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Isolation, purification and characterization of chymosin from riverine buffalo (*Bubalos bubalis*)

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Chymosin, an aspartyl proteinase, is used for curdling of milk and manufacture of cheese. We report the purification and the physicochemical properties of chymosin isolated from the abomasal tissue of buffalo calves. The enzyme preparation extracted from buffalo abomasal tissues could be purified 29–fold using anion exchange and gel filtration chromatography. The molecular weight of the purified enzyme was 35·6 kDa on SDS-PAGE. Partial N-terminal amino acid sequence of the first eight amino acid sequences of buffalo chymosin was identical to the first eight amino acid sequences of cattle chymosin. Buffalo chymosin exhibited a skewed bell-shaped stability profile as a function of temperature with maximum activity near 55 °C. Milk clotting activity decreased gradually as pH increased. The enzyme became completely inactive, however, above pH 7·0. The ratio of milk clotting to proteolytic activity was 3·03. When compared with cattle chymosin, there were subtle differences in the stability and relative proteolytic activity of buffalo chymosin.

Keywords: Chymosin, N-terminal sequencing, purification, physicochemical factors.

Chymosin is an aspartyl proteinase secreted in the abomasum of the sucking ruminant. Bovine (cattle) calf chymosin is the milk-clotting enzyme present predominantly in the rennet, which has been traditionally used for cheese-making. The purification and properties of cattle calf chymosin have been studied extensively (Foltman, 1970, 1992 & 1993). Milk clotting enzymes from microbial (Beppu, 1983) and plant (Lo Piero, 2002) sources have also been used in the dairy industry for some time. However, the quality of processed products using these enzymes has been found to be inferior to that made from cattle calf rennet (Beppu, 1983). Microbial rennet usually possesses low milk-clotting and high proteolytic activity leading to proteolysis of milk proteins, especially casein, releasing a large number of small peptides, which can produce a bitter taste (Neelakantan et al. 1999; Li & Walsh, 2000). Such products have limited acceptability. Presently, recombinant rennet is extensively used for cheese production, as a substitute for native bovine rennet and microbial rennet (Mohanty et al. 1999). While the physicochemical properties of bovine rennet and recombinant bovine rennet have been extensively studied, there is a lack of information on chymosin from species such as buffalo, goat, sheep and camel, which are also thought to be good sources of rennet. In a country like India, buffaloes are the major source of milk. The coagulation of buffalo milk requires more bovine rennet than does cows' milk owing to inherent compositional differences (Singh et al. 1975). Similar observations have been reported for camel milk (Elagamy, 2000). In addition, buffalo chymosin might be expected to have an inherent compatibility with buffalo milk and might be better in milk-clotting than chymosin from other sources. Therefore, the use of buffalo chymosin might be a preferred option for quality cheese production from buffalo milk. Use of buffalo chymosin is limited owing to a lack of information on the purification and physicochemical properties of the enzyme. Hence, in the present investigation, we purified buffalo chymosin and examined its properties.

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Materials and Methods

Extraction and purification of buffalo chymosin

The steps involved were: extraction of enzyme from abomasal tissue; clarification of tissue extracts; precipitation, dialysis, DEAE cellulose ion exchange chromatography; and gel filtration chromatography. Buffalo calf abomasal tissues, excised within 12 h of death from calves 1–2 months old, were flushed with distilled water and minced into small

pieces. The minced pieces were freeze-dried under vacuum and 100 g of the minced tissue was homogenized in 2 l distilled water before straining through muslin. The remaining debris from the homogenate was removed by centrifugation at 5000 ***g*** at 4 °C for 10 min and re-extracted with 1 l distilled water. Aluminium sulphate solution (0·33 M) was added with continuous stirring until the pH of the solution reached 4·0. The pH was then immediately raised to 5·8 by addition of 0·5 M-disodium hydrogen phosphate, which resulted in the formation of a gelatinous precipitate. The precipitate was removed by centrifugation at 5000 ***g*** at 4 °C for 10 min and the supernatant was subjected to ultra filtration using a 100 kDa nominal molecular weight cut-off hollow fibre cartridge (Millipore Corporation, Bedford, Massachusetts, USA). The filtrate was again ultra filtered using a 10 kDa membrane (Millipore Corporation) to concentrate the extract to a final volume of 100 ml. This resulted in the retention of chymosin within the molecular weight range 10–100 kDa. The retentate was dialysed using a dialysis membrane (Sigma Chemical Co., St Louis, MO 63118, USA) of 10-kDa cut-off size against 2 l of distilled water at 4 °C.

