Purification and characterization of three extracellular (1 → 3)- β -D-glucan glucohydrolases from the filamentous fungus *Acremonium persicinum*

Stuart M. PITSON,* Robert J. SEVIOUR,*§ Barbara M. McDOUGALL,* James R. WOODWARD,† and Bruce A. STONE‡

*Biotechnology Research Centre, La Trobe University, Bendigo, P.O. Box 199, Bendigo, Victoria 3550, and †Plant Science and Biotechnology Unit, Victorian Department of Agriculture, and ‡School of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia

Three (1 → 3)- β -D-glucanases (GNs) were isolated from the culture filtrates of the filamentous fungus *Acremonium persicinum* and purified by (NH₄)₂SO₄ precipitation followed by anion-exchange and gel-filtration chromatography. Homogeneity of the purified proteins was confirmed by SDS/PAGE, isoelectric focusing and N-terminal amino acid sequencing. All three GNs (GN I, II and III) are non-glycosylated, monomeric proteins with apparent molecular masses, estimated by SDS/PAGE, of 81, 85 and 89 kDa respectively. pI values for the three enzymes are 5.3, 5.1, and 4.4 respectively. The pH optimum for GN I is 6.5, and 5.0 for GN II and III. All three purified enzymes displayed stability over the pH range 4.5–10.0. Optimum activities for GN I, II and III were recorded at 65, 55 and 60 °C respectively, with both GN II and III having short-term stability

up to 50 °C and GN I up to 55 °C. The purified GNs have high specificity for (1 → 3)- β -linkages and hydrolysed a range of (1 → 3)- β - and (1 → 3)(1 → 6)- β -D-glucans, with laminarin from *Laminaria digitata* being the most rapidly hydrolysed substrate of those tested. *K_m* values for GN I, II, and III against *L. digitata* laminarin were 0.1, 0.23 and 0.22 mg/ml respectively. D-Glucono-1,5-lactone does not inhibit any of the three GNs, some metals ions are mild inhibitors, and *N*-bromosuccinimide and KMnO₄ are strong inhibitors. All three GNs acted in an exo-hydrolytic manner, determined by the release of α -glucose as the initial and major product of hydrolysis of (1 → 3)- β -D-glucans, and confirmed by viscometric analysis and the inability to cleave periodate-oxidized laminarin, and may be classified as (1 → 3)- β -D-glucan glucohydrolases (EC 3.2.1.58).

INTRODUCTION

Enzymes hydrolysing (1 → 3)- β -D-glucans occur widely in filamentous fungi [1–6]. They can be classified, according to their action on linear (1 → 3)- β -D-glucan substrates, as exo- or endo-(1 → 3)- β -D-glucanases (exo- or endo-GNs) [7]. Typically, exo-GNs like those of *Sporotrichum dimorphosporum* (formerly *Basidiomycete* sp. QM806) [(1 → 3)- β -D-glucan glucohydrolase; EC 3.2.1.58] sequentially cleave glucose residues from the non-reducing end of (1 → 3)- β -D-glucans [8–13]. However, recently a novel exo-GN [(1 → 3)- β -D-glucan laminaribiohydrolase; EC 3.2.1.-] has been isolated from *Bacillus circulans* that hydrolyses linear (1 → 3)- β -D-glucans by successive removal of laminaribiose units from their non-reducing ends [14]. In contrast, endo-GNs usually cleave β -glucosidic linkages at random sites along the β -D-glucan chain [5] and may be of the *Nicotiana glutinosa* [(1 → 3)- β -D-glucan glucanohydrolases; EC 3.2.1.39] [15] or *Rhizopus arrhizus* [(1 → 3)-[(1 → 3)(1 → 4)]- β -D-glucan 3(4)-glucanohydrolase; EC 3.2.1.6] [16–18] types. Although many fungal GNs have been purified and partially characterized, surprisingly few have been extensively studied with respect to their mode of substrate attack. Among those that have received considerable attention are the *S. dimorphosporum* exo-GN and the *R. arrhizus* endo-GN.

The filamentous fungus *Acremonium persicinum* produces an extracellular β -D-glucan when grown in nitrogen-limiting conditions [19,20]. In the absence of glucose, *A. persicinum* can utilize this β -D-glucan, and others such as laminarin, carboxymethyl (CM)-pachyman and pustulan, as the sole carbon source with the secretion of both GNs and (1 → 6)- β -D-glucanases into the culture filtrate [21]. Studies into the regulation of these enzymes [21] suggested the presence of multiple GNs. This is

common in fungi, where the synthesis of several GNs, often with independent regulation and different suspected cellular functions, has been well documented [6].

In this paper the purification and characterization of the GNs secreted by *A. persicinum* is reported, and forms part of a study into the synthesis of β -D-glucans and β -D-glucan hydrolases and their role.

EXPERIMENTAL

Enzyme substrates

Laminarin from *Laminaria digitata*, glucan from baker's yeast (*Saccharomyces cerevisiae*), lichenin from *Cetraria islandica*, 4-nitrophenyl β -D-glucoside, 4-nitrophenyl β -D-cellobioside, 4-nitrophenyl β -D-lactoside, 4-nitrophenyl β -D-xyloside, 4-nitrophenyl β -D-galactoside, 4-methylumbelliferyl β -D-glucoside, 2-nitrophenyl β -D-glucoside, methyl β -D-glucoside, phenyl β -D-glucoside, gentiobiose, salicin, sophorose, β , β' -trehalose, pullulan, maltose, and isomaltose were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hydroxyethylcellulose and laminarin from *Eisenia bicyclis* were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). Pustulan from *Umbilicaria papulosa* was supplied by Calbiochem (San Diego, CA, U.S.A.), and (1 → 3)(1 → 4)- β -D-glucan from barley (*Hordeum vulgare*) was obtained from Biocon Biochemicals (Kilnagary, Irish Republic). Laminarin from *Laminaria hyperborea* and curdlan from *Alcaligenes faecalis* were supplied by Serva (Heidelberg, Germany). Scleroglucan from *Sclerotium glaucum* was purchased from Pillsbury Chemical Co. (Minneapolis, MN, U.S.A.), and CM-cellulose [degree of substitution (DS) 0.54] from ICI (Dingley, Vic., Australia). (1 → 3)-

Abbreviations used: CM, carboxymethyl; DP, degree of polymerization; DS, degree of substitution; PMSF, phenylmethanesulphonyl fluoride; GN, (1 → 3)- β -D-glucanase.

§ To whom correspondence should be addressed.

