

Crystal Structure of a Barley β -D-Glucan Exohydrolase. Catalytic Mechanism, Substrate Binding and Homology Modelling

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Introduction

The (1 \rightarrow 3,1 \rightarrow 4)- β -glucans are important components of cell walls in members of the Poaceae family of higher plants. They consist of long, linear chains of glucosyl residues that are linked *via* (1 \rightarrow 3)- and (1 \rightarrow 4)- β -glucosidic linkages. As wall components, the (1 \rightarrow 3,1 \rightarrow 4)- β -glucans make a relatively minor contribution to the total weight of cereal grains, but they can have a disproportionately large impact on grain technology, utilization and nutrition. This impact is attributable to the propensity of these polysaccharides to form aqueous solutions of high viscosity. Thus, in malting and brewing they can adversely affect the efficiency of malt extraction, filtration processes and the quality of the final beer. Similarly, (1 \rightarrow 3,1 \rightarrow 4)- β -glucans can have undesirable effects on the digestibility of cereal-based stockfeeds by monogastric animals such as pigs and poultry. In contrast, they are important constituents of the 'dietary fibre' component of human foods, which is considered to be of salutary importance in several areas of human digestion and health (Bhatty, 1993).

The (1 \rightarrow 3,1 \rightarrow 4)- β -glucans from barley generally contain about 70% (1 \rightarrow 4)- β -glucosyl residues and 30% (1 \rightarrow 3)- β -glucosyl residues (Parrish *et al.*, 1960; Bacic *et al.*, 1988). Adjacent (1 \rightarrow 3)- β -glucosyl residues are seldom detected in these polysaccharides. The high viscosities of barley (1 \rightarrow 3,1 \rightarrow 4)- β -glucan solutions are caused not only by the high molecular weights of the polysaccharides, but also by their high degree of molecular asymmetry. Woodward *et al.* (1983) (4) showed that the water-soluble barley (1 \rightarrow 3,1 \rightarrow 4)- β -glucan has an axial ratio (average length:average width) of about 100.

Enzymes that catalyse the hydrolysis of (1 \rightarrow 3,1 \rightarrow 4)- β -glucans and their degradation products have been mostly extracted from germinated barley grain, young barley seedlings, or from coleoptiles. Several different types of enzyme are required to completely depolymerize (1 \rightarrow 3,1 \rightarrow 4)- β -glucans to glucose. Candidate enzymes and their action patterns are summarized in Figure 1. In germinated grain, the glucose could be translocated as an energy source to the developing seedling.

The first step shown in Figure 1 involves a hypothetical endo-acting enzyme that releases polymeric wall (1 \rightarrow 3,1 \rightarrow 4)- β -glucan into solution, without hydrolyzing it to low molecular weight oligosaccharides. An enzyme with this action pattern has been the subject of many reports in the malting and brewing literature, and has been given the name ' β -glucan solubilase' (Bamforth and HL, 1981). The most important enzymes in (1 \rightarrow 3,1 \rightarrow 4)- β -glucan degradation in germinated grain are probably the (1 \rightarrow 3,1 \rightarrow 4)- β -glucan endohydrolases of the EC 3.2.1.73 group. These enzymes catalyse the hydrolysis of (1 \rightarrow 4)- β -glucosyl linkages, but only where these linkages are immediately adjacent to (1 \rightarrow 3)- β -glucosyl residues (Figure 1). Thus, this group of enzymes has a strict requirement for adjacent (1 \rightarrow 3)- and (1 \rightarrow 4)- β -

glucosyl residues (Figure 1) and the enzymes release oligosaccharides that contain (1→4)- β -glucosyl residues and a single (1→3)- β -glucosyl residue at the reducing terminus (Figure 1).

The released (1→3,1→4)- β -oligoglucosides can be further hydrolysed to glucose (Figure 1). This process will be particularly important in the germinated grain. Although the enzymes that hydrolyse the (1→3,1→4)- β -oligoglucosides have not been identified unequivocally, β -glucosidases (EC 3.2.1.21) and a group of broad specificity β -glucan exohydrolases are likely to be involved (Figure 1). In this report, the crystallization of barley β -glucan exohydrolase isoenzyme ExoI is described (Hrmova *et al.*, 1998), together with its three-dimensional structure (Varghese *et al.*, 1999). The structural data allows the molecular basis of substrate binding, specificity and mechanisms of catalysis to be defined. The availability of structural information also provides opportunities for a 'rational re-design' of enzymes for enhanced performance in the industrial context.

