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Mutual conversion of substrate specificities of *Thermoactinomyces vulgaris* R-47 α-amylases TVAI and TVAII by site-directed mutagenesis

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Abstract

Thermoactinomyces vulgaris R-47 produces two α-amylases, TVAI and TVAII, differing in substrate specificity from each other. TVAI favors high-molecular-weight substrates like starch, and scarcely hydrolyzes cyclomaltooligosaccharides (cyclodextrins) with a small cavity. TVAII favors low-molecular-weight substrates like oligosaccharides, and can efficiently hydrolyze cyclodextrins with various sized cavities. To understand the relationship between the structure and substrate specificity of these enzymes, we precisely examined the roles of key residues for substrate recognition by X-ray structural and kinetic parameter analyses of mutant enzymes and successfully obtained mutants in which the substrate specificity of each enzyme is partially converted into that of another.

Keywords: α-Amylase; X-ray structures; Site-directed mutagenesis; Cyclodextrin; Cyclodextrinase activity

1. Introduction

α-Amylase [α-(1→4)-D-4-glucanohydrolase] catalyzes the hydrolysis of α-(1→4)-D-glucosidic linkages in starch to release α anomer products and has been studied with respect to structure, function and industrial applications.[1,2] *Thermoactinomyces vulgaris* R-47 produces two α-amylases, TVAI (637 amino acid residues, $M_w = 71{,}000$ Da)[3,4] and TVAII (585 amino acid residues, $M_w = 67{,}500$ Da).[5] In addition to hydrolyzing starch, they have unique hydrolyzing activities for pullulan with the structure [α-(1→4)-Glc-α-(1→4)-Glc-α-(1→6)-Glc]$_n$ and cyclomaltooligosaccharides (cyclodextrins, CDs), which are scarcely hydrolyzed by other α-amylases.[6] TVAI as an extracellular enzyme favors high-molecular-weight substrates like starch and pullulan and can hydrolyze γ-CD (eight glucose units), but cannot hydrolyze α- and β-CDs (six and seven glucose units) with a small cavity. On the other hand, TVAII as an intracellular enzyme favors low-molecular-weight substrates and can efficiently hydrolyze small maltooligosaccharides including α- and β-CDs by what is called cyclodextrinase activity.[7] We have reported the crystal structures of wild-type TVAI,[8] wild-type TVAII[9] and inactive TVAII mutant enzymes complexed with β-CD[10] and maltohexaose.[11] They belong to α-amylase family 13 of the sequence-based classification of glycoside hydrolyses. However, they have an extra domain (domain N) in addition to the three domains (domains A, B and C) common to all known α-amylases.[12] Recently, Maltogenic amylase (ThMA) and *Bacillus stearothermophilus Neopullulanase* (NPL) have been reported, which also have a domain N and can hydrolyze CDs.[13,14] In Fig. 1, the active site structures of TVAI and TVAII with substrates are shown. Several insights about their unique substrate specificities were deduced from X-ray structures, as follows; (1) The

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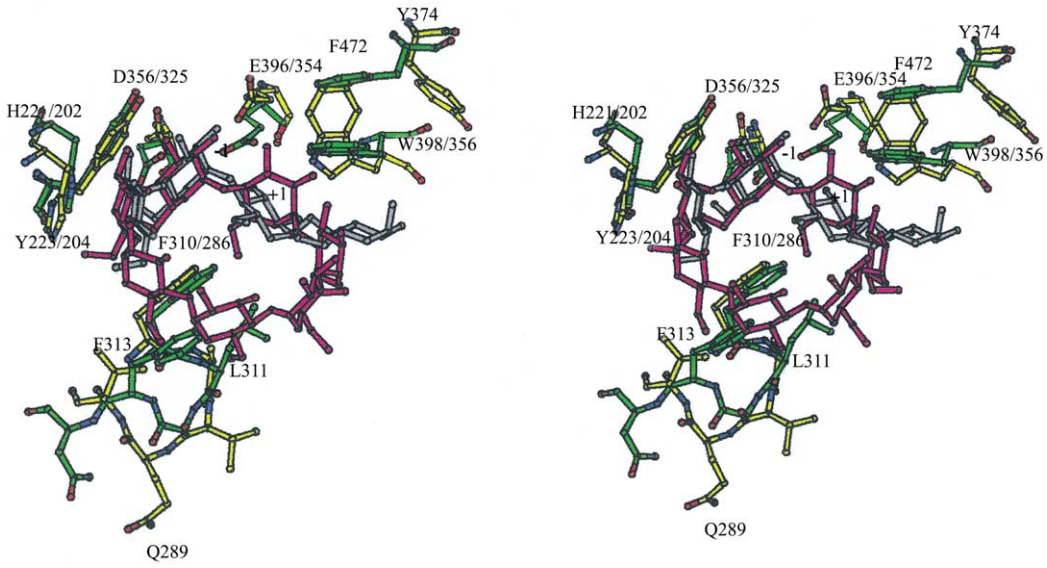


