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Monohydroxamates of Aspartic Acid and Glutamic Acid Exhibit Antioxidant and Angiotensin Converting Enzyme Inhibitory Activities

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Two monohydroxamates of L-aspartic acid β-hydroxamate (AAH) and L-glutamic acid γ-hydroxamate (GAH) were used for testing antioxidant and angiotensin converting enzyme (ACE) inhibitory activities in comparison with those of asparagine and glutamine, respectively. The half-inhibition concentrations, IC_{50}, of scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) were 36 and 48 μM and against superoxide radicals were 18.99 and 6.33 mM, respectively, for AAH and GAH. However, no activities of asparagine and glutamine were found. AAH and GAH also exhibited activities against peroxynitrite-mediated dihydrorhodamine 123 oxidations and hydroxyl radical-mediated DNA damage. For ACE inhibitory activities, the IC_{50} values were 4.92 and 6.56 mM, respectively, for AAH and GAH. The ACE hydrolyzed products on the TLC chromatogram also confirmed the inhibitory activities of the two amino acid hydroxamates on ACE. When 1.23 mM AAH was added, AAH showed competitive inhibitions against ACE, and the apparent inhibition constant (K_i) was 2.20 mM.

KEYWORDS: Angiotensin converting enzyme (ACE); antioxidant activity; L-aspartic acid β-hydroxamate (AAH); L-glutamic acid γ-hydroxamate (GAH)

INTRODUCTION

Active oxygen species (or reactive oxygen species) and free radical-mediated reactions are involved in degenerative or pathological processes such as aging (*1*, *2*), cancer, coronary heart disease, and Alzheimer's disease (*3*−*6*). There have been several reports concerning natural compounds in fruits and vegetables for their antioxidant activities, such as anthocyanin (*7*), water extracts of roasted *Cassia tora* (*8*), and the storage proteins of sweet potato root (*9*) and yam tuber (*10*).

Hypertension is considered to be the central factor in stroke, with ∼33% of deaths due to stroke attributed to untreated high blood pressure (*11*). There are several classes of pharmacological agents that have been used in the treatment of hypertension (*11*); one class of antihypertensive drugs known as angiotensin I converting enzyme (ACE) inhibitors (i.e., peptidase inhibitors) has a low incidence of adverse side effects and is the preferred class of antihypertensive agents for the treatment of patients with concurrent secondary diseases (*12*). ACE (peptidyldipeptide hydrolyase, EC 3.4.15.1) is a dipeptide-liberating Zn-containing exopeptidase, which removes a dipeptide from the C terminus of angiotensin I to form angiotensin II, a very hypertensive compound. Several food-derived peptides can inhibit ACE (*13*) including α-lactalbumin and β-lactoglobulin (*14*−*16*) and yam dioscorin (*17*). Several antioxidant peptides (reduced glutathione and carnosine-related peptides) also exhibited ACE inhibitory activities (*18*).

A variety of hydroxamic acid derivatives have been reported to have pharmacological and biological activities toward cancer, cardiovascular diseases, Alzheimer's disease, tuberculosis, etc. (*19*). Succinimide hydroxamic acids were proved to be potent inhibitors of histone deacetylase and tumor cell proliferation (*20*). Hydroxamic acid derivatives of salicylic acid were cyclooxygenase (COX)-1 and COX-2 inhibitors (*21*). Oxal hydroxamic acid derivatives were potent inhibitors of matrix metalloproteinases (*22*). The aspartic acid β-hydroxamate exhibited antitumor activity on L5178Y leukemia (*23*), therapeutic effect on friend erythroleukemia (*24*), and antiproliferative activity on friend virus-infected erythropoietic progenitor cells (*25*). We also reported that the different degrees of esterification of pectin hydroxamates exhibited both semicarbazide-sensitive amine oxidase and ACE inhibitory activities (*26*). In this paper, two hydroxamates of L-aspartic acid β-hydroxamate (AAH) and L-glutamic acid γ-hydroxamate (GAH), amino acid analogues, were used for testing antioxidant and ACE inhibitory activities in comparison with asparagine and glutamine, respectively. The results showed that AAH and GAH exhibited antioxidant, antiradical, and ACE inhibitory activities.

