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An approach to the characterization of a novel thermophilic *Bacillus thermoamylovorans* lipase

Maria A. Longo, Elisabete Carvalho, Francisco J. Deive, M. Angeles Sanromán
Department of Chemical Engineering. University of Vigo.
Campus Lagoas Marcosende 36310 Vigo. Spain
e-mail: mlongo@uvigo.es http://bioprocesos.uvigo.es/

The aim of this work was the purification and tentative characterization of a thermophilic lipolytic enzyme from a thermophilic *Bacillus thermoamylovorans* strain, isolated from a Galician hot spring. This organism showed high extracellular enzyme activity. The extracellular extract was concentrated using several protein precipitation protocols including ammonium sulfate and organic solvents. Further purification was attempted by hydrophobic interaction chromatography (HIC). Also, identification through mass spectrometry techniques was attempted.

1. Introduction

Thermophilic enzymes are sought after due to their stability towards higher temperatures. Also, their high optimal activity temperatures are associated to remarkable processing benefits, since the higher solubility, decreased viscosity and increased diffusion rates allow the use of higher substrate concentrations and usually lead to higher reaction rates. Also, as the reaction temperatures are generally higher than optimal growth temperature of mesophilic organisms, there is a lower risk of contamination (Littlechild et al. 2007).

Microbial lipases work at the oil-water interface and catalyse hydrolysis of large chain triglycerides, normally insoluble in water. Apart their hydrolytic action, lipases present the interesting ability to catalyse several reactions in low water activity conditions, namely esterification, interesterefication, transesterification and aminolysis. Lipolytic enzymes have received considerable attention, both in research and industry, due to their many potential applications on detergent, food, flavour, pharmaceutical, fine chemicals, and cosmetics industries (Jaeger and Eggert, 2002; Hasan et al. 2006).

A wide diversity of lipases can be found in nature, with very different molecular weights, optimum pH and temperature, and substrate and reaction specificities. The search for novel enzymes from extremophiles appears a suitable way of obtaining lipases with improved biocatalytic abilities (Gupta et al. 2004; Ferrer et al. 2007).

Commercial application of enzymes usually requires suitable downstream processing steps to be designed. Purification of lipases has been reviewed by several authors. Most microbial lipases are extracellular and the majority of purification processes start by removal of cells, and a preliminary concentration step that is usually precipitation.

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Generally, it is followed by one or more chromatographic steps, such as ion exchange, gel filtration, affinity or hydrophobic interaction chromatography. Novel techniques have also been used, namely immunopurification, phase separation through aqueous two phase systems, and chromatography using specific stationary phases (Saxena et al. 2003; Gupta et al. 2004).

This work describes the preliminary purification of the lipase from a *Bacillus thermoamylovorans* CH6B strain recently isolated from a Galician hot spring. Concentration of the extracellular extract and further chromatographic purification is undertaken. Also, identification of the enzyme through mass spectrometry techniques is attempted.

2. Materials and Methods

2.1 Microorganism and culture

The strain was isolated from samples collected in the hot spring A Chavasqueira, located in the province of Ourense (North West Spain). Submerged cultures were carried out in 250 mL shake flasks with 50 mL of medium. The medium was composed of (g/L, in distilled water): 8 trypticase, 4 yeast extract and 3 sodium chloride. The medium was autoclaved at 121 ºC for 20 min. The flasks were inoculated (3% v/v) with previously obtained cells pellets, and incubated in an orbital shaker at 55 ºC and 100 rpm. After 12 days of growth, the culture broth was centrifuged (10 min, 5000×g) to remove the biomass.

2.2 Extracellular postincubate concentration

The extracellular extracts were concentrated by several precipitation methods that are briefly described below:

- Ammonium sulphate: 30-90% salt saturation, mixing for 16 h at 4 ºC.
- Ethanol: 9:1 volume ratio cold ethanol:enzyme extract, mixing for 1 hour at -20 ºC.
- Acetone: 4:1 volume ratio cold acetone:enzyme extract, mixing for 1 hour at -20 ºC.
- Sodium cholate-ethanol-ether: one volume of extract was mixed with 0.5% sodium cholate (30 min, 4 ºC). Afterwards 3 volumes of a cold mix of ethanol-diethyl ether (1:1 v:v) were added.

In all cases, the resulting mixtures were centrifuged (10000xg) for 15 min at 4 ºC, and the supernatants discarded.

The extracellular extract was also concentrated by ultrafiltration, using an Amicon Ultra-15 unit with 10 kDa MW cut-off, centrifuged for 45 min at 5000xg and 4ºC.

