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Semicarbazide-sensitive amine oxidase inhibitory and hydroxyl radical scavenging activities of aspartic acid β-hydroxamate and glutamic acid γ-monohydroxamate

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ABSTRACT. It was reported that 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, two amino acid hydroxamates, namely aspartic acid β-hydroxamate (AAH) and glutamic acid γ-monohydroxamate (GAH), showed dose-dependently inhibitory against SSAO from bovine plasma. The IC_{50} of AAH and GAH was 0.7 mM and 0.023 mM, respectively, in the comparisons with 0.035 mM of semicarbazide (positive controls). The AAH showed mixed noncompetitive inhibition against bovine SSAO in the respect to benzylamine (substrate) and benzylamine-SSAO (substrate-enzyme complex). It was found that the V'_{max} value was reduced and K'_m value was increased in the presence of AAH (0.4 mM). Using electron spin resonance to investigate the hydroxyl radical scavenging activities, AAH and GAH showed effectively scavenging activities and IC_{50} was 0.66 mM and 1.21 mM, respectively.

Keywords: Aspartic acid β-hydroxamate (AAH); Electron spin resonance; Hydroxyl radical; Glutamic acid γ-monohydroxamate (GAH); Semicarbazide-sensitive amine oxidase (SSAO).

INTRODUCTION

Amine oxidases (AOs) have been divided into two main groups (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) and the other contains a cofactor possessing one or more topaquinones and copper ions [diamine oxidase, lysyl oxidase, or semicarbazide-sensitive AO (SSAO)], (Klinman and Mu, 1994; Klinman, 1996; Lyles, 1996). MAO-A and MAO-B were well known mitochondria 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, and MAO inhibitors were recently used to treat anxiety disorders and Alzheimer's disease (Yamada and Yasuhara, 2004). The SSAO (EC 1.4.3.6), arises from the inhibition by semicarbazide, is a name for a group of heterogenous enzymes widely distributed in nature, including plants, microorganisms, and the organs of mammals (vasculature, dental pulp, eye, and plasma) (Boomsma et al., 2000).

AO converts 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. It has been found that endogenous compounds, aminoacetone and methylamine, are good substrates for most SSAOs (Precious et al., 1988; Lyles and Chalmers, 1992; Jalkanen and Salmi, 2001). In recent research, it was found that plasma SSAO was elevated in diabetes mellitus and heart failure, and is implicated in atherosclerosis, endothelial damage, and glucose transport into adipocytes (Boomsma et al., 1995, 1997, 1999; Yu and Zuo, 1993, 1996, 1997).

The galactosamine, mannosamine, and glucosamine were showed to have dose-dependent inhibitory activities against SSAO (O'Sullivana et al., 2003). In our published results, several purified natural products, synthetic compounds, and crude extracts showed SSAO inhibitory activities. For examples, the myricetin galloylglycoside

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(A)

(B)

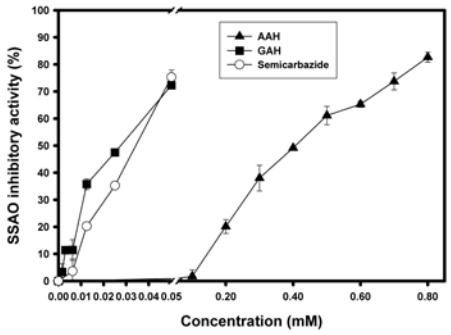

Figure 1. The effects of AAH and GAH on SSAO from bovine plasma. (A) The structure of AAH and GAH, and (B) inhibition of SSAO activities by AAH, GAH, and semicarbazide, respectively. The SSAO inhibition (%) was calculated with the equation: (ΔA420 nm/min$_{blank}$ – ΔA420 nm/min$_{sample}$) ÷ ΔA420 nm/min$_{blank}$ × 100%.

isolated from leaves of *Acacia confusa* (Lee et al., 2006) and geraniin isolated from *Phyllanthus urinaria* (Lin et al., 2008) showed dose-dependent inhibitory activities against SSAO. The hydroxamic acid derivative of hydroxyurea showed MAO and SSAO inhibitory activities (Liu et al., 2010). The synthesized alginic acid hydroxamate (Liu et al., 2007), synthesized galacturonic acid hydroxamate (Liu et al., 2011a) and glucuronic acid hydroxamate (Liu et al., 2011b) were reported to have SSAO inhibitory activities. The extracts of *Taiwanofungus camphoratus* (Chang-Chih) showed dose-dependent inhibitory activities against SSAO (Wang et al., 2007).

