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EXPRESSION AND CHARACTERIZATION OF BOVINE CHYMOSIN IN *PICHIA (KOMAGATAELLA) PASTORIS*

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ABSTRACT

Bovine chymosin (EC 3.4.23.4), which belongs to a family of aspartic proteases, is mainly derived from mammalian gastric mucosal cells of the abomasum of unweaned calves. Chymosin contains two residues of Asp in an active site, which catalyze the selective cleavage of a peptide bond in κ-casein between Phe^{105} and Met^{106}. This catalytic process yields insoluble *para-κ*-casein, which causes milk coagulation. In this study, we sought to identify an alternative milk coagulant that is safe and efficient and, at the same time, can produce cheese with a good taste. Bovine prochymosin B was chosen and constitutively expressed at a high level in *Pichia pastoris*. The recombinant chymosin protein was expressed as a secretory form and was found to exhibit milk-clotting activity. It was stable at 25–50°C and had optimal activity at 37°C and pH 6.27. The activity of the recombinant chymosin was activated by cations such as Ca^{2+}, Mg^{2+}, Fe^{3+}, Cd^{2+}, Co^{2+}, and Mn^{2+}, but inhibited by Ni^{2+}, and was not affected by Na^{+}, K^{+}, Cs^{2+}, and Li^{+}. These results suggest that recombinant bovine chymosin is an acid milk coagulant, and that it could be a safe and efficient enzyme suitable for use in cheese production.

Keywords: bovine, chymosin, casein, milk coagulation, *Pichia pastoris.*

INTRODUCTION

Rennet is a complex of enzymes consisting of two endopeptidases: pepsin and chymosin, which is used in the production of cheese products. It is mainly derived from mammalian gastric mucosal cells in abomassum which are taken from unweaned calves. Its key component, chymosin (EC 3.4.23.4), belongs to a family of aspartic proteases [1]. Their three-dimensional conformation, where the active site is located between both lobules, confers on them the ability to act against the Phe^{105}-Met^{106} peptide bond present in milk *k*-casein molecules, originating insoluble *para-κ*-casein, which finally leads to milk coagulation [2]. Bovine chymosin is widely used for cheese production in the dairy industry. Traditional bovine chymosin production, however, requires the slaughter of a large numbers of calves; which keeps the price of chymosin high and also raises ethical issues [1]. Isolation and purification of active chymosin from bovine abomassum is time consuming and technically complex, involving affinity and ion exchange chromatography. Low purity (only 4-8% active chymosin) and contamination of the rennet with potential infectious agents also can be a concern. Therefore, the production of recombinant bovine chymosin is an attractive alternative method for obtaining the enzyme as the consumption of cheese increases globally. Currently, recombinant bovine chymosin, produced in yeast [3],*Escherichia coli* [4] and fungal cells [5], has been extensively used by cheese manufactures in many countries. The *Pichia(Komagataella)*pastoris expression platform has enabled a wide range of protein products to enter the market for therapeutic and industrial uses [6]. *P. pastoris* also produces only a relatively low level of native secreted proteins, which greatly assists the purification of recombinant protein.

The aim of this study was the production of a recombinant *Pichia pastoris* strain constructed in our laboratory, transformed with a bovine prochymosin sequence and secreting an inducible active chymosin activity to the fermentation medium.

MATERIALS AND METHODS

Strains, vectors, and reagents

For the cloning purposes Pfu and Taq DNA-polymerases, *E. coli* DH5α cells were obtained from laboratory stocks, all restriction enzymes, ligase, were purchased from Thermo Scientific (Fermentas, USA). Intermediate cloning TA vector pGEM-t was purchased from Promega (Wisconsin, USA). The yeast expression vectors pGAPZαA and pPICZαA and the yeast *P. pastoris* GS115 were obtained from Life Technologies, Inc. (Grand Island, NY, USA). Oligonucleotides synthesized at the National Center for Biotechnology (Astana, KZ) on the ASM-800 automatic synthesizer using the phosphoamidite method, followed by ammonolysis and purification in PAGE. Dry skim milk powder was purchased from Bio-Rad (USA). Commercial bovine chymosin («Dr.Guber», Russia) was used as a standard for determining of milk clotting activity, SDS-PAGE and Western blot controls. Anti-chymB antibodies were obtained by using immunized rabbit. All other chemicals used during work process were purchased from Sigma-Aldrich, AppliChem, Promega, and Amresco with quality of molecular biology grade.

