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Exploration of Cellulose Surface-Binding Properties of *Acidothermus cellulolyticus* Cel5A by Site-Specific Mutagenesis

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Abstract

Understanding the interactions between cellulases and cellulosic substrates is critical to the development of an efficient artificial cellulase system for conversion of biomass to sugars. We directed specific mutations to the interactive surface of the *Acidothermus cellulolyticus* EI endoglucanase catalytic domain. The cellulose-binding domain is not translated in these mutants. Amino acid mutations were designed either to change the surface charge of the protein or to modify the potential for hydrogen bonding with cellulose. The relationship between cellulase-to-cellulose (Avicel PH101) binding and hydrolysis activity was determined for various groupings of mutations. While a significant increase in hydrolysis activity was not observed, certain clusters of residues did significantly alter substrate binding and some interesting correlations emerged. In the future, these observations may be used to aid the design of endoglucanases with improved performance on pretreated biomass.

Index Entries: Cellulase; endoglucanase; site-directed mutagenesis; *Acidothermus cellulolyticus* Cel5A.

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Introduction

The Cellulase Cost Problem

We now understand that the cellulase production system used for the economic production of bioethanol from biomass must be made considerably more efficient than those currently available from commercial *Trichoderma reesei* technologies. The US Department of Energy's future goals to produce bioethanol for less than $1.00/gal require substantial improvements in the current production efficiency for cellulase activity or equally large improvements in other unit operations of the overall process, or both *(1)*. To improve cellulase use efficiency dramatically, we must either increase the volumetric production or conversion yield efficiency of *T. reesei* cellulase activity 10-fold—an improbable task—or increase the specific activity of the cellulase system by an equivalent degree. We feel that combinations of these options are the most promising in a mid- to long-range research scenario *(2)*. An enzyme engineering approach has another distinct advantage: once the principles governing cellulase action on cellulose are known, enzyme and enzyme systems may be "tuneable" for maximum efficiency on various biomass feedstocks *(3,4)*.

Increasing the Specific Activity of Cellulases

The specific activity of cellulases may be increased by a suite of biotechnical approaches, some of which may be applied alone or used in concert with other solutions. The cellulase system is a true synergistic enzyme complex requiring at least one endoglucanase, two different exoglucanases (one cellulose reducing terminus specific and one cellulose nonreducing terminus specific), and a cellobiase. The native *T. reesei* cellulase system is indeed a difficult standard to beat; however, this enzyme complex has some important limitations for economic application to bioethanol production. One limitation is common to all enzymes that act on insoluble substrates: low specific activity. The fungal cellulase complex is produced at the tips of growing hyphae and is thus concentrated in pockets in the plant tissue defined by this small exposure zone. The protein concentration can be predicted to be very high in these "enzyme pockets," and natural selection pressure for cellulases of high specific activity may have been lacking (D. Eveleigh, personal communication). However, there is no convincing evidence to date that bacterial cellulase component enzymes exhibit higher specific activities than their fungal counterparts. Fungal cellulases are also distinctly mesophilic enzymes, often encountering irreversible denaturation at temperatures above 55°C. Thus, increasing thermal tolerance may be another strategy to improve performance. Other strategies include reducing end product inhibition (by cellobiose, glucose, and perhaps xylose), reducing nonspecific binding to substrate, and enhancing enzymatic cellulose decrystallization. To some extent, all these topics involve the direct interaction of enzymes with an insoluble substrate *(4,5)*.

Probing the Performance of Endoglucanases

In 1984, *Acidothermus cellulolyticus* was isolated from Yellowstone National Park hot springs *(6)*. EI endoglucanase (Cel5A) was the first endoglucanase isolated from *A. cellulolyticus* culture broth and was described by US patent no. 5,275,944 *(7)*. EI hydrolyzes cellulose and cellodextrins by the retaining-type, two-step general mechanism of Koshland *(8)*. This mechanism is highly conserved throughout glycosyl hydrolase family 5 *(9)*. Although EI has been expressed at laboratory scale from *Streptomyces lividans* and *Pichia pastoris*, *Escherichia coli* is the expression system of choice for rapid and convenient production of mutant enzymes. However, rEI is expressed from *E. coli* in a truncated form (i.e., indigenous proteases cleave the catalytic domain [CD] from the linker peptide leaving the EI_{CD}). We have determined that EI_{CD} is not only more thermally stable than native EI *(10)*, but also comparably active on pretreated biomass relative to the full-length enzyme (when assayed in the presence of *T. reesei* cellobiohydrolase I [CBHI]). EI is also an excellent candidate for site-directed mutagenesis because a high-resolution three-dimensional structure of the CD was resolved in 1996 *(11)*. A general consideration for EI site-directed mutagenesis is that EI is a member of the 4/7 subclass of family 5; thus, the key catalytic residues (Glu 282 and Glu 162) are located in the β-turns on the active site side of β-strands 4 and 7, and modification of these regions can be expected to have a strong negative impact on active-site geometry *(12)*.

