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Semicarbazide-sensitive amine oxidase inhibitory activity of galacturonic acid hydroxamate

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ABSTRACT. In the literature, semicarbazide-sensitive amine oxidase (SSAO) in plasma was elevated in diabetes mellitus and heart failure. SSAO catalyzed the oxidative deamination of various amines to produce hydrogen peroxide, which is one possible source of oxidative stresses. In this research, galacturonic acid hydroxamate (GalA-NHOH), but not GalA, effectively inhibited SSAO activities from bovine plasma and porcine plasma in a dose-dependent manner, and the IC_{50} values were 0.041 and 0.018 mM, respectively. The SSAO activities from bovine plasma and pig plasma were confirmed by the SSAO specific inhibitor of semicarbazide. Using AO activity staining in the 7.5% native-PAGE gels, it was also revealed that GalA-NHOH had inhibitory activities against SSAO from bovine plasma. GalA-NHOH showed mixed noncompetitive inhibition against bovine SSAO in respect to benzylamine (substrate) and benzylamine-SSAO (substrate-enzyme complex). It was found that the V'_{max} and K'_m values were reduced in the presence of GalA-NHOH (0.03 mM).

Keywords: Galacturonic acid; Galacturonic acid hydroxamate (GalA-NHOH); Semicarbazide-sensitive amine oxidase (SSAO).

INTRODUCTION

Amine oxidases (AOs) have traditionally been divided into two main groups, based on the chemical nature of the attached cofactor (Jalkanen and Salmi, 2001). One is the flavin adenine dinucleotide-containing enzymes (monoamine oxidase A (MAO-A), -B, and polyamine oxidase) (Shih et al., 1999). MAO-A and MAO-B are well known mitochondrial enzymes (EC 1.4.3.4) that have firmly established roles in the metabolism of neurotransmitters (Shih et al., 1999). They can be distinguished selectively by specific inhibitors of clorgyline and deprenyl, respectively. MAO inhibitors have recently been used to treat anxiety disorders and Alzheimer's disease (Yamada and Yasuhara, 2004). The other AO contains a cofactor possessing one or more topaquinones [diamine oxidase, lysyl oxidase, or semicarbazide-sensitive AO (SSAO)], (Klinman and Mu, 1994; Klinman, 1996; Lyles, 1996). SSAO (EC 1.4.3.6) is the name for a group of heterogenous enzymes widely distributed in nature, in plants, microorganisms, and the organs (vasculature, dental pulp, eye, and plasma) of mammals (Boomsma et al., 2000). It converts primary amines into the corresponding aldehydes, generating hydrogen peroxide and ammonia. Benzylamine appears to be a good substrate for all SSAOs and MAO-B (Lyles, 1996), and a variety of other amines (e.g. serotonin, tyramine, tryptamine, polyamine, and dopamine) have been reported to be substrates for some but not all SSAOs. The endogenous compounds aminoacetone and methylamine have proven to be good substrates for most SSAOs (Precious et al., 1988; Lyles and Chalmers, 1992; Jalkanen and Salmi, 2001). Recent research has found plasma SSAO to be elevated in diabetes mellitus and heart failure, and it is implicated in atherosclerosis, endothelial damage, and glucose transport into adipocytes (Yu and Zuo, 1993, 1996, 1997; Boomsma et al., 1995, 1997, 1999).

In the literature, galactosamine, mannosamine, and glucosamine were shown to have dose-dependent inhibitory activities against SSAO (O'Sullivana et al., 2003); myricetin galloylglycoside showed dose-dependent inhibitory activities against SSAO (Lee et al., 2006); alginic acid hydroxamates showed dose-dependent inhibitory activities against SSAO (Liu et al., 2007); the extracts of

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Taiwanofungus camphoratus (Chang-Chih) showed dose-dependent inhibitory activities against SSAO (Wang et al., 2007); geraniin isolated from *Phyllanthus urinaria* showed dose-dependent inhibitory activities against SSAO (Lin et al., 2008). The galacturonic acid hydroxamate (GalA-NHOH) was previously reported to have antioxidant activities (Liu et al., 2008). In this research, GalA-NHOH, but not GalA, effectively inhibited the SSAO activities of bovine plasma and porcine plasma in a dose-dependent manner. The GalA-NHOH showed mixed noncompetitive inhibition against bovine SSAO with respect to benzylamine (substrate) and benzylamine-SSAO (substrate-enzyme complex).

