The Relationship Between The Affinity and The Kinetics of Cellulase for Its Substrate

M. Kanie[1] and S. Karita[1]

[1] Graduate School of regional innovation studies, Mie University, Tsu, Mie, Japan
609M006@m.mie-u.ac.jp

Abstract—**The recombinant endoglucanase of family 44 (Cel44A) isolated from *Clostridium thermocellum* F1 has activities for cellulase, xylanase, and xyloglucanase. Previous studies using mutant Cel44A (E186Q) had suggested that Trp64 play a crucial role in binding to cellooligosaccharides. This study confirmed previous findings, and provided further studies understanding the role of each subsite in the cleft of the catalytic domain. Isothermal titration calorimetry (ITC) provided a direct measure of binding enthalpy (*ΔH*) and allowed the determination of the association constants (*K*a) of the mutated Cel44As with various degrees of cellooligosaccharides. Experimental binding studies with the mutants lacking aromatic binding residues (W64A/E186Q and W64A/Y71A/E186Q) suggest that the each amino acid residues didn't dominate whole enzymatic affinity. However, W64A/E186Q and W64A/Y71A/E186Q showed a higher affinity for cellohexaose and cellopentaose than did E186Q. These high affinities are probably seemed to be related to the rates of hydrolysis and product pattern as demonstrated by enzyme assay and thin layer chromatography of hydrolytic products. Our result suggests that the high affinity of catalytic domain causes its products to remain in the cleft and delay the proceeding of hydrolysis.**

Keywords— **cellulase, ITC**

I. Introduction

Degradation and conversion of lignocellulosic biomass is attracting intensive attention because of its potential for the development of sustainable and environment-friendly energy and materials industries. In nature, a vast group of enzymes, glycoside hydrolases, can break down the lignocellulose into oligosaccharides. There are some factors involved in the efficiency of glycoside hydrolases activity. They are affinity for its substrate, specificity, and inhibition and dissociation of production [1]. These factors are affected by the three dimensional structure of enzyme, which is so diverse and determined by the amino acid configuration. That is, what kind of amino acid it is, for example, polar or non-polar and how far the distance between substrate and amino acid is [3]. Since the hydrolysis occurs when the substrate approaches the degradation point, it is important to understand how the enzyme binds to its substrate in order to achieve the effective degradation. The goal of this study is to reveal how cellulase degrades its substrate and to search for an efficient way to degrade.

Isothermal titration calorimetry (ITC) provides a direct measure of binding enthalpy (ΔH) allowing the simultaneous determination of the binding parameters, association constants (Ka), entropy (ΔS), free energy (ΔG), and the binding stoichiometry. These thermodynamics parameters are related to each other by the following equations:

$$\Delta G = \Delta H - T\Delta S$$
$$\Delta G = -RT\ln Ka$$

For spontaneous reactions, ΔG is negative, and ΔG is directly related to the binding affinity. The tighter the binding, the more negative the ΔG. Enthalpy and entropy both contribute to ΔG. Hydrogen bonds and hydrophobic interactions are the dominant interactions in protein-carbohydorate complexes. ΔH is negative, if a binding is enthalpically favored. Favorable enthalpy requires correct placement of hydrogen bond acceptor and donor groups at the binding interface. A positive $T\Delta S$ results in entropically favored binding. Favorable entropy changes are primarily due to hydrophobic interactions, such as interaction between an aromatic ring and a sugar ring [2-4]. In brief, association constants evaluate the enzyme's affinity and the way of binding is.

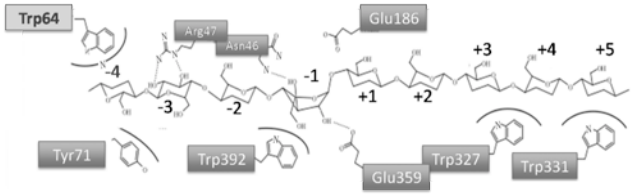

Fig 1. Cel44A cleft. Aromatic amino acid residues show stacking effect site and dotted lines show hydrogen bonding. Plus and minus indicate number of subsites. Glu186 and Glu359 are reaction centers of the enzyme.

