C_7(P32) and C_6(P34) PR proteins induced in tomato leaves by citrus exocortis viroid infection are chitinases

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Two chitinases induced in tomato leaves (*Lycopersicon esculentum* Mill cv. Rutgers) by citrus exocortis viroid (CEV) infection were purified. Their molecular masses determined by sodium dodecyl sulfate-gel electrophoresis were 32 kDa and 34 kDa and by filtration through Sephadex G-100 were 23 kDa and 25 kDa, respectively. These chitinases (P32 and P34) have been shown to be identical to the tomato pathogenesis-related proteins C_7 and C_6. They were purified in three stages: ammonium sulphate fractionation, chitin affinity chromatography and CM-Sepharose chromatography. The characterization of P32, the major component of the CEV-induced chitinase activity, revealed a basic protein (pI, 8·5), with a low optimum pH of 5·2 for hydrolytic activity, a high thermal stability and resistance to proteolytic degradation. These tomato chitinases have been shown to be serologically related to the tobacco chitinases. Our results lend further support to the hypothesis that enhanced chitinase activity is a component of a general mechanism of response to pathogens and other stress agents.

INTRODUCTION

Pathogenesis-related (PR) proteins are a group of plant encoded proteins whose synthesis is induced by infection with viroids, viruses, and other pathogens, as well as in response to chemically induced stress and even natural senescence [3, 29, 30].

Infection of tomato plants by citrus exocortis viroid (CEV) results in the accumulation of 10 PR proteins whose synthesis seems to be mediated by ethylene [6].

Studies on different plant species have shown that chitinases can be induced by pathogen attack [12, 16, 21, 32], elicitors from fungal cell walls [12, 21, 25], or treatment with the stress-associated hormone ethylene [1, 12].

To date, all PR proteins biochemically characterized have been found to be hydrolases. *In vitro* studies have indicated the possible functions of several of the induced proteins in tobacco. For example, PR proteins P and Q have chitinase activity [11], while PR-2, N and O have β-1,3-glucanase activity [9], suggesting that these hydrolytic enzymes could be involved in the degradation of bacterial and fungal cell walls. Two PR proteins from *Cladosporium fulvum* infected tomato plants have been purified and shown to be a β-1,3-glucanase and a chitinase, respectively [8]. Several potato PR proteins are also chitinases [10] and a cucumber PR protein has also been reported to be a chitinase [16].

The first assignment of a biochemical function to a viroid induced PR protein was

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the demonstration that P69 is an alkaline endoprotease [31]. In this paper, we described the purification of two further PR proteins, P32 and P34, from CEV-infected and ethephon treated plants and demonstrate that they have chitinase activity. We also provide evidence that these chitinases are identical to the tomato PR proteins C_7 and C_6 [6]. The 32 kDa chitinase, the major component of the CEV-induced chitinase activity has been studied in detail.

MATERIALS AND METHODS

Plant materials

Tomato plants (*Lycopersicon esculentum* Mill, cv. Rutgers) were grown from seeds in a greenhouse at 20° to 30 °C. Inoculation with CEV was performed as described previously [27]. The ethephon (2-chloroethylphosphonic acid) treatment was applied as a 0·02 M foliar spray to 4 week-old healthy plants as described previously [6]. Apical leaves (four top leaves) showing characteristic symptoms of viroid infection were harvested 4 weeks after inoculation. Ethephon treated tissues were normally harvested 4 days after treatment. All harvested tissues were frozen in liquid nitrogen and stored at −70 °C until required for analysis.

Protein extraction

Leaves were homogenized in cold (4 °C) 50 mM phosphate-citrate buffer at pH 2·8 containing 15 mM 2-mercaptoethanol (1 ml buffer per gram fresh weight) in a Polytron homogenizer for 3 × 1 min at top speed. After filtration through a double layer of cheese-cloth, the homogenate was centrifuged at 15000 ***g*** for 30 min. Ammonium sulphate was added to the supernatant to give a 20% saturated solution, and after stirring for 1 h at 4 °C, the precipitate formed was removed by centrifuging at 20000 ***g*** for 15 min. An additional amount of ammonium sulphate was added to the supernatant to give a 75% saturated solution and this was centrifuged at 20000 ***g*** for 15 min. The pellet was resuspended in a small amount of 50 mM sodium acetate buffer (pH 5·2) and the suspension dialysed against several changes of the same buffer.

