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Inhibitory Activities of Semicarbazide-Sensitive Amine Oxidase and Angiotensin Converting Enzyme of Pectin Hydroxamic Acid

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Solutions of 100 mL of 1% commercial pectin each with a different degree of esterification (DE), DE94, DE65, and DE25, were reacted with 100 mL of 2 M alkaline hydroxylamine (pH 12.0) at room temperature for 4 or 18 h. These pectin hydroxamic acids (PHAs; DE94T4, DE94T18, DE65T4, and DE25T4) were used to test the inhibitory activities against semicarbazide-sensitive amine oxidase (SSAO) and angiotensin-converting enzyme (ACE). Compared to different DE pectins (DE94, DE65, and DE25), the PHAs of DE94T4, DE94T18, DE65T4, and DE25T4 showed different inhibition activities against SSAO or ACE. Commercial pectins with different DE values showed negligible SSAO or ACE inhibitions. The order of SSAO inhibition was DE65T4 > DE94T18 ≈ DE25T4 ≫ DE94T4. However, the order of ACE inhibition was DE94T4 > DE94T18 ≫ DE65T4 > DE25T4. The SSAO activity staining or ACE-hydrolyzed products on TLC chromatogram also confirmed the inhibitory activities of PHAs against SSAO or ACE.

KEYWORDS: Angiotensin converting enzyme (ACE); degree of esterification (DE); semicarbazide-sensitive amine oxidase (SSAO); pectin; pectin hydroxamic acid (PHA)

INTRODUCTION

Several risk factors are associated with stroke, including age, gender, elevated cholesterol, smoking, alcohol, excessive weight, race, family history, and hypertension (*1*). Although some of these risk factors cannot be modified, one factor that can be controlled and has the greatest impact on etiology of stroke is high blood pressure (*2*). There are several classes of pharmacological agents that have been used in the treatment of hypertension (*1*); one class of antihypertensive drugs known as angiotensin I converting enzyme (ACE) inhibitors (ACEI, i.e., peptidase inhibitors) has a low incidence of adverse side effects and are the preferred class of antihypertensive agents for the treatment of patients with concurrent secondary diseases (*3*). ACE (peptidyldipeptide hydrolyase, EC 3.4.15.1) is a dipeptide-liberating zinc-metallopeptidase, which has been classically associated with the renin−angiotensin system regulating peripheral blood pressure (*4*). ACE removes a dipeptide from the C terminus of angiotensin I to form angiotensin II, a very hypertensive compound. Several endogenous peptides such as enkephalins, β-endorphin, and substance P were reported to be competitive substrates and inhibitors of ACE (*4*). Several food-derived peptides can inhibit ACE (*5*) including α-lactalbumin and β-lactoglobulin (*6*−*8*), casein (*9*−*11*), zein (*12*, *13*), gelatin (*14*), and yam dioscorin (*15*), all of which were hydrolyzed by pepsin, trypsin, or chymotrypsin. Several antioxidant peptides (reduced glutathione and carnosine-related peptides) also exhibited ACEI activities (*16*).

The semicarbazide-sensitive amine oxidase (SSAO, EC 1.4.3.6) is a common name of copper-containing metalloprotein for a group of heterogeneous enzymes widely distributed in nature, including plants, microorganisms, and organs of mammals (vasculature, dental pulp, eye, and plasma) (*17*). SSAO converts primary amines into the corresponding aldehydes, generating hydrogen peroxide and ammonia. It was found that the endogenous compounds aminoacetone and methylamine are good substrates for most SSAOs (*18*, *19*). In recent works, it was found that plasma SSAO activity increased in diabetes mellitus and heart failure. SSAO activity was implicated to have roles in atherosclerosis, endothelial damage, and glucose transport into adipocytes (*20*−*22*). Recently, it was also postulated that SSAO was a cardiovascular risk factor in nondiabetic obese patients (*23*).

