Methanol-soluble, β-elimination products from preparations of alginic acid hydroxamate exhibited DPPH scavenging and angiotensin converting enzyme inhibitory activities

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ABSTRACT. The methanol-soluble sugar hydroxamic acid derivatives obtained during the preparation of alginic acid hydroxamates (AAH) were separated by BioSil-ODS HPLC column (10×250 mm) in acetonitrite: 0.05% trifluoroacetic acid, 10:90 (V/V). The absorbance at 235 nm was set for monitoring β-elimination products. Each fraction was collected and assayed for hydroxamic acid contents, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, and inhibitory activity against angiotensin converting enzyme (ACE). The fraction with higher hydroxamic acid content was further separated by Sephadex G-15 column. Fractions containing both ACE inhibitory activity and DPPH scavenging activity overlapped with fractions containing high levels of hydroxamic acid derivatives. The chromatogram showed little tailing when judged by hydroxamic-acid content but significant tailing when judged by biological activity. Each fraction from above was further separated by silica-TLC in acetonitrite: distilled water, 95:5 (V/V). It was found that a methanol soluble fraction prepared from alginic acid hydroxamates (AAH) exhibited DPPH scavenging activity.

Keywords: Alginic acid hydroxamates; Angiotensin converting enzyme (ACE); DPPH; β-elimination.

INTRODUCTION

Free radical-mediated reactions have involved in degenerative or pathological processes such as aging (Harman, 1995), cancer, coronary heart disease and Alzheimer's disease (Ames, 1983; Smith et al., 1996; Diaz et al., 1997). Meanwhile there are many epidemiological results revealing an association between a diet rich in fresh fruit and vegetable and a decrease in the risk of cardiovascular diseases and certain forms of cancer in humans (Salah et al., 1995). Several reports were concerned natural compounds in fruit and vegetable for their antioxidant activities, such as phenolic compounds (Rice-Evans et al., 1997), anthocyanin (Espin et al., 2000), echinacoside in *Echinaceae* root (Hu and Kitts, 2000), methanolic and hot-water extracts of *Liriope spicata* L. (Hou et al., 2004), the storage proteins of sweet potato root (Hou et al., 2001a), yam tuber (Hou et al., 2001b), potato tuber (Liu et al., 2003b) and yam mucilages (Hou et al., 2002; Lin et al., 2005).

There are several classes of pharmacological agents which have been used in the treatment of hypertension (Mark and Davis, 2000), one class of anti-hypertensive drugs known as angiotensin I converting enzyme (ACE) inhibitors (i.e. peptidase inhibitors) has a low incidence of adverse side-effects and are the preferred class of anti-hypertensive agents when treating patients with concurrent secondary diseases (Fotherby and Panayiotou, 1999). ACE (EC 3.4.15.1) is a dipeptide-liberating exopeptidase, which has been classically associated with the renin-angiotensin system regulating peripheral blood pressure (Mullally et al., 1996). The potent ACE inhibitors were frequently derived from food proteins (Ariyoshi, 1993; Hsu et al., 2002). However, pomegranate juice (Aviram and Dornfeld, 2001), flavan-3-ols and procyanidins (Actis-Goretta et al., 2003), yam mucilages (Lee et al., 2003) and tannins (Liu et al., 2003a) were reported to have ACE inhibitory activity.

Alginic acids, extracted from brown seaweeds or *Phaeophyceae*, are unbranched high molecular-weight polymers containing two types of uronic acid residues of β-(1→4)-linked D-mannuronic acid and α-(1→4)-linked L-guluronic acid acid. Its derivatives have wide

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applications in different industry (Anderson et al., 1991; Taqieddin and Amiji, 2004). We recently reported that monohydroxamates of aspartic acid and glutamic acid exhibit antioxidant and angiotensin converting enzyme inhibitory activities (Liu et al., 2004), and the pectin hydroxamic acids exhibited both semicarbazide-sensitive amine oxidase and ACE inhibitory activities (Hou et al., 2003), and antioxidant activities (Yang et al., 2004). In our recent report, the methyl ester of alginic acid was used to react alkaline hydroxylamine in methanol to produce alginic acid hydroxamates (AAH), which was proven to exhibit antioxidant and semicarbazide-sensitive amine oxidase inhibitory activities (Liu et al., 2006). However, the filtrates of AAH reaction solution by a G3 glass filter after being adjusted to neutral pH exhibited potent antioxidant activities. In this report, the small molecule of methanol-soluble alginic acid hydroxamate (MSAAH) was further separated by BioSil-ODS HPLC column and Sephadex G-15 column. Each fraction was analyzed for its DPPH scavenging and ACE inhibitory activities.