Purification of the chitinases

Sodium bicarbonate was added to the dialysed protein extract (40 ml) to give a concentration of 20 mM, and the pH was raised to pH 8·4 with 0·1 M NaOH. The preparation was loaded onto a column (30 × 3 cm) of regenerated chitin, synthesized by acetylation of chitosan [18], equilibrated with 20 mM sodium carbonate buffer, pH 8·4 [19]. The column was eluted with 250 ml of 20 mM sodium carbonate buffer (pH 8·4), followed by 250 ml of 20 mM sodium acetate buffer (pH 5·5) and finally 200 ml of 20 mM acetic acid (pH 3·2). Fractions of the acetic acid eluate (10 ml) were pooled, concentrated on Centrifugal-Ultrafree (Millipore Corporation, Bedford) at 2500 ***g*** for 45 min and dialysed against 50 mM sodium acetate buffer (pH 5.0).

The dialysed chitinase fraction was applied on a CM-Sepharose (Pharmacia Fine Chemicals) column (20 × 3 cm) equilibrated with 20 mM sodium acetate (pH 5.2), and the adsorbed protein fraction was eluted with 200 ml of a linear gradient from 0 to 0·3 M NaCl in the same buffer. Active fractions (8 ml) were pooled and concentrated on Ultrafree-PF (Millipore Corporation, Bedford).

Chitinase assay

Endo- and exo-chitinase activity was measured by a colorimetric assay [1]. The reaction mixture contained 2 mg of colloidal chitin and various amounts of enzyme solution to give a final volume of 1 ml of 0·1 M sodium acetate (pH 5.2). The mixtures were incubated on a test tube rotator at 37 °C for 1 h and then centrifuged at 15000 ***g*** for 30 min. For the determination of endo-chitinase, 0·3 ml of the supernatant was incubated at 37 °C with 0·02 ml of 3% (w/v) snail gut enzyme (Cytohelicase, IBF) to hydrolyse the chitin oligomers. The resulting N-acetyl-glucosamine (GlcNAc) was determined using the method of Reissig *et al.* [23] with modifications described by Legrand *et al.* [11]. Controls included enzyme and substrate blanks as well as 50 μM and 100 μM GlcNAc in the assay buffer. Since the amount of reaction product was not a linear function of enzyme concentration [1], a dilution series of the enzyme (in duplicate) was tested, and the activity, calculated from a standard curve. One unit of activity was defined as that which catalysed the formation of 1 mol of GlcNAc (or its equivalent) s^{-1}.

The exo-chitinase assay omitted incubation with cytohelicase and the amount of GlcNAc was estimated in 0·3 ml of the supernatant obtained after centrifugation of the reaction mixture as described above.

Electrophoretic analysis

Sodium-dodecyl-sulphate (14%) polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Conejero & Semancik [5]. Non-denaturing polyacrylamide electrophoresis was carried out in cylindrical tubes (80 × 5 mm) as described by Reisfeld *et al.* [22] for basic proteins.

The marker proteins used for the estimation of molecular masses with SDS–PAGE were: lactalbumin (14 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), catalase (57 kDa) and bovine serum albumin (67 kDa).

Protein determination

Protein content was measured by the method of Bradford [4] using bovine serum albumin as a standard.

Molecular mass determinations of the chitinases

The molecular masses of the native enzymes were determined by gel filtration on a Sephadex G-100 column (75 × 1·5 cm). The enzymes and molecular markers were separately applied to the column which had been equilibrated with 50 mM Tris-HCl (pH 7·5), 100 mM NaCl. The molecular masses of the markers used were bovine serum albumin (67 kDa), ovalbumin (45 kDa), trypsin inhibitor (20 kDa), and cytochrome C (12·4 kDa). The void volume was determined using Blue Dextran (2000 kDa).

