Chapter 3

Identification of Enzyme Sources

3.1. Introduction

As discussed previously, most flavonoids (except catechins) naturally exist in plants as glycosides, i.e. conjugated with a sugar moiety, and the most frequently occurring forms are β-glycoside derivatives (Harborne *et al.*, 1975; Havsteen, 1983). Studies show that processing methods such as canning, frying and boiling do not result in the deglycosylation of the flavonoids, though some may be lost through leaching (Crozier *et al.*, 1997; Price *et al.*, 1997; Price *et al.*, 1998a; Price *et al.*, 1998b). However, in food that has undergone microbial fermentation, for example red wine, black tea, tempeh and miso, the aglycone isomers are the major form present (Wang & Murphy, 1994b; McDonald *et al.*, 1998; Chun *et al.*, 2008). Deglycosylation can be catalysed by enzymes such as β-glycosidases.

The enzyme β-D-glycosidase (β-D-glycoside glucohydrolase, Enzyme Commission number: EC 3.2.1.21) can catalyse the hydrolysis of glycosidic linkages in aryl and alkyl β-glycosides and cellobiose, and occurs ubiquitously in plants, animals, fungi and bacteria (Esen, 1993).

Plant β-glycosidases have been known for over 170 years since the description of the action of almond β-glycosidase by Liebig and Wohler in 1837. Besides almond, maize, white clover and cassava are also well-studied sources of β-D-glycosidases (Esen, 1993).

Current research on β-glycosidases has significant scientific, medical, and economic implications. Human acid β-glycosidases have potential in the development of therapeutic and diagnostic procedures that will be useful in the treatment of Gaucher's disease, an inherited disorder caused by the deficiency of acid β-glycosidase localized in the lysosome (Esen, 1993). Cytosolic neutral β-glycosidase is implicated in the metabolism of pyridoxine-5′-β-D-glycosidise, as well as the hydrolysis of β-glycosidises ingested from foods of plant and animal sources. Plant β-glycosidases have been implicated in a variety of growth, productivity, defence, and feed toxicity-related reactions, and may be involved in the metabolism of plant hormones such as auxin, gibberellin, and cytokinin, whose storage forms occur as β-glycosidases and

are activated upon cleavage by β-glycosidase (Esen, 1993). In fungi and bacteria, the enzyme is involved in cellulose and cellobiose catabolism as part of the cellulose complex. Cellulases break down cellulose to cellobiose, and β-glycosidases hydrolyse cellobiose to two glucose molecules. Thus, fungi and bacterial β-glycosidases appear to be ideal candidates for engineering a β-glycosidase to be used as part of the cellulose complex in the industrial-scale conversion of cellulose to glucose.

Because β-glycosidises and β-glycosidases are ubiquitous in the living world, one expects to find structural and catalytic properties shared by all β-glycosidases. In fact, a review of the literature indicated that almost all β-glycosidases have subunit molecular weights of 55 to 65 kD, acidic pH optima around pH 5-6, (from plant sources especially), and an absolute requirement for a β-glycoside (i.e. glucoside, and to a much lesser extent fucoside and galactoside) as substrate (Esen, 1993).

β-D-Glycosidase is a kind of hydrolase. The assay of β-D-glycosidases is most commonly undertaken using para-nitrophenyl-β-D-glucopyranoside (abbreviated as p-NP-Glc) as artificial substrate other than its natural substrates. Using p-NP-Glc is very much quicker and more convenient for the enzyme assay. After p-NP-Glc has been hydrolysed, para-nitrophenol (abbreviated as p-NP) is produced. p-NP-Glc shows strong absorption at 400nm, primarily due to the ionized form of p-NP, and exhibits a bright yellow colour in alkaline or basic conditions. This allows quantification to be made colorimetrically using spectroscopy, where results can be obtained much faster than using high performance liquid chromatography (HPLC). The reaction where the p-NP-Glc is hydrolysed by β-D-glycosidases to produce p-NP is shown in figure 3-1 (adapted from www.sigma-aldrich.com). The bond being broken is the glycosidic bond.

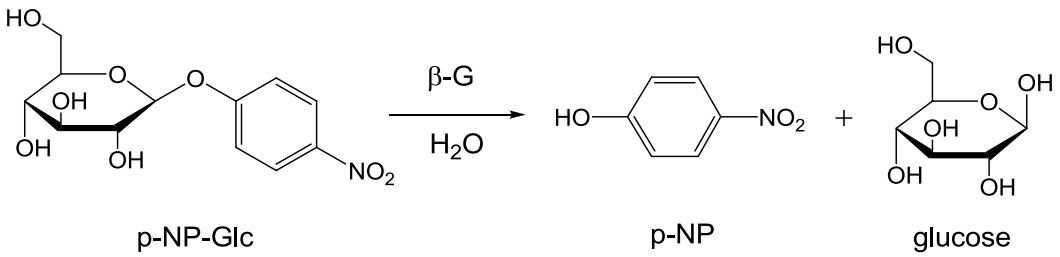

Figure 3-1 Enzyme catalysed reaction of p-NP-G producing p-NP

3.2. Aims of Chapter

The aims of this chapter are:

- To extract β-glycosidase from almonds
- To investigate properties of extracted β-glycosidase from almonds
- To optimize reaction conditions for extracted β-glycosidase from almonds
- To identify other plant sources of β-glycosidase

3.3. Materials and Methods

3.3.1. Chemicals

The following products and artificial substrates for enzyme assay were obtained from Sigma-Aldrich Chemicals plc, Poole, Dorset, UK:

- para-nitrophenyl-β-D-glucopyranoside ($C_{12}H_{15}NO_8$; MW: 301.3; CAS number: 2492-87-7) N7006-1g
- para-nitrophenol ($O_2NC_6H_4OH$; MW: 139.11; CAS number: 100-02-7)
- pure β-D-glycosidase from almond (β-D-glucoside glucohydrolase; CAS number: 9001-22-3) crude lyophilized powder, ≥ 2 units/mg solid; One unit will liberate 1.0μmole of glucose from salicin per min at pH 5.0 at 37°C

3.3.2. Stock Solutions

- Stock solutions of para-nitrophenol (p-NP) in different pH buffers: Stock solutions of p-NP (2.5M) were prepared by dissolving solid p-NP (0.0348g) in 100ml buffer (buffers used: 0.1M citrate buffer, pH 3.5; 0.1M acetate buffer, pH 4.5; 0.1M acetate buffer, pH 5.5; 0.1M phosphate buffer, pH 6.5; 0.1M phosphate buffer, pH 7.5; 0.1M carbonate buffer, pH 10.5)

