A Thesis for the Degree of Doctor of Philosophy

Characterization of α- and β-glucosidase inhibitors from jujube leaf and their applications to foods

대추 잎 유래 알파-, 베타-글루코시데이즈 저해제의 특성 규명과 식품에의 적용에 관한 연구

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Abstract

The jujube leaf has been used for medicinal purposes throughout the ancient history of several Asian countries due to its antioxidant and sedative properties and regulatory effects on blood pressure. Although the leaf is more abundant in flavonoids and polyphenols as compared to the fruit, the majority of recent research efforts have focused on the fruit and the leaf is regarded as a by-product or waste.

Flavonoids and polyphenols are known to inhibit the activity of α-glucosidase. Inhibitors of α-glucosidase have received growing interest because of their potential of being used as treatment for diabetes and obesity. Many research groups are trying to find α-glucosidase inhibitors from natural products because of the disadvantages of commercial drugs that possess α-glucosidase inhibitory activity. However, despite the many unknown compounds and various flavonoids in the jujube leaf, no studies have reported on an α-glucosidase inhibitory activity from the jujube leaf. Therefore, in this study, I investigated α-glucosidase inhibitory activity of the jujube leaf.

The inhibitory activity of jujube leaf on α-glucosidase was expected to be low compared to acarbose, but preliminary experiments showed that

jujube leaf α-glucosidase inhibition was approximately twice as strong as acarbose. Therefore, this strong inhibition was further investigated in this work, which ultimately revealed that the strong inhibition was caused by 3',5'-di-C-β-D-glucosyl phloretin. This substance was not previously reported to inhibit α-glucosidase; thus, the α-glucosidase inhibitory activity of this inhibitor was characterized. It was confirmed that the α-glucosidase inhibitory activity of this substance was approximately 13.5-times stronger than that of acarbose. This inhibitor can selectively inhibit α-glucosidase, as it does not inhibit α-amylase. Because of this inhibition specificity, 3',5'-di-C-β-D-glucosyl phloretin is expected to have less side effects than acarbose. In addition, *in vitro* experiments showed that the rate of α-glucosidase inhibition was not statistically significantly different.

The functional rice that can control postprandial blood glucose through the use of jujube leaf inhibitory activity on α-glucosidase was produced. Rice is a staple food in many Asian countries. However, some people who require control of postprandial blood glucose are more likely to eat brown rice than white rice, but many patients avoid brown rice due to the texture. Therefore, in this study, I aimed to produce functional rice with similar texture to white rice that can use α-glucosidase inhibitory activity of jujube leaf to control postprandial blood glucose. It was observed that the

addition of jujube leaf extract did not affect the texture of rice including hardness and stickiness. As the amount of jujube leaf extract increased from 0 to 10 mg/mL, the time required for glucose degradation reached its maximum value, increasing from 180 to 360 min. Although the rice possessed the flavor and color of jujube leaf, there was no statistically significant difference from conventional instant rice when sensory evaluation was performed by preference degree. Thus, the possibility of producing functional rice by using the α-glucosidase inhibitory activity of jujube leaf was confirmed.

I demonstrated the presence of a β-glucosidase inhibitor in the process of converting the rutin present in jujube leaf into isoquercitrine. The expected molecular weight of this β-glucosidase inhibitor was 392 g/mol and the predicted molecular formula was $C_{17}H_{23}O_{13}N$. It was confirmed that this inhibitor showed mixed non-competitive inhibition. This substance is a new inhibitor, so further studies of its structure and inhibition mechanism analysis are needed.

A functionally enhanced grapefruit juice was produced using the β-glucosidase inhibitory activity of jujube leaf. Naringin, a bitter compound in citrus fruit juice, is one of the main reasons for a deterioration in the production quality of citrus fruit juice. In the food industry, naringinase is

used to remove naringin and then naringenin is produced from naringin. Naringenin is tasteless and has various functionalities. The main drawback of naringenin, despite its different functionalities, is its low bioavailability due to low water solubility. The aim of the present study was to produce grapefruit juice with enhanced functionality by degrading naringin and enhancing the production of prunin using the β-glucosidase inhibitory activity of jujube leaf. Naringin was effectively removed and 2.47 mmol/mL of prunin was produced, which was approximately 1.31 times higher than that of grapefruit juice without jujube leaf extract. Moreover, it has been observed that the optimum condition of prunin production was reached with lower temperature, lower amount of enzyme, and this result is expected to be an advantage when applied to the food industry.

In summary, in this study, novel α- and β-glucosidase inhibitors derived from jujube leaf were isolated and characterized and their applicability to the food industry was confirmed.

Keywords: α-glucosidase inhibitor, β-glucosidase inhibitor, jujube leaf, natural compounds

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Chapter I. Literature review

I-1. Introduction

Glucosidase is an enzyme (EC. 3. 2. 1.-) that catalyzes the cleavage of glycosidic bonds of oligosaccharides or glycoconjugates. Glucosidases play a vital role in many important biological processes such as post-translation modification of glycoproteins, lysosomalization of glycoconjugates, and digestion (de Melo, da Silveira Gomes, & Carvalho, 2006). Glucosidase inhibitors could be used to control these biological processes. For these reasons, significant effort has been put into the development of therapeutic agents based on inhibitors of glucosidases and to find and produce new inhibitors (L.-P. Guo, Jiang, Lv, & Wang, 2010).

Glucosidase inhibitors have attracted significant attention because of their therapeutic potential for treating diseases such as diabetes, HIV infections, metastatic cancer, and lysosomal storage diseases (Butters, Dwek, & Platt, 2005; Cheng & Josse, 2004; Kordik & Reitz, 1999; Qi, Zhao, Lu, Wang, & Jin, 2016). Glucosidase inhibitors have also been useful for understanding the relationship between structure and activity, which is needed in order to effectively explore biochemical pathways and mimic enzyme transition states.

Glucosidase inhibitors are not used extensively in the food industry because of various limitations, with poor solubility in water being the major

limitation, as it is with rutin (Gullón, Lú-Chau, Moreira, Lema, & Eibes, 2017; Pujara, Jambhrunkar, Wong, McGuckin, & Popat, 2017). Therefore, many research groups are studying ways to increase the water solubility of glucosidase inhibitors, such as encapsulation, use of ionic liquids, and chemical synthesis (de Faria, Shabudin, Claúdio, Válega, Domingues, Freire, et al., 2017; Wang, Hu, Zoghbi, Huang, & Xia, 2017; Z. Zhou, Li, Sun, Lu, Tong, Sun, et al., 2016). However, these methods require additional research before they are ready for use in the food industry. Furthermore, side effects and instability in light and heat complicates the use of glucosidase inhibitors in the food industry. Thus, there are many efforts being made to find novel inhibitors with reduced side effects and better applicability to the food industry. This review provides information on glucosidase inhibitors obtained from natural products. Furthermore, in this study, the application of these substances to the food industry was investigated.

I-2. Glucosidase Inhibitors

I-2-1. α-Glucosidase inhibitors

α-Glucosidase is an enzyme that catalyzes the cleavage of the terminal glucose from starch that is linked by an α-linkage. α-Glucosidases are essential for digestion of carbohydrates in the small intestine because only monosaccharides can be taken up easily (Diamond, Karasov, Cary, Enders, & Yung, 1984). These enzymes degrade carbohydrates in the intestine, thus facilitating their absorption. Inhibitors of α-glucosidases can retard the absorption of glucose during digestion and therefore can be used to control postprandial blood glucose levels (Du, Shi, & Qiu, 2005). In 1990, acarbose was the first α-glucosidase inhibitor to be approved for treating type 2 diabetes (Joshi, Standl, Tong, Shah, Kalra, & Rathod, 2015). Since then, other α-glucosidase inhibitors such as miglitol and voglibose have been used as therapeutic agents for type 2 diabetes. However, therapeutic agents for type 2 diabetes based on α-glucosidase inhibition have gastrointestinal side effects such as abdominal distention, diarrhea, and stomach aches (L.-P. Guo, Jiang, Lv, & Wang, 2010). For these reasons, many efforts have been made to find new inhibitors with reduced side effects and better applicability.

Recently discovered α-glucosidase inhibitors from natural products are shown in Table I-1.

Table I-1. The lists of α-glucosidase inhibitors which is obtained from nature recently

Plant	Part	Extract/active constituent	IC_{50}	Kinetic study	Comparison	Reference
Myristica cinnamomea King	bark	giganteone D	5.05 μM	Mixed type	4 times higher than acarbose	(Sivasothy, Loo, Leong, Litaudon, & Awang, 2016)
Pluchea indica (L.) Less.	leaf	3,5-Di-*O*-caffeoylquinic acid	1166 μM	-	-	(Arsiningtyas, Gunawan-Puteri, Kato, & Kawabata, 2014)
		4,5-Di-*O*-caffeoylquinic acid methyl ester	208 μM	-	-	
		3,4,5-Tri-*O*-caffeoylquinic acid methyl ester	2 μM	-	-	
		3,4,5-Tri-*O*-caffeoylquinic acid	13 μM	-	-	
		1,3,4,5-Tetra-*O*-caffeoylquinic acid	11 μM	-	-	
Quercus gilva Blume	leaf	compound 1	168.5 μM	Uncompetitive	12 times lower than quercetin	(Indrianingsih, Tachibana, Dewi, & Itoh, 2015)
		compound 2	920.6 μM	Uncompetitive	66 times lower than quercetin	
		compound 3	28.36 μM	Non-competitive	2 times lower than quercetin	
grape	pomace	6-*O*-(p-coumaroyl)-D-	68% (0.5	-	1.36 times	(Sun, Kadouh,

		glucopyranoside	mg/mL)		higher than acarbose	Zhu, & Zhou, 2016)
		methyl 6-*O*-(p-coumaroyl)-β-D -galactopyranoside	75% (0.5 mg/mL)	-	1.5 times higher than acarbose	
Ligustrum robustum (Rxob.) Blume		extract	37.68 μg/mL	-	1.12 times higher than acarbose	(Yu, Gao, Zhang, He, He, Jia, et al., 2015)
Ipomoea batatas	leaf	*trans*-*N*-(p-Coumaroyl)tyramine	4.47 μM	-	37 times higher than acarbose	(L. Zhang, Tu, Yuan, Wang, Xie, & Fu, 2016)
		trans-*N*-Feruloyltyramine	9.04 μM	-	19 times higher than acarbose	
		cis-*N*-Feruloyltyramine	14.35 μM	-	12 timeshigher than acarbose	
		3,4,5-TriCQA	4.61 μM	-	37 times higher than acarbose	
		3,4-DiCQA	163.49 μM	-	1.03 times higher than acarbose	
		3,5-DiCQA	181.86 μM	-	1.07 times lower than	

					acarbose	
		4,5-DiCQA	108.26 μM	-	1.6 times higher than acarbose	
		4,5-Feruloylcourmaoylquinic acid	215.93 μM	-	1.28 times lower than acarbose	
		Caffeic acid	2250.32 μM	-	13.32 times lower than acarbose	
		Caffeic acid ethyl ester	355.34 μM	-	2.1 times lower than acarbose	
		7-Hydroxy-5-methoxycoumarin	64.14 μM	-	2.63 times higher than acarbose	
		Quercetin-3-*O*-glucoside	22.38 μM	-	7.55 times higher than acarbose	
		Rhametin	432.04 μM	-	2.55 times lower than acarbose	
Poncirus trifoliata (L.) Raf.	Fruit	Extract	81.27 μg/mL	-	2.29 times lower than acarbose	(Tundis, Bonesi, Sicari, Pellicanò,
	Seed	Extract	170.54	-	4.8 times	Tenuta,

			µg/mL		lower than acarbose	Leporini, et al., 2016)
	peel	essential oil	300.17 µg/mL	-	8.45 times lower than acarbose	
Rhododendron arboreum	bark	3-β-acetoxyurs-11-en-13 β, 28-olide	14.7±1.3 µM	-	37 times higher than acarbose	(Raza, Ilyas, Sajid, Nisar, Khokhar, & Iqbal, 2015)
		3-*O*-acetylbetulinic acid	12.3±2.6 µM	-	44 times higher than acarbose	
		Betulin	26.9±1.2 µM	-	20 times higher than acarbose	
		Betulinic acid	16.5±0.6 µM	-	33 times higher than acarbose	
		Ursolic acid	12.4±2.1 µM	-	43 times higher than acarbose	
		Lupeol	27.4±3.6 µM	-	19.89 times higher than acarbose	
		3-*O*-acetylursolic acid	3.3±0.1 µM	-	165 times higher than acarbose	

I-2-1-1. α-Glucosidase inhibitors present in jujube leaf

I-2-1-1-1. Rutin

Rutin is a class of naturally occurring flavonoids that have antioxidative, anti-inflammatory, and anti-cancer effects and also reduce the fragility of blood vessels (Ihme, Kiesewetter, Jung, Hoffmann, Birk, Müller, et al., 1996). Rutin is also called rutoside, quercetin-3-o-rutinoside, and sophorin. The structure of rutin contains aglycone and glycosyl groups. The glycosyl group of rutin is called rutinose and the aglycone group is called quercetin (Chua, 2013). Plants contain high levels of rutin, especially buckwheat (Li, Zhou, Gao, Bian, & Shan, 2009), the flower buds of *Tussilago farfara* L. (Gao, Huang, Gao, Xu, Inagaki, & Kawabata, 2008), capers (Tlili, Khaldi, Triki, & Munné-Bosch, 2010), olive tree leaves (Savournin, Baghdikian, Elias, Dargouth-Kesraoui, Boukef, & Balansard, 2001), and jujube leaf (Elaloui, Laamouri, Ennajah, Cerny, Mathieu, Vilarem, et al., 2016). The α-glucosidase IC_{50} value of rutin from tartary buckwheat was 0.196 mmol/L, which is two-times less than that of acarbose (0.091 mmol/L) (Li, Zhou, Gao, Bian, & Shan, 2009). Rutin showed moderate inhibition of mammalian α-glucosidase whereas quercetin showed weak inhibition (Gao, Huang, Gao, Xu, Inagaki, & Kawabata, 2008), so it is expected that the rutinoside in rutin could affect inhibition activity. The

inhibition type of rutin to α-glucosidase was mixed type of non-competitive and anti-competitive inhibition. These kinds of inhibition could be an advantage to use the pharmaceutical and food industries. The *Ki* value of rutin to α-glucosidase was about 28.3 mol/L. It was higher value than that of quercetin and isoquercitrin (Rutin ≈ Isoquercitrin > Quercetin). From these results, α-glucosidase inhibitory activity could be affected by the C3-OH on flavonoid (Li, Zhou, Gao, Bian, & Shan, 2009).

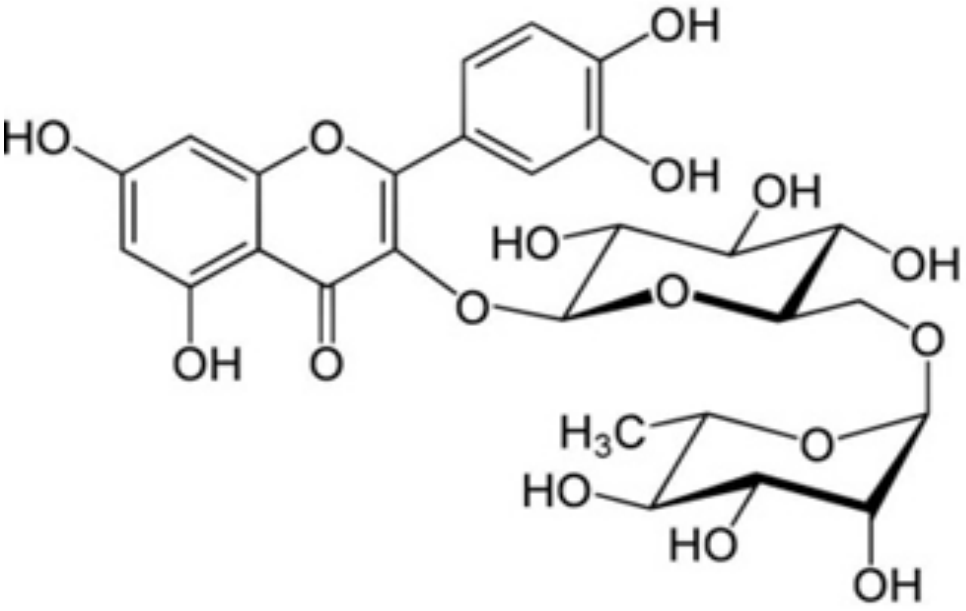
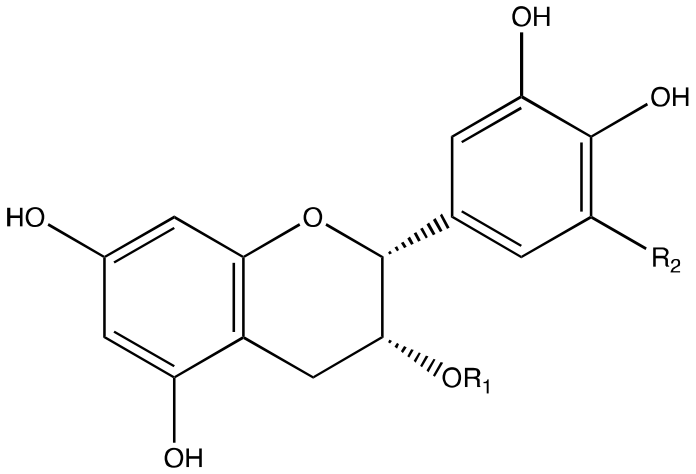


Figure I-1. The structure of rutin.

I-2-1-1-2. Catechin

Catechin is one of the most well-known flavonoids. Flavan-3-ols is a known part of flavonoids and is commonly known as catechins. In plants, there are four major catechins: (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin, and (-)-epigallocatechin gallate (Cabrera, Artacho, & Giménez, 2006) (Figure I-2). These are colorless, water-soluble compounds that contribute bitterness and astringency to green tea (Balentine, Wiseman, & Bouwens, 1997). Catechins have an anti-obesity effect, immunomodulatory activity, antitumorigenic effect, antiproliferative effect, anticancer activity, and reduction of cardiovascular disease (Crespy & Williamson, 2004; Nie & Xie, 2011; Rains, Agarwal, & Maki, 2011). Catechins are present in various types of tea, chocolate, apples, and grapes (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). There is approximately 1.99 mg/g of catechin in jujube leaf (Bai, Cui, Cheng, Cao, Wu, Guo, et al., 2017). The α-glucosidase inhibition rate of catechin is 2.91-times higher than that of acarbose (Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012). Inhibition type of epigallocatehchi gallate is mixed type, close to non-competitive inhibition. Catechin, epicatechin, and epigallocatechi gallate could inhibit both α-glucosidase and α-amylase.



	R_1	R_2
Epicatechin	H	H
Epicatechin gallate	Gallate	H
Epigallocatechin	H	OH
Epigallocatechin gallate	Gallate	OH

Figure I-2. Four major catechins in plants.

I-2-1-1-3. Isoquercitrin

Isoquercitrin (quercetin-3-O-β-d-glucopyranosidase) is one of the major glycosidic forms of rutin and possesses antioxidant, antiradical, anti-inflammatory, and antidiabetic activities (Valentová, Vrba, Bancířová, Ulrichová, & Křen, 2014). Isoquercitrin is ubiquitously distributed throughout the plant kingdom; for example, tea and wine, estern redbud, *Eucommia ulmoides* Oliv. leaves, Chinese hawberry fruits, and pistachio nuts (Dai, Huang, Zhou, Gong, Liu, & Shi, 2013; Douglass, Howard, & Wender, 1949; Fabani, Luna, Baroni, Monferran, Ighani, Tapia, et al., 2013; Jurikova, Sochor, Rop, Mlcek, Balla, Szekeres, et al., 2012). However, as it is difficult to obtain pure isoquercitrin, many researchers use naringinase, hesperinase, and rhamnosidase to enzymatically cleave rutin to obtain isoquercitrin (Hasumura, Yasuhara, Tamura, Imai, Mitsumori, & Hirose, 2004) (Figure I-3).

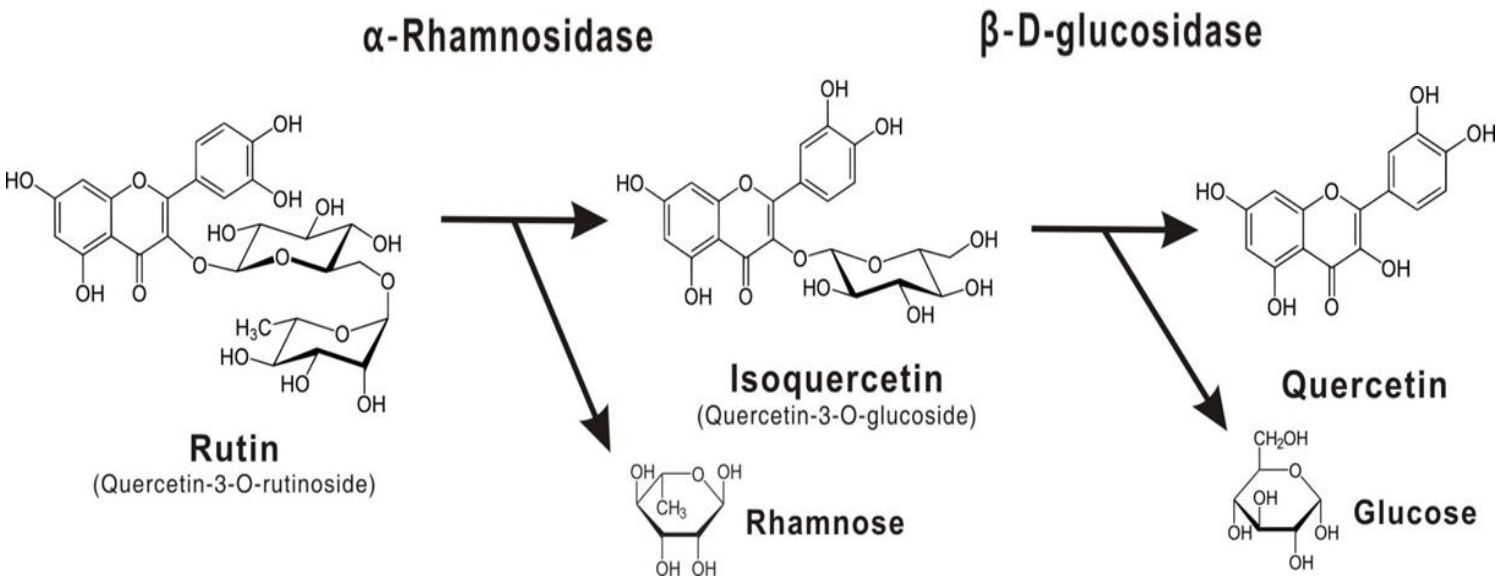


Figure I-3. Hydrolysis of rutin into isoquercitrin, and quercetin by hesperidinase/naringinase containing α-L-rhamnosidase activities and β-D-glucosidase activities.

