A Truncated *Fibrobacter succinogenes* 1,3−1,4-β-D-Glucanase with Improved Enzymatic Activity and Thermotolerance†

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ABSTRACT: As an approach to improving *Fibrobacter succinogenes* 1,3−1,4-β-D-glucanase (Fsβ-glucanase) for use in industry and to studying the structure−function relationship of the C-terminus in the enzyme, a C-terminally truncated (∼10 kDa) Fsβ-glucanase was generated using a PCR-based gene truncation method and then overexpressed in either *Escherichia coli* BL21(DE3) or *Pichia pastoris* strain X-33 host cells. The initial rate kinetics, protein folding, and thermostability of the wild-type and truncated glucanases were characterized. The truncated enzyme expressed in *Pichia* cells was found to be glycosylated and composed of two dominant polypeptide bands as judged by SDS−PAGE. An approximate 3−4-fold increase in the turnover rate (k_{cat}), relative to that of the full-length enzyme, was detected for the purified truncated glucanases produced in *E. coli* (designated TF-glucanase) or *Pichia* host cells (designated glycosylated TF-glucanase). The glycosylated TF-glucanase is the most active known 1,3−1,4-β-D-glucanase, with a specific activity of 10 800 ± 200 units/mg. Similar binding affinities for lichenan (K_m = 2.5−2.89 mg/mL) were detected for the full-length enzyme, TF-glucanase, and glycosylated TF-glucanase. Both forms of truncated glucanase retained more than 80% of their original enzymatic activity after a 10 min incubation at 90 °C, whereas the full-length enzyme possessed only 30% of its original enzymatic activity after the same treatment. This report demonstrates that deletion of the C-terminal region (∼10 kDa) in Fsβ-glucanase, consisting of serine-rich repeats and a basic terminal domain rich in positively charged amino acids, significantly increases the catalytic efficiency and thermotolerance of the enzyme.

1,3−1,4-β-D-Glucanase (lichenase, EC 3.2.1.73) is an endo-β-D-glucanase that specifically hydrolyzes 1,4-β-D-glucosidic bonds adjacent to 1,3-β-linkages in mix-linked β-glucans, yielding mainly cellobiosyltriose and cellotriosyltetraose (*1*). The enzyme has received much attention in both basic and applied research, because of its enzymatic functions and importance in industrial applications. Supplementation of this fibrolytic enzyme in animal feed is one of the approaches for increasing the feed conversion efficiency and growth rate of nonruminal animals (*2*, *3*). 1,3−1,4-β-D-Glucanase has also been used in the beer industry to replace or supplement malt enzymes, thereby reducing the industrial processing problem(s) caused by β-glucans from plant cell walls, including a reduced yield of barley seed extract, lowered rates of wort separation and beer filtration, and formation of hazes and gelatinous precipitates in beer (*4*). However, a major drawback to widespread use of 1,3−1,4-β-D-glucanase as an industrial enzyme is the thermal stability of the enzyme during industrial processes. For instance, the elevated temperatures employed in malting processes (50−70 °C) or feed pelleting and/or expansion processes (65−90 °C) can cause a severe inactivation of the enzyme. Therefore, the creation of a heat-resistant 1,3−1,4-β-D-glucanase would circumvent the aforementioned problem. Moreover, a 1,3−1,4-β-D-glucanase enzyme with high catalytic activity could reduce the quantity of enzyme supplement during industrial processing that potentially leads to an increase in enzyme utilization efficiency through cost reduction.

Fibrobacter succinogenes has been shown to play a major role in plant fiber degradation in the rumen, and several enzymes related to the degradation of cellulose or hemicellulose polymers of the plant cell wall from this organism have been isolated and studied (*5*). An *F. succinogenes* 1,3−1,4-β-D-glucanase (Fsβ-glucanase)[1] was first isolated and characterized by Erfle and co-workers (*6*, *7*). This enzyme consists of a protein sequence with circular permutation at the catalytic domain in which two highly conserved catalytic regions (A and B) of the enzyme are in a reverse orientation, as compared to that of other 1,3−1,4-β-D-glucanases (*7*−*9*). Moreover, a quintet repeat serine-rich region, PXSSSS, was observed to be unique and existing only at the C-terminal, nonhomologous region of the amino acid sequence

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[1] Abbreviations: Fsβ-glucanase, *F. succinogenes* 1,3−1,4-β-D-glucanase; TF-glucanase, truncated glucanase; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; Ni−NTA, nickel−nitrilotriacetic acid; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; DNS, 3,5-dinitrosalicylic acid; CD, circular dichroism; BTD, basic terminal domain; CBM, carbohydrate binding modules.

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of the *F. succinogenes* 1,3−1,4-β-D-glucanase. The five PXSSSS repeats in Fsβ-glucanase separate the catalytic domain and a basic terminal domain (BTD) rich in positively charged amino acids, and this unique protein structural feature is similar to an endo-β-1,4-glucanase D, encoded by a *celD* gene, identified from the same bacterial strain, *F. succinogenes* S85 (*10*). The serine-rich periodic sequences (SRPS) in endo-β-1,4-glucanase D, composed of six serine-rich repeats, were predicted to function as flexible linkers, to facilitate interactions between the BTDs and acidic membrane proteins from *F. succinogenes*. The possible roles of these serine-rich repeat segments in 1,3−1,4-β-D-glucanase in terms of the biological or structural and enzymatic functions of the enzyme, however, have not been well characterized to date.

Bacterial 1,3−1,4-β-D-glucanase enzymes have been proposed to follow a general acid−base catalytic mechanism whereby specific amino acid residues acting as a general acid or a nucleophile are required for enzymatic catalysis (*11*). Recently, we have identified a number of amino acid residues in Fsβ-glucanase that play important roles in the catalysis and thermostability of the enzyme. Glu^{56} and Glu^{60} are thought to be the essential catalytic acid−base residues in the retaining glycosidase activity of Fsβ-glucanase, while replacement of Gly^{63} with alanine can greatly reduce the thermostability of the enzyme (*12*). We have also demonstrated that several tryptophan residues, i.e., Trp^{54}, Trp^{141}, Trp^{148}, and Trp^{203}, play important roles in maintaining the structural integrity of the substrate-binding cleft and the catalytic efficiency of the enzyme (*13*).

