Structure and Function of a Family 10 β-Xylanase Chimera of *Streptomyces olivaceoviridis* E-86 FXYN and *Cellulomonas fimii* Cex

Satoshi Kaneko1*, Hitomi Ichinose1, Zui Fujimoto2, Atsushi Kuno3, Kei Yura4, Mitiko Go4, Hiroshi Mizuno2, Isao Kusakabe5, and Hideyuki Kobayashi1

From the 1National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan, the 2National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan, the 3Department of Material & Biological Chemistry, Faculty of Science, Yamagata University, Yamagata 990-8560, Japan, the 4Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8602, Japan, and the 5Institute of Applied Biochemistry, University of Tsukuba, 1-1-1 Tennoodai, Tsukuba, Ibaraki 305-8572, Japan

**Keywords:** xylanase; glycoside hydrolase family 10; (β/α)$_8$-barrel; chimeric enzyme; topology of substrate binding cleft; module shuffling

**Running Title:** Structure and function of a chimeric xylanase

*To whom correspondence should be addressed. Tel.: +81-298-38-8063; Fax: +81-298-38-7996; E-mail: sakaneko@nfri.affrc.go.jp

**The abbreviations used are:** GH, glycoside hydrolase family; 3D, three-dimensional; dp, degree of polymerization; SoXyn10A, *Streptomyces olivaceoviridis* E-86 family GH10 xylanase (formerly known as FXYN); CfXyn10A, *Cellulomonas fimii* family
GH10 xylanase (formerly known as Cex); BCF, bond cleavage frequency; PNP-G2, \(p\)-nitrophenyl-\(\beta\)-D-cellobioside; PNP-X2, \(p\)-nitrophenyl-\(\beta\)-D-xylobioside; PCR, polymerase chain reaction; TIM, triose phosphate isomerase; 4MeGlcAX4, 4-O-methyl-\(\alpha\)-D-glucuronosyl-(1\(\rightarrow\)2)-\(\beta\)-D-xylopyranosyl-(1\(\rightarrow\)4)-\(\beta\)-D-xylopyranosyl-(1\(\rightarrow\)4)-\(\beta\)-D-xylopyranoside; 4MeGlcAX3, 4-O-methyl-\(\alpha\)-D-glucuronosyl-(1\(\rightarrow\)2)-\(\beta\)-D-xylopyranosyl-(1\(\rightarrow\)4)-\(\beta\)-D-xylopyranosyl-(1\(\rightarrow\)4)-\(\beta\)-D-xylopyranoside; HPAEC-PAD, high performance anion-exchange chromatography with pulsed amperometric detection; ESI-MS, electrospray ionization mass spectrometry; NMR, nuclear magnetic resonance; McIlvaine buffer, 0.1 M citric acid-0.2 M Sodium phosphate buffer; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; FC-14-15, a chimeric xylanase comprising modules M1 to M14 from SoXyn10A and modules M15 to M22 from CfXyn10A; CF-14-15, a chimeric xylanase comprising modules M1 to M14 from CfXyn10A and modules M15 to M22 from SoXyn10A.

**PDB code deposition information:** Crystal structure of the chimera of SoXyn10A and CfXyn10A (FC-14-15), 1v6y. The atomic coordinates (code 1v6y) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
ABSTRACT

The catalytic domain of xylanases belonging to glycoside hydrolase family 10 (GH10) can be divided into 22 modules (M1 to M22; Sato, Y., Niimura, Y., Yura, K., and Go, M. (1999) Gene 238, 93-101). Inspection of the crystal structure of a GH10 xylanase from Streptomyces olivaceoviridis E-86 (SoXyn10A) revealed that the catalytic domain of GH10 xylanases can be dissected into two parts, an N-terminal larger region and C-terminal smaller region, by the substrate binding cleft, corresponding to the module border between M14 and M15. It has been suggested that the topology of the substrate binding clefts of GH10 xylanases are not conserved (Charnock, S.J., Spurway, T.D., Xie, H., Beylot, M.H., Virden, R., Warren, R.A.J., Hazlewood, G.P., and Gilbert, H.J. (1998) J. Biol. Chem. 273, 32187-32199). To facilitate a greater understanding of the structure-function relationship of the substrate binding cleft of GH10 xylanases, a chimeric xylanase between SoXyn10A and Xyn10A from Cellulomonas fimi (CfXyn10A) was constructed and the topology of the hybrid substrate binding cleft established. At the three-dimensional level, SoXyn10A and CfXyn10A appear to possess 5 subsites, with the amino acid residues comprising subsites –3 to +1 being well conserved, while the +2 subsites are quite different. Biochemical analyses of the chimeric enzyme along with SoXyn10A and CfXyn10A indicated that differences in the structure of subsite +2 influence bond cleavage frequencies and the catalytic efficiency of xylooligosaccharide hydrolysis. The hybrid enzyme constructed in this study displays fascinating biochemistry, with an interesting combination of properties from the parent enzymes, resulting in a low production of xylose.
INTRODUCTION