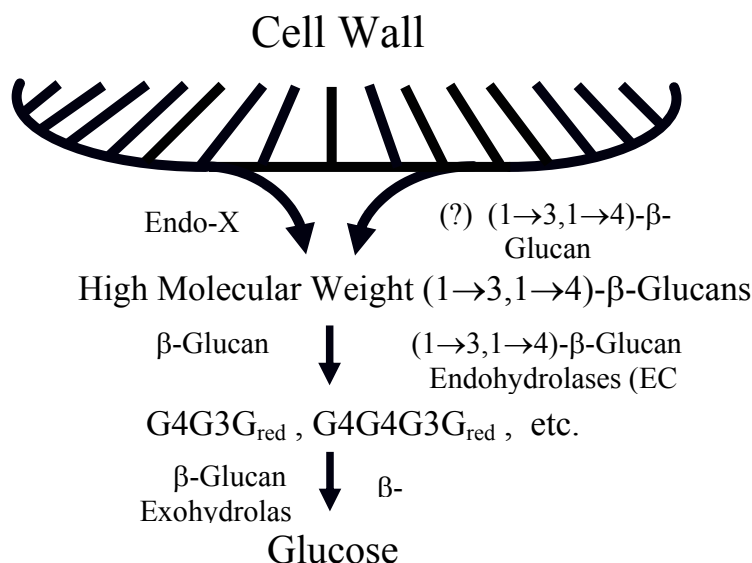


Figure 1. Enzymic hydrolysis of cell wall (1→3,1→4)- β -glucans

Materials and Methods

The barley β -glucan exohydrolase isoenzyme ExoI was purified by fractional precipitation with ammonium sulphate, cation and anion exchange chromatography, chromatofocussing, hydrophobic interaction chromatography and size exclusion chromatography (Hrvova *et al.*, 1996). The purified enzyme was subsequently crystallized by vapour diffusion in the presence of ammonium sulphate and polyethylene glycol (Hrmova *et al.*, 1998). Mercury and platinum derivatives were subsequently obtained and the 3D structure of the enzyme was solved by X-ray crystallography at 2.2 Å resolution (Varghese *et al.*, 1999). The crystallization was effected in 8-10 μ l 'hanging droplets' containing enzyme at concentrations of up to 6-8 mg/ml, in ammonium sulphate solutions. The droplets adhered to glass cover slips and were suspended over a well of a microtitre plate. Water was gradually removed from the droplets by vapour diffusion into a slightly more concentrated ammonium sulphate solution in the well of the microtitre plate. The enzyme crystallized from the super-saturated solution. Crystals took up to 100 days to grow to the 0.6-1 mm size required for X-ray crystallography (Hrvova *et al.*, 1998). Good quality native data collected from the X-ray

diffraction patterns, together with data sets for heavy metal derivatives of the enzymes, allowed the structure of the enzyme to be solved.

Results

Barley β -glucan exohydrolase isoenzyme ExoI has two distinct domains. The first consists of 357 amino acid residues that fold into a $(\beta/\alpha)_8$ barrel. A 16-amino acid linker connects the first domain to the second, which consists of residues 374-559 arranged in an $(\alpha/\beta)_6$ sandwich. The $(\alpha/\beta)_6$ sandwich has a six-stranded β -sheet, with three α -helices on either side of the sheet (Figure 2) (Varghese *et al.*, 1999). At the COOH-terminal region of the enzyme (residues 560-605) is a long, antiparallel loop. Carbohydrate is found on each of three potential *N*-glycosylation sites at Asn221, Asn498 and Asn600 (Varghese *et al.*, 1999). Of particular interest is the presence of a glucose molecule that is located in a surface pocket on the enzyme. The glucose is tightly bound to the enzyme and is likely to be the product of the enzymic reaction that has not been released after hydrolysis. The pocket occupied by the glucose clearly represents the substrate-binding region of the enzyme.

Specific labelling experiments and comparisons with other family 3 hydrolases indicate that Asp285 is the catalytic nucleophile of the β -glucan exohydrolases and that Glu491 is likely to be the catalytic acid (Varghese *et al.*, 1999).

