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Extracellular Production of a Functional Soy Cystatin by *Bacillus subtilis*

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A recombinant *Bacillus subtilis* producing soy cystatin was developed by subcloning with a soy cystatin gene cloned in *Escherichia coli*. An active form of cystatin against the cysteine protease from Pacific whiting fillets contaminated with *Myxosporidia* parasite was constitutively expressed and secreted extracelluarly into the medium. Two gene fragments of signal peptides from *kerA* and *sacB* were introduced and compared for secretion efficiency of cystatin. The secretion level of active cystatin improved with the signal peptide of *kerA* when compared to that of *sacB*. Inhibitor activity was reduced rapidly after peak expression of the target protein at 36 h of fermentation. The addition of 1% glucose, a suppressor of protease, into the medium sustained the increase of the cystatin activity during fermentation. This study introduced a potential new method for fermentation production of cystatin.

KEYWORDS: Protease inhibitor; cloning; signal peptide; secretion; surimi

INTRODUCTION

A soybean gene (N_2) that induces production of a cysteine proteinase inhibitor, cystatin, has previously been isolated and expressed in *Escherichia coli* (*E. coli*) (*1*). The inhibitory activity of the cystatin produced by this gene was reported to be higher than that of epoxide *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino)butane(E-64), a strong chemical inhibitor (*2*). Kang and Lanier (*2*) found this cystatin to be effective against the heat-stable cysteine proteases present in Pacific whiting (*Merluccius productus*) surimi that induce a mushy texture upon cooking.

Several cysteine protease inhibitors have previously been expressed in *E. coli*, such as chicken cystatin (*3*, *4*), human cystatin C (*5*, *6*), and oryzacystatin (*7*). The products from *E. coli*, however, require extensive efforts to convert them to a pure and bioactive form because they are intracellularly produced, being released only by cell disruption.

Commercial production of proteins by microbes for edible food ingredients must be from organisms generally recognized as safe (GRAS). One such GRAS microorganism, the yeast *Pichia pastoris*, has recently been cloned for the production of a chicken cystatin (*8*). *Bacillus subtilis* (*B. subtilis*), which is also GRAS, has historically been an attractive host for the expression of protein on an industrial scale because it secretes foreign proteins directly into the culture medium (*9*, *10*). The secretory system thus facilitates protein recovery in a sufficiently pure form in a relatively simple and inexpensive fashion. In the present study, *B. subtilis* was used as a host for the production of soy cystatin to eliminate problems associated with its production in *E. coli* such as cytotoxin contamination, higher costs of purification, and regulatory concerns. The procedure and results of gene isolation, transformation, introduction of a new signal sequence, and glucose addition to improve the yield of cystatin are described in this paper.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. *E. coli.* strain DH5α, previously cloned with a soy cystatin gene (N_2) (*1*), was provided by Dr. S. Nielsen of Purdue University. *B. subtilis* WB600 (trpC2 nprA apr epr bfp mpr:ble nprB::ery) (*11*) was used as the host for cloning and expression studies. Plasmid pWB980 carrying a P43 promoter was used for the insertion of the cystatin gene into *B. subtilis*. Two signal peptides from genes *sacB* (*11*) and *kerA* (*12*) were used to study the secreting efficiency.

Cystatin Gene (DNA Insert) Manipulations and Vector Constructions. Minipreparation of the plasmids harbored in *B. subtilis* was by a rapid alkaline sodium sulfate method (*13*). Chromosomal DNA of the N_2 gene was isolated from *E. coli* using the method of Sambrook et al. (*14*). Restriction enzymes and DNA ligases were purchased from Promega (Madison, WI) and Boehringer-Mannheim (Mannheim, Germany), respectively, and used as recommended by the manufacturers.

Amplification of N_2 Gene and Isolation. To amplify the cystatin gene by a Polymerase Chain Reaction (PCR), four synthetic primers were specifically designed to contain two different restriction sites (*15*). The two forward primers contain the restriction sites *Bam*HI for *SacB* and *Xba*I for *KerA* at the 5′ end; the other two backward primers carry the restriction site *Sph*I at the 3′ end:

(1) 5′-CGCACGC**GGATCC** ATGGAATTAGGTGGCATCACC -3′ (*Bam*HI forward primer).

