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Multimeric Hemicellulases Facilitate Biomass Conversion▽†

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Two highly active trifunctional hemicellulases were constructed by linking the catalytic portion of a xylanase with an arabinofuranosidase and a xylosidase, using either flexible peptide linkers or linkers containing a cellulose-binding domain. The multifunctional enzymes retain the parental enzyme properties and exhibit synergistic effects in hydrolysis of natural xylans and corn stover.

Bio-depolymerization of lignocellulosic biomass requires a large number of enzymes, many of which work synergistically to degrade complex polysaccharides. For example, the hydrolysis of corn stover cellulose by cellobiohydrolase I is significantly enhanced by the presence of small quantities of hemicellulases (15). Important hemicellulases for biomass hydrolysis include xylanases, xylosidases, and arabinofuranosidases (14). These enzymes commonly work in concert, and their synergistic effects in xylan degradation have been studied (4, 7, 17). We recently constructed two chimeric xylan-degrading enzymes, demonstrating that engineering bifunctional hemicellulases is a feasible strategy for reducing the number of proteins required for biomass conversion (5). In this study, we focused on producing a trifunctional hemicellulase and explored the feasibility of using a cellulose binding domain (CBD) as both a spacer and a functional module.

Recombinant multimeric hemicellulases are produced in *Escherichia coli*. Overlapping PCR (4) was used to construct two in-frame fusion genes: (i) the gene encoding *a*rabinofuranosidase-*x*ylanase-*x*ylosidase (the AXX gene), consisting of an α-arabinofuranosidase gene (*deAFc*; GenBank accession no. DQ284779 [18]), the *Clostridium thermocellum* xylanase gene (*xynZ*; GenBank accession no. M22624), and the *Thermoanaerobacterium* sp. strain JW/SL YS485 xylosidase gene (GenBank accession no. AF001926); and (ii) a gene encoding a CBD-containing AXX protein (the CBD-AXX gene), in which the 471-bp *C. cellulovorans* CBD sequence (*cbpA*; GenBank accession no. M73817) was inserted into the two peptide linkers in AXX (Fig. 1). The soluble parental and multimeric enzymes were produced in *E. coli* and purified by nickel affinity chromatography to high homogeneity as determined by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The recombinant production levels of AXX (~165 kDa) and CBD-AXX (~200 kDa) were estimated to be approximately 15 and 10 mg/liter of cell culture, respectively.

The multimeric enzymes have pH profiles and temperature profiles similar to those of the parental enzymes. When assayed using Remazol brilliant blue-xylan as a substrate, AXX, CBD-AXX, and a mixture of the three parental enzymes generated similar pH profiles, although CBD-AXX showed an apparent shift toward pH 6 (Fig. 2A). A shift in pH optimum was observed previously for the xylanase-arabinofuranosidase and xylanase-xylosidase chimeras (5). We attribute this shift in pH optimum to either the change of pH in the microenvironment generated by the polymeric proteins or altered tertiary structure. Similar pH profiles for arabinofuranosidase activity were also observed for the multimeric enzymes and DeAFc in an assay using 4-nitrophenyl-α-L-arabinofuranoside (4NPA) as a substrate (Fig. 2B). When assayed using 2-nitrophenyl-xylopyranoside (2NPX) as a substrate, the xylosidase activity profiles were essentially identical for AXX, CBD-AXX, and xylosidase (Fig. 2C). In all three assays, CBD-AXX is consistently more active than AXX. One explanation is that the positions of the CBDs between the enzyme subunits provide optimal spacing for the three catalytic subunits and thereby improve the overall activities of the enzymes.

AXX and CDB-AXX have temperature profiles for xylanase, arabinofuranosidase, and xylosidase similar to those for their respective parental enzymes when assayed at pH 6.0 (Fig. 2D to F). These results, together with results from the pH study, indicate that the enzymatic components of the two multimeric enzymes retain their parental properties.

