

Inhibitory Effects of Epigallocatechin Gallate and Its Glucoside on the Human Intestinal Maltase Inhibition

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Abstract Human intestinal maltase (HMA) is an α -glucosidase responsible for the hydrolysis of α -1,4-linkages from the non-reducing end of malto-oligosaccharides. HMA has become an important target in the treatment of type-2 diabetes. In this study, epigallocatechin gallate (EGCG) and EGCG glucoside (EGCG-G1) were identified as inhibitors of HMA by an *in vitro* assay with IC_{50} of 20 ± 1.0 and 31.5 ± 1.0 μ M, respectively. A Lineweaver-Burk plot confirmed that EGCG and EGCG-G1 were competitive inhibitors of maltose substrate against HMA and inhibition kinetic constants (K_i) calculated from a Dixon plot were 5.93 ± 0.26 and 7.88 ± 0.57 μ M, respectively. Both EGCG and EGCG-G1 bound to the active site of HMA with numerous hydrophobic and hydrogen bond interactions.

Keywords: alpha-glucosidase, human intestinal maltase, EGCG, EGCG glucoside, molecular docking

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1. Introduction

Green tea has attracted significant attention, both in the scientific and in the consumer communities, because of its health benefits for a variety of disorders, ranging from cancer to weight loss [1]. Epigallocatechin gallate (EGCG) is a major catechin in green tea that accounts for 50 ~ 80% of the total tea components [2]. EGCG possesses a range of biological and medicinal properties, including anti-oxidant [3], anti-carcinogen [2,4], anti-obesity [5], anti-bacterial, anti-viral, anti-enzymatic [1,6] and anti-metastasis effects. The latter effects are directed at matrix metalloproteinases (MMPs) including MMP2, MMP7, MMP9, and MMP12 [7]. However, EGCG is not very soluble in water, and degrades easily in aqueous solutions [8]. Due to these disadvantages, the uses of EGCG in the food, drug, and cosmetic industries remains somewhat limited. To circumvent these disadvantages, enzymatic transglycosylation using *Leuconostoc mesenteroides* glucansucrases has been applied to the modification of EGCG and a variety of nature bioactive substances to improve their functionality and physicochemical properties [9-11]. Transglycosylated compounds display enhanced functionalities in water solubility, light stability, increased resistance to oxidation, and less bitter taste [9-12].

Small intestinal α -glucosidase [EC 3. 2. 1. 20] and pancreatic α -amylase [EC 3. 2. 1. 1] are key enzymes of dietary carbohydrate digestion in humans. Inhibitors of these enzymes may be effective in retarding carbohydrate digestion and glucose absorption to suppress post prandial hyperglycemia [13]. α -Glucosidase is an attractive target for treatment of type 2 diabetes and obesity [14,15]. Human intestinal maltase (HMA) is a N-terminal catalytic domain of human intestinal maltase-glucoamylase (MGA)

and is responsible for α -glucosidase activity, which hydrolyzes α -1,4-linkages of maltose, resulting in production of glucose [15]. According to the glycoside hydrolase (GH) classification scheme, HMA is a member of GH31 [16,17].

In this study, we examined the inhibition activity of EGCG and EGCG glucoside (EGCG-G1) against HMA expressed from *Pichia pastoris* X-33. The detailed inhibition mechanism of EGCG and EGCG-G1 was investigated based on enzyme inhibition kinetic and molecular docking studies using Autodock 3.0.5 [18].

2. Material and Methods

2.1. Materials

P. pastoris X-33 as the expression host and plasmid pPICZ α A were purchased from Invitrogen (Carlsbad, CA, USA). EGCG was obtained from Sigma-Aldrich (St. Louis, MO, USA). The Sephadex LH-20 gel was acquired from Amersham Biosciences (Uppsala, Sweden).

2.2. Preparation and activity of recombinant HMA

HMA protein (MW 126,000 Da) was expressed in *P. pastoris* X-33 and purified using Ni-Sepharose resin (GE Healthcare, Buckinghamshire, UK) as described previously [17]. Purified sample of HMA solutions were stored at -80°C .