The dialysed enzyme extract was applied to a column packed with DEAE-Cellulose-52 (20 cm × 2·4 cm) and equilibrated with 0·05 M-sodium phosphate buffer, pH 5·8. Most other proteins did not bind to the column under these conditions. The column was then washed with the same equilibration buffer until the absorbance of the eluant at 280 nm was <0·05. The bound proteins were eluted with a linear gradient of 0·05 M–0·35 M-phosphate buffer, pH 5·4 at a flow rate of 30 ml/h. At each step of purification, fractions were monitored for chymosin by assay of milk-clotting activity (Berridge, 1945) and caseogram (Foltman, 1981). Fractions containing milk-clotting activity were pooled and concentrated to 15 ml by ultra filtration using a 10-kDa membrane. The retentate was desalted by washing with several volumes of 0·05 M-sodium phosphate buffer, pH 5·5.

For further purification, the retentate was again applied to a high-resolution anion exchange column, Mono Q (HR-5/5) attached to FPLC system (Pharmacia Corporation, Peapack, New Jersey, USA). An aliquot of 0·5 ml sample containing 34·5 units of chymosin was injected into Mono Q column equilibrated with 0·05 M-sodium phosphate buffer, pH 5·5 (solution A) and eluted with a linear gradient of 0·6 M-NaCl in solution A at flow rate of 0·5 ml/min. The milk-clotting activity of individual peak fractions was measured by the method of Berridge (1945). Fractions showing milk-clotting activity were pooled and concentrated to a final volume of 1·0 ml using 10-kDa cut-off membrane.

The purified buffalo chymosin was further refined by injecting 50 µl (50 units/ml) onto a gel filtration superose 12 column pre-equilibrated with 150 mM-NaCl in 0·05 M-sodium phosphate buffer, pH 5·5. Individual peak fractions were collected at a flow rate of 0·5 ml/min. Fractions were monitored for milk-clotting activity. The molecular weight of buffalo chymosin was determined from the elution profile of standard proteins, namely phosphorylase, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme and dextran blue under similar elution conditions, and by comparision with standard cattle chymosin.

SDS-PAGE of pooled and concentrated fractions of chymosin from Mono Q and gel filtration column was carried out as described by Laemmli (1970). The protein from the SDS-PAGE gel was electroblotted onto the PVDF membrane and microsequencing was done using an automated protein sequencer (Model 476 A, Applied Biosystem, Lincoln center drive, Foster city, USA).

The purified enzyme preparation was electrophoresed on 1% agarose in 0·05 M-sodium acetate buffer (pH 5·3) at 15 volts/cm of electrode length using LKB-Pharmacia mini gel apparatus. The enzymic activity of chymosin in the agarose gel was monitored by overlaying three volumes of 1·3% agarose in 0·15 M-sodium acetate (pH 5·3) and one volume of skim milk reconstituted to 4% in distilled water (Foltman et al. 1985). Milk-clotting activity was indicated by the appearance of precipitation zones around the protein band.

The milk-clotting activity of buffalo chymosin was determined as described by Berridge (1945). Spray-dried skim milk powder (12 g) was reconstituted with 100 ml 0·01 M-calcium chloride solution. An aliquot of 1 ml of the purified enzyme or its dilution was added to 10 ml substrate and mixed thoroughly before incubating at 30 °C. The time required for the first appearance of flakes was recorded.

One unit of milk-clotting activity was defined as the amount of enzyme required to clot 10 ml of milk in 100 s at 30 °C. Total protein in fractions obtained from ion exchange chromatography and FPLC was measured by the method of Lowry et al. (1951). The proteolytic activity of the purified enzyme preparation was estimated using soluble casein as a substrate (Rao, 1984), as described by Kobayashi & Murakami (1978). Casein (2 g) was dissolved in the minimum amount of 0·1 M-NaOH at room temperature in a beaker using a magnetic stirrer. After adjusting the pH to 6·5 with 0·1 M-HCl, the solution was transferred to a volumetric flask and the final volume was made up to 100 ml with 0·1 M-phosphate buffer, pH 5·5. The assay mixture, comprising 5 ml of the substrate, 5 ml of 0·1 M-phosphate buffer, pH 5·5 and 0·5 ml enzyme solution was incubated at 37 °C for 1 h, followed by addition of 12% trichloroacetic acid (TCA) and subsequent incubation on ice for 20 min. Precipitated proteins were separated by centrifugation and the tyrosine content in the supernatant was estimated by the method of Lowry et al. (1951). One unit of proteolytic activity was defined as the amount of enzyme required to liberate 100 µg tyrosine in 60 min.

