Short Paper

Nucleotide sequence of cDNA encoding for preprochymosin in native goat (*Capra hircus*) from Iran

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Summary

Prochymosin is one of the most important aspartic proteinases used as a milk-clotting enzyme in cheese production. In the present investigation we report sequence of cDNA encoding goat (*Capra hircus*) preprochymosin and compare its nucleotide and deduced amino acid sequences with sequences of other ruminants preprochymosin. As bovine prochymosin, the caprine prochymosin cDNA encodes 365 amino acids with a prosegment of 42 amino acids and the mature goat chymosin begins with glycine. The preprochymosin nucleotide sequence reported in this study differs from other reported goat sequence (AY389343) in three nucleotides, two of which alter the amino acids at positions 19p and 139.

Key words: Goat, Prochymosin

Introduction

Chymosin (Rennin, EC 3.4.23.4) is the major proteolytic enzyme in the stomach of all nursing mammals (Rao *et al*., 1998). The enzyme that is an aspartyl proteinase, is secreted by the chief cells of the gastric mucosa, as a zymogene called prochymosin (365 amino acids, MW. 40777 Da). Prochymosin is irreversibly converted under acidic condition into active enzyme (323 amino acids, MW. 35652 Da) by cleavage of forty-two amino acids from the NH_2-terminus (Harris *et al*., 1982; Nishimori *et al*., 1984).

Chymosin is used in cheese manufacturing primarily as a milk coagulant because it cleaves κ-casein in a specific manner, at Phe^{105}-Met^{106} bond, with low proteolytic activity (Mohanty *et al*., 1999; Vega-Hernandez *et al*., 2004).

Calf chymosin is found in three major forms: A, B and C. Chymosin A and B, differ at only one amino acid residue, but chymosin A has a significantly higher specific activity. These three forms have also some other different enzymatic properties (Chitpinityol and Crabbe, 1998).

In our attempt to clone and produce the recombinant goat (*Capra hircus*) prochymosin, we found some differences in nucleotide sequences with those recovered by Vega-Hernandez *et al*. (2004). We present here the cDNA sequence encoding for caprine preprochymosin because of the importance of amino acid substitution effect on enzymatic properties.

Materials and Methods

RNA isolation and RT-PCR

Total RNA was isolated from the abomasums of 7–10-day-old cross-breed goats (*Capra hircus*) reared in Fars province, Iran; using RNX (plus™) solution (CinnaGen Inc., Iran), by following the manufacturer's recommended protocol. RNA was extracted from about 100 mg of tissue using RNX solution and chloroform and then was precipitated by isopropanol.

The first strand cDNA was synthesized using 200 U of M-MuLV reverse transcriptase (Fermentas, Lithuania) and primer ReOutR (5' CGCGTGGGGACAGT GAGGTTCTTG), designed based on the caprine preprochymosin nucleotide sequence (GenBank™ accession number: AY389343). The reaction also contained 1000 pmol of each dNTP and 40 U ribonuclease inhibitor and was performed at 42°C for one h. The single strand cDNA was used as template for double strand cDNA synthesis. PCR was carried out using *pfu* DNA polymerase (Fermentas, Lithuania) in a 25 µl reaction containing one unit of enzyme, 8 mM $MgSO_4$, 400 pmol of each dNTP and 20 pmol of each primers ReOutR and ReOutF (5' CAGCGGCCGGACCCAAGATGA). The reaction mixture was heated to 94°C for 3 min and then incubated for 35 cycles of 94°C for 50 sec, 60°C for 50 sec and 72°C for 1 min, with a final incubation of 72°C for 5 min.

cDNA cloning

The PCR products were run on 1% agarose gels. The DNA fragment of interest was excised from the gel and purified with the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The purified blunt-end PCR products were A-overhanged by adding the *Taq* DNA polymerase and dATP and then heating at 72°C for 15 min. The treated fragments were cloned into the cloning vector pTZ57RT (Fermentas, Lithuania) using T4 DNA ligase (Fermentas, Lithuania) at 16°C for overnight. The ligation reaction was transformed to *E. coli* XL1-Blue via heat shock treatment using calcium chloride (42°C for 45 sec).

