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Purification and partial characterization of milk-clotting enzyme extracted from glutinous rice wine mash liquor

Yanping Wang†, Qiaoling Cheng, Zaheer Ahmed, Xiaoxue Jiang, and Xiaojia Bai

Key Laboratory of Food Nutrition and Safety, Ministry of Education,
Tianjin University of Science & Technology, Tianjin 300457, China
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Abstract−Glutinous rice wine mash liquor is a traditional food of south of China and its ability to coagulate the milk has been proved. The aim of this work was to extract milk-clotting enzyme from glutinous rice wine mash liquor. A partial purified extract of enzyme was obtained by fractional precipitation with $(NH_4)_2SO_4$. The fractions obtained by precipitation, 40-90% possessed the milk-clotting activity (MCA) (145.72 U/mg). The 40-90% $(NH_4)_2SO_4$ fraction was further purified by sephadex G-100 and DEAE-sephadex A-50 with MCA (4,360±50 U/mg), which was confirmed by SDS-PAGE that showed only one band with a molecular mass of 36.0 kDa. Highest MCA was attained at 36 °C. The enzyme was completely inactivated by heating for 20 min at 60 °C. The MCA increased with the decreasing of milk pH from 8.0 to 5.5, and it was active at the wide range of pH 1 to 7. The metal ions Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Al^{3+}, Fe^{2+} had a very clear function to accelerate milk coagulation whereas Na^{+} and K^{+} decelerated the activity slightly. The curd effect of the milk-clotting enzyme has primarily been studied.

Key words: Milk-clotting Enzyme, Activity, Purification, Glutinous Rice Wine Mash Liquor, Enzyme Properties

INTRODUCTION

Enzymes are as good as chemical catalyst for catalyzing a variety of chemical reactions. Generally, more than 3000 reactions catalyzed by enzymes are known. Some of them are even more effective than corresponding chemical catalysts [1]. Microbial enzymatic hydrolysis is generally considered better than chemical hydrolysis because of high product specificity, simplicity of preparation procedures, and low environmental contamination [2]. As the development of industrial enzymes has depended heavily on the use of microbial sources [3], scientists are paying much more attention on it and new sources of enzymes such as cellulolytic enzymes by *Trichoderma inhamatum* KSJ1 [4] chitinase by *Aeromonas* sp. DYU-Too7 [2], Lipase by *Candida rugosa* [5] and milk-clotting aspartic proteinases by *Centaurea calcitrapa* [6] have been reported recently.

With growing urbanization in China, the demand for processed dairy foods has increased considerably, in particularly for different cheese varieties and low-lactose milk due to increasing intolerance of human beings to lactose in milk and other milk products. Cheese is a fermented dairy product which may have the highest nutritional value as it is enriched in proteins, fats, vitamins, minerals and other bioactive components. Rennet (EC 3.4.23.4) is the key enzyme in the manufacturing process of cheese. Calf rennet (bovine chymosin) is conventionally used as a milk-clotting agent in the dairy industry for the production of quality cheeses with good flavor and texture [7]. Owing to an increase in demand for cheese production worldwide, coupled with the reduced supply of calf rennet, there is a need to search for rennet substitutes.

Various plants and microbial proteases have been suggested as milk coagulants. Plant sources for milk-clotting enzymes have been identified from *Cynara scolymus* [8], *Helianthus annuus* [9], *Centaurea calcitrapa* [6] and *Ongokea gore tree* [10]. Unfortunately, most of these plant milk-clotting enzymes have been found to be unsuitable because they produce extremely bitter cheeses. Proposed microbial substitutes for animal proteases include those from fungi and bacteria, such as *Thermoascus aurantiacus* [11], *Aspergillus* [12], *Penicillium* [13], *Muco*r [14], *Rhizopus oryzae* [15] and *Bacillus sphaericus* [16]. At present, microbial rennet is used for one-third of the entire cheese produced worldwide.

Glutinous rice wine mash liquor, which can coagulate milk, is a traditional food in southern China. Liu and Luo [17] reported that the milk-clotting by glutinous rice wine mash liquor was a special process of chemical catalysts by rennet-like protease, an acid protease produced by molds of rice starter during glutinous rice fermentation. Therefore, glutinous rice wine mash liquor can coagulate milk because of the presence of milk-clotting enzyme in glutinous rice wine mash liquor. The organisms involved in the fermentation of glutinous rice have been isolated and identified [18-20]. In the previous work we reported the isolation and fermentation condition of milk-clotting enzyme producing strain from glutinous rice wine mash liquor [21]. Although Jiang et al. [22] have carried out the purification and proteolytic property and the cleavage site on κ-casein of milk-clotting enzyme from glutinous rice wine, but complete characteristics of the enzyme have not been explored. The aim of the present work was to extract, purify and to investigate the characteristics of milk-clotting enzyme from glutinous rice wine mash liquor, and later on its milk curding ability was also carried out to estimate its potential to use in cheese-making industry.

