Identification of nicotianamine as a potential inhibitor of angiotensin I-converting enzyme in aqueous extract of okara dry powder

NAOYOSHI NISHIBORI – TAKEFUMI SAGARA – KYOJI MORITA

Summary
A powder was prepared from an autoclaved okara extract to increase the concentration of potential angiotensin I-converting enzyme (ACE) inhibiting substances contained in the extract, thereby enhancing its inhibitory activity to characterize active substances more easily. On the other hand, nicotianamine, one of phytosiderophores, is now drawing attention for its ACE-inhibiting activity and anti-hypertensive effect among foods of plant origin, and therefore anticipated as an active component other than short-chain peptides contained in okara extract. Then, to investigate potential ACE-inhibiting substances contained in okara, the extract of the powder was fractionated using reverse-phase high performance liquid chromatography, and nicotianamine in the active fraction separated with strong cation exchange column was identified using liquid chromatography-mass spectrometry. As a result, nicotianamine was detected in the active fraction regarding ACE inhibition, but not in the inactive fractions, thus providing indirect evidence for suggesting nicotianamine as a potential ACE-inhibiting substance in okara extract powder.

Keywords
nicotianamine; angiotensin I-converting enzyme inhibitor; okara extract, soya

Nicotianamine is one of low molecular weight amphoteric compounds contained in a variety of plants and known as a key intermediate compound in the biosynthesis of a lant phytosiderophore, mugineic acid, which is a natural iron (Fe) chelator secreted from roots of graminaceous plants participating in dissolution and transport of Fe to the rhizosphere. Regarding other than graminaceous plants, nicotianamine has also been localized, and considered to play an important role in the internal transport of Fe and other metal cations in higher plants. Thus, nicotianamine has generally been considered as a major substance contributing to nutrient metal metabolism in all higher plants [1–4].

Nicotianamine, on the other hand, has recently been receiving the most attention thanks to its inhibitory effect on angiotensin-I converting enzyme (ACE), one of the key enzymes constituting the renin-angiotensin-aldosterone system, which has been established as the physiologically important system controlling blood pressure in mammals [5, 6]. Chronic high blood pressure is one of the typical pathologic conditions in human modern lifestyle and commonly recognized as a risk factor of cardiovascular disease, which is a serious health issue. ACE is the enzyme catalysing the conversion of inactive angiotensin I to active angiotensin II, which is well known as a potent vasoconstrictor active in elevation of blood pressure through interaction with its receptor AT1. Furthermore, angiotensin II was shown to stimulate the release of aldosterone from the adrenal cortex, resulting in elevation of blood pressure [7]. Therefore, the inhibition of ACE activity can be anticipated to reduce the blood pressure through suppression of angiotensin II production, thereby improving the physical condition of hypertensive patients as a result. Thus, nicotianamine is considered to be essential in the plant metal metabolism, and also anticipated to be beneficial in the improvement of high blood pressure in humans.

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Okara is a by-product of tofu (known as soybean curd) making process. It contains mostly insoluble dietary fibre as well as proteins and minerals of soybean. The use of waste is an important process to promote the full use of agricultural products and has been recognized to promote added value as well as alleviate environmental impacts [8]. Finding the health effects of okara, the waste of tofu making process, is expected to not only help to improve the health of people, but also promote the lean use of soybeans with economically favourable effects. In a previous study [9], the autoclaved extract of okara was shown to cause inhibition of ACE, which resulted in reduction of blood pressure, thus proposing short-chain peptides as potential ACE-inhibiting substances contained in okara. However, it would be understandable to anticipate the possibility that potentially active ACE-inhibiting substances other than short-chain peptides might be contained in okara extract as well. Practically, insoluble dietary fibre is well known as a major component and polyphenolic compounds, such as isoflavones, are also identified as a minor component in okara powder [10]. It therefore seems possible to presume that okara extract may contain bioactive low-molecular substances derived from soybean and ACE-inhibiting substance can be considered as one of those. The powder of okara extract was prepared to increase the concentration of low-molecular substances for the detection and identification of putative active substances in the autoclaved extract. Then, the extract was fractionated and analysed using liquid chromatography-mass spectrometry (LC-MS) to investigate whether nicotianamine was distributed in the fractions exhibiting ACE-inhibiting activity.

