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REVIEW

Screening Research Methods for α-glucosidase Inhibitors and Angiotensin-converting Enzyme Inhibitors in Fermented Soybean Products and Fermented Milk Products

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Abstract

A simple and highly sensitive assay method for α-glucosidase inhibitory activity was devised and a microplate method using absorbance at 450 nm for angiotensin-converting enzyme activity was introduced. Successful examples using these methods are shown, in which active components from Chinese traditional fermented foods and Mongolian fermented milk products were identified. These traditional fermented foods are expected to elicit effective components against lifestyle-related diseases.

Discipline: Food science
Additional key words: diabetes, hypertension, lifestyle-related disease, traditional foods

Introduction

Many traditional foods are available worldwide and are believed to be healthy for humans. It has also been suggested that some traditional foods even impact on present day lifestyle-related diseases. Natto, soy paste, and soy sauce are fermentation products of the soybean, which have been used for preservation since ancient times. Consequently, various effective compounds are produced due to the action of microbes in these products. Milk products, including sour milk, yogurt, and cheese are also produced by fermentation and are known to have various effects on human health. The anti-cholesterol agent was found in products fermented by *Aspergillus terreus*, and similar structural compounds are used as first-selection drugs worldwide[1]. Various traditional fermented foods exist in each district in China and Mongolia, both of which have very long histories. In China and Mongolia, many fermented soybean and milk products are consumed, respectively. The microorganism in these traditional foods is safe and boosts the development of functional foods because they have been consumed since ancient times.

In this personal review, simple and highly sensitive assay methods for α-glucosidase inhibitory activity and the microplate method using absorbance at 450 nm for angiotensin-converting enzyme activity, are introduced. In addition, successful examples using these methods are shown in which active components were identified.

α-Glucosidase inhibitory activity as an anti-diabetes food

1. A method of measuring α-glucosidase inhibitory activity

Disaccharides such as sucrose are hydrolyzed to glucose by α-glucosidase in the intestinal epithelial cell membrane. Glucose is then rapidly absorbed into the epithelial cells and transferred to the blood, increasing blood glucose levels. Reducing α-glucosidase in intestinal tissue is a very effective treatment for diabetic patients or as a preventive measure for individuals concerned about their blood sugar levels.

Methods to measure the inhibitory activity of natural foods and products have previously been reported [3,8,12]. However, it is not easy to detect the inhibitory activity of α-glucosidase in these samples because

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natural foods and products are often dark, hindering efforts to measure the absorbance. This causes interference with the conventional measurement methods using synthetic substrates for α-glucosidase. Furthermore, although the IC_{50} value is used as a marker to express the intensity of the enzyme's inhibitory activity, it is often difficult to determine the IC_{50} value, as the intensity of α-glucosidase inhibition with the positive control varies widely between some measurements. Therefore, I aimed to develop a new method of measurement that could be applied to colored samples.

This new method was applied to fermented foods, which are almost always colored, to seek highly active products[16]. Rat small intestine acetone powder was used as a source of α-glucosidase. This enzyme solution was placed in a 96-well microplate. Eight serial dilutions of the sample were made and added to each well, followed by warming at 37 °C for 40 min with the synthetic substrate 4-nitrophenyl-β-D-glucopyranoside (4-NGP). The reaction was stopped by adding an alkali solution, whereupon the reaction mixture turned yellow. The absorbance at 405 nm was measured after the color change using a microplate reader and a blank sample without the enzyme was simultaneously measured. A typical strong inhibitory compound from natural sources is 1-deoxynojirimycin (DNJ), which is contained in the mulberry leaf, hence mulberry leaf extract was used as the positive control. The slope of the absorbance versus the dilution plot of the mulberry leaf extract was −18.23, indicating that the sample showed strong suppressive activity. In contrast, coffee was used as the negative control. The slope of the absorbance versus the dilution plot of coffee solution was 0.176, which was interpreted as being close to zero. The variation index in this assay method was 3.39% (n = 4) for intra-assay and 2.68% (n = 3) for inter-assay. From these results, a simple and sensitive α-glucosidase assay method was developed for colored samples.

