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Miou TODA[a], Jun KAWABATA[a] & Takanori KASAI[a]
[a] Laboratory of Food Biochemistry, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University
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Inhibitory Effects of Ellagi- and Gallotannins on Rat Intestinal α-Glucosidase Complexes

Miou TODA, Jun KAWABATA,† and Takanori KASAI

Laboratory of Food Biochemistry, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kitaku, Sapporo 060-8589, Japan

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The clove ellagitannins and their related polygalloylglucoses inhibited maltase activity of rat intestinal α-glucosidases. The structure-activity relationship study of those galloylglucoses, varying the extent of galloylation on the glucose core, with the ellagitannins, indicated that an increasing number of galloyl units in the molecule lead to an increase in the inhibitory activity. Penta-*O*-galloyl-β-D-glucose, with five galloyl groups showed the highest inhibitory activity. On the other hand, hexahydroxydiphenoyl units contained in the ellagitannins had little effect on the activity. After separation of maltase-glucoamylase and sucrase-isomaltase complexes from the crude mixture of the rat α-glucosidases, the inhibitory activities of the galloylglucose derivatives against each complex were examined. The inhibitory influence on the maltase-glucoamylase complex was more potent than on the sucrase-isomaltase complex.

Key words: α-glucosidase; inhibitor; galloyl; hexahydroxydiphenoyl; hydrolyzable tannin

The main nutrition energy of the human diet is supplied by carbohydrates. Dietary carbonhydrates are broken down to monosaccharides by hydrolytic enzymes, α-glucosidases, and absorbed into the intestinal brush border membrane, so intestinal α-glucosidases are physiologically important enzymes in the digestive process of dietary carbohydrates. The intestinal α-glucosidases are divided into four hydrolase types, namely, maltase (EC 3.2.0.20), glucoamylase (EC 3.2.1.3), sucrase (EC 3.2.1.48), and isomaltase (EC 3.2.1.10). These four enzymes from two complexes having the different substrate specificities, maltase-glucoamylase (M-G) and sucrase-isomaltase (S-I) complexes. Maltose derived from dietary starch is hydrolyzed by M-G and S-I complexes. On the other hand, sucrose and isomaltose are hydrolyzed only by S-I complex.[1)]

Diabetic and most obese patients suffer from abnormal blood glucose and required control of carbohydrate metabolism. Suppression of intestinal α-glucosidase activity can retard the digestion and absorption of carbohydrates, and therefore, α-glucosidase inhibitors have a possibility to reduce the postprandial elevation of blood glucose levels. In fact, a potent α-glucosidase inhibitor, acarbose, has been therapeutically used for diabetic and obese patients.

In our previous paper, 50% aqueous methanol extracts of dried flower buds of clove (*Syzgium aromaticum*) had potent inhibitory activity against rat intestinal maltase, and two ellagitannins, casuarictin (**1**) and eugeniin (**2**) were isolated as active principles.[2)] Furthermore, it was suggested that an increasing number of galloylation on the glucose core of the molecule would improve the enzyme inhibitory activity in the structure-activity relationship study.

In this paper, we prepared a series of galloylglucoses, varying the extent of galloylation, and compared the relative inhibitory activity of those together with the clove tannins to further clarify the importance of the number and regiochemical aspects of galloyl and hexahydroxydiphenoyl (HHDP) groups

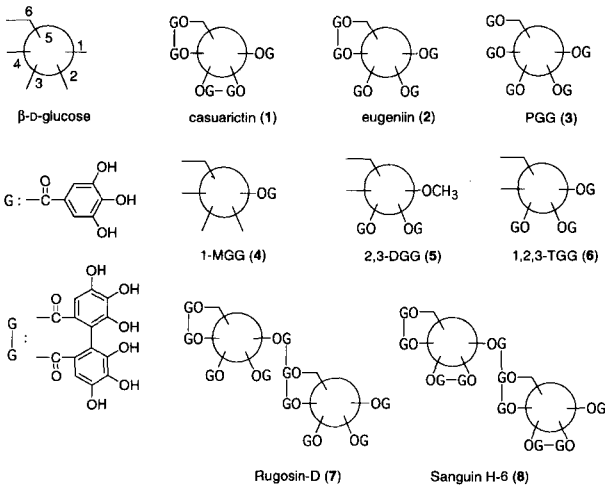

Fig. 1. Schematic Structures of Tannins Examined in This Study.

