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Note

Purification of an α-L-arabinofuranosidase from *Aspergillus niger* by substrate affinity chromatography

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Arabinan-splitting enzymes, arabanases and arabinosidases, have received much attention in connection with structural studies of hemicelluloses and also maceration studies of plant tissues, as arabinose containing hemicelluloses are known to be integral components of most of the plant cell walls[1]. α-L-Arabinofuranosidase (E.C. 3.2.1.55) has been shown to be of interest in connection with a new concept of cancer chemotherapy[2].

For all such purposes, highly purified enzymes are required. Various methods for the purification of the arabinan-degrading enzymes have been reported[3]. Different separation techniques were used to purify the enzymes, such as ion-exchange chromatography[4], chromatography on hydroxyapatite[5] and gel chromatography[6]. In all instances a high degree of purification could be achieved only by using multi-step separations, which led to the recovery of extremely low yields of pure enzyme.

This paper describes an affinity chromatography technique that allows the preparation of highly purified α-L-arabinofuranosidase by using either arabinan cross-linked by epichlorohydrin or arabinan linked to epoxy-activated Sepharose 6B via ether linkages as affinity carriers. These specific insoluble adsorbents allow a selective single-step separation of this enzyme from a mixture of extracellular enzymes of *Aspergillus niger* preparations.

EXPERIMENTAL

Enzyme preparations

Two commercial *Aspergillus niger* enzyme preparations, Pectinol R 10 (Röhm & Haas, Darmstadt, G.F.R.) and Ultrazym 20 (Ciba-Geigy, Basel, Switzerland), were used.

Crude enzymes were prepared by dissolving Pectinol and Ultrazym in cold, distilled water, filtering and freeze-drying.

Partially purified enzymes were obtained after precipitation of the crude enzymes with methanol, redissolution in cold water and reprecipitation in 40–70% ammonium sulphate. The precipitate was dialysed against water and freeze-dried.

Substrates

The following substrates were used.

(a) Nitrophenyl derivatives of monosacharides. The α-L-arabinofuranoside

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and -pyranoside derivatives were prepared according to Fielding and Hough[7] and the β-D-mannopyranoside derivative according to Garegg and Iversen[8]. All other derivatives were commercial products (Sigma, St. Louis, MO, U.S.A.).

(b) The arabinan was either prepared from sugar beet chips by extraction with hot lime water[9] or was a commercial product (Koch-Light Labs., Colnbrook Great Britain). The polysaccharides were purified by ion-exchange chromatography on DEAE-Sephacel (Pharmacia, Uppsala, Sweden) in the borate[10] or the hydroxide form[11].

(c) Sucrose, soluble starch, pectin (degree of esterification 28%), sodium pectate, O-carboxymethylxylan, O-carboxymethylcellulose and galactomannans (guar gum and locust bean gum) were commercial preparations and were used without further purification.

Affinity carriers

(a) Epoxy-activated Sepharose 6B and arabinan were cross-linked according to the procedure of Sundberg and Porath[12]. A 3-g amount of freeze-dried epoxy-activated Sepharose 6B (Pharmacia) was mixed with 400 mg of purified arabinan in 30 ml of 0.01 *N* sodium hydroxide solution (pH 12) for 24 h at 40°C. Excess active groups were blocked by leaving the gel overnight in 1 *M* ethanolamine solution. The gel was washed with bicarbonate buffer (pH 8.0) and finally with acetate buffer (pH 4.0). Before use, the affinity carrier was washed with about 10 volumes of 0.01 *M* phosphate buffer (pH 7.0) containing 0.5 *M* potassium chloride.

(b) Arabinan cross-linked by epichlorohydrin was prepared by the method of Vijayalakshmi *et al.*[13]. A 0.5-ml volume of epichlorohydrin and 2 ml of 30% sodium hydroxide solution were added with vigorous stirring to 1 g of dry arabinan powder. The reaction mixture was stirred for 4 h at room temperature and then neutralized with 15 ml of 1 *N* acetic acid. The gelled arabinan was filtered, washed with water and alcohol, resuspended in water and finally freeze-dried. By this procedure a more porous structure of the gel could be obtained. Before use, the affinity carrier was washed with 10 volumes of 0.01 *M* phosphate buffer (pH 7.0) containing 0.5 *M* potassium chloride.

Enzyme assays

Glycanase activities. A 0.5-ml volume of a 2% (w/v) solution of the different polysaccharides was incubated with 0.1 ml of enzyme solution (crude or partially purified enzyme or fractions from the affinity chromatography) for 10 min at 40°C. The reaction was stopped by adding 5 ml of *p*-hydroxybenzoic acid hydrazide reagent (PAHBAH) to the incubation medium. The polysaccharide splitting activities of the enzymes were determined by measuring the increase in reducing sugar end-groups with the aid of the PAHBAH method[14,15].

