한 국 축 산 식 품 학 회 지
Korean J. Food Sci. Ani. Resour.
Vol. 27, No. 3, pp. 329~336(2007)

Optimum Conditions for Glycoside Conversion to Aglycone by γ-Galactosidase

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유당분해효소에 의한 Glycoside의 Aglycone으로 전환을 위한 최적 조건 확립

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ABSTRACT

This study was designed to find the optimum conditions for converting isoflavone glycoside to aglycone by β-galactosidase. Three different forms of the enzyme were tested and the optimum enzyme concentration, incubation temperature, pH, and incubation time were determined. Before treatment with enzyme, isoflavone contained 89.4% glycoside including daidzin, glycitin and genistin, and only 10.6% aglycone including daidzein, glycitein and genistein. Among the enzymes tested, the highest rate of isoflavone hydrolysis to aglycone, 35%, was observed when 3 unit/g Fungal Lactase (Amano Enzyme) was used. Higher incubation temperatures resulted in a higher rate of hydrolysis along with a greater loss of isoflavone mass. Therefore, body temperature (37°C) may be adequate for isoflavone conversion, with 44.9% hydrolysis and less than 10% loss of mass. As expected, a higher amount of aglycone was produced at pH 7 compared with other pH values. During 5 hr of incubation, the conversion of glycoside to aglycone increased dramatically from 0 to 1 hr, and plateaued thereafter. In addition, commercial soy-based milk was hydrolyzed more effectively with β-galactosidase when incubated for 5 hr. Based on the above results, the optimum conditions for isoflavone hydrolysis by β-galactosidase were for 3 hr at 37°C, pH 7 with 3 unit/g Fungal Lactase (Amano Lactase), yielding an average total amount of aglycone ranging from 40 to 47%.

Key words : isoflavone, aglycone, β-galactosidase, hydrolysis

INTRODUCTION

Soybeans, which have long been part of the diet in Asian countries, contain a variety of biologically active compounds (Messina and Messina, 1991; Izumi *et al.*, 2000). Interest in soy ingredients has increased recently all over the world. Epidemiologic studies have shown that the consumption of soybeans decreases the risk of various diseases and conditions, including breast cancer (Adlercreutz *et al.*, 1991a, 1991b; Lee *et al.*, 1991), prostate cancer (Severson *et al.*, 1989), colon cancer (Watanabe and Koessel, 1993), osteoporosis (Knight and Eden, 1996), menopausal symptoms (Aldercreutz *et al.*, 1991) and coronary heart disease (Clarkson *et al.*, 1995).

At present, the study on soy isoflavones has focused on the exertion of weak estrogenic activities by virtue of structural similarity to a female hormone, estrogens (Hwang *et al.*, 2003; Tikkanen and Aldecreutz, 2000); it may thus be possible that dietary soy isoflavones affect a regulation of hormonal homeostasis in women and are an effective dietary factor in preventing bone loss (Morabito *et al.*, 2002; Uesugi *et al.*, 2002) by an enhanced calcium absorption especially in women after manopause.

Isoflavones in soybean exist about 0.1-0.3 mg/100 mg protein (Kudou *et al.*, 1991), and primarily as glucoside forms: 6"-O-malonylglucosides and 6"-O-acetylglucosides (Izumi *et al.*, 1997; Kodou *et al.*, 1991; Ohta *et al.*, 1980), and rarely as aglycone form. The primary soy isoflavones are genistein, daidzein and much lower amounts of glycitein,

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and their respective β-glucosides, genistin, daidzin and glycitin (Kudou *et al.*, 1991).

