Appendix

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CAN GYNOPHORE OF *ARACHIS HYPOGAEA* BE A GOOD SOURCE OF CHITOSANASE?

PALANI, P. AND R. BALASUBRAMAN! N*

Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai-600 025.

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Chitosanase activity has been monitored in the extracts of leaf, stem, cotyledon, hypocotyl and root of 10 day old seedling of *A. hypogaea*, imbibed seeds and gynophore extract using salting out, gel filtration, ion-exchange chromatography and preparative, native PAGE techniques. The partially purified enzyme hydrolysed glycol chitosan incorporated with gel matrix. The apparent moleculer mass of partially purified chitosanase of *A. hypogaea* is around 30kDa. The chitosanase of groundnut gynophore inhibited condial germination of *Myrothecium verrucaria*, *Acremonium obclavatum* and *Fusarium solani*.

Key Words: Arachis hypogaea, chitosanase

Plants have been endowed with complex defence mechanisms to protect themselves against phytopathoginic microorganisms. Antifungal hydrolytic enzymes produced by plants are one of the important components of defence mechanisms in plants (Boller, 1987) Among the hydrolytic enzymes chitinases and glucanases have been well established and they act synergistically to inhibit the growth of certain plant pathogenic fungi (Mauch *et al.*, 1988a. 1988b; Trudel *et al.*, 1989). Besides chitinase and glucanases plants do have other hydrolytic enzymes. One of such enzyme is chitosanase (EC.3.2.1.99), a part of chitinolytic system which acts on chitosan, a deacetylated form of chitin (Monaghan *et al.*, 1973) Chitosan occurs naturally in the cell walls of zygomycetous fungi (Bartincki-Garcia, 1968). Another source of natural chitosan is through the major deacetylation of chitin which forms the principal component of shellfish or crustacean exoskeleton.

Chitosanases have been studied extensively in bacteria (Managhan *et al.*, 1973; Davis and Eveleigh, 1984; Palletier and Sygnsch, 1990) and fungi (Fenton and Eveleigh, 1981; Alfonso *et al.*, 1992; and Shimosaka *et al.*, 1993). The presence of chitosanase has been reported only recently in plants. Grenier and Asslein (1990) have detected chitosanase isoforms in the intercellular washing fluid (IWF) of stressed and non-stressed leaves of barely, tomato and cucumber cotyledons. Purified chitosanases of stressed barely and tomato plants have been shown to have lytic activity against *Fusarium oxysporum*, *Verticillium albo-atrum* and *Ophiostoma ulmi*. Subsequently, organ specific isoenzymes of chitosanase have been found during different developmental stages of cucumber (Souad El Quakfauai and Asselin, 1992). Chitosanase activity have also been detected in leek and onion roots colonized by different VAM fungi (Eliane Dumas Gaudot *et al.*, 1992) As far as we are aware, there is no report on the presence chitosanase in *Archis hypogaea*. In the present investigation, chitosanase enzyme has been detected in imbibed seeds and different organs of 10d old seedlings of groundnut and gynophore of *A. hypogaea*. Partial purification and properties of chitosanase from gynophore extract have been described in this paper.

MATERIALS AND METHODS

Groundnut pods (cv TMV-7) were procured from Oil Seed Research Station (TNAU) Tindivanam, Tamil Nadu. The seeds were sufrace sterilized with mercuric chloride (0.01% w/v) and were sown in earthen pots containing the mixture of soil, sand, and farmyard manure. The pots were maintained in the glass house of CAS in Botany, University of Madras, Guindy campus, Chennai. Different plant organs like leaf, stem, cotyledon, hypocotyl and root were collected from the 10 day old seedlings. Gynophores were collected form the standing crop of TMV-7 from the research field of Oil Seed Research Station, Tindivanam. Different organs of the plant were homogenised in a pre-cooled mortar and pestle with Tris-HCL buffer (0.05M; pH 7.2), with ascorbic acid (0.01%w/v) and polyvinyl polyprrolidone (PVPP) (0.1% w/v). The homogenate was filtered through 4 layers of cheese cloth and the filtrate was centrifuged at 15, 000xg for 20 min. at 4°C. The supernatant was collected and used for protein estimation and enzyme activity. The supernatant was preceipitated with ammonium sulphate

*Corresponding author.
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(50% w/v; saturation) followed by 80% (w/v) saturation. The precipitate was collected by centrifugation and the pellet was dialysed in Tris-HCL buffer (0.01 M; pH 7.2) The precipitate was dissolved and dialysed against the same buffer for 48h at 4°C. The dialyzed sample was lyophilised and stored at 4°C until further use.

