Regular Paper

Degradation of Crystalline Celluloses by *Phanerochaete chrysosporium* Cellobiohydrolase II (Cel6A) Heterologously Expressed in Methylophilic Yeast *Pichia pastoris*

(Received February 24, 2012; Accepted March 31, 2012)
(J-STAGE Advance Published Date: April 12, 2012)

Kiyohiko Igarashi,^{1,*} Michiko Maruyama,¹ Akihiko Nakamura,¹ Takuya Ishida,¹
Masahisa Wada^{1,2} and Masahiro Samejima¹

¹Department of Biomaterial Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo
(1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan)

²Department of Plant & Environmental New Resources, College of Life Sciences, Kyung Hee University
(Yongin-si, Gyeonggi-do 446-701, Republic of Korea)

Abstract: Hydrolysis of crystalline cellulose is a crucial step in utilization of cellulosic biomass and is generally the bottleneck in the biochemical process. The combination of pre-treatment and the use of suitable enzymes is the key to effective saccharification, and ammonia pretreatment is a promising technique to enhance the velocity and yield in saccharification of crystalline cellulose. In the present study, we heterologously expressed recombinant cellobiohydrolase II from *Phanerochaete chrysosporium* (PcCel6A) in *Pichia pastoris*. We then employed surface density analysis to compare the velocities of degradation of crystalline cellulose III_I, which was prepared from algal cellulose I by supercritical ammonia treatment, by the recombinant enzyme and *Trichoderma reesei* cellobiohydrolase I (TrCel7A). The hydrolytic velocity of crystalline cellulose III_I by PcCel6A was approximately 4 times faster than that by TrCel7A, though velocity of cellulose I degradation by PcCel6A was almost half of that by TrCel7A. Since adsorption of both enzymes on cellulose III_I is no more than twice that on cellulose I, we speculate that the enhanced hydrolysis of cellulose III_I by PcCel6A than TrCel7A is not simply due to the increased surface area, but also reflects higher accessibility of cellulose III_I to PcCel6A.

Key words: cellobiohydrolase, crystalline cellulose, *Phanerochaete chrysosporium*, glycoside hydrolase

Cell wall of terrestrial plants consists mainly of cellulose, hemicellulose and lignin, though cellulose is predominant, accounting for up to 50% by weight. Cellulose in the cell wall has an approximately 70% of crystallinity, so that about one-third of the plant cell wall consists of crystalline cellulose, which is generally called cellulose I.¹⁾ In order to utilize this cellulose as a raw material for fuels and plastics, it must be hydrolyzed into its component sugar, glucose.²⁾ Initially, strong acids such as sulfuric and hydrochloric acids were used for this purpose. These chemical processes are inexpensive, but are difficult to regulate owing to the heterogeneity of biomass, and are also environmentally problematic.³⁾ Recently, therefore, there has been a trend to combine chemical and biochemical processes. The chemical reaction is mainly used as a pre-treatment to convert cellulose into a form that is more easily hydrolyzed. For example, diluted acids or weak acids are used to convert cellulose to soluble cellooligosaccharides, which can be hydrolyzed to glucose by β -glucosidase. However, as cellulases have become less expensive, the balance between pretreatment and enzymatic hydrolysis has tilted in favor of enzymatic processes.^{4–6)}

Among many pretreatment procedures, ammonia treatment is favorable because it drastically enhances the enzymatic digestability of cellulosic biomass without causing loss of sugar components.^{7,8)} It removes substituents such as acetyl and feruloyl groups in xylan, like other alkaline treatments, and it promotes the enzymatic degradation of hemicelluloses.⁹⁾ In addition, crystalline cellulose is converted from cellulose I to III_I,¹⁰⁾ which is significantly more degradable by cellulase, and consequently saccharification of the pretreated biomass is greatly enhanced.^{11,12)} Various factors may be involved, including decrease of crystal density, increase of hydrophobic surface area, weakening of the interaction between hydrophobically packed cellulose sheets, and so on. However, we previously showed by means of high-speed atomic force microscopic observations that the velocity of movement of glycoside hydrolase (GH) family 7 cellobiohydrolase I (CBH; EC 3.2.1.176) from cellulose-degrading ascomycete *Trichoderma reesei* (TrCel7A) on cellulose I and III_I surfaces was not significantly different, suggesting that the processivity of the enzyme on these crystals is the same and that the hydrolysis of single cellulose chains is not affected by the treatment.¹³⁾ Therefore, we suggested that the treatment serves to improve accessibility of the substrate to the enzyme, but does not enhance the hydrolytic reaction.

