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Overexpression, purification and preliminary X-ray analysis of pullulanase from *Bacillus subtilis* strain 168

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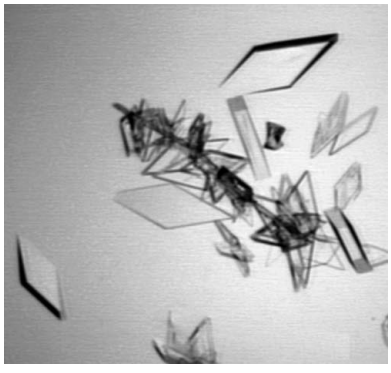

The *AmyX* gene encoding pullulanase from the common spore-forming bacterium *Bacillus subtilis* strain 168 was cloned, overexpressed in *Escherichia coli*, purified and crystallized. The recombinant pullulanase was purified to homogeneity using ammonium sulfate precipitation, hydrophobic chromatography and anion-exchange chromatography, resulting in a specific activity of 24.10 U per milligram of protein. SDS–PAGE analysis showed that the molecular weight of the protein is approximately 81.0 kDa, which is similar to the calculated molecular weight, 81.1 kDa, from its translated cDNA sequence. The k_{cat} and K_m of the purified enzyme with pullulan as substrate were approximately 79 s^{-1} and 1.284 mg ml^{-1}, respectively. X-ray crystallographic analysis of the pullulanase crystal showed that the crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 70.568, b = 127.68, c = 189.25 Å. The crystal contains two molecules of pullulanase in the asymmetric unit, with a solvent content of 53.15%. The crystal diffracted to 2.1 Å resolution at a synchrotron and is suitable for structure determination.

1. Introduction

Pullulanase (pullulanase type I, pullulan 6-glucanohydrolase, EC 3.2.1.41), also known as limit dextrinase, is a debranching enzyme that specifically breaks up α-1,6-glycosidic linkages of pullulan, starch and amylopectin. Pullulanases can be found in higher plants (Nakamura *et al.*, 1996; Renz *et al.*, 1998; Kristensen *et al.*, 1999; Beatty *et al.*, 1999), as well as in bacteria, particularly the genera *Bacillus* (Jensen & Norman, 1984; Kuriki *et al.*, 1990; Suzuki *et al.*, 1991; Ara *et al.*, 1992; Kim *et al.*, 1993), *Fervidobacterium* (Bertoldo *et al.*, 1999), *Klebsiella* (Michaelis *et al.*, 1985; Eisele *et al.*, 1972; Dupuy *et al.*, 1992), *Thermotoga* (Kriegshauser & Liebl, 2000) and *Thermus* (Tomiyasu *et al.*, 2001).

After the recent discovery of a new type of pullulanase that can hydrolyze both α-1,6- and α-1,4-linkages, the enzymatic nomenclature of pullulanases was divided into two groups: (i) pullulanase type I, which specifically cleaves the α-1,6-glycosidic linkages in pullulan and branched oligosaccharides, forming maltotriose and linear oligosacharides, respectively, and (ii) pullulanase type II, or amylopullulanase, which hydrolyzes both α-1,6-glycosidic linkages and α-1,4-glycosidic linkages in branched and linear oligosaccharides (Bertoldo & Antranikian, 2002). In addition, based on amino-acid sequence similarities these pullulanases are classified into glycoside hydrolase (GH) family 13 together with α-amylases and isoamylases (Henrissat, 1991; http://afmb.cnrs-mrs.fr/CAZY/GH_13.html).

In the food industry, pullulanases are used in the brewing process and starch hydrolysis together with β-amylases in order to produce a starch syrup that is high in maltose content (Belitz & Grosch, 1999).

To comprehensively understand the function of pullulanases at the cellular level, it is important to resolve their structures in native and complexed forms. Preliminary studies have been reported on crystals of pullulanase from *Pyrococcus woesei* (Knapp *et al.*, 1995) and *Fervidobacterium pennivorans* (Lebbink *et al.*, 2000). However, to our knowledge, no data on the three-dimensional structures of pullulanases (type I or II) have yet been published or deposited in the Protein Data Bank (PDB). We now report the cloning and expression of the pullulanase (*AmyX*) gene from *Bacillus subtilis* strain 168 in

Escherichia coli as well as the purification and preliminary crystallographic analysis of the enzyme.

