Analytical Characterization of Commercial Recombinant Endoglucanase E1

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Introduction

To characterize new enzymes and draw conclusions about their possible use on an industrial scale, sensitive and high-throughput analytical methods are necessary that provide a data set about thermal and chemical stability, activity, potential inhibitors or activators and structure-function relationships. Well-characterized enzymes can serve as reference and benchmark for new or optimized molecules. We therefore present here the in-depth characterization of a commercially available EndoE1 (endo-1,4-ß-D-glucanase from *Acidothermus cellulolyticus*, without carbohydrate binding domain, lot SLCQ2012) expressed recombinantly in corn[1].

We show the characterization of this enzyme with respect to activity, thermal stability at different pH values, primary sequence confirmation by mass spectrometry and changes in enzyme conformation upon inhibitor binding by using HDX-MS. The analytical method panel presented can be used as platform to screen novel enzymes with limited sample amount in a reasonable time frame.

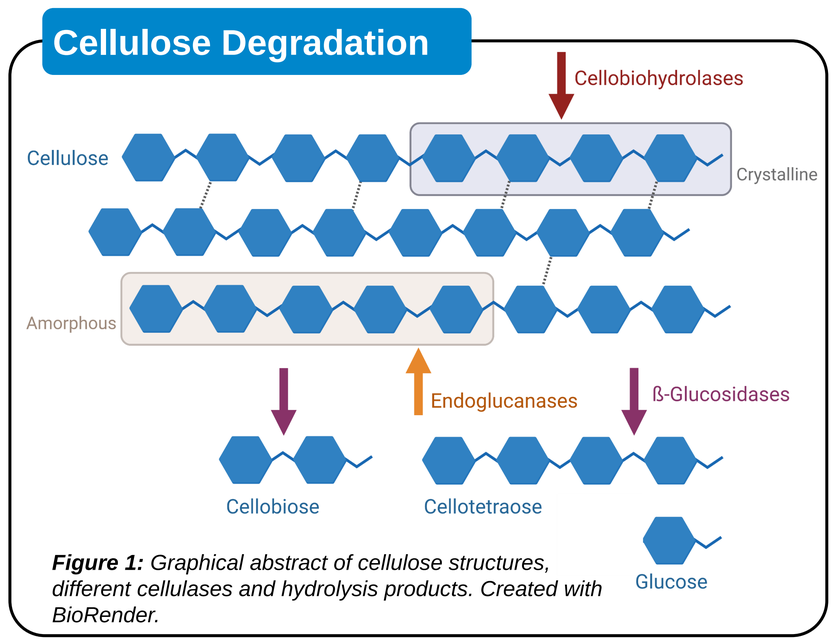


Figure 1: *Graphical abstract of cellulose structures, different cellulases and hydrolysis products. Created with BioRender.*

Enzyme Activity

EndoE1 activity was assessed by a 3,5-Dinitrosalicylic acid **DNS** assay, which is based on hydrolysis of cellulose to reducing sugars and by a **pNP** assay based on hydrolysis of 4-Nitrophenyl-β-1,4-cellobioside.

The commercial EndoE1 preparation showed no to very low activity (Figure 2). Moreover, it also inhibited the activity of a positive control cellulase mixture from *T. reesei*. However, when impurities found by **MS** and **SEC** (Figure 3) were removed, activity could be shown (Figure 2).

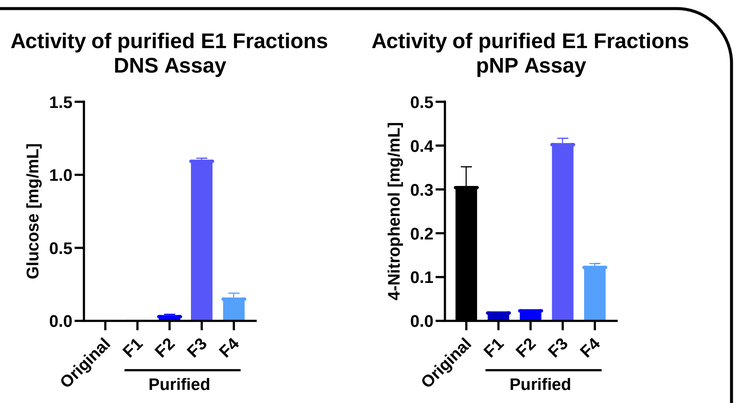
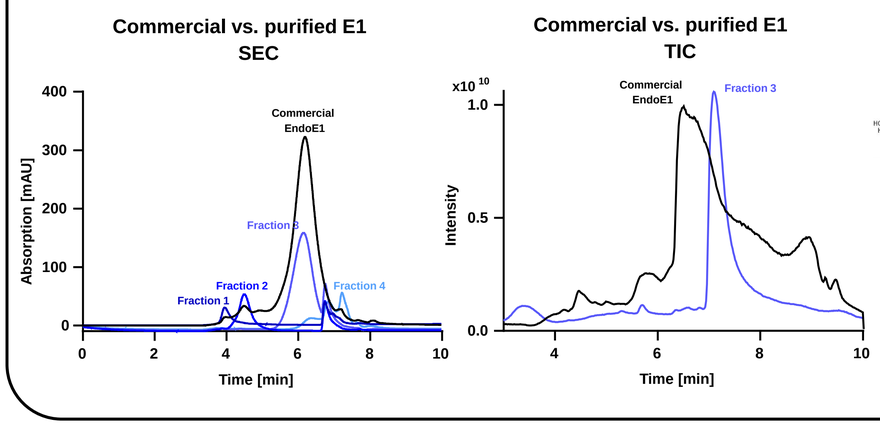


