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Modification of Tryptophan Residues of Plant Class III Chitinases Involved in Enzyme Activity

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Chemical modification of tulip bulb chitinase–1 (TBC–1) and pokeweed leaf chitinase (PLC–B) with N–bromosuccinimide (NBS) suggested the involvement of tryptophan residue (s) in the activity. In the case of TBC–1, at pH 4.0, about 1 mol of tryptophan residues out of 4 mol was oxidized with 5 mol of NBS per mol of TBC–1 and all 4 mol of tryptophan residues were oxidized with 15 mol of NBS. At pH 4.5, about 3 mol of tryptophan were finally oxidized although the oxidation rate of the first tryptophan residue was same as at pH 4.0. In both cases, the oxidation of 1 mol of tryptophan residues caused 85% activity loss. Analysis of the oxytryptophan–containing peptides afforded to identify that Trp172 and Trp255 were those which reacted first with NBS at pH 4.0.

When PLC–B was reacted with 16–folds molar of NBS at pH 4.0, all seven tryptophan residues were oxidized with a concomitant decrease of chitinase activity. Oxidized tryptophan residues were also determined as those at positions of 165 and 256 in PLC–B. From these results, it was inferred that the rapidly oxidized tryptophan residues in plant class III chhitinases were involved in their chitinase activity.

INTRODUCTION

Chitinases (EC 3.2.1.14) are enzymes catalyzing the hydrolysis of chitin, a β –1,4–linked homopolymer of N–acetylglucosamine (GlcNAc) and are considered to be self–defense–related proteins for protection against fungal pathogens (Schlumbaum *et al.*, 1986, Broekaert *et al.*, 1988, Stintzi *et al.*, 1993, Gooday, 1999). Five classes of plant chitinases have been proposed on the basis of the amino acid sequence (Henrissat and Bairoch, 1993). Class I chitinase consists of a chitin–binding domain and a catalytic domain linked with a linker peptide; class II chitinase shows sequence similarity to the class I chitinase but lacks the chitin–binding domain; class III chitinase shows no sequence similarity to class I nor II chitinase but has distant sequence similarity to bacterial and fungal chitinases; class IV chitinase shows sequence similarity to the class I chitinase but it is smaller due to four deletions; and class V chitinase shows distant sequence similarity to bacterial and fungal chitinases as class III chitinase does, but it is larger than class III chitinase.

We previously isolated two bulb chitinases, GBC–a from gladiolus (*Gladiolus gan-*

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davensis) (Yamagami *et al.*, 1997) and TBC-1 from tulip (*Tulipa bakeri*) (Yamagami *et al.*, 1998b), and characterized their enzymatic properties and amino acid sequences (Yamagami *et al.*, 1998a, Yamagami and Ishiguro, 1998) to find that bulb chitinase belongs to the class III chitinase. We later cloned cDNA of TBC-1, sequenced and expressed it in *E. coli* cells (Yamagami *et al.*, 2000). We also isolated two plant chitinases from the pokeweed leaves (PLC-A and -B) and characterized (Ohta *et al.*, 1995). From their amino acid sequences, PLC-A belongs to class II (Yamagami *et al.*, 1998c) and PLC-B to class III (Tanigawa *et al.*, 1995), respectively. However, there are major differences in properties between the bulb chitinases and so far reported plant class III chitinases: 1) bulb chitinase had no disulfide bond but plant class III had conserved three disulfide bonds (Tanigawa *et al.*, 1995); 2) bulb chitinases had no lysozyme activity while hevamine (Jeckel *et al.*, 1991) and PLC-B (Ohta *et al.*, 1995) had; and 3) a hydrolysis of $(GlcNAc)_5$ yielded $(GlcNAc)_2$ and $(GlcNAc)_3$ by bulb chitinases and GlcNAc and $(GlcNAc)_4$ by PLC-B (Ohta *et al.*, 1995).

In this paper, we have exploited a role of tryptophan residue of plant class III chitinase (TBC-1 and PLC-B) by chemical modification with N-bromosuccinimide. We have also determined their positions and discussed activity and structure relationship of class III chitinase.

MATERIALS AND METHODS

Materials.

