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New aqueous two-phase system based on cashew-nut tree gum and poly(ethylene glycol)

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Abstract

The characterisation of a new system based on cashew-nut tree gum, a branched acidic heteropolysaccharide found in Brazil, and poly(ethylene glycol) (PEG) was studied. Phase diagrams are provided for the PEG–cashew-nut tree gum system. The influence of PEG molecular mass, tie-line length and pH on bovine serum albumin (BSA) partition was investigated. Protein partition coefficient was little influenced by changing PEG molecular mass. Increasing the tie-line length decreased the partition. Increasing the pH also raised the BSA partition coefficient. It is shown that systems formed by PEG and cashew-nut tree gum may be considered as an interesting alternative for use in protein purification.

Keywords: Aqueous two-phase systems; Cashnew-nut tree gum; Poly(ethylene glycol)

1. Introduction

Aqueous two-phase systems (ATPSs) have found widespread use in biochemical research for separation and purification of macromolecules cells and cell particles [1]. In recent years the ATPSs have also found applications in various areas of biotechnology.

The two-phase systems are obtained by combining aqueous solutions of two (water-soluble) polymers, differing in their chemical structure, or by addition of an organic salt (e.g. sulphate or phosphate), at high concentration, to a solution of a polymer. For separation purposes in the laboratory the polymer–polymer type of system has been almost exclusively used, preferentially those consisting of fractionated dextran and poly(ethylene glycol) (PEG) [1,2]. The properties of these systems are well studied but high cost of fractionated dextran prevents the use of this system in large-scale processes [3]. For the large-scale isolation of enzymes the inexpensive PEG–salt systems are been used. This choice has been indicated by economic reasons due to the high cost of fractionated dextran. However, the properties of the PEG–salt systems strongly limit their usefulness for applications in biotechnology mainly due to the high concentration of salt, which may cause the denaturation of sensitive biological structures [4]. Another problem related to the use of PEG–salt systems is waste disposal. ATPSs based on dextran, starch and cellulose derivatives have the advantage of biodegradability [5]. As a consequence, there is a need to

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develop new biphasic systems suitable for large-scale processes. So, it is necessary to find inexpensive substitutes for fractionated dextran with equivalent partition properties. Several polymers, such as starch derivatives [6], maltodextrin [7] and cellulose derivatives [8] have been tested as an alternative to fractionated dextran. The utilisation of polysaccharides may have a big impact in the development of two-phase systems for large-scale purification [6,9].

Exudate gum from *Anacardium occidentale* L. is a branched acidic heteropolysaccharide largely found in Brazil. The polysaccharide is produced in epithelial cells which border the gum ducts and is known to be part of the biochemical defences of the plant [10]. The polysaccharide molecular mass (M_r) is ≈110 000 [11]. The gum contains galactose (73%), glucose (11%), glucuronic acid (6%), arabinose (5%), rhamnose (4%) and mannose (1%) [10]. Nut production in trees older than 25 years increases after gum extraction. Given the importance of cashew tree culture to some regions of Brazil, especially the north-west, a study of the gum for biotechnological application is of potential industrial interest. A structural fragment of the gum is shown in Fig. 1.

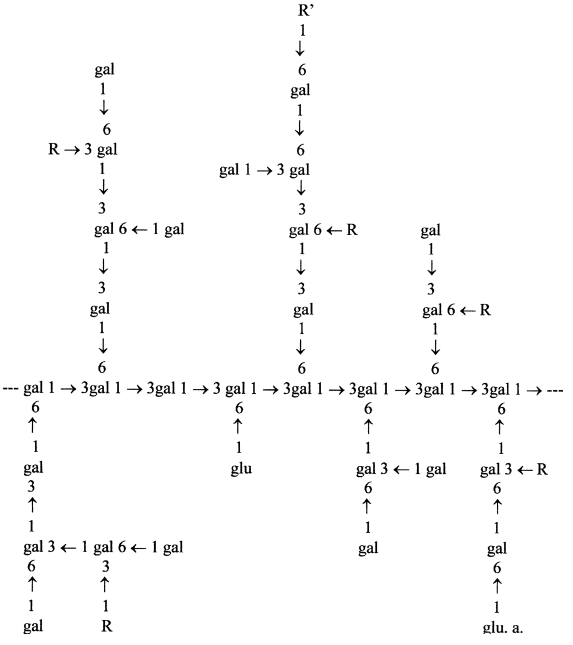

Fig. 1. Structural fragment of cashew-nut tree gum. Gal = galactose, R = D-mannose, L-rhamnose, L-arabinose or arabinose chains with 1,2 linkage. R′ = D-glucose (glu) or D-glucuronic acid (glu. a.) [12].

In this work we report on the use of cashew-nut tree gum. The potential utilisation of this gum as an aqueous-phase-forming polymer, as well as its capacity for protein separation is evaluated.

