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Ucon–benzoyl dextran aqueous two-phase systems: protein purification with phase component recycling

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Abstract

Benzoyl dextran with a degree of substitution of 0.18 was synthesized by reacting dextran T500 with benzoyl chloride. A new type of aqueous two-phase system composed of benzoyl dextran as bottom phase polymer and the random copolymer of ethylene oxide and propylene oxide (Ucon 50-HB-5100) as top phase polymer has been formed. The phase diagram for the system Ucon 50-HB-5100–benzoyl dextran with a degree of substitution of 0.18 was determined at room temperature. This two-phase system has been used to purify 3-phosphoglycerate kinase from baker's yeast. The top-phase polymer (Ucon) can be separated from target enzyme by increasing the temperature. The bottom-phase polymer (benzoyl dextran) could be recovered by addition of salt. Yeast homogenate was partitioned in a primary Ucon 50-HB-5100–benzoyl dextran aqueous two-phase system. After phase separation the top phase was removed and temperature-induced phase separation was used for formation of a water phase and a Ucon-rich phase. The benzoyl dextran-enriched bottom phase from the primary system was diluted, and the polymer was separated from water by addition of Na_2SO_4.

Keywords: Aqueous two-phase system; Partitioning; Protein; Benzoyl dextran; Ucon

1. Introduction

Aqueous two-phase systems composed of poly(ethylene glycol) (PEG) and dextran have been widely used for separation and purification of biomaterials. These systems create an efficient and a mild separation method which is suitable for many biological substances, e.g. cells, cell organelles, proteins and nucleic acids [1,2]. However, the high cost of dextran prevents the application of this method on a large scale [3]. Also, the separation of biomolecules from phase-forming polymers can be a problem for this system. Several methods have been studied to reduce the cost of phase-forming polymers, e.g. dextran has been substituted by a lower-cost polymer, hydroxypropyl starch [4]. Recovery of the phase-forming polymer by temperature-induced phase separation can both reduce the cost of polymer and separate the desired biological molecules from the polymer solution [5–7].

Ucon 50-HB-5100 is an inexpensive, linear non-ionic random co-polymer of ethylene oxide and propylene oxide composed of equal amounts by weight of each monomer. This polymer has a lower cloud point compared with poly(ethylene glycol) and the cloud point can be decreased by addition of salts.

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Ucon has been used to form aqueous two-phase systems with dextran or hydroxypropyl starch. These systems were applied to purify different enzymes from baker's yeast, where temperature-induced phase separation was used to separate Ucon from target enzyme and to recycle the Ucon polymer [5–7].

Dextran is a relatively hydrophilic polymer and the properties of this polymer can be changed by different methods. Hydrophobically modified dextrans have been prepared with benzoyl or valeryl groups [8,9]. Aqueous two-phase systems can be obtained either in mixtures of PEG and the modified dextrans or in mixtures of an unmodified dextran and a modified dextran. The phase behaviour of these two-phase systems and the partitioning of proteins in these systems have been studied. The hydrophobically modified dextran polymers show hydrophobic association in aqueous solution, and the association is affected by the degree of substitution and the concentration of polymer [8,9].

In this work we describe a new type of aqueous two-phase system composed of Ucon 50-HB-5100 and benzoyl dextran with a degree of substitution (DS) of 0.18. The phase diagram for the Ucon–benzoyl dextran system was determined at room temperature. The application of this two-phase system for purification of 3-phosphoglycerate kinase (PGK) from baker's yeast has been studied. In this work, we also show a possibility of recovering both phase-forming polymers, Ucon and the modified dextran.

2. Experimental

2.1. Chemicals

Dextran T500 with an average molecular mass of 500 000, was obtained from Pharmacia (Uppsala, Sweden). Ucon 50-HB-5100, average molecular mass 4000, was obtained from Union Carbide, New York, USA. Benzoyl chloride and triethylamine were obtained from Aldrich-Chemie (Steinheim, Germany). Benzoyl dextran with a degree of substitution of 0.18 was synthesized according to our previous work [8]. All other chemicals were of analytical grade.

