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LOUISE LACALENDOLA TUNDISI

BIOPROCESSOS ALTERNATIVOS DE PURIFICAÇÃO DE L-ASPARAGINASE TERAPÊUTICA PARA LEUCEMIA LINFÓIDE AGUDA (LLA)

THERAPEUTIC L-ASPARAGINASE FOR ACUTE LYMPHOID LEUKEMIA (ALL) PURIFICATION EMPLOYING ALTERNATIVE BIOPROCESS

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Dissertação apresentada ao instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestra em Ciências, na área de Fármacos, Medicamentos e Insumos para Saúde.

Dissertation presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the Master degree in Sciences, in the area of Drugs, Medicine and Health Supplies.

ORIENTADORA: Profª Drª Priscila Gava Mazzola

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“Nobody knows what the future holds on
Said it’s bad enough just getting old”
Vampire Weekend

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RESUMO

A L-asparaginase (L-ASNase) é um biofármaco utilizado principalmente no tratamento da leucemia linfoide aguda (LLA) que atinge principalmente crianças de 3 a 5 anos e adolescentes, aumentando sua sobrevida de 20% para 90%. A obtenção de um biofármaco envolve uma série de processos que vão desde sua produção a sua purificação. A purificação de um biofármaco, como a L-ASNase, geralmente consiste em várias etapas que representam até 80% dos custos totais de produção, e se faz necessário para que haja uma diminuição dos seus efeitos colaterais quando aplicado. Por ser uma etapa de extrema importância, este trabalho teve como objetivo principal extrair e purificar a L-ASNase por processos alternativos, utilizando a precipitação com etanol e sistemas de duas fases aquosas. Para isso, foi realizada a caracterização da L-ASNase de *Aspergillus oryzae* e a avaliação dos fatores que afetam sua recuperação e purificação em sistema aquoso de duas fases. Ambas as etapas estão descritas nos capítulos II e III do presente trabalho. Os resultados obtidos mostram que os processos alternativos podem levar a uma produção com um menor número de etapas e menos onerosa de um medicamento extremamente importante no tratamento de LLA. Com mudanças nas propriedades do sistema, é possível modificar a partição da molécula podendo atingir alto grau de purificação.

Palavras-chave: Bioprocessos; L-asparaginase; Precipitação; Leucemia Linfóide Aguda; Sistema bifásico aquoso

ABSTRACT

L-asparaginase (L-ASNase) is a biopharmaceutical used mainly for acute lymphoblastic leukemia (ALL) treatment, which affects mostly children 3 to 5 years old and adolescents, increasing their survival from 20% to 90%. Obtaining a biopharmaceutical involves a series of processes ranging from its production to its purification. Purification of a biopharmaceutical, such as L-ASNase, generally consists of several steps which account for up to 80% of the total production costs, and is necessary to decrease its side effects when applied. As an extremely important step, the main objective of this work was to extract and purify the L-ASNase by alternative processes, using ethanol precipitation and two-phase aqueous systems. For this, the characterization of *Aspergillus oryzae* L-ASNase and the evaluation of the factors that affect its recovery and purification in a two-phase aqueous system were performed. Both stages are described in Chapters II and III of this work. The results obtained show that alternative processes can lead to a less steps and cheaper production of an extremely important drug in the treatment of ALL. With changes in the property of the systems, it is possible to modulate a partition of a molecule and being able to reach a high purification degree.

Keywords: Bioprocess; L-Asparaginase; Precipitation; Aqueous two-phase system, Acute Lymphoid Leukemia

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ABREVIATURAS E SIGLAS

L-ASNase – L-asparaginase

L-ASN – L-asparagina

LLA – Leucemia Linfoide Aguda

PEG – Polietilenoglicol

SDFA - Sistemas de Duas Fases Aquosas

SUMÁRIO

1. Introdução

O presente trabalho encontra-se dividido em capítulos redigidos sob forma de artigos, a saber:

- CAPÍTULO I: Revisão da literatura *"L-asparaginase Purification"*
- CAPÍTULO II: "*Characterization of L-asparaginase from Aspergillus oryzae"*
- CAPÍTULO III: "*L-asparaginase in aqueous-two phase system, factors affecting recovery and purification"*

Desta forma, determinadas informações que constam em um capítulo poderão ser repetidas nos capítulos seguintes, assim como na discussão e na conclusão.

2. Justifcativa

No processo de produção de um biofármaco, os padrões de exigência da Agencia Nacional de Vigilância Sanitária, ANVISA, são rigorosos e o tempo de estudos clínicos são maiores, levando ao um custo mais elevado de produção, quando comparado a outros fármacos de origem sintética. Por outro lado, uma das mais promissoras frentes de inovação tecnológica com grande potencial de impulsionar a produção industrial brasileira é a biotecnologia, pois além de sermos consumidores de biofármacos, há forte tendência mundial em buscar rotas alternativas usando processos biotecnológicos para produção de princípios ativos farmacêuticos.

A produção de biofármacos no Brasil é ainda inexpressiva se comparada com os Estados Unidos da América (EUA) e Europa. O Brasil se mostra atrasado em mais de 30 anos em um mercado que move US$ 160 bilhões por ano no mundo e cresce 12% ao ano (Neto, 2016). De acordo com um estudo feito pelo Grupo FarmaBrasil, os biofármacos são responsáveis por um pouco menos do que um terço dos US$ 6,6 bilhões de déficit na balança comercial de medicamentos de 2015 (CRF, 2016). Segundo Souza (2012), a continuidade do fornecimento de medicamentos usados em oncologia, em especial onco-hematologia, tem sido causa de grande preocupação desde o ano de 2011, pois a oferta de muitos fármacos foi interrompida, causando grande preocupação em todo o sistema de cuidados do paciente onco-hematológico. A L-ASNase é um dos medicamentos cuja produção foi diminuída pelo distribuídos de biofármacos para o Brasil.

A L-ASNase é tratamento padrão-ouro para a leucemia linfoide aguda, doença maligna proliferativa do sistema hematopoiético da linhagem linfoide (Avramis & Tiwari, 2006; INCA, 2016; Kuldeep Kumar, Jagjeet Kaur, Shefali Walia, Teena Pathak, & Diwakar Aggarwal, 2014; Pui & Evans, 2006; Singh, Gundampati, Jagannadham, & Srivastava, 2013; Verma, Kumar, Kaur, & Anand, 2007). A interrupção do fornecimento destes fármacos reduziria, anualmente, as chances de cura de aproximadamente 5 mil crianças e adolescentes que, utilizando integralmente os protocolos atuais, é de 80%. A LLA nas crianças evolui rapidamente, de forma que a interrupção ou adiamento de qualquer etapa da terapia implica prejuízo irremediável no prognóstico que atualmente é promissor.

O gargalo da produção de um produto biotecnólogico é o processo *downstream,* por ser a parte mais onerosa do processo. O processo *Downstream* é definido como uma série de etapas que resulta em uma biomolécula purificada como produto. Desenvolver técnicas eficientes, minimizando o número de operações necessárias para a purificação desejada tem sido uma das maiores preocupações nesta área. Além desses aspectos, deve-se considerar o desenvolvimento de técnicas de baixo custo e viáveis no aumento de escala, já que a biotecnologia deve transpor a bancada do laboratório de pesquisa para a indústria (Belter, Cussley, & HU, 1988; GUPTA, 1994). O número de etapas de purificação pode resultar em até a 80% de custo final da produção de produtos de origem biotecnológica, além de implicar perdas da molécula alvo, tanto por sua obtenção em baixas concentrações quanto por fatores relacionados à sensibilidade das biomoléculas à fatores externos (BENAVIDES, 2005; Pessoa Jr & Kilikian, 2005; Pessoa-Jr & Kilikian, 2015; K. S. M. S. e. a. RAGHAVARAO, 1995).

A definição do processo de purificação varia de acordo com o local de produção da enzima (intra- ou extracelular) com a finalidade da molécula produzida e de suas características físico-químicas. Ácidos orgânicos e enzimas não utilizadas em humanos por vezes não requerem elevado grau de pureza, não havendo necessidade de operações cromatográficas. Por outro lado, produtos de uso farmacêutico, tanto para uso diagnóstico quanto terapêutico, tem de atender requisitos de pureza, como a L-ASNase. As exigências de pureza podem ser bastante elevadas (até 99,98% para substâncias de uso humano) requerendo a remoção de contaminantes presentes, demandando várias etapas e complexidade no processo de purificação (Mazzola et al., 2008; Molino, Viana Marques, Júnior, Mazzola, & Gatti, 2013; Pessoa Jr & Kilikian, 2005; Pessoa-Jr & Kilikian, 2015; Wheelwright, 1989b). A purificação industrial de enzimas ocorre, geralmente, através de processos múltiplos que consistem em técnicas de custo elevado tais como cromatografia de afinidade e cromatografia de troca iônica (Molino et al., 2013; C. O. Rangel-Yagui et al., 2003).

Acrescendo-se a isso, purificação de enzimas nos permite determinar sua estrutura primária e tridimensional (Khushoo, Pal, Singh, & Mukherjee, 2004; Vidya, Ushasree, & Pandey, 2014), e com isso estabelecer com mais exatidão sua função, mecanismo cinético e outros parâmetros (Zuo, Xue, Zhang, Jiang, & Mu, 2014)

Para cumprir a demanda industrial, a tecnologia enzimática necessita de uma abordagem biotecnológica tanto em termos de qualidade, quanto em quantidade.

Para o aumento da produção, é possível utilizar-se de técnicas de intensificação de processo, o qual vai desde a seleção de microrganismos produtores, até a otimização e modelagem do processo de produção (Weuster-Botz, 2000).

Buscando viabilizar pureza, baixo custo, demanda e qualidade há um esforço por parte dos pesquisadores em estudar bioprocessos alternativos de purificação.

3. Objetivos

3.1. Objetivo Geral

Extrair e purificar L-asparaginase por precipitação com Etanol e sistemas de duas fases aquosas (polimérico)

3.2. Objetivos Específicos

- Estudar o processo de precipitação da L–asparaginase utilizando etanol;
- Utilizar planejamento experimental para analisar o processo de extração da L-asparaginase por sistemas bifásicos aquosos compostos por polietilenoglicol (PEG) de diferentes massas moleculares;
- Avaliar a estabilidade da enzima L- asparaginase pré purificação frente as condições operacionais dos sistemas de purificação;
- Avaliar a estabilidade da enzima L-asparaginase frente as mudanças de temperatura e pH.

4. Execução

Capitulo I – "L-asparaginase Purification"

Louise L. Tundisi; Diego F. Coêlho; Beatriz Zanchetta; Patricia Moriel; Adalberto Pessoa- Jr.; Elias B. Tambourgi; Edgar Silveira; Priscila G. Mazzola

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Abstract: L-asparaginase (L-ASNase) is the gold standard enzyme used to treat acute lymphoblastic leukemia. This disease primarily affects children; however, treatment increases survival from 20% to 90%. As a biomolecule, it is obtained via a biotechnological process and its purification usually consists of several steps that account for up to 80% of the total production costs. This review discusses available strategies for the purification of L-ASNase and highlights a process with fewer steps, and consequently, lower cost and higher yield. This process emphasizes the possibility of using a novel aqueous two-phase system extraction process to purify L-ASNase.

Keywords: Aqueous-two phase system, Biotechnology, Downstream Process, L-asparaginase, Purification.

Introduction

L-Asparaginase (L-aspartate amidohydrolase, EC 3.5.1.1) is an enzyme that catalyzes the conversion of L-asparagine in its free amino acid form to L-aspartate and ammonia. For over 30 years, L-ASNase has been a mainstay of multidrug chemotherapeutic regimens extensively used for the treatment of malignancies of the lymphoid system, acute lymphoblastic leukemia, Hodgkin's lymphoma and melanosarcoma (Singh et al., 2013). The use of L-ASNase in anti-cancer therapy is based on its catalytic activity because asparagine is essential for lymphoblast growth. These cells are unable to produce endogenous L-asparagine, therefore, starvation for this amino acid leads to death of these cells (Kotzia & Labrou, 2007).

Alternatively, L-ASNase has a great potential as a food processing aid. During food processing, L-asparagine and reducing sugars that are present in the raw material undergo a Maillard-type reaction, forming acrylamide, which is a potential carcinogen and is highly toxic in its monomer form (Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002). This type of reaction usually occurs in cooked plant-based foods, such as potato products, short dough biscuits and cereals (Anese, Quarta, Peloux, & Calligaris, 2011; Pedreschi, Kaack, & Granby, 2008).

Currently, several L-ASNase agents are commercially available. These are either derived from *Escherichia coli* in its native form (native *E. coli* asparaginase) or as a PEGylated enzyme (PEG asparaginase). Otherwise, L-ASNase is extracted from *Erwinia chrysanthemi* (*Erwinia* asparaginase). Unfortunately, a therapeutic response by patients rarely occurs without some evidence of toxicity (Narta, Kanwar, & Azmi,

2007), and contamination with glutaminase is one cause of this toxicity (Gallagher, Marshall, & Wilson, 1989; Manna, Sinha, Sadhukhan, & Chakrabarty, 1995).

Most commercial applications of enzymes do not require highly purified enzyme preparations; highly purified L-ASNase, however, would enable more efficient and successful use with reduced risk of toxicity and allergies. Additionally, enzyme purification allows for the determination of its primary and three-dimensional structures (Khushoo et al., 2004; Vidya et al., 2014) and therefore a more accurate establishment of its function and kinetic mechanisms and parameters (Zuo, Zhang, Jiang, & Mu, 2015).

Alternatively, as biotechnology moves from the research lab bench to the marketplace, the need for large-scale purification processes becomes obvious. The classical purification strategies result in low yields and require long processing times, reducing productivity and increasing process costs. The purification process, i.e., a downstream process, is defined as a series of steps that, when followed, result in a purified protein product (Wheelwright, 1989b). As a general guideline, the purification process can be described as a four stage process that includes (a) removal of insoluble material, (b) concentration, (c) fractionation and (d) purification. However, there is no algorithm by which one can proceed, step-by-step, to develop a fool-proof large-scale purification process.