Cel44A is endoglucanase, which produces cellotetraose as its main product and one of the domains of CelJ derived from *Clostridium thermocellum* strain F1. Its gene was cloned previously [5]. The recombinant Cel44A domain, with molecular weight of 58 kDa, consists of 519 amino acid residues. Cel44A has an activity of cellulase, xylanase, and xyloglucanase. The site-directed mutant (E186Q), which had no cellulase activity was constructed and its

crystal structure binding to substrates was shown [6]. Figure.1 shows the substrates recognition pattern in the cleft structure of Cel44A. The amino acid residues presented in the cleft recognize the sugar rings of substrate. The position of amino acid residuses, in the cleft, which binds the sugar ring, is called subsite. As is shown in Figure 1, in the cleft, with its active site at center, 'plus' represents the reducing side of substrate and 'minus' represents non-reducing side.

II. Materials and methods

Preparation of plasmids

Site-directed mutagenesis was performed on the cel44A from C. thermocellum F-1 by polymerase chain reaction (PCR) with DNA polymerase KOD-FX (Toyobo, Japan). The mutagenic primers for the mutation were as follows: Cel44A, 5'-**AGATCT**CTGGTTCCGCGT-3' and 5'-**GGTACC**TTAGGGCTCC-3'; W64A/E186Q, 5'-GACGCACTGCATTACAGTG-3' and 5'-CACTGTAATGCAGTGCGTC-3'; W64A/Y71A/E186Q, 5'-ATACAGCACTTTTGGAGGA-CG -3' and 5'-AAGTGGTGTATCACTGTAATGVAG-3'. They were designed according to Ref. 5 E186Q was templated to amplify the coding resion of W64A/E186Q by PCR, then the W64A/E186Q was templated to amplify the coding resion of W64A/Y71A/E186Q. The resulting PCR products were inserted between the BamHⅠ and KpnⅠ site of pQE30 (Quiagen, Germany). W64A/Y71A was prepared to exchange the region coding proton donor from Cel44A wild type and W64A/Y71A/E186Q. The restriction fragment was treated by *Age* Ⅰ and *Sal* Ⅰ.

Protein Production and Purification

All proteins were over expressed by using the expression system in *Escherichia coli* strain JM109. The cell-free extracts were prepared with sonication for 30 min. The expressed proteins were purified by Ni-NTA column (Quiagen, Germany) from the cell-free extracts. Protein samples were dialyzed against 50mM sodium phosphate buffer (pH7.0) at 4℃.

The three-dimensional (3D) structures of mutants

To ensure that the amino acid replacement did not cause structural alteration, mutants and wild type Cel44A were monitored with the circular dichroism (CD) spectra (J-720, JASCO) and differential scanning calorimeter (DSC) (VP-DSC, Microcal) measurement. Protein concentration of the sample ranged 1.0 to 3.0 μM. The range of wavelength was 185-250nm on CD spectra. The scanning temperature is from 50 to 100℃ on DSC.

Microcalorimetry Titration Studies

Titration calorimetry measurements were performed with an ITC200 calorimeter. Ligand solutions of glucose (G1), cellobiose (G2), cellotriose (G3), cellotetraose (G4), cellopentaose (G5) and cellohexaose (G6) were prepared by diluting with same buffer used for the protein dialysis. Aliquots (2.0μl) of the 0.3-0.8 mM ligand solution were added by means of a 40 μl rotating stirrer-syringe (1000 rpm) to the reaction cell, containing almost 200μl of the 0.015-0.08 mM protein solution. The heat of dilution was determined by same way dropping the ligand solution into the buffer solution without proteins. Calorimetric data analysis was carried out with ORIGIN 70 software (MicroCal). Binding parameters such as the number of binding sites (n), the association constant (Ka, M^{-1}), and the binding enthalpy (ΔH, kcal/mol of bound ligand) were determined by fitting the experimental binding isotherms. The slope of the isotherm in the equivalence point primarily determined Ka.