Physicochemical factors affecting buffalo chymosin activity

The enzyme preparation was subjected to heat treatments at 30, 40, 50, 55 and 60 °C for 15 min and assayed for milk-clotting activity. The temperature of enzyme treatment at

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Table 1. Procedures used in the purification of buffalo chymosin

Purification steps	Volume ml	Units/ml	Total units	Protein mg/ml	Total protein mg	Specific Activity units/mg	Relative Purification fold
Extraction	2200	8·77	19 294	5·2	11 440	1·686	1·0
Clarification	2150	8·65	18 597	4·0	8600	2·16	1·28
Ultrafiltration 10-kD (U/F)	100	41·65	4165	7·2	720	6·78	3·44
DEAE-cellulose chromatography U/F	15	34·5	517·5	1·24	18·6	27·82	16·55
FPLC (mono Q)	1·5	50	74·63	1·548	2·322	48·21	28·59

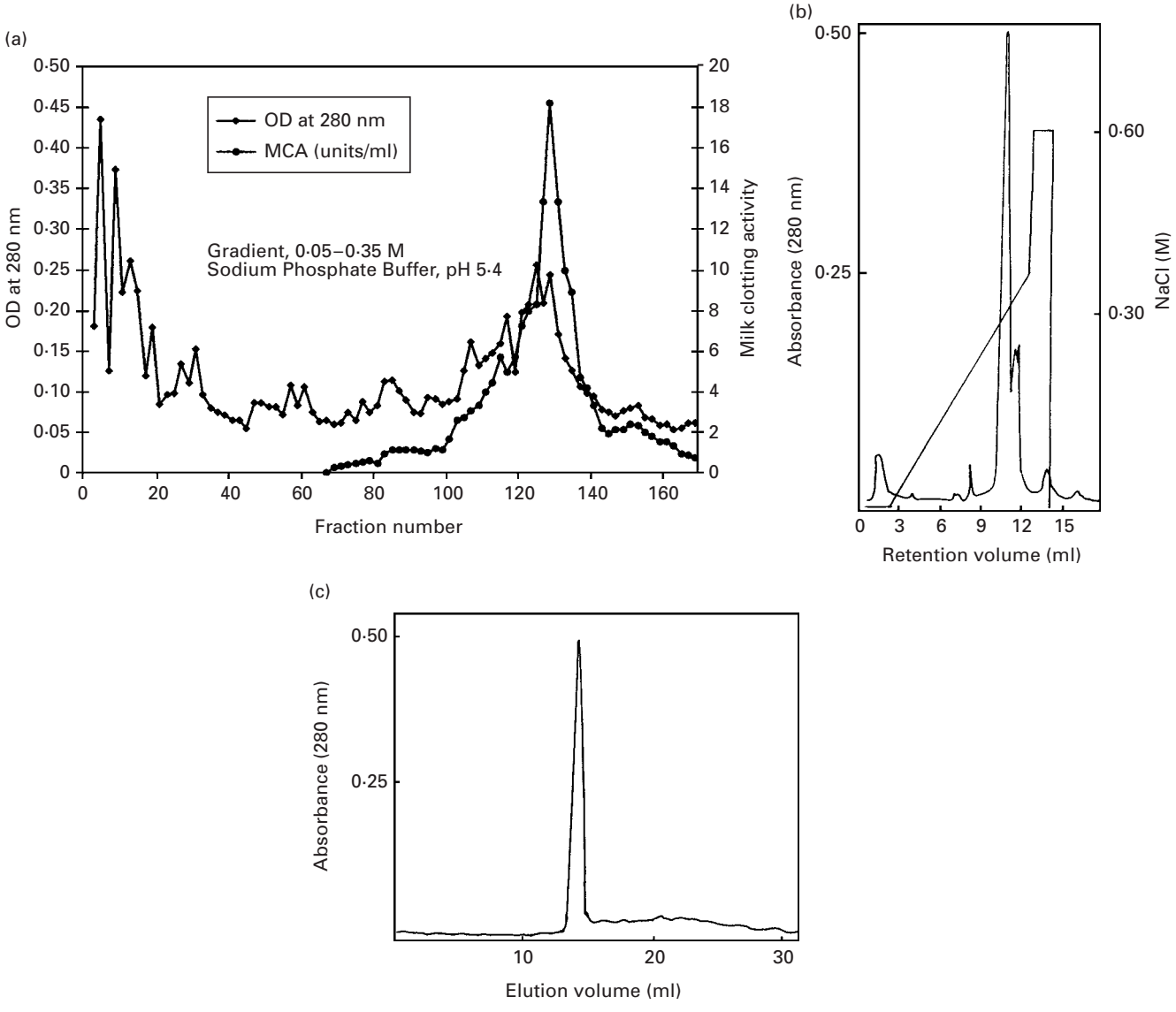

Fig. 1. (a) Elution profile of buffalo chymosin on DEAE cellulose ion exchange column. (b) Elution profile of buffalo chymosin using FPLC HR 5/5 Mono Q column. (c) Elution profile of buffalo chymosin on Superose 12 (HR 10/30) column.

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which there was maximum activity was taken as 100%. The enzyme solution treated at the above temperatures was used for assaying proteolytic activity (Kobayashi & Murakami, 1978) and the temperature of enzyme treatment at which there was maximum proteolytic activity was taken as 100%.

Aliquots of spray-dried skim milk (12 g in 100 ml 0·01 M-calcium chloride) were incubated at 30, 40, 50, 55 and 60 °C for 15 min. The milk-clotting activity of the purified chymosin was tested by addition of the enzyme preparation (chymosin in 50 mM-sodium phosphate buffer, pH 5·5) to the incubated substrate samples by following the method as described above.

Aliquots of reconstituted spray-dried skim milk as above were adjusted to pH 5·5, 5·8, 6·0, 6·25, 6·5, 6·8, 7·0, 7·5 and 8·0. The milk-clotting activity of the purified chymosin was tested by addition of the enzyme preparation (chymosin in 50 mM-sodium phosphate buffer, pH 5·5) into the above incubated samples by the method described above. The pH of substrate showing maximum activity was taken as 100%.