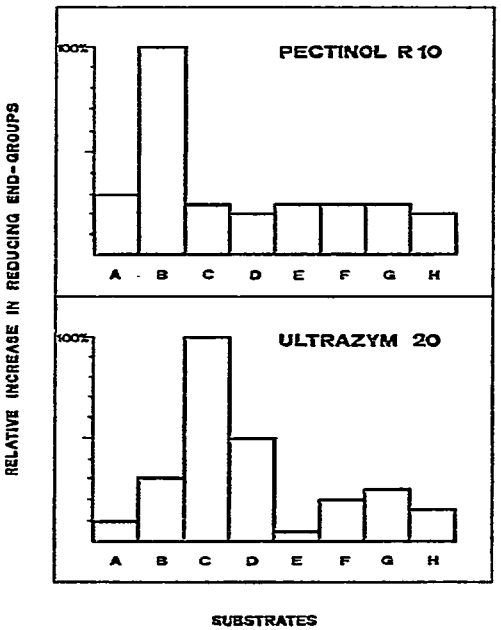
Glycosidase activities. By using the appropriate nitrophenyl sugar derivative as a substrate, the glycosidase activities of the different enzyme fractions were measured[16]. A 0.2-ml volume of substrate solution (25 mg of sugar derivative dissolved in 100 ml of 0.1 *M* buffer, pH 4.0) were incubated with 0.1 ml of enzyme preparation for 10 min at 30°C. The reaction was stopped by adding 0.2 ml of 1 *M* sodium carbonate solution. The concentration of the liberated nitrophenol was determined by measuring the absorbance at 405 nm.

Affinity chromatography of Pectinol R-10 and Ultrazym

Samples of 2–4 mg of crude or partially purified enzyme solutions were applied to the two different affinity carriers in 0.2 ml of 0.01 *M* phosphate buffer (pH 7.0) containing 0.5 M potassium chloride (buffer A). The column (7.5 × 0.9 cm) was first eluted at 2°C with buffer A at the rate of 1.5 ml/h (fractions of 1.2 ml were collected). The adsorbed enzyme α-L-arabinofuranosidase was subsequently eluted from the column with either 0.2% of soluble arabinan in buffer A or 0.2% of *p*-nitrophenyl-α-L-arabinofuranoside in buffer A (= buffer B).

RESULTS AND DISCUSSION

Fig. 1 shows the enzymic activities of the partially purified Pectinol R 10 and Ultrazym 20 on different polysaccharides and sucrose. It is interesting that the activity patterns of the two commercial preparations differ considerably even though both are produced by *Aspergillus niger* cultures. Similar observations were made for the glycosidase activities of the two enzyme preparations (Fig. 2). Presumably Pectinol R 10 and Ultrazym 20 are obtained in slightly different ways, using different substrates for the induction of the extracellular glycanases and glycosidases of *A. niger*.



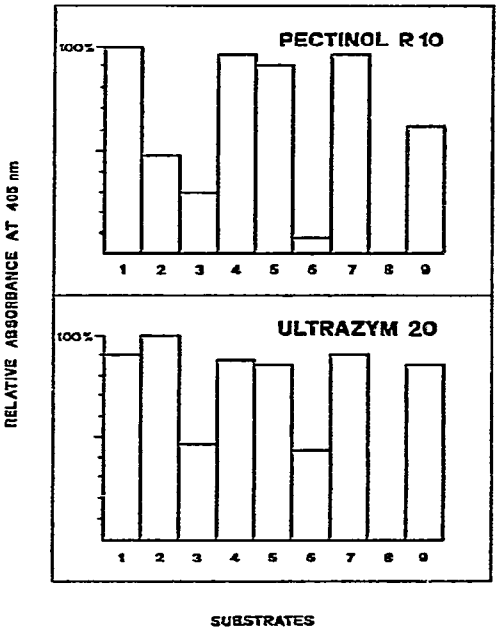


Fig. 1. Polysaccharide-degrading activities in the starting materials: A = pectin; B = sodium pectate; C = CM-xylan; D = CM-cellulose; E = arabinan; F = galactomannan; G = sucrose; H = soluble starch.

Fig. 2. Nitrophenylglycoside-splitting activities in the starting materials: 1 = α-L-Araf; 2 = α-L-Arap; 3 = β-D-Xylp; 4 = α-D-Galp; 5 = β-D-Galp; 6 = α-D-Glcp; 7 = β-D-Glcp; 8 = α-D-Manp; 9 = β-D-Manp.

Using substrate affinity chromatography it was possible to isolate and purify an

α-L-arabinofuranosidase from the complex enzyme mixture. Both affinity carriers under investigation (cross-linked arabinan and epoxy-activated Sepharose 6B–arabinan) give specific adsorption of the enzyme. The arabinan-splitting activity of Pectinol R 10 is separated into a minor non-adsorbing and a major adsorbing fraction (Fig. 3). This finding suggests the presence of at least two different arabinan-degrading enzymes in Pectinol R 10. The adsorbed enzyme is an α-L-arabinofuranosidase, as could be demonstrated by its action on *p*-nitrophenylarabinofuranoside and on sugar beet arabinan, where only arabinose residues were released during the enzymatic breakdown. No activities on other nitrophenyl sugar derivatives could be demonstrated. The non-adsorbed arabinan-degrading enzyme is probably an isoenzyme and its nature will be further investigated.

