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Immobilized 4-aminophenyl 1-thio-β-D-galactofuranoside as a matrix for affinity purification of an exo-β-D-galactofuranosidase

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Abstract

An alternative and fast method for the purification of an exo-β-D-galactofuranosidase has been developed using a 4-aminophenyl 1-thio-β-D-galactofuranoside affinity chromatography system and specific elution with 10 mM D-galactono-1,4-lactone in a salt gradient. A concentrated culture medium from *Penicillium fellutanum* was chromatographed on DEAE–Sepharose CL 6B followed by chromatography on the affinity column, yielding two separate peaks of enzyme activity when elution was performed with 10 mM D-galactono-1,4-lactone in a 100–500 mM NaCl salt gradient. Both peaks behaved as a single 70 kDa protein, as detected by SDS-PAGE. Antibodies elicited against a mixture of the single bands excised from the gel were capable of immunoprecipitating 0.2 units out of 0.26 total units of the enzyme from a crude extract. The glycoprotein nature of the exo-β-D-galactofuranosidase was ascertained through binding to Concanavalin A–Sepharose as well as by specific reaction with Schiff reagent in Western blots. The purified enzyme has an optimum acidic pH (between 3 and 6), and K_m and V_{max} values of 0.311 mM and 17 μmol h^{-1} μg^{-1} respectively, when 4-nitrophenyl β-D-galactofuranoside was employed as the substrate.

Keywords: Affinity chromatography; β-D-Galactofuranose; exo-β-D-Galactofuranosidase; *Penicillium fellutanum*; 1-thio-β-D-Galactofuranoside

1. Introduction

D-Galactose in the pyranose form is a common component of glycoconjugates, whereas D-galactofuranose (Gal*f*) has a more restricted distribution, being described in glycoconjugates isolated from bacteria, fungi and protozoa [1].

The absence of galactofuranose in mammal glycoconjugates suggests that the enzymes involved in the metabolism of this sugar in pathogenic organisms could be a good target for the design of drugs. A specific exo-β-D-galactofuranosidase was first purified from the culture medium of *Penicillium fellutanum* [2] and later described in *Helminthosporium sacchari* [3] and *Penicillium* and *Aspergillus* species [4].

The synthesis and evaluation of 1-thio-β-D-galactofuranosides as exo-β-D-galactofura-

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nosidase inhibitors has recently been described [5]. The 4-aminophenyl 1-thio-β-D-galactofuranoside was the best inhibitor found and was considered a suitable ligand for the preparation of an affinity phase for the isolation of β-D-galactofuranosidases. The use of a specific ligand for binding the enzyme was expected to provide sufficient amounts of pure protein to permit the raising of antibodies for immunochemical studies and as reagents for future detection of clones expressing β-D-galactofuranosidase coding sequences.

2. Results

Isolation and purification of exo-β-D-galactofuranosidase.—Previous attempts to concentrate the enzyme by either ammonium sulfate fractionation or direct lyophilization of the culture medium met with failure. In the first case there was an extensive loss of activity (90%) and in the second the concentrated medium became too viscous due to the concomitant concentration of extracellular pepti-