MATERIALS AND METHODS

Materials

ACE (I unit, rabbit lung) was purchased from Fluka Chemie GmbH (Switzerland); Alginic acid (low viscosity, A-2158), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), N-(3-[2-furyl] acryloyl)-Phe-Gly-Gly (FAPGG), and other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO, USA). Silica gel 60 F_{254} were purchased from E. Merck Inc. (Darmstadt, Germany). Sephadex G-15 powder was purchased from Amersham Biosciences (Uppsala, Sweden).

Preparation of small molecules of methanol-soluble alginic acid hydroxamate (MSAAH)

The preparation of AAH was previously reported (Liu et al., 2006). In brief, the 8 g of methyl ester of alginic acid 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. The filtrates from AAH reaction medium by a G3 glass filter after being adjusted to neutral pH were collected and dried by the rotary evaporator. The salt in dried powder was removed through filtration process by being dissolved in methanol repeatedly to get the small molecules of methanol-soluble alginic acid hydroxamate (MSAAH).

Separation of methanol-soluble alginic acid hydroxamate (MSAAH)

Chromatographic separation of MSAAH (dissolved in distilled water) was carried out in the Hitachi (Japan) HPLC system equipped with a photodiode array detector (L-2450) and a BioSil-ODS HPLC column (10×250 mm). The MSAAH was separated isocratically with a mobile phase consisting of a mixture of acetonitrile: 0.05% trifluoroacetic acid, 10:90 (V/V). The flow rate was 1 ml/min and each fraction contained 1 ml. The absorbance at 235 nm was set for monitoring β-elimination products. Each fraction was collected and assayed for hydroxamic acid contents, DPPH scavenging activity, and ACE inhibitory activity. The fraction with the highest hydroxamic acid content was further separated by Sephadex G-15 column (1×75 cm) and eluted by distilled water. The flow rate was 0.5 ml/min and 1 ml was saved for 50 fractions. Each fraction was collected and assayed for hydroxamic acid contents, DPPH scavenging activity, and ACE inhibitory activity.

Determination of hydroxamic acid contents

The hydroxamic acid contents were determined by acidic ferric chloride solution (Soloway and Lipschitz, 1952) with some modifications as followed. 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, and the acetohydroxamic acid was used to plot the standard curve and was expressed as μmole NHOH equivalent/ml.

Scavenging activity against DPPH radicals

The scavenging activity against DPPH radical was measured according to the method of Hou et al. (2001a, b) and Lee et al. (2006). Each 10 μl of sample solution was added to 0.1 ml of 1 M Tris-HCl buffer (pH 7.9), and then mixed with 500 μM DPPH in methanol for 20 min under light protection at room temperature. Means of triplicates were measured. The solvent system in each separation process was used as a blank experiment. The scavenging activity of DPPH radicals (%) was calculated with the equation: $(\triangle A517_{blank} - \triangle A517_{sample}) \div \triangle A517_{blank} \times 100\%$.

Determination of ACE inhibitory activity

The ACE inhibitory activity was measured according to the method of Holmquist et al. (1979) and Lee et al. (2003) with some modifications. The 15 μl (15 mU) commercial ACE (1 U/mL, rabbit lung) were mixed with 200 μl of sample solution 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 ($\triangle A_{inhibitor}$) was recorded during 5 min at room temperature. The solvent system in each separation process was used instead of sample solution for blank experiments ($\triangle A_{blank}$). The ACE activity was expressed as $\triangle A$ 345 nm and the ACE inhibition (%) was calculated as followed: $[1 - (\triangle A_{inhibitor} \div \triangle A_{control})] \times 100\%$. Means of triplicates were determined.

Qualitative determination of sugar, hydroxamic acid, and DPPH scavenging activity by TLC

Each fraction (fractions 34 to 45) separated by

the Sephadex G-15 column was further qualitative determination of total sugar, hydroxamic acid, and DPPH scavenging activity by TLC. The 10 μl of each fraction was spotted on a silica gel 60 F$_{254}$ TLC plate and was separated by acetonitrite: distilled water, 95:5 (V/V). For sugar staining, the plate was dipped in cerium molybdate solution (in 10% sulfuric acid), and then heated at hot plate (Ren et al., 2002), the white zones against blue background showed the position of sugar. For hydroxamic acid staining, the acidic ferric chloride solution in methanol was used (Soloway and Lipschitz, 1952), and the reddish color against the yellow background showed the position of hydroxamic acid. For DPPH scavenging activity, the plate was dipped in 500 μM DPPH solution (in methanol) and then was protected from light for a suitable time, the white zones against purple background showed the position of anti-DPPH radicals.

