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Antifungal Hydrolases in Pea Tissue[1]

I. PURIFICATION AND CHARACTERIZATION OF TWO CHITINASES AND TWO β-1,3-GLUCANASES DIFFERENTIALLY REGULATED DURING DEVELOPMENT AND IN RESPONSE TO FUNGAL INFECTION

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ABSTRACT

Chitinase and β-1,-3-glucanase activities increased coordinately in pea (*Pisum sativum* L. cv "Dot") pods during development and maturation and when immature pea pods were inoculated with compatible or incompatible strains of *Fusarium solani* or wounded or treated with chitosan or ethylene. Up to five major soluble, basic proteins accumulated in stressed immature pods and in maturing untreated pods. After separation of these proteins by chromatofocusing, an enzymic function could be assigned to four of them: two were chitinases and two were β-1,3-glucanases. The different molecular forms of chitinase and β-1,3-glucanase were differentially regulated. Chitinase Ch1 (mol wt 33,100) and β-1,3-glucanase G2 (mol wt 34,300) were strongly induced in immature tissue in response to the various stresses, while chitinase Ch2 (mol wt 36,200) and β-1,3-glucanase G1 (mol wt 33,500) accumulated during the course of maturation. With a simple, three-step procedure, both chitinases and both β-1,3-glucanases were purified to homogeneity from the same extract. The two chitinases were endochitinases. They differed in their pH optimum, in specific activity, in the pattern of products formed from [^{3}H]chitin, as well as in their relative lysozyme activity. Similarly, the two β-1,3-glucanases were endoglucanases that showed differences in their pH optimum, specific activity, and pattern of products released from laminarin.

An attack by potential pathogens elicits profound changes in the metabolism of a plant. In recent years, particular attention has been paid to the strongly altered pattern of protein synthesis in plant cells treated with pathogens or pathogen-derived elicitors. Analysis of proteins (9), of translation products of mRNA (7, 9, 21), and of translation products of newly formed mRNA (7) indicate that 20 or more proteins are newly formed or strongly induced upon infection or elicitor treatment. What is the significance of these proteins for the defense of the plant against pathogens? The function of most of them is still entirely unknown. Some of them, the pathogenesis-related (PR-) proteins (21, 23) and the resistance-related proteins (9), have obtained suggestive names. However, their relation to pathogenesis or to induced resistance remains a challenging, unsolved puzzle.

The function of a small number of the newly synthesized proteins has been identified. Certain key enzymes of secondary metabolism, such as phenylalanine ammonia lyase and chalcone synthase, belong to this group. While these enzymes do not harm microoganisms directly, they contribute to the synthesis of antibiotic, secondary metabolites, called phytoalexins (8). We have become interested in another group of proteins: namely, hydrolytic enzymes with the potential to directly attack and degrade the cell walls of potential pathogens.

Many pathogenic microorganisms attack their host plants by secreting hydrolases, such as pectinase and cellulase, which are able to degrade components of the plant cell wall. Correspondingly, plants possess hydrolases capable of attacking and degrading the cell walls of potential pathogens. The widespread occurrence of lysozyme and chitinase is particularly intriguing, since these enzymes appear to lack endogenous substrates in higher plants (2). Abeles *et al.* (1) have observed that ethylene induces chitinase and β-1,3-glucanase in bean leaves. Since many fungi have cell walls rich in chitin and β-1,3-glucan, they hypothesized that these two hydrolases might help the plants defend against pathogenic fungi. Subsequently, it was shown, in a number of plant tissues, that chitinase and β-1,3-glucanase are coordinately induced not only by ethylene but also by pathogen attack (13, 18) and by elicitors (11, 13, 19). The co-occurrence and co-regulation of chitinase and β-1,3-glucanase point to a common function for these two enzymes. We have shown previously that increased ethylene production is not required for the induction of chitinase and β-1,3-glucanase in pathogen-infected pea pods (13), indicating that a more direct connection exists between pathogen attack and induction of the two hydrolases.

In this article we report that chitinases and β-1,3-glucanases are prominent among the stress-induced proteins in pea pods. Of the five major basic proteins that accumulate in response to pathogen attack, two are chitinases and two are β-1,3-glucanases. We present a purification scheme for all major isozymes and describe their differential regulation during development and in response to several stresses such as wounding, pathogen attack, and treatment with chitosan or ethylene. We have obtained direct evidence for the antifungal activity of combinations of purified chitinase and β-1,3-glucanase (our unpublished observations).

MATERIALS AND METHODS

Biological Material. Seeds of *Pisum sativum* L. cv "Dot" (an Alaska-type variety), were sown in vermiculite and grown in a greenhouse or in a growth chamber under a 16:8 h, 21°C:17°C light:dark regime. The plants were fertilized every 3 d with 2 ml/L Greenzyt (CIBA-GEIGY, Switzerland). *Fusarium solani* f.sp. *phaseoli*, strain W-8 (American Type Culture Collection, ATCC 38135), and *Fusarium solani* f.sp. *pisi*, strain P-A (ATCC 38136),

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were grown on malt extract agar.

Inoculation of Plant Material. Freshly harvested immature pea pods (about 4 d after anthesis, 220 ± 50 mg fresh weight) were carefully split in half. The exposed endocarp tissue of each half pod was treated with 25 μl of the following solutions: sterile water, 0.1% (w/v) chitosan, or a suspension of macroconidia (1×10^6 spores per ml) of *F. solani* f.sp. *phaseoli* (a nonpathogen of peas) or *F.solani* f.sp *pisi* (a pathogen of peas). The inoculated samples were incubated at room temperature in a moist, dark chamber for the appropriate time period. Mature pea pods were harvested 3 to 4 weeks after anthesis. For the ethylene treatment, the pods were treated with sterile water and incubated in an atmosphere containing 20 ppm ethylene. After incubation, the pea pods were either homogenized immediately or frozen and kept at −80°C until used.

Enzyme Extraction. Plant material was ground in liquid nitrogen with a mortar and pestle. The resulting fine powder was extracted (4 ml g^{-1}) with cold 0.1 M Tris-HCl (pH 7.5), containing 1 mM phenylmethylsulfonyl fluoride. Insoluble material was removed by centrifugation at 20,000*g* for 20 min. Reducing sugars, which would have interfered with the glucanase assay, were removed by precipitation of the proteins in the extract with ammonium sulfate (95% saturation) at 0°C for 2 h. The precipitate was collected by centrifugation (20 min, 20,000*g*) and suspended in 10 mM Tris-HCl, pH 8.0.

