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Isolation of a Novel Isozyme of Tobacco BY-2 Chitinase Induced by a Fungal Elicitor

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Abstract

Three isozymes of chitinase were induced by a fungal elicitor in cultured tobacco BY-2 cells. The most acidic one, designated TBC-3, was described here. The molecular mass of TBC-3 was estimated to be 28.5 kDa. The N-terminal amino acid sequence of TBC-3 was analyzed and a homology search was performed. Fifteen amino acids at the N-terminus showed 60% homology to class II chitinases from other plants but not higher than 27% homology to already known chitinases from tobacco. Therefore, TBC-3 is thought to be a novel tobacco chitinase that may be classified into class II.

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In higher plants, chitinase is thought to be a typical pathogenesis related (PR) protein (Nishizawa *et al.*, 1999; Arakane *et al.*, 2000; Pozo *et al.*, 2002). Chitinase catalyzes the hydrolysis of chitin, a polysaccharide of β-1,4-linked *N*-acetylglucosamine, which is a major component of fungal cell wall. The accumulation of chitinase in plant cells is therefore regarded as defense response to the invasion of pathogenic fungi. However, the defense response does not seem to be so specific to a particular fungus species, but rather it often causes a simultaneous induction of many isozymes (Rothe *et al.*, 1998). Therefore, it is vital to analyze the molecular properties of respective isozymes and their functional relationships.

We discovered that in cultured tobacco BY-2 cells at least 3 isozymes of chitinase were induced by an elicitor derived from *Alternaria alternata*. We designated these 3 major isozymes TBC (tobacco BY-2 chitinase)-1, -2, and -3. In this paper, isolation and the molecular characterization of the most acidic isoform, TBC-3, was investigated and compared with other chitinase genes reported from tobacco (Neuhaus *et al.*, 1996; Brunner *et al.*, 1998).

Cultured tobacco cell line BY-2 (Saito *et al.*, 1996) derived from *Nicotiana tabacum* L. cv. Bright Yellow-2 was treated for 3 h with cell-free extract from autoclaved *A. alternata* culture. Then the cells were frozen in liquid nitrogen and homogenized. The homogenate was suspended in a 25 mM imidazole/HCl buffer solution (pH 6.8) containing 1 mM EDTA and 1 mM DTT and centrifuged (8,400*g*, 60 min) to obtain the crude protein fraction in the supernatant. The supernatant was heated at 50℃ for 30 min, and resulting denatured protein precipitates were removed by centrifugation (8,400*g*, 60 min). Then the pH of the supernatant was adjusted to 5.0 with acetic acid, and protein precipitates formed were removed by centrifugation (8,400*g*, 60 min). The chitinase activity appeared to remain in the supernatant.

The supernatant was mixed with colloidal chitin (Huynh *et al.*, 1992; Hon *et al.*, 1995) and stood still at 4℃ for 30 min until coprecipitation occurred. The precipitate was washed and suspended in 10 mM NaOH to release chitinase fraction. After centrifugation (4,100*g*, 4 min), the supernatant was collected and its pH was adjusted to 5.0. After centrifugation again (8,400*g*, 60 min), the supernatant was collected and put in a SP Sephadex C-50 column (3.1 cm x 12.4 cm). The elution was done with sodium acetate buffer solution (50 mM, pH 5.0). Non-adsorptive fraction was collected and

applied in a QAE Sephadex A-50 column. Chitinase activity was detectable in the fractions eluted with 50 mM sodium acetate buffer (pH 5.0) containing 0.2 M and 0.4 M NaCl. These two fractions were combined and concentrated by the ultrafiltration (cut off MW: 5,000 Da) and finally dissolved in sodium phosphate buffer solution (0.1 M, pH 7.0) containing 1.2 M $(NH_4)_2SO_4$. This solution was applied in a hydrophobic HPLC column (HIC PH-814, Showa Denko K.K., Tokyo) and eluted with a linear gradient of 1.2-0.3 M $(NH_4)_2SO_4$ in 0.1 M sodium phosphate buffer solution (pH 7.0). Three distinct peaks were obtained and named TBC-1, -2, and -3. Among them, TBC-3 was selected and further purified by anion exchange column (HiTrap Q HP 0.7 cm x 2.5 cm) (**Fig. 1A**).

Table 1 summarizes the total protein yield, the specific activity, and the recovery rate of chitinase at the respective purification steps. Chitinase activity of each fraction was determined by chitinase active staining method reported elsewhere (Trudel and Asselin, 1989). Protein content was determined by the Bradford method using γ-globlin as a standard (Bradford, 1976).

From 2.4 kg (wet matter) of BY-2 cells, 56 g of crude extract was obtained. The final pure fraction of TBC-3 was 1 μg. The recovery of enzyme activity was 0.13%. The purified TBC-3 fraction appeared as single band on SDS-PAGE and also on native-PAGE (**Fig. 1B, C**). These results indicated that TBC-3 was purified almost to homogeneity and its molecular weight was estimated to be 28.5 kDa.

