Asian-Aust. J. Anim. Sci.
Vol. 23, No. 10 : 1369 - 1379
October 2010

www.ajas.info

Comparison of Milk-clotting Activity of Proteinase Produced by *Bacillus Subtilis var, natto* and *Rhizopus oligosporus* with Commercial Rennet

Ming Tsao Chen*, Ying Yu Lu and Tien Man Weng

Department of Bioindustry Technology, Da Yeh University, 168 University Road, Dacun, Changhua, Taiwan, 51591

ABSTRACT : This study investigated purification and milk-clotting activity of the enzymes produced by *Bacillus subtilis var, natto* and *Rhizopus oligosporus* compared with that of commercial rennet. The clotting time, viscosity, tension and microstructure of the curd and electrophoretic patterns of milk proteins were determined. The milk-clotting activity/proteolytic activity ratios (MCA/PA ratio) of *B. subtilis, R. oligosporus* and commercial rennet were also compared. The results revealed that the curd formed by the commercial rennet had the highest viscosity and curd tension and the shortest clotting time among the three enzymes. However, curd produced by *Rhizopus* enzymes was ranked as second. From the MCA/PA ratio and electrophoretogram analyses it could be concluded that the enzyme produced by *B. subtilis* had the highest proteolytic activity, while the commercial rennet had the highest milk-clotting activity. Observations of microstructures of SEM showed that the three-dimensional network for curd formed by commercial rennet was denser, firmer and more smooth. The milk-clotting activity, specific activity, purification ratio and recovery of the purified enzymes produced by both the tested organisms were also determined with ion exchange chromatography and gel filtration. (**Key Words :** Microbial Rennet, Milk Clotting, *Bacillus subtilis*, *Rhizopus oligosporus*, Purification)

INTRODUCTION

Rennet, the milk-clotting enzyme used in cheese-making is obtained from the stomach contents of the unweaned calf. Because of the difficulty with acquiring sufficient quantity of rennet for the cheese industry, especially the incidence of mad cow disease, other sources for the enzyme have been sought for many years (Arima et al., 1970) from some strains of bacteria (Babbar et al., 1965), fungi (Knight, 1966; Sardinas, 1966; Richardson et al., 1967) and high plants (Berkowittz-Hundert et al., 1964) were investigated to produce a suitable milk-clotting enzyme. The enzymes produced by *Rhizopus spp.*, *Mucor* and *Rhizomucor* having milk-clotting activity have been reported (Wang et al., 1969; Preetha and Boopathy, 1997; Seker et al., 1999; Kumar et al., 2005). However, during our investigation on natto fermentation, we found that *Bacillus subtilis var, natto* produced enzyme having milking-clotting activity. Natto is a traditional soybean fermented food in Japan and Asian countries. Its functional properties and fermentation technology have been documented by many authors. However, the milking-clotting activity has not been reported. Therefore, the purpose of this study was to investigate the milk-clotting activity of enzyme produced from soybeans fermented by *B.subtilis var, natto* and properties of milk curd as compared with the enzyme produced by *Rhizopus oligosporus* and the commercial rennet.

* Corresponding Author: Ming Tsao Chen. Tel: +886-4-8511325, Fax: +886-4-8511325, E-mail: michen@mail.dyu.edu.tw

Received December 17, 2008; Accepted July 11, 2009

MATERIALS AND METHODS

Preparation of soybean as fermentation medium

Soybeans were washed and soaked in three times their weight of tap water at 4°C for 18 h and then drained. The soaked soybeans were cooked for 20 min in an electrical cooker and cooled. Then, the cooked beans were placed in a steaming shelf and steamed at 121°C (1.2 kg/cm^2) for 15 min in a retort (HILXLEY, Model:HL-341, USA). The steamed beans were cooled to 40°C and 100 g of which were allotted in Styrofoam box (11×11×8 cm) and covered immediately with an aluminum foil which was punched several small holes used for inoculation.

Preparation of *natto*

Natto fermentation was carried out according to the procedure described by Bernard et al. (2004). *Bacillus subtilis var, natto* starter was obtained from Yuzo Takahashi Laboratory Co., Japan (Nattomoto). Inoculum was prepared with 0.1 g nattomoto plus 10 ml sterilized distilled water and inoculated in 100 g of the steamed soybeans and incubated at 43°C for 20 h. During fermentation the punched aluminum foil was covered after inoculation. The natto samples were left at 4°C for 24 h to complete maturation prior to analysis.

Preparation of tempeh

Tempeh fermentation was also carried out using the method described by Bernard et al. (2004). Hundred grams of steamed soybeans were inoculated with 0.2 g of a commercial starter of *Rhizopus oligosporus* which was obtained from P.T. Aneka Fermentasi Industri, Indonesia. After inoculation the mixture was incubated at 37°C for 20 h under humidity control. Fresh tempeh was stored at 4°C 24 h for maturation.

Crude enzyme preparation

The fermented products of natto and tempeh were separately added with distilled water by the ratio of 1:1, and homogenized using a blendor (Osterizer Co. Ltd.), then centrifuged by 10,000×g, 10 min. at 4°C. The supernatant was collected and stored at 4°C for analysis.

Purification of milk-clotting enzymes from *natto* and tempeh

The enzyme preparation obtained from the above step was further purified by the procedures of ion-exchange chromatography of DEAE-Sepharose CL-6B and size-exclusion chromatography of Sephacryl S-100HR. The fractions were collected for enzyme activity and protein content analyses (Kumar et al., 2005).

Curdling test

The reconstituted milk was prepared with 30 grams of skim milk powder added 70 ml of sterilized distilled water in 100 ml glass beaker(idxheight = 6×7 cm). Crude enzyme solution was adjusted pH by diluted HCl or NaOH solution to 5, 6, 7, and 8, then added milk by the ratio of 1:5 (v/v, enzyme solution:milk), and incubated at 25, 30, 35 and 40°C in water bath, respectively. The clotting time was recorded.

