The Inhibition of α -Amylase by Tea Polyphenols

Yukihiko HARA and Miwa HONDA

Food Research Laboratories, Mitsui Norin Co., Ltd.,
2-1-17, Fujieda, Fujieda-shi, Shizuoka 426, Japan

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The inhibition of α -amylase from human saliva by polyphenolic components of tea and its specificity was investigated *in vitro*. Four kinds of green tea catechins, and their isomers and four kinds of their dimeric compounds (theaflavins) produced oxidatively during black tea production were isolated. They were (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECg), (–)-epigallocatechin gallate (EGCg), (–)-catechin (C), (–)-gallocatechin (GC), (–)-catechin gallate (Cg), (–)-gallocatechin gallate (GCg), theaflavin (TF1), theaflavin monogallates (TF2A and TF2B), and theaflavin digallate (TF3). Among the samples tested, EC, EGC, and their isomers did not have significant effects on the activity of α -amylase. All the other samples were potent inhibitors and the inhibitory effects were in the order of TF3 > TF2A > TF2B > TF1 > Cg > GCg > ECg > EGCg. The inhibitory patterns were noncompetitive except for TF3.

α -Amylases (EC 3.2.1.1) catalyze the hydrolysis of α -1,4-glucosidic linkages of starch, glycogen, and various oligosaccharides. The inhibition of their activity in the digestive tract of humans is considered to be effective to control obesity or diabetes by diminishing the absorption of glucose decomposed from starch by these enzymes.^{1–4)} Therefore, effective and nontoxic inhibitors of α -amylases have long been sought.

On the other hand, tannins or vegetable polyphenols are known to have strong affinities for peptides or protein and these characteristics were exemplified and reported as inhibitors of amylase or trypsin.^{5–8)}

However, few studies have yet been done on whether the polyphenols of green tea or black tea, consumed daily in the commonest drinks, influence the activities of α -amylase. We investigated how the activities of α -amylase are affected by tea polyphenols extracted from teas in pure forms.

Materials and Methods

α -Amylase preparation. α -Amylase (220 units/mg solid) from human saliva was purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. One unit of α -

amylase liberates 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20°C. A stock solution of the enzyme was prepared at a final concentration of 1.0 mg/ml in 80 mM 3,3-dimethylglutaric acid buffer (pH 6.9) containing 40 mM NaCl and 5.0 mM CaCl₂. The test enzyme solution (20 μ g/ml) was obtained by diluting the stock solution before each examination.

Catechins and theaflavins. (–)-Epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECg), and (–)-epigallocatechin gallate (EGCg) were prepared from crude catechins (containing 90% catechins) as described in our previous paper.⁹⁾ Isomers of the above compounds were made by heating the corresponding catechin solution.¹⁰⁾

Crude theaflavins, theaflavin (TF1), theaflavin monogallate A (TF2A), theaflavin monogallate B (TF2B), and theaflavin digallate (TF3) were prepared from black tea.¹¹⁾

The structural formulas of these compounds are shown in Fig. 1.

Enzyme assay. The test enzyme solution (150 μ l, final conc. 2 μ g/ml) was diluted with the buffer (1230 μ l) and incubated for 10 min at 37°C. Soluble starch solution (120 μ l) heated to 37°C was added to the above preparation. At predetermined times, 200 μ l of the reaction mixture was transferred to a test tube containing 200 μ l of DNS reagent.¹²⁾ Immediately the mixture was boiled for 5 min and cooled in tap water. After the addition of 2.0 ml of distilled water, the absorbance of the solution was measured at 540 nm with a Shimadzu model UV-240 spectrophotometer. α -Amylase activity was expressed in terms of molar of maltose liberated in one minute by one

Fig. 1. Structures of Catechins and Theaflavins.

unit of α -amylase.

The inhibitory effects of samples were measured by incubating α -amylase solution (150 μ l) with inhibitor solution (1230 μ l) for 10 min at 37°C. The enzyme activity was assayed as described above.

The concentrations of samples for 50% inhibition of the enzyme were calculated as follows:

$$\text{Activity (\%)} = \frac{(\text{control} - \text{sample activity})}{\text{control activity}} \times 100$$

Other materials. Soluble starch was purchased from Wako Pure Chemical Ind., Ltd., Osaka, Japan. All other chemicals were of reagent grade.