MATERIALS AND METHODS

1. Materials

Culture used: Chiu-Yao, a famous culture used in glutinous rice

†To whom correspondence should be addressed.
E-mail: ypwang40@yahoo.com

wine mash liquor was collected from Hubei province, whereas glutinous rice powder was collected from local market of Tianjin, China.

2. Methods

2-1. Preparation of Culture Filtrate from Glutinous Rice Wine Mash Liquor

Chiu-Yao, which is a traditional culture for glutinous rice wine mash liquor, was collected from Hubei, China and was grown in glutinous rice powder medium with the following components: glutinous rice power 5 g, distilled water 50 mL. The mixture was cultured at 30 °C for 52 hours by shaking on an orbital shaker (140 rpm). The culture supernatant was obtained by filtering through a pledget and followed by centrifugation at 4,000×g for 10 min.

2-2. Purification of Milk-clotting Enzyme

2-2-1. Ammonium Sulfate Fractionation

The culture supernatant was subjected to precipitation by increasing concentration of $(NH_4)_2SO_4$ as described by Green and Hughens [23]. Milk-clotting enzyme in crude extract was precipitated with ammonium sulfate (40-90% saturation). The precipitate obtained after centrifugation at 8,000×g for 15 min at 4 °C was suspended in phosphate buffer (5 mM, pH 6.2) and dialyzed overnight to remove the salt.

2-2-2. Size-exclusion Chromatography

The enzyme fraction precipitated by 40-90% $(NH_4)_2SO_4$ was loaded onto a Sephadex G-100 column pre-equilibrated with phosphate buffer (5 mM, pH 6.2). Enzyme fractions eluted with the same buffer were analyzed for enzyme activity and protein content. Active enzyme fractions were pooled and stored at 4 °C for further studies.

2-2-3. Ion-exchange Chromatography

The concentrated enzyme obtained from the above step was further purified by passing through DEAE-Sephadex A-50 previously equilibrated with phosphate buffer (20 mM, pH 7.4). The fractions were eluted at a flow rate of 30 mL/h with a linear gradient of 0.15-0.55 M NaCl in phosphate buffer (2 mM, pH 7.4). Fractions were collected and analyzed for enzyme activity and protein content. Active enzyme fractions were pooled and stored at 4 °C for further studies.

2-3. Enzyme Assay

The milk-clotting activity (MCA) was determined as described by Arima et al. [24]. The unit of MCA (SU) is defined as the amount of enzyme capable of clotting 1.0 mL of substrate in 40 min at 35 °C. A 10% suspension of skim milk powder was used as the substrate. Protein determination was done according to the method of Bradford [25], and SDS-PAGE was done by following the method of Laemmli [26].

2-4. Effect of Temperature on Activity and Thermostability

To study the effect of temperature on milk-clotting activity assay, the reaction mixture containing 10% skim milk was incubated at a temperature range of 25-65 °C.

The enzyme thermostability was determined by preincubating the enzyme in the temperature range of 25-60 °C. The incubation time of samples varied from 10 to 60 min. After incubation, the samples were submitted for determination of milk-clotting activity.

2-5. Effect of pH on Activity and pH Stability

To study the effect of pH on milk-clotting activity assay, the reaction mixture containing 10% skim milk was adjusted to different pH (3.5-8.5). The buffers used were: 0.1 M citrate-phosphate (pH 3.5-6.0) and 0.1 M sodium phosphate (pH 6.0-8.5).

For pH stability, the enzyme was dispersed (1 : 1) in the following 0.1 M buffer solutions: HCl-KCl (pH 1.0-2.0), citrate-phosphate (pH 3.5-7.0), sodium-phosphate (pH 6.0-7.5), Tris-HCl (pH 7.0-8.5), carbonate-bicarbonate (pH 9.0-12.0) and maintained at room temperature for 24 h, and afterwards MCA was determined.

