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Purification of a Sugarbeet Pectin Modifying Enzyme System from *Aspergillus niger* by Antibody Affinity Column Chromatography

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ABSTRACT

Polyclonal antibodies were raised against polygalacturonases partially purified from culture filtrates of Aspergillus niger *grown on sugarbeet pectin or polygalacturonic acid. An enzyme-linked immunosorbent assay (ELISA) for polygalacturonase was developed and the antisera were exploited in immuno-affinity chromatography to remove polygalacturonase from a mix of sugarbeet modifying enzymes. This approach has facilitated the preparation of an enzyme mix, containing a pectin deacetylase and demethoxylase together with an arabinofuranosidase, free from polygalacturonase, in a rapid, simple procedure.*

INTRODUCTION

Pectin as derived from sugarbeet pulp is a poor gelling agent and this has been attributed to its high acetyl content (Pippen *et al.*, 1950). However, other factors such as the non-uronide content and the degree and type of branching of the molecule also influence its gelling properties (Matthew *et al.*, 1990). In a previous paper (Matthew *et al.*, 1990) an enzyme system was used to modify sugarbeet pectin in such a way as to enhance its gelling properties. The changes in pectin structure were monitored by chemical techniques and ^{13}C-NMR studies. Guillon and Thibault (1990) have reported a modification of sugarbeet pectin using pectolytic enzymes from *A. niger* which results in a pectin with enhanced gelling properties in a system using persulphate as the gel inducer. In this laboratory (Williamson *et al.*, 1990), a commercially available enzyme extract

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from orange peel was used to enhance the gelling properties of sugarbeet pectin.

When *Aspergillus niger* is grown in a medium containing sugarbeet pectin as the sole carbon source (Matthew *et al.*, 1990) the crude culture filtrate contains a polygalacturonase, a deacetylase, an arabinofuranosidase and a demethoxylase. The polygalacturonase must be removed before the remaining mix of desirable enzyme activities can be used to treat sugarbeet pectin to improve its gelling properties. Purification of the desirable enzymes using conventional techniques results in poor yields so an alternative strategy was sought.

Affinity chromatography is now widely used for separations of biological substances which are difficult to achieve by other methods. A specific binding substance is immobilised on an insoluble support material and this reversibly binds the molecule of interest. In this paper the use of antibody affinity column chromatography to remove the polygalacturonase from an enzyme mixture is reported.

A. niger was grown either in medium containing sugarbeet pectin or in medium in which the carbon source was polygalacturonic acid. Both crude culture filtrates contain a polygalacturonase but, when *A. niger* is grown on sugarbeet pectin, deacetylase, arabinofuranosidase and demethoxylase are also present. The polygalacturonases were purified by FPLC and used to raise polyclonal antibodies. The antisera were then exploited in immuno-affinity chromatography to remove the polygalacturonase from an enzyme preparation containing deacetylase, arabinofuranosidase and demethoxylase.

MATERIALS AND METHODS

Preparation of sugarbeet pectin

Pectin was extracted from sugarbeet pulp by acid treatment (Matthew *et al.*, 1990) and stored in freeze-dried form. The sugarbeet pectin so obtained has been fully characterised by a ^{13}C-NMR study (Keenan *et al.*, 1985).

Preparation of polygalacturonase

At all stages fractions were assayed for enzyme activity. Routine testing for deacetylase and arabinofuranosidase was performed as previously described (Matthew *et al.*, 1990) by a small-scale assay on microtitration plates: the hydrolysis of, respectively, *p*-nitrophenyl acetate and

p-nitrophenyl arabinofuranoside (0·5 μmole in 0·2 ml incubation) was monitored using a Dynatech MR700 microplate reader (Dynatech Laboratories, Billingshurst, UK). Demethoxylase was measured using a method based on Hills and Speiser (1945), released methanol being estimated by the method of the Leatherhead Food Research Association (1982). Polygalacturonase activity was monitored by a cup plate assay. A mix of 2 g pectin and 3 g agar dissolved in 100 ml acetate buffer pH 5·2 was used to prepare 9 cm diameter, 3 mm thick gels. Plugs, 1 cm diameter, were removed from the gel and 100 μl enzyme was placed in the well. The area of clearance was measured after 18 h at room temperature.

