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Regular paper

Conserved Cys residue influences catalytic properties of potato endo-(1→3)-β-glucanase GLUB20-2

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The synthesis and degradation of (1→3)-β-glycosidic bonds between glucose moieties are essential metabolic processes in plant cell architecture and function. We have found that a unique, conserved cysteine residue, positioned outside the catalytic centre of potato endo-(1→3)-β-glucanase — product of the *gluB20-2* gene, participates in determining the substrate specificity of the enzyme. The same residue is largely responsible for endo-(1→3)-β-glucanase inhibition by mercury ions. Our results confirm that the spatial adjustment between an enzyme and its substrate is one of the essential factors contributing to the specificity and accuracy of enzymatic reactions.

Keywords: *Solanum tuberosum*, substrate specificity, inhibition, structure-function relationships, endo-(1→3)-β-glucanase; GLUB20-2

INTRODUCTION

The proper functioning of the plant cell often demands rapid deposition and precise degradation of (1→3),(1→6)-β-glucan callose, in a temporally and spatially coordinated way. There are numerous endo-(1→3)-β-glucan hydrolases (glucan endo-1,3-beta-D-glucosidases, 3.2.1.39) of various specificities, localizations, and modes of action, which most probably facilitate the proper turnover of callose (Stone & Clarke, 1992).

The three-dimensional structures, as well as the mechanisms of catalysed reactions, are conserved within the families of glycoside hydrolases (Davies & Henrissat, 1995). Most plant endo-(1→3)-β-glucanases, together with the endo-(1→3),(1→4)-β-glucanases (EC 3.2.1.73), are classified in the family GH17 (glycoside hydrolase 17; Henrissat & Davies, 2000). Such differences in specificity within one structural group indicate the gain of new functions by evolutionary divergence without a change in the mechanism of catalysis (Chen *et al.*, 1995).

The substrate specificity of an enzyme originates from the shape complementarity and chemical compatibility of the substrate and the binding site of the enzyme (Koshland, 1958). Thus, the physical barriers created by evolution *via* single amino-acid substitutions should eliminate any accidental binding of non-cognate molecules (Varghese *et al.*, 1994; Høj & Fincher, 1995). Crystallographic analyses published by Varghese *et al.* (1994) of two barley endo-β-glucanases from the family GH17, with distinct substrate specificities, showed that their three-dimensional structures were almost equivalent, both assuming a $(\beta/\alpha)_8$ barrel conformation. The most pronounced feature is a long groove running along the upper surface of the molecule, which can accommodate seven glucose residues of a (1→4)-β chain or eight residues of a (1→3)-β chain (Varghese *et al.*, 1994; Hrmova & Fincher, 2001). Because of the internal architecture of the groove, endo-(1→3)-β-glucanases and endo-(1→3),(1→4)-β-glucanases do not hydrolyse each other's substrates or (1→4)-β-glucans (Høj *et al.*, 1988; Hrmova & Fincher, 2001).

Hg^{2+}-driven inhibition has been observed for a number of plant endo-(1→3)-β-glucanases (Moore & Stone, 1972; Wong & Maclachlan, 1979; Felix & Meins, 1985), which raises questions about the con-

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Abbreviations: Ba, barley β-glucan; Ch, chitin; CM-Cu, carboxymethylated curdlan; CM-Pa, carboxymethylated pachyman; Cu, curdlan; La, laminarin; Li, lichenan; Pa, pachyman; Pu, pustulan; Y, yeast β-glucan.

tribution of the Cys residue(s) to this phenomenon. Hg^{2+} ions are potent toxic compounds acting against enzymes containing Cys residues. The high affinity of mercury ions and metallic mercury for –SH groups allows them to disrupt cell metabolism. Inducible, endogenous processes of detoxification, involving the metallothioneins and glutathione, are also based on this mechanism (Cobbett & Goldsbrough, 2002).

The amino-acid sequences of numerous mature plant β-glucanases contain a single, highly conserved Cys residue as part of the short conserved N-terminal motif, GVCY (see Supplementary data, Fig. 1S at www.actabp.pl). In barley endo-(1→3)-β-glucanase GII, the GVCY motif is recognized as a secondary structural element, the β1-strand. The Cys residue is positioned at the bottom of the catalytic groove and is not exposed at the surface of the molecule (Varghese *et al.*, 1994). It is uncertain whether such localization has any effect on the properties of the enzyme.