The proteolytic activity of both bovine and buffalo chymosin was measured at incubation times 10, 20, 30, 50, 70 and 90 min. The incubation time at which there was maximum proteolytic activity was taken as 100%.

Results and Discussion

The summary of the sequence of the purification steps beginning with extraction and clarification of the enzyme followed by ultra filtration, ion exchange chromatography and FPLC is shown in Table 1. The extraction step yielded a total of 19 294 units with specific activity of 1·686 units/mg protein. There was a slight reduction in enzyme recovery after the clarification step, which yielded 18 587 units with specific activity of 2·16. Ultra filtration of the clarified extract was carried out to retain the protein within a molecular weight range of 10–100 kDa. Both the retentate and the permeate obtained from the ultra filtration were tested for milk-clotting activity. The retentate from 100-kDa cut-off filtration membrane did not show any milk-clotting activity whilst the retentate of the 10-kDa cut-off filtration retained most of the original milk-clotting activity. The ultra filtration step, on the other hand, resulted in significant reduction in the recovery of enzyme as only 4165 units could be recovered with specific activity of 6·78, thereby showing 3·44-fold purification.

The elution profile on DEAE-cellulose is shown in Fig.1a. Elution of buffalo chymosin was achieved with 0·22 M-sodium phosphate buffer, pH 5·4. The major part was eluted in fractions corresponding to a linear gradient range of 0·24–0·30 M. These results are comparable to those of Foltman (1966) and Houen et al. (1996), who also achieved elution with 0·25–3·0 M under similar elution conditions. Fractions numbering 72 to 160 were subsequently pooled and concentrated by ultrafiltration through a 10-kDa cut-off membrane. DEAE cellulose chromatography led to a 16·55–fold purification of buffalo chymosin with specific activity