2. Identification of strong activity components in α-glucosidase of Chinese traditional fermented foods

Screening experiments were performed on various traditional fermented foods using the simple measurement of α-glucosidase inhibition. Antioxidative activity and α-glucosidase inhibitory activity were found in "okara" fermented by *Bacillus subtilis B2*[19,20]. The *Bacillus subtilis B2* microorganism produced the highest inhibitory activity of α-glucosidase in comparison to that of three other microorganisms, including a *Rhizopus* sp., an *Asperigillus* sp., and *Bacillus subtilis* (natto) (Figure 1). However, the active compounds inhibiting α-glucosidase activity have yet to be elucidated. The active component from the Chinese traditional fermented okara was identified through the following steps:

B. subtilis B2 was isolated from "Meitauza", a Chinese traditional fermented okara. The bacterial culture was maintained on a nutrient agar slant and stored at 4 °C. The culture was inoculated into 30 mL of Luria-Bertani (LB) medium, and allowed to grow at 40 °C for 16 h. The enriched culture was then diluted with sterile, distilled water containing 0.9% NaCl and 0.1% peptone to prepare a culture suspension of approximately 106 colony-forming units (CFU)/mL, and served as an inoculation culture for the fermentation of okara.

Soybeans (2,000 g), harvested in 2005 at the Center of Soybean Research, Agricultural Academy of Jilin Province (Jilin, China), were soaked in distilled water (3 times the amount of soybeans, w/w) at room temperature for 12 h, which brought the soaked soybeans to a final weight of approximately 2.2 times exceeding their original weight. The soaked soybeans were then used to produce fresh okara. The hydrated soybeans were ground and filtered with distilled water. Fresh okara (85% moisture) was obtained after removing the liquid containing the water-soluble components from soybeans.

Distilled water was added to the fresh okara to adjust the okura concentration in the growth medium to 4.5%. The growth medium (30 mL), containing 4.5% of okara, was placed in a 150-mL Erlenmeyer flask and sterilized at 121 °C for 20 min. The sterilized growth medium was inoculated with 1% (v/v) of the *B. subtilis* culture suspension and incubated at 40 °C with shaking at 150 rpm for 96 h. Four milliliters of the culture broth was

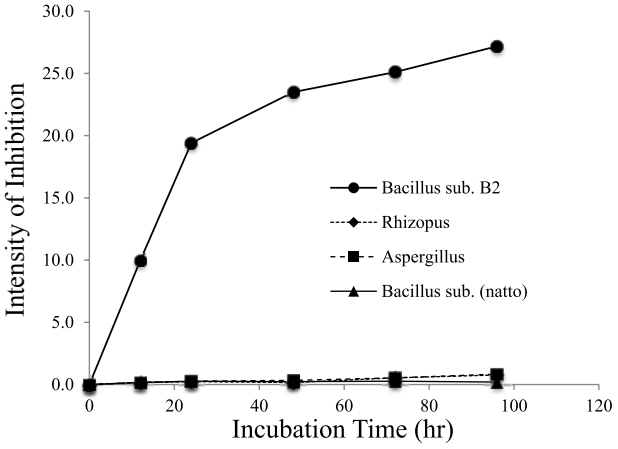

Fig. 1. α-Glucosidase inhibitory activity of fermented okara broth by some microorganisms

Values represent the mean of duplicate assays from a single fermentation. The intensity of inhibition is expressed as the slope of the regression curve using the dilution method of measuring α-glucosidase activity.

taken from the flask after incubating for 4, 12, 24, 48, 72, and 96 h for analysis. To analyze α-glucosidase inhibition activity, the culture broth was centrifuged at 3000 ×*g* for 15 min at 4 °C, and the supernatant was filtered through a 0.45-μm membrane. The filtrate was collected as the fermentation broth and analyzed for α-glucosidase inhibitory activity.

To prepare a sample large enough for purification and to identify the α-glucosidase inhibitor, 1 L of sterilized growth medium containing 4.5% of okara was prepared and fermented for 72 h at 40 °C with shaking at 150 rpm. After 72 h, the fermented liquid was centrifuged at 3000 ×*g* for 15 min at 4 °C, and the supernatant was filtered through a 0.45-μm membrane under a vacuum. The filtrate was immediately freeze-dried for further purification and analysis.

The inhibitory activity of the fermentation broth against α-glucosidase was determined by the reaction between α-glucosidase and 4-NPG, according to the protocol using a 96-well plate (flat bottom wells). The fermentation broth was serially diluted with an equal volume of distilled water and dispensed into the wells of the plates (20 μL/well), whereupon 50 μL of rat intestine powder suspension (25 mg/mL), 50 μL of 4-NPG (0.91 mg/mL) as substrate, and 120 μL of phosphate buffer (pH 6.7) were added. The mixture was incubated at 37 °C for 45 min to allow the reaction between α-glucosidase and 4-NPG to produce 4-nitrophenol. The reaction was terminated by adding 50 μL sodium carbonate (0.67 M). The formation of 4-nitrophenol in each well was measured by the intensity of absorbance at 405 nm using a microplate reader. The α-glucosidase inhibitory activity of the fermentation broth was then computed as the slope from the absorbance curve versus the concentration of the fermentation broth. A higher slope indicates stronger α-glucosidase inhibitory activity of the fermentation broth.

