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Carbohydrate Research 297 (1997) 365–374

CARBOHYDRATE RESEARCH

Purification and characterization of a (1 → 3)-β-D-glucan endohydrolase from rice (*Oryza sativa*) bran [1]

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Received 28 June 1996; accepted 10 October 1996

Abstract

A (1 → 3)-β-glucanase with an apparent M_r of 29,000 and an isoelectric point of 4.0 has been purified 2000-fold from extracts of rice bran, using fractional precipitation with ammonium sulfate, anion exchange chromatography, size-exclusion chromatography, chromatofocussing, and hydrophobic interaction chromatography. The enzyme can be classified with the EC 3.2.1.39 group, because it releases laminarabiose and higher laminara-oligosaccharides from linear (1 → 3)-β-D-glucans with an action pattern that is typical of (1 → 3)-β-D-glucan endohydrolases. However, the introduction of substituents or branching in the (1 → 3)-β-D-glucan substrates causes a marked decrease in the rate of hydrolysis. Thus, substituted or branched (1 → 3)-β-D-glucans of the kind commonly found in fungal cell walls are less susceptible to hydrolysis than essentially linear (1 → 3)-β-D-glucans. Kinetic analyses indicate an apparent K_m of 42 μM, a k_{cat} constant of 67 s^{-1}, and a pH optimum of 5.0 during hydrolysis of the (1 → 3)-β-D-glucan, laminaran, from *Laminaria digitata*. The first 60 NH_2-terminal amino acid residues of the purified rice (1 → 3)-β-glucanase contain blocks of amino acids that are conserved in other cereal (1 → 3)-β-glucanases. Although the precise tissue location and function of the enzyme in rice bran are not known, it is likely that it is concentrated in the aleurone layer and that it plays a pre-emptive role in the protection of ungerminated grain against pathogen attack.

Keywords: (1 → 3)-β-Glucanase; Rice; Substrate specificity; Kinetic analysis

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[1] This work was supported by grants from the Ministry of Agriculture, Forestry, and Fisheries of Japan, the Australian Research Council, and the Grains Research and Development Corporation of Australia.

PII S0008-6215(96)00291-1

1. Introduction

Plants have evolved a wide array of defence mechanisms against invasion by potentially pathogenic microorganisms. One such mechanism is the rapid expression of a group of soluble proteins, designated 'pathogenesis-related' or PR proteins, in response to microbial attack or tissue wounding [1–3]. Although the functions of all PR proteins have not been defined, they include (1 → 3)-β-D-glucan glucanohydrolases (EC 3.2.1.39), or (1 → 3)-β-glucanases. These enzymes are thought to contribute to the plant's defence capability by hydrolysing the (1 → 3)- and (1 → 3,1 → 6)-β-D-glucans that are major components of cell walls of many fungi [4]. Indeed, in vitro studies have shown that (1 → 3)-β-glucanases can inhibit fungal growth by causing extensive degradation of hyphal walls and lysis of hyphal tips [5,6]. Furthermore, partial hydrolysis of fungal cell walls by (1 → 3)-β-D-glucan endohydrolases releases branched or substituted (1 → 3,1 → 6)-β-D-oligoglucosides that in turn can elicit a variety of other plant defence responses [3,7].

Although most attention has been focussed on the role of (1 → 3)-β-glucanases in plant defence responses, they also function in normal growth and development of the plant. The (1 → 3)-β-glucanases are required for the removal of wound or dormancy callose, in microsporogenesis, in pollen tube growth and in senescence [8]. In some instances the accumulation of (1 → 3)-β-glucanases during normal development might still be related to its role in plant protection, particularly in tissues that are especially vulnerable to pathogen attack. Thus, there are relatively high amounts of (1 → 3)-β-glucanases in barley grain, both before and after germination [9–11]. It can be argued that the presence of starch and storage proteins in ungerminated grain, together with the rapid accumulation of sugars and amino acids following germination, renders the grain particularly susceptible to invasion by a broad spectrum of soil-borne microorganisms [12]. Synthesis of (1 → 3)-β-glucanases and other anti-microbial proteins in cereal grains may therefore represent pre-emptive or constitutive expression of important PR proteins in preparation for pathogen attack, as distinct from the inducible expression which follows actual invasion by microorganisms.

Here, we describe the purification of a (1 → 3)-β-glucanase from rice bran, its action pattern, substrate specificity, and its kinetic properties. Despite the commercial importance of rice and its central role in human nutrition, rice (1 → 3)-β-glucanases have not been extensively studied. However, the sequence of a rice gene encoding a putative (1 → 3)-β-glucanase has been reported [13]. Amino-terminal sequence analysis of the rice bran (1 → 3)-β-glucanase purified here reveals that the enzyme is not a product of the gene cloned by Simmons et al. [13].

