A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Optimization of Enzymatic Production of Isoquercetin and

Possible Existence of β-D-glucosidase Inhibitor in Jujube Leaf Extract

대추잎 추출물로부터 효소를 활용한 아이소쿼시틴 생산의 최적화와

베타글루코시데이즈 저해제의 존재 가능성 제시

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석사학위논문

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이 논문을 석사학위 논문으로 제출함

2014년 2월

서울대학교 대학원 농생명공학부

조 영 제

조영제의 석사 학위논문을 인준함

2014년 2월

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ABSTRACT

Recently, quercetin and its glucosides including isoquercetin (quercetin-3-β-D-glucoside, Q3G) and rutin (quercetin-3-rutinoside) draw attention due to healthful bioactivities such as antiproliferative, antioxidant, anti-iflammatory. However, isoquercetin exhibits the highest bioavailability, which varies according to the type of sugar moiety. Rutin is the most common flavonoid in plant resources, especially in jujube leaves which are the waste of food industry. To increase the availability of isoquercetin, the enzymatic biotransformation of isoquercetin from rutin in the jujube leaf extract using hesperidinase from *Aspergillus niger* was optimized. Hesperidinase is an enzyme complex containing α-L-rhamnosidase and β-D-glucosidase activities. Employing response surface methodology (RSM), the isoquercetin yield was optimized concerning the temperature (40~60℃, X_1) time (24~72 h, X_2) and pH (2~6, X_3). The second-order polynominal model was developed by experiments based on Boc-behnken design containing 15 experimental runs with three replicates at the center point. The coefficient (R^2) and *p*-value of response surface regression equation for isoquercetin yield were 0.93 and 0.01, respectively. The results showed the statistical significance. Consequently, the optimum condition was predicted at stationary point as to produce 2.65 mg/mL of isoquercetin by processing at

47.3℃, 52.16 h, and under pH 5.31. The verification of the model was carried out at the optimal conditions. The experimental value of 2.57 mg/mL of isoquercetin showed good agreement with the predicted one. These results might provide important information for utilization of jujube leaf as a waste in the food industry.

From the optimization study, one interesting phenomenon was observed. Under various treatment conditions, the bioconversion of isoquercetin from rutin (α-L-rhamnosidase activity) was well-performed but the accumulation of quercitin (β-D-glucosidase activity) was not. A further study was carried out to ensure the cause of the loss of β-D-glucosidase activity of hesperidinase whether it is affected by enzyme kinetics or possible enzyme inhibitor. Firstly, to examine the effect of enzyme kinetics, enzymatic biotransformation was conducted at 40℃ and pH 3.8 with enzyme concentration ranging from 0.03125 mg/mL to 160.0 mg/mL for 24, 48 and 72 h. Regardless of reaction time, quercetin was not produced in the concentration of enzyme below 5 mg/mL, while the activity of rhamnosidase was observed. The esculin hydrolysis test was conducted by phenolic compounds to appraise whether it is competitive inhibition or not. Secondly, to evaluate the existence of possible inhibitors, jujube leaf extracts with various concentrations (0 – 20%) were mixed with 0.5 mM of 4-nitrophenyl β-D-glucospyranoside solution containing 0.3 unit/mL of β-D-glucosidase

from Almonds. The Hanes-Woolf plot showed non-competitive inhibition. Therefore, these results strongly suggested that certain components in the jujube leaf extract act like an inhibitor of β-d-glucosidase. Further study is necessary to identify and confirm the possible inhibitor.

Keywords: jujube leaf extract, hesperidinase, rutin, isoquercetin, β-D-glucosidase inhibitor, response surface methodology

Part 1. Optimization of enzyme biotransformation of isoquercetin from jujube leaf extracts

I. INTRODUCTION

Quercetin, one of the most common flavonoid in plants (Formica & Regelson, 1995), and its glycosides including isoquercetin (quercetin-3-O-glucoside) and rutin (quercertin-3-O-rutinoside) were well-known phenolic compounds for healthful benefits (Muir, Collins, et al., 2001). Recently, isoquercetin is of great interest for stronger bioactivities than quercetin and rutin (You, Ahn, et al., 2010). For instance, isoquercetin exhibits better protective effect against lipid peroxidation and oxidative stress, and more potent antiproliferative effect on various cancer cell lines (You, Ahn, & Ji, 2010). Furthermore, bioavailability of isoquercetin is higher than that of quercetin for higher water solubility, and rutin for better intestinal absorption (Manach, Scalbert, et al., 2004), (Makino, Shimizu, et al., 2009). This is the reason why isoquercetin exhibits more effective on some *in vivo* bioactivities, on which quercetin, the aglycone, shows stronger effect *in vitro*. Especially, isoquercetin exhibits lower inhibitory effect on α-glucosidase activity, but was investigated to show stronger anti-diabetic activity on rat and human type 2 diabetes mellitus than quercetin (Muir, et al., 2001)

Subsequently, methods allowing production of isoquercetin from natural resources have been pursuit for food and drug applications. On the same aspect, only methods using chemicals acceptable for food and drug are

allowed. Although a chemical method was reported recently (Wang, Zhao, et al., 2011) , thus, the most of the efforts were exerted to attain high yield biotransformation using microorganisms or enzymes (Gerstorferová, Fliedrová, et al., 2012). Among those reports, enzymatic biotransformation has been preferred as the most prospective method, because microbial transformation is characterized by low selectivity, yield, and productivity (Rajal, Cid, et al., 2009) .

Selective derhamnosylation of rutin is considered as the most feasible food-grade production method of isoquercetin (Weignerová, Marhol, et al., 2012), since rutin is the predominant flavonoid in foods, plant-based beverages, diverse parts of various plants (Xie, You, et al., 2007). However, the selective derhamnosylation of rutin requires sophisticated techniques to enhance the yield, because virtually all preparations of α-L-rhamnosidase include β-glucosidase or sometimes rutinosidase activity, which leads undesirable production of quercetin from the isoquercetin produced or directly from rutin as shown in Fig. 1, respectively. Further, only two α-L-rhamnosidases are commercially available: hesperidinase and naringinase, which exhibit β-glucosidase activity (You, Ahn, & Ji, 2010).

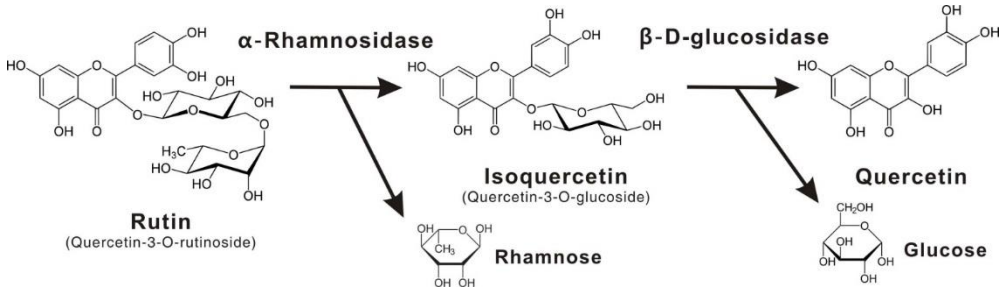

Figure 1. Biotransformation of rutin to isoquercetin and quercetin by hesperidinase.

The purification of α-L-rhamnosidase from those complexes with β-glucosidase is yet technically unavailable (Yadav, Yadav, et al., 2011) and possible recombinant enzymes are not allowed for food-grade applications. Therefore, followings are proposed as feasible ways to obtain high yield production of isoquercetin. Unwanted β-D-glucosidase activity of α-L-rhamnosidase preparations could be knocked-out by using selective inhibitors, or by exposing at specific physical conditions based on the difference in the resistance of rhamnosidase and glucosidase to conditional stress. For instance, rhamnosidase exhibit better thermostability than glucosidase (Zverlov, Hertel, et al., 2000). Based on kinetic principle between rhamnosidase and glucosidase activities, the maximum yield of isoquercetin, which is possible without additional treatments, can be obtain by optimizing process conditions, i.e. time, temperature, pH. With the help of response surface methodology (RSM), the optimization of conditions for enzymatic process conditions is the simplest way to achieve high yield process.

Rutin and quercetin is typical in leaf of jujube (*Ziziphus* Spp.), which belongs to Rhamnaceae family (San, Yıldırım, et al., 2009). However, leaf of jujube were treated as the industrial waste, though entire organs of jujube such as fruits, flowers, leaves, seeds, roots, even bark, etc. have been used for the source of traditional medicines or that of such beverages as tea (Zhao,

Liu, et al., 2008). Thus, biotransformation methods enabling high yield production of isoquercetin from leaf of jujube should be highly advantageous to food industry for both environmental and economic sakes.

On the aspect of possible for food and drug industrial applications, here, we explored the optimum conditions of biotransformation methods for producing isoquercetin from rutin in jujube leaf by employing hesperidinases from *Aspergillus niger* and *Penicillium* sp., widely discovered commercial α-L-rhamnosidase complexes.

Ⅱ. MATERIAL AND METHODS

2.1. Chemicals and reagents

Hesperidinase from *Aspergillus niger* (EC. 3.2.1.168 Hesperidin-α 1,6-rhamnodiase), hesperidinase from *Penicillium sp.*, β-glucosidase from Almonds, 0.2% w/v solution of esculin, and the standards of rutin (quercetin-3-rutinoside trihydrate), isoquercetin (quercetin 3-β-D-glucoside), and quercetin (quercetin dihydrate) were purchased from Sigma-Aldrich (Sigma Chemical, St. Louis, MO, USA). These chemicals were dissolved in McIlvine buffer before use. To make pH 3.8 McIlvaine buffer, 0.1 M citric acid (YAKURI PURE CHEMICALS) and 0.2 M sodium phosphate dibasic anhydrous (Na_2HPO_4, DUKSAN PURE CHEMICALS) were mixed.

