Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–5, 2017

www.elsevier.com/locate/jbiosc

Investigation of storage time-dependent alterations of enantioselective amino acid profiles in kimchi using liquid chromatography-time of flight mass spectrometry

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Received 5 March 2017; accepted 27 April 2017
Available online xxx

Although naturally abundant amino acids are represented mainly by L-enantiomers, fermented foods are known to contain various D-amino acids. Enantiospecific profiles of food products can vary due to fermentation by bacteria, and such alterations may contribute to changes in food properties that would not be dependent exclusively on L-amino acids. Therefore, more attention should be paid to the study of temporal alterations of D-amino acid profiles during fermentation process. However, there have been very few studies reporting time-dependent profiling of D-amino acids because enantioseparation of widely targeted D-amino acids is technically difficult. This study aimed to achieve high throughput profiling of amino acids enantiomers. Enantioselective profiling of amino acids using CROWNPAK CR-I(+) column, liquid chromatography, time of flight mass spectrometry, and principle component analysis was performed to investigate time-dependent alterations in concentrations of free D- and L-amino acids in kimchi stored at 4°C or 25°C. We demonstrated significant changes in D- and L-amino acid profiles in kimchi stored at 25°C. In particular, concentrations of the amino acids D-Ala, D-Ser, D-*allo*-Ile, D-Leu, D-Asp, D-Glu, and D-Met became higher in kimchi with storage time. This is the first report of time-dependent alterations of D- and L-amino acid contents in kimchi. This study showed that our analytical method of enantioselective detection of amino acids using liquid chromatography time-of-flight mass spectrometry (LC-TOFMS) with CROWNPAK CR-I(+) enables high throughput food screening and can be recommended for advanced studies of the relationship between D-amino acid content and food properties.

[**Key words:** D-Amino acids; Enantioselective analysis; Time-dependent profiling; Food analysis; High throughput profiling; Metabolomics; Kimuchi]

Amino acid content is an important nutritional characteristic of various foods. Thus, determination of amino acids is routinely performed as a part of food component analysis (1,2). Most amino acids may exhibit different biological properties, depending whether they are D- or L-enantiomers. However, the contribution of D-amino acids to various biological processes has been almost completely ignored because of their low occurrence. Nevertheless, some fermented foods are known to include high amounts of D-amino acids (3,4), and some microorganisms are reported to produce several D-amino acids. D-Amino acid content may be altered during the storage process due to the fermentation by living microorganisms. Thus, enantioselective amino acid analysis would be useful for elucidation of time-dependent alterations of D- and L-amino acid profiles in fermented foods. However, very few studies on time-dependent changes of D-amino acid profiles in fermented foods have been reported, likely due to the technical difficulty of such analysis. D- and L-amino acids have almost the same physico-chemical properties, except for optical rotation. Previously, chiral high performance liquid chromatography (HPLC) technique was developed to enable separation of several target amino acids. However, simultaneous enantioseparation widely targeted amino acids is still difficult, especially in natural samples, such as foods or biological fluids, due to impurities (5–7). At present, two-dimensional liquid chromatography coupled with pre-derivatization is often used as a promising solution for removing crucial sample impurities in enantioselective exhaustive profiling of D- and L-amino acids (8). However, this method is not a realistic platform for food screening due to its extremely low throughput.

Recently, we developed an extra facile method for D- and L-amino acid high throughput profiling (9). This new method allowed for simultaneous analysis of all optically active proteinogenic amino acids, except for proline, using a chiral column, liquid chromatography (LC), and time-of-flight mass spectrometry (TOFMS). In addition to producing good peak shapes and providing high resolution in chromatography, this method only requires 10 min per run. Thus, combined with an established method of sample preparation for quantitative analysis, it can provide a high throughput method for exhaustive profiling of D- and L-amino acids in food samples.

In the present study, we examined practical utility of our novel analytical method to distinguish between D- and L-amino acids. In particular, we determined whether it can detect time-dependent alterations of enantiospecific amino acid profiles in fermented food. Kimchi, a Korean traditional food made by fermenting vegetables using microorganisms, such as *Lactobacteria* (10), was selected as the target fermented food for this study. Previously, we revealed that kimchi contains many D-amino acids at relatively

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1389-1723/$ – see front matter © 2017, The Society for Biotechnology, Japan. All rights reserved.
http://dx.doi.org/10.1016/j.jbiosc.2017.04.019

higher concentrations compared to other fermented foods (9). Because kimchi products include living microorganisms, a drastic time-dependent enantio-modulation of D- and L-amino acids during kimchi storage is expected. However, to the best of our knowledge, such changes have not been studied previously. Therefore, this study is the first report of significant time-dependent alterations of exhaustive D- and L-amino acid profiles in kimchi during storage.