β -Linked and (1 $\rightarrow$ 4)- β -linked oligoglucosides of degree of polymerization (DP) 2–7 were supplied by Seikagaku Kogyo Co. (Tokyo, Japan). Pachyman from *Poria cocos* was kindly supplied by Dr. A. B. Blakeney, Yanco Agricultural Institute, Yanco, N.S.W., Australia. The *A. persicinum* β -D-glucan was obtained from the culture filtrate of *A. persicinum* grown under nitrogen-limiting conditions, as previously described [19]. Culture filtrates containing about 5 mg/ml of the glucan were extensively dialysed against 50 mM sodium acetate buffer, pH 5.0, adjusted to 2 mg/ml, and used directly in the enzyme assays. This kept the β -D-glucan in solution, as ethanol precipitation renders it water-insoluble. Lutean was prepared from *Penicillium luteum* culture filtrates [22]. Reduced pneumococcal glucan RSIII was prepared from *Streptococcus pneumoniae* glucan SIII as described by Anderson and Stone [17]. Schizophyllan M-2 was provided by Dr. S. Kitamura, Kyoto Prefectural University, Kyoto, Japan. Insoluble yeast glucan from *S. cerevisiae* and soluble CM-yeast glucan (DS 0.42) were obtained from Dr. J. Sandula, Institute of Chemistry, Bratislava, Slovakia. A summary of the proposed structures of these β -D-glucans is given in [23].

CM-pachymans (DS 0.23, 0.29 and 0.36) were prepared from pachyman by the method of Stone [24]. Periodate-oxidized laminarin, and periodate-oxidized/borohydride-reduced laminarin from *L. digitata* were prepared by the method of Goldstein et al. [25] and Nelson and Lewis [26]. Borohydride-reduced laminarin was prepared by the method of Valent et al. [27].

Other chemicals

Phenylmethanesulphonyl fluoride (PMSF), 2-mercaptoethanol, dithiothreitol, orcinol, BSA and pI marker proteins were obtained from Sigma (Sigma-Aldrich Pty Ltd., Castle Hill, N.S.W., Australia). Coomassie Brilliant Blue R250 and Coomassie Brilliant Blue G250 were supplied by Bio-Rad Laboratories (North Ryde, N.S.W., Australia). Molecular-mass markers were obtained from Pharmacia LKB (AMRAD-Pharmacia Biotech, Melbourne, Vic., Australia). All other chemicals were of analytical grade and obtained from commercial sources.

Organism and culture conditions

Acremonium persicinum Nicot and W. Gams QM107a was maintained as a soil culture at 4 °C and recovered by growth for 4 days at 28 °C on malt-extract agar (Oxoid CM59). Spores, suspended in water, were inoculated into liquid media containing 2.5 g/l *E. bicyclis* laminarin, 0.6 g/l $(\text{NH}_4)_2\text{SO}_4$, 0.5 g/l yeast extract (Oxoid L21), and mineral salts [28]. The medium, as 350 ml aliquots in 1 litre flasks, was shaken in an orbital incubator (Paton Industries, Adelaide, S.A., Australia) at 180 rev./min at 28 °C for 6 days. Culture filtrates from shake flask cultures (4 $\times$ 350 ml) were collected by removing cells by centrifugation (7500 g, 30 min) and filtration of the supernatant through Whatman GF/C filters (1.2 μ m pore size).

Enzyme assays and other determinations

GN activities were routinely determined, in duplicate, using laminarin from *L. digitata* as substrate. Assays were performed in 50 mM sodium acetate buffer, pH 5.0, with 2 mg/ml substrate at 40 °C for 30 min, and the amount of reducing sugar released determined by the Somogyi–Nelson method [29,30]. One unit of activity is defined as 1 μ mol of reducing sugar released (as glucose equivalents)/min. Viscometric assays were performed using 5 mg/ml CM-pachyman (DS 0.23) as substrate in an Ostwald viscometer by the method of Clarke and Stone [31]. The

CM-pachyman was dispersed in alkali before use [32]. Protein was determined by the Coomassie Brilliant Blue reagent [33] using BSA as a standard. Determinations of protein during chromatography were by absorbance at 280 nm. The carbohydrate content of the purified enzymes was determined by the phenol/ H_2SO_4 method [34] using D-mannose and D-glucose as standards. Fungal biomass was determined by filtration of aliquots of culture medium through pre-dried, pre-weighed Whatman GF/C filters (1.2 μ m pore size) that were then dried to constant weight. Residual laminarin concentrations in culture filtrates were determined by measuring total reducing sugars released after incubation of samples of culture filtrate with *S. dimorphosporum* exo-GN, kindly supplied by the late Dr. E. T. Reese, U.S. Army Natick Research and Development Command, Natick, MA, U.S.A. Samples (0.1 ml) were hydrolysed with 100 munits of enzyme at 37 °C in sodium acetate buffer, pH 4.8 [8] for 3 h, after which time no further reducing sugars were liberated. Reducing-sugar values were corrected to account for any sugars present in the samples prior to hydrolysis, and converted into laminarin concentrations through the use of laminarin standards hydrolysed in parallel with the culture filtrates.

Purification of GNs

All operations for the preparation of enzyme extracts were performed at 4 °C. Before concentrating the culture filtrates, PMSF (1 mM), 2-mercaptoethanol (20 mM), and EDTA (10 mM) were added to give the final concentrations indicated. The culture filtrates were then concentrated to about 100 ml by ultrafiltration with an Amicon CH4A concentrator using a 10–20 kDa cut-off hollow-fibre cartridge (HIP10-20, Amicon). The concentrated extracts were then fractionated by slow addition of solid $(\text{NH}_4)_2\text{SO}_4$ at pH 6.0 to give the desired degree of saturation. Precipitates were collected by centrifugation (22000 g, 30 min), redissolved in 20 mM Bistris buffer, pH 6.0, containing PMSF, 2-mercaptoethanol, and EDTA at the above concentrations, centrifuged again (22000 g, 30 min) to remove undissolved material, and desalted in the same buffer using a PD-10 desalting column (Pharmacia LKB). Extracts were stored at –70 °C prior to further use.

All chromatographic procedures were performed using an FPLC system and pre-packed columns (Pharmacia LKB). For anion-exchange chromatography a Mono-Q column HR5/5 (5 mm $\times$ 50 mm) equilibrated with 20 mM Bistris buffer, pH 6.0, was used. Extracts prepared as indicated were first filtered through Millipore-GV (0.22 μ m-pore-size) filters (Millipore) and applied to the column in the equilibration buffer. Absorbed protein was eluted with a linear NaCl gradient (0–0.5 M) in the same buffer with a flow rate of 1 ml/min, and 1 ml fractions were collected. Active fractions were further purified by FPLC gel-filtration chromatography with a Superose 12 column HR10/30 (10 mm $\times$ 300 mm) equilibrated with 50 mM sodium acetate buffer pH 5.0 containing 150 mM NaCl. Samples (0.5 ml) were applied and eluted with the equilibration buffer at a flow rate of 0.4 ml/min and collected in 0.4 ml fractions. Molecular masses of the purified GNs were estimated using phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa) as standards.

