An investigation of the nature and function of module 10 in a family F/10 xylanase FXYN of *Streptomyces olivaceoviridis* E-86 by module shuffling with the Cex of *Cellulomonas fimi* and by site-directed mutagenesis

Satoshi Kaneko[a,*], Atsushi Kuno[b,c], Zui Fujimoto[d], Daisuke Shimizu[c,f], Sachiko Machida[a], Yoko Sato[e], Kei Yura[e], Mitiko Go[e], Hiroshi Mizuno[d], Kazunari Taira[c,f], Isao Kusakabe[f], Kiyoshi Hayashi[a]

[a] *National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8642, Japan*
[b] *Department of Material and Biological Chemistry, Faculty of Science, Yamagata University, Yamagata 990-8560, Japan*
[c] *National Institute for Advanced Interdisciplinary Research, Ministry of International Trade and Industrial Science, Tsukuba, Ibaraki 305-8562, Japan*
[d] *National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan*
[e] *Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan*
[f] *Institute of Applied Biochemistry, University of Tsukuba, 1-1-1 Tennoodai, Tsukuba, Ibaraki 305-8572, Japan*

Received 23 August 1999

Abstract **Although the amino acid homology in the catalytic domain of FXYN xylanase from *Streptomyces olivaceoviridis* E-86 and Cex xylanase from *Cellulomonas fimi* is only 50%, an active chimeric enzyme was obtained by replacing module 10 in FXYN with module 10 from Cex. In the family F/10 xylanases, module 10 is an important region as it includes an acid/base catalyst and a substrate binding residue. In FXYN, module 10 consists of 15 amino acid residues, while in Cex it consists of 14 amino acid residues. The K_m and k_{cat} values of the chimeric xylanase FCF-C10 for PNP-xylobioside (PNP-X_2) were 10-fold less than those for FXYN. CD spectral data indicated that the structure of the chimeric enzyme was similar to that of FXYN. Based on the comparison of the amino acid sequences of FXYN and Cex in module 10, we constructed four mutants of FXYN. When D133 or S135 of FXYN was deleted, the kinetic properties were not changed from those of FXYN. By deletion of both D133 and S135, the K_m value for PNP-X_2 decreased from the 2.0 mM of FXYN to 0.6 mM and the k_{cat} value decreased from the 20 s^{-1} of FXYN to 8.7 s^{-1}. Insertion of Q140 into the doubly deleted mutant further reduced the K_m value to 0.3 mM and the k_{cat} value to 3.8 s^{-1}. These values are close to those for the chimeric enzyme FCF-C10. These results indicate that module 10 itself is able to accommodate changes in the sequence position of amino acids which are critical for enzyme function. Since changes of the spatial position of these amino acids would be expected to result in enzyme inactivation, module 10 must have some flexibility in its tertiary structure. The structure of module 10 itself also affects the substrate specificity of the enzyme.**

Key words: Cex; Chimeric xylanase; Family 10 xylanase; Module; *Cellulomonas fimi*; *Streptomyces olivaceoviridis*

1. Introduction

Xylan is a major component of the hemicelluloses in plant cell walls, it consists of a linear backbone of β-1,4-linked xylopyranose units and often has side chains composed of other sugar residues such as arabinose and glucuronic acid. β-Xylanase (EC 3.2.1.8) hydrolyzes β-1,4-glycosidic linkages within the xylan backbone to yield short chain xylo-oligosaccharides of varying length. On the basis of the amino acid sequences of the catalytic domains, β-xylanases have been classified into two glycanase families (F/10 and G/11) [1,2]. These two families of xylanases differ in their patterns of cleavage of various heteroxylans and also in their structure. Family F/10 xylanase is known to have the $(\beta/\alpha)_8$ barrel structure which is the one of the most common protein structures, and belongs to the so-called 4/7 superfamily of glycosyl hydrolases [3]. The enzyme FXYN from *Streptomyces olivaceoviridis* E-86 has been purified and its substrate specificity characterized [4–8]. We recently succeeded in crystallizing the intact FXYN [9] and also in isolating its gene [10]. The catalytic domain of FXYN indicates that it belongs to family F/10 of the glycosyl hydrolase families. The catalytic domain of FXYN, along with members of 11 other families of glycoside hydrolases, is also classified into clan GH-A on the basis of structural and mechanistic information [Coutinho, P.M. and Henrissat, B. (1999) Carbohydrate-Active Enzymes server at URL: http://afmb.cnrs-mrs.fr/–pedro/CAZY/db.html].

