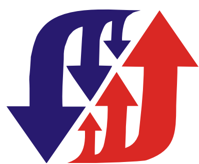

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Structural and functional characterization of a highly secreted α-L-arabinofuranosidase (GH62) from *Aspergillus nidulans* grown in sugarcane bagasse

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ABSTRACT

α-L-Arabinofuranosidases that belong to the glycoside hydrolase family 62 (GH62) present important applications in biofuel and food industries. In this work, an α-L-arabinofuranosidase (AxhA, GH62) from *A. nidulans* was more highly secreted when cultivated in sugar-cane bagasse than when cultivated in xylan. Mass spectrometry analysis confirmed that the enzyme produced by *A. nidulans* is N-glycosylated at asparagine 86. However, using circular dichroism, we found that the melting temperature and structural profile of the enzyme produced by the fungus and the one produced by *E. coli* (not glycosylated) were highly similar indicating that the N-glycan did not alter the structure of the protein. The crystallographic structure of AxhA was obtained and the final model evidences a five-bladed β-propeller fold, which is conserved in family GH62. The enzyme produced by *E. coli* showed Vmax, Km, Kcat and catalytic efficiency equal to 546.10 U/mg of protein, 3.22 mg/mL, 323.10 s^{-1} and 100.34 mg/mL/s, respectively in substrate rye arabinoxylan. The optimal activity of the enzyme was observed at 60°C and pH 5.0. These results are of great relevance, considering the lack of studies on structural characterization of enzymes that belong to GH62 family.

Palavras-chave: α-L-arabinofuranosidase, *Aspergillus nidulans*, N-glycosylation

INTRODUCTION

Carbohydrate-Active enzymes (CAZymes) are categorized in different classes according to their function in the CAZy database (www.cazy.org). Glycoside hydrolases (GH) are the most important class of enzymes used in plant biomass deconstruction, since they hydrolyze cellulose and hemicellulose (Lombard et al., 2014).

α-L-Arabinofuranosidases, which belong to GH62 family, release arabinose residues from arabinoxylan by the hydrolysis α-1,3 and α-1,2 arabinose linked to xylan backbone. Thus, they can be applied to second-generation bioethanol production, as well the production of the sweetener arabinose (Numan and Bhosle, 2006). Different types of microorganisms produce α-L-arabinofuranosidase (GH62), such as different *Aspergillus* species that secrete large amount of proteins. The species *A. nidulans* is a genetic model and has been studied for homologous and heterologous protein production. Additionally, fungi can secrete N-glycosylated enzymes, what can influence drastically the structure, secretion, activity and stability of proteins (Larkin and Imperiali, 2011). N-glycosylation of enzymes deserves attention since this pattern depends on each organism, being absent in some bacteria, like some *E. coli* strains.

The aim of this work was to study the α-L-arabinofuranosidase (AxhA, GH62) from *A. nidulans* A773. This enzyme was highly secreted by *A. nidulans* grown in pretreated sugarcane bagasse and detected by glycoproteomics approach. The enzyme was cloned and transformed in *A. nidulans* and in *E. coli*. The crystallographic structure of α-L-arabinofuranosidase (GH62) from *A. nidulans* is described for the first time.

MATERIAL AND METHODS

Microorganisms, vectors, gene and cloning

All *E. coli* strains were maintained in Luria-Bertani (LB). *A. nidulans* A773 (A773) were maintained in minimal medium (MM) (salt solution, trace elements and pyridoxine) and 1% glucose, pH 6.5. The gene AN7908, which encodes for the protein α-L-arabinofuranosidase (AxhA), was amplified and cloned in the vector pEXPYR for the production of enzyme in A773. *E. coli* DH5α was used for propagation of plasmids. For the production of the enzyme in *E. coli* the AN7908 gene was cloned in the pET28A. *E. coli* BL21, Arctic and Origami were used for AxhA production.

Transformation procedures

The DNA was transformed in the calcium competent *E. coli* cells by heat shock. The colonies obtained were tested for colony PCR and the genetic material was extracted. AxhA production by *E. coli* BL21, Origami and Arctic were cultivated in LB and the protein production induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) and evaluated by SDS-PAGE.

For fungal transformation the protoplasts of the A773 were obtained and the exogenous DNA was introduced in the competent cells using polyethileneglycol (PEG) solution. For the enzyme production the spores of the transformants (10^7–10^8 de esporos/mL) were inoculated in MM, 2% maltose, 100 mM HEPES, pH 6.5 for 72h at 37°C.

Protein purification and identification

The proteins produced by A773 were purified by ionic exchange chromatography (DEAE-sepharose Fast Flow). The proteins produced by *E. coli* were purified by affinity chromatography (Histrap HP) followed by gel filtration chromatography (Superdex G-75). Proteins were quantified by Bradford method (Bradford, 1976), using bovine serum albumin and evaluated by SDS-PAGE. For glycosylation confirmation, the proteins were treated with the enzyme endo-H before digestion with Asp-N to generate peptides. These peptides were separated by C18 column coupled with the mass spectrometer Q-Tof Ultima (Waters) with nano-eletrospray as ionization source.

Structural characterization

For the structural analysis, it was used circular dichroism far-UV in the espectropolarimeter JASCO J-810 using wave length in the range 195 - 240 nm, and quartz cuvettes of 0.1-cm.