In the present study, we have cloned the gene coding

*Corresponding author (Tel. +81-3-5841-5258, Fax. +81-3-5841-5273, E-mail: aquarius@mail.ecc.u-tokyo.ac.jp).
Abbreviations: AOX, alcohol oxidase; CBH, cellobiohydrolase; DO, dissolved oxygen; GH, glycoside hydrolase; HS-AFM, high-speed atomic force microscopy.

another type of fungal CBH classified into GH family 6 (EC 3.2.1.91) from the basidiomycete *Phanerochaete chrysosporium* (PcCel6A). The recombinant enzyme was heterologously expressed in the methylotrophic yeast *Pichia pastoris* in order to obtain a large amount of the enzyme uncontaminated with other cellulases. We found that this enzyme shows higher hydrolytic activity than TrCel7A towards cellulose III_I. The reason for this difference between the two enzymes is discussed.

MATERIALS AND METHODS

Materials. *Phanerochaete chrysosporium* strain K-3¹⁴⁾ was used as a source of the target gene. *Escherichia coli* strain JM109 (Takara Bio Inc., Shiga, Japan) and *Pichia pastoris* strain KM71H (Invitrogen Ltd., Carlsbad, USA) were used as the subcloning host and for heterologous production of the recombinant protein, respectively. Crystalline cellulose I and III_I were prepared from green algae *Cladophora* spp. according to our previous reports¹¹⁾ and stored as 0.2% suspensions in MilliQ water. GH family 7 cellobiohydrolase from *Trichoderma reesei* (TrCel7A) was purified from a commercial cellulase mixture, Celluclast® 1.5L (Novozyme, available from Sigma-Aldrich Corporation, St. Louis, USA) as described previously.¹⁵⁾

Cloning cDNA encoding GH family 6 cellobiohydrolase (PcCel6A). *P. chrysosporium* was grown on the Kremer and Wood medium¹⁶⁾ containing 2% cellulose (CF11; Whatman Laboratory Products, Fairfield, USA) as a sole carbon source based on the previous report.¹⁷⁾ After 3 days of cultivation, culture filtrate and mycelia (containing unused cellulose particles) were separated using a glass filter membrane (ADVANTEC® GA-100; Toyo Roshi Kaisha Ltd., Tokyo, Japan), and the mycelia were frozen with liquid nitrogen. Total RNA was extracted from approximately 200 mg of frozen mycelial powder using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from mRNA, which was purified from 1 µg of total RNA extracted above with Oligotex™-dT30<Super> (Takara Bio Inc.), using ReverTraAce (Toyobo Co., Ltd., Osaka, Japan) and 3' RACE adaptor primer (Invitrogen Ltd.), according to the manufacturers' instructions. The oligonucleotide primers for the amplification of the cDNA fragment encoding GH family 6 CBH (*cel6A*) were designed based on the total genomic sequence of *P. chrysosporium* as follows; *cel6A*-F: 5'-CAGGCGTCGGAGTGGGGACAGTGC-3', *cel6A*-R: 5'-CTACAGCGGCGGGTTGGCAGCCGA-3' and PCR was performed using KOD-Plus (version 2; Toyobo Co., Ltd.) according to the manufacturer's instructions. PCR products were cloned by using a Zero Blunt® TOPO® PCR cloning kit (Invitrogen Ltd.) and *E. coli* JM109, and sequenced using a Thermo Sequenase Primer Cycle Sequencing Kit (GE Healthcare UK Ltd., Buckinghamshire, UK) with a DNA sequencer SQ5500E (Hitachi High Technologies Corporation, Tokyo, Japan). Since 3 nucleic acids encoding a proline residue at the linker region were missing from the sequence of CBH II (Cel6A) of *P. chrysosporium* strain ME-446¹⁸⁾ (AAB32942) as shown in Fig. 1, the nucleotide sequence of Cel6A from strain K-3 was submitted to the DDBJ/EMBL/ GeneBank databases under accession number AB699568.

