Zeitschrift: Botanica Helvetica
Herausgeber: Schweizerische Botanische Gesellschaft
Band: 104 (1994)
Heft: 1

Artikel: Purification and properties of apoplastic chitinases from rust infected leaves of Arachis hypogaea L.

Autor: Govindasamy, V. / Balasubramanian, R.

DOI: https://doi.org/10.5169/seals-71617

Bot Helv 104: 79–86

0253-1453/94/010079-08 $ 1.50 + 0.20/0

Purification and properties of apoplastic chitinases from rust infected leaves of *Arachis hypogaea* L.

V. Govindasamy, and R. Balasubramanian*

Centre for Advanced Study in Botany, University of Madras, Guindy Campus, Madras 600025, India.

Manuscript accepted March 10, 1994

Abstract

Govindasamy V. and Balasubramanian R. 1994. Purification and properties of apoplastic chitinases from rust infected leaves of *Arachis hypogaea* L. Bot. Helv. 104: 79–86.

Apoplastic chitinase from rust infected leaves of *Arachis hypogaea* (peanut) has been isolated and purified by ammonium sulphate precipitation, DEAE-Cellulose, Sephadex gel filtration and preparative isoelectric focusing (IEF) techniques. IEF indicated the isotype and acidic nature of the purified chitinase. The apparent molecular mass of purified IWF chitinase-1 from rust infected leaves was 29 kDa. The pH and temperature optima were 5.0 and 45 °C respectively. Mercury chloride inhibited chitinase activity completely while $NaNO_3$ activated the enzyme activity. Purified chitin was better hydrolysed than impure chitin. *A. hypogaea* chitinase inhibited germination, germ tube growth and appressorium formation of *Puccinia arachidis* uredospores. There was no lysis of uredospore germ tubes. Growth of *Trichoderma harzianum* and *Fusarium solani* was not inhibited. Besides, this enzyme also shows a weak *lysozyme* activity. Based on acidic chitinase/lysozymic property, peanut IWF chitinase can be grouped under class III chitinases of plants.

Key words: Arachis hypogaea, chitinase, purification, intercellular washing fluid, *Puccinia arachidis*.

Introduction

Chitinases are induced in tissues following pathogen infection and there is indirect evidence that this induction is important in the biochemical defense of plants against potential pathogens (Boller 1987). Chitinases and β-1,3 glucanases have been shown to be induced coordinately in pea (*Pisum sativum*) pods when inoculated with compatible or incompatible strains of *Fusarium solani* and wounded or treated with chitosan (Mauch et al. 1988a). Increased chitinase activity has been observed in various plants after infection with the powdery mildew fungus (*Erysiphe graminis f. sp hordei*, Krag et al. 1990), *Puccinia sp.* (Fink et al. 1988) and *Uromyces phaseoli* on bean leaves (Schlumbaum

* Corresponding author.

et al. 1986). Chitinase activity has been monitored in different subcellular fractions of leaves of cucumber (Boller and Métraux 1988). Most of the plant chitinases possess antifungal property (Roberts and Selitrennikoff 1988, Mauch et al. 1988 b). Besides, some chitinases of plant origin have lysozyme activity as well (Boller et al. 1983, Mauch et al. 1988 a).

The groundnut (peanut) rust, *Puccinia arachidis* colonizes susceptible leaves of *A. hypogaea* intercellularly. Increased protein and chitinase activity from rust infected detached groundnut leaves have been reported from this laboratory (Sathiyabama and Balasubramanian 1991). Better understanding of this host-biotrophic parasite interaction may require isolation and analysis of properties of chitinases from intercellular washing fluids (IWF) of *A. hypogaea*. In this paper we present our results in purification and properties of chitinase from IWF of rust infected leaves of the susceptible cv. (TMV 7) of *A. hypogaea*.

Materials and methods

Seeds of *Arachis hypogaea* L. cv. TMV 7 obtained from Pyoor Research Station, Pyoor, (TNAU) were used. Raising of groundnut plants and maintaining of rust infections on leaves were followed as described (Govindasamy and Balasubramanian 1989). Leaves of 40 d old *A. hypogaea* plant maintained in a glasshouse were used. About 7 kg of rust diseased (12 d) leaves of *A. hypogaea* was used for the purification of IWF chitinase. IWF was collected by the method of Rohringer et al. (1983) using cold glass distilled water as an infiltration medium. The collected fluid was used after centrifugation (20 000 *g* for 15 min at 4 °C).

