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Crystallization and preliminary X-ray diffraction studies of a family 26 endo-β-1,4 mannanase (ManA) from *Pseudomonas fluorescens* subspecies *cellulosa*

M. Scott,[a] R. W. Pickersgill,[a] G. P. Hazlewood,[b] D. Bolam,[c] H. J. Gilbert[c] and G. W. Harris[a]*
at [a]Institute of Food Research, Earley Gate, Whiteknights Road, Reading, Berkshire RG6 6BZ, England, [b]Department of Cellular Physiology, The Babraham Institute, Babraham, Cambridge CB2 4AT, England, and [c]Department of Biological Sciences, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne NE1 7RU, England. E-mail: gillian.harris@bbsrc.ac.uk

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Abstract

Crystals of an endo-β-1,4-mannanase (1,4-β-D-mannohydrolase, E.C. 3.2.1.78) from *Pseudomonas fluorescens* sub species *cellulosa* have been grown by the hanging-drop technique at 291 K over a period of one to two weeks to maximal dimensions of 0.17 × 0.17 × 0.25 mm. These crystals belong to the space group *R*32 (or *R*3) with cell dimensions of $a = b = 155.4$ and $c = 250.8$ Å (hexagonal setting) and contain three (six) molecules in the asymmetric unit. The crystals diffract to at least 3.2 Å using a laboratory source and are suitable for structure determination.

1. Introduction

Biodegredation of mannan, a major polysaccharide component of the ubiquitous hemicellulose, is achieved in nature by endo-1,4-β-D-mannanases (E.C. 3.2.1.78) which randomly hydrolyse the β-1,4-mannopyranosyl linkages within the main chain of mannans. Usually a degree of polymerization of at least four is required, although some mannanases are able to hydrolyse mannotriose. Small oligosaccharides produced by the action of mannanases can then be further hydrolysed by β-mannanosidases to liberate mannose. Hemicelluloses are the second most abundant polysaccharides in nature usually associated with cellulose and lignin in plant cell walls (Dekker, 1985). The major constituents of hemicelluloses are the hetero-1,4-β-D-xylans and hetero-β-D-mannans.

β-Mannan is a polysaccharide containing 95% or more of mannose units. It can exist as a linear polymer of mannose units which are (1–4) linked in the β-pyranosyl form or as a heteropolymer consisting of a backbone of β-1,4-mannose units which carry substitutions in the form of other sugars such as glucose and galactose to form glucomannan, galactomannan and galactoglucomannan. In glucomannans, the main polymer chain also contains glucopyranose and mannopyranose units randomly distributed within the molecule. In galactoglucomannans, D-galactose side groups are linked (1–6) to the mannose or glucose units of the backbone chain and the hexose units of the backbone are frequently acetylated at the C2 or C3 positions. The average degree of acetylation is 20–30% (Lindberg *et al.*, 1973).

Endo-β-1,4-mannanases have been isolated and characterized from various sources such as the bacteria *Bacillus pumilus* (Akino *et al.*, 1989) and *Streptomyces lividans* (Arcand *et al.*, 1993), yeast (Oda & Tonomura, 1996), fungi (Arisan-Atac *et al.*, 1993) and even a mud snail, *Pomacea insularus* (Yamaura & Matsumoto, 1993). To date the primary sequences of ten mannanases from eubacteria and aerobic and anaerobic fungi have been determined (Bolam *et al.*, 1996). Hydrophobic cluster analysis (HCA) and comparison of the primary structures of these enzymes have placed them in two distinct families of glycosyl hydrolases, namely family 5 and family 26 (Henrissat, 1991; Henrissat & Bairoch, 1993). Family 5 is a member of the GH-A clan (Henrissat *et al.*, 1995; Henrissat & Bairoch, 1996) or 4/7 superfamily (Jenkins *et al.*, 1995) of glycosyl hydrolases (families 1, 2, 5, 10, 17, 30, 35, 39 and 42) which all have the same tertiary fold, namely the $(\beta/\alpha)_8$-barrel structure. In addition HCA suggests that family 26 is also related to the GH-A clan of glycosyl hydrolases (Bolam *et al.*, 1996).