Separation and Purification of Chitinases and β-1,3-Glucanases. All operations were carried out on ice or at 4°C in a cold room. The redissolved proteins were dialyzed against 10 mM Tris-HCl (pH 8.0), and then loaded onto a column (4 × 12 cm) of Trisacryl-DEAE (IBF, Clichy, France) equilibrated with the same buffer. The column was run at a flow rate of 50 cm h^{-1}. The basic proteins, which passed through the column in the void volume, were collected. The extract was processed further according to one of the following two methods (Fig. 1) depending on the aim of the experiment. In method A, chitinase activity was separated from the rest of the basic proteins by affinity chromatography on a chitin column. The extract containing the basic proteins was loaded onto a column (10 ml volume/30 units chitinase loaded) of regenerated chitin (2) equilibrated with 20 mM Tris-HCl (pH 8.0). The proteins that passed through the column in the void volume and the chitinase activity that was eluted after a wash with 20 mM sodium acetate buffer (pH 5.5) by lowering the pH to 3.2 with 20 mM acetic acid were collected separately. Each preparation was concentrated to a volume of about 3 to 4 ml by ultrafiltration (PM 10 diaflo membrane, Amicon-Grace). In method B, the eluate from the anion exchange column was directly concentrated by ultrafiltration.

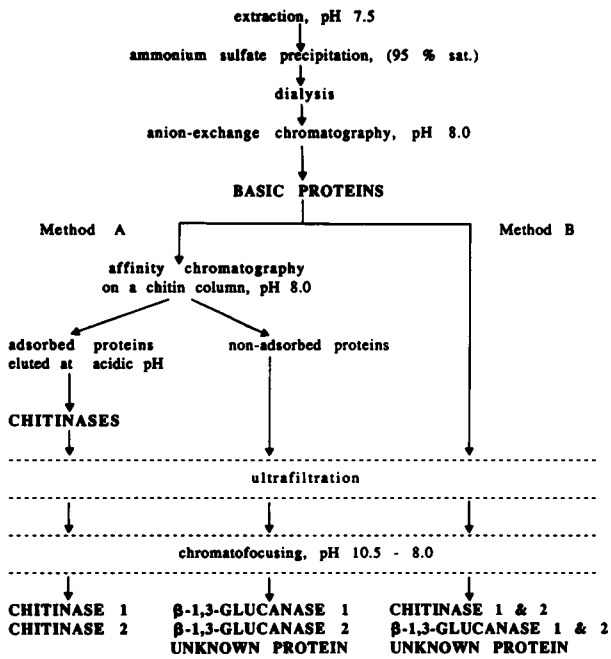

FIG. 1. Flow chart of the purification and separation protocols. Details are described in the text.

The desalted and concentrated preparations were adjusted to pH 11.0 by the addition of triethylamine-HCl (pH 11.0) to a final concentration of 5 mM. The solutions (3–4 ml) were applied to a chromatofocusing column (0.9 × 30 cm) containing PBE 118 resin (Pharmacia), equilibrated with 25 mM triethylamine-HCl (pH 11.0). The column was eluted at a flow rate of 38 cm h^{-1} with Pharmalyte 8 to 10.5 (Pharmacia), diluted 1:45 (v/v) with water, and adjusted to pH 8.0 with 1 N HCl. Each chromatofocusing run was controlled by monitoring A_{280} and the pH gradient at 4°C. Fractions of 2.1 ml were collected and assayed for chitinase and β-1,3-glucanase activity. Fractions containing individual isozymes were pooled, dialyzed against water, and lyophilized. Since the ampholytes used for chromatofocusing absorbed slightly at 280 nm, the A_{280} values from each run were corrected by subtracting the A_{280} pattern of a corresponding run without protein.

Enzyme Assays. Chitinase activity was assayed radiometrically with [^{3}H]chitin as a substrate (13). The [^{3}H]chitin was synthesized by acetylation of chitosan (Sigma) with [^{3}H]acetic anhydride (2). The regenerated [^{3}H]chitin was dissolved in cold hydrochloric acid and made colloidal by precipitation in 50% (v/v) ethanol. The specific radioactivity of the final product was 10,000 Bq mg^{-1}. The activity of β-1,3-glucanase was determined by a modification of a colorimetric assay (13). The assay mixture contained, in a total volume of 250 μl: enzyme solution, 0.5 mg of reduced laminarin, 5 μmol of sodium acetate buffer (pH 5.5), and 50 μg of BSA. After incubation at 37°C for 20 min, the amount of reducing sugars that had been released was determined. Laminarin (United States Biochemical Corporation) was reduced as described (13).

In both assays, product formation was not a linear function of enzyme activity. Therefore, activities were calculated for an enzyme concentration approaching zero with standard curves of the purified enzymes (2). The amount of enzyme producing 1 μmol min^{-1} GlcNAc equivalents or 1 μmol min^{-1} glucose equivalents, respectively, at infinite dilution was defined as 1 unit. Each value is the mean of at least two replicate assays performed at different enzyme concentrations. The average standard deviation was less than 10% of the mean in both assays.

Lysozyme activity was determined in a reaction mixture consisting of enzyme, 0.6 mg of lyophilyzed cells of *Micrococcus lysodeikticus* (Calbiochem), and 16 μmol of sodium acetate buffer (pH 4.5) in a total volume of 2 ml. After incubation at 37°C for the appropriate time, the reaction was stopped by the addition of 1 ml of 0.5 M Na_2CO_3 and the A_{645} was measured.

Determination of the pH Optimum of Chitinases and β-1,3-Glucanases. The pH dependence of the enzymic hydrolysis of chitin by purified chitinase and of laminarin by purified β-1,3-glucanase was determined by replacing the buffer used in the respective standard enzyme assays with 10 mM sodium citrate buffer (for pH values between 3 and 6) or 10 mM sodium phosphate buffer (for pH values between 6 and 8). The pH value was determined in an aliquot of the reaction mixture. The amounts of soluble radioactivity (chitinase) and of reducing sugars (β-1,3-glucanase) were used directly as a measure of activity. The experiments were repeated twice and gave identical results.