2. Experimental

2.1. Chemicals

Crude gum was collected as a natural exudate from cultivated *Anacardium occidentale* trees in various localities in Pernambuco State, Brazil. Common type plants about 20 years old, yellow cashew producers were utilised. PEG 8000 and PEG 4000 were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Protein

Bovine serum albumin (BSA) with an M_r of 67 500 was obtained from Sigma.

2.3. Purification of gum

Clear nodules free of bark were selected to be purified via ethanol purification by use of the Rinaudo-Millas method as previously described [13]. Precipitation with ethanol permitted the isolation of the polysaccharide from the monosaccharides and oligosaccharides, which remained in solution.

2.4. Phase diagrams

Phase diagrams were determined at room temperature according to Albertsson's [1] procedure. The binodal of the phase diagram, the demarcation between PEG–cashew-nut tree gum compositions showing monophasic and biphasic behaviour was obtained by direct observation of two-phase formation for a large number of solutions containing varying concentrations of PEG and cashew-nut tree gum. Systems that displayed a distinct phase–phase interface were considered biphasic. The polymer compositions of the top and bottom phases of various

systems were analysed. PEG concentration was determined according to Skoog [14]. Polysaccharide concentration was determined by measuring reducing sugars (DNS method [15] — concentration after a hydrolysis step with sulphuric acid).

2.5. Two-phase systems

The systems (total mass 5 g) were prepared from stock solutions of the polymers in water, 50% (w/w) cashew-nut tree gum and 50% (w/w) PEG. The polymers solutions were weighed out and mixed with water and phosphate buffer (pH 6.0, 7.0 and 8.0), to the desired pH. The buffer concentration was 15 m*M*. Visual estimates of the volumes of top and bottom phases were made in graduated centrifuge tubes. The volumes of the phases were then used to estimate the volume ratio (V_r = volume of the top phase/volume of the bottom phase). The partition experiments were performed at room temperature (27±2°C) by mixing systems with 500 μl of a 2 mg/ml albumin solution. The systems were well vortex-mixed for 5 min and then centrifuged at 236 *g* for 5 min to obtain two clear phases.

The study of protein partitioning in PEG–cashew-nut tree gum systems was carried out at pH 6.0, 7.0 and 8.0 with PEG 4000 and 8000 and for different tie-line lengths (TLLs) by varying the PEG and gum concentrations. The TLLs were measured directly from the phase diagrams. The assays were performed in triplicate.

2.6. Protein partition coefficient

Protein concentration on the top phase was determined according to Bradford [16] and partition coefficient (*K*) was defined as the ratio between BSA in the upper (PEG) and lower (cashew-nut tree gum) phases. Samples of the top phase were diluted in water, when necessary, and the protein concentration analysed. The protein concentration of the bottom phase, due to its viscosity, was calculated from mass balance, according to Venâncio et al. [6] and Almeida et al. [17]. The protein concentration of the phases was determined for a set of three independent systems.

3. Results and discussion

In order to use polymer–polymer ATPSs in large scale biotechnical processes it is necessary to develop low cost polymers, which can replace fractionated dextran. Crude dextran has been used as phase-forming polymer for enzyme extractions [18]. Crude dextran, however, forms bottom phases with higher viscosity compared to the corresponding phases with dextran. Thus, crude dextran has to be hydrolysed in order to be efficient. The PEG–salt systems, which have been preferentially used for large-scale extractions [19], have several limitations. Most proteins partition strongly to the salt-rich bottom phase and the salt concentration in both phases is so high that it may destroy sensitive biological structures. We have studied a low-cost polymer, the cashew-nut tree gum, with the aim of finding a new ATPS.

Several ATPSs formed by cashew-nut tree gum and PEG were tested for their effectivness in the separation process. For each system, the following effects were studied: pH (6.0, 7.0 and 8.0), PEG average molecular mass (4000, 8000) and tie-line length.

3.1. Phase diagrams

The molecular mass of PEG is an important factor in equilibrium distribution. Phase diagrams for PEG–cashew-nut tree gum with different molecular mass PEG are displayed in Fig. 2. Once the binodal did not show a significant displacement with pH changes (data not shown), characterisation of phase diagrams

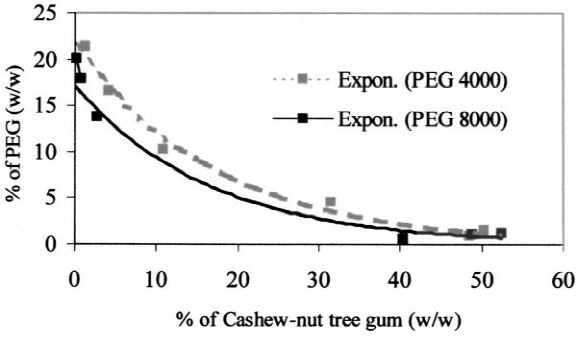

Fig. 2. Phase diagrams for PEG–cashew-nut tree gum systems at 27±2°C, pH 6.0.

