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A primary study on texture modification and proteolysis of mao-tofu during fermentation

Xinhuai Zhao* and Xiaoting Zheng

Key Lab of Dairy Science, Ministry of Education, Northeast Agricultural University, Harbin 150030, PR China.

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A strain of *Mucor* was isolated from mao-tofu, a local fermented soybean product in central China, and used in this study to evaluate its role on texture modification and proteolysis of mao-tofu. Texture and microstructure of mao-tofu was monitored by texture analyzer and scanning electron microscopy (SEM). Proteolysis occurred during fermentation was evaluated by SDS-PAGE and chemical analysis. Results from Texture Profile Analysis showed that adhesiveness of mao-tofu had an increase trend while hardness, cohesiveness and springiness had a decrease trend as fermentation progressed. SEM analysis showed that the microstructure of mao-tofu was modified from a typical network structure to a more homogeneous structure. SDS-PAGE profiles indicated that cleavage of native soybean proteins occurred during fermentation and that molecular weights of most peptides were less than 20 kDa. The contents of water-soluble protein, total free amino groups and water-soluble free amino groups in mao-tofu fermented for 6 days were increased from 0.33%, 47.3 mmol/100 g and 120.7 mmol/100 g to 5.46%, 158.2 mmol/100 g protein and 338.2 mmol /100 g protein, respectively. All these results indicated that the texture or microstructure of mao-tofu was greatly modified as the results of soybean protein degradation during fermentation.

Key words: *Mucor* sp., mao-tofu, fermentation, texture, proteolysis.

INTRODUCTION

Soybean, originated from China and cultivated for more than 5000 year, is an important agricultural commodity and has wide applications in food, livestock and others because it is rich in protein and oil (Liu, 1999). Soybean foods are very important and popular diets to Chinese, for many non-fermented soybean foods and fermented soybean foods are produced in city or countryside. Fermented soybean foods, such as well-known sufu, are traditional foods that originated from ancient China and some might have history before Qing dynasty (300 BC) (Fu, 1994). Typically, sufu is made from soybean protein curd (well known as tofu) and fermented with different microorganisms, including *Mucor*, *Bacteria* and *Aspergillus* (Wang, 1998). Suhu has firm texture and characteristic flavor, and is now considered as Chinese cheese. Sufu fermented by *Mucor* is the earliest type and is now West, but with a much shorter maturing time. Typically,

produced widely in China (Wang, 1998). Some food factories in China today have specialized plants to manufacture their sufu products with different flavor characteristics, which depend on the type of microbe and processing conditions used. Mao-tofu is also a popular soybean food produced in central China, especially in countryside. Mao-tofu is a mould-fermented soybean protein curd (tofu) covered by white fungous mycelia, and has different cooking recipes. Technically, mao-tofu is farmhouse-prepared by mould fermentation, primarily *Mucor* sp.. Fermentation of tofu with *Mucor* sp. has some helpful impacts on the quality of final product, such as the improvement in bioavailability of some nutrients or the modification in texture or flavor. Fermentation of mao-tofu gives final product desirable texture and flavor that are different to tofu. These effects had been well studied and reported for typical fermented dairy products in West, such as cheese (Aryana and Haque, 2005; Muir et al.1997; Verdini and Rubiolo, 2002), but were rarely studied or reported for mao-tofu. The traditional production of mao-tofu is much similar to the production of mould-matured cheese, which also had widely studied in

*Corresponding author. E-mail: zhaoxh@mail.neau.edu.cn. Tel: 86 451 55191813. Fax: 86 451 55190340.

the production period for mao-tofu is from 3 to 5 days. During fermentation, mould grows in the surface of tofu at ambient temperature, which leads to the production and excretion of some enzymes such as proteases, peptidases and others. As a result of fermentation, soybean proteins in mao-tofu are degraded gradually and some flavor compounds are formed. The final product is covered by white fungous mycelia and has a smooth texture and unique flavor. These characteristics give mao-tofu an attractive appearance favored by customers. Maotofu might be considered as a mould-matured soybean cheese. While mao-tofu has been farmhouse-prepared in central China for thousands of years and is consumed by local peoples, few researches have studied the chemical changes and texture modification occurred during mould-fermentation. There exists a need to know these changes because they are related to the quality of mao-tofu. These characteristics might be important references that could be helpful to the industrial production of mao-tofu. In our previous study, a strain of fungi was isolated from a mao-tofu product and identified as *Mucor* sp. With this *Mucor* sp., we prepared mao-tofu samples with pure culture fermentation in the laboratory under simulated processing conditions as traditional procedure. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and some chemical parameters involved in proteolysis were analyzed to reveal the degradation extent of soybean proteins. Also texture profile analysis (TPA) and scanning electron microscopy (SEM) were applied to show how texture modification in mao-tofu occurred during fermentation.