I-2-2. β-Glucosidase inhibitors

Recently, the synergistic effects between β-glucosidase and chemotherapy for treating liver and breast cancer have been studied (Y. Zhang, Zhu, Miao, Hu, & Wang, 2016; X. Zhou, Huang, Yang, Jiang, Wei, Li, et al., 2017). Therefore, these inhibitors are potential therapeutic agents for Gaucher disease (Parmeggiani, Catarzi, Matassini, D'Adamio, Morrone, Goti, et al., 2015). However, studies of the synergistic or therapeutic effect of β-glucosidase inhibitors have only just started. Such studies have mainly used conduritol β-epoxide, which is a chemically synthesized β-glucosidase inhibitor. Chemically synthesized materials have limited use for long-term treatment because of side effects and high cost. For these reasons, significant efforts have been made to find new inhibitors from natural products. β-glucosidase inhibitors recently obtained from natural products are shown in Table I-2. The efficacy of β-glucosidase inhibitors has yet to be confirmed, so there have not been discovered or studied. In addition, some jujube leaf studies did not report a substance exhibiting β-glucosidase inhibitory activity (Elaloui, et al., 2016; S. Guo, Duan, Tang, Qian, Zhao, Qian, et al., 2011).

Table I-2. The lists of β-glucosidase inhibitor which obtained from natural product

Plant	Part	Extract/bioactive compounds	IC_{50}	Comparison	References
Rhododendron arboreum	bark	3-β-acetoxyurs-11-en-13 β, 28-olide	13.80%	4.3 times lower than Castanospermine	(Raza, Ilyas, Sajid, Nisar, Khokhar, & Iqbal, 2015)
		3-*O*-acetylbetulinic acid	10.08%	5.9 times lower than Castanospermine	
		Betulin	6.91%	8.6 times lower than Castanospermine	
		Betulinic acid	5.38%	11.1 times lower than Castanospermine	
		Ursolic acid	7.20%	8.3 times lower than Castanospermine	
		Lupeol	4.48%	13.4 times lower than Castanospermine	
		3-*O*-acetylursolic acid	4.54%	13.2 times lower than Castanospermine	
		β-sitosterol-3-*O*-beta-D-glucoside	2.84%	21.1 times lower than Castanospermine	

I-3. Applications of Glucosidase Inhibitors

There are few applications about α-glucosidase inhibitor. Acarbose was the first α-glucosidase inhibitor to become available in the United States. Acarbose is an oligosaccharide complex from microbes. Its structure is similar to oligosaccharides that result from the digestion of starch (Coniff & Krol, 1997) (Figure I-4). The structure of acarbose allows competitive and reversible inhibition of α-glucosidase. Acarbose is the most widely used as a treatment for diabetes in the management of hyperglycemia in the last 20 years (Weng, Soegondo, Schnell, Sheu, Grzeszczak, Watada, et al., 2015). However, acarbose is a competitive inhibitor of α-amylase in the small intestine and also of α-glucosidase. α-Amylase is required for the hydrolysis of carbohydrates and starches to oligosaccharides. If inhibited, this enzyme would be unable to digest carbohydrates and starches in the small intestine. These undigested carbohydrates and starches would move to the large intestine and result in production of gas by microorganisms (Tharanathan, 2002). Eventually, this would cause gastrointestinal side effects such as flatulence, diarrhea, or abdominal cramps (Chiasson, Josse, Gomis, Hanefeld, Karasik, Laakso, et al., 2002). Glucosidase inhibitors that are marketed as being edible and medicinal products are comprised of acarbose, miglitol, and voglibose.

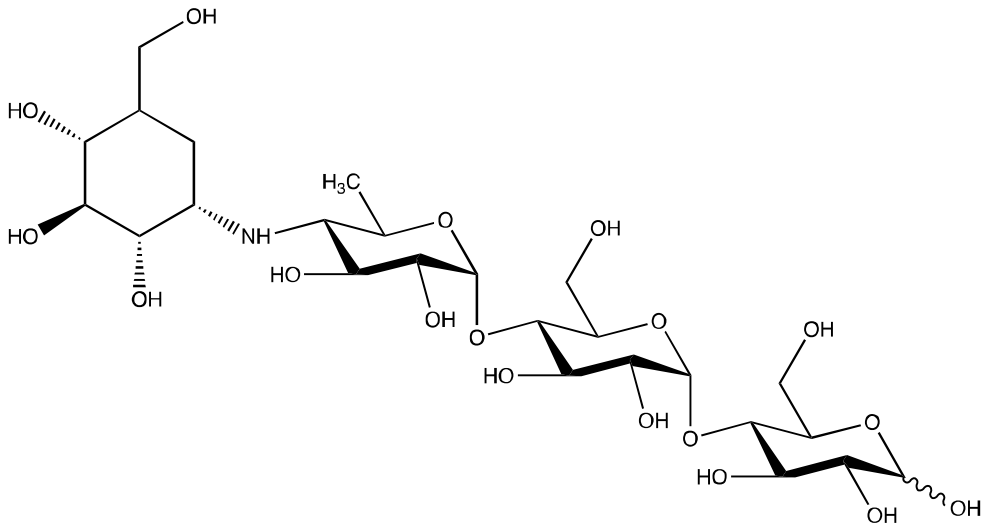


Figure I-4. The structure of acarbose.

I-4. References

Arsiningtyas, I. S., Gunawan-Puteri, M. D., Kato, E., & Kawabata, J. (2014). Identification of α-glucosidase inhibitors from the leaves of *Pluchea indica* (L.) Less., a traditional Indonesian herb: promotion of natural product use. *Nat. Prod. Res., 28*(17), 1350-1353.

Bai, L., Cui, X., Cheng, N., Cao, W., Wu, Y., Guo, S., Zhang, L., Ho, C. T., & Bai, N. (2017). Hepatoprotective standardized EtOH–water extract of the leaves of *Ziziphus jujuba*. *Food Funct., 8*(2), 816-822.

Balentine, D. A., Wiseman, S. A., & Bouwens, L. C. (1997). The chemistry of tea flavonoids. *Critical Reviews in Food Sci. Nutr., 37*(8), 693-704.

Butters, T. D., Dwek, R. A., & Platt, F. M. (2005). Imino sugar inhibitors for treating the lysosomal glycosphingolipidoses. *Glycobiology, 15*(10), 43R-52R.

Cabrera, C., Artacho, R., & Giménez, R. (2006). Beneficial effects of green tea—a review. *J. Am. College Nutr., 25*(2), 79-99.

Cheng, A. Y., & Josse, R. G. (2004). Intestinal absorption inhibitors for type 2 diabetes mellitus: prevention and treatment. *Drug Disc. Today: Therap. Strat., 1*(2), 201-206.

Chiasson, J. L., Josse, R. G., Gomis, R., Hanefeld, M., Karasik, A., Laakso, M., & Group, S.-N. T. R. (2002). Acarbose for prevention of type 2

diabetes mellitus: the STOP-NIDDM randomised trial. *The Lancet, 359*(9323), 2072-2077.

Chua, L. S. (2013). A review on plant-based rutin extraction methods and its pharmacological activities. *J. Ethnopharm., 150*(3), 805-817.

Coniff, R., & Krol, A. (1997). Acarbose: a review of US clinical experience. *Clin. Therap., 19*(1), 16-26.

Crespy, V., & Williamson, G. (2004). A review of the health effects of green tea catechins in *in vivo* animal models. *J. Nutr., 134*(12), 3431S-3440S.

Dai, X., Huang, Q., Zhou, B., Gong, Z., Liu, Z., & Shi, S. (2013). Preparative isolation and purification of seven main antioxidants from *Eucommia ulmoides* Oliv.(Du-zhong) leaves using HSCCC guided by DPPH-HPLC experiment. *Food Chem., 139*(1-4), 563-570.

de Faria, E. L., Shabudin, S. V., Claúdio, A. F. M., Válega, M., Domingues, F. M., Freire, C. S., Silvestre, A. J., & Freire, M. G. (2017). Aqueous solutions of surface-active ionic liquids: remarkable alternative solvents to improve the solubility of triterpenic acids and their extraction from biomass. *ACS Sustainable Chem. Eng., 5*(8), 7344-7351.

de Melo, E. B., da Silveira Gomes, A., & Carvalho, I. (2006). α-and β-Glucosidase inhibitors: chemical structure and biological activity. *Tetrahedron, 62*(44), 10277-10302.

Diamond, J. M., Karasov, W. H., Cary, C., Enders, D., & Yung, R. (1984). Effect of dietary carbohydrate on monosaccharide uptake by mouse small intestine *in vitro*. *J. Phys., 349*(1), 419-440.

Douglass, C. D., Howard, W. L., & Wender, S. H. (1949). The isolation of isoquercitrin from the seed pods of *Cercis canadensis*. *J. Am. Chem. Soc., 71*(8), 2658-2659.

Du, W., Shi, X., & Qiu, M. (2005). Progress in treatment of diabetes drugs. *Chin. Hosp. Pharm. J., 25*, 67-69.

Elaloui, M., Laamouri, A., Ennajah, A., Cerny, M., Mathieu, C., Vilarem, G., Chaar, H., & Hasnaoui, B. (2016). Phytoconstituents of leaf extracts of *Ziziphus jujuba* Mill. Plants harvested in Tunisia. *Indust. Crops Prod., 83*, 133-139.

Fabani, M. P., Luna, L., Baroni, M. V., Monferran, M. V., Ighani, M., Tapia, A., Wunderlin, D. A., & Feresin, G. E. (2013). Pistachio (*Pistacia vera* var Kerman) from Argentinean cultivars. A natural product with potential to improve human health. *J. Funct. Food., 5*(3), 1347-1356.

Gao, H., Huang, Y. N., Gao, B., Xu, P. Y., Inagaki, C., & Kawabata, J. (2008). α-Glucosidase inhibitory effect by the flower buds of *Tussilago farfara* L. *Food Chem., 106*(3), 1195-1201.

Gullón, B., Lú-Chau, T. A., Moreira, M. T., Lema, J. M., & Eibes, G. (2017). Rutin: a review on extraction, identification and purification methods, biological activities and approaches to enhance its bioavailability. *Trends Food Sci. Technol., 67*, 220-235.

Guo, L.-P., Jiang, T. F., Lv, Z. H., & Wang, Y. H. (2010). Screening α-glucosidase inhibitors from traditional Chinese drugs by capillary electrophoresis with electrophoretically mediated microanalysis. *J. Pharm. Biomed. Anal., 53*(5), 1250-1253.

Guo, S., Duan, J. A., Tang, Y., Qian, Y., Zhao, J., Qian, D., Su, S., & Shang, E. (2011). Simultaneous qualitative and quantitative analysis of triterpenic acids, saponins and flavonoids in the leaves of two *Ziziphus* species by HPLC–PDA–MS/ELSD. *J. Pharm. Biomed. Anal., 56*(2), 264-270.

Hasumura, M., Yasuhara, K., Tamura, T., Imai, T., Mitsumori, K., & Hirose, M. (2004). Evaluation of the toxicity of enzymatically decomposed rutin with 13-weeks dietary administration to Wistar rats. *Food Chem. Toxic., 42*(3), 439-444.

Ihme, N., Kiesewetter, H., Jung, F. a., Hoffmann, K., Birk, A., Müller, A., & Grützner, K. (1996). Leg oedema protection from a buckwheat herb tea in patients with chronic venous insufficiency: a single-centre, randomised, double-blind, placebo-controlled clinical trial. *European J. Clin. Pharm., 50*(6), 443-447.

Indrianingsih, A. W., Tachibana, S., Dewi, R. T., & Itoh, K. (2015). Antioxidant and α-glucosidase inhibitor activities of natural compounds isolated from *Quercus gilva* Blume leaves. *Asian Pacific J. Trop. Biomed., 5*(9), 748-755.

Joshi, S. R., Standl, E., Tong, N., Shah, P., Kalra, S., & Rathod, R. (2015). Therapeutic potential of α-glucosidase inhibitors in type 2 diabetes mellitus: an evidence-based review. *Expert Opi. Pharm., 16*(13), 1959-1981.

Jurikova, T., Sochor, J., Rop, O., Mlcek, J., Balla, S., Szekeres, L., Adam, V., & Kizek, R. (2012). Polyphenolic profile and biological activity of Chinese hawthorn (*Crataegus pinnatifida* BUNGE) fruits. *Molecules, 17*(12), 14490-14509.

Kordik, C. P., & Reitz, A. B. (1999). Pharmacological treatment of obesity: therapeutic strategies. *J. Med. Chem., 42*(2), 181-201.

Li, Y. Q., Zhou, F. C., Gao, F., Bian, J. S., & Shan, F. (2009). Comparative evaluation of quercetin, isoquercetin and rutin as inhibitors of α-glucosidase. *J. Agri. Food Chem., 57*(24), 11463-11468.

Manach, C., Williamson, G., Morand, C., Scalbert, A., & Rémésy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr., 81*(1), 230S-242S.

Nie, S. P., & Xie, M. Y. (2011). A review on the isolation and structure of tea polysaccharides and their bioactivities. *Food Hydrocol., 25*(2), 144-149.

Parmeggiani, C., Catarzi, S., Matassini, C., D'Adamio, G., Morrone, A., Goti, A., Paoli, P., & Cardona, F. (2015). Human acid β-glucosidase inhibition by carbohydrate derived iminosugars: towards new pharmacological chaperones for gaucher disease. *ChemBioChem, 16*(14), 2054-2064.

Pujara, N., Jambhrunkar, S., Wong, K. Y., McGuckin, M., & Popat, A. (2017). Enhanced colloidal stability, solubility and rapid dissolution of resveratrol by nanocomplexation with soy protein isolate. *J. Col. Interface Sci., 488*, 303-308.

Qi, Y., Zhao, Y., Lu, H., Wang, X., & Jin, N. (2016). Comparative analysis of the bonding modes between two antidiabetic drugs with β-

glucosidases from different species. *Indian J. Pharm. Sci., 78*(4), 512-524.

Rains, T. M., Agarwal, S., & Maki, K. C. (2011). Antiobesity effects of green tea catechins: a mechanistic review. *J. Nutr. Biochem., 22*(1), 1-7.

Raza, R., Ilyas, Z., Sajid, A., Nisar, M., Khokhar, M. Y., & Iqbal, J. (2015). Identification of highly potent and selective α-glucosidase inhibitors with antiglycation potential, isolated from rhododendron arboreum. *Record. Nat. Prod., 9*(2), 262.

Savournin, C., Baghdikian, B., Elias, R., Dargouth-Kesraoui, F., Boukef, K., & Balansard, G. (2001). Rapid high-performance liquid chromatography analysis for the quantitative determination of oleuropein in *Olea europaea* leaves. *J. Agri. Food Chem., 49*(2), 618-621.

Sivasothy, Y., Loo, K. Y., Leong, K. H., Litaudon, M., & Awang, K. (2016). A potent alpha-glucosidase inhibitor from *Myristica cinnamomea* King. *Phytochemistry, 122*, 265-269.

Sun, S., Kadouh, H. C., Zhu, W., & Zhou, K. (2016). Bioactivity-guided isolation and purification of α-glucosidase inhibitor, 6-OD-glycosides, from tinta Cão grape pomace. *J. Funct. Food., 23*, 573-579.

Tharanathan, R. N. (2002). Food-derived carbohydrates—structural complexity and functional diversity. *Crit. Rev. Biotech., 22*(1), 65-84.

Tlili, N., Khaldi, A., Triki, S., & Munné-Bosch, S. (2010). Phenolic compounds and vitamin antioxidants of caper (*Capparis spinosa*). *Plant Food. Human Nutr., 65*(3), 260-265.

Tundis, R., Bonesi, M., Sicari, V., Pellicanò, T., Tenuta, M., Leporini, M., Menichini, F., & Loizzo, M. (2016). *Poncirus trifoliata* (L.) Raf.: Chemical composition, antioxidant properties and hypoglycaemic activity via the inhibition of α-amylase and α-glucosidase enzymes. *J. Funct. Food., 25*, 477-485.

Valentová, K., Vrba, J., Bancířová, M., Ulrichová, J., & Křen, V. (2014). Isoquercitrin: pharmacology, toxicology, and metabolism. *Food Chem. Toxic., 68*, 267-282.

Wang, Q., Hu, C., Zoghbi, A., Huang, J., & Xia, Q. (2017). Oil-in-oil-in-water pre-double emulsions stabilized by nonionic surfactants and silica particles: A new approach for topical application of rutin. *Col. Surf. A: Physicochem. Eng. Aspects, 522*, 399-407.

Weng, J., Soegondo, S., Schnell, O., Sheu, W. H. H., Grzeszczak, W., Watada, H., Yamamoto, N., & Kalra, S. (2015). Efficacy of acarbose in different geographical regions of the world: analysis of a real-life database. *Diabetes/metabolism Res. Rev., 31*(2), 155-167.

Yilmazer-Musa, M., Griffith, A. M., Michels, A. J., Schneider, E., & Frei, B. (2012). Grape seed and tea extracts and catechin 3-gallates are potent inhibitors of α-amylase and α-glucosidase activity. *J. Agric. Food Chem., 60*(36), 8924-8929.

Yu, Z. L., Gao, H. X., Zhang, Z., He, Z., He, Q., Jia, L. R., & Zeng, W. C. (2015). Inhibitory effects of Ligustrum robustum (Rxob.) Blume extract on α-amylase and α-glucosidase. *J. Funct. Food., 19*, 204-213.

Zhang, L., Tu, Z. C., Yuan, T., Wang, H., Xie, X., & Fu, Z. F. (2016). Antioxidants and α-glucosidase inhibitors from *Ipomoea batatas* leaves identified by bioassay-guided approach and structure-activity relationships. *Food Chem., 208*, 61-67.

Zhang, Y., Zhu, K., Miao, X., Hu, X., & Wang, T. (2016). Identification of β-glucosidase 1 as a biomarker and its high expression in hepatocellular carcinoma is associated with resistance to chemotherapy drugs. *Biomarkers, 21*(3), 249-256.

Zhou, X., Huang, Z., Yang, H., Jiang, Y., Wei, W., Li, Q., Mo, Q., & Liu, J. (2017). β-Glucosidase inhibition sensitizes breast cancer to chemotherapy. *Biomed. Pharm., 91*, 504-509.

Zhou, Z., Li, W., Sun, W. J., Lu, T., Tong, H. H., Sun, C. C., & Zheng, Y. (2016). Resveratrol cocrystals with enhanced solubility and tabletability. *Int. J. Pharmac., 509*(1-2), 391-399.

Chapter II. A novel α-glucosidase inhibitor from jujube leaf extract

II-1. Introduction

Ziziphus jujuba Mill, which is known as the jujube, belongs to the Rhamnacea family. Traditionally, jujube has been used to treat biliousness, diarrhea, asthma, and obesity (Gao, Wu, & Wang, 2013). In addition, jujube leaves traditionally have been used to make teas and medicines to treat fever, cardiovascular disease, and insomnia (Elaloui, Laamouri, Ennajah, Cerny, Mathieu, Vilarem, et al., 2016). Although jujube leaves contain more flavonoids and polyphenols than jujube fruits, jujube fruit and seeds have attracted more attention and there are only a few reports in the literature regarding the efficacy of jujube leaves (S. Guo, Duan, Tang, Qian, Zhao, Qian, et al., 2011; Y. J. Kim & Son, 2011). For these reasons, jujube leaves are currently regarded as by-products and are discarded.

Flavonoids and polyphenols are known to inhibit α-glucosidase activity. α-Glucosidase inhibitors are promising agents for treating type 2 noninsulin-dependent diabetes mellitus (Asano, Yamashita, Yasuda, Ikeda, Kizu, Kameda, et al., 2001). α-Glucosidases are essential for the digestion of carbohydrates in the small intestine, because only monosaccharides can be taken up easily. α-Glucosidases degrade carbohydrates in the small intestine, thus facilitating their absorption (Diamond, Karasov, Cary, Enders, & Yung,

1984). α-Glucosidase inhibitors can delay the production of glucose during digestion and can therefore be used to regulate the activity of α-glucosidase and control postprandial blood glucose levels (Holman, Cull, & Turner, 1999; K. Kim, Nam, Kurihara, & Kim, 2008). Therefore, α-glucosidase inhibitors are promising agents for the treatment of metabolic disorders, such as type 2 noninsulin-dependent diabetes mellitus, obesity, and hyperglycemia (Asano, et al., 2001). Most of these metabolic disorders require long-term treatment. Chemically synthesized drugs often have gastrointestinal side effects, such as abdominal pain, meteorism, and diarrhea (L. P. Guo, Jiang, Lv, & Wang, 2010). Therefore, significant research efforts have been made toward identifying novel α-glucosidase inhibitors derived from natural sources. A number of studies sought to identify natural α-glucosidase inhibitors in foods derived from plants, such as tea (Kumar, Narwal, Kumar, & Prakash, 2011; Sun, Kadouh, Zhu, & Zhou, 2016).

However, only a few α-glucosidase inhibitors are commercially available, namely acarbose, voglibose, and miglitol. The synthesis of these materials involves a tedious multistep process. Moreover, these compounds have gastrointestinal side effects such as abdominal pain, meteorism, and diarrhea (L. P. Guo, Jiang, Lv, & Wang, 2010). Therefore, many research groups are looking for alternative α-

glucosidase inhibitors with reduced side effects. In particular, a number of studies sought to identify natural α-glucosidase inhibitors in foods derived from plants, such as herbal teas and other medicinal plants (Kumar, Narwal, Kumar, & Prakash, 2011; Sun, Kadouh, Zhu, & Zhou, 2016).

