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Reppal PES — a Starch Derivative for Aqueous Two-Phase Systems

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ABSTRACT

Purification of biomolecules in aqueous two-phase systems has been found to be an interesting method, though the cost of the most commonly used phase systems has deterred its use for large-scale work. This paper describes a modified starch polymer as used together with poly(ethylene glycol) for formation of two-phase systems.

INTRODUCTION

The method of using aqueous two-phase systems for separation of biomolecules has been known for about 30 years (Albertsson, 1971). Initially the technique was primarily used for analytical work, and phase systems composed of poly(ethylene glycol) (PEG) and dextran have become the most widespread. For work on a larger scale (10–1000 litres) the use of aqueous two-phase systems has been hampered by the high cost of fractionated dextran (Kroner *et al.*, 1984). However, the potential advantages of partitioning have encouraged the search for other phase systems. Crude dextran is cheap but gives phase systems of unacceptably high viscosity. Systems composed of PEG and salt have been found to be useful in some applications. However, when biochemical binding reactions based on electrostatic or bioaffinity interactions are to take place in the phase system, the high ionic strength often prevents efficient binding.

PEG has been found to be an almost ideal component for aqueous two-phase systems because of its high hydrophilicity and thus high degree of biocompatibility. There are several polymers that can be used

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as constituents in aqueous two-phase systems together with PEG; however, the natural polysaccharides have proven to be the most promising. The price level as well as the availability restricts the possible range to products available in bulk quantities, but the standard qualities of starch or cellulose products commercially available do not conform to the physico-chemical requirements (Ling & Mattiasson, 1984).

In the starch industry, several ways of modification are used for products of different usage. We have investigated how potato starch can be modified to be appropriate for use in aqueous two-phase systems by hydrolysis and modification with propylene oxide.

MATERIALS AND METHODS

Preparation of hydroxypropyl starch derivatives

The modification of starch with propylene oxide was carried out to different degrees of substitution (DS) according to the method of Kesler & Hjermstad (1964). The hydroxypropyl derivative was prepared by the reaction between starch and propylene oxide in the presence of aqueous alkali. The temperature was held well below the swelling temperature of starch.

The hydroxypropyl derivative was partly hydrolysed with hydrochloric acid to produce a suitable molecular weight distribution. This reaction took place in a continuous tube reactor heated with steam. Different molecular weight distributions could be obtained by altering the acid concentration and/or reaction time. After hydrolysis the solution was purified by charcoal filtration and ion-exchange adsorption. The molecular weight distribution can then be made more narrow by different methods of fractionation. Finally the polymer solution was spray-dried to a white powder with about 95% dry weight. The preparations with a molecular weight of 100 000 and 200 000 were used for further investigations. These preparations are now available as Reppal® PES.

Hydroxypropyl starch derivatives with a more narrow molecular weight distribution were obtained by fractionation of Reppal PES in a DDS Mini-Lab 10 ultrafiltration unit equipped with different membranes of polysulfone.

Analyses

The molecular weight distribution was determined by gel filtration (Praznik, 1985) on a Superose® 6 column (Pharmacia, Sweden),

calibrated with fractionated dextrans (Pharmacia, Sweden) of known molecular weight.

The degree of substitution (DS) was determined spectrophotometrically following hydrolysis of the starch polymer with sulphuric acid (Johnson, 1969).

^{1}H-NMR spectra were recorded on a Bruker AM-500 FT spectrometer. Reppal PES 200 was exchanged with ^2H_2O, lyophilised and dissolved in high-purity ^2H_2O (99·95%). The chemical shifts are expressed relative to internal acetone set to 2·225 ppm. Spectra were recorded at 27 and 70°C.

Mass spectrometry was carried out as follows: 5 g of Reppal PES with a DS value of 0·85 was dissolved in 100 ml 1 M sulphuric acid and hydrolysed for 7 h in a boiling water bath. The pH was adjusted to 6 by addition of 2M NaOH. The sample was deionised by addition of 0·5 g ion-exchange resin (BioRad A6 501-X8 (D)). After stirring for 10 min the resin was removed by filtration and the clear solution lyophilised. The hydrolysed saccharide sample was reduced by deuterated sodium borohydride and methylated according to Hakomori (1964). GLC–MS was carried out on a VG 12-250 quadrupole instrument fitted with a SE-30 WCOT capillary column. Spectra were recorded at 70 eV with an ion source temperature of 200°C.