Isoflavone glycosides are the major forms found in the soybean grains and in non-fermented foods (Carr-Panizzi *et al.*, 2004; Coward *et al.*, 1998). The glucoside conjugates of isoflavones are converted into aglycones during soybean processing by the effect of β-glucosidase (Toda *et al.*, 2001; Yin *et al.*, 2005) including β-galactosidase. To establish a relation between isoflavone intake and its proposed biological activity, the absoption, distribution, metabolism, and excretion of isoflavones from the glucoside and aglycone forms have been investigated in animals and humans (Jackson *et al.*, 2002; Yin *et al.*, 2005). After ingestion, the glucoside forms of isoflavones are hydrolyzed by β-galactosidase to the aglycone forms in the jejunum (Matsuura *et al.*, 1989). The released aglycone forms of isoflavones are either absorbed intact by the intestine or further metabolized by intestinal microflora into several other products (Han *et al.*, 2001; Piskula *et al.*, 1999). Even though the hydrolyisis of glucoside isoflavones were investigated from the past, specific optimum conditions have not been reported, therefore, the objective of the present study was to find the possibility and optimum conditions of β-galactosidase for the conversion of isoflavone glycosides to aglycones.

MATERIALS AND METHODS

Materials

Isoflavone was obtained from Amore Pacific Co. Ltd. (Seoul, Korea). Daidzin (4'-hydroxyisoflavone-7-glucoside), genistin (4',5,7-trihydroxyisoflavone-7-glucoside), daidzein (4',7-dihydroxyisoflavone, 7-*O*-β-D-glucopyranoside), glycitein (4',7-dihydroxy-6-methoxyisoflavone) and genistein (4',5,7-trihydroxyisoflavone) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Commercial soy-based milk (Vegemil, Chung's Food Co., Ltd., Chungju, Korea) was purchased. Three different kinds of β-galactosidase were Fungal Lactase (activity: 80,000 unit/g, Amano Enzyme, Inc., Nagoya, Japan), Fungal Lactase (activity: 100,000 units/g, Bio-Cat, Inc., Troy, VA, USA) and Lactoles N10 (activity: 10,000 units/g, Daiwa Kasei K. K., Shiga, Japan) were also purchased from Sigma Chemical Company.

Assay of enzyme activity

The activity of β-galactosidase was monitored with isoflavone and a synthetic substrate, ortho-nitrophenol-β-D-galactopyranoside (ONPG, Sigma Chemical Company). In the standard assay, enzyme activity was measured by determining the aglycone concentration after 20 min incubation at 37°C in 0.1 M acetic buffer, pH 4.5.

The isoflavone-hydrolyzing activity was measured as follows: The reaction mixture was prepared using the 10 mL substrate solution (1,000 ppm) and 10 mL enzyme solution (4 unit/mL). The mixture was incubated for 1, 2 or 3 hr at 37°C, and taken as 1 mL, after which 4 mL Na_2CO_3 was added to stop the reaction. The mixture was filtered through a 0.22 μm membrane for the analysis of aglycone by HPLC.

The estimation of the hydrolyzing activity of the synthetic substrate, the ONPG was follows. A 4 mL sample of 0.05 M ONPG in 0.1 N acetic buffer, pH 4.5 was mixed with 1 mL of enzyme solution, and incubated at 37°C for 20 min. The reaction was stopped by addition of 0.5 mL of Na_2CO_3 solution (10%). The resulting yellow color was immediately measured at 420 nm with a spectrophotometer (DU 650, Beckman Inc., Fullerton, CA, USA). The hydrolyzed ONPG was determined by referring to a calibration curve prepared, concurrently in the same manner, with 5-300 μM of ONPG.

Isoflavone extraction

One gram of isoflavone sample was weighed into 125 mL flat bottom flasks, and 12 mL extraction solution (0.1% acetic acid) was added to flask. The solution was mixed using a rotary shaker (120 rpm) overnight at room temperature. Extracts were suction filtered through Whatman no. 42 filter paper into 250 mL round bottom flasks and washed twice with 12 mL extraction solution. Samples were condensed to approximately 1 mL using a vacuum rotary evaporator (EYELA, Rikakiai Co. Ltd., Tokyo, Japan) with a water bath at 30°C. The dried material was re-dissolved in a mixture of methanol:water (80:20, v/v) to a final volume of 10 mL and filtered through a 0.22 μm PTFE syringe filter (Millex-LCR, Millipore Co., Billerica, MD, USA) prior to HPLC analysis (Wang and Murphy, 1994). All steps were performed in reduced light conditions to minimize light-induced isoflavone degradation.