- 0.05M para-nitrophenyl-β-D-glucopyranoside(p-NP-Glc) solutions at different pH: 0.1507g p-NP-Glc were dissolved in 10ml appropriate buffer
- 0.075M para-nitrophenyl-β-D-glucopyranoside (p-NP-Glc) solutions in acetate buffer pH 5.5: 0.2260g p-NP-Glc were dissolved in 10ml acetate buffer (0.1M, pH 5.5)
- Stock solutions of flavonoid glucosides: stock flavonoid glucoside solutions were made as follows: apigin-500µg/ml; daidzin-500 µg/ml; genistin-500µg/ml; naringin-500µg/ml; quercetin-3-glucoside-100µg/ml; quercetin-4'-glucoside-200µg/ml; quercetin-3, 4'-diglucoidse-200µg/ml; rutin-500µg/ml.
- Stock solutions of flavonoid aglycone: stock flavonoid aglycone solutions were made as follows: apigenin-500µg/ml; daidzein-500µg/ml; equol-500µg/ml; genistein-500µg/ml; naringenin-500µg/ml; quercetin-500µg/ml.

3.3.3. p-NP Standard Curves

In order to quantify the p-NP liberated, a series of p-NP standard curves under different pH conditions were prepared.

From the 2.5mM stock solution of p-NP, dilutions were carried out with the appropriate buffers to obtain stock solutions of 0.1mM for pH 4.5, 5.5, 6.5, 7.5, and 10.5.

A series of dilutions were made using the 0.1mM solutions, 0.10ml, 0.20ml, 0.40ml, 0.60ml, 0.80ml, 1.00ml, plus relevant volume of buffer to 1.0ml, and 1.0ml of sodium carbonate-bicarbonate (Na_2CO_3-$NaHCO_3$, 0.1M, pH10.5), to give the desired amounts of p-NP over the range of 0.01 to 0.10µmoles (concentration ranging from 0.005mM to 0.050mM) in a final volume of 2.0ml. Then the absorbance readings were measured at a wavelength of 400nm.

For p-NP at pH 3.5, a series of dilutions were made by directly using the 2.5mM stock solutions 0.08ml, 0.10ml, 0.20ml, 0.40ml, 0.60ml, 0.80ml, relevant citrate buffer (0.1M, pH 3.5) and 1.0ml Na_2CO_3 buffer, to give the amounts of p-NP over the range

of 0.2 to 2.0μmoles in a final volume of 2.0ml. Then the absorbance readings were measured at a wavelength of 400nm.

The absorbance readings were taken using a Cecil CE2021 spectrophotometer. A solution made up of equal volumes of appropriate buffers and Na_2CO_3 buffer was used as the sample blank.

3.3.4. Crude Enzyme Extracts

Plant tissues were extracted as soon as possible after purchase and some fresh materials needed to be dealt with on the day of purchase. The materials were either separated as peel/skin, leaf, flesh, core, seed, or extracted as a whole.

5.0g of tested plant tissues were blended with 100ml of acetate buffer (0.1mM, pH 5.5, stored at 4ºC at least 1 day before use) until fine particles were obtained (approximately 5 minutes). Then the mixture was centrifuged at 15000r/min for 20minutes at 4ºC. The supernatants were filtered through Whatman No.40 filter paper and keep on ice. The extract was stored in a stoppered flask at 4°C.

3.3.5. Hydrolysis Reactions

3.3.5.1. Artificial substrate

The reaction was carried out using 0.70ml of buffer (0.1M) of the appropriate pH value as described subsequently, 0.20ml of the p-NP-Glc solution (0.05M, same pH value as buffer), and 0.10ml of the β-glycosidase solution (either purified β-glycosidase, 0.1mg/ml, pH5.5, or enzyme extract, 50mg/ml, pH5.5). After incubation, 0.02ml of the reaction mixture was diluted in 0.98ml of the appropriate buffer and the reaction was quenched with 1.0ml Na_2CO_3 buffer (0.1M, pH10.5). For pH 3.5, 0.10ml of reaction mixture was diluted in 0.90ml buffer and 1.0ml Na_2CO_3 buffer. The p-NP liberated was measured colorimetrically using spectroscopy at a wavelength of 400nm. Controls were prepared under the same conditions, except that deionised water

(0.10ml) was added instead of the enzyme solution. Then according to the absorbance reading, the percentage of hydrolysis could be calculated.

$$\text{Percentage of hydrolysis} = \frac{\text{Amount of p-NP produced}}{\text{Initial amount of p-NP-Glc used}} \times 100\ \%$$

3.3.5.2. Purified flavonoid substrates

The flavonoid glycosides apigenin-7-glucoside (apigin), diadzein-7-glucoside (diadzin), genistein-7-glucoside (genistin), naringenin-7-rhamnoglucoside (naringin), quercetin-3-glucoside (Q3), quercetin-4'-glucoside (Q4'), quercetin-3, 4'-diglucoside (Q34'), and rutin (quercetin-3-rhamnoglucoside) were used. The reactions were conducted in the presence of 100μM of the flavonoid glycoside and 150μl of the enzyme extract. The stock flavonoid glucoside (1mM or 500μg/ml, 75μl) was pipetted into a micro-centrifuge tube and left to dry overnight in the fumehood. Then flavonoid glucoside was redissolved in 600μl of acetate buffer (0.1M, pH 5.5) and the reaction started by adding 150μl of the enzyme extract. The reaction mixture was incubated at 50°C for 1.0hr. Methanol (750μl) was added to stop the reaction. The controls were prepared under the same conditions, except that 150μl of the acetate buffer (0.1M, pH 5.5) was used instead of the enzyme. The samples were filtered through 0.20μm PTFE filter into 2ml HPLC vials for HPLC analysis.

$$\text{Percentage of hydrolysis} = \frac{\text{Peak area of aglycone formed}}{\text{Total peak area (aglycone + glycoside)}} \times 100\ \%$$

3.3.6. Optimization

Experiments were designed to find out optimum conditions for enzyme reaction catalysed by whole almond extracts.

On the basis of previous studies, an orthogonal experiment which contained three factors and three levels was designed for optimization of the reaction conditions using the orthogonal table $L_9(3^4)$. The percentage of p-NP-Glc hydrolysed to p-NP was used

as results of each experiment and the variance analysis was carried out for statistical analysis. The established optimum reaction conditions were used in subsequent experiments to identify novel sources of enzyme.