2-6. Effect of Metal Ions

The effect of some metal ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Al^{3+}, Fe^{2+}) at 1 mM, 5 mM, 10 mM concentration on milk-clotting activity was tested. The enzyme was incubated at room temperature for 30 minutes with metal ions. Then the MCA was measured by the standard assay procedure.

2-7. Preparation of Milk Curd

The purified enzyme and commercial cheese rennet were used for the preparation of comparative milk curds. 400 mL fresh milk was poured into sterile cheese slurry vats, heated at 65 °C for 30 min, and then cooled rapidly by immersing it in an ice bath up to the time it attained a temperature of 37 °C. Milk was acidified by inoculation with a 2% inoculum *S. thermophilus* strain which was taken from our lab. Once pH was reduced to 6.4, 10 mL of milk-clotting enzyme or commercial cheese rennet was added, curded for 2 h which was followed by cutting and removing the whey. The protein content, fat content and moisture content of the curds obtained with the milk-clotting enzyme from glutinous rice wine mash liquor was compared with that of the reference curds obtained with the commercial rennet.

RESULTS AND DISCUSSION

1. Purification of Milk-clotting Enzyme

Partial purification of the milk-clotting enzyme was carried out by fractional precipitation with $(NH_4)_2SO_4$ and six fractions were obtained. The fraction precipitated with 0-20% and 20-40% $(NH_4)_2SO_4$ did not show MCA. Of all the six fractions obtained, precipitant obtained by 40-60% $(NH_4)_2SO_4$ was the most active milk-clotting

Table 1. Milk-clotting activity of precipitation fraction of culture liquid

Precipitation step (%)	Protein content (mg/mL)	MCA (U/mL)	Recovered MCA (%)	Specific MCA (U/mg)	Yield (%)
CF[a]	2.48±0.052	55.75±3.35	100.00	22.58±2.45	100
0-20	0.87±0.025	0.00	0.00	0.00	35.08±2.02
20-40	0.44±0.009	0.00	0.00	0.00	17.74±1.44
40-60	0.48±0.007	87.36±2.89	156.69±10.09	182.65±3.44	15.32±1.03
60-80	0.31±0.016	17.23±2.11	30.95±4.83	55.58±1.72	12.50±1.29
80-90	0.28±0.011	7.53±1.85	13.51±2.67	26.88±3.17	11.29±2.03
40-90	1.38±0.334	205.77±3.83	369.25±38.55	145.72±4.56	57.98±5.66

[a]CF=Culture filtrate

Table 2. Purification of chymosin extracted from glutinous rice wine

Purification step	Total MCA (U)	Total protein (mg)	Specific MCA (U/mg)	Purification fold	Yield (%)
CF[a]	27999	1238	22.6	-	100
$(NH_4)_2SO_4$ (40-90%) fractionation	16240	111	145.7	6.45	57.98
Sephadex G-100	9240	4.2	2200.0	97.4	33.07
DEAE-sephadex A-50	7000	1.6	4360.0	193.1	25.21

[a]CF=Culture filtrate

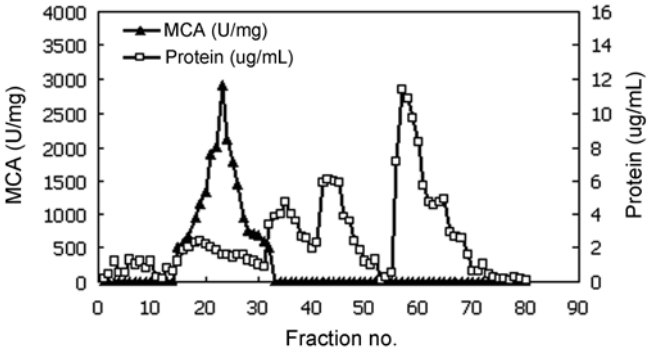

Fig. 1. Chromatographic purification of the milk-clotting enzyme by SephadexG-100 pre-equilibrated with phosphate buffer (5 mM, pH 6.2) and eluted with the same buffer. Flow rate was 12 mL/h. Enzyme activity and protein content were measured.

