Agric. Chem. Biotechnol. **49**(3), 110-113 (2006)

Note

Prolyl Endopeptidase Inhibitory Activity of 6-*O*-Palmitoyl L-Ascorbic Acid

Yoon-Seok Park and Young-Sook Paik*

College of Environment and Applied Chemistry and Plant Metabolism Research Center, Kyung Hee University, Yongin 446-701, Korea

Received August 11, 2006; Accepted September 19, 2006

Prolyl endopeptidase (PEP, EC 3.4.21.26, also referred to as prolyl oligopeptidase) degrades proline-containing, biologically active neuropeptides such as vasopressin, substance P and thyrotropin-releasing hormone by cleaving peptide bonds on carboxyl side of prolyl residue within neuropeptides of less than 30 amino acids. Evaluation of PEP levels in postmortem brains of Alzheimer's disease patients revealed significant increases in PEP activity. Therefore, a specific PEP inhibitor can be a good candidate of drug against memory loss. Upon our examination for PEP inhibitory activity from micronutrients, ascorbic acid (vitamin C) showed small but significant PEP inhibition (13% PEP inhibition at 8 μg · ml^{-1}). Palmitic acid showed almost no PEP inhibition. However, 6-*O*-palmitoyl ascorbic acid (1) showed 70% PEP inhibition at 8 μg · ml^{-1} indicating that hydrophobic portion of the compound 1 may facilitate the inhibitory effect. IC_{50} value of compound 1 was 12.6 ± 0.2 μM. The primary and secondary Lineweaver-Burk and Dixon plots for compound 1 indicated that it is a non-competitive inhibitor with inhibition constant (*Ki*) value of 23.7 μM.

Key worlds: *prolyl endopeptidase inhibitor, memory loss, 6-O-palmitoyl L-ascorbic acid, ascorbic acid, palmitic acid*

Prolyl endopeptidase (PEP, EC 3.4.21.26) widely distributed in various organs, particularly in the 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] PEP has been suggested to participate in learning and memory processes.[1] Evaluation of the PEP levels in postmortem brains obtained from Alzheimer's disease patients revealed significant increases in the enzyme activity, suggesting that PEP plays a functional role in the brain.[5] Thus, a specific PEP inhibitor can be a good candidate for anti-amnestic drugs for curing memory loss and neuropathological disorders by blocking the catabolism of endogenous neuropeptides.

At present, drug treatments for Alzheimer's disease are primarily focused on treating the symptoms rather than preventing mental deterioration. Mental illness in elderly people has been found to be associated with poor diet. In particular, deficiency of micronutrients is associated with cognitive impairment. Studies showed that a diet rich in antioxidants, such as vitamins C (ascorbic acid) and E (tocopherol) and polyphenols, can help reduce the risk of dementia by eliminating harmful free radicals and decreasing the level of oxidative stress.[6,7] Vitamin C, as an antioxidant, plays an important role in protecting against the heart disease, high cholesterol, high blood pressure, common cold, cancer, Alzheimer's disease and other types of dementia.[8-13] Human body does not produce vitamin C on its own, nor does it store it. Eating adequate amounts of vitamin C in the daily diet may help reduce the risk of developing some of these conditions. The objective of our work was to search vitamin C for the potential health benefits associated with age-related memory loss in terms of PEP inhibition.

*Corresponding author
Phone: 82-31-201-2422; Fax: 82-31-202-7337
E-mail: paikys@khu.ac.kr

Abbreviations: PEP, prolyl endopeptidase

Materials and Methods

Materials. 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). All chemicals including vitamins C and E, 6-*O*-palmitoyl L-ascorbic acid, palmitic acid, and solvents, either reagent or HPLC grade, were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA)

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.[14,15] A mixture of 800 μL of 0.1 M phosphate buffer (pH 7.0), 80 μL of 2 mM *Z*-Gly-Pro-*p*NA in 40% 1,4-dioxane, and 40 μL sample solution (1 mg · ml^{-1} MeOH stock solution diluted with 0.1 M phosphate buffer, pH 7.0) was preincubated at 37°C for 10 min. The reaction was started by adding 80 μL of 0.1 unit · ml^{-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 of the solution (A)

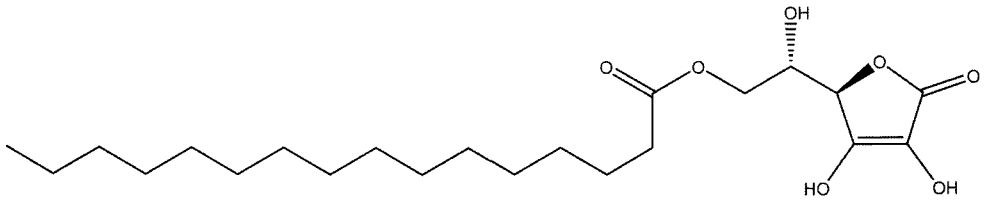

Fig. 1. Structure of 6-O-palmitoyl ascorbic acid (1).

