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Extracellular production of human cystatin S and cystatin SA by *Bacillus subtilis*

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Abstract

We herein describe the development of a *Bacillus subtilis* system that can be used to produce large quantities of recombinant (r-) human salivary cystatins, a cysteine protease inhibitor of family 2 in the cystatin superfamily. The *B. subtilis* that lacked the alkaline protease E gene (Δ*aprE* type mutant strain) was prepared by homologous recombination. The cDNA fragments coding for mature cystatins (S and SA) were ligated in frame to the DNA segment for the signal peptide of endoglucanase in the pHSP-US plasmid vector that was then use to transform the Δ*aprE* type mutant strain of *B. subtilis*. The transformants carrying the expression vectors were cultivated in 5-L jar fermenters for 3 days at 30 °C. Both r-cystatin S and r-cystatin SA were successfully expressed and secreted into the culture broth, and were purified using a fast performance liquid chromatography system. The first use of Δ*aprE* type mutant strain of *B. subtilis* made it possible to obtain a high yield of secreted protein, which makes this system an improvement over expression in *Escherichia coli*. We conclude that this system has high utility for expression of commercial quantities of secreted proteins.

Keywords: *Bacillus subtilis*; Endoglucanase; Human salivary cystatin

Cysteine protease inhibitors belonging to the cystatin superfamily [1] are found in the fluids and tissues of both plants and animals. They are generally tight-binding inhibitors of cysteine proteases such as papain, ficin, and the cathepsins and as such, should regulate the activity of endogenous cysteine proteases that mediate proteolysis and tissue damage [2,3]. Human cystatins play a role in inflammatory diseases [4], cytokine induction [5–7], tumor progression [8], bone resorption [9], and microbial infection [10–12], the latter of which may be mediated by the inhibition of essential microbial and viral proteases.

Production of r-human family 2 cystatins (S, SA, SN, C, D, E, F, and 11) by *Escherichia coli* cells have been studied [8,13–20] because of their therapeutic potentials [21,22]. The *E. coli* systems, however, require extensive efforts or higher costs to convert expressed proteins to a pure and bioactive form since they are intracellular preparations, being released only by cell disruption. Commercial production of proteins by microbes must be generally recognized as safe. As a safety microorganism, the yeast *Pichia pastoris* has been introduced for the production of a chicken cystatin [23]. *Bacillus subtilis* has also been historically an attractive host for the expression of protein on an industrial scale because it secretes foreign protein directly into the culture medium [24,25]. To eliminate problems associated with their production in *E. coli* such as cytotoxin contamination, higher costs of purification, and regulatory concerns, *B. subtilis* was used as a host for the production of a soy cystatin [26]. It has been demonstrated that the system supplied about 0.3 g/L of a functional cystatin.

In this study, we prepared a strain lacking the alkaline protease E gene of *B. subtilis* by homologous recombination. The DNA sequences for promoter and signal of the

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alkaline endoglucanase gene in *Bacillus* sp. KSM-S237 were employed to accomplish higher expression levels of r-human salivary cystatins by the first use of Δ*aprE* type mutant strain of *B. subtilis*. Our system yielded approximately 1.1 g/L of r-cystatin S and 0.8 g/L of r-cystatin SA.

Materials and methods

Bacterial strain and plasmids

Bacillus subtilis Marburg 168 and *B. subtilis* Δ*aprE* were used as host strains for cystatin S production. *B. subtilis* Δ*aprE*, which is a genetically engineered alkaline protease (AprE) deleted strain, was prepared in our laboratory. *E. coli* HB101 was used as the host for subcloning while the plasmid pHY300PLK was used as the vector. Genomic DNAs from *B. subtilis* M. 168 and *Bacillus* sp. KSM-S237, and plasmid DNA were prepared as described [27–30]. Transformation of *E. coli* and *B. subtilis* with plasmids was carried according to the conventional method [31,32].