MATERIALS AND METHODS

Materials

Soybean protein curd (tofu) prepared by soybean processors was purchased from local market. A strain of *Mucor* sp. was previously isolated from a mao-tofu product collected from countryside in central China. The collected mao-tofu sample was prepared as traditional procedure and fermented at ambient temperature in a woven bamboo tray, with some rice straw as natural inoculum. The spore suspension of *Mucor* was prepared as previously described (Sparringa and Owens, 1999). The *Mucor* grew on Potato Dextrose Agar (PDA) medium and its spore suspension was stored at 5°C until mao-tofu preparation. Standard protein maker used in SDS-PAGE was purchased from Solarbio Science and Technology Co., Ltd. (Beijing, China). All chemicals used were of analytical grade. Water used was redistilled water.

Preparation of mao-tofu

Fresh tofu purchased for mao-tofu preparation was cut into cubes (about 18 × 14 × 10 cm) by knife with smooth surface. After being subjected to sterilization at 100°C for 20 min to inactivate microorganisms or enzymes contaminated, the cubes were cut into dices (about 3 cm × 3 cm × 3 cm) aseptically in lab. Then, the dices were inoculated with *Mucor* inoculum (105 spores mL⁻¹) over their surface. The inoculum had been prepared before as a pure starter.

All dices were placed in sterilized bamboo trays separately to facilitate air circulation and mycelia development, and cultured in an incubator at 20 ± 1°C with a relative humidity of 73 to 76% for 6 days. During fermentation, some dices were randomly selected every day as analysis samples for texture examination and chemical analysis.

Texture analysis of mao-tofu

A Texture Analyzer (TA-XT PLUS, US) with operating software Texture Expert was used in texture analysis. The texture profile analysis (TPA) option applied in cheese analysis (Kailasapathy and Lam, 2005; Özer et al, 2003) was used with some modifications to monitor the physical characteristics of nonfermented tofu or fermented mao-tofu. From the TPA curves, following textural parameters were obtained or calculated to describe the texture of tofu or mao-tofu: hardness, adhesiveness, cohesiveness and springiness. All samples were held at ambient temperature for 1 h before testing. The mycelia cover in the surface of mao-tofu was removed by hand prior to sampling. In sampling, a stainless steel corer with an internal diameter of 23 mm was used. The prepared sample was cut to a height of 20 mm and placed on the sample retaining plate in Texture Analyzer. An acrylic probe of 10 mm diameter (P/0.5) was used in analysis. Each sample was compressed axially to 50% of their original height in two consecutive compression cycles. Test velocity, time, distance and trigger force were 1.0 mm·s⁻¹, 5.0 s, 10.0 mm and 5.0 g, respectively. The numbers of samples analyzed were six. Hardness, adhesiveness, cohesiveness and springiness were calculated by the instrument's software from the generated force-time curves.

Microstructure examination

Microstructure examination was performed with scanning electron microscopy as an earlier described (Lorenzen et al., 2002). Small pieces were cut from tofu or mao-tofu samples. After overnight fixation with 2% glutaraldehyde in 0.1 mol·L⁻¹ sodium phosphate buffer (pH 7.2), all samples were transferred into microporous specimen capsules (Plano, Wetzlar, Germany). Dehydration (in graded series of ethanol and acetone, respectively), critical point drying with liquid carbon dioxide, and coating with gold in a sputter coater was also same to reference description. The specimens were viewed in the microscope chamber in a scanning electron microscope (S-3400N, Hitachi, Japan), using an accelerating voltage of 5.0 kV.