Preparation of antisera

Polyclonal antibodies against chitinase were obtained as follows. The purified chitinase (P32), 0·5 mg in 1 ml of phosphate buffered saline (PBS), was mixed with an equal volume of complete Freund's adjuvant (Difco) and injected intra-muscularly into rabbits. Five booster injections, each containing 0·1 mg of chitinase in 0·5 ml of PBS

and 0·5 ml of incomplete Freund's adjuvant were administered at 10 day intervals. Blood collections were made 8–10 days after the booster injection. A preimmune serum was taken from the rabbit prior to the initial injection of enzyme protein. The polyclonal antibody against tobacco chitinases was generously provided by Dr Meins (Friedrich-Miescher-Institut, Basel).

Purification of immunoglobulins was performed according to the method of Harboe & Ingild [7]. Non-immune (control) serum was similarly fractionated for use in comparative experiments with the chitinase IgG.

Immunodiffusion analyses

Double-diffusion analyses [20] were carried out in 1% (w/v) agarose in 50 mM Tris-HCl, (pH 7·5) containing 0·9% NaCl. Immunoprecipitin lines were allowed to develop for 24 h at room temperature and then stained with Coomassie Brilliant Blue, as described by Mayer & Walker [15], after washing the gel to remove unprecipitated protein.

Immunoblotting (Western blots)

Immunoblots were performed according to the method of Towbin *et al.* [28]. Proteins were transferred electrophoretically to nitrocellulose membranes. Chitinases were detected with the appropriate antisera (1 $\mu l\ ml^{-1}$ buffer), followed by goat anti-rabbit antibody coupled to horseradish peroxidase and then by the colour-development reagent (4-chloro-1-naphthol). All materials employed were obtained from Bio-Rad.

RESULTS

The effects of CEV-infection and ethylene treatment on chitinase activity

Exo- and endo-chitinase activity was measured in extracts of tomato leaves from CEV-infected, ethylene treated and control plants (Table 1). Both viroid infection and ethylene treatment produced an enhancement of exo-chitinase activity (5 and 4-fold, respectively) as compared with control healthy plants. Endo-chitinase activity, which was greater by a factor of 95 than exo-chitinase activity in control healthy leaves, was also enhanced by factors of about 12 and 19 in CEV infected and ethylene treated plants, respectively.

Purification of viroid-induced chitinase activity

Chitinase was purified from acidic extracts of tomato leaves showing symptoms of CEV infection [6] by ammonium sulphate precipitation, followed by affinity and ion exchange chromatography. The changes in specific activity and recovery of the enzyme after each stage of the purification process are summarized in Table 2.

Chitinase precipitated with ammonium sulphate was purified by affinity chromatography using regenerated chitin as the adsorbent. The active fractions were eluted by changing the pH of the buffer (Fig. 1). Chitinase activity was found in two fractions after ion exchange chromatography on a CM-Sepharose column (Fig. 2). The active fractions were eluted by changing the ionic strength of the buffer. After this procedure, the specific activities of the enzymes had increased 103·8 fold in fraction I (P32) and 2·1 fold in fraction II (P34).

TABLE 1
The effects of CEV-infection and ethylene treatment on chitinase activity

	Activity ($nkat\ ml^{-1}$ crude extract)			Increase in activity relative to the control	
	Control	Ethylene-treated	CEV-infected	Ethylene-treated	CEV-infected
Exo-chitinase	0·02	0·099	0·086	05·1	4·3
Endo-chitinase	1·90	36·970	19·400	19·4	12·5

Desalted homogenates of tomato apical leaves (4 top leaves) from: CEV-infected plants harvested 1 week after symptom emergence, 0·02 M ethephon-treated (ethylene-treated) plants harvested 4 days after treatment and healthy control plants. All plants were 6 weeks old at harvest. See Materials and Methods for details.

TABLE 2
Purification of CEV-induced chitinase from tomato leaves

Fraction	Total protein (mg)	Total chitinase (nkat)	Specific activity ($nkat\ mg^{-1}$ protein)	Yield (%)	n-Fold
Crude extract pH 2·8	86·0	4042·0	47·0	100·0	1·0
Precipitated by 75% ammonium sulphate	42·0	2192·4	52·2	54·2	1·1
Chitin column eluate; pH 3·3	2·1	1930·5	919·3	47·8	19·6
CM-Sepharose column eluate:					
0·16 M NaCl	0·4	1806·0	4881·1	44·7	103·8
0·28 M NaCl	0·2	20·9	99·3	0·5	2·1

Purification of chitinase from CEV-infected, symptom expressing, tomato apical leaves 1 week after emergence of symptoms. Protein content of each fraction was determined by the method of Bradford [4] and the activity of the enzyme was measured by a colourimetric assay. See Materials and Methods for details.