The plant cell wall consists mainly of a complex mixture of polysaccharides such as cellulose, pectins and hemicellulose (1), where the latter is comprised mainly of xylan. The backbone of xylan is formed by β-1,4-linked d-xylopyranose units to which several side groups such as α-1,2-linked 4-O-methyl d-glucuronic acid and α-1,3-linked l-arabinofuranose are attached (2). β-Xylanases (EC 3.2.1.8) randomly hydrolyze β-1,4-glycosidic linkages within the xylan backbone via a double displacement mechanism involving a carboxylate functioning as a proton donor (the acid/base catalyst), which facilitates the dissociation of the poor glycosidic leaving group, and a nucleophilic carboxylate involved in formation of a covalently linked enzyme-substrate intermediate (3). On the basis of primary structure homology, the majority of xylanases have been classified into glycoside hydrolase families 10 and 11 (GH10 and GH11, respectively) (4). The three-dimensional (3D) structures of ten GH10 xylanases have now been solved (5-11). They all have very similar structures, comprising (β/α)₈-barrels as well as additional helices and loops which are arranged in a basic TIM-barrel structure forming the active site cleft (9). The cleft forms deep grooves consistent with the endo-mode of action, and comprises a series of subsites, each one tailored towards the binding of a single xylose moiety (11). The subsites that bind the glycone and aglycone regions of the substrate are prefixed by (-) and (+), respectively, and their numbers are related to the proximity to the site of bond cleavage (the glycosidic bond between the +1 and -1 subsites is cleaved by the enzyme (12)). Analysis of active site amino acids playing important roles in substrate binding and catalysis have been extensively facilitated by the elucidation of crystal structures of GH10 xylanases covalently linked to mechanism-based cellobiosyl and xylobiosyl inhibitors (13) and also by site directed mutagenesis (14, 15). Although GH10 xylanases display the same
gross fold, the topology of their substrate binding clefts is not conserved (15), and thus differences in substrate specificity could be expected.

*Cellulomonas fimi* endo-xylanase Xyn10A (CfXyn10A, formerly known as Cex) is one of the most characterized xylanases (13, 16-22). Based on the 3D structure of CfXyn10A, the catalytic domain has been divided into 22 modules (M1 to M22) (23), where a module is defined as a contiguous polypeptide segment of a protein which has a compact conformation within a globular domain. A module is defined by the distance between Cα atoms, and on average a module is about 15 amino acid residues long (24). Interestingly, thirty-one intron sites in fungal genes encoding GH10 xylanases were found to correlate to module boundaries with considerable statistical significance (23). This indicates that the location of introns in eukaryotic xylanase genes are not random and supports the concept that introns play an important role in protein evolution as mediators of exon shuffling (25). Therefore, module shuffling *in vitro* mimics one of the natural mechanisms of protein evolution. Thus, module shuffling was selected as a tool to study the structure-function relationships of xylanases.

The GH10 enzyme Xyn10A from *Streptomyces olivaceoviridis* E-86 (SoXyn10A, formerly known as FXYN) was selected as one of the parent enzymes as this enzyme is amenable to crystallization (11, 26), and has been cloned (27). In addition, the substrate specificity of SoXyn10A has been well characterized (28-36). Further, we have succeeded in soaking xylotriose into both the glycon and aglycon region of the substrate binding cleft (37). As shown in Figure 1, the catalytic domain of GH10 xylanases is sub-divided into two parts which consist of the N-terminal larger region and the C-terminal smaller region. The boundary between module M14 and M15 corresponds to the border between these two regions. In the present investigation, we selected the boundary between modules M14 and M15 for shuffling to understand the
topology and function of the substrate binding cleft. In this paper, the structure and function of the resultant chimeric GH10 xylanases are described.

MATERIALS AND METHODS

Construction of chimeric enzymes—The catalytic domain of SoXyn10A and CfXyn10A were separately subcloned into the pQE60 vector (QIAGEN, Hilden, Germany). Construction of the chimera was performed by the polymerase chain reaction (PCR) using overlapping primers at their respective module boundaries (Figure 2). The DNA fragment from SoXyn10A encoding modules M1 to M14 and the DNA fragment from CfXyn10A encoding modules M15 to M22 were amplified using the following sets of primers for FC-14-15 (M1-M14 sense: 5’-CCA TGG GCT CCT ACG CCC TTC CCA GAT CAG-3’; M1-M14 antisense: 5’-GCG ACT GGA AGC CAA CGC AGT CGA TTG GCA CGC C-3’; M15-M22 sense: 5’-CTG CGT CGG CTT CCA GTC GCA CCT CAT CGT CGG CC-3’; M15-M22 antisense: 5’-GGA TCC GAA GGC TTC CAT CAC GGC GGC GTA GG-3’) and for CF-14-15 (1-14 sense: 5’- CCA TGG CTA GGA CCA CGC CCG CAC CCG -3’; M1-M14 antisense: 5’-GCG ACT GGA ATC CTA CAC AGT CGA GCG GGA CGC C-3’; M15-M22 sense: 5’-CTG CGT CGG GTT CCA GTC ACA CTT CAA CAG CGG CAG C-3’; M15-M22 antisense: 5’- GGA TCC AGC GTT GAG GAC GGC GGT GTA GGC AGC -3’). Each of the 25 amplification cycles consisted of denaturation at 98°C for 1 min and annealing and primer extension at 72°C for 1 min. The 10 bp overlapping regions (underlined) of the primers were designed to be complementary at their respective module boundaries. The first round of PCR products were separated by agarose gel electrophoresis, followed by gel extraction, and used for the second round PCR without primer. Each of the 20 amplification cycles consisted of denaturation at 98°C for 1 min, annealing at 60°C for
25 min and primer extension at 72°C for 5 min. The strands having matching sequences at their respective module boundaries overlapped and acted as primers for each other. On the 3rd round of PCR, the combined fragment was amplified with PCR primers for FC-14-15 (sense: 5’-CCA TGG GCT CCT ACG CCC TTC CCA GAT CAG-3’ and antisense: 5’-GGA TCC GAA GGC TTC CAT CAC GGC GGC GTA GG-3’) for CF-14-15 (sense: 5’-CCA TGG CTA GGA CCA CGC CCG CAC CCG-3’ and antisense: 5’-GGA TCC AGC GTT GAG GAC GGC GGT GTA GGC AGC-3’) by 25 cycle of shuttle PCR with denaturation at 98°C for 1 min and annealing and primer extension at 72°C for 1 min.