Structure-function relationships and the engineering of glycosidases have attracted considerable current interest [11–15]. However, most previous work has concentrated on only a limited number of amino acids by using site-directed mutagenesis. Of necessity such work only determines the function of isolated amino acids and cannot investigate the nature of larger regions of protein. Rather than concentrating on one or two amino acids at a time by using site-directed mutagenesis, an alternative approach is to use gene shuffling to investigate the function of larger regions of protein. In the present study, we chose the concept of 'module' for the chimeric enzyme study. A module is a contiguous polypeptide segment of a protein, which has a compact conformation within a globular domain. A module is defined by using the distance between Cα atoms and on average is about 15 amino acid residues long. Based on the three-dimensional structure of Cex [16], the catalytic domain of Cex was divided into 22 modules [17] (Fig. 1). Module boundaries in Cex were found to be closely related to intron-exon boundaries [17]. This indicates that the locations of the introns in eukaryotic xylanase genes are not random and supports the idea that introns play an important role in protein evolution as a mediator of exon shuffling [18]. Therefore, module shuffling in vitro mimics

*Corresponding author. Fax: (81) (298) 38-8122.
E-mail: sakaneko@nfri.affrc.go.jp

0014-5793/99/$20.00
PII: S0014-5793(99)01318-6

one of the natural mechanisms of protein evolution. Furthermore, it is known that xylanase folds in an $(\beta/\alpha)_8$ barrel (TIM barrel) structure. As well as the basic TIM barrel structure, there are additional helices and loops which are arranged so as to form the active site cleft [19]. Module 10 is one of these additional helices and is arranged in a loop to form part of the substrate binding cleft. It is an important module for family 10 xylanase because it contains amino acid residues which act as acid/base catalysts and it forms the −1 site of the substrate binding cleft (Fig. 2). To understand the function of this module, we constructed a chimeric xylanase and some related mutants. In this paper, the function of module 10 will be discussed.

2. Materials and methods

2.1. Construction of chimeric and mutant enzymes

The catalytic domains of FXYN and Cex were separately subcloned into the pQE60 vector. Construction of the chimera was performed by the polymerase chain reaction (PCR) using overlapping primers at their respective module boundaries. DNA fragments from FXYN encoding modules 1–9 and modules 11–22 were amplified by using the following sets of primers (1–9 sense: 5′-CCA TGG GCT CCT ACG CCC TTC CCA GAT CAG-3′; 1–9 antisense: 5′-ACG CCT CGT TCA CGT CCC ACT GAG CGA TCT TG-3′; 11–22 sense; 5′-GCA GGA CTC GAA CCT GCA GCG CAC CGG CAA CGA CTG-3′; 11–22 antisense: 5′-GGA TCC GCC ACC GTT GAG TGC GTT GAG GAC G-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. DNA encoding module 10 of the Cex gene was synthesized (5′-GGA CGT CGT GAA CGA GGC GTT CGC CGA CGG CGG CGG CCG CCG GCA GGA CTC GAA CCT GCA GC-3′). The 10 bp overlapping regions (underlined) of the primers were designed to be complementary at their respective module boundaries. The first round 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 third round of PCR, the combined fragment was amplified with PCR primers (sense: 5′-CCA TGG GCT CCT ACG CCC TTC CCA GAT CAG-3′ and antisense: 5′-GGA TCC GCC ACC GTT GAG TGC GTT GAG GAC G-3′) by 25 cycles of shuttle PCR with denaturation at 98°C for 1 min and annealing and primer extension at 72°C for 1 min.

Site-directed mutagenesis was performed by the improved megaprimer PCR mutagenesis strategy that was originally described by Séraphin et al. [20]. The primers used to delete or insert Asp-133, Ser-135 or Gln-140 were (D133$^-$) 5′-AGG CCT TCT CGG ACG GTT CGG GCG-3′, (S135$^-$) 5′-TCG GAC GAC GGT GGA GGA CGG CGG-3′, (D133$^-$S135$^-$) 5′-CCT TCT CGG ACG GTG GTG GTC GCC-3′ and (D133$^-$S135$^-$Q140$^+$) 5′-CGG TCG TCG GCA GGA TTC CAA CCT-3′, respectively. The details of the method are as described previously [21].