Transformants were plated on LB agar plates containing 100 µg/ml ampicillin. Ten transformants were grown in LB broth with ampicillin at 37°C with shaking. Plasmid DNA was extracted and then purified using AccuPrep® Gel Purification Kit (Bioneer, South Korea). The identity of recombinant plasmid was ascertained by PCR using M13 universal primers. One authentic clone was used to determine the nucleic acid sequence of goat preprochymosin. Both strands of the DNA insert were sequenced using M13 universal primers.

Results from sequencing of goat preprochymosin gene were analysed and aligned with nucleotide sequences of preprochymosin reported from sheep and cattle and also with a sequence from another goat submitted in NCBI GenBank using software DNAstar.

Results

The numbering of the amino acid residues in the manuscript is based on the sequence of porcine pepsin A (Tang *et al*., 1973). Similar to the situation for the calf preprochymosin, the cDNA encodes a 16 amino acid residue signal peptide (pre-part) that is followed by a 42 amino acid peptide proenzyme region and also 323 amino acids for mature enzyme. The deduced caprine prochymosin sequence contained three disulphide bond linking Cys^{45} to Cys^{50}, Cys^{206} to Cys^{210}, and Cys^{250} to Cys^{283} (Foltmann *et al*., 1977). The first two cysteine residues are located in the N-terminal domain of the chymosin molecule and the others in the C-terminal domain.

In addition, two aspartic residues, Asp^{32} and Asp^{215} that work as catalytic residues in aspartic proteinase are present in goat chymosin.

Phylogenetic tree and also percentage identities, prepared using the DNAstar software, showed the closest identity between the sequences of nucleotide and amino acid of goat obtained in this study and those of reported by Vega-Hernandez *et al*. (2004) (AY389343). They showed 99.7 and 99.5% similarities in nucleotide and amino acid sequence, respectively. This is followed by sheep (98.9 and 98.7%) and cattle (95.1 and 94%).

The sequence of prochymosin reported in the current study differs from those reported by Vega-Hernandez *et al*. (2004) in three codons at positions 11p (CCT), 19p (CAT) and 139 (AGT) instead of CCG, CGT and AAC, respectively. The two last substitutions alter the codons Arg and Asn to His and Ser, respectively.

Discussion

Chymosin is an aspartic proteinase that is synthesized in the stomach of neonatal ruminants. This enzyme possesses a very

high milk clotting activity with low proteolytic activity and this makes chymosin practically suitable for the manufacture of cheese (Chitpinityol and Crabbe, 1998; Mohanty *et al*., 1999).

World shortage of natural rennet due to increased demand for cheese production has intensified the attempts to produce recombinant calf chymosin in microorganisms (Emtage *et al*., 1983; Hidaka *et al*., 1986; Rao *et al*., 1998; Mohanty *et al*., 1999).

For calf chymosin three iso-enzymes A, B and C have been identified. Chymosin A and B, differ at only one amino acid residue, but chymosin A has higher specific activity and different technological properties; hence, the isoenzyme with higher enzymatic activities should be chosen for production of recombinant prochymosin. Different isoenzymes for goat prochymosin have not been reported. The deduced amino acid sequence revealed in this study differed in some residues from those reported by Vega-Hernandez *et al*. (2004), namely the substitutions of Arg-19p (strongly basic residue) with histidine (slightly basic residue) and Asn-139 with serine. Since amino acid substitution in prochymosin might cause a major alteration in its enzymatic and other technological properties (Chitpinityol *et al*., 1998; Chitpinityol and Crabbe, 1998; Richter *et al*., 1998), the effects of substitutions, found in the current study, on the enzymatic and other properties of caprine prochymosin require further investigation.

On the basis of an amino acid sequence alignment of aspartic proteinase zymogens, it has been suggested that a basic residue at position 36p, Lys in most cases (porcine pepsinogen numbering), is essential for the correct folding and subsequent processing of the zymogen molecule; but despite to the other mammals, in sheep (classified into the caprine subfamily) the residue Lys^{36} is substituted with Glu (Francky *et al*., 2001) and the present study showed the same substitution for caprine prochymosin.

Variation in bovine prochymosin gene sequence has been reported by other investigators (Pungercar *et al*., 1991). Genetic polymorphism can be considered as one of the reasons for these variations (Donnelly *et al*., 1986; Hallerman *et al*., 1988), although using different DNA polymerases to construct of cDNA could insert errors in the sequence as we obtained errors in using *Taq* DNA polymerase with the same sample amplified by *pfu*.

The cDNA obtained in this study will be used for expression of prochymosin protein in *E. coli* BL21 (DE3).

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