Bacterial expression and functional characterization of a naturally occurring exon6-less preprochymosin cDNA

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Abstract
Chymosin (Rennin EC 3.4.23.4), an aspartyl proteinase, is the major proteolytic enzyme in the fourth stomach of the unweaned calf, and it is formed by proteolytic activation of its zymogene, prochymosin. Following the cloning of synthesized cDNAs on mRNA pools extracted from the mucosa of the calf fourth stomach, we have identified an alternatively spliced form of preprochymosin cDNA (AS6 preprochymosin). Sequencing data analysis showed that the exon six has been spliced out and, therefore the gene product is 114 bp shorter in length. In order to determine the biological significance of the AS6 preprochymosin, we expressed the encoding cDNA together with a complete chymosin cDNA in E. coli. Under the same expression conditions, we found at least a 5-fold higher expression of AS6 preprochymosin protein in comparison to a full-length recombinant chymosin. Protein prediction program analyses showed that the missing exon contain groups of amino acids with high hydrophobicity score. Therefore, the deletion of this exon may explain the higher expression of the recombinant product in E. coli. Most importantly, the biological activity of the purified AS6 preprochymosin, was confirmed in an assay of chymosin milk-clotting activity using the recombinant preprochymosin and commercial rennet as positive controls. The expression of the biologically active preprochymosin lacking exon 6 may have important implications on the existence of this splicing form of mRNA in vivo and on its biotechnological applications in cheese manufacture.
Keywords*: Preprochymosin; Aspartyl proteinase; Alternatively spliced transcript; Milk-clotting; Microbial rennet; Recombinant chymosin; Hydrophobicity.*

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INTRODUCTION

The use of rennet, an extract from the fourth stomach (abomasums) of milk-fed calves in cheese manufacture was among the earliest applications of exogenous enzymes in food processing, dating back to approximately 6000 B.C (Foltmann, 1966). The major milk clotting component of standard rennet is chymosin (88 to 94%) which specifically cleaves the milk kappa-casein at the Phe105-Met106 peptide bond (Fig. 1 a and b). Other proteolytic enzymes such as pepsin (about 6-10%) are also present in rennet and play an important role in cheese production, especially in cheese ripening and may be a cause of the development of bitterness during storage (Rogelj *et al.*, 2001).

Microorganisms like *Rhizomucour pusillus*, *R. miehei*, *Endothia parasitica*, *Aspergillus oryzae*, and *Irpex lactis* have been used extensively for rennet production in cheese manufacture since the 1970s (Fox, 1999). The rennets from microbial sources are more proteolytic in nature in comparison to rennet from animal sources, resulting in production of some bitter peptides during the process of cheese ripening (Green, 1984). Therefore, attempts have been made to clone the gene for calf and other ruminant chymosins, and to express them in various microorganisms from bacteria to yeast and molds (Emtage *et al.*, 1983; Harris, *et al.*, 1982; Ward *et al.*, 1990; Housen *et al.*, 1996; Goff *et al.*, 1984; Hakki *et al.*, 1989 and Vega-Hernandez *et al.*, 2004). Indeed, the gene for calf chymosin was one of the first genes for mammalian enzymes that was cloned and expressed in microorganisms such as bacteria (Nishimori *et al.*, 1982).

Protein and cDNA sequence analyses have shown that chymosin is synthesized by mucosal cells as pre-

