

Characters of Chymosin Gene Isolated from Different Animal Sources at Molecular Level

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Abstract: Four different animal tissues as a source for chymosin gene and three set of primers were used in this study. SDS-PAGE Polyacrylamide gel electrophoresis and PCR amplification techniques were used to differentiate between these animal rennet. After electrophoresis, the molecular weight of the enzyme was 40 kDa on SDS-PAGE for Buffalo and Cow but appears 43kDa and 30 kDa for Camel and Pig chymosin, respectively. Two prominent proteins were found in cow and buffalo rennet, while only one protein was observed in camel and pig. These patterns provided a basis for distinguishing animal rennet and the other enzymes as well as a means of identifying each type of enzyme by the characteristic pattern were shown. Also PCR product were identified.

Keywords: Chymosin gene, SDS-PAGE, PCR and Animals.

INTRODUCTION

Chymosin is an aspartic proteinase (EC 3.4.23.4) that is responsible for the coagulation of milk in the fourth stomach (abomasum) of unweaned calves^[10]. This enzyme (323 amino acids, 35.6 kDa) is secreted by the chief cells of the gastric mucosa as an inactive precursor, known as prochymosin (365 amino acids, 40.8 kDa). In the acidic conditions of the lumen, prochymosin is converted into the active form by autocatalytic cleavage of the 42-amino acid N-terminal prosequence^[15]. There are two allelic forms of calf chymosin designated as chymosins A and B, respectively, differing by a single amino acid substitution (Asp/Gly)^[5].

Chemistry of Chymosin is synthesized in vivo as preprochymosin. It is characterized as a protein of 365 amino acids. The 16 amino acids hydrophobic leader pre sequence^[21] is a signal sequence which is important in secretion of chymosin across the cell membranes. This is followed by a 42 amino acid pro sequence (Fig.1). It has long been known that chymosin is secreted as an inactive zymogene called prochymosin^[7], having a molecular weight of 40,777 kDa whose inactive state is maintained by the N-terminal propeptide^[5]. At acidic pH, the precursor undergoes autocatalytic activation to chymosin (35,600 kDa molecular weight, 323 amino acids, observed at pH around 5.0) or pseudo-chymosin (337 amino acids, observed at pH around 2). There are two naturally allelic forms of calf chymosin designated as chymosin A and B, respectively. These forms differ by a single amino acid residue an aspartate for a glycine at

position 244^[5, 6, and 16]. Chymosin exhibits a range of pH optima. The pH optima for calf stomach chymosin A and B have been reported to be pH 4.2 and 3.7 respectively^[8 and 16].

MATERIALS AND METHODS

Gene Source: Chymosin gene obtained from the abomasum of unweaned calves, bovine, Camel and Pig has traditionally been used as milk clotting enzyme for cheese manufacture. Although animals such as goat, sheep, and rabbit are believed to be good sources of rennet, these sources have not been investigated extensively for commercial exploitation^[17].

SDS-Polyacrylamid Gel Electrophoresis: Total protein extraction and patterns SDS-PAGE were performed according to Sheri *et al*^[22]. Protein samples were denaturated in denaturation buffer by boiling for 5 min in water bath and were subjected to 10% polyacrylamid gel electrophoresis in the presence of SDS(SDS-PAGE). Gels were stained with Coomassie blue R-250 and washed in acetic acid /water solution (1:9,v/v), molecular mass marker ranging from 14.4 to 200 kDa were used as control(Bio-Rad).

Amplification of Genomic DNA: The amplification products were generated using genomic DNA isolated from abomasum of different animal sources Buffalo, Cow, Camel and Pig tissues using a direct DNA and mRNA isolation kit (Qiagen), for PCR amplification forward and reverse primers were used which are based on sequences from the prochymosin gene.

The PCR reactions were carried out in a Gen Amp PCR System 9700 (PE Applied Biosystems) with incubation at 94°C for 4min; 35 cycles of 94 °C for 1 min, 58 °C for 30s, and 72 °C for 1min and incubation at 72 °C for 4min. The PCR products were run on 1% (w/v) agarose gels, and ethidium bromide staining.

