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The Latex of *Hevea brasiliensis* Contains High Levels of Both Chitinases and Chitinases/Lysozymes[1]

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ABSTRACT

The latex of the commercial rubber tree, *Hevea brasiliensis*, was fractionated by ultracentrifugation as described by G. F. J. Moir ([1959] Nature 184: 1626–1628) into a top layer of rubber particles, a cleared cytoplasm, and a pellet that contains primarily specialized vacuoles known as lutoids. The proteins in each fraction were resolved by two-dimensional gel electrophoresis. Both the pellet fraction and cleared cytoplasm contained large amounts of relatively few proteins, suggesting that laticifers serve a very specialized function in the plant. More than 75% of the total soluble protein in latex was found in the pellet fraction. Twenty-five percent of the protein in the pellet was identified as chitinases/lysozymes, which are capable of degrading the chitin component of fungal cell walls and the peptidoglycan component of bacterial cell walls. Both the chitinase and lysozyme activities were localized exclusively in the pellet or lutoid fraction. The chitinases/lysozymes were resolved into acidic and basic classes of proteins and further purified. An acidic protein (molecular mass 25.5 kD) represented 20% of the chitinase activity in latex; this protein lacked the low level of lysozyme activity that is associated with many plant chitinases. Six basic proteins, having both chitinase and lysozyme activities in various ratios and molecular mass of 27.5 or 26 kD, were resolved. Two of the basic proteins had very high lysozyme specific activities which were comparable to the specific activities reported for animal lysozymes. Like animal lysozymes, but unlike previously characterized plant chitinases/lysozymes, these basic chitinases/lysozymes were also capable of completely lysing or clearing suspensions of bacterial cell walls. These results suggest that laticifers may serve a defensive role in the plant.

Latex is the cytoplasm of highly specialized cells known as laticifers. Most of the volume of the latex from *Hevea brasiliensis* is filled by rubber particles and specialized vacuoles known as lutoids (5, 20). In fact, rubber has been reported to represent from 20 to 60% of the wet weight of latex (5). Laticifers are anastomosed as a result of partial hydrolysis of adjacent walls, and thus form a tube-like network or paracirculatory system through the plant (5). When laticifers are wounded, their collective latex or cytoplasm flows from the wound site. This feature of laticifers is exploited commercially to collect rubber from trees of *H. brasiliensis*, the sole commercial source of natural rubber.

Because laticifers appear to be dedicated primarily to the biosynthesis of the isoprenoid, rubber, it is reasonable to expect the major proteins in latex to be enzymes involved in the biosynthesis of rubber. Indeed, mRNA transcripts encoding enzymes involved in rubber biosynthesis were recently shown to be 20- to 100-fold higher in laticifers than in leaves (11). However, transcripts of several putative defense genes including chitinases, pathogenesis-related proteins, phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase, and 5-enolpyruvylshikimate 3-phosphate synthase were also expressed at 10- to 50-fold higher levels in laticifers than in leaves (11).

In the present paper, data are presented showing that chitinases/lysozymes represent in excess of 20% of the soluble protein in the latex of *H. brasiliensis*. Furthermore, the chitinase/lysozyme activity in the latex of *H. brasiliensis* was resolved into seven proteins and the physical and catalytic properties of each were further characterized. Chitinases/lysozymes have also been identified in the laticifers of numerous other latex-containing species, including monocotyledons, dicotyledons, and a gymnosperm (6, 7, 14, 15). In many of these species chitinases/lysozymes also appear to represent from 2 to 30% of the total soluble protein in the latex (15) (M Martin, manuscript in preparation).

The high level of chitinases/lysozymes found in the laticifers of *H. brasiliensis* contrasts sharply with the very low levels found in tissues such as leaves, stems, and roots of young health dicotyledonous plants (3). In these tissues, chitinase/lysozymes and several other proteins rapidly increase following exposure of the plant to elevated levels of the phytohormone ethylene, bacterial, viral or fungal infection or a number of other biotic or abiotic stresses leading to their classification as "defense" or "pathogenesis-related" proteins (3, 9, 10, 12, 17).

MATERIALS AND METHODS

Plant Material

Seeds of *Hevea brasiliensis* were obtained from I.N.C.A., CIRAD, Abidjan, Ivory Coast, and were grown under greenhouse conditions at approximately 26 to 28°C with supplemental lighting. Additional grafted plants were also obtained from Aracruz Florestal S.A., Brazil. Lyophilized pellet or bottom fraction and cleared cytoplasm from the latex of mature trees were obtained from Malaysia. Most of the results

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[2] Abbreviations: CEW, chicken egg white; NEFGE, nonequilibrium pH gradient electrophoresis.

presented herein were obtained using the latex from 4 to 12 month old trees grown from seeds obtained from Ivory Coast. Exceptions are noted in the text.

