Directed Mutagenesis of Specific Active Site Residues on Fibrobacter succinogenes 1,3–1,4-β-D-Glucanase Significantly Affects Catalysis and Enzyme Structural Stability*

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The functional and structural significance of amino acid residues Met50, Glu56, Asp58, Glu60, and Gly63 of Fibrobacter succinogenes 1,3–1,4-β-D-glucanase was explored by the approach of site-directed mutagenesis, initial rate kinetics, fluorescence spectroscopy, and CD spectrometry. Glu56, Asp58, Glu60, and Gly63 residues are conserved among known primary sequences of the bacterial and fungal enzymes. Kinetic analyses revealed that 240-, 540-, 570-, and 880-fold decreases in $k_{cat}$ were observed for the E56D, E60D, D58N, and D58E mutant enzymes, respectively, with a similar substrate affinity relative to the wild type enzyme. In contrast, no detectable enzymatic activity was observed for the E56A, E56Q, E58A, E60A, and E60Q mutants. These results indicated that the carboxyl side chain at positions 56 and 60 is mandatory for enzyme catalysis. M39F, unlike the other mutants, exhibited a 5-fold increase in $K_m$ value. Lower thermostability was found with the G63A mutant when compared with wild type or other mutant forms of F. succinogenes 1,3–1,4-β-D-glucanase. Denatured wild type and mutant enzymes were, however, recoverable as active enzymes when 8 M urea was employed as the denaturant. Structural modeling and kinetic studies suggest that Glu56, Asp58, and Glu60 residues apparently play important role(s) in the catalysis of F. succinogenes 1,3–1,4-β-D-glucanase.

1,3–1,4-β-D-Glucanase (1,3–1,4-β-D-glucan 4-glucohydrolases, EC 3.2.1.73; lichenase) specifically hydrolyzes 1,4-β-D-glucosidic bonds adjacent to 1,3-β-D-glucosidic linkages in mixed linked b-D-glucans, yielding mainly cellulobiosyltriose and cellotriosyltetraose (1). This group of enzymes represents a distinct family of glucanohydrolases with similar substrate specificities widely observed among different organisms, including bacilli, clostridium, ruminal bacteria and fungi, and higher plants (2–5). The β-D-glucan substrate for the enzyme is a major cell wall component of endospore tissues in cereal grains. Chemically it is mostly a linear homopolymer of glucose molecules linked via β-1,3- and β-1,4-glycosidic bonds at a ratio of ~1:2.5. Various bacterial or fungal 1,3–1,4-β-D-glucanases show good sequence similarity with endo-β-1,3-glucanases (laminarases) (see Fig. 1), and together they are classified as a member of family 16 glycosyl hydrolases (2, 3). A high degree of amino acid sequence homology (70–90%) was observed among the various 1,3–1,4-β-D-glucanases isolated from different Bacillus species (6, 7). However, little or no homology between the bacteria and plant (barley) 1,3–1,4-β-D-glucanases was detected, neither at the primary nor at the tertiary structure levels of the compared proteins (4, 8). The bacterial enzymes are, in general, more thermostolerant than their barley counterparts.

With respect to the kinetic properties of bacterial 1,3–1,4-β-D-glucanases, these enzymes reside with the retaining glycosidase activity leading to the hydrolysis of glycosidic bonds with a net retention of the anomeric configuration during β-glucan hydrolysis (9). The 1,3–1,4-β-D-glucanase enzyme has been proposed to follow a general acid-base catalytic mechanism in which specific amino acid residues acting as a general acid or a nucleophile are required for completing a catalytic reaction of the enzyme (10). A number of methodologies have been previously applied to the characterization of catalytic mechanism(s) of Bacillus 1,3–1,4-β-D-glucanases, including site-directed mutagenesis, affinity labeling, and x-ray crystallography analysis. The Glu134 and Glu138 amino acid residues within the Bacillus licheniformis enzyme have been identified as the nucleophile and the catalytic acid/base, respectively (11, 12). The Glu135 and Glu139 residues of Bacillus macerans and H(A16-M)1,3–1,4-β-D-glucanases have also been shown to confer a catalytic function similar to that observed for Glu134 and Glu138 of the B. licheniformis enzyme (4, 12, 13).