In an attempt to improve Fsβ-glucanase for industrial utilization as a feed or food processing aid, gene truncation, initial rate kinetics, and overexpression of the enzyme in bacterial (*Escherichia coli*) and yeast (*Pichia*) cells were investigated. Our results demonstrate that engineered, truncated forms of Fsβ-glucanase, with approximately 10 kDa deleted from the C-terminus, possess more industrially favorable properties, i.e., higher specific activity (4−5-fold increase) and thermotolerance, compared to the wild-type enzyme. Strikingly, the truncated enzyme expressed in the *Pichia* host cell system can efficiently recover ≥80% of its activities even after being boiled for 10 min.

EXPERIMENTAL PROCEDURES

Materials. Lichenan, phenylmethanesulfonyl fluoride (PMSF), and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Sigma (St. Louis, MO). Lichenan was from Sigma, and barley β-glucan was from Megazyme. A mixture of four dNTPs was from Amersham Pharmacia Biotech, and *pfu* polymerase was from Strategene (La Jolla, CA). Other chemicals were reagent grade or the equivalent.

Construction of Two Truncated 1,3−1,4-β-D-Glucanase Genes in an E. coli Expression Vector. The pFsNcE plasmid carrying the full-length *F. succinogenes* 1,3−1,4-β-D-glucanase gene in the pET26b(+) vector as described previously (*12*) was used as the template for the gene truncation experiment. To comparatively evaluate the kinetic properties of the recombinant full-length Fsβ-glucanase and those of other truncated enzymes created in this study, we designated this full-length enzyme as the "wild-type" enzyme. The gene encoding a truncated 1,3−1,4-β-D-glucanase containing a His tag at its C-terminus (designated PCR-TF-glucanase) was generated using PCR with a pair of specific primers (Oligo A, 5′-CAGCCGGCGATGGCCATGGTTAGCGCA-3′; and Oligo B, 5′-CTGCTAGAAGAATTCGGAGCAGGTTCGTC-3′), designed to amplify both strands of the gene corresponding to the amino acid sequence from methionine 1 to proline 248. The amplified DNA fragment was digested with *Nco*I and *Eco*RI and then ligated with a pET26b(+) vector predigested with the two restriction enzymes. The amplified PCR-TF-glucanase gene was confirmed by DNA sequencing. In addition to the coding sequence of truncated 1,3−1,4-β-D-glucanase, this DNA construct contained a *pel* B signal sequence at its N-terminus to allow the truncated glucanase to be extracellularly expressed in culture medium, and 19 extra amino acid residues with a six-histidine tag at the C-terminus to facilitate protein purification.

Another truncated form of Fsβ-glucanase (designated TF-glucanase), without a six-histidine tag at the C-terminus, was created using PCR-based site-directed mutagenesis by introducing a stop codon at amino acid residue 249 of the PCR-TF-glucanase gene. A pair of complementary mutagenic primers, 5′-CCTGCTCCG**TAA**TCGAGCTCC-3′, corresponding to the sense strand sequence, were designed for this experiment. The mutagenic PCRs were performed using the method published elsewhere (*13*). The resulting PCR 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 of LB/kanamycin liquid culture. Plasmids were isolated from the culture using a QIAprep Spin Miniprep kit (Qiagene, Hilden, Germany), and the mutation was confirmed by DNA sequencing.

Finally, the recombinant plasmid encoding either TF-glucanase (consisting of 246 amino acid residues) or PCR-TF-glucanase (consisting of 267 amino acid residues) was then transformed into *E. coli* BL21(DE3) host cells (Novagen, WI).

Construction of the Truncated 1,3−1,4-β-D-Glucanase Gene in a Pichia Expression Vector. A pair of specific primers, Oligos C and D, were designed to amplify both strands of the TF-glucanase gene, corresponding to the amino acid sequence from N-terminal residue Val2 to Pro 248 at the C-terminus, and to generate two cutting sites for the restriction enzymes, *Pst*I and *Xba*I, at the 5′ and 3′ ends, respectively. Oligos C and D are 5′-TACGCTGCAGTTAGCGCAAAGGATTTTAGC-3′ and 5′-TAGTTCTAGATCACGGAGCAGGTTCGTCATCTCTC-3′, respectively. The PCR-amplified DNA products were digested with *Pst*I and *Xba*I and then ligated into the *Pichia* expression vector, pPICZαB (Invitrogen), which was predigested with *Pst*I and *Xba*I. This newly created recombinant plasmid, containing a truncated *F. succinogenes* glucanase gene, was designated pPICZ-TFGlu and was then transformed into *Pichia pastoris* strain X-33 host cells (Invitrogen). This construct allowed the TF-glucanase to be extracellularly expressed in the culture medium, because the vector contains an α factor signal sequence fused upstream of the TF-glucanase.

Purification of Wild-Type and Truncated Forms of 1,3−1,4-β-D-Glucanases Expressed in E. coli Cells. Overexpression of the wild-type and truncated Fsβ-glucanases in *E. coli*

cells was performed using the method reported by Cheng et al. (*13*). The wild-type and truncated forms of 1,3−1,4-β-D-glucanases were extracellularly secreted into LB culture medium at 33 °C. The enzyme in the culture supernatant was collected by centrifugation at 8000*g* for 15 min at 4 °C, and reduced 10-fold in volume on 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 7.8) (buffer A) and loaded onto a Q-Sepharose FF column (Pharmacia, Uppsala, Sweden) pre-equilibrated with the same buffer. 1,3−1,4-β-D-Glucanase proteins, the wild-type or truncated forms, were collected from eluants of the column eluted with a 0 to 1 M NaCl salt gradient in buffer A. To obtain high-purity protein, the enriched TF-glucanase protein fraction from the first Q-Sepharose column was then further purified on a second Q-Sepharose column with the same buffer systems for equilibration and elution. The enriched PCR-TF-glucanase protein fraction obtained from the first Q-Sepharose column was further purified using a Ni−NTA affinity column. Homogeneous enzyme preparations were obtained from a 10 to 300 mM imidazole gradient eluant, as verified by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) according to the method of Laemmli (*14*). Protein concentrations were quantified using a dye binding assay (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin (BSA) as the standard.