2.3 Chromatographic separation

Chromatography was performed on an AKTA Purifier apparatus (Amersham Pharmacia Biotech) equipped with an UV detector. A HiTrap Hydrophobic Interaction Chromatography (HIC) kit (GE Healthcare) containing 1 mL of several resins: Phenyl Sepharose High Performance, Butyl Sepharose High Performance, Butyl Sepharose 4 Fast Flow, Octyl Sepharose 4 Fast Flow, was used in preliminary studies.. For higher scale separation, an Octyl Sepharose 4 Fast Flow resin was used (10 mL column, 1.6 cm diameter). The protein pellet after ammonium sulphate precipitation was resuspended in

50 mM sodium phosphate buffer (pH 7.0). Samples were injected into the column previously equilibrated in 50 mM sodium phosphate, pH 7.0 (eluant A). Elution was performed with a linear gradient of increasing concentration of ethylene glycol (eluent B: 50% of ethylene glycol in A).

2.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoretic analysis was performed according to the method of Laemmli (1970), using 4-10% Tris-HCl gels. In order to detect hydrolytic activity, after the electrophoresis the gels were submitted to a renaturalization process, as described by Fuciños et al. (2005). Esterase activity was detected with α-naphthyl acetate and Fast-Red stain, at pH 7.0 (phosphate buffer), 55 ºC (Schmidt-Dannert et al. 1996). After the reaction, the gels were briefly subjected to a cycle of staining/destaining with Coomassie Blue R-250 to determine molecular weights.

2.5 Lipolytic activity assay

Lipolytic activity was routinely determined spectrophotometrically using 2.5 mM *p*-nitrophenyl laurate as substrate (Sigurgísladóttir et al. 1993), at pH 7.5, 55 ºC and 20 min reaction time, in a 1 mL total reaction volume. One activity unit was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol per minute under standard assay conditions. The activities were expressed in U/L.

2.6 Protein concentration assay

Protein concentration was determined colorimetrically using a commercially available Bradford assay (Biorad) and bovine serum albumin (Sigma Chemical Co, St Louis USA) as standard.

2.7 Mass spectrometric analysis using an ion trap

Mass spectrometry analysis was performed at the Unidad de Proteómica of Universidad Complutense de Madrid. Digested peptides were analyzed using liquid chromatography-MS/MS (LC-MS/MS) on an LTQ linear ion trap mass spectrometer (Thermo Electron). The peptides were separated on a BioBasic C-18 PicoFrit column at a flow rate of 200 nL/min, using water and acetonitrile with 0.1% formic acid each as solvents A and B, respectively. Peptides were trapped and desalted in the trap column for 5 min. The gradient was started and kept for 5 min at 5% B, then ramped to 70% B in 60 min, and finally kept at 90% B for another 5 min. The search of peptides was performed with a licensed version of MASCOT, in SwissProt database (405506 sequences).

3. Results and Discussion

Bacillus thermoamylovorans was cultivated in shake flasks for 12 days. This culture time allowed attaining the maximum level of lipolytic activity, around 1700 U/L, and an extracellular protein concentration of 0.36 g/L.

3.1 Enzyme concentration

Several precipitation and concentration protocols were tested as a preliminary method to

concentrate the crude enzyme solution. Ammonium sulphate at several concentrations, ethanol and acetone were employed as precipitating reagents. Also, an experiment was carried out in which the extract was treated with sodium cholate prior to the precipitation step, in order to prevent interfering interactions between microbial polysaccharides and the target enzymes (Fuciños et al. 2005). The purification levels (defined as the ratio between the specific activity of the precipitate by that of the crude extract) attained are shown in Figure 1. The results obtained by ultrafiltration are also included.

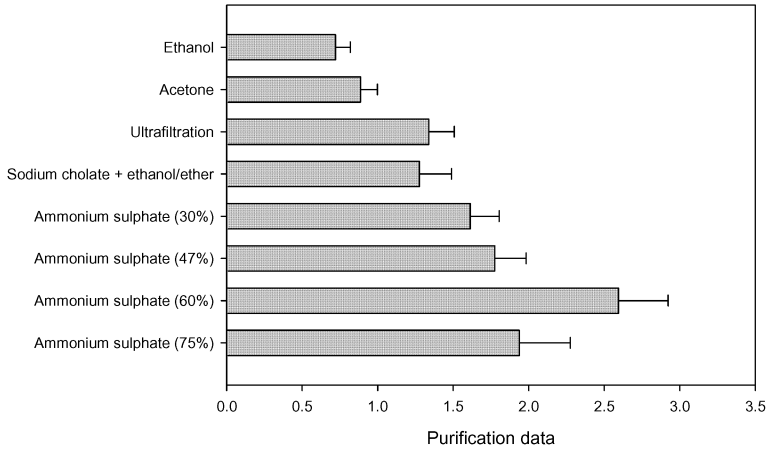

Figure 1 Purification levels obtained with the different concentration methods (experiments by duplicate, analytical determinations by triplicate)

The purification levels using the organic solvents (ethanol or acetone), were very close or inferior to 1, meaning that organic solvents work mainly as concentrators, and don't provide any purification degree, similarly to what happened using ultrafiltration. The use of sodium cholate treatment did not seem to improve the process. On the other hand, ammonium sulphate provides some degree of enrichment of the precipitate in the desired enzyme, giving purification levels superior to 1 for all the concentrations tested. The purification level seems to increase until it reaches a maximum at 60% of ammonium sulphate saturation. With higher amounts (75%) the purification level achieved seems to be lower. This trend maintains for higher concentrations of ammonium sulphate. The results were also confirmed by electrophoresis and zymogram analysis of the precipitates.