A key ruminal bacterial enzyme producer, Fibrobacter succinogenes, plays a major role in plant fiber degradation in the rumen of major livestock species. Several of the enzymes related to the degradation of cellulose or hemicellulose polymers of plant cell walls from this organism have been isolated and partially characterized (14). A F. succinogenes 1,3–1,4-β-D-glucanase was isolated and investigated by Erfle and co-workers (15, 16). This enzyme consists of a protein sequence with a circular permutation in which two highly conserved catalytic domains (namely A and B) of the enzyme are in a reverse orientation as compared with that of other 1,3–1,4-β-D-glucanases (6, 8, 16). A 5 times repeated segment, PXSSSS, was only observed in the C-terminal, nonhomologous region of the amino acid sequence of the Fibrobacter enzyme relative to the bacilli or other bacterial and fungal 1,3–1,4-β-D-glucanases. Nevertheless, alignment of the amino acid sequences of the F. succinogenes enzyme with other 1,3–1,4-β-D-glucanases suggests that a number of amino acid residues in the highly conserved region may play important roles in catalysis of the enzyme (see Fig. 1). In an attempt to identify the possible involvement of specific amino acid residues in the catalytic...
activity of \textit{F. succinogenes} 1,3-1,4-\(\beta\)-D-glucanase, we evaluated the potential functional significance of the Met\textsuperscript{59}, Glu\textsuperscript{56}, Asp\textsuperscript{58}, Glu\textsuperscript{60}, and Gly\textsuperscript{63} residues of the enzyme, using a combination of various approaches including site-directed mutagenesis, fluorescence spectroscopy, circular dichroism spectroscopy, kinetics, and structural modeling. Several lines of evidence were obtained, providing some useful information about the structural and functional relationship of \textit{F. succinogenes} 1,3-1,4-\(\beta\)-D-glucanase. The most significant findings of this investigation are: 1) substitutions of Glu\textsuperscript{56}, Asp\textsuperscript{58}, and Glu\textsuperscript{60} with either alanine or glutamine can completely abolish enzymatic activity; 2) replacement of Gly\textsuperscript{63} with alanine can greatly reduce catalytic efficiency for the enzyme. This study provides new information that may be used to improve this ruminal enzyme for industrial utilization as a feed or food-processing aid, using either rational design or DNA shuffling approaches.

**EXPERIMENTAL PROCEDURES**

**Subcloning of \textit{F. succinogenes} 1,3-1,4-\(\beta\)-D-Glucanase Gene**—The full-length cDNA of \textit{F. succinogenes} 1,3-1,4-\(\beta\)-D-glucanase (Fs\(\beta\)-glucanase)\textsuperscript{4} in a pBl10 plasmid was amplified and introduced with NcoI and EcoRI restriction enzyme recognition sites at the 5’ and 3’ ends, respectively, by using a PCR-based method. The two primers designed for the NcoI and EcoRI sites were 5’-TACACGACATGTAAGCCGAAA-3’ and 5’-GGAACGGATCCTGTTCAAGTGC-3’, respectively. The PCR reaction was performed with a thermocycling program as follows: 94°C for 5 min, 55°C for 1 min, and 72°C for 1 min for 1 cycle; 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles; and 94°C for 1.5 min, 55°C for 1.5 min, and 72°C for 10 min for 1 cycle. The resulting amplified DNA fragments were digested with NcoI and EcoRI, purified, and ligated onto the pET28b(+) vector, which was predigested with NcoI and EcoRI. The recombinant gene sequence for Fs\(\beta\)-glucanase, designated “pFsNcE,” was confirmed by DNA sequencing using the chain termination method (17). In this DNA construct, a petB leading peptide at the N terminus plus 19 amino acid residues including a His\textsubscript{6} tag at the C terminus to facilitate protein purification were included. The recombinant plasmid encoding for the wild type enzyme was then transformed into \textit{Escherichia coli} BL21(DE3) host cells.