Viscosity was measured with a Brookfield RV viscosimeter using spindle No. 1.

Phase diagrams were determined according to Albertsson (1971).

The time required for phase separation was determined by observation of the formation of an interface in the phase systems. A turbid mixture of phase components was obtained and the system was mixed by shaking in a test tube. The velocity of the phase settling process was noted and plotted as a function of time. Separation was considered complete when 2% of the height of the system was still turbid. This point is indicated with an arrow in the diagrams.

Enzyme degradations by hydrolysis were performed under the following conditions: A solution of Reppal PES 200 (15%) was incubated at 55°C and a pH of 5·5 with 0·2 g Fungamyl per kg dry substance. Fungamyl is a fungal α-amylase from NOVO A/S, Denmark. The degradation was controlled by HPLC analysis carried out on an ion-exchange column (Aminex HPX 42 A from BioRad Ltd). This indicates distinct peaks for monosaccharides (DP 1), disaccharides (DP 2), etc., up to DP 4. DP 5 to DP 7 are calculated together and finally higher saccharides are given as a sum (DP= degree of polymerisation).

The following definitions of molecular weight distribution were used. The average molecular weight of a polymer such as starch is defined by

the number average molecular weight,

$$M_n = (\Sigma n_i M_i)/(\Sigma n_i)$$

and by the weight average molecular weight,

$$M_w = (\Sigma n_i M_i^2)/(\Sigma n_i M_i)$$

M_w is greater than M_n for heterogeneous samples and the ratio M_w/M_n can be taken as an indication of heterogeneity.

RESULTS AND DISCUSSION

Hydrolysis

In order to make starch soluble, it must be hydrolysed to give fragments with an appropriate molecular weight. A polymer that is to be used as a phase constituent in aqueous two-phase systems must also conform to further requirements. Fragments of low molecular weight do not contribute to the formation of a phase, while very long chains give a solution a high viscosity that slows down separation.

In a series of preparations having different degrees of hydrolysis, two were considered useful for formation of aqueous two-phase systems together with PEG and thus indicate a suitable range of molecular weights (Table 1).

The molecular weight distribution, given as the M_w/M_n ratio, can be brought down to 10–20 by a single hydrolytic step. For native starch, the ratio is around 102–103. A value of 2–4 can be obtained using ultrafiltration or other methods of fractionation.

The viscosities of Reppal PES 100 and 200 were nearly independent of the shear rate, indicating Newtonian behaviour. The results are shown in Table 2.

TABLE 1
Molecular Weight Determinations for Reppal PES

Types	M_w	M_n	M_w/M_n
Reppal PES 100	100 000	10 000	10
Reppal PES 200	200 000	20 000	10
Reppal PES 60 UF	123 000	29 000	4·3
Reppal PES 200 UF	326 000	79 000	4·1

Substitution

A solution of hydrolysed starch is not stable but forms a white precipitate after a while; a process known as retrogradation. In order to obtain stable solutions the starch must be derivatised with a small group that impedes or inhibits interchain binding. Both hydroxyethyl and hydroxypropyl groups have been used to produce this effect; however, hydroxypropyl has the advantage of a more straightforward chemistry.

By alteration of temperature, reaction time and concentrations, derivatives of different DS can be obtained. We have prepared hydroxypropyl derivatives with DS values ranging from 0·1 to 0·9 as measured by the ninhydrin reaction (Johnson, 1969).

The positions of the hydroxypropyl group of the substituted starch were analysed by GC–MS. 2-Hydroxypropyl (shown in Fig. 1), 2,3-hydroxypropyl and 2,6-hydroxypropyl derivatives of D-glucose were all identified. The relative distribution was estimated at 3:1:1.