HMA activity was measured by the amount of released glucose from maltose as a substrate by a glucose oxidase-peroxidase (GOP) method using a Glucose-E kit (BMI, Sungnam, Korea). The enzymatic reaction was composed of 20 μL of enzyme (0.04 U/mL), 40 μL of maltose of various concentrations, and 140 μL of 50 mM potassium phosphate buffer (pH 6.5) at 37°C for 15 min. The reaction was stopped by adding 0.4 mL of 2 M Tris-Cl (pH 8.0) and 0.1 mL of glucose-oxidase assay reagent. Reactions were allowed to develop at 37°C for 1 h and absorbance was measured at 505 nm to determine the amount of glucose produced by HMA activity in the reaction.

2.3. Preparation of EGCG-G1

The acceptor reaction mediated by *L. mesenteroides* glucosyltransferase and purification of EGCG-G1 using Sephadex LH-20 chromatography were performed as described previously [9,19].

2.4. Inhibition assay

Primary inhibitory activity of each test compound was determined by measurement of the remaining activity of HMA at a concentration of 100 μM . EGCG and EGCG-G1 were dissolved in water as a 10 mM stock solution. The enzymatic reaction, composed of 20 μL of enzyme

(0.04 U/mL), 40 μL of 10 mM maltose, 1 μL of test compound, and 139 μL of 50 mM potassium phosphate buffer (pH 6.5), was incubated at 37°C for 15 min and the control reaction was carried out by following the inhibition assay protocol, except for the use of 1 μL of buffer instead of test compound. Enzymatic activity was measured by the glucose-oxidase assay described above. Samples and blanks were prepared in triplicate. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of HMA inhibitor necessary to reduce HMA activity by 50% relative to a reaction mixture containing HMA enzyme but no inhibitor.

2.5. Enzyme kinetics

Kinetic parameters of recombinant HMA were determined using the glucose-oxidase assay to detect the release of glucose upon the addition of enzyme at increasing maltose concentrations (2.5 ~ 15 mM) with a reaction time of 15 min. Reactions products were produced linearly within this time frame. All methods were similar to those used in the initial studies, except that multiple concentrations of the inhibitors were used (0, 10, and 20 μM). The type of inhibition was determined using Lineweaver-Burk plots calculated using the SigmaPlot program (SigmaStat, San Diego, CA, USA). K_i values for the competitive inhibitors were determined by Dixon plot ($1/v$ as a function of $[I]$) from the different inhibitor concentrations.

2.6. Molecular docking study of HMA with EGCG and EGCG-G1

The Autodock 3.0.5 docking software [18] was used to perform the automated molecular docking between HMA and EGCG and EGCG-G1. The HMA crystal structure [PDB code 2QMJ with inhibitor Acarbose (ACR)] [20] at 1.9 \AA resolution was prepared for docking and post-docking refinement. For the docking experiment of EGCG and EGCG-G1 with 2QMJ, all water molecules and the inhibitor (ACR) located in the active site of 2QMJ were removed, and the structure information containing only the amino acid residues of the HMA enzyme was used. The process of molecular docking between EGCG or EGCG-G1 and HMA has been described previously [21-23]. The hydrogen interaction between EGCG or EGCG-G1 and HMA active site pocket was visualized by Ligplot software [24].

3. Results and Discussion

3.1. HMA inhibition by EGCG and EGCG-G1

In the effort to develop effective anti-diabetic agents, a variety of α -glucosidase inhibitors have been discovered [25]. Due to the limited availability of the human α -