2.2. Yeast homogenate

Yeast homogenate was prepared from commercial baker's yeast. A 50-ml volume of triethanolamine–HCl buffer (50 m*M*), pH 8.0, was mixed with 50 g of yeast. Ethylene diamine tetraacetic acid (EDTA) and β-mercaptoethanol were added to 2.0 m*M* and 1.23 m*M*, respectively. Sonication was for 16 min in a Branson CP-30 sonifier. The homogenate was then centrifuged at 12 000 *g* for 10 min to remove cell debris. The pellet was discarded and the supernatant was used for enzyme purification.

2.3. Two-phase systems

Aqueous two-phase systems composed of Ucon and benzoyl dextran were prepared from stock solutions of 40% Ucon 50-HB-5100 (all compositions are given in weight percentage) and 14.18% benzoyl dextran with a degree of substitution of 0.18. The stock solutions were weighed out and mixed together with buffer, salt, yeast homogenate and water. The systems were left standing at different temperatures until phase separation. Suitable amounts of each phase were withdrawn; after dilution the samples were analysed. The partition coefficients (K) of the substances are defined as the ratio of their respective concentrations in top and bottom phase.

2.4. Phase diagram

Phase diagram was determined by analysis of the composition of top and bottom phases. The concentration of benzoyl dextran was determined by polarimetry. The specific rotation of dextran is $[\alpha]_D^{25} = +199°$ ml/g/dm [8]. The concentration of Ucon was determined by refractive index. The concentration of benzoyl dextran in each sample was measured first by polarimetry and its contribution was subtracted from the refractive index readings [5].

2.5. Recovery of phase forming polymers

In a primary Ucon–benzoyl dextran aqueous two-phase system, Ucon polymer was enriched in the top phase and benzoyl dextran in the bottom phase. Desired enzyme was first extracted to the Ucon top

phase. After phase separation the Ucon top phase was removed. The temperature-induced phase separation was performed by addition of Na_2SO_4 to 0.2 *M* and increasing the temperature to 45°C. At this temperature a Ucon-enriched bottom phase and a water top phase were formed. The enzyme was extracted in the water phase. The Ucon can be recovered in the Ucon-enriched bottom phase. The benzoyl dextran bottom phase from a primary Ucon–benzoyl dextran two-phase system was diluted by salt additions to lower polymer concentrations. By addition of Na_2SO_4 to 1 *M*, the solution was separated in a benzoyl dextran-enriched bottom phase and a water–salt top phase.

2.6. Assays of proteins and enzyme activity

Proteins were assayed by using Coomassie Brilliant Blue G and measuring at 595 nm with bovine serum albumin as standard [10]. 3-Phosphoglycerate kinase activity was measured according to Scopes [11].

3. Results and discussion

3.1. Ucon–benzoyl dextran two-phase system and phase diagram

In a Ucon–benzoyl dextran aqueous two-phase system, Ucon is enriched in the top phase and benzoyl dextran in the bottom phase. The phase diagram for the Ucon–benzoyl dextran system (DS= 0.18) is shown in Fig. 1. The binodal curve for Ucon and dextran system is also shown in Fig. 1. Dextran is a relatively hydrophilic polymer. However, benzoyl dextran is more hydrophobic than the non-substituted dextran and therefore more compatible with the hydrophobic Ucon. Thus, to form a two-phase system, a higher polymer concentration was required.

