Agric. Chem. Biotechnol. **48**(4), 207-212 (2005)

Article

Prolyl Endopeptidase Inhibitory Activity of Ursolic and Oleanolic Acids from Corni Fructus

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Received November 18, 2005; Accepted December 5, 2005

Prolyl endopeptidase (PEP, EC 3.4.21.26), also referred to as prolyl oligopeptidase, has been suggested to participate in learning and memory processes by cleaving peptide bonds on carboxyl side of prolyl residue within neuropeptides of less than 30 amino acids, and is abundant in brains of amnestic patients. Therefore, compounds possessing PEP inhibitory activity can be good candidate of drug against memory loss. Upon examination for PEP inhibition from traditional medicinal plants having tonic, stimulating, and anti-amnestic effects, Corni Fructus (*Cornus officinallis*) showed significant PEP inhibition. Ursolic and oleanolic acids, components of Corni Fructus, inhibited PEP with IC_{50} values of 17.2 ± 0.5 and 22.5 ± 0.7 μM, respectively.

Key words: *prolyl endopeptidase inhibitor, anti-amnestic, Corni Fructus, ursolic acid, oleanolic acid*

Prolyl endopeptidase (PEP, EC 3.4.21.26) has been suggested to participate in learning and memory processes, because it can degrade proline containing neuropeptides such as vasopressin, substance P, and thyrotropin-releasing hormone.[1)] This enzyme, widely distributed in various organs including human brain, cleaves peptide bond on the carboxylic side of prolyl residue within polypeptides of less than 30 amino acids and, therefore, is also referred to as prolyl oligopeptidase.[1-4)] Studies have revealed that PEP activities of Alzheimer's patients are significantly higher than those of the control group.[5)] Thus, a specific PEP inhibitor can be a good candidate of anti-amnestic drug by blocking the metabolism of endogenous neuropeptides.

Herbal medicine is an increasingly common form of complementary and alternative therapies. According to the theory of Chinese medicine, amnesia is caused by breathing stagnation and blood stasis or insufficiency of the liver and kidney functions.[6,7)] Thus, a blood-quickening and stasis-transforming formula or a kidney-tonifying formula is clinically used for the treatment of amnesia. Our search for anti-amnestic constituents from raw medicinal materials possessing tonic, stimulating, and anti-amnestic effects revealed almost all medicinal plants with powerful PEP inhibitory activity contain phenolic compounds of catechol or pyrogallol group. For example, PEP inhibitory principle of moutan cortex is 1, 2, 3, 4, 6-pentagalloyl-β-D-glucopyranose[8)] and that of sheng-di-hong-jing-tian is 3-*O*-galloylepigal-locatechin-(4β → 8)-epigallocatechin 3-*O*-gallate.[9)] Although their inhibitory potencies are very powerful, their effectiveness may come from interaction with proteins, thereby giving false positive effect.

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Through hundreds of years of medicinal practices, some traditional medicines including Ba Wei Di Huang Wan[10)] in China and Yook-Mee-Tang[11)] in Korea have been confirmed to be effective for the treatment of dementia. Corni Fructus (*Cornus officinallis*), one of the ingredients in Ba Wei Di Huang Wan and Yook-Mee-Tang, contains various hydrolysable tannins including cornusiins A and B, two isomeric triterpenic acids (ursolic and oleanolic acids), monoterpenes, and gallic, malic, and tartaric acids.[11,12)] The purpose of the present work was to search for non-phenolic PEP inhibitors from Corni Fructus.

Materials and Methods

Materials. Corni Fructus was purchased from a local herb drug market. PEP (from *Flavobacterium meningosepticum*) and benzyloxycarbonyl-glycyl-L-prolyl-*p*-nitroanilide (*Z*-Gly-Pro-*p*NA) were purchased from Seikagaku Co. (Tokyo, Japan). Benzyloxycarbonyl-L-prolyl-prolinal (*Z*-Pro-prolinal) as a positive control was purchased from Biomol Research Laboratories Inc. (Philadelphia, PA, USA). The silica gel for column chromatography (Kiesel gel, 230-400 mesh) and TLC plates (Kiesel gel 60 F_{254}) were purchased from Merck (Darmstadt, Germany). All chemicals including oleanolic and ursolic acids and solvents, either reagent or HPLC grade, were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Spectroscopic analysis. UV-VIS spectra were recorded on a Varian Cary 300 and Jasco V-530 spectrophotometers. ^{1}H-, ^{13}C-, and 2D-NMR spectra in CD_3OD were measured on a 400 MHz FT NMR (Varian Inova-AS 400) at 400 and 100 MHz, respectively. EIMS was obtained on a JEOL JMS-700 spectrometer.