RESULTS AND DISCUSSIONS

Molecular Characterization of Rennet: Chymosin is an aspartyl protease, which is located in the fourth stomach (abomasum), and carries along with a limited proteolysis of milk k-casein that leads to its coagulation. The chymosin is synthesized in the calf cell as a precursor, the preprochymosin. The preprochymosin is secreted and a leader peptide of 16 amino acids is removed to give rise to prochymosin (inactive) with a molecular mass of 46 kDa. Under the acidic condition of the stomach prochymosin catalyzes the removal of peptide of 42 amino acids from the amino terminal end of the protein by self-processing mechanisms, forming the active chymosin (molecular mass of 6 kDa)^[5,6,15].

The results illustrated in Fig. (2) show that SDS-PAGE electrophoresis patterns of bovine calf rennet and cow calf rennet protein are quit similar but different from pig rennet and camel calf rennet. Each pattern had one major band except cow calf rennet pattern that had one minor band. The molecular masses of the major bands reached 43 kDa, 40 kDa, 40 kDa and 30 kDa for camel calf rennet, buffalo calf rennet, cow calf rennet and pig rennet respectively. The apparent molecular weight of Camel chymosin was about 43 kDa and it was somewhat higher than the weight observed for pig, cow and buffalo chymosin (Fig. 2). Another band was found with the cow chymosin that measured 16 kDa.

SDS-PAGE- electrophoresis profile (Fig. 2) shows that the major band of Camel chymosin is 43 kDa, whereas the minor band is ≥ 16 kDa for Calf chymosin. This minor band that was observed in the SDS-PAGE- gel indicates that possible autocatalytic

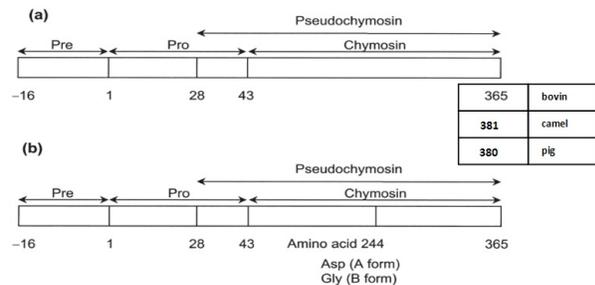


Fig. 1: Chymosin: (a) protein structure and (b) protein variants

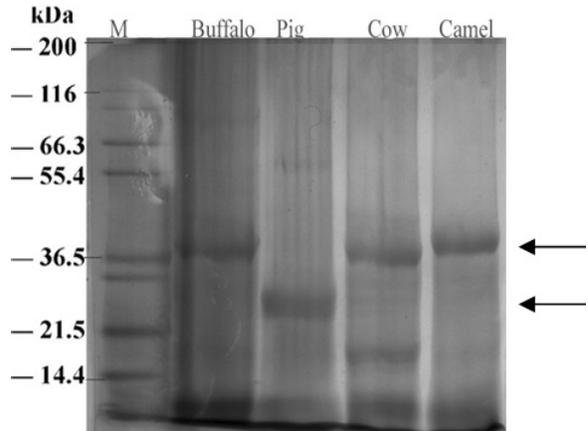


Fig. 2: SDS-PAGE analysis of tissues extracts of buffalo, camel, cow and pig. The arrows indicate the position of the protein. The 12% polyacrylamide gels were stained with Coomassie blue. The positions of molecular mass markers (SDS-PAGE standards; Bio-Rad) are indicated on the left.

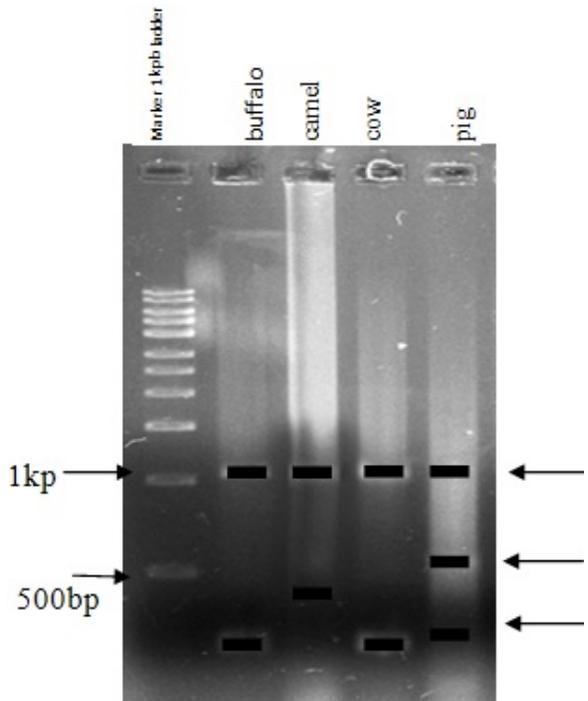


Fig. 3: M: 1kpb DNA ladder; lane 2-5: PCR products of DNA extracted from different animal tissues.