Purification and identification of the α-glucosidase inhibitor in the fermented samples were performed according to the following methods.

(1) Ethanol precipitation

The lyophilized powder of the fermented liquid sample was resolved in 10 mL distilled water and adjusted to 80% ethanol. To precipitate the polysaccharide and protein sufficiently, the sample in 80% ethanol solution was placed at 4 °C for 1 h. After centrifuging, the corresponding supernatant was concentrated to 5 mL by vacuum evaporation and immediately freeze-dried to obtain the lyophilized sample for the next purification step.

(2) Dialysis

Two types of dialysis membrane were used for the current purification: 100-Dalton (Da) and 1000-Da molecular weight cutoff (MWCO) from Spectra/Por Biotech Cellulose Ester (CE) Dialysis Membrane Tubing, Spectrum Labs, Inc., USA). The lyophilized sample after ethanol precipitation was resolved in 5 mL distilled water and filtered under a vacuum with a 0.45-μm membrane (Millex-HX; Millipore, MA, USA). The filtrate was dialyzed using the membrane with 100-Da MWCO, and the dialysate was exchanged at regular intervals of 2, 6, and 12 h respectively. The dialysate containing compounds with molecular weight (MW) below 100 Da were collected and mixed and then concentrated to a lyophilized powder to analyze the α-glucosidase inhibitory activity. The dialyzed sample was transferred to the dialysis membrane with 1000-Da MWCO. The dialysis process was equivalent as with the 100-Da MWCO. The dialysate and dialyzed solution were collected and freeze-dried for further analysis and purification.

(3) Active charcoal chromatography

Activated charcoal was packed in a column. Before applying the sample, the column was equilibrated with distilled water. After applying the sample, 5–30% of ethanol solution was used for the gradient wash. The eluted solution was collected after an interval of 5 min, and each collected fraction was analyzed for α-glucosidase inhibitory activity. Fractions with high α-glucosidase inhibitory activity were combined and freeze-dried for further steps.

(4) Carboxymethyl (CM)-sepharose chromatography

CM-sepharose chromatography was used to purify large amounts of sample using a formic acid solution with a fixed pH and at a flow rate of 5 mL/min. The eluted fractions with strong α-glucosidase inhibitory activity were then combined and dried for further purification.

(5) Preparative thin layer chromatography (TLC)

The active sample was resolved in a small amount of methanol-water solution (1:1) and spotted on the preparative thin layer plates (Silica gel 60 F254, 2 mm; Merck Ltd., Germany). The plate was developed with 3 runs of a 30:30:10 chloroform:methanol: ammonium solution. The area of silica with α-glucosidase inhibitory activity was collected, and water was used to extract the active compound bound to the silica. The extraction water containing the active compound was then immediately freeze-dried for further analysis.

(6) Purity determination

The purity of the active compound was determined by both the TLC and high-performance liquid chromatography (HPLC) methods. The purified sample was spotted on thin layer plates (Silica gel 60 F254, 0.05 mm; Merck Ltd., Germany), accompanied with DNJ as a reference standard. Plates were developed in a 30:30:10 chloroform:methanol:ammonium solution, followed by

heating for a few minutes in a 130 °C oven after spraying the plates with a ninhydrin solution.

HPLC analysis was modified according to a method with an amide-type column coupled to an evaporative light scattering detector (ELSD). A TSK-Gel-amide-80 column (4.6 × 250 mm, 4 μm; Tosoh, Tokyo, Japan) was used in the Shimadzu LC-10A HPLC system. Separation was performed using a mixture of acetonitrile and distilled water (81:19, v/v, containing 6.5 mM ammonium acetate; pH 5.5). The flow rate was adjusted to 1 mL/min, and the column temperature was maintained at 70 °C. The eluate was split post-column, with one portion of the eluate sent to a SEDEX 55 ELSD detector (Sedere, Alfortville, France). The conditions were optimized for maximum sensitivity.

