Determination of Chiral Amino Acids in Various Fermented Products Using a Two-Dimensional HPLC-MS/MS System

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Abstract
Chiral amino acids in fermented products including Japanese traditional black vinegar, a processed cheese and nam pla were determined using an on-line two-dimensional (2D) HPLC-MS/MS system. As the target amino acids, Ala, Asp, Glu, Leu, Pro and Ser were selected. Prior to the HPLC separation, the fermented products were appropriately homogenized and deproteinized, then amino acids were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). Using the 2D-HPLC system, the target NBD-amino acids were individually purified by a microbore reversed-phase column in the first dimension and further separated by a narrowbore enantioselective column in the second dimension. The detection was performed by a fluorescence detector and also by a tandem mass spectrometer. Compared to the 2D-HPLC with fluorescence detection, the target chiral amino acids in complicated real world matrices were successfully determined using the 2D HPLC-MS/MS system without interference by the co-elution of unknown intrinsic compounds. In all the tested fermented products, various D-amino acids were observed, and the obtained values were 0.02-6.21 mmol/L (%D=0.7-29.2%) in the black vinegar, 0.02-0.73 µmol/g (0.2-24.8%) in the processed cheese and 0.07-2.30 mmol/L (0.5-23.5%) in the nam pla.

Keywords: D-Amino acids; Two-dimensional HPLC-MS/MS; Fermented products

1. Introduction
D-Amino acids, the minor stereoisomers of amino acids in the living beings, have been discovered in higher organisms along with the advances in analytical techniques [1-3]. In 1986, the presence of non-negligible levels of D-aspartate (Asp) in rodents and humans was reported [4]. Since then, it has been clarified that D-Asp regulates the hormonal synthesis/secretion, such as testosterone and melatonin, in a variety of endocrine glands [5-7]. D-Serine (Ser) was found in the rat brain in 1992 [8], and has been revealed to play a significant role for acquiring memories by modulating the neuronal transmission via the N-methyl-D-aspartate subtype of the glutamate receptor in the frontal brain area [9,10]. In recent years, it was demonstrated that D-Asp has an anti-oxidative effect and D-alanine (Ala) was related to the repair/maintenance of the basement membrane in the skin [11]. These findings have indicated that D-amino acids have physiological activities in mammals, and might have beneficial effects on our healthy lives.

As for the origins of the D-amino acids in mammals, several sources including enzymatic synthesis, dietary uptake and production by the intestinal microbes were reported [2]. D-Amino acids are synthesized by enzymes (such as the racemase) expressed in bacteria, archaea and eukaryotes including humans [12-14]. Although the amino acid racemase discovered in humans is only the Ser racemase until now, the intestinal microorganisms produce various D-amino acids by a variety of enzymes [15,16]. Therefore, the other origins (derived from diet [17-21] and intestinal microbes [15,16,22]) are significant for most of the D-amino acids observed in mammals. Because the orally administrated D-amino acids are able to be distributed in various tissues [23], taking foods and/or beverages which

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Received: 28 April 2019
Accepted: 21 May 2019
J-STAGE Advance Published: 30 May 2019
DOI: 10.15583/jpchrom.2019.011
naturally contain high levels of D-amino acids is considered to be an effective way to ingest these D-amino acids as functional ingredients. In particular, the products fermented by microorganisms are considered to contain large amounts of various D-amino acids, and the analysis of chiral amino acids in various fermented products is highly required.

For the determination of the amino acid enantiomers, a variety of chromatographic methods using chiral derivatizing reagents, chiral stationary phases and chiral mobile phases has been reported [2,24]. Among the previously reported analytical techniques, the combination of the reversed-phase separation (the purification of target amino acids as the D plus L mixtures) and the enantioselective separation by a chiral stationary phase (two-dimensional (2D) HPLC analysis) is one of the most sensitive and selective approaches [2,3,25-29]. The combination of the one-dimensional chromatographic separation and detection by a tandem mass spectrometer (LC-MS/MS) is also widely used as the highly sensitive and selective analysis [30,31]. However, the determination of chiral amino acids in biological samples is still frequently disturbed by the co-elution of unknown interfering compounds even using the previously mentioned 2D-HPLC or LC-MS/MS methods. Therefore, improvement of the selectivity of the analytical method is still eagerly required for the determination of amino acid enantiomers in real world samples.