Cloning of bovine prochymosin inintegral vectors and its expression in P.pastoris

Full length, bovine prochymosin DNA (1098 bp) (Genbank accession no. j00003.1) was synthesized *de novo* by PCR-based accurate synthesis of long DNA sequences [7]. Gene was cloned into the TA cloning vector, pGEM-T, and sequenced by Sanger method using «BigDye Terminanor v 3.1 Cycle sequencing Kit» with pUC/M13 forward and reverse primers to observe the correct assembling and any mutations in gene sequence. Mutations were eliminated by Quick Change protocol [8].Prochymosin gene *prochymB* was cloned into the *P.pastoris* expression vectors pPICZαA and pGAPZαA by EcoRI and NotI restriction sites. *P.pastoris GS115* cells were transformed according to the manufacturer's protocol (Life Technologies, Grand Island, NY, USA). Tansformants were selected on zeocin (200 μg/mL) YPD-agar (1% yeast extract, 2% peptone, 2% glucose) with additional screening of 15 colonies from each plate were analyzed for insertion by PCR and using milk-clotting activity enzyme assay. Clones with the highest clotting activity was selected and cells were inoculated in 10 ml of YPD medium with 200 μg/mL of zeocin and cultured for 24h, on the next day cells were collected by centrifugation at 2000×g for 15 minutes at +4°C and growth media was changed to BMGY or BMMY and were grown for 5 days. Every 24h cell-free supernatant was collected and stored on ice. Then cell-free supernatants were used for analysis in the enzymatic assay and SDS-PAGE [9,10] and Western Blot.

SDS-PAGE and Western Blotting

Polyacrylamide gel electrophoresis was accomplished according to the method of Laemmli [11] in a Mini-PROTEAN tetra cell (Bio-Rad). The anti-chymB polyclonal antibody was raised against the full-length recombinant His-tagged *Bostaurus*preprochymosin B protein. About 1 mg of the purified recombinant preprochymosin B was mixed with Freund's complete adjuvant and injected into rabbit. Three additional injections were made at 2-week intervals. One week after the last injection, the blood was collected and the immune serum was purified by precipitation with 33% ammonium sulphate. The purified rabbit anti-ARP polyclonal antibody was used as a primary antibody, and horseradish peroxidase-conjugated goat anti-rabbit IgG, as a secondary antibody. Cultural media proteins were separated in a 10% SDS-polyacrylamide gel and then electroblotted onto a polyvinyl difluoride membrane (Pierce) using a BioRad Mini-transblot Cell according to the manufacturer's instructions. After the protein transfer, the membrane was gently shook in blocking solution containing 5% milk and 0.1% Tween-20 in 1×TBS (Tris-buffered saline: 50 mMTris–HCl (pH 7.5), 20 mMNaCl) for 1 h at room temperature. After removing the blocking solution, the membrane was incubated in 10 mL of the affinity-purified anti-chymB polyclonal antibody (1:10,000 dilutionin the blocking solution with 0.1% Tween-20) overnight at 4°C. The membrane was washed five times in 10 mL of wash buffer (1×TBS with 0.1% Tween-20), for 5 min each time. After washing, the membrane was incubated in 10 mL of the secondary antibody (1:20,000 dilution in the blocking solution) for 1 h at room temperature. Then the membrane was washed five times in 10 mL of wash buffer, for 5 min each time. The working substrate solution was prepared by mixing an equal volume of peroxide solution and luminal/enhancer solution and used at 0.1 mL•cm^2 of the blot area. The membrane was incubated in the working solution for 2 min in darkness and exposed to Agfa CP-BU x-ray film [12].