3. Identification of the α-glucosidase inhibitor structure

(1) Positive mass-mass spectrometry (MS-MS)

Electrospray ionization (ESI) mass spectra were recorded on an LCQ Classic mass spectrometer (Thermo Fisher Scientific, Waltham, MA) in the positive- and negative-ion modes. Fast atom bombardment (FAB) mass spectra were recorded on a HX-110/110A mass spectrometer (JEOL Ltd., Tokyo, Japan) in the positive-ion mode. The MS equipment used was an LCQ spectrometer (Thermo Fischer Scientific) with an ESI interface. MS was carried out in the positive-ion measurement mode. The sample was resolved in an acetonitrile-water solution (1:1, v/v). Full scan spectra were obtained by scanning masses with m/z between 50 and 500.

(2) Nuclear magnetic resonance (NMR) spectroscopy

The TLC fraction (1 mg) was dissolved in D_2O (1.0 mL), and nuclear magnetic resonance (NMR) spectra were measured at 298 K on an Avance 800 spectrometer (Bruker Biospin, Karlsruhe, Germany). The active component of this fraction was identified in comparison with an authentic DNJ using ^{1}H-NMR, ^{13}C-NMR, and two-dimensional NMR, including double-quantum-filtered correlation spectroscopy, total correlation spectroscopy, heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond correlation. One milligram of purified lyophilized sample was resolved in D_2O and analyzed by proton NMR. ^{1}H-NMR, ^{13}C-NMR, and HSQC-NMR were performed.

(3) α-Glucosidase inhibitory activity of the fermented okara

A previous study showed that the fermentation broth of *B. subtilis B2* in LB, with added okara as the additional nitrogen source and exhibiting strong α-glucosidase inhibitory activity might be a strategy for preparing functional foods for diabetic patients[20]. The initial unfermented okara exhibited minimal α-glucosidase inhibitory activity, while the activity of the fermented okara broth increased gradually over a prolonged fermentation period. The α-glucosidase inhibitory activity increased drastically during the first 48 h of fermentation and continued to increase slightly from 48 to 96 h. The fermentation time selected for further large-scale preparation of the α-glucosidase inhibitor was 48 h.

(4) Purification of the α-glucosidase inhibitor

The lyophilized powder from the supernatant and the precipitate formed after ethanol precipitation were resolved in 5 mL distilled water, and their α-glucosidase inhibitory activities were determined. The supernatant retained approximately 83% activity, while the α-glucosidase inhibitory activity of the precipitate, containing mostly protein and polysaccharide, was minimal (data not shown), indicating that most of the active compound remained in the supernatant and is likely not a protein or polysaccharide.

After dialysis using the 100-Da and 1000-Da MWCO membranes in sequence, the samples were separated into 3 parts: part I contained compounds with MW less than 100 Da, part II contained compounds with MW from 100 to 1000 Da, and part III contained the compounds with MW exceeding 1000 Da. Each part exhibited α-glucosidase inhibitory activity. Comparatively, part II showed an α-glucosidase IC_{50} value of 13 μg/mL, which was much lower than activities exhibited by parts I and III, with 250 and 64 μg/mL, respectively (data not shown). Accordingly, part II was used for further purification to obtain the α-glucosidase inhibitor.

In active charcoal chromatography, the α-glucosidase inhibitory activity of the eluate was checked from a gradient ethanol concentration of 10%. During the following 200 min, the active compounds were washed off continuously and eluate with high activity was concentrated and applied to the CM-sepharose ion-exchange column. Most of the active compounds were washed off using a formic acid solution with pH 3.7.

After preparative TLC separation, the IC_{50} of the sample reduced to 0.2 μg/mL, and the final recovery was 40.3%.

(5) Purity determination

The DNJ standard and sample were compared by performing HPLC chromatography. The retention time of the main peak in the sample was 17.2 min, which resembled that of the DNJ standard.

TLC chromatography showed that the Rf value of the purified sample was almost identical to that of the DNJ standard. Based on these Rf values, the active compound in the purified sample may be DNJ, but is inconclusive. MS and NMR analyses were then performed to

identify the accurate structure of the active compound.

(6) Identification of the structure of the α-glucosidase inhibitor

The mass spectrum showed a peak at m/z = 164.1 (M+1) (Figure 2). The chemical shifts observed by ^{1}H-NMR and ^{13}C-NMR were compared with those of the DNJ standard. These data show that the purified active compound is identified as DNJ.