The multimeric enzymes are kinetically comparable to the parental enzymes. To compare the kinetic parameters of the multimeric enzymes with those of the parental enzymes, the chromophore-tagged synthetic substrate 4NPA was used for kinetic analysis of arabinofuranosidase activity, following the procedures described elsewhere (19). The 4NPA substrate hydrolysis data were subjected to nonlinear curve fitting to the Michaelis-Menten equation by using GraphPad Prism 5 software (GraphPad Software, CA). When assayed at 45°C with 50 mM phosphate buffer (pH 6.0) containing 100 μg/ml bovine serum albumin, the K_m values for DeAFc, AXX, and CBD-AXX were 188 ± 12 μM, 200 ± 29 μM, and 194 ± 12 μM,

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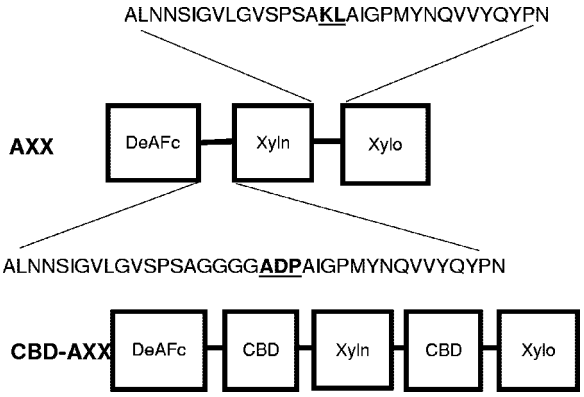

FIG. 1. Schematic representation of the organization of AXX and CBD-AXX. The amino acid sequences of the two peptide linkers are shown. PCR primers used in the construction are listed in Table S1 in the supplemental material. The residues corresponding to a BamHI site (ADP) and a HindIII site (KL) where the CBDs are inserted are underlined. Xyln, xylanase; Xylo, xylosidase.

respectively; the k_{cat} values for DeAFc, AXX, and CBD-AXX were determined to be $1.1 \pm 0.02\ s^{-1}$, $0.9 \pm 0.03\ s^{-1}$, and $1.0 \pm 0.01\ s^{-1}$, respectively. The similar K_m and k_{cat} values for DeAFc and the multimeric enzymes with 4NPA indicate that the DeAFc component of the multimeric enzymes maintains its parental catalytic efficiency.

The multimeric enzymes are highly active on natural arabinoxylans. An enzyme-coupled assay (19) was used to measure xylose release by the enzymes, using water-soluble wheat and rye arabinoxylan as substrates. Similar to the parental enzyme mixture, AXX and CBD-AXX were highly active in hydrolyzing natural xylans (Fig. 3). The specific activities of CBD-AXX are approximately 5% and 12% higher than those of AXX for wheat and rye arabinoxylan, respectively, which is consistent with results obtained using synthetic substrates. Because the *cbpA* CBD used in CBD-AXX does not bind xylan (6), the increased activity of CBD-AXX on arabinoxylans is likely due