Gel electrophoresis and isoelectric focusing of GNs

The homogeneity and molecular masses of the purified enzymes were determined by SDS/PAGE with pre-poured gradient gels (ExcelGel SDS Gradient 8-18; Pharmacia LKB) using a

Multiphor II electrophoresis system (Pharmacia LKB). Approx. 1 μ g of the purified enzymes was applied to the gel after treatment by the method of Laemmli [35]. Molecular masses were determined with the same standards as those used for gel filtration, and gels were stained with Coomassie Brilliant Blue R250. Analytical isoelectric focusing was performed with pre-poured Immobiline gels (Immobiline DryStrip, pH 3.0–10.5; Pharmacia LKB) using a Multiphor II flat-bed apparatus (Pharmacia LKB). Gels containing approx. 3 μ g of protein were stained with Coomassie Brilliant Blue R250. Apparent pI values for the purified enzymes were determined with the following marker proteins; amyloglucosidase (3.6), trypsin inhibitor (4.6), β -lactoglobulin (5.1), carbonic anhydrase II (5.9), carbonic anhydrase I (6.6), myoglobin (6.8, 7.2), L-lactate dehydrogenase (8.6) and trypsinogen (9.3).

Glycoprotein staining with the periodic acid/Schiff reagent [36] was also performed on SDS/PAGE gels to which about 2 μ g of each protein was applied and run under the conditions outlined above.

Enzyme characterization

The effect of pH on activity of the purified GNs was measured at 40 °C over the pH range 3.0–11.5 in 50 mM buffers (citrate, pH 3.0–4.0; acetate, pH 4.0–5.5; Mes, pH 5.5–6.5; Mops, pH 6.5–8.5; Ches [2-(*N*-cyclohexylamino)ethanesulphonic acid], pH 8.5–10.0; Caps [3-(cyclohexylamino)propane-1-sulphonic acid], pH 10.0–11.5). pH-stability was determined by assaying the residual activity after preincubation of the enzymes in the various buffers for 20 h at 4 °C. Thermal stabilities of the purified GNs were determined by measuring the activity remaining after pre-incubation of the enzymes at various temperatures (4–80 °C) for 30 min in 50 mM sodium acetate buffer, pH 5.0. Long-term thermal stabilities of crude extracts were determined by incubation of culture filtrates (about 30 μ g/ml protein), containing 0.02 % (w/v) sodium azide, and adjusted to pH 5.5 with sodium acetate buffer, at the various temperatures (–80 to +40 °C) for 5 weeks. Aliquots of enzyme extracts were stored at –20 °C and –80 °C and removed at intervals, thawed and assayed. Temperature optima were determined over the same temperature range by incubating for 30 min at pH 5.0 (50 mM sodium acetate buffer). Kinetic determinations were performed at 40 °C and at the respective optimum pH for the various enzymes. All substrates were used over the concentration range 0.05–2.0 mg/ml, and data were analysed by Michaelis–Menten kinetics using a weighted non-linear regression program [37]. Substrate specificities of the purified GNs were determined against a range of β -D-glucans and β -D-glucosides at 2 mg/ml. Activities against most substrates were determined reductometrically [29,30], although the hydrolysis of 4-nitrophenyl β -glycosides was monitored by the release of 4-nitrophenol, measured by its absorbance at 400 nm in alkaline solution [38]. The effect of various metal ions and enzyme inhibitors (listed in Table 4 below) on GN activity was measured by pre-incubation of the enzymes with 1 mM of the compounds at 25 °C for 60 min at pH 5.0 (50 mM sodium acetate buffer). The residual activity was then determined under standard enzyme assay conditions and corrected for any effect of the various compounds on the reductometric assay procedure.

N-terminal amino-acid-sequence analysis

Automated amino-acid-sequence analysis was performed using trifluoroacetic acid chemistry in an Applied Biosystems 470A gas-liquid-phase sequencer with an on-line model 130A HPLC for identification of phenylthiohydantoin derivatives.

Analysis of hydrolysis products

Hydrolysis products after incubation of the enzymes with *L. digitata* laminarin, *E. bicyclis* laminarin, schizophyllan, *S. cerevisiae* CM-(1 → 3)(1 → 6)- β -D-glucan and CM-pachyman (DS 0.23) were analysed by TLC. Reaction mixtures were the same as for substrate-specificity assays, except that the buffer concentration was decreased from 50 mM to 10 mM to avoid salt effects during TLC analysis. The products released were applied to silica-gel 60 TLC sheets (Merck) and developed in ethyl acetate/acetic acid/water (2:1:1, by vol.). Reducing-sugar products were stained with the orcinol reagent [39]. Qualitative determinations of glucosyltransferase activities were also performed by TLC analysis. The purified enzymes were incubated with 2 and 10 % (w/v) solutions of glucose, laminaribiose, and laminaritriose at 40 °C in 50 mM sodium acetate buffer, pH 5.0. Samples were taken at intervals up to 120 min and analysed by TLC as described above.

Polarimetric analysis of hydrolysis products

Optical rotations of enzyme reaction mixtures were measured in a 10 cm polarimeter cell with a D4 Polarimeter (Bellingham and Stanley Ltd., London, U.K.). The purified enzymes were added to 10 mg/ml solutions of *L. digitata* laminarin in 50 mM sodium acetate buffer, pH 5.0, and optical rotations at 589 nm (sodium D lamp) were measured during a 60 min period at 26 °C. After 60 min, a drop of concentrated NH_4OH was added to the polarimeter tube to catalyse the mutarotation of glucose [10] and the optical rotation measured again.

RESULTS

Enzyme production

Growth of *A. persicinum* on laminarin (from *E. bicyclis*) as the sole carbon source, and the production of GN, are shown in Figure 1. Under the conditions used, fungal biomass increased for about 72 h until the laminarin substrate was exhausted, and subsequently decreased slowly over the remainder of the culture period. After an initial small increase, the pH decreased and remained relatively constant at about pH 5.5 after growth had ceased. GN was detected in the culture filtrate from the beginning

Figure 1 Growth and GN production of *A. persicinum* on laminarin in batch culture

See the text for further details. ●, GN activity; ○, biomass; □, pH; ▽, protein concentration; and △, residual laminarin.

Figure 2 Anion-exchange chromatography by FPLC

The 50–70%-satd.-(NH₄)₂SO₄ fraction of crude culture filtrate was desalted and applied to a Mono-Q column with 20 mM Bistris, pH 6.0, and a linear gradient of 0–0.5 M NaCl. ●, GN activity; —, A₂₈₀; and ----, NaCl concentration.

of the incubation, and it continued to increase for some time after the exhaustion of laminarin and the biomass production had ceased, possibly due to its release from autolysed cells. Maximum GN levels were detected after 144 h (the usual incubation period), after which time a small decrease in activity was observed. The levels of extracellular protein also increased during the culture period, following the increase in GN activity up to 144 h.

