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Structural and functional analysis of *Aspergillus niger* xylanase to be employed in polyethylenglycol/salt aqueous two-phase extraction

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ABSTRACT

The structure and enzymatic activity of *Aspergillus niger* xylanase were evaluated in different media to establish an appropriate protocol for the extraction of the enzyme in polymer/salt aqueous two-phase systems. Different factors were studied: the concentration and molecular weight (1000, 2000, 4600 and 8000) of polyethyleneglycol, the concentration and type of salt (sodium citrate and potassium phosphate) and pH, time and temperature. Xylanase was stable for 5 h at pH between 2.7 and 9.0 and at temperatures up to 50 °C. Fluorescence spectroscopy and circular dichroism experiments showed that neither the secondary/tertiary structure of the enzyme nor its catalytic activity were significantly altered in the presence of either salt or PEG. Xylanase partitioned into the PEG-rich phase driven by the excluded volume effect. Partitioning was more favorable to the polymer phase in the PEG1000/NaCit system, where *Kp* was 12 times higher than in the others aqueous two-phase systems. These results demonstrate the potential application of the PEG1000/NaCit system as a first step for the extraction of *Aspergillus niger* xylanase.

1. Introduction

The biotechnological potential of xylanases in various industries, such as brewery and wine, animal feed, textile and laundry, pulp and paper, biofuel, agriculture, as well as in research and development has been shown in the last few years. The actual demand and use of xylanase in many industrial applications requires an efficient and cost-effective downstream operation to perform a high-yield enzyme separation (Polizeli et al., 2005).

Endo-1,4-β-xylanase (EC 3.2.1.8) randomly hydrolyzes the β-1,4-glycosidic bonds of xylan into xylooligomers with different lengths. Xylans, the most abundant hemicellulose, are heteropolysaccharides found in the cell walls of all land plants and in almost all plant tissues (Biely, 1985). Endo-1,4-β-xylanase is the major component of xylanolytic systems produced by biodegrading microorganisms such as fungi and bacteria. *Aspergillus niger* is one of the most potent organisms used in biotechnology for xylanase production and secretion (Khan et al., 2003), considered in the status of Generally Recognised As Safe (GRAS) by the United States Food and Drug Administration (FDA) (Schuster et al., 2002).

Traditional methods, such as ammonium sulfate precipitation, size exclusion or ion exchange chromatography, allow the purification of xylanase from a crude hydrolase mixture produced by fermentation broth.

Separation techniques based on aqueous two-phase extraction (ATPE) have been increasingly used as a first step of clarification, concentration and purification of important biomolecules from their natural source (Porfiri et al., 2011; Rocha and Nerli, 2013).

Aqueous two-phase systems (ATPSs) present several advantages such as, high biocompatibility (70–80% water content), low biomolecular degradation, high resolution low cost and easy scale-up (Albertsson, 1986). In order to form the two phases, aqueous solutions of either two polymers or a polymer and a salt are required. In ATPE, protein distribution between the two phases is influenced by system properties like type, size and concentrations of polymers and salts used, pH and temperature, and by partitioned-biomolecule properties such as: molecular weight, isoelectrical points, conformation and hydrophobicity.

In different works, polyethyleneglycol (PEG)/phosphate or PEG/sulfate ATPSs were found to be the most suitable for isolating fungal xylanase (Antov et al., 2006; Garai and Kumar, 2013). However, for industrial purposes, potassium phosphate represents a waste disposal problem. In the last few years sodium citrate has been used as a substitute for potassium phosphate since citrates are biodegradable and non-toxic and can be discharged into biological wastewater treatment plants (Tubio et al., 2009).

The aim of this article was to describe the effect of different polymers and salts on the structure and activity of *A. niger*

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xylanase with an emphasis on the effect of temperature and pH on its stability. In-depth characterization of the enzyme will be useful to evaluate xylanase partitioning in polymer/salt ATPS.

2. Materials and methods

2.1. Chemicals

Endo-1,4-β-xylanase from *A. niger* (Xyl) was obtained from Megazyme Int. (Wicklow, Ireland). According to the data sheet, the molecular weight is 25,000, the specific activity is 79.3 U/mg and the purity is 99.99%. Sodium citrate (NaCit), potassium phosphate (KPi), birchwood xylan, xylose, polyethyleneglycols of average molecular weight: 1000, 2000, 4600 and 8000 (PEG1000, PEG2000, PEG4600, PEG8000) and 1-anilino-8-naphthalene sulfonate (ANS), were purchased from Sigma Chem. Co. and used without further purification. All the other reagents were of analytical quality.

2.2. Methodologies

2.2.1. Determination of Xyl activity

Xyl activity was determined by the 3,5-dinitrosalicilic acid (DNS) method (Miller, 1959), measuring the amount of reducing sugars liberated when birchwood xylan (1% w/w) and Xyl were mixed in 50 mM citrate buffer pH 5.30, according to the Bailey et al. method (Bailey, 1992). Each sample was incubated at 50 °C for 10 min. Then 1 ml of DNS reagent was added to each tube, and the samples were boiled for 10 min. The absorbance of each sample was measured at 560 nm. A calibration curve of absorbance at 560 nm against the xylose concentration was used to calculate the μmoles of xylose liberated. Xyl activity was expressed as units (U) per milliliter of enzyme (U mL^{-1}). One unit is defined as the amount of enzyme required to release 1 μmol per minute of xylose reducing equivalent (μmol xylose min^{-1}). Solutions of substrate alone and enzyme alone were used as controls. All the experiments were performed in triplicate.