Enzymatic assay

Enzymatic activity of chymosin was assessed using the acidified to pH 4.0 growth media, which supposed to autocatalytic hydrolysis of "pro" 42 amino acids from N-terminal region of prochymosin. Substrate was prepared by mixing skim milk powder (12% w/v) in potassium phosphate buffer (pH 6.4). For screening purposes, 200 μL of 12% milk solution in potassium phosphate buffer supplemented with 40 mM $CaCl_2$ was mixed with 50 μL of supernatant, and incubated at room temperature. Clotting time (s) was determined when the first flocculation was observed in the substrate film on the wall of the test

tube[3].Finally, 4 mL of supernatant containing active chymosin was employed to elaborate milk curds using 1 L of pasteurized low-fat cow's milk.

One unit (SU) of enzymatic activity was defined as the amount of enzyme needed to clot 1 ml of skim milk in 40 min at 37°C. This method was also used to determine the enzymatic activity in supernatants at different pH values, the optimal substrate pH, temperature, and Ca^{2+} concentration.

Determination of optimal substrate pH, temperature for enzymatic reaction and enzyme thermal stability, effect of calcium and other metals on enzyme activity

To determine the optimal substrate pH, skim milk powder was resuspended in Milli-Q water with 40 m$MCaCl_2$, pH values was regulated with 1M citric acid or 0.1M NaOH in range of pH from 6.18 to 6.75 (6.18; 6.23; 6.27; 6.33; 6.38; 6.43; 6.48; 6.54; 6.60; 6.71; 6.75).

To determine optimal temperature for enzymatic reaction, 1 ml of substrate (12%-milk solution in 25 mM potassium-phosphate buffer with pH 6.27, and 40 m$MCaCl_2$) was preheated at temperature from 25°C to 60°C for 10 minutes, and then enzyme in amount of 20 µl was added, mixed well avoiding foam formation and kept at appropriate temperature. Thermal stability was determined by incubation the cultural media with expressed enzyme within a temperature range 25°-65°C for 1h. At the same time enzyme solution was incubated at 4°C, 25°C and 37°C for 24h and then incubated within a temperature range 25°-65°C for 1h with recording a milk clotting time (s).

To study the effect of calcium and different metals on enzyme activity skim milk solution (12% w/v milk powder resuspendedin 25 mM potassium phosphate buffer, pH 6.27) was supplemented with 0-90 mM $CaCl_2$, preheated for 3 minutes at 37°C. The enzyme solution was applied in amount of 20 µl, reaction was mixed well and a milk clotting time (s) was recorded. To determine effect of other metals 10 mM (KCl, NaCl, $MgCl_2$, CsCl, $FeCl_3$, $CdCl_2$, LiCl, $CoCl_2$, $MnCl_2$, $NiCl_2$) metals was added instead of calcium.

RESULTS AND DISCUSSION

The full-length bovineprochymosin gene was successfully synthesized, all mutations was eliminated by quick change PCR. The list of used oligonucleotides is presented in supplementary materials A. Gene was cloned into the yeast expression vectors, pPICZαA and pGAPZαA, under the control of the AOX1 and GAP promoters, to derive the expression vectors pPICZαA/ProchymB and pGAPZαA/ProchymB. Vectors were transformed in *Pichia pastoris* GS115, and clones were selected. In vivo, chymosin is synthesized as preprochymosin, which has a 16-amino acid lead peptide that is subsequently removed to produce the prochymosin. Under the acidic condition of the stomach, prochymosin is then converted to the mature enzyme, chymosin, by removal of the N-terminal 42-amino acid propeptide[13]. In order to obtain active bovine chymosin, we constructed recombinant plasmids contained only the sequence coding for the pro enzyme. The recombinant expression vectors pPICZαA/ProchymB and pGAPZαA/ProchymB were linearized with the restriction enzymes PagI and BspHI, respectively, and transformed into *P. pastoris* strain GS115 to express the target protein. After centrifugation, the culture supernatant was analyzed for milk-clotting activity. The molecular mass of bovine prochymosin predicted from the DNA sequence was 41 kDa, whereas the molecular mass of the mature enzyme was 35.6 kDa.

