Overexpression of *celB* Gene Coding for β-Glucosidase from *Pyrococcus furiosus* Using a Baculovirus Expression Vector System in Silkworm, *Bombyx mori*

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β-Glucosidase is a member of the glycosyl hydrolases that specifically catalyze the hydrolysis of terminal nonreducing β-D-glucose residues from the end of various oligosaccharides with the release of β-D-glucose. *CelB* gene, encoding the thermostable β-glucosidase, was amplified from the *Pyrococcus furiosus* genome and then cloned into the baculoviral transfer vector under the control of the *polyhedrin* gene promoter. After co-transfection with the genetically modified parental *Bombyx mori* nucleopolyhedrovirus (BmNPV), the recombinant virus containing *celB* gene was used to express β-glucosidase in silkworm. The recombinant β-glucosidase was purified to about 81% homogeneity in a single heat-treatment step. The optimal activity of the expressed β-glucosidase was obtained at pH 5.0 and about 105 °C; divalent cations and high ionic strength did not affect the activity remarkably. This suggested that the enzymatic characteristics of recombinant β-glucosidase were similar to the native counterpart. The expressed β-glucosidase accounted for more than 10% of silkworm total haemolymph proteins according to the protein quantification and densimeter scanning. The expression level reached 10,199.5 U per ml haemolymph and 19,797.4 U per silkworm larva, and the specific activity of the one-step purified crude enzyme was 885 U per mg. It was demonstrated to be an attractive approach for mass production of thermostable β-glucosidase using this system.

Key words: β-Glucosidase, *Pyrococcus furiosus*, Baculovirus Expression Vector System

Introduction

A variety of hyperthermophilic archaea and bacteria have been isolated from many ecosystems, mainly from water-containing terrestrial and marine high temperature areas (Stetter, 1992). Hyperthermophilic microorganisms have their optimal growth temperature at about 90 °C, and grow fast at temperatures between 80 °C and 110 °C (Stetter *et al*., 1990). Due to the metabolic flexibility and their outstanding heat resistance, they are of great value for basic research as well as for biotechnological applications (Huber *et al*., 2000). The discovery of hyperthermophilic microorganisms led to the isolation of enzymes that could function optimally at temperatures exceeding their host growth (Adams *et al*., 1995; Niehaus *et al*., 1999). *Pyrococcus furiosus*, isolated from a shallow marine hydrothermal vent, with an optimal growth temperature at above 100 °C, is one of the most well studied hyperthermophilic microorganisms (Woese *et al*., 1990). *P. furiosus* forms complex food webs, which use complex peptide mixtures or sugars as carbon and energy sources, *e.g.* starch, glycogen, maltose and cellobiose, so it must secrete various relevant enzymes to assimilate these compounds (Laderman *et al*., 1993; Dong *et al*., 1997). These enzymes are a useful extension of the enzyme application in industry for their extremophilic properties (like temperature, pH and pressure). Hyperthermophilic β-glucosidase has a variety of applications and is widely used in numerous fields, such as food, medicine, dairy products, environment protection and organic chemical industries (Fischer *et al*., 1996; Hansson *et al*., 2001; Splechtna *et al*., 2002). β-Glucosidase is one of the key enzymes of *P. furiosus* involved in growth on β-linked sugars. This enzyme serves as a model system for studying the molecular mechanisms that are employed by hyperthermophilic organisms to optimize enzyme stability and catalysis (Lebbink *et al*., 2001). β-Glucosidase purified from *P. furiosus* is composed of four identical subunits,

and is one of the most thermostable glycosyl hydrolases described to date, which is very active and stable at 105 °C (Kengen *et al.*, 1993). The substrate specificity of the wild-type enzyme was found to be relaxed, cleaving glycosidic linkages when glucose, galactose, xylose, cellobiose, lactose and mannose were in the terminal, noninducing position (Bauer and Kelly, 1998; Bauer *et al.*, 1999). Hyperthermophilic β-glucosidase, glycosyl hydrolase family 1, from *P. furiosus* was successfully cloned and expressed in *E. coli* (Voorhorst *et al.*, 1995).