glucosidase, most searches for inhibitors have been based on commonly used α -glucosidases, such as those found in bacteria, yeast, plants, and animal tissues [13,26-30]. In this study, we used human intestinal maltase expressed in *P. pastoris* X-33 and purified by Ni-Sepharose resin for inhibition assay of EGCG and EGCG-G1. Its K_m and V_{max} values were 3.3 ± 0.25 mM and 62 ± 2 U/mg, respectively. The yeast *P. pastoris* has been established as a host organism for the expression of heterologous proteins from prokaryotes, eukaryotes, and viruses. The increasing popularity of this particular expression system can be attributed to several factors, most importantly: (1) the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris* and their similarity to those of *Saccharomyces cerevisiae*, one of the most well-characterized experimental systems in modern biology; (2) the ability of *P. pastoris* to produce foreign proteins at high levels, either intracellularly or extracellularly; (3) the capability of performing many eukaryotic posttranslational modifications, such as glycosylation, disulfide bond formation, and proteolytic processing; and (4) the availability of the expression system as a commercially available kit [31,32].

EGCG-G1 was synthesized using glucansucrase from *L. mesenteroides*, which contains a glycosyl residue in the B ring of EGCG (Fig. 1). The inhibitory activities of EGCG and EGCG-G1 toward the recombinant HMA activity were measured by the GOP method. At 100 μ M of each compound, EGCG and EGCG-G1 inhibited 95.4% and 82% of the HMA activity, respectively (Table 1). The IC_{50} values of EGCG and EGCG-G1 in inhibiting the catalytic

activity of HMA were calculated to be 20 ± 1.0 and 31.5 ± 1.0 μ M, respectively (Table 1).

To characterize the inhibitory mechanism, the inhibition constants (K_i) of EGCG and EGCG-G1 against recombinant HMA activity were determined by measurement of the initial rates of maltose hydrolysis in the presence of different inhibitor concentrations. Both Lineweaver-Burk and Dixon plots were used. As shown in Fig. 2A, EGCG and EGCG-G1 exhibited competitive inhibition toward HMA because the Lineweaver-Burk plot of $1/v$ versus $1/[S]$ resulted in a family of straight lines with the same y-axis intercept. On the basis of linear regression analysis of the Dixon plot (Fig. 2B), the inhibitor constants (K_i) were determined to be 5.93 ± 0.26 and 7.88 ± 0.57 μ M, respectively. Comparison with acarbose, one of commercial drug for treatment of diabetes, EGCG and EGCG-G1 showed 3.22 times and 2.42 times higher inhibitory activity against HMA than that of acarbose [17].

3.2. Molecular modeling studies for inhibitory mechanism

To understand the inhibitor binding mode of EGCG and EGCG-G1 with HMA, we performed a molecular docking simulation and hydrogen bonding interaction between these compounds and the active site pocket of HMA. EGCG and EGCG-G1 showed strong binding to the active site pocket of HMA with free binding energies of -14.20 and -15.13 kcal/mol, respectively. EGCG and EGCG-G1 showed numerous hydrogen bond (H-bond) interactions and hydrophobic interactions with various amino acid residues in active site pocket of HMA. Figs. 3A and 3B show the details of the specific interactions between EGCG and EGCG-G1 with HMA. EGCG displayed numerous hydrophobic interactions with Tyr299, Trp441, Met444, Asp542, His600, Gln603, and Tyr605 in the active site of HMA. The O atom of the 4-OH and 5-OH group of 3,4,5 trihydroxybenzoate (gallate ring) formed H-bonds with the main chain carboxyl group of Gln603 with a distance 2.64 Å and with the side chain amine group of Gln603 with a distance of 2.54 Å , respectively. The O atom of the 5-OH group and 7-OH group of ring A of chroman group had 2.67 Å and 3.03 Å H-bond with side chain carboxyl group of Asp327 and N of imidazol group of His600, respectively. The O atom of the 3'-OH group of ring B

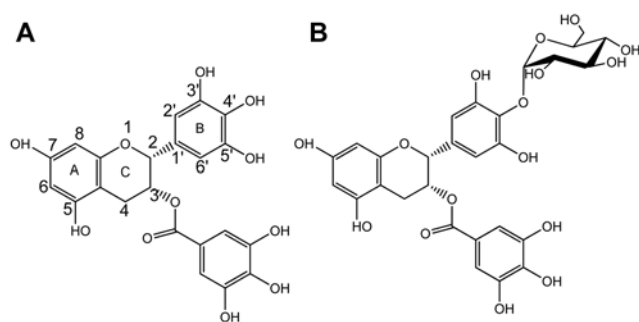


Fig. 1. Chemical structures of EGCG and EGCG-G1.