Analysis of Chitinase Reaction Products. The enzymic hydrolysis of highly labeled [^{3}H]chitin (100,000 Bq mg^{-1}) was per-

formed in 1.5 ml of the standard chitinase assay mixtures and was stopped by boiling for 15 min. After centrifugation at 2000*g* for 15 min, the supernatant was desalted by addition of 50 mg ml^{-1} ion exchanger (type V, Merck). Preliminary experiments had shown that chitooligosaccharides did not bind to this ion exchanger. The desalted samples were applied to silica gel TLC plates (type 60, Merck) and developed with *n*-propanol: water:ammonia solvent (70:30:1, v/v). A chitooligosaccharide standard series was prepared by acid hydrolysis of [^{3}H]chitin (2). For fluorography, the dry plates were immersed in diethylether containing 7% (w/v) 2,5-diphenyloxazole for 5 s, dried, and then incubated in contact with an x-ray film (Kodak X-Omat 5) at −80°C for 5 d. For quantitative analysis, individual bands were scratched out after their location by fluorography and their radioactivity was determined. The R_F value of the monomer GlcNAc was determined by spraying a corresponding plate with a solution of 1 g dimethylaminobenzaldehyde, 30 ml ethanol, 30 ml concentrated HCl, and 180 ml of *n*-butanol. The plate was incubated at 90°C for 5 min.

Analysis of β-1,3-Glucanase Reaction Products. The enzymic hydrolysis of laminarin was performed in 1 ml of a standard reaction mixture and was stopped by the addition of 50 μl 1 N NaOH. Aliquots of 6 μl were applied to a silica gel plate (type 60, Merck). The plates were developed three times with acetonitrile:water (4:1, v/v) (25). The sugars were detected by spraying with a solution of 3 g naphthol in a mixture of 100 ml methanol and 30 ml concentrated H_2SO_4 and incubating the plate at 110°C for 5 min. Oligosaccharide standards were prepared by acid hydrolysis of laminarin (25). The developed TLC plates were scanned with a Shimadzu Scanner model CS-390.

Protein Determination. Protein concentration was measured according to Bradford (4) with BSA as a standard.

Electrophoresis. SDS-PAGE was performed according to Laemmli (12). Phosphorylase B (92.5 kD), BSA (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD) and egg white lysozyme (14.3 kD) served as mol wt markers. The gels were stained with Coomassie brilliant blue R-250.

RESULTS

β-1,3-Glucanase and Chitinase in Pea Pods during Ripening. Changes in the protein content and in chitinase and β-1,3-glucanase activities were determined in pea pods during the course of fruit development and maturation (Fig. 2). During the first 10 d after anthesis, the pods grew vigorously and the seeds remained small. Protein content decreased considerably, and chitinase and β-1,3-glucanase activities remained at a low level (on a fresh weight basis). At this stage, chitinase and β-1,3-glucanase could be strongly induced by infection or by treatment with chitosan or ethylene (see below). Later, during the period of seed filling, *i.e.* from 10 to 30 d after anthesis, the activities of chitinase and β-1,3-glucanase increased dramatically. The protein concentration in the pods, however, continued to decline, so that the specific activity of both enzymes increased by a factor of about 20 during this period.

β-1,3-Glucanase and Chitinase: Major Soluble Basic Proteins of Pea Pods. The basic proteins in an extract of pea pods were isolated by anion exchange chromatography on Trisacryl-DEAE at pH 8. Basic proteins, which passed through the column in the void volume, represented about 0.4% of the total soluble protein in untreated immature pods, but 2 to 5% of the total soluble protein in infected immature pods or untreated mature pods. The void volume also contained 60 to 70% of the chitinase and β-1,3-glucanase activity loaded onto the column. Thus, only a small fraction of the total soluble proteins, but most of the chitinase and β-1,3-glucanase, were basic proteins.

The basic proteins were further analyzed by chromatofocusing.

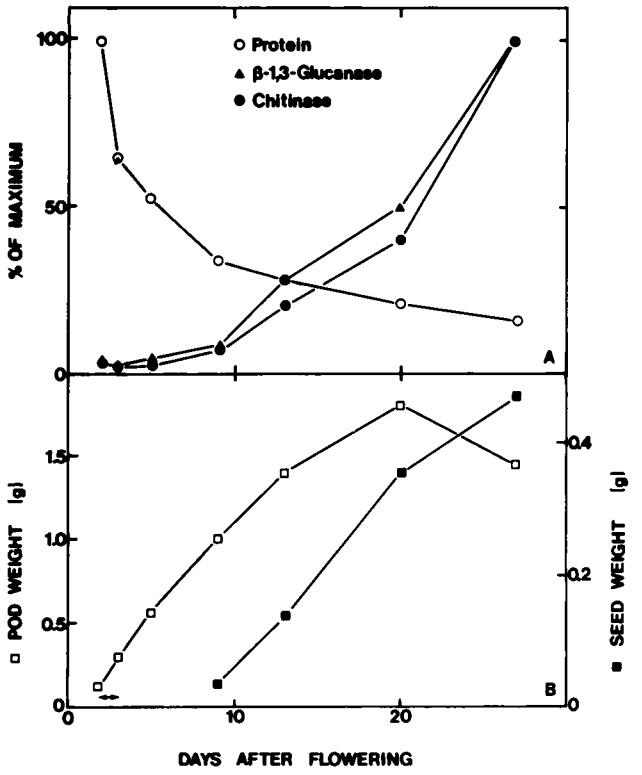

FIG. 2. A, Chitinase and β-1,3-glucanase activities and protein in pea pods in the course of maturation. Values of 100% correspond to 6.5 mg protein, 1800 munits β-1,3-glucanase, and 550 munits chitinase per g fresh weight, respectively; B, pod fresh weight and seed fresh weight per fruit. The arrows on the abscissa mark the time period at which immature pods were excised for experimentation.

A maximum of four to five major protein peaks were resolved; two of these exhibited β-1,3-glucanase activity (G1 and G2), and two exhibited chitinase activity (Ch1 and Ch2). Each peak appeared to consist of a single protein species when assayed by SDS-PAGE (data not shown). However, preparations of G2 and Ch1 showed some cross-contamination. Our results indicate that different forms of chitinase and β-1,3-glucanase make up a large proportion of the soluble basic proteins in pea pods. In addition to the major protein peaks, a small but distinct chitinase peak (Ch3) was observed which slightly preceded G1. Since this chitinase never made up more than 1% of the total chitinase activity, it was not studied further.