(2) 5′-TACATACA**GCATGC**TDTAGGTGCTACAAACATTGAC-3′ (*Sph*I backward primer).

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(3) 5′-GCTAGC**TCTAGA** GAATTCGGTGGCATCACCGAT-3′ (*Xba*I forward primer).

(4) 5′-TACATACA**GCATGC**TTAGGTGCTAGAAACATTGAC-3′ (*Sph*I backward primer).

The PCR reaction was performed with *Taq* (Promega) DNA polymerase under the following conditions: 94 °C for 30 s, 54 °C for 1.5 min, 72 °C for 1.5 min (30 cycles), and 72 °C for 5 min. The resulting product was digested by *Bam*HI/*Sph*I to separate the DNA fragments on a 0.8% agarose gel. Desired DNA fragments from the PCR product were recovered and purified by the QIAquick gel extraction kit and PCR purification kit (Qiagen Inc., Valenicia, CA), respectively.

Vector Preparation. Plasmid pWB980 was used for cystatin expression in *B. subtilis* WB600 (*trpC2 nprA apr epr bfp mpr:ble nprB::ery*) (*11*, *16*). Following a small-scale isolation of the plasmid harbored in *B. subtilis*, plasmid digestion was conducted with *Bam*HI/*Sph*I for a single strand overhang at each end that matched with that of the cystatin insert gene. For ligation, the excised plasmid and insert genes were incubated in a ligation mixture (*14*) overnight at 14 °C. Two plasmids, one carrying *sacB* and one carrying *kerA* signal sequences, were constructed as pINH1 and pINH2.

Transformation and Screening of *B. subtilis* WB600. Transformation of *B. subtilis* WB600 was carried out by the competent cell method as described by Lin et al. (*17*). The plasmid DNA (1 μg) in 50 μL was added to the solution (500 μL) of competent cells prepared fresh from *B. subtilis* WB 600 (*18*). Following shaking at 150 rpm for 90 min, cells were plated for overnight incubation at 37 °C on tryptose blood agar base (TBAB) plates containing kanamycin (200 μg/mL). Colonies grown on the plate were picked and frozen for plasmid isolation and protease inhibitory assay. They were confirmed for the gene insertion by digestion with restriction enzymes and PCR amplification.

Expression of N_2 in *B. subtilis*. The host cells were grown in a shake incubator at 37 °C in Luria−Bertani (LB) medium containing 20 μg/mL kanamycin for routine gene expression. At designated time periods, 50 mL of the medium was centrifuged at 8000*g* for 20 min, and both supernatant and cells were saved. Prior to assay, the cells were sonicated and centrifuged after suspension in a 20 mM potassium phosphate buffer, pH 6.0, with 20 mM sodium azide.

Expression of N_2 in *E. coli*. *E. coli* cells containing the soy cystatin gene were isolated from a single colony following agar plate streaking. Following preparation of an overnight preculture from the isolated colony, the culture was diluted 1:100 in LB broth for bulk incubation at 37 °C. When an optical density (A_{600}) of 0.5 was reached, the culture was quickly cooled, on ice, to room temperature, and isopropyl β-D-thiogalactoside (IPTG, 0.4 mM) was introduced for the expression of the recombinant protein during continued incubation overnight at 25 °C.

The cells thus cultured were harvested by centrifugation (8000*g*) for 20 min and sonicated after suspension in a 20 mM potassium phosphate buffer, pH 6.0, with 20 mM sodium azide. Following separation of cell debris by a similar centrifugation, proteins precipitated between 20 and 80% ammonium sulfate saturation were pooled, dissolved in buffer, and frozen and stored at −70 °C for future purification and measurement of inhibitory activity.

Purification of Cystatin Expressed by *E. coli*. A HisTrap kit purchased from Pharmacia Biotech (Piscataway, NJ) was used to obtain a high-purity histidine-tagged inhibitor protein. According to the recommendations of the manufacturer, a column (1 mL, 0.7 × 2.5 cm) was prepared for activation and washing prior to sample loading. The column was loaded with the cellular protein of *E. coli* previously prepared by ammonium sulfate precipitation at 20−80% saturation. The second and third fractions having the two highest A_{280} values were saved for a positive control sample for SDS-PAGE and Western blot.