**Site-directed Mutagenesis**—Plasmid pFsNcE was used as the template for a PCR-based mutagenesis (18) of Fs\(\beta\)-glucanase. For this purpose, a pair of complementary mutagenic primers was designed for the mutation of each desired amino acid residue. Eleven pairs of mutagenic primers, as shown in Table I, were used to mutate Met\textsuperscript{59} → Phe, Glu\textsuperscript{56} → Ala, Glu\textsuperscript{56} → Asp, Glu\textsuperscript{56} → Gln, Asp\textsuperscript{58} → Ala, Asp\textsuperscript{58} → Glu, Asp\textsuperscript{58} → Asn, Glu\textsuperscript{60} → Ala, Glu\textsuperscript{60} → Gln, Gly\textsuperscript{63} → Ala. The mutagenic PCR reaction mixture consisted of 10 ml TCR, 10 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100, 0.1 mM nucleoside-free bovine serum albumin, 10 mg of template DNA, 0.5 mM dNTPs, 0.5 μM each of the complementary primers, and 2.5 units of cloned Turbo Pfu DNA polymerase.

Table 1

| Mutants | Mutagenic primer sequences |
|--------|---------------------------|
| M39F   | 5’-CCATTCTCTTCTGAGAAGAAGAACATCTGTTCC-3’ |
| E56A   | 5’-GGAAGGCCCCTCTGCCCTGGAATGGAATTAGCC-3’ |
| E56Q   | 5’-GGAGCCCTCCAGCAATGGTTTCCAAGTGC-3’ |
| D58A   | 5’-CCCTGGGTAAGAATGCTTGGCCG-3’ |
| D58E   | 5’-CCCTGGGTAAGAATGCTTGGCCG-3’ |
| D58N   | 5’-CCCTGGGTAAGAATGCTTGGCCG-3’ |
| E60A   | 5’-GGGATGGAATGTAATGCTTGGCCG-3’ |
| E60Q   | 5’-GGGATGGAATGTAATGCTTGGCCG-3’ |

Mutant DNA was generated with a thermocycling program of 2 min at 95°C and 16 cycles of 30 s at 95°C, 60 s at 55°C, and 12 min at 68°C on a Hybaid Touchdown thermal cycler. The PCR-generated products were digested with 10 units of DpnI at 37°C for 1 h, prior to their use for transformation into XL-1 Blue cells. Mutations were confirmed by fluorescent dideoxy chain termination DNA sequencing using T7 promoter, T, terminator primers. The mutagenesis plasmid was then transformed into BL21(DE3) host cells for the overexpression of mutant enzyme.

**Protein Production and Cellular Localization of Fs\(\beta\)-glucanase in \textit{E. coli}**—Optimal protein production conditions and cellular localization of Fs\(\beta\)-glucanase in \textit{E. coli} cells were investigated. 5 ml of pregrown culture in BL21(DE3) bacterial strain carrying pET28b(+) containing the Fs\(\beta\)-glucanase gene was added to 500 ml of fresh LB broth containing 30 μg/ml kanamycin. The culture was shaken vigorously at 33 °C until the A\textsubscript{600} nm reached 0.4–0.6. Addition of 1 mM isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) to the culture then was performed, and the culture was further incubated for 12–24 h at 33 °C. Small amounts of culture aliquots were collected with a constant time interval after the IPTG induction. The culture medium and cell extract prepared from the collected cell pellet at different time periods of IPTG induction were then employed for enzymatic activity assay, SDS-polyacrylamide gel electrophoresis (PAGE) according to Laemmli (19), and zymogram analysis.

**Purification of Wild Type and Mutant Fs\(\beta\)-glucanase**—The wild type or mutant forms of Fs\(\beta\)-glucanase produced in the abovementioned procedure were further purified. Approximately 80–85% of the total Fs\(\beta\)-glucanase expressed in \textit{E. coli} host cells was secreted into the culture medium. The extracellular secreted enzyme was collected by centrifugation at 8,000 × g for 15 min at 4°C and concentrated 10 times by volume using a Pellicon Cassette concentrator (Millipore, Bedford, MA) with a 10,000 M\textsubscript{r} cut-off membrane. The concentrated supernatant was then dialyzed against 50 mM Tris-Cl buffer, pH 7.8 (buffer A), and loaded onto a Q-Sepharose FF (Amersham Pharmacia Biotech) column pre-equilibrated with the same buffer. 1,3-1,4-\(\beta\)-D-Glucanase proteins, either wild type or mutants, were collected from eluants of the column using a 0–1 M NaCl salt gradient in buffer A. A second nickel-nitri-triacetic acid affinity column equilibrated with 50 mM sodium phosphate, pH 8.0, 0.5 M NaCl, and 10 mM imidazole buffer (buffer B) was then employed for further purification of the enzymes. From a 10–300 mM imidazole gradient eluant, a homogeneous enzyme preparation was obtained, as verified by SDS-PAGE. Protein concentration was quantified as described by Bradford (20) with bovine serum albumin as the standard.