Purification of Truncated Fsβ-Glucanase Expressed in Pichia Host Cells. The expression of the TF-glucanase gene in *P. pastoris* strain X-33 was carried out as indicated in the instruction manual of the *Pichia* expression kit (Invitrogen). To induce target enzyme expression, the cells of *P. pastoris* strain X-33 carrying the pPICZ-TFGlu gene, which had been cultured overnight, were resuspended to an A_{600} of 1 in BMMY medium, containing 0.5% (v/v) methanol, in a 1 L baffled flask and shaken at 250 rpm at 28 °C. Methanol (0.5%) was added to the culture every 24 h. One milliliter was withdrawn from the culture at 1−2 day intervals, and after centrifugation, the supernatant was stored at −20 °C for further analysis. The enzyme in the culture supernatant was collected by centrifugation at 8000*g* for 15 min at 4 °C, and its volume was reduced 10-fold on the Pellicon Cassette concentrator (Millipore) with a 10 000 M_r cutoff membrane. The concentrated culture supernatant was then dialyzed against 50 mM Tris-HCl buffer (pH 7.8) (buffer A) and loaded onto a Q-Sepharose FF column (Pharmacia) pre-equilibrated with the same buffer. Glycosylated TF-glucanase was eluted from the column with a 0 to 1 M NaCl salt gradient in buffer A. The homogeneity of the purified enzyme was verified by SDS−PAGE, and its protein concentration was quantified with a dye binding assay. The enzyme deglycosylation reaction was performed using the Denaturing Protocol of an Enzymatic Deglycosylation kit according to the supplier's instruction (Bio-Rad) using deglycosylation enzymes, NANase II, *O*-glycosidase, and PNGase F. The efficiency of deglycosylation was examined using SDS−PAGE.

Mass (MS) Analysis. The molecular masses of the purified glucanases were determined by triple-quadrupole MS using a Micromass Quattro-Bio-Q mass spectrometer (Waters, Milford, MA) equipped with an electrospray ionization (ESI) source. A LC Packings (Amsterdam, The Netherlands) Ultimate capillary- and nano-LC system was interfaced with the mass spectrometer for on-line protein analysis. Purified protein samples (100 pmol) were injected into the LC system with a microbore reversed-phase column (C8, 1.0 mm × 250 mm, Vydac, Vesperia, CA) and washed with a mobile phase of 70% solvent A (0.08% TFA in H_2O) and 30% solvent B (0.072% TFA in 80% acetonitrile) for 10 min, followed by a elution with a linear gradient from 30 to 100% solvent B for the next 70 min. The eluted proteins were also monitored by the UV absorbance at 214 nm. A flow rate of 35 μL/min was used.

Zymogram Analysis. An enzymatic activity assay using zymogram analysis was performed using the method reported by Piruzian et al. (*15*) with minor modifications. Protein samples in sample buffer (*14*) were heated at 90 °C for 10 min and separated on a 12% SDS−polyacrylamide gel containing lichenin (1 mg/mL). 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. The gel was then incubated at 40 °C for 10 min. The protein bands with 1,3−1,4-β-D-glucanase activity were visualized using Congo red staining (0.5 mg/mL).

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 band(s) on the membrane was visualized by staining with 0.1% amido black staining solution and 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-β-D-glucanase was measured by determining the rate of reducing sugar production from the hydrolysis of substrate (soluble form of lichenan or barley β-glucan). The 3,5-dinitrosalicylic acid (DNS) method (*16*) with glucose as the standard, a commonly used method for assaying reducing sugar and determining glucanase activities (*12*, *13*, *17*−*19*), was adapted and employed in this study. A standard enzyme activity assay was performed in a 0.3 mL reaction mixture, containing 50 mM sodium citrate buffer (pH 6.0) and 0.4−8 mg/mL lichenan, by starting the reaction with the addition of enzyme. After incubation at 50 °C for 10 min, the reaction was terminated by the addition of a DNS solution (*16*). One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar (glucose equivalent) per minute. Specific activity was expressed in micromoles of glucose per minute per milligram of protein. Various amounts of the purified enzymes (5−12 μg/mL) were used in each kinetic assay reaction, depending on the enzymatic activity of the enzyme. The results of kinetic studies in this report were performed independently at least three times, and every data point (the variables include substrate concentration, temperature, pH, etc.) in each independent experiment was determined in triplicate. Kinetic data were analyzed using ENZFITTER (BIOSOFT) and Enzyme Kinetics (SigmaPlot 2000, SPSS Inc.).

Circular Dichroism (CD) Spectrometry. CD spectrometric studies on the wild-type and truncated forms of *F. succino-*

genes glucanase were carried out in a Jasco J715 CD spectrometer and a 1 mm cell at the indicated temperatures. Spectra were collected from 200 to 260 nm in 1.3 nm increments, and each spectrum was blank-collected and smoothed using the software package provided with the instrument.

Protein Reactivation Profile of the Full-Length and Truncated Form of Fsβ-Glucanases. Enzyme samples were pretreated at 90 and 100 °C for 10 and 30 min, respectively, and then incubated at room temperature (25 °C). Recovery of enzymatic activity in heat-treated samples, following incubation for 0−24 h at room temperature, was measured using standard enzyme activity assays.

Protease Digestion of Fsβ-Glucanase. The purified wild-type and truncated forms of Fsβ-glucanase were incubated with a 1 mg/mL trypsin protease solution in 20 mM MOPS buffer (pH 7.0) or with a 1 mg/mL pepsin protease solution in 20 mM glycine-HCl buffer (pH 2.5), at 37 °C for 1, 2.5, or 5 h. The residual enzymatic activities of the glucanases, after trypsin or pepsin digestion, were then determined with the standard enzymatic assay.

Fluorescence Spectrometry. The fluorescence emission spectra of native, 90 °C-treated, and 90 °C-treated−renatured wild-type Fsβ-glucanase and TF-glucanase were recorded on an Amico-Bowman Series 2 spectrofluorimeter (Spectronic Instruments, Inc.) at 25 °C with a 1 cm × 1 cm cuvette. Emission spectra were recorded from 305 to 430 nm by excitation at 295 nm, with a 4 nm slit for both spectra. Protein samples were diluted to 0.03 mg/mL in 50 mM sodium phosphate (pH 7.0). The 90 °C-treated samples were recovered at 25 °C for 0, 3, and 10 min, respectively, and emission spectra were then recorded immediately.

