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Article

Two-Step Purification of L-Asparaginase from Acrylaway® L

Louise Lacalendola Tundisi[1], Diego de Freitas Coêlho[2], Alessandra Valéria de Sousa Faria[3], Adalberto Pessoa Junior[4], Elias Basile Tambourgi[2], Laura de Oliveira Nascimento[5], Edgar Silveira[6], Priscila Gava Mazzola[5]

[1]Graduate Program in Biosciences and Technology of Bioactive Products, Institute of Biology, University of Campinas, Campinas, Brazil, [2]Chemical Engineering School, University of Campinas, Campinas, Brazil, [3]Department of Biochemistry and Tissue Biology – Institute of Biology, University of Campinas, Brazil, [4]Pharmaceutical Sciences School, University of São Paulo, São Paulo, Brazil, [5]Pharmaceutical Sciences School, University of Campinas, Campinas, Brazil, [6]Genetics and Biochemistry Institute, Federal University of Uberlândia, Uberlândia, Brazil

L-Asparaginase (L-ASNase) is a biopharmaceutical used for acute lymphoblastic leukaemia (ALL) treatment, dramatically increasing the patients' chance of cure. However, its production and distribution in developing countries were disrupted because of its low profitability, which caused great concern among patients. This study evaluates the feasibility of combining fractional precipitation and aqueous two-phase systems (ATPS) to purify L-ASNase from a low-grade product, commercially known as Acrylaway® L. The ATPS purification results were not particularly expressive compared to the two-step purification process composed of ethanol precipitation and gel filtration, which was able to recover the target molecule with a purification factor over 5 fold. Thus, we studied a purification process capable of manufacturing pharmaceutical grade L-ASNase from a commercially available low-grade raw material; however, improvements regarding its throughput must be achieved, and high purity is the first step to apply it as a new biopharmaceutical product. The proposed process could pose as a short-time solution to mitigate its shortage while a cost-effective production plant is being developed.

Keywords: Aqueous two-phases system. L-Asparaginase. Downstream processes. Bioprocessing. Fractional precipitation.

INTRODUCTION

L-Asparaginase (L-ASNase) is an enzyme used for both cancer treatment and food manufacturing (Tundisi et al., 2017). As a drug, it is used as an antineoplastic agent in multidrug chemotherapeutic regimens against acute lymphoblastic leukaemia (ALL), lymph sarcoma, and many other malignancies of the lymphoid system (Shanmugaprakash et al., 2015; Lopes et al., 2017, Santos et al., 2017). This enzyme catalyses the hydrolysis of extracellular asparagine, which produces L-aspartate and ammonia. As many malignant cells do not have the ability to synthesize L-ASNase, the rapid depletion of this amino acid damages cell functions by blocking protein synthesis, finally leading to apoptosis (Bussolati et al., 1995).

ALL is the most common leukaemia among paediatric patients (Asselin, Gaynon, Whitlock, 2013). The survival rate of patients with ALL increases up to 90% with a multidrug treatment, including L-ASNase (Kamps et al., 2009; Tundisi et al., 2017). L-ASNase is accepted by the Brazilian Ministry

***Correspondence:** D. de F. Coêlho. Faculdade de Engenharia Química. Departamento de Engenharia de Sistemas Químicos. Universidade Estadual de Campinas. Av. Albert Einstein, 500, Cidade Universitária. CEP.: 13083-852, Campinas, SP, Brazil. Phone: +55 19 3788 3952. Fax: +55 19 3788 3894. Email: diegofcoelho@gmail.com. ORCID: https://orcid.org/0000-0002-2123-5891. Louise Lacalendola Tundisi: ORCID: https://orcid.org/0000-0002-3374-6103. Alessandra Valéria de Sousa Faria: ORCID: https://orcid.org/0000-0003-1161-5724. Adalberto Pessoa Junior.: ORCID: https://orcid.org/0000-0002-5268-8690. Elias Basile Tambourgi: ORCID: https://orcid.org/0000-0003-3275-0810. Laura de Oliveira Nascimento: ORCID: https://orcid.org/0000-0002-4741-8493. Edgar Silveira: ORCID: https://orcid.org/0000-0001-7249-639X. Priscila Gava Mazzola**:** ORCID: https://orcid.org/0000-0002-3795-8189

of Health as gold standard treatment for ALL, and it is sold commercially as Elspar® (Merck, *Escherichia coli*) or Erwinase® (Speywood, *Erwinia chrysanthemi*) (Singh et al., 2013; Kumar et al., 2014; INCA, 2016).

