Antioxidant and semicarbazide-sensitive amine oxidase inhibitory activities of alginic acid hydroxamates

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Abstract: The commercial polysaccharides of alginic acid (medium (3500 cps, 2% solution) and low (250 cps, 2% solution) viscosities) were esterified with acidic methanol (1 mmol L^{-1} HCl) at 4 °C with gentle stirring for 5 days to obtain methyl esters of medium-viscosity alginic acid (ME-MVA) and low-viscosity alginic acid (ME-LVA). These ME-MVA and ME-LVA were reacted with alkaline hydroxylamine to obtain medium-viscosity alginic acid hydroxamates (MVA-NHOH) and LVA-NHOH. The percentages of hydroxamic acid content in MVA-NHOH and LVA-NHOH were calculated as 25% and 20%, respectively. The hydroxamate derivatives of alginic acid were used to test the antioxidant and semicarbazide-sensitive amine oxidase (SSAO) inhibitory activities in comparison with original materials (MVA and LVA). The half-inhibition concentrations, IC_{50}, of scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) were 24.5 and 29.8 μg mL^{-1} for MVA-NHOH and LVA-NHOH, respectively. However, few scavenging activities of the MVA and LVA were found at the same concentrations. The IC_{50} of the positive control of butylated hydroxytoluene was 5 μg mL^{-1}. The scavenging activity of DPPH radical was pH-dependent, and the optimal pH for both of MVA-NHOH and LVA-NHOH was the Tris-HCl buffer (pH 7.9). Using electron spin resonance (ESR) to detect the activity of scavenging hydroxyl radicals, both alginic acid hydroxamates showed dose-dependent scavenging activities, and the IC_{50} was 90 and 92 μg mL^{-1}, respectively, for MVA-NHOH and LVA-NHOH. Both alginic acid hydroxamates also exhibited protection against hydroxyl radical-mediated DNA damage. Both MVA-NHOH and LVA-NHOH showed dose-dependent inhibitory activities against bovine SSAO (2.53 units); the IC_{50} was 0.16 and 0.09 μg mL^{-1}, respectively, for MVA-NHOH and LVA-NHOH, compared with 3.81 μg mL^{-1} of semicarbazide (positive controls). Amine oxidase activity staining also revealed that both MVA-NHOH and LVA-NHOH exhibited SSAO inhibitory activities. Both MVA-NHOH and LVA-NHOH showed mixed non-competitive inhibition against bovine SSAO. It was found that the V'_{max} value was reduced and the K'_m value was either increased (added MVA-NHOH, 0.05 μg mL^{-1}) or reduced (added LVA-NHOH, 0.11 μg mL^{-1}) in the presence of alginic acid hydroxamate.

Keywords: alginic acid hydroxamate; antioxidant activity; electron spin resonance (ESR); semicarbazide-sensitive amine oxidase (SSAO)

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 the antioxidant activities of natural compounds in fruit and vegetables, such as anthocyanin,[7] the storage proteins of sweet potato root,[8,9] yam tuber,[10] yam mucilage[11] and potato tuber.[12]

The semicarbazide-sensitive amine oxidase (SSAO, EC 1.4.3.6) contains a cofactor possessing one or more topaquinones, which is a common name for a group of metalloproteins widely distributed in nature, including plants, microorganisms and mammalian organs (vasculature, dental pulp, eye and plasma).[13] SSAO converts primary amines into the corresponding aldehydes, generating hydrogen peroxide and ammonia. In recent research, it was found that plasma SSAO was raised in diabetes mellitus and heart failure and is implicated in atherosclerosis, endothelial damage[14–18] and glucose transport into adipocytes.[19,20]

Alginic acids, extracted from brown seaweeds or Phaeophyceae, are unbranched high-molecular polymers containing two types of uronic acid residues of β-(1 → 4)-linked D-mannuronic acid and α-(1 → 4)-linked L-guluronic acid. Its derivatives have wide applications in various industries.[21–24] We recently reported that monohydroxamates of aspartic acid and glutamic acid exhibit antioxidant and angiotensin-converting enzyme inhibitory activities,[25] and the pectin hydroxamic acids exhibited both semicarbazide-sensitive amine oxidase and ACE

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inhibitory activities,[26] and antioxidant activities.[27] In this report, the commercial polysaccharides of alginic acid with different viscosities, medium (3500 cps, 2% solution) and low (250 cps, 2% solution), were first esterified with acidic methanol (1 mmol L^{-1} HCl) at 4 °C to produce methyl esters of medium-viscosity alginate (ME-MVA) and low-viscosity alginate (ME-LVA). Both esters were reacted with alkaline hydroxylamine to produce medium-viscosity alginic acid hydroxamates (MVA-NHOH) and low-viscosity alginic acid hydroxamates (LVA-NHOH). These self-prepared MVA-NHOH and LVA-NHOH were used to test the antioxidant and semicarbazide-sensitive amine oxidase (SSAO) inhibitory activities in comparison with starting materials (MVA and LVA). The present result showed that both MVA-NHOH and LVA-NHOH exhibited antioxidant, antiradical activities and SSAO inhibitory activities.