Purified AxhA produced by *E. coli* was crystallized using crystallization kits (Crystal Screen HT, JCSG+ Suite, PACT Suite, Precipitant Synergy, SaltRx HT and Wizard I&II) and sitting drop vapor diffusion technique. The crystals were diffracted in MX-2 beam line at LNLS-CNPEM, Campinas-SP. Data processing and refinement were carry out with Program Packages CCP4 and Phenix.

Biochemical characterization

Enzymatic activity was measured with different substrates. The release of reducing sugars were determined by DNS method (Miller, 1959). The kinetic constants were determined using different concentrations of the substrate rye arabinoxylan (0.5 – 8 mg/mL).

The effect of pH on enzymatic activity of AxhA was determined using different pH values (2.6, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) at 40°C. The effect of temperature was evaluated after the measurement of the enzymatic activity in different temperatures (30, 35, 40, 45, 50, 55, 60, 65, 70 and 75°C) at pH 5.0.

The products of hydrolysis were analyzed, using the equipment P/ACE MQD (Beckman Coulter, Pasadena, CA) equipped with the fluorescence induced by laser and a fused silica capillary with diameter of 50 µm and length of 31 cm.

RESULTS AND DISCUSSION

Transformation of α-L-arabinofuranosidase in *A. nidulans*

After transformation in A773, we obtained four strains that super secreted AxhA. AxhA was analyzed by mass spectrometry (LC-MS/MS) and a N-glycosylation site was validated at the asparagine 86. This recombinant AxhA was purified and used for further experiments of circular dichroism. Considering this enzyme is N-glycosylated, the protein was also produced in *E. coli* strains that do not glycosylate proteins, for comparison.

Transformation and production of α-L-arabinofuranosidase in *E. coli*

We observed that all the three strains (Arctic, BL21 and Origami) produced the enzyme, although better production was observed by *E. coli* Arctic that was selected for further studies. AxhA purification led to a single band in SDS-PAGE and was used for further structural, and biochemical studies.

Structural characterization of α-L-arabinofuranosidase

In order to investigate the influence of N-glycosylation in the structure of AxhA produced by *A. nidulans* in comparison to the not glycosylated enzyme produced by *E. coli,* we carried out the analysis with circular dichroism. It was possible to observe that the proteins are composed mainly by β-sheet, in agreement with crystallographic structure. Moreover, the melting temperature of both AxhA was 60°C. This is a strong evidence that the N-glycosylation in the amino acid 86 did not influence the structure of AxhA from *A. nidulans*, compared to the not glycosylated protein.

The purified AxhA produced in *E. coli* was crystallized at 18°C after 15 days using 15% PEG8000, 30% 2-Methyl-2,4-pentanediol (MPD), 0.1M calcium chloride, 0.1M sodium acetate and pH 5.5 as crystallization solution. The structure was solved to a resolution of 1.57 and the initial phases were obtained by molecular replacement using an α-L-arabinofuranosidase from *Streptomyces thermoviolaceus* as a model. The final model evidences a conserved folding in family GH62, known as five-bladed β-propeller. The structure is highly similar with others from the same family, including the residues that compose the binding site. It is interesting to observe that AxhA (from *A. nidulans)* presents more structural similarity with an α-L-arabinofuranosidase from the bacterium S. *thermoviolaceus.*

Determination of α-L-arabinofuranosidase activity in different substrates and hydrolysis profile

The α-L-arabinofuranosidase activity was measured using the substrates rye arabinoxylan, wheat arabinoxylan, arabinan sugarbeeet, debranched arabinan, linear arabinan and arabino-galactan. The enzyme showed activity only towards the soluble carbohydrate, rye arabinoxylan, resulting in 230 U/mg protein. After capillary electrophoresis analysis, we observed the hydrolysis of only rye arabinoxylan releasing arabinose.

Biochemical characterization of α-L-arabinofuranosidase

The kinetic studies were carried out using rye arabinoxylan (0.5 – 8 mg/mL) and 0.1 µg of pure enzyme. Michaelis Menten constant (Km) maximum velocity (Vmax) were 3.22 mg/mL and 546,1 U/mg of protein, respectively. The turnover number, Kcat was equal to 323.1 s^{-1} and catalytic efficiency was equal to 100.34 mg/mL/s. The enzyme AxhA showed optimum activity in the range 60-65°C and pH 5.0 using the substrate rye arabinoxylan.

De La Mare (2013) found that Km of an α-L-arabinofuranosidase (GH62) from *Penicillium funiculosum* was equal to 2.7 mg/mL, while for the other two α-L-arabinofuranosidases from the fungus the value of Km values were 0.33 e 0.58 mg/mL. One of the three α-L-arabinofuranosidase showed optimal activity at pH 2.8 and 50°C. The other two were found in the pH range 2.6 – 4.5 and 40 °C, respectively.

CONCLUSIONS

The recombinant α-L-arabinofuranosidase AxhA (GH62) was cloned and produced by *A. nidulans* A773 and *E. coli*. Although the enzyme produced in fungi presented a N-glycan, it apparently did not affect the structure of the protein. AxhA presented Vmax, Km equal to 546.1 U/mg of protein and 3.22 mg/mL, respectively, and optimal activity at 60°C and pH 5.0 using the substrate rye arabinoxylan. The crystal structure of the enzyme was solved showing a conserved folding in the family and a structure more similar to a bacterial enzyme than to fungal enzymes.

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