2.2. Preparation of jujube leaf extract

The jujube leaves were collected on April 2012 from Boeun, South Korea. Samples were dried by natural light and stored in a freezer at -80℃ before smash. Samples were smashed with blender (Hanil, HMF-1000, Korea). The powder of jujube leaf was filtered through a sieve which size was from 150 μm to 300 μm. This powder was stored in a freezer at 3°C.

The optimal conditions for extraction were 0.5 g of filtered jujube leaf powder with 10 mL of 45% ethanol (v/v; water/ethanol) at 45℃ for 15 min with 120 rpm of agitation. These optimal conditions were acquired by the former member of the laboratory (data unpublished). The extract was centrifuged at 15000 rpm for 10 min to concentrate the substrate and then filtered with filter paper (110 mm ϕ). To prevent denaturation by ethanol, the ethanol was evaporated with reducing pressure at 40℃ and 150 rpm. The concentration of the jujube leaf extracts were measured by UV-visible spectrophotometer (Shimadzu UV-1700) at the absorbance for pure rutin, 357 nm (Manach, Morand, et al., 1995).

2.3. Enzymatic biotransformation

2.3.1 Effect of the enzyme concentration

The effect of enzyme concentration on the enzymatic biotransformation was evaluated with hesperidinase ranging from 0.03 unit/mL (0.01 g/mL) to 0.6 unit/mL (0.2 g/mL). These various concentrations of enzyme were mixed with 3 mL of jujube leaf extract. These mixtures (pH 3.8) were reacted in a shaking water bath at 40℃ for 24 h with 120 rpm of agitation. To denature the enzyme, these samples were put into 70℃ of water bath for 10 min (SORIA & ELLENRIEDER, 2002).

2.3.2 Effect of the enzyme reaction time

To evaluate the effect of enzyme reaction time on the enzymatic biotransformation, the extract was reacted with fixed concentration of hesperidinase [0.3 unit/mL (0.1 g/mL)] in a water bath at 45℃ (120 rpm of agitation). The pH of the mixture was 3.8. The samples were collected at every 12 h for 3 days.

2.3.3 Effect of pH on the enzyme biotransformation.

The effect of pH was studied with different pH conditions of pH 2, 3.8, 6 and 8. The optimal pH provided by the manufacturer was 3.8. The pH of the enzyme solution and jujube leaf extracts was modified with McIlvaine buffer and citrate buffer, respectively. The reaction temperature was 40℃ and reaction time was 24 h.

2.3.4 Effect of enzyme reaction temperature on the enzyme biotransformation.

To evaluate the effect of reaction temperature, the reaction temperature was varied from 40℃, 50℃ and 60℃. The pH and reaction time was stabilized as 3.8 and 24 h, respectively.

2.4. Analytical methods

The concentration of rutin, isoquercetin, and quercetin were calculated using standard curve. These flavonoids, which were in the jujube leaf extracts or the products produced by the enzymatic biotransformation, were analyzed by HPLC system (600 Controller, WATERS, Milford, MA, USA) interfacing to UV detector (486 Tunable absorbance detector, WATERS, Milford, MA, USA) with Hibar 250-4 HPLC column LiChrospher 100 RP-18(5-μm).. The wavelength of the detection was 280 nm (Delourdesmatabilbao, Andreslacueva, et al., 2007).The mobile phase consisted of 2.5% (v/v, acetic acid distilled water) (solvent A) and acetonitrile (solvent B). The chromatographic conditions were as follows: initial 95% A,5% B; 0-25 min 75%A, 25% B; 25-40 min 50%A, 50% B; 40-50 min 20%A, 80% B; 50-55 min 50%A, 50% B; 55-60 min 95%A, 5% B; 60-65 min 95%A, 5% B with a flow rate of 1 mL/min (Table 1). Before injection, the samples were filtered by syringe filters (syringe filter PVDF 0.22 μm, OlimPeak, Barcelona, Spain).

Table 1. Instrument and operating conditions for HPLC analysis

Column	C18 LiChrospher 100 analytical column (4 mm X 250 mm I.D., 5um)
Solvent	A (2.5% acetic acid), B (acetonitrile)
Elution condition	0-20 min, 95-75% A 20-40 min, 75-50% A 40-50 min,50-20% A 50-60 min, 20-95% A
Flow rate	1.0 mL/min
UV range	280 nm

2.5. Response surface methodology (RSM)

To optimize the reaction condition of hesperidinase for producing isoquercitrin, the response surface experiment was performed with the Box-Behnken design (BBD). For response surface methodology (RSM), the experimental data using statistical software SAS program (SAS Institute, Cary, NC., U.S.A.) was used. Based on the results of the experiments mentioned above, reaction temperature (X1, 40-60℃), processing time (X2, 24-72 h), and pH (X3, pH 2-6) were selected as the independent variables. The concentration of isoquercitrin (mg/mL) was the response variable. Variable level was coded -1 (low), 0 (central point) and 1 (high). To fit the experimental data, quadratic polynomial equation was given as follow:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y is the response; X1, X2, and X3 are variables; β0 is the model intercept coefficient; β1, β2, and β3 are linear coefficients; β12, β13, and β23

are interaction coefficients between the three factors; β11, β22, and β33 are quadric coefficients. The range and levels of experimental design was listed in Table 2.

The statistical analysis was conducted by ANOVA. The quality of fit of the polynomial model was determined by the coefficient of determination R2, and its statistical analysis was examined by the F-test in the SAS program (9.3 version).

Table 2. Central Composite design for optimization of the enzyme reaction condition of isoquercetin from jujube leaf extracts

Run	Variable levels (coded)			Isoquecetrin yield (mg/mL)	
	X_1	X_2	X_3	Experimental	Predicted
	Temperature (℃)	Time (h)	pH		
1	40(-1)	24(-1)	4(0)	1.4377	1.1981
2	40(-1)	72(1)	4(0)	1.8628	1.5984
3	60(1)	24(-1)	4(0)	0.4545	0.7174
4	60(1)	72(1)	4(0)	0.4556	0.6934
5	50(0)	24(-1)	2(-1)	0.1026	0.2551
6	50(0)	24(-1)	4(1)	2.0315	1.8538
7	50(0)	72(1)	2(-1)	0.1360	0.3120
8	50(0)	72(1)	6(1)	2.3274	2.1732
9	40(-1)	48(0)	2(-1)	0.1508	0.2365
10	60(1)	48(0)	2(-1)	0.1397	-0.2776
11	40(-1)	48(0)	6(1)	1.7295	2.1452
12	60(1)	48(0)	6(1)	1.3609	1.2736
13	50(0)	48(0)	4(0)	2.3269	2.3145
14	50(0)	48(0)	4(0)	2.3720	2.3145
15	50(0)	48(0)	4(0)	2.2470	2.3145

Ⅲ. Results and Discussion

3.1. Effect of the concentration of the enzyme

Hesperidinase is an enzyme mixture containing two active enzymes (α-L-rhamnosidase and β-D-glucosidase). α-L-rhamnosidase produce isoquercetin from rutin, and β-D-glucosidase splits the isoquercetin into glucose and quercetin. However, as it can be seen in Figure 2, increasing the concentration of enzyme from 0.03 unit/mL to 0.6 unit/mL increased the concentration of isoquercetin while the concentration of quercetin was effectively constant. When the more hesperidinase was used, the activity of α-L-rhamnosidase was increased. On the other hand, the β-D-glucosidase did not have activity. In other words, the more enzymes were treated, the more isoquercetin can be produced Despite of the 0.6 unit of enzyme was four times higher than the one used by Wang et al. (Wang, Ma, et al., 2012) (0.05 g/mL), quercetin was not produced. This concentration of enzyme was good enough to hydrolyze isoquercetin to quercetin.

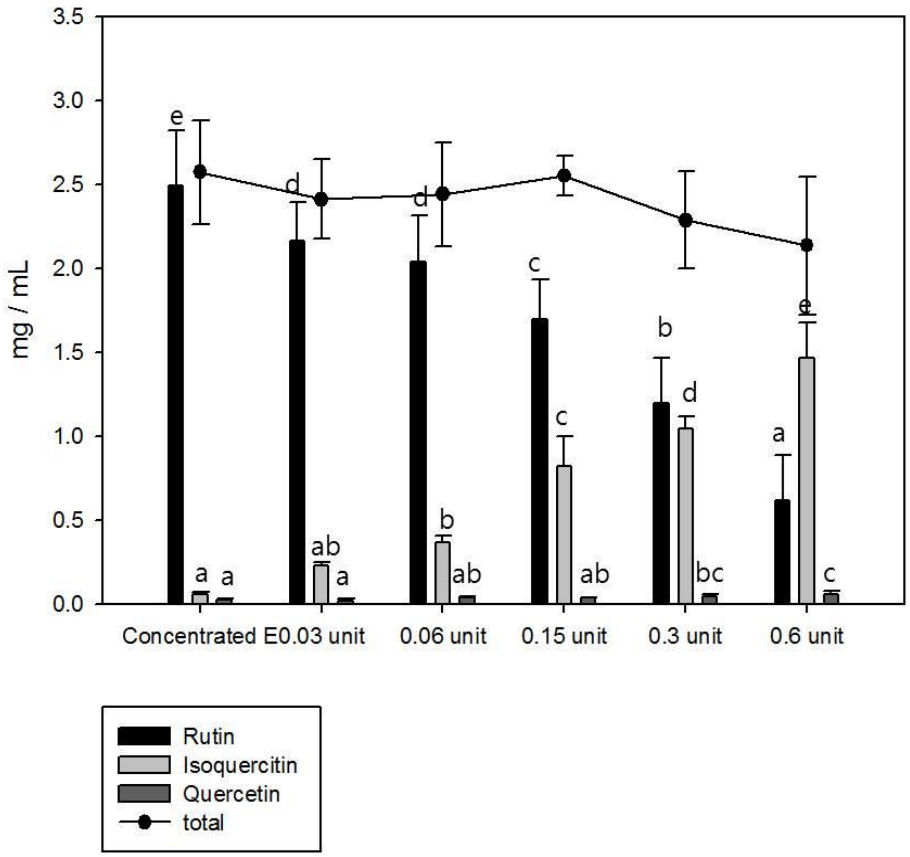

Figure 2. The effect of hesperidinase concentration on the flavonoid yields (Alphabets represent Duncan's grouping).

