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# Inhibitory effects of chlorogenic acids from green coffee beans and cinnamate derivatives on the activity of porcine pancreas α-amylase isozyme I

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ABSTRACT

Nine kinds of chlorogenic acids (CGAs) account for 80% of the total CGA content in green coffee beans. They consist of three subgroups of caffeoylquinic acids (CQAs), feruloylquinic acids (FQAs), and dicaffeoylquinic acids (diCQAs). We previously reported the inhibitory effects of 5-CQA on porcine pancreas α-amylase (PPA) isozymes, PPA-I and PPA-II. In this paper, we investigated the PPA-I inhibition by eight kinds of CGAs. The $IC_{50}$ values of CQAs, FQAs, and diCQAs against the PPA-I-catalysed hydrolysis of *p*-nitrophenyl-α-D-maltoside were 0.08–0.23 mM, 1.09–2.55 mM, and 0.02–0.03 mM, respectively. All CQAs and FQAs and 3,5-diCQA showed mixed-type inhibition with binding to the enzyme–substrate complex (ES) being stronger than to the enzyme (E). 3,4-DiCQA and 4,5-diCQA showed mixed-type inhibition, but, conversely are suggested to bind to E stronger than ES.



## 1. Introduction

The chlorogenic acids (CGAs) refer to a family of esters between quinic acid and one or more cinnamate derivatives, such as caffeic, ferulic and *p*-coumaric acids. CGAs are widely distributed in plants, such as yacon, prune, potato, and sweet potato (Islam et al., 2002; Takenaka et al., 2003). Various biological activities of CGAs have been reported, such as antioxidant activity, antimutagenicity, cancer suppression, matrix metalloproteinase inhibition, tyrosinase inhibition, DNA methylation inhibition, and so forth (Iwai, Kishimoto, Kakino, Mochida, & Fujita, 2004).

Coffee is one of the most consumed drinks in the world, and a major source of polyphenols, in particular, chlorogenic acids (CGAs), in the human diet. Green coffee beans are high in CGAs; their contents are 3.5–7.5% (w/w dry matter) for *Coffea arabica* and 7.0–14.0% (w/w dry matter) for *Coffea canephora* (Ky et al., 2001). The nomenclature of CGAs is based on the IUPAC numbering system (1976), and 5-caffeoylquinic acid (5-CQA) is generally called chlorogenic acid. Thirty-four kinds of CGAs have been reported in green coffee beans (Clifford, Knight, Surucu, & Kuhnert, 2006). The CGAs in green coffee beans consist of three main classes: caffeoylquinic acids (CQAs) with three isomers (3-, 4-, and 5-CQA), dicaffeoylquinic acids (diCQAs) with three isomers (3,4-, 3,5-, and 4,5-diCQA), and feruloylquinic acids (FQAs) with three isomers (3-, 4-, and 5-FQA). These nine kinds of CGAs account for 80% of the content of total CGAs in green coffee beans. During roasting, there is a progressive destruction and transformation of CGAs with some 8–10% being lost for every 1% loss of dry matter (Clifford, 1999). The colour, flavour, and aroma of coffee originate from CGAs in green coffee beans by roasting. There are 70–200 mg and 70–350 mg of CGAs per cup (200 ml) of Arabica coffee and Robusta coffee, respectively.

Porcine pancreas α-amylase (EC 3.2.1.1, hereafter abbreviated as PPA) is an endo-glucanase catalysing the hydrolysis of α-1,4-glucosidic linkages in starch, amylose, amylopectin, and glycogen. PPA is composed of 496 amino acid residues and shows 83% homology with human pancreas α-amylase (Pasero, Mazzei-Pierron, Abadie, Chicheportiche, & Marchis-Mouren, 1986). Two active components were separated from crystalline preparation of PPA by anion-exchange chromatography; the rapidly-eluted component was designated PPA-I and the slowly-eluted one was PPA-II (Marchis-Mouren & Pasero, 1967; Sakano, Takahashi, & Kobayashi, 1983). These isozymes are secreted in about equal amounts in pancreatic juice. The amino acid sequence of PPA-I is known (Kluh, 1981), whereas only a partial sequence is reported for PPA-II (Meloun, Kluh, & Moravek, 1980). PPA-I and PPA-II have almost the same molecular mass. The optimum pH values and temperatures for both isozymes in their starch hydrolysis activity are 6.9 and 53 °C, and their isoelectric points are 6.5 and 6.1, respectively (Sakano et al., 1983).

