Analysis of Water-soluble Constituents in Fermented Brown Rice and Rice Bran by Aspergillus oryzae (FBRA)

Ken Tanaka, a, Yukiko Horie, b Hideyuki Nemoto, b Hiroaki Kosaka, b Michiko Jo, c Yasuhiro Tezuka d

a College of Pharmaceutical Science, Ritsumeikan University, 1-1-1 Noji-Higashi, Kusatsu, Shiga 525-8577, Japan
b Genmaikoso co., Ltd., kita 12 nishi 1, kita-ku, Sapporo, Hokkaido 001-0012, Japan
c Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
d Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920-1181, Japan

(Received June 1, 2017; Accepted June 20, 2017)

In a previous paper, we analyzed the amounts of ferulic acid and its derivatives produced in the fermentation of brown rice and rice bran by Aspergillus oryzae (FBRA). Ferulic acid and its derivatives are considered to be biologically active constituents in FBRA and the amounts of these compounds increase remarkably depending on the fermentation time. Another benefit of fermentation is that it is considered to increase the nutritional value of the food. In this study, we examined changes in the nutritional components, such as dipeptides and the free forms of water soluble vitamins, in FBRA using LC-MS analysis.

Key Words: fermented brown rice by Aspergillus oryzae, dipeptides, water-soluble vitamins, LC-MS

*ktanaka@fc.ritsumei.ac.jp
**Introduction**

In a previous paper, we described the production of ferulic acid and its derivatives, which have antioxidative properties, in the fermentation process of brown rice and rice bran by *Aspergillus oryzae* (FBRA). Phutthaphadoong et al. have reported on the chemopreventive effect of FBRA on inflammation-related colorectal carcinogenesis in ApcMin/+ mice [1]. They also mentioned the nutritional advantages of fermented food. In recent years, many dipeptides have been found in fermentation products, and the activities of these, such as ACE inhibition, have been reported [2]. In addition, Rajalakshmi et al. have reported on changes in the chemical compositions of fermented and unfermented traditional Indian food and the water-soluble vitamins thiamine and riboflavin, and showed the amounts of these increased significantly with fermentation [3]. These results indicate an increase in nutritional value due to the fermentation of the food.

In this study, we used LC-MS analysis to study changes in nutritional components, such as dipeptides and the free forms of water soluble vitamins, arising as a result of the fermentation of brown rice and rice bran by *A. oryzae*.

**Experimental**

**Materials and sample preparation**

FBRA was produced by the fermentation of 10% brown rice and 90% rice bran by *A. oryzae* at 40 °C for 44 hr. Every 4 hr., 25 g of the sample was collected from the fermentation batch.

**Standard samples and reagents**

Vitamin B₁, vitamin B₂, niacin, pantothenic acid, vitamin B₆, biotin, Folic acid and vitamin B₁₂ were purchased from Nacalai tesque Co., Ltd. (Kyoto, Japan). All chemicals were of analytical grade, and the chromatographic solvents were of LC-MS grade (Wako Pure Chemical Ind. Co. Ltd., Tokyo, Japan).

**Analytical instruments**

LC-MS analyses were performed with a Shimadzu LC-IT-TOF mass spectrometer equipped with an ESI interface. The ESI parameters were as follows: source voltage, +4.5 kV, capillary temperature, 200 °C, nebulizer gas, 1.5 l/min. The mass spectrometer was operated in positive ion mode scanning from m/z 200 to 2000. A Waters Atlantis HILIC (2.1 mm i.d. x 150 mm) was used and the column temperature was maintained at 40 °C. The mobile phase was a binary eluent of (A) 0.1% HCOOH solution and (B) CH₃CN under the following gradient conditions: 0-30 min linear gradient from 95% to 50% B, 30-40 min isocratic at 100% B. The flow rate was 0.2 ml/min.

