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INHIBITORS OF POLYGALACTURONASE IN CALLI OF *OROBANCHE AEGYPTIACA*

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Key Word Index—*Orobanche aegyptiaca*; Orobanchaceae; calli; polygalacturonase; inhibitors.

Abstract—The presence of at least three distinct polygalacturonases (PGase) in callus of *Orobanche* was demonstrated. The PGase activity is labile and at pH 4.5 does not require activation by cations. It can be partially purified on Biogel P 100 columns and can be resolved by PAGE into several bands. Broomrape callus tissue also contains inhibitors of PGase activity. One of these is a low M_r compound, stable to boiling and removable by dialysis. An additional inhibitor precipitable by ammonium sulphate is also present. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

During attachment to and penetrating of the root parasite *Orobanche* (broomrape) into its host there is little evidence for the destruction of host root cells. The parasite apparently forces its way between host cells, a process which may be facilitated by dissolution of the pectins which bind adjacent cells. Pectin methyl esterase (PME) may be involved in this process [1]. PGase (polygalacturonase) could be another enzyme taking part in this dissolution process. PGase activity is present in all parts of mature *Orobanche* tissue, e.g. in the tubercle, but the enzyme has never been characterised and its function during infection of the host has not been studied [2].

Both endo- and exo-polygalacturonases are widespread enzymes involved in fruit ripening, cell growth and in fungal pathogen–plant–host interactions [3–10]. Both usually attack only polygalacturonic acid and not its methyl ester, so that *in vivo* their activity is closely linked with that of PME.

The presence in plant tissues of inhibitors of polygalacturonases is well documented. Some of these inhibitory substances were shown to be proteins [11–14]. The ability of phenolic compounds or their oxidation products to inhibit polygalacturonase is also well known [15, 16]. It is usually assumed that these PGase inhibitors are involved in the host–parasite interaction during infection of plant tissues by pathogens. The possibility that phenolic compounds are involved in the interaction of *Orobanche* with its host must, therefore, be considered. The presence of caffeic acid glycoside esters, which have pharmacological activity, in *Orobanche* tissues is well established [17]. In addition, *Orobanche* seeds are known to contain a flavonoid compound, tricin [18]. In the following we show the occurrence of three polygalacturonases in calli of *Orobanche* and provide evidence for the presence of inhibitors in the tissue.

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RESULTS AND DISCUSSION

In order to show unequivocally the presence of polygalacturonase in calli of *Orobanche* we used three different methods: the cup-plate method [19, 20], viscometry [21] and the liberation of reducing groups using the Bernfeld procedure [22].

Demonstration of PGase activity using the cup-plate method

For rapid screening of activity we employed the cup-plate method. Such screening indicated that extracts of calli had low PGase activity. PGase activity has been reported to increase in response to treatment with ethylene in the abscission zone [10]. We examined whether PGase in *Orobanche* responded to ethylene. The addition of ethrel to liquid media did not increase their PGase activity. More PGase activity diffused out from calli grown on solid agar than from calli obtained from liquid culture medium. The results also indicated that when the tissue is fractured, activity decreases, which points to the liberation of an inhibitor or instability of the enzyme or both.

Calli grown on agar containing 1.44 μM GA_3, with polygalacturonic acid as substrate showed PGase

Table 1. Dependence of polygalacturonase activity in *Orobanche* extracts on pH using pectin or polygalacturonic acid as substrate. Assay using the cup-plate procedure

Substrate	Polygalacturonic acid		Pectin	
	Activity after (hr)			
pH	24	72	24	72
4.0	++	++	−	−
4.5	++	++	−	−
5.0	+	+	−	−
5.5	++	++	−	−
6.0	±	±	−	−
6.5	±	±	−	−
7.0	−	±	−	+
7.5	++	++	±	++
8.0	+	++	+	++

A control using the commercial PGase from *Aspergillus niger* (Sigma) gave activity, as expected only at pH 5.5, and only when the substrate was polygalacturonic acid. This confirmed the legitimacy of the method.

The calli or extracts prepared by crushing the calli were placed on 1% Difco agar, in Petri dishes. The various pH values were attained by the use of phosphate citrate buffer, 20 mM.

− Total lack of activity.

± indicates a white zone at site of application indicating very weak activity.

+ Some activity, with a small clear zone.