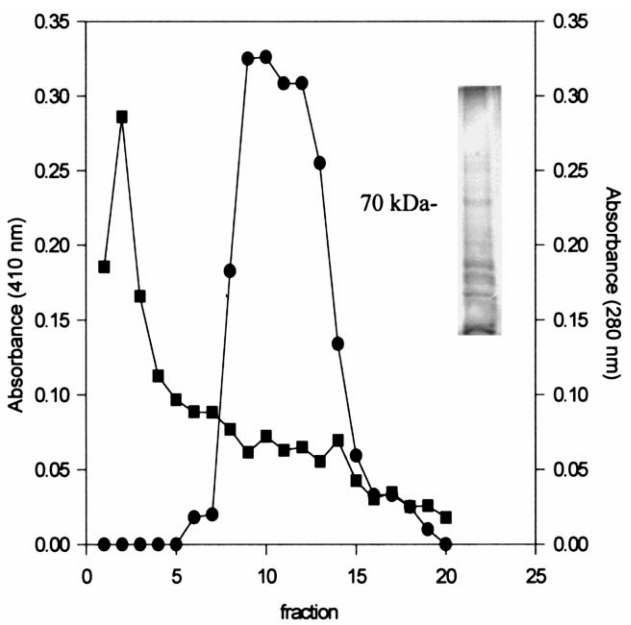

Fig. 1. Ion-exchange chromatography of exo-β-D-galactofuranosidase from *P. fellutanum*. The DEAE–Sepharose CL 6B column (1.5 × 5 cm) was previously equilibrated and washed with 10 mM acetate buffer (pH 5.0). Enzyme activity was eluted in 1 mL fractions with a 0–0.5 M NaCl gradient at a flow rate of 1 mL/min. (■-■) Protein (280 nm); (●-●) enzyme activity (410 nm). Inset: 10 μg of a pool of Fractions 9–12 were subjected to SDS-PAGE and stained by Coomassie Brilliant Blue.

dophosphogalactomannan, which is known to be secreted into the medium by the growing fungus [2]. Thus, after filtration of the *P. fellutanum* culture in Whatman no. 1 paper (30 min, room temperature (rt)), the crude supernatant (pH 4.5) was concentrated under pressure (Amicon) for 4 h at 25 °C. Most fragments of the peptidophosphogalactomannan are probably eliminated by the Amicon filtration step, as a sharp decrease in molecular mass has been previously correlated with the increase of incubation time [2]. The material was extensively dialyzed against water in order to further eliminate the remnants of saccharides. The concentrated material, with a pH of 5.2, was adsorbed on a DEAE–Sepharose CL 6B column, washed with 10 mM acetate buffer (pH 5.0), and the enzyme eluted with a linear gradient of 0–0.5 M NaCl in the same buffer (Fig. 1). The fractions that contained exo-β-D-galactofuranosidase activity were pooled, dialyzed against 0.1 M NaCl in 66 mM acetate buffer, pH 5.0, and loaded onto a 4-aminophenyl 1-thio-β-D-galactofuranoside–Sepharose column. After extensive washing, the enzyme was eluted with 10 mM D-galactono-1,4-lactone in the same buffer in a gradient of 100–500 mM NaCl (Fig. 2). Two peaks of enzyme activity were eluted from the affinity column, with a total recovery of 63% of the original enzyme activity.

In non-denaturing gels, both purified peaks migrated as a 150 kDa band (not shown) and under denaturing conditions in SDS-PAGE both peaks migrated as a single 70 kDa band (Fig. 3(A)). In addition, both migrated as a single band, even when they were mixed together (Fig. 3(B)). The glycoprotein nature of the purified enzyme was ascertained by a positive staining of the SDS-PAGE with the Schiff reagent (Fig. 3(B)) and by the fact that both mixed peaks bind to Concanavalin A–Sepharose. In the latter case, the enzyme activity was specifically eluted by a mixture of 0.1 M methyl α-D-mannopyranoside and 0.1 M methyl α-D-glucopyranoside in a broad peak, suggesting microheterogeneity in the carbohydrate moieties (Fig. 4). Possibly, the appearance of two peaks of enzyme activity with different affinities for the column ligand was due to differences in composition and size of