To overcome long processing times, new technologies such as membrane processes and Aqueous Two-Phase Systems (ATPS) integrated with precipitation and/or fractionation steps are emerging as viable processes for enzyme purification. Industries require purification strategies that are inexpensive, rapid, high-yield, amenable to large-scale operations, and above all, environmentally friendly.

Although L-ASNase has been extensively reviewed (Emadi, Zokaee, & Sausville, 2014; Müller & Boos, 1998; Narta et al., 2007; Savitri, Asthana, & Azmi, 2003), here we focus on the purification of L-ASNase for its pharmaceutical and/or food industries usage.

Purification Stages

Removal of Insoluble Material

L-ASNase is produced by several microorganisms, such as *E. coli* (Vidya et al., 2014), *Erwinia chrysanthemi* (Tong et al., 2014), *Pseudomonas stutzeri* (Manna et al., 1995), *Serratia marcescens* (Boyd & Phillips, 1971) and *S. cerevisiae* (Pauling & Jones, 1980), and the fermentation processes involved are always followed by the removal of insoluble materials, e.g., cells from the culture or cell debris from crude broth, either by centrifugation or by filtration.

Centrifugation is a standard unit operation in some downstream recovery processes and is primarily used to separate solids from liquids. It can remove particles as small as 0.5 μm from whole cells to organisms. It has an excellent separation efficiency, high perfusion capacity, causes little clogging and is easy to scale-up; however, it is susceptible to mechanical failure when used in long-term continuous operation (Wheelwright, 1991). Centrifugation is the process of choice for the removal of cells and/or cells debris (Badoei-Dalfard, 2015; Khushoo et al., 2004; Kotzia & Labrou, 2007; Mohan Kumar & Manonmani, 2013).

Filtration is used to remove small particles from solutions or to concentrate or separate soluble molecules. It is used in biotechnology processes when the target molecule has a suitable size. This technique uses a selective barrier through which part of the flow is retained (the retentate) and part passes through (the filtrate). The process can be arranged so that the substance of interest is found either in the filtrate or in the retentate (Wheelwright, 1989b). Filters are available in many forms, with different filter compositions, different mechanisms of filtration and for diverse applications.

In downstream processes, the challenge lies in processing of high cell concentration broths/extracts using traditional solid-liquid techniques (i.e., centrifugation and filtration). A common drawback found in centrifugation and/or filtration systems is that complex broths usually contain more than one protein with similar or higher molecular masses, leading to poor resolution. One way to overcome this problem is by using alternative enrichment and concentration processes, such as fractional precipitation and ATPS (Silva, Fernandes-Platzgummer, Aires-Barros, & Azevedo, 2014; R. R. G. Soares, Azevedo, Van Alstine, & Aires-Barros, 2015).

Product Enrichment and Concentration

Many industrial biotechnology processes require an early recovery step for removal and/or concentration of the target molecule from a variety of complex upstream contaminants. However, the low concentration and the similarity of the target molecule's physicochemical properties to those of other proteins within the media render the selective separation quite difficult and expensive (Coelho, Silveira, Pessoa Junior, & Tambourgi, 2013).

Partitioning of proteins in aqueous systems by precipitation is a traditional method for the recovery and partial purification of biomolecules. This method is aggressive toward the biomolecule due to the potential modification of its three dimensional structure, resulting in deformation of the protein. Therefore, it must be applied only when refolding of the protein is possible.

The solubility of a protein depends on the distribution of ionized groups and the hydrophobic and hydrophilic zones on the molecule's surface. These properties are responsible for polar interactions with the aqueous solvent and ionic interactions with salt in the medium as well as can promote electrostatic repulsion among the molecules with the same charge. Few proteins are soluble in pure water; most require a low concentration of salt to maintain their native form and promote stability. Normally, proteins that have positively or negatively charged regions tend to self-aggregate in solutions with very low concentrations of salt. The salt can either neutralize the charges on the protein surface, blocking aggregation, or it can promote aggregation when the concentration is raised, as the protein surface becomes over-charged (Green & Hughes, 1955).

The addition of organic solvents to an aqueous medium containing protein causes a variety of effects that culminates in precipitation of the protein. The solvent destroys the hydrophobic hydration layer of hydrophobic regions and interacts with these regions due to their greater affinity for the solvent. This allows interactions between superficial regions with negative or positive charges, leading to the formation of aggregates. The solvent may also interact with the internal hydrophobic regions of the protein, leading to an irreversible deformation. Reducing the temperature can minimize this effect because, at low temperatures, molecular flexibility is lower, thus reducing the extent of solvent penetration. The use of shorter chain alcohols can also

reduce deformation because shorter chain alcohols produce less denaturation compared with longer chain alcohols (Scopes, 1994).

The use of ammonium sulfate as a precipitation agent for L-ASN is well known; however, there is no standard protocol for L-ASNase precipitation. Manna et al. (1995) precipitated L-ASNase from *Pseudomonas stutzeri* by fractional precipitation and recovered the enzyme in the ammonium sulfate fraction at 35-55% of saturation. Meena et al. (2015) claimed that 45% ammonium sulfate would precipitate all of the L-ASNase from *Nocardiopsis alba*; on the other hand, Badoei-Dalfard (2015) used ammonium sulfate starting at 70% of saturation to precipitate L-ASNase from *Pseudomonas aeruginosa*. The use of organic solvents or short chain alcohols, such as methanol and ethanol, for precipitation is well known. Mohan Kumar and Manonmani (2013) studied precipitation with different solvents and concluded that the L-ASNase that was precipitated with methanol was observed to have better activity than enzyme that was obtained using other precipitants.

Although the selectivity obtained with precipitation is poor when compared with other techniques, such as chromatography (Wheelwright, 1991), some researchers have reported good results (Coelho et al., 2013; Cortez, Pessoa, & Assis, 1998; Singh et al., 2013). Its disadvantage, when compared with other low resolution techniques, is the likelihood of irreversible inactivation of the biomolecule during the precipitation process. On the other hand, ATPS has been extensively exploited as an inexpensive method for the recovery of biological products.

ATPS can be formed when two chemically different polymers (e.g., poly (ethylene glycol) (PEG) and dextran) or one polymer and a specific salt (e.g., PEG and potassium phosphate) are mixed together at certain concentrations in a solution (Hatti-Kaul, 2001; Zaslavsky, 1994). One phase is rich in one polymer, and the second phase is rich in the other component (polymer or salt) with water as a solvent in both phases. Several ATPS that are used for protein purification are listed in Table 1.

Table 1.1 – ATPS for protein purification

Top Phase	Bottom Phase	Purified protein	Reference
Poly(Ethylene Glycol)	Phosphate salts	Pectinases	(Maciel et al., 2014)
		Royal Jelly Protein 1	(Ibarra-Herrera, Torres-Acosta, Mendoza-Ochoa, Aguilar-Yañez, & Rito-Palomares, 2014)
		Monoclonal Antibody	(Eggersgluess, Richter, Dieterle, & Strube, 2014)
		C-phycocyanin	(Chethana, Nayak, Madhusudhan, & Raghavarao, 2014)
		Bromelain	(J. F. Ferreira, Santana, & Tambourgi, 2011)
	Sulphate salt	Bromelain	(Coelho et al., 2013)
	Citrate salts	Papain	(Y. Lu, 2014)
		Lysozyme	(Yanmin Lu, Lu, Wang, Guo, & Yang, 2013)
		α-lactoalbumin	(Kalaivani & Regupathi, 2013)
		Proteases	(Porto et al., 2008)

	Sodium polyacrylate	Amyloglucosidase	(Pereira Alcântara, Do Nascimento, Mourão, Minim, & Minim, 2013)
	Dextran	Glucosyltransferase	(Shibusawa et al., 2006)
		ß-glucosidase	(Johansson & Reczey, 1998)
	Starch	α-amylase	(Pietruszka, Galaev, Kumar, Brzozowski, & Mattiasson, 2000)
Etylene Oxide-Propylene Oxide	Phosphate salts	Lipase	(Show et al., 2012)
		Bromelain	(Rabelo, Tambourgi, & Pessoa, 2004)

The different types of ATPS have many diverse variables. Using the phase diagram for specific types of systems, as an example, is habitually susceptible to conditions such as temperature, pH, added components such as salts or organic buffers, as well as the component polymers' molecular weights, among others. Manipulating and optimizing the equilibrium compositions in each of the immiscible phases can induce differential partitioning behavior in a mixture. Knowledge of a higher affinity of certain molecules for one phase with respect to the other can be exploited for using phase partitioning for the purification of proteins (R. R. G. Soares et al., 2015).

PEG-phosphate ATPS are the most common systems used for protein separations since Albertsson (1977) first described the process in 1977, and polymer-polymer and PEG-salt systems have since been used conventionally by researchers. Although ATPS are easy to scale up, there is a low protein solubility problem, possibly related to crowding effects in such aqueous systems (L. A. Ferreira et al., 2016; Stepanenko et al., 2016).

High Resolution Purification

After the first steps of purification, chromatographic steps are often used to achieve maximum purification and/or to polish the target molecule. Selection of the procedure to use should be based on its capacity; the general rule is to proceed from a high to a low capacity method (i.e., from ion-exchange chromatography to affinity chromatography) (Linn, 2009).

Ion-Exchange Chromatography (IEC) is the most popular chromatographic method because of its versatility. A generic chromatographic process can recover different types of products using a simple buffer or resin change. IEC is based on the attraction of molecules with different surface charges, and a protein's surface charge is dependent on its pI and the pH of the surrounding buffer. When the pH of the buffer is above the pI of the protein, the protein is negatively charged, and it will bind to an anion exchange resin. Conversely, when the pH of the buffer is below the pI of the protein, the protein is positively charged, and it will bind to a cation exchange resin.

After the protein is bound to a resin, the elution step can be chosen based on the characteristics of each target molecule. Elution of the protein can be controlled by the elution mode used, such as a linear salt gradient, a step salt gradient, a pH gradient or by counter-buffer displacement. The elution mode can be optimized to obtain the most effective elution profile for a given molecule. In the early 1970s, Law and Wriston Jr (1971a) purified L-ASNase using a two-step IEC method that consisted of two anion exchange matrices at different pHs, resulting in a 14.2-fold purification and a 55.2% yield. Kotzia and Labrou (2007) purified L-ASNase from *Erwinia chrysanthemi* using single-step cation exchange chromatography and achieved greater than 15.4-fold purification with a 69.8% yield.

Considered as a medium-capacity chromatography method, Gel Filtration Chromatography is based on the fractionation of molecules according to their size. Gel filtration chromatography differs from conventional filtration in that there is no retentate within the column.

Gel filtration makes use of porous beads as a stationary phase, in which small proteins would equilibrate within pores of the beads and larger proteins would pass by the beads. Larger protein molecules would, therefore, elute from the column first, due to their limited path within the column, and small proteins, which can access

the internal path in the beads, would elute later. Manna et al. (1995) used gel filtration chromatography early in the purification of L-ASNase from *Pseudomonas stutzeri* and recovered 79% of the activity recovery with a 12-fold purification for the overall process. Furthermore, Mohan Kumar and Manonmani (2013) used gel filtration chromatography as a polishing step in the purification of L-ASNase from *Cladosporium sp.*, obtaining a 4.55-fold purification in this step and a greater than 865-fold purification for the overall process. Kavitha and Vijayalakshmi (2014), purified L-ASNase from *Streptomyces tendae* TK-VL_333 using Sephadex G-100 and obtained a specific activity of 51.7 U/mg with a 17.23–fold purification and a recovery of 30.5%. The active enzyme fractions were pooled, and the enzyme was further purified using CM-Sephadex C-50 column chromatography. The purity of the enzyme was increased 87.2-fold with a recovery of 25.7%.

Affinity chromatography can be viewed as a counter method to IEC because the latter is a diverse method in which one can purify a protein using a simple buffer change, while the former is a more specific method in which one can change the ligand and then purify a specific protein. Affinity chromatography is based on the interaction of two molecules that bind to each other with high affinity, such as enzyme-substrate, receptor-ligand, and antigen-antibody interactions. An understanding of the interactions between the stationary phase ligand and the target molecule is the key to success with affinity chromatography. Khushoo et al. (2004) used a nickel charged affinity resin to purify a recombinant *E. coli* L-ASNase that contained a polyhistidine (6 x His) tag. They were able to recover 86% of its initial activity with a purification factor of 3.3-fold.

Chromatographic processes are well established in the biotechnology and pharmaceutical industries. These processes are highly reliable, reasonably accurate and precise. However, the equipment used with such techniques is expensive, require highly trained personnel and are not easily scaled-up.

Aqueous two-phase systems (ATPS)

The processes of purification and separation of biotechnological products have not experienced the same rate of development in recent years, when compared with the optimization of fermentative processes. Current processes used in the

purification of bioproducts consist of several operations with low efficiency and are expensive from an industrial point of view (Carlota O. Rangel-Yagui et al., 2003).

Products from biotechnological processes are commonly obtained in a dilute form and in the presence of complex media containing several contaminants such as cells, cell components, contaminating proteins, culture medium components, pigments, polysaccharides, etc. These contaminants can diminish the biological activity of a molecule, or even inactivate it (Asejo, 1990).

Aqueous liquid phases from ATPS are formed under certain “critical” conditions of temperature, ionic strength or polymer concentration. These are complex coacervate systems consisting of two immiscible phases that are formed (R. R. G. Soares et al., 2015). Such systems are obtained by mixing two (or more) components in an aqueous solution above a critical concentration, which separate spontaneously into two-phases (Rosa, Ferreira, Azevedo, & Aires-Barros, 2010). Due to the high water content, and consequently a low denaturation effect, they have been used extensively to obtain biologically active proteins. Hydrophilic polymers, nonionic surfactants, salts and ionic liquids are species that are commonly used to form ATPS. Another alternative is to use microemulsions of surfactants in organic solvents, which constitutes a reverse -micellar system.