A novel potato (*Solanum tuberosum* L. cv. Desirée) basic endo-(1→3)-β-glucanase, product of the *gluB20-2* gene (GenBank accession no. **AJ586575**), has been cloned and analysed *in planta* (Barabasz, 2005). Homology modelling using the available data (Varghese *et al.*, 1994; Receveur-Bréchot *et al.*, 2006) demonstrated a high degree of similarity between the three-dimensional structure of GLUB20-2, and the reference structures of the GH17 family. The similarity includes the location of the Cys residue. The substrate specificity and substrate binding, and the susceptibility of the enzyme to Hg^{2+}-driven inhibition, were examined for recombinant, His-tagged GLUB20-2 protein and its mutant variant, GLUB20-2 C^6→A (GLUB C^6→A). The contributions of the C^6 residue to both the protein's Hg^{2+}-driven inhibition and its substrate specificity were analysed.

MATERIALS AND METHODS

Polysaccharides. Polysaccharides were purchased from the following companies: laminarin (*Laminaria digitata*) and chitin (crab shells) were from Sigma-Aldrich Corp; CM-curdlan (*Alcaligenes faecalis*), pachyman, CM-pachyman (*Poria cocos*), lichenan (*Cetraria islandica*), also barley (*Hordeum vulgare*) and yeast (*Saccharomyces cerevisiae*) β-glucans were from Megazyme International Ireland Ltd; and pustulan (*Umbilicaria papullosa*) was from Merck & Co., Inc. The polysaccharides were prepared as previously described (Denault *et al.*, 1978) or according to the manufacturers' protocols. For the details of the polysaccharide properties, see Table 1.

Bacterial strains, plasmids, and culture media. *Escherichia coli* strains DH5α F′ and BL21 (Novagen, EMD Chemicals Inc) and the vectors pGEM-T Easy (Promega Corporation) and pET30a (Novagen, EMD Chemicals Inc) were used in this study. The source of the *gluB20-2* coding sequence was the pTOPOgluB(20-2)ORF plasmid obtained by introduction of an open reading frame encoding *GLUB20* into pCRII-TOPO vector (Invitrogen Corp; Barabasz, 2005). The bacteria were grown in solid or liquid LB or TB medium (Sambrook *et al.*, 1989). Ampicillin or kanamycin (both 50 μg/ml) was used to select cells carrying one of the plasmids.

Recombinant DNA techniques. Preparation of plasmid DNAs, PCR, site-directed mutagenesis, restriction enzyme digestion, ligation, and transformation were performed as described previously (Ho *et al.*, 1989; Sambrook *et al.*, 1989).

Protein purification. Recombinant His-tagged proteins GLUB20-2 and GLUB C^6→A were expressed in *E. coli* strain BL21 and purified by affinity chromatography using Ni–NTA agarose (Qiagen), according to the manufacturer's procedures. The hydrolytic activities of the purified proteins were tested using La as the substrate (Lever, 1972). The reaction conditions for activity analyses were established before further experiments. For details, see Supplementary data at www.actabp.pl.

Hg^{2+} effect on enzyme activity. Samples containing 0.015 μg of enzyme were supplemented with $HgCl_2$, pre-incubated for 10 min at 28°C, and supplemented with 1.5 mg of La in solution, to a final volume of 1 ml. The standard procedure was applied and the product was assayed as described previously (Lever, 1972).

Determination of substrate specificity. The reaction mixture contained 0.5 μg of enzyme and 3 mg of polysaccharide per time point. An aliquot (1 ml) of reaction mixture was withdrawn at each time point and the reaction was stopped by heat denaturation. Samples were taken every 30 s for 0–5 min, and every 5 min for 5–40 min, whereas the zero point sample was withdrawn immediately after the enzyme had been added. The product was detected as described previously (Miller, 1959). Specific activities were calculated using the initial linear parts of the hydrolysis curves.

Binding assay. Suspensions containing 2 mg of insoluble polysaccharide and 1 μg of enzyme in 300 μl of an appropriate buffer were incubated and centrifuged as described previously (Yamamoto *et al.*, 1998). The control samples lacked insoluble polysaccharides. Supernatant samples were used to measure the residual activity of the free enzyme against La. The product was detected ac-

cording to Lever (1972). Data were calculated as relative values (control sample activities = 100%).

Equipment. All spectrophotometric measurements were performed in Cary 50 spectrophotometer (Varian, Inc).