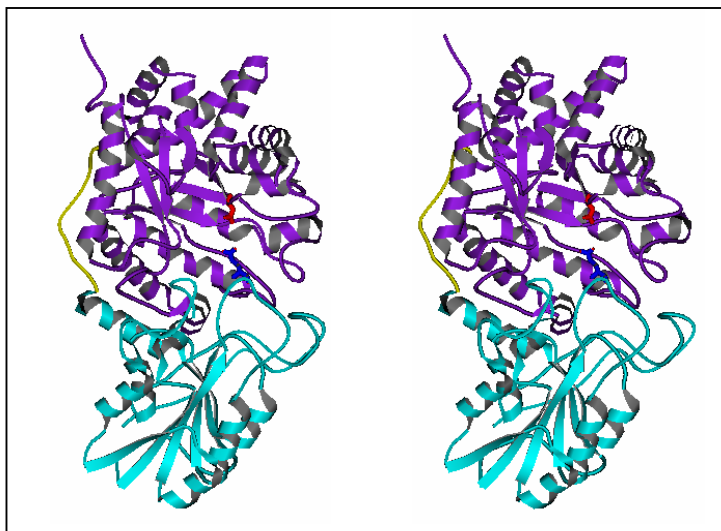


Figure 2. Three-dimensional structure of barley β -D-glucan exohydrolase isoenzyme ExoI

Discussion

The three-dimensional structure of the barley β -glucan exohydrolase and the positions of catalytic residues can be reconciled with the enzyme's broad substrate specificity. At 13 Å in depth, the catalytic pocket could accommodate 2-3 glucosyl residues, given that the distance between glycosidic oxygen atoms of two adjacent residues in (1 \rightarrow 3)- or (1 \rightarrow 4)- β -glucans is approximately 5 Å (Tvaroska *et al.*, 1983). If the pocket is deep enough for only two glucosyl residues, the remainder of the oligomeric or polymeric substrate would project from the pocket, away from the enzyme surface. Thus, substrate binding would be relatively

independent of the overall substrate shape for the β -glucan exohydrolases, in contrast to the (1 \rightarrow 3,1 \rightarrow 4)- β -glucan endohydrolases where the substrate has to have the correct shape to fit into the much longer substrate-binding cleft (Varghese *et al.*, 1994). Because substrate shape is determined by the type of glycosidic linkage, the lack of strict shape requirements for substrates of the β -glucan exohydrolases could explain why substrates with many different linkage types can be hydrolysed.

The barley β -glucan exohydrolases hydrolyse polysaccharides such as (1 \rightarrow 3,1 \rightarrow 4)- β -glucans and laminarin, as well as a range of β -oligoglucosides, including (1 \rightarrow 3,1 \rightarrow 4)- β -oligoglucosides. This broad specificity, together with a distribution that includes not only the Poaceae but also many dicotyledons, makes it difficult to assign a single, unequivocal function to the enzymes. Because they hydrolyse (1 \rightarrow 4)- β -glucosyl linkages considerably more slowly than (1 \rightarrow 3)- β -glucosyl linkages (Hrmova *et al.*, 1998), it might be argued that these enzymes are less likely to play a role in hydrolysis of (1 \rightarrow 3,1 \rightarrow 4)- β -oligoglucosides in germinated grain. The genes encoding the β -glucan exohydrolases are transcribed in the scutellum of germinated grain, but their mRNAs are most abundant in elongating coleoptiles (Harvey *et al.*, 1999). The latter observation has led to the suggestion that β -glucan exohydrolases function in auxin-mediated cell elongation in growing coleoptiles (Harvey *et al.*, 1999; Hoson and Nevins, 1989; Kotake *et al.*, 1997). The amount of (1 \rightarrow 3,1 \rightarrow 4)- β -glucan in walls decreases markedly during coleoptile growth (Sakurai and Masuda, 1978), in a process that has been linked to the wall 'loosening' believed to be necessary for cell elongation (Labrador and Nevins, 1989). We conclude therefore that the barley β -glucan exohydrolases may perform multiple functions that could include wall loosening in the growing seedling and the complete conversion of cell wall (1 \rightarrow 3,1 \rightarrow 4)- β -glucans to glucose in the germinated grain. The latter function could have important implications in the brewing process, especially where high-alcohol, low-calorie beers are required.

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