Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

Partitioning of *Canavalia brasiliensis* Lectin in Polyethylene Glycol - Sodium Citrate Aqueous Two-Phase Systems

Kelany S. Nascimento[a]; A. M. Azevedo[a]; B. S. Cavada[b]; M. R. Aires-Barros[a]
[a] Institute for Biotechnology and Bioengineering (IBB), Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisbon, Portugal [b] Biochemistry and Molecular Biology Department, Federal University of Ceará (UFC), Fortaleza, Ceará, Brazil

Online publication date: 24 November 2010

To cite this Article Nascimento, Kelany S. , Azevedo, A. M. , Cavada, B. S. and Aires-Barros, M. R.(2010) 'Partitioning of *Canavalia brasiliensis* Lectin in Polyethylene Glycol - Sodium Citrate Aqueous Two-Phase Systems', Separation Science and Technology, 45: 15, 2180 — 2186
To link to this Article: DOI: 10.1080/01496395.2010.507446
URL: http://dx.doi.org/10.1080/01496395.2010.507446

Separation Science and Technology, 45: 2180–2186, 2010

ISSN: 0149-6395 print/1520-5754 online
DOI: 10.1080/01496395.2010.507446

Taylor & Francis
Taylor & Francis Group

Partitioning of *Canavalia brasiliensis* Lectin in Polyethylene Glycol – Sodium Citrate Aqueous Two-Phase Systems

Kelany S. Nascimento,[1] A. M. Azevedo,[1] B. S. Cavada,[2] and M. R. Aires-Barros[1]

[1]*Institute for Biotechnology and Bioengineering (IBB), Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisbon, Portugal*
[2]*Biochemistry and Molecular Biology Department, Federal University of Ceará (UFC), Fortaleza, Ceará, Brazil*

The partitioning of *Canavalia brasiliensis* (ConBr) lectin from *Canavalia brasiliensis* seeds was carried out in aqueous two-phase systems composed of polyethylene glycol (PEG) and sodium citrate. The effect of PEG molecular weight (600, 1000, 2000, and 3350), pH (6.0, 7.0, and 8.0) and ionic strength (0, 2, 5, and 10% NaCl) on the partition behavior of ConBr in ATPS was systematically investigated. Liquid-liquid equilibrium data for the PEG 600-sodium citrate system at pH 6.0, 7.0, and 8.0 were determined at 25°C. The effect of the pH on the position of the binodal curves was not significant, but it had a strong influence in the partitioning of the ConBr. An increase in the NaCl concentration significantly favored the partitioning of ConBr to the top phase, probably due the dehydration of the protein molecules triggered by high ionic strength. The best system for purification of ConBr was composed by 17.54% PEG 600, 15.14% sodium citrate, 5% NaCl at pH 8.0 with recovery yield 70% and 98% purity. This system was found suitable for the recovery of ConBr, since it not only allowed the partitioning of the lectin towards the PEG-rich phase but also the concentration of contaminants in the salt-rich phase.

Keywords aqueous two-phase systems; *Canavalia brasiliensis*; lectin; polyethylene glycol; sodium citrate

INTRODUCTION

Plant lectins are known as carbohydrate reversible binding proteins and possess the ability to agglutinate cells or to precipitate polysaccharides and glycoconjugates (1). Due to their specific binding properties, lectins can be considered important biotechnological tools with wide applications including blood group typing, erythrocyte polyaglutination, lymphocyte subpopulation characterization, and histochemical evaluation of healthy and pathological conditions. Lectins are also recognized as dynamic contributors to differentiate malignant tumor cells from benign cells, being associated with events such as metastasis glycosylation, cell adhesion and localization, signal transduction across membranes, mitogenic stimulation, host immune defense potentiation, cytotoxicity, and apoptosis (2,3).

Legume lectins are one of the most extensively studied plant lectin families, in particular the family Leguminosae, subtribe Diocleinae due to its molecular basis of the protein–carbohydrate interaction. Among these lectins, *Canavalia brasiliensis* (ConBr) is a typical Diocleinae lectin which entails mannose/glucose-binding specificity and its monomer contains 237 amino acid residues and most arranged in two large anti-parallel β-sheets. Its multimeric structure is made up of identical monomers of 25.5 kDa, and it characteristically exhibits pH-dependent dimer-tetramer equilibrium, displaying a mixture of dimers (31%) and tetramers (69%) even at pH 8.5. It is a metalloprotein requiring divalent ions (Ca^{2+} and Mn^{2+}) to exhibit full biological activity (4).

In the past few years, several hundred of these proteins have been purified and characterized in detail, taking into consideration their biochemical properties, sugar-binding specificities, and several biological activities, including anti-tumor, antifungal, and antiviral activities (3,5,6).