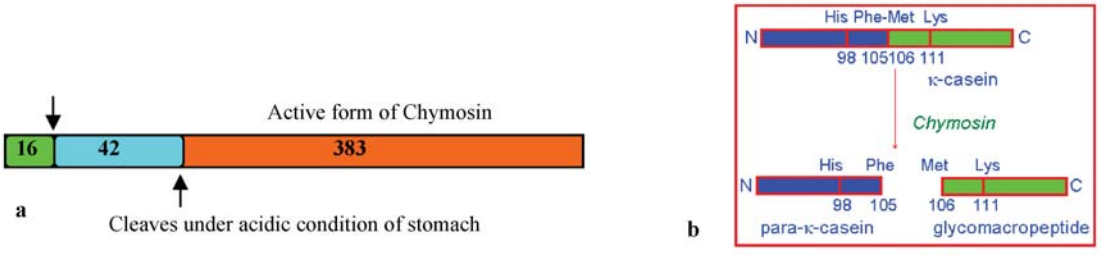

Figure 1. a: Diagrammatic representation of structure of preprochymosin protein. The arrows show the cleavage sites and the numbers correspond to the number of amino acids in the putative protein. The green box shows the signal peptide after translocation of protein to plasma membrane or periplasmic space, the blue box shows the peptide which is cleaved upon exposure of the protein to acidic condition of stomach and the orange box shows active form of enzyme. b: Diagram showing cleavage site of calf chymosin.

prochymosin (Foltmann *et al.*, 1979) and autocatalytic activation at pH 4 to 5, resulting in cleavage of a 42 AA fragment at the amino terminal end of prochymosin (Harris *et al.*, 1982 and Emtage *et al.*, 1983). Structural studies have shown that the principal catalytic residues are Asp 32 and Asp 215 (pepsin numbering) and the bilobular chymosin structure is maintained through a network of hydrogen bonds involving amino acids Thr33, Ser35, Thr216, Thr218, NHGly217, and NHGly34 (Zhang *et al*., 1997 and Newman *et al.*, 1991).

The gene encoding bovine preprochymosin (bPC) is 10.5 kb in length and includes 9 exons. A mature mRNA is generated after splicing of all eight introns (Hidaka *et al.*, 1986). However, no expressed protein was shown and biological importance of various isoforms has not been explained, multiple splicing forms of bPC mRNA in the stomach tissues of suckling calves has recently been reported (Zinovieva *et al.*, 2002). It was suggested that the mature mRNA lacking exons six and/or eight can be translated into mature proteins since these lower molecular weight enzymes still harbor chymosin active site residues as well as all conserved cystein and hydrogen bonding residues.

In this study, we isolated preprochymosin and one of the alternatively spliced forms of preprochymosin mRNA from the stomach tissues of suckling calves. The synthesized cDNA was cloned into a bacterial expression vector and expression pattern of these forms of preprochymosin were analyzed by SDS-PAGE. The nucleotide sequence data showed that exon 6 has been spliced out in mature mRNA. The hydrophobicity profiles of the two exon6-less preprochymosin and native preprochymosin were compared, and the effects of hydrophobic amino acid content on bacterial expression of the recombinant proteins was discussed. Finally, proteolytic activities of both recombinant preprochymosins were performed in an assay of milk clotting.

MATERIALS AND METHODS

Tissue preparation: The stomach tissues were derived from a suckling calf (about 14 days). Mucus membranes from the fourth stomach were scrapped by clean scalpel, aliquoted and kept in -70°C until RNA extraction.

Total RNA extraction and cDNA synthesis: 100 mg of tissue was powdered in liquid nitrogen and homogenized in TRIZOL solution (GIBCO-BRL, Germany). Total RNA was extracted according to manufacturer's instruction. The integrity of the RNA was analyzed by 1% (w/v) RNase-free agarose gel and its concentration was measured by spectophotometery. First strand cDNA was synthesized at 42°C for 1h using oligo-dT primer and AMV reverse transcriptase (Roche, Applied Science, Germany).

PCR amplification and cDNA cloning: The resultant first strand cDNA was used as template for PCR. The sense primer Ky1 (+) (5´-gggaattccat ATGAGGTGTCTCGTGGTGCTA-3´) corresponds to the beginning of leader sequence of preprochymosin cDNA including NdeI restriction site (underlined). The antisense Ky2 (-) (5´-gtcagcct CGAGATCAGATGGCTTTGGCCAGC-3´) hybridizing to the C-terminal end of calf preprocymosin cDNA and contains an additional XhoI restriction site at its 5´-end (underlined).