2.2.2. Effect of pH, time and temperature on the stability of Xyl

The pH stability assay was carried out by pre-incubating the enzyme (1.04 μM) in 50 mM citrate buffer at different pHs between 2.7 and 9.0 at 25 °C. Xyl activity was measured every 30 min for 5 h.

Thermal stability was evaluated by pre-incubating the enzyme (1.04 μM) in 50 mM citrate buffer pH 5.30 at different temperatures (from 20 to 60 °C) in a thermostatic bath. Incubation was carried out in sealed vials to prevent changes in the sample volume and, hence, in enzyme concentration due to evaporation. Aliquots of enzyme solution were taken every 30 min for 5 h and catalytic activity was measured. The residual Xyl activity was calculated as the percentage of activity remained after incubation respect to the activity of the enzyme without heat treatment.

2.2.3. Fluorescence and circular dichroism studies of Xyl in the presence of PEGs and salts

Fluorescence measurements were performed on an Aminco Bowman Serie 2 spectrofluorometer using a thermostatized quartz cuvette of 1 cm of optical pathway. Xyl (1.3 μM) was excited at 280 nm and the emission was recorded from 290 to 430 nm. The scanning rate was 3 nm min^{-1} and the bandwidth was 4 nm. Fluorescence quenching experiments were performed by using acrylamide. This is an efficient quencher of tryptophan fluorescence able to distinguish between buried and exposed side chains. Xyl was titrated with acrylamide (40 mM) in the absence and presence of PEG. After each addition, fluorescence was measured at 340 nm (exciting at 280 nm).

Circular dichroism spectra (CD) at 5.2 μM final concentration of Xyl were performed in a Jasco J-810 spectropolarimeter, using a thermostatized cuvette of 1 mm of pathlength, the scan rate was of 50 nm min^{-1} and the bandwidth was of 1 nm. Repetitive scanning of five cycles was used. All the experiments were performed in triplicate. Data were corrected by using the software provided by the instrument manufacturer.

CD data are presented in terms of mean residue ellipticity (MRE, expressed as deg cm^2 $dmol^{-1}$) as a function of wavelength, calculated by Eq. (1) according to the procedure described earlier (Kelly et al., 2005):

$$[\theta]_{MRE} = \frac{MRW \cdot \theta_{obs}}{10 \cdot d \cdot c} \quad (1)$$

where $[\theta]_{MRE}$ is the calculated mean residue ellipticity (deg cm^2 $dmol^{-1}$), θ_{obs} is the observed ellipticity (expressed in degrees), d is the pathlength (cm), and c is the protein concentration (g mL^{-1}). The Mean Residue Weight (MRW) for the peptide bond is calculated from $MRW = M/(N-1)$, where M is the molecular weight of the polypeptide chain (in Da), and N is the number of amino acids in the chain; the number of peptide bonds is $N-1$. All the CD spectra were corrected for polymer contributions. The secondary structure composition was estimated by using CDPro software package (http://lamar.colostate.edu/~sreeram/CDPro/main.html) developed by Sreerama and Woody, 2000.

2.2.4. Protein surface hydrophobicity (S_0)

Relative surface hydrophobicity of Xyl was determined by applying the optical method reported by Haskard and Li-Chan (1998). Stock solution (6 mM) of the anionic fluorescence probe 1-anililo-naphthalene-8-sulfonate (ANS) was prepared in 50 mM phosphate buffer pH 6.00. Aliquots of the protein were added to a sample containing 2551 μL of buffer solution with a final ANS concentration of 119.95 μM and the relative fluorescence intensity (I_f) was measured after each addition, the protein concentration ([protein]), varying from 0 to 10 μM. Excitation and emission wavelengths were 382 and 466 nm respectively with a bandwidth of 4 mm. Under the above-mentioned experimental conditions (with ANS excess), the surface hydrophobicity S_0 was determined from the initial slope of the linear regression analysis of the I_f vs. [protein] plot (Hatti-Kaul, 2001). The temperature of the sample was maintained at 25 °C by a HAAKE DC3 thermostatic bath and measured with a thermocouple immersed inside the cuvette. The heating rate was 0.01 °C/min.

2.2.5. Aqueous two-phase diagrams

The cloud point and turbidimetric titration methods (Hatti-Kaul, 2001) were used to determine the binodal curve of the PEG/salt systems at 22 °C. In the cloud point method, either PEG or salt solutions were added drop-wise and mixed until the resulting mixture became cloudy. In the turbidimetric titration method, water was added drop-wise to several biphasic systems with different compositions, until a biphasic system was formed, after mixing in a vortex mixer. Tie Line Length (TLL) was calculated using the Eq. (2):

$$TLL = \sqrt{[\Delta PEG]^2 + [\Delta Salt]^2} \quad (2)$$

where [ΔPEG] and [ΔSalt] are the differences between the concentrations of PEG and Salt in the top and bottom phases, respectively expressed in % (w/w).

2.2.6. Preparation of the aqueous biphasic systems

To prepare the ATPSs, stock solutions of the phase components