Fig. 1. Stereoview of the active-site structures of TVAI (green) and TVAII (yellow) illustrated by the programs MOLSCRIPT[15] and RASTER3D.[16] The β-CD (magenta) and four glucose units of maltohexaose (gray) are superimposed. The selected residues in TVAI/TVAII are labeled.

active site structure of TVAII is more suitable for the binding of α- and β-CD than that of TVAI, where Phe286 mainly recognizes α- and β-CDs;[17] (2) Phe313 and Trp398 of TVAI are expected to cause steric hindrance with the superimposed β-CD to inhibit its binding, suggesting that these residues may inhibit the binding of β-CD; (3) The lower part of the active site in Fig. 1 is very different between TVAI (the region from Leu311 to Leu316) and TVAII (the region from Ala287 to Met293), suggesting that these residues are closely related to the difference in substrate specificity between TVAI and TVAII. To elucidate precisely why TVAII has strong hydrolyzing activities for α- and β-CDs and TVAI has not, and why TVAI can more efficiently hydrolyze starch and pullulan than TVAII, studies on mutant enzymes in which the key residues for substrate recognition have been replaced are very useful. We report here the X-ray structures of mutant TVAIs (F313A (Phe313 → Ala) and W398V (Trp398 → Val)), and kinetic analyses of mutant TVAIs (F313A, W398A (Trp398 → Ala) and W398V) and mutant TVAII (A287M293-TVAII-L311L316), where the region from Ala287 to Met293 of TVAII has been replaced by the region from Leu311 to Leu316 of TVAI.

Table 1
Crystallographic parameters and refinement statistics

Enzyme	F313A	W398V
Temperature (K)	100	100
Space group	*C*2	*C*2
Resolution (Å)	2.2	2.2
Completeness (%)	96.0	99.4
R_{merge}	0.069	0.072
Cell dimensions		
a (Å)	120.94	122.7
b (Å)	50.42	50.82
c (Å)	107.92	108.8
β (°)	103.6	104.2
Measured References.	162,092	412,389
Unique References.	31,175	33,199
Structure refinement		
Resolution range (Å)	25–2.2	25–2.2
References	31,175	33,199
Completeness (%)	96.0	99.4
$R_{crystal}$ (%)	17.9	19.1
R_{free} (%)	22.0	23.7
r.m.s.d. bond lengths (Å)	0.006	0.007
r.m.s.d. bond angles (°)	1.3	1.3
Amino acids	637	637

