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Structural and catalytic roles of residues located in β13 strand and the following β-turn loop in *Fibrobacter succinogenes* 1,3-1,4-β-D-glucanase

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ABSTRACT

Background: Fibrobacter succinogenes 1,3-1,4-β-D-glucanase (Fsβ-glucanase) is the only naturally occurring circularly permuted β-glucanase among bacterial glucanases with reverse protein domains. We characterized the functional and structural significance of residues 200–209 located in the domain B of Fsβ-glucanase, corresponding to the major surface loop in the domain A region of *Bacillus licheniformis* glucanase.
Methods: Rational design approaches including site-directed mutagenesis, initial-rate kinetics, and structural modeling analysis were used in this study.
Results: Our kinetic data showed that D202N and D206N exhibited a 1.8- and 1.5-fold increase but G207N, G207−, F205L, N208G and T204F showed a 7.0- to 2.2-fold decrease, in catalytic efficiency (k_{cat}/K_M) compared to the wild-type enzyme. The comparative energy $\Delta\Delta G_b$ value in individual mutant enzymes was well correlated to their catalytic efficiency. D206R mutant enzyme exhibited the highest relative activity at 50 °C over 10 min, whereas K200F was the most heat-sensitive enzyme.
Conclusions: This study demonstrates that Phe205, Gly207, and Asn208 in the Type II turn of the connecting loop may play a role in the catalytic function of Fsβ-glucanase.
General significance: Residues 200–209 in Fsβ-glucanase resided at the similar structural topology to that of *Bacillus* enzyme were found to play some similar catalytic function in glucanase.

1. Introduction

1,3-1,4-β-D-Glucanase or lichenase (1,3-1,4-β-D-glucan 4-glucanohydrolase, EC 3.2.1.73) is an endo-glycosidase that specifically cleaves β-1,4-glycosidic bonds in 3-*O*-substituted glucopyranose units through a double-displacement reaction assisted by general acid–base catalysis [1]. The natural substrates for this enzyme are β-glucans from grain endosperm cell walls or lichenan from the Iceland moss *Cetraria islandica*. Structurally, these substrates are linear homopolymers of glucose molecules linked via β-1,3- and β-1,4-glycosidic bonds at a ratio of ~1:2.5 [2,3]. When the substrates are hydrolyzed by 1,3-1,4-β-D-glucanase, trisaccharide 3-*O*-β-cellobiosyl-D-glucopyranose and tetrasaccharide 3-*O*-β-cellotriosyl-D-glucopyranose are the major products [4]. Various 1,3-1,4-β-D-glucanase genes from bacterial origins, including different *Bacillus* species [5–10], *Fibrobacter succinogenes* [11], *Ruminococcus flavefaciens* [12], and *Clostridium thermocellum* [13], and from higher plants such as barley [14,15], have been isolated, cloned and characterized. The microbial glucanases are classified as members of glycosyl hydrolase family 16 with a β-jellyroll structure, whereas the plant 1,3-1,4-β-D-glucanases belong to family 17 with a $(\beta/\alpha)_8$ three-dimensional structure. In general, the bacterial 1,3-1,4-β-D-glucanases are intrinsically more thermostable than the plant enzymes, and are important biotechnological aids in the brewing industry for reducing viscosity during mashing [16,17], and in the animal feeds industry for improving the digestibility of a plant-based diet [17,18].

Among bacteria that secrete 1,3-1,4-β-D-glucanase, *F. succinogenes* plays a major role in plant fiber degradation in the rumen of most livestock species. The protein sequence (349 amino acids; gene accession number: M33676) of *F. succinogenes* glucanase (Fsβ-glucanase) contains two highly conserved domains (A and B) and is circular permutated as well as oriented in a reverse order (B to A) to that of other 1,3-1,4-β-D-glucanases (A to B) from different origins [11,19–21]. Moreover, Fsβ-glucanase contains a five PXSSSS (P: proline; S: serine; X: possibly any amino acid) repeated segment and a basic terminal domain (BTD) rich in positively charged amino acids at its C terminus, which is not observed in other bacterial or fungal 1,3-1,4-β-D-glucanases [11,22]. Recently, we observed that a truncation on the C-terminal region containing the quintet repeat and BTD domain can greatly increase the specific activity and thermotolerance of Fsβ-glucanase [22]. In our previous studies of Fsβ-glucanase, we also demonstrated that Glu56, Asp58 and Glu60 residues played important role(s) in the catalysis of Fsβ-glucanase

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[23], and Trp54, Trp141, Trp148 and Trp203 are crucial residues for maintaining structural integrity in the substrate-binding cleft and for the catalytic efficiency of the enzyme [24]. However, issues concerning the structural and functional relation of catalysis in Fsβ-glucanase as well as specific amino acids involving in substrate binding still remain to be further clarified.