The molecular masses of these cationic chitinases, estimated by SDS-PAGE, were 32 kDa and 34 kDa, respectively (Fig. 3). The native enzymes exhibited molecular masses of 23 kDa and 25 kDa by gel filtration on Sephadex G-100. These results suggest that both enzymes are monomeric.

Using a non-denaturing (10%) PAGE system for basic proteins [22] the two chitinases, P32 and P34, were shown to correspond to the two PR proteins C_7 and C_6 (Fig. 4) previously described [6]. In an attempt to acquire further evidence on the relationship between P32 and C_7 and P34 and C_6, non stained gels were sliced into 5 mm fractions, 1 ml of distilled water was added to each fraction and the fractions were incubated at room temperature for 1 h. Samples of 50 μl of each fraction were then assayed separately for chitinase activity and this activity was only found in the bands corresponding to PR proteins C_7 (P32) and C_6 (P34).

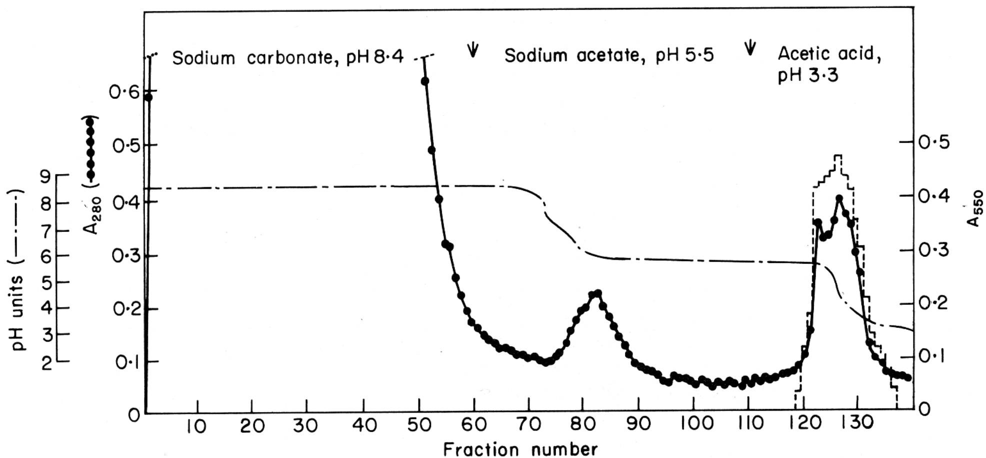

FIG. 1. Fractionation of chitinase activity on a chitin column. Extracts were loaded onto a chitin column (30 × 3 cm) equilibrated with 20 mM sodium carbonate buffer, pH 8·4. The column was run at room temperature with a flow rate of 100 ml per hour. Fractions containing 8 ml were collected. The activity was eluted by changing the pH of the buffer. pH gradient (●–●–). Protein was measured spectrophotometrically at 278 nm (●——●). Chiltinase activity (histogram) was assayed as described in Materials and Methods.

