Isolation of chitin deacetylase from *Mucor rouxii* by immunoaffinity chromatography

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ABSTRACT

The purification of chitin deacetylase from *Mucor rouxii* to homogeneity employing conventional methods has already been described. However, a lengthy protocol is required resulting in a low yield and specific activity for the enzyme. A 169-fold one-step purification of chitin deacetylase by immunoaffinity chromatography is reported, resulting in a homogeneous enzyme preparation. The enzyme purified using this procedure was judged to be electrophoretically homogeneous as tested by both native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate PAGE. Using antibodies of lower affinity, less severe chemical conditions were required for the desorption of immunoadsorbents. Chitin deacetylase purified by immunoaffinity chromatography exhibited a specific activity of 13 U mg^{-1} while a 30% yield was obtained, both much higher than the respective values obtained using conventional methodology.

INTRODUCTION

Chitin, a polymer of N-acetyglucosamine and the second most abundant polymer in nature after cellulose, occurs widely in the exoskeletons of many invertebrates and in the cell walls of most fungi and some algae [1,2]. Chitin is an insoluble material and has limited industrial use, whereas chitosan, a partially deacetylated form of chitin, occurring in several Zygomycetes species [3,4], is water soluble and has a large number and a wide variety of important applications [5–7]. Chitosan is produced by the thermochemical deacetylation of chitin, which leads to a highly heterogeneous end-product owing to the severity of the treatment [8]. An alternative

or complementary procedure exploiting the enzymatic deacetylation of chitin could potentially be employed, especially when a controlled, nondegradative and well defined process is required.

Chitin deacetylase (CDA) catalyses the conversion of chitin to chitosan by deacetylating N-acetylglucosamine residues. It was first identified and partially purified from extracts of the fungus *Mucor rouxii* [9]. Since then, a similar enzyme activity has been reported in several other fungi [10,11] and in some insect species [12].

Chitin deacetylase from *Mucor rouxii* has been purified to homogeneity and further characterized by employing conventional chromatographic procedures [13]. However, conventional methods require the use of a series of purification steps, which is time consuming and results in low yield and specific activity for the enzyme.

Although there are some fundamental limitations, immunoaffinity chromatography has been used increasingly and successfully for enzyme

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purification [14]. A main drawback of this technique is that the deforming agents usually required for desorption often denature and inactivate labile proteins. However, low-affinity antibodies require less severe desorption conditions. A review of the literature suggests that a proper choice of intermediate affinity antibodies for the preparation of an immunoaffinity column can lead to high yields and rapid purification procedures for enzymes [14,15].

This paper describes the isolation of chitin deacetylase from *Mucor rouxii* to homogeneity employing immunoaffinity chromatography in a one-step procedure.

EXPERIMENTAL

Materials

Mucor rouxii (ATCC 24905) was obtained from American Type Culture Collection. Glycol chitosan and glass beads (425–600 μm) were purchased from Sigma, [^{3}H]acetic anhydride from DuPont New England Nuclear and enzymes and reagents for acetic acid determination from Boehringer Mannheim Biochemica. All chromatographic media (Phenyl Sepharose CL-4B, Q Sepharose Fast Flow, S Sepharose Fast Flow, cyanogen bromide-activated Sepharose) and molecular mass markers were obtained from Pharmacia and ultrafiltration membranes from Amicon. Chitohexaose was purchased from BioCarb, enzyme-linked immunosorbent assay (ELISA) microtitre plates from Nunc and anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase from NIDA Biotechnology. All other chemicals were of the highest purity commercially available.

Enzyme assays

We employed two different assays for the determination of chitin deacetylase activity, as follows.

(I) Chitin deacetylase activity was determined using as the substrate partially O-hydroxyethylated chitin (glycol chitin), radiolabelled in the N-acetyl groups. The substrate was prepared according to Araki and Ito [9] from glycol chitosan and [^{3}H]acetic anhydride. Enzyme assays were performed as described previously [9], with the following modifications: 25 mM sodium glutamate (pH 4.5) was used as the buffer and BSA (0.1 mg ml^{-1}) was added to the assay mixture. The incubation time was 10 min at 50°C.