However, the continuity of the supply of oncology drugs has been a cause of great concern since 2011, when the distribution of an important fraction of these drugs was interrupted. Since then, L-ASNase national production and distribution has become completely dependent on importation (Anvisa, 2017), causing great concern throughout the system of onco-hematologic patient care. The following years saw the surfacing of several research projects targeting the study and development of biotechnological processes, from traditional microbiological screening of suitable organisms (Erva et al., 2017; Sanjay et al., 2017) to genetic engineering well-known species (Chityala et al., 2015; Vidya et al., 2017).

On the other hand, high-purity L-ASNase could be obtained by its recovery and purification from commercially available low-grade sources. Industrially known as Acrylaway® L, L-ASNase is used to reduce the acrylamide content of baked starchy foods (Xu, Oruna-Concha, Elmore, 2016) because of their suspected carcinogenic effect. Among other suspected carcinogens, acrylamide and heterocyclic amines are products of the Maillard reaction (Rannou et al., 2016), the latter being responsible for the brown and crusty aspects, with distinctive flavour, characteristic of baked or fried food. The addition of L-ASNase prior to thermal processing converts (up to 90%) asparagine into aspartic acid and ammonium, dramatically reducing acrylamide formation (Xu, Oruna-Concha, Elmore, 2016).

In any case, a complex multistep purification process is often required for the complete removal of contaminants, and subsequently to achieve the standard of purity for human use, usually up to 99.98% (Mazzola et al., 2008; Molino et al., 2013; Tundisi et al., 2017). Ideally, a feasible process would employ high-efficiency techniques to obtain L-ASNase at the required purity while using the smallest number of purification steps. It is important to highlight that achieving a required purity level is just one of the steps in the process to use this L-ASNase source as a new biopharmaceutical product.

A straightforward approach is to recover the target-molecule from its crude extract using a high-throughput method, which usually lacks in selectivity but concentrates the raw material several fold. Among other methods suitable for the high volume processing (Coelho et al., 2013), fractional precipitation has proved to be able to selectively purify compounds based on their molecular mass and hydrophobicity (Cortez, Pessoa Jr, 1999).

Ethanol is one of the precipitation agents used for fractional precipitation because of its low dielectric constants (compared to water), which destroy the hydrophobic hydration layer of hydrophobic regions and outset to enclose such regions due to their greater affinity with the solvent. This allows interaction between surface regions with either negative or positive charges, leading to precipitation by aggregation (Scopes, 1994). The physicochemical properties of ethanol, such as its complete miscibility with water, ability to decrease the freezing-point of water mixtures, chemical inertness, and low toxicity, creates a wide range of potential applications and makes it one of the most important organic solvents (Cortez, Pessoa, Assis, 1998). As the second largest producer of bioethanol worldwide (Proskurina et al., 2018), Brazil offers a favourable scenario to develop a suitable low-cost bioprocess based on a fractional precipitation using it as precipitant agent. The obtained precipitate, partially free of impurities and concentrated, can then be purified by selective separation methods based on physicochemical differences between the target molecule and other compounds present in the extract (Carpio, Simone de Souza, 2017).

Purification of biomolecules by Aqueous Two-Phase Systems (ATPS) offers well-known benefits, such as high interfacial contact area and biocompatibility, and is able to achieve very selective separation by adjusting the physicochemical properties of the phases (Coelho et al., 2013). This method is based on the observations made by Albertsson (1977), which found that an aqueous mixture containing salt and a soluble polymer, above certain component's concentration, would spontaneously separate into two immiscible phases. The target molecule will partition preferably to one of the phases according to its affinity, yielding a process with less purification steps, at a lower cost (Lopes et al., 2018) and shorter processing time than conventional purification techniques, such as affinity chromatography and size-exclusion or ion exchange chromatography (Ketnawa, Rungraeng, Rawdkuen, 2017).

ATPS has been used in the purification of several biomolecules, including proteins and enzymes (Cabrera-Padilla et al., 2013; Kalaivani, Regupathi, 2013), viruses (Nazer, Dehghani, Goliaei, 2017), plasmid DNA (Gomes et al., 2009), and monoclonal antibodies (Campos-Pinto et al., 2017), from plant and animal cells, as well as from several microorganisms.

Considering the above, developing new biotechnological processes to produce L-ASNase and purifying the produced enzyme are steps of utmost importance for the Brazilian scenario. Thus, L-ASNase purification from a commercially available low-grade enzyme was studied using fractional precipitation, followed by an ATPS purification step or by gel filtration. The recovered gel filtration fraction was analysed by electrophoresis and the enzyme activity was characterized. Results show that fractional precipitation was able to purify the target molecule and can be used as an alternative to obtain medical-grade L-Asparaginase from a commercially available low-purity lysate. If implemented, this process would have the advantage to drastically cut down the L-ASNase time-to-market preparation, favouring the scenario for acute lymphoblastic leukaemia patients.