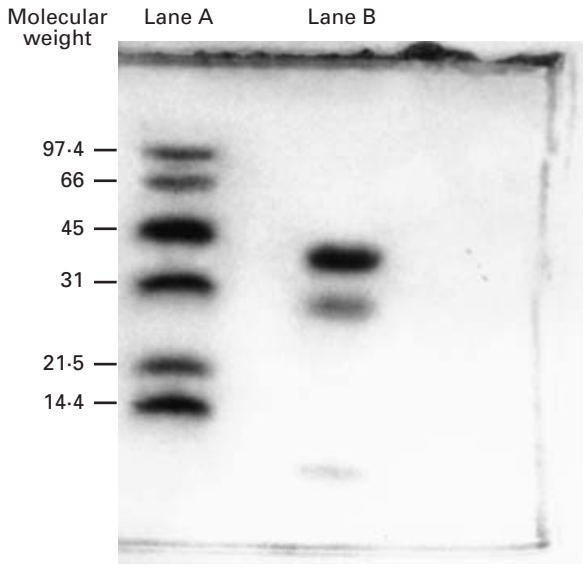

Fig. 2. SDS-PAGE (12%) profile of purified buffalo chymosin. A: Protein molecular weight markers, B: Purified buffalo chymosin.

of 27·82 units/mg protein. During the course of this study, buffalo chymosin was observed to resolve into at least three components on DEAE-cellulose ion exchange chromatography, indicating possible heterogeneity. Others have reported heterogeneity of cattle calf chymosin on passing through a DEAE-cellulose ion exchange column. Foltman (1970) used commercial preparations of crystalline cattle calf rennet, which was found to resolve into at least three components in DEAE-cellulose ion exchange chromatography, and similar results have been reported by others (Castle & Wheelock, 1971; Shindo & Arima, 1979; Jensen et al. 1982; Foltman, 1992).

Elution of buffalo chymosin from Mono Q column occurred at 40% 0·6 M NaCl in 0·05 M-sodium phosphate buffer, pH 5·5 (Fig. 1b). The elution profile revealed two closely spaced chromatographic peaks showing milk-clotting activity. The elution profile also indicated a few minor peaks, which did not show any milk-clotting activity. Purification of buffalo chymosin achieved by FPLC was 28·59–fold with specific activity of 48·21 units/mg protein. These results agree closely with those of Bernakova et al. (1988), who used similar elution conditions. Our results further corroborate those of Foltman (1992) who purified cattle chymosin from pooled fractions obtained by ion exchange chromatography on Mono Q column and also observed two chromatographically homogeneous major components, when eluted with a linear gradient of 0·05 M-ammonium acetate, pH 5·3.

Figure 1c shows the elution profile of buffalo chymosin from a superose-12 (HR 10/30) gel filtration column. Buffalo chymosin eluted as a single symmetrical peak with an elution volume of 13·47 ml, which was similar to that of cattle chymosin (13·45 ml), suggesting that both are of similar molecular weight.

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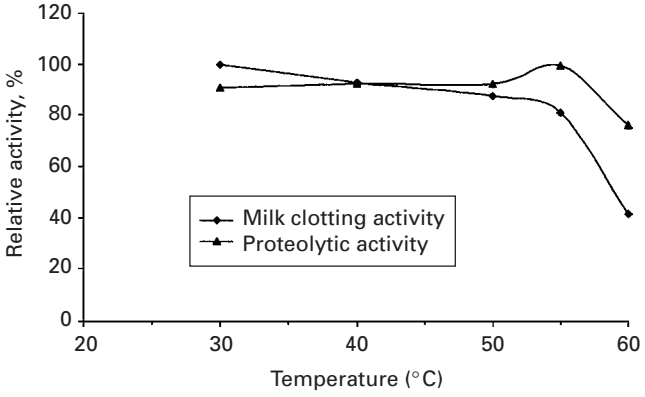

Fig. 3. Effect of heat treatment on its relative milk clotting and proteolytic activity of buffalo chymosin.

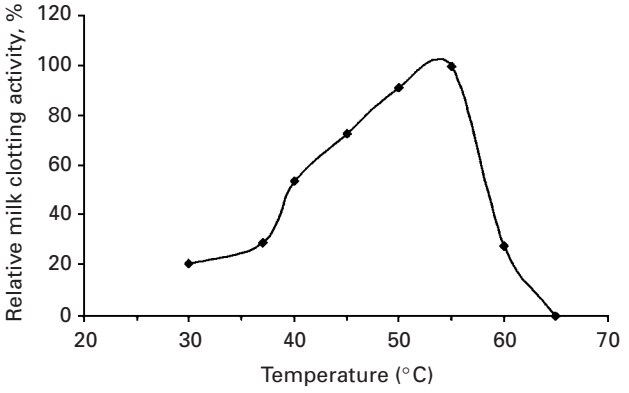

Fig. 4. Effect of substrate temperature on relative milk-clotting activity of buffalo chymosin.