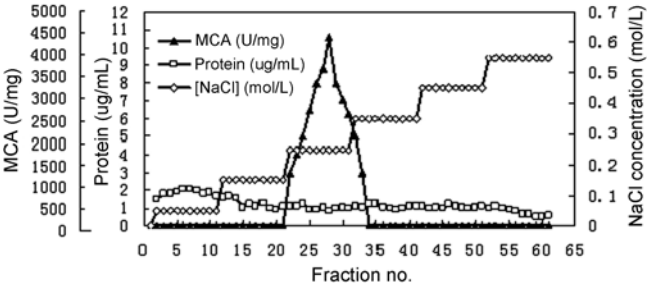

Fig. 2. Further purification chromatography on DEAE-Sephadex A-50 column previously equilibrated with phosphate buffer (20 mM, pH 7.4). The fractions eluted at the flow rate of 30 mL/h with linear gradient of 0.15-0.55 M NaCl in phosphate buffer (20 mM, pH 7.4). Fractions were collected and analyzed for enzyme activity and protein content.

enzyme fraction and its MCA was equivalent to 182.65 U/mg, with a recovery in activity of 156.69% (Table 1). On comparing the fraction precipitated by $(NH_4)_2SO_4$ at 40-60% with 40-90%, the later fraction was considered better because of higher recovered MCA (369.25%) and higher protein yield (57.98%). Sephadex G-100 column purified the enzyme to about 97.4-fold purification with specific activity of 2,200 U/mg (Table 2). The active fractions were further purified by DEAE-Sephadex A-50 and resulted in 193.1-fold purification of the enzyme (specific activity 4,360 U/mg). The elution diagrams of milk-clotting enzyme during fractionation of the crude enzyme using Sephadex G-100 (Fig. 1) and DEAE-Sephadex A-50 columns (Fig. 2) yielded a single active peak of MCA. Many previous studies have reported milk-clotting enzyme from different

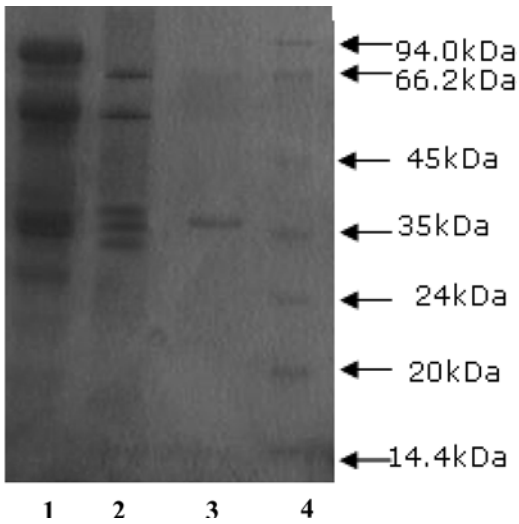

Fig. 3. SDS-PAGE of milk-clotting enzyme purification steps. Lane 1, 2, 3 and 4 was glutinous rice wine mash liquor, milk-clotting enzyme purified by sephadex G-100, milk-clotting enzyme purified by DEAE-sephadex A50 and marker.

sources using a range of chromatographic techniques; some of them showed single active peak [27-30]. On the other hand, two fractions exhibiting MCA were reported when the milk-clotting enzyme was purified from Myxococcus xanthus strain 422 [31], *Bacillus sphaericus* [16] and *Mucor baciliformis* [32]. The molecular weight of our milk-clotting enzyme was determined as 36.0 kDa by 12% SDS-PAGE gel (Fig. 3). This result is in accordance with the findings of other researchers who also reported enzymes with almost similar molecular weight, like milk-clotting enzymes of *Enterococcus faecalis* TUA2495L (34.0-36.0 kDa) [33], *E. paraitica* (37.5 kDa) [34] and *M. miehei* (34.0-39.0 kDa) [24]. Furthermore, calf rennet which is considered to be the best milk-clotting enzyme for cheese production, also has a similar molecular weight of 35.65 kDa [35].

2. Temperature Optima and Thermostability

The optimal temperature for the milk clotting enzyme activity was also investigated. The highest MCA of our purified milk-clotting enzyme was at 36 °C (Fig. 4). This result differs from the milk-clotting enzyme produced by *Nocardiopsis* sp., which showed the maximum activity at 55 °C [36]. The maximum MCA was recorded at 65 °C for *Rhizomucor miehei* [37], *Bacillus sphaericus* [16] and *Penicillium oxalicum* [38]. The optimum temperature for milk clotting enzyme to make cheese is 36 °C as at this enzyme can retain its maximum activity, and which is also an optimum temperature for most of the thermophilic lactic acid bacteria. Therefore, in this aspect this enzyme