Determination of isoflavone concentration

The concentration of isoflavone was measured by a slight modification of the procedure of Wang and Murphy (1994). Two grams of finely ground isoflavone samples were stirred in 10 mL of 80% aqueous methanol solvent for 2 h at room temperature. The extract was filtered through Whatman no. 42 filter paper. A rotary evaporator was used to dry the filtrate under vacuum at a room temperature. The residue was redissolved in 10 mL 80% MeOH solution and filtered through a 0.22 μm filter unit.

Analysis of isoflavone by high performance liquid chromatography (HPLC)

A liquid chromatograph (Waters 600, ETL Testing Lab. Inc., Cortland, NY) equipped with a Prontosil Eurobond C_{18} column (5 μm, 250 × 4.0 mm i.d., BISCHOFF, Leonberg, Germany) and an absorbance detector at a 254 nm was used. The mobile phase for HPLC was consisted of solvent (A) 20% (v/v) methanol in filtered Milli Q H_2O, and (B) 60% (v/v) methanol in water. The solvent gradient was as follows: solvent A was decreased from 100 to 0% over 50 min, and finally 100% held for the next 10 min. The flow rate was 1 mL/min and 20 μL of sample was injected.

Optimum conditions for isoflavone hydrolysis

Four different conditions (enzyme concentration, incubation temperature, pH, and time) were examined for hydrolysis activity of β-galactosidase. For determination of enzyme concentration, 10 mL solution containing 500 ppm isoflavone and 50, 40, 30, 20 or 10 units enzyme was adjusted to pH 7 and incubated at 30°C for 2 hr. For incubation temperature, 10 mL same solution as described above containing 60 unit enzyme was incubated with 6 different temperatures (30, 35, 37, 40, 45 or 50°C) for 2 hr. Same procedure was applied for incubation pH determination (pHs 2, 3, 4, 5, 6, 7, 8 or 9), and for incubation time (1, 2, 3, 4 or 5 hr). Then 1 mL was taken and 4 mL Na_2CO_3 was added to stop reaction.

Application to soy-based milk

To examine the isoflavone content in soy-based milk, 20 mL was dissolved in 25 mL of methanol for 60 min at 60°C, and centrifuged at 1,200 × g for 15 min. Then, the supernatant was filtered and injected to HPLC as described above.

For hydrolysis, 1.5 unit/mL β-galactosidase was added into 10 mL Vegemil and incubated for 1, 2, 3, 4 or 5 hr, and Na_2CO_3 was added to stop the reaction. The mixture was filtered through a 0.22 μm membrane for the analysis of aglycone by HPLC.

Statistical analysis

Data from each experiment were analyzed by analysis of variance (ANOVA) using a SAS program (1985) and differences among treatments were determined by Duncan's multiple test at $p < 0.05$, unless otherwise stated.

RESULTS

Hydrolytic activity of β-galactosidase enzymes

To evaluate the enzyme activity of different commercial β-galactosidases (Fungal Lactase from Amano enzyme, Fungal Lactase from Bio-cat, and Lactoles N10), three enzymes were reacted at pH 7 and 37°C for 20 min (data not shown). The activities of both Fungal Lactases decreased over 25% compared with those described. In the case of Lactoles N10, activity decreased from 10,000 to 8,696.8 unit/g, which may be due to the adequate reaction conditions for enzyme.