2.2. Production of enzymes in Escherichia coli

For expression in *E. coli* and purification of the FXYN, Cex, FCF-C10 and the four mutants, the pET expression system (Novagen, Madison, WI, USA) was employed. Thus, each gene was individually inserted into the pET28 vector (to yield pET*fxyn*, pET*cex*, pET*fcf-c10*, pET*d133*$^-$, pET*s135*$^-$, pET*d133*$^-$*s135*$^-$, and pET*d133*$^-$*s135*$^-$*q140*$^+$, respectively). The enzymes were expressed as fusion proteins that consisted of each enzyme plus a carboxy-terminal tag with six histidine residues. The 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. The cells were harvested and lysed, and the cell lysate was then loaded on a HiTrap chelating column (Pharmacia, Uppsala, Sweden). 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 SDS-PAGE [22]. The enzyme eluted as a homogeneous protein by SDS-PAGE, and the relevant fractions were pooled and dialyzed against de-ionized water.

2.3. Circular dichroism and steady-state kinetic studies

The circular dichroism of FXYN, Cex and FCF-C10 were measured using conditions reported previously [21]. Steady-state kinetics were investigated as previously reported [21]. Briefly, the reaction mixture containing the substrate, at various concentrations, in 25% McIlvaine buffer (a mixture of 0.1 M citric acid and 0.2 M Na_2HPO_4, pH 7.0) that contained 0.05% 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 absorbance at 400 nm with a spectrometer (DU-7400; Beckman, Palo Alto, CA, USA). *p*-Nitrophenyl-β-D-xylobioside (PNP-X_2) was synthesized by the method described in a previous paper [23]. The xylobiose used in the synthesis was purified from 'Xylobiose Mixture' (Suntory Ltd., Osaka, Japan). *p*-Nitrophenyl-β-D-cellobioside (PNP-G_2) was kindly gifted from Yaizu Suisan Co. Ltd. (Yaizu, Japan).

2.4. Differences of hydrolysis rates between FXYN and FCF-C10

Reaction mixtures containing 150 μl of McIlvaine buffer (0.2 M Na_2HPO_4-0.1 M citric acid), 50 μl of 1% BSA (v/w) and 250 μl of PNP-X_2 solution (4 mM or 0.4 mM) were equilibrated at 30°C for

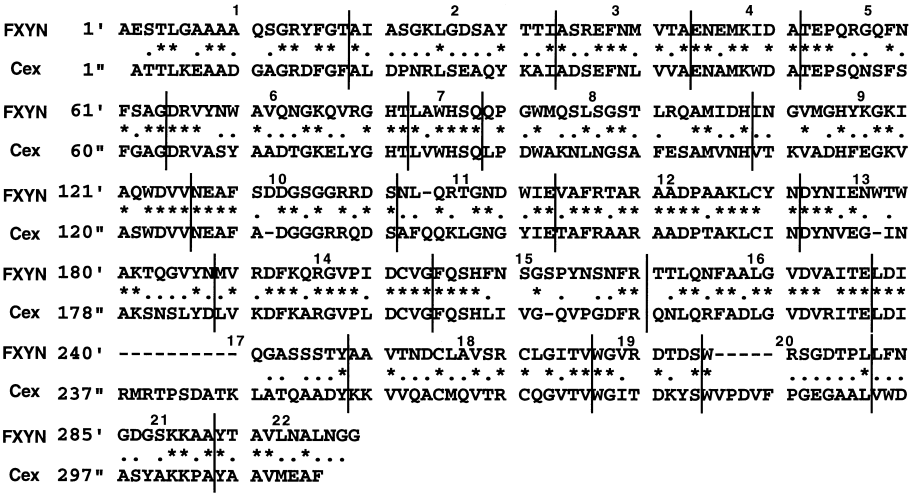

Fig. 1. Amino acid alignment of the FXYN and Cex catalytic domains based on module analysis. Module boundaries are indicated by vertical lines and modules are numbered from 1 to 22.

Table 1
Kinetic parameters of mutant xylanases

	PNP-G_2			PNP-X_2		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM)
FXYN	116	1.2	0.010	2.0	20	10
Cex	0.7	1.1	1.6	0.015	1.8	120
FCF-C10	64	1.8	0.028	0.24	1.9	7.9
$D133^-$	91	2.5	0.027	2.4	27	11
$S135^-$	105	2.7	0.026	2.3	30	13
$D133^-S135^-$	88	2.5	0.028	0.6	8.7	15
$D133^-S135^-$ $Q140^+$	55	2.0	0.036	0.3	3.8	13

5 min and, then reactions were initiated by the addition of 50 μl of enzyme solution (the final concentrations of FXYN and FCF-C10 were 0.005 mg/ml). The amount of *p*-nitrophenol released was determined by monitoring the absorbance at 400 nm with a spectrometer (DU-7400; Beckman).

