Purification and Partial Characterization of a Developmentally Regulated 1,3- β -Glucanase from *Penicillium italicum*

By MIGUEL SÁNCHEZ,¹* CÉSAR NOMBELA,² JULIO R. VILLANUEVA¹
AND TOMÁS SANTOS¹

¹ Departamento de Microbiología, Facultad de Biología, Universidad de Salamanca, Spain

² Departamento de Microbiología, Facultad de Farmacia, Universidad de Madrid, Spain

(Received 23 November 1981; revised 26 January 1982)

1,3- β -Glucanase I is one of the three 1,3- β -glucanase enzymes which are developmentally regulated in *Penicillium italicum*. On incubation of the fungus in derepressing medium (limited in glucose), 1,3- β -glucanases II and III are produced first, followed by 1,3- β -glucanase I. We have purified the latter enzyme 290-fold from cell-free extracts of derepressed mycelium. The purification involved Sephadex G-100 chromatography, adsorption to DEAE-Sephadex, concanavalin A-Sepharose and preparative isoelectric focusing. The purified enzyme appeared homogeneous on SDS-PAGE. The apparent molecular weights estimated by Sephadex G-100 filtration and SDS-PAGE were 65000 and 68000 respectively. The enzyme appears to be an acidic glycoprotein (pI 4.7) with an endo-splitting mode of action, clearly different from 1,3- β -glucanases II and III. The K_m on a standard, slightly branched 1,3- β -glucan (laminarin) was 0.04 mg ml⁻¹, at least ten times lower than the average K_m for β -glucanases. It showed almost no activity on heavily branched substrates (yeast wall glucan). An evaluation of the physico-chemical properties and other characteristics suggests a metabolic role for this endo-1,3- β -glucanase.

INTRODUCTION

The wide occurrence of β -glucanases in fungi has been well established (Reese *et al.*, 1962; Chesters & Bull, 1963*a,b*; Clarke & Stone, 1965; Abd-El-Al & Phaff, 1969). Many of them have been purified and characterized (see Villanueva *et al.*, 1976), so that their properties are generally well known. However, data on their regulation and function are scarce. The fact that many β -glucanase-producing fungi have β -glucans among the structural components of their cell walls has led to the hypothesis that some of these enzymes are involved in morphogenic-morpholytic processes during fungal development and differentiation (Wessels, 1966; Wessels & Koltin, 1972; Mahadevan & Mahadkar, 1970; Bartnicki-Garcia, 1973). Recent reports on their regulation and genetics (Friebe & Holldorf, 1975; Santos *et al.*, 1978*a, b*; Rey *et al.*, 1979) support the idea of diversity in β -glucanase function so that, within the same organism, certain enzymes may be involved in cell wall dynamics, with others playing only a metabolic role. For example, the mutation *exb1-1*, which abolishes exo- β -glucanase production in *Saccharomyces cerevisiae* does not affect cell wall dynamics during the life cycle of that organism (Santos *et al.*, 1979*b*). This would suggest that this enzyme is not critically involved in cell wall morphogenesis. Fungal β -glucanases are generally constitutive and subject to developmental regulation so that maximum levels of enzyme activity are attained during idiophase, when hyphal growth ceases. This occurs after consumption of the carbon source, which acts as a repressor of β -glucanase synthesis (Friebe & Holldorf, 1975; Santos *et al.*, 1978*a*). This means that levels of β -glucanase activity are minimal and frequently undetectable at the time of maximum morphogenic activity (Santos *et al.*, 1978*a*), and the increase in enzyme activity which takes place in resting cells, under glucose deprivation, would fulfil a metabolic role (most probably directed towards the mobilization of potential carbon sources in glucose-starved cells). In our laboratory we have focused on the 1,3-

β -glucanase system of *Penicillium italicum*, which consists of three enzymes subject to catabolite repression (Santos *et al.*, 1978*a, b*). During active growth only enzymes II and III are present in very low levels, whereas exhaustion of glucose, which leads to cell-growth arrest, results in the synchronous derepression of both enzymes and triggers the production of 1,3- β -glucanase I which is absent during active growth, and whose synthesis is not co-ordinated with enzymes II and III (Santos *et al.*, 1978*b*). In addition to the different pattern of derepression, preliminary data on this 'late' 1,3- β -glucanase revealed some interesting properties such as non-periplasmic localization and lack of lytic activity on *P. italicum* cell walls, which clearly distinguishes this enzyme from the other two (Santos *et al.*, 1979*a*). In this communication we describe the purification and properties of this developmentally regulated 1,3- β -glucanase.