MATERIALS AND METHODS

Materials. ACE (I unit, rabbit lung), asparagine, L-aspartic acid β-hydroxamate, dihydrorhodamine 123 (DHR), DPPH, glutamine,

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L-glutamic acid γ-hydroxamate, *N*-(3-[2-furyl]acryloyl)-Phe-Gly-Gly (FAPGG), NADH, and phenazine methosulfate (PMS) were purchased from Sigma Chemical Co. (St. Louis, MO). Silica gel 60 F_{254} was purchased from E. Merck Inc. (Darmstadt, Germany). Peroxynitrite was obtained from Calbiochem Co. (Darmstadt, Germany). Calf thymus DNA (activated, 25 A_{260} units mL^{-1}) was purchased from Amersham Biosciences (Uppsala, Sweden). Other chemicals and reagents were from Sigma Chemical Co.

Scavenging Activity of DPPH Radical by Spectrophotometry. Every 0.3 mL of AAH, GAH, asparagine, and glutamine (6−60 μM) was added to 0.1 mL of 1 M Tris-HCl buffer (pH 7.9) and then mixed with 0.6 mL of 100 μM DPPH in methanol to the final concentration of 60 μM for 20 min under light protection at room temperature (*27*, *28*). The decrease of absorbance at 517 nm was measured and expressed as ΔA_{517nm}. Deionized water was used as a blank experiment. The scavenging activity of DPPH radicals (%) was calculated with the equation $(\Delta A_{517nm,blank} - \Delta A_{517nm,sample}) \div \Delta A_{517nm,blank} \times 100\%$. IC_{50} stands for the concentration of half-inhibition.

Scavenging Activity of Superoxide Radicals by Spectrophotometry. The superoxide radical was determined by the PMS−NADH generating system (*29*). Every 0.2 mL of sample containing different amounts of AAH (10−20.5 mM), GAH (2.5−12.5 mM), asparagine, and glutamine (2.5−12.5 mM) was added in sequence to 0.2 mL of 630 μM nitroblue tetrazolium, 0.2 mL of 33 μM PMS, and 0.2 mL of 156 μM NADH in 100 mM phosphate buffer (pH 7.4). Deionized water was used as a blank experiment. The changes of absorbance at 560 nm were recorded during 2 min and expressed as ΔA_{560nm}/min. The scavenging activity of superoxide radicals was calculated as follows: $(\Delta A_{560nm}/min_{blank} - \Delta A_{560nm}/min_{sample}) \div \Delta A_{560nm}/min_{blank} \times 100\%$. IC_{50} stands for the concentration of half-inhibition.

Protecting Peroxynitrite-Mediated Dihydrorhodamine 123 Oxidation by AAH and GAH. The protection of peroxynitrite-mediated DHR oxidation was according to the method of Kooy et al. (*30*). The total 180 μL reaction mixture included different amounts of AAH (0.3, 0.45, and 0.65 mM) or GAH (0.1, 0.16, and 0.22 mM), 0.9 mM DHR, and 5 μL of peroxynitrite in 50 mM phosphate buffer (pH 7.4) containing 90 mM NaCl. After 5 min of reaction, the fluorescent intensity was measured at the excitation and emission wavelengths of 500 and 536 nm, respectively, and excitation and emission slit widths of 2.5 and 3.0 nm, respectively. The control test was without sample additions.

Protecting Hydroxyl Radical-Induced Calf Thymus DNA Damage by AAH and GAH. The hydroxyl radical was generated by Fenton reaction according to the method of Kohno et al. (*31*). The 45 μL reaction mixture included AAH (1.35, 1.65, 2.12, 2.96, and 4.93 μg/mL) or GAH (1.46, 1.80, 2.32, 3.24, and 5.40 μg/mL), 15 μL of calf thymus DNA, 18 mM $FeSO_4$, and 60 mM hydroxygen peroxide at room temperature for 15 min. Ten microliters of 1 mM EDTA was added to stop the reaction. Only calf thymus DNA was used for blank test, and the control test was without sample addition. After agarose electrophoresis, the treated DNA solutions were stained with ethidium bromide and observed under UV light.