2. Experimental

Materials.—Laminaran (from *Laminaria digitata*), baker's yeast glucan, lichenans (from *Usnea barbata* and *Cetraria islandica*), BSA, phenylmethanesulfonyl fluoride, orcinol, Coomassie Brilliant Blue R-250, larchwood xylan, and 4-nitrophenyl β-D-glucoside were purchased from Sigma (St. Louis, MO, USA). Laminarans (from *Laminaria hyperborea* and *Eisenia bicyclis*) and curdlan (from *Alcaligenes faecalis*) were purchased from Tokyo Kasei Koggo (Tokyo, Japan), pachyman (from *Poria cocos*) was from Calbiochem (San Diego, CA, USA), barley (1 → 3,1 → 4)-β-D-glucan (from *Hordeum vulgare*) was from Biocon Biochemicals (Kilnagleary, Ireland), *O*-(carboxymethyl)cellulose (CM-cellulose; degree of substitution, ds 0.54) was from ICI (Dingley, Australia) and Kieselgel 60 thin-layer plates were from Merck (Darmstadt, Germany). Pneumococcal SIII polysaccharide (from *Streptomyces pneumoniae*), pustulan (from *Umbilicaria popullosa*), and CM-pachyman (ds 0.29) were generously provided by Professor B.A. Stone, La Trobe University, Melbourne, Australia. Insoluble yeast glucan (from *Saccharomyces cerevisiae*) and soluble CM-yeast glucan (ds 0.42) were donated by Dr J. Sandula (Institute of Chemistry, Bratislava, Slovakia) and schizophyllan M-2 was provided by Dr S. Kitamura (Kyoto Prefectural University, Kyoto, Japan). (1,3)-β-Oligoglucosides of degree of polymerization (dp) 2–7 were purchased from Seikagaku Kogyo (Tokyo, Japan).

Enzyme purification.—Rice (*Oryza sativa* L. cv. Amaroo) bran was provided by Mr A.B. Blakeney, NSW Agriculture, Yanco, NSW. The bran (800 g dry wt) was homogenized in 4 L 50 mM NaOAc buffer, pH 5.0, containing 10 mM NaN_3, 10 mM EDTA, 3 mM β-mercaptoethanol, and 3 mM phenylmethanesulfonyl fluoride. The extraction and all subsequent procedures were performed at 4 °C. After stirring the homogenate gently for 1 h, insoluble material was removed by centrifugation and the supernatant fractionated with $(NH_4)_2SO_4$. Proteins precipitating in the 0–40% saturated $(NH_4)_2SO_4$ fraction were redissolved in extraction buffer, dialysed against the same

buffer, and applied to a 2.5 cm × 30 cm column of DEAE-Sepharose (Pharmacia Biotech, Uppsala, Sweden) that had been equilibrated in 50 mM NaOAc buffer, pH 5.0. After elution of unbound proteins at a flow rate of 9.8 cm h^{-1}, bound proteins were eluted with a linear 0–0.2 M NaCl gradient in the same buffer. Fractions containing (1 → 3)-β-glucanase were pooled, concentrated by ultrafiltration on a YM10 membrane (Amicon Corporation, Danvers, MA, USA) and applied to a 1.5 cm × 100 cm column of Bio-Gel P-60 (Bio-Rad, Richmond, CA, USA) equilibrated in 50 mM NaOAc buffer, pH 5.0, containing 0.2 M NaCl. Proteins were eluted at a flow rate of 4 cm h^{-1}, fractions containing (1 → 3)-β-glucanase were pooled and concentrated by ultrafiltration as described above, and dialysed against 25 mM imidazole–HCl buffer, pH 7.4. The dialysed enzyme preparation was applied to a 0.7 cm × 15 cm chromatofocussing column of PBE 74 (Pharmacia Biotech) equilibrated in the same buffer and bound proteins were eluted at a flow rate of 40 cm h^{-1} with 11% (v/v) Polybuffer 74 (Pharmacia Biotech) adjusted to pH 2.8 with HCl. Fractions containing (1 → 3)-β-glucanase activity were pooled, dialysed against 50 mM NaOAc buffer, pH 5.0, containing 2 M $(NH_4)_2SO_4$, and applied to a 0.7 cm × 10 cm column of Phenyl-Sepharose (Pharmacia Biotech) equilibrated in the same buffer. Bound proteins were eluted with 50 mM NaOAc buffer, pH 5.0 at a flow rate of 0.5 cm h^{-1}.

Enzyme assay.—(1 → 3)-β-Glucanase activity was determined reductometrically by monitoring the increase in reducing sugars [14] released in a 0.25% (w/v) solution of *Laminaria digitata* laminaran in 50 mM NaOAc buffer, pH 5.0, at 37 °C. One unit of enzyme activity is defined as the amount of enzyme required to release 1 μmol glucose equivalents per min. One unit corresponds to 16.67 nkat.

Table 1
Structural features of β-glucan substrates

Substrate (source)	Major linkages	Structure	References
Laminaran (*Laminaria digitata*, *Laminaria hyperborea*)	(1 → 3;1 → 6)-β 7:1	Glc |1→6 [-(Glc1→3Glc)$_3$-1→3Glc-]$_n$	[18]
Laminaran (*Eisenia bicyclis*)	(1 → 3;1 → 6)-β 3:2	Glc |1→6 [-Glc1→3Glc Glc |1→6 |1→6 Glc1→3Glc1→3Glc-]$_n$	[19]
Curdlan (*Alcaligenes faecalis*) Pachyman (*Poria cocos*)	(1 → 3)-β	[-Glc1 → 3Glc-]$_n$	[20]
Yeast glucan (*Saccharomyces cerevisiae*)	(1 → 3;1 → 6)-β 4:1	(Glc1→3Glc)$_{1-2}$ |1→6 [-(Glc1→3Glc)$_2$]$_n$	[21]
Schizophyllan (*Schizophyllum commune*)	(1 → 3;1 → 6)-β 3:1	Glc |1→6 [-Glc1→3Glc1→3Glc-]$_n$	[22]
Lichenan (*Usnea barbata*, *Cetraria islandica*)	(1 → 4;1 → 3)-β 2:1	[-Glc1 → 4Glc1 → 4Glc1 → 3Glc-]$_n$	[23]
Pustulan (*Umbilicaria populosa*)	(1 → 6)-β	[-Glc1 → 6Glc-]$_n$	[24]
Barley glucan (*Hordeum vulgare*)	(1 → 4;1 → 3)-β 2.3–2.7:1		[25]

Protein determination.—Protein was measured using the Coomassie Brilliant Blue reagent [15], with BSA as a standard. Protein in column eluates was monitored by absorbance at 280 nm.