Pectin, a macromolecule of galacturonic acid polymer with different methyl esters, exists in the middle lamella of plant cell wall and acts as cells' adhesions (*24*). The ratio of esterified galacturonic acid units to total galacturonic acid units is called the degree of esterification (DE) (*24*). DE was correlated with firming effects of vegetables during cooking (*25*). Except for the food industry of jams and jellies (*26*), there are reports concerning the physiological activities of pectins on the interactions between fibroblast growth factors and receptors (*27*), on the modulation of lung colonization of B16-F1 melanoma

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cell (*28*), and on the inhibition of human cancer cell growth and metastasis in nude mice (*29*). Pectin diets could also reduce the incidence of colon cancer in rats (*30*). In this paper, we used alkaline hydroxylamine (pH 12.0) to react the different DE (DE94, DE65, and DE25) pectins at room temperature for 4 or 18 h to produce different pectin hydroxamic acids (PHAs; DE94T4, DE94T18, DE65T4, and DE25T4). Compared to the different DE pectins, the PHAs showed different inhibitory activities against SSAO or ACE. The SSAO activity staining or ACE-hydrolyzed products on the TLC chromatogram also confirmed inhibitory effects of PHAs on SSAO or ACE.

MATERIALS AND METHODS

Materials. Commercial pectins (from citrus fruit, DE94, DE65, and DE25), *N*-(3-[2-furyl]acryloyl)-Phe-Gly-Gly (FAPGG), and bovine plasma (P-4639, reconstituted with 10 mL of deionized water) were purchased from Sigma Chemical Co. (St. Louis, MO); ACE (I unit, rabbit lung) was purchased from Fluka Chemie GmbH (Buchs, Switzerland); Tris, electrophoretic reagents, and silica gel 60 F_{254} were purchased from E. Merck Inc. (Darmstadt, Germany); other chemicals and reagents were from Sigma Chemical Co.

Different DE Pectins Reacted with Alkaline Hydroxylamine. Solutions of 100 mL of 1% commercial pectin each with different DE, DE94, DE65, and DE25, were reacted with 100 mL of 2 M alkaline hydroxylamine (pH 12.0) at room temperature for 4 or 18 h (*25*). The pH during reaction was ~11.6. After the pH had been adjusted to 6.5, 3 volumes of 2-propanol was added to precipitate the modified pectins. These PHAs were redissolved in water and then dialyzed against deionized water (with three changes of the deionized water) overnight. After precipitation with 2-propanol, washing with methanol, and rinsing with acetone, these PHAs (DE94T4, DE94T18, DE65T4, and DE25T4) were used for further investigations.

SSAO Inhibitory Activity of PHAs Determined by Fluorometric Spectrophotometry. SSAO inhibitory activity was determined by fluorometric spectrophotometry (*31*). The 200 μL reaction mixture contained 20 μL of PHAs (0.5−2.5 mg/mL) or different DE pectins (0.5−1.25 mg/mL) or acetohydroxamic acid [0.0188 mg/mL (0.25 mM) to 0.094 mg/mL (1.25 mM)] or clorgyline (10 μM), deprenyl (10 μM), the mixtures of clorgyline (10 μM) and deprenyl (10 μM) or semicarbazide (100 μM), 20 μL of bovine serum (SSAO is 31.7 units/mL), 1 unit/mL horseradish peroxidase, 1 mM benzylamine, and 50 μM Amplex Red. The excitation and emission filters were 560 ± 10 and 590 ± 10 nm, respectively. SSAO reacted with benzylamine to produce hydrogen peroxide that was coupled with Amplex Red catalyzed by horseradish peroxidase to produce fluorescence. SSAO activity was expressed as nanomoles of hydrogen peroxide formed per hour per milliliter of plasma. After incubation for 60 min at room temperature, SSAO activity was measured and expressed as the percentage of activity relative to the control experiment conducted simultaneously without addition of these inhibitory compounds. Means of triplicates were determined.

SSAO Inhibitory Activity of PHAs on 7.5% Native Polyacrylamide Gels. Fifty microliters of PHAs (DE25T4, DE65T4, 10 μg/μL) or pectins (DE25, DE65, 10 μg/μL), or 40 mM semicarbazide was mixed with 40 μL of bovine plasma in 50 mM phosphate buffer (pH 7.5) overnight, and then electrophoresis on native PAGE gels was done for SSAO activity staining. When PAGE on 7.5% native gels was finished, the gels were dipped in 50 mM phosphate buffer (pH 7.5) for 20 min twice for pH change before activity staining. The process of SSAO activity staining was as below (*32*). Twenty milligrams of benzylamine and 10 mg of 3-amino-9-ethylcarbazole were dissolved in 3 mL of dimethylformamide and then added to 50 mL of 50 mM phosphate buffer (pH 7.5) as the substrate solution, in which gels were submerged and shaken for 5 min. Then, 200 μL horseradish peroxidase (5 mg/mL) was added. The gels were gently shaken in darkness at room temperature for 30 min. The gels were then destained with 10% acetic acid and washed with distilled water.