† To whom correspondence should be addressed. Fax: +81-11-716-0879; Tel: +81-11-706-4140; E-mail: junk@chem.agr.hokudai.ac.jp

on interaction between the hydrolyzable tannins and the rat intestinal α-glucosidase. In addition, after purification of each rat intestinal α-glucosidase complex, the inhibitory specificity of those tannins was examined.

Materials and Methods

General experimental procedures. ^{1}H-NMR spectra were measured with a Bruker AMX-500 spectrometer (500 MHz); chemical shifts are expressed relative to the residual signals of methanol-d_4 (δ 3.30) and acetone-d_6 (δ 2.04). FAB-MS data were obtained with a JEOL JMS-AX500 instrument using triethanolamine as a matrix.

Materials. All reagents were of analytical grade and were purchased from Wako Pure Chem. Co. Osaka, Japan, unless otherwise stated. Rat intestinal acetone powder, Sephadex G-200, and 2,3,4,6-tetra-*O*-benzyl-D-glucose were supplied by Sigma Aldrich Japan Co., Tokyo, Japan. Sep-pack Light Alumina B Cartridges were purchased from Waters Corp., Massachusetts, U.S. DEAE-Toyopearl 650M and Toyopearl HW65F were supplied by Tosoh Co. Tokyo, Japan. Eugeniin and casuarictin were isolated from dried flower buds of clove,[2] Penta-*O*-galloyl-β-D-glucose (PGG, **3**) was as described in the literature.[3]

1-O-galloyl-β-D-glucose (4). 1-*O*-galloyl-β-D-glucose (1-MGG, **4**) was prepared from 2,3,4,6-tetra-*O*-benzyl-D-glucose by the method of Kawamoto *et al.*[3] with a slight modification. A mixture of both anomers were separated by the preparative HPLC[4] [column, Inertsil SIL 20.0×250 mm; mobile phase, hexane-ethyl acetate (6:1); flow rate, 10 ml/min; detection, UV 254 nm] before debenzylation. The resultant benzylated β-ester was deprotected by hydrogenelysis to give **4** as a dark white green amorphous substance; FAB-MS (negative) *m/z* (%): 331 $[M-H]^-$; ^{1}H-NMR δ (methanol-d_4) (ppm, J=Hz): 3.38–3.49 (4H, m, 2–5-H), 3.84 (1H, dd, J=12.0, 1.9, 6-H), 5.64 (1H, d-like, J=8.0, 1-H), 7.11 (2H, s, galloyl-H).

1-O-methyl-2,3-di-O-galloyl-β-D-glucose (5). A mixture of dried (3 h at 60°C *in vacuo*) 1-*O*-methyl-β-D-glucose (9.7 g, 50 mmol), benzaldehyde dimethyl acetal (7.5 ml, 50 mmol) and *p*-toluenesulfonic acid monohydrate (25 mg, 0.12 mmol) in dimethylformamide (DMF) (40 ml) was stirred vigorously for 1 h at 60°C under reduced pressure to remove methanol. Triethylamine (0.3 ml) was then added to the mixture to stop the reaction. After solvent removal at 90°C *in vacuo*. The mixture was directly chromatographed on silica gel by elution with ethyl acetate-triethylamine (100:0.1). The white crystal of 1-*O*-methyl-4,6-*O*-benzylidene-β-D-glucopyranose (3.3 g, 24% yield) obtained was subjected to 2,3-*bis*-*O*-galloylation by the same procedure as **4**. Debenzylation by hydrogenolysis yielded 1-*O*-methyl-2,3-di-*O*-galloyl-β-D-glucose (2,3-DGG, **5**) as a dark brown amorphous substance; FAB-MS (negative) *m/z* (%): 497 $[M-H]^-$; ^{1}H-NMR δ (methanol-d_4) (ppm, J=Hz): 3.43 (3H, s, 1-Me), 3.71–3.89 (4H, m, 4–6H), 4.99–5.02 (2H, m, 1 and 2-H), 5.65 (1H, t, J=9.2, 3-H), 6.97 and 7.02 (2×2H, each s, galloyl-H).