Viscosity and tension of curd measurements

Viscosity and tension of curd indicate mechanical properties of curd formed by action of clotting enzyme. They are very important physical properties for fermented dairy products. They are affected by pH and temperature. The suitability of viscosity and tension of curd may affect casein grain production while curd cutting of cheese-making. Samples were prepared by the same procedure as curdling test. Viscosity was measured according to the method described by Argia et al. (1992). Viscosity of curd was measured by a viscometer (Brookfield Digital Viscometer Model DV-E) using Brookfield spindle No. LV-4(S64), loading the spindle on viscometer and lowering the spindle to the level as the same as the milk sample and starting the spindle for 10 s, then record its viscosity number. Curd tension was measured according to the method described by Huang and Chen (2006). Curd tension was measured by a rheometer (Sun Rheo meter Compac-199II), the conditions for test were as follows:

Prob: No. 11, loading weight: 2 kg, loading stage speed: 5 cm/min and press depth: 2 cm/min.

SDS-PAGE of milk proteins in curd

Extraction of milk proteins was performed using the procedure of Mujoo et al. (2003). Twenty milligrams of curd were extracted with 500 μl of 0.03 M Tris [Tris (hydroxymethyl)aminomethane] buffer (pH 8.0) containing 0.01 M β-mercaptoethanol (β-ME) for 1 h with vortexing every 10 min. Samples were then centrifuged at room temperature for 20 min at 11,000×g; the supernatant contained the total milk proteins.

SDS-PAGE was carried out by the method of Harlow and Lane (1988). The protein content of the supernatant was analyzed according to the Bradford method (Bradford, 1976). The protein extract was diluted to 4 mg/ml with distilled water, and 20 μl of the diluted extract were mixed with 20 μl of SDS-sample buffer (0.15 M Tris-HCl, pH 6.8, 4% w/v SDS, 5% v/v β-ME) and heated at 96°C for 3 min. Fifteen μl of the solution cooled to room temperature (20°C), containing 40 μg of protein, were loaded onto a gradient gel containing 15% polyacrylamide.

Phosphorylase b (MW 94000), albumin (MW 67000), ovalbumin (MW 43000), carbonic anhydrase (MW 30000), trypsin inhibitor (MW 20100), α-lactalbumin (MW 14400) were employed as protein standards (markers) for the identification and estimation of the molecular weight of milk proteins.

Purification of clotting enzyme

Purification of clotting enzyme was carried out by the procedure of Kumar et al. (2005). The crude enzyme solution was precipitated with 20-80% saturated ammonium sulfate solution and dialyzed for 24 h, then through ionic exchange chromatography and gel filtration. The protein concentration was determined with the method of Bradford (1976).

Table 1. Effects of pH and temperature on clotting time by the crude enzyme from soybean fermented by *Bacillus subtilis* var. *natto*

Temp. (°C) \ pH	pH 5	pH 6	pH 7	pH 8
25	27.0±1.0 ^{ax}	24.0±0.2 ^{ax}	21.3±2.8 ^{ax}	28.0±3.6 ^{ax}
30	24.6±3.7 ^{axy}	20.3±4.5 ^{axy}	18.6±4.0 ^{axy}	22.0±3.4 ^{axy}
35	20.6±0.5 ^{ay}	16.0±1.7 ^{bcy}	14.0±1.7 ^{cy}	18.6±0.5 ^{by}
40	14.6±1.5 ^{az}	12.0±1.0 ^{az}	10.6±1.5 ^{az}	14±1.0 ^{az}

$^{a-c}$ Different superscripts within the same row indicate significantly different ($p<0.05$).
$^{x-z}$ Different superscripts within the same column indicate significantly different ($p<0.05$).
Mean±SD; n = 3. Time:min.

Microstructure of curd

Microstructure of the curd was studied with field emission scanning electron microscope (FE-SEM; JEOL JSM-7401F). The dehydrated sample curd was fixed on double sides sticking gel tape of aluminum stage and coated with golden film. Then the coated sample was placed on the sample stage in the chamber, vacuumed and then scanned the microstructure of its surface.

Milking clotting and proteolytic activities of enzyme

The milk-clotting activity of enzyme was determined using the procedures described by Kumar et al. (2004) with modification. Five milliliters of assay milk (10% skim milk powder containing 0.01 M $CaCl_2$) was taken in a test tube and the contents were brought to the temperature of 37°C. 0.5 ml of enzyme extract was then added and the curd formation was observed while manually rotating the test tube from time to time. The end point was recorded when discrete particles were discernible. One milk-clotting unit is defined as the amount of enzyme present in 1 ml of extract clotting 10 ml substrate in 40 min i.e.

$$\text{Milk-clotting unit (U/ml)} = 2{,}400/t \times DF$$

Where t was clotting time and DF is dilution factor.

In this test, the commercial rennet from *Mucor miehei*, type II purchased from Merck Co. Ltd. (USA) was used to compare with the clotting activity of crude enzyme solution extracted from *Bacillus subtilis var, natto* and *Rhizopus oligosporus*. The commercial rennet of *Mucor miehei* was prepared by dissolving 1 g of compound in 100 ml distilled water.

The proteolytic activity of the enzyme was assayed using the method described by Arima et al. (1970). To 2.5 ml of 1% (w/v) alkali soluble casein in 0.02 M potassium phosphate buffer (pH 6. 5) 0. 5 ml of the enzyme extract was added. The reaction mixture was incubated at 37°C in a water bath for 10 min and the reaction was terminated by adding 2.5 ml of 0.44 M trichloroacetic acid. The precipitates formed were removed by filtration through Whatman No. 1 filter paper. One milliter of 1 N Folin-Ciocalteu reagent and 2.5 ml of 0.55 M sodium carbonate solution was added to 1 ml of the above clear filtrate. This was further incubated at 37°C for 20 min for color development. The optical density at 660 nm expresses activity in term of proteolytic units (PU).

