Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Preparation and crystallization of selenomethionyl dextranase from *Penicillium minioluteum* expressed in *Pichia pastoris*

Anna M. Larsson,[a] Jerry Ståhlberg[b] and T. Alwyn Jones[a]*

[a]Department of Cell and Molecular Biology, Uppsala University, Biomedical Centre, Box 596, SE-751 24 Uppsala, Sweden, and [b]Department of Molecular Biology, Swedish University of Agricultural Sciences, Biomedical Centre, Box 590, SE-751 24 Uppsala, Sweden

Correspondence e-mail: alwyn@xray.bmc.uu.se

Received 16 October 2001
Accepted 28 November 2001

Dextranase from the fungus *Penicillium minioluteum* hydrolyses α-1,6-glycosidic bonds in dextran polymers. The enzyme has been expressed in *Pichia pastoris* in the presence of selenomethionine (SeMet). The level of SeMet incorporation was estimated by amino-acid analysis to be 50%. The protein has been crystallized in space group $P2_12_12$, with unit-cell parameters $a = 103.6$, $b = 115.3$, $c = 49.8$ Å and one molecule per asymmetric unit. The crystals diffract to 2.0 Å and the presence of SeMet in the crystals has been confirmed by an X-ray absorption spectrum.

1. Introduction

Dextranase (α-1,6-glucan 6-glucanohydrolase; E.C. 3.2.1.11) hydrolyses the α-1,6-glycosidic linkage within the dextran molecule and releases smaller isomaltosaccharides, usually 3–5 glucose units long. In the sugar industry, contamination by dextran constitutes a significant problem owing to the high molecular weight of the dextran polymer. It is synthesized by soil bacteria, mainly *Leuconostoc* species, that infect canes and damage beets during delays between harvest and processing. The use of dextranase in processing has reduced the problem by cleaving the dextran polysaccharides, thus lowering the juice viscosity (Clarke, 1997). The presence of dextranase activity has been reported in bacteria (Dewar & Walker, 1975), yeast (Koenig & Day, 1988) and other fungi (Kosaric *et al.*, 1973), but no enzyme structure is available. Dextranase from the fungus *Penicillium minioluteum* is a 67 kDa glycoprotein containing three potential N-glycosylation sites. It is a member of glycosyl hydrolase family 49 (Coutinho & Henrissat, 1999), which also includes bacterial dextranases and isopullulanase and dextranase from the closely related *Penicillium funiculosum*. The enzyme has an optimal activity at pH 5 and 323 K and an isoelectric point of 3.88 (Raíces *et al.*, 1991). The DEX gene has previously been isolated (GenBank accession number L41562; García *et al.*, 1996) and recombinantly expressed in *Pichia pastoris* at a level of 3.2 g l^{-1} (Roca *et al.*, 1996).

The use of the methylotrophic yeast *Pichia pastoris* as a vector for recombinant expression of protein has increased dramatically in recent years. The powerful and tightly regulated promoter of the methanol-induced AOX1 gene allows controlled expression at high levels. The high capacity for secretion of heterologous protein into the medium and the post-translational modifications make it a suitable host for many eukaryotic proteins that cannot be expressed in *Escherichia coli*.

Selenomethionine (SeMet) incorporation has become a standard method for obtaining the phases in protein crystallography using single- or multiwavelength anomalous dispersion (Hendrickson, 1991). Incorporation in proteins expressed in *E. coli* is routinely made with methionine auxotrophic strains, where a complete substitution of methionine to selenomethionine is achieved (Hendrickson *et al.*, 1990). Expression of selenomethionine-containing protein in *Saccharomyces cerevisiae* has been shown to yield partial incorporation (Bushnell *et al.*, 2001), where approximately 50% incorporation can be obtained with a non-auxotrophic strain. Because of the resemblance between the two yeasts, we have used a similar protocol to express dextranase in *Pichia pastoris*.