Chitinase Assay

Chitinase activity was measured radiometrically at 37°C in a reaction mixture containing enzyme, 0.5 mg [^{3}H]chitin, 0.6 mM sodium azide, and 25 mM sodium citrate (pH 5.0) in a total volume of 285 μL. The reaction was stopped after 30 min by the addition of 15 μL of 100% (w/v) TCA. After centrifugation for 5 min at 14,000*g* in a Beckman Microfuge, the radioactivity of 150 μL of the supernatant was determined. Activity was calculated for an enzyme concentration approaching zero as described by Boller *et al.* (3), because the release of soluble products was not a linear function of enzyme concentration in all cases. Chitinase specific activity is expressed as nkatal/mg of protein where nkatal is defined as nanomoles of *N*-acetylglucosamine equivalents solubilized per second. Tritium-labeled, regenerated chitin was synthesized as described elsewhere (21) using 25 mCi of ^{3}H-labeled acetic anhydride per gram of chitosan from crab shell. The regenerated chitin was washed to neutrality with water, washed with methanol, dried *in vacuo,* and stored as a dried powder. Specific radioactivity activity of the chitin was determined as described elsewhere (21).

Lysozyme Assay

Lysozyme activity of the chitinases/lysozymes from latex was measured as the rate of lysis of *Micrococcus lysodeikticus* cell walls (Sigma Chemical Co.). The reaction was monitored continuously as decrease in light scattering at 570 nm using a Cary model 219 Spectrophotometer (Varian Instruments) at 37°C. The reaction contained, in a volume of 1 mL, 20 mM sodium acetate (pH 4.8), 0.2 mg of cell walls, and enzyme. CEW[2] lysozyme (Sigma) was assayed in 20 mM sodium citrate (pH 6.0) at 37°C. One unit is defined as a decrease in *A* of 0.001/min. Specific activities were calculated from initial rates.

Exochitinase Assay

Exochitinase activity was measured as described above for the radiometric chitinase assay using the same chitin preparation. However, the reaction was terminated by boiling for 5 min, and exochitinase activity was measured colorimetrically as the formation of *N*-acetylglucosamine (26).

Acid Phosphatase Assay

Activity of acid phosphatase was measured at 37°C as the rate of increase in absorbance at 405 nm due to the formation of *p*-nitrophenol from *p*-nitrophenylphosphate. Assays were performed in a volume of 1.0 mL and contained 0.1 M sodium acetate (pH 5.0), 3 mM *p*-nitrophenylphosphate, and enzyme. Prior to measuring *A* at 405 nm, the pH was adjusted to 8.0 by the addition of 1 M $NaHCO_3$ to permit detection of the colored product.

Purification of Chitinase/Lysozyme

Latex was harvested from a spiral cut in the bark of the rubber trees directly into a centrifuge tube on ice and was centrifuged for 1 h at 50,000*g* in a Beckman Table Top ultracentrifuge at 5°C. The latex was separated into a pellet, cytosol (cleared cytoplasm), and rubber fraction and each fraction was diluted 1:2 into 25 mM Tris·Cl, pH 7.6 (buffer A), containing 4 mM EDTA, 10 mM 2-mercaptoethanol, 1.6 mM PMSF, 1 mM benzamidine, 5 mM ϵ-amino-*n*-caproic acid, 3 μg mL^{-1} pepstatin, and 0.2 mg mL^{-1} *p*-tosyl-L-arginine methyl ester, 0.2 mg mL^{-1} L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone, 0.3 μM aprotinin, and 0.1 mg mL^{-1} soybean trypsin inhibitor. Both pellet and cytosol were dialyzed against the same buffer. A significant amount of precipitation formed during dialysis and was removed by centrifugation at 14,000*g* for 10 min at 5°C. Chitinase and lysozyme activity were not found in the precipitate. The dialyzed fractions were each loaded to a Pharmacia Mono Q 5/5 anion exchange column in buffer A. After washing the column with buffer A, bound proteins were eluted with a 0 to 0.3 M gradient of NaCl in buffer A. The unbound proteins were dialyzed against 50 mM Mes, pH 6.5 (buffer B), loaded onto a Pharmacia Mono S 5/5 cation exchange column in buffer B, and eluted with a gradient of 0 to 0.5 M NaCl in buffer B. Protein was determined using Bio-Rad (Richmond, CA) protein assay reagent.

Determination of pH Optimum

The pH dependence of the enzymatic hydrolysis of both chitin and *M. lysodeikticus* cell walls was determined in 20 mM sodium acetate and 20 mM sodium citrate for pH values between 4 and 6 and in 20 mM Tris·Cl for pH values between 6 and 7.