α-Amylase inhibitors (AAIs) seem to be effective for the prevention and therapy of metabolic syndromes, such as type II diabetes and obesity, controlling the elevation of plasma blood glucose

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levels by delaying postprandial carbohydrate digestion and absorption. AAI from white beans (*Phaseolus vulgaris*) was reported to reduce glycaemia in both non-diabetic and diabetic animals and reduced their intake of food and water (Tormo, Gil-Exojo, Romero de Tejada, & Campillo, 2006). AAI from chestnut astringent skin extract was reported to suppress the rise in plasma glucose level after boiled-rice loading in a dose-dependent manner in humans (Tsujita, Yakaku, & Suzuki, 2008). Various AAIs, such as acarbose (Al Kazaz, Desseaux, Marchis-Mouren, Prodanov, & Santimone, 1998) and protein AAI from wheat kernel (Oneda, Lee, & Inouye, 2004), are discussed in the inhibition mechanism against PPA.

We previously reported the inhibitory effect of 5-CQA, and its components, caffeic acid (CA) and quinic acid (QA), on the PPA isozymes, PPA-I and PPA-II, using *p*-nitrophenyl-α-D-maltoside ($G_2$-*p*NP) as substrate at pH 6.9 and 30 °C (Narita & Inouye, 2009). The effects of other CGAs contained in green coffee beans have yet to be elucidated.

In this study, based on our previous report, the inhibition effects of eight kinds of CGAs (3-CQA, 4-CQA, 3-FQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA) and eight cinnamate derivatives [cinnamic acid (CiA), *m*-methoxycinnamic acid (*m*-MCiA), *p*-methoxycinnamic acid (*p*-MCiA), ferulic acid (FA), isoferulic acid (IFA), *p*-coumaric acid (*p*-CoA), *m*-coumaric acid (*m*-CoA), and dihydrocaffeic acid (DHCA)] against PPA-I were examined and their structure–function relationships were evaluated. Fig. 1 shows the structures of CGAs and cinnamic acid derivatives used for this study. We describe the kinetic and thermodynamic analysis of the inhibitory effects of 5-FQA, 3,5-diCQA, and 4,5-diCQA (as the representatives of FQAs and diCQAs in green coffee beans) and discuss their inhibitory mechanisms. We also provide some insights into the nutritional significance of the inhibitors and their preventive effects against diabetes and obesity.

## 2. Materials and methods

### 2.1. Materials and reagents

$G_2$-*p*NP (lot B76297-1) was purchased from Calbiochem (San Diego, CA). 3,4-Dihydroxyhydrocinnamic acid [synonym: dihydrocaffeic acid (DHCA)] was from Sigma (St. Louis, MO). *m*-Hydroxycinnamic acid [*m*-coumaric acid (*m*-CoA)], 3-hydroxy-4-methoxycinnamic acid [isoferulic acid (IFA)], *p*-methoxycinnamic acid (*p*-MCiA), and 3-methoxycinnamic acid (*m*-MCiA) were from Wako Pure Chemical (Osaka, Japan). Pancreatin, chlorogenic acid hemihydrate, caffeic acid (CA), D-(−)-quinic acid (QA), *trans*-cinnamic acid (CiA), 4-hydroxy-3-methoxycinnamic acid [ferulic acid (FA)], *trans*-*p*-coumaric acid (*p*-CoA), and all other chemicals were of reagent grade and were from Nacalai Tesque (Kyoto, Japan). Decaffeinated green coffee bean extract (DGCBE) was obtained from Ominedo Pharmaceutical Industry (Nara, Japan).