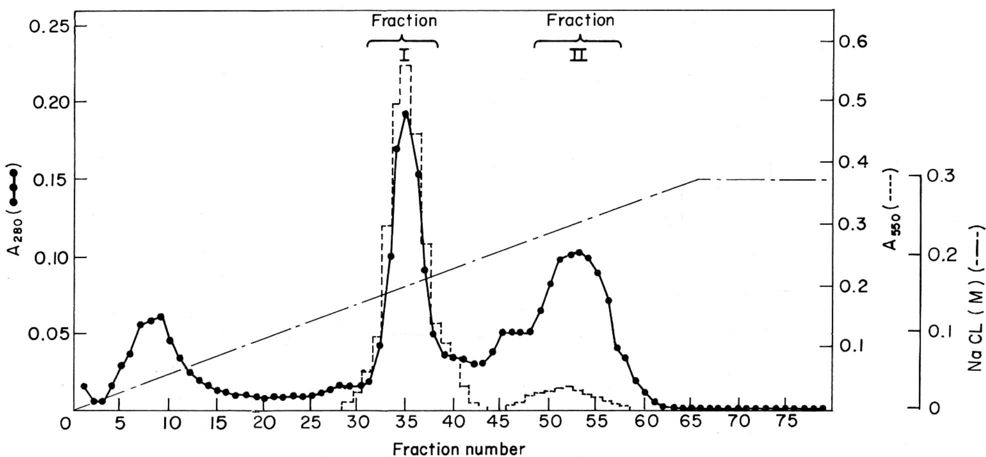

FIG. 2. Elution profile of chitinase activity after cation exchange chromatography. A CM-Sepharose column was equilibrated, loaded and eluted as described in Materials and Methods. NaCl gradient (-—-—). Protein was measured spectrophotometrically at 278 nm (●——●). Chitinase activity (histogram) was assayed as described in Materials and Methods.

Specificity of the anti-serum

Using a Western blot assay it was found that an antiserum raised in rabbits against the purified chitinase P32 reacted with both chitinases but not with any other protein present in the gel (Fig. 5a). The serological relationship between P32 and P34 and tobacco chitinase, P33, was also studied using IgG against tobacco chitinase. The test showed that this IgG had a highly specific affinity with the two chitinases from tomato (Fig. 5b).

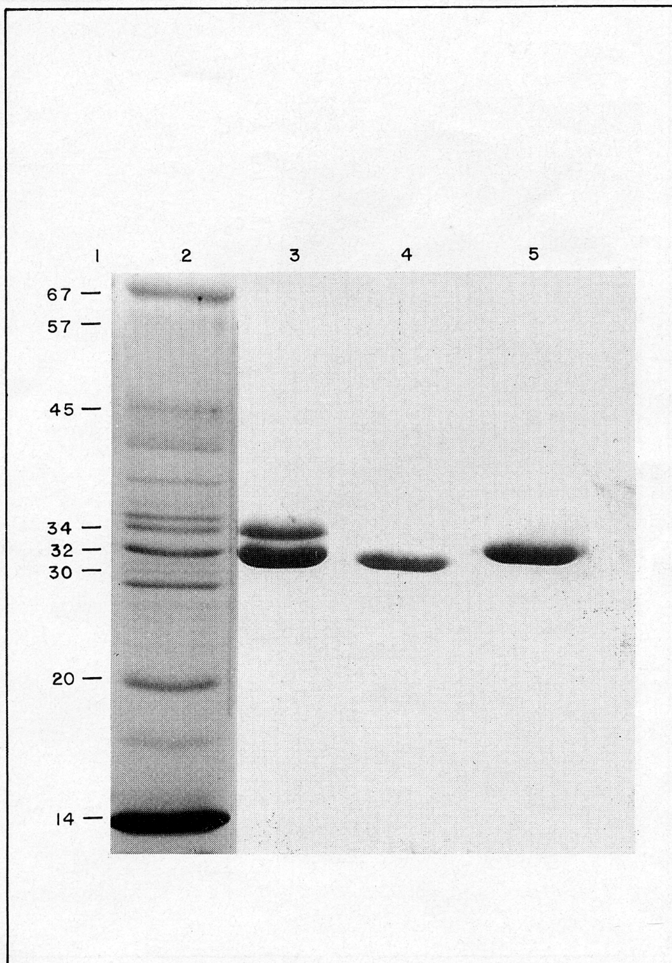

FIG. 3. SDS–PAGE of chitinases. Lane 1, molecular masses (kDa); lane 2, crude extract from CEV-infected tomato leaves; lane 3, chitinase preparation after affinity chromatography on regenerated chitin; lanes 4 and 5, purified chitinases, fraction I (P32) and II (P34), respectively, after cation-exchange chromatography on CM-Sepharose.

Double immunodiffusion tests confirmed the serological relationship between the two tomato CEV-induced chitinases (Fig. 6) with the tobacco chitinase (data not shown).