2. Results and discussion

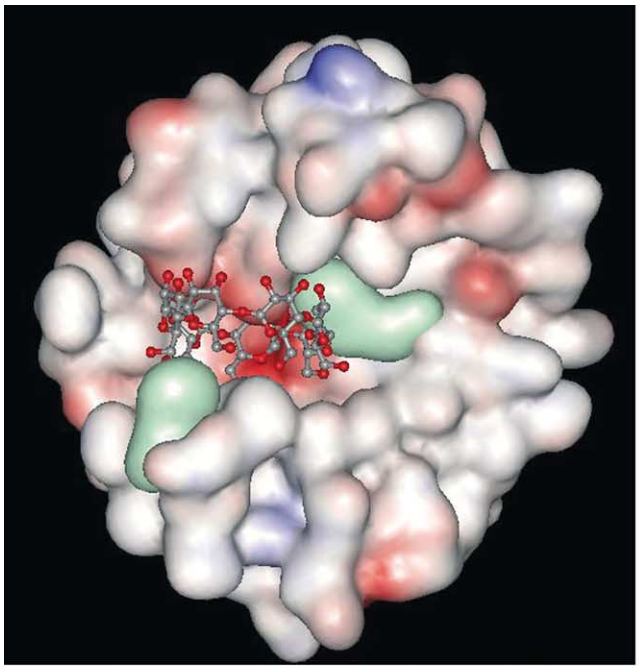
2.1. X-ray structures of F313A and W398V

The structure refinement statistics are listed in Table 1. The stereochemistry of all amino acids was verified with the program PROCHECK,[18] and in a Ramachandran plot,[19] 85.2% of residues in F313A and 86.3% of residues in W398V were found to be in the most favored regions. In overall three-dimensional structure, F313A and W398V are almost the same as the wild-type TVAI. The r.m.s. deviations of the Cα atoms compared to wild-type TVAI are 0.39 Å for F313A, and 0.59 Å for W398V. As the three-dimensional structure of both

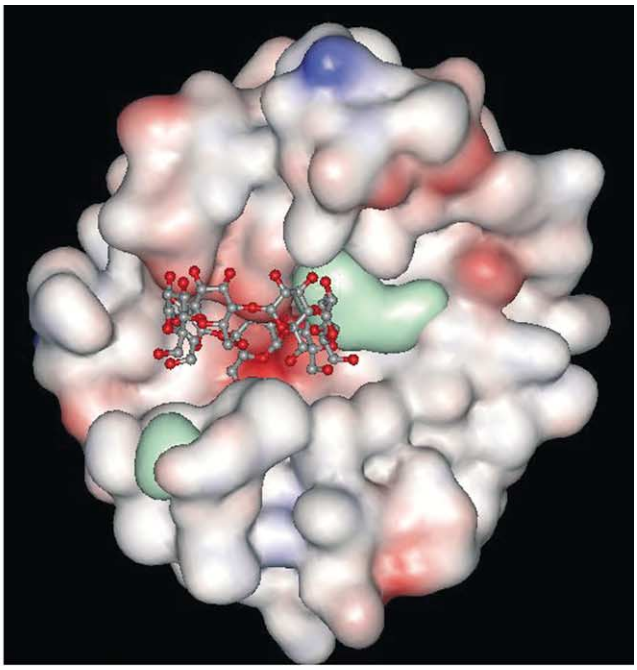
mutants is very similar to that of wild-type TVAI, the replacements of these two residues is expected not to lead to drastic structural changes in TVAI. The active site surface structures together with those of wild-type TVAI are shown in Fig. 2. One β-CD from the X-ray structure of the E354A–β-CD complex[10] is also superimposed in Fig. 2. As expected, the replaced Ala and Val residues make no short contacts with the β-CD. However, it is expected that Phe313 in W398V and Trp398 in F313A still have steric hindrance with the β-CD.