RESULTS

Hg^{2+}-dependent inhibition

New insight into the structure–function relationships of endo-(1→3)-β-glucanases was obtained while examining the single amino-acid substitution in the GLUB20-2 molecule.

The modified GLUB C^6→A protein was used to analyse the effect of Hg^{2+} ions on the hydrolytic activity of endo-(1→3)-β-glucanase. The specific activity of the GLUB C^6→A variant against standard substrate laminarin (La) under control conditions was about four times lower than the activity of the original enzyme, GLUB20-2 (Fig. 1A). We observed that GLUB20-2 activity was strongly affected by Hg^{2+} ions. However, the C^6→A substitution seemed to effectively enhance the enzyme's resistance to moderate concentrations of Hg^{2+} (Fig. 1B). At low $HgCl_2$ concentrations (5–10 μM), GLUB C^6→A retained over 50% of its initial activity, whereas the activity of the source enzyme underwent a dramatic reduction (Fig. 1B). The activity of GLUB20-2 decreased even more at higher concentrations of the inhibitor, reaching 3.7% of its initial value at 200 μM $HgCl_2$, at which concentration GLUB C^6→A still retained 16% of its activity (Fig. 1B). Moreover, in the range of 10–50 μM Hg^{2+}, the hydrolytic activity of GLUB C^6→A against La was, in absolute terms, significantly higher than that of GLUB20-2 (Fig. 1A).

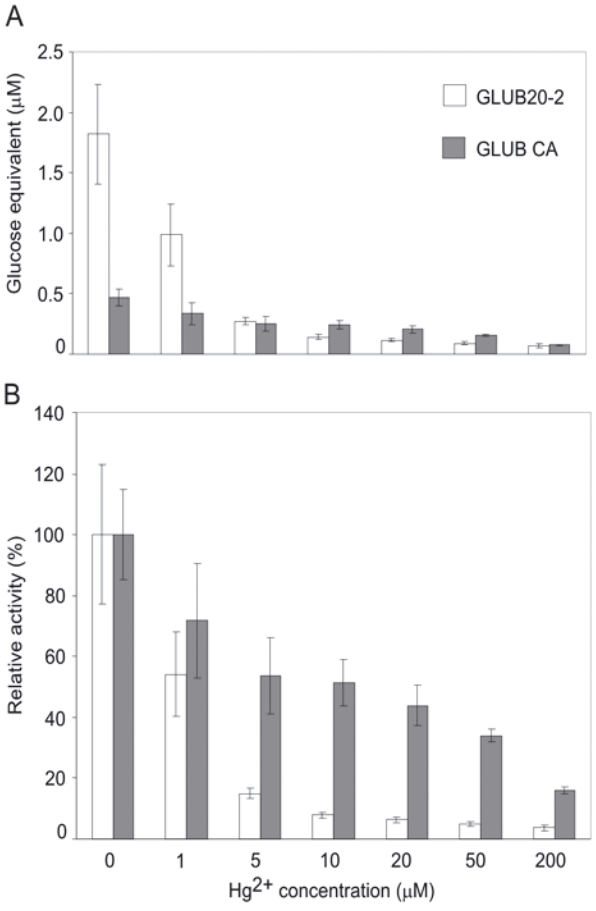

Figure 1. Effects of Hg^{2+} on GLUB20-2 and GLUB C^6→A activities.
The activities of GLUB20-2 (□) and GLUB C^6→A (■) were measured in the presence of increasing concentrations of $HgCl_2$. The control samples did not contain $HgCl_2$. Data are the means of 10 independent experiments. Panel A: absolute values; Panel B: relative values (control value = 100%). Bars represent S.D.

Substrate specificity

Figure 2 and Table 2 describe the hydrolysis of four soluble substrates with various properties. The hydrolytic ability of GLUB20-2 was confirmed to be strictly limited to (1→3)-β bonds. Both (1→3)-β- and (1→3),(1→6)-β-glucans were readily hydrolysed by the enzyme, although the highest enzyme–substrate affinity was observed for GLUB20-2 and the (1→3)-β-glucan CM-curdlan (CM-Cu). The time course of the release of glucose equivalents can be depicted as a logarithmic curve, with most of the