Proteolytic and Inhibitory Activity Assays. Proteases, most originating from the Myxosporean parasite *Kuda paniformis* in Pacific whiting fillets (*19*), were crudely purified for measurement of inhibitory activity of the culture media using the method of Kang and Lanier (*2*). Inhibitory activity was measured on the supernatant of the unprocessed *B. subtilis* culture media and the supernatant of the sonicated *B. subtilis* transformants as well as the supernatant of the sonicated *E. coli*. Crude protease at 0.35−0.45 unit was used to determine the linear assay range for each inhibitor sample, and various aliquot volumes of each inhibitor solution were added to determine inhibitory activity. One unit of protease activity was defined as the amount of enzyme that induced an increase of 1.0 absorbance unit at 440 nm after 30 min of incubation at 55 °C. Inhibitory activity was defined as the amount that inhibited one unit of the crude protease.

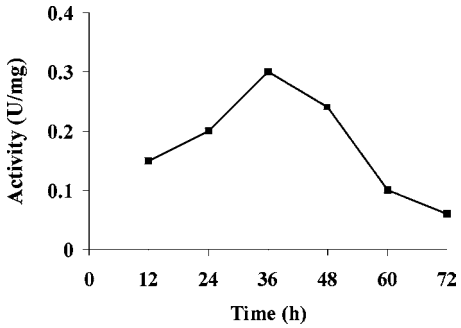

Figure 1. Inhibitory activity in the media of *B. subtilis* cystatin-producing bacteria with N_2 gene and signal peptide *sacB* (CPB-1) during fermentation (incubation at 37 °C for 72 h). One unit of activity was defined as the amount of inhibitor that reduced 1 unit of protease activity purified from Pacific whiting filets.

Gel Electrophoresis. The culture media of *B. subtilis* transformants was collected for gel electrophoresis. Protein was concentrated by precipitation with 5% trichloroacetic acid (TCA; St. Louis, MO) and resuspended in phosphate buffer. Electrophoresis was performed using 18% precast Tris−glycine gels (Novex, San Diego, CA) loaded with 20 μg of protein per lane.

Western Blot Analysis. Western blot was conducted according to a standard procedure described by Towbin et al. (*20*). Following electrophoresis, proteins were electroblotted onto nitrocellulose film with a Trans-Blot cell. The primary antibody against soy cystatin was a gift from S. Nielsen at Purdue University. Secondary antibody was rabbit anti-chicken IgY conjugated with alkaline phosphatase from Jackson Immunoresearch Laboratory Inc. (West Grove, PA). Novex multicolor markers were used as size standards.

RESULTS AND DISCUSSION

Construction and Expression of N_2 in *B. subtilis*. The pINH1 and pINH2 plasmids were created by inserting the N_2 gene into the plasmid pWB980 harboring *sacB* and *kerA* signal peptide sequences, respectively. The insertion was confirmed by restriction digestion and PCR analysis (data not shown). Plasmids were used to transform *B. subtilis* WB600. Inhibitory assay from the supernatant of cystatin-producing *B. subtilis* with *sacB* (CPB-1) indicated that the organism was capable of producing and secreting cystatin in LB medium (**Figure 1**). During 72 h of fermentation, the assay showed that the inhibitor reached a maximum level of activity at 36 h followed by a rapid decrease thereafter.

Inhibitory activity in *B. subtilis* culture media was compared to that of the *E. coli* lysate. The activity (per gram of protein) in the *E. coli* lysate was 186-fold higher than the cystatin from the media of *B. subtilis* (**Table 1**). Overexpression of foreign genes by *E. coli* is well documented for a high yield up to 50% of the total cellular protein. Within a few hours of induction, cloned *E. coli* usually started to fully accumulate the target protein under the control of pET plasmid and bacteriophage T7 promoter (*21*, *22*). It was unclear if the low activity from the *B. subtilis* was due to an inefficient expression of N_2 by the organism or whether the signal peptide in the microbe was not suitable to secrete the foreign protein.

