Endochitinases from *Castanea crenata* Cotyledons

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Three constitutive endochitinase isoforms were purified from the cotyledons of *Castanea crenata*. All three isoforms are basic with p*I* between 8.5 and 9, possess approximate molecular weights of 25 000, 26 000, and 25 500, and have been named Cha, Chb, and Chc, respectively. All isoforms cross-reacted with antibodies raised against Ch1 chitinase previously purified from *Castanea sativa*. The molecular weights and amino acid compositions of Cha, Chb, and Chc suggest that these proteins belong to the class II endochitinases.

INTRODUCTION

Chitinases play a role in the defense of plants against infection by fungal pathogens (Boller, 1988; Bowles, 1990; Bol et al., 1990). This statement is supported by the known induction of chitinases by fungal infection or other stress conditions, their *in vitro* antifungal activity, and the observed fact that in the course of incompatible interaction chitinases accumulate much earlier than in the case of compatible reaction. (Schlumbaum et al., 1986; Roberts and Selitrennikoff, 1988; Mauch et al., 1988; Benhamou et al., 1990). Furthermore, transgenic plants constitutively expressing a chitinase gene showed enhanced resistance to *Rhizoctonia solani* (Broglie et al., 1991).

The constitutive presence of chitinases in the seed storage tissue of monocotyledonous plants has been confirmed (Roberts and Selitrennikoff, 1988). Barley seed class I and class II endochitinases have been well characterized (Leah et al., 1991; Swegle et al., 1989; Kragh et al., 1991; Huynh et al., 1992). For dicotyledonous seed tissue, less information exists with respect to this enzyme system, though other tissues have been extensively studied. Chitinases have been partially characterized from soybean seeds (Wadsworth and Zikakis, 1984) and cucumber seeds (Majeau et al., 1990), and recently we have purified three basic isoforms, Ch1, Ch2, and Ch3, from the cotyledons of *Castanea sativa* (European chestnut) (Collada et al., 1992). Ch1 and Ch2 are class II endochitinases with approximate molecular weigths of 25 000 and 26 000, whereas, Ch3 belongs to class I and has a molecular weight of 32 000.

We here report the isolation of three major endochitinases from cotyledons of *Castanea crenata* (Japanese chestnut). Hybrids between the two cited *Castanea* species are extensively grown in Europe because of the enhanced resistance to *Phytophthora cambivora* (Schad et al., 1952; Vieitez et al., 1986). The study of the defense proteins of seeds of *Castanea* species is important in two main areas: the basic mechanisms of protection and possible improved storage conditions of chestnuts destined for commerce.

MATERIALS AND METHODS

Plant Material. Mature seeds of *C. crenata* Lieb. and Zucc. were collected from Lourizán (Spain) and stored at −40 °C. Partially defatted flour was obtained from the cotyledons as described by Collada et al. (1991).

Purification of Chitinases. All purification steps were performed at 4 °C. Salt-soluble flour proteins were extracted with 50 mM Tris-HCl, pH 8.2, and 0.5 M NaCl (10:1 v/w; 1 h). After centrifugation for 30 min at 30000*g*, the supernatant was precipitated with 70% (saturated) $(NH_4)_2SO_4$, collected by centrifugation (30 min; 30000*g*), and dissolved in extraction buffer, and the pH was brought to 3.5 by addition of 0.1 M HCl. After an additional centrifugation, the supernatant, containing the endochitinase activity, was dialyzed against water and lyophilized. This preparation was chromatographed on a SP-Sephadex C-50 column (40 × 2.5 cm) equilibrated with 10 mM sodium acetate and 0.08 M NaCl, pH 5.3, using a 400-mL linear gradient of NaCl from 0.08 to 0.2 M in acetate buffer. Homogeneity of the active fractions was tested by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and those appropiate were pooled and concentrated using a Centrifugal Ultrafree filter unit.

Endochitinase Assay and Protein Determination. Endochitinase activity was determined according to the method of Boller and Mauch (1988). Protein estimation was carried out by the method of Smith et al. (1985).

Electrophoretic Methods and Immunoblotting. SDS–PAGE was performed using the method of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue G-250 (Blakesley and Boezi, 1977). For immunoblotting, proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane as described previously (Collada et al., 1992) and reacted with anti-*C. sativa* Ch1 endochitinase monospecific antibodies, as described in Collada et al. (1992). The antigens were detected immunochemically with alkaline phosphatase-coupled secondary antibodies.