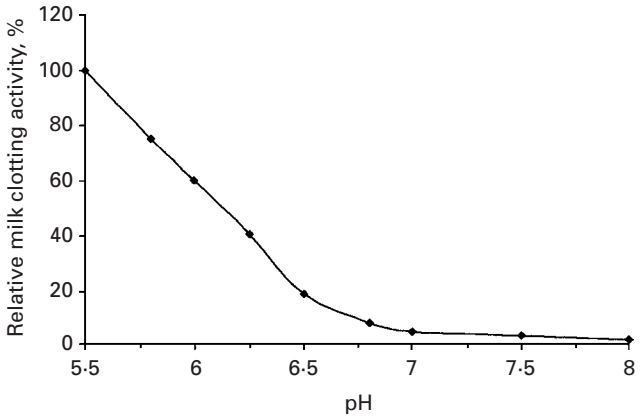

Fig. 5. Effect of substrate pH on relative milk-clotting activity of buffalo chymosin.

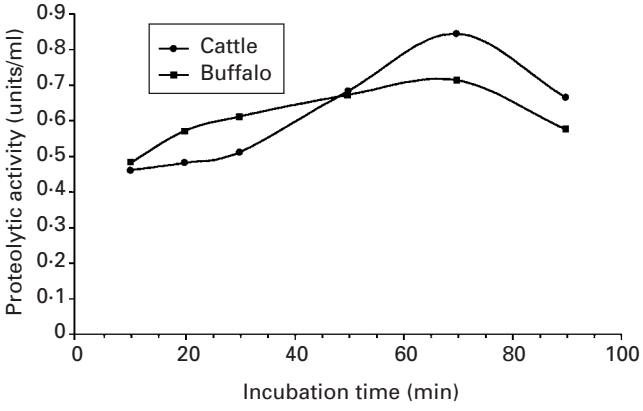

Fig. 6. Effect of incubation period on relative proteolytic activity of cattle and buffalo chymosin.

The purified protein was heterogeneous in nature as shown by the presence of a major protein band representing buffalo chymosin, a closely spaced minor protein band and a small molecular weight protein band moving along with the dye front in the SDS-PAGE profile (Fig. 2). The major band corresponded to the molecular weight of standard cattle chymosin (35·6 kDa) whereas the first minor band was <30 kDa. To rule out the possibility of protein contaminants, the pooled and concentrated fractions of buffalo chymosin were ultra filtered using 30-kDa molecular weight cut-off membrane and the retentate was again electrophoresed in 12% SDS-PAGE, which exhibited the same profile as observed earlier. The minor bands observed in the SDS-PAGE gel suggest possible autocatalytic degradation of buffalo chymosin, leading to electrophoretic heterogeneity. Our results in this regard are consistent with those of Abdel Malak et al. (1996), who also reported a molecular weight of 36±1 kDa for buffalo (*Bos buffali*, L.) chymosin with similar electrophoretic heterogeneity. In contrast, a preparation of buffalo chymosin with a molecular weight of 23 kDa has been reported (Rao, 1984). Those results, however, were not based on advanced separation techniques, which might explain the discrepancy. A similar electrophoretic heterogeneity of bovine calf chymosin was reported by Donnelly (1986) and Foltman (1992) due to the presence of two isozymes of bovine chymosin, such as chymosin A and B and chymosin C (molecular weight of around 8·5 kDa, a degradation product of chymosin A) that moves along with the dye front in SDS-PAGE. The heterogeneity of buffalo chymosin may be presumed to be caused by the presence of similar types of isozymes, as buffaloes and cattle are close to each other on the evolutionary ladder, as they descend from the same *bovidae* family. Others have also observed the presence of different electrophoretically distinguishable components of cattle rennin in SDS-PAGE (Melachouris, 1968; Bakri & Ashworth, 1976; Shindo & Arima, 1979).

The partial N-terminal sequence of the first eight amino acids of the purified preparation of buffalo chymosin was found to be Gly-Glu-Val-Ala-Ser-Val-Pro-Leu. This sequence is identical to the N terminal first eight amino acid residues of cattle chymosin. Our findings agree with those

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of Abdel Malak et al. (1996), who reported the N terminal sequences of the first seven amino acids of the chymosin from Egyptian buffalo (*Bos buffali* L.).

The u.v. absorption spectrum of buffalo chymosin from 260 nm to 310 nm indicated that the maximal absorption of the purified enzyme occurred at precisely 276·8 nm, similar to the value of 278 nm found for cattle chymosin by Foltman (1970).