Statistical analysis

The data were analysed the general linear models procedure of the Statistical analysis Software (MINTAB 10). Signifivant differences were determined using Duncan's multiple range test: the level of significance employed for all tests was p = 0.05. And Sigma Plot 2001 was used for plotting.

RESULTS AND DISCUSSION

Milk-clotting time

Effects of pH and temperature on the milk-clotting time for the enzymes obtained from soybean fermented by *B.*

Table 2. Effects of pH and temperature on clotting time by the crude enzyme from soybean fermented by *Rhizopus oligosporus*

Temp. (°C) \ pH	pH 5	pH 6	pH 7	pH 8
25	12.0±2.6 ^{ax}	11.6±2.5 ^{ax}	12.3±1.5 ^{ax}	15.0±1.7 ^{ax}
30	10.3±3.7 ^{ax}	10.0±3.4 ^{axy}	11.0±1.0 ^{axy}	14.3±2.1 ^{axy}
35	10.0±1.0 ^{bx}	9.0±1.7 ^{bxy}	10.6±1.5 ^{bxy}	14.0±2.6 ^{axy}
40	8.0±1.0 ^{ax}	7.6±1.1 ^{ay}	8.3±2.3 ^{ay}	10.0±2.6 ^{ay}

$^{a-c}$ Different superscripts within the same row indicate significantly different ($p<0.05$).
$^{x-y}$ Different superscripts within the same column indicate significantly different ($p<0.05$).
Mean±SD; n = 3. Time:min.

subtilis var, natto and *R. oligosporus* were shown in Table 1 and 2. From Table 1 it could be found the optimal conditions for milk-clotting time of enzyme produced by *B. subtilis* were at pH 7 and 40°C. Table 2 indicated the optimal conditions for milk-clotting time of enzyme produced by *R. oligosporus* were at pH 6 and 40°C. The clotting speed of enzymes from both organisms increased with temperature in spite of pH. However, decrease in milk pH will shorten clotting time (Storry and Ford, 1982). But McMahon and Brown (1984) elucidated that the effect of pH on clotting activity of enzyme at nonenzymatic action stage than enzyme action stage. Cheryan et al. (1975) indicated that clotting time for rennin from calf could shorten 30 times when milk pH value lowered from 6.7 to 5.6. As pH is lowered it can enhance casein occurring hydrophobic effect, and leading hydrophobic group to expose out, then resulting in casein micelle unstable and shorten the clotting time (Dalgleish and Law, 1988).

Viscosity and tension of curd

Rheological properties of curd are the important index of the texture of yoghurt (Labropoulos et al., 1984). Physical properties of curd of milk are expressed in many ways such as viscosity, hardness, elasticity and firmness (Modler et al., 1983; Parnell-Clunies et al., 1986). Viscosity and curd tension were used to indicate the texture of milk curd formed by the enzymes obtained from *B. subtilis* and *R. oligosporus* in this study. Figure 1 and 2 were shown that viscosity of curd increased with clotting temperature increased. It was found viscosity of curd formed at 40°C, pH 5 for enzymes obtained from both organisms was higher than those of other temperatures (25 to 35°C) and pH.

In generally, the viscosity of curd is correlated with the size of casein micelle which is affected by the denaturated whey proteins and the covalent bonding between sulfhydryl group and disulfides of casein micelle (Parnell-Clunieset et al., 1986).

Effects of temperature and pH value on curd tension

McMahon and Brown (1984) proposed that increase in clotting temperature could fasten enzymatic and non-enzymatic clotting reaction, and enhance casein micelles assembled and bonding to make firmer curd (Dalgleish, 1987). Lagoueyte et al. (1994) observed curd formed at different clotting temperatures and found casein micelles assembled firmlier and protein chain became denser.

Figure 3 and 4 indicated that curd tension became firmer with clotting temperature increased. It was found the firmness of curds formed at clotting temperature 40°C for the enzymes obtained from both organisms was higher than those of curds formed at other temperatures (25 to 35°C). The trends in effects of temperature and pH value on curd tension were in agreement with viscosity of curd.

Comparison on milk-clotting time and rheological properties of curd fromed by enzyme action

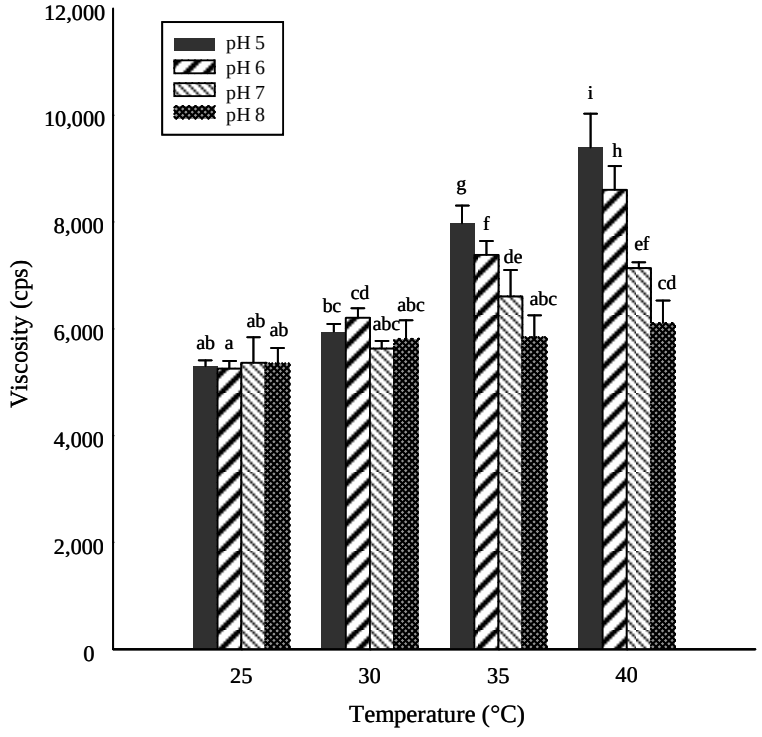

Figure 1. Effects of pH and temperature on clotting viscosity by the crude enzyme from soybean fermented by *Bacillus subtilis* var. *natto* (mean±SD; n = 3; $p<0.05$).

