Purification and characterization of an extracellular (1 → 6)-β-glucanase from the filamentous fungus *Acremonium persicinum*

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An endo-(1 → 6)-β-glucanase has been isolated from the culture filtrates of the filamentous fungus *Acremonium persicinum* and purified by $(NH_4)_2SO_4$ precipitation followed by anion-exchange and gel-filtration chromatography. SDS/PAGE of the purified enzyme gave a single band with an apparent molecular mass of 42.7 kDa. The enzyme is a non-glycosylated, monomeric protein with a pI of 4.9 and pH optimum of 5.0. It hydrolysed (1 → 6)-β-glucans (pustulan and lutean), initially yielding a series of (1 → 6)-β-linked oligoglucosides, consistent with endo-hydrolytic action. Final hydrolysis products from these substrates were gentiobiose and gentiotriose, with all products released as β-anomers, indicating that the enzyme acts with retention of configuration. The purified enzyme also hydrolysed *Eisenia bicyclis* laminarin, liberating glucose, gentiobiose, and a range of larger oligoglucosides, through the apparent hydrolysis of (1 → 6)-β- and some (1 → 3)-β-linkages in this substrate. K_m values for pustulan, lutean and laminarin were 1.28, 1.38, and 1.67 mg/ml respectively. The enzyme was inhibited by *N*-acetylimidazole, *N*-bromosuccinimide, dicyclohexylcarbodi-imide, Woodward's Reagent K, 2-hydroxy-5-nitrobenzyl bromide, $KMnO_4$ and some metal ions, whereas D-glucono-1,5-lactone and EDTA had no effect.

INTRODUCTION

(1 → 6)-β-Glucans do not occur as commonly as β-glucans composed of (1 → 4)- or (1 → 3)-glucosidic linkages [1]. In fact, only a few linear (1 → 6)-β-glucans have been identified, the best known being pustulan from *Umbilicaria* species [2] and lutean from *Penicillium luteum* [3]. However, (1 → 6)-β-linkages occur frequently in the glucans of yeast and filamentous-fungal cell walls [4-9] and, not surprisingly, enzymes hydrolysing (1 → 6)-β-glucosidic linkages are common in these organisms [10–13].

Although many fungi secrete (1 → 6)-β-glucan hydrolases [10], very few of these enzymes have been purified and adequately characterized in terms of their mode of substrate attack. The action of typical (1 → 6)-β-glucanases [(1 → 6)-β-D-glucan glucanohydrolase, EC 3.2.1.75] has been described as involving the random hydrolysis of (1 → 6)-linkages in (1 → 6)-β-glucans [14]. However, the (1 → 6)-β-glucanase from *Gibberella fujikuroi* has been studied in some detail [15–17], and its mode of action shown to differ, since although it has a principal specificity for (1 → 6)-β-glucosidic linkages, it also efficiently hydrolyses (1 → 3)-β-linkages adjacent to 6-substituted glucose residues. This enzyme, and a similar one from *Rhizopus chinensis* [18], act in a endo-hydrolytic manner and can hydrolyse (1 → 3)(1 → 6)-β-glucans like the *Eisenia* laminarin.

Although several other microbial endo-(1 → 6)-β-glucanases have also been described [15,19–27], the lack of information on their hydrolytic activities against *Eisenia* laminarin makes it difficult to satisfactorily specify their action patterns. However, most appear to display low activity against (1 → 3)(1 → 6)-β-glucans such as yeast glucan and the *Laminaria* laminarins, and therefore, possibly have a similar action to the *Gibberella* enzyme. By contrast, a (1 → 6)-β-glucan hydrolase isolated from *Flavobacterium* [28] has a high specificity for (1 → 6)-β-glucosidic linkages in (1 → 6)-β-glucans, but acts by an exo-hydrolytic mechanism. However, this enzyme has a preference for short-chain (1 → 6)-β-linked oligosaccharides, has glucosyltransferase activity and is inhibited by D-glucono-1,5-lactone, and thus is best described as a (1 → 6)-β-glucosidase with activity against long-chain (1 → 6)-β-glucans. A similar group of enzymes, but with broader specificity, are the yeast-type β-glucan exo-hydrolases which generally hydrolyse both (1 → 6)-β-glucans and (1 → 3)-β-glucans ([29]; see also [11,12]).

