Purification of a new galactanase from *Penicillium oxalicum* catalysing the hydrolysis of β -(1→5)-galactofuran linkages

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An endo β -(1→5)-galactofuranase from *Penicillium oxalicum* has been purified 91-fold. The enzyme is a basic glycoprotein with a pI 7.9 and 20 % (w/w) carbohydrate content, galactose being the principal sugar. The apparent M_r of the enzyme estimated by denaturing gel electrophoresis was 77000. The optimum pH was 5.0, and the enzyme was stable over the pH range 4.0–7.5. This enzyme hydrolyses specifically (1→5)-linked β -D-galactofuranose residues in homo- and hetero-galactans, but did not hydrolyse *o*-nitrophenyl galactose and β -(1→5)-galactofuranbiose. K_m and V_{max} values were 1.2 mg · ml⁻¹ and 0.55 μ mol · h⁻¹ respectively when *Eupenicillium crustaceum* β -(1→5)-galactofuran was used as substrate. The enzyme showed high affinity for different separation gels and proteins. The enzyme specificity and its mode of action showed that it could be an useful tool for analysing the fine structure of polysaccharides.

INTRODUCTION

Specific galactanases for β -(1→3)- and β -(1→4)-galactopyranosyl linkages have been described in micro-organisms [1–5]. Generally these enzymes degrade D-galactan randomly to afford D-galactose and D-galacto-oligosaccharides, and they are therefore endo-D-galactanases, although exo-D-galactanases have also been described [3,5]. A specific galactosidase for β -(1→5)-galactofuranosyl linkages has been studied [6], but no galactanase specific for this linkage has been described. During autolysis, *Penicillium oxalicum* produces lytic enzymes capable of degrading its own cell walls [7–10] and polysaccharides from different sources. In the present work we report on the purification of a new enzyme, an endo- β -(1→5)-D-galactofuranase, from this fungus.

MATERIALS AND METHODS

Organisms and culture conditions

Penicillium oxalicum Curie & Thom, A.T.C.C. (American Type Culture Collection) 64.198, *Talaromyces wortmanii* (Klöcker) C. R. Benjamin CBS (Centraalbureau voor Schimmelcultures) 235.38, *Eupenicillium crustaceum* Ludwig, CBS 635.70 and *Talaromyces flavus* Stolk & Samson, CBS 352.72, were maintained on potato/dextrose agar slants. For inoculum, spore suspension in sterile distilled water (10⁶ spores · ml⁻¹) were obtained from sporulated cultures grown on malt-agar slants. *P. oxalicum* was grown on the mineral medium described by Reyes & Byrde [11] supplemented with yeast extract (1 g · l⁻¹) at 25 °C in stationary culture. *T. wortmanii*, *E. crustaceum* and *T. flavus* were grown as previously described [12].

Preparation of enzymic substrates

The water-soluble fraction (F1S) obtained from mycelial cell walls of *T. wortmanii*, *E. crustaceum* and *T. flavus*, as described by Gómez-Miranda *et al.* [13], was used as a galactan source. β -(1→3)-Galactan was obtained by Smith degradation [1] of gum arabic (from acacia tree; Sigma). The recovered product was obtained in 80 % yield and gave galactose after acid hydrolysis.

β -(1→4)-Galactan was obtained by chemical reduction of citrus-fruit pectin with 100 mM-NaBH₄ in 5 M-NaOH at 90 °C [3]. The yield was 30 %, and the specific rotation $\alpha_{589}^{20} = +52.6^\circ$,

(c 1 in water) according to the values described by Labavitch *et al.* [2].

β -(1→5)-Galactan oligomers were obtained by 6 M-HCl hydrolysis of *E. crustaceum* galactan at 25 °C for 30 min. After HCl was eliminated under vacuum, galactan oligomers were separated with two Bio-Gel P2 columns connected in tandem (first column 1.6 cm × 70 cm; second column 2.6 cm × 40 cm) previously equilibrated with distilled water. Their molecular masses were calculated with a calibration curve for these columns. Raffinose, gentiobiose and *N*-acetylglucosamine oligomers were used as standards [14].

o-Nitrophenyl galactopyranoside, a chromogenic substrate, was purchased from Sigma.