PAGE

SDS-PAGE was performed as described elsewhere (24) using a 7.5 to 15% gradient of acrylamide. Two-dimensional gel electrophoresis was performed with NEFGE in the first dimension (23) and SDS-PAGE as described above in the second dimension. Proteins were detected by staining with Coomassie brilliant blue R250.

Immunoblotting

Proteins were transferred from polyacrylamide gels to Immobilon (Millipore Corp., Bedford, MA) using an American Bionetics Inc. Polyblot Apparatus (Emeryville, CA). The buffer system and protocol were those described by the manufacturer of the blotting apparatus except for the omission of methanol. The chitinases/lysozymes from *H. brasiliensis* latex transferred very poorly or not at all from the polyacrylamide gels to nitrocellulose or Immobilon membranes when methanol was included in the transfer buffer. The blots were incubated with antibody prepared in rabbit against endochitinase from *Phaseolus vulgaris*. The antigen-antibody complex was visualized as described elsewhere (2) using alkaline phosphatase conjugated with goat anti-rabbit IgG.

RESULTS

Localization of Chitinase and Lysozyme Activities

Fresh latex from *H. brasiliensis* can be fractionated by ultracentrifugation at 50,000*g* without the aid of an osmoticum into: (a) a top layer of polymerized rubber particles; (b) a cleared cytoplasm or cytosol; and (c) a large pellet or bottom fraction which has been reported to consist primarily of specialized vacuoles known as lutoids (20). The activity of acid phosphatase, which is a marker enzyme for soluble vacuolar proteins, was measured in both the cleared cytosolic and pellet fractions. Greater than 97% of that activity was localized in the pellet, confirming the presence of intact vacuoles in that fraction (Fig. 1). Greater than 98% of both the chitinase and lysozyme activities was also found in the pellet, showing that in latex these activities also are located exclusively in the vacuoles or lutoids. Not every preparation of fractionated latex yielded 98% intact vacuoles, but the percent of activity found in the pellet versus the cytosol was the same for each of the three activities. In fact, the vacuoles from frequently bled plants lysed readily during centrifugation.

Protein determinations showed that 70 to 80% of the soluble protein in latex was in the pellet or bottom fraction. Two-dimensional gel electrophoresis of the soluble proteins from the both cytosol and pellet revealed that both fractions contain large amounts of relatively few proteins (Fig. 2). Four polypeptides (a 59 kD polypeptide with a pI of approximately 4.5 and three polypeptides with molecular masses of 25.5, 26, and 27.5 kD and isoelectric points of approximately 5, 7.5, and 8) comprised the bulk of the protein in the pellet fraction. The 25.5, 26, and 27.5 kD polypeptides (indicated by arrows on Fig. 2) reacted very weakly with antibody against *P. vulgaris* endochitinase. Proteins immunoreactive with antibody to *P. vulgaris* endochitinase were not detected in the cytosolic fraction.

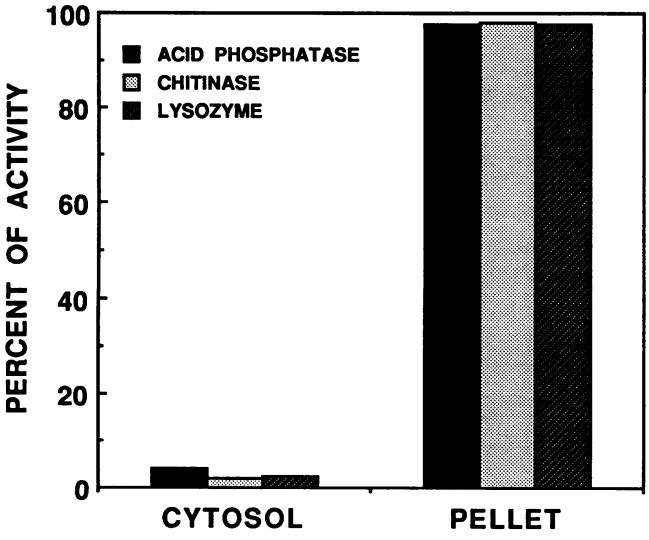

Figure 1. Localization of chitinase, lysozyme, and acid phosphatase activities in fractionated latex from *H. brasiliensis*. Latex was separated by ultracentrifugation into a rubber, cytosolic (cleared cytoplasm), and pellet fraction, and chitinase, lysozyme and acid phosphatase activities were assayed in each fraction as described in "Materials and Methods." Activity in each fraction is expressed as the percentage of the total activity in whole latex.