The filamentous fungus *Acremonium persicinum* produces an extracellular β-glucan when grown in nitrogen-limiting conditions [30]. In the absence of glucose, *A. persicinum* can utilize this β-glucan and others, like laminarin, carboxymethyl (CM)-pachyman and pustulan, as the sole carbon source, with the secretion of both (1 → 3)-β-glucanases and (1 → 6)-β-glucanases into the culture filtrate [31]. A recent study [32] has described the purification and characterization of three extracellular (1 → 3)-β-glucanases from this fungus. In the present paper the purification and characterization of a (1 → 6)-β-glucanase secreted by *A. persicinum* is reported.

EXPERIMENTAL

Organism and culture conditions

Growth of *Acremonium persicinum* Nicot and Gams QM107a in liquid media and collection of the culture filtrates were as described previously [32].

Enzyme substrates and other chemicals

Dicyclohexylcarbodi-imide, diethyl pyrocarbonate, *N*-acetylimidazole, 2-hydroxy-5-nitrobenzyl bromide and Woodward's Reagent K (*N*-ethyl-5-phenylisoxazolium-3′-sulphonate) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Abbreviations used: CM, carboxymethyl; DP, degree of polymerization; CWF, cell-wall fraction.

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All other chemicals and enzyme substrates were obtained from sources previously specified [32]. A summary of proposed structures for the β-glucan substrates used is given by Hrmova and Fincher [33].

(1 → 6)-β-Glucanase assay and other determinations

(1 → 6)-β-Glucanase activities were determined routinely in duplicate using pustulan from *Umbilicaria papullosa* (Calbiochem, San Diego, CA, U.S.A.) as substrate. Assays were performed in 50 mM sodium acetate buffer, pH 5.0, with 4 mg/ml substrate at 40 °C for 30 min, and the amount of reducing sugar released estimated by the Somogyi–Nelson method [34,35]. A unit of activity is defined as 1 μmol of reducing sugar released (as glucose equivalents)/min. Fungal biomass, protein concentrations and the carbohydrate content of the purified enzyme were determined as previously described [32].

Purification of the (1 → 6)-β-glucanase

Purification of the (1 → 6)-β-glucanase from *A. persicinum* culture filtrates, concentrated 14-fold to ≈ 100 ml by ultrafiltration [32], was achieved using $(NH_4)_2SO_4$ fractionation followed by FPLC® chromatography with anion-exchange (Mono Q) and gel filtration (Superose 12) columns as described previously for purification of the extracellular (1 → 3)-β-glucanases of *A. persicinum* [32].

Characterization of the (1 → 6)-β-glucanase

The homogeneity, molecular masses and pIs of the purified (1 → 6)-β-glucanase were determined by SDS/PAGE and analytical isoelectric focusing as previously described [32]. Glycoprotein staining with the periodic acid/Schiff reagent [36] was also performed on SDS/PAGE gels as described previously [32].

The effects of pH, and temperature on the activity of the purified (1 → 6)-β-glucanase were determined by methods previously described [32]. Similarly, substrate specificity and kinetic characteristics were also determined as detailed previously [32], except that substrates were used at 4 mg/ml in substrate-specificity determinations and over the range of 0.2–10 mg/ml for kinetic determinations. The enzymic hydrolysis products from pustulan, lutean, and *E. bicyclis* laminarin were analysed by TLC as previously described [32]. (1 → 6)-β-Linked oligosaccharide standards for TLC analysis were obtained by hydrolysing pustulan with 0.5 M HCl at 100 °C for 120 min [25,37].

^{1}H NMR analysis of pustulan hydrolysis by the purified (1 → 6)-β-glucanase

Both the purified (1 → 6)-β-glucanase (0.83 unit in distilled water) and pustulan (5 mg in 500 μl of 0.2 M sodium acetate buffer, pH 5.0) were freeze-dried three times from ^2H_2O (99.96 atom % ^{2}H; Cambridge Isotope Laboratories, Andover, MA, U.S.A.) to exchange labile H atoms for ^{2}H. This freeze-dried pustulan was then redissolved in 0.7 ml of ^2H_2O and equilibrated at 30 °C, and initial spectra recorded. The purified (1 → 6)-β-glucanase was then added and the stereochemical course of hydrolysis followed by collection of ^{1}H-NMR spectra at intervals during the incubation.