MATERIALS AND METHOD

Preparation of aqueous extract of spray-dried okara powder

Dried okara (kindly donated from Shikoku Kakoki, Tokushima, Japan) was suspended in distilled water at a concentration of 100 g·l^{-1} and autoclaved at 121 °C for 30 min. Then it was left in the autoclave chamber overnight to allow it to cool down. The autoclaved extract was filtered through a nylon mesh (74 μm opening) to remove the precipitate, and the filtrate was then dried and solidified to powder using a spray-dryer to concentrate okara components for further analysis. Next, the components were extracted by suspending the powder in distilled water at a concentration of 40 g·l^{-1} and kept at 5 °C overnight. The suspension was centrifuged at 5 000 ×g for 20 min and the supernatant was filtered through a syringe-top polytetrafluoroethylene filter (0.2 μm pore size) to remove the dregs.

Determination of ACE activity

The enzyme activity was determined as described previously [11–13]. Briefly, the reaction mixture containing 0.1 mol·l^{-1} borate buffer (pH 8.0), 1 mol·l^{-1} NaCl, 1 mmol·l^{-1} hippuryl-L-histidyl-L-leucine (HHL Fujifilm Wako, Osaka, Japan), 0.5 mU ACE (Sigma-Aldrich, St. Louis, Missouri, USA) and 100 μl of the extract or the obtained fractions, in a total volume of 150 μl, was incubated at 37 °C for 60 min. The reaction was terminated by adding 10 μl of 5 mol·l^{-1} hydrochloric acid. Then, hippuric acid formed enzymatically during the reaction period was determined using a reverse-phase high performance liquid chromatography (HPLC) system equipped with a UV-VIS detector. Briefly, hippuric acid in 10 μl of the reaction mixture was separated on C18 column Capcell Pak ACR 150 mm × 1.5 mm, 3 μm particle size (Osaka Soda, Osaka, Japan) with a mobile phase containing 25 % MeOH and 0.1 % trifluoroacetic acid at a flow rate of 0.15 ml·min^{-1}, and detected by monitoring the absorbance at 230 nm. ACE activities were expressed as a percent of control that contained no extracts of the powder.

Fractionation of ACE-inhibiting substances

The extract of the powder was subjected to dialysis overnight against distilled water in a quantity of 1 : 100 and the dialysate was concentrated up to the almost same volume as the original extract (designated as the dialysate sample). The inhibiting effects of both dialysed extract and dialysate sample on ACE activity were examined to estimate the molecular size of inhibiting substances in the aqueous okara extract. Moreover, cationic substances in okara extract were separated using cation exchange column. Briefly, the dialysate sample (10 ml) was evaporated under vacuum conditions and the residue was dissolved in 2.5 ml of water. The prepared sample solution (2 ml) was loaded onto a column (150 mm length × 24 mm internal diameter packed with AG 50W-X8 (Bio-Rad, Hercules, California, USA), and the column was washed twice with 100 ml of water. The solutions passing through the column and washing the resin were combined and dried up under vacuum, resulting in non-cationic fraction. Following the washing process, the column was rinsed with 150 ml of 2 mol·l^{-1} ammonium hydroxide and dried up under vacuum, resulting in cationic fraction. These two residues were then dissolved in 2.0 ml of distilled water.

ACE-inhibiting activities of the two fractions were determined after dilution of a portion of samples to the original concentration, and then cationic fraction was roughly fractionated to separate nicotianamine from short-chain peptides contained in the okara powder extract by a reverse-phase HPLC system using the eluent containing a low concentration of methanol. In brief, the sample (200 μl) was loaded onto an octadecylsilyl (ODS) column Capcell Pak AQ 150 mm × 4.6 mm, 5 μm particle size (Osaka Soda) and eluted with a mobile phase containing the gradient increase in the ratio of solution A (formic acid and water, 1:1000, v/v) and solution B (formic acid and methanol, 1:1000, v/v) at a flow rate of 0.7 ml·min^{-1}. The mobile phase composition started at 1 % solution B followed by a linear increase of solution B to 40 % for 60 min, the elution pattern being monitored by determining the absorbance at 210 nm. The eluates collected at 0–15 min, 15–25 min and 25–50 min (designated as ODSF1, ODSF2 and ODSF3, respectively) were dried up under reduced pressure, and the residue was dissolved in the original volume (0.8 ml) of distilled water. Then, the effects of these three samples on ACE activity were determined as described above. The fraction containing the highest ACE-inhibiting activity (ODSF1, 500 μl) was dried up under reduced pressure and the residue was dissolved in 250 μl of the elution solution Then, a portion (100 μl) of this solution was re-fractionated using a polymer-coated strong cation exchange column Capcell Pak SCX column 250 mm × 4.6 mm, 5 μm particle size (Osaka Soda) and 0.1 mmol·l^{-1} potassium dihydrogen phosphate solution as an eluent at a flow rate of 0.6 ml·min^{-1}, and the chromatograms were recorded as stated above. The eluates were collected every 2 min and an aliquot of each fraction (50 μl) was then used for the analysis of ACE-inhibiting activity.

