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A cofactor requirement for polygalacturonase from *Cuscuta campestris*

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Abstract

The occurrence of a polygalacturonase in tissue of *Cuscuta* is reported. The enzyme activity requires the presence of a cofactor, which can be removed by dialysis. The cofactor is heat stable and extractable in 80% methanol or in petroleum ether. It appears to be a low molecular weight peptide, since its activity is lost on incubation with proteinase K or papain. The exact nature and the possible function of this co-factor in the breakdown of pectins by *Cuscuta* are under investigation.

Keywords: *Cuscuta campestris*; Convolvulaceae; Polygalacturonase; Cofactor for activity; Peptide; Characterisation

1. Introduction

We believe that pectin breakdown is an important step during the invasion of the host by dodder (*Cuscuta campestris*). Endo- and exo-polygalacturonases are among the enzymes, which may be involved in this process. We have previously shown the involvement of these enzymes in host infection by the root parasite *Orobanche* (Ben-Hod, Bar Nun, Tzaban & Mayer, 1997). Much of the literature relevant to PGase in parasitic plants is discussed in Ref. (Ben-Hod et al., 1997). We have already described some of the properties of a pectin methyl esterase from *Cuscuta* and shown that it had some unusual features (Bar Nun & Mayer, 1999). This led us to study of the involvement of polygalacturonase in host infection by *Cuscuta*, since it is the second enzyme in the sequence of steps involved in the degradation of pectin. The occurrence of this enzyme in *Cuscuta* has been reported, but it was never adequately characterised (Nagar, Singh & Sanwal, 1985). The presence of an inhibitor of polygalacturonase in the host of a *Cuscuta* has been reported by Singh and Singh (1997), who also show that an activator of xylanase may be present in the host tissue. The presence of an inhibitor of polygalacturonase has also been shown in *Orobanche* tissue (Ben-Hod et al., 1997). Here, we report on what appears to be a co-factor requirement for activity of a polygalacturonase from *Cuscuta*.

2. Results

It was first shown that crude extracts from *Cuscuta* released reducing groups when incubated with polygalacturonic acid, suggesting the presence of a polygalacturonase. This was confirmed using a viscometric assay. In the presence of 0.5% polygalacturonic acid, M_w 25,000–50,000 (Serva) 0.4 ml crude extract reduced viscosity by 11.76% after incubation for 30 min at pH 7.2 in 10 mM Pi buffer. This result suggest that the activity followed was due to an endogalacturonase. However, a viscometric assay was not possible as a

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Table 1
PGase activity of crude dialysed extracts of *Cuscuta* in presence of boiled crude extracts[a]

Reaction mixture	Enzyme activity/1.0 ml dialysed extract
Crude extract	4.6
Crude extract, dialysed	0
Dialysed extract + 0.1 ml boiled extract	0
Dialysed extract + 0.2 ml boiled extract	3.6
Dialysed extract + 0.3 ml boiled extract	7.6
Dialysed extract + 0.4 ml boiled extract	7.6
Dialysed extract + 0.5 ml boiled extract	10.8

[a] Enzyme activity of dialysed extracts was examined in the presence of various amounts of boiled extracts. Incubation for 22 h with 0.5% polygalacturonic acid. Activity as micromol reducing groups released per ml dialysed extract per 22 h.

routine procedure. Routinely release of reducing groups was, therefore, followed.

The first indication of the unusual behaviour of polygalacturonase from *Cuscuta* was the observation that crude extracts completely lost the ability to release reducing groups from polygalacturonic acid if they were dialysed for 24 h against 10 mM NaCl in dialysing tubing with a cut off of 12,000. This was not due to instability of enzyme activity, which was retained for 6 days at 5°C. Similarly, precipitation of protein with 80% saturated ammonium sulphate, followed by resuspension of the precipitates in buffer resulted in total loss of activity.

