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## Proteolysis of Milk and Casein Fractions by *Streblus asper* (Kesainai) Extract

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### Abstract

Whole milk (12.5% total solids) and casein fractions (1% w/v) were reacted with *Streblus asper* (Kesainai) leaf extract Maxiren and Rennilase, separately. Their electrophoretic profiles were determined and compared. Close similarity electrophoretic profile of milk coagulum and whey were observed for milk treated with *Streblus asper* extract and Maxiren Rennilase hydrolysed more milk casein, resulting in comparatively higher amount of macro peptide bands in the whey fraction. Lower molecular weight macro peptides were formed by *Streblus asper* extract compared with Maxiren, when a casein was used as substrate. Excessive proteolysis of  $\alpha$ -casein was observed when Rennilase was used. However, obvious difference between the electrophoretic profile of *Streblus asper* extract and Maxiren treated  $\beta$ -casein was observed Rennilase on the other hand, hydrolysed  $\beta$ -casein excessively. Higher molecular weight macro peptides were obtained when  $\kappa$ -casein was reacted with *Streblus asper* extract compared with Maxiren. Whereas Rennilase, resulted in excessive hydrolysis of  $\kappa$ -casein. Casein loss studies showed that casein protein retained in coagulum after proteolysis was 1.2, 2.3 and 3.1 percent for Rennilase, *Streblus asper* extract and Maxiren, respectively. Proteolytic activity at 5 percent enzyme concentration was found to increase in the following order: Maxiren, *Streblus asper* and Rennilase.

### Introduction

Calf rennet has been used for centuries as milk coagulant in cheese making and it is regarded as the best milk coagulant against which other rennets are measured. However decreased supply of calf rennet coupled with a worldwide increase in cheese demand has stimulated research in rennet substitutes (Idris *et al.*, 1998). Several extracellular proteases of bacterial and fungal origin had been investigated. Milk coagulating enzymes from *Streptococcus liquifaciens*, *Micrococcus caseolyticus*, *Bacillus cereus*, *Bacillus coagulans* and *Bacillus subtilis* have received considerable attention (Campos *et al.*, 1990, Lopez-Fandino *et al.*, 1991). However, no bacterial protease has been used commercially. Fungal proteases have been investigated extensively and milk coagulating enzymes from *Endothia parasitica* (Sardinas, 1968), *Mucor pusillus* var. *Lindt*. (Arima and Iwasaki, 1969) and *Mucor miehei* (Aunstrup *et al.*, 1970) have been patented. Some peptides produced by proteinases bound to cell walls can cause bitterness, which is an objectionable off flavour in cheese (Hill and Gasson, 1986).

It has been suggested that enzymes from higher plants might be useful in cheese making and some have been investigated. They include papain (EC 3.4.4.10) from the latex of the plant *Carica papaya*, ficin (EC 3.4.4.12) from the latex of *Ficus carica* and bromelin (EC 3.4.4.24) from pineapple, *Ananas sativa* (Birkkjaer and Jonk, 1985). Extract from the flower petals of *Cynara cardunculus* (Campos *et al.*, 1990) were traditionally used in Portugal for the production of cheese from sheep milk. A leaf extract from Sodom apple (*Calotropis procera*) is used in Nigeria for production of Ware, a traditional cheese (Aworh and Muller, 1986). However, it was found that most plant proteases are strongly proteolytic and caused extensive digestion of the curd (Stadhouders *et al.*, 1983), resulting in reduced

yield, high fat loss, soft texture, acid arid bitter flavor (Visser *et al.*, 1983). According to Vieira de Sa and Barbosa (1972), the strong proteolytic activity, of plant protease was responsible for increased nitrogen and dry matter losses in whey and subsequent reduction of cheese yield. The leaf extract of *Streblus asper* is used in Malaysia to coagulate milk for the production of a yoghurt-like product locally known as "dadih". *Streblus asper* extract was found to contain a thermostable milk coagulating protease (Manap *et al.*, 1992), that could be used as a rennet substitute. This study was carried out to compare milk protein hydrolysis by *Streblus asper* extract to commercial milk coagulating enzymes, namely Rennilase and Maxiren.