METHODS

Reagents. Bovine serum albumin (fraction V), ovalbumin, trypsin, lysozyme, paranitrophenyl- β -D-glucoside, cellobiose, salicin, oxidized glutathione, iodoacetamide and *N*-ethylmaleimide were obtained from Sigma. Laminarin was from Koch-Light. Acrylamide, *N,N'*-methylenebisacrylamide and ampholites were from Bio-Rad. Sephadex G-100, DEAE-Sephadex and concanavalin A-Sepharose 4B were from Pharmacia. Yeast 1,3- β -glucan was prepared according to Northcote & Horne (1952) as modified by Sentandreu *et al.* (1975). All other chemicals were reagent grade.

Organism and growth conditions. *Penicillium italicum*. *Penicillium italicum* CECT 2294 (Colección Española de Cultivos Tipo, Departamento de Microbiología, Facultad de Ciencias, Valencia, Spain) was the organism used in this study. Growth conditions were as described previously (Santos *et al.*, 1978*a*). The standard derepression system consisted of a two step incubation. Conidia were first inoculated in Czapeck-glucose medium (Santos *et al.*, 1978*a*). After 48 h incubation, actively growing cells were transferred to fresh medium limited in glucose, and incubated for a further 24–30 h. Mycelia were harvested and homogenized as published (Santos *et al.*, 1978*a*).

Determinations. The activity of 1,3- β -glucanase on different substrates was assayed as previously described (Santos *et al.*, 1978*b*). One unit of activity is defined as the amount of enzyme that catalyses the release of reducing groups equivalent to 1 nmol glucose, min⁻¹ at 37 °C under standard conditions. Specific activity is defined as units of activity (mg protein)⁻¹. Protein was determined by the Lowry method, using bovine serum albumin as standard.

Paper chromatography. Paper chromatography was performed on Whatman no. 1 filter paper using ethyl acetate/pyridine/water (8:7:1, by vol.) as solvent. Reducing sugars were detected according to Trevelyan *et al.* (1950).

Polyacrylamide gel electrophoresis. Native gel electrophoresis was performed at pH 8.5 in Tris/glycine buffer on 7.5% (w/v) acrylamide gels (0.6 × 10 cm) at 2 mA per gel, according to Davis (1964). Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed as described by Weber & Osborn (1969). Molecular weight standards used were bovine serum albumin (67 000), ovalbumin (45 400), trypsin (23 800) and catalase (140 000). The enzyme activity was measured directly on gels, in parallel with protein and carbohydrate staining, as described by Notario *et al.* (1976). Densitometric scans of gels were obtained using a densitometer coupled to a Pye-Unicam P spectrophotometer. Glycoprotein staining was performed according to Zacharius *et al.* (1969).

Purification of 1,3- β -glucanase I. All procedures were carried out at 0–4 °C.

(i) **Lyophilization and Sephadex G-100 chromatography.** Crude extracts from 500 g (wet wt) derepressed mycelium were centrifuged at 40 000 *g* for 30 min. The supernatant was dialysed against water, lyophilized, redissolved in 10 ml 50 mM-acetate buffer pH 5.5 and loaded on to a 80 × 5 cm column of Sephadex G-100 equilibrated in the same buffer. Fractions (10 ml) were collected at a rate of 20 ml h⁻¹. Molecular weight standards were as for SDS-PAGE (above) plus lysozyme (14 700) and omitting catalase.

(ii) **Concanavalin A-Sepharose chromatography.** Fractions from the Sephadex G-100 column containing 1,3- β -glucanase activity were pooled and applied to a 10 × 1.5 cm column of concanavalin A-Sepharose 4B equilibrated in 50 mM-acetate buffer pH 5.5, 1 M-NaCl. The column was first washed with this buffer until non-adsorbed material had come through, and then eluted with the same buffer plus 7% (w/v) α -methyl-D-mannoside. Fractions (10 ml) were collected at a flow rate of 20 ml h⁻¹.