LC-MS analysis of nicotianamine in okara extract

ACE-inhibiting activity in each fraction obtained from strong cation exchange (SCX) column was determined and nicotianamine in active SCX fractions was then analysed by LC-MS using a system consisting of SI-2 pump (Osaka Soda), SI-1 auto-sampler (Osaka Soda) and LCQ Fleet ion trap MS (Thermo Fisher Scientific, Waltham, Massachusetts, USA), with positive electrospray ionization single ion monitoring (ESI-SIM) mode after derivatizing the analysis objects with 9-fluorenyl methoxycarboxyl chloride (FMOC-Cl) as described previously [14] with slight modification. In brief, an aliquot of each fraction (50 μl) was mixed with 100 μl of sodium borate (pH 8.0), 100 μl of 50 mmol·l^{-1} ethylendiaminetetraacetic acid (EDTA, pH 8.0), 50 μl of 50 mmol·l^{-1} FMOC-Cl acetonitrile solution and then incubated at 60 °C for 20 min. At the end of incubation, 100 μl of 5% formic acid was added to stop the reaction. The FMOC-derivatized samples were separated using C18 column Capcell Pac MG-II 150 mm × 2.0 mm, 5 μm particle size (Osaka Soda) at a flow rate of 0.2 ml·min^{-1}. Elution was carried out using a linear gradient of solution B (formic acid and HPLC grade methanol, 1:1000, v/v) in solution A (formic acid and water, 1:1000, v/v), the gradient elution starting with solution A containing 20 % of solution B, and solution B was then linearly increased up to 100 % for 15 min and subsequently kept for additional 5 min.

RESULTS AND DISCUSSION

Nicotianamine is a natural Fe chelator participating in the metal metabolism in a variety of plants, and studied with interest as an ACE-inhibiting substance, thereby anticipating to be beneficial to the improvement of hypertension [5, 6]. Previously, we found ACE-inhibiting activity in the autoclaved extract of okara, which is the residuum obtained in the process of manufacturing tofu from soybeans [9]. Then, a spray-dried powder of the autoclaved extract was prepared for further experiments. The extract of this powder was prepared and subjected to determination of active fractions to identify potentially active components, as described above.

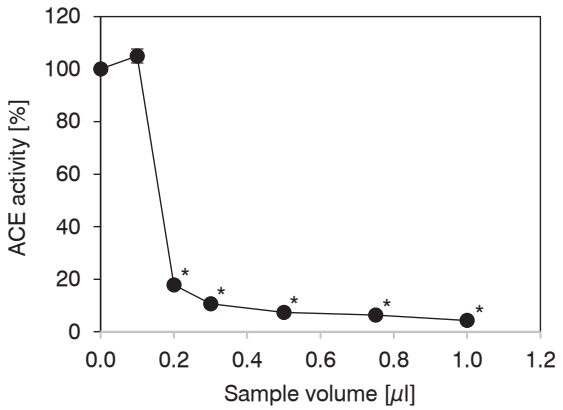

Fig. 1. Inhibitory effect of spray-dried okara powder extract on angiotensin-I converting enzyme activity.

Values are mean ± standard error of mean (n = 6).
ACE – angiotensin I-converting enzyme, * – the difference between experimental plots was significant at $P < 0.05$.