(II) Acetic acid released by the action of chitin deacetylase on various chitinous substrates was determined by the enzymatic method of Bergmeyer [16] via three coupled enzyme reactions. Units of enzyme activity were determined using 166 nmol of chitohexaose in a total volume of 500 μl buffered with 25 mM sodium glutamate (pH 4.5). The incubation time was 10 min at 50°C and the reaction was terminated by heating at 100°C prior to acetic acid determination.

Protein assay and electrophoretic techniques

Protein content was determined by the method of Lowry et al. [17] using bovine serum albumin as a standard. Slab-gel electrophoresis using gradient or homogeneous polyacrylamide gels and discontinuous buffer systems under denaturing and reducing conditions were performed as described by Laemmli [18]. Non-denaturing polyacrylamide gels were prepared by the method of Davis [19]. Protein markers were α-lactalbumin ($M_r = 14\,400$), soybean trypsin inhibitor (20 100) carbonic anhydrase (30 000), ovalbumin (43 000), bovine serum albumin (67 000) and β-phosphorylase (94 000). Protein bands were revealed by staining with Coomassie Brilliant Blue R.

Production of antibodies

An adult male white New Zealand rabbit was immunized with 500 μg of CDA [1 mg/ml in 0.010 M phosphate-buffered saline (PBS) containing 0.15 M NaCl (pH 7.4)], purified from the fungus *Mucor rouxii*, emulsified with an equal volume of Freund's complete adjuvant in a total volume of 1 ml. It was administered intradermally as described previously [20]. Further booster doses of 150 μg of CDA emulsified in Freund's incomplete adjuvant were administered at 4-week intervals. Test bleeds from the marginal ear vein were used to monitor the serum anti-

body titre by ELISA. The blood was allowed to clot at 37°C for 1 h and then stored at 0°C overnight. After the blood clot had been removed, the rabbit sera were aliquoted and stored at −20°C. Control serum was taken prior to immunization.

The antibody titre was monitored using a non-competitive ELISA [21]. CDA was immobilized on flat-bottomed microtitre plates at 2 μg ml^{-1} of buffer containing 0.05 M sodium carbonate and sodium hydrogencarbonate (pH 9.6) by incubation overnight at 4°C. The wells were washed with a 0.05% aqueous solution of Tween 20, followed by two washes with distilled water. Subsequently, 200 μl of blocking agent solution per well were incubated for 1 h at room temperature. The blocking agent consisted of 1 g of bovine serum albumin dissolved in 100 ml of 0.010 M PBS containing 0.15 M NaCl (pH 7.4). The wells were washed as described above. An anti-rabbit IgG antiserum conjugated to horse-radish peroxidase was used to detect indirectly specific IgG bound to immobilized CDA. The conjugate was diluted 10 000-fold in 0.010 M PBS containing 0.15 M NaCl (pH 7.4) and incubated at 100 μl per well for 1 h at room temperature. The wells were washed with aque-ous Tween 20 solution as described above, followed by two washes with distilled water. The wells were aspirated and incubated with 100 μl of substrate–chromogen solution made up just prior to use as follows: 3 ml of substrate [0.08% (v/v) hydrogen peroxide solution] were mixed with 12 ml of 0.25 mM chromogen (3,3',5,5'-tetramethylbenzidine–HCl) and the reaction was stopped after 15 min by addition of 50 μl of 4 M sulphuric acid per well. The absorbance was read at 450 nm using an ELISA reader (Titertek, Multiskan Plus).

The concentration of antibody in solution was determined by measuring the absorbance at 280 nm using an average molar absorptivity for antibodies of 1.45 l mol^{-1} cm^{-1} for a solution of 1 mg ml^{-1} protein using a 1-cm path-length cell [22].

The enzyme activity of a defined amount of purified CDA (control activity 0.45 mU μl^{-1}) was measured after incubation with various amounts of antiserum [23]. The difference be-tween the measured enzyme activity and the control activity was expressed as percentage inhibition of the control activity.

