Volume 290, number 1,2, 221–223 FEBS 10207 September 1991

ADONIS 0014579391009182

Overproduction, purification and crystallization of *Bacillus cereus* oligo-1,6-glucosidase

Kunihiko Watanabe[1], Kazuhisa Kitamura[1], Yasuo Hata[2], Yukiteru Katsube[2] and Yuzuru Suzuki[1]

[1]*Department of Agricultural Chemistry, Kyoto Prefectural University, Shimogamo, Sakyo, Kyoto 606, Japan* and [2]*Institute for Protein Research, Osaka University, Yamada-oka, Suita, Osaka 565, Japan*

Received 23 July 1991

The gene coding for oligo-1,6-glucosidase from *Bacillus cereus* ATCC7064 has been overexpressed in *Escherichia coli* MV1184 cells under the control of the *lac* promoter in the genetically engineered plasmid pBCE4-2. Oligo-1,6-glucosidase was purified in large quantities and was crystallized at 25°C by using a hanging drop vapor diffusion method with 53% saturated ammonium sulfate. The crystals have the shape of hexagonal bipyramids and belong to the space group $P6_2$ or $P6_4$ with lattice constants of $a=b=106.1$ Å, $c=120.0$ Å and $\gamma=120°$.

Oligo-1,6-glucosidase; Proline theory; Thermostabilization; Crystallization; X-Ray analysis; Overexpression

1. INTRODUCTION

The proline theory for increasing protein thermostability has been proposed from the comparative analysis on five *Bacillus p*-nitrophenyl-α-D-glucopyranoside-hydrolyzing oligo-1,6-glucosidases (dextrin 6-α-D-glucanohydrolase; EC 3.2.1.10) [1,2]. This theory suggests that a protein would be thermostabilized by increasing the frequency of proline occurrence at β-turns (presumably their second sites) and the total number of hydrophobic residues present in the protein [1]. Recently primary sequences of oligo-1,6-glucosidases from mesophile *Bacillus cereus* ATCC7064 and obligately thermophile *Bacillus thermoglucosidasius* KP1006 were compared and indicated that proline residues preferably occur within the loops of the thermostable enzyme, replacing the corresponding ionic or polar residues in the thermolabile enzyme [3–5]. So a knowledge of a high resolution structure of the oligo-1,6-glucosidase protein is needed to explain the significance of the proline theory for protein thermostability from the structural viewpoint. Moreover, the understanding of the three-dimensional structure of the enzyme is important to provide information on the active site of enzymes hydrolyzing an α-1,6-glucoside linkage. A prerequisite for both of these studies is the ability to produce pure samples of the protein in large quantities to obtain crystals suitable for X-ray crystallography. In this report, we describe the construction of an over-expression system for producing *B. cereus* oligo-1,6-glucosidase and the purification and crystallization of the enzyme.

Correspondence address: Y. Suzuki, Department of Agricultural Chemistry, Kyoto Prefectural University, Shimogamo, Sakyo, Kyoto 606, Japan. Fax: (81) (75) 781-1841.

2. MATERIALS AND METHODS

2.1. *Host and plasmids*

Escherichia coli strain MV1184 was used as a host for DNA manipulation and expression [6,7]. The vector used was pUC118 [6]. The original plasmid containing the *B. cereus* oligo-1,6-glucosidase gene was pBCE4 [3]. *E. coli* cells were grown on L-broth medium containing 1% peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose and ampicillin (50 μg/ml) (pH 7.2).

