

# Evaluation of Crude Hydroxypropyl Starch As a Bioseparation Aqueous-Phase-Forming Polymer

Armando Venâncio and José A. Teixeira\*

Centro de Engenharia Química da Universidade do Porto, R. Bragas, 4099 Porto Codex, Portugal

Manuel Mota

Departamento de Engenharia Biológica, Universidade do Minho, Largo do Paço, 4719 Braga Codex, Portugal

A new aqueous two-phase system based on crude hydroxypropyl starch (HPS) and poly(ethylene glycol) (PEG) is described. Unlike other polymer-polymer aqueous two-phase systems, the HPS used is commercially available. Previous works have characterized almost exclusively purified carbohydrates or their derivatives, having neglected the large amount of gums and carbohydrates already used in the paper, food, or textile industries. Two-phase aqueous polymer systems based on such commercial polymers may have as a drawback their degree of purity, which may affect the separation procedure. However, their lower cost should encourage studies on their use in aqueous two-phase polymer extraction. The influence of temperature, pH, and ionic strength on the partition of several different molecular weight and isoelectric point proteins and whole cells was studied. The results obtained were compared with corresponding dextran-PEG systems, Aquaphase PPT-PEG systems, crude dextran-PEG systems, maltodextrin-PEG systems, and Klucel L-pluronic systems. Protein partition coefficient results exhibited higher values than those in corresponding systems. It is shown that this system, based on a crude hydroxypropyl starch, is highly attractive as an initial purification step.

## Introduction

Aqueous two-phase systems have found use in biochemical research for the separation and purification of macromolecules, cells, and cell fragments (Albertsson, 1971). In recent years the aqueous two-phase systems have also found applications in various areas of biotechnology. These phase systems have been used for enzyme purification on a large scale (Veide et al., 1983; Tjerneld et al., 1987) affinity precipitation (Kamihira et al., 1992), affinity purification (Plunkett and Arnold, 1990), and extractive bioconversions (Larsson and Mattiasson, 1988; Andersson and Hähn-Hägertal, 1990).

Aqueous two-phase systems not only provide a gentle environment for bioactive proteins (Albertsson, 1971) but also offer unique possibilities for downstream processing. A very difficult mechanical separation step can be replaced by an extraction process, allowing the separation of cells and cell debris from a soluble protein by partition into opposite phases. Besides physical extraction, a reactive extraction may be applied. This is accomplished by confining a ligand to one phase by covalent binding to one of the polymers forming the system, usually poly(ethylene glycol) (PEG). So far, most of the laboratory work has been performed with a system composed of fractionated dextran and PEG (Albertsson, 1971). The properties of this system are well studied, but despite the ease of scale-up, the high cost of fractionated dextran prevents the use of this system in large-scale processes (Kroner et al., 1984).

As an alternative for large-scale enzyme processing, the PEG-salt systems have been used (Albertsson, 1971). Although inexpensive, the high salt concentration in both

phases of this system limits its usefulness. The PEG-salt phase forms only at rather high ionic strength, which may cause the denaturation of sensitive biological structures and the dissociation of most ligand-protein complexes (Kula, 1989). Another problem related with the use of PEG-salt systems is waste disposal. Aqueous two-phase systems based on dextran, starch derivatives, and cellulose derivatives have the advantage of their biodegradability (Sturesson et al., 1990).

As a consequence, there is a need to develop new aqueous two-phase systems suitable for large-scale processes. By allowing processes to be carried out at low salt concentrations, polymer-polymer systems may be more useful than PEG-salt systems. Since polymers and salts used to generate two-phase systems alone can account for as much as 75% of the total production cost of an aqueous-phase extraction (Datar et al., 1986), it is necessary to find inexpensive substitutes for fractionated dextran with equivalent partition properties. Several polymers, such as crude unfractionated dextran (Kroner et al., 1982), starch derivatives (Tjerneld et al., 1986), poly(vinyl alcohol) (Tjerneld, 1989), maltodextrin (Szlak and Giuliano, 1988), and cellulose derivatives (Skuse et al., 1992) have already been tested, indicating that they must be considered as an alternative to fractionated dextran. However, their high cost is also a drawback for large-scale utilization.