Recently, we solved the crystal structure of TFsβ-glucanase, a truncated form of Fsβ-glucanase, using the multiple-wavelength anomalous dispersion (MAD) method [21]. The crystal structure of TFsβ-glucanase in complex with β-1,3-1,4-cellotriose (CLTR), a major product of the enzyme reaction, was also elucidated [25]. The TFsβ-glucanase is composed mainly of two antiparallel β-sheets with seven and eight strands arranged on top of each other to form a jellyroll β-sandwich structure (Fig. 1A). The substrate-binding site in the enzyme was located in a channel of approximately 25 Å long at the concave side of the protein molecule, which could accommodate five glucopyranose residues. The amino acid residues from Lys200 to Thr204 were located at the C-terminal end of strand β13, and residues from Phe205 to Arg209 followed in a β-turn loop connecting strands β13 and β14 and located at up-right of the concave side of the enzyme

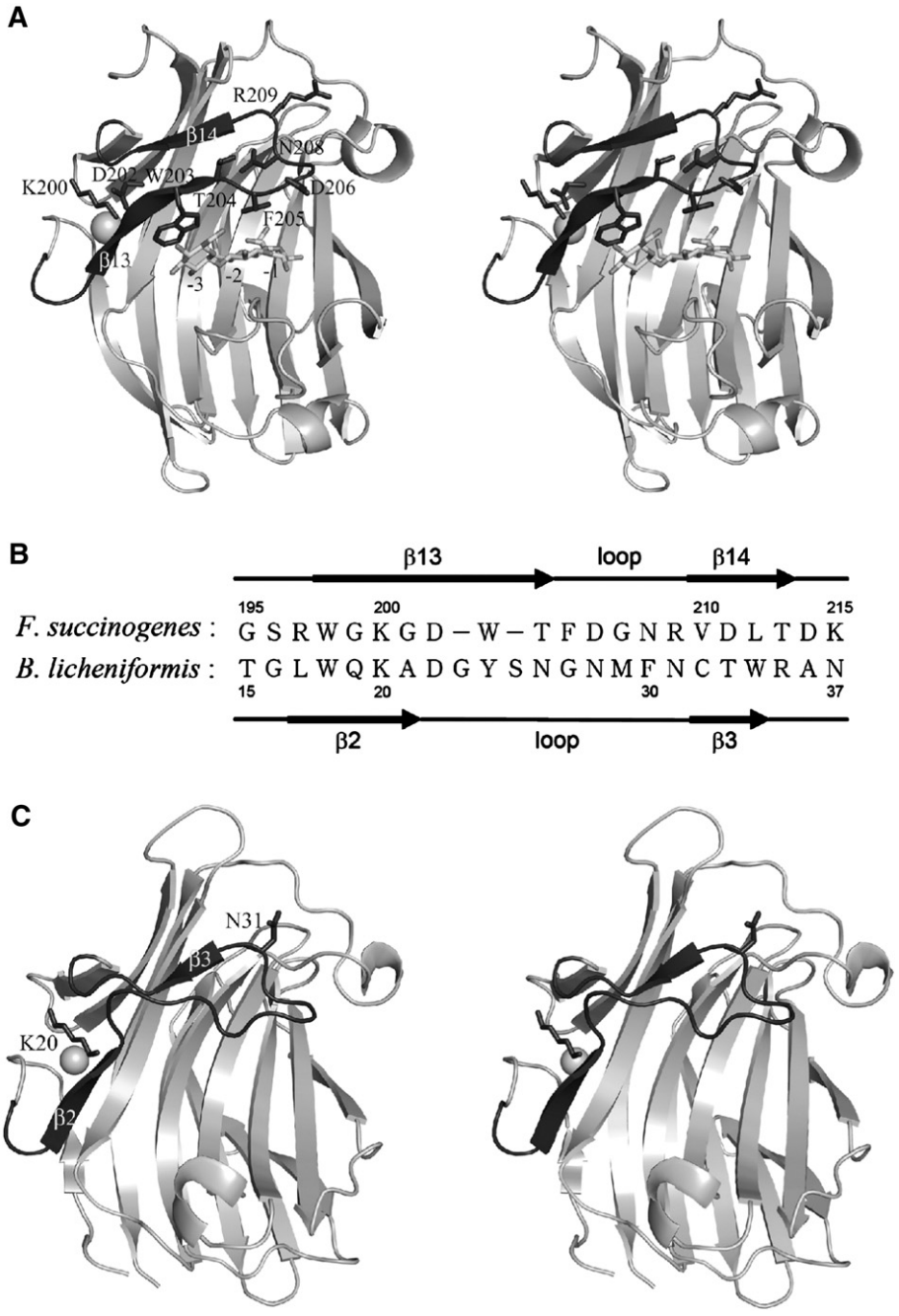

Fig. 1. (A) A ribbon drawing of the structure of truncated *Fibrobacter succinogenes* 1,3-1,4-β-D-glucanase (TFsβ-glucanase) in complex with β-1,3-1,4-cellotriose (CLTR). The overall topology of the TFsβ-glucanase–CLTR complex consists mainly of two eight-stranded anti-parallel β-sheets arranged in a jellyroll β-sandwich, and both β-sheets are twisted and bent, which results in a convex and a concave side of the molecule. The sugar product β-1,3-1,4-cellotriose is labeled as −3 to −1 (−1 is the reducing end). The calcium ion is displayed as a ball located on the convex side of the protein. (B) Structure-assisted sequence alignment of 1,3-1,4-β-D-glucanases from *F. succinogenes* and *B. licheniformis* was performed with use of ALSCRIPT [34]. Residues 200-209 located in the C-terminal half of strand β13 and the following loop to connect strand β14 of Fsβ-glucanase were related to the residues 20–31 located in the loop region between strands β2 and β3 in *B. licheniformis* 1,3-1,4-β-D-glucanase. (C) A ribbon drawing of *B. licheniformis* 1,3-1,4-β-D-glucanase structure showing the sequence of Lys20–Asn31, which forms a surface loop connecting β2 and β3 and covers the partial carbohydrate-binding cleft of the enzyme [27].