MATERIALS AND METHODS

Materials

Benzylamine, 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, ABTS), bovine plasma (P-4639, reconstituted with 10 mL deionized water), pig plasma (P-2891, reconstituted with 10 mL deionized water), 3-amino-9-ethylcarbazole (AEC), clorgyline, deprenyl, horseradish peroxidase (148 units/mg solid), galacturonic acid, and semicarbazide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (33%) was from Wako Pure Chemical Industry (Osaka, Japan). Other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

Preparations of GalA-NHOH from GalA

The preparation of GalA-NHOH from GalA has been reported previously (Liu et al., 2008). In brief, commercial GalA was esterified with 500 mL of ice-cold acidic methanol (1 N HCl) at 4°C with gentle stirring for 5 days (Sajjaanatakul et al., 1989) to get galacturonic acid methyl ester. After being adjusted to neutral pH by KOH in methanol, the reacted solution was dried by a rotary evaporator. The reacted substances containing galacturonic acid methyl ester were stirred in 500 ml methanol 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 to get GalA-NHOH. After being adjusted to neutral pH by HCl in methanol, the reacted solution was dried by a rotary evaporator. The dried substances containing GalA-NHOH were stirred in methanol, and the vast amounts of insoluble salt were filtered by a G3 glass filter and then discarded, the filtrates were dried by a rotary evaporator. This procedure was repeated in triplicate. After being separated by reverse phase C18 HPLC column using 99% methanol as eluting solvent, each fraction was determined by acidic ferric chloride solution for hydroxamic acid contents (Soloway and Lipschitz, 1952) as follows: Each 0.2 ml of separated fraction was mixed with 0.3 mL of 4 N HCl and 0.5 mL of 10% ferric chloride in 0.1 N HCl. The absorbance at 540 nm was determined after 10 min standing. The active fractions were collected and dried by a rotary evaporator. The recovery was about 50%.

Determination of AO inhibitory activity

AO inhibitory activities were determined by spectrophotometric method according to Szutowicz et al. (1984) with modifications using hydrogen peroxide to plot the AO standard curve. The major modification was the use of heating at 100°C boiling water for 5 min instead of sodium azide to stop the reaction. The total 200 μL reaction solution [containing 50 μL of 200 mM phosphate buffer, pH 7.4, 50 μL of 8 mM benzylamine, AO, and different amounts of GalA, GalA-NHOH, and specific inhibitors (clorgyline, deprenyl, and semicarbazide, 1 mM)] was placed at 37°C for one hour and then heated at 100°C in boiling water for 5 min to stop the reaction. After cooling and a brief centrifugation, the 90 μL reaction solution was isolated and added to a 710 μL of spectrophotometric solution containing 200 μL of 200 mM phosphate buffer (pH 7.4), 100 μL of 2 mM ABTS solution, and 25 μL of horseradish peroxidase (10 μg/mL). Means of triplicates were measured. Deionized water was used instead of GalA, GalA-NHOH, and specific inhibitors as a blank experiment. One unit of AO activity was defined as amounts of enzyme to produce one nanomole of hydrogen peroxide during one hour under the reaction conditions above. The changes of absorbance at 420 nm were recorded during 1 min and expressed as $\Delta A420_{nm/min}$. The relative AO activity (%) was calculated with the equation: $(\Delta A420_{nm/min, blank} - \Delta A420_{nm/min, sample}) \div \Delta A420_{nm/min, blank} \times 100\%)$.