^{1}H-NMR spectra were recorded on a Bruker DRX-400 spectrometer with a 5 mm inverse broad-band probe, operating at 400 MHz and 30 °C. This temperature was chosen to minimize the interference of the residual $^1H^2HO$ resonance with resonances of interest, and the spectra were referenced to the residual $^1H^2HO$ at 4.72 p.p.m. (measured indirectly with respect to sodium 2,2-dimethyl-2-silapentane-5-sulphonate at 30 °C). Complex data points (8192) were acquired over the 3531 Hz spectral width, with 32 transients and eight dummy transients per spectrum. The recycle time was 2.16 s, and the data were processed with a 1 Hz line-broadening exponential window function. The plotted spectra were normalized to the peak height (and area) of the $^1H^2HO$ resonance.

Inhibitor assays

Inhibition by dicyclohexylcarbodi-imide and 2-hydroxy-5-nitrobenzyl bromide were assayed by the method of Moore and Stone [38], while the effect of Woodward's Reagent K on enzyme activity was determined as described by Lombardo [39]. In all three assays, after incubation at 25 °C for 30 min, the reactions were quenched by diluting 1 in 4 with 1 M sodium acetate buffer, pH 5.5, containing 100 μg/ml BSA, left at room temperature for 15 min, and desalted by FPLC® into 50 mM sodium acetate buffer, pH 5.0, with a fast-desalting column (AMRAD–Pharmacia Biotech).

Inhibition by diethyl pyrocarbonate and *N*-acetylimidazole were assayed as described by Pradel and Kassab [40] and Moore and Stone [38] respectively. After incubation for 30 min at 25 °C, the reactions were stopped by desalting by FPLC®.

For substrate-protection assays, 4 mg/ml pustulan was added to the reaction mixtures prior to the introduction of inhibitors, and in all cases the residual activity was assayed against pustulan. The effects of other inhibitors and metal ions were determined as previously described [32].

Fractionation of *A. persicinum* cell walls

For the preparation of cell walls, *A. persicinum* was grown for 4 days in liquid media containing 30 g/l glucose, 8 g/l $NaNO_3$, 1 g/l yeast extract (Oxoid L21), and mineral salts [41] at 28 °C in an orbital incubator (Patons Industries, Adelaide, S.A., Australia) at 180 rev./min. Mycelia were harvested by centrifugation (7500 ***g***, 30 min) and washed four times with distilled water to remove loosely attached cell-surface polysaccharides, and the cell walls then extracted and fractionated by a modification of the method of Hiura et al. [21,42]. The washed cells were homogenized for 10 min in a Braun MSK homogenizer with 0.25–0.30 mm diameter Ballotini beads, and the homogenate autoclaved for 60 min at 121 °C. The soluble extract (CWF 1) was removed by centrifugation (15000 ***g***, 30 min), and the insoluble material was washed four times with distilled water and further extracted by heat treatment (121 °C, 60 min). The hot-water-insoluble material was then extracted with 1 M NaOH for 24 h at 4 °C, and the alkali-insoluble extract (CWF 2) removed by centrifugation (15000 ***g***, 30 min) and the sediment washed four times with 1 M NaOH. The alkali-soluble material was neutralized with acetic acid, and the precipitate formed (CWF 3) was separated from the soluble material (CWF 4) by centrifugation (15000 **g**, 30 min) and washed four times with distilled water. All extracts were then exhaustively dialysed against distilled water and stored at 4 °C in sodium azide solution (0.2 mg/ml).

RESULTS

Enzyme production

Growth of *A. persicinum* on *E. bicyclis* laminarin as the sole carbon source, and production of (1 → 6)-β-glucanase is shown

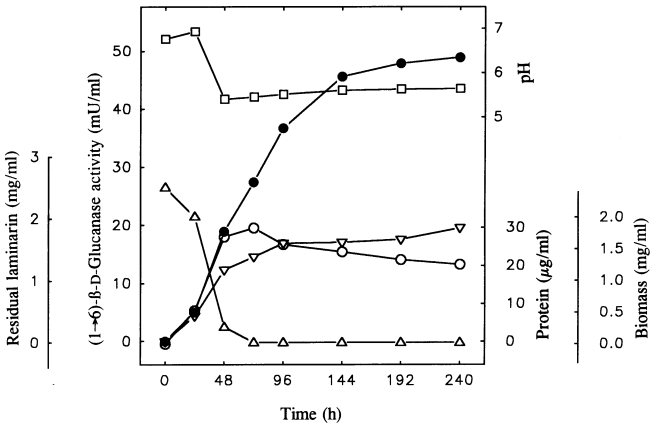

Figure 1 Growth and (1 → 6)-β-glucanase production of *A. persicinum* on laminarin in batch culture