Aqueous Two-phase Polymer Systems

ATPS are formed by mixing two or more water-soluble polymers such as PEG and dextran, or a single polymer and a particular salt in water above concentration limits (Zaslavsky, 1994). These systems consist of two coexisting aqueous phases which are separated by an interface. Each of these phases is preferably enriched by one of the components of the system while the aqueous medium offers benefits for biomolecules, such as a high interfacial contact area of operation, biocompatibility and simplicity of the procedure (P.Å. Albertsson, 1986; Coelho et al., 2013; Rosa, Azevedo, Sommerfeld, Bäcker, & Aires-Barros, 2011).

This process is based on mass transfer between the two immiscible liquid phases, and separation occurs due to the physicochemical characteristics of the target molecule (Coelho et al., 2013). Protein purification is achieved by manipulating the partition coefficient, the molecular weight of the polymers, the types of ions in the system and the ionic strength of the saline phase by addition of salt. The partition into

two aqueous phases is a mild method of protein purification, where denaturation or loss of biological activity is not commonly observed. This is mainly due to the high water content in both phases of approximately 70 to 90%, and to low interfacial tension, which protects the proteins (Asenjo & Andrews, 2011; Rabelo et al., 2004; Santos, e Silva, Coutinho, Ventura, & Pessoa Jr, 2015). The combination of PEG and dextran is a useful system for the separation of macromolecules (Nishimura et al., 1995). PEG and polyacrylic acid (PAA) are non-ionic polymers used in the preparation of aqueous two-phase systems; in addition to providing a stabilizing effect on biomolecules, these molecules are advantageous in terms of biodegradability, toxicity and cost (Santos et al., 2015).

Aqueous Two-phase Micellar Systems

Separation methods for biomolecules that use aqueous two-phase micellar systems explore the characteristics of some micellar systems that, when subjected to certain conditions, can spontaneously separate into two immiscible liquid phases (C.-l. Liu, Kamei, King, Wang, & Blankschtein, 1998). There is considerable interest in using these systems to purify and concentrate substances such as Bovine Serum Albumin, bacteriophages, antibiotics, cholesterol oxidase, lysozyme and other enzymes, fat soluble vitamins and organics (Lee & Su, 1999; C.-l. Liu et al., 1998; Carlota O. Rangel-Yagui et al., 2003; Sánchez-ferrer, Bru, & García-carmona, 1994; Sirimanne, Patterson Jr, Ma, & Justice Jr, 1998). This technique has been used to separate contaminants with hydrophobic characteristics that are present in enzymatic solutions. For example, Núñez-Delicado, Bru, Sánchez-Ferrer, and García-Carmona (1996) used this technique to remove 92% of the phenolic compounds present in a tyrosinase solution without affecting the enzyme's structure.

Surface-active agents are molecules that are typically composed of two chemically distinct portions: a hydrophilic portion and a hydrophobic portion. Due to their distinct chemical structures, when molecules of surfactants are dissolved in water, aggregate structures known as micelles form spontaneously. In a micelle, the hydrophobic tails attract one another to minimize their unfavorable contact with water, while the hydrophilic heads remain on the outer surface of the micelle to maximize their contact with water (Chevalier & Zemb, 1990).

At certain temperatures and concentrations of surfactants, a homogeneous micellar aqueous solution can be separated into two macroscopic phases, both containing micelles, but with one having a higher concentration of these micelles. This phase separation is induced by a "temperature increase" of the system. It is important to highlight that the water content in each of the phases is high (in general it is above 90% w/w) and is favorable for biomolecules (C. L. Liu, Nikas, & Blankschtein, 1996). The phase separation phenomenon that is induced by a "temperature increase" can be represented by a bell shaped curve, referred to as a binodal curve, which is constructed by varying the surfactant concentration and the temperature of the medium. The binodal curve represents, therefore, a separation limit which is based on the temperature and the concentration of surfactant, wherein the micellar solution separates into two macroscopic phases. This process of phase separation according to Blankschtein, Thurston, and Benedek (1986) is a result of the effects of the "internal energy", which promotes the separation of the micelles and the water, and the entropic effects that promote miscibility of the micelles in water. These interactions are predominantly controlled by temperature.

Although surfactants, in particular ionic surfactants, may bind to proteins resulting in denaturation, uncharged surfactants do not bind strongly to biomolecules and, thus, do not distort them (Makino, Reynolds, & Tanford, 1973). Therefore, micellar aqueous two-phase systems that are composed of non-charged surfactants can provide a mild environment that is compatible with biomolecules (C.-l. Liu et al., 1998; Sirimanne et al., 1998) The micelles, because they are susceptible to modifications in their structure, enable the control and optimization of the partitioning of biomolecules by adjustment of characteristics such as size and shape by varying the temperature, concentration of the surfactant and salt content. The amphiphilic character of the micelles can offer hydrophobic and hydrophilic environments to solutes, allowing selectivity in the separation of biomolecules based on hydrophobicity. Furthermore, the selectivity of the partition can be improved using a specific binding affinity to the target biomolecule or mixtures of ionic and non-ionic surfactants.

Process Design and Equipment Selection

Any separation process that is intended to be used industrially in a downstream process for purification of L-ASNase must be examined regarding its feasibility, its possibility of damaging the enzyme and its throughput (King, 1980).

While process feasibility can be considered for its potential to provide product in the desired quality under the required process conditions; avoiding degradation or loss of product is a major concern during bioprocess design selection because the target-molecule(s) may be sensitive to contact with surfaces, heat or separating agents. Nevertheless, each factor can be optimized during the selection of a processing approach by screening empirically on a laboratory scale.

However, the process throughput can change significantly depending on whether a continuous or a batch mode of operation is chosen for production. The decision must consider not only operational costs (automation and workforce required), capital cost for plant implementation and an appropriated rate of production but also fluid properties, number of theoretical stages, available space and residence time in the extractor (Dutta, 2007).

Liquid-liquid extraction itself, in its simplest form, would consist of the transfer of one component from a binary mixture into a second phase, in which the first solvent is insoluble and the solute has a higher affinity. The lack of conceptual complexity provides a wide range of applications and an even larger variety of liquid-liquid extraction devices. A detailed guideline for extractor selection was given by Lo, Baird, and Hanson (1983).

Some equipment designs are derived from, or have incorporated features from, those used in absorption, stripping and distillation (although a small phase density difference and high viscosity usually restrict its applications). Generally, mechanically agitated and centrifugal devices are preferred (Dutta, 2007).

Mechanical high-speed centrifugal devices have been used for many years to separate liquid-liquid dispersions by enhancing the settling rates and, consequently, reducing settling time. This category includes the Podbielniak centrifugal extractor, the annular centrifugal extractor and countercurrent chromatography devices. The latter has shown excellent results in purifying natural products (Qi et al., 2010; I. Sutherland

et al., 2013) and its use is being increased, especially after reports have been published concerning successful scale-up studies (I. Sutherland et al., 2011).

Countercurrent Chromatography

The term “Countercurrent Chromatography” was chosen by Ito to name a technique that combines all the advantages from its parent methods (countercurrent distribution and liquid-liquid partition chromatography) without suffering complications such as sample loss, contamination, solute peaks or excessive dilution of samples (Yoichiro Ito & Bowman, 1970). When compared with commonly used purification techniques, this method has several advantages. It eliminates the use of solid supports and, consequently, the irreversible adsorption and loss of sample. It also allows the injection of high sample loads at 10-30% of total column volume (Grudzien, 2011) and provides high sample recovery with high purity, with excellent reproducibility (Yoichiro Ito, 1981).

Despite numerous different column designs that are proposed to increase phase retention or phase mixing, they can be grouped into the following two main types: hydrodynamic and hydrostatic columns.

Hydrodynamic columns derived directly from the work of Y. Ito et al. (1966) and are essentially a long tube wound in a drum that rotates on a planetary axis and generates a variable centrifugal force field. The field has its lowest value in the region proximal to the planetary coil, where there is mixing between the phases, and its maximum value at the tidal side where there is settling between phases (I. A. Sutherland, 2007).

On the other hand, Centrifugal Partition Chromatography retains the stationary phase by a constant gravitational field that is generated by the rotational speed of a single axis in which the column is assembled. The CPC column is essentially a stack of disks in which a series of chambers interconnected by narrow ducts are carved circumferentially to form a pattern that is assembled in the rotor of a centrifuge. The mobile phase will flow through the stationary phase in each chamber in a cascade-mixing mode due to the Coriolis acceleration effect (Ikehata et al., 2004). Because of this design, the pressure across each chamber can be as high as 200 bar, depending on its patterns, density differences, viscosity and G field (I. A. Sutherland, 2007). However, hydrodynamic CCC exhibits a low-pressure drop process with no

rotating seals in which mass transfer occurs by wave-mixing in each coil unit. Nevertheless, hydrodynamic CCC lacks separating phases due to its sensitivity to changes in interfacial tension, and, consequently, it is not stable when being used with aqueous two-phases systems (I. Sutherland, Hawes, Ignatova, Janaway, & Wood, 2005).

While some researchers are still searching for ATPS that are capable of being used in hydrodynamic CCC systems (Shinomiya et al., 2013; Zhou, Zhang, Xu, Ma, & Zhang, 2014), CPC has been proved to be reliable by successfully fractionating and isolating several natural compounds (Bezold, Goll, & Minceva, 2015; Foucault & Nakanishi, 1990; Ward, Cárdenas-Fernández, Hewitson, Ignatova, & Lye, 2015). For instance, Oelmeier, Ladd Effio, and Hubbuch (2012) have successfully investigated the separation and purification of four monoclonal antibodies from host cell proteins using PEG-PO_4 systems. Their research has demonstrated that precipitation experiments performed with phase forming components provide a reasonable correlation with protein partitioning in ATPS.

Ward et al. (2015) studied the separation of a synthetic mixture of sugars that are normally found in the hydrolyzed pectin fraction from Sugar Beet Pulp (SBP) using an ethanol/$(SO_4)_2NH_4$ ATPS supplemented with dimethyl sulfoxide as a phase modifier and obtained two, out of four, components with purities greater than 90%. As the separation was reported to take under 2 hours and the purified sugars are valuable for the production of pharmaceutical intermediates, CPC was shown to be a promising technique for process integration (within a crop refinery).

Cao, Tian, Zhang, and Ito (1998) purified 10-deacetylbaccatin III (10-DAB) from an aqueous extract of Chinese yew (*T. chinensis*) needles using a multilayer coil instrument (260 ml) and a two-step protocol. The compound, which is a precursor in the production of Taxol (paclitaxel), was first extracted using an n-hexane–ethyl acetate–methanol–water (2:5:2:5) solvent system and the fraction that was obtained was subjected to a polishing step using an n-hexane–chloroform–methanol–water (5:25:34:20) system. The process yielded 10-DAB at 98% purity.

In fact, there are an extensive number of very diverse applications for both CCC techniques. In particular, the manuscripts written by Marston and Hostettmann (2006), Neves Costa and Leitão (2010) and Marston, Slacanin, and Hostettmann

(1990) compiled reviews of remarkable applications, which included purification of natural products such as saponins, polyphenols, furanocoumarins, flavonoids and tannins.

The process integration characteristic is one of its main features and is able to control esterification (Nioi et al., 2015) and hydrolysis (Krause, Oeldorf, Schembecker, & Merz, 2015) reactions, thus reducing the number of steps during the recovery of its products.

Scale-up

ATPS processes are attractive for industrial implementation because their scale-up is simple and accurate, as a linear scaling and relatively simplistic equipment and facility demands, as showed by Rosa et al. (2010). A huge economic advantage is that this process allows the integration of clarification, concentration and purification in just one step and that scale-up is possible by using equipment that is traditionally used in the chemical industry for liquid–liquid extraction, such as mixer-settlers, column contactors and centrifugal contactors (Azevedo, Rosa, Ferreira, & Aires-Barros, 2009; Rosa et al., 2010).

Concluding Remarks

The choice of ATPS as part of a purification process enables the control of overall purification cost issues and can provide a viable alternative for L-ASNase purification. The ATPS protein partitioning process is well-known to be a non-burdensome technique, which is also convenient and easily scalable. ATPS has been successfully used in the development of bioprocesses for the recovery and purification of various biomolecules. Thus, two-phase partitioning can be used to separate proteins from cellular debris or to purify proteins and is suitable for the purification of L-ASNase that is obtained from fermentation.

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Conflict of Interest

The authors declare no commercial or financial conflicts of interest.

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Capitulo II – Characterization of L-Asparaginase from *Aspergillus oryzae*

Louise L. Tundisi; Alessandra V. de S. Faria; Adalberto Pessoa- Jr.; Elias B. Tambourgi; Edgar Silveira; Priscila G. Mazzola

Submetido à Separation Science and Technology

Abstract:

L-Asparaginase, one of the major antileukemic agent for pediatric patients, has some main factors influencing its activity as Km, glutaminase activity, clearance of the enzyme and development of resistance. Most of the commercialized enzyme present an intrinsic glutaminase activity responsible for life threatening side effects. In this study, glutaminase free asparaginase produced from *Aspergillus oryzae* presented low Km, after precipitation with ethanol presented activity of 135.45 U/mg and after gel filtration, 322.02 U/mg. Purified enzyme showed optimum activity between pH 5.8–9.0 and also showed activity from 5 ºC to 55 ºC which can be convenient for human use.

Keywords: biopharmaceuticals; bioprocessing; bioproducts; biotechnology; enzyme activity; ethanol precipitation

Introduction

L-asparaginase (ASNase), as an effective antineoplastic agent, is an enzymatic drug and an essential component in the treatment when combined with chemotherapy against acute lymphoblastic leukemia (ALL) and of some other diseases such as lymphosarcoma, Hodgkin's disease, acute myelogenous leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, reticulosarcoma and melanosarcoma (Avramis & Tiwari, 2006; K. Kumar, J. Kaur, S. Walia, T. Pathak, & D. Aggarwal, 2014; Pui & Evans, 2006; Verma et al., 2007).