Extraction and Isolation. Corni Fructus (600 g) was

extracted with methanol three times, and the solvent was removed under reduced pressure to yield dark brown mass. The crude methanol extract was then partitioned between chloroform and water. A portion of chloroform soluble fraction (1.8 g) was chromatographed on a silica gel column, eluting with a gradient of chloroform/ethyl acetate (9 : 1 → 2 : 1), to give six fractions (Fr. A-F). Fr. E (83.7 mg) was subjected to column chromatography using chloroform/methanol (98 : 2) as the eluent to give three fractions (Fr. E1-E3). Fr. E1 (36.2 mg, 4 : 1 mixture of compounds **1** and **2**): R_f 0.56 $CHCl_3$: MeOH = 95 : 5, EIMS *m/z* (rel. int.) 456 $[M]^+$ (20), 438 (8), 410 (8), 249 (100), 248 (100), 207 (100), 203 (100), 189 (64), 133 (100). The major compound **1** was identified as ursolic acid by comparison with an authentic sample (Sigma) and the reported data.[13] ^{1}H NMR (400 MHz, CD_3OD) δ 5.22 (1H, t, *J* = 3.6 Hz, H-12), 3.14 (1H, dd, *J* = 11.2, 4.8 Hz, H-3), 2.19 (1H, d, *J* = 11.2 Hz, H-18), 1.11 (3H, s, H-27), 0.97 (3H, s, H-23), 0.96 (3H, d, *J* = 6.8 Hz, H-30), 0.95 (3H, s, H-24), 0.88 (3H, d, *J* = 6.8 Hz, H-29), 0.84 (3H, s, H-26), 0.77 (3H, s, H-25). ^{13}C NMR (100 MHz, CD_3OD): δ 180.48, 138.41, 125.59, 78.49, 55.57, 53.21, 47.64, 47.64, 42.09, 39.65, 39.29, 39.29, 38.86, 38.70, 36.99, 36.98, 33.20, 30.66, 28.10, 27.65, 26.77, 24.21, 23.25, 22.98, 20.48, 18.37, 16.71, 16.56, 15.28, 14.92. The minor compound **2** was identified as oleanolic acid by comparison with an authentic sample (Sigma) and the reported data.[14] ^{1}H NMR (400 MHz, CD_3OD) δ 5.23 (1H, t, *J* = 3.6 Hz, H-12), 3.14 (1H, dd, *J* = 11.2, 4.8 Hz, H-3), 2.82 (1H, d, *J* = 9.2 Hz, H-18), 1.15, 0.96, 0.93, 0.93, 0.90, 0.81, 0.77 (each 3H, s, H-23, 24, 25, 26, 27, 29, 30).

PEP inhibition assay. UV-VIS spectra were recorded on Varian Cary 300 and Jasco V-530 spectrophotometers. PEP activity was assayed using the method of Yoshimoto *et al.* with minor modifications.[11,15] A mixture of 800 μ*l* of 0.1 M phosphate buffer (pH 7.0), 80 μ*l* of 2 mM *Z*-Gly-Pro-*p*NA in 40% 1,4-dioxane, and 40 μ*l* sample solution (1 mg · m*l*$^{-1}$ MeOH stock solution diluted with 0.1 M phosphate buffer) was pre-incubated at 37°C for 10 min. The reaction was started by adding 80 μ*l* of 0.1 unit · m*l*$^{-1}$ PEP in 0.1 M phosphate buffer (pH 7.0) at 37°C. After incubation for 30 min, the amount of released *p*-nitroaniline was determined colorimetrically based on the absorbance at 380 nm (A). A_{380} of the mixture containing 960 μ*l* of 0.1 M phosphate buffer (pH 7.0) and 40 μ*l* sample solution was separately measured as mentioned above (B). A control was made by adding 40 μ*l* of 0.1 M phosphate buffer instead of 40 μ*l* of the sample solution of (A). The percentage of inhibition was calculated using the following equation: percentage of inhibition = [{A_{380} of control-(A-B)}/A_{380} of control] × 100. Three distinct experiments were carried out, and IC_{50} values were determined graphically based on the curves of the enzyme activity versus inhibitor concentrations.