See the text for details. ●, (1 → 6)-β-glucanase activity; ○, biomass; □, pH; ▽, protein concentration; △, residual laminarin. U, units.

in Figure 1. Laminarin was chosen as the carbon source for these experiments as it is known to support the production of both (1 → 3)-β- and (1 → 6)-β-glucanase activity by this fungus [31]. (1 → 6)-β-Glucanase was detected in the culture filtrate from the onset of growth, and steadily increased to near maximum levels after 144 h, which was the usual incubation period.

Enzyme purification

On $(NH_4)_2SO_4$ fractionation of the concentrated culture filtrate, 92% of the (1 → 6)-β-glucanase activity was recovered in the 50–70%-satd. $(NH_4)_2SO_4$ fraction. Anion-exchange chromatography of the desalted 50–70%-satd. $(NH_4)_2SO_4$ fraction on a Mono Q column gave a single peak of (1 → 6)-β-glucanase activity. This was then chromatographed on a Superose 12 column, and the elution profile revealed a single major protein peak with corresponding (1 → 6)-β-glucanase activity. The purified (1 → 6)-β-glucanase was analysed by SDS/PAGE (Figure 2) and isoelectric focusing (not shown) with Coomassie Blue staining, and in each case a single protein band was observed, suggesting that the preparation was homogeneous. The purification factors and yields of the (1 → 6)-β-glucanase at each step are summarized in Table 1.

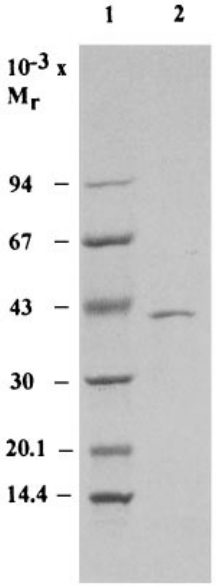

Figure 2 SDS/PAGE of the purified *A. persicinum* (1 → 6)-β-glucanase

Lane 1, molecular-mass markers; lane 2, purified (1 → 6)-β-glucanase.

Properties of the purified glucanase

The molecular masses of the purified (1 → 6)-β-glucanase estimated by SDS/PAGE and gel-filtration chromatography was 42.7 and 44.3 kDa respectively, suggesting that the enzyme is a monomeric protein. The pI, determined by isoelectric focusing, was 4.9. The lack of periodic acid/Schiffs staining after SDS/PAGE indicated that the enzyme is not a glycoprotein, and this was further supported by quantitative carbohydrate determination on the purified enzyme in solution by the phenol/H_2SO_4 method [43] which failed to detect any carbohydrate.

Maximum activity was observed at pH 5.0, although the (1 → 6)-β-glucanase has a broad pH stability, retaining greater than 80% of its activity over the tested pH range of 4.5–9.5 after 24 h at 4 °C. The purified enzyme also displayed short-term stability up to 50 °C. Crude (1 → 6)-β-glucanase preparations were stable, retaining greater than 90% of their activity after 5 weeks at 30 °C and pH 5.5, while the purified (1 → 6)-β-glucanase (at ≈20 μg/ml protein) showed no detectable loss of activity during 4 months storage at 4 °C or 12 months at −70 °C.

The purified (1 → 6)-β-glucanase obeyed Michaelis–Menten kinetics over the concentration range 0.2–10.0 mg/ml for all substrates tested. A K_m of 1.28 mg/ml was obtained against pustulan, which corresponds to a value of 75.3 μM based on a molecular mass for pustulan of 17 kDa [degree of polymerization (DP) 105] [44]. Similar K_m values of 1.38 mg/ml and 1.67 mg/ml were obtained for lutean and *E. bicyclis* laminarin respectively (Table 2).

