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A Brief Investigation on the Proteolysis and Textural Modification of a Semi-hard Cheese Ripened by *Mucor* spp.

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Abstract: In the present study, the proteolysis of a semi-hard cheese induced by a strain of *Muco*r spp. was briefly investigated. The cheese samples were smeared with the suspension of the *Muco*r in the surface and kept at 4°C and a relative humidity of 85-90% for ripening. Soluble or insoluble nitrogen fractions were separated from cheese samples ripened for different times and assayed by electrophoresis and RP-HPLC analysis to show the proteolysis occurred in the cheese samples. Electrophoresis analysis of the pH 4.6-insoluble nitrogen fractions showed that some protein fractions in the cheese samples were degraded into the peptides of lower molecular weights. RP-HPLC analysis results for the water-soluble nitrogen fractions also confirmed protein degradation and the formation of some new peptides. Chemical analysis revealed that the ratio of pH 4.6-soluble nitrogen to the total nitrogen of the cheese samples had a 3-fold increase after a ripening time of 90 days. Observation results under scanning electron microscopy clearly showed textural modification in the ripened cheeses. It is thus demonstrated that the *Mucor* spp. might be a potential starter for semi-hard cheese to degrade protein fractions and modify cheese texture.

Key words: *Mucor* sp., semi-hard cheese, ripening, proteolysis, microstructure

INTRODUCTION

During the ripening of a surface mould-ripened cheese, complex biochemical processes occurred are induced by the starter or nonstarter microflora and secondary organisms smeared in its surface. The moulds have more complex enzyme system than the bacteria and have been used to ripen Brie, Camermbert, Gamalost or Neufchatel cheese (Voigt *et al*., 2010; Sienkiewicz-Szapka *et al*., 2009). For example, the mutant and no mutation of *P. camemberti*, *P. roqueforti*, *G. candidum* and *P. caseicolum* are among those used frequently in cheese production (Le Drean *et al*., 2010; Seratlic *et al*., 2011). The mould smeared in the surface of the cheese can produce some enzymes. Degradation of milk proteins by the excreted exogenous and indigenous proteases from the mould is referred as proteolysis and can lead to the improved textural, flavor and nutritional quality of the cheeses. Proteolysis gives rise to the gradual formation of some peptides of lower molecular weights (Chen *et al*., 2012) and thus is considered as the most important chemical changes during cheese ripening.

Mao-tofu, one of traditional soybean foods in China, is fermented by some microorganisms mainly *Mucor* spp. The *Mucor* can produce some proteases that induce in protein degradation in the Mao-tofu, resulting in the formation of bioactive peptides having better antioxidative and angiotensin I converting enzyme inhibitory activities (Hang and Zhao, 2011, 2012). The ripening of the Mao-tofu shares a similarity to the ripening of the mould-ripened cheeses but with a shorter ripening time (Zhao and Zheng, 2009). It is obviously that the *Mucor* has the potential application for cheese ripening. Unfortunately, no much information is available in the present time about the practical application of the *Mucor* spp. in cheese ripening yet. In the present study, a strain of the *Muco*r previous separated from Mao-tofu was used to ripen a semi-hard cheese. Some instrumental or chemical analyses were carried out to reveal the protein degradation and textural modification induced by the *Mucor* during cheese ripening. The aim of the present study was to show the applicability of the *Mucor* in cheese processing.

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MATERIALS AND METHODS

Materials: Fresh milk was obtained from local dairy farm of Harbin, China. Chymosin (Maxiren-180, 1:10000 strength) was obtained from DSM Food Specialties, Holland. Casein was purchased from Sigma (St. Louis, Mo, USA). Water used was purified by Milli-Q Plus (Millipore Corp., Bedford, MA, USA). The chemicals used in RP-HPLC analysis were of HPLC grade while others were of analytical grade.

Strain and culture conditions: The *Muco*r spp. was isolated from local Mao-tofu product with casein plate medium by a transparent ring method (Zhao and Zheng, 2009) and identified as *Mucor Micheli ex Fries*. The *Mucor* was cultured in slope medium (in g L^{-1}): $NaNO_3$ 3, KH_2PO_4 1, $MgSO_4$ 0.5, Kcl 0.5, $FeSO_4$ 0.01, sucrose 30, agar 20. The culture was maintained at (28±1)°C for 48 h in an incubator and then kept at 4 °C before use. The spore suspension of the *Mucor* was prepared as a previous study (Zhang and Zhao, 2010).