ACE Inhibitory Activity of AAH and GAH by Spectrophotometry. The ACE inhibitory activity was measured according to the method of Holmquist et al. (*32*) with some modifications. Twenty microliters (20 mU) of commercial ACE (1 U/mL, rabbit lung) was mixed with 200 μL of different amounts of AAH, GAH, asparagine, and glutamine (2−10 mM), and then 1 mL of 0.5 mM FAPGG [dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl] was added. The decreased absorbance at 345 nm ($\Delta A_{inhibitor}$) was recorded during 5 min at room temperature. Deionized water was used instead of sample solution for blank experiments (ΔA_{blank}). The ACE activity was expressed as ΔA_{345nm}, and the ACE inhibition (%) was calculated as follows: $[1 - (\Delta A_{inhibitor} \div \Delta A_{blank})] \times 100\%$. Means of triplicates were determined.

Determination of ACE Inhibitory Activity of AAH and GAH by TLC. The ACE inhibitory activities of PHAs were determined by the TLC method (*32*). The reactions of commercial ACE with AAH or GAH were according to the method of Anzenbacherova et al. (*33*) with some modifications. Each 100 μL sample (AAH, 12.7 mM; GAH, 15.87 mM) was mixed with 20 mU of ACE, and then 200 μL of 0.5 mM FAPGG was added and reacted at room temperature for 10 min. One thousand microliters of methanol was added to stop the reaction. The blank experiment was FAPGG only; the control experiment was ACE reacted with FAPGG under the same conditions. Each was dried under reduced pressure and redissolved with 400 μL of methanol, and 50 μL was spotted on a silica gel 60 F_{254} by CAMAG Linomat IV spray-on technique. The FAPGG and FAP (ACE-hydrolyzed products) were separated by TLC in water-saturated *n*-butanol/acetic acid/water, 4:1:1 (v/v/v) and observed under UV light (*32*).

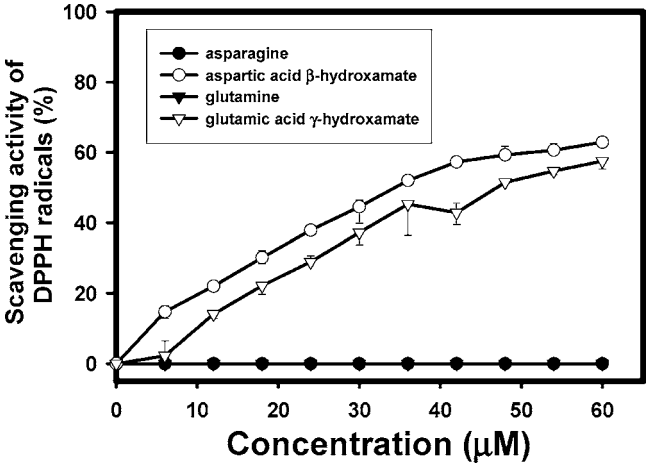

Figure 1. Effects of different concentrations of L-aspartic acid β-hydroxamate (AAH), L-glutamic acid γ-hydroxamate (GAH), asparagine, and glutamine (6, 12, 18, 24, 30, 36, 42, 48, 54, and 60 μM) on the scavenging activities of DPPH radicals with spectrophotometry. The scavenging activity of DPPH radicals (%) was calculated with the equation $(\Delta A_{517nm,blank} - \Delta A_{517nm,sample}) \div \Delta A_{517nm,blank} \times 100\%$.

Determination of the Kinetic Properties of ACE Inhibition by AAH. The kinetic properties of ACE (15 mU) without or with AAH (1.23 mM) were determined using different concentrations of FAPGG as substrates (0.1, 0.125, 0.25, and 0.5 mM). The K_m (without AAH) was calculated from Lineweaver−Burk plots, and the K_i (with AAH) was calculated using the equation $K_i = [I]/(K_m'/K_m) - 1$, where [I] is the concentration of AAH, 1.23 mM, added and K_m' is the Michaelis constant in the presence of inhibitor at concentration [I].