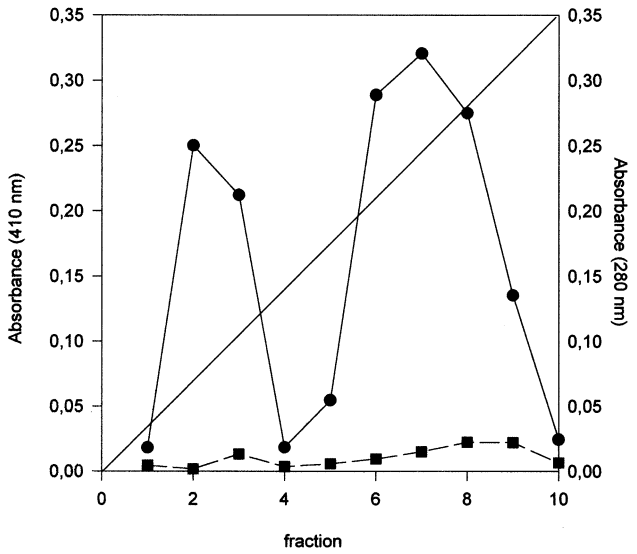

Fig. 2. Elution profile of exo-β-D-galactofuranosidase from Sepharose–4-aminophenyl 1-thio-β-D-galactofuranoside. Eluted fractions from the DEAE–Sepharose CL 6B column were dialyzed and passed onto a Sepharose–4-aminophenyl 1-thio-β-D-galactofuranoside column (0.5 × 10 cm) equilibrated with 66 mM acetate buffer, pH 5.0, and eluted with a gradient of 100–500 mM NaCl (diagonal line) in the same buffer and containing 10 mM D-galactono-1,4-lactone. Fractions of 1 mL were collected. β-D-Galactofuranosidase activity was determined as described in Section 4. (■-■) Protein (280 nm); (●-●) enzyme activity (410 nm).

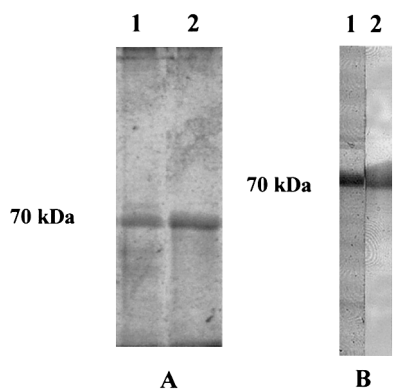

Fig. 3. SDS-PAGE of purified exo-β-D-galactofuranosidase. Electrophoreses were performed in 10% polyacrylamide gels under denaturing conditions as described in Section 4. Samples containing ~10 μg of protein were used. Following electrophoresis, gels were stained with: A, silver nitrate, lanes 1 and 2 correspond, respectively, to both peaks eluted from the affinity column; B, mixed peaks stained with Coomassie Brilliant Blue (lane 1) or Schiff reagent (lane 2). A commercial standard mixture was run in parallel to determine molecular weight.

the carbohydrate moieties, which might affect enzyme conformation. The total carbohydrate content of the purified exo-β-D-galactofuranosidase was estimated as 17% (w/w) as compared with the amount of enzyme protein. At the end, recovery of enzyme total units was ~40% of that from the original supernatant.

All subsequent experiments were carried out with a mixture of both peaks which, in addition to having the same molecular mass and the same general properties, have also been shown in preliminary experiments to demonstrate very similar kinetic properties.

Antibodies raised against the 70 kDa band from the SDS-PAGE reacted with paraformaldehyde-fixed fungi (Fig. 5) and only recognized a 70 kDa protein in Western blots when a concentrated supernatant fraction was employed (Fig. 6). Moreover, 78% of the enzyme activity (0.2 U) was immunoprecipitated by the antibodies from an affinity-purified preparation (0.26 U), confirming both the β-D-galactofuranosidase identity of the 70 kDa polypeptide and the specificity of the antibody.

*Properties of the exo-β-*D*-galactofuranosidase.*—The enzyme has maximal activity between pH 3 and 6, with a sharp drop in

