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Metaproteomic analysis of microbiota in the fermented fish, *Siniperca chuatsi*

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Trometamol (PubChem CID: 6503)
Dithiothreitol (PubChem CID: 446094)
Egtazic acid (PubChem CID: 6207)
Phenylmethylsulfonyl fluoride (PubChem CID: 4784)
Acetone (PubChem CID: 180)
Ethanol (PubChem CID: 702)
Acetic acid (PubChem CID: 176)
Formic acid (PubChem CID: 284)
Acetonitrile (PubChem CID: 6342)

ABSTRACT

The microbial community of spontaneously fermented fish plays an important role in product sensory quality. Recently, microbiota research has seen a shift in perspective from taxonomy to function. The investigation of the microbial community metaproteome will reveal information on strains as well as the expressed proteins to better understand the roles of the microbiota in fermented fish.

In this study, shotgun metaproteomic methods were used to identify and annotate 2175 proteins from the traditional Chinese fermented fish *Siniperca chuatsi*. These proteins belonged to 553 bacterial strains of 19 phyla, including 10 strains of lactic acid bacteria. Based on the KEGG annotation system, 1217 proteins were found to be involved in metabolic pathways, among which 352 proteins were related to amino acid metabolism. Specifically, 63 amino acid degradation-related proteins were identified in *Streptococcus* sp., *Bacillus* sp., *Escherichia* sp., and *Pseudoalteromonas* sp., which indicated that these strains were potential candidates for generating aroma compounds and thus contributors to the special flavour of the fermented fish. This information on metaproteomes in fermented *S. chuatsi* increases the understanding of microbial metabolic modes and leads to knowledge-based improvements of the stability and palatability of fermented fish.

1. Introduction

Fishes are preserved traditionally by various methods, including salting, drying, or both. When such processes are prolonged, natural fermentation begins. The co-function of multi-species microbial communities and endogenous enzymes provide fermented fish with desired properties such as an extended shelf life and good

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organoleptic properties. Previous studies (Jung, Lee, & Jeon, 2013; Shivanne Gowda, Narayan, & Gopal, 2016) have demonstrated that the diversity of the microbial community is of importance to the safety, flavour, texture, and health aspects of fermented fish. As a result, the structure profiles of the species composing the microbial diversity of fermented fish products have been widely investigated (Lee, Jung, & Jeon, 2015, 2014; Guan, Cho, & Lee, 2011; Zeng, Xia, Jiang, & Yang, 2013).

In addition to microbial structure, it is equally important to research functional traits (Krause et al., 2014; Wolfe & Dutton, 2015) and determine the physiological, morphological, and metabolic characteristics that affect functions of the microbial community in the mixed culture fermentation system. Recently, microbiota research has seen a shift in perspective from taxonomy to function. Among all the systematic approaches for the characterization of microbial ecosystems functions, the advantage of the metaproteomic method (Armengaud, 2016; Jansson & Baker, 2016; Wilmes, Heintz-Buschart, & Bond, 2015) is to find out what genes are actually expressed in a mixed culture, which makes metaproteomic analysis an ideal tool to better understand the roles of the microbiota in fermented fish. There are several metaproteomic methods, but the LC/LC-MS/MS and GeLC-MS/MS approaches are the most commonly used and are expected to produce more protein identification numbers (Herbst et al., 2016). Several metaproteomics studies (Andrés-Barrao et al., 2016; Lu et al., 2016) have been recently performed to identify the proteins that control metabolic activities in microbial communities or to identify the microbial functional groups present in fermented food. Metabolic profiling of the microbiota could reveal how the microbial consortia influence food qualities, and in the future, lead to knowledge-based improvements of fermentation stability.

To understand the sources of the special flavours of fermented fish, investigation of the protein and amino acid metabolism of the microbiota requires primary consideration. The microbial communities in fermented fish are able to catabolize proteins and other substrates to alcohols, aldehydes, and volatile fatty acids (Liu, Nauta, Francke, & Siezen, 2008). In particular, branched-chain amino acids, aromatic amino acids, and sulfur-containing amino acids are the main substrates for flavour compounds in fish fermentation (Liu et al., 2008). Lactic acid bacteria (LAB) are involved in many fish fermentation processes because of their unique metabolic characteristics. Additionally, LAB in fermented fish generate various aroma compounds (Smid & Kleerebezem, 2014) through different metabolic pathways.