(GIBCO-BRL, Germany). These primers were designed based on sequences from the bovine pre-prochymosin gene (Carey *et al.,* 1983 and GenBank accession number A15633). The PCR was carried out in a GeneAmp PCR system 9600 (PE Applied Biosystems, USA) using *Taq* DNA polymerase (Roche, Germany) with incubation at 94°C for 3 min; 35 cycles of 94°C for 30s, 54°C for 30s, and 72°C for 1 min; and incubation at 72°C for 10 min. Each PCR experiment included one positive and negative control, interposed between the samples tested.

The PCR products were run on 1% gel (w/v) agarose gels, purified with the QIAquick PCR purification kit (Qiagen GMBH, Germany), and cloned into the *NdeI-XhoI* sites of plasmid pET 28a(+), just downstream of T7 promoter region (Novagen). *E. coli* BL21 (DE3) (Novagen) cells were transformed with ligated plasmids according to $CaCl_2$ method (Sambrooke *et al.,* 2001).

DNA sequencing and bioinformatics analysis: Positive colonies were selected in a Colony-PCR screening assay. Briefly, the colonies on plates were picked up, dissolved in 50 µl of dH_2O, boiled for 2 min and 1µl of that was used as a template in the PCR reaction mentioned above. Following the restriction enzyme analyses on individual plasmid DNA, two different DNA samples from two clones (called hereafter C4 and CN4) were selected and their nucleotide sequences were determined (MWG, Germany). Both strands of the DNA inserts were sequenced.

The DNA sequences were compared using BLAST program (Altschul *et al.,* 1990). A protein sequence alignment was then generated using the SIM program with the default parameters (Huang *et al.,* 1991).

Regions of high hydrophobicity in preprochymosin, AS6 preprochymosin, and for the missing exon (exon6) were determined using ProtScale Tool (Kyte and Doolittle, 1982) in which hydropathic (hydrophobic) regions achieve a positive value. Setting window size to 5-7 is suggested to be a good value for finding putative surface-exposed regions.

Expression and purification of calf preprocymosin and AS6 preprochymosin: 5 ml overnight pre-cultures of individual clones (C4 and NC4) were used to inoculate (1:500 dilution ratio) 50 ml cultures of 2xTY-(Amp 100 µg/ml) (glucose 1%) (Sigma, USA). The bacterial cultures were grown at 37°C for 2-4h until OD_{600} of 0.5. Then the cultures were spin down and washed twice to remove traces of glucose. Finally, the cells were resuspended in the same volume of original medium and isopropylthio-β-D-galactoside (IPTG) (Sigma, USA) was added to final concentration of 1mM to induce expression of proteins under T7 promoter. Growth was continued at 37°C for different time points and cells were harvested by centrifugation at 10,000 ×g) for 1 min after 2, 4 and 8h post-induction. OD was measured at each time and the pellets were dissolved in the suitable amount of sample loading buffer, and analyzed by the SDS-PAGE (10%). The large-scale protein purification was also done. Expression of recombinant chitinase was induced by adding IPTG at a final concentration of 1mM to the actively growing culture of *E. coli*. The culture was further grown for 2h and the cells harvested by centrifugation. The cells were disrupted by sonication and the induced proteins were purified using Ni-NTA beads from Qiagen (Qiagen, Gmbh, Germany) according to manufacturer's instruction. For western blot analysis, bacterial lysate from C4 and NC4 clones were subjected to SDS-PAGE, transferred to nitrocellulose membrane (Roche, Germany). The membrane was then incubated with primary mouse monoclonal anti-histidine tag (Roche, Germany). As secondary antibody conjugate, goat anti-mouse HRP (Sigma, USA) was used, and the blot was developed with diaminobenzidine tetrahydrocholride (DAB) substrate (Sigma, USA). It should be noted that in all the experiments regarding expressed proteins, equal amount of proteins were used.