Differential Induction of β-1,3-Glucanase and Chitinase Isozymes during Development and in Response to Stress. Basic proteins isolated from pea pods of different ages or subjected to different stress conditions were analyzed by chromatofocusing (Table I).

Chromatofocusing of the basic proteins from untreated immature pea pods yielded only one major protein peak which coincided with the peak of β-1,3-glucanase G1 (Fig. 3A). Low levels of β-1,3-glucanase G2 and of all three chitinases were also observed. The total chitinase activity eluted was evenly distributed between chitinase Ch1 and Ch2.

The pattern of basic proteins was drastically changed in pea pods treated with *F. solani* f.sp. *phaseoli* (Fig. 3B). Chitinase Ch1 increased about 60-fold within 28 h; nearly all of the chitinase activity eluted as Ch1. The level of chitinase Ch2 remained low. β-1,3-Glucanase G1 and G2 increased by 6-fold and more than 100-fold, respectively. In addition to these peaks, another protein peak appeared that could not be assigned an enzymic function.

Table I. *Induction of Different Forms of Chitinase and β-1,3-Glucanase in Pea Pods by Various Stimuli*

Batches of 220 appropriately treated pea pods corresponding to 50 g of immature or 270 g of mature pods, respectively, were processed according to method B described in Figure 1. The treated pods were incubated for 28 h. The experiments were repeated at least once with similar results except for the ethylene treatment, which was performed only once.

Treatment	Chitinase					β-1,3-Glucanase				
	Total activity			Proportion of eluted activity		Total activity			Proportion of eluted activity	
	Crude extract	After anion exchange	After chromato-focusing	Ch1	Ch2	Crude extract	After anion exchange	After chromato-focusing	G1	G2
	units			*% units eluted*		*units*			*% units eluted*	
Immature pods										
None	3.9	2.2	1.3	46	54	32	12	9	93	7
Water	24.3	16.2	11.4	82	18	118	73	51	60	40
F. solani phaseoli	121.4	82.4	62.5	95	4	543	343	269	31	69
F. solani pisi	114.4	69.8	54.0	96	3	444	293	237	35	65
Chitosan	ND[a]	ND	16.9	93	7	189	117	105	23	77
Ethylene	58.5	40.4	29.2	95	4	233	156	96	39	61
Old pods										
None	90.8	ND	31.2	16	84	542	307	212	96	4

[a] Not determined.

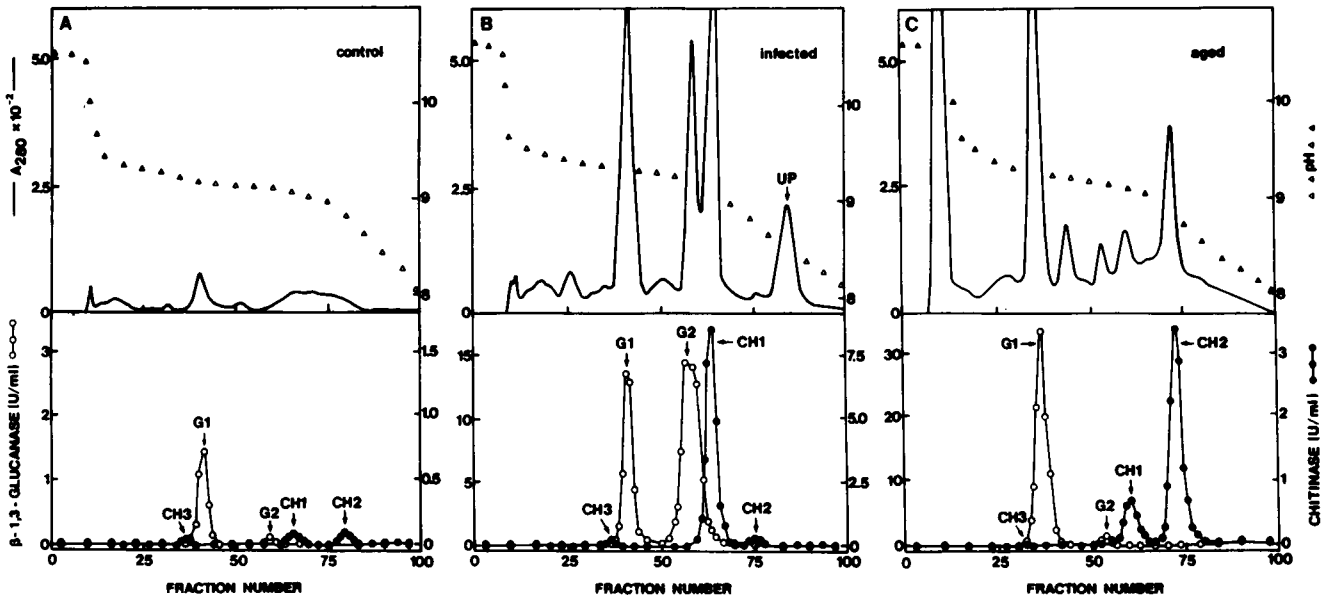

FIG. 3. Chromatofocusing of the basic proteins from (A) immature, untreated pea pods, (B) immature pea pods treated with *Fusarium solani* f.sp. *phaseoli* for 28 h, and (C) untreated mature pods. Batches of 220 pea pods corresponding to 50 g of immature or 270 g of mature pods were processed according to method B described in Figure 1. The chromatofocusing column had a volume of 19 ml; the fraction size was 2.1 ml.

This peak yielded a single protein band when assayed by SDS-PAGE and was designated unknown protein (UP).

The pattern in ripe pods (Fig. 3C) differed markedly from the pattern in healthy or infected immature pods. The major basic proteins in ripe pods were β-1,3-glucanase G1 and chitinase Ch2. Chitinase Ch1 and β-1,3-glucanase G2 were also present but were much less abundant than in infected tissue.