TBC-1 was purified from tulip (*Tulipa bakeri*) bulbs as described (Yamagami *et al.*, 1998b), and PLC-B from the leaves of pokeweed (*Phytolacca americana*) as reported (Ohta *et al.*, 1995). N-Bromosuccinimide (NBS) was purchased from Wako Pure Chemicals and recrystallized from deionized water before use. Lysylendopeptidase and thermolysin were from Wako Pure Chemicals, and TLCK-chymotrypsin from Sigma Chemical Co.

Modification of Class III plant chitinases.

Modification of TBC-1 and PLC-B with NBS was done at 15 °C as reported previously for class I and II chitinases (RSC-a and -c) (Yamagami and Funatsu, 1996 and 1997), which was essentially based on the method described by Spande et al. (Spande *et al.*, 1966). Ten μl of 2.8 mM NBS solution in 0.1 M sodium acetate buffer of the desired pH was added to 2 ml of protein solution (each 0.4 mg of TBC-1 or PLC-B/ml of the same buffer) in a quartz cell with stirring. After 1 min, the UV absorption spectrum of the reaction mixture was recorded using a Hitachi U-3210 spectrophotometer and a decrease in absorbance at 280 nm was measured. This procedure was repeated until the decrease at 280 nm ceased. The number of the tryptophan residue oxidized was calculated from the decrease in the absorbance at 280 nm (Spande and Witkop, 1967).

Chitinase activity measurement

The routine assessment of chitinase activity was performed colorimetrically with glycolchitin as a substrate. Enzymatic reaction was monitored by an increase in the reducing sugar according to the method of Imoto and Yagishita (Imoto and Yagishita,

1971). Ten μl of the enzyme solution (1.6 pmol) was added to 500 μl of 0.2% (w/v) glycolchitin solution in 0.1 M sodium acetate buffer, pH 5.0. After incubation at 37 °C for 15 min, the reducing power of the mixture was measured with ferri–ferrocyanide reagent. One unit of activity was defined as the enzyme activity that produced 1 μmol of GlcNAc per min at 37 °C. Concentrations of TBC–1, NBS–oxidized TBC–1, PLC–B and NBS–oxidized PLC–B were determined by amino acid analysis after acid hydrolysis.

Identification of oxidized tryptophan residues of TBC–1 or PLC–B

To identify rapidly oxidized tryptophan residue (s) in TBC–1 or PLC–B, TBC–1 or PLC–B was separately reacted with NBS under the conditions where about 85% of activity was lost. The reaction mixtures were dialyzed and lyophilized. Reduction and carboxymethylation, proteolytic digestion, fractionation of peptides were carried out as described previously (Yamagami and Funatsu, 1996 and 1997). Determination of peptides by UV spectrum, amino acid analysis and N–terminal sequence analysis afforded the identification of oxidized tryptophan residue (s).

RESULTS

NBS–oxidation of TBC–1

When TBC–1 was treated with NBS at pH 4.0, as shown in Fig. 1, 3.9 of 4 tryptophan

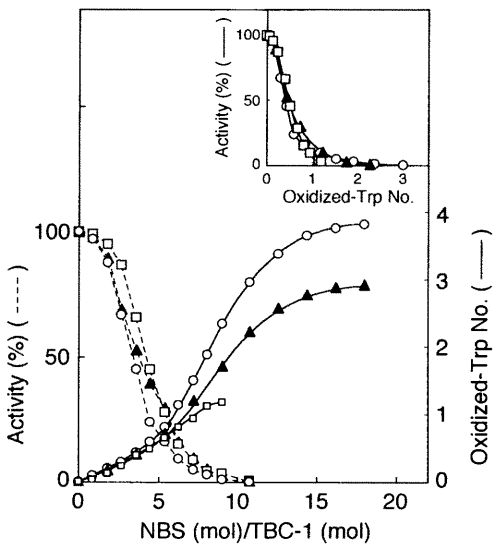

Fig. 1. Effects of NBS–oxidation of Tryptophan Residues on Chitinase Activity of TBC–1
TBC–1 was oxidized with NBS at pH 4.0 (○), pH 4.5 (▲) and pH 5.0 (□). The number of tryptophan residues oxidized was calculated spectrophotometrically, based on the molar absorbance coefficient of 5,500 $M^{-1} \cdot cm^{-1}$ at 280 nm (—). Chitinase activity was measured at pH 5.0 with glycolchitin as a substrate and the activity of native TBC–1 was taken to be 100 % (···). In the inset, activity vs oxidized tryptophan residues was replotted.