++ Activity, with defined clear zone.

activity at three pH values, 4.0–4.5, 5.0–5.5 and 7.5–8.0 (Table 1). When pectin, 68% esterified, was used as the substrate, activity could only be detected at pH 7.5–8.0 and activity increased with time of incubation. This observation is consistent with the idea that at the higher pH, degradation of substrate is dependent on the prior action of pectin methylesterase, which has a pH optimum around 7.5 as shown previously.

Increased PGase activity could not be induced by adding polygalacturonic acid or pectin with various degrees of esterification to cultures of calli for periods of 24–48 hr prior to assay of activity.

Demonstration of PGase activity using viscometry

The cup-plate method does not give a completely quantitative measure of PGase activity. We, therefore, compared the results of this assay with those obtained using viscometry (Table 2). PGase activity in *Orobanche* extracts was clearly present. These results also confirm those of the experiments using the cup-plate method, showing that three distinct enzymes differing in pH optima are present. The presence of 10 mM KCl in the extraction medium reduced activity by 75%, either by inhibiting acitivty or due to failure to extract the enzyme. NaCl improved extraction of the activity at pH 5, but decreased extraction at pH 4.2. We have previously shown that pectinmethyl esterase

Table 2. PGase activity in extracts from *Orobanche*. Results as % change in viscosity of substrate after 30 min

Extraction medium	pH 4.2	pH 5.0	pH 8.0
Pi–citrate buffer only	13	6.7	8.7
Pi–citrate + 10 mM NaCl	5.5	19	—
Pi–citrate + 10 mM KCl	0	0	—

—Not examined.

activity from *Orobanche* was stimulated by K^+ [1]. The inhibition of PGase by K^+ was therefore unexpected. Usually PGase is tightly bound to the cell wall and extracted with high Na^+ concentrations. However, the PGase which we suggest is involved in host–parasite interaction, should not be bound so tightly. It was readily extracted with 10 mM NaCl. The relation between K^+ inhibition and possible biological function remains unclear.

Effect of cations on PGase activity

The inhibition by KCl during extraction prompted us to study the effect of cations in the reaction mixture. Passing extracts through a HiTrap (Pharmacia) column to remove inorganic salts reduced enzyme activity from 23.4% decrease in viscosity before treatment to 2.6% after passage through the column. Addition of 40 mM KCl to the reaction mixture increased the activity slightly to 8.1%, but did not restore it to the level prior to HiTrap treatment. Repeti-tion of these experiments, using the Bernfeld method, showed that PGase activity was almost totally inhibited by KCl. The cause of the discrepancy between the effect of KCl in the two methods is not clear. The use of the Bernfeld procedure confirmed the presence of PGase activity at pH 4.5. Dialysis of crude extracts, using tubing with a cut-off point of 12 000–14 000 reduced PGase activity almost to zero, whether assayed viscometrically or by the Bernfeld assay. Dialysis on Amicon YM 3 membranes, cut-off point 3000, also caused a loss of 90% of the activity. The addition of 40 mM K^+, or 1 mM Mg^{2+}, Mn^{2+} or Ca^{2+}, to extracts dialysed through YM 3 membranes did not restore activity to the levels before dialysis, and the effects observed were marginal, within the limit of error of the Bernfeld procedure following 22 hr incubation. The loss of activity following dialysis, and also loss of part of the activity during concentration using ultrafiltration, cannot be ascribed to the loss of a cation during treatment. The PGase active at pH 4.5 does not require the presence of cations. High concentrations of Na^+, above 0.1 M, inhibited enzyme activity.

Purification of PGase on Biogel P 100 Columns

A typical separation is shown in Fig. 1. Concentrations of the crude extracts before application to