Cellulases are known to interact with the cellulose surface through one or both faces of the cellulose-binding domain (CBD) and the cellulose-proximal surface of the CD. The entrance to the active-site channel also plays a role in stabilizing the glycosyl residues as they proceed toward the final docking position for hydrolysis. Thus, the amino acid residues defining the active site, and in immediate contact with the cellulose surface, are candidates for modification. We assume that for cellulases to function at maximal specific activity, they must bind to cellulose for the correct period of time, and, although somewhat subjective, too weak binding probably leads to inadequate substrate fit into the active site too strong binding probably leads to enzyme loss to the surface and even poor "leaving group" formation. Hence, the stage is clearly set for productive modification of the surface-interactive residues to effect higher catalytic rates on pretreated biomass. The question is, how must the chemistry be changed, and is this surface optimally engineered in nature already? Note that there are currently no reports of cellulases being enhanced, by engineering surface amino acid residues, to be more effective on crystalline cellulose *(13–15)*. Previous efforts related to this work have focused on the cellulose-binding characteristics of native *(16,17)* and modified CBD's *(18–20)*. Others have examined native, full-length, and truncated cellulase enzymes *(21–24)*. Several recent reviews on cellulase/cellulose-binding theory and experimental design include those by Bothwell and Walker *(25)* and Lee et al. *(26)*.

In the present study, the first phase of our strategy to improve EI action on pretreated biomass was the systematic replacement of the key surface amino acid residues that are expected to interact through hydrogen bonding with cellulose. These are charged residues and those with amide or hydroxyl functionalities. Key surface residues were replaced with arginines, alanines, aspartates, and tyrosines (see Results for notes on mutation strategy). The two hydrophobic surface residues, Trp42 and Tyr82, are positioned to interact with the target cellodextrin (probably at the – 4 and – 7 positions) and were not modified. For the purpose of our study, we have identified surface residues that fall into three classes: those located directly in the active site, those stabilizing the target cellodextrin (extended site), and those available for surface interaction.

Materials and Methods

Polymerase Chain Reaction Mutation

A 2.3-kb *Bam*HI fragment, containing the CD of the EI gene, was subcloned into pUC19. This *Bam*HI fragment includes the EI native promoter, which functions in both *E. coli* and *S. lividans*, and has approx 800 bp of upstream sequence. The downstream *Bam*HI site cleaves the EI coding sequence at a point such that the protein is truncated near the beginning of the CBD. Thus, the construct encodes a protein that includes a signal peptide, the N-terminal CD, the entire linker region, and the first few amino acids of the C-terminal CBD. The construct was further modified by introduction of a TAATGA stop codon following the CD so that only the CD was expressed.

QuickChange™ Site-Directed Mutagenesis (Stratagene, San Diego, CA) was used to generate mutants with targeted amino acid substitutions. Mutagenic primers, containing the desired mutation(s), were designed and synthesized (DNA Sequencing Facility, Iowa State University). The double-stranded DNA vector with EI insert, combined with the two complementary synthetic oligonucleotide primers containing the desired mutation, underwent temperature cycling with *Pfu*Turbo DNA polymerase for primer extension and amplification. Three percent dimethyl sulfoxide was added to the standard polymerase chain reaction (PCR) mixture to facilitate EI amplification. Incorporation of the oligonucleotide primers generated a mutated gene. Following temperature cycling, the product was treated with *Dpn*I to digest the parental methylated DNA template, and the PCR product was used to transform Epicurian Coli XL1-Blue supercompetent cells (Stratagene). Transformed Amp® colonies were picked, and plasmid DNA was extracted (QIAprep spin mini prep kit; Qiagen) from several transformants for sequencing (DNA Sequencing Facility, Iowa State University) and confirmation of the mutations (sequence alignments with OMIGA).

Fermentation

Transformed *E. coli* XL1/Blue cells were grown overnight on LB plates with 100 µg/mL of ampicillin at 37°C. A single colony was then used to inoculate a 5-mL LB-Amp^{100} culture that was incubated for 8 h at 37°C, and 250 rpm. Two milliliters of the seed were transferred to 500 mL of LB-Amp^{100} broth and incubated overnight. The entire contents were then aseptically transferred to a 10-L NBS BioFlow 3000 fermentor (9-L working volume). The conditions maintained throughout the experiment were as follows: pH 7.0, 30°C, 250 rpm agitation, and 30% dissolved oxygen. The cells were harvested, after 24 h, using an NBS bench model CEPALE continuous centrifuge at 30,000*g*.

