). Some researchers investigated the relationship between the urine excretion, plasma concentration, phytoestrogen levels and isoflavone intake, and found the urine excretion of isoflavone could be used as a biomaker of isoflavone intake, absorption and metabolism (Ritchie *et al.*, 2004). Franke *et al.* (2008a) found that the urine isoflavone excretion rate reflects circulating isoflavones accurately when area-under-curve (AUC) and identical time intervals are used ($r = 0.93$; $p < 0.001$), and concluded that urine isoflavone excretion is an adequate surrogate for determining isoflavone bioavailability and for measuring soy

or isoflavone exposure in epidemiologic or other studies. They also suggested using apparent bioavailability when using urinary excretion data, since bioavailability can be defined based on circulating levels.

The benefits of using urine to measure isoflavones include its non-invasiveness compared with blood sampling, as well as the ability to collect highly concentrated urine in large amounts, which leads to low quantification limits. Urine can be obtained by participants themselves without medical supervision. Most importantly, urine can be accumulated over many hours (even days) reflecting exposures over much longer time periods, therefore a descriptive time-course of excretion curve can be conducted, compared to data from blood, which only reflects one given point in time per collection. Also urine can be collected as frequently as desirable, samples are easier to handle and store than blood. As a result, it will take a lot of time both analysing samples and calculating results.

7.2.5. Soy Isoflavone Metabolites in Human Urine

For many years the studies on isoflavones metabolism have been focused on daidzein and genistein, the principal isoflavones of soy, while the red clover isoflavones formononetin and biochanin A, which have 4'-methoxyl groups at B-ring, are believed to be demethylated and converted to their metabolites daidzein and genistein (Heinonen *et al.*, 2002). For glycitin, there is less information available, which may be due to its low concentration in nature and lower oestrogenic activity (Setchell *et al.*, 2001; Heinonen *et al.*, 2003). The metabolic pathways of isoflavones so far are based on the identification of the metabolites. Figure 7-2 and 7-3 show the colon metabolic pathways of daidzein and genistein.

Isoflavones are generally very stable and their main absorption site is believed to be the colon (Williamson, 2004). Most of isoflavones reach the colon and then take microbial deglycosylation by various bacteria such as *E. coli* (Hur *et al.*, 2000), then can be further metabolised into dihydrodaidzein and dihydrogenistein respectively. Dihydrodaidzein may be further converted into equol and *O*-desmethylangolensin (O-DMA) (Heinonen *et al.*, 1999; Hwang *et al.*, 2006). Equol would then be absorbed

like other isoflavone aglycone by the re-absorption mechanism and enter the blood system. O-DMA may be degradated continuously into smaller molecules like phenolic acids or phenylacetic acids before being eliminated.