When a crude extract, boiled for 30 min, was added back to a dialysed crude extract in a ratio of one to one, PGase activity was fully restored to the extract, as measured by the release of reducing groups from polygalacturonic acid. The ability to restore activity was strictly proportional to the amount of boiled extract added to the dialysed preparations (Table 1). The restoration of activity was not related to simple cofactors. Addition of the adjuvants present in the grinding medium (1 mM DTT and 2 mM DIECA) did not restore activity to dialysed extracts. Addition of cations such as calcium, or potassium at 5 mM was also ineffective. These results seemed to indicate that a putative factor was responsible for restoration of PGase activity which had a M_w of less than 12,000. When dialysis was carried out in tubing with a cut off of 1200 for 48 h, full PGase activity was retained in the extract, indicating a M_w between 1200 and 12,000. We were able to define the size more precisely using either Pharmacia HiTrap desalting columns (containing Sephadex G25) or Vivaspin tubes (disposable ultrafiltration devices with a polysulphone membrane). Passage of boiled extracts through a HiTrap column with a cut off of approximately 6000 resulted in loss of the ability to restore PGase activity. Centrifugation of active extracts through a Vivaspin tube with a cut off of 5000 resulted in retention of activity. These results together indicate that our active factor had a M_w between 1200 and 5000. The factor might be a carbohydrate. To explore this question boiled extracts were passed through a ConA Sepharose column. The column was equilibrated with Pi buffer, pH 6.5 containing 0.15 M NaCl, 1 mM $CaCl_2$ and $MnCl_2$. It was eluted with 10 mM Pi buffer, pH 7.1, and the eluate then tested for its ability to restore PGase activity to dialysed extracts. The eluates from the column did restore PGase activity of dialysed extracts. This makes it unlikely that a carbohydrate containing either mannose or glucose is involved, but does not rule out other carbohydrates. Examination of the active fractions from boiled extracts before and after acid hydrolysis for the presence of carbohydrates, using TLC on Polygram Cel 400 plates did not reveal any sugars, the detection limit being 2 μg.

Incubation of methanolic extracts (see below) with a

Table 2
Effect of proteinase K on the ability of boiled extracts to restore PGase activity to dialysed preparations from *Cuscuta*[a]

Treatment of extract	Enzyme activity/1.0 ml extract
Crude extract	3.0
Boiled crude extract	0
Dialysed crude extract	0
Dialysed crude extract + boiled crude extract	7.2
Dialyed crude extract + boiled crude extract + 5 units of proteinase K	0

[a] Crude dialysed extracts were prepared as described. Boiled extracts were incubated for 30 min with or without proteinase K at 37°C. After incubation the extracts were again boiled and then added to dialysed extracts and PGase activity followed. Incubation for 22 h with 0.5% polygalacturonic acid. Activity as micromol reducing groups released per ml dialysed extract per 22 h.

Table 3
Methanolic extraction of the activating factor[a]

Treatment of extract	Activity/1.0 ml original extract
Dialysed crude	0
Dialysed crude + 80% MeOH extract from tissue dissolved in buffer	4.6
Extraction of tissue with 100% MeOH. The residue extracted with buffer and extract tested for activity	1.6
Residue from 100% MeOH extraction extracted with 80% MeOH, and then suspended in buffer. Suspension tested for activity	0.46
Residue of the extract with 100% MeOH redissolved in buffer and added to dialysed crude	2.92

[a] Tissue was extracted with methanol. The methanolic extracts were evaporated to dryness and the residues taken up in buffer and their activity tested on dialysed crude preparations. Incubation for 22 h with 0.5% polygalacturonic acid. Activity as micromol reducing groups released per ml dialysed extract per 22 h.

mixture of one unit each of glycosidase a (Type IV from Brewers yeast, Sigma) and b (from almonds, Fluka) at 29°C in 10 mM Pi buffer, pH 7.0 for 45 min did not change their ability to restore activity. In contrast incubation of boiled extracts with proteinase K (form *Tritirachium album*, Boehringer Mannheim) destroyed their ability to restore PGase activity of dialysed crude extracts (Table 2). Extracts were incubated with one, two or five units of proteinase K for 30 min at 37°C. Suitable controls were run at all times which included measurement of reducing groups at zero time (control) and after 22 h incubation (experiment). In all cases we incubated enzyme (with or without activator), alone, substrate alone and a mixture of enzyme and substrate in the presence or absence of activator. The proteinase K alone did not act as a factor restoring PGase activity, although it apparently caused a small release of reducing groups after 22 h from boiled crude extracts. In these experiments, extracts treated with proteinase K were boiled before addition to dialysed extracts in order to avoid inactivation of enzymatic activity by proteolysis. Similar experiments using papain (from papaya latex, Sigma), one or two units at 25°C for 30 min gave the same result, i.e. loss of activity. All these experiments were carried out at pH 7.0. All together these results would indicate that the factor lost during dialysis of crude extracts is a low molecular weight peptide, stable to boiling.