Corresponding analyses were performed with pea pods treated with sterile water, with spores of *F. solani* f.sp. *pisi* (compatible interaction), with chitosan, or with ethylene (Table I). Incubation of wounded immature pea pods with sterile water alone caused an approximately 10-fold increase of G2 and Ch1 but only a 2-fold increase in G1 and Ch2. When wounded pods were exposed to additional stress (pathogens, chitosan, or ethylene), the levels of G1 and Ch2 were not much affected, but the levels of G2 and Ch1 increased by 3- to 8-fold over the levels in wounded tissue alone (Table II). Clearly, ethylene and chitosan induced qualitatively similar changes in the isozyme pattern as an infection with *F. solani*. The unknown protein UP was found only after fungal infection or chitosan treatment but not after wounding or ethylene treatment. No significant difference with respect to the induced pattern of basic proteins was found between compatible and incompatible interaction.

In summary, the increase of chitinase and β-1,3-glucanase activities in immature pea pods that occurs in response to different stresses is based mainly on the increases of chitinase Ch1 and β-

Table II. *Survey of the Changes in the Pattern of Basic Proteins Induced by Various Stimuli*

Extracts from pea pods treated with water for 28 h (wounded) served as a reference to compare the increase of individual isozymes induced by different treatments. The comparison was made on a per pod basis.

Treatment	Protein				
	G1	G2	Ch1	Ch2	UP[a]
Untreated	0.4	0.1	0.1	0.5	−
Wounded[b]	1	1	1	1	−
Infected	2	8	6	1	+
Chitosan	1	3	3	1	+
Ethylene	1	3	3	1	−
Maturation	7	1	1	17	−

[a] +, Induced; −, not induced. [b] The amounts of the individual isozymes present in wounded tissue were standardized to one.

1,3-glucanase G2. In mature pea pods, on the other hand, primarily chitinase Ch2 and β-1,3-glucanase G1 accumulate.

Purification of β-1,3-Glucanase and Chitinase Isozymes. To purify reasonable quantities of the different isozymes of β-1,3-glucanase and chitinase, the chitinases were separated from the β-1,3-glucanases by affinity chromatography on regenerated chitin prior to chromatofocusing (Method A, Fig. 1). About 90% of the chitinases Ch1 and Ch2 was retained on the chitin column. The bound chitinases were eluted as a single protein peak when the column was washed with a low pH buffer and were further separated by chromatofocusing. β-1,3-Glucanases and the unknown protein passed through the chitin column in the void volume and were separated by subsequent chromatofocusing (data not shown). Table III summarizes the purification of G1, Ch1, and Ch2 from old pods and of G1, G2, and Ch1 from infected immature pods. Between 20 to 40% of the activity in a crude extract was isolated as homogeneous preparations of separate isozymes.

Protein preparations from healthy and infected immature pea pods at different stages of the purification were analyzed by SDS-PAGE (Fig. 4). In crude extracts from infected pods, new bands appeared at mol wt of about 18, 30, and 34 kD (Fig. 4, lanes 2–4). After passage of the preparations through Trisacryl-DEAE and a chitin column, three bands were found to be most prominent in the extract of infected pods (Fig. 4, lanes 6 and 7). These three proteins were identified as G1, G2, and UP and were purified to homogeneity by chromatofocusing (Fig. 4, lanes 8–10). SDS-PAGE of the chitinases eluted from the chitin column resolved two bands representing Ch1 and Ch2 as shown by chromatofocusing (data not shown). Both isozymes were represented about equally in uninfected control pods, while after infection Ch1 strongly predominated (Fig. 4, lanes 11–13).

SDS-PAGE of the basic proteins from mature pods after removal of the chitinases revealed only one dominating protein

Table III. *Purification of β-1,3-Glucanases and Chitinases from Mature and from Infected Immature Pea Pods*

The starting material consisted of 520 g of healthy mature or 50 g of immature pea pods which were incubated with *F. solani phaseoli* for 48 h. The purification protocol followed Method A described in Figure 1.

Step	Mature Pods				Infected Immature Pods			
	Total		Specific activity	Recovery	Total		Specific activity	Recovery
	Protein	Activity			Protein	Activity		
	mg	*units*	*units mg*$^{-1}$	%	*mg*	*units*	*units mg*$^{-1}$	%
β-1,3,-Glucanase								
Ammonium sulfate	608	988	1.6	100	310	514	1.7	100
Trisacryl-DEAE	22.1	675	30.5	68	ND	315		61
Chitin column pH 8	14.8	603	40.7	61	4.3	296	68.5	58
Chromato-focusing								
G1	4.2	330	78.0	33	0.9	70	76.0	14
G2	0.6	28	43.1[a]	3	0.8	83	111.1	16
Chitinase								
Ammonium sulfate	608	150	0.3	100	310	132	0.4	100
Trisacryl-DEAE	22.1	117	5.3	78	ND[b]	116		88
Chitin column pH 3.2	4.5	58	12.8	38	1.9	32	16.8	24
Chromato-focusing								
Ch1	0.7	12	17.7	8	1.1	22	19.0	16
Ch2	2.7	34	12.7	23	0.1	1	9.5[a]	1

[a] Not purified to homogeneity. [b] Not determined.