Plasma SSAO activity stains on 10% native polyacrylamide gels

The SSAO activity staining on a 10% polyacrylamide gel was according to the method of Lee et al. (2002). Bovine plasma (23.45 units) was premixed with GalA or GalA-NHOH (0, 0.1, 1, 10, and 100 μM) overnight and prepared for electrophoresis. When native PAGE was finished, the gels were balanced for 20 min twice in 50 mM Tris-HCl buffer (pH 7.9) before activity staining. The process of plasma SSAO activity staining was as below. Eighty mg benzylamine and 40 mg AEC was dissolved in 10 mL dimethylformamide and then added to 40 mL, 50 mM Tris-HCl buffer (pH 7.9) as the substrate solution, in which the gel was submerged and shaken for 5 min. Then, 200 μL horseradish peroxidase (5 mg/mL) was added, and the gel was gently shaken at room temperature. It was subsequently destained with 10% acetic acid and then washed with distilled water.

Determination of the kinetic constant of bovine SSAO in the presence of GalA-NHOH

The K_m and V_{max} of bovine SSAO (18.76 units) and the K_m' and V_{max}' of bovine SSAO in the presence of GalA-NHOH (0.03 mM) were calculated from Lineweaver-Burk plots using different concentrations of benzylamine as substrate (0.8, 1, 2, 4, and 8 mM).

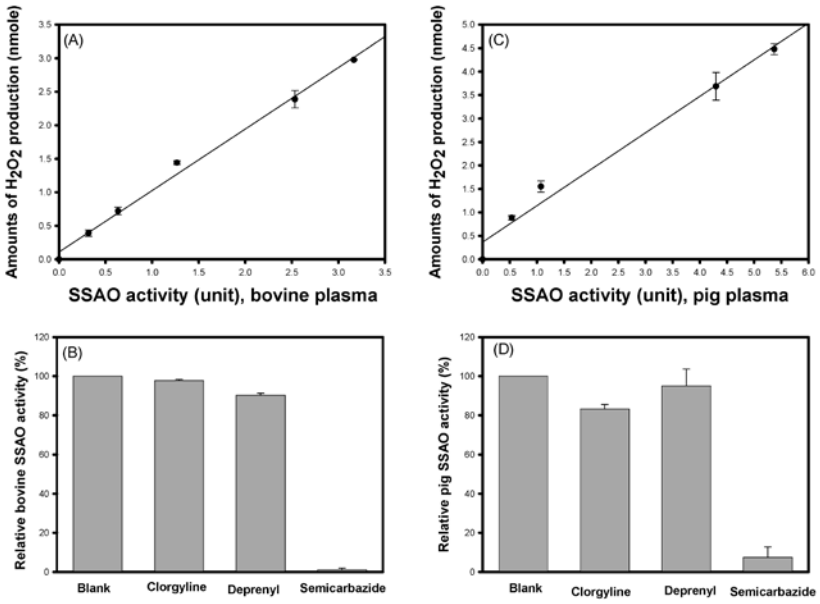

Figure 1. The modified ABTS method for AO activity determinations. The correlations of hydrogen peroxide production and AO activity from bovine plasma (A) and pig plasma (C) in the standard curve. The AO type of bovine plasma (C) and pig plasma (D) were investigated by specific inhibitors, including clorgyline (final concentration, 1 mM), deprenyl (final concentration, 1 mM), and semicarbazide (final concentration, 1 mM).

RESULTS AND DISCUSSION

A variety of hydroxamic acid derivatives have been proven to have biological activities toward cancer, cardiovascular diseases, Alzheimer's disease, and tuberculosis (Muri et al., 2002). The hydroxamate moiety was reported to chelate metal ions and therefore to inhibit metalloenzyme activities (Yale, 1943; Neilands, 1967; Hou et al., 2003; Liu et al., 2004). SSAO (EC 1.4.3.6) is a common name for a group of metalloproteins widely distributed in nature, in plants, microorganisms, and the organs (vasculature, dental pulp, eye and plasma) of mammals (Boomsma et al., 2000). Therefore, the synthesized GalA-NHOH was used to evaluate the SSAO inhibitory activities in comparison with the original material of GalA.

