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Crystallization and X-ray analysis of native and selenomethionyl β-mannanase Man5A from blue mussel, *Mytilus edulis*, expressed in *Pichia pastoris*

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The glycohydrolase family 5 β-mannanase Man5A from *Mytilus edulis* has been expressed in *Pichia pastoris* and purified in a form suitable for X-ray crystallographic analysis. Crystals were grown by the hanging-drop technique at 293 K using polyethylene glycol 5000 monomethylether as precipitant and dioxane as additive. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 61.8, b = 64.8, c = 90.7 Å. Diffraction to 1.4 Å resolution has been obtained at 100 K. Expression was also performed in the presence of selenomethionine. The incorporation of SeMet was estimated at 40% by amino-acid analysis and its presence in crystals was confirmed from the X-ray absorption scanning spectrum.

1. Introduction

Polysaccharides containing mannan are major components of the hemicellulose fraction in both hardwoods and softwoods as well as in the endosperm of many leguminous seeds and some mature seeds of non-leguminous plants (Dekker, 1979). The gums extracted from many leguminous seeds are rich in mannan and are used as stabilizers in food production (Christgau *et al.*, 1994). Mannanases (EC 3.2.1.78) have received industrial attention because of their ability to modify and degrade mannan-containing polysaccharides, for example in paper manufacturing (Paice & Jurasek, 1984). β-1,4-Mannanases catalyze the hydrolytic cleavage of the β-1,4-glycosidic linkages in mannan, galactomannan, glucomannan and galactoglucomannan (Matheson & McCleary, 1985; McCleary & Matheson, 1986). Several mannanases have been purified and characterized from several different sources such as plants, fungi and bacteria (Shimahara *et al.*, 1975; Reese & Shibata, 1965; Arcand *et al.*, 1993; Araujo & Ward, 1990; Johnson, 1990; McCleary, 1988). Recently, a mannanase from blue mussel has been purified and characterized (Xu *et al.*, 2001).

The mannanase gene from *M. edulis* consists of a 1104 bp open reading frame (ORF) which encodes a 41 kDa protein (Xu *et al.*, manuscript in preparation). It belongs to glycoside hydrolase family 5 and was therefore assigned the name Man5A. Sequence similarity is evident with mannanase A from *Clostridium cellulovorans*, man5-K from *C. cellulolyticum*, the β-mannan endohydrolase precursor from *Coffea arabica* and the Cel4A mannanase precursor from *Agaricus bisporus*, with sequence identities in the 20–25% range. The three-dimensional structures of two other family 5 mannanases have been solved, namely those of *Thermobifida fusca* (formerly *Thermomonospora fusca*; PDB code 3man; Hilge *et al.*, 1998) and *Trichoderma reesei* (PDB code 1qnr; Sabini *et al.*, 2000). However, they appear to be more distantly related to the *M. edulis* mannanase (Xu *et al.*, manuscript in preparation).

Incorporation of selenomethionine into expressed proteins is now widely used for phase determination by multiwavelength anomalous dispersion (Walsh *et al.*, 1999). Prokaryotic expression is commonly performed using methionine-auxotrophic strains of *Escherichia coli* (Doublie, 1997). With eukaryotic hosts, partial SeMet incorporation has been reported with a non-auxotrophic strain of *Saccharomyces cerevisiae* (Bushnell *et al.*, 2001) and recently also with the methylotrophic yeast *P. pastoris* (Larsson *et al.*, 2002). In this paper, we report the expression of recombinant *M. edulis* β-mannanase in *P. pastoris*, with and without selenomethionine, together with purification, crystallization and preliminary X-ray diffraction analysis.