**Gene Expression and Protein Purification**—For expression in *E. coli* and purification of the SoXyn10A, CfXyn10A, FC-14-15 and CF-14-15, the pET expression system (NOVAGEN, Madison, WI, USA) was employed. Thus, each gene was individually inserted into the pET28 vector (to yield pETfxyn, pETcex, pETFc-14-15, and pETcf-14-15, respectively). The enzymes were expressed as fusion proteins that comprised each enzyme plus a carboxyl-terminal tag of six histidine residues. The recombinant plasmids were used to transform *E. coli* BL21 (DE3) and transformants were cultivated at 25°C in LB medium (1 liter) that contained kanamycin (20 µg/ml) until the optical density at 600 nm reached 0.4. After addition of isopropyl-1-thio-β-D-galactoside (IPTG) to a final concentration of 1 mM, the culture was incubated at 25°C for 24 h. After the *E. coli* cells were removed from the culture by centrifugation (6,000 x g, 10 min), ammonium sulphate was added to give a 70% saturation level and the resulting mixture was kept at 4°C for 16 h. The precipitate was collected by centrifugation (10,000 x g, 20 min) and dissolved in a small amount of distilled water followed by dialysis against deionized water. After removal of insoluble
material by centrifugation (12,000 g, 30 min), the obtained solution was then loaded on a HisTrap™ chelating column (Amersham Bioscience, Piscataway, NJ, USA). The bound enzyme was eluted with a 50 mM phosphate buffer (pH 7.0) containing 250 mM imidazole. The elution of the enzyme was monitored by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (38). The enzyme eluted as a homogenous protein as detected by SDS-PAGE, and the relevant fractions were pooled and dialyzed against deionized water.

**Crystallization and data collection**—Crystallization trials of FC-14-15 were conducted using the modified crystallization conditions for SoXyn10A (26). FC-14-15 was crystallized by the hanging drop vapor diffusion method at room temperature using a 20 mg/ml protein solution and a reservoir solution comprising 1.2 M ammonium sulfate and 2% McIlvaine buffer (a mixture of 0.1 M citric acid and 0.2 M Na₂HPO₄, pH 5.5). After two weeks, thin needle crystal clusters grew to a size of 0.1 x 0.1 x 0.4 mm.

Diffraction experiments were conducted at beam-line 6A at the Photon Factory in Tsukuba, Japan, with a Weissenberg camera for macromolecules (39) at room temperature. The collimator used was 0.1 mm and the wavelength was 1.0 Å. Intensity data covering a total rotation of 180° was collected using a single crystal. Diffraction data were detected using a imaging plate (20 x 40 cm) and digitized by BA2000 (Fuji film, Tokyo, Japan). Crystals of FC-14-15 diffracted beyond 2.0 Å resolution. The data sets were processed using the program DENZO, and scaled using the program SCALEPACK (40). The crystals are orthorhombic and belong to space group P2₁2₁2₁, with cell dimensions of \( a = 48.48 \text{Å} \), \( b = 57.19 \text{Å} \), and \( c = 106.67 \text{Å} \), containing one molecule in the asymmetric unit. The collected native intensity data set included a total
of 52,375 observations, which were reduced to 13,853 unique reflections with a completeness of 88.3% and a merging R-factor of 0.065 against the data from 100 to 2.2 Å resolution.

Structure determination—The structural analysis was performed using the molecular replacement method with the program AMoRe (41) in the CCP4 suite (42) (Table 1). Two structures of SoXyn10A (PDB accession code 1xyf) and CfXyn10A (2exo) were superimposed based on topological alignment. A 3D model of FC-14-15 was built by connecting the C-terminal 1-203 residues of SoXyn10A and C-terminal 202-310 of CfXyn10A and was used as a search model. This model was subjected to a rotation search using the 8.0-3.5 Å resolution data. A single high peak had correlation coefficients of 0.189 and the following translation search resulted in a correlation coefficient of 0.510 and an R-value of 41.3%. The model thus positioned was subjected to cycles of rigid-body refinement against the data from 30 to 2.2 Å resolution using the program CNS (43), resulting in proper structure models. Ten percent of the observed reflections were randomly removed for cross-validation (44). The structure was refined by iterative cycles of simulated annealing and manual model rebuilding, conducted on the resultant $2F_{obs} - F_{calc}$ and $F_{obs} - F_{calc}$ maps, using the program CNS. The structure was refined to an R-factor of 15.3 % and an $R_{free}$-factor of 19.8%. The stereochemistry of the model was analyzed with the program PROCHECK (45). In a Ramachandran plot (46), 91.7 % of the non-glycine residues were in the most favoured regions of phi-psi plot and the remainder were in additional allowed regions.