3.3.7. Stability of Crude Enzyme Extracts

The stability of enzyme extracts was monitored every day for a period of five days from the day of extraction. Monitoring was carried out through the determination of the percentage of hydrolysis after assaying with the substrate p-NP-Glc incubated at pH 5.5 and 50°C for one hour. Activity was subsequently monitored at five day intervals to a maximum of 22 days.

3.4. Results and Discussion

3.4.1. p-NP Standard Curves

Para-nitrophenol (abbreviated as p-NP) is the product of the enzyme reaction with para-nitrophenyl-β-D-glucopyranoside (abbreviated p-NP-Glc). In order to estimate the amount of p-NP in unknown samples, standard curves for p-NP had to be established.

Figures 3-2 to 3-7 show standard curves for p-NP at different pH values. Because p-NP exhibits a bright yellow colour in alkaline or basic conditions, equivalent amounts of p-NP give much lower absorbance values at pH 3.5. In order to obtain proper data in the suitable range of absorbance reading, not only was more reaction mixture used for colour reaction (0.10ml for pH 3.5 and 0.02ml for others), but also a higher concentration standard curve was made (2.5mM for pH 3.5 and 0.1mM for others).

The standard curves for absorbance readings at 400nm against amount of p-NP were plotted and shown in figures 3-2 to 3-7. The R^2 values showed the linearity was very good.