ASNase catalyzes extracellular L-asparagine (Asn) hydrolysis to L-aspartate and ammonia, and the effective depletion of Asn in blood leads to a block of protein synthesis in cancer cells. As a result, protein synthesis is blocked in cancer cells resulting in apoptosis (Bussolati et al., 1995). Normal cells, however, are able to synthesize Asn and are less affected by its depletion with ASNase treatment.

Preparations of ASNase employed in therapy also promote a rapid and effective conversion of L-glutamine to L-glutaminate. The transportation of amino nitrogen in the blood and an amino group donor for many biosynthetic reactions is due to glutamine. An extended decrease of plasma glutamine levels can compromise liver's functions, cause leucopenia, immunosuppression, acute pancreatitis,

thromboembolysis, hyperglycemia and neurological seizures (Islam Husain, Anjana Sharma, Suresh Kumar, & Fayaz Malik, 2016). Therefore, enzymes with high activity, low K_m, and strong preference for asparagine over glutamine, are the most appropriate for ALL treatment (Derst, Henseling, & Röhm, 2000). To diminish or avoid side effects it is important to produce L-ASNase with those characteristics (Ali et al., 2016; I. Husain, A. Sharma, S. Kumar, & F. Malik, 2016; Nguyen, Su, & Lavie, 2016).

ASNase is accepted by the Brazil's Ministry of Health as gold standard treatment for ALL, and it is sold commercially as Elspar® (Merck, *Escherichia coli*) or Erwinase® (Speywood, *Erwinia chrysanthemi*). The use of ASNase increases the survival in pediatric patients from 20% to 90% (Mila, 2014).

Large-scale production of biomolecules are important for pharmaceutical applications. In order to achieve or to produce a pure, marketable product, several processing steps are required, such as dialysis, ionic and affinity chromatography, and others. The various stages that comprise the purification of bioproducts are the bottleneck of the process. There is a need for a massive separation to remove a large part of cellular components in both laboratory and industrial scale..

Separation of proteins from aqueous media by precipitation is one of the traditional methods for the recovery and partial purification of biomolecules (Mazzola et al., 2008; Tundisi et al., 2017). Ethanol precipitation is a promising technique, since it can be applied on industrial scale. Because of their low dielectric constants (compared to water), organic solvents 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. As Cortez et al. (1998) described, ethanol is the most important of the solvents due to its good physicochemical properties, as complete miscibility with water, good freezing-point depression, no explosive mixtures, high volatility, chemical inertness, low toxicity, and low cost, especially in Brazil.

This paper reports the purification and partial characterisation of ASNase from *Aspergillus oryzae* as a new enzyme source for ALL treatment. ASNase purification of by ethanol purification and gel filtration chromatography. The partial characterisation of ASNase will be hereafter described.

Material and Methods

Reagents and chemicals

Crude L-ASNase (E.C. 3.5.1.1) from *Aspergillus oryzae* was donated by Novozymes (Araucária, PR, Brazil). Standard L-ASNase and BCA was purchased from (Sigma-Aldrich, USA). SDS – PAGE was done in the *In vitro* Bioassays & Signal Transduction Laboratory Department of Biochemistry and Tissue Biology. All others reagents were of analytical grade. All experiments were performed at least in triplicate, and the data presented is the average of these independent measurements.

Enzymatic Assay

L-ASNase activity was determined by estimating the amount of ammonia produced during hydrolysis. Enzyme assay mixture of 1.05 mL in 50 mmol.l^{-1} Tris–HCl (pH 8.6) with 50 µL of L-asparagine solution, and 50 µL enzyme solution. The reaction was conducted at 37 ºC for 30 minutes when 50 µL of 1.5 mol.l^{-1} trichloroacetic acid (TCA) was added to stop reaction. 200 µL of reaction mixture supernatant was added to 4.3 mL of deionized water and 500 µL of Nessler's reagent to measure the released ammonia after L-asparagine hydrolysis. Analysis 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 purification by ethanol precipitation

Precipitation of crude L-ASNase was performed in a single step using different concentrations of ethanol (0–80%). Test tubes containing 1.0 mL of crude extract were cooled to 0 °C using a refrigerated water bath (Ethik Technology, SP, Brazil). The volumes of absolute ethanol at 0 °C were added to the tubes in order to achieve desired final ethanol concentration. After precipitation, the sample was centrifuged at 2,000g for 30 min at 4 °C, and the precipitate was solubilised in 1.0 mL of Tris HCl buffer (0.1 mol.l^{-1}, pH 8.6). After determination of optimum ethanol concentration, 100 mL of crude L-ASNase were purified under constant agitation and temperature. All precipitations were performed in triplicate.

Gel filtration chromatography

L-ASNase was further purified by gel filtration chromatography using Sephadex G-75 as stationary phase with a column height of 60 cm, preequilibrated with Tris HCl buffer 0.1 mol.l^{-1}, pH 8.6. The proteins were eluted at 0.5 mL.min^{-1} with the same buffer. The apparent molecular mass of the purified L-ASNase fraction was estimated by 10% SDS-PAGE according to Laemmli (1970). The proteins were stained with Coomassie Brilliant Blue R-250.The molar mass range was from 10 kDa to 250 kDa.

Effects on pH and Temperature

The optimum pH was determined by assessing L-ASNase activity at different pHs at 37ºC. Four buffer systems, including citrate-phosphate buffer (50 mmol.l^{-1}, pH 2.6–5.8), phosphate buffer (50 mmol.l^{-1}, pH 5.8–7.4), Tris-HCl buffer (50 mmol.l^{-1}, pH 7.4–9.0), and carbonate buffer, (50 mmol.l^{-1}, pH 9.8–10.6), were used for measuring the optimum pH of enzyme activity.

Optimum temperature for enzyme activity was measured in optimum pH conditions (Tris-HCl buffer 50 mmol.l^{-1}, pH 7.4; and phosphate buffer 50 mmol.l^{-1}, pH 7.4) at different temperatures ranging from 5–55 ºC.

Enzymatic kinetics

L-ASNase kinetic parameters were performed incubating the enzyme in various substrate concentrations (ranging from 0.945 mmol.l^{-1} to 18.9 mmol.l^{-1}) in 50 mmol.l^{-1} phosphate buffer or Tris-HCl buffer, pH 7.4. The Michaelis-Menten constant (K_m), and maximum velocity (V_{max}) was determined through fitting from the Hill equation:

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

where *[S]* is the substrate concentration, and *n* is the Hill coefficient. When n < 1, there is a negative cooperativity; when n = 1, there is no cooperativity (i.e. Michaelis-Menten mechanism); and when n > 1, there is a positive cooperativity. All statistical analyses and mathematical fitting were performed using Origin 8.0 (Origin Lab, USA).

Results

Ethanol Purification and Gel Filtration Chromatography

In this study ethanol precipitation profile of L-ASNase was made (figure 2.1). Precipitation starts near 50% (w/w) ethanol, however highest L-ASNase activity was recovered at 60% (w/w) ethanol. Total protein was precipitated from 50% to 80% (w/w) ethanol.

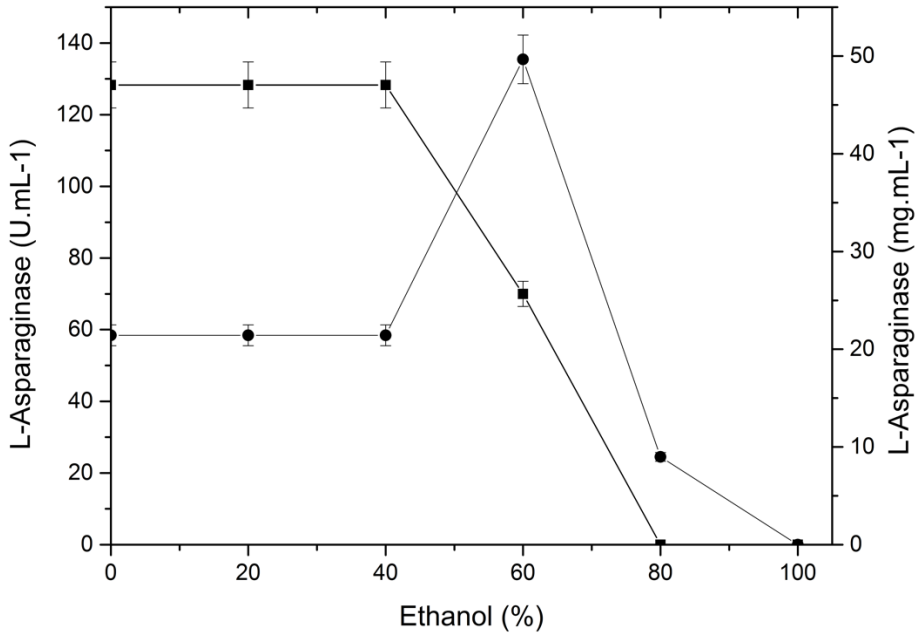

Fig. 2.1 - Ethanol Precipitation Profile

Figure 2.2 presents results of the chromatographic step of L-ASNase purification. The elution profile of the gel filtration presented four peaks, but only one peak presented L-ASNase activity, achieving a purification fold of 2.38. Although the peaks overlapped, the third peak was homogeneous.

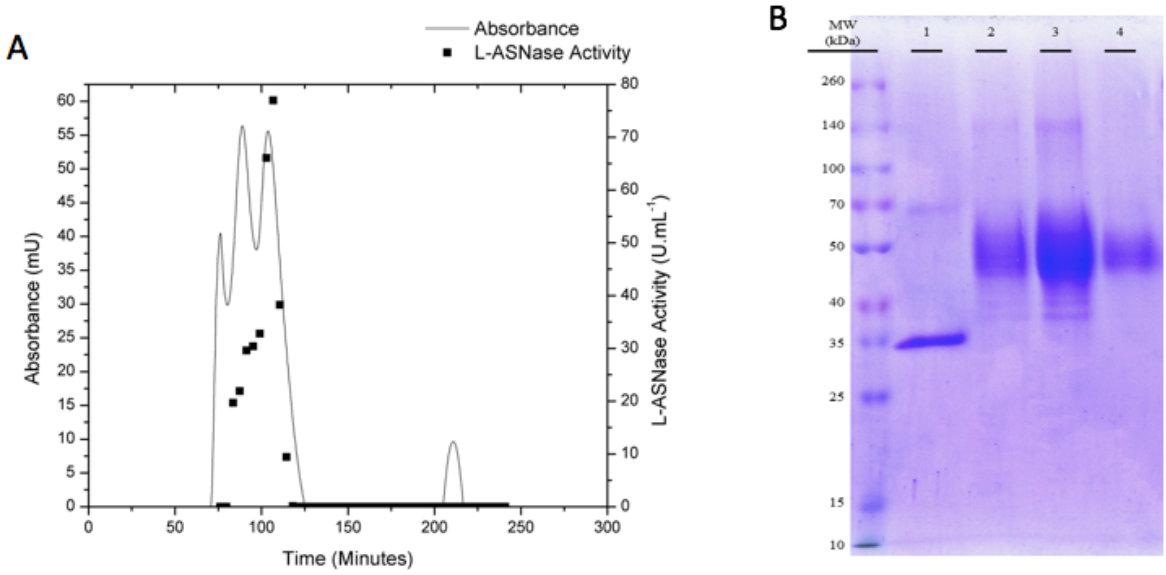

Fig.2.2 – a) Gel filtration chromatography elution profile b) SDS-PAGE of purified L-ASNase. Lane 1 - molecular markers; Lane 2 - standard L-ASNase from *E. coli*; Lane 3 - crude broth from *A. oryzae*; Lane 4 - ethanol precipitated L-ASNase from *A. oryzae*; Lane 5 - gel filtration chromatography purified L-ASNase fr om *A. oryzae.*

Table 2.1 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 2.32-fold, and further purified to an overall purification factor of 5.52-fold.

Table 2.1 - Summary of steps used at ethanol precipitation of L-ASNase

Steps	Total Activity (U/mL)	Total Protein (mg/mL	Specific Activity (U/mg)	Purification Fold	Recovery (%)
Crude	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	2.38	6.99

n.a.: non-applicable

Enzyme characterisation

Figure 2.3 shows the effect of pH on the enzyme activity. It is newsworthy that no L-ASNase activity was detected at high acidic pHs, its activity was detectable when pH 5.0 was reached. It is clear that L-ASNase from *A. oryzae* has an optimum pH range from pH 5.8 to 9.0. Phosphate buffer pH 7.4 and Tris-HCl pH 7.4 showed the best results for L-ASNase activity.

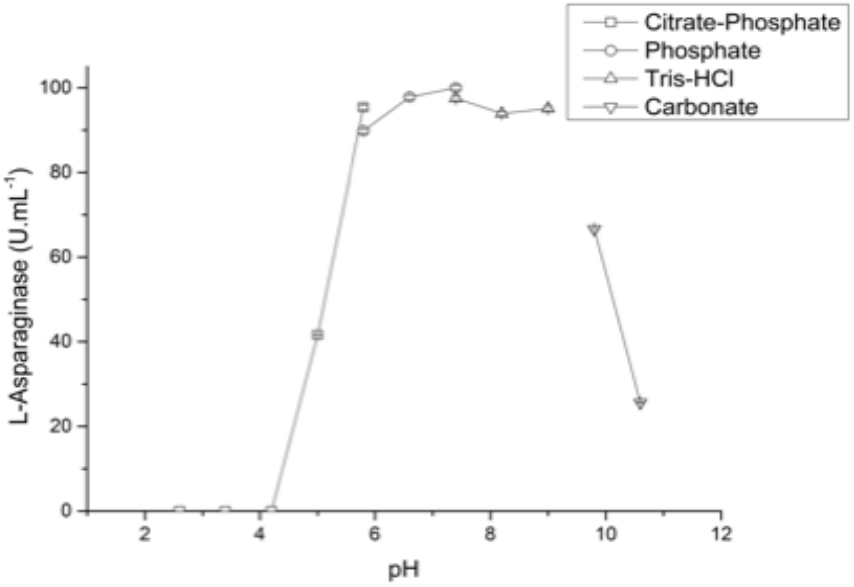

Fig.2.3 - Effect of pH change of L-ASNase activity

As shown in figure 2.4, L-ASNase from *A. oryzae* exhibited optimum activity after 15 ºC, maintaining its maximum activity at 55 ºC. L-ASNase from *A. oryzae* has a wide range of working pH, 4,8 to 10,4, and also presents its activity from 5 ºC to 55 ºC.