3. Results

3.1. Construction and characterization of chimeric xylanase

As shown in Fig. 1A, the homology of the catalytic domains of Cex and FXYN was 49%. The catalytic domain of Cex can be divided into 22 modules based on its three-dimensional structure. Modules 4, 6, 7, 10, 15, 19 and 20 include amino acid residues which make up the substrate binding cleft [17]. Module 10 is considered to be one of the most important modules for xylanase because it contains an acid/base catalyst and a substrate binding residue. Module 10 in FXYN was replaced with module 10 of Cex to give a chimeric enzyme (Fig. 3). The chimera was expressed in *E. coli* as an active enzyme. The chimeric enzyme was purified so that it gave a single band on SDS-PAGE. Circular dichroism indicated that the constructed chimeric enzyme folded in the same way as the parent enzymes (Fig. 4). The effect of replacing module 10 in FXYN with that of Cex was to delete one amino acid residue and to change three other amino acids in the enzyme. To investigate the effect that this change had on the chimeric xylanase (FCF-C10), the kinetic parameters were determined for PNP-G_2 and PNP-X_2 (Table 1). The kinetic parameters for PNP-G_2 were not very different from FXYN. To our surprise however, K_m and k_{cat} values of FCF-C10 for PNP-X_2 (K_m: 0.2 mM and k_{cat}: 1.9 s^{-1}) were decreased 10-fold from those of FXYN (K_m: 2.0 mM and k_{cat}: 20 s^{-1}). However, the effectiveness of the enzyme was not reduced at low substrate concentration (Fig. 5). To investigate which residue had the most effect on the kinetic parameters, various mutants of FXYN were constructed.

3.2. Construction and characterization of FXYN mutant

Module 10 of FXYN and Cex consisted of 15 amino acids and 14 amino acids respectively. The identity of the two module 10 sequences was 66.7%. By considering the non-identical amino acids in module 10 of FXYN and Cex (Fig. 3), we designed four mutants of FXYN ($D133^-$, $S135^-$, $D133^-S135^-$, $D133^-S135^-Q140^+$) (Fig. 6). The mutants constructed were purified and their kinetic parameters were determined for PNP-G_2 and PNP-X_2 (Table 1). When D133 or S135 was deleted ($D133^-$ and $S135^-$), the kinetic parameters for PNP-X_2 were largely unchanged when compared with FXYN. In the case of the double deletion of D133 and S135 ($D133^-S135^-$), the K_m value for PNP-X_2 was 0.6 mM, representing a three-fold decrease from FXYN (2.0 mM). The k_{cat} value was also decreased to 8.7 s^{-1}. Insertion of Q140 into the double mutant ($D133^-S135^-Q140^+$) gave an enzyme with kinetic parameters close to those of FCF-C10.

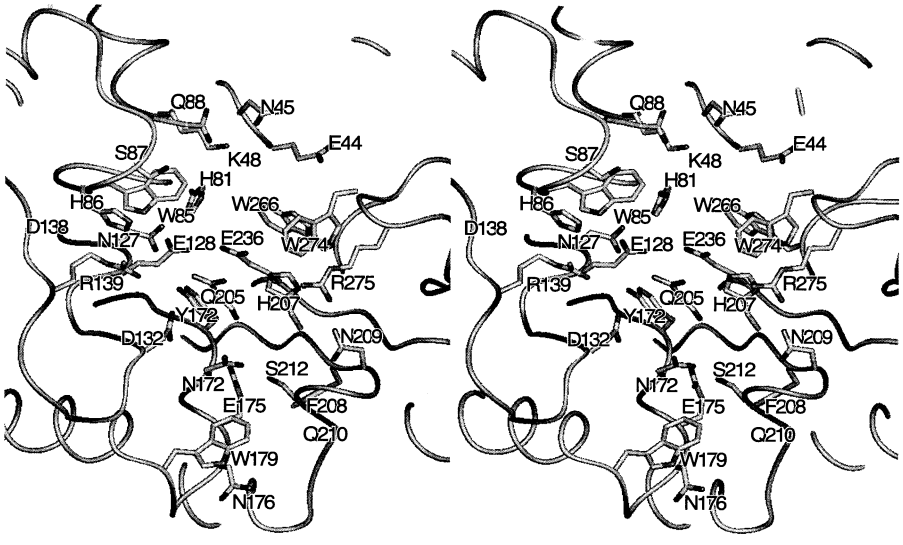

Fig. 2. The substrate binding cleft of FXYN. The structure of FXYN was modeled by using the crystal structure of the xylanase A from *Streptomyces lividans* [28].