A low proteolytic activity to milk-clotting ratio is a prerequisite for an acceptable rennet substitute (Fox, 1969; Elagamy, 2000). In our study, the purified preparation of buffalo chymosin exhibited higher milk-clotting activity and lower proteolytic activity with the ratio being 3·03, which may be attributed to high purity of the buffalo chymosin. The high milk-clotting to low proteolytic activity of buffalo chymosin suggests that it can be used as an alternative rennet substitute, particularly for making cheese from buffalo milk.

The potential for commercial application of this enzyme in cheese manufacture was explored by examining the effects of some physicochemical factors on milk-clotting activity. Holding the chymosin at different temperatures from 30 °C to 60 °C for 15 min prior to the milk-clotting assay induced a relative decline of milk-clotting activity as the temperature of the enzyme solution was increased, as shown in Fig. 3. The enzyme was relatively stable up to 55 °C and a relative milk-clotting activity of 50% was recorded when the temperature was raised to 60 °C. However, Shindo & Arima (1979) reported that cattle chymosin was relatively stable up to 50 °C and a relative decline in the milk-clotting activity of almost 55% was observed at 55 °C, there being a total loss of activity at 60 °C. Our finding suggests that buffalo chymosin is more stable than cattle chymosin. The heat stability of chymosin from different sources appears to be an intrinsic property and is species-dependent, as reported by Elagamy (2000) who recorded high heat resistance of camel chymosin compared with that from cattle and buffalo.

Similarly, in our study the proteolytic activity of buffalo chymosin treated at different temperatures exhibited a relatively stable proteolytic activity curve up to a temperature of 55 °C after which there was a continuous decline. On the other hand, in a related study on cattle chymosin, Shindo & Arima (1979) observed stable proteolytic activity up to 50 °C and a substantial decline at 55 °C and above. Measurement of proteolytic activity at different temperatures also suggests that buffalo chymosin is more stable than cattle chymosin.

Heat treatment of the substrate appears to have a significant effect on milk-clotting activity of buffalo chymosin. Activity gradually increased as the heat treatment of milk was increased from 30 °C, with maximum activity at 55 °C. However, at 60 °C, milk-clotting activity declined sharply, the relative activity being around 30% of maximum. When the temperature of milk was increased to 65 °C, activity was completely lost (Fig. 4). In related studies on buffalo chymosin (Elagamy, 2000), recombinant bovine chymosin (Li & Walsh, 2000) and native bovine chymosin (Fox, 1969), maximum activities were recorded at 45 °C, after which the milk-clotting activity declined up to 55 °C. The discrepancy might be explained by the pH-dependency of the enzymes: the above investigators standardized the enzyme at pH 6·5, whereas we standardized at pH 5·5.

Milk-clotting activity was substantially influenced by milk pH (Fig. 5). At pH 5·5, activity of the buffalo chymosin was highest and as the pH of the milk was increased, milk-clotting activity gradually declined. At pH 7·0 and above, milk did not clot. This might be due to the complete inactivation of buffalo chymosin at pH 7·0, as seen for bovine chymosin (Foltman, 1970) and for chymosin from transgenic sheep (Mezina et al. 2001). These findings agree with those of Elagamy (2000) who measured the milk clotting activity over a pH range 5·8–7·0.

Proteolytic activity of buffalo chymosin followed an almost linear curve with time at 37 °C while that of cattle chymosin followed an S shaped curve (Fig. 6). Maximum proteolytic activity was observed after 70 min of incubation, after which it declined gradually.

It can be concluded that the nature of buffalo chymosin after purification is more or less similar to bovine chymosin, although there appear to be subtle differences in physicochemical characteristics between the two. Nevertheless, buffalo chymosin could serve as an alternative source of milk-clotting enzyme for the manufacture of cheese from buffalo milk.

