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Heterologous Expression of Bovine Prochymosin in *Pichia pastoris* GS115

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ABSTRACT

Background: Aqueous extract of dried fourth stomach of unweaned bovine contains prochymosin, that subsequently is converted to active chymosin, a milk-coagulating enzyme in the cheese industry. Recombinant chymosin has been found to be a suitable replacement for natural bovine chymosin. Meanwhile it possesses several advantages to other plant, fungal and bacterial milk-clotting enzymes.
Objectives: In present research we evaluate the expression of this critical enzyme in a eukaryotic system for future use in cheese industry.
Materials and Methods: We have cloned bovine prochymosin gene in methylotrophic yeast, *P. pastoris,* using pPIC9K as an expression vector. The recombinant plasmid was transformed into the host by electroporation, and it was expressed in optimum conditions (temperature 29ºC, 200 rpm, 2% methanol for induction, and 5 days of incubation). Transcription and expression of the recombinant prochymosin was evaluated by the reverse transcription polymerase chain reaction (RT-PCR), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis as well as western blotting and enzyme-linked immunosorbent assay (ELISA).
Results: In optimum conditions, only a low level of this heterologous protein was detected using ELISA method and subsequently confirmed by RT-PCR.
Conclusions: Since it has been reported that *P. pastoris* is an appropriate host for the expression of recombinant proteins, a low level of expression of prochymosin in this host should be explored in our future research.
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▸ *Implication for health policy/practice/research/medical education:*
Eukaryotic expression of an important enzyme used in dairy industry.

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1. Background

Chymosin is an aspartic proteinase (EC 3.4.23.4) that is responsible for the coagulation of milk in the fourth stomach of unweaned calves (1). This enzyme (35.6 kDa) is secreted by the cells of the gastric mucosa as an inactive precursor, known as prochymosin (40.8 kDa). In the acidic conditions of the lumen, it is subsequently converted to chymosin, by autocatalytic cleavage of the 42-amino

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acid N-terminal prosequence (2). Chymosin is used extensively in cheese production because of specific cleavage of κ-casein, at the Phe105 – Met106 bond. Due to a shortage of calf stomachs, it is possible to clone the gene for calf chymosin in appropriate vectors and express them in different hosts including *E. coli*, (Chy-Max, Pfizer, Milwaukee, USA), Kluyveromyces lactis (Maxiren, Gist-Brocades, Delft, Holland) and *Aspergillus niger* var. awamori (Chymogen, Genencor, Palo Alto, CA, USA) (3). The methylotrophic yeast Pichia pastoris is suitable for the expression of eukaryotic proteins. *P. pastoris* has numerous advantages of higher eukaryotic expression systems, but the ease of its genetic manipulation is similar to *E. coli* and *S. cerevisiae*. The other advantage of *P. pastoris* is that the ultrahigh cell densities are easily achieved at minimal costs (4, 5). The *P. pastoris* expression system has been used successfully for the production of different recombinant heterologous proteins, such as A. *awamori* glucoamylase, *Aspergillus oryzae Tannase*, *Rhizopus oryzae lipase, E. coli* L-galactosidase, dengue virus structural protein, human granulocyte-colony stimulating factor and human eosinophil peroxidase (6, 7). In most cases, the alcohol oxidase I promoter (PAOXI) was used for the expression of heterologous genes, and vectors integrate into the Pichia genome. PAOXI is completely repressed when cells have used glucose as carbon source, and induced in the presence of methanol (4).

2. Objectives

Ahmadian et al. (1) have shown that the expression of recombinant prochymosin in *E.coli* system was acceptable, but the formation of inclusion bodies and the necessity for refolding and activation of the produced enzyme, caused to evaluate the expression of this important enzyme in a eukaryotic system for meet the dairy industry's needs.

