Mutagenesis of Trp^{54} and Trp^{203} Residues on *Fibrobacter Succinogenes* 1,3−1,4-β-D-Glucanase Significantly Affects Catalytic Activities of the Enzyme†

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ABSTRACT: The possible structural and catalytic functions of the nine tryptophan amino acid residues, including Trp^{54}, Trp^{105}, Trp^{112}, Trp^{141}, Trp^{148}, Trp^{165}, Trp^{186}, Trp^{198}, and Trp^{203} in *Fibrobacter succinogenes* 1,3−1,4-β-D-glucanase (Fsβ-glucanase), were characterized using site-directed mutagenesis, initial rate kinetics, fluorescence spectrometry, and structural modeling analysis. Kinetic studies showed that a 5−7-fold increase in K_m value for lichenan was observed for W141F, W141H, and W203R mutant Fsβ-glucanases, and approximately 72-, 56-, 30-, 29.5-, 4.9-, and 4.3-fold decreases in k_{cat} relative to that for the wild-type enzyme were observed for the W54F, W54Y, W141H, W203R, W141F, and W148F mutants, respectively. In contrast, W186F and W203F, unlike the other 12 mutants, exhibited a 1.4- and 4.2-fold increase in k_{cat}, respectively. W165F and W203R were the only two mutants that exhibited a 4−7-fold higher activity relative to the wild-type enzyme after they were incubated at pH 3.0 for 1 h. Fluorescence spectrometry indicated that all of the mutations on the nine tryptophan amino acid residues retained a folding similar to that of the wild-type enzyme. Structural modeling and kinetic studies suggest that Trp^{54}, Trp^{141}, Trp^{148}, and Trp^{203} play important roles in maintaining structural integrity in the substrate-binding cleft and the catalytic efficiency of the enzyme.

Enzymatic hydrolysis of polysaccharides is a critical process in the various metabolisms of plant, animal, and microbes. 1,3−1,4-β-D-Glucanase (1,3−1,4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.73; lichenase) specifically cleaves β-1,4-glucosidic bonds adjacent to β-1,3-linkages in mixed linkage β-glucans, such as lichenan or barley β-glucan, resulting in cellobiosyltriose and cellotriosyltetraose as major products (*1*). Genes encoding 1,3−1,4-β-D-glucanases have been isolated from bacteria, fungi, and higher plants (*2*−*5*). On the basis of amino acid sequence similarities, various bacterial and fungal enzymes are classified as members of family 16 of the glycosyl hydrolases and the plant enzymes as family 17 (*2*). The feasibility of using this enzyme for bioindustrial applications has promoted studies into the structure−function relationship of the enzyme in order to obtain biochemical and biostructural information that could improve the enzyme, with regard to industrial applications, e.g., increased specific activity, thermal stability, and tolerance to extreme pH environments.

In a previous study, a 1,3−1,4-β-D-glucanase was cloned from *Fibrobacter succinogenes* (*6*), a key bacterium that can effectively degrade plant fiber in the rumen of major livestock species. Various key residues involved in the catalysis of the enzyme were subsequently characterized by Chen et al. (*7*). Bacterial 1,3−1,4-β-D-glucanases are categorized as retaining glycosidases, which function through a double-displacement mechanism via formation and hydrolysis of a glycosyl−enzyme intermediate (*8*). Essential acidic amino acid residues act as general acid−base, and the catalytic nucleophiles in the enzyme catalysis (*1*, *9*). Glu^{56} and Glu^{60} in *Fibrobacter succinogenes* 1,3−1,4-β-D-glucanase (Fsβ-glucanase) were suggested to function as the essential catalytic acid/base residues in retaining glycosidase activity of the enzyme (*7*). These specific amino acid residues, acting as general acids or nucleophiles, are required for completing the catalytic reaction of the enzyme. Relative to other 1,3−1,4-β-D-glucanases, the Fsβ-glucanase enzyme consists of a primary amino acid sequence with a circular permutation, in which the two regions in the catalytic domain (region A to B in other enzymes) are in a reverse orientation, region B to A, in Fsβ-glucanase (*10*−*12*). Structural modeling of Fsβ-glucanase has shown a global three-dimensional structure similar to that of the *Bacillus* counterpart, but with differences in the connecting loops and random coils (*4*, *7*). These structural deviations among the various 1,3−1,4-β-D-glucanases may confer different functions or physiochemical characteristics upon the enzymes.

In the present study, alignment analysis of the primary sequence of the *Fibrobacter* 1,3−1,4-β-D-glucanase with other bacterial and fungal enzymes revealed that a number

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[1] Abbreviations: Fsβ-glucanase, *Fibrobacter succinogenes* 1,3−1,4-β-D-glucanase; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethanesufonyl fluoride; Ni−NTA, nickel-nitrilotriacetic acid; SDS−PAGE, SDS−polyacrylamide gel electrophoresis.

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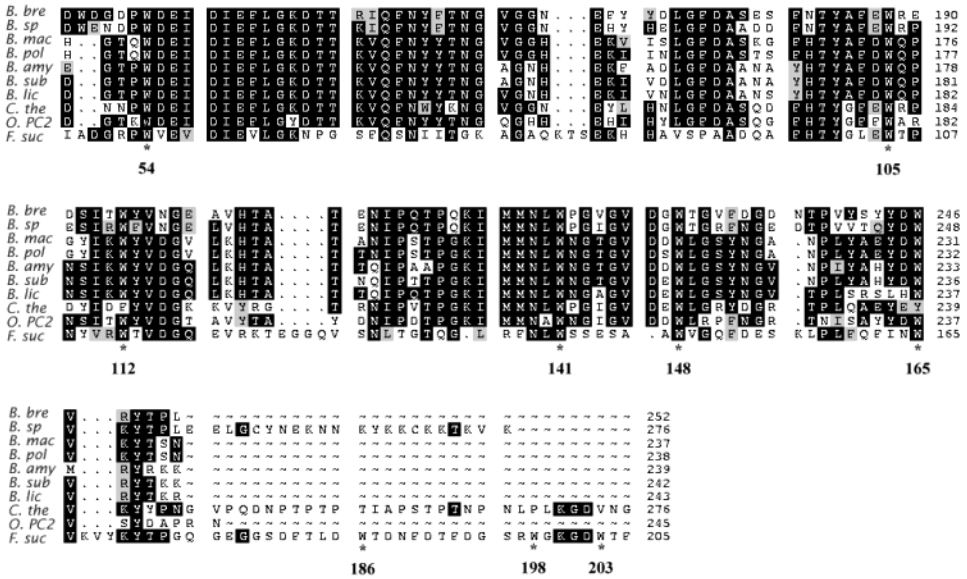