### Materials and Methods

*Streblus asper* (Kesainai) extract was prepared by grinding 20 g dry leaves in distilled water and fine sand. One hundred ml of 10 mM Tris-HCl buffer, pH 7.1 was added and the extract was centrifuged at 10,000 rpm, for 30 min. The crude extract was concentrated 15 fold 1:1 ultrafiltration (20 KDa membrane). The U.F. extract was centrifuged at 13,000 rpm for 10 min in the cold room Maxiren (Gist-brocades, The Netherlands) 1.0 percent w/v in distilled water and Rennilase (Novo, Denmark) will be obtained from the initial liquid stock without further dilution. Milk was prepared by reconstituting skimmed milk powder (12.5% total solids). Casein fraction solution (1 w/v) was prepared by dissolving lyophilised powder of bovine casein (Sigma, USA). Minimum purity of  $\alpha_s$ -case,  $\beta$ -casein and  $\kappa$ -casein was 85, 90 and 80 percent, respectively.

**Electrophoretic study:** Active enzyme treatment of milk was done by the addition of 2.0 ml enzyme (*Streblus asper* Maxiren and Rennilase) to 8.0 ml milk and left to coagulate at 30°C. The coagulum was subsequently scalded to 38°C

within 1 hr. It is then incubated at 30°C for 30 min after which, the whey was separated from coagulum. 0.1 ml whey was mixed with an equal volume of treatment buffer (0.125 M Tris-HCl, pH 6.8, 4 percent SDS, 20 percent glycerol and 10 percent mercaptoethanol). To 0.01g coagulum, 1.0 ml treatment buffer was added. Whey and coagulum buffer mixtures were boiled at 100°C for 90 seconds, then 2 drops of methyl red were added to each mixture. Samples were kept in ice till used in SDS-PAGE. Boiled enzyme treatment of milk was done by addition of 2.0 ml boiled enzyme (100°C for 15 min) to 8.0 ml milk. The mixture was incubated but no coagulation was observed. The incubated mixture was diluted to 10<sup>-1</sup> and treated using the same procedure described above for active enzyme.

Active enzyme treatments of casein fractions were the same as active enzyme treatment of milk except that, samples used were  $\alpha$ -casein,  $\beta$ -casein and  $\kappa$ -casein (1% w/v) and no coagulation occurred in these treatments. Boiled enzyme treatments of casein fractions were the same as that of boiled enzyme treatment of milk. However dilution to 10<sup>-1</sup> was not done with treated casein samples. Two ml boiled enzyme (100°C for 15 min) was added to 8.0 ml treatment buffer and 2 drops of methyl red were added to the mixture and the mixture used as the control for these experiments without addition of substrate.

SDS-PAGE was done to fractionated protein in 1.5 mm slabs prepared as described by Laemmli (1970). Sample (10-20  $\mu$ l) was loaded in each well. Electrophoresis was at 80 w/v for 6 hours at room temp. The gels were stained with Coomassie Brilliant Blue R 250, overnight and destained for 6 hours.

**Casein Loss study:** Milk (12.5% total solids) was coagulated using *Streblus asper*, Maxiren and Rennilase at 5 percent concentrations. Whey was separated from coagulum and then total protein (TP), Non-protein Nitrogen (NPN) and Casein-protein (CP) of both whey and coagulum were determined, separately. Nitrogen content was determined according to Kjeldahl method described by Barbano and Clark (1990) using micro Kjeldahl Instrument (Gerhardt, Germany).

## Results and Discussion

The electrophoretic profiles of protein in the coagulum and whey fraction following treatment with *Streblus asper* extract, Maxiren and Rennilase are shown in Fig. 1. The first 3 lanes were for  $\alpha$ -,  $\beta$ - and  $\kappa$ -casein, respectively. None of the casein fractions in lane 1, 2 and 3 gave a single solid band, as the lyophilised powder used was 80-90 percent pure. Electrophoretic profiles of *Streblus asper* and Maxiren were comparable. Coagulum fractions of both enzymes (lane 4 and 7) differed only in one or two bands. The same result was observed in the whey fractions (lane 5 and 8). However, Rennilase exhibited whey protein profile with a major band that was missing in the other enzyme treatments.

This major band might be due to high Rennilase concentration used. Bands for casein fractions were also observed in lane 11, whereas for other enzyme it could not be detected in whey fractions. This could be explained by the fact that Rennilase did not give a strong coagulum texture, as a result separation of whey and coagulum could not be complete and some coagulum was mixed with whey fraction, giving casein bands in lane 11. However, it was clear that Rennilase was more proteolytic than the other two enzymes since more caseins were hydrolysed (lane 10) giving comparably higher amount of macro peptides in lane 11. Lanes 6, 9 and 12 showed profile for boiled enzyme, which was similar to whole milk electrophoretic pattern and served as control.