Table 1. Inhibitory activity of EGCG and EGCG-G1 against HMA

| Compound | Inhibition ^a (%) | IC_{50} (μ M) ^b | K_i (μ M) (μ M \pm SD) ^b | Type of inhibition | Docking energy (kcal/mol) |
|----------|--------------------------------|--------------------------------------|--|--------------------|------------------------------|
| EGCG | 95.4 | 20.0 ± 1.0 | 5.93 ± 0.26 | Competitive | -14.20 |
| EGCG-G1 | 82.0 | 31.5 ± 1.0 | 7.88 ± 0.57 | Competitive | -15.13 |

^aInhibition at a concentration of 100 μ M.

^bData are expressed as mean \pm SD (n = 6).

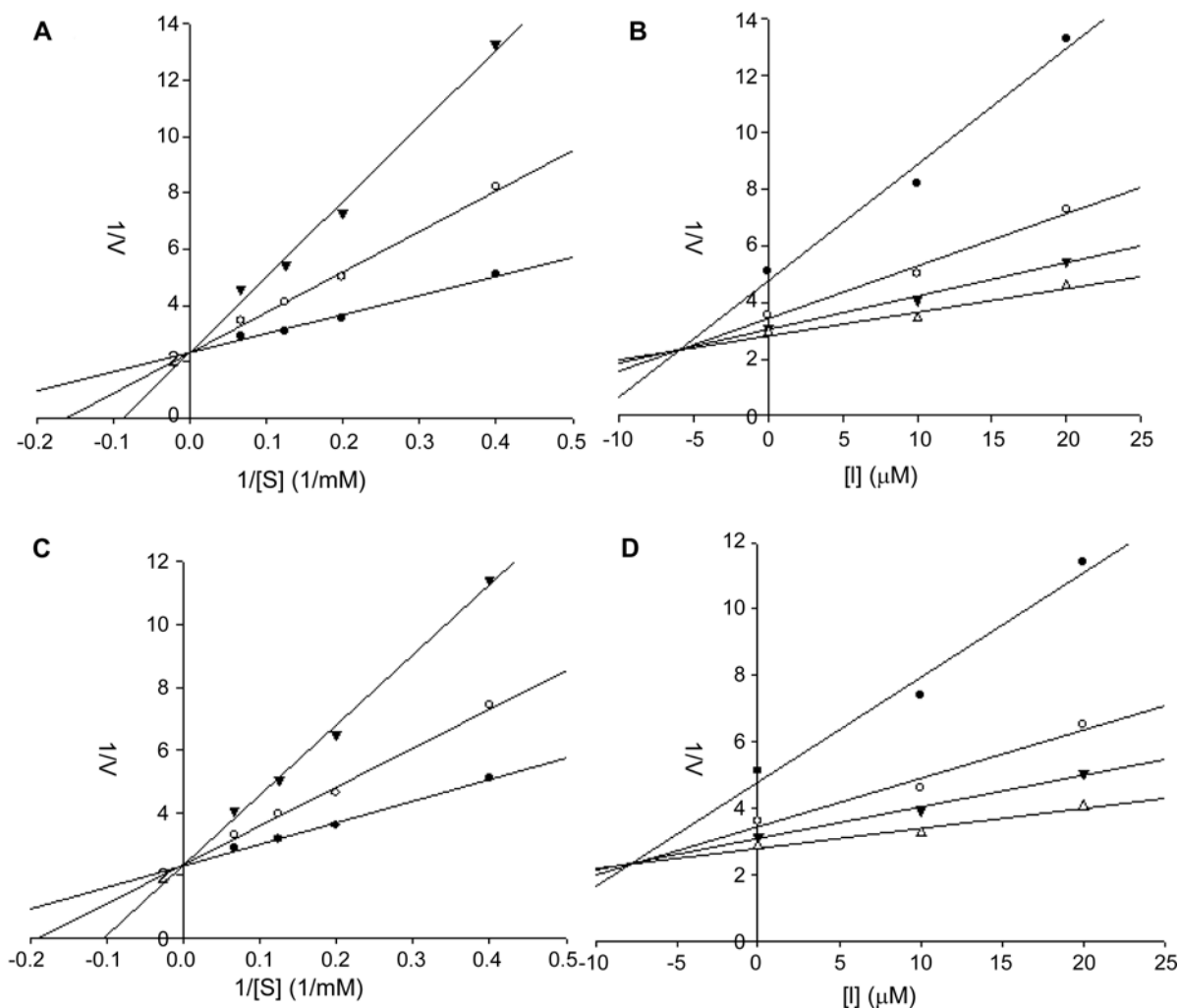


Fig. 2. Graphical determination of the inhibition types for EGCG and EGCG-G1. (A, C) Lineweaver-Burk plots for the inhibitions of EGCG or EGCG-G1 against HMA. (B, D) Dixon plot analyses for the inhibition of HMA by EGCG and EGCG-G1. The kinetic constants, K_i , were calculated using linear regression analysis. (A, C) EGCG and EGCG-G1 concentration 0 μM (●), 10 μM (○), and 20 μM (▼). (B, D) maltose concentrations 2.5 mM (●), 5.0 mM (○), 8 mM (▼), and 15 mM (△).

(catechol) accepted a 3.22 Å H-bond of side chain carboxyl group of Asp542. These results showed that EGCG bound strongly to substrate binding sites of HMA through H-bonds with key residues in the active site pocket of HMA: Asp32, acid/base catalysts Asp542 and His600. Like EGCG, EGCG-G1 has numerous hydrophobic interactions with