FIGURE 1: Amino acid sequence alignment of selected bacterial and fungal 1,3−1,4-β-D-glucanases. The primary sequence of *Fibrobacter succinogenes* 1,3−1,4-β-D-glucanase (*F. suc*) (*11*) is compared with those of *Bacillus brevis* (*B. bre*)(*23*), *Bacillus* sp (*B. sp*)(*28*), *Bacillus macerans* (*B. mac*)(*29*), *Bacillus polymyxa* (*B. pol*)(*30*), *Bacillus amyloliquefaciens* (*B. amy*)(*31*), *Bacillus subtilis* (*B. sub*)(*32*), *Bacillus licheniformis* (*B. lic*)(*33*), *Clostridium thermocellum* (*C. the*)(*10*), and *Orpinomyces* strain PC-2 (*O. PC2*)(*5*). The alignment was optimized by introducing gaps, denoted by dots; the residues with positional identity in at least three of these compared enzymes are shaded. *Asterisks* denote the candidate tryptophan residues for mutation in this study. The numbers on the right are the residue numbers of the last amino acid in each line.

of tryptophan (Trp) residues are strictly conserved within these enzymes (Figure 1). The specific roles for most of these tryptophan residues in 1,3−1,4-β-D-glucanases have not been characterized. In contrast, some of the conserved tryptophan residues in the cellulose-binding domains of various β-1,4-glucanases and xylanases have been studied and have been proposed to be involved in substrate-binding (*13*, *14*) or to form the foundation of a substrate binding subsite (*15*). To identify possible involvement of specific Trp residues in the catalytic activity or structural stability of the *F. succinogenes* 1,3−1,4-β-D-glucanase enzyme, we evaluated and characterized all of the nine Trp residues in the enzyme, namely, Trp^{54}, Trp^{105}, Trp^{112}, Trp^{141}, Trp^{148}, Trp^{165}, Trp^{186}, Trp^{198}, and Trp^{203}, using a number of experimental approaches. Our results obtained from site-directed mutagenesis, initial-rate kinetics, fluorescence spectroscopy, and structural modeling studies suggest that among the test Trp residues, Trp^{54} may play an important role in facilitating the catalytic function of Fsβ-glucanase. Surprisingly, mutation of Trp^{203} with either arginine or phenylalaine caused significant yet opposite effects on the catalysis of the enzyme.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Culture Media, and the Expression Vector. The plasmid pET26b(+) (Stratagene, CA) was used as the vector for expression of glucanase in *Escherichia coli* cells. *E. coli* XL1-Blue was used for plasmid propagation and plasmid isolation. *E. coli* BL21(DE3) was used for protein expression and purification. Transformed *E. coli* cells were grown in LB medium containing 30 μg/mL kanamycin at 33−37 °C.

Site-Directed Mutagenesis. The plasmid pFsNcE carrying the *F. succinogenes* 1,3−1,4-β-D-glucanase gene in the pET26b(+) vector as described (*7*) was used as the template for site-directed mutagenesis using a PCR-based method. Pairs of complementary mutagenic primers, as shown in Table 1 (primers corresponding to the sense strand sequence are shown), were designed for the mutations of Trp54 → Phe or Tyr, Trp105 → Phe or His, Trp112 → Phe or His, Trp141 → Phe or His, Trp148 → Phe, Trp165 → Phe or His, Trp186 → Phe, Trp198 → Phe or His, and Trp203 → Phe or Arg. The mutagenic PCR reaction mixture contained 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 0.1% Triton^R X-100, 0.1 mg/mL nuclease-free BSA, 10−15 ng of template DNA, 0.2 mM dNTPs, 0.25 μM each of the complementary primers, and 2.5 units of cloned Turbo *Pfu* DNA polymerase (Stratagene, La Jolla, CA).

The mutagenic PCR reactions were preceded with a thermocycling program of 2 min at 95 °C, 16 cycles of 1 min at 55 °C, 13 min at 68 °C, and 45 s at 95 °C on a Hybaid TouchDown thermal cycler. The resulting products were digested with 10 units of *Dpn* I at 37 °C for 1 h and subsequently transformed into *E. coli* XL-1 Blue competent cells by electroporation. The resulting cells were grown on LB agar plates containing 30 μg/mL kanamycin at 37 °C. Colonies were selected randomly from the plates and amplified at 37 °C for 16 h in 5 mL LB/kanamycin liquid culture. Plasmids were isolated from the culture using a QIAprep Spin Miniprep kit (Qiagene, Hilden, Germany), and mutations were confirmed by DNA sequencing. In these DNA constructs, a *pel* B leading peptide at the N terminus plus 19 extra amino acid residues, including a His_6 tag at the C terminus to facilitate protein purification, were included. The mutated plasmids were then transformed into *E. coli* BL21 (DE3) host cells for the overexpression of mutant enzymes.