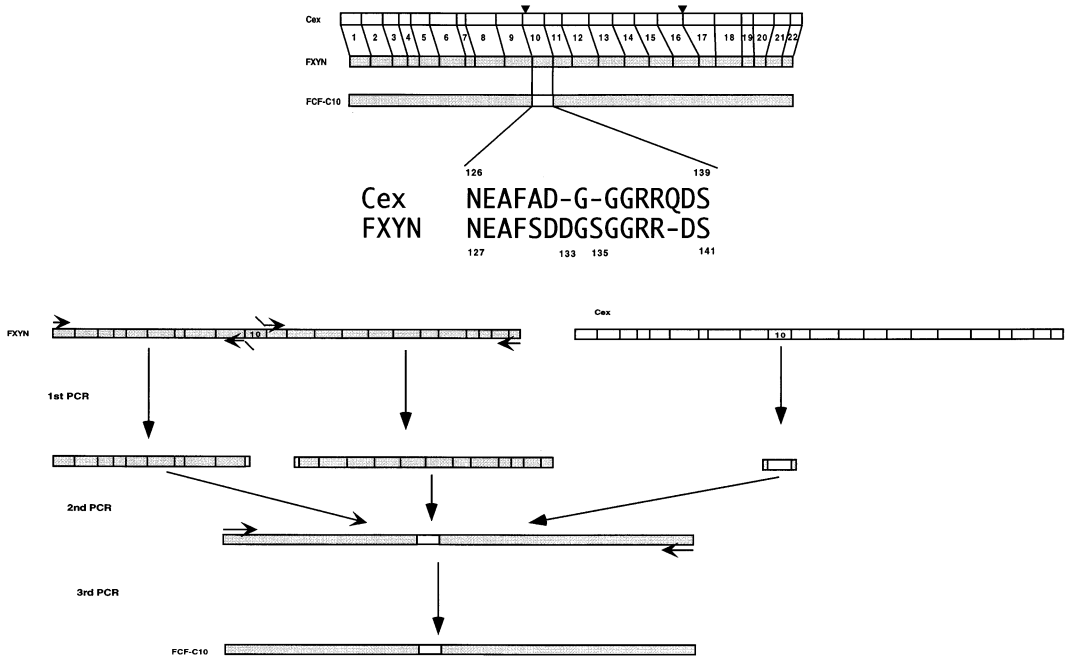

Fig. 3. Construction of the chimeric xylanase FCF-C10 from FXYN and Cex by module shuffling.

4. Discussion

Based on the three-dimensional structure of Cex, the catalytic domain of Cex was divided into 22 modules which had an average of 15 amino acid residues each [17]. Substrate binding residues were identified by co-crystallization of Cex with an inhibitor [24], they are located in modules 4, 6, 7, 10, 15, 19 and 20 [17]. Module 10 includes catalytic and substrate binding amino acid residues. E128 of FXYN is an acid/base catalyst [21] of the double displacement mechanism type [25] and N127 surrounds the −1 site in the substrate binding cleft [26–28]. Since these amino acid residues are conserved in all family 10 xylanases, replacement of module 10 did not change these amino acids. When module 10 of FXYN was replaced with that of Cex, neither the secondary structure of the enzyme (Fig. 4) nor the k_{cat}/K_m value changed (Table 1). These results indicate that the shuffling did not affect the location of the N127 and E128 residues. However, the K_m and k_{cat} values decreased almost 10-fold. To investigate this further, four kinds of mutants were constructed. The length of module 10 is different in FXYN (15 amino acids) and Cex (14 amino acids), but the D133 or S135 deleted mutant of FXYN had similar kinetic parameters to FXYN. When D133 and S135 were both deleted, the K_m and k_{cat} values for PNP-X_2 were significantly reduced. Addition of Q140 to the $D133^{-}S135^{-}$ mutant resulted in an enzyme similar in character to the chimeric xylanase. This indicates that the conformation of module 10 was changed slightly by the deletion of the two amino acids. As shown in Fig. 7, there are differences in the environment of module 10 between FXYN and Cex. The distances from the side chains of D132 of FXYN to those of Y172 and N173 are 2.6 Å and 4.1 Å, indicating D132 hydrogen bonds with Y172 and Y172 also hydrogen bonds with R139 which exists in module 10. In contrast, in Cex, the distance from D131 to Y171 is 3.6 Å, which indicates that D131 cannot form a hydrogen bond with Y171. Instead, D131 hydrogen bonds with N172 and R136 forms hydrogen bonds with Y171 and D138. In FXYN, Y172 forms the + side of the substrate

