Prochymosin expression in *Bacillus subtilis*

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1. SUMMARY

Prochymosin (PC) sequence was cloned in *Bacillus subtilis* using two kinds of plasmid constructions. In plasmid pSM316 the cDNA was inserted to obtain the intracellular expression of the enzyme. The enzyme turned out to be expressed in an insoluble form which could be converted to native enzyme under proper denaturing and refolding conditions. The levels of intracellular expression of PC were further enhanced by modifying the 5' region of the gene in a way that a two-cistron expression system was created. For the PC secretion, the cDNA was fused to the subtilisin leader sequence and expressed under the control of the *B. subtilis* neutral protease promoter. A properly folded PC was secreted by the cells, although to low levels.

2. INTRODUCTION

Chymosin is a protein of great commercial interest for its use in the alimentary industry as the milk-clotting agent for cheese production. Its decreasing availability from natural sources attracted the attention of the biotechnology industry to attempt the expression of this eukaryotic gene in various microorganisms.

The active form of the enzyme derives in nature from the autocatalytic processing of PC, the inactive precursor which has an extension of 42 amino acids at its N-terminus. The processing of PC can be obtained in vitro by incubating the precursor in solution at pH 4.5, or with a shorter incubation at pH 2, which results in the removal of part of the N-terminal extension and in the formation of enzymatically active pseudochymosin [1,2].

PC has been obtained intracellularly in *Escherichia coli* in large amounts (about 5% of the total proteins) [3] where it accumulates in the insoluble protein fraction, as cytoplasmic inclusion bodies [4]. Such aggregates however can be easily purified from the disrupted cells, solubilized with strong denaturing agents and a good portion of the protein can be recovered in an activatable form by careful refolding of the polypeptide. Others have obtained intracellular expression of PC in yeast [5]. In all cases PC accumulates in prokaryotes as insoluble aggregate, and the reasons for this have not been identified [6]. Secretion of the enzyme in the culture medium represents an attractive alternative to intracellular production;
even moderate levels of expression from suitable microorganisms can in fact be commercially interesting if the solubilization/refolding procedure can be avoided. Recently secretion of PC has been reported from fungi [7], from yeast [8,9] and from L-forms of *Proteus mirabilis* [10].

We have introduced the PC gene into expression vectors designed for the expression of foreign genes in *Bacillus subtilis*, a non-pathogenic Gram-positive microorganism widely used for industrial fermentation processes. We report here both the intracellular and extracellular production of PC by *B. subtilis* as identified by detection with specific antibodies and by its enzymatic activity.

3. MATERIALS AND METHODS

3.1. Bacterial strains and media

For the plasmids construction and PC production the following strains were used: *E. coli* JM101 [11]; *B. subtilis* SMS118 [12]. *E. coli* cells were grown in LB medium (NaCl 10 g l⁻¹, yeast extract 5 g l⁻¹, bacto tryptone 10 g l⁻¹), while VY medium (Veal infusion broth 25 g l⁻¹, yeast extract 5 g l⁻¹) was used for the growth of *B. subtilis*.

3.2. Plasmid construction and transformation

All the enzymes used in plasmid construction were purchased by Boehringer Mannheim and used according to the supplier's instructions. Transformation of *E. coli* strain was performed by high voltage electroporation using a Bio-Rad gene pulser apparatus [13]. Transformation of *B. subtilis* strain was carried out as described [14].

3.3. DNA synthesis

The oligodeoxynucleotides were synthesized on a Beckman System 1 Plus DNA Synthesizer using the β-cyanoethyl phosphoramidites chemistry and purified either by HPLC [15] or by urea–PAGE.

3.4. RNA preparation and dot blot analysis

*E. coli* JM101 strain containing the plasmids pSM316, pSM341, pSM342 and pSM343 were used to inoculate flasks containing 10 ml LB with 15 μg ml⁻¹ kanamycin. After overnight growth, these cultures were used to inoculate fresh medium to an initial OD₆₀₀ of 0.05. After reaching an OD₆₀₀ of 0.6–0.8 the cells were harvested and the pellets immediately frozen in a dry ice-ethanol bath. Then the pellets were resuspended in 1 ml of cold 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, containing 500 μg ml⁻¹ of proteinase K and mixed with 1 ml of 0.2% SDS. *B. subtilis* strain SMS118 lysates were obtained as described [16].