Enzyme Assays and Source of the Substrate—Steady-state kinetics were investigated as previously reported (33). Briefly, the reaction mixture containing 250 µl
of substrate solution ($p$-nitrophenyl-$\beta$-D-xylobioside (PNP-X2) or $p$-nitrophenyl-$\beta$-D-celllobioside (PNP-G2)) at various concentrations, 150 µl of McIlvaine buffer (a mixture of 0.1 M citric acid and 0.2 M Na$_2$HPO$_4$, pH 7.0) and 50 µl of 1% (w/v) BSA (bovine serum albumin) was incubated at 30°C for 5 min and then 50 µl of enzyme solution was added. The amount of $p$-nitrophenol released was determined by monitoring the absorbance at 400 nm as a function of time with a spectrometer (DU-7400; Beckman, Palo Alto, CA). The kinetic parameters $k_{cat}$ and $K_m$ were determined by Eadie-Hofstee plot from three independent experiments, and at a five substrate concentrations. PNP-X2 was synthesized by the method described in a previous paper (47). The xylobiose used in the synthesis was purified from ‘Xylobiose Mixture’ (SUNTORY Ltd., Osaka, Japan). PNP-G2 was a generous gift from Yaizu Suisan Co. Ltd. (Yaizu, Japan).

For the hydrolysis of soluble birchwood xylan, reaction mixtures containing 150 µl of McIlvaine buffer, 50 µl of 1% BSA (w/v) and 250 µl of birchwood xylan solution (0.2 mg/ml; Fulka, Neu-Ulm, Switzerland) were equilibrated at 30°C for 5 min, and then reactions were initiated by the addition of 50 µl of enzyme solution (the final concentrations of SoXyn10A, CfXyn10A and FC-14-15 were 0.002, 0.004 and 0.005 mg/ml). The increase in reducing power was measured by the method of Fox and Robyt (48). The hydrolysis products were also analyzed using a Carbo Pac PA-1 column (Dionex Co. Ltd., Sunnyvale, CA, USA) with high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) system with a flow rate of 1 ml/min and elution with 0.1 M NaOH (0-5 min), followed by a linear gradient (5-35 min) of sodium acetate (0-0.4 M).

Substituted xylooligosaccharides such as 4-0-methyl-$\alpha$-D-glucuronosyl-$(1\rightarrow2)$-$\beta$-D-xylopyranosyl-$(1\rightarrow4)$-$\beta$-D-xylopyranosyl-$(1\rightarrow4)$-$\beta$-D-xylopyranosyl-$(1\rightarrow4)$-$\beta$-D-xylopyranosyl-$(1\rightarrow4)$-$\beta$
-D-xylopyranoside (4MeGlcUAX4) and 4-O-methyl-α-D-glucuronosyl-(1→2)-β-D-
xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-xylopyranoside (4MeGlcUAX3)
were prepared from the final reaction products of birchwood xylan hydrolysis by
FC-14-15 according to the method described previously (30) and the structure of the
oligosaccharides determined by nuclear magnetic resonance (NMR) and electrospray
ionization mass spectrometry (ESI-MS). 1H and 13C NMR experiments were recorded
at 303 K with a Bruker Avance-500 spectrometer. ESI-MS was performed in the
negative ion mode. Solutions of oligosaccharides (100 µg) in aqueous 30% methanol
containing 0.75% HCl (100 µl) were infused into the electrospray source at 4 µl per min.
The ion spray was operated at 5000 V with an orifice potential of 35 V. Ten scans
(100-2500 amu) were collected and averaged.

Bond cleavage frequencies (BCFs) and activities for the hydrolysis of
xylooligosaccharides, and the calculation of subsite binding energies were performed as
described previously (15). These values were determined from three independent
experiments. To evaluate the catalytic efficiencies of the xylanases against
xylooligosaccharides, 0.3–800 nM of enzyme were incubated with 10 µM of substrate
in McIlvaine buffer, pH 7.0 for up to 200 min at 30°C. At regular time intervals, a
0.1-ml aliquot was removed, the enzyme was inactivated by adding sodium hydroxide
to the concentration of 0.1 M, and the xylooligosaccharides in the samples were
quantified by HPAEC-PAD as described above using L-fucose as an internal standard.
The progress curves of oligosaccharide cleavage were used to determine the $k_{cat}/K_m$
of the reaction using the following equation described by Matsui et al. (49, 50),

\[
k \cdot t = \ln([S_0] / [S_t]) \quad \text{(Eq. 1)}
\]
where \( k = \frac{k_{\text{cat}}}{K_m}[E] \), \( t \) represents time, and \([S_0]\) and \([S_t]\) represent substrate concentrations at time 0 and \( t \), respectively. This relationship is only valid when \([E] \ll [S] \ll K_m\). The concentration of substrate in these reactions was \( \ll K_m \) and was low enough to prevent transglycosylation reactions from occurring. To determine the BCF, 0.3–800 nM of each of the enzymes were incubated with the different substrates (10 \( \mu \)M) in McIlvaine buffer, pH 7.0 at 30 °C. After 30 and 60 min incubations, a 0.1-ml aliquot was removed, the enzyme was inactivated by adding sodium hydroxide to the concentration of 0.1 M, and the xyloooligosaccharides and methylated xyloooligosaccharides in the samples were quantified by HPAEC-PAD as described above using L-fucose as an internal standard. The BCF and \( k_{\text{cat}}/K_m \) data obtained from these experiments were used to calculate the \( \Delta G \) of xylose binding at each of the subsites, following the method of Saganuma et al. (51). The equation used to calculate the free energy of ligand binding was as follows.