(iii) **DEAE-Sephadex column chromatography.** 1,3- β -Glucanase I is not adsorbed to concanavalin A, thus, fractions not retained by the concanavalin A-Sepharose column were pooled, dialysed extensively against 50 mM-Tris/HCl pH 7.2 and loaded on to a DEAE-Sephadex column (20 × 1.5 cm) pre-equilibrated in the same buffer. After washing thoroughly, materials adsorbed to the column were eluted in 10 ml fractions with a 500 ml gradient of NaCl (0–0.2 M) in the same buffer, at a flow rate of 15 ml h⁻¹.

(iv) **Isoelectric focusing.** Fractions from the DEAE-Sephadex column were subject to isoelectric focusing in a 110 ml column (LKB). The pH gradient (3–10) was generated with a 1% ampholite solution and stabilized in a discontinuous gradient of sucrose (Notario *et al.*, 1976). After 36 h electrofocusing at 350 V, 5 ml fractions were

collected and activity determined after fivefold dilution of samples, to minimize interference of ampholites with the enzyme assay. After extensive dialysis against 50 mM-acetate buffer, pH 5.5, samples of the enzyme preparation were quickly frozen and stored at -25°C .

Characterization of the enzyme:

pH optimum. About 30 units of dialysed enzyme was incubated with the substrate buffered in different solutions (50 mM-glycerol/HCl for pH 2.2–3.5; 50 mM-citrate/phosphate for pH 4–6.5; 50 mM-Tris/HCl for pH 7–8; glycine/NaOH for pH > 8), under standard conditions (Santos *et al.*, 1978*a*).

Temperature optimum. About 30 units of enzyme was incubated with substrate in 50 mM-acetate buffer, pH 5.5 at different temperatures (10–80 $^{\circ}\text{C}$) for 10 min.

Thermal stability. About 30 units of enzyme in 50 mM-acetate buffer pH 5.5 was pre-incubated at 50, 60 or 70 $^{\circ}\text{C}$ for different periods, quickly chilled in an ice-water-bath and the remaining activity determined as usual.

Inhibition studies. About 30 units of enzyme was pre-incubated with the different inhibitors (see text) for 30 min. The substrate was then added and enzyme activity determined.

K_m determination. About 20 units of enzyme was incubated for 20 min with laminarin at concentrations ranging from 0.01 to 2 mg ml $^{-1}$, in 50 mM-acetate buffer pH 5.5 at 37 $^{\circ}\text{C}$. Under these conditions the reaction was linear for at least 60 min. The K_m was estimated graphically from double-reciprocal plots; the value quoted represents the average of results from three different experiments.

Substrate specificity. The spectrum of substrates hydrolysed by the enzyme was determined under standard conditions as published (Santos *et al.*, 1978*b*).

RESULTS

Enzyme purification

Table 1 summarizes the steps followed in the purification of the 'late' β -glucanase. All 1,3- β -glucanase activity eluted in a single peak from the Sephadex G-100 column with a V_e/V_0 of 1.6, which demonstrates that all three enzymes have a similar molecular weight. Since 1,3- β -glucanase I is not retained by concanavalin A-Sepharose (Fig. 1), this step ensured the separation of 1,3- β -glucanase I (45% of total activity) from glucanases II and III which were retained, and were released only after elution with a 7% solution of α -methyl-mannoside. The enzyme activity eluted from DEAE-Sephadex in a single peak at NaCl 0.07 M. Figure 2 shows the elution profiles in DEAE-Sephadex, of 1,3- β -glucanases from crude extracts of derepressed mycelium compared with the activity remaining after concanavalin A-Sepharose chromatography. As can be seen, at this step, 1,3- β -glucanase I is free from 1,3- β -glucanases II and III. After DEAE-Sephadex chromatography only three major proteins were detected in the enzyme preparation by native gel electrophoresis (Fig. 3*a*), one of which was fully coincident with enzyme activity and gave a strong band with carbohydrate-specific stain. A final preparative electrofocusing step was included to purify the enzyme further. The activity eluted as a single peak which, after SDS-PAGE, appeared to be homogeneous as judged by the presence of a single polypeptide band

Table 1. *Purification of 1,3- β -glucanase I from derepressed mycelium of P. italicum*