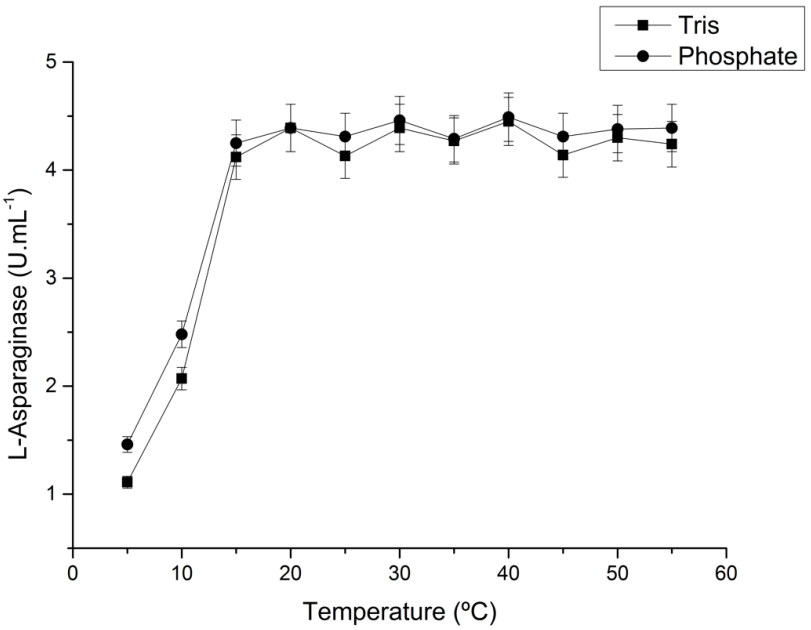

Fig. 2.4 - Effect of temperature change of L-ASNase activity. (closed square) Tris-HCl buffer; and (closed circle) Phosphate buffer.

The kinetic properties of L-ASNase from *A. oryzae* were assessed at pH 7.4 at 37 ºC, using L-asparagine in phosphate buffer or Tris-HCl as substrate (Table 2.2). The kinetic plots of L-ASNase activity against substrate concentration are in figure 2.5. Once again, phosphate buffer showed better results when compared to Tris-HCl buffer. In phosphate buffer, L-ASNase shows to be slightly more active than in Tris-HCl buffer, therefore, its V_{max} and K_m are also barely higher.

Table 2.2 - Kinetic parameters of *Aspergillus oryzae* L-ASNase in different buffers

Buffer	V_{max}	K_m (mM)	n	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

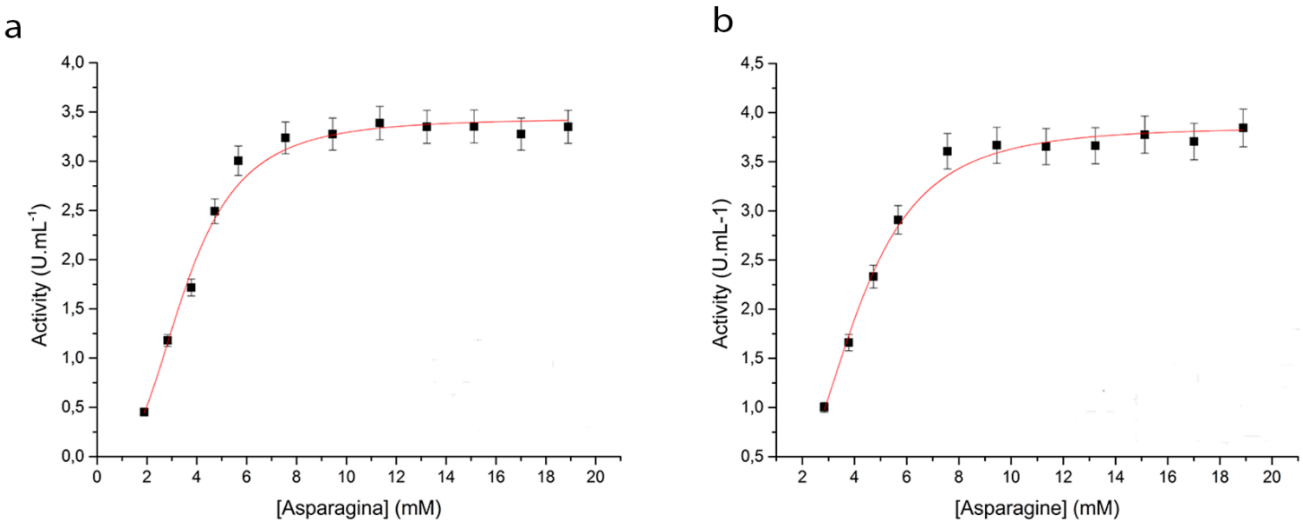

Fig.2.5 - L-ASNase kinetics using Hill equation fitting on (a) Tris-HCl buffer, and (b) Phosphate buffer

Non-specificity of a ASNase could decrease the L-glutamine plasma levels and, therefore, cause liver problems (Derst et al., 2000), as well as leucopenia, neurological seizures, pancreatitis and coagulation abnormalities leading to intracranial thrombosis or haemorrhage (Duval et al., 2002), ASNase from *A. oryzae* was tested against L-glutamine, and L-Asparagine. Purified samples showed no glutaminase activity, while it presented high activity against L-ASN.

Discussion

Ethanol Purification and Gel Filtration Chromatography

L-asparaginase precipitation was detected at 50% ethanol, reaching its highest result at 60% ethanol. Total protein precipitated from 50% to 80% (w/w) ethanol. This difference between L-ASNase and total protein precipitation is the key to achieve purification through a single step of fractional precipitation.

Croisfelt et al. (2015) stated that it was possible to purify enzymes (i.e. bromelain) through ethanol precipitation, in a two-step process. In these findings, the enzyme was recovered after 30% ethanol concentration. L-ASNase starts to precipitate near 50% (w/w) ethanol.

In this study, it was used ethanol as the precipitant agent; as it seemed to be the most suitable one for the intended purpose, considering cost and eco-friendly characteristics as it is recoverable by distillation. (Grethlein & Lynd, 1992; Lopes et al., 2015; P. A. G. Soares, Vaz, Correia, Pessoa Jr, & Carneiro-da-Cunha, 2012; Vander Griend, 2007). Mohan Kumar and Manonmani (2013) studied several organic solvents as precipitant agents for L-ASNase purification, and stated that methanol was the best solvent for the precipitation. However, it was not clear what was the recovery yield achieved. The most common initial step for L-ASNase purification is ammonium sulphate precipitation. L-ASNase was purified by ammonium sulphate fractional precipitation at 60-80% saturation by Kumar, Venkata Dasu, and 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, Elshal, Kumosani, and Aldahlawi (2015) also achieved a 2-fold purification factor using a chromatographic step, i.e. DEAE-Sepharose. Mohan Kumar and Manonmani (2013) could achieve a much higher purification factor using chromatographic steps, however, their L-ASNase had only 84 $U.mg^{-1}$ of specific activity. Similar results are also described by Singh et al. (2013).

A generic purification process requires a quick and mild step as first step, aiming to remove contaminants, cell debris, and proteases from crude extract. Most proteins are only soluble in water, or need a small concentration of salts to remain on its native state. The addition of organic solvents and/salts would disrupt the surface charge balance within proteins, leading to its precipitation. Distinct proteins have distinct surface charge, which could be selectively precipitated at different organic solvent concentration

Enzyme characterisation

pH and Temperature

L-ASNase from *A. oryzae* showed an optimum pH range from pH 5.8 to 9.0. Phosphate buffer pH 7.4 and Tris-HCl pH 7.4 showed the best results for L-ASNase activity, among all the other buffers tested. Similar results were described by Singh et al. (2013); however, there is no overlapping on the buffer systems. Zuo et al. (2014)

found that their recombinant L-ASNase from *Thermococcus gammatolerans* had an optimum pH at 50 mmol.l^{-1} Tris-HCl, pH 8.5. It is important to note that at neutral pH, phosphate buffer had better activity than Tris-HCl buffer at neutral pH, just like the results described here. Although, their activity assay was measured at 85 ºC, which could lead to a discrepancy at the results, as ours were measured at 37 ºC.

L-ASNase from *A. oryzae* exhibited optimum activity after 15 ºC, maintaining its maximum activity at 55 ºC. It also presented its activity from 5 ºC to 55 ºC. Zuo et al. (2014) described a recombinant L-ASNase from *T. gammatolerans* with an optimum temperature of 85 ºC, and it only exhibit 10% of its activity at 40 ºC. Vidya et al. (2014) reported that wild L-ASNase displayed a low thermic tolerance. Meanwhile, Singh et al. (2013) reported that L-ASNase from *Bacillus aryahattai* had an optimum temperature at 40 ºC, and after that threshold, the enzyme activity declined substantially.

Phosphate buffer reactions and Tris-HCl buffer reactions showed the same profile in the temperature range, and confirmed the findings of the optimum pH, phosphate buffer yields better results than Tris-HCl. Since L-ASNase can be employed in the food industry as an additive (Anese et al., 2011; Pedreschi et al., 2008), and as a pharmaceutical enzyme, these results clearly show that L-ASNase from *A. oryzae* can be used in both.

Kinetics

L-ASNase from *A. oryzae* with Tris-HCl buffer and Phosphate buffer showed Vmax of 3,5 and 4,01 Km of 3,5 and 4,01 and n of 3,45 and 3,41 respectively. L-ASNase from *Bacillus coagulans* (Law & Wriston Jr, 1971b), *Arabidopsis thaliana* (Gabriel, Telmer, & Marsolais, 2012), *Pyroccocus furiosus* (Bansal et al., 2012), and *Escherichia coli* (Derst et al., 2000), presented similar results to the *A. oryzae* L-ASNase described here. These results are in accordance with mesophilic L-ASNase, where the Km values are be around 10 µmol.l^{-1} and 4 mmol.l^{-1} (Bansal et al., 2012; Kotzia & Labrou, 2007; Li et al., 2007). Futhermore, according to Bansal et al. (2012), these results could be related to an increased substrate accessibility due to a missing salt bond.

The Hills coefficient, higher than 1, describes that the *A. oryzae* L-ASNase has a positively cooperative binding mechanism, i.e. once one ligand molecule is bound to the enzyme, its affinity for other ligand molecules increases. Note that this does not state that L-ASNase presents an allosteric mechanism, but that, as many L-ASNases, *A. oryzae* L-ASNase could be in dimers or tetramers, which could confer this pseudo-allosteric mechanism. Law and Wriston Jr (1971b) described an L-ASNase from *Bacillus coagulans* with similar sigmoidal kinetic behaviour.

This behaviour relates to a multi-interaction binding site, such as a sigmoidal kinetics plot, i.e. non-Michaelis-Menten kinetics. Since L-ASNase does not require effectors, its multi-binding sites should be characterised as the monomers linked together in an enzyme complex, mimicking an allosteric kinetics. Yun, Nourse, White, Rock, and Heath (2007) suggested that *E. coli* type I L-ASNase have four allosteric sites, which were distinct from the catalytic sites, and the allosteric effector been the ASNase itself. Apart from that, Anishkin et al. (2015) supported the idea that *E. coli* type II L-ASNase is in fact a cooperative behaviour.

Conclusion

The purification of a glutaminase-free L-ASNase could help the treatment of ALL patients, since it reduces the side effects of L-ASNase use. On the other hand, the use of ethanol for the purification of noble biomolecules such as L-ASNase, can reduce costs and processing time, increasing productivity.

The present study demonstrated the purification of L-ASNase from *Aspergillus oryzae* by ethanol precipitation and gel filtration chromatography. L-ASNase is stable in a wide range of pH and temperature, and it is specific for L-asparagine, therefore, it has potential to be used as a drug for ALL.

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Capítulo III – Purification of L-asparaginase in aqueous two-phase system

Louise L. Tundisi; Diego F. Coêlho, Adalberto Pessoa- Jr.; Elias B. Tambourgi; Edgar Silveira[6]; Priscila G. Mazzola

A ser submetido à Bioresource technology

Abstract:

L-asparaginase (L-ASNase) belongs to the gold standard treatment for acute lymphoblastic leukemia that manly affects pediatric patients, and treatment increases survival from 20% to 90%. There is a strong worldwide tendency to seek alternative routes to produce pharmaceutical active ingredients obtained by biotechnological processes. Highly purified L-ASNase, would enable more efficient and successful use with reduced risk of toxicity and allergies. There is a urge for cheaper purification processes. Aqueous two-phases system (ATPS), requires less steps, less processing time and scaling up operation is easier, thus requiring a lower cost if compared to the classics processes. Partitioning on a aqueous two-phases system, depends on biomolecule properties likewise system properties (such as type of phase forming salt, molecular weight and concentration of phase forming polymer, concentration of salt and phase volume ratio). In this study, PEG 4000 and 6000 and sodium sulfate were used to form different systems varying its concentrations from 15% - 20% (w/w) and 8% - 13% (w/w) respectively. L-asparaginase partitioned to salt-rich bottom phase, showing a negative Kp log, purification factors of 0.8 – 1.58, and a satisfactory recovery, 86% being the highest.

Keywords: aqueous two-phase system, protein extraction, L-asparaginase, purification.

Introduction

L-asparaginase (ASNase) is an enzyme used as antineoplastic agent in multidrug chemotherapeutic regimens against acute lymphoblastic leukemia (ALL), lymph sarcoma and many others malignancies of the lymphoid system. (Avramis & Tiwari, 2006; Lopes et al., 2015). This enzyme catalyzes extracellular asparagine (Asn) hydrolysis, which produces L-aspartate and ammonia; since many malignant cells lose their ability to synthetize Asn, the rapid depletion damage cell functions, blocking protein synthesis leading to apoptosis (Bussolati et al., 1995).