To answer the question, inhibitor activity in the supernatant of nutrient media was compared to the activity in the lysate solution of *B. subtilis* CPB-1. The activities in the cell lysate at

Table 1. Comparison of Inhibitory Activities Expressed by *B. subtilis* CPB-1 Media and *E. coli* Lysate

host	protein concn (mg/mL)	activity[a] (units/mL)	specific activity (units/g)	*X*-fold
B. subtilis (CPB-1)[b]	0.29	0.27	931	1
E. coli	2.6	450	173077	186

[a] One unit of activity was defined as the amount of inhibitor that inhibited 1 unit of protease activity purified from Pacific whiting filets. [b] Cystatin-producing bacteria with N_2 gene and signal peptide *sacB*.

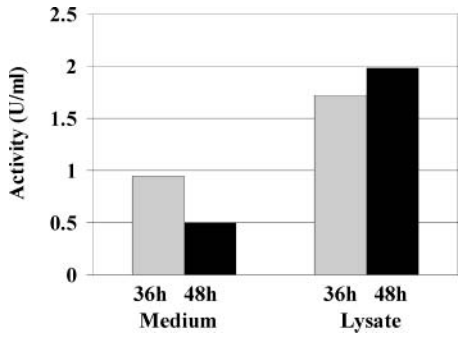

Figure 2. Comparison of inhibitory activities of the secreted cystatin in medium and the uncreated cystatin in lysate of *B. subtilis* with N_2 gene and signal peptide *sacB* (CPB-1).

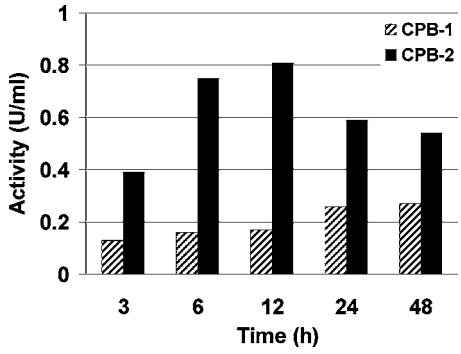

Figure 3. Secretion of cystatin by *B. subtilis* with different signal peptides. CPB-1 contains the *sacB* signal peptide; CPB-2 contains the *kerA* signal peptide.

36 and 48 h of fermentation were 2 and 4 times higher than those from the media at the same times per unit of protein, although they are still less than in the *E. coli* lysate. The results indicated that the signal peptide of *sacB* (*11*) may not be efficient for the secretion of the inhibitor protein.

Comparison of Secretion Systems with *sacB* and *kerA*. Cystatin-producing *B. subtilis* 2 (CPB-2) was created by switching the *sacB* signal peptide with a signal peptide from *kerA* (*12*) and compared to a second preparation of CPB-1. The CPB-2 appeared to secrete the inhibitor into the media at a significantly higher rate than did CPB-1 for the entire 48 h fermentation period (**Figure 3**). CPB-2 activity reached a maximum level at 12 h as compared to 36 h with CPB-1, and the activity was 3−4 times higher. These results were supported by gel electrophoresis that revealed thicker and darker bands at 14 kDa (target protein) from CPB-2 than from CPB-1 media (**Figure 4a**). Analysis by western blot showed no target protein from the negative control having no insertion, a weak band from CPB-1, and a strong band from CPB-2. As a positive control, cystatin was purified from *E. coli*, and the resulting band was located at 14 kDa for both gel electrophoresis and western blot (**Figure 4**).

Rapid Degradation of Cystatin after Peak Expression. One substantial advantage of expression via *B. subtilis* over *E. coli* is its ability to secrete foreign proteins directly into the culture medium (*9*, *23*), which could lead to a reduction in cost for purification of the target protein (*23*−*26*). This advantage, however, may not be useful if the secreted protein is being degraded by coexpressed proteases from host cells.