Asp203, Tyr299, Trp406, Met444, Lys480, Asp542, and Gln603. EGCG-G1 displayed one H-bond interaction between the O atom of 5-OH group of ring A of chroman group and the side chain carboxyl group of Asp327 at a distance of 2.49 Å. The O atom of glucose in EGCG-G1 also formed two H-bonds with the amino group of Thr205 and the hydroxyl group of the side chain Thr205 at 2.86 and 3.06 Å, respectively. EGCG-G1 is EGCG with glycosylation at 4'-OH of ring B. The glycosylation at 4'-OH of ring B would increase solubility in water 55 times

more than EGCG [9], but it would change the physicochemical properties of EGCG molecule, such as surface area, acidity, hydrophobicity, and solvation. With glycosylation at the 4'-OH of ring B of EGCG, some potential H-bonds were removed. These included the H-bond between the N of the imidazol group of His600 and the 7-OH group of ring A of the chroman group, the O atom of the 3'-OH group of ring B (catechol) and side chain carboxyl group of Asp542, and the O atom of 4-OH and 5-OH groups of the gallate ring and main chain carboxyl group of Gln603. However, glycosylation at the 4'-OH of ring B of EGCG produced two H-bonds between the Thr205 and the O atom of glucose in EGCG-G1. The hydrophobic interaction of EGCG or EGCG-G1 also differed. EGCG-G1 had unique hydrophobic interactions with Asp203, Trp406 and Lys480 compared with EGCG having Trp441, Gln603 and Tyr605