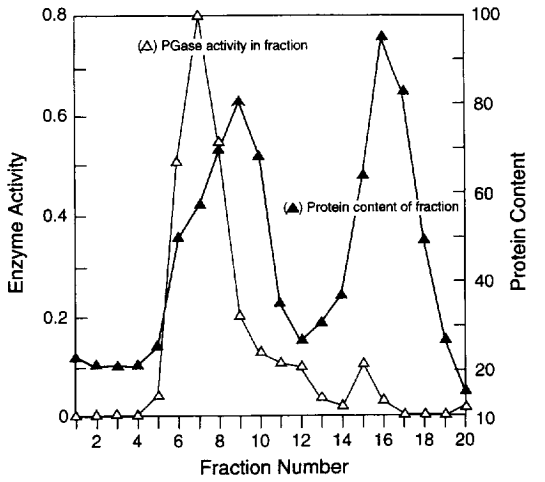

Fig. 1. Separation of a crude, concentrated extract from *Orobanche* on a Biogel P 100 fine column. Column 1.4 cm diameter × 50 cm. Two millilitre fractions collected △—△ PGase activity as δ OD per 0.5 ml eluate after 22 hr incubation with substrate, 0.5% polygalacturonic acid, M_r 4000 (Sigma). ▲—▲ Protein μg 0.5 ml^{-1}. Sp. act. of crude extract–3.2; Sp. Act. of concentrated extract–1.14; Sp. act. of fraction 6–10.08; fraction 7–14.0; fraction 8–7.8.

Table 3. Distribution of PGase activity along an acrylamide gel. The resolving gel, was cut into sections after removal of the stacking gel. Incubation was with 0.5% polygalacturonic acid M_r 4000 for 22 hr at pH 4.5 in acetate buffer. All results were brought to a common denominator of equal volume. Sp. act. is expressed as δOD–mg protein in the gel section

Position on gel	δOD of gel section	Sp. act. in section
Crude extract	0.319	1.6
Top 1 cm	0.192	4.0
2nd cm from top	—	—
3rd cm from top	0.0024	—
4th cm from top	0.019	—
Top cm subdivided		
3.3 cm from top	0.26	1.08
3.3–6.6 mm from top	0.067	1.395
6.6–10 mm from top	0.069	5.75

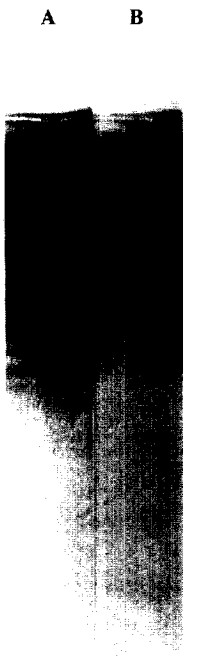

Fig. 2. Electrophoretic separation and immunoblotting of crude extract of *Orobanche* from liquid culture. Resolution of gel using a 4% stacking gel and 10% acrylamide resolving gel. All other procedures as in the Experimental. (A) Gel stained with Coomassie Blue. (B) Immunoblot of the gel after Western blotting using *Botrytis* PGase antibody.

the column was accompanied by a loss of activity. Separation on the Biogel resulted in a 14-fold purification compared with the material loaded on the column. The most active fraction from this separation was separated into two protein bands following native PAGE, one with M_r just above, and one just below 66 000 (results not shown). These were detected by silver and Coomassie Blue staining.

Using electrophoretic separations considerable purification of the PGase present in the calli or suspension cultures could be obtained, but recovery was always low. All attempts at staining the gels for enzyme activity were unsuccessful, due to the very small amounts of enzyme which were present. Enzyme protein did not penetrate far into the gel, indicating either that it was of high M_r or that it was a glycoprotein. Because of the apparently very small amounts of enzyme present, staining for glycoprotein gave questionable results.

Crude extracts were separated on acrylamide gels, using the entire width of the gel. Strips of such a gel were stained for protein or immuno blotted, using antibodies against *Botrytis* PGase. The remaining gel was adjusted to pH 4.5 (see Experimental) and then cut into horizontal strips. The strips were suspended in buffer and activity determined using the Bernfeld method (Table 3 and Fig. 2). Enzyme activity is located in a very well-defined section of the gel with a purification of more than five-fold (Table 3).

In the region of the gel which contains PGase activity several protein bands were still present (Fig. 2). Immuno-blotting of the gels shows a number of reacting bands in the region shown in Fig. 2, as well as reacting bands where no activity was found. In the absence of pre-immune serum for the antibody, the relevance of the reaction in regions in which no enzyme activity can be detected is not clear. The antibody available to use was against endo PGase I, produced by *Botrytis cinerea*, prepared in rabbit. This PGase has a low sugar content [4]. The occurrence of the many diffuse bands indicated rather low specificity. We have previously observed multiplicity of bands in pre-immune serum from rabbit when tested against extracts of *Orobanche* [1].