1,2,3-tri-O-galloyl-β-D-glucose (6). 1,2,3-tri-*O*-galloyl-β-D-glucose (1,2,3-TGG, **6**) was prepared from D-glucose by 4,6-benzylidene acetalization, galloylation and deprotection as mentioned above. Dried D-glucose (4.5 g, 25 mmol) was treated with a mixture of benzaldehyde dimethyl acetal (3.5 ml, 25 mmol) and *p*-toluensulfonic acid monohydrate (6.5 mg, 0.03 mmol) in DMF (20 ml). After 1 h, triethylamine (0.15 ml) was added. 4,6-*O*-benzylidene-D-glucopyranose was purified by chromatography on silica gel [ethyl acetate-triethylamine (100:0.1)] to yield white crystals (3.0 g, 45% yield). This acetal was then subjected to 1,2,3-tris-galloylation (tri-*O*-galloyl). A β-anomer was separated by preparative HPLC [column, Inertsil SIL 20.0×250 mm; mobile phase, hexane-ethyl acetate (75:28); flow rate, 10 ml/min; detection, UV 254 nm] prior to final deprotection. Hydrogenolysis of the β-triester acetal afforded **6** as a dark brown amorphous substance; FAB-MS (negative) *m/z* (%): 635 $[M-H]^-$; ^{1}H-NMR δ (acetone-d_6) (ppm, J=Hz): 3.74–4.02 (4H, m, 4, 5 and 6-H), 5.39 (1H, dd, J=9.8, 8.3, 2H), 5.58 (1H, t, J=9.8, 3-H), 6.08 (1H, d, J=8.3, 1-H), 7.06, 7.08 and 7.98 (3×2H, each s, galloyl-H).

Purification of the two α-glucosidase complexes. The two rat intesitanl α-glucosidase complexes, M-G and S-I, were separated from the solubilized crude enzyme preparations obtained from rat intestinal acetone powder by the method of Kolinska and Kraml[5] and Semenza *et al.*[6] with a slight modification.

The commercial rat intestinal acetone powder was homogenized in 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM EDTA at 4°C. The homogenate was centrifuged at 27,000×*g*, for 60 min, at 4°C. The sediment was treated with 0.1 M potassium phosphate buffer (pH 7.0) containing 1.0% TritonX-100 at 4°C. The resultant suspension was centrifuged at 32,000×*g* for 60 min at 4°C and the supernatant was dialyzed against 0.05 M potassium phosphate buffer (pH 7.0) containing 1.5 mM EDTA at 4°C for 2 days. The concentrated crude enzyme solution thus obtained was put on a Sephadex G-200 column and eluted with 0.05 M potassium phosphate buffer (pH 7.0) containing 2.5 mM EDTA. Sucrase and maltase active fractions were dialyzed

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against 0.01 M potassium phosphate buffer (pH 7.0) at 4°C for 1 day and then put on DEAE-Toyopeal 650M, equilibrated with this buffer solution. The column was eluted by NaCl linear garadient (0–0.8 M). The active fractions were concentrated, put on Toyopeal HW-65F and eluted with 0.01 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.5 M NaCl. Then polyacrylamide preparative electrophoresis with the gel concentaration of 5% was done using DL-asparagine-imidazole buffer. Two glucosidase complexes were detected by an enzyme reaction using 4-methylumbelliferyl α-D-glucoside as a fluorescent substrate. Two bands corresponding to M-G (R_f= 0.5–0.6) and S-I (R_f=0.6–0.7) complexes showed fluorescence. The gels cut out were sliced into small pieces and each complex was extracted with 0.01 M potassium phosphate buffer (pH 7.0) for 2 days. For the enzyme assay, each enzyme complex solution was adjusted to 1 U/ml as a maltase, sucrase, or isomaltase activity.