Although jujube leaves contain more flavonoids than the fruit, jujube fruit and seeds have received more attention and there are only a few reports regarding the efficacy for healthiness of jujube leaves in the literature (S. Guo, et al., 2011). To my knowledge, only a few studies have been performed regarding the identification and characterization of bioactive compounds in jujube leaves, but these studies could not fully explain their efficacy for treating various diseases (Elaloui, et al., 2016; R. Zhang, Chen, Shi, Li, Peng, Zheng, et al., 2014a, 2014b). Therefore, the rate of α-glucosidase inhibition by jujube leaves was examined. In these experiments, a stronger than expected α-glucosidase inhibition activity of jujube leaves was observed. To confirm this observation, the compound in jujube leaves that mediates α-glucosidase inhibition was searched.

The preliminary experiments demonstrated that jujube leaf extracts had an α-glucosidase inhibitory activity which was much stronger than

what we initially expected; thus, we considered the possibility that jujube leaf extracts contain a novel α-glucosidase inhibitor. In this work, the structure of this novel jujube leaf α-glucosidase inhibitor and its inhibitory characteristics were investigated.

II-2. Materials and Methods

II-2-1. Chemicals

α-Glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), α-amylase from porcine pancreas (EC 3.2.1.1), 4-nitrophenyl α-D-glucopyranoside (*p*NPG), 2-chloro-4-nitrophenyl α-d-maltotrioside (CNP-G3), pepsin from porcine gastric mucosa (EC 3.4.23.1), lipase from porcine pancreas (EC 3.1.1.3), and pancreatin from porcine pancreas were purchased from Sigma-Aldrich (St. Louis, MO, USA).

II-2-2. Sample preparation

Sun-dried jujube leaf, grown in Boeun, South Korea, was stored at -80 °C. Frozen jujube leaves were pulverized with a household blender (HMF-1000, Hanil Electric, Seoul, Korea) and filtered through a fine mesh (150-300 μm). The powder was stored at -18 °C. For extraction, jujube leaf powder (0.5 g) was mixed with distilled water (20 mL) and stirred at 95 °C for 15 min. The extract was filtered through filter paper (No. 4, 110 mm ø, Whatman International Ltd, Maidstone, UK) and dried using a rotary vacuum evaporator (EYELA, N-N series, Tokyo, Japan). Jujube leaf extract powder was re-dispersed into buffer at a concentration of 10 mg/mL

II-2-3. α-Glucosidase inhibition assay

Inhibition assays contained 50 μL of α-glucosidase from *Saccharomyces cerevisiae* (1 U/mL, pH 6.8, in 0.1 M phosphate buffer) and 100 μL of various concentrations of jujube leaf extract (0–0.07 mg/mL, pH 6.8, in 0.1 M phosphate buffer). Reactions were incubated in 96-well plates at 37 °C for 15 min. Subsequently, 50 μL of *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) (0.5 mmol/mL, pH 6.8, in 0.1 M phosphate buffer) was added to each well and the absorbance at 400 nm was measured with a 96-well plate spectrophotometer (Multiskan GO, Thermo Scientific, MA, USA) at 37 °C for 60 min. The inhibition rate was calculated as follows:

$$Activity\ (\%) = \left\{\left(\frac{Inhibitor\ (OD_{400}) - Background\ (OD_{400})}{Control\ (OD_{400}) - Background\ (OD_{400})}\right)\right\} \times 100$$

where *Control* (OD_{400}) is the absorbance of the control (without sample), *Inhibitor* (OD_{400}) is the absorbance of the sample, and *Background* (OD_{400}) is the absorbance of sample (without *p*NPG).

II-2-4. α-Amylase inhibition assay

Assays contained 100 μL of α-amylase from porcine pancreas (5 U/mL, pH 6.8, in 0.1 M phosphate buffer) and 50 μL of different concentrations of jujube leaf extract (0-0.1 mg/mL, in 0.1 M phosphate buffer). Reactions incubated in 96-well plates at 37 °C for 15 min. Then, 100 μL of 2-chloro-4-nitrophenyl-α-maltotrioside (CNP-G3) (1 mM, pH 6.8, in 0.1 M phosphate buffer) was added to each well and the absorbance at 405 nm was measured at 37 °C for 60 min with a 96-well plate spectrophotometer. The inhibitory activity was calculated as follows:

$$Activity\ (\%) = \left\{\left(\frac{Inhibitor\ (OD_{400}) - Background\ (OD_{400})}{Control\ (OD_{400}) - Background\ (OD_{400})}\right)\right\} \times 100$$

where *Control* (OD_{400}) is the absorbance of the control (without sample), *Inhibitor* (OD_{400}) is the absorbance of the sample, and *Background* (OD_{400}) is the absorbance of the sample (without CNP-G3).

II-2-5. Isolation of α-glucosidase inhibitor

Samples were purified by size exclusion chromatography using a PD-10 column packed with Sephadex G-25 medium (GE Healthcare, Piscataway, NJ, USA) in DW (distilled water). Aliquots of 10 mL of each fraction were dried at 65°C. Fractions 8–14, which showed the strongest α-glucosidase inhibitory activity, were redispersed in DW. The samples were further purified by preparative high-performance liquid chromatography (HPLC) (Ultimate 3000; Thermo Scientific Dionex, Sunnyvale, CA, USA) with a reversed phase C_{18} column (YMC-Triart; YMC, Co., Ltd., Kyoto, Japan). The mobile phase was maintained at 0.8 mL/min with a linear gradient composed of acetonitrile (solvent A) and 0.5% formic acid (solvent B) under the following conditions: solvent B was decreased from 98% to 95% over 10 min, then decreased from 95% to 20% over 10 min, then held at 20% for 5 min, after which solvent B was increased to 98% over 1 min and then held at 95% for 4 min (30-min run time).

II-2-6. Structure Analysis

The α-glucosidase inhibitor present in jujube leaf was characterized by HPLC diode array detection (DAD) mass spectrometry (MS) analysis. A 5 μL aliquot of each sample was injected onto an analytical reverse phase column (YMC-Triart C18). The mobile phase was composed of acetonitrile (A) and 2.5% acetic acid (B) and the gradient conditions were as follows: solvent B was decreased from 95% to 75% for 15 min and then was increased to 95% for 5 min (20 min run time), with a flow rate of 1 mL/min.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to conduct metabolite profiling of the jujube leaf extract, using an Ultimate 3000 RSLC HPLC system (Thermo Fisher Scientific) connected to a Triple TOF 5600 (AB SCIEX; Concord, ON, Canada). A Capcell Pak MGII column (2*100 mm, 5 μm; Shiseido, Tokyo, Japan) was used and the experimental conditions were as follows: the mobile phase was composed of 0.1% formic acid in DW (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Solvent B was increased from 5% to 50% over 14 min and then was increased to 100% over 3 min and maintained for 2 min; then, solvent B was decreased to 5% over 0.5 min (19.5 min run time). The data were measured in positive mode, with an ionization energy of 5,500 V. The source temperature was 500 °C, and the pressures of curtain gas, ion source gas 1,

and ion source gas 2 were 50, 50, and 25 psi, respectively. The data were analyzed using AnalystOR TF 1.7, PeakViewOR 2.2, and MarkerViewOR 1.2.1.1.

II-2-7. Enzyme kinetic study

Enzyme kinetic studies were conducted to examine the type of inhibition. A 100 μL aliquot of α-glucosidase (0.1 U/mL, pH 6.8) was incubated with various concentrations of jujube leaf extract (0, 0.075, and 1 mg/mL) for 15 min at 37 °C. Then, various concentrations of the substrate *p*NPG (0, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 mM) were added to the reactions. The absorbance of these samples at 405 nm was measured using a 96-well plate spectrophotometer at 37 °C for 60 min.

II-2-8. *In vitro* digestion

Simulated *in vitro* digestion experiments were conducted in accordance with the method pioneered by Minekus et al. (2014).

I. *Mouth* (pH 7.0; 5 min): A 5-mL volume of jujube leaf extract was blended with 4 mL of simulated salivary fluid and then 25 μL 0.3 M $CaCl_2$ and 975 μL distilled water were added.

II. *Stomach* (pH 3.0; 2 h): An 8-mL volume of simulated gastric fluid and 5 μL of 0.3 M $CaCl_2$ were added, followed by 1 mL of porcine pepsin stock solution and the volume was adjusted to 10 mL with distilled water.

III. *Small intestine* (pH 7.0; 2 h): An 11-mL volume of simulated intestinal fluid, 40 μL of 0.3 M $CaCl_2$, and 2.5 mL of bile juice (160 mM) were added. Pancreatin solution (100 U/mL of trypsin in the final mixture) and pancreatin lipase (2,000 U/mL in the final mixture) were added to the mixture.

The composition of the simulated saliva, gastric, and intestinal fluids are shown in Table II-1. All samples were mixed (60 rpm) in a shaking water bath (BS-31, JEIO Tech., Seoul, Korea) during the *in vitro* digestion tests and were maintained at 37 °C to simulate conditions of the gastrointestinal

tract. After each digestion step (I-III above), the samples were boiled to denature the digestive enzymes. After the final digestion step, each sample was freeze-dried.

Table II-1. Composition of stock solution of simulated digestion fluids

Constituent	Stock concentration (mol/L)	SSF		SGF		SIF	
		pH 7		pH 3		pH 7	
		Volume of stock (mL)	Final concentration in SSF (mmol/L)	Volume of stock (mL)	Final concentration in SGF (mmol/L)	Volume of stock (mL)	Final concentration in SIF (mmol/L)
KCl	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	1	6.8	13.6	12.5	25	42.5	85
NaCl	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	0.15	0.5	0.15	0.4	0.12	1.1	0.33
$(NH_4)_2CO_3$	0.5	0.06	0.06	0.5	0.5	-	-

II-2-9. Statistical analysis

All results were analyzed using Tukey's significant difference test with IBM SPSS Statistics version 21.0 (IBM Co., Armonk, NY, USA). At least three independent replicates were performed in each experiment.

II-3. Results and Discussion

II-3-1. α-Glucosidase inhibition

α-Glucosidases are essential for digestion of carbohydrates in the small intestine, because only monosaccharides can be taken up easily. These enzymes degrade carbohydrates into monosaccharides in the intestine, thus facilitating their absorption (Diamond, Karasov, Cary, Enders, & Yung, 1984). α-Glucosidase inhibitors can delay the production of glucose during digestion and can therefore be used to regulate the activity of α-glucosidases to control postprandial blood glucose levels (Holman, Cull, & Turner, 1999; K. Kim, Nam, Kurihara, & Kim, 2008). In several studies, the therapeutic effectiveness of various agents with inhibitory activity against α-glucosidase against diabetes and obesity was evaluated (Figueiredo-González, Grosso, Valentão, & Andrade, 2016). To compare α-glucosidase inhibitory activity of jujube leaf extract with acarbose (a well-established anti-diabetes drug), the IC50 values of jujube leaf extract and acarbose against α-glucosidase and α-amylase were determined. The extract of jujube leaves was expected to inhibit the activity of α-glucosidase because it contains polyphenols such as catechin, quercetin-3-rutinoside, quercetin-3-O-β-d-glucoside,

protocatechuic acid, caffeic acid, and kaempferol-7-O-glucoside, which exhibit α-glucosidase inhibitory activity (Table II-2) (R. Zhang, et al., 2014a). I expected that jujube leaves should have lower α-glucosidase inhibitory activity than that of acarbose (Kwon, Apostolidis, & Shetty, 2008; Li, Zhou, Gao, Bian, & Shan, 2009; Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012; B. Zhang, Deng, Ramdath, Tang, Chen, Liu, et al., 2015), judging by the content of α-glucosidase inhibitors in jujube leaves in relation to their inhibition rate.

However, the α-glucosidase inhibitory activity of jujube leaf extract (IC50 value: 0.67 ± 0.25 mg/mL) was approximately double than that of acarbose (IC50 value: 1.22 ± 0.35 mg/mL) (Figure II-2). These results suggest that jujube leaves contain a previously unidentified substance that strongly inhibits α-glucosidase. Therefore, several experiments were conducted to identify the source of this strong α-glucosidase inhibition that exceeded expectations.

Table II-2. The compound, contents, and inhibition rate of α-glucosidase inhibitors present in jujube leaf

Compound	**Contents (mg/g)**	**Inhibition rate (vs. acarbose)**
Catechin	1.99	2.91 times high
Quercetin-3-Rutinoside	4.4	2.15 times low
Quercetin-3-*O*-β-D-glucoside	0.06	2.03 times low
Protocatechuic acid	0.01	1.6 times high
Caffeic acid	0.01	2.6 times high
Kaempferol-7-*O*-glucoside	0.03	4.03 times high

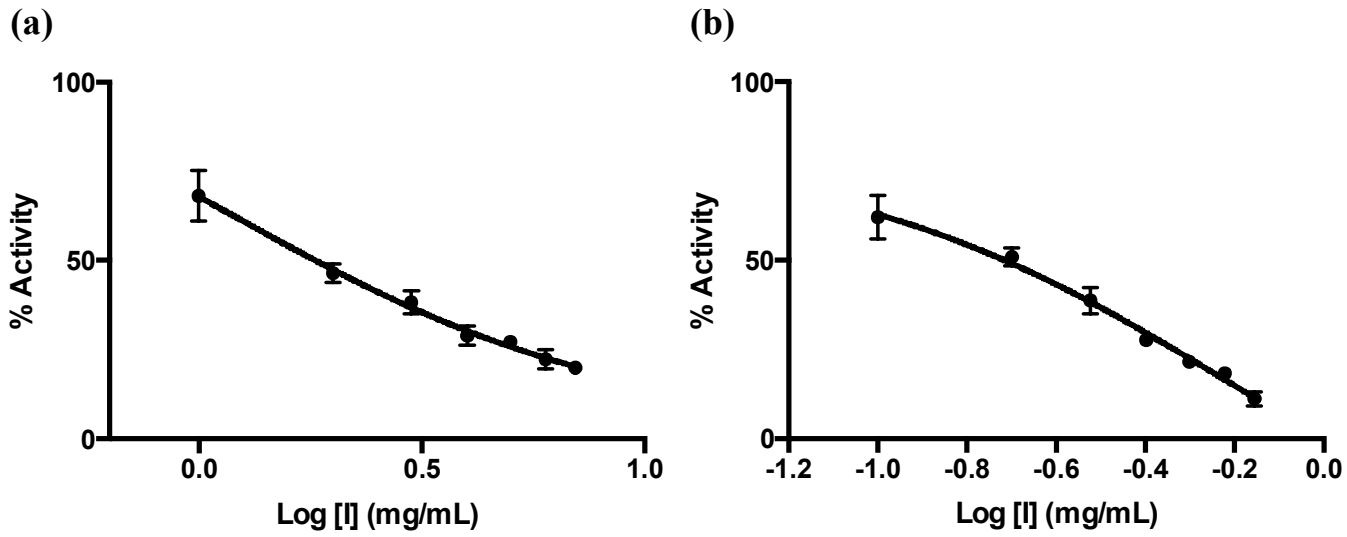


Figure II-1. α-Glucosidase inhibitory activity of acarbose (a) and jujube leaf extract (b).

II-3-2. Isolation and purification of novel α-glucosidase inhibitor

Twenty fractions of the crude water extract of jujube leaves were generated by size exclusion chromatography using Sephadex G-25. Among these 20 fractions, fractions 8-14 showed stronger inhibitory activity than the other fractions (Figure II-2). Fractions 8-14 at 0.5 mg/mL inhibited more than 85.68% of α-glucosidase activity. Prior to structural analysis, a purification experiment was conducted using preparative HPLC to increase the purity of this novel α-glucosidase inhibitor. As shown in Figure II-3, a single substance with ≥ 90% purity was obtained.

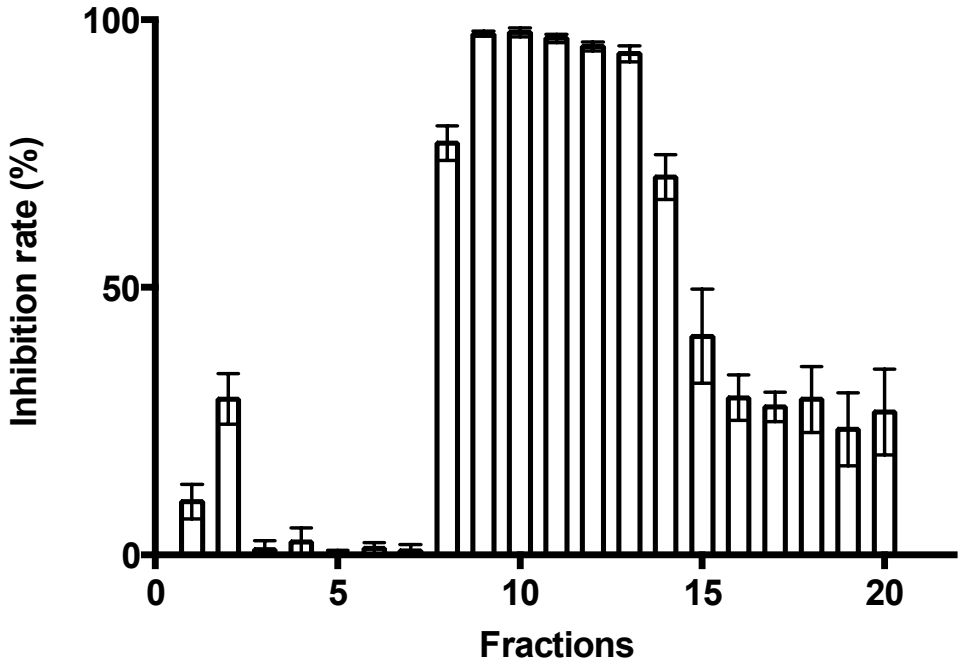
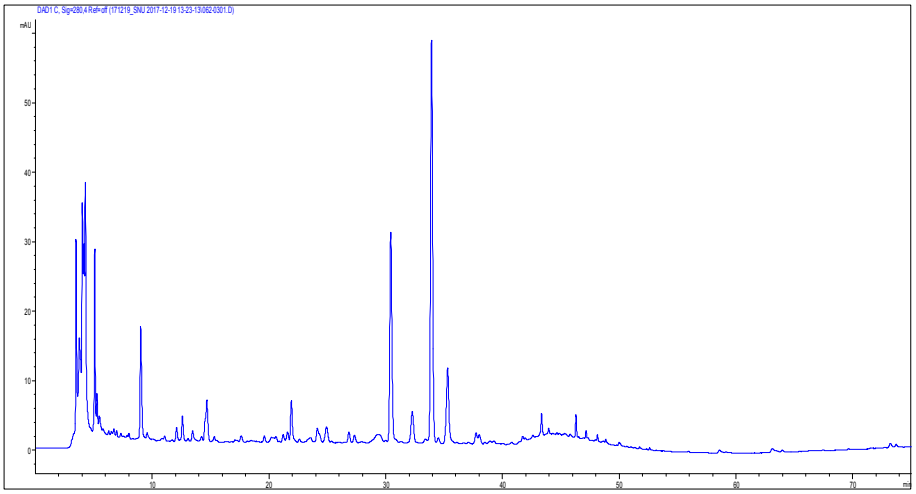


Figure II-2. Size-exclusion chromatography of jujube leaf extract using Sephadex G-25 column. Fractions were collected and assayed for the inhibition rate of α-glucosidase.

(a)



(b)

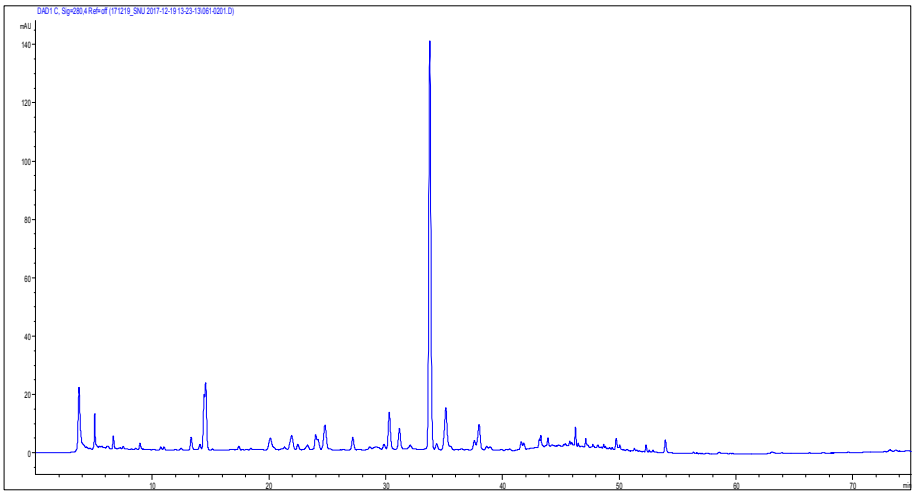


Figure II-3. HPLC chromatogram of crude jujube leaf extract (a) and sample separated and purified with semi-preparative LC (b).

II-3-3. Structure analysis of novel α-glucosidase inhibitor

LC-ESI/MS and LC/MS/MS were performed to identify the α-glucosidase inhibitor in the fractions. One major peak was identified on the chromatogram, which appeared at a retention time (t_R) of 7.859 min (Figure II-4 a). MS analysis of this major peak (t_R = 7.859 min) showed an $[M-H]^-$ ion at *m/z* 597.18 (Figure II-4 b), and the MS/MS fragments were 579, 507, and 417 (*m/z*) (Figure II-4 c). These results were consistent with previously published data and indicated that the compound was 3',5'-di-C-β-D-glucosyl phloretin, the structure of which is shown in Figure II-5. The α-glucosidase inhibitory activity of 3',5'-di-C-β-D-glucosyl phloretin has not yet been examined in the research papers. Therefore, experiments were performed to characterize its inhibitory activity.