Cell Lysis by Bead Milling

In aliquots of approx 50 g (wet wt), cells were added to the chamber of a Biospec (Bartlesville, OK) bead mill containing 100 g of 0.1-mm glass beads, and the head space was filled with 20 m*M* Tris, pH 8.0. Cells were lysed for 5 min while the chamber was chilled with ice. The lysate was clarified by centrifugation at 27,000*g* (Sorvall RC-5B, GSA rotor) and 10°C for 20 min. The supernatant was decanted and centrifuged again for 10 min.

Protein Purification

In general, the purification scheme proceeded as follows: In preparation for hydrophobic interaction chromatography, the clarified supernatant was brought to 0.5 *M* $(NH_4)_2SO_4$ and filtered through a glass fiber filter (Whatman GF/F), followed by a 0.45 µm PES filter. The filtrate was loaded onto a HiLoad phenyl sepharose fast protein liquid chromatography (FPLC) column, the column was washed with 3 to 5 column vol of the starting buffer, and the rEI protein was eluted by a 4-column vol descending linear gradient to 0% salt in 20 m*M* Tris, pH 8.0. Fractions (20 mL) were collected and assayed for p-nitrophenol cellobioside (pNPC) hydrolysis activity. In a 96-well microtiter plate, 100 µL of select fractions and 50 µL of 50 m*M* acetate buffer, pH 5.0 with 0.8 mg/mL of pNPC were combined and incubated at 50°C. After appropriate incubation (1 h to overnight, depending on activity level), 15 µL of 1 *M* Na_2CO_3 was added to the wells to increase pH, enhancing color development in the positive wells. Appropriate fractions were pooled and desalted in an Amicon PM10 stirred cell concentrator to ≤400 µS/cm for loading onto a HiLoad Q-Sepharose ion-exchange FPLC column. After washing, the rEI protein was eluted with a 4-column vol ascending linear gradient to 100% 20 m*M* Tris, pH 8.0, with 1 *M* NaCl. The fractions were again assayed for pNPC activity, the active fractions were pooled, and the volume was reduced to <2 mL in a stirred cell concentrator with a PM10 membrane. In rare cases, ion-exchange chromatography was conducted at pH 9.0 to enhance binding to the Q-Sepharose

column. For final purification and buffer exchange, the concentrate was loaded onto a Superdex 200, HiLoad prep-grade SEC column with a flow rate of 1 mL/min in 20 m*M* acetate, pH 5.0, buffer with 100 m*M* NaCl. Protein purity was confirmed with sodium dodecylsulfate polyacrylamide gel electrophoresis and colloidal Coomassie brilliant blue protein staining. The ultraviolet absorbance at 280 nm was measured, and the protein concentration was calculated based on the extinction coefficient calculated from the primary amino acid sequence of the mutant protein by using the ExPASy ProtParam tool (http://www.expasy.ch/tools/protparam.html).

Cellulose Binding Assay

In 300-µL aliquots, a continuously mixed suspension of 40 mg/mL of Avicel PH101 (final assay concentration of 20 mg/mL of microcrystalline cellulose; FMC), in 50 m*M* sodium acetate, buffer pH 5.0, was added to 2 mL of Safe-Lock Eppendorf tubes via a wide-bore pipet tip. In triplicate, 20–70 µg/mL of purified rEI protein and an appropriate volume of buffer, to bring the total volume to 600 µL, was added. Protein was added last to start the reaction, and the tubes were immediately placed into cassette holders, which were inserted into a rotary mixer submerged in a 50 ± 0.1°C water bath. Mixing at 3 rpm was maintained for 30 min, after which the cassette was quickly removed and the tubes were microfuged at 14,000 rpm for 4 min. The supernatants were decanted and microfuged again to remove residual cellulose. Supernatants were analyzed for reducing sugars by the bicinchoninic acid method of Doner and Irwin *(27)* and for activity on pNPC. For quantitative analysis, the pNPC assay was conducted basically as just described, except in covered test tubes and a 50°C water bath, and the absorbance was read at 410 nm. The concentration of unbound rEI was calculated from the slope of a pNPC standard curve generated with the particular mutant. Bound EI was calculated by subtracting the unbound amount from the starting enzyme concentration. The range of protein loadings was used to bracket 1% hydrolysis, as the benchmark for comparison. This level of hydrolysis was chosen to ensure excess of substrate throughout the assay, to minimize feedback inhibition, and because EI alone would not be expected to extensively hydrolyze Avicel. The maximum extent of hydrolysis in these assays was 2%, at the highest protein loadings.