Further information, confirming this possibility was obtained as follows. *Cuscuta* tissue was extracted with 80% methanol. The methanol was removed in a stream of nitrogen and the residue dissolved in phosphate buffer, 10 mM, pH 7.0. The resultant solution was then added to crude dialysed extracts. PGase activity was fully restored to dialysed crude extracts by this preparation (Table 3) consistent with the idea that a methanol soluble, heat stable low molecular weight peptide is responsible for restoration of activity in dialysed extracts. In further experiments we prepared a boiled crude extract, which was then concentrated five fold by using a Vivaspin tube with 5000 cut-off. Attempts to further define the molecular weight of the active factor using gel filtration on Biogel P-6, Fine, columns were not successful. Elution resulted in recovery of activity in a single peak. However, the molecular weight of this peak is in doubt, although it eluted after one of the markers used, the oxidised b chain of insulin, the other markers used being aprotinin, cytochrome c and vitamin B12. Since the markers did not give clear peaks and we did not adequately determine the void volume of the column, this result is of limited value, although consistent with a M_w of about 3000–5000.

In order to better understand the nature of the activating factor extracted using 80% methanol, further experiments were conducted. *Cuscuta* tissue was

Table 4
Effect of extraction with petroleum ether on PGase activity of crude extracts from *Cuscuta*[a]

Treatment	Enzyme activity/1.0 ml extract
Experiment 1	
Crude extract	2.6
Crude dialysed extract	0
Crude extract, treated with petroleum ether	1.6
Petroleum ether extract, evaporated and tested after resuspension	0
Experiment 2	
Crude extract	3.0
Crude dialysed extact	0
Petroleum ether extract, evaporated and redissolved in buffer pH 4.5 + dialysed extract	1.72
Petroleum ether extract, evaporated and redissolved in buffer pH 7.0 + dialysed extract	1.96

[a] Tissue was extracted with buffer and the boiled homogenate treated with petroleum ether bp 40–60°C at room temperature. The petroleum extracts were evaporated in a stream of nitrogen and the residue redissolved in buffer at pH 4.5 or 7.0. Incubation for 22 h with 0.5% polygalacturonic acid. Activity as micromol reducing groups released per ml dialysed extract per 22 h.

extracted directly with either 100% or 80% methanol, or sequentially with 100% and then 80% methanol. The alcoholic extract discarded and the residue extracted with buffer as before. The activity was then tested (Table 3). Extraction of the tissue using 100% methanol appeared to inactivate enzyme activity. The activity of extracts of residues from the methanol treatment was negligible. The addition of extracts of this residue to dialysed crude extracts restored about 60% of the activity (Table 3). Residues after extraction with 100% MeOH were re-extracted with 80% MeOH. The resultant residue after suspension in buffer had negligible enzyme activity. However, the methanol extract itself was able to restore activity to dialysed crude preparations. It, therefore, seems that activity was indeed 80% methanol soluble, but methanol treatment inactivates the enzyme.

The nature of the flavanoids and phenolics in *Cuscuta* is still not fully established, but it has been shown by Loffler, Sahm, Wray, Czygan and Proksch (1995), that some of them are extractable with petroleum ether. These phenolics interfere in purification of enzymes and give background colour development in estimation of PGase activity. We, therefore, conducted some experiments using petroleum ether to treat our crude extracts, with unexpected results.

Extraction was carried out using petroleum ether bp 40–60°C. Extraction of crude preparation with petroleum ether results in a 33% loss of their PGase activity. Some of the ability to restore the activity of dialysed preparations could be recovered from the petroleum ether extract, when these were evaporated and resuspended in buffer (Table 4) but these petroleum ether extracts were not able to completely restore the PGase activity of dialysed preparations. These results in combination could indicate that a least part of the ability to restore PGase activity to dialysed preparations could be ascribed to a factor, which is partially soluble in an apolar solvent. Electrophoresis of such preparations in 4–17.5% acrylamide gels, after concentration, showed the presence of a number of low molecular weight bands, which stained with Coomassie Blue indicating that they were protein or protein derived. Analysis of such preparations, using a modified Folin–Ciocalteu reaction (Shakir, Audilek, Drake & Shakir, 1994) also indicated the presence of protein. Unfortunately, due to the presence of phenolics in these extracts, which interfere with assay of protein, absolute amounts of protein were difficult to establish, even after dialysis. In these experiments it should also be noted that the crude extracts themselves showed some variability in their PGase activity. This is probably due to the fact that the plant material collected in the field was not homogeneous and different samples differed in their turgor, which would affect the weight of the tissue actually used for the different experiments.