Results and Discussion

The kinetic parameters of α -amylase for soluble starch

The kinetic parameters such as Michaelis constants (K_m) and maximum velocity ($V_{\max}$) were obtained through Lineweaver-Burk plots (Fig. 2). K_m was 1.9 mg/ml and $V_{\max}$ was 2.6×10^{-7} M/U.

Inhibition of enzyme activity by various samples

The percentage of α -amylase activity in the presence of various inhibitors is shown in Table I. Among twelve samples, free catechins (EC and EGC) and their isomers as well as gallic acid did not have significant effects on the activity of α -amylase. All other samples were inhibitors and the inhibitory effects were in the order of TF3 > TF2A > TF2B > TF1 >

Cg > GCg > ECg > EGCg.

Inhibition constants of α -amylase inhibitors

The inhibition of the α -amylase activity by four kinds of catechins (ECg, EGCg, and their isomers) and four kinds of theaflavins (TF1, TF2A, TF2B, and TF3) were measured and their inhibition constants (K_i) were calculated from Dixon plots (Figs. 3 and 4). These figures showed noncompetitive inhibition patterns for these polyphenols but TF3. The K_i values are listed in Table II. The results are that galloyl (3,4,5-trihydroxybenzoyl) groups at 3-OH (ECg and EGCg) showed potent inhibition and their inhibitory activity was potentiated 10 times by isomerization of these gallated compounds (ECg $\rightarrow$ Cg and EGCg $\rightarrow$ GCg). Among these compounds, catechol catechins (Cg and ECg) were 2 times more inhibitory than pyrogallol catechins (GCg and EGCg) respectively.

From these facts it is assumed that a 3,4,5-trihydroxybenzoyl moiety at 3-OH of these catechins is essential for the inhibition and their activities are affected by the stereostructure of the B ring.

In the case of theaflavins, their inhibitory activities were much stronger than those of catechins. Further their inhibitory potency was enhanced as the number of galloyl moiety increased (TF3 > TF2 > TF1).

Fig. 2. Lineweaver-Burk Plots for Hydrolysis of Soluble Starch by α -Amylase from Human Saliva.

Table I. INHIBITION OF α -AMYLASE BY TEA POLYPHENOLS AND GALLIC ACID

Concentration of soluble starch was 2.0 g/l.

Samples	Configuration	MW	ID ₅₀ (μ M)
(-)-Epicatechin (EC)	2 <i>R</i> ,3 <i>R</i>	290	> 1000
(-)-Catechin (C)	2 <i>S</i> ,3 <i>R</i>	290	> 1000
(-)-Epigallocatechin (EGC)	2 <i>R</i> ,3 <i>R</i>	306	> 1000
(-)-Gallocatechin (GC)	2 <i>S</i> ,3 <i>R</i>	306	> 1000
(-)-Epicatechin gallate (ECg)	2 <i>R</i> ,3 <i>R</i>	442	130
(-)-Catechin gallate (Cg)	2 <i>S</i> ,3 <i>R</i>	442	20
(-)-Epigallocatechin gallate (EGCg)	2 <i>R</i> ,3 <i>R</i>	458	260
(-)-Gallocatechin gallate (GCg)	2 <i>S</i> ,3 <i>R</i>	458	55
Theaflavin (TF1)		565	18
Theaflavin monogallate A (TF2A)		717	1.0
Theaflavin monogallate B (TF2B)		717	1.7
Theaflavin digallate (TF3)		869	0.6
Gallic acid		188	> 1000

By the way, among the samples tested, only theaflavin digallate (TF3) was not spotted on a line by the Dixon plot. This fact implied possible interactions of the substrate with inhibitors. Therefore we examined the influence of the substrate in the different reaction sys-

tems. First, the inhibitors (at concentrations of the calculated IC₅₀) and the substrate were incubated, followed by the addition of the enzyme. Second, the inhibitors, the substrate, and the enzyme (all of them at the above concentrations) were mixed in the solution at

Fig. 3. Dixon Plots for Hydrolysis of Soluble Starch by α -Amylase in the Presence of Catechins.

(a), ECg; (b), EGCg; (c), Cg; (d), GCg. Concentration of soluble starch was 1.5 g/l (●), 2.0 g/l (○), and 3.0 g/l (▲).