Activation and qualitative assay of chymosin milk-clotting activity: The preprochymosin and AS6 pre-prochymosin were activated at pH 4.7 according to procedure of Pedersen and his co-workers (Pedersen *et al*., 1979). Briefly, both purified preprochymosin solutions were activated at 25°C by immediate mixing of preproenzyme solutions with 1 ml of 200 mM sodium acetate, pH 4.7. The enzyme solutions were then diluted 20-fold with 50 mM phosphate buffer, pH 6.5 to stop activation, concentrated by ultrafiltration through a YM-10 Amicon centrifugal filter unit (Millipore Corporation, USA) and stored on ice until milk-clotting activity was measured.

Commercial rennet from a fungal source (Rennilase^R) was used as a positive control in a milk-clotting assay as described by van den Berg and his co-workers (van den Berg *et al.,* 1990). In this protocol, a simple qualitative microtiter (Nunc, Denmark) plate assay was developed for the measurement of milk-clotting activity. The final assay mixture (100 µl per well) contained 12% (w/v) dried skim milk; 20 mM $CaCl_2$; 25 mM phosphate buffer (pH 6.3) and activated chymosin, and was incubated at 37°C for 30 min.

RESULTS

Cloning and characterization of the preprochymosin cDNAs: RT-PCR was performed with a set of primers deduced from bovine sequences (Carey *et al.*, 1983) and RNA from abomasums as a template, resulted in bands of approximately 1.1 kb and 900 bp (Fig. 2a). These fragments were purified from gel, cloned just downstream of T7 promoter region in pET28a expression vector, and transformed into *E. coli* BL21 (DE3) cells. The gene encoding T7 RNA polymerase carried on the bacteriophage λDE3 is integrated into genome of the bacterial cells and was used to drive the expression of the protein under the control of T7 promoter.

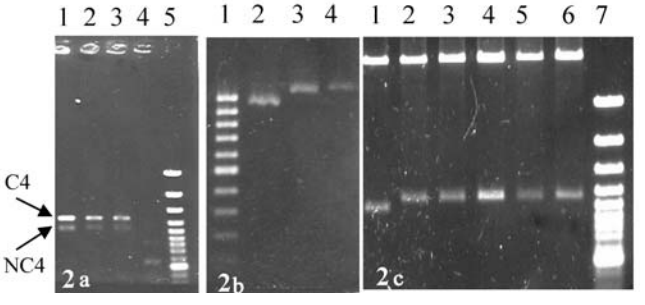

Figure 2. 2a: Photograph showing the RT-PCR products, lane 1, the PCR product with 1.5 µl cDNA (template); lane 2, PCR product with 1 µl cDNA; lane 3, PCR product with 0.5 µl cDNA; lane 4, PCR product with 0.25 µl cDNA and Lane 5 is DNA size marker (100 bp). 2b: Photograph showing the colony-PCR products, lane1, 1 kb DNA ladder (100 bp); lane 2, truncated form of preprochymosin (clone NC4) and lane 3 and 4 is the full-length of amplified preprochymosin (clones C4 and C5). 2c: The same clones that were positive for colony-PCR, were grown up overnight for plasmid minipreparation and they were digested with restriction enzymes *NdeI* and *XhoI*. lane1: clone NC4; lane 2 to 6 are different colonied containing the full-length C4 and lane 7 is DNA size marker.

Following a colony-PCR assay, restriction enzyme analysis was performed on a few recombinant clones (C1-C4) (Figs. 2a and 2b). As shown, one of these clones, NC4, has smaller fragment (about 110 bp in length).