2. Experimental

2.1. Cloning and expression

Taq DNA polymerase (Amersham Pharmacia Biotech) and the oligonucleotide primers 5′-G**CTGCAG**ACAGACTGAGTGTTAGTGGGACCA-3′ and 5′-TG**GCGGCCG**CTTAAAGGCCAAACTGAACTTGTCC-3′ were used to amplify the gene coding for the mannanase. An *M. edulis* cDNA library synthesized from mRNA isolated from the

hepatopancreas (digestive gland) of blue mussel was used as a template (Xu *et al.*, manuscript in preparation). Engineered unique restriction sites for *Pst*I and *Not*I were introduced (shown in bold). After amplification, the PCR product was digested with the two restriction enzymes and purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech).

An EasySelect *Pichia* Expression Kit from Invitrogen was used for transformation and expression of the recombinant mannanase. The engineered mature mannanase coding sequence was directionally cloned into the *E. coli*/*P. pastoris* shuttle vector pPICZα B, which was digested by the same restriction enzymes and purified. The ligation product was transformed into *E. coli* competent cells TOP10F' (Invitrogen). The *E. coli* transformants were selected on plates containing the antibiotic Zeocin (Invitrogen) at a concentration of 25 µg ml^{-1}. The mannanase-pPICZα B plasmid DNA was purified from *E. coli* cells and linearized with the restriction enzyme *Sac*I to allow integration of the vector DNA into the chromosomal DNA of *P. pastoris*. The wild-type X-33 strain of *P. pastoris* was used as a host strain and the *Pichia* EasyComp Kit was used to produce chemically competent *Pichia* cells according to the Invitrogen instruction manual. The transformants were selected on YEPD (1% yeast extract, 2% peptone, 2% dextrose and 2% agar) plates containing Zeocin (100 µg ml^{-1}). The presence of the mannanase gene in the transformants was confirmed by PCR amplification using yeast genomic DNA as a template for the reaction.

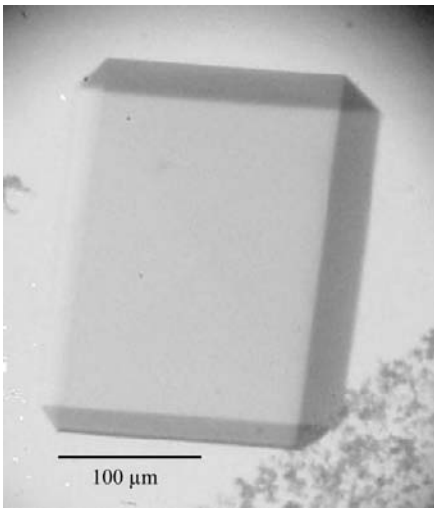

Figure 1
Crystal of the SeMet-containing recombinant β-mannanase from blue mussel (*M. edulis*).

Mannanase-producing *P. pastoris* cells were grown in 50 ml buffered complex medium containing glycerol (BMGY, Invitrogen) at 301 K to a cell density of 4.5. Induction was performed by transferring the cells into 500 ml buffered complex medium containing methanol (BMMY, Invitrogen) at 290 K. Every 12 h, methanol and NH_3 were added to final concentrations of 0.5 and 0.25%, respectively. The induction was interrupted when the enzyme concentration in the medium levelled off, usually after a period of 6 d. A mannanase-containing cell-free culture supernatant was obtained by centrifugation.

2.2. Expression of selenomethionine-containing mannanase

The same strain was used for the expression of SeMet-incorporated mannanase. The growth conditions before induction were the same as those described above. The cells were then washed three times with PBS and resuspended in 500 ml buffered minimal methanol medium according to the Invitrogen instruction with the following modification. The medium contained 100 m*M* potassium phosphate at pH 6.0, 1.34% YNB without amino acids (Difco), 0.3 mg l^{-1} biotin, 3 mg l^{-1} folic acid, 3 mg l^{-1} riboflavin, 3 mg l^{-1} niacinamide, 3 mg l^{-1} thiamine, 3 mg l^{-1} calcium pantothenate, 6 mg l^{-1} pyridoxal, 0.1 mg l^{-1} selenomethionine, 0.1 mg l^{-1} L-histidine and 0.1 mg l^{-1} L-tryptophan. The induction took place at 290 K. As before, methanol and NH_3 were added every 12 h to a final concentration of 0.5 and 0.25%, respectively. In this case, the induction was interrupted already after 3 d owing to levelling off of the enzyme concentration. A mannanase-containing cell-free culture supernatant was obtained by centrifugation.