Methods of characterization of fungal galactans

F1S fractions were hydrolysed with 0.05 M- or 2 M-H₂SO₄ at 100 °C for 5 h. The neutral sugars obtained were converted into their corresponding alditol acetates [15], and their identification and quantification was done by g.l.c. as described by Gómez-Miranda *et al.* [16].

For structural analysis, periodate oxidation was performed by the method of Aspinall & Ferrier [17]. The Smith degradation products were converted into alditol acetates and identified by g.l.c.. Methylation analysis was performed by the Hakomori method as described by Prieto *et al.* [18]. The partially methylated alditol acetates were analysed by g.l.c. in a Perkin–Elmer gas chromatograph with a SP-2100 fused-silica capillary column (30 m × 0.25 mm) programmed from 160 °C (1 min) to 200 °C with a temperature gradient of 2 °C · min⁻¹, maintaining the final temperature for 10 min, and identifying the final products using a Perkin–Elmer ITD electron-impact mass detector.

Analytical techniques

Slab-gel electrophoresis was carried out by the method Laemmli [19] with 7.5 % acrylamide (PAGE), and under denaturing conditions (SDS/PAGE). Gels were stained by the method of Morrissey [20]. Isoelectric focusing was carried out with 0.1 mm-thick 5 %-polyacrylamide gels and a pH range from 3.5 to 10 (LKB Ampholines). An isoelectric-focusing protein-calibration kit (Pharmacia) ranging from pH 3.5 to 10 was used. Anode and cathode solutions were 0.025 M-aspartic acid and 2 M-ethylene-diamine respectively. Gels were stained with Coomassie Brilliant Blue R-250.

Total carbohydrate concentration in the protein was determined by the anthrone method [21], and sugar composition was determined by hydrolysing the protein with 0.5 M-H₂SO₄ for 16 h at 100 °C; alditol acetates of the hydrolysis products were identified and quantified by g.l.c.

T.l.c. on silica-gel 60 F₂₅₄ (Merck) plates was performed with propan-1-ol/ethanol/water (7:1:2, by vol.) as the ascendent irrigant. Detection was done by charring with methanol/conc.H₂SO₄ (9:1, v/v) [3]. A mixture of galactose oligosaccharides obtained by hydrolysis with HCl was used as standard.

Enzymic assays

β -(1 $\rightarrow$ 5)-Galactofuranase activity was assayed as follows. Each activity assay contained 1 mg of substrate/ml and from 10 to 50 μ g of enzyme/ml in 10 mM-sodium phosphate buffer, pH 5.5. Activity was assayed by incubation for 30 min at 37 °C. Soluble reducing sugars were measured by the combination of the methods of Somogyi [22] and Nelson [23]. One unit of enzyme activity was defined as the amount which release 1 μ mol of soluble reducing sugars/min.

β -Galactosidase activity was determined in the same conditions by measuring the amount of *o*-nitrophenol released from *o*-nitrophenyl galactoside. The reaction was stopped by the addition of 0.2 M-sodium/potassium borate buffer, pH 9.8.

RESULTS

Characterization of polysaccharides

FIS fractions were mainly composed of galactose (most of it in furanose form) and smaller amounts of mannose and glucose.

By the analysis of the results from Smith degradation and methylation analysis it was not possible to distinguish between (1 $\rightarrow$ 4)-galactopyranose and (1 $\rightarrow$ 5)-galactofuranose.