Purification of IgG

CDA was purified to homogeneity from mycelial extracts of the fungus *Mucor rouxii* as described previously [13]. The mycelial extract was subjected to 52% ammonium sulphate frac-tionation and 50°C heat treatment and sub-sequently chromatographed on Phenyl-Sepha-rose CL-4B, Q-Sepharose Fast-Flow and S-Sepharose Fast-Flow.

Specific anti-CDA IgG was affinity purified from rabbit serum using a CDA–Sepharose-4B adsorbent. CDA was immobilized on cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions, resulting in 1.3 mg of CDA immobilized per ml of gel (90% cou-pling efficiency).

Rabbit antiserum against CDA obtained from two bleedings was subjected to 40% ammonium sulphate fractionation. The immunoglobulin-con-taining precipitate was dissolved and dialysed extensively against 0.025 M Tris–HCl–0.2 M sodium chloride (pH 7.4) and subsequently loaded on to the CDA-linked Sepharose 4B column (2.2 × 2 cm I.D.; 7.5 ml). The adsorbent was initially washed with ten column volumes of 0.025 M Tris–HCl–0.2 M NaCl (pH 7.4) and subsequently with two column volumes of 0.025 M Tris–HCl–1 M NaCl (pH 7.4) in order to remove nonspecifically bound proteins. A batch of IgG was eluted with two column volumes of 0.2 M glycine–HCl buffer (pH 2.8). A further batch of higher affinity IgG was eluted with two column volumes of 0.2 M glycine–HCl buffer (pH 2.2) (data not shown). All fractions were immediately adjusted to pH 7.0 with 1 M Tris–HCl (pH 9.0). The two populations of IgG fractions were pooled separately and concen-trated by ultrafiltration, prior to dialysis against 0.025 M Tris–HCl (pH 7.4). The purified specific IgG shows the characteristic rabbit IgG pattern in sodium dodecyl sulphate–poly-acrylamide gel electrophoresis (SDS-PAGE) (data not shown). Pure specific IgG was stored at −20°C at a concentration >1 mg ml^{-1} in 0.010 M Tris–HCl–0.1 M sodium chloride (pH 7.4).

Purification of CDA

The immunoadsorbent was prepared as follows. Pure lower affinity IgG (5.9 mg) was dialysed against buffer containing 0.1 M sodium hydrogencarbonate and 0.5 M sodium chloride pH 8.3 and mixed with preswollen cyanogen bromide-activated Sepharose 4B (5 ml) and subsequently immobilized according to the manufacturer's instructions. The ligand concentration was estimated to be 1 mg IgG per ml gel (85% coupling efficiency).

Frozen mycelia (2 g) were thawed, minced and homogenized in 10 ml of 0.05 M Tris–HCl buffer (pH 7.4) containing 0.5 mM phenylmethylsulphonyl fluoride, 0.1 mM N-ethylmaleimide and 150 mM NaCl, using an improvised glass-bead miller (2 g of glass beads per gram of wet mycelia). All steps were performed at 4°C. The resulting homogenate was centrifuged at 15 000 g for 30 min at 4°C. The supernatant (12.2 ml; 4.6 mg ml^{-1}; 56.0 mg) was then incubated in a water-bath set at 50°C for 15 min and rapidly cooled in ice. Precipitated protein was removed by centrifugation at 136 000 g for 45 min at 4°C. The supernatant (11.5 ml; 0.54 mg ml^{-1}; 6.2 mg) was loaded on to the immunoadsorbent (2 × 1.6 cm I.D.; 5 ml) previously equilibrated in 25 mM Tris–HCl buffer (pH 7.4) containing 150 mM NaCl (buffer A). The column was washed with buffer A (50 ml) and subsequently with 25 mM Tris–HCl (pH 7.4)–1 M NaCl (10 ml) in order to remove non-specifically bound proteins. CDA was eluted using 0.2 M glycine–HCl buffer (pH 2.8) at a flow-rate of 35 ml h^{-1}. The eluate was immediately adjusted to pH 7.0 with 1 M Tris–HCl (pH 9.0), dialysed against buffer A and concentrated by ultrafiltration (300 μl; 40 μg ml^{-1}; 12 μg; 160 mU).