RESULTS

Expression and Purification of Wild-Type (full-length) and Truncated Forms of Fsβ-Glucanase in E. coli or Pichia Host Cells. The cDNA coding sequences of recombinant wild-type (*12*) and truncated Fsβ-glucanases were successfully subcloned and expressed by use of a T_7 promoter-driven protein expression system in *E. coli* cells. The truncated forms of *F. succinogenes* 1,3−1,4-β-D-glucanase (PCR-TF-glucanase and TF-glucanase), as well as the wild-type enzyme (Fsβ-glucanase), were effectively expressed in *E. coli* cells. Full-length or truncated glucanases made up more than 60% of the secreted protein from induced cells, as determined using SDS−PAGE and zymogram analyses (data not shown). Homogeneous wild-type and truncated glucanase enzymes were then purified by Q-Sepharose anion exchange and Ni−NTA affinity columns, as described in Experimental Procedures. Figure 1A shows the purified wild type (lane 1), TF-glucanase (lane 2), and PCR-TF-glucanase (lane 3) were all more than 96% homogeneous from SDS−PAGE and zymogram analyses.

The expression of TF-glucanase in *P. pastoris* strain X-33 was evaluated for its protein yield and enzymatic activity. Our results showed that the enzyme expression was approximately 1.94×10^6 units per 427 mg/L after induction for 15 days and 1.76×10^6 units per 469 mg/L after induction for 23 days in culture medium. SDS−PAGE further demonstrated that more than 90% of secreted proteins in the culture medium of transformed *Pichia* cells were of the two

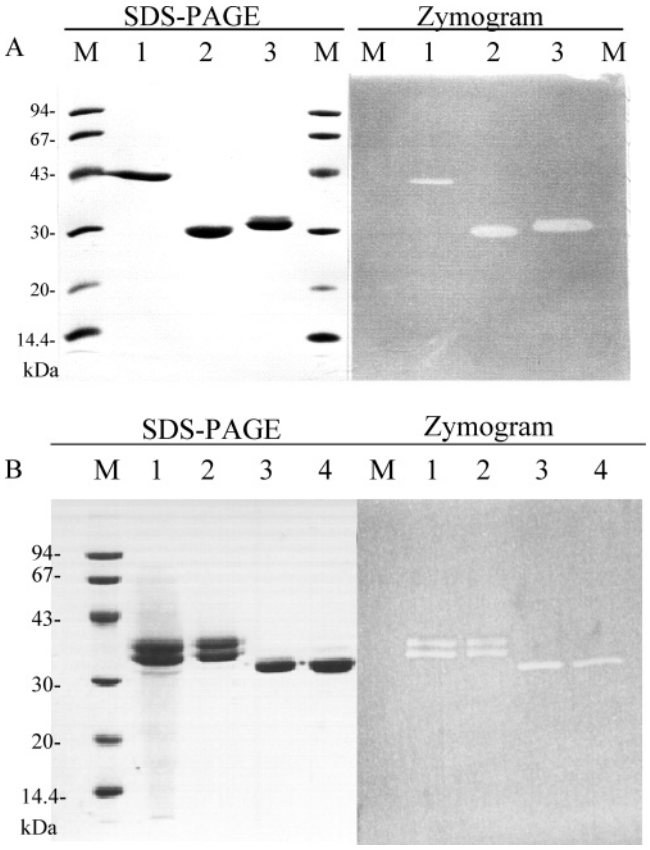

FIGURE 1: (A) SDS−PAGE and zymogram analysis of the full-length (wild-type) and truncated forms of *Fibrobacter* 1,3−1,4-β-D-glucanase expressed in *E. coli* cells: lane M, molecular weight markers; lane 1, wild-type Fsβ-glucanase; lane 2, TF-glucanase; and lane 3, PCR-TF-glucanase. Three micrograms and 0.15 μg of the wild-type and truncated enzymes were employed for SDS−PAGE and zymogram analysis, respectively. (B) SDS−PAGE and zymogram analysis of the truncated forms of *Fibrobacter* 1,3−1,4-β-D-glucanases expressed in *Pichia* host cells: lane M, low-molecular weight markers; lane 1, aliquot from the culture medium of *Pichia* cells containing the TF-glucanase gene; lane 2, purified glycosylated TF-glucanase from *Pichia* host cells; lane 3, deglycosylated TF-glucanase from lane 2; and lane 4, purified TF-glucanase expressed in *E. coli* cells. Approximately 3 and 0.15 μg of the truncated enzymes were employed for SDS−PAGE and zymogram analysis, respectively.

dominant forms of glycosylated TF-glucanase (designated glycosylated TF-glucanase) (Figure 1B, lane 1). Homogeneous glycosylated TF-glucanase (>96% pure) was obtained after Q-anion exchange column chromatography (lane 2). The glycoside moiety of the glycosylated TF-glucanase was effectively removed after digestion with glycosidases using an enzymatic deglycosylation kit (Bio-Rad) as described in Experimental Procedures, resulting in a single form of enzyme (lane 3), which has a mobility on the SDS gel similar to that of the nonglycosylated TF-glucanase expressed in *E. coli* (lane 4).

Biochemical Characterization of Wild-Type and Truncated Forms of Fsβ-Glucanase. The first 25 amino acid residues of the N-terminal sequence of the purified wild-type Fsβ-glucanase were determined to be MVSAKDFSGAELYT-LEEVQYGKFEA, which represents a mature form of the Fsβ-glucanase enzyme without the presence of a *pel* B leader peptide at its N-terminus. The N-terminal amino acid sequences of PCR-TF-glucanase, TF-glucanase, and glycosylated TF-glucanase were identical to that of the wild-type

Table 1: Kinetic Properties of *F. succinogenes* 1,3− 1,4-β-D-Glucanases[a]

enzyme	specific activity (units/mg)	k_{cat} (s^{-1})	K_m(lichenan) (mg/mL)	k_{cat}/K_m (s^{-1} M^{-1})	optimum temperature (°C)
wild-type	2065 ± 82	1296 ± 51	2.50 ± 0.09	518	50 (at pH 6.0)
PCR-TF-glucanase	7833 ± 334	3911 ± 166	2.88 ± 0.12	1358	50 (at pH 6.0)
TF-glucanase	7980 ± 341	3695 ± 157	2.89 ± 0.05	1279	50 (at pH 6.0)
glycosylated TF-glucanase	10800 ± 200	5360 ± 90	2.83 ± 0.09	1894	50 (at pH 6.0)