2.2. *Plasmid construction for overproducing oligo-1,6-glucosidase*

Unless otherwise stated, DNA manipulations were performed as described in [8]. Plasmid pBCE4 was digested with *Eco*RI and filled in with Klenow fragment. After a subsequent digestion with *Sal*I, the blunt-*Sal*I fragment containing the oligo-1,6-glucosidase gene was isolated. The vector pUC118 was digested with *Bam*HI and also filled in as well. After a subsequent digestion with *Sal*I, the plasmid was ligated with the blunt-*Sal*I fragment containing the oligo-1,6-glucosidase gene. The resulting plasmid (pBCE4-1) was cleaved with *Kpn*I and *Sma*I, and then subjected to progressive digestion with exonuclease III [9]. The transformants obtained by deletion were transferred onto two L-broth plates in the presence or absence of isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM). The *p*-nitrophenyl-α-D-glucopyranoside-hydrolyzing activity of each colony was assessed after blotting onto a filter paper using the substrate [3,10]. The colonies showing the more activity on the IPTG-containing plate were individually incubated at 37°C in 200 ml L-broth in the presence of 0.5 mM IPTG [3]. The cellular enzyme activity was photometrically determined by using *p*-nitrophenyl-α-D-glucopyranoside and cell extracts sonically prepared in buffer A (50 mM potassium phosphate buffer/5 mM EDTA, pH 7.0) as described before [3,4]. The deleted segments of the plasmids were sequenced by the dideoxy chain termination method [7,11].

2.3. *Overproduction and purification of* B. cereus *oligo-1,6-glucosidase*

All purification procedures were performed at 4°C. Protein determination was done by the Lowry method with bovine serum albumin as

standard [12]. *E. coli* MV1184 containing the plasmid pBCE4-2 was aerobically grown for 18 h in L-broth/IPTG (0.5 mM). The cell extract (270 ml) was prepared by sonication from 16 g wet cells (total 2.6 liters of culture) suspended in buffer A. The precipitates from 35–85% saturated $(NH_4)_2SO_4$ fractionation were collected by centrifugation, dissolved in buffer A and dialyzed against the same buffer. The dialyzate was applied to a DEAE-cellulose column (4.5 × 26 cm), followed by elution with 0–0.4 M NaCl linear gradient (total 2.2 liters). The active fractions (500 ml) were concentrated to 50 ml by ultrafiltration through a Diaflo-Amicon membrane PM-10 (Amicon Corp.). The concentrate was dialyzed against 30% saturated $(NH_4)_2SO_4$/buffer A and then was applied to a butyl-Toyopearl 650M column (3.0 × 13 cm) equilibrated with the dialysis buffer. Elution was performed at a flow rate of 10 ml/h with a linear gradient of 30–0% saturated $(NH_4)_2SO_4$ (total 600 ml). The active fractions (total 150 ml) were combined, concentrated to 30 ml by ultrafiltration and dialyzed against 10 mM phosphate buffer (pH 7.0). The dialyzate was applied to a hydroxylapatite (Bio-Gel HTP) column (3.0 × 11 cm) equilibrated with the same buffer. The column was developed with a linear 10–70 mM phosphate gradient (total 1.0 liters; pH 7.0) with a flow rate of 12 ml/h. The active fractions (400 ml) were concentrated to 10 ml by ultrafiltration, and dialyzed against buffer A. The purified protein solution was finally concentrated to 40 mg/ml with ultrafiltration using Centricon-10 (Amicon Corp.).

2.4. *Crystallization of* B. cereus *oligo-1,6-glucosidase*

Crystals of oligo-1,6-glucosidase were grown at 25°C by a hanging drop vapor diffusion method [13]. Crystallization droplets were prepared on siliconized coverslips by mixing 10 μl of a purified protein solution and an equal volume of a 53% $(NH_4)_2SO_4$-saturated buffer A solution (reservoir solution), and were equilibrated against 1.0 ml of the reservoir solution by sealing up the reservoir wells of a tissue culture dish (24 wells) with the coverslips. The crystals obtained in this way were kept once in a 70% $(NH_4)_2SO_4$ saturated buffer A solution (mother liquor). Then they were sealed in glass capillaries with a bit of the mother liquor for X-ray experiments. Precession photographs were taken with a precession camera (Charles Supper) using Ni-filtered CuKα radiation (λ=1.54182 Å) produced by a Rigaku RU-200 rotating anode X-ray generator run at 40 kV and 100 mA. The space group and the cell dimensions were determined from Laue symmetry, absence rule ($l \neq 3n$ for (hkl)) and spacing of diffraction spots on precession photographs.

