

THERMOSTABLE ENDO-1,5- α -L-ARABINANASE FOR ISOLATING FUNCTIONAL OLIGOSACCHARIDES FROM SUGAR BEET PULP

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Abstract:

Sugar beet pulp is a rich source of structurally-complex cell wall polysaccharides, particularly branched arabinan. The goal of this project is to develop an efficient biochemical platform for generating functional oligosaccharides, specifically, feruloylated arabino-oligosaccharides (FAOs), from sugar beet pulp. FAOs may promote healthful colon functioning through prebiotic, anti-inflammatory, and mucosal immuno-modulatory activities. Enzyme treatment may improve FAO bioavailability in food and feed applications. We hypothesize beet root-expressed endo-arabinanase can selectively cleave the arabinan chain *in situ* in beet tissue to release soluble FAOs. In this study, a thermostable endo-1,5- α -L-arabinanase (TS-ABN) from *Bacillus thermodenitrifican* TS-3 was expressed in yeast cells (*Pichia pastoris*) and functional enzyme was secreted into media. We present our characterization of the yeast-expressed enzyme in terms of molecular structure, specific activity, sensitivity to pH and temperature, and tolerance to high temperature. Continuing studies will investigate the enzyme's ability to generate FAOs for evaluating bioactivity in a colon epithelial cell model.

Introduction:

The United States produced 31.4 million tons of sugar beets (*Beta vulgaris L.*) in 2014 (National Agricultural Statistics Service, 2015), and this accounts for about 10% of the world total production (Food and Agriculture Organization, 2011). Sugar beet processing in the U.S generates over 1.5 million tons of beet pulp annually (Grohmann et al., 1994). Beet pulp is used primarily as animal feed (a high energy and fiber source in animal diets) and represents a largely under-utilized source of functional polysaccharides. While beet roots are non-lignified tissue, it contains the phenolic metabolite ferulic acid, which can cross-link cell wall polysaccharides (unique to dicot plants). Ferulic acid is ester-linked to O-2 or O-5 of arabinofuranose residues, which is part of α -(1,5) linked arabinan chains on rhamnogalacturonan I (RG I) (Saulnier & Thibault, 1999; Williamson et al., 1998; Zykwiniska et al., 2006) (Fig. 1). Better utilization of beet pulp's unique structural chemistry for production of value-

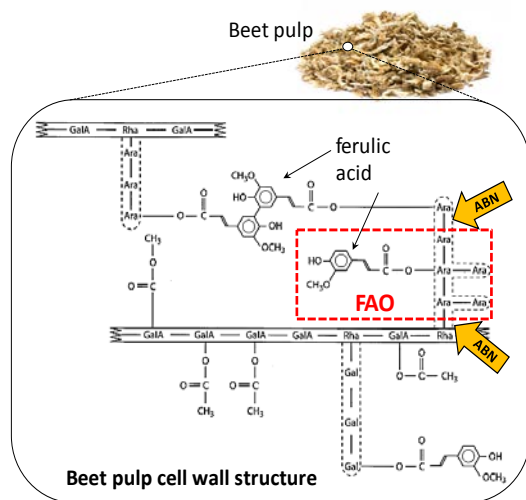


Figure 1. Schematic of FAOs immobilized in the plant cell wall fibers. FAOs can be released through selectively cleaving the arabinan chain by ABN.

added bioproducts, particularly those beneficial to animals in feed or to human health in nutraceutical applications, can improve the economic viability and competitiveness of sugar beet industry.

Feruloylated arabino-oligosaccharides (FAOs), in which the potent antioxidant ferulic acid is esterified to arabinofuranosides, can be generated from sugar beet pulp through selectively cleaving the arabinan chain of the cell wall polysaccharides by a key glycohydrolase – endo-arabinanase (ABN) (**Fig. 1**) (Saulnier & Thibault, 1999). FAOs may be useful in food and feed applications for healthful colon functioning as implicated by prebiotic (Holck et al., 2011), antioxidant (Ohta et al., 1994), and anti-inflammatory activities (Vigsnaes et al., 2011). While free ferulic acid is readily absorbed in the stomach, ferulic acid linked to larger arabino-oligosaccharides can reach the colon (Vitaglione et al., 2008). FAOs may therefore provide a novel means to deliver high antioxidant activity to the colon.

In this study, a thermostable endo-1,5- α -L-ABN (EC 3.2.1.99) from *Bacillus thermodenitrificans* TS-3 was expressed in yeast cells (*Pichia pastoris*) in order to produce enzyme in quantities sufficient for characterization and pulp-treatment studies. We present our progress to characterize the yeast-expressed enzyme in terms of molecular structure, specific activity, sensitivity to pH and temperature, and tolerance to high temperature. Continuing studies will investigate pulp-treatments with ABN to determine the enzyme's ability to generate FAOs, which will subsequently be isolated and evaluated for bioactivity in a colon epithelial cell model.