Heterologous expression of *cel6A* and purification of PcCel6A. Two different oligonucleotide primers were designed, based on the nucleotide sequence of the mature protein, for ligation into the *Eco*RI and *Not*I sites of the *Pichia* expression vector pPICZα-A (Invitrogen Ltd.): *cel6A*-*Eco*RI-F: 5'-TTTGAATTCAGGCGTCGGAGTGGGGACAGTGC-3' and *cel6A*-*Not*I-R: 5'-TTTTCGCGCCGCTACAGCGGCGGGTTGGCAGCCGA-3'. After the construction of pPICZα/*cel6A* vector, we eliminated the Ste13 signal peptide cleavage site and N-terminal extension generated from the *Eco*RI sequence by the combination of inverse PCR and *Dpn*I treatment using primers *cel6A*-Ste(-)-F: 5'-GTATCTCTCGAGAAAAGACAGGCGTCGGAGTGGGGA-3' *cel6A*-Ste(-)-R: 5'-TCCCCA-CTCCGACGCCTGTCTTTTCTCGAGAGATAC-3', to obtain the recombinant protein with the native N-terminus. Approximately 5 µg of the DNA construct in pPICZα/*cel6A*-Ste(-) was linearized with *Bgl*III (Takara Bio Inc.) prior to transformation of *P. pastoris*. Electroporation and selection of transformants were carried out according to the instruction manual of the EasySelect™ *Pichia* expression kit (version G; Invitrogen Ltd.) as described previously.^{19–21)} The recombinant protein was produced using a Mini jar-fermentor (TSC-M5L; Takasugi Seisakusho K.K., Tokyo, Japan) equipped with a DO controller (DJ-1033; ABLE Corporation, Tokyo, Japan) according to the *Pichia* Fermentation Process Guidelines (Invitrogen Ltd.). After 4 days in methanol-fed batch culture, the medium was collected by centrifugation (30 min, 5,000 × G), and ammonium sulfate and sodium acetate buffer, pH 5.0, were added to the cell-free culture medium to final concentrations of 1 M and 20 mM, respectively. The solution was fractionated on a Phenyl-TOYOPEARL 650S column (26 mm × 120 mm; Tosoh Corporation, Tokyo, Japan) equilibrated with 20 mM sodium acetate buffer containing 1 M ammonium sulfate, pH 5.0. The protein was eluted with a 300 mL reverse gradient to 20 mM sodium acetate buffer, pH 5.0. The fractions containing the recombinant protein were collected, equilibrated against 20 mM Tris-HCl buffer, pH 8.0, and applied to a SuperQ-TOYOPEARL 650S column (9 mm × 120 mm) equilibrated with the same buffer. The protein was eluted from the column with a linear gradient from 0 to 0.5 M NaCl in 100 mL. Its purity was confirmed by SDS-PAGE analysis on 12% polyacrylamide gel, and the N-terminal amino acid sequence was determined with a protein sequencer (Model 491 cLC; Applied Biosystems Inc., Foster City, USA).

Fig. 1. Domain architecture of PcCel6A (upper) and sequence comparison of linker regions from Cel6A of *P. chrysosporium* strain K3 and ME446 (AAB32942).

One proline residue is missing from the cct-repeated region in strain K3.