\[
\Delta G (\text{kcal/mol}) = R \cdot T \cdot \ln \left( \frac{k_{\text{cat}}}{K_m(x_a)} \cdot \text{BCF}(X_a)^b / k_{\text{cat}}/K_m(x_{a-1}) \cdot \text{BCF}(X_{a-1})^b \right) / 4183 \quad \text{(Eq. 2)}
\]

The kinetic parameters are as follows: \( k_{\text{cat}}/K_m \) (\( X_a \)) represents \( k_{\text{cat}}/K_m \) for a xyloooligosaccharide of degree of polymerization (dp) \( a \); \( \text{BCF}(X_a)^b \) represents bond cleavage frequency for glycosidic bond \( b \) of a xyloooligosaccharide of dp \( a \); \( R \) is the gas constant (8.314 Jmol\(^{-1}\)K\(^{-1}\)); and \( T \) is temperature (K). The series of methylated xyloooligosaccharides from methyl-\( \beta \)-D-xyloside to methyl-\( \beta \)-D-xylohexaoside were synthesized (Kitaoka et al. manuscript in preparation).

For the hydrolysis of 4MeGlcUAX4, reaction mixtures (total 1 ml) containing 100 \( \mu \)l of McIlvaine buffer, 10 \( \mu \)l of 1% BSA (w/v), 71 \( \mu \)l of 4MeGlcUAX4 solution...
(final concentration 10 µM), 70 µl of 0.005% fucose and 699 µl of deionized water were equilibrated at 30°C for 5 min, and then reactions were initiated by the addition of 50 µl of enzyme solution (the final concentrations of SoXyn10A, CfXyn10A and FC-14-15 were 400 nM). After incubation for 0, 20, 40, 80, 120, and 200 min at 30°C, the reaction was stopped by boiling for 30 min. The reaction mixtures were analyzed by HPAEC-PAD as described above. Hydrolysis extents against 4MeGlcUAX4 were calculated from peak areas using L-fucose as an internal standard. The assay was performed in triplicate.

**RESULTS AND DISCUSSION**

*Construction of chimeric xylanase*—At present, ten GH10 xylanase structures have been solved. The fold of the catalytic domain of all these enzymes is well conserved, however, the topologies of the substrate binding cleft are quite different (15). In the known GH10 structures, the structure of the subsites on the glycon side of the substrate binding cleft is highly conserved, while the aglycon regions are quite different. Therefore, the substrate specificity of GH10 xylanases is probably determined by the structure of the aglycon side of the substrate binding cleft. The catalytic domain of GH10 xylanases can be structurally subdivided into the N-terminal larger part and the C-terminal smaller part by the substrate binding cleft. This boundary corresponds with the module boundary between modules M14 and M15 (Figure 1). Therefore, in this study, we used the boundary of M14 and M15 for the gene shuffling study to understand the topology and function of substrate binding cleft. Two kinds of chimeric enzymes such as FC-14-15 and CF-14-15 were constructed and the topology of the substrate binding cleft was characterized. The chimera FC-14-15 construct was composed of modules M1 to M14 from SoXyn10A and modules M15 to M22 from CfXyn10A, while
the construct CF-14-15 was composed of modules M1 to M14 from CfXyn10A and modules M15 to M22 from SoXyn10A. The chimeric xylanases were constructed by splicing PCR (Figure 2). CF-14-15 was purified as a soluble protein, but was not active. Circular dichroism spectra of CF-14-15 indicated that the enzyme did not fold in a similar way to the parental enzymes (data not shown). Attempts to refold the protein were unsuccessful. Therefore, only FC-14-15 was further characterized.

Structure of FC-14-15—The crystal structure of FC-14-15 was determined at 2.2 Å resolution by the molecular replacement method (Figure 1). This model includes 316 amino acids and 240 water molecules. Two N-terminal residues (Ala-1 and Glu-2) were disordered and could not be observed in the electron density. Four C-terminal residues (Gly-Ser-Arg-Ser) which originate from the vector were visible in the electron density and were added to the model. The overall architecture was a β/α-TIM barrel motif as commonly seen in the catalytic domain of GH10 xylanase. Root-mean-square (rms) difference for all the corresponding Cα-carbon atoms between M1-M14 of the chimera and SoXyn10A was 0.248 Å and that between M15-M22 atoms of the chimera and CfXyn10A was 0.193 Å, indicating that the structure of FC-14-15 was basically conserved from that of the parent proteins.

Figure 3 shows the superposition around the catalytic cleft of FC-14-15 with both parent proteins. The bound xylooligosaccharides shown are from the structure of SoXyn10A in complex with xylotriose (37). It is apparent that the structure of the subsites on the glycon side is highly conserved between SoXyn10A and CfXyn10A. However, the structure of the aglycon side is very different. In SoXyn10A, the aglycon side of the catalytic cleft is different from CfXyn10A, as shown in Figure 3, especially in the vicinity of the loop region Asn-209-Pro-213, which protruded out to the end of
the glycon side of the catalytic cleft compared to the other xylanase. In the xylotriose complex with SoXyn10A (37), Asn-209 and Ser-212 in this loop together with Arg-275 coordinate to the +2 xylose unit and the position of the bound xylose at subsite +2 was different from that of xylose binding to the other xylanases (52, 53).

