Carbohydrate Research, 165 (1987) 93–104
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

PURIFICATION AND SOME PROPERTIES OF CHITINASES FROM MELON PLANTS INFECTED BY *Colletotrichum lagenarium*

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(Received November 21st, 1986; accepted for publication, February 17th, 1987)

ABSTRACT

Chitinases have been purified from melon plants infected with the fungal pathogen *Colletotrichum lagenarium*. Six different chitinases have been separated through a 4-step purification procedure. The two major enzymes, chitinases I and II, have been purified to homogeneity, and have molecular weights of 29,000 and 34,000, basic pI values of 8.4 and 10.0, and similar amino acid compositions. Chitinase I acts as an endo-enzyme, and chitinase II as an exo-enzyme, hydrolysing preferentially short oligosaccharide substrates.

INTRODUCTION

Chitinase occurs in a number of higher plants[1–4], and catalyses the hydrolysis of chitin, a (1→4)-linked polymer of 2-acetamido-2-deoxy-β-D-glucose. Since chitin is not present in plants, the enzyme has no apparent function in the primary metabolism, but indirect evidence suggests a defence role against fungal pathogens or insects since chitin is an important component of fungal cell-walls and insect cuticles. Chitinase hydrolyses the cell wall of fungal pathogens[5,6], releases elicitors of defence reactions[7,8], and is highly stimulated in various plants upon infection with fungal pathogens[6,9–11] and by treatment with the plant hormone, ethylene, or with fungal elicitors[1,6,12–14].

Chitinase from plants has been poorly characterised hitherto, and there are few reports on its purification. More information is available on chitinase from insects: it comprises a multicomponent system[15,16], including endo-chitinase, exo-chitinase, and β-*N*-acetylglucosaminidase. Endo-chitinase hydrolyses the internal linkages of chitin, producing oligosaccharides which are then hydrolysed by exo-chitinase and/or β-*N*-acetylglucosaminidase, these two latter enzymes differing by their substrate specificities. So far, only endo-chitinases have been purified from plants[3,4,11,12], probably because they correspond to the major activity.

We now report on the purification and characterisation of two chitinases from

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0008-6215/87/$ 03.50

the multicomponent chitinolytic system present in melon plants infected with *Colletotrichum lagenarium*.

RESULTS AND DISCUSSION

Chitinase purification. — Two assays were used: (*a*) a colorimetric assay based on the assessment of 2-acetamido-2-deoxy-D-glucose possibly released by exo-chitinase and/or β-*N*-acetylglucosaminidase; and (*b*) a radiochemical assay based on the release of soluble radioactive fragments from [H^3]chitin, which allowed endo-chitinase activity to be determined, plus the subsequent hydrolytic effect of exo-chitinase and β-*N*-acetylglucosaminidase. By using the colorimetric assay, it was found that chitinase activity in *Colletotrichum lagenarium*-infected plants begins to increase 3 days after inoculation, and reaches a 10-fold value after 6 days (Fig. 1). A similar phenomenon was observed when the radiochemical assay was used, but, in one instance, there was a much higher rate of increase. Chitinase activities declined 7 days after inoculation, which was coincident with seedling death.

Thus, at least two different chitinolytic activities were present in the healthy melon plants and were highly increased in 6 day-infected plants. Purification of the chitinases from healthy and infected plants was then undertaken.

The first two steps of the purification, namely, acid and ammonium sulfate fractionation, resulted in a 2–4-fold increase in specific activities (Table I). The

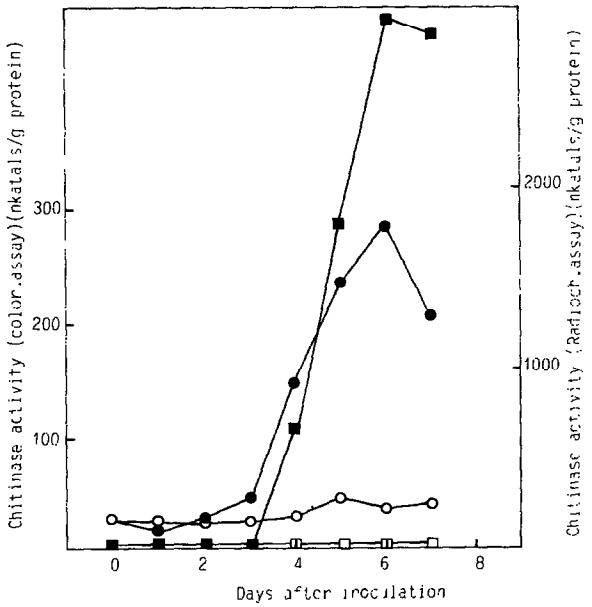

Fig. 1. Colorimetric (○,●) and radiochemical (□,■) assays of chitinase activity in the aerial parts of healthy (○,□) or infected (●,■) melon plants, during the course of infection.

partially purified extract, after desalting by chromatography on Sephadex G-25, was submitted to ion-exchange chromatography. The elution profile (Fig. 2a) showed 2 major peaks (1 and 2) of radiochemically assessed chitinase activity, and 1 major and 2 other peaks (3–5) of colorimetrically assessed chitinase activity, in infected plants. The corresponding activities were low or undetectable in control plants (Fig. 2b). All of the activity was not adsorbed on the ion-exchanger, probably reflecting additional minor chitinase activities, which were not considered

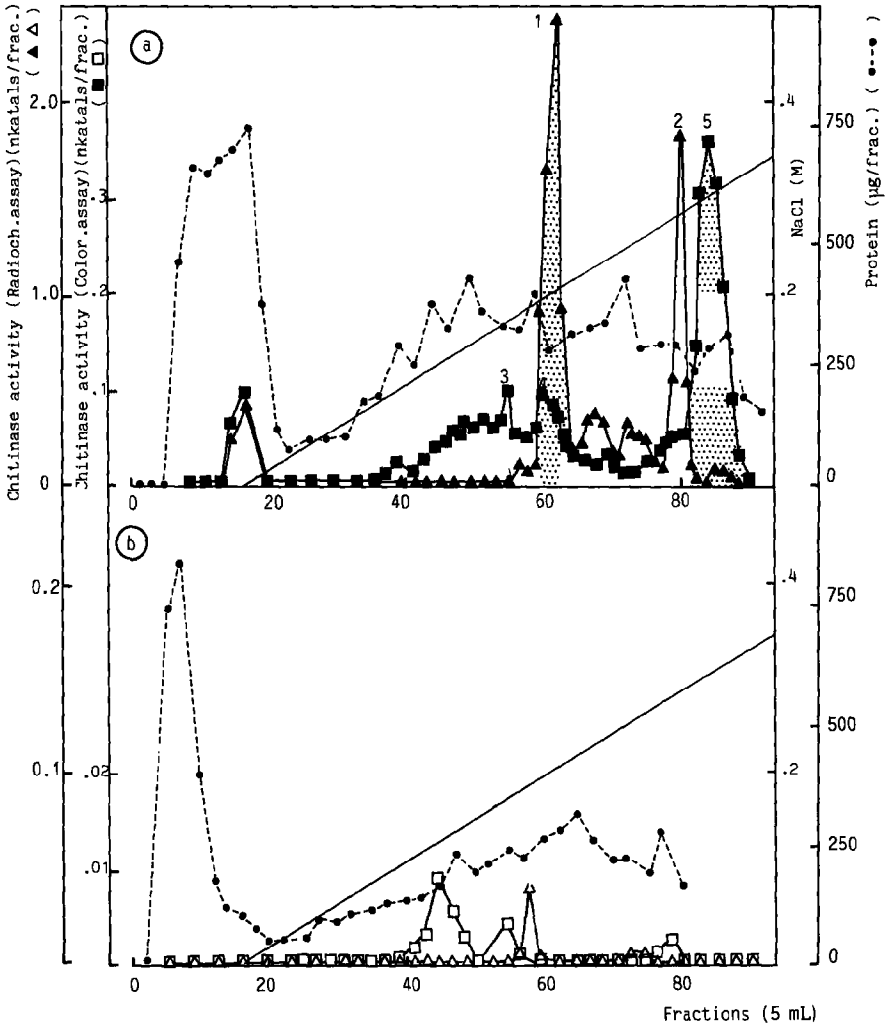

Fig. 2. Ion-exchange chromatography of a partially purified extract from (a) infected melon plants and (b) healthy controls. After acid and ammonium sulfate fractionation, the fraction obtained from 50 g fresh-weight of plant material was applied to a column (14 × 2.5 cm) of CM-cellulose 32 (Whatman) previously equilibrated with 20mM acetate buffer (pH 5.0) and eluted with 700 mL of a linear gradient of NaCl (0.02→0.5M); 5-mL fractions were collected.

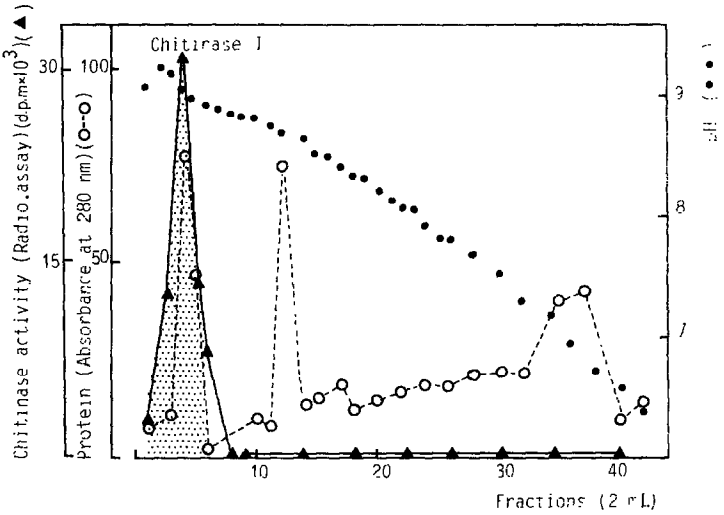

Fig. 3. Chromatofocusing of chitinase I. Concentrated, dialysed fractions from the shaded area of peak 1 in Fig. 2 were applied to a column (10 × 1 cm) of Polybuffer Exchanger 94 (Pharmacia) equilibrated with 25mM ethanolamine–HOAc (pH 9.4) and eluted at 15 mL/h with a 10-fold diluted Polybuffer 96; 2-mL fractions were collected.

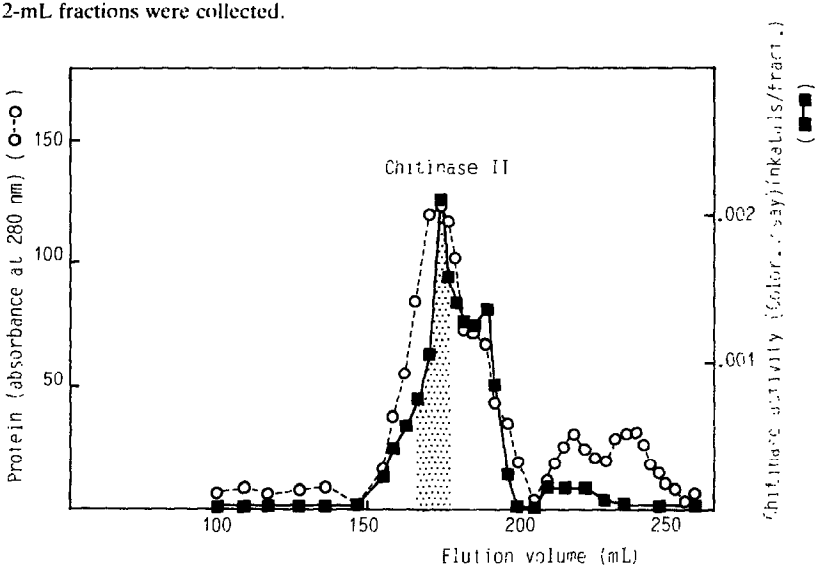

Fig. 4. Gel filtration of chitinase II. Concentrated and dialysed fractions from the shaded area of peak 5 in Fig. 2 were applied to a column (104 × 2.6 cm) of Sephacryl S-200 previously equilibrated with 20mM acetate buffer (pH 5.0) and eluted with the same buffer; 2-mL fractions were collected.

further. After this purification step, each chitinolytic activity was low, possibly indicating that the different chitinases have been separated and their synergistic action suppressed[15,17]. The two major chitinase activities (I and II, peaks 1 and 5, respectively) were further purified by chromatofocusing and gel filtration. Chromatofocusing of chitinase I (Fig. 3) resulted in a 15-fold increase in specific

TABLE I

PURIFICATION OF CHITINASES FROM INFECTED MELON PLANTS

Purification step		*Specific activity (nkat/g of protein)*		*Purification*	
		Colorimetric assay	*Radiochemical assay*	*Colorimetric assay*	*Radiochemical assay*
Crude extract		289.8	2280.3	1.00	1.00
Precipitation at pH 4.5		361.3	3775.2	1.3	1.7
Ammonium sulfate fractionation		1065.5	4711.2	3.7	2.1
CM-Cellulose chromatography	(1)[a]	201	35610		15.6
	(2)	105	16250		7.1
	(3)	633	1500	2.2	
	(4)	258	15400	0.9	
	(5)	684	1050	2.4	
Sephacryl S-200 chromatography	(5)	2802		9.7	
Chromatofocusing	(1)		541250		237.4

[a]Data for the separated activities as shown in Fig. 2.

activity and indicated a high pI value. Gel filtration of chitinase II (Fig. 4) gave two active fractions and only that of higher activity was retained.

The two purified enzymes were essentially homogeneous as judged by sodium dodecyl sulfate(SDS)-gel electrophoresis (Fig. 5). The final purifications of the two chitinases I and II were 10- and 230-fold, respectively. It should be emphasised that only two of the six components of the chitin-degrading system were retained during the purification procedure. Attempts to purify the two enzymes more quickly by affinity chromatography[3,12] were unsuccessful when purified chitin, regenerated chitin, or chitin oligosaccharides attached to Sepharose 6B were used.

The presence of a multicomponent chitin-degrading enzyme system in infected melon plants is consistent with the report[18] on bean chitinase that a multigene family, consisting of 3 or 4 members, encodes for the enzyme. Chitinases I and II are also found with low activity in healthy melon plants and are highly enhanced upon infection and ethylene- or elicitor-treatment[19].

Properties of chitinases I and II. — The molecular weights, estimated by SDS-polyacrylamide gel electrophoresis, were 34,000 for chitinase I and 29,000 for chitinase II. When determined by chromatography on Sephacryl S-200, the value for chitinase II was 27,000. The isoelectric points of chitinases I and II were 8.4 and 10.0, respectively, as indicated by chromatofocusing analysis (Fig. 6).

Chitinases I and II were similar to other plant chitinases, but markedly different from animal enzymes. Chitinases from wheat germ[3], bean[1,12], yam[4], tomato[11], and fungi[20,21] have molecular weights of ~30,000. Chitinases from animals[22–24] have molecular weights ranging from 45,000 to 120,000. Chitinases I and II had basic pI values similar to those of most chitinases from plants, whereas chitinases from animal origin have acidic pI values.

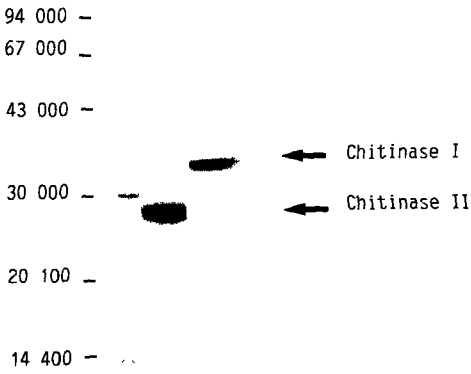

Fig. 5. SDS-polyacrylamide gel electrophoresis of purified chitinases I and II from infected melon plants: A, mixture of marker proteins; B, chitinase II; C, chitinase I.

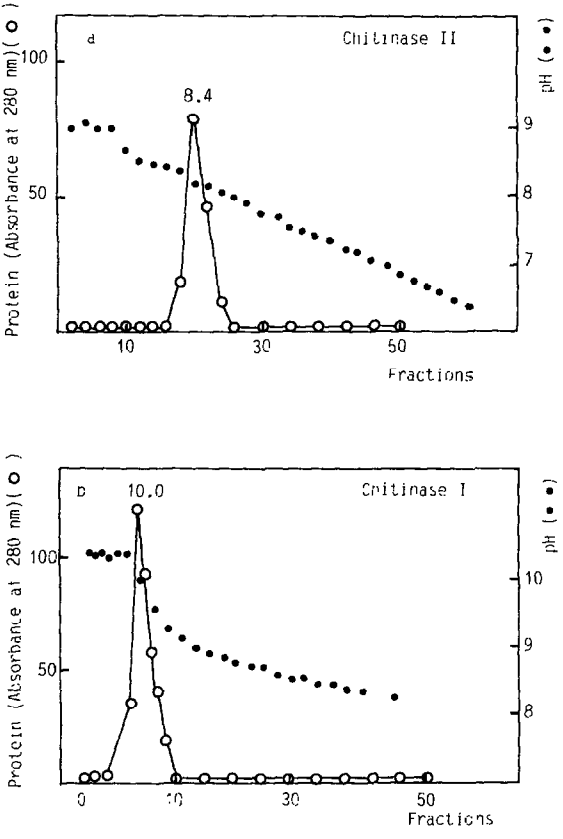

Fig. 6. Chromatofocusing of purified chitinases (a) II, (b) I. Purified chitinase I was applied to a column (10 × 1 cm) of Polybuffer Exchanger 118, and eluted with a 4.5-fold diluted Pharmalyte 8 to 10.5–HCl. Purified chitinase II was applied to a column (10 × 1 cm) of Polybuffer Exchanger 94, and eluted with a 10-fold diluted Polybuffer 96; 2-mL fractions were collected.

The amino acid compositions of chitinases I and II are listed in Table II. There were differences in histidine, arginine, phenylalanine, proline, serine, and threonine. Aspartic acid (or asparagine), glutamic acid (or glutamine), glycine, and alanine were the most abundant amino acids in both enzymes. Sugar residues were absent, since staining with thymol–sulfuric acid reagent[25] after SDS-polyacrylamide gel electrophoresis was negative and since the amount of sugar detected by the anthrone assay[26] was negligible.

Considering the values of the isoelectric points of both proteins, a large proportion of the acidic amino acids are probably present in the amide form, as suggested by the presence of a large peak for ammonia in the amino acid analyses. These data would be consistent with those for bean chitinase where the amino acid composition deduced from the nucleotide sequence of a cDNA clone[18] shows that Gln represents ~80% of Glx, and Asn ~40% of Asx.

Hydrolytic activity of chitinases I and II. — A kinetic study of the hydrolysis of purified chitin or regenerated [^{3}H]chitin by purified chitinases I and II was conducted, using h.p.l.c. with detection on the basis of refractive index and liquid scintillation counting. Similar results were obtained for either substrate. The main products released after incubation with chitinase I for 15 min and 30 min were chitotriose and chitobiose (30 and 40–50%, respectively), plus higher oligosaccharides. After 3 and 18 h, chitobiose was the main product (50–60%) together with

TABLE II

AMINO ACID COMPOSITION OF THE PURIFIED CHITINASES[a]

Amino acid	*Chitinase I*	*Chitinase II*
	(nmol percentage)	
Asx	8.2	12.5
Thr	5.3	9.5
Ser	14.2	6.7
Glx	12.8	7.2
Pro	4.5	8.1
Gly	15.3	12.1
Ala	8.7	9.1
Val	4.9	6.0
Met	1.1	0.3
Ile	3.5	3.7
Leu	5.1	4.3
Tyr	1.9	3.3
Phe	2.9	4.9
Lys	4.3	3.2
His	3.7	0.8
Arg	3.2	5.7
Cys	1.1	1.3

[a]Mean values from two analyses of protein hydrolysed for 24 h under vacuum, in 6M HCl containing 0.1% of phenol.

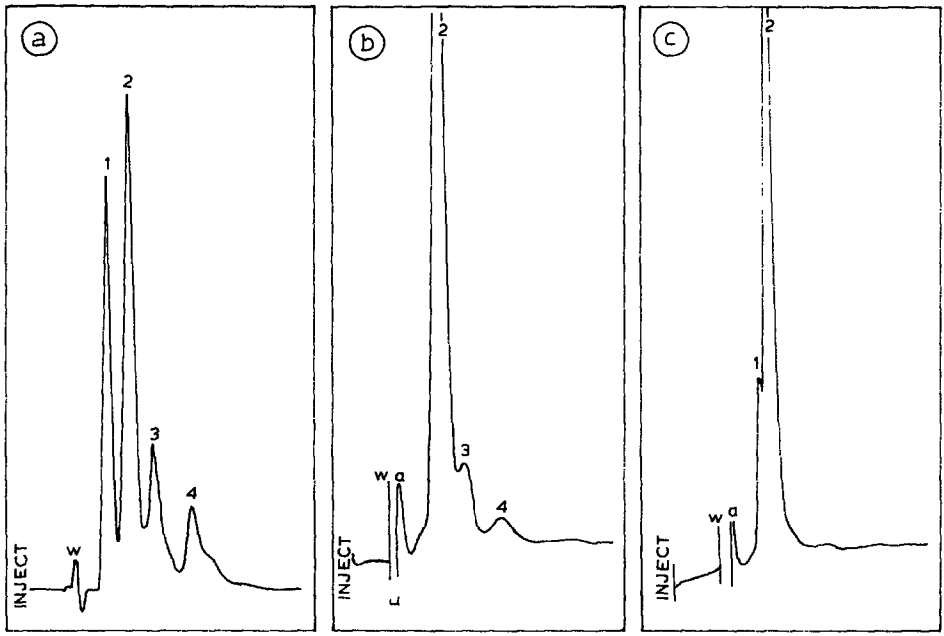

Fig. 7. H.p.l.c. of the chitinase products. The chito-oligosaccharides were detected on the basis of refractive index and identified by their retention time: *w*, water (2.5 min); *a*, acetate (3.5 min); 1, GlcNAc (5.0 min); 2, $(GlcNAc)_2$ (6.5 min); 3, $(GlcNAc)_3$ (8.3 min); 4, $(GlcNAc)_4$ (10.5 min). Each analysis was conducted for 18 min: (a) standard calibration solution of chito-oligosaccharides (5 mg/mL); (b) reaction mixture from 3-h incubation of chitin with chitinase I (60 μg/mL); (c) reaction mixture from 3-h incubation of chitin with chitinase II (10 μg/mL). The peak area/mole coefficients were 2.279 for 1, 2.500 for 2, 3.980 for 3, and 5.436 for 4.

10–20% of monosaccharide after 18 h. Chitinase II was less active than chitinase I and incubation times of 3–18 h were required. The main products were the mono- and di-saccharides. The colorimetric assay used does not allow measurement of the total chitinase II activity, since only monosaccharide was assessed, but allows it to be distinguished from endo-enzymic activities. Typical analyses are shown in Fig. 7. Similar product profiles have been obtained with chito-oligosaccharides up to chitoheptaose.

Thus, chitinase I seems to act as an endo-enzyme, its initial products being di-, tri-, and higher oligo-saccharides. The characterisation of chitinase II is more difficult. The main products were mono- and di-saccharides, but the data in Table I indicate that its activity decreased during purification, which could reflect the loss of a synergistic effect or the use of an inappropriate substrate (chitin) since exo-enzymes exhibit higher affinity for oligosaccharides. Thus, chitinase II is thought to be an exo-enzyme.

In order to clarify this point, the affinities of chitinases I and II for chitin and chito-oligosaccharides were compared. [^{3}H]chitin was used as the substrate, and

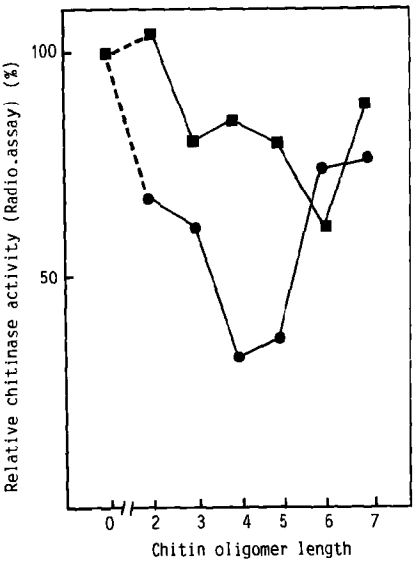

Fig. 8. Action of chitinases I (■) and II (●) on [^{3}H]chitin. 0.2mM Chito-oligosaccharides of increasing lengths were added as competitive substrates.

chito-oligosaccharides of increasing length were added as competitive substrates. The data show (Fig. 8) that the chito-oligosaccharides were the more effective substrates for chitinase II, since activity towards [^{3}H]chitin was reduced by 40–70% in the presence of chito-oligosaccharides up to the pentaose. The activity of chitinase I towards [^{3}H]chitin was affected to ≤25% by the presence of short oligosaccharides and to ~40% for the hexaose. These data were confirmed by the determination of the kinetic constants for chitinases I and II. For chitinase I, the K_m for the hexamer was 0.098 ±0.020mM and 1.110 ±0.104mM for the trisaccharide, whereas the corresponding values for chitinase II were 1.70 ±0.15 and 0.54 ±0.07mM.

Thus, chitinase I is an endo-enzyme, exhibiting a good affinity for chitin or, to a lesser extent, for long chito-oligosaccharides. Chitinase II has a higher affinity for the shorter chito-oligosaccharides than for chitin, has no detectable activity on *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, and therefore is neither an endo-enzyme nor a β-*N*-acetylglucosaminidase, but an exo-enzyme as defined by Koga *et al.*[15].

EXPERIMENTAL

General. — Melon seedlings (*Cucumis melo*, cv. Cantaloup charentais), healthy and inoculated with *Colletotrichum lagenarium*, were obtained as previously described[27]. Protein was determined by the method of Lowry *et al.*[28] and carbohydrate by the anthrone assay[26].

Chitinase activities were measured[14] by (*a*) a colorimetric assay with purified chitin as the substrate[29] and (*b*) a radiochemical assay[30] with radiolabelled chitin (specific activity, 10.80 kBq/μmol GlcNAc equiv.).

Enzyme purification. — All steps were carried out at 0–5°, except where otherwise indicated.

Enzyme extraction was performed on the aerial part of seedlings by homogenisation, with a mortar and pestle, in 0.1M acetate buffer (pH 4.65) (2 mL/g fresh weight) made 5% in Polyclar AT (Sigma), 0.5% in polyethyleneglycol (PEG 6,000), and 0.1% in 2-mercaptoethanol. The homogenate (pH 6.0) was filtered through cheesecloth and centrifuged at 20,000*g* for 20 min. The pellet was discarded and the supernatant solution was assayed for chitinase activity.

The pH of the crude extract was adjusted to 4.5 with 2M acetic acid. After storage at room temperature for 15 min, the suspension was centrifuged for 20 min at 20,000*g*. The pellet was discarded, the pH of the supernatant solution was adjusted to 5.0 with 3M NaOH, and ammonium sulfate fractionation was then performed. The fraction obtained between 40 and 75% saturation, after stirring for 30 min at 0°, was recovered by centrifugation (20 min at 20,000*g*), then dissolved in 20mM acetate buffer (pH 5.0), desalted on Sephadex G-25, and purified on a column (14 × 2.5 cm) of CM-cellulose (CM-32, Whatman) previously equilibrated with 20mM acetate buffer (pH 5.0). The enzyme was eluted with 700 mL of a linear gradient of NaCl in the buffer (0.02→0.5M), and 5-mL fractions were collected.

The fractions containing the major chitinase activities, which were detected by both the colorimetric and the radiochemical assays, were combined, lyophilised, dialysed against 20mM acetate buffer (pH 5.0), applied to a column (104 × 2.6 cm) of Sephacryl S-200 previously equilibrated with 20mM acetate buffer (pH 5.0), and eluted with the same buffer (2-mL fractions). The fractions corresponding to the major, radiochemically assessed chitinase activity were also combined, lyophilised, dialysed, added to a column (10 × 1 cm) of Polybuffer Exchanger 94 (Pharmacia) equilibrated with 25mM ethanolamine–acetic acid buffer (pH 9.4), and eluted at 15 mL/h with 10-fold diluted Polybuffer 96 (Pharmacia) which generated a linear gradient of pH 9.4→6.5; 2-mL fractions were collected.

Product analysis. — The chitinase assays were carried out as previously described, except that acetone was used to stop the reaction when [^{3}H]chitin was used as the substrate. After centrifugation, the supernatant solution was concentrated or lyophilised. The residue was dissolved in 200 μL of H_2O, and an aliquot (50 μL) was filtered through a 0.45-μm Millipore HV filter and then analysed by h.p.l.c.[31] on a Spectra-Physics SP 8000 chromatograph equipped with an Altex 10-μm Ultrasil NH_2 column (4.6 × 250 mm), using acetonitrile–H_2O (70:30) at 1.3 mL/min. The sugars were detected by using a refractive index detector or by liquid scintillation counting. Chito-oligosaccharides, kindly provided by Dr. A. Gadelle, were obtained by partial hydrolysis of chitin with hydrogen fluoride and purified by gel-permeation chromatography[32].

Kinetic parameters. — Purified chitinases I and II (60 and 10 μg/mL, respec-

tively) were assayed by using various substrates (0.05–1mM). Concentrations of substrate and product were then determined by h.p.l.c. as described above, and K_m values were deduced from Lineweaver–Burk plots.

Determination of molecular weights. — SDS-Polyacrylamide gel electrophoresis was carried out as described by Laemmli[33], with a resolving gel containing 12.5% of acrylamide and a stacking gel containing 3% of acrylamide. The markers α-lactalbumin, trypsin inhibitor, carbonic anhydrase, ovalbumin, albumin, and phosphorylase b were obtained from Pharmacia. Coomassie Brillant Blue was used as the protein stain. Gel filtration was performed using a column (104 × 2.6 cm) of Sephacryl S-200 equilibrated with 20mM acetate buffer (pH 5.0). Blue dextran 2000 was used to determine the void volume, and ribonuclease A, chymotrypsinogen, ovalbumin, and bovine serum albumin were used as markers.

Amino acid analysis. — Purified enzymes were hydrolysed[34] at 110° in 6M HCl containing 0.1% of phenol under vacuum for 24 h. The acid hydrolysates were processed by using a Beckman 119 BL Amino Acid Analyzer.

Determination of isoelectric points. — Purified chitinase II was applied to a column (10 × 1 cm) of Polybuffer Exchanger 94 equilibrated with 25mM ethanolamine–acetic acid (pH 9.4) and eluted with 10-fold diluted Polybuffer 96, which generated a pH gradient 9.4→6.5. Purified chitinase I was applied to a column (10 × 1 cm) of Polybuffer Exchanger 118 equilibrated with 25mM triethylamine–HCl buffer (pH 11) and eluted with 4.5-fold diluted Pharmalyte 8–10.5–HCl. The resulting pH gradient was 10.5→8.5.

ACKNOWLEDGMENTS

We thank Dr. G. Abravanel and Dr. M. Rossignol for technical assistance, Dr. A. Gadelle for the purified chitin oligosaccharides, and Dr. A. Toppan and Pr. R. Ranjeva for helpful discussions.

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