0.8 g of CsCl was then added to the lysates which were layered over a 2 ml 5.7 M CsCl cushion in an ultracentrifuge tube and centrifuged for 16 h at 35 000 rpm at 20°C. The pellets, containing RNA, were then suspended in TE and aliquots of each preparation were treated with RQ1 DNase (Promega) according to the supplier's instructions.

For RNA dot blot we used Zeta Probe Membranes by Bio-Rad and the complete PC gene as probe. Nonradioactive labeling of probe and detection of mRNA were performed with Chemi-Probe kit by FMC.

3.5. Proteins extraction and analysis

10-ml cultures cells were grown overnight at 37°C. Cells were then collected by centrifugation, washed with TE (10 mM Tris-HCl, pH 8, 1 mM EDTA), resuspended in 120 μl of 25% sucrose in TE and incubated 45 min at 37°C with 10 μl lysozyme 40 mg ml⁻¹. The suspension was sonicated to homogeneity and the soluble fraction was separated from the insoluble pellet by centrifugation in an Eppendorf centrifuge for 10 min at 4°C. The extracellular proteins were collected by addition of TCA (at a final concentration of 5%) and the precipitate was washed with acetone. The extracts were analyzed with SDS–PAGE [17] and by Western analysis as described by Towbin et al. [18] except for the color reaction which was performed with 4-chloro-1-naphthol.

3.6. Enzymatic assay of chymosin

The isolation of the inclusion bodies and the solubilization of the PC produced by *E. coli* and *B. subtilis* strains were carried out according to Marston et al. [19] except for the cells lysis procedure, which was performed on a French Press apparatus (Aminco, IL, U.S.A.).
The activation of PC and assay of its milk clotting activity were performed as previously described [3].

4. RESULTS

4.1. Construction of recombinant plasmids

Plasmids containing the PC gene cloned in constructs suitable for the intra- and extracellular expression of the enzyme in *B. subtilis* were derived from the ‘shuttle’ vector pSM214 [20] and from pSM308 [21]. pSM214 carries the replication origins for both *E. coli* and *B. subtilis* and places the inserted gene under the control of a modified T5 phage promoter, while pSM308 is able to replicate only in *B. subtilis* and the cloned gene is transcribed from the *B. subtilis* neutral protease promoter [22].

To obtain intracellular synthesis of PC, the bovine gene derived from plasmid pWHA43 [23] was first cloned in a pUC plasmid (pC1 in Fig. 1A), thus facilitating the subsequent constructions, and then inserted in the pSM214 shuttle vector as an EcoRI-HindIII fragment, and the plasmid obtained was named pSM316 (Fig. 1A).

To enhance PC production we have also modified the untranslated region upstream 5′ end of the gene, to create a more efficient ribosome binding site (RBS). The pSM316 SacI-EcoRI fragment containing the RBS region was replaced with synthetic oligodeoxynucleotides to obtain the constructions shown in Fig. 2. pSM341 construction, based on the two-cistron expression system previously described [24], carries she RBS and the first 8 codons of the *E. coli* lpp. The efficient ribosome attachment to the mRNA containing the *lpp* RBS provides for efficient translation initiation. Plasmid pSM342 was constructed by a replacement of the pSM316 Shine-Dalgarno (SD) sequence with the pSM164 one which was demonstrated to increase the h-GH production by *B. subtilis* [25]. The fragment SacI-EcoRI of pSM343 contains instead a slightly modified sequence derived from the parental plasmid pWHA43 linked to the pSM316 SD sequence.

The secretion of the enzyme in both the inactive (PC) and active (chymosin) forms was attempted by linking the corresponding sequences to a synthetic oligonucleotide carrying the subtilisin signal peptide sequence (Fig. 1B), which is recognized and cleaved by a membrane peptidase and is responsible for the secretion of *B. subtilis* subtilisin [26]. The synthetic linkers were appropriately ligated to the pSM308 vector and to the PC and chymosin sequences in plasmids pSM318 and pSM319, respectively (Fig. 1A).