2.2. Kinetic analyses of mutant TVAIs and TVAII

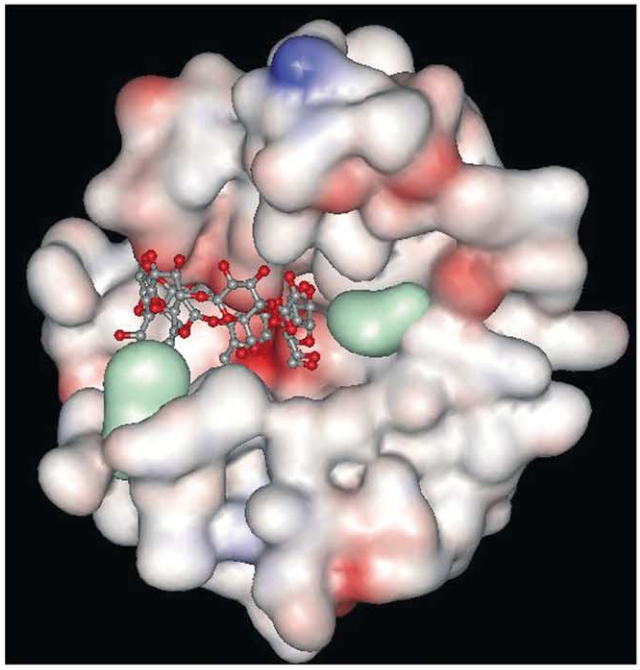
The kinetic parameters of wild-type TVAI, mutant TVAIs (F313A, W398A and W398V), wild-type TVAII,



(A) Wild-type TVAI



(B) F313A



(C) W398V

Fig. 2. The structures of the active site surface of: (A) wild-type TVAI; (B) F313A; and (C) W398V illustrated by the program VIEWLITE.[20] The β-CD (ball and stick style) is also superimposed. In A–C, the 313th and the 398th residues are shown in light green.

Table 2
Kinetic parameters of wild-type and mutant enzymes

	TVAI			
	Wild-type	F313A	W398A	W398V
K_m (mM)				
α-CD	49.8	135	111.8	ND [c]
β-CD	103.4	40.7	15.8	ND
Starch [a]	0.52±0.02	0.56±0.03	0.97±0.05	0.72±0.08
k_{cat} (s^{-1})				
α-CD	7.4	23.2	1.2	ND
β-CD	18.3	83.1	0.33	ND
Starch	674	444	57.8	263
k_{cat}/K_m [b]				
α-CD	0.16 (100)	0.18 (113)	0.011 (6.9)	ND
β-CD	0.18 (100)	2.8 (1550)	0.02 (12)	ND
Starch	1297 (100)	796 (61)	60.3 (4.6)	357 (28)
	TVAII			
	Wild-type	A287M293-TVAII-L311L316		
K_m (mM)				
α-CD	0.43	ND		
β-CD	0.99	ND		
Starch [a]	0.25	1.7		
k_{cat} (s^{-1})				
α-CD	22	ND		
β-CD	22	ND		
Starch	12.2	69.3		
k_{cat}/K_m [b]				
α-CD	51 (100)	ND		
β-CD	22 (100)	ND		
Starch	49 (100)	41 (84)		

[a] K_m values of starch were percentage (w/v).
[b] The ratio of k_{cat}/K_m for each variant relative to wild-type enzyme (%) is given in parentheses.
[c] ND means 'not determined'.

and mutant TVAII (A287M293-TVAII-L311L316), are listed in Table 2.

The replacement of Phe313 by Ala (F313A) caused a more drastic increase of activity for β-CD, while the enzymatic activity for α-CD and starch was not so affected. The k_{cat}/K_m value of F313A for β-CD is almost 15 times as high as that of wild-type TVAI, indicating a trend of TVAII-type substrate specificity. From the X-ray structures, there is expected to be steric hindrance between Phe313 in wild-type TVAI and C-6 atoms of the binding β-CD, but not the Ala313 in F313A. In spite of the expected steric hindrance between Trp398 of F313A and β-CD, F313A significantly hydrolyzes β-CD, indicating that Phe313 is possibly one of the main residues responsible for the poor hydrolyzing activity of TVAI for β-CD. In the binding of α-CD, it is thought that there is still steric hindrance between amino acid residues close to Phe313 and α-CD, due to its smaller cavity, that serves to inhibit the binding of α-CD. This is because the hydrolyzing activity for α-CD of F313A is almost the same as that of wild-type TVAI. To confirm this, we attempted to obtain a mutant TVAII without the hydrolyzing activity for α- and β-CDs, by introducing the region around Phe313 of TVAI to TVAII. The TVAII mutant, A287M293-TVAII-L311L316, has the sequence Leu-Gly-Phe-Asn-Ser-Leu, including Phe313 from TVAI instead of the corresponding loop of Ala-Val-Gln-Val-Pro-Ala-Met of TVAII. As shown in Table 2, the hydrolyzing activity of A287M293-TVAII-L311L316 for starch remains, but that for α- and β-CDs all but disappeared (less than 0.1% of the activity of wild-type TVAII), indicating a trend of TVAI-type substrate specificity. These results suggest that the structures in the region from Leu311 to Leu316 in TVAI and from Ala287 to Met293 in TVAII recognize the cavity of the CDs as substrates.