Assay for rat intestinal α-glucosidase inhibitory activity. The α-glucosidase inhibitory activity assay was done as previously described.[7] In the case of two α-glucosidase complexes, the following modification was used to make the assay scale smaller. Each enzyme complex solution prepared from rat intestinal acetone powder was adjusted to 1 U/ml of its specific activity for maltase, sucrase, and isomaltase using maltose, sucrose, and isomaltose, respectively, as a substrate. The reaction mixture for enzyme activity consisted of the enzyme complex solution (0.01 ml), 56 mM substrate in 0.1 M potassium phosphate buffer (pH 6.3, 0.15 ml) and test sample in 50% aqueous dimethylsulfoxide (DMSO, 0.04 ml). The 2 M Tris-HCl buffer for stopping the enzyme reaction was 0.3 ml. Basic alumina treatment before the glucose oxidase method was done in both assays using a commercial Sep-Pak Light Alumina B Cartridge. The terms, maltase, sucrase and isomaltase activities represent the activity of the enzyme solution measured using maltose, sucrose, and isomaltose, respectively, as a substrate throughout the test.

Results and Discussion

The inhibitory effects of the hydrolyzable tannins on rat intestinal α-glucosidases were investigated using ellagitannins, casuarictin (**1**) and eugeniin (**2**) isolated from clove, together with synthetic galloylglucoses, PGG (**3**), 1-MGG (**4**), 2,3-DGG (**5**), and 1,2,3-TGG (**6**) (Fig. 1). In our previous paper, the comparison of an inhibitory activity against rat intestinal α-glucosidase mixture using maltose as a substrate showed that the inhibitory activity was in the order of PGG > eugeniin > casuarictin.[2] Considering the structure-activity relationship among those compounds, it was indicated that an increasing number of uncoupled galloyl units in the molecule led to an increase of the inhibitory activity and that hexahydroxydiphanoyl (HHDP) esters, bridged between two galloyls, had less influence on the activity than galloyl esters when comparing the compounds with similar molecular weights. The comparison of only three compounds, however, seemed insufficient to demonstrate what extent of contribution the number of galloyl units in the molecule gives toward the enzyme inhibitory activity and it is still unclear how influence HHDP gives to the activity. To disclose the effects of HHDP on the inhibitory activity, 1,2,3-TGG and 1-MGG were prepared and compared with eugeniin (4,6-HHDP-1,2,3-TGG) and casuarictin (2,3:4,6-di-HHDP-1-MGG), respectively. The maltase inhibitory activities of eugeniin (**2**), 1,2,3-TGG (**6**), casuarictin (**1**), and 1-MGG (**4**) at 1 mM were 50%, 29%, 27%, and 15%, respectively. The difference in the inhibitory values between **2** with an additional HHDP and **6** was 21% and that between **1** with two additional HHDPs and **4**, 12%. Hence, HHDP might have a weak influence to the inhibitory activity. Moreover, the activity of 2,3-DGG was also examined, and the inhibitory values were in the order of PGG > eugeniin > 1,2,3-TGG > casuarictin > 2,3-DGG > 1-MGG (Fig. 2). This result clearly supports our previous finding that an increasing level of galloylation in the molecule would increase its inhibitory activity. It was unclear, however, whether the solvent used, 10% aqueous DMSO, influenced the complexation of the enzyme and inhibitors, though it had negligible effects on the enzyme activity.[7]

In the polygalloyl glucoses such as PGG, galloyl groups exist together in the same molecule. We next evaluated the importance of the polygalloyl structure

