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A Prolyl Endopeptidase-Inhibiting Antioxidant from *Phyllanthus ussurensis*

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A prolyl endopeptidase inhibitor was isolated from the ethyl acetate soluble fraction of *Phyllanthus ussurensis*. The active compound was identified as an ellagitannin, corilagin. It was shown to non-competitively inhibit prolyl endopeptidase (PEP) with the IC_{50} value of 1.17×10^{-6} μM. The *Ki* value was 6.70×10^{-7} M. Corilagin was less inhibitory to other serine proteases such as chymotrypsin, trypsin, and elastase, indicating that it was relatively a specific inhibitor of PEP. Corilagin also effectively inhibited reactive oxygen species such as hydroxide and superoxide anion radical, hydrogen peroxide, and DPPH. Especially, corilagin showed potent scavenging activity on the superoxide anion radical in the ESR method (IC_{50} = 3.79×10^{-6} M) as well as xanthine oxidase system.

Key words: *Phyllanthus ussurensis*, Prolyl endopeptidase, Inhibitor, Antioxidant, Corilagin, Alzheimer

INTRODUCTION

A major histopathological characteristic of Alzheimers disease (AD) is the deposition of amyloid protein in the parenchyma of the amygdala, hippocampus, and neocortex (Sisodia and Price, 1995). The major component of the amyloid is the β-amyloid protein (Aβ), a 39-43 amino acid peptide composed of a portion of the transmembrane domain and the extracellular domain of the amyloid precursor protein (APP) (Glenner and Wong, 1984). The neurotoxicity of the Aβ has been detected in several cell systems, including primary cultured neurons (Mattson *et al.*, 1993). The Aβ having an alanine *C*-terminus is derived from the proteolytic cleavage of the APP by the action of the endoproteolytic enzymes, β- and γ-secretase (Checler, 1990). Recent studies have suggested that prolyl endopeptidase [PEP; EC 3.4.21.26] could be involved in the processing of the *C*-terminal portion of the APP in AD (Sugita, 1990).

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The PEP is a serine protease, which is known to cleave peptide substrates in the *C*-terminal side of proline residues (Yaron and Naider, 1993). It plays an important role in degradation of the proline-containing neuropeptides such as oxytocin, vasopressin, substance P, neurotensin and angiotensin, which were suggested to participate in learning and memory processes (Rennex *et al.*, 1991; Aoyagi *et al.*, 1990). It was found that the PEP activity of AD patients is significantly higher than that of the normal person (Portevin *et al.*, 1996). It has been suggested that specific PEP inhibitors could prevent memory loss and increase attention span in patients suffering from senile dementia. For example, some natural and synthetic PEP inhibitors have been reported to show dose-dependant cognition-enhancing activity in rats with scopolamine-induced amnesia (Yoshimoto *et al.*, 1987). Therefore, much effort has been devoted to developing PEP inhibitors as anti-dementia drugs.

On the other hand, recent evidence in the field of AD research has highlighted the importance of oxidative process in its pathogenesis. Based on laboratory and clinical studies, it appears that reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are generated extracellulary and intracellulary by various mechanisms

are among the major intermediary risk factors that initiate and promote neurodegeneration in idiopathic AD (Prasad *et al.*, 2000). Thus, antioxidant supplements could be useful in the prevention of AD, and as an adjunct to standard therapy in the treatment of AD.

MATERIALS AND METHODS

General

Optical density was measured with a Bio-TEK ELISA autoreader ELX 808 (USA). ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance Digital 400 spectrometer (Germany) at 400 and 100 MHz, respectively. Chemical shifts were given in δ (ppm) from TMS. EIMS was recorded on VG QUATTRO II (UK). ESR was measured with a JES-TE 300 (JEOL, Japan) at 9.78 GHz of microfrequency (microwave power; 10 mW, modulation 100 kHz; modulation amplitude, 0.495 G; time constant 0.16 ms). TLC was performed on a precoated silica gel plate (Merck, Art. 5715). Sephadex LH-20 was a product of Sigma (USA).