The nucleotide sequence of the two selected clones (C4 and NC4) was determined and used as queries to search for related nucleotide sequences in other mammals using the BLAST program and GenBank database. Comparison of deduced nucleotide sequences of C4 and NC4 clones with their counterparts from other mammals indicated almost 100% sequence identity between cloned preprochymosin cDNA and that of bPC sequences available in GenBank database. However, for clone NC4 we found a gap in nucleotide sequence 650 to 763 (the nucleotide sequence is numbered from the beginning of preprochymosin cDNA) when it is aligned with a full-length bPC (Fig. 3). We found a complete match of nucleotide sequence of this shorter cDNA sequence with an alternatively spliced bPC mRNA recently reported by Zinovieva and coworkers (Zinovieva *et al*., 2002). Comparison of deduced amino acid sequences of C4 and NC4 clones is shown in figure 3.

Hydrophobicity studies: There are various tools to analyze and predict the hydrophobicity pattern of a

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Full-length(C4)and Truncated(NC4)form of preprochymosin.

C4  GEVASVPLTNYLDSQYFGKIYLGTPPQEFTVLFDTGSSDFWVPSIYCKSNACKNHQRFDP
NC4 GEVASVPLTNYLDSQYFGKIYLGTPPQEFTVLFDTGSSDFWVPSIYCKSNACKNHQRFDP

C4  RKSSTFQNLGKPLSIHYGTGSMQGILGYDTVTVSNIVDIQQTVGLSTQEPGDVFTYAEFD
NC4 RKSSTFQNLGKPLSIHYGTGSMQGILGYDTVTVSNIVDIQQTVGLSTQEPGDVFTYAEFD

C4  GILGMAYPSLASEYSIPVFDNMMNRHLVAQDLFSVYMDRNGQESMLTLGAIDPSYYTGSL
NC4 GILGMAYPSLASEYSIPVFDNMMNRHLVAQDLFSVYMD————————————————————

C4  HWVPVTVQQYWQFTVDSVTISGVVVACEGGCQAILDTGTSKLVGPSSDILNIQQAIGATQ
NC4 —————SVTISGVVVACEGGCQAILDTGTSKLVGPSSDILNIQQAIGATQ

C4  NQYGEFDIDCDNLSYMPTVVFEINGKMYPLTPSSYTSQDQGFCTSGFQSENHSQKWILGD
NC4 NQYGEFDIDCDNLSYMPTVVFEINGKMYPLTPSSYTSQDQGFCTSGFQSENHSQKWILGD

C4  VFIREYYSVFDRANNLVGLAKAI
NC4 VFIRGYYSVFDRANNLVGLAKAI
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Figure 3. Alignment of the deduced amino acid sequences of C4 and NC4.