One of the practical strategies to overcome the co-elution of unknown compounds is increasing the analytical dimension. For this purpose, we have developed in a previous study an on-line 2D HPLC-MS/MS system having higher selectivity for the determination of 6 amino acid enantiomers including Ala, Asp, glutamate (Glu), leucine (Leu), proline (Pro) and Ser [32]. The 2D HPLC-MS/MS system is considered to be a promising technique for the enantioselective analysis of chiral ingredients in complicated real world samples. In the present study, the proposed 2D HPLC-MS/MS system was applied to the determination of chiral amino acids in a variety of fermented products including Japanese traditional black vinegar, processed cheese and nam pla.

2. Experimental

2.1. Reagents

The D-enantiomer of Asp and L-enantiomers of Ala, Asp, Leu, and Ser were purchased from Nacalai Tesque (Kyoto, Japan). The other D- and L-amino acids were products of FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), the reagent for the pre-column derivatization of amino acids, was obtained from Tokyo Chemical Industry (Tokyo, Japan). Acetonitrile (MeCN) and methanol (MeOH) of LC-MS grade were acquired from Merck (Darmstadt, Germany). Tetrahydrofuran (THF), trifluoroacetic acid (TFA) and formic acid (FA), the mobile phase additives for the HPLC analysis, were the products of FUJIFILM Wako. Water was purified by a Milli-Q Integral 3 system from Merck. All other reagents were of the highest grade and used without further purification.

2.2. Sample preparation

The Japanese traditional black vinegar (Kurozu) made with steamed rice, koji and underground water was obtained from Sakamoto Kurozu, Inc. (Kagoshima, Japan). The processed cheese was purchased from Megmilk Snow Brand Co., Ltd. (Tokyo, Japan). Nam pla, which is a seasoning made from the mixture of anchovies extract, water, salt and sugar, was the product of Monty & Totco Co., Ltd. (Bangkok, Thailand). All of the fermented products analyzed in the present study were commercially available. Black vinegar was diluted 100 times with 400 mmol/L sodium borate buffer (pH 8.0). To the aliquot (20 µL), 5 µL of 200 mmol/L NBD-F in MeCN was added and heated at 60ºC for 2 min. The reaction was stopped by adding 75 µL of an aqueous 0.2% (v/v) TFA 30% (v/v) MeCN solution. The cheese was homogenized in 20-fold volumes of water using a micro homogenizing system (Micro Smash™ MS-100R, Tommy, Tokyo, Japan) at 3,500 rpm for 2 min and centrifuged at 12,000 x g for 10 min. To the supernatant obtained from the cheese-water suspension, 4-fold volumes of MeOH was added and centrifuged at 12,000 x g for 5 min. To 20 µL of nam pla, 20 µL of water and 360 µL of MeOH were added and centrifuged at 12,000 x g for 10 min and the supernatant was diluted 10 times with MeOH. A portion of the supernatant obtained from the fermented products-MeOH suspensions (50 µL was used for the cheese and 10 µL was used for nam pla) was evaporated to dryness under reduced pressure at 40ºC. The residues were dissolved in 10 µL of water, and the amino acids were derivatized by adding 400 mmol/L sodium borate buffer (pH 8.0) and 200 mmol/L NBD-F. After these reaction mixtures were diluted 5 times with water, their solutions (10 µL) were subjected to the HPLC system described in Section 2.3.