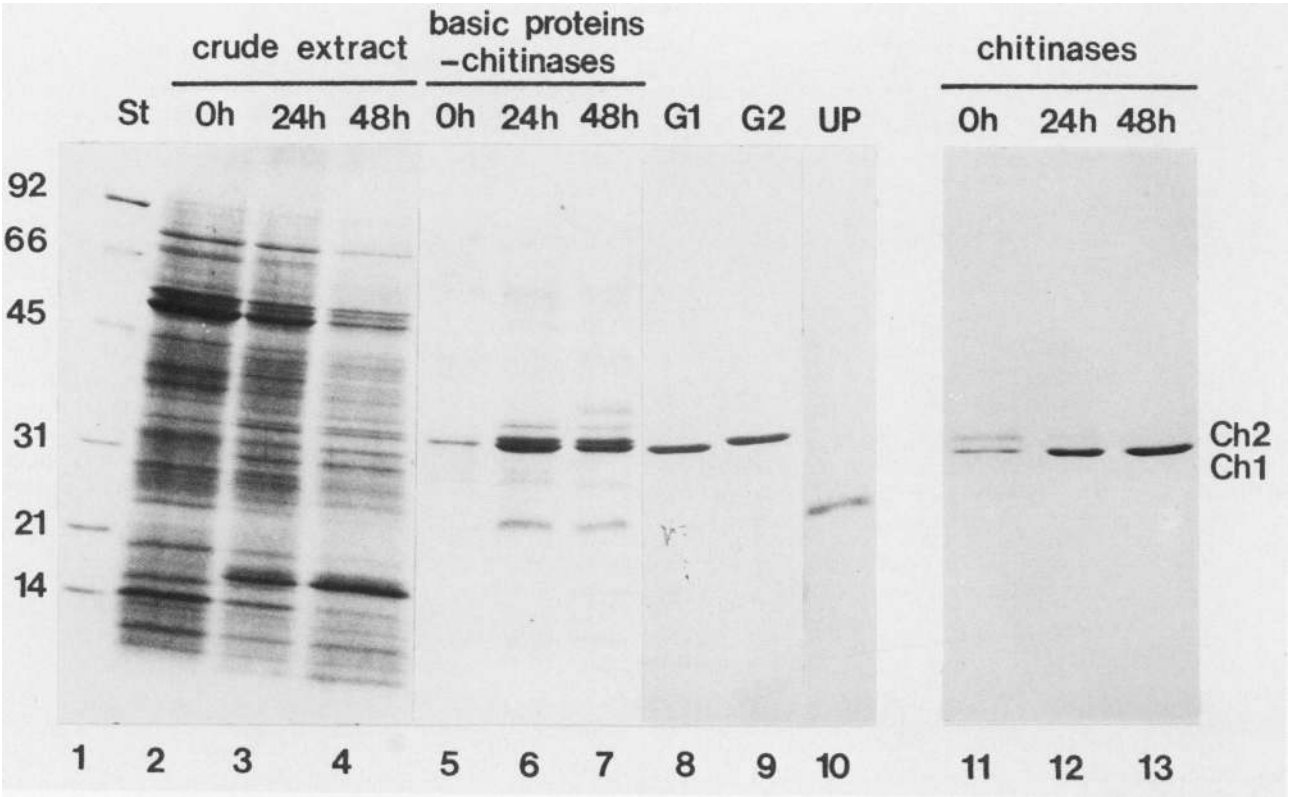

FIG. 4. SDS-PAGE on a 10 to 16% (T) gradient gel of protein preparations from immature pea pods, incubated with *Fusarium solani* f.sp. *phaseoli* at various stages of purification. Lane 1, mol wt standards; lanes 2 to 4, 80 μg crude protein extract; lanes 5 to 7, 50 μg protein after anion exchange chromatography and passage through a chitin column at pH 8.0; lanes 8 and 9, 5 μg of β-1,3-glucanase G1 and G2, respectively, separated by chromatofocusing from the preparation shown in lane 7; lane 10, 5 μg unknown protein (UP), separated by chromatofocusing from the preparation shown in lane 7; lanes 11 to 13, 5 μg protein eluted from the chitin column at pH 3.2 (*i.e.* chitinase Ch1 and Ch2). The mol wt in kD of the standard proteins is indicated on the left.

band (Fig. 5, lane 3). This protein was purified to homogeneity by chromatofocusing and was identified as β-1,3-glucanase G1 (Fig. 5, lane 4). As in infected tissue, two different chitinases were retained on the chitin column (Fig. 5, lane 5) and could be separated by chromatofocusing yielding homogeneous preparations of Ch1 and Ch2 (Fig. 5, lanes 6 and 7). In this case, however, the heavier form, Ch2, predominantly accumulated.

The characteristics of chitinases Ch1 and Ch2, β-1,3-glucanases G1 and G2, and the protein UP were examined using our purified preparations (Table IV). The specific activities of the purified enzymes allowed us to estimate how much enzyme was present in crude extracts. In untreated immature pea pods, β-1,3-glucanase and chitinase represented 0.2% of the total soluble protein. Within 28 h after inoculation with *F. solani*, this value increased to 4%. During this time period, up to 100μg of β-1,3-glucanase and chitinase, respectively, were produced per g fresh weight. In mature tissue the combined β-1,3-glucanases and chitinases constituted more than 6% of the total soluble proteins.

Biochemical Characterization of the Purified Chitinases. The two forms of chitinase exhibited a slightly different pH optimum (Table IV). The pattern of products formed from radioactive chitin was studied (Fig. 6). Both chitinases were identified as endochitinases, since they initially catalyzed the release of chitooligosaccharides. With increasing incubation time, both chitinases started to break down the initially released larger oligosaccharides into smaller units. Hydrolysis by Ch2 ultimately produced a mixture of diacetylchitobiose and triacetylchitotriose. Hydrolysis by Ch1 also produced these two saccharides; however, only Ch1 appeared to be capable of hydrolyzing triacetylchitotriose into GlcNAc and diacetylchitobiose.

The lysozyme activity of the two chitinases was very different (Fig. 7). Ch1 was approximately 10 times more effective than Ch2 against the cell walls of *Micrococcus lysodeikticus*. An inhibitor of lysozyme activity did not appear to be present in the Ch2 preparation, since Ch1 was just as effective when Ch1 and Ch2 preparations were mixed.

Biochemical Characterization of the Purified β-1,3-Glucanases. The two forms of β-1,3-glucanase differed slightly in their pH optimum (Table IV). The pattern of product formation from reduced laminarin was studied (Fig. 8). Both β-1,3-glucanases were endoglucanases. Neither released glucose from laminarin. G1 formed about 4 times more of the trisaccharide than of the di- and tetrasaccharide. On the other hand, G2 produced about equal amounts of di-, tri-, and tetrasaccharides.