residues in TBC-1 were oxidized with 18 mol of NBS per mol of TBC-1. The reactivity of tryptophan residues toward NBS decreased with the increase in pH. At pHs 4.5 and 5.0, 3.0 and 1.2 tryptophan residues were oxidized with 18 and 9 mol of NBS, respectively. Chitinase activity of TBC-1 toward glycolchitin was lost by oxidation of one tryptophan residue in any cases (Fig. 1, inset). These results suggested that one tryptophan residue oxidized first was involved in the chitinase activity.

Identification of the tryptophan residue oxidized first in TBC-1

The tryptophan residue oxidized first was identified by using 1W-oxid-TBC-1 prepared by treatment with 8 mol of NBS at pH 5.0. 1W-oxid-TBC-1 was digested with lysylendopeptidase followed by chymotrypsin. Lyophilized digest was dissolved in 5 mM potassium phosphate buffer, pH 6.0, and separated into the soluble (Cs) and the pellet (Cp) fractions, and then the Cp fraction was further digested with thermolysin (CTs fraction). The resulting peptides were first separated by reverse-phase HPLC on a YMC-Gel C4 column using a phosphate acetonitrile elution system, and then purified by reverse-phase HPLC on YMC-Gel C18 column using a trifluoroacetic acid-acetonitrile elution system. From the UV-absorption spectra of the peptides, five and two peptides containing oxytryptophan residue were obtained from the Cs and CTs fractions, respectively. Amino acid compositions and N-terminal sequences of these peptides are shown in Table 1. From these results, it was seen that Trp172 and Trp251 of TBC-1 were equally oxidized by NBS.

Table 1. Amino acid compositions of peptides containing oxytryptophan residue

Peptide	Cs2	Cs3a	Cs4	Cs5	Cs6	CTs1a	CTs3
Asp				2.83 (3)	1.88 (2)		
Glu		2.12 (2)	2.21 (2)				1.13 (1)
Ser				1.35 (1)	1.26 (1)		
Gly							
His							
Arg							
Thr							
Ala		2.08 (2)	1.05 (1)	1.18 (1)	1.15 (1)	0.77 (1)	0.43 (0)
Pro							
Tyr							
Val	1.00 (1)			1.00 (1)	1.00 (1)	1.00 (1)	
Met							
Cys							
Ile							
Leu		0.68 (1)	0.95 (1)	0.60 (1)			0.94 (1)
Phe				0.62 (1)			
Trp	+(1)	+(1)	+ (1)	+ (1)	+(1)	+(1)	+(1)
Lys			1.00 (1)				1.00 (1)
Yield (n mol)	3	0.5	2.3	1.2	1.5	2.2	0.9
N-terminal	V	Q	Q	F	V	V	A
Corresponding sequence	VW	QALWEK	QALWEK	FVWSADDSL	VWSADD	VW	ALWEK
Position of Trp in TBC-1	251	172	172	251	251	251	172

NBS-oxidation of PLC-B

When PLC-B was treated with NBS, as shown in Fig. 2, tryptophan residue (s) were oxidized linearly with the increasing amount of NBS and ended up with all seven tryptophan residues oxidized with 16-folds molar NBS. The modified PLC-B in which 2 mol of 7 mol of tryptophan residues were oxidized, showed almost complete loss of chitinase activity at pH 7.0 and 80% loss at pH 3.5 while oxidation of the third tryptophan residue resulted in the complete loss of activity at pH 3.5 (Fig. 2, inset).

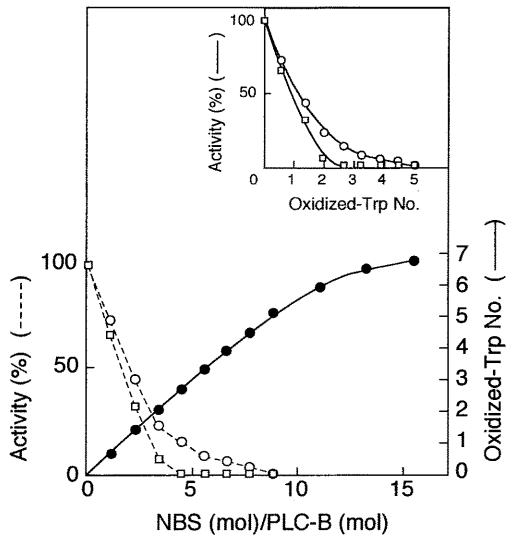

Fig. 2. Effects of NBS-oxidation of Tryptophan Residues on Chitinase Activity of PLC-B