ALL is the most common leukemia among pediatric patients (Asselin, Gaynon, & Whitlock, 2013), its survival rate has increased to almost 90% with the

multidrug treatment containing L-asparaginase (Kamps et al., 2009). Even the good out weighing the bad, if used for a long-term treatment, it's likely to occur some side effects as hypersensitivity, usually followed by allergic reactions (Pieters et al., 2011) or even hepatotoxicity (Narta et al., 2007; Özdemir, Turhan, Eren, & Bor, 2017)

Whilst organic acids and industrial enzymes do not require a high degree of purity, and subsequently no need for chromatographic operations. On the other hand, pharmaceutical products must meet purity requirements for both medical and therapeutic use. Highly purified L-ASNase, would enable more efficient and successful use with reduced risk of toxicity and allergies. As purity requirements can be quite high (up to 99.98% for human use), it demands a thorough removal of contaminants, which might yield a complex multistep purification process (Mazzola et al., 2008; Molino et al., 2013; Tundisi et al., 2017)

A downstream process with elevated number of purification steps can account to up to 80% of the final production cost of biotechnological products and increases losses of the target molecule, as much for its obtaining in low concentrations as for factors related to the sensitivity of biomolecules to external factors (Iqbal et al., 2016; K. S. M. S. Raghavarao, Rastogi, Gowthaman, & Karanth, 1995; Wheelwright, 1989a)

Thus, studying efficient techniques and minimizing the number of operations required for the desired purification has been a major concern in this area. In addition to these aspects, the development of low-cost and viable techniques in scale-up should be considered, as biotechnology moves from the laboratory bench to the industry (Belter et al., 1988; GUPTA, 1994; Tundisi et al., 2017). This urge to an economically advantageous production encourages seeking alternative purification methods.

Conventional high-purity purification methods, such as chromatography, crystallization and dialysis, are time consuming, demanding, expensive and result in low yields (P. Å. Albertsson, 1986; Molino et al., 2013). Aqueous two-phases system (ATPS), a liquid-liquid extraction (LLE) method, requires less steps, less processing time and scaling up operation is easier. ATPS gives some advantages over conventional purification techniques, such as a higher recovery with pharmaceutical-grade purity and a shorter processing time (Ketnawa, Rungraeng, & Rawdkuen, 2017)

and has been used in purification of proteins, products from microorganism, virus and others (Mazzola et al., 2006; Nazer, Dehghani, & Goliaei, 2017; Spir et al., 2015; Yücekan & Önal, 2011).

Liquid-liquid extraction is a process of partitioning a solute from a liquid phase to another liquid immiscible phase in contact with the first one, where the solute has more affinity. (Asenjo, 1990). ATPS can be formed when two water-soluble polymers are combined or one polymer and a specific salt are mixed above a certain concentration in a solution, which separates spontaneously into two-phases, resulting in a biomolecule partitioning and recovery (Fig. 3.1). Protein partitioning in two-phase systems, is commonly susceptible to conditions such as the isoelectric point, surface hydrophobicity, temperature, molar weight and concentration of system's components, pH and salt addition (R. R. G. Soares et al., 2015).

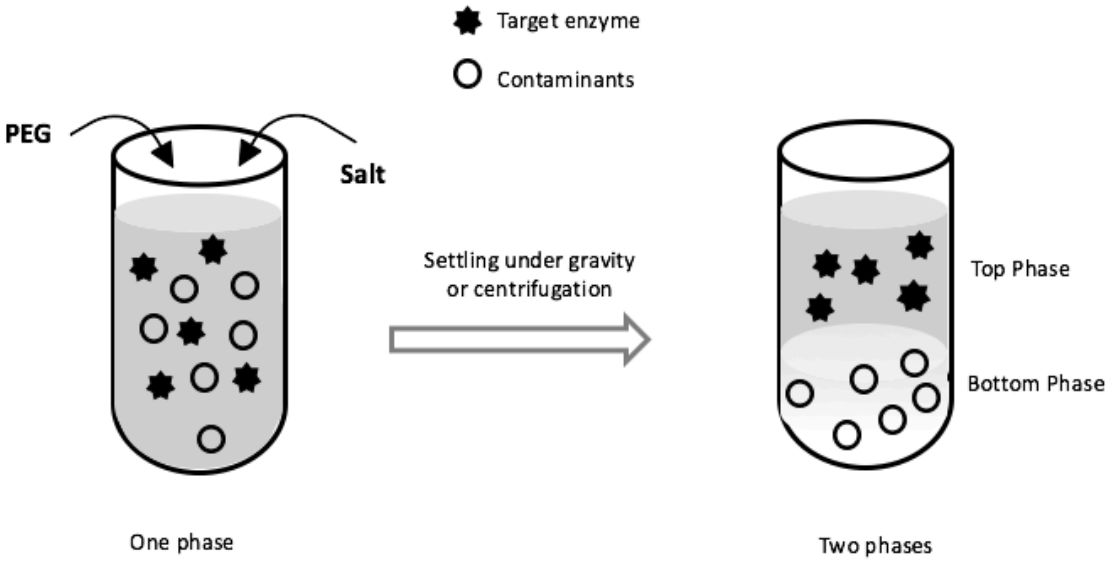

Fig. 3.1 - Schematic diagram of aqueous two-phases partitioning system (ATPS)

Phase partitioning is a mild method for recovering biological materials because of its low interfacial tension, non-toxicity, high water content of up to 70-90% in both liquid phases. It is a rapid separation with minimal enzyme denaturation and loss of biological activity, selective separation and low interfacial tension, resulting in high mass transfer(P.Å. Albertsson, 1986; Coelho et al., 2013; Lopes et al., 2015; Rosa et al., 2011; Walter & Fisher, 1985).

The objective of this work is to study L-asparaginase partition using aqueous two-phases systems with different compositions. Varying concentrations and MW of PEG and different concentrations of sodium sulphate (Na_2SO_4) selected based on the binodal reported for the respective system.

Materials and Methods

L-asparaginase

L-ASNase (E.C. 3.5.1.1) from *Aspergillus oryzae* was donated by Novozymes (Araucária, PR, Brazil). The inicial material was precipitated using ethanol in concentration of 60%, in order to have a free glycerol L-asparaginase to be used in the essay.

Nessler assay for L-asparaginase enzymatic activity

L-ASNase activity was determined by estimating the amount of ammonia produced during hydrolysis. Enzyme assay mixture of 1.05 mL in 50 mM Tris–HCl (pH 8.6) with 50 µL of L-asparagine solution, and 50 µL enzyme solution. The reaction was conducted at 37 ºC for 30 minutes when 50 µL of 1.5 M trichloroacetic acid (TCA) was added to stop reaction. 200 µL of reaction mixture supernatant was added to 4.3 mL of deionized water and 500 µL of Nessler's reagent to measure the released ammonia after L-asparagine hydrolysis. Analysis 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.

Protein Measurement

Protein concentration was determined by UV 280 nm. The calibration curve was obtained from stock solutions of bovine serum albumin (BSA).

Aqueous two-phases system preparation

PEG solutions with different molar masses, specifically 4000 and 6000 g/mol, were dissolved (50% w/w) in distillated water and transferred to 15-mL

graduated tubes together with sodium sulphate. Aliquots of the asparaginase preparation corresponding to 10% (w/w) of the total mass of system were later added, water was added to form a 5 g system. After 15 s of vortex, the two phases were separated by 10 min centrifugation at 2000G and at 4°C. The volume of each phase was then measured, followed by protein concentration and L-asparaginase activity determination. To avoid interference of PEG, dichloromethane was used to extract it from each phase.

Experimental design

A 2^2 factorial design with central point was used to evaluate the influence of two independent variables, namely PEG concentration (X_1) and sulphate salt concentration (X_2) on two systems, evaluating: partition coefficient, activity yield and purification factor of the L-asparaginase.

The adjust of the model was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA); the first-order model equation was determined by Fischer's test. The experimental values were analized with Design Expert (© Stat-Ease, Inc. 2017, Minneapolis, MN, United States (U.S.).

Determination of the partition coefficient, activity yield and purification factor

L-Asparaginase partition coefficient was determined as the ratio of the collagenase activity in the top phase (A_t) to that in the bottom phase (A_b):

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

The activity yield was defined as the ratio of A_b to the initial activity in the asparaginase preparation (A_i) and expressed as a percentage:

$$Y(\%) = \left(\frac{Ai}{Ab}\right).100$$

The purification factor was calculated as the ratio of the specific activity in the bottom phase (A_b/C_b) to the initial specific activity in the asparaginase preparation before partition (A_i/C_i):

$$PF = \frac{\left(\frac{Ab}{Cb}\right)}{\left(\frac{Ai}{Ci}\right)}$$

where C_b and C_a are the protein concentrations, expressed in mg/mL, in the bottom phase and the asparaginase preparation, respectively.

Results and Discussion

At first, we calculated the total amount of protein in each phase and, after the partition, the total amount of protein in the system decreased (table 3.1). System number four of both systems, with the highest concentrations of PEG and salt, presented a visual precipitation at the interface (figure 3.2), explaining the protein loss. The protein precipitation itself is a consequence of high PEG and salt concentration, which caused an increase of excluded volume effect of the polymeric phase and salting-out effect of the salt-rich bottom phase (Priyanka, Rastogi, Raghavarao, & Thakur, 2012). We could not detect visual precipitates in others systems, but submicron agglomerates are not visual and might have been formed at the interface, causing lower protein values (Amin, Barnett, Pathak, Roberts, & Sarangapani, 2014). The content of precipitated protein layer at the interface could not be collected and analyzed due to its reduced thickness.

According to the pareto analyses, the salt concentration was the only factor that had a significant negative effect, augmenting protein loss with salt increase in PEG 4000 systems (above bonfferoni, 4.86, t-value, 3,53). No factors influenced significantly protein loss in PEG 6000 systems. We then evaluated the partition of the protein

content in the system. Protein partitioned to the salt-rich bottom phase, as showed by the negative log Kp values (Table 3.2 and 3.3).

Table 3.1 – Protein Loss

	Run	%PEG (w/w)	%Na_2SO_4 (w/w)	Total protein loss in the system (%)	Total protein loss bottom phase (%)
PEG 4000	1	15	8	-	35,13
	2	20	8	-	-
	3	15	13	26,65	9,58
	4	20	13	24,77	42,82
	5	17.5	10.5	9,41	18,63
	6	17.5	10.5	4,10	13,59
	7	17.5	10.5	6,21	6,10
PEG 6000	1	15	8	5,76	14,52
	2	20	8	-	-
	3	15	13	-	31,24
	4	20	13	11,38	29,56
	5	17.5	10.5	-	6,74
	6	17.5	10.5	2,56	6,98
	7	17.5	10.5	3,86	8,36

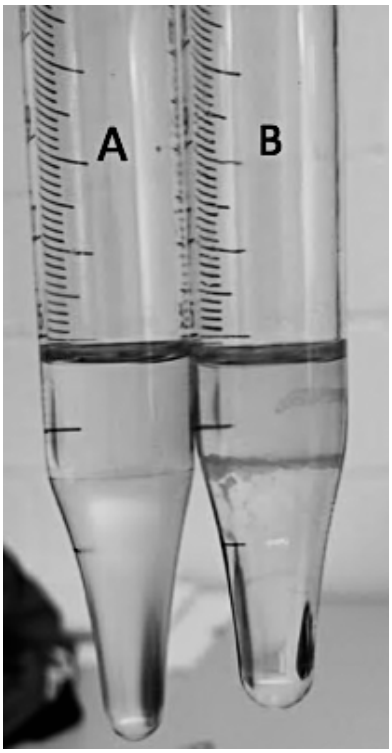

Fig. 3.2 – Picture of ATPS tubes, showing the interface formed in the system number 4. A) System without visual precipitation B) System with visual precipitation

Polyethylene glycol causes interference in Nessler activity essay (Ingham & Ling, 1978), so dichloromethane (CH_2Cl_2) was used to extract PEG traces from ATPS phases. However, PEG removal resulted in a loss of up to 42,8% of total protein in the salt-rich bottom phase (table 3.1). Therefore, the activity partition coefficient of the enzyme could not be calculated. We can, however, affirm that active enzyme partitioned preferentially to the salt-rich bottom phase, as shown by the high activity of this phase and insignificant (below 10U/mL) activity of the PEG-rich top phase (Table 3.2 and 3.3).

Table 3.2 - Effect of phase composition in PEG 4000/salt ATPS on partitioning of L-asparaginase from *A. Oryzae*

	Run	%PEG (w/w)	$\%Na_2SO_4$ (w/w)	At (U/mL)	Ab (U/mL)	Log Kp	Y (%)	PF
PEG 4000	1	15	8	-	234.65	-0.489	67.33	1.22
	2	20	8	-	333.08	-0.307	86.88	1.28
	3	15	13	-	199.45	-0.208	57.23	1.40
	4*	20	13	-	48.78	0.464	14.63	1.33
	5	17.5	10.5	-	202.50	-0.213	58.10	1.27
	6	17.5	10.5	-	215.97	-0.147	61.97	1.28
	7	17.5	10.5	1.17	268.78	-0.208	77.12	1.42

At: Top phase activity; Ab: Bottom phase activity; Log Kp: Log of partition coefficient; Y: activity recovery of the bottom phase; PF: Purification factor of the bottom phase; -: activity values were lower than the quantification limit (1 U/mL); * system with visual precipitation at the interface

Considering that sulphate salts have an ability to promote hydrophobic interactions between the target and contaminant proteins (Yue, Yuan, & Wang, 2007), L-asparaginase would preferentially partitionate to the PEG-rich top phase. L-asparaginase partition pattern towards the salt-rich bottom phase could be a result of high molecular weight PEGs, leading to an exclude-volume effect in the PEG-rich top phase; it is important to highlight that PEG concentration is another factor that influence this effect (Priyanka et al., 2012; Shkel, Knowles, & Record, 2015; Yücekan & Önal, 2011; Yue et al., 2007). Partitioning also depends on biomolecule properties; for example, Cisneros, Benavides, Brenes, and Rito-Palomares (2004) had lutein's higher recovery at top phase using PEG 8000 in its higher concentration (22.9% w/w).