Culture conditions and preparation of cell extract

Bacillus subtilis harboring the plasmids was propagated at 30 °C for 72 h, with shaking, in 50-ml aliquots of optimized medium in 500-ml flasks. The medium consisted of (w/v) 7% maltose, 0.05% yeast extract (Difco), 4.6% dried corn steep liquor, 0.5% fish meal extract, 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 15 μg/ml tetracycline (pH 6.8). The supernatant was collected from the culture broth by centrifugation at 10,000*g* for 20 min, after which the pellet were resuspended with 1 mM EDTA, 1 mM PMSF, 1 mM DTT, and 5% glycerol in 50 mM potassium phosphate buffer (pH 7.4) and sonicated (model 7500; BIOMIC). The supernatants were then collected following centrifugation. To evaluate whether this system was suitable for large-scale fermentation, we cultivated the *B. subtilis* producing r-cystatins (S and SA) in 3 L of medium using a 5-L jar fermenter. Transformed *E. coli* strains were then grown in LB broth containing tetracycline (5 μg/ml).

DNA sequencing

Sequencing was carried out by the dideoxy chain termination method using a DNA sequencing kit—BigDye terminator cycle sequencing ready reaction (Applied Biosystems) and automated DNA sequencer.

Disruption of the alkaline protease E gene in B. subtilis M. 168

A *B. subtilis* strain was prepared that lacked the alkaline protease E gene [33] using homologous recombination that disrupted the targeted gene. A 1194 bp DNA fragment (A), which contained the upstream region of the alkaline protease E gene (nucleotides −1226 to −33 upstream from the initiation codon), was amplified by PCR using the genomic DNA from *B. subtilis* M. 168 as the template and primers 1 (having a *Xba*I site) and 2. A 1219 bp DNA fragment (B), which contains a part of the alkaline protease E gene (nucleotides 268–1487), was also amplified by PCR using genomic DNA from *B. subtilis* M. 168 as the template and primers 3 and 4 (having a *Xba*I site). The fragment for the *aprE* deletion (Δ*aprE*), which lacks a 299 bp-DNA segment carrying the Shine-Dalgarno (SD)[1] sequence, initiation codon, and signal peptide sequence of the *aprE* gene, was prepared by recombinant PCR using two primers (1 and 4) and fragments (A and B) as the template. A chloramphenicol resistant gene (*Cmr*) was amplified by PCR using primers 5 (having a *Hin*cII site) and 6 (having a *Hin*dIII site) and plasmid pC194 as the template. The *Cmr* PCR fragment was then inserted between the *Hin*cII and *Hin*dIII sites of the pUC18 plasmid (Takara shuzo) and the Δ*aprE* PCR fragment was inserted at the *Xba*I site of the same plasmid. The resultant plasmid, designated pUC*Cm*^{r}Δ*aprE* (6.8 kbp), was used to disrupt *aprE*. Eventually, the M. 168 (wild) strain cells were transformed by the plasmid. These transformants were then grown at 37 °C on a DM3 agar plate supplemented with chloramphenicol. Growing colonies were replicated to an LB-agar plate without chloramphenicol that allowed for the selection of chloramphenicol sensitive strains.

Construction of the expression vector

Using primers 7 and 8 and the genomic DNA from the *Bacillus* sp. KSM-S237 as the template, a DNA fragment representing the promoter and signal peptide of endoglucanase was amplified by PCR. DNA fragments coding for mature cystatins S and SA were amplified by PCR using three sets of primers (9 and 10 for S; 11 and 12 for SA) and the *E. coli* expression vectors for the cystatins as templates [13,14]. Using the overlap extension method, the DNA segments for mature cystatin S (^{1}SSSKEENRIIPGGIYDADLND…) and SA (^{1}WSPQEEDRIIEGGIYDADLND…) consisting of 121 amino acids [13,14], were ligated in frame to the DNA segment for the signal peptide ($^{-30}$MMLRKKTKQLISSILILVLLLSLFPAALAA) of endoglucanase, after which the chimeric genes were inserted between the *Bam*HI and *Hin*dIII sites of the pHY300PLK plasmid (Yakult Co). The plasmid, which we named pHSP-US, was used to produce r-cystatins in *B. subtilis*. Fig. 1 summarizes construction of the vector.

Preparation of anti r-cystatin S antibody

The suspension of r-cystatin S produced by *E. coli* cells [13] in PBS was used as the immunogen to induce the formation of antibodies in female BALB/c mice.

[1] *Abbreviations used:* SD, Shine-Dalgarno; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis; BAPA, Benzoyl-L-arginine-4-nitroanilide.