Determination of ACE Inhibitory Activity of PHAs by Spectrophotometry. The ACE inhibitory activity was measured according to the method of Holmquist et al. (*33*) with some modifications. Twenty microliters (20 milliunits) of commercial ACE (1 unit/mL, rabbit lung) was mixed with 200 μL of different amounts of PHAs or commercial pectins (DE94T4 and DE94T18, 0.4098−3.2784 mg/mL; DE94, 0.8197−3.2784 mg/mL; DE65T4, 0.8137−3.2784 mg/mL; DE65, 0.4098−1.6392 mg/mL; DE25T4, 0.8197−3.2784 mg/mL; DE25, 0.4098−1.6329 mg/mL) or acetohydroxamic acid [0.031 mg/mL (0.4098 mM) to 0.49 mg/mL (6.577 mM)], and then 1 mL of 5×10^{-4} M FAPGG [dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 0.3 M NaCl] was added. When FAPGG was hydrolyzed by ACE to produce FAP, the absorbance at 345 nm decreased. The decreased absorbance at 345 nm ($\Delta A_{\text{inhibitor}}$) 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 $\Delta A_{345\text{nm}}$ and the ACE inhibition (percent) was calculated as follows: $[1 - (\Delta A_{\text{inhibitor}} \div \Delta A_{\text{control}})] \times 100\%$. Means of triplicates were determined.

Determination of ACE Inhibitory Activity of PHAs by TLC. The ACE inhibitory activities of PHAs were determined according to a TLC method (*33*). The reactions of commercial ACE with DE94, DE94T4, and DE94T18 were according to the method of Anzenbacherova et al. (*34*) with some modifications. Each 100 μL sample (10 μg/μL) was mixed with 20 milliunits of ACE, and then 200 μL of 5×10^{-4} M FAPGG was added and reacted at room temperature for 10 min. Then 1000 μL 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 (CAMAG, Muttenz, Switzerland). The FAPGG and FAP (ACE-hydrolyzed products) were separated by TLC in butanol/acetic acid/water, 4:1:1 (v/v/v), and observed under UV light (*33*).

RESULTS AND DISCUSSION

SSAO Inhibitory Activity of Acetohydroxamic Acid and PHAs Determined by Fluorometric Spectrophotometry. SSAO was inhibited by semicarbazide, other hydrazine derivatives, and hydroxylamine (*35*). No report was found for the potent inhibitory activities of hydroxylamine derivatives, hydroxamic acid, on SSAO. Under alkaline conditions, the esters, anhydrides, imides, and amides could react with hydroxylamine (NH_2OH) to produce their corresponding hydroxamic acid derivatives (RCONHOH) (*36*). The simplest hydroxamic acid, acetohydroxamic acid, was used to test its inhibitory activity on SSAO. Bovine amine oxidase, being reported to belong to SSAO, was purified to homogeneity (*37*). **Figure 1A** showed the inhibitory activity of acetohydroxamic acid (0.0188−0.094 mg/mL corresponding to 0.25−1.25 mM, respectively) on the SSAO (31.69 units/mL). From the results, it was found that acetohydroxamic acid exhibited dose-dependent inhibition on SSAO. Compared with SSAO, amine oxidases A and B were well-known mitochondria enzymes having firmly established roles in the metabolism of neurotransmitters (noradrenaline) that were inhibited by clorgyline and deprenyl, respectively (*38*). **Figure 1B** showed inhibitory activities of clorgyline (10 μM), deprenyl (10 μM), and the mixtures of clorgyline (10 μM) and deprenyl (10 μM) or semicarbazide (100 μM) on SSAO from bovine plasma. It was clear that bovine plasma amine oxidase was of SSAO type and inhibited by semicarbazide. Therefore, the different DE pectins (DE94, DE65, and DE25) were used to react with alkaline hydroxylamine (*39*) to produce PHAs. After dialysis overnight to remove the remaining free hydroxylamine in PHAs, DE94T4, DE94T18, DE65T4, and DE25T4 (**Figure 1C**) were used to investigate their inhibitory activities against SSAO in comparison with DE94, DE65, and DE25 (**Figure 1D**). It was found that the different DE pectins showed negligible inhibition on SSAO (**Figure 1D**). In contrast, PHAs showed dose-dependent and different inhibitory activities against