2.3. Protein purification and analysis

20 ml Chelating Sepharose Fast Flow (Amersham Pharmacia Biotech) was saturated with nickel ions and equilibrated in 20 m*M* sodium phosphate buffer pH 6.5 containing 0.5 *M* NaCl. 250 ml culture supernatant was applied directly, without pre-treatment, to the column at a flow rate of 5 ml min^{-1}. After washing with equilibration buffer to remove unbound contaminants, a 20 column volumes imidazole gradient from 0 to 500 m*M* was introduced at a flow rate of 2 ml min^{-1}. The mannanase eluted at an imidazole concentration of around 350 m*M*. The purity was analyzed by SDS–PAGE and a single well defined band was obtained. The mannanase solution was diafiltrated three times in 20 m*M* sodium acetate buffer pH 5.5 using a Ultrafree-15 Centrifugal Filter Device (Millipore). The mannanase activity was assayed using a locust bean gum substrate as described by Stålbrand *et al.* (1993).

Amino-acid composition analysis of both mannanase and selenomethionine-containing mannanase was performed by the Amino Acid Analysis Laboratory, Department of Biochemistry, Uppsala University, Sweden. SeMet does not contribute to the methionine content and so the level of incorporation is given by the ratio between the relative amounts of methionine in the SeMet protein and in the native protein.

2.4. Crystallization

Crystallization was performed at room temperature using the hanging-drop vapour-diffusion technique (McPherson, 1982). The drops were prepared by mixing 2 µl each of the protein (in 20 m*M* sodium acetate buffer pH 5.5) and the reservoir solutions. Initially, commercially available kits from Hampton Research (Crystal Screen, Crystal Screen II, Grid Screens MPD and Grid Screens PEG/LiCl) were used at a protein concentration of 10 mg ml^{-1}. After 5 d, needle-shaped crystals had formed with solution No. 26 from Crystal Screen II, which contains 0.2 *M* ammonium sulfate, 0.1 *M* MES pH 6 and 30% monomethyl ether polyethylene glycol (mme PEG) 5000. These initial conditions were further optimized by varying the pH between 6.0 and 7.0, the ammonium sulfate concentration between 0 and 0.5 *M* and the PEG concentration between 10 and 30%. However, even under the best conditions tested the crystals grew as aggregates. In the next optimization phase we used Additive Screen 1 from Hampton Research. Larger crystals and a larger fraction of single crystals formed with dioxane and to some extent also with ethanol, 2-propanol, hexanediol, glycerol and $MgCl_2$. The best crystals so far have been obtained with a protein concentration of 8 mg m l^{-1} and a reservoir containing 0.1 *M* MES pH 6.5, 0.2 *M* ammonium sulfate, 25%(*w*/*v*) mme PEG 5000 and 3% dioxane.

Microseeding (Stura & Wilson, 1992) was shown to improve the crystal formation and was best performed after 1 d of equilibration against the reservoir. An acupuncture needle was first dipped briefly either directly into a hanging drop with crystals previously formed under the optimized conditions above or into a diluted microcrystal preparation prepared as follows: a crystal-containing drop was dispersed in 200 µl of

the reservoir against which it had been equilibrated, the solution was mixed vigorously on a Vortex for 1 min, centrifuged at 13 000*g* for 1–5 min and the resultant supernatant was used for seeding. The surface of the new crystallization drop was then gently touched with the needle. The two sources of microcrystals gave equivalent results. Useful crystals (Fig. 1) appeared within 5 d of microseeding being initiated.