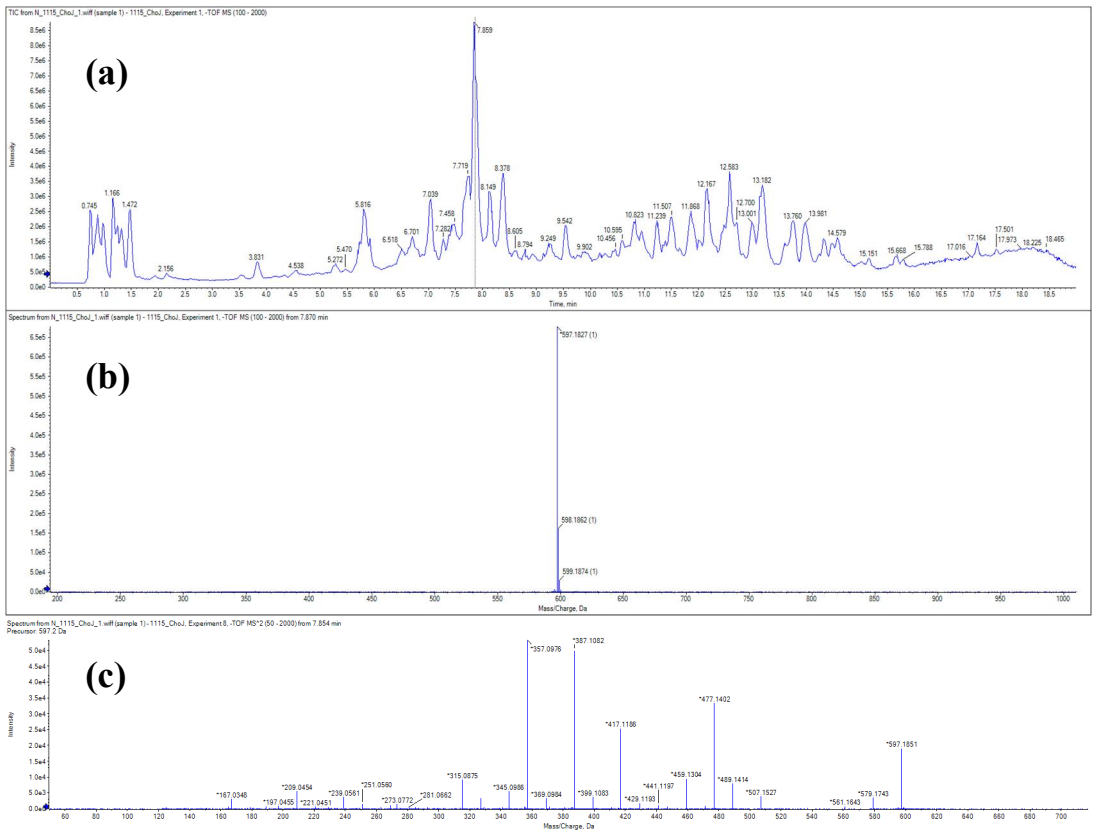


Figure II-4. The chromatogram of jujube leaf extract after size exclusion chromatography (a), LC-ESI/MS of the major peak (7.859 min) (b), and LC-MS/MS of this substance (c).

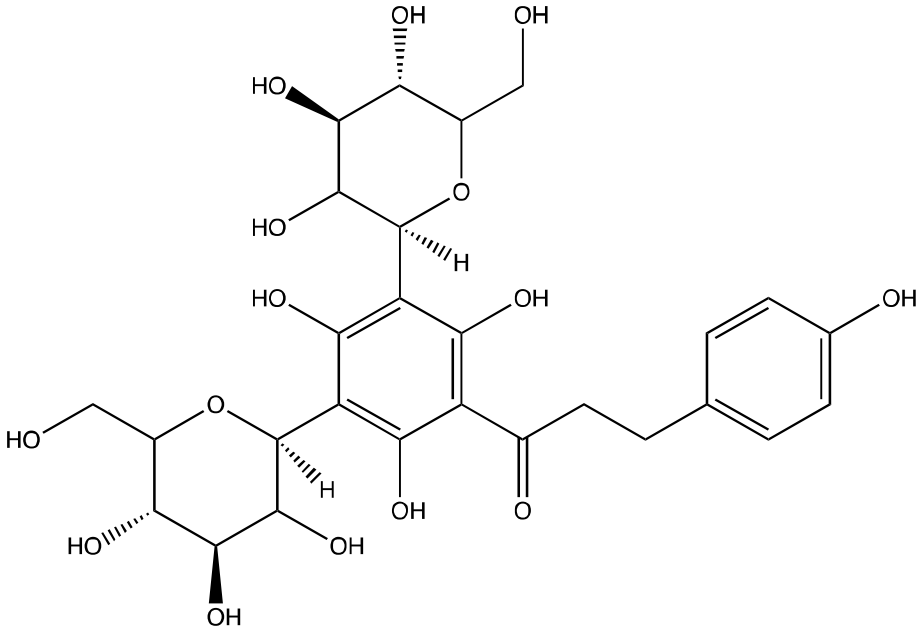


Figure II-5. Structure of a novel α-glucosidase inhibitor (3', 5'-di-C-β-D-glucosyl phloretin) from jujube leaf.

II-3-4. Characterization of novel α-glucosidase inhibitor

II-3-4-1. α-Glucosidase inhibitory activity

To characterize this novel α-glucosidase inhibitor, its IC_{50} values against α-glucosidase and α-amylase were measured (Figure II-6). The 3',5'-di-C-β-D-glucosyl phloretin α-glucosidase IC_{50} value (0.09 ± 0.02 mg/mL, equal to 0.16 ± 0.04 μmol/mL) was approximately 20 times larger than that of acarbose (1.88 ± 0.54 μmol/mL) (Table II-3). This represents very strong inhibition, even compared to the aglycone of 3',5'-di-C-β-D-glucosyl phloretin, namely phloretin. Manaharan *et al.* (Manaharan, Appleton, Cheng, & Palanisamy, 2012) reported that phloretin has α-glucosidase inhibitory activity, but the inhibitory effect is only approximately 2.3-times stronger than that of acarbose. The strong inhibitory effect could allow lower concentrations of the inhibitor to be used, which would be beneficial from not only a cost perspective but would also facilitate low levels of side effects during long-term treatment.

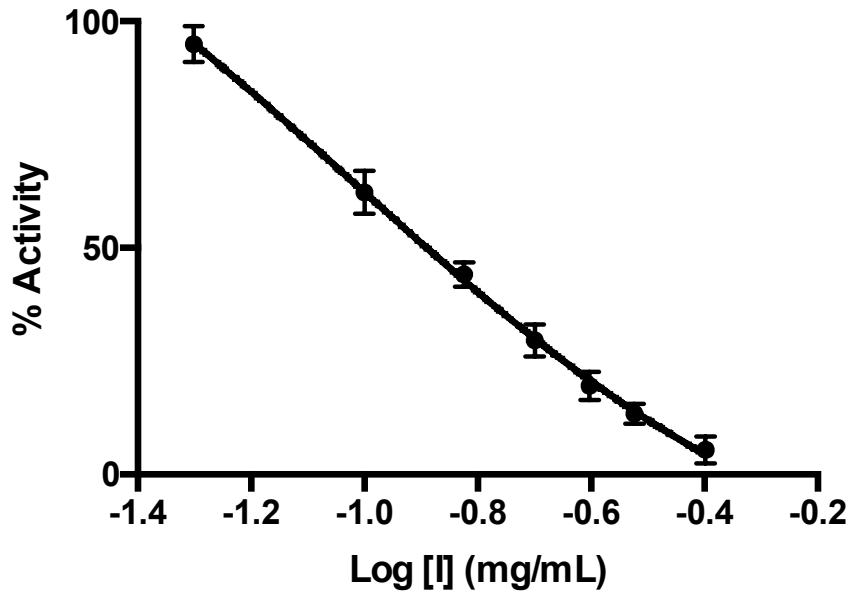


Figure II-6. α-Glucosidase inhibitory activity of 3', 5'-di-C-β-D-glucosyl phloretin.

Table II-3. α-glucosidase inhibitory activity of jujube leaf extract, acarbose, and 3', 5'-di-C-β-D-glucosyl phloretin

	IC_{50} values (mg/mL) for α-glucosidase inhibition
Jujube leaf extract (JLE)	0.67±0.25
Acarbose	1.22±0.35
3', 5'-di-C-β-D-glucosyl phloretin	0.09 ± 0.02

The values are the mean ± standard error (SE) (n=3).

II-3-4-2. α-Amylase inhibitory activity

Side effects of therapeutic agents based on α-glucosidase inhibitory activity can be caused by various factors. The major reason of side effects of therapeutic agents based on α-glucosidase inhibitory activity is nonspecific α-glucosidase inhibition (Hogan, Zhang, Li, Sun, Canning, & Zhou, 2010). The non-specific inhibition of α-glucosidase activity, such as that seen with acarbose, can cause inhibition of pancreatic α-amylase in the small intestine, which is required for hydrolysis of carbohydrates and starches to oligosaccharides. If inhibited, pancreatic α-amylase would be unable to digest carbohydrates and starches in the small intestine. These undigested carbohydrates and starches would move to the large intestine and result in production of gas by microorganisms, in turn resulting in side effects (Tharanathan, 2002).

The IC_{50} value of 3',5'-di-C-β-D-glucosyl phloretin for α-amylase was measured to examine the possibility that this inhibitor would have less side effects as compared to acarbose. The IC_{50} value of acarbose for α-amylase was 0.08 ± 0.001 mg/mL (Table II-4), while α-amylase maintained 95% or more activity in the presence of 3',5'-di-C-β-D-glucosyl phloretin, even at the α-glucosidase inhibitory concentration of 2 mg/mL (Figure II-7).

Thus, it can be concluded that 3',5'-di-C-β-D-glucosyl phloretin selectively inhibited α-glucosidase without inhibiting α-amylase, which confirmed its potential for use as a therapeutic agent that may not cause abdominal pain, gas, or diarrhea, all of which are disadvantages of acarbose.

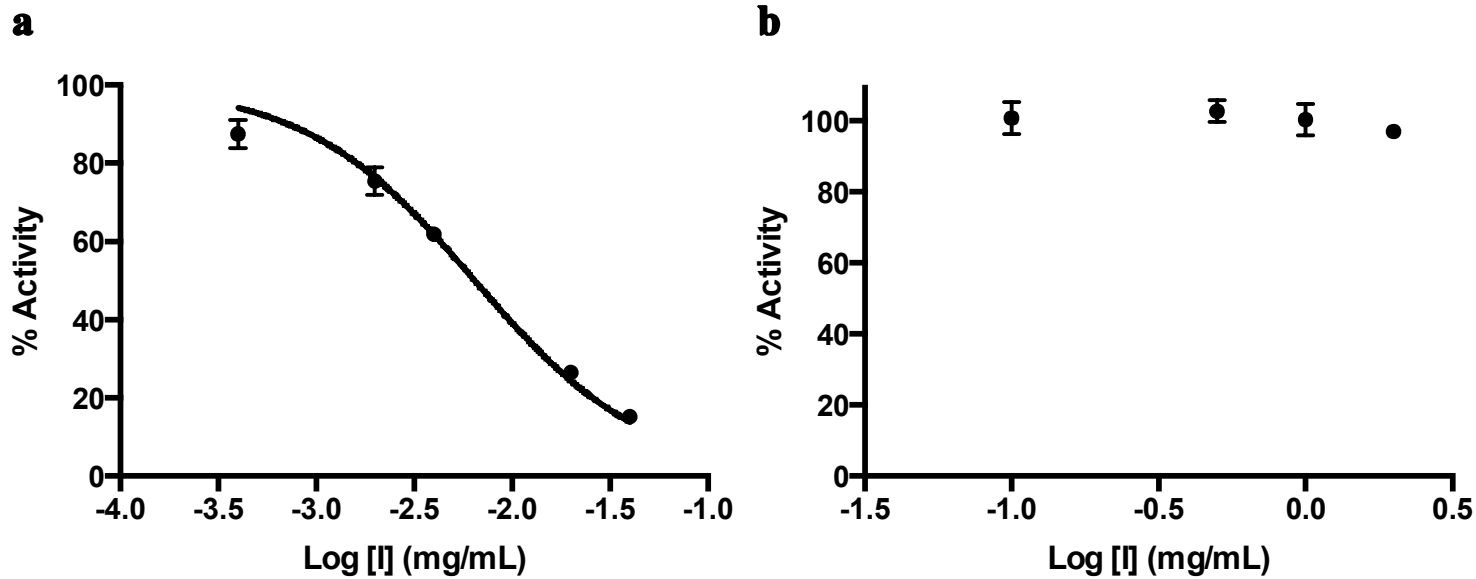


Figure II-7. α-Amylase inhibitory activity of acarbose (a) and 3', 5'-di-C-β-D-glucosyl phloretin (b).

Table II-4. α-Amylase inhibitory activity of jujube leaf extract and acarbose

	IC_{50} values (mg/mL) for α-amylase inhibition
Jujube leaf extract (JLE)	-
Acarbose	0.08±0.001

The values are the mean ± standard error (SE) (n=3).

- : Inhibition was not observed.

II-3-5. Kinetic studies

As the results of kinetic studies, the K_m value increased to 1.97, 2.33 and 2.83 mg/mL as the concentration of inhibitor was increased to 0, 0.075 and 0.1 mg/mL, and the V_{max} values were decreased to 0.029, 0.015 and 0.011 μmol/mM·mL·min, respectively. From these results, it could be confirmed that this inhibitor has a mixed-noncompetitive inhibition. The inhibitory mechanism of phloretin and acarbose involves binding to the active site of α-glucosidase (i.e., competitive inhibition) (Bischoff, 1995; Dong, Li, Zhu, Liu, & Huang, 2012), whereas the novel inhibitor showed mixed non-competitive inhibition close to non-competitive inhibition (Figure II-8). Competitive inhibition is overcome by a higher concentration of substrate, which is a disadvantage in many applications. However, increased substrate concentrations have no effect on non-competitive inhibitors. Therefore, this novel inhibitor is expected to provide more advantages during production of therapeutic agents as compared to the other inhibitors described above. Then the *Ki* value is dissociation constants of enzyme-inhibitor complex, it could be obtained as 2.1 mg/mL using by Sigmaplot software (version 12.5).

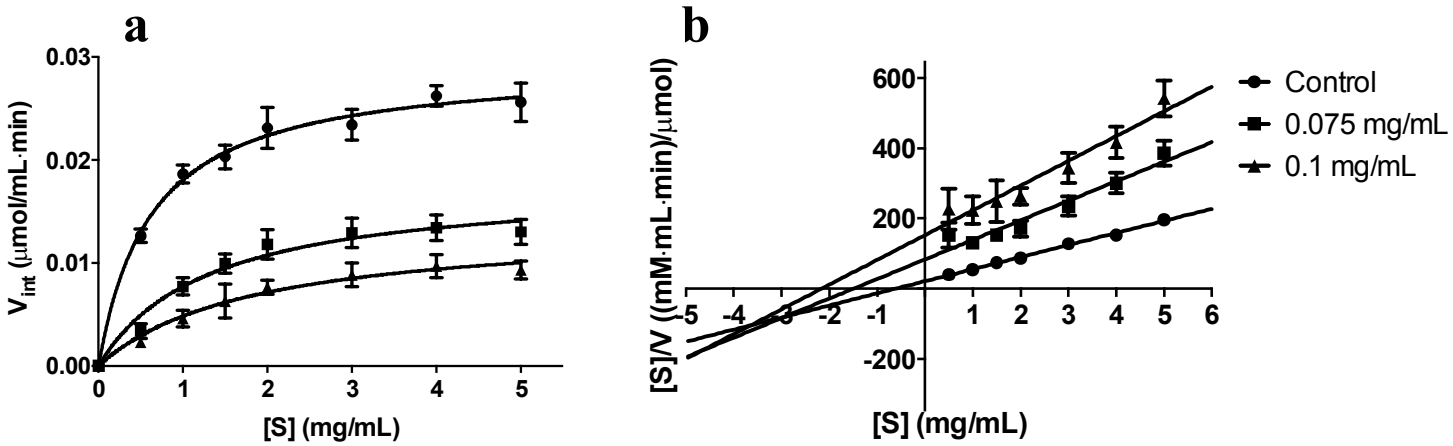


Figure II-8. Michaelis-Menten plot (a) and Hanes-Woolf plot (b) for kinetic study of α-glucosidase inhibition on 3', 5'-di-C-β-D-glucosyl phloretin.

II-3-6. *In vitro* digestion

pH and electrolyte conditions change during digestion and many bioactive substances are often denatured by pH or metal ions. This denaturation may either increase or eliminate the activity of the bioactive substance. When activity is lost as a result of electrolytes or pH changes or other digestion enzymes in the human body, its use is limited, even if the activity is excellent under some conditions. *In vitro* digestion studies are widely used to predict the behavior of food components in the digestive tract.

To evaluate the α-glucosidase and α-amylase inhibitory activities of jujube leaf extract after *in vitro* digestion, simulated *in vitro* digestion test conditions were established. The percent inhibition rates of α-amylase and α-glucosidase are shown in Table II-5. The α-glucosidase inhibition rate of jujube leaf extract (0.2 mg/mL) was 42.76 ± 5.04% before *in vitro* digestion and 58.33 ± 15.17% after *in vitro* digestion. In contrast, no α-amylase-inhibitory activity of jujube leaf extract was detected before or after *in vitro* digestion. Therefore, jujube leaf extract retained α-glucosidase inhibitory activity after *in vitro* digestion and did not inhibit α-amylase activity. The jujube leaf extract could be used by itself without any further treatment

because its inhibitory activity is maintained during the pH and ionic changes that occur during digestion.

Table II-5. α-Glucosidase and α-amylase inhibition rate of samples before and after *in vitro* digestion

	Samples (0.2 mg/mL)	
	Before *in vitro*	After *in vitro*
α–Glucosidase activity (%)	42.76±5.04*	58.33±15.17*
α-Amylase activity (%)	-	-

The values are the mean ± standard error (SE) (n=3).

* The difference was not statistically significant.

- : Inhibition was not observed.

II-4. Conclusion

In this study, the strongest inhibitor of α-glucosidase was separated and purified from jujube leaf extract. The purified α-glucosidase inhibitor was 3', 5'-di-C-β-D-glucosyl phloretin, and the inhibitory activity of this substance has not been yet reported. Then, the study was carried out to identify α-glucosidase inhibitory characteristic of this substance. This α-glucosidase inhibitor has non-competitive inhibition and it can inhibit α-glucosidase selectively. A selective α-glucosidase inhibition can be expected to be useful in the pharmaceutical and food industries due to it could be expected to have lower side effect than that of non-selective α-glucosidase inhibitors. And the non-competitive inhibition also has more advantages to the pharmaceutical and food industries than competitive inhibition. Furthermore, as this novel inhibitor can be obtained by hot water extraction, it is apparently highly soluble in water and stable against high temperatures and thus is expected to be applicable to many fields.

II-5. References

Asano, N., Yamashita, T., Yasuda, K., Ikeda, K., Kizu, H., Kameda, Y., Kato, A., Nash, R. J., Lee, H. S., & Ryu, K. S. (2001). Polyhydroxylated alkaloids isolated from mulberry trees (*Morus alba* L.) and silkworms (*Bombyx mori* L.). *J. Agri. Food Chem., 49*(9), 4208-4213.

Bischoff, H. (1995). The mechanism of alpha-glucosidase inhibition in the management of diabetes. *Clin. Invest. Med, 18*(4), 303-311.

Diamond, J. M., Karasov, W. H., Cary, C., Enders, D., & Yung, R. (1984). Effect of dietary carbohydrate on monosaccharide uptake by mouse small intestine *in vitro*. *J. Physiol., 349*(1), 419-440.

Dong, H. Q., Li, M., Zhu, F., Liu, F. L., & Huang, J. B. (2012). Inhibitory potential of trilobatin from *Lithocarpus polystachyus* Rehd against α-glucosidase and α-amylase linked to type 2 diabetes. *Food Chem., 130*(2), 261-266.

Elaloui, M., Laamouri, A., Ennajah, A., Cerny, M., Mathieu, C., Vilarem, G., Chaar, H., & Hasnaoui, B. (2016). Phytoconstituents of leaf extracts of *Ziziphus jujuba* Mill. Plants harvested in Tunisia. *Indust. Crop. Prod., 83*, 133-139.

Figueiredo-González, M., Grosso, C., Valentão, P., & Andrade, P. B. (2016). α-Glucosidase and α-amylase inhibitors from *Myrcia* spp.: a stronger alternative to acarbose? *J. Pharm. Biomed. Anal., 118*, 322-327.

Gao, Q. H., Wu, C. S., & Wang, M. (2013). The jujube (Ziziphus jujuba Mill.) fruit: a review of current knowledge of fruit composition and health benefits. *J. Agri. Food Chem., 61*(14), 3351-3363.

Guo, L. P., Jiang, T. F., Lv, Z. H., & Wang, Y. H. (2010). Screening α-glucosidase inhibitors from traditional Chinese drugs by capillary electrophoresis with electrophoretically mediated microanalysis. *J. Pharm. Biomed. Anal., 53*(5), 1250-1253.

Guo, S., Duan, J. A., Tang, Y., Qian, Y., Zhao, J., Qian, D., Su, S., & Shang, E. (2011). Simultaneous qualitative and quantitative analysis of triterpenic acids, saponins and flavonoids in the leaves of two *Ziziphus* species by HPLC–PDA–MS/ELSD. *J. Pharm. Biomed. Anal., 56*(2), 264-270.

Hogan, S., Zhang, L., Li, J., Sun, S., Canning, C., & Zhou, K. (2010). Antioxidant rich grape pomace extract suppresses postprandial hyperglycemia in diabetic mice by specifically inhibiting alpha-glucosidase. *Nutr. Metabol., 7*(1), 71.

Holman, R. R., Cull, C. A., & Turner, R. C. (1999). A randomized double-blind trial of acarbose in type 2 diabetes shows improved glycemic

control over 3 years (UK Prospective Diabetes Study 44). *Diabetes Care, 22*(6), 960-964.

Kim, K., Nam, K., Kurihara, H., & Kim, S. (2008). Potent α-glucosidase inhibitors purified from the red alga *Grateloupia elliptica*. *Phytochemistry, 69*(16), 2820-2825.