Statistics. Means of triplicates were measured. Student's *t* test was used for comparison between two treatments. A difference was considered to be statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Scavenging Activity against DPPH Radicals of AAH and GAH. Two commercial hydroxamates of L-aspartic acid β-hydroxamate and L-glutamic acid γ-hydroxamate, amino acid analogues, were used to test antioxidant properties in comparison with asparagine and glutamine, respectively. **Figure 1** shows the results of scavenging activity of DPPH radicals. The DPPH radicals were widely used in the model system to investigate the scavenging activities of several natural compounds. The colors change from purple to yellow, and its absorbance at wavelength 517 nm decreases. Both AAH and GAH showed dose-dependent DPPH radical scavenging activities. The IC_{50} values of scavenging activity against DPPH were 36 and 48 μM, respectively, for AAH and GAH. However, no DPPH scavenging activities were found for either asparagine or glutamine in the same concentrations (**Figure 1**). Under the same condition, the IC_{50} values were 12.98 and 24.80 μM, respectively, for butylated hydroxytoluene (BHT) and reduced glutathione (*27*). As the DPPH radical is scavenged by antioxidants through the donation of a hydrogen atom, it forms the reduced DPPH−H (*29*). The side chains of $R-CONH_2$ in asparagine and glutamine and R−CONHOH in AAH and GAH

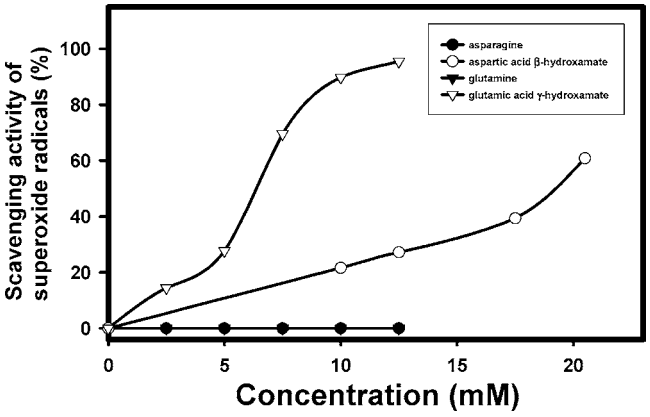

Figure 2. Effects of different concentrations of L-aspartic acid β-hydroxamate (AAH, 10–20.5 mM), L-glutamic acid γ-hydroxamate (GAH, 2.5–12.5 mM), asparagine, and glutamine (2.5–12.5 mM) on the scavenging activities of superoxide radical generating by the PMS–NADH generating system.The changes of absorbance at 560 nm were recorded during 2 min and expressed as ΔA_{560nm}/min. The scavenging activity of superoxide radicals was calculated as follows: $(\Delta A_{560nm}/min_{blank} - \Delta A_{560nm}/min_{sample}) \div \Delta A_{560nm}/min_{blank} \times 100\%$.

might explain the differences of scavenging activities against DPPH radicals.

Scavenging Activity against Superoxide Radicals of AAH and GAH. Superoxide radicals were used to evaluate antiradical activities of AAH and GAH (**Figure 2**). Both AAH and GAH showed dose-dependent superoxide radical scavenging activities. The IC_{50} values of scavenging activity against superoxide were 18.99 and 6.33 mM, respectively, for AAH and GAH. However, no superoxide scavenging activities were found for both asparagine and glutamine in the same concentrations (**Figure 2**). The IC_{50} of scavenging activity against superoxide was 3.5 mM for carnosine (β-alanylhistidine) (*34*). This result together with **Figure 1** revealed that amino acid derivatives of AAH and GAH exhibited antioxidant or antiradical activities.