We can see that FIS from *T. wortmanii* has mainly (1 $\rightarrow$ 2)- and/or (1 $\rightarrow$ 3)-linked galactofuranose and smaller amounts of (1 $\rightarrow$ 6) linkages. These results are confirmed by methylation analysis, which gives two main peaks: 2,5,6-Me₃-1,3,4-Ac₃-galactose and 3,5,6-Me₃-1,2,4-Ac₃-galactose (Me = methyl; Ac = acetyl).

FIS from *E. crustaceum* contains the highest proportion of (1 $\rightarrow$ 5) galactofuranose linkages as shown by Smith degradation and confirmed by n.m.r. (M. Bernabé, personal communication).

FIS from *T. flavus* has a considerable amount of (1 $\rightarrow$ 5)-galactofuranose [12]. In addition it has (1 $\rightarrow$ 2)- and (1 $\rightarrow$ 3)-linked galactofuranose, and also (1 $\rightarrow$ 6) linkages.

Isolation and purification of β -(1 $\rightarrow$ 5)-galactofuranase activity

The purification procedure is summarized in Table 1.

Isolation and filtration through Sephadex G-200

Centrifugation supernatants from 14-day-old *Penicillium oxalicum* autolysed cultures were concentrated 10-fold under vacuum at 25 °C and then frozen in 4 ml fractions (fraction I). These fractions were used as lytic enzyme source. There was not an appreciable loss of β -(1 $\rightarrow$ 5)-galactofuranase activity by freezing and thawing.

Each 4 ml fraction was filtered through a Sephadex G-200 column (2.5 cm $\times$ 90 cm) equilibrated with 14 mM-sodium phosphate buffer, pH 6.5, to remove a phenolic yellow pigment, previously described in different strains of *P. oxalicum* [24], that showed a high absorbance at 280 nm; 3.5 ml fractions were collected. The protein elution profile is shown in Fig. 1(a). Three fractions with β -(1 $\rightarrow$ 5)-galactofuranase activity were obtained

(A, B and C), with percentages of 5.4, 73.5 and 20.6 % of the total activity. The pigment was co-eluted with fraction C. This fraction, concentrated and filtered through the column, gave an elution profile identical with that of fraction I (Fig. 1a).

Table 1. Purification of β -(1 $\rightarrow$ 5)-galactofuranase from *P. oxalicum*

For experimental details, see the Results section. Enzyme activity [with *Eupenicillium crustaceum* β -(1 $\rightarrow$ 5)-galactofuran as substrate] and protein were determined as described in the Materials and methods section. Units of activity are expressed as μ mol $\cdot$ min⁻¹.

Step	Total activity (units)	Protein (mg)	Specific activity (units $\cdot$ mg ⁻¹)	Purification (fold)	Recovery (%)
Fraction I	0.211	6.00	0.035	1	100
Sephadex G-200	0.148	0.60	0.247	7	70
Desalting PD-10	0.133	0.60	0.222	6	63
Mono Q HR 5/5	0.062	0.13	0.477	14	29
Superose 6 HR 10/30	0.192	0.06	3.200	91	91

Fig. 1. Gel-filtration chromatography through Sephadex G-200 (a) and Superose 6 (b)

Gel-filtration chromatography was carried out as described in the Materials and methods section. The elution profile of proteins (continuous line) and endo- β -(1 $\rightarrow$ 5)-galactofuranase activity (dotted line) are displayed. In (b) 'slash' marks indicate the fraction collected as purified enzyme. G is the galactofuranase; I is an inhibitor.

Fig. 2. PAGE and SDS/PAGE of the purified endo- β -(1 $\rightarrow$ 5)-D-galactofuranase

Electrophoresis in native (a) and denaturing conditions (b) was carried out as described in the Materials and methods section, protein concentrations of 8 and 5 μ g respectively being used. The SDS/PAGE M_r standards (lane 2) used were egg albumin (M_r 45000), BSA (M_r 66000), phosphorylase b from rabbit muscle (M_r 97400), *Escherichia coli* β -galactosidase (M_r 116000) and rabbit myosin (M_r 200000). The open arrow points to the purified enzyme.