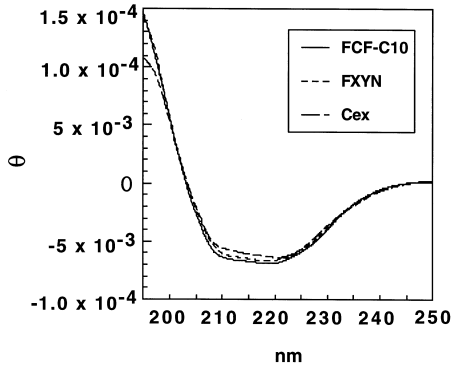

Fig. 4. Circular dichroism of chimeric xylanase with the parental enzymes.

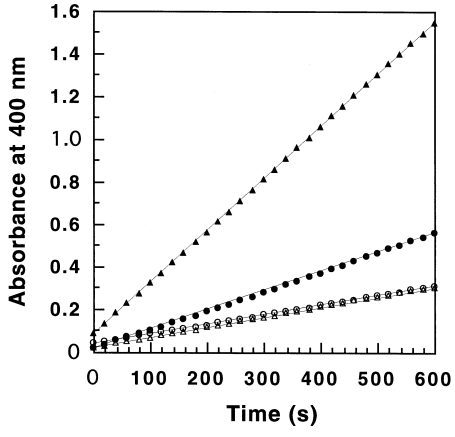

Fig. 5. The hydrolysis of PNP-xylobioside by FXYN and FCF-C10 xylanases. FXYN (▲) and FCF-C10 (●) were incubated with 2 mM PNP-X_2, FXYN (△) and FCF-C10 (○) were incubated with 0.2 mM PNP-X_2.

	127 133 135 141
FXYN	NEAFSDDGSGGRR-DS
$D133^-$	NEAFSD-GSGGRR-DS
$S135^-$	NEAFSDDG-GGRR-DS
$D133^-S135^-$	NEAFSD-G-GGRR-DS
$D133^-S135^-Q140^+$	NEAFSD-G-GGRRQDS
Cex	NEAFAD-G-GGRRQDS
	126 139

Fig. 6. Amino acid sequences of module 10 of Cex, FXYN of *S. olivaceoviridis* E-86 and FXYN mutant xylanases.

binding cleft together with N173 [28] (Fig. 2). Y172 seems to be an important residue for recognizing the phenolic rings of PNP-X_2 and PNP-G_2. This can be concluded because Y172 in FXYN corresponds to F181 of xylanase A from *Pseudomonas fluorescens*. F181 of xylanase A from *P. fluorescens* is located on the +1 subsite and forms a weak stacking interaction with the phenolic ring of PNP-G_2 [14]. This interaction is important for the hydrolysis of PNP glycosides [14]. The double deletion of amino acids in module 10 must change the position of D132 which in turn will affect the position of Y172. Due to the change in position or orientation of Y172, it appears that the enzyme is able to form stronger interactions with the phenolic ring of PNP-X_2 and PNP-G_2. Further changes in kinetic parameters occurred with the addition of Q140 and may be explained by an elimination of hydrogen bonds between R139 and Y172 which would result in a more Cex-like environment around module 10. Therefore