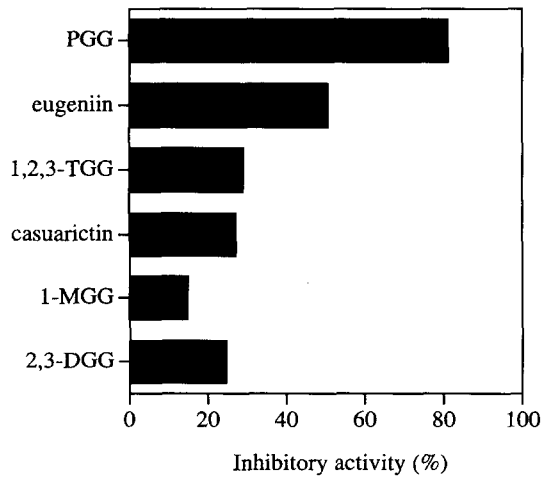

Fig. 2. Rat Intestinal Maltase Inhibitory Activities of Ellagitannins (Eugeniin and Casuarictin) and Galloylglucoses (1-MGG, 2,3-DGG, 1,2,3-TGG, and PGG) Using Maltose as a Substrate. The concentration of each tested compound was 10^{-3} M.

on the inhibitory activity. The inhibitory activities of 5 mM each of gallic acid and 1-MGG, which correspond to the same galloyl equivalents as 1 mM PGG in the reaction mixture, were compared with PGG (Table 1). Gallic acid (4%) and 1-MGG (16%) showed far lower activity than PGG (81%) against maltase. This result strongly indicates that it is advantageous for the inhibitory activity that two or more galloyls exist in the same molecule to form the polygalloyl structure. PGG could interact more effectively with the enzyme protein than gallic acid itself, since five galloyl esters bind to a D-glucose core in the molecule of PGG. Bacon and Rhodes reported that gallic acid showed lower affinity to proline-rich proteins (PRP) in the binding competition assay between hydrolyzable tannin and PRP, but PGG showed higher affinity.[8]

In the inhibitory study of sweet almonds α-glucosidase by hydrolyzable tannins, it has been demonstrated that the order of inhibitory activity is PGG > casuarictin > β - 1,2,3,6 - tetra - *O* - galloyl - D-glucose > β-1,2,6-tri-*O*-galloyl-D-glucose. In addition, rugosin D (**7**), a dimeric form of eugeniin carrying 4,6-HHDP group, and sanguin H6 (**8**), a dimeric form of casuarictin carrying 2,3- and 4,6-HHDP groups, showed even higher activity than monomeric tannins due to their high affinity to the enzyme.[9] The HHDP esters present in monomeric gallotannins with molecular weights smaller than 1000 have a little influence on the interaction with an enzyme protein as shown in the comparison of **1** and **4**. On the other hand, in the case of dimeric tannins, HHDP would have considerable effect on the enzyme inhibitory activity since the higher activity was observed in **8** than **3** although **8** has only one uncoupled galloyl compared to **3**. It is supposed that how effective gallotannins bind to protein might depend not only on the number of galloyl groups, but also on their whole molecular structure. The details, however, remain to be clarified.

We have suggested that tested gallotannins had influence only on maltase activity, but not on sucrase activity of crude α-glucosidase mixture.[2] In this experiment with synthesized galloyl glucoses, PGG, which showed the highest activity against maltase, inhibited the sucrase activity as low as 40% and also eugeniin did 17%. These inhibitory activities were significantly lower than those against maltase, PGG (81%) and eugeniin (50%) (Fig. 3). The same tendency was seen at the concentration-dependency assay (Fig. 4). The IC_{50} values were 0.16 mM of PGG and 1 mM of eugeniin in maltase activity but the inhibitory activity of PGG against sucrase did not reach to 50% even at the concentration of 1 mM. In the rat intestinal α-glucosidase, there are two complexes, maltase-glucoamylase (M-G) and sucrase-isomaltase (S-I) complexes. The tested compounds might have an effect on the sites which were responsible only for maltose-hydrolyzing activity but not for sucrose-hydrolyzing activity. This could be the reason in which the inhibitory activity using maltose as a substrate was higher than sucrose. There is an interesting difference in substrate spicificity observed between the two enzyme complexes. The M-G complex does not have sucrose-hydrolyzing activity but S-I complex can cleave maltose. Thereby, we propose the

Table 1. The Inhibitory Activities of Gallic Acid, 1-MGG, and PGG against Rat Intestinal α-Glucosidases

