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# Culture of pectin methylesterase and polyphenoloxidase in *Cuscuta campestris*

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**Abstract**

A method for growing *Cuscuta campestris* aseptically *in vitro* was developed. Polyphenoloxidase activity in extracts of *Cuscuta* was partially characterised and ways for inhibiting it during extraction were determined. Pectin methylesterase activity in extracts was demonstrated. The enzyme, with activity at pH 7.0, was purified using column chromatography on Con A-Sepharose and Biogel P-100. The enzyme was shown to be a glycoprotein, with a molecular weight of about 40–45 kD, which seemed to be able to associate sometimes to what appears to be a dimer. Its possible role in host infection by *Cuscuta* is discussed. 

*Keywords:* *Cuscuta campestris*; Convolvulaceae; *in vitro* culture; polyphenoloxidase; phenolics; pectin methylesterase; Con A-Sepharose; Biogel P-100

## 1. Introduction

*Cuscuta* species are regarded as holoparasites, which attach to and parasitise the aerial parts of a very wide range of host plants. The incidence of *Cuscuta* (dodder) worldwide appears to be increasing and damage to and loss of yield of the host can be very severe. The biology of *Cuscuta* has been reviewed repeatedly (Schmucker, 1959; Ashton & Santana, 1976; Raven, 1983; Dawson, Musselman, Wolswinkel, & Dorr, 1994). The ability of dodder to carry out photosynthesis is still disputed and appears to differ between different species of the genus. However, it is clear that the chlorophyll content of the parasite is much lower than that of its host (Dinelli, Bonetti, & Tibiletti, 1993). *Cuscuta* has been cultured *in vitro* but reports indicate considerable differences in the nutrient conditions required for its *in vitro* growth (Loo, 1996; Ehrenfeld, Baumel, Czygan, & Proksch, 1996; Furuhashi, Kanno, & Morita, 1995; Bakos, Fari, Toldi, & Lados, 1995). A routine system for growing *Cuscuta* in the laboratory does not appear to have been described. To what extent cultured *Cuscuta* forms haustoria, by which it attaches to its host, is also not certain.

It seems probable that penetration into host tissue requires the activity of pectin-degrading enzymes, secreted by the parasite, as also seems to be the case for the root parasite, broomrape (Losner-Goshen, Portnoy, Mayer, & Joel, 1998). In the case of *Orobanche*, both polygalacturonase and pectin methylesterase may be involved in the infection process and the latter enzyme was studied during infection (Losner-Goshen et al., 1998).

Pectin methylesterase (PME) in a number of plant tissues has been described. Most of the papers describe a PME which is extracted with high, 1 M, NaCl in buffer. Bordenave and Goldberg (1994) describe the extraction of different isoforms of PME from mung bean hypocotyl with different concentrations of NaCl ranging from 0.1–1.0 M. In most cases, the enzyme is said to be a glycoprotein, existing in a number of isoforms differing in Mr and electrophoretic mobility. PME in pods and seeds of green beans and the corresponding cDNas were studied by Ebbelaar, Tucker, Laats, van Dijk, Stolle-Smits, and Recourt (1996). However, Glover and Brady (1994) describe a PME from peach fruit which is either not glycolsylated or had a low level of glycosylation. It could not be retained on Con A-Sepharose columns and existed in several isoforms, with basic isoelectric point. The enzyme from mung bean had an apparent Mr of 32000 on SDS electrophoresis and of 75000 in native gels (Goldberg, Pierron, Durand, & Mutaftschiev, 1992). In this case, different isoforms seemed to differ in their activity towards *p*-nitrophenyl acetate and pectin. Different isoforms had different iso-electric points (Bordenave &

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Goldberg, 1993). The different isoforms, originating in a small multigene family, differed in their response to the presence of cations in the assay medium (Bordenave, Breton, Goldberg, Huet, Perez, & Pernollet, 1996). The PME from tomato pericarp was also resolved into four distinct isoforms which, although similar in their immunological characteristics, differed in their behaviour, indicating structural differences (Warrilow, Turner, & Jones, 1994). The PME from tomato appears to be even more variable since Gaffe, Tieman and Handa (1994) describe two groups of the enzyme which also differ in their immuno-cross reactivity. Thus, PME is by no means a simple enzyme and shows great variability within and between species.

The presence of pectin-degrading enzymes in *Cuscuta* has been previously reported (Nagar, Singh, & Sanwal, 1984), but the enzymes, one of which probably is a pectin methylesterase, were not adequately characterised. The preparations examined were not obtained from aseptic tissue. A mutiple form of pectin methylesterase in mature stems of *C. reflexa* has also been described, which was solubilised at high salt concentrations (Srivasta, Nighjkar, & Kumar, 1994).

In order to confirm that pectin methylesterase is indeed involved in host infection, we wished to characterise and isolate this enzyme from dodder and, if possible, to localise it at the electron microscopal level during the infection process.

In this paper, we report on some of the properties of pectin methylesterase from *C. campestris* and ways of growing the parasite under axenic conditions.

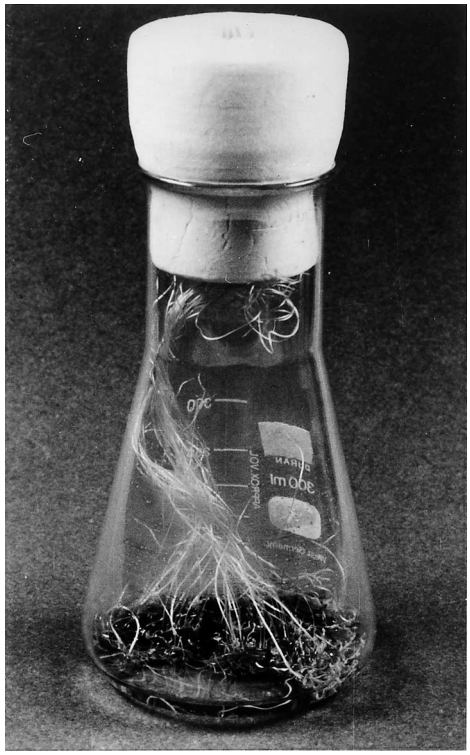

Fig. 1. Culture of *Cuscuta campestris* in the laboratory.