Materials and Methods:

In vitro* expression of ABN in *P. pastoris

The gene encoding thermostable ABN (EC 3.2.1.99) from *B. thermodenitrificans* TS-3 (Takao et al., 2002b) and C-terminal 6 \times His tag was synthesized by GenScript (Piscataway, NJ), subcloned into the *pPICZ α B* yeast expression vector (Invitrogen, CA) (**Fig. 2**), and then stably integrated into the genome of *Pichia pastoris* (X-33 strain, Invitrogen, CA). Inducible protein expression (by methanol) was conducted by following the protocol of *EasySelect™ Pichia Expression Kit* (Invitrogen, CA).

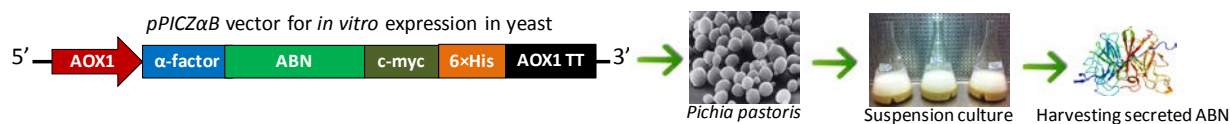


Figure 2. Schematic of the ABN gene cloned into the yeast expression vector *pPICZ α B*. AOX1: alcohol oxidase-1 promoter; α -factor: secretion signal; c-myc: c-myc peptide epitope; AOX1 TT: AOX1 transcription terminator.

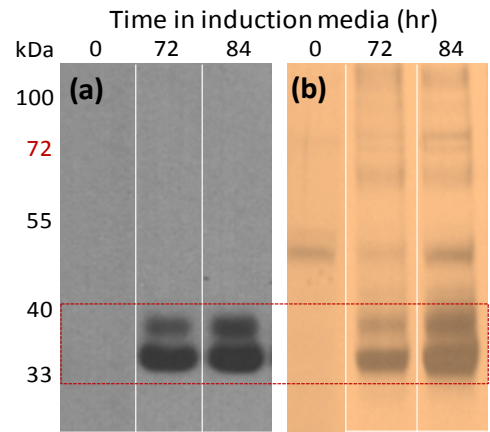
Characterization of recombinant enzyme

Recombinant ABN secreted into yeast culture media was recovered by ultrafiltration or IMAC column. His-tagged ABN was detected with anti-6 \times His antibodies by Western blotting. Enzyme activity was measured based on reducing groups released from de-branched arabinan (Megazyme, Ireland) using the BCA reagent as described by Doner and Irwin (1992). One unit of enzyme activity is defined as the amount of the enzyme that catalyzes the conversion of 1 μ mol of L-arabinose per min per milliliter of reaction mixture at 70°C. The sensitivity of the enzyme activity to temperature and pH was determined as described by Takao et al. (2002a).

Results and Discussion:

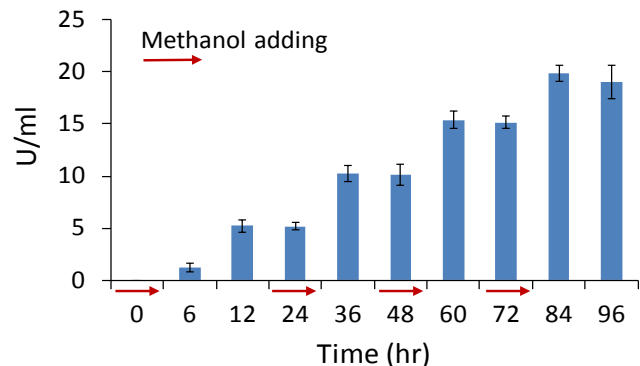
Expression of ABN in *P. pastoris* was driven by the *AOX1* promoter, which was induced by methanol. As shown in **Fig. 3**, recombinant ABN was secreted into *P. pastoris* culture media over a 4-day time course with methanol (0.5%) added every 24 hr. ABN was accumulated with a maximum of ~70 mg/L at 84 hr. There were two bands of ABN detected on the gels by Western blotting (**Fig. 3a**); while the lower band (~35 kDa) is the expected one since it matches the molecular weight of native ABN, the upper band (~37 kDa) is presumably the ABN with an uncleaved signal peptide (α -factor). No glycosylated form of recombinant ABN was detected based on ConA lectin-affinity column separation (data not shown), even though one potential N-glycosylation site was identified in ABN. In addition, it was found that the recombinant ABN was the dominant protein product detected in the culture media at the late stage of culture (72 and 84 hr), accounting for >85% of total soluble proteins (TSP) (**Fig. 3b**). This greatly simplifies the separation/purification of the enzyme for industrial use.

Figure 3. Time course of recombinant ABN secretion from *P. pastoris*. (a) Anti-6xHis Western blotting detection of ABN; (b) Separation of culture media with SDS-PAGE gel (silver stained).



The enzyme activity of recombinant ABN increased with the time up to 20 U/ml at 84 hr (**Fig. 4**). The specific enzyme activity of ABN was calculated to be ~350 U/mg (ABN), comparable to that reported in the literature (Takao et al., 2002a). In addition, the highest enzyme activity was detected at 77°C and pH 7. Continuing studies will investigate the enzyme's ability to generate FAOs, and recovering FAOs for evaluating bioactivity in a colon epithelial cell model.

Figure 4. Time course of the enzyme activity accumulated in culture media after adding methanol every 24 hr.



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