MATERIAL AND METHODS

Reagents and chemicals

L-ASNase from *A. oryzae* (Acrylaway® L, composed by 4% asparaginase, 50% glycerol, 46% water) was donated by Novozymes (Araucária, PR, Brazil). Standard L-ASNase from *Escherichia coli* (E.C. 3.5.1.1) and Bicinchoninic Acid (BCA) were purchased from Sigma-Aldrich (St. Louis, USA). All other reagents were of analytical grade. All experiments were performed at least in triplicate, and the data presented is the average of these independent measurements.

Protein content and L-ASNase activity analysis

Protein concentration was determined by ultraviolet absorption at 280 nm (Thermo Scientific – Multiskan GO). The calibration curve was obtained using a stock solution of bovine serum albumin (1 mg/mL).

L-ASNase activity was determined by the quantification of ammonia produced during L-Asparagine hydrolysis. In general, 50 µL of L-Asparagine solution and 50 µL of enzyme solution were mixed in 1.05 mL of Tris HCl buffer (50mM, pH 8.6). The reaction was conducted at 37 °C for 30 min, and then 50 µL of 1.5 M trichloroacetic acid was added to stop the reaction. Reaction mixture supernatant (200 µL) was added to 4.3 mL of deionized water, and 500 µL of Nessler's reagent was added to measure released ammonia.

Analyses were performed at 436 nm in a spectrophotometer (Micronal AJX-1900, Campinas, São Paulo). One unit (U) of L-ASNase activity was defined as the amount of enzyme required to release 1.0 µmol of ammonia per minute at pH 8.6 at 37 °C.

L-ASNase ethanol fractional precipitation

Fractional precipitation was performed by mixing 1.0 mL of enzyme extract to different volumes of ethanol to achieve the desired ethanol concentration (0-80%, v/v). The ethanol-extract mixture was kept at 0°C and under constant agitation for 10 min, when it was centrifuged at 2,000g for 30 min at 4 °C. Ethanol was added in a step-wise manner to the extract and samples from the supernatant were collected in-between precipitation steps for analysis. A larger extract sample (100mL) was used in a subsequent precipitation, which used the optimal ethanol concentration found by the fractional precipitation. As previously described, the L-ASNase precipitate was centrifuged at 2,000g for 30 min at 4 °C. All assays were performed in triplicate. Precipitation resulted in a glycerol-free L-ASNase, used in the enzymatic and ATPS assays.

GEL FILTRATION CHROMATOGRAPHY

L-ASNase was further purified by gel filtration chromatography (GFC) using Sephadex G-75 as stationary phase (60 cm height), pre-equilibrated with Tris HCl buffer (0.1 M, pH 8.6). Proteins were eluted at 0.5 mL/min with the same buffer. The apparent molecular mass of purified L-ASNase fraction was estimated by 10% SDS-PAGE according to Laemmli (1970). Proteins

were stained with Coomassie Brilliant Blue R-250; molar mass range was from 10 kDa to 250 kDa.

Effects of pH and Temperature on Enzymatic Activity

Optimum pH was determined by assessing L-ASNase activity at different pHs at 37ºC. Four buffer systems, including citrate-phosphate buffer (50 mM, pH 2.6–5.8), phosphate buffer (50 mM, pH 5.8–7.4), Tris HCl buffer (50 mM, pH 7.4–9.0), and carbonate buffer (50 mM, pH 9.8–10.6), were used for evaluating optimum pH of enzyme activity.

Optimum temperature for enzyme activity was determined in optimum pH conditions (Tris HCl buffer 50 mM, pH 7.4; phosphate buffer 50 mM, pH 7.4) at different temperatures ranging between 5–55 ºC.

Both pH and temperature analysis were performed to observe possible differences between the standard L-ASNase and the fraction purified by fractional precipitation and GFC. These purification steps were employed prior to characterization to avoid interference by any undesired species.

Enzymatic kinetics

L-ASNase kinetic parameters were performed incubating the enzyme in various substrate concentrations (ranging from 0.945 mM to 18.9 mM) in 50 mM phosphate buffer or Tris HCl buffer, pH 7.4. Michaelis-Menten constant (K_m), and maximum velocity (V_{max}) were determined by fitting Hill equation:

$$v = V_{max} \frac{[S]^n}{Km^n + [S]^n} \quad (1)$$

where *[S]* is substrate concentration and *n* is Hill coefficient. All statistical analyses and mathematical fitting were performed using Origin 8.0 (Origin Lab, USA).

