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Cloning and expression of β-glucosidase from *Flavobacterium meningosepticum*: A new member of family B β-glucosidase

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A β*-glucosidase gene from* Flavobacterium meningosepticum *has been cloned and expressed in* Escherichia coli*. The entire nucleotide sequence was determined and analyzed. An open-reading frame of 2,178 bp encoding a polypeptide of 726 amino acids with a calculated* M_r *of 79,952 was derived from the sequence. The coding region is flanked by a putative promoter and transcription terminator sequences. The nucleotide sequence accession number of the complete gene sequence in Genbank is AF015915. Based on the comparison of amino acid sequences, this enzyme is classified as a family B* β*-glucosidase and is highly homologous with sequences from* Clostridium thermocellum, Kluyvermyces fragilis, *and* Agrobacterium tumefaciens*. The cloned enzyme was purified to near homogeneity by ammonium sulfate fractionation (35 ~ 75%) and chromatography on a cation-exchanged column at pH 6.9. This expressed* β*-glucosidase has optimum activity at pH 4.2 ~ 5.0 and 50°C and is stable in the pH range of 5.0 ~ 8.1 at 25°C. It showed a high specificity on the glycone portion of aryl-*β*-*D*-glycosides. All of these characteristics are highly consistent with those of the native* β*-glucosidase.* © *1998 Elsevier Science Inc.*

Keywords: *Flavobacterium meningosepticum*; cloning; expression; β-glucosidase

Introduction

β-Glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) catalyzes the hydrolysis of a variety of β-glucosides and oligosaccharides. The enzyme is widely distributed in nature. Several of these enzymes have been purified from different sources including human tissue,[1,2] plant,[3–5] fungi,[6–9] and bacteria.[10–12] In recent years, a number of β-glucosidase genes have been cloned from various microorganisms and more than 20 nucleotide sequences have been analyzed in organizing β-glucosidase families and catalytic domains. From the amino acid sequence comparison, β-glucosidase can be divided into two families: A and B[13,14] (or family 1 and 3 by Henrissat's classification[15,16]). The family A enzymes were found in organisms of all three primary kingdoms and possess a distinctive feature in their low substrate specificity with respect to the C-4 configuration; thus, they are active on both β-glucosides and β-galactosides.[17–19] Family B enzymes are found in rumen bacteria as well as in fungi. Another feature of this classification is that family A β-glucosidases contain fewer than 540 amino acids ($M_r < 60$ kDa per subunit) while family B β-glucosidases are longer than 700 amino acids ($M_r > 77$ kDa per subunit).[13,14] We have recently purified a β-glucosidase from *F. meningosepticum* (Li, unpublished data). This enzyme is a dimeric protein with a M_r of 78 kDa for each subunit. The enzyme is active on PNPG and cellobiose although the activity (k_{cat}/K_m) for PNPG is at least 100-fold better than that for cellobiose. In celluloytic processes, β-glucosidase (more specifically, cellobiase) plays an important role in hydrolyzing cellobiose to glucose. Cellobiose inhibits the catalytic functions of cellobiohydrolase (EC 3.2.1.91) and endo-β-glyconase (EC 3.2.1.4). Because of their biotechnological importance, microbial β-glucosidases have attracted a great deal of attention from biochemical researchers. For further study on *F. meningosepticum* β-glucosidase, we have cloned the gene and expressed the gene product. The molecular cloning of genes not only provides a powerful tool for producing protein on the large scale but also offers the possibility of applying protein engineering

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methods in analyzing enzymatic action and improving enzyme functions. In this paper, we report the nucleotide sequence of the *F. meningosepticum bgl* gene, the deduced amino acid sequence, and the purification of the encoded β-glucosidase. Furthermore, the deduced amino acid sequence was compared with those from other microbes.

Materials and methods

Materials

Buffers were either purchased from Sigma Chemical (St. Louis, MO) or E. Merck Co. (Gibbstown, NJ). The buffer system consisted of 100 mM NaCl and 50 mM of the following buffers: NaOAc (3.5 ~ 5.6); MES (5.6 ~ 7.0); MOPS (6.5 ~ 7.9); phosphate (7.0 ~ 7.9); HEPES (6.8 ~ 8.2); and BICINE (7.6 ~ 9.0). Substrates were purchased from Sigma. Restriction enzymes, calf intestinal alkaline phosphatase, and T4 DNA ligase were purchased from either New England Biolabs (Beverly, MA) or Boehringer Mannheim (Mannheim, Germany) and used according to the recommendation of the supplier. Oligonucleotide primers were synthesized by PE Applied Biosystems (Perkin-Elmer, Taiwan).