4.2. Intracellular synthesis of prochymosin

The intracellular protein fraction of *B. subtilis* cells transformed with the recombinant plasmid pSM316 (designed to direct intracellular synthesis of PC), was analyzed for presence of the protein by crossreactivity with anti-chymosin antibodies on Western blots. Cells containing pSM316 accumulate a polypeptide which cross-reacts with the specific antibodies and comigrates with PC produced in *E. coli* by the parental plasmid pWHA43 (Fig. 3). This protein is accumulated intracellularly in an insoluble form, as inclusion bodies (data not shown), as it has been shown to happen in *E. coli* [4]. To see if activatable enzyme could be recovered from the *B. subtilis* insoluble protein fraction, the inclusion bodies were isolated and solubilized in urea following the procedure described for the *E. coli*-produced chymosin [19]. Solubilized fractions were activated and tested for activity in a simple microwell milk-clotting assay [3], revealing that the protein accumulated in the inclusion bodies could be recovered in the active enzyme form. Despite the use of the strong modified T5 promoter, pSM316 cells produced relatively scarce amounts of PC both in *E. coli* and *B. subtilis*. We established that this was mostly due to inefficient translation of mRNA on the basis of two observations. First of all, the plasmid constructs designed to increase mRNA translation by RBS substitution directed increased amounts of PC. The most efficient synthesis of the enzyme was obtained (both in *E. coli* and *B. subtilis*) by forced translational coupling in the two-cistron construct pSM341 (Figs. 2–4) with a 10-fold increase relative to pSM316 in both microorganisms. In addition, by Northern blot analysis, similar levels of steady-state PC mRNA were measured from all constructions (data not shown).
Fig. 1. (A) Construction of pSM316, pSM318 and pSM319 plasmids. For a detailed explanation see Results. E, EcoRI; B, BamHI; C, CfrI; H, HindIII; ApR, CmR and KmR, resistances to ampicillin, cloramphenicol and kanamycin respectively; Pnpr, B. subtilis neutral protease gene promoter; PT5, modified T5 promoter. (B) Synthetic linkers used for pSM318 and pSM319 constructions. Large letters indicate subtilisin leader sequence.
PLASMID | SEQUENCE | PROCYMONIN PRODUCTION (mg l⁻¹)
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>pSM316</td>
<td>5' GAGCTCAAGGAGGAATTC ATG SD</td>
<td>E.coli</td>
</tr>
<tr>
<td>pSM341</td>
<td>5' GAGCTCAGAGGTAATAATGCGATTAAAATTGGAGGAAATTG ATG SD1 Met1 SD2 Stop</td>
<td>30</td>
</tr>
<tr>
<td>pSM342</td>
<td>5' GAGCTGTTAACAGGATTTAATG ATG SD</td>
<td>3</td>
</tr>
<tr>
<td>pSM345</td>
<td>5' GAGCTGAATCTAAGGATTTAATG ATG SD</td>
<td>10</td>
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Fig. 2. RBS containing regions of recombinant plasmids from SacI restriction site to PC start codon ATG. On the right side, relative amounts of PC synthesized in E. coli and B. subtilis.

4.3. Secretion of PC in B. subtilis

To a fragment containing the bovine PC sequence and missing the initiator methionine the subtilisin signal peptide sequence was linked and placed under the control of the npr promoter (plasmid pSM318; see Fig. 1B). Upon transformation of B. subtilis cells with this plasmid, chlor-

Fig. 3. Western blot analysis of PC synthesis in B. subtilis. Lane 1, insoluble PC from E. coli containing the parental plasmid pWHA43; lane 2, intracellular insoluble PC from B. subtilis containing pSM316; lane 3, intracellular PC-subtilisin leader peptide fusion from B. subtilis containing pSM318; lane 4, extracellular soluble PC from the same strain.

Fig. 4. SDS-PAGE analysis of soluble-and insoluble fractions from B. subtilis recombinant strains harbouring the following plasmids: pSM316 (lanes 1, 2); pSM341 (lanes 3, 4); pSM342 (lanes 5, 6); pSM343 (lanes 7, 8). Lane 9, molecular mass standards; lane 10, insoluble fraction from E. coli/pWHA43. The arrow indicates the position of PC migration. Molecular mass standards (BioRad): 66, 42, 31, 21.5, 14 kDa.
amphenicol resistant colonies were isolated, grown in liquid culture and assayed for PC synthesis by Western blot analysis. Fig. 3 shows that pSM318 directs the synthesis and secretion of a polypeptide which comigrates with PC produced intracellularly by B. subtilis and E. coli and cross-reacts with antibodies directed against PC. Not all of the hybrid PC synthesized intracellularly is processed and a protein migrating slower than PC is accumulated in the cell, with the size corresponding to the unprocessed form of the precursor (about 44 kDa). The reactivity of the 60 kDa protein present in the culture supernatant turned out to be aspecific since the same reactivity was visible in the culture medium of the recipient strain (data not shown). The secreted protein was treated at pH 2 directly in the culture medium and responded positively when tested for enzymatic activity in the milk-clotting assay.