RESULTS AND DISCUSSION

Chitosan is produced from chitin by thermochemical deacetylation. This process results in a polymer product having a broad distribution of molecular mass and a heterogeneous extent of deacetylation. However, for many potentially important applications, uniform material with specific physical and chemical properties is required. The development of a controllable

process, employing the enzymatic deacetylation of chitinous substrates, presents an attractive alternative as degradation of chitin is prevented and control of the degree of deacetylation can be effected after optimization of the enzymatic reaction.

The purification of chitin deacetylase from *Mucor rouxii* to homogeneity employing conventional purification methodology has been reported [13]. The enzyme is a high-mannose glycoprotein and exists as a monomer with an apparent molecular mass of 75 000–80 000. However, this purification scheme is time consuming and results in low yields (11.8%) and specific activity (2.9 U mg^{-1}) for the enzyme, which is a limiting factor for large-scale applications.
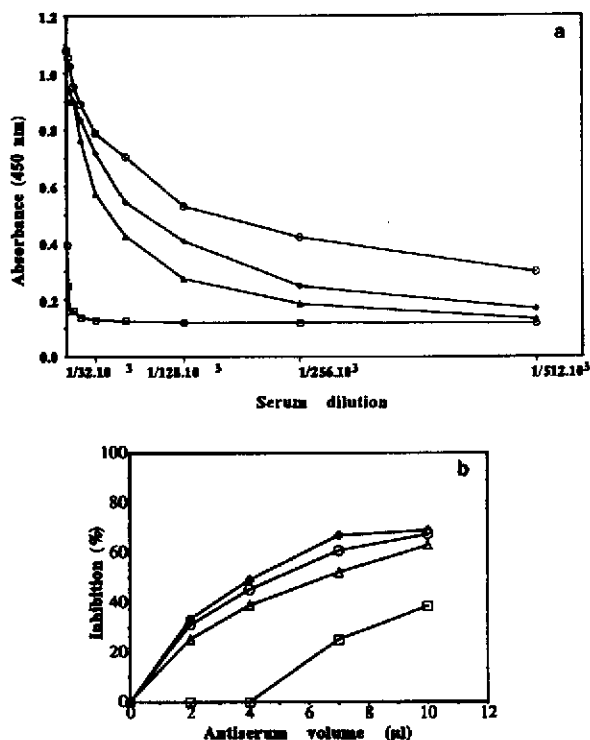

Fig. 1. Determination of the titre of the antiserum. Antisera were obtained at (□) 0 (normal serum), (○) 6, (◇) 23 and (△) 40 weeks after priming. (a) By a non-competitive ELISA: titre was determined at the serum dilution used to obtain half of the maximum absorbance value observed. (b) By immunoinhibition of chitin deacetylase (control activity: 0.45 mU ml^{-1}), after incubation with various amounts of anti-CDA immune sera. The difference between the measured enzyme activity and control activity was expressed as percentage inhibition of the control activity.

In this study, immunoaffinity chromatography was examined as an alternative purification procedure for the rapid isolation of the enzyme in large amounts. We immunized a rabbit with a purified antigen preparation in order to obtain specific antibodies of the enzyme for the development of an immunoaffinity column. The titre of the antisera obtained was exceptionally good, as evidenced by non-competitive ELISA studies (Fig. 1a), probably owing to the glycosylated nature of the enzyme [13]. As the antiserum titre as determined by ELISA is high (>1:50 000 against 800 for normal serum), it is expected that a fraction of the specific antibodies will recognize the epitope at or near the active site of the enzyme (Fig. 1b). The antisera obtained showed no detectable cross-reactivity against other proteins contained in the crude extract of *Mucor rouxii*.