PLC-B was oxidized with NBS at pH 4.0 (●) and the number of tryptophan residues oxidized was calculated spectrophotometrically, based on the molar absorbance coefficient of 5,500 $M^{-1} \cdot cm^{-1}$ at 280 nm. Chitinase activity was measured at pH 3.5 (○···○) and pH 7.0 (□···□) with glycolchitin as a substrate and the activity of native PLC-B was taken to be 100%. In the inset, the numbers of oxidized tryptophan residues are plotted against chitinase activity at pH 3.5 (○−○) and pH 7.0 (□−□).

Identification of rapidly reacted tryptophan residues in PLC-B

2W-oxi-PLC-B was reduced, carboxymethylated and subjected to proteolytic digestion with trypsin and thermolysin. Oxytryptophan-containing peptides were separated and analyzed as above demonstrating that oxidation by NBS occurred at Trp165 and Trp256 of PLC-B (Fig. 3).

DISCUSSION

Since Hayashi *et al.* first reported that Trp62 of lysozyme was oxidized with NBS with the concomitant 100% loss of lysozyme activity (Hayashi *et al.*, 1965), and Phillips et

T1 Tyr-Gly-Gly-Val-Met-Leu-Trp*-Asp-Arg (Tyr250~Arg258)
→ → →

T2 Tyr-Gly-Gly-Val-Met-Leu-Trp*-Asp-Arg (Tyr250~Arg258)
→ → →

T3 Asp-Ala-Trp*-Asn-Gly-Trp*-Thr-Ser-Gln-Ile-Pro-Ala-Gln-Lys
→ → →
(Asp200~Lys213)

T4 Val-Tyr-Leu-Thr-Ala-Ala-Pro-Gln-Cys-Pro-Phe-Pro-Asp-His-
→ → →
Trp*-Leu-Asn-Lys (Val151~Lys168)

T5 Asp-Ala-Trp*-Asn-Gln-Trp*-Thr-Ser-Gln-Ile-Pro-Ala-Gln-Lys
→ → →
(Asp200~Lys213)

Th1 Trp-Gly-Gly-Asn-Gly (Trp7~Gly11)
→ → →

Th2 Val-Trp (Val178~Trp179)
→ →

Th3 Leu-Trp*-Asp-Arg (Leu255~Arg258)
→ → →

Th4 Ile-Tyr-Trp-Gly-Gln-Asn-Gly-Gly-Glu-Gly-Thr (Ile5~Thr15)
→ → →

Th5 Leu-Trp*-Asp-Arg (Leu255~Arg258)
→ → →

Th6 Leu-Trp-Asp-Asn-Phe (Leu100~Phe104)
→ → →

Th7 Phe-Val-Trp (Phe177~Trp179)
→ → →

Fig. 3. Tryptophan-containing Peptides from RCM-2W-oxi-PLC-B
T and Th represent peptides purified from tryptic and thermolytic digests, respectively, and Trp* indicates the presence of oxytryptophan. Arrows under the sequence show that amino acids were determined by the Edman method. Description in the blankets are the corresponding positions in PLC-B.

al. later showed that Trp62 was really involved in substrate-binding in lysozyme by X-ray crystallographic study (Blake *et al.*, 1965), NBS oxidation became one of the prominent methods to exploit amino acid residues that consist an active site in glycosyl hydrolases. With regard to the active site of the enzymes that hydrolyze glycolchitin and N-acetyl-chitooligosaccharides, only lysozyme has been extensively investigated and little information concerning the active site of plant chitinases by chemical modification has been available. Yamagami and Funatsu examined the effects of chemical modifications on the activity of rye seed chitinase-c (RSC-c, class II) (Yamagami and Funatsu, 1996) and chitinase-a (RSC-a, class I) (Yamagami and Funatsu, 1997), and found that tryptophan and glutamic/aspartic acid residues might be involved in the activity. They, then, identified that Trp72 of RSC-c and Trp23 and Trp131 of RSC-a (Trp131 of RSC-a corresponds to Trp72 of RSC-c) are involved in substrate-binding.