2.3. 2D HPLC-MS/MS determination of NBD-amino acid enantiomers

Enantiomers of the NBD-amino acids were determined by the previously reported 2D HPLC-MS/MS system [32] combining a reversed-phase separation, an enantioselective separation and the detection using a tandem mass spectrometer (Triple Quad™ 5500, Sciex, Framingham, MA, USA) with slight modifications. The target NBD-amino acids were separated from other compounds by a microbore reversed-phase column (KSAARP, 1.0 x 500
mm, 45°C, an original C18 column produced by collaboration with Shiseido, particle size 3 µm) in the first dimension. As the mobile phases for the reversed-phase separation, aqueous 10% (0-180 min), 25% (180-330 min) MeCN solutions containing 0.05% TFA and an aqueous 28% MeCN 2% THF solution containing 0.05% TFA (330-450 min) were pumped by the stepwise gradient elution mode (25 µL/min). Elution of the NBD-amino acids in the first dimension was monitored by the absorbance at 470 nm, and the target fractions were collected in an on-line loop-transfer device having 6 loops. All of the fractionated NBD-amino acids were automatically introduced into the second dimension, and the enantioseparations were separated by an original enantioselective column (KSAACSP-001S, 1.5 x 250 mm, 25°C, produced by collaboration with Shiseido, particle size 5 µm). As the mobile phases for the enantioselective separation of NBD-Asp, Glu and Leu, mixtures of MeCN-MeOH (75/25, v/v) containing 0.2% FA was used (150 µL/min). The eluted NBD-amino acid enantiomers were detected by a fluorescence detector (ex. 470 nm, em. 530 nm) and also by a tandem mass spectrometer.

As for the MS/MS conditions (positive-ion mode), the precursor and product ion pairs for NBD-Ala, Asp, Glu, Leu, Pro and Ser were 253/190, 297/173, 311/230, 295/233, 279/217 and 269/206, respectively. The ion spray voltage, turbo gas temperature and entrance potential were 5500 V, 700°C and 10 V for all the NBD-amino acids. The curtain gas pressure was 25 psi (172 kPa) for NBD-Ala, 20 psi (138 kPa) for NBD-Asp/Glu/Ser, 30 psi (207 kPa) for NBD-Leu and 35 psi (241 kPa) for NBD-Pro. The ion source gas 1 was 80 psi (552 kPa) for NBD-Asp/Glu/Ser, 60 psi (414 kPa) for NBD-Pro and 70 psi (483 kPa) for NBD-Pro. The collision gas pressure was 8 psi (55 kPa) for NBD-Ala/Glu/Pro and 10 psi (69 kPa) for NBD-Asp/Leu/Ser. The declustering potential for NBD-Ala, Asp, Glu, Leu, Pro and Ser was 121, 116, 131, 156, 91 and 91 V, respectively, and the collision energy was 21, 37, 21, 29, 27 and 31 eV, respectively. As the collision cell exit potential, 20 V was selected for NBD-Ala/Asp/Glu, 16 V for NBD-Leu/Pro and 24 V for NBD-Ser.

3. Results and discussion

The amounts of the NBD-amino acids in the fermented products were determined using the 2D HPLC-MS/MS system with the conditions described in Section 2.3. Typical chromatograms obtained by the analysis of a

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Fig. 1. Reversed-phase separation of NBD-amino acids in Japanese traditional black vinegar (A). The reagent peak is indicated by an asterisk. Enantioselective separations of 6 target amino acids detected by an MS/MS (B) and x20 magnified chromatograms (C). Japanese traditional black vinegar sample are shown in Fig. 1. In the first dimension, the reversed-phase separation was performed by an ODS column, KSAARP (1.0 x 500 mm), to isolate the target NBD-amino acids from the other intrinsic compounds within 450 min. Although a variety of peaks was observed in the black vinegar in the first dimension, all of the target fractions were collected at the same retention times of the standard NBD-amino acids represented by the closed bars (shown in Fig. 1A), and on-line transferred to the next dimension. In the second dimension, all of the NBD-amino acids were separated into their d-forms (former peak) and L-forms (latter peak) by an original Pirkle-type column, KSAACSP-001S (1.5 x 250 mm), in about 30 min. The target NBD-amino acids were detected by the fluorescence detector and also by the tandem mass spectrometer after the enantioselective separations. By adopting the MS/MS (2D HPLC-MS/MS) technique, the interfering peaks derived from the intrinsic compounds were hardly observed even in the 20-times magnified chromatograms, and the target chiral amino acids were successfully determined. Without using MS/MS as a detector (2D-HPLC followed by the fluorescence detection),
several unknown peaks were still observed besides the peaks of the NBD-amino acids in many cases having the risk to interfere the determination of the target analytes (Fig. 2).