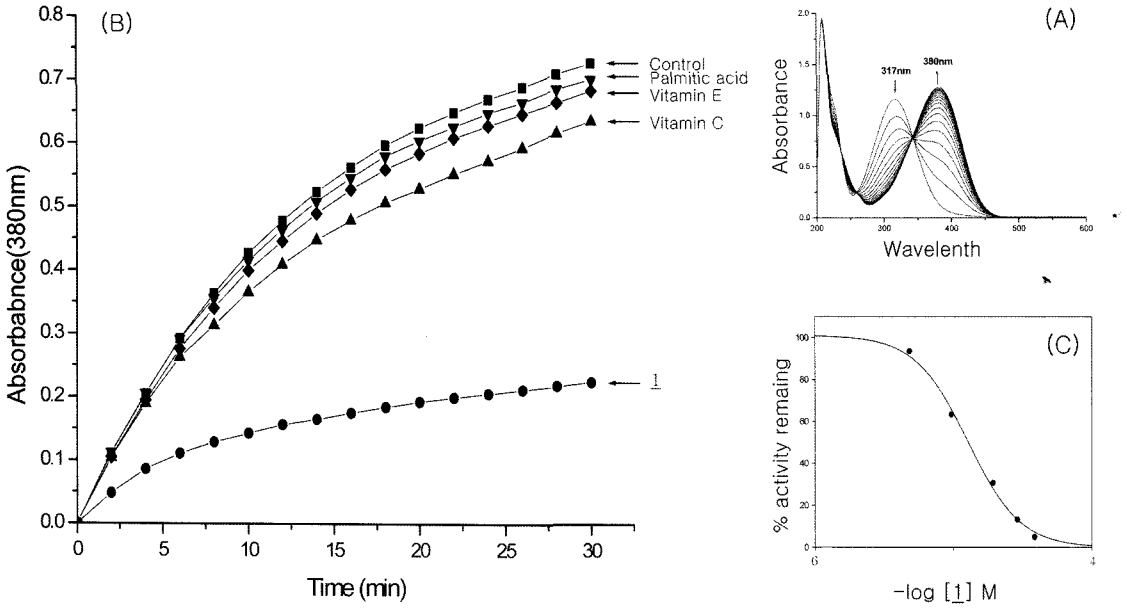

Fig. 2. (A) UV-vis spectra for the production of *p*-nitroaniline. *p*-Nitroaniline was produced by reacting *Z*-Gly-Pro-*p*NA with PEP in 0.1 M phosphate buffer (pH 7.0; 37; scanning interval, 10 min). (B) Typical inhibition pattern of *p*-nitroaniline produced from the reaction of *Z*-Gly-Pro-*p*NA with PEP in 0.1 M phosphate buffer (pH 7.0, 37°C, 2-min scanning interval) at 380 nm in the absence and presence of vitamins C and E, palmitic acid, and 6-*O*-palmitoyl ascorbic acid (8 μg · mℓ$^{-1}$). (C) Plots of % activity remaining from the reaction of *Z*-Gly-Pro-*p*NA with PEP versus -log [6-*O*-palmitoyl ascorbic acid]. Each data point represents the average of triplicate measurements.

was determined colorimetrically based on the absorbance at 380 nm. The A_{380} value of the mixture containing 960 μL of 0.1 M phosphate buffer (pH 7.0) and 40 μL sample solution was separately measured as mentioned above (B). A control was made by adding 40 μL of 0.1 M phosphate buffer instead of 40 μL of the sample solution used in 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. Triplicate samples were analyzed, and IC_{50} value was determined graphically based on the curves of the enzyme activity versus inhibitor concentrations.