Thin layer chromatography (TLC)

TLC of the hydrolysis products was done on a silica gel 60-plastic sheet (Merck) developed with a solvent of *n*-butanol—acetic acid—water (2:1:1), and the oligosaccharides were made visible by spraying the plate with a diphenylamine aniline-phosphate reagent.

Enzyme assays

Enzyme activity of wild type Cel44A and W64A/Y71A toward CMC (carboxymethylcellulose) (SIGMA, USA) was assayed. Each reaction mixture contained 200μl of 1.25% solution of CMC in 50 mM sodium phosphate buffer (pH7.0) and 100 μl of enzyme solutions. The mixture was incubated at 60℃ for 10 min and the amount of reducing sugars released from each substrate was measured with dinitrophthalic acid reagent. The absorbance of solution was measured at optical density was 570 nm.

III. Results

Preparation of mutant cellulases

We confirmed that the each mutant plasmids contained target mutation and there were not any extra mutations leading to amino acid displacement in the coding region amplified by PCR. The purified proteins gave a single band on SDS-PAGE and it's sure that each molecular weight of the mutants was 58kDa. And then W64A/E186Q, W64A/Y71A/E186Q, W64A, W64A/Y71A were manufactured. It was ascertained that the three-dimensional (3D) structures of mutants were almost the same as those of wild type.

Thermodynamics of Substrate Binding for Mutant cellulases

Table 1, 2 and 3 summarize the thermodynamic parameters and the association constants extracted from the binding isotherms. These parameters are given at a temperature of 25℃.

Table 1. Thermodynamic parameters for cellooligosaccharide binding to E186Q

Ligand	$Ka\times10^4$ (M^{-1})	ΔH^0 (kcal mol^{-1})	ΔG^0 (kcal mol^{-1})	$T\Delta S^0$ (kcal mol^{-1})	N
G6	19.6	-16.3	-7.25	-9.1	0.93
G5	15.5	-13.0	-7.07	-6.0	1.16
G4	20.3	-11.1	-7.24	-3.8	0.67
G3	1.1	-16.8	-5.53	-11.2	0.45
G2	ND	ND	ND	ND	ND
G1	ND	ND	ND	ND	ND

ND: no detection

Table 2. Thermodynamic parameters for cellooligosaccharide binding to W64A/E186Q

Ligand	$Ka\times10^4$ (M^{-1})	ΔH^0 (kcal mol^{-1})	ΔG^0 (kcal mol^{-1})	$T\Delta S^0$ (kcal mol^{-1})	N
G6	33.4	-12.13	-7.53	-4.60	0.77
G5	16.7	-13.91	-7.13	-6.78	0.77
G4	7.9	-2.52	-6.68	4.16	1.18
G3	ND	ND	ND	ND	ND
G2	ND	ND	ND	ND	ND
G1	ND	ND	ND	ND	ND

Table 3. Thermodynamic parameters for cellooligosaccharide binding to W64A/Y71A/E186Q

Ligand	$Ka\times10^4$ (M^{-1})	ΔG^0 (kcal mol^{-1})	ΔH^0 (kcal mol^{-1})	$T\Delta S^0$ (kcal mol^{-1})	N
G6	49.1	-7.76	-11.92	-4.2	0.81
G5	26.9	-7.42	-11.32	-3.9	0.97
G4	ND	ND	ND	ND	ND
G3	ND	ND	ND	ND	ND

E186Q could bind G3, G4, G5 and G6, and W64A/E186Q could bind G4, G5 and G6. In the case of W64A/Y71A/E186Q could bind only G5 and G6. All of the binding interactions were exothermic, and the enthalpy values ranged from -16.8 to -2.52 kcal/mol. For E186Q, entropy values were negative and for W64A/E186Q, they ranged from $T\Delta S$=-6.78 to +4.16 kcal/mol. The heat of dilution was measured for respective cellooligosaccharides. The heat detected for G3, G4, G5 and G6 was -373.23 cal, -1326.99 cal, -546.06 cal and -599.14 cal respectively.