RESULTS AND DISCUSSION

In our recent report, the methyl ester of alginic acid was used to react alkaline hydroxylamine in methanol to produce alginic acid hydroxamates (AAH), which was proven to exhibit antioxidant and semicarbazide-sensitive amine oxidase inhibitory activities (Liu et al., 2006). It was found that the AAH exhibited a smaller molecular size than the original materials identified by gel filtration. It was proposed that the β-elimination might be occurred (Sajjaanatakul et al., 1989) during alkaline hydroxylamine reaction and resulted in polymer breakdown and produced a smaller molecule. In this report, the filtrates from AAH reaction medium, adjusted to neutral pH, were collected and dried by the rotary evaporator to get methanol-soluble alginic acid hydroxamates (MSAAH) that were further separated by BioSil-ODS HPLC column and Sephadex G-15 column. It was found that small molecule of MSAAH exhibited DPPH scavenging and ACE inhibitory activities.
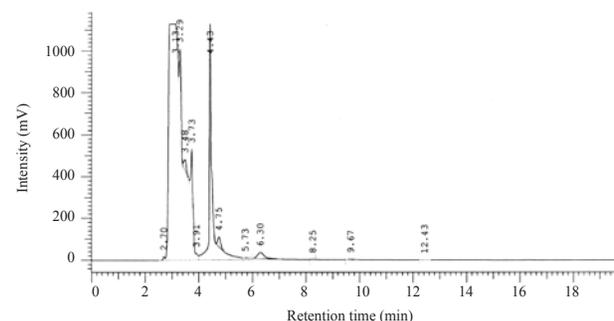
Separations of methanol-soluble alginic acid hydroxamate (MSAAH) by BioSil-ODS HPLC column

The suitable amounts of MSAAH (dissolved in distilled water) were separated by a BioSil-ODS HPLC column isocratically with a mobile phase consisting of a mixture of acetonitrite: 0.05% trifluoroacetic acid, 10:90 (V/V). The absorbance at 235 nm was set for monitoring β-elimination products (Figure 1). From the result of Figure 1, it was found that the separation was complete within 10 min, and the major peaks were found between 3 to 5 min. This chromatographic process (within 10 min) was repeated several times to collect the available amounts of separated fractions for further biological assays.
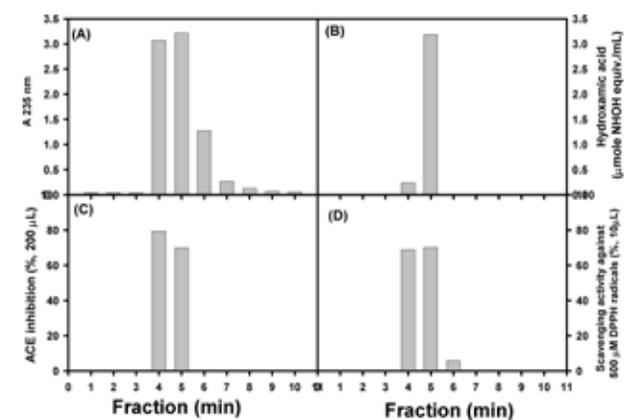
Properties and biological activities of separated MSAAH

Figure 2 showed (A) the absorbance at 235 nm; (B)