Statistics. Intergroup 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

The role of micronutrients in age-related cognitive decline is of great interest. For example, a diet rich in antioxidants such as vitamins C and E can help reduce the risk of elderly cognitive impairment by eliminating harmful free radicals and decreasing the level of oxidative stress.[6,7] Age-related cognitive decline (ARCD) refers to mild deterioration in memory performance, executive functions, and speed of cognitive processing.[16] Avoidance of cardiovascular and other chronic diseases, a flexible personality during middle age, and maintenance of vision and hearing have been identified as protective factors to the progressive cognitive decline.[16] Eating adequate amounts of vitamin C in the daily diet may help reduce the risk of developing some of these conditions since studies showed that vitamin C can play a role in protecting against the heart disease, high cholesterol, high blood pressure, osteoarthritis, cataracts, diabetes, common cold, cancer, Alzheimer's disease and other types of dementia.[8-13]

Vitamins C and E, 6-*O*-palmitoyl L-ascorbic acid (1; Fig. 1), and palmitic acid were chosen to examine the relationship between these compounds and the inhibitory activity of PEP, which has been suggested to participate in the learning and memory processes. PEP inhibitory activity was measured using *Z*-Gly-Pro-*p*NA as a substrate. Fig. 2A shows the UV-vis spectra for the production of *p*-nitroaniline by the enzymatic reaction of PEP on *Z*-Gly-Pro-*p*NA at 10-min