The protein content of IWF was determined by the dye binding method of Bradford (1976) using bovine serum albumin fraction V (Sigma Chemical Co., USA) as a standard. In order to rule out the possibility of mixing up of isolated IWF with cellular proteins, a marker enzyme such as glucose-6-phosphate dehydrogenase (G-6-PDH; EC 1.1.1.49) was assayed by the method of Simcox et al. (1977).

Colorimetric assay of endochitinase activity described by Boller et al. (1983) was followed. Colloidal chitin prepared from crab shells (Skujins et al. 1965) was used as substrate. One unit of chitinase activity is defined as the amount of enzyme which produces 100 µg of GlcNac/ml chitin reaction mixture at 37 °C under the assay conditions. Specific activity was calculated per mg protein.

The following methods were used commonly for the sequential purification of IWF chitinase from rust infected leaves of *A. hypogaea*. All purification procedures were performed at 4 °C. Ammonium sulphate precipitated and dialysed chitinase protein from IWF was passed on DEAE-Cellulose column (bed: 10 × 2.5 cm) which was pre-equilibrated with Tris-HCl buffer, (0.02 M, pH 7.6 containing 0.02% NaN_3). After washing the bed with the same buffer, chitinase protein was eluted with 110 ml of phosphate-citrate buffer (0.015 M, pH 5.5) followed by the same buffer (100 ml) with a linear gradient of NaCl (0.0–0.2 M). The column was further washed with Tris-HCl buffer, 0.125 M, pH 7.6 with a linear gradient of NaCl (0.0–0.6 M). Five ml fractions were collected, dialysed and assayed. Absorbance at 280 nm, protein content (Bradford 1976) and chitinase activity were monitored for each fraction.

DEAE-Cellulose eluted chitinase active fractions were passed through a Sephadex G-100 column (bed: 85 × 2.0 cm, id, 40–120 µm, Pharmacia Fine chemicals, Uppsala Sweden) equilibrated with sodium acetate buffer (0.05 M, pH 5.2) and eluted with the same buffer. Sephadex G-100 eluted chitinase-active fractions were subjected to preparative isoelectric focusing in a LKB 8100-1 column (110 ml) in a sucrose density gradient using the long range ampholine (pH 3.5–10, Servalyte, Serva, Germany) as described by LKB Producter AB, Bromma, Sweden. After a 17 h run, 2.7 ml fractions were collected tested for their pH (Expandable Ion Analyzer, Orion Research EA 940, USA), dialysed against glass distilled water for 48 h and then assayed for protein and chitinase activity.

Native polyacrylamide gel electrophoresis (PAGE) was done by the method of Davis (1964). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in poly-

acrylamide slab (12%, w/v separating and 5%, w/v stacking) gel according to the method of Laemmli (1970). Standard proteins for molecular mass determination were purchased from Bio-Rad (Bio-Rad, Richmond, USA). Gels were stained in 0.2% (w/v) Brillant Blue R 250 (Sigma Chemical Co., USA).

For pH optima, sodium acetate buffer from pH 3.5 to 6.0, sodium phosphate buffer for pH 6.5 and Tris-HCl buffer from pH 7.0 to 9.0 (0.05 M each) were used with colloidal chitin (0.1%) as the substrate. A range of temperatures from 20 °C to 60 °C were tried. Using 0.18 units of enzyme, endochitinase activity was measured throughout. Effect of metal ions and inhibitors (10 mM) on purified chitinase and by using Lineweaver-Burk plot, Michaelis-Menten constant (K_m) of the enzyme were also determined.