Amino Acid Analysis. Samples were hydrolyzed with 5.7 N HCl and 5 mM phenol at 110 ± 1 °C for 24 h. Performic acid oxidation of proteins was as described by Hirs (1967). Amino acid analysis was carried out by reversed-phase high-performance liquid chromatography following the method of Bidlingmeyer et al. (1984). An Ultrasphere ODS column (0.46 × 25 cm) was used at a temperature of 40 °C.

RESULTS AND DISCUSSION

Analysis by SDS–PAGE (reducing conditions) of salt-soluble proteins from *C. crenata* cotyledons [precipitated with 70% (saturated) $(NH_4)_2SO_4$] showed three major bands with approximate molecular weights of 22 000–23 000, 25 000–26 000, and 31 000 (Figure 1, track 1). The 22 000–23 000 and 31 000 proteins are principal components of reserve globulins (Collada et al., 1986, 1991) and precipitate at pH 3.5 (Figure 1, track 2), leaving the endochitinase activity in the supernatant. The preparation obtained by salting out followed by differential precipitation, enriched in endochitinases, was used for the purification of the most abundant isoenzymes of this system. This preparation was fractionated by chromatography on SP-Sephadex (Figure 2). The majority of endochitinase activity is contained in three fractions,

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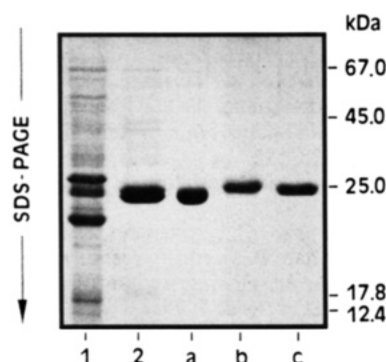

Figure 1. SDS–PAGE of (1) protein fraction obtained by salting-out with ammonium sulfate from salt-soluble proteins of *C. crenata* cotyledons; (2) protein fraction obtained by differential precipitation at pH 3.5; (a–c) peaks a, b, and c of SP-Sephadex chromatography corresponding to Cha, Chb, and Chc, respectively. The molecular weights of marker proteins appear on the right.

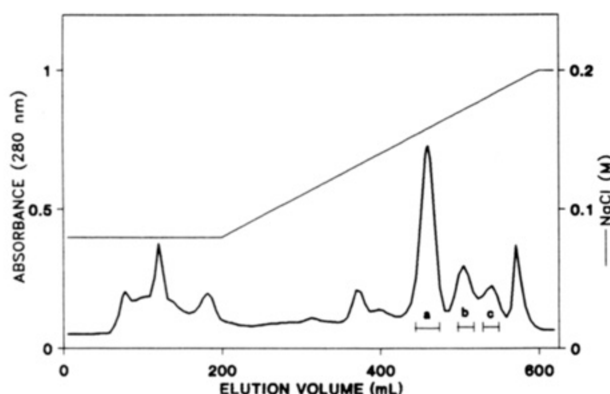

Figure 2. Fractionation on SP-Sephadex C-50 of the protein fraction from *C. crenata* cotyledons enriched in endochitinase activity. The proteins were eluted in a linear gradient of 0.08–0.20 M NaCl in 10 mM sodium acetate, pH 5.3.

labeled a, b, and c, which make up more than 50% of total recovered protein. When analyzed by SDS–PAGE, each fraction revealed the presence of a single band of 25 000, 26 000, 25 500 (Figure 1). These proteins have been named Cha, Chb, and Chc endochitinases, respectively. The sodium chloride concentrations required to elute the Cha and Chb endochitinases from the SP-Sephadex column (0.16 and 0.17 M) are the same as those required to elute, under the same conditions, the chitinases Ch1 and Ch2 of *C. sativa* (Collada et al., 1992). The specific activity of Cha was 240 milliunits/mg of protein, whereas that of chitinase Chb and Chc was 150 milliunits/mg of protein. These activities are similar to those described for *C. sativa* chitinases.