## 2. Results

### 2.1. *Characterisation of* in vitro *cultured* Cuscuta

When using plant tissue cultured *in vitro* in order to use it for biochemical studies, the characteristics of the tissue should be established. The general appearance of the cultured *Cuscuta* tissue is shown in Fig. 1. The vertical stems had a lower chlorophyll content, 53.9 μg.g.fresh wt$^{-1}$, compared to the thickened plagiotrophic ones, 142.3 μg.g.fr.wt$^{-1}$. Acetone extracts showed absorption peaks for chlorophyll and, in addition, peaks indicating the presence of flavonoids and carotenoids (data not shown). The levels of chlorophyll resemble those reported in the literature (Dinelli et al., 1993). While no haustoria could be detected in the vertically growing stems, typical self infection was detected in the plagiotrophic stems, which twined and showed haustoria (Figure 2). The *Cuscuta* cultured *in vitro* also flowered in some cases. This seems to show that the cultured material was similar to that collected in the field and justifies its use for biochemical work.

Preliminary screening of crude extracts of laboratory grown *Cuscuta* showed the presence of polygalacturonase in such extracts with activity at pH 6.0, 7.0 and 8.0. This activity was rather labile during and after extraction, but was stable in the intact tissue at $-20^{\circ}$. Similar screening for PME activity showed activities at two pH values, 6.0 and between pH 7.0 and 8.0. Since the substrate used for our assays is unstable above pH 7.5, all further experiments were carried out at pH 7.0. PME activity was very stable both in frozen tissue and in extracts kept in the cold. We also established that the activity hydrolysing *p*-nitrophenyl acetate was a pectin methyl esterase, by showing titrimetrically the formation of acidic groups on hydrolysis of polygalacturonic acid.

### 2.2. *Polyphenoloxidase in extracts from dodder*

During the screening of crude extracts, we noticed marked browning in enzyme preparations indicating the apparent presence of a polyphenol oxidase (PPO). This browning was deleterious and ways to prevent it were sought. *Cuscuta* sp. have also been reported to contain various phenolic compounds, including caffeoyl quinic acid derivatives and derivatives of quercetin and kaemferol. In the case of *C. campestris*, the presence of azaleatol and isorhamnetol has been reported (Istudor, Predescu, Popa, Badoi, & Sialvara, 1984). However, preliminary observations on the tissues we used could not confirm this. In contrast, caffeoyl quinic acid derivatives

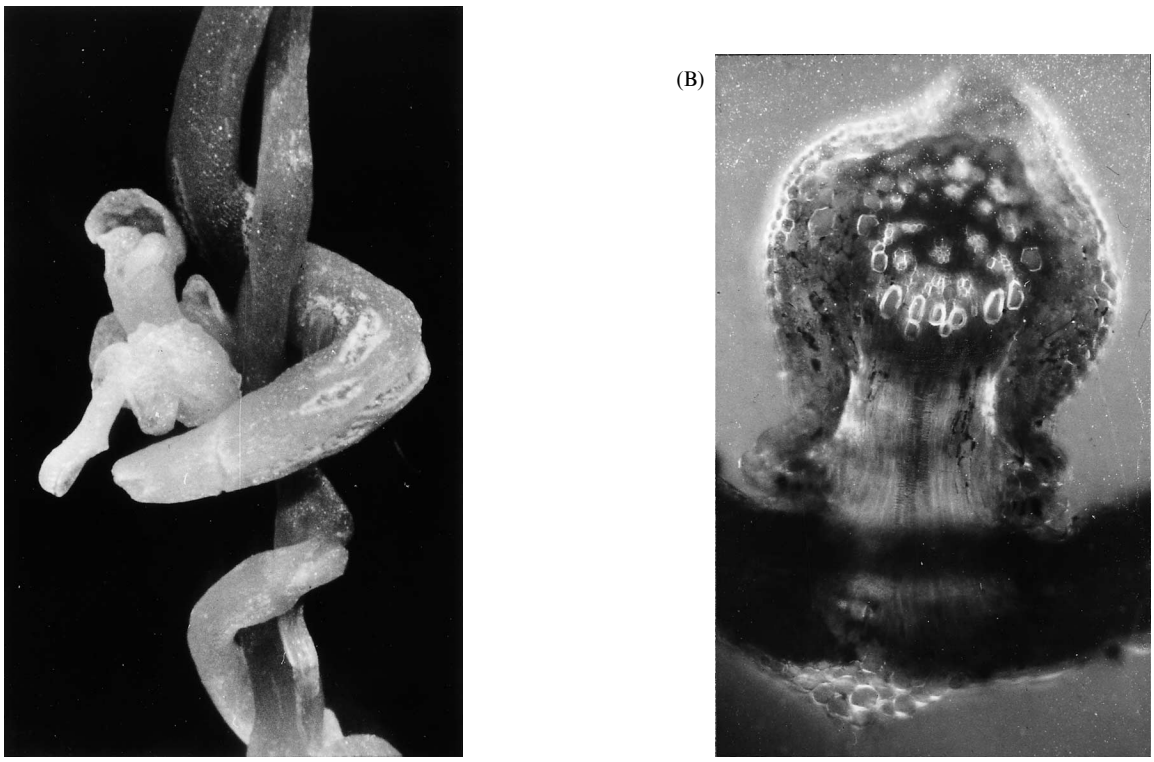


Fig. 2. Coiling of plagiotrophic parts of *Cuscuta* cultured *in vitro*. A, general aspect; B, appearance of a haustorium.

and a flavonoid were present (C. Andary, personal comm.).