Properties of CEV-induced tomato chitinase (P32)

Since the endo-chitinase P32 was the major CEV-induced chitinase we investigated its properties in detail.

The activity of the purified endo-chitinase was not a linear function of enzyme concentration (data not shown), behaviour which coincides with that of the tobacco chitinases [1]. Its activity was inhibited by its specific antiserum and also by tobacco antiserum (Fig. 7). About 3 μl of P32 antiserum (2 mg ml^{-1}) and 10 μl of tobacco

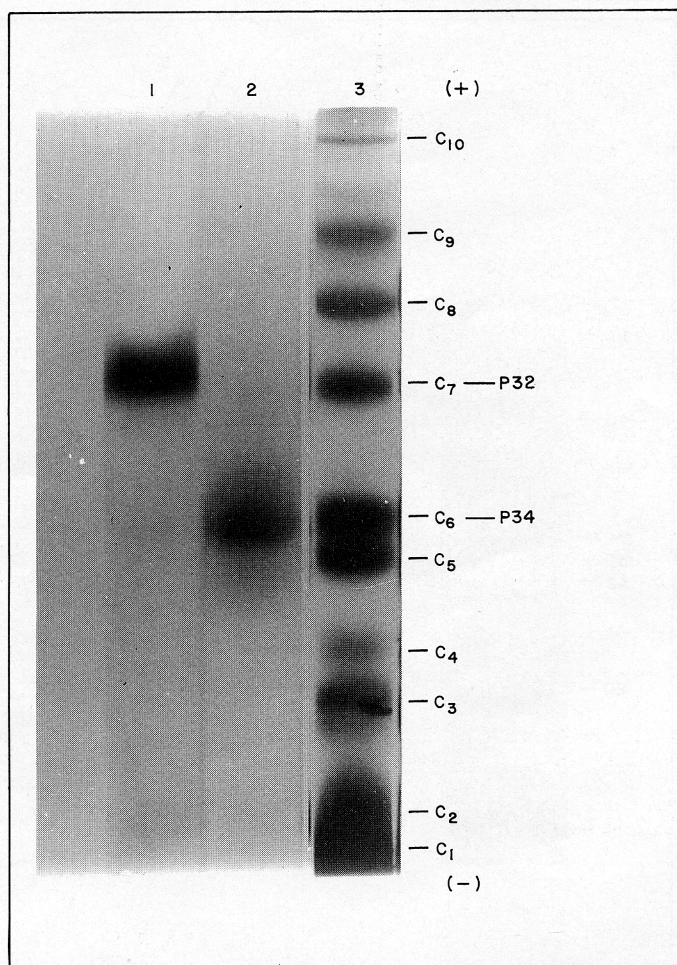

FIG. 4. Cathodic electrophoresis of the chitinases using the non-denaturing system of Reisfeld *et al.* [22]. Lane 1 and 2, purified chitinases, fraction I (P32) and II (P34), respectively, after cation-exchange chromatography on CM-Sepharose; lane 3, acidic extract of CEV-infected tomato leaves with PR proteins labelled.

antiserum (2 mg ml^{-1}) were needed to inhibit (> 90% and > 60%, respectively) the activity of 1 μl of fraction I which contained 35 μg of P32 chitinase.

The endo-chitinase has a pH optimum around 5·2 and at this pH its activity is stable at temperatures up to 40 °C for up to 2 h. Maximum activity occurs between 35 °C and 40 °C. It has a pI of 8·5 to 9·0 as ascertained by electrofocusing.

The resistance of the PR proteins to proteinase attack was also investigated. P32 was treated with six proteases, including pronase, proteinase K, trypsin, α-chymotrypsin, papain and tomato aspartyl proteinase [24] and the two glycosidases, α-mannosidase and β-glucosidase. The results (not presented) showed that P32 was resistant to all the carbohydrases and proteases tested except proteinase K which produced a 30%