Enzyme assay. Crystalline cellulose (0.1% w/v) was incubated with various concentrations of enzymes (0.25–8.0 μM) in 1.0 mL of 50 mM sodium acetate buffer, pH 5.0, at 30°C with inversion. The mixture was centrifuged (10,000 G $\times$ 30 s) to remove insoluble cellulose, and the supernatant (150 μL) was collected in 96-well microplates. The concentration of free enzyme [*F*] was determined by HPLC (LC-2000 series; Jasco Corporation, Tokyo, Japan) with a UV detector (UV-2075), based on absorption coefficients at 280 nm of 92,000 and 83,000 $\text{M}^{-1}\text{cm}^{-1}$ for *PcCel6A* and *TrCel7A*, respectively. The soluble products were similarly analyzed on an HPLC system equipped with microplate auto-sampler (AS-2057; Jasco Corporation) and a Corona^{TM} Charged Aerosol Detector^{TM} (ESA Biosciences Inc., Chelmsford, USA). The supernatant was separated on Shodex® Asahipak NH₂P-50 (Showa Denko K.K., Kawasaki, Japan) with a linear gradient of acetonitrile/H₂O (60/40 to 50/50, v/v). Products were quantified using cellooligosaccharides with degree of polymerization (DP) = 2–7 (Seikagaku Corporation, Tokyo, Japan) as standards. Data analysis was performed using the surface density (ρ) according to the previous report.²²⁾

RESULTS AND DISCUSSION

One of the advantages of using *P. pastoris* for protein expression is its high productivity of heterologous protein (at the gram per liter of culture level). In this study, we over-expressed the gene coding *PcCel6A* in *P. pastoris* under control of the alcohol oxidase promoter (*AOX1*) as shown in Fig. 2. The wet weight of glycerol-fed yeast cells increased continuously up to 48 h of cultivation. When the wet weight reached 250 g/L at about 42 h of cultivation, methanol, a strong inducer of *AOX1*, was added, while dissolved oxygen was maintained at a rather low level (2–3 ppm). The protein concentration in the culture increased after 60 h of cultivation and reached 4.6 g/L at 160 h.

The effect of substrate characteristics on the hydrolysis of cellulose has been widely discussed, but there has been less consideration of the effect of differences of surface area among substrates, probably because of the difficulty of estimating the cellulase-accessible surface area of cellulose samples. To remove the effect of differences of surface area among various substrates, therefore, we have previously developed a method for evaluating cellulase activity that takes account of the surface density (ρ) of enzymes acting at a crystalline surface.^{12,22)} Surface density is easily calculated by dividing the actual amount of adsorbed enzyme by the maximum amount (A/A_{max}), and it enables us to distinguish differences of hydrolytic rate from differences of accessible surface area. When the adsorption of *PcCel6A* was measured

at various concentrations of free enzyme, typical hyperbolic plots were obtained, as shown in Fig. 3. The parameters obtained from Fig. 3 are compared those for *TrCel7A* in Table 1. The plots for cellulose I as a substrate were fitted with Langmuir's two-binding site model²³⁾ and the A_{max} value of *PcCel6A* was estimated to be 2.2 ± 0.2 nmol/mg-cellulose. However, for cellulose III_I, simulation using the two-site model did not fit the data, and a single hyperbolic function (Langmuir's equation) gave a better fit. The A_{max} value of *PcCel6A* for cellulose III_I was 3.3 ± 0.2 nmol/mg-cellulose, and the ratio of A_{max} -cellulose III_I/ A_{max} -cellulose I of *PcCel6A* was 1.5, clearly indicating that cellulose III_I used in the present study has a greater surface area for *PcCel6A*, as was the case for *TrCel7A*. In a previous report,¹¹⁾ crystalline cellulose III_I had a 1.7 times larger surface area than cellulose I when *TrCel7A* was used, though the increase of surface area for *TrCel7A* did not fully explain the enhanced hydrolysis of cellulose III_I. In the case of *PcCel6A*, the

Fig. 2. Production of recombinant *PcCel6A* by the methylotrophic yeast *P. pastoris* in jar-fermenter culture.

The time courses of wet weight of yeast cells (open square), concentration of dissolved oxygen (cross) and protein concentration in the culture medium (filled circle) were monitored. Solid and open arrowheads indicate the starting points of glycerol and methanol feeds, respectively.