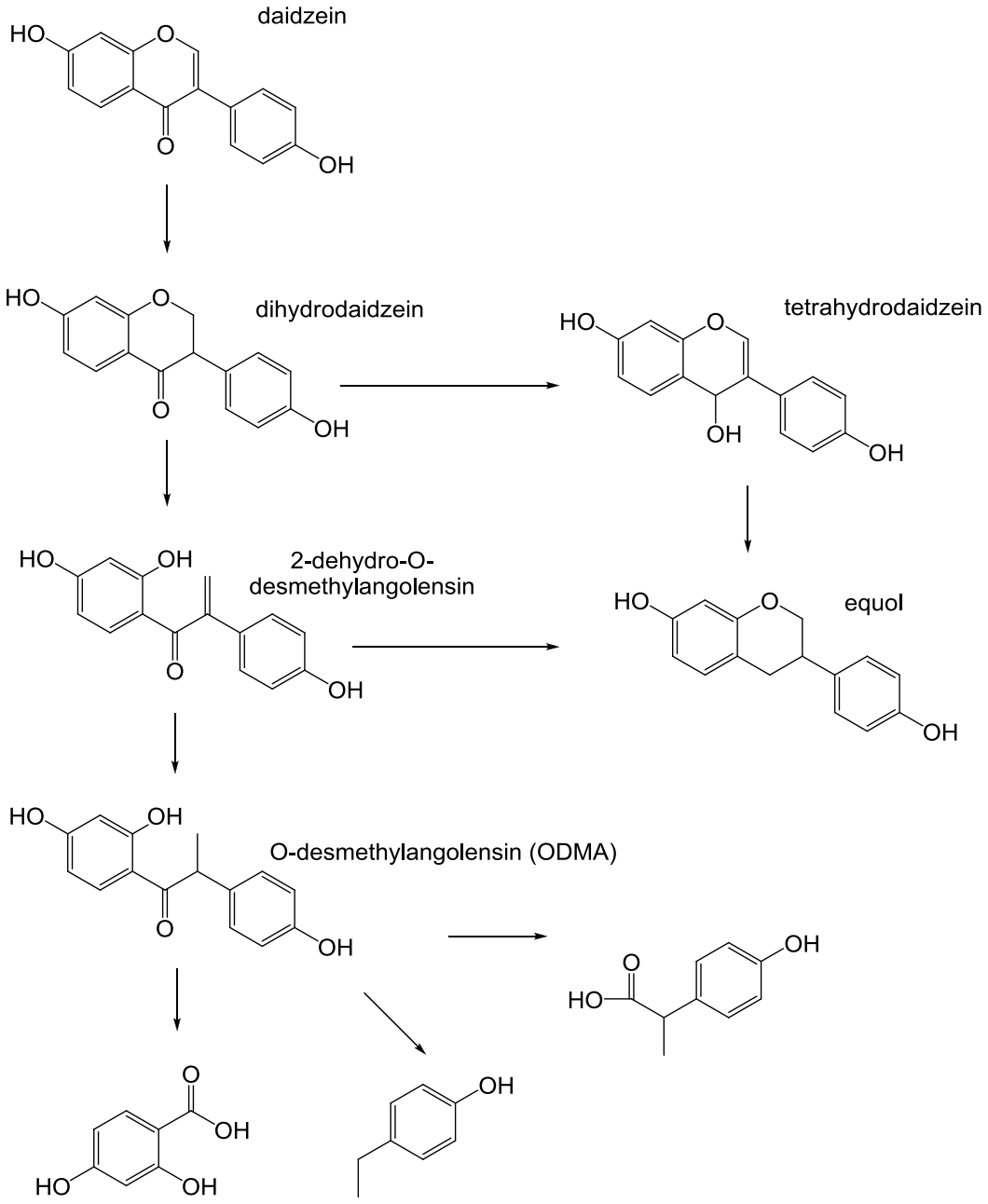

Figure 7-2 Degradation of daidzein in the colon (adapted from (Day *et al.*, 2004)).

Equol was first identified in human urine in 1982, followed by the identification of *O*-desmethylangolensin (O-DMA) in 1984 (Heinonen *et al.*, 2002), which two have been considered as the end-products of metabolism of daidzein. Then some other

metabolites of intermediates were identified in late 1980s. Comprehensive studies on soy isoflavone metabolism were carried out by Kelly *et al.* (1993) and Joannou *et al.* (1995). The main metabolites of daidzein were reported to be equol, dihydrodaidzein (Int-O-D) and O-DMA. The minor metabolites of daidzein, identified as 2-dehydro-*O*-desmethylangolensin (2-de-O-DMA), and two isomers of tetrahydrodaidzein (Tetra-D), better known as 4-OH-equol, were reported. Two metabolites of genistein, dihydrogenistein (Int-O-G), and 6'-hydroxyl-*O*-desmethylangolensin (6'-OH-O-DMA), were also identified (Joannou *et al.*, 1995).

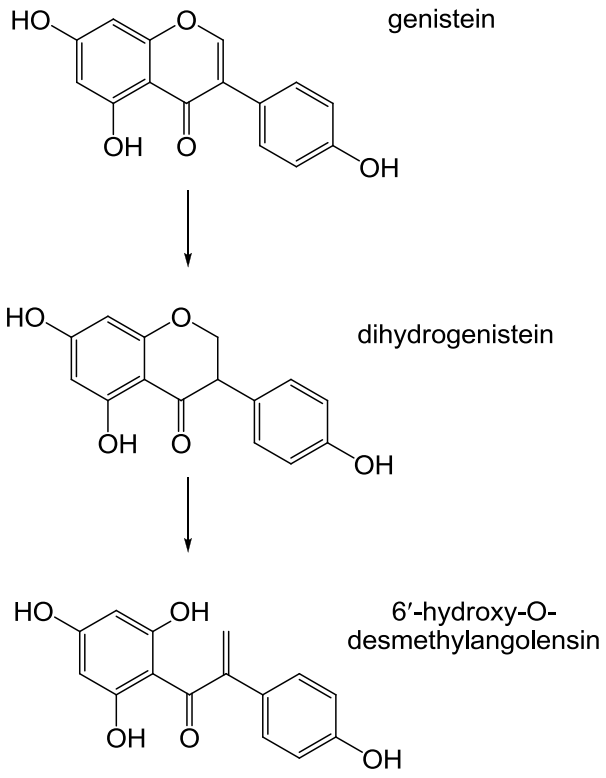

Figure 7-3 Postulated metabolic breakdown of genistein (Joannou *et al.*, 1995).