2. Experimental methods

2.1. Expression

A mutated variant of the dextranase-encoding DEX gene from *Penicillium minioluteum* strain HI-4 (Guilarte *et al.*, 1985) was used for expression. In this construction, the asparagine residues in all three potential N-glycosylation sites have been substituted by alanine to give a glycosylation-free protein. To obtain secreted protein, the *Pichia pastoris* vector MP36 (Yong *et al.*, 1992) was used with the mutated DEX gene inserted to give the phenotype methanol-utilization slow (MutS).

The cells were grown in 150 ml YP medium (1% yeast extract, 2% peptone) containing 1% glycerol and 100 m*M* potassium phosphate buffer pH 6 at 301 K to a cell density of OD_{600}

4.5. The culture was centrifuged and the pelleted cells were washed three times with 0.9%(*w*/*v*) NaCl and resuspended in 50 ml synthetic complete medium [0.09 mg ml^{-1} each of adenine sulfate, uracil, L-tryptophan, L-histidine–HCl, L-arginine–HCl, L-tyrosine, L-leucine, L-isoleucine and L-lysine–HCl, 0.15 mg ml^{-1} L-phenylalanine, 0.3 mg ml^{-1} L-glutamic acid, 0.3 mg ml^{-1} L-aspartic acid, 0.45 mg ml^{-1} L-valine, 0.6 mg ml^{-1} L-threonine, 1.2 mg ml^{-1} L-serine, 0.34 mg ml^{-1} thiamine, 0.12 mg ml^{-1} L-cysteine, 0.3 mg ml^{-1} L-glutamine, 0.3 mg ml^{-1} succinic acid, 0.2 mg ml^{-1} L-proline, 0.2 mg ml^{-1} L-alanine, 0.01 mg ml^{-1} inositol and 1.34%(*w*/*v*) yeast nitrogen base without amino acids (Invitrogen)] containing 0.1 mg ml^{-1} selenomethionine instead of methionine. For induction of the expression, 1% methanol was added initially and after 24 h 0.5% methanol was added each 12 h. The expression proceeded for 82 h at 295 K. The supernatant was decanted after centrifugation and passed through a Whatman GF/B filter.

2.2. Purification

The buffer was changed to 10 m*M* sodium acetate pH 5 by repeated concentration and dilution in an Omegacell concentrator (Filtron). The protein sample was loaded on a DEAE-Sepharose ion-exchange column (Pharmacia) equilibrated with the same buffer. Dextranase was eluted using a linear gradient with an increasing concentration of sodium acetate. Fractions containing dextranase were concentrated to 15 mg ml^{-1}. The dextranase activity was determined according to Kosaric *et al.* (1973) by quantifying the amount of reducing sugar released from polymeric dextran.

2.3. Crystallization

Crystals were grown by the hanging-drop vapour-diffusion method (McPherson, 1982) at 293 K. Initial crystallization attempts were made with a monomethylether polyethyleneglycol (mme PEG) 5000 grid screen (10, 15, 20 or 30%) at six different pH values (4–9). $CaCl_2$ or $CdCl_2$ was used as an additive. The protein sample was prepared with a concentration of 7.5 mg ml^{-1} in 0.1 *M* sodium acetate pH 5.5, 0.1 *M* NaCl and 10 m*M* $CaCl_2$. The NaCl was necessary to prevent precipitation of the protein. The crystallization drop setup consisted of 4 µl protein mixed with 2 µl reservoir solution comprising 20% mme PEG 5000 (Fluka), 0.1 *M* sodium acetate pH 5.5, 0.1 *M* NaCl and 10 m*M* $CaCl_2$. These drops were allowed to equilibrate for 24 h, after which they were microseeded with previously obtained crystals. The microseeds were prepared by vortexing the crystals in mother liquor, followed by centrifugation. An acupuncture needle was dipped into the supernatant to transfer the microcrystals to the equilibrated drops. Crystals appeared within 2 d and were fully grown in a couple of weeks. Prior to data collection, the crystal was transferred to a cryoprotectant solution containing 25% mme PEG 5000, 12.5% glycerol, 0.2 *M* sodium acetate pH 5.5, 0.2 *M* NaCl and 20 m*M* $CaCl_2$ and then immediately flash-frozen in liquid nitrogen.

