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Purification and characterization of milk clotting enzyme from goat (*Capra hircus*)

Ashwani Kumar [a,*], Jitender Sharma [b], Ashok Kumar Mohanty [a], Sunita Grover [a], Virender Kumar Batish [a]

[a] *Division of Dairy Microbiology, National Dairy Research Institute, Karnal-132 001, India*
[b] *Department of Biotechnology, Kurukshetra University, Kurukshetra-136 119, India*

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Abstract

Chymosin, the major component of rennet (milk clotting enzyme), is an acid protease produced in the fourth stomach of milk-fed ruminants including goat and sheep in the form of an inactive precursor prochymosin. It is responsible for hydrolysis of κ-casein chain in casein micelles of milk and therefore, used as milk coagulant in cheese preparation. The present investigation was undertaken to purify and characterize goat (*Capra hircus*) chymosin for its suitability as milk coagulant. The enzyme was extracted from abomasal tissue of kid and purified nearly 30-fold using anion exchanger and gel filtration chromatography. Goat chymosin resolved into three major active peaks, indicating possible heterogeneity when passed through DEAE-cellulose ion exchange column. The purified enzyme had a molecular mass of 36 kDa on SDS-PAGE, which was further confirmed by Western blot analysis. The purified enzyme preparation was stable up to 55 °C with maximum activity at 30 °C. The milk clotting activity was decreased steadily as pH is increased and indicated maximum activity at pH 5.5. Proteolytic activity of goat chymosin increased with incubation time at 37 °C. Goat chymosin was found to be more thermostable than cattle chymosin and equally stable to buffalo chymosin.

Keywords: Milk clotting enzyme; Kid chymosin; Cheese preparation; Purification; Abomasal tissue; Anion exchanger; Gel filtration; Physiological factors

1. Introduction

Chymosin, the major component of rennet, is an acid protease produced in the fourth stomach of milk-fed ruminants including goat and sheep in the form of an inactive precursor prochymosin. It is used to clot milk in cheese preparation by cleaving peptide bond between Phe 105 and Met 106 in the κ-casein chain. Chymosin and pepsin are the two major gastric proteases. Chymosin secretion is maximal during the first few days after birth, and declines thereafter, replaced by pepsin. Unavailability of calf stomach and ethical problems associated with animal slaughtering has necessitated finding other alternatives to calf chymosin. In this regard, various microbial alternatives are used for chymosin production but these sources too are not suitable for production of quality cheese as they produce a bitter taste (Neelakantan et al., 1999; Walsh and Li, 2000). Recombinant chymosin production has been achieved in different microorganisms (Mohanty et al., 1999; Rogelj et al., 2001; Vega-Hernandez et al., 2004; Kappeler et al., 2006) due to consumer push for vegetarian cheese. Buffalo, sheep, goat and rabbit are believed to be good sources of rennet. Bovine (*Bos tarus*) chymosin (Foltman, 1970, 1992, 1993), buffalo (*Bubalos bubalis*) chymosin (Mohanty et al., 2003) and lamb (*Ovis platyurea*) chymosin (Baudys et al., 1988) have been characterized extensively. However, there is limited information on the purification and characterization of kid chymosin (Amourache and Vijayalakshmi, 1984; Moschopoulou et al., 2006). While, milk products such as cheese and paneer produced from goat milk are of good taste and good nutritional value, it is worthwhile to test the use of goat chymosin as a suitable rennet substitute for the preparation of cheese from milk of goat, cow

* Corresponding author. Present address: 245, Brahampury, Muzaffarnagar-251 001 (U.P.), India. Tel.: +91 981 307 9015; fax: +91 131 244 1236.
E-mail addresses: ashwanindri@rediffmail.com, ashwanindri@gmail.com (A. Kumar).

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and buffalo. Therefore, in the present investigation, an attempt has been made to purify and characterize goat chymosin for its suitability as milk coagulant.

2. Materials and methods

2.1. Extraction of enzyme

Fifteen-day-old kids (*Capra hircus*) fed milk for chymosin secretion and, thereafter, slaughtered for collection of abomasal tissue. The 50 g tissue was flushed with distilled water and minced manually into small pieces. The minced tissue was freeze dried under vacuum. The tissue was extracted with 600 ml of distilled water in a homogenizer. After homogenization, the tissue sample along with homogenate was strained through muslin cloth. The crude enzyme extract was taken after centrifugation at 6000×*g* for 10 min at 4 °C. The tissue was further re-extracted with 100 ml of distilled water under the same conditions. The crude extracts were pooled from the two successive extractions.

