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PURIFICATION OF PECTIN METHYLESTERASE FROM *OROBANCHE AEGYPTIACA*

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Key Word Index—*Orobanche aegyptiaca*; Orobanchaceae; pectin methylesterase; purification; antibodies; Biogel P-100.

Abstract—The purification of pectin methylesterase by the use of acrylamide gel electrophoresis followed by electro-elution and Biogel column chromatography is described. Antibodies were prepared against the purified enzyme and their specificity tested against crude extracts of *Orobanche* calli and purified enzyme.

INTRODUCTION

In a previous paper [1] we reported on the presence of a pectin methylesterase in the tissue of *Orobanche aegyptiaca* calli and seedlings. Since we postulated that pectin methylesterase (PME) is one of the factors involved in the infection of the host by parasite seedlings, it is necessary to follow the presence of PME and its activity in the fusion zone between the root of the parasite seedling and host tissue. This can be achieved using immuno-histochemical methods, provided a parasite PME antibody is available. We observed that antibodies from other plant tissues were not specific enough for this purpose although the presence of PME could be observed. In order to obtain antibody to *Orobanche* PME it is essential to purify the enzyme. Since germinating seeds are not a satisfactory source of enzyme and are severely limited in availability we decided to purify the enzyme from callus tissue. We report here such a purification, which has proved to be far more refractory than we had envisaged. Although there are numerous recent reports on the purification of PME [2–5], we found that we had to develop our own procedures, since the published methods did not give satisfactory results.

RESULTS AND DISCUSSION

A variety of methods have been reported for the purification of PME in plant tissues. CM sepharose CL 6B, was used successfully for purifying PME from Papaya [2] and *Vigna radiata* [6], but was ineffective for broomrape. The use of CM Sephadex C50 and DEAE Sephacell columns was also ineffective. DEAE Sephadex A 50 columns have frequently been used to purify PME under non-absorbing conditions. We were able to achieve some success using such columns by applying crude extracts and eluting with 0.3 M NaCl. A seven-fold purification could be attained but recovery of protein was very low, so that the use of this column also had to be abandoned.

Some success was obtained using Biogel P-150 columns. A crude extract of callus in 10 mM phosphate buffer, containing 2% PVPP and 0.15 M NaCl was dialysed and loaded directly onto the column (30 × 0.9 cm). The column was eluted with 10 mM phosphate buffer, pH 6.0. We obtained appreciable purification in this one step procedure. Unfortunately Biogel P-150 is no longer available so that we resorted to the use of Biogel P-100.

While examining the assay procedure of Moustacas *et al.* [7] on fractions obtained from the Biogel P-150 column, we observed that the PME of *Orobanche* did not have optimal activity in the presence of NaCl. Addition of 40 mM KCl resulted in an appreciable increase in enzyme activity in the crude extract (the results were 0.011, *cf* to 0.027 absorbance units without and with the addition of KCl, respectively). In fractions obtained by precipitation with ammonium sulphate, 30–70% saturation, addition of 40 mM KCl increased activity by almost 50%, and omission of NaCl and the use of only KCl gave a further 10% increase in activity. The use of KCl was adopted in all subsequent experiments.

Purification of PME was achieved as follows. *Orobanche* callus was ground as described in the Experimental at pH 4.2, in a solution containing ascorbic acid and NaCl. The crude extract so obtained was applied directly to a 3-mm-thick acrylamide gel, and then electrophoresis carried out. The region containing PME activity was located using ruthenium red staining on a strip of the gel. The region of the gel containing the activity was cut out of the gel (top 1 cm of the resolving gel, immediately below the stacking gel). At the front of the gel an intense yellow region was detected, probably due

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Table 1. Purification of PME on Biogel P-100 column

	PME activity (units ml^{-1})	Protein ($\mu g\ ml^{-1}$)	Spec. act. (units μg^{-1})	Purification
Extract 12000 × *g* supernatant	14.6	1700	8.6	
Fraction 17	11.9	14	853	99.4
Fraction 18	6.7	23.6	286	33.4