Enzyme assays

Prolyl endopeptidase (from *Flavobacterium meningosepticum*) and its substrate (*Z*-Gly-Pro-pNA) were purchased from Seikagaku Co. (Japan). Z-Pro-Prolinal was used as a positive control and synthesized according to the reference (Bakker *et al.*, 1990). Chymotrypsin, trypsin, and elastase were purchased from Sigma (USA). PEP activity and inhibition percent of samples were determined according to the reported method (Song and Raskin, 2002). Briefly, a mixture of 210 μL of 0.1 M Tris-HCl buffer (pH 7.0), 20 μL of 2 mM *Z*-Gly-Pro-*p*NA (in 40% dioxane), 10 μL of the sample solution (in MeOH), and 10 μL of 0.1 unit/mL PEP was incubated at 30°C for 30 min, and A410 of the reaction mixture was then measured (A). The A410 of the mixture containing 240 μL of 0.1 M Tris-HCl (pH 7.0) and 10 μL of the sample was separately measured as above (B). A control was made by adding 10 μL of MeOH instead of the sample solution to 240 μL of the buffer. The percent inhibition was calculated by the following equation: inhibition (%) = [{A410 of the control − (A − B)}/A410 of the control] × 100. Chymotrypsin, trypsin, and elastase were assayed according to the protocols described in Sigma catalog (Sigma, USA) using *N*-benzoyl-L-Arg-*p*NA, *N*-benzoyl-L-Tyr-*p*NA, and *N*-succinyl-Ala-Ala-Ala-*p*NA as substrates, respectively.

Reactive oxygen species scavenging activity

Hydrogen peroxide scavenging activity was measured according to the Müller's method (Müller, 1985). Hydroxyl and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity were analyzed by the method of Chung (Chung *et al.*, 1997) and Blois (Blois, 1985), respectively. Xanthine-xanthine oxidase system was used for the evaluation of superoxide anion scavenging activity (Iio *et al.*, 1985). ESR was measured in xanthine-xanthine oxidase system according to the previous method (Luo *et al.*, 2001). Briefly, 4 μL sample, 25 μL 2 mM xanthine, 25 μL xanthine oxidase (0.5 U/mL), and 50 μL 900 mM DMPO (5,5-dimethylpyrroline-*N*-oxide) were mixed and its volume was adjusted to 1 mL with 100 mM sodium phosphate buffer (pH 7.4). The mixture was stood exactly for one minute and introduced to the ESR spectrophotometer.

Plant material, extraction, and isolation

P. ussuriensis was cultivated in Kyungsan, Kyungsangbuk-Do, Korea. The specimen (voucher no. knunpc-pu02) is stored at the Division of Applied Biology and Chemistry, Kyungpook National University, Daegu, Korea. After being air-dried in the fume hood at room temperature, the aerial parts of the plant (5 kg) were refluxed in water thrice. The water extract was lyophilized and the extract (1 kg) was suspended in MeOH. The MeOH soluble fraction (90 g) was chromatographed on a Sephadex LH-20 (7×100 cm, 40%-100% MeOH) to yield ten fractions (Fr. 1-10). Re-chromatography of Fr. 4 using a Sephadex LH-20 (4×60 cm, 40%-100% MeOH) column and subsequent Lobar chromatography (LiChroprep RP-18, Merck, 3×27 cm, 40-63 μM, 20% MeOH, 5 mL min^{-1}) of the active fraction afforded 2.7 g of corilagin.

RESULTS AND DISCUSSION

Structure determination

Corilagin was obtained as a pale grayish powder that was positive to $FeCl_3$ reagent, suggesting that it had phenolic OH group(s) in its structure. Its molecular formula was determined as $C_{27}H_{22}O_{18}$ on the basis of EI-MS and NMR spectra. Molecular ion peak was detected at *m/z* 634. The fragment ion at *m/z* 466 ([M-galloyl+H]$^{+}$) indicated the presence of a galloyl group. In the ^{1}H-NMR spectrum, an aromatic singlet (δ 7.12, 2H), which could be assignable to the aromatic protons in a symmetrical structure of galloyl moiety, was appeared with two singlets (δ 6.84 and 6.69, each 1H) characteristic to the HHDP (hexahydroxydiphenoyl) group. Signals corresponding to glucose moiety were found at δ 4.08 to δ 6.38. In the ^{13}C-NMR spectrum, three carbonyl carbons (δ 165.05, 167.14, and 168.53) were evident. An overlapped aromatic carbon signal was detected at δ 110.75. Considering these data, the active compound was finally identified as corilagin (Fig. 1) by comparing its spectral data with those in the literature (Ham *et al.*, 2001). The NMR data are listed in the Table I.