Our previous report showed that aspartic acid β-hydroxamate (AAH) and glutamic acid γ-monohydroxamate (GAH) (Figure 1A) exhibited angiotensin converting enzyme inhibitory activities, and scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide radicals (Liu et al., 2004). The AAH exhibited antitumor activity on L5178Y leukemia (Thomasset et al., 1991), therapeutic effect on friend erythroleukemia (Tournaire et al., 1994a) and antiproliferative activity on friend virus-infected erythropoietic progenitor cells (Tournaire et al., 1994b). In this research, AAH and GAH were effectively to inhibit SSAO activities from bovine plasma in a dose-dependent manners, and also showed effectively scavenging activities against hydroxyl radicals.

MATERIALS AND METHODS

Materials

L-Aspartic acid β-hydroxamate (AAH, A6508), benzylamine, 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, ABTS), bovine plasma (P-4639, reconstitute with 10 mL deionized water), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), ferrous sulfate, L-glutamic acid γ-monohydroxamate (GAH, G2253), horseradish peroxidase (148 units/mg solid), 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).

Determination of SSAO inhibitory activity

SSAO inhibitory activities were determined by spectrophotometric method according to Szutowicz et al. (1984) with modifications (Liu et al., 2011a,b) using hydrogen peroxide to plot the SSAO standard curve. The total 200 μL reaction solution (containing 50 μL of 200 mM phosphate buffer, pH 7.4, 50 μL of 8 mM benzylamine, SSAO, and different amounts of AAH or GAH, and semicarbazide) was placed at 37°C for one hour and then heated at 100°C boiling water for 5 min to stop reaction. After cooling and brief centrifugation, the 90 μL reaction solution was isolated and added to 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 AAH or GAH or semicarbazide as a blank experiment. One unit of SSAO activity was defined as amounts of enzyme to produce one nanomole of hydrogen peroxide during one hour under above-mentioned reaction condition. The changes of absorbance at 420 nm were recorded during 1 min and expressed as $\Delta A420_{nm/min}$. The relative SSAO activity (%) was calculated with the equation: ($\Delta A420_{nm/min,\ blank} - \Delta A420_{nm/min,\ sample}$) ÷ $\Delta A420_{nm/min,\ blank}$ × 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 AAH or GAH (0, 1.5, 1.75, 2, 2.25, and 2.5 mM) 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 shaked for 5 min. Then, 200 μL horseradish peroxidase (5 mg/mL) was added. The gel was gentle shaking at room temperature. The gel was destained with 10% acetic acid and then washed with distilled water.

Determination of the kinetic constant of bovine SSAO in the presence of AAH

The K_m and V_{max} of bovine SSAO (18.76 units) and the

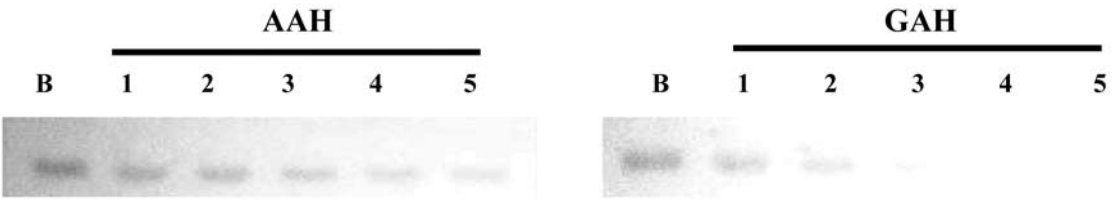

Figure 2. Effects of different concentrations of AAH and GAH on SSAO activity from bovine plasma using activity staining in 10% native-PAGE gels. Lane 1 to lane 5 were 1.5, 1.75, 2, 2.25, and 2.5 mM, respectively.

K_m' and V_{max}' of bovine SSAO in the presence of AAH (0.4 mM) were calculated from Lineweaver-Burk plots using different concentrations of benzylamine as substrate (0.67, 0.83, 1, 1.33, 2, and 4 mM).

Scavenging activity of hydroxyl radicals as measured by electron spin resonance (ESR) spectrometry

Hydroxyl radicals were generated by means of the Fenton reaction (Liu et al., 2010). The reaction mixture (500 μL) included AAH (0.5 mM and 2.5 mM) or GAH (1.0, 1.5, and 2.5 mM), 5 mM DMPO, and 0.05 mM ferrous sulfate. After mixing, the solution was transferred to an ESR quartz cell and placed at the cavity of the ESR spectrometer. Hydrogen peroxide was added to give a final concentration of 0.25 mM. Deionized water was used instead of the sample solution as the blank. After 40 s, the relative intensity of the signal of the DMPO-OH spin adduct was measured. All ESR spectra were recorded at ambient temperature (25°C) 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; and scan time, 1.5 min.