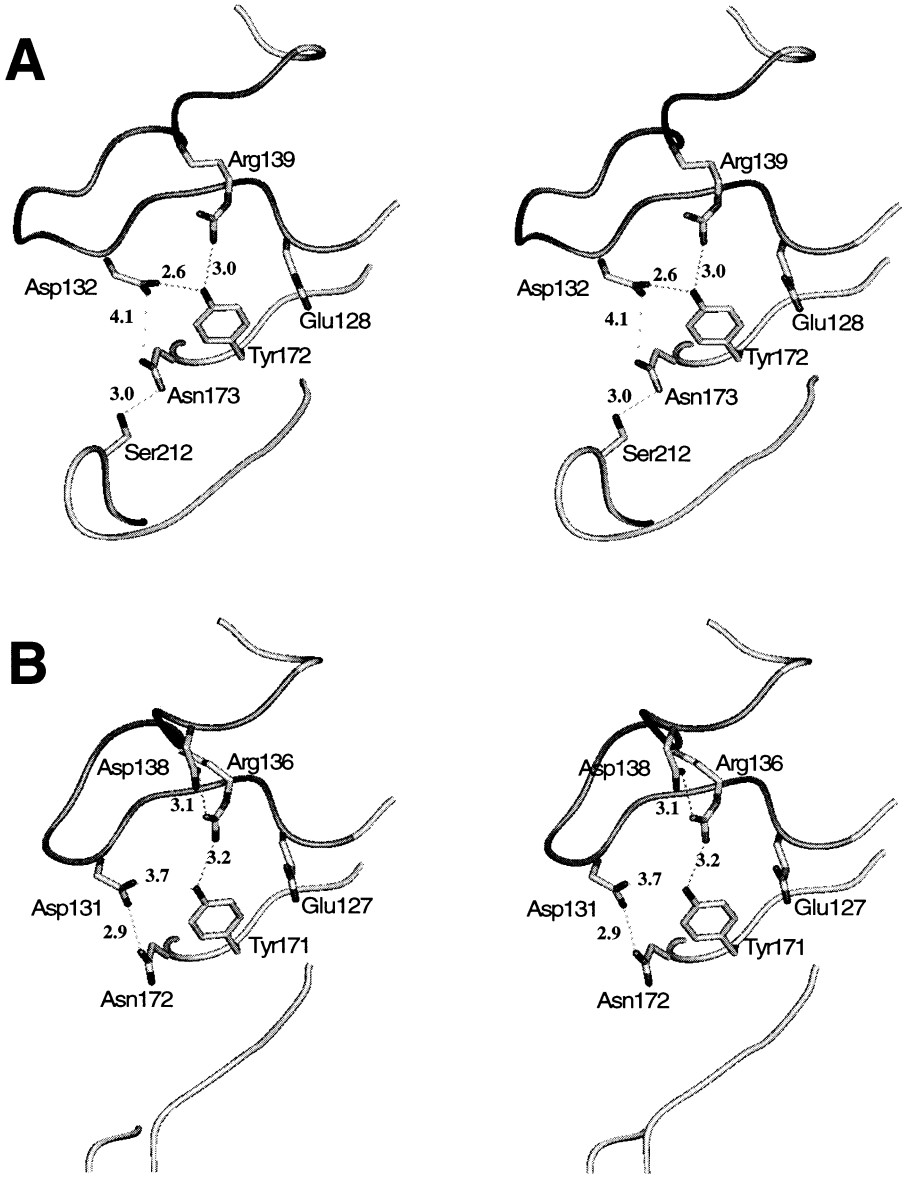

Fig. 7. The environment of module 10 in FXYN and Cex. A: FXYN. B: Cex. Hydrogen bonds are shown as dashed lines. The numbers written beside hydrogen bonds indicate the distance between the atoms in Å. The structure of FXYN was modeled using the crystal structure of the xylanase A from *Streptomyces lividans* [28].

the kinetic properties of FCF-C10 changed from FXYN to Cex-like. The kinetic properties for PNP-G_2 were not greatly affected in this study. However, in a similar way to PNP-X_2, the K_m for PNP-G_2 decreased in the order FXYN > $D133^-S135^-$ > $D133^-S135^-Q140^+$. Module 10 is close to, and interacts with the +1 site of the substrate binding cleft and it also includes N127 which forms part of the −1 site of the cleft. In FXYN, the key residue for recognizing the C-6 position of glucose is W274, this residue is also located at the −1 site, but on the opposite side of the cleft to N127 [24,29] (Fig. 2). N127 recognizes the OH groups at the C-2 positions of xylose and glucose, and does not distinguish between them. Since FXYN has a large K_m value for PNP-G_2 which is due largely to the − side of the subsite, and the environment on the − side of the subsite has not changed significantly in the shuffled and mutated enzymes, the kinetic parameters for PNP-G_2 were not expected to be greatly affected by the shuffling and mutations of FXYN.

In conclusion, our results indicate that in xylanase, module 10 is not only related directly to enzyme activity in that it includes catalytic (E128 in FXYN) and substrate binding (N127 in FXYN) residues, but it also interacts with adjacent modules which are involved with the substrate binding cleft. Despite the fact that module 10 contains amino acids critical for the catalytic process, it can be readily modified so as to change the substrate specificity of the xylanase without inactivating it.

Acknowledgements: The authors thank Suntory Ltd. for supplying the 'xylobiose mixture' and Dr. Yasuo Gama, NIMC, AIST, for his help in the synthesis of PNP-X_2. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences.