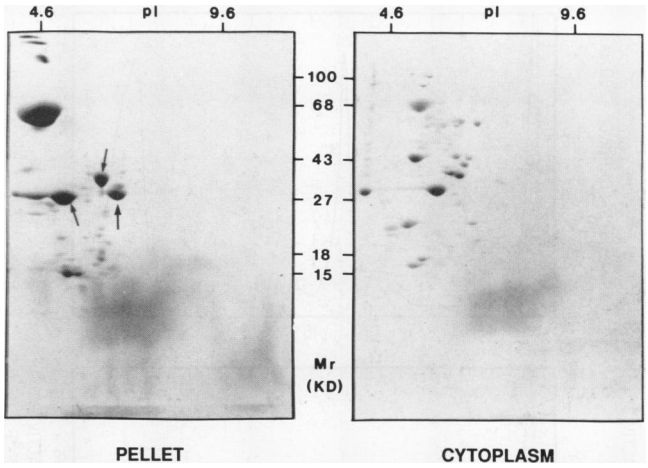

Figure 2. Profile of the total soluble proteins in the pellet and cytosolic (cleared cytoplasm) fractions of the latex from *H. brasiliensis*. Proteins were resolved by NEFGE followed by SDS-PAGE as described in "Materials and Methods" and stained with Coomassie brilliant blue. Isoelectric points and molecular masses in kilodaltons of standard proteins are indicated. Bands which reacted with antibody against *P. vulgaris* chitinase are indicated by arrows. The gels contained 175 μg of protein from the pellet of 5 μL of latex or 55 μg of protein from the cytosol of 5 μL of latex. Latex was from Brazilian trees.

Purification of Chitinase and Chitinases/Lysozymes

The protein in the pellet or bottom fraction of *H. brasiliensis* latex was resolved into three major fractions (designated 1A, 2A, and 3A) when applied to a Mono Q anion exchange column at pH 7.5 and eluted with a gradient of NaCl (Fig. 3). Fractions 1A and 2A each represented approximately 10% of the total soluble protein in latex and both had chitinase activity. Fraction 2A contained one-fourth of the recovered chitinase activity but none of the lysozyme activity. That protein was assumed to be acidic by virtue of its binding to the Mono Q column at pH 7.5. Nearly 60% of the chitinase activity and 80% of the lysozyme activity were recovered in fraction 1A which was presumed to be basic since it failed to bind to the Mono Q column at pH 7.5. The proteins in fraction 1A was further resolved by cation exchange chromatography into six major fractions (1–6B); each had both chitinase and lysozyme activities. Although not readily apparent in Figure 4, several of the minor peaks also have chitinase and lysozyme activity. Thus, nearly all of the basic proteins in the pellet appeared to be chitinases/lysozymes; the Mono S column effectively provided resolution of these chitinases/lysozymes with little further purification. In that step, 60% of protein was recovered, but only 41% of the chitinase activity and 20% of the lysozyme activity were recovered. Results are summarized in Table I.

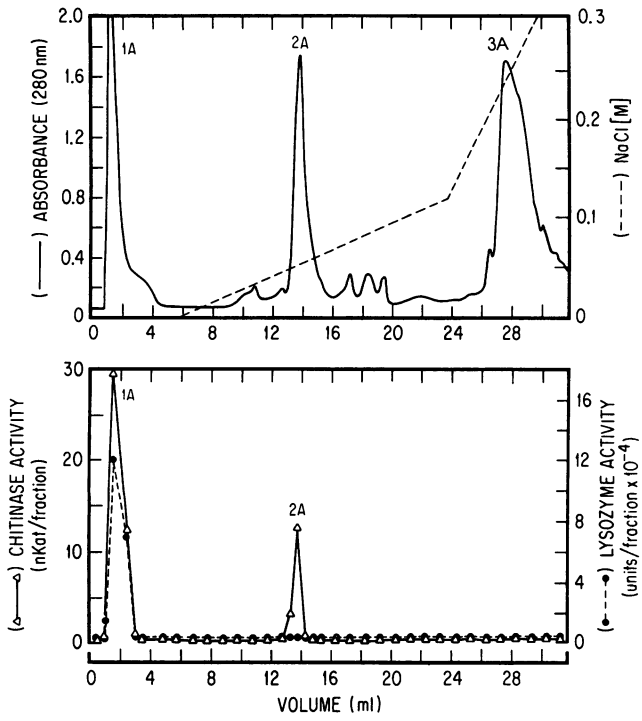

Figure 3. Elution profile of chitinase (△) and lysozyme (●) activities from a Pharmacia Mono Q 5/5 anion exchange column. Proteins in the pellet fraction of 0.5 mL of latex from *H. brasiliensis* were applied to the column in 25 mM Tris·Cl (pH 7.5) and eluted as shown with a gradient of NaCl (-----) in the same buffer. Fraction sizes of 0.5 mL were collected. Elution of protein from the column was monitored at 280 nm. Major fractions of protein are designated 1A, 2A, and 3A.