[a] Kinetic assays for wild-type and truncated glucanases were performed with lichenan as the substrate in 50 mM citrate buffer (pH 6.0).

enzyme. Glycosylated TF-glucanase could not be digested by the NANase II and *O*-glycosidase DS enzyme mixture (data not shown); however, after treatment of the enzyme with PNGase F, which cleaves Asn-linked oligosaccharides from glycoproteins, and under denaturing conditions a single protein band with mobility similar to that of the TF-glucanase was observed (Figure 1B). This result indicates that the two forms of TF-glucanase produced from *P. pastoris* were N-link glycosylated. Two potential N-glycosylation sites at Asn^{44} and Asn^{129} were predicted on the basis of the consensus sequence of protein glycosylation [Asn-Xaa-Ser/Thr, where Xaa can be any amino acid except Pro (*20*)].

The molecular masses of the two glycosylated forms of glycosylated TF-glucanase and its deglycosylated form were determined by LC−MS. The purified glycosylated TF-glucanase was separated and analyzed using LC−MS. Two peaks, monitored using UV, were detected, and their molecular masses were determined to be 31 850 and 29 983 Da with an accuracy of 0.01−0.02%. The molecular mass of the deglycosylated TF-glucanase was 27 957 Da. The net differences between the molecular masses of the two glycosylated forms of TF-glucanase and the deglycosylated enzyme (2026 and 3893 Da, respectively) indicate that the 29 983 Da TF-glucanase contains possibly a main oligosaccharide chain of $Man_{10}GlcNAc_2$. The 31 850 Da TF-glucanase possibly contains two oligosaccharide chains, mainly composed of $Man_xGlcNAc_2$ and $Man_yGlcNAc_2$ ($x + y = 19$), linked at two putative Asn glycosylation sites. The wild type, PCR-TF-glucanase, and TF-glucanase, without a six-His tag at its C-terminus, expressed in *E. coli* cells have molecular masses of 37 669, 29 722, and 27 744 Da, respectively.

The full-length enzyme, PCR-TF-glucanase, TF-glucanase, and glycosylated TF-glucanase exhibited similar resistance to trypsin protease digestion. After the enzymes were coincubated with trypsin for 5 h at 37 °C, more than 60% of the enzymatic activity was still detected (data not shown). This result demonstrates that neither protein glycosylation nor gene truncation at the C-terminus of *F. succinogenes* 1,3−1,4-β-glucanase affects its resistance to protease attack by trypsin. In contrast, neither the wild type nor truncated glucanases were detected as being active after being coincubated with pepsin protease in glycine buffer (pH 2.5) (data not shown).

Kinetic Analysis of Wild-Type and Truncated Forms of Fsβ-Glucanase. Kinetic studies were mainly performed using lichenan as the substrate, with a standard enzyme activity assay as described in Experimental Procedures. The specific activity of the wild-type (full-length) Fsβ-glucanase, PCR-TF-glucanase, and TF-glucanase from *E. coli* cells and glycosylated TF-glucanase from *Pichia* cells were 2065 ± 82, 7833 ± 334, 7980 ± 341, and 10 800 ± 200 U/mg, respectively (Table 1). Approximate 4−5-fold and 3−4-fold increases in specific activity and turnover rate (k_{cat}), respectively, were obtained for the truncated enzymes compared to the values for the wild-type enzyme. Similar K_m values for lichenan (2.5−2.89 mg/mL) were detected for the full-length and truncated glucanases (Table 1). Therefore, the catalytic efficiency (k_{cat}/K_m) of TF-glucanase and PCR-TF-glucanase was 2.47−2.62-fold higher than that of the wild-type enzyme. Moreover, glycosylation of the truncated glucanase exhibited an additive effect (3.7-fold higher than that of the wild-type enzyme) on the catalytic efficiency of the enzyme (Table 1). These results indicated that the protein truncation for ~10 kDa at the C-terminal region of Fsβ-glucanase resulted in a substantial improvement in its enzymatic activity, and the TF-glucanase and PCR-TF-glucanase with a 19-amino acid residue (1978 Da) difference at their C-termini have very similar catalytic activities.

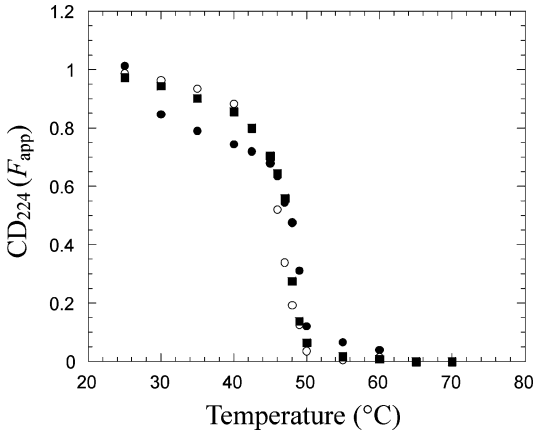

FIGURE 2: Titration curves of the CD at 224 nm represented as the apparent fraction of native protein, CD_{224} (F_{app}), at various temperatures in 50 mM sodium phosphate buffer (pH 7.0). CD_{224} signals from the full-length glucanase (■), TF-glucanase (○), and glycosylated TF-glucanase (●) at the indicated temperatures.

CD Spectrometric Analysis of the Melting Points of the Full-Length and Truncated Forms of 1,3−1,4-β-D-Glucanase. CD spectroscopy was employed for the determination of the melting points of wild-type (full-length) and truncated 1,3−1,4-β-D-glucanases. Far-UV (200−260 nm) CD spectra of the full-length and truncated glucanases were monitored at the indicated temperatures ranging from 25 to 70 °C, and the results at CD_{224} are presented in Figure 2. CD_{224} (F_{app}) represents the apparent fraction of native protein, which was calculated using the equation $F_{app} = (Y_{obsd} - Y_U)/(Y_N - Y_U)$ (*21*). Y_{obsd} represents the observed values of CD at 224 nm of the wild type, TF-glucanase, and glycosylated TF-glucanase at various temperatures. Y_N and Y_U represent the CD values at 224 nm of the wild type, TF-glucanases, and glycosylated TF-glucanase at 25 and 70 °C, respectively. As shown in Figure 2, similar melting points at ~47−48 °C

were observed for the full-length and truncated glucanases, suggesting that the serine-rich and basic terminal domain (BTD) at the C-terminus of Fsβ-glucanase does not play a direct or critical role in the structural stability of the enzyme.