A preliminary characterisation of the putative PPO in extracts was therefore attempted. Activity of extracts towards 4-methyl catechol was greatest at pH 5.0 (2.99 µl $O_2$/min at pH 5.0, 1.84 at pH 6.0 and 0.92 at pH 7.0). Surprisingly, there was some activity towards quinol at pH 7.0, which might be due to laccase activity We attempted to differentiate between PPO and laccase activity using 2,3-naphthalene diol, which supposedly inhibits the former, but not the latter (Table 1). Instead of inhibition of PPO, stimulation by naphthalene diol was observed. Other inhibitors were therefore studied. Addition of DIECA, DTT or phenylthiourea to the grinding medium reduced PPO activity in the extracts significantly. A combination of 2 mM Dieca and 1 mM DTT reduced PPO activity by 80%. Grinding in the presence of 1 mM phenylthiourea reduced activity by almost 45%. By means of the addition of catalase and ethanol (Table 1), the involvement of peroxidase activity could be ruled out. PPO activity was also inhibited by addition of the inhibitors to the assay mixture, as opposed to their addition during extraction (Table 2). Activity at pH 7.0 was low towards 4-methyl catechol. These results point to a typical reaction catalysed by PPO. The enzyme activity of PPO showed a typical dependence on concentration of 4-methyl catechol, with a $K_m$ of $5.5 \times 10^{-4}$ M and a $V_{max}$ of 4 µl $O_2$/min/ml. Addition of 2,3-naph-

Table 1
Polyphenoloxidase activity in the presence of catalase. Enzyme activity in 0.1 M $P_i$-citrate buffer, pH 7.0, was examined in the presence or absence of 95 units catalase and ethanol in a total reaction mixture vol. of 3.3 ml. Substrates $10^{-3}$ M 4-methyl catechol or $10^{-2}$ M quinol. 2,3-Naphthalene diol concentration $5 \times 10^{-3}$ M.

| Reaction mixture | $O_2$ uptake (µl/min) |
|---|---|
| Enzyme + 4-Me catechol | 1.15 |
| Enzyme + 4-Me catechol + naphthalene diol | 5.75 |
| Enzyme + 4-Me catechol + naphthalene diol + ethanol | 5.75 |
| Enzyme + 4-Me catechol + naphthalene diol + catalase + ethanol | 4.60 |
| Enzyme + 4-Me catechol + naphthalene diol + catalase | 4.60 |
| Enzyme + quinol + naphthalene diol | 1.73 |

thalene diol to the reaction mixture during assay of activity, in some instances, greatly stimulated oxygen uptake in the presence of 4-methyl catechol. Naphthalene diol alone was not oxidised. At a constant methyl catechol concentration with changing naphthalene diol concentration, the $V_{max}$ increased to 50 µl $O_2$/min/ml and the $K_m$ remained unchanged at $8 \times 10^{-4}$ M for concentrations up to $5 \times 10^{-3}$ M of the compound, the $1/S$ against $1/V$ being linear. However, at higher naphthalene diol concentrations, the curve deviated completely from linearity and 50% inhibition of activity was observed instead of stimulation. This could indicate the presence of two binding sites for the naphthalene diol, one site being responsible for the stimulation, due to some conformational change, the other for inhibition, by competition for the active site of the enzyme. The affinity for the putative inhibitor was $1.5 \times 10^{-3}$ M, when plotting $1/V$ against 1/inhibitor concentration, in the region of stimulation. No previous stimulation of PPO by naphthalene diol has been reported (Mayer & Harel, 1979; Mayer, 1987). We also tested PPO in dodder collected in the field to ensure that we were not dealing with an artefact resulting from laboratory culture. PPO of field grown dodder behaved in the same way as that from the laboratory. Stimulation of oxygen uptake by naphthalene diol was lost when enzyme preparations were kept in the cold for a few days. Further examination of the naphthalene diol response showed that complete inhibition by naphthalene diol could be achieved if supernatant from a boiled extract was added to a partially purified PPO, obtained by ammonium sulphate fractionation. Apparently, the naphthalene diol response results from the presence of some factor in crude extracts. It is clear that dodder contains a very active PPO which can, however, be inhibited to a considerable extent by addition of DTT, DIECA and PVP during extraction. In subsequent procedures for extracting PME, these inhibitors were routinely added to the extraction buffer.