**Zymogram Analysis**—A zymogram was used to measure the enzymatic activity of the wild-type and mutant forms of Fs\(\beta\)-glucanase which was performed essentially according to a reported method (21) with minor modifications. A 12% SDS-polyacrylamide gel containing lichenan (1 mg/ml) and protein samples in sample buffer (19) pretreated at 90°C for 10 min was prepared for zymogram analysis. After electrophoresis, the gel was rinsed twice with 20% isopropanol alcohol in 50 mM sodium citrate buffer, pH 6.0, for 20 min to remove SDS, and then stained 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-\(\beta\)-D-glucanase activity were then visualized using the Congo red staining.

**N-terminal Amino Acid Sequencing**—Protein samples for N-terminal sequence determination were resolved on a 12% SDS-polyacrylamide gel followed by electrophoretic transfer onto a polyvinylidene difluoride membrane, using a Mini-Trans-Blot cell system (Bio-Rad). Transferred protein bands on the membrane were visualized using the 0.1% Amido Black staining and then excised with a clean sharp razor blade. N-terminal amino acid sequencing was carried out on an Applied Biosystems model 492 gas phase sequencer equipped with an automated on-line phenylthiohydantoin analyzer.

**Kinetic Studies**—The enzymatic activity of 1,3-1,4-\(\beta\)-D-glucanase was measured by determining the rate of reducing sugar production from the hydrolysis of substrate (lichenan). The reducing sugar was measured and quantified by the method of Miller (22) with glucose as the standard. A standard enzyme activity assay was performed in a 0.3-m1 reaction mixture containing 50 mM sodium citrate buffer (pH 6.0) and 2.7–8 mg/ml lichenan by stirring the reaction with the enzyme added. After incubation at reaction at 40°C for 10 min, the reaction was terminated by the addition of a salicylic acid solution (22). 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 as μmol of glucose/min/mg 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 using enzyme kinetics.

Circular Dichroism Spectrometry—CD studies on the wild type and mutant forms of \( F.\) succinogenes \( \beta\)-glucanase were carried out in a Jasco J715 CD spectrometer and a 1-mm cell at 25 °C. Spectra were collected from 200 to 260 nm in 1.3-nm increments, and each spectrum was blank collected and smoothed by using the software package provided with the instrument.

Fluorescence Spectroscopy—The fluorescence emission spectra of the wild type and mutant forms of \( F.\) succinogenes \( \beta\)-glucanase were carried out in an Amico-Bowman series 2 spectrometer (Spectronic Instruments, Inc.) at 25 °C with 1-cm cuvette. Excitation spectra were taken at 282 nm, and emission spectra were recorded at 302–440 nm, with a 4-nm slit for both spectra. Protein samples were treated with 20–260 nm in 1.3-nm increments, and each spectrum was blank collected and smoothed by using the software package provided with the instrument.

RESULTS

Expression and Cellular Localization of the Recombinant \( \beta\)-Glucanase in \( E.\) coli Cultures—The conditions for expression of \( \beta\)-glucanase in engineered \( E.\) coli cells were optimized in this study. The wild type and mutant forms of the enzyme were effectively expressed and secreted into the LB medium as a soluble protein when host cells were grown at 33 °C with IPTG induction. Production of the enzyme in the whole \( E.\) coli culture was detected 2 h after IPTG induction and reached a plateau of maximum activity 8–16 h postinduction (data not shown). It was also found that enzymatic activity was detected primarily in the cell-associated fraction (cytosolic form) during the early stage (2–8 h) of induction, and upon prolonged IPTG induction for up to 16 h, enzymatic activity was mostly detected in the conditioned culture medium (extracellular form). The pelB leader sequence in the plasmid DNA construct was apparently fully functioning and facilitated the effective secretion of the \( \beta\)-glucanase into the culture medium. Approximately 80% of the extracellular secretion form of the protein was found as \( \beta\)-glucanase after IPTG induction for 16–20 h, as determined using SDS-PAGE and zymogram analyses (data not shown). The results from SDS-PAGE and zymogram analysis were also in good agreement with data from enzymatic activity assays (data not shown), suggesting that the \( E.\) coli expressed enzyme can be collected as either a cytosolic or an extracellular form from the host bacterial culture.