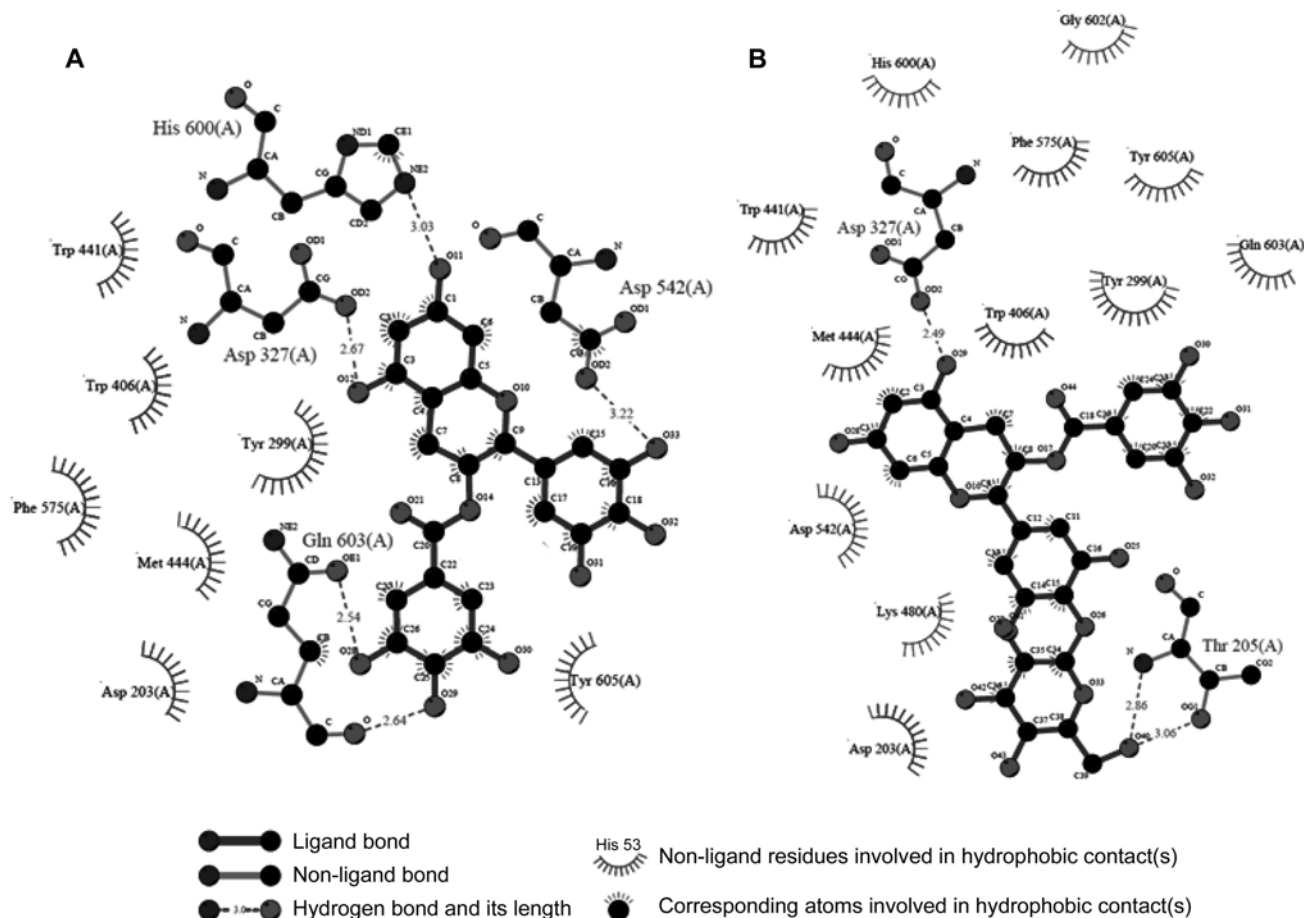


Fig. 3. Hydrogen bond interactions of EGCG and EGCG-G1 with various amino acid residues in the active site pocket of HMA. Hydrogen bond interactions of EGCG (A) and EGCG-G1 (B) with amino acid residues in the active site of HMA are represented by green dashed lines.

(Fig. 3).

This study reports for the first time that EGCG and EGCG-G1 competitively inhibit HMA expressed in *P. pastoris* X-33 with a K_i values of 5.93 ± 0.26 and $7.88 \pm 0.57 \mu\text{M}$, respectively. EGCG and EGCG-G1 were shown to be bound to the active site of HMA by docking simulation using the Autodock 3.0.5 software, and numerous hydrophobic interactions as well as H-bond interactions with various amino acid residues were observed.

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