Protecting Peroxynitrite-Mediated DHR Oxidation by AAH and GAH. Peroxynitrite is formed from nearly diffusion-limited reaction between nitric oxide and superoxide and is an initiator of potentially harmful oxidation reactions (*35*). From the results of **Figure 3**, it was found that the protective effect of peroxynitrite-mediated DHR oxidation of either AAH (**Figure 3A**) or GAH (**Figure 3B**) was dose-dependent. Except for cysteine and urate (*30*), the amino acid derivatives of AAH and GAH with peroxynitrite scavenging activities were first reported. The IC_{50} values of scavenging activity against peroxynitrite-mediated DHR oxidation were 2.5 and 25 μM, respectively, for urate and cysteine (*30*). The protection capacities of AAH were 27.03, 30.76, and 39.07% for 0.3, 0.45, and 0.65 mM, respectively, and those of GAH were 7.20, 14.47, and 20.41% for 0.1, 0.16, and 0.22 mM, respectively. A significant difference was observed between the peroxynitrite, peroxynitrite plus 0.3, 0.45, or 0.65 mM AAH, and peroxynitrite plus 0.22 mM GAH ($p < 0.05$).

Protecting Hydroxyl Radical-Induced Calf Thymus DNA Damage by AAH and GAH. Free radicals could damage macromolecules in cells, such as DNA, protein, and lipids in membranes (*36*). **Figure 4** shows the amino acid derivatives of AAH and GAH protected against hydroxyl radical-induced calf thymus DNA damage. Only calf thymus DNA was used for the blank test, and the control test was without AAH (top, **Figure 4**) or GAH (bottom, **Figure 4**) additions. Compared to blank test and control test, it was found that added AAH (lanes

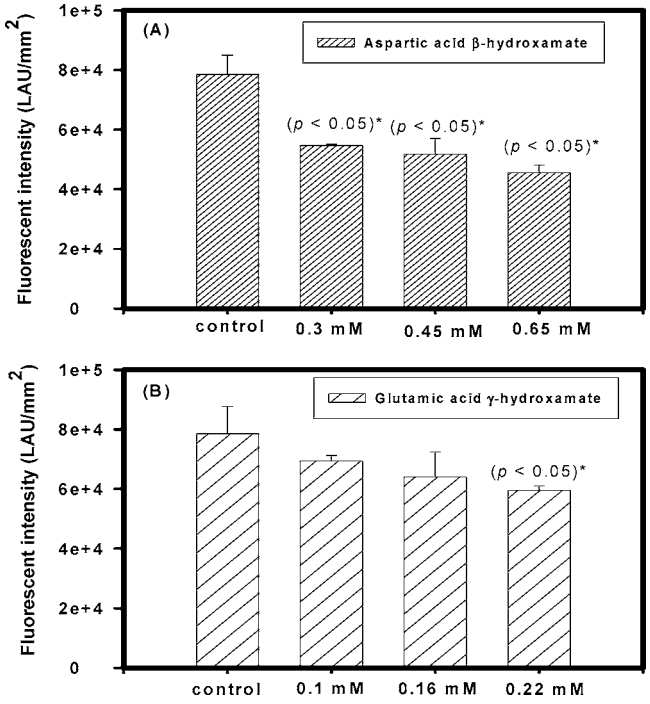

Figure 3. (**A**) L-Aspartic acid β-hydroxamate (AAH, 0.3, 0.45, and 0.65 mM) or (**B**) L-glutamic acid γ-hydroxamate (GAH, 0.1, 0.16, and 0.22 mM) protected peroxynitrite-mediated dihydrorhodamine 123 (DHR) oxidation. The total 180 μL reaction mixture included different amounts of AAH or GAH, 0.9 mM DHR, and 5 μL of peroxynitrite in 50 mM phosphate buffer (pH 7.4) containing 90 mM NaCl. After 5 min of reaction, the fluorescent intensity was measured. The control test was without AAH or GAH additions. A difference was considered to be statistically significant when $p < 0.05$ (*).

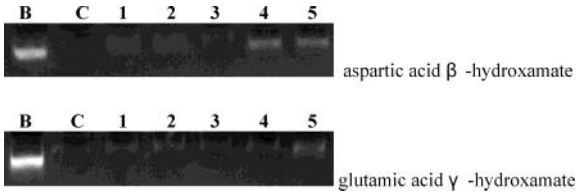

Figure 4. Effects of L-aspartic acid β-hydroxamate (top, AAH, lanes 1–5 were 1.35, 1.65, 2.12, 2.96, and 4.93 μg/mL, respectively) or L-glutamic acid γ-hydroxamate (bottom, GAH, lanes 1–5 were 1.46, 1.80, 2.32, 3.24, and 5.40 μg/mL, respectively) on the protections of hydroxyl radical-induced calf thymus DNA damage after 15 min of reaction. Only calf thymus DNA was used for the blank test, and the control test was without AAH or GAH addition.