3.2 Effect of the reaction time

The effect of reaction time on the isoquercetin yield was shown in Figure 3. The yield of isqoercetun was increased greatly, and then the increase rate was decreased when the enough time (36 h) was passed. However, the activity of β-D-glucosidase did not appear. The concentration of isoquercetin was increased from 0.07±0.02 mg/mL to 1.88±0.39 mg/mL. After 36 h, conversion rate did not change significantly. At 36 h, the amount of isoquercetin was 1.62 mg/mL, and at 72 h the amount of isoquercetin was 1.88 mg/mL, which showed 16% increase. Since the enzyme is a protein, the activity can be affected by the tertiary and quaternary structure of proteins, and in case of the less stable enzymes, a longer reaction time causes the decrease of activity (Mozhaev, Berezin, et al., 1988) .

(Day, DuPont, et al., 1998) showed that the deglycosylation rate of isoqueretin was 90% after 90 min, at 37℃. However, in this study, quercetin was not produced after even 72 h.

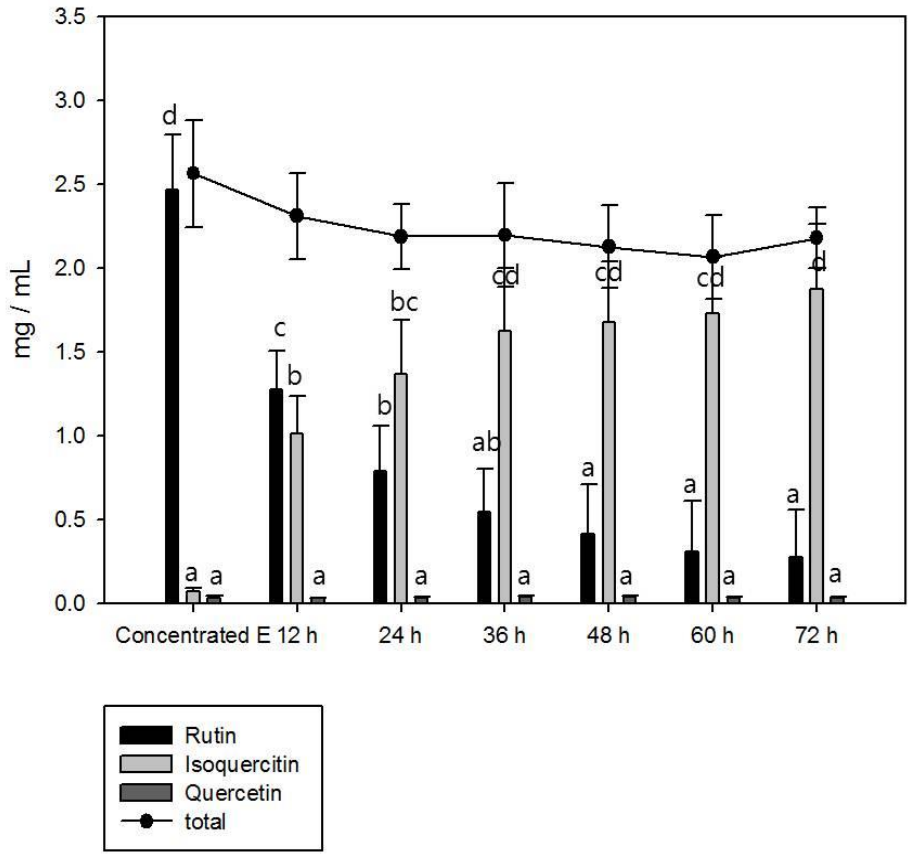

Figure 3. Concentration of products (rutin, isoquercetin, quercetin) with increased reaction time (from 12 to 72 every 12 h), at 50℃, pH 3.8, and enzyme concentration is 0.3 unit/mL.

4.3 Effect of the pH

To evaluate the effect of pH, biotransformation experiments were conducted at various pH's (Figure 4). It is known by the manufacturer that the optimal pH of hesperidinase was 3.8. The highest activity was observed at pH 3.8. In this condition, 1.12 mg/mL of isoquercetin was produced. At pH 6, the isoquercetin was produced 1.01±0.11 mg/mL. At the other pH's, the conversion rate of isoquercetin was very low as compared with pH 3.8 and pH 6. Nevertheless, the concentration of quercetin did not increase significantly at any other pH's.

The pH is one of the most important factors to control the biotransformation to isoquercetin. Depending on the (Wang, et al., 2012), at the pH 7, α-L-rhamnosidase activity was high while β-glucosidase activity was low. However, in this study, the quercetin did not produce at any pH.

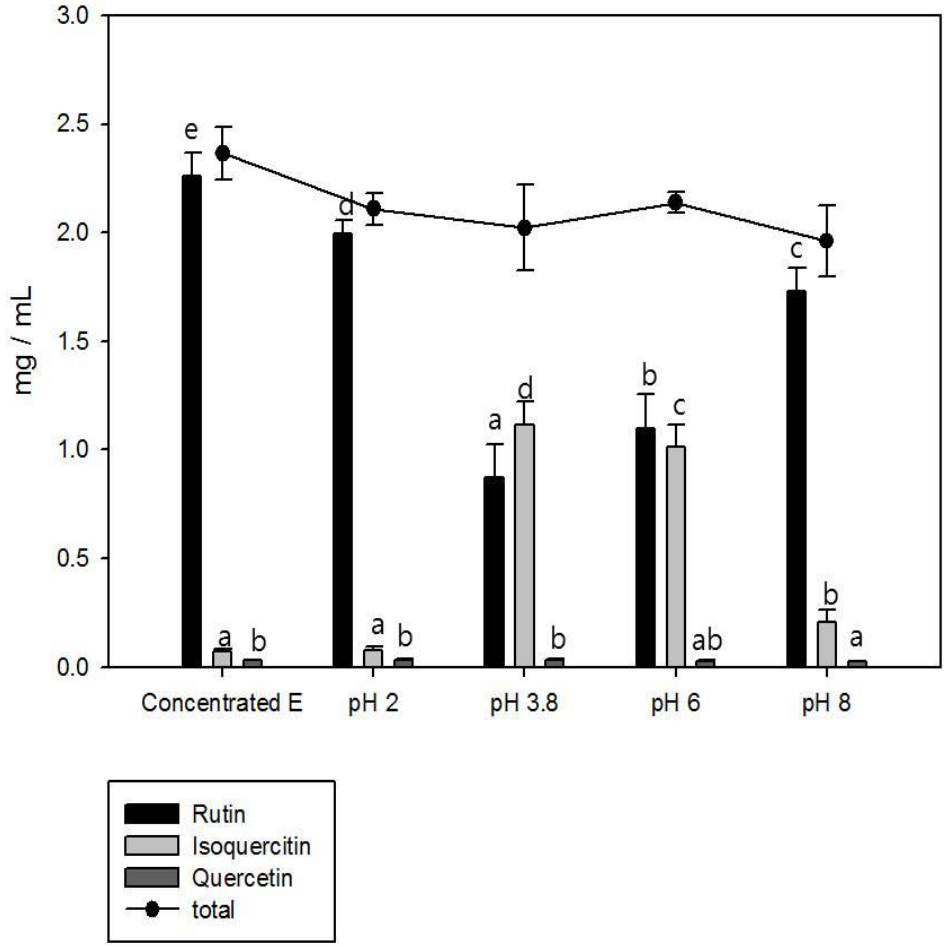

Figure 4. The concentration of concentrated extract (no enzyme treatment), concentrated extract treated by 0.3 unit/mL hesperidinase from *Aspergillus niger* at each pH (pH 2-8) at 40℃ for 24 h (Alphabet is meaning Duncan's grouping).

4.4 Effect of the reaction temperature

Hesperidinase contains two active enzymes, which are α-L-rhamnosidase and β-D-glucosidase. The enzyme is an organocatalyst, which consisted of protein, so the optimum pH and optimum temperature exist. Thus, to confirm the effect of reaction temperature, the jujube leaf extracts and enzyme mixture was treated at 40℃, 50℃ and 60℃ for 24 h in a hot water bath. The highest amount of isoquercetin (1.46±0.23 mg/mL) was produced at 50℃. The lowest amount of isoquercetin (0.17±0.06 mg/mL) was produced at 60℃. However, the amount of quercetin did not increase significantly as compared with no enzyme treated one at each temperature.