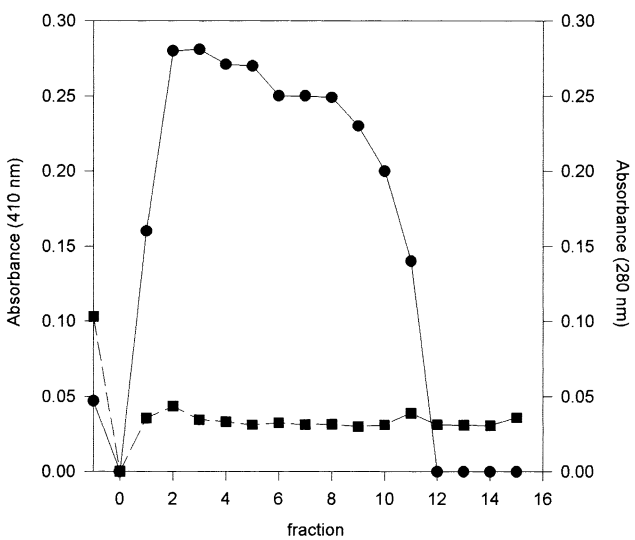

Fig. 4. Chromatography of exo-β-D-galactofuranosidase on Concanavalin A–Sepharose. Eluted fractions from the Sepharose–4-aminophenyl 1-thio-β-D-galactofuranoside column were dialyzed and passed onto a Concanavalin A–Sepharose column (0.5 × 1 cm) equilibrated with 500 mM NaCl in 50 mM Tris–HCl, pH 7.4, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. The column was washed with 30 mL of the same buffer and the bound material was eluted with 10 mL of 0.1 M methyl α-D-glucopyranoside and 0.1 M methyl α-D-mannopyranoside. Fractions of 1 mL were collected and β-D-galactofuranosidase activity was determined as described in Section 4. (■-■) Protein (280 nm); (●-●) enzyme activity (410 nm).

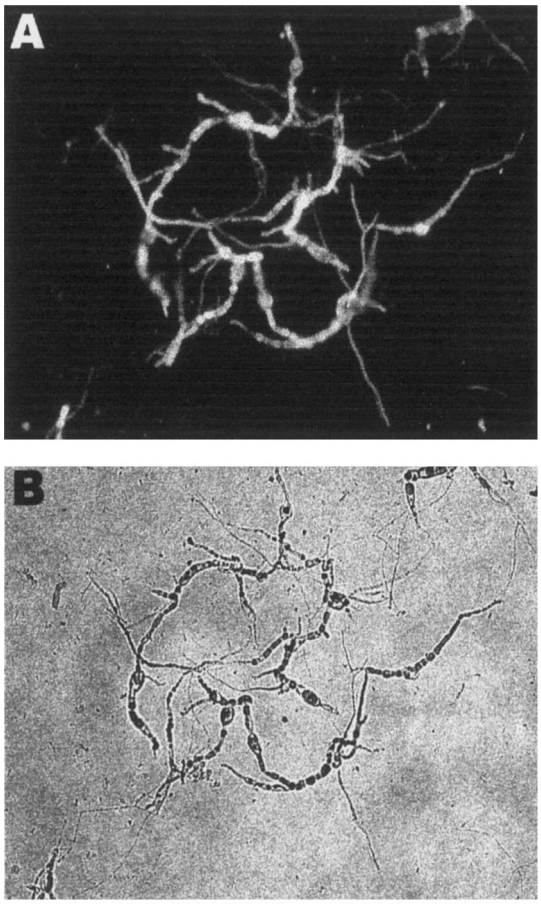

Fig. 5. Indirect immunofluorescence of *P. fellutanum* with a polyclonal antibody against β-D-galactofuranosidase. (A) Fluorescence of mycelium forms of *P. fellutanum*; (B) phase contrast.

activity at pH 8.0 or above (Fig. 7) and is stable after several cycles of freezing and thawing, as reported [2]. Values for K_m and V_{max} of 0.311 mM and 17 μmol h^{-1} μg^{-1}, respectively, were obtained for 4-nitrophenyl β-D-galactofuranoside as substrate (Fig. 8). Strong inhibition was observed when D-galactono-1,4-lactone was employed, as reported elsewhere [5].