The protein content of the extract of different organs of 10d seedling, imbibed seeds and gynophore extracts were determined by the dye binding method of Bradford (1976) with bovine serum albumin fraction (V) as the reference protein. Chitosanase activity was assayed from the above samples following the procedure of Dygert *et al.* (1965) for the determination of reducing sugars.

The lyophilized protein sample obtained after 50% (w/v) ammonium sulphate saturation from the gynophore extract was passed through Bio-Gel P60 column chromatography (60 cmx4.5 cm id.). A known amount of protein was loaded and fractions (5ml each) were collected using an automatic fraction collector. The absorbance of each fraction was read at 280 nm in a UV spectropotometer and fractions having absorbancy values were pooled together. Pooled fractions showing high enzyme activity were further separated by ion exchange chromatography. Anion exchanger, DEAE-Sephadex was prepared according to the manual provided by the manufacturer and packed in a column tube (5 cm x 1.5 cm i.d). The column was equilibrated with Tris-HCl buffer (0.05 M; pH 7.2). Proteins of pooled fraction 2 and 3 obtained after Bio-Gel P60 column was adsorbed into the column. Unbound proteins were washed with the same buffer. Bound proteins were desorbed with a linear gradient of NaCl (0.05 M-1.0 M) with Tris-HCl buffer (0.05 M; pH 7.2). Each fraction was collected separately and dialyzed against the same buffer (0.01 M; pH 7.2) for 24h with 4 changes.

Preparative native PAGE

Protein fractions eluted from ion-exchange column with 0.2, 0.3, and 0.4 M NaCl were electrophoresed in native PAGE (15% w/v). Bands with hydrolytic activity in the gel incorporated with glycol chitosan were cut and enzyme was eluted by grinding in a pro-cooled mortar and pestle with Tris-HCl buffer (0.05M; pH 7.2). The ground gel was centrifuged at 15,000 xg for 15min at 4°C. The supernatant was collected and dialyzed extensively in Tris-HCl buffer (0.01M; pH 7.2) at 4°C.

Analytical native PAGE

The protein obtained from preparative PAGE was electrophoresed in 15% (w/v) gel, incorporated with glycolchitosan (0.02% w/v) in the separating gel. After separation, the gel was incubated in sodium acetate buffer (0.01M; pH 5) for 4h at 37°C in a moist condition. The gel was then rinsed with sterile water and stained with coomassie brilliant blue (0.2% w/v), prepared in a mixture of methanol, acetic acid and water in 4:1:5 ratio. Destaining was carried out with the same solvent without the dye.

Table 1: Chitosanase activity of various organs of 10d old seedling imbibed seeds and gynophore of *Arachis hypogaea*

Plant organs	Protein content (mg/g.f.wt)	Enzyme activity (units/g.f.wt)
10d old seedling		
Leaf	5.10	142.0
Stem	3.40	134.4
Cotyledon	6.10	167.3
Hypocotyl	0.80	126.0
Root	2.70	113.4
Imbibed seed		
Seed coat	1.01	no activity
Cotyledon	9.60	240.0
Gynophore	6.30	180.0

*Each value is mean of two experiments.

Molecular weight determination of partially purified chitosanase

Partially purified chitosanase was subjected to SDS-PAGE (15% w/v), as described by Laemmli, (1970). The gel was then stained with silver nitrate following the procedure of Blum *et al.* (1987). One of the gel lanes was loaded with marker proteins. After staining the molecular mass was determined.

Effect of partially purified protein on conidial germination

Condial suspensions of *Acremonium obclavatum, Fusarium soloni, Myrothecium verrucaria* and *Trichoderma harziamum* were prepared from 10 day old cultures grown in potato dextrose agar in sterile water. The conidial suspension was filtered through sterile buchner funnel using a cotton pad. A known concentration of spore suspension (10/ml) was incubated with the partially purified enzyme in a sterile cavity slide for 12-16h at 25±2°C. Control was maintained with the buffer alone.

RESULTS

Extracts of different organs of 10d old seedling, imbibed seed and gynophore of mature plant showed chitosanase activity. Table 1 shows chitosanase activity of extracts of organs 10d old seedling of *A. hypogaea*. Among the extracts, extract of cotyledon showed

maximum chitosanase activity which was followed by leaf, stem, hypocotyl and root. However, chitosanase activity in the gynophore extract was significant among all the extracts tested for enzyme activity and hence this extract was taken for partial purification. Extracts of the above organs were analysed for chitosanase activity on 15% (w/v) native PAGE. Prior to electrophoresis the polyacrylamide gel was incorporated with glycolchitosan (0.2%w/v). Almost all the extracts except seed coat of imbibed seeds showed hydrolytic activity on native PAGE (Fig 1). Enzyme activity regions appeared as clear and translucscent bands whereas the remaining part of the gel was intensely stained with coomassie brilliant blue.