Step	Total protein (mg)	Volume (ml)	Total activity (units)	Specific activity	Purification factor (fold)	Yield (%)
1. Crude extract (40000g supernatant)	2380	2000	86117*	36.18	1	100
2. Lyophilization and Sephadex G-100	23.10	40	23294*	1008.39	27	27
3. Concanavalin A- Sepharose	6.82	161.5	17724	2598.82	71	20
4. DEAE-Sephadex	1.05	60	5647	5378.09	148	6
5. Isoelectric focusing	0.20	30	2100	10500.00	290	2

* 1,3- β -glucanase I activity in crude extracts and the Sephadex G-100 fraction was calculated by estimating that activity as 45% of total 1,3- β -glucanase. This assumption was based on the profile of glucanase activities after DEAE-Sephadex chromatography of crude extracts (starting material), and was further confirmed by concanavalin A-Sepharose chromatography (step 3), which separated β -glucanase I from enzymes II and III, showing that the former still represented 40–45% of total activity.

Fig. 1. Concanavalin A binding by the different 1,3- β -glucanases. Set amounts (30 units) of 1,3- β -glucanase I (○), and of 1,3- β -glucanases II and III (●) (separated by DEAE-Sephadex chromatography), were each mixed with the indicated amounts of concanavalin A-Sepharose 4B in 50 mM-acetate pH 5.5, 1 M-NaCl. After 30 min gentle shaking at room temperature, the gel was removed by centrifugation and the activity remaining in the supernatant determined.

Fig. 2. DEAE-Sephadex chromatography of 1,3- β -glucanases: (a) crude extracts of derepressed mycelium; (b) 1,3- β -glucanase remaining after Sephadex G-100 and concanavalin A-Sepharose chromatography.

which was also stained by carbohydrate-specific reagents (Fig. 3b). This is preliminary evidence that 1,3- β -glucanase I is a glycoprotein. The source of enzyme for additional characterization was the homogeneous preparation obtained after isoelectric focusing. The molecular weight of the enzyme as determined by Sephadex G-100 filtration was 68 000, and by SDS-gel electrophoresis, 65 200. Hence the enzyme appears to be a single polypeptide with an isoelectric point of 4.7, according to the elution pattern from the isoelectric focusing column.

Fig. 3. Electrophoretic analysis (densitometric trace) of the enzyme preparation. (a) Native gel electrophoresis of the DEAE-Sephadex fraction. Three gels were run in parallel. Two of them were stained for protein and carbohydrate, respectively, and the third was quickly frozen, cut into 2 mm pieces and the enzyme activity determined in the different fractions as published (Notario *et al.*, 1976). —, A_{560} (protein staining); ----, A_{575} (carbohydrate staining); ●, A_{520} (enzyme activity). (b) SDS-PAGE of the isoelectric focusing fraction. Symbols as in (a) (enzyme activity not tested).

Table 2. *Substrate specificity of 1,3- β -glucanase I from P. italicum*

Substrate	Structure	Relative activity (%)
Laminarin	Slightly branched 1,3- β -glucan	100
Pustulan	Linear 1,6- β -glucan	17
Yeast glucan	Branched 1,3- β -glucan	3
PNPG*	<i>p</i> -Nitrophenyl- β -D-glucopyranoside	0
Salicin*	Hydroxyphenyl- β -D-glucopyranoside	0
Cellobiose*	1,4- β -D-Glucopyranosyl- β -D-glucopyranoside	0

* β -Glucosidase substrate. PNPG is also hydrolysed by *exo*- β -glucanases (see Villanueva *et al.*, 1976).

Characterization of 1,3- β -glucanase I

pH optimum. The optimum pH was 6 (for the buffers used), with a reduction to 70–65% of maximum activity at pH values of 5 and 7, and only 20% of maximum activity at pH values of 3 and 9.

Temperature optimum and thermal stability. With short incubation times (10 min) the optimum temperature for the enzyme was 50 °C, with a sharp reduction to 20% of maximum activity at 60 °C. After 10 min preincubation at 60 °C the enzyme had lost 90% of its activity, while 3 min at 70 °C resulted in complete inactivation.