Using the present 2D HPLC-MS/MS system, the concentrations of the target chiral amino acids in a variety of fermented products were determined and the obtained values are summarized in Table 1. In the Japanese traditional black vinegar sample fermented in the earthenware jar, high levels of various D-amino acids were found besides the L-amino acids. The amounts of the target L-amino acids were 1.54-15.05 mmol/L, while the amounts of the d-amino acids were 0.02-6.21 mmol/L. The obtained %D values (D/(D+L) x 100) of Ala, Asp, Glu, Leu, Pro and Ser were 29.2, 19.6, 10.6, 0.7, 1.0 and 5.5%, respectively. In the processed cheese sample, the concentrations of the L-amino acids were also high, and most of the d-amino acids (except for D-Pro) were observed. Especially, the %D values of Ala and Asp were high (24.8 and 18.5%, respectively). For the nam pla sample, besides the high concentrations of the L-amino acids, a large amount of D-Pro (23.5%) was found. To confirm the approximately equal %D values were obtained in all cases.

Until now, the existence of D-amino acids including Ala, Asp, Glu, Leu, Pro and Ser has been investigated in various fermented foods and beverages [17-21]. By using this system, a wide range of target amino acid enantiomers (Ala, Asp, Glu, Leu, Pro and Ser) were successfully determined without any visible co-elution (Fig. 2) and the obtained values in the fish souses made in Japan, China and Thailand were 2.96-3.19, 2.76-2.89 and 0.66-0.70 mmol/L, respectively [19]. In the Japanese black vinegar, all of the proteinogenic chiral amino acids were determined by employing a method combining ultra-high performance liquid chromatography and detection using circular dichroism [20] and also by using a 2D-HPLC-FL system [21]. These reports described that the values of d-Ala, Asp, Glu, Leu, Pro and Ser in the Japanese black vinegar were 45.1-3898 (%D=1.1-38.3%), 27.9-395 (1.3-35.3%), 22.8-420 (1.0-21.0%), less than 10 to 46.8 (0.5-0.8%), less than 10 to 80.6 (1.1-2.6%) and 17.5-95.9 µmol/L (0.9-10.0%), respectively. In the present study, the amounts of the target D-amino acids in the Japanese traditional black vinegar, a processed cheese and nam pla were analyzed using the 2D HPLC-MS/MS system and the obtained values are roughly consistent with those in previous reports. Since the target amino acid enantiomers were successfully determined without any visible co-elution of unknown compounds in the present study, the 2D HPLC-MS/MS system is useful as a practical tool for the simultaneous determination of chiral amino acids in real world samples. Further applications of the present 2D HPLC-MS/MS system to various food/biological/clinical samples are currently ongoing.

4. Conclusion A highly-selective on-line 2D HPLC-MS/MS system combining the reversed-phase separation, enantioselective separation and determination using a tandem mass spectrometer, was applied to the simultaneous analysis of chiral amino acids in various fermented products. By using this system, a wide range of target amino acid enantiomers (Ala, Asp, Glu, Leu, Pro and Ser) were successfully determined in Japanese black vinegar, processed cheese and nam pla without any interference by intrinsic substances. The results obtained in the present...
study indicate that the 2D HPLC-MS/MS system is practically useful for the precise determination of chiral amino acids in complicated matrices such as foods and beverages. D-Amino acids have recently been the focus as physiologically active molecules, and further applications of the present 2D HPLC-MS/MS system to various natural products are expected.

Acknowledgement
This study was partly supported by JSPS KAKENHI Grant Number 15H05749 and the JSPS Research Fellowships for Young Scientists (C.I.). The authors appreciate Shiseido Co., Ltd. (Tokyo, Japan) for their technical support and funds.

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