### 2.3. Partial purification of PME

PME from *Cuscuta* tissue, whether grown in the laboratory or collected in the field, was easily extracted from the tissue. The extraction medium finally adopted was 10 mM acetate buffer containing 10 mM NaCl, together with inhibitors of PPO, 2 mM DTT, 1 mM DIECA and 10% solid PVP. This ease of extraction contrasts sharply with most reported procedures in which PME was solubilised only in the presence of fairly high, >0.1 M, NaCl. The ease of solubilisation was confirmed by using a procedure which did not involve grinding of the tissue. Stems of *Cuscuta* were infiltrated under vacuum with the standard extraction buffer. Pressure was then applied to the stems by placing them in a syringe and the activity measured in the expressed liquid. The activity was the same as that of extracts ground in buffer and then centrifuged. Clearly, part of the PME activity is very loosely bound and easily removed from the tissue. The extract obtained by grinding was centrifuged and then treated with pectolyase (see Methods) to remove part of the pectic substances. Following this treatment, the extracts were again centrifuged and fractionated with ammonium sulphate. The bulk of activity was recovered in the fraction precipitating between 40–80% ammonium sulphate. This fraction was resuspended and then applied to a column of Con A-Sepharose (Figure 3). Step wise elution of this column with buffer and α-methyl mannoside resulted in separation of the activity of PME into two, part being eluted directly with buffer and not bound to the column, and a fraction which was eluted with 0.2 M α-methyl mannoside. Clearly, part of the PME activity was present as a glycoprotein fraction, while some activity was not glycosylated. The glycoprotein-containing fraction was applied to a Biogel P-100 column and eluted with 50 mM acetate buffer, pH 4.5. Activity with good purification was obtained (Figure 4). The steps of the purification procedure are shown in Table 3 and the elution pattern from Biogel is shown in Fig. 4.

At various steps in the purification procedure, we examined the active fractions using PAGE. We performed dozens of electrophoretic separations using either native or SDS PAGE. After SDS separation, gels were washed with buffered isopropanol (Blank, Sugyama, & Dekker, 1982). The gels were then brought to the required pH by exposure to buffer in an electric field and stained for activity. Whilst on native gels, enzyme activity could be detected in crude extracts, on the gels using ruthenium red, after purification, no activity could be detected using this dye. However, staining with naphthyl acetate also permitted us to show activity in purified extracts even after SDS electrophoresis.

Native PAGE of purified fractions from a Biogel column always resolved into a number of protein fractions, some with high Mr, others with low Mr.

In some cases, we used Sephacryl S 200 for purifying the enzyme after separation on a Con A-Sepharose column. SDS electrophoresis of the purified fractions from the Sephacryl column, showed the presence of a glycoprotein in the region which stained for esterase activity (Figure 5).

Table 2
Effect of inhibitors on PPO activity when added to reaction mixture. Extracts were prepared at pH 5.0 in 0.1 M phosphate citrate buffer and activity followed with $5 \times 10^{-3}$ M 4-methyl catechol as substrate, at pH 5.0.

| Addition to extract | Activity (µl $O_2$/min/1.0 ml) |
|---|---|
| None | 5.2 |
| Dieca 2 mM | 1.15 |
| DTT 1 mM | 4.4 |
| Dieca 2 mM + DTT 1 mM | 0.35 |
| Naphthalenediol $5 \times 10^{-3}$ M | 46 |
| Naphthalene diol + Dieca 2 mM | 28.7 |

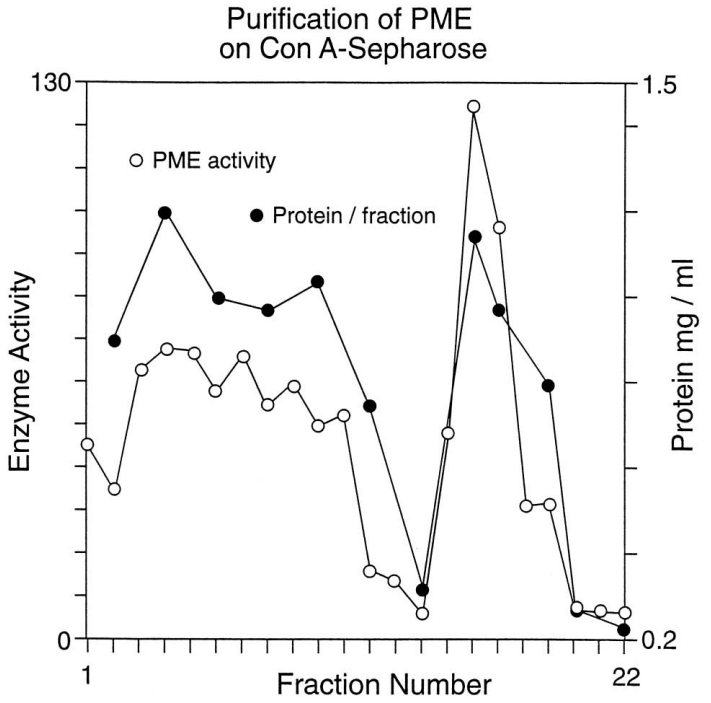


Fig. 3. Fractionation of *C. campestris* extract on Con A-Sepharose. Extract prepared from material collected in the field. Extraction using 10 mM $P_i$ buffer, pH 4.5, containing 10 mM NaCl, DTT, Dieca and PVP, treated with pectolyase and then fractionated using ammonium sulphate. The active fraction was loaded onto a column (0.9 × 14 cm). Elution using 10 mM $P_i$ buffer, pH 6.5, containing 0.15 M NaCl. Fractions 1–6, buffer only, fractions 6–19, buffer with 0.2 M α-mannoside and fractions 20–22, buffer containing 0.5 M α-mannoside. Sp. activity (δO.D./mg protein/20 min) were: Crude (after centrifugation)—15.3 units; Fraction 16—108.4.