SDS-PAGE

Aliquots were taken from tofu or mao-tofu samples and mixed well with an equal volume of buffer (pH 6.8) containing β-mercaptoethanol. After being boiled and centrifuged at 10000 g for 10 min, supernatants were separated. An SDS-PAGE analysis method from Laemmli (1970) was used for evaluation of protein degradation using gradient (15% w/v) gels. The gels were 1.5 mm thick and consisted of a 2 cm stacking gel and a 10 cm running gel. 20 μg protein was applied to sample slots. The period of electrophoresis was 4 h at 120 V. After the end of electrophoresis, the gel was separated. Protein bands were stained with 0.25% Coomassie Brilliant Blue R-250 in methanol/water/acetic acid (5:5:1, v/v/v), and then destained in the same solvent. Standard protein markers used and their molecular weights (in kDa) were as follow: egg albumin lysozyme, 14.4; trypsin inhibitor, 20.1; bovine carbonic anhydrase, 31.0; ovalbumin, 43.0; bovine serum albumin, 66.2; phosphorylase b, 97.4. The gel images were visualized and photo-



Figure 1. Growth of *Mucor* sp. in the surface of mao-tofu fermented for 4 days.

Table 1. Changes of textural parameters of mao-tofu during fermentation^a.

Parameter	Fermentation times					
	0 day	2 days	3 days	4 days	5 days	6 days
Hardness (g)	239.1 ± 2.46	256.3 ± 1.73**	292.4 ± 4.10**	307.8 ± 2.92**	283.7 ± 3.25**	269.9 ± 1.37**
Springiness	0.943 ± 0.021	0.951 ± 0.017*	0.932 ± 0.023*	0.905 ± 0.019*	0.884 ± 0.038*	0.860 ± 0.050*
Cohesiveness	0.518 ± 0.014	0.537 ± 0.030*	0.442 ± 0.036*	0.446 ± 0.016*	0.435 ± 0.025*	0.428 ± 0.013*
Adhesiveness (g.s)	20.92 ± 3.03	65.57 ± 6.02**	80.87 ± 2.02**	78.32 ± 9.51**	90.68 ± 5.07**	108.10 ± .05**

^a Values represent the mean ± SD ($n=6$), and tofu (0 day) was used as control.

*** Value is significantly different from the value of tofu (** $P<0.01$ and * $P<0.05$).

graphed by PhotoDoc-It Imaging System (UVP Inc., San Gabriel, CA, USA).

Chemical analysis of mao-tofu

All samples were analyzed in triplicate for moisture by AOAC methods 926.08 (AOAC, 2000), or for total protein by the Kjeldahl method 920.123 (AOAC, 2000) on a Kjeltec 2300 Analyzer (Foss, Sweden). Moisture was expressed as weight of water in 100-gram sample (g/100 g). Conversion factor 6.25 was used to calculate total protein (TP). TP was expressed as weight of protein in 100-g sample (g/100 g).

Extraction of water-soluble total protein (WSTP) from tofu or mao-tofu was as reference (Moatsou et al, 2004): 10 g of sample was homogenized with 50 mL distilled water using a Stomacher blender (Seward, London SE1 1PP, UK) for 5 min, then centrifuged at 4000 g for 20 min and filtered through filter paper (Whatman 40). The nitrogen in supernatant obtained was estimated in triplicate by Kjeldahl method as before. WSTP was also calculated with conversion factor 6.25 and expressed as weight of water-soluble proteins in 100-g sample (g/100 g). Proteolysis of mao-tofu during fermentation was also monitored by measuring the content of free amino groups (-NH₂) in tofu and mao-tofu. The content of free amino groups and the content of water-soluble free amino groups in all samples were determined in triplicate by a formaldehyde titration method described in literature (Zhang et al., 2007), because sample solutions for tofu or mao-tofu had some insoluble particulates that led other analysis methods such as spectrophotometry unavailable. Both indexes were expressed as mmol -NH₂ in 100-g proteins (mmol/100 g protein).

Statistical analysis

All data were expressed as means ± standard error (SE) from at least three independent experiments. One-way analysis of variance (ANOVA) was used to evaluate the significance levels of the analysis data obtained. $P \leq 0.05$ was considered as significantly difference.