Lactose is the main compound of dairy products, and it is an important nutrition for the growth of body, but lactose intolerance is a prevalent clinical problem; about 70% of the world population, especially Asian and African, can't use it effectively because of the absence of glucosidase in the small intestine (Harju, 1987). This can be eliminated by infusion of lactose-prehydrolyzed milk and enzyme-added milk (Rosado *et al.*, 1989).

At present, the β-glucosidase used in the dairy industries is almost from viscera of animals or other microorganisms, which still remains low yield, costly and sensitive to high temperature. Hyperthermophilic glucosidase is prior due to the high temperature for substrate degradation, high reaction performance, labor force, time and energy effective. Presently, the hyperthermophilic glucosidase is mainly expressed in *E. coli* or a yeast expression system. It was demonstrated that there were some problems in the expression product yields using these systems, *e.g.* a majority of the recombinant glucosidase retained its inactive counterpart intracellular because of its secreting bottleneck and most of the protein expressed in the yeast system was misfolding. Although the dissolubility of glucosidase expressed in *E. coli* was more favorable than in the yeast system, the expression yield was low (Smith and Robinson, 2002). While the silkworm baculovirus expression system is an inexpensive high-level expression system, it has been used effectively for the expression of recombinant proteins (Liu *et al.*, 2005; Lü, 1998). This system has a great deal of advantages, such as the host range is virtually limited to the mulberry silkworm, *Bombyx mori*, and it also increases protein yields because of the large size, short life cycle, easiness to rear and manipulate (Maeda, 1989). Here we report the overexpression, simple purification and characterization analysis of the thermostable β-glucosidase encoded by the *celB*-gluc and expressed in silkworm larvae using a baculovirus expression vector system (BEVS).

Materials and Methods

Enzymes and chemicals

Unless otherwise indicated, restriction endonucleases, T4 DNA ligase and platinum *pfx* polymerase were purchased from Invitrogen (Carlsbad, CA, USA). DNA purification kit was obtained from Promega Corporation (Madison, WI, USA). *Para*-nitrophenyl-β-d-glucopyranoside (pNPG) was from Amresco (Solon, OH, USA). Lipofectin kit, TC-100 insect cell medium, fetal bovine serum and 10 kDa protein ladder were from Gibco BRL (Gaithersburg, PA, USA). pGEM-3Z and pVL1393 were maintained in our laboratory. Primers used in this experiment were synthesized by Invitrogen (Beijing, China). All other chemicals were of analytical grade.

Organisms and culture conditions

P. furiosus (strain JCM8422) was obtained from Japan Collection of Microorganisms, RIKEN (Saitama, Japan), and cultured at 98 °C according to a previous report (Kengen *et al.*, 1993). The chromosomal DNA of *P. furiosus* was isolated as described (Laderman *et al.*, 1993). *E. coli* TG1 was preserved in our laboratory. The parental baculovirus BmBacPAK6 was a kind gift from Professor X. F. Wu (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences; Chinese patent 1242428). Bm-5 cells were propagated at 27 °C in TC-100 insect medium, supplemented with 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (Summers and Smith, 1987). The hyperexpression variety of silkworm (JY1) was bred and maintained in the laboratory. The larvae were fed on mulberry leaves and routinely reared at about 23–25 °C.

Construction of plasmids

The *celB*-gluc gene was amplified by the PCR method using the following two primers, which were designed according to the reported *celB* gene (Genbank accession No. AF013169):
5′-GAGGGATCCAATATGAAGTTCCCAAAA AACTTCATGT-3′ (forward), 5′-AAAGAATTC TGGCTACTTTCTTGTAACAAATTTGAG-3′ (reverse). A *Bam*HI site upstream of the start codon and an *Eco*RI site downstream of the stop

codon were introduced, respectively (the *Bam*HI and *Eco*RI sites are underlined). PCR reaction was carried out using high fidelity DNA polymerase according to manufacturer's instructions. The PCR product was separated on agarose gel and purified using a DNA purification kit. The fragment was inserted into pGEM-3Z using *Bam*HI and *Eco*RI sites. The recombinant plasmid was sequenced to confirm if there were insertion or deletion mutation occurred during the PCR reaction. The confirmed *celB* gene was subcloned into the baculoviral transfer vector pVL1393 to generate pVL-celB.