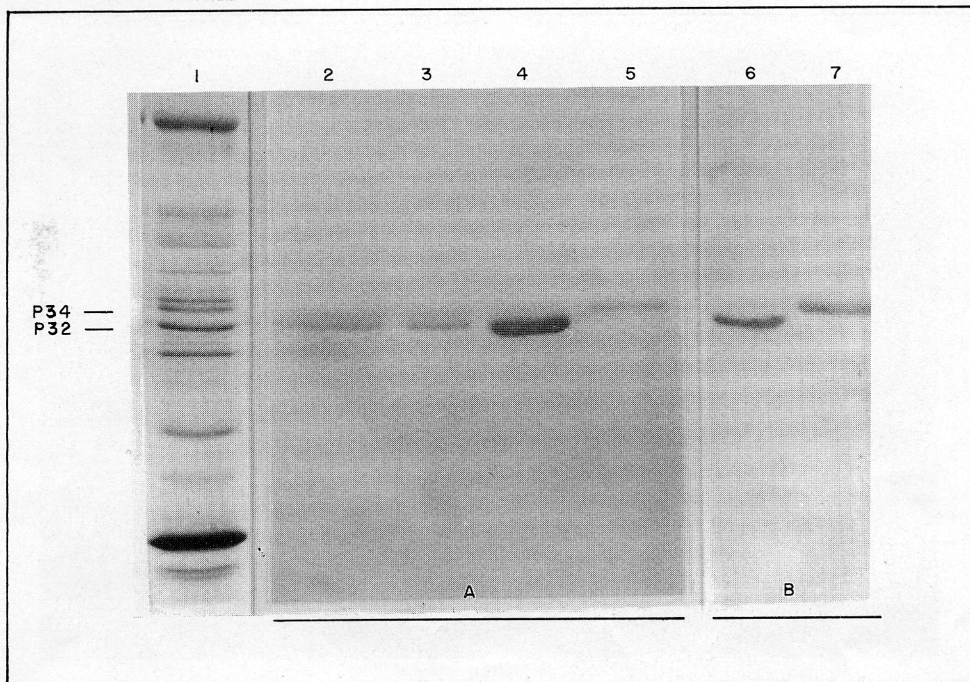

FIG. 5. Immuno blot of crude extract and purified chitinases from CEV-infected tomato leaves reacted with antisera against tomato and tobacco chitinase. Lane 1, SDS–PAGE of crude extract of CEV-infected tomato leaves. Lanes 2–7 reacted with (A) tomato chitinase and (B) tobacco chitinase. Lane 2, crude extract; lane 3, chitinase preparation after affinity chromatography on regenerated chitin; lanes 4 and 6, purified chitinase fraction I (P32); lanes 5 and 7, purified chitinase fraction II (P34).

reduction in activity and tomato aspartyl proteinase, which gave complete inhibition at its optimum pH of 2·8.

DISCUSSION

In this paper we report the purification of the two PR proteins, P32 and P34 from CEV infected and ethephon treated tomato plants. They appear to be identical to the tomato PR proteins C_7 and C_6 described previously [6]. Both proteins possess chitinase activity and they share a number of properties with chitinases from other plant species [2, 10]. They are both endo-chitinases and are immunologically related to each other and to P33 chitinase from tobacco. Both proteins have low molecular masses (32 kDa and 34 kDa, respectively). The more detailed study of the P32 chitinase showed further similarities to other chitinases in that it was a basic protein (pI, 8·5), had a low optimum pH for hydrolytic activity (5·2), high thermal stability and was relatively resistant to degradation by proteases and carbohydrases.

Chitinase induction by pathogen attack has been generally associated with incompatible or hypersensitive host reactions [29] and, has been implicated in defence reactions against invading fungi [8, 21, 25]. Consistent with this role is our previous