Cheese preparation and ripening: The fresh milk was pasteurized at 63°C for 30 min. After acidification of the milk with lactic acid solution of 10% (w/w) to a pH value of 5.7, $CaCl_2$ and KNO_3 were added at a level of 0.16 and 0.14 g kg^{-1}, respectively. Chymosin was added to the milk at 33°C at a level of 0.034 g kg^{-1} as the producer recommended. The formed curd was cut into the cubes about 1 cm^3 and the whey was drained three times at 15 min interval. The curd was pressed at 0.3 MPa for 2 h in a cylindrical mould (15 cm diameter, 30 cm height), removed from the mould and cut into cubes (8×4×4 cm). The obtained cubes (fresh cheeses) were smeared with the prepared suspension of the *Muco*r on the surfaces after salting in refrigerated brine (5%, w/w) for 18 h and cultured at (28±1)°C for 24 h. All prepared cheese samples then were put to a ripening chamber, kept at (4±1)°C and a relative humidity of 85-90% and ripened for a period of 90 days.

Some chemical and instrumental analysis: Three nitrogen fractions, including Water-soluble Nitrogen (WSN), pH 4.6-Soluble Nitrogen (SN) and pH 4.6-insoluble nitrogen (ISN) fractions, were prepared from the central and external zones of the cheese samples ripened for different times. The preparing procedures used were the same as the reported methods (Wang *et al*., 2011; Pino *et al*., 2009). Nitrogen content in the assayed samples was determined by the Kjeldahl method (IDF, 2001) and a conversion factor of 6.38 was used for the calculation of protein content. All evaluations were carried out at three times.

A tricine-urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-urea-SDS-PAGE) as described by Schagger and von Jagow, 1987 was applied to analyze the peptide profiles of pH 4.6-ISN fractions. The gel images were visualized and photographed by PhotoDoc-It Imaging System 4.5 (UVP Inc., San Gabriel, USA).

A Waters Alliance 2695 HPLC coupled with a 2996 UV detector was used in RP-HPLC analysis to show proteolysis of the cheese samples at different ripening times, reflected by the peptide profiles in the separated WSN fractions. The separation of the peptides was carried out at a Hypersil C18 column (4.6×250 mm, 5 μm). The solvent A was water containing 0.1% (v/v) trifluoroacetic acid (TFA) while the solvent B was acetonitrile containing 0.1% (v/v) TFA. Protein content of the analyzed samples was fixed at 10 g L^{-1}. The flow rate of elution was 0.75 mL min^{-1}. Injection volume of the samples and monitoring wavelength were set at 10 μL and 280 nm, respectively.

Observation of microstructure: The morphology and the microstructure of the cheese samples were characterized by using a Scanning Electron Microscope (SEM). Sample preparation was carried out as the method of Sanchez-Macias *et al*. (2013). The samples were mounted on metal stubs by silver paint and the surface was coated by gold. The images were taken at 5 kV with a Hitachi S-3400N model SEM (Tokyo, Japan). The micrographs were obtained at magnification of 300 and compared visually.

RESULTS AND DISCUSSION

Soluble nitrogen fractions of the ripened cheese samples: To investigate the proteolysis occurred in the external and central zone of the ripened cheese samples during ripening period, pH 4.6-SN fractions were separated from the ripening cheese samples and analyzed. The ratio of pH 4.6-SN to Total Nitrogen (TN) was thus calculated and is given in Table 1. The ratio values increased from 4.59 or 4.16 to 19.38 or 18.55% in the external and central zone of the samples, respectively, resulting in an increase level of 3.2 or 3.4-fold. This result indicated that some milk protein fractions were soluble in the medium of pH 4.6, due to the occurrence of proteolysis in the cheese samples. Also, it was also shown that the proteolysis was greater in the external zone than that in the central zone of the cheese samples. The growth of the *Mucor* and much protease production at the cheese surface attributed to this different proteolysis.