3. Materials and Methods

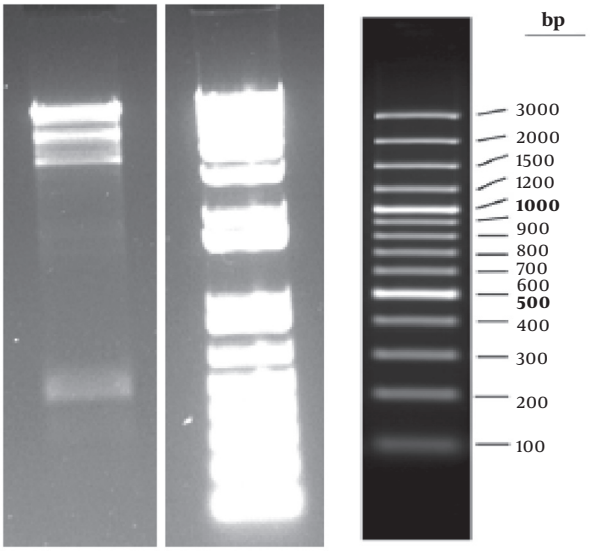
In the present study, the bovine prochymosin gene, was cloned in pPIC9K, at *NotI-SnaBI* site, under the control of alcohol oxidase I promoter (PAOXI) and with Saccharomyces cerevisiae alpha factors (α-MF) to secrete proteins into the medium. The recombinant plasmid (pPIC9K/Prochymosin) was transformed into *E. coli* strain TOP10 for amplification of plasmid. Approximately 10 μg of recombinant expression plasmid was linearized with SacI. Transformation of *P. pastoris* strain GS115 was made using electroporation following manufacturer's recommendations (Invitrogen) by a Gene Pulser (Bio-Rad) using 80 μg of competent cells. The recombinant GS115 strain was cultured on Yeast Extract Peptone Dextrose (YPD) Medium plus 2% agar. The strain GS115 has a mutation in the histidinol dehydrogenase gene (his4) that prevents it from synthesizing histidine. The expression plasmid carries the HIS4 gene that complements his4 in this host, so the transformed cells are selected for their ability to grow on a histidine-deficient medium (Minimal Dextrose or MD: 1.34% yeast nitrogen base without amino acids, 4×10^{-5} % biotin, 2% dextrose and 2% agar) by incubation at 28 - 30°C for 4 days. PCR amplification was used to verify the prochymosin gene integration into the AOX1 locus in the chromosome of the transformed *P. pastoris*. For PCR amplification, the reactions were carried out in a GeneAmp PCR system 9700 (PE Applied Biosystems) with conditions of denaturation at 94° C for 3 min; 30 cycles for amplification (at 94°C / 60 sec, 53°C / 60 sec, and 72°C / 150 sec); and the final extension at 72°C for 300 sec. The reaction mixture contained 0.2 mM of each primers (F primer 5′-AOX1: 5´-GACTGGTTCCAATTGACAAGC-3´; and R primer 3′-AOX1: 5′-GCAAATGGCATTCTGACATCC-3´), 2 mM $MgCl_2$, 2.5 U of PFU DNA polymerase (Fermentas) and extracted genome of recombinant pichia strains as a template. Gene insertion events at the AOX1 (GS115) loci arise from a single crossover event between the loci and any of the three AOX1 regions on the vector. These events result in the insertion of one or more copies of the vector upstream or downstream of the AOX1. The phenotype of such a transformant is His^+ Mut^+ that can utilize methanol quickly. For confirmation of gene insertion, two different PCR products from two clones were selected and their nucleotide sequences were determined (MWG, Germany). The pPIC9K vector contains bacterial kanamycin gene that confers resistance to geneticin in *P. pastoris*. Because of the genetic linkage between the kanamycin gene and the "expression cassette", a single copy of pPIC9K integrated into the Pichia genome confers ~ 0.25 $mg.mL^{-1}$ resistance to geneticin. Multiple integrated copies of pPIC9K can increase the geneticin resistance level from 0.5 $mg.mL^{-1}$ (1 - 2 copies) up to 4 $mg.mL^{-1}$ (7 - 12 copies). Protein expression may upsurge as a result of the gene dosage effect; it involves growing colonies in microtiter plates until all colonies reach to the same density. The colonies were then spotted on the YPD-geneticin plates (in different concentrations of geneticin: 0.25, 0.5, 1.0, and 2.0 $mg.mL^{-1}$) and scored for geneticin resistancy. For expression of the recombinant protein, selected colonies were inoculated into 10 mL Buffered Minimal Glycerol-complex (BMGY) Medium (1% (w/v) yeast extract, 2% (w/v) peptone, 0.1M phosphate buffer (pH 6.0), 1.34% yeast nitrogen base, 4×10^{-5} % biotin and 1% (v/v) glycerol), and were incubated at 28°C in a shaker incubator at 180 rpm until it reached A600 of 2-6 as per the manufacturer's recommendation. The cells were harvested by centrifugation and resuspended in 50 mL of Buffered Minimal Methanol-complex (BMMY) Medium (the same as BMGY except that glycerol was replaced by 2% v/v Methanol), to A600 of 1.0 in a 250 mL conical flask. Incubation was continued at 29°C in a shaker incubator at 200 rpm with the addition of methanol every 24 hours to achieve a concentration of 2% to sustain the induction for 5 days (based on our optimization experiments). Samples with high copy numbers of