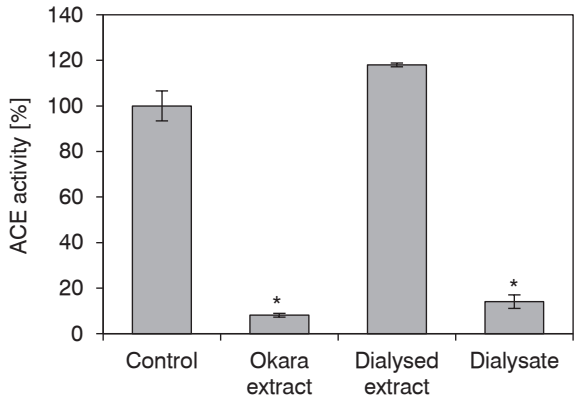

Fig. 2. Inhibitory effects of dialysed extract and dialysate on angiotensin-I converting enzyme activity.

Values are mean ± standard error of mean (n = 6).
ACE – angiotensin I-converting enzyme, * – the difference between two groups was significant at $P < 0.05$.

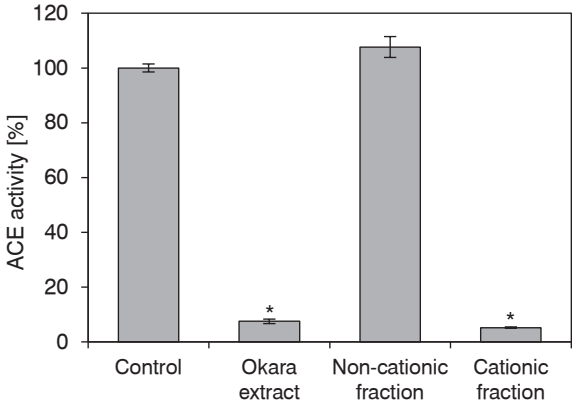

Fig. 3. Inhibitory effects of non-cationic and cationic fractions separated using cation exchange column on angiotensin-I converting enzyme activity.

Values are mean ± standard error of mean (n = 6).
ACE – angiotensin I-converting enzyme, * – the difference between two groups was significant at $P < 0.05$.

In the present study, the effect of the powder extract on ACE activity was first examined, this extract being found to inhibit strongly the enzyme activity (Fig. 1). ACE-inhibiting activity of the powder extract was almost 20-fold higher than that of the autoclaved extract in our previous work [9]. Since putative ACE-inhibiting substances other than short-chain peptides might be presumed to be small molecules, the extract of the powder was dialysed overnight and the inhibitory effects of both the dialysed extract and the dialysate sample on ACE activity were then examined to estimate roughly the molecular size of the active substances in the powder extract. Consequently, the inhibitory effect of the dialysed extract (inner) on ACE activity was completely lost, but the inhibiting activity of the dialysate sample (outer) was almost identical to the original powder extract (Fig. 2). Therefore, it seems reasonable to speculate that ACE-inhibiting substances contained in this extract may mostly pass the dialysis, thereby suggesting these substances in the extract to be small molecules.

Nicotianamine, a small-size cationic substance, has been reported to inhibit ACE activity, thereby proposing its possible effectiveness and usefulness in the improvement of high blood pressure. We presumed that nicotianamine might be one of potential inhibiting substances contained in the extract of the powder and tried to isolate small-size cationic substances from the active dialysate sample using a cation-exchange resin. Then, the fractions passing through and those adsorbed on the resin column were obtained for the analysis of ACE-inhibiting activity. The effects of these fractions on ACE activity were determined, the enzyme inhibiting activity being detected in the resin-adsorbed fraction but not in the non-adsorbed fraction (Fig. 3). Therefore, it seemed possible to speculate that potential ACE inhibiting substances might be cationic small molecules, thus providing evidence in support of the assumption that the active substance in the extract of the dry powder might be nicotianamine. Short-chain peptides belong to substances with small molecules contained abundantly in okara extract and generally known to have ACE-inhibiting activity. In addition, these peptides are known to contain a hydrophobic amino acid residue within the molecular structures, and can be considered to be substantially retained in an ODS column.

In contrast, nicotianamine is speculated to be hardly retained in an ODS column, due to its hydrophilic property as an amphoteric compound. Therefore, this compound is practically separable from ACE-inhibiting short-chain peptides by HPLC using an ODS column as a stationary phase and a methanolic-aqueous solution as a mobile phase. In this study, the fraction of cationic small molecules obtained from a cation exchange column (AG 50W-X8) was further fractionated using an ODS column and low concentrations of methanol as an eluent. As shown in Fig. 4, almost all ACE-inhibiting activity was recovered in the fraction (ODSF1) collected at 0–15 min. Therefore, it seemed possible to eliminate the possibility that ACE inhibiting activity of this fraction might be due to short-chain peptides. Then, the ODSF1 fraction was further separated with a cation ex-

change SCX column, the elution pattern of cationic substances is shown in Fig. 5. Furthermore, ACE-inhibiting activity in individual SCX fractions was also determined, the inhibitory activity being detected only in fraction 4 (SCXF4) obtained by elution at 6–8 min, but no inhibitory activity could be detected in other fractions (Fig. 6).