The milkclotting activity of the bovine chymosin was determined to be 540 SU/ml. Clotting activity of chymosin was strongly affected by temperature, with optimum activity occurred at 37°C, although the enzyme was stable at temperature ranging from 25°C to50°C. At temperature above 60°C, bovine chymosin was completely inactivated after 60 min. The activity of bovine chymosin was highly-dependent on pH, with optimum activity at pH 6.27.

Bovine chymosin was thus similar to buffalo and goat chymosins by having optimum activity at acidic pH, a property that would make the enzyme suitable for use in the production of cheese.

The effect of various metal ions on the milk-clotting activity of bovine chymosin was also evaluated. The activity of the enzyme was three times suppressed by 10 mM Ni^{2+}, but was not affected by Na^{+}, K^{+}, Cs^{2+}, Li^{+}. The interesting thing is that the enzyme activity was increased in the presence of, except Ca^{2+}, ions of Mg^{2+}, Fe^{3+}, Cd^{2+}, Co^{2+}, and Mn^{2+}. The process of milk coagulation carried out by chymosin is divided into two stages. In the first stage, k-casein is hydrolyzed into para casein. In the second stage, Ca^{2+}can combines with para casein to form a firm clot. The maximum milk clotting activity of bovine chymosin could be obtained at a CaCl2 concentration of 40 mM.

The enzymatic characteristic showed that this recombinant chymosin was similar to native calf chymosin in terms of optimum conditions; 37°C, pH 6.27 and 40 mM CaCl2. The enzyme was stable at temperature ranging from 25 to 50°C. The recombinant bovine chymosin described in this study exhibited satisfactory level of activity in k-casein hydrolysis. This study may provide an alternative strategy valuable for the preparation of milk coagulant suitable for the cheese making processing.