The α-glucosidase inhibitory activity could be produced by *B. subtilis B2* using only okara as the culture. The active compound was purified by chromatographic steps and identified as DNJ using MS and NMR analyses. Although a few researchers reported DNJ synthesized by *Bacillus* spp. isolated from soil[13,15], this is the first report Bacillus capable of producing DNJ isolated from food sources. Fermentation of okara by *B. subtilis B2* might be used to produce food-derived DNJ products as functional foods for diabetic patients. Recently it was reported that DNJ had not only anti-glucosidase activity but also adiponectin and GLUT4 expression activities[9]. Therefore the fermented foods contained with the DNJ are anticipated as overall function foods for diabetes and obesity.

Detection of angiotensin-converting enzyme inhibitory activity from fermented milk products for future anti-hypertension foods

Many studies have been performed concerning the role of food in enhancing the quality of life. It has been found that many food components (e.g. active peptides and proteins from milk or eggs, various flavonoids, and related compounds from vegetables or cereals) have properties that improve the quality of life.

Angiotensin-converting enzyme (ACE) is the main target enzyme in blood pressure regulation, and the key enzyme responsible for converting angiotensin I to II. Angiotensin II is a very strong vasoconstrictor, which increases blood pressure and causes hypertension. There are many papers reporting the isolation and detection of anti-hypertensive peptides from milk and fish. Moreover, it has also been reported that sardine peptides have a strong inhibitory activity on ACE, based on a study of alkaline protease-hydrolyzed sardine muscle[11]. Identification of isoleucine-proline-proline (IPP) and valine-proline-proline (VPP) as anti-hypertensive peptides from