Quantitative analysis of the water-soluble vitamins was performed by LC–MS using a Thermo Scientific TSQ-7000 mass spectrometer equipped with an ESI interface. The ESI parameters were as follows: source voltage, -4.0 kV, capillary temperature, 200°C, nebulizer gas, 1.5 l/min. The mass spectrometer was operated in the selected reaction monitoring (SRM) mode: vitamin B₁; m/z 169.91 → m/z 133.5.134.4, vitamin B₂; m/z 377.05 → m/z 272.5-273.4, niacin; m/z 122.91 → m/z 79.5-80.4, pantothenic acid; m/z 219.95 → m/z 89.5-90.4, vitamin B₆; m/z 169.91 → m/z 133.5-134.4, biotin; m/z 244.94 → m/z 226.5-277.4, Folic acid; m/z 442.05 → m/z 294.5-295.4 and vitamin B₁₂; m/z 1355.8.

A Waters Atlantis T3 column (2.1 mm i.d.× 150 mm) was used and the column temperature was maintained at 40°C. The mobile phase was a binary eluent of (A) 0.1 % HCOONH₄ solution and (B) CH₃CN under the following gradient conditions: 0–30 min linear gradient from 0% to 50% (B), 30–40 min isocratic at 100% (B). The flow rate was 0.15 mL/min.

**Sample preparation**

**Analysis of water-soluble compounds using the HILIC column**

One gram of fine powder of the samples was accurately weighted and extracted three times with 50 ml of water under reflux conditions for 30 min. After centrifugation, the extracts were combined and lyophilized. The extract was dissolved in 70% methanol to give a 20 mg/ml solution and then filtrated through a 0.2 μm Millipore filter. Two μl of this solution was injected into the LC-MS.

**Quantitative analysis of water-soluble vitamins**

One gram of fine powder of the samples was accurately weighted and extracted four times with 10 ml of water by ultrasonication at room temperature for 30 min and left for one night. After centrifugation, the solvents were combined and lyophilized.

The extract was dissolved in methanol to give a 20 mg/ml solution, which was then filtrated through a 0.2 μm Millipore filter. Two μl of this solution was injected into the LC-MS.
Results and discussion

Figure 1 shows LC-MS total ion chromatograms and a 3D-chromatogram of the sample taken every 4 hours after the start of fermentation. The concentrations of trisaccharide and tetrasaccharide were highest 10 hours after starting the fermentation, and monosaccharide and disaccharide concentrations increased after 14 hours. Furthermore, the formation of dipeptides was observed after 14 hours from the start of fermentation. The type of dipeptide was estimated based on the exact mass number of the ion and its composition data observed in the mass spectra (Table 1). Recently, several bioactive dipeptides have been discovered in food. Suetsuna at al. reported that the dipeptides, Tyr-His, Lys-Tyr, Phe-Tyr, and Ile-Ty, showed significant hypotensive effects in hypertensive rats [4]. In addition, Hellier et al. indicated that the constituent amino acids were absorbed faster when presented as dipeptides rather than as free form amino acids [5]. These results indicate that dipeptides produced by the fermentation of brown rice should be important in terms of amino acid absorption as well as their functionality.

---

**Table 1 Dipeptides detected in FBRA extract**

| Dipeptide | Formula | Exact mass | Observed | Calculated |
|-----------|---------|------------|----------|------------|
| Leu-Leu   | C_{12}H_{22}N_{2}O_{5} | 244.1787 | 245.1850 | 245.1860 |
| Val-Leu   | C_{12}H_{22}N_{2}O_{5} | 230.1631 | 231.1682 | 231.1703 |
| Val-Glu   | C_{12}H_{22}N_{2}O_{5} | 246.1216 | 247.1271 | 247.1288 |
| Val-Thy   | C_{12}H_{22}N_{2}O_{5} | 280.1423 | 281.1481 | 281.1496 |
| Val-Val   | C_{12}H_{22}N_{2}O_{5} | 216.1474 | 217.1536 | 217.1547 |
| Ala-Phe   | C_{12}H_{22}N_{2}O_{5} | 236.1161 | 237.1230 | 237.1234 |
| Asp-Lys   | C_{12}H_{22}N_{2}O_{5} | 260.1485 | 261.1509 | 261.1557 |
| Ala-Leu   | C_{12}H_{22}N_{2}O_{5} | 202.1318 | 203.1380 | 203.1390 |
| Thr-Leu   | C_{12}H_{22}N_{2}O_{5} | 232.1423 | 233.1496 | 233.1496 |
| Ala-Tyr   | C_{12}H_{22}N_{2}O_{5} | 252.1110 | 253.1179 | 253.1183 |
| Ser-Leu   | C_{12}H_{22}N_{2}O_{5} | 218.1267 | 219.1326 | 219.1339 |
| Gly-Leu   | C_{12}H_{22}N_{2}O_{5} | 188.1161 | 189.1222 | 189.1234 |
| Pro-Leu   | C_{12}H_{22}N_{2}O_{5} | 228.1474 | 229.1539 | 229.1547 |
| Pro-Thr   | C_{12}H_{22}N_{2}O_{5} | 214.1318 | 215.1380 | 215.1390 |
| Ala-Ala   | C_{12}H_{22}N_{2}O_{5} | 160.0848 | 161.0917 | 161.0921 |
| Ala-Thr   | C_{12}H_{22}N_{2}O_{5} | 190.0954 | 191.1021 | 191.1026 |
| Leu-Gln   | C_{12}H_{22}N_{2}O_{5} | 259.1532 | 260.1596 | 260.1605 |