Chitin oligosaccharides produced by peanut IWF chitinase was analysed by high performance liquid chromatography (HPLC) as described by Broekaert et al. (1988). Regenerated chitin prepared by the method of Molano et al. (1977) was used as the substrate for HPLC analysis. Samples were analysed by HPLC on a column of Shim-Pack CLC-NH_2 (5 µm, 6.0 mm × 150 mm, Shimadzu, Kyoto, Japan). The column was eluted with 73% acetonitrile at a flow rate of 1.5 ml min^{-1}. The elution position of the GlcNAc oligomers was determined with a refractive index detector (RID-6A, Shimadzu, Japan) and compared with that of GlcNAc oligomer standards of different chain lengths. N-acetyl-D-glucosamine, N,N′diacetyl chitobiose, N,N,N′triacetyl chitotriose (Sigma Chemical Co., USA) were used as standard chitin oligomers.

Effect of chitinase on germination and germ tube growth of *P. arachidis* was determined as described (Govindasamy and Balasubramanian 1989). Lysozyme activity of purified chitinase-I (10 µg) was tested against *Micrococcus lysodeikticus* cells (Sigma Chemical Co., USA) as described by Martin (1991). Hen egg-white *lysozyme* (HEW, 10 µg, EC 3.2.1.17), histamine and GlcNAc (10 mM, Sigma Chemical Co., USA) were also included as a reference (HEW) and inhibitors of lysozyme activity, respectively.

Results

Apoplastic chitinase activity was similar in control as well as rust infected leaves. Negligible amount of G-6-PDH activity found in IWF suggests minimal contamination of cytosolic protein. Ammonium sulphate of 90% saturation was used to precipitate the protein from IWF.

DEAE-Cellulose column chromatography: Tris-HCl buffer-washed fractions contained a single peak of protein which did not show any chitinase activity. Chitinase protein was eluted by phosphate-citrate buffer which showed a single protein peak with high chitinase activity and these fractions (between 8–13) were pooled and used for Sephadex G 100 gel chromatography. Tris-HCl buffer (0.125 M, pH 7.6) eluted another chitinase protein which was not processed further.

Sephadex G-100 gel chromatography: Of the two protein peaks obtained in gel filtration, the first major peak exhibited high chitinase activity whereas the second minor peak showed low enzyme activity. Fractions (20–25) of the first, major, colourless protein peak were pooled and used for preparative IEF.

Preparative Iso-electric Focusing (IEF): Results presented in Fig. 1 show that there were three major protein peaks focused at different pI points. Of the three peaks, the one focused around pH 3.0 (fractions 27–30) and the other peak below 3.0 (fractions 34–36) showed chitinase activity. The peak focused around pH 3.0 showed higher protein and enzyme activity was hereafter designated as chitinase-I and the other (fractions 34–36) as chitinase-II. Only chitinase-I was taken for further characterization in this study. Table 1 shows an increase of chitinase specific activity after each purification step.