Starch is a natural inexpensive polymer available in large quantities. The characteristics of a starch can be modified by chemical, physical, and/or enzymatic treatment to enhance or repress its intrinsic properties or to impart new ones. This capability for modification has been an asset in developing new uses for starch. The main problem for its utilization is the strong gel-forming tendency (retrogradation) in solution (Rutenberg, 1980). To prevent

\* Author to whom correspondence should be addressed.

or repress retrogradation, it is necessary to modify the molecular alignment or the intermolecular hydrogen-bond formation. This is accomplished with the insertion of a few ester or ether groups by reacting some of the hydroxyl groups of the starch molecule with monofunctional agents (Rutenberg, 1980). Previous studies (Tjerneld, 1989) showed that the best stability in solution is obtained with hydroxypropyl derivatives, which are currently used in the paper, textile, and food industries as thickening and stabilizing agents.

The use of a hydroxypropyl starch, commercialized under the trade name Aquaphase PPT, with a price one-tenth of the dextran price was reported by Tjerneld (1986). Its utilization, compared with traditional PEG-dextran systems, has reduced the cost of a two-phase system by a factor of 7. Reppal PES, the commercial trade name of a starch hydroxypropyl derivative similar to Aquaphase PPT (Reppe AB, 1987), has also been successfully used as a substitute for dextran in extractive bioconversions (Johansson and Joelsson, 1985). These starch derivatives were especially developed to two-phase aqueous separations, which makes them, although cheaper than fractionated dextran, still quite expensive. The utilization of low-price, commercially available starch derivatives used in the paper, food, and textile industries must be taken into consideration. In this work, we report on the use of a hydroxypropyl derivative of starch currently used in local textile industries, with a price one-tenth of the Reppal PES price (November, 1992, prices). The potential utilizations of this starch hydroxypropyl derivative as an aqueous-phase-forming polymer, as well as its capacity for protein separation, are evaluated. The possibility of its use for cell separation is also tested.

## Materials and Methods

**Polymers.** Poly(ethyleneglycol) (PEG 8000), number-average molecular weight  $M_n = (7-9) \times 10^3$ , was purchased from Sigma Chemical Co. (St. Louis, MO). Hydroxypropyl starch (HPS), commercialized under the trade name, C-Film 05392 [number-average molecular weight  $M_n = (60-70) \times 10^3$ , with a substitution degree of 0.03-0.04 (mol of hydroxypropyl groups/mol of glucose units)], was a generous gift from Sarcol (Porto, Portugal). It is a modified corn starch, obtained by the esterification of raw corn starch.

**Cells.** Yeast cells were obtained from fresh, commercial pressed yeast (baker's yeast).

**Proteins.** Lysozyme from chicken egg white with a molecular weight (MW) of 14 400, pepsin from porcine stomach mucosa with an MW of 35 000, egg albumin (grade III) with an MW of 44 000, bovine serum albumin (BSA) with an MW of 67 500, alcohol dehydrogenase from baker's yeast with an MW 150 000, and catalase from bovine liver with an MW of 232 000 were used. All proteins were obtained from Sigma.

**Two-Phase Systems.** The systems were prepared from stock solutions of the polymers in water: 20% (w/w) HPS and 30% (w/w) PEG. The polymer solutions were weighed out and mixed with water, buffer, and protein sample.

For a system pH lower than 6.0, acetate buffer was used, and for higher pH, phosphate buffer was employed. In both cases, the total buffer concentration was 10 mM.