On the other hand, residues which are expected to be involved in substrate binding in FC-14-15 and which originated from CfXyn10A, are indicated in orange letters in Figure 3. Gln-203 and Trp-281 of CfXyn10A are conserved in SoXyn10A, and have contacts to xylose at subsites -1 and +1. Arg-237 seems to hydrogen bond to the xylose at subsite +2. The position of Arg-237 is close to that of Asn-209 of SoXyn10A. Phe-286 is located close to subsite +2, and its position is occupied by Arg-275 in SoXyn10A. Therefore, xylose at subsite +2 in FC-14-15 is probably sandwiched by two aromatic residues, Phe-286 from CfXyn10A and Tyr-172 from SoXyn10A, and is expected to bind tightly to the cleft as in the parent CfXyn10A, which has two aromatics in equivalent positions. The tight binding at subsite +2 by CfXyn10A is demonstrated by high binding energy at this subsite compared to Xyn10A from Cellvibrio japonicus (formerly Pseudomonas cellulosa) (15).

As shown in Figure 3, the crystal structure of FC-14-15 revealed that all amino acid residues in the catalytic cleft retained their structures from their parent enzyme. Consequently, the structure of the glycon subsites of FC-14-15 were conserved from both parent enzymes, while that of the subsite +1 was hybrid between SoXyn10A and CfXyn10A and that of the subsite +2 was similar to that of CfXyn10A.

Kinetics—Kinetic data were measured for FC-14-15 along with parental SoXyn10A and CfXyn10A using PNP-G2 and PNP-X2 as the substrates (Table 2). Compared with SoXyn10A, CfXyn10A displays much a higher level of catalytic
efficiency towards both substrates. The SoXyn10A enzyme displays $k_{cat}/K_m$ values some 157-fold (for PNP-G2) and 29-fold (for PNP-X2) lower than the corresponding CfXyn10A values. More than 100-fold difference between the $K_m$ values of the two parental enzymes against PNP-G2 and PNP-X2 was observed, however, $k_{cat}$ values against these aryl sugars were not so different. $K_m$ values of the chimera were between those of the parents while $k_{cat}$ was larger than for both parents.

The Topology of the Substrate Binding Site of CfXyn10A, SoXyn10A, and FC-14-15—The topology of the substrate binding cleft of FC-14-15 was investigated. The catalytic efficiency of the chimeric xylanase along with SoXyn10A and CfXyn10A against different degrees of polymerization (dp) of xylooligosaccharides are shown in Figure 4. All of the enzymes showed a similar trend toward xylooligosaccharide length. Enzymes demonstrated optimal catalytic efficiency around dp 4 indicating the number of the major subsites of FC-14-15, SoXyn10A and CfXyn10A was four. The catalytic efficiency for xylotriose of FC-14-15 was similar to that of SoXyn10A, however the efficiencies for xylopentaose and xylohexaose were the same as those of CfXyn10A indicating the property of the chimera become CfXyn10A like, when the subsite +2 is occupied by the substrate.

Bond cleavage frequencies (BCFs) of FC-14-15, SoXyn10A and CfXyn10A were investigated by using the series of methyl xylotrioside to methyl xylohexaoside (Figure 5). We utilized low substrate concentrations to prevent transglycosylation reactions. In the case of the hydrolysis of xylotrisaccharide, the subsites -1, -2, and +1 are used to produce the major hydrolysis product. The amino acids which comprise subsites -1, -2, and -3 of the structure are almost completely conserved and the structure of subsite +1 is hybrid between the parents, but substrate binding is mainly achieved by
a stacking interaction with a Tyr (Tyr-172 in SoXyn10A and Tyr-171 in CfXyn10A) (Figure 3). Consistently, there was not a great difference between both the parental and chimeric enzymes with regard to bond cleavage frequency. In contrast, there were differences when oligosaccharides longer than the xylotetraose were used, due to the influence of the properties of the parental enzymes on subsite +2. In the case of the hydrolysis of xylotetraose, SoXyn10A cleaved the first and second linkages from the reducing end, whereas CfXyn10A only hydrolyzed the second linkage, releasing exclusively xylobiose from xylotetraose. The chimeric enzyme had the same BCF for xylotetraose as CfXyn10A. The BCF of the chimera for xylopentaose again showed similar properties to CfXyn10A while, to our surprise, the BCFs of the chimera for xylohexaose showed rather hybrid properties between the parental enzymes.

The binding energies at each of the subsite for SoXyn10A, CfXyn10A, and FC-14-15 were calculated as previously described (15) and are displayed in Figure 6. The data indicate that the substrate binding cleft of SoXyn10A and CfXyn10A contain five xylose subsites, two that bind to aglycone (+1 and +2) and three that interact with the glycone region of the substrate (-3, -2 and -1). The binding energy of subsite +2 of FC-14-15 was close to CfXyn10A whereas the energy of subsite -2 of the chimera was close to SoXyn10A. The higher binding energy at subsite +2 of FC-14-15 compared to SoXyn10A would affect the BCF for xyooligosaccharides, resulting in a similar tendency as seen with CfXyn10A. The high binding energy at subsite +2 for the chimera can be explained by the fact that both Phe-286 and Tyr-172 can contribute to binding. Trp-179 does not make a direct contact with xylotriose in the aglycone side of the cleft of the SoXyn10A/xylotriose complex (37), but this could be due to the presence of the long loop Asn-209-Ser-212 which could make it difficult for an extended xylan chain to go in the appropriate direction. Trp-179 interacts with a distal
xylobiose molecule in a complex with SoXyn10A (37), and could have a role in binding xylohexaose at subsite +3 in the FC-14-15 chimera, where the loop 209-212 is missing. A binding contribution by Trp-179 could explain the higher positive energy for subsite +3 in the chimera, and the fact that the chimera's BCF for xylohexaose is more similar to SoXyn10A, which has only slightly negative binding energy at subsite +3, as opposed to CfXyn10A which has a larger negative binding energy.