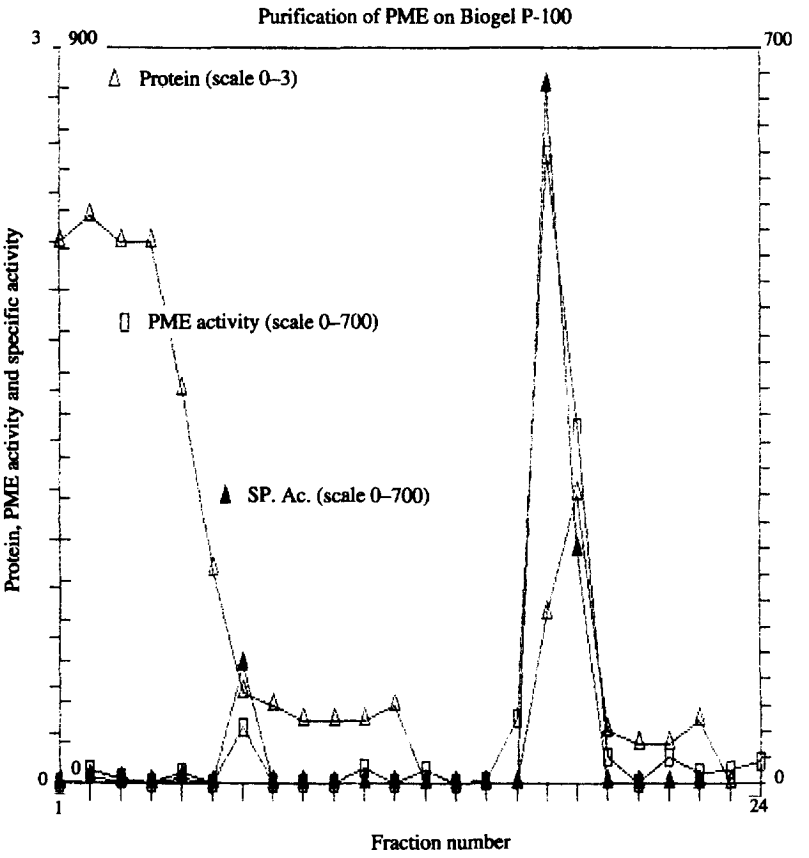

Fig. 1. Separation of PME on a Biogel P-100 column. Elution with phosphate buffer, pH 6.0, 10 mM.

to the presence in the extract of caffeic acid esters, which are characteristic of *Orobanche* [8]. Their presence may well be the reason for the difficulty in purifying PME from *Orobanche*. The purification is summarized in Table 1 and the elution pattern shown in Fig. 1. The active fractions (17 and 18, Table 1) were run on PAGE and stained for activity and for protein (Fig. 2).

The antibodies prepared against PME were tested on Western blots prepared after separating purified PME and crude extracts from *Orobanche* calli. In the purified enzyme preparations two bands were found to react with the antibody and on the crude preparations two to three bands reacting with antibody were observed (Fig. 3). Pre-immune serum tested under identical conditions, and at the same dilution did not show any reactive bands. The antibodies prepared therefore appear to meet the requirements for their use in immuno-electron microscopy. Nevertheless, it is possible that these antibodies could also react with host PME. A crude extract of tomato roots, containing 19 μg per lane protein did not react with our antibody under the conditions used for the western blot. Since *O. aegyptiaca* parasitizes a very wide range of hosts, belonging to many genera and families, we did not test our antibodies against other hosts.

EXPERIMENTAL

Tissue. Callus tissue of *Orobanche aegyptiaca* was grown as described in Ben-Hod *et al.* [9].