The storage stability was tested using five different hydroxypropyl starch derivatives at concentrations of 20%, 30% and 40% (w/w),

TABLE 2
Viscosity (mPa s) of a Reppal PES Solution at 20°C

	Concentration			
	5%	*10%*	*15%*	*20%*
Reppal PES 100	10	15	25	50
Reppal PES 200	10	20	35	75

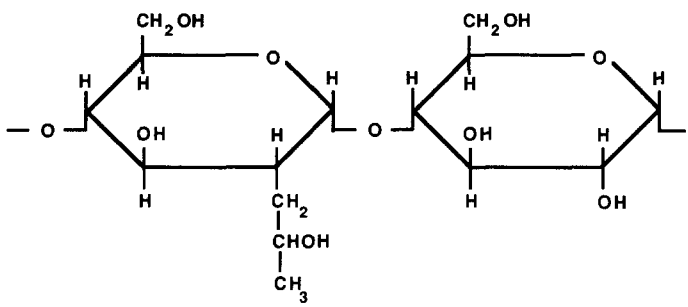

Fig. 1. Structure of a segment of hydroxypropyl starch, showing the hydroxypropyl group in the 2 position.

respectively, over a period of 14 days at 4°C. The final results are shown in Table 3. The retrogradation, as determined by visual observation of the formation of a white precipitate or gel, developed within 4 days. No further development was observed after that time.

One of the starch preparations, PES 200 (with a DS of 0·14 using the ninhydrin test) was analysed by NMR and GC–MS. Figure 2 shows the ^{1}H-NMR spectrum. The hydroxypropyl group is easily identified with a chemical shift of 1·1 ppm and a coupling constant (J) of 6·7 Hz. The

TABLE 3
Stability of Reppal PES at 4°C

DS	*Concentration*		
	20%	*30%*	*40%*
0·14	Clear solution	Opalescent	Gel
0·29	Clear solution	Clear solution	Clear solution
0·35	Clear solution	Clear solution	Opalescent
0·48	Clear solution	Clear solution	Clear solution
0·85	Clear solution	Clear solution	Opalescent

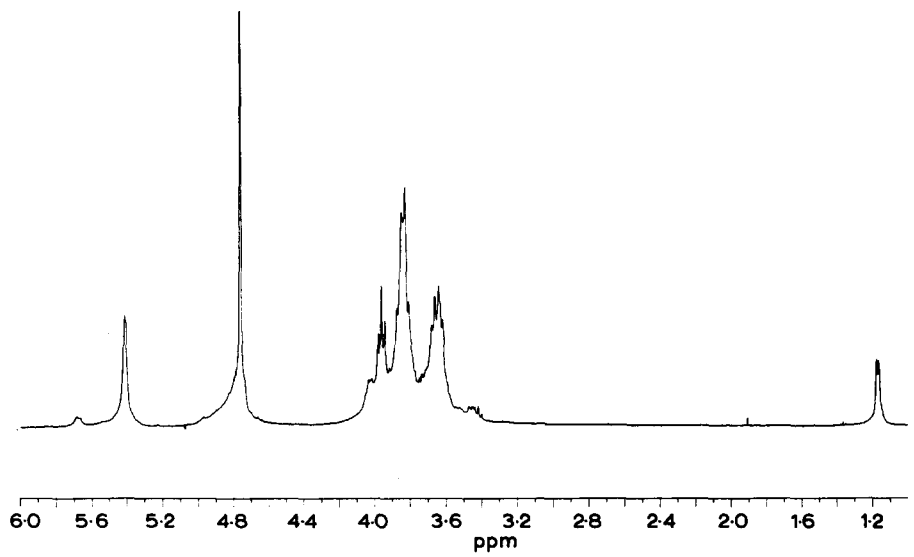

Fig. 2. ^{1}H-NMR spectrum of Reppal PES 200, recorded at 27°C.

degree of modification was estimated at 0·16 by comparison with the signals in the anomeric region.

Formation of two-phase systems

The suitability of Reppal PES for formation of two-phase systems was investigated by determination of phase diagrams and measurement of the speed of separation.

Phase diagrams were obtained for two-phase systems composed of PEG 8000 and Reppal PES 100 or PES 200 (Fig. 3). The influence of the molecular weight distribution is seen from a comparison with the line for the PEG 8000/PES 200 UF system. A phase diagram of a PEG/dextran system is included for reference.

In Fig. 4 is shown a series of phase diagrams for the substituted Reppal PES 200. The conclusion can be drawn that for increasing DS, higher concentrations are needed to produce two-phase systems. This effect is probably due to the fact that the hydrophobicity of the polysaccharide is increased by substitution and approaches the hydrophobicity of PEG. The difference in hydrophobicity between the two phases affects the speed at which they separate from each other. The separation behaviour in two-phase systems of the different starch derivatives and PEG 8000 was studied in respect of time (Fig. 5). The results show that an increase of the degree of substitution results in a decrease of the speed of separation.