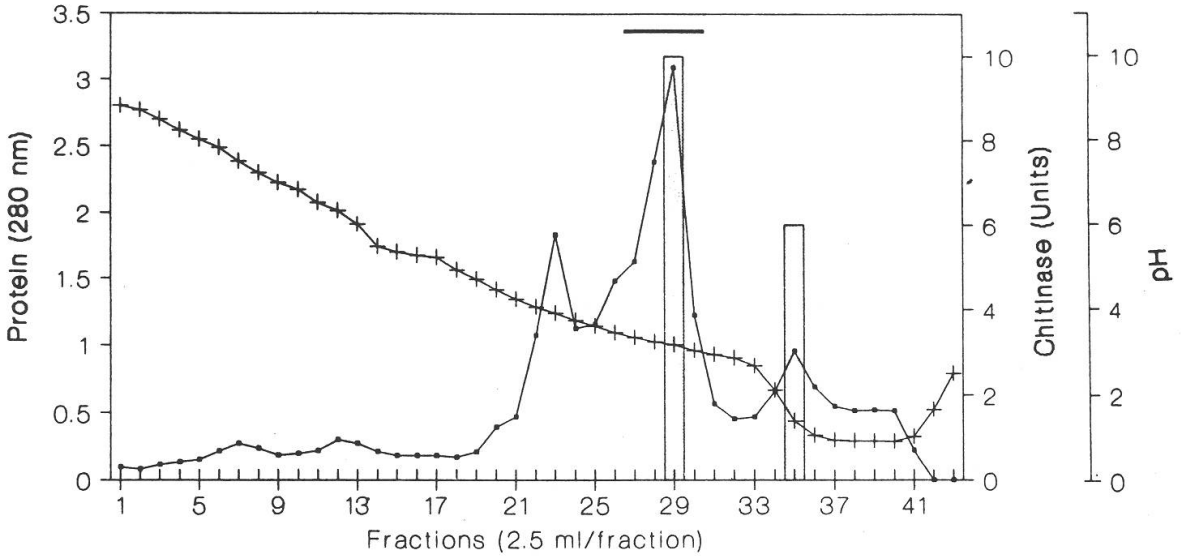

Fig. 1. Preparative isoelectric focusing of Sephadex G-100 active fractions: —●— protein, —|— pH □ CNase activity (× 10).

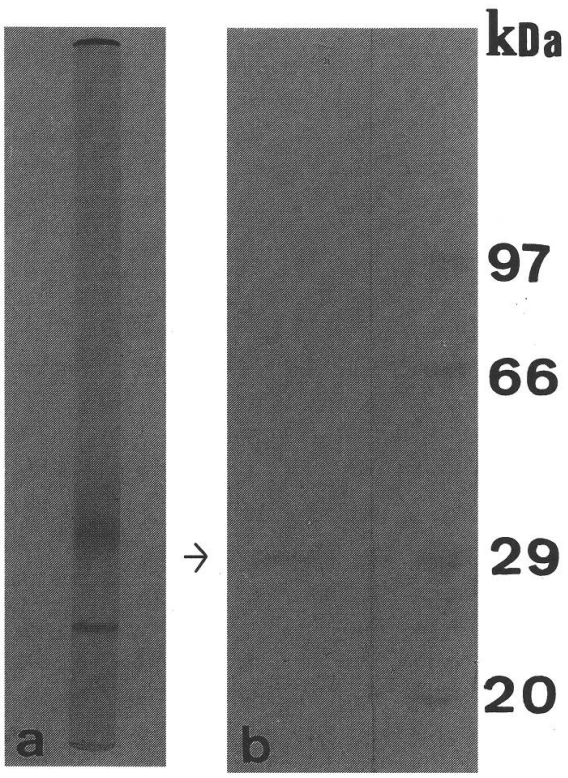

Fig. 2. a) Native PAGE (Davis system) of purified chitinase from IWF of rust infected leaves of *Arachis hypogaea*. b) SDS-PAGE of purified chitinase (shown by arrow). Standard marker proteins (Bio-Rad, USA) were: soybean trypsin inhibitor, 20 100; carbonic anhydrase, 29 000; bovine albumin, 66 000; phosphorylase-b, 97 000 daltons.

Table 1. Summary of chitinase purification from IWF of rust infected *Arachis hypogaea* leaves.

Step	Stages	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg protein)	Purification (fold)	Yield (%)
1.	Clarified IWF	420	280.0	0.66	1	100
2.	Ammonium sulphate precipitate	200	172.50	0.86	1.3	47.6
3.	DEAE-Cellulose (PO_4-citrate buffer wash)	20.5	30.80	1.50	2.2	4.8
4.	Sephadex G-100	15.0	52.50	3.50	5.1	3.5
5.	Preparative IEF Chitinase-I	3.5	16.00	4.57	6.6	0.8

Table 2. Effect of purified chitinase on germination and germ tube growth of *Puccinia arachidis*.

Control		Chitinase-I added (10 µg) at time zero	
Germination (%)	Germ tube length (µm)	Germination (%)	Germ tube length (µm)
70 ± 4.1 [1, 3]	29 ± 8.2	4.0 ± 0.8	16.4 ± 7.0
71 ± 4.0 [2]	32 ± 4.2	4.2 ± 3.3	22.0 ± 5.5

[1] After a 5 h incubation period.
[2] Two h post-incubation after distilled water wash.
[3] $\pm$SD (N = 50).

Electrophoresis: Chitinase-I purified from IWF of infected leaves showed a single coomassie blue stained protein band on native PAGE (Fig. 2a). The apparent molecular mass of purified chitinase-I was ca. 29 kDa on SDS-PAGE (Fig. 2b, lane 1).