Purification and Biochemical Characterization of Wild Type and Mutant Forms of \( \beta\)-Glucanase—Homogeneous preparations of various recombinant enzymes were obtained by fractionation with a Q-Sepharose cation exchange column and followed by a separation with nickel-nitrilotriacetic acid affinity column, as described under “Experimental Procedures.” The first 25-amino acid sequence at the N terminus of the purified \( \beta\)-glucanase was determined to be MVSAKDFSGAELYTLEEVQYGKPEA, which represents a matured form of the \( \beta\)-glucanase enzyme without the presence of a pelB leader peptide at the N terminus. The wild type enzyme has a molecular mass of 37,669 Da, as determined by mass spectrometry. The estimated isoelectric point of the recombinant enzyme is pH 6.7, as analyzed by a Genetics Computer Group, Inc. (Madison, WI) computer program. The three-dimensional structure of this enzyme, to our knowledge, has not been solved so far. Therefore, for the current study the target amino acid residues for mutation were chosen based on the evaluation and comparison of the amino acid sequence of the 1,3,1,4-\( \beta\)-glucanases isolated from different organisms and on the prediction of their possible roles in catalysis. Fig. 1 shows that several of the amino acid residues of \( \beta\)-glucanase, including Glu 56, Asp 58, Glu 60, and Gly 63, are all conserved in the compared amino acid sequences. Methionine 39 is the only nonconservative amino acid residue observed in \( \beta\)-glucanase; in other words, the equivalent residues to position 39 of \( \beta\)-glucanase among other compared bacterial or fungal enzymes are all hydrophobic residues, including phenylalanine, isoleucine, and leucine. The expression conditions and purification protocol for the 11 mutant enzymes, namely M39F, E56A, E56D, E56Q, E56K, E56T, E56R, E56N, D58A, D58E, and G63A, were similar to that of the wild type enzyme. Purity of the wild type and mutant enzymes was evaluated by SDS-PAGE analysis (Fig. 2). The wild type and the 11 mutant enzymes exhibited identical mobility and are present as greater than 96% homogeneity when using electrophoresis as a criterion. Zymogram analysis revealed that the mutant enzymes showed a similar or reduced level of enzymatic activity as compared with the wild type enzyme (data not shown). Similar protein expression profiles and yield levels were obtained from the culture supernatants collected for the wild type and the 11 mutant forms of \( \beta\)-glucanase, as judged by SDS-PAGE analysis, and protein concentrations were determined by Bradford assay.

Fluorescence and Secondary Structure Analysis—The emission fluorescence spectra of the native, urea-denatured, and denatured-renatured wild type and mutant forms of \( \beta\)-glucanase were analyzed. Fig. 3 shows the superimposed emission spectra of both native wild type and G63A enzymes, with a maximum emission peaked at 336 nm. The emission spectra of 8 M urea-denatured wild type and mutant enzymes were bimo-
structure/functions of Fsβ-glucanase

FIG. 2. SDS-polyacrylamide gel electrophoresis of the purified wild type and mutant forms of F. succinogenes 1,3-1,4-β-D-glucanase. A 12% SDS-polyacrylamide gel was used and stained with Coomassie Brilliant Blue R-250. Lane M, molecular mass standards; lane 1, wild type; lanes 2–12, M39F, E56A, E56D, E56Q, D58A, D58E, D58N, E60A, E60D, E60Q, and G63A mutants, respectively.

renatured forms of the wild type, E56Q, and G63A glucanases showed CD spectra between 200 and 280 nm similar to their correspondent native forms of the proteins (data not shown). These results suggest that the single amino acid substitution of wild type Fsβ-glucanase in this study did not cause a global conformational change or aberrant folding of the enzyme. Folded structures were observed for the individual mutant enzymes as evidenced by analysis of the fluorescent and CD spectra, relative to those of the wild type enzyme. Therefore, the observed differences in the kinetic properties between the mutant and wild type forms of the Fsβ-glucanase enzyme apparently are not due to the disruption of the structural integrity of the enzyme.