The methods used involved usually an ammonium precipitation, followed by ionic and affinity chromatographic steps and a final protein concentration step (7). Chromatographic methods can be time-consuming and expensive, leading to the loss of biological activity and a poor yield of the entire process. The affinity chromatography maintenance is a hardy task when plant crude extracts are loaded into columns because such samples contain pigments, oily components, proteolytic enzymes and other complex substances which could damage the column and impair the purification (8).

Therefore, due to the great importance of the lectins in the biological and medical field, it is essential to develop new purification methods which are more economical and selective, that can handle high contents of solids and complex substances, and allow process integration.

Received 29 November 2009; accepted 24 February 2010.
Address correspondence to M. R. Aires-Barros, IBB, Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, São João de Deus, 1000 Lisbon, 218 417 000, Portugal. Tel.: +351 21 8419065; Fax: +351 21 8419062. E-mail: rabarros@ist.utl.pt

Aqueous two-phase systems (ATPS) have been successfully used in the partition and the purification of human antibodies (9,10), whey proteins (11), amylases (12), amino acids and peptides (13,14), and nucleic acids (15), among many other biomolecules, due to their advantages over traditional methods. One of the main advantages is the high water content in each phase (between 80–90%), which provides a suitable environment for the preservation of biological activity. Additionally, the low interfacial tension, which facilitates the migration of biomolecules through the interface, the easy scale-up, the possibility of processing high content of solids and integrate separation and concentration in just one step, turns this technique particularly attractive for biomolecules purification (16,17).

The present work proposes a new methodology for the purification of ConBr, extracted from the *Canavalia brasiliensis* seeds based in ATPS of polyethylene-glycol (PEG) and citrate salt. Citrate salt will be used as an alternative to phosphate salt commonly used in ATPS since citrates are biodegradable and non-toxic and can be discharged into biological wastewater treatment plants.

EXPERIMENTAL

Chemicals

Analytical grade polyethylene glycols (PEGs) with molecular mass average of 600, 1000, and 2000 were purchased from Fluka (Buchs, Switzerland). However, PEG 3350, citric acid monohydrate, trisodium citrate dihydrate, and sodium chloride (NaCl) were obtained from Sigma (St. Louis, MO, USA). Polymers were used without further purification. Ultrapure water for the experiments was obtained from a Milli Q-system. Finally, crude extracts and the lectin from *Canavalia brasiliensis* seeds were kindly provided by Biologically Active Molecule Laboratory (BioMol-Lab/UFC) from the Federal University of Ceará-Brazil.

Aqueous Two-Phase Diagrams

The determination of the binodal curve was carried out by the turbidimetric titration method (18). Stock aqueous solutions of PEG of 50% (w/w) and sodium citrate of 35% (w/w) were prepared from known quantities of the polymer and salt, respectively. The pH of the sodium citrate stock solutions was adjusted to 6.0, 7.0, and 8.0, by the addition of appropriate quantities of citric acid 35% (w/w). Small aliquots of the sodium citrate stock solution, approximately (0.01 to 0.05) g, were added to 1 g polymer stock solution in a glass tube until the solution became turbid, which was indicative of the formation of a second liquid phase. After the equilibrium was achieved, the two phases became clear and transparent, and the interface became well defined. The mixture composition was determined using an analytical balance at 25°C to measure the weight. For the tie lines determination, at least a series of ATPSs of four different known total compositions were prepared in graduated glass tubes and placed in a thermostatic bath at 25°C. The equilibrium phase composition and tie-line length (TLL) of the PEG 600-sodium citrate systems are shown in Table 1. The biphasic systems were prepared weighted, using an analytical balance with a precision of ±0.0001 g. Samples from the top and the bottom phases were collected, by syringes. The polymer concentration in both phases was determined by refractive index measurements performed at 25°C using a refractometer 30PX from Mettler Toledo (Greifensee, Switzerland) and the concentration of citrate was determined using a conductivimeter from Oaklon Instruments. The TLL was calculate by Eq. (1):

$$\mathrm{TLL} = \sqrt{[\Delta\mathrm{Polymer}]^2 + [\Delta\mathrm{Salt}]^2} \quad (1)$$

where [ΔPolymer] is the difference between PEG concentration in the two phases and [ΔSalt] is the difference between salt concentration in the two phases.