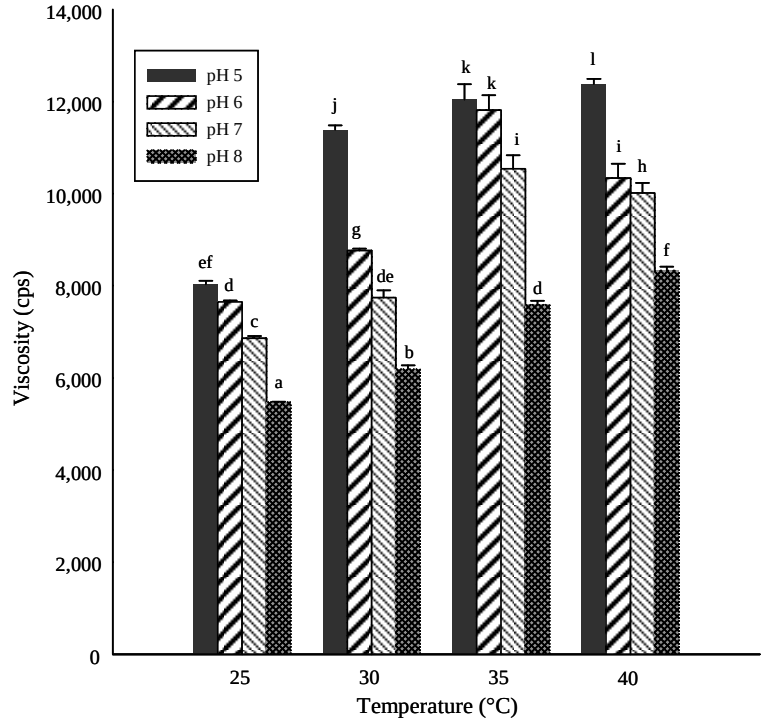

Figure 2. Effects of pH and temperature on clotting viscosity by the crude enzyme from soybean fermented by *Rhizopus oligosporus* (mean±SD; n = 3; $p<0.05$).

From Table 3, it was found that the rennet produced by *Mucor meihei* (Merck Co.) had the shortest milk-clotting time, *Rhizopus* was the second and *B. subtilis* was the longest. The viscosity and curd tension of the curds caused by the clotting enzymes were obtained as the following order:rennet>*Rhizopus*>*Bacillus.* From these results we

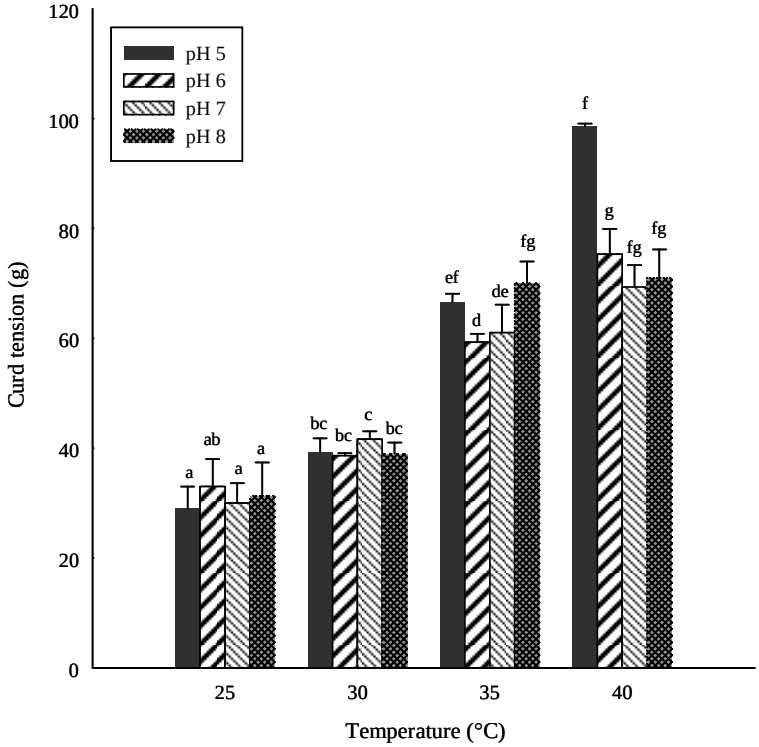

Figure 3. Effects of pH and temperature on curd tension by the crude enzyme from soybean fermented by *Bacillus subtilis* var. *natto* (mean±SD; n = 3; $p<0.05$).

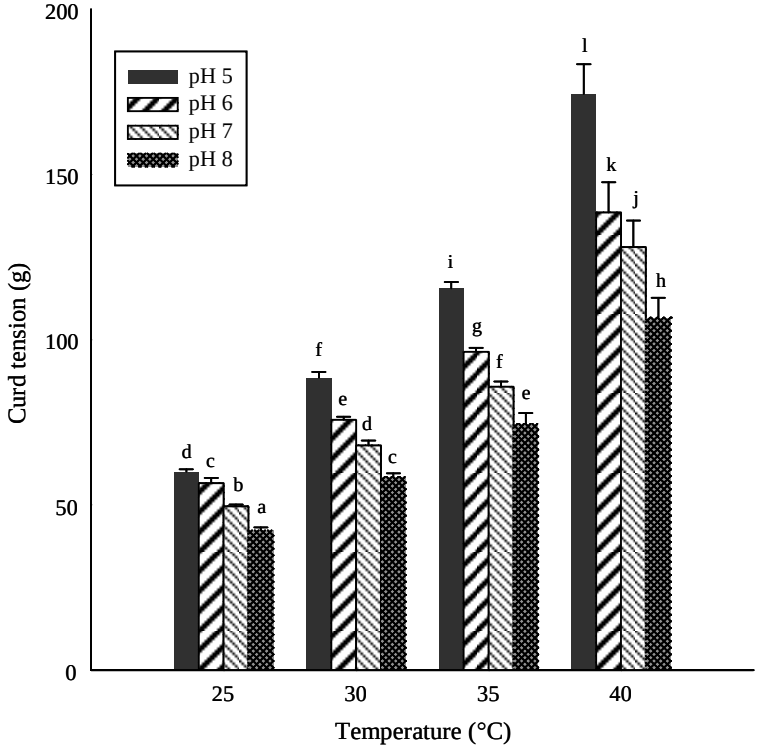

Figure 4. Effects of pH and temperature on curd tension by the crude enzyme from soybean fermented by *Rhizopus oligosporus* (mean±SD; n = 3; $p<0.05$).

