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Changes in proteases and chemical compounds in the exterior and interior of sufu, a Chinese fermented soybean food, during manufacture

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ABSTRACT

Sufu is a pleasant-tasting, traditional Chinese fermented soybean food that is rich in nutrients. The manufacture of sufu includes four steps: preparation of tofu, preparation of pehtze, salting of pehtze and ripening of salted pehtze in a dressing mixture. Based on electrophoresis and ^{1}H-NMR methods, the changes in protease and chemical compounds in the exterior and interior of sufu were investigated during fermentation. The results indicated that proteases with various molecular masses (162.6, 124.6, 104.3, 66.9, and 44.9 kDa) existed in both the exterior and interior of pehtze, salted pehtze, and ripened sufu, which proved that proteolytic reactions in the exterior and interior of sufu were occurring simultaneously. Furthermore, ethanol, ethylene glycol, glucose, isopropanol, and mannitol were the major carbonic compounds present in sufu, and amino acids were the predominant nitrogen compounds present in sufu. Correlation analysis revealed important links between protease activity and chemical compounds, e.g., succinate, histamine, and trimethylamine, were significantly positively correlated with protease activity in sufu. Through these results, the effect of protease on chemical compounds in sufu was identified, which can be useful for the improvement of enzymatically ripened sufu.

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1. Introduction

Sufu, a form of Asian fermented soybean, is a soft cheese-type product, which has been widely consumed as an appetizer in China for centuries (Han, Cao, Rombouts, & Nout, 2004). Nutritionally, sufu has the same significance to Asians as cheese does to the people of the Western Hemisphere (Han, Rombouts, & Nout, 2001). In addition to being consumed for its nutritional value, sufu has antioxidative activity, antimutagenicity, and many other beneficial bioactivities (Moy, Lu, & Chou, 2012), due to its components such as essential amino acids, fatty acids, organic acids, and vitamins. Generally, the commercial manufacture of sufu comprises four steps: (I) preparation of tofu, a fresh soybean curd made via salt precipitation from boiled soymilk, (II) preparation of pehtze (tofu overgrown with the mycelium of molds) via spray-inoculation of diced tofu with a pure culture inoculum of mold, such as *Actinomucor elegans*, (III) salting of the pehtze (the NaCl content of salted pehtze can reach more than 12%), and (IV) ripening of salted pehtze in a dressing mixture (containing 10–12% salt, 8–12% ethanol, 5–10% sugar, and some spices) for approximately 2–3 months (Han, Beumer, Rombouts, & Nout, 2001). Although a pure culture inoculum is used in pehtze preparation, the process of sufu manufacture itself is carried out under non-sterile conditions, and consequently, other microbes may be involved in sufu production. In addition, during the ripening of sufu, soybean proteins are broken down into small peptides and free amino acids, mainly due to the action of proteases from various microorganisms present in sufu (Feng et al., 2014).

Research on fermented food has indicated that taste compounds are generated through primary proteolysis of the raw material by endogenous enzymes or proteases from microorganisms, followed by secondary proteolysis and enzymatic or chemical conversion of amino acids into derivatives (Zhao, Schieber, & Gänzle, 2016). Similarly, in sufu analogues such as cheese, the extent of proteolysis during manufacturing and ripening and the level of casein degradation of peptides and amino acids can change the body, texture, and color of the product (Soltani, Sahingil, Gokce, & Hayaloglu, 2016). Enzymatically ripened sufu is generally sweeter and less

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salty than mold-fermented sufu (Moy & Chou, 2010). Therefore, protease is one of the most important parameters that determine the quality of sufu. Protease degrades the proteins to smaller nitrogen compounds such as peptides, amino acids, amines, and ammonia, resulting in increase of flavor (Lehninger, Nelson, & Cox, 2009; Han, Rombouts, & Nout, 2004). Examining protease and chemical compound changes simultaneously is crucial for a better understanding of protease function in sufu. Moreover, research on the metabolic composition of fermented food will enhance our understanding of the role of specific compounds that contribute to food flavor and relate food flavor to the technology of food production (Lee, Jung, & Jeon, 2015; Li et al., 2017a, 2017b; Mazzei & Piccolo, 2012; Piras et al., 2013; Vidal et al., 2016).

Earlier investigations on fermented tofu and other soybean products (Feng et al., 2015; Jung, Lee, & Jeon, 2014; Lee, Cho, & Lee, 2014) revealed information about microbes; however, to date, less information is available regarding the changes in protease and chemical compounds during the fermentation process of sufu and their interrelationship. A better understanding of the protease and chemical compound profiles in sufu will facilitate technological progress and the improvement of product quality, and provide guidance for screening functional strains for enzymatically ripened sufu.

2. Materials and methods

2.1. Sampling and preparation of protein extracts from the commercial processes of sufu manufacture

Samples were taken randomly at different stages of sufu production, including pehtze preparation, salting, and ripening (samples were drawn every 5 d during ripening) from a sufu company in Beijing, China. Tofu was treated with a pure culture inoculum of *A. elegans*. Before performing protease and chemical compound analyses, each piece of sufu was separated into the red-colored surface layer (2 mm thick), and the remaining pink-colored inner portion. Samples of 5 g were homogenized in 45 mL of 20 mM phosphate buffer, pH 7.5. The samples were then centrifuged (10,000×*g* for 10 min) and the supernatant was collected and used as an extract.