In the beginning, the AO activities of bovine plasma and pig plasma were investigated by specific inhibitors, including clorgyline (MAO-A inhibitor), deprenyl (MAO-B inhibitor), and semicarbazide (SSAO inhibitor) (Figure 1B and D) using the modified ABTS method. The hydrogen peroxide was used to plot the standard curve. After heating at 100°C for 5 min, the change in ABTS absorbance ($\Delta A420_{nm/min}$) was positively correlated with concentrations of hydrogen peroxide, and the value of R square was 0.992 (data not shown). Using this modified ABTS method, the different amounts of bovine plasma (R^2=0.993, Figure 1A) and pig plasma (R^2=0.981, Figure 1C) were positively correlated with the generated hydrogen peroxide. The AO activity from bovine plasma and pig plasma was specifically inhibited by semicarbazide, but not clorgyline or deprenyl (Figure 1B and D). Therefore, it was confirmed that the AO activity from bovine and pig plasma belonged to SSAO.

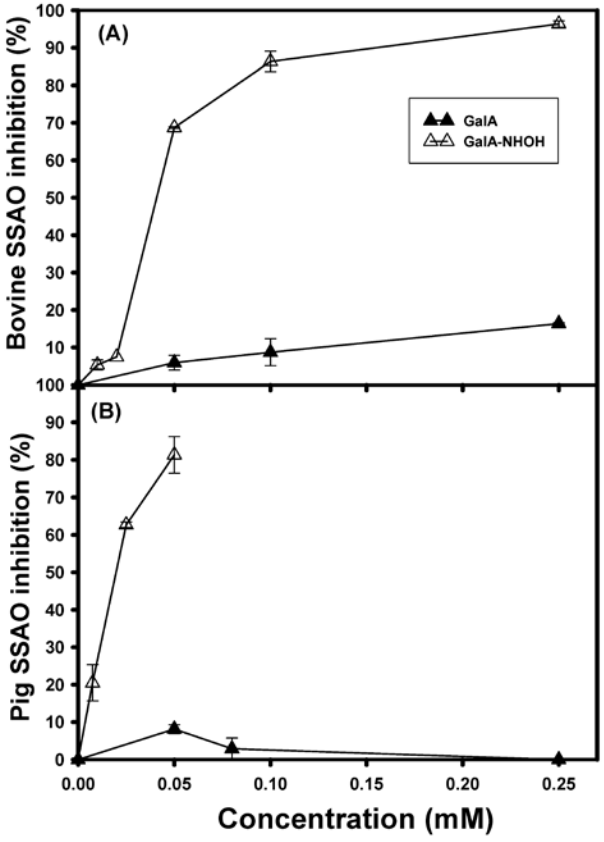

Figure 2. The effects of GalA and GalA-NHON on SSAO from bovine plasma (A) and pig plasma (B). The SSAO inhibition (%) was calculated with the equation: ($\Delta A420$ nm/min$_{blank}$ – $\Delta A420$ nm/min$_{sample}$) ÷ $\Delta A420$ nm/min$_{blank}$ × 100%.

Figure 2 showed the inhibitory activities of GalA and GalA-NHOH against SSAO from bovine plasma (18.76 units, Figure 2A) and pig plasma (11.05 units, Figure 2B). From the results of Figure 2A and B, it was found that GalA-NHOH, but not GalA, showed dose-dependently inhibitory activities against SSAO from bovine plasma and pig plasma. The IC_{50} values were calculated to be 0.041 and 0.018 mM, respectively. Pectin hydroxamate (Hou et al., 2003), myricetin galloylglycosides (Lee et al., 2006), alginic acid hydroxamate (Liu et al., 2007), geraniin (Lin et al., 2008), and hydroxyurea (Liu et al., 2010) have also been reported to exhibit SSAO inhibitory activities.

Using AO activity staining in 7.5% native-PAGE gels, it was also found that GalA-NHOH, but not GalA, exhibited inhibitory activities against SSAO from bovine plasma (higher than 1 μM, Figure 3).