References

Abdel Malak CA, Abou El-Adab IFG, Vukashinovic V, Zalunin IA, Timokhina EA, Lavrenova GI & Stepanov VM 1996 Buffalo (*Bos buffali* L) chymosin purification and properties. *Comparative Biochemistry and Physiology B Biochemistry and Molecular Biology* **113** 57–62

Asato N & Rand AG 1971 Resolution of prorennin and rennin by polyacrylamide gel electrophoresis. *Analytical Biochemistry* **44** 32–41

Bakri M & Ashworth US 1976 Chymosin purification by gel permeation chromatography of rennet. *Milchwissenschaft* **31** 477–479

Beppu T 1983 The cloning and expression of chymosin (rennin) genes in micro-organisms. *Trends in Biotechnology* **1** 85–89

Bernakova E, Sajdok J, Rauch P & Kas J 1988 Determination of chymosin and bovine pepsin contents of bovine rennets by high performance liquid chromatography. *Netherlands Milk and Dairy Journal* **42** 337–340

Berridge NJ 1945 Purification and crystallization of rennin. *Biochemical Journal* **39** 179–186

Castle AV & Wheelock JV 1971 Purification of rennin. *Journal of Dairy Research* **38** 69–71

Donelly UK, Carroll DP, O'Callaghan DM & Walls D 1986 Genetic polymorphism of bovine chymosin. *Journal of Dairy Research* **53** 657–664

Elagamy EI 2000 Physicochemical, molecular and immunological characterization of camel calf rennet: a comparison with buffalo rennet. *Journal of Dairy Research* **67** 73–81

Foltman B 1966 A review on prorennin and rennin. *Comptes Rendus des Travaux du Labotoire Carlsberg* **35** 143–231

Foltman B 1970 Prochymosin and chymosin (prorennin and rennin). *Methods in Enzymology* **19** 421–443

Foltman B 1981 Gastric proteinases. Structure, function, evolution and mechanism of action. *Essays in Biochemistry* **17** 52–84

Foltman B, Szecsi PB & Tarasova NI 1985 Detection of proteases by clotting of casein after gel electrophoresis. *Analytical Biochemistry* **146** 353–360

Foltman B 1992 Chymosin: A short review on foetal and neonatal gastric proteases. *Scandinavian Journal of Clinical and Laboratory Investigation* **52** suppl 210, 65–79

Foltman B 1993 General and molecular aspects of rennets. In *Cheese: Chemistry, Physics and Microbiology. Vol 1*, pp. 37–88 (Ed. PF Fox). London: Chapman and Hall

Fox PF 1969 Milk clotting and proteolytic activities of rennet, and of bovine pepsin and porcine pepsin. *Journal of Dairy Research* **36** 427–433

Houen G, Madsen MT, Harlow KW, Lonbald P & Foltman B 1996 The primary structure and enzymatic properties of porcine prochymosin and chymosin. *International Journal of Biochemistry and Cell Biology* **28** 667–675

Kobayashi H & Murakami T 1978 Rapid and large scale isolation of chymosin (rennin) by pepstatin amino hexylagarose. *Agricultural Biological Chemistry* **42** 2227–2258

Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **227** 680–685

Lo Piero AR, Puglisi I & Petrone G 2002 Characterization of ''Lettucine'', a Serine-like Protease from Lactuca sativa Leaves, as a Novel enzyme for Milk Clotting. *Journal of Agricultural Food Chemistry* **50** 2439–2443

Lowry OH, Rosenborough NJ, Farr AL & Randall RI 1951 Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193** 265–275

Melachouris N 1968 Discontinuous gel electrophoresis of whey proteins, casein and clotting enzymes. *Journal of Dairy Science* **52** 456–459

Mezina MN, Lavrenova GI, Prokof'ev MI, Starovoitova VV, Ermolaev VI, Chernyh VY, Balandina GN & Demidovich SS 2001 Isolation of milk-clotting enzyme from transgenic sheep milk and its comparison with calf chymosin. *Biochemistry* (*Moscow*) **66** 378–383

Mohanty AK, Mukhopadhyay UK, Grover S & Batish VK 1999 Bovine chymosin: Production by rDNA technology and application in cheese manufacture. *Biotechnology Advances* **17** 205–217

Neelakantan S, Mohanty AK & Kaushik JK 1999 Production and use of dairy enzymes for food processing: A review. *Current Science* **77** 143–148

Rao S 1984 Extraction and purification of chymosin from buffalo calves. *PhD thesis* National Dairy Research Institute, Karnal, India

Shindo K & Arima S 1979 Studies on chymosin: I. Chromatographic purification and some properties of chymosin. *Agricultural Biological Chemistry* **28** A177–A180

Singh J, Chander H & Rao VRB 1975 Extraction of rennet with 5% NaCl from buffalo, cow, goat and pig. *Journal of Food Science and Technology* (*India*) **12** 317–319

Walsh MK & Li X 2000 Thermal stability of acid proteinases. *Journal of Dairy Research* **67** 637–640