Enzyme and Microbial Technology 98 (2017) 58–66

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Oxidative cleavage and hydrolytic boosting of cellulose in soybean spent flakes by *Trichoderma reesei* Cel61A lytic polysaccharide monooxygenase

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ARTICLE INFO

Article history:
Received 3 October 2016
Received in revised form 1 December 2016
Accepted 26 December 2016
Available online 29 December 2016

Keywords:
Lytic polysaccharide monooxygenase
Soy spent flake
Trichoderma reesei Cel61A (*Tr*Cel61A)
Soy polysaccharides
Cellulose hydrolysis

ABSTRACT

The auxiliary activity family 9 (AA9) copper-dependent lytic polysaccharide monooxygenase (LPMO) from *Trichoderma reesei* (EG4; *Tr*Cel61A) was investigated for its ability to oxidize the complex polysaccharides from soybean. The substrate specificity of the enzyme was assessed against a variety of substrates, including both soy spent flake, a by-product of the soy food industry, and soy spent flake pretreated with sodium hydroxide. Products from enzymatic treatments were analyzed using mass spectrometry and high performance anion exchange chromatography. We demonstrate that *Tr*Cel61A is capable of oxidizing cellulose from both pretreated soy spent flake and phosphoric acid swollen cellulose, oxidizing at both the C1 and C4 positions. In addition, we show that the oxidative activity of *Tr*Cel61A displays a synergistic effect capable of boosting endoglucanase activity, and thereby substrate depolymerization of soy cellulose, by 27%.

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1. Introduction

Soy polysaccharides are a carbohydrate-rich by-product resulting from the manufacture of soy milk, tofu, and soy protein isolate. They are composed predominantly of water-insoluble cell wall polysaccharides of high molar mass, with constituent polysaccharide types including type I arabinogalactan, cellulose, arabinoxylan, rhamnogalacturonan, arabinan, xyloglucan, and homogalacturonan [1]. This material is generally referred to as okara when resulting from soy milk or tofu production, and soy spent flake following soy protein isolate production [2]. Both of these products are produced in large quantities globally with approximately 2.8×10^6 tons of okara produced by the Chinese tofu industries each year [3] and 8×10^5 tons of okara discarded annually in Japan [4]. Currently the vast majority of this material is disposed of as waste at significant cost, utilized in low value applications such as fertilizer or animal feed, or burned [5,6]. For these reasons, there is significant interest in increasing the utilization of this material, and recent efforts with this aim have centered on the solubilization of the recalcitrant polysaccharides utilizing acidic extraction [7], enzymatic degradation [8], chelating agents [9], and/or oxidizing agents [1].

The use of lytic polysaccharide monooxygenases (LPMOs) has been an emerging focal point for research on the enzymatic degradation of polysaccharides. These enzymes are classified as auxiliary activity (AA) enzymes in the Carbohydrate-Active enZyme database (CAZy; http://www.cazy.org) [10], corresponding to families AA9 (originally GH61), AA10 (originally CBM33), AA11, and AA13 [11]. LPMOs are copper-dependent enzymes that are capable of catalyzing the cleavage of glycosidic bonds through oxidation of sugar residues. While oxygen-dependent enzymes were already suggested as an important part of fungal cellulose degradation as early as the 1970s [12], investigation into their importance and functional impact on cellulose degradation has risen dramatically in recent years. Over the past decade, there has been a wide array of research into these enzymes, with current research demonstrating LPMO activity on a variety of substrates including cellulose [13], xylan [14], xyloglucan [15], chitin [16], starch [17], β-glucan [18],

Abbreviations: LPMO, lytic polysaccharide monooxygenase; MALDI, matrix assisted laser desorption ionization; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; PASC, phosphoric acid swollen cellulose; DP, degree of polymerization.

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http://dx.doi.org/10.1016/j.enzmictec.2016.12.007
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glucomannan [15], and lichenan [15]. To date, LPMOs have been shown to oxidize at the C1 position [19], the C4 position [20], or at both the C1 and C4 positions [21] of polysaccharide sugar residues to result in glycosidic cleavage and degradation of the polysaccharide [22].

One of the main drivers of the growth in research for LPMOs has been their demonstrated ability to boost the activity of traditional glycoside hydrolases (GHs) in the degradation of recalcitrant polysaccharides [11]. This has been particularly relevant for biomass conversion, where there is a strong desire to maximize the overall efficiency of the enzymes used in order to minimize production costs [23]. This enhancing effect has been demonstrated for a wide variety of substrates including cellulose [24], lignocellulose [23], spruce [25], kenaf and oak [26]. The current understanding is that this GH boosting effect is the result of the LPMOs introduction of chain breaks in the crystalline and less accessible portions of the substrate [25].

The filamentous fungi *Trichoderma reesei* (*Hypocrea jecorina*) is one of the most well studied cellulolytic systems in nature, and it is known to produce two family AA9 LPMOs, *Tr*Cel61A and *Tr*Cel61B. *Tr*Cel61A was first investigated through expression in *S. cerevisiae* in the late 1990s and was characterized as a weakly acting endoglucanase at that time [27]. Due to this observed activity, it was given the name EGIV and this activity was also confirmed through homologous expression in *T. reesei* [28]. The LPMO activity of *Tr*Cel61A was confirmed through recombinant expression in *Pichia pastoris* by Tanghe, et al. [29]. In the latter study, both neutral and oxidized cello-oligosaccharides were observed following *Tr*Cel61A treatment of phosphoric acid swollen cellulose (PASC). Of the oxidized products, C1 oxidation was the most abundant; however evidence of C4 and C1-C4 oxidation led to their classification of *Tr*Cel61A as a type-3 LPMO [21].