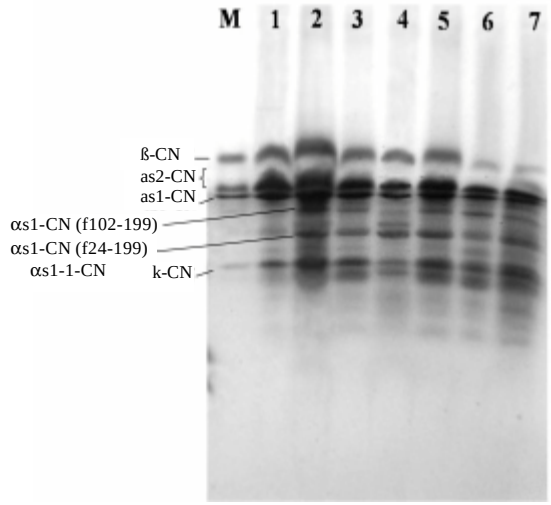

Fig. 1: Tricine-urea-SDS-PAGE profiles of pH 4.6-insoluble nitrogen (ISN) fractions of the ripened cheese samples. Lane 1-7, pH 4.6-ISN fractions separated from the cheese samples ripened for 1, 7, 14, 21, 35, 60 and 90 days, respectively, lane M: Casein, CN: Casein

Table 1: The measured ratio of pH 4.6 soluble nitrogen (pH 4.6-SN) to total nitrogen (TN) of the cheese samples ripened for different times

Ripening time (days)	Sample location	pH 4.6-SN/TN (%)
1	External	4.59±0.66
	Central	4.16±0.34
14	External	9.83±0.43
	Central	8.15±1.59
35	External	17.65±0.67
	Central	16.53±1.21
60	External	17.90±0.73
	Central	16.90±0.92
90	External	19.38±1.31
	Central	18.55±0.93

All values were expressed as Means±standard deviations, The number of trials was three

Gradual proteolysis in the ripened cheese samples: When the separated pH 4.6-ISN fractions from the cheese samples were subjected to electrophoresis analysis, the obtained profiles (Fig. 1) also showed the proteolysis induced by the *Mucor*. According to the obtained distributing profiles of the peptides, the nomenclatures of α_s-casein, β-casein, sub-κ-casein and the sub-groups of α_s-casein were carried out, in referring to the reported results from Voigt *et al.* (2010), Santillo *et al.* (2007), Milesi *et al.* (2007) and Sheehana *et al.* (2007).

It was seen form the Fig. 1 that the color of the α_{s1}-casein band became weaker when the cheese samples were ripened for 7 days; however, the color of the β-casein band was not weakened. This fact indicated that the α_{s1}-casein was easier to be degraded than the β-casein by the applied *Mucor* in the early ripening stage. Two new peptide fractions, α_{s1}-casein (f102-199) and α_{s1}-casein (f24-199), were generated (lane 2) as the result of the proteolysis of α_{s1}-casein. The similar result was also reported by Saeman *et al.* (1988), Van den Berg and Exterkate (1993) and Auldist *et al.* (1996). When the cheese samples were ripened for 14 days, the color of the β-casein band was weakened obviously (i.e. proteolysis of β-casein) and more α_{s1}-casein (f102-199) and α_{s1}-casein (f24-199) were generated (lane 2 vs. lane 3). If the cheese samples were ripened for longer time (e.g. 21-60 days, lane 4-6), some new peptides having lower molecular weights showed an increasing trend in their band color intensities (i.e. their amount), especially those peptides having molecular weights less than κ-casein. The appearance of these peptides revealed gradual degradation of milk protein fractions in the cheese samples. When the cheese samples were ripened for 90 days, a large amount of these new peptides were generated (lane 7). This result shared a similarity to the reported result of De Wit *et al.* (2005) and Sadat-Mekmene *et al.* (2013). I.e., the cheese samples got much proteolysis at this ripening time, as it was expected.

Peptide profiles of water soluble nitrogen fractions separated from cheese samples: The separated Water Soluble Nitrogen (WSN) fractions were also analyzed by a RP-HPLC procedure to reveal the protein degradation in the cheese samples during ripening. The obtained results are given in Fig. 2. Some peptides with retention time of 5-30 min were found in these WSN fractions and their peak area (or amount) increased as ripening time progressed. This fact indicated much proteolysis occurred.

Michaelidou *et al.* (2003) had found that there were some new peaks with retention time of 5-40 min in the WSN fractions of a Kefalograviera cheese during HPLC analysis and the peak heights of these fractions increased with ripening time. Verdini *et al.* (2004) studied the proteolysis of Port Salut Argentino cheeses by HPLC analysis and also found that the peak areas with retention time of 20-28 min had 3 or 4-fold increase in the external zone of the cheese during ripening. These two reported studies showed similar conclusion to the present study, i.e. some peptides of lower molecular weights were formed during cheese ripening. The composition and characteristics of the peptides in the WSN fractions of the ripened cheese samples were not known and should be identified or determined in further study.