Isoflavone content

Six forms of isoflavone were found such as daidzin, glycitin, genistin, daidzein, glycitein, and genistein. Although four different concentrations (50, 100, 150, and 200 ppm) were tested (data not shown), no significant difference was found ($p>0.05$). Among isoflavone components, 89.4% was glycosides, which were daidzin, glycitin, and genistin, and only 10.6% was aglycone consisting of daidzein, glycitein, and genistein. Total amount of glycoside was 323.9 mg/g isoflavone, which was consisted by daidzin 151.2 mg, glycitin 126.8 mg and genistin 45.0 mg. Comparative aglycone form were daidzein 6.7 mg, glycitein 11.3 mg and genistein 16.5 mg, respectively.

Hydrolytic activity

Among three different enzymes, the highest hydrolytic activity was found in Fungal Lactase (Amano enzyme), which high conversion of glycoside to aglycone was found when it was reacted at 30°C for 3 hr (Fig. 1). The amount of aglycone increased from 15.6 to 34.9% during 3 hr incubation.

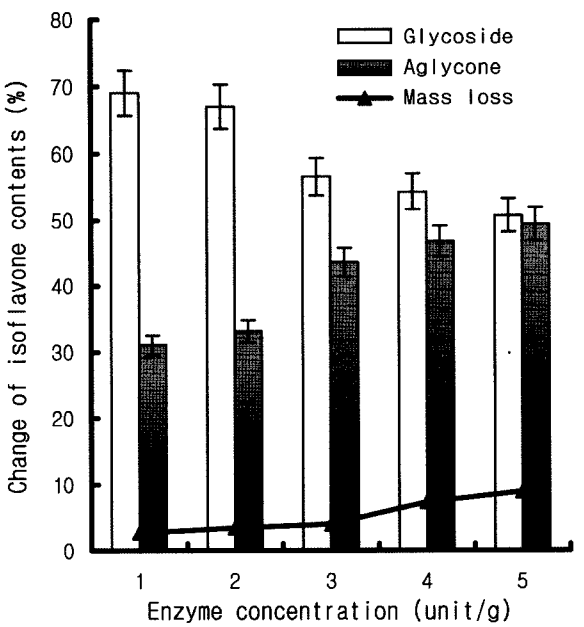

Fig. 1. Hydrolysis of isoflavone incubated with various concentrations of Amano Lactase at 30°C for 2 hr. Glycosides contained daidzin, glycitin, and genistin. Aglycones contained daidzein, glycitein, and genistein.

Comparatively, 28.9% of aglycone was produced by Fungal Lactase (Bio-cat) and only 6% with Lactoles N10. Therefore, Fungal Lactase (Amano Enzyme) showed a high efficiency of conversion of glycoside to aglycone.

Optimum conditions for isoflavone hydrolysis

1) Enzyme concentration

The hydrolysis efficiency of Fungal lactase (Amano Enzyme) was examined and shown when different concentrations (1, 2, 3, 4 or 5 unit/g) were added (Fig. 1). When 3, 4 or 5 unit/g enzyme was added, aglycone components were converted from glycone consisted of 40.5, 43.8, and 45.3% of total isoflavone, respectively. Based on results, higher concentration of enzyme showed greater hydrolysis rate of isoflavone, however, more enzyme was resulted in more mass loss of isoflavone. In comparison of 3 and 5 unit/g enzyme addition, mass loss of isoflavone was 4.0 and 8.9%, respectively. Therefore, the optimum enzyme concentration for isoflavone hydrolysis was estimated as 3 unit/g.

2) Incubation temperature

The optimum incubation temperature for isoflavone hydrolysis is shown in Fig. 2. Higher incubation temperatures were resulted in higher hydrolysis rates and mass loss of total isoflavone contents. Enzyme-untreated isoflavone contained 90.5% glycone and 9.6% aglycone component, however, aglycone content was increased up to 52.4% when reacted with 3 unit/g enzyme at 45°C for 2 hr. Even at 37°C as body temperature, 44.9% of aglycone was produced. Incubation over 40°C for enzyme reaction could not be acceptable in the present study since about 10% of mass loss was found. Therefore, high amount of glycone conversion to aglycone in body could be produced and this condition was highly effective on isoflavone application.

