Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
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Published online: 22 May 2014.

Biosci. Biotechnol. Biochem., **67** (2), 341–346, 2003

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Received August 16, 2002; Accepted October 6, 2002

We expressed chitinase-1 (TBC-1) from tulip bulbs (*Tulipa bakeri*) in *E. coli* cells and used site-directed mutagenesis to identify amino acid residues essential for catalytic activity. Mutations at Glu-125 and Trp-251 completely abolished enzyme activity, and activity decreased with mutations at Asp-123 and Trp-172 when glycolchitin was the substrate. Activity changed with the mutations of Trp-251 to one of several amino acids with side-chains of little hydrophobicity, suggesting that hydrophobic interaction of Trp-251 is important for the activity. Molecular dynamics (MD) simulation analysis with hevamine as the model compound showed that the distance between Asp-123 and Glu-125 was extended by mutation of Trp-251. Kinetic studies of Trp-251-mutated chitinases confirmed these various phenomena. The results suggested that Glu-125 and Trp-251 are essential for enzyme activity and that Trp-251 had a direct role in ligand binding.

Key words: chitinase; tulip bulb; *Tulipa bakeri*; site-directed mutagenesis

Plant chitinases (EC 3.2.1.14) catalyze the hydrolysis of β-1,4-linked homopolymers or oligomers of GlcNAc; the enzymes seem to be self-defense-related proteins for protection against fungal pathogens.[1–3] Class III chitinase has no sequence similarity to class I or II chitinases but is similar in sequence to bacterial and fungal chitinases.[4] We previously isolated two bulb chitinases, GBC-a from gladiolus (*Gladiolus gandavensis*)[5] and TBC-1 from tulip (*Tulipa bakeri*),[6] and characterized their enzymatic properties and amino acid sequences,[7,8] finding that bulb chitinases are in class III. There are, however, differences in properties between bulb chitinases and plant class III chitinases characterized so far. Bulb chitinases have no disulfide bonds, but plant class III chitinases have three conserved disulfide bonds.[9] Bulb chitinases do not lyse *Micrococcus luteus* cell walls but hevamine[10] and PLC-B[11] do lyse such cell walls. Hydrolysis of $(GlcNAc)_5$ yields mainly $(GlcNAc)_3$ and $(GlcNAc)_2$ by bulb chitinases, but PLC-B hydrolyzes $(GlcNAc)_5$ mainly to $(GlcNAc)_4$ and GlcNAc. We propose therefore that bulb chitinases should be classified into a new subclass, class IIIb, within class III.[5,6] Elsewhere, we cloned TBC-1 cDNA and overexpressed it in *E. coli*.[12] To understand the structural basis for activity of class IIIb chitinase, enzymatic characterization, molecular dynamics simulation, and kinetic analysis of mutated TBCs-1 prepared by site-directed mutagenesis at six amino acid residues were done.

Materials and Methods

Materials. TBC-1 was prepared from tulip (*Tulipa bakeri*) bulbs as described previously.[6] GlcNAc oligomers were prepared by the method of Rupley.[13] Oligonucleotides were purchased from Amersham Pharmacia Biotech. Restriction endonucleases and DNA-modifying enzymes were from Takara Shuzo or New England Biolabs. The plasmid vectors used were as follows: pBluescript II from Stratagene, pGEM-T Easy Vector from Promega, and pET-22b expression vector from Novagen. A Quikchange site-directed mutagenesis kit was purchased from Stratagene. A fluorescence-labeled primer cycle sequencing kit (Thermo Sequenase[tm]) with 7-deaza-dGTP was from Amersham. All other chemicals were of analytical grade for biochemical use.