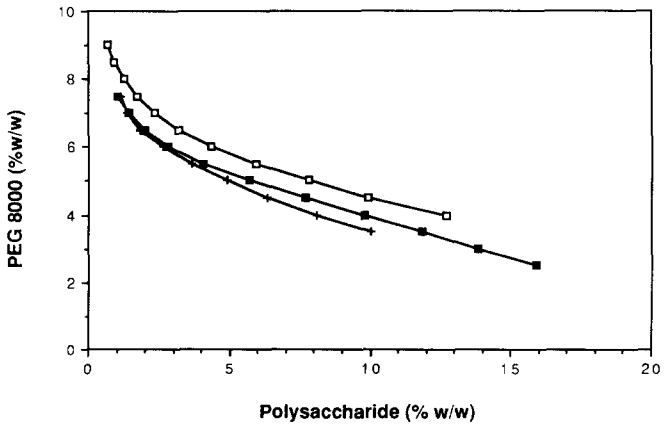

Fig. 3. Phase diagrams of three aqueous two-phase systems based on poly(ethylene glycol) and different polysaccharides, Reppal PES 100 (□), PES 200 (■) and dextran T70 (+), respectively.

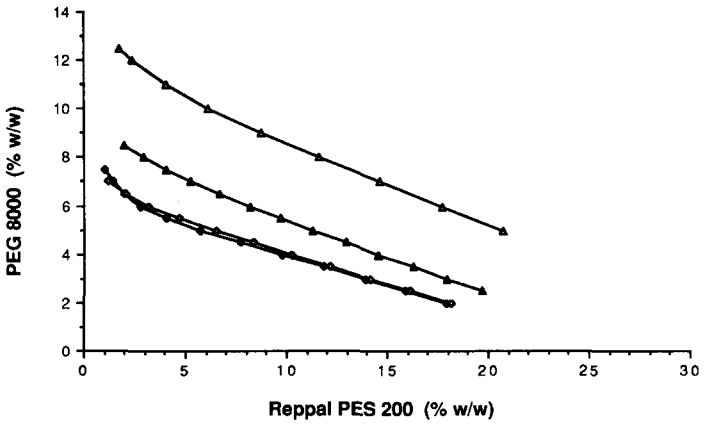

Fig. 4. Phase diagrams of PEG and substituted starch at different degrees of substitution of hydroxypropyl groups, DS = 0·13 (◆); 0·17 (◇); 0·29 (▲); 0·85 (△).

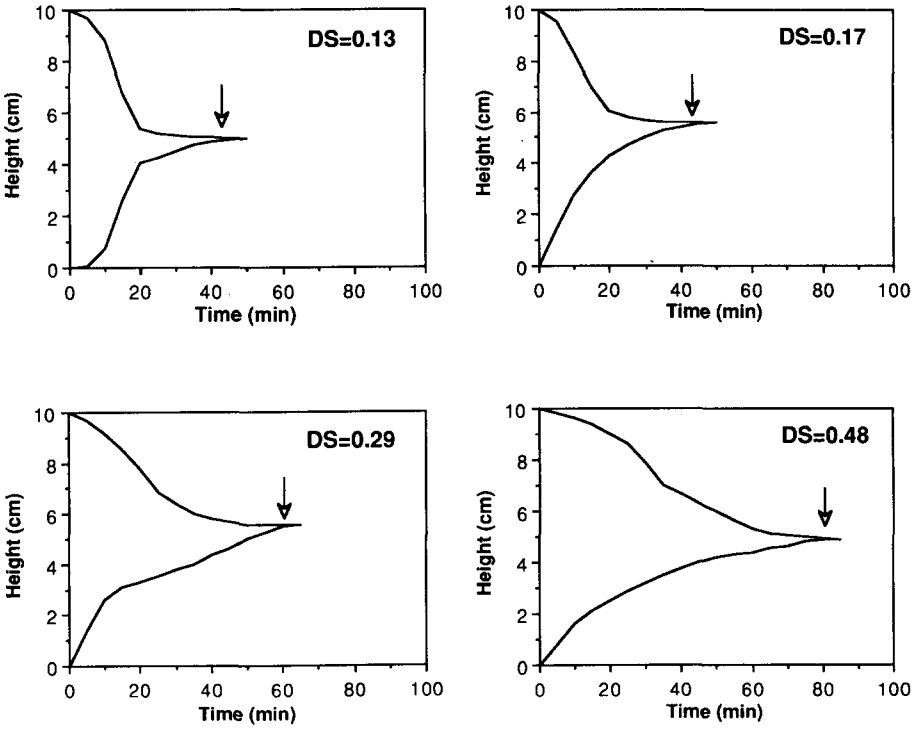

Fig. 5. Time study of the dynamics of phase separation. The time at which phase settling is considered complete is indicated with an arrow.