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Supplementary materials A

Table 1. Oligonucleotides list for bovine preprochymosin B synthesis

Name	Sequence 5'-3'
ChymF1	atgagatgtttggtggttttgttggctgttttcgctttgtctcaaggtgctgaaattactaggattccattgtacaaggg
ChymR2	tgcttttgcaagaaatcttccaacaaaccatgttccttcaaagcctttctcaaagacttacccttgtacaatggaatcct
ChymF3	gaagatttcttgcaaaagcaacaatacggtatttcttctaagtactctggttttggtgaagttgcttctgttccattgac
ChymR4	cccaagtaaatcttaccaaagtattgagaatccaagtagttagtcaatggaacagaagcaac
ChymF5	tttggtaagatttacttgggtactccaccacaagaatttactgttttgtttgatactggttcttctgatttctgggttcc
ChymR6	gactttcttggatcaaatctttgatggttcttacaagcgttagacttacagtaaatagatggaacccagaaatcagaaga
ChymF7	agatttgatccaagaaagtcttctacttttcaaaacttgggtaagccattgtctattcattacggtactggttctatgca
ChymR8	acagtttgttgaatatcaacaatgttagaaacagtaacagtatcgtaacccaaaataccttgcatagaaccagtaccgta
ChymF9	gttgatattcaacaaactgttggtttgtctactcaagaaccaggtgatgttttcacttacgctgaatttgatggtatttt
ChymR10	atcatgttatcgaaaactggaatagagtattcagaagccaaagatgggtaagccatacccaaaataccatcaaattcagc
ChymF11	ccagttttcgataacatgatgaacagacatttggttgctcaagatttgttttctgtttacatggatagaaacggtcaaga
ChymR12	acccaatgcaaagaaccagtgtagtaagatgggttaatagcacccaaagtcaacatagattcttgaccgtttctatccat
ChymF13	actggttctttgcattgggttccagttactgttcaacaatactggcaatttactgttgattctgttactatttctggtgt
ChymR14	accaacttagaagtaccagtatccaaaatagcttgacaaccaccttcacaagcaacaacaacaccagaaatagtaacaga
ChymF15	actggtacttctaagttggttggtccatcttctgatattttgaacattcaacaagctattggtgctactcaaaaccaata
ChymR16	atttcaaaaacaacagttggcatgtaagacaagttatcacaatcaatatcaaattcaccgtattggttttgagtagcacc
ChymF17	ccaactgttgtttttgaaattaacggtaagatgtacccattgactccatctgcttacacttctcaagatcaaggtttttg
ChymR18	ataaaaacatcacccaaaatccacttttgagaatggttttcagattgaaaaccagaagtacaaaaaccttgatcttgaga
ChymF19	attttgggtgatgtttttattagagaatactactctgtttttgatagagctaacaacttggttggtttggctaaggctatttaa
Chymfw1	atgagatgtttggtggttttgttggctgttttcgctttgt
Chymrv1	atcatgttatcgaaaactggaatagagtattcagaagcca
Chymfw2	ccagttttcgataacatgatgaacagacatttggttgctc
Chymrv2	ttaaatagccttagccaaaccaaccaagttgttagctcta
ChymF1fw1	atgagatgtttggtggttttgt
ChymF1rv1	cccaagtaaatcttaccaaagt
ChymF1rv2	atcatgttatcgaaaactggaat
ChymF2fw2	tttggtaagatttacttgggtac
ChymF2rv1	acccaatgcaaagaaccagtgtag

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БҰЗАУ ПРОХИМОЗИНДЫ *PICHIA (KOMAGATAELLA) PASTORIS* ЖАСУШАЛАРЫНДА ЭКСПРЕССИЯСЫ ЖӘНЕ СИПАТТАМАСЫ

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ТҮЙІН

Бұзау химозині (EC 3.4.23.4) аспарагинді проеазалар тобына жатады, химозин жаңа туған бұзаулар асқазанының ұлтабар бөлімінің шырышты жасушаларынан алынады. Химозиннің белсенді сайтындағы екі Asp к-казеин молекуласындағы Phe^{105} және Met^{106} арасындағы таңдамалы пептидті байланысын ыдыратады, бұл өз кезегінде сүт коагуляциясына әкелетін, ерімейтін *пара-к*-казеинді тудырады. Берілген жұмыста, тиімді және қауіпсіз сүт коагулянтының баламасын алу үшін және дәмі жақсы ірімшік жасай алатын, бұзау В прохимозині таңдалып, жоғары деңгейде *Pichia pastoris* жасушаларында конститутивті түрде өндірілді. Рекомбинантты химозин секреторлы күйде экспрессияланып, сүт-ұйытқыш қасиетін көрсетті. Химозин 25-50°С тұрақтылық көрсетіп, 37°С және pH 6.27 көрсеткіштерінде оңтайлы белсендікке ие болды. Рекомбинантты химозин белсендігі Ca^{2+}, Mg^{2+}, Fe^{3+}, Cd^{2+}, Co^{2+}катиондарымен белсендірілді, бірақ Ni^{2+} керісінше баяулатты, Na^{+}, K^{+}, Cs^{2+}, Li^{+}еш әсер етпеді. Алынған нәтижелер рекомбинантты бұзау химозинінің қышқылды

сүт коагулянты екенін көрсетіп, оны ірімшік өндірісі үшін тиімді және қауіпсіз фермент ретінде қарастыруға мүмкіндік берді.

Негізгі сөздер: Бұзау, химозин, казеин, сүт коагуляциясы, *Pichia pastoris.*