3. RESULTS AND DISCUSSION

3.1. *Overexpression of the* B. cereus *oligo-1,6-glucosidase gene and purification*

A progressive deletion between the initiation codon of oligo-1,6-glucosidase gene and the *lac* promoter of pBCE4-1 generated about 300 transformants. Ten colonies showing the strong hydrolytic activity of *p*-nitrophenyl-α-D-glucopyranoside on the L-broth/IPTG (0.5 mM) plate were screened. One clone containing the most enzyme activity was selected for study of enzyme production [3]. The clone produced 4–5-5-fold more *B. cereus* oligo-1,6-glucosidase (30–40 U/ml culture at 15–20 h) than *E. coli* cells bearing pBCE4 (7.5 U/ml culture at 10–12 h). The plasmid contained in this clone (named pBCE4-2) was found to be deleted between nucleotide positions 1–266 of the *Eco*RI–*Sal*I fragment containing the *B. cereus* oligo-1,6-glucosidase gene as judged from DNA sequencing [3]. The intact promoter system of the *B. cereus* oligo-1,6-glucosidase gene was disrupted between the −35 and −10 regions by the deletion. Therefore, the oligo-1,6-glucosidase gene of the plasmid pBCE4-2 seemed to be expressed predominantly under the control of the *lac* promoter in the presence of IPTG.

Table I

Purification of oligo-1,6-glucosidase from *E. coli* MV1184 bearing pBCE4-2 in large quantities

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)
Cell extract	1900	86500	45.5
$(NH_4)_2SO_4$ fractionation	1020	69600	68.2
DEAE-cellulose eluate	295	52500	178
Butyl-toyopearl eluate	125	31900	255
Hydroxylapatite eluate	104	29300	282

Table I summarizes the purification of oligo-1,6-glucosidase produced by *E. coli* cells containing the plasmid pBCE4-2. The final enzyme preparation (2.6 ml, 104 mg protein) was purified in large quantities, 6.2-fold over the cell extract with yield being 33.9%. The protein was judged to be homogeneous from PAGE analysis (data not shown). The trace amount of impurities in the DEAE-cellulose eluate was effectively eliminated by the combination of butyl-Toyopearl and hydroxylapatite column chromatographies in place of the immunosorbent chromatography coupled with rabbit antiserum against *B. cereus* oligo-1,6-glucosidase [3]. The immunosorbent chromatography had required an alkaline buffer (pH 11) for elution so that the enzyme activity was significantly lost. The overall purification indicates that the oligo-1,6-glucosidase comprised more than 15% of the total cellular proteins in *E. coli* harboring the pBCE4-2 plasmid.

3.2. *Crystallization and characterization*

The hanging drop method was adopted to establish the best condition for crystallization of *B. cereus* oligo-

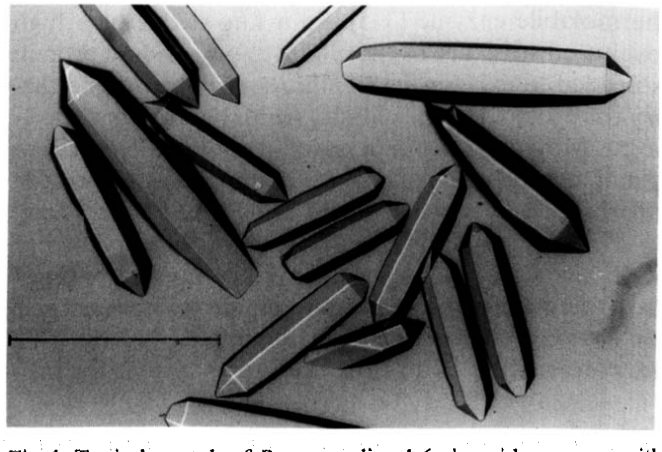

Fig. 1. Typical crystals of *B. cereus* oligo-1,6-glucosidase grown with ammonium sulfate as precipitant. The photograph is a 40-fold magnification of the crystals. Bar = 1 mm.

