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Article

Effects of Synergistic Inhibition on α-Glucosidase by Phytoalexins in Soybeans

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Abstract: To determine the mode of action of the effects of phytoalexins in soybeans, we analyzed enzyme inhibition kinetics using Michaelis–Menten plots and the Lineweaver–Burk plots. The results showed that glyceollin showed competitive inhibition, genistein showed noncompetitive, daidzein was uncompetitive, and luteolin showed a mixed mode of action. The *Ki* values were determined using a Dixon plot as: glyceollin, 18.99; genistein, 15.42; luteolin, 16.81; and daidzein, 9.99 μM, respectively. Furthermore, potential

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synergistic effects between glyceollin and the three designated polyphenols were investigated. A combination of glyceollin and luteolin (the ratio of 3:7 of glyceollin and luteolin) had synergistic effects on α-glucosidase inhibition according to combination index (CI)-isobologram equation. Collectively, these results showed that a combination of glyceollin and luteolin has the potential to inhibit α-glucosidase activity via a synergistic mode of action.

Keywords: α-glucosidase; glyceollin; genistein; luteolin; daidzein; phytoalexins; enzyme kinetics; combination index

1. Introduction

Soybeans are most human-friendly food produces in the world endowing beneficial purposes as ‘meat in the field’ because of its plentiful nutritional value. Presently, most countries produce soybeans to use as food ingredients, feed additive, or biomaterials etc. Annual consumption of soybeans is marked as tremendous tons a year and most important counties for production are China, USA, and Brazil etc. Research has shown that soybeans contain various compounds with beneficial functions. A high dietary intake of soybeans can reduce the risk of breast cancer and coronary heart disease, and has anti-diabetic effects by enhancing glucose uptake [1-3]. The soybeans especially contain effective compounds: phytoalexins such as genistein, luteolin, and daidzein. Originally, phytoalexins are known for substances when environmental triggers induce. It is noticed that several phytoalexins are actively produced when soybeans are exposed to various stresses such as microbes, UV, and other physical attack. There are biological activities of soybean-derived polyphenol compounds: anti-oxidant, antitumor, anti-inflammatory, anti-obesity and moreover anti-

atopic effects [4-6].

Glyceollins, which are synthesized from daidzein in soybeans infected with fungi, have potent antioxidant functions via inhibition of reactive oxygen species (ROS) production. Glyceollins also display anti-inflammatory effects by suppressing the nuclear factor kappa B (NF-κB) signaling pathway. The compounds not only have antitumor potential by inhibiting angiogenesis, but also have antimelanogenic activity in alleviating melanin biosynthesis [7-10]. Daidzein is converted into glyceollins in soybeans during fermentation after fungal infection, such as by *Aspergillus sojae*. In the anti-diabetic *in vitro* assay, α-glucosidase is well established to determine absorption of monosaccharide from small intestine. In addition, it is important to define enzymatic inhibitory mechanism for understanding drug metabolism. Previously, various mechanistic studies on isoflavonoid compounds in soybeans were performed [7-10]. However, there are little data on the enzymatic approaches of glyceollins derived from phytoalexins, or on how glyceollins and other anti-diabetic agents are associated with the inhibition to *α*-glucosidase.

Originally, α-glucosidase (EC 3.2.1.20; alternatively named maltase, glucoinvertase, or glucosidosucrase) is one of pivotal enzymes whose specificity is directed mainly toward the exohydrolysis of 1,4-α-glucosidic linkages, and that hydrolyze oligosaccharides rapidly, relative to polysaccharides. Since α-glucosidase induces postprandial hyperglycemia in type 2 diabetes by breaking di-, tri-, and oligosaccharides into monosaccharides, their inhibitors delay carbohydrate digestion and absorption, thereby attenuating post-prandial hyperglycemia [11]. On the other hand, the α-glucosidase inhibitory activity has been established using *p*-nitrophenyl α-D-glucopyranoside (p-NPG) as substrate and the inhibition makes easier to detect its activity as an anti-diabetic potential. There are mounting evidences for the anti-diabetic effects of various polyphenol compounds by plants [12-14]. Of them, soybeans are most human-friendly food resources in the world endowing beneficial purposes.

In the present study, we first compared the **α**-glucosidase inhibitory activities of four polyphenol compounds (glyceollin, genistein, luteolin, and daidzein) derived from **soybeans,** and then studied the mode of action of the inhibitory effects by deducing the K_i values of the four polyphenol compounds. We also investigated the synergistic effects between glyceollin and the other three polyphenol compounds. A deeper understanding of the enzyme kinetics and mode of action of glyceollin could help to develop nutraceuticals that prevent diabetes without side effects. Our results indicated that a combination of glyceollin and luteolin has synergistic effects on α-glucosidase inhibition.

2. Materials and Methods

2.1. Reagents

α-Glucosidase (from baker's yeast), *p*-nitrophenyl α-D-glucopyranoside (pNPG), acarbose, genistein, daidzein, and luteolin were purchased from Sigma-Aldrich (St, Louis, MO, USA). Glyceollins, which have three isomers, were semi-purified from elicited soybeans, as described previously [12]. All samples were prepared at various concentrations by dissolution in dimethyl sulfoxide (DMSO), except for water-soluble acarbose. The structures of the compounds are shown in Fig. 1.