Substrates and mode of action. As shown in Table 2, the spectrum of substrates hydrolysed by the enzyme is rather narrow, with good activity restricted to laminarin (a slightly branched 1,3- β -glucan) and almost no activity on yeast 1,3- β -glucan (a heavily branched molecule). The K_m for laminarin was low (0.04 mg ml⁻¹). No activity could be detected against β -glucosidase substrates. As determined by paper chromatography, the products of laminarin hydrolysis were

laminaritriose, laminaribiose and glucose, which indicates that this 1,3- β -glucanase is an endo-splitting enzyme.

Inhibition. A variety of compounds were examined for their ability to affect the action of the purified enzyme. All reagents that act on SH groups affected the 1,3- β -glucanase activity. Oxidized glutathione, Hg^{2+} , iodoacetamide and *N*-ethylmaleimide, at 1 mM, resulted in 12%, 71%, 14%, and 6% inhibition, respectively. At 5 mM, Hg^{2+} , iodoacetamide and *N*-ethylmaleimide gave inhibitions of 90%, 55% and 80%, respectively. No inhibition was observed with δ -gluconolactone (1 mM) which at low concentrations is a potent inhibitor of exo- β -glucanases (Notario *et al.*, 1976). Cations such as Ca^{2+} , Mg^{2+} and Mn^{2+} , at concentrations of 1 mM and 5 mM, had almost no effect on enzyme activity.

DISCUSSION

Both the molecular weight and isoelectric point of 1,3- β -glucanase I are within the range found for other β -glucanases (Villanueva *et al.*, 1976). In this regard, 1,3- β -glucanase I does not differ grossly from β -glucanases II and III which are also acidic proteins (as judged from their behaviour on DEAE-Sephadex) of a similar molecular weight. Besides the difference in the pattern of derepression, there are several properties which clearly distinguish β -glucanase I from enzymes II and III. All three enzymes appear to be glycoproteins, but whereas 1,3- β -glucanases II and III bind to concanavalin A, 1,3- β -glucanase I does not (Fig. 1). Unlike 1,3- β -glucanases II and III, this enzyme behaves as an endo-splitting hydrolase as judged from the products of reaction on laminarin and the inability to hydrolyse substrates such as *p*-nitrophenyl- β -D-glucoside, which is hydrolysed by both glucanases II and III (Santos *et al.*, 1978*b*). The most significant properties of enzyme I are its uncommonly low K_m on laminarin (0.04 mg ml⁻¹), which is at least ten times lower than the average K_m for other glucanases (Villanueva *et al.*, 1976), and its low activity on branched 1,3- β -glucans (such as yeast 1,3- β -glucan). The fact that endo-1,3- β -glucanase I is never localized in the cell wall periplasmic space and cannot digest *P. italicum* cell walls (Santos *et al.*, 1979*a*) (while glucanases II and III are both periplasmically located and able to digest cell walls of *P. italicum*), together with the aforementioned properties such as high affinity for slightly branched glucans and low activity on branched molecules, makes it very unlikely that this enzyme has any morphogenetic role and indicates a metabolic function. The enzyme is in fact secreted to the medium (Santos *et al.*, 1978*b*), most probably to mobilize potential carbon sources in the environment when glucose is no longer available. The purification and characterization of glucanases II and III may well provide additional evidence for diversity of β -glucanase function within the same organism.

M. Sánchez was the recipient of a fellowship from the Ministerio de Educación y Ciencia. This investigation was aided by a grant from Comisión Asesora de Investigación y Ciencia, to J. R. Villanueva.