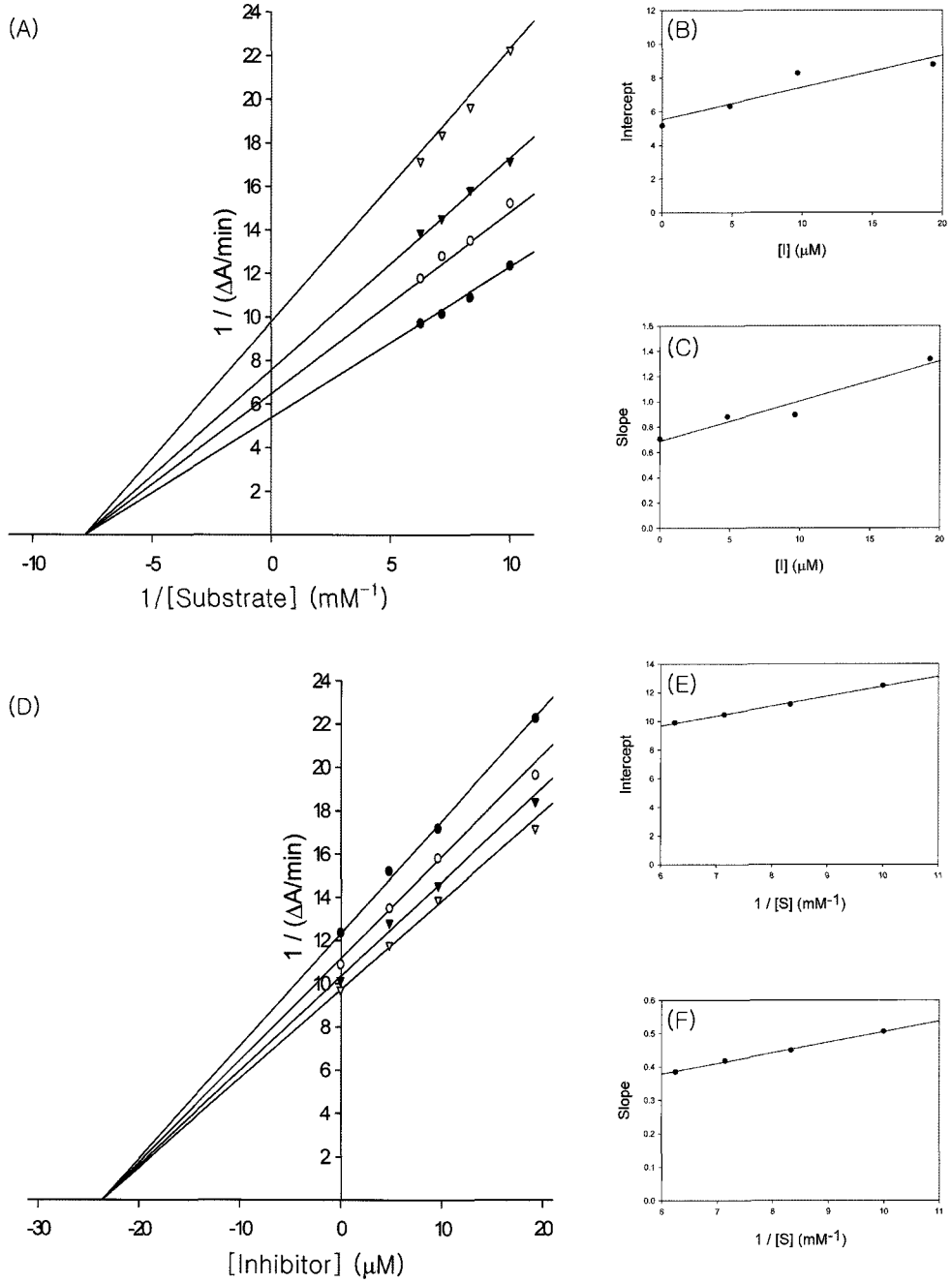

Fig. 3. Kinetic analysis of PEP inhibition. (A) Lineweaver-Burk plots of PEP inhibition by 6-*O*-palmitoyl ascorbic acid (1) in the absence (●) and presence of 4.82 (○), 9.65 (▼), and 19.30 (▽) μM 6-*O*-palmitoyl ascorbic acid. (B) Secondary plot of intercepts (*i*) taken from Lineweaver-Burk plots versus 6-*O*-palmitoyl ascorbic acid concentration. (C) Secondary plot of slopes (*s*) taken from Lineweaver-Burk plots versus 6-*O*-palmitoyl ascorbic acid concentration. (D) Dixon plots of PEP inhibition by 6-*O*-palmitoyl ascorbic acid. [S] = 0.1 mM (●), 0.12 mM (○), 0.14 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.

scanning intervals. The λ_{max} of *Z*-Gly-Pro-*p*NA at 317 nm decreased, whereas that of *p*-nitroaniline at 380 nm increased. *Z*-Pro-prolinal (IC_{50} = 2.19 nM), a synthetic PEP inhibitor, was used as a reference compound of the positive control.[17]

Upon preliminary examination of vitamins C and E, 6-*O*-palmitoyl L-ascorbic acid (1), and palmitic acid at 8 μg · ml^{-1} for the inhibition of PEP, vitamin C showed small but significant inhibitory activity with the value of 13% PEP inhibition, whereas vitamin E and palmitic acid showed almost no inhibitory activity (Fig. 2B). However, 6-*O*-palmitoyl L-ascorbic acid (1) showed 70% inhibition of PEP activity at 8 μg · ml^{-1} (Fig. 2B), indicating that hydrophobic portion of compound 1 may facilitate the inhibitory effect. Compound 1 was further investigated at various concentrations

to evaluate IC_{50} value and showed a dose-dependent PEP inhibitory effect with IC_{50} value of 12.6 ± 0.2 μM (Fig. 2C).

Lineweaver-Burk plots of the PEP inhibition by compound **1** indicate that it is a noncompetitive inhibitor (Fig. 3A). The secondary Lineweaver-Burk plots show linear relationship for both intercepts (*i*) versus compound **1** concentration (Fig. 3B) and slope (s) versus compound **1** concentration (Fig. 3C). Dixon plots of the PEP inhibition by compound **1** also indicate that it is a noncompetitive inhibitor with an inhibition constant (K_i) value of 23.7 μM (Fig. 3D). 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 as shown in Figs. 3E and 3F, respectively.

Although IC_{50} value of compound **1**, 12.6 μM, was higher than those reported for strong natural inhibitors, such as ginkgolic acid (IC_{50}, 0.62 μM),[17] staurosporine (IC_{50}, 0.77 μM),[18] and kynapsin 24 (IC_{50}, 1.14 μM),[19] compound **1** was similar or more effective PEP inhibitor than triterpenic acids possessing a variety of physiological effects, such as ursolic and oleanolic acids (IC_{50}, 17.2 and 22.5 μM, respectively).[20] Compound **1** was also more effective PEP inhibitor than unsaturated fatty acids such as oleic and linoleic acids, DHA, arachidonic acid, and EPA (IC_{50}, 23.6, 43.8, 46.2, 53.4, 99.4 μM, respectively),[15] whose deficiency is associated with retarded visual acuity, cognitive impairment, cerebellar dysfunction, and various other neurological disorders.[21]

In summary, 6-*O*-palmitoyl L-ascorbic acid (**1**) inhibited PEP activity and its IC_{50} was 12.6 ± 0.2 μM and K_i was 23.7 μM, while ascorbic and palmitic acids showed small or little PEP inhibition. Therefore, both components, ascorbyl and palmitoyl group, may function synergically on the PEP inhibition. These results suggest compound **1** has potential use in the prevention of memory loss and could be used as memory-enhancing principles.

Acknowledgments

This research was supported by the Kyung Hee University Research Fund in 2006.