Physical Properties

Chitinase (2A) and chitinases/lysozymes 1–6B from both the Mono Q and the Mono S columns appeared to consist of a single major protein when analyzed by SDS-PAGE (Fig. 5). The chitinases/lysozymes in fraction 1–5B each had molecular masses of 27.5 kD, while fraction 2A and 6B had molecular masses of 25.5 and 26 kD, respectively.

Serological Relationships

The acidic chitinase and each of the basic chitinases/lysozymes showed a very weak reaction with antibody to the basic endochitinase from *P. vulgaris*. Thus, all of the proteins apparently have some common antigenic determinants in spite of differences in molecular weight, charge, and catalytic properties.

Catalytic Properties

The specific activities as both chitinases and lysozymes of each of the proteins resolved on the Mono Q and Mono S columns are summarized in Table II along with the ratio of chitinase to lysozyme activity. As chitinases, the acidic protein (2A) and basic proteins (1-, 3-, 4-, and 5B) had very similar specific activities ranging from 4 to 7 nkatals/mg. The specific activity of 2B was 2- to 3-fold higher and the specific activity of 6B was 6- to 10-fold higher. The chitinase activity of each of these proteins exhibited a very broad pH optimum between 4.5 and 6.0. Only chitinase 2A, 3B, and 4B were able to hydrolyze chitin to *N*-acetylglucosamine. *N*-Acetylglucosamine was not released by 6B and 2B which exhibited the highest specific activity in the radiometric assay suggesting that they preferentially hydrolyze larger polymers. The exochitinase activity of 1B was not measured due to insufficient protein.

The behavior of these proteins as lysozymes was more dramatically different. The lysozyme specific activity of the basic proteins varied by more than 45-fold. Protein 1B had a very low lysozyme specific activity of 1800 units/mg. In contrast, protein 3B has a lysozyme specific activity of nearly 80,000 units/mg, which is comparable to the specific activity reported for chicken egg white lysozyme and other animal lysozymes. The acidic chitinase (2A) lacked lysozyme activity under all assay conditions examined. These included a range of pH values between 3.5 and 7.0 and a range of molar concentration of buffers between 20 and 200 mM in several buffer systems. The high percentage of lysozyme activity

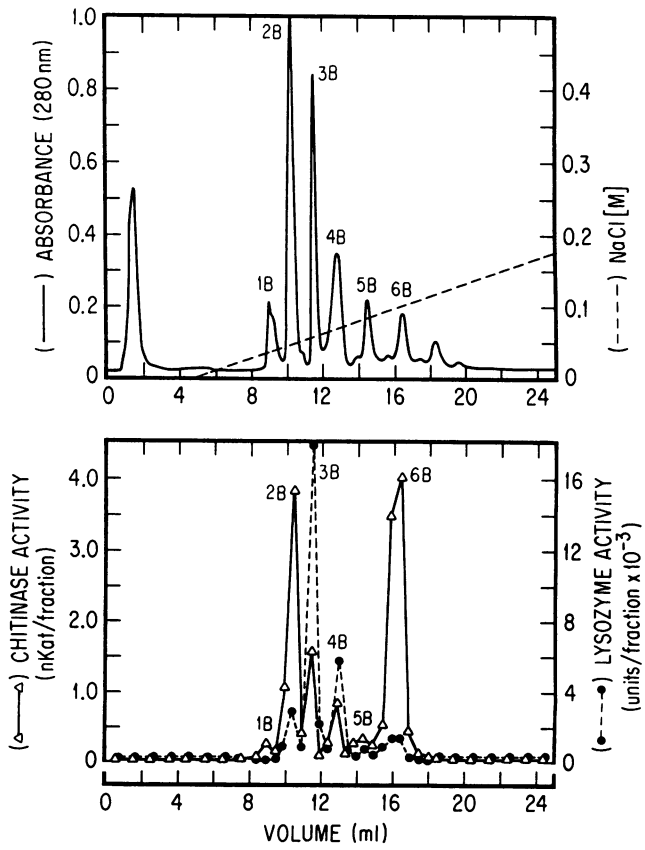

Figure 4. Elution profile of basic chitinase (△) and lysozyme (●) activities from a Pharmacia Mono S 5/5 cation exchange column. Proteins in peak 1A that failed to bind to the Mono Q column were applied to a Mono S 5/5 column in 50 mM Mes (pH 6.0) and eluted with a gradient of NaCl (-----) as shown. Elution of protein from the column was monitored at 280 nm and 0.5 mL fractions were collected. The fractions with chitinase and/or lysozyme activities are designated 1–6B.