REFERENCES

- ABD-EL-AL, A. & PHAFF, H. J. (1969). Purification and properties of endo- β -glucanases in the yeast *Hanseniaspora valbyensis*. *Canadian Journal of Microbiology* **15**, 697-701.
- BARTNICKI-GARCIA, S. (1973). Fundamental aspects of hyphal morphogenesis. *Symposia of the Society for General Microbiology* **23**, 245-267.
- CHESTERS, C. G. C. & BULL, A. T. (1963*a*). The enzymic degradation of laminarin. 1. The distribution of laminarinase among micro-organisms. *Biochemical Journal* **86**, 28-31.
- CHESTERS, C. G. C. & BULL, A. T. (1963*b*). The enzymic degradation of laminarin. 2. The multicomponent nature of fungal laminarinases. *Biochemical Journal* **86**, 31-38.
- CLARKE, A. E. & STONE, B. A. (1965). β -Glucan hydrolase from *Aspergillus niger*. Isolation of a 1,4- β -glucan hydrolase and some properties of the 1,3- β -glucan hydrolase components. *Biochemical Journal* **96**, 793-801.
- DAVIS, B. J. (1964). Disc electrophoresis II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **122**, 407-427.
- FRIEBE, B. & HOLLDORF, A. W. (1975). Control of extracellular 1,3- β -glucanase activity in a basidiomycete species. *Journal of Bacteriology* **122**, 818-825.
- MAHADEVAN, P. R. & MAHADKAR, V. R. (1970). Role of enzyme in growth and morphology of *Neurospora crassa*: cell wall-bound enzymes and their possible role in branching. *Journal of Bacteriology* **101**, 941-947.
- NORTHCOTE, D. H. & HORNE, R. D. (1952). The chemi-

- cal composition and structure of the yeast cell wall. *Biochemical Journal* **51**, 232–236.
- NOTARIO, V., VILLA, T. G. & VILLANUEVA, J. R. (1976). Purification of an exo- β -glucanase from cell-free extracts of *Candida utilis*. *Biochemical Journal* **159**, 555–562.
- REESE, E. T., PARRISH, F. W. & MANDELS, M. (1962). 1,6- β -Glucanases in fungi. *Canadian Journal of Microbiology* **8**, 327–334.
- REY, F., ACHA, I. G. & NOMBELA, C. (1979). The regulation of β -glucanase synthesis in fungi and yeasts. *Journal of General Microbiology* **110**, 83–89.
- SANTOS, T., VILLANUEVA, J. R. & NOMBELA, C. (1978*a*). Regulation of 1,3- β -glucanase synthesis in *Penicillium italicum*. *Journal of Bacteriology* **133**, 542–548.
- SANTOS, T., SÁNCHEZ, M., VILLANUEVA, J. R. & NOMBELA, C. (1978*b*). Regulation of the 1,3- β -glucanase system of *Penicillium italicum*: glucose repression of the various enzymes. *Journal of Bacteriology* **133**, 465–471.
- SANTOS, T., SÁNCHEZ, M., VILLANUEVA, J. R. & NOMBELA, C. (1979*a*). Derepression of 1,3- β -glucanases in *Penicillium italicum*: localization of the various enzymes and correlation with cell wall glucan mobilization and autolysis. *Journal of Bacteriology* **137**, 6–12.
- SANTOS, T., REY, F., CONDE, J., VILLANUEVA, J. R. & NOMBELA, C. (1979*b*). *Saccharomyces cerevisiae* mutant defective in exo-1,3- β -glucanase production. *Journal of Bacteriology* **139**, 333–338.
- SENTANDREU, R., ELORZA, M. V. & VILLANUEVA, J. R. (1975). Synthesis of yeast wall glucan. *Journal of General Microbiology* **90**, 13–20.
- TREVELYAN, W. E., PROCTER, W. E. & HARRISON, J. S. (1950). Detection of sugars on paper chromatograms. *Nature, London* **166**, 444–445.
- VILLANUEVA, J. R., NOTARIO, V., SANTOS, T. & VILLA, T. G. (1976). β -Glucanases in nature. In *Microbial and Plant Protoplasts*, pp. 323–355. Edited by J. F. Peberdy, A. H. Rose, H. J. Rogers & E. C. Cocking. London: Academic Press.
- WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determination by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* **244**, 4406–4412.
- WESSELS, J. G. H. (1966). Control of cell-wall glucan degradation during development in *Schizophyllum commune*. *Antonie van Leeuwenhoek* **32**, 341–355.
- WESSELS, J. G. H. & KOLTIN, Y. (1972). R-glucanase activity and susceptibility of hyphal walls to degradation in mutants of *Schizophyllum* with disrupted nuclear migration. *Journal of General Microbiology* **71**, 471–475.
- ZACHARIUS, R. M., ZELL, T. E., MORRISON, J. H. & WOODLOCK, J. J. (1969). Glycoprotein staining following electrophoresis on acrylamide gels. *Analytical Biochemistry* **30**, 148–152.