Employing immunoaffinity chromatography, a homogeneous enzyme preparation could be obtained within a day (Figs. 2 and 3). The enzyme purified with this procedure was judged to be electrophoretically homogeneous, as tested by both native PAGE and SDS-PAGE. Enzyme

Fig. 3. Denaturing PAGE of the eluate containing chitin deacetylase activity and obtained from the immunoadsorbent. Lanes: 1 = crude protein mixture obtained from *Mucor rouxii* strain after cell lysis (150 μg); 2 = protein mixture obtained after 50°C treatment (150 μg); 3 = fractions with chitin deacetylase activity eluted from the immunoadsorbent (15 μg); M = molecular mass markers (molecular masses are given on the right).
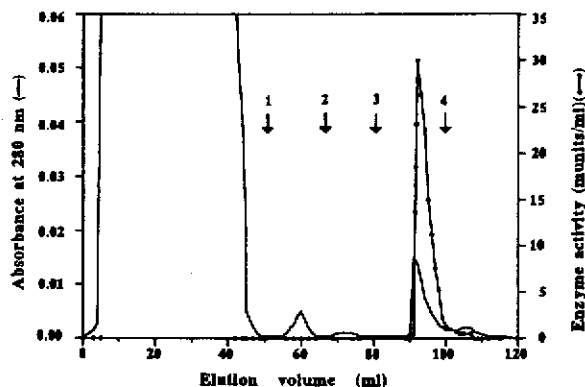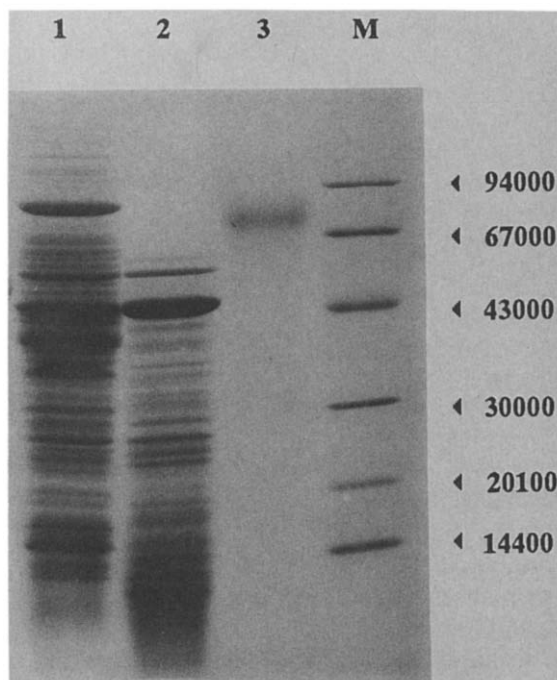

Fig. 2. Purification of chitin deacetylase by immunoaffinity chromatography. A sample (11.5 ml, 6.2 mg; 0.49 U) of partially purified chitin deacetylase was applied to an immunoadsorbent (2 × 1.6 cm I.D.; 5 ml) previously equilibrated in buffer A. The column was washed with buffer A and subsequently with buffer A containing 1 M NaCl (1), followed by buffer A (2), 0.2 M Glycine–HCl (pH 2.8) (3) and finally with 25 mM Tris–HCl (pH 7.4) (4). Fractions (1 ml) with chitin deacetylase activity were collected during the 0.2 M glycine–HCl (pH 2.8) wash. The protein content was followed by UV spectrophotometry at 280 nm.

activity assays using the gel slices obtained from gradient (5–20%) native PAGE exhibited chitin deacetylase activity coinciding with the detected protein band. Purified chitin deacetylase subjected to gel filtration on Sephacryl S-200 HR was eluted as a single peak exhibiting chitin deacetylase activity. Purified enzyme preparations exhibited a specific activity of 13.3 U mg^{-1} while a 30% overall yield was obtained (Table I), which represents increases of four- and threefold, respectively, in comparison with the previously reported purification scheme [13]. Further, employing conventional methodology for the isolation of the enzyme, traces of chitinase activity were detected which could degrade the polymer substrate (chitin). In contrast, enzyme preparations obtained from the immunoadsorbent were free of chitinase activity. The immunoadsorbent could be used repeatedly