Table II. INHIBITION CONSTANTS (K_i) OF TEA POLYPHENOLS FOR α -AMYLASE

Samples	K_i (M)
(-)-Epicatechin gallate	1.4×10^{-4}
(-)-Catechin gallate	3.2×10^{-5}
(-)-Epigallocatechin gallate	2.3×10^{-4}
(-)-Gallocatechin gallate	4.5×10^{-5}
Theaflavin	1.7×10^{-5}
Theaflavin monogallate A	7.8×10^{-7}
Theaflavin monogallate B	1.6×10^{-6}

the same time. The results showed that all the inhibitors did not show a marked decrease of inhibitory activity compared with the figures in the original system except for the reactions involving TF3, where the inhibitory activity in both systems became apparently nil. To see this phenomenon in more detail, the direct

binding capacity of TF3 to substrate was measured by incubating TF3 with the substrate (Fig. 5). The enzyme activity curve obtained in Fig. 5 shows a certain latent phase at lower TF3 concentrations where TF3 seems to bind predominantly to the substrate. Even in this system the IC_{50} of TF3 is as low as 9.8×10^{-7} M, indicating still more the potent inhibitory activity of this compound.

In addition to the findings of this paper, tea polyphenols are known to inhibit hypertensive angiotensin I converting enzyme (ACE)¹¹⁾ and plaque forming glucosyltransferase (GTF).¹⁵⁾ It deserves notice if there is any difference of inhibitory pattern in each instance. In every case theaflavins inhibited enzymes more strongly than catechins. This seems to be a common phenomenon and due in part to the strong affinity of theaflavins for enzymes.

Fig. 4. Dixon Plots for Hydrolysis of Soluble Starch by α -Amylase in the Presence of Theaflavins.

(a), TF1; (b), TF2A; (c), TF2B; (d), TF3. Concentration of soluble starch was 1.5 g/l (●), 2.0 g/l (○), and 3.0 g/l (▲).

Among catechins, gallated catechins were better inhibitors than free catechins. Differences were observed among gallated catechins and their isomers against different enzymes.

In the case of ACE inhibition, the EGCg isomer (GCg) showed little potency as against notable EGCg's. And EGCg was more potent than ECg (EGCg > ECg > GCg). This is just the opposite of the case in α -amylase (GCg > ECg > EGCg). In the case of GTF inhibition, no noticeable difference was observed between EGCg and its isomer. This is probably due to the fact that the GTF used was a mixture of at least three different types of enzymes.¹⁵⁾ From these observations it was assumed that stereostructural differences of these catechins specify the inhibitory potency against each enzyme.

The fact that tea polyphenols inhibit the activity of α -amylase at concentrations (IC_{50}) ranging 0.5–120 μ g/ml is of interest since a cup of tea contains more or less 100 mg of polyphenols at more or less 1000 μ g/ml concentration. It deserves notice if a daily cup would actually affect the enzyme activity. Two grams each of green tea and black tea were extracted with 150 ml of boiling distilled water for 1 min. In the assay system previously described, these daily cup solutions were incubated with the starch solution and the enzyme solution was mixed afterwards. As a result, green tea and black tea inhibited the enzyme activity 87% and 100% respectively. Dilution of the black tea cup by half still inhibited the enzyme 100%. Tea is one of the most commonly consumed beverages in the world. In the case of Japanese,

Fig. 5. Inhibition of α -Amylase by TF3.

TF3 was incubated with the soluble starch (●) and with the enzyme (○) for 10 min at 37°C. Concentration of soluble starch was 2.0 g/l.

people consume on the average almost one kg of made tea (dry leaves) a year, meaning nearly 100 g consumption of tea polyphenols a year.

Today, foods are abundant in the advanced countries and obesity due to overeating is a serious problem to be faced and solved. It is of great interest if tea polyphenols could control body weight without sacrificing the appetite.

A mixture of green tea polyphenols and the major component of it, (–)-epigallocatechin gallate (EGCg) or minor components of black tea, theaflavins, have been demonstrated to have a variety of biological activities on top of this enzyme inhibition, such as antioxidant activity,¹⁶⁾ antibacterial activity,^{17,18)} antiviral activity,¹⁹⁾ a hypocholesterolemic action in rats,²⁰⁾ hypotensive action in rats,²¹⁾ anti-ulcer action in rats,²²⁾ and suppression of the growth of inoculated tumor cells in animals.²³⁾ Our result found an additional example of the biological activity of tea polyphenols and will open a new prospect for further use of them.

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