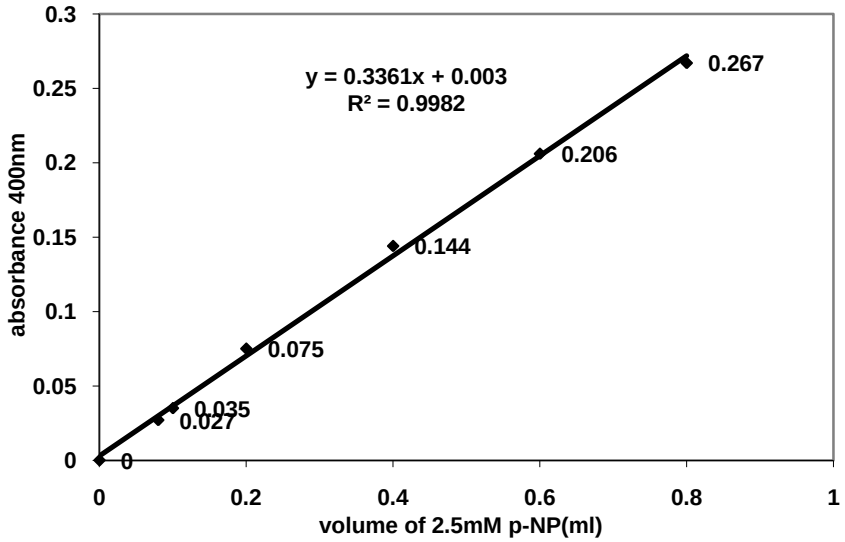

Figure 3-2 Standard curve for p-NP at pH 3.5

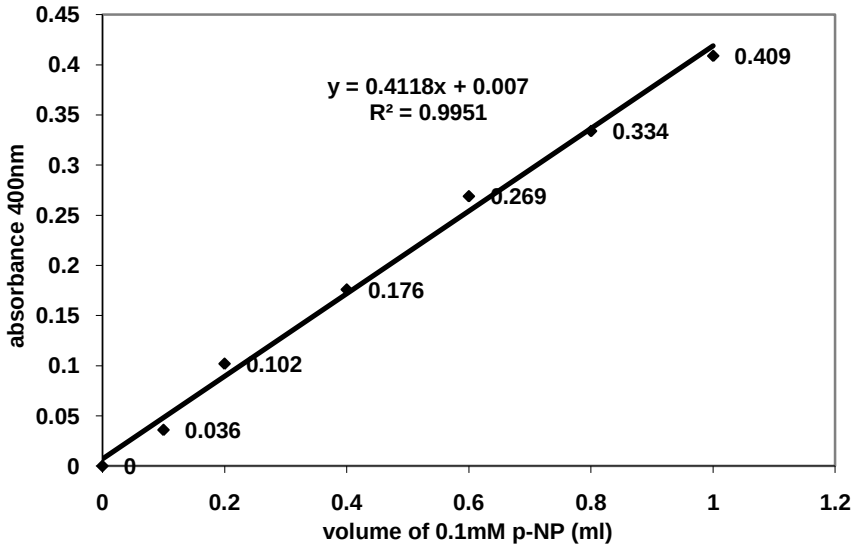

Figure 3-3 Standard curve for p-NP at pH 4.5

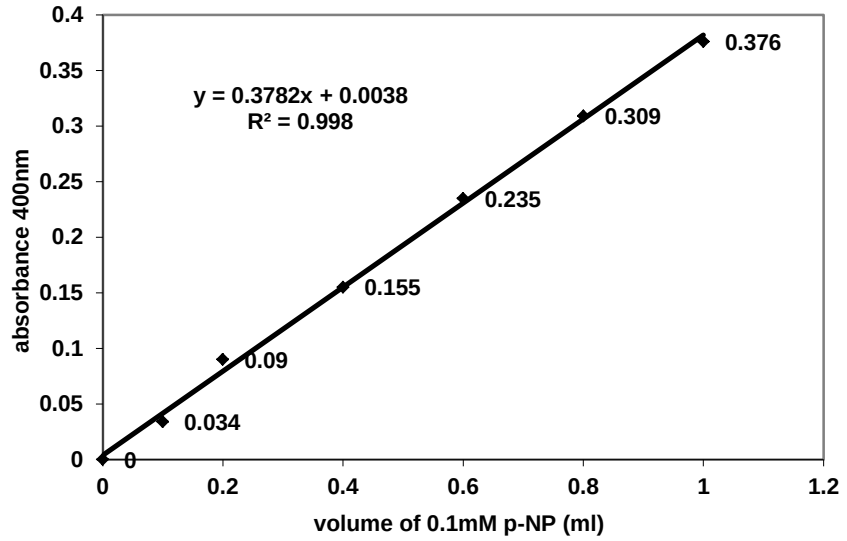

Figure 3-4 Standard curve for p-NP at pH 5.5

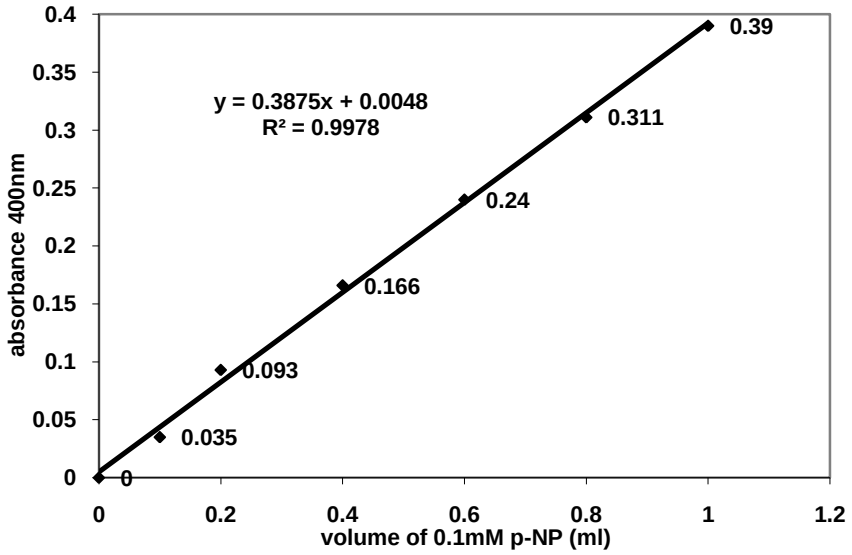

Figure 3-5 Standard curve for p-NP at pH 6.5

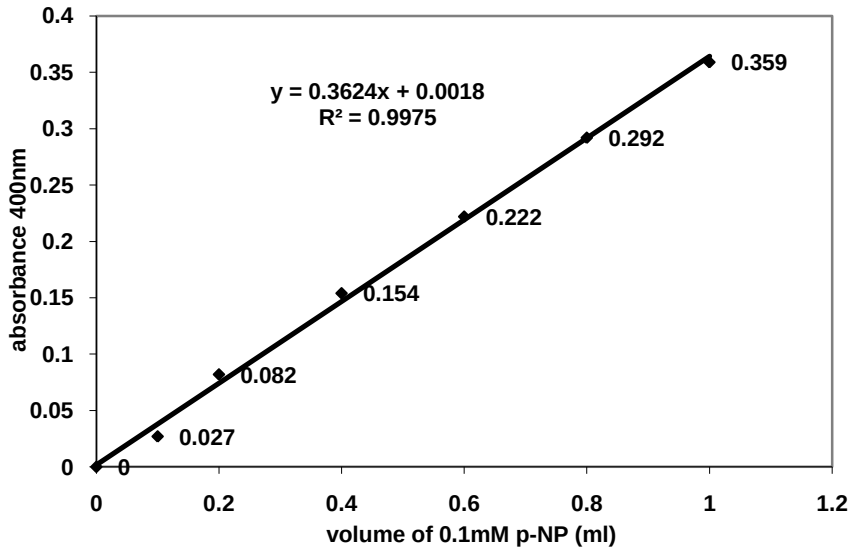

Figure 3-6 Standard curve for p-NP at pH 7.5

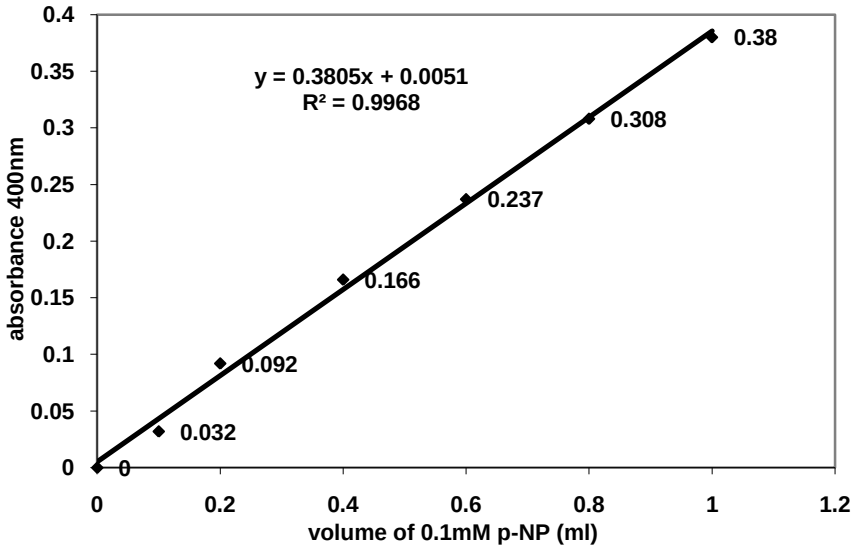

Figure 3-7 Standard curve for p-NP at pH 10.5

3.4.2. Effects of pH on the Enzyme Activities of Whole Almond Extracts

The study of the effect of pH on the β-glycosidase activities from whole almond extracts was carried out by using p-NP-Glc as substrate and colorimetrically measured

absorbance at λ 400nm. Initial reaction conditions were incubations at 40°C for 1.0hr, under different pH conditions. The results showed that the highest percentage of hydrolysis of p-NP-Glc appeared at pH 5.5, which was from the average of triplicate experiments (shown in figure 3-8). Subsequent experiments explored a wider range of temperature.

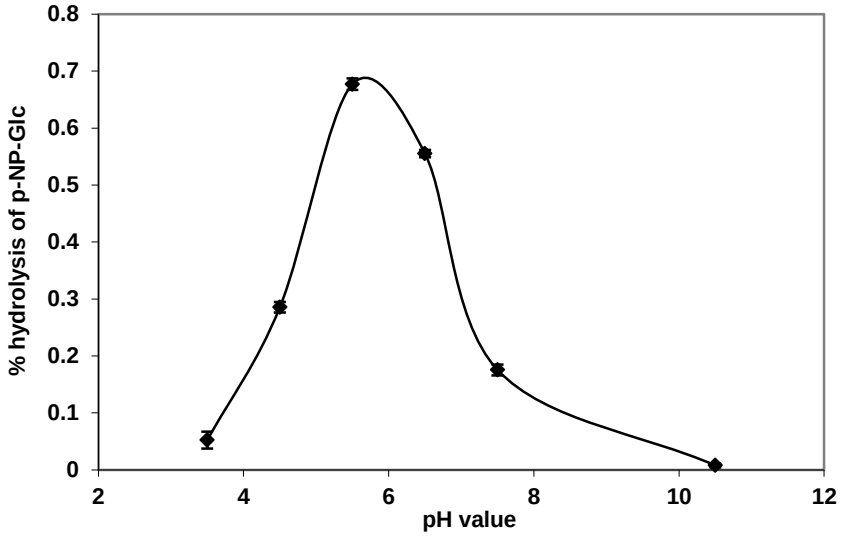

Figure 3-8 Effects of pH on the enzyme from almond extracts

3.4.3. Effects of Temperature on the Enzyme from Whole Almond Extracts

The study of the effect of temperature on the β-glycosidase activities from whole almond extracts was carried out by using p-NP-Glc as substrate and colorimetrically measured absorbance at λ 400nm. Reaction conditions were incubations at pH 5.5 for 1.0hr, at different temperatures. The results showed that the best absorbance was at 50°C, which was from the average of triplicate experiments (figure 3-9).