Aspergillus niger CS180 (CMI 1298302)

This was grown in liquid medium as previously described (Matthew *et al.*, 1990) with either sugarbeet pectin (A) or polygalacturonic acid (Sigma, Poole, UK) (B) as carbon source at 6 g/litre. The crude culture filtrate was fractionated with solid ammonium sulphate. In the case of (A) the fraction precipitated between 25 and 50% saturation was used as partially purified sample and for (B) the fraction precipitated between 25 and 90% saturation was collected to give maximum yield of the enzyme. The precipitates were resuspended in 2 M ammonium sulphate and stored at 6°C until required.

FPLC

Samples of the stock solutions of partially purified enzymes stored in 2 M ammonium sulphate were dialysed overnight against buffer, 20 mM 2-(*N*-morpholino) ethane sulphonic acid (MES) pH 6·0. The dialysed solution was passed through a 0·5 cm × 20 cm column of Sephadex A50 (Pharmacia LKB, Uppsala, Sweden) to remove residual pigment. The column was eluted with 20 mM MES pH 6·0 followed by 0·5 M sodium chloride in the same buffer.

Polygalacturonase was eluted by the salt. Relevant fractions were pooled and dialysed overnight against the MES buffer. 2·0 ml of dialysed sample (0·5 mg total protein) was loaded onto a Mono Q HR 5/5 column connected to an FPLC system fitted with a GP 250 gradient programmer (all Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was eluted with a linear gradient of 0–0·5 M sodium chloride in 20 mM MES pH 6·0 buffer at 0·5 ml/min over 50 min. 1·0 ml fractions were collected and assayed for polygalacturonase activity. When the starting sample was derived from the sugarbeet pectin-containing medium then the fractions

were also assayed for deacetylase, arabinase and demethoxylase activities. With each of the two polygalacturonase preparations fractions from several FPLC runs were pooled, freeze dried and stored at 6°C until used.

Preparation of other enzymes

A. niger was grown in liquid medium in which sugarbeet pectin (Matthew *et al.*, 1990) was the carbon source. The crude culture filtrate was fractionated with ammonium sulphate. The fraction precipitating between 50 and 90% saturation contained the deacetylase, the arabinofuranosidase, the demethoxylase and some polygalacturonase and was applied to the affinity chromatography columns.

To obtain samples for cross-reaction tests with the antisera, the precipitate was resuspended in 50 mM phosphate buffer, pH 6·3, and subjected to gel filtration as already described (Matthew *et al.*, 1990). Fractions containing both deacetylase and arabinofuranosidase but no demethoxylase or polygalacturonase were bulked and stored as freeze-dried powder. Fractions containing only demethoxylase were bulked and stored in the same way.

Production of antisera

New Zealand White male rabbits were injected both subcutaneously and intramuscularly with a total of 100 μg polygalacturonase preparation per animal, dissolved in 2·0 ml Freund's complete adjuvant (Difco, East Molesey, UK) and sterile saline (7:3, v:v). After 6 weeks booster injections were given as above, but using Freund's incomplete adjuvant. The animals were bled from the marginal ear vein twice during the period 10–14 days after boosting. Further boosting and bleeding were performed as necessary. Plasma was separated from the whole blood and stored at −20°C.

Screening of antisera

Microtitration plates (Nunc Immunoplate 1, Gibco Ltd, Paisley, UK) were coated (250 μl/well) with the enzyme preparations (10 μg in 1·0 ml carbonate buffer, 0·05 M pH 9·6) and unreacted sites subsequently blocked with bovine serum albumin (10 g/litre in the same buffer). Coated plates were stored at room temperature over desiccant. Titration curves were performed using an enzyme-linked immunosorbent assay (ELISA). Antisera were diluted in phosphate-buffered saline, pH 7·4,

containing Tween 20 (0·5 ml/litre) (PBST), to give a 10-fold dilution series. Dilutions of antiserum (200 μl/well) were incubated in the coated plates for 3 h at 37°C. After washing five times with PBST using an automatic plate washer, anti-rabbit IgG horseradish peroxidase conjugate (200 μl/well, diluted 1:1000, v:v, in PBST; Sigma Chemical Co.) was added and the plate incubated for 2 h at 37°C. After further washing, peroxidase substrate (200 μl/well; Cambridge Life Sciences, Luton, UK) was added and the reaction allowed to proceed for 15–20 min before addition of 2 M sulphuric acid (50 μl/well). Well optical densities at 450 nm were read on a plate reader (Titertek Multiscan, Flau Laboratories, Richmansworth).