Fig. 3. Plot of free protein concentration vs. amount of adsorbed protein for *PcCel6A* on cellulose I (filled circle) and cellulose III_I (open circle).

The plots were fitted to Langmuir's two-binding site and one-binding site models for cellulose I and III_I, respectively, and the parameters obtained from the fitting are summarized in Table 1.

Table 1. Parameters for the adsorption of *PcCel6A* and *TrCel7A* on crystalline cellulose.

		A_1 (nmol/mg-cellulose)	K_{ad1} (μM^{-1})	A_2 (nmol/mg-cellulose)	K_{ad2} (μM^{-1})
<i>PcCel6A</i>	Cellulose I	0.59 ± 0.03	11 ± 1	1.6 ± 0.2	0.44 ± 0.03
	Cellulose III _I	3.3 ± 0.2	0.54 ± 0.06	n.d. ^b	n.d. ^b
<i>TrCel7A</i> ^a	Cellulose I	0.22 ± 0.02	8.5 ± 0.7	2.0 ± 0.2	0.44 ± 0.04
	Cellulose III _I	1.8 ± 0.5	13 ± 2	1.8 ± 0.5	0.27 ± 0.04

Cellulobiohydrolases were incubated with 0.1% crystalline cellulose for 2 h in 50 mM Na-acetate buffer pH 5.0. ^aThese values were taken from our previous report.¹¹⁾ ^bNot determined because a single hyperbolic function gave a better fit to the results.

difference of surface area between cellulose I and III_I was less than in the case of *TrCel7A*, suggesting that the difference of hydrolysis by the enzyme is partly related to a difference in reactivity with the cellulose samples, as discussed below.

The hydrolysis of cellulose I and III_I by various concentrations of *PcCel6A* was monitored by measuring cellobiose concentration in the supernatant of the reaction mixture as shown in Fig. 4. When cellulose III_I was used as a substrate in Fig. 4 (B), the concentration of product was more than 10 times higher than in the case of cellulose I (Fig. 4), indicating that the hydrolysis is accelerated similarly to that of *TrCel7A*. In the case of *TrCel7A*, however, increasing the concentration of enzyme caused a decrease of hydrolysis as previously reported, whereas this inhibition was not observed for *PcCel6A* in both cases (Figs. 4 (A) and (B)), suggesting that over-crowding of molecules on the surface does not occur for *PcCel6A*. The maximal value of hydrolytic velocity of crystalline cellulose III_I by *PcCel6A* was 19.5 $\mu\text{M}/\text{min}$ at 8.0 μM , which is 3.7 times higher than that of *TrCel7A* (5.33 $\mu\text{M}/\text{min}$ at 4.0 μM). Normally the velocity of hydrolysis has been evaluated in the region where product formation is linearly related to reaction time in order to characterize CBHs. A linear increase of cellobiose concentration was observed after 120 min of incubation at all concentrations tested when cellulose I was used as the substrate, whereas product formation was linear only at low conversion of cellulose III_I and the velocity was drastically decreased with increasing incubation time at high levels of enzyme loading ($>2.0 \mu\text{M}$). This may be because of the shortage of crystalline cellulose as a substrate and the accumulation of cellobiose, a strong inhibitor of CBHs, with progress of the reaction. After 360 min of incubation, the degradation of crystalline

Fig. 4. Time course of cellobiose formation by *PcCel6A* from cellulose I (A) and cellulose III_I (B).

0.2 (filled square), 0.5 (filled circle), 1.0 (filled triangle), 2.0 (open square), 4.0 (open circle) and 8.0 (open triangle) μM *PcCel6A* was incubated with 0.1% crystalline cellulose in sodium acetate buffer, pH 5.0 and the amount of soluble products was measured using HPLC based on the previous report,¹⁹⁾ as described in the Materials and Methods section.

cellulose III_I reached 77% with 8.0 μM *PcCel6A* and 2,370 μM cellobiose was produced in the reaction mixture, which is 15 times more than in the case of cellulose I under the same conditions.