MATERIALS AND METHODS

Materials

Alginic acid (low viscosity, A-2158, LVA; medium viscosity, A-2033, MVA), alginate lyase (from *Flavobacterium* spp., A-1603), benzylamine, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, ABTS), bovine plasma (P-4639, reconstituted with 10 mL deionized water), butylated hydroxytoluene (BHT), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-amino-9-ethylcarbazole (AEC), ferrous sulfate, horseradish peroxidase (148 units mg^{-1} solid) and semicarbazide were purchased from Sigma Chemical Co. (St Louis, MO, USA). Hydrogen peroxide (33%) was from Wako Pure Chemical Industry (Osaka, Japan). Calf thymus DNA (activated, 25 A260 units mL^{-1}) was purchased from Amersham Biosciences (Uppsala, Sweden). Other chemicals and reagents were from Sigma Chemical Co. (St Louis, MO, USA).

Preparation of alginic acid hydroxamates (LVA-NHOH and MVA-NHOH)

Each of the commercial alginic acids (from *Macrocystis pyrifera*) of medium (3500 cps, 2% solution) and low (250 cps, 2% solution) viscosity were suspended in acidic methanol (1 mmol L^{-1} HCl) at 4 °C with gentle shaking for 5 days.[28] After filtration by a G3 glass filter, each sample was washed with 70% methanol until negative chloride ion was detected with silver nitrate. After rinsing with 100% methanol, both ME-LVA and ME-MVA were dried at 37 °C for further use of hydroxamic acid derivatives. 8 g each of ME-LVA and ME-MVA suspended in 500 mL methanol were stirred at room temperature for 20 h with a mixed solution (insoluble salt was removed by filtration) containing 13 g of potassium hydroxide in 50 mL methanol and 12 g of hydroxylamine-HCl in 150 mL methanol[26,27,29] to obtain LVA-NHOH and MVA-NHOH. After filtration by a G3 glass filter, each sample was washed with 70% methanol and readjusted to neutral pH value. After rinsing with 100% methanol, the LVA-NHOH and MVA-NHOH were dried at 37 °C for biological activity assays. The changes in molecular size after alkaline hydroxylamine reaction were monitored by Sephacryl S-200 (1.0 cm × 100 cm, fractionation range of polysaccharide 1–80 kDa) gel filtration using 100 mmol L^{-1} Tris-HCl (pH 7.9) as eluting buffers. 0.8 mL each of 2% LVA, MVA, LVA-NHOH and MVA-NHOH were loaded on to a Sephacryl S-200 column and 2 mL was collected for each tube and assayed for total sugars,[30] and the dextran (50 kDa, fraction 22; 12 kDa, fraction 26, and 5 kDa, fraction 31) was used for molecular size standards. The void volume was determined at fraction 15 using 270 kDa dextran as a standard. Viscosity was measured using a Rheostress 1 double-cone viscometer (HAAKE Mess-Technik, Karlsruhe, Germany), with a cone angle of 1° at 298 K. The viscosities of each 2% solution (LVA, LVA-NHOH, MVA and MVA-NHOH) at different shear rates (from 800 to 1 s^{-1}) were continuously measured by computer-controlled testing programs.

Determination of hydroxamic acid percentage in LVA-NHOH and MVA-NHOH

500 μL each of 1% MVA-NHOH and LVA-NHOH was hydrolyzed with 100 μl of alginate lyase (50 mg mL^{-1} in 500 mmol L^{-1} Tris-HCl buffer, pH 6.8) at 37 °C for 24, 30 and 48 h. The sugar contents in each hydrolysate of MVA-NHOH and LVA-NHOH were determined by phenol-sulfuric acid,[30] and galacturonic acid (GA) was used to plot the standard curve. Sugar contents of hydrolysates in MVA-NHOH and LVA-NHOH were expressed as μmol.equivalent GA. Hydroxamic acid contents were determined by acidic ferric chloride solution,[31] with some modifications as follows. Each 0.2 mL of MVA-NHOH and LVA-NHOH hydrolysates was mixed with 0.3 mL of 4 mmol L^{-1} HCl and 0.5 mL of 10% ferric chloride in 0.1 mmol L^{-1} HCl. Absorbance at 540 nm was determined after 10 min standing, and the acetohydroxamic acid was used to plot the standard curve. Hydroxamic acid contents of hydrolysates in MVA-NHOH and LVA-NHOH were expressed as μmol.equivalent acetohydroxamic acid. The hydroxamic acid content was divided by sugar contents to give the hydroxamic acid percentage in LVA-NHOH and MVA-NHOH.

Scavenging activity of alginic acid hydroxamates against DPPH radicals

0.3 mL each of LVA, MVA, LVA-NHOH and MVA-NHOH (final concentrations 0.0125, 0.025, 0.05, 0.1 and 0.2 mg mL^{-1}) was added to 0.1 mL of 1 mol L^{-1} Tris-HCl buffer (pH 7.9), and then mixed with 0.6 mL of 100 μmol L^{-1} DPPH in methanol to final concentrations of 60 μmol L^{-1} for 20 min under light protection at room temperature.[11,12,25,27] The decrease of absorbance at 517 nm was measured and expressed as ΔA517 nm. Deionized water was used as a blank experiment. BHT (0.001, 0.00125,

0.0025, 0.005, 0.01 and 0.0125 mg mL^{-1}) was used as a positive control. Means of triplicates were measured. The scavenging activity of DPPH radicals (%) was calculated from the equation ($\Delta A517_{blank} - \Delta A517_{sample}$) ÷ $\Delta A517_{blank} \times 100\%$. IC_{50} stands for the concentration of 50% scavenging activity.