Preparation of ATPS

Polyethylene glycol (PEG) solutions with different molecular weights, specifically 4000 and 6000 g/mol, were dissolved [50% (w/w)] in distilled water and transferred to 15-mL graduated tubes with Na_2SO_4. L-ASNase solution was added corresponding to 10% (w/w) of the total mass of the system, and water was added to reach 5-g system. The total L-ASNase activity added initially to the system was 766.7539 U, which was used to calculate the purification yield using Equation 3. After mixing for 15s, phases were separated by centrifugation at 2,000g for 10 min at 4 °C. As Acrylaway® L is a glycerol-enriched suspension of L-ASNase, the solids obtained as optimal fraction in the fractional precipitation step were resuspended in an equal volume of phosphate buffer (pH 7.4, 50mM) and used as L-ASNase crude extract. The volume of each phase was measured, followed by the determination of protein concentration and L-ASNase activity. To avoid PEG interference, dichloromethane was used to extract PEG from each phase prior to any activity or protein analysis.

Experimental design

A 2^2 factorial design with a central point was used to evaluate the influence of two independent variables, namely PEG concentration (X_1) and Na_2SO_4 concentration (X_2). The partition coefficient, activity yield, and purification factor (PF) of L-ASNase were used as outputs. Experimental results were analysed with Design Expert (©Stat-Ease, Inc. 2017, Minneapolis, MN, United States).

Determination of partition coefficient, yield, and purification factor

L-ASNase partition coefficient (K) was determined as the logarithmic ratio between L-ASNase activity in top (A_t) and bottom (A_b) phases, as shown in Equation 2. The logarithmic function was applied to allow fast phase partitioning analysis, where a negative value implies that enzyme was partitioned to the bottom phase, while a positive value indicates otherwise.

$$K = log\left(\frac{At}{Ab}\right) \quad (2)$$

Activity yield (Y) was defined as the ratio between total activity in the bottom phase (T_b) and total initial activity in L-ASNase preparation (T_i) and expressed as a percentage. The total activity is calculated by multiplying the activity of a given sample per its volume, as shown in Equation 3:

$$Y(\%) = \frac{T_b}{T_i} = (A_b \cdot V_b) \cdot (A_i \cdot V_i)^{-1} \cdot 100\% \quad (3)$$

Purification factor (PF) was given as the ratio between specific activity in bottom phase and initial specific activity in L-ASNase preparation prior to the partition, as presented in Equation 4, and specific activity was obtained by dividing enzymatic activity by protein concentration.

$$PF = \left(\frac{A_b}{C_b}\right) \cdot \left(\frac{A_i}{C_i}\right)^{-1} \quad (4)$$

where A_b and A_i are enzymatic activity, C_b and C_i are protein concentrations, expressed in mg/mL, in the bottom phase and in L-ASNase preparation, respectively.

RESULTS

Ethanol purification and gel filtration chromatography

Croisfelt et al. (2015) stated that it was possible to purify enzymes (i.e., bromelain) by ethanol precipitation, in a two-step process. In these findings, the enzyme was recovered after 30% ethanol concentration. L-ASNase starts to precipitate near 40% (w/w) ethanol (Figure 1). While the total protein concentration of the supernatant reached undetectable levels at 80% (w/w) ethanol, the highest L-ASNase activity was observed at 60% (w/w) ethanol. Thus, undesired proteins were removed by performing a precipitation at 40% ethanol. Subsequently, the L-ASNase fraction was recovered as precipitate at 60% of ethanol and the supernatant was discarded.

Mohan Kumar, Manonmani (2013) studied several organic solvents as precipitant agents for L-ASNase purification and concluded that methanol was the best solvent for the precipitation. For this study, ethanol was chosen as the precipitant agent because of its suitability for the intended purpose, considering cost and eco-friendly features (Lopes et al., 2017).