3.2. Purification of 3-phosphoglycerate kinase from yeast homogenate

The application of a Ucon–benzoyl dextran two-phase system for purification of 3-phosphoglycerate kinase from baker's yeast was affected by several

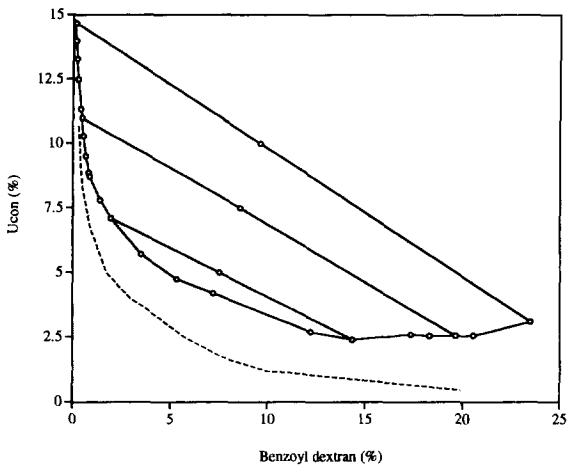

Fig. 1. Phase diagram for the Ucon 50-HB-5100–benzoyl dextran system (DS=0.18) at room temperature (21°C). The dashed line shows the binodal curve for the Ucon–dextran T500 system at 22°C, the data are from Harris et al. [5].

factors. Table 1 shows the effect of polymer concentration on the partition coefficient of 3-phosphoglycerate kinase and protein. With a two-phase system composed of 5.0% Ucon and 7.5% benzoyl dextran, at 21°C with 10 m*M* triethanolamine–HCl and 20 m*M* triethylammonium phosphate, pH 7.4, the *K* values were 0.81 and 0.44 for PGK and proteins, respectively. When the polymer concentration was increased to 5.8% Ucon and 7.8% benzoyl dextran, the *K* values were decreased to 0.48 and 0.24 for PGK and protein, respectively. This is due to the fact that with increased polymer concentration, the tie-line length was increased and the proteins were partitioned more to one phase [12].

Table 2 shows the effect of temperature on the partition coefficient of 3-phosphoglycerate kinase.

Table 1
Effect of polymer concentration on the purification of 3-phosphoglycerate kinase (PGK) from yeast homogenate

Ucon (%)	BzDx (%)	*K* (PGK)	*K* (protein)
5.0	7.5	0.81	0.44
5.5	8.5	0.69	0.40
5.8	7.8	0.48	0.24

The system was composed of Ucon 50-HB-5100 and benzoyl dextran (DS=0.18), 10 m*M* triethanolamine–HCl, 20 m*M* triethylammonium phosphate, pH 7.4, and 10% supernatant from yeast homogenate. Room temperature (21°C).

Table 2
Effect of temperature on partition coefficient of 3-phosphoglycerate kinase in polymer aqueous two-phase systems

Temperature (°C)	K (PGK)
4	0.58
21	0.81
23	0.84

The system composition was: 5.0% UCON 50-HB-5100 polymer, 7.5% benzoyl dextran (DS=0.18), 10 m*M* triethanolamine–HCl, 20 m*M* triethylammonium phosphate, pH 7.4, 10% supernatant from yeast homogenate.

The buffer and salt composition were the same as in Table 1. The K value for PGK was increased with increasing temperature. However, when the temperature was increased above 23°C, a longer time was required to form the two-phase system.

The effect of triethylammonium phosphate concentration on the partition coefficient of PGK was studied. In a system containing 5% Ucon and 7.5% benzoyl dextran without triethylammonium phosphate, the K value was 0.72. By addition of 20 m*M* triethylammonium phosphate, the K value was increased to 0.92. The cation of triethylammonium phosphate, $(CH_3CH_2)_3N^+H$, is relatively hydrophobic and has been shown to partition negatively charged proteins to the hydrophobic Ucon phase in Ucon–hydroxypropyl starch aqueous two-phase systems [13]. The isoelectric point of yeast PGK is 7.0 [14]. At a pH value higher than 7.0 the PGK is a negatively charged molecule, so it could be partitioned more to the top phase by the triethylammonium counterions. A concentration of 20 m*M* triethylammonium phosphate was chosen for the purification of PGK.