Temperature and pH Effects on the Enzymatic Activity of Wild-Type and Truncated 1,3−1,4-β-D-Glucanases. The effects of temperature and pH on wild-type, PCR-TF-glucanase, TF-glucanase, and glycosylated TF-glucanase enzymes were further investigated to evaluate their biophysical properties. The optimum temperature for enzymatic activity of the wild-type and truncated 1,3−1,4-β-D-glucanases was found to be 50 °C when they were assayed with 50 mM citrate buffer (pH 6.0) (Table 1). The wild type and three forms of truncated enzymes were also found to exhibit similar thermostabilities between 30 and 90 °C. When incubated for 10 min at 45 and 50 °C, the full-length as well as the truncated enzymes exhibited approximately 80−90 and 50% activities, respectively, as compared to the enzymes without heat pretreatment. A dramatic loss of enzymatic activity occurred in full-length and truncated glucanases at temperatures higher than 55 °C (data not shown).

Various buffers at different pH values were employed for evaluating the optimum pH and pH tolerance of the full-length and truncated glucanases. The optimum pH values for enzymatic activity were determined between pH 6 and 8 for wild-type glucanase, PCR-TF-glucanase, and TF-glucanase and between pH 6 and 7 for glycosylated TF-glucanase. The effects of pH on the stability of the wild-type and truncated enzymes were also compared. The truncated glucanases, like the wild-type enzyme, were stable at pH 5−10, with a sharp decrease in activity observed, however, at low pH values (i.e., pH $\leq$4.0). All enzymes retained more than 90% of their original enzymatic activity after a 1 h incubation in buffers with pH values between pH 6 and 10 (data not shown). Approximately 65% enzymatic activity was noted in the full-length and truncated forms of glucanase after incubation for 1 h in 50 mM sodium acetate buffer (pH 4.0), and little enzymatic activity ($<$10%) could be detected when the enzymes were preincubated with buffers with a pH lower than 3.0.

Reactivation Profile of 1,3−1,4-β-D-Glucanase after Heat Treatment. To evaluate the efficiency of Fsβ-glucanase activity recovery after high-temperature treatment, the enzymatic activity of the wild-type, TF-glucanase, and glycosylated TF-glucanase enzymes, as a function of time at 25 °C, was examined after a 90 or 100 °C treatment. The wild-type enzyme, TF-glucanase, and glycosylated TF-glucanase recovered 8, 30, and 60%, respectively, of their original activity after the transfer of the 90 °C-pretreated enzymes to 25 °C for 3 min. However, more than 80% of the original activity was regained in the TF-glucanase and glycosylated TF-glucanase after a 20 min recovery at 25 °C; in comparison, only 30% of the original enzyme activity was recovered for the wild-type enzyme under the same conditions (Figure 3 inset). The restored enzymatic activity of the wild-type enzyme gradually decreased to less than 10% after prolonged recovery at 25 °C for 4 h; in contrast, TF-glucanase and glycosylated TF-glucanase were still able to retain $\geq$70% of their original activity after a 24 h recovery at 25 °C (Figure 3). PCR-TF-glucanase exhibited a reactivation profile very similar to that of TF-glucanase (data not shown).

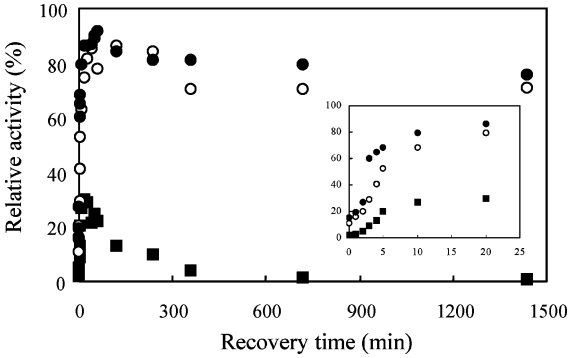

FIGURE 3: Recovery of specific enzymatic activity of wild-type Fsβ-glucanase (■), TF-glucanase (○), and glycosylated TF-glucanase (●) as a function of time at 25 °C after treatment at 90 °C for 10 min. Various enzyme samples at concentrations ranging from 0.042 to 0.005 μg/mL were pretreated at 90 °C for 10 min and then incubated at room temperature (25 °C). The enzymatic activity in test samples after heat treatment was determined by the standard enzyme assay described in Experimental Procedures. Relative enzyme activity represents the ratio of the recovered activity in heat-treated enzymes to the activity of the untreated enzyme.

Although the glycosylated TF-glucanase exhibited a reactivation profile very similar to that of TF-glucanase after heating at 90 °C for 10 min, a higher relative enzymatic activity was recovered in the glycosylated TF-glucanase than in the TF-glucanase after treatment at 100 or 90 °C for 30 min. After being heated at 90 °C for 30 min, glycosylated TF-glucanase regained 89% of its original activity after a recovery period of 20 min at 25 °C; however, TF-glucanase recovered only 67% of its original enzyme activity under the same conditions. After heat treatment at 100 °C for 10 or 30 min and a 20 min 25 °C recovery period, the glycosylated form of the enzyme recovered 88 and 66% of its original activity, respectively; TF-glucanase produced from *E. coli* cells, however, could only recover 72 and 56%, respectively, of its original enzymatic activity under the same conditions.