Table 2. Enzyme activity of the purified β -(1 $\rightarrow$ 5)-galactofuranase towards different substrates

The activity of the purified β -(1 $\rightarrow$ 5)-galactofuranase was determined as described in the Materials and methods section, with an enzyme concentration of 10 μ g $\cdot$ ml $^{-1}$

Substrate	Composition	Relative rate of hydrolysis (%)
FIS from:		
<i>E. crustaceum</i>	β -(1 $\rightarrow$ 5)-Galactofuran	100
<i>T. flavus</i>	(1 $\rightarrow$ 5)-, (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 2)-Galactofuran	33
<i>T. wortmanii</i>	(1 $\rightarrow$ 3)- and/or (1 $\rightarrow$ 2)-Galactofuran	0
Gum arabic from the acacia tree	Branching (1 $\rightarrow$ 3)-galactopyran residues	0
Smith degradation of gum arabic	β -(1 $\rightarrow$ 3)-Galactan	0
Citrus pectin	β -(1 $\rightarrow$ 4)-Galacturonic acid	0
Chemical reduction of citrus pectin	β -(1 $\rightarrow$ 4)-Galactan	0
<i>o</i> -Nitrophenyl galactopyranoside	—	0
β -(1 $\rightarrow$ 5)-Galactofuranbiose	—	0
Chitin	Poly[β -(1 $\rightarrow$ 4)- <i>N</i> -acetylglucosamine]	0
Chitosan	Poly[β -(1 $\rightarrow$ 4)-glucosamine]	0
Cotton cellulose	Poly[β -(1 $\rightarrow$ 4)-glucose]	0
Carboxymethyl-cellulose	Poly[β -(1 $\rightarrow$ 4)-carboxymethylglucose]	0
Xylan	Poly[β -(1 $\rightarrow$ 4)-xylopyranose]	0
Lichenan	β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-Glucan	0

Ion-exchange chromatography

Fraction B obtained from the Sephadex G-200 column was filtered through a PD-10 desalting column, and then loaded on to a Mono Q HR 5/5 f.p.l.c. ion-exchange column

Fig. 3. Analysis by t.l.c. of the product derived from the hydrolysis of the β -(1 $\rightarrow$ 5)-D-galactofuran from *E. crustaceum* by the endo- β -(1 $\rightarrow$ 5)-D-galactofuranase

Lanes from 1 to 6 (left to right) show the reaction hydrolysis product after incubation times of 30 min, 1 h, 2 h, 4 h, 8 h and 22 h. Lane 7 shows a D-galactose standard (M), and lane 8 a mixture of β -(1 $\rightarrow$ 5)-galactan oligomers from the chemical hydrolysis of β -(1 $\rightarrow$ 5)-galactofuran (H). The positions of the monomer (M), dimer (D), trimer (T) and tetramer (Tt) of β -(1 $\rightarrow$ 5)-galactan are shown.

(5 mm $\times$ 50 mm) equilibrated with 10 mM-sodium phosphate buffer, pH 7.2. Under these conditions most of the activity was not retained (76%), although 24% was eluted with a 0–0.5 M-NaCl gradient.

Filtration through Superose 6

The non-retained fraction from the Mono Q ion-exchange column was filtered through a Superose 6 HR 10/30 f.p.l.c. gel-filtration column (1.3 cm $\times$ 30.8 cm) equilibrated with 50 mM-sodium phosphate buffer (pH 5.2)/150 mM-NaCl.

The enzyme β -(1 $\rightarrow$ 5)-galactofuranase (G) was purified 91-fold, and a yield of 91% was obtained. This high yield is due to the elimination of an inhibitor (I) in the last purification step (Fig. 1b). This inhibitor was characterized as a polysaccharide [21]. In the last purification step (Table 1), this polysaccharide (30 μ g) inhibited 70% of the activity of the purified galactanase (60 μ g). The purified enzyme migrated as a single protein band in PAGE (Fig. 2) and under denaturing conditions (SDS/PAGE) (Fig. 2).