Table 3.3 - Effect of phase composition in PEG 6000/salt ATPS on partitioning of L-asparaginase from *A. Oryzae*

	Run	%PEG (w/w)	%Na_2SO_4 (w/w)	At (U/mL)	Ab (U/mL)	Log Kp	Y (%)	PF
PEG 6000	1	15	8	-	531.01	-0.209	69.25	1.39
	2	20	8	1.00	387.86	0.132	50.58	1.29
	3	15	13	-	590.22	-0.164	76.98	1.19
	4*	20	13	-	244.68	0.212	31.91	0.84
	5	17.5	10.5	-	589.89	-0.002	76.93	1.58
	6	17.5	10.5	-	491.58	-0.161	64.11	1.34
	7	17.5	10.5	-	432.07	-0.121	56.35	1.12

At: Top phase activity; Ab: Bottom phase activity; Log Kp: Log of partition coefficient; Y: recovery of the bottom phase activity; PF: Purification factor of the bottom phase; - activity values were lower than the quantification limit (1 U/mL).* System with visual precipitation at the interface

Zhu, Yan, Chen, and Wang (2007) described a L-asparaginase from *E. coli* in situ extraction using thermoseparating aqueous two-phases system. In his study, he also presented data of systems using PEG 6000 and KHP salt (KHP is the mixture of KH_2PO_4 and K_2HPO_4). The results showed that L-asparaginase partitioned to the PEG-rich top phase, with K_a values of 2.9 – 7.4, a recovery of 75.8% – 87.3% and a purification factor of 0.97-1.69. Compared to our study, the PEG concentration used was lower (8.5% (w/w)) and almost all salt concentrations were higher than those used in this paper (11.6%; 16.3%; 21.5%; 26.9%). These different concentrations might have increased the hydrophobicity of the bottom phase and, together with a higher top phase solvation capability (due to low PEG concentration), contributed to L-asparaginase partition to such phase. Noteworthy, Zhu et al. (2007) work lacks mentioning PEG's interference with the activity quantification by Nessler method, although our group and previous work reported the issue and highlighted the need for

PEG extraction (Ingham & Ling, 1978). PEG should be extracted even when the enzyme partitionates mostly to the salt-rich bottom phase, which contains low PEG amounts, to collect reliable results of enzyme activity.

The systems tested in this work did not present high purification factors (PF) if compared to other authors that reported at least a 2-fold increase (de Albuquerque Wanderley et al., 2017; Nascimento et al., 2016). Purification factor was calculated using total protein obtained after PEG extraction. Low purification factors in this case can be a result of the effect of the high PEG MW. When L-asparaginase is forced to the bottom phase because of the exclude-volume effect (Shkel et al., 2015) on the top PEG-rich phase, other contaminants can be affected by the same effect. Thus, every molecule is forced to partition to the bottom phase, building up a pool of undesired proteins (contaminants) and resulting in a low purification factor. The best system to purify the enzyme was the system with 17.5 % PEG 6000 and 10.5 % Na_2SO_4 (table 3.3).

An alternative scenario is on in which L–asparaginase would not be partitioned altogether with contaminants, but with inactive L-asparaginase. If the specific activity of the bottom phase (table 3.4) is high enough and the “impurities” causing a low purification factors are mostly inactive L-asparaginase, this partitioning could be considered efficient, but would still need to improve the observed activity loss.

Table 3.4 - Effect of phase composition in PEG 4000/6000 and salt ATPS on specific activity of bottom phase.

	Run	% PEG (w/w)	% Salt (w/w)	SA (U/mg)
PEG 4000	1	15	8	188.0282
	2	20	8	196.7314
	3	15	13	214.4883
	4	20	13	204.2712
	5	17.5	10.5	194.9720
	6	17.5	10.5	196.5932
	7	17.5	10.5	217.6790
PEG 6000	1	15	8	213.4446
	2	20	8	128.9547
	3	15	13	243.2593
	4	20	13	206.4198
	5	17.5	10.5	198.5688
	6	17.5	10.5	183.5029
	7	17.5	10.5	172.4095

Conclusion

The use of aqueous two-phases system for L-asparaginase extraction, shows a viability in terms of recovery and specific activity values.

Pareto analyses showed that the salt concentration was the only factor that had significant influence in protein loss on systems with PEG 4000. In PEG 6000 systems any factor studied had significant influence.

As partitioning of enzymes are strongly dependent on the MW of the PEG, by choosing a wider range for the polymer molecular mass and the others studied variables (PEG and salt concentration, and different salts) this extraction method could reach higher values for its purification factor and thus achieve the required pharmaceutical-grade purity.

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DE ALBUQUERQUE WANDERLEY, M. C.; WANDERLEY DUARTE NETO, J. M.; CAMPOS ALBUQUERQUE, W. W.; DE ARAÚJO VIANA MARQUES, D.; DE

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5. Discussão geral

Por haver a necessidade de buscar rotas alternativas para purificação de produtos biotecnológicos, este trabalho utilizou processos como a precipitação por etanol e a extração por sistema bifásico aquoso. Esses processos foram considerados por possuirem as características adequadas, como baixo custo e *eco-friendly* (Grethlein & Lynd, 1992; Lopes et al., 2015; P. A. G. Soares et al., 2012; Vander Griend, 2007).

O passo inicial mais comum para a purificação da L-ASNase é a precipitação com sulfato de amónio. A L-ASNase foi purificada por precipitação fracional de sulfato de amônio a 60-80% de saturação por Kumar et al. (2011), no entanto, seus resultados apresentaram quase 30% de perda da atividade inicial de L-ASNase durante o processo. Proteinas totais precipitaram em um intervalo de concentração de etanol de 50% a 80% (p/p). A precipitação por etanol da L-ASNase proveniente de *A. Oryzae* foi detectada na concentração de 50%, atingindo o seu maior resultado em etanol a 60%. Após precipitação, L-ASNase apresentou um fator de purificação de 2,32 vezes e após a filtração através de gel um fator de purificação de 5,52 vezes. Houve ganho de 6,08% em relação a atividade inicial de L-ASNase de 2748,05 U/mL, após precipitação com etanol 60% (p/p), ao contrário do resultado apresentado por Kumar et al. (2011) ao utilizar sulfato de amonio como agente precipitante, onde teve uma redução de 29,84% da atividade inicial.

Croisfelt et al. (2015) realizou a precipitação por etanol da bromelina, que teve inicio em 30% de etanol. Este resultado juntamento com o obtido no estudo, evidencia que, diferentes proteínas precipitam com diferentes concentrações ótimas de etanol. Esta diferença entre as concentrações ótimas de etanol necessárias para a precipitação de proteínas, como na precipitação de L-ASNase e a precipitação de proteínas totais, por exemplo, é a chave para conseguir a purificação através de um único passo.

Com relação a estabilidade da enzima em diferentes valores de pH, a L-ASNase produzida por *A. oryzae* mostrou-se ativa entre pH 5,8 a 9,0. Porém sua atividade ótima foi alcançada em tampão fosfato pH 7,4 e Tris-HCl pH 7,4 entre todos

os outros tampões testados (tampão citrato-fosfato (50 mM, pH 2,6-5,8), tampão fosfato (50 mM, pH 5,8-7,4), tampão Tris-HCl (50 mM, pH 7,4-9,0) e tampão carbonato (50 mM, pH 9,8 -10,6)). Singh et al. (2013) apresentou resultados semelhantes, atividade ótima em pH 8,5, no entanto, não há sobreposição nos sistemas tampão como foi realizado neste trabalho.

Zuo et al. (2014) demonstraram em seu trabalho que a L-ASNase, recombinante de *Thermococcus gammatolerans,* apresentou uma atividade ótima a 50 mM de Tris-HCl, pH 8,5. É importante notar que em pH neutro, o tampão fosfato apresentou maior atividade do que o tampão Tris-HCl, assim como os resultados descritos neste trabalho. No entanto, o seu ensaio de atividade em diferentes pHs foi medido a 85 ºC, o que poderia levar a uma discrepância nos resultados, já que o nosso foi medido a 37 ºC.

A atividade enzimática também foi medida em diferentes temperaturas em condições ótimas de pH (tampão Tris-HCl 50 mM, pH 7,4 e tampão fosfato 50 mM, pH 7,4). A enzima apresentou atividade entre 5 ºC - 55 ºC, porém exibiu ótima atividade após 15 ºC, mantendo sua atividade máxima até 55 ºC. Singh et al. (2013) relataram que a temperatura ideal da atividade da L-ASNase, de *Bacillus aryahattai*, foi de 40 ºC, e após esse limiar, a atividade enzimática diminuiu substancialmente. Estes paramêtros analisados mostram que a L-asparaginase proveniente de *A. oryzae* pode ser conveniente para uso humano, já que a mesma apresenta atividade a 37^{o}C.

A L-ASNase de *Bacillus coagulans* (Law & Wriston Jr, 1971b), *Arabidopsis thaliana* (Gabriel et al., 2012), *Pyroccocus furiosus* (Bansal et al., 2012) e *Escherichia coli* (Derst et al., 2000), apresentaram resultados semelhantes a L-ASNase de *A. oryzae* descritos neste estudo. A cinética de L-ASNase de *A. oryzae* foi realizada com tampão Tris-HCl e tampão fosfato e mostrou Vmax de 3,43 e 3,85 Km baixo de 3,55 e 4,04 e n de 3,01 e 3,05, respectivamente. Parâmetros cinéticos da L-Asnase proveniente de *A. oryzae* não haviam sido relatadas anteriormente.

Outras purificações também foram feitas através de precipitação com etanol integrada com sistemas bifásicos aquosos. Nos sistemas bifásicos aquosos estudados, o log do coeficiente de partição de proteínas totais com valores negativos indica que a partição da L-Asnase foi preferencialmente para fase contendo sal.

Entretanto, considerando que os sais de sulfato têm uma capacidade de promover interações hidrofóbicas entre as proteínas alvo e contaminantes (Yue et al., 2007) a L-ASNase, preferencialmente, particionaria para a fase superior,rica em PEG. A partição de L-ASNase para a fase inferior pode ser um resultado de um aumento da massa molecular de PEG, levando a um efeito de volume de exclusão na fase superior. É importante ressaltar que a concentração de PEG também pode influenciar neste efeito (Shkel et al., 2015; Yücekan & Önal, 2011; Yue et al., 2007).

O fator de purificação obtido foi > 1, porém não podendo ser considerado alto quando comparados com outros autores que observaram pelo menos duas vezes a atividade específica inicial, 2, 74 – 27,62 vezes (de Albuquerque Wanderley et al., 2017; Nascimento et al., 2016). Podemos considerar que este fator de purificação baixo se deve ao efeito de exclusão causado pelo PEG com alto peso molecular utilizado no estudo (Shkel et al., 2015), ou mesmo pela hipótese de que a L-ASNase não teria sido particionada com juntamente com contaminantes, mas com L-ASNase inativa.

A recuperação alcançada foi satisfatória, todas acima de 50%, sendo o maior valor 86,88%. Exceto para o sistema com maiores concentrações de sal (13% (p/p)) e PEG (20% (p/p), pois esse sistema mostrou precipitação na interface.

A precipitação em si é uma consequência das altas concentrações de PEG e sal utilizadas, o que causou um aumento do volume da fase polimérica e da fração salina na fase inferior e subsequentemente um efeito *salting-out* da proteína presente (Priyanka et al., 2012).

(Zhu et al., 2007) descreveu uma extração de L-asparaginase *in situ* de *E. coli* usando o sistema bifásico aquoso de termo separação. Em seu estudo, ele também apresentou dados de sistemas utilizando PEG 6000 em concentrações menores que 8,5% (p/p), porém utilizando KHP como sal (KHP é a mistura de KH2PO4 e K2HPO4) em diferentes concentrações, (11,6%, 16,3%, 21,5%, 26,9%).. Os resultados mostraram que a L-ASNase particionava para a fase superior, rica em PEG, com valores de Ka de 2,9-7,4, uma recuperação de 75,8% a 87,3% e um fator de purificação de 0,97-1,69. Diferentemente deste trabalho, a concentração de PEG utilizada por Zhu foi menor e quase todas as concentrações de sal foram maiores do que as utilizadas neste trabalho. Combinadas, essas diferenças entre o sal e as

concentrações de PEG, pode ter ocorrido um aumento na hidrofobicidade da fase inferior e, juntamente com uma capacidade de solvatação maior da fase superior, devido à baixa concentração de PEG, conduziu a L-asparaginase a partição para essa fase.

A dificuldade encontrada com o uso do PEG foi sua interferência no método de quantificação de atividade utilizado. Os testes evidenciaram que o PEG precisa ser extraído para que se possa quantificar a atividade da L-asparaginase (Ingham & Ling, 1978). No entanto, Zhu et al. (2007) não menciona a interferência do PEG no método de quantificação da atividade –Nessler em seu trabalho. Ao particionar seletivamente a enzima para fase inferior, permite-se que o ensaio enzimático L-ASNase seja realizado após a remoção de PEG, como foi feito neste trabalho, que também está presente (muitas vezes em traços) na fase rica em sal. O uso do diclorometano para a extração do PEG de ambas as fases do sistema, resultou em uma perda na quantidade total de proteínas, 2-52%.

Como a partição de enzimas é fortemente dependente do peso molecular do PEG, escolhendo uma gama mais ampla para a massa molecular do polímero e as demais variáveis estudadas (concentrações de PEG e sal e diferentes) este método de extração pode atingir valores mais elevados de purificação, e assim conseguir a pureza farmacêutica exigida.

6. Conclusão

A obtenção de um biofármaco envolve uma série de processos que vão desde sua produção a sua purificação. Ao utilizar métodos alternativos na purificação, pode-se reduzir o custo total de produção.