DISCUSSION

Chitinase and β-1,3-glucanase are stable enzymes and represent a relatively large portion of the soluble proteins in infected immature and uninfected mature pea pods. Because of their abundance and their unusually high isoelectric points we have been able to purify all the major forms of these enzymes to homogeneity using a relatively simple protocol. Chitinases and β-1,3-glucanases have previously been purified from various sources (reviewed in Ref. 3). Most of these enzymes are similar to the chitinases and β-1,3-glucanases present in pea pods. Typically, they are basic proteins with a mol wt around 30 kD. Acidic forms of chitinases have been found in yam tubers (22) and in infected cucumber seedlings (14). Acidic forms of β-1,3-glucan-

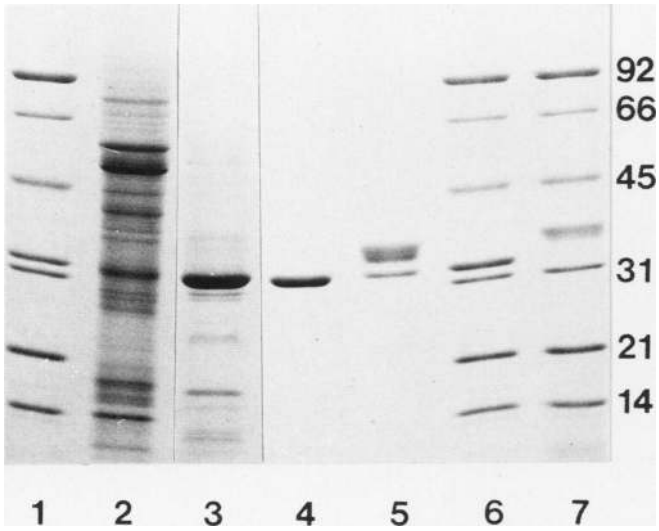

FIG. 5. SDS-PAGE on a 9 to 18% (T) gradient gel of protein preparations from mature pea pods at various stages of purification. Lane 1, mol wt standard, including 2 μg of β-1,3-glucanase G1; lane 2, 60 μg crude protein extract; lane 3, 15 μg protein after anion exchange chromatography and passage through a chitin column at pH 8.0; lane 4, 10 μg β-1,3-glucanase G1, separated by chromatofocusing from the preparation shown in lane 3; lane 5, 5 μg protein eluted at pH 3.2 from the chitin column (*i.e.* chitinase Ch1 and Ch2); lanes 6 and 7, 3 μg chitinase Ch1 and Ch2, respectively, included in the mol wt standards. Numbers on the right refer to the mol wt of the standard proteins in kD.

ases are known from tobacco leaves (17) and pea seedlings (26). The acidic forms of β-1,3-glucanases in pea seedlings have been hypothesized to function in cell wall metabolism and cell expansion of the plant itself (27). About half of the β-1,3-glucanase activity extracted from healthy immature pods bound to the Trisacryl-DEAE column and could only be eluted with salt solutions, indicating that acidic forms of β-1,3-glucanase are present as well. They were not further investigated because they were only found in low proportion in infected immature pea pods.

Both chitinases and both β-1,3-glucanases purified from pea pods are endohydrolases, releasing oligosaccharides as initial products. Thus, these enzymes are similar to other plant chitinases (2, 16) and most β-1,3-glucanases (10, 17,). An endohydrolytic activity would appear to be more suitable for the postulated defense function than an exohydrolytic activity, since the former could destroy the polymer fabric of the pathogen cell wall more effectively. Exohydrolases such as the β-glucosyl hydrolase described from soybean cell walls (6) may be more important in terminal hydrolysis of polymer fragments in the plant's own metabolism.

The induction of β-1,3-glucanase and chitinase activities in response to infection and elicitor treatment is an indirect argument for their role in defense. β-1,3-Glucanase and chitinase activities are induced with similar kinetics in ethylene-treated bean leaves (1, 2, 24), infected tomato stems (18), elicitor-treated parsley cell cultures (11), and tobacco callus cultures transferred to hormone-free media (15, 20). We have shown that β-1,3-glucanase and chitinase activities increase in a closely parallel manner in pea pods, both during fruit development and in response to various stimuli. No stimulus was found to induce only one of the two enzyme activities. We hypothesize that the coordinated induction of β-1,3-glucanase and chitinase in various situations has evolved because the two enzyme activities inhibit fungal growth most effectively when acting in combination (our unpublished data). The hydrolases accumulating in maturing pea pods may protect the growing seeds from bacteria, fungi, and insects. It is noteworthy that during development β-1,3-glucanase and chitinase start to accumulate in the pods at the same time as the seed filling begins. Very little β-1,3-glucanase and chitinase is present in developing seeds.

The increase of β-1,3-glucanase and chitinase during development and in response to stress is based on different molecular forms of β-1,3-glucanase and chitinase. Thus, the accumulation of β-1,3-glucanase and chitinase in infected immature pea tissue is not the result of premature aging. The presence of the different molecular forms in healthy pea tissue and their accumulation during maturation or in response to abiotic stress shows that all isozymes are formed by the pea tissue. It is not known whether the different molecular forms of chitinase and β-1,3-glucanase are products of different genes or the result of different processing of the same gene products. In beans, several genes coding for chitinase have been found (5).

Clearly, the increase in β-1,3-glucanase and chitinase activities observed in response to various stresses and during development is the result of a net accumulation of the enzyme proteins. Accumulation of β-1,3-glucanase and chitinase in ethylene-treated bean leaves (24) and in cell cultures of tobacco (15, 20) was found to be regulated at the mRNA level. It remains to be seen if the regulation mechanism in pea pods is similar. Regulation of gene expression has previously been studied in pea pods by using two-dimensional gel electrophoresis of the proteins present *in vivo* and formed by *in vitro* translation (9). About 20 new proteins appeared in infected or chitosan-treated pods and were described as resistance-related proteins. Their synthesis was shown to be regulated at the mRNA level. Unfortunately, basic proteins were not analyzed in these studies.

The significance of differentially regulated molecular forms of β-1,3-glucanases and chitinases is unclear. The respective iso-

Table IV. *Survey of Physical and Enzymic Properties of the Basic Proteins Purified from Pea Pods*

Enzyme	Mol Wt[a]	pI[b]	pH Optimum[c]	Specific Activity[d]
β-1,3-Glucanase G1	33,500 ± 900	9.26 ± 0.06	5.1	77 ± 8
β-1,3-Glucanase G2	34,300 ± 1400	9.15 ± 0.05	5.4	111 ± 9
Chitinase Ch1	33,100 ± 500	9.12 ± 0.04	4.2	18.6 ± 1.4
Chitinase Ch2	36,200 ± 500	8.87 ± 0.07	4.7	12.6 ± 1.6
Chitinase Ch3	39,000 ± 1400[e]	9.30 ± 0.06	4.7	ND[f]
Unknown protein (UP)	23,400 ± 1400	8.65 ± 0.08		

[a] Determined by SDS-PAGE; mean values and SD from eight (G1, G2, Ch1, and Ch2) or three (Ch3 and UP) independent determinations, respectively. [b] Determined by chromatofocusing; mean values and SD from eight independent measurements. [c] Determined as described in "Materials and Methods." [d] Units mg^{-1}; mean values and SD from five independent measurements. [e] Not purified to homogeneity. [f] Not determined.

