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# Prolyl Endopeptidase Inhibitors from Green Tea

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Three prolyl endopeptidase (PEP) inhibitors were isolated from the methanolic extract of green tea leaves. They were identified as (-)-epigallocatechin gallate, (-)-epicatechin gallate, and (+)-gallocatechin gallate with the  $IC_{50}$  values of  $1.42 \times 10^{-4}$  mM,  $1.02 \times 10^{-2}$  mM, and  $1.09 \times 10^{-4}$  mM, respectively. They were non-competitive with a substrate in Dixon plots and did not show any significant effects against other serine proteases such as elastase, trypsin, and chymotrypsin, suggesting that they were relatively specific inhibitors against PEP. The isolated compounds are expected to be useful for preventing and curing of Alzheimer's disease.

Key words: Prolyl endopeptidase inhibitor, Green tea leaves, (-)-Epigallocatechin gallate, (-) -Epicatechin gallate, (+)-Gallocatechin gallate

#### INTRODUCTION

The deposition of the amyloid  $\beta$  protein (A $\beta$ ) is extracellular plaques, and cerebrovascular amyloid is one of the major histopathological features of Alzheimer's disease (Selkoe, 1991). A $\beta$  (1-42) with an alanine C-terminus is derived from the proteolytic cleavage of a large protein, known as an amyloid precursor protein (APP), by the action of the yet unidentified endo-proteolytic enzyme 'sectatase' (Checler, 1995). The neurotoxicity of A $\beta$  has been detected in several cell systems, including primarycultured neurons (Mattson et al., 1993). Accordingly, in the development of anti-dementia drugs, it would be important to identify agents that can prevent the formation of A $\beta$ . Prolyl endopeptidase [PEP; EC 3.4.21.26] is a serine protease which is known to cleave peptide substrates on the C-terminal side of proline residues. PEP also plays an important role in the degredation of proline-containing neuropeptides such as oxytocin, vasopressin, substance P, neurotensin and angiotensin, which have been suggested as participants in learning and memory processes (Rennex et al., 1991; Yoshimoto et al, 1983). It was previously found that the PEP activity of Alzheimer's patients was significantly higher than that of

a normal person (Aoyagi et al., 1990). In addition, recent studies have suggested that PEP could be involved in the processing of the C-terminal portion of the amyloid precursor protein in Alzheimer's disease (Ishiura et al., 1990). As a result, it has been postulated that specific PEP inhibitors could prevent memory loss and increase attention span in patients suffering from senile dementia. Some natural and synthetic PEP inhibitors have been reported to show dose-dependant cognition-enhancing activity in rats with scopolamine-induced amnesia (Portevin et al, 1996; Yoshimoto et al., 1987). PEP inhibitors such as eurystatin (Toda et al., 1992), poststatin (Aoyagi et al., 1991), staurosporine (Kimura et al., 1990), SNA-8073-B (Kimura et al., 1997a), propetin (Kimura et al., 1997b) and polyozellin (Hwang et al., 1997) have been isolated from microbial origin but rarely been found from plant sources. In the course of screening for PEP inhibitors from edible plants, we found that EtOAc soluble fraction of green tea leaves showed significant activity. In this paper, isolation, structure determination, and the biological activity against PEP are discussed.

#### MATERIALS AND METHODS

#### General

Green tea leaves (*Thea sinensis*) were obtained in October, 1999 from the cultivation field located at Kangjin, Chunnam, Korea. Authentic catechins were purchased from Sigma. OD was measured with ELISA autoreader

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(ELX, 808Bio-TEK). <sup>1</sup>H- and <sup>13</sup>C-NMR spectra were recorded on a Avance Digital 400, Bruker. Chemical shifts were given in  $\delta$  (ppm) from TMS. EIMS was measured on JMS 700, JEOL at 70 eV. Silica gel (Kieselgel 60, Art. 7734) and pre-coated TLC plates (Kieselgel 60 F<sub>254</sub>, Art. 5715 and Art. 1.15685) were the products of Merck. Sephadex LH-20 was purchased from Sigma.

## **Enzyme** assay

Prolyl endopeptidase (PEP) activity was determined using the method of Yoshimoto, *et al.*, 1980. PEP (from *Flavobacterium meningosepticum*) and its substrate (Z-Cly-Pro*p*NA) were purchased from Seikagaku Co. (Japan). Z-Pro-Prolinal was synthesized according to Bakker *et al.*, 1990 and used as a positive control. Trypsin, chymotrypsin, and elastase (from Sigma) were measured according to the references (Chung *et al.*, 1983; Hubert *et al.*, 1992; Bieth *et al.*, 1974) using *Bz*-L-Arg-pNA, *Bz*-L-Tyr-pNA, *N*-Suc-Ala-Ala-Ala-pNa as substrates, respectively.