Results

Selection of Sites for Mutation

The surface of EI endoglucanase was mapped manually using MSI InsightII software. Glycine substitutions are expected to result in peptide backbone perturbation according to phi/psi angle predictions, and thus they were not replaced. The other amino acids suggested for testing are expected to cause little or no backbone distortion *(28)*. Additionally, there

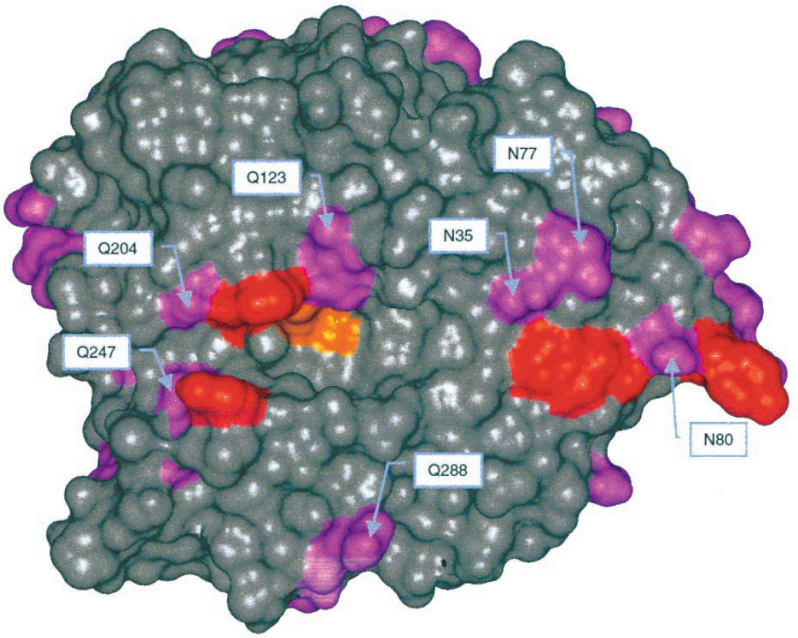

Fig. 1. Mutation strategy for group I hydrogen-bonding residues.

are no prominent Ala residues on the EI_{CD} cellulose interactive surface, so it was not possible to easily make amino acid replacements that increased hydrogen bonding. The following positions of key interactive surface amino acid residues in EI endoglucanase are based on the criteria discussed:

1. EI surface (central): Gln204; Gln247; Gln123; Asn35; Asn80; Asn77; Thr248; Ser210; Ser121; Asp327; Asp324.
2. EI Surface (right): Asp252; Gln288; Ser251; Thr290; Asp342; Asp334.
3. EI Surface (left): Thr129; Ser130, Ser131; Asp165; Asp173; Asp119; Asp68.
   Note that these positions are viewed from the cellobiose leaving side of the active site, interactive surface up.

The locations of the amino acid residues just given are also shown in Figs. 1–3. Several residues were considered unlikely to have a profound effect on surface binding because their locations were more to the side of the domain and more than 15 Å. away from cellulose. These were Asp173, Asp209, Asp252, Asp342, Ser210, and Thr290. Residues likely to affect active-site function, Gln204 and Gln247, were also not changed. Because modifications to Asp327 were shown experimentally to affect activity (data not shown), residue Asp324 was not modified. Asp327 has been shown to interact with the O6 of glucose, which indicates the importance of Asp327 in the hydrolysis of cellulose (but probably not xylan) *(11)*. These residues, along with R62 (the only arginine residue lying directly

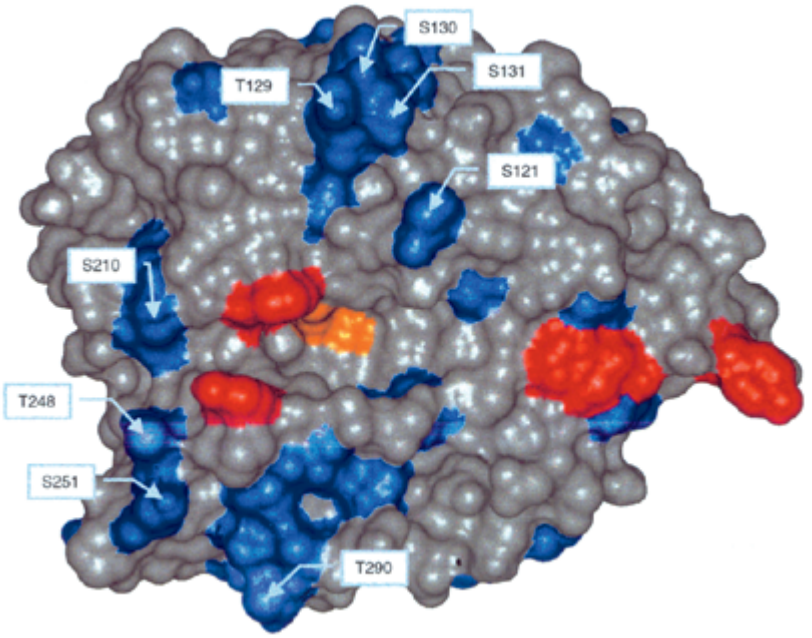

Fig. 2. Mutation strategy for group II hydrogen-bonding residues.