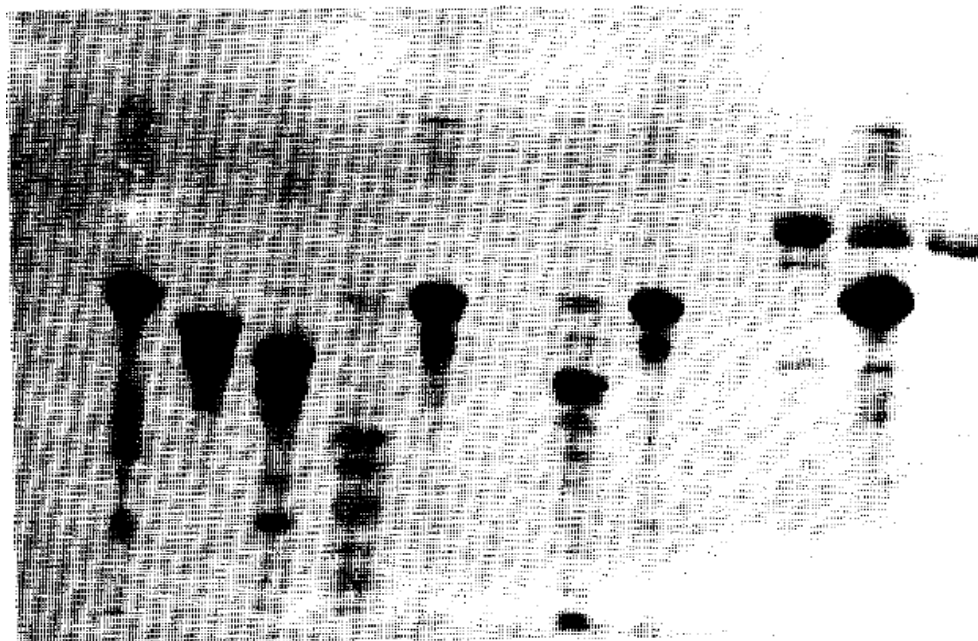
The electrophoretic profile of the products resulting from treatment of  $\alpha$ -casein with *Streblus asper* extract, Maxiren and Rennilase were shown in Figure 2. Studies by Mulvihill and Fox (1979) on chymosin (comparable to Maxiren) showed that it hydrolysed  $\alpha_{s1}$ -casein under three different conditions to yield different peptide fractions. The resulting peptide obtained were  $\alpha_{s1}$ -11 and  $\alpha_{s1}$ -III/ $\alpha_{s1}$ -IV with molecular weights of 21,000, 17,000 and 15,000 Dalton, respectively. Since no standards were used in the present experiment, it could not be proven that the bands in lane 7 for Maxiren were actually the fractions mentioned above. However, it can be observed that active Maxiren nearly hydrolysed all  $\alpha$ -casein to produce several peptide bands. Active *Streblus asper* extracts on the other hand produced more minor bands than Maxiren. Active Rennilase however, totally hydrolysed  $\alpha$ -casein but there was no clear band (lane 10), showing the presence of any low molecular weight macro peptide. Control Rennilase (60.5  $\mu$ g protein) gave one major and two minor bands in lane 12. High Rennilase concentration used might have affected the result in lane 10, due to the high proteolytic activity of the enzyme at this particular concentration.

The electrophoretic profile of the product resulting from treatment of  $\beta$ -casein with *Streblus asper* extract, Maxiren and Rennilase were shown in Figure 3. Previous studies by Lopez-Fandino *et al.* (1991) showed that treatment of bovine  $\beta$ -casein with a fungal protease produced several bands on electrophoretic gels. Their molecular weight is in the range 27,000-13,000, with 15,000 and 13,000 predominated. Referring to Fig. 3 lane 7, it could be seen that there is one major band and several minor bands detected for active Maxiren. However, at least four major bands were detected in lane 4 Fig. 3, corresponding to active *Streblus asper* extract. Molecular weights of macro peptides produced by Maxiren and *Streblus asper* extract, were lower than  $\kappa$ -casein. The electrophoretic profile of  $\beta$ -casein treated separately with the two enzymes showed very close similarity. Rennilase treatment (lane 10) showed that it has hydrolysed  $\beta$ -casein excessively, yielding low molecular weight peptides, which were not detected with SDS-PAGE. This suggested that Rennilase was more proteolytic than Maxiren and *Streblus asper* extract.