Substrate specificity

The purified (1 → 6)-β-glucanase hydrolysed a range of (1 → 6)-β- and (1 → 3)(1 → 6)-β-glucans (Table 3). The highest activity was detected against pustulan, with lutean and the *E. bicyclis* laminarin also hydrolysed rapidly. Only very low activities were observed against gentiobiose, an insoluble (1 → 3)(1 → 6)-β-glucan from *Saccharomyces cerevisiae*, and its CM derivative. Similarly, the other yeast glucan preparation tested, which is mainly composed of a mixture of the insoluble (1 → 3)(1 → 6)-β-glucan and a soluble (1 → 6)-β-glucan containing some (1 → 3)-substituted β-glucose residues [4,5], was also hydrolysed slowly. The purified (1 → 6)-β-glucanase also had only slight activity against the extracellular glucan from *A. persicinum*, but hydrolysed some *A. persicinum* cell-wall fractions more readily. No activity was detected when several other β-glucans, β-linked oligoglucosides or aryl and alkyl β-glycosides were used as substrates.

Products of enzyme action and mode of substrate cleavage

Examination of the products released from the hydrolysis of pustulan by TLC (Figure 3a) revealed an initial release of a range of (1 → 6)-β-linked oligosaccharides which, as the incubation continued, were further cleaved to yield eventually only gentiobiose and gentiotriose as final hydrolysis products. Only trace amounts of glucose were detected late in the incubation. Action

Table 1 Summary of purification and yields of the (1 → 6)-β-glucanase from *A. persicinum*

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification factor
Crude culture filtrate	39	63	1.6	100	1
Ultrafiltration	29.3	61	2.1	96	1.3
$(NH_4)_2SO_4$ precipitation	6.3	51	8.3	82	5.1
Mono-Q anion-exchange chromatography	0.061	4.1	67	6.5	41
Superose 12 gel-filtration chromatography	0.022	3.3	146	5.0	89

Table 2 Kinetic properties of the purified (1 → 6)-β-glucanase from *A. persicinum*

Kinetic parameters were determined at 40 °C in 100 mM sodium acetate buffer, pH 5.0, using 14.5 munit of (1 → 6)-β-glucanase.

Substrate	Parameter	Value
Pustulan	K_m	
	(mg/ml)	1.28
	(μM)*	75.3
	k_{cat} (s^{-1})	172
	k_{cat}/K_m ($10^{-6} \times s^{-1} \cdot M^{-1}$)†	2.29
Lutean	K_m (mg/ml)	1.38
E. bicyclis laminarin	K_m (mg/ml)	1.67

* Based on a molecular mass for pustulan of 17 kDa [44].
† Calculations based on molar glucose content.

Table 3 Relative rates of hydrolysis of β-glucans and oligosaccharides by the purified *A. persicinum* (1 → 6)-β-glucanase

The relative rate of hydrolysis of the (1 → 6)-β-glucanase against pustulan was arbitrarily set at 100% and corresponds to 14.5 munits of enzyme activity. No activity was detected against *Laminaria* laminarins, pachyman, CM-pachyman, curdlan, schizophyllan, scleroglucan, lichenin, barley β-glucan, reduced pneumonococcal RSIII glucan, CM-cellulose, hydroxyethylcellulose, pullulan, *A. persicinum* cell-wall fraction 4, aryl and alkyl β-glycosides, sophorose, and (1 → 3)-β- or (1 → 4)-β-oligosaccharides.

Substrate	Relative activity (%)
Pustulan	100
Lutean	86
Laminarin (from *E. bicyclis*)	46
Gentiobiose	0.5
(1 → 3)(1 → 6)-β-Glucan (*S. cerevisiae*)	0.2
CM-(1 → 3)(1 → 6)-β-glucan (*S. cerevisiae*)	0.7
Yeast glucan (*S. cerevisiae*)	1.6
A. persicinum extracellular glucan	0.3
A. persicinum cell-wall fractions	
CWF 1	13.4
CWF 2	7.2
CWF 3	3.9