Physico-chemical properties: Although there was enzyme activity between pH 4.0–7.5, maximum activity was found in the range of pH 4.5–5.0. The temperature profile showed that maximum chitinase-I activity was around 45 °C. More than 80% enzyme activity was retained till 60 °C.

Purified chitinase-I hydrolysed several chitin substrates used with substrate specificities in the following sequence: chitin powder > regenerated chitin > colloidal chitin. Among the various substrates, crude chitin was the least hydrolysed. Mercury chloride (10 mM) completely inhibited chitinase-I activity. Among other metal ion solutions, $ZnSO_4$ (10 mM) inhibited 80% of activity followed by $MnCl_2$ (10 mM) (70%). By contrast, $NaNO_3$ (10 mM) activated (20–30%) chitinase-I activity. The approximate K_m (Michaelis-Menten constant) for regenerated chitin was 0.8 mg ml^{-1}.

Products of chitin hydrolysis: Chitinase-I produced dimer, trimer and other higher oligomers after 3 h incubation with the substrate. Further increase in incubation time of 24 h resulted in the production of monomers and dimers only (data not shown). However, a 3 h reaction mixture incubated with β-glucuronidase (HP-2, from snail juice) showed both monomers and dimers.

Table 3. Effect of purified chitinase on germlings (3 h old) of the groundnut rust *Puccinia arachidis*.

Parameter	Control	Chitinase-I	
		5 μg · ml^{-1}	10 μg · ml^{-1}
Germ tube length (μm)	6.0±1.2	3.6±0.6	2.6±0.8
Branched germlings with appressoria (%)	50.0±2.3	1.6±0.5	1.6±0.7

– 80–85% of the uredospores had germinated.
– results were obtained 1 h after chitinase-I treatment and are given ±SD (N=50).

Inhibition of germination of P. arachidis uredospores: Results presented in Table 2 show that there was a significant inhibition in germination (64%) and germ tube growth (45%) of uredospores of *P. arachidis*. Chitinase-I (0.045 units) treated uredospores did not germinate even after repeated rinses with water. In contrast to germination, germ tube growth resumed 2 h after incubation with distilled water rinse (Table 2). The germ tubes of *P. arachidis* tend to branch and form appressoria after the process of germination. In this experiment, uredospores were germinated for 3 h and germlings were incubated with the purified chitinase-I to find out its effect on branching and appressoria formation of uredospores. Results presented in Table 3 show that the number of branched germlings with appressoria – like structures were drastically reduced (96%) in the enzyme treated germlings, irrespective of enzyme concentration used. There was also inhibition of germ tube growth.

The antifungal activity of chitinase-I was tested by its ability to inhibit growth of certain chitin containing fungi. A weak inhibition of hyphal growth of *Penicillium* sp. at 0.045 units of chitinase was observed. Chitinase-I did not inhibit the growth of *T. harzianum* and *F. solani*. When *Pseudomonas fluorescens* seeded agar plates were incubated with chitinase-I for 24 h, inhibition was evident as transparent clearing around the chitinase (0.045 units) disc (data not shown). There was no clearing zone around the paper disc with sterile water as a control.

Lysozyme activity: Purified IWF chitinase-I of *A. hypogaea* showed lysozyme activity. The lysozyme activity of groundnut chitinase-I was inhibited completely by histamine and GlcNAc at 10 mM.

Discussion

Results of the present study indicate that there was no alteration in apoplastic chitinase activity of control and infected leaves. Fink et al. (1990) reported that there was no difference in apoplastic chitinase activity in the compatible interaction of oat and *Puccinia coronata f. sp. avena*. Chitinase-I purified from IWF of rust infected leaves showed a molecular mass of 29 kDa on SDS-PAGE (Fig. 2b). The molecular mass of plant chitinase usually falls in this range (Fink et al. 1990, Jacobsen et al. 1990). IWF of peanut leaf chitinase showed two isoenzymes with a pI of 3.0 and below 3.0. It will be interesting to know whether the different isoenzymes of peanut leaves are products of the same gene or the result of different processing of the same gene products. There have

been some similarities in pH, temperature optima and sensitivity to inhibitors between *A. hypogaea* leaf chitinase and chitinases of tomato (Pegg 1988), bean plants (Boller et al. 1988) and barley grains (Jacobsen et al. 1990).