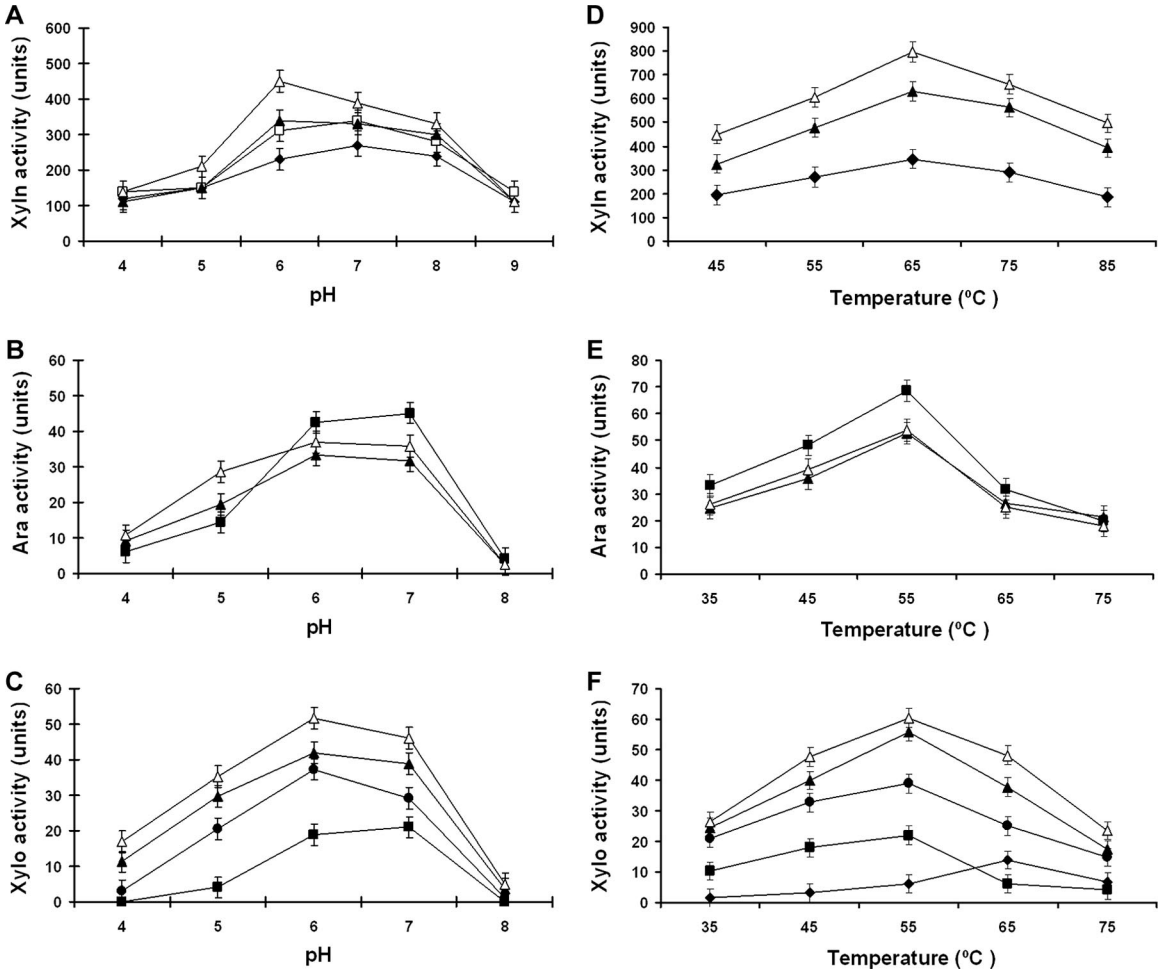

FIG. 2. pH and temperature profiles of the multimeric and parental enzymes. Enzyme activities were determined at different pHs at 45°C. (A) Xylanase (Xyln) activities on substrate Remazol brilliant blue-xylan (1 enzyme unit is defined as 1 μmol dye released/nmol enzyme/min). (B) Arabinofuranosidase (Ara) activities on substrate 4-nitrophenyl-α-L-arabinofuranoside (4NPA; 1 enzyme unit is defined as 1 nmol 4NP released/nmol enzyme/min). (C) Xylosidase (Xylo) activities on substrate 2-nitrophenyl-β-D-xylopyranoside (2NPX; 1 enzyme unit is defined as 1 nmol 2NP released/nmol enzyme/min). Enzyme activities were measured at different temperatures at pH 6.0. (D) Xylanase activity. (E) Arabinofuranosidase activity. (F) Xylosidase activity. Triplicate assays were performed using purified xylanase (◆), DeAFc (■), xylosidase (●), enzyme mixture (□), AXX (▲), and CBD-AXX (△).