3.2 Hydrophobic interaction chromatography

Further purification of the enzyme was attempted using hydrophobic interaction chromatography, a technique that is commonly used in lipase purification sequences (Saxena et al. 2003; Royter et al. 2009). Several HIC resins were screened, in order to establish the best chromatography support and elution conditions.

First, Phenyl Sepharose and Butyl Sepharose High Performance supports were assayed, and elution at decreasing NaCl concentration (3 M to 0 M) was attempted. Elution was

not possible in these conditions, and the columns were rapidly saturated. Addition of Triton X-100 (0 to 1%) to the eluent did not improve the results. Two polarity changing solvents (i.e. ethylene glycol and isopropanol) were also employed, and both gave good results in terms of elution, ethylene glycol providing the best activity recovery.
Butyl Sepharose and Octyl Sepharose 4 Fast Flow were also employed as chromatographic supports, and elution was assayed with Triton X-100 and sodium cholate (0 to 1% in both cases) in the former, and isopropanol (0 to 30%) and ethylene glycol (0 to 50%) in the latter. The best results were achieved with Octyl Sepharose 4 Fast Flow, which showed a somewhat better binding than other supports, and the best elution of activity was possible using a gradient of increasing ethylene glycol concentration. This support was chosen for scaling up of the chromatography, and a 10 mL column was prepared. Extracts were injected corresponding to a total protein of 2-9 mg and total activity of 10-13 units. Elution was performed with a gradient volume of 20 column volumes from 0 to 50% ethylene glycol (in phosphate buffer 50 mM). Eluate fraction collection and lipase activity analysis indicated that a major percentage of the enzyme activity was recovered at 80-130 mL elution volume (Figure 2).

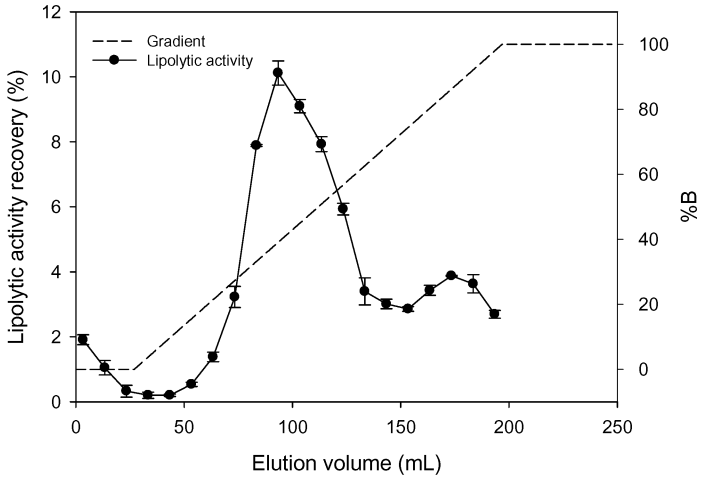

Figure 2 Elution of lipolytic active fractions from an *Octyl Sepharose 4 Fast Flow* column with increasing concentration of 50% ethylene glycol (enzyme activity expressed as percentage with respect to the total amount)

The crude extract and eluted fractions were subjected to electrophoresis and zymogram analysis, and a band with lipolytic activity was detected at around 40 kDa.

3.3 Enzyme identification through mass spectrometry using an ion trap

The band presenting lipolytic activity was excised from the SDS-PAGE gel, and the proteins were extracted from the gel, digested with trypsin and subjected to analysis by LC–MS/MS. The gel band appeared to contain a mix of proteins, since several significant hits were obtained. The highest scores after a search on Mascot were a 35452 Da Bacillus pyruvate dehydrogenase E1, a 66074 Da chaperone protein dnaK, and a 77152 Da staphylococcal lipase. The latter is specially relevant, since the lipolytic activity of the excised band had been unequivocally ascertained.

4. Conclusions

Partial purification and preliminary characterization of the lipase produced by a recently isolated *Bacillus thermoamylovorans* strain has been attempted. Ammonium sulphate precipitation was selected as concentration method, and a hydrophobic interaction chromatography protocol was designed for partial purification of the enzyme. Application of Maldi-TOF techniques on protein bands with lipolytic activity, excised from SDS-PAGE gels, revealed that the enzyme presents homology with staphylococcal lipases.

Acknowledgements

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