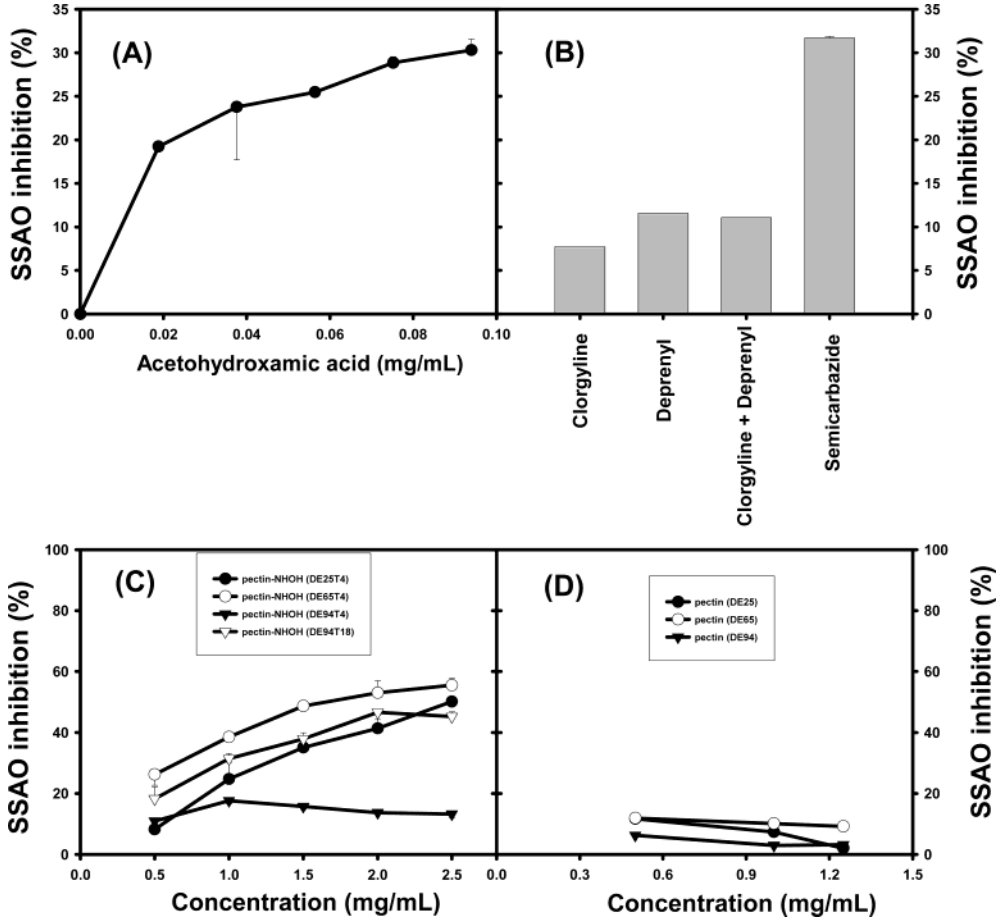

Figure 1. Effects of (A) acetohydroxamic acid [0.0188 mg/mL (0.25 mM) to 0.094 mg/mL (1.25 mM)]; (B) clorgyline (10 μM), deprenyl (10 μM), the mixture of clorgyline (10 μM) and deprenyl (10 μM) or semicarbazide (100 μM); (C) PHA (0.5–2.5 mg/mL) of DE25T4, DE65T4, DE94T4, and DE94T18; and (D) pectin (0.5–1.25 mg/mL) of DE25, DE65, and DE94 on SSAO (31.7 units/mL) activity from bovine plasma.

SSAO. The order of inhibitory activities was DE65T4 > DE94T18 ≈ DE25T4 ≫ DE94T4 (**Figure 1C**). This is the first report that PHAs exhibit inhibitory activities against SSAO. The inhibitory activities on SSAO were not due to the interference of enzyme−substrate interaction by PHAs. Both pectin and PHAs are macromolecules, but only the PHAs exhibit dose-dependent inhibitory activities against SSAO. Besides the hydroxamic acid groups in galacturonic acid moiety, other factors, such as the effectors of molecular size that might be responsible for the observed inhibitory activities, will be further investigated.