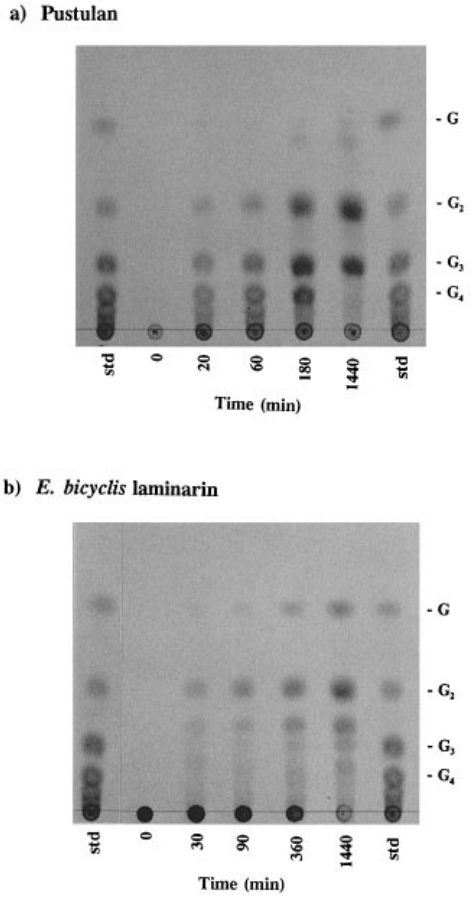

Figure 3 TLC analysis of hydrolysis products released over time by action of the purified *A. persicinum* (1 → 6)-β-glucanase on (a) pustulan and (b) *E. bicyclis* laminarin

A portion of (1 → 6)-β-glucanase amounting to 14.5 munits of activity was used. Standards were glucose (G), gentiobiose (G_2), gentiotriose (G_3), and gentiotetraose (G_4).

on lutean produced identical product patterns to those observed with pustulan (results not shown), while hydrolysis of *E. bicyclis* laminarin (Figure 3b) resulted in the release of a more complex mixture of products, including glucose, gentiobiose, and a range of larger unidentified oligosaccharides.

Stereochemistry of hydrolysis of the purified (1 → 6)-β-glucanase

The partial ^{1}H-NMR spectra recorded after the addition of the purified *A. persicinum* (1 → 6)-β-glucanase illustrating the stereochemical course of the reaction are shown in Figure 4. During the first few minutes of the incubation, doublets at 4.65 p.p.m. (*J* 8 Hz) and 4.66 p.p.m. (*J* 8 Hz), characteristic of H-1β from (1 → 6)-β-oligoglucosides of different chain length, appeared and rapidly increased in intensity. A small doublet at 5.23 (*J* 3.8 Hz), assigned to H-1α, only became noticeable later in the incubation, and almost certainly arose from the mutarotation of the initially formed β-anomers. After 17 min the relative intensities of these α- and β-anomer resonances (calculated after integration of the peaks) were 1:15.4, far from the expected mutarotational equilibrium value of about 1:2 [45], which was

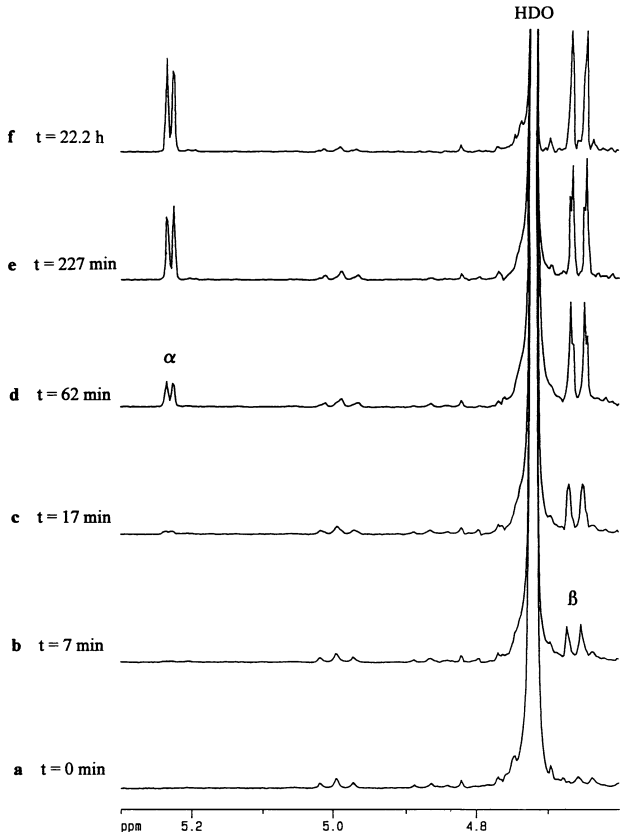

Figure 4 Partial ^{1}H-NMR spectra of the anomeric region showing the stereochemical course of pustulan hydrolysis by the purified *A. persicinum* (1 → 6)-β-glucanase

(**a**) Spectrum of pustulan before addition of enzyme. (**b**–**e**) Spectra of pustulan hydrolysis at the indicated times after enzyme addition. (**f**) Spectrum of the anomerically equilibrated reaction mixture after 22.2 h. HDO, $^1H^2HO$.

observed after the reaction was left overnight (22.2 h). This clearly indicates that the enzyme hydrolysis occurs with retention of configuration.