Optimal pH of alginic acid hydroxamates for scavenging DPPH radical

0.05 mg mL^{-1} each of LVA-NHOH and MVA-NHOH was used to investigate the optimal pH for DPPH scavenging activities. 0.1 mL each of 1 mol L^{-1} buffer containing KCl-HCl buffer (pH 2.0, 2.5, and 3.0), acetate buffer (pH 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5), phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, and 8.0) and Tris-HCl buffer (pH 7.0, 7.5, 8.0, 8.5, and 9.0) were used for comparison. The scavenging activity of DPPH radicals (%) was calculated from the equation ($\Delta A517_{blank} - \Delta A517_{sample}$) ÷ $\Delta A517_{blank} \times 100\%$.

Scavenging activity of hydroxyl radicals by ESR spectrometry

The hydroxyl radical was generated by Fenton reaction according to the method of Kohno *et al*.[32] The total 500 µl mixture included 0.0625, 0.0925, 0.125, and 0.5 mg mL^{-1} each of LVA-NHOH and MVA-NHOH, 5 mmol L^{-1} DMPO and 0.05 mmol L^{-1} ferrous sulfate. After mixing, the solution was transferred to an ESR quartz cell and placed in the cavity of the ESR spectrometer; hydrogen peroxide was then added to a final concentration of 0.25 mmol L^{-1}. Deionized water was used instead of sample solution for blank experiments. After 40 s, the intensity of the signal of DMPO-OH spin-adduct ($ISA_{DMPO-OH}$) was measured. All ESR spectra were recorded at the ambient temperature (298 K) on a Bruker EMX-6/1 ESR spectrometer equipped with WIN-ESR SimFonia software version 1.2. The conditions of ESR spectrometry were as follows: center field 345.4 ± 5.0 mT; microwave power 8 mW (9.416 GHz); modulation amplitude 5 G; modulation frequency 100 kHz; time constant 0.6 s; scan time 1.5 min. The intensities of DMPO-OH spin signal in ESR spectrometry were used to evaluate the scavenging activity against hydroxyl radical. The scavenging activity of hydroxyl radicals (%) was calculated from the equation ($ISA_{blank,\ DMPO-OH} - ISA_{sample,\ DMPO-OH}$) ÷ $ISA_{blank,\ DMPO-OH} \times 100\%$. IC_{50} stands for the concentration of 50% scavenging activity.

Protecting hydroxyl radical-induced calf thymus DNA damage by LVA-NHOH and MVA-NHOH

The hydroxyl radical was generated by Fenton reaction according to the method of Kohno *et al*.[32] The 45 µL reaction mixture included LVA-NHOH and MVA-NHOH (final concentrations 0.033, 0.164, 1.316 and 2.632 mg mL^{-1}), 15 µL of calf thymus DNA, 18 mmol L^{-1} $FeSO_4$ and 60 mmol L^{-1} hydrogen peroxide at room temperature for 15 min. 10 µl of 1 mmol L^{-1} EDTA was added to stop the reaction. Only calf thymus DNA was used for the blank test, and the control test was without LVA-NHOH or MVA-NHOH additions. After agarose gel electrophoresis, the treated DNA solutions were stained with ethidium bromide and observed under UV light.

SSAO inhibitory activities of LVA-NHOH and MVA-NHOH

SSAO inhibitory activity was determined by the spectrophotometric method according to Szutowicz *et al*.[33] with some modifications. The total 200 µL reaction solution (containing 50 µL of 200 mmol L^{-1} phosphate buffer, pH7.4, 50 µL of 8 mmol L^{-1} benzylamine, bovine plasma (containing 2.53 units SSAO) and different amounts of LVA-NHOH (0.0313, 0.0625, 0.1094, 0.1563 and 0.2031 µg mL^{-1}), MVA-NHOH (0.1094, 0.1563, 0.2031 and 0.3125 µg mL^{-1}), semicarbazide, LVA and MVA (0.697, 1.394, 2.788 and 5.576 µg mL^{-1})) was kept at 37 °C for 1 h and then heated at 100 °C to stop the reaction. After cooling and brief centrifugation, 90 µL of reaction solution was isolated and added to 710 µL of solution containing 200 µL of 200 mmol L^{-1} phosphate buffer (pH 7.4), 100 µL of 2 mmol L^{-1} ABTS solution, and 25 µL of horseradish peroxidase (10 µg mL^{-1}). Changes of absorbance at 420 nm were recorded during 1 min and expressed as $\Delta A420\ nm\ min^{-1}$. Means of triplicates were measured. Deionized water was used as a blank experiment. The SSAO inhibition (%) was calculated from the equation ($\Delta A420\ nm\ min^{-1}{}_{blank} - \Delta A420\ nm\ min^{-1}{}_{sample}$) ÷ $\Delta A420\ nm\ min^{-1}{}_{blank} \times 100\%$. IC_{50} stands for the concentration of 50% inhibitions.

Plasma SSAO activity stains on 10% native polyacrylamide gels

SSAO activity staining on a 10% polyacrylamide gel was according to the method of Lee *et al*.[34] Bovine plasma was pre-mixed with clorgyline (1 mmol L^{-1}, MAO-A inhibitor), deprenyl (1 mmol L^{-1}, MAO-B inhibitor), semicarbazide (1 mmol L^{-1}, SSAO inhibitor), LVA-NHOH (80 and 125 µg), or MVA-NHOH (80 and 125 µg) overnight and prepared for electrophoresis. When native PAGE was completed, the gels were balanced for 20 min twice in 50 mmol L^{-1} Tris-HCl buffer (pH 7.9) before activity staining. The process of plasma SSAO activity staining was as below. 20 mg of benzylamine and 10 mg of AEC were dissolved in 10 mL of dimethylformamide and then added to 45 ml of 50 mmol L^{-1} Tris-HCl buffer (pH 7.9) as the substrate solution, in which the gel was submerged and shaken for 5 min. Then, 200 µL of horseradish peroxidase (5 mg mL^{-1}) was added. The gel was shaken gently at room temperature, destained with 10% acetic acid and then washed with distilled water.