	Activity (%)	
	Maltase	Sucrase
Gallic acid (5 mM)	4.3	8.12
1-MGG (5 mM)	16.3	34.4
PGG (1 mM)	80.9	39.6

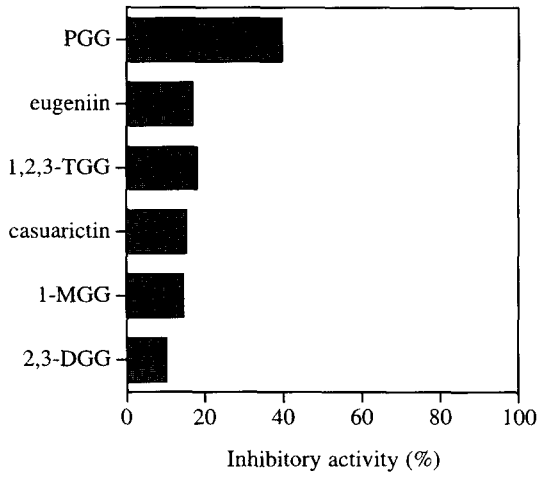

Fig. 3. Rat Intestinal Sucrase Inhibitory Activities of Ellagitannins (Eugeniin and Casuarictin) and Galloylglucoses (1-MGG, 2,3-DGG, 1,2,3-TGG, and PGG) Using Sucrose as a Substrate.
The concentration of each tested compound was 10^{-3} M.

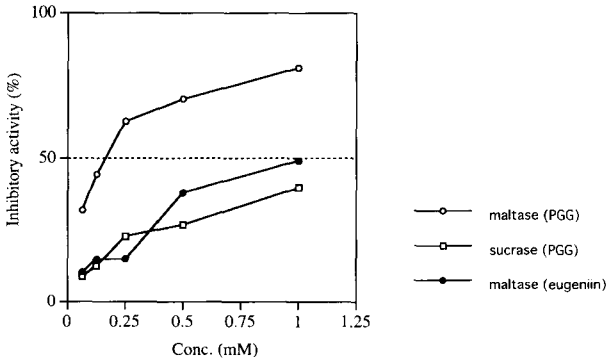

Fig. 4. Rat Intestinal Maltase and Sucrase Inhibitory Activity of Eugeniin and PGG at Various Concentration (0.064 mM^{-1} mM).
Maltase activity of PGG (open circle), sucrase activity of PGG (open square), maltase activity of eugeniin (filled circle). IC_{50} values of eugeniin and PGG were 1 mM and 0.16 mM, respectively.

hypothesis that the inhibitory influences of the galloylglucoses might be more effective on M-G complex than S-I complex. The fact that the concentration-dependence curve of the inhibitory activity of PGG against maltase because nearly flat at 1 mM and did not exceed 75% inhibition could be accounted for by the residual maltose-hydrolyzing activity of the unaffected S-I complex.

To test this hypothesis, we examined the inhibitory activity of gallotannins by purified two complexes from crude rat intestinal α-glucosidase preparations. The activity of M-G complex was measured using maltose (MGm). On the other hand, maltose (SIm), sucrose (SIs) and isomaltose (SIi) were used for S-I complex, as a substrate. The specific activity of two complexes prepared from rat intestinal acetone powder in this study, M-G and S-I, was 2.68 U/mg protein and 1.29 U/mg protein, respectively, using maltose as a substrate. In the maltase reaction mixture with crude enzyme, the rate of each activity would be M-G:S-I = 2:1. In the inhibitory assay of MGm, PGG (**3**), eugeniin (**2**) and 1,2,3-TGG (**6**) showed inhibition of 88%, 71% and 45%, respectively. On the other hand, in the assay of SIm and SIs, a weak inhibitory activities were shown for PGG (53% and 61%), eugeniin (27% and 25%) 1,2,3-TGG (17% and 17%), respectively (Fig. 5). This result emphasizes our assumption described above. Using isomaltose as a substrate for S-I complex (SIi), PGG, eugeniin, and 1,2,3-TGG showed high inhibition of 73%, 45%, and 34%, respectively. Other galloyltannins, 1-MGG, 2,3-DGG, and casuarictin, showed lower inhibitory activity as shown with crude α-glucosidase (data not shown). Free S-unit of S-I complex shows high maltase activity, but I-unit has little specificity for maltose.[10] Therefore, the tannins might particularly have influence on M-G complex and I-unit of S-I complex. Furthermore, in the sucrase inhibitory assay of PGG, there is an interesting difference between using crude enzyme (40%) and purified S-I complex (61%). The crude enzyme solution contains various proteins, and tannin might have interaction not only with the targeted enzyme protein but also with other proteins. Thus, the inhibitors could have an affinity more directly to the M-G or S-I complex when enzyme was purified, and thus the inhibitory activity against the M-G or S-I complex was higher than against crude enzyme.