MATERIALS AND METHODS

Kimchi sample Kimchi [Yoshinoya hakusai (Chinese cabbage)] was purchased from Bingo Tsukemono Co., Ltd. (Hiroshima, Japan).

Storage of kimchi Kimchi sample was transferred into four 50-mL conical tubes on the day designated as storage day 0. For the next 28 days, two tubes were stored at 25°C (room temperature), to allow typical biochemical changes in D- and L-amino acid profiles, whereas the remaining two tubes were stored at 4°C (refrigerator temperature) as a control group, in which biochemical changes would be minimal.

DL-Amino acid reagents DL-Alanine (DL-Ala), DL-serine (DL-Ser), DL-valine (DL-Val), DL-threonine (DL-Thr), DL-cysteine hydrochloride monohydrate (DL-Cys), DL-isoleucine (DL-Ile, mixture of four stereoisomers containing DL-*allo*-Ile), DL-leucine (DL-Leu), DL-asparagine monohydrate (DL-Asn), DL-aspartic acid (DL-Asp), DL-glutamine (DL-Gln), DL-lysine monohydrochloride (DL-Lys), DL-glutamic acid (DL-Glu), DL-methionine (DL-Met), DL-histidine (DL-His), DL-phenylalanine (DL-Phe), DL-arginine hydrochloride (DL-Arg), DL-tyrosine (DL-Tyr), and DL-tryptophan (DL-Trp) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). DL-Alanine-2,3,3,3-d_4, used as internal standard (IS), was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Reagents for sample preparation and mobile phase Ultrapure water (Water) for liquid chromatography/mass spectrometry (LC/MS), ethanol (EtOH), 0.1 mol/L hydrochloric acid, and trifluoroacetic acid (TFA) for HPLC were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methanol (MeOH) and acetonitrile (ACN) for LC/MS were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Chloroform for HPLC was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan).

Preparation of DL-amino acid calibration standard solutions and internal standard solution To make amino acid standard solutions, DL-Glu, DL-His, and DL-Trp were dissolved in 50% solution of MeOH in Water (1:1, v/v) that contained 0.02 mol/mL HCl, DL-Asp and DL-Tyr were dissolved in 50% MeOH that contained 0.05 mol/mL HCl, and other amino acids were dissolved in 50% MeOH. DL-Amino acid standard mixture was prepared by combining all DL-amino acid solutions to a final concentration of 1000 nmol/mL. Then, this standard mixture was serially diluted by 50% MeOH to prepare solutions that contained 400, 100, 40, 10, 4, and 1 nmol/mL in 50% MeOH (DL-amino acid calibration standard solutions).

DL-Alanine-2,3,3,3-d_4 was dissolved in 50% MeOH to make a 20 μmol/mL internal standard (IS) solution.

Preparation of kimchi samples Kimchi samples stored at 4°C or 25°C were collected at 0, 3, 7, 14, and 28 days. A piece of Chinese cabbage was taken as a sample and crushed with a spatula before centrifugation at 16,000 ×*g* for 10 min at 4°C to collect only the liquid part of kimchi (kimchi soup). The resulting supernatant was collected and diluted with an equal volume of water. Fifty microliters of the diluted kimchi soup was mixed with 50 μL of 50% MeOH, 10 μL of IS solution, and 150 μL of MeOH before vortex mixing for 10 s. After centrifugation at 16,000 ×*g* for 10 min at 4°C, 180 μL of the supernatant was mixed with 90 μL of water and 180 μL of chloroform, and vortexed for 10 s. After another centrifugation at 16,000 ×*g* for 10 min at 4°C, 40 μL of the upper (MeOH-Water) layer was mixed with 160 μL of ACN and EtOH mixture (8:2, v/v) and vortexed for 10 s. After final centrifugation at 16,000 ×*g* for 10 min at 4°C, the supernatant was transferred to a vial for analysis.

Preparation of calibration samples Fifty microliters of Water, 50 μL of DL-amino acid calibration standard mixture (1000, 400, 100, 40, 10, 4, 1, or 0 nmol/mL in 50% MeOH), 10 μL of IS solution, and 150 μL of MeOH were mixed and vortexed for 10 s. After centrifugation at 16,000 ×*g* for 10 min at 4°C, 180 μL of the supernatant was mixed with 90 μL of Water and 180 μL of chloroform before vortex mixing for 10 s. After another centrifugation at 16,000 ×*g* for 10 min at 4°C, 40 μL of the upper (MeOH-Water) layer was mixed with 160 μL of ACN and EtOH mixture (8:2, v/v) and vortexed for 10 s. After final centrifugation at 16,000 ×*g* for 10 min at 4°C, the supernatant was transferred to a vial for analysis.