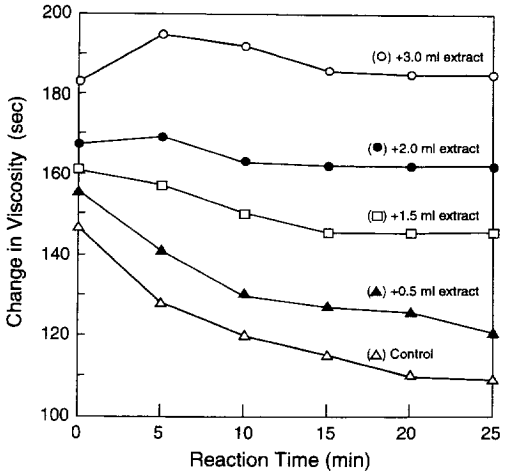

Fig. 3. Effect of varying amounts of *Orobanche* extracts on the activity of commercial PG from *Aspergillus niger*. Extracts were prepared as described in Table 4 and activity followed viscometrically. Note that due to the addition of extracts the reading at zero time, i.e. initial viscosity, increased with increasing amount of extract.

The behaviour of the PGase active at pH 4.5 both on Biogel columns (Fig. 2), and on native gels (Table 3 and Fig. 2), indicates that it has a relatively high M_r, probably between 60 000 and 80 000.

The apparent instability of the PGase we studied supports the view that the PGase has a limited function, during the infection process when the parasite penetrates into the host. A very stable enzyme could perhaps lead to autolysis of the parasite cells, while a labile one would merely act on host cells and then lose activity.

Since some of the results indicated the presence of an inhibitor of polygalacturonase activity attempts were made to verify this.

Demonstration of the presence of an inhibitor

We followed the activity of commercial PGase by viscometry in the presence of extracts of calli. The results in Fig. 3 clearly show that PGase from *Aspergillus niger* is inhibited by extracts from calli from *Orobanche*, and that the degree of inhibition is dependent on the amount of extract added. The inhibitor present could be either a phenolic compound or a polypeptide or protein which have been reported to inhibit PGase activity [12, 15, 23, 24].

The inhibitor present in the extracts of *Orobanche* was partially removed by dialysis and completely so by passage through a HiTrap desalting column (Table 4). This indicated that the inhibitory substance is of low M_r. The fact that boiling the extracts did not significantly reduce inhibition mitigates against a protein being the major factor involved in the inhibition. However, some contribution by a proteinaceous factor cannot be excluded (see later).

Table 4. Decrease in viscosity (%) of a solution of polygalacturonic acid treated with PGase from *Aspergillus niger* in the presence of extracts of calli of *Orobanche*, after various treatments

Treatment	% change in viscosity
Extract + buffer + substrate	0
PGase + buffer + substrate	80.5
PGase + extract + buffer substrate	47.5
PGase + boiled extract + buffer + substrate	58.7
Extract (after HiTrap) + buffer + substrate	2
PGase + extract (after HiTrap) + buffer + substrate	74.4
PGase + boiled extract (after HiTrap) + buffer + substrate	78.8
PGase + extract after dialysis against 10 mM NaCl + buffer + substrate	64.3

Extract = 3 g calli of *Orobanche* ground with 5 ml buffer, pH 4.5, containing 10 mM NaCl and 10 mM Na ascorbate, centrifuged for 10 min at 1000*g* and supernatant used.

Substrate = polygalacturonic acid, Serva M_r 25 000–50 000, 0.4% assay after 20 min, using 0.5 μl commercial PGase in reaction in reaction mixture.

HiTrap = passage of extract through a Pharmacia HiTrap desalting column.

Studies to establish the nature of the PGase inhibitor

The Bernfeld assay of the activity of commercial PGase was used to follow the behaviour of the putative inhibitor present in extracts of *Orobanche* tissue (Table 5). The results in Table 5 confirm that inhibition of the activity of commercial PGase is proportional to the amount of crude *Orobanche* extract added. Again we found that the inhibitor was stable to boiling, and removed by dialysis. The apparent M_r of the inhibitor is below 12 000–14 000. Dialysis against buffer through Amicon YM 3 filters caused a loss of inhibitory activity. Inhibitory activity is partly retained if the extracts are concentrated using ultrafiltration. This could indicate that more than one factor is involved.

Ethanolic extracts of calli did not inhibit commercial PGase activity. When the residue of the cells, after ethanol extraction, was suspended in buffer, part of the inhibitory activity, 25–45% was recovered, under conditions identical to those described in Table 5.