Inhibitory activity against PEP

The prolyl endopeptidase (PEP) inhibitory activity of

corilagin (IC_{50} = 1.17×10^{-6} M) was less than that of a synthetic positive control, Z-Pro-Prolinal (IC_{50} = 5.16×10^{-8} M) but similar to those of polyozellin (IC_{50} = 2.72×10^{-6} M), kynapcin-12 (IC_{50} = 1.25×10^{-6} M), and kynapcin-24 (IC_{50} = 1.14×10^{-6} M) (Hwang *et al.*, 1997; Song and Raskin, 2002; Lee *et al.*, 2000), which have been previously isolated from natural products. Corilagin was non-competitive with a substrate in the Dixon plot (Fig. 2) and the inhibition constant (*Ki*) was 6.7×10^{-7} M. 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, corilagin did not show significant inhibition of chymotrypsin, trypsin, and elastase (Table II). Thus, corilagin appeared to be relatively a specific inhibitor of PEP, as is the case with other natural inhibitors (Hwang *et al.*, 1997; Song and Raskin, 2002; Lee *et al.*, 2000; Lee *et al.*, 1998).

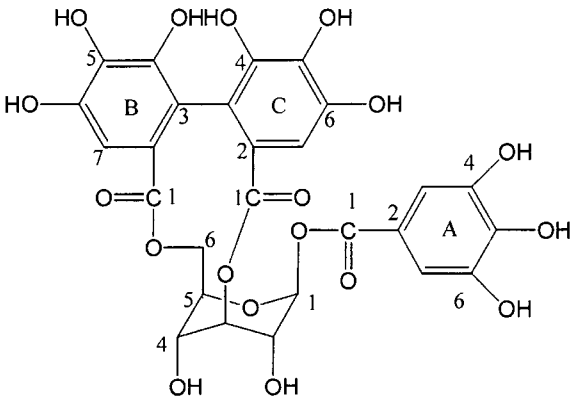

Fig. 1. Structure of corilagin

Table I. NMR data of corilagin

NO.		^{1}H (δ)[1)]	^{13}C (δ)
Glucose	1	6.38 (1H, *s*)	94.05
	2	4.08 (1H, *m*)	70.57
	3	4.96 (1H, *m*)	64.26
	4	4.56 (1H, *m*)	68.89
	5	4.53 (1H, *m*)	75.57
	6	4.11, 4.93 (2H, *m*)	62.17
Galloyl (ring A)	1		165.05
	2		120.71
	3	7.12 (1H, *s*)	110.75
	4		145.79
	5		139.21
	6		145.79
	7	7.12 (1H, *s*)	110.75
HHDP[4)] (ring B)	1		168.53
	2		125.65
	3		116.40
	4[2)]		145.29
	5		136.62
	6[3)]		144.87
	7	6.84 (1H, *s*)	109.83
HHDP[4)] (ring C)	1		167.14
	2		125.47
	3		115.78
	4[2)]		144.69
	5		137.12
	6[3)]		144.87
	7	6.69 (1H, *s*)	107.99

NMR spectra were measured in acetone-d_6.
[1)]Integral and multiplicity. [2,3)]Assignment may be exchangeable.
[4)]Hexahydroxydiphenoyl.

Many pyrrolidine derivatives such as Z-Pro-Prolinal and JTP-4819 have been synthesized as potent PEP inhibitors (Arai *et al.*, 1993). On the other hand, staurosporine (Kimura *et al.*, 1990), poststatin (Aoyagi *et al.*, 1991), and eurystatin (Toda *et al.*, 1992) were isolated from microbial sources. Plant-derived flavonoids containing a catechol ring (Lee *et al.*, 1998) and tannins with a pyrogallol moiety (Fan *et al.*, 1998) have also been reported to effectively inhibit the activity of PEP. The presence of a carbonyl group with a catechol or pyrogallol moiety has been suggested as the essential structural feature for PEP inhibitory activity (Lee *et al.*, 1998; Kim and Song, 2000).

Corilagin, which was expected to have an antioxidative

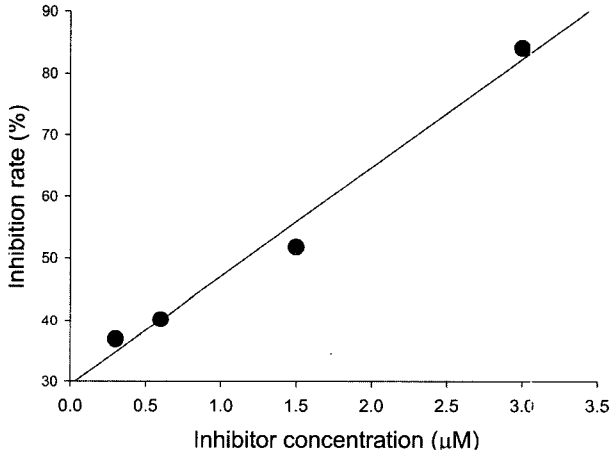

Fig. 2. Dose-dependant inhibition of PEP by corilagin

Table II. Inhibitory activity of corilagin against other serine proteases

Enzyme	IC_{50} (M)
PEP	1.17×10^{-6}
Trypsin	>1×10^{-3}
Chymotrypsin	>1 ×10^{-3}
Elastase	>1 ×10^{-3}