Expression and Purification of Mutant Enzymes. One liter of *E. coli* cells harboring the plasmid encoded for the wild-type or mutagenized Fsβ-glucanase was grown at 33 °C with

Table 1: Oligonucleotide Primers for Site-Directed Mutagenesis

mutants	sequences of mutagenic primers
W54F	5′-GATGGAAGGCCC**TTC**GTAGAAGTGGATATTGAAG-3′
W54Y	5′-GATGGAAGGCCC**TAC**GTAGAAGTGGATATTGAAG-3′
W105F	5′-CTACGGTCTCGAA**TTC**ACTCCGAATTACGTCCG-3′
W105H	5′-CCTACGGTCTCGAA**CAC**ACTCCGAATTACGTCCG-3′
W112F	5′-CCGAATTACGTCCGC**TTC**ACTGTTGACGGTC-3′
W112H	5′-CCGAATTACGTCCGC**CAC**ACTGTTGACGGTCAG-3′
W141F	5′-CCGTTTTAACCTT**TTC**TCGTCTGAGAGTGCGGC-3′
W141H	5′-CTCCGTTTTAACCTT**CAC**TCGTCTGAGAGTGCGGCT-3′
W148F	5′-GAGAGTGCGGCT**TTC**GTTGGCCAGTTCG-3′
W165F	5′-CCGCTTTTCCAGTTCATCAAC**TTC**GTCAAGGTTTATAAG-3′
W165H	5′-CCAGTTCATCAAC**CAC**GTCAAGGTTTATAAGTATACGC-3′
W186F	5′-GACTTTACGCTTGAC**TTC**ACCGACAATTTTGAC-3′
W198F	5′-GATGGCTCCCGC**TTC**GGCAAGGGTGAC-3′
W198H	5′-GATGGCTCCCGC**CAC**GGCAAGGGTGACTG-3′
W203F	5′-CTGGGGCAAGGGTGAC**TTC**ACATTTGACGGT-3′
W203R	5′-TGGGGCAAGGGTGACC**GTA**CATTTGACGGT-3′

vigorous shaking to $OD_{600} = 0.4-0.6$. Protein expression was then induced with 0.4 mM isopropyl-β-D- thiogalactopyranoside (IPTG) for 14 to 16 h at 33 °C. The culture supernatant containing expressed proteins was harvested by centrifugation and then added with 1 mM phenylmethanesufonyl fluoride (PMSF) and 1 μg/mL leupeptin. Subsequently, the medium was concentrated to one-tenth of the original volume using a Pellicon Cassette concentrator (Millipore, Bedford, MA) with a 10 000 M_r cutoff membrane. The concentrated culture supernatant was then dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM PMSF and 1 μg/mL leupeptin (buffer A) and loaded onto a Sepharose Q FF (Pharmacia, Sweden) column pre-equilibrated with the same buffer. 1,3−1,4-β-D-Glucanase proteins, either wild-type or mutants, were collected from the eluants of the column using a 0−1 M NaCl salt gradient in buffer A. The collected eluants containing most of the Fsβ-glucanase enzyme were then concentrated and dialyzed at 4 °C overnight against 50 mM sodium phosphate (pH8.0) containing 1 mM PMSF and 1 μg/mL leupeptin. Imidazole and NaCl were added afterward to the dialyzed sample and adjusted to 10 mM and 0.3 M, respectively. The resulting sample solution was then applied to a 1.0 × 10 cm^2 nickel−nitrilotriacetic acid (Ni−NTA) affinity column pre-equilibrated with 50 mM sodium phosphate (pH 8.0) containing 10 mM imidazole, 0.3 M NaCl, 1 mM PMSF, and 1 μg/mL leupeptin. The column was washed with five column-volumes of 50 mM sodium phosphate (pH 8.0) containing 20 mM imidazole, followed with a linear gradient of 20−250 mM imidazole in the same buffer for the elution of proteins. Aliquots (2−5 μL) of selected fractions were employed for glucanase activity assay and SDS−polyacrylamide gel electrophoresis (SDS−PAGE). Fractions containing desired protein with at least 95% purity were then pooled and dialyzed at 4 °C overnight against 50 mM sodium phosphate buffer, pH 7.0. The protein concentration was quantified as described by Bradford (*16*) with bovine serum albumin (BSA) as the standard.

Zymogram Analysis. A zymogram was used to measure the enzymatic activity of wild-type and mutant forms of Fsβ-glucanase and was performed essentially according to a previously reported method (*17*) with minor modifications. A 12% SDS−polyacrylamide gel containing lichenan (1 mg/mL) and protein samples in sample buffer (*18*) pretreated at 90 °C for 10 min were prepared for zymogram analysis. After electrophoresis, the gel was rinsed twice with 20% 2-propanol in 50 mM sodium citrate buffer (pH 6.0) for 20 min to remove SDS, and then equilibrated in 50 mM sodium citrate buffer for 20 min. Before staining with Congo red solution (0.5 mg/mL), the gel was preincubated at 40 °C for 10 min. The protein bands with 1,3−1,4-β-D-glucanase activity were then visualized using the Congo red staining.

Kinetic Analysis. The enzymatic activities of purified wild-type and mutant 1,3−1,4-β-D-glucanases were measured by determining the rate of reducing sugar production from the hydrolysis of the substrate (lichenan). Reducing sugar was quantified using 3,5-dinitrosalicylic acid reagent (*19*) with glucose as the standard. A standard enzyme activity assay was performed in a 0.3 mL reaction mixture containing 0.6−8 mg/mL lichenan in 50 mM sodium citrate buffer (pH 6.0) or 50 mM sodium phosphate buffer (pH 7.0) by starting the reaction with an appropriate amount of enzyme. After optimal temperature (40−50 °C) incubation for 10 min, the reaction was terminated by the addition of 0.6 mL of a dinitrosalicylic acid solution (1% 3,5-dinitrosalicylic acid, 0.2% phenol, 1% NaOH) (*19*). The resulting solution was incubated at boiling temperature for 10 min, mixed with 150 μL of 40% sodium potassium tartrate, and cooled at room temperature for 15 min, and the absorbance was detected at 575 nm. One unit of enzyme activity was defined as the amount of enzyme required for releasing 1 μmol of reducing sugar (glucose equivalent). The specific activity is expressed in micromoles of glucose per minute per milligram of protein. Various amounts of the purified enzymes (0.24−82.7 μg/mL) were used in each kinetic assay reaction, depending on the enzymatic activity of the enzyme. Kinetic data were analyzed using the computer program ENZFITTER (BIOSOFT) and Enzyme Kinetics (SigmaPlot 2000, SPSS Inc.).