Instrument D,L-Amino acids were analyzed using a Nexera system (pump: LC-30AD, autosampler: SIL-30A, degasser: DGU-20A5, column oven: CTO-30A, Shimadzu, Kyoto, Japan) and TripleTOF 5600 System (AB SCIEX, Concord, Canada) with a CROWNPAK CR-I(+) column (Daicel CPI, Osaka, Japan).

LC-TOFMS analysis Extracted sample (1 μL) was injected onto CROWNPAK CR-I(+) column (3.0 mm i.d. × 150 mm; particle size, 5 μm). The mobile phase used was a mixture of ACN, EtOH, Water, and TFA (80:15:5:0.5, v/v/v/v), and the flow rate was 0.4 mL/min. The autosampler and column oven temperatures were maintained at 4°C and 30°C, respectively.

Column eluent was analyzed in positive ionization mode using a TripleTOF 5600 system under the following conditions: ion source gas 1 (50 psi), ion source gas 2 (50 psi), curtain gas 1 (30 psi), temperature (600°C), ion spray voltage floating (5500 V), declustering potential (60 V), collision energy (5 V), and mass range (60–600 *m*/*z*). Data processing was performed using MultiQuant (AB SCIEX).

Statistical analysis Principal components analysis (PCA) was performed with MarkerView (AB SCIEX) using values of peak areas of D,L-amino acids from samples of kimchi stored for 0, 3, 7, 14, or 28 days. Then, each peak area was normalized by the average IS peak area in all kimchi samples. Logarithmic transformation and pareto scaling were performed prior to PCA analysis.

Calculation of amino acid concentrations The regression equation ($Y = aX + b$; with weighting factor of $1/X^2$) was calculated by the least-squares method using MultiQuant. Then, ratios of peak areas (amino acid peak area/IS peak area) obtained from measurements of the calibration samples were used as Y values, and the concentrations of the calibration samples were used as X values. D-Amino acid concentration was corrected by the recovery rates of corresponding D-amino acids in kimchi (Table S1). The recovery rates of amino acids in kimchi samples were estimated by the ratios of the peak area of 1000 nmol/mL amino acid standard solution spiked in kimchi soup to the corresponding peak area of 1000 nmol/mL amino acid standard solutions spiked in Water. As several endogenous amino acids are present in kimchi, their peak area ratios were subtracted before calculating their recovery rates. Then, peak area of amino acids was normalized by the peak area of IS.

RESULTS AND DISCUSSION

Time-dependent profiling of D,L-amino acids in kimchi during storage at 4°C and 25°C.

Our analytical method of enantioselective amino acid detection using LC-TOFMS with CROWNPAK CR-I(+) enables simultaneous analysis of 18 proteinogenic amino acids enantiomers with high resolution and high throughput (9). In this study, time-dependent alterations of D,L-amino acid profiles in kimchi during storage were investigated. Although kimchi contains many impurities, LC-TOFMS method provided reliable enantioselective amino acid profiling of kimchi by separating endogenous impurities, as shown in Fig. 1. Consequently, 12 D-amino acids (D-Ala, D-Ser, D-*allo*-Ile (Fig. S1), D-Leu, D-Asn, D-Asp, D-Glu, D-Met, D-His, D-Phe, D-Arg, D-Tyr) and 17 L-amino acids (L-Ala, L-Ser, L-Val, L-Thr, L-Ile, L-Leu, L-Asn, L-Asp, L-Gln, L-Lys, L-Glu, L-Met, L-His, L-Phe, L-Arg, L-Tyr, L-Trp) were detected in kimchi stored at 25°C for 28 days.

Principle component analysis PCA is a non-supervised clustering method that enables a reduction of dimensions of multivariable data and simultaneous maintenance of the variance using synthetic variables called principle components (PCs) (11). In the present study, peak areas of D- and L-amino acids obtained by LC-TOFMS analysis were subjected to PCA to visualize the relationship between D- and L-amino acid profiles and kimchi storage period. As shown in the PCA score plot generated by combining PC1 (86.0% of total variance) with PC2 (6.7% of total variance) in Fig. 2A, samples of kimchi stored at 4°C showed no separation of profiles according to storage period. In contrast, samples of kimchi stored at 25°C were clearly clustered according to storage period. Therefore, PCA score plot successfully demonstrated the change in amino acid profiles at different storage periods at 25°C. This result shows that storage temperature affects amino acid profiles, and that amino acid profiles in kimchi gradually change during storage at 25°C. The loading plot indicates metabolites, which contributed most to the separation between groups obtained in the score plot. Metabolites that did not appreciably change between groups are plotted near the origin, whereas metabolites specific to different groups are plotted at areas further from the origin (11). As shown in PCA loading plot in Fig. 2B, some D-amino acids (D-Ala, D-Ser, D-*allo*-Ile, D-Leu, D-Asp, D-Glu, D-Met, and D-Tyr) were located far from the origin. This indicated that the difference in amino acid profiles of