Analysis of the primary sequence of ManA from *P. fluorescens* subsp. *cellulosa* and comparison with the sequences of other mannanases in the SWISSPROT database places this enzyme in the glycosyl hydrolase family 26 (Bolam *et al.*, 1996) according to the classification of Henrissat (1991).

Studies on the plant cell wall hydrolases produced by *P. fluorescens* subsp. *cellulosa* have shown that with the exception of ManA they are all modular in structure, comprising a catalytic domain linked by serine-rich sequences to a non-catlaytic cellulose binding domain (CBD). However, inspection of the primary structure of ManA did not reveal any obvious linker sequences or protein motifs characteristic of the non-catalytic domain of other plant cell wall hydrolases of *P. fluorescens* subsp. *cellulosa* indicating that ManA is a non-modular protein consisting of a single catalytic domain (Braithwaite *et al.*, 1995).

To determine the substrate specificity of endo-β-1,4-mannanase A (ManA) from *P. fluorescens* subsp. *cellulosa* the gene encoding this enzyme was expressed in *Escherichia coli*, a host that has no endogenous polysaccharidase activity. The data obtained showed that ManA (M_r = 43 116 Da) has a very narrow substrate specificity. It hydrolyses mannan and galactomannan but shows no activity towards other plant cell-wall polysaccharides. This characteristic is very similar to other plant cell-wall hydrolases expressed by *P. fluorescens* subsp. *cellulosa* (Braithwaite *et al.*, 1995).

We report the crystallization and preliminary X-ray diffraction study of ManA, an endo-β-1,4-mannanase from *P. fluorescens* subsp. *cellulosa*.

2. Materials and methods

2.1. *Purification and enzyme assay*

ManA expressed in *E. coli.* strain BL21 containing the plasmid pDB1 was purified to homogeneity in a single step using anion-exchange chromatography (Bolam *et al.*, 1996). Purity was evaluated by SDS–PAGE as described by Laemmli (1970). Using a dyed substrate, Azo-carob galactomannan

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(Megazyme Ltd), the activity of ManA was determined by measuring the release of soluble dyed oligosaccharides at 590 nm (Bolam *et al.*, 1996).

2.2. *Crystallization*

For crystallization, fractions of purified ManA were dialysed overnight against water containing 2 m*M* sodium azide (NaN_3). The protein was then concentrated in a 10 ml stirred cell concentrator (Amicon) fitted with a 10 kDa membrane (Flowgen). Using the hanging-drop vapour-diffusion technique in Linbro plates over 1 ml reservoirs, many different crystallization conditions were surveyed by the protocol of Jancarik & Kim (1991) using both crystal screens I and II supplied by Hampton Research.

Assuming 1 mg ml^{-1} of protein gives an OD_{280} of 1.0, an estimated concentration of 12 mg ml^{-1} was used to set up droplets of 3 µl of protein and 3 µl of reservoir solution. After one to two weeks very tiny needles were observed in one droplet of the screen where 2-methyl 2,4-pentanediol (MPD) was the precipitant. After optimization the best crystals were obtained at 291 K from hanging drops containing 4 µl of protein and 2 µl of reservoir solution which consisted of 50–60% MPD and 0.1 *M* Tris–HCl buffer pH 7.5–8.5.

2.3. *Data collection*

A native data set has been collected from a crystal of ManA using a 300 mm imaging plate (MAR Research, Hamburg) mounted on a Rigaku generator (courtesy of S. Wood, Southampton University). The resolution of these data is 3.2 Å. However, a crystal of ManA was examined using the synchrotron at LURE (Orsay, France) at a wavelength of 1.386 Å using a 180 mm imaging plate (MAR Research, Hamburg) and diffraction was observed to at least 2.7 Å resolution.