Enzymic properties and kinetics.—The pH optimum of the rice (1 → 3)-β-glucanase was determined over the pH range 3–8 in 0.1 M NaOAc and 0.1 M sodium phosphate buffers containing 160 μg/mL BSA, using the *Laminaria digitata* laminaran as a substrate. Heat stability was determined by measuring residual activity after incubating the purified enzyme in 0.1 M NaOAc buffer, pH 5.0 for 10 min at temperatures ranging from 20 to 90 °C.

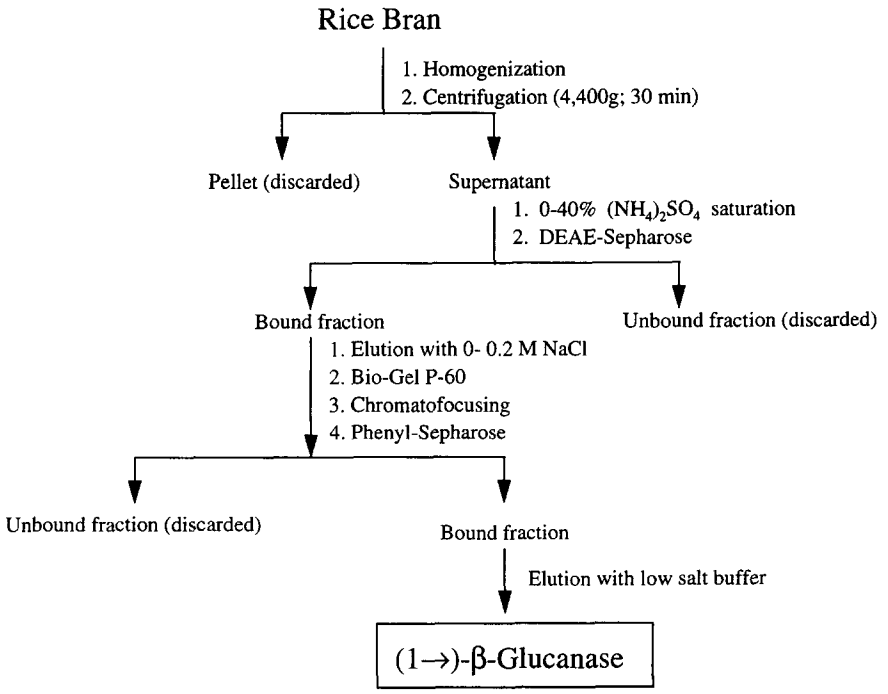
Kinetic parameters were determined at 37 °C by incubating 83.4 pkat of purified rice bran (1 → 3)-β-glucanase in 0.1 M NaOAc buffer, pH 5.0 containing *Laminaria digitata* laminaran over the concentration range 0.2–1 mg/mL. Kinetic data were processed with a non-linear regression analysis program based on Michaelis–Menten kinetics [16].

Polyacrylamide gel electrophoresis.—Proteins were separated by SDS-PAGE on 12.5% gels (total acrylamide 12.17%; cross-linker 0.33%) containing a 6% stacker gel [17]. Molecular-size marker proteins (Pharmacia Biotech) were phosphorylase b (M_r 94,000), BSA (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soyabean trypsin inhibitor (M_r 20,000), and α-lactalbumin (M_r 14,400). Gels were stained with Coomassie Brilliant Blue R-250 and destained as described by Laemmli [17].

Substrate specificities and action pattern.—A range of soluble and insoluble β-D-glucans, differing in linkage type and in the ratio of their linkage types [18–25] (Table 1), were prepared at a final concentration of 0.25% (w/v) in 50 mM NaOAc buffer, pH 5.0 containing 160 μg/mL BSA. Substrates were incubated with 333.4 pkat rice bran (1 → 3)-β-glucanase at 37 °C and activity was measured reductometrically [14]. Activity against 1 mM 4-nitrophenyl β-D-glucoside in 50 mM NaOAc buffer, pH 5.0 at 37 °C was determined spectrophotometrically [26].

The action pattern of the enzyme was examined by TLC of the products released at intervals up to 40 h when 666.8 pkat purified enzyme was incubated with 0.25% (w/v) *Laminaria digitata* laminaran in 10 mM NaOAc buffer, pH 5.0. The reaction was stopped by heating for 2 min to 100 °C and insoluble products were removed by centrifugation. Concentrated hydrolysis products were applied to Kieselgel 60 TLC plates, developed in 75% (v/v) acetonitrile and reducing sugars were detected with the orcinol reagent [11].

Amino acid sequence analysis.—Automated amino acid sequence analysis was performed in a Hewlett–Packard G1005A protein sequencer (Palo Alto, CA, USA) using the Hewlett–Packard 3.0 sequencing routine, which is based on Edman degradation chem-



Scheme 1. Summary of purification procedures for the (1 → 3)-β-glucanase from rice bran.

Table 2
Enzyme yields during purification of (1 → 3)-β-glucanase from rice bran

Purification step	Yield				Purification factor [c]-fold
	Protein (mg)	Activity [a] (Units)	Specific activity (Units mg^{-1})	Recovery [b] (%)	
Crude homogenate	1004	252	0.25	100	1
0–40% $(NH_4)_2SO_4$	556	194	0.35	77	1.4
DEAE-Sepharose	23	88	3.8	35	15
Bio-Gel P-60	1.6	58	36.3	23	145
PBE 74	0.48	36	75	14	300
Phenyl-Sepharose	0.04	20	500	8	2000

[a] As recovered enzyme units assayed on *Laminaria digitata* laminaran.
[b] Expressed in % of enzyme units.
[c] Calculated on the basis of specific activity (Units mg protein^{-1}).

istry. Phenylthiohydantoin (PTH) derivatives of amino acids were identified by HPLC on a narrow bore C_{18} reversed-phase PTH Vydac column (2.1 mm × 250 mm) [26].