After SDS-PAGE, TBC-3 band was transferred to a PVDF membrane and the band was analyzed using a protein sequencer. The N-terminal 15 amino acids of TBC-3 were identified and compared with those of other proteins by the BLAST search. The results were summarized in **Table 2**. The N-terminal sequence of TBC-3 (15 amino acid residues) showed 60-67% identity with class IV chitinases, and 60% identity with class II chitinases from *Sambucus nigra* (Coupe *et al.*, 1997) and *Phytolacca americana* (Yamagami *et al.*, 1998). On the other hand, the amino acid sequence of TBC-3 showed no marked identity to those of already reported tobacco chitinases in the BLAST search. In tobacco, multiple chitinase isozymes have been found and divided into six classes (I-VI) based on the amino acid sequence features (Neuhaus *et al.*,

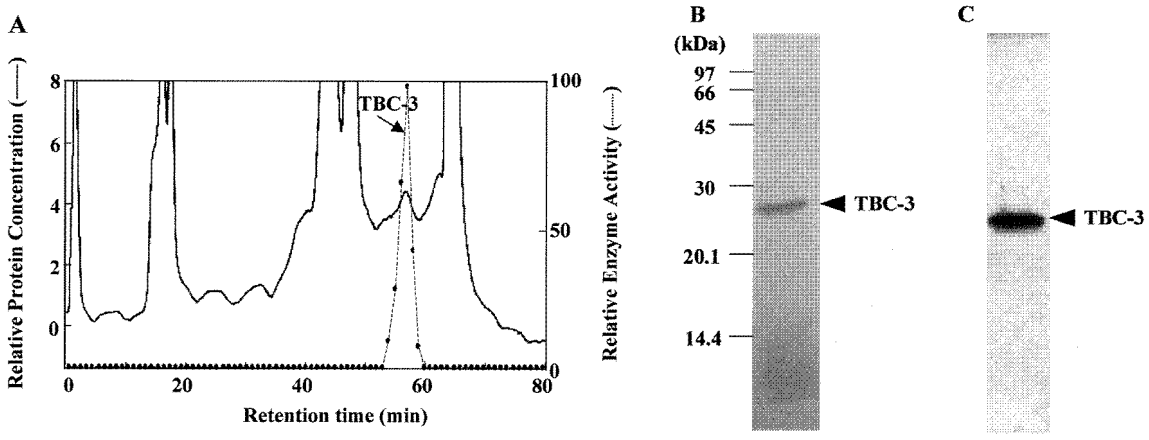

Fig. 1 Purification of TBC-3. (A) Chromatograms of the anion exchange HPLC. Relative protein concentration was measured at 280 nm. (B) Electrophoretic analysis of TBC-3 fraction obtained by the anion exchange HPLC. SDS-PAGE was performed and stained with silver staining. (C) Enzyme activity staining done on native-PAGE.

Table 1 Chitinase activity obtained at each step of purification

Purification step	Total protein (mg)	Specific activity	Recovery(%)
Crude extract	55916	1	100
Heating treatment	8721.3	6.17	96.2
Acetic acid treatment	3087.5	14.2	78.4
Colloidal chitin affinity	405.66	28.3	20.5
SP-Sephadex C-50	344.92	32.0	19.7
QAE-Sephadex A-50	79.131	119.5	16.9
Hydrophobic HPLC	0.470	4646.1	3.90
Anion-exchange HPLC	0.001	72260	0.13

Table 2 Alignment of amino acid sequences of TBC-3 and similar proteins using the BLAST search

	Left end residue No.		Right end residue No.	homology (%)
TBC-3	1	SNGGSVADVVSNAFF	15	-
Saccharum officinarum Chitinase (class IV)*	58	SGGGNVASVVTDAFF	72	67
Sorghum halepense Chitinase (class IV)*	58	SGGGNVASVVTDAFF	72	67
Phytolacca americana Chitinase (class II)	1	GGVSVANVVSQGFF	14	64
Vigna unguiculata Chitinase (class IV)*	43	SNNVNVADIVTDAFF	57	60
Daucus carota Chitinase (class IV)*	62	GNGVSVADIVTDDFF	76	60
Zea mays Chitinase (class IV)*	75	SGGANVANVVTDAFF	89	60
Beta vulgaris Chitinase (class IV)*	82	GGGSSVSDIVSQAFF	96	60
Sorghum bicolor Chitinase (class IV)*	59	SGGGNVGSVVTDAFF	73	60
Sorghum arundinaceum Chitinase (class IV)*	59	SGGGNVGSVVTDAFF	73	64
Sabucus nigra Chitinase (class II)*	37	VSGQSVADIVTDAFF	51	60

* These sequences were deduced from cDNA

1996; Brunner *et al.*, 1998). As the result of amino acid sequence alignments, 15 amino acid sequence of TBC-3 showed less than 27% sequence identity with tobacco chitinases in any region of their sequences. These results suggested that TBC-3 was a novel tobacco chitinase isozyme.

Chitinases analyzed so far are classified into 6 classes (classes I, II, III, IV, V and VI) according to their sequential properties (Neuhaus *et al.*, 1996; Henrissat, 1999). The classes I and IV chitinases consist of an N-terminal chitin binding domain. Class II chitinases lack the chitin binding domain and have amino acid sequence identity with the main structure of class IV. On the other hand, the sequences of classes III, V and VI chitinases lack the chitin binding domain and have no sequential similarity to the enzymes in class II. In the case of TBC-3, the chitin-binding domain was not exist at the N-terminal region. Therefore, TBC-3 may be classified into class II rather than class IV.

To understand the significance of the multiple isozymes that are induced simultaneously by a single elicitor, it is essential to investigate the functional roles of respective isozymes separately. The present study is the first step focused on the most acidic one. The developmental study is directed towards other isozymes in the same way.

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