The 20% HPS stock solution was prepared by dissolving the powder in hot water. The solution was allowed to stay, under continuous agitation, at 80 °C for 45 min, after which a 15-min centrifugation step at 2000g was performed in order to remove insoluble material. The amount of insoluble material removed by this centrifugation step was

compared with the amount removed by further centrifugation at 20000g for 1 h. The total amount of insoluble material was determined as 6.0% (w/w). From this amount, 85% was removed by the 2000g centrifugation step. All of the insolubles that were not removed by centrifugation were retained in the lower phase of the two-phase system, leaving the top phase clean for further processing.

**Phase Diagrams.** Each phase was weighed after separation. The polymer compositions of the top phase and of each polymer stock solution were analyzed by a combination of refractometry and spectrophotometric techniques [DNS method (Chaplin and Kennedy, 1986), after a 1-h hydrolysis step, with sulfuric acid (1:5) in boiling water]. The bottom phases of a set of three independent systems were also subjected to the described analytical procedure, and the results were compared to the compositions determined by mass balance. Since the results were in close agreement, it was decided to determine bottom phase compositions using mass balance instead of an analytical determination.

**Phase Densities.** Phase densities were measured using a PAAR DMA 6000 densimeter at  $19 \pm 1$  °C, using air and water as calibrants.

**Phase Viscosities.** Phase viscosities were measured using a Carri-Med CS rheometer at  $19 \pm 1$  °C. Upper phase viscosities were also measured using a capillary viscometer. The capillary constant of the viscometer was determined using water and glycerol as calibrants.

**Protein Partition Coefficient.** The partition of proteins between the two phases was determined by two different methods. For the systems with a composition closer to the critical point, samples were taken from the top and bottom phases and assayed for protein concentration, according to Bradford (1976), using BSA as standard. The bottom phase protein concentration was also calculated by mass balance, and an error lower than 10% was achieved in the resulting partition coefficients. The protein partition coefficient was defined as the ratio between the protein concentrations in the top and bottom phases.

For systems with a composition distant from the critical point, it was not possible to collect a sample from the bottom phase due to its viscosity. In this case, protein concentration was measured after appropriate dilution of a portion of the bottom phase. For all proteins tested, only catalase showed a large difference between the protein concentration determined this way and that determined by mass balance, indicating that protein precipitation occurred at the interface. This quantity was not quantified. For other proteins there was close agreement between the protein concentrations determined in this way and by mass balance. Thus, all partition coefficient values were determined on the basis of mass balance, after three independent experiments performed in duplicate.

**Time of Phase Separation.** The time required for phase separation was determined by a set of five parallel experiments performed in test tubes containing 7.5 g of a two-phase system. The systems were allowed to separate under gravity until a clear interface was noticed.

**Cell Partition Experiments.** Cell partition was determined by taking appropriate amounts from both phases and counting the cells, after dilution, using a Neubauer slide. A total cell concentration of 10% (w/w) was used.

**Phase Separation.** For phase composition determinations, 30 g of a two-phase system was centrifuged for 30 min at 140g and constant temperature. For partition coefficient determination, the total mass of the two-phase system was reduced to 7.5 g. For cell partition experiments,

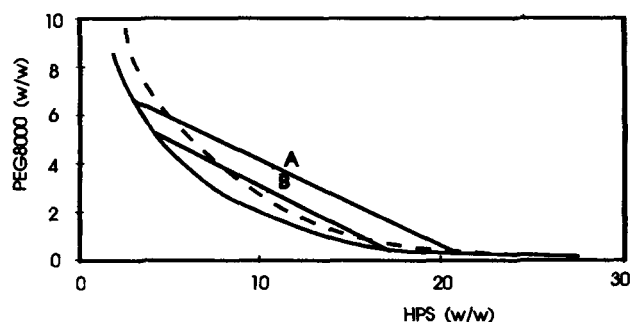


Figure 1. Phase diagrams for the system HPS-PEG 8000-water with 10 mM phosphate buffer (pH 6.8) at 19 °C (—) and 50 °C (---).