Standard curves for the polygalacturonases and determination of cross-reactivity with the other enzymes were carried out by competitive ELISA. A dilution of antiserum was chosen from plots of titration curves such that the antibody concentration was limiting, but still sufficient to give a high optical density reading at 450 nm in the competitive ELISA. Samples of enzyme preparations diluted in PBST to give a range of concentrations from 1 ng/ml to 1 mg/ml were added to coated plates (100 μl/well) followed immediately by a suitable antiserum dilution (100 μl/well). After incubation at 37°C for 2 h the plates were washed five times with PBST; enzyme-labelled antibody was added and the assay completed as for the titration curves. The degree of cross-reactivity was expressed (as a percentage) as the concentration of the homologous sample at 50% displacement (i.e. 50% maximum OD_{450}) divided by the concentration of the cross-reactant at 50% displacement.

Affinity chromatography

Antisera raised against (A) and (B) were purified in turn to obtain an IgG fraction with which affinity chromatography columns were prepared.

Purified IgG was prepared from antisera by serial precipitation using sodium sulphate (Weir, 1978). The final precipitate was resuspended in distilled water and then freeze-dried overnight. The IgG was coupled to CNBr-activated Sepharose 4B (Pharmacia LKB, Uppsala, Sweden) using methods outlined in the suppliers booklet, *Affinity Chromatography*. The coupling buffer was 0·2 M sodium bicarbonate pH 8·5, 0·5 M sodium chloride with 0·5% Tween 80. The washing buffers were 0·1 M acetate buffer pH 4·0 with 0·5 M sodium chloride and the coupling buffer. Columns 0·5 cm × 20 cm were packed and kept in 0·05% thiomersal in phosphate-buffered saline at 6°C when not in use.

Samples of the 50–90% ammonium sulphate cut which contained deacetylase, arabinofuranosidase, demethoxylase and some

polygalacturonase were dialysed overnight against 0·1 M phosphate buffer pH 6·0. Approximately 2 mg protein in 100 μl was applied to the affinity column. Elution was with 7·0 ml of the phosphate buffer (as above), 3·0 ml 1·0 M propionic acid, and then 3·0 ml same buffer. Fractions (1·0 ml) were collected and those in propionic acid were dialysed overnight against the phosphate buffer; all fractions were then assayed for deacetylase, arabinofuranosidase and polygalacturonase activities. The optical density at 280 nm was measured to monitor protein elution. The remainder of fractions 4–7 were pooled, as were fractions 10–13. The pooled fractions were assayed for demethoxylase activity.

RESULTS

Antisera titres

Strong immune responses were produced by each of the two polygalacturonase preparations, with antibody titres (defined as that dilution used for competitive ELISA) ranging from 1:20 000, down to 1:100 000 for different rabbits (data not shown).

Cross-reactivity of antisera

Neither antiserum showed a high degree of cross-reactivity with the heterologous polygalacturonase preparation (Table 1). When tested against the other partially purified enzyme preparations (deacetylase plus arabinofuranosidase and demethoxylase) the antiserum raised against preparation (A) (derived from sugarbeet pectin) was the more specific (Table 1). An ELISA standard curve for polygalacturonase prepared from a culture grown on polygalacturonic acid, and cross-reactivity against the demethoxylase-containing preparation is shown in Fig. 1.

Affinity chromatography

IgGs prepared from the two antisera, (i.e. that raised against polygalacturonase derived from sugarbeet pectin containing medium (A) and that raised against the polygalacturonase obtained on polygalacturonic acid containing medium (B)), were bound to the affinity support material and small columns were prepared.

