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Process Biochemistry 42 (2007) 263–266

Process Biochemistry

www.elsevier.com/locate/procbio

Short communication

Metabolomic profiling of Cheonggukjang during fermentation by ^{1}H NMR spectrometry and principal components analysis

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Received 21 March 2006; received in revised form 6 July 2006; accepted 8 July 2006

Abstract

Metabolomic analysis of extracts of Cheonggukjang was carried out using ^{1}H nuclear magnetic resonance (NMR) spectrometry and principal components analysis (PCA). The major peaks in the ^{1}H NMR spectra of the 50% methanol fraction were assigned to isoleucine/leucine, lactate, alanine, acetic acid, citric acid, choline, fructose, sucrose, tyrosine, phenylalanine and formic acid. The first two principle components (PC1 and PC2) of the ^{1}H NMR spectra of the aqueous fraction allowed discrimination of Cheonggukjang extracts of samples obtained after different periods of fermentation. These two principal components cumulatively accounted for 98.5% of the total variation of all variables. The major peaks within the ^{1}H NMR spectra that contributed to discrimination of different samples were assigned to isoleucine/leucine, lactate, acetic acid, citric acid, choline, fructose, glucose and sucrose. This metabolomic analysis of samples of Cheonggukjang extract demonstrates that NMR and PCA can be used to obtain standard trajectory plots and related information for Cheonggukjang and other fermented foods.

Keywords: Metabolomic analysis; Cheonggukjang; ^{1}H NMR; Principal component analysis

1. Introduction

Cheonggukjang is a traditional Korean food that is the product of the fermentation of boiled soybeans with rice straw. Cheonggukjang has a characteristic flavor, taste and nutritional composition [1]. In addition to being consumed for its nutritional value, Cheonggukjang has antioxidative, antimicrobial and many other beneficial bioactivities [2,3]. Analysis of the chemical components of samples of Cheonggukjang from nine regions in Korea revealed that Cheonggukjang contains many biochemical components including fatty acids, amino acids, carbohydrates and organic acids [4]. However, no metabolomic profiles of Cheonggukjang have been reported to date.

The term 'metabolome' has been used to describe the observable chemical profile or fingerprint of the metabolites present in whole tissues [5]. Chemical analysis techniques that are used to obtain metabolite profiles preferably should be rapid, reproducible and stable over time. In addition, these techniques should not require complex sample preparation procedures. Nuclear magnetic resonance (NMR) is a technique that may meet the aforementioned requirements. NMR has been used widely as a fingerprinting tool for quality control analysis of industrial and natural products. Because NMR produces highly complex sets of data, multivariate or pattern recognition techniques such as principal components analysis (PCA) have been designed specifically to analyze NMR-derived data. The concept of PCA is to describe the variance in a set of multivariate data in terms of a set of underlying orthogonal variables and the orthogonal variables representing metabolite concentrations can be expressed as a particular linear combination of the principal components (PC). PCA is a linear additive model that each PC accounts for a portion of the total variance of the data set [6]. Recently, a combination of NMR and PCA has been applied to the metabolic profiling of various kinds of coffee, juice, wine, beer and plants [7–14].

In this study, we used ^{1}H NMR spectroscopy followed by PCA for the metabolomic analysis of extracts of Cheonggukjang samples obtained after fermentation for various periods of time. Our technique not only revealed the major components of Cheonggukjang, but also demonstrated that these components can be used to discriminate among samples of Cheonggukjang that have been fermented for different periods of time.

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doi:10.1016/j.procbio.2006.07.014

2. Materials and methods

2.1. Cheonggukjang samples

Samples of Cheonggukjang were obtained from Chunbuk National University. To summarize the traditional fermentation method, white beans were submerged in 15 °C of water for 15 h before being steamed in a commercially available steamer (NK, Eunkwang Machinery, Korea) at a pressure of 1.7 kg/cm^2 for 30 min. Thereafter, the beans were allowed cool to 50 °C. The beans were then fermented with rice straw at 42 °C for 40 h in a fermentation room. Samples of Cheonggukjang produced by the fermenting beans were obtained 0, 5, 10, 20 and 40 h after the start of the fermentation process. The samples of Cheonggukjang were freeze-dried and stored at −80 °C.

2.2. Solvents and chemicals

First-grade chloroform, methanol and D_2O (99.9%) were purchased from Sigma (St. Louis, MO, USA) and $CDCl_3$ (99.8%) and NaOD were purchased from Cambridge Isotope Laboratories (Miami, FL, USA) and Cortec (Paris, France), respectively.

2.3. Extraction of Cheonggukjang samples

Three hundred milligrams of ground Cheonggukjang samples was transferred into a centrifuge tube. Five milliliters of a 50% water–methanol mixture and 5 ml of chloroform were added to the antler sample in the tube and vortexed for 30 s and sonicated for 1 min. The materials were then centrifuged at 3000 rpm for 20 min. The extraction was performed twice. The aqueous and organic fractions were transferred separately into a 50 ml round-bottomed flask and dried with a rotary vacuum evaporator. Each experiment was performed in triplicate.