Table 1
Tie-lines compositions (w/w) and system volume ratio (V_r) of PEG 4000–cashew-nut tree gum system at pH 6.0

Tie-line length	System V_r	System composition % (w/w)			Top phase composition % (w/w)			Bottom phase composition % (w/w)		
		Gum	PEG	H_2O	Gum	PEG	H_2O	Gum	PEG	H_2O
1	1.7	18	9	73	5.52	13.54	80.94	36.35	2.37	61.28
2	1.6	20	11	69	1.66	19.23	79.11	43.22	0.63	56.15
3	1.5	22	13	65	0.59	20.24	79.17	47.84	1.68	50.48

was performed at pH 6.0. The two curves, which represent the borderline between one and two phases, have the same shape but there is a parallel displacement between the two. This means that higher polymer concentrations are needed to obtain two phases with PEG 4000, which can be correlated to its lower molecular mass, compared with PEG 8000. This effect of PEG molecular mass on the binodal is in accordance with the results of Albertsson [1]. The same displacement of the binodals was observed by Venâncio et al. [9] for PEG–solvitose gum of ATPSs.

The polymer compositions and volume ratios (V_r) of the tested systems are shown in Tables 1 and 2. It can be seen that this system forms two phases with high polymer concentrations. The cashew-nut tree gum is enriched in the denser bottom phase while PEG is found in the upper phase. The ratio between the volumes indicate a higher volume of the upper phase when compared to the bottom phase and that the volume ratio decreases as the TLL increases.

3.2. Protein partition

The partitioning of molecules between the two phases is a complex phenomenon because of the involvement of many factors in the interactions between the solute and the phase forming components. This makes the molecular mass and chemical properties of the polymer and the size and the chemical properties of the partitioned solute of extreme importance. Due to the complexity of the partitioning phenomenon, it is difficult to predict protein behaviour and select separation conditions for a rational planning of experiments [18].

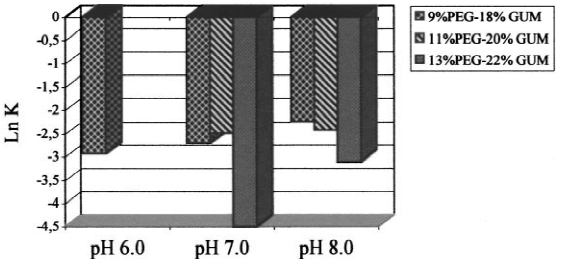

Fig. 3. Effect of tie-line length and pH on BSA partition coefficient in PEG 4000–cashew-nut tree gum system at 27±2°C. BSA partition coefficient was not determined for the second (11% PEG–20% cashew gum) and third (13% PEG–22% cashew gum) tie-lines since BSA concentrated in the cashew gum-rich bottom phase at pH 6.0. Typical uncertainty in the K values obtained from triple samples were approximately 4%.

3.2.1. Effect of PEG molecular mass

The molecular mass of PEG is an important factor in equilibrium distribution. Figs. 3 and 4 show the effect of TLL and pH on BSA partition coefficient for PEG 4000–cashew-nut tree gum and PEG 8000–cashew-nut tree gum, respectively. In all of the tested situations, BSA was mainly in the bottom phase

Table 2
Tie-lines compositions (w/w) and system volume ratio (V_r) of PEG 8000–cashew-nut tree gum system at pH 6.0

Tie-line length	System V_r	System composition % (w/w)			Top phase composition % (w/w)			Bottom phase composition % (w/w)		
		Gum	PEG	H_2O	Gum	PEG	H_2O	Gum	PEG	H_2O
1	2.2	16	9	75	3.64	13.33	83.03	39.15	0.86	59.99
2	1.9	18	11	71	1.33	19.39	79.28	43.88	0.85	55.27
3	1.8	20	13	67	0.35	21.56	78.09	47.37	0.99	51.64

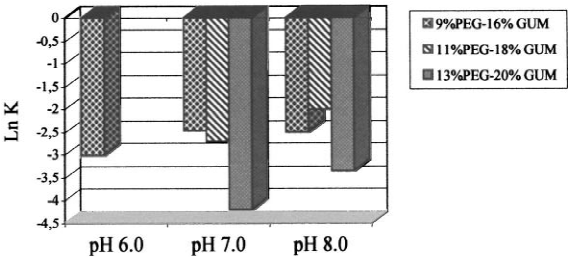

Fig. 4. Effect of tie-line length and pH on BSA partition coefficient in PEG 8000–cashew-nut tree gum system at 27±2°C. BSA partition coefficient was not determined for the second (11% PEG–18% cashew gum) and third (13% PEG–20% cashew gum) tie-lines since BSA concentrated in the cashew gum-rich bottom phase at pH 6.0. Typical uncertainty in the *K* values obtained from triple samples was ≈3.5%.

under the experimental conditions studied. It was not possible to determine BSA partition coefficient for the second and third tie lines at pH 6.0 for either PEG 4000 or 8000 since BSA was not detected in the PEG-rich upper phase.