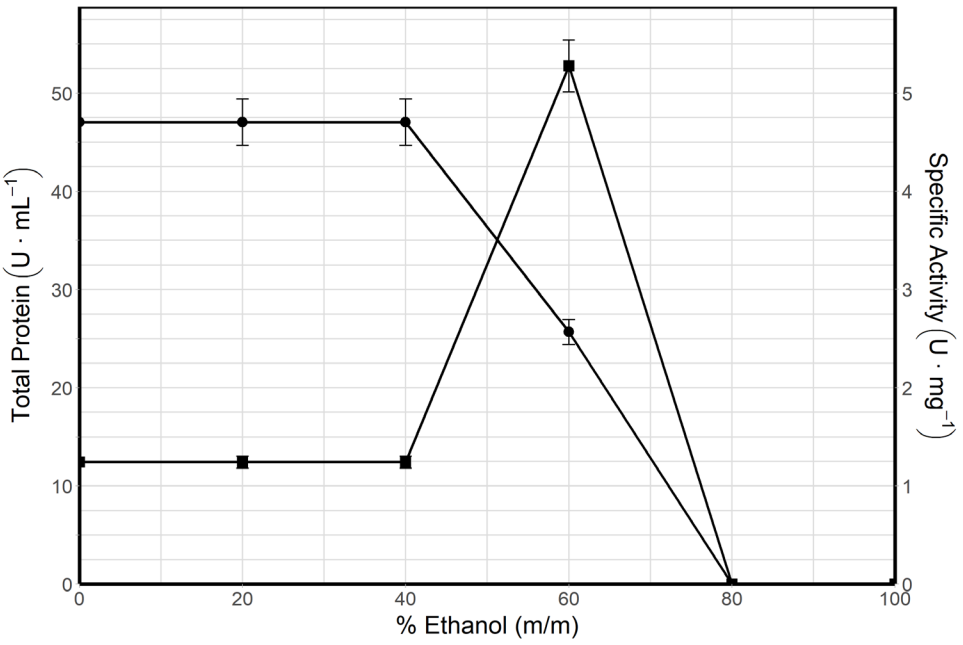

Figure 1 - L-Asparaginase's (L-ASNase) ethanol precipitation profile. The plot presents the amount of total protein (●) and L-ASNase specific activity (■) at the supernatant, respectively.

A specific activity peak observed in the range between 40-60% of ethanol implies that most of the target molecule (or a smaller fraction of contaminants) precipitated, resulting in a purified L-ASNase fraction. Fractional precipitation is an often performed preliminary step for bulky processing, since it is capable of recovering target molecules from highly complex mixtures (Coelho et al., 2013). Thus, the precipitated L-ASNase fraction was resuspended in phosphate buffer 50 mM pH 7.4 and used subsequently in the chromatographic, characterization, and ATPS steps.

Figure 2 presents results of the chromatographic step of L-ASNase purification. The elution profile of gel filtration presented four peaks, with L-ASNase activity spread between peaks two and three and reaching its maximum on the latter, where it achieved a purification factor of 2.38-fold. Although the peaks overlapped, the third peak stood out in both absorbance and L-ASNase activity. The overlapping peaks observed while using Sephadex G-75 are a result of close similarities between the species present in the purified extract and is consistent with the purification capability of precipitation methods (Lee, Kim, 2017; Gong et al., 2018).

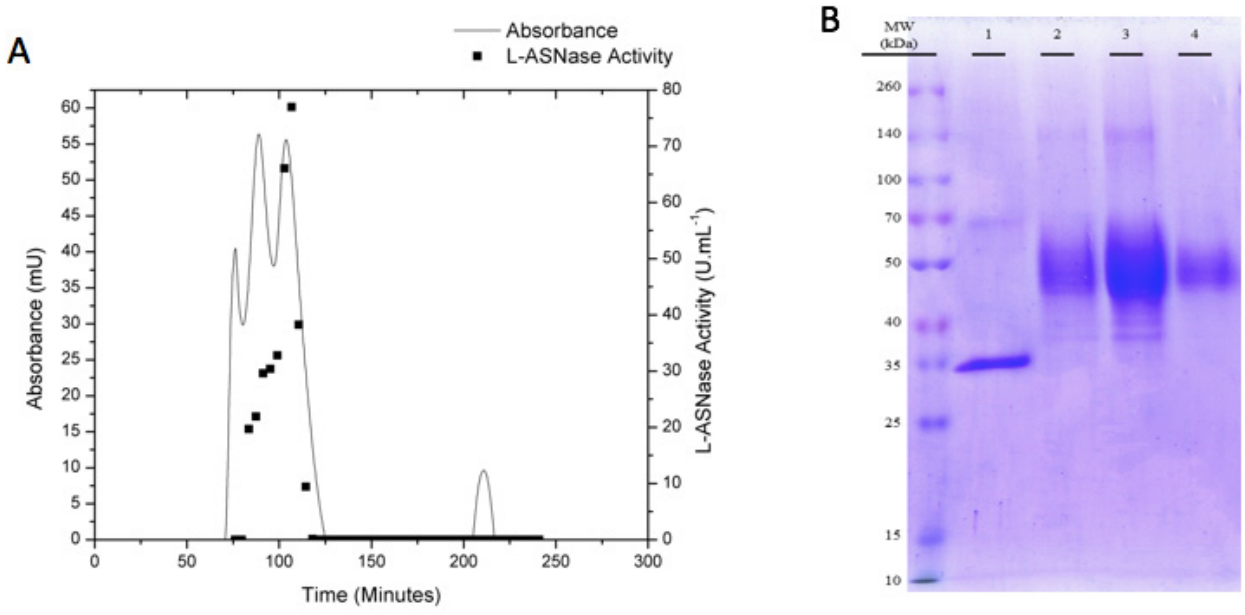

Figure 2 - (A) Gel filtration chromatography elution profile. (B) SDS-PAGE of purified L-Asparaginase (LASNase), from left to right: molecular markers; Lane 1 - standard L-ASNase from E. coli; Lane 2 - crude broth from A. oryzae; Lane 3 - ethanol precipitated L-ASNase from A. oryzae; Lane 4 - gel filtration chromatography purified L-ASNase from A. oryzae.