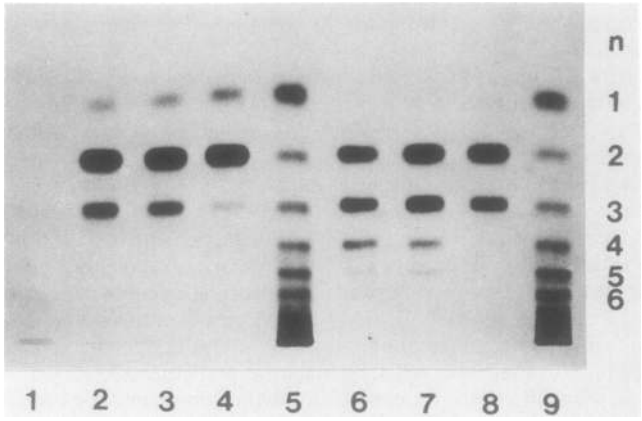

FIG. 6. Analysis of water-soluble products formed by chitinase Ch1 and Ch2 from [^{3}H]chitin. Fluorography of a TLC plate. Lane 1, control incubation in the absence of enzymes for 24 h; lanes 2 to 4, incubation with 7 μg chitinase Ch1 for 1, 3, and 24 h; lanes 6 to 8, incubation with 7 μg chitinase Ch2 for 1, 3, and 24 h; lanes 5 and 9, products of a partial acid hydrolysis of [^{3}H]chitin. Numbers on the left refer to the degree of polymerization (n) of the degradation products.

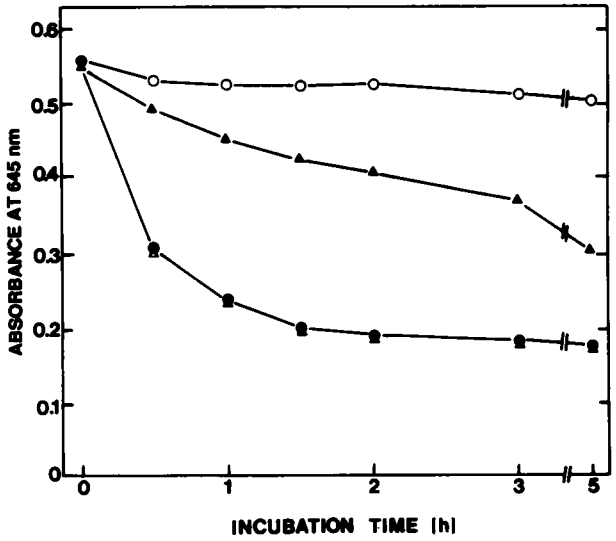

FIG. 7. Lysozyme activity of chitinases Ch1 and Ch2. A suspension of *M. lysodeikticus* was incubated with water (○), with 0.43 μg purified chitinase Ch1 (●), or with 0.61 μg purified chitinase Ch2 (▲). These quantities of the chitinases had equal activity against [^{3}H]chitin (8 munits). In a mixing experiment (△), 0.43 μg chitinase Ch1 and 0.61 μg chitinase Ch2 were used in combination.

zymes differ not only in their isoelectric points and mol wt, but also in a number of enzymic properties such as pH-optimum, specific activity, and mode of action. This suggests that different forms might have different functions. With the exception of the different specific lysozyme activity of the two purified chitinases, the differences found appear to be rather small. This might be partly because the experiments were performed with simple linear substrates. The nature of the polymeric substrate can have a strong influence on the mode of action. The products formed from newly synthesized chitin by chitinase were shown to have a higher chain length compared to the products released from preformed chitin (16). Since the release of elictors from pathogen cell walls is one of the proposed functions of β-1,3-glucanase and chitinase, subtle differences in the mode of action could have important consequences.

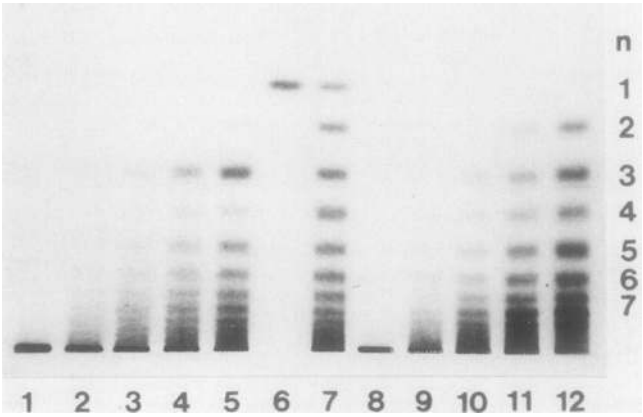

FIG. 8. TLC analysis of products formed by β-1,3-glucanases from laminarin. Lanes 1 to 5 and 8 to 12, incubation with 1.5 μg β-1,3-glucanase G1 and G2, respectively, for 0, 10, 30, 90, and 210 min; lane 6, 6 μg glucose; lane 7, products of partial acid hydrolysis of laminarin (400 nmol glucose equivalents). Numbers on the right indicate the degree of polymerization (n) of the degradation products.

In immature pea pods, the pathogen *F. solani* f.sp. *pisi* and the non-pathogen *F. solani* f.sp. *phaseoli* induce the same isozymes as ethylene and chitosan. Clearly, the induction of particular isozymes of β-1,3-glucanase and chitinase is not correlated with susceptibility or resistance. We hypothesize that the induction of β-1,3-glucanase and chitinase is a general, nonspecific antifungal defense. Combinations of β-1,3-glucanase and chitinase strongly inhibit growth of many potentially pathogenic fungi (our unpublished data). It remains to be seen how successful fungal pathogens cope with the rapid accumulation of β-1,3-glucanase and chitinase in infected tissue.

CONCLUSIONS

We have identified four major soluble basic proteins induced by pathogen attack and other stresses in pea pods. Two are β-1,3-glucanases and two are chitinases. The individual forms of the enzymes are differentially regulated during development and in response to stress but always in a way that leads to a coordinated increase in both enzymic activities. We believe that this parallel increase is related to the antifungal defense function of chitinase and β-1,3-glucanase, a function that requires both activities in combination (our unpublished data).

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