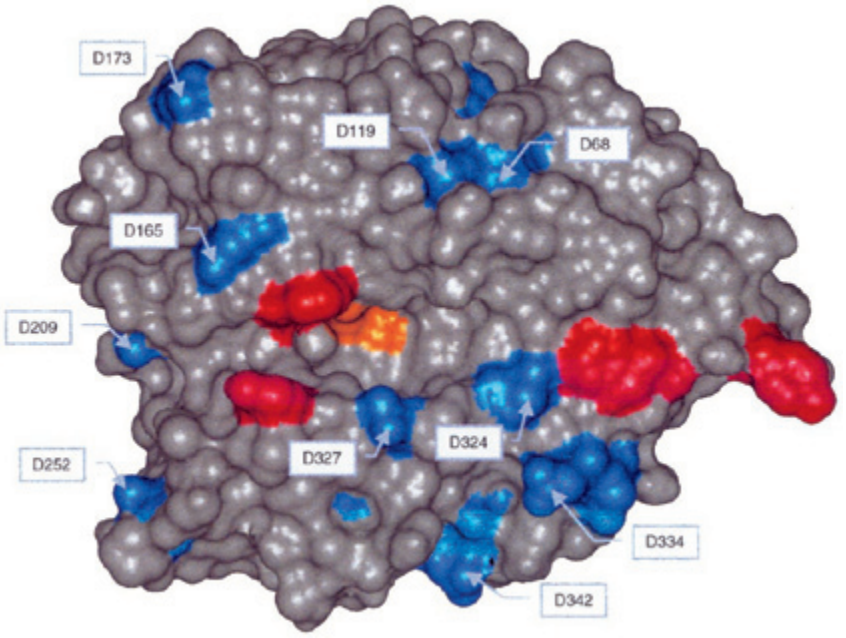

Fig. 3. Mutation strategy for negatively charged surface residues.

on the interactive surface), were not modified because of possible interference with binding and location of the cellodextrin substrate. Two other lysines (not shown), K333 and K71, lie on the side of the domain, to the right and left side, respectively. Note that some amino acid residues are found in distinct "clusters" on the interactive surface of EI (see Figs. 1–3), specifically the serines and threonines in Fig. 2.

Based on this strategy, three groups of amino acid residues were chosen for mutation: the negatively charged group (Asp334, Asp327, Asp68, Asp119, and Asp165); the group I neutral hydrogen-bonding residues (Asn77, Asn80, Asn35, and Gln288) and the group II neutral hydrogen bonding residues (Thr248, Ser251, Ser130, Ser251, Ser121, Thr129, and Ser131). Arginines or lysines were not modified. The cellodextrin platform aromatic residues Tyr245, Trp213, Trp42, and Tyr82 were also not modified. Various combinations of mutations were constructed from these groups. Our strategy was to combine as many promising mutations as possible in single clones to test the concept of disruption to native charge and hydrogen-bonding patterns. Single residue mutations were found not to be adequate for testing activity and binding performance.

Mutant Production and Purification

The results of the production and testing of 22 individual and sets of EI surface mutations are given in Table 1. For comparison, the activity, binding data, and fermentation yield/purification yield of the *E. coli*– and *S. lividans*–expressed wild-type EI are also given. The calculated p*I* values for the mutations show that many mutants would be expected to be somewhat more basic than the wild-type enzyme. These non-wild-type charges had profound effects on the purification procedures. Mutants with a p*I* >5.6 required pH 9.0 Q-Sepharose chromatography loading buffer (wild-type enzyme protocol uses pH 8.0 buffer). The mutant with a p*I* >6 lost activity after ion-exchange chromatography. The amino acid sites Asp327 and Asp165 were found to be detrimental to enzyme stability and activity, (see Table 1). Another mutant, EI-T248A/S251A/S130A/S121A/T129A/S131A, demonstrated especially low protein yields, possibly owing to the cluster of hydroxyl residues 129, 130, and 131. This observation is especially interesting considering that a less extensively mutated enzyme, EI-T248A/S251A/S130A/S121A, showed no yield problem, nor did mutants in which the same Ser and Thr residues were changed to either Arg or Asp.