The replacement of Trp398 by Ala or Val leads to a decrease in the hydrolyzing activity, not only for α- and β-CDs, but also for starch. However, the hydrolyzing activity of W398A and W398V for CDs almost dis-

appeared, while that for starch remained with residual activity of 4.6 and 28%, respectively. When a substrate binds to α-amylase, the torsion angles of the glucosidic bonds at the catalytic site are proposed to deviate from those in the regular helical structure of amylose at the first step in the catalytic reaction.[21–23] In the X-ray structure of inactive mutant TVAII–maltopentaose complexes,[11] a largely deviated the torsion angle of glucoside bonds between subsite −1 and +1 at the catalytic site was found. Trp398 was expected to make nicely stacking interactions with the distorted linear substrates, as shown in Fig. 1. On the replacement of Trp398 with Ala or Val, interactions between glucose units of the substrate and the 398th residue should decrease. Maybe this is why the hydrolyzing activity of W398A and W398V for starch is 4 and 28%, respectively, compared to wild-type TVAI. On the other hand, α- and β-CDs with a stable circular structure cannot easily change their conformations, and the electron density of the omit map in the X-ray structure of the E354A–β-CD complex strongly suggested that the β-CD at the active site maintains its circular structure.[10] Although Trp398 is expected to exhibit steric hindrance with β-CD having a circular shape, given the X-ray structure of wild-type TVAI, the replacement of Trp398 with Ala or Val causes the near disappearance of hydrolyzing activity for CDs, indicating that Trp398 is indispensable for the catalytic reaction on CDs. Also, as discussed above, F313A having Trp398 can hydrolyze β-CD. Therefore, it is proposed that Trp398 can change its side-chain conformation to fit a circular CD through binding, which plays an important role in locating CDs in the proper position and orienting them for the hydrolyzing reaction. Unfortunately, the X-ray structures of TVAI–CD complexes are not yet available; however, in the X-ray structure of mutant TVAII (E354A)–β-CD complex,[10] such an induced fit of Trp356 (corresponding to Trp398 in TVAI) to β-CD was found. The Trp residue at this position is strictly conserved in the enzymes with the hydrolyzing activity for CDs (Trp359 in ThMA and Trp359 in NPL), while other α-amylase family enzymes without the hydrolyzing activity have a residue other than Trp at this position. These findings also support our proposal about the role of the Trp residue.

3. Experimental

3.1. Site-directed mutagenesis and purification of the mutant TVAIs and TVAII

All of the mutant TVAIs were prepared from recombinant *Escherichia coli* JM 109 cells. Oligonucleotide-directed mutagenesis was carried out using the plasmid pTV 93 with the Quick Change Kit (Stratagene) for the construction of mutant TVAIs, F313A, W398A and W398V. The oligonucleotides used to produce these mutants were F313A: 5′-TCT TTT CTC GGC GCC AAC AGT TTG CCC-3′and 5′-GGG CAA ACT GTT GGC GCC GAG AAA AGA-3′, W398A: 5′-GGG GAG TAT GCC GGC AAC GCC AAT CCC TGG ACG-3′ and 5′-CGT CCA GGG ATT GGC GTT GCC GGC ATA CTC CCC-3′, W398V: 5′-GGG GAG TAT GTG GGC AAC GCC AAT CCC TTG ACG-3′ and 5′-CGT CCA GGG ATT GGC GTT GCC CAC ATA CTC CCC-3′. The preparation of mutant TVAII, A287M293-TVAII-L311L31, has already been reported.[4] All mutant enzymes were purified in the same way as the wild-type TVAI or TVAII.[24]