On a Rigaku generator a total of 100 frames of 1° oscillations were measured at a wavelength of 1.5418 Å using a crystal-to-detector distance of 250 mm. The images were processed with *DENZO* (Otwinowski, 1993). *DENZO*, in conjunction with the display program *XDISPLAYF* (Minor, 1993), was used to autoindex an image.

3. Results and discussion

Crystals of ManA have a rhombohedral lattice with unit-cell dimensions $a = b = 155.4$ and $c = 250.8$ Å (hexagonal setting). The space group is *R*32 (or *R*3), with 2–4 (or 4–8) molecules in the asymmetric unit. Table 1 shows the number of molecules per asymmetric unit in *R*32 and *R*3 required to give a value of the crystal packing parameter V_m within the accepted range for proteins (Matthews, 1968).

The data were scaled and merged using the program *SCALEPACK* from the *HKL* program suite (Otwinowski, 1993). Because of crystal deterioration only the first 63 frames (63° of data) were scaled and merged to yield 19 226 independent reflections, 98.2% of a complete data set to 3.2 Å resolution, from a total of 111 324 measurements with an overall merging *R* factor on intensity of 0.157 for 18 666 reflections measured more than once. The data set was 99.8% complete in the last resolution shell of 3.35–3.20 Å. As the merging *R* factor was rather high in the last shell, the resolution was cut to 3.35 Å and the data scaled and merged to yield 16 750 independent reflections, 98.0% of a complete data set to 3.35 Å resolution, from a total of 97 110 measurements with an overall merging *R* factor on intensity of 0.143 for 16 232 reflections measured more than once. The data were also scaled and merged in the space group *R*3, to yield 36 660 independent reflections, 98.0% of a complete data set to 3.2 Å resolution with an overall merging *R* factor on intensity of 0.141 for 27 785 reflections measured more than once. Although *R*32 is the most probable space group we cannot exclude *R*3 with a pseudo twofold axis as a possibility.

Table 1. *Number of molecules in the asymmetric unit for space groups R32 and R3 for acceptable values of the crystal packing parameter* V_m

V_m ($Å^3$ Da^{-1})	Number of molecules in the asymmetric unit	
	*R*32	*R*3
3.38	2	4
2.25	3	6
1.69	4	8

Endo-β-1,4-mannanase cleaves the backbone of mannan, one of the major polysaccharides of the plant cell wall. Recently there have been major advances in our understanding of the structures and catalytic mechanisms of cellulases and xylanases (Davies & Henrissat, 1995), but other important hemicellulases such as mannanases have received little attention.

Glycosyl hydrolases have been classified into families on the basis of amino-acid sequence similarity and hydrophobic cluster analysis (Henrissat, 1991). According to this classification, mannanases belong to two distinct families, namely 5 and 26 (Henrissat & Bairoch, 1993), with endo-β-1,4-mannanase from *P. fluorescens* subsp. *cellulosa* included in family 26. Recently, several glycosyl hydrolase families, including family 5, have been grouped into the 4/7 superfamily or GH-A clan (Jenkins *et al.*, 1995; Henrissat *et al.*, 1995) members of which all have the eightfold β/α-barrel architecture. Recently the crystallization of two isoforms of a β-mannanase from *Thermomonaspora fusca* has been reported (Hilge *et al.*, 1996). This mannanase belongs to the glycosyl hydrolase family 5 (Henrissat & Bairoch, 1993). The X-ray structures of three family 5 cellulases have been reported to date (Dominguez *et al.*, 1995; Ducros *et al.*, 1995; Sakon *et al.*, 1996). As was predicted (Jenkins *et al.*, 1995), all have the eightfold β/α-barrel architecture. There has been a suggestion that family 26 is also a member of the GH-A clan (Bolam *et al.*, 1996). Knowledge of the structure of ManA from *P. fluorescens* subsp. *cellulosa* would show whether this hypothesis is valid and would add greatly to the understanding of this class of glycosyl hydrolase enzymes.

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