2.2. α-Glucosidase inhibitory assay

The α-glucosidase activity was measured as described previously with slight modifications [13]. In brief, polyphenols or acarbose (A8980, Sigma) were placed in a 96-well plate, and then 100 μL of α-glucosidase per well was added. The substrate, pNPG (*p*-nitrophenyl α-D-glucopyranoside, N1377, Sigma) was then added into each well as a final concentration of 0.1 M with total 200 μl volume. The absorbance at 405 nm was measured immediately using a spectrophotometer (Victor3 multi-label counter, Wallac, Turku, Finland)

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104 at 37°C and then every 2 min for 40 min. The absorbance values were plotted against time,
105 and the rate (velocity) of product generation (α-glucosidase activity) was calculated from the
106 straight-line part of the graph.
107
108 *2.3. Enzyme kinetics for α-glucosidase*
109 The enzyme reaction was performed according to the above reaction conditions with
110 samples at various concentrations. pNPG was placed with polyphenol samples in 96-well
111 plates and α-glucosidase was added to initiate the enzyme reaction. The absorbance
112 variations for each concentration of pNPG were then obtained spectrophotometrically. The
113 inhibition modes of the polyphenols were determined using Michaelis–Menten and
114 Lineweaver–Burk plots using GraphPad Prism 6.0 and the SigmaPlot 10.0 software
115 programs [14-15], respectively. The inhibitor constant K_i is an indication of an inhibitor's
116 potency. The constant is the concentration required to produce half maximum inhibition, and
117 could be determined by a Dixon plot [16] using Graphpad Prism 6.0 and SigmaPlot 10.0
118 software programs [14-15]. To describe how the agents inhibit α-glucosidase, the
119 Lineweaver–Burk equations, in double reciprocal form, were expressed as follows:

120 $$\frac{1}{v} = \frac{Km}{Vmax}\left(1 + \frac{1}{Ki}\right) \times \frac{1}{[S]} + \frac{1}{Vmax} \quad \text{(Competitive inhibition)}$$

121 $$\frac{1}{v} = \frac{Km}{Vmax} \times \frac{1}{[S]} + \frac{1}{Vmax}\left(1 + \frac{[I]}{Ki}\right) \quad \text{(Uncompetitive inhibition)}$$

122 $$\frac{1}{v} = \frac{Km}{Vmax} \times \left(1 + \frac{[I]}{Ki}\right) \times \frac{1}{[S]} + \frac{1}{Vmax}\left(1 + \frac{[I]}{Ki}\right) \quad \text{(Noncompetitive inhibition)}$$

123 $$\frac{1}{v} = \frac{Ks}{Vmax}\left(1 + \frac{[I]}{Ki}\right) \times \frac{1}{[S]} + \frac{1}{Vmax}\left(1 + \frac{[I]}{\alpha Ki}\right) \quad \text{(Mixed inhibition)}$$

124
125 Where v is the enzyme reaction rate in the absence and presence of samples; V_{max} and [S]
126 are the maximum reaction velocity and the substrate concentration, respectively; and K_m and
127 K_s are the Michaelis–Menten constant and the dissociation constant for the affinity of the

128 substrate, respectively. The α symbol is the ratio of the uncompetitive inhibition constant to
129 the competitive inhibition constant, and has a value of 1 for noncompetitive inhibition. The
130 αKi value is the inhibitor constant when inhibitor (I) occupies the enzyme-substrate (ES)
131 complex [16].
132
133 *2.4. Docking studies*
134 To investigate the inhibition modes of the individual polyphenols with α-glucosidase,
135 docking calculation was performed by Autodock Vina, an improved program for molecular
136 docking and virtual screening, compared to the average accuracy of the binding mode
137 predictions of AutoDock 4.0 [17]. Three-dimensional coordinates of α-glucosidase used as
138 the input structure were prepared by a structure modeling using the SWISS-MODEL server
139 [18]. The protein modeling used the structure of isomaltase from *S. cerevisiae* (PDB code
140 3AJ7) as a template, approaching 73% amino acid identity with the commercial α-
141 glucosidase, MAL12. For the docking calculation, the *pdbqt* files were generated using
142 AutoDock Tools version 1.5.4 and determination of the grid box size was also carried out
143 using the program (http://mgltools.scripps.edu/). Default parameters except the
144 exhaustiveness option were used as described in the AutoDock Vina manual. The best
145 theoretical binding modes of inhibitors are displayed with their ΔG_{bind} scores.
146
147 *2.5. Synergistic effects on α-glucosidase inhibition*
148 Synergistic effects on α-glucosidase inhibition were measured using the same methods as
149 above. A designed concentration of pNPG together with a combination treatment of two
150 polyphenols was added into wells of a 96-well plate, and the enzyme reaction was started by
151 the addition of α-glucosidase. We calculated the IC_{50} values of each polyphenol and their

combinations, and used the statistical differences of these values to assess any synergistic effects.