The results indicate that the system studied is slightly influenced by the PEG molecular mass. The partition coefficients show no regular tendency and are similar in all cases.

The effect of polymer molecular mass is usually attributed to the increasing number of hydrophilic end groups on shorter PEG chains, which reduces the overall hydrophobicity [19], and to the excluded volume effects that increase with increasing polymer molecular mass. Due to these effects, the general tendency expected would be an increase of the partition coefficients as the PEG molecular mass decreases. However, it is important to bear in mind that by changing the polymer molecular mass, one needs to alter the polymer concentration, which influences the partition coefficients in the opposite way. Still, as the PEG molecular chain decreases, its hydrophilic character becomes stronger [7]. Therefore, and considering the hydrophilic character of albumin, PEGs of lower molecular mass should increase the protein affinity for the PEG-rich phase. However, this tendency was not observed for BSA partitioning, probably due to the charge of the cashew-nut tree gum. This fact could explain the little influence of variation of PEG molecular mass from 8000 to 4000 in the BSA partition coefficient. Christian et al. [20] also studied the influence of PEG molecular mass upon the partition coefficient of BSA in PEG–arabinogalactan systems. Altering the molecular mass of the PEG had no very significant effect upon the partition coefficient.

3.2.2. Effect of tie-line length

This effect is related to the influence of the distance from the critical point on the partitioning behaviour of proteins in aqueous two-phase systems. Near the critical point, the *K* value should be close to 1 [1] which is confirmed by most of the results shown in Figs. 3 and 4. The increase of the TLL leads, in most cases, to a decrease of *K*. This increase is due to the increasing PEG concentration, which causes a molecular exclusion of the BSA from the top to the bottom phase. These results are in accordance with the data presented by Sturesson et al. [5] with two different proteins in phase systems based on PEG–hydroxypropyl starch and Almeida et al. [17] with cutinase in also PEG–hydroxypropyl starch systems.

3.2.3. Effect of pH

Although the *K* value shows little changes by changing the system pH, an increase of this parameter can be detected as the pH is increased from 6.0 to 8.0 (Figs. 3 and 4). Similar results for BSA partitioning were observed by Venâncio et al. [9] in PEG–solvitose gum systems and Christian et al. [20] in PEG–arabinogalactan systems.

3.3. Economics

The cost of the polymer–polymer two-phase system studied was compared to that of other systems (Table 3). The cost of this new system shows that

Table 3
Costs of 1 kg of different aqueous two-phase systems

System	$US
9% PEG 8000–16% cashew-nut tree gum	5.92[a]
9% PEG 4000–18% cashew-nut tree gum	6.01[a]
5% PEG 8000–7% dextran T500	35[b]
5% PEG 8000–7% crude dextran	5.7[b]
5% PEG 8000–14% hydroxypropyl starch	8.4[c]
5% PEG 8000–16% arabinogalactan	12[d]

[a] CNPCa-EMBRAPA experimental plantation, Pacajus, Ceará, Brazil.
[b] Sigma, St. Louis, Mo, USA.
[c] Shearwater Polymers, Huntsville, AL, USA.
[d] Larex, St. Paul, MN, USA.

cashew-nut tree gum possesses an economic advantage. The PEG–cashew-nut tree gum system has a competitive price, almost the same of PEG–crude dextran system. Also, cashew-nut tree gum is a natural product largely found in Brazil, which facilitates its use in industries that require products free of chemical contaminants.

4. Conclusions

Aqueous polymer phase diagram and biomolecule partitioning can be influenced by many factors including polymer concentration, molecular mass and pH.

It was observed that increasing polymer molecular mass from 4000 to 8000 led to lower polymer concentrations required for phase separation. The partition coefficient of BSA was little influenced by changing PEG average molecular mass. Increasing the TLL (polymer concentrations) promoted a decrease in partition coefficient of the protein. Increasing the pH also raised the BSA partition coefficient.

The initial characterisation of this new system based on cashew-nut tree gum and PEG showed that cashew gum is of potential utilisation as an aqueous-phase-forming polymer.

The availability of a new inexpensive polymer for phase partitioning makes it economically possible to use it together with PEG for the separation and purification of biomolecules. Research on the influence of other parameters on the protein partitioning in this system is in progress.

Acknowledgements

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