3. Discussion

The present study shows that 4-aminophenyl 1-thio-β-D-galactofuranoside is a suitable ligand for affinity chromatography and that D-galactono-1,4-lactone is an adequate compound for elution of the exo-β-D-galactofuranosidase bound to the column, as previously suggested [5]. Elution with the lactone only was subject to unpredictable behavior, probably because of differences of affinity to the ligand in the enzyme population. The introduction of a salt gradient together with the lactone gave more reproducible results and showed the existence of at least two pools of enzyme isoforms. Purification of exo-β-D-galactofuranosidase from *P. fellutanum* has been previously achieved by a combination of

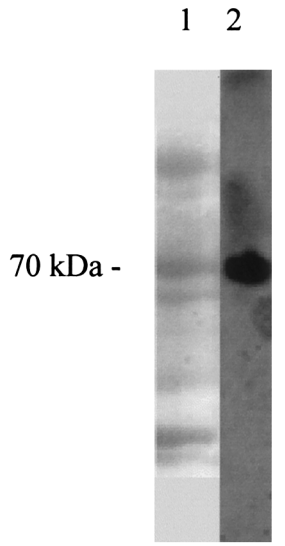

Fig. 6. Western blot of the β-D-galactofuranosidase with the polyclonal antibody. The concentrated culture supernatant was subjected to electrophoresis and transferred to a nitrocellulose membrane as described in Section 4. Lane 1 was stained with 0.2% Ponceau; lane 2 was incubated with the antibody and developed as described in Section 4.

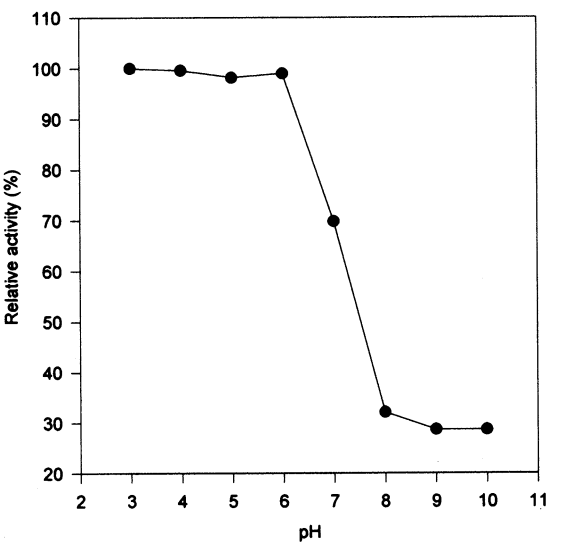

Fig. 7. Effect of pH on the enzyme activity. The enzyme was assayed under standard conditions in 66 mM buffers varying from pH 3.0 to 10.0. Buffers: (pH 3–5) acetate; (pH 6–7) phosphate; (pH 8–10) borate.

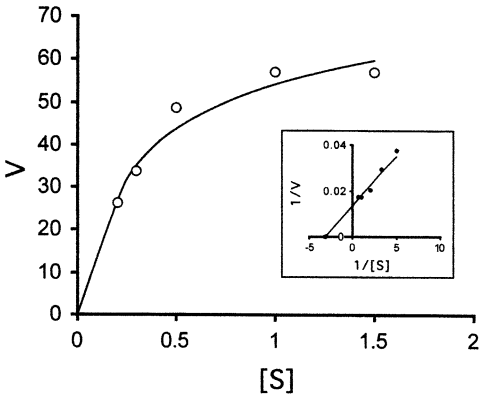

Fig. 8. Plot of V in μmol h^{-1} vs. [S] (mM) of β-D-galactofuranosidase from *P. fellutanum*. Assay conditions: 0.5 mL and 8.5 μg of enzyme protein. Inset: Lineweaver–Burk reciprocal plot.