Table I. Total Composition of the Systems Mentioned in Figure 1

tie-line	system composition (% w/w)		tie-line length (%)
	PEG	HPS	
A	3.8	10.6	19.2
B	2.8	10.3	14.4

Table II. Densities and Viscosities of Systems A and B

tie-line	density (g cm <sup>-3</sup> )		viscosity (mPa·s) upper phase
	lower phase	upper phase	
A	1.074	1.024	4.3
B	1.094	1.023	4.0

the system was allowed to separate under the action of gravity, and samples were collected after a 60-min separation.

## Results and Discussion

The phase diagram for the system HPS-PEG 8000-water at two temperatures is shown in Figure 1. It may be seen that, for higher temperatures, higher polymer concentrations were present in the upper phase. This result has already been reported for other systems (Albertsson, 1971). The total system compositions (systems A and B) of the tie-lines marked on Figure 1 are given in Table I.

The densities of both phases of systems A and B as well as upper phase viscosities are shown in Table II. Studies on the dynamic viscosity of these systems' bottom phases clearly demonstrate that they must be regarded as non-Newtonian liquids (Figure 5). Upper phase dynamic viscosity was also tested, and it was proved that in these systems, they behave as Newtonian liquids (Figure 5). However, since the values for upper phase viscosity were near the lower detection point of the rheometer, a capillary viscometer was used for accurate determination. This system shows upper phase viscosities (Table II) close to the values of other systems. Sawant (1990) has reported an upper phase viscosity between 2.9 and 4.4 mPa·s for a dextran-PEG system. Maltodextrin-PEG systems present higher values between 7.7 and 20 mPa·s (Raghav Rao et al., 1991).

The lower phase viscosity of the HPS-PEG 8000 system is higher than that of dextran-PEG (Table III), Aquaphase-PEG (Table III), or maltodextrin-PEG (Raghav Rao et al., 1991). For corresponding dextran-PEG systems, Kroner and Hustedt (1986) have reported lower phase viscosities of 145 mPa·s for a tie-line length of 14.4% and 303 mPa·s for a tie-line length of 19.8%. Klucel L-pluronic systems (Skuse et al., 1992) and crude dextran-PEG systems (Kroner et al., 1982) show higher viscosities than the tested system.

The results relating protein molecular weight and the protein partition coefficient (Figure 2) indicate that increasing protein molecular weight decreases the partition

Table III. Lower Phase Dynamic Viscosity of Several Systems

shear rate (s <sup>-1</sup> )	viscosity (mPa·s)			
	tie-line A	tie-line B	Aquaphase-PEG <sup>a</sup>	dextran-PEG <sup>a</sup>
4.6	1090	800	55.7	102
11.5	840	650	53.4	104
29	630	550	52.8	104
110	360	530		

<sup>a</sup> Data from Tjernelid et al. (1986).

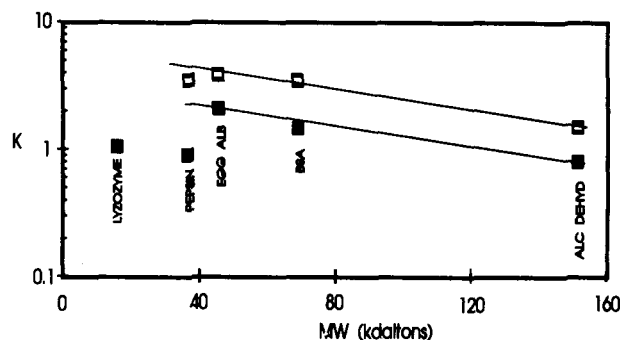


Figure 2. Relationship between protein partition coefficient, *K*, at 19 °C and pH 6.3, and protein molecular weight, for a two-phase system with composition corresponding to tie-line A, with 0.10 M NaCl (■) and without NaCl (□).