Enzymatic activity and kinetics. The assay of chitinase activity and calculation of steady-state kinetic parameters were done colorimetrically with

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Abbreviations: TBC-1, chitinase-1 of tulip bulbs; GBC-a, chitinase-a from gladiolus bulbs; PLC-B, pokeweed leaf chitinase-B; rTBC-1, recombinant TBC-1; NBS, *N*-bromosuccinimide; MD, molecular dynamics

Table 1. Primers Used to Construct Recombinant DNA and TBC-1 Mutants

Primer	Sequence (5′→3′)[a]
S9A-5′	5′-CGTGAGTACATCGGCGCGCAGTTTAACGATGTC-3′
S9Q-5′	5′-CCGTGAGTACATCGGCCAGCAGTTTAACGATGTCAAG-3′
D121E-5′	5′-GTACCATCTTGATGGAATTGAAATCGACTACGAGCAC-3′
D121N-5′	5′-CAGTACCATCTTGATGGAATTAATATCGACTACGAGCAC-3′
D123E-5′	5′-GATGGAATTGATATCGAATACGAGCACTTCAAAGGTGAC-3′
D123N-5′	5′-GATGGAATTGATATCAACTACGAGCACTTCAAAGGTGAC-3′
E125Q-5′	5′-GAATTGATATCGACTACCAGCACTTCAAAGGTGACC-3′
E125D-5′	5′-GGAATTGATATCGACTACGATCACTTCAAAGGTGACC-3′
W172A-5′	5′-GCCACTACCAGGCGTTGGCGGAGAAGTACG-3′
W251F-5′	5′-GGGGAAGCTTCATGGCATCTTTGTTTTCTCGGCTGATG-3′
W251Y-5′	5′-CATCAGCCGAGTAAACAAAGATGCCATGAAGCTTCCCC-3′
W251L-5′	5′-GGAAGCTTCATGGCATCTTTGTTTTGTCGGCTGATG-3′
W251A-5′	5′-GCATCTTTGTTGCGTCGTCGGCTGATG-3′

[a] Underlined codons encode amino acid changes.

glycolchitin as the substrate. The enzymatic reaction was monitored in terms of changes in the amount of reducing sugars by the method of Imoto and Yagishita.[14] Ten microliters 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 reduction by 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 minute at 37°C. Concentrations of TBC-1, recombinant TBC-1 (rTBC-1), and mutant proteins were measured by amino acid analysis.

Site-directed mutagenesis. Mutants of TBC-1 were generated by site-directed mutagenesis of the appropriate regions of the wild-type chitinase gene, *tbc-1*, with the mutagenesis kit. The nomenclature of TBC-1 mutants and the oligonucleotide primers used for mutagenesis are shown in Table 1. Mutations were introduced into an amplified mature TBC-1 cDNA fragment that had been subcloned into the pGEM-T Easy Vector. After mutagenesis, the mature fragment was sequenced to verify the presence of the desired mutation, excised by digestion with *Nde*I and *Bam*HI, and ligated into an expression vector, pET-22b, that had been digested with the same enzymes. The mutant plasmids were used to transform *E. coli* BL21 (DE3). All mutant forms and rTBC-1 were obtained from culture media in soluble form and were purified to homogeneity by the method as reported previously.[12]

CD spectra of native TBC-1 and its mutant proteins. Far ultraviolet CD spectra of the chitinases were obtained with a JASCO J-720 spectropolarimeter. The spectra were measured at protein concentrations of 10 μM in 50 mM sodium phosphate buffer, pH 7.0, with a 0.1-cm cell path length at 25°C.

MD simulation. MD simulation of hevamine and three mutants, W255F, W255L, and W255Y, was done with the Discover 95.0 program (Accerlrys Inc.). The structure of each mutant was modeled on the basis of the X-ray crystal structure of hevamine (PDB code, 1HVQ). The parameter used was the consistent valence Forcefield (cvff). The parameters are provided for amino acids, water, and a variety of other functional groups. After Trp-255 of hevamine was replaced with the InsightII package (Accerlrys Inc.), the optimum dihedral angles of mutated side chains were searched for by avoidance of steric conflicts. Next, the following three steps were applied for energy minimization. First, the coordinates of the heavy atoms of proteins were tethered loosely (1,047 $kJmol^{-1} nm^{-1}$) and the positions of hydrogen atoms were optimized by the steepest-descents method and conjugate gradients method until the maximum derivative was less than 2.1 $kJmol^{-1} nm^{-1}$. Second, the coordinates of the main-chain atoms of proteins were tethered loosely (1,047 $kJmol^{-1} nm^{-1}$) and the positions of the amino acid residues were optimized. Third, the positions of all atoms were optimized without being tethered until the maximum derivative was less than 2.1 $kJmol^{-1} nm^{-1}$.