2.6. Determination of combination index

Combination index values were calculated by the method of Chou [19]. The equation offers the theoretical basis for the combination index (CI)-isobologram equation that permits quantitative determination of compound interactions, where CI < 1, = 1, and > 1 show synergism, additive effect, and antagonism, respectively. Based on the algorithm, computer software has been established to admit automated simulation of synergism and antagonism at all levels.

2.7. Statistical analysis

The results are presented as means ± standard deviation (SD). Statistical differences between mean values ± SD were determined by the Tukey's one-way ANOVA test using IBM SPSS Statistics (Armonk, NY, USA). The differences were considered significant at $p < 0.05$.

3. Results

3.1. Phytoalexins derived from soybeans have potential in inhibiting α-glucosidase compared to acarbose

The α-glucosidase inhibitory activities of polyphenol compounds derived from soybeans (Fig. 1B~E) were measured to compare which compound is major potential and effective against α-glucosidase activity. Acarbose (Fig. 1A), which has been used for a positive control of α-glucosidase assay and also known as effective agent for anti-diabetes [20], was used for a standard agent in this study. Including acarbose, all compounds reduced α-

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glucosidase activity in a concentration-dependent manner (Fig. 1F, grey columns). To compare the inhibitory activity on α-glucosidase among acarbose (a well-known α-glucosidase inhibitor) and four polyphenol compounds elicited from soybeans, the half maximal inhibitory concentrations (IC_{50}s) were determined. The IC_{50} values of acarbose, glyceollin, genistein, luteolin, and daidzein were 530.50 ± 100.13, 13.22 ± 2.31, 23.66 ± 3.54, 11.94 ± 1.63, 20.16 ± 6.17 μM, respectively. These results showed that the four soybean-derived polyphenol compounds had more than 20 times higher α-glucosidase inhibitory activity than acarbose.

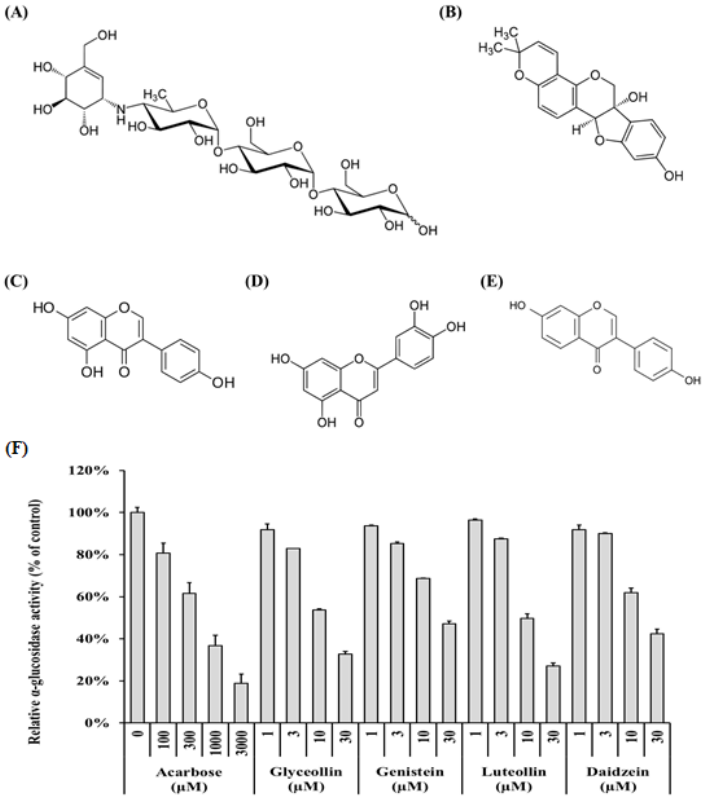

Fig. 1. Structures of soybean-derived polyphenol compounds used in this experiment and their α-glucosidase inhibitory activity. (A) Acarbose, a positive control, (B) glyceollin (C) genistein, (D) luteolin, and (E) daidzein. α-Glucosidase inhibitory activities (F) were calculated according to relative percentage of the control group (100%; grey columns).

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3.2. Determination of inhibition modes and K_i values on polyphenols derived from soybeans on α-glucosidase

To define the mode of inhibition of the polyphenols, enzyme kinetics was performed with designated concentrations of pNPG. The saturated velocity for α-glucosidase was calculated from the nonlinear regression curve as five different concentration of substrate (Fig. 2). To determine the inhibition modes, Lineweaver-Burk plots were constructed and were selected as the most suitable mode after the determination of all types of inhibition modes using the SigmaPlot 10.0 software. The results revealed that acarbose and glyceollin used competitive inhibition, while genistein showed non-competitive inhibition. Luteolin showed mixed inhibition and daidzein used an uncompetitive mode of inhibition (Fig. 3).