3. Results

Purification of a (1 → 3)-β-glucanase from rice bran.—The procedure developed for the purification of rice bran (1 → 3)-β-glucanase is summarised in Scheme 1, while purification factors, yields and specific activities are shown in Table 2. Selected column chromatography profiles are presented in Fig. 1 and SDS-PAGE gels of proteins remaining at various stages of the purification procedure are shown in Fig. 2.

Although the majority of the initial protein was removed during $(NH_4)_2SO_4$ fractional precipitation and DEAE-Sepharose ion exchange chromatography

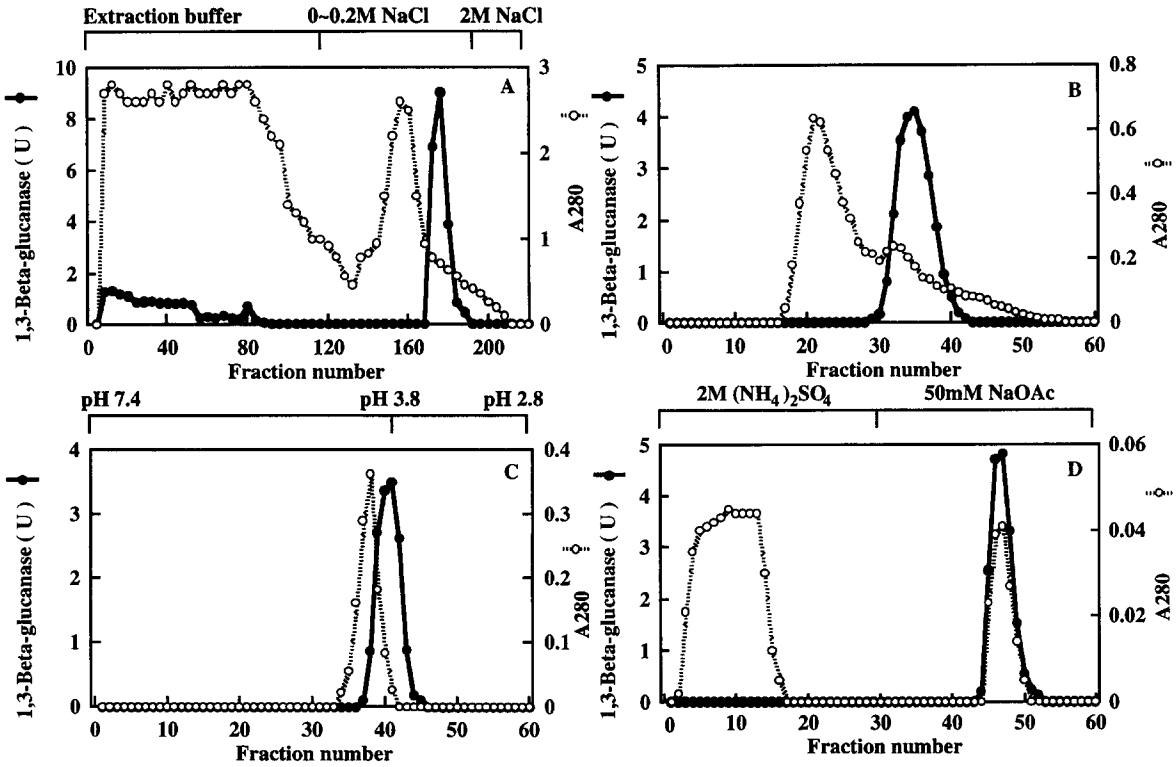


Fig. 1. Sequential chromatography of rice bran extracts on DEAE-Sepharose (A), Bio-Gel P60 (B), PBE 74 (C), and Phenyl-Sepharose (D). Material precipated by 0–40% saturated ammonium sulfate was desalted, applied to the DEAE-Sepharose column and eluted with 0–0.2 M NaCl. Active fractions were separated on Bio-Gel P60 and applied to the PBE 74 chromatofocussing column, and finally fractionated on Phenyl-Sepharose with 50 mM NaOAc buffer, pH 5.0. Fractions were assayed for protein (○) and activity against *Laminaria digitata* laminaran (●).

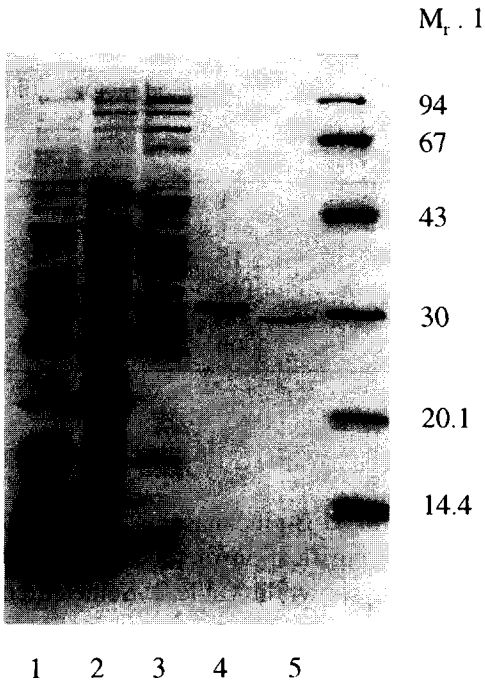


Fig. 2. SDS-PAGE of samples taken at various stages during the purification (Scheme 1) of the rice bran (1 → 3)-β-glucanase. Lane 1, crude extract; lane 2, 0–40% ammonium sulfate precipitate; lane 3, fraction eluted from DEAE-Sepharose with NaCl; lane 4, pooled (1 → 3)-β-glucanase fractions from the Bio-Gel P60 column; lane 5, pooled (1 → 3)-β-glucanase fractions from Phenyl-Sepharose. Standard proteins are shown (M_r).