RESULTS

Textural modification of mao-tofu during *Mucor* fermentation. The fermentation of mao-tofu requires two stages, the first being the growth of mold mycelia and the second being protein hydrolysis by enzymes excreted by mycelia. In the first stage, *Mucor* was allowed to grow as many mycelia as possible. At the same time, various enzymes including proteases were excreted. *Mucor* grew very quickly in the surface of tofu during fermentation. When mao-tofu was fermented for 2 days, mycelia appeared at surface of mao-tofu. A white-mycelia cover was formed at the fourth day (Figure 1). The growth of *Mucor* in tofu had a definitive effect on the texture of mao-tofu. TPA results for nonfermented tofu and fermented maoto-fu samples are listed in Table 1. There were significant differences between tofu and mao-tofu for all the textural parameters determined by TPA. Hardness of mao-tofu increased first (second day to fourth day) and then showed a decrease trend (fifth day to sixth day) as

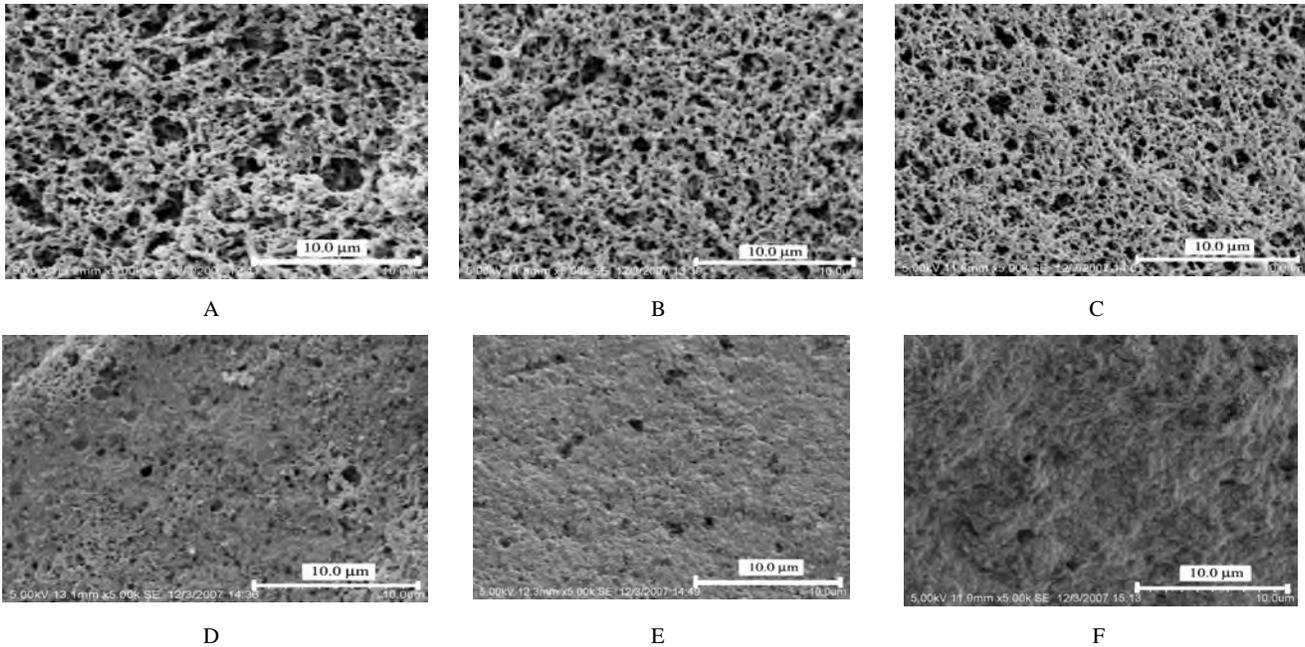


Figure 2. Microstructures of mao-tofu at different fermentation times (magnification $\times 5000$). A, tofu; B to F, mao-tofu fermented with *Mucor* sp. for 2, 3, 4, 5 or 6 days respectively.

fermentation progressed. Mao-tofu fermented for 6 days also had a different hardness to tofu ($P < 0.01$). Adhesiveness of mao-tofu was increased significantly ($P < 0.01$), indicated by a fourfold increase (from 21 g.s at first day to 108 g.s at sixth day). Cohesiveness and springiness of mao-tofu showed a decrease trend as fermentation progressed ($P < 0.05$). All these changes indicated that the texture of mao-tofu was different from the texture of tofu. This resulted from the modification in structure, because key structure element of tofu are proteins that were degraded gradually during fermentation.