According to all of these preliminary experimental results, the treatment temperature and pH were the strongest factors. Besides one quite interesting observation was that quercetin was not produced at any combination of the treatment conditions. These results might suggest the potential existence of the β-D-glucosidase inhibitor in jujube leaf extracts.

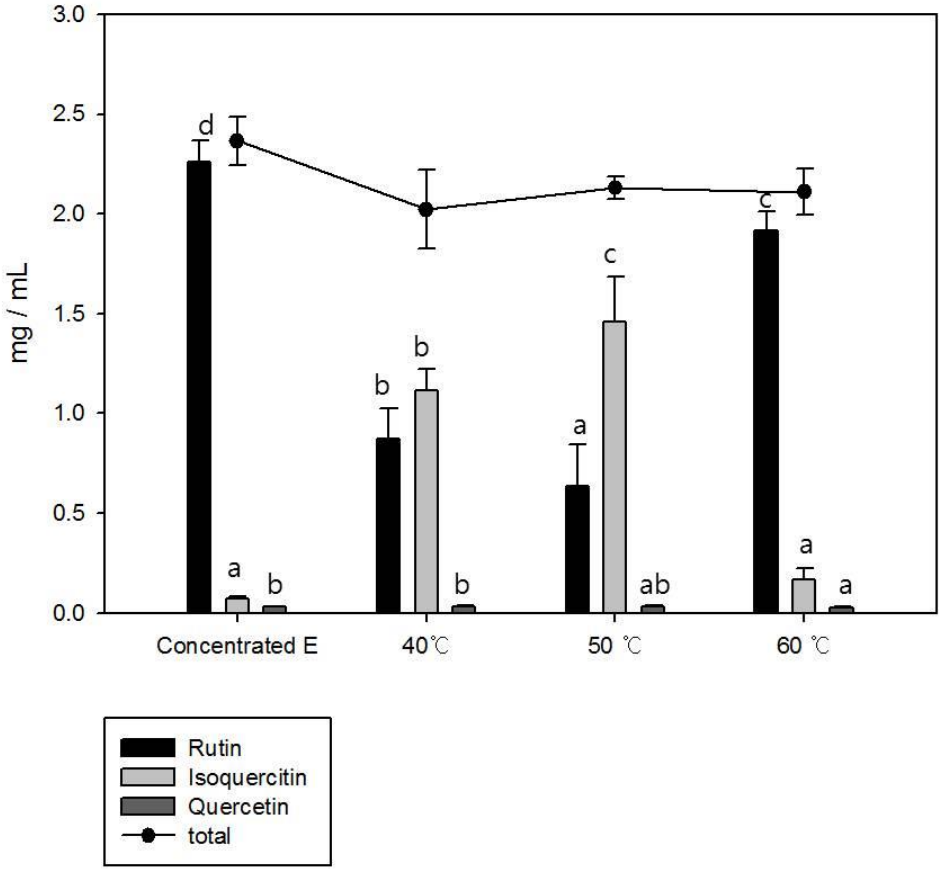

Figure 5. The concentration of concentrated extract (no enzyme treatment), concentrated extract treated by 0.3 unit/mL hesperidinase from *Aspergillu niger* at each temperature (40-60℃) in pH 3.8 for 24 h. (Alphabet is meaning Duncan's grouping).

4.5 Response Surface Methodology (RSM)

The results of the experiments which were conducted under the coded condition, the yield of isoquercitrin did not have a great contrast to predicted value except some conditions which were conducted under pH 2 (Table 2).

In this study, a Box-Behnken design was conducted to determine the optimum conditions for production of isoquercitrin. Independent and response variables were analyzed to get regression equation for prediction of the response under the given range of the conditions. The polynomial regression equation obtained for isoquecetrin yield (Y) was as follows:

$$Y = -23.812263 + 0.787849X_1 + 0.100424X_2 + 1.963675X_3 - 0.007834X_1^2 - 0.000442X_1X_2 - 0.000832X_2^2 - 0.004469X_3X_1 + 0.001376X_3X_2 - 0.171669X_3^2 \quad (2)$$

From the results of ANOVA, the p-value of total regression was 0.0144 which means statistically significant ($p<0.05$), so the result showed that the assumed model was a proper model and the linear and quadratic regression were statically significant as well ($p<0.05$) (Table 3). The p-value of crossproduct regression did not have significant influence ($p>0.1$), which means that the interaction between the variables did not influence the

response. R2 value of more than 0.60 in RSM generally means statistically significant (Velts, Uibu, et al., 2011). In our study, the R^2 value was 0.9394 (Table 3). It indicated that this statistical model can be explained 93.94% of the variability in the response.

Based on this polynomial equation, three-dimensional (3D) graph for predicted response was drawn (Figure 6). As shown in Figure 6, the concentration of isoquercitrin was increased until temperature, time, and pH reached the optimum condition and then decreased when temperature, time, and pH were passed over the optimum condition. The three-dimensional graph of the produced concentration of isoquercitrin was demonstrated the effect of different variables. Two variables were varied and third one was kept constant. Figure 6a shows the three-dimensional graph of the effect of treatment temperature and time on the concentration of isoquercitrin (mg/mL). The effect of reacting time and pH on isoquercitrin concentration (mg/mL) was shown in Figure 6b. Figure 6c shows the effect of temperature and pH on the concentration of isoquercitrin (mg/mL). The convex curvature shows well-defined optimum variables.

The p-values of the time (X_2) was larger than 0.05, so this can be accepted null hypothesis (Table 4). The RSM analyses showed that temperature (X_1) and pH (X_3) significantly affected the production of isoquercitrin. The result of canonical analysis, the amount of isoquercitrin was maximum at the

stationary point. The optimum combination of factors was at maximum peak. The optimum condition was 47.3℃, 52.16 h and pH 5.31 (Table 5). At optimum condition, 2.65 mg/mL of isoquercitrin was expected. The value of R^2 was 0.94.

The verification of the model for predicting the optimal conditions was tested using the optimal conditions of 47.3℃, 52.16 h and pH 5.31. The experimental result (2.57 mg/mL of isoquercitrin) was found to be in good agreement with the predicted ones.

Table 3. Analysis of variance for isoquercetin as linear, quadratic term and interactions on response variables

Regression	DF	Type I Sum of Squares	R-Square	F-value	Prob>F	Remark
Linear	3	7.0167	0.5804	15.97	0.0054	Significant
Quadratic	3	4.2464	0.3512	9.66	0.0160	Significant
Crossproduct	3	0.0941	0.0078	0.21	0.8826	
Total Model	9	11.3572	0.9394	8.61	0.0144	Significant

Table 4. Analysis of variance of the factors for isoquercetin

Factor	DF	Sum of squares	Mean Square	F-value	Prob>F
Temperature (X_1, ℃)	4	3.3021	0.8255	5.64	0.0428
Time (X_2, h)	4	0.9816	0.2454	1.68	0.2903
pH (X_3)	4	7.7763	1.9441	13.27	0.0071

Table 5. Predicted maximum values of isoquercetin, the response variables of jujube leaves extract treated by hesperidinase

Factor	Optimum conditions
	Isoquercitrin
Temperature (X_1, ℃)	47.3 ℃
Time (X_2, h)	52.16 h
pH (X_3)	5.31
Predicted value	2.65 mg/mL
Experimental value at the above conditions	2.57 mg/mL

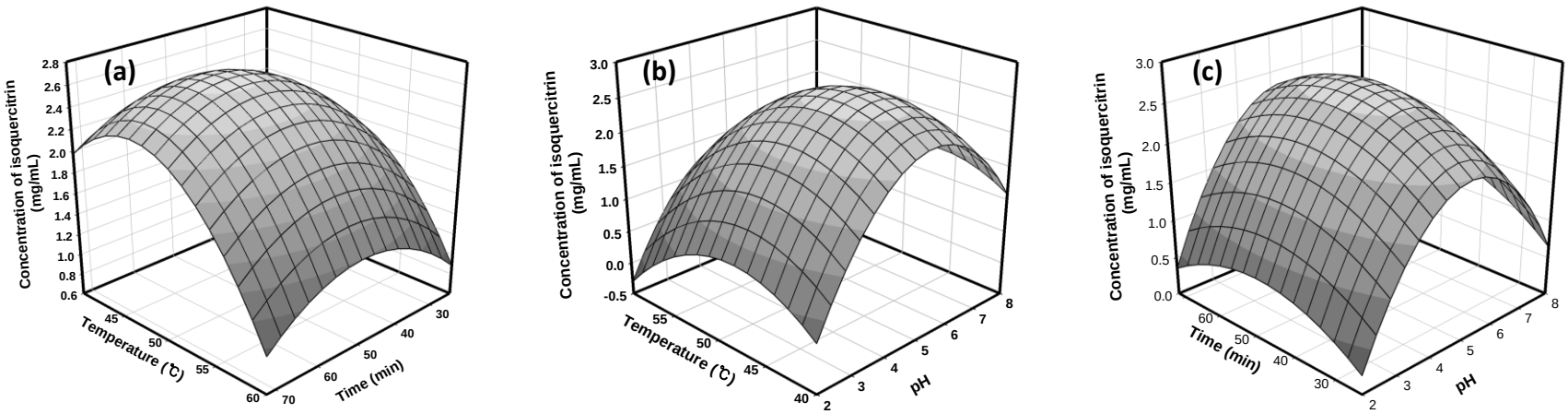

Figure 6. Response surface plot showing the effect of temperature and pH on amount of isoquercetin in jujube leaf extract teated by hesperidinase.