Figure 4 showed the kinetic parameter of SSAO from bovine plasma with or without 0.03 mM GalA-NHOH additions. Without GalA-NHOH additions, the calculated K_m of bovine SSAO was 2.01 mM, which approximated the 2.23 mM of the previous report (Liu et al., 2007). In the presence of GalA-NHOH, the calculated K_m' was 1.39 mM (K_m' < K_m). From the results of kinetic data, the GalA-NHOH showed mixed noncompetitive inhibition against bovine SSAO with respect to benzylamine (substrate) and benzylamine-SSAO (substrate-enzyme complex). The GalA-NHOH did not act as a bovine SSAO substrate. However, the hydroxamic acid moiety in GalA-NHOH might partially compete with benzylamine in the SSAO active site or benzylamine-SSAO complex and then change the SSAO kinetic properties.

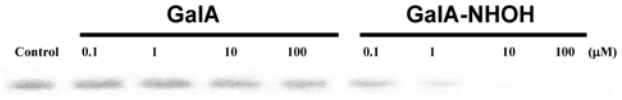

Figure 3. Effects of different concentrations of GalA and GalA-NHOH on SSAO activity from bovine plasma using activity staining in 7.5% native-PAGE gels.

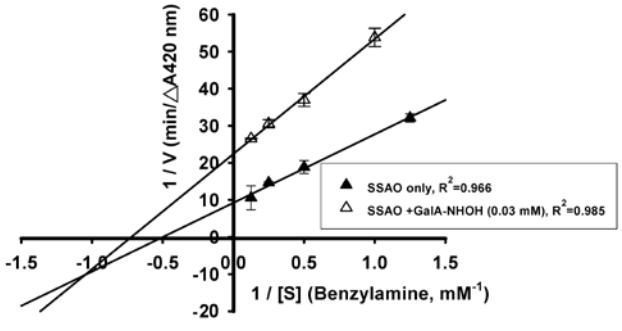

Figure 4. The kinetic properties of bovine SSAO (18.76 units) in the absence and presence of GalA-NHOH (0.03 mM) in Lineweaver-Burk plots using different concentrations of benzylamine as substrate (0.8, 1, 2, 4, and 8 mM).

In conclusion, GalA-NHOH exhibited SSAO inhibitory activities in this report. Ferrer et al. (2002) reported that SSAO activity was higher in the cerebral blood vessels of subjects with Alzheimer's disease. Active hydrogen peroxide might interact with ferrous ions *in vivo* to produce hydroxyl radicals (the Fenton reaction), which is a possible source of oxidative stresses.Taken together with a previous report on antioxidant activities (Liu et al., 2008), a case can be made that GalA-NHOH might possess anti-aging properties, a possibility which merits further investigation.

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半乳糖醛酸羥肟酸具有抑制 semicarbazide-敏感型胺酶活性

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文獻報導，semicarbazide- 敏感型胺酶（SSAO）在糖尿病患與心臟受損病患的血漿中有增加。SSAO 催化氧化去胺反應，過程中產生過氧化氫，可能是細胞內產生氧化逆境來源之一。本研究發現，合成物之半乳糖醛酸羥肟酸（GalA-NHOH），而非原料之半乳糖醛酸，具有有效且濃度相關抑制商品化牛血漿或豬血漿來源之 SSAO，抑制 SSAO 活性一半所需濃度分別為 0.041 及 0.018 mM。利用電泳膠體活性染色技術，也顯示半乳糖醛酸羥肟酸抑制商品化來源之 SSAO 活性。半乳糖醛酸羥　酸在 0.03 mM 濃度下抑制牛血漿來源之 SSAO 之動力學研究，發現其 V'_{max} 及 K'_m 數值都明顯降低。顯示半乳糖醛酸羥肟酸為混合型非競爭型抑制（mixed noncompetitive inhibition），其競爭對象為 SSAO 反應基質與 SSAO- 基質複合體。

關鍵詞：半乳糖醛酸；半乳糖醛酸羥肟酸 (GalA-NHOH); semicarbazide- 敏感型胺酶 (SSAO)。