noted that the commercial rennet had the highest milk-clotting activity. Although the enzyme from *B. subtilis* took longer time to coagulate milk, it might not be suitable for cheese-making use. However, the enzyme can coagulate milk and delay it passing through the aliment tract for completely digestion, especially for baby formula.

Purification of clotting enzyme produced by *B. subtilis var, natto*

The protein peak with milk-clotting activity was 52 U/ml in the fraction number 14 to 21 (Figure 5) which were collected together and lyophilized for further gel filtration. The lyophilized enzyme powder was redissolved in small amount of 50 mM phosphate buffer.

Then repeated gel filtration on Sephacryl S-100HR column and eluted in the same buffer solution. The active fractions were pooled and protein concentration was measured at 595 nm wave length. The enzyme activity was also analyzed. The active peak of the milk-clotting enzyme was found in fraction number 19 to 21 which was 20 U/ml (Figure 6), and collected together.

The changes in milk-clotting and proteolytic activities

Table 3. Comparison on time, viscosity and curd tension of clotting milk by the milk-clotting enzymes produced by *Bacillus subtilis* var. *natto*, *Rhizopus oligosporus* and rennet (mean±SD; n = 3)

Treatment	Time (s)	Viscosity (cps)	Curd tension (g)
Rennet	170±33	11,835±1,038	284±13
Enzyme from *Rhizopus oligosporus*	201±7	7,307±82	62±1
Enzyme from *B. subtilis* var. *natto*	437±5	5569±190	39±0.5

Table 4. Purification of milk-clotting enzyme from soybean fermented by *Bacillus subtilis* var. *natto*

Step	Milk clotting activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude enzyme	60	675	0.088	1	100
Ammonium sulfate	40	443	0.090	1.02	66.7
DEAE-sepharose CL-6B	26	27	0.963	10.94	43
Sephacryl 100 HR	22	14.79	1.487	16.89	36.7

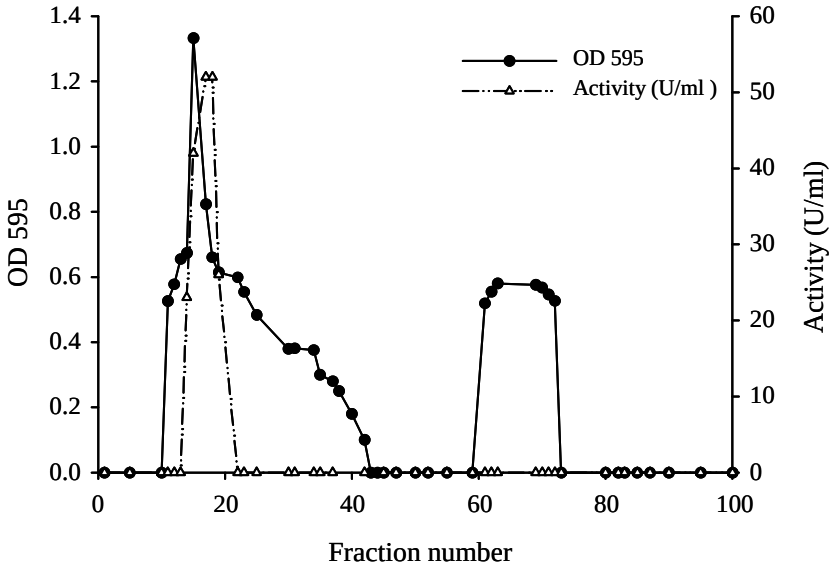

Figure 5. Chromatographic pattern of milk-clotting enzyme from soybean fermented by *Bacillus subtilis* var. *natto* on DEAE-Sepharose CL-6B gel

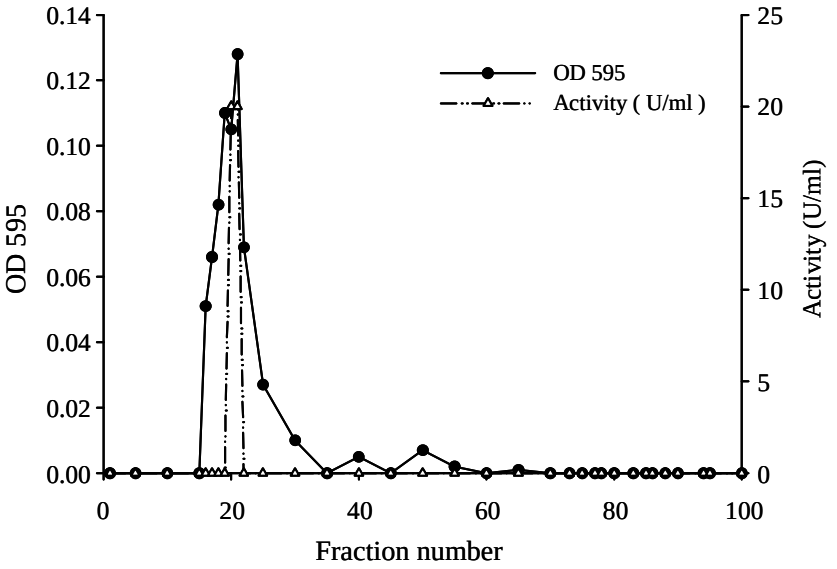

Figure 6. Chromatographic pattern of milk-clotting enzyme from soybean fermented by *Bacillus subtilis* var. *natto* on Sephacryl S-100HR gel.

of the enzyme produced by *B. subtilis var, natto* after purification were shown in Table 4. The specific activity of crude enzyme before purification was 0.088 U/ml protein, and 1.487 U/ml protein after purification. The recovery was 36.7% and the ratio of purification was 16.89.