It seems that purified chitinase-I of *A. hypogaea* is an endochitinase, in view of the assay system used in the present study. Results on HPLC analysis of products of hydrolysis by purified chitinase substantiate this result.

The observed inhibition of germination and germ tube growth of *Puccinia arachidis*, causal agent of the groundnut rust, indicates an antifungal action of *A. hypogaea* chitinase (Tables 2 & 3). Tobacco leaf chitinase significantly inhibited the spore germination rate of *Trichoderma hamatum* (Broekaert et al. 1988). When germlings were treated with chitinase-I, the formation of infection structures, branching and appressoria, was affected. But there was no lysis of uredospore germ tubes. It has been reported that growing hyphal tips contain 'nascent chitin' which could be easily affected by chitinase (Wessels 1986, Rast et al. 1991). Instead of lysis, growth of germ tube could be arrested by interfering with 'nascent chitin' present at its tip. By interfering with the 'nascent chitin', the delicate balance between lytic and synthetic process of wall growth might be altered by the addition of chitinase, at least in part (Bartnicki-Garcia 1973; Farkas 1979).

There have been considerable variations regarding the susceptibility of the different fungal species to inhibition by plant chitinases. In *Botrytis cinerea* chitinase did not affect growth at concentrations lower than 320 $\mu m\ ml^{-1}$ (Broekaert et al. 1988). Also purified chitinase of *A. hypogaea* did not inhibit growth of *Trichoderma harzianum* and *Fusarium solani* (data not shown). As mentioned by Schlumbaum et al. (1986), inhibition of some fungi may require the combined action of chitinases and β-1,3 glucanases. *A. hypogaea* chitinase-I showed weak lysozyme activity which was inhibited by histamine-HCl and N-acetylglucosamine. Chitinases of plants showing lysozyme activity have been reported (Boller et al. 1983, Mauch et al. 1988a, Audy et al., 1990, Martin 1991, Lynn 1989). Inhibition of lysozyme activity of bean chitinase by GlcNAc and histamine is very well documented (Boller et al. 1983). This bifunctional property (chitinase/lysozyme) of peanut chitinase may widen its spectrum of defense response against pathogenic microorganisms.

The authors thank Prof. A. Mahadevan, Director, for facilities and one of us (VG) thanks UGC, New Dehli, for a fellowship.

References

Audy P., Le Quere D., Leclerc D. and Asselin A. 1990. Electrophoretic forms of lysozyme activity in various plant species. Phytochem. 29: 1143–1159.

Bartinicki-Garcia S. 1973. Fundamental aspects of hyphal morphogenesis. Symposia of the Society for General Microbiology 23: 245–267.

Boller, T., Gehri A., Mauch F. and Vögeli U. 1983. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta 157: 22–31.

Boller T. 1987. Hydrolytic enzymes in plant disease resistance In: Plant-Microbe Interactions, Molecular and Genetic perspectives, Vol.: 2, ed. T. Kosuge and E. W. Nester. Macmillan, New York, pp. 385–413.

Boller T. and Métraux J. P. 1988. Extracellular localization of chitinase in cucumber. Physiol. Mol. Plant Pathol. 33: 11–16.

Boller, T., Gehri A., Mauch F. and Vögeli U. 1988. Chitinase from *Phaseolus vulgaris* leaves. In: Methods in Enzymology Vol. 161. Part B. ed. W.A. Wood, S. T. Kellogg Academic Press Inc., pp. 479–484.