When the enzyme mix was loaded onto these columns both types were able to selectively bind polygalacturonase, the other enzymes being

TABLE 1
Cross-Reactivity (%)[a] of Polygalacturonase Antisera

Cross-reactant	*Antiserum*[b]	
	A	*B*
Polygalacturonase A	100	24
Polygalacturonase B	<1	100
Deacetylase + arabinase	2	208
Demethoxylase	<1	<1

[a]For calculation see text.
[b]A: antiserum to polygalacturonase A (derived from sugarbeet pectin). B: antiserum to polygalacturonase B (derived from polygalacturonic acid).

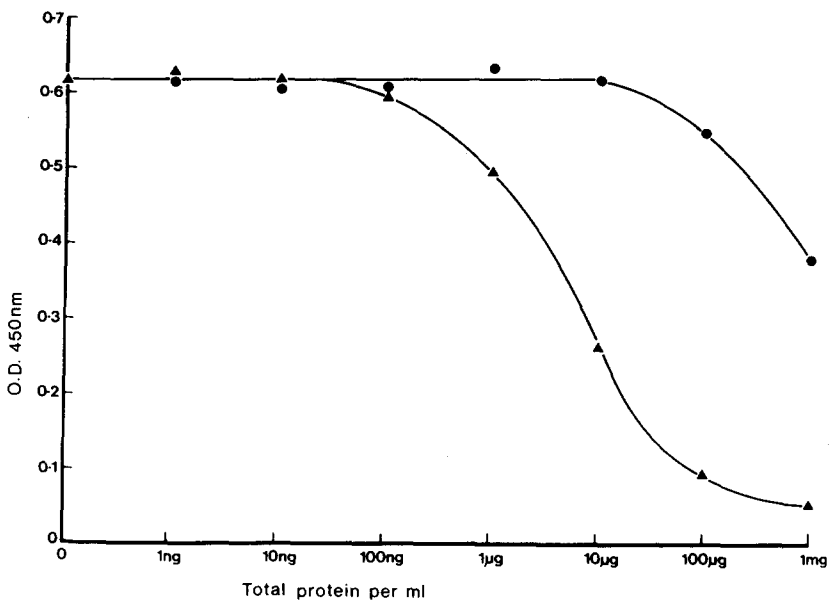

Fig. 1. Standard curve in competitive ELISA for polygalacturonase derived from growth on polygalacturonic acid (▲——▲), and cross-reactivity against demethoxylase (●——●).

eluted first in the phosphate buffer. The elution patterns are shown in Fig. 2 and Fig. 3. Because of limitations on the scale of the assay technique, demethoxylase was measured only for pooled fractions, viz. pooled fractions 4–7 and 10–13 in each instance. In each case pooled

fractions 4–7 gave a positive result for demethoxylase activity, there being no detectable activity in the later running fractions. In each instance no detectable polygalacturonase was present before fraction No. 10.

DISCUSSION

The gelling properties of sugarbeet pectin can be considerably enhanced by enzymic modification (Matthew *et al.*, 1990). This modification effects some deacetylation, a reduction in the arabinose content and some demethoxylation. The crude culture filtrate from *A. niger* from which these enzymes are derived contains a polygalacturonase. This enzyme breaks down the backbone of the pectin molecule and renders the pectin unsuitable for gelling. Using conventional separation techniques (Matthew *et al.*, 1990) we were able to obtain only very small amounts of a polygalacturonase-free enzyme preparation with which to treat sugarbeet pectin; a fresh approach was therefore taken.

Antisera were raised against two polygalacturonase-containing preparations from culture filtrates of *A. niger*. Both polygalacturonase preparations were free of detectable deacetylase, arabinofuranosidase and demethoxylase activities but were not further characterised. The