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Determination of the kinetic constant of bovine SSAO in the presence of LVA-NHOH and MVA-NHOH

The K_m and K'_m of bovine SSAO (2.53 units) in the absence and presence of LVA-NHOH (0.11 μg mL^{-1}) and MVA-NHOH (0.05 μg mL^{-1}), respectively, was calculated from Lineweaver–Burk plots using different concentrations of benzylamine as substrates (0.67, 0.8, 1, 1.33, 2 and 4 mmol L^{-1}).

RESULTS AND DISCUSSION

Properties of alginic acid hydroxamates

A variety of hydroxamic acid derivatives have been reported to have biological activities toward cancer, cardiovascular diseases, Alzheimer's disease and tuberculosis.[35] In our previous report,[26,27] pectin with different methyl esters was reacted with alkaline hydroxylamine[29] to produce different pectin hydroxamate derivatives. The alginic acid was composed of two uronic acid residues, and pectin was composed of galacturonic acid with different methyl esters. Therefore, alginic acid was first esterified with acidic methanol to obtain methyl esters of alginic acid (ME-LVA and ME-MVA). Both ME-LVA and ME-MVA were then reacted with alkaline hydroxylamine to obtain LVA-NHOH and MVA-NHOH. Figure 1 shows the molecular size (A, B) and viscosity (C, D) of hydroxamate derivatives of alginic acid and their starting materials. From the results of Fig. 1(A) and (B) it was found that broader molecular size distributions from 1 to 80 kDa (available fractionation ranges) were found after alkaline hydroxylamine reaction. The MVA was located at the void volume (fraction 15) and the LVA was located (fraction 16) close to the void volume; however, LVA-NHOH and MVA-NHOH exhibited a smaller molecular size than the original materials. β-Elimination might be occurring[28] during alkaline hydroxylamine reaction and resulting in polymer breakdown, producing a smaller molecule. Figure 1(C) and (D) showed the viscosity changes at different shear rates. It was clear that at any shear rate the level of appearance viscosity of MVA was higher than that of LVA. This meant that the molecular size of MVA was higher than that of LVA. It was also found that at any shear rate the levels of appearance viscosity of MVA-NHOH and LVA-NHOH were much lower than those of MVA and LVA, respectively. The 2% LVA-NHOH was kept around 6 cps during 10–800 shear rate; 2% MVA-NHOH was around 15–8 cps during 10–800 shear rate. Both LVA-NHOH and MVA-NHOH produced reddish precipitates with acidic ferric chloride solution, which was the main evidence for hydroxamic acid–ferric complex;[31] however, the LVA and MVA remained yellowish with acidic ferric chloride solution (data not shown). The alginic acid hydroxamate was further hydrolyzed by alginate lyase (from *Flavobacterium* spp.) at different time intervals for the calculation of hydroxamic acid percentage in LVA-NHOH and MVA-NHOH. The hydroxamate contents in MVA-NHOH were increased from 0.351, 0.458 to 0.463 μmol.equivalent galacturonic acid, respectively, for 24, 30 and 48 h hydrolysis. The percentage of hydroxamic acid content in alginic acid derivatives was calculated as follows. The hydroxamic acid content was divided by sugar contents to make 20% and 25%, respectively, for LVA-NHOH and MVA-NHOH.

Scavenging activity of LVA-NHOH and MVA-NHOH against DPPH radicals

Figure 2 shows the results of scavenging activity of LVA-NHOH and MVA-NHOH against DPPH