Enzyme degradation

Aqueous two-phase systems can be used in biotechnological production (Kaul & Mattiasson, 1986). Both of the phases will then be in contact with the microorganisms and their products. It is thus of interest to find out how the polymers are affected by this contact. Reppal PES is manufactured from starch and can be degraded by amylases. The degradation depends on the DS and has been examined for four different degrees of substitution. These results are compared to a starch that is only hydrolysed to solubility but not substituted.

Samples were analysed after 2·5, 5, 24 and 48 h of degradation by α-amylase and the results can be seen in Fig. 6. It can be seen that the higher the degree of substitution, the harder the product is to degrade and a DS of 0·85 protects the starch polymer from degradation.

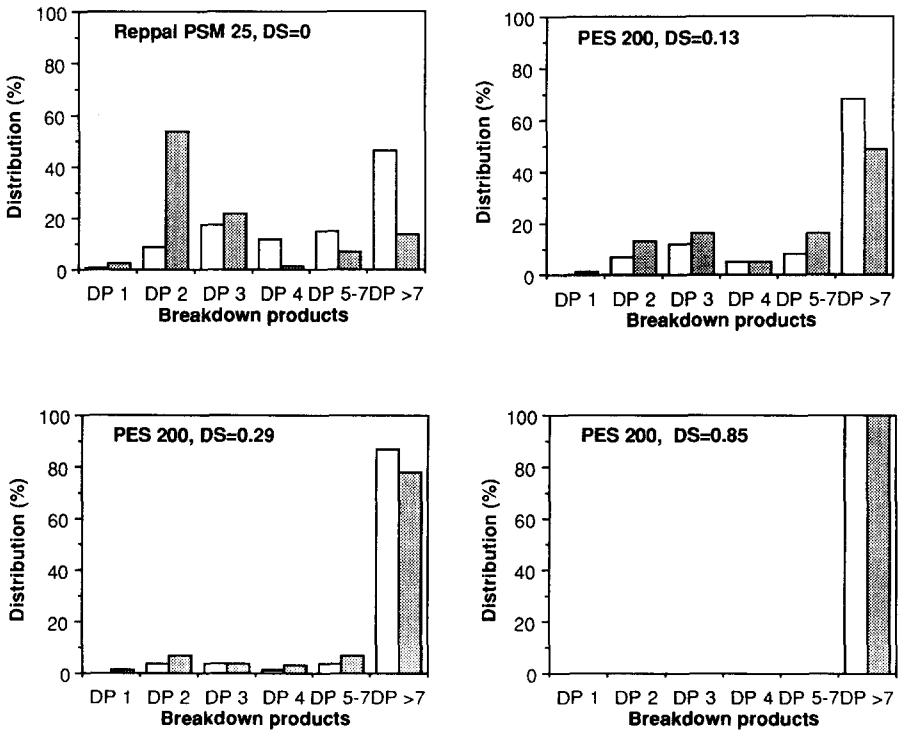

Fig. 6. Susceptibility of hydroxypropyl-substituted starch to enzymatic hydrolysis. Initially all starch is more than 7 units long; in time a distribution of low molecular weight units emerges. □, 5 h of hydrolysis; ▨, 48 h of hydrolysis.