2.4. Analysis of selenomethionine content

The incorporation of selenomethionine was estimated by quantitative amino-acid analysis as a decrease in the relative methionine content. Amino-acid analysis was performed by the Amino Acid Analysis Laboratory, Department of Biochemistry, Uppsala University, Sweden.

An X-ray absorption spectrum of dextranase crystals was collected near the Se *K* absorption edge by measuring the fluorescent signal perpendicular to the beam during an energy scan performed at beamline ID14 EH4 (ESRF, Grenoble).

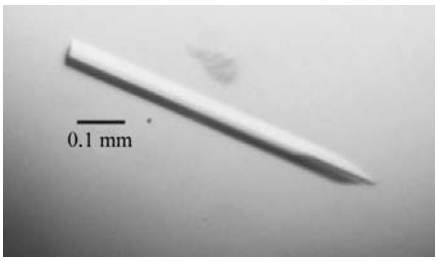

Figure 1
Crystal from an N-glycosylation-free variant of *Penicillium minioluteum* dextranase with about 50% incorporation of selenomethionine.

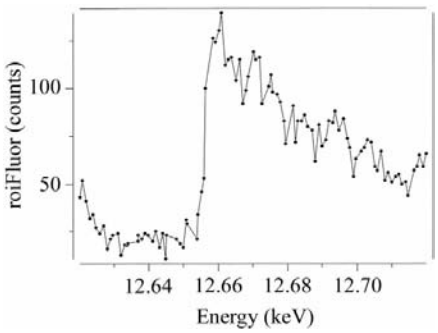

Figure 2
Se *K* edge fluorescence scan of selenomethionyl dextranase crystals performed at ESRF beamline ID14 EH4.

3. Results and discussion

Dextranase from recombinant expression in *Pichia pastoris* (Roca *et al.*, 1996) yielded thin needle-like crystals that aggregated in star formations, possibly owing to glycosylation heterogeneity. Some improvement was achieved after enzymatic deglycosylation, but the problem of disorder persisted. In an attempt to reduce heterogeneity, a glycosylation-free mutant was used for expression. The mutated variant is about 60 kDa and contains 12 methionine residues.

Since the highest expression level and incorporation in *S. cerevisiae* has been obtained with a non-auxotrophic vector (Bushnell *et al.*, 2001), the same *Pichia pastoris* vector (MP36; Yong *et al.*, 1992) as was used for expression of native dextranase was also used for expression with selenomethionine. The amount of expressed dextranase was considerably lower in the medium containing SeMet compared with that in methionine-containing medium. The major expression in SeMet medium occurred during the first 24 h, while the expression in the control continued for about 84 h. As expected for the phenotype MutS, neither of the two cultures showed significant growth during expression. The more rapid decrease in expression in the selenomethionine-containing culture probably reflects a toxic effect of selenomethionine in yeast.

The crystals containing selenomethionine (Fig. 1) grew under the same conditions and time interval and with the same space group and unit-cell parameters as native crystals. Both native and selenomethionine-containing crystals diffracted to about 2 Å.

From amino-acid analysis, a 50% incorporation of selenomethionine was estimated. The peak at 12 660 eV in the absorption-edge scan shows the presence of selenium in the crystals (Fig. 2). Dextranase crystallized in the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 103.6, b = 115.3, c = 49.8 Å and one molecule per asymmetric unit.

We thank Marie Sundqvist and Dr David Eaker, Department of Biochemistry, Uppsala University for analysis and quantification of the amino-acid composition and Drs Ed Mitchell and Raimond Ravelli at beamline ID14 EH4, ESRF, Grenoble for help with the X-ray absorption scan. We are grateful to Drs José Cremata and Bianca García at the Center for Genetic Engineering and Biotechnology, Havana, Cuba for providing the glycosylation-free

dextranase clone. Financial support is gratefully acknowledged from the Swedish Natural Science Research Council (NFR) for grants to TAJ, the Swedish Foundation for Strategic Research *via* the Centre for Forest Biotechnology and Chemistry and the Swedish Council for Forestry and Agricultural Research for salary support to JS.

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