2. Experimental procedures

2.1. Bacterial strains, plasmid, enzymes and reagents

The genomic DNA of *B. subtilis* strain 168 was used to amplify the *AmyX* gene. Expression hosts HMS 174 (DE3), BL21 (DE3), AD 494 (DE3) and Origami (DE3) were obtained from Novagen (UK). The plasmid pET2d was purchased from Novagen (UK). Restriction enzymes *Nco*I and *Xho*I were from Nippon Gene (Japan), while Ligation High was from Toyobo (Japan). PCR reagents were from Toyobo (Japan). IPTG, X-gal, agarose, ampicillin and other reagents for protein purification were purchased from Wako (Japan).

2.2. Construction of recombinant expression plasmid

Genomic DNA of *B. subtilis* strain 168 was isolated according to the method of Sambrook *et al.* (1989). PCR was performed to amplify the entire *AmyX* gene of 2157 bp coding for pullulanase using the genomic DNA as a template and two primers, 5′-GG**CCATGG**T-CAGCATCCGCCGCAGCTTCGA-3′ (forward) and 5′-GG**CTC-GAG**TCAAGCAAAACTCTTAAGATCT-3′ (reverse) containing *Nco*I and *Xho*I restriction-enzyme sites (bold), respectively. The two primers correspond to the N- and C-terminal amino-acid sequence of BSP, respectively. PCR amplification was carried out using the KOD Plus system (Toyobo, Japan), with a thermal profile consisting of denaturation at 367 K for 2 min and 30 cycles of 367 K for 15 s, 333 K for 30 s and 341 K for 4 min. The amplified gene (*AmyX*) was digested with *Nco*I and *Xho*I. The digestion product was then ligated with the *Nco*I and *Xho*I double-digested pET21d expression vector and transformed into HMS 174 (DE3) cells. The DNA sequences were confirmed by automated DNA sequencing.

2.3. Expression of the recombinant *B. subtilis* pullulanase (rBSP)

E. coli strain HMS 174 (DE3) harbouring pEBSP was inoculated in a 2500 ml flask containing 500 ml LB medium supplemented with 100 µg ml^{-1} ampicillin and was then incubated at 310 K with orbital shaking. The culture was induced with IPTG to a final concentration of 0.5 m*M* when the absorbance at 600 nm reached 0.6–0.8. The culture was further incubated at 291 K for 60 h. The cells was harvested by centrifugation at 6000 rev min^{-1} for 20 min at 277 K. The cell pellets were suspended in 20 m*M* Tris–HCl buffer pH 8.1 containing 5 m*M* EDTA, 20 m*M* β-mercaptoethanol (ME) and 200 µ*M* PMSF and then disrupted by sonication at 200 µA for 30 min at 277 K. Finally, the sonicated mixture (lysate) was centrifuged at 14 000 rev min^{-1} for 30 min at 277 K.

2.4. Purification of the rBSP

The crude extract was first fractionated with 30% saturated ammonium sulfate. The mixture was centrifuged and the supernatant was further fractionated with ammonium sulfate to a final saturation of 60%. After centrifugation, the supernatant was discarded and the pellets were collected and dissolved with the same buffer to a final saturation of 20%.

The protein solution was applied onto a Toyopearl Butyl-650M column (Tosoh, Japan) previously equilibrated with the same buffer containing 20% saturated ammonium sulfate. Proteins were eluted with a decreasing gradient of ammonium sulfate concentration from 20 to 0%. The fractions showing pullulanase activity were pooled and dialyzed against 20 m*M* Tris–HCl buffer pH 8.1 containing 5 m*M* EDTA, 20 m*M* ME and 200 µ*M* PMSF. The resultant solution was loaded onto a previously equilibrated Hiload Q-Sepharose column (GE Healthcare Bio-Sciences, NJ, USA). The proteins were eluted with a linear gradient of NaCl from 0 to 0.5 *M*. Active fractions were pooled and dialyzed against the same buffer to remove the salt. The desalted protein solution was applied onto a Mono-Q column (GE Healthcare Bio-Sciences, NJ, USA) which was previously equilibrated with the same buffer used in the previous step. The proteins were eluted with a linear gradient of NaCl from 0 to 0.5 *M*.