1−5 were 1.35, 1.65, 2.12, 2.96, and 4.93 μg/mL, respectively) above 2.96 μg/mL (lane 4, top, **Figure 4**) or added GAH (lanes 1−5 were 1.46, 1.80, 2.32, 3.24, and 5.40 μg/mL, respectively) above 5.40 μg/mL (lane 5, bottom, **Figure 4**) could protect against hydroxyl radical-induced calf thymus DNA damage after 15 min reactions. The purified potato root protein, patatin, also exhibited protecting roles against hydroxyl radical-induced calf thymus DNA damage (*28*) under the concentrations of 0.91−3.64 mg/mL.

AAH and GAH Exhibited ACE Inhibitory Activity. Several antioxidant peptides (reduced glutathione and carnosine-related peptides) exhibited ACEI activities (*18*). Our recent study also confirmed that acetohydroxamic acid (the simplest hydroxamic acid, $CH_3CONHOH$) and the different degrees of esterification of pectin hydroxamates exhibited ACE inhibitory activities (*26*). Therefore, the amino acid derivatives of AAH and GAH were used for determinations of ACE inhibitory

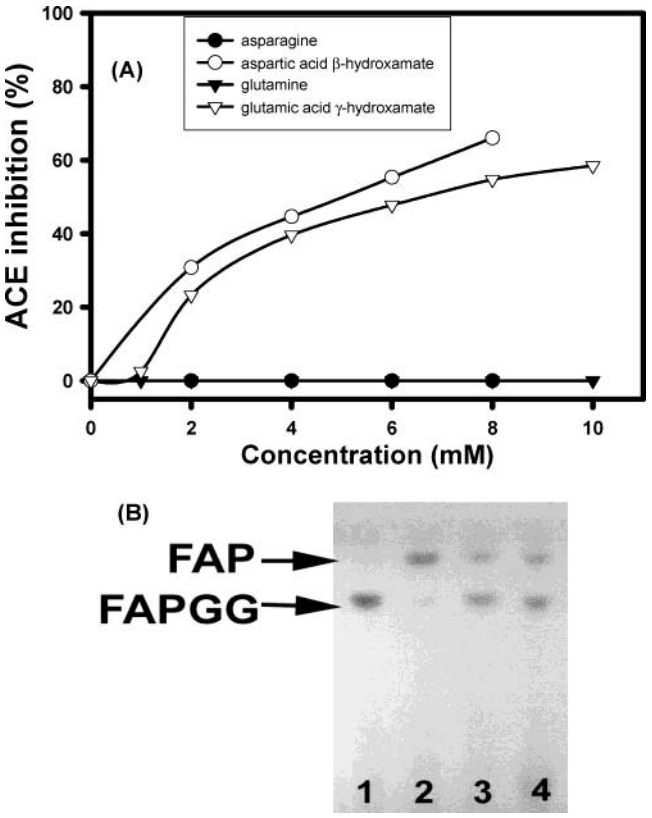

Figure 5. Effects of (**A**) of L-aspartic acid β-hydroxamate, L-glutamic acid γ-hydroxamate, asparagine, and glutamine (2–10 mM) on 20 mU ACE activities from rabbit lung. The ACE activity was expressed as ΔA_{345nm}, and the ACE inhibition (%) was calculated as follows: $[1 - (\Delta A_{inhibitor} \div \Delta A_{blank})] \times 100\%$. Means of triplicates were determined. (**B**) TLC chromatograms of a silica gel 60 F_{254} on the effects of L-glutamic acid γ-hydroxamate (lane 3, 15.87 mM) or L-aspartic acid β-hydroxamate (lane 4, 12.7 mM) on 20 mU ACE: lane 1, blank test (FAPGG only); lane 2, control test (ACE hydrolyzed FAPGG to produce FAP). Each solution was dried under reduced pressure and redissolved with 400 μL of methanol. Each 50 μL was spotted on a silica gel 60 F_{254} by CAMAG Linomat IV spray-on technique. FAPGG and FAP were separated by water-saturated *n*-butanol/acetic acid/water, 4:1:1 (v/v/v).