Enzyme purification

(NH₄)₂SO₄ fractionation of the concentrated culture filtrate showed that most of the GN activity (89%) was recovered in the 50–70%-satd.-(NH₄)₂SO₄ fraction. For anion-exchange chromatography the desalted 50–70%-satd.-(NH₄)₂SO₄ fraction was applied to the Mono-Q column in 20 mM Bistris buffer, pH 6.0, and eluted with a linear NaCl gradient of 0–0.5 M. The elution profile (Figure 2) shows three peaks of GN activity, designated GN I, II and III, corresponding to fractions 13, 18 and 38–39. Fractions containing GN III also contained a considerable amount of β -D-glucosidase activity (as determined against 4-nitrophenyl β -D-glucoside). Additionally, fraction 22 displayed (1 $\rightarrow$ 6)- β -D-glucanase activity, determined against

Figure 3 Gel-filtration chromatography by FPLC

GN I, II and III from the Mono-Q column were applied to a Superose 12 column and eluted with 50 mM sodium acetate buffer, pH 5.0. ●, GN activity; —, A₂₈₀.

pustulan. Fractions from the Mono-Q column with GN activity were chromatographed on a Superose 12 column. The elution profiles (Figure 3) for GN I and II revealed single protein peaks with corresponding GN activity. Gel filtration of GN III fractions that also contained β -D-glucosidase activity resulted in the resolution of two protein peaks. The first peak, corresponding to fraction 19, displayed β -D-glucosidase activity (results not shown), and the second peak contained GN (Figure 3), but no β -D-glucosidase activity.

Purity of enzyme preparations

The purification factors and yields of the three GNs are shown in Table 1. On analysis by SDS/PAGE (Figure 4) and isoelectric focusing, each GN showed a single protein band, suggesting their homogeneity. This was confirmed by N-terminal sequence analysis, which showed the absence of secondary sequences. The N-terminal amino acid sequences of the three β -glucanases are shown below ('X' indicates 'residue not identifiable'):

	1	10	20
GN I	ATDWWFGN	VERTGKVP	FPGTNTVDYP
GN II	ATDWWFGN	VERTGKVP	FXTNTXDY
GN III	AXEWWFANI		

A database search for comparable peptides and corresponding nucleotide sequences was performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service [40]. No similar sequences were identified.

Properties of the purified glucanases

The molecular masses of GN I, II and III were estimated by SDS/PAGE to be 81, 85 and 89 kDa respectively (Figure 4). Quite similar values, 79, 83 and 90 kDa for GN I, II and III respectively were obtained by using gel-filtration chromatography, suggesting the enzymes are monomeric proteins. Isoelectric focusing resulted in single protein bands that corresponded to pI values of 5.3, 5.1 and 4.4 for GN I, II and III respectively. The absence of a positive reaction with periodic acid/Schiff

Table 1 Summary of purification and yields of GNs from *A. persicinum*

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification factor
Crude culture filtrate	39	463	12	100	1
Ultrafiltration	29	448	15	97	1.3
(NH ₄) ₂ SO ₄ precipitation	6.3	361	57	78	4.8
GN I					
Mono-Q	0.29	66	229	14	19
Superose 12	0.23	60	261	13	22
GN II					
Mono-Q	0.53	113	213	24	18
Superose 12	0.35	102	292	22	25
GN III					
Mono-Q	0.17	31	179	6.5	15
Superose 12	0.07	24	335	5.1	28

Figure 4 SDS/PAGE of the purified *A. persicinum* GNs

Lane 1, molecular-mass (*M*) markers; lane 2, GN III; lane 3, GN II; lane 4, GN I.

reagent after SDS/PAGE indicated that the enzymes were not glycoproteins. This was further supported by quantitative carbohydrate determinations on the purified enzymes in solution by the phenol/H₂SO₄ method.

Maximum activity was observed at pH 6.5 for GN I, and at 5.0 for GN II and III (Figure 5). All three glucanases appeared to have broad pH optima, with greater than 80% of maximum activity observed between pH 5.0 and 7.5 for GN I, and between 4.5 and 7.0 for GN II and III. The pH stability range was also broad, with all three enzymes retaining more than 90% of their activity over the pH range of 4.5–10.0 after 24 h at 4 °C. With short incubation times (15 min) the optimum temperatures for GN I, II and III were 65, 55 and 60 °C respectively. At higher temperatures the activity decreased rapidly (Figure 5). Some differences in the thermal stability of the purified β -glucanases were also observed (Figure 5). GN I appeared relatively stable at 55 °C, retaining more than 90% of its activity after 30 min. At 60 °C, however, rapid inactivation occurred, with less than 50%

Figure 5 Effects of pH and temperature on the purified *A. persicinum* GNs

●, pH and temperature optima; ○, pH- and temperature-stability. Maximum activities recorded for each enzyme were arbitrarily set at 100% and correspond to 5.2, 5.8, and 6.7 munits of GN I, II and III respectively.

Table 2 Kinetic properties of purified GNs from *A. persicinum*

Parameters were determined at 40 °C in 50 mM sodium acetate buffer, pH 5.0, using 5.2, 5.8 and 6.7 munits of GN I, II and III respectively.

Substrate	GN ...	I	II	III
<i>L. digitata</i> laminarin				
K_m (mg/ml)		0.10	0.23	0.22
$(\mu M)^*$		18	43	41
k_{cat} (s^{-1})		375	482	571
k_{cat}/K_m ($10^{-7} s^{-1} \cdot M^{-1}$)†		2.04	1.13	1.41
<i>L. hyperborea</i> laminarin				
K_m (mg/ml)		0.36	0.60	0.72
<i>E. bicyclis</i> laminarin				
K_m (mg/ml)		0.86	0.78	0.82

* Based on a molecular mass for *L. digitata* laminarin of 5.3 kDa (DP 32) [43].

† Calculations based on molar glucose content.

of its activity remaining after 15 min (results not shown) and 16 % after 30 min (Figure 5). GN II and III retained 80–90 % of their activities at 50 °C, but were almost totally inactivated after 30 min at 55 °C. In all cases the thermal stability appears to be enhanced by the presence of substrate [41]. Crude GN preparations retained more than 95 % of the activity after 5 weeks at 30 °C (pH 5.5). The purified GNs (at about 30 µg/ml protein) also appeared very stable, with no detectable loss of activity during 4 months storage at 4 °C. Similar stabilities have been reported with the exo- β -glucanase from *Schizosaccharomyces japonicus* var. *versatilis* [42]. Crude and purified *A. persicinum* GN preparations could be stored at –80 °C in 50 mM sodium acetate buffer, pH 5.0, for at least 12 months without appreciable loss in activity. However, at –20 °C loss of activity was detected in some samples, while others were unaffected, indicating this is not an effective method for storage of these enzymes.