(Fig. 1A), SDS-PAGE revealed that many proteins remained at this stage (Fig. 2, lane 3). However, after size-exclusion chromatography on Bio-Gel P60 (Fig. 1B) a single protein band of apparent M_r 30,000 was detected on SDS gels (Fig. 2, lane 4), and this protein was assumed initially to be the rice (1 → 3)-β-glucanase. However, NH_2-terminal sequence analysis of the first 20 amino acid residues of this protein revealed the following sequence:

GNGYLFPEYIGAQFTGVRFS.

This sequence did not match any other sequence in the DNA or protein databases, and bore no similarity to the sequences of previously characterized plant (1 → 3)-β-glucanases.

It appeared, therefore, that the single protein band detected after gel-filtration chromatography (Fig. 2, lane 4) was a contaminant. Subsequent chromatofocussing on PBE94 confirmed this, because the (1 → 3)-β-glucanase activity did not line up with the major protein peak (Fig. 1C). However, by carefully pooling fractions from the chromatofocussing column, based on both protein and activity profiles, and subjecting them to hydrophobic interaction chromatography on Phenyl-Sepharose, the (1 → 3)-β-glucanase was successfully separated from the major contaminating protein (Fig. 1D). The apparent M_r of the purified rice bran (1 → 3)-β-glucanase is approximately 29,000, which is slightly lower than that of the contaminating protein (Fig. 2, lanes 4 and 5). The NH_2-terminal amino acid sequence of the purified (1 → 3)-β-glucanase is similar to those of other plant (1 → 3)-β-glucanases [13,27–32]; selected sequences are aligned in Table 3. The amino acid sequence analysis also suggested that the final (1 → 3)-β-glucanase preparation was substantially free of other contaminating proteins, because no secondary sequences could be detected.

Action pattern and substrate specificity.—During the hydrolysis of laminaran from *Laminaria digitata* by the purified rice bran (1 → 3)-β-glucanase, oligosaccharides of relatively high degree of polymerization (dp) were initially released. These were progressively reduced in size over a 40 h incubation period; laminarabiose, higher laminara-oligosaccharides of dp 3–8 and some glucose were among the final hydrolysis products (Fig. 3). This action pattern is typical of an endohydrolase and warrants the classification of the rice bran enzyme as a (1 → 3)-β-D-glucan glucanohydrolase (EC 3.2.1.39).

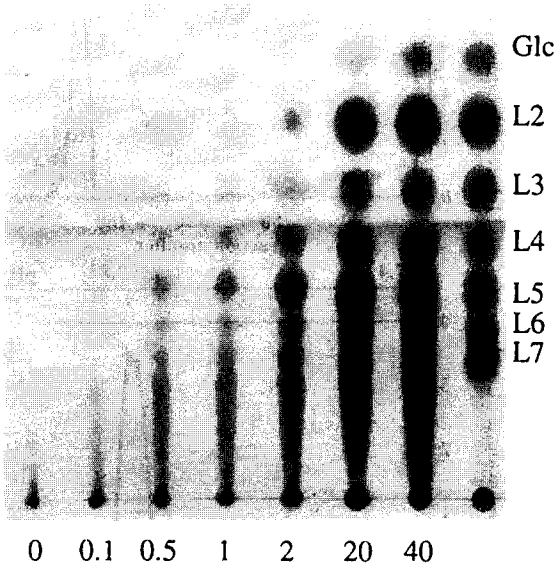


Fig. 3. Thin-layer chromatography of the hydrolysis products of *Laminaria digitata* laminaran by the purified rice bran (1 → 3)-β-glucanase after 0, 0.1, 0.5, 1, 2, 20, and 40 h hydrolysis. Standards were glucose (Glc) and (1 → 3)-β-oligoglucosides of dp 2–7 (L2–L7).

Table 3
NH_2-Terminal amino acid sequence alignments of plant (1 → 3)-β-D-glucan endohydrolases [a]

	1	10		20		30		40		50		60
rice bran	IGV-Y	GVIGN	NLPSP	SDVVQ	LYKSN	GIDSM	RIYFP	RSDIL	QALSG	-PI-L	TMDVG	NDQLG
rice grain	***-*	**L**	****R	*E***	**R*-	*						
barley	***C*	*****	****R	*****	**R*K	**NG*	****A	DGQA*	S**RN	SG*G*	IL*I*	****A
tobacco	A**C*	*RQ**	G****	A***S	*CNR*	N*RR*	***D*	DQPT*	E**R*	SN*EM	LLG*P	*PD*E
bean	***C*	*MM**	****A	NE*IN	**R**	N*RR*	*L*D*	NGAA*	G**RN	SG*E*	ILG*P	*SD*Q
pea	**IC*	*MM**	***PA	NE*IA	***A*	N*KR*	*L*D*	NQPA*	N**RD	SG*E*	ILGIP	*SD*Q
tomato	***C*	*K*A*	****D	Q**IK	**N**	N*KK*	*****	ETNVF	N**K*	SN*EI	IL**P	*QD*E
barley EII	***C*	*MSA*	***AA	*T**S	MF***	**K**	*L*A*	NQAA*	**VG*	TG*NV	VVGAP	**V*S
rice gene	***C*	*MSA*	***PA	*S**G	M*R**	**T**	*L*A*	DRRLA	*SVG*	TG*SV	VVGAP	**V*S

[a] References: rice bran (acidic form) (this paper); rice grain (basic form) [27]; barley (isoenzyme GII) [28]; tobacco (acidic form) [29]; bean [30]; pea [31]; tomato (acidic form) [32]; barley (1 → 3;1 → 4)-β-glucanase (isoenzyme EII) [28]; rice (Gns1 gene) [13].