2.2. Determination of protease activity

Protease activity was determined according to the method described by Anson (1938) with minor modification. Suitably diluted enzyme solution was added to 2.5 mL of 1% (w/v) casein solution in different buffers (50 mM lactate buffer, pH 3.0; 50 mM phosphate buffer, pH 7.5; and 50 mM borax-sodium hydroxide buffer, pH 10.5) at 30 °C for 10 min before adding 2.5 mL 44 mM trichloroacetic acid solution. The mixture was centrifuged at 12,000 g for 10 min. For every l mL of supernatant, 5 mL of 0.5 M Na_2CO_3 and 1 mL of 2 N Folin–Ciocalteu reagent were added and mixed thoroughly. The absorbance values were measured at 660 nm. One unit (U) of protease activity was defined as the amount of enzyme that was required to release 1 μg tyrosine per g sufu per min under the conditions described above (Sun, Zhang, Yan, & Jiang, 2016).

2.3. SDS-PAGE and zymogram analysis of leaching from sufu

SDS-PAGE was performed using 7.5% (w/v) acrylamide in gels as described by Laemmli (1970). Protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma Chemicals, Perth, Australia). The molecular weight standards (Pharmacia) used for the calibration of the molecular mass included myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.2 kDa), albumin (66.4 kDa), and ovalbumin (44.3 kDa). Protease zymograms of the protein extracts were performed in a SDS-polyacrylamide gel that was copolymerized with 0.1% gelatin for 10 min. After electrophoresis, the gel was washed 3 times with different buffers (50 mM lactate buffer, pH 3.0; 50 mM phosphate buffer, pH 7.5; and 50 mM borax-sodium hydroxide buffer, pH 10.5) containing 2.5% Triton X-100 for 30 min. Then, the gel was incubated in different buffers (50 mM lactate buffer, pH 3.0; 50 mM phosphate buffer, pH 7.5; and 50 mM borax-sodium hydroxide buffer, pH 10.5) for 3 h at 30 °C, followed by staining with Coomassie Brilliant Blue R-250.

2.4. Chemical compound analysis using ^{1}H-NMR spectroscopy

The chemical compounds produced during sufu fermentation, including carbohydrates, organic acids, amino acids, biogenic amines, and nitrogen compounds, were analyzed by using ^{1}H-NMR spectroscopy according to the method described by Lee et al. (2017) with minor modification. Samples of 1 g of sufu were homogenized with 1.5 mL of ultrapure dH_2O in a mini-beadbeater for 60 s and then kept in ice for 10 min. The mixtures were centrifuged at 13,400×*g* at 4 °C for 10 min. For every l mL of supernatant, 1 mL of 0.2 mM phosphate buffer pH 7.0 containing 20% (w/v) of deuterium oxide (D_2O, 99.9%), 1 mM 3-trimethylsilyl-2,2,3,3-d4-propionate (TSP, 98%), 10 mM imidazole and 0.2% (w/v) azide sodium were added and mixed thoroughly. Once again, the mixtures were centrifuged at 13,400×*g* at 4 °C for 10 min and then, the supernatants were transferred into NMR tubes. ^{1}H-NMR spectra were obtained using an Avance III 600-MHz NMR spectrometer (Bruker, Germany) operating at a proton NMR frequency of 600.13 MHz and a temperature of 300 K, using a 5 mm PATXI probe. For each sample, 128 scans were recorded, with the following parameters: pulse sequence, NOESYGPPR1D (RD–90°–t_1–90°–t_m–90°-acquisition); relaxation delay, 2.00 s; mixing time (for NOESY), 1.00 s; acquisition time, 2.28 s; number of steady states transients (dummy scans), 4; gradient pulse times, 1 ms; solvent suppression, presaturation with spoil gradient; spectral width, 7184 Hz; time domain size, 32 k (Yan et al., 2015).

2.5. Statistical analysis

All spectral data were scaled to total intensity of the corresponding spectrum, and then analyzed via principal component analysis (PCA) with Pareto scaling. PCA was performed with AMIX software (version 3.9.9). The output from the PCA consists of score plots giving an indication of the differentiation of the classes in terms of metabolome similarity, and score plots giving an indication as to which NMR spectral regions were important with respect to the classification obtained in the score plots. Compounds were identified and quantified with Chenomx software (version 7.5; Chenomx, Edmonton, AB, Canada) with the reference of internal standard 3-(Trimethylsilyl) propionic acid-D4 sodium salt (TSP).

Correlations between protease and chemical compounds variables were analyzed via variation partitioning analysis (VPA), based on redundancy analysis. Significant differences ($p < 0.05$) were tested by one-way ANOVA using SPSS software (SPSS v19.0, Inc.).

3. Results and discussion

3.1. Changes in protease activity

Sufu was ripened in an incubation room with controlled temperature (~30 °C), relative humidity (~90%), and air circulation to ensure adequate aeration. pH was in the range of 6.18–7.23 during

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