hydroxamic acid contents (expressed as μmole equiv./ml); (C) ACE inhibition (%, 200 μl); and (D) scavenging activity against 500 μM DPPH radical (%, 10 μl) of the separated MSAAH. From the results of Figure 2(A), it was found that the fractions 4 and 5 exhibited the major absorbance at 235 nm. The double bond in the position of C4 and C5 was characterized to have major absorbance at 235 nm (Deng et al., 2006). From the results of Figure 2(B), it was found that the fraction 5 contained the highest amounts of hydroxamic acid moiety (3.18 μmole NHOH equivalent/ml). The fraction 4 contained 0.2415 μmole NHOH equivalent/ml. Each fraction was used to test the ACE inhibitory (Figure 2C) and DPPH scavenging (Figure 2D) activities. The fractions 4 and 5 were found to have 79.44% and 70%, respectively, ACE inhibitory activities (Figure 2C, 200 μl). In our previous report, the hydroxamates of L-aspartic acid β-hydroxamate (AAH)
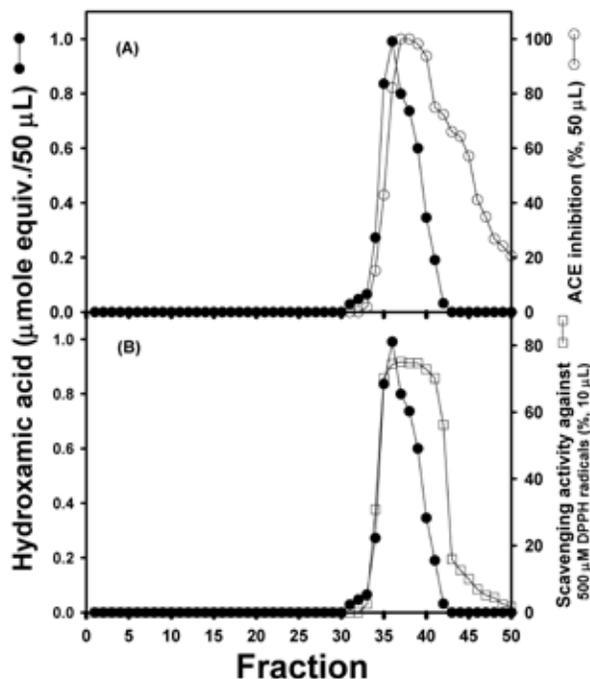

Figure 1. The HPLC chromatogram of BioSil-ODS column (10×250 mm) for the separation of methanol-soluble alginic acid hydroxamates (MSAAH). The MSAAH was separated isocratically with a mobile phase consisting of a mixture of acetonitrite: 0.05% trifluoroacetic acid, 10:90 (V/V). The flow rate was 1 ml/min and each fraction contained 1 ml. The absorbance at 235 nm was set for monitoring β-elimination products.

Figure 2. Properties and biological activities of the separated MSAAH by BioSil-ODS HPLC column. (A) The absorbance at 235 nm; (B) hydroxamic acid contents (expressed as μmole equiv/ml); (C) ACE inhibition (%, 200 μl); and (D) scavenging activity against 500 μM DPPH radical (%, 10 μl). The separation method was described at Figure 1.

and L-glutamic acid γ-hydroxamate (GAH) showed dose-dependent ACE inhibitory activities, and the IC_{50} was 4.92 mM and 6.56 mM, respectively, for AAH and GAH (Liu et al., 2004). The pectin hydroxamic acids exhibited ACE inhibitory activities (Hou et al., 2003), however, the ACE inhibitory activity was less active as MSAAH did. It was also found that fractions 4 and 5
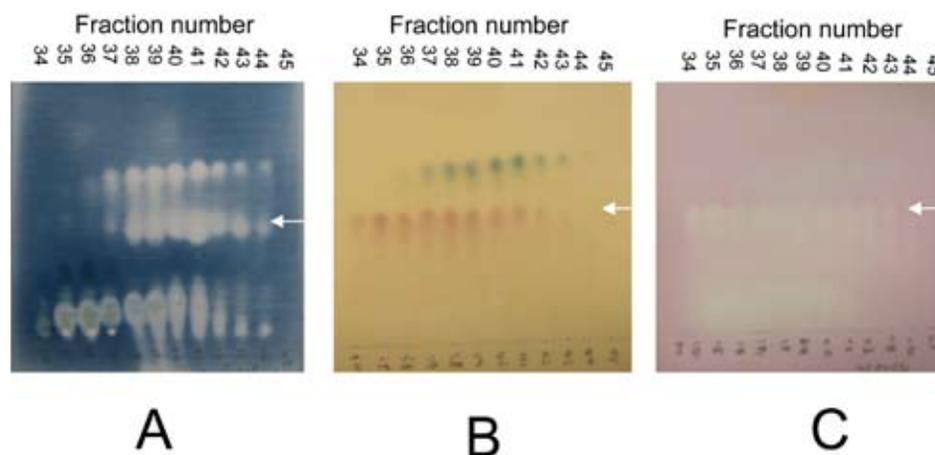

Figure 3. The Sephadex G-15 chromatograms of the fraction 5 of the MSAAH that was separated by BioSil-ODS column. (A) The hydroxamic acid contents (μmole equiv/50 μl), and ACE inhibition (%, 50 μl); (B) The scavenging activity against DPPH radical (%, 10 μl) were assayed in each fraction of Sephadex G-15 column. The column (1×75 cm) was eluted by distilled water. The flow rate was 0.5 ml/min and 1 ml was saved for 50 fractions.

