Biocatalysis and Agricultural Biotechnology 7 (2016) 118–126

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

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

Single method of purification for endoglucanase from *Aspergillus niger* by polyelectrolyte precipitation

María Julia Boggione, Ramiro Becher, Beatriz Farruggia *

IPROByQ-CONICET, Laboratory of Chemical-Physical Applied to Bioseparation Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Suipacha 531, 2000 Rosario, Argentina

ARTICLE INFO

Article history:
Received 26 February 2016
Received in revised form
14 April 2016
Accepted 26 May 2016
Available online 27 May 2016

Keywords:
Endoglucanase
Anionic polyelectrolyte
Cationic polyelectrolyte
Precipitation
Bioseparation

ABSTRACT

The precipitation of endoglucanase from *Aspergillus niger* with synthetic and natural electrically charged polymers -poly vinyl sulfonate (PVS) and chitosan (CHS)- was characterized and applied to a simple method of purification of an enzymatic extract obtained from fungal culture under solid-state fermentation (SSF).

The kinetics of complex formation was determined. The results of the kinetic profile obtained for CHS and PVS indicated an exothermic mechanism for the formation of the non-soluble complex. CHS exhibited a marked stabilizing effect on endoglucanase.

The enzyme precipitated successfully with both polymers. The precipitation method applied to commercial endoglucanase and the fungal extract showed similar patterns with high purification factors. The recovery of the activity in the re-dissolved precipitate from the fungal extract was close to 40% at pH 5.3 using PVS (1% w/w) as precipitating agent and the purification factor was near 9. The purification factor of *endo*glucanase in the precipitate of the enzymatic extract from SSF with CHS (0.05% w/v) was around 7. These parameters make this precipitation method appropriate to be included in the last stages of a downstream process, with advantages such as simplicity, scalability and ability to concentrate and stabilize the enzyme.

1. Introduction

Cellulases are hydrolases which comprise a complex of endoglucanase (3.2.1.4), exoglucanase and β-glucosidases (Sohail et al., 2009). Cellulases have a wide range of applications in various industries such as textile, laundry, pulp and paper, food and feed products, as well as in bioethanol production (Bhat, 2000; Dave et al., 2013; Silva et al., 2013). Cellulases have great potential in the saccharification of lignocellulosics into fermentable sugars which can be used for the production of bioethanol (Boggione et al., 2016; Maki et al., 2009; Pandey et al., 2016). It is critical to consider the conditions that contribute to enzyme stability for the design and development of a biotechnological process where the function of the enzyme is relevant. Highly polluting chemical processes can be replaced by environmentally-friendly biotechnological methodologies using cellulases.

Endoglucanase is one of the main components of the enzyme complex (Sohail et al., 2009). Traditional methods for endoglucanase purification are the precipitation with ammonium sulfate, ion-exchange chromatography, affinity chromatography and gel filtration (Bakare et al., 2005; Bischoff et al., 2006; Calza et al., 1985; Gupta et al., 2012; Kaur et al., 2007; Murashima et al., 2002; Saha, 2004; Yin et al., 2010). The latter is costly, time-consuming and ammonium sulfate cannot be disposed into the environment. Besides, chromatography is not a suitable technique to be used in a downstream process from the economic point of view. Therefore, a new protocol which considers the costs and reduces the environmental impact is needed.

In the downstream processing of enzymes, the choice of the purification technique should ensure the preservation of the enzyme structure associated with its biological function. One technique for large-scale protein separation involves the addition of polyelectrolytes to achieve selective protein separations. Proteins interact strongly with both synthetic and natural polyelectrolytes (Romanini et al., 2007). These interactions are modulated by a broad array of variables such as pH and ionic strength, and may result in soluble complexes (Morawetz and Hughes Jr, 1952; Xia and Dubin, 1994), complex coacervation (Ahmed et al., 1994), precipitation (Sternberg and Hershberger, 1974), or gelation (Petit et al., 1995).

Abbreviations: PVS, poly vinyl sulfonate; CHS, Chitosan; CE, commercial endoglucanase; PF, purification factor; %R, percentage of recovery

* Correspondence to: IPROByQ, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Suipacha 531, S2002 LRK, Rosario, Argentina.
E-mail address: bfarrug@fbioyf.unr.edu.ar (B. Farruggia).

http://dx.doi.org/10.1016/j.bcab.2016.05.014
1878-8181/

Several interactions such as electrostatic, hydrophobic, hydrogen bonds may occur between proteins and polymers, resulting in protein folding and complexation (Radeva, 2001). Polyelectrolyte-protein complex formation includes recovery and protein purification (Roy et al., 2005), stabilization and immobilization of enzymes (Manrich et al., 2008), modification of protein–substrate affinity (Mattiasson et al., 1998). The phase boundaries of different proteins can be used in systems containing several proteins as a means of selecting the optimal pH and ionic strength for maximum yield and purity. The light scattered by the samples, detected through turbidity measurements, appears to be the simplest and most sensitive technique to follow the progress of any kind of aggregation (Radeva, 2001).

Precipitation by the formation of polymer-protein complex is a novel alternative potentially applicable in order to recover endoglucanase from *Aspergillus niger*. This method has different advantages over traditional ones, such as simplicity, low cost and speed.

The first aim of this work was to investigate the formation of the polyelectrolyte-endoglucanase complex. Two polymers were selected to evaluate complex formation: anionic poly vinyl sulfonate (PVS) and cationic chitosan (CHS). The mechanism of polymer-endoglucanase interaction and the conditions for precipitation were determined in order to obtain the optimum conditions for precipitation.