degradation of Cow chymosin. These results are consistent with those of Abdel Malak *et al.*,^[1] who also reported that a molecular weight of 36 ± 1 kDa for buffalo chymosin has the similar electrophoretic heterogeneity that reached 35.6 kDa^[13].

Table 1: Sequences of the three primers used in this study for PCR amplification.

primer	Nucleotide sequences
camel	CACGTGGCGGAGTGGGATCACCAGGATCCCTCTG TAGAGGATCAGATGGCCTTGGCCAGCCCCACG
Pig	TCTCCAGGGCAGTGGGATCACC TCCACGATGCCGGCACCATGAC
Buffalo and cow	CCAGGGCTTCTGTACCAGTGGCTTC TGAGAATCATCTGTCTGGAAACCTC

In contrast, preparation of buffalo and cow chymosin with molecular weight of 23 kDa has been reported^[18]. These 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^[3] and Foltmann^[4] due to the presence of two isozymes of bovine chymosin, such as chymosin A and B, while chymosin C (molecular weight of around 8.5 kDa, a degradation product of chymosin A) that moves a long with the dye in front of SDS-PAGE. The heterogeneity of buffalo chymosin may be presumed to be caused by the presence of similar of isozymes, where buffaloes and cattle are close to each other on the evolutionary ladder, and belonging to the same family. Other investigators have also observed that the presence of different electrophoretically distinguish components of cattle rennin in SDS-PAGE^[19].

Characteristics of the Chymosin Gene: PCR was performed with the three sets of primers deduced from chymosin gene sequences^[11,12] (Table 1) resulted in different bands of approximately 1.2 and 0.2 kb according to the source of the chymosin gene (Fig. 3). The length of pre-prochymosin gene isolated from stomach mucosa of different animal sources and amplified by PCR was 1.1 kb, 1.2 kb, 0.5 and 0.5 kb for camel, pig, buffalo and cow respectively and it was corresponding with the sequence obtained from genomic analysis of pre-prochymosin gene^[14].

Fig. (3) shows that PCR profiles of each primer we found that two main bands of approximately 1.2 kb and 0.25 kb for buffalo chymosin, 1.1 kb and 0.23 kb for cow, 1.1 kb and 0.35 kb for camel and 1.16 kb, .0.5 kb and 0.2 kb for pig chymosin were obtained. These different bands due to calf chymosin are found in three major forms. A, B and C, chymosin B being the most abundant. Chymosin A and B differ at only one amino acid position but chymosin C appears to be a degradation product of product of chymosin A that lack three residues^[2]. It is likely that chymosin A and chymosin B are synthesized from different alleles of the same polymorphic gene rather than a multiple gene family as only one locus of chymosin gene is found from the hybridization of the calf gene with a chymosin gene^[3].

These results are in agreement with Stefan *et al.*,^[20] who illustrated the length of camel

pre-prochymosin gene isolated from stomach mucosa and amplified by PCR was 1172bp. Also these results are in harmony with Houen^[13], who found that pig and calf chymosin possessed 80% amino acid sequence identity but showed considerable differences in their enzymatic properties. A comparison of their structures may therefore contribute to an understanding of the significance of the amino acid residues responsible for the differences in these properties.

On the other hand, like many other gastric proteases, pig chymosin occurs as several genetic variants. The apparent homozygosity in some animals suggests that, for calf chymosin^[14], there is only one cistron for pig chymosin with co-dominant expression of the two alleles in heterozygotic animals. In comparison with the electrophoretic mobilities of human pepsinogenes^[9], it was found that there is a charge difference between the two predominant forms, these differences must reside in the mature proteins. In conclusion, the nature of buffalo chymosin is more or less similar to cow and camel chymosin and there appear to be different from pig chymosin. Pig chymosin in relation to the other chymosin sources, the two bands of PCR product, have the same electrophoretic mobility and are clearly related to calf chymosin.

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