Comparison of product pattern

The degradation patterns of Cel44A wild type and W64A/Y71A were qualitatively analyzed on cellooligosaccharides. As shown in Fig. 2, wild type hydrolyzed G6 to yield G4, G3, and G2 as major products, and G5, and G1 as minor products. It hydrolyzed G5 to produce mainly G4, and G1. On the other hand, W64A/Y71A hydrolyzed G6 to yield G4, and G3 mainly, but G6 and G5 exist in the solution. In addition, there was no more G2 and G1.

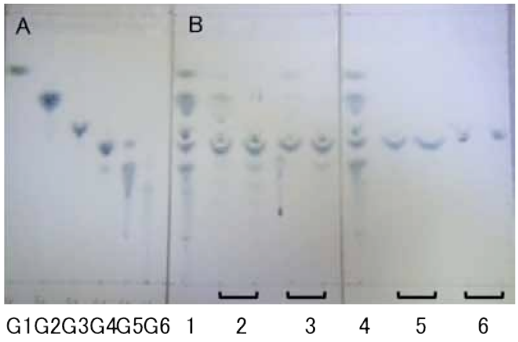

Fig2. TLC of products from Hydrolysis of cellooligosaccharides.
A: Each cellooligosaccharide (20μg)
B: Each cellooligosaccharide was incubated with Cel44A wild type or W64A/Y71A for 10 hour at 60℃. 1 and 4. G1-G6 cellooligosaccharade, 2. Mix G6 with enzyme (left is wild type and right is mutant), 3. Mix G5, 5. Mix G4, 6. Mix G3

Comparison of enzyme activity

Using CMC as the substrate, enzyme activity was detected at pH 7.0 and 60℃. The activities of Cel44A wild type and W64A/Y71A toward CMC were tested (Fig. 3). Both enzymes were active on CMC, but W64A/Y71A showed week activity. Its rate of hydrolysis was lower and its amount of reducing sugar in suturation point was less than that of wild type.

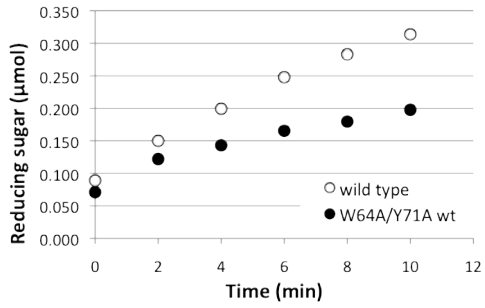

Fig. 3 Enzyme activity. The amount of reducing sugar generated from CMC hydrolysis by wild type (○) and W64A/Y71A (●) during the course of the reaction is indicated.

IV. Discussion

ITC data shown that E186Q could associate with G3, G4, G5 and G6, and its association constants for the different cellooligosaccharides ranged from 10^3 M^{-1} for the substrate

G3 to $10^5\,M^{-1}$ for the substrates G4, G5 and G6. The decline of affinity for G3 was remarkable because G3 was not long enough to reach the amino acid involved in the interaction. Presuming that non-reducing end of substrate attaches to subsite -4, the amino acid residues (Asn46, Glu359 and Trp392) can associate with each substrate, when the substrate is G6, G5 or G4, whereas in the case of G3, they cannot. From this point of view, non-reducing side of each cellooligosaccharides seems to be pulled strongly by Trp 64 and Tyr 71. Two previous experiments also suggested that Trp64 and Tyr71 play a significant role on binding to cellooligosaccharides. First, Cel44A cleaves G6 into G4 and G2, and G5 into G4 and G1 [7]. Therefore the main product of Cel44A is cellotetraose because of the strong affinity two aromatic residues have. Second, the observation of the crystal structure of E186Q complexed with G6 through X-ray crystal structure analysis showed that the substrate was clearly observed binding at subsite -4 [6]. Third, as you can see in Figure 1, Cel44A dose not have recognition-site for sugar ring around subsites +1 and +2, and have pretty weak interactions of one-side aromatic stacking around subsites +3, +4 and +5. These features indicate that the affinity around minus side subsites is higher than that plus side subsites. All these three evidences indicate that our research results are more reasonable. As regards the ΔH values, the shorter the substrates were, the more unfavorable enthalpy they had, except for G3. Hydrogen bond formation or van der Waals interactions between protein and cellooligosaccharides create favorable enthalpy. Hydrogen bond formation is a result of optimal placement of hydrogen bond donor and accepter groups on the protein and substrate, and is highly directional and specific [3]. The increase of enthalpy means that hydrogen bond formation or van der Waals interactions decrease. Although the hydrogen bonds reduced, the affinity of E186Q for G4, G5 and G6 remained approximately the same. Hydrophobic stacking interactions, presumably resulting from the dehydration of highly ordered water molecules surrounding the hydrophobic surface such as the face of sugar ring and aromatic residue, were perhaps involved in the association to compensate reducing hydrogen bond, so that the ΔS was increased. Hence, it is estimated that the shorter the sugar chain become, the less specificity the enzyme has. G3 is smaller enough than the cleft of the protein so that two G3 molecules bind to one protein, but stoichiometry of binding was not two.