Fluorescence Spectrometry. The fluorescence emission spectra of native, 8 M urea-treated, and denatured−renatured wild-type and mutant forms of Fsβ-glucanase were taken on an AMICO−Bowman Series 2 spectrofluorimeter (Spectronic Instruments, Inc., NY) at 25 °C with a 1 × 1 cm^2 cuvette. Protein samples were diluted to 0.03 mg/mL in 50 mM sodium phosphate, pH 7.0 with or without the presence of 8 M urea. The urea-denatured samples were then renatured by dialysis against 50 mM sodium phosphate, pH 7.0 at 4 °C for 24 h. Excitation spectra were taken at 295 nm, and emission spectra were recorded from 330 to 430 nm, with a 4 nm slit for both spectra.

RESULTS

Purification and Biochemical Characterization of Wild-Type and Mutant Forms of Fsβ-Glucanase. The nine tryptophan residues in Fsβ-glucanase, namely, Trp^{54}, Trp^{105}, Trp^{112}, Trp^{141}, Trp^{148}, Trp^{165}, Trp^{186}, Trp^{198}, and Trp^{203} were all chosen for mutation. The result of amino acid sequence comparison (Figure 1) shows that residues Trp^{54}, Trp^{105}, Trp^{112}, Trp^{141}, Trp^{148}, and Trp^{165} of Fsβ-glucanase are all conserved among the compared bacterial and fungal enzymes, whereas Trp^{186}, Trp^{198}, and Trp^{203} are nonhomologous residues.

Wild-type and W54F, W54Y, W105F, W105H, W112F, W141F, W141H, W148F, W165F, W165H, W186F, W198F, W203F, and W203R mutant Fsβ-glucanases were effectively expressed and secreted from *E. coli* BL21(DE3) host cells upon induction at 33 °C with IPTG, as judged by SDS−PAGE and zymogram analysis (data not shown). Approximately 80% of the total Fsβ-glucanase expressed in *E. coli* host cells was secreted into the culture medium. Homogeneous preparation of the enzymes was performed using either a Q-Sepharose cation exchange column followed by separation with a nickel−nitrilotriacetic acid (Ni−NTA) affinity column or by one-step purification using a Ni−NTA affinity column. A greater than 95% purity of wild-type and mutant enzymes was obtained (data not shown). Similar protein expression profiles and yield levels were routinely obtained for wild-type and the 14 mutant forms of Fsβ-glucanase, as judged by SDS-PAGE analysis and Bradford assay. Zymogram analysis revealed that mutant enzymes showed a similar or reduced level of enzymatic activity as compared to the wild-type enzyme (data not shown). The purified wild-type or mutant enzymes collected from the culture supernatant were found no longer to contain the *pel*B leading peptide anymore, as determined by N-terminal amino acid sequence analysis.

Temperature and pH Effects on Enzymatic Activity of Fsβ-Glucanase. The effect of temperature and pH on the enzymatic activity of the purified wild-type and mutant enzymes was examined. The optimal temperature for the enzymatic activity of wild-type and mutant Fsβ-glucanases was observed between 45 and 50 °C. The temperature sensitivity of the wild-type and mutant Fsβ-glucanases was further investigated to characterize potential effects of introduced mutations. Figure 2 shows some results from this study. The replacement of amino acid residues in Trp^{54}, Trp^{112}, Trp^{141}, Trp^{148}, Trp^{186}, and Trp^{203} did not result in any significant change in thermal stability between 30 and 90 °C. The wild-type and all mutant enzymes, excluding W105F, W105H, W165H, and W198F, exhibited similar enzymatic activity (80−100% relative activity) when incubated for 10 min at 20−42 °C. Although more than 60% of the enzymatic activity (in comparison to wild-type) was detected at 47 °C, a dramatic loss of enzymatic activity occurred at temperatures higher than 50 °C (Figure 2A). In contrast, the W105F, W105H, W165H, and W198F variants exhibited a significant decrease in thermostability. Less than 50% enzymatic activity was detected for the four mutant enzymes after incubation at 42 °C for 10 min, and less than 10% of the original activity was detectable when the enzymes were treated at 47 °C (Figure 2A).