Next, the structure was filled by overlaying a 12.0-Å layer of single point charge (SPC) water with the SOAK procedure of the InsightII program. Before MD simulation was started, the following two steps were applied for energy minimization. First, all atoms of proteins were fixed and the positions of hydrogen atoms of water molecules were optimized by the steepest-descents method until the maximum derivative was less than 2.1 $kJmol^{-1} nm^{-1}$. Second, the all atoms of proteins were fixed while the positions of water molecules were optimized until the maximum derivative of water molecules was less than 2.1 $kJmol^{-1} nm^{-1}$.

The conformation optimized by the energy minimization was used as a starting conformation for NVT (N, number of particles, V, constant volume

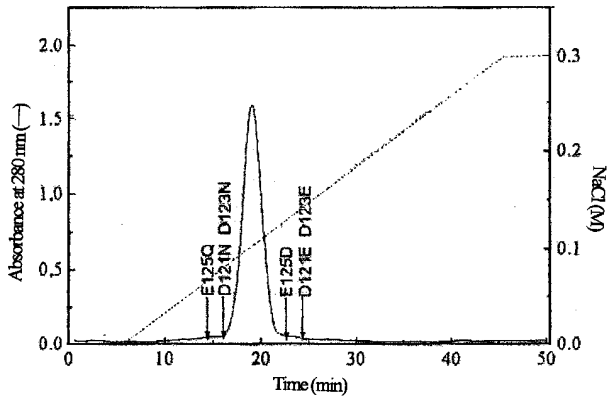

Fig. 1. Mono-Q Column Chromatography (pH 7.5) of Chitinase Fractions Obtained in Sephacryl S-200HR Gel Filtration.

The chitinase fractions obtained in Sephacryl S-200HR gel chromatography of chitinase mutant preparations expressed in *E. coli* were separately put on a Mono-Q column equilibrated with 10 mM Tris-HCl buffer (pH 7.5). Elution was done with a linear gradient of NaCl from zero to 0.3 M in the same buffer. The solid line indicates the elution profile of rTBC-1 (280 nm), and arrows indicate the positions of the peaks where each mutant protein was eluted. The names of the mutant proteins are given in Table 1.

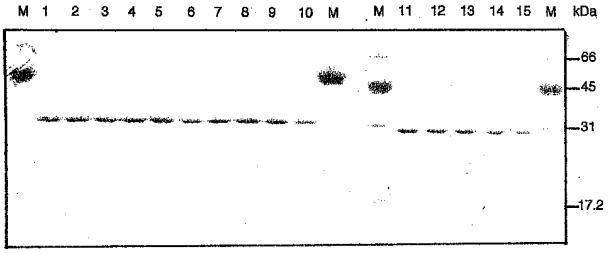

Fig. 2. SDS-PAGE Analysis of rTBC-1 and Its Mutant Proteins.

Homogeneity of the rTBC-1 and its mutant proteins was confirmed by SDS-PAGE as described by Laemli. Lanes 1-15 contained rTBC-1, S9A, S9Q, D121N, D121E, D123N, D123E, E125Q, E125D, W172A, rTBC-1, W251F, W251Y, W251L, and W251A, respectively, and M contained bovine serum albumin (66 kDa), ovalbumin (45 kDa), wild-type TBC-1 (31 kDa), and myoglobin (17.2 kDa).

and T, constant temperature) molecular dynamics at 300 K to generate possible stable conformations. The system was warmed to 300 K. After a 100-fs equilibration stage, simulation was continued at 300 K for 300 ps with a 1.0-fs time step. The simulations were done with the Verlet velocity algorithm[15] and the structures were stored in the computer every 1.0 ps.