Kim, Y. J., & Son, D. Y. (2011). Antioxidant effects of solvent extracts from the dried jujube (*Zizyphus jujube*) sarcocarp, seed, and leaf via sonication. *Food Sci. Biotech., 20*(1), 167-173.

Kumar, S., Narwal, S., Kumar, V., & Prakash, O. (2011). α-glucosidase inhibitors from plants: A natural approach to treat diabetes. *Pharmacognosy Rev., 5*(9), 19.

KWON, Y. I., Apostolidis, E., & Shetty, K. (2008). Inhibitory potential of wine and tea against α-Amylase and α-Glucosidase for management of hyperglycemia linked to type 2 diabetes. *J. Food Biochem., 32*(1), 15-31.

Li, Y. Q., Zhou, F. C., Gao, F., Bian, J. S., & Shan, F. (2009). Comparative evaluation of quercetin, isoquercetin and rutin as inhibitors of α-glucosidase. *J. Agri. Food Chem., 57*(24), 11463-11468.

Manaharan, T., Appleton, D., Cheng, H. M., & Palanisamy, U. D. (2012). Flavonoids isolated from *Syzygium aqueum* leaf extract as potential antihyperglycaemic agents. *Food Chem., 132*(4), 1802-1807.

Pawlowska, A. M., Camangi, F., Bader, A., & Braca, A. (2009). Flavonoids of *Zizyphus jujuba* L. and *Zizyphus spina-christi* (L.) Willd (Rhamnaceae) fruits. *Food Chem., 112*(4), 858-862.

Sun, S., Kadouh, H. C., Zhu, W., & Zhou, K. (2016). Bioactivity-guided isolation and purification of α-glucosidase inhibitor, 6-OD-glycosides, from tinta Cão grape pomace. *J. Funct. Food., 23*, 573-579.

Tharanathan, R. N. (2002). Food-derived carbohydrates—structural complexity and functional diversity. *Crit. Rev. Biotech., 22*(1), 65-84.

Yilmazer-Musa, M., Griffith, A. M., Michels, A. J., Schneider, E., & Frei, B. (2012). Grape seed and tea extracts and catechin 3-gallates are potent inhibitors of α-amylase and α-glucosidase activity. *J. Agri. Food Chem., 60*(36), 8924-8929.

Zhang, B., Deng, Z., Ramdath, D. D., Tang, Y., Chen, P. X., Liu, R., Liu, Q., & Tsao, R. (2015). Phenolic profiles of 20 Canadian lentil cultivars and their contribution to antioxidant activity and inhibitory effects on α-glucosidase and pancreatic lipase. *Food Chem., 172*, 862-872.

Zhang, R., Chen, J., Shi, Q., Li, Z., Peng, Z., Zheng, L., & Wang, X. (2014a). Phytochemical analysis of Chinese commercial *Ziziphus jujube* leaf tea using high performance liquid chromatography–electrospray ionization-time of flight mass spectrometry. *Food Res. Int., 56*, 47-54.

Zhang, R., Chen, J., Shi, Q., Li, Z., Peng, Z., Zheng, L., & Wang, X. (2014b). Quality control method for commercially available wild Jujube leaf tea based on HPLC characteristic fingerprint analysis of flavonoid compounds. *J. Separ. Sci., 37*(1-2), 45-52.

Chapter III. Application of α-glucosidase inhibitory activity of jujube leaf to produce the functionalized rice

III-1. Introduction

The glycemic index (GI) of food is a numerical measurement used to indicate the level and speed with which a food raises the level of glucose in the blood (Wolever & Jenkins, 1986). Foods can be classified as low GI foods, medium GI foods, and high GI foods with GI values of less than 55, 56-69, and 70 or more, respectively (Mohan, Anjana, Gayathri, Ramya Bai, Lakshmipriya, Ruchi, et al., 2016). The GI value of the food was the percentage of the test food's incremental area under curve (IAUC) for standard glucose IAUC (Wolever, T.M., 2004).

Rice is an important crop because it is a major food staple in many countries including India, Korea, Japan, and China (Sugiyama, Tang, Wakaki, & Koyama, 2003). However, the GI value of white rice is very high, about 98. Therefore, people who need to control postprandial glucose, such as diabetics, should avoid eating white rice. Brown rice is recommended for diabetics, because the GI value of brown rice is lower than white rice (about 65). Brown rice is unpolished rice with just the bark removed.

However, due to the texture of brown rice, most diabetics are reluctant to eat it (Kozuka, Yabiku, Sunagawa, Ueda, Taira, Ohshiro, et al., 2012). In addition, previous research suggested that heavy metals, such as

mercury (Hg), on the surface of brown rice could cause public health problems if people continuously consume brown rice (Meng, Feng, Qiu, Anderson, Wang, Zhao, et al., 2014).

The previous study demonstrated that a natural α-glucosidase inhibitor could be obtained from jujube leaves. Therefore, the aim of this study is to produce functional rice that can control postprandial blood glucose by using the α-glucosidase inhibitory activity of jujube leaves and examined the texture of this functional rice.

III-2. Materials and Methods

III-2-1. Chemicals

Rice (Icheon, Korea) was purchased at a local market. Two kinds of instant rice were purchased from CJ Cheiljedang (Seoul, Korea): Hatban and instant rice that can help control postprandial blood sugar levels. Intestinal acetone powders from rat and the GOD-POD assay kit were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Embeil Co. (Gunpo, Korea), respectively. All other reagents and chemicals were of analytical grade.

III-2-2. Sample preparation

Twelve grams of rice were washed three times. After washing, the rice was mixed with various volumes of water (8, 10, 12, and 14 mL) and then the samples were swelled for 30 min with water. The jujube leaf rice was swelled with 5 and 10 mg/mL of jujube leaf extract instead of water (JRice). The rice was cooked at 121 °C for 15 min under 2 atm. The instant rice (Hatban), which was used as a positive control, was cooked in a microwave for 2 min.

III-2-3. Texture analysis

The hardness and stickiness of rice were measured using a texture analyzer (TA-XT, Stable Micro system, Surrey, UK) with a 5 kg load cell when the central temperature of all samples reached 50 °C. Five grains of cooked rice were placed as a single layer on the base plate. A two-cycle compression method was conducted using a 10 mm diameter probe, which was set above 25 mm from plate. The pre-test speed and test-speed were set at 1 mm/s. The compression was set to 80% strain. For each cooked rice, six times replicate measurement were conducted. The parameters were recoded from the test curves were hardness and stickiness.

III-2-4. Preparation of rat intestinal enzyme solution

Intestinal acetone powder from rat (0.3 g) were suspended in 9 mL of 0.1 M sodium acetate buffer (pH 5.2, 4 mM $CaCl_2$, made with benzoic acid saturated distilled water) and the suspension was sonicated 12 times for 30 s at 37 °C. The solution was then centrifuged at 13,000 rpm for 30 min at 4 °C and the supernatant was used to measure the enzyme inhibition rate.

III-2-5. Measurement of degree of carbohydrate hydrolysis

The degree of carbohydrate hydrolysis throughout the incubation period (0-360 min) was determined, based on the method of Ha Ram Kim et al., with modifications. The rice sample (90 mg) was transferred into a 4 mL vial and suspended in 2.25 mL of 0.1 M sodium acetate buffer. After sample dispersion was equilibrated in a 37 °C shaking incubator (600 rpm) for 10 min, the rat intestinal enzyme solution (2.25 mL) was added to each vial. The samples incubated for certain amounts of time in a shaking incubator (600 rpm, 37 °C) and were then transferred to 95 °C for 10 min to terminate the enzymatic reactions. The glucose in the supernatant, released by the hydrolysis of carbohydrates, was measured using a GOD-POD kit after centrifugation at 5,000 × g for 10 min.

III-2-6. Sensory analysis

Sensory analysis was performed to evaluate attributes (appearance, flavor, taste, texture, and total) of the four types of cooked rice. The degree of preference of each attribute was examined. In all sensory tests, panelists consisted of 20 members of the Department of Food Biotechnology, Hoseo University (Asan, Chungnam, Korea). The panel rated the different parameters on a 9-point scale (1 = very poor and 9 = very good). The cooked rice samples were labeled with three-digit random numbers that were placed on four dishes and the panelists drank water to cleanse the palate between samples.

III-2-7. Statistical analysis

All results were analyzed using Tukey's significant difference test with IBM SPSS Statistics version 21.0 (IBM Co., Armonk, NY, USA). At least three independent replicates were performed in each experiment.

III-3. Results and Discussion

III-3-1. Texture of rice

To determine the amount of water in rice, 12 g of rice was added to various amounts of water (8, 9, 10, and 12 mL) to make cooked rice and the texture of the cooked rice was measured. Hardness and stickiness were measured relative to instant rice (Hatban). As a result, when the amount of water increased from 8, 10, 12, to 14 mL, the hardness was approximately 6,958 g at 8 mL, but there was no statistically significant difference of approximately 2,926 g in 10 mL of water. As the amount of water increased from 8 to 14 mL, the stickiness increased from 16.32 to 273.97 g·s (Figure III-1). Based on these two factors, rice was prepared by adding 12 mL of water to 12 g of rice, which represents the rice cooking method that generates a texture most similar to instant rice.

To determine whether jujube leaf affects texture, 5 and 10 mg/mL of jujube leaf extract were added before cooking to cook rice and then hardness and stickiness were measured. As shown in Figure III-2, the hardness and stickiness of rice along with 5 and 10 mg/mL jujube leaf extract were the same as cooked rice alone. These results indicate that jujube leaf extract did

not affect the hardness and stickiness of cooked rice. Although it is known that cooking instant rice with cyclodextrin helps to control postprandial blood glucose levels, there is a statistically significant difference between hardness and stickiness in general instant rice.

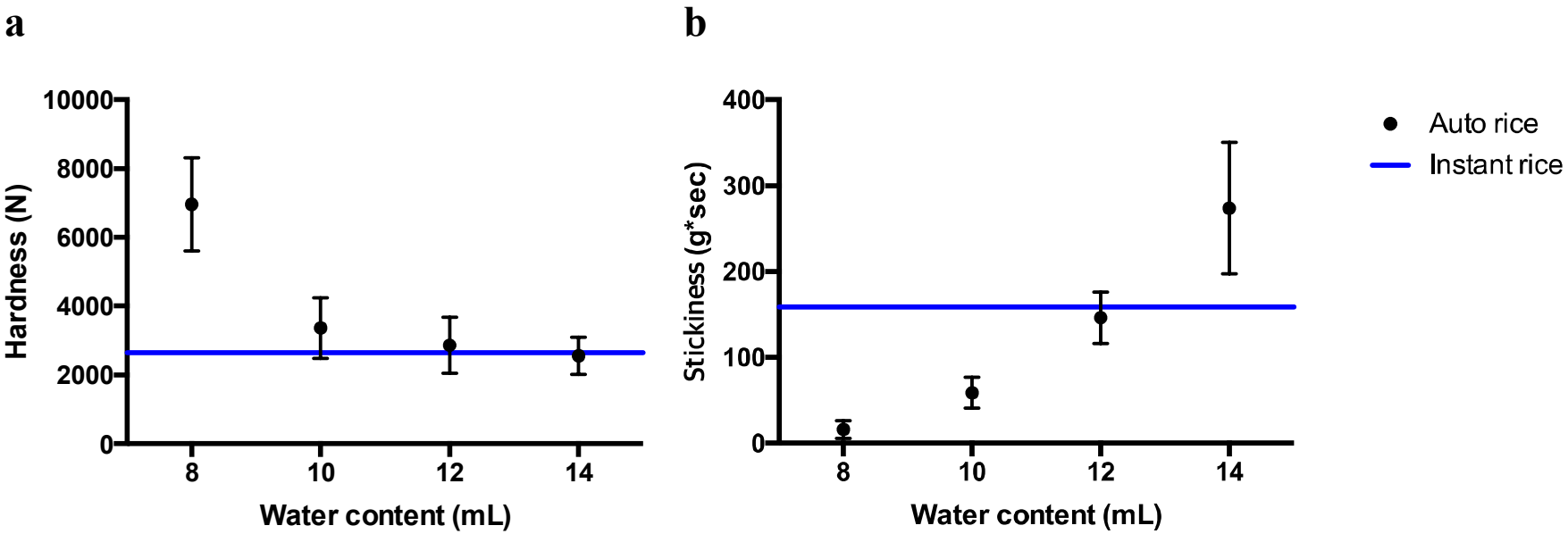


Figure III-1. The hardness (a) and stickiness (b) of cooked rice by the amount of water (8, 10, 12, and 14 mL water for 12 g of rice). Instant rice (Hatban) was used as control.

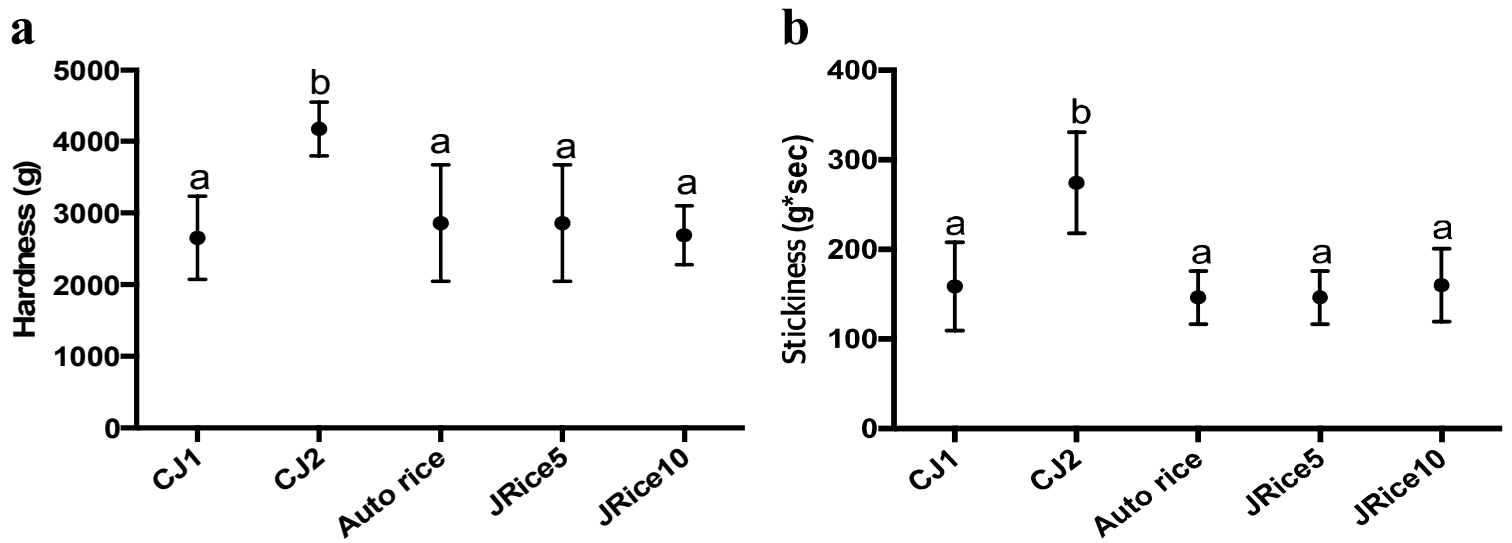


Figure III-2. The hardness (a) and stickiness (b) of five different cooked rice (CJ1: Hatban; CJ2: Rice that can help control postprandial blood sugar; Auto rice: Rice cooked using autoclave without jujube leaf; JRice 5: Rice cooked using autoclave with 5 mg/mL of jujube leaf; JRice 10: Rice cooked using autoclave with 10 mg/mL of jujube leaf).

III-3-2. Degree of carbohydrate hydrolysis

The GOD-POD assay was used to measure the degree of digestion. Normal cooked rice reached the maximum degree of hydrolysis at 180 min. The time to reach the maximum degree of hydrolysis increased to 210 and 360 min as the concentration of jujube leaf containing α-glucosidase inhibitor was increased to 5 and 10 mg/mL, respectively (Figure III-3). Although purified α-glucosidase inhibitor could not be used for production of rice, it was confirmed that the α-glucosidase inhibitory effect was maintained even at high temperature and high-pressure during rice production. Previous studies have shown that purified α-glucosidase inhibitor (0.09 ± 0.02 mg/mL) is approximately 7.4-times stronger than the α-glucosidase inhibition of jujube leaf (0.67 ± 0.25 mg/mL). It is expected that the use of purified α-glucosidase inhibitors will further delay the time required to reach the maximum degree of hydrolysis at the same concentration of α-glucosidase inhibitor.

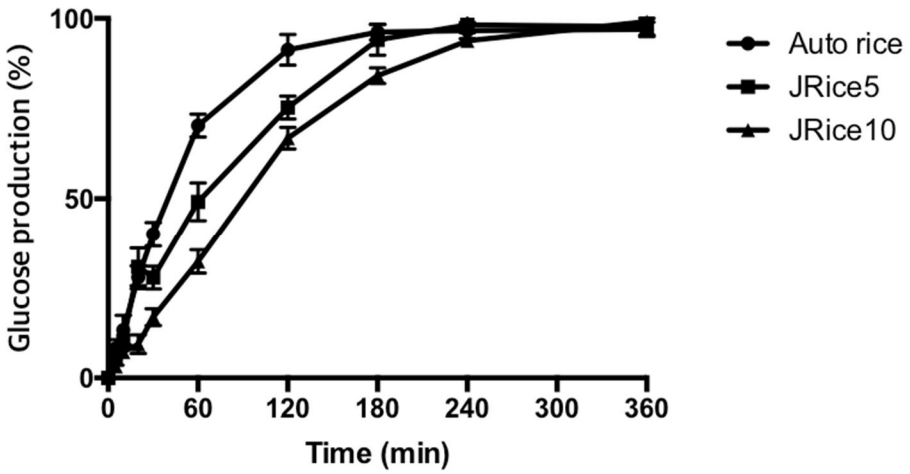


Figure III-3. Digestion profile of cooked rice using autoclave (auto rice) and cooked rice using autoclave with 5 and 10 mg/mL of jujube leaf (JRice 5 and 10).

III-3-3. Sensory test

Sensory evaluation was performed to evaluate the preference for four different kinds of cooked rice. Table III-1 shows the sensory tests used to evaluate five attributes (appearance, flavor, taste, texture, and total) of four different preparations of cooked rice. There was no significant difference (P >0.05) between all samples. Although the color and flavor of jujube leaf were retained by the jujube leaf rice, it was confirmed that it is not the color and flavor that has an adverse effect on the taste of cooked rice (Figure III-4).

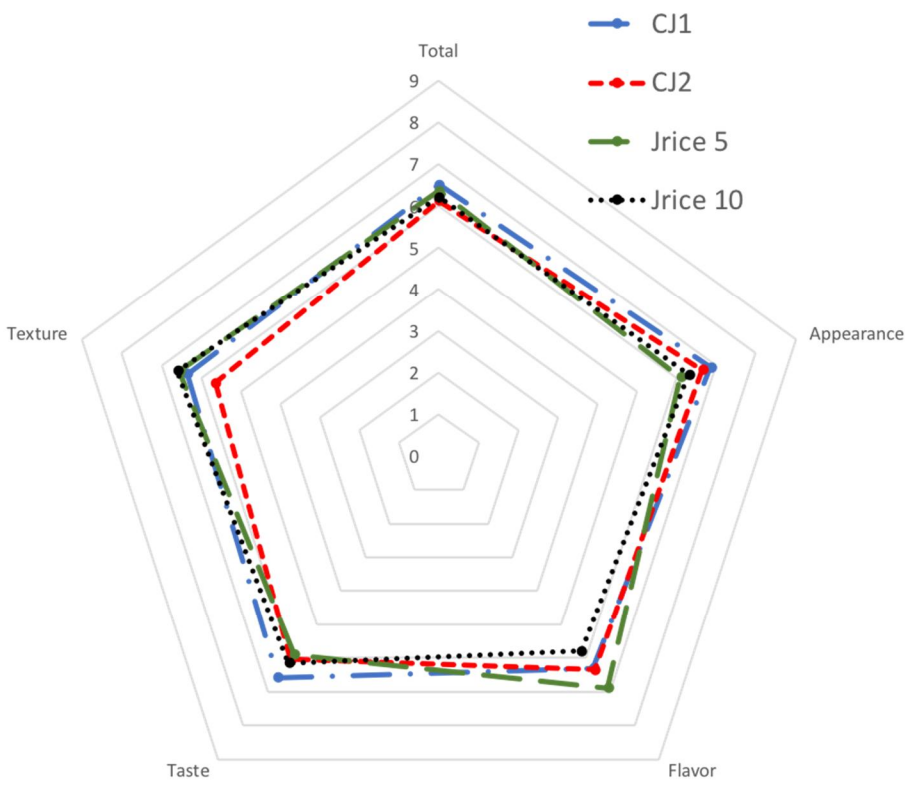


Figure III-4. Sensory test of two type of instant rice (Hatban and Rice that can help control postprandial blood sugar), and cooked rice with jujube leaf (5 and 10 mg/mL).

Table III-1. Sensory attributes of four different cooked rice

	Sensory attributes score [a]				
	Appearance	**Flavor**	**Taste**	**Texture**	**Total**
CJ1	6.85±1.63 *a	6.3±1.78 a	6.58±1.46 a	6.35±1.66 a	6.52±1.18 a
CJ2	6.65±1.73 a	6.35±1.31 a	6.05±1.57 a	5.65±1.87 a	6.125±0.86 a
JRice5	6.1±1.29 a	6.9±1.17 a	5.9±2.17 a	6.55±1.54 a	6.36±1.17 a
JRice10	6.3±1.45 a	5.8±1.58 a	6.15±1.63 a	6.6±0.99 a	6.21±1.01 a

[a] Results are from panelist scored analysis on a 9-scale (1=very poor and 9=very good).

The values are means ± standard deviation.

*Values followed by the same letters within each column did not differ significantly ($P > 0.05$).

III-4. Conclusion

Functional rice was produced that can control postprandial blood glucose by using glucosidase inhibitory activity of jujube leaf. Prior to produce functional rice, an experiment was conducted to confirm whether the α-glucosidase inhibitory activity of jujube leaf was maintained at a high temperature and high pressure. As a result, it was confirmed that the α-glucosidase inhibitory activity was maintained even at the temperature 121 C, 3 atm, which is the conditions for producing the instant rice. Even though the concentration of jujube leaf extract (0, 5, 10 mg/mL), the texture of rice was not changed. In addition, the results of GODPOD method was expected to help control the postprandial blood glucose. Although the flavor and color of jujube leaf was remained, it did not affect the preference. From these results, α-glucosidase inhibitory activity of jujube leaf extract is expected to be applicable to the food industry.