The extract of gynophore were passed through Bio-Gel P60 column and fractions showing chitosanase activity were pooled for further purification.

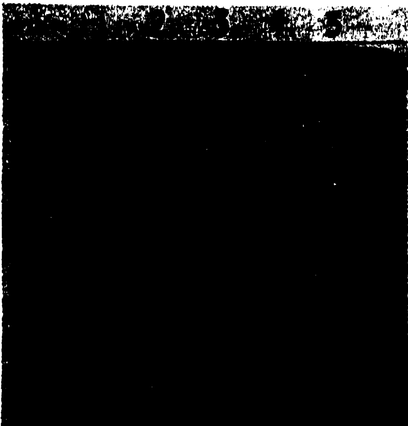

Figure 1. Enzyme activity staining of chitosanase of 10d old seedling on native PAGE
Lanes: 1-Leaf, 2-Stem, 3-Cotyledon, 4-Hypocotyl, 5-Root.

Fractions showing enzyme activity were subjected to DEAE-Sephadex ion exchange chromatography. Fractions eluted with 0.2M and 0.3M NaCl with the elution buffer contained maximum enzyme activity. Subsequently, fractions obtained with 0.2 and 0.3M NaCl concentration were subjected to preparative, native PAGE. The region showing chitosanase band was cut and enzyme was eluted. Eluted enzyme was analyzed for its hydrolytic activity on 15%(w/v) native PAGE (Fig. 2). The partially purified enzyme had hydrolysed, the glycol chitosan (0.02% w/v) incorporated with the gel matrix and the hydrolysed region, appeared as a single band (Fig. 2).

The purified enzyme was analyzed further on 15% (w/v) SDS-PAGE to check for its homogeneity. The enzyme band, appeared as a single and prominent

Figure 2. Enzyme activity staining of partially purified chitosanase on native PAGE with the substrate.

band (Fig. 3). The apparent molecular mass of the purified enzyme was 30 KDa using the standard authentic marker proteins.

The partially purified chitosanase of gynophore of groundnut inhibited conidial germination of *M. verrucaria* and *A. obclavatum* and *F. solani*.

DISCUSSION

Like chitinase, chitosanase also has no known endogenous substrate in higher plants and a role of the

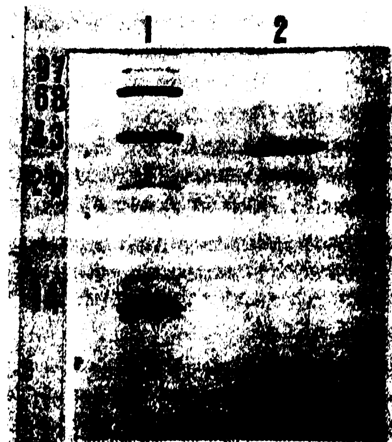

Figure 3. Molecular mass determination of partially purified chitosanase on SDS-PAGE
Lane 1. Standard protein markers
Lane 2. partially purified chitosanase of gynophore of *A. hypogaea*.

enzyme in tissue differentiation is still under debate. Nevertheless, Souad El Quakfaoui and Asselin (1992) have detected isoforms of chitosanase specific to different organs of cucumber during their development and they have suggested a role for chitosanase in differentiation of organs.

Data presented in this study indicate the presence of chitosanase in not only different organs of 10 d seedling of *A. hypogaea* but in gynophore of 60d old plant as well. The presence of chitosanase in gynophore assumes significance in view of the fact that gynophore develops after post anthesis and then enters the soil crust. It faces a new environment when it pierces the soil. It may be prone to soil borne phytopathogens and upon contact this enzyme may protect from fungi such as *Rhizopus*, which is known to induce damping off and seed rot in groundnut (Kenneth *et al.*, 1973). The genus *Rhizopus* belongs to Zygomycetes and fungi belonging to this group contain chitosan in their cell walls (Bartnicki-Garcia, 1968).

The partially purified chitosanase inhibited conidial germination of *Acremonium obclavatum, Myrothecium verrucaria* and *Fusarium solani.* Antifungal activity of chitosanase isoforms have been explained by Grenier and Asselin (1990). When the gel matrix was embedded with spores of *F. oxysporum, Verticillium albo-atrum* and *Ophiostoma ulmi* lytic activity was observed in the gel matrix as clear zones through the opaque spore suspension.

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