the prochymosin gene were analyzed for transcription of the prochymosin gene. RNA was extracted from the disrupted cells according to the RNAfast protocol following the manufacturer's recommendation. Isolated RNA was used as the template in reverse transcription reaction. First strand cDNA synthesis was performed using Revert AidTM First Strand cDNA Synthesis Kit (Fermentase). The cDNA produced was used as a template for amplification by PCR. After every induction, recombinant proteins secreted into the medium, were precipitated using ammonium sulphate and 80% TCA solutions. Protein samples were dialyzed against a solution of 100mM Tris-Hcl pH 8.0 for removing the salts. The solution was loaded onto Ni-NTA column for purifying 6XHis-tagged proteins. The protein samples were separated by electrophoresis on a 12% SDS-PAGE gel. The expression of prochymosin was determined using a monoclonal anti-6Xhis-tag antibody (Serotec, USA) as a first antibody (2 $\mu g.mL^{-1}$) and a 1:2000 dilution of polyclonal rabbit anti-mouse immunoglobulin/HRP (Roche, Germany) as a second through western blotting. Finally, protein samples were visualized using DAB/H_2O_2 chromogen-substrate solution. Expression of the recombinant prochymosin was also confirmed by Enzyme Linked Immunosorbent Assay (ELISA) using anti-prochymosin polyclonal antibody as a first antibody (of 1/500 in PBS) and a polyclonal rabbit anti-mouse immunoglobulin/HRP (diluted 1:2000 in PBS) as a second. Finally, protein samples were visualized using freshly prepared chromogen-substrate mixture (ABTS/H_2O_2). The positive results are shown as green color, but we used ELISA-reader (for reading absorption in 405 nm) for quantitative evaluation.

4. Results

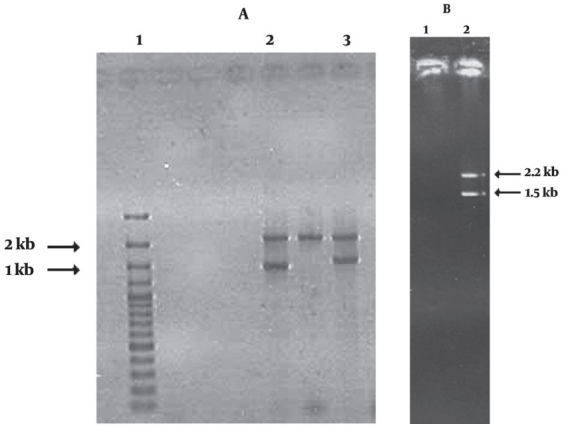
To confirm the transformation of recombinant plasmid (pPIC9K/ the approximately 1100 bp fragment of bovine prochymosin gene which fused to six histidine tag at its 3' end) in *E. coli* strain TOP10, we have used digest check as described in Figure 1. In results of PCR experiments, two expected bands (a 1.5 kb and another 2.2 kb) were detected (Figure 2A). The results of sequencing showed that recombinant prochymosin is the same as Bos taurus chymosin precursor (mRNA, complete cds) present in the gene bank (Accession number: FJ768675.1), and also the one which was previously cloned and sequenced by Ahmadian et al. (1). In our studies only a few high-geneticin resistant colonies were observed (colonies number 3, 6, 7, 8, 14, and 24), which were smaller in sizes than low-geneticin resistant colonies but their morphology were similar. Colony number 6, which has grown on 2 $mg.mL^{-1}$ geneticin, was selected for expression of recombinant protein in a shake flask; and colony number 10 for comparison.