Protein partitioning

An indirect way of characterising a phase system is to determine the partitioning of model substances. Bovine serum albumin and human immunoglobulin were partitioned in phase systems of different concentrations as shown in Fig. 7. The dependence of the partition coefficient on

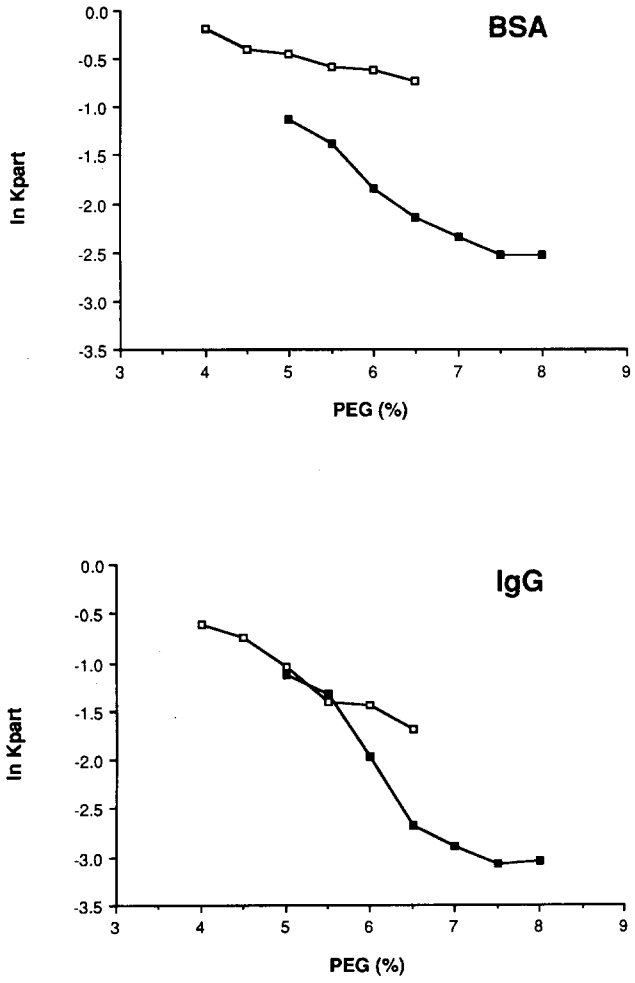

Fig. 7. Partitioning of bovine serum albumin (BSA) and human immunoglobulin G (IgG) in phase systems composed of PEG 8000 and Reppal PES 200 (□), and PEG 8000 and dextran T70 (■), respectively of different polymer concentrations. At 5% PEG, the PES 200 concentration is 14% and dextran is 7%. Both the PEG and the polysaccharide concentration increase linearly.

the concentration of PES 200 is lower than for dextran T70, which means that PES 200 has a greater tolerance for small deviations in phase composition.

Glucose-6-phosphate dehydrogenase was partitioned in different phase systems, composed of PEG and Reppal PES of different molecular weight, in order to study the range of attainable partition coefficients (Table 4).

Suitability

Most water-soluble polymers will form an aqueous two-phase system together with some other polymer. If partitioning is to be useful some requirements should be met:

ability to form two phases at relatively low polymer concentrations
low viscosity at these concentrations
quick separation after mixing
no detrimental effects on biological structures

Dextran has been used for a large number of partition experiments and is known as an excellent choice, though expensive. One criterion for the suitability of the use of a polysaccharide as a component in aqueous two-phase systems is therefore that it should have properties that resemble those of dextran.

Results from the different experiments shown here all indicate a dextran-like behaviour. Direct evidence comes from the phase diagrams and other physical properties. Indirect evidence is shown by the fact that the partitioning of proteins and other molecules in PEG/Reppal PES follows the same pattern as in PEG/dextran. Two differences were noted. First, Reppal PES has a somewhat higher viscosity, which is reflected by

TABLE 4
Partition of Glucose-6-phosphate Dehydrogenase in Phase Systems with Polymers of Different Molecular Weight

	PEG 8000	*PEG 3350*	*PEG 1000*
Reppal PES 100	1·8	8	18
Reppal PES 200	2·3	8	21
Composition (PEG, PES)	5%, 14%	7·5%, 15%	14%, 15%

The composition of a phase system is given as the w/w % concentration of PEG and Reppal PES in the total system.

an increased separation time. Secondly, dextran withstands microbial attack, whereas Reppal PES is biodegradable, which may be of advantage in large-scale operations.

The main point for usage on a preparative scale is the price of the polymer. Attempts to solve this problem include the introduction of hydrolysed dextran of intermediate quality, modified starch (Tjerneld *et al.*, 1987) and polyacrylate (Hughes & Lowe, 1988). The cost of aqueous two-phase systems is now only a few per cent of that for dextran-based systems, thus making the use of polysaccharides in aqueous two-phase systems economically feasible.

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