Bacterial strains and plasmids

All microorganisms including *F. meningosepticum* (ATCC 13253, also known as *Chryseobacterium meningosepticum*) and *Escherichia coli* strains were obtained from CCRC (Taiwan). *E. coli* strains JM109 and BL21 (DE3) served as cloning and expression hosts, respectively. Plasmid pUC19 was used as an *E. coli* cloning vector for sequencing. Plasmid pCR-Scrip™ Amp SK (+) obtained from Stratagene Co. was employed for expression in BL21 (DE3).

Isolation of genomic DNA from F. meningosepticum

F. meningosepticum was precultured on a solid M3 medium containing meat extract (3 g l^{-1}), peptone (5 g l^{-1}), and 1.5% agar. A single colony was selected and inoculated to 10 ml M3 medium. Cells for isolating genomic DNA were harvested from a 50-ml overnight culture and resuspended in 6 ml Tris/EDTA (10 mM Tris-HCl pH 8; 1 mM EDTA). Proteinase K and SDS were added to a final concentration of 0.2 mM and 1%, respectively. After incubation for 2 h at 37°C, 1 ml of 5 M NaCl and 0.87 ml of 10% CTAB were added. The resulting mixture was incubated at 65°C for 40 min and then extracted with an equal volume of chloroform. The mixture was centrifuged (10,000 *g* for 15 min), and the aqueous phase was extracted twice with phenol/chloroform. The nucleic acid was precipitated in 0.3 M sodium acetate and 2.5 volumes ethanol at −20°C. The genomic DNA was resuspended in Tris/EDTA and stored at −20°C for later applications.

Cloning and screening for positive colonies

The genomic DNA of *F. meningosepticum* was partially digested with *Sau3A* I. DNA fragments between 6–9 kb were obtained through the ultracentrifugation of a 10–40% sucrose gradient. The DNA fragments were ligated into the cloning vector pUC19 and pretreated with *Bam*H I and calf alkaline phosphatase. Transformation of *E. coli* strain JM109 was accomplished by the $CaCl_2$ method.[20] Clones harboring the recombinant plasmid with the β-glucosidase gene were first selected on Luria broth (LB) plates containing 100 μg ml^{-1} ampicillin and screened by overlaying 4 ml top agar containing 0.1% 4-methylumbelliferyl-β-D-glucoside (MUG) over the colonies. After 2–4 h incubation at 37°C, positive clones were fluorescent when observed under UV light. To confirm activity, positive colonies were restreaked on LB agar plates containing MUG.

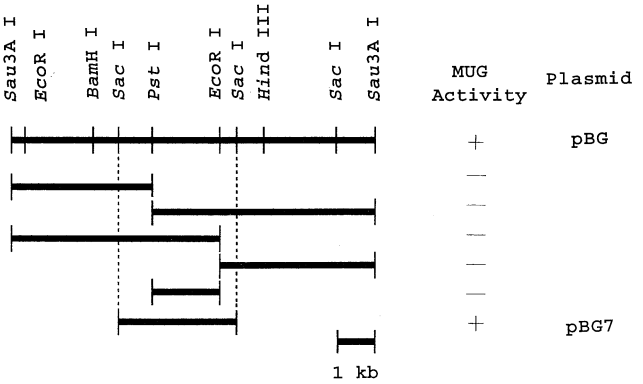

Figure 1 Restriction maps of cloned segments and localization of essential regions. pBG is the original plasmid. The others are subcloned plasmids. Only pBG and pBG7 have β-glucosidase activity.

Nucleotide sequence analysis

The DNA sequencing reactions were conducted by using PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) with supercoiled plasmids as templates. The data were collected and analyzed with a ABI PRISM™ 310 DNA Sequencer. The sequencing protocol was employed as described in the manufacturer's protocol. Sequence data and multiple protein sequence alignments were analyzed with GenBank data by using the DNASIS PRO V3.2 (Hitachi) software package.