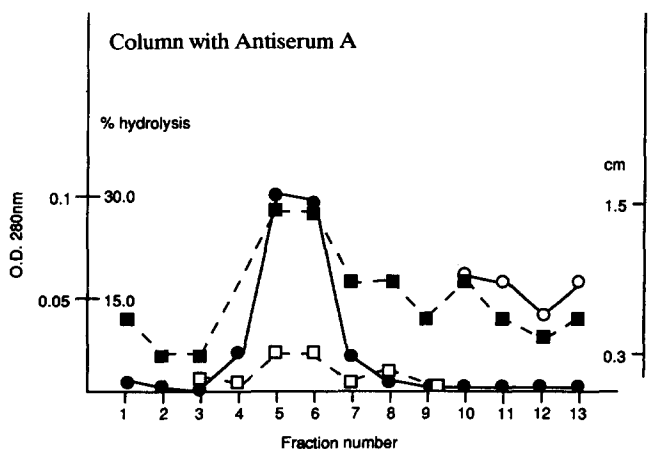

Fig. 2. Elution patterns for antiserum A. ■ Total protein, optical density at 280 nm; ● deacetylase, % hydrolysis of *p*-nitrophenyl acetate; □ arabinofuranosidase, % hydrolysis of *p*-nitrophenyl arabinofuranoside; ○ polygalacturonase, clearance around 1 cm well.

antisera were intended for use in immuno-affinity columns for the removal of polygalacturonase from a sugarbeet pectin modifying enzyme mixture. Such an approach overcomes the difficulties of conventional removal of polygalacturonase (Matthew *et al.*, 1990) when yields of the desirable enzyme activities are low. In the ELISA system, the level of cross-reaction of antiserum B with the enzyme fraction enriched in deacetylase and arabinofuranosidase is presumably due to the presence of antibodies which recognise the uncharacterised components of the antigen preparation. On SDS polyacrylamide gels both polygalacturonase preparations showed many polypeptide bands and several of these bands were recognised by the antisera after electroblotting onto Immobilon P (Millipore, Watford, UK), (data not shown).

Affinity purification of pectic enzymes has been used by previous workers (Ward & de Boer, 1989), although in rather a different format. In their work, culture supernatant from *Erwinia carotovora* was pretreated with a monoclonal antibody specific for pectic lyase and the antibody-enzyme complexes so formed were then captured by anti-species antibodies bound to Sepharose beads in aqueous suspension. Subsequently the enzyme was separated from the antibody in an iso-electric focusing gel. In the present work the specific antibody was bound directly to the Sepharose and the matrix packed into columns, giving a very simple system. This format allows, in theory, large volumes of dilute

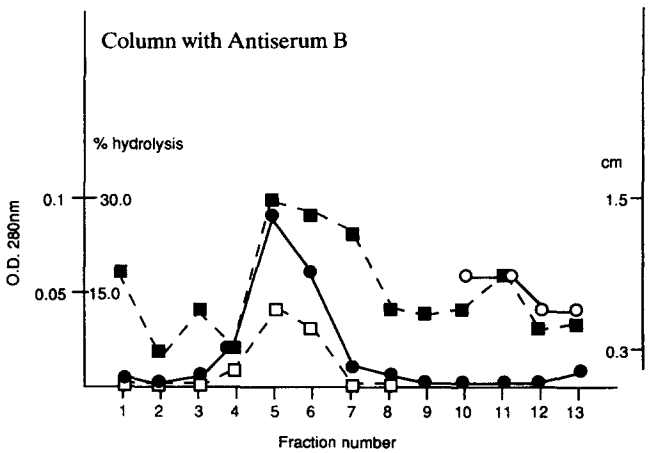

Fig. 3. Elution patterns for antiserum B. ■ Total protein, optical density at 280 nm; ● deacetylase, % hydrolysis of *p*-nitrophenyl acetate; □ arabinofuranosidase, % hydrolysis of *p*-nitrophenyl arabinofuranoside; ○ polygalacturonase, clearance around 1 cm well.

solutions of the molecule of interest to be processed rapidly. Washing away unbound material and elution of bound analyte is technically easy. The advent of monoclonal antibody technology could allow almost unlimited production of specific antibody for column manufacture instead of the limited supply of polyclonal antiserum used here. However, we have shown that the polygalacturonase can be specifically bound and that in the affinity chromatography systems cross-reaction with the other enzymes is low. Using conventional chromatography systems, the yield of purified deacetylase, arabinofuranosidase and demethoxylase was tiny, around 5% or less. In the immunoaffinity system described here recovery of these enzymes was between 50 and 60%. This is a potentially useful method which deserves further exploitation.

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