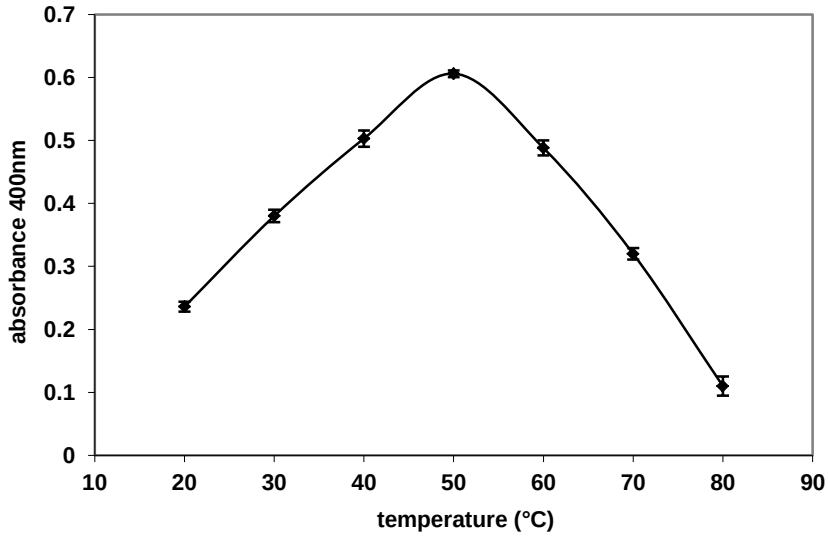

Figure 3-9 Effects of temperature on the enzyme from almond extracts

3.4.4. Stability of Whole Almond Extracts

The study of the stability of whole almond extracts and pure β-D-glucosidase from almond was also carried out by using p-NP-Glc as substrate and colorimetrically measured absorbance at λ 400nm. Incubations were carried out at pH 5.5, at 50°C for 1.0hr according to previous experiments (3.4.2. and 3.4.3.).

The stability results for almond extracts are shown in figure 3-10, which were from the average of triplicate experiments. It was found that storing at 4°C was sufficient to maintain the stability of the enzyme since it was stable under these conditions for at least 22days.

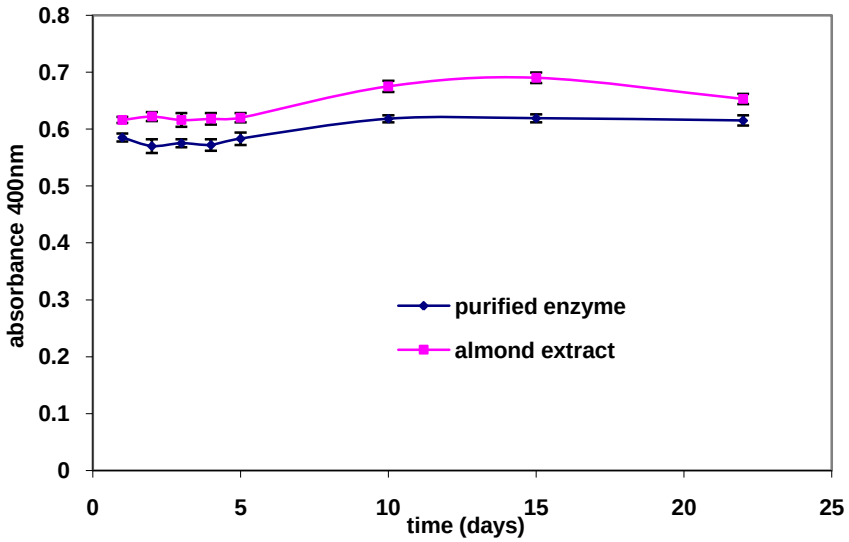

Figure 3-10 Stability of β-glucosidase from almond (for conditions of storage, see text)

3.4.5. Optimization

On the basis of previous experiments, an orthogonal experiment was designed for optimization of the reaction conditions. The experimental points are shown in the factor-level table (table 3-1) and the experiments were carried out according to the $L_9(3^4)$. The absorbance of p-NP formed and percentage of p-NP-Glc hydrolysed were shown in table 3-2 as results and the range of each factor were calculated.

Table 3-1 Factor-level table for almond extract optimizations

Factor	pH Value	Temperature	Reaction Time
Level 1	4.5	40 °C	1.0 hr
Level 2	5.5	50 °C	1.5 hr
Level 3	6.5	60 °C	2.0 hr

From table 3-2, according to the $R_{\sum}$, it was shown that pH value was the most critical factor, but both temperature and reaction time were less important since they were at same level as the error given by the blank column (column 4). The initial optimum

conditions could be given by pH5.5, 50°C, 2.0 hr from the information displayed in table 3-2 only.

Table 3-2 Design of orthogonal experiment, results and statistics ($L_9(3^4)$) (whole almond extracts)

No. of experiment	Column 1 (pH)	Column 2 (Temp.)	Column 3 (Time)	Column 4	Absorbance	Hydrolysis%
1	1 (4.5)	1 (40°C)	1 (1.0hr)	1	0.250	29.45
2	1 (4.5)	2 (50°C)	2 (1.5hr)	2	0.268	31.64
3	1 (4.5)	3 (60°C)	3 (2.0hr)	3	0.267	31.52
4	2 (5.5)	1 (40°C)	3 (2.0hr)	2	0.607	82.94
5	2 (5.5)	2 (50°C)	1 (1.0hr)	3	0.628	85.84
6	2 (5.5)	3 (60°C)	2 (1.5hr)	1	0.580	79.22
7	3 (6.5)	1 (40°C)	2 (1.5hr)	3	0.454	57.96
8	3 (6.5)	2 (50°C)	3 (2.0hr)	1	0.480	61.32
9	3 (6.5)	3 (60°C)	1 (1.0hr)	2	0.326	42.68
Σ_1 (level 1)	92.61	170.35	157.97	169.99	3.860	502.57
Σ_2 (level 2)	248.00*	178.80*	168.82	157.26		
Σ_3 (level 3)	161.96	153.42	175.78*	175.32		
$R_\Sigma = \Sigma_{max}-\Sigma_{min}$	155.39	25.38	17.81	18.06		

Variance analysis was then carried out, since it could quantify the effect of factors including error. The results are shown in table 3-3.