Figure 2: Left: *Comparison of original EndoE1 and purified fractions 1 to 4 in DNS Assay.* ***Right:*** *Comparison of original EndoE1 and purified fractions 1 to 4 in pNP Assay.*



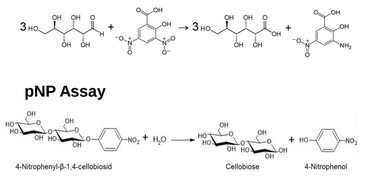


Figure 3: Left: *Size exclusion chromatographic analysis of commercial EndoE1 and its purified fractions.* ***Right:*** *Total ion current of reverse phase chromatographic analysis.*

Mass Spectrometry

Characterization by mass spectrometry was performed at the level of the intact, non-reduced enzyme (Figure 4) and at the level of a tryptic enzyme digest (Figure 5).

Intact mass analysis confirmed the primary sequence with a Δ mass of +30 Da (N-terminal formylation). In addition, contaminants and adduct were found, e.g. PEG.

Tryptic peptide map confirmed the primary sequence at amino acid level, a formylated N-terminus, heterogeneity of the C-terminus and contaminating proteins.

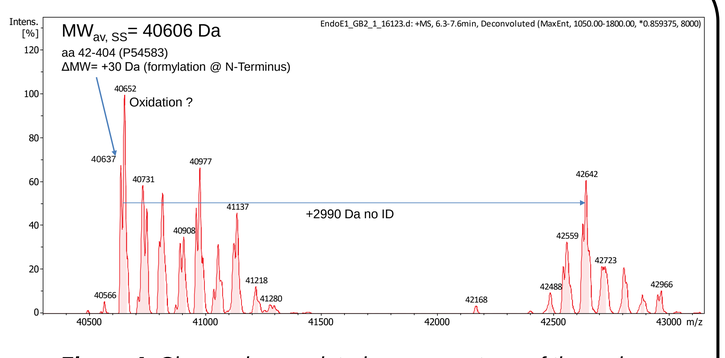


Figure 4: *Charge-deconvoluted mass spectrum of the main chromatographic peak (Figure 3, right).*

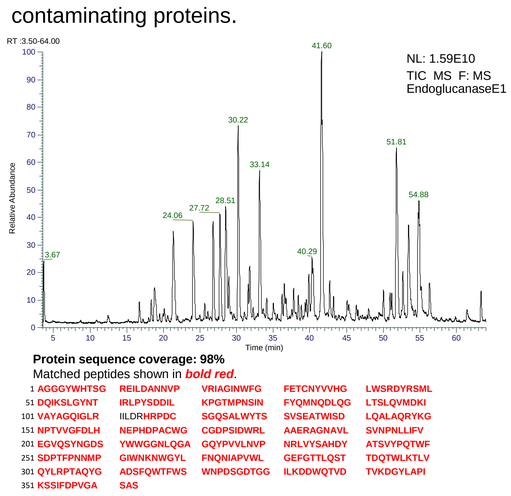


Figure 5: *Tryptic peptide map and LC-MS/MS analysis of EndoE1.*

Table 1: *Proteins identified in commercial EndoE1 preparation using tryptic peptide map and BioPharmaFinder analysis.*

Proteins	Peak Area	Abund. (mol)
EndoglucE1|EndoglucE1	65.6%	91.3%
Zeamatin|ZEAM_Maize	1.2%	4.6%
Superoxidedismutase|SODC4_Maize	1.0%	3.4%
EndochitinaseA|CHIA_Maize	0.3%	0.6%
Triosephosphateisomerase|TIPS_Maize	0.1%	0.2%

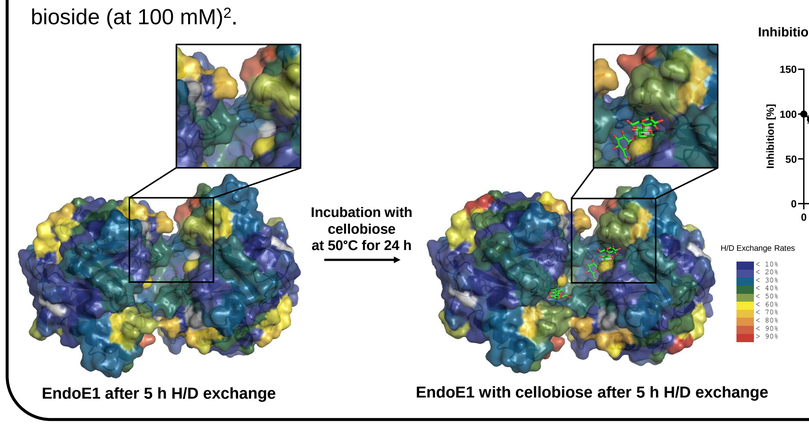
Purity of the product was 91.3%, with five different proteins as contaminants.