Part 2

Identification of β-D-glucosidase inhibitor in jujube leaf extracts

I. Introduction

Glucosidase inhibitors receive attention as one of the most important therapeutic agent because they interrupt vital metabolic function in biological systems, and they are available to apply in diverse fields like pharmacology, food and agriculture (Pandey, Sree, et al., 2013). Discovery of new glucosidase inhibitor is momentous due to their potential capability in the treatment of diabetes, HIV, metastatic cancer, and lysosomal storage disease (Asano, 2003). The inhibition on intestinal α-glucosidases would delay the digestion and absorption of carbohydrates and consequently suppress the postprandial hyperglycemia (Li, Wen, et al., 2005) (Frantz, Calvillo, et al., 2005) .

Glucosidase inhibitors can be obtained from chemical modification, microorganism, and plants. The glucosidase inhibitor, 1-Deozynojirimycin and 3-Azido-3-deoxythymidine can be obtained in the manner of chemical modification. However, 3-Azido-3-deoxythymidine shows significant side effect (Sunkara, Bowlin, et al., 1987). Acarbose and voglibose, one of the most famous natural glucosidase inhibitor, are commonly used as a drug for type 2 diabetes mellitus. Acarobose is from plants, for example pine bark extract (Kim, Wang, et al., 2004).

Judging from the results of previous experiments, there is a β-D-

glucosidase inhibitor in jujube leaf. Because at any conditions of previous experiments, β-D-glucosidase activity was blocked.

Here, the objective of this study was to examine the possible existence of β-D-glucosidase inhibitors in the jujube leaf extract using esculin hydrolysis test and to elucidate the inhibition mechanism. The β-D-glucosidase inhibitor, which is get from jujube leaf, can be used in pharmaceutical industry and food industry. It does not have no side effect due to this inhibitor is from nature. Moreover jujube leaves are by-products, so it has a powerful advantage in the aspect of economy.

Ⅱ. Materials and Methods

2.1 Chemicals and reagents

Hesperidinase from *Penicillium sp.* (EC. 3.2.1.168 Hesperidin-α 1,6-rhamnosidase), β-D-glucosidase from almonds, and 0.2% w/v solution of esculin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The esculin was dissolved in 0.1 M sodium acetate buffer (pH 5). Iron (III) chloride ($FeCl_3$, DUKSAN PURE CHEMICALS) was dissolved in 0.1 M sodium acetate buffer to make 0.5% w/v solution.

2.2. Verification of existence of β-D-glucosidase inhibitor

From the previous study, the jujube leaf extract showed β-D-glucosidase inhibition. In order to confirm this inhibition was caused by certain inhibitors existing in the jujube leaf extract, enzymatic reaction was carried out with various concentration of hesperidinase (0.3125 mg/mL - 160 mg/mL). The reaction was performed in a 40°C shaking water bath for maximum reaction time of 72 h.

2.3. Analysis of phenolic compounds

Many well-known inhibitors of glucosidase are phenolic compounds. It

was investigated that the jujube leaf extract contained glycosilamines, spirooxathiazoles, and glycoconjugates which were well-known inhibitors of β-D-glucosidase (Pérez, Muñoz, et al., 2008). These inhibitors have a similar structure with a substrate, isoquercetin, showing a competitive inhibition. To evaluate the competitive inhibition, the fractions of phenolic compounds were acquired using Ultimate 3000 HPLC (Dionex) under the conditions shown in Table 6. The 20μL of fractions were acquired every 10 s for 18 min and repeated 10 times so that the total amount of fraction became about 2 mL.

Table 6. Operating condition for HPLC to analysis phenolic compounds

Column	Inno C-18 column (4.6*150, 5 μm)
Solvent	A (0.3% TFA), B (Acetonitrile)
Elution condition	0-25 min, 90-40% A 25-30 min, 40-0% A 30-35 min,0-0% A 35-36 min, 0-90% A 35-40 min, 90-90% A
Flow rate	0.8 mL/min
UV range	280 nm (190-800 nm DAD scanning)

2.4. Esculin hydrolysis test

Esculin hydrolysis test was applied to check the inhibition of β-D-glucosidase activity (Salazar & Furlan, 2007). An enzyme agar solution was prepared in 7 mL of sodium acetate buffer with 0.07 g of agar powder was dissolved at 200℃. Before adding $FeCl_3$ solution and enzyme, this agar solution was cooled at 60℃ for preventing denaturation of enzyme. When the temperature of agar solution was 60℃, 1.2 mL of $FeCl_3$ and 40 μL of β-glucosidase from almonds (0.01 unit/mL) were added. This solution was poured into petri dish, and the fractions (5μL), were spot inoculated on the surface of enzyme agar solution. This petri dish was incubated at room temperature for 15 min. When the agar solution and the inoculated fractions were dried, 6 - 7 mL of esculin solution was poured into the surface of agar, and incubated at room temperature for 30 min (Pandey, Sree, Dash, & Sethi, 2013). In esculin hydrolysis test, the esculin was used as substrate. The esculin was hydrolyzed to glucose and esculetin by β-D-glucosidase. At this time, esculetin was bound with ferric citrate, the color was changed to dark brown and black (Pandey, Sree, Dash, & Sethi, 2013).

2.5. Enzyme kinetics

The enzyme kinetics was evaluated to examine the mechanism of inhibition.

4-nitrophenyl-β-D-glucophyranoside was used as substrate. The concentration of substrate was 0, 4.5, 9, 13.5, 18, 27, and 36 mM. The concentration of β-D glucosidase from almonds was fixed as 0.0138 g/mL. The ratio of substrate, enzyme, and buffer was 1 : 1 : 8. The reaction was carried out at 40 ℃,and the solution was stirred 600 rpm with magnetic bar. The sample was taken every 5 min, for 40 min. For denaturation of enzyme, 400 mM of sodium carbonate was used. After dilution to 1/10, these samples were measured with UV-spectrometer at 400 nm. The jujube leaf extracts as inhibitors were added instead of buffer (0%, 10%, and 20%). The Michaelis-Menten plots were transformed Hanes-Woolf plots for determining the enzyme kinetics properties (Voegele, Bardin, et al., 1997).

2.6 Analysis of metal ions

Non-competitive inhibitions are not combined with binding site of enzyme, it is combined to other sites, and then the enzyme has a low affinity (Rothman, Bowen, et al., 1985). Metal ions and surfactants are well-known non-competitive inhibitors.

Metal ions concentrations in the jujube leaf extract were investigated to inquire the inhibitory activity. According to (Wang, et al., 2012), Cu, Zn, Mn, Fe, Al, Li, Ca, Na, Mg, K, and Co have inhibitory activity. The concentration

of these metal ions was measured with ICP-730-ES (VARIAN, USA).

Ⅲ. Results and Discussion

3.1 Verification of existence of β-D-glucosidase inhibitor

Since possible inhibitor concentration should be constant in a fixed concentration of jujube leaf extract, quercetin tends to be produced when the concentration of enzyme reached at certain level (10 mg/mL).

The amount of rutin was reduced from 0.173 mol/mL at 24 h to 0.028 mol/mL at 72 h when the concentraton of enzyme was 5 mg/mL. And the amount of isoquercetin was increased from 2.506 mol/mL at 24 h to 2.729 mol/mL at 72 h, but the amount of quercetin was not changed (Figure 7). In this enzyme concentration, the activity of α-L-rhamnosidase was remained for 72 h, but the activity of β-D-glucosidase was not observed. However, when the concentration of enzyme was 10 mg/mL, it started to produce quercetin. And the amount of quercetin was increased from 0.0327 mol/mL at 24 h to 0.0514 mol/mL at 72 h.

From these results, the reason why quercetin was not produced at less than 5 mg/mL of the enzyme concentration was that the amount of enzyme was less than the amount of inhibitor. It was neither a short treatment time nor unsuitable reaction conditions. To sum up, there is a β-D-glucosidase inhibitor in jujube leaves.

