High Temperature Enzymatic Biomass Breakdown Systems

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Agricultural and forest waste products are abundant and low-cost biomass sources useful in renewable fuel energy and feedstock preparation. Cellulose is an abundant and renewable biopolymer that can be used for biofuel generation, however structural entrapment with other cell wall components hinders enzyme substrate interactions, a key bottleneck for ethanol production. Biomass is routinely subjected to treatments that facilitate cellulase-cellulose contacts.

Cellulases and glucosidases act by hydrolyzing glycosydic bonds of linear glucose beta1,4-linked polymers producing glucose. Here we describe six high temperature-operating cellulases (TCel) and two beta glucosidases identified from a survey of thermo-bacterial and archaeal genomes. Three TCel enzymes preferentially hydrolyzed soluble cellulose while two preferred insoluble cellulose such as cotton linters and filter paper. TCel enzymes had temperature optima ranging from 85^0C to 102^0C. TCel enzymes were stable retaining 80% of initial activity after a 120h, at 85^0C. Two modes of cellulose breakdown were detected, endo- and exo-acting glucanases, and with two-enzyme combinations at 85^0C, synergistic cellulase activity was observed for some enzyme combinations, indicating that these enzymes would be useful in high-temperature, hydrothermal based biomass treatments.

Hydrolysis of hemicellulose is accomplished by the action of endo-xylanases. Reaction products vary in composition and degree of polymerization as a function of both feedstock and the enzyme activities utilized, ranging from single sugars to complex branched polysaccharides.

Endo-xylanases depolymerize xylan by degrading hemicellulose useful in biofuel production as well as in paper bleaching. Here we describe the molecular basis and mode of action of xylanase 10B from the hyperthermophilic bacterium *Thermotoga petrophila* using biochemical, biophysical and crystallographic methods. The crystal structure of xylanase 10B RKU-1 (TpXyl10B) has been solved in the native state and in

complex with xylobiose. The protein-xylobiose complex crystal showed a classical binding mode shared among other xylanases, which encompasses the -1 and -2 sub-sites. Interestingly, TpXyl10B displayed a temperature-dependent mode of action producing xylobiose and xylotriose at 20°C and exclusively xylobiose at 90°C as determined by capillary zone electrophoresis. Moreover, circular dichroism spectroscopy (CD) suggested a coupling effect of temperature-induced structural changes with this particular enzymatic behavior. Molecular dynamics simulations supported the CD analysis suggesting that an open conformational state adopted by the catalytic loop (Trp297-Lys326) triggers modifications in the product release area (+1, +2 and +3 subsites), which drives the enzymatic activity to the specific release of xylobiose at high temperatures.

Arabinan is a biomass structural polysaccharide degraded by two enzymes, arabinofuranosidase and arabinanase. We characterized an arabinanase and arabinofuranosidase isolated from *Thermotoga petrophila* with unique thermostable properties such as the insignificant decrease of residual activity after incubation up to 90 degrees. We determined the mode of operation through capillary zone electrophoresis, which accumulates arabinotriose and arabinobiose as end products after hydrolysis of arabinan-containing polysaccharides. Spectroscopic analyses by Far-UV circular dichroism and intrinsic tryptophan fluorescence emission demonstrated that arabinanase is folded and formed mainly by beta-sheet structural elements. *In silico* molecular modeling showed that the arabinanase structure encompasses a five-bladed beta-propeller catalytic core juxtaposed by distorted up-and-down beta-barrel domain. The low-resolution structure determined by small angle X-ray scattering indicated that arabinanase is monomeric in solution and its molecular shape is in full agreement with the model. Arabinofuranosidase was over expressed, purified and biochemically characterized. The enzyme had optimum activity at pH 6.0 and 70°C with linear α-1,5-linked arabinoheptaose as substrate. The substrate cleavage pattern monitored by capillary zone electrophoresis showed that TpAraF is a classical exo-acting enzyme producing arabinose as its end product. Far-UV circular dichroism analysis displayed a typical spectrum of α/β barrel proteins analogously observed for other GH51 α-L-arabinofuranosidases. Moreover, TpAraF was crystallized in two crystalline forms, which can be used to determine its crystallographic structure.

Other enzymes that degrade hemicellulose components include mannanase laminarinase which degrade mannans and 1,3 beta glucans. Crystals of the catalytic domain of *Thermotoga petrophila* RKU-1 mannanase were obtained from three different conditions, resulting in two crystalline forms. Crystals from conditions with phosphate or citrate salts as precipitant (CryP) belonged to space group P2(1)2(1)2(1), with unit-cell parameters a=58.76, b=87.99, c=97.34 A, while a crystal from a condition with ethanol as precipitant (CryE) belonged to space group I2(1)2(1)2(1), with unit-cell parameters a=91.03, b=89.97, c=97.89 A. CryP and CryE diffracted to resolutions of 1.40 and 1.45 A, respectively. Here we also describe a functional characterization and low-resolution structure of 1,3-β-Glucan-depolymerizing enzyme laminarinase from *Thermotoga petrophila* (TpLam). We determine TpLam enzymatic mode of operation, which specifically cleaves internal β-1,3-glucosidic bonds. The enzyme most frequently attacks the bond between the 3rd and 4th residue from the non-reducing end, producing glucose, laminaribiose and laminaritriose as major products. Far-UV circular dichroism

demonstrates that TpLam is formed mainly by beta structural elements, and the secondary structure is maintained after incubation at 90°C. The structure resolved by small angle X-ray scattering, reveals a multi-domain structural architecture of a V-shape envelope with a catalytic domain flanked by two carbohydrate-binding modules.
Finally, all of these proteins are operative at high temperatures actively degrading biomass components and therefore useful in hot water pretreatments. Moreover, the use of ionic liquids in biomass pretreatments has recently been shown to be efficient and only hyperthermophilic enzymes can withstand the presence of residual amounts (up to 15%) of ionic liquids.

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