III-5. References

Wolever, T. M. (2004). Effect of blood sampling schedule and method of calculating the area under the curve on validity and precision of glycemic index value. *Brit. J. Nutr., 91(2),* 295-300.

Kim, H. R., Choi, S. J., Park, C.-S., & Moon, T. W. (2017). Kinetic studies of *in vitro* digestion of amylosucrase-modified waxy corn starches based on branch chain length distributions. *Food Hydrocol., 65*, 46-56.

Kozuka, C., Yabiku, K., Sunagawa, S., Ueda, R., Taira, S.-i., Ohshiro, H., Ikema, T., Yamakawa, K., Higa, M., & Tanaka, H. J. D. (2012). Brown rice and its component, γ-oryzanol, attenuate the preference for high-fat diet by decreasing hypothalamic endoplasmic reticulum stress in mice. *Diabetes*, *61*, 3084-3093.

Meng, B., Feng, X., Qiu, G., Anderson, C. W., Wang, J., Zhao, L. J. E. s., & technology. (2014). Localization and speciation of mercury in brown rice with implications for pan-Asian public health. *Environ. Sci. Technol., 48*(14), 7974-7981.

Mohan, V., Anjana, R. M., Gayathri, R., Ramya Bai, M., Lakshmipriya, N., Ruchi, V., Balasubramaniyam, K., Jakir, M. M., Shobana, S.,

Unnikrishnan, R. J. D. t., & therapeutics. (2016). Glycemic index of a novel high-fiber white rice variety developed in India—A randomized control trial study. *Diabetes Technol. Ther. 18*(3), 164-170.

Sugiyama, M., Tang, A., Wakaki, Y., & Koyama, W. J. E. j. o. c. n. (2003). Glycemic index of single and mixed meal foods among common Japanese foods with white rice as a reference food. *Eur. J. Clin. Nutr., 57*(6), 743.

Wolever, T. M., & Jenkins, D. J. J. T. A. j. o. c. n. (1986). The use of the glycémie index in predicting the blood glucose response to mixed meals. *Am. J. Chlin. Nutr., 43*(1), 167-172.

Chapter IV. A natural β-glucosidase inhibitor from jujube leaf extract

IV-1. Introduction

Enzyme inhibitors play a key role in many bioprocesses and are thus an essential part of a wide-range of biological applications, including applications in agriculture, clinical care, cosmetics, and food processing. Therefore, inhibitors of glycosidases, which are extremely common throughout nature that have crucial roles in diverse metabolic processes, have been attracting significant interest (Asano, 2003). In particular, inhibitors of glucosidases are being explored for their therapeutic effects in diabetes, HIV infection, cancer, and lysosomal storage disease (Rempel & Withers, 2008). Additionally, extensive efforts are being made to discover novel inhibitors of glucosidases.

In the previous study, β-glucosidase inhibitors were obtained from jujube leaf extract (JLE), a natural source (Jo, Lim, Chang, & Choi, 2016). Inhibitors of β-glucosidase are used as a chemotherapy agent for hepatocellular carcinoma (Y. Zhang, Zhu, Miao, Hu, & Wang, 2016) and as a treatment for patients with type 1 Gaucher disease (Lieberman, Wustman, Huertas, Powe, Pine, Khanna, et al., 2007). In addition, β-glucosidase inhibitors have been used to modify the unexpected activities of enzyme complexes to produce substances with higher bioavailability (Vila-Real,

Alfaia, Bronze, Calado, & Ribeiro, 2011; Wang, Ma, Wu, Yu, Xia, Sun, et al., 2012; Wang, Sun, Yu, Wu, & Guo, 2013).

For economic reasons, the glucosidase inhibitors commonly used in industry are obtained mostly via chemical synthesis. However, these chemically synthesized products occasionally cause serious side effects (Venkatesh, Reddy, Reddy, Ramesh, & Rao, 2003). Substances from natural sources are considered to be safer than their synthesized counterparts and are thus significant for the purposes of food-grade usage (Valiathan, 1998). This is the main reason why natural products have received increased attention. Several natural products of high cost that effectively provide health benefits for chronic and degenerative global health problems, such as obesity, cancer, Alzheimer's disease, and diabetes mellitus, are currently available on the global market (Ganem, 1996; Kwon, Apostolidis, & Shetty, 2008; Nishimura, 2003). Thus, it should be both economically and environmentally advantageous to produce these valuable substances, including glycosidase inhibitors, from the waste or by-products of natural resources. Many natural resources, especially whole parts of many plants, have been explored in the drug and pharmaceutical industries for many years now (Newman & Cragg, 2016).

Jujube leaves, used since ancient times as a tea in east Asia and India, are considered to be an agricultural by-product or waste, because the fruit is

the main purpose for cultivating the jujube plant. Moreover, although some ancient documents state that jujube leaf tea exhibits several health benefits, including anti-obesity activity, few research studies on the health benefits of jujube leaf can be found (R. Zhang, Chen, Shi, Li, Peng, Zheng, et al., 2014). Water extraction is a suitable process for obtaining valuable substances from natural resources for the purposes of food-grade usage. Tea, a water extract of natural resources, is the second most popular beverage in the world next to water (Chen, Kitts, & Ma, 2017) and it is a traditional beverage with various beneficial effects.

In the process of searching for β-glucosidase inhibitors from natural materials, I identified such a substance in water extracts of jujube leaves. Initially, the inhibitory properties of JLE fractions were evaluated to identify the one most responsible for this activity. Then, the major substance with the strongest inhibition activity in a series of fractions was isolated and purified and its inhibitory characteristics were verified.

IV-2. Materials and Methods

IV-2-1. Chemicals

The β-glucosidase from almonds (2.18 U/mg of β-glucosidase activity, EC. 3.2.1.21) and *p*-nitrophenyl-β-D-glucopyranoside (5.5 mM) were reconstituted in McIlvaine buffer (pH 5.31). Sephadex G-25 was obtained from GE Healthcare (Boulder, CO, USA).

IV-2-2. Preparation of jujube leaf extract

Sun-dried jujube leaves, harvested in June 2013 in Boeun, South Korea, were stored at -80 °C. Frozen jujube leaves were pulverized using a household blender (HMF-1000, Hanil Electric, Seoul, Korea) and filtered with a fine mesh (150-300 μm). The pulverized samples were stored at -18 °C.

Extraction of the jujube leaves was performed using food-grade distilled water. A mixture of 0.5 g of pulverized jujube leaves per 20 mL of distilled water was stirred at 95 °C for 15 min. The extract was filtered with filter paper (filter paper No. 4, 110 mm ϕ, Whatman International Ltd., Maidstone, UK). This filtered extract was evaporated using a vacuum centrifuge (centrifugal evaporator, CVE-2200, EYELA, Bohemia, NY, USA) and the powdered samples were dissolved in McIlvaine buffer.

IV-2-3. β-Glucosidase inhibition assay

The reaction mixture contained 5.5 mM of *p*NPG (50 μL), 0.3 U/mL of almond β-glucosidase (100 μL), and inhibitors (50 μL). The enzyme and inhibitors incubated for 10 min at 37 ℃, then substrate (*p*NPG) was added, followed by incubation for 40 min at 37 ℃. The released *p*-nitrophenol in the reaction was measured at 400 nm using a microplate reader (MULTISKAN GO, Thermo Scientific, Waltham, MA, USA). The inhibition rate was calculated as follows:

$$Inhibition\ rate\ (\%) = \left\{1 - \left(\frac{Inhibitor\ (OD_{400}) - Background\ (OD_{400})}{Control\ (OD_{400}) - Background\ (OD_{400})}\right)\right\} \times 100$$

where *Control* (OD_{400}) is the absorbance of control (without sample), *Inhibitor* (OD_{400}) is the absorbance of sample, *Background* (OD_{400}) is the absorbance of sample (without *p*NPG).

IV-2-4. Isolation of β-glucosidase inhibitor

IV-2-4-1. Size exclusion chromatography

The samples were subjected to size exclusion chromatography using a PD-10 column packed with Sephadex G-25 medium in distilled water. Ten milliliters of each fraction were dried at 65 °C. The dried fractions were dissolved in 1 mL of McIlvaine buffer (pH 3.8) prior to analysis in the glucosidase inhibition assays.

IV-2-4-2. Semi-preparative HPLC

Fractions 6, 7, and 8, which displayed the strongest inhibition of β-glucosidase activity, were dispersed in distilled water. The samples were further purified by semi-preparative high-performance liquid chromatography (HPLC, Ultimate 3000, Thermo Scientific Dionex, Sunnyvale, CA, USA). The conditions of the semi-preparative HPLC are shown in Table IV-1.

Table IV-1. The semi-preparative LC conditions for purifying the β-glucosidase inhibitor from jujube leaf extract

System	Ultimate 3000 (Thermo Dionex, USA)			
Column	VDS C18 column (25X0.46 cm, 5 μm, VDS Optilab, Germany)			
Temperature	30°C			
Solvent	A (Acetronitrile), B (0.5% Acetic acid)			
Gradient Timetable	Time (min)	A (%)	B (%)	Flow (mL/min)
	0.0	2	98	0.8
	10.0	5	95	0.8
	20.0	80	20	0.8
	25.0	80	20	0.8
	26.0	2	98	0.8
	30.0	2	98	0.8

IV-2-5. Characteristic analysis

IV-2-5-1. Prediction of the empirical formula

The empirical formula of the substance was predicted based on its molecular weight and constituent elements. The molecular weight was measured using direct injection probe mass spectrometry (DIP-MS, JMS-700, JOEL, Tokyo, Japan). Constituent elements were measured using an elemental analyzer (Flash EA 1112, Thermo Scientific). DIP-MS was performed at the Center for Research Facility (Kyunghee University, Kyunggi, Korea) and elemental analysis used was performed at the National Instrumentation Center for Environmental Management (NICEM, Seoul National University, Seoul, Korea).

IV-2-5-2. Enzyme kinetic study

The substrate (4-nitrophenyl-β-D-glucopyranoside) was used at concentrations of 0, 1, 3, 5, and 7 mM in the reactions. The concentration of enzyme (β-glucosidase from almonds) was 0.3 U/mL. The ratio of substrate, enzyme, and buffer (or inhibitor) was 1:1:2. Reactions incubated at 37 °C. The enzyme and inhibitors (2 and 4 mg/mL, respectively) incubated for 15 min prior to addition of 100 μL of substrate. Samples were measured with UV-spectrometer at 400 nm every 3 min for 90 min.

IV-2-6. Statistical analysis

All results were analyzed using Tukey's significant difference test with IBM SPSS Statistics version 21.0 (IBM Co., Armonk, NY, USA). At least three independent replicates were performed in each experiment.

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IV-3. Results and Discussion

IV-3-1. Isolation of β-glucosidase inhibitor

The jujube leaves were extracted with water to simulate the jujube leaf tea (Figure IV-1 b), and then the size exclusion chromatography and semi-preparative LC were used to separate and purity the substance which is the most responsible for β-glucosidase inhibition.

IV-3-1-1. Size exclusion chromatography

In the first separation process, a PD-10 column packed with Sephadex G-25 medium was used to collect 35 fractions from the JLE based on molecular size and the β-glucosidase inhibition assay was used to confirm the inhibition rate of each fraction. The concentrations of each fraction in the enzyme activity assay were not equalized, because the purpose of this study was to identify the main inhibitor in jujube leaf tea. Two large peaks (fractions 7 and 20) were identified, as shown in Figure IV-2. Fractions 5-8 displayed stronger inhibition of β-glucosidase activity (approximately 15% or greater) than the other fractions. Because of the characteristics of size exclusion chromatography, these other fractions may contain lower

molecular weight substances that also inhibit β-glucosidase activity. These lower molecular weight substances could even have stronger β-glucosidase inhibitory activity because they were not of the same concentration. However, this study focused on the main β-glucosidase inhibitor in jujube leaf tea; thus, we only investigated fractions 5, 6, 7, and 8. From these fractions, the main inhibitor was separated and purified and further characterized.

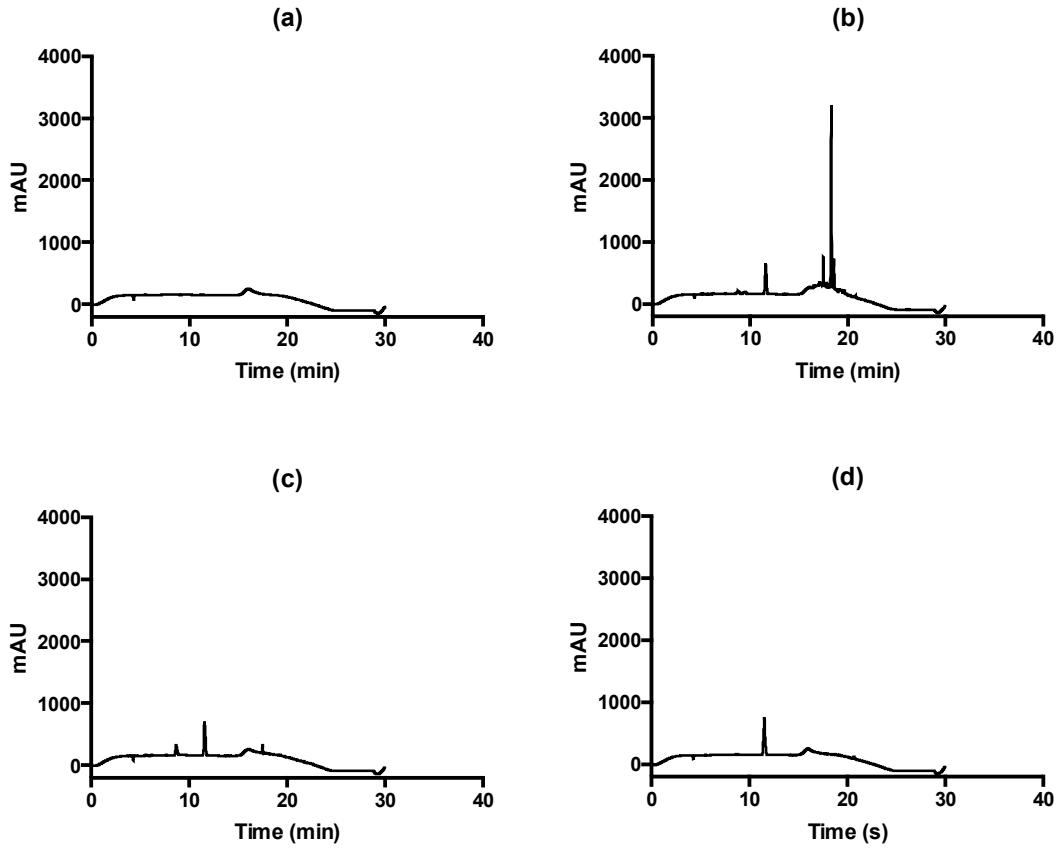

Figure IV-1. HPLC chromatograms of each step of isolation and purification. (a) distilled water (DW) which was a solvent of each step, (b) jujube leaf extract, (c) crude β-glucosidase inhibitor after size-exclusion chromatography, (d) purified β-glucosidase inhibitor after preparative HPLC.

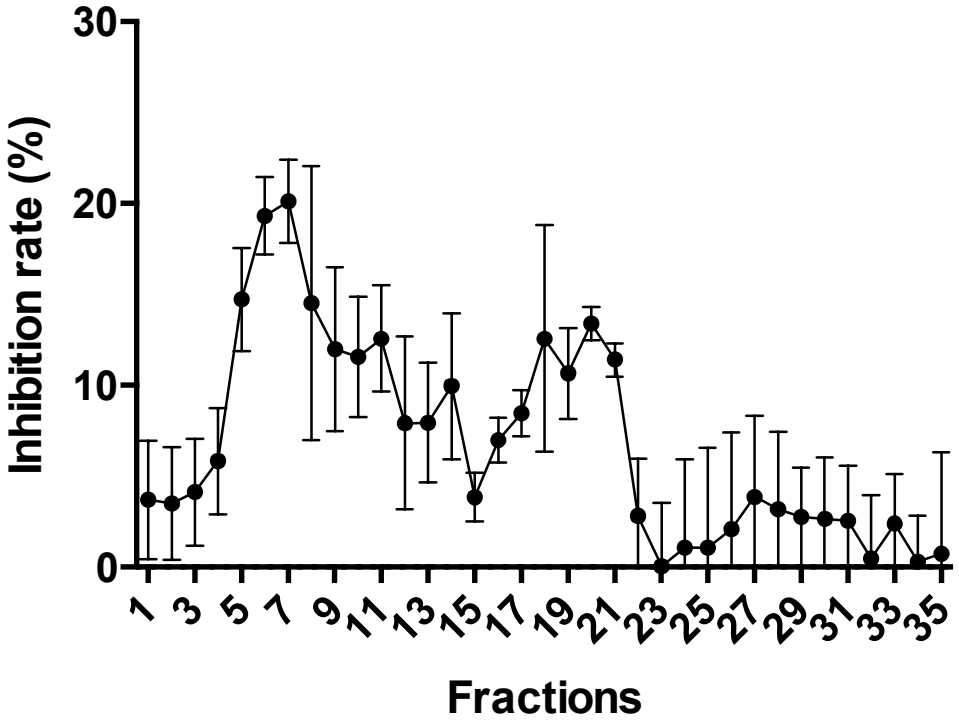

Figure IV-2. Size-exclusion chromatography of jujube leaf extract using Sephadex G-25 column. Fractions were collected and assayed for the inhibition rate of β-glucosidase.

IV-3-1-2. Semi-preparative LC

The size exclusion chromatography fractions (fractions 5-8) that contained the majority of β-glucosidase inhibitory activity had three peaks, as shown in Figure IV-1 c. Each peak was separated and purified using semi-preparative LC, then each peak's rate of inhibition was measured to identify which peak was the strongest β-glucosidase inhibitor. The inhibition rate of each peak (at retention times 8.80, 11.58, and 17.51 min) was 2.81 ± 0.72%, 45.03 ± 0.75%, and 4.15 ± 1.16%, respectively. Of these, the second peak with the retention time of 11.58 min showed the strongest inhibition of 45.03 ± 0.75% (Figure IV-3). This substance was isolated and purified using semi-preparative LC to a purity of over 90% as compared to the baseline, as shown in Figure IV-1 a and Figure IV-1 d.

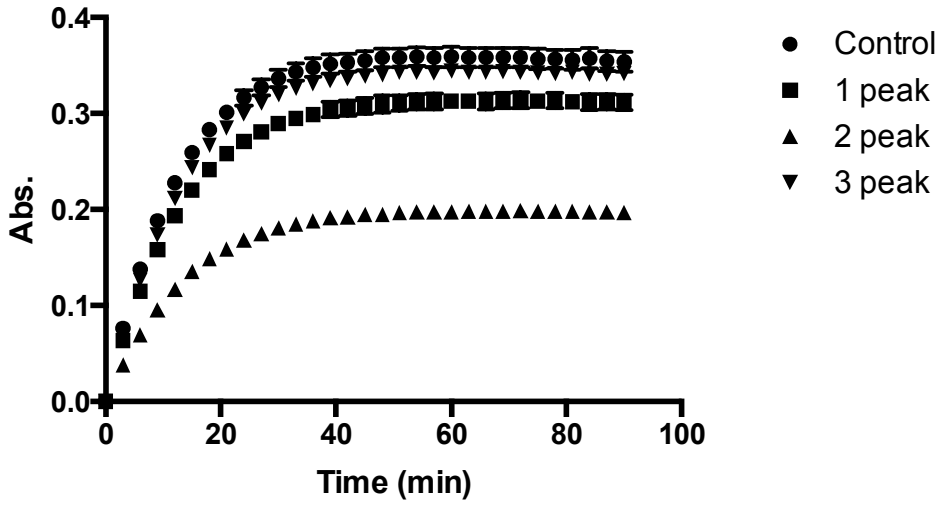

Figure IV-3. The comparison of β-glucosidase activity of each peak obtained from semi-preparative LC for purification.

IV-3-2. Characteristic of β-glucosidase inhibitor from jujube leaf

IV-3-2-1. Prediction of empirical formula

To determine whether this substance is present in jujube leaves, the molecular weight was measured using DIP-MS. The FAB-MS exhibited $[M+K]^+$ ion at *m/z* 431, $[M+Na]^+$ ion at *m/z* 415, and $[M+H]^+$ ion at *m/z* 393; thus, the predicted molecular weight was approximately 392 g/mol (Figure IV-4). No previous studies have reported a substance with this molecular weight in jujube leaves. Moreover, most of the well-known inhibitors of β-glucosidase have a molecular weight of less than 200 g/mol, except bromoconduritol (209.04 g/mol) (Table IV-2). For predicting the empirical formula, the constituent elements were identified using an elemental analyzer. The elemental composition of this novel β-glucosidase inhibitor from jujube leaves is shown in Table IV-3. Based on the molecular weight and elemental composition, the empirical formula of this novel β-glucosidase inhibitor is predicted to be $C_{17}H_{23}O_{13}N$. This structure has not been referenced in previous β-glucosidase inhibitor studies. Therefore, it is considered to be a new substance not previously described in any study of natural products. It is expected that much time and effort will be needed because there is a lack of information on the properties of this novel β-

glucosidase inhibitor (e.g., solubility and reactivity with solvent) and the molecular weight is large to analyze the structure Furthermore, there is a limitation to collect sufficient amount of β-glucosidase inhibitor of high purity (90% or more) to measure NMR. So, before studying its structure, studies were first conducted to analyze the characteristics of this inhibitor.