Kinetic properties

The three GNs obey Michaelis–Menten kinetics over the concentration range 0.05–2 mg/ml for all substrates used for kinetic determinations. The K_m values for the three GNs differed slightly with laminarin from *L. digitata* as substrate. GN I had a K_m of 0.10 mg/ml, whereas GN II and III had similar K_m values of 0.23 and 0.22 mg/ml respectively (Table 2). These correspond to substrate concentrations of 18.4, 42.6 and 40.6 µM (on the basis of a molecular mass for *L. digitata* laminarin of 5.3 kDa (DP 32) [43]). Catalytic rate constants (k_{cat}) and specificity constants (k_{cat}/K_m) of the three enzymes with *L. digitata* laminarin as substrate were similar (Table 2), reflecting the similarities in their respective specific activities (Table 3). The K_m values for all three GNs with laminarin from *L. hyperborea* as substrate were approx. 3-fold higher than those with *L. digitata* laminarin. This correlates with the lower specific activity of the three GNs against *L. hyperborea* laminarin (28–46 % of the activity against *L. digitata* laminarin). Higher K_m values were also obtained when *E. bicyclis* laminarin was used.

Substrate specificities

All three GNs hydrolysed a wide range of (1 → 3)- β - and (1 → 3)(1 → 6)- β -D-glucans (Table 3). Laminarin from *L. digitata* was hydrolysed at the highest rate by all three enzymes, although several other substrates, including schizophyllan, scleroglucan,

Table 3 Relative rates of hydrolysis of β -D-glucans, oligosaccharides and chemically modified laminarins by purified *A. persicinum* GNs

The relative rates of hydrolysis of the three glucanases against laminarin from *L. digitata* were arbitrarily set at 100% and correspond to 5.2, 5.8, and 6.7 munits for GN I, II and III respectively. No activity was detected against lichenin, barley β -glucan, pustulan, lutean, reduced pneumococcal RSIII glucan, CM-cellulose, hydroxyethylcellulose, pullulan, aryl and alkyl β -D-glycosides and non-(1 → 3)- β -linked oligosaccharides. All chemical modifications were made to *L. digitata* laminarin.

Substrate	Relative rate (%)		
	GN I	GN II	GN III
<i>L. digitata</i> laminarin	100	100	100
Laminaribiose	0.6	2.0	1.7
Laminaritriose	37	44	39
Laminaritetraose	52	65	60
Laminaripentaose	58	71	67
Laminarihexaose	68	76	78
Laminariheptaose	80	86	85
Oxidized laminarin	0.1	0	0.3
Oxidized/reduced laminarin	0.2	0.1	0.3
Oxidized/reduced/mild acid-hydrolysed laminarin	72	66	81
Reduced laminarin	89	86	91
<i>L. hyperborea</i> laminarin	46	28	37
<i>E. bicyclis</i> laminarin	24	19	22
Curdian	0.4	1.3	1.4
Pachyman	0.2	0.3	1.1
CM-pachyman			
DS 0.23	20	17	21
DS 0.29	8.5	7.6	9.3
DS 0.36	0.3	0.8	1.4
Schizophyllan	45	38	47
Scleroglucan	38	36	40
<i>A. persicinum</i> extracellular glucan	18	19	17
(1 → 3)(1 → 6)- β -D-Glucan (<i>S. cerevisiae</i>)	0.2	0.1	0.3
CM-(1 → 3)(1 → 6)- β -D-glucan (<i>S. cerevisiae</i>)	4.4	6.2	3.7
Yeast glucan	8.7	5.3	5.2

and laminarins from *L. hyperborea*, and *E. bicyclis*, were also degraded significantly. Hydrolysis of CM-pachyman (DS 0.23) was also substantial, although a decrease in activity was detected as the DS increased. In contrast, the water-insoluble (1 → 3)- β -D-glucans, pachyman and curdlan, were degraded much more slowly, as was the insoluble (1 → 3)(1 → 6)- β -D-glucan from *S. cerevisiae* and its soluble CM derivative. The other yeast glucan preparation, which is mainly composed of a mixture of the insoluble (1 → 3)(1 → 6)- β -D-glucan and a soluble (1 → 6)- β -D-glucan that contains some (1 → 3)-substituted β -D-glucose residues [44,45], was also hydrolysed slowly. No activity was detected with (1 → 6)- β -D-glucans (pustulan and lutean), gentiobiose, (1 → 4)- β -D-glucans (CM-cellulose and HE-cellulose), cello-oligosaccharides (DP 2–7), sophorose, (1 → 3)(1 → 4)- β -D-glucans (lichenin, barley β -D-glucan and reduced pneumococcal glucan), or aryl and alkyl β -D-glycosides (methyl β -D-glucoside, phenyl β -D-glucoside, 4-methylumbelliferyl β -D-glucoside, salicin, 4-nitrophenyl β -D-glucoside, 4-nitrophenyl β -D-cellobioside, 4-nitrophenyl β -D-lactoside, 4-nitrophenyl β -D-xyloside, 4-nitrophenyl β -D-galactoside, and 2-nitrophenyl β -D-glucoside).

The glucan from the parent organism, *A. persicinum*, was a good substrate for all three GNs. The fine structure of this β -D-glucan is currently being investigated [19].

Figure 6 TLC analysis of hydrolysis products released over time (0–1440 min) by the action of the purified *A. persicinum* GNs on (a) *L. digitata* laminarin, (b) schizophyllan and (c) *E. bicyclis* laminarin

Amounts of enzymes used were 5.2, 5.8, and 6.7 munits for GN I, II, and III respectively. Standards (Std) were glucose (G), laminaribiose (L_2), laminaritriose (L_3), laminaritetraose (L_4), laminaripentaose (L_5), laminarihexaose (L_6), and laminariheptaose (L_7), gentiobiose (G_2), and gentiotriose (G_3).

Activity profiles against (1 → 3)- β -D-linked oligoglucosides (laminari-oligosaccharides) were similar for all three GNs, with laminaribiose hydrolysed at a low rate, but laminaritriose and larger oligosaccharides degraded more rapidly, with the activity increasing with increasing chain length (Table 3).

Mode of substrate cleavage: endo or exo

The mode of substrate attack of the purified glucanases was initially determined by viscometric analysis with CM-pachyman (DS 0.23) as substrate. The activity of all three enzymes on CM-pachyman resulted in a slow linear decrease in viscosity, but a rapid increase in reducing-sugar liberation (results not shown). The enzymes were also assayed reductometrically against chemically modified laminarin with the rates of hydrolysis shown in Table 3. Neither periodate-oxidized laminarin nor periodate-

Table 4 Effect of metal ions and inhibitors on the activity of purified *A. persicinum* GNs

Activities are expressed as relative percentages of the activity of the respective enzymes with no inhibitors or metal ions added and correspond to 5.2, 5.8, and 6.7 munits of GN I, II and III respectively. No effect on activity was observed with the addition of 1 mM LiCl, NaCl, KCl, CaCl_2 , CoCl_2 , NiCl_2 , AgCl, PbCl_2 , urea, EDTA, dithiothreitol, sodium diethyldithiocarbamate or D-glucono-1,5-lactone. Abbreviation: pHMB, *p*-hydroxymercuribenzoate.