The second goal of this work was to apply the above mentioned information to design a simple downstream process that requires a low amount of chemicals and is able to recover and concentrate endoglucanase from a solid culture medium.

2. Materials and methods

2.1. Chemicals and materials

Commercial endoglucanase (CE) from *Aspergillus niger* was purchased from Sigma-Aldrich (USA). Solutions of CE were prepared in 50 mM citrate buffer (pH 5.3). PVS of 170000 Da molecular mass, 1.267 g/cm^3 density was purchased from Sigma-Aldrich (USA) and was used in solutions of 1% w/w in 50 mM citrate buffer (pH 2.7).

CHS was purchased from Sigma-Aldrich (USA) with a minimum deacetylation degree of approximately 75%, and used without further purification. Solutions of CHS (0.05% w/v) in 50 mM phosphate buffer (pH 5.1) were prepared. All the other chemicals were of analytical grade. Carboxymethylcellulose (CMC) was acquired from Sigma-Aldrich (USA).

Soy hull pellets, a by-product of soybean processing, were used in this work as support for SSF.

2.2. Determination of endoglucanase activity and total proteins

Endoglucanase activity was determined by the enzymatic capacity to hydrolyze CMC (Miller, 1959), which releases reducing sugars. Under certain conditions, 3,5-dinitrosalicylic acid (DNS) is reduced by the generated sugars and changes its color from orange to red. The reduced DNS can be detected by measuring absorbance at 560 nm. The reaction was carried out by mixing 900 μL of 1% w/v CMC solution with an adequate aliquot of enzymatic sample at 50 °C for 10 min. Then, 1 mL of 1% w/v DNS was added and maintained at 100 °C for 10 min and the absorbance was measured at 560 nm. Blanks without enzyme and without CMC were assayed as controls. A calibration curve of glucose was carried out under the same experimental conditions as the samples. One unit of endoglucanase activity is the amount of enzyme that liberates 1 μmol of glucose/min under the above mentioned conditions (Bansal et al., 2012; Gouka et al., 1997). Total protein concentration was estimated by the Warburg and Christian method (Warburg and Christian, 1941).

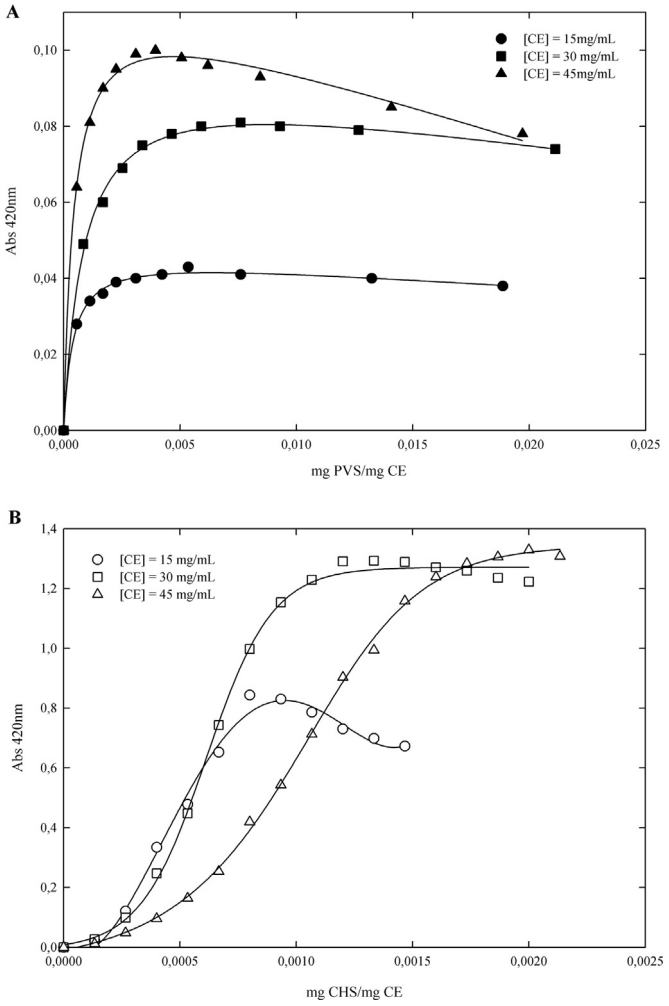

Fig. 1. A: Titration curves for three CE concentrations with 1% w/w PVS. Medium: 50 mM citrate buffer (pH 2.7). Temperature 15 °C. B: Titration curves for three CE concentrations with 0.05% w/w CHS. Medium: 50 mM phosphate buffer (pH 5.1). Temperature 15 °C.

2.3. Endoglucanase turbidimetric titration curves with polymers at different pH and ionic strengths

The non-soluble polymer-endoglucanase complex was monitored by turbidimetric titration (Cooper et al., 2005; Kokufuta et al., 1981). CE solutions with a fixed protein concentration were titrated at 20 °C in a glass cell with the polymer solution in the absence and presence of NaCl. Both protein and polyelectrolyte solutions were adjusted to the same pH value in order to avoid pH changes in the course of the titration. Complex formation was monitored by measurement of the absorbance at 420 nm vs. the added amount of polymer. Stoichiometric polymer/protein ratio was defined as the minimum ratio needed to achieve a full protein-precipitation by forming a non-soluble complex. Stoichiometric polymer/protein ratio was obtained from the turbidimetric curves as the minimal polymer concentration required to achieve a plateau. Data have been expressed as mg of polyelectrolyte per mg of CE.

The time required to form the complex was determined by measuring the time demanded in order to obtain the maximum absorbance (Cooper et al., 2005).