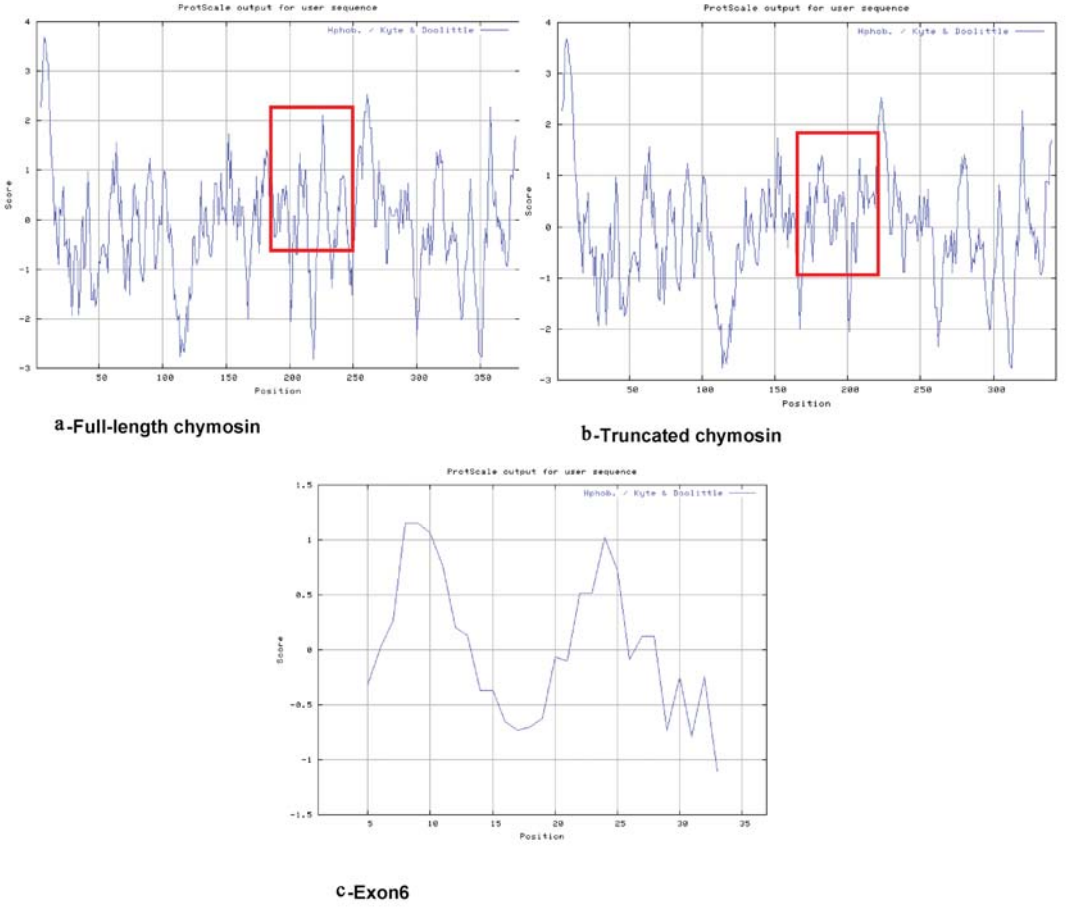

Figure 4. Hydropathy plot of the full-length chymosin (a), truncated (b) and Exon 6 only (c).

protein, among which the hydrophobicity criteria of Kyte-Doolittle is a widely applied scale for delineating hydrophobic character of a protein. Each amino acid is given a hydrophobicity score between -4.5 and 4.5. A score of 4.5 is the most hydrophobic and a score of -4.5 is the most hydrophilic. Regions values above zero are hydrophobic in character. Using these criteria and ProtScale tool with a window size of 7 amino acids, two regions (residues 6-13 and 21-30 within the exon 6 with total number of 38 amino acids) have been scored with high hydrophobic values (Fig. 4a-c). Comparison of these graphs shows some changes in the hydrophobicity maxima in the exon 6 region.

Expression of the preprochymosin cDNAs: The expression of preprochymosin and AS6 preprochymosin under control of the T7 promoter was performed in *E. coli*. protein assay was performed on samples and equal amount of proteins were loaded onto the wells. The result of SDS-PAGE is shown in figure 5. It should be noted that the expression of the full-length preprochymosin using clone C4 is very low and therefore it is detectable by western blot analysis more eas-

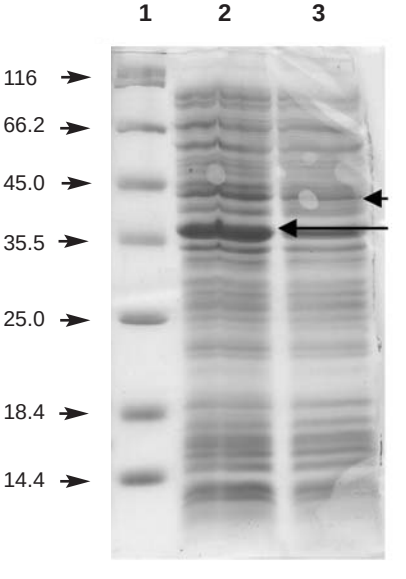

Figure 5. SDS-PAGE analysis of expression of two clones of preprochymosin (NC4 and C4) in *E. coli*. Lane 1: molecular Size marker, lane 2: NC4 clone and lane 3: C4 clone.