coefficient. Although this result was expected, caution must be taken in extrapolating these data, since all protein partition experiments were performed at a fixed pH (pH 6.3) and the *pI* values for each protein are different. It is interesting to note that the expected linear relationship (Abbott et al., 1990) is obtained for the proteins with *pI* values close to the tested pH [egg albumin, *pI* 4.63 (*Merck Index*, 1989); BSA, *pI* 4.7–4.9 (Albertsson, 1971); alcohol dehydrogenase, *pI* 5.4 (Hames and Rickwood, 1981)]. For the other proteins tested [lysozyme, *pI* 10.5–11.0 (*Merck Index*, 1989); pepsin *pI* < 1 (*Merck Index*, 1989)], the partition values lie outside the straight line.

It is known that salt concentrations alter the protein partition in aqueous two-phase systems. To obtain a more complete characterization of protein partition, the effect of the addition of sodium chloride to the system was studied. It can be seen from Figure 2 that the presence of this salt decreased the partition coefficient of negatively charged proteins. As in other systems (Albertsson, 1971), it might be expected that salts do not distribute evenly between the two phases and that a slight predominance of the chloride anion in the upper phase may occur. The upper phase becomes negatively charged and repels negatively charged proteins to the lower phase.

A comparative analysis between literature results and this commercial HPS-PEG system was performed. BSA partition coefficients, displayed in Table IV, clearly indicate that larger BSA partition coefficients are observed in the tested system. It was also possible to verify similar partition behavior with other operating pH values.

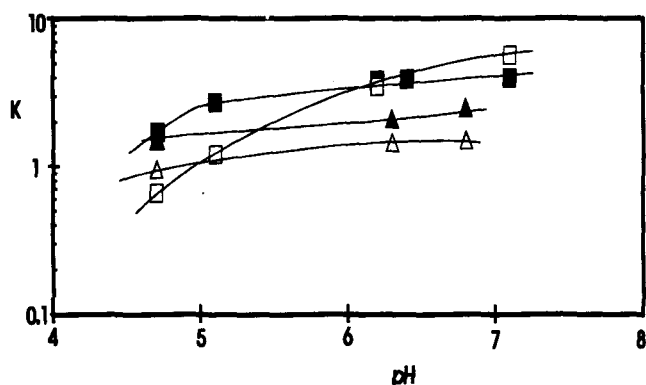
The decrease in partition coefficients of negatively charged proteins is strongly dependent on the pH of the two-phase system. As the pH decreases (Figures 3 and 4), getting closer to the *pI* of the protein, the protein becomes less negative and its partition coefficients in the presence and absence of sodium chloride tend to correlate. This can be used to determine the isoelectric points of proteins. From our experiments, a *pI* of 5.0 was obtained for BSA and 4.7 for egg albumin, which are close to literature values (Albertsson, 1971).

The polymer concentration for phase formation was also studied. As was expected, the increase in total polymer

**Table IV. Partition of BSA on Different Two-Phase Systems**

pH	buffer	partition coefficient		
		system B and BSA	Aquaphase-PEG <sup>a</sup> and BSA	dextran-PEG <sup>a</sup> and BSA
5.0	potassium acetate	1.37 ± 0.05	0.34	0.085
5.0	potassium phosphate + 100 mM NaCl	1.47 ± 0.05		
6.0	potassium phosphate	2.04 ± 0.10	0.56	0.18
6.0	potassium phosphate + 100 mM NaCl	1.57 ± 0.04		
7.0	potassium phosphate	2.55 ± 0.15	1.50	0.41
7.0	potassium phosphate + 100 mM NaCl	1.67 ± 0.06	0.21	0.06