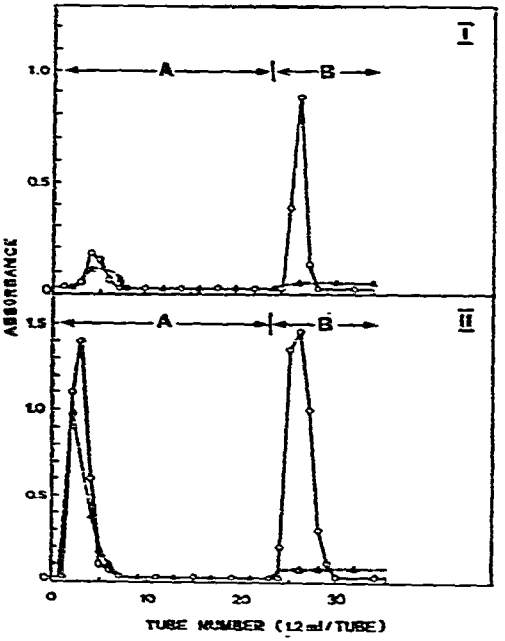


Fig. 3. Affinity chromatographic elution profiles of Pectinol R 10 (for details, see text). I, Sepharose 6B–arabinan; II, cross-linked arabinan. ○, Absorbance at 405 nm (nitrophenol); △, absorbance at 280 nm. A, Buffer A; B, buffer B.

The effectiveness of the affinity chromatographic technique for the purification of an α-L-arabinofuranosidase was demonstrated by applying crude and partially purified Pectinol R 10 to both affinity columns. It was found that it is not necessary to purify the enzyme before the affinity chromatographic step, as no differences in the elution patterns were obtained. This means that the described technique permits the purification of an α-L-arabinofuranosidase in a single step.

In further experiments different amounts of enzyme mixtures were applied on a small cross-linked arabinan column (6.0 × 0.9 cm). Amounts of up to 24 mg of the enzyme mixture could be loaded on the affinity carrier without any influence on its separation ability.

The chromatographic behaviour of Ultrazym 20 on both affinity carriers is very similar to that of Pectinol R 10, as indicated in Fig. 4(I) for the epoxy-activated Sepharose 6B–arabinan column. The main part of the arabinan-degrading activity was not adsorbed by the affinity carriers, and it passed through the columns with the bulk of the other enzymes. This result is different from that obtained with Pectinol R 10 (Fig. 3). Rechromatography of the non-adsorbed enzyme fraction indicated that all of its arabinan-degrading activity is again eluted in the front peak, as shown int Fig. 4 (II), thus clearly demonstrating the presence of at least two different arabinan-degrading enzymes in Ultrazym 20. The adsorbed enzyme had the same specificity as the α-L-arabinofuranosidase from Pectinol R 10.

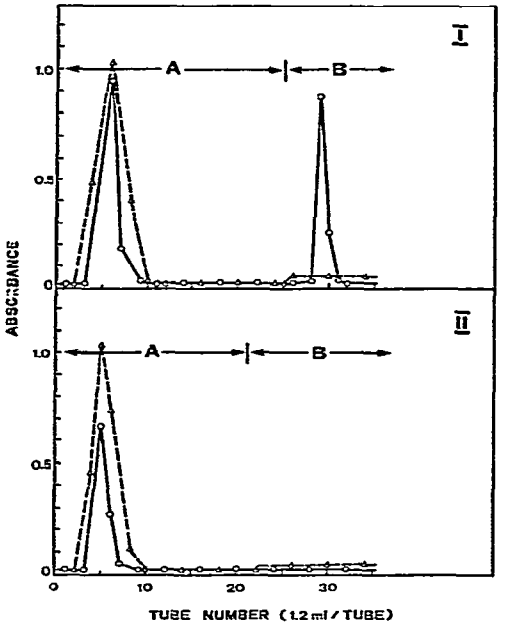


Fig. 4. Elution profiles of Ultrazym 20 with epoxy-activated Sepharose 6B arabinan. (I) Purified enzymic solution; (II) rechromatography of the non-adsorbed peak (for details, see text). ○, Absorbance at 405 nm; △, absorbance at 280 nm. A, Buffer A; B, buffer B.

Both enzyme preparations showed maximum activity for arabinan and the *p*-nitrophenyl-α-L-arabinofuranoside at pH 4.0[5,17]. At this pH it has been shown that during loading and elution arabinose was immediately hydrolysed off both affinity carriers. It is therefore very important to perform the affinity chromatography at pH 7.0, where no arabinan-degrading activity is detectable.

In conclusion, both epoxy-activated Sepharose 6B–arabinan and arabinan corss-linked by epichlorohydrin have been shown to be very useful affinity carriers for the preparation of an α-L-arabinofuranosidase completely free of other enzymatic activities. This technique has the advantage that no preliminary purification steps are necessary.

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