Mutant Characterization

Profiles showing activity of the mutant EI enzymes on cellulose and their respective binding behavior are presented in Figs. 4–6. For the group I hydrogen-bonding mutations, replacement of amide-containing residues, Asn and Gln, with Ala, Tyr, or Arg reduced binding of the enzyme to cellulose but had a minimal impact on hydrolysis activity (Fig. 5). For the group II hydrogen-bonding mutations, replacement of hydroxyl-contain-

Table 1
Summary of E1$_{CD}$ Surface Mutations

Mutation site(s)	Molecular weight (kDa)	p*I*	Fermentation yield (g)[a]	Protein yield (µg)	pNPC activity[b]	Avicel Activity[c]	Bound at protein loading for 1% hydrolysis(%)
Wild-type *A. cellulolyticus* E1							
S. lividans–produced E1$_{CD}$	40,294	4.87			0.23	23.3	96.2
E. coli–produced E1$_{CD}$	40,294	4.87	87	1019	0.24	23.4	96.8
Group I H-bonding mutants							
N77R/N80R/N35R/Q288R	40,448	5.33	91	910	0.26	31.1	50.1
N77A/N80A/N35A/Q288A	40,294	4.87	NA	4377	0.25	25.4	70.2
N77Y/N80Y/N35Y/Q288Y	40,476	4.87	253	299	0.17	21.4	54.3
N77D/N80D/N35D/Q288D	40,284	4.62	103	1199	0.24	21.2	68.8
Group II H-bonding mutants							
T248A/S251A/S121A/S130A	40,216	4.87	NA	3050	0.21	22.2	98.9
T248A/S251A/S121A/S130A/T129A/S131A	40,170	4.87	434	170	[d]		
T248D/S251D/S121D/S130D	40,392	4.62	100	755	0.14	40.6	83.9
T248D/S251D/S121D/S130D/T129D/S131D	40,434	4.53	366	1381	0.16	80.1	65.9
T248R/S251R/S121R/S130R	40,556	5.33	196	555	0.18	30.3	98.4
T248R/S251R/S121R/S130R/T129R/S131R	40,681	5.68	410	671	0.15	41.0	87.8
Surface negative charge mutants							
D334R	40,335	5.06	90	691	0.22	24.5	92.6
D334R/D68R/D119R/D165R	40,458	6.08	182	No activity recovered in Q-sepharose fractions			
D334R/D68R/D119R	40,417	5.66	300	840	0.20	18.9	92.2
D334Y	40,342	4.95	74	458	0.16	32.5	98.2
D334Y/D68Y/D119Y/D165Y	40,486	5.30	Not done, inactive mutant				
D334Y/D68Y/D119Y	40,438	5.16	362	1937	0.22	25.6	92.9
D165Y	40,342	4.95	91	17	[d]		
D334A	40,250	4.95	164	475	0.16	22.8	96.6
D334A/D68A/D119A/D165A	40,118	5.30	307	146	[d]		
D334A/D68A/D119A	40,162	5.16	443	3330	0.17	75.1	88.2
D327R	40,335	5.06	96	370	0.05	252.8	87.4
D327Y	40,342	4.95	NA	1257	0.07	97.2	74.8

[a]NA, data not available.
[b]Units are µmol pNP/(µg protein/min).
[c]Units are µg/mL of protein loading required for 1% Avicel hydrolysis.
[d]Insufficient quantity purified for assays.

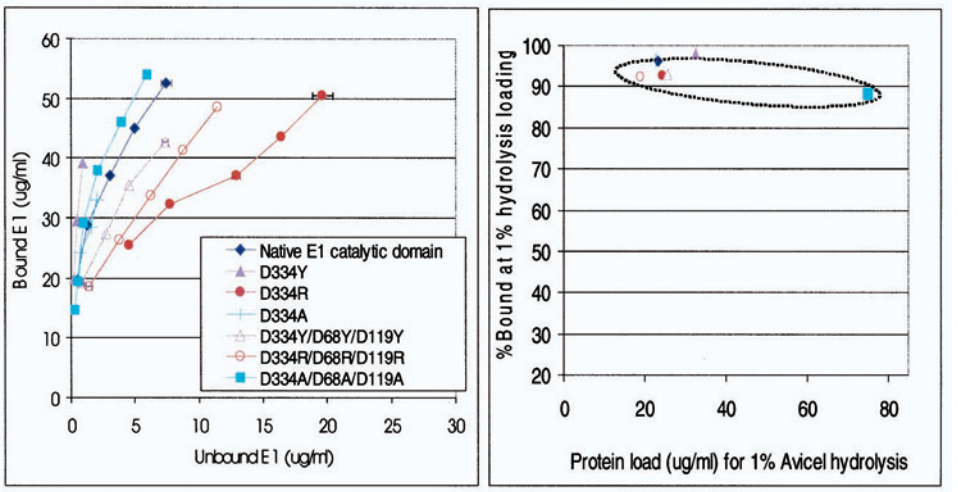

Fig. 4. Cellulose-binding data for surface charge residues.