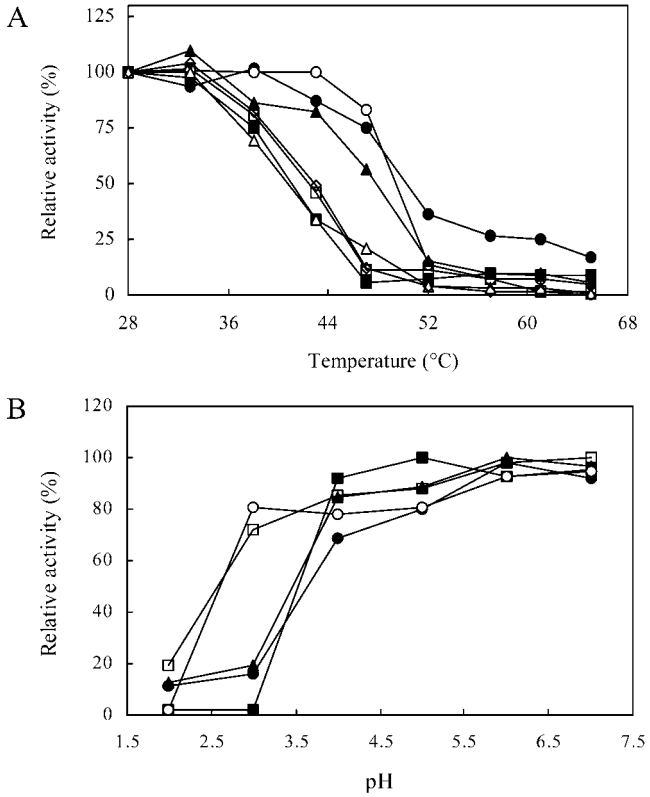

FIGURE 2: Effect of temperature (A) and pH (B) on enzymatic activity of wild-type and mutant *F. succinogenes* 1,3−1,4-β-D-glucanases. Panel A: The purified wild-type (●), W54Y (○), W105F (■), W105H (□), W165F (▲), W165H (△), and W198F (◇) enzymes at a protein concentration of 0.007−0.49 mg/mL were incubated for 10 min at 28, 33, 38, 43, 47, 52, 57, 61, and 65 °C, respectively, in 50 mM phosphate buffer, pH 7.0. Panel B: The purified wild-type (●), W165F (○), W198F (■), W203F (▲), and W203R (□) mutant enzymes at a protein concentration of 0.007−0.49 mg/mL were incubated for 1 h at pH 2, 3, 4, 5, 6, and 7, respectively. Various buffer solutions were prepared for this study, including 50 mM glycine−HCl (pH 2−3), 50 mM sodium citrate (pH 4−6), and 50 mM sodium phosphate (pH 7). Enzymatic activities were measured immediately at the optimal temperature and pH values with respect to the individual enzyme variants immediately after incubation, and relative activity is expressed as a percentage of the native (optimum) enzyme activity. Each assay was performed in triplicate.

We observed that most of the recombinant enzymes with a single mutated tryptophan residue exhibited a similar mode of pH dependency as that of the wild-type enzyme, with the optimal pH for enzymatic activity existing between a range of pH 5−8. The effects of pH on the stability of the wild-type and mutant enzymes were also compared. Most of the mutant enzymes, like the wild-type enzyme, were stable in pH 5−10, but became unstable when the pH was less than 5. The W165F and W203R mutants, however, exhibited 70−85% enzymatic activity even at pH 3. In comparison, the wild-type and all other test mutant enzymes lost most of their enzymatic activity (less than 18% of activity retained) at pH 3. The results obtained for the wild-type, W165F, W198F, W203F, and W203R enzymes are represented in Figure 2B.

Kinetic Analysis of Wild-Type and Mutant Forms of Fsβ-Glucanase. The effects of specific tryptophan-residue mutations on the catalytic ability and other functions in Fsβ-glucanase were evaluated using a comparative study of the kinetic properties of the wild-type and mutant enzymes with mainly lichenan as the substrate. The specific activity of the

Table 2: Kinetic Parameters of Wild-Type and Tryptophan Mutants of *F. Succinogenes* 1,3-1,4-β-D-Glucanase[a]

enzyme	specific activity (units/mg)	k_{cat} (s^{-1})	K_m, lichenan (mg/mL)	k_{cat}/K_m (mL s^{-1} mg^{-1})
wild-type	2065 ± 82	1296 ± 51	2.50 ± 0.09	518
W54F	29 ± 1.1	18 ± 0.7	1.84 ± 0.17	10
W54Y	37 ± 0.1	23 ± 0.0	1.34 ± 0.06	17
W105F	1621 ± 58	1018 ± 36	1.90 ± 0.23	536
W105H	1519 ± 2	953 ± 1	3.09 ± 0.01	308
W112F	916 ± 1.5	586 ± 1	2.34 ± 0.20	250
W141F	419 ± 3.3	263 ± 2	11.58 ± 0.12	23
W141H	69 ± 0.2	43 ± 0.1	12.84 ± 0.05	3
W148F	480 ± 30.3	302 ± 19	3.61 ± 0.45	84
W165F	1791 ± 34	1125 ± 21	2.43 ± 0.11	463
W165H	1572 ± 1.8	987 ± 1	1.81 ± 0.01	545
W186F	2941 ± 90	1846 ± 56	2.74 ± 0.1	674
W198F	1755 ± 4.4	1127 ± 3	3.64 ± 0.27	310
W203F	8726 ± 147	5476 ± 92	4.37 ± 0.14	1253
W203R	70 ± 0.1	44 ± 0.0	17.32 ± 0.03	2.5

[a] Reactions were performed with lichenan (8 mg/mL) as the substrate in 50 mM sodium citrate buffer (pH 6.0) or 50 mM phosphate buffer (pH 7.0) and at optimum temperatures of the individual enzymes as characterized in this study.

recombinant wild-type enzyme expressed in *E. coli* was 2065 ± 82 units/mg when assayed at 50 °C in 50 mM sodium phosphate, pH 7.0. The affinity for lichenan (K_m), turnover rate (k_{cat}), and catalytic efficiency (k_{cat}/K_m) of the wild-type enzyme were estimated to be 2.50 ± 0.09 mg/mL, 1296 ± 51 s^{-1}, and 518 s^{-1}(mg/mL)$^{-1}$, respectively (Table 2).

A dramatic change in specific activity was found for the W54F and W54Y mutants with a 72- and 56-fold decrease, respectively, whereas these mutations caused only a slight effect on substrate affinity as compared to the wild-type enzyme (Table 2). The catalytic efficiency, k_{cat}/K_m, for the W54F and W54Y enzymes was approximately 52- and 30-fold, respectively, lower than that of the wild-type enzyme. Only marginal changes (1.2−2.2-fold decrease) in specific activity or binding affinity for lichenan were found in the substitution of Trp^{105}, Trp^{112}, Trp^{165}, and Trp^{198} residues with phenylalanine or histidine (Table 2). The W148F mutation resulted in a 6-fold decrease in catalytic efficiency. The W141F mutant exhibited a 4.3-fold decrease in k_{cat}; however, when Trp^{141} was mutated to histidine, the turnover rate for the resulting recombinant mutant enzyme (W141H) was 30-fold lower than that for the wild-type enzyme. A lower binding affinity for lichenan was also found associated with the mutated Trp^{141} residue of Fsβ-glucanase, with an approximate 5-fold decrease in K_m value for the W141F and W141H mutants as compared to that for the wild-type enzyme. The catalytic efficiencies of W141F and W141H were thus significantly reduced by 22.5- and 173-fold, respectively, when compared to that of the wild-type enzyme.