Table I. *Purification of Chitinase and Chitinases/Lysozymes from the Latex of H. brasiliensis*

Purification	Total Protein	Chitinase			Lysozyme		
		Total activity	Specific activity	Recovery	Total activity	Specific activity	Recovery
	mg	*nkat*	*nkat mg*$^{-1}$	%	*units* × 10^{-3}	*units/mg* × 10^{-3}	%
Whole latex[a]	47.0	162	3.5		509	10.8	
Pellet	36.1	159	4.4	98	503	13.9	99
Mono Q							
Fraction 1A	4.8	91	19.0	56	400	83.3	79
Fraction 2A	4.8	33	6.9	20	0	0	0
Mono S[b]	2.9	37		23	79.1		16

[a] The results shown are for 1 mL of latex. [b] These values were obtained by adding total protein and total activity in fractions 1-6B.

recovered from the Mono Q column with the basic proteins (in this purification 80% and in some purifications as high as 90%) does not suggest that lysozyme activity associated with the acidic chitinase was lost in the course of the purification.

The plant chitinases/lysozymes characterized to date not only have low lysozyme specific activities, but also stop after 25 to 60% hydrolysis (decrease in absorbance) of a suspension of *M. lysodeikticus* cell walls (3, 16). Animal lysozymes, such as CEW lysozyme, are able to effect as much as 95% lysis or reduction in absorbance of a suspension of *M. lysodeikticus* cell walls (Fig. 6). However, the time course for hydrolysis of bacterial cell walls by chitinase/lysozyme 3B and 4B looked much like that of CEW lysozyme. Fractions 3B and 4B effected 95% lysis or hydrolysis of bacterial cell walls at approximately half the rate observed with chicken egg white lysozyme (Fig. 6). Lysozyme 3B and 4B completely clear a bacterial cell wall suspension with little or no change in rate, suggesting that they do not discriminate between large and small oligomers. Fractions 2B and 6B also effected 85% reduction in absorbance of bacterial cell wall suspension, but at a much lower rate. The reaction required 70 min for completion and is not shown. With chitinase/lysozyme 5B, the reaction stopped after only 70% lysis after 40 min (complete reaction not shown). In every case, the extent of the lysozyme reaction was highly pH dependent. Each of the proteins (2–6B) showed a pH optimum at 4.6, for the maximum initial rate of hydrolysis. However, less than 60% lysis or hydrolysis of the cell walls was observed at pH 4.6. Although the initial rate was lower, the extent of the reaction increased with increasing pH up to 5.2. The pH range for each of the latex lysozymes was very narrow with an optimum in each case of 4.6 and with very little activity below 4.4 or above 5.6. The reaction rate of lysozyme 1–6B also dropped dramatically with increasing molarity of the buffer above an optimum of 10 to 20 mM. Activity was not observed in 100 mM buffer.

DISCUSSION

The chitinases/lysozymes isolated from the latex of *H. brasiliensis* and described in this paper are exceptions to many of the generalizations which were initially made regarding the physical and catalytic properties, mode of regulation, and function of chitinases/lysozymes in plants. In fact, it has become apparent that several forms of chitinases/lysozymes

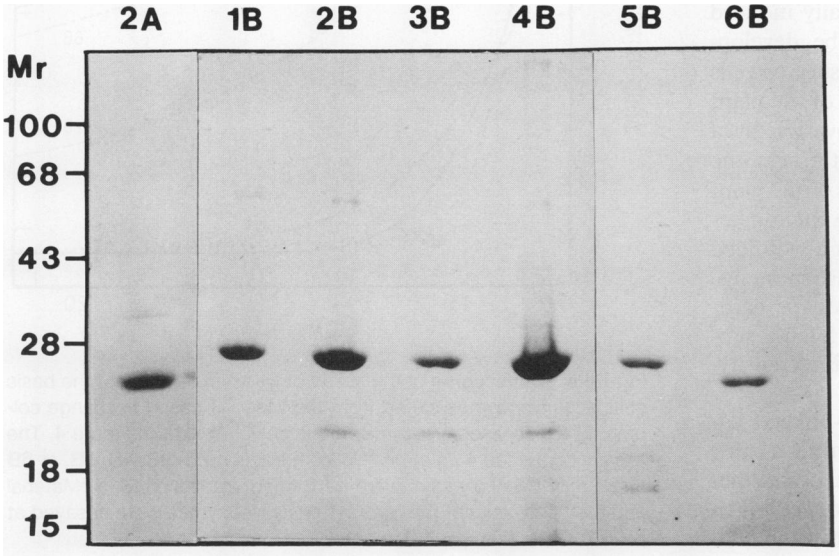

Figure 5. Protein profiles on SDS-PAGE of the acidic chitinase and basic chitinases/lysozymes from the latex of *H. brasiliensis* fractionated by anion and cation exchange chromatography. The lanes correspond to fraction 2A from the Mono Q column and fraction 1–6B from the Mono S column. Molecular masses in kilodaltons of standard proteins are indicated.