Protein Partitioning in Aqueous Two-Phase Systems

Partitioning of ConBr protein from *Canavalia brasiliensis* crude extracts seeds was carried out in PEG (600, 1000, 2000, 3350)-sodium citrate systems. The partition experiments were performed at different pH levels, molecular

TABLE 1
Phase compositions for the PEG 600/sodium citrate/water system at 25°C, pH 6, 7, and 8. Concentrations expreseed in % (w/w)

	Total composition		Top phase		Bottom phase		
Tie-line	Citrate	PEG	Citrate	PEG	Citrate	PEG	TLL
pH 6.0							
1	17.53	20.31	4.44	40.65	30.26	1.83	46.6
2	16.69	19.34	5.44	37.16	27.61	3.16	40.6
3	15.89	18.41	7.30	32.12	24.82	4.95	32.2
4	15.14	17.54	10.30	25.49	19.96	10.01	18.2
pH 7.0							
1	17.52	20.31	4.09	41.20	30.40	0.09	48.8
2	16.69	19.34	5.16	37.38	28.03	0.65	43.3
3	15.90	18.42	6.60	33.07	25.38	1.67	36.6
4	15.14	17.54	9.32	26.32	21.87	5.23	24.5
pH 8.0							
1	17.53	20.31	3.82	41.86	29.15	2.71	46.6
2	16.69	19.34	4.78	38.28	27.04	3.36	41.4
3	15.90	18.42	6.15	34.17	24.58	4.69	34.8
4	15.16	17.54	8.26	28.42	21.03	8.03	24.1

weight, and NaCl concentrations at constant temperature (25°C). Initially the concentrations of NaCl investigated were 0, 5, and 10% and taking into account the results obtained it was decided to study the region between 0% and 5% NaCl using 2% NaCl.

Phase systems were prepared in 15 ml graduated centrifuge tubes by weighting appropriate amounts of PEG of desired molecular weight and of sodium citrate from stock solutions and filling in with water, until a final weight of 5 g was achieved. On the other hand, for the systems with sodium chloride, the powdered salt was directly dissolved into the systems. The amount of extract added to these systems was 5 mg and the contents were thoroughly mixed.

After the mixture was prepared, the systems were centrifuged at $3000 \times g$ for 10 min (Eppendorf, Hamburg, Germany). Afterwards the tubes were brought into equilibrium in a thermostatic bath at 25°C for 12 hours. Finally, the volumes of the top and bottom phases were measured and the samples from each solution were collected using a syringe to determine the protein concentration. It was necessary to dilute the samples due to the high viscosity of the polymer solution.

The ConBr partition coefficient was defined as the ratio of equilibrium concentration of the protein in the top phase $[C]_{top}$ and in the bottom phase $[C]_{bottom}$,

$$K = \frac{[C]_{top}}{[C]_{bottom}} \quad (2)$$

The recovery yield of ConBr in the top phase (Y_C) was defined as the ratio between the mass of ConBr in the top phase and the total mass of ConBr added to the system expressed in percentage

$$Y = \frac{mConBr_{Top}}{mConBr_{Total}} \times 100\% \quad (3)$$

Purity (P) was defined by ratio of the area of the ConBr peak to the total area of the chromatogram subtracted by the total area of the blank chromatogram. All partitioning experiments were carried out in duplicate.

Size Exclusion Chromatography (SEC)

The purity and the concentration of both phases was evaluated by size exclusion chromatography (SEC) using a TSK-GEL Super SW3000 column (30 cm × 4.6 mm I.D., 4 μm) and a TSK-GEL super SW guard column (3.5 cm × 4.6 mm I.D.) from Tosoh Bioscience (Stuttgart, Germany). Samples from both phases were diluted at least 10 times in SEC buffer and run in isocratic mode at 0.35 ml/min with 20 mM sodium phosphate buffer at pH 7.0 with 150 mM NaCl for 25 min. Absorbance was monitored at 215 nm.

RESULTS AND DISCUSSION

Phase Diagram for the PEG 600-Sodium Citrate Systems

Phase diagrams for the PEG 600-sodium citrate system at pH 6.0, 7.0, and 8.0 at 25°C were obtained in order to define the best system for ConBr selective extraction. Four tie-lines were chosen for each pH value, according to the data shown in Table 1. Figure 1 presents the phase diagrams obtained for the PEG 600-sodium citrate system at pH 6.0, 7.0, and 8.0 at 25°C. The influence of pH on the binodal curves of PEG-citrate systems was also analyzed, in order to understand if ConBr partitioning could be affected by binodals displacement. Figure 2 shows the