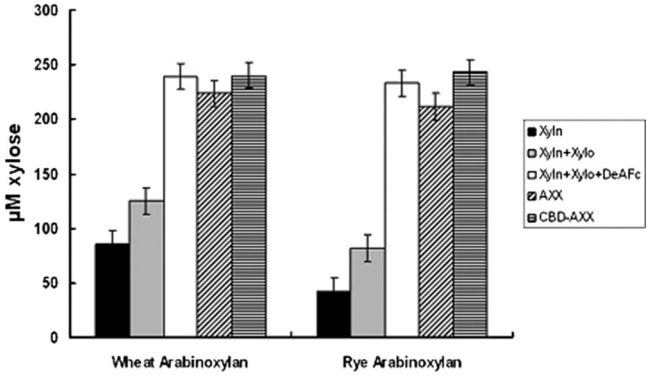

FIG. 3. Enzyme activities toward natural arabinoxylans. Equal molar concentrations (25 nM) of the parental xylanase (xyln), mixtures of two or three parental enzymes (xyln+xylo and xyln+xylo+DeAFc, respectively), and AXX or CBD-AXX were assayed for their activities on water-soluble wheat arabinoxylan and rye arabinoxylan. An enzyme-coupled assay (19) was used to measure xylose release in 50 mM phosphate buffer (pH 6) containing 50 μg/ml bovine serum albumin at 45°C.

to optimization of the overall structure by the CBDs rather than from enhanced substrate binding.

CBD-AXX is more efficient in hydrolysis of corn stover. The broad-substrate specificities of the parental enzymes enable the liberation of sugars from corn stover by the enzyme mixture and the multimeric enzymes (Fig. 4A), as determined using a colorimetric assay described previously (1). The combined effects of the multimeric enzymes in the presence of cellulases were also determined. In the presence of purified recombinant cellulase E1 of *Acidothermus cellulolyticus* (GenBank accession U33212; purified in our laboratory), considerably more sugars were released by both the enzyme mixture and the multimeric enzymes (Fig. 4B). A similar result was also observed when a commercial cellulase preparation (Alltech, Nicholasville, KY) was used (Fig. 4C).

In corn stover assays, AXX resembles the parental enzyme mixture except when assayed with the commercial cellulase, which resulted in a relative decrease in AXX activity after 24 h (Fig. 4C). In all cases, however, CBD-AXX is distinctly more active than either AXX or the enzyme mixture in corn stover hydrolysis. Thermostability assays of AXX and CBD-AXX indicated little difference between them; after 48 h, both proteins retained 60 to 80% of their activities, depending on the substrates used in the assay (data not shown). Therefore, protein stability is unlikely to be responsible for the superior performance observed for CBD-AXX. The carbohydrate-specific binding modules greatly enhance the enzyme activities against insoluble substrates, such as cellulose (2, 13). Protein engineering fusing CBDs with carbohydrate hydrolases has led to increased cellulosic binding efficiency (16). Our results are consistent with a higher affinity for binding of CBD-AXX than non-CBD-containing enzymes to corn stover, leading to higher hydrolysis efficiency. The observations that CBD-AXX does not possess significantly higher activity than AXX or the enzyme mixture toward arabinoxylans, which have very low cellulose content, provide further support for the specific role of CBDs in cellulosic binding. Nonetheless, it is possible that the CBDs not only enhance substrate binding but also improve the