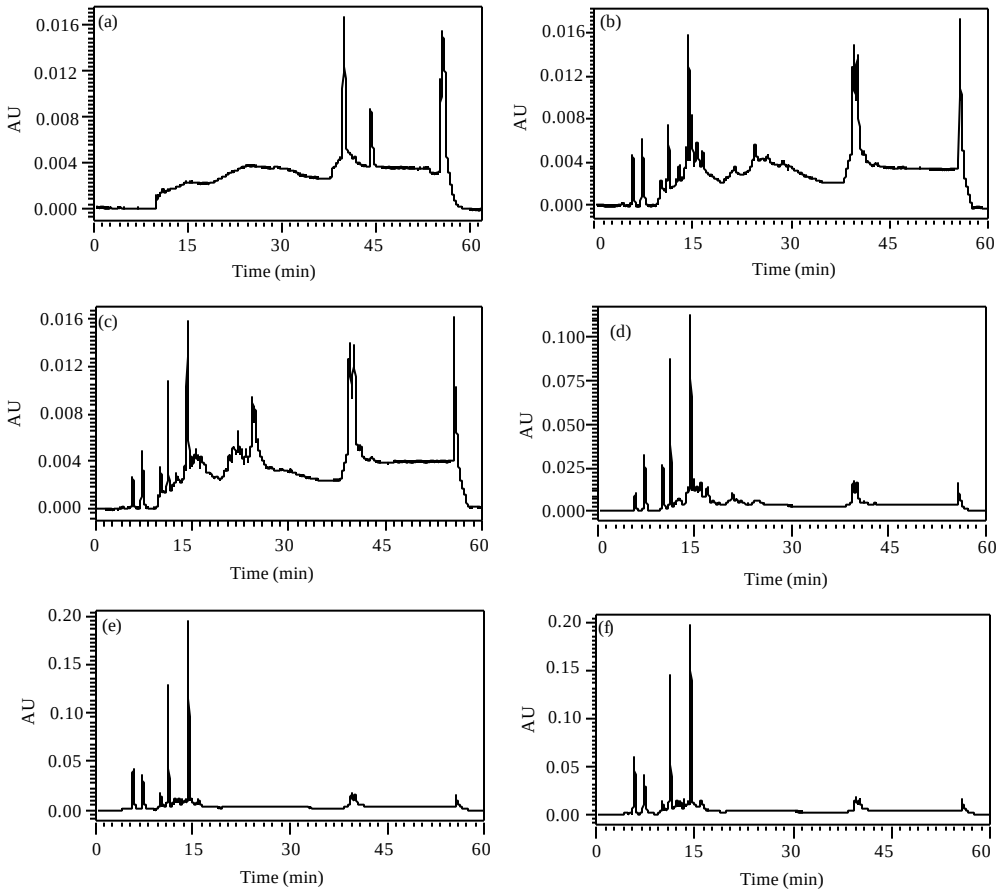

Fig. 2(a-f): RP-HPLC profiles of the water soluble nitrogen fractions separated from the cheese samples ripened for 1, 7, 14, 35, 60 and 90 days (a-f), respectively

Microstructural modification of the cheese samples during ripening: Microstructural features of the observed cheeses under SEM are shown in Fig. 3a-f. A smooth protein matrix was found for the 1-day-old cheese sample and several pore spaces were interspersed on the surface (Fig. 3a). The size and shape of these pore spaces were irregular and asymmetric, with estimated diameters in the range of 5-40 μm. The microstructural features of the cheeses samples ripened for a longer period (14, 21, 35, 60 and 90 days) were clearly modified (Fig. 3b-f), indicating the important role of the applied *Mucor* for cheese ripening. When the cheese samples were ripened for 35 or 60 days, they had a large number of smaller cellular pore spaces, which brought about uniformity and compactness in the microstructure (Fig. 3d-e). As the aggregates of casein micelles were degraded to numerous small aggregates during ripening, the microstructure of the cheese samples was thoroughly destroyed. After a ripening of 90 days the cheese samples showed a protein network in looser and symmetrical state (Fig. 3f). These images visually suggest that the protein degradation induced by the *Mucor* conferred microstructural modification on the cheese samples, as it was found that much pH 4.6-SN fractions were formed during cheese ripening (Table 1). Atia *et al.* (2004) had investigated microstructural changes of the Cheddar cheese during its ripening. They found that major microstructural change in the Cheddar cheese was the appearance of a compact, dense and homogeneous structure after a ripening time of 150 days.