### 2.2. HPLC analysis of nine kinds of CGAs in DGCBE

HPLC analysis of CQAs, FQAs, and diCQAs in DGCBE was performed according to the procedures previously reported (Iwai et al., 2004; Matsui et al., 2007), with some modifications. The sample solution was applied to reversed-phase column chromatography in a preparative HPLC 7400 system (GL Science, Tokyo) on an Inertsil ODS-3 [4.6 mm ID × 15.0 cm] column (GL Science) at a column temperature of 35 °C. The mobile phase was composed of solvents **A** (50 mM acetic acid in $H_2O$) and **B** [50 mM acetic acid in acetonitrile], and the gradient programme was as follows: 0–30.0 min, 5–20% (v/v) **B**; 30.0–45.0 min, 20–35% (v/v) **B**; 45.0–50.0 min, 35–80% (v/v) **B**; 50.0–50.1 min, 80–5% (v/v) **B**; 50.1–60 min, 5% (v/v) **B**. The injection volume of the sample solution was 10 μl and the flow rate was 1.0 ml/min. CGAs were detected by absorption at 325 nm with a photodiode array. The spectra of CQAs, FQAs, and diCQAs exhibit an absorption maximum close to 325 nm and a shoulder at 300 nm (Murata, Okada, & Homma, 1995). CGAs in DGCBE were identified by comparing the retention times and the UV spectra of nine standard materials; 5-CQA purchased from Nacalai Tesque and 8 CGAs (3-CQA, 4-CQA, 3-FQA, 4-FQA, 5-FQA, 3,5-diCQA, 3,4-diCQA, and 4,5-diCQA) purified in our laboratory according to the method previously reported (Iwai et al., 2004; Matsui et al., 2007).

### 2.3. Purification of eight kinds of CGAs and PPA-I

3-CQA, 4-CQA, 3-FQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA were purified from DGCBE according to the procedures previously reported (Iwai et al., 2004; Matsui et al., 2007) with some modifications. Two grams of DGCBE were dissolved in 5 ml of distilled water. Five millilitres of the DGCBE solution were applied to a Sephadex LH-20 column (26 mm ID × 45 cm) (GE Healthcare Bio-Science, Piscataway, NJ) using 17 mM acetic acid at a flow rate of 1.0 ml/min and 25 °C. First, the non-adsorbed fractions (10 ml each) having absorption at 325 nm were collected and packed into one fraction, which was named as the NF fraction. NF was concentrated under reduced pressure at 40 °C with a rotary evaporator, freeze-dried, and stored at −20 °C. Then, the adsorbed material on the Sephadex LH-20 column was eluted with 70% (v/v) ethanol, and collected as 10 ml fractions. The fractions having absorption at 325 nm were packed into one fraction, which was named the AF fraction. AF was concentrated, freeze-dried, and stored in the same way as NF.

NF (0.4 g) was dissolved in 2 ml of distilled water. The NF sample (2 ml) underwent size-exclusion chromatography on a Toyopearl HW-40F column (26 mm ID × 45 cm; Tosoh, Tokyo, Japan), and eluted with 17 mM acetic acid at a flow-rate of 0.5 ml/min and 25 °C. The fractions (3 ml each) were collected using a fraction collector. Three peaks (peaks 1–3, hereafter designated as PF-1, PF-2, and PF-3) were obtained (see Fig. 3A). Each peak fraction was concentrated under reduced pressure at 40 °C with an evaporator.

AF (0.4 g) was dissolved in 2 ml of distilled water. Two millilitres of the solution were applied to a Toyopearl HW-40F size-exclusion chromatography column (Tosoh), and successively eluted with a stepwise gradient of 25% (v/v) and 30% (v/v) ethanol containing 17 mM acetic acid at a flow rate of 1.0 ml/min and at 25 °C (see Fig. 3B). Two peaks (peaks 4 and 5, hereafter designated as PF-4 and PF-5) were obtained. Each peak fraction was concentrated at 40 °C with an evaporator.

The PFs, from PF-1 to PF-5, were applied to reversed-phase column chromatography with a preparative HPLC PLC-561 system (GL Science) on an Inertsil ODS-3 column (20 mm ID × 25 cm; GL Science) at a column temperature of 40 °C. The mobile phase was composed of solvents **A** (20% methanol in 35 mM acetic acid) and **B** (methanol), and the gradient programme was as follows: 0–60.0 min, 0–50% (v/v) **B**; 60.0–70.0 min, 50–100% (v/v) **B**; 70.0–70.1 min, 100–0% (v/v) **B**; 70.1–80 min, 0% (v/v) **B**. The injection volume of the sample solution was 5 ml and flow rate was 15.0 ml/min. CGAs were detected by absorption at 325 nm. The purified CGAs were freeze-dried and stored at −20 °C.

PPA-I was purified from pancreatin according to the procedures previously reported (Narita & Inouye, 2009).

### 2.4. Inhibition of PPA-I by CGAs using $G_2$-pNP as substrate

PPA-I hydrolyses $G_2$-*p*NP to produce *p*-nitrophenol (*p*NP) and maltose (Oneda et al., 2004). Hydrolysis of $G_2$-*p*NP by 0.8 μM PPA-I in 20 mM sodium phosphate buffer (pH 6.9, buffer B) containing 25 mM NaCl was measured continuously following the