Construction of expression vector

Since there were no convenient restriction sites with which to subclone the β-glucosidase gene into the expression vector, two oligonucleotide primers designated as PS1 and PT1 were designed and synthesized to direct PCR amplification of the full-length protein coding region. The sequences are listed with the bold-type letters representing the mismatching positions for insertion of new restriction sites: PS1 (−154 ~ −125): 5′-AATAT**GGT**A**C** **CAT**ATGAAAAAATTATCAAT-3′; PT1 (+2,330 ~ +2,304): 5′-GGAT**GG**AT**CC**AAATTTCTTAAATAAAG-3′. The PCR products were ethanol precipitated, blunt-end ligated into a *srf* I-digested pCR-Scrip™ Amp SK (+) vector, and further transformed into BL21. The plasmid obtained with correct orientation was designated pCRS1.

Expression and purification of cloned β*-glucosidase*

All purification steps were performed at ambient temperature (approximately 25°C). A 1-l culture of *E. coli* BL21 (DE3) with pCRS1 was grown at 37°C to mid-log phase in LB medium containing 100 μg ml^{-1} ampicillin. The transcription was then induced by adding IPTG to a final concentration of 0.5 mM, and growth was continued for 6 h. After centrifugation, the cell pellet was resuspended in 15 ml 50 mM Tris-HCl pH 7.6 containing 0.1 mM PMSF and sonicated for a total of 10 min with four intervals of 2 min each. The lysate was centrifuged at 10,000 *g* for 30 min, and the cellfree extract was subjected to ammonium sulfate fractionation. The precipitate between 35 ~ 75% ammonium

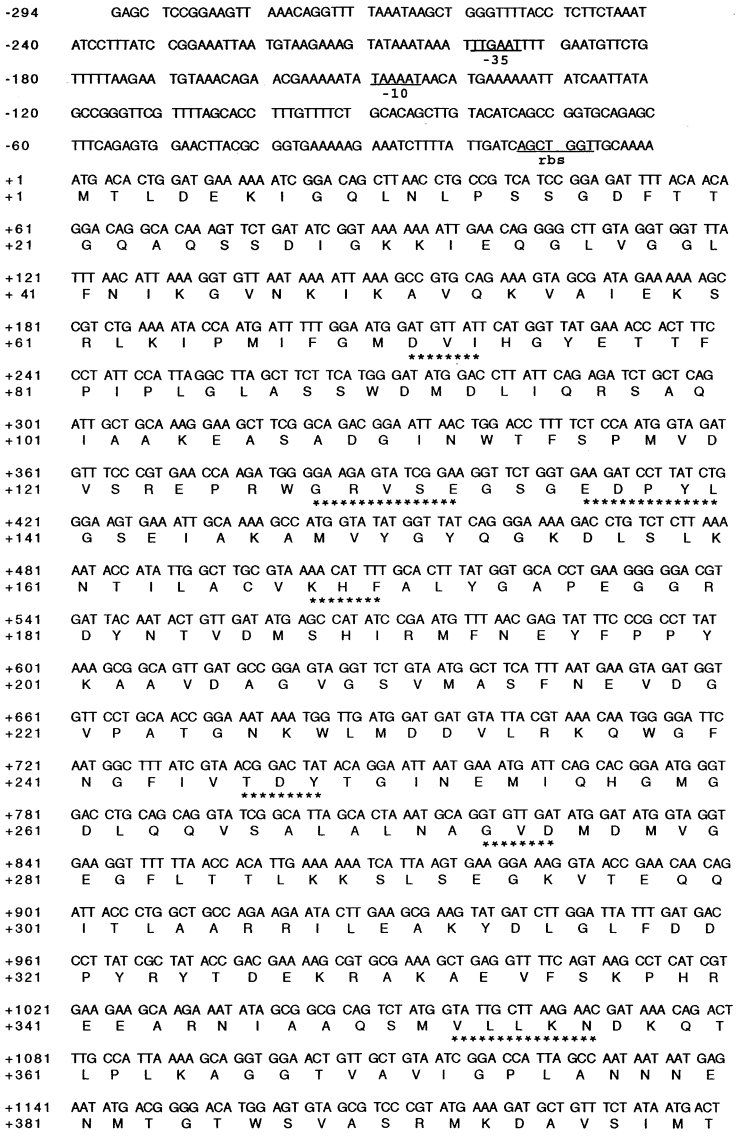

Figure 2 (A and B) Nucleotide sequence of the *F. meningosepticum bgl* gene and deduced amino acid sequence of β-glucosidase. The putative Shine-Dalgarno ribosomal binding site (rbs) is underlined. The potential −10 and −35 promoters are also indicated. The possible transcription terminator is underlined by facing arrows. The conserved amino acid sequences are marked by asterisks. Nucleotide (amino acid) numbers start at the first residue of the coding sequence and refer to the first nucleotide (amino acid) of each line.