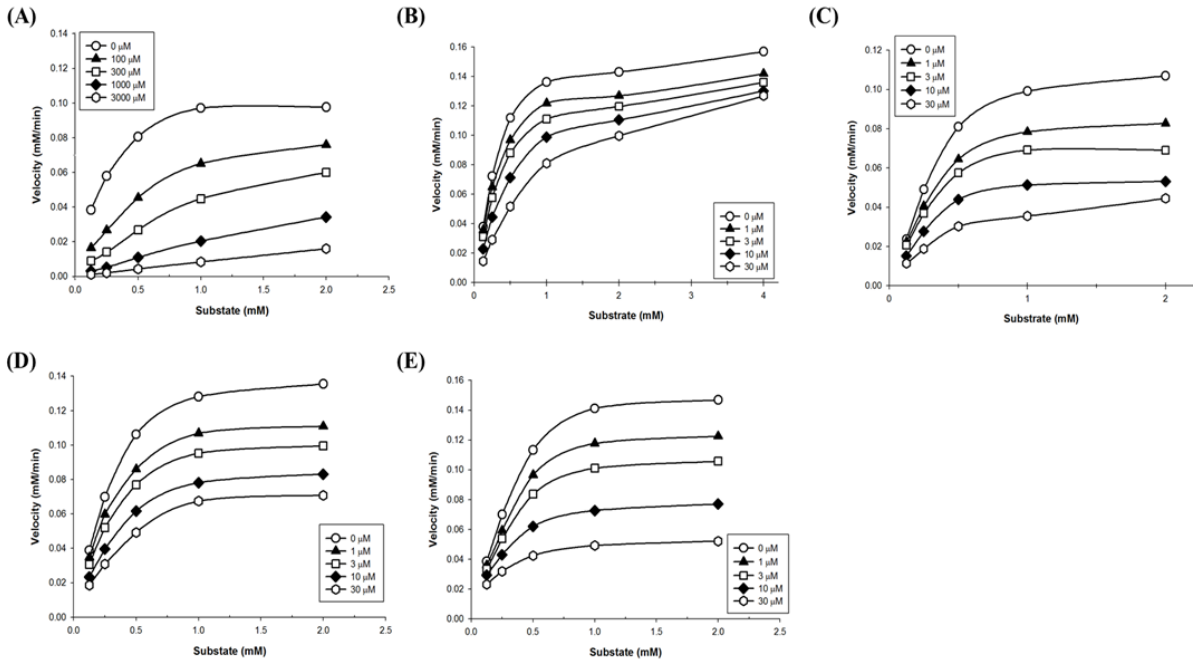

Fig. 2. Non-linear regression analysis of α-glucosidase. The inhibitors applied at the indicated concentrations, (A) acarbose, (B) glyceollin, (C) genistein, (D) luteolin, and (E) daidzein. A mixture of 100 μL of each concentration of *p*-nitrophenyl α-D-glucopyranoside (pNPG) was added to 96-well plates that contained polyphenols, and treated with α-glucosidase to initiate the enzyme reaction. The Lineweaver-Burk plots show four kinds of classical inhibition modes of action on enzyme kinetics. Each plot was carried out in three independent experiments.

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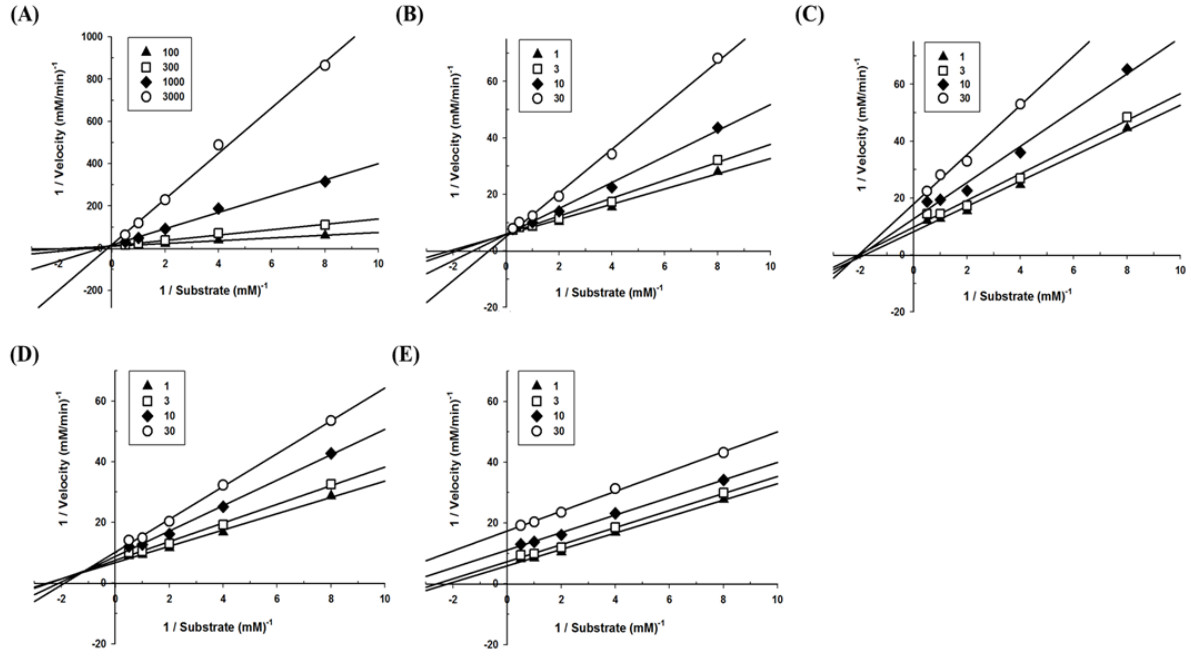

Fig. 3. Modes of action of polyphenols derived from soybeans in α-glucosidase inhibition. To calculate the mode of action, Lineweaver–Burk plots were constructed. (A) Acarbose, (B) glyceollin, (C) genistein, (D) luteolin, and (E) daidzein.