3) Incubation pH

The change of hydrolysis rate with different incubation pHs (2-9) is shown in Fig. 3. The aglycone content was the highest when incubated at pH 7, which was the optimum pH for Fungal Enzyme (Amano Enzyme). At lower pH in 2-5, aglycone conversion to aglycone was sufficiently high, but isoflavone mass loss increased up to 18%. The reason for relatively higher hydrolysis could be due to acid hydrolysis instead of enzyme process. Therefore, the optimum pH existed between pH 6 to 8, which showed 41.9, 47.2, and 40.7%, respectively. This result provided strong evidence that conversion of isoflavone from glycone to aglycone form could be efficiently processed within the intestine.

4) Incubation time

The change of hydrolysis rate depending on incubation time is shown in Fig. 4. After 1 hr incubation, glycone content including daidzin and genistin decreased significantly, except glycitin, which was slightly increased. However, aglycone content dramatically increased, especially in daidzein and genistein. The reason why the amount of glycitin and glycitein changed into small range may be due to

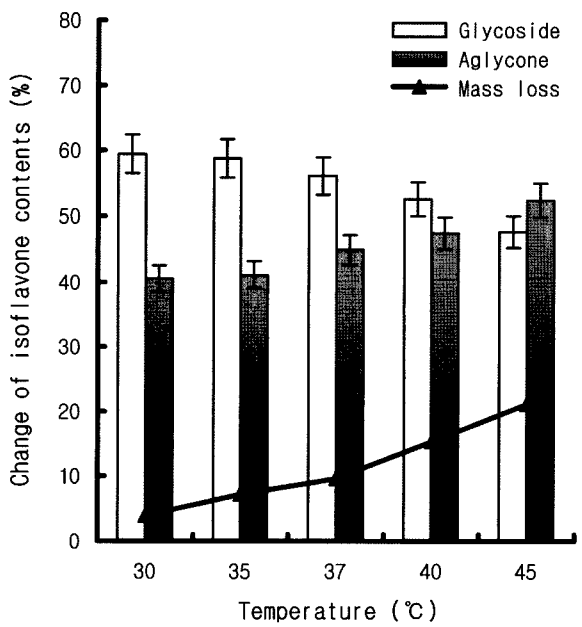

Fig. 2. Hydrolysis of isoflavone incubated with various temperatures with Amano Lactase for 2 hr. Glycosides contained daidzin, glycitin, and genistin. Aglycones contained daidzein, glycitein, and genistein.

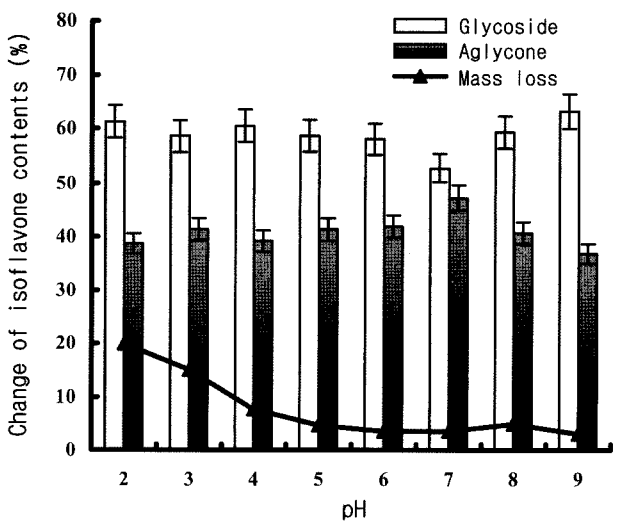

Fig. 3. Hydrolysis of isoflavone incubated with various pHs with Amano Lactase at 37°C for 2 hr. Glycosides contained daidzin, glycitin, and genistin. Aglycones contained daidzein, glycitein, and genistein.