activity from its chemical structure, was tested for radical scavenging activity on several reactive oxygen species such as hydrogen peroxide, superoxide anion, DPPH, and hydroxyl radicals. Corilagin showed almost the same activity with positive controls BHA (butylhydroxyl anisol) and α-tocopherol in the hydrogen peroxide, DPPH, and hydroxyl radical scavenging assays: however, its scavenging activity on the superoxide anion radical was almost three times higher than those of the positive controls (Table III). In addition, corilagin effectively reduced the superoxide radicals in the ESR study (Fig. 4). The IC_{50} value of corilagin was 3.79×10^{-6} M, while that of a positive control vitamin C was 78.06×10^{-6} M.

The immune response is very active in AD and may contribute to the disease rather than help. The brain's immune cells (microglia) respond to the plaques and tangles and attempt to clean up this debris. This is a natural response. However, plaques and tangles are very difficult to dissolve. In the process of trying to digest the material within plaques and tangles, microglia also release pro-inflammatory proteins and free radicals, which cause secondary damage (Mattson, 2002). Especially, the superoxide anion radicals have been known to play a great role in the pathogenesis of AD (Qin *et al.*, 2002; Liu *et al.*, 2002). The non-peptidyl and small molecular-weight antioxidant corilagin may have potential use in the prevention and treatment of AD.

Table III. Reactive oxygen species scavenging activity[1] of corilagin

Compounds (μM)		Inhibition (%)			
		DPPH[2]	H_2O_2	HR[3]	SOA[4]
Corilagin	0.01	30.7	9.0	74.6	40.7
	0.1	58.6	13.2	69.8	64.3
	1.0	–	96.5	–	–
BHA[5]	0.01	27.4	9.2	73.0	14.5
	0.1	30.7	9.0	79.4	22.0
	1.0	–	95.7	–	–
V-E[6]	0.01	26.5	6.9	75.6	22.1
	0.1	32.7	2.7	83.6	25.6

[1]Presented as an inhibition %
[2]α,α-Diphenyl-β-picrylhydrazyl
[3]Hydroxyl radical
[4]Superoxide anion
[5]Butylhydroxyl anisol
[6]α-Tocopherol

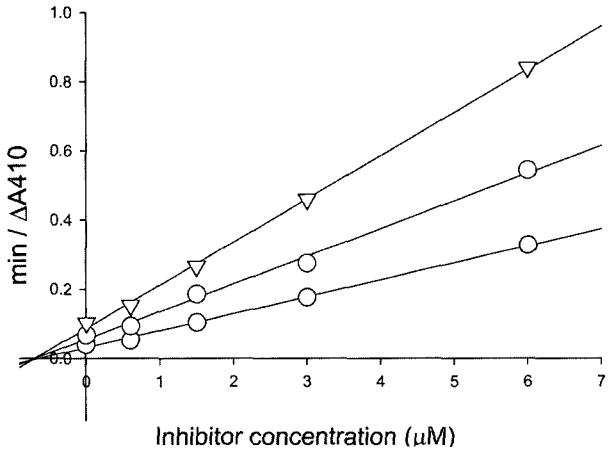

Fig. 3. Dixon plot of the inhibition of PEP by corilagin. Concentration of substrate: 1.0 mM (–▽–), 1.5 mM (–○–), 2.0 mM (–○–). 1/V was indirectly estimated by taking reciprocal value of the changes in OD at 410 nm per minute.

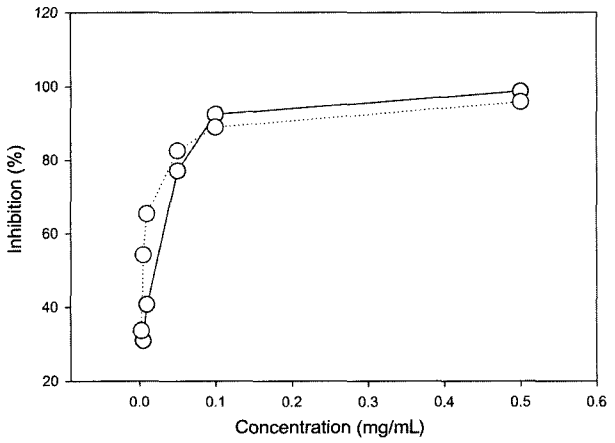

Fig. 4. Inhibition of superoxide radical by corilagin in ESR spectroscopy. Corilagin, –○–; ascorbic acid, –○–.

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