The mutation on the Trp^{203} residue of Fsβ-glucanase resulted in interesting changes to the kinetic properties of the enzyme. W203F showed a 4.2-fold increase in specific activity as opposed to a 29.5-fold decrease observed for W203R, relative to the wild-type enzyme. W203R also exhibited a more pronounced (6.9-fold) decrease in binding affinity for lichenan than that observed for W203F (1.7-fold). W203F and W203R thus exhibited approximately a 2.4-fold more and 207-fold less catalytic efficiency, respectively, as compared to the wild-type enzyme.

In addition to lichenan, the substrate specificity of the wild-type and mutant enzymes was also examined using various substrates, including laminarine, carboxymethyl cellulose, and xylan. No detectable activity was observed for the wild-type enzyme or for any of the fourteen tested mutant enzymes using these substrates in activity assays.

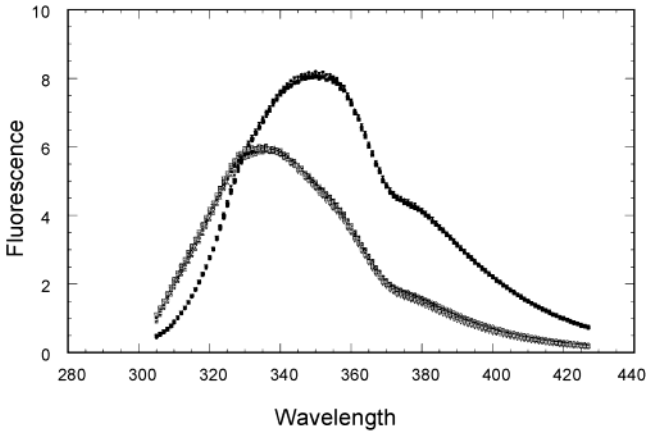

FIGURE 3: Fluorescence emission spectra of wild-type and mutant *F. succinogenes* 1,3−1,4-β-D-glucanases. The fluorescence emission spectra of native, 8M urea-treated, and the denatured−renatured Fsβ-glucanase enzymes were quantitatively assayed upon excitation at 295 nm using a spectrofluorimeter. Each enzyme was measured at a concentration of 0.03 mg/mL in 50 mM phosphate buffer (pH 7.5), or in 8 M urea−phosphate buffer. The fluorescence emission spectra for the native form of wild-type (○), W54F (□), W141H (◇), W198F (△), and W203F (▽), denatured form of wild-type (●), W54F (■), W141H (◆), W198F (▲), and W203F (▼), and the denatured−renatured wild-type (×), W54F (+), W141H (square with x in the center), W198F (−), W203F (⊡) enzymes are represented.

Fluorescence Spectrometric Analysis of Wild-Type and Mutant Forms of Fsβ-Glucanase. The structural integrity of the native, urea-denatured, and denatured−renatured wild-type and mutant forms of the Fsβ-glucanase were analyzed using fluorescence spectrometric analysis. Figure 3 shows the superimposed emission spectra of the native form of the wild-type and mutant enzymes (W54F, W141F, W198F, and W203F) with a maximum emission peak at 335 nm. After unfolding in 8 M urea, the fluorescence spectra of the enzymes all showed a bathochromic (red) shift with a maximum emission peak at 349 nm and a slight shoulder at 376 nm (Figure 3). All of the urea-denatured enzymes were then dialyzed against phosphate buffer at 4 °C to remove the denaturant. The resulting renatured samples were analyzed for their fluorescence emission spectra using identical experimental conditions that were employed for the native and urea-denatured protein samples. The data in Figure 3 show that after refolding, the maximum emission spectra of the renatured-wild-type and W54F, W141F, W198F, and W203F mutant enzymes all shifted back to 335 nm and superimposed well with each other. Similar results were obtained for other tryptophan mutant Fsβ-glucanase (data not shown). Enzymatic activities of the native and renatured wild-type and tryptophan mutant Fsβ-glucanases were also determined in parallel. Approximately 92−100% activity, relative to the native enzyme, was recovered for the renatured enzymes. These results suggest that a single amino acid substitution of any one of the nine tryptophan residues in the Fsβ-glucanase does not cause a global conformational change or aberrant folding of the enzyme. Therefore, the observed differences in the kinetic properties between the wild-type and mutant forms of the Fsβ-glucanase enzyme

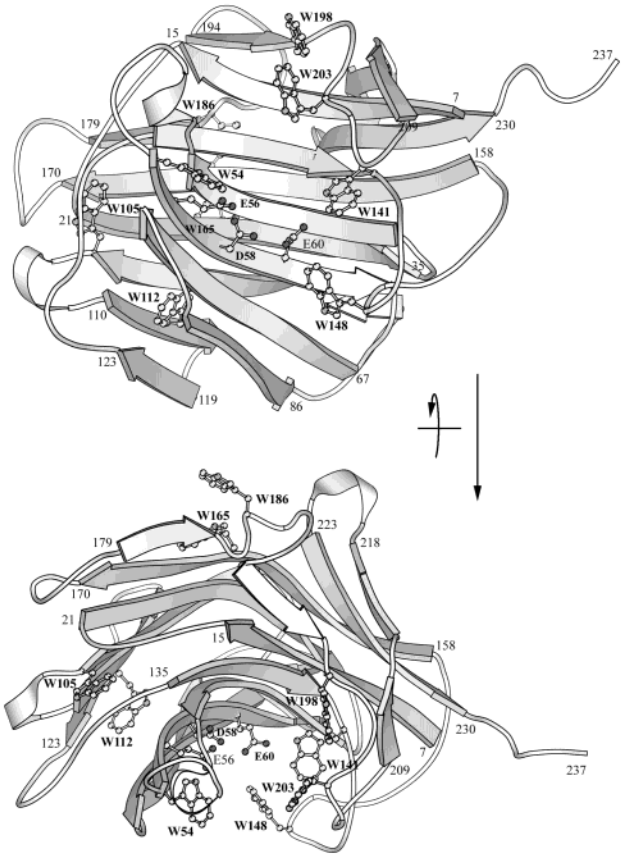

FIGURE 4: Ribbon diagram of the *F. succinogenes* 1,3−1,4-β-D-glucanase structure model. Two views of the model are shown here with the second view rotated approximately 90° to the first one. β-Strands are drawn as arrows, and residues Trp^{54}, Trp^{105}, Trp^{112}, Trp^{141}, Trp^{148}, Trp^{165}, Trp^{186}, Trp^{198}, and Trp^{203} that were subjected to mutational analysis are displayed as ball-and-stick models. The positions of the catalytic center Trp^{54}, Glu^{56}, Asp^{58}, and Glu^{60} are also indicated. The protein structure of cpA16M-59 (*19*) was used as a template for generating the Fsβ-glucanase model. This figure was generated using the program MOLSCRIPT (*34*).

apparently are not due to the disruption of the structural integrity of the enzyme.