References

[1] Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller Jr., R.C. and Warren, R.A.J. (1991) Microbiol. Rev. 55, 303–315.
[2] Henrissat, B. and Bairoch, A. (1993) Biochem. J. 293, 781–788.
[3] Jenkins, J., Leggio, L.L., Harris, G. and Pickergill, R. (1995) FEBS Lett. 362, 281–285.
[4] Kusakabe, I., Kawaguchi, M., Yasui, T. and Kobayashi, T. (1977) Nippon Nogeikagaku Kaishi 51, 429–437.
[5] Kusakabe, I., Ohgushi, S., Yasui, T. and Kobayashi, T. (1983) Agric. Biol. Chem. 47, 2713–2723.
[6] Yoshida, S., Kusakabe, I., Matsuo, N., Shimizu, K., Yasui, T. and Murakami, K. (1990) Agric. Biol. Chem. 54, 449–457.
[7] Yoshida, S., Ono, T., Matsuo, N. and Kusakabe, I. (1994) Biosci. Biotechnol. Biochem. 58, 2068–2070.
[8] Matsuo, N., Yoshida, S., Kusakabe, I. and Murakami, K. (1991) Agric. Biol. Chem. 55, 2905–2907.
[9] Fujimoto, Z., Mizuno, H., Kuno, A., Yoshida, S., Kobayashi, H. and Kusakabe, I. (1997) J. Biochem. (Tokyo) 121, 826–828.
[10] Kuno, A., Shimizu, D., Kaneko, S., Koyama, Y., Yoshida, S., Kobayashi, H., Hayashi, K., Taira, K. and Kusakabe, I. (1998) J. Ferment. Bioeng. 86, 434–439.
[11] MacLeod, A.M., Tull, D., Rupitz, K., Warren, R.A.J. and Withers, S.G. (1996) Biochemistry 35, 13165–13172.
[12] Lawson, S.L., Wakarchuk, W.W. and Withers, S.G. (1997) Biochemistry 36, 2257–2265.
[13] Charnock, S.J., Lakey, J.H., Virden, R., Hughes, N., Sinnott, M.L., Hazlewood, G.P., Pickersgill, R. and Gilbert, H.J. (1997) J. Biol. Chem. 272, 2942–2951.
[14] Charnock, S.J., Spurway, T.D., Xie, H., Beylot, M.H., Virden, R., Warren, R.A., Hazlewood, G.P. and Gilbert, H.J. (1998) J. Biol. Chem. 273, 32187–32199.
[15] Warren, R.A.J. (1999) in: Protein Engineering in Industrial Biotechnology (Alberghina, L., Ed.) (in press), IPD, Singapore.
[16] White, A., Withers, S.G., Gilkes, N.R. and Rose, D.R. (1994) Biochemistry 33, 12546–12552.
[17] Sato, Y., Niimura, Y., Yura, K. and Go, M. (1999) Gene (in press).
[18] Gilbert, W. (1978) Nature 271, 501.
[19] Schmidt, A., Schlacher, A., Steiner, W., Schwab, H. and Kratky, C. (1998) Protein Sci. 7, 2081–2088.
[20] Séraphin, B. and Kandels-Lewis, S. (1996) Nucleic Acids Res. 24, 3276–3277.
[21] Kuno, A., Shimizu, D., Kaneko, S., Hasegawa, T., Gama, Y., Hayashi, K., Kusakabe, I. and Taira, K. (1999) FEBS Lett. 450, 299–305.
[22] Laemmli, U.K. (1970) Nature 227, 680–685.
[23] Takeo, K., Ohguchi, Y., Hasegawa, R. and Kitamura, S. (1995) Carbohydr. Res. 277, 231–244.
[24] White, A., Tull, D., Johns, K., Withers, S.G. and Rose, D.R. (1996) Nature Struct. Biol. 3, 149–154.
[25] Sinnott, M.L. (1990) Chem. Rev. 90, 1171–1202.
[26] Roberge, M., Dupont, C., Morosoli, R., Shareck, F. and Kluepfel, D. (1997) Protein Eng. 10, 399–403.
[27] Moreau, A., Shareck, F., Kluepfel, D. and Morosoli, R. (1994) Eur. J. Biochem. 219, 261–266.
[28] Derewenda, U., Swenson, L., Green, R., Wei, Y., Morosoli, R., Shareck, F., Kluepfel, D. and Derewenda, Z.S. (1994) J. Biol. Chem. 269, 20811–20814.
[29] Notenboom, V., Birsan, C., Warren, R.A.J., Withers, S.G. and Rose, D.R. (1998) Biochemistry 37, 4751–4758.