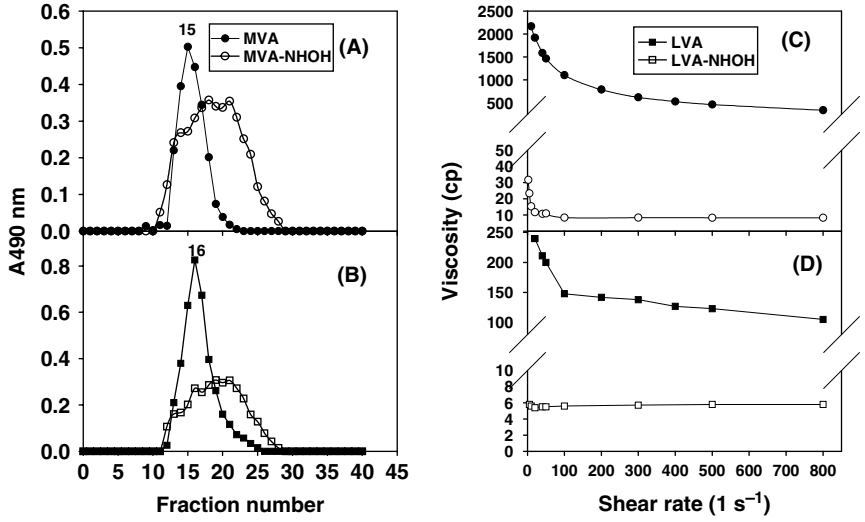

Figure 1. Molecular size (A, B) and viscosity at different shear rate (C, D) of hydroxamate derivatives of alginic acid and their starting materials. 0.8 mL each of 2% MVA and MVA-NHOH (A, C) and LVA and LVA-NHOH (B, D) were loaded on to a Sephacryl S-200 column, 2 mL was collected for each tube, and the dextran (50 kDa, fraction 22; 12 kDa, fraction 26; and 5 kDa, fraction 31) was used for molecular size standards. The void volume was determined at fraction 15 using 270 kDa dextran standard. Viscosity was measured using a Rheostress 1 double-cone viscometer (HAAKE Mess-Technik, Karlsruhe, Germany), with a cone angle of 1° at 298 K. The viscosities of each 2% solution (LVA, LVA-NHOH, MVA, and MVA-NHOH) at different shear rates (from 800 to 1 s^{-1}) were continuously measured by computer-controlled testing programs.

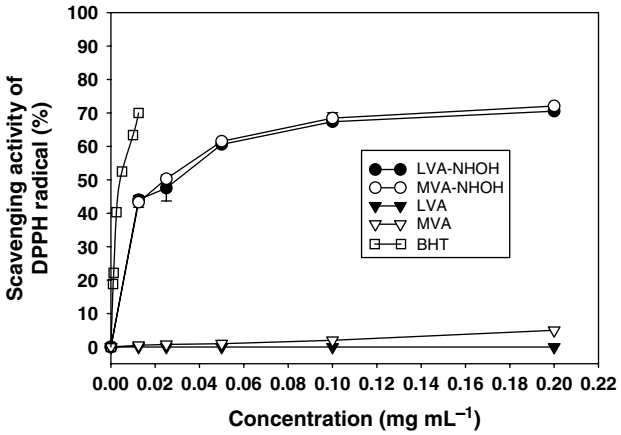

Figure 2. Effects of different concentrations of alginic acid (low viscosity, LVA; medium viscosity, MVA) and corresponding hydroxamate derivatives (LVA-NHOH and MVA-NHOH) on the scavenging activity of DPPH radicals using spectrophotometry.

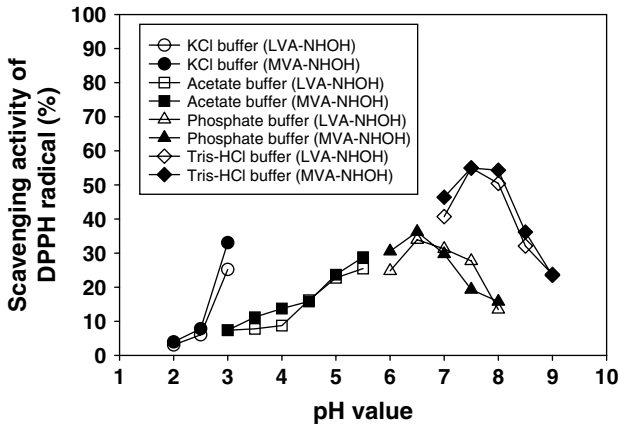

Figure 3. Optimal pH for DPPH scavenging activities of alginic acid hydroxamates (LVA-NHOH and MVA-NHOH) in different buffer systems: KCl buffer (pH 2.0, 2.5 and 3.0), acetate buffer (pH 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5), phosphate buffer (pH 6.0, 6.5, 7.0, 7.5 and 8.0) and Tris-HCl buffer (pH 7.0, 7.5, 8.0, 8.5 and 9.0).

radicals. DPPH radicals were widely used in the model system to investigate the scavenging activities of several natural compounds. Colors change from purple to yellow, and absorbance at wavelength 517 nm decreases as the result of forming DPPH-H through donation of hydrogen by antioxidants. Both LVA-NHOH and MVA-NHOH showed dose-dependent DPPH radical scavenging activities. The IC_{50} of scavenging activity against DPPH was 0.0298 and 0.0245 mg mL^{-1} for LVA-NHOH and MVA-NHOH, respectively. Leaf polysaccharides of hsian-tsao[36] were reported to exhibit anti-DPPH radical activity, and the IC_{50} was 0.86 mg mL^{-1}, which was much higher than that of LVA-NHOH or MVA-NHOH. No or little DPPH scavenging activity of LVA or MVA (original material) was found under the same concentrations. The IC_{50} of the positive control of BHT was 5 μg mL^{-1}. The anti-DPPH radical capacities of LVA-NHOH and MVA-NHOH were about 1/6 and 1/5 that of BHT. The prepared LVA-NHOH and MVA-NHOH in this research had much lower IC_{50} than those of the pectin hydroxamic acids reported, and efficiencies of DPPH scavenging activity of alginic acid hydroxamates were about 20-fold higher than those of pectin hydroxamates.[27]

Optimal pH for scavenging DPPH radical

Yale[37] reported that in aqueous solution the monohydroxamic acids behaved as weak acids. The dissociation of hydroxamic acid moiety in alginic acid derivatives might influence DPPH scavenging activity. Therefore, both LVA-NHOH and MVA-NHOH (0.025 mg mL^{-1}) were used for optimal pH of DPPH scavenging assay (Fig. 3). LVA-NHOH and MVA-NHOH had similar pH-dependent scavenging behavior against DPPH radicals. The optimal pH for LVA-NHOH and MVA-NHOH was with Tris-HCl buffer of pH 7.5 or 7.9.