Effects of metal ions and inhibitors

A range of metal ions and other inhibitors were examined for their effects on the activity of the purified (1 → 6)-β-glucanase with the results shown in Table 4. Almost complete inhibition was observed with 1 mM $KMnO_4$, while moderate inhibition was seen with 1 mM Sn^{2+} Cu^{2+}, Zn^{2+} and Mn^{2+}. Activity was unaffected by 1–20 mM D-glucono-1,5-lactone or 1 mM EDTA.

The effects of a range of relatively group-specific potential inhibitors of the *A. persicinum* enzyme were investigated by determining the residual activity after incubation with the compounds in the presence or absence of substrate. The results are shown in Table 4. All inhibited (1 → 6)-β-glucanase activity to various extents, but the presence of substrate (pustulan at 4 mg/ml) decreased the inhibition observed with *N*-bromosuccinimide and *N*-acetylimidazole.

DISCUSSION

Although very few microbial (1 → 6)-β-D-glucanases have been extensively characterized, a comparison of their physico-chemical properties with those found for the *A. persicinum* (1 → 6)-β-glucanase, reveals many similarities. For example, a molecular mass of 43 kDa for the *A. persicinum* enzyme is similar to that of several other fungal (1 → 6)-β-glucanases, which are commonly in the range of 30–45 kDa (see [11,13,22]), while their bacterial counterparts are slightly larger at 50–55 kDa [22,27]. Similarly, all described microbial (1 → 6)-β-glucanases are monomeric, the majority have optimal activity between pH 4.5 and 5.5, and K_m values are commonly in the range of 1–3 mg/ml for linear (1 → 6)-β-glucans [11,22].

The purified *A. persicinum* (1 → 6)-β-glucanase acts on pustulan and lutean by typical endo-hydrolytic action, giving a series of (1 → 6)-β-linked oligoglucosides as early hydrolysis products, and gentiobiose and gentiotriose as the only significant final hydrolysis products. This type of action on (1 → 6)-β-glucans has been described previously for the endo-(1 → 6)-β-glucanases of *G. fujikuroi* [17], *Mucor hiemalis* [19], *Aspergilus usamii* [15], *R. chinensis* [18] and a cell-wall lytic enzyme of *Bacillus circulans* [27]. However, the action of the *A. persicinum* enzyme differs from that of the endo-(1 → 6)-β-glucanases isolated from *Penicillium italicum* [20], *Neurospora crassa* [21], *P. brefeldianum* [10,22], *Acinetobacter* sp. [25] and a non-cell-wall lytic enzyme from *B. circulans* [27] as these enzymes released glucose and gentiobiose as the major products and appeared to hydrolyse gentiotriose.

In addition to its action on (1 → 6)-β-glucans, the *A. persicinum* enzyme also displayed significant activity against the (1 → 3)(1 → 6)-β-glucan from *E. bicyclis*, releasing glucose and a range of oligoglucosides. This pattern of activity has again been observed

Table 4 Effects of metal ions and potential inhibitors on the activity of the purified *A. persicinum* (1 → 6)-β-glucanase

Activities are expressed as relative percentages of the activity of the (1 → 6)-β-glucanase with no inhibitors or metal ions added and corresponds to 14.5 munits. Values given in parentheses are from substrate-protection experiments and are the relative residual activities after incubation of the inhibitor and enzyme in the presence of 4 mg/ml pustulan. No effect on activity was observed with the addition of 1 mM $BaCl_2$, NaCl, LiCl, $NiCl_2$, $PbCl_2$, $FeCl_3$, $CoCl_2$, KCl, AgCl, $CaCl_2$, NH_4MoO_4, urea, EDTA, acetamide, iodoacetic acid, dithiothreitol, sodium diethyl dithiocarbamate, D-glucono-1,5-lactone (1–20 mM) or diethyl pyrocarbonate (100 mM). Compounds were added at 1 mM unless otherwise indicated.