Fluorescence Spectrometric Analysis of Wild-Type and Truncated Fsβ-Glucanases. The structural integrity of the native, heat-denatured, and denatured−recovered wild-type and PCR-TF-glucanase was analyzed using fluorescence spectrometry. The emission spectra of full-length and truncated enzymes had a maximum emission peak at 335 nm (◇ symbols in Figure 4A,B). After the heat-denatured wild-type enzyme and the heat-denatured PCR-TF-glucanase had been heated at 90 °C for 10 min, their emission spectra exhibited slight bathochromic (red) shifts with a maximum peak at 338 nm, and the emission spectral values between 325 and 400 nm for both denatured forms of glucanase significantly increased (◆ symbols), as compared to those of the native form of the enzyme (◇ symbols). In light of the CD results (Figure 2), these data indicate that the structural integrities of wild-type and truncated glucanase proteins were both changed after high-temperature treatment. A room-temperature (25 °C) recovery process was then performed to evaluate the protein refolding efficiency of heat-treated full-length or truncated glucanases by measuring their fluorescence emission spectra. After the denatured−recovered wild-type enzyme had been heated at 90 °C for 10 min followed by recovery at 25 °C for 3 (▲) or 10 min (▽), its emission spectrum was unable to shift back to that of the native enzyme (◇); instead, it was more superimposed with

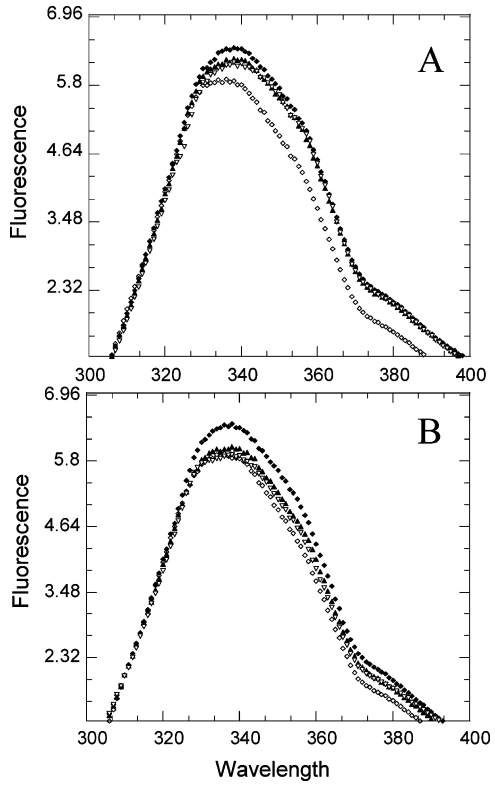

FIGURE 4: Fluorescence emission spectra of wild-type Fsβ-glucanase and PCR-TF-glucanase. The fluorescence emission spectra between 305 and 430 nm of full-length (A) and truncated glucanases (B) were quantitatively assayed upon excitation at 295 nm using a spectrofluorimeter: (◇) native enzyme, (◆) 90 °C-treated enzyme, (▲) 90 °C-treated enzyme, recovered at 25 °C for 3 min, and (▽) 90 °C-treated enzyme, recovered at 25 °C for 10 min.

the spectrum of the heat-denatured enzyme without a recovery process (◆) (Figure 4A). However, after recovery for 3 (▲) or 10 min (▽) at 25 °C, the emission fluorescence spectra of the denatured−recovered PCR-TF-glucanase had become closely superimposed with that of the native PCR-TF-glucanase enzyme (◇), especially at the emission peak area between 325 and 350 nm (Figure 4B). These results indicate that the protein refolding efficiency of the truncated enzyme after heat treatment is much superior to that of the full-length enzyme; this effectively explained the observations in Figure 4 in which 70−80 and 30% of enzymatic activities in the truncated and full-length enzymes, respectively, were recovered after heating at 90 °C for 10 min.

DISCUSSION

F. succinogenes 1,3−1,4-β-D-glucanase (Fsβ-glucanase) is classified as a member of the family 16 glycosyl hydrolases (*22*). A high degree of amino acid sequence homology (50−70%) exists between the various 1,3−1,4-β-D-glucanases isolated from bacterial and fungal species (*9*, *18*, *23*). Fsβ-glucanase, however, only shares approximately 30% of its primary protein sequence with the other family 16 glucanases, namely, the natural circularly permuted protein sequence in its catalytic domain and a unique segment of five PXSSSS repeats and a basic terminal domain (BTD) at the C-terminus (*7*). Although serine-rich regions are found in a number of 1,4-β-glycanases and are usually found in linker sequences connecting functional domains (*24*), the specific function of serine-rich regions in these microbial glucanases is still not clear. The 1,3−1,4-β-D-glucanase from *F. succinogenes* is the only lichenase so far identified possessing a serine-rich protein sequence and a BTD. To explore the structural and enzymatic function of the C-terminal repeat segment in Fsβ-glucanase and to improve the enzyme's industrial properties, we constructed truncated forms of recombinant Fsβ-glucanase by deleting its C-terminal serine-rich repeat segment and BTD using PCR gene truncation. We carried out comparative studies on initial rate kinetics and protein folding of the full-length and truncated glucanases.

Kinetic analyses of the truncated glucanase in this study, namely, PCR-TF-glucanase, TF-glucanase, and glcosylated TF-glucanase, revealed that they possessed more enzymatic activity than the wild-type enzyme. An approximately 2.85−4.1-fold increase in the turnover rate (k_{cat}) was found in the three forms of truncated Fsβ-glucanase, as compared to that of the wild-type (full-length) enzyme, while very similar K_m values were found for the full-length and truncated enzymes (Table 1). Therefore, deletion of the C-terminal serine-rich and BTD sequence of Fsβ-glucanase did not deleteriously affect enzyme catalysis. This observation is similar to the finding reported by Hall and Gilbert (*25*) concerning a carboxymethylcellulase from *Pseudomonas fluorescens* ssp. *cellulose*. This carboxymethylcellulase contains two regions rich in serine residues at its C-terminus, and the deletion of these serine-rich regions did not cause a concomitant loss of catalytic activity. However, a previous report (*26*) has demonstrated that the presence of a C-terminal BTD termed domain C in xylanase C from *F. succinogenes* appeared to decrease the overall catalytic activity of the enzyme 7-fold with birch wood xylan as the substrate. In the case of Fsβ-glucanase presented here, the deletion of the serine-rich segment and BTD did significantly increase its catalytic efficiency. Hydrophobic cluster analysis revealed a similar pattern of secondary structure between the BTD of Fsβ-glucanase and domain C of xylanase C (data not shown) that seems likely to have similar interference effects on the catalytic activities of the respective enzyme. Most importantly, with regard to the need of high-temperature industrial processes (e.g., feed pelleting and/or expansion at 65−90 °C), this deletion on Fsβ-glucanase significantly increased its efficiency of protein refolding after heating. From enzymatic activity and fluorescence spectrometry assays (results shown in Figures 3 and 4), the denatured−recovered full-length Fsβ-glucanase exhibited only 30% of its original, native enzymatic activity, and its fluorescence emission spectrum was more like that of the denatured form of the enzyme. The truncated enzymes, however, recovered more than 80% of their original activities and possessed a native form-like fluorescence emission spectrum after a heating and recovery process.