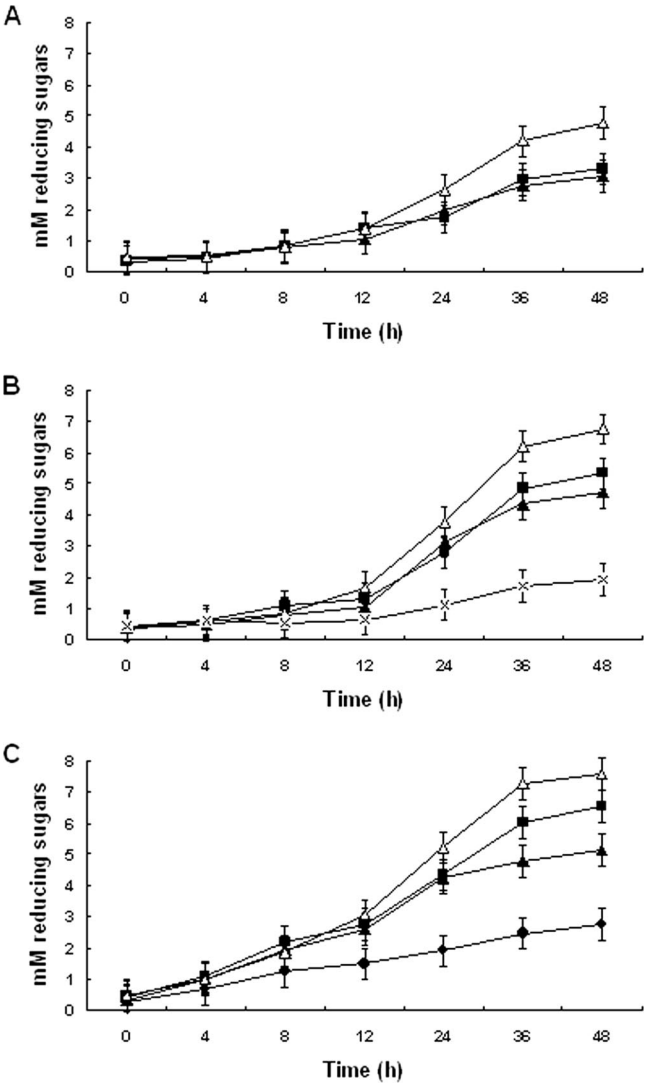

FIG. 4. Hydrolysis of corn stover by the enzyme mixture or the multimeric enzymes in the presence or absence of cellulases. Assays were performed at 45°C with 50 mM phosphate buffer (pH 6) containing 20 nM of each enzyme and 30 mg/ml of corn stover in the presence or absence of 10 nM cellulase E1 or 1.5 μg commercial cellulase. (A) Reducing sugar release by enzyme mixture (■), AXX (▲), and CBD-AXX (△) in a course of 48 h. (B) Reducing sugar release by cellulase E1 (×), the enzyme mixture plus E1 (■), AXX plus E1(▲), and CBD-AXX plus E1 (△). (C) Reducing sugar release by the commercial cellulase (●), the enzyme mixture plus the commercial cellulase (■), AXX plus the commercial cellulase (▲), and CBD-AXX plus the commercial cellulase (△).

overall structure of CBD-AXX, leading to the observed increase in corn stover hydrolysis.

Naturally occurring and artificial multidomain enzymes are useful in metabolic engineering (3). We and others have demonstrated that the creation of artificial, multifunctional, lignocellulosic hydrolases is a realistic and practical approach for the improvement of biomass conversion (5, 8, 9, 12). These novel enzymes can be used alone, when a 1:1:1 stoichiometry is preferred, or in mixtures with small quantities of other enzymes to create an optimal enzyme cocktail when enzyme ratio

is critical. Because of the potential synergy and ease for transformation, engineered fusion enzymes can be used to improve industrial microbes (11) or transgenic plant feedstock (10). However, artificial enzymes with three to five functional subunits are not commonly reported. The multimeric enzymes characterized in this study closely resemble the parental enzymes or their mixtures with regard to their enzymatic properties. CBD-AXX surpasses both AXX and the parental enzyme mixture in hydrolysis of corn stover, providing support for our design strategy of using CBDs to enhance substrate binding. The multifunctional enzymes have the potential to be more cost-effective in industrial enzyme production than mixtures of multiple single enzymes.

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