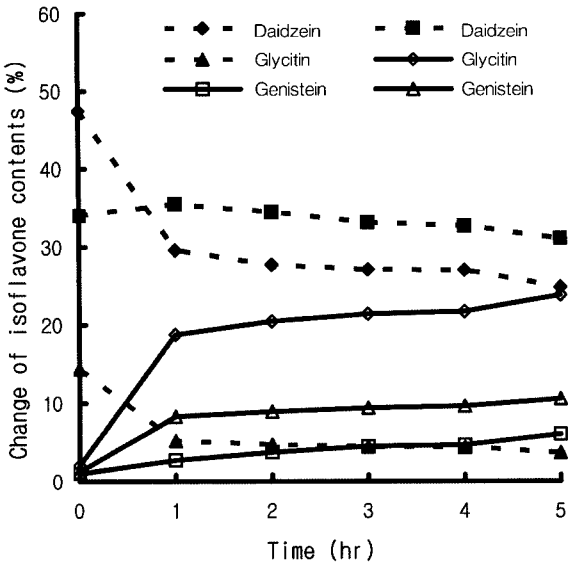

Fig. 4. Hydrolysis of isoflavone incubated with various times with Amano Lactase at 30°C. Glycosides contained daidzin, glycitin, and genistin. Aglycones contained daidzein, glycitein, and genistein.

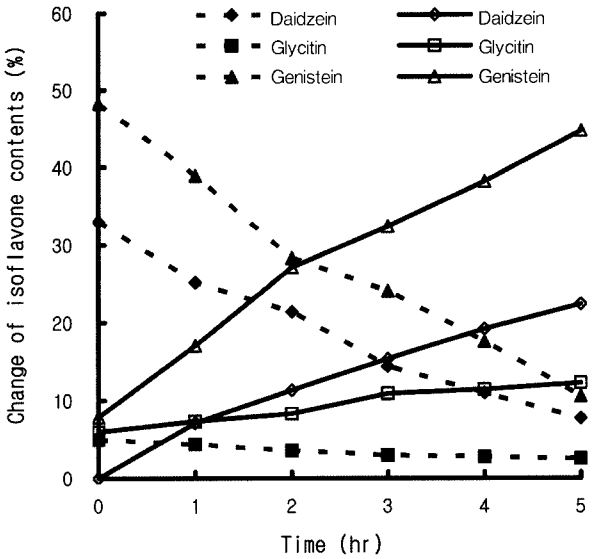

Fig. 5. Hydrolysis of isoflavone in soy-based milk during incubation with Amano Lactase at 30°C. Glycosides contained daidzin, glycitin, and genistin. Aglycones contained daidzein, glycitein, and genistein.

enzyme specificity or structure of glycitin. Although with prolonged incubation time resulted in high hydrolysis of isoflavone, the difference was not significant between 1 and 5 hr. Therefore, even 1 hr incubation could be sufficient for optimum hydrolysis of isoflavone in the present study.

Application to soy-based milk

Based on above conditions, we examined how β-galactosidases hydrolyze the isoflavone in soy-based commercial milk (Fig. 5). The percentage of total isoflavone contents were daidzin 33.9, glycitin 5.0 and genistin 48.2% as glycoside forms, while glycetin 6.0 and genistein 7.9% without daidzein as aglycone forms.

With longer incubation, greater amounts of aglycones were produced in soy-based milk as expected. During 1 hr incubation only, the percentage of glycosides decreased to daidzin 25.2%, glycitin 4.4%, and genistin 38.9%. However, daidzein, glycetein, and genistein increased to 7.1, 7.4, and 17.0%, respectively.

During 5 hr incubation, the daidzin amount was changed from 33.9 to 7.7% and genistin decreased from 48.2 to 10.6%. In the case of aglycone forms, 22.4% of daidzein

Table 1. Hydrolysis of isoflavone glycoside by various commercial β-galactosidases incubated at 30°C for 3 hr[1].