Microstructure modification of mao-tofu during *Mucor* fermentation

The microstructure of tofu or mao-tofu at different fermentation stages was examined by SEM technique. Scanning electron micrographs obtained are shown in Figure 2, which revealed clearly how microstructure modifications of tofu occurred during fermentation. Tofu exist as a typical protein network structure with more open space (Figure 2A). During fermentation, the microstructure of tofu was modified or destroyed gradually, for protein network was diminished gradually during fermentation (Figure 2B to Figure 2F). After mao-tofu being fermented for six days, a compact and uniform structure was formed, as shown in Figure 2F. Mao-tofu fermented for 4 to 6 days had a more compact microstructure than tofu, which reflected or confirmed texture modifications.

The modification in microstructure of mao-tofu might show directly how the degradation of soybean proteins occurred during fermentation.

Proteolysis of mao-tofu during *Mucor* fermentation

It was well known that the degradation of proteins would lead to the formation of peptides and free amino acids. Such fragments could be monitored with many techniques. The degradation of proteins and formation of peptides in mao-tofu during *Mucor* fermentation were first evaluated with SDS-PAGE. The distributions of protein bands for tofu and mao-tofu samples at different fermentation stages are shown in Figure 3. It could be seen clearly that proteolysis occurred in mao-tofu during fermentation. Molecular weights of protein subunits in tofu were mainly in the range of 20 to 66 kDa (see Lane 0), characterized by three bands (labeled as a, b, c). As fermentation progressed, soybean proteins in mao-tofu were degraded by proteases to small peptides (see protein bands in Lane 2 to Lane 6). Some peptides had a molecular weight less than 20 kDa when mao-tofu was fermented for 2 or 3 days (shown in Lane 2 to 3). When mao-tofu was fermented for 6 days, most peptides had molecular weight less than 20 kDa, indicating that most soybean proteins in mao-tofu were degraded into small peptides because little native soybean proteins were left (shown in Lane 6). Results from chemical analysis also showed some composition changes occurred in mao-tofu,

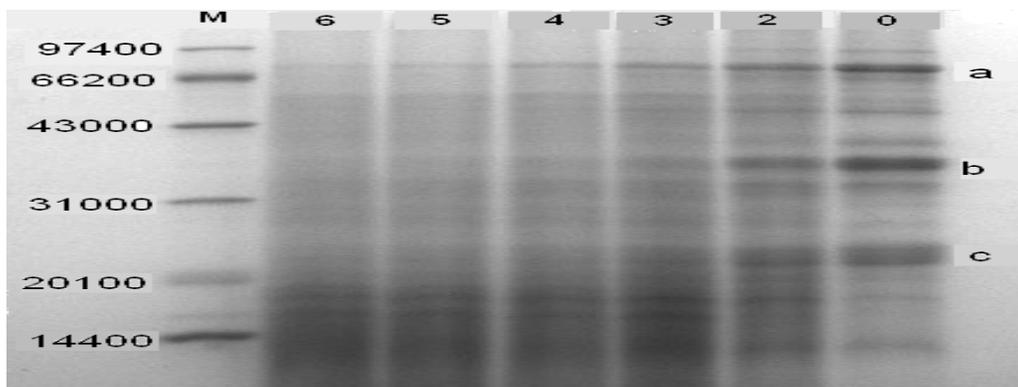


Figure 3. SDS-PAGE analysis of proteins and degraded products in mao-tofu. Lane M, protein markers with molecular weight ranged from 14.4 k to 97.4 kDa; Lane 0, tofu; Lane 2 to Lane 6, mao-tofu fermented with *Mucor* sp. for 2, 3, 4, 5 or 6 days, respectively.

especially those related to degradation of soybean proteins. These results are listed in Table 2. As fermentation progressed, some moisture was lost (from 84.46 to 75.64%), leading to an increase in total proteins (from 8.1 to 12.6%). The content of water-soluble protein also increased clearly (from 0.33 to 5.46%), which reflected protein degradation during fermentation. The content of free amino groups and the content of water-soluble free amino groups in mao-tofu fermented for 6 days had a twofold increase. The content of free amino groups increased from 47.3 to 158.2 mmol/100 g proteins while the content of water-soluble free amino groups increased from 120.7 to 338.2 mmol/100 g proteins. These two indexes indicated the formation of free amino groups in mao-tofu, and were two direct evidences for protein degradation.