## **Extraction and Isolation**

Dried green tea leaves (100 g) were refluxed in MeOH and the extract was evaporated to dryness. The MeOH extract (16 g) was suspended in water and the suspension was partitioned with  $CH_2Cl_2$  and EtOAc, consecutively. The EtOAc extract (5 g) was chromatographed on a silica gel column (4.5 × 80 cm,  $CH_2Cl_2$ -MeOH-HCOOH=10: 1:0.5→0:100:5) and the resultant active fraction was applied on a Sephadex LH-20 column (2.5 × 38 cm, 30 →70% MeOH) to give Fr A~E. Fr B was suspended in 30 ml of acetone-chloroform mixture (1:1). Concentration of the soluble fraction gave compound **1** (252 mg). HPLC (µBondapak C18, 7.8 × 300 mm, Waters, 1% HOAc in 30 % MeOH) of Fr. D and E afforded 5 mg of **2** and 4 mg of **3**, respectively.

### Compound 1 [(-)-epigallocatechin gallate]

Amorphous pale pinkish white powder; FeCl<sub>3</sub> positive; C<sub>22</sub>H<sub>18</sub>O<sub>11</sub> (M.w. 458); FABMS *m/z*: 459 [M<sup>+</sup>+1], 289 [M<sup>+</sup>-galloyl]; <sup>1</sup>H-NMR (400 MHz, acetone-*d*<sub>6</sub>) : 8.32 (brs, -OH), 8.16 (brs, -OH), 7.01 (2H, s, H-2", 6"), 6.61 (2H, s, H-2',6'), 6.04 (1H, d, *J*=2.0 Hz, H-8), 6.02 (1H, d, *J*= 2.0 Hz, H-6), 5.53 (1H, m, H-3), 5.05 (1H, brs, H-2), 3.02 (1H, dd, *J*=17.4, 4.6 Hz, H-4a), 2.89 (1H, dd, *J*=17.4, 2.2 Hz, H-4b); <sup>13</sup>C-NMR (100MHz, acetone-*d*<sub>6</sub>) δ: Table I.

## Compound 2 [(-)-epicatechin gallate]

Amorphous pale pinkish white powder; FeCl<sub>3</sub> positive;  $C_{22}H_{18}O_{10}$  (M.w. 442); FABMS *m*/z: 443 [M<sup>+</sup>+1], 273 [M<sup>+</sup>-galloyl]; <sup>1</sup>H-NMR (400 MHz, acetone-*d*<sub>6</sub>) : 7.08 (1H, d, *J*=2.0 Hz, H-2'), 6.94 (2H, s, H-2",6"), 6.91 (1H, dd, *J*=8.5, 2.0 Hz, H-6'), 6.78 (1H, d, *J*=8.5 Hz, H-5'), 6.07 (1H, d, *J*=2.0 Hz, H-8), 6.04 (1H, d, *J*=2.0 Hz, H-6),

Table I. <sup>13</sup>C-NMR data of compounds 1, 2, and 3

No.	<sup>13</sup> C ( $\delta$ ) in acetone- $d_6$				
	1	2	3		
2	78.5 (d)	76.7 (d)	79.0 (d)		
3	69.7 (d)	68.0 (d)	70.8 (d)		
4	25.2 (t)	25.9 (t)	24.9 (t)		
5	157.8 (s)	156.1 (s)	157.6 (s)		
6	96.8 (d)	95.1 (d)	96.7 (d)		
7	158.2 (s)	156.4 (s)	158.4 (s)		
8	96.2 (d)	94.4 (d)	95.8 (d)		
9	157.5 (s)	155.7 (s)	156.6 (s)		
10	99.4 (s)	97.6 (s)	99.5 (s)		
1'	131.1 (s)	130.0 (s)	131.3 (s)		
2'	107.1 ( <i>d</i> )	113.5 (d)	106.6 (d)		
3'	146.6 (s)	144.1 (s)	146.9 (s)		
4'	133.5 (s)	144.2 (s)	133.7 (s)		
5'	146.6 (s)	114.2 (d)	146.9 (s)		
6'	107.1 ( <i>d</i> )	117.8 (d)	106.6 (d)		
1"	122.2 (s)	120.4 (s)	122.0 (s)		
2",6"	110.3 (d)	108.5 ( <i>d</i> )	110.3 (d)		
3",5"	146.3 (s)	144.6 (s)	146.5 (s)		
4"	139.2 (s)	137.5 (s)	139.4 (s)		
COO	166.5 (s)	169.6 (s)	166.5 (s)		