The velocity of cellobiose production was then plotted versus surface density ρ as shown in Fig. 5. The cellobiose-producing velocity of *PcCel6A* was not influenced by surface density for either cellulose I or cellulose III_I, whereas the velocity of cellulose III_I hydrolysis was approximately 40 times higher than that of cellulose I. In the case of *PcCel6A*, however, the velocity was slightly enhanced, but not reduced, with increase of ρ ; this is in marked contrast with *TrCel7A*, whose velocity is lowered with increase of ρ . Although the velocity of *TrCel7A* showed a maximum at $\rho = 0.5$, the hydrolytic activity of *PcCel6A* continuously increased with increase of ρ . Since cellobiose production is affected not only by ρ , but also by the actual amount of adsorption (Fig. 5 (A)), we also plotted the surface density dependence of specific activity for *PcCel6A* (Fig. 5 (B)), and compared the parameters obtained with those of *TrCel7A*, as shown in Table 2. In this analysis, the k_0 -value is the ideal velocity when $\rho \rightarrow 0$, i.e., the apparent velocity of the enzyme at

Fig. 5. Surface density (ρ) dependence of velocity for cellobiose production (A) and specific activity of bound enzyme (B).

The velocity and specific activity for cellulose I (filled circle) and cellulose III_I (open circle) were estimated using the data from Figs. 3 and 4 as described in Materials and Methods. The parameters obtained from B are summarized and compared with those from *TrCel7A* in Table 2.

Table 2. Specific activity of bound enzyme (k) at $\rho \rightarrow 0$ (k_0) and reduction rate of k (B) for hydrolysis of crystalline celluloses.

		k_0 (min^{-1})	B
<i>PcCel6A</i>	Cellulose I	0.85 ± 0.05	-1.6 ± 0.2
	Cellulose III _I	18 ± 1	-1.1 ± 0.1
<i>TrCel7A</i> ^a	Cellulose I	1.7 ± 0.1	-2.5 ± 0.2
	Cellulose III _I	4.5 ± 0.4	-1.3 ± 0.1

^aThese values were taken from our previous report.¹¹⁾ These parameters were calculated from the ρ - k plots in Fig. 5(B).

infinite surface area, and the *B*-value is the reduction rate of the velocity with increase of ρ . The k_0 -value of *PcCel6A* was almost half of that of *TrCel7A* for cellulose I as a substrate, whereas k_0 of *PcCel6A* is 4.3 times higher than that of *TrCel7A* for cellulose III_I as a substrate. The *B*-value was enhanced for cellulose III_I compared with cellulose I for both enzymes, indicating that conversion of cellulose I to III_I prevents the reduction of the velocity at higher density of enzyme molecules.

Recently, we have observed *TrCel7A* molecules using high-speed atomic force microscopy (HS-AFM), and our results indicated that the velocities of processive movement of *TrCel7A* molecules on cellulose I and III_I are similar, but the congestion of enzyme molecules at the surface of crystalline cellulose III_I is apparently less than that in the case of cellulose I, as seen in real-time movie recordings.¹³⁾ We speculate this is mainly because of the difference of surface area available for cellulases between the two crystalline celluloses. However, simple comparison of the amounts of adsorbed enzyme on cellulose I and III_I could not explain the drastic enhancement of hydrolysis of cellulose III_I, clearly indicating that other mechanisms are also involved. Moreover, the degradation of crystalline cellulose III_I by *PcCel6A* was greatly enhanced compared with that by *TrCel7A* in the present study. The difference between the modes of action of GH family 6 and 7 CBHs has been frequently discussed, and the major difference between these enzyme families is that GH family 6 CBHs hydrolyze cellulose chains from the non-reducing to the reducing-end (EC 3.2.1.91), whereas GH family 7 CBHs act in the opposite direction (EC 3.2.1.176). However, we recently observed by means of HS-AFM that CBH II from *T. reesei* (*TrCel6A*) does not move on cellulose III_I during hydrolysis, and a possible endo-type hydrolysis of crystalline cellulose by GH family 6 enzymes was proposed.¹³⁾ Since production of hydrolytic products (cellobiose) by *TrCel6A* is higher than that by *TrCel7A*, cellulose III_I may be more susceptible to endo-type attack of CBHs than cellulose I, although typical endo-glucanases can not degrade crystalline cellulose (data not shown).