Purification of clotting enzyme produced by *R. oligosporus*

The processes of purification with ion exchange chromatography and gel filtration were the same as those of the enzyme produced by *B. subtilis var, natto*. The protein peak with milk-clotting activity was 100 U/ml in the fraction number 15 to 42 through ion exchange chromatography (Figure 7). And the active peak of the milk-clotting enzyme was 88 U/ml which was found in the fraction number 22 to 25 (Figure 8). The changes in milk-clotting and proteolytic activities of the enzyme produced by *R. oligosporus* were shown in Table 5. The specific activity of crude enzyme before purification was 0.230 U/ml protein, and 6.32 U/ml protein after purification. The recovery was 19.7% and the ratio of purification was 27.47.

Comparison on milk-clotting and proteolytic activities

The ratio of milk-clotting and proteolytic activities of

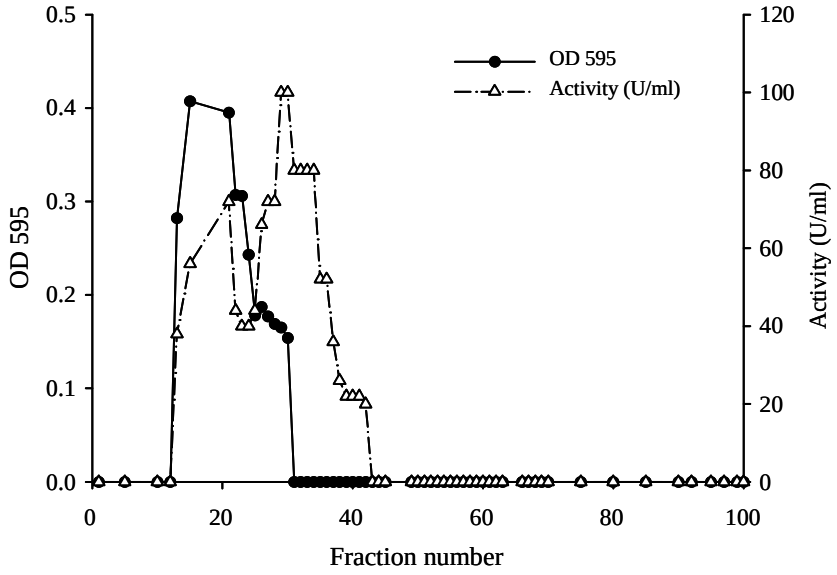

Figure 7. Chromatographic pattern of milk-clotting enzyme from soybean fermented by *Rhizopus oligosporus* on DEAE-Sepharose CL-6B gel.

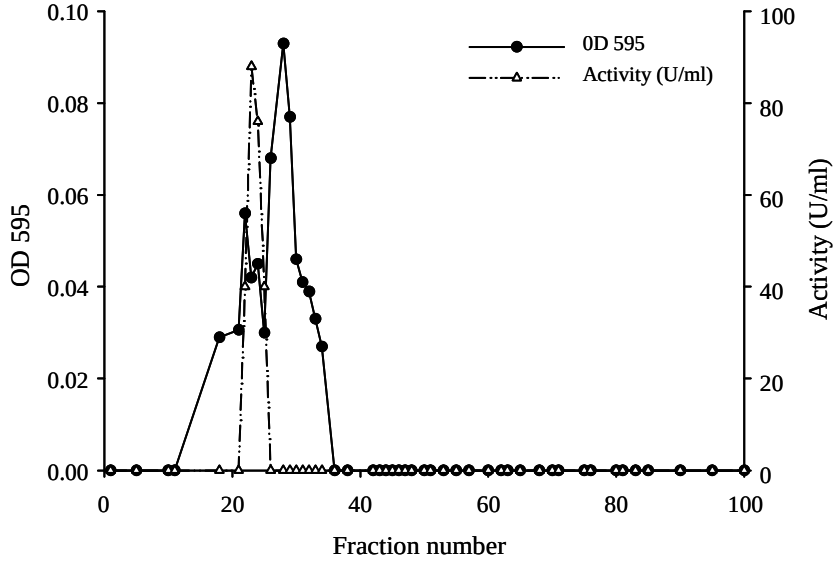

Figure 8. Chromatographic pattern of milk-clotting enzyme from soybean fermented by *Rhizopus oligosporus* on Sephacryl S-100HR gel

enzyme (MCA/PA) is an important standard for replacement of calf rennet. Rao and Mathur (1979) proposed that microbial milk clotting enzymes generally showed greater proteolytic activity than calf rennet and produced lower yields of cheese. Enzymes with lower MCA/PA ratio used as clotting agent for cheese making will produce the cheese of lower yields, soft body and biter taste (Sardinas, 1972). Almost all proteases are able to clot milk in suitable conditions. Therefore, it must be considered MCA/PA ratio as searching calf rennet replacers. The higher MCA/PA ratio enzyme which has higher milk-clotting activity and lower proteolytic activity is suitable for cheese-making. Thus, clotting time is shorter and the biter tasty peptides are not easily produced.

