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Saccharomyces cerevisiae asparaginase II, a potential antileukemic drug: Purification and characterization of the enzyme expressed in *Pichia pastoris*

Luciana Facchinetti de Castro Girão [a, b], Surza Lucia Gonçalves da Rocha [b], Ricardo Sposina Sobral [a], Ana Paula Dinis Ano Bom [c], André Luiz Franco Sampaio [d], José Godinho da Silva Jr. [c], Maria Antonieta Ferrara [e], Elba Pinto da Silva Bon [a, **], Jonas Perales [b, *]

[a] Federal University of Rio de Janeiro, Institute of Chemistry, Department of Biochemistry, Enzyme Technology Laboratory, 21941-909, Rio de Janeiro, Rio de Janeiro, Brazil
[b] Oswaldo Cruz Foundation, Oswaldo Cruz Institute, Toxinology Laboratory, 21040-900, Rio de Janeiro, Rio de Janeiro, Brazil
[c] Oswaldo Cruz Foundation, Biomanguinhos, Laboratory of Macromolecules, 21040-900, Rio de Janeiro, Rio de Janeiro, Brazil
[d] Oswaldo Cruz Foundation, Farmanguinhos, Institute of Pharmaceutical Technology, 21041-250, Rio de Janeiro, Rio de Janeiro, Brazil
[e] Oswaldo Cruz Foundation, Farmanguinhos, Drugs Technology Laboratory, 21041-250, Rio de Janeiro, Rio de Janeiro, Brazil

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ABSTRACT

Asparaginase obtained from *Escherichia coli* and *Erwinia chrysanthemi* are used to treat acute lymphocytic leukaemia and non-Hodgkin's lymphoma. However, these agents cause severe adverse effects. *Saccharomyces cerevisiae* asparaginase II, encoded by the *ASP3* gene, could be a potential candidate for the formulation of new drugs. This work aimed to purify and characterize the periplasmic asparaginase produced by a recombinant *Pichia pastoris* strain harbouring the *ASP3* gene. The enzyme was purified to homogeneity with an activity recovery of 51.3%. The estimated molecular mass of the enzyme was 136 kDa (under native conditions) and 48.6 kDa and 44.6 kDa (under reducing conditions), suggesting an oligomeric structure. The recombinant asparaginase is apparently non-phosphorylated, and the major difference between the monomers seems to be their degree of glycosylation. The enzyme showed an isoelectric point of 4.5 and maximum activity at 46 °C and pH 7.2, retaining 92% of the activity at 37 °C. Circular dichroism and fluorescence analyses showed that the enzyme structure is predominantly α-helical with the contribution of β-sheet and that it remains stable up to 45 °C and in the pH range of 6–10. *In vitro* tests indicated that the recombinant asparaginase demonstrated antitumoural activity against K562 leukaemic cells.

1. Introduction

Drugs containing the antitumour enzyme L-asparaginase have been used for the treatment of acute lymphocytic leukaemia, chronic lymphocytic leukaemia, non-Hodgkin's lymphoma, lymphosarcoma and melanosarcoma for over 30 years [1–10]. The main enzyme sources for the antitumour drug are the bacteria *Escherichia coli* and *Erwinia chrysanthemi*. However, its use is limited by severe immunological reactions and side effects such as thrombosis, bleeding, immunosuppression and anaphylaxis [11,12].

Considering the drawbacks of the bacterial enzymes and the need for options for patients who have developed hypersensitivity to the bacterial asparaginase, the stable *Saccharomyces cerevisiae* asparaginase II, encoded by the *ASP3* gene, is optimally active at pH and temperature ranges close to the human physiological conditions and could be a potential candidate for enzyme replacement in

* Corresponding author. Toxinology Laboratory, Instituto Oswaldo Cruz, Pavilhão Ozório de Almeida, Sala 5, Fundação Oswaldo Cruz-FIOCRUZ, Avenida Brasil, 4365 – Manguinhos, Rio de Janeiro, Rio de Janeiro 21040-900, Brazil.
** Corresponding author. Universidade Federal do Rio de Janeiro, Instituto de Química, Laboratório de Tecnologia Enzimática, Avenida Athos da Silveira Ramos, 149 – Centro de Tecnologia, Bloco A, Sala 539, Cidade Universitária, Rio de Janeiro, Rio de Janeiro 21941-909, Brazil.
E-mail addresses: elba1996@iq.ufrj.br (E. Pinto da Silva Bon), jperales@ioc.fiocruz.br (J. Perales).

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the formulation of new drugs [13].

To overcome the low asparaginase II production in *S. cerevisiae*, in previous work performed in our laboratory, the *ASP3* gene was successfully expressed in the methylotrophic yeast *Pichia pastoris* under the control of the *AOX1* gene promoter. The enzyme yield per dry cell mass for the recombinant *P. pastoris* strain reached 800 Ug^{-1}, which was 7-fold higher than that obtained for *S. cerevisiae*. Subsequently, high-density cell cultures resulted in a volumetric yield of 85,600 UL^{-1} and global volumetric productivity of 1083 UL^{-1} h^{-1} [14]. The selected process for the enzyme extraction from the yeast periplasm under alkaline conditions in the presence of cysteine resulted in 100% enzyme recovery [15].

The advantages of using *P. pastoris* machinery to express heterologous proteins, in general, are well known [16]. For the production of therapeutic proteins, *P. pastoris* is particularly well suited due to its GRAS (Generally Recognized as Safe) status. Moreover, the glycosylation pattern of the proteins secreted by *P. pastoris* is more similar to that of mammalian proteins than to that of *S. cerevisiae*, suggesting that these molecules may be less antigenic in humans [17].