SSAO Inhibitory Activity of PHAs on 7.5% Native Polyacrylamide Gels. The effects of PHAs on SSAO activity staining are shown in **Figure 2**. It was found that the different DE pectins (lanes 3 and 5, **Figure 2**) could not inhibit SSAO; however, PHAs (DE25T4 and DE65T4, lanes 4 and 6, **Figure 2**) exhibited SSAO inhibitory activities compared to lane 2 of semicarbazide added as a positive control. These results confirmed that PHAs exhibited SSAO inhibitory activities.

Determination of ACE Inhibitory Activity of PHAs by Spectrophotometry. Raash et al. (*40*) found that ACE inhibitor could reduce amine oxidase activity. Therefore, acetohydroxamic acid and PHAs were used to test ACE inhibitory activities (**Figure 3**). **Figure 3A** shows the dose-dependent inhibitory activity of acetohydroxamic acid on ACE (0.031−0.49 mg/mL corresponding to 0.4098−6.577 mM, respectively). Parts B, C, and D of **Figure 3** show, respectively, the effects of pectin hydroxamic acid (DE94T4 and DE94T18, 0.4098−3.2784 mg/mL) and pectin (DE94, 0.8197−3.2784 mg/mL), of pectin

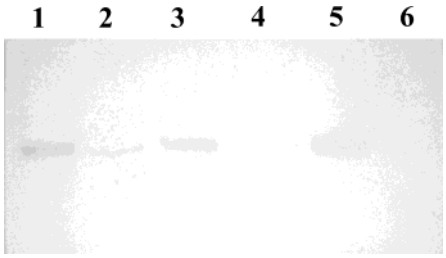

Figure 2. Effects of PHA on SSAO activity staining: (lane 1) control; (lane 2) semicarbazide added (400 μM); (lane 3) pectin (DE25) added; (lane 4) PHA (DE25T4) added; (lane 5) pectin (DE65) added; (lane 6) PHA (DE65T4) added. Bovine plasma (40 μL) was premixed with compounds mentioned above overnight and then assayed with SSAO (31.7 units/mL) activity staining on 7.5% polyacrylamide gels.

hydroxamic acid (DE65T4, 0.8137−3.2784 mg/mL) and pectin (DE65, 0.4098−1.6392 mg/mL), and of pectin hydroxamic acid (DE25T4, 0.8197−3.2784 mg/mL) and pectin (DE25, 0.4098−1.6329 mg/mL) on the activity of 20 milliunits of ACE from rabbit lung. It was found that different DE pectins showed negligible ACE inhibitions (**Figure 3B−D**). However, PHAs of DE94T4 and DE94T18 showed dose-dependent ACE inhibitory activities (**Figure 3B**). The order of ACE inhibition was DE94T4 > DE94T18 ≫ DE65T4 > DE25T4. Both pectin and PHAs were macromolecules; however, only PHAs showed dose-dependent ACE inhibitory activities. Besides the hydroxamic acid groups in galacturonic acid moiety, other factors that might be responsible for the observed inhibitory activities against ACE should be further investigated.