Table 4
Relative rates of hydrolysis of β-glycans by rice (1 → 3)-β-D-glucan endohydrolase

Substrate	Relative rate (%) [a]
laminaran	
Laminaria digitata	100
Laminaria hyperborea	33
Eisenia bicyclis	3
CM-pachyman (*Poria cocos*)	119
curdlan (*Alcaligenes faecalis*)	60
CM-(1,3;1,6)-β-D-glucan (*Saccharomyces cerevisiae*)	31
schizophyllan (*Schizophyllum commune*)	12
lichenan	
Usnea barbata	2
Cetraria islandica	2

[a] The relative rates of hydrolysis of (1 → 3)-β-D-glucan endohydrolase on *L. digitata* laminaran, measured reductometrically, were arbitrarily set at 100% and correspond to 500 units mg^{-1} protein, respectively. No activity was detected against barley (1 → 3;1 → 4)-β-D-glucan, SIII polysaccharide (*Streptococcus pneumoniae*), pustulan (*Umbilicaria popullosa*), CM-cellulose, pachyman (*Poria cocos*), xylan (larchwood), and 4-nitrophenyl β-D-glucoside.

The activities of the rice bran (1 → 3)-β-glucanase on a range of potential substrates are compared in Table 4. Preferred substrates are the essentially unbranched (1 → 3)-β-D-glucans, CM-pachyman, *Laminaria digitata* laminaran, and curdlan (Table 4). Laminaran from *Laminaria hyperborea*, curdlan, CM-yeast glucan, and schizophyllan were hydrolysed at a lower rate (Table 4). The enzyme has no activity on barley (1 → 3,1 → 4)-β-D-glucan, the SIII polysaccharide from *Streptococcus pneumoniae*, pustulan, CM-cellulose, insoluble pachyman, (1 → 4)-β-D-xylan or 4-nitrophenyl β-D-glucoside, and hydrolyses the (1 → 3,1 → 4)-β-D-glucan, lichenan, very slowly (Table 4).

Kinetic properties.—The purified rice bran (1 → 3)-β-glucanase obeys linear Michaelis–Menten kinetics at *Laminaria digitata* laminaran concentrations of 0.2–1.0 mg/mL (data not shown). The K_m value calculated from these data is 0.17 mg/mL which, based on a dp of 25 for this substrate, corresponds to 42 μM. The catalytic rate constant, k_{cat}, is 67 s^{-1} and the specificity or catalytic efficiency factor, k_{cat}/K_m, 1.6×10^6 s^{-1} M^{-1}.

The pH optimum of the enzyme is 5.0 (Fig. 4A) and activity decreases sharply at pH values above 6 or below 3.5, as measured using the *Laminaria digitata* laminaran as substrate.

When the temperature stability of the enzyme was monitored by heating at various temperatures for 10 min and measuring residual activity thereafter, the rice bran (1 → 3)-β-glucanase retained most of its activity at 60 °C. However, activity was lost very rapidly between 60 °C and 70 °C (Fig. 4B). Thus, the stability of the rice (1 → 3)-β-glucanase is relatively high (cf. [11]), but this is not unusual for PR proteins and is thought to be related to their need to survive in the hostile environment created by invading microorganisms [33].

4. Discussion

A (1 → 3)-β-D-glucan endohydrolase has been purified 2000-fold from extracts of rice bran, using ammonium sulfate precipitation, anion-exchange and size-exclusion chromatography, chromatofocussing, and hydrophobic interaction chromatography (Scheme 1; Table 2). The purified enzyme has an apparent M_r of 29,000 and an isoelectric point of 4.0. This acidic isoelectric point may be contrasted to (1 → 3)-β-

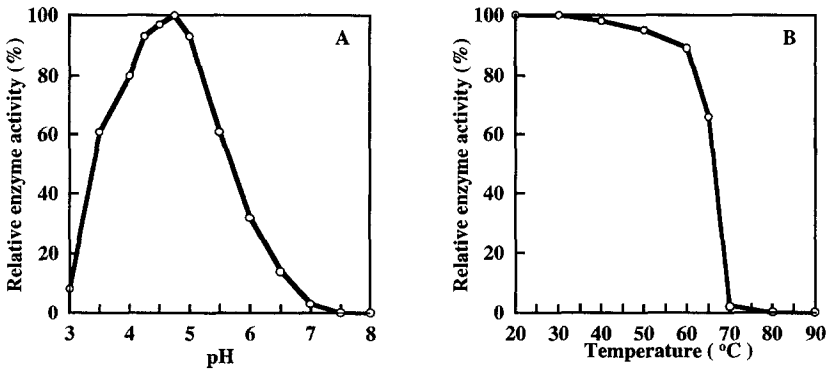


Fig. 4. Effect of pH on the activity (A) and the temperature stability (B) of the purified rice bran (1 → 3)-β-glucanase.

glucanases from other cereals, which are often, but not always, basic proteins [11,28,34]. Although NH_2-terminal amino acid sequence analysis indicated that the purified rice bran enzyme is a single acidic protein that is free from other isoforms or from contaminating proteins, it is also known that a highly basic (1 → 3)-β-glucanase is found in rice grain [27]. The detection of enzyme activity in the fraction which did not bind to DEAE-Sepharose at pH 5.0 (Fig. 1A) suggests that other isoforms exist in rice bran. Similarly, Southern blots of rice genomic DNA probed with a barley (1 → 3)-β-glucanase cDNA show that several (1 → 3)-β-glucanase genes are present in rice (T. Akiyama, H. Kaku, N. Shibuya, and G.B. Fincher, unpublished data).