DISCUSSION

Like casein's role in cheese, soybean proteins play a key role in the textural properties of tofu, a semisolid food. The changes in hardness and other textural parameters in cheese were most likely due to the degradation of casein as a result of the activities of the enzymes. Hardness is the force required to compress a food between the molars. It had been shown in cheese that proteolysis was probably responsible for the weakening of the protein matrix (Aryana and Haqu, 2005). Fermented by *Mucor* sp., soybean proteins were degraded into low molecular weight protein fragments by proteases excreted by *Mucor*, and network structure formed by proteins was destroyed. Degradation of soybean proteins and destruction of protein network structure in mao-tofu might result in the decrease of hardness as fermentation progressed. Meanwhile, moisture loss occurred during fermentation (Table 2). The increase in the protein concentration due to moisture loss

in mao-tofu might cause an increase in viscosity and lead to a firmer bodied product. Therefore, hardness of mao-tofu may have increased because of moisture loss. These two different effects resulted in irregular changes of hardness during fermentation. Hardness increased at early fermentation stages (2 to 4 days), mainly because of moisture loss and less proteolysis, and then decreased at 5 to 6 days, mainly because of much proteolysis. Cohesiveness is a parameter describing the force needed to stimulate the strength of the internal bonds making up the body of the product (Vliet, 1991). Because proteolysis occurred during fermentation, which led to the destruction of the number and strength of bonds making up of the protein matrix, cohesiveness of fermented mao-tofu changed clearly to a lower level compared to that of tofu ($P < 0.05$).

Springiness is a parameter used to describe the extent to which a compressed food returns to its original size when the load is removed. It was expected that the springiness of mao-tofu would be changed because of proteolysis. Analysis showed that springiness of mao-tofu was modified significantly ($P < 0.05$), compared to that of tofu. Water act as a plasticizer; it could be expected that the elastic modulus might decrease with the decrease of moisture (Özer et al., 2003). During fermentation, moisture of mao-tofu was lost significantly. Degradation of soybean proteins and loss of moisture had a synergistic effect to decrease springiness. Textural modification occurring in cheese ripening has been well studied, and some textural parameters (including cohesiveness, hardness and springiness) were assayed. Pollard et al. (2003) found that textural parameters of commercial natural cheddar cheese during maturation were reduced (Pollard et al., 2003). It was also found that when encapsulated proteases were applied in accelerated cheese ripening, changes in textural properties of cheeses ripened for 5 months were significant different between the experimental cheeses and control

Table 2. Changes of chemical compositions of mao-tofu during fermentation^a.

Parameter	Fermentation times					
	0 day	2 days	3 days	4 days	5 days	6 days
Moisture (g /100g)	84.46 ± 0.05	80.18 ± 0.12**	78.01 ± 0.07**	76.72 ± 0.02**	76.27 ± 0.06**	75.64 ± 0.05**
Total proteins (g/100g)	8.11 ± 0.09	10.34 ± 0.09**	11.47 ± 0.05**	12.11 ± 0.13**	12.28 ± 0.03**	12.59 ± 0.04**
Water-soluble proteins (g /100g)	0.329 ± 0.002	0.983 ± 0.001**	1.826 ± 0.008**	3.195 ± 0.007**	3.784 ± 0.001**	5.463 ± 0.003**
Free amino groups (mmol /100g protein)	47.3 ± 1.83	63.3 ± 1.12**	80.1 ± 0.87**	110.1 ± 3.41**	146.4 ± 1.62**	158.2 ± 2.70**
Water-soluble free amino groups (mmol /100g protein)	120.7 ± 3.12	207.5 ± 5.34**	294.7 ± 2.45**	310.1 ± 1.32**	389.4 ± 1.82**	338.2 ± 4.19**

^a Values represent the mean ± SD (n=3) and tofu (0 day) was used as control.