Unlike other flavonoids, both the phase I and phase II metabolites of isoflavones have been identified in human body fluids. Although the metabolism of isoflavones in humans is diverse and individual variation does exist, usually isoflavone metabolites are formed by deglycosylation, reduction, oxidation, methylation, demethylation, and conjugation with glucuronic and/or sulfate acids prior to excretion (Heinonen *et al.*, 2002). The most abundant phase I metabolites of isoflavones seem to be formed by reduction. In terms of phase II metabolism, there were fewer studies in humans have

been carried out, but it has been suggested that isoflavones and their metabolites occur mainly as glucuronide conjugates, with sulfates and sulfoglucuronides have also been found (Adlercreutz *et al*., 1995). In general, the levels of phase I metabolites are low, dominant biotransformation products of isoflavones in humans are glucuronides, followed by sufates and sulfoglucuronides (Hendrich, 2002). The favourite position of glucuronidaton for daidzein and genistein may be 7-*O*-glucuronides, and then 4'-*O*-glucuronides (Heinonen *et al.*, 2002).

So, from the literature, all of the urine analysed used enzymatic extraction for isoflavone measurement, and glucuronidases and sulfatases are very popular for such purposes. In human plasma or urine, only small amounts of free isoflavone aglycones have been identified. Glucuronide conjugates made up about 70-80% of urinary isoflavones and 50-60% of plasma isoflavones, whereas isoflavone aglycones made up 5% of urinary isoflavones and 20-30% of plasma isoflavones, although individual variations exist (Hendrich, 2002). The proportion of these 3 forms in plasma and urine is shown in figure 7-4.

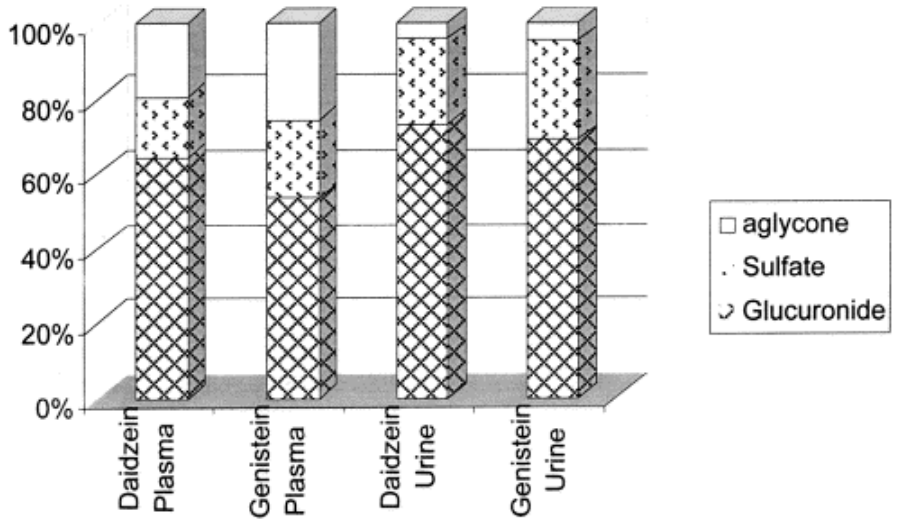

Figure 7-4 Percentages of glucuronide and sulfate conjugates and isoflavone aglycones in urine and plasma from women fed soymilk isoflavones (Hendrich, 2002).

Since some metabolites of isoflavone, such as equol and ODMA, have been reported to possess important bioactivities (Frankenfeld *et al.*, 2004; Raschke *et al.*, 2006; Nestel *et al.*, 2007), the pharmacokinetics of the metabolites have also been

investigated (Watanabe *et al.*, 1998; Richelle *et al.*, 2002; Franke *et al.*, 2004; Franke *et al.*, 2008a; Franke *et al.*, 2008b; Rüfer *et al.*, 2008), but much lesser and incomplete data are available accompanied by huge individual variations. More rapid analysis method(s) with lower detection limits are urgently required because of the lower levels present either in plasma or in urine.

7.2.6. Inter-individual Differences

7.2.6.1. Urine Excretion

Urine excretion has been reported to show variation due to inter-individual differences. Richelle *et al.* (2002) analyzed the urine samples of 6 postmenopausal women fed isoflavone supplement-enhanced soy beverages at the level of 1mg isoflavone aglycone equivalent/kg body weight and found the total isoflavone excretions were: daidzein 50% and genistein 18% for aglycone isoflavone beverage; daidzein 56% and genistein 20% for glycoside isoflavone beverage, but the equol excretions in Richelle's study were less than 1% of daidzein intake. However, the intake used by Richelle *et al.* was relatively high, and reached more than 60mg of individual isoflavones, for example, 156μmol = 44.3mg of glycitein, which may be too high to be natural situations.