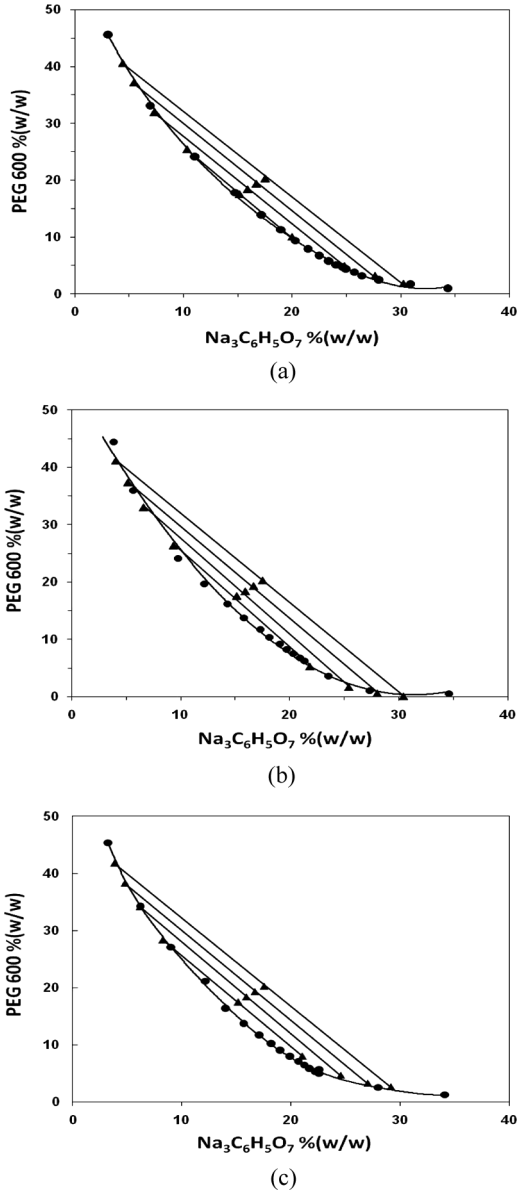

FIG. 1. Liquid-liquid equilibrium diagrams for PEG 600-sodium citrate ATPS, (a) pH 6.0, (b) pH 7.0, and (c) pH 8.0 at 25°C.

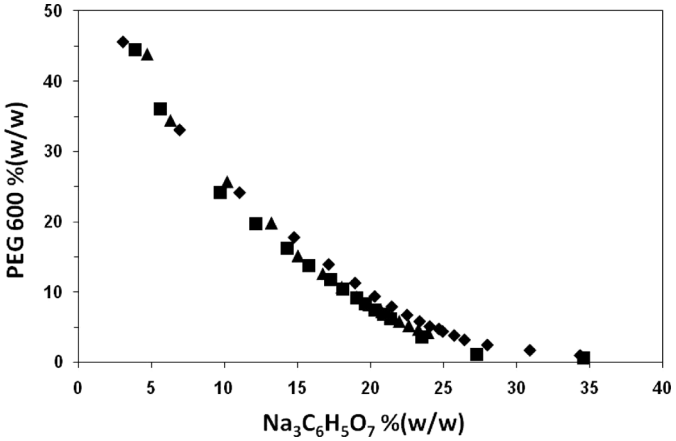

FIG. 2. Effect of pH on the phase diagram for PEG 600-sodium citrate ATPS: pH 6.0 (◆), 7.0 (■), and 8.0 (▲) at 25°C.

effect of the pH values, varying from 6.0 to 8.0, on the binodal curves. For the three pH values tested the binodals obtained were almost coincident, and thus the displacement of binodals was considered negligible at the pH values tested.

Effect of PEG Molecular Weight and pH on Lectin ConBr Partitioning

This study was carried out using phase systems formed by 17.54% w/w PEG 600 15.54% w/w sodium citrate. For the higher tie-line lengths it was not possible to perform partition assays due to protein precipitation and to the difficulty in obtaining an effective phase separation after centrifugation.

Thus the effects of the polymer molecular weight (600, 1000, 2000, and 3350) were evaluated at pH 6.0, 7.0, and 8.0 only for the shortest tie-line (17.54% PEG 600, and 15.14% sodium citrate). It was observed that there was a decrease in the polymer molecular weight leading to an increase in the partitioning coefficient (Kp), although ConBr remained essentially in the citrate phase (Fig. 3). The decrease in the protein partition coefficient with increasing PEG molecular weight in PEG-salt systems has been described previously and might be attributed to the excluded volume effects (19). The best results were obtained with the lower molecular weight polymer, PEG 600 at pH 8.0, with recovery yields on the PEG top phase of about 37%.

The effect of pH on the partition coefficient and recovery yield of ConBr was also studied, as shown in Fig. 3. According to the results obtained the partition coefficient of ConBr increases with pH and a higher recovery of ConBr was achieved in the PEG-rich phase. Extraction yields of 13.6, 15.0, and 37.1% were achieved in an ATPS composed by 17.54% (w/w) PEG 600 and 15.14% (w/w)