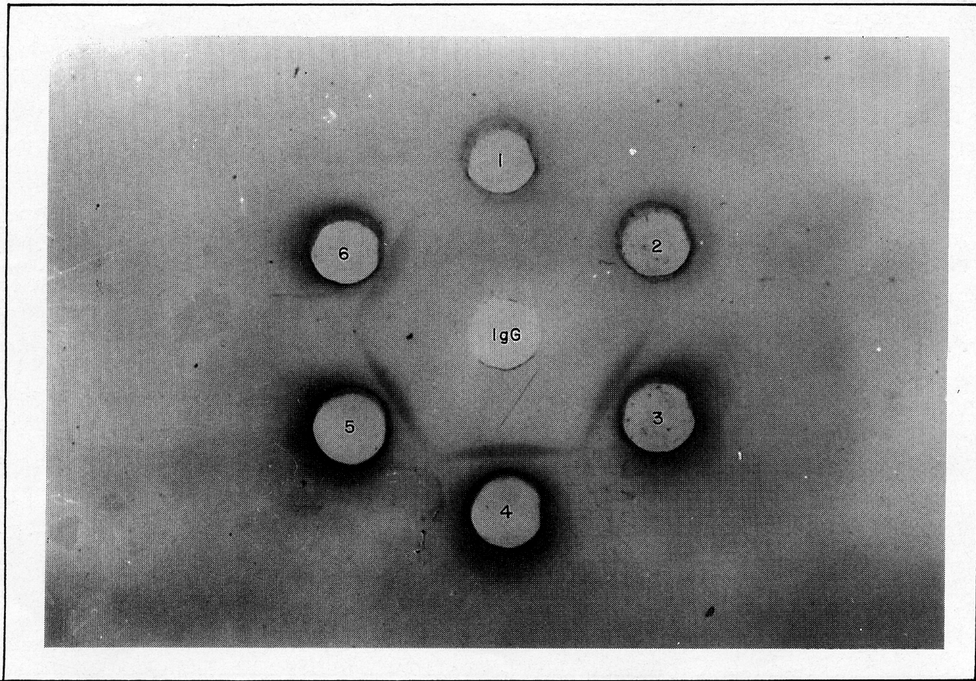

FIG. 6. Double immunodiffusion precipitin reactions between chitinase P32 and the antiserum prepared against it. The centre well contained 10 μl of IgG obtained from rabbits immunized against purified chitinase (P32). The outside wells, contained: (1) control well, no sample present; (2) 10 μl crude extract of healthy tomato leaves; (3) 10 μl crude extract of ethephon-treated tomato leaves; (4) 10 μl crude extract of CEV-infected tomato leaves; (5) 10 μl purified chitinase (P32); (6) 10 μl purified chitinase (P34). After 24 h at 6 °C, samples were stained with Coomassie Brilliant Blue as described by Mayer & Walker [15].

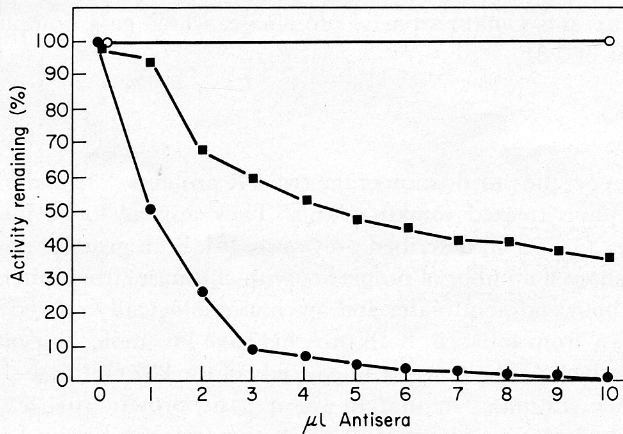

FIG. 7. Inhibition of purified chitinase, fraction I (P32), by antisera. Different amounts of tomato chitinase antiserum (●) and tobacco chitinase antiserum (■) were added to a given amount of the purified enzyme in a standard assay 5 min before the addition of substrate. The activity is recorded as a percentage of that preimmune serum (○).

finding [13, 14, 26] that the growth of plant pathogenic fungi in culture is inhibited by the purified chitinases. As has been pointed out previously [13], endo-chitinases of the type described in this paper are more likely to play a defensive role than exo-chitinases.

This is the first report that a viroid infection induces chitinase production and it is also the first report of a systemic, non-necrotic reaction causing production of chitinases. This fact lends support to the suggestion [12, 16, 21, 32] that chitinase induction is a component of a general mechanism of response to pathogen attack.

Chitinases cannot be directly involved in defence against viroids. They are most likely to be components of a specific defence mechanism which evolved during coevolution with pathogens containing chitin as a part of their structure. Our finding that a viroid stimulates chitinase production indicates that the induction of this specific antifungal defence is linked to a network of events that are coordinately triggered as a response to a wide variety of pathogens and stress factors.

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