different purification techniques, including an affinity chromatography step on Sepharose 4B–peptidophosphogalactomannan. In this case, the detection of three major bands was reported after the column step [2]. Despite the existence of two peaks of enzyme activity released by the affinity column, our results show that only one 70 kDa polypeptide was detected by SDS-PAGE after the 4-aminophenyl 1-thio-β-D-galactofuranoside affinity chromatography. Although this molecular mass is in the same range as the 77 kDa described for the endo-(1 → 5)-β-D-galactofuranosidase of *P. oxalicum* [6] and the 67 kDa for the endo-β-galactofuranosidase from *Bacillus* sp. [7], these are different enzymes since Rietschel–Berst et al. [2] observed that the β-D-galactofuranosidase from *P. fellutanum* has no endo-galactofuranosidase activity. The exo-β-D-galactofuranosidase activity of the enzyme was recently confirmed with the hydrolysis of the synthetic disaccharide β-D-Gal*f*-(1 → 3)-α-D-Man*p* [8]. The 150 kDa molecular mass for the enzyme subjected to non-denaturing conditions suggests that the molecule is a dimer. The glycoprotein nature of the purified enzyme was confirmed by Schiff staining and binding to Concanavalin A–Sepharose. In addition, 17% total carbohydrate content was estimated in relation to the protein. The purified enzyme has similar optimal temperature (not shown) and pH (acidic), as previously reported [2].

The adoption of the affinity-chromatography step using a specific ligand for the purification of β-D-galactofuranosidase requires less manipulation and, together with the availability of a specific antibody, may facilitate the detection and purification of equivalent enzymes. This is especially critical when small amounts of starting material are available, as is frequently the case with pathogenic organisms.

4. Experimental

Culture of P. fellutanum.—An inoculum of *P. fellutanum* Biouge was kindly provided by Dr J.E. Gander (University of Florida, USA) and maintained in CzapeK-Dox agar medium [9,10]. Cultures were obtained by inoculating spores on 500 mL of Raulin–Thom medium containing 5% D-glucose [2], kept at 24 °C under constant light and vigorously shaken on a New Brunswick Environmental Controlled Shaker at 200 rpm. On day 3, the medium was supplemented with 7.5 mM $(NH_4)_2CO_3$, as recommended [11].

Enzyme assays.—The substrate 4-nitrophenyl β-D-galactofuranoside was prepared by the method previously reported [12], and modified as described for the synthesis of other galactofuranosyl glycosides [13].

exo-β-D-Galactofuranosidase activity was measured using 62 μL of 5 mM 4-nitrophenyl β-D-galactofuranoside, 100 μl of 66 mM acetate buffer, pH 4.0 and the enzyme source in a final volume of 0.5 ml, as previously described [5]. After 1 h at 37 °C, the reaction was stopped by the addition of 0.25 M Na_2CO_3–0.25 M $NaHCO_3$ (1 ml) and 4-nitrophenol was estimated by the absorbance at 410 nm. The molar extinction coefficient used for unit calculation was 17,800. K_m and V_{max} values were determined by Lineweaver–Burk plots. One unit of activity was defined as the amount of *p*-nitrophenol formed per h per μg of protein at 37 °C.

Preparation of the 4-aminophenyl 1-thio-β-D-galactofuranoside affinity column.—4-Aminophenyl 1-thio-β-D-galactofuranoside [5] was coupled to CH-Sepharose 4B (Pharmacia Fine Chemicals) by the carbodiimide procedure. A total of 1 g of the solid phase, previously swollen as recommended by the

manufacturer, was mixed with 60 mg of 4-aminophenyl 1-thio-β-D-galactofuranoside dissolved in 5 mL of water, followed by the addition of 0.4 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The suspension was stirred gently for 24 h at 4 °C and the unreacted sites in the gel were blocked as recommended by the supplier. The gel was then packed into a column and equilibrated with 66 mM acetate buffer, pH 4.0, before use. The amount of ligand bound to the resin was ~6 μmol/mL of swollen resin, as estimated by the chromotropic acid method [14].