Xylan Hydrolysis—Activities for the hydrolysis of xylan by SoXyn10A, CfXyn10A, and FC-14-15 and their hydrolysis products were examined. The enzyme reaction mixtures which produced the same levels of reducing power were subjected to HPAEC-PAD (Figure 7). The hydrolysis products of FC-14-15 were similar to those of CfXyn10A. However, no xylose peak was detected from the hydrolysis product of FC-14-15, whereas parental SoXyn10A and CfXyn10A both produced xylose. The hydrolysis products of SoXyn10A and CfXyn10A differed the amounts of substituted xylooligosaccharides. Substituted xylooligosaccharides were purified from final reaction mixture of FC-14-15 and the structure determined by ESI-MS and NMR. Two kinds of substituted oligosaccharides were obtained. The ESI-mass spectrum of the oligosaccharides contained an intense ion at \( m/z \) 603 and 735, respectively, indicating oligosaccharides consisting of three xylose moieties and one 4-O-methyl glucuronic acid and four xylose residues and one 4-O-methyl glucuronic acid, respectively. The chemical shifts of NMR indicated that 4-O-methyl glucuronic acid was attached \( O-2 \) position of xylose at the non-reducing end. Thus, the structures of the isolated oligosaccharides were defined as 4MeGlcUAX3 and 4MeGlcUAX4. This result suggested that for substituted substrates the activity of SoXyn10A and CfXyn10A would be different. To test this hypothesis, the activities of SoXyn10A, CfXyn10A and
FC-14-15 in the hydrolysis of 4MeGlcUAX4 were determined (Figure 8).

4MeGlcUAX4 was hydrolyzed by the enzymes to xylose and 4MeGlcUAX3 with the following decreasing order of reactivity; SoXyn10A > CfXyn10A > FC-14-15. The hydrolysis rates of the substrate by FC-14-15 and CfXyn10A were similar, whereas SoXyn10A hydrolyzed 4MeGlcUAX4 with a significantly higher extent than the other enzymes. This can be explained from the result of BCF (Figure 5) and the structures of complexes with substituted oligosaccharides (54). According to the xylotriose-bound structure of SoXyn10A, the $O\text{-}2$ atoms of bound xylose at subsite -1 and -2 were buried into the cleft and could not have attached branches. Therefore, to hydrolyze 4MeGlcUAX4, the enzymes have to cleave the first linkage from reducing end to produce xylose and 4MeGlcUAX3. From the BCF (Figure 5), it can be seen that SoXyn10A is able to hydrolyze the first linkage from reducing end but the other enzymes cannot, explaining why CfXyn10A and FC-14-15 are not good at hydrolyzing 4MeGlcUAX4.

In the case of CfXyn10A and FC-14-15, the production of xylose was dependent on the hydrolysis of xylotriose and 4MeGlcUAX4 since xylose was not produced by the degradation of xylooligosaccharides longer than xylotriose (Figure 5). In contrast, SoXyn10A produced xylose when the enzyme cleaved xylotetraose in addition to the above substrates (Figure 5). CfXyn10A hydrolyzes xylotriose more efficiently than SoXyn10A and FC-14-15 (Figure 4), while SoXyn10A hydrolyzes 4MeGlcUAX4 more efficient than CfXyn10A and FC-14-15 (Figure 8). Since FC-14-15 had the weakest reactivity for both substrates, a lower production of xylose was observed than with SoXyn10A and CfXyn10A.

Conclusion— The results of this study suggest that the topology of the
substrate binding cleft is determined by the environment of the glycon side especially in subsite +2 which is not conserved in GH10 xylanases. The hybrid enzyme that we constructed in this study is fascinating, with an interesting combination of properties from the parent enzymes, resulting in low production of xylose.

It is already known that the structures of the glycon side of the active site in GH10 xylanases are highly conserved however in the aglycon side the structures are very different. The effects of these differences on substrate specificity have not previously been elucidated, in spite of the topology of the substrate binding cleft of GH10 xylanases not being conserved. The structure of subsites -3 to +1 of the substrate binding clefts of SoXyn10A and CfXyn10A were not significantly different, however differences in the topology of the clefts between the two enzymes could be found. Especially, these differences were remarkable when the enzymes used subsite +2. Not only the properties of hydrolysis of linear xylooligosaccharides, but also of hydrolysis of substituted oligosaccharides were affected.

Acknowledgments—The authors are grateful to Dr. Simon J. Charnock for the useful comments on analysis of BCFs. The authors are also grateful to SUNTORY Ltd. for supplying the xylobiose mixture. We wish to thank Dr. Leila Lo Leggio for valuable discussions and critically reading this manuscript. This work was supported in part by Grants for the Program for Promotion of Basic Research Activities for Innovative Biosciences and Grant-in-Aid for Scientific Research.