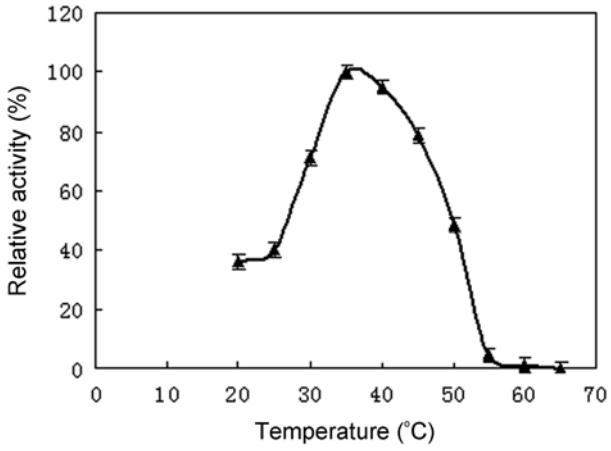

Fig. 4. Effect of temperature on the milk-clotting enzyme activity.

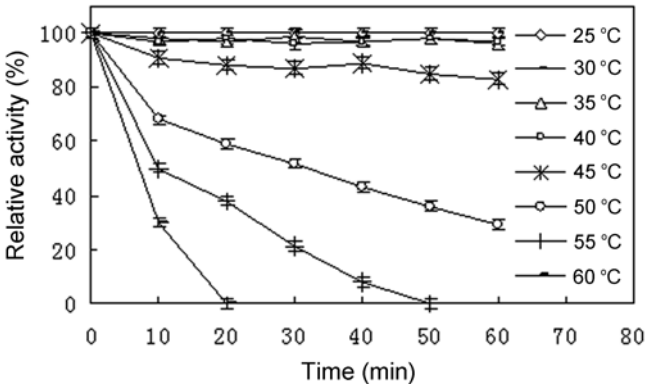

Fig. 5. Thermal stability of the milk-clotting enzyme.

is superior over many other enzymes reported in the literature whose optimum activity is at very high temperature. Higher temperature always accelerates the speed of curd stiffening and makes cutting of curd difficult.

The enzyme is quite stable at 40 °C for more than 60 min. Incubation of the enzyme at 45 °C for 20 min resulted in loss of 20% of its activity, while it was fully inactivated upon heating at 60 °C for 20 min (Fig. 5). This result was similar to the result of O'Leary and Fox [39] who found that acid protease produced by *M. pusillus* was completely inactivated after heating at 60 °C for 20 min. Also, milk-clotting enzyme from *Rhizopus oryzae* remains fully active even after 60 min of incubation at 40 °C, but at 60 °C it losses 62% of its initial activity [40]. Calf rennet is stable up to 50 °C and loses its activity at 60 °C [35]. The thermo stability of milk-clotting enzyme from glutinous rice wine mash liquor is a little lower than that of calf rennet, which is propitious to reusing the whey after making the cheese.

3. pH Optima and pH Stability

pH always has a significant effect on the milk clotting activity of enzyme. The milk-clotting enzyme from glutinous rice wine mash liquor exhibited the maximal MCA in milk at a pH of 5.5, and the MCA increased with the decreasing of milk pH from 8.0 to 5.5 (Fig. 6). Lin et al. [41] studied a culture filtrate from lao-chao with *Rhizopus oryzae* which was used as a milk-clotting agent; its maximum

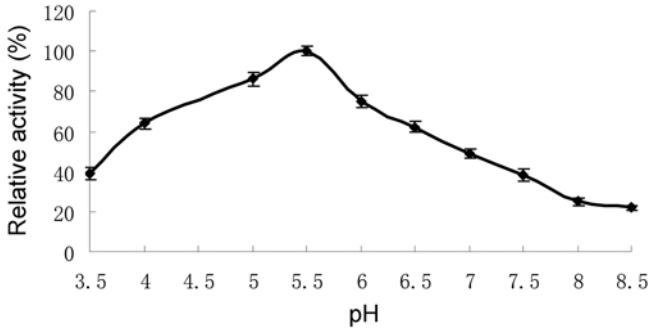

Fig. 6. Effect of pH on the milk-clotting enzyme activity.