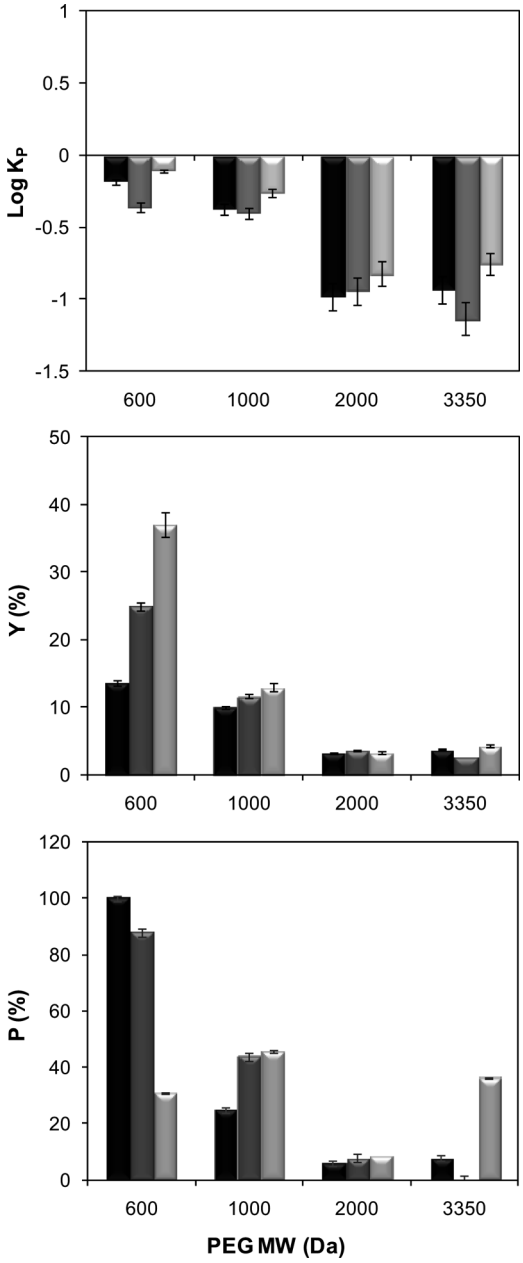

FIG. 3. Effect of polymer molecular weight, at pH 6.0 (■), 7.0 (■), and 8.0 (■), on the partition coefficient (Log Kp), yield (Y) and purity (P) of ConBr, with 17.54% PEG 600, 15.14% sodium citrate ATPS.

sodium citrate, for pH 6.0, 7.0, and 8.0, respectively. The variation of pH values did not affect the phase ratio, which was kept around 1.0.

The partitioning behavior of the ConBr probably is governed by a combination of hydrophobic and electrostatic interactions. It has been reported that the lectin of *Canavalia brasiliensis* seeds, is structurally active in a state of dimer-tetramer equilibrium in the range of pH around 5.0 to 8.5 with the content of tetrameric form increasing

with the pH (20). Hence, one could expect a decrease in the partitioning of ConBr with an increase in the pH due to size exclusion effects. On the other hand, for pH higher than 7.4, the protein has an overall negative charge and repulsions are expected to occur between the protein and the negatively charged citrate anion. In addition, the more hydrophobic tetramer form probably interacts preferentially with the more hydrophobic PEG-rich phase, enhancing the extraction of ConBr to the polymer-rich phase and explaining the increase of the recovery yield with the pH. Nevertheless, ConBr remains preferentially in the citrate-rich phase at all the conditions tested, giving low recovery yields, ranging between 13% and 37%, and the partition coefficients lower than 1.0.

Effect of Ionic Strength on the ConBr Partitioning

In order to improve the partition of ConBr to the PEG-rich phase different concentrations of NaCl were added to an ATPS containing 17.54% PEG 600 and 15.14% sodium citrate at pH 6.0, 7.0, and 8.0. The effect of NaCl concentration on the partition coefficient and recovery yield of ConBr was studied in the range from 0 to 10% (w/w) and is illustrated in Fig. 4. It can be observed that the partition coefficient of ConBr increased with an increase in the NaCl concentration. In addition the logarithm of the Kp became positive for concentrations higher than 5%, meaning that ConBr partitioned preferentially to the top phase at higher ionic strength.

It is well known that protein molecules are associated with a hydration shell in solution and that the bound water hinders protein molecules from binding to the hydrophobic molecules. When NaCl is added to the system the lectin becomes dehydrated due to the hydration effect of the salt molecules surrounding the protein. Thus, the hydrophobic zones of the lectin will be naked gradually with increasing of the salt concentration. In other words, the naked hydrophobic surface increases with increasing salt concentration, making the hydrophobic interactions between the lectin and the hydrophobic molecules stronger (21). At pH 8.0, ConBr is mainly a tetramers form, whose association between the molecules is higher, thus the protein solubility in the salt phase decreases and consequently the protein affinity with polymer phase increases. Besides, the addition of NaCl to a PEG–phosphate biphasic system increases the hydrophobicity difference between the phases and thus promotes the partition of the more hydrophobic proteins to the PEG rich phase (22).

Using 5% of NaCl the recovery yield of ConBr in the top phase was higher than 70% and the purity was about 98%, with contaminant proteins partitioning towards the bottom phase, as it can be seen in the electrophoresis gel in Fig. 5 (lane 7). For 10% of NaCl, 96% of ConBr was recovered on the top phase, although with a lower purity of 65%, as shown in Fig. 5 (lane 9).