REFERENCES


2. Timell, T. E. (1965) in Advances in Carbohydrate Chemistry Vol. 20 (Wolforom, M.


Table 1  X-ray diffraction data and refinement statistics for FC-14-15

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value 1</th>
<th>Value 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell parameters (P2₁2₁2₁)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a (Å)</td>
<td>48.48</td>
<td></td>
</tr>
<tr>
<td>b (Å)</td>
<td>57.19</td>
<td></td>
</tr>
<tr>
<td>c (Å)</td>
<td>106.67</td>
<td></td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>30-2.2</td>
<td>2.34-2.20</td>
</tr>
<tr>
<td>No. of reflections in refinement</td>
<td>13714</td>
<td>1178</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>88.3</td>
<td>(78.1)</td>
</tr>
<tr>
<td>R-factor (%)</td>
<td>15.3</td>
<td>(16.2)</td>
</tr>
<tr>
<td>Rfree-factor (%)</td>
<td>19.8</td>
<td>(21.6)</td>
</tr>
<tr>
<td>No. of protein non-hydrogen atoms</td>
<td>2458</td>
<td></td>
</tr>
<tr>
<td>No. of water molecules</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>rmsd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Bond angles (deg.)</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Dihedral angles (deg.)</td>
<td>21.5</td>
<td></td>
</tr>
</tbody>
</table>
Table 2  Kinetic parameters of chimeric xylanase

<table>
<thead>
<tr>
<th></th>
<th>PNP-G₂</th>
<th>PNP-X₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$k_{cat}$ (s⁻¹)</td>
</tr>
<tr>
<td>SoXyn10A</td>
<td>97±5</td>
<td>2.2±0.2</td>
</tr>
<tr>
<td>CfXyn10A</td>
<td>0.71±0.04</td>
<td>2.5±0.1</td>
</tr>
<tr>
<td>FC-14-15</td>
<td>13±1</td>
<td>3.4±0.8</td>
</tr>
</tbody>
</table>
Figure 1  Structure of SoXyn10A, CfXyn10A and FC-14-15
A: SoXyn10A (RCSB Protein Data Bank accession number 1ISX), B: FC-14-15, C: CfXyn10A (RCSB Protein Data Bank accession number 2XYL). The crystal analysis of the FC-14-15 were performed at 2.2Å resolution. The coordinate for FC-14-15 have been deposited in the RCSB Protein Data Bank (accession number 1v6y). The crystal structure of GH10 xylanases indicate that the catalytic domain of the enzymes are subdivided into two parts separated by the module boundary between module M14 and M15 (modules M1-M14, purple; modules M15-M22, yellow). The boundary between M14 and M15 correspond with the substrate binding cleft of GH10 xylanases.

Figure 2  Construction of FC-14-15
A: Schematic representation of the boundary of module M1-M22 and the constructed chimeric enzymes. B: The chimeric xylanases were constructed by three times PCR with overlapping primers.

Figure 3  Substrate binding cleft of SoXyn10A, CfXyn10A, FC-14-15
Superposition of the substrate binding cleft of FC-14-15 (green), SoXyn10A (modules from M15 to M22; pink) and CfXyn10A (modules from M1 to M14; orange). Xylotriose inserted into the SoXyn10A cleft were modeled from the SoXyn10A/xylotriose complex structure (37). The numbering of amino acid residues in black referred to the amino acid number of SoXyn10A and the numbering in orange referred to the amino acid number of CfXyn10A.

Figure 4  Rate of xylooligosaccharide hydrolysis by CfXyn10A, SoXyn10A and FC-14-15
SoXyn10A (________), CfXyn10A (________), and FC-14-15 (_____ _) were incubated with xylooligosaccharides of different lengths and the rate of substrate hydrolysis was used to calculate $k_{cat}/K_m$.

**Figure 5** Bond cleavage frequencies of xylooligosaccharides by CfXyn10A, SoXyn10A, and FC-14-15

**Figure 6** Binding energies of the subsites of the substrate binding sites of CfXyn10A, SoXyn10A and FC-14-15

The binding energies of the subsites of SoXyn10A (A), CfXyn10A (B), and FC-14-15 (C) were calculated using the method described previously (15), substituting the $k_{cat}/K_m$ data displayed in Figure 4 and the BCFs exhibited in Figure 5.

**Figure 7** HPAEC-PAD analysis of soluble birchwood xylan hydrolysis by CfXyn10A, SoXyn10A and FC-14-15

Birchwood xylan hydrolysate by SoXyn10A (A), CfXyn10A (B), and FC-14-15 (C) were applied to HPAEC-PAD system. The positions at which xylose (a), xylobiose (b), xylotriose (c), xylotetraose (d), and xylooligosaccharides substituted by 4-O-methyl glucuronic acid (e) were eluted from the HPAEC column are indicated.

**Figure 8** Hydrolysis of 4MeGlcUAX4 by CfXyn10A, SoXyn10A and FC-14-15

SoXyn10A (________), CfXyn10A (________), and FC-14-15 (_____ _) were incubated with 4MeGlcUAX4, and the extent of substrate hydrolysis was detected by HPAEC-PAD.
Fig. 1   Kaneko et al.
Fig. 2 Kaneko et al
Fig. 4 Kaneko et al.
Fig. 5 Kaneko et al.
Structure and function of a family 10 beta-xylanase chimera of streptomyces olivaceoviridis E-86 FXYN and cellulomonas fim Cex
Satoshi Kaneko, Hitomi Ichinose, Zui Fujimoto, Atsushi Kuno, Kei Yura, Mitiko Go, Hiroshi Mizuno, Isao Kusakabe and Hideyuki Kobayashi

J. Biol. Chem. published online April 12, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M308899200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2004/04/12/jbc.M308899200.citation.full.html#ref-list-1