Statistics. Inter-group comparisons of data were made using Enzyme Kinetics Module (Add-on software for SigmaPlot 2000) from SPSS Science (San Francisco, CA, USA).

Results and Discussion

Age-related cognitive function decline, a common occurrence in old age, generally refers to mild deterioration in memory performance, executive functions, and speed of cognitive processing.[16] Avoidance of cardiovascular and other chronic diseases, high educational level, a flexible personality during middle age, and maintenance of vision and hearing have been identified as protective factors, whereas a family history of dementia, hypertension, diabetes mellitus, depression, smoking, head injury, low linguistic ability early in life, low-complexity occupation, higher density of persons per bedroom in the home, and low level of physical activity have been identified as factors contributing to the progressive cognitive decline.[16-18]

Some traditional Chinese herbal medicines have a long history as remedies for elderly cognitive decline. For example, Ba Wei Di Huang Wan is one of the most common herbal medicine for dementia in China.[10] It is a combination of eight raw medicinal materials, i.e., Alismatis Rhizomae (*Alisma orientale*), Corni Fructus (*Cornus officinallis*), Dioscoreae Rhizomae (*Dioscorea batatas*), Moutan Cortex Radicis (*Paeonia suffruticosa*), Hoelen (*Poria cocos*), Rehmanniae Radix (*Rehmannia glutinosa*), Cinnamoni Cassiae Cortex (*Cinnamomum cassia*), and Aconiti Tuber (*Aconitum carmichaelii*). Yook-Mee-Tang consisting of six herbs (Alismatis Rhizomae, Corni Fructus, Dioscoreae Rhizomae, Moutan Cortex, Hoelen, and Rehmanniae Radix; already mentioned in Ba Wei Di Huang Wan) is also used as a remedy for the elderly with cognitive decline in Korea.[11] In our search for anti-amnestic constituents in Yook-Mee-Tang, both methanol extracts of Moutan Cortex and Corni Fructus showed significant PEP inhibitory activity.[11] Tannin, 1, 2, 3, 4, 6-pentagalloyl-β-D-glucopyranose (IC_{50} 0.077 μM), is the PEP inhibitory principle of Moutan Cortex.[8] However, the effectiveness of tannin may come from interaction with proteins, thereby giving false-positive effect. Corni Fructus

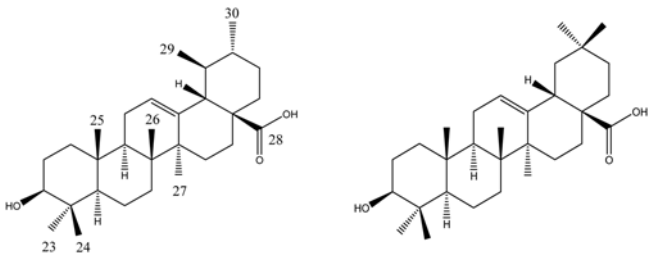

Fig. 1. Structures of ursolic and oleanolic acids.

Table 1. IC_{50} and K_i values of ursolic and oleanolic acids

Compound	IC_{50} (μM)[a]	K_i (μM)
ursolic acid	17.2 ± 0.5	51.6
oleanolic acid	22.5 ± 0.7	36.1
Z-Pro-prolinal	0.00219 ± 0.00022	-

[a]Data are means ± S.E. of three separate experiments.