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 were reported to chelate metal ions and therefore to inhibit metalloenzyme activities (Neilands, 1967; Hou et al., 2003; Liu et al., 2004). SSAO (EC 1.4.3.6) is a common name for a group of copper-containing metalloproteins widely distributed in nature, included plants, microorganisms, and organs of mammals (vasculature, dental pulp, eye and plasma) (Boomsma et al., 2000). Therefore, the amino acid hydroxamates of AAH and GAH (Figure 1A) were used to evaluate the SSAO inhibitory activities in the comparison with positive control of semicarbazide.

In our previous report, it was proved that bovine plasma contained SSAO activities which the AO activity in bovine plasma was specifically inhibited by semicarbazide,

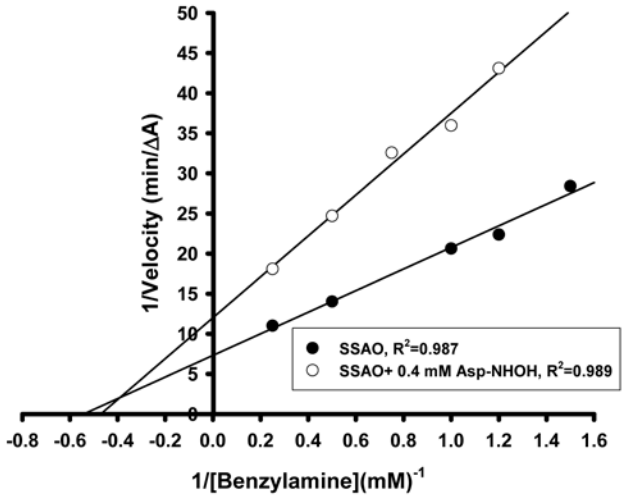

Figure 3. The kinetic properties of bovine SSAO (18.76 units) in the absence and presence of AAH (0.4 mM) in Lineweaver-Burk plots using different concentrations of benzylamine as substrate (0.67, 0.83, 1, 1.33, 2, and 4 mM).

but not clorgyline or deprenyl, therefore, it was confirmed that the AO activity from bovine plasma was belonged to SSAO (Liu et al., 2011a). Figure 1B showed the effects of AAH and GAH on bovine plasma SSAO inhibitory activities. It was found that AAH or GAH showed dose-dependly inhibitory against SSAO from bovine plasma. In calculations, the IC_{50} of AAH and GAH was 0.7 mM and 0.023 mM, respectively, in the comparisons with 0.035 mM of semicarbazide (positive controls).

Using AO activity staining in 10% native-PAGE gels, the intensity of brown color showed AO activity. It was found that GAH higher than 1.75 mM (lane 2) in tested concentrations showed inhibitory activities against SSAO from bovine plasma (Figure 2) compared to the SSAO only (lane B in Figures 2), and the AAH in tested concentrations showed minor inhibitory activities against SSAO from bovine plasma (Figure 2).

Figure 3 showed the kinetic parameter of SSAO from bovine plasma with or without 0.4 mM AAH additions. Without AAH additions, the K_m of bovine SSAO was calculated to be 1.84 mM which was closed to 2.23 mM (Liu et al., 2007) and 2.01 mM (Liu et al., 2011a) of the previous report, and the V_{max} was 0.1368 (ΔA/min). In the presence of 0.4 mM of AAH, the calculated K_m' was 2.13