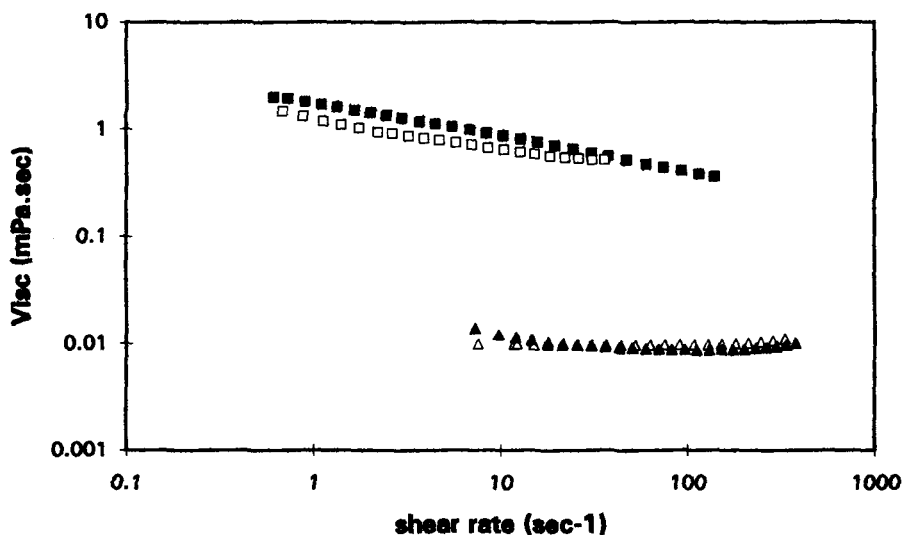
<sup>a</sup> Data from Sturesson (1990).



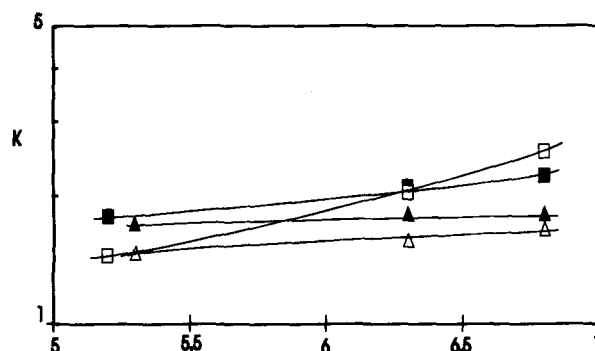
**Figure 3.** Relationship between egg albumin (filled symbols) and BSA (open symbols) partition coefficients ( $K$ ) and pH. The system composition corresponds to tie-line A without NaCl (■, □) and with 0.10 M NaCl (▲, △).

concentration increased the uneven distribution of proteins between the phases.

Two-phase aqueous systems may also find practical application in the separation of whole cells. For this purpose, the partition of baker's yeast in the presence of different amounts of sodium chloride and sodium sulfate was assayed. To determine the cell partition, the system was allowed to separate under the action of gravity. The time required for phase separation was assayed on system B (Table I) both without the presence of yeast cells and in the presence of 10% (w/w) yeast cells. As Kroner and Hustedt (1986) have already observed, the presence of yeast cells increases the time required for phase separation. In our experiments, 30 min was necessary for phase



**Figure 5.** Dynamic viscosity of the upper phase (■, □) and bottom phase (▲, △) of systems A (filled symbols) and B (open symbols).



**Figure 4.** Relationship between egg albumin (filled symbols) and BSA (open symbols) partition coefficients ( $K$ ) and pH. The system composition corresponds to tie-line B without NaCl (■, □) and with 0.10 M NaCl (▲, △).

**Table V. Partition of Baker's Yeast, on Tie-Line B, in the Presence of Different Salts<sup>a</sup>**

salt	upper phase	interface	lower phase
0.05 M Na <sub>2</sub> SO <sub>4</sub>	2	18	80
0.05 M NaCl	1	10	90
0.10 M NaCl	0	2	98

<sup>a</sup> Data are expressed in % of total cells in the system.