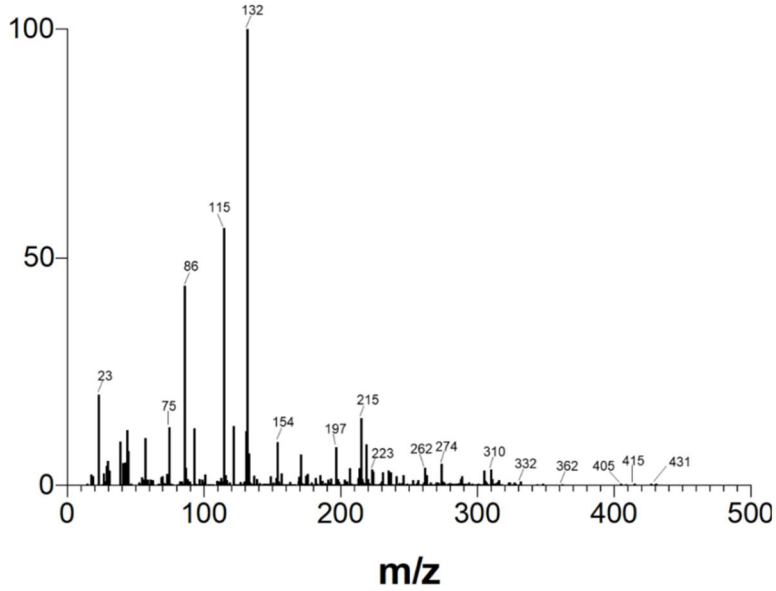

Figure IV-4. Direct injection probe-MS data of β-glucosidase inhibitor from jujube leaf extract.

Table IV-2. The molecular weight of well-known β-glucosidase inhibitors

Name	Molecular Weight (g/mol)
Conduritol β Epoxide	162.14
Castanospermine	189.21
Bromoconduritol	209.04
Deoxynojirimycin	163.17
Conduritol B	146.14
Cyclophellitol	176.17

Table IV-3. The element composition of β-glucosidase inhibitor from jujube leaf

Element Name	Retention Time (min)	Weight %
Nitrogen	47	2.58
Carbon	74	43.82
Hydrogen	204	4.39
Oxygen	143	46.41
Total		97.2

IV-3-2-2. Enzyme kinetic study

Kinetic studies of this novel inhibitor were performed to investigate its characteristics. Michaelis-Menten plots were transformed to Hanes-Woolf plots to determine enzyme kinetic properties (Figure IV-5). With increased concentrations of the inhibitor from 0 to 4 mg/mL, the V_{max} (1/slope) decreased from 0.0062 to 0.0039, whereas the K_m value (x-axis) increased from 1.87 to 2.75. These trends in K_m and V_{max} represent mixed non-competitive inhibition (Javaid, Saad, Rasheed, Moin, Syed, Fatima, et al., 2015). Then, the K_i value is dissociation constants of enzyme-inhibitor complex, it could be obtained as 0.026 mg/mL using by Sigmaplot software (version 12.5).

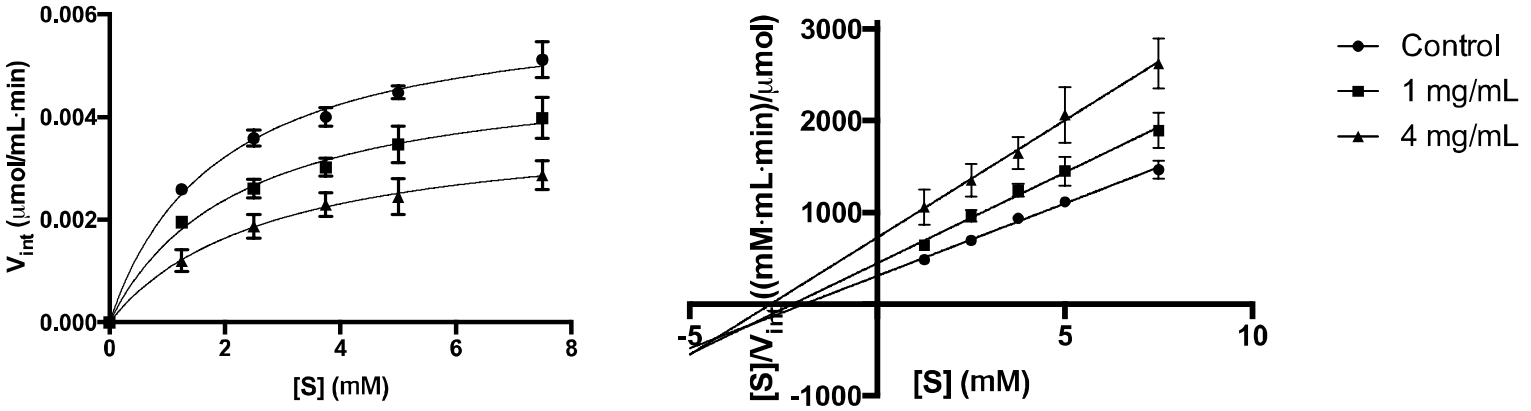

Figure IV-5. Michaelis-Menten plot (a) and Hanes-Woolf plot (b) for kinetic study of β -glucosidase inhibition on the novel β-glucosidase inhibitor from jujube leaf extract.

Despite the fact that the novel inhibitor is a single compound, a mixed inhibition pattern was revealed. There are many examples in which mixed inhibition is observed for a single inhibitor compound. The novel inhibitor is a non-competitive inhibitor that binds to the allosteric site, and it is anticipated that the inhibitor also binds to an allosteric site that is close to the active site (exosite), resulting in a mixed inhibition pattern (Blat, 2010).

To further characterize the inhibitory properties of this inhibitor, its IC_{50} value was determined and compared to that of a well-known β-glucosidase inhibitor, conduritol β-epoxide (Figure IV-6). The IC_{50} value of conduritol β-epoxide was 0.68 μmol/mL, whereas the IC_{50} value of the novel inhibitor from JLE was 6.6 μmol/mL (Table IV-4).

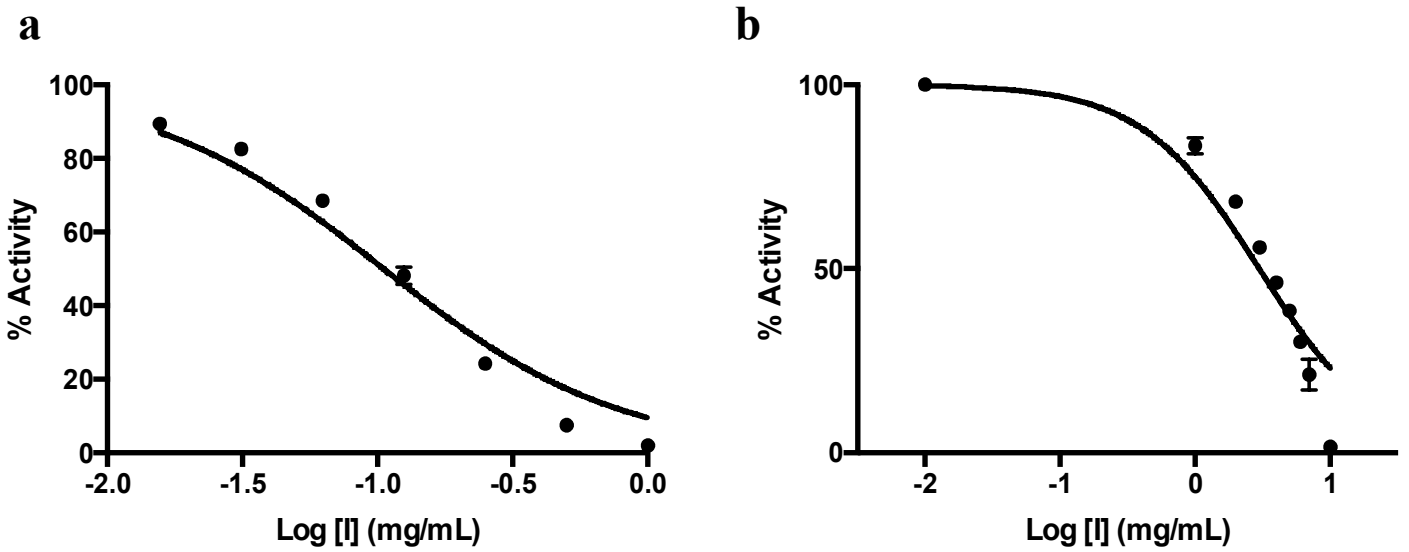

Figure IV-6. The inhibition rate of β-glucosidase with β-epoxide (a) and β-glucosidase inhibitor from jujube leaf extract (b).

Table IV-4. β-Glucosidase inhibitory activity of conduritol β-epoxide and inhibitor from jujube leaf extract

	IC_{50} **value**
Conduritol β-epoxide	0.11 mg/mL
Inhibitor from JLE	2.98 mg/mL

Although its degree of inhibition was weaker than that of conduritol β-epoxide, it is expected that the novel inhibitor will be used in food because of its history as being part of tea in ancient times. Furthermore, the novel inhibitor is expected to have fewer side effects than its chemically synthesized counterparts because it is a naturally derived substance. In addition, the competitive inhibition effect of other inhibitors such as conduritol β-epoxide could be overcome by a higher concentration of substrate, which is a disadvantage in many applications (de Melo, da Silveira Gomes, & Carvalho, 2006; Moitra, 2015). However, increased substrate concentrations have no effect on non-competitive inhibitors, which is an apparent advantage over competitive inhibitors.

IV-4. Conclusion

The β-glucosidase inhibitor was isolated and purified from jujube leaf extract. The predicted molecular weight of this inhibitor was 392 g/mol. This molecular weight has not been reported as β-glucosidase inhibitor. In addition, it has a larger molecular weight than known β-glucosidase inhibitors. Prior to the analysis of the structure of this inhibitor, the inhibitory properties of this β-glucosidase inhibitor were confirmed. This β-glucosidase inhibitor has mixed non-competitive inhibition and it has weaker inhibition than that of β-epoxide. And this novel inhibitor has several advantages, such as its ease of use in the food and pharmaceutical industries because it can be extracted with water and its temperature and heat stability. The discovery of novel inhibitors from natural products could provide new information that can be used in various aspects of research in the food, phytochemical, and pharmaceutical sciences. However, further studies of the structure of inhibitor and inhibition mechanisms of inhibitor are needed.

IV-5. References

An, H., Wang, H., Lan, Y., Hashi, Y., & Chen, S. (2013). Simultaneous qualitative and quantitative analysis of phenolic acids and flavonoids for the quality control of *Apocynum venetum* L. leaves by HPLC–DAD–ESI–IT–TOF–MS and HPLC–DAD. *J. Pharm. Biomed. Anal., 85*, 295-304.

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

Blat, Y. (2010). Non-competitive inhibition by active site binders. *Chem. Bio. Drug Des., 75*(6), 535-540.

Chen, X.-M., Kitts, D. D., & Ma, Z. (2017). Demonstrating the importance of phytochemical profile of different teas on antioxidant and anti-inflammatory capacities. *Funct. Food. Health Disease, 7*(6), 375-395.

de Melo, E. B., da Silveira Gomes, A., & Carvalho, I. (2006). α-and β-Glucosidase inhibitors: chemical structure and biological activity. *Tetrahedron, 62*(44), 10277-10302.

Ganem, B. (1996). Inhibitors of carbohydrate-processing enzymes: design and synthesis of sugar-shaped heterocycles. *Acc. Chem. Res., 29*(7), 340-347.

Javaid, K., Saad, S. M., Rasheed, S., Moin, S. T., Syed, N., Fatima, I., Salar, U., Khan, K. M., Perveen, S., & Choudhary, M. I. (2015). 2-Arylquinazolin-4 (3H)-ones: a new class of α-glucosidase inhibitors. *Bioorga. Medi. Chem., 23*(23), 7417-7421.

Jo, Y., Lim, S., Chang, P.-S., & Choi, Y. J. (2016). The possible presence of natural β-d-glucosidase inhibitors in jujube leaf extract. *Food Chem., 194*, 212-217.

Kwon, Y.-I., Apostolidis, E., & Shetty, K. (2008). *In vitro* studies of eggplant (*Solanum melongena*) phenolics as inhibitors of key enzymes relevant for type 2 diabetes and hypertension. *Biores. Tech., 99*(8), 2981-2988.

Lieberman, R. L., Wustman, B. A., Huertas, P., Powe, A. C., Pine, C. W., Khanna, R., Schlossmacher, M. G., Ringe, D., & Petsko, G. A. (2007). Structure of acid β-glucosidase with pharmacological chaperone provides insight into Gaucher disease. *Nature Chem. Bio., 3*(2), 101-107.

Moitra, K. (2015). Overcoming multidrug resistance in cancer stem cells. *Bio. Med. Res. Int., 2015*.

Newman, D. J., & Cragg, G. M. (2016). Natural products as sources of new drugs from 1981 to 2014. *J. Nat. Prod., 79*(3), 629-661.

Nishimura, Y. (2003). gem-Diamine 1-N-iminosugars and related iminosugars, candidate of therapeutic agents for tumor metastasis. *Curr. Topics Med. Chem., 3*(5), 575-591.

Rempel, B. P., & Withers, S. G. (2008). Covalent inhibitors of glycosidases and their applications in biochemistry and biology. *Glycobiology, 18*(8), 570-586.

Valiathan, M. S. (1998). Healing plants. *Current science,* 75(11), 1122-1126.

Venkatesh, S., Reddy, G. D., Reddy, B. M., Ramesh, M., & Rao, A. A. (2003). Antihyperglycemic activity of Caralluma attenuata. *Fitoterapia, 74*(3), 274-279.

Vila-Real, H., Alfaia, A. J., Bronze, M. R., Calado, A. R., & Ribeiro, M. H. (2011). Enzymatic synthesis of the flavone glucosides, prunin and isoquercetin, and the aglycones, naringenin and quercetin, with selective α-L-rhamnosidase and β-D-glucosidase activities of naringinase. *Enz. Res., 2011*.

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. *J. Mole. Catal. B: Enz., 81*, 37-42.

Wang, J., Sun, G.-X., Yu, L., Wu, F.-A., & Guo, X.-J. (2013). Enhancement of the selective enzymatic biotransformation of rutin to isoquercitrin using an ionic liquid as a co-solvent. *Biores. Tech., 128*, 156-163.

Zhang, R., Chen, J., Shi, Q., Li, Z., Peng, Z., Zheng, L., & Wang, X. (2014). Phytochemical analysis of Chinese commercial *Ziziphus jujube* leaf tea using high performance liquid chromatography–electrospray ionization-time of flight mass spectrometry. *Food Re. Int., 56*, 47-54.

Zhang, Y., Zhu, K., Miao, X., Hu, X., & Wang, T. (2016). Identification of β-glucosidase 1 as a biomarker and its high expression in hepatocellular carcinoma is associated with resistance to chemotherapy drugs. *Biomarkers, 21*(3), 249-256.

Chapter V. Application of β-glucosidase inhibitory activity of jujube leaf to the production of functionalized grapefruit juice

V-1. Introduction

Naringin is a compound that is a major contributor to the bitterness of citrus juices such as grapefruit and bitter orange. Naringin is one of the factors that degrades the quality of grapefruit juice (Puri, Marwaha, Kothari, & Kennedy, 1996). Therefore, the food industry uses a glycoside hydrolase enzyme, naringinase, to remove naringin (Puri & Banerjee, 2000). Naringinase is an enzyme complex that cleave rhamnose and glucose from naringin as shown in Figure V-1; the final product is naringenin (Ribeiro, 2011).

Naringinase can improve the quality of grapefruit juice because naringenin is tasteless and has a variety of useful properties such as antioxidant, free radical scavenger, anti-inflammatory, anti-carcinogenic, anti-mutagenic, and immune system modulator (Cavia-Saiz, Busto, Pilar-Izquierdo, Ortega, Perez-Mateos, Muñiz, et al., 2010; Puri, Marwaha, Kothari, & Kennedy, 1996). Despite these functionalities, the solubility of naringenin in water is very low (0.214 mg/mL), resulting in poor bioavailability (Borzova, Gudzenko, & Varbanets, 2018; Hsiu, Huang, Hou, Chin, & Chao, 2002).

However, the intermediate product of naringin, prunin, has a water

solubility of 1.53 mg/mL, which is higher than that of naringenin. Like naringenin, prunin has many useful functionalities such as anti-inflammatory, antiviral, sweetening agent in diabetes therapy, prevention of gastric mucosal ulceration, treatment of cardiovascular disease, obesity, hepatic steatosis, and cardiomyopathy associated with doxorubicin (Yadav, Yadav, Yadav, & Yadav, 2010). To produce prunin, which has better solubility in water than naringenin and has a variety of functions, it is necessary to inhibit the β-glucosidase activity of naringinase. There are various methods for inhibiting the activity of β-glucosidase (Wang, Sun, Yu, Wu, & Guo, 2013), but the method most suitable for the food industry is the use of food-grade inhibitors.

In the previous study, a β-glucosidase inhibitory activity was identified from jujube leaves, and it is available as food (Jo, Lim, Chang, & Choi, 2016). The aim of the present study was to produce grapefruit juice with enhanced functionality by degrading naringin and enhance the production of prunin using the β-glucosidase inhibitory effect of jujube leaves

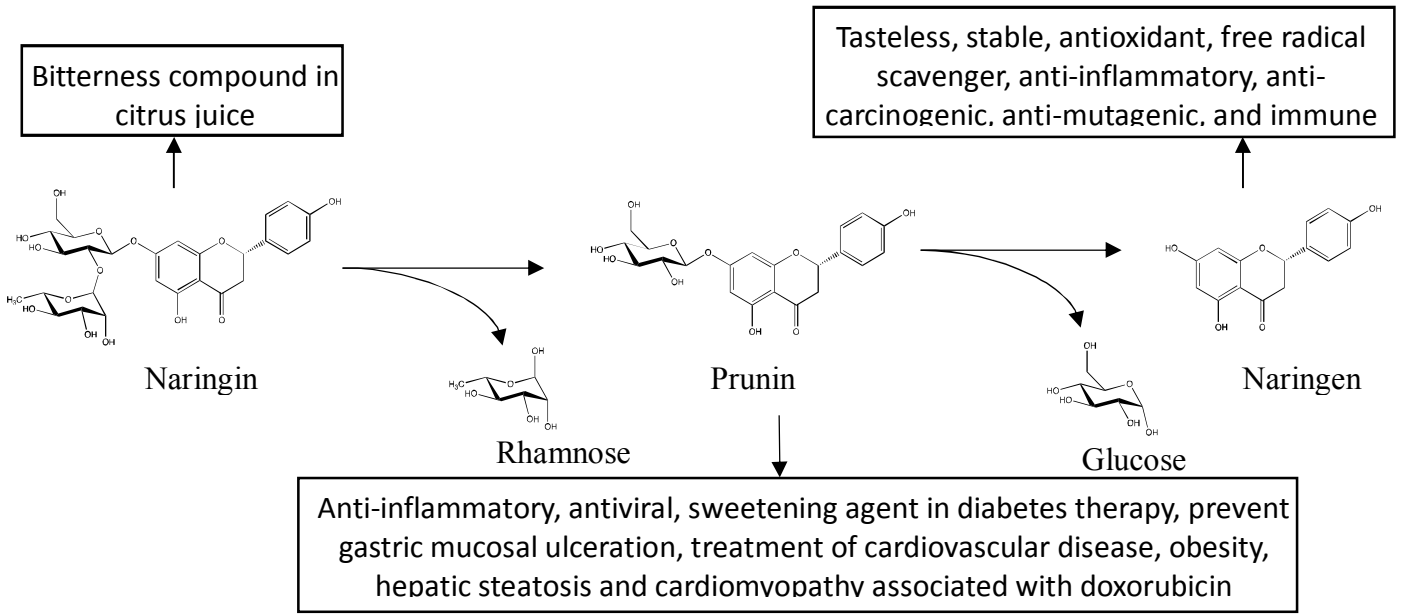

Figure V-1. Hydrolysis of naringin into prunin, and naringenin by naringinase containing α-L-rhamnosidase activities and β-D-glucosidase activities.

V-2. Materials and Methods

V-2-1. Chemicals

Grapefruits were purchased from the local market. Naringinase, an enzyme used for enzymatic treatment of debittering, was purchased from Vision Biochem Co. (Sungnam, Korea). HPLC standards, namely naringin, prunin, and naringenin, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

V-2-2. Enzymatic biotransformation

All enzymatic biotransformation experiments were conducted in a temperature-controlled shaking water bath 50 °C with an agitation rate of 600 rpm. An enzyme solution was prepared by adding 30 mg of naringinase to 1 mL of water. For making grapefruit juice, the grapefruit peel was removed, and the grapefruit was ground with a mixer (HMF-1000, Hanil Electric, Seoul, Korea), followed by filtration with filter No. 4. First, 0.2 mL of enzyme solution were mixed with 0.1 mL of various jujube leaf extract concentrations (0, 5, and 10 mg/mL), followed by incubation for 10 min. Then, 2.7 mL of grapefruit juice were added. Samples were collected every 30 min for 120 min and were transferred to a 90 °C water bath for 15 min to terminate the enzymatic reactions.

V-2-3. HPLC analysis

HPLC analysis was performed to observe the enzymatic biotransformation of naringin in grapefruit juice by naringinase. The concentrations of naringin, prunin, and naringenin were measured by HPLC (Ultimate 3000, Thermo Scientific Dionex, Sunnyvale, CA, USA) coupled to a UV detector. A C-18 column was used to separate and detect the naringin, prunin, and naringenin at 250 nm. The gradient condition of HPLC is shown in Table V-1.

Table V-1. HPLC gradient condition for analyzing naringin, prunin, and naringenin in grapefruit juice

Flow rate	1 mL/min	
Solvent	A: Acetonitrile, B: DW	
Temperature	25°C	
Time (min)	A (%)	B(%)
0	5.0	95.0
4	5.0	95.0
14	40.0	60.0
16	40.0	60.0
24	70.0	30.0
26	5.0	95.0
32	5.0	95.0

V-2-4. Response surface methodology

To optimize the reaction conditions for producing prunin via of naringinase activity, response surface methodology was performed using the Box-Behnken design (BBD). Based on the experimental results, the concentration of enzyme (X_1, 10-30 mg/mL), processing time (X_2, 10-50 min), and reaction temperature (X_3, 30-70 °C) were selected as independent variables and the concentration of prunin (mmol/mL) was considered to be the responsive variable. The levels of independent variables were coded as -2, -1, 0, 1, or 2 (Table V-2). The range and levels of experimental design are listed in Table V-3. The following quadratic polynomial equation was used to fit the experimental data:

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

where Y is the responsible variable; X_1, X_2, and X_3 are the independent variables; β_0 is the model intercept coefficient; β_1, β_2, and β_3 are the linear coefficients; β_{12}, β_{13}, and β_{23} are interaction coefficients among the three factors; and β_{11}, β_{22}, and β_{33} are the quadratic coefficients.