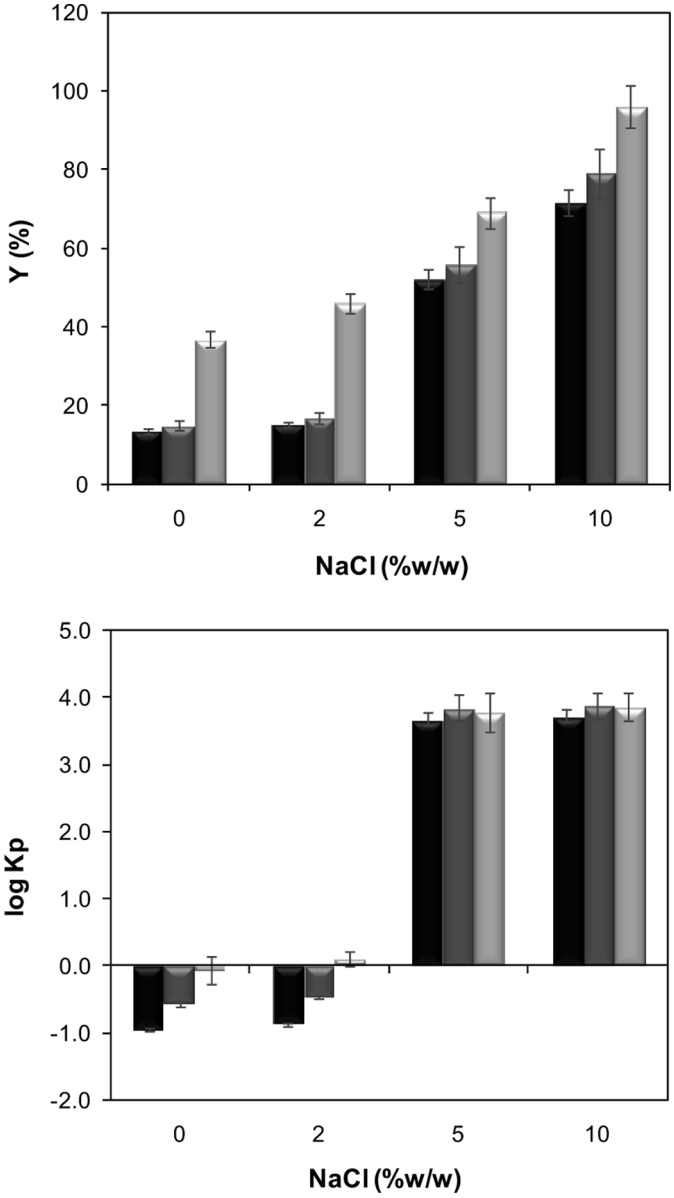

FIG. 4. Effect of NaCl concentration in the partition coefficient (Kp) and recovery yield (Y) of ConBr with 17.54% PEG 600, 15.14% sodium citrate ATPS at pH 8.0 and NaCl ranging from 0 to 10%.

Hence, 5% NaCl concentration allows a complete separation between ConBr (top phase) and contaminants (bottom phase), the first partitioning mostly to the top phase and the latter remaining in the bottom phase. Nevertheless, the recovery yield of ConBr obtained for 5% NaCl (70%) was lower than the one obtained for 10% NaCl (96%).

In a further evaluation of the effect of NaCl concentration on both the purity and the recovery yield of ConBr in the PEG-rich phase, one can conclude that NaCl has an opposite effect, with purity decreasing while recovery increasing as the NaCl concentration increases. The effect