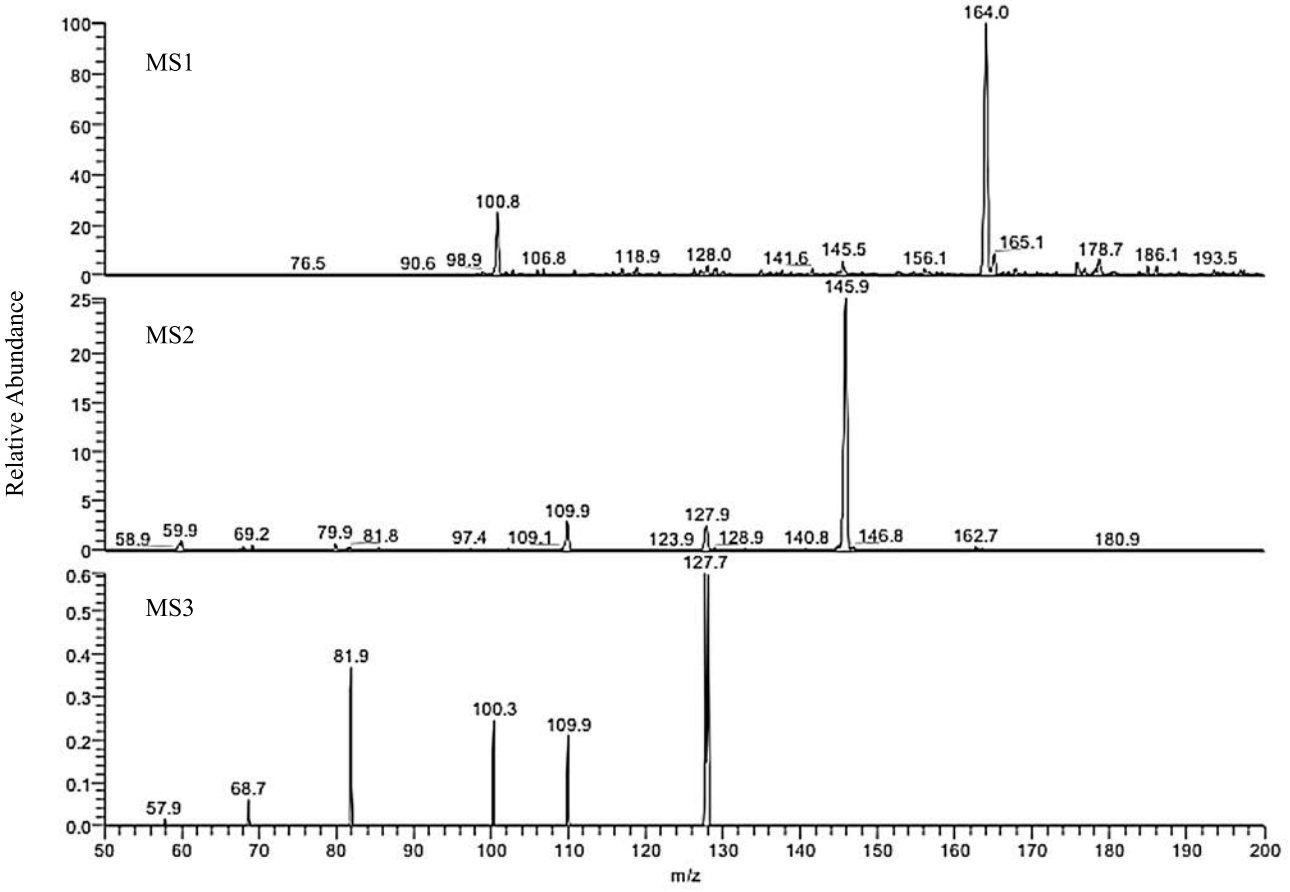

Fig. 2. Full scan MS-MS spectrum of the active compound. MS1 is the original one
MS2 and MS3 are the fractions of the parent and daughter ions respectively (m/z = 164 and 146).

sour milk was also reported [14]. Zhu *et al.* recently reported the identification of other active peptides from tryptic casein hydrolysate using a new purification method [18]. From egg white protein, a novel ACE inhibitory peptide (glutamine-isoleucine-glycine-leucine- phenylalanine, QIGLF) was also recently detected[17]. ACE inhibitory peptides isolated from the milk of other animals, including the yak[7], goat[4], and sheep[5], have been reported. In particular, it has been reported that a few peptides in mare milk have strong inhibitory activity on ACE[2].

Traditional foods, which contain many unidentified active compounds, are a key source of functional foods. In addition, Mongolian traditional foods may have newfound benefits for human health. In rural Mongolian areas in particular, about 60 products, including traditional fermented milk products and essential foods for native Mongolians, are consumed on a daily basis.

It is well known that there are many types of fermented milk products in local areas of Mongolia; typically aaruul, eezgii, and byaslag. Aaruul is made from the milk of various species, including the cow, mare, and yak.

The following steps are involved in manufacturing aaruul:

(1) Raw milk mixed with alkali is heated to 80–85 °C and then mixed well; (2) the partially skimmed milk is fermented by lactic acid bacteria, more milk is added, and alcoholic fermentation is performed; and (3) the residue is squeezed and then sun-dried. The product thus obtained is called aaruul. Eezgii and byaslag are also made from the milk of various species. Boiled milk is mixed with acidic yogurt and after curdling, the supernatant is removed. The dried product thus formed is called byaslag. If the mixture is condensed by heating (without removing the supernatant) and then dried, eezgii is produced.

Following a sequence of complicated steps such as these, various microorganisms are cultured by classical fermentation methods. In the Mongolian area, mare milk is preferred to produce such dairy products and is always used by the local aborigines for their dairy products or as a direct drink. Milk from mares is produced in lesser quantities than that from cows and is thus considered a very rare product worldwide. In this section, studies on the ACE inhibitory activity of Mongolian milk products were performed.

1. Preparation of solubilized extracts from samples and their IC_{50} measurements

Mongolian milk products — aaruul, eezgii, and byaslag — were purchased from a local market in Ulaanbaatar, Mongolia. The samples were pulverized into small particles and mixed with distilled water, whereupon the mixture was homogenized and centrifuged and the supernatant was filtered and used to study inhibitory activity. The ACE inhibitory activity (IC_{50} value) was assayed using a commercially available ACE kit, the WST® assay kit (Dojindo Co. Ltd.).

2. Purification of fractions with ACE inhibitory activity by HPLC

The dissolved solution was applied to a Capcell Pak C_{18} MG MS-II column (4.6 × 250 mm; Shiseido Co., Tokyo, Japan) and separated by reverse-phase high-performance liquid chromatography (RP-HPLC). Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in filtered, deionized water, while solvent B was 0.1% (v/v) TFA in acetonitrile. A linear gradient from 0 to 75% of solvent B was applied at a flow rate of 1.0 mL/min for under 75 min. Active fractions were applied to the column a second time for further purification, and fractions were subsequently assayed by mass spectrometry.

3. Mass spectrometry analysis for molecular mass identification

The molecular masses of the purified ACE inhibitory compounds were determined using ESI Fourier transform ion cyclotron resonance mass spectrometry (ESI FTICR MS). All experiments were performed on a Bruker Daltonics APEX Ultra FTICR mass spectrometer (Billerica, MA, USA) equipped with a 7.0 T actively shielded superconducting magnet.

