博士論文（要約）

Quantitative Analysis and Metabolic Profiling of Organic Compounds in Traditional Functional Foods by NMR Spectroscopy

（NMR による伝統的機能性食品の成分定量分析及びメタボリックプロファイリング）

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論文題目

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Introduction

The quantitative analysis and metabolic profiling of two groups of traditional functional foods, fermented milk products and caterpillar fungi, were performed by NMR spectroscopy, which can observe signals from multiple components in intact samples in a non-destructive manner and thus is suitable to analyze the component changes over time. In Part 1, the component changes during fermentation processes of Bulgarian yogurt, Caspian Sea yogurt and kefir were monitored by NMR spectroscopy to characterize each fermentation process. In Part 2, the component changes of several kinds of caterpillar fungi during the growth were monitored by NMR spectroscopy and principal component analysis (PCA), to identify the important factor for increasing the content of cordycepin (3-deoxyadenosine), the major anti-cancer agent in caterpillar fungi.

Quantitative Analysis and Metabolic Profiling of Organic Compounds in Three Kinds of Fermented Milk Products by NMR Spectroscopy

Fermented milk products, usually made by using lactic acid bacteria, have a number of well-established benefits such as improving digestion, support of the immune system. Chemical compositions of fermented milk products were analyzed by LC- and/or GC-MS. These analytical methods need pre-preparations: appropriate extraction, purification and chemical derivatization for each component. To study the intact state of fermented milk, I have analyzed chemical compositions of Bulgarian yogurt, Caspian Sea yogurt and kefir during their fermentation processes by NMR

spectroscopy. Firstly, ^{1}H, ^{13}C, and ^{31}P NMR spectra as well as ^{1}H-^{13}C HSQC, ^{1}H-^{1}H DQF-COSY, ^{1}H-^{31}P CT-HMBC, and ^{1}H-^{13}C CT-HMBC spectra of Bulgarian yogurt were measured, and the observed ^{1}H, ^{13}C, and ^{31}P NMR signals were assigned to 10 organic compounds: α/β-lactose, α/β-galactose, lactic acid, citric acid, lecithin, creatine, acetic acid, alanine, cephalin and *N*-acetyl-D-glucosamine-1-phosphate, of which the last compound was identified for the first time as a component of Bulgarian yogurt. The molar ratios of α/β-anomers of lactose and galactose were determined based on the ^{1}H NMR spectra. In addition, the signals from the acyl chains of milk fats were successfully identified in spite of being overlapped with many other signals. For accurate quantitative analysis of fermented metabolites, the difference spectrum that was obtained by subtracting the diffusion-ordered spectroscopy (DOSY) spectrum from the quantitative ^{1}H NMR spectrum was used [1].

Based on the NMR assignment data of Bulgarian yogurt, the following seven components were also found in Caspian Sea yogurt and kefir by their 1D and 2D NMR spectra: α/β-lactose, α/β-galactose, lactic acid, citric acid, lecithin, creatine and ethanol. Then, the amounts of these seven compounds were monitored during the fermentation processes of the three kinds of fermented milk products by the aforementioned quantitative difference spectra, which revealed the characteristics of each fermentation process. In addition, the metabolism of lactic acid bacteria was also studied.

Quantitative Analysis and Metabolic Profiling of Organic Compounds in Extracts from Natural and Cultured *Cordyceps* by NMR Spectroscopy

In recent years, considerable evidence has shown that *Cordyceps*, also known as caterpillar fungus or tochukaso, exhibits several bioactivities such as anti-angiogenesis effects, tumor growth suppression and regulation of apoptotic homeostasis. *Cordyceps* products have gained increasing popularity not only in clinical trials but also in the global market for herbal supplements. However, natural *Cordyceps* has gradually become unable to meet the consumer demands, because high quality natural *Cordyceps*, such as *Cordyceps sinensis*, is very rare and large amounts are difficult to obtain. Although there are some studies on the cultivation process of *Cordyceps*, the actual quality of the cultured *Cordyceps* has not been fully studied in terms of their ingredients. This is one of the major barriers to the development of cultivation methods to obtain cultured *Cordyceps* of high quality that can act as a replacement for naturally grown *Cordyceps*. In addition, many components have been isolated from natural and cultured *Cordyceps* in recent studies, such as cordycepin, adenosine, proteins, amino acids, carbohydrates, carboxylic acids, lipids, glycosides and minerals. Among them, some functional components, such as amino acids, mannitol, uridine, adenosine, cordycepin, ergosterol and β-glucans, were separated and analyzed by LC/GC-MS and/or UV-visible spectrophotometry, which need pre-preparation and separation of the samples to be analyzed. In contrast, I have analyzed water extracts of *Cordyceps* by solution NMR without any separation or purification, because consumers usually drink water extracts of *Cordyceps*. Moreover, I have

performed NMR-based metabolic profiling to investigate and evaluate newly improved cultivation processes of *Cordyceps*.