Compound added (1 mM)	Relative rate (%)		
	GN I	GN II	GN III
None	100	100	100
MgSO_4	71	74	99
MnCl_2	18	64	85
FeCl_3	47	5.5	14
CuCl_2	75	71	53
ZnSO_4	56	96	84
SnCl_2	75	25	56
BaCl_2	115	101	106
HgCl_2	90	80	0.6
KMnO_4	0	0	0
NH_4MoO_4	90	79	99
pHMB	75	76	69
<i>N</i> -Bromosuccinimide	0.5	0.2	0
Iodoacetic acid	79	83	73
Acetamide	88	79	90

oxidized/borohydride-reduced laminarin was substantially degraded by any of the three purified β -glucanases. Subsequent mild acid hydrolysis of the modified laminarins [26], however, rendered them susceptible to degradation. These results also are consistent with exo-hydrolytic attack from the non-reducing end of the laminarin molecule.

Products of enzyme action

The action patterns of the purified GNs were determined by examination of the products released from the hydrolysis of *L. digitata* laminarin, *E. bicyclis* laminarin, schizophyllan, *S. cerevisiae* CM-(1 → 3)(1 → 6)- β -D-glucan, and CM-pachyman (DS 0.23). All three purified enzymes gave similar reaction products on the same substrate, although the major hydrolysis products differed with the substrate used. The first, and major product of hydrolysis of *L. digitata* laminarin was glucose (Figure 6a), with some minor products appearing, one with an R_f value corresponding to that of gentiobiose. Similar products were also released during the hydrolysis of schizophyllan, but more gentiobiose was observed due to the higher proportion of (1 → 6)- β -glucose side-branch residues on the main (1 → 3)- β -D-glucan chain [46] (Figure 6b). Hydrolysis of *E. bicyclis* (Figure 6c) laminarin resulted in the release of a more complex mixture of products, but, again, glucose was a major component, together with considerable amounts of various short- and long-chain oligosaccharides. Glucose was the sole hydrolysis product from both CM-pachyman and the *S. cerevisiae* CM-(1 → 3)(1 → 6)- β -D-glucan (results not shown).

Stereochemistry of hydrolysis

The stereochemical course of laminarin hydrolysis was followed by determining polarimetrically the anomeric form of the glucose released. Similar results were obtained for the three GNs where, in each case, the optical rotation increased gradually in a positive direction during the enzymic hydrolysis. Addition of NH_4OH

resulted in a negative shift in optical rotation (decreasing to the equilibrium value of a mutarotated glucose solution), indicating that the enzymes release glucose in the α -configuration, and, hence, act with inversion of configuration. The lack of glucosyltransferase activity with glucose, laminaribiose and laminaritriose as potential acceptors also supports these results.

Effects of metal ions and inhibitors

A range of metal ions were examined for their effect on the activity of the purified GNs. The results are shown in Table 4. The effects of the metal ions on activity varied for the different GNs, with GN I most inhibited by Mn^{2+} , Fe^{3+} and Zn^{2+} , GN II by Fe^{3+} , Sn^{2+} and Mn^{2+} , while GN III was inhibited by a wider range of ions, with the greatest effects observed with Hg^{2+} , Fe^{3+} , Cu^{2+} and Sn^{2+} . All three GNs were totally inhibited by 1 mM $KMnO_4$ and *N*-bromosuccinimide, whereas moderate inhibition was observed with *p*-hydroxymercuribenzoate, iodoacetic acid and ammonium molybdate (Table 4). The activity of the three GNs was unaffected by 1–20 mM D-glucono-1,5-lactone.

DISCUSSION

Three GNs were separated from the culture filtrates of *A. persicinum* grown on laminarin as the sole carbon source and purified to homogeneity by $(NH_4)_2SO_4$ precipitation, anion-exchange and gel-filtration chromatography. The purity of the final enzyme preparations was confirmed by the appearance of single bands after SDS/PAGE and the absence of any secondary sequences during N-terminal amino-acid-sequence analysis. Purification factors for the three enzymes (22, 24.6 and 28.2 for GN I, II and III respectively) are relatively low compared with similar results with other fungal β -glucanases [47–49], suggesting that, under the growth conditions used, the GNs constitute a high proportion of the total extracellular protein.

Although relatively few fungal GNs have been as extensively characterized, a comparison of their physico-chemical properties with those found for the *A. persicinum* GNs reveals many similarities. The pI values, molecular masses, pH and temperature optima and stabilities of each of the three purified *A. persicinum* enzymes are similar and fall well within the range of values previously reported for other β -glucanases from filamentous fungi [4–6]. For example, the molecular masses of filamentous fungal GNs are commonly in the range 20–80 kDa [6], although values as high as 230 kDa [41,50] and, surprisingly, as low as 2.1 kDa [51], have been reported. Similarly, the majority of fungal β -glucanases have optimal activity between 30 and 60 °C and pH 4.0–6.0 [4,6]. The K_m values for the three purified GNs, with *L. digitata* laminarin as substrate, were similar (0.10–0.22 mg/ml or 20–45 μ M), and are again comparable with values obtained with exo-GNs from other filamentous fungi, including *Penicillium oxalicum* (0.2 mg/ml) [52] and *Trichoderma reesei* (0.28 mg/ml) [53]. All three enzymes had K_m values with *L. hyperborea* laminarin about three times higher than those with *L. digitata* laminarin (Table 2), which is probably a reflection of the poor solubility of this substrate [26]. K_m values with *E. bicyclis* laminarin, although similar for all three *A. persicinum* glucanases, were again higher than for *L. digitata* laminarin (Table 2). This is probably due to the presence of intrachain (1 $\rightarrow$ 3)- β - and (1 $\rightarrow$ 6)- β -linkages, as well as occasional (1 $\rightarrow$ 6)- β -linked glucose and gentiobiose substituents in this substrate [54].

The high N-terminal amino-acid-sequence identity between GN I and II, and, as far as the sequence permits, GN III, and the similarities of their substrate specificities and action patterns raise the possibility that these enzymes are encoded by a family

of closely related genes. Alternatively, GN I and II may be the product of a single gene and subsequently modified by proteinases present in the culture filtrate. This type of proteolytic processing has been reported previously with the production of multiple molecular-mass forms of peptidoglycan hydrolases from a single proprotein of *Staphylococcus simulans* [55]. However, the observed differences in physico-chemical properties of the *A. persicinum* glucanases, such as pH and temperature optima and stability, kinetic parameters, effects of metal ions and inhibitors and pI values, all suggest that they are separate distinct enzymes and not artefacts of proteolytic activity. Although only a small portion of the N-terminal sequence of GN III was identifiable, obvious differences exist between it and the sequences of GN I and II, which clearly indicate that it is the product of a different gene.