References

1. Cunningham, D. F. and O'Connor, B. (1997) Proline specific peptidase. *Biochem. Biophys. Acta* **1343**, 160-186.
2. Walter, R., Shlank, H., Glass, J. D., Schwartz, I. L. and Kerenyi, T. D. (1971), Leucylglycinamide released from oxytocin by human uterine enzyme. *Science* **173**, 827-829.
3. Kalwant, S. and Porter, A. G. (1991) Purification and characterization of human brain prolyl endopeptidase. *Biochem. J.* **276**, 237-244.
4. Odaka, C., Mizuochi, T., Shirasawa, T., Morain, P. and Checler, F. (2002) Murine T cells expressing high activity of prolyl endopeptidase are susceptible to activation-induced cell death. *FEBS Lett.* **512**, 163-167.
5. Aoyagi, T., Wada, T., Nagai, M., Kojima, F., Harada, S., Takeuchi, T., Takahashi, H., Hirokawa, K. and Tsumita, T. (1990) Deficiency of kallikrein-like enzyme activities in cerebral tissue of patients with Alzheimer's disease. *Experientia* **46**, 94-97.
6. Engelhart, M. J., Geerlings, M. I., Ruitenberg, A., van Swieten J. C., Hofman, A., Witteman, J. C. and Breteler, M. M. (2002) Dietary intake of antioxidants and risk of Alzheimer disease. *J. Amer. Med. Assoc.* **287**, 3223-3229.
7. Markesbery, W. R. (1997) Oxidative stress hypothesis in Alzheimer's disease. *Free Radical Bio. Med.* **23**, 134-147.
8. Parle, M. and Dhingra, D. (2003) Ascorbic acid: a promising memory-enhancer in mice. *J. Pharmacol.* **93**, 129-135.
9. Frank, B. and Gupta, S. (2005) A review of antioxidants and Alzheimer's disease. *Ann Clin. Psychiatr.***17**, 269-286.
10. Morris, M. C., Beckett, L. A. and Scherr, P. A. (1998) Vitamin E and vitamin C supplement use and risk of incident Alzheimer disease. *Alz. Dis. Assoc. Disord.* **12**, 121-126.
11. Masaki, K. H., Losonczy, K. G., Izmirlian, G., Foley, D. J., Ross, G. W., Petrovitch, H., Havlik, R. and White, L. R. (2000) Association of vitamin E and C supplement use with cognitive function and dementia in elderly men. *Neurology* **54**, 1265-1272.
12. Duffy, S. J., Gokce, N., Holbrook, M., Huang, A., Frei, B., Keaney Jr, J. F. and Vita, J. A. (1999) Treatment of hypertension with ascorbic acid. *The Lancet* **354**, 2048-2049.
13. Padayatty, S. J. and Levine, M. (2000) Reevaluation of ascorbate in cancer treatment: emerging evidence, open minds and serendipity. *J. Am. Coll. Nutr.* **19**, 423-425.
14. Yoshimoto, T., Walter, R. and Tsuru, D. (1980) Proline-specific endopeptidase from *Flavobacterium*. Purification and properties. *J. Biol. Chem.* **255**, 4786-4792.
15. Park, Y.-S., Jang, H.-J., Lee, K.-H., Hahn, T.-R. and Paik, Y.-S. (2006) Prolyl endopeptidase inhibitory activity of unsaturated fatty acids. *J. Agric. Food Chem.* **54**, 1238-1242.
16. Solfrizzi, V., Panza, F., Torres, F., Mastroianni, F., Del Parigi, A., Venezia, A. and Capurso, A. (1999) High monounsaturated fatty acids intake protects against age-related cognitive decline. *Neurology* **52**, 1563-1569.
17. Lee, J.-H., Lee, S.-Y., Lee, K.-S., Jang, H.-J., Lee, K.-H., Hahn, T.-R. and Paik, Y.-S. (2004) Prolyl endopeptidase inhibitors from the leaves of *Ginkgo biloba*. *Planta Med.* **70**, 1228-1230.
18. Kimura, K., Kawaguchi, N., Yoshihama, M. and Kawanishi, G. (1990) Staurosporine, a prolyl endopeptidse inhibitor. *Agric. Biol. Chem.* **54**, 3021-3022.
19. Song, K.-S. and Raskin, I. (2002) A prolyl endopeptidase-inhibiting benzofuran dimer from *Polyozellus multiflex*. *J. Nat. Prod.* **65**, 76-78.
20. Park, Y.-S., Jang, H.-J. and Paik, Y.-S. (2005) Prolyl endopeptidase inhibitory activity of ursolic and oleanolic acids from corni fructus. *Agric. Chem. Biotechnol.* **48**, 207-212.
21. Haag, M. (2003) Essential fatty acids and the brain. *Can. J. Psychiatr.* **48**, 195-203.