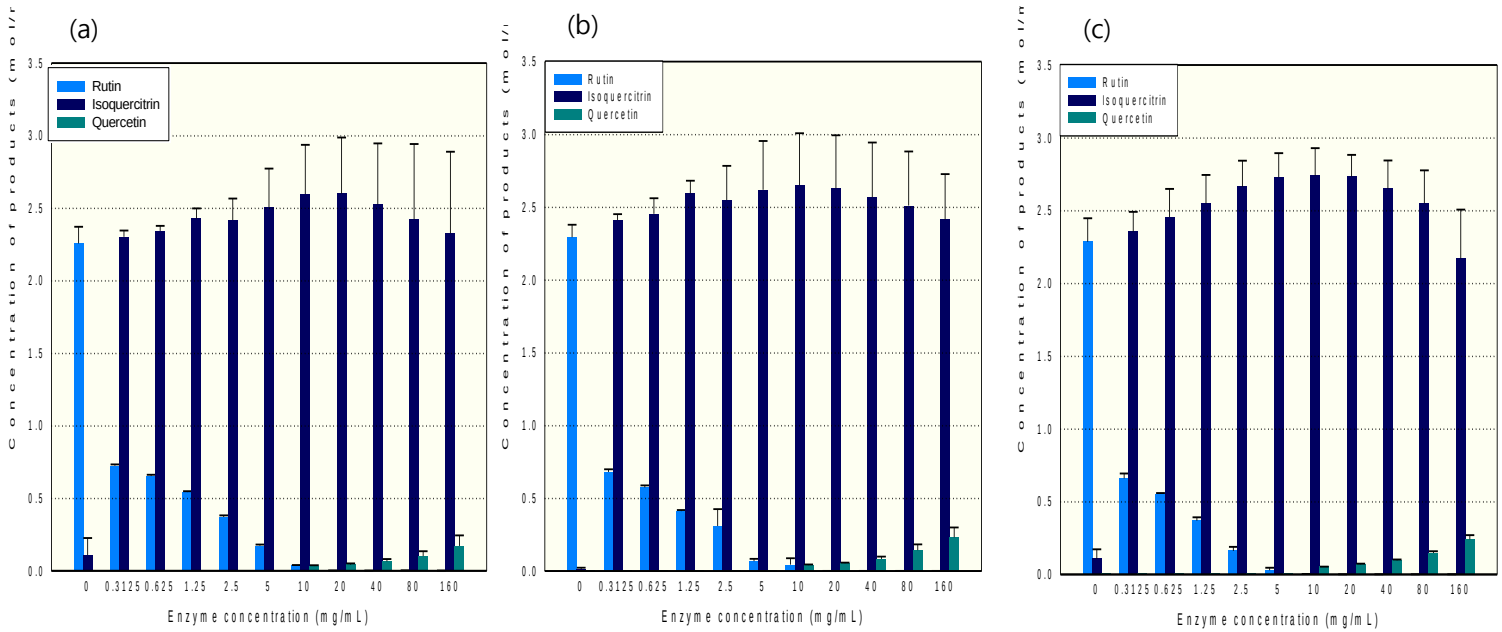

Figure 7. The effect of enzyme concentration of the concentration of products. The samples were taken every 24 h for 3 days. The treatment time of a, b, and c were 24, 48, and 72 h, respectively.

3.2 Phenolic compounds in the jujube leaf extract

The fractions which were get from jujube leaf extracts (Figure 8.) were spotted into the esculin agar. When spotting all of the fractions of phenolic compounds into the esculin agar, the fractions, which were the part of phenolic compounds (9, 10, 14, 15, 35, 45, 47, 49, 53, 56, 57, 58, 60, and 76), did not have inhibitory activity (Figure 9. a, c, d, e, and g, peak point). However, other fractions, which part did not have any phenolic compounds (28, 29, 30, 34, 39, and 41), there had inhibitory activity (Figure 9. c, and d). The inhibitory activity did not occurred at the part of phenolic compounds. From these results, the kind of inhibition was not a competitive inhibition. The inhibition section was from 5.5 min to 7.5 min. The inhibitory activity area can be predicted the broad peak at the other absorbance.

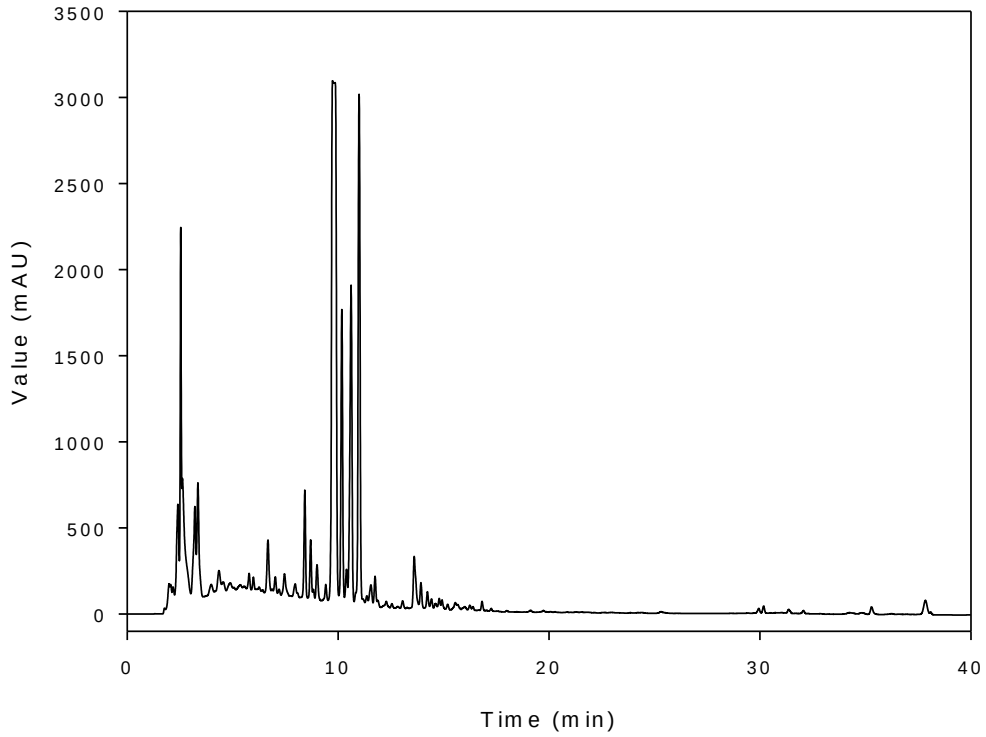

Figure 8. Analysis of phenolic compounds for getting the fractions from jujube leaf extracts.

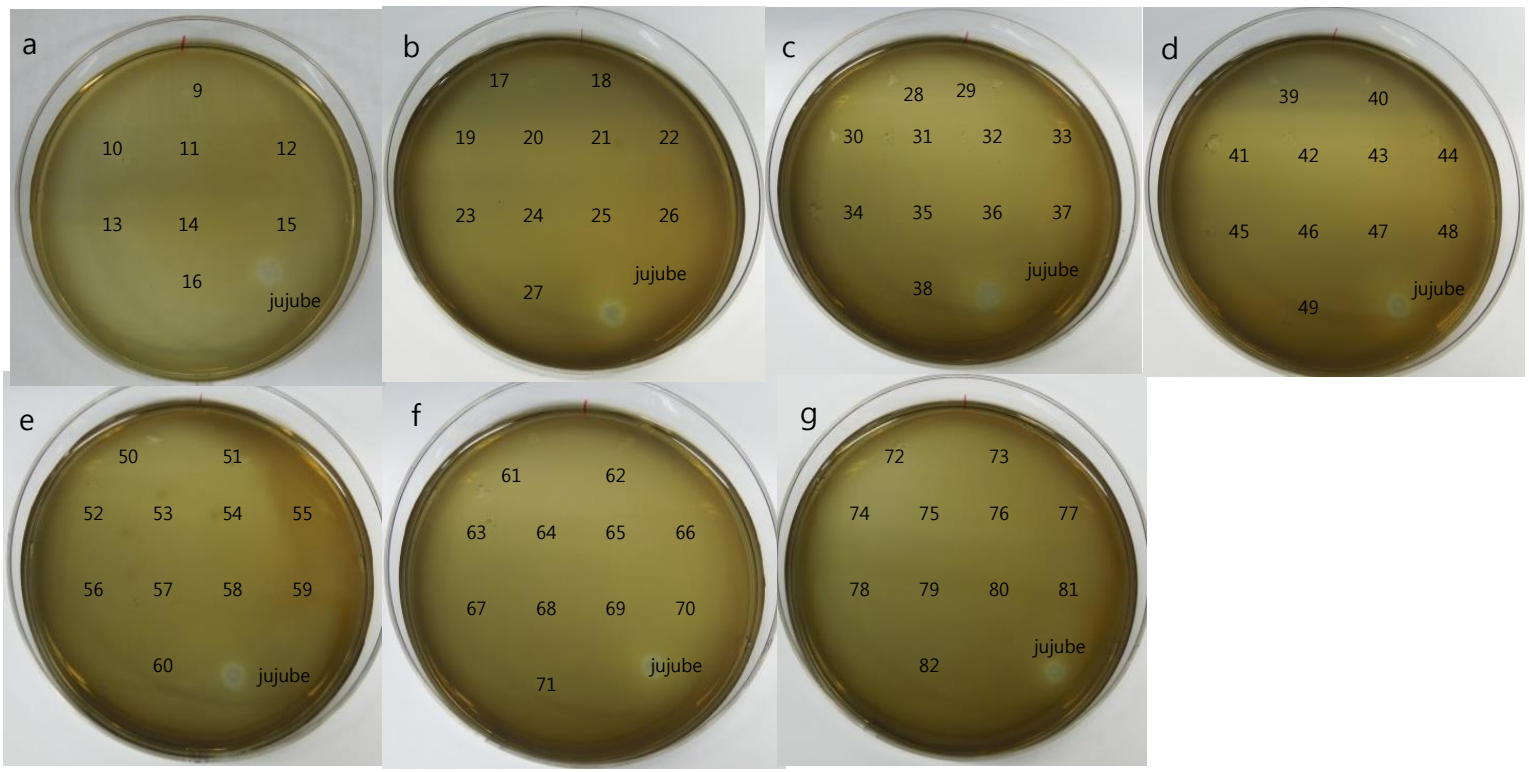

Figure 9. Esculin hydrolysis test. Peak point, which had phenolic compounds, was 9, 10, 14, 15, 35, 45, 47, 49, 53, 56, 57, 58, 60, and 76. However, the inhibition activity was occurred at 28, 29, 30, 34, 39, and 41. This section was from 5.5 min to 7.5 min.