Apparently, the inhibitor is not soluble in ethanol, and it may be bound rather tightly to some component in the cells. The extraction with ethanol was therefore repeated, after prior grinding of the cells in acetate buffer, pH 4.6, 10 mM containing 10 mM NaCl. After grinding the tissue, absolute ethanol was added, the homogenate centrifuged and the supernatant concentrated using a flash evaporator. The residual solution, 2.5 ml, was again tested as described in Table 5. In this case 45% inhibition of PGase activity was obtained, compared with 60% inhibition with an equi-

Table 5. Effect of various amounts of extract of *Orobanche* from suspension culture on the activity of commercial pectinase.

Treatment	Enzyme activity after 1 hr	(δ OD)
Commercial enzyme		0.601
Commercial enzyme	+86 μl extract	0.663
Commercial enzyme	+173 μl extract	0.571
Commercial enzyme	+345 μl extract	0.249
Commercial enzyme	+345 μl boiled crude	0.245
Commercial enzyme	+345 μl crude concn 5× on YM 10	0.385
Commercial enzyme	+345 μl crude concn 5× on YM 30	0.428
Commercial enzyme	+345 μl crude after dialysis	0.704
Commercial enzyme	+345 μl crude, dialysed and conc. 5×	0.693

15 g tissue was ground with 10 mM acetate buffer, pH.4.5, containing 10 mM NaCl and centrifuged at 17000*g* for 10 min. The supernatant, 43 ml, was tested for inhibitory activity. Parts of the crude extract were concentrated five-fold on either a YM 10 or YM 30 Amicon membrane. The reaction mixture contained 125 μl polygalacturonic acid, 0.5% 30 μl pectinase with varying amounts of crude or concentrated extract and buffer to make up the volume to 500 μl.

valent amount of crude extract (see Table 5). This indicates, but does not prove, that the inhibitor is bound to some cell component and is liberated by grinding the tissue in buffer. However, part of the activity could not be recovered.

The experiments using viscometry had already indicated that more than one inhibitor is involved. Crude extract of *Orobanche* tissue were fractionated with ammonium sulphate and two fractions precipitated, between 0 and 35%, and between 35 and 70% saturation. These were tested for inhibitory activity. The 0–35% fraction was devoid of any inhibitory activity. However, the 35–70% saturation fraction gave a small but significant 5% inhibition of commercial PGase. From these experiments it can be deduced that two inhibitors are present, one with M_r below 3000 and one of a protein or polypeptide nature.

Calli grown on solid agar substrate had much lower levels of caffeic acid glycoside esters, compared with those grown in liquid medium. These estimations were made for us by Professor C. Andary (Montpellier). The inhibitory activity of extracts from both types of tissue was therefore compared. Inhibition by the two types of extracts was identical. The low M_r inhibitory compound does not seem to belong to this group of phenolic compounds.

Crude extracts from calli from solid or liquid media, as well as ethanolic extracts gave UV absorption spectra characteristic of caffeic acid glycoside esters. Since the former had inhibitory activity, while the latter did not, this supports the above conclusion. At this stage we have no idea of the nature of the low M_r inhibitor, nor of its cellular location.

The inhibitory compound may be bound to some cell fractions, possibly the cell wall, or released by a chemical or enzymic reaction occurring during grinding. In addition to the low M_r inhibitor we have a clear indication that an additional inhibitor is present. The presence of inhibitors of PGase in various plant tissues is well documented and their occurrence in broomrape is, therefore, not surprising. However, it remains to be shown that such inhibitory compounds have a function in *Orobanche*. One may speculate that such inhibitors protect the parasite from its own PGases during invasion of the host. Again we have no direct evidence for this.

EXPERIMENTAL

Culture of Orobanche. Calli of *Orobanche* were cultured as described in ref. [25], either in liquid medium or on solid medium, with or without addition of GA_3, 0.1 ppm. After harvest, intact tissue, cell suspension or calli were stored at −20°. Over a period of several months no loss of enzyme activity was detected.