Experimental design and statistical analysis were conducted using the Statistical Analysis System (SAS, Version 9.3, Cary, NC, USA). ANOVA and *F*-tests were used for statistical assessments. The quadratic models for the independent variables are shown as 3D and 2D contour plots.

Table V-2. Levels of independent variables for experimental design

X_i	Independent variables	Levels				
		-2	-1	0	1	2
X_1	Concentration of enzyme (mg/mL)	10	15	20	25	30
X_2	Processing time (min)	10	20	30	40	50
X_3	Reaction temperature (°C)	30	40	50	60	70

Table V-3. Box Behnken design for optimization of production of prunin

	X_1 (Con. Enz.)	X_2 (Time)	X_3 (Temp.)
1	15	20	40
2	15	20	60
3	15	40	40
4	15	40	60
5	25	20	40
6	25	20	60
7	25	40	40
8	25	40	60
9	20	30	50
10	20	30	50
11	10	30	50
12	30	30	50
13	30	30	50
14	20	10	50
15	20	50	50
16	20	30	30
17	20	30	70

V-2-5. Statistical analysis

All results were analyzed using Tukey's significant difference test with IBM SPSS Statistics version 21.0 (IBM Co., Armonk, NY, USA). At least three independent replicates were performed in each experiment.

V-3. Results and Discussion

V-3-1. Enzymatic biotransformation

The concentration of naringin, a bitter ingredient in grapefruit juice, was approximately 1.64 ± 0.07 mol/mL before enzyme treatment and decreased to 0.12 ± 0.01 mol/mL after a 30 min enzymatic treatment, regardless of the enzyme inhibitor concentration. The concentration of prunin and naringenin increased until 30 min after enzyme reaction, the concentration of prunin decreased after 30 min of enzyme treatment, and the concentration of naringenin increased continuously under all experiment conditions. As the concentration of inhibitor increased from 0, 5, to 10 mg/mL, the amount of prunin produced during a 30-min reaction time was 0.88 ± 0.59, 1.00 ± 0.08, and 1.10 ± 0.02 mol/mL, respectively (Figure V-2). These results suggest that the jujube leaf extract specifically inhibited β-glucosidase without inhibiting the activity of α-rhamnosidase. Jujube leaf extract at 10 mg/mL was able to produce enhanced functionalized grapefruit juice containing an approximately 1.25-fold higher concentration of prunin as compared to the conventional grapefruit juice production process.

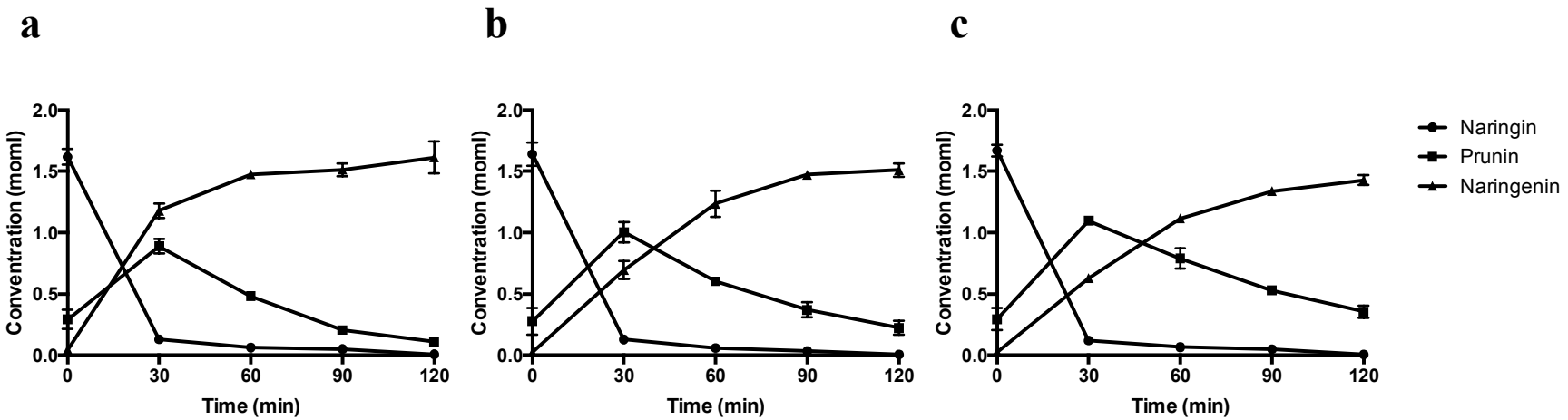

Figure V-2. Enzyme biotransformation of naringin to prunin and naringenin with various jujube leaf extract concentration (a) 0, (b) 5, (c) 10 mg/mL.

V-3-2. Response surface methodology

To investigate the effect of jujube leaves on the production of prunin, the production of prunin was optimized for samples with jujube leaf and those without jujube leaf. The optimal level of factors (concentration of enzyme, processing time, and reaction temperature) and the effect of their interaction on prunin production were determined by Box-Behnken design.

Naringinase converted the naringin of grapefruit juice to prunin in the range of 0.39 to 1.89 mg/mL. In the presence of jujube leaf, the concentration of prunin was in the range of 0.38 to 2.47 mg/mL. The supplementation of grapefruit juice with 5 mg/mL of jujube leaf increased the yield of prunin 1.31-fold.

The independent and response variables were analyzed in this study to obtain regression equations to predict the response under the given conditions. The polynomial regression equations for prunin yield without jujube leaf (Y_1) are as follows:

$$Y_1 = 0.227949x_1 + 0.022937x_2 + 0.081937x_3 - 0.004059x_1^2 + 0.00325x_1x_2 - 0.000112x_2^2 - 0.001025x_3x_1 - 0.00313x_3x_2 - 0.000550x_3^2 - 3.288287$$

The polynomial regression equations obtained for prunin yield with jujube leaf (Y_2) are as follows:

$$Y_2 = 0.230486x_1 + 0.054688x_2 + 0.091938x_3 - 0.003389x_1^2 - 0.000125x_1x_2 + 0.0001x_2^2 - 0.001475x_3x_1 - 0.001012x_3x_2 - 0.000337x_3^2 - 3.836806$$

Based on ANOVA, the *p*-values of total regressions were 0.105 and 0.2137 and are statistically significant (p <0.5); thus, the assumed model was appropriate. The linear regression of enzyme concentration, processing time, and reaction temperature on prunin yield was highly significant (0.059) at Y_1 and the quadratic regression of concentration of enzyme, processing time, and reaction temperature on prunin yield was highly significant (0.164) at Y_2. A coefficient of determination (R^2) greater than 0.60 in RSM generally represents statistical significance. In this study, the R^2 values were 0.71 at Y_1 and 0.77 at Y_2. These results indicated that these statistical models could explain 71% and 77% of the variability in the response variable and experimental and predicted yields have good agreement.

The 3D plots for Y_1 and Y_2 are shown in Figure V-3 (a-c) and Figure V-3 (d-f), respectively. The optimum conditions for prunin production are shown in Table V-4. The production of prunin increased approximately 1.31-

times, from 1.89 to 2.47 mmol/mL, when jujube leaf was added. Furthermore, the amount of enzyme required decreased from 27.1 mg/mL to 26.5 mg/mL and the treatment temperature decreased from 35 °C to 30.1 °C.

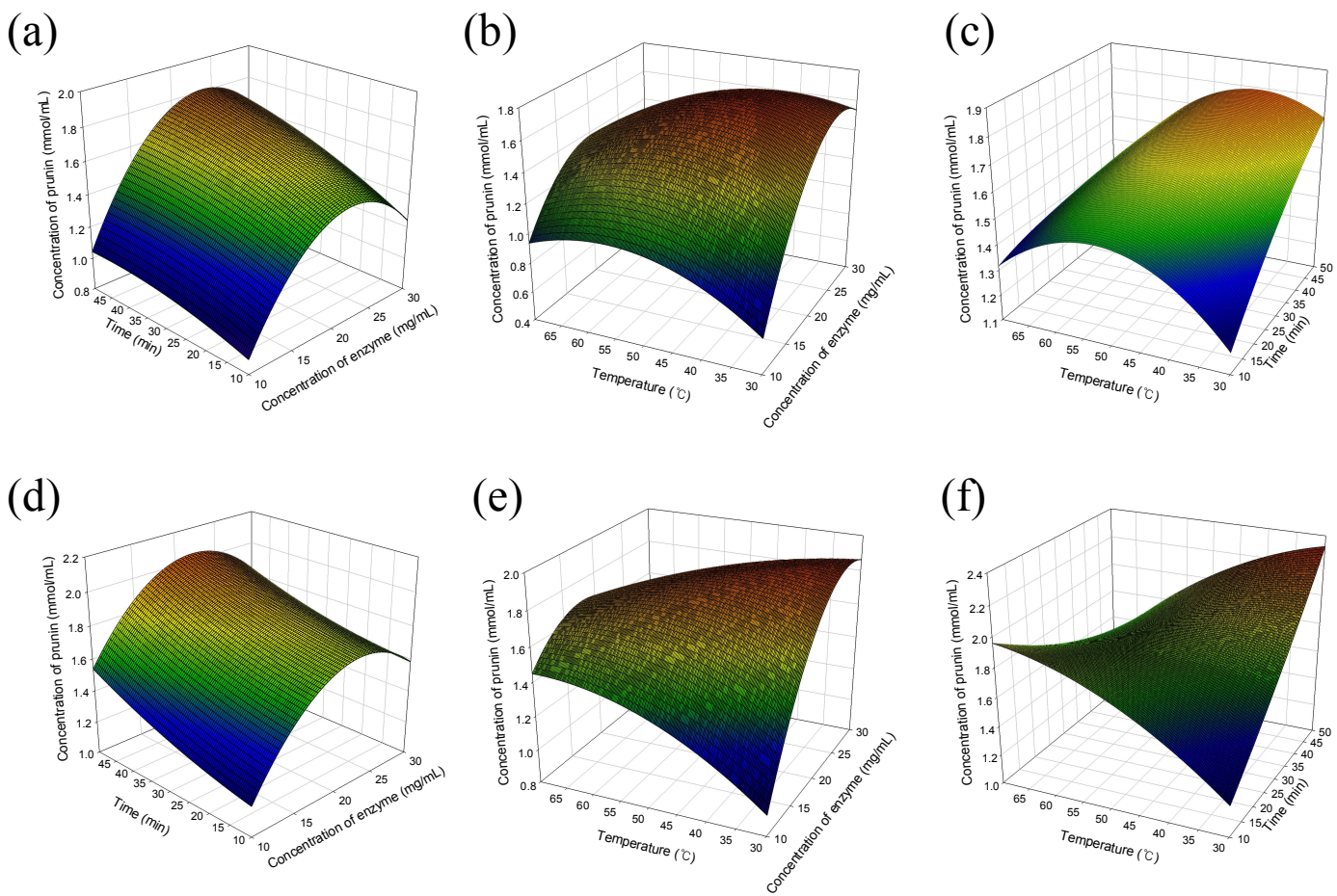
(a)
Concentration of prunin (mmol/mL)
Time (min)
Concentration of enzyme (mg/mL)
(b)
Concentration of prunin (mmol/mL)
Temperature (℃)
Concentration of enzyme (mg/mL)
(c)
Concentration of prunin (mmol/mL)
Temperature (℃)
Time (min)
(d)
Concentration of prunin (mmol/mL)
Time (min)
Concentration of enzyme (mg/mL)
(e)
Concentration of prunin (mmol/mL)
Temperature (℃)
Concentration of enzyme (mg/mL)
(f)
Concentration of prunin (mmol/mL)
Temperature (℃)
Time (min)

Figure V-3. The 3D plots for predicted responses. Response surface for the effect of concentration of enzyme and reaction time (a), treatment temperature and concentration of enzyme (b), reaction time and treatment temperature on producing prunin concentration without jujube leaf (c). Response surface for the effect of concentration of enzyme and reaction time (d), treatment temperature and concentration of enzyme (e), and reaction time and treatment temperature on producing prunin concentration with jujube leaf (f).

Table V-4. The optimum conditions of prunin production with/without jujube leaf during grapefruit juice production process

	With jujube leaf	Without jujube leaf
X_1 (Concentration of Enzyme (mg/mL))	26.5	27.1
X_2 (Reaction time (min))	50.0	50.0
X_3 (Treatment temperature (°C))	30.1	35.0
Y (Concentration of prunin (mmol/mL))	2.47	1.89

V-4. Conclusion

The functionalized grapefruit juice with increased prunin content was produced using the β-glucosidase inhibitory activity of jujube leaf. In the grapefruit juice producing process, the naringinase was used to remove the bitter ingredient, naringin. In order to produce grapefruit juice with increased the content of prunin as a functional material, jujube leaf extract was used in this process. It was confirmed that the content of prunin was effectively increased when the jujube leaf extract was added. The maximum prunin concentration and its optimum conditions were investigated by RSM according to the presence or absence of jujube leaf extract. The addition of jujube leaf allows grapefruit juice containing a higher amount of prunin to be produced using a lower treatment temperature and less amount of enzyme as compared to without jujube leaf. The water extract of jujube leaf is expected to be widely used in the food industry.

V-5. References

Borzova, N., Gudzenko, O., & Varbanets, L. (2018). Purification and Characterization of a Naringinase from Cryptococcus albidus. *App. Biochem. Biotech., 184*(3), 953-969.

Cavia□Saiz, M., Busto, M. D., Pilar□Izquierdo, M. C., Ortega, N., Perez□Mateos, M., Muñiz, P. (2010). Antioxidant properties, radical scavenging activity and biomolecule protection capacity of flavonoid naringenin and its glycoside naringin: a comparative study. *J. Sci. Food Agri., 90*(7), 1238-1244.

Hsiu, S.-L., Huang, T.-Y., Hou, Y.-C., Chin, D.-H., & Chao, P.-D. L. (2002). Comparison of metabolic pharmacokinetics of naringin and naringenin in rabbits. *Life Sci., 70*(13), 1481-1489.

Jo, Y., Lim, S., Chang, P.-S., & Choi, Y. J. (2016). The possible presence of natural β-d-glucosidase inhibitors in jujube leaf extract. *Food Chem., 194*, 212-217.

Puri, M., & Banerjee, U. C. (2000). Production, purification, and characterization of the debittering enzyme naringinase. *Biotech. Advan., 18*(3), 207-217.

Puri, M., Marwaha, S., Kothari, R., & Kennedy, J. F. (1996). Biochemical basis of bitterness in citrus fruit juices and biotech approaches for debittering. *Crit. Rev. Biotech., 16*(2), 145-155.

Ribeiro, M. H. (2011). Naringinases: occurrence, characteristics, and applications. *Applied microbiology and biotechnology, 90*(6), 1883.

Wang, J., Sun, G.-X., Yu, L., Wu, F.-A., & Guo, X.-J. (2013). Enhancement of the selective enzymatic biotransformation of rutin to isoquercitrin using an ionic liquid as a co-solvent. *Biores. Tech., 128*, 156-163.

Yadav, V., Yadav, P. K., Yadav, S., & Yadav, K. (2010). α-L-Rhamnosidase: a review. *Proc. Biochem., 45*(8), 1226-1235.

국문 초록

대추 잎은 항산화, 혈압조절, 등의 효능이 있는 것으로 알려져 있어서 고대에는 한국, 중국 등 아시아 지역에서 약으로 이용되어왔다. 하지만 잎에는 열매에 비해 더욱 풍부한 플라보노이드와 폴리페놀이 존재함에도 불구하고 최근 대부분의 연구가 열매로 집중되면서 잎은 부산물로 여겨져 버려지고 있다.

플라보노이드와 폴리페놀은 녹차를 포함한 잎을 이용한 차류에 풍부하다고 알려져 있으며, 이들은 α-글루코시데이즈의 활성을 저해하는 것으로 알려져 있다. 이러한 α-글루코시데이즈 저해제들은 당뇨와 비만의 치료제로 활용 가능성이 있어 꾸준히 관심을 받고 있다. 최근 아카보스와 같이 α-글루코시데이즈 저해활성을 이용한 시판약들이 가진 단점들 때문에 천연의 α-글루코시데이즈 저해제를 찾는 연구가 활발히 진행되고 있다. 하지만 대추 잎에는 알려지지 않은 물질들이 다수 존재하며, 다양한 플라보노이드와 폴리페놀이 존재함에도 불구하고, 대추 잎에 대한 α-글루코시데이즈 저해활성에 관한 연구가 진행되어 있지 않았다. 그래서 이번 연구에서 대추 잎의 α-글루코시데이즈 저해활성에 대해 알아보았다.

기존에 알려진 대추 잎에 존재하는 α-글루코시데이즈 저해제의 α-글루코시데이즈 저해활성은 아카보스에 비해 약할 것으로 예상되었지만, 예비 실험 결과 아카보스보다 약 2 배 강한 저해를 나타냈다. 이와 같이 예상보다 강한 저해가 나타나게 된 원인에 대해 알아보는 연구를 진행하였다. 가장 저해가 강한 물질을 분리 정제해 본 결과 3',5'-di-*C*-β-D-glucosyl phloretin 이라는 물질에 의해 강한 저해가 나타나는 것으로 확인되었다. 이 물질은 기존에 α-글루코시데이즈 저해능력에 관하여 보고된 바가 없는 물질이었으며, α-글루코시데이즈 저해능력을 확인해 본 결과 아카보스에 비해 약 13.5 배 강한 저해를 하였다. 그리고 이 저해제는 α-아밀레이즈는 저해하지 않고, 선택적으로 α-글루코시데이즈만을 저해하여 아카보스보다 부작용이 적을 것으로 기대되었다. 게다가 모방 체외 위장관 환경하에서 실험한 결과 통계적으로 유의미한 차이 없이 α-글루코시데이즈를 저해하는 것을 확인하였다.

대추 잎의 α-글루코시데이즈 저해활성을 이용하여 식후 혈당 조절에 도움을 줄 수 있는 기능성 밥의 제조를 해 보았다. 밥은 한국, 인도, 중국 등 여러 나라에서 주식으로 하고 있다. 하지만 혈당지수가 매우 높은 식품에 속하여 식후 혈당 조절이

필요한 당뇨환자들은 백미보다 현미를 권하고 있다. 하지만 현미의 식감 때문에 많은 환자들이 현미를 기피하고 있다. 그래서 이번 연구에서 대추 잎의 α-글루코시데이즈 저해활성을 이용하여 식후 혈당 조절에 도움을 줄 수 있고, 식감은 백미와 유사한 기능성 밥을 제조하는 것을 목표로 하였다. 대추 잎 추출물의 양이 0, 5, 10 mg/mL 첨가되어도 밥의 하드니스와 스티키니스에는 영향이 없는 것을 관찰하였다. 대추 잎 추출물의 양이 0, 5, 10 mg/mL 로 증가할수록 당 분해가 최대치에 도달하는 시간이 180, 210, 360 분으로 증가하였다. 향과 색은 있었지만 기호도에 의한 관능평가를 하였을 때 기존의 즉석밥과 통계적으로 유의미한 차이가 없었다. 이를 통해 대추 잎의 α-글루코시데이즈 저해 활성을 이용하여 기능성 밥 제조의 가능성을 확인할 수 있었다.

대추 잎에 풍부한 루틴을 아이소퀘시틴으로 전환하는 과정에서 β-글루코시데이즈 저해제의 존재 가능성에 대해 연구하기 시작하였고, β-글루코시데이즈 저해제의 존재를 증명하였다. 대추 잎에 존재하는 β-글루코시데이즈 저해제를 분리 정제하였다. 그 저해제의 예상 분자량은 392 g/mol 이었으며, 분자식은 $C_{17}H_{23}O_{13}N$ 으로 예상되었다. 그리고 이 저해제는 혼합 비경쟁적 저해의 양상을 나타내는 것으로 확인되었다. 이 물질은 기존에

알려진 저해제가 아닌 새로운 저해제로 구조, 저해 기작에 대한 추가 연구가 필요하다.

대추 잎의 β-글루코시데이즈 저해활성을 이용하여 기능성이 강화된 자몽주스를 제조하였다. 쓴맛 성분인 나린진은 시트러스계 과일 주스 제조 시 품질저하의 주요한 원인이 된다. 이를 제거하기 위해 식품산업에서 나린지네이즈라는 효소를 사용하게 된다. 이때 나린지네이즈에 의해 나린진은 나린제닌으로 전환이되고, 이때 생성된 나린제닌은 아무런 맛이 없고, 다양한 기능성을 가지고 있는 것으로 알려져 있다. 하지만 이 성분은 다양한 기능성에도 불구하고 물에 대한 용해도가 낮아 생체내 이용율이 낮다는 단점이 있다. 여기서 대추 잎의 β-글루코시데이즈 저해 활성을 이용하여 나린진의 중간생성물이며, 다양한 기능성을 가지고 있고, 물에 대한 용해도가 나린제닌 보다 높은 푸르닌으로 전환이 되어 기능성이 강화된 자몽주스를 제조하는 것을 목표로 하였다. 나린지네이즈 처리 시 쓴맛 성분인 나린진은 효과적으로 제거되었고, 푸르닌의 함량이 대추 잎을 처리하지 않은 자몽주스에 비해 약 1.31 배 많은 2.47 mmol/mL 가 생성되었다. 게다가 더 낮은 온도, 더 적은 양의 효소로 최적점에 도달하는 것이

관찰되었고, 이 결과를 통해 식품산업에 적용 시 이점이 될 것으로 기대된다.

요컨대, 본 연구에서 대추 잎 유래의 새로운 α-, β-글루코시데이즈 저해제들을 얻을 수 있었고, 이들의 특성을 규명하였으며, 식품산업에 적용가능성도 확인할 수 있었다.

핵심어: α-글루코시데이즈 저해제, β-글루코시데이즈 저해제, 대추 잎, 천연물

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