The compound with a retention time of 6.5 min has been annotated as kojic acid glucoside (m/z 305.0843, (C$_{12}$H$_{23}$O$_{6}$+H)$^+$. Mitsue et al. reported the synthesis of kojic acid 7-O-D-glucopyranoside by α-amylase from Aspergillus albus [6]. The compound showed tyrosinase inhibitory activity and superior properties to kojic acid for its solubility in water and stability against light. The bioactive components detected in FBRA were present with relatively high concentrations, which should suggest the importance of FBRA as nutritional source.
Water-soluble vitamins are present in association with enzyme proteins, and it is known that the absorption rate is poor in people with declining digestive abilities such as the elderly [7]. Rajalakshmi et al. reported on changes in the chemical composition of fermented traditional food in Indian households, and the increasing amounts of the free forms of thiamine and riboflavin [3]. Therefore, in order to evaluate the increase in nutritional value resulting from the fermentation of brown rice and rice bran, the changes in the amounts of free form water-soluble vitamins were analyzed. In Figure 2, SRM chromatograms of water-soluble vitamin standards and FBRA extracts are shown. Except for vitamin B12, 7 water-soluble vitamins were detected without any interference peaks using simple extraction methods and analytical conditions. Fig. 3 shows the amount of free form water-soluble vitamins depending on fermentation time. Significant increases of free form vitamins B2, B6 and biotin were observed. These results also indicate an increase in nutritional function due to the fermentation of brown rice and rice bran by A. oryzae.

**Conclusion**

In this study, we used LC-MS to study the changes in nutritional components arising in the fermentation of brown rice and rice bran by A. oryzae. In the final FBRA product, several dipeptides were detected. It is reported that constituent amino acids were absorbed faster when presented as dipeptides rather than as free form amino acids. In addition, significant increases of the free form vitamins B2, B6 and biotin were observed in the FBRA product. These results indicate that an increase in nutritional function should be achieved by fermenting brown rice and rice bran with A. oryzae.
References and Notes

[1] S. Phutthaphadoong, Y. Yamada, A. Hirata, H. Tomita, A. Hara, P. Limtrakul and H. Mori, Oncology reports, 23, 53-59 (2010).
[2] A. H. Pripp, T. Isaksson, L. Stepaniak and T. Sørhaug, T. European Food Research and Technology, 219, 579-583 (2004).
[3] R. Rajalakshmi and K. Vanaja, British Journal of Nutrition, 21, 467-473 (1967).
[4] K. Suetsuna, K. Maekawa and J. R. Chen, The Journal of nutritional biochemistry, 15(5), 267-272 (2004).
[5] M. D. Hellier, C. D. Holdsworth, I. McColl and D. Perrett, Gut, 13(12), 965-969 (1972).
[6] T. Mitsue, K. Egoshi, K. Okazaki, and T. Hara, Abstracts of Papers, the Annual Meeting of the Japan Society for Fermentation and Bioengineering, Hiroshima, November, 1991, p. 248.
[7] www.mhlw.go.jp/file/05-Shingikai../0000067134.pdf