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Some citrus chitinases also possess chitosanase activities

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Abstract. Several acidic chitinase and chitosanase isoforms were found in 4-week-old nonembryogenic sweet orange ('Valencia' [*Citrus sinensis* (L.) Osbeck]) callus tissue. Two isoforms (designated A1-CF1 and A1-CF2) were purified to homogeneity using HPLC size exclusion, anion exchange, and chromatofocusing techniques. Both hydrolase isoforms exhibited activity with either colloidal chitin or solubilized shrimp shell chitosan. Specific activities for the purified isoforms could not be calculated because of the lack of protein and contamination of ampholytes. However, the specific activities for chitinase and chitosanase after anion exchange were respectively 404 nmol GlcNAc per min per mg protein and 2,475 nmol GlcN per min per mg protein. The M_r for both enzymes was 30,500. The homogeneous proteins cross-reacted in western blots with antiserum against a basic class I potato leaf chitinase.
Key words. Chitosanases; chitinases; hydrolases; sweet orange, callus tissue; immunoblotting; pathogenesis-related proteins.

Chitinases (EC 3.2.1.14) have been reported in many plant varieties and have been found in most plant parts including leaves, roots, fruit, and seeds[1]. Plant chitinases and other hydrolases can either be constitutive or are induced in monocots and dicots after infection by different plant viruses[2], bacteria[3], and fungi[4–6] and are consequently called plant pathogenesis-related (PR) proteins. Chitinase activity can also be induced by the use of elicitors such as insoluble fungal cell wall fractions[7,8] and ethylene and ozone fumigation[9,10]; however, induction by an elicitor is not a criterion for being a PR protein. Chitinases can inhibit fungal growth, either alone[11] or in combination with β-1,3-glucanases[12], probably by attacking the fungal cell wall[1] which contains glucan, chitin and chitosan[13].

Chitosanases (EC 3.2.1.99) hydrolyze deacetylated chitin (chitosan). Chitosanases are relatively new plant PR-protein members[14–17], although they were first reported in bacteria and fungi in 1973 by Monaghan et al.[18]. Chitosanase isoforms have been reported in leaf, and seed, fruit extracts of several plant species using polyacrylamide gel activity assays[14,15,17] and have been induced in plants treated with chemicals or different pathogens[15]. Collectively, these reports suggest that plant chitosanases, chitinases, and β-1,3-glucanases, working separately or in concert, might be involved in defending the host plant by degrading the cell walls of fungal invaders.

Our laboratory has recently been interested in hydrolases resident in citrus. In particular, we have been interested in the chitinases and chitosanases and the possibility of using these enzymes either alone or in conjunction with other hydrolases (e.g., β-1,3-glucanases) to reduce citrus production and postharvest losses caused by pathogens and insects. Plant chitinases have been cited in only two reports[19,20] as being active against chitosan as has been reported for chitinases from *Streptomyces griseus*[21]. During the course of our investigations we observed that some of the chitinases from 'Valencia' callus tissue were able to hydrolyze chitin as well as chitosan. The present report details the purification of two chitinase/chitosanase isoforms from citrus and suggests that such enzymes would be advantageous to plant defense systems by increasing effectiveness against a broader number of pests.

Materials and methods

Chemicals. Shrimp shell chitosan (18% acetylation, determined by IR spectroscopy[16]) was purchased from Atomergics Chemetals Corp. (Farmingdale, NY; Lot LO729). Fluorescamine (Fluram) was obtained from Fluka (Buchs, Switzerland), and [^{3}H] acetic anhydride (sp. act. 50 mCi/mmol) was from NEN (Wilmington, DE). The low molecular weight markers (M_r 14,500, 21,500, 31,000, 45,000, 66,000 and 97,400) were from BioRad (Richmond, CA), and the Polybuffer 74 from Pharmacia (Piscataway, NJ). Bovine serum albumin (BSA; fraction V), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and nitro blue tetrazolium (NBT) were purchased respectively from Sigma (St. Louis, MO) and U.S. Biochemicals (Cleveland, OH).