2.5. Enzyme assay

Pullulanase activity was measured in 0.1 *M* acetate buffer pH 5.4 according to Bernfeld (1955) with slight modification using pullulan as substrate. Briefly, a mixture of 500 µl 1% pullulan and 450 µl 0.2 *M* acetate buffer pH 5.4 was equilibrated at 310 K for 15 min. 50 µl enzyme solution was then added and the mixture was incubated for a further 4 min. The reaction was stopped by the addition of 1 ml 3,5-dinitrosalicylic (DNS) acid solution and the mixture was heated for another 5 min in a boiling-water bath. The mixture was then placed in an iced-water bath. The absorbance was measured at 540 nm. One unit of pullulanase activity is defined as the amount of enzyme needed to liberate reducing sugar equivalent to 1 µmol maltotriose per minute at 310 K. The kinetic parameters K_m and V_{max} for pullulan of the purified pullulanase were analyzed using a series of pullulan concentrations: 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 mg ml^{-1} in 0.1 *M* acetate buffer pH 5.4. The K_m and V_{max} values for the enzyme were calculated using the Michaelis–Menten equation with *KaleidaGraph* v.3.6 (Synergy Software, PA, USA).

The protein content was determined spectrophotometrically at 280 nm or by the Bradford method (Bradford, 1976) with BSA as a standard.

2.6. Characterization of rBSP

Protein was analyzed by SDS–PAGE using an ATTO AE-6530 Dual mini-slab system using a 10% acrylamide gel (Laemmli, 1970). Broad molecular-weight standards (APRO, Japan) were used to estimate the size of the recombinant protein. Gels were stained using Coomassie Brilliant Blue R-250.

The effect of pH and temperature on the activity of the purified pullulanase was analyzed using approximately 90 U mg^{-1} enzyme. For the pH–activity profile, 0.1 *M* Britton–Robinson buffer was used instead of the acetate buffer in order to obtain a wide range of pH values (3.0–10.0). For the effect of temperature, the assay mixture (at the pH optimum) was incubated at temperatures between 298 and 348 K.

2.7. Crystallization and X-ray data collection

Initial screening for BSP crystallization was performed with commercially available crystallization kits from Emerald Biostructures Inc. (Bainbridge Island, WA, USA) and Hampton Research (Laguna Niguel, CA, USA) using the sitting-drop vapour-diffusion method with 96-well plates. The crystals were grown at 293 K. Further screening was carried out by modification of the PEG molecular weight and concentration and by replacement of the buffer and salt. Final crystal preparation was performed by the hanging-drop vapour-diffusion method using 24-well plates. The hanging drop (6 µl) contained 3 µl enzyme solution and 3 µl reservoir solution (1 ml) consisting of 10% PEG 4000, 0.1 *M* acetate buffer pH 5.2, 0.2 *M* $Mg(CH_3COO)_2$. The concentration of rBSP was adjusted to 10 mg ml^{-1} from the initial concentration.

Table 1
Purification of *B. subtilis* pullulanase.

Purification step	Protein (mg ml^{-1})	Activity (U ml^{-1})	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification fold (%)
Crude extract†	71.72	38.03	3232.84	0.53	100.00	1.00
60% ammonium sulfate fraction	54.32	33.41	2004.62	0.62	62.01	1.16
Toyo Butyl-650M	5.17	24.67	1726.85	4.77	53.42	9.00
Q-Sepharose	2.24	29.25	1316.40	13.06	40.72	24.63
Mono-Q	1.68	40.48	404.81	24.10	12.52	45.44

† From approximately 15 g cells (wet weight).