The fermented fish *Siniperca chuatsi*, also called “Chouguiyu,” is one of the most famous Chinese fish products. Traditionally, fresh or frozen *Siniperca chuatsi* is salted, sealed and stored at room temperature for 7–15 days. Spontaneous fermentation provides the final product with its special smell and desirable taste. Fermented *S. chuatsi* was made without heat-sterilized treatments that destroy microorganisms in fish. As a result, the microbial diversity in the products was complex. Previously, several publications reported the LAB structure (Dai, Li, Wu, & Zhao, 2013) and flavour properties of the products (Li, Wu, Li, & Dai, 2013). Until now, there have been no reports concerning the metabolic characteristics and other functional properties of the microbial communities in fermented *S. chuatsi*. In this study, we will investigate the expressed microbial proteins in fermented *S. chuatsi* with an HPLC-MS/MS based metaproteomic approach. Our goals are to link physiological and metabolic features with phylogenetic diversity as well as to identify the key enzymes and microbial strains that contribute to the process of aroma formation in fermented *S. chuatsi* products.

2. Materials and methods

2.1. Sample collection

S. chuatsi (average net weight 750 ± 20 g) was purchased at fish markets in Dalian, China. The fish were previously caught and were stored in ice within 24 h from being transported from the fishing grounds. The fish were then gutted, soaked in salted water (6% (w/v)) and covered with heavy bags (average weight: 6 kg for each fish). The containers of the fish were sealed and stored at a temperature of 8 ± 1 °C for 15 days for natural fermentation.

After fermentation, a 10 mL liquid sample was taken from the fermented fish containers. Then, a gradient differential centrifugation method (Tang, Underwood, Gielbert, Woodward, & Petrovska, 2014) was used to obtain bacterial fractions with different sedimentation coefficients. Five pelleted fractions were collected sequentially when the liquid was centrifuged as follows: 300 g for 10 min (F1), 1000 g for 10 min (F2), 3000 g for 10 min (F3), 8000 g for 10 min (F4) and 14,000 g for 10 min (F5) at 4 °C. The recovered pellets were washed 3 times in pre-cooled PBS buffer (140 mmol/L NaCl, 2.7 mmol/L KCl, 6.5 mmol/L $Na_2HPO_4 \cdot H_2O$, and 1.5 mmol/L $KH_2PO_4 \cdot H_2O$, pH adjusted to 7.4) plus 0.1% Tween 80. The pellets were then resuspended, centrifuged and stored at −80 °C until further processing.

2.2. Protein extraction and digestion

Protein extraction and digestion were performed according to our previous method (Ji, Cao, Yao, Xue, & Xiu, 2014). Five fractions of bacterial cells were disrupted ultrasonically (90 times for 3 s on ice, 300 W) in 1 mL lysis buffer (8 mol/L urea, 150 mmol/L NaCl, 1 mmol/L EDTA, 50 mmol/L Tris (pH 7.4), 65 mmol/L DTT, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L PMSF, cocktail inhibitor). Lysates were centrifuged at 13,000 g and 4 °C for 15 min. The proteins within the sample supernatant were precipitated with 5 mL acidic acetone (acetone/ethanol/acetic acid = 50/50/1) at −20 °C overnight. The pellets were then resuspended in 1 mL denaturing buffer containing 50 mmol/L Tris - HCl (pH 7.4) and 8 mol/L urea, and the protein concentration was determined via Bradford assay. Thereafter, the sample proteins were resuspended and digested by trypsin with a weight ratio of trypsin to protein of 1/25 and incubated at 37 °C overnight. The hydrolytic peptides were desalted by a solid phase extraction column (Oasis HLB SPE, Waters) and lyophilized by a vacuum concentrator.

2.3. LC-MS/MS analysis

The tryptic peptides were analysed by a 2D nano LC–MS/MS system according to the method described by Wang et al. (2010). A strong cation exchange – reverse phase chromatography (SCX–RP) multidimensional HPLC system was applied for peptide separation. For SCX separation, 20 μg of peptides were loaded onto the SCX column, and then ion-exchange separation was performed with seven salt pulses containing ammonium acetate at concentrations of 50 mmol/L, 100 mmol/L, 150 mmol/L, 200 mmol/L, 300 mmol/L, 500 mmol/L and 1000 mmol/L. Each salt pulse was followed by a 130 min reverse phase gradient from 100% solvent A (water, containing 21.7 mmol/L formic acid) to 35% solvent B (ACN, containing 21.7 mmol/L formic acid). The LTQ–Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) was used for peptide detection. The parameter setups were as follows: the ion transfer capillary temperature was set to 250 °C, and the spray voltage was 2.0 kV. Full mass scans were performed followed by the collection of data-dependent MS/MS acquisition mode results. The MS data were