Compound added	Residual activity (%)
None	100
$HgCl_2$	90
$SnCl_2$	47
$MgSO_4$	86
$CuCl_2$	20
$ZnSO_4$	44
$MnCl_2$	54
$KMnO_4$	0.5
p-Hydroxymercuribenzoate	93
N-Bromosuccinimide	
0.1 mM	3.7 (12)
1 mM	0 (2.6)
N-Acetylimidazole	
10 mM	24 (76)
100 mM	18 (45)
Woodward's Reagent K	
2 mM	33 (36)
20 mM	29 (34)
Dicyclohexylcarbodi-imide	
10 mM	35 (39)
100 mM	34 (36)
2-Hydroxy-5-nitrobenzyl bromide	
10 mM	35 (44)
100 mM	35 (38)

previously with the *Gibberella* endo-(1 → 6)-β-glucanase and was attributed to the ability of this enzyme to hydrolyse (1 → 3)-β-linkages involving 6-substituted glucose residues [17]. Thus 3-*O*-β-gentiobiosyl gentiobiose (Glcβ1-6Glcβ1-3Glcβ1-6Glc) is hydrolysed to give gentiobiose as the only product, while 3-*O*-β-gentiotriosyl gentiobiose (Glcβ1-6Glcβ1-6Glcβ1-3Glcβ1-6Glc) yields gentiobiose and gentiotriose or 6-*O*-β-laminaribiosyl glucose (Glcβ1-3Glcβ1-6Glc) which are not further cleaved. The *E. bicyclis* laminarin is hydrolysed to yield a range of (1 → 3)(1 → 6)-β-linked oligosaccharides with 6-substituted reducing residues, as well as glucose and (1 → 6)-β-linked oligoglucosides. This type of action pattern is common for other glucanohydrolases and is directly analogous to the *Rhizopus arrhizus* (1 → 3)-β-glucan hydrolase [(1 → 3)-[(1 → 3)(1 → 4)]-β-D-glucan 3(4)-glucanohydrolase, EC 3.2.1.6] [46–48]. Therefore, on the basis of its apparent mode of substrate attack, instead of being referred to as a '(1 → 6)-β-D-glucan glucanohydrolase (EC 3.2.1.75)', the *A. persicinum* enzyme should, more correctly, be described as a (1 → 6)-[(1 → 3)(1 → 6)]-β-D-glucan 6(3)-glucanohydrolase (EC 3.2.1.-).

The (1 → 6)-β-glucanase acts with retention of anomeric centre (*e* → *e*), releasing products in the β-configuration. A search of the literature did not reveal any similar studies investigating the stereochemical course of hydrolysis of other (1 → 6)-β-glucanases, although the results obtained here are consistent with data obtained from other endo-β-glucanases which have been investigated [see 12].

Inhibition of the *A. persicinum* enzyme with *N*-acetylimidazole suggests the importance of tyrosine (or lysine) residues [49], possibly at or near the active site (catalytic or binding sites), since presence of the substrate decreases inhibition. Inhibition with dicyclohexyl carbodi-imide, Woodward's Reagent K, 2-hydroxy-5-nitrobenzyl bromide and *N*-bromosuccinimide may also suggest the importance of carboxy groups and tryptophan or histidine residues [49,50], although the presence of the substrate gave little protection against inhibition by these reagents.

Under the conditions used in this study *A. persicinum* secretes a single (1 → 6)-β-glucanase into the culture fluid. This is common, since only a few filamentous fungi are known which produce multiple (1 → 6)-β-glucanase activities [10]. In contrast, two or more extracellular (1 → 3)-β-glucanases are usually synthesized in fungi [11,13], as is the case in *A. persicinum*, where three (1 → 3)-β-glucanases have been isolated [32]. Although the *A. persicinum* (1 → 6)-β-glucanase can hydrolyse isolated polysacharides from its own cell wall, the physiological significance of its production by this fungus is not yet clear. Further understanding may be gained by studies, currently underway, into the location and regulation of synthesis of this enzyme in *A. persicinum*.

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4 December 1995/20 February 1996; accepted 26 February 1996