Texture and flavor are two of the key quality attributes of the ripened cheeses. The present result showed the potential of the *Mucor* to improve the texture of the semi-hard cheese. Whether the *Mucor* can give the ripened cheese a desired flavor is needed to be investigated.

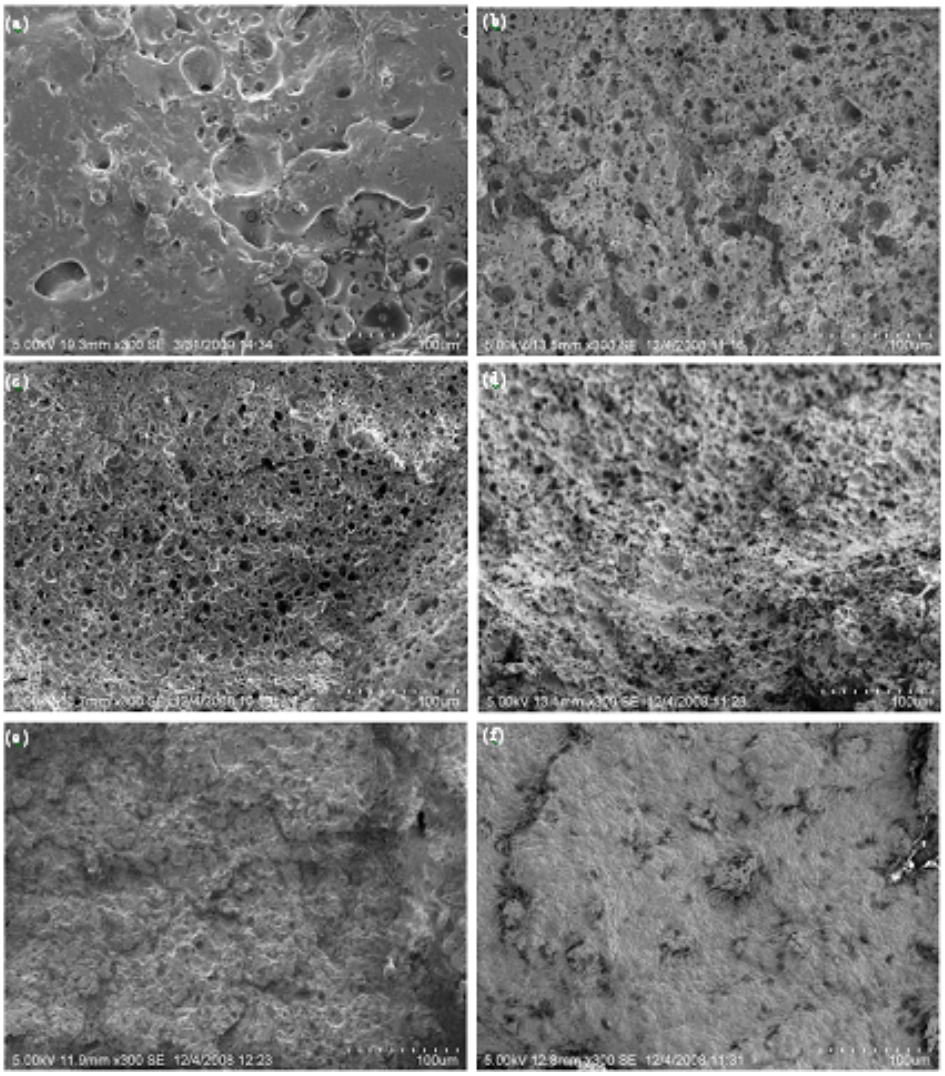

Fig. 3(a-f): Microstructural characteristics of the cheese samples observed under Scanning Electron Microscopy (SEM), The used magnification of SEM was 300, The assayed cheese samples were ripened for 1, 14, 21, 35, 60 and 90 days (A-F), respectively

CONCLUSION

During the *Mucor*-induced ripening of the semi-hard cheese, some milk proteins were degraded with the formation of some peptides of lower molecular weights, reflected by the carried out tricine-urea-SDS-PAGE and RP-HPLC analyses. At the same time, the ratio of pH 4.6-soluble nitrogen to total nitrogen exhibited a 3-fold increase after a ripening time of 90 days. SEM micrographs also showed that the ripened cheeses had more uniform and compact network structure. The *Mucor* spp. investigated induced proteolysis and textural modification in the cheese samples, thus may be a potential starter for surface-smeared mould ripened cheeses.

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