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Characterization of the Endoxylanase from *Humicola grisea* var. *thermoidea*

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Abstract

The extracellular xylanases were produced by Humicola grisea, as it was seen in gel activity assay, and the xylanase of 23 kDa, was purified by precipitation with ammonium sulphate followed by ion exchange chromatography in Q sepharose and gel filtration in Sephacryl S-100. The optimum pH and temperature were 5.5 and 65°C respectively.

Keywords: Enzyme production, hemicellulosic substrates, *Humicola grisea*, sugarcane bagasse, xylanase.

Introduction

The use of sugarcane bagasse in biotechnological process for enzyme production has been also described by the use of its cellulosic and hemicellulosic fractions. Actually, xylan is the major hemicellulose present in the plant cell wall and is a polysaccharide composed of β-1,4-linked xylopyranose units (Pulls, 1997). In nature, the complete hydrolysis of xylan to monosaccharides occurs by the combined action of enzymes released by fungi and bacteria, including the xylanases (EC.3.2.1.8) and β-D-xylosidases (EC.3.2.1.37). The existence of a multiplicity of xylanases in microorganisms may be a strategy to achieve superior xylan hydrolysis (Agger, 2014).
In this context, was obtained xylanases, purified by gel filtration and characterized in relation to its biochemical properties.

Materials and methods

Microorganism and xylanases production

The harvested conidia ($0.35x10^6$ spores ml^{-1}) of the wild-type strain of *H. grisea*, isolated from Brazilian soil (Almeida *et al*., 1995) was inoculated in 250 mL Erlenmeyer flasks containing 100 ml of minimal medium (Pontecorvo *et al*., 1953) added with 1% (w/v) of the carbon source. The flasks were maintained in a rotatory shaker at 120 rpm, 42ºC and the samples were taken out at each 24 h to analise the xylanase production. At the end of fermentation (after the observation of the maximum xylanase activity), the mycelium was harvested by filtration through filter paper, and the filtered of culture was used as a source of xylanase.

Enzyme assay and protein determination

Xylanase activity was assayed, according to Miller (1959), using oatspelt xylan 1% (Sigma-Aldrich®, Steinheim, Germany) as substrate by determining the amount of reducing sugar released. One unit (U) of xylanase activity was defined as the amount of enzyme that produced 1 µmol of reducing sugar per minute under the assay conditions. Protein concentration was determined by Bradford's method (Bradford, 1976), using bovine serum albumin as standard. Absorbance at 280 nm was used to monitor protein profile during chromatographic procedures.

Humicola grisea xylanase purification

The mycelium-free fermented medium, produced in the optimized conditions, was filtered in cellulose acetate membrane (0.45 µm) followed by precipitation with ammonium sulphate at 20% of saturation to remove the pigments. The supernatant was colleted by centrifugation at 10,000 *g* for 30 min at 4ºC, and ammonium sulphate was added to obtain 50% of saturation. The precipitate was colleted as described above, re-dissolved in distilled water to obtain about 300 U/ml of xylanase activity, and dialyzed overnight against 2 L of distilled water.

This concentrated crude protein was applied to an Q Sepharose (Amersham Biosciences, Uppsala, Sweden) column (15 x 2.5 cm) previously equilibrated with 50 mM Tris-HCl pH 8.0 buffer and eluted at a flow rate of 40 ml h^{-1} with the same buffer. Fractions of 3.0 ml were collected and monitored for protein (A_{280}) and xylanase activity. Fractions containing xylanase activity were pooled, concentrated with ammonium sulphate (50%) and applied onto a Sephacryl S-100 (Amersham Biosciences, Uppsala, Sweden) column (87 x 1.5 cm) equilibrated, eluted and monitored as described for the previous column. In this case, the collected fractions volume was 1 ml and those containing xylanase activity were pooled and stored at -20ºC.

Electrophoresis and xylanase activity assay

Polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 13% acrylamide gel, as described by Laemmli (1970), and the gel was silver stained as described by Blum *et al*. (1987).

For the activity assay, proteins from the mycelium-free fermented medium were dialyzed against water overnight, vacuum dried (Eppendorf Concentrator 5301) and separated by SDS-PAGE in 13% gel containing 0.1% (w/v) oat-spelt xylan (Sigma-Aldrich®, Steinheim, Germany). After the separation of proteins, the gel was incubated for 1 h in 2.5% (v/v) Triton X-100 to remove SDS; this was followed by incubation in 100 mM MES (USB™, Cleveland, USA) buffer, pH 6.5 at 50°C for 1 h. Xylanase activity was visualized as clearing around the protein band(s) after incubation of gels in 0.1% (w/v) Congo Red for 20 min, followed by a washing step as
Faria *et al*. (2002).