3. X-ray analysis

Prior to data collection, a single crystal was transferred to a cryoprotectant stabilizing solution consisting of the growth buffer supplemented with 20%(*v*/*v*) glycerol. The crystal was then mounted in a Hampton CryoLoop and flash-cooled using liquid nitrogen. Diffraction data were collected under cryoconditions. 154 consecutive 1° oscillation images (30 min exposure) were recorded on our rotating-anode X-ray source (Rigaku/MAR345). The data were processed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Native data from the mannanase were also collected at the synchrotron beamline ID14-EH1, ESRF, Grenoble. With SeMet mannanase an X-ray absorption spectrum was collected by measuring the fluorescent signal perpendicular to the beam during an energy scan performed at beamline ID-29, ESRF. The same crystal was then used for multiple-wavelength data collection to 2.4 Å resolution.

4. Results and discussion

The *M. edulis* β-mannanase was succesfully expressed and secreted in *P. pastoris* with expression levels exceeding 100 mg l^{-1} under standard conditions. The enzyme was purified to apparent homogeneity in a single step using immobilized metal-ion affinity chromatography (IMAC). With the SeMet mannanase the expression level was lower, around 20 mg l^{-1}. One possible reason is that a rich medium was used in the expression of normal mannanase, whereas a minimal medium was used for the SeMet mannanase. Another possibility is that SeMet might be toxic to the yeast cell.

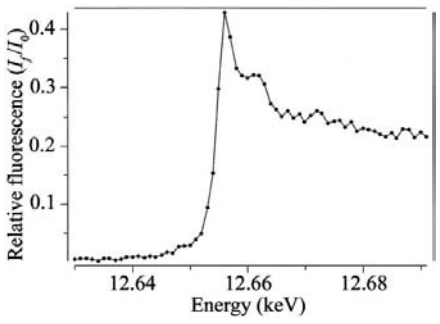

Figure 2
Se *K* edge fluorescence scan of a crystal of SeMet-containing β-mannanase performed at the ESRF beamline ID-29.

Table 1
Amino-acid composition of recombinant *M. edulis* β-mannanase expressed in the absence (native) and presence of selenomethionine (SeMet).

Amino acid	No. of residues (theoretical)	No. of residues (native)	No. of residues (SeMet)
A	26	30.9	26.6
C	2	2.0	2.0
D + N	41	40.8	40.8
E + Q	33	35.5	35.2
F	17	17.5	16.9
G	36	35.1	36.0
H	19	18.0	18.4
I	14	11.4	12.3
K	17	17.5	17.1
L	24	24.5	24.3
P	8	7.8	7.8
R	13	12.5	12.6
S	33	31.8	32.2
T	18	17.4	17.7
V	16	16.1	17.6
Y	13	12.4	12.5
M	10	11.4	6.9

Among the crystallization conditions tested for mannanase, several drops containing organic solvents (*e.g.* dioxane, 2-propanol and ethanol) as additives gave single crystals suitable for X-ray diffraction analysis. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 61.8, b = 64.8, c = 90.7 Å and one molecule per asymmetric unit (Matthews number V_M = 2.2 $Å^3$ Da^{-1}). The diffraction data collected on our rotating-anode X-ray source had (values for the highest resolution shell, 2.31–2.20 Å, are given in parentheses) a completeness of 98% (93%), a multiplicity of 6.0 (4.1) and 93% (87%) of the reflections with $I > 3\sigma(I)$. The internal agreement of the data, R_{merge}, was 0.06 (0.15). Recently, diffraction to 1.4 Å resolution was obtained at the ESRF synchrotron beamline ID14-EH1. The variation of the unit-cell parameters is within 0.5 Å between the crystals tested so far.

Crystals of the same space group, size and appearance were grown from the SeMet mannanase under the same conditions (Fig. 1). The crystal used here had unit-cell parameters a = 62.3, b = 65.0, c = 91.1 Å. The peak at 12 656 eV in the X-ray absorption spectrum confirms the presence of selenium in the crystal (Fig. 2). From the amino-acid composition analysis an incorporation of around 40% SeMet was estimated (Table 1).

The resolution of the three-dimensional structure of the *M. edulis* β-mannanase will give further insight in the structural relationship of mannanases and other enzymes in family 5 of the glycoside hydrolases.

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