The result of milk-clotting and proteolytic activities for the enzymes produced by *B. subtilis var, natto* and *R. oligosporus* as compared with the commercial rennet were shown in Table 6. From the data we could find that the commercial rennet had the highest clotting activity and lowest proteolytic activity, its MCA/PA ratio was 4,669.6. This finding was the same as the result of Arima et al. (1979). However, MCA/PA ratio for the crude enzyme produced by *R. oligosporus* was 609.7 which was lower than the commercial rennet, but higher than those of papain

Table 5. Purification of milk-clotting enzyme from soybean fermented by *Rhizopus oligosporus*

Step	Milk clotting activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude enzyme	132	575	0.230	1	100
Ammonium sulfate	114	269	0.424	1.84	86
DEAE-sepharoe CL-6B	66	12	5.5	23.91	50
Sephacryl 100 HR	26	4.1	6.32	27.47	19.7

Table 6. Comparison of milk-clotting activity to proteolytic activity ratio

Treatment	Milk clotting activity (U)	Proteolytic activity (OD660)	Ratio (U/OD660)
rennet	537±1.8	0.115±0.002	4,669.6
Enzyme from *Rhizopus oligosporus*	239±5.6	0.392±0.001	609.7
Enzyme from *B. subtilis* var. *natto*	110±1.2	0.440±0.009	250.0

Mean±SD. n = 3.

(367), ficin (393) and the crude enzyme produced by *B. subtilis var, natto* (250) which was higher than that of Biodiastase (138). From these results we also noted that the proteolytic activity of the crude enzyme produced by *B. subtilis var*. *natto* was higher than those of proteases of *R. oligosporus* and the commercial rennet. Rao and Mathur (1979) reported that the proteases prepared from *B. subtilis* produced lower yield of cheese due to continued proteolysis beyond milk-clotting. Puhan and Irvine (1973) also reported that the milk-clotting enzyme for cheese-making prepared from a mutant of *B. subtilis* lowered the yield and made a softer curd. Therefore, they suggested that the best cheese was made from milk acidified to pH 6.3 to 6 or the bacterial enzyme combined with the commercial rennet by the ratio of 1:1 when the *B. subtilis* proteases were used as coagulants.

SDS-PAGE patterns of milk proteins in curd

Figure 9 indicated the electrophoretograms of the proteins in the curds of the commercial rennet, enzymes produced by *B. subtilis* and *R. oligosporus*. Lane 1 was the pattern of milk (the reference), Lane 2 was the pattern obtained from the curd obtained from rennet, Lane 3 was the pattern of the curd obtained from *B.subtilis* and Lane 4 was the pattern of the curd obtained from *Rhizopus.* From the electrophoretograms we found that two components of molecular weight near 75 kDa disappeared and the bands of other two components of 32 kDa (α-casein) and 26 kDa (β-casein) became small after enzyme reaction. The bands of 32 kDa and 26 kDa components almost disappeared and two bands appeared at 15 kDa and below 10 kDa of the electrophoretogram of the curd by *B. subtilis*. This indicated the enzyme produced by *B. subtilis* had the highest in proteolytic activity among three enzymes. The results were in the agreement with MCA/PA ratio.

Microstructure of the curds

The curds from three enzymes tested were used to analyze their microstructure with SEM. From Figure 10 it was observed that the microstructure of the curd from *B. subtilis* surface of network became looser and rougher and the granules were bigger, while the granules on the microstructure of the curd from *R. oligosporus* were smaller and the surface was less rough. However, the microstructure of the curd from the commercial rennet showed denser and smooth network and less granules. These results indicated that the commercial rennet used for cheese-making could get better quality curd.

CONCLUSION

The crude milk-clotting enzyme produced by *B. subtilis var, natto* had the shortest clotting time at pH 7, 40°C and optimum conditions for viscosity and curd tension were

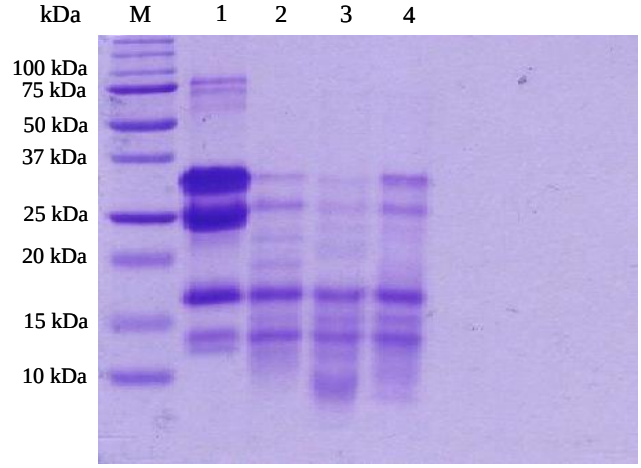

Figure 9. SDS-PAGE electrophoretograms of milk and curd formed by enzymes. M = marker, 1 = milk, 2 = commercial rennet, 3 = *B. subtilis*, 4 = *R. oligosporus*.