Recently, we determined the crystal structure of a truncated Fsβ-glucanase containing amino acid residues 1−258 of the wild-type enzyme, which exhibits a specific activity (7792 ± 424 U/mg), as well as a thermostability, very similar to that of TF-glucanase (containing residues 1−248) or PCR-TF-glucanase (containing residues 1−267), by the multiple-wavelength anomalous dispersion method (*27*). The overall topology of the truncated Fsβ-glucanase consists mainly of two eight-stranded antiparallel β-sheets arranged in a jellyroll

Table 2: Comparison of Kinetic Properties of Bacterial and Fungal 1,3−1,4-β-D-Glucanases[a]

enzyme (organism/source)	specific activity (units/mg)	k_{cat} (s^{-1})	optimum temperature (°C)	pH optima
glycosylated TF-glucanase[b]	10800 ± 200 (lichenan)	5360 ± 90	50	6.0−7.0
Orpinomyces strain PC-2[c]	3790 (lichenan)	1764	45	~6.0
	5320 (barley glucan)	2476		
B. macerans[d]	−	1880 ± 70 (lichenan) (at 50 °C)	65	7.0
H(A16-M)[d]	3731 ± 91	1860 ± 50 (at 50 °C)	64	6.5−7.0
	4890 ± 120 (lichenan)	2445 ± 60 (at 64 °C)		
CPA16M-59[d]	2833 ± 69	1450 ± 90 (at 50 °C)	62	6.5−6.8
	3930 ± 100 (lichenan)	2015 ± 51 (at 62 °C)		
Clostridium thermocellum[e]	214 (barley glucan)	135	80	8−9
	202 (lichenan)			
B. subtilis[f]	2600 (barley glucan)	1101	55	6.5
B. amyloliquefaciens[f]	2490 (barley glucan)	1077	55	6.5
Bacillus licheniformis[g]	900 ± 60 (barley glucan)	411 ± 27	55	7.0
lichenase (Megazyme)[h]	336 (barley glucan)	−	60	6.5−7.0

[a] All of the kinetic data from this study or from previous publications as indicated were determined using the dinitrosalicylic acid (DNS) assay method (*16*). Glucose was used as a standard for all enzymatic assays. [b] Data determined in this report. [c] Data from ref *18*. [d] Data from refs *17* and *28*. [e] Data from ref *9*. [f] Data from ref *19*. [g] Data from ref *30*. [h] Data from Megazyme (lot 30501).

β-sandwich that is conserved in families 16 and 7 in Clan-B and families 11 and 12 in Clan-C (http://afmb.cnrs-mrs.fr/CAZY/). On the basis of our current structural model (*27*), we predict that the deleted C-terminal peptide containing the five PXSSSS repeats and the basic terminal domain (BTD) segment is very likely to fold as an independent structural domain in Fsβ-glucanase. Moreover, the deleted 10 kDa peptide at the C-terminus may cause steric hindrance or electrostatic impulsion toward the concave cleft of the catalytic region in full-length Fsβ-glucanase that may result in a lower activity and a less efficient protein folding compared to those of the truncated enzyme after heat treatment. We have compared the sequence identity of the C-terminal extension of Fsβ-glucanase (~10 kDa) with those of all carbohydrate binding modules (CBM) reported at http://afmb.cnrs-mrs.fr/CAZY/. We found that our C-terminal 10 kDa peptide fragment exhibited a very low level of sequence identity (15−25%) with those CBM members containing serine repeats or serine-rich regions. In addition, deletion of the C-terminal peptide segment did not affect the K_m value for lichenan in the truncated enzyme, compared to that of the full-length enzyme (Table 1). Whether the C-terminal extension indeed is not involved in the interaction of the substrate with the Fsβ-glucanase enzyme warrants further elucidation. To shed more light on the functional or structural role(s) of the C-terminal PXSSSS repeats and BTD, we are investigating the three-dimensional structure of full-length Fsβ-glucanase using X-ray crystallography.

In summary, we created improved 1,3−1,4-β-D-glucanases (e.g., TF-glucanase) with increased catalytic activity and enhanced heat-tolerance capabilities. This truncated enzyme was also successfully expressed in an industrially acceptable host system, *Pichia* host cells. To our knowledge, the glycosylated form of TF-glucanase, created in this study, is the most active known 1,3−1,4-β-D-glucanase, with a specific activity of 10 831 ± 185 U/mg. A list of kinetic properties of bacterial and fungal 1,3−1,4-β-D-glucanases reported previously is given in Table 2. For example, a hybrid 1,3−1,4-β-D-glucanase, H(A16-M), constructed from the *Bacillus macerans* and *Bacillus amyloliquefaciens* enzymes (*28*), has a specific activity of 4890 ± 120 U/mg, with lichenan as a substrate at 65 °C (*29*). The V_{max} values for the fungal 1,3−1,4-β-D-glucanase from *Orpinomyces* strain PC-2, with lichenan and barley β-glucan as substrates at pH 6.0 and 40 °C, are 3790 and 5320 U/mg, respectively (*18*). A commercially available 1,3−1,4-β-D-glucanase (Megazyme International Ireland Ltd.) produced from *Bacillus subtilis* has a stated specific activity of only 336 U/mg. In comparison, the truncated enzymes created and characterized in this study have k_{cat} (s^{-1}) values 2.2−40-fold higher than those of other 1,3−1,4-β-D-glucanases listed in Table 2. The improved performance of this enzyme (compared to existing enzymes) with regard to the thermotolerance and catalytic activity could be of significance to the farm industry, as a feed or as a food processing aid. The potential benefits of this truncated Fsβ-glucanase thus warrant further evaluation, e.g., in animal field tests as a feed supplement.

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