Scavenging activity of hydroxyl radicals by ESR spectrometry

The hydroxyl radical was generated by Fenton reaction according to the method of Kohno *et al*.[32] and was trapped by DMPO to form DMPO-OH adducts. The intensities of DMPO-OH spin signal in ESR spectrometry were used to evaluate the scavenging activity of LVA-NHOH (Fig. 4A) and MVA-NHOH (Fig. 4B) against hydroxyl radicals. From the results of DMPO-OH intensities in Fig. 4(A), LVA-NHOH showed dose-dependent anti-hydroxyl radical activities. There were 14.77%, 50.69%, 57.65% and 86.68% scavenging activities, respectively, for 0.0625, 0.0925, 0.125 and 0.5 mg mL^{-1} of LVA-NHOH. The IC_{50} of LVA-NHOH for scavenging activity against hydroxyl radical was 0.092 mg mL^{-1}. MVA-NHOH also showed dose-dependent anti-hydroxyl radical activities (Fig. 4B). There were 13.92%, 53.10%, 69.89% and 89.25% scavenging activities, respectively, for 0.0625, 0.0925, 0.125, and 0.5 mg mL^{-1} of MVA-NHOH. The IC_{50} of MVA-NHOH for scavenging activity against hydroxyl radical was 0.09 mg mL^{-1}.

From above results, both MVA-NHOH and LVA-NHOH showed anti-radical or antioxidant activities (Figs 2 and 4). The resonance properties of hydroxamic acid moieties (R-CONHOH)[37] might explain the antioxidant activities. MVA-NHOH (25%) had higher amounts of hydroxamic acid moiety than LVA-NHOH (20%), which might be explained by the lower IC_{50} of MVA-NHOH in the radical-scavenging activities.

Protecting hydroxyl radical-induced calf thymus DNA damage by hydroxamates of alginic acid

Free radicals could damage macromolecules in cells, such as DNA, protein and lipids in membranes.[38] Fig. 5 shows LVA-NHOH (upper panel) and MVA-NHOH (lower panel) against hydroxyl radical-induced calf thymus DNA damage. Only calf thymus DNA was used for the blank test, and the control

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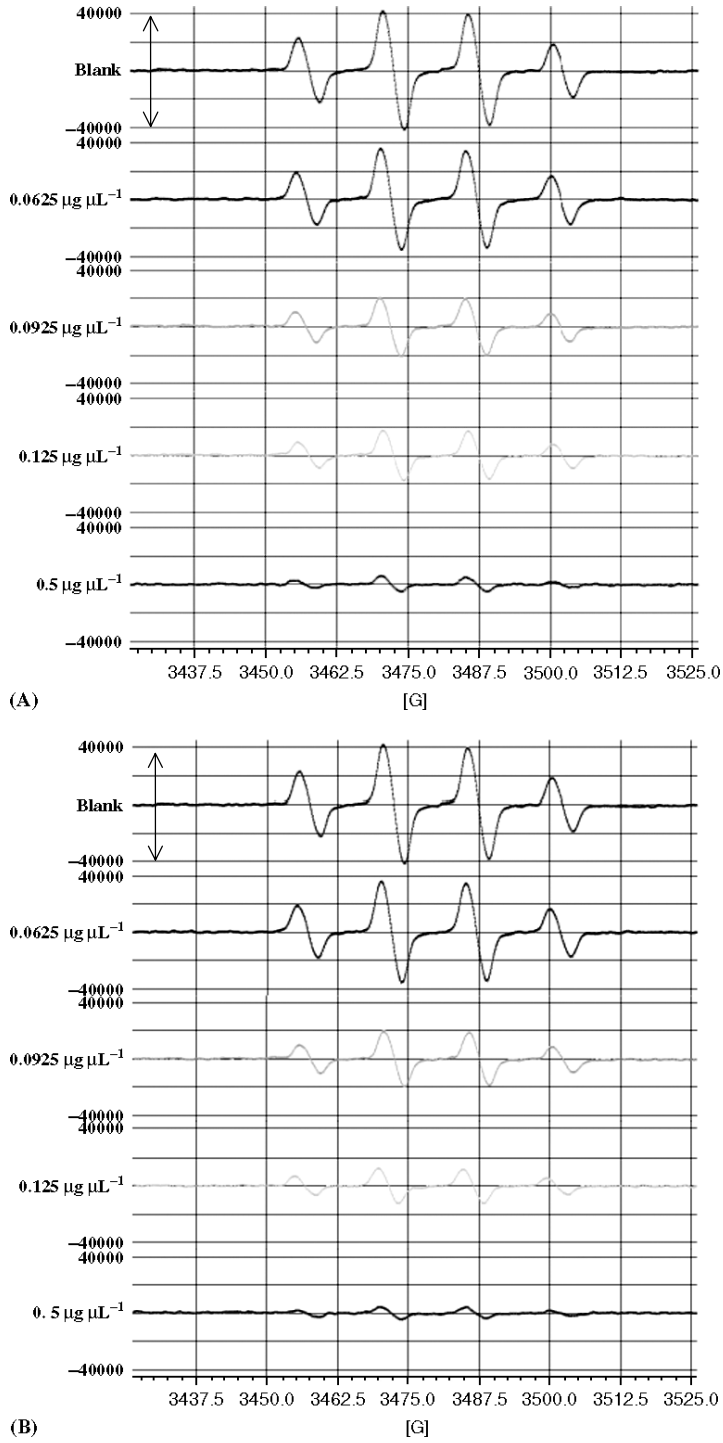