Meanwhile, the inhibitor constant K_i is an indication of an inhibitor's potency. The constant is the concentration required to produce half maximum inhibition, and could be determined by a Dixon plot [16]. The initial slope v was determined for each concentration of the polyphenols. The reciprocal velocity ($1/v$) versus the substrate concentration (for each 0.25, 0.5, 1, and 2 mM pNPG) was plotted. A single regression line for each concentration of substrate was obtained, and the K_i was calculated from the intersection of the four lines. The K_i values were determined by GraphPad Prism 6.0 software as 45.88 ± 3.75, 18.99 ± 4.45, 15.42 ± 2.48, and 16.81 ± 9.60 μM, for acarbose, glyceollin, genistein, and luteolin, respectively (Table 1). It is not possible to calculate the K_i in the case of uncompetitive inhibition where the four lines do not intersect. Therefore, for daidzein, the αKi value as calculated, which is the inhibitor constant when the inhibitor occupies the enzyme-substrate complex. The αKi value of daidzein was calculated as 9.99 ± 1.24 μM.

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Table 1. K_i values of polyphenols derived from soybeans on α-glucosidase inhibition. K_i values of acarbose, glyceollin, genistein, luteolin, and daidzein were calculated using GraphPad Prism 6.0 software. For daidzein only an α*Ki* value is provided instead of the *Ki* value

Inhibitor	Mode of inhibition	K_m (μM)	K_i (μM)
Acarbose	Competitive	0.21±0.02	45.88±3.75
Glyceollin	Competitive	0.24±0.03	18.99±4.45
Genistein	Noncompetitive	0.36±0.06	15.42±2.48
Luteolin	Mixed	0.30±0.06	16.81±9.60
Daidzein	Uncompetitive	0.40±0.05	9.99±1.24

3.3. Structural basis for the modes of inhibition

To understand the modes of inhibition in molecular level, we next performed docking studies using the model structure of α-glucosidase. Docking models of acarbose, glyceollin, genistein, luteolin, and daidzein were generated, and the best models of each inhibitor were chosen (Fig. 4A-E). First, we compared the docking model of acarbose with homologous structure in complexed with maltose. The superimposition result displayed that acarbose was docked into pocket in which substrate maltose experimentally binds, and a nitrogen atom was located near the glycosidic bond of the substrates to block the hydrolysis (Fig. 4A). The result well explains why the acarbose competitively binds to α-glucosidase and inhibits the enzyme. Then, we used the acarbose model as an indicator of substrate-binding site, superposing with other docking models.

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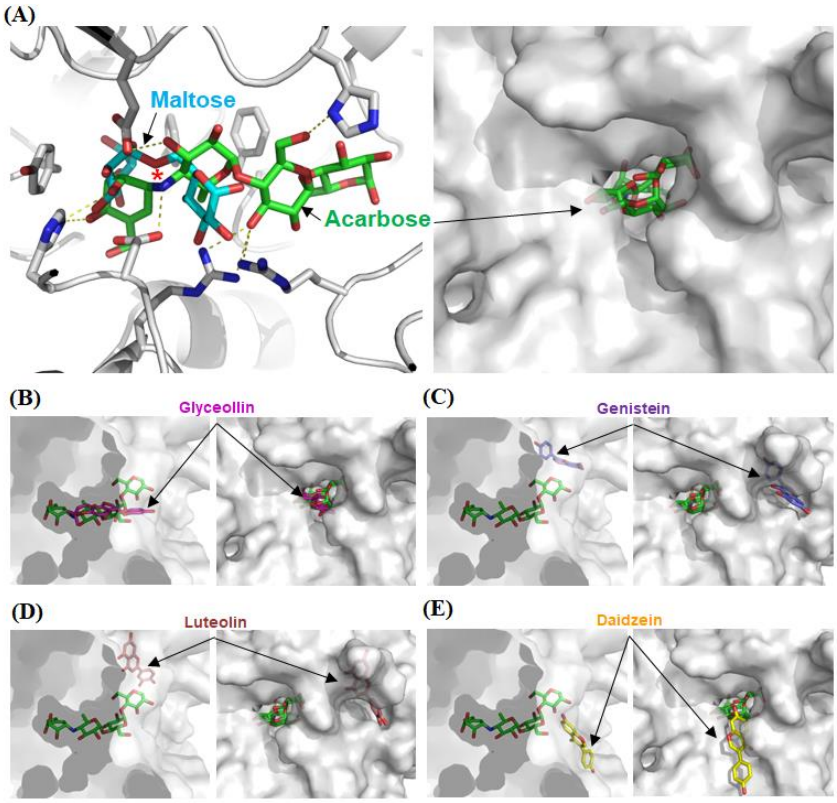