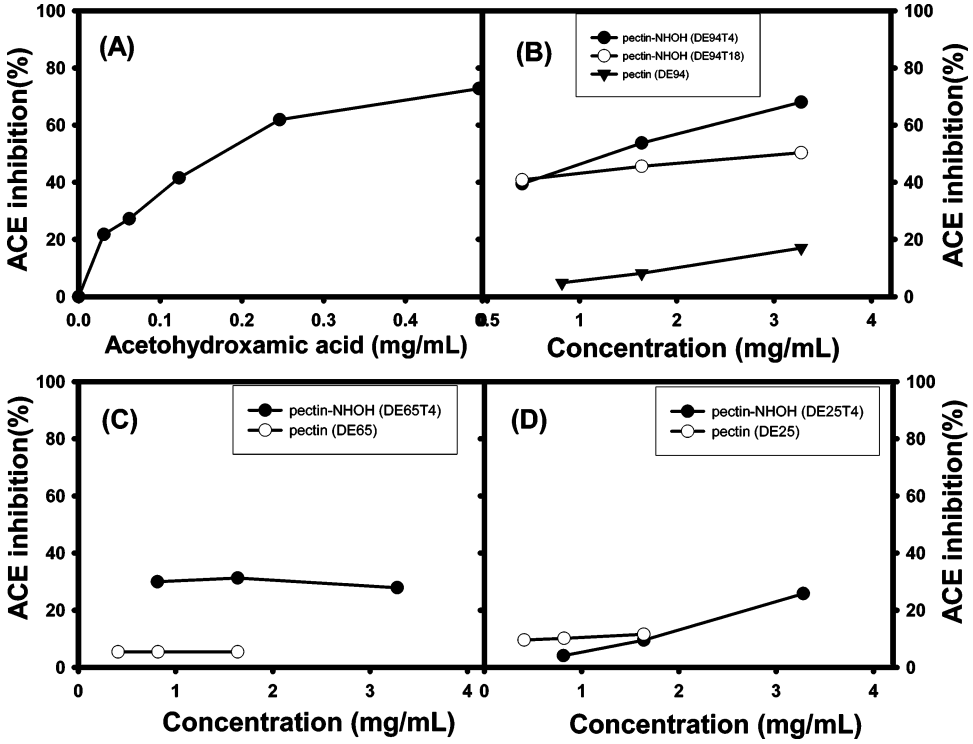

Figure 3. Effects of (A) acetohydroxamic acid [0.031 mg/mL (0.4098 mM) to 0.49 mg/mL (6.577 mM)]; (B) PHA (DE94T4 and DE94T18, 0.4098–3.2784 mg/mL) and pectin (DE94, 0.8197–3.2784 mg/mL); (C) PHA (DE65T4, 0.8137–3.2784 mg/mL) and pectin (DE65, 0.4098–1.6392 mg/mL); and (D) PHA (DE25T4, 0.8197–3.2784 mg/mL) and pectin (DE25, 0.4098–1.6329 mg/mL) on 20 milliunits of ACE activity from rabbit lung.

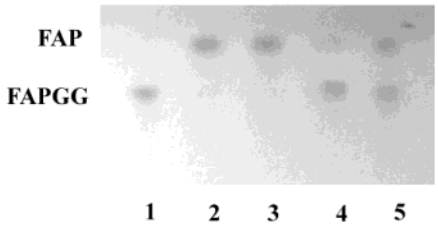

Figure 4. TLC chromatograms of a silica gel 60 F_{254} showing the effects of 2 mg of pectin (DE94, lane 3) or PHA (DE94T4, lane 4; DE94T18, lane 5) on 20 milliunits of ACE: (lane 1) blank test (FAPGG only); (lane 2) control test (ACE hydrolyzed FAPGG to produce FAP). Each solution was dried under reduced 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 on TLC developed by solvents butanol/acetic acid/water, 4:1:1 (v/v/v).

Determination of ACE inhibitory activity of PHAs on TLC. The inhibitory effects of PHAs on ACE activity are shown on TLC chromatogram of a silica gel 60 F_{254} in **Figure 4**. Compared with the blank (lane 1) and control (lane 2) tests, it was found that DE94 pectin (lane 3) did not inhibit ACE; however, DE94T4 (lane 4) and DE94T18 (lane 5) showed ACE inhibitory activities because most FAPGG was not hydrolyzed and fewer FAP were produced. This confirmed that PHAs exhibited ACE inhibitor activities.

The PHAs exhibited different inhibitory orders against SSAO and ACE. From the literature, PHAs were reported to have metal chelating activities (*36*). Because both SSAO and ACE were metalloproteins, the different inhibitory orders of PHAs on SSAO and ACE might reflect the different metal chelating capacities of the effectors. Acetohydroxamic acid exhibited an ACEI activity similar to those of carnosine, homocarnosine, and anserine (*16*). The pectins have been reported to have different biological activities in addition to those for the food industry of jams and jellies (*26*), such as on the interactions between fibroblast growth factors and receptors (*27*), on the modulation of lung colonization of B16-F1 melanoma cell (*28*), and on the inhibition of human cancer cell growth and metastasis in nude mice (*29*). Our results, showing the modified pectins as a new source of inhibitors for SSAO and ACE, suggest possible benefits of the modified pectins to general public health, which is interesting and deserving of further investigations.

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