Table I shows the purification summary of L-ASNase. The enzyme was partially purified by ethanol precipitation with 106.81% recovery of its initial activity, and a purification factor of 2.32-fold, and further purified to an overall purification factor of 5.52-fold.

TABLE I - Summary of steps used at ethanol precipitation of L-ASNase

Steps	L-ASNase Activity (U/mL)	Protein Concentration (mg/mL)	Specific Activity (U/mg)	Purification Fold	Recovery (%)
Crude Extract	2,748.05	47.04	58.42	n.a.	100.00
0-60%	2,935.06	21.67	135.45	2.32	106.81
60-80%	629.11	25.67	24.51	0.42	22.89
GF	192.32	0.597	322.02	5.51	6.99

* n.a. non-applicable; GF gel filtration.

L-ASNase was previously purified by ammonium sulphate fractional precipitation at 60-80% saturation by Kumar, Venkata Dasu, Pakshirajan (2011)¢, however, their results presented almost 30% loss of the initial L-ASNase activity during the process. Singh et al. (2013) described an ammonium fractional precipitation at 30-80%, with an enzymatic activity recovery increase; however, it could not reach over 75% recovery. Mohamed et al. (2015) also achieved a 2-fold purification factor using a chromatographic step, i.e., DEAE-Sepharose. Mohan Kumar, Manonmani (2013) achieved a higher purification factor using chromatographic steps, but only 84 $U.mg^{-1}$ of specific activity was observed. Thus, our results showed a higher initial activity recovery and overall purification factor than the previously reported studies.

Enzyme characterization

Figure 3a shows the effect of pH on the enzyme activity. It is newsworthy that no L-ASNase activity was detected at high acidic pH (below pH 4), and its activity became detectable when pH 5.0 was reached. L-ASNase from *A. oryzae* showed an optimum pH range from pH 5.8 to 9.0, with a maximum, represented as 100%, at pH 7.4., which confirms similar results (Singh et al., 2013). Zuo et al. (2014) found that their recombinant L-ASNase from *Thermococcus gammatolerans* had an optimum pH of 8.5 at 50 ºC Tris HCl. Differences between the latter and this study (higher activity and different temperature) were bound to be observed, since enzyme origin may significantly alter its activity and optimum conditions.

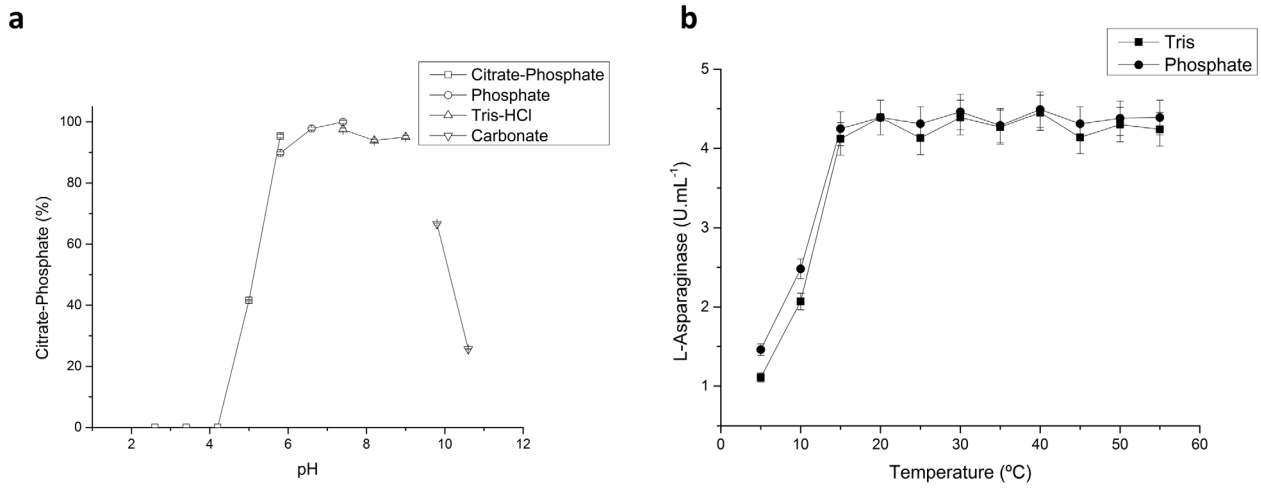

Figure 3 - Effect of pH and temperature in L-Asparaginase activity. Representing the effects of the a) pH using different buffers to overlap pH ranges and b) temperature Tris-HCl buffer (■) and Phosphate buffer (●).