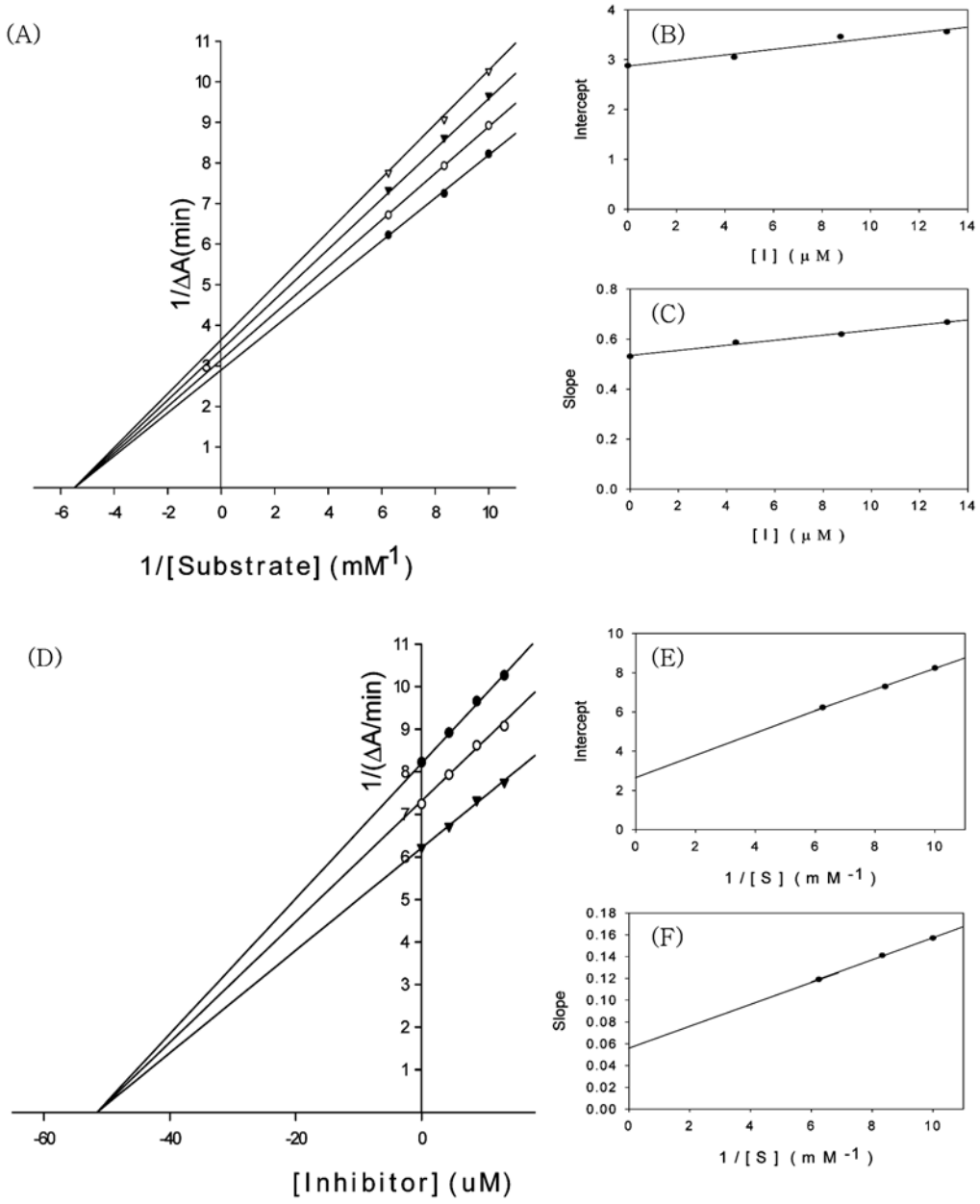

Fig. 2. (A) Lineweaver-Burk plots of PEP inhibition by ursolic acid [in the absence (●) and presence of 4.38 (○), 8.76 (▼), and 13.14 (▽) μM ursolic acid]. (B) Secondary plot of intercepts (*i*) taken from Lineweaver-Burk plots versus ursolic acid concentration. (C) Secondary plot of slopes (*s*) taken from Lineweaver-Burk plots versus ursolic acid concentration. (D) Dixon plots of PEP inhibition by ursolic acid. [S] = 0.1 mM (●), 0.12 mM (○), 0.16 mM (▼). (E) Secondary plot of intercepts (*i*) taken from Dixon plots versus reciprocal of the *Z*-Gly-Pro-*p*NA concentration. (F) Secondary plots of slopes (*s*) taken from Dixon plots versus reciprocal of the *Z*-Gly-Pro-*p*NA concentration.

contains various hydrolysable tannins, including cornusiins A and B, gallic, malic, and tartaric acids, monoterpenes, and triterpenes.[11,12] For the search of non-phenolic PEP inhibitory principles, methanol extracts of Corni Fructus was further fractionated and chromatographed as described in Methods section.