Table II. *Catalytic Properties of the Chitinase and Chitinases/Lysozymes from H. brasiliensis*

Enzyme	Chitinase Activity[a]	Lysozyme Activity[b]	Ratio Lysozyme/Chitinase
	nkat/mg	*units/mg* × 10^{-3}	
Chitinase 2A	6.9	0	0
Chitinase/lysozyme			
1B	4.0	1.8	450
2B	14.8	11.2	757
3B	6.8	79.6	11,706
4B	6.5	44.9	6,908
5B	4.2	8.8	2,095
6B	38.7	13.5	349
CEW lysozyme	0.69	110.0	159,351

[a] Assays were performed at pH 5.0. [b] Assays were performed at pH 4.8. CEW was assayed at pH 6.0.

are present in most plants (10, 12, 14, 16, 27) and that they defy generalization.

Most chitinases/lysozymes have been characterized as defense proteins because they are often barely detectable in healthy plants but are induced as much as 30-fold by ethylene or various abiotic or biotic stresses (3, 16). Likewise, a substrate for chitinases/lysozymes is not known to be present in the plant; rather the chitin component of fungal cell walls or the peptidoglycan component of bacterial cell walls is thought to serve as a substrate. In a few plants, chitinases/lysozymes induced by stresses may represent as much as 1 to 2% of the total soluble protein in tissues such as leaves. In contrast, chitinases and chitinases/lysozymes represent more than 20% of the total soluble protein in the latex of *H. brasiliensis* which have not knowingly been subjected to stress. Similarly, high levels of chitinases or chitinase/lysozymes were found in the laticifers of numerous other latex-containing plants (15) (M Martin, manuscript in preparation). Chitinases have also been isolated from seeds (22), and were shown to accumulate in a development-dependent manner in the seed pods of pea (16) and in several floral parts of tobacco (13). In pea pods, distinct chitinase/lysozyme isozymes were developmentally induced and stress-induced. Chitinase/lysozymes may be developmentally or constitutively expressed in these tissues or cells because they are particularly critical for survival of the plant or because they are unusually susceptible to invasion by a pathogen. Laticifers form a tubelike network through the plant which gives a pathogen access to large areas of the plant unobstructed by the cell wall. Thus, an invading microorganism might be particularly difficult to contain in a wounded laticifer without constitutively expressed defense proteins. Alternatively, unidentified endogenous substrates for chitinase/lysozymes may be present in these tissues. Such a substrate might be present in laticifer walls which undergo hydrolysis as the laticifers expand.

Chitinases preferentially hydrolase the β-1,4-linkage between the *N*-acetyl-β-D-glucosamine moieties in chitin, a component of fungal cell walls and insect exoskeletons. Lysozymes preferentially cleave the β-1,4-linkage between *N*-acetyl-β-D-glucosamine and muramic acid which are found in the peptidoglycan component of bacterial cell walls. The generalization has made that animals contain lysozymes with low associated chitinase activity or no chitinase activity and plants contain chitinases with low associated lysozyme activity (8). The basic endochitinases from *P. vulgaris* and *P. sativum* (3, 16), which effect less than 25 to 50% hydrolysis of bacterial cell walls, are examples of the plant chitinases with low levels of lysozyme activity. However, three classes of chitinases/lysozymes were isolated from the latex of *H. brasiliensis:* (a) those having chitinase activity but lacking lysozyme activity; (b) those behaving as "typical" plant chitinases with low lysozyme activity; and (c) those having both high chitinase activity and very high lysozyme activity. Proteins with high lysozyme activity have also been reported in *Parthenocissus* (1) and in the latex of both a *Ficus* species and *Carica papaya* (6, 7, 15). A protein having only lysozyme activity was not found in the latex of *H. brasiliensis*. However, lysozymes lacking chitinase activity have been purified from the latex of *Asclepias syriaca* (14). Lysozyme activities, if measured, have not been reported for most chitinases from plants.

Little progress has been made toward characterizing the catalytic properties of chitinases/lysozymes in plants. Most, if further characterized, were reported to be endohydrolytic enzymes based on their failure to release *N*-acetylglucosamine from chitin or smaller oligomers. However, nearly half of the chitinases or chitinases/lysozymes in the latex of *H. brasiliensis* are capable of hydrolyzing chitin to *N*-acetylglucosamine. It is of interest to note that the same chitinases/lysozymes (3B and 4B) which release *N*-acetylglucosamine from chitin also completely clear suspensions of bacterial cell walls. Both

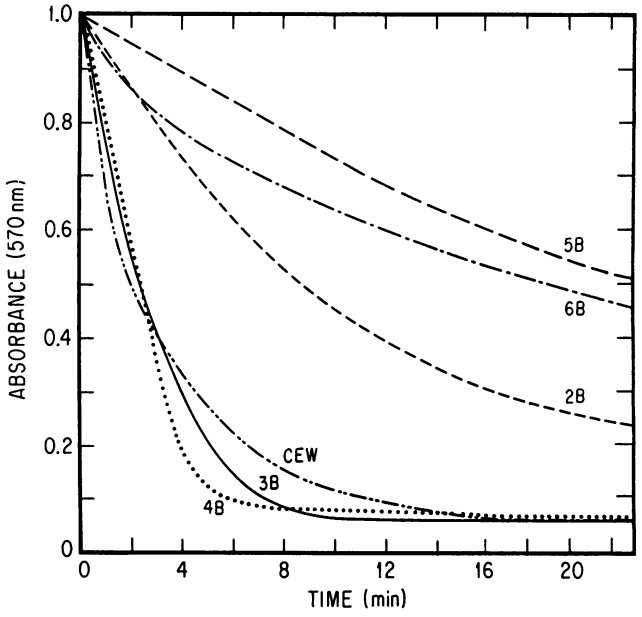

Figure 6. Time course of the assay of lysozyme activity of the basic chitinases/lysozymes eluted from the Mono S cation exchange column. The chitinases/lysozymes are is designated as in Figure 4. The assays contained 4 μg of protein from fraction 2B, 3B, 4B, 5B, or 6B or 2 μg of CEW. Assays were performed as described in "Material and Methods" except the *H. brasiliensis* lysozymes were assayed at pH 5.2 rather than pH 4.8.

endochitinases and exochitinases were also reported to be present in pea pods (16) and in melon (27). In melon those chitinases which release *N*-acetylglucosamine from chitin were found to degrade preferentially small oligomers. The large loss in both chitinase and lysozyme activities upon separation of the basic isozymes found in the latex of *H. brasiliensis* suggests that these enzymes may be acting synergistically on chitin and bacterial cell walls. When the endohydrolytic enzymes which hydrolyze large polymers without release of *N*-acetylglucosamine are separated from the exohydrolytic enzymes or enzymes which degrade smaller polymers, the exohydrolytic enzymes may lack their preferred substrate.

The majority of chitinases/lysozymes identified to date in plants have been basic proteins. This may be due in part to the ease with which basic proteins are separated from the bulk of the cellular protein and further purified. Nevertheless, acidic chitinases have now been isolated from in yam tubers (29), infected cucumber seedlings (18), infected tobacco (12), and tomato leaves (9). In the latex of *H. brasiliensis,* an acidic chitinase represents over 10% of the total soluble protein and 20% of the chitinase activity. This acidic chitinase lacked lysozyme activity. An extracellular acidic chitinase purified from cucumber (19) and an acidic chitinase purified from yam tubers also lacked lysozyme activity (29). The amount of acidic chitinase in latex varied with source. The acid chitinase represented 20% of the chitinase activity in trees obtained from Ivory Coast but more than 50% of the chitinase activity in trees obtained from Brasil; the acidic chitinase was not present in lyophilized latex preparations (M Martin, unpublished results). Likewise, a 59 kD polypeptide which represents more than 50% of the protein in the pellet fraction from young plants, was absent in lyophilized latex. Other researchers have reported that the pellet fraction from fresh and lyophilized latex of mature trees was predominantly basic proteins. Research is underway to establish whether these differences are due to processing of the latex, plant age, stress, or other factors.

My research is presently directed toward further characterization of the catalytic properties of the chitinases/lysozymes in latex and establishing what, if any, structural relatedness exists. The latex of *H. brasiliensis* may in fact contain two classes of chitinases/lysozymes with no homology in common. A basic chitinase/lysozyme has been purified previously from the latex of *H. brasiliensis* (28). The N-terminal sequence of that protein was shown to have homology with an acidic chitinase from *Cucumis sativis* (19) and a chitinase/lysozyme from *Parthenocissus quinquifolia* (1); it did not show homology with the basic endochitinase from *P. vulgaris* (4). A 5 kD, basic protein referred to as hevein was also isolated from the latex of *H. brasiliensis* (30). Although no enzymatic function was assigned to this protein, it was shown to have a high degree of homology with the N terminus of wheat germ agglutinin (25) and the N terminus of a basic endochitinase form *P. vulgaris* (4). In fact, hevein may be a proteolytic fragment of a chitinase/lysozyme.

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