sulfate saturation was collected and desalted using a HiTrap desalting column (Pharmacia, Uppsala, Sweden). The filtrate was then applied onto a cation-exchanged column (5 ml HiTrap SP, Pharmacia) pre-equilibrated with 20 mM phosphate buffer pH 6.9. The column was eluted with a 100 ml linear gradient of NaCl (0 ~ 300 mM) at a flow rate of 1 ml min^{-1}. The enriched enzyme was collected and stored at 4°C for further studies.

Enzyme assays

In the activity assay, 0.08 ~ 0.16 μg purified protein was added to 0.5 ml 50 mM phosphate buffer pH 7.0 containing 1 mM *p*-nitrophenyl-β-D-glucopyranoside. In all assays, an enzyme unit was defined as the amount of enzyme required for releasing 1 μmol *p*-nitrophenol from substrate in 1 min. The absorbance of *p*-nitrophenolate was measured at 400 nm. The extinction coefficient of *p*-nitrophenolate/*p*-nitrophenol is 8,100 M^{-1} cm^{-1} at pH 7.0.

Results and discussion

Isolation and characterization of genomic gene encoding β-glucosidase

One positive transformant which was fluorescent under UV light was found from 4,000 colonies. The recombinant plasmid from this clone was denoted as pBG.

In order to locate the β-glucosidase gene in the pBG 9-kb (*Sau3A* I partially digested) fragment, a series of subclones was constructed from a restriction map of pBG using

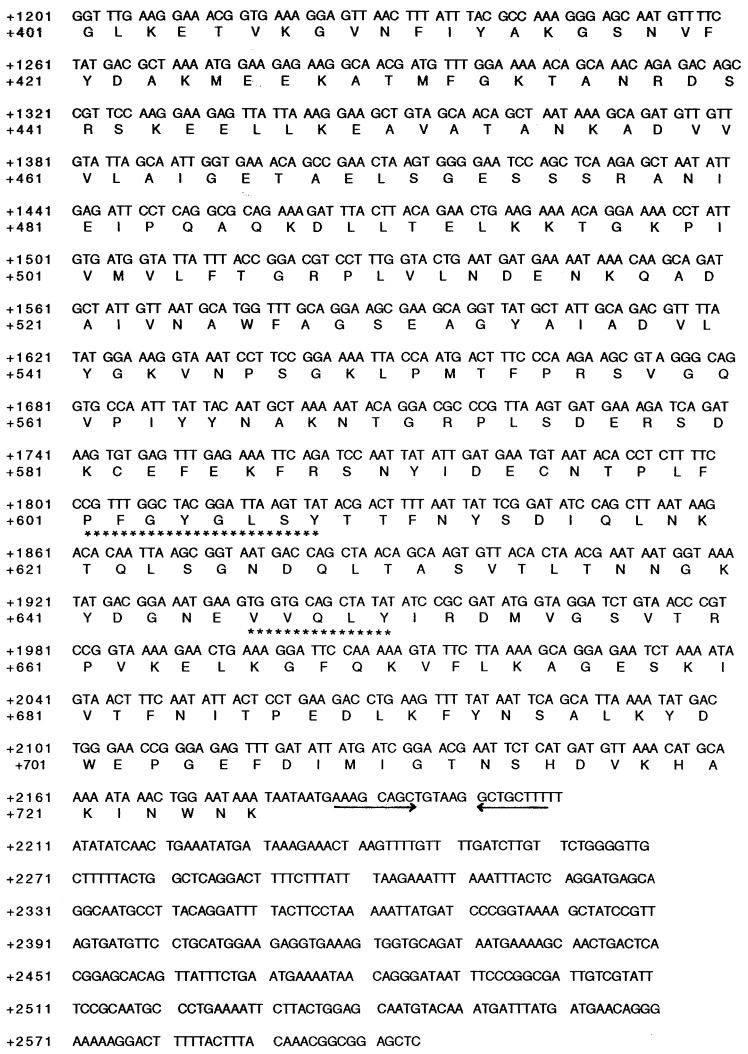

Figure 2 (Continued)