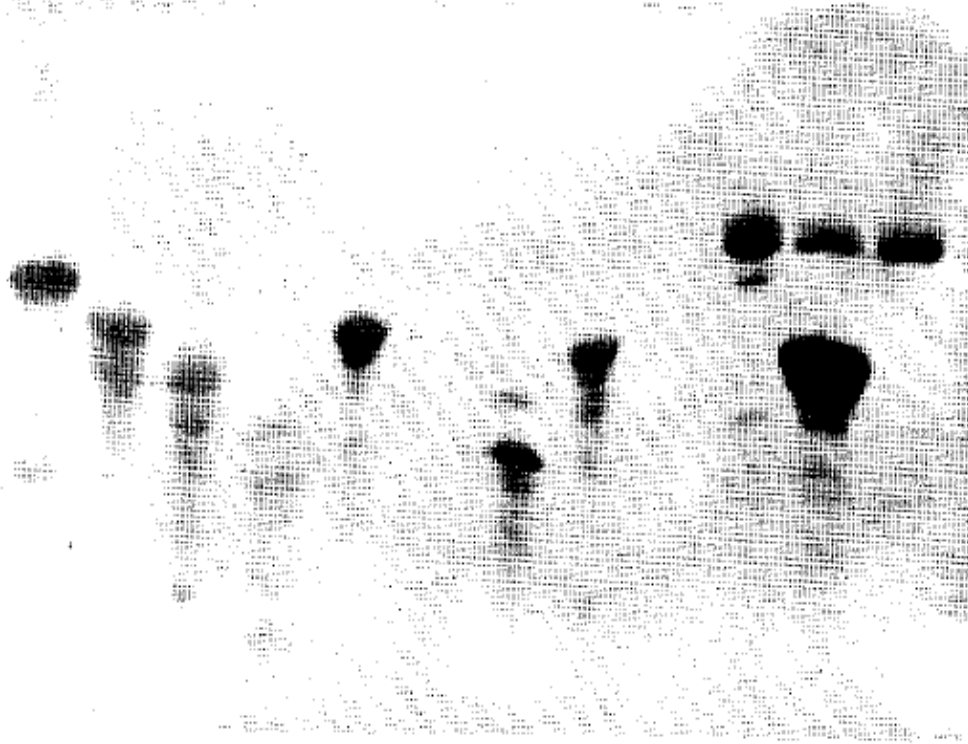
C: Coagulum      W: Whey      E: Boiled enzyme + whole milk (control)

Fig.1: Electrophoretogram of the coagulum and whey fraction of milk coagulated Using *Streblus asper* (Kesinai) extra Maxiren and Rennilase.



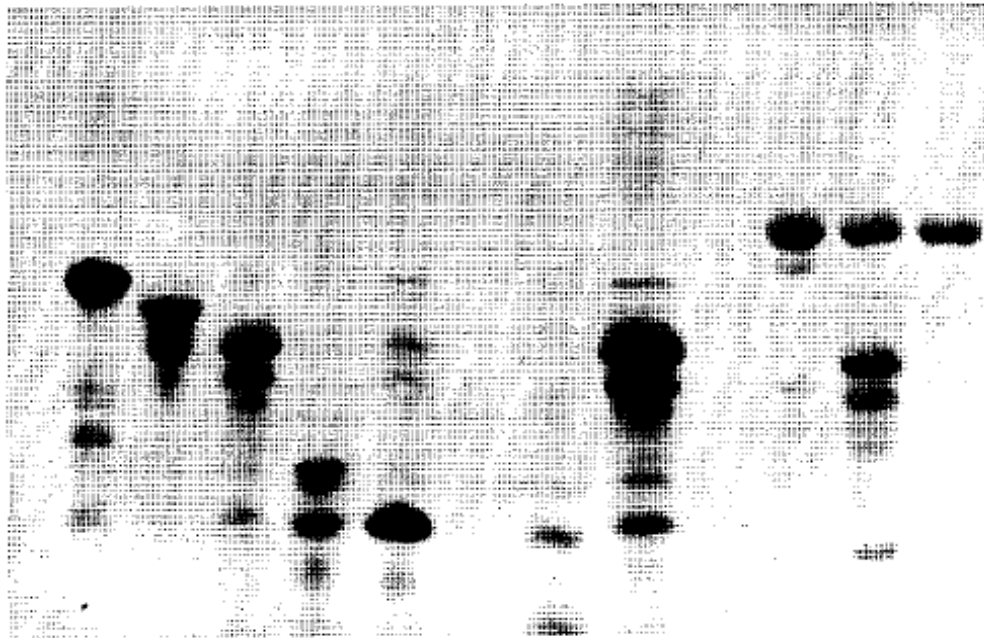
A: Active enzyme +  $\alpha$ -casein      B: Boiled enzyme +  $\alpha$ -casein      E: Boiled enzyme (without  $\alpha$ -casein)