L-ASNase from *A. oryzae* exhibited activity between 5 ºC and 55 ºC, and optimum activity after 15 ºC. Wild L-ASNase displayed a low thermic tolerance (Vidya, Ushasree, Pandey, 2014), while L-ASNase from *Bacillus aryahattai* had an optimum temperature at 40 ºC, and enzyme activity declined substantially after that threshold (Singh et al., 2013).

The kinetic properties (Table II) and profiles (Figure 4) of L-ASNase from *A. oryzae* were assessed at pH 7.4 and 37 ºC, using L-Asparagine as substrate in phosphate (Figure 4.A) or Tris HCl buffers (Figure 4.B). The non-linear regression of data allowed us to obtain the parameters of the Michaelis-Menten's equation, as follows: V_{max} of 3.43 and 3.85, K_m of 3.55 mM and 4.04 mM, and n of 3.01 and 3.05, corresponding to the kinetic data in Tris HCl and phosphate buffers, respectively.

TABLE II - Hill's equation kinetic parameters for the *Aspergillus oryzae* L-Asparaginase in different buffers obtained through non-linear fitting

Buffer	V_{max}	K_m **(mM)**	**n**	$\mathbf{R^2}$
Tris-HCl, pH 7.4	3.43	3.55	3.01	0.99
Phosphate, pH 7.4	3.85	4.04	3.05	0.99

L-ASNase from *Bacillus coagulans* (Law, Wriston Jr, 1971), *Arabidopsis thaliana* (Gabriel, Telmer, Marsolais, 2012), *Pyrococcus furiosus* (Bansal et al., 2012), and *Escherichia coli* (Derst, Henseling, Röhm, 2000) presented similar results to the *A. oryzae* L-ASNase found in this study. These results are also in accordance with mesophilic L-ASNase, where K_m values were around 5.3 mM and 12 mM (Li et al., 2007; Bansal et al., 2012).

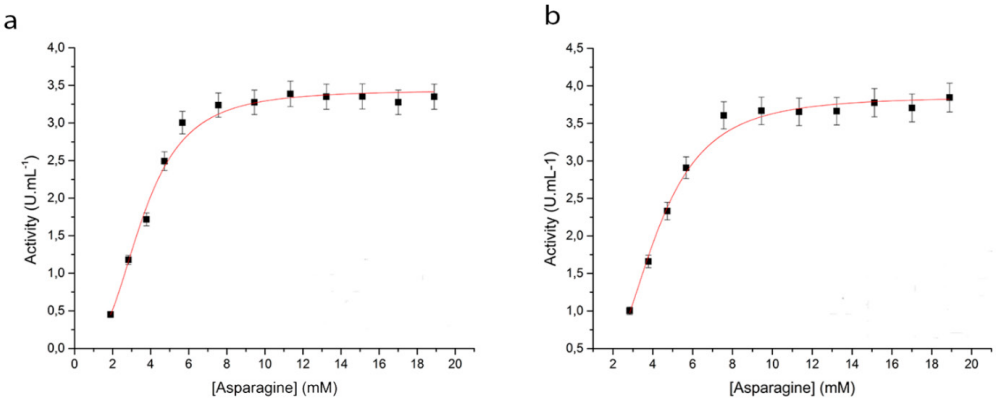

Figure 4 - L-Asparaginase kinetics using Hill's equation fitting on (a) Tris-HCl buffer, and (b) Phosphate buffer.

Aqueous two-phase systems partitioning

The L-ASNase produced by *Aspergillus oryzae* showed a stronger affinity for the bottom salt-rich phase, where the highest level of L-Asparagine hydrolysis was observed (Table III), resulting in a near zero (below 10 U/mL) enzymatic activity on its upper phase. Therefore, data and analysis regarding the upper phase were intentionally suppressed.

It is worth highlighting that a study using a design of experiment matrix was chosen because of its exploratory nature and, although the model was not statistically significant, it is suitable for prospecting promising tie-lines. Once global compositions capable of feasible purification are identified, an in-depth study can evaluate the effect of tie-line length and phases ratio on L-ASNase purification.