Tannins (polyphenols) have a specific property such as the ability to precipitate some proteins. Protein precipitation occurs as a surface phenomenon in which phenolic groups of tannins bind to the protein surface. Concerning the principle of association between polyphenols and protein at the low protein concentration, it was suggested that polyphenols bind to one or more sites of the protein surface and form a hydrophobic mono-layer, which then causes aggregation and precipitation. In contrast, at high

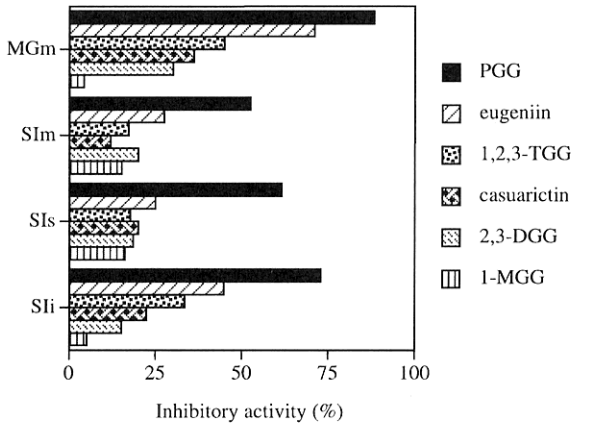

Fig. 5. Inhibitory Activity of 1,2,3-TGG, Eugeniin and PGG against Two α-Glucosidase Complexes, Maltase-Glucoamylase (M-G complex) and Suclase-Isomaltase (S-I complex).
The concentration of each tested compound was 10^{-3} M.
MGm, maltase activity of M-G complex; SIm, maltase activity of S-I complex; SIs, sucrase activity of S-I complex; SIi, isomaltase activity of S-I complex.

protein concentrations, polyphenols make complexation and cross-linking with protein and by this multiple interaction, they form a hydrophobic surface and precipitation.[11] In a similar manner, the mechanism of the inhibitory action of the galloyl glucoses against α-glucosidases could be the tannin-protein binding which makes changes of conformation of the enzyme, and hence, reduce the enzyme activity by association and precipitaion.

Baxter *et al.* have reported about importance of the galloyl-prolyl residues interaction.[12] Polyphenols tend to seek out binding sites such as hydrophobic parts on protein surface and make hydrophobic binding. Those hydrophobic parts are mainly prolyl residues in polypeptide chain. At the next stage, the hydrogen bonds between phenolic residues and polar groups of protein forces the association. Moreover, they suggested that the linkage of polyphenols with proline-rich proteins were in a multidentate fashion and proteins precipitated by themselves. In addition, large polyphenols such as tannins tend to bind with consecutive proline residues in a polypeptide chain. Therefore, if M-G and S-I enzyme complexes had different contents and arrangements of proline residues, it might lead to the difference in inhibitory activity against the both complexes.

It has already been known that aspartic residues are necessary to α-glucosidase activity.[13,14] Although the site, where tannins bind, might not exactly be active the center, if it were near active aspartic residues, such tannins could influence the enzyme activity.

In conclusion, our results suggest that the inhibitory activity of the galloyltannins against the rat intestinal α-glucosidases are influenced by not only the number of galloyl units but also the whole structure of tannins, and the inhibitory influence on the M-G

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complex was more potent than on the S-I complex.

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