3.2. X-ray structural analysis

F313A and W398V were crystallized under the same conditions as wild-type TVAI.[24] X-ray diffraction data for F313A were collected at 100 K using an R-AXISIIc imaging plate system[25] with CuK_α radiation on a Rigaku rotating anode X-ray generator operated at 100 mA and 40 kV. The data were processed using the programs DENZO and SCALEPACK.[26] The diffraction data for W398V were collected at 100 K using an ADSC/CCD detector system on the BL6A beam line in the Photon Factory (Tukuba, Japan). Data were processed using the program DPS/MOSFLM[27] and the routines from the CCP4 program suite.[28] Initial phases were determined by a molecular replacement method with the structure of the wild-type TVAI as a probe model, using the program CNS.[29] Refinements of both structures using 2.2 Å resolution data were carried out by the program CNS. Inspection of the refined model for the electron density map was done using the program XFIT.[30] Solvent molecules were gradually introduced if the peaks above 4.0σ in the (F_o-F_c) electron density map were in the range of a hydrogen bond. To avoid overfitting of the diffraction data, a free R-factor with 10% of the test set excluded from refinement was monitored.[31] Refinements of the final structures were converged at an R-factor of 0.179 ($R_{free}=0.220$) for F313A, and an R-factor of 0.191 ($R_{free}=0.237$) for W398V. The coordinates and structure factors of F313A (code 1IZJ) and W398V (code 1IZK) have been deposited in the Protein Data Bank.

3.3. Enzyme assays

The activities for starch and CDs were assayed as described.[3,5] The products were analyzed by the Nelson–Somogyi method by monitoring the reducing power of the hydrolysate. CDs and soluble starch were purchased from Wako Pure Chemical Industries Ltd. and were used without further purification. The assay conditions were as follows: Enzymatic reactions were

carried out in 100 mM phosphate buffer (pH 6.0) at 40 °C, under which conditions each mutant enzyme showed maximum activity. The range of the concentrations of the substrates were: α-CD, 40–120 mM; β-CD, 10–120 mM; starch, 0.05–1.0% (w/v). Typically, the enzyme concentration was 0.5–1 μM; however, when the level of activity was too low, the concentration was gradually increased to obtain reproductive measurements. The measurement of activity for each set of conditions was carried out at least five times to determine the kinetic parameters.