Figure 4. Scavenging activity of (A) low-viscosity alginic acid hydroxamate (LVA-NHOH) and (B) medium-viscosity alginic acid hydroxamate (MVA-NHOH) against hydroxyl radicals measured by electron spin resonance spectrometry. The total 500 L mixture included 0.0625, 0.0925, 0.125 and 0.5 mg mL^{-1} each of LVA-NHOH and MVA-NHOH, and deionized water was used instead of sample solution for blank experiments. All ESR spectra were recorded at ambient temperature (298 K) on a Bruker EMX-6/1 ESR spectrometer equipped with WIN-ESR SimFonia software version 1.2. ESR spectrometry conditions were as follows: center field 345.4 ± 5.0 mT; microwave power 8 mW (9.416 GHz); modulation amplitude 5 G; modulation frequency 100 kHz; time constant 0.6 s; scan time 1.5 min.

test was without added LVA-NHOH (upper panel, Fig. 5) or MVA-NHOH (lower panel, Fig. 6). Lanes 1–4 show 0.033, 0.164, 1.316, and 2.632 mg mL^{-1} of LVA-NHOH (upper panel) or MVA-NHOH (lower panel) added, respectively. Compared with blank and control tests, it was found that the hydroxamates of alginic acid above 2.632 mg mL^{-1} (lane 4, both panels, Fig. 5) could protect against hydroxyl radical-induced calf thymus DNA damage during 15 min reactions.

SSAO Inhibitory activity of LVA-NHOH and MVA-NHOH

Hydroxamate derivatives have been reported to have metal-chelating activity.[25,26,38] In recent research, plasma SSAO was raised in diabetes mellitus and heart

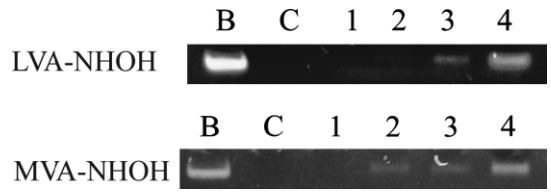

Figure 5. Effects of alginic acid hydroxamates (upper panel: LVA-NHOH; lower panel: MVA-NHOH) on protection of hydroxyl radical-induced calf thymus DNA damage after 15 min reaction. After agarose gel electrophoresis, the treated DNA solutions were stained with ethidium bromide and observed under UV light. Only calf thymus DNA was used for the blank test (B), and the control test (C) was without LVA-NHOH or MVA-NHOH addition. Lanes 1–4 show 0.033, 0.164, 1.316, and 2.632 mg mL^{-1}, respectively.

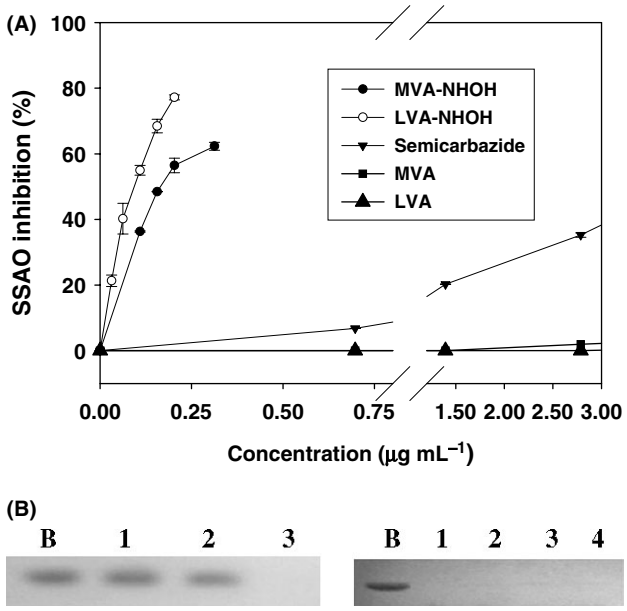

Figure 6. The effects of alginic acid hydroxamates on bovine SSAO. (A) Each of LVA-NHOH (0.0313, 0.0625, 0.1094, 0.1563 and 0.2031 μg mL^{-1}), MVA-NHOH (0.1094, 0.1563, 0.2031 and 0.3125 μg mL^{-1}), semicarbazide, LVA and MVA (0.697, 1.394, 2.788 and 5.576 μg mL^{-1}) was reacted with 2.53 units of bovine SSAO at 37 °C for 1 h. SSAO inhibition (%) was calculated from the equation (ΔA420 nm min$^{-1}{}_{\text{blank}} - \Delta$A420 nm min$^{-1}{}_{\text{sample}}$) ÷ ΔA420 nm min$^{-1}{}_{\text{blank}} \times 100\%$. (B) Bovine plasma was pre-mixed with clorgyline (panel A, lane 1, 1 mmol L^{-1}), deprenyl (left: lane 2, 1 mmol L^{-1}) and semicarbazide (left: lane 3, 1 mmol L^{-1}), LVA-NHOH (right: lanes 1 and 3, 80 and 125 μg) or MVA-NHOH (right: lanes 2 and 4, 80 and 125 μg) overnight and prepared for electrophoresis and then stained for SSAO.