**Table VI. Costs of Six Concurrent Systems**

system	reference	cost (\$ kg <sup>-1</sup> )
tested system		0.18–0.20
7% dextran T500–5% PEG 8000	Tjerneld et al., 1986	6.40
14% Reppal PES–5% PEG 8000	Tjerneld et al., 1986	1.67
1.25% crude dextran–9% PEG 4000	Kroner et al., 1982	0.55
22% maldextrin–4% PEG 8000	Szlag and Giuliano, 1988	0.32–0.65
4% Klucel L–10% pluronic P105	Skuse et al., 1992	0.80

separation when no cells were present; this is similar to the results presented for other systems: 30 min for dextran–PEG (Kroner and Hustedt, 1986) and 20–40 min for Klucel L–pluronic (Skuse et al., 1992). Cell-containing systems required 60 min for 90% phase separation. Comparison of the separations achieved in systems with and without yeast cells was not possible since the polymers introduced with the cell homogenate shifted the binodal of the system to the left and contributed to phase separation (Kula, 1986).

It may be seen from Table V that, for all of the tested salt concentrations, the yeast cells accumulated in the lower phase. The amount of cells found in the lower phase was

higher when the salt added was sodium chloride, a phenomenon comparable to the previously described charge effects. Chloride anions, as previously mentioned, are present in a slightly higher concentration in the upper phase and, consequently, repel to the lower phase the negatively charged cells. In turn, as sulfate anions are known to accumulate in the lower phase (Albertsson, 1971), their presence repels the negatively charged cells to the upper phase and interface. Thus with this system, a significant concentration of cells can be obtained.

The cost of the polymer-polymer two-phase system studied was compared to that of five other systems focused on in the literature (Table VI). The price of the tested HPS was, in 1992, \$1 kg<sup>-1</sup> (Sarcot). The cost of dextran 500 was \$89.56 kg<sup>-1</sup>, that of crude dextran \$25.82 kg<sup>-1</sup> (Skuse et al., 1992), that of Reppal PES \$11 kg<sup>-1</sup> (Reppe AB, Sweden), that of pluronic \$2.94 kg<sup>-1</sup> (BASF Portugal), that of Klucel \$12.74 kg<sup>-1</sup> (Skuse et al., 1992), that of maltodextrin \$0.98–2.48 kg<sup>-1</sup> (Szlak and Giuliano, 1988), and that of PEG 8000 \$2.58 kg<sup>-1</sup> (Skuse et al., 1992).

It can be seen that the HPS-PEG 8000 system is the least expensive, having a slightly lower price than crude dextran-PEG 8000, maltodextrin-PEG 8000, or Klucel L-pluronic and being far less expensive than fractionated dextran-PEG 8000 or Aquaphase PPT-PEG 8000. This fact, associated with its performance in protein and cell separation, makes it highly attractive.

### Conclusions

It is shown that the HPS-PEG system compares well with traditional systems. Furthermore, it has the advantage of the low cost of HPS. A great disadvantage of the use of such a commercial polymer may be its degree of purity, since such a polymer has a large amount of insoluble material. This material was only partially removed by the centrifugation step. Total removal (>99%) required a 10-fold greater centrifugation speed. The presence of such a high amount of insoluble material may be responsible for the high value of lower phase viscosity, which was 5–6 times higher than that of corresponding dextran-PEG systems and 10 times higher than that of Aquaphase PPT-PEG. This drawback is counterbalanced by the favorable partition coefficient values obtained for the tested proteins and, above all, by its extremely low price.

Since, after centrifugation, the top phase is clean for further processing, it may be concluded that there will be no need for further HPS purification if the operating conditions are designed in such a way that the exocellular products will be the only ones transferred to the top phase.

### Acknowledgment

A.V. was supported by a JNICT (Junta Nacional de Investigaçao Científica e Tecnológica) grant.

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Accepted July 8, 1993.\*

\* Abstract published in *Advance ACS Abstracts*, October 1, 1993.