Examination of the time course of release of oligosaccharide products from the *Laminaria digitata* laminaran showed that the enzyme hydrolyses this substrate with a typical endo-action pattern (Fig. 3). It was important to confirm this action pattern because highly active (1 → 3)-β-D-glucan exohydrolases of the type found in germinated barley and in maize coleoptiles [26,35] might also be present; the exohydrolases hydrolyse laminaran but release glucose as the major hydrolysis product. The observation that laminarabiose, laminarapentaose, and higher laminara-oligosaccharides of dp 6–8 are major hydrolysis products suggests that the rice bran (1 → 3)-β-glucanase has a relatively long substrate-binding domain and that oligosaccharides containing fewer than 5–7 glucosyl residues are therefore hydrolysed relatively slowly (Fig. 3). Subsite mapping of three barley (1 → 3)-β-glucanases shows that these enzymes have eight β-glucosyl-binding subsites [36]. The X-ray crystal structure of one barley (1 → 3)-β-glucanase isoenzyme has been solved. The enzyme forms an $(\alpha/\beta)_8$ barrel structure and has a deep cleft approximately 40 Å long running over the surface of the molecule [37]. The length of the cleft, which represents the substrate-binding region of the enzyme, corresponds to 7 or 8 residues of an extended (1 → 3)-β-D-glucan chain [36,37]. Varghese et al. [37] have suggested that the three-dimensional conformations of (1 → 3)-β-glucanases are likely to be conserved in many higher plants, and the hydrolysis products released by the rice bran (1 → 3)-β-glucanase (Fig. 3) are consistent with a substrate-binding region 7–8 glucosyl residues in length.

Kinetic analyses of the rice bran (1 → 3)-β-glucanase during hydrolysis of *Laminaria digitata* laminaran allowed the calculation of a K_m value of 42 μM, a k_{cat} of 67 s^{-1} and a k_{cat}/K_m of 1.6×10^6 s^{-1} M^{-1}. These are at the lower end of the range of values obtained for three barley (1 → 3)-β-glucanase isoenzymes [11]. It should be emphasised that K_m and k_{cat} values for polysaccharide hydrolases are best considered as approximations only, because many polysaccharide substrates are heterogeneous in size and structure, and because the products of a reaction can also act as additional substrates.

The pH optimum of the rice enzyme is approximately 5.0 (Fig. 4A) and the bell-shaped activity curve suggests that catalysis is mediated by two amino acid residues. Based on the pH-activity curve shown in Fig. 4A, the catalytic nucleophile would have a pK_a of approximately 3.5 and the catalytic acid would have a pK_a of approximately 5.5. Two catalytic glutamic acid residues with similar pK_a values have been identified in barley (1 → 3)-β-glucanase isoenzyme GII [38] and again it is likely that these two residues are conserved in the rice enzyme [34].

The substrate specificity of the rice bran (1 → 3)-β-glucanase has been investigated using a range of linear, substituted, and branched β-D-glucans (Tables 1 and 4). The preferred substrates are the essentially linear (1 → 3)-β-D-glucans CM-pachyman, *Laminaria digitata* laminaran, and curdlan (Table 4). As the degree of substitution or branching increases, the rate of hydrolysis generally decreases (Tables 1 and 4), suggesting that the substrate-binding cleft in the enzyme cannot easily accommodate a (1 → 3)-β-D-glucan chain carrying projecting glucosyl residues or polymeric side chains on O-6 atoms. Thus, the enzyme hydrolyses (1 → 3,1 → 6)-β-D-glucans of fungal cell-wall origin more slowly than linear (1 → 3)-β-D-glucans. These apparent conformational constraints to substrate specificity are somewhat difficult to reconcile with the proposed PR function of (1 → 3)-β-glucanases, where it might be expected that an enzyme capable of hydrolysing highly branched or highly substituted (1 → 3)-β-D-glucans would provide better protection against a broad spectrum of potentially pathogenic fungi than would an enzyme with more limited specificity. Alternatively, a more limited specificity of the type observed for the rice bran (1 → 3)-β-glucanase (Table 4) could result in the release of relatively complex, branched (1 → 3,1 → 6)-β-oligoglucosides that could themselves elicit secondary responses in the cascade that accompanies pathogen invasion of plant tissues [3,7].

The final consideration here relates to the possible functions of (1 → 3)-β-glucanases in rice bran. The precise tissue location of the enzyme is not known,

but it is unlikely to be associated with husk, pericarp-testa or other tissues of maternal origin, because these tissues are non-living in the mature grain and are comprised predominantly of cell-wall remnants. Thus, the enzyme is probably located in the aleurone layer. It is possible that the (1 → 3)-β-glucanase functions in germinated grain to remove the callosic material in the plasmodesmata of aleurone layer cells or in the extracellular space of the subaleurone starchy endosperm [39,40]. However, levels of (1 → 3)-β-glucanases in both ungerminated and germinated grain are considered more than adequate to remove the small deposits of callosic material in the grain and it is likely that they also make a major contribution to the grain's ability to defend itself against pathogen attack [9,11,34].

Acknowledgements

We are grateful to Mr Tony Blakeney for providing the rice bran and to Professor Bruce Stone for generously providing many polysaccharide substrates. We thank Dr Neil Shirley for assistance with the amino acid sequence analyses and Professor J.D. Bewley and Professor P.B. Høj for helpful comments.