3.3. Enzyme kinetics

Enzyme kinetics were measured to verifying the type of inhibition. In hanes-woolf plot (Figure 9.), the ratio of jujube leaf extracts were increased from 0% to 20%, the slope of plots were increased from 0.0021 to 0.0051, respectively. However, the x-intercept was fixed at -7.5. In this case, slope means $1/V_{max}$, x-intercept means $-K_m$ value. Like this, the x-intercepts are fixed at one spot, and the slope is changed, this means non-competitive inhibition. Non-competitive inhibitors do not bind to binding site, it is bound to another site of enzyme, and then the enzyme has a low affinity with substrate (Rothman, Bowen, Herkenham, Jacobson, Rice, & Pert, 1985). The famous non-competitive inhibitors are metal ions and surfactants (Seifert & Domka, 2005).

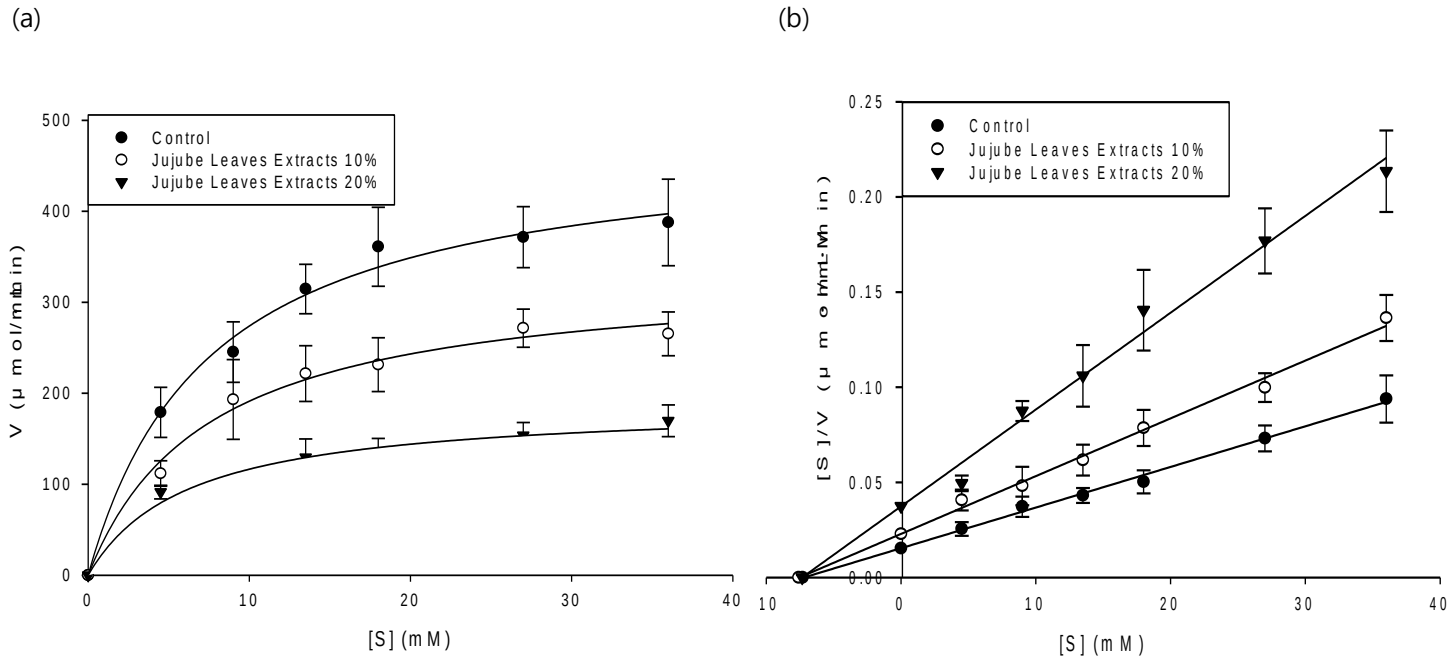

Figure 10. The ratio of jujube leaf extracts were increased from 0% to 20%. The enzyme concentration was fixed on 0.3 unit/mL. (a) Michaelis-Menten plot and (b) Hanes-Woolf plot.

3.4. Analysis of metal ions

Metal ions were famous non-competitive inhibitors. In this study, to evalute whether the inhibitory activity comes from metal ions or not, the concentration of some metal ions were determined. In terms of the results of (Wang, et al., 2012), Na^{+}, K^{+}, Li^{+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Al^{3+}, and Fe^{+} have inhibitory activity. So in this study, these metal ions were investigated. In jujube leaf extracts, the amount of metal ions were like below table (table 8). The highest concentration of metal ions was K^{+}, but it was below 0.01 mM. According to (Wang, et al., 2012), the concentration of metal ions needed to 1.0 mM for observing the inhibitory activity. It seemed to have no β-D-glucosidase inhibition on metal ions, since the concentrations were very low.

Table 8. Analysis the metal ions, which is famous non-competitive inhibitiors

Metal ions	Concentration (mM)
Cu	4.09E-06
Zn	3.99E-05
Mn	5.28E-06
Fe	2.69E-06
Al	1.11E-06
Li	ND
Ca	0.000244274
Na	5.53E-05
Mg	0.002274018
K	0.011198195
Co	ND

*: ND : Not Detected

Ⅳ. CONCLUSIONS

Optimization the enzymatic producing isoquercetin from jujube leaf extracts was performed using RSM. In this study, the quercetin did not produced at all conditions. The hypothesis, there is a β-D-glucosidase inhibitor in jujube leaf extracts, was set up from these results. To demonstrate this hypothesis, some experiments like esculin hydrolysis test, analysis phenolic compounds, and enzyme kinetics were carried out. It can be seen that this inhibitor was not a competitive inhibitor in esculin hydrolysis test. Furthermore, according to the enzyme kinetics, this inhibition was non-competitive inhibition. Well-known noncompetitive inhibitors were metal ions and surfactants. Metal ions, one of the well-known non-competitive inhibitor, was very slight. To sum up, there is a non-competitive inhibitor in jujube leaf extracts. And surfactant can be predicted as inhibitor in that the rutin is hydrophobic, but it was dissolved in water. In further study, identifying the inhibitor, and investigating the mechanism of this inhibitor. Natural glucosidase inhibitor can be seen to get from jujube leaf extracts according to this study. This glucosidase inhibitor will play an important role in the field of food industry and phamaceuticals in that the glucosidase inhibitor is natural material not a chemical compound. Furthermore, it can be attained from by-product, so it is economical.

Ⅴ. REFERENCES

Asano, N. (2003). Glycosidase inhibitors: update and perspectives on practical use. *Glycobiology, 13*(10), 93R-104R.

Day, A. J., DuPont, M. S., Ridley, S., Rhodes, M., Rhodes, M. J., Morgan, M. R., & Williamson, G. (1998). Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver β-glucosidase activity. *FEBS letters, 436*(1), 71-75.

Delourdesmatabilbao, M., Andreslacueva, C., Jauregui, O., & Lamuelaraventos, R. (2007). Determination of flavonoids in a citrus fruit extract by LC–DAD and LC–MS. *Food Chemistry, 101*(4), 1742-1747.

Formica, J., & Regelson, W. (1995). Review of the biology of quercetin and related bioflavonoids. *Food and chemical toxicology, 33*(12), 1061-1080.

Frantz, S., Calvillo, L., Tillmanns, J., Elbing, I., Dienesch, C., Bischoff, H., Ertl, G., & Bauersachs, J. (2005). Repetitive postprandial hyperglycemia increases cardiac ischemia/reperfusion injury: prevention by the α-glucosidase inhibitor acarbose. *The FASEB journal, 19*(6), 591-593.

Gerstorferová, D., Fliedrová, B., Halada, P., Marhol, P., Křen, V., & Weignerová, L. (2012). Recombinant α-l-rhamnosidase from *Aspergillus terreus* in selective trimming of rutin. *Process Biochemistry, 47*(5), 828-835.

Kim, Y.-M., Wang, M.-H., & Rhee, H.-I. (2004). A novel α-glucosidase inhibitor from pine bark. *Carbohydrate research, 339*(3), 715-717.

Li, Y., Wen, S., Kota, B. P., Peng, G., Li, G. Q., Yamahara, J., & Roufogalis, B. D. (2005). *Punica granatum* flower extract, a potent α-glucosidase inhibitor, improves postprandial hyperglycemia in Zucker diabetic fatty rats. *Journal of Ethnopharmacology, 99*(2), 239-244.

Makino, T., Shimizu, R., Kanemaru, M., Suzuki, Y., Moriwaki, M., & Mizukami, H. (2009). Enzymatically modified isoquercitrin, α-oligoglucosyl quercetin 3-O-glucoside, is absorbed more easily than other quercetin glycosides or aglycone after oral administration in rats. *Biological and Pharmaceutical Bulletin, 32*(12), 2034-2040.

Manach, C., Morand, C., Texier, O., Favier, M.-L., Agullo, G., Demigné, C., Régérat, F., & Rémésy, C. (1995). Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. *The Journal of nutrition, 125*(7), 1911-1922.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American journal of clinical nutrition, 79*(5), 727-747.

Mozhaev, V. V., Berezin, I. V., Martinek, K., & Nosoh, Y. (1988). Structure-Stability Relationship in Proteins: Fundamental tasks and strategy for the development of stabilized enzyme catalysts for biotechnolog. *Critical Reviews in Biochemistry and Molecular Biology, 23*(3), 235-281.

Muir, S. R., Collins, G. J., Robinson, S., Hughes, S., Bovy, A., De Vos, C. R., van Tunen, A. J., & Verhoeyen, M. E. (2001). Overexpression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonols. *Nature biotechnology, 19*(5), 470-474.

Pérez, M., Muñoz, F. J., Muñoz, E., Fernández, M., Sinisterra, J. V., & Hernáiz, M. J. (2008). Synthesis of novel glycoconjugates and evaluation as inhibitors against β-glucosidase from almond. *Journal of Molecular Catalysis B: Enzymatic, 52*, 153-157.