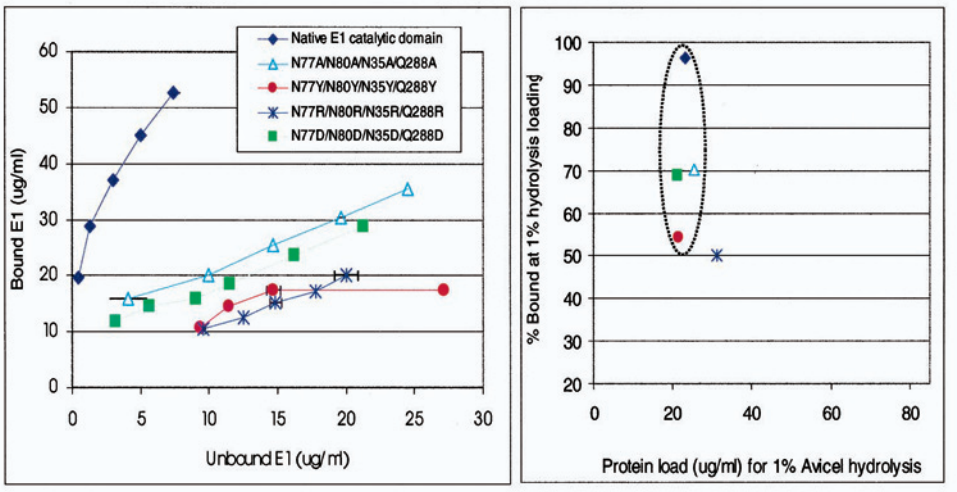

Fig. 5. Cellulose-binding data for group I residues.

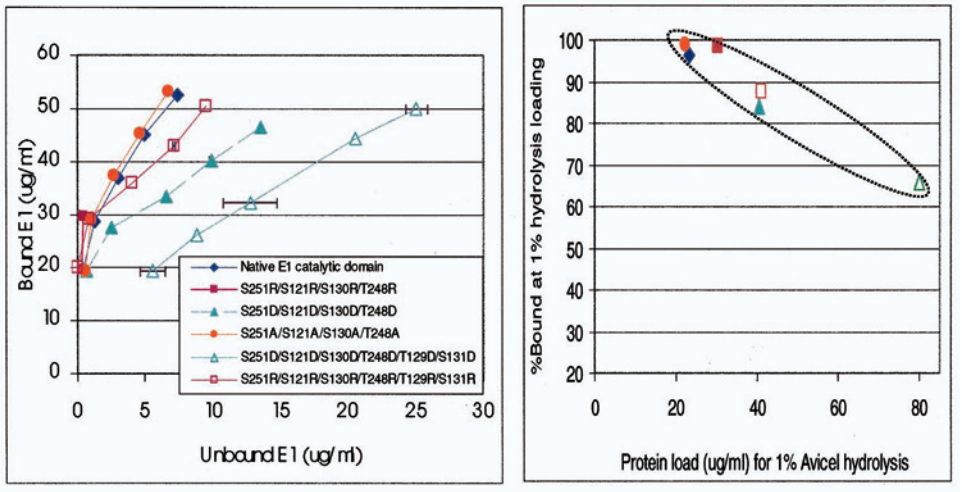

Fig. 6. Cellulose-binding data for group II residues.

ing residues, Ser and Thr, reduced cellulose binding as well as hydrolysis activity (Fig. 6). In the case of the surface negative charge mutations, replacement of Asp residues 68, 119, and 334 with Tyr and Arg had a limited effect on either cellulose binding or hydrolysis. Replacement of these residues with Ala resulted in a mutant with significantly reduced cellulose hydrolysis activity (Fig. 4). Replacement of Asp165 and Asp327 had a profound detrimental effect on both hydrolysis activity and stability during purification. Interestingly, one mutation set, Asp334Arg/Asp68Arg/Asp119Arg, showed greater activity on cellulose than the wild-type enzyme (see Table 1). Curiously, this improved mutant exhibited a lower pNPC activity than the wild type. The pNPC activities were used as an indirect measure of unbound enzyme. We did look for correlations between pNPC and Avicel activity and found nothing consistent. No correlation was expected since a soluble substrate (pNPC) may never "see" the changes that were made to the cellulose interactive surface. This mutation set also resulted in one of the greatest changes in p*I*, from 4.87 to 5.66.