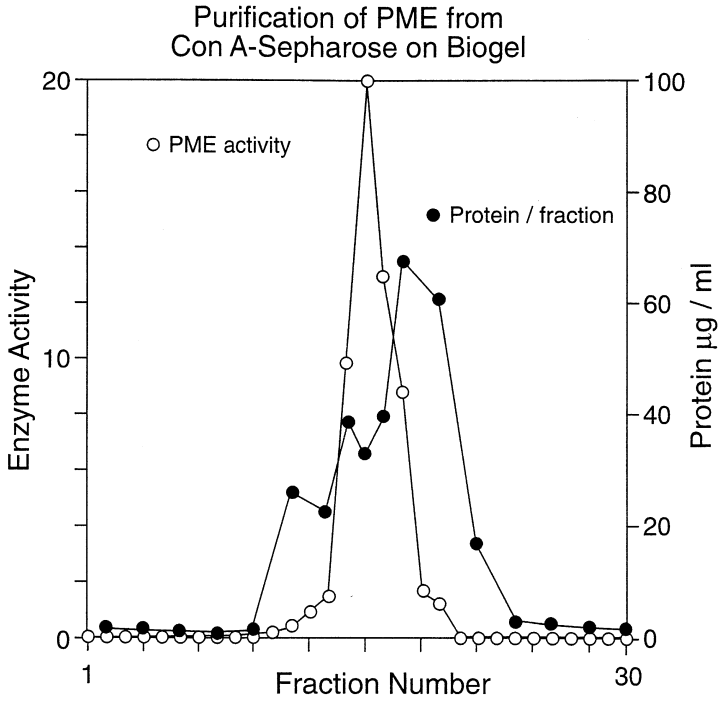


Fig. 4. Fractionation of PME on a Biogel P-100 column. Extract from *C. campestris*, collected in the field, using 10 mM acetate buffer, pH 4.5, containing 10 mM NaCl, 2 mM Dieca, 1 mM DTT and 10% insol. PVP. Incubation with 1 µg/ml pectolyase (Sigma P-3026) for 24 hr followed by centrifugation and ammonium sulphate fractionation. The active fractions eluted from a Con A-Sepharose column were applied to a Biogel P-100 column 1.4 × 27 cm. Elution with 50 mM actetate buffer, pH 4.5. Sp. activity (δO.D./mg protein): Crude—12.0; Fraction 16—592; Fraction 17—323.

Table 3
Purification of PME from *Cuscuta* collected in the field. Tissue ground in 10 mM acetate buffer, pH 4.5, containing 10 mM NaCl and 10% PVP as well as 2 mM Dieca and 1 mM DTT.

| Fraction | Specific activity |
|---|---|
| Crude extract filtered through cheese cloth | 2.1* |
| Extract after centrifugation at 17000 × *g* for 10' | 10.46 |
| 40–80% saturation with $(NH_4)_2SO_4$ | 41*,* |
| Fraction 8 Con A-Sepharose | 81 |
| Fraction 9 Con A-Sepharose | 88.6 |
| Fraction 15 from Biogel P-100 | 185 |
| Fraction 16 from Biogel P-100 | 272 |
| Fraction 17 from Biogel P-100 | 525 |

* Purification estimated from a number of other experiments.
*,* Purification estimated from a number of experiments using ammonium sulphate fractionation.

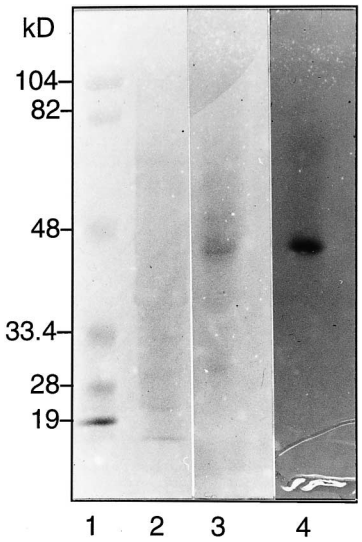


Fig. 5. Electrophoretic separation on SDS gels of active fractions eluted off a Sephacryl S 200 column (22.5 × 1.4 cm), with 50 mM acetate buffer, pH 4.5, after extraction, ammonium sulphate fractionation and passage through a Con A-Sepharose column. Lane 1, markers; Lane 2, Protein staining using Coomassie Blue; Lane 3, Staining for glycoprotein; Lane 4, Staining for activity, using naphthyl acetate.

### *2.4. Electrophoretic separation of PME after extraction and purification*

In the electrophoretic pattern of a crude extract of *Cuscuta*, after treatment with pectolyase and concentration using a Vivaspin tube, a considerable number of protein bands can be discerned with, however, considerable background staining. Extracts treated in order to inhibit PPO activity and with pectolyase gave a similar pattern with slightly less smearing. This pattern was reproducible on native gels. A rough indication of the Mr of the native PME was obtained using Mr markers. Staining such gels for PME activity usually showed a smear when using Ruthenium Red. However, when native gels were stained using naphthyl acetate, three well defined bands around 45 kD were observed (Figure 6).

Activity eluted off Biogel columns in a well defined peak, but on native gels, this peak was resolved into a number of protein bands, despite an apparent purification of at least 70 fold.

SDS gels of the same partially purified extracts from a Biogel columns still showed the presence of a number of protein bands, but PME activity was now found in one very strongly staining region at around 45 kD, with a minor band at about 30 kD (Figure 7).

When activity staining using naphthyl acetate was applied to the active fractions eluting from the Con A-Sepharose column, enzyme activity was detected with a molecular weight of just below 40 kD, with 20 fold

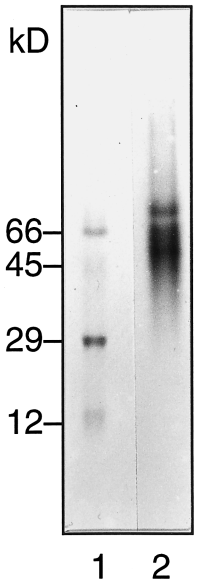


Fig. 6. Electrophoretic separation on native gels of active fractions from Biogel. Lane 1, markers; Lane 2, Staining for activity using naphthyl acetate.