PEP inhibitory activity was measured using *Z*-Gly-Pro-*p*NA as a substrate, and the amount of released *p*-nitroaniline was determined colorimetrically at 380 nm. *Z*-*P*ro-*p*rolinal, a synthetic PEP inhibitor, was used as a reference compound of the positive control (IC_{50} = 2.19 nM).[19] Upon preliminary examination of the chloroform-soluble fraction of Corni Fructus for the PEP inhibitory activity at 8 μg · m*l*$^{-1}$, Fr. E1 (4 : 1 mixture of ursolic and oleanolic acids) showed 37% inhibition. Further study of PEP inhibitions of these isomeric triterpenic acids, ursolic and oleanolic acids, at 8 μg · m*l*$^{-1}$ showed dose-dependent inhibitions of 49.6 and 45.1%, with IC_{50} values of 17.2 ± 0.5 and 22.5 ± 0.7 μM, respectively. (Fig. 1 and Table 1)

Lineweaver-Burk plots of the PEP inhibition by ursolic acid

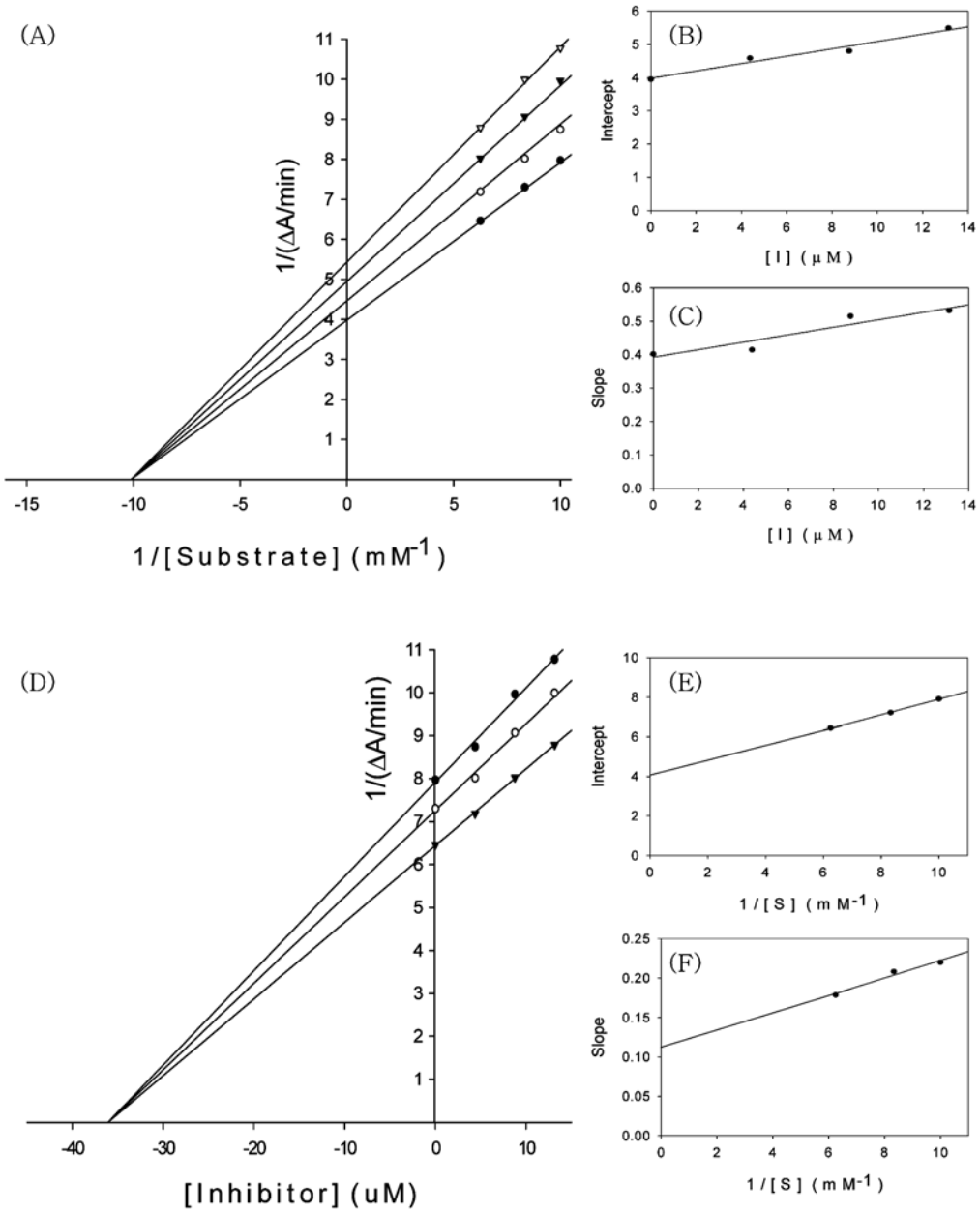

Fig. 3. (A) Lineweaver-Burk plots of PEP inhibition by oleanolic acid [in the absence (●) and presence of 4.38 (○), 8.76 (▼), and 13.14 (▽) μM oleanolic acid]. (B) Secondary plot of intercepts (*i*) taken from Lineweaver-Burk plots versus oleanolic acid concentration. (C) Secondary plot of slopes (*s*) taken from Lineweaver-Burk plots versus oleanolic acid concentration. (D) Dixon plots of PEP inhibition by oleanolic acid. [S] = 0.1 mM (●), 0.12 mM (○), 0.16 mM (▼). (E) Secondary plot of intercepts (*i*) taken from Dixon plots versus reciprocal of the *Z*-Gly-Pro-*p*NA concentration. (F) Secondary plots of slopes (*s*) taken from Dixon plots versus reciprocal of the *Z*-Gly-Pro-*p*NA concentration.

indicate that ursolic acid is a noncompetitive inhibitor (Fig. 2A). The secondary Lineweaver-Burk plots show linear relationship for both intercepts (*i*) versus ursolic acid concentration (Fig. 2B) and slope (s) versus ursolic acid concentration (Fig. 2C). Dixon plots of the PEP inhibition by ursolic acid also indicate that it is a noncompetitive inhibitor with an inhibition constant (K_i) value of 51.6 μM (Fig. 2D and Table 1). The secondary Dixon plots show linear relationships between intercepts (*i*) versus reciprocal of the *Z*-Gly-Pro-*p*NA concentration, and slope (s) versus reciprocal of the *Z*-Gly-Pro-*p*NA concentration (Figs. 2E and F, respectively).