Pandey, S., Sree, A., Dash, S. S., & Sethi, D. P. (2013). A novel method for screening beta-glucosidase inhibitors. *BMC Microbiol, 13*, 55.

Rajal, V. B., Cid, A. G., Ellenrieder, G., & Cuevas, C. M. (2009). Production, partial purification and characterization of α-L-rhamnosidase from Penicillium ulaiense. *World Journal of Microbiology and Biotechnology, 25*(6), 1025-1033.

Rothman, R. B., Bowen, W., Herkenham, M., Jacobson, A., Rice, K., & Pert, C. (1985). A quantitative study of [3H] D-Ala2-D-Leu5-enkephalin binding to rat brain membranes. Evidence that oxymorphone is a noncompetitive inhibitor of the lower affinity delta-binding site. *Molecular pharmacology, 27*(3), 399-409.

Salazar, M. O., & Furlan, R. L. (2007). A rapid TLC autographic method for the detection of glucosidase inhibitors. *Phytochemical Analysis, 18*(3), 209-212.

San, B., Yıldırım, A., Polat, M., & Yıldırım, F. (2009). Mineral composition of leaves and fruits of some promising jujube (Zizyphus jujuba Miller) genotypes. *Asian Journal of Chemistry, 21*(4), 2898-2902.

Seifert, K., & Domka, F. (2005). Inhibiting effect of surfactants and heavy metal ions on the denitrification process. *Polish Journal of Environmental Studies, 14*(1), 87-93.

SORIA, F., & ELLENRIEDER, G. (2002). Thermal inactivation and product inhibition of Aspergillus terreus CECT 2663 α-L-rhamnosidase and their role on hydrolysis of naringin solutions. *Bioscience, biotechnology, and biochemistry, 66*(7), 1442-1449.

Sunkara, P. S., Bowlin, T. L., Liu, P. S., & Sjoerdsma, A. (1987). Antiretroviral activity of castanospermine and deoxynojirimycin, specific inhibitors of glycoprotein processing. *Biochemical and biophysical research communications, 148*(1), 206-210.

Velts, O., Uibu, M., Kallas, J., & Kuusik, R. (2011). Waste oil shale ash as a novel source of calcium for precipitated calcium carbonate: Carbonation mechanism, modeling, and product characterization. *Journal of hazardous materials, 195*, 139-146.

Voegele, R. T., Bardin, S., & Finan, T. M. (1997). Characterization of the Rhizobium (Sinorhizobium) meliloti high-and low-affinity phosphate uptake systems. *Journal of bacteriology, 179*(23), 7226-7232.

Wang, J., Ma, Y.-L., Wu, X.-Y., Yu, L., Xia, R., Sun, G.-X., & Wu, F.-A. (2012). Selective hydrolysis by commercially available hesperidinase for isoquercitrin production. *Journal of Molecular Catalysis B: Enzymatic, 81*, 37-42.

Wang, J., Zhao, L.-L., Sun, G.-X., Liang, Y., Wu, F.-A., Chen, Z., & Cui, S. (2011). A comparison of acidic and enzymatic hydrolysis of rutin. *Afr J Biotechnol, 10*(8), 1460-1466.

Weignerová, L., Marhol, P., Gerstorferová, D., & Křen, V. (2012). Preparatory production of quercetin-3-β-d-glucopyranoside using alkali-tolerant thermostable α-l-rhamnosidase from *Aspergillus terreus*. *Bioresource technology, 115*, 222-227.

Xie, S., You, L., & Zeng, S. (2007). Studies on the flavonoid substrates of human UDP-glucuronosyl transferase (UGT) 2B7. *Die Pharmazie-An International Journal of Pharmaceutical Sciences, 62*(8), 625-629.

Yadav, V., Yadav, S., Yadava, S., & Yadav, K. D. (2011). α-l-Rhamnosidase from Aspergillus flavus MTCC-9606 isolated from lemon fruit peel. *International Journal of Food Science & Technology, 46*(2), 350-357.

You, H. J., Ahn, H. J., & Ji, G. E. (2010). Transformation of Rutin to Antiproliferative Quercetin-3-glucoside by Aspergillus niger. *Journal of Agricultural and Food Chemistry, 58*(20), 10886-10892.

Zhao, Z., Liu, M., & Tu, P. (2008). Characterization of water soluble polysaccharides from organs of Chinese Jujube (Ziziphus jujuba Mill. cv. Dongzao). *European Food*

Research and Technology, 226(5), 985-989.

Zverlov, V. V., Hertel, C., Bronnenmeier, K., Hroch, A., Kellermann, J., & Schwarz, W. H. (2000). The thermostable α-l-rhamnosidase RamA of Clostridium stercorarium: biochemical characterization and primary structure of a bacterial α-l-rhamnoside hydrolase, a new type of inverting glycoside hydrolase. *Molecular microbiology, 35*(1), 173-179.

국 문 초 록

대춧잎에는 rutin, sapponin과 같은 flavonoid계의 성분이 많이 함유되어 있어 예로부터 중국과 한국 등 동양에서 약재로 많이 사용되어 왔지만 최근엔 대부분이 폐기되고 있다. 대춧잎의 주요 플라보노이드계의 성분인 rutin에서 rhamnose가 떨어진 것은 isoquercetin이고, isoquercetin에서 glucose가 떨어진 것은 quercetin이다. Rutin, isoquercetin, quercetin 모두 다양한 생리활성작용이 보고되어 있지만 특히 isoquercetin이 생물학적 활용에 있어서 가장 뛰어난 것으로 알려져 있다. 이러한 isoquercetin의 활용도를 높이기 위하여 *Aspergillus niger*에서 유래한 hesperidinase를 이용하여 대춧잎 추출물 내 rutin을 isoquercetin으로 전환하는 공정을 최적화 하였다. Hesperidinase는 α-L-rhamnosidase와 β-D-glucosidase 활성을 가진 효소 복합체이다. 사전 실험을 통하여 처리 온도(40~60℃, X_1), 처리 시간(24~72 h, X_2), 처리 pH(2~6, X_3)를 조작변수로 선택하고 isoquercetin 전환수율을 종속변수로 하여 반응표면분석을 실시하였다. Box-behnken 방법으로 설계된 실험은 중심점을 기준으로 15번의

실험을 3회 반복하는 것으로 이를 통해 2차 다항식을 얻었다. 반응표면 회귀식의 결정계수와 p 값은 각각 0.93과 0.01로 통계적으로 유의함을 알 수 있었다. 결과적으로 최적 조건은 47.3℃, 52.16 시간, pH 5.31에서 반응하였을 때 반응표면의 정상점에서 2.65 mg/mL의 isoquercetin을 생산하는 것으로 예측되었다. 최적 조건에서 실시한 검증 실험 결과는 2.57 mg/mL isoquercetin으로서 예측값과 잘 부합되었다. 이런 결과는 식품산업에서 폐기물인 대춧잎을 이용할 때 중요한 자료로서 활용될 수 있을 것이다.

최적화 실험 중 한 가기 흥미로운 현상은 다양한 처리 조건에도 불구하고 rutin으로부터 isoquercetin으로의 전환(α-L-rhamnosidase 활성)은 일어났지만, quercetin(β-D-glucosidase 활성)은 생성되지 않았다. 따라서 이러한 β-D-glucosidase 활성의 손실이 효소 동역학에 의한 것인지 저해제의 존재 때문인지 밝히기 위한 추가 연구를 실시하였다. 우선 처리 시간(24~72시간)과 효소 농도(0~3.0 unit/mL)를 변화시켜가며 실험한 결과 효소 농도 5 mg/mL 이하에서는 quercetin이 시간이 아무리 많이 지나도 생성되지 않는 것을 관찰할 수 있었다. 이러한 결과는 대춧잎 추출물 내 β-D-glucosidase의 저해제가 있다는 것을 시사하는 것이었다. 따라서 β-D-glucosidase의 저해제로 알

려진 페놀화합물을 기준으로 분획물을 얻어 esculin hydrolysis test를 해본 결과 페놀화합물이 있는 peak에서는 저해 반응이 없는 반면, peak가 없는 시간대에서 저해반응이 있는 것 관찰할 수 있었다. 저해 기작을 알아보기 위해 유사기질인 4-nitrophenyl β-d-glucospyranoside을 사용하여 대춧잎 추출물의 농도를 0 – 20%로 변화를 주면서 아몬드 유래의 β-D-glucosidase와 반응시켜 활성을 측정하여 구한 Michaelis-Menten 도표를 Hanes-Woolf 도표로 변화시켜 본 결과 기울기가 변하고, $-K_m$ 값을 의미하는 x절편이 변하지 않는 것을 통해 비경쟁적 저해인 것을 확인할 수 있었다. 대표적인 비경쟁적 저해제로는 금속이온과 계면활성제가 있다. 금속이온은 저해작용을 할 만큼 충분한 양이 있지 않다는 것을 확인하였으며 물에 녹지 않는 rutin이 수용액 상태로 안정적으로 있다는 점과 계면활성제가 비경쟁적 저해제의 대표적인 예라는 점에서 대추잎 추출물 속의 β-D-glucosidase의 저해제가 계면활성제의 한 종류라고 추측되며, 저해제의 동정을 위한 추가 실험이 필요하다.

주요어 : 대추잎 추출물, hesperidinase, rutin, isoquercetin, β-D-glucosidase 저해제, 반응표면분석