Vergne *et al.* (2007) investigated the effects of matrix on the link between soy isoflavone supplementation and equol production. Two formulations, A and B, were soy extract capsules mixed with cellulose as the excipient and adjusted with soy flour respectively. They found that 65% of daidzein was eliminated through urine for both formulations, 51.4% and 33.2% of genistein was eliminated in urine for both formulations, and the peaks of absorption for both daidzein and genistein were at 12hr. For equol, it appeared in urine from 6hr and reached a maximum at 18hr. The authors did not calculate the percentage of equol excretion, but found that daidzein excretion in urine of the "equol-producers" was lower than for the "equol non-producers", and suggested that the "equol producers" daidzein excretion plus "equol producers" equol excretion was equivalent to "equol non-producers" daidzein excretion.

Differences in the chosen subjects themselves bring natural variations. From a health perspective, isoflavones may be of greater benefit to those who are able to retain biologically active isoflavone metabolites in their circulatory system for longer periods of time after discontinuing the intake of soya foods. Tsangalis *et al*. (2005) found that the woman who excreted the greatest level of isoflavone after two 14 days washout periods also had the highest BMI, at approximately 39kg/m^2. They suggested women with a higher percentage of body fat may have a greater tendency to retain isoflavones because isoflavones in an aglycone form (absorbed) are lipid soluble.

Some differences may be caused by the bigger time interval between 2 experimental points, in other words, the lack of data between 2 sample collection times, since most research focused on plasma concentration, when samples cannot be collected too frequently. So, although some scientists believe that the urine content of flavonoids cannot be used as a biomarker of bioavailability or dietary intake (Williamson, 2004), it is useful for urine analysis to be evidence and a supplementary parameter of flavonoid absorption and metabolism.

Up to now, bioavailability studies gave contradictory results, which may be due to the different experimental designs and the huge inter-individual differences. Such findings make this area highly attractive and challenging.

7.2.6.2. Equol

There is a great inter-individual variability in the capacity to produce equol. Only 30-40% of the western population are equol-producers (Frankenfeld *et al.*, 2004; Cassidy *et al.*, 2006; Wiseman, 2006), while the corresponding percentage among Asian populations may be as high as 60% (Morton *et al.*, 2002; Song *et al.*, 2006; Ko *et al.*, 2010). The ability or inability of persons to produce equol seems to remain the same for at least several years (Karr *et al.*, 1997; Setchell *et al.*, 2002b), since the composition of the intestinal flora is believed to be playing a major role causing this variability, which may not be changed in a short time (Manach *et al.*, 2004).

Much research has been carried out to investigate this inter-individual difference (Lampe *et al.*, 1998; Rowland *et al.*, 2000; Lampe *et al.*, 2001; Morton *et al.*, 2002;

Ohta *et al.*, 2002; Bowey *et al.*, 2003; Frankenfeld *et al.*, 2004; Saitoh *et al.*, 2004; Song *et al.*, 2006; Vergne *et al.*, 2007; Ko *et al.*, 2010), and found that equol producers tend to consume less fat and more carbohydrates as percentages of energy than do non-equol producers (Manach *et al.*, 2004; Wiseman, 2006). Consumption of dietary fibre has been suspected to affect equol production by favouring the growth of certain bacterial species. However, supplementation with 16g wheat bran did not increase equol production in young women (Lampe *et al.*, 1998). The effect of adaptation of the intestinal flora to the consumption of isoflavones is not clear. Lu & Anderson (1998) observed an increase in equol production after 1 month of isoflavone consumption. Some non-equol-producing women even acquired the ability to produce equol after consuming soymilk for 2 weeks. Lampe *et al* (2001) did not observe any effect on equol production of a 1-month adaptation in comparison with a 4-days supplementation. A recent study carried out in Taiwan by Ko *et al.* (2010) showed that 8 of 20 non-producers were induced to become equol producers by ingestion of soymilk weekly for 16 weeks. In a preliminary study, the subject who could excrete 6.65μmol and 6.03μmol equol or more in urine at 48hr experimental period after ingesting either the glycoside form or the aglycone form of soy isoflavone (biscuits A & B) respectively, is definitely an equol-producer, according to the definition of Lampe *et al.* (1998).

7.3. Concluding Statements

- Glycosidases from plant sources have been compared.
- Apple seeds have been found to be a novel source of a glycosidase.
- The novel enzyme has been studied and may be an alternative commercial source of glycosidase activity.
- The novel enzyme has been used in the production of a food material with high isoflavone aglycone content.
- Foods rich in isoflavone aglycones might have potential health benefits.