Prior to data collection, the crystals were treated with cryoprotectant [30%(*v*/*v*) glycerol in reservoir solution] and flash-cooled in a nitrogen-gas stream (100 K). Diffraction data were collected at beamline BL38B1, SPring-8 synchrotron (Hyogo, Japan). The collected images were processed with *HKL*2000 (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Overexpression in *E. coli* and purification of recombinant BSP

Two specific primers were derived from the complete sequence of the *B. subtilis* strain 168 genome for the putative *AmyX* gene (GenBank Accession No. NC_000964 or GeneID No. 937292) coding for pullulanase. Comparison of the nucleotide sequence of BSP cDNA with the putative *AmyX* gene showed a 100% match, indicating that the gene was stable and no mutation had occurred. The plasmids were then cloned into *E. coli* HMS 174 (DE3), BL21 (DE3), AD 494 (DE3) and Origami (DE3) cells for expression.

Expression of recombinant BSP was screened under various growth conditions by analyzing the activity of the enzyme. Four different expression hosts were used for expression screening. However, only BL21 (DE3) was unable to express the target gene. Expression of BSP was dependent on the incubation temperature. No BSP activity was found in the culture grown at 310 K, but high activity was seen at 291–293 K. The lack of activity might arise from misfolding of the protein or from the formation of inclusion bodies, which can be avoided by lowering the incubation temperature to allow correct folding of the protein. In this work, HMS 174 (DE3) host bearing pETBSP was used as the expression source for further analysis.

The purification procedure is summarized in Table 1. The expressed pullulanase was successfully purified to homogeneity as seen on the SDS–PAGE gel (Fig. 1). The molecular weight of the enzyme was estimated to be approximately 81 kDa, which is similar to the calculated molecular weight based on its translated cDNA sequence (81.1 kDa). Ten amino-acid residues of the purified protein, MVSIRRSFEA, obtained after automatic Edman degradation perfectly matched the first ten amino-acid residues of BSP N-terminal sequence, indicating that the protein is exactly pullulanase.

The V_{max} value for pullulan of the purified enzyme was determined to be 27.609 U min^{-1} per milligram of protein, which is equivalent to a k_{cat} of 97 s^{-1}; the K_m value was 1.284 mg ml^{-1}. In *Bacillus* sp. S-1, the K_m for pullulan was 7.92 mg ml^{-1} (Kim *et al.*, 1993), while in *F. pennavorans* Ven5, the pullulanase (type I) showed V_{max} and K_m values of 1.58 U mg^{-1} and 0.4 mg ml^{-1}, respectively, using pullulan as substrate (Dupuy *et al.*, 1992). In addition, *Aerobacter aerogenes* (currently known as *Klebsiella pneumoniae*) was reported to have K_m, V_{max} and k_{cat} values of 0.000017 g ml^{-1}, 52.9 U per milligram of protein and 7700 min^{-1}, respectively (Yokobayashi *et al.*, 1973). This study was used glucose to determine the enzymatic activity. However, we carried out a separate study on the kinetic parameters of commercial *K. pneumoniae* pullulanase (Hayashibara Biochemical Laboratories Inc., Japan) using maltotriose as the standard to determine the enzymatic activity, which showed k_{cat} and K_m values of 116 s^{-1} and 0.617 mg ml^{-1}, respectively.

The optimum pH of pullulanase was found to be 6.0, which is common to pullulan-degrading enzymes from various bacteria such as *Thermus* strain IM6501 (Tomiyasu *et al.*, 2001) and *F. pennovorans* Ven5 (Lebbink *et al.*, 20000). The temperature optimum for activity of the recombinant pullulanase was 313 K.