Figure 1. The Presence of Target Gene Was Checked by Digesting of Plasmids with EcoRI and NotI



As EcoRI has a restriction site at nucleotide number 478 in prochymosin gene, and NotI at 3' end of the gene; cleavage of recombinant plasmid with these two enzymes produced a 550 bp fragment.

Figure 2. The Integration of Target Gene into the Genome of GS115 Was Determined by PCR and RT-PCR Amplification



A) PCR amplification: Lane 1 contains a 1 kb ladder. Lane 2 shows the wild-type AOX1 gene from GS115. Lane 3 shows the native AOX1 gene of yeast genome (2.2 kb) and a 1.5 kb product containing the gene of interest (prochymosin, 1.1 kb) flanked by AOX1 sequences (from GS115 or pPIC9K); B) RT-PCR analysis: Lane1 shows PCR of extracted mRNA without reverse transcription with specific primers (negative control). Lane2 shows PCR of Prochymosin cDNA that has been made from prochymosin mRNA with specific primers (the result is same as part A).

The expression of precipitated 40 kDa protein (prochymosin) from the supernatant of growing medium of the colony number 6 was detected only with ELISA analysis as a green color with A405 = 0.051 with an average of three repeats. The low level of expression was below the limit of detection sensitivity of Western blotting. The colony number 10 didn't show any detectable expression of target protein. The result of RT-PCR analysis was same as the PCR amplification of target gene as described previously (2 bands), and confirms the result of ELISA analysis (Figure 2B).

5. Discussion

Hence, in optimum conditions for the expression of prochymosin gene in *P. pastoris*, only a low level of recombinant protein was detected using ELISA method and subsequently confirmed by RT-PCR. Although it has been reported that *P. pastoris* represents an appropriate host for the expression of recombinant proteins, the low level of expression in this host could be attributed to several factors. These potential impediments include, the lack of consistency of codon usage of the bovine and pichia (8), copy number of the gene (9), the efficiency and strength of promoters (10), efficiency of translation signals (11) and signal peptides (12), processing and folding in the endoplasmic reticulum and Golgi apparatus (13), environmental factors of expression (14), extracellular secretion (15), and protein turnover by proteolysis (16, 17). As the recombinant, colony number 6 is high geneticin resistant (which grown on 2 mg/mL of this Antibiotic), the copy number of the gene was not restrictive for recombinant protein production. We have used some protease inhibitors such as EDTA (5 mM) to reduce proteolysis in production process. Therefore, it remains to be determined which of these factors resulted in the enhancement of prochymosin production existing in the machinery of *P. pastoris*. The fermentation can be easily scaled up to achieve greater level of expression. Meanwhile, the parameters influencing the growth and protein productivity of *P. pastoris* (such as pH, aeration, carbon and nitrogen source feed rate) can be controlled. According to some reports, the expression level could be increased by growing the recombinant Pichia in a fermentor, as the higher cell density could improve the production rate of prochymosin. In the fermentor, 3 - 5 times higher transcription levels can be obtained as a consequence of the controlled methanol concentration (18). Additionally, it is shown that codon optimization of prochymosin gene can further improve the expression level (8, 19).

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Authors' Contribution

Gholamreza Ahmadian was corresponding author. Sara Sadr Mohammad Beigi wrote the manuscript and is guarantor. Sara Sadr Mohammad Beigi and Fatemeh Ramezani equal contributed to the development of the protocol, abstracted data, and repaired the manuscript. Soheila Ghandili and Mohammadreza Soudi were technical assistant and advisor respectively.

Financial Disclosure

We have no financial interests related to the material in the manuscript.

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