Fig. 4. Molecular docking studies on the inhibitiory modes. Docking models for the inhibitory modes of the α-glucosidase inhibitors. The model of MAL12, a structure of α-glucosidase, was shown as cartoon or surface diagram in a color scheme as white. Maltose, acarbose, glyceollin, daidzein, luteolin, and genistein were shown as stick diagram. (A) The inhibitory mode of acarbose. The maltose molecule was prepared by superposing the model of MAL12 with the structure of isomaltase (PDB code 3AXH), and shown with a cyan color. Residues involved in substrate-binding were displayed as stick model. Yellow dashed line indicates hydrogen bond interactions. A glucosidic bond to be hydrolyzed was indicated by a star symbol. (B)-(E) The inhibitory modes of polyphenols. Glyceollin, genistein, daidzein, and luteolin are shown as color schemes with magenta, purple, yellow, and brown, respectively.

The α-glucosidase accommodated the docking model of glyceollin, a pterocarpan which structurally different compared to other isoflavones and flavone, with its substrate binding pocket (Fig. 4B). The theoretical affinity of the binding (ΔG_{bind}) was -10.3 kcal/mol. This structural model indicates the competitive inhibition mode of glyceollin. The other polyphenols showed alike structures each other. However, in spite of the similar conformations, they were docked into the enzyme differently. Interestingly, the model of

daidzein showed that it covers the entrance of the pocket with ΔG_{bind} of -7.6 kcal/mol (Fig. 4C). Although uncompetitive inhibitor binds only to enzyme-substrate complex, the relatively low affinity of the binding compared to that of glyceollin might intimates a need of *p*-nitrophenyl α-D-glucopyranoside. Thus, the model showed the cavity of the binding of the uncompetitive inhibior and might explain the rare uncompetitive inhibition mode as described above. Luteolin and genistein were proposed as mixed and non-competitive inhibitors before. Mixed inhibitor binds both apo enzyme and enzyme-substrate complex, and it binds a different site from the substrate-binding pocket, altering the active-site configuration and turnover. Non-competitive inhibition is a special case of mixed inhibition under unchanged K_m. Surprisingly, the docking models showed a different cavity that harbors luteolin and genistein with ΔG_{bind}s of -8.4 and -8.7 kcal/mol, respectively, intimating that mixed or non-competitive inhibition might occur altering the catalytic cleft with their attachment (Fig. 4D, E).

3.4. Combined effects with glyceollin plus luteolin on α-glucosidase inhibition

Next, we studied the α-glucosidase inhibitory effect between pairs of these polyphenols by mixing glyceollin with luteolin, genistein, or daidzein. As shown in Table 2, we obtained IC_{50} values of each polyphenol singly and for the combination treatments on α-glucosidase inhibition at each concentration of pNPG. Interestingly, luteolin showed combined effects on α-glucosidase inhibition when combined with glyceollin, while genistein or daidzein did not (Table 2, bold characters).

In Fig. 5A, we further investigated the effects of the combination by mixing glyceollin and luteolin at ratios of 0:10 to 10:0, and then testing their inhibition of *α*-glucosidase. The results showed that a ratio of glyceollin to luteolin of 3:7 caused the highest inhibition of *α*-glucosidase. We then plotted the kinetic mode of action in Fig. 5B. Surprisingly, the data

296 showed that the mode of action was competitive inhibition. These results indicate that a
297 combination of competitive and mixed inhibition modes might be more potent inhibitory to
298 α-glucosidase activity, whereas a combination of competitive and uncompetitive or
299 noncompetitive modes are not.

300

301 **Table 2.** Synergistic effects of glyceollin and three existing polyphenols derived from
302 soybeans on α-glucosidase inhibition. IC_{50} values were obtained using GraphPad Prism 6.0
303 software. $^{*}p < 0.05$, $^{\#}p < 0.05$, denote significant difference from glyceollin alone and
304 luteolin alone, respectively

Inhibitor	Mode of inhibition	K_m (μM)	K_i (μM)
Acarbose	Competitive	0.21±0.02	45.88±3.75
Glyceollin	Competitive	0.24±0.03	18.99±4.45
Genistein	Noncompetitive	0.36±0.06	15.42±2.48
Luteolin	Mixed	0.30±0.06	16.81±9.60
Daidzein	Uncompetitive	0.40±0.05	9.99±1.24