Generation of recombinant baculovirus

BmBacPAK6, a genetically modified BmNPV (*Bombyx mori* nucleopolyhedrovirus), was digested with *Bsu*36I (New England Biolabs, Ipswich, MA, USA) and employed as parental virus in this study. Transfer plasmid containing the *celB* gene was co-transfected into Bm-5 cells with digested BmBacPAK6 DNA mediate by lipofectin following manufacturer's protocol. The cells were allowed to grow at 27 °C for 4–5 d and the medium supernatant was collected as the primary viral stock for screening of recombinant virus. 24 isolated virus plaques from the plaque assays were cultivated in a 24-well plate for about 4–5 d and recombinant virus were further identified *via* adding X-Gal (5-bromo-4-chloro-3-indolyl-*beta*-D-galactopyranoside) to every well. PCR amplification was also conducted to confirm whether the *celB* gene was incorporated into the viral genome. Primers were designed as follows: *celB* reverse primer and a baculovirus general primer 5′-ACTGTTTTCGTA ACAGTTTTGTAA-3′. The recombinant virus was used to inject the silkworm larva and the plaque of recombinant virus expressed β-glucosidase at maximal activity was selected to perform another two rounds of purification. The pure recombinant virus was used to generate a high titer viral stock for protein expression.

Expression of celB in silkworm larvae

Early fifth instar silkworm larvae were infected with the purified recombinant virus by subcutaneous injection at about 2×10^5 plaque forming units (PFU) per worm and routinely reared on mulberry leaves. The larval haemolymph was collected at 24, 36, 48, 60, 72, 84, 96, 108, 120 and 132 h post infection (h.p.i.) by puncturing abdominal legs.

According to the time course, when β-glucosidase expressed at the highest level, the larval bodies were ground and sonicated in ice-cold 0.1 M sodium acetate buffer (pH 5.0), then centrifuged at 4 °C to produce a supernatant for further analysis. Haemolymph and tissue supernatant were stored at −20 °C.

Protein quantification and SDS-PAGE analysis

Protein was quantitatively determined with Coomassie Brilliant Blue G-250 as described by Bradford (1976). Bovine serum albumin was used as a standard. The properly diluted heat-treated and nature haemolymph samples were denatured and run on 10% SDS-PAGE gel (with 5% stacking gel) using standard procedures as described by Sambrook *et al.* (1989). After electrophoresis, the gel was stained using Coomassie Brilliant Blue R-250.

Partial purification of β-glucosidase

Appropriate larval haemolymph containing expressed hyperthermophilic β-glucosidase was prepared by incubating the haemolymph sample at 90 °C for 15 min to denature the host protein. The heat-denatured sample was then cooled on ice and centrifuged at 4 °C to produce a supernatant that was assayed for activity and enzymatic characteristics.

CelB activity assay

β-Glucosidase activity was assayed with the modified method previously described by Kengen *et al.* (1993). pNPG (1 mM final concentration) was used routinely as substrate. The temperature of 1.8 ml 0.1 M sodium acetate buffer (pH 5.0) containing appropriate substrate was raised to 90 °C, then 200 μl properly diluted sample were added. The reaction was incubated for 10 min at 90 °C, and stopped by placing the tubes on ice. Then 2 ml 1 M Na_2CO_3 were added immediately for color development, the increase in absorbance at 405 nm as a result of *para*-nitrophenyl (pNp) liberation was measured. All activities were corrected for nonenzymatic hydrolysis of pNPG at 90 °C, which was below 0.5% of the enzyme hydrolysis rate. The pNp concentrations were determined using a pNp calibration curve (0–2 mM).

One unit (U) of β-glucosidase activity was defined as the amount of enzyme required to liberate

1 μmol pNp per min under the given assay conditions. All activity assays were carried out in triplicate at least.

pH profile, optimal temperature and enzyme thermostability

Based on the assay method described above, the samples were incubated in a water bath from 70 to 100 °C and in an oil bath from 100 to 140 °C in 0.1 M sodium acetate buffer (pH 5.0) to determine the temperature optimum of expressed β-glucosidase. The effect of pH value on β-glucosidase was determined at 90 °C in different buffers covering the pH range from 2.5 to 8.0. The different buffers were as follows: 0.1 M sodium phosphate and sodium acetate (pH 2.5–3.0), 0.1 M sodium acetate (pH 2.5–6.5) and 0.1 M sodium phosphate (pH 6.0–8.0). The thermostability of the BEVS-derived β-glucosidase was measured by dissolving the enzyme solution in 0.1 M sodium acetate (pH 5.0) at different temperatures for 10 min. After that, the samples were renatured at room temperature for 30 min; then a standard activity assay was conducted to estimate the activity remained.