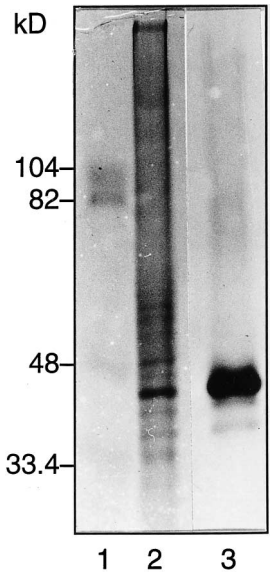


Fig. 7. Electrophoretic separation on SDS gels of active fractions from Biogel. Lane 1, Markers; Lane 2, Protein staining using Coomassie Blue; Lane 3, Staining for activity using naphtyl acetate.

purification. The active fraction still contained, at least, some half dozen bands of protein. When the active fractions were combined and passed through a Biogel P-100 column, a further purification was obtained, to give final purification of over 70 fold. The number of bands staining for protein was reduced. However, PME activity now appeared as two distinct bands, one around 40 kD and a second one at 70–80 kD (Figure 8). From this result, for which we had previous indications, it appears that the enzyme is undergoing aggregation, probably dimerization during concentration, prior to application to the Biogel column. It must be remembered that the eluates from Con A Sepharose, after passage through Biogel, are concentrated about 10 fold prior to application to the gels, using Viva spin tubes. This procedure and the fact that we are dealing with a glycoprotein is perhaps the cause for the aggregation, although this is a rather unusual phenomenon. The band staining for esterase activity gave a clear reaction with the antibody for pectin methylesterase prepared from *Orobanche aegyptiaca* cell cultures (Bar Nun, Ben Hod, Lavi, & Mayer, 1996).

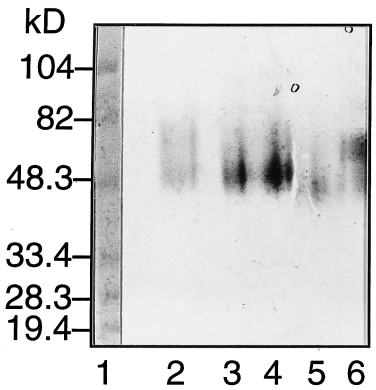


Fig. 8. Electrophoretic separation on SDS gels of active fractions from Con A-Sepharose and from Biogel. Lane 1, markers; Lane 2, fraction after 40–80% ammonium sulphate saturation; Lanes 3, 4 and 5, active fractions from Con A-Sepharose; Lane 6, Active fraction from Biogel after concentrating the active fractions from Con A-Sepharose and elution from Biogel. Activity staining for lanes 2–6 using naphthyl acetate.

## 3. Discussion

Our results show that it is possible to use axenically grown *Cuscuta campestris* tissue in order to study the enzymology of the parasitic plant, without being dependent on collection in the field.

The polyphenol oxidase of *Cuscuta*, seems to be a normal PPO, showing typical responses to all inhibitors, with the exception of 2,3-naphthalene diol. The response to naphthalene diol, which is not itself oxidised is difficult to understand. Since it is almost certain that no coupled oxidation reactions are involved in the stimulation of the oxidation of 4-methyl catechol, we suggest that in this case, the inhibitor induces a conformational change in the PPO and that this change is dependent on the presence of some, as yet, unidentified factor in the tissue extracts. This observation indicates that caution is required when using 2,3-naphthalene diol as a specific inhibitor of PPO as we had previously suggested (Mayer & Harel, 1979).

The PME we detected in *Cuscuta* shows some characteristics which clearly show it to be different from PMEs previously reported. The PME is very easily extracted from the tissue, and its solubilisation does not require high salt concentrations. In this respect it resembles the PME from *Orobanche* (Losner-Goshen et al., 1998). There are several PMEs in *Cuscuta*, differing in pH optimum and also in the amount of sugars attached to the enzyme. At least one form is a typical glycoprotein, as indicated by binding to Con A-Sepharose and by staining for glycoprotein after electrophoresis. The PME described here clearly differs from that described for *C. reflexa*. The PME in the latter was only extracted using high salt concentrations, almost 2 M. Whether this PME was a glycoprotein was not investigated. The PME from *C. reflexa* was resolved into two forms, both with molecular weight of around 60 kD, only one of which was retained on DEAE, cellulose higher than that reported here. It appears as if we are dealing with a PME significantly different from that described in Srivasta et al. (1994).

The enzyme described by us seems to be extremely labile and to undergo aggregation deaggregation, depending on its concentration, during the isolation procedure. Because of this lability, we have still not been able to purify the enzyme to homogeneity. It is possible that such lability is part of the biological function which

we suggest for this enzyme. An enzyme which is involved in penetration into host tissue should be loosely attached to the cell walls and should not be able to damage cells of the parasite while entering into host tissue. To resolve this question, further studies are clearly required, which would demonstrate the location of this enzyme in the host using electron microscopy as we have done for *Orobanche*.