pUC19 again as a vector (*Figure 1*). Among the six subclones, pBG7 containing a 2.9-kb *Sac* I-digested DNA insert was shown to include a β-glucosidase (*bgl*) gene. This plasmid DNA was then sequenced. The nucleotide sequence and the deduced protein sequence of *bgl* gene are shown in *Figure 2*. A 2,178 bp open reading frame (ORF) initiating at ATG (position 1) and terminating at TAA (position 2,179) was identified within this sequence. The ORF encodes a polypeptide of 726 amino acids with a calculated M_r of 79,952. This molecular mass is consistent with the value measured from the purified *F. meningosepticum* enzyme (*Figure 3*). A putative ribosomal-binding site of seven nucleotides (AGCTGGT) was located seven nucleotides upstream of the translational start codon (ATG). Within the 5′-noncoding region, a possible −10 promoter sequence (TAAAAT) is located at position −145, which is preceded by a potential −35 sequence (TTGAAT) at position −187. A palindromic sequence of a possible transcription terminator is located 6 bp downstream of the stop codon (TAA) between nucleotide positions 2,187 and 2,208.

Nucleotide sequence accession number

The Genbank nucleotide sequence accession number of the complete gene sequence is AF015915.

Comparative analysis of amino acid sequences of family B β-glucosidase from different organisms

β-glucosidases are found in organisms such as mammals, plants, fungi, and bacteria. The diversity of β-glucosidases might serve as a useful tool for tracing an evolutionary event or to make a possible prediction of the active site position. The mechanistic actions of glycosyl hydrolases are thought to be a general-acid catalysis in which two amino acids participate in a single-displacement or double-displacement reaction resulting in inversion or retention of configuration, respectively, at the anomeric carbon atom of the hydrolyzed glycoside.[21] By analyzing the primary structure of enzymes and finding the invariant amino acid residues, it is possible to locate the potential active site

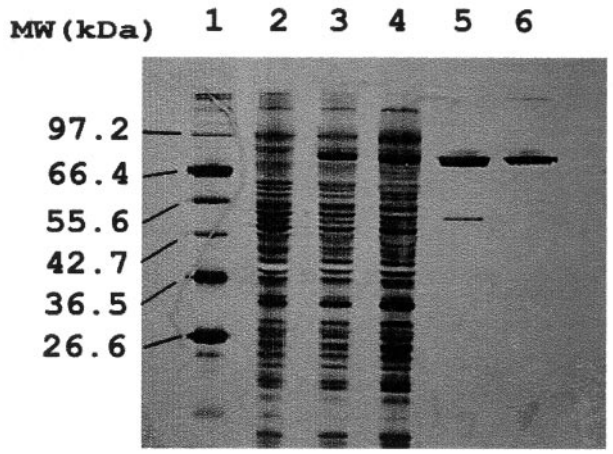

Figure 3 Electrophoretic analysis of the expressed β-glucosidase enzyme at various stages of purification. Separation was performed on a 12.5% (w/v) SDS-polyacrylamide gel. Lanes: Marker (1); crude extract from IPTG-induced BL21 (DE3) without pCRS1 (2); crude extract from IPTG-induced BL21 (DE3) containing pCRS1 (3); precipitate of 35 ~ 75% ammonium sulfate fractionation (4); purified β-glucosidase from HiTrap chromatography (5); and purified β-glucosidase from *F. meningosepticum* (6).

position.[22] More than ten nucleotide sequences related to β-glucosidase were retrieved from GenBank and compared with the *F. meningosepticum bgl*. The amino acid multiple alignment revealed several family B β-glucosidases including those from *Clostridium thermocellum*, *Kluyvermyces fragilis*, and *Agrobacterium tumefaciens* with high homology to *F. meningosepticum* β-glucosidase (*Table 1*). The greatest homology was to *C. thermocellum* β-glucosidase B (β-glu B) with a 33.8% identity. Sequences (DPYL, KHF, VLLKN, and FGYGLSY) found in *F. meningosepticum* β-glucosidase are identical with four of the eight conserved patterns in family B enzymes.[14] The rest of the eight sequences (DVI, GRVSE, TDY, and GVD) also showed high similarity to the correspondent conserved patterns. The positions of conserved sequences are indicated (by asterisks) in *Figure 2*. The *F. meningosepticum* β-glucosidase is therefore classified into family B.