Structural Modeling. F. succinogenes 1,3−1,4-β-D-Glucanase is the only native, circularly permutated protein reported in the family 16 endoglucanases. The primary amino acid sequence of the enzyme is oriented in a reverse order of domain B followed by domain A when compared to the 1,3−1,4-β-D-glucanases from different origins (*10*−*12*). In the present study, we built a structural model of Fsβ-glucanase based on the three-dimensional structure of a de novo circularly permutated variant, cpA16M-59 (*20*), using the Modeler 4 and PROCHECK programs (*21*, *22*). The modeled *Fibrobacter* glucanase structure is composed mainly of two antiparallel β-sheets with seven and eight strands, arranged on top of each other to form a compact, sandwich-like structure (Figure 4). Homologous structural modeling of Fsβ-glucanase suggested that Trp^{54}, Trp^{141}, and Trp^{148} are located at the cleft on the concave side of the protein molecule, which is the putative substrate-binding site and catalytic center, as was previously also suggested by the structural analysis of an enzyme−inhibitor complex of the *Bacillus* 1,3−1,4-β-D-glucanase (*4*). Trp^{198} and Trp^{203} are likely located at the loop that partially covers one of the terminals of the putative substrate-binding tunnel. Structural modeling analysis suggests that Trp^{105}, Trp^{112}, Trp^{165}, and Trp^{186} are localized in the β-sheet of the convex side of the protein molecule and away from the putative substrate-binding cleft.

DISCUSSION

A high degree of amino acid sequence homology (50−70%) has been observed among the various 1,3−1,4-β-D-glucanases isolated from *Bacilli*, *Clostridium*, and fungus *Orpinomyces* (*5*, *10*, *23*). The *Fibrobacter succinogenes* 1,3−1,4-β-D-glucanase (Fsβ-glucanase) is also classified as a member of family 16 endoglucanases; however, it only shows approximately 30% amino acid sequence identity with other bacterial and fungal glucanases. Amino acid sequence alignments have revealed several conserved tryptophan residues in the family 16 glucanases (Figure 1). On the basis of our structural model of Fsβ-glucanase (Figure 4), Trp^{54}, Trp^{141}, Trp^{148}, Trp^{198}, and Trp^{203} residues are located on the concave side of the enzyme, which is the proposed substrate-binding site. Trp^{165} and Trp^{186} are located on the convex side opposite from the substrate-binding side, while Trp^{105} and Trp^{112} are located between the β-sheets within the protein core. By employing a site-directed mutagenesis strategy, we explored the potential functional and structural significance of all nine of the tryptophan residues of Fsβ-glucanase.

In this study, we mutated the nine tryptophan residues to phenylalanine, tyrosine, or histidine, and varied phenotypes in their kinetic properties were observed (Table 1). The k_{cat}/K_m values for the Trp mutants located on the convex side or between the β-sheets in the protein, i.e., W105F, W105H, W112F, W165F, W165H, and W186F, were very similar to that of the wild-type Fsβ-glucanase, resulting in marginal effects on the catalytic efficiency of the enzyme. These results indicate that Trp^{105}, Trp^{112}, Trp^{165}, and Trp^{186} do not play important roles in substrate binding or in enzyme catalysis. This is consistent with what was predicted from the structural model (Figure 4) in that Trp^{105}, Trp^{112}, Trp^{165}, and Trp^{186} are located in regions far from the putative catalytic center on the enzyme's concave side. The slightly reduced (or increased) catalytic efficiency of these mutants is likely due to minor local structural arrangements of the protein. Surprisingly, mutating Trp^{165} increased the stability of the enzyme to acidic (pH 3) conditions. This result was not predicted from the structural model. One possible explanation is that the replacement of Trp with Phe changes the interactions surrounding residue 165 in the protein, resulting in a local rearrangement of the β-sheet of the enzyme's convex side that has a favorable effect on enzyme stabilization in an acidic environment.

Directed mutagenesis performed on Trp residues located on the concave side of the enzyme had significant effects upon the turnover rate, substrate-binding affinity, and catalytic efficiency of Fsβ-glucanase. Substitution of Trp^{54}, Trp^{141}, and Trp^{148} resulted in a 6.2−173-fold decrease in the catalytic efficiency of Fsβ-glucanase. The crystal structure of *Bacillus macerans* 1,3−1,4-β-D-glucanase (*25*) possesses a catalytic site comprised of residues Trp^{101}, Glu^{103}, Asp^{105}, and Glu^{107}, which correspond to the Trp^{54}, Glu^{56}, Asp^{58}, and Glu^{60} residues found in Fsβ-glucanase. In our structural model of Fsβ-glucanase, Trp^{54} hydrogen bonds to Glu^{56}

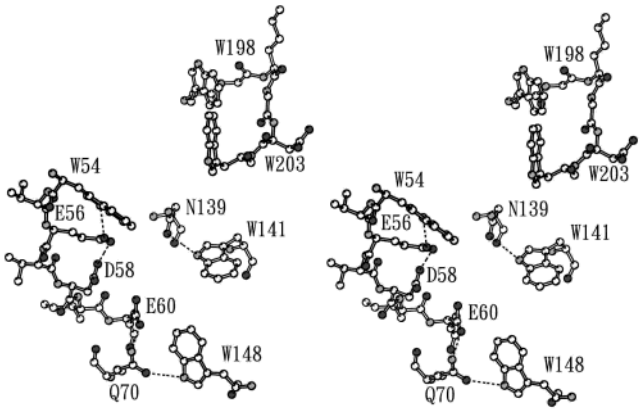

FIGURE 5: Stereoviews of the candidate tryptophan residues for the mutational study in *F. succinogenes* 1,3−1,4-β-D-glucanase. Amino acid residues are drawn as ball-and-stick diagrams. The hydrogen bonds between the side chains of residues Trp^{54}, Trp^{141}, and Trp^{148}, and the neighboring residues in the Fsβ-glucanase structure model are displayed as dashed lines.