Table 3-3 Results of variance analysis (whole almond extracts)

Source of Variances	Sum of Variances	Freedom	Variance	F Value	$F_\alpha(2,2)$
pH value	4039.82	2	2019.91	70.38 *	$F_{0.05}(2,2)$ =19.0 $F_{0.10}(2,2)$ = 9.0 $F_{0.25}(2,2)$ = 3.0
Temperature	111.35	2	55.68	1.94	
Time	53.71	2	26.85	0.94	
Error	57.40	2	28.70		
Total	4262.28	8			

The conclusion from the variance tests was the same as made from table 3-2. The significance order of the considered factors was:

pH Value * > Temperature > Error ≈ Time of Hydrolysis

(*: the effect of the factor is significant)

The effect of pH was highly significant because its F value was bigger than $F_{\alpha}(f_1, f_e)$ ($\alpha = 0.05$). The effects of other two factors considered, temperature and time, were smaller. Their F values were 1.94 and 0.94 respectively. These two factors did not make a significant contribution towards the total variance, with the effect of time being smaller than that of the error. This meant it was unnecessary to consider this factor apart from the convenience. So the optimum conditions could be ensured at pH 5.5, 50°C for 1.0hr.

Considering that the range between temperatures tested was big, an additional experiment regarding temperature was performed at 40°C, 45°C, 50°C, 55°C, 60°C. The results showed that the optimum temperature was 50°C, as expected (figure 3-11). Finally the optimum conditions were established as pH 5.5, 50°C for 1.0hr.

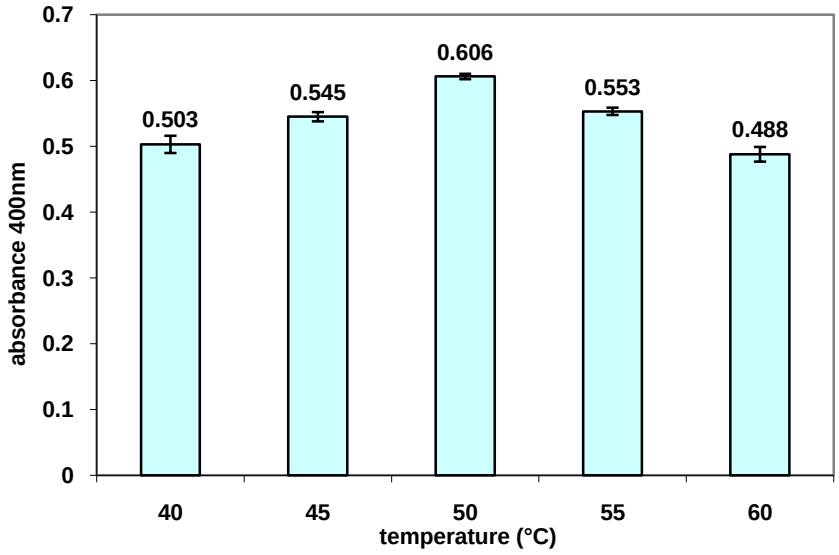

Figure 3-11 Additional experiment on the effects on temperature on activity of β-glucosidase from almond extracts

3.4.6. Identification of Alternative Enzyme Sources

Enzyme β-glycosidases were extracted from different plant tissues, ideally from possible food waste, i.e. uneatable parts. Different parts of the materials were tested by using daidzin and genistin as substrates, and some were tested with quercetin-4'-glucoside. The enzyme catalysed reaction was described in 3.3.5.2. The percentage hydrolysis of daidzin, genistin, and quercetin-4′-glucoside were calculated from the determination by HPLC. The results were shown in table 3-4 and figure 3-12 with standard deviations obtained from triplicate experiments.

By comparing percentage hydrolysis for flavonoid glycosides, apple seeds, pumpkin seeds, pomegranate seeds and honeydew melon seeds were found to be likely good sources of β-glucosidase other than whole almond. Apple seeds and pumpkin seeds appeared to be best. However, pumpkin seed is a “normal food” while apple seed is food waste, so apple seed was chosen as the source of enzyme β-glycosidase for further study.

Table 3-4 Percentage hydrolysis of flavonoid glycosides catalysed by different plant tissues

Tested materials	Daidzin					Genistin					Quercetin-4'-glucoside				
(%hydrolysis)	1	2	3	M	SD	1	2	3	M	SD	1	2	3	M	SD
Almond	98.52	100	98.87	99.13	0.77	64.88	65.52	69.13	66.51	2.3	100	100	100	100	0
Apple core	3.44	4.22	2.72	3.46	0.75	0	0	0	0	0	0	0	0	0	0
Apple flesh	1.89	2.72	2.92	2.51	0.55	0	0	0	0	0	0	0	0	0	0
Apple seeds	77.63	77.22	74.92	76.59	1.5	54.70	53.22	50.18	52.70	2.3	85.75	87.85	86.38	86.66	1.1
Apple skins	2.22	2.48	2.68	2.46	0.23	0	0	0	0	0	0	0	0	0	0
Honeydew melon seeds	16.88	17.92	18.36	17.72	0.76	29.88	33.20	31.21	31.43	1.7	21.88	23.28	22.70	22.62	0.70
Orange flesh	3.12	4.18	3.71	3.67	0.53	0	0	0	0	0	--	--	--	--	--
Orange seeds	6.34	5.88	6.98	6.40	0.55	3.32	3.48	4	3.60	0.36	5.56	6.01	5.71	5.76	0.23
Orange peel	5.90	6.12	5.35	5.79	0.40	4.86	5.21	4.84	4.97	0.21	3.32	3.86	2.75	3.31	0.56
Pineapple peels	5.22	6.78	3.78	5.26	1.5	0	0	0	0	0	0	0	0	0	0
Pomegranate seeds	56.82	56.60	59.8	57.74	1.8	18.86	20.08	21.36	20.10	1.3	17.02	18.33	17.96	17.77	0.68
Pumpkin seeds	96.38	93.77	97.28	95.81	1.8	40.19	39.04	40.86	40.03	0.92	70.82	74.28	72.55	72.55	1.7
Soybean	19.47	19.87	19.94	19.76	0.25	5.81	5.2	6.34	5.78	0.57	8.22	9.01	8.69	8.64	0.40
Soy flour	5.88	5.78	5.38	5.68	0.26	0	0	0	0	0	--	--	--	--	--
Strawberry flesh	2.28	1.92	2.34	2.18	0.23	0	0	0	0	0	--	--	--	--	--
Strawberry seeds	3.32	4.04	3.02	3.46	0.52	1.62	2.01	1.83	1.82	0.20	--	--	--	--	--
Sunflower seeds	20.80	22.60	20.80	21.40	1.0	5.77	6.28	5.14	5.73	0.57	2.89	3.04	3.70	3.21	0.43
Tomato flesh	5.22	6.08	4.54	5.28	0.77	1.44	1.83	1.14	1.47	0.35	--	--	--	--	--
Tomato seeds	8.82	9.08	9.73	9.21	0.47	6.35	7.01	6.47	6.61	0.35	--	--	--	--	--
Tomato skin	5.65	6.83	4.83	5.77	1.0	3.06	3.98	3.10	3.38	0.52	--	--	--	--	--