Properties of the purified β -(1 $\rightarrow$ 5)-galactofuranase

An M_r value of about 77000 was determined by SDS/PAGE (Fig. 2). When determining the apparent M_r by gel permeation on Superose 6, there was an increase in the retention time with increasing NaCl concentration. This effect was observed with pH values ranging from 5.5 to 7.2. It could be due to a hydrophobic interaction of the protein with the gel. The protein also showed a high affinity for the yellow pigment and for the proteins found in centrifugal supernatants of *P. oxalicum* autolysed cultures.

The pI was 7.9. The enzyme is a glycoprotein with 18–22% carbohydrate, galactose being the principal sugar.

The enzyme hydrolyses FIS fractions from *E. crustaceum* and *T. flavus* which contain β -(1 $\rightarrow$ 5)-galactofuran and has no activity towards *o*-nitrophenyl galactopyranoside, β -(1 $\rightarrow$ 5)-galactofuranbiose or the other polysaccharides assayed (Table 2).

Optimum pH, pH-stability, variation of activity with enzyme concentration and incubation time, and K_m (Lineweaver–Burk plot) were determined using *E. crustaceum* β -(1 $\rightarrow$ 5)-galactan as substrate.

The enzyme has optimum activity at pH 5.0 and was stable over the pH range 4.0–7.5 (at 25 $^{\circ}$ C with a preincubation time of 2 h).

The variations of the rate of substrate hydrolysis with the incubation time and with the enzyme concentration showed a linear relationship at the initial stages of the reaction (30 min). K_m and V_{max} values of $1.2 \text{ mg} \cdot \text{ml}^{-1}$ and $0.55 \mu\text{mol} \cdot \text{h}^{-1}$ respectively were obtained. When the effect of different ions at 50 mM concentration on enzymic activity was studied, a very high inhibition by most of the assayed ions, such as acetate, borate and citrate anions, was observed. This inhibition was total with Fe^{3+} .

Characterization of the reaction products

The enzymic degradation products obtained when incubating the enzyme with *E. crustaceum* β -(1 $\rightarrow$ 5)-galactofuran from 0.5 to 24 h were analysed by t.l.c. and g.l.c. Although total reducing substances increased with incubation time, the concentration of released monomer determined by g.l.c. remained constant after 4 h of incubation. T.l.c. revealed the production of monomer, dimer and trimer of galactose. After 24 h of incubation the only product detected was galactose (Fig. 3).

DISCUSSION

Filamentous fungi synthesize lytic enzymes during autolysis [25]. In *P. oxalicum* autolysed cultures we have found a β -(1 $\rightarrow$ 5)-galactofuranase activity. The presence of this activity could be explained by the facts that *P. oxalicum* and other *Penicillium* spp. have galactose as a component of their cell walls [26] and that *P. charlesii* synthesized a β -(1 $\rightarrow$ 5)-galactofuran, galactocarolose [27], whose production increased by the addition of tartrate to the mineral medium.

The purified enzyme is a glycoprotein with approx. 20% carbohydrate (mainly galactose), has a pI of 7.9 and an apparent M_r of 77 000 determined by electrophoresis.

Evidence is provided that the purified β -(1 $\rightarrow$ 5)-galactofuranase is an endoenzyme which hydrolyses internal galactoside linkages with production of mono-, di- and tri-saccharides of galactose. The reaction product after 24 h of incubation was galactose, although the monomer concentration did not increase from 4 h of incubation. The enzyme did not degrade purified galactofuranbiose or *o*-nitrophenyl galactopyranoside, specific substrates of β -galactosidase and exo-type enzymes. Furthermore, the enzyme is specific for β -(1 $\rightarrow$ 5)-galactofuran linkages in homo-

and hetero-polysaccharides. It does not hydrolyse β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-galactan and other polysaccharides (Table 2), indicating that it has a high specificity.

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