The amino acid compositions of the three purified proteins were partially characterized by their relatively high levels of Asx and Gly, typical of plant endochitinases (Table I). These compositions were compared between themselves and with those of previously characterized chitinases of *C. sativa* (Collada et al., 1992), using the compositional divergence index of Cornish-Bowden (1980) (Table II). The values obtained for all binary comparisons between Ch1, Ch2, Cha, Chb, and Chc are those expected for homologous proteins with a high degree of sequence similarity. These values, along with their molecular weights and p*I*s (between 8.5 and 9, data not shown), indicate that Cha, Chb, and Chc are class II basic endochitinases. The relationship of the three isoenzymes of *C. crenata* with Ch1 was further analyzed by immunoblotting with monospecific polyclonal antibodies raised

Table I. Amino Acid Composition and Molecular Weights of Purified Chitinases from *C. crenata* Cotyledons

	mol/100 mol of amino acid			residues/mol[a]		
amino acid	Cha	Chb	Chc	Cha	Chb	Chc
Lys	4.0	4.0	4.6	9	10	11
His	1.8	1.6	2.2	4	4	5
Arg	4.6	4.7	4.8	11	12	11
Asx	12.4	12.7	12.3	29	32	29
Thr	8.0	8.7	8.1	19	22	19
Ser	6.9	8.1	7.7	16	20	18
Glx	5.6	5.9	6.5	13	15	16
Pro	5.5	5.5	5.5	13	14	13
Gly	12.9	12.8	12.4	30	32	29
Ala	9.8	9.4	9.5	23	24	23
Val	4.0	3.9	3.9	9	10	9
Cys	2.6	2.8	2.3	6	7	6
Met	1.1	0.8	1.0	3	2	2
Ile	4.9	4.6	4.5	12	12	11
Leu	4.6	4.4	4.8	11	11	11
Tyr	6.4	6.3	5.9	15	16	14
Phe	4.8	3.8	4.0	11	10	10
total residues/mol				234	253	237
MW (1)[b]				25 000	26 000	25 500
MW (2)[b]				24 906	26 813	25 249

[a] Number of residues adjusted by the method of Delaage (1968) to the molecular weight determined by SDS–PAGE. Trp was not analyzed. [b] Molecular weights (1) determined by SDS–PAGE or (2) calculated from the adjusted amino acid composition.

Table II. Values of the Compositional Difference Index for the Binary Comparison of Purified Chitinases from the *C. crenata* and *C. sativa* Cotyledons[a]

	$S\Delta N_r$					
	Cha	Chb	Chc	Ch1	Ch2	Ch3
Cha	0.00					
Chb	0.23	0.00				
Chc	0.13	0.22	0.00			
Ch1	0.06	0.21	0.09	0.00		
Ch2	0.23	0.03	0.18	0.21	0.00	
Ch3	1.32	0.86	1.23	1.36	0.90	0.00

[a] Compositional difference index: $S\Delta N_r = S\Delta N/0.42N$; based on Cornish-Bowden (1980) as modified by Paz-Ares et al. (1983). $S\Delta N_r < 1$, 95% confidence of homology.

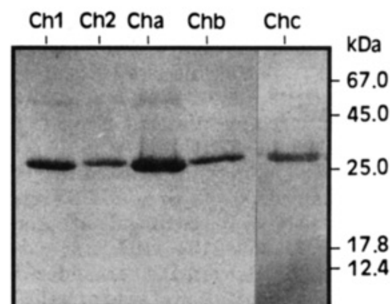

Figure 3. Western blot showing the cross-reactivity of *C. sativa* (Ch1 and Ch2) and *C. crenata* (Cha, Chb, and Chc) endochitinases. Proteins were subjected to SDS–PAGE, transferred to nitrocellulose, and reacted with antibodies raised against Ch1. The quantity of Ch1 and Ch2 loaded on the gel (1 μg) was half that of Cha, Chb, and Chc (2 μg).

against this protein (Figure 3). The antibodies showed significant cross-reactivity with Cha, Chb, and Chc. The low Cys content of Cha, Chb, and Chc and the compositional divergence indices between Ch3 (the class I endochitinase from *C. sativa*) and each of the three proteins suggest that none possess the Cys-rich domain (hevein domain) characteristic of class I endochitinases.

The low values found for the Cornish-Bowden index in the comparisons Cha–Ch1 and Chb–Ch2 and the similar

molecular weights might indicate that each pair corresponds to equivalent proteins of the two *Castanea* species. This suggests that gene duplication probably gave rise to the genes coding for Cha and Chb, Ch1 and Ch2, prior to the appearance of the two species, in the common ancestor of both. However, it is also possible that in each species one of the proteins is a posttranslational modification or product of the other protein. The data for Chc suggest that an allelic variant of Cha (the seeds came from trees whose genotype had not been controlled) exists or could be coded for by a gene that arose during the evolution of *C. crenata* once this species diverged from *C. sativa*.

The results show that endochitinases are abundant proteins in the cotyledons of *C. crenata* as is the same for *C. sativa*. We are presently comparing the antifungal activities of the chitinases from both species.

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