1,6-glucosidase, using the purified and highly concentrated protein. Crystals with the shape of hexagonal bipyramids appeared within one week and were grown in two weeks to approximate dimensions of 0.3 × 0.3 × 1.2 mm (Fig. 1).

The precession photographs showed that the crystals belonged to a hexagonal space group $P6_2$ or $P6_4$ with unit cell dimensions $a=b=106.1$ Å, $c=120.0$ Å and $\gamma=120°$ (data not shown). There is one enzyme molecule per asymmetric unit of the crystal. By using the cell parameters and the protein partial specific volume of 0.717 cm^3/g calculated from its amino acid composition, one oligo-1,6-glucosidase molecule is calculated to occupy 40% of the volume of the crystal asymmetric unit with a volume per mass ratio (V_M) of 2.95 $Å^3/Da$ [3,14]. The V_M value is in the higher range for protein crystals, which may explain the fragility. However, the crystals give X-ray diffraction spots up to at least 2.8 Å resolution and are suitable for a higher resolution X-ray analysis.

B. cereus oligo-1,6-glucosidase was predicted to have an $(\alpha/\beta)_8$-barrel super-secondary structure common to those of α-1,4-D-glucan-cleaving enzymes as α-amylases, cyclodextrin glucanotransferases (CGTases) and α-glucosidase from various sources [3,15]. The high resolution X-ray analyses of *Aspergillus oryzae* and porcine pancreatic α-amylases [16,17] and *Bacillus circulans* CGTase [18] revealed their $(\alpha/\beta)_8$-barrel folds with rigid assignments of amino acid sequence. This information will be of great help to determine the tertiary structure of the *B. cereus* oligo-1,6-glucosidase protein and to consider the environment around the residues to be replaced by proline in the thermostable *B. thermoglucosidasius* enzyme. We are in the process of developing further native data collections at a higher resolution and a search for heavy-atom derivatives.

Acknowledgements: We deeply thank Dr. Hiroaki Kato and Professor Dr. Jun-ichi Oda, Institute for Chemical Research, Kyoto University, for useful advice about crystallization techniques. This work was supported in part by Grant-in-Aid 01560104 for scientific research from the Ministry of Education, Science and Culture in Japan.

REFERENCES

[1] Suzuki, Y., Oishi, K., Nakano, H. and Nagayama, T. (1987) Appl. Microbiol. Biotechnol. 26, 546–551.
[2] Suzuki, Y. (1989) Proc. Japan Acad. 65, Ser. B 146–148.
[3] Watanabe, K., Kitamura, K., Iha, H. and Suzuki, Y. (1990) Eur. J. Biochem. 192, 609–620.
[4] Watanabe, K., Iha, H., Ohashi, A. and Suzuki, Y. (1989) J. Bacteriol. 171, 1219–1222.
[5] Watanabe, K., Chishiro, K. and Suzuki, Y. (1989) Proceedings of the annual meeting of the agricultural chemical society of Japan, p. 371.
[6] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.
[7] Vieira, J. and Messing, J. (1987) Methods Enzymol. 153, 3–11.
[8] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[9] Henikoff, S. (1984) Gene 28, 351–359.
[10] Paoni, N.F. and Arroyo, R.L. (1984) Appl. Environ. Microbiol. 47, 208–209.
[11] Sanger, F. (1981) Science 274, 1205–1210.
[12] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 1265–1275.
[13] McPherson, A. (1990) Eur. J. Biochem. 189, 1–23.
[14] Matthews, B.W. (1968) J. Mol. Biol. 33, 491–497.
[15] MacGregor, E.A. and Svensson, B. (1989) Biochem. J. 259, 145–152.
[16] Matsuura, Y., Kusunoki, M., Harada, W. and Kakudo, M. (1984) J. Biochem. 95, 697–702.
[17] Buisson, G., Duée, E., Haser, R. and Payan, F. (1987) EMBO J. 13, 3909–3916.
[18] Klein, C. and Schulz, G.E. (1991) J. Mol. Biol. 217, 737–750.