In conclusion, GH family 6 cellobiohydrolase from the basidiomycete *P. chrysosporium* (*PcCel6A*) was heterologously expressed in *P. pastoris*, and its kinetic properties were characterized by means of surface density analysis. Ammonia treatment, which converts crystalline cellulose I to cellulose III_I, apparently increases the surface area and the susceptibility to *PcCel6A*. This is quite similar to the adsorption and degradation behavior of GH family 7 cellobiohydrolase from *T. reesei* (*TrCel7A*) on crystalline cellulose, but the enhancement of hydrolysis of crystalline cellulose III_I was much greater for *PcCel6A* than for *TrCel7A*. We speculate that this difference may be due to a difference in the susceptibility of cellulose III_I to endo-type hydrolysis by the cellobiohydrolases, rather than a difference in processivity. The present results clearly indicate the importance of appropriate conversion of crystalline polymorphs for better saccharification of cellulosic biomass.

ACKNOWLEDGMENTS

This research was supported by Grants-in-Aid for Scientific

Research to K.I. (19688016 and 21688023) from the Japanese Ministry of Education, Culture, Sports, and Technology; by a Grant for Development of Technology for High Efficiency Bioenergy Conversion Project to M.S. (07003004-0) from the New Energy and Industrial Technology Development Organization; and by a Advanced Low Carbon Technology Research and Development Program from the Japan Science and Technology Agency to K.I.

REFERENCES

- 1) D.N.S. Hon: Cellulose - a random-walk along its historical path. *Cellulose*, **1**, 1–25 (1994).
- 2) M.E. Himmel, S.Y. Ding, D.K. Johnson, W.S. Adney, M.R. Nimlos, J.W. Brady and T.D. Foust: Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science*, **315**, 804–807 (2007).
- 3) M.E. Himmel, M.F. Ruth and C.E. Wyman: Cellulase for commodity products from cellulosic biomass. *Curr. Opin. Biotechnol.*, **10**, 358–364 (1999).
- 4) J. Sheehan and M. Himmel: Enzymes, energy, and the environment: A strategic perspective on the U.S. Department of Energy's Research and Development Activities for Bioethanol. *Biotechnol. Prog.*, **15**, 817–827 (1999).
- 5) C.E. Wyman: Twenty years of trials, tribulations, and research progress in bioethanol technology: selected key events along the way. *Appl. Biochem. Biotechnol.*, **91–93**, 5–21 (2001).
- 6) A.K. Chandel, G. Chandrasekhar, M.B. Silva and S.S. da Silva: The realm of cellulases in biorefinery development. *Crit. Rev. Biotechnol.*, doi: 10.3109/07388551.2011.595385, Posted on line on September 19 (2011).
- 7) B.L. Foster, B.E. Dale and J.B. Doran-Peterson: Enzymatic hydrolysis of ammonia-treated sugar beet pulp. *Appl. Biochem. Biotechnol.*, **91–93**, 269–282 (2001).
- 8) A. Ferrer, F.M. Byers, B. Sulbaran-de-Ferrer, B.E. Dale and C. Aiello: Optimizing ammonia pressurization/depressurization processing conditions to enhance enzymatic susceptibility of dwarf elephant grass. *Appl. Biochem. Biotechnol.*, **84–86**, 163–179 (2000).
- 9) H.K. Murnen, V. Balan, S.P. Chundawat, B. Bals, C. Sousa Lda and B.E. Dale: Optimization of ammonia fiber expansion (AFEX) pretreatment and enzymatic hydrolysis of *Miscanthus × giganteus* to fermentable sugars. *Biotechnol. Prog.*, **23**, 846–850 (2007).
- 10) M. Wada, H. Chanzy, Y. Nishiyama and P. Langan: Cellulose III_I crystal structure and hydrogen bonding by synchrotron X-ray and neutron fiber diffraction. *Macromolecules*, **37**, 8548–8555 (2004).
- 11) K. Igarashi, M. Wada and M. Samejima: Activation of crystalline cellulose to cellulose III_I results in efficient hydrolysis by cellobiohydrolase. *FEBS J.*, **274**, 1785–1792 (2007).
- 12) K. Igarashi, M. Wada and M. Samejima: Kinetic analysis of cellobiohydrolase: Quantification of enzymatic reaction at a solid/liquid interface applying the concept of surface density. *Trends Glycosci. Glycotechnol.*, **21**, 13–22 (2009).
- 13) K. Igarashi, T. Uchihashi, A. Koivula, M. Wada, S. Kimura, T. Okamoto, M. Penttilä, T. Ando and M. Samejima: Traffic jams reduce hydrolytic efficiency of cellulase on cellulose surface. *Science*, **333**, 1279–1282 (2011).
- 14) S.C. Johnsrud and K.E. Eriksson: Cross breeding of selected and mutated homokaryotic strains of *Phanerochaete chrysosporium* K-3: New cellulase deficient strains with increased ability to degrade lignin. *Appl. Microbiol. Biotechnol.*, **21**, 320–327 (1985).
- 15) K. Igarashi, A. Koivula, M. Wada, S. Kimura, M. Penttilä and M. Samejima: High speed atomic force microscopy visualizes processive movement of *Trichoderma reesei* cellobiohydrolase I on crystalline cellulose. *J. Biol. Chem.*, **284**, 36186–36190 (2009).
- 16) S.M. Kremer and P.M. Wood: Evidence that cellobiose oxidase from *Phanerochaete chrysosporium* is primarily an Fe(III) reductase. Kinetic comparison with neutrophil NADPH oxidase and yeast flavocytochrome *b₂*. *Eur. J. Biochem.*, **205**, 133–138 (1992).