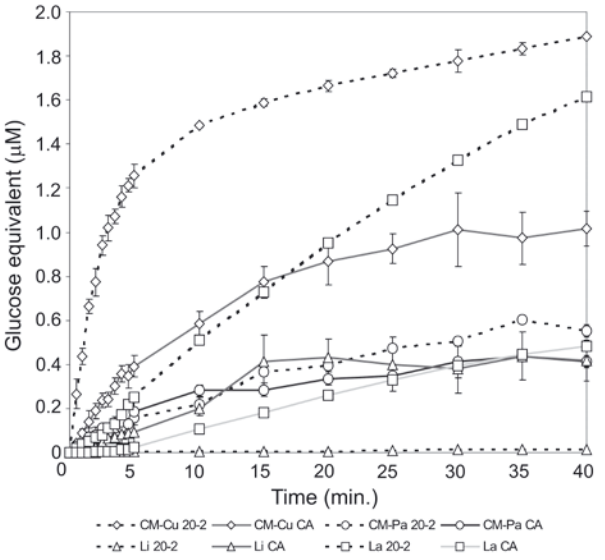

Figure 2. Hydrolysis of soluble β-glucans by GLUB20-2 and GLUB C^6→A.
The activities of both enzymes were measured against various soluble polysaccharides. Data are the means of four independent experiments. Bars represent S.D. 20-2, GLUB20-2; CA, GLUB C^6→A.

Table 1. Polysaccharides used in the study

Name	Description
Barley β-glucan	Linear (1→3),(1→4)-β-glucan; (1→4):(1→3) ~ 2.3–2.7
Chitin	(1→4)-β-*N*-acetyl-glucosamine
Curdlan	Linear (1→3)-β-glucan; ~ 100% (1→3); degree of polymerization (DP) ~ 450
CM-Curdlan	Modified by O-carboxymethylation with chloroacetic acid to confer water solubility
Laminarin	Branched (1→3),(1→6)-β-glucan; (1→3):(1→6) ~ 7; DP ~ 26–32
Lichenan	Linear (1→3),(1→4)-β-glucan; (1→4):(1→3) ~ 2
Pachyman	Linear (1→3)-β-glucan; ~ 100% (1→3); DP ~ 250–690
CM-Pachyman	Modified by O-carboxymethylation with chloroacetic acid to confer water solubility
Pustulan	Linear (1→6)-β-glucan; ~ 100% (1→6)
Yeast β-glucan	Branched (1→3),(1→6)-β-glucan; (1→3):(1→6) ~ 4

product appearing in the first 5 min of the reaction. The furthest part of the curve probably reflects the progress of substrate depletion. Another (1→3)-β-glucan tested, CM-pachyman (CM-Pa), was a poor substrate for GLUB20-2. Despite its high initial activity towards this polysaccharide, the hydrolysis curve tended to saturate around 0.5 μmol of glucose equivalent ml^{-1} (Fig. 2). For the (1→3),(1→6)-β-glucan La, no curve saturation appeared during the experiment. No glucose equivalent release was observed when the (1→3),(1→4)-β-glucan lichenan (Li) was used as the substrate, even when the amount of enzyme was increased five-fold (not shown).

Table 2. Specific activities of GLUB20-2 and GLUB C^6→A

Substrate	Specific activity (μmol min^{-1} mg^{-1})	
	GLUB20-2	GLUB C^6→A
CM-Cu	484.00 ± 40.45	174.00 ± 4.84
La	90.00 ± 4.17	24.00 ± 0.53
CM-Pa	62.00 ± 8.61	82.00 ± 25.39
Li	0.08 ± 0.07	62.00 ± 14.02

Specific activities were calculated as described in the Materials and Methods. Results are the means of four experiments ± S.D.

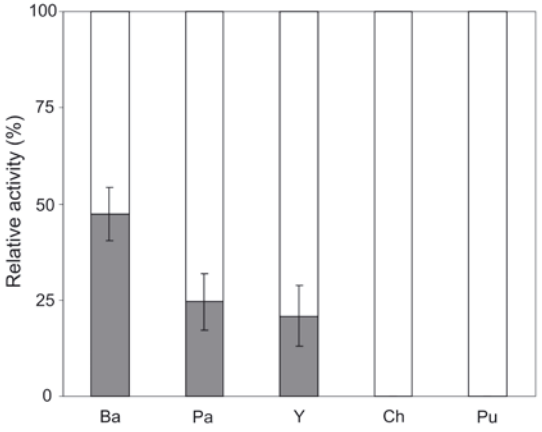

Figure 3. Binding of GLUB20-2 to insoluble β-glucans. Enzyme samples were incubated with or without insoluble polysaccharides and subsequently centrifuged. Hydrolytic activity against La was measured for supernatant aliquots and presented in relative values (samples incubated without polysaccharides (□) = 100%). The bound enzyme activities (■) are the means of 16 independent values. Bars represent S.D.