Modulation of β-glucosidase activity by ions

To identify if there were any changes of β-glucosidase expressed using BEVS relative to the native counterpart, activity assays were performed in the presence of several salts, such as $CaCl_2$, $CoCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, $ZnSO_4$ and $NaWO_4$, at the concentration of 1 mM. To measure the dependence of the activity on high ionic strength, the enzyme reactions were carried out at 90 °C for 10 min in 0.1 M sodium acetate buffer (pH 5.0) containing NaCl varying from 0.1 M to 1.7 M.

Results and Discussion

Construction of the recombinant baculovirus

The *celB* gene was amplified by the PCR method and inserted into the pGEM-3Z to generate the plasmid p3Z-celB. Sequence analysis showed that there has no mutation occurred in the *celB* coding region compared with the reported *celB* gene. Since the *celB* fragment was inserted into the transfer vector pVL1393 with *Bam*HI and *Eco*RI sites, and there was a *Pst*I in the *celB* gene, the plasmid was digested with these enzymes, to further confirm the recombinant pVL-celB.

The baculoviral transfer plasmid pVL-celB was co-transfected with *Bsu*36I digested BmBacPAK6 genomic DNA into Bm-5 cells. The linearized BmBacPAK6 virus lacked an essential gene for propagation, which can be rescued *via* co-transfecting linearized BmBacPAK6 with a baculoviral transfer vector by homologous recombination. More than 90% virus was recombined and further represented in a plaque assay. Recombinant virus was also confirmed by PCR using *celB* reversed primer, and a forward primer designed according to the −40 nucleotides with respect to the initial translational site of *polyhedrin* gene. After another two rounds of purification, the pure and high expression viral clone was obtained.

Expression of β-glucosidase in silkworm larvae

Haemolymph of silkworm larvae was harvested on ice every 12 h from 24 h.p.i. Although a small quantity of β-glucosidase was expressed before 60 h, the accumulation of recombinant product was dramatically increased during the late phase of infection. Up to 10,199.5 U of the β-glucosidase activity was detected in 1 ml haemolymph and also 19,797.4 U in one silkworm larva, which were harvested at 120 h.p.i., while the expression yield in *E. coli* was about 50–80 mg/l (equal to 13,000–20,800 U). The specific activities were about 260 U per mg in *E. coli* and 885 U per mg in BEVS-silkworm system. It is possible that the relatively simple purification process of BEVS-silkworm results in the insignificant decrease of β-glucosidase activity. Alternatively, it is possible that some unknown post-translational modifications make up the increase of activity. On the SDS-PAGE gel, the β-glucosidase was about 55 kDa according to the protein ladder, with remarkable increment of protein amount represented on the gel (Fig. 1). The haemolymph of parental BmBacPAK6-infected silkworm was collected and subjected to SDS-PAGE analysis as the control of native and heat-denatured samples. The expressed thermostable β-glucosidase is about 10% of total protein in haemolymph, based on the densimeter scanning and protein quantity.

Simple purification of β-glucosidase

High purification cost is a major factor restricting the use of β-glucosidase in the industry and analyses. Two difficulties are the low yields and the need to remove contaminating activities. In

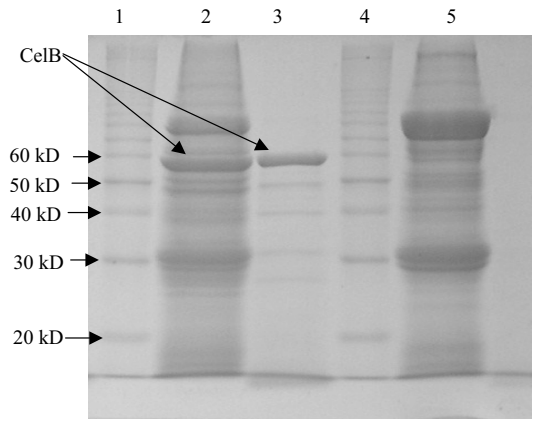

Fig. 1. SDS-PAGE analysis of the BEVS-derived β-glucosidase. Lanes 1 and 4, 10 kDa standard protein ladder; lane 2, silkworm haemolymph with expressed β-glucosidase; lane 3, heat-treated silkworm haemolymph with expressed β-glucosidase; lane 5, haemolymph of silkworm infected with BmBacPAK6. The arrows indicate a part of the protein ladder bands and the β-glucosidase band.