Purification and characterization of cloned β-glucosidase

The recombinant β-glucosidase, encoded by the pCRS1 plasmid, was purified to near homogeneity by ammonium sulfate fractionation and HiTrap SP (*Table 2*). With this simple process, an 85-fold purification and 30% yield was reached. Further purification by HiTrap SP or Mono-S chromatography at pH 6.6 did not enhance purity. Electrophoretic analysis of enzyme purity from various steps of purification is shown in *Figure 3*. The molecular mass of the cloned enzyme was determined to be 78 kDa which is consistent with the size predicted from the nucleotide sequence data and the native enzyme from *F. meningosepticum*. The physical properties of the cloned β-glucosidase were investigated and compared with those of the native enzyme (Li, unpublished data). For thermostability experiments, 300-μl portions of purified β-glucosidase were heated in glass tubes at 30, 37, 50, and 60°C. The concentration of recombinant enzyme was 0.1 μg ml^{-1} in 50 mM phosphate buffer pH 7.0 with 100 mM NaCl. After being heated for the appropriate time interval, 50 μl of samples were then removed to assay the residual activity in phosphate buffer pH 7.0 at 25°C. For pH stability experiments, enzyme samples (same as above) were incubated in a series of buffers with pH values of 3.8, 5.0, 6.0, 7.0, 8.1, and 9.3 at 25°C for 60 min. Samples were removed for assay at different time intervals. The activity measured at pH 7.0 and 25°C served as control. Results showed that the recombinant enzyme has optimum activity at pH 4.2 ~ 5.0 and 50°C and is stable in the pH range of 5.0–8.1 at 25°C. The K_m value of PNPG was 0.68 mM. The activity of this enzyme was also tested on a group of *p*-nitrophenyl-β-D-glycosides including galactoside, mannoside, N-acetylglucosaminide, and xyloside. No significant activity (less than 1% of that of PNPG) can be detected. This cloned enzyme presented a high specificity on the glycon portion of aryl-β-D-glycosides. All of these characteristics are highly consistent with those of the native β-glucosidase.

Table 1 Comparisons of *F. meningosepticum* β-glucosidase with various microbial β-glucosidases[a]

Protein	Species	Family[b]	Identity (%)	Similarity (%)	GenBank	Reference
β-glu A	*Clostridium thermocellum*	A1	19.4	44.4	X60268	23
β-glu A	*Bacillus polymyxa*	A1	18.3	44.8	M60210	24
β-glu B	*Agrobacterium* sp.	A1	19.0	43.4	M19033	25
β-glu 1	*Trifolium repens*	A2	16.4	42.3	X56734	26
β-glu	*Manihot esculenta*	A2	24.0	47.8	S35175	27
β-glu 1	*Saccharomyces fibuligera*	B2	26.8	50.5	M22475	28
β-glu	*Candida pelliculosa*	B2	24.8	51.6	X02903	29
β-glu	*Agrobacterium tumefaciens*	B3	33.0	55.5	M59852	30
β-glu B	*Clostridium thermocellum*	B3	33.8	55.1	X15644	31
β-glu	*Kluyveromyces fragilis*	B3	32.4	55.1	X05918	32

[a]The comparison of amino acid sequences was analyzed by using the BESTFIT program from the Genetics Computer Group (GCG) package.[33]
[b]The classification is adapted from reference 13.

Table 2 Purification of β-glucosidase from BL21 (DE3) containing plasmid pCRS1[a]

Step	Total protein (mg)	Total activity (unit)	Specific activity (unit mg^{-1})	Purification (fold)	Yield (%)
Crude extract	975	26,940	27.6	1	100.0
35 ~ 75% sat. $(NH_4)_2SO_4$	436	15,517	35.6	1.3	57.6
HiTrap SP	3.1	7,408	2,369	85.7	27.5

[a]Protein concentration was determined by the BCA method.[34] An enzyme unit is defined as the amount of enzyme required to release 1 μmol *p*-nitrophenol from substrate in 1 min.

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List of symbols

CTAB Hexadecyltrimethyl ammonium bromide;
PNPG *p*-Nitrophenyl-β-D-glucopyranoside;
MUG 4-methylumbelliferyl-β-D-glucopyranoside;
CIP Calf intestinal alkaline phosphatase;
IPTG Isopropylthio-β-D-galactopyranoside

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