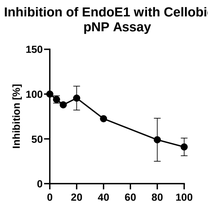
HDX MS

Hydrogen deuterium mass spectrometry (**HDX-MS**) is used to investigate protein structures, dynamics and interactions. It tracks the rate of hydrogen to deuterium exchange within the amide backbone of proteins to give information regarding solvent accessibility and can be used to help understand binding mechanisms and substrate interactions.

Here we investigated possible structural changes of EndoE1 upon binding of the inhibitor cellobioside (at 100 mM)[2].

Our results revealed a very slow overall deuterium uptake, which could be attributed to a highly compact protein folding. Small but significant differences in deuterium uptake were found in the region of substrate binding pocket. The differences were most prominent after 5 hours of deuterium exchange.





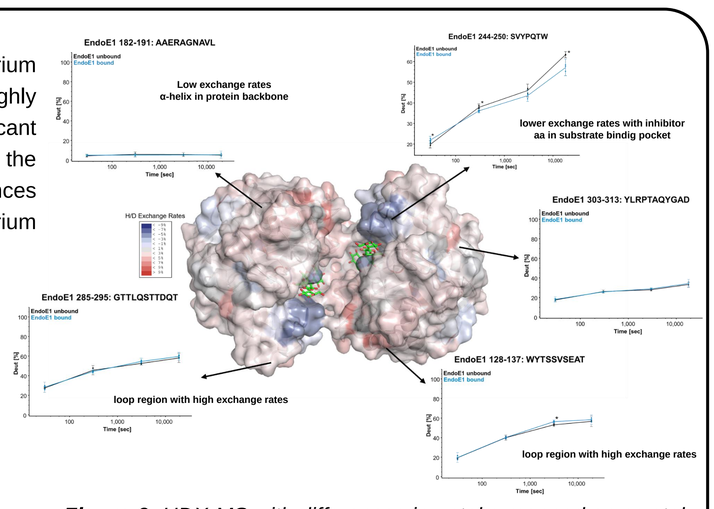


Figure 6: *HDX-MS with differences in uptake mapped on crystal structure of EndoE1 (PDB 1ECE[3]). Blue rates show slower uptake with bound inhibitor, red parts exchange faster without inhibitor. Uptake curves are shown as examples.*

Figure 7: Left: *Differences in exchange rates of H/D mapped on the surface of crystal structures of EndoE1 with (PDB 1ECE[3]) and without inhibitor (PDB 1VRX[4]). EndoE1 was incubated with Cellobiose for 24 h at 50°C.* ***Right:*** *Inhibition of EndoE1 with Cellobiose at different concentrations, measured with pNP assay.*

nanoDSF

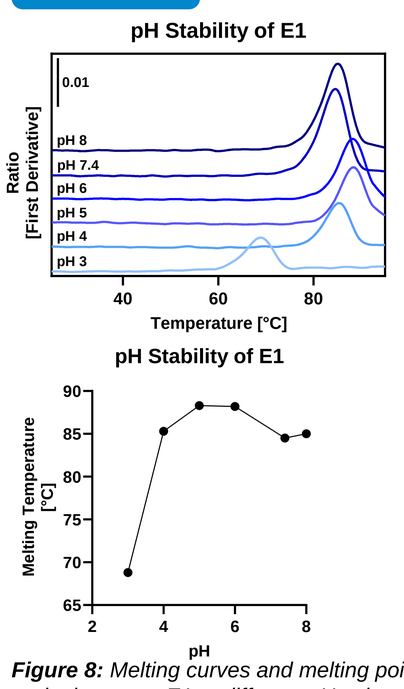


Figure 8: *Melting curves and melting points of endoglucanase E1 at different pH values.*

For the recombinant EndoE1, which has a globular, very compact three-dimensional structure, the temperature and pH stability was determined by measuring the intrinsic shift in tryptophan fluorescence during unfolding (**nanoDSF**).

Figure 8 shows that the enzyme has a rather stable three-dimensional structure in a pH range between 4 and 8 and starts to unfold at temperatures >80 °C. At pH 3, however, the stability decreases.

Summary and Outlook

Our results revealed a batch-to batch variation of commercial enzyme preparations with regards to microheterogeneity, purity and activity. Structural-based assays such as HDX-MS will be further optimized to show subtle differences upon binding of inhibitory components.

The analytical methods presented can be used to characterize enzyme preparations and/or novel enzyme candidates. They are useful to identify batch-to-batch differences and to screen enzyme candidates for stability, activity and special properties. Furthermore, they can assist enzyme engineering to optimize the enzyme structure according to the desired properties using low sample amount and within a reasonable time frame.

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