TABLE I

SUMMARY OF THE PURIFICATION PROTOCOL FOR CHITIN DEACETYLASE

Step	Total protein (mg)	Enzyme activity (U)[a]	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude extract	56.000	0.55	0.010	1.0	100.0
50°C treatment	6.200	0.49	0.079	7.9	89.0
Immunoadsorbent	0.012	0.16	13.333	1333.0	29.1

[a] One unit of the enzyme activity is defined as the amount of the enzyme required to produce 1 μmol of acetic acid per minute when incubated with 16.6 nmol of chitohexaose under optimum pH (4.5) and temperature (50°C) conditions.

more than twenty times to isolate chitin deacetylase without any decrease in its enzyme-purifying ability. The successful isolation of the enzyme is mainly due to the effectiveness of the immunoadsorbent where the enzyme was enriched 169-fold (Table I).

The binding capacity of the CDA–Sepharose 4B for anti-CDA antibodies was determined to be *ca.* 1.2 mg of IgG per mg of immobilized CDA. This high binding capacity is due to the fact that a number of IgG molecules can bind to a CDA molecule depending on antigenic valency.

The capacity of the immunoadsorbent used in this study, although not optimized, was determined to be higher than 42 μg of chitin deacetylase per ml of immunoadsorbent (4% of the theoretical maximum capacity). The maximum theoretical binding capacity of the immunoadsorbent occurs when both binding sites of each immobilized antibody molecule bind a molecule of CDA. Assuming that the molecular masses of CDA and IgG are 80 000 and 160 000, respectively, 1 mg of immobilized IgG could bind 1 mg of CDA. However, in practice only a small percentage of antigen binding sites remain available for binding the antigen after covalent immobilization on a matrix.

After isolation and subsequent immobilization of lower affinity antibodies, less severe chemical conditions were required for the desorption of the immunoadsorbent. Two separate batches of IgG were eluted from the CDA–Sepharose 4B column. The first batch was eluted with 0.2 *M* glycine–HCl buffer (pH 2.8) and the second

batch with 0.2 *M* glycine–HCl buffer (pH 2.2). As elution of the immunoadsorbent at pH 2.2 represents a more drastic procedure compared with the pH 2.8 elution procedure, IgG fractions eluted with 0.2 *M* glycine–HCl (pH 2.8) (lower affinity IgG) were used for the preparation of the immunoadsorbent. An immunoadsorbent having immobilized specific IgG instead of crude polyclonal antibody was employed in order to increase the specificity of the adsorbent for the enzyme. It is expected that the half-life of the immunoadsorbent having immobilized lower affinity antibodies will be increased by preventing irreversible binding of chitin deacetylase to higher affinity IgG after successive chitin deacetylase purifications [the term "affinity" used in the text refers to the functional affinity (avidity) as polyclonal antibodies are used].

The same immunoadsorbent could potentially be employed for the isolation of chitin deacetylases from other Zygomycetes species, as an inter-species homology has been observed by Western blot assays (data not shown). In this way, a comparative study of chitin deacetylases from various sources can be performed.

During the isolation of the enzyme by conventional methods, a second chitin deacetylase activity of molecular mass 38 000 was identified, which is more likely a degradation product of full-length chitin deacetylase [13]. Employing immunoaffinity chromatography only one form of the enzyme could be identified, of molecular mass 75 000–80 000, suggesting that degradation products of the protein can be avoided using this rapid purification scheme.

This study has demonstrated the effectiveness of immunoaffinity chromatography for the isolation of chitin deacetylase from *Mucor rouxii* in a one-step procedure. Bearing in mind the potential application of the enzyme in large-scale deacetylation processes, we are presently focusing on the evaluation of other enzyme sources for the identification of the best producer species, overproduction of the enzyme in a suitable system after cloning the gene from a cDNA library we have constructed and optimization of substrate pretreatment requirements.

CONCLUSIONS

Chitin deacetylase from *Mucor rouxii* has been purified to homogeneity in a one-step procedure employing immunoaffinity chromatography. Using antibodies of lower affinity, less severe chemical conditions were required for the desorption of immunoadsorbents. A 169-fold purification of the enzyme was achieved while purified enzyme preparations exhibited a specific activity of 13 U mg^{-1} and a 30% yield was obtained.

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