Enzymatic activity assays were further carried out for the native and renatured form of the wild type and mutant enzymes. After renaturation, the wild type and mutant enzymes showed a recovery of ~85% activity relative to their original native forms of the enzyme. The results from the fluorescence, CD, and activity assays suggest that a great majority of the denatured Fsβ-glucanases, either as wild type or mutants, was able to effectively reassociate to their correspondent native forms of the protein. This study hence concludes that a reversible denaturation capability was observed for Fsβ-glucanase after the treatment with a high concentration of urea.

Kinetic Analysis of Wild Type and Mutant Forms of Fsβ-glucanase—Experiments on kinetic studies were mainly performed by using lichenan as the substrate in standard enzyme assays, as described under “Experimental Procedures.” The specific activity of the recombinant, wild type Fsβ-glucanase expressed in E. coli cells is 1388 ± 82 units/mg, estimated with lichenan at 40 °C in 50 mM sodium citrate buffer, pH 6.0. This value is slightly higher than that (960 units/mg) reported for the native enzyme (15). The affinity for lichenan (Km), the turnover number (kcat), and the catalysis efficiency (kcat/Km) of the wild type enzyme were 1.91 mg/ml, 871 s⁻¹, and 456 s⁻¹ (mg/ml)⁻¹, respectively.

In an attempt to evaluate the effect of specific amino acid substitutions on enzyme activity or other functions, the kinetic properties of the mutant enzymes were characterized. A dramatic change in the turnover rate was found for the mutations on amino acid residues Glu56, Asp58, and Glu60. For the E56A, E56Q, and G63A glucanases, either as wild type or mutants, we showed that no enzymatic activity was detectable for these variant protein forms. 2.5-, 241-, 570-, 880-, 540-, and 2.8-fold decreases in kcat were observed for M39F, E56D, D58N, D58E, E60D, and G63A, respectively, relative to the kcat value of the wild type enzyme. These results suggest that amino acid residues Glu56, Asp58, and Glu60 may play an important role(s) for enzymatic activity and may be directly involved in the catalysis of Fsβ-glucanase.

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Small changes in Km values for lichenan were observed for the E56D, D58E, D58N, E60D, and G63A mutant enzymes; however, a 5.3-fold higher Kcat for lichenan was found for M39F as compared with the wild type enzyme. Comparison of the specificity constants, kcat/Km, is shown in Table II. The specificity constants decreased 4.5- and 14-fold in G63A and M39F, respectively. However, more significant changes (~210–970-fold decrease) were observed in the single mutations of Glu56, Asp58, and Glu60, relative to the wild type enzyme. These results indicate that the three acidic amino acid residues in Fsβ-glucanase may play important roles in the catalysis of the enzyme. We have also examined the substrate specificity of the wild type and mutant forms of Fsβ-glucanase, by using lichenan, barley β-glucan, larminarine, carboxymethyl cellulose, and xylan as test substrates in enzymatic activity assays. No detectable binding affinity was observed for the wild type or mutant Fsβ-glucanase enzymes when larminarine, carboxy-
methyl cellulose, or xylan was used as the substrate.

Various buffers at different pH values were employed for evaluating the optimum pH and pH tolerance of the Fsβ-glucanase enzyme. Mutant and wild type enzymes exhibited similar pH response profiles between pH 4 and pH 10 with the pH optimum value being between 6 and 8 (conferring ~100% enzyme activity). At pH 5.0 and pH 10.0, the wild type and mutant enzymes were shown at only ~20 and 40% of their optimal activities, respectively (data not shown). The optimum temperatures for the wild type and D58N Fsβ-glucanase were observed at 50 °C; however, for the M39F, E56D, D58E, and E60D mutants the optimum temperatures were found to have shifted to 40 °C as compared with the wild type enzyme (data not shown). The G63A mutant shows an optimum temperature at 30 °C, which is 20 °C lower than that of the wild type enzyme.