Results

Construction and expression of TBC-1 mutants, and purification of mutant proteins

To identify the amino acid residues responsible for the chitinase activity of TBC-1, 13 TBC-1 mutants were engineered by site-directed mutagenesis and expressed in *E. coli* cells. The mutated amino acid residues were selected by comparison of the amino acid sequences of TBC and hevamine as well as the result of NBS-oxidation[16] of TBC-1. The resulting mutant proteins were independently purified from the soluble fraction of the sonic extracts by the procedures described for rTBC-1.[12] The elution profiles are shown in Fig. 1 as mutant E125Q, D123N, and D121N were eluted earlier than rTBC-1, which was eluted at the same position as wild TBC-1, but the mutant proteins (E125D, D121E and D123E) were eluted later from the Mono-Q column. Other mutant proteins were eluted at the same position as rTBC-1. Figure 2 shows SDS-PAGE profiles of a typical preparation of rTBC-1 and its mutant purified proteins. All mutant proteins gave CD spectra similar to the spectrum of TBC-1, suggesting that the expressed proteins were unaltered from the wild-type enzyme (not shown). All mutant proteins were overexpressed to the same extent as rTBC-1, the yields were close to 25 mg of protein from the soluble fraction in 1 l of the culture.

Characterization of mutant proteins

The specific activities toward glycolchitin of the enzymes were 106% for rTBC-1, 100% for S9A, 94.5% for S9Q, 32.7% for D121N, 64.8% for D121E, 8.5% for D123N, 1.0% for D123E, 0% for E125Q, and E125D, 72.5% for W172A, 89.8% for W251F, 50.0% for W251Y, 2.2% for W251L, and 0% for W251A, when the enzyme activity of the native TBC-1 was taken to be 100%. The Glu-125 of TBC-1, which corresponds to the catalytic residue Glu-127 in hevamine (only the three-dimensional structure of which has been solved as a class III chitinase), was changed to glutamine (E125Q). The Trp-251 of TBC-1, which corresponds to Trp-255 of hevamine being related to substrate binding, and which was rapidly oxidized with NBS at pH 4.0 in TBC-1,[16] was replaced with phenylalanine, tyrosine, leucine, and alanine, respectively. The proteins E125Q, E125D, W251A, and W251L had no chitinase activity. When Trp-251 was replaced with phenylalanine, tyrosine, leucine, and alanine, the chitinase activity decreased in proportion to the decrease in hydrophobicity of the side chain of the amino acid residues introduced. When Asp-123, which corresponds another catalytic carboxylate in hevamine, was changed to asparagine or glutamic acid, activity decreased much, but a change at Asp-121, which might be a catalytic carboxylate positioned near the catalytic center, to asparagine or glutamic acid caused a moderate loss of activity. A moderate effect was observed when Trp-172, which was one of the first rapidly reacting tryptophan residues with NBS, was changed to alanine. No activity loss was observed when Ser-9 was replaced with alanine or glutamine.

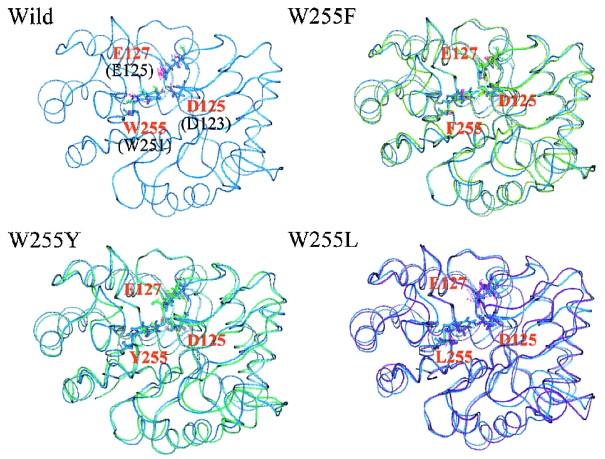

Fig. 3. Backbone Structures of Hevamine and Its Mutant Forms Seen by MD Simulation.