To identify nicotianamine in the fractions obtained from SCX column, the FMOC derivative of authentic nicotianamine was analysed with LC-MS with SIM mode, and the ion peak was detected at 17 min (Fig. 7A), which had a mass of 748 *m/z* consistent with di-FMOC-derivatized nicotianamine $[NA+2FMOC+H]^+$ (Fig. 7B). SCX fractions were analysed by LC-MS and the ion peak was detected in a single fraction (SCXF4), but not in other SCX fractions (Fig. 7A). The retention time and the molecular mass of this ion peak were shown to be identical to those of authentic nicotianamine (Fig. 7C). Thus, only the SCX fraction containing nicotianamine was shown to have ACE-inhibiting activity, but other fractions without nicotianamine caused no significant effect on ACE activity. Therefore, these findings provided evidence for suggesting that nicotianamine might be the most hopeful candidate for another ACE-inhibiting substance contained in the extract of spray-dried okara powder. From the practical point of view, it should be noted that half maximal inhibitory concentration (IC_{50}) of this extract on ACE activity was calculated as 0.15 μl, which was approximately equivalent to 6 μg of the powder. On the other hand, the IC_{50} values of several commercial products containing ACE-inhibiting peptides were calculated at 2–3 μg (data not shown). Therefore, the spray-dried powder of okara ex-

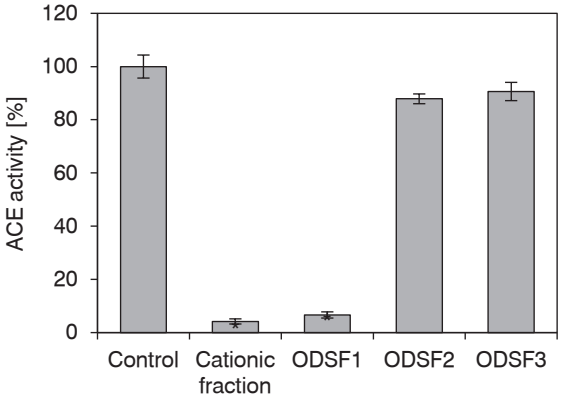

Fig. 4. Inhibitory effects on angiotensin-I converting enzyme activity of whole extract and three fractions separated using octadecylsilyl column.

Values are mean ± standard error of mean (n = 6).
ACE – angiotensin I-converting enzyme; ODSF1, ODSF2 and ODSF3 – the fractions collected at 0–15 min, 15–25 min and 25–50 min, respectively; * – the difference between two groups was significant at $P < 0.05$.

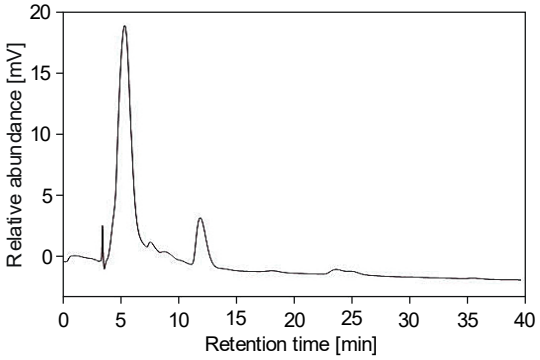

Fig. 5. Elution pattern of the active octadecylsilyl column fraction ODSF1 separated with HPLC using strong cation exchange column.