Discussion

Our ultimate goal is to improve the performance (increase the specific activity) of cellulases acting on pretreated biomass used for bioethanol production. We are focusing efforts on the highly active *A. cellulolyticus* EI endoglucanase, which has been shown to work with a high degree of synergism with fungal exoglucanases including *T. reesei* CBHI. Our strategy has been to explore site-specific mutations that change the surface chemistry of EI, thus permitting new binding behavior with cellulose. These mutations must not cause inactivation or loss of solubility of the recombinant enzyme. Mutations to highly conserved amino acid residues in the active site were not made in this study.

Examination of Figs. 4–6 reveals three distinct relationships between mutant EI binding to cellulose and hydrolysis activity (i.e., see the shapes of data grouping). When Asp was replaced with Tyr or Arg (Fig. 4), little change in binding compared to the wild type was observed. However, the Ala replacement resulted in substantial loss of activity. We cannot infer however, from these data, that loss of cellulose-binding strength results in decreased activity. In Fig. 5, the replacement of the wild-type residues (Asn and Gln) with Ala, Arg, or Tyr resulted in substantially decreased binding. Curiously, activity on cellulose was not affected much. Shown in Fig. 6, replacement of four wild-type Ser residues and two wild-type Thr residues with Ala, Arg, and Tyr had an effect unlike that of the other mutant groups. For example, Ser/Thr replacements with the charged residues, Arg and Asp, resulted in loss of activity and reduced binding to cellulose. In the case of the Asp replacement mutant, the negative charges added to the surface seemed to have a dramatic effect. In summary, replacement of wild-type, surface neutral hydrogen-bonding residues with Ala, Arg, or Tyr appears to decrease binding to cellulose. In some cases, this did not result in signifi-

cant loss of activity (i.e., group I). When wild type aspartic acid residues were replaced with Ala, Arg, or Tyr, only the nonhydrogen-bonding, hydrophobic Ala mutant showed a substantial reduction in activity.

Thus far, we have assumed that all surface mutations made do not affect protein structure, but this has not been proven. Therefore, the possibility that certain mutants display characteristics resulting from unforeseen structural effects must be entertained. Furthermore, activity assays were carried to approx 2% hydrolysis of Avicel, which permits access to both amorphous and crystalline cellulose.

Conclusion

We have examined the interaction of one endoglucanase with clean microcrystalline cellulose and found no profound correlation between enzyme surface charge and cellulose-binding characteristics in the case of wild-type Asp residues. Replacement of neutral, surface hydrogen bond–forming residues with the charged residues Asp and Arg resulted in loss of binding and/or activity. The surface charge changes accompanying these modifications did not appear to cause distinguishable patterns of binding or activity reduction, however. Because the Tyr replacement mutants also result in reduction of binding (group I), the mere introduction of charge cannot be implicated for this overall effect. One mutant, EI-Asp334Arg/Asp68Arg/Asp119Arg, showed modest but measurably higher activity on cellulose than the wild-type enzyme and, more importantly, little reduction in apparent cellulose-binding strength. Thus, for the EI endoglucanase mutants made in this study, modified binding properties do not predict activity on cellulose.

For future work, application of this experimental query to cellulases and biomass feedstocks for bioethanol production is apparent. There are aspects of the surface chemistry of chemically treated biomass that must differ considerably from the native plant tissue normally encountered by cellulases acting in the biosphere *(29)*. Pretreated biomass differs from natural biomass in two important ways: (1) pretreated biomass has been heated past the phase transition temperature of lignin (about 140°C), and (2) pretreated biomass has been relieved of most of its acetylated hemicellulose content. The implications of this treatment history are that cellulose fibers in pretreated biomass are probably "coated" with displaced and modified lignin, and it is reasonable to assume that this lignin impedes enzyme action through strong, nonproductive binding. This hypothesis follows, because several studies have shown a strong tendency for lignin in wood to interfere with cellulase action *(29–32)*. We also recognize that pretreated hardwood pulp harbors a weak yet negatively charged surface not associated with native wood, because regions of hemicellulose nascent to 4-*O*-Me-glucuronic acid branches are protected during dilute acid hydrolysis and probably remain in the fiber *(33,34)* and glucuronic acid content in cellulose increases as a function of normal wood oxidation *(35)*.

The result of the alteration of wood following pretreatment most certainly alters interactions between cellulases and the biomass surface. Thus, efforts to understand more fully such interactions at the molecular level are important.

Acknowledgment

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