Lineweaver-Burk plots of the PEP inhibition by oleanolic acid indicate that oleanolic acid is a noncompetitive inhibitor (Fig. 3A). The secondary Lineweaver-Burk plots show linear relationship for both intercepts (*i*) versus oleanolic acid concentration (Fig. 3B) and slope (s) versus oleanolic acid concentration (Fig. 3C). Dixon plots of the PEP inhibition by oleanolic acid also indicate that it is a noncompetitive inhibitor with an inhibition constant (K_i) value of 36.1 μM (Fig. 3D and Table 1). The secondary Dixon plots show linear

relationships between intercepts (*i*) versus reciprocal of the *Z*-Gly-Pro-*p*NA concentration, and slope (s) versus reciprocal of the *Z*-Gly-Pro-*p*NA concentration (Figs. 3E and F, respectively).

Both ursolic and oleanolic acids are triterpenoid compounds that exist widely in medicinal herbs and possess a variety of physiological effects. They have cytotoxicity against cancer cells, anti-inflammatory, antiprotozoal, and antimicrobial properties, and hepatoprotective and anti-ulcer activities.[20] In our study, both ursolic and oleanolic acids also showed PEP inhibitory activity and, therefore, could be used as memory-enhancing principles.

In summary, ursolic and oleanolic acids inhibited PEP with IC_{50} values of 17.2 and 22.5 μM, and K_i values of 51.6 and 36.1 μM, respectively. Although IC_{50} values of ursolic and oleanolic acids were higher than those reported for strong natural inhibitors such as ginkgolic acid (IC_{50}, 0.62 μM),[19] staurosporine (IC_{50}, 0.77 μM),[21] and kynapsin 24 (IC_{50}, 1.14 μM),[22] they were more effective PEP inhibitors than ω-3 polyunsaturated fatty acids such as DHA (IC_{50}, 62.2 μM) and EPA (IC_{50}, 67.0 μM),[23] whose deficiency is associated with retarded visual acuity, cognitive impairment, cerebellar dysfunction, and various other neurological disorders.[24] These results suggest ursolic and oleanolic acids have potential use in the prevention of memory loss.

Acknowledgments. This research was supported by the Kyung Hee University Research Fund in 2005.

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