The three *A. persicinum* GNs have a similar action pattern and specificity to the exo-GN from *S. dimorphosporum* [8–10,56,57] and several other less well-characterized exo-GNs from filamentous fungi (see [5]). All three enzymes act in an exo-hydrolytic manner, removing single glucose residues from the non-reducing ends of linear (1 $\rightarrow$ 3)- β -D-glucans, as determined by product analysis and their inability to hydrolyse periodate-oxidized laminarin. They are highly specific for (1 $\rightarrow$ 3)- β -D-glucosyl linkages in β -D-glucans, but appear able to hydrolyse (1 $\rightarrow$ 3)(1 $\rightarrow$ 6)- β -D-glucans such as schizophyllan, scleroglucan and *Laminaria* laminarins, liberating glucose and gentiobiose, the latter presumably arising by the cleavage of (1 $\rightarrow$ 3)- β -linkages involving (1 $\rightarrow$ 6)- β -glucose-substituted residues, as shown for the *S. dimorphosporum* exo-GN [9]. The three enzymes hydrolyse the *Eisenia* laminarin with an apparent endo-hydrolytic action. This behaviour has been observed previously in the action of the exo-GN from *S. dimorphosporum* on *E. bicyclis* laminarin [13,58]. This was shown to be due to the ability of the exo-hydrolase to cleave 'internal' (1 $\rightarrow$ 3)- β -linkages adjacent to (1 $\rightarrow$ 6)- β -glucosyl residues, both in the main glucan backbone and as branches, resulting in the liberation of glucose, gentiobiose and higher oligosaccharides. However, like most other fungal exo-GNs, the *A. persicinum* β -glucanases showed no comparable ability to 'bypass' (1 $\rightarrow$ 4)- β -glucosyl residues in (1 $\rightarrow$ 3)(1 $\rightarrow$ 4)- β -D-glucans such as lichenin, barley β -D-glucan and reduced pneumococcal glucan. The relatively slow hydrolysis of CM-pachyman is presumably due to the interference by CM substituents with productive enzyme-substrate interactions, as the rate of hydrolysis decreases as the DS increases. This type of steric hindrance has been previously noted with the endo-GNs from *N. glutinosa* [15] and barley [23], the exo-GN of the unicellular alga *Euglena gracilis* [59], and in the hydrolysis of CM-cellulose by cellulases [60]. Similarly, the highly branched structure of the yeast (1 $\rightarrow$ 3)(1 $\rightarrow$ 6)- β -D-glucan [44] may prevent enzyme binding by the *A. persicinum* GNs.

The *A. persicinum* GNs can be easily distinguished from β -D-glucosidases by their high linkage specificity, their inability to hydrolyse aryl and alkyl β -D-glucosides, their inversion of anomeric centre and their increasing activity with increase of substrate chain length. Further discrimination can be made from the lack of inhibition by D-glucono-1,5-lactone, which is a powerful competitive inhibitor of β -D-glucosidases and non-specific exo- β -D-glucanases, but has little effect on specific exo-GNs [5,61–63]. In these respects their action patterns and specificities are similar to most other filamentous-fungal exo-GNs, but differ substantially from those observed with yeast exo- β -glucanases, which often display many of the characteristics of β -D-glucosidases [4,5,64,65].

Most fungi appear to secrete both exo- and endo-GNs [6], and these may act synergistically for efficient degradation of simple

and complex β -D-glucans [66]. However, *A. persicinum* appears to differ in that only exo-hydrolytic activity was detected in the culture fluid under the growth conditions used in the present study. The physiological significance of the secretion by *A. persicinum* of multiple GNs with similar physical properties and the same substrate specificities and action patterns is not yet known. It is possible that the synthesis of each of the GNs described in the present study may be controlled by a different regulatory mechanism, hence explaining the occurrence of several enzymes. Such a system appears to control GN synthesis in *S. cerevisiae*, where constitutive and sporulation-specific enzymes are found [67,68]. For *P. italicum* it has been suggested that the three GNs secreted by this organism are produced at different stages of cell growth. Two are produced in low levels during active growth and are thought to be involved in hyphal growth and extension because of their activity against isolated *P. italicum* cell walls [69,70]. The third enzyme is produced only after active growth has ceased, and it is implicated in the utilization of reserve materials such as extracellular glucans, since it has no detectable cell-wall-degrading ability [47]. A similar regulation may also occur in *A. persicinum*, since it produces an extracellular β -D-glucan that may serve as a reserve material. However, this β -D-glucan is degraded by all three isolated GNs (Table 3). Other possibilities exist: one or more of the GNs studied here may occur as intracellular or wall-bound enzymes and be released into the culture fluid only during cell autolysis. GN activities are found in both these cellular locations in *A. persicinum* [21].

We thank Dr. Maria Hrmova for her assistance with the enzyme characterization, and Ms. Rosemary Condon for the amino-acid-sequence analyses.