**Temperature Sensitivity of Wild Type and Mutant Fsβ-glucanases**—The temperature sensitivity of wild type and mutant Fsβ-glucanases was investigated to further characterize the effect of introduced mutations. Replacement of amino acid residues in Met39, Glu56, Asp58, and Glu60 did not cause significant changes in thermostability, as evaluated with a temperature range between 30 and 90 °C. The wild type and mutant enzymes with specific mutations of acidic amino acid residues were shown to exhibit similar enzymatic activity between 20 and 45 °C, but a dramatic loss of enzymatic activity was observed at temperatures higher than 50 °C. In contrast, the G63A variant exhibited a greatly impaired thermostability, served at temperatures higher than 50 °C. In contrast, the M39F, E56D, D58E, and E60D mutants were shown at only ~20% of their nonglycyl and nonpropyl residues falling in the most favored or in the additionally allowed regions in the Ramachandran plot, as analyzed by the PROCHECK program (25). The modeled Fsβ-glucanase structure consists mainly of two antiparallel β-sheets with seven and eight strands, respectively (Fig. 5), arranged atop each other to form a compact, sandwich-like structure. The overall β-sheet model structure of the Fsβ-glucanase is similar to that of cpA16M-59 with only minor changes in the surface loop regions. The two β-sheets are bent to give rise to a concave and a convex side of the molecule. The Glu56, Asp58, and Glu60 amino acid residues are located at the cleft on the concave side of the protein molecule (Fig. 5), and this cleft was likely the substrate binding side as previously suggested based on the structure analysis of a protein-inhibitor complex of the Bacillus 1,3–1,4-β-d-glucanase with epoxyalkyl β-oligoglucosides (4).

**DISCUSSION**

Bacterial 1,3–1,4-β-d-glucanases belong to the category of retaining enzymes and are classified into family 16 endoglucanases (3). More than 50% protein sequence homology was found among the bacterial enzymes, including those from Clostridium and Bacilli and the ruminal fungus Orpinomyces (5). In comparison, only ~30% homology was found for the F. succinogenes 1,3–1,4-β-d-glucanase with respect to the other bacterial enzymes. In addition, a naturally occurring circular permutation structure was only found for the F. succinogenes 1,3–1,4-β-d-glucanase. Although the biochemical properties of the native F. succinogenes enzyme (15) and its encoding cDNA sequence have been characterized (16), further studies on the structure-function relationship were not reported. In this study, a number of conserved amino acid residues, as revealed by sequence comparison studies, were investigated for their potential involvement in the catalysis of the enzyme.

Studies on the three-dimensional structure of a covalent protein-inhibitor complex of H(A16-M) with 3,4-epoxybutyl-β-d-cellobioside (4) have shown that the inhibitor binds to the Glu105 amino acid residue, which corresponds to the Glu106 residue of the Fsβ-glucanase enzyme, based on the amino acid sequence alignment of these two proteins. Site-directed mutagenesis and chemical modification of the glutamic acid residues, e.g. Glu105 and Glu106 in H(A16-M), Glu105 in Bacillus amyloliquefaciens (26), Glu103 in B. macerans (27), and Glu134 and Glu138 in B. licheniformis (11), suggested that the glutamic acid residue of the Fsβ-glucanase enzyme was likely the substrate binding side as previously suggested based on the structure analysis of a protein-inhibitor complex of the Bacillus 1,3–1,4-β-d-glucanase with epoxyalkyl β-oligoglucosides (4).