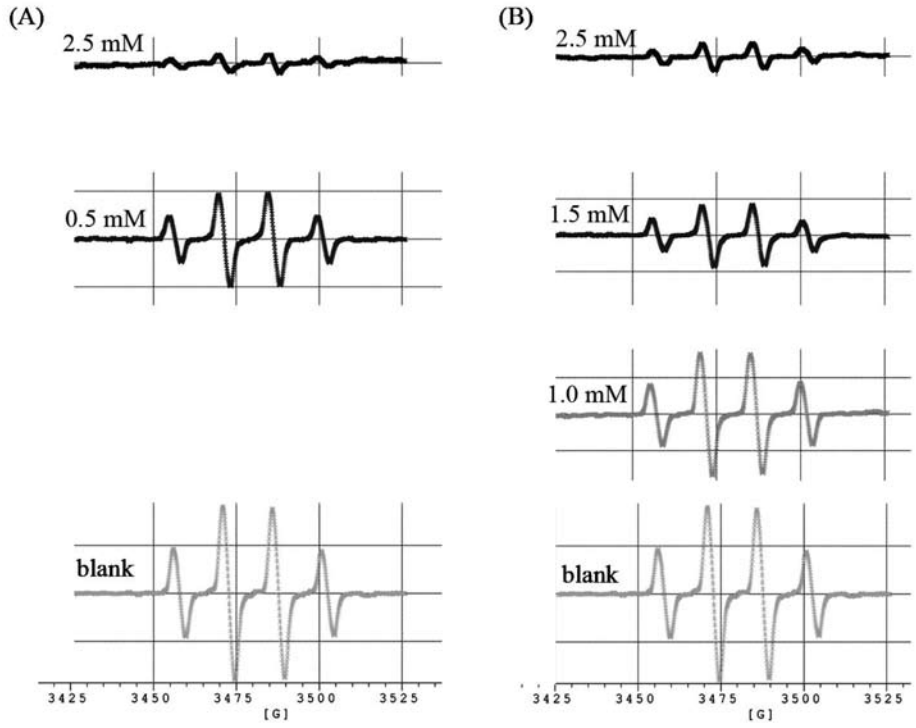

Figure 4. Scavenging activities of (A) AAH (0.5 mM and 2.5 mM), and (B) GAH (1 mM, 1.5 mM and 2.5 mM) against hydroxyl radicals as measured by electron spin resonance spectrometry.

mM ($K_m' > K_m$) and calculated V_{max}' was 0.0833 (ΔA/min) ($V_{max}' < V_{max}$). From the results of kinetic data, the AAH showed mixed noncompetitive inhibition against bovine SSAO in the respect to benzylamine (substrate) and benzylamine-SSAO (substrate-enzyme complex). It was suggested that the hydroxamic acid moiety in AAH might partially compete with benzylamine in the SSAO active site or benzylamine-SSAO complex and then change the SSAO kinetic properties.

The problem of aging-related diseases, such as neurodegenerative diseases (ex. Alzheimer, Parkinson, and Huntington disease), have been emphasized recently. The intricate causes of the aging process are still a matter of extensive speculation, giving rise to many theories. In particular, the role of reactive oxygen species is a prerequisite nowadays for understanding this process (Muller et al., 2007). SSAO catalyzed the oxidative deamination of various amines to produce hydrogen peroxide which is one possible source of oxidative stresses. Ferrer et al. (2002) reported that SSAO activity was higher in the cerebral blood vessels of subjects with Alzheimer's disease. Jiang et al. (2008) reported that products of aldehyde in SSAO-catalyzed deamination might be correlated with cerebral amyloid angiopathy in Alzheimer's disease througt beta amyloid aggregation and advanced glycation end-product formation. 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. Therefore, except from SSAO inhibition by AAH and GAH above-mentioned, the hydroxyl radical scavenging activites of both amino acid hydroxamates were evaluated by ESR. Figure 4 showed the scavenging activities of AAH (Figure 4A) and GAH (Figure 4B) against hydroxyl radicals. From the changes of DMPO-OH adduct intensities, it was clear that both amino acid hydroxamates exhibited hydroxyl radical scavenging activities. In calculations, IC_{50} of AAH and GAH was 0.66 mM and 1.21 mM, respectively.

In conclusion, SSAO catalyzed the oxidative deamination of various amines to produce the corresponding aldehydes, ammonia and hydrogen peroxide. AAH and GAH exhibited SSAO inhibitory and anti-hydroxyl-radical activities *in vitro*. It might be beneficial for anti-aging properties, and requires further investigation.

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天門冬胺酸羥肟酸與麩胺酸羥肟酸具有抑制 semicarbazide-敏感型胺酶與清除氫氧自由基活性

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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。因此，本研究想探討兩種胺基酸羥肟酸衍生物對於 SSAO 抑制活性與其清除氫氧自由基活性。結果顯示，天門冬胺酸羥肟酸 (AAH) 與麩胺酸羥肟酸 (GAH)，具有有效且濃度相關抑制商品化牛血漿來源之 SSAO，抑制 SSAO 活性一半所需濃度 (IC_{50}) 分別為 0.7 及 0.023 mM，其對照比對為 semicarbazide (IC_{50} 為 0.035 mM)。AAH 在 0.4 mM 下探討抑制牛血漿 SSAO 之動力學變化，結果顯示發現其 V'_{max} 數值降低及 K'_m 數值增加。顯示 AAH 對於牛血漿 SSAO 的抑制反應屬於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。利用電子自旋共振探討氫氧自由基清除實驗，AAH 與 GAH 都具有濃度相關清除活性，其 IC_{50} 分別為 0.66 mM 與 1.21 mM。

關鍵詞：天門冬胺酸羥肟酸；電子自旋共振；氫氧自由基；麩胺酸羥肟酸；Semicarbazide- 敏感型胺酶 (SSAO)。