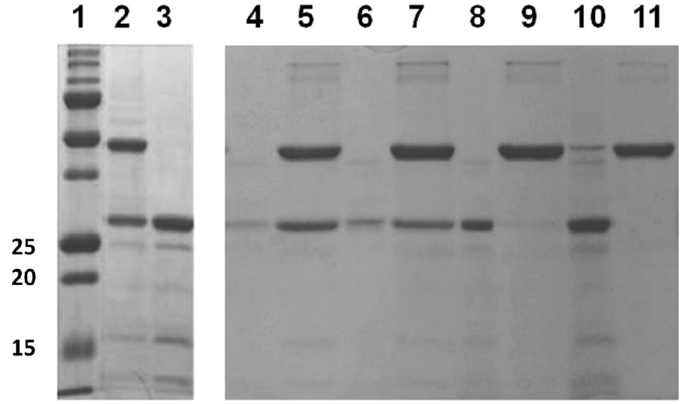

FIG. 5. SDS-PAGE for the variation of the addition of NaCl (0, 2, 5, and 10% w/w) to the top and bottom phase system composed by 17.54% PEG 600/15.14% sodium citrate buffer pH 8.0. Lane 1: molecular weight markers (from top to bottom: 250, 150, 100, 75, 50, 37, 25, 20, and 15 kDa); lane 2: extract of ConBr seeds; lane 3: pure ConBr lane 4: top phase (TP) of the system without NaCl; lane 5: bottom phase (BP) of the system without NaCl; lane 6: TP composed by 2% NaCl; lane 7: BP composed by 2% NaCl; lane 8: TP composed by 5% NaCl; lane 9: BP composed by 5% NaCl; lane 10: TP composed by 10% NaCl; lane 11: BP composed by 10% NaCl. ConBr is characterized by three bands of 30 kDa (chain α), 18 kDa (chain β) and 12 kDa (chain γ).

of increasing NaCl concentration upon protein partition behavior has been explained based upon the protein hydrophobicity and electrostatic interactions. In the case of ConBr, the decrease in protein purity when high NaCl concentrations were used may be explained by a migration of contaminant proteins also to the top phase. ATPS with 5% NaCl concentration exhibited the best protein purity but the recovery was lower than 10% NaCl concentration, may be due the precipitation that was observed on the interface.

The positive effect of NaCl on driving biomolecules partitioning to the polymer top phase has been already demonstrated, namely in our group for the purification of antibodies with PEG-phosphate systems (23) and PEG-citrate systems (24). Thus, by changing the concentration of NaCl and choosing the right system composition it can be possible to selectively target the partition of the target biomolecule to the phase with fewer impurities. An increase in the partition coefficient with an increase of NaCl could have also been observed with the higher molecular weight PEGs; however, the results would never be as good as the ones obtained with PEG 600, as these systems presented already a very high precipitation at low salt concentrations.

CONCLUSIONS

An aqueous two-phase system based on PEG-sodium citrate was successfully used for the separation of ConBr lectin from *Canavalia brasiliensis* extracts. Various factors have been found to influence the partitioning of ConBr to the top phase, including PEG molecular weight, ionic strength, and system pH. It was shown that lower molecular weights, e.g., PEG 600, high pH values, and high ionic strengths are beneficial for the extraction of ConBr from seed extracts. The most favorable composition for the selective recovery of ConBr was determined to be 17.54% PEG 600, 15.14% sodium citrate, 5% NaCl, at pH 8.0. For this system, approximately 70% of ConBr could be extracted from *Canavalia brasiliensis* extracts seed with 98% purity, revealing the potential of aqueous two-phase extraction as an alternative process in the downstream processing of lectins.

ACKNOWLEDGEMENTS

K. S. Nascimento appreciates the financial support provided for this work by CNPq (National Counsel of Technological and Scientific Development) – Brazil. A. M. Azevedo acknowledges the program "Ciência 2007" of the Portuguese Ministry for Science, Technology and Higher Education.

REFERENCES

1. Sharon, N.; Lis, H. (1990) Legume lectins – a large family of homologous proteins. *FASEB J.*, 4: 3198–3208.
2. Wang, H.; Ng, T.B.; Ooi, V.E.C.; Liu, W.K. (2000) Effects of lectins with different carbohydrate-binding specificities on hepatoma, choriocarcinoma, melanoma, and osteosarcoma cell lines. *Int. J. Biochem. Cell Biol.*, 32 (3): 365–372.
3. Gorelik, E.; Galili, U.; Raz, A. (2001) On the role of cell surface carbohydrates and their binding proteins (lectins) in tumor metastasis. *Cancer Metastasis Rev.*, 20: 245–277.
4. Cavada, B.S.; Barbosa, T.; Arruda, S.; Grangeiro, T.B.; Barral-Netto, M. (2001) Revisiting proteus: Do minor change in lectin structure matter in biological activity? Lessons from and potential biotechnological uses of the Diocleinae subtribe lectins. *Curr. Protein Pept. Sci.*, 2 (2): 123–135.
5. Tian, Q.; Wang, W.; Miao, C.; Peng, H.; Liu, B.; Leng, F.; Dai, L.; Chen, F.; Bao, J. (2008) Purification, characterization, and molecular cloning of a novel mannose-binding lectin from rhizomes of Ophiopogon japonicus with antiviral and antifungal activities. *Plant Sci.*, 175 (6): 877–884.
6. Sitohy, M.; Doheim, M.; Badr, H. (2007) Isolation and characterization of a lectin with antifungal activity from Egyptian Pisum sativum seeds. *Food Chem.*, 104 (3): 971–979.
7. Spelzini, D.; Farruggia, B.; Picó, G. (2005) Features of the acid protease partition in aqueous two-phase systems of polyethylene glycol-phosphate: Chymosin and pepsin. *J. Chromatogr. B*, 821 (1): 60–66.
8. de Santana, M.A.; Santos, A.M.C.; Oliveira, M.E.; de Oliveira, J.S.; Baba, E.H.; Santoro, M.M.; de Andrade, M.H.G. (2008) A novel and efficient and low-cost methodology for purification of Macrotyloma axillare (Leguminosae) seed lectin. *Int. J. Biol. Macromol.*, 43 (4): 352–358.
9. Azevedo, A.M.; Rosa, P.A.J.; Ferreira, I.F.; de Vries, J.; Visser, T.J.; Aires-Barros, M.R. (2008) Integrated process for the purification of antibodies combining aqueous two-phase extraction, hydrophobic interaction chromatography, and size-exclusion chromatography. *J. Chromatogr. A*, 1213: 154–161.