The C^6→A substitution significantly affected the hydrolytic ability of the modified enzyme. The reaction velocity dropped considerably for CM-Cu and La, which reflects the massive reduction in the specific activity of the enzyme towards these substrates (Table 1). Moreover, the effect of the substitution was sufficient to allow the (1→3),(1→4)-β-glucan Li to be hydrolysed by GLUB C^6→A, with the product release rate and the specific activity becoming similar to those for CM-Pa (Fig. 2, Table 2). No effect of the C^6→A substitution was noted on the enzyme's affinity for CM-Pa, which remained as moderate as that of the unmodified enzyme.

Additional data were obtained from the binding assay of GLUB20-2 to insoluble polysaccharides performed to analyse interactions between the enzyme and insoluble glucans. In a series of experiments, the capacities of various polysaccharides in suspension to bind the enzyme were tested. Figure 3 presents the results of the binding assay applied to five insoluble compounds. We observed that the (1→3),(1→4)-β-glucan Ba representing the class of compounds shown previously to be non-hydrolysable by GLUB20-2 (Fig. 2), bound over 50% of the enzyme, about twice as much as did the Pa or yeast (1→3),(1→6)-β-glucan (Y). In agreement with our predictions, neither chitin (Ch) nor (1→6)-β-glucan pustulan (Pu) bound GLUB20-2 under the experimental conditions used.

DISCUSSION

As previously reported, the activity of His-tagged recombinant barley endo-(1→3)-β-glucanase is very similar to the activity of the native protein, probably because the barley enzyme lacks post-translational modifications (Hrmova *et al.*, 2002).

A

QPIGVCYGKIANNLPSDQDVIKLYNANNIKKMRIYYPHT
NVFNALKGSNIEIILDVPNQDLEALANPSNANGWVQDNI
RNHFPDVKFKYAVGNEVDPGRESGKYARFVGPAMENINN
ALSSAGLQNQIKVSTSTYSGLLTNTYPPRDSIFREEYKS
FINPIIGFLARHNLPLLANIYPYFGHIDNTNAVPLSYAL
FNQQRRNDTGYQNLFDALVDSMYFATEKLGGQNIEIIVS
ESGWPSEGHPAATLKNARTYYTNLINHVKRGAGTPKKPG
KTIETYLFAMFDENEKKGEASEKHFGLFNPDQRPKYQLN
FNLEHHHHHH

B

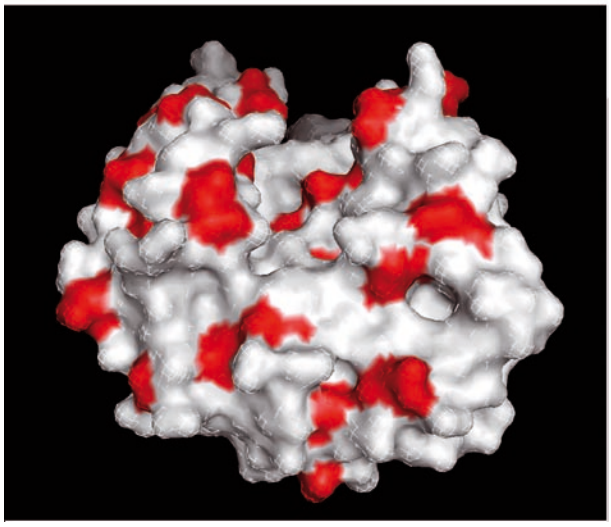

Figure 4. Amino acids possibly involved in Hg^{2+}-driven inhibition of recombinant GLUB20-2 activity.
(A) GLUB20-2 residues that are potential targets of Hg^{2+} are shown in red typeface. (B) The spatial positions of some Hg^{2+}-sensitive residues (red) in the GLUB20-2 molecule in a side view of the GLUB20-2 molecule from the southern end of the catalytic groove. The image was drawn with the PyMOL application (DeLano, 2002), using the molecule model derived from the SWISS-MODEL automated homology modelling server (http://swissmodel.expasy.org/SWISS-MODEL.html). Templates: 1ghs and 2cyg (www.rcsb.org/pdb).