## 4. Experimental

### 4.1. Plant material

Seeds of *Cuscuta campestris*, obtained from Dr. D. Joel, Neve Ya-ar, were treated with 95% (v/v) $H_2SO_4$ for 20 min, rinsed with distilled water 3 × for 5 min and then placed in 1.5% NaOCl containing 0.2% Tween 20 for 5 min. They were then rinsed 4 × with sterile distilled water and sown on culture medium (Murashige & Skoog, 1962) containing 2% sucrose and 1% agar. Germination was 80–90%. The seedlings were transferred to sterile culture medium containg full M & S medium and M & S vitamins, 2% sucrose, 100 mg.l$^{-1}$ inositol, 10% coconut water (Sigma) and 0.1 mg.l$^{-1}$ benzyl adenine, 0.04 mg.l$^{-1}$ indolebutyric acid and 0.1 mg.l$^{-1}$ GA3, pH 5.6

In this culture medium, a dense network of plagiotropically growing stems developed from which slender negatively geotrophic twining stems emerged above the medium. These cultures could be maintained for 3–4 month in fluorescent light 5.5 μEinstein.m$^2$.sec$^{-1}$, with transfer, after which they began to degenerate. Cultured material was collected and kept at −20°. During the growing season, we also collected *Cuscuta* from the field, near Beit Guvrin (31° 35′N 34° 53′E) where it appeared in profusion. The stems were cleaned in the laboratory and then immediately frozen and again kept at −20°. The laboratory grown material was used for enzyme extraction without further treatment, whilst the material collected in the field was first surface sterilised with 1% NaOCl for 10 min before treatment. Comparison of the enzyme in the two sources showed them to be equivalent.

*Polyphenoloxidase* activity was followed as described in Mayer, Harel, and Ben-Shaul (1966), using an oxygen electrode.

*PME* activity was determined as previously described by following the hydrolysis of *p*-nitrophenyl acetate (Bar Nun et al., 1996), using double controls, of substrate alone and enzyme alone. This was necessary because the enzyme preparations contained phenolic compounds and a flavonoid, which developed yellow colours at pH 7.0, over time.

*Polygalacturonase* activity was followed by measuring the release of reducing groups from polygalacturonic acid (Bernfeld, 1955), again using two controls, enzyme alone in buffer and substrate alone in buffer, because the substrate polygalacturonic acid was not stable over time and the enzyme preparations released reducing groups over time in the absence of subtrate.

### 4.2. Enzyme extraction

After testing different ways of extracting enzyme from the tissue, we adopted the following procedure. Tissue (3 g) was ground with 5 ml 10 mM acetate buffer, pH 4.5, containing 10 mM NaCl. To this buffer we added 2 mM Dieca, 1 mM DTT and 10% solid polyvinyl pyrrolidone in order to reduce polyphenoloxidase activity as far as possible. Such extracts were extremely viscous, due to a high content of pectin in the tissue. The extracts were incubated with pectolyase (Sigma-P-3026, pectin-transeliminase) 1 μg/ml overnight and then centrifuged to remove precipitates. Such extracts were used for further purification of PME. In the case of polygalacturonase, crude extracts were used for preliminary screening.

### 4.3. Electrophoresis

We routinely followed the separation of crude and partially purified extracts using electrophoresis under non-denaturing or denaturing conditions according to Laemmli (1970), using 4% stacking and 10% resolving acrylamide gel. Gels were stained for protein using Coomassie Blue R 250, or silver staining (Morrissey, 1981) and glycoprotein was detected as described in Zaccharius, Zell, Morrison, and Woodlock (1969).

For detection of PME the gels were brought to the correct pH as described by Ben-Hod, Bar Nun, Tzaban, and Mayer (1997), then incubated with 0.6% pectin (Sigma P-9135, 8.9% methoxy groups) and then stained with Ruthenium Red (Karmona, Bar Nun, & Mayer, 1990; Lisker & Retig, 1974). However, the detection of PME activity on acrylamide gels, after PAGE–SDS separation and removal of the SDS according to Blank et al. (1982) was extremely difficult. In the presence of SDS it was impossible to detect activity on the gels and inhibition of PME by SDS has been reported (Zimmerman, 1978). We compared three different methods of detecting PME activity on gels. One method was that using Ruthenium Red staining after incubation with pectin, the second was that using exposure of the gels to *p*-nitrophenyl acetate (Moustacas, Nari, Diamantidis, Noat, & Crasnier, 1986) and the third was the method using detection of esterase activity, with a mixture of a and b naphthyl acetate followed by staining with Fast Blue BB (Rosendahl & Sen, 1900). Various amounts of pectin (from orange—Sigma P-0764) were applied to gels and the gels run in the usual way. SDS was removed from the gels using isopropanol, brought to pH 7.0 and then stained with each of the three methods.

Using the Ruthenium Red method, the minimal amount of PME activity on the gel which could be detected was

approximately ten units. Using the *p*-nitrophenyl acetate method, commercial PME could be detected using about 1 unit of activity per lane. However, staining was smeared and no clear bands could be detected. In contrast, the naphthyl acetate method detected esterase activity of PME at as little as 0.27 units per lane.

We calibrated the colourimetric method of determining PME activity, after measuring the specific absorbtion of pure *p*-nitrophenol which was found to be 8300 at 400 nm, at pH 7.0 in 10 mM phosphate buffer. From this we determined the enzyme activity of our extracts in absolute units. The results showed that our purified fractions contained of the order of 1.5 units/ml (a unit being defined as the hydrolysis of substrate at the rate of 1 μmol/min). This activity was in the range of sensitivity of the esterase method, but a least one order of magnitude too low for the Ruthenium Red method.

### 4.4. Column chromatography

A variety of columns were tested in order to purify PME from dodder. Among the columns tested were: Sephacryl S 200, CM Sephadex, DEAE Sephadex A 25 and CM Sephadex C 50. None of these gave satisfactory results, except for Sephacryl, and this was used in some of the experiments. In most cases, we resorted to separation on Biogel P-100, fine. Biogel columns were eluted with 50 mM acetate buffer, pH 4.5. Biogel columns were calibrated using thyroglobulin, carbonic anhydrase, albumin and alcohol dehydrogenase.

Con A Sepharose columns were equilibrated with $P_i$ buffer, pH 6.5 containing 0.15 M NaCl and 1 mM $CaCl_2$ and $MnCl_2$. Columns were eluted stepwise, with equilibration buffer followed by 0.2M and 0.5 M α-methyl mannoside.