Interestingly, B. subtilis cells transformed with the recombinant plasmid pSM319 designed for the secretion of activated enzyme did not seem to contain any protein cross-reacting with the antibody nor displayed any enzymatic activity in the medium, even in the absence of plasmid rearrangements, as analyzed by restriction enzyme mapping and sequencing of the cloned synthetic sequence.

5. DISCUSSION

Bacillus subtilis is a microorganism particularly suitable for the synthesis of proteins to be used in the food industry because of its non-pathogenicity and established industrial usage. For this reason we tested the ability of this microorganism to express the milk clotting enzyme chymosin. The experiments reported here describe both the intracellular production of PC by B. subtilis. PC produced intracellularly in B. subtilis accumulates in cytoplasmic inclusion bodies in the insoluble fraction of cellular proteins. Others have shown similar insolubility of PC produced in E. coli. The inclusion bodies can be quantitatively solubilized by alkali-urea treatment, upon which the polypeptides fold in a proper tridimensional conformation to allow the subsequent auto-processing at acidic pH. Refolding of the polypeptide chain obtained in this way restores the enzymatic activity responsible for milk clotting at the same specific activity as the native enzyme.

Notable increase in PC production was obtained by modification of the mRNA RBS region to obtain efficient translation of the PC mRNA. A large increase was observed when translation of the PC sequence was coupled to the translation of the lpp cistron in both E. coli and B. subtilis. A measure of the relative mRNA levels indicated that in both E. coli and B. subtilis equivalent amounts of steady-state mRNAs directed much efficient heterologous protein accumulation in cells containing the modified constructs, thus showing that the plasmid modifications lead directly to an increment in protein synthesis at the level of initiation. This system has been shown to work well for the production of a few heterologous proteins [24,27] and is probably based on efficient 'feeding' of ribosomes to the second cistron RBS forced by coupling to an efficiently translated cistron upstream from it.

In laboratory conditions, where roughly 4 g l⁻¹ of cells (wet weight) were obtained, the B. subtilis strain containing pSM341 synthesized about 10 mg l⁻¹ of PC, a level which is about 3- to 4-times lower than what is obtained with the same construct in E. coli. This, as suggested by the results of the mRNA level measurements, appears to be due to a more efficient translation of PC mRNA in E. coli than in B. subtilis.

While activation of the enzyme synthesized intracellularly requires cell disruption and solubilization of the inclusion bodies, enzyme activity can be recovered directly from the medium of B. subtilis strain containing pSM318 which directs the intracellular synthesis of a hybrid precursor and secretes it as PC. The structure of the precursor 44 kDa protein must be at least partially compatible with the secretory apparatus of B. subtilis, and the subsequent refolding of the polypeptide must have recreated enough of the native structure to be auto-processed quantitatively into the active form by acid treatment of the culture medium. Unfortunately, the level of PC secretion was very low, being estimated in the order of 100 µg l⁻¹ of culture. The low accumulation of the enzyme in
the culture supernatant might be due to inefficiency of the secretion process and/or protein degradation, as in the case of other heterologous proteins whose secretion has been attempted in *B. subtilis* [21].

We have also attempted to clone a plasmid designed to direct the secretion of the enzyme in the active form, as chymosin, but several colonies containing this plasmid were never shown to produce the enzyme or any cross-reacting polypeptide. The reason for this lack of accumulation and/or secretion is unknown but we cannot rule out instability of the product or a deleterious effect of its accumulation for bacterial growth, and we are not aware of any other host system where cloning of mature chymosin as such was accomplished.

Another possible explanation for the lack of chymosin expression could be that the pro region is required for guiding the enzyme to a proper folded conformation, as has been found to be the case for other proteases [28].

REFERENCES