Assay of enzyme activity. Enzyme activity was followed viscometrically [21] or by the cup-plate assay as described in refs [19, 20]. Some difficulties were met when following the liberation of reducing groups due to hydrolysis of polygalacturonic acid [22]. Extracts of *Orobanche* browned rapidly, due to the presence of PPO in them. Neither ascorbate nor cyanide could be used to prevent browning, since both interfere in the assay for reducing groups. The use of tropolone as an inhibitor of PPO also did not improve results. Tissue extracts contained large amounts of reducing groups which gave high background readings, which were not stable and changed with time. The use of Pharmacia High Trap minicolumns reduced background readings. Prolonged incubation of enzyme extracts with substrate, 22 hr at 29° with a suitable zero time blank control, gave reliable results.

Extraction of enzyme. The calli or cells were ground in 10 mM NaOAc buffer, pH 4.5 containing 10 mM NaCl. The homogenate was centrifuged at 17000*g* 10 min and the supernatant used as source of enzyme and of inhibitor activity. In some cases other grinding media were used as described in each case.

Substrates for assay of PGase activity were either polygalacturonic acid from Serva, M_r 25 000–50 000 or from Sigma (P 1879, M_r 4000–6000). In tests of PGase activity against pectin, Pomosin, 40% esterification, was used [21]. Amicon membranes and Pharmacia HiTrap columns for desalting were used as recommended by the manufactures.

Purification of enzyme activity on Biogel P 100 columns. An extract of *Orobanche* tissue from a liquid suspension culture was applied after concn on an Amicon YM 30 membrane to a Biogel P 100 column, equilibrated with extraction buffer (10 mM NaOAc buffer, pH 4.5). The column was eluted with the same buffer, frs collected and activity determined using the Bernfeld procedure. Protein in the frs was determined after dialysis for 24 hr against 10 mM NaCl. This sepn was repeated a number of times. Only partial purification was obtained, and a number of PGase peaks were observed, which showed variability with regard to their exact elution pattern.

Electrophoresis of extracts was according to ref. [26] under either non-denaturing conditions or in the presence of SDS. Gels were stained for protein in the usual way with Coomassie Blue R, or with silver stain [27].

Staining for enzyme activity was as described in refs [28, 29]. We calibrated the ruthenium red staining method, using a standard commercial polygalacturonase prepn (Sigma, Pectinase from *Aspergillus niger*). Known amounts were applied to acrylamide gels, sepd and then stained for PGase activity, after incubation of the gels for 1 hr. The minimal amount of commercial enzyme which could just be detected on the gels was 0.0023 units cm^{-1} pocket of the gel on a 1 mm thick gel. This corresponded to a prepn which releases 5.6 μmol galacturonic acid in 24 hr under optimal conditions. The amount of enzyme activity we detected, using the Bernfeld method (or the viscosimetric method) was well below this value.

The very alkaline gels were adjusted to pH 4.6 for assay of enzyme activity by placing them between two electrodes made of stainless steel netting immersed in appropriate buffer and exposed them for 2–3 hr to a low (24 V) constant voltage. This procedure adjusted the pH to that of the buffer without affecting the proteins, as determined by staining with Coomassie Blue. A slight diffusion was observed in the very low M_r proteins, below 15 000.

Immunoblotting was carried out as described in refs [30, 31], using an antibody prepared in rabbits against polygalacturonase from *Botrytis cinerea*, obtained from D. J. Johnston, Scottish Crop Research Institute. Unfortunately we did not have a pre-immune serum for this antibody prepn.

Proteins sepd by PAGE were transferred to nitrocellulose. The nitrocellulose was blocked using non-fat dried milk 1% (Marvel, Cadbury's, U.K.). The antibodies were dilute 1000-fold in Tris buffered saline with 0.01% Tween 20. The nitrocellulose blots were incubated for 1 hr and binding visualized using goat anti-rabbit IgG alkaline phosphatase conjugate, diluted 1000×. The NBT/BCIP colour development system was used.

Extraction of inhibitor. Orobanche tissue (3 g), from liquid culture were extracted directly with 10 ml EtOH, and the whole kept overnight at 4°. The EtOH was sepd from the tissue by centrifugation, and the EtOH removed by flash evapn. The residue was resuspended in NaOAc buffer, to give a soln equal in concn to the extract previously shown to inhibit PGase.

Concentration of enzyme extracts. Attempts to concentrate the enzyme preps were made, using either Me_2CO or $(NH_4)_2SO_4$), pptn in the standard fashion, or Amicon membranes. The use of PM 10 or PM 30 membranes was invariably accompanied by a very appreciable loss of enzyme activity. We were more successful using YM 10 or YM 30 membranes, which usually gave a four-fold concn.

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