The crude extract was clarified with 0.33 M aluminium sulphate solution with continuous stirring until the pH of the solution reached 4.0. The pH was then instantly raised to 5.8 with 0.5 M disodium hydrogen phosphate. The gelatinous precipitate formed including most of the impurities was removed by centrifugation at 6000×*g* at 4 °C for 10 min.

2.2. Ultrafiltration

The supernatant obtained in above step was subjected to ultrafiltration using a 100 kDa nominal molecular weight cut-off hollow fibre cartridge (Millipore Corporation, Bedford, Massachusetts, USA). The filtrate obtained was then ultrafiltered using a 10 kDa membrane (Millipore Corporation) to retain the chymosin with in the molecular range of 10–100 kDa and to concentrate the extract to a final volume of 58 ml.

2.3. DEAE-cellulose ion exchange chromatography

The ultrafiltrate enzyme extract was applied to a column packed with DEAE-cellulose-52 equilibrated with 0.05 M sodium phosphate buffer (pH 5.8) and washed with the same equilibration buffer until the absorbance of the eluant at 280 nm reached to <0.05. The majority of the proteins were washed away since they did not bind to the column under these conditions. The bound proteins were eluted with a linear gradient of 0.05 M (buffer A)–0.35 M (buffer B) phosphate buffer (pH 5.4) at a flow rate of 30 ml/h, monitored with the help of peristaltic pump (Pharmacia). The gradient was 100% buffer A for 25 min, 0–100% buffer B for 35 h, then washing with 100% buffer B and final washing with 100% buffer A for 15 min. At each step of purification, the fractions having milk clotting activity were pooled and concentrated to 11 ml by ultrafiltration using a 30 kDa membrane. The retentate was desalted by washing with 0.05 M sodium phosphate buffer (pH 5.4).

2.4. Superose 12HR 10/30 gel filtration column

The fraction with milk clotting activity was further refined in terms of its purity and molecular weight by injecting 50 µl (13 units/ml) on to a gel filtration Superose column attached to FPLC system (Pharmacia Cop., Peapack, New Jersey, USA) and equilibrated with 150 mM NaCl in 0.05 M sodium phosphate buffer (pH 5.4). Individual peak fractions were collected at a flow rate of 0.5 ml/min for 40 min and the peak fractions were ascertained by milk clotting activity. The fraction containing milk clotting activity was concentrated using 30 kDa cut-off centricon tubes.

2.5. Fast protein liquid chromatography

The purified goat chymosin from gel filtration column was further applied to a high resolution anion exchange column, Mono Q (HR-5/5) attached to FPLC system. An aliquot of 0.2 ml sample containing 22 units/ml of chymosin was injected into Mono Q column equilibrated with 0.05 M phosphate buffer (pH 5.4). The buffers used were 0.05 M phosphate buffer pH 5.4 (buffer A) and 0.5 M phosphate buffer pH 5.4 (buffer B). The elution was performed at a flow rate of 1 ml/min and the gradient was 100% buffer A for 10 min, 0–100% buffer B for 40 min, then washing with 100% buffer B for 10 min and final washing with 100% buffer A for 5 min. Protein content of crude extract and fractions were determined by Lowry et al. (1951).

2.6. SDS-PAGE and Western blot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following Laemmli (1970). SDS-PAGE was performed by mixing a sample with sample buffer, incubated at 100 °C for 5 min, centrifuged for 1 min and loading on to a 12% T slab gel. Proteins were fixed with 15% TCA, stained with Coomassie blue R250 (Sigma) and destained with

Table 1
Purification index of goat chymosin

Purification steps	Volume (ml)	Units/ml	Total enzyme unit	Protein (mg/ml)	Total protein (mg)	Specific activity (units/mg)	Relative purification fold	Yield (%)
Extraction	600	1.67	1002	4	2400	0.4175	1.0	100
Clarification	450	1.54	693	3	1350	0.5133	1.2	69.16
Ultrafiltration 10 kDa (U/F)	58	5.55	321.9	2.5	145	2.22	5.3	32.15
DEAE-cellulose chromatography (U/F)	11	13.25	145.75	2.07	22.77	6.4	15.32	14.5
Gel filtration	1.5	21	31.5	2.17	3.255	9.677	23.17	3.14
Mono Q	0.45	29	13.05	2.32	1.044	12.5	29.9	1.30