5.55 (1H, brs, H-2), 5.14 (1H, m, H-3), 3.06 (1H, dd, J=17.5, 5.0 Hz, H-4a), 2.93 (1H, dd, J=17.5, 2.5 Hz, H-4b); <sup>13</sup>C-NMR (100 MHz, acetone- $d_6$ ) : Table 1

#### Compound 3 [(+)-gallocatechin gallate]

Amorphous pale pinkish white powder; Positive to FeCl<sub>3</sub>;  $C_{22}H_{18}O_{11}$  (M.w. 458); FABMS m/z: 459 [M<sup>+</sup>+1], 289 [M<sup>+</sup>-galloyl]; <sup>1</sup>H-NMR (400 MHz, acetone- $d_6$ ) : 6.94 (2H, s, H-2",6"), 6.38 (2H, s, H-2',6'), 5.95 (1H, d, J=2.0 Hz, H-8), 5.88 (1H, d, J=2.0 Hz, H-6), 5.29 (1H, m, H-3), 5.02 (1H, d, J=5.5 Hz, H-2), 2.72 (1H, dd, J=17.0, 5.0 Hz, H-4a), 2.64 (1H, dd, J=17.0, 5.5 Hz, H-4a); <sup>13</sup>C NMR (100 MHz, acetone- $d_6$ )  $\delta$ : Table I

#### **RESULTS AND DISCUSSION**

The activity-guided purification of EtOAc soluble fraction afforded three inhibitors, **1**, **2**, and **3**. **1** was obtained as a slightly pinky white powder, and positive to FeCl<sub>3</sub>. In FABMS spectrum,  $[M+1]^+$  was observed at m/z 459 along with the fragment ion at m/z 289  $[M^+$ -galloyl], indicating that **1** has a galloyl moiety in its structure. UV spectrum showed the catechin absoprtion at 220 and 280 nm (Es-Safi *et al.*, 2000). In <sup>1</sup>H NMR spectrum, two aromatic singlets appeared at  $\delta$  7.01 (2H) and 6.61 (2H), which could be assigned as the symmetric protons in catechin B-ring and galloyl moiety, respectively. Two *meta*-coupled protons, originated from catechin A-ring, were observed at  $\delta$  6.04 (1H, d, J=2.0 Hz) and 6.02 (1H, d, *J*=2.0 Hz). In addition, the signals at  $\delta$  5.05, 5.54, and 3.05 showed the typical resonances of H-2, -3, and -4 of catechin skeleton. Considering the coupling constant of H-2 (broad singlet), the relative stereochemistry of H-2 and -3 should be a *cis*-form (Nonaka *et al.*, 1983; Saeki *et al.*, 2000). A carbonyl carbon ( $\delta$  166.5) and three *sp*<sup>3</sup> carbons ( $\delta$ 78.5, 69.7, and 25.2) were detected in <sup>13</sup>C-NMR spectrum. From above data, 1 was assumed to be a (-)-epigallocatechin gallate. This was finally confirmed by comparing NMR data with those in the reported data (Nonaka *et al.*, 1983; Saeki *et al.*, 2000).

Compound 2 was positive to  $FeCl_3$  and showed  $[M^++$ 1] at m/z 443 in positive FABMS spectrum. The <sup>1</sup>H-NMR data were very similar to those of 1 except for the resonances at  $\delta$  6.78 (1H, d, J=8.5 Hz), 6.91 (1H, dd, J= 8.5, 2.0 Hz), and  $\delta$  7.08 (1H, d, J=2.0 Hz), suggesting that B-ring of 1 was substituted with a catechol moiety instead of a pyrogallol. In <sup>13</sup>C-NMR spectrum, a carbonyl ( $\delta$  169.6), three  $sp^3$  ( $\delta$ 76.7, 68.0, and 25.9), and eighteen aromatic carbon signals ( $\delta$ 94.4 to 155.7) were detected. The coupling constant between H-2 and -3 was almost zero as in the case of 1. The final structure was identified as (-)-epicatechin gallate by referring reported data (Nonaka et al., 1983; Saeki et al., 2000) and direct comparison with an authentic sample. Compound 3 was also positive to FeCl<sub>3</sub>. The proton resonances at  $\delta$  6.94 (2H, s) and  $\delta$  6.38 (2H, s) suggested the presence of a galloyl and a pyrogallol group. The aromatic signals at  $\delta$ 5.95 and 5.88 (each 1H, d, J=2.0 Hz) and  $sp^3$  proton signals at  $\delta$ 5.02, 5.29, and 2.75 indicated that **3** had also gallocatechin gallate skeleton. The major difference between 1 and 3 was the coupling constant of H-2. The 5.5 Hz of coupling constant implied that the relative stereochemistry of H-2 and -3 of compound 3 was trans (Nonaka et al., 1983; Saeki et al., 2000). In <sup>13</sup>C-NMR, an ester carbonyl carbon was shown at  $\delta$ 166.5 with three sp<sup>3</sup> carbons ( $\delta$ 79.0, 70.8, and 24.9) and eighteen aromatic carbons ( $\delta$ 95.8 to 146.9). From these data, **3** was identifed as (+)-gallocatechin gallate, a 3-epimer of **1**, and finally confirmed by the comparison of reported NMR data (Saeki et al., 2000).