Time (hr)	Commercial Lactase	Glycoside			Aglycone			Sum of aglycone
		Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein	
		(%)						
1	Control[2]	47.42^{a}	33.97^{b}	14.38^{a}	1.99^{d}	1.03^{c}	1.21^{c}	4.23^{d}
	Fungal Lactase (Amano enzyme)	39.17^{b}	35.65^{a}	9.62^{d}	9.49^{b}	1.51^{b}	4.56^{a}	15.56^{b}
	Fungal Lactase (Bio-cat)	38.82^{b}	32.97^{b}	11.37^{c}	9.88^{a}	2.14^{a}	4.82^{a}	16.84^{a}
	Lactoles N10	46.39^{a}	32.97^{b}	13.37^{b}	2.88^{c}	2.21^{a}	2.22^{b}	7.31^{c}
2	Control	47.30^{a}	33.82^{a}	14.25^{a}	2.17^{c}	1.12^{d}	1.36^{c}	4.63^{d}
	Fungal Lactase (Amano enzyme)	23.61^{c}	35.43^{a}	7.41^{d}	20.88^{a}	2.59^{c}	10.08^{a}	33.55^{a}
	Fungal Lactase (Bio-cat)	31.65^{b}	31.74^{a}	9.78^{c}	14.88^{b}	2.89^{a}	6.79^{b}	24.54^{b}
	Lactoles N10	46.00^{a}	32.17^{a}	12.87^{b}	3.46^{c}	2.79^{b}	2.57^{c}	8.82^{c}
3	Control	47.25^{a}	33.70^{ab}	14.18^{a}	2.17^{d}	1.30^{c}	1.40^{d}	4.87^{d}
	Fungal Lactase (Amano enzyme)	23.14^{c}	34.60^{a}	7.36^{d}	21.54^{a}	2.67^{b}	10.67^{a}	34.88^{a}
	Fungal Lactase (Bio-cat)	29.89^{b}	30.46^{c}	8.13^{c}	17.37^{b}	3.15^{a}	8.34^{b}	28.86^{b}
	Lactoles N10	45.89^{a}	31.46^{bc}	12.13^{b}	4.37^{c}	3.15^{a}	3.34^{c}	10.86^{c}

[1]Values represent the means; n=3. Values in a column with different superscripts were significantly different ($p<0.05$).
[2]Control : Content of isoflavone hydrolysis without lactase incubated at 37°C.

was produced from 0% and 12.2% and 44.7% of glycetein and genistein were produced, respectively.

This result showed the similar trend to that in Fig. 4, which isoflavone sample was hydrolyzed. In Fig. 4, no difference in isoflavone hydrolysis was found among various incubation times, while more isoflavone was hydrolyzed into aglycone forms in Fig. 5. The highest amounts or components were also different between isoflavone sample and soy-based milk, which is probably due to different sources of isoflavone and various kinds of food processing. However, this study indicated that β-galactosidases could increase the isoflavone hydrolysis from glycosides to aglycones in soy-based food system.

DISCUSSION

Large quantities of potentially beneficial or toxic plant phenolics are present in human diets. It is well known that the consumption of soy-based foods is associated with a number of health benefits, including lower risk of cardiovascular disease, breast and prostate cancer, attenuated menopausal symptoms, and prevention of bone loss with age (Richelle *et al.*, 2002).

A study demonstrated that enzyme hydrolysis of a purified and concentrated extract of isoflavones does not enhance the absorption of isoflavones in postmenopausal women (Richelle *et al.*, 2002). Even though the effect of aglycone isoflavones to enhance the health is still controversial, chronic ingestion of soy products would result in a gradual rise in plasma concentrations due to the relatively efficient uptake and the low elimination rates of isoflavones.

Soy isoflavones are found mainly as glycosides in soybeans and in unfermented soy foods (Wang and Murphy, 1994). Effective absorption of the isoflavone likely requires the conversion of glycosides into aglycones via the action of β-galactosidases obtained from bacteria that colonize in the small and the large intestine (Xu *et al.*, 1995). To increase the effective form of isoflavone in soy-foods, exogenous enzyme treatment could be the major key step for processing. Therefore, higher rate of hydrolysis of isoflavone conversion due to exogenous β-galactosidase with the optimum conditions shown in the present study may provide useful information for soy-product processing.