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References

1. Yamamoto, T.; Shinke, R, In *Enzyme Chemistry and Molecular Biology of Amylases and Related Enzymes*; The Amylase Research Society of Japan, Eds; CRC Press: Boca Raton, 1995; pp. 3–5.
2. Komaki, T. In *Handbook of Amylase and Related Enzymes. Their Sources, Isolation Methods, Properties and Applications*; The Amylase Research Society of Japan, Eds; Pergomon Press; 1988.
3. Tonozuka, T.; Mogi, S.; Shimura, Y.; Ibuka, A.; Sakai, H.; Matsuzawa, H.; Sakano, Y.; Ohta, T. *Biochim. Biophys. Acta* **1995**, *1252*, 35–42.
4. Ibuka, A.; Tonozuka, T.; Matsuzawa, H.; Sakai, H. *J. Biochem.* **1998**, *123*, 275–282.
5. Tonozuka, T.; Sakai, H.; Ohta Sakano, Y. *Carbohydr. Res.* **1994**, *261*, 157–162.
6. Tonozuka, T.; Ohtsuka, M.; Mogi, S.; Sakai, H.; Ohta, T.; Sakano, Y. *Biosci. Biotechnol. Biochem.* **1993**, *57*, 395–401.
7. Park, K. H.; Kim, T. J.; Cheong, T. K.; Kim, J. W.; Oh, B. H.; Svensson, B. *Biochim. Biophys. Acta* **2000**, *1478*, 165–185.
8. Kamitori, S.; Abe, A.; Ohtaki, A.; Kaji, A.; Tonozuka, T.; Sakano, Y. *J. Mol. Biol.* **2002**, *318*, 443–453.
9. Kamitori, S.; Kondo, S.; Okuyama, K.; Yokota, T.; Shimura, Y.; Tonozuka, T.; Sakano, Y. *J. Mol. Biol.* **1999**, *287*, 907–921.
10. Kondo, S.; Ohtaki, A.; Tonozuka, T.; Sakano, Y.; Kamitori, S. *J. Biochem.* **2001**, *129*, 423–428.
11. Yokota, T.; Tonozuka, T.; Shimura, Y.; Ichikawa, K.; Kamitori, S.; Sakano, Y. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 619–626.
12. Henrissat, B. *Biochem. J.* **1991**, *280*, 309–316.
13. Kim, J. S.; Cha, S. S.; Kim, H. J.; Kim, T. J.; Ha, N. C.; Oh, S. T.; Cho, H. S.; Cho, M. J.; Kim, M. J.; Lee, H. S.; Kim, J. W.; Choi, K. Y.; Park, K. H.; Oh, B. H. *J. Biol. Chem.* **1999**, *274*, 26279–26286.
14. Hondoh, H.; Kuriki, T.; Matsuura, Y. *J. Mol. Biol.* **2003**, *326*, 177–188.
15. Kraulis, P. J. *J. Appl. Crystallogr.* **1991**, *24*, 946–950.
16. Merritt, E. A.; Bacon, D. J. *Methods Enzymol.* **1997**, *277*, 505–524.
17. Ohtaki, A.; Kondo, S.; Tonozuka, T.; Sakano, Y.; Kamitori, S. *Carbohydr. Res.* **2001**, *334*, 309–313.
18. Laskowski, R. A.; MacArthur, M. W.; Moss, D. S.; Thornton, J. M. *PROCHECK v.2: Programs to check the stereochemical quality of protein structures*; Oxford Molecular Ltd: Oxford, UK, 1992.
19. Ramachandran, G. N.; Sasisekharan, V. *Adv. Protein Chem.* **1968**, *23*, 283–437.
20. The program VIEWERLITE: distributed from Accelys, Inc., http://www.accelrys.com
21. Sinnot, M. L. *Chem. Rev.* **1990**, *90*, 1171–1202.
22. McCarter, J. D.; Withers, S. G. *Curr. Opin. Struct. Biol.* **1994**, *4*, 885–892.
23. Fujimoto, Z.; Takase, K.; Doui, N.; Momma, M.; Matsumoto, T.; Mizuno, H. *J. Mol. Biol.* **1998**, *277*, 393–407.
24. Kondo, S.; Kaji, A.; Yuguchi, K.; Tonozuka, T.; Sakano, Y.; Kamitori, S. *Protein Peptide Lett.* **2000**, *7*, 197–200.
25. Higashi, T. *J. Appl. Crystallogr.* **1990**, *23*, 253–257.
26. Otwinowski, Z. In *Proceedings of the CCP4 Study Weekend: Data Collection and Processing*; Sayer, L.; Isaacs, N.; Bailey, S., Eds; Daresbury Laboratory: Warrington, UK; 1993; pp. 56–62.
27. Leslie, A. G. W. In *Crystallographic Computing V*; Moras, D.; Podjarny, A. D.; Thierry, J. C., Eds.; Oxford University Press: Oxford, 1991; pp 27–38.
28. Collaborative Computational Project 4. *Acta Crystallogr. Sect. D* **1994**, *50*, 760–763.
29. Brünger, A. T.; Adams, P. D.; Clore, G. M.; DeLano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J. S.; Kuszewski, J.; Nilges, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson, T.; Warren, G. L. *Acta Crystallogr. Sect. D* **1998**, *54*, 905–921.
30. McRee, D. E. *Practical Protein Crystallography*; Academic Press: San Diego, 1993.
31. Brünger, A. T. *Nature* **1992**, *355*, 472–475.