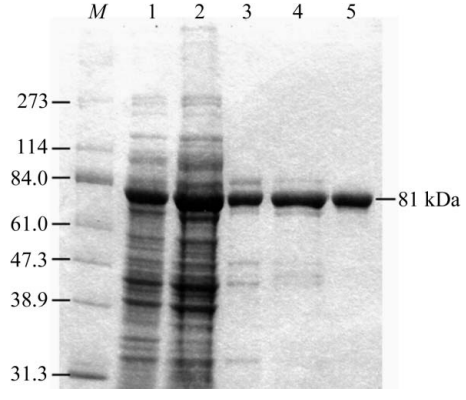

Figure 1
SDS–PAGE protein profile of fractions collected after successive purification steps of the recombinant pullulanase of *B. subtilis* strain 168. Lane *M*, marker proteins (kDa); lane 1, culture supernatant; lane 2, 60% ammonium sulfate fraction; lane 3, pooled fractions from Toyopearl Butyl-650M column; lane 4, pooled fractions from Q-Sepharose column; lane 5, pooled fractions from Mono-Q column.

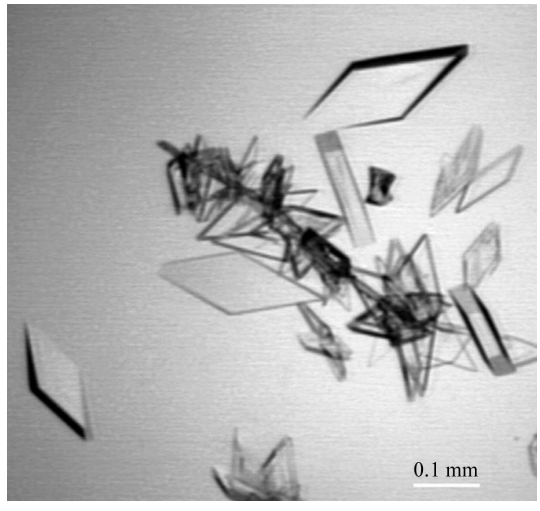

Figure 2
Crystals of recombinant pullulanase from *B. subtilis* strain 168.

Table 2
Data-collection statistics for *B. subtilis* pullulanase.

Values in parentheses are for the highest resolution shell.

X-ray source	SPring-8 BL38B1
Wavelength (Å)	0.8
Detector	Jupiter 210 CCD
Crystal-to-detector distance (cm)	19.5
$\Delta\varphi$ per frame (°)/total no. of frames	1.0/150
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 70.568, b = 127.68, c = 189.25
Resolution range (Å)	46.3–2.1 (2.18–2.10)
Measured reflections	590744 (38618)
Unique reflections	99003 (9775)
Multiplicity	6.0 (5.7)
Completeness (%)	99.6 (99.6)
$R_{\rm merge}$ (%)	7.8 (31.4)
$I/\sigma(I)$	6.8 (2.77)

3.2. Crystallization and X-ray analysis

Small thin crystals were seen after incubation for 24 h using Wizard II screen condition No. 8, which contained 10% PEG 8000, 0.1 *M* phosphate buffer pH 6.2 and 0.2 *M* NaCl. After further modification, favourable conditions for the growth of BSP crystals were achieved and consisted of 10–12% PEG 4000, 0.1 *M* acetate buffer pH 5.2–5.4 and 0.2 *M* $Mg(CH_3COO)_2$ or $Ca(CH_3COO)_2$. Diamond-shaped crystals were obtained under these conditions (Fig. 2). The inclusion of metal ion (Mg^{2+}/Ca^{2+}) improves the physical appearance of the crystals.

Diffraction data sets were collected at liquid-nitrogen temperature using a synchrotron-radiation source (λ = 0.80 Å). A complete X-ray diffraction data set was collected to 2.1 Å resolution with 96.6% completeness. The crystal belongs to the orthorhombic crystal system, space group $P2_12_12_1$, with unit-cell parameters a = 70.568, b = 127.68, c = 189.25 Å. A summary of data statistics is shown in Table 2. The crystal volume per unit protein molecular weight or the Matthews coefficient ($V_{\rm M}$) was determined to be 2.63 Å^3 Da^{-1} and the solvent content ($V_{\rm s}$) to be 53.15%, assuming a dimer of rBSP in the asymmetric unit (Matthews, 1968; Kantardjieff & Rupp, 2003).

To obtain full insight into the three-dimensional structure of BSP, we are now working to elucidate its structure by multiple isomorphous replacement or multiwavelength anomalous scattering methods.

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