As expected, the present study showed that much higher amount of glycoside form existed compared with aglycone form in isoflavone used. Glycoside was 89.4% and only 10.6% was aglycone, therefore, it is proven that isoflavone hydrolysis is required for conversion to an effective form of isoflavone. Also, higher conversion occurred with more concentration of enzyme added, however, more mass loss of isoflavone was found. Thus, 3 unit/g of enzyme addition appeared to be an optimum concentration for both more hydrolysis rate and less mass loss of isoflavone. The increase in genistein content may be attributed to the increased amount of genistin, which was readily transformed to genistein through hydrolysis by the β-glucosidase. The transformation of malonyl genistin to genistein may provide an explanation for the increased content of genistein.

The effect of pH on the enzyme activity was observed at 37°C since this temperature was optimum for the isoflavone hydrolysis. The sample with addition of exogenous β-galactosidase resulted in maximum amount of total aglycone (47.2%) at pH 7.0 (Fig. 3), but most samples incubated in other pHs showed a significant low rate of the hydrolysis.

Certain flavonoid glycosides are absorbed from the small intestine in human by either hydrolysis of the β-glucoside or a specific active transport mechanism. The only mammalian β-glucosidase to have an activity within the gut lumen is lactase phlorizin hydrolyse (LPH). The study by Day *et al.* (2000) demonstrated that LPH is active on various (iso)flavonoid glucosides with a high affinity towards the flavonoid glucosides in particular. All of mammalian LPHs have two catalytic sites (Semenza, 1987), one to hydrolyze lactose, which mainly responsible for the hydrolysis of the flavonol glucosides, and other involved in the deglycosylation of more hydrophobic substrates (Day et al, 2000). Lactase has a preference for hydrophilic substrates, but will accept a broad range of aglycones. They concluded that LPH is capable of hydrolyzing various flavonol and isoflavone glucosides (Day *et al.*, 2000). Based on above data, isoflavone could be effectively converted by β-galactosidase into aglycone forms, which is considered as an effective dietary factor in preventing bone loss and in decrease of various disease risks. Most of women with menopause women have been suffered by lack of β-galactosidase.

Above results could be applied as an effective way to attenuate the lactose intolerance symptoms, requires the person to quit drinking milk and eating other dairy products (Newcomer and McGill, 1984). A large majority of non-Caucasians and elderly people in Western Europe and United States, and various ethnic population groups cannot properly digest the lactose because they lack sufficient quantities of lactase (β-galactosidase, EC 3.2.1.23) that breaks down lactose in their gastric tract (Simmons, 1978). Researchers who have studied lactose deficiency report that symptoms appear more frequently as age increases (Simmons, 1978).

Lactose requires lactase, which is produced in the small

intestine, to hydrolyze lactose into glucose and galactose. In the absence of lactase, lactose maldigestion usually shows symptoms of digestive tract discomfort, cramping and/or diarrhea (Bayless *et al.*, 1975). To solve this problem, an approach has been tried in which lactose was reduced in milk by lactase before consumption (Scrimshaw and Murray, 1988). However, hydrolysis of lactose during the process resulted in taste changes in milk because glucose and galactose were about four times sweeter than lactose, and many lactose maldigesters did not like the taste of lactose-hydrolyzed milk. Our previous study found the satisfied efficiency of β-galactosidase microencapsulation and the possibility of acceptable milk products fortified β-galactosidase (Kwak *et al.*, 2001). Therefore, β-galactosidase microencapsulation could offer two advantages, which prevents lactose intolerance, and increases calcium absorption affected by an estrogenic effect of isoflavone.

ACKNOWLEDGEMENT

This research was supported by a grant from the Korea Research Foundation, Seoul, Republic of Korea (KRF-2003-041-F00038).

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(2007. 7. 11. 접수/2007. 9. 2. 채택)