This suggests that extrapolation of the recombinant plant endo-(1→3)-β-glucanase characteristics to the native protein might be valid and informative.

Our results demonstrate that the C^6 residue, which does not belong to the catalytic centre of the endo-(1→3)-β-glucanase, can at least partially contribute to the mercury ion toxic effect on enzyme activity. Because the target Cys residue occurs directly below the surface of the catalytic groove (Varghese *et al.*, 1994), inhibition by $HgCl_2$ may simply originate from the spatial interference in the substrate binding in the groove and/or the reduced accessibility of the substrate to the catalytic residues. Similarly, the occlusion of a water pore by the covalent attachment of Hg^{2+} to the free sulphydryl of a Cys residue has been shown to be the main mechanism of inhibition of water-channel permeability (Preston *et al.*, 1993). It is also very likely that the covalent modification of the sulphydryl group by Hg^{2+} influences the enzyme–substrate interaction *via* local or global conformational changes in the GLUB20-2 molecule. The exposure of a previously inaccessible proteolytic site in the presence of mercury chloride was demonstrated in the plasma membrane protein PMIP31 from red beet (Barone *et al.*, 1998). Conformational changes are also believed to contribute to the inhibition of water transport by aquaporins (Preston *et al.*, 1993) and have been regarded as the major mechanism of mercury action on enzymes (MacGregor & Clarkson, 1974).

However, more general ways of mercury-ion-driven effects should also be considered. Despite the extremely high affinity of mercury ions for sulphydryl groups, their interactions with other residues cannot be excluded, especially with those that are readily accessible (MacGregor & Clarkson, 1974). The C^6 residue displays limited accessibility. Moreover, even when Cys^6 is substituted with Ala, the reduced but progressive decrease in the activity of the enzyme suggests the possible involvement of

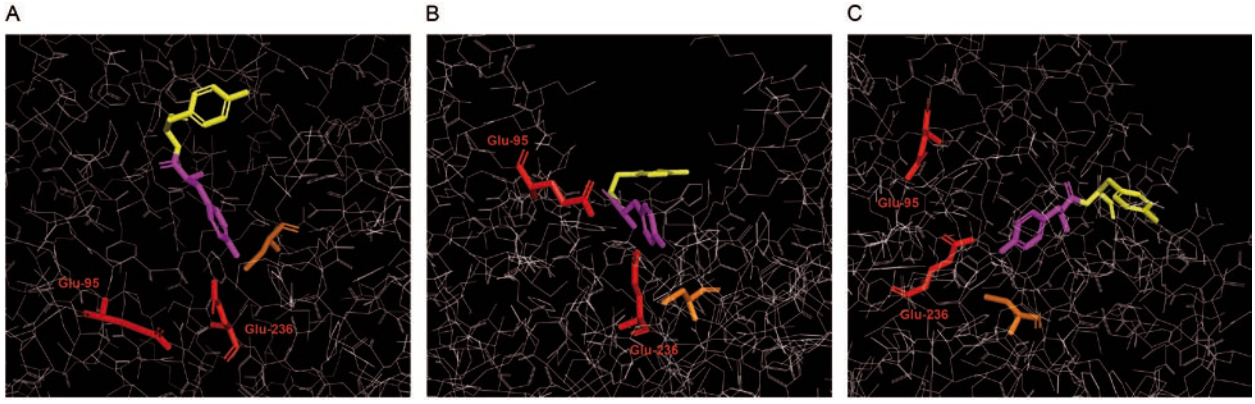

Figure 5. Spatial relationship of C^6 to the catalytic residues and other crucial elements in the GLUB20-2 molecule.
(A) View of the catalytic groove from above (arranged parallel to the NS axis). (B) A side view of the GLUB20-2 molecule from the southern end of the catalytic groove. (C) A view of the catalytic groove from above (arranged parallel to the SW–NE axis). Orange, C^6; red, catalytic residues E^{95} and E^{236}; magenta, Y^{35}; yellow, Y^{36}. Data are adapted from Varghese *et al.* (1994). The picture was drawn as Fig. 4B.

other residues, among them probably Glu, Asp, and His (Dixon & Webb, 1964). It can be assumed that the affinities of the catalytic carboxyl groups of the two Glu residues in the catalytic centre of the molecule for mercury ions, in their local context, is low enough to retain traces of activity, even at the highest concentrations of the toxic compound tested in our study. Figure 4 shows the potential target residues in the GLUB20-2 protein sequence (A) and the positions of some residues that contact the surface of the molecule in the three-dimensional structure of GLUB20-2 (B).