--: not determined; 1, 2, 3: triplicate determinations; M: mean; SD: standard deviation

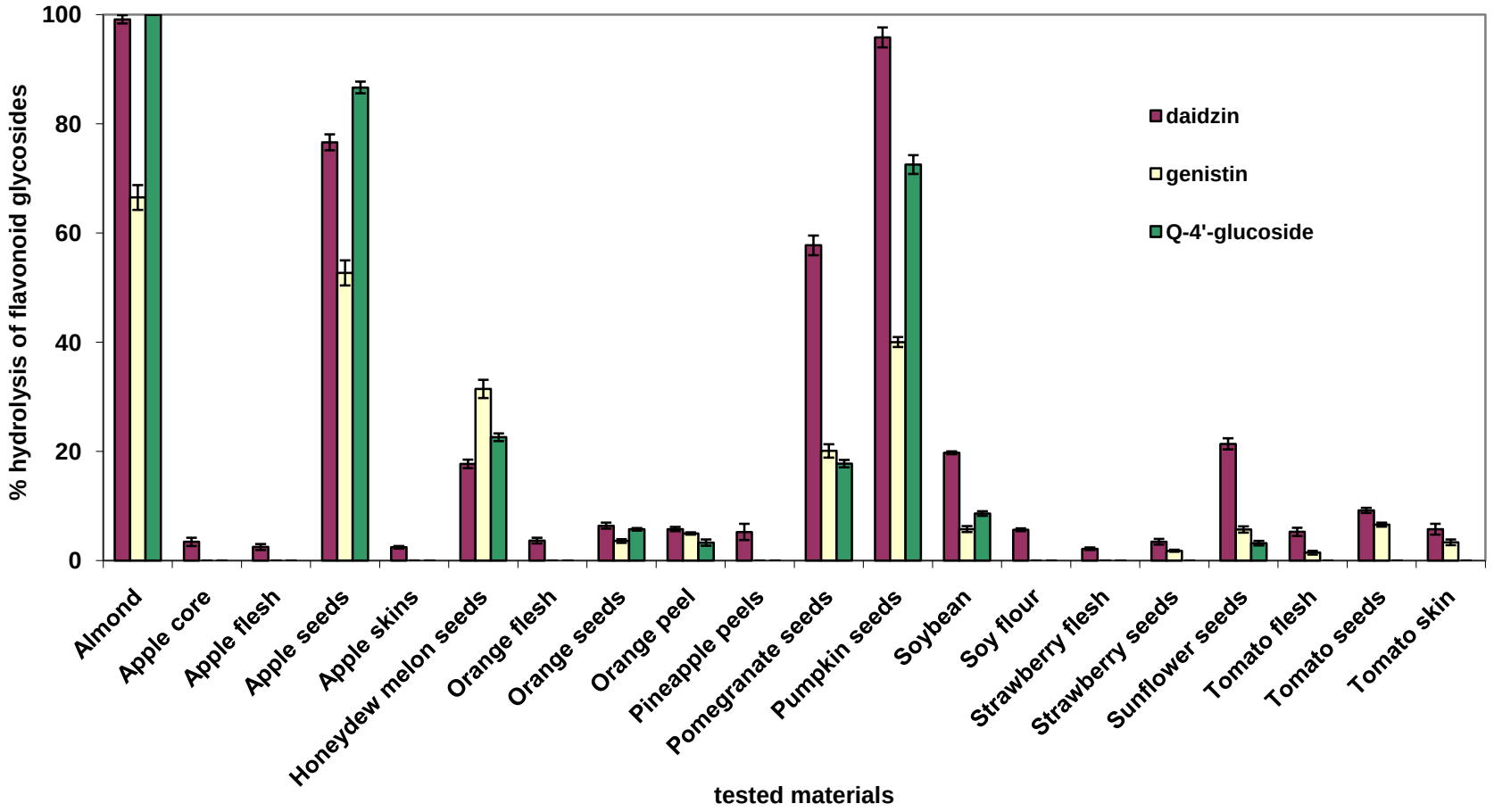

Figure 3-12 Percentage hydrolysis of flavonoid glycosides catalysed by different plant tissues

3.4.7. Substrate Analysis Using Additional Substrates

As a hydrolase, β-glycosidases show broad specificity with some selection at the binding site. In order to test the specificity and get some information about the hydrolysis site interaction with substrate, experiments were carried out to extend the range of substrates tested. Hydrolysis % for other flavonoid glycosides are shown in table 3-5. The reaction conditions were as described in 3.3.5.2 at optimum conditions for almond. Figure 3-13 shows the HPLC chromatogram for a mixture of quercetin and its glycosides. Figure 3-14 shows the HPLC chromatogram where quercetin-3, 4′-diglucoside was hydrolysed to quercetin-3-glucoside by apple seed extracts.

Table 3-5 Effect of crude enzyme extracts on hydrolysis of other flavonoid glycosides

Flavonoid glycosides	By apple seeds		By whole almond	
	% hydrolysis	SD	% hydrolysis	SD
Apigenin-7G	78.45	0.02	75.56	0.05
Naringenin-7G	0	0	0	0
Quercetin-3G	0	0	37.02	0.01
Quercetin-3,4'G*	14.32	0.001	16.49	0.008
Quercetin-4′G	86.66	0.01	100	0
Rutin	0	0	0	0

* Quercetin-3, 4′-diglucoside was hydrolysed to quercetin-3-glucoside

SD: standard deviation

Concentration of tested flavonoid glycosides were: quercetin-3G: 100μg/ml; quercetin-3, 4′G: 200μg/ml; quercetin-4′G: 200μg/ml; others: 500μg/ml

Reaction conditions were: pH 5.5, 50°C, 1.0hr.