REFERENCES

- Reese, E. T. and Mandels, M. (1959) *Can. J. Microbiol.* **5**, 173–185
- Kawai, M. (1973) *Nippon Nogei Kagaku Kaishi* **47**, 473–477
- Halliwell, G. (1979) *Prog. Ind. Microbiol.* **15**, 1–60
- Bielecki, S. and Galas, E. (1991) *Crit. Rev. Biotechnol.* **10**, 275–304
- Stone, B. A. and Clarke, A. E. (1992) *Chemistry and Biology of (1 → 3)- β -Glucans*, LaTrobe University Press, Melbourne
- Pitson, S. M., Seviour, R. J. and McDougall, B. M. (1993) *Enzyme Microb. Technol.* **15**, 178–192
- International Union of Biochemistry (1992) *Enzyme Nomenclature; Recommendations of the Nomenclature Committee of the International Union of Biochemistry*, Academic Press, Orlando
- Huotari, F. I., Nelson, T. E., Smith, F. and Kirkwood, S. (1968) *J. Biol. Chem.* **243**, 952–956
- Nelson, T. E., Johnson, J., Jantzen, E. and Kirkwood, S. (1969) *J. Biol. Chem.* **244**, 5972–5980
- Nelson, T. E. (1970) *J. Biol. Chem.* **245**, 869–872
- Bochkov, A. F., Sova, V. V. and Kirkwood, S. (1972) *Biochim. Biophys. Acta* **258**, 531–540
- Nelson, T. E. (1975) *Biochim. Biophys. Acta* **377**, 139–145
- Yamamoto, R. and Nevins, D. J. (1983) *Carbohydr. Res.* **122**, 217–226
- Kanzawa, Y., Kurasawa, T., Kanegae, Y., Harada, A. and Harada, T. (1994) *Microbiology (U.K.)* **140**, 637–642
- Moore, A. E. and Stone, B. A. (1972) *Biochim. Biophys. Acta* **258**, 248–264
- Marshall, J. J. (1974) *Carbohydr. Res.* **34**, 289–305
- Anderson, M. A. and Stone, B. A. (1975) *FEBS Lett.* **52**, 202–207
- Clark, D. R., Johnson, J., Chung, K. H. and Kirkwood, S. (1978) *Carbohydr. Res.* **61**, 457–477
- Stasinopoulos, S. J. and Seviour, R. J. (1989) *Mycol. Res.* **92**, 55–60
- Stasinopoulos, S. J. (1990) *M. Appl. Sci. Thesis*, College of Advanced Education, Bendigo, Vic.
- Pitson, S. M., Seviour, R. J., Bott, J. and Stasinopoulos, S. J. (1991) *Mycol. Res.* **95**, 352–356
- Lloyd, P. F., Pon, G. and Stacey, M. (1956) *Chem. Ind. (London)* **??**, 172–173
- Hrmova, M. and Fincher, G. B. (1993) *Biochem. J.* **289**, 453–461
- Stone, B. A. (1972) *Methods Carbohydr. Chem.* **6**, 384–385
- Goldstein, I. L., Hay, G. W., Lewis, B. A. and Smith, F. (1965) *Methods Carbohydr. Chem.* **5**, 361–369
- Nelson, T. E. and Lewis, B. A. (1974) *Carbohydr. Res.* **33**, 63–74
- Valent, B. S., Darvill, A. G., McNeil, M., Robertsen, B. K. and Albersheim, P. (1980) *Carbohydr. Res.* **79**, 165–192
- Pitt, D. E. and Bull, A. T. (1982) *Exp. Mycol.* **6**, 41–51
- Nelson, N. (1944) *J. Biol. Chem.* **153**, 375–380
- Somogyi, M. (1952) *J. Biol. Chem.* **195**, 19–23
- Clarke, A. E. and Stone, B. A. (1962) *Phytochemistry* **1**, 175–188
- Krishna-Murti, C. R. and Stone, B. A. (1961) *Biochem. J.* **78**, 715–723
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.* **28**, 350–356
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Zacharius, M. R., Zell, T. E., Morrison, J. H. and Woodlock, J. J. (1969) *Anal. Biochem.* **30**, 148–152
- Easterby, J. S. (1993) *Hyper 1.02a: Hyperbolic Regression Analysis of Enzyme Kinetic Data*, Liverpool University Press, Liverpool
- Berghem, L. E. R. and Pettersson, L. G. (1973) *Eur. J. Biochem.* **37**, 21–30
- Farkas, V. and MacLachlan, G. (1988) *Carbohydr. Res.* **184**, 213–219
- Altschul, S. F., Gish, W., Miller, W., Meyers, E. W. and Lipman, D. J. (1990) *J. Mol. Biol.* **215**, 403–410
- Bodenmann, J., Heiniger, U. and Hohl, H. R. (1985) *Can. J. Microbiol.* **31**, 75–82
- Fleet, G. H. and Phaff, H. J. (1975) *Biochim. Biophys. Acta* **401**, 318–332
- Friedlaender, M. H. G., Cook, W. H. and Martin, W. G. (1954) *Biochim. Biophys. Acta* **14**, 136–144
- Manners, D. J., Masson, A. J., Patterson, J. C., Bjorndal, H. and Lindberg, B. (1973) *Biochem. J.* **135**, 19–30
- Manners, D. J., Masson, A. J., Patterson, J. C., Bjorndal, H. and Lindberg, B. (1973) *Biochem. J.* **135**, 31–36
- Tabata, K., Ito, W., Kojima, T., Kawabata, S. and Misaki, A. (1981) *Carbohydr. Res.* **43**, 772–776
- Sanchez, M., Nombela, C., Villanueva, J. R. and Santos, T. (1982) *J. Gen. Microbiol.* **128**, 2047–2053
- Ohno, N., Hashimoto, Y. and Yadomae, T. (1986) *Carbohydr. Res.* **158**, 217–226
- Tangerone, B., Royer, J. C. and Nakas, J. P. (1989) *Appl. Environ. Microbiol.* **55**, 177–184
- Hien, N. H. and Fleet, G. H. (1983) *J. Bacteriol.* **156**, 1204–1213
- Notario, V., Villa, T. G. and Villanueva, J. R. (1976) *Biochem. J.* **159**, 555–562
- Copa-Patiño, J. L., Monstrol, I. F., Laborda, F. and Perez-Leblic, M. I. (1987) *Trans. Br. Mycol. Soc.* **88**, 317–321
- Bamforth, C. W. (1980) *Biochem. J.* **191**, 863–866
- Usui, T., Toriyama, T. and Mizuno, T. (1979) *Agric. Biol. Chem.* **43**, 603–611
- Neumann, V. C., Heath, H. E., LeBlanc, P. A. and Sloan, G. L. (1993) *FEMS Microbiol. Lett.* **110**, 205–212
- Nelson, T. E., Scaletti, J. V., Smith, F. and Kirkwood, S. (1963) *Can. J. Chem.* **41**, 1671–1678
- Copa-Patiño, J. L., Zhang, Y., Padmaperuma, B., Marsden, I., Broda, P. and Sinnott, M. L. (1993) *Biochem. J.* **293**, 591–594
- Nanjo, F., Usui, T. and Suzuki, T. (1984) *Agric. Biol. Chem.* **48**, 1523–1532
- Barras, D. R. and Stone, B. A. (1969) *Biochim. Biophys. Acta* **191**, 342–363
- Halliwell, G. (1963) in *Enzymic Hydrolysis of Cellulose and Related Materials* (Reese, E. T., ed.), pp. 71–92, Pergamon Press, Oxford
- Conchie, J., Gelman, A. L. and Levvy, G. A. (1967) *Biochem. J.* **103**, 609–615
- Reese, E. T., Maguire, A. H. and Parrish, F. W. (1968) *Can. J. Biochem.* **46**, 25–34
- Levy, G. A. and Snaith, S. M. (1972) *Adv. Enzymol. Relat. Areas Mol. Biol.* **26**, 151–181
- Brock, T. D. (1965) *Biochem. Biophys. Res. Commun.* **19**, 623–629
- Abd-El-Al, A. T. H. and Phaff, H. J. (1968) *Biochem. J.* **109**, 347–360
- Copa-Patiño, J. L., Rodriguez, J., Reyes, F. and Perez-Leblic, M. I. (1990) *FEMS Microbiol. Lett.* **70**, 233–240
- Del Rey, F., Santos, T., Garcia-Acha, I. and Nombela, C. (1979) *J. Bacteriol.* **139**, 924–931
- Del Rey, F., Santos, T., Garcia-Acha, I. and Nombela, C. (1980) *J. Bacteriol.* **143**, 621–627
- Santos, T., Sanchez, M., Villanueva, J. R. and Nombela, C. (1978) *J. Bacteriol.* **133**, 465–471
- Santos, T., Sanchez, M., Villanueva, J. R. and Nombela, C. (1979) *J. Bacteriol.* **137**, 6–12