- 17) N. Habu, K. Igarashi, M. Samejima, B. Pettersson and K.E. Eriksson: Enhanced production of cellobiose dehydrogenase in cultures of *Phanerochaete chrysosporium* supplemented with bovine calf serum. *Biotechnol. Appl. Biochem.*, **26**, 97–102 (1997).
- 18) C.A. Tempelaars, P.R. Birch, P.F. Sims and P. Broda: Isolation, characterization, and analysis of the expression of the *cbhIII* gene of *Phanerochaete chrysosporium*. *Appl. Environ. Microb.*, **60**, 4387–4393 (1994).
- 19) K. Igarashi, T. Ishida, C. Hori and M. Samejima: Characterization of an endoglucanase belonging to a new subfamily of glycoside hydrolase family 45 of the basidiomycete *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.*, **74**, 5628–5634 (2008).
- 20) K. Igarashi, M. Yoshida, H. Matsumura, N. Nakamura, H. Ohno, M. Samejima and T. Nishino: Electron transfer chain reaction of the extracellular flavocytochrome cellobiose dehydrogenase from the basidiomycete *Phanerochaete chrysosporium*. *FEBS J.*, **272**, 2869–2877 (2005).
- 21) R. Kawai, K. Igarashi, M. Yoshida, M. Kitaoka and M. Samejima: Hydrolysis of β -1,3/1,6-glucan by glycoside hydrolase family 16 endo-1,3(4)- β -glucanase from the basidiomycete *Phanerochaete chrysosporium*. *Appl. Microbiol. Biotechnol.*, **71**, 898–906 (2006).
- 22) K. Igarashi, M. Wada, R. Hori and M. Samejima: Surface density of cellobiohydrolase on crystalline celluloses. A critical parameter to evaluate enzymatic kinetics at a solid-liquid interface. *FEBS J.*, **273**, 2869–2878 (2006).
- 23) J. Ståhlberg, G. Johansson and G. Pettersson: A new model for enzymatic hydrolysis of cellulose based on the two-domain structure of cellobiohydrolase I. *Bio/Technology*, **9**, 286–290 (1991).