were exhibited 68.84% and 70.17%, respectively, DPPH (500 μM) scavenging activities (Figure 2D, 10 μl). The hydroxamates of L-aspartic acid β-hydroxamate (AAH) and L-glutamic acid γ-hydroxamate (GAH) exhibited scavenging activities against DPPH radicals, and the IC_{50} for AAH and GAH against DPPH (60 μM) was 36 μM and 48 μM, respectively (Liu et al., 2004). The IC_{50} of scavenging activity against DPPH (60 μM) were 1.51, 5.43 and 5.63 mg/mL for DE94T4, DE65T4, DE25T4, respectively (Yang et al., 2004). The fraction with the highest hydroxamic acid content (fraction 5) was chosen for further separation.

Separation of methanol-soluble alginic acid hydroxamate (MSAAH) by Sephadex G-15 column

The fraction 5 was further separated by Sephadex G-15 column and eluted by distilled water. The hydroxamic acid contents, ACE inhibition, and scavenging activity against DPPH radical were assayed in each fraction (Figure 3). It was found that both ACE inhibitory (Figure 3A) and DPPH scavenging activities (Figure 3B) were overlapped with fraction containing hydroxamic acid moiety (Figures 3A and 3B). The available fractionation range of Sephadex G-15 was below 1500 Da. The methylglucopyranoside (MW 194.2) was eluted in the fraction 35. However, the hydroxamic acid fraction was eluted in a broader manner (fractions 34 to 41), and the tailing phenomenon in the biological activity assay was found (Figures 3A and 3B).

Qualitative determination of sugar, hydroxamic acid, and DPPH scavenging activity on the TLC

The fractions 34 to 45 in the Sephadex G-15 were further analyzed to qualitatively determine the total sugar, hydroxamic acid, and DPPH scavenging activity on the TLC which was separated by acetonitrite: distilled water, 95:5 (V/V). From the results of Figure 4A, there were

Figure 4. Thin-layer chromatography for the determination of sugar (A), hydroxamic acid (B), and DPPH scavenging activity (C) in the fractions 34 to 45 that was separated by the Sephadex G-15 column. The 10 μl of each fraction was spotted on a silica gel 60 F_{254} TLC plate and was separated by acetonitrite: distilled water, 95:5 (V/V). The arrow indicated the position of sugar-hydroxamic acid derivatives with DPPH scavenging activities.

several white zones in each fraction which indicated the positions of sugar. It meant that each fraction contained sugar mixtures. From the results of Figure 4B, there was only one position (arrow indicated) in fractions 35 to 41 with reddish color after spraying the acidic ferric chloride. It meant that in the sugar mixtures (Figure 4A) only one was belonged to hydroxamic acid derivatives (Figure 4B, arrow indicated), and which exhibited the potent DPPH scavenging activities (Figure 4C, arrow indicated).

In conclusion, the small molecule of methanol-soluble AAH exhibited both DPPH scavenging and ACE inhibitory activities in the Sephadex G-15 column (Figure 3). From the results of TLC qualitative assay, it was found that only one sugar was belonged to hydroxamic acid derivatives (Figure 4B, arrow indicated), and which exhibited the potent DPPH scavenging activities (Figure 4C, arrow indicated). The isolation and structure identification of AAH with potent biological activity will be investigated further.

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β-移除反應所產生之甲醇可溶小分子糖類羥肟酸衍生物具有清除 DPPH 自由基與抑制血管收縮素轉換酶活性

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　　在製備褐藻酸羥肟酸過程中，因為 β-移除反應而產生甲醇可溶小分子產物。利用 BioSil-ODS 高效液相層析管柱（10×250 mm）以乙腈：0.05% 三氟醋酸為 10：90（體積比）進行分離，以波長 235 nm 之吸光值進行檢測 β-移除反應產物。收集的區分液分別檢測羥肟酸含量、清除 DPPH 自由基活性與抑制血管收縮素轉換酶活性。較高羥肟酸含量的區分液則進一步以 Sephadex G-15 膠濾層析管柱進行分離。結果顯示，各區分的 DPPH 清除活性與抑制血管收縮素轉換酶活性與各區分液中羥肟酸含量重疊，但管柱出現拖尾的現象。此重疊區分液再以乙腈：水為 95：5（體積比），在矽膠薄層層析板進行展開分離。結果顯示，因為 β-移除反應而產生小分子產物具有 DPPH 清除活性。

關鍵詞：褐藻酸羥肟酸；血管收縮素轉換酶；DPPH 自由基；β-移除反應。