From table 3-5, it was shown that:

- Almond extracts could partly hydrolyse quercetin-3-glucoside. The resulting quercetin peak coincided with the Q3G peak. Apple seed extracts could not hydrolyse Q3G at all under these conditions.

- For quercetin-4'-glucoside, both extracts could hydrolyse Q4'G efficiently, with 100% by almond and 86.66% by apple seeds when tested under optimum conditions for the almond extracts.

- For quercetin-3, 4'-diglucoside, only one glucose has been hydrolysed partly, which is on the 4' position. So after hydrolysis, two peaks appeared which represent Q34'DG and Q3G respectively (see figure 3-14). However, although almond extracts could partly hydrolyse quercetin-3-glucoside, it looked like almond did not hydrolyse the Q3G produced from Q34'DG since the peak representing quercetin did not appear.

- No extracts could hydrolyse rutin (quercetin-3-rhamnoglucoside) and naringin (naringenin-7-O-rhamnoglucoside), while more than 75% of apigin (apigenin-7-O-glucoside) could be hydrolysed.

The results show that the extract from almond and apple seeds requires a glucose moiety while rhamnose does not work. β-D-Glycosidase can only hydrolyse a terminal glucose unit but not an in-the-chain glucose (Mabry *et al.*, 1970). For the conjugation position, β-glycosidase may require either 7 or 4' positions.

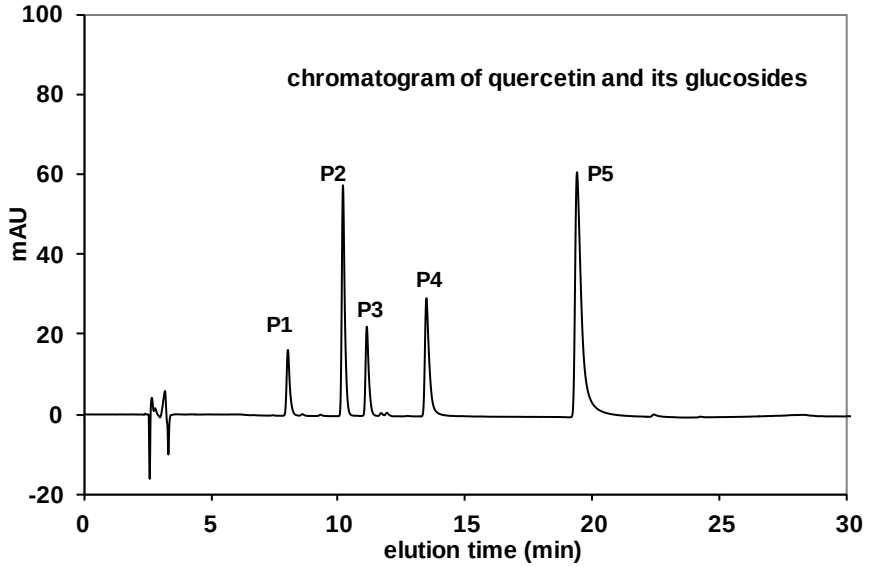

Figure 3-13 Chromatogram of quercetin and its glucosides. Peaks identified: P1: quercetin-3, 4′-diglucoside; P2: rutin; P3: quercetin-3-glucoside; P4: quercetin-4′-glucoside; P5: quercetin

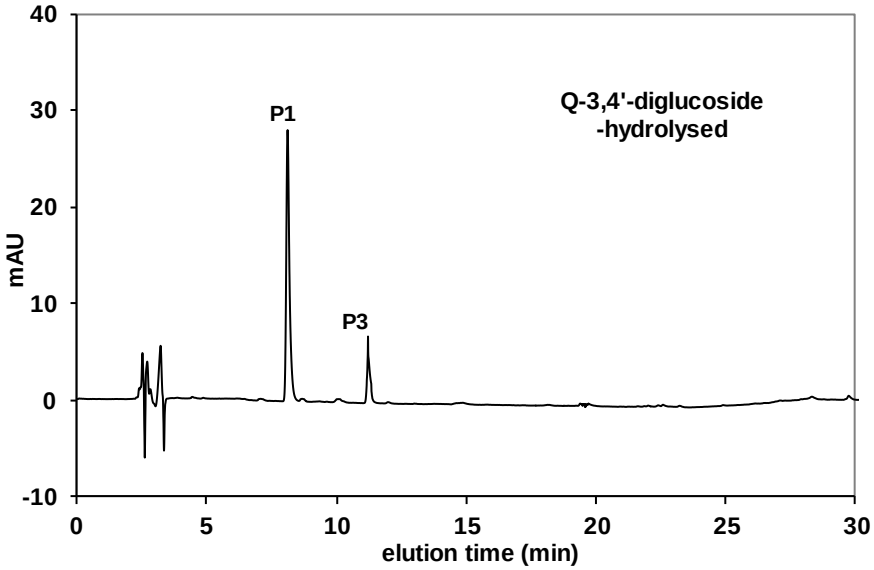

Figure 3-14 Chromatogram of quercetin-3, 4′-diglucoside after hydrolysis. Peaks identified: P1: quercetin-3, 4′-diglucoside; P3: quercetin-3-glucoside. For further details, see text.

3.5. Conclusions

- Whole almond is a good source of β-glucosidase, and can hydrolyse the flavonoid glycosides tested fully. The optimum reaction conditions were to incubate at pH 5.5, at 50°C for 1.0 hr, conditions which were used for selecting enzyme sources from different plant tissues.

- By looking at percentage hydrolysis of flavonoid glycosides, apples seeds, pumpkins seeds, pomegranate seeds and honeydew melon seeds were found to be good sources of β-glucosidase along with whole almond.

- Besides daidzin (daidzein-7-O-glucoside) and genistin (genistein-7-O-glucoside), β-glucosidase from apple seed extracts and whole almond extracts could hydrolyse apigin (apigenin-7-O-glucoside) and quercetin-4'-glucoside efficiently but could not hydrolyse narigin (narigenin-7-O-rhamnoglucoside) and rutin (quercetin-3-rhamnoglucoside). They can partly hydrolyse quercetin-3, 4'-diglucoside into quercetin-3-glucoside.

- Apple seeds appear to be a good source of β-glucosidase activity when compared to β-glucosidase extracted from almonds – a common source of commercially-available enzyme

- The β-glucosidase from apple seeds will be investigated further (chapter 4) as a novel source of the enzyme, particularly since apple seeds are often a food waste product in apple processing.