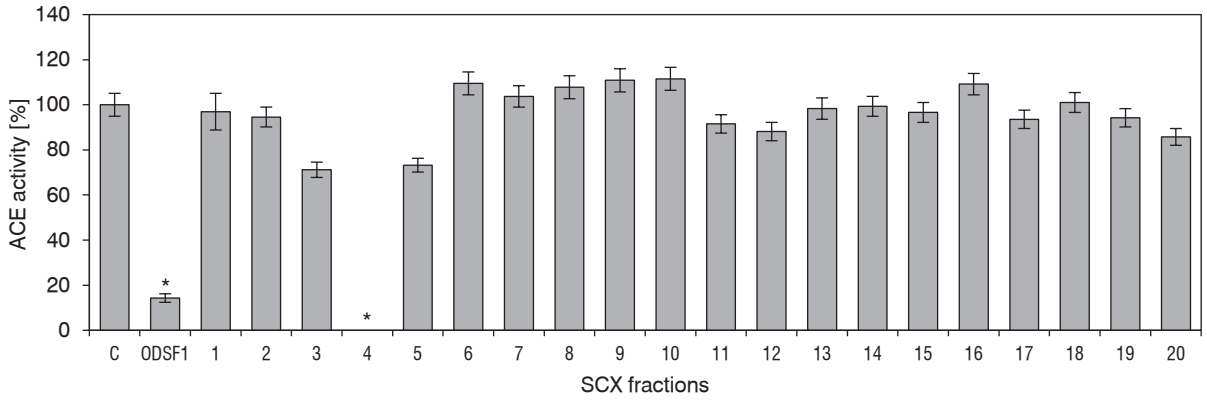

Fig. 6. Effects of eluted fractions from strong cation exchange column on angiotensin-I converting enzyme activity.

Values are mean ± standard error of mean (n = 6).
ACE – angiotensin I-converting enzyme, SCX – strong cation exchange, C – control, ODSF1 – octadecylsilyl column fraction ODSF1, * – the difference between two groups was significant at $P < 0.05$.

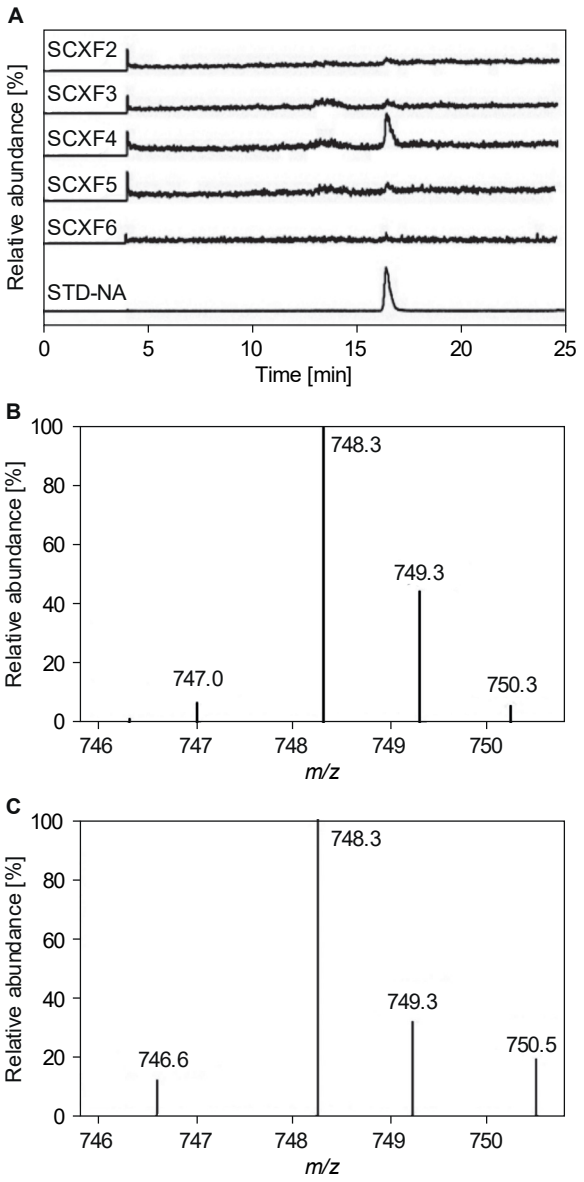

Fig. 7. Nicotianamine analysis.

A – LC-MS chromatograms with single ion monitoring of *m/z* 748 showing detection of nicotianamine derivatized with 9-fluorenyl methoxycarboxyl chloride in fractions (SCXF2–SCXF6), B – mass spectrum of authentic nicotianamine, C – mass spectrum of SCXF4 peak.
Relative abundance is normalized to the strongest ion.
STD-NA – authentic nicotianamine.

tract could be speculated to have almost identical potency to these commercial products, and might be effective in the reduction of blood pressure, just like commercially available ACE inhibitors derived from foodstuff.

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