### 4.5. Concentration of the enzymes

A variety of methods were used, including the use of Amicon PM 30 or YM 30 membranes. Ultrafiltration invariably resulted in considerable loss of enzyme activity, presumably due to attachment to the membrane. Concentration using Viva spin tubes was used in a number of steps during the purification procedure.

Since *Cuscuta* extracts contain large amounts of phenolics, as far as possible these had to be removed prior to protein determination using a modified Lowry method (Shakir, Audilek, Drake III, & Shakir, 1994). The use of Pharmacia Hi-Trap columns was adequate, but diluted the enzyme and we therefore routinely used dialysis overnight for this purpose.

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## References

Ashton, F. M., & Santana, D. (1976). *Bull. 1880*. University of California: Department of Agriculture, 24 pp.
Bakos, A., Fari, M., Toldi, O., & Lados, M. (1995). *Plant Science*, *109*, 95.
Bar Nun, N., Ben Hod, G., Lavi, E., & Mayer, A. M. (1996). *Phytochemistry*, *41*, 403.
Ben-Hod, G., Bar Nun, N., Tzaban, S., & Mayer, A. M. (1997). *Phytochemistry*, *45*, 1115.
Bernfeld, P. (1955). *Methods in enzymology* (Vol 1, p. 144). S. P. Colowick, & N. O. Kaplan (Eds.). London: Academic Press.
Blank, A., Sugyama, R. H., & Dekker, C. A. (1982). *Analyt. Biochem.*, *120*, 267.
Bordenave, M., & Goldberg, R. (1993). *Phytochemistry*, *33*, 999.
Bordenave, M., & Goldberg, R. (1994). *Plant Physiol.*, *106*, 1151–1156.
Bordenave, M., Breton, C., Goldberg, R., Huet, J.-C., Perez, S., & Pernollet, J.-C. (1996). *Plant Mol. Biol.*, *31*, 1039.
Dawson, J. H., Musselman, L. J., Wolswinkel, P., & Dorr, I. (1994). *Rev. Weed Sci.*, *6*, 265.
Dinelli, G., Bonetti, A., & Tibiletti, E. (1993). *Weed Research*, *33*, 253.
Ebbelaar, M. E. M., Tucker, G. A., Laats, M. M., van Dijk, C., Stolle-Smits, T., & Recourt, K. (1996). *Plant Mol. Biol.*, *31*, 1141.
Ehrenfeld, K., Baumel, P., Czygan, F.-C., & Proksch, P. (1996). In M. T. Moreno, J. I. Cubero, D. Berner, D. Joel, L. J. Musselman, & C. Parker (Eds.), *Advances in Parasitic Plant Research* (pp. 347–352). Junta de Andalucia Consejero de Agricultura y Pesca.
Furuhashi, K., Kanno, M., & Morita, T. (1995). *Plant Cell Physiol.*, *36*, 533.
Gaffe, J., Tieman, D. M., & Handa, A. K. (1994). *Plant Physiol.*, *105*, 199.
Glover, H., & Brady, C. (1994). *Phytochemistry*, *37*, 949.
Goldberg, R., Pierron, M., Durand, L., & Mutaftschiev, S. (1992). *J. Expt. Bot.*, *43*, 41.
Istudor, V., Predescu, I., Popa, E., Badoi, F., & Sialvara, S. (1984). *Farmacia*, *32*, 173.
Karmona, Z., Bar Nun, N., & Mayer, A. M. (1990). *Journal Intern. de Science de la Vigne et du Vin*, *34*, 103.
Laemmli, U. K. (1970). *Nature*, *227*, 680.
Lisker, N., & Retig, N. (1974). *J. Chromatography*, *96*, 245.
Loo, S. W. (1996). *Am. J. Bot.*, *33*, 294.
Losner-Goshen, D., Portnoy, V. H., Mayer, A. M., & Joel, D. M. (1998). *Ann. Bot.*, *81*, 319.
Mayer, A. M. (1987). *Phytochemistry*, *26*, 11.
Mayer, A. M., & Harel, E. (1979). *Phytochemistry*, *18*, 193.
Mayer, A. M., Harel, E., & Ben-Shaul, R. (1966). *Phytochemistry*, *5*, 783.
Morrissey, J. H. (1981). *Analyt. Biochem.*, *117*, 307.
Moustacas, A.-M., Nari, J., Diamantidis, G., Noat, G., & Crasnier, M. M. (1986). *Eur. J. Biochem.*, *155*, 191.
Murashige, T., & Skoog, F. (1962). *Physiol. Plant*, *15*, 473.
Nagar, R., Singh, M., & Sanwal, G. B. (1984). *J. Expt. Bot.*, *35*, 1104.
Raven, J. A. (1983). *Adv. Ecological Res.*, *13*, 136.
Rosendahl, S., & Sen, R. (1900). *Methods in Microbiology*, *24*, 176.
Shakir, F. K., Audilek, D., Drake III, A. A., & Shakir, K. M. M. (1994). *Analyt. Biochem.*, *216*, 232.
Schmucker, Th. (1959). *Encyclopedia of Plant Physiology* (Vol. 11, p. 481).
Srivasta, S., Nighjkar, A., & Kumar, A. (1994). *Phytochemistry*, *37*, 1233.
Warrilow, A. G. S., Turner, R. J., & Jones, M. G. (1994). *Phytochemistry*, *35*, 863.
Zaccharius, R. M., Zell, T., Morrison, J. H., & Woodlock, J. J. (1969). *Anal. Biochem.*, *30*, 148.
Zimmerman, R. E. (1978). *Anal. Biochem.*, *85*, 219–223.