The structures are presented in Fig. 1 and <sup>13</sup>C-NMR data are listed in Table I.

All three compounds inhibited PEP in a dose-dependent manner and were non-competetive with a substrate in Dixon plots (Fig. 2). The inhibition constant (Ki) of **1**, **2**, and **3** were  $1.66 \times 10^{-3}$  mM,  $3.80 \times 10^{-3}$  mM,  $14.60 \times 10^{-3}$  mM, respectively. The IC<sub>50</sub> values are presented in Table II. Their activities were lower than that of *Z*-Pro-Prolinal, a positive control, but similar to those of natural inhibitors such as staurosporine ( $7.70 \times 10^{-4}$  mM; Kimura *et al.*, 1990) and propeptin ( $1.10 \times 10^{-3}$  mM; Kimura *et al.*, 1997b). To check the enzyme specificity, the inhibitory activities on other serine proteases such as chymotrypsin, trypsin, and elastase were compared with that of PEP. Up to 1 mM of

the isolated compounds inhibited only less than 50% of above enzyme activities. Thus, they were thought to be relatively specific inhibitors of PEP.

Many pyrrolidine derivatives such as Z-Pro-Prolinal and JTP-4819 had been synthesized (Arai *et al.*, 1993). The flavonoids containing a catechol ring (Lee *et al.*, 1998) and tannins having pyrogallol moiety (Fan *et al.*, 1999) from plant sources have been reported to effectively inhibit the activity of PEP. It has been suggested that the presence of carbonyl group with catechol or galloyl moieties were essential for the activity (Lee *et al.*, 1998; Kim *et al.*, 2000). Compound **1** and **3** showed ca 100 times of higher activity than **2**, suggesting that galloyl moiety played an important role in PEP inhibition. No significant differences in inhibitory activity were found between diastereomers, **1** and **3**. Therefore, the stereochemistry of C-3 seemed not to be important for the activity.

AD has been known as a multi-disorder disease caused by variety of reasons, for example, active oxygen species (Behl, 1999; Miranda *et al.*, 2000), accumulation of  $\beta$ amyloid, and cholinergic neurodegeneration (Selkoe, 1994). The isolated compounds, which inhibited not only PEP but active oxygen species (Mitscher, 1997; Yukiaki, 1999), are expected to be used in the prevention and treatment of Alzheimer's disease.



Fig. 1. Structures of compounds 1, 2 and 3



**Fig. 2.** Dixon plots of compounds **1**, **2** and **3**. From the top to bottom, compound **1** [(-)-epigallocatechin gallate], **2** [(-)-epicatechin gallate], and **3** [(+)-gallocatechin gallate]. Substrate concentration:  $\bullet$ , 0.25 mM;  $\bigcirc$ , 0.50 mM;  $\checkmark$ , 1.00 mM.

Table II. IC<sub>50</sub> values against PEP and related enzymes

IC <sub>50</sub> values	Compound				
(mM)	1	2	3	Control <sup>b</sup>	
PEP <sup>a</sup>	$1.42 \times 10^{-4}$	$1.02 \times 10^{-2}$	$1.09 \times 10^{-4}$	$0.70 \times 10^{-4}$	
Elastase	>1.60	>1.0	>1.2	ND	
Trypsin	>1.60	>1.0	>1.2	ND	
Chymotrypsin	>1.60	>1.0	>1.2	ND	

<sup>a</sup>Prolyl endopeptidase

<sup>b</sup>Positive control, Z-Pro-Prolinal.

ND, not determined.

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