Firstly, NMR spectral analyses of the natural *Cordyceps* and related species were performed to choose a fungus species suitable for the analysis of cultivation processes. Twenty four compounds were identified by 1D and 2D NMR spectroscopy in the extracts from the natural *Cordyceps* and *Paecilomyces tenuipes*, which is considered to be an anamorph of *Cordyceps*. Twenty-one compounds out of the 24 compounds were quantified by ^{1}H NMR spectra on the basis of signal assignments. Combined with the ^{1}H NMR data, PCA analyses were performed to identify the differences among the natural *Cordyceps* and *P. tenuipes* rapidly. The results showed that natural *Cordyceps militaris* is a good substitute for natural *Cordyceps sinensis* in terms of the content of cordycepin, the major anti-cancer agent in *Cordyceps*.

Secondly, NMR spectral analyses of cultured *C. militaris* in two kinds of growth media, the silkworm and rice media, were performed to determine a better medium for the cultivation of *C. militaris*. The results showed that the types of cultivation media had a large effect on the amounts of functional components. The silkworm chrysalis medium was chosen as the better medium to cultivate *C. militaris* because the *C. militaris* cultivated in this silkworm medium possessed more amounts than that cultivated in rice medium, and the composition of the cultured *C. militaris* in the silkworm medium was the most similar to that of natural *C. militaris*.

Based on the data on natural and cultured *Cordyceps*, it is concluded that natural *C. militaris* was deemed to be a proper natural *Cordyceps* fungus for cultivation, because it has higher amounts of cordycepin and other functional components than the other natural ones, and the silkworm chrysalis medium was regarded as a better medium in which the cultured *Cordyceps* with richer content could be obtained. For stable and successful cultivation, appropriate disinfection method has to be used to avoid contamination. To compare the effects of two disinfection methods on the growth of cultured *C. militaris*, growth-dependent changes of the chemical components during the cultivation process of *Cordyceps* were analyzed. In disinfection method #1, the mycelium samples were directly disinfected. In disinfection method #2, the silkworm chrysalis media were disinfected before the seeding of *C. militaris*. After the disinfection by the two different methods, twenty NMR-visible compounds in cultured *C. militaris* were simultaneously monitored during the cultivation process. The results showed that the different disinfection methods influenced the amounts of functional components of *Cordyceps*. I discovered that cordycepin could be synthesized at different growth stages, even at the stage of mycelia, in different cultivation conditions and that the *C. militaris* cultivated in the silkworm medium and disinfected by method #2 contained three times higher quantity of cordycepin than natural *C. militaris*.

Conclusion

To study the intact state of traditional functional foods, I have performed the quantitative

analysis and metabolic profiling of organic compounds in fermented milk products and *Cordyceps*.

In Part 1, 1D (^{1}H, ^{13}C, and ^{31}P) and 2D (^{1}H-^{13}C and ^{1}H-^{31}P) NMR spectra of Bulgarian yogurt were successfully obtained without any separation or pretreatment. In addition, the components of yogurt were quantified using the ^{1}H NMR spectra and the difference spectra between the quantitative ^{1}H NMR spectra and the DOSY spectra. Furthermore, this study confirms the new quantitative method for monitoring chemical changes in various fermented milk products during fermentation. The measurements of quantitative ^{1}H NMR spectra and DOSY NMR spectra are simple and quick, and the pre-preparation of samples and organic solvent are not necessary. Moreover, this quantitative method with the elimination of overlapping signals proved to be promising for monitoring of chemical changes during fermentation.

In Part 2, the non-separated metabolic profiling analysis of natural and cultured *Cordyceps* were performed by NMR spectroscopy. Since consumers usually drink the water extracts of *Cordyceps* as a supplement, the non-destructive analysis of water extracts of *Cordyceps* is suitable as the analytical method. As a result, I discovered that the *C. militaris* fungus and silkworm chrysalis medium were suitable for the cultivation of *Cordyceps*. Furthermore, cordycepin can be synthesized at different growth stages under different cultivation conditions, even at the stage of mycelia, and that the *C. militaris* cultured in the silkworm medium and disinfected before the seeding contained three times higher quantity of cordycepin than the natural *C. militaris*. These findings will help develop not only the cultivation methods of *Cordyceps* for industrial uses but also for food engineering, specifically mushroom cultivation and microbial fermentation, and then increase the content of functional components such as cordycepin, the major anti-cancer agent in *Cordyceps*.

Reference

1. Lu, Y., Hu, F., Miyakawa, T., & Tanokura, M. (2016). Complex Mixture Analysis of Organic Compounds in Yogurt by NMR Spectroscopy. *Metabolites* **6**, 19.