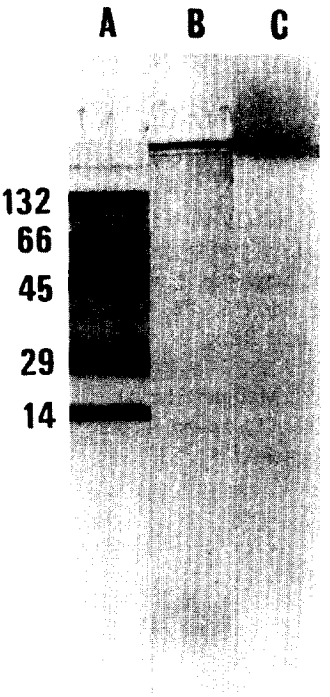

Fig. 2. Electrophoretic separation of active fraction (pooled fractions 17 and 18, Fig. 1) from Biogel P-100 column on native gels. (A) Molecular weight markers; (B) staining for protein, using silver stain; (C) staining for activity using ruthenium red.

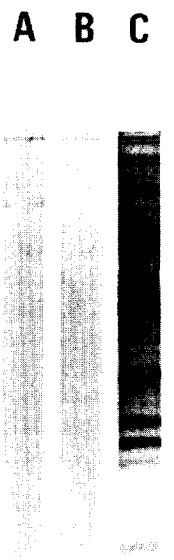

Fig. 3. Western blots using antibodies prepared against purified PME. (A) Purified enzyme; (B) crude extract of *Orobanche* callus; (C) protein in crude extract (Coomassie Blue R 250 stained).

Assay methods. PME activity was measured either by the methods described in Ben-Hod *et al.* [1] or by following the hydrolysis of *p*-nitrophenyl acetate as described in Ref. [7], by monitoring *A* at 400 nm. Protein was examined using a rapid Lowry assay [10].

Columns and absorbents. Columns of the various types were prepared by standard procedures.

Extraction and processing and purification of enzyme. The callus was ground with 10 mM NaCl, containing 0.2% sodium ascorbate, and the extract brought to pH 4.2 with lactic acid, using 50 ml solution for 30 g fresh weight of callus. This gave good yields and virtually prevented browning. Crude extracts were applied directly to 3-mm-thick acrylamide gel and electrophoresis carried out according to Laemmli [11], under non-denaturing conditions. The gels were stained for protein using Coomassie Blue R-250 or with silver according to Morrissey [12]. The gel was finely chopped and electro-eluted in Tris-glycine buffer pH 8.3 as used for electrophoresis. Electro-elution was carried out at 100 V for 20 hr, at room temp. The eluted enzyme extract was removed from the cell, placed in dialysis tubing and concd by placing the tubing in solid PEG 8000. The concentrated extract was directly applied to a Biogel P-100 column (0.9 × 30 cm). The column was eluted with phosphate buffer pH. 6.0, 10 mM and 1.0 ml frs collected.

Western blotting. Western blotting was onto nitrocellulose using standard methods [13].

Preparation of antibodies. Initially we attempted to use rabbits for the preparation of antibodies. We used goat anti-rabbit IgG complexed with alkaline phosphatase and detection was with nitrotetrazolium blue and 5-bromo-4-chloro-3-indolyl phosphate to test pre-immune serum. Pre-immune serum from rabbits was tested on crude extracts of *Orobanche* calli, separated by PAGE, and transferred to nitrocellulose. The Western blots were developed. On these blots the pre-immune serum detected some 10–12 non-specific bands. This indicates that rabbit serum contained a large number of epitopes reacting with our extracts and it was therefore unsuitable for our purpose. We therefore resorted to the use of guinea pigs in order to obtain our antibodies. This proved to be satisfactory and no non-specific bands were observed in the pre-immune serum using goat-antiguinea pig IgG linked to horseradish peroxidase, and using 4-chloro-1-naphthol as the detecting agent. Western blots were carried out by standard methods. The blocking protein was spray-dried skimmed milk (1.0% fat) (Marvel–Cadbury's, U.K.). Staining of the gels for PME activity was by the method of Lisker and Retig [14] or using the 'sandwich technique' of Bertheau *et al.* [15].

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