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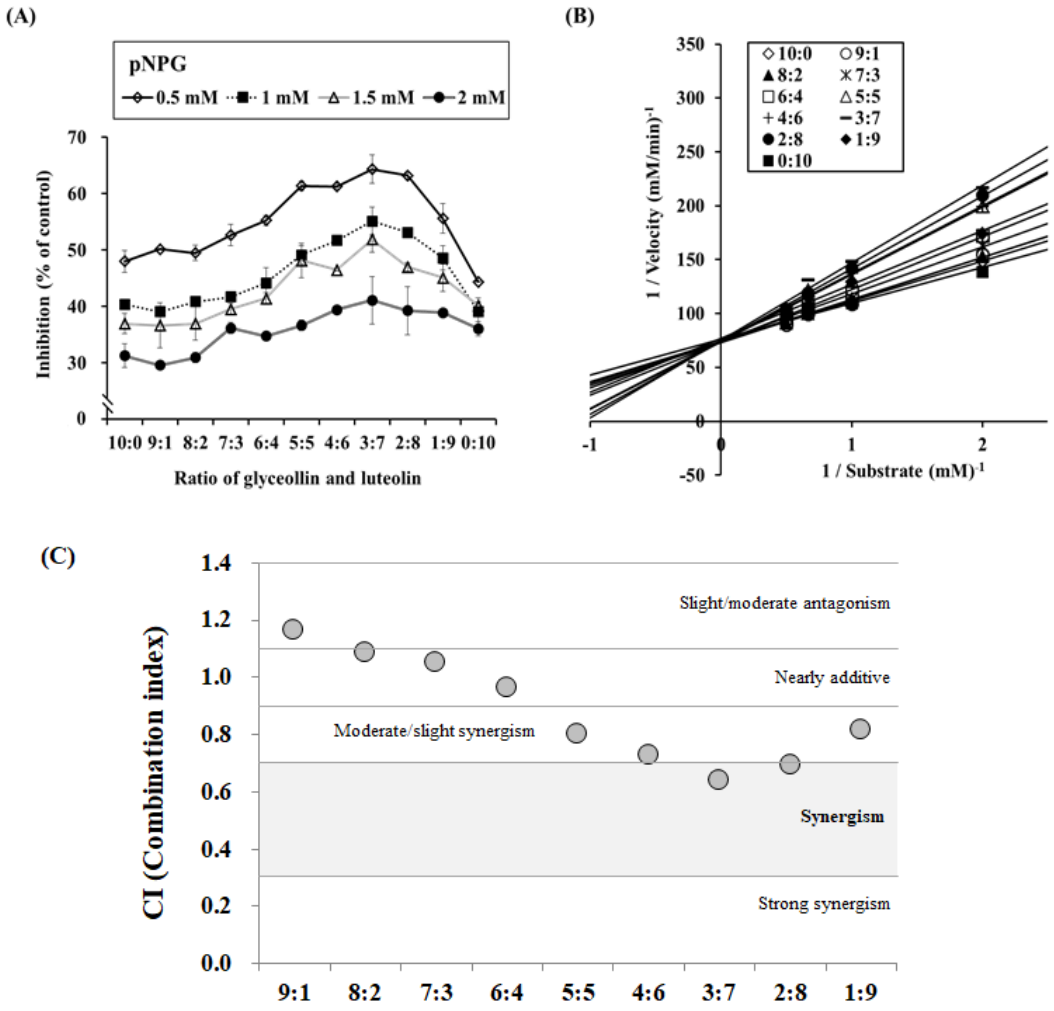

Fig. 5. Synergistic effect of glyceollin plus luteolin on α-glucosidase inhibition. Glyceollin or luteolin were prepared with at 2 μM concentrations and mixed as 0:10 to 10:0 ratios. Therefore, a 5:5 ratio indicates mixture of 1 μM glyceollin plus 1 μM luteolin. (A) Pattern of α-glucosidase inhibition according to four concentrations of substrate (0.5, 1, 1.5, and 2 mM). (B) Lineweaver–Burk plot of the effects of glyceollin plus luteolin mixture at various ratios of the two compounds. (C) Combination index plot of various ratios of glyceollin and luteolin. Combination index (CI)-isobologram equation that permits quantitative determination of compound interactions represents that CI < 1, = 1, and > 1 show synergism, additive effect, and antagonism, respectively.

3.5. Confirmation of synergism by combination index equation with glyceollin plus luteolin

It is important for determining the mode of action between compound(s) with synergism, additive effect, and antagonism, respectively, in that plant classically has lots of active and/or inactive components such as polyphenol compounds and alkaloids. To scrutinize whether the

inhibition mode of action between glyceollin and luteolin is associated with synergism, we applied to CI values for actual experiments points by Chou method [19]. As a result, for glyceollin or luteolin, 4 data points (1, 3, 10, and 30 μM) entered in the equation, and got 13.6215 and 12.2697 of Dm value, respectively. As shown in Fig. 5C, the ratios of glyceollin to luteolin between 9:1 to 7:3 got >1.0 of CI value. Nevertheless, the ratios of glyceollin to luteolin between 6:4 to 1:9 triggered <1.0 of CI value. Interestingly 3:7 ratio remarkably produced lowest CI value, suggesting the ratio ranked most synergistic effect on *α*-glucosidase inhibition.