We removed part of the interfering phenolics using Polyclar AT (insoluble polyvinyl pyrolidone). Absorption of the 80% methanolic extract on the column followed by elution with 80% methanol, resulted in 46–75% recovery of the activating compounds in a single fraction. Removal of the bulk of the phenolics could be confirmed from UV absorption spectra of the purified fraction.

Attempts at purifying the cofactor using reverse phase HPLC procedures are now underway. The cofactor was applied to a C4 reverse phase column and the column eluted with water and a gradient of acetonitrile, containing trifluoroacetic acid. Little or no binding, occurred and the cofactor was eluted as a single fraction, with a retention time of 6.59 min. Continuation of elution for upto 90 min did not reveal any additional active fractions.

3. Discussion

The occurrence of a cofactor requirement for the polygalacturonase activity of extracts of dodder, appears to be novel and unexpected. At this stage the data indicate that the cofactor is a low molecular weight hydrophilic peptide or a very small protein molecule. The fact that some activation activity could be extracted into petroleum ether is at variance with this conclusion. However, the amount of activator extractable into petroleum ether was quite small. It is possible that more than one factor is involved. However, until the cofactor has been isolated and identified conclusions about its precise nature remains tentative. For this reason it also seems premature to speculate about the possible function of a cofactor needed for PGase, although it could easily play a role in regulating the activity of pectin breakdown. Previous results with broomrape (Ben-Hod et al., 1997; Bar Nun & Mayer, 1999) lend some plausibiliy to such a suggestion. The biological function of the cofactor was supported by a simple experiment. One of the hosts of *Cuscuta* is *Pelargonium graveolens*. The stems of the parasitic plant coil around and infest the petioles of the leaves of the host. We extracted such petioles with 25% MeOH and added the extracts, before or after boiling, to dialysed preparations from *Cuscuta*. Addition in both cases resulted in reactivation of PGase activity of the dialysed extracts. This finding has a major implication. The heat stable factor activating PGase is present not only in tissue of the parasite but also in host tissue. This could imply that the activity of PGase, secreted by the parasite, may be regulated by substances present in the host. It may be speculated that such a regulatory mechanism might be involved in preventing dissolution of cells of the parasite by its own secretory enzymes.

4. Experimental

In this study we used *Cuscuta campestris* collected in the field as previously described (Bar Nun & Mayer, 1999). The tissue was extracted with 10 mM acetate buffer, pH 4.5 containing 10 mM NaCl and 2 mM Dieca, 1 mM DTT and 10% solid polyvinyl pyrrolidone as previously described (Bar Nun & Mayer, 1999). Such crude extracts were dialysed for 24 h against 10 mM NaCl to provide the dialysed extract. Such extracts contained between 150 and 250 μg protein/ml depending on the precise batch of extract. Polygalacturonase activity in extracts was followed by measuring the release of reducing groups from polygalacturonic acid using the Bernfeld (1955) reaction as previously described (Ben-Hod et al., 1997). In all cases controls of enzyme alone, or enzyme with addition of cofactor as well as substrate alone were run at zero time and after the 22 h incubation period. Activity was determined from colour formation in the full reaction mixture minus the two controls, resulting perhaps in an underestimate of activity. The zero values reported are due to this calculation. In some experiments, we also followed reduction of viscosity of solutions of polygalacturonic acid as described in Karmona, Bar Nun & Mayer, (1990).

Extraction of the cofactor with methanol or petroleum ether was carried out in the cold, and the solvents evaporated in a stream of nitrogen to prevent possible oxidative processes. Gel filtration was carried out on Biogel P-6, using 50 mM acetate buffer, pH 7.0 as the eluant. Electrophoresis was carried out as described in Laemmli, (1970), but using 4–17.5% gels.

Conditions for HPLC separation of the co-factor.

Dried samples were dissolved in water containing 0.1% trifluoracetic acid (TFA), briefly sonicated and centrifuged prior to analysis by reverse phase HPLC (Alliance-Waters), equipped with an automatic injector, photodiode array UV detector and Millenium integration software. HPLC runs were performed on an analytical column (VYDAC, C4, 250 × 4.6 mm) using a linear gradient 0–60% of acetonitrile/water (1% per min, starting 5 min after sample injection, flow rate 0.5 ml/min.) both solvents containing 0.1% TFA. Alternatively, runs were performed on a Narrow Bore column (VYDAC, C18, 250 × 2.1 mm, flow rate 0.1 ml/min) under isocratic conditions (100% water 0.1% TFA).

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