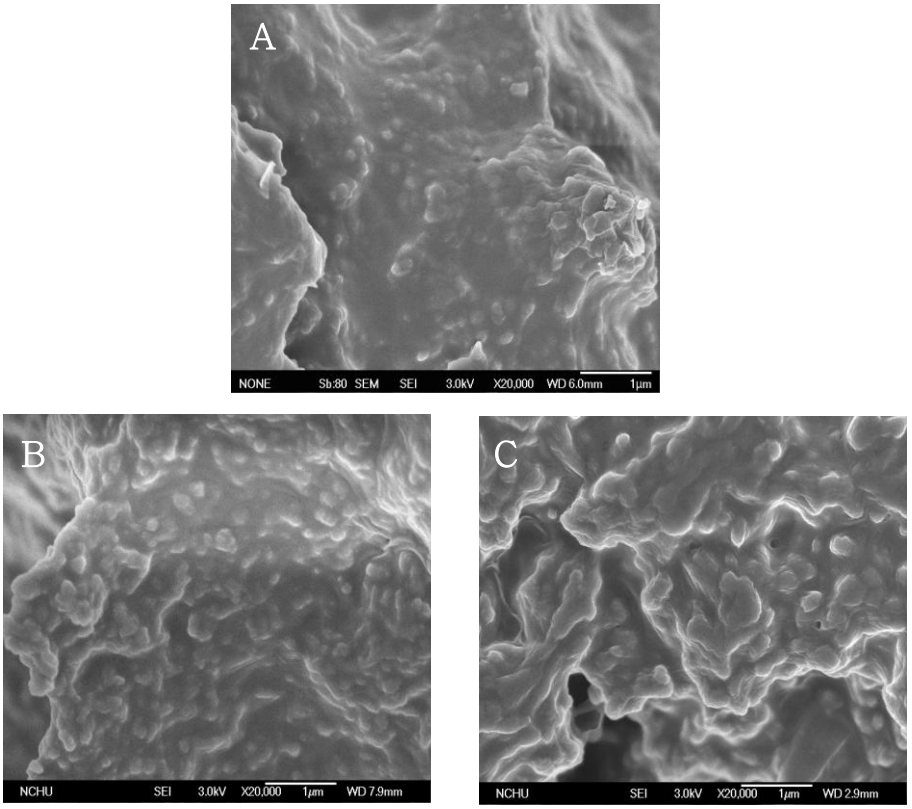

Figure 10. The microstructures of (A) rennet (B) *Rhizopus oligosporus* (C) *Bacillus subtilis* var. *natto*.

40°C, pH 5. The specific activity of the purified enzyme was 1.487 U/ml protein, the recovery was 36.7%, purification ratio was 16.89.

The crude milk-clotting enzyme produced by *R. oligosporus* had the shortest clotting time at 40°C, pH 6, and the optimum conditions for curdling were 40°C, pH 5. The specific activity of the purified enzyme was 6.32 U/ml protein, the recovery was 19.7%, purification ratio was 27.47.

However, the commercial rennet was the best milk-clotting agent for both clotting time and curd quality as compared with the tested enzymes produced by *R. oligosporus* and *B. subtilis var, natto*.

REFERENCES

Arima, K., J. Yu and S. Iwasaki. 1970. Milk-clotting enzyme from *Mucor pursillus var. Lindt*. In: Methods in enzymology, Vol. 19, (Ed. G. E. Perlmann and L. Lorand). pp. 446-459. Academic Press, New York.

Babbar, I. J., R. A. Scrinivasan, S. C. Chakravorty and A. T. Dudani. 1965. Microbial rennet substitutes-a review. Indian J. Dairy Sci. 18:89-95.

Berkowitz-Hundert, R., J. Leibowitz and J. Ilany-Feigenbaum. 1964. Researches on milk-clotting enzymes from Palestinian plant sources. Enzymologia 27:332-342.

Bernard, F. G., Z. Alexandre, M. Robert and M. Catherine. 2004. Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. Food Res. Intern. 37:123-131.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Cheryan, M., P. J. van Wyk, N. F. Olson and T. Richardson. 1975. Secondary phase and mechanism of enzymatic milk coagulation. J. Dairy Sci. 58:477-481.

Dalgleish, D. G. and A. J. R. Law. 1988. pH-induced dissociation of bovine casein micelles. I. Analysis of liberated caseins. J. Dairy Res. 55:529-538.

Harlow, E. and D. Lane. 1988. Antibodies. pp. 636-69, pp. 685. Cold Spring Harbor Laboratory, New Yor.k, USA.

Huang, M. H. and M. Y. Chen. 2006. Effects of addition of activated carbon and chitosan on characteristics of yoghurt. J. Chin. Soc. Anim. Sci. 35:187-200.

Knight, S. G. 1966. Production of a rennin-like enzyme by molds. Can. J. Microbiol. 12:420-422.

Labropoulos, A. E., W. F. Collins and W. K. Stone. 1984. Effects of ultra-high temperature and vat processes on heat-induced rheological properties of yogurt. J. Dairy Sci. 67:405-409.

Lagoueyte, N., J. Lablee and B. Tarodo de la Fuente. 1994. Temperature affects microstructure of renneted milk gel. J. Food Sci. 59:956-959.

McMahon, J. D. and R. J. Brown. 1984. Enzymatic coagulation of casein micelles: a review. J. Dairy Sci. 67:919-929.

Modler, H. W., M. E. Larmond, C. S. Lin, D. Froehich and B. Emmons. 1983. Physical and sensory properties of yogurt

stabilized with milk proteins. J. Dairy Sci. 66:422-429.

Parnll-Clunies, E. M. Y. Kauda and J. M. Deman. 1986. Influence of heat treatment of milk on the flow properties of yogurt. J. Food Sci. 51:1459-1462.

Preetha, S. and R. Boopathy. 1997. Purification and characterization of a milk clotting protease from *Rhizomucor miehei*. World J. Microbiol. Biotechnol. 13:573-578.

Puhan, Z. and D. M. Irvine. 1973. Reduction of proteolytic activity of *B. subtilis* proteases by acidification of milk cheddar cheese manufacture. J. Dairy Sci. 56:323-327.

Rao, L. Krishna and D. K. Mathur. 1979. Assessment of purified bacterial milk clotting enzyme from *bacillus subtilis* K-26 for cheddar cheese making. J. Dairy Sci. 62:378-383.

Richardson, G. H., J. H. Nelson, R. E. Lubnow and R. L. Schwarberg. 1967. Rennin-like enzyme from *Mucor pusillus* for cheese manufacture. J. Dairy Sci. 50:1066-1072.

Sardinas, J. L. 1969. Milk-curdling enzyme elaborated by *Endothia parasitica.* US. Plant 316:275-453.

Sardinas, J. L. 1972. Microbial rennets. Adv. Appl. Microl. 15:39-73.

Seker, S., H. Beyenal and A. Tanyolac. 1999. Modeling milk clotting activity in the continuous production of microbial rennet from Mucor miehei. J. Food Sci. 3:525-529.

Sushil Kumara, Neeru S. Sharmaa, Mukh R. Saharanb and Randhir Singh. 2005. Extracellular acid protease from *Rhizopus oryzae*: purification and characterization. Process Biochem. 40:1701-1705.

Wang, H. L., D. I. Ruttle and C. W. Hesseltine. 1969. Milk-clotting activeity of proteinases produced by *Rhizopus*. Can. J. Microbiol. 15:99-104.