**TABLE II**

Kinetic properties of wild type and mutant forms of F. succinogenes 1,3–1,4-β-d-glucanase

| Enzyme | Specific activity | $k_{cat}$ | $K_m$ | $V_{cat}$ | $V_{max}$ |
|--------|------------------|----------|------|----------|----------|
|        | units/mg s$^{-1}$ | mg/ml    |       | mg/ml    | s$^{-1}$ |
| Wild type | 1388 ± 82  | 871   | 1.91 ± 0.01 | 456  |
| M39F   | 545 ± 33    | 342   | 10.3 ± 0.05 | 33.2  |
| E56A   | ND          | ND    | ND    | ND       | ND       |
| E56D   | 5.75 ± 0.24 | 3.61  | 1.69 ± 0.09 | 2.14  |
| E58Q   | ND          | ND    | ND    | ND       | ND       |
| D58A   | ND          | ND    | ND    | ND       | ND       |
| D58E   | 1.57 ± 0.11 | 0.99  | 2.10 ± 0.06 | 0.47  |
| D58N   | 2.43 ± 0.13 | 1.33  | 0.98 ± 0.06 | 1.56  |
| E60A   | ND          | ND    | ND    | ND       | ND       |
| E60Q   | ND          | ND    | ND    | ND       | ND       |
| G63A   | 502 ± 42    | 315   | 3.10 ± 0.01 | 101   |

$^a$ ND, not detected.

![FIG. 4. Temperature sensitivity of wild type and mutant forms of F. succinogenes 1,3–1,4-β-d-glucanase. The purified wild type ( ), M39F ( ), E58Q ( ■), and G63A (□) enzymes at a enzyme concentration of 0.007–1.24 mg/ml were incubated for 10 min at 35, 40, 45, 50, 55, 60, 65, 70, 75, and 80 °C, respectively, in 50 mM citrate buffer, pH 6.0. Enzyme activity was assayed by the method of Miller (22), as described under “Experimental Procedures,” immediately after incubation and is expressed as a percentage of the relative activity at 40 °C. The protein concentration was 0.24–82.7 μg/ml in each assay. Each assay was performed either in duplicate or in triplicate.](image)
In the present Fsβ-glucanase structure model, Met39 is located in a helical region, which may be involved in maintaining the structural integrity of the enzyme. The presence of an additional cysteine residue (Cys30) in the Fsβ-glucanase structure suggests the possibility of disulfide bond formation, which could help stabilize the enzyme's tertiary structure. Further studies are needed to confirm the exact role of these additional residues in the Fsβ-glucanase enzyme. Overall, the Fsβ-glucanase structure model provides valuable insights into the enzyme's catalytic mechanism and its potential applications in biotechnology and medicine.
The same heat treatment, was detected after a heat treatment at 45 °C for 10 min. With the amino acid residue Met39 of Fs-β-glucanase, moreover, this mutation apparently has resulted in a more effective catalytic efficiency for the enzyme. Glu60 may function as general acid/base residues based on the relationship of the naturally occurring, circular permutated protein structure, and this interpretation also supported the results of fluorescence spectrometry assays (data not shown).

Slight decreases in specific activity and substrate binding affinity were observed for the replacement of Gly63 with alanine; however, significant reduction of the enzyme stability was also observed for this mutant. After incubation at 35 °C for 10 min, the G63A mutant only retains ~25% of its original enzymatic activity. Therefore, the conserved Gly63 residue among various bacterial and fungal 1,3-1,4-β-D-glucanases (Fig. 1) is first reported in this study as an important residue for enzyme stability. This residue is located at the end of a β-strand, which contains three active site residues, Glu58, Asp58, and Gly60. It has been shown that the thermal stability of the Fis protein (28) and the Arc repressor (29) was largely reduced when glycines located in turn regions were mutated to alanines. Therefore, it is possible that the Gly63 plays a similar role in 1,3-1,4-β-D-glucanases in that it stabilizes the folding of the entire protein. We found that only ~5% residual activity was detected in the mutant enzyme treated with 40 °C for 10 min, whereas the wild type was still fully active after the same heat treatment.

In summary, this report first identified several key amino acid residues that are involved in the catalysis of F. succinogenes 1,3-1,4-β-D-glucanase. It is proposed here that Glu56 and Glu60 may function as general acid/base residues based on the detailed comparison in kinetics. We also demonstrated that Gly63 is important for the stability of the enzyme. Our study has provided useful information on the structure-function relationship of the naturally occurring, circular permuted protein structure of a bacterial 1,3-1,4-β-D-glucanase isolated from rumen F. succinogenes. Further studies of the three-dimensional structure of the enzyme using the x-ray crystallography approach are in progress.

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