Our results indicated that the cystatin in the nutrient media was probably being degraded rapidly after reaching a maximum level of inhibitory activity for both CPB-2 and CPB-1 (**Figures 3** and **5a**), indicating a possible involvement of proteases originating from the host cells during fermentation. To determine whether the degradation was due to host-produced proteases or autolysis during extended fermentation, inhibitory activity was measured on the medium of a negative control having no insertion as well as the medium from CPB-1. Inhibitory activity from the control medium showed a slight negative value at 24 h of fermentation and a more substantial negative value at 36 h, which was maintained throughout the 72 h of measurements, whereas the CPB-1 medium showed increased inhibitory activity up to 36 h of fermentation that decreased thereafter (**Figure 5a**).

In an inhibitor assay, a negative activity value is achieved when more breakdown products are detected in the inhibitor-containing sample than in the sample containing no inhibitor. Although the negative control certainly would not have contained the inhibitor, the expected inhibitory activity would be 0 units/mg throughout the whole time period. Because more proteolytic breakdown products were detected in the negative control as fermentation time increased, it was apparent that proteases were present in the medium after 24 h of fermentation or earlier.

When protein concentration of the media was measured throughout the fermentation period, the total protein increased in both media up to 36 h of fermentation, but then leveled off (**Figure 5b**). With the same total protein during the last 72 h of fermentation, it would be expected that cystatin activity in CPB-1 medium would remain the same, unless the inhibitor were not autolyzed and the inhibitor unit of the negative control would be zero, too. However, the inhibitory activity in the medium decreased rapidly after 36 h and the negative control showed negative values.

The measurement of lower inhibitory activity due to proteases expressed by the host can be caused by two mechanisms (1) direct proteolytic attack of the cystatin during fermentation or (2) extra degradation of the azocasein substrate by the host protease during the inhibitor assay that can mask the inhibitor activity. Although the actual mechanism for the inhibitory reduction was not clear in this study, one of the two or both can explain the rapid loss of inhibitory activity after peak expression.

The *B. subtilis* strain, WB600, used in this study was designed to be deficient in expression of six major proteases (*11*), yet this deficiency appeared to be not enough to preserve the target inhibitor protein in this case. The host cell had previously been used to express other proteases (*11*, *17*) rather than inhibitor so unknown proteases coexpressed by the host may not have been easily detected.

Improved Cystatin Production by Glucose. To compensate for the likely proteolytic attack on the cystatin, glucose, a common catabolic repressor for protease production by *B. subtilis* (*27*, *28*), was added to the growth medium for CPB-2. Cystatin production was compared for CBP-2 grown in (1) 1%

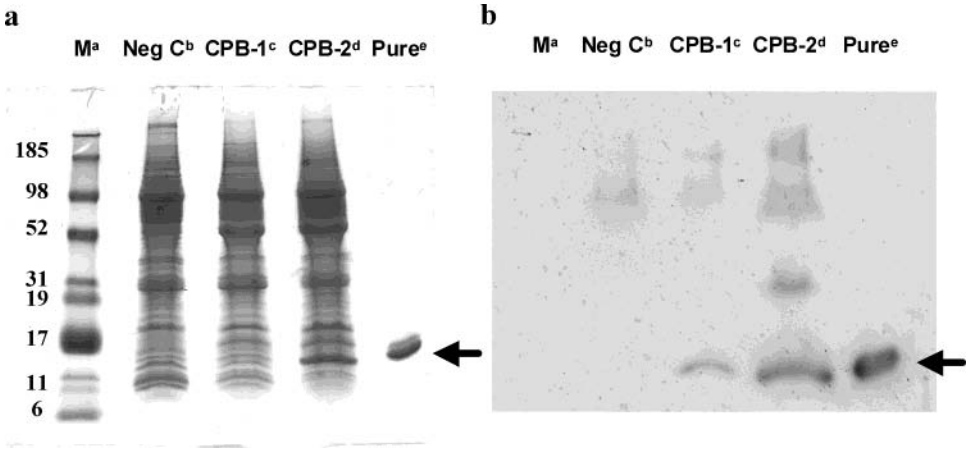

Figure 4. Analysis of amount of cystatin expressed into culture media by *B. subtilis* recombinants compared with cystatin purified from *E. coli* lysate: (**a**) SDS-PAGE; (**b**) western blot; (lanes M) molecular weight markers; (lanes Neg C) negative control (*B. subtilis* containing a plasmid with no N_2 gene); (lanes CPB-1) *B. subtilis* containing a plasmid with the N_2 gene and the *SacB* signal peptide; (lanes CPB-2) *B. subtilis* containing a plasmid with the N_2 gene and the *kerA* signal peptide; (lanes Pure) purified *E. coli* cystatin.