failure and is implicated in atherosclerosis, endothelial damage and glucose transport into adipocytes.[14–18] Therefore, hydroxamate derivatives of alginic acid were used to evaluate SSAO inhibitory activities (Fig. 6). From the results of Fig. 6(A), both MVA-NHOH and LVA-NHOH showed dose-dependent inhibitory activities against bovine SSAO (2.53 units). Semicarbazide showed 6.78%, 20.26%, 35.24% and 75.30% inhibitions, respectively, for 0.697, 1.394, 2.788, and 5.576 μg mL^{-1}. The IC_{50} was 0.16 and 0.09 μg mL^{-1}, respectively, for MVA-NHOH and LVA-NHOH in comparison with 3.81 μg mL^{-1} of semicarbazide (positive controls). No SSAO inhibitory activity of LVA and MVA was found. Figure 6B (left) shows that bovine serum AO was inhibited by semicarbazide (lane 3), but not by clorgyline (lane 1) or deprenyl (lane 2), and it was clear that bovine serum contained SSAO. Figure 6B (right) shows the effects of LVA-NHOH (lanes 1 and 3: 80 and 125 μg) and MVA-NHOH (lanes 2 and 4: 80 and 125 μg) on bovine SSAO. Compared to blank, it was found that both LVA-NHOH and MVA-NHOH could inhibit SSAO activity on 10% PAGE gels.

Determination of the kinetic constant of bovine SSAO in the presence of LVA-NHOH and MVA-NHOH

The K_m and K'_m of bovine SSAO (2.53 units) in the absence and presence of MVA-NHOH (0.05 μg mL^{-1}, Fig. 7A) and LVA-NHOH (0.11 μg mL^{-1}, Fig. 7B), respectively, was calculated from Lineweaver–Burk plots using different concentrations of benzylamine as substrate (0.67, 0.8, 1, 1.33, 2, and 4 mmol L^{-1}). The results indicated that MVA-NHOH or LVA-NHOH

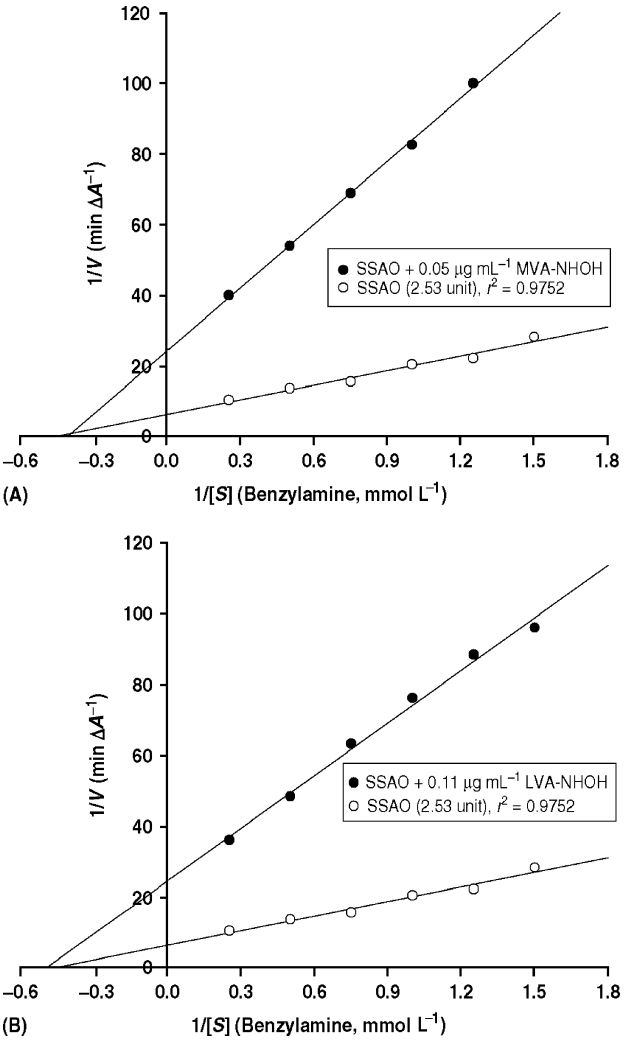

Figure 7. Kinetic properties of bovine SSAO (2.53 units) in the absence and presence of (A) MVA-NHOH (0.05 μg mL^{-1}) and (B) LVA-NHOH (0.11 μg mL^{-1}) in Lineweaver–Burk plots using various concentrations of benzylamine as substrate (0.67, 0.8, 1, 1.33, 2 and 4 mmol L^{-1}).

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acted as a mixed noncompetitive inhibitor toward bovine SSAO with respect to benzylamine (substrate) and benzylamine–SSAO (substrate–enzyme complex). In the absence of inhibitor, the calculated K_m was 2.227 mmol L^{-1} for bovine SSAO. In the presence of MVA-NHOH (0.05 μg mL^{-1}, Fig. 7A), the calculated $K'_m{}'$ was 2.403 mmol L^{-1} ($K'_m > K_m$). In the presence of LVA-NHOH (0.11 μg mL^{-1}, Fig. 7B), the calculated $K'_m{}'$ was 1.983 mmol L^{-1} ($K'_m < K_m$). From the results of kinetic data, the inhibitors (MVA-NHOH or LVA-NHOH) had different affinities toward bovine SSAO and bovine SSAO–benzylamine complex. The alginic acid hydroxamate did not act as bovine SSAO substrates. However, the hydroxamic acid moiety in MVA-NHOH or LVA-NHOH might partially compete with benzylamine in the SSAO active site or benzylamine–SSAO complex and then change the SSAO kinetic properties.

CONCLUSION

The alginic acid hydroxamates (LVA-NHOH and MVA-NHOH) showed antioxidant and anti-radical activities (Figs 2–5) and SSAO inhibitory activity (Figs 6 and 7) in this report. The small molecules of alginic acid hydroxamates from alginate lyase hydrolysis will be investigated further.

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