Fig. 2: Electrophoretogram of the fraction of  $\alpha$ -casein treated with *Streblus asper* (Kesinai) extract, Maxirenand Rennila



A: Active enzyme +  $\beta$ -casein      B: Boiled enzyme  $\beta$ -casein      E: Boiled enzyme iwithout  $\beta$ -casein)

Fig. 3: Electrophoretogram of the fraction of  $\beta$ -casein treated with *Streblus asper* (Kesinai extract, Maxioren and Rennilase).



A: Active enzyme +  $\kappa$ -casein      B: Boiled enzyme +  $\kappa$ -casein      E: Boiled enzyme (without  $\kappa$ -casein)

Fig. 4: Electrophoretogram of the fraction of  $\kappa$ -casein treated with *Streblus asper* (Kesinai) extract, Maxirenand Rennilase.

Table 1: Percentage of Total Protein (TP), Non-Casein Protein (NCP), Non-protein Nitrogen (NPN) and Casein Protein (CP) in Milk Coagulum and Whey Using *Streblus asper* Extract, Maxiren and Rennilase

Enzyme source	Coagulum				Whey		
	TP	NCP	NPN	CP	TP	NCP	NPN
<i>Streblus asper</i>	3.00 <sup>a</sup>	0.739 <sup>a</sup>	0.178 <sup>a</sup>	2.26 <sup>b</sup>	0.719 <sup>d</sup>	0.447 <sup>c</sup>	0.218 <sup>c</sup>
Max iren	3.48 <sup>a</sup>	0.332 <sup>b</sup>	0.172 <sup>a</sup>	3.15 <sup>a</sup>	0.838 <sup>d</sup>	0.336 <sup>c</sup>	0.178 <sup>c</sup>
Rennilase	2.16 <sup>b</sup>	0.952 <sup>a</sup>	0.244 <sup>a</sup>	1.21 <sup>c</sup>	0.951 <sup>c</sup>	0.370 <sup>c</sup>	0.216 <sup>c</sup>

Values, in columns with the same heading, which are significantly different at 5% level, are followed by superscripts of different alphabet.

The electrophoretic profiles of  $\kappa$ -casein after treatment with *Streblus asper* extract, Maxiren and Rennilase were shown in Figure 4. Several investigators has focused their attention on hydrolysis of  $\kappa$ -casein due to its role in stabilising milk casein (Dalgleish, 1979; Addeo *et al.*, 1988; Deshmukh *et al.*, 1989). Rennin cleaves K-casein at Phe<sub>105</sub>-Met<sub>106</sub> peptide bond to produce para- $\kappa$ -casein and a macro peptide soluble in whey. Maxiren (lane 7 Figure 4) hydrolysed  $\kappa$ -casein to yield one major and several minor bands. The major band could riot be proved to be the macro peptide suggested by previous researchers (Lawrence and Creamer, 1969; McMahon *et al.*, 1984) as the same band appears in lane 8 for inactivated enzyme. Two major bands resulted from hydrolysis of  $\kappa$ -casein by *Streblus asper* extract (lane 4). The lower major band produced by *Streblus asper* extract appears to be similar to that in lane 7 for Maxiren, suggesting that the two enzymes would react on  $\kappa$ -casein in a very similar way. Rennilase on the other hand hydrolysed  $\kappa$ -casein excessively as shown in lane 10 and appears to be more proteolytic than Maxiren and *Streblus asper* extract. Inactivated Rennilase (lane 11) produced the same macro peptide bands as the other enzymes. In a study by Van Hooydonk *et al.* (1984), a minor band just below  $\kappa$ -casein could be detected when  $\kappa$ -casein was reacted with rennin it was designated as para- $\kappa$ -casein, although existed in very small quantity. In this study this particular band could not be detected, probably due to low para-K-casein concentration.