The substitution of the highly conserved Cys residue with Ala significantly reduced the enzyme's susceptibility to mercury ions. Moreover, in absolute terms, GLUB $C^6 \rightarrow A$ was similarly or even more active than the unmodified form of the enzyme at Hg^{2+} concentrations of 5 μM and higher. This phenomenon should be discussed in terms of at least two mechanisms. First, the elimination of mercury-ion binding to the Cys residue abolished the main site of the interaction with the ion (MacGregor & Clarkson, 1974). Second, the potential impact of the $C^6 \rightarrow A$ substitution on the molecular conformation might also compromise the spatial positions of other amino-acid moieties that participate in the protein–mercury ion interaction.

The structural features that influence water solubility and gel-forming ability are extremely important for enzyme–substrate affinity. Many polysaccharides are insoluble compounds and require chemical modifications to be readily hydrolysed (Stone & Clarke, 1992). Both CM-Pa and CM-Cu were O-carboxymethylated to make them water-soluble. However, CM-Cu was totally soluble in water (CM degree about 0.4), whereas CM-Pa (CM degree about 0.2) formed a colloidal solution. This difference in solubility probably explains their different patterns of hydrolysis, especially because there was no significant difference in the amounts of released product when the unmodified, insoluble Cu and Pa were subjected to prolonged hydrolysis (not shown).

The observed changes in the patterns of soluble polysaccharide hydrolysis are an intriguing manifestation of the influence on substrate specificity of a residue that is neither catalytic nor involved in substrate binding. Such an effect could appear at the time of substrate binding or during the reaction itself, and results from a reduction in the substrate affinity of the enzyme or any type of inhibition (Cornish-Bowden, 2004). Because no additional elements were introduced into the reaction, it is highly probable that the binding between the enzyme and the substrates was affected by conformational changes introduced into the GLUB20-2 molecule by the $C^6 \rightarrow A$ substitution.

It is clear that the strong binding between GLUB20-2 and Ba contradicts the conclusions drawn from the comparison of the three-dimensional structures of barley glucanases (Varghese *et al.*, 1994). However, even if some binding occurred between the unmodified protein and (1→3),(1→4)-β-glucan, the glucan position in relation to the catalytic residues was supposedly incorrect and thus prevented its hydrolysis. The conformational change caused by the $C^6 \rightarrow A$ substitution appears to be sufficient to allow the correct accommodation of the (1→3),(1→4)-β-glucan in the catalytic groove and its hydrolysis by GLUB $C^6 \rightarrow A$. As shown in Fig. 5, the C^6 residue probably does not contact the surface of the GLUB20-2 molecule. However, it is a part of a secondary structural element (Varghese *et al.*, 1994). It is also positioned in proximity to Y^{35}, which functions to stabilize the catalytic residue E^{236} in its proper position. The introduced modification could cause changes in substrate specificity by acting on either the spatial stability of the catalytic residue or on the shape of the catalytic cleft. It could affect the structure of the β_1-strand and therefore disturb the shape of the whole molecule, including the position of another crucial residue, Y^{36}, which seems to be the main element blocking the proper accommodation of the (1→3),(1→4)-β-glucan in the GLUB20-2 groove (Varghese *et al.*, 1994). Although an N-terminal Cys residue also occurs in endo-(1→3),(1→4)-β-glucanases (www.cazy.org), Y^{36} is usually then replaced by a residue with a small side chain, which does not restrict (1→3),(1→4)-β-glucan accommodation (Varghese *et al.*, 1994).

The results obtained using GLUB $C^6 \rightarrow A$ once again confirm the relationships between the three-dimensional structure, function, and enzymatic activity of the protein. The difference in structure between the endo-(1→3)-β- and endo-(1→3),(1→4)-β-glucanases, leading to altered substrate specificity, is very subtle and suggests a common ancestry of the two enzyme types. In fact, it is assumed that the endo-(1→3),(1→4)-β-glucanases diverged from the (1→3)-β-glucanases during the appearance of graminaceous monocotyledons (Høj & Fincher, 1995). We believe that this assumption is justified even in the light of the latest discovery of mixed-linked glucans outside the order *Poales* (Sørensen *et al.*, 2008), which is of crucial consequence for the understanding of plant cell wall evolution.

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