References

[1] T. Boller, *Plant-Microbe Interactions: Molecular and Genetic Perspectives*, Vol. 2, Macmillan, New York, 1987, pp 385–413.
[2] S. Kauffmann, M. Legrand, P. Geoffroy, and B. Fritig, *EMBO. J.*, 6 (1987) 3209–3212.
[3] R.A. Dixon and C.J. Lamb, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 41 (1990) 339–367.
[4] J.G.H. Wessels, *New Phytol.*, 123 (1993) 397–413.
[5] F. Mauch, L.A. Hadwiger, and T. Boller, *Plant Physiol.*, 87 (1988) 325–333.
[6] K. Skriver, F.L. Olsen, J.C. Rogers, and J. Mundy, *Proc. Natl. Acad. Sci. USA*, 88 (1991) 7266–7270.
[7] F. Côte and M.G. Hahn, *Plant Mol. Biol.*, 26 (1994) 1379–1411.
[8] B.A. Stone and A.E. Clarke, *The Chemistry and Biology of (1 → 3)-β-glucans*, La Trobe University Press, Bundoora, Vic., 1992, pp 422–426.
[9] G.M. Ballance, W.O.S. Meredith, and D.E. Laberge, *Can. J. Plant Sci.*, 56 (1976) 459–466.
[10] P.B. Høj, D.J. Hartman, N.A. Morrice, D.N.P. Doan, and G.B. Fincher, *Plant Mol. Biol.*, 13 (1989) 31–42.
[11] M. Hrmova and G.B. Fincher, *Biochem. J.*, 289 (1993) 453–461.
[12] G.B. Fincher, *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 40 (1989) 305–346.
[13] C.R. Simmons, J.C. Litts, N. Huang, and R.L. Rodriguez, *Plant Mol. Biol.*, 18 (1992) 33–45.
[14] M. Lever, *Anal. Biochem.*, 47 (1972) 273–279.
[15] M.M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
[16] F. Perella, *Anal. Biochem.*, 174 (1988) 437–447.
[17] U.K. Laemmli, *Nature (London)*, 227 (1970) 680–685.
[18] A.T. Bull and C.G.C. Chesters, *Adv. Enzymol.*, 28 (1963) 325–364.
[19] T. Usui, T. Toriyama, and T. Mizuno, *Agric. Biol. Chem.*, 43 (1979) 603–611.
[20] T. Harada, A. Misaki, and H. Saito, *Arch. Biochem. Biophys.*, 124 (1968) 292–298.
[21] G. Kogan and J. Alfoldi, *Biopolymers*, 27 (1988) 1055–1063.
[22] K. Tabata, W. Ito, T. Kojima, S. Kawabata, and A. Misaki, *Carbohydr. Res.*, 89 (1981) 121–135.
[23] A.S. Perlin and S. Suzuki, *Can. J. Chem.*, 40 (1962) 50–56.
[24] B. Lindberg and J. McPherson, *Acta Chem. Scand.*, 8 (1954) 985–988.
[25] J.R. Woodward, G.B. Fincher, and B.A. Stone, *Carbohydr. Polym.*, 3 (1983) 207–225.
[26] M. Hrmova, A.J. Harvey, J. Wang, N.J. Shirley, G.P. Jones, B.A. Stone, P.B. Høj, and G.B. Fincher, *J. Biol. Chem.*, 271 (1996) 5277–5286.
[27] T. Akiyama, H. Kaku, and N. Shibuya, *Plant Cell Physiol.*, 37 (1996) 702–705.
[28] P. Xu, J. Wang, and G.B. Fincher, *Gene*, 120 (1992) 157–165.
[29] G. Payne, E. Ward, T. Gaffney, P.A. Goy, M. Moyer, A. Harper, F. Meins Jr., and J. Ryals, *Plant Mol. Biol.*, 15 (1990) 797–808.
[30] B.V. Edington, C.J. Lamb, and R.A. Dixon, *Plant Mol. Biol.*, 16 (1991) 81–94.
[31] M.M. Chang, L.A. Hadwiger, and D. Horowitz, *Plant Mol. Biol.*, 20 (1992) 609–618.
[32] J.A.L. van Kan, M.H.A.J. Joosten, and C.A.M. Wagemakers, *Plant Mol. Biol.*, 20 (1992) 513–527.
[33] A. Stinzi, T. Heitz, V. Prasad, S. Wiedemann-Merdinoglu, S. Kauffmann, P. Geoffroy, M. Legrand, and B. Fritig, *Biochimie*, 75 (1993) 687–706.
[34] P.B. Høj and G.B. Fincher, *Plant J.*, 7 (1995) 367–379.
[35] T. Hoson and D.J. Nevins, *Plant Physiol.*, 90 (1989) 1353–1358.
[36] M. Hrmova, T.P.J. Garrett, and G.B. Fincher, *J. Biol. Chem.*, 270 (1995) 14556–14563.
[37] J.N. Varghese, T.P.J. Garrett, P.M. Colman, L. Chen, P.B. Høj, and G.B. Fincher, *Proc. Natl. Acad. Sci. USA*, 91 (1994) 2785–2789.
[38] L. Chen, G.B. Fincher, and P.B. Høj, *J. Biol. Chem.*, 268 (1993) 13318–13326.
[39] L. Taiz and R.L. Jones, *Planta*, 92 (1970) 73–82.
[40] R.G. Fulcher, G. Setterfield, M.E. McCully, and P.J. Wood, *Aust. J. Plant Physiol.*, 4 (1977) 917–928.