TABLE III - Results of Protein Concentration, Activity (A_B), Specific Activity (SA_B), Purification Factor (P_F), Purification Yield (Y_E^B and Percentage of Interfacial Precipitated Protein (I_{PPT}) obtained from the analysis of the bottom phase after L-asparaginase partitioning in both PEG4000 and PEG6000

	%PEG (w/w)	%Na_2SO_4 (w/w)	Protein Concentration (µg/mL)		A_B (U/mL)	SA_B (U/µg)	P_F	Y_E^B	I_{PPT}
			BDE	ADE					
PEG 4000	15	8	1.92	1.25	234.65	188.02	1.23	46.66%	35.57%
	20	8	1.69	1.69	333.08	196.73	1.28	63.30%	27.30%
	15	13	1.03	0.93	199.45	214.49	1.40	34.77%	43.14%
	20	13	0.42	024	48.78	204.27	1.33	8.12%	59.89%
	17.5	10.5	1.28	1.04	202.50	194.97	1.27	38.83%	49.39%
	17.5	10.5	1.27	1.10	215.97	196.59	1.28	42.94%	42.74%
	17.5	10.5	1.32	1.23	268.78	217.67	1.42	46.17%	37.69%
PEG 6000	15	8	1.62	1.38	295.00	213.45	1.39	42.28%	45.74%
	20	8	1.85	1.85	245.79	132.86	0.87	75.46%	0.00%
	15	13	1.41	0.97	245.79	253.26	1.65	32.99%	42.57%
	20	13	0.70	0.49	277.04	560.93	3.65	11.75%	56.30%
	17.5	10.5	1.33	1.24	236.09	190.73	1.24	52.59%	36.24%
	17.5	10.5	1.44	1.34	101.95	76.12	0.50	54.63%	30.38%
	17.5	10.5	1.52	1.39	240.04	172.41	1.12	42.59%	37.17%

* BDE and ADE are respectively protein concentration before and after PEG extraction using dichloromethane.

To prevent PEG interference on the Nessler's enzymatic assay (Ingham, Ling, 1978), dichloromethane (CH_2Cl_2) was used to extract PEG traces from both ATPS phases prior to L-ASNase activity analysis. Protein precipitation was not observed during PEG extraction using dichloromethane. All shown results refer to analyses post PEG extraction. Protein concentration also decreased significantly after PEG removal, as shown in Table III, and was a result of precipitation at the interface between the ATPS phases. Protein precipitation in ATPS is not only often reported, but also well documented and known to be used as a tool to increase the target molecule purification factor by selectively precipitating contaminants (Coelho et al., 2013). Since sample collection at the interface with no contaminants from either phase could not be realistically performed, both protein concentration and biological activity were not analysed.

While not particularly expressive in terms of yield (31.91%), the best purification factor result (P_F = 3.65)) was obtained for an ATPS composed by 20% PEG 6000 and

13% Na_2SO_4, thus making these techniques a promising alternative to prospect L-ASNase purification. The obtained results are consistent with the purification of other enzymes purified using ATPS (Nascimento et al., 2016; de Albuquerque Wanderley et al., 2017), but clearly present an inferior performance compared to those provided by fractional precipitation and gel filtration.

As sulphate salts have the ability to promote hydrophobic interactions between the target molecule and contaminant proteins (Yue, Yuan, Wang, 2007), one would expect that L-ASNase would preferentially partition to the PEG-rich upper phase. Instead, L-Asparaginase partitioned towards the salt-rich bottom phase, which can be a result of PEG's high molecular weight. An increase in PEG fraction in a given phase leads to an exclude-volume effect, which partially overcomes its hydrophobic interaction with compatible protein patches and subsequently drives them to the opposite phase (Omidinia et al., 2010; Shkel, Knowles, Record, 2015). Partitioning also depends on biomolecule properties; for example, Cisneros et al. (2004) showed higher recovery of lutein at the top phase using PEG 8000 22.9% (w/w).

CONCLUSION

Obtaining a biopharmaceutical is only possible by associating a series of processes of synthesis and purification to provide the bioactive compounds with the required features. Alternative low-cost processes, such as fractional precipitation, and use of available resources provide the means to produce and supply the market with essential drugs previously considered not profitable. The recovery of *A. oryzae* L-ASNase by aqueous two-phase systems using polyethylene glycol and sodium sulphate proved to be inadequate, since it required a preliminary precipitation step and achieved low yield and resolution. However, the use of ethanol for the purification of bioactive molecules, such as L-Asparaginase, has the potential to decrease costs throughout its recovery from low-grade raw-materials available commercially. Coupled with a non-optimized gel filtration chromatography, this study showed that it was possible to obtain a 5.5-fold purification factor in the purification of L-Asparaginase from commercial low-grade extracts.

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AUTHORS' CONTRIBUTIONS STATEMENT

Edgar Silveira conceived the original idea. Louise L. Tundisi carried out the experiments. Alessandra V. de S. Faria contributed to the analysis of the results. Diego F. Coêlho wrote the manuscript with support from Adalberto Pessoa Junior and Elias B. Tambourgi. All authors discussed the results and contributed to the final manuscript. Laura de O. Nascimento and Priscila G. Mazzola helped supervise the project.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that this research did not involve animal or human participants during its studies.

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