Table 3 shows the purification of PGK from yeast homogenate using a Ucon–benzoyl dextran two-phase system. The system was composed of 5% Ucon and 7.5% benzoyl dextran, 10 m*M* triethanolamine–HCl, 20 m*M* triethylammonium phosphate, pH 7.4. At 21°C the partition coefficient of PGK was 0.81 and protein 0.44. After phase separation the Ucon top phase was removed, Na_2SO_4 was added to 0.2 *M*. Temperature-induced phase separation was performed to separate Ucon polymer from water. The K value for PGK in a Ucon–water system was larger than 100. From the enzyme in the primary top phase 100% of enzyme activity was recovered in the water phase. The purification factor for PGK was 2, and 47% of the original PGK activity was recovered in the water phase.

Table 3
Purification of PGK from baker's yeast homogenate using an aqueous two-phase system at 21°C and temperature-induced phase separation at 45°C

	K (21°C)	K (45°C)	Purification factor	% Units recovered in water phase
PGK	0.81	>100	2	47
Protein	0.44			

The system composition was: 5% Ucon, 7.5% BzDx (DS=0.18), 10 m*M* triethanolamine–HCl, 20 m*M* triethylammonium phosphate, pH 7.4, 10% supernatant from yeast homogenate.

3.3. The recovery of phase-forming polymers

Fig. 2 shows the scheme for enzyme purification using a Ucon–benzoyl dextran system and the

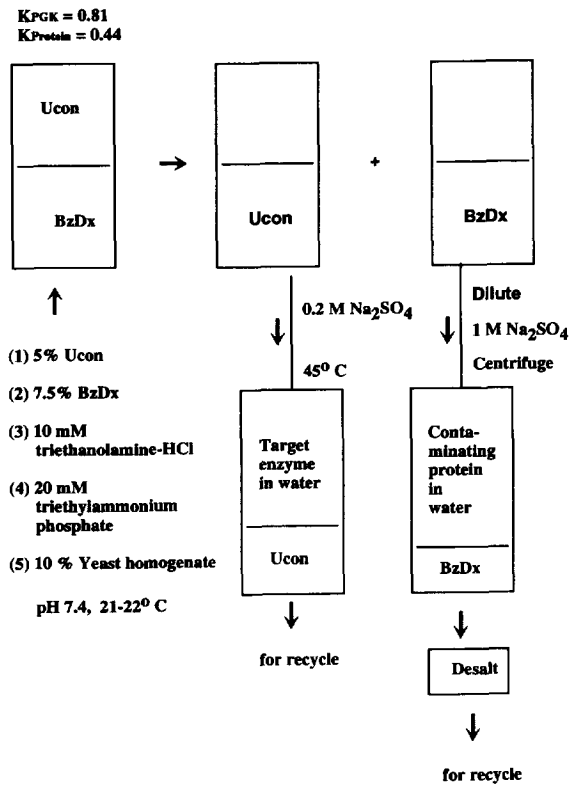

Fig. 2. Enzyme purification scheme using the Ucon–benzoyl dextran system and recovery of Ucon polymer and benzoyl dextran.

recovery of phase-forming polymers. Ucon, benzoyl dextran, buffer, salt and yeast homogenate were mixed first. After phase separation the Ucon top phase was removed. By addition of Na_2SO_4 to 0.2 *M* and increasing the temperature to 45°C, the Ucon top phase could be separated to a Ucon-enriched bottom phase and a water top phase containing target enzyme. Ucon can be recovered from the Ucon-rich phase and the enzyme from the water phase. This procedure has been applied for purification of enzymes using Ucon–dextran or Ucon–hydroxypropyl starch systems. The cloud point of Ucon was decreased by addition of salt [5]. The benzoyl dextran from the primary Ucon–benzoyl dextran two-phase system was diluted to a lower concentration polymer solution. By addition of Na_2SO_4 to 1 *M* the benzoyl dextran could be separated from water to form a benzoyl dextran-enriched bottom phase and a water top phase. The separation of benzoyl dextran from water is due to the hydrophobic association between benzoyl dextran molecules in aqueous solution [9]. Na_2SO_4 has a salting-out effect on proteins. By addition of this salt the hydrophobic interaction between benzoyl dextran molecules was strengthened and the result