In the present study, the recombinant asparaginase produced by *P. pastoris* was purified and characterized with respect to molecular mass, isoelectric point, glycosylation, phosphorylation, secondary and tertiary structures and optimum activity pH and temperature. The knowledge of the chemical and physicochemical characteristics of the protein is necessary for the development of an antileukemic biopharmaceutical. A preliminary evaluation of the antileukemic potential of the homogeneous asparaginase preparation was also performed.

2. Material and methods

2.1. Material

Standards (low molecular weight-LMW proteins, gel filtration calibration kit, Peppermint Stick) and chromatographic columns were purchased from GE Healthcare (Piscataway, NJ, USA). Acrylamide/bis-acrylamide, Coomassie Brilliant Blue R-250 and Coomassie G-250 were obtained from Bio-Rad, USA. Bovine serum albumin (BSA) and PNGase F were purchased from Sigma–Aldrich, USA. GelCode Glycoprotein Staining Kit was from Thermo Scientific, USA, and Pro-Q Diamond was from Invitrogen, USA. Endoglycosidase H was from New England Biolabs, UK. All other reagents were of at least analytical grade.

2.2. Microorganism and enzyme production

Asparaginase production was performed using a recombinant strain of *P. pastoris* (GS115) expressing the *S. cerevisiae ASP3* gene as previously described [14] and the extraction of the yeast periplasmic enzyme to obtain the asparaginase crude extract, as already described [15].

2.3. Enzyme purification

Enzyme purification was performed in three sequential steps. First, seven cycles of ultrafiltration at 4500 × g and 4 °C for 7 min using a 50 kDa ultrafiltration membrane (Millipore) were used for enzyme concentration and buffer exchange. The enzyme concentrate was then loaded onto a Superdex 200 (1.0 × 30 cm) column equilibrated with 50 mM sodium phosphate plus 150 mM NaCl, pH 7.0. The fractions with enzymatic activity were pooled and ultrafiltered for buffer exchange as described above.

The pooled active fraction from Superdex 200 was subjected to a third purification step using a Mono-Q column (0.5 × 5 cm) equilibrated with 20 mM Tris–HCl buffer, pH 7.0 and eluted using a linear gradient of 0–1 M NaCl in the same buffer. The fractions with enzymatic activity were pooled and stored at 4 °C for subsequent analysis.

All fractions of the purification steps were verified by SDS-PAGE on 12% acrylamide gels as described by Laemmli [18]. The total protein concentration was determined using the Bradford method [19] with BSA as the standard, and the asparaginase enzymatic activity was measured using the hydroxylamynolysis reaction [15].

2.4. Peptide analysis by mass spectrometry

The SDS-PAGE 12% gel bands were excised, destained and trypsinized as described by Schevchenko [20]. The peptides from the tryptic digest were analysed using a MALDI-TOF/TOF mass spectrometer (ABI 5800 Applied Biosystems) in reflectron mode. The samples were mixed with the matrix (10 mg mL^{-1} α-cyano-4-hydroxycinnamic acid in 50% acetonitrile with 0.3% trifluoroacetic acid), 1:1 (v/v). The mixtures were applied individually on the MALDI plate (1 μL) and dried at room temperature. The MALDI plate was calibrated with a mixture of the following peptides: arg-bradykinin (*m/z* 904.46), angiotensin I (*m/z* 1296.68), GLU1-fibrinopeptide B (*m/z* 1570.67), ACTH (1–17) (*m/z* 2093.08) and ACTH (18–39) (*m/z* 2465.19). In the MS/MS mode, the five most abundant ions were selected (timed ion selector) after fragmentation by CID (collision-induced dissociation). The masses of the generated fragments (MS/MS) were subjected to a search of the non-redundant (nr) database of the NCBI (National Center for Biotechnology Information) using the MASCOT (Matrix Science, London, England) program. The search allowed the following parameters: two missed trypsin cleavages, ± 0.8 Da error in MS mode, ± 0.6 Da error in MS/MS mode and variable changes in relation to carbamidomethylation (Cys), oxidation (Met) and pyro-Glu (N-terminal Glu). The specification of the instrument was "MALDI-TOF/TOF" to search using the MASCOT program. The results were manually checked.

2.5. Enzyme characterization

2.5.1. Molecular weight

The molecular weight was estimated using two different methods: SDS-PAGE 12% in denaturing conditions as described by Laemmli [18] using LMW standards and molecular exclusion chromatography using a Sephacryl S-200 column (1.6 × 60 cm). The column was previously equilibrated with a 50 mM sodium phosphate buffer, pH 7.0 plus 150 mM NaCl and calibrated with bovine serum albumin, chymotrypsinogen, egg albumin, ribonuclease, aldolase and blue dextran.

2.5.2. Isoelectric point

Three micrograms of a selected fraction of the ion exchange chromatography (FS-TI) were resuspended and solubilized in 125 μl of a rehydration solution containing 8 M urea, 2% CHAPS, 0.002% bromophenol blue, 1% ampholytes and 0.1 M DTT for 30 min. This solution was applied to a 7 cm isoelectric focusing strip (GE Healthcare, USA) with a linear pH gradient ranging from 3 to 10. The electrical conditions used were 30 V/12 h, 200 V/1 h, 500 V/1 h, 1000 V/1 h, 3500 V/30 min and up to 14,000 V/h. The strip was subsequently incubated for 15 min with stirring in 10 mL equilibration solution (1.5 M Tris–HCl pH 8.8, 6 M urea, 30% glycerol (v/v) 2% SDS (w/v), bromophenol blue 0.002% (w/v) containing DTT (10 mg/mL) and then incubated for 15 min with stirring, in 10 mL of another equilibrium solution containing iodoacetamide (25 mg mL^{-1}). The strip was then positioned on a 12% polyacrylamide gel with SDS, and the system was sealed with agarose