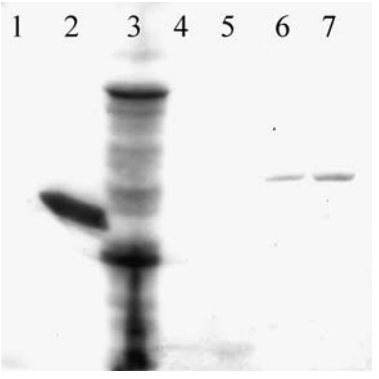

Figure 6. Western blot analysis of different colonies showing expression of preprochymosin using anti-6X His tag monoclonal antibody. The lanes were loaded as follows: lane 1: the positions of the protein size marker; lane 2: total protein of clone CN4 (4h post-induction with IPTG); lane 3: a protein containing 6x his tag used as a positive control for western blotting; lanes 4, 5: a clone containing only pET28 vector used a negative control. Total protein of induced 2h and 4h post-induction respectively; lanes 6, 7: total protein of clone C4 (total protein of 2h and 4h post-induction respectively).

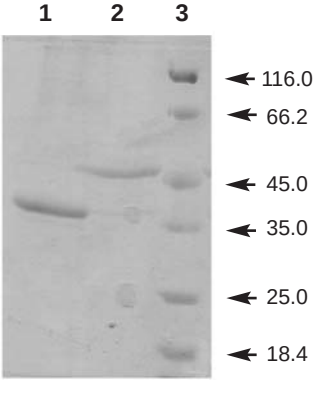

Figure 7. Analysis of the expression and purification of both full-length (C4) and truncated form (NC4) of preprochymosin. The C4 and NC4 forms were expressed in *E. coli* and purified by affinity chromatography using Ni-NTA beads. Lane 1, purified NC4 protein; lane 2, purified C4 protein and lane 3 is molecular size marker.

Figure 8. Milk-clotting assay: The clotting activity of preprochymosin and exon6-less preprochymosin are showed in this picture. Lane 1, exon6-less preprochymosin and lane 2, preprochymosin. In each lane up to down, respectively: unpurified protein, dilution 1, 1/2, 1/4, 1/16, 1/32 of purified proteins, positive control and negative control.

ily as shown in figure 6 (lanes 6 and 7).

We observed a higher expression of a specific protein band with expected size for exon6-less clone (NC4) in comparison with full-length preprochymosin produced from clone C4. The immunoreactivity of these bands was shown by Western blot analysis using mouse monoclonal antibody against His-tag as the expressed recombinant proteins contain 6x his tag at their N-terminal domain (Fig. 6).

Purification and activation of preprochymosins: After induction of bacterial cells from both clones, large scale protein purification was performed. Inclusion bodies containing preprochymosins His-tag were dissolved in denaturing buffer and purified by a His-tag affinity column. The purity of the recombinant products were analyzed by SDS-PAGE (Fig. 7).

Activation and processing of the recombinant preprochymosins: Equal amount of purified preprochymosins were activated according to Pedersen *et al.* (1979). After acid activation and neutralization, the purified fractions were tested in a qualitative milk-clotting assay. The clotting activity of preprochymosin and exon6-less preprochymosin were comparable. These results were compared to that obtained with the commercial fungal rennet (Fig. 8) as a positive control, and bacterial lysate harboring pET28a without any insert as a negative control.

DISCUSSION

Milk coagulants are principally divided in two different groups of animal and microbial rennets. Due to shortage of calf stomach and the economic value of animal-based rennet, gene for calf chymosin was one of the first genes for mammalian enzymes that was cloned and expressed in microorganisms (Nishimori *et al.*, 1982 and Teuber, 1993). The enzymatic properties of recombinant chymosin are indistinguishable from those of native calf chymosin. The recombinant products have unique specificity in hydrolyzing milk kappa casein and has no other proteolytic activity, an associated problem observed in both animal-based rennet extracts as well as in microbial rennets resulting in production of some bitter peptides during the prolonged process of cheese ripening (Rogelj *et al.*, 2001).