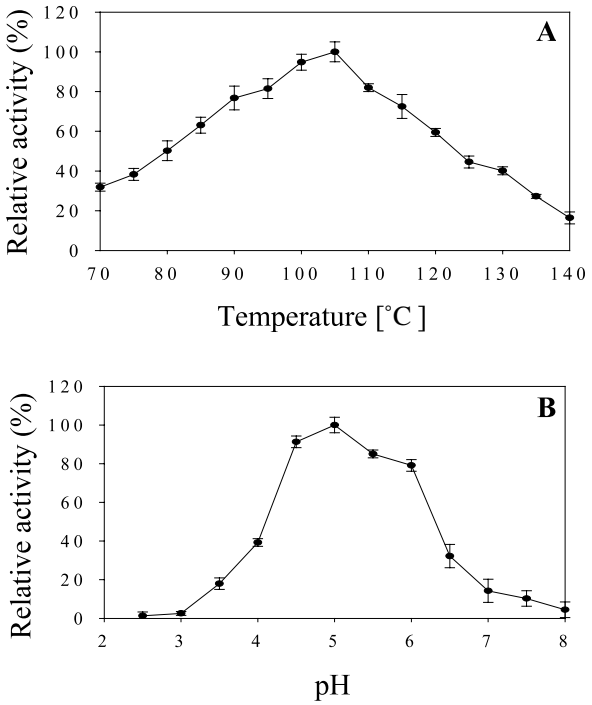

Fig. 2. The temperature and pH profiles of the BEVS-derived β-glucosidase. (A) Using pNPG as substrate the enzymatic activity of β-glucosidase expressed in silkworm was measured at pH 5.0 and a temperature range from 70 to 140 °C in a water or oil bath; the optimal temperature for enzymatic reaction was 105 °C. (B) The enzymatic activity was measured at 90 °C and different pH values from 2.5 to 8.0; the optimal pH value for enzymatic reaction was 5.0.

this experiment, the silkworm haemolymph samples were heated at 90 °C for 15 min, resulting in the denaturation and precipitation of the majority of haemolymph proteins. Centrifugation of the heat-treated samples produced supernatants containing about 81% of β-glucosidase in the total protein according to densimeter scanning. This method has primary application as a means of obtaining a majority of thermotolerant enzymes from silkworm body, which also achieves a rapid, simply established procedure of purification. Expressing β-glucosidase using BEVS can improve enzyme yield, and subsequent purification by heat-treatment will also save time and reduce expenditure.

Enzymatic characterization of β-glucosidase

All experiments concerning the optimization of the reaction were performed with pNPG as substrate. The optimum temperature and pH value of β-glucosidase were recorded at 105 °C and pH 5.0, respectively (Fig. 2). β-Glucosidase displayed a maximum activity at 105 °C, similar to that reported previously (Kengen *et al.*, 1993; Pouwels *et al.*, 2000). More than 40% β-glucosidase activity remained in a pH range from 4 to 6.5, and there was a sharp increase from pH 4 to 4.5. In the thermostability assay, there was no significant change when the enzyme was pre-incubated at 90, 100 and 110 °C for 10 min, and 80% β-glucosidase activity remained when pre-incubating at 120 and 130 °C for 10 min. It has a sharp drop when the sample was incubated at 140 °C for 10 min. Furthermore, the entire β-glucosidase activity still remained when the diluted haemolymph samples were kept at room temperature for 4 months. There was also no remarkable difference in the β-glucosidase activity in the presence of 1 mM divalent cations. Previous reports showed that several enzymes from thermophiles are stimulated by high ionic strength. It was proved that insignificant influence of high ionic strength on the activity of BEVS-derived β-glucosidase occurred in the presence of varying concentrations of NaCl.

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