Enzyme characterization

pH and temperature influence on the enzymatic activities: The pH influence on the purified xylanase activity was studied by carrying out the previously described enzyme assay under different pH values for the medium. The following buffers were used: 50 mM sodium citrate (pH 3.0-5.0), 50 mM sodium phosphate (pH 6.07.4), 50 mM Tris-HCl (pH 7.7-8.2) and 50 mM sodium carbonate (pH 9.2-10.9). A similar procedure was performed to evaluate the influence of temperature in xylanase activity, varying the temperature from 0 to 100ºC under the assay conditions at optimum pH.

Results and discussion

The purification procedure was carried out using two precipitations with ammonium sulphate, followed by ion exchange chromatography in QSepharose and gel filtration in Sephacryl S-100. The fractions correspondent to these peaks were pooled into groups and applied into SDSPAGE in order to observe the protein profile and detect the xylanases by in-gel activity assay. In peak I it was observed, through the gel analysis, an 34 kDa xylanase in high concentration, indicating that xylanolitic activity is mainly correlated to the presence of this xylanase. In peak II the 23 kDa and the 25 kDa xylanases were observed in high concentrations. In peak III the purified enzyme was observed as a single band with an estimated molecular mass of 23 kDa, as showed by SDS-PAGE and in-gel activity assay (Figure 1). The purification procedure resulted in a yield of 4.4% of the activity and an 12.2-fold increase in specific activity (Table 1).

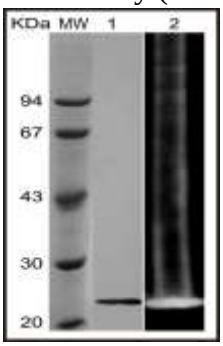

Figure 1 - Electrophoresis of pooled fractions 144 until 151 eluted from gel filtration column Sephacryl S100. MW - Molecular weight; 1 - SDS-PAGE (13%) stained with silver nitrate; 2 - In-gel (13%) xylanolitic activity. Molecular weight markers were phosphorylase b (94 kDa); bovine serum albumin (67 kDa); ovoalbumin (43 kDa); carbonic anhydrase (30 kDa); and trypsin inhibitor (20.1 kDa).

Table 2 – Purification of 23 kDa xylanase from *H. grisea*

Step	Total protein (µg ml^{1})	Total activity (U ml_{1})	Specific activity (U $µg_{1}$)	Purification fold	Yield (%)
Culture supernatant	122	5,580	45.7	1	100
$(NH_4)_2SO_4$ (20%)	118	2,520	21.3	0.5	45
$(NH_4)_2SO_4$ (50%)	81	3,105	38.3	0.8	56
Q-Sepharose	2.3	318	138.3	3.0	5.7
Sephacryl S-100™	0.44	246	559.1	12.2	4.4

Enzymatic characterization

In Figure 2 it shows the influence of pH and temperature and stability of the purified xylanase at incubated different conditions. The 23 kDa xylanase was active over a pH range of 3.0 to 9.0, with an optimum pH at 5.5 (Figure 2a), and it showed to be stable at various pH values, retaining 97 and 88% of the initial activity after 60 min of incubation in pH 5.0 and 5.5, respectively (Figure 2b). These results are similar to other fungal xylanases which are more

effective in pH 4-6 range (Sá-Pereira *et al*., 2002). The thermostability at optimum temperature can be compared to xylanases of many mesophilic and thermophilic fungi, which presented a good stability at temperatures from 40ºC to 60ºC (Techapun *et al*., 2002).

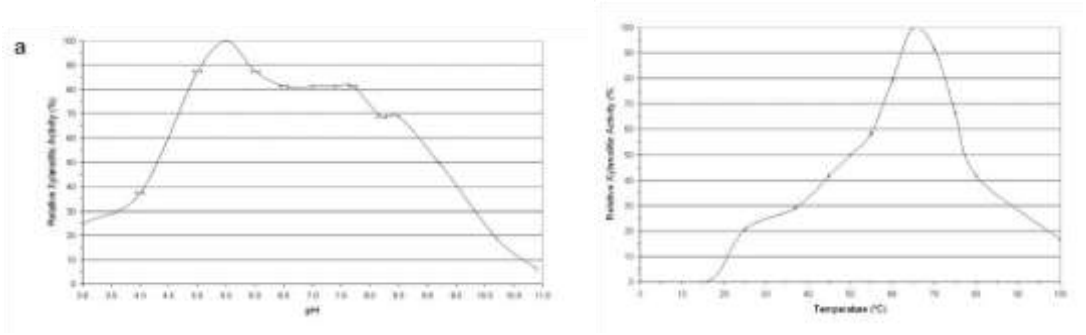

Figure 2– Influence of temperature and pH on xylanase activity. A - Optimum pH. B - - Optimum temperature.

Conclusion

The *H. grisea* produced at least three proteins with xylanases activities and a 23 kDa xylanase was purified and exhibited high affinity for the substrate oat-spelt xylan. The optimum pH and temperature values for the purified enzyme were 5.5 and 65ºC respectively, and the thermostability was comparable to xylanases of many mesophilic and thermophilic fungi.

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