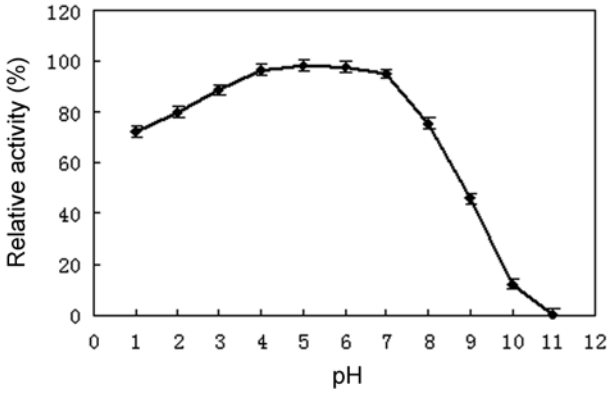

Fig. 7. Stability of milk-clotting enzyme at different pH.

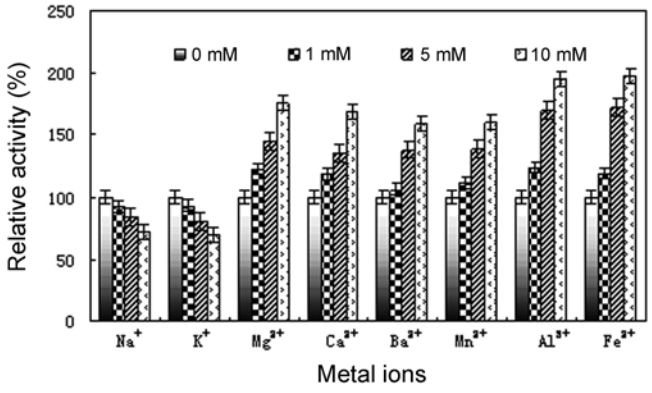

Fig. 8. Effect of metal ions on the milk-clotting enzyme activity.

activity was about pH 3. An optimum pH of 6.0 was reported for the partially characterized rennet-like proteases from Australian cardoon *Cynara cardunculu*s [42]. Purified milk-clotting aspartic protease from the stigma of artichokes showed maximum activity at pH 5.0 [43]. Amal et al. [38] and Cavalcanti et al. [36] studied the milk-clotting enzyme from *Penicillium oxalicum* and *Nocardiopsis* sp. and found that maximum activity was at pH 4 and 7.5, respectively. The enzyme from glutinous rice wine mash liquor was active at a wide range of pH, i.e., 1-7 and the activity was lost about 40% when pH was 8.0 (Fig. 7). Similar results have been reported for milk-clotting enzyme from *Mucor miehe* [44] and *Mucor bacilliformis* [45]. Lin et al. [41] also found the same result on a milk-clotting agent from lao-chao with *Rhizopus oryzae*.

Table 3. Comparison of curd made by different chymosin

Curd reagent	Protein content (%)	Fat content (%)	Moisture content (%)
The milk-clotting enzyme form glutinous rice wine mash liquor	22.8±1.2	27.9±2.4	46.5±3.2
Commercial cheese rennet	16.0±1.3	14.8±1.9	53.5±2.8

4. Effect of Metal Ions

The effects of different metal ions on MCA always exhibit differently. We found that Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Al^{3+}, Fe^{2+} are potent activators, whereas Na^{+}, K^{+} are inhibitors of the MCA (Fig. 8). The Al^{3+} and Fe^{2+} at 10 mM were the most effective, resulting in more than 2-fold increase of the MCA. Contrary to our results Magda et al. reported that Fe^{2+} ion did not show any effect on the enzyme activity [16]. Many researchers have reported that MCA increases with the increase of Ca^{2+} ion concentration. Ca^{2+} can accelerate para-κ-casein aggregation and it can make the tertiary structure of enzyme more stable. It has the function of protecting the enzyme, especially for the enzymes which have low thermostability.

5. Effect of Milk Curd

The effect of milk curd can influence the texture and quality of cheese.

In this study, the curding effect of the milk-clotting enzyme from glutinous rice wine mash liquor has been primarily studied. The results for total amount of protein content, fat content and moisture content are depicted in Table 3 in comparison with curd obtained by commercial cheese rennet and the milk-clotting enzyme from glutinous rice wine mash liquor. The curd obtained by the milk-clotting enzyme from glutinous rice wine mash liquor showed a little higher content of protein and fat content, good flavor and aroma; thus it has the potential to be used in the cheese industry.

In this report, the milk-clotting enzyme was extracted from glutinous rice wine mash liquor and its characteristics were investigated. The milk-clotting enzyme showed high activity with good characteristics. Moreover, media used for production of enzyme was rice powder which is very cheap, reducing the production cost, making it a viable candidate to substitute commercial rennin-like enzymes.

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