The main chain was drawn with a blue line for wild-type hevamine, a yellow line for W255F, a green line for W255Y, and a violet line for W255L. The structures of the mutant forms have been superimposed on that of the wild-type enzyme, and amino acid residues responsible for the active site of hevamine are shown in orange.

Table 2. Average Intermolecular Distances between Asp-125 and Glu-127 in the Active Sites of Wild-type and Three Mutant Proteins

$C_\alpha - C_\alpha$: Asp-125C_α – Glu-127C_α (Å)

	Wild-type	W255F	W255Y	W255L
Mean	6.40	6.67	6.48	6.70

Hydrogen bond: Asp-125OH – Glu-127O_2 (Å)

	Wild-type	W255F	W255Y	W255L
Mean	2.04	2.95	3.81	5.59

The intermolecular distances, Asp-125C_α-Glu-127C_α and Asp-125OH-Glu-127O_2, were calculated using the sampled structures by MD simulation.

Table 3. Kinetic Parameters of Wild-type and Tryptophan-mutated TBC-1

Enzyme	K_m (mg/ml)	k_{cat} (s^{-1})	k_{cat}/K_m (ml/s/mg)
Wild-type	0.72	0.76	1.06
W251F	0.86	0.54	0.63
W251Y	1.56	0.36	0.23
W251L	2.93	0.02	0.01

MD simulation

Asp-123, Glu-125, and Trp-251 of TBC-1 corresponded to Asp-125, Glu-127, and Trp-255 of hevamine in sequence alignment, respectively. The main-chain of the wild type and the main chains of the mutant proteins, W255F, W255Y, and W255L were quite similar (Fig. 3). On the other hand, the distance between Glu-127 and Asp-125, which may be in the catalytic site of chitinase, seemed to increase when Trp-255 was replaced with phenylalanine, tyrosine, or leucine. The distance calculated between α-carbons of two acidic amino acids (Asp-125 and Glu-127) showed that the main chain of hevamine changed little with a mutation of Trp-255 with phenylalanine, tyrosine, or leucine (Table 2). In fact, however, the distance between side chains of Asp-125 and Glu-127 was increased by mutation.

Kinetic parameters

The replacement of Trp-251 (Trp-255 of hevamine) with phenylalanine, tyrosine, or leucine increased K_m and decreased k_{cat}, respectively (Table 3).

Discussion

Of plant class III chitinases, hevamine from *Hevea brasiliensis* has been studied in most detail, including X-ray crystallographic analysis of hevamine-substrate ($(GlcNAc)_3$) complexes[17] and hevamine-inhibitor (allosamidin) complexes.[18] Hevamine has been classified as a family 18 glycosyl hydrolase, with a $(\beta/\alpha)_8$ barrel structure with an active center that catalyzes hydrolysis of β-1,4-glycosidic bonds by a substrate-assisted mechanism.[19] This active center consists of Glu-127 and Asp-125 for catalytic function and Trp-255 for ligand binding.[17]

Ser-9 in TBC-1 is an alanine in TBC-2 with a specific activity 1/50 that of TBC-1, and Ser-9 corresponds to a glutamine in hevamine, which residue seems to be hydrogen-bonded to a substrate, so Ser-9 was mutated here to alanine or glutamine. Because Asp-121, Asp-123, and Glu-125 were in conserved positions in class III chitinases, these residues were mutated to asparagine (D121N), glutamic acid (D121E), asparagine (D123N), glutamic acid (D123E), glutamine (E125Q) and aspartic acid (E125D). As the oxidation of Trp-172 and Trp-251 with NBS resulted in the complete loss of enzymatic activity,[16] and as the importance of the Trp residues in other plant chitinases was reported,[20] Trp-172 and Trp-251 were mutated. CD spectral determination of all mutant proteins suggested that the overall structures were not altered (not shown). The relative enzyme activities of the mutant forms showed that Asp-123 and Glu-125 were responsible for catalytic activity and that Trp-251 was needed for substrate binding. The chitinase activity of the Trp-251 mutant enzymes decreased in proportion to the decrease in hydrophobicity of the side chains of the amino acid residues that changed. Mutation of Ser-9 to alanine or glutamine had little effect on chitinase activity, suggesting that this residue was not essential; Ser-9 may not be involved in hydrogen bonding as it is in hevamine. The effects of a mutation of Asp-121 could be related to disturbance of the hydrophobic field around the corresponding Asp-123 of hevamine by Tyr-6. Because this field was not formed around one of the active site residue, chitinase activity was