activities in comparison with asparagine and glutamine, respectively (**Figure 5**). From the results of **Figure 5A**, it was found the AAH and GAH showed dose-dependent ACE inhibitory activities. However, no ACE inhibitory activities were found for both asparagine and glutamine in the same concentrations. The IC_{50} values were 4.92 and 6.56 mM, respectively, for AAH and GAH. The ACE inhibitory activity of AAH and GAH might be attributed to the chelating properties of hydroxamic acid moieties (*19*, *26*). **Figure 5B** shows the TLC chromatogram of a silica gel 60 F_{254} on the effects of GAH (lane 3, 15.87 mM) or AAH (lane 4, 12.7 mM) on ACE activity: lane 1, blank test (FAPGG only); lane 2, control test (ACE hydrolyzed FAPGG to produce FAP). Compared with the blank (lane 1) and control (lane 2) tests, it was found that GAH (lane 3) and AAH (lane 4) showed ACE inhibitory activities for FAPGG retention and fewer FAP of ACE products. It was again confirmed that AAH and GAH exhibited ACEI activities.

Kinetic Properties of ACE Inhibition by AAH. Lineweaver−Burk plots of ACE (15 mU) without or with AAH (1.23 mM) in the different concentrations of FAPGG are shown in **Figure 6**. The results indicate that the AAH acted as a competitive inhibitor with respect to the substrates (FAPGG). Without the AAH, the calculated K_m was 0.407 mM FAPGG

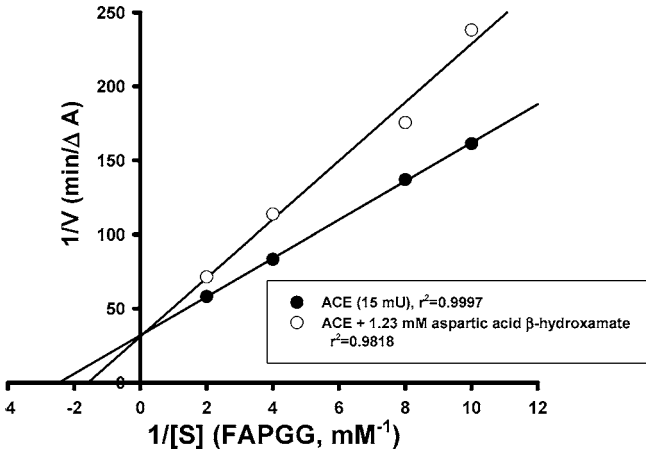

Figure 6. Lineweaver–Burk plots of ACE (15 mU) without or with L-aspartic acid β-hydroxamate (1.23 mM) in the different concentrations of FAPGG (0.1, 0.125, 0.25, and 0.5 mM).

for ACE, which was close to the result (0.3 mM) of Holmquist et al. (*32*). In the present study of AAH (1.23 mM), the calculated K_m' was 0.635 mM. From the equation $K_i = [I]/(K_m'/K_m) - 1$, the calculated K_i was 2.20 mM for FAPGG.

In conclusion, the amino acid hydroxamates of AAH and GAH showed antioxidant (**Figures 1**−**4**) and ACE inhibitory activities (**Figures 5** and **6**). The resonance properties of hydroxamic acid moieties (R−CONHOH) in the amino acid side chain might explain the differences between L-aspartic acid β-hydroxamate (AAH) and L-glutamic acid γ-hydroxamate (GAH) and asparagine and glutamine. Hydroxamates of AAH and GAH were reported to be involved in the microbial transport of iron and were therefore applied therapeutically in conditions of iron deficiency (*37*). Some synthetic peptides containing hydroxamic acid derivatives or dihydroxamates of aspartic acid and glutamic acid will be prepared and investigated further.

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