(Figure 5), previously suggested as a neucleophile in Fsβ-glucanase catalysis (*7*). It is likely that any hydrogen bond between the Nϵ1 of Trp^{54} and the Oϵ2 of Glu^{56} would be abolished upon substitution of Trp^{54} with either phenylalanine or tyrosine, resulting in a decrease in the enzyme's catalytic ability. Therefore, our study suggests that hydrogen bonding between Trp^{54} and Glu^{56} could be structurally important for the enzymatic activity of Fsβ-glucanase.

Substitution of Trp^{141} effectively reduced both the affinity for the substrate and the specific activity of the enzyme (Table 2). Mutation of Trp^{148} also had a pronounced deleterious effect on the specific activity of Fsβ-glucanase. Trp^{141} and Trp^{148} are proposed to locate in a loop crossing the β-strand in which Glu^{56} and Glu^{60} reside, with their side chains pointing toward the center of the cleft and facing those of Trp^{54}, Glu^{56}, and Asp^{58} on the opposite side of the cleft (Figure 4). Trp^{141} hydrogen bonds to the carboxyl group of Asn^{139} (OδD1 atom) in our structural model of Fsβ-glucanase. This Trp residue is highly conserved in the 1,3−1,4-β-D-glucanases from bacterial origins. Trp^{126} in cpA16M-59 glucanase and Trp^{184} in H(A16-M) glucanase from *Bacilli*, corresponding to the Trp^{141} of Fsβ-glucanase, hydrogen bond to conserved Asn residues (Asn^{124} and Asn^{182}, respectively) in their enzymes (*20*, *4*). When further compared with *B. licheniformis* 1,3−1,4-β-D-glucanase (*26*), the Trp^{213} residue of the *Bacillus* enzyme, which corresponds to the Trp^{141} of the *Fibrobacter* enzyme, has been proposed to play an important role in maintaining the structural integrity of the substrate-binding cleft and in creating a hydrophobic environment that favors substrate binding (*27*). This indicates that the Trp^{141} residue in Fsβ-glucanase may play an important structural role in stabilizing the enzyme to facilitate proper substrate binding or catalytic efficiency.

The W148F mutant shows a 6.3-fold decrease in its k_{cat}/K_m value relative to the wild-type enzyme. In our structural model, Trp^{148} forms a hydrogen bond with the oxygen atom in the amide side chain of Gln^{70} (Oϵ1 atom). The amide nitrogen atom of Gln^{70} (Nϵ2) hydrogen bonds to the carboxyl group of Glu^{60} (Oϵ1 atom). Glu^{60} is likely the general acid in the catalytic reaction (*7*), and Trp^{148} is likely the third layer residue around the active site. This indicates that disruption of a hydrogen bond network around the active site environment could result in the deterioration of enzymatic activity in Fsβ-glucanase.

According to our structural model of Fsβ-glucanase, Trp^{198} and Trp^{203} are positioned on a loop, which is located at the very end of the putative substrate-binding tunnel. The replacement of Trp^{198} with phenylalanine did not have a significant effect on k_{cat} and K_m values. Surprisingly, the mutation of Trp^{203} with either phyenlalanine or arginine had markedly different effects on enzymatic activity (Table 2) and led to the W203R enzyme being more stable at acidic pH (pH 3−5) than the wild-type or W203F mutant enzymes. Replacement of residue 203 with phenylalanine, an amino acid residue with a less bulky side chain than Trp, effectively facilitated the enzyme's catalytic efficiency, whereas introduction of a positive charge group such as arginine in residue 203 apparently interrupts the catalytic function of Fsβ-glucanase. W203F shows a 4.2- and 1.7-fold increase in the specific activity and K_m for lichenan, respectively, whereas the W203R enzyme exhibited a 29.5-fold decrease in specific activity and 6.9-fold increase in the K_m value when compared with those of the wild-type enzyme. These results indicate that maintaining an aromatic side chain in residue 203 is crucial for enzymatic activity and also for binding with the substrate.

In summary, the present study systematically characterized the potential catalytic and structural role(s) of the nine tryptophan residues in *Fibrobacter* 1,3−1,4-β-D-glucanase and, for the first time, identified several tryptophan residues involved in the catalysis of Fsβ-glucanase. These findings correlating structure and function may be applicable to other forms of 1,3−1,4-β-D-glucanases, since most of these residues are highly conserved among the various 1,3−1,4-β-D-glucanase enzymes isolated from different organisms, including bacteria and fungi. Trp^{54}, Trp^{141}, and Trp^{203} apparently play crucial roles regarding the catalytic function of the enzyme. The substitution of Trp^{203} and Trp^{186} with phenylalanine resulted in increases in catalytic efficiency (k_{cat}/K_m) without disturbing other properties examined. W165F exhibited a minor decrease in its specific activity compared to the wild-type enzyme but was found to be an acidic pH (pH 3−5) tolerable enzyme. The potential application of W203F, W186F, and W165F mutant Fsβ-glucanases in bioindustry thus warrant further evaluation. Studies of the three-dimensional structure of the enzyme using an X-ray crystallography approach are currently being pursued in our laboratory to address the three-dimensional interactions and the functional or structural roles of specific amino acid residues with regards to potential biotechnological applications.

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