With respect to class III chitinases, which have very distant similarity to bacterial and fungal chitinases but no sequence similarity to plant class I nor II chitinase, little information is available on the structure-activity relationship. TBC-1 has 15% similarity to hevamine and 14% to PLC-B, whereas PLC-B has 63% similarity to hevamine suggesting

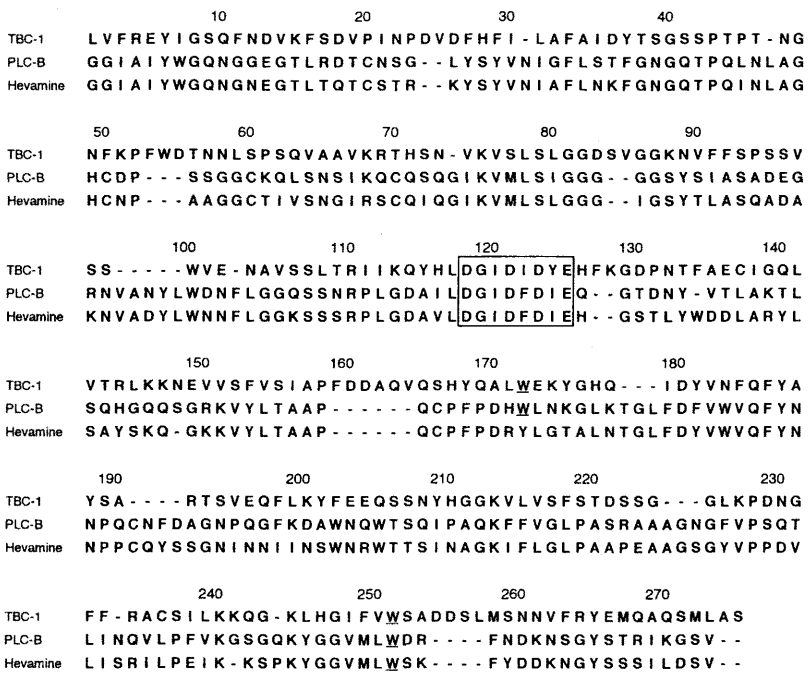

Fig. 4. Comparison of the Amino Acid Sequences of TBC–1, PLC–B and Hevamine.
Amino acid sequences of TBC-1 and PLC-B are aligned to compare with that of hevamine. Several gaps (-) have been inserted to give an optimal alignment of the proteins. Amino acids homologous to the sequence known as the catalytic site of hevamine are shown in the box. The underlined residues indicate tryptophan residues rapidly oxidized with NBS.

that PLC–B belongs to the same class as hevamine (Fig. 4). We, therefore, undertook this modification study to see whether tryptophan residue (s) be also involved in the activity of class Ⅲ chitinases. As a result, it was shown that oxidation reaction of tryptophan residue (s) in TBC–1 or PLC–B occurred as the amount of NBS increased, finally ended up all tryptophan residues oxidized as did in class I or class Ⅱ chitinase. All tryptophan residues in TBC–1 and PLC–B seem to be located on the surface or the accessible part of the enzyme molecules. Both in TBC–1 and PLC–B, chitinase activity was lost when one or two tryptophan residues, respectively, were oxidized. From these results, it was inferred that most rapidly oxidized tryptophan residue (s) are responsible for the activity as in the cases of RSC–a (class I) and RSC–c (class Ⅱ). Tryptophan residues that were rapidly oxidized were identified to be Trp172 and Trp251 of TBC–1 and Trp165 and Trp256 of PLC–B. As shown in Fig. 4, these tryptophan residues are located at the same position in the alignment of both chitinases and hevamine. Of these residues, Trp251 of TBC–1 (or Trp256 of PLC–B) is conserved in hevamine, whose three dimensional structure was solved. In hevamine, tyrosine is located at the same position of Trp172 of TBC–1 and Trp165 of PLC–B. It is very interesting to investigate how the tryptophan residue (s) is

involved in substrate-binding. Mutational analysis employing site-directed mutagenesis of these class III chitinases are being done.

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