Using gene specific primers designed based on nucleotide data in GenBank, the predominant RT-PCR corresponding to full-length preprochymosin was amplified. Furthermore, a weaker additional PCR product of decreased length (approximately 110 bp

shorter in length) was observed. To confirm the origin and to determine the structures of the transcripts, sequence analysis of the cloned RT-PCR products was performed. A database homology search using NCBI BLAST network server showed high degree of sequence identity with those of preprochymosin nucleotide sequences available in GenBank. However, we found a nucleotide gap at position 650 to 763 corresponding to the exon 6 of the shorter transcript. The molecular analysis at the exon-intron junction showed that the exon 6 was precisely spliced out without change in the reading frame. This product corresponds to the A2 form identified by Zinovieva *et al*. (2002).

An open reading frame beginning at the first ATG codon of alternatively spliced preprochymosin mRNA predicts a protein of 343 amino acids with a molecular mass of approximately 37.7 kDa. Similar to its full-length preprochymosin, the protein has an N-terminal leader sequence and a proenzyme region of 16 and 42 amino acids, respectively (Fig. 1a). Both cloned preprochymosin are type B allelic variants forms of chymosin. It is necessary to mention that anion-exchange chromatography reveals three major forms of the enzyme: chymosins C, B, and A, in order of their elution (Foltmann, 1970). This terminology originates with determinations that the A form has the highest specific milk-clotting activity of the three forms, and the C form has the lowest (Foltmann, 1970). Chymosins A and B are allelic variants which differ in a single amino acid residue; the A form has Asp286, whereas the B form has Gly (Foltmann *et al.*, 1979). Chymosin C, however, has until now been described only as a mixture of which a major component is partly autolyzed chymosin A (Foltmann *et al.*, 1966).

Exon 6 has 38 residues of which 12 are very hydrophobic and 17 are less hydrophobic amino acids. We used hydrophobicity criteria of Kyte-Doolittle to analyze and predict the hydrophobicity pattern of these two forms of chymosin. Using this program, two regions (residues6-13 and 21-30 within the exon 6 with total number of 38 amino acids) have been scored with high hydrophobic values (Fig. 4). The comparison of these profiles shows some shifts in the hydrophobicity profile from preprochymosin to AS6 reprochymosin. One of the important factors in determining the expression levels of eukaryotic proteins in bacterial hosts is hydrophobic nature of polypeptide. There are reports of improved expression in bacteria by exchanging of hydrophobic residues into neutral or hydrophilic residues (Linder *et al.*, 2004; Strub *et al*., 2004). These amino acid replacements are especially important for surface residues where exposed hydrophobic residues may increase aggregations and misfolding inside host expression system and loss of protein during purification stage.

Milk clotting activities of the activated recombinant products were compared with that of commercial microbial rennet in a qualitative manner. Although the strongest milk clotting activity was observed for microbial rennet, there was a clear milk-clotting activity for chymosin and AS6 chymosin in ELISA wells. As it was suggested by Zinovieva and co-workers (2002), the deletion of exon six and/or eight would keep both chymosin active Asp residues and hydrogen bonding amino acid residues.

It is also likely that low clotting activity of chymosin and AS6 chymosin is due to improper re-folding or conditions set for the activation of AS6 preprochymosin. Therefore, further confirmations and standardization of the milk-clotting activity of this alternatively spliced protein product is required in order to explore its potential biotechnological applications. However, high expression of this alternatively expressed transcript in bacteria, and proper folding of the AS6 chymosin protein molecule in the absence of exon six are the two most important aspects distinguished in this research work and may have certain implications in the design of new protein motifs for bacterial expression.

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