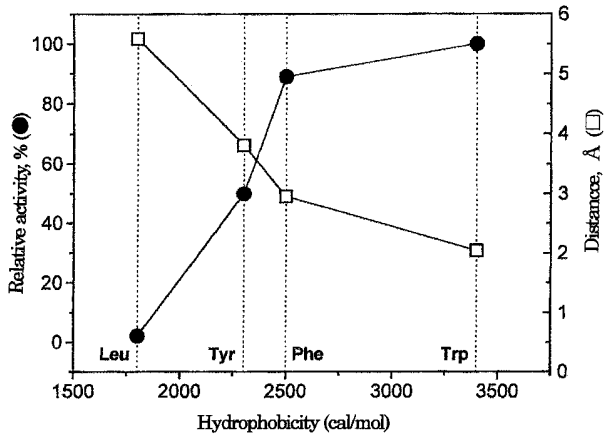

Fig. 4. Effects of Hydrophobicity of Side Chains at Residue 251 on Chitinase Activity of Mutant Enzymes.

The hydrophobicity (Δg_t, cal/mol) of amino acid residues introduced into TBC-1 (abscissa) was plotted against chitinase activity (left ordinate) and the simulated distance between side chains of Asp-123 and Glu-125 (right ordinate). The hydrophobicity of tryptophan, phenylalanine, tyrosine, and leucine are taken from Nozaki and Tanford.[21]

indirectly influenced when Asp-121 was mutated to asparagine.

No study on the role of the Trp-255 residue of hevamine (Trp-251 of TBC-1) by site-directed mutagenesis has appeared, although it has been reported that Trp-255 is important for the activity of hevamine. MD simulation of TBC-1 mutant forms suggested that the decrease of chitinase activity of W251F, W251Y, and W251L corresponded to the different hydrophobicity of their side chains, although the main chains of the mutated forms seemed unaltered. These results suggested that Trp-251 of TBC-1 interacted with a substrate *via* hydrophobic force. Another important result is that the distance between the side chains of Asp-123 and Glu-125 (Asp-125 and Glu-127 of hevamine), both residues of which are needed for activity, was greater when the side chains of amino acids introduced were more hydrophobic (Fig. 4). These results showed that Trp-251 indirectly governed the spatial positions of Asp-123 and Glu-125. Correct positioning would make possible interactions needed for catalysis. Kinetic parameters were compatible with our suggestions above. The K_m for W251L was fourfold that for the wild type, suggesting that affinity for binding decreased; k_{cat} decreased with mutation. Hydrophobic stacking, especially of aromatic rings, seemed therefore to be needed for substrate binding. At the same time, an amino acid residue with high hydrophobicity was needed for correct distance and orientation of Asp-123 and Glu-125 for catalytic activity.

Experiments on hydrolysis of $(GlcNAc)_6$ by TBC-1 and Trp-mutated TBC-1 and on the precise positioning of substrates in the active center of this enzyme by X-ray crystallographic analysis of (GlcNAc) oligomer-TBC-1 complex are being in progress and will in the future give more detail information on the binding site of substrate to the active site of TBC-1 and on a local structure assisting the enzyme catalysis from behind the catalytic center.

Acknowledgment

Computation time was provided by the Supercomputer Laboratory, Institute for Chemical Research, Kyoto University. This study was partially supported from the Research for the Future Program of the Japan Society for the Promotion of Science.

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