Total protein (TP), Non-Casein Protein (NCP), Non-protein Nitrogen (NPN) and Casein Protein (CP) in milk coagulum and whey produced by Maxiren, *Streblus asper* extract and Rennilase were shown in Table 1. It was found that %TP was highest in coagulum of milk treated with Maxiren, followed by *Streblus asper* extract and Rennilase, respectively. The values for Maxiren and *Streblus asper* extract are not significantly different at 5 percent level. This suggests that the ability of *Streblus asper* extract to retain protein in milk curd was similar to that of Maxiren. Rennilase on the other hand showed values, which are significantly lower than that for the other two enzymes. This indicates that less protein was retained in its coagulum compared with the other two enzymes. Percentage of TP was highest in whey of milk treated with Rennilase, followed by Maxiren and *Streblus asper* extract, respectively. The value for Rennilase was significantly

different from that for *Streblus asper* extract. The result can be explained by referring to the value for the coagulum Rennilase hydrolysed more protein fractions than the othe, two enzymes, giving a higher rise in %TP values in whey Streblus as per extract on the other hand did not hydrolyse as much protein as Rennilase, therefore giving a lower value of %TP in whey fraction. This property is desirable it cheese making where the coagulum formed would be collected and higher cheese yield could be obtained Previous studies showed that %TP in whey fraction of milk treated with enzyme from *Mucor miehei* varied frok 0.9029-0.9043 percent, whereas for calf rennet, the approximate value was 0.8786 percent (Emmons *et al.*, 1990). The result in this study is similar to the aforementioned findings.

Non-casein protein consists of serum protein and not protein nitrogen. Serum proteins such as  $\alpha$ -lactalbur min are  $\beta$ -lactoglobulin would remain unchanged during the enzym coagulation process. Therefore, the increase of NCP value after enzyme coagulation is totally caused by the increase in NPN. Table 1 shows that %NCP is highest in mi coagulum from Rennilase treatment, followed by *Strebles asper* extract and Maxiren, respectively. However in values for Rennilase and *Streblus asper* extract showed significant difference. Maxiren, however, gave significantly lower value than the other two enzymes. The high %NCP for Rennilase might be due to excess proteolysis of casein protein. Maxiren on the other hand showed a lower %NCP value due to its lower proteolyte activity.

Non-protein nitrogen (NPN) consists of nitrogen from amino acids, peptides and ammonia). The %NPN value show increase in whey fraction after the milk coagulation process as proteolytic enzymes would cleave casein at various bonds to yield peptides and macro peptides, which soluble in whey. Therefore the amount of NPN would increase in whey (Emmons *et al.*, 1990). In this study NPN value in milk coagulum showed no significant difference all enzyme treatment. The same result is also observed whey fractions. Emmons *et al.* (1990) showed that %NPN in coagulum treated with Rennilase was 0.1663 percent whereas for Maxiren, it was reported as 0.1662 percent. The values for whey fraction varied from 0.2289-0.22 percent for *Mucor miehei* rennet and 0.2108-0.2187 percent for calf rennet (Emmons *et al.*, 1990). In this study

differences between values were small and could not be detected using analysis of variance. However, %NPN of whey treated with Rennilase was indeed higher than with Maxiren, suggesting that proteolytic activity of Rennilase was higher than that of Maxiren, this is in agreement with Rauch *et al.* (1989).

Casein protein (CP) value was obtained by subtracting NCP from TP. Casein protein (CP) value is important in determining the suitability of any protease for commercial use in cheese making. Proteases with high proteolytic activity might hydrolyse more casein protein, producing macro peptides that are soluble in whey. Hence, casein yield would be low, which is undesirable in cheese making. Table 1 shows that %CP was highest in coagulum from milk treated with Maxiren, followed by *Streblus asper* extract and Rennilase, respectively. The values were significantly different at 5 percent level. The result suggests that their proteolytic activity increase in the same order. Results of proteolytic and casein loss studies showed that *Streblus asper* extract is comparable to Maxiren in many aspects and is also less proteolytic than Rennilase. Since Rennilase, which is more proteolytic than *Streblus asper* extract, had already been used commercially in cheese making, *Streblus asper* extract is therefore potentially useful in cheese-making. Further studies from other aspects of *Streblus asper* protease should be carried out to determine its suitability for commercialisation.

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