2.4. NMR measurements

KH_2PO_4 was added to D_2O as a buffering agent. The pH of the D_2O used for NMR measurements was adjusted to 6.0 using a 1N NaOD solution. All spectra were obtained by a NMR spectrometer (Avance 600 FT-NMR, Bruker, Germany) operating at a proton NMR frequency of 600.13 MHz. For each sample, 128 scans were recorded with the following parameters: 0.155 Hz/point, pulse width of 4.0 μs (30°) and relaxation delay of 1.0 s. Free induction decays were Fourier transformed with line broadening (LB) = 0.3 Hz, Gaussian maximum position for Gaussian function (GB) = 0 and peak detection sensitivity for peak picking (PC) = 1.0. The spectra were referenced to trimethyl silane propionic acid sodium salt (TSP) at 0.00 ppm for aqueous fractions and, for $CHCl_3$ fractions, to residual solvent at 7.26 ppm. Hexamethyl disilane (HMDS, 0.01%, v/v) and TSP (0.01%, w/v) were used as internal standards for $CDCl_3$ and D_2O, respectively. The peak intensities in 0.04 ppm bins in the ^{1}H NMR spectra for δ = 0.52–10.00 were used as variables.

2.5. Data analysis

The spectral region δ = 0.52–10.00 was segmented into regions of 0.04 ppm width giving a total of 237 integrated regions per NMR spectrum. The region from 4.60 to 4.90 was excluded from the analysis because of the residual signal of water in aqueous extracts, whereas that from 7.00 to 7.50 was excluded because of the residual signal of $CHCl_3$ in organic fractions. All spectral data were mean centered with no scaling, then analyzed by PCA based on the covariance matrix. PCA was performed with SIMCA-P software (Umetrics, Umeå, Sweden). The output from the PCA analysis consisted of score plots giving an indication of the differentiation of the classes in terms of metabolomic similarity and loading plots giving an indication as to which NMR spectral regions were important with respect to the classification obtained in the score plots. The statistical significances of the mean values of acetic acid and citric acid were tested by Tukey's multiple *t*-test of one-way ANOVA using SPSS 12.0 software (SPSS Inc., Chicago, USA).

3. Results and discussion

3.1. Visual inspection of ^{1}H NMR spectra and assignment of compounds

Because there was no obvious difference between the spectra of the $CHCl_3$ extracts of the various samples (data not shown), only the aqueous fraction was analyzed further. Representative ^{1}H NMR spectra of aqueous extracts of Cheonggukjang are presented in Fig. 1. The NMR signals in the aromatic region (δ = 6.0–8.0) were smaller than those in the aliphatic (δ = 0.5–3.3) and sugar region (δ = 3.3–6.0). The signals of the main aromatic compounds were assigned as follows: formic acid at δ = 8.46 (s), tyrosine at δ = 6.90 (d, J = 8.5 Hz) and phenylalanine at δ = 7.42 (m) (Fig. 1). In addition, the following signals were assigned based on comparisons with the chemical shifts of standard compounds and two-dimensional NMR using ^{1}H–^{1}H correlation spectroscopy, heteronuclear multiple quantum coherence and heteronuclear multiple bond coherence: lactate at δ = 1.34 (d, J = 6.6 Hz), alanine at δ = 1.48 (d, J = 7.3 Hz), acetic acid at δ = 1.90 (s), citric acid at δ = 2.58 (d, J = 6.6 Hz), choline at δ = 3.22 (s), fructose at δ = 4.22 (d, J = 8.7 Hz) and sucrose at δ = 5.42 (d, J = 3.9 Hz). The metabolomic profile changed according to the fermentation time (Fig. 2). In the early stage of fermentation, the amount of sugars such as sucrose (δ = 5.42) and fructose (δ = 4.22) were relatively high compared to the concentrations of these substances in the samples obtained during the later stage of fermentation, while the concentration of acetic acid was lower during the early stage than during the later stage of fermentation. The concentration of sugar and citric acid (δ = 2.58) decreased gradually during fermentation. As shown in Fig. 2e, the amount of aromatic compounds such as tyrosine and phenylalanine were higher in the samples obtained after 40 h of fermentation, than in earlier stage samples (0, 5 and 10 h).

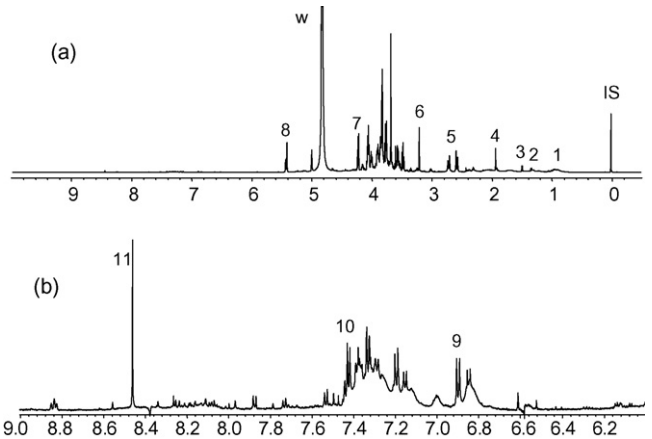

Fig. 1. Representative ^{1}H nuclear magnetic resonance (NMR) spectra of the total (a) and aromatic (b) region of the aqueous fraction of Cheonggukjang extracts from samples obtained at the start of fermentation. IS, internal standard; 1, isoleucine/leucine; 2, lactate; 3, alanine; 4, acetic acid; 5, citric acid; 6, choline; 7, fructose; 8, sucrose; 9, tyrosine; 10, phenylalanine; 11, formic acid; w, water. Values on the *X*-axis are the chemical shift in ppm relative to TSP.