Bradford M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.
Broekaert W. F., van Parihs J., Allen A. K. and Peumans W. J. 1988. Comparison of some molecular, enzymatic and antifungal properties of chitinases from thorn-apple, tobacco and wheat. Physiol. Mol. Plant Pathol. 33: 319–331.
Davis B. J. 1964. Disc electrophoresis: II Method and application to human serum proteins. Ann. New York Acad. Sci. 121: 404–427.
Farkas V. 1979. Biosynthesis of cell walls of fungi. Microbiol. Rev. 43: 117–144.
Fink W., Liefland M. and Mendgen K. 1988. Chitinases and β-1,3 glucanases in the apoplastic compartment of oat leaves (*Avena sativa* L.). Plant physiol. 88: 270–275.
Fink W., Liefland M. and Mendgen K. 1990. Comparison of various stress responses in oat in compatible and nonhost resistant interactions with rust fungi. Physiol. Mol. Plant Pathol. 37: 309–321.
Govindasamy V. and Balasubramanian R. 1989. Biological control of groundnut rust, *Puccinia arachidis, by Trichoderma harzianum* Z. Pflkrank. Pfl. Schutz 96: 337–345.
Huynh Q. K., Jeffry R. B. and James F. Z. 1992. Isolation and characterization of a 22 kDa protein with antifungal properties from maize seeds. Biochem. Biophy. Res. Commu. 182: 1–5.
Jacobsen S., Mikkelsen J. D. and Hejgaard J. 1990. Characterization of two antifungal endo-chitinases from barley grain. Physiol. Plant. 79: 554–562.
Kragh K. M., Jacobsen S. and Mikkelsen J. D. 1990. Induction, purification and characterization of barley leaf chitinase. Plant Sci. 71: 55–68.
Laemmli U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277: 680–685.
Lynn K. R. 1989. Four lysozymes from the latex of *Asclepias syriaca*. Phytochem. 28: 1345–1348.
Martin M. N. 1991. The latex of *Hevea brasiliensis* contains high levels of both chitinases and chitinases/lysozymes. Plant physiol. 95: 469–476.
Mauch F., Hadwiger L. A. and T. Boller 1988 a. Antifungal hydrolases in pea tissues. I. Purification and characterization of two chitinases and two β-1,3 glucanases differentially regulated during development and in response to fungal infection. Plant. Physiol. 87: 325–333.
Mauch F., Mauch-Mani B. and Boller T. 1988 b. Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combination of chitinase and β-1,3 glucanase. Plant Physiol. 88: 936–942.
Molano J., Duran A. and Cabib E. 1977. A rapid and sensitive assay for chitinase using tritiated chitin. Anal. Biochem. 83: 648–656.
Pegg G. F. 1988. Chitinase from tomato, *Lycopersicon esculentum*. In: Methods in Enzymology Vol 161 Part B, ed. W. A. Wood, S. T. Kellogg, Academic Press Inc. pp 484–488.
Rast D. M., Horsch, M., Furter R. and Gooday G. W. 1991. A complex chitinolytic system in exponentially growing mycelium of *Mucor rouxii* – Properties and function. J. Gen. Microbiol. 137: 2797–2810.
Roberts W. K. and Selitrennikoff C. P. 1988. Plant and bacterial chitinases differ in antifungal activity. J. Gen. Microbiol. 134: 169–176.
Rohringer R., Ebrahim-Nesbat F. and Wolf G. 1983. Proteins in intercellular washing fluids from leaves of barley (*Hordeum vulgare* L.). J. Expt. Bot. 34: 1589–1605.
Sathiyabama M. and Balasubramanian R. 1991. Chitinases and glucanases in *Arachis hypogaea* L. in detached leaves infected with *Puccinia arachidis* Speg. J. Pl. Dis. Prot. 98: 305–316.
Schlumbaum A., Mauch F., Vögeli U. and Boller T. 1986. Plant chitinases are potent inhibitors of fungal growth. Nature 324: 365–367.
Simcox P. D., Reid E., Canvin D. T. and Dennis D. T. 1977. Enzymes of the glycolytic and pentose phosphate pathways in proplastids from the developing endosperm of *Ricinus communis*. Plant Physiol. 59: 1128–1132.
Skujins J. J., Potgieter H. J. and Alexander M: 1965. Dissolution of fungal cell walls by a streptomycete chitinase and β-(1-3)glucanase. Arch. Biochem. Biophys. 11: 358–364.
Wessels J. G. H. 1986. Cell wall synthesis in apical growth. Int. Rev. Cytol. 104: 37–79.