4. Discussion

In a study of α-glucosidase related to postprandial hyperglycemia in type 2 diabetes, glyceollin showed a similar effect to three known polyphenols (genistein, luteolin, and daidzein (Table 1). Moreover, for the first time, we revealed that glyceollin is a competitive inhibitor of α-glucosidase (Fig. 3B). Competitive and mixed inhibitors produce their effects by combining with free enzyme to prevent substrate binding, thus producing an enzyme-inhibitor (EI) complex. By contrast, non-competitive or uncompetitive inhibitor cannot directly interrupt the binding of the enzyme to its substrate. We hypothesized that a synergistic effect would occur between competitive and noncompetitive (or uncompetitive) inhibitors, because the binding sites of the inhibitors are different. However, the combinations of competitive and noncompetitive (or uncompetitive) inhibitors decreased rather increased the inhibition activity (Table 2). Interestingly, glyceollin (a competitive inhibitor) and luteolin (a mixed inhibitor) displayed a significant synergistic effect, with decreased IC_{50} values at all concentrations of the substrate (Table 2).

The biological mechanism is not limited to reaction of a single compound, therefore,

complicated synergistic mechanisms must be involved in most of biological events [21]. Liu *et al*. demonstrated that a combination of inhibitors improves their inhibitory activity against α-glucosidase [22]. They selected two typical xanthone derivatives (1,3,7-trihydroxyxanthone and 1,3-dihydroxybenzoxanthone), and observed their synergistic effect. In their study, 2 μM of 1,3,7-trihydroxyxanthone exhibited approximately 15 % inhibition, while 2 μM of 1,3-dihydroxybenzoxanthone did approximately 10 % inhibition. Interestingly, the synergistic effect of combining the two inhibitors at a 3:7 ratio produced 40 % maximal inhibition [22]. Another study on α-glucosidase demonstrated that genistein synergistically inhibited with some metal ions such as copper and zinc ions [21]. Therefore, we next hypothesized that a mixture of two polyphenols would have a synergistic inhibitory effect against α-glucosidase. Indeed, glyceollin showed a synergistic effect with luteolin on α-glucosidase inhibition (Table 2). When acarbose, which is a competitive inhibitor like glyceollin, was used in combination with luteolin or glyceollin, no synergistic effects were detected. Combined treatment with genistein and daidzein also produced no synergistic effects.

It is shown that dose and effect are interchangeable via defined parameters derived from the unified theory for the Michaelis-Menten equation, Hill equation, Henderson-Hasselbalch equation, and Scatchard equation. The equation provided the theoretical basis for the combination index (CI)-isobologram equation that allows quantitative determination of compound interactions, where CI < 1, = 1, and > 1 denote synergism, additive effect, and antagonism [19], respectively. In this study, by approving a unique and effective systemic method, we disclosed that the combination of glyceollin (a competitive inhibitor) and luteolin (a mixed inhibitor) displayed a significant synergistic effect, with decreased maximal CI value (0.64244) with the ratio of 3:7 of glyceollin and luteolin, theoretically (Fig. 5C). This theoretical approach can gain increased applications in food sciences, from how to

effectively evaluate an extract by food and/or its ingredient(s), entity to how to beneficially use multiple compounds, or modalities in combination therapies.

To verify whether the combination of glyceollin and luteolin affect the mode of action of α-glucosidase inhibition, it was necessary to analyze the affinity of each of the polyphenol compounds. When soybeans are infected with various elicitors, varieties of polyphenol compounds are naturally produced. Those polyphenol compounds called as phytoalexins; although the identification of the polyphenolics is still needed. This study is initially to investigate the synergistic potency of the two polyphenols by comparison of their K_i values; however, we do not know how the enzyme's structure is changed after binding of inhibitors. Therefore, it would be useful to perform molecular docking simulation studies and protein structure analysis, including X-ray crystallography and nuclear magnetic resonance (NMR), to determine the synergistic inhibition mode of soybean-derived polyphenols, if we can measure, which will shed light on their inhibitory mechanisms.

5. Conclusions

By analyzing enzyme inhibition kinetics using Michaelis–Menten plots and the Lineweaver–Burk plots, we found that glyceollin showed competitive inhibition, genistein showed noncompetitive, daidzein was uncompetitive, and luteolin showed a mixed mode of action. These results indicated that glyceollin, genistein, luteolin, and daidzein could be promising α-glucosidase inhibitors for anti-diabetic approaches in soybeans. A combination of glyceollin and luteolin had synergistic effects on *α*-glucosidase inhibition, showing that a combination of glyceollin and luteolin has the potential to inhibit *α*-glucosidase activity via a synergistic mode of action. The inhibition of α-glucosiadase by polyphenol compounds in soybeans is not so high when the soybeans are not elicited by *Aspergillus*. However, the selective glyceollin produced by the fermentation by the fungus or other fungi is thought to

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enhance the antidiabetic effect rapidly. Collectively, we believe that fermentation of soybeans induce various phytoalexins (e.g., glyceollins in soybeans); therefore, the intake of fermented soybeans, such as soy sauce, soy paste, Koji, and Natto might be useful to synergistically prevent and/or control type 2 diabetes mellitus.

Conflict of interests

The authors declare no competing financial interest.

Ethical approval

This article does not include any studies with human participants or animals performed by any of the authors.

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