Thus, we mutated these two aromatic residues, Trp64 and Tyr71, to examine their roles. W64A/E186Q has two mutations about active site and the aromatic residue in the cleft. W64A/E186Q could bind to G6, G5 and G4. The *K*a was two times higher for G6 than G5 and for G5 than G4. Concerning thermodynamics parameter (ΔH, ΔS), the alteration of *K*a between G6 and G5 was caused by reduction of ΔS. While in case of the reduction of *K*a between G5 and G4 was caused by increase of ΔH. As described above, increase of ΔH means cleaving hydrogen bond and decrease of ΔS means release of hydrophobic interaction. These results indicate that G6 can interact with Trp327 but G5 can't when Tyr71 pulled non-reducing end. Therefore, the localization of Tyr71 suggested that its side chain contact with non-reducing end of cellooligosaccharides and is responsible for substrate binding on W64A/E186Q. W64A/Y71A/E186Q was examined by ITC to confirm the significance of Tyr71 for substrate binding. This mutant, however, could bind to G6 and G5; contrary to expectation, which is W64A/Y71A/E186Q could not bind to anyone. Moreover, these *K*as were higher than that of E186Q and this force of binding was derived from hydrophobic interaction.

As shown in Fig. 2, the product pattern of W64A/Y71A was different from that of wild type. When G6 was hydrolyzed by mutant, G6 was not degraded enough by comparison with wild type and G2 was not produced (Fig. 2 lane. 2) while the main products of Cel44A are G4 and G2. In addition, the rates of hydrolysis by mutant were lower than that of wild type (Fig. 3). These results suggest that the high affinity of mutant W64A/Y71A cause the product such as G4 to keep in plus side of the cleft after the degradation of cellooligosaccharides, then, G6 is synthesized from this G4 and produced G2 eventually. Therefore, enzyme activity was depressed because the hydrolysis cannot be proceeded sufficiently. This shows that appropriate affinity is favorable because the enzymatic activity is alternated when its affinity for its substrates is high.

Acknowlegements

We thank Akiyoshi Tanaka for using ITC and the technical discussion, Tomiko Chikada for DNA sequencing, and all other members of our laboratory for their valuable advice.

References

1. N. Karim, and S. Kidokoro. (2004) Thermochim. Acta., **412**: 91-96
2. MicroCal, Isothermal titeration calorimetry and deug design. Application note (2006)
3. G. Zolotnitsky, U. Cogan, N.Adir, V. Solomon, G. Shoham and Y. Shoham. (2004) Proc. Natl. Acad. Sci USA., **101**: 11275-11280.
4. F. A. Quiocho, D. K. Wilson and N.K. Vyas. (1989) Nature. 340 (6232): 404-407.
5. M. M. Ahsan, T. Kimura, S. Karita, K. Sakka and K. Ohmiya. (1996) J. Bacteriol., **178**: 5732-5740.
6. Y. Kitago, S. Karita, N. Watanabe, M. Kamiya, T. Aizawa, K. Sakka and I. Tanaka. (2007) J. Biol. Chem., **282**: 35703-35711.
7. M. M. Ahsan, M. Matsumoto, S. Karita, T. Kimura, K. Sakka and K. Ohmiya. (1997) Biosci. Biotech. Biochem., **61**: 427-43