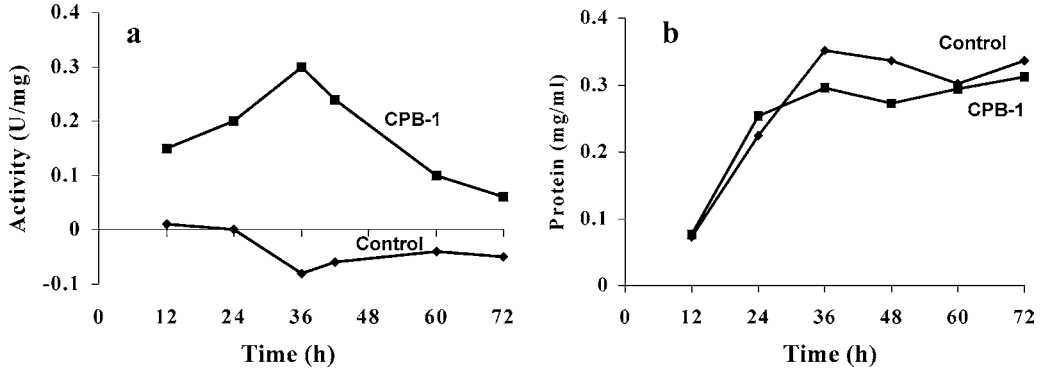

Figure 5. Comparison of inhibitory activity and protein concentration in the media of *B. subtilis* cystatin-producing bacteria with N_2 gene and signal peptide *sacB* (CPB-1) and *B. subtilis* containing no cystatin gene (control).

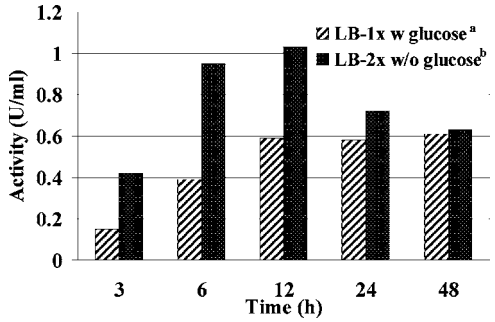

Figure 6. Effect of glucose (1%) on the inhibitory activity of *B. subtilis* CPB-2 medium: (slashed bars) LB media (25 g/L) with 1% glucose; (black bars) LB media (50 g/L) with no glucose.

glucose in a final concentration of 25 g/L LB (LB-1X) and (2) 0% glucose in a final concentration of 50 g/L LB (LB-2X). CBP-2 grown in the LB-1X with glucose expressed the inhibitor at a relatively lower rate than did the CBP-2 grown in the LB-2X for the entire period of fermentation. This was probably due to lower nutrients in the LB-1X. However, inhibitory activity in the LB-1X medium with glucose continuously increased with no subsequent reduction, whereas the activity in the medium without glucose showed a rapid reduction after 12 h of fermentation (**Figure 6**). As a result, the inhibitory activity from the two media reached approximately the same level at the end of fermentation. These results indicated that the lowered inhibitory activity after peak expression was probably due to host-produced proteases during fermentation and that glucose demonstrated a repressive effect on protease production by *B. subtilis*.

This study demonstrated the ability of a GRAS microorganism, *B. subtilis*, to produce and secrete a food-grade soy cystatin into culture medium. The activity, however, was lower than that produced in *E. coli* lysate per unit of protein. A significant improvement in inhibitory activity was obtained by replacing the signal peptide from *sacB* with one from *kerA* and adding glucose to the medium. The yield was still inferior to that of *E. coli*.

Although this method provides easy purification of the target protein for an edible food component, it would be advantageous if the present expression system yielded inhibitor contents comparable to or higher than that from *E. coli*. Further research is recommended to further improve cystatin production in *B. subtilis*.

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