O uso de etanol para a purificação de biomoléculas nobres, como a L-Asparaginase, pode reduzir custos e aumentar a produtividade.

A L-ASNase é estável em uma ampla faixa de pH e temperatura, e é específico para L-asparagina, portanto, tem potencial para ser usado como um fármaco para LLA. A purificação de uma L-ASNase livre de glutaminase poderia ajudar no tratamento de LLA, uma vez que reduz os efeitos secundários do uso de L-ASNase.

O processo de extração da L-ASNase proveniente de *A. Oryzae* por sistemas bifásicos aquosos com a utilização de PEG 4000 e 6000 e sulfato de sódio se mostrou viável. Além disso, como a literatura sobre a recuperação da L- ASNase utilizando sistemas aquosos de duas fases é escassa e nenhum processo sobre a obtenção de L- ASNase de *A. Oryzae* foi relatado, estes resultados intensificam o preenchimento das lacunas existentes e contribui para construir sua literatura de referência.

A purificação de L- ASNase proveniente de *A. Oryzae* por sistema bifásico aquoso se mostra possível com a variação de peso molecular do PEG e de sua concentração, é importante ressaltar que a partição depende das propriedades das biomoléculas, bem como das propriedades do sistema (como o tipo de fase que forma sal, peso e concentração de polímero formador de fase, concentração de sal e proporção de volume de fase).

Os resultados mostram que os processos alternativos podem levar a uma produção mais rápida e barata de um medicamento extremamente importante no tratamento de LLA.

L-asparaginase proveniente de *A. Oryzae* foi purificada com o uso de métodos alternativos, obtendo bons resultados, contribuindo para o estudo do processo de purificação da enzima e sua literatura. É necessário que sejam feitos outros estudos para os sistemas bifásicos aquosos analisando-se os efeitos que outros compostos e sua concentrações possam ter sobre a partição da L-asparaginase. Assim, podendo atingir grau de purificação e recuperação adequados

para produção em escala industrial de um medicamento, visando atingir produção nacional e posterior registro e comercialização.

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8. Apêndice

Estes dados estão sendo apresentados pois foram utilizados durante a elaboração dos resultados de todos os artigos apresentados na dissertação. Como não haverá a redação de um artigo exclusivo sobre a estabilidade da L-asparaginase e os estudos de estabilidade foram feitos exclusivamente para que pudessemos garantir a realização dos experimentos, estes serão apresentados em forma de apêndice.

8.1. Teste de estabilidade

O teste de estabilidade foi realizado com L-asparaginase (E.C. 3.5.1.1) de *Aspergillus oryzae* doada pela Novozyme (Araucária, PR, Brazil). As concentrações utilizadas dos compostos, foram as maiores concentrações que seriam utilizadas posteriormente nas extrações. PEG 4000 e PEG 6000 20% (p/p) e sulfato de sódio 13% (p/p). As soluções foram colocadas em tubos falcon de 15 mL, 0,5 mL da amostra de L-asparaginase foi adicionada em cada tubo e depois os mesmos foram agitados por inversão.

Foi retirada de cada tubo uma alíquota a qual imediatamente foi realizado o teste de nessler para medir a atividade, admitindo este ponto como o T0. O mesmo foi realizado após 2 e 4 horas, tempos escolhidos de acordo com o intervalo de tempo necessário para a extração. As leituras foram feitas em triplicatas, a 436 nm utilizando espectrofotômetro (Micronal AJX-1900, Campinas, São Paulo).

Tabela 1 – Absorbâncias obtidas de acordo com o tempo, a partir da leitura utilizando o método de nessler.

	PEG 4000	**PEG 6000**	**Sulfato de Sódio**
0h	5,2787	5,3452	5,0461
2h	5,4841	5,6623	5,5053
4h	5,0854	5,3331	5,0189

As variações das absorbâncias não se mostram estatisticamente diferentes, mostrando que não ouve queda na atividade de acordo com o tempo.

9. Anexos

9.1. Publicações

9.1.1. Artigos Publicados

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L-Asparaginase Purification

Louise L. Tundisi,[1] Diego F. Coêlho,[2] Beatriz Zanchetta,[2] Patricia Moriel,[3] Adalberto Pessoa Jr.,[4] Elias B. Tambourgi,[2] Edgar Silveira,[5,6] and Priscila G. Mazzola[3]

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

L-asparaginase (L-ASNase) is the gold standard enzyme used to treat acute lymphoblastic leukemia. This disease primarily affects children; however, treatment increases survival from 20% to 90%. As a bioproduct, it is obtained via a biotechnological process and its purification usually consists of several steps that account for up to 80% of the total production costs. This review discusses available strategies for the purification of L-ASNase and highlights a process with fewer steps, and consequently, lower cost and higher yield. This process emphasizes the possibility of using a novel aqueous two-phase system extraction process to purify L-ASNase.

Keywords: Aqueous-two phase system, biotechnology, downstream process, L-asparaginase, purification

INTRODUCTION

L-Asparaginase (L-ASNase, also called L-asparagine amidohydrolase with Enzyme Commission number 3.5.1.1) is an enzyme that catalyzes the conversion of L-asparagine in its free amino acid form to L-aspartate and ammonia. For over 30 years, L-ASNase has been a mainstay of multidrug chemotherapeutic regimens extensively used for the treatment of malignancies of the lymphoid system, acute lymphoblastic leukemia, Hodgkin's lymphoma and melanosarcoma (1). The use of L-ASNase in anti-cancer therapy is based on its catalytic activity because asparagine is essential for lymphoblast growth. These cancerous cells are unable to produce endogenous L-asparagine, therefore, starvation for this amino acid leads to death of these cells (2).

Alternatively, L-ASNase has a great potential as a food processing aid. During food processing, L-asparagine and reducing sugars that are present in the raw material undergo a Maillard-type reaction, forming acrylamide, which is a potential carcinogen highly toxic in its monomer form (3, 4). This type of reaction usually occurs in cooked plant-based foods, such as potato products, short dough biscuits and cereals (5, 6).

Currently, several L-ASNase agents are commercially available. These are either derived from *Escherichia coli* in its native form (native *E. coli* asparaginase) or as polyethylene glycol derived enzyme (PEG asparaginase). Otherwise, L-ASNase is extracted from *Erwinia chrysanthemi* (*Erwinia* asparaginase). Unfortunately, a therapeutic response by patients rarely occurs without some evidence of toxicity (7), and contamination with glutaminase is one cause of this toxicity (8, 9).

Most commercial applications of enzymes do not require highly purified enzyme preparations; highly purified L-ASNase, however, would enable more efficient and successful use with reduced risk of toxicity and allergies. Additionally, enzyme purification allows for the determination of its primary and three-dimensional structures (10, 11) and therefore a more accurate establishment of its function and kinetic mechanisms and parameters (12).

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Address correspondence to Edgar Silveira, Genetics and Biochemistry Institute, Federal University of Uberlândia, Av. Pará, 1720, Uberlândia-MG, Brazil, 38400-902. E-mail: edgar@ufu.br

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Review

Biopharmaceuticals from microorganisms: from production to purification

Angela Faustino Jozala[a], *Danilo Costa Geraldes*[b], *Louise Lacalendola Tundisi*[b], *Valker de Araújo Feitosa*[c], *Carlos Alexandre Breyer*[d], *Samuel Leite Cardoso*[e], *Priscila Gava Mazzola*[f], *Laura de Oliveira-Nascimento*[f,g], *Carlota de Oliveira Rangel-Yagui*[c], *Pérola de Oliveira Magalhães*[h], *Marcos Antonio de Oliveira*[d], *Adalberto Pessoa Jr*[c,*]

[a] *Universidade de Sorocaba (UNISO), Departamento de Tecnologia e Processo Ambiental, Sorocaba, SP, Brazil*
[b] *Universidade de Campinas (UNICAMP), Instituto de Biologia, Programa de Pós-Graduação em Biociências e Tecnologia de produtos bioativos, Campinas, SP, Brazil*
[c] *Universidade de São Paulo, Departamento de Bioquímica e Tecnologia Farmacêutica, São Paulo, SP, Brazil*
[d] *Universidade Estadual de São Paulo (UNESP), Instituto de Biociências, Campus do Litoral Paulista, SP, Brazil*
[e] *Universidade de Brasília, Faculdade de Ciências da Saúde, Programa de Pós-Graduação em Ciências Farmacêuticas, Brasília, DF, Brazil*
[f] *Universidade Estadual de Campinas, Faculdade de Ciências Farmacêuticas, Campinas, SP, Brazil*
[g] *Universidade Estadual de Campinas, Instituto de Biologia, Departamento de Bioquímica e Biologia Tecidual, Campinas, SP, Brazil*
[h] *Universidade de Brasília, Faculdade de Ciências da Saúde, Departamento de Farmácia, Brasília, DF, Brazil*

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ABSTRACT

The use of biopharmaceuticals dates from the 19th century and within 5–10 years, up to 50% of all drugs in development will be biopharmaceuticals. In the 1980s, the biopharmaceutical industry experienced a significant growth in the production and approval of recombinant proteins such as interferons (IFN α, β, and γ) and growth hormones. The production of biopharmaceuticals, known as bioprocess, involves a wide range of techniques. In this review, we discuss the technology involved in the bioprocess and describe the available strategies and main advances in microbial fermentation and purification process to obtain biopharmaceuticals.

* Corresponding author.
E-mail: pessoajr@usp.br (A. Pessoa Jr).
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9.1.2. Artigos submetidos

Characterization of L-Asparaginase from *Aspergillus oryzae*

Autores: Louise L. Tundisi; Alessandra V. de S. Faria; Adalberto Pessoa- Jr.; Elias B. Tambourgi; Edgar Silveira; Priscila G. Mazzola

Revista: Separation Science and Technology

L-asparaginase in aqueous-two phase system, factors affecting recovery and purification

Autores: Louise L. Tundisi; Diego F. Coêlho, Adalberto Pessoa- Jr.; Elias B. Tambourgi; Laura de O. Nascimento; Edgar Silveira; Priscila G. Mazzola

Revista: Bioresource technology

9.1.3. Anais de congressos

CHARACTERIZATION OF L-ASPARAGINASE FROM *Aspergillus oryzae*

TUNDISI, L. L.[1]; PESSOA-JR., A.[2]; TAMBOURGI, E.B.[3]; SILVEIRA, E.[4,5]; MAZZOLA, P. G.[6]

[1]Institute of Biology, Campinas State University (UNICAMP), Campinas, São Paulo, Brazil; [2]Faculty of Pharmaceutical Sciences, Campinas State University, Campinas, São Paulo, Brazil; [3]School of Chemical Engineering, Campinas State University, Campinas, São Paulo, Brazil; [4]Genetics and Biochemistry Institute, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil; [5]Faculty of Medical Sciences, Campinas State University, Campinas, São Paulo, Brazil; [6]School of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil

L-asparaginase (L-ASNase) is the gold standard treatment for acute lymphoblastic leukemia that manly affects pediatric patients. The L-ASNases employed in treatment are from *Escherichia coli* and *Erwinia chrysanthemi*. The characterization of other L-ASNases has been reported, but the choice of the most appropriate is still on debate. This choice should be based on its pharmacokinetics, immune hypersensitivity, doses, prices, pharmacodynamics. The main factors influencing the antileukemic activity of ASNase are enzymatic activity, Km, glutaminase activity, clearance of the enzyme and development of resistance. In this study, L-ASNase from *Aspergillus oryzae* was characterized aiming possible therapeutic use. Four different buffers (phosphate-citrate buffer pH 2.6 to 5.8; phosphate buffer pH 5.8 to 7.4; Tris - HCl pH 7.4 to 9.0; and carbonate buffer pH 9.8 to 10.6) were used to measure the optimum pH for L-ASNase activity. The optimum temperature for enzyme activity was measured at optimal pH conditions (Tris-HCl and phosphate buffer, pH 7.4) at different temperatures ranging from 5 to 55°C. The activity was calculated by quantifying the free ammonia after the enzimatic reaction, using the Nessler reagent. The kinetic parameters calculation, e.g. Michaellis-Menten constant (Km), maximum velocity (Vmax) and Hills coefficient (n), were performed by incubating the enzyme in different concentrations of substrate at optimum conditions of pH and fitted on Hill's equation. This glutaminase free asparaginase showed a low Km (3.39 mM and 3.81 mM), optimum activity between pH 5.8 - 9.0, best activity results with phosphate buffer pH 7.4 and Tris-HCl pH 7.4 and also showed activity from 5°C to 55°C. These results indicate that L-ASNase from *A. oryzae* has potential for use in humans.

Acknowledgments: FAPESP, FAPEMIG, CAPES, CNPq

9.2. Declaração

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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Dissertação de Mestrado, intitulada "***BIOPROCESSOS ALTERNATIVOS DE PURIFICAÇÃO DE L-ASPARAGINASE TERAPÊUTICA PARA LEUCEMIA LINFÓIDE AGUDA (LLA)***", desenvolvida no Programa de Pós-Graduação em Biociências e Tecnologia de Produtos Bioativos do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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Nome do(a) aluno(a): Louise Lacalendola Tundisi

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Data: 05/09/2017

DECLARAÇÃO

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou Anais de congressos sujeitos a arbitragem, que constam da minha Dissertação de Mestrado, intitulada **BIOPROCESSOS ALTERNATIVOS DE PURIFICAÇÃO DE L-ASPARAGINASE TERAPÊUTICA PARA LEUCEMIA LINFÓIDE AGUDA (LLA),** não infringem os dispositivos da Lei n.º 9.610/98, nem o direito autoral de qualquer editora.

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Nome do(a) autor(a): **Louise Lacalendola Tundisi**
RG n.º 338131772 SSP/SP

Profa. Dra. Priscila G. Mazzola
CRF 33311 - Matr. 293994
Faculdade de Ciências Farmacêuticas
UNICAMP

Assinatura:_____
Nome do(a) autor(a): **Priscila Gava Mazzola**
RG n.º 306978775 SSP/SP