10. Azevedo, A.M.; Rosa, P.A.J.; Ferreira, I.F.; Aires-Barros, M.R. (2008) Downstream processing of human antibodies integrating an extraction capture step and cation exchange chromatography. *J. Chromatogr. B*, 877 (1–2): 50–58.
11. Boaglio, A.; Bassani, G.; Picó, G.; Nerli, B. (2006) Features of the milk whey protein partitioning in polyethyleneglycol-sodium citrate aqueous two-phase systems with the goal of isolating human alpha-1 antitrypsin expressed in bovine milk. *J. Chromatogr. B*, 837 (1–2): 18–23.
12. Kammoun, R.; Chouayekh, H.; Abid, H.; Naili, B.; Bejar, S. (2009) Purification of CBS 819.72 [alpha]-amylase by aqueous two-phase systems: Modelling using response surface Methodology. *Biochem. Eng. J.*, 46 (3): 306–312.
13. Berggren, K.; Wolf, A.; Asenjo, J.A.; Andrews, B.A.; Tjerneld, F. (2002) The surface exposed amino acid residues of monomeric proteins determine the partitioning in aqueous two-phase systems. *Biochim. Biophys. Acta*, 1596 (2): 253–268.
14. Zaslavsky, A.; Gulyaeva, N.; Zaslavsky, B. (2000) Peptides partitioning in an aqueous dextran-polyethylene glycol two-phase system. *J. Chromatogr. B*, 743 (1–2): 271–279.
15. Gomes, G.A.; Azevedo, A.M.; Aires-Barros, M.R.; Prazeres, D.M.F. (2009) Purification of plasmid DNA with aqueous two phase systems of PEG 600 and sodium citrate/ammonium sulfate. *Sep. Purif. Technol.*, 65 (1): 22–30.
16. Azevedo, A.M.; Rosa, P.A.J.; Ferreira, I.F.; Aires-Barros, M.R. (2009) Chromatography-free recovery of biopharmaceuticals through aqueous two-phase processing. *Trends Biotechnol.*, 27 (4): 240–247.
17. Rosa, P.A.J.; Ferreira, I.F.; Azevedo, A.M.; Aires-Barros, M.R. (2010) Aqueous two-phase systems: A viable platform in the manufacturing of biopharmaceuticals. *J. Chromatogr. A*, 1217 (6): 2296–2305.
18. Hatti-Kaul, R. (2000) *Aqueous Two-Phase Systems: Methods and Protocols (Methods in Biotechnology)*; Humana Press: New Jersey, USA.
19. Sebastião, M.J.; Cabral, J.M.S.; Aires-Barros, M.R. (1994) Partitioning of recombinant Fusarium solani pisi cutinase in polyethylene glycol-aqueous salt solution two-phase systems. *J. Chromatogr. A*, 668 (1): 139–144.
20. Gabius, H.J.; Andre, S.; Kaltner, H.; Siebert, H.C. (2002) The sugar code: functional lectinomics. *Biochim. Biophys. Acta*, 1572 (2–3): 165–177.
21. Chen, J.; Sun, Y. (2003) Modeling of the salt effects on hydrophobic adsorption equilibrium of protein. *J. Chromatogr. A*, 992 (1–2): 29–40.
22. Rosa, P.A.J.; Azevedo, A.M.; Aires-Barros, M.R. (2007) Application of central composite design to the optimisation of aqueous two-phase extraction of human antibodies. *J. Chromatogr. A*, 1141 (1): 50–60.
23. Azevedo, A.M.; Rosa, P.A.J.; Ferreira, I.F.; Aires-Barros, M.R. (2007) Optimisation of aqueous two-phase extraction of human antibodies. *J. Biotechnol.*, 132 (2): 209–217.
24. Azevedo, A.M.; Gomes, A.G.; Rosa, P.A.J.; Ferreira, I.F.; Pisco, A.M.M.O.; Aires-Barros, M.R. (2009) Partitioning of human antibodies in polyethylene glycol-sodium citrate aqueous two-phase systems. *Sep. Purif. Technol.*, 65 (1): 14–21.