*** Value is significantly different from the value of tofu (** $P < 0.01$ and * $P < 0.05$).

cheeses, for reduction in the mean score of textural parameters was observed (Kailasapathy and Lam, 2005). SEM analysis is a common technique applied in texture analysis, and was also applied in our study to monitor the microstructure modifications that occurred in mao-tofu at different fermentation times. The microstructure appeared relatively porous in tofu, but the microstructure appeared to be less porous and more homogeneous in mao-tofu (comparing Figure 2A to Figure 2F). During fermentation, soybean proteins acting as key structure elements in tofu was hydrolyzed gradually by proteases excreted by *Mucor*, which resulted in the destruction of protein network and texture modification. When fermented for 4 to 6 days, mao-tofu had different microstructure characteristics, showing by a more compact, dense and uniform microstructure. The micro-structure changes occurring in Cheddar cheese during ripening process were also reported to be a more compact structure (Stanley and Emmons, 1977). In another observation reported by Atia et al. (2004), it was found that the microstructure of young Cheddar type cheese was characterized by an open, irregular and fibrous protein matrix; after 150 days

of ripening, the protein matrix had become more compact, dense and homogeneous. The most important biochemical reaction in mao-tofu during fermentation might be proteolysis. Proteolysis is also an important reaction in cheeses ripening (Fernández-Salguero, 2004; Fox et al., 1990). To elucidate the hydrolysis extent of soybean proteins at different fermentation times, the degraded soybean proteins fragments were analyzed by SDS-PAGE. It was found that native soybean proteins in tofu were hydrolyzed gradually into peptones or peptides (Figure 3). The longer the fermentation progressed, the more peptides formed in mao-tofu. This trends was confirmed clearly in SDS-PAGE because much protein bands with lower molecular weight were found in mao-tofu samples fermented for 5 to 6 days, or supported by the changes in the contents of free amino groups and water-soluble free amino groups (Table 2) because more free amino groups were determined in mao-tofu fermented for 5 to 6 days. Water-soluble proteins reflect the content of total soluble proteins, small and medium-sized peptides, free amino acids separated from large peptides and protein (Christensen et al., 1991). Content of water-soluble proteins in

mao-tofu increased during fermentation (Table 2), indicating that proteolysis occurred. In the early period of fermentation, soybean proteins were hydrolyzed, giving peptides as breakdown products that were largely water-soluble. It was also found in low-fat Cheddar cheese that the amount of water-soluble nitrogen increased during four months of ripening (Küçüköner and Haque, 2006).

The levels of free amino groups and water-soluble free amino groups in mao-tofu increased significantly as fermentation progressed. These two indices relate to the breakdown of soybean proteins. The content of free amino groups or water-soluble free amino groups in mao-tofu fermented for 6 days had a twofold increase compared to that in tofu, indicating directly that proteolysis occurred. Kailasapathy and Lam (2005) reported that levels of free amino groups in cheeses increased as ripening progressed. Fernández-Salguero (2004) found the gradual hydrolysis of proteins to soluble low molecular weight compounds in mould-ripened cheeses. Cinbas and Kilic (2006) reported a similar change in white cheese manufactured by traditional production method, in which water-soluble free amino groups increased during storage from 43 to

232 g nitrogen kg⁻¹ total nitrogen. These study confirmed those changes occurred in mao-tofu.

Conclusions

During fermentation of tofu with *Mucor*, the texture of mao-tofu was modified. Data from TPA show that adhesiveness increased while hardness, cohesiveness and springiness decreased as fermentation progressed, indicating that textural modification occurred. SEM analysis showed that protein network was destroyed during fermentation, with a homogeneous and dense microstructure formed in mao-tofu. Results from SDS-PAGE analysis indicated clearly that soybean proteins were degraded into peptide fragments (molecular weight < 20.1 kDa) during fermentation. Chemical analysis also showed that content of water-soluble protein, free amino groups and water-soluble free amino groups in mao-tofu fermented for 6 days increased to much higher levels of 5.46%, 158.2 and 338.2 mmol/100 g proteins, respectively, similar to those that occurred in cheese ripening. Proteases produced by mao-tofu during fermentation which were key element in texture modification and proteolysis, need further study.

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