Isolation and purification of exo-β-D-galactofuranosidase.—The presence of exo-β-D-galactofuranosidase was detected in the culture filtrate after 10 days of incubation, with the highest activity observed around day 20 as previously described [2]. The supernatant of the *P. fellutanum* culture (pH 4.5) was initially filtered through Whatman no. 1 paper (30 min, rt) and concentrated 10-fold on Amicon using a P10 membrane (cutoff 12,000–14,000 molecular mass) at 25 °C during ~4 h. After extensive dialysis against distilled water (final pH 5.2), the material was loaded onto a DEAE–Sepharose CL 6B column and the enzyme was eluted with a linear gradient of 0–0.5 M NaCl in 10 mM acetate buffer, pH 5.0. The active fractions were pooled and dialyzed against 66 mM acetate buffer, pH 5.0. The dialyzate was applied on a column (1 mL) of 4-aminophenyl 1-thio-β-D-galactofuranoside–Sepharose, washed with 20 mL of 0.1 M NaCl in 66 mM acetate buffer, pH 5.0 and eluted with 10 mM D-galactono-1,4-lactone in a 100–500 mM NaCl gradient. The active fraction was dialyzed in 66 mM acetate buffer, pH 5.0, and concentrated on Amicon model 8200 using a P10 membrane.

SDS-PAGE and Western blot analysis.—Samples were resuspended in sample buffer containing 6 M urea, separated on 10% SDS-PAGE [15] and stained alternatively with $AgNO_3$ [16], Coomassie Brilliant Blue R-250 [17], or Schiff reagent [18]. Proteins were transferred to nitrocellulose membranes, which were incubated overnight at 4 °C with anti-galactofuranosidase antibody (1:200) and developed by a chemiluminescent method (ECL, Amersham Pharmacia Biotech).

Antibody preparation.—Antibodies were raised in mice by inoculating the 70 kDa band excised from gels slightly stained with Coomassie Blue in which the affinity-purified enzyme had been subjected to electrophoresis. The excised band (1–2 μg, as estimated by the staining intensity) was washed with water, resuspended in phosphate-buffered saline (PBS), pH 7.2, and homogenized in a Potter vessel. The soluble material was mixed with an equal volume of Freund's adjuvant and 0.2 mL aliquots were inoculated into mice. Serum was collected after four weekly intraperitoneal inoculations of the same amount of antigen mixed with incomplete Freund's adjuvant.

Immunofluorescence.—Indirect immunofluorescence was performed with 4% paraformaldehyde-fixed fungi. After 30 min at rt, the fungi were washed with PBS and added to slides. The slides were air dried, flooded in PBS (2 × 5 min) and incubated subsequently with β-D-galactofuranosidase antibodies (1/200 in PBS) or with an unrelated antibody, followed by FITC-anti-mouse antibodies (Sigma). After each antibody incubation (30 min, 37 °C), the slides were washed with PBS (3-fold, 5 min).

Immunoprecipitation.—Immunoprecipitation of the affinity-purified enzyme was performed by incubating 7 μg of the enzyme with 40 μL of immune serum and 50 μL of protein A–Sepharose in 200 μL of PBS. Unrelated sera or PBS were used as controls. After 18 h at 4 °C, the immunocomplexes bound to the resin were centrifuged and washed with PBS (3 × 1 mL) and resuspended in 66 mM acetate buffer, pH 4.0, in order to measure the enzyme activity.

Lectin affinity chromatography.—Concanavalin A–Sepharose (Pharmacia Fine Chemicals) was packed in a 1-mL column and extensively washed with 500 mM NaCl in 50 mM Tris–HCl, pH 7.4, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. Three units of enzymatic activity were incubated with the resin (1 h at rt), washed with 30 mL of the same buffer and the bound material eluted with 10 mL of 0.1 M methyl α-D-glucopyranoside and 0.1 M methyl α-D-mannopyranoside. Fractions of 1 mL were collected and 100 μL of each fraction was employed for enzyme activity determination.

Protein and carbohydrate determination.— Protein concentration was determined by the method of Bradford [19] or estimated by spectrometry at 280 nm. The amount of neutral carbohydrate was measured by the phenol–H_2SO_4 method [20], using glucose as standard.

Acknowledgements

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