4. Detection of active fractions and identification of the active components

The ACE inhibitory activity of various traditional milk products, including 6 samples of aaruul, 3 samples of eezgii, and 2 samples of byaslag, were estimated using the assay kit. Aaruul is a type of coagulated milk produced in Mongolia, eezgii is a kind of cottage cheese also produced there, and byaslag is a different kind of Mongolian cheese. The IC_{50} value of the aaruul samples ranged from 2.59 to 5.30 mg/mL, while that of eezgii was between 4.82 and 28.04 mg/mL, and that of the byaslag samples ranged from 123.37 to 141.47 mg/mL. It has been previously reported that many peptides in sour milk have strong ACE inhibitory activity. The IC_{50} of the active peptide VPP, which is common in sour milk, is approximately 9 μmol/L [14], while that of the flavonoid kaempferol-3-*O*-galacto pyranoside is approximately 260 μmol/L [10]. Therefore, it seems likely that these IC_{50} values of milk samples considerably exceed those of the milk products. This finding may indicate that the samples have very weak ACE inhibitory activity.

There are many types of fermented milk products in Mongolia, and differences in the IC_{50} value between the different types of milk products were compared. Relatively high IC_{50} values (4.02 ± 0.81 [mean ± standard deviation] mg/mL) were found in the aaruul (mare) group. Aaruul (cow) showed almost equivalent inhibitory activity at 4.05 ± 1.37 mg/mL whereas that of eezgii (cow) was 14.36 ± 12.15 mg/mL. The lowest IC_{50} (132.42 ± 12.80 mg/mL) was found in byaslag (cow). These differences may depend on the fermentation processes involved. The production of aaruul involves 2 fermentation steps, of lactic acid and alcohol respectively. Conversely, the production of eezgii and byaslag involves only a single fermentation step.

Purification of active components from aaruul (mare) was attempted. To purify the active components from aaruul, ultrafiltration was performed using a centrifuging type ultrafilter membrane (Sartorius Vivaspin® 3000-Da MWCO). The inhibitory activity of the low MW fraction (bottom layer, MW <3000 Da) exceeded that of the high MW fraction (upper layer). The lower MW fraction of aaruul was then selected for the next purification step, in which the active components were purified using reverse-phase HPLC.

In HPLC chromatogram, many peaks were detected by UV at 210 nm (Figure 3). The first fraction, "No. 1" (with a retention time of around 5 min), exhibited the highest absorbance of all fractions in the HPLC chromatogram. Normally, the initial peak (retention time is 4-5 min) may be contaminated with some compounds not absorbed on the HPLC column. Fraction No. 1 contained little protein content and was thus not used for the next purification step. Instead, the second active fraction, "No. 2," with a retention time of around 11 min, was selected as the target fraction to identify active components by mass spectrometry.

Based on the result of ESI FTICR MS, 2 main sample peaks were detected (Figure 4). According to the MW and other data, the highest peak of 362.05 Da was identified as 5′-GMP, while the second highest peak of 346.06 Da corresponded to 5′-AMP. Furthermore, the retention time of 5′-GMP was determined by reverse-phase HPLC under equivalent conditions, and the 5′-GMP standard was detected at an almost equivalent retention time.

The ACE inhibitory activity of nucleotide compounds identified as active components of aaruul were determined. Authentic standard 5′-monophosphates of various nucleotides were tested using the ACE inhibitory assay kit. We used 5′-AMP, 5′-CMP, 5′-GMP, and 5′-IMP as test samples. The IC_{50} of 5′-GMP was the highest among the agents tested (IC_{50} = 6.2 mmol/L), followed by 5′-CMP (IC_{50} = 14.7 mmol/L) and 5′-IMP (IC_{50} = 24.7 mmol/L), while the weakest agent was 5′-AMP (IC_{50} = 146.8 mmol/L). From the IC_{50} experimental results and data from MS and HPLC, it was evident that 5′-GMP was the only ACE inhibitor found in the Mongolian aaruul (mare). Generally, mare milk is more concentrated than milk from other species, such as the cow, yak, and sheep. In addition, the production steps of Mongolian milk products require a longer fermentation period. For these reasons, milk products obtained from mares contain more active components than other daily products. 5′-GMP was a weak ACE inhibitor in the aaruul (mare); however, this is the first report that 5′-AMP is an ACE inhibitor. There may be potential to make new anti-hypertensive foods from such dairy products, especially from Mongolian fermented mare milk products.

This report on the inhibitory effect of 5′-GMP on ACE is likely very valuable as it is the first of its kind, even though the determined inhibitory activity is very low. It is thought that some nucleotides in the mare milk product are due to microorganisms such as lactic acid bacteria, yeast, edible fungi, etc. It seems that milk products without filtration have more nucleotides, and aaruul is made from fermented milk without filtration. However, when we take aaruul as a normal daily food, signifi-

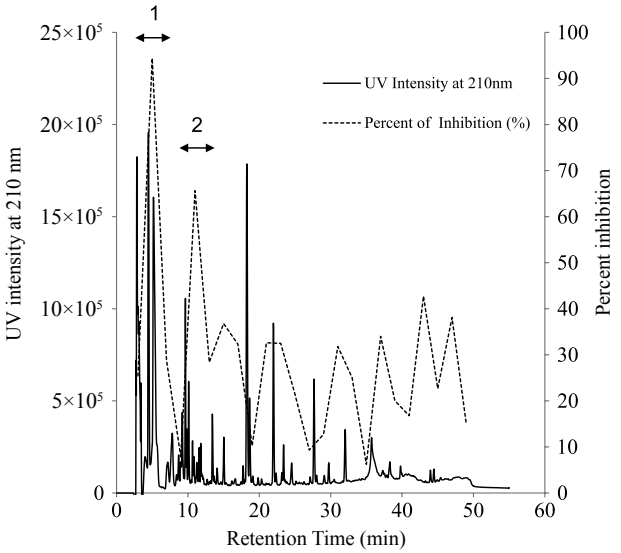

Fig. 3. HPLC chromatogram of aaruul derived from mare milk and the activity of ACE inhibition

The solid line shows absorbance at 210 nm, while the dashed line shows the percentage inhibition of ACE activity by these fractions. HPLC was used with a reverse-phase C_{18} column (4.6 × 250 mm, 5 μm) and an acetonitrile linear gradient system (0–50%, 50 min). The percentage inhibition was expressed as the sample activity of 0.3 μg protein equivalent. The arrows indicate the 2 most active fractions.

cant quantities must be ingested to show the inhibitory effect on ACE.

On marine products, the IC50 values of some peptides (4 amino acids) are 21-315 μM, while the IC50 value of the 2 amino acid peptides are 322 and 1214 μM[6]. The activity of 5′-GMP is not much higher than that of the active peptide in sour milk, the marine peptides or some flavonoids. However, this is the first report showing that 5′-GMP has an inhibitory effect on ACE. It is believed that these results will provide crucial information to develop new agents for hypertension therapy.

5. In future

The healthy functionalities of extracts from traditional fermented foods were measured, and identification of the active compound contained in the foods is described in this review. Due to the relatively strong activities in the food samples, the active components could be easily identified. Despite sufficient health function activity in the food samples used, it is often difficult to detect the functional activity under many purification steps. In recent years however, structural determination has become possible using a smaller volume of sample thanks to the development of analytical instruments such as HPLC, mass spectrometry, and NMR. Nevertheless, it is still difficult to precisely identify active component structures (especially isomeric). In future, there is likely to be scope to help produce value-added farm products by suggesting cultivation or processing methods to supplement the components. For example, we could seek active components from farm products and identify them using the assay system described. Moreover, if we can discover a microorganism producing functional components by fermentation, it is very likely that we could create products whose components are more concentrated by fermentation. The study of components of traditional fermented foods is thus especially significant.

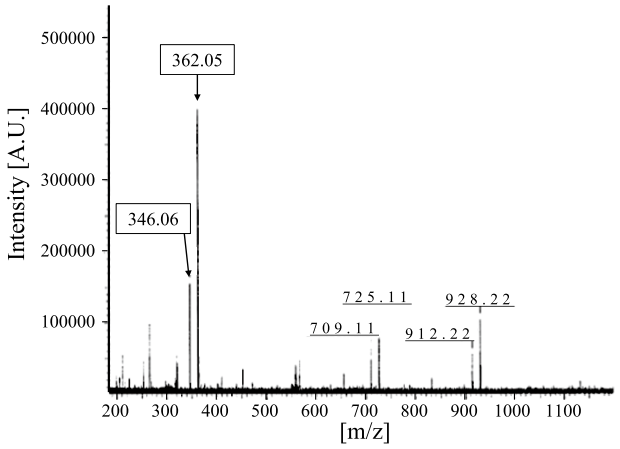

Fig. 4. Mass spectrometry analysis of aaruul-containing mare active components

Fourier transform ion cyclotron resonance mass spectrometry was performed by electrospray ionization. The 2 most active peaks were 362.05 and 346.06 Da, respectively.

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