In this study, the LPMO activity of *Tr*Cel61A expressed in *T. reesei* was investigated against a range of substrates and the oxidation products of these treatments were analyzed with MALDI-TOF, ESI mass spectrometry and high performance anion exchange chromatography (HPAEC). The effect of *Tr*Cel61A addition on endoglucanase treatment of soy spent flake was also investigated, and a synergistic effect was observed resulting in increased glucose yields compared to enzyme addition alone. We present, to our knowledge for the first time, demonstration of LPMO activity on soybean cellulosic polysaccharides and the utility of this activity in the improved enzymatic degradation of this abundant and underutilized raw material.

2. Materials and methods

2.1. Materials

Soy spent flakes were obtained from DuPont™ Nutrition & Health – Protein Solutions (St. Louis, MO) as a by-product of the soy protein isolate production process. The material was lyophilized and ground to pass a USA-Standard ASTM 40-mesh screen. Avicel® PH-101 microcrystalline cellulose, L-fucose (Fuc), L-rhamnose (Rha), D-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-xylose (Xyl), D-glucuronic acid (GlcA), D-galacturonic acid (GalA), D-(+)-cellobiose, D-(+)-cellotriose, cellotetraose, cellopentaose, and cellohexaose were obtained from Sigma-Aldrich (St. Louis, MO). Galactan (potato) was obtained from Megazyme International (County Wicklow, Ireland). GE Healthcare PD MidiTrap G-25 columns were purchased from VWR (Radnor, PA). Maltodextrin 20DE (item no. 019046) was purchased from Cargill (Wayzata, MN). PASC was obtained from DuPont™ Industrial Biosciences (Palo Alto, CA) and was prepared as described by Wood [30].

2.2. Chemicals

Concentrated sodium hydroxide (50%; NaOH), 96% glacial acetic acid, sodium acetate trihydrate, Tris base, copper(II) sulfate pentahydrate, 2,5-dihydroxybenzoic acid MALDI-MS matrix, methanol, acetonitrile, urea, ammonium bicarbonate, DL-dithiothreitol (DTT), iodoacetamide, formic acid, and trifluoroacetic acid (TFA) were all purchased from Sigma-Aldrich (St. Louis, MO). Concentrated sulfuric acid (95–98% w/v; H_2SO_4) was purchased from VWR (Radnor, PA) and used to generate a 12 M H_2SO_4 stock solution. Concentrated hydrochloric acid (30%; HCl) was purchased from Merck Millipore (Darmstadt, Germany) and used to generate a 2 M HCl solution. L(+)-Ascorbic acid AnalaR NORMAPUR® was obtained from VWR (Radnor, PA). Sodium azide (NaN_3) was purchased from Merck Performance Materials (Darmstadt, Germany).

2.3. Enzymes

Lytic polysaccharide monooxygenase *Tr*Cel61A from *Trichoderma reesei* (EGIV;UniProt:O14405; AA9) was obtained from DuPont™ Industrial Biosciences (Palo Alto, CA) following recombinant expression in a *Trichoderma reesei* (*Hypocrea jecorina*) strain deleted for the major cellulase genes and beta-glucosidase as described in PCT Pat. Appl. Publ. No. WO2009/048488 [31]. Cell debris was removed by centrifugation and the supernatant was concentrated using a VivaFlow50 ultrafiltration membrane (MWCO 30 kDa) from Sartorius (Göttingen, Germany), resulting in a final protein concentration of 100 mg/mL. The purity of the enzyme was assessed using an SDS-PAGE gel (Supplementary Information Fig. S1), and N-terminal characterization was performed as described in 2.5.5 to verify the expected presence of an N-terminal histidine with methylation of the imidazole [18]. Endo-1,4-β-D-glucanase from *Bacillus amyloliquefaciens* (EC 3.2.1.4; GH5) with 3500U activity (17.07 mg/mL protein) and beta-glucosidase from *Agrobacterium sp*. (EC 3.2.1.21; GH1) with 600U activity (2.48 mg/mL protein) were obtained from Megazyme International (County Wicklow, Ireland). α-Chymotrypsin from bovine pancreas (EC 3.4.21.1) was obtained from Sigma-Aldrich (St. Louis, MO).

2.4. Soy spent flake NaOH pretreatment

NaOH pretreated soy spent flakes were produced as described in Mittal, Katahira, Himmel & Johnson [32]. In brief, dry soy spent flakes were combined with 16.5% w/w NaOH in an amount of 8.57 mg per mL of 16.5% w/w NaOH. This mixture was stirred under nitrogen at 25 °C for 2 h. Upon completion, the material was separated by centrifugation and the soluble fraction was decanted off with the remaining insoluble fraction re-diluted in MilliQ water to the original reaction volume and vortexed. The centrifugation, separation and dilution described above were repeated twelve times until the pH of the soluble fraction was neutral. The final, rinsed insoluble material was lyophilized and ground to pass a USA-Standard ASTM 40-mesh screen.

2.5. Material characterization

2.5.1. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The free monosaccharides in each sample were quantified by HPAEC-PAD using a Dionex ICS-3000 Ion Chromatography system with a CarboPac® PA100 guard column (50 × 2 mm) followed by a CarboPac® PA100 analytical column (250 × 2 mm) and a PAD, all from Dionex Corporation (Sunnyvale, CA). The flow rate used was 0.25 mL/min under the following elution profile, with MilliQ water as eluent A and 600 mM NaOH as eluent B: 0–18 min, 1.5% B;

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