Citrus tissue culture. A nonembryogenic cell line (Val 88-1) was developed from immature fruit vesicles of

Citrus sinensis (L.) Osbeck cultivar 'Valencia' as reported recently[16]. After 8 months of selection, a rapidly growing callus was obtained. For maintenance, Val 88-1 was grown for 28 days on Murashige and Tucker's[22] basal medium, supplemented with 1 μM 6-benzylaminopurine, 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 100 mg per liter casein hydrolysate.

Chitinase assay. Chitinase activity was measured according to Molano et al.[23] using tritiated chitin prepared by acetylation of shrimp shell chitosan with [^{3}H]-acetic anhydride. The specific activity of the prepared chitin was determined after acid hydrolysis[24] via fluorometric analysis[25]. The specific activity was 605 Ci per mmol GlcNAc. Radioassays were conducted as previously reported[16]. One unit of chitinase activity is defined as the release of 1 nmol GlcNAc per min.

Chitosanase assay. The chitosanase assay followed Osswald et al.[16] using solubilized shrimp shell chitosan. One unit of chitosanase activity is defined as the release of 1 nmol GlcN per min.

Immunoblotting. All manipulations were performed at room temperature. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 2-mercaptoethanol was performed as described by Laemmli[26] in a 14% (w/v) acrylamide gel which was overlaid with a stacking gel of 4% (w/v) acrylamide. The separated proteins were blotted onto an Immobilon-P transfer membrane (PVDF, pore size 0.45 μm, No. IPVH 304FO, Millipore, Bedford, MA) using a semidry electroblotting system (Pharmacia). Blotting was carried out in blotting buffer (48 mM Tris, 39 mM glycine, 0.0375% (w/v) SDS, 20% methanol (v/v), pH 8.3) for 1.5 h at 0.8 mA/cm^2 gel. Afterwards, the blot was rinsed in 20 mM Tris, pH 7.5 150 mM NaCl (TBS), and then blocked with blocking solution (TBS + 1% BSA) for 1.5 h. The blot was then incubated with the primary antibody (1:2500) diluted with the blocking solution) for 1 h. The primary antibody was raised in rabbits against a basic (class I) potato leaf chitinase and was donated by Dr. E. Kombrink, Max Planck Institute, Cologne, Germany. Information on the cross-reactivity and preparation of the antibody has been reported[27]. Afterwards the blot was washed three times for 5 min each with TBS supplemented with 0.05% (v/v) Tween-20 (TBST) and transferred into the alkaline phosphatase-bound goat anti-rabbit IgG antiserum (Kirkegaard and Perry Labs. Inc., Gaithersburg, MD) for 1 h. The secondary antibody was diluted 1:1500 with the blocking solution. After an additional three washes (5 min each) in TBST and one wash for 5 min in TBS the blot was transferred to 25 ml freshly prepared color development solution (100 mM Tris, pH 9.5; 100 mM NaCl, 5 mM $MgCl_2$) supplemented with 100 μl NBT-solution (stock: 75 mg per ml in 70% N,N-dimethylformamide) and 75 μl BCIP-solution (50 mg per ml water). The blots were developed in the dark for 10–15 min.

Enzyme extraction and purification. All operations were performed on ice or at 4 °C in a cold room. Callus tissue (2.8 kg) was harvested 4 weeks after transfer. The tissue was powdered in liquid nitrogen, extracted with 5 L acetate buffer (100 mM, pH 5) for 30 min, and the extract was centrifuged for 15 min at 20,000 *g*. The supernatant was brought to 40% (w/v) ammonium sulfate (AS), stirred for 1 h, and centrifuged for 15 min at 15,000 *g*. Subsequently, enough AS was added to the supernatant from the 40% AS treatment to bring the concentration of AS to 60%. After 1 h incubation with AS, the suspension was centrifuged for 15 min at 15,000 *g*, and the pellet was resuspended in 440 ml of 20 mM phosphate buffer, pH 6.7. Aliquots (100 ml) of the AS preparation were desalted on a G-25 column (5 × 25 cm; flow rate 1.5 ml per min) equilibrated with 20 mM phosphate buffer, pH 6.7. The active fractions were combined and concentrated with centriprep-10 concentrators (Amicon, Danvers, MA). Aliquots (100 μl; 1.2 mg protein) were subject to HPLC size exclusion chromatography (SEC; TSK-gel GS2000SW, TosoHaas, Montgomeryville, PA). The HPLC system consisted of a HPLC pump (SP8000 Ternary, SpectraPhysics, San Jose, CA), an autosampler (AS 3500, SpectraPhysics), an integrator (SP4290, SpectraPhysics), a fraction programmer (Model 3100, ISCO, Lincoln, NE), a fraction collector (Cygnet Model, ISCO), and a UV detector (Dupont Instruments, Wilmington, DE). The chitinase/chitosanase active fractions from SEC were pooled, dialyzed for 5 h in a 30 mM Tris buffer (pH 8), and concentrated with centriprep-10 concentrators. Aliquots (800 μl; 1.5 mg protein) were separated on an HPLC anion exchange column (Protein-Pak Q 8HR, 10 × 100 mm, Waters, Milford, MA). The column was equilibrated with 20 mM Tris buffer, pH 8 and the proteins were eluted using a combination of three elution buffers: buffer A was 20 mM Tris, pH 8; buffer B was 0.1 M sodium phosphate, pH 6.7, 0.1 M Na_2SO_4; buffer C was 20 mM Tris, pH 8, 0.5 M NaCl (fig. 1).

The first fraction containing chitinase/chitosanase activity collected during anion exchange chromatography (fraction A1) was further purified on a chromatofocusing column (Mono P RH 5/20, Pharmacia) with a linear gradient starting with 25 mM bis-Tris, pH 6.3 and ending with Polybuffer 74 (1:10 dilution), pH 3.3.

Results and discussion

The results of the purification of two chitinase/chitosanase isoforms (A1-CF1 and A1-CF2) are presented in the table. Enzyme recovery after AS fractionation was 50 to 58% depending on whether chitinase or chitosanase activity was monitored. The desalted AS preparation was then subjected to SEC and fractions with chitinase/chitosanase activities following SEC were combined and concentrated. Aliquots (800 μl) of the

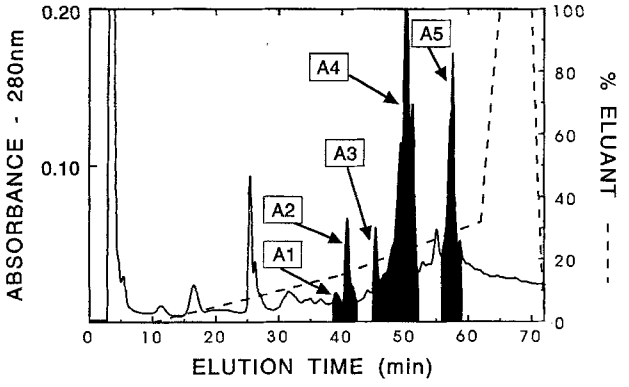

Figure 1. HPLC anion exchange chromatography elution pattern of chitinase and chitosanase isoforms. The anion exchange column was equilibrated with buffer A (see 'Materials and methods'). The loaded proteins from the SEC pooled fractions were eluted using a two step gradient that started 10 min post-injection and ended at 40 min increasing to 15% buffer B. The second step started at 40 min post-injection and ended at 62 min with an increase to 40% B. The column was washed (5 min) after each run with buffer C. The flow rate was 1 ml per min. Each fraction (shaded areas) was collected separately and the chitinase and chitosanase activities were determined.
——— Absorbance at 280 nm; – – – – – Buffers B and C

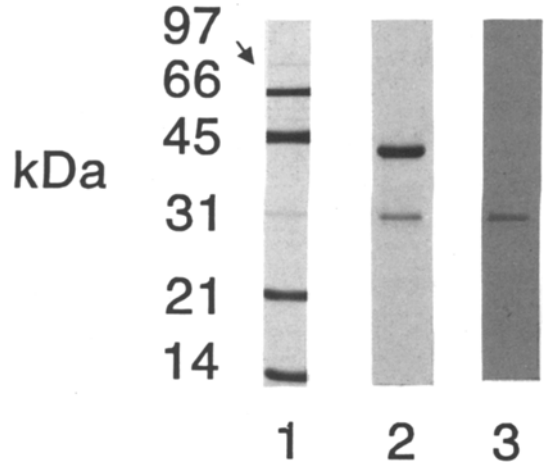

Figure 2. SDS-PAGE and immunoblot of protein fraction A1 after anion exchange chromatography. The SDS-PAGE and the immunoblot were run as described in 'Materials and methods'. Lane 1, molecular weight markers; lane 2, SDS-PAGE of protein fraction A1; lane 3, western blot of A1.

pooled SEC fractions were subjected to HPLC anion exchange chromatography. The anion exchange chromatography elution pattern is shown in figure 1. The basic chitinases and chitosanases eluted in the void volume after 4.5 min. Acidic chitinases and chitosanases eluted at 39 min (fraction A1), 41 min (fraction A2), 45 min (fraction A3), 49 min (fraction A4), and 57 min (fraction A5). Fractions A1 through A4 exhibited both chitinase and chitosanase activities while fraction A5 exhibited chitinase activity exclusively. Fraction A1 was further purified (characterization of the proteins in the other fractions will be reported elsewhere). Fraction A1 was concentrated and the proteins were separated on 14% SDS-PAGE. After silver staining two major protein bands were visible (fig. 2) and only the protein with a 30,500 M_r cross-reacted with the antibody raised against the basic class I potato leaf chitinase. Fraction A1 was concentrated and then was subjected to chromatofocusing (fig. 3). Two peaks (A1-CF1, A1-CF2) with chitinase and chitosanase activity were detected with eluent pH values of pH 5.4 and 5.29. The proteins in these two fractions were concentrated, separated on a 14% SDS-PAGE, and determined to be homogeneous proteins with M_rs of 30,500 (fig. 4). Both A1-CF1 and A1-CF2 cross-reacted with the potato leaf chitinase antibody (fig. 4). Recovery of the two proteins was about 0.03%. It was not possible to determine specific activities for the purified proteins due to interference with ampholytes from the chromatofocusing step.

Cross-reaction of the two chitinase/chitosanase isoforms with the antibody suggests that the citrus callus tissue enzymes may be similar to the class I potato leaf chitinases. Proof of whether or not A1-CF1 and A1-CF2 are actually class I chitinases can only be established by determining the primary structures. Otherwise, the two proteins are similar in regard to M_rs and pIs. Only the slight differences in the pIs of the enzymes allowed them to be purified to homogeneity using a chromatofocusing column, because the two proteins

Purification summary for Chitinases/Chitosanases A1-CF1 and A1-CF2

	CHITINASE					CHITOSANASE			
Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg prot.)	Purif. factor	% Recovery	Total activity (U)	Specific activity (U/mg prot.)	Purif. factor	% Recovery
Crude Homog.	2,180	65,742	30	1	100	179,476	82	1	100
40–60% AS	1,025	38,364	37.4	1.24	58.3	89,771	87	1.1	50
SEC	270	17,448	65	1.6	26.5	34,466	128	1.6	19.2
Anion Exchg.	0.12	49	404	13.5	0.07	297	2,475	30	0.2
Chromato-focusing									
A1-CF1	nd[a]	14.6	nd[a]	nd[a]	0.02	55.1	nd[a]	nd[a]	0.03
A2-CF2	nd[a]	20	nd[a]	nd[a]	0.03	58	nd[a]	nd[a]	0.03

[a]Not determined.

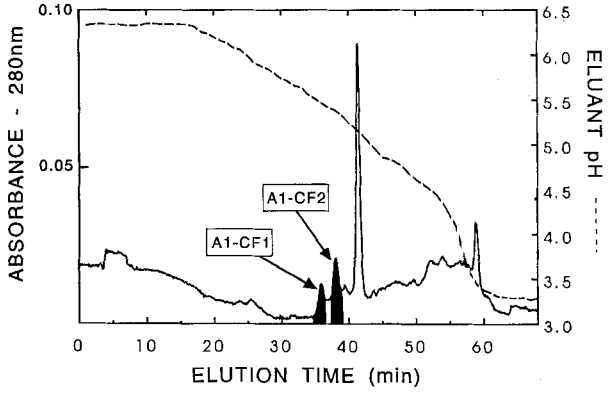

Figure 3. Elution pattern of acidic A1 hydrolases on a chromatofocusing column. The column was equilibrated with 25 mM bis-Tris buffer pH 6.3 prior to applying the A1 proteins (see 'Materials and methods'). The flow rate was 1 ml per min. The two fractions (shaded areas) were collected and the chitinase and chitosanase activities were determined.
——— Absorbance at 280 nm; - - - - - Eluant pH on the column

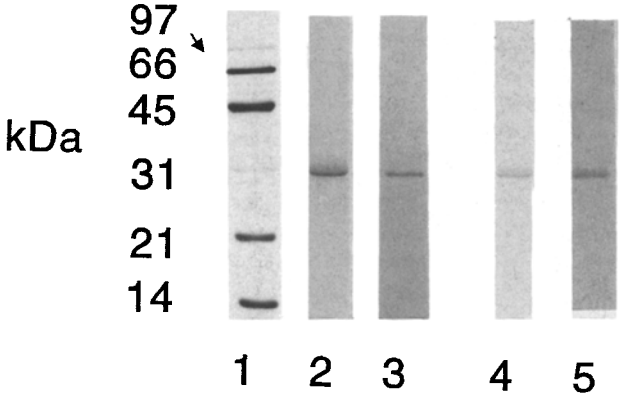

Figure 4. SDS-PAGE and western blots of protein fractions A1-CF1 and A1-CF2 with antiserum raised against a basic, class I potato leaf chitinase. Lane 1, molecular weight markers; lane 2, SDS-PAGE of fraction A1-CF1; lane 3, western blot of A1-CF1; lane 4, SDS-PAGE of A1-CF2; lane 5, western blot of A1-CF2.

had similar molecular weights and they could not be distinguished using 14% SDS-PAGE at the conditions specified here.

We have shown that two purified hydrolases from nonembryogenic 'Valencia' callus tissue degraded both chitin and chitosan and therefore, can be defined as chitinases and chitosanases. Chitosan is a cell wall component of Zygomycete fungi[13] and is thought to be produced by the action of chitin deacetylases on chitin[28]. Chitin deacetylases are known for several fungi, such as *Mucor* or *Rhizopus* sp.[29], or *Colletotrichum lindemuthianum*[30]. Although the function(s) of chitosanases in higher plants is unknown, some plant chitosanases have been identified as PR proteins and degrade the chitosan cell wall component of fungal invaders[14,15]. Also, plant chitosanases produce chitosan oligomers, which could act as either elicitors or molecular signals. Kauss et al.[30] have shown that chitosan induced the 1,3-β-glucan synthesis in plants via the induction of 1,3-β-glucan synthase. Furthermore, chitinase activity in carrot cells or pea pods has been reported to increase in response to treatment with chitosan[31,32]. Recently Hirano et al.[33] showed an increase in chitinase activity in several seedlings when seeds were coated with chitosan derivatives. Chitinases that are also chitosanases (or vice versa) would be valuable assets in a plant's defensive arsenal as they would be active against a wider variety of fungal pathogens and could generate oligosaccharide elicitors from chitin or chitosan.

Thus far, there have been few reports on plant chitosanases. Most of these reports have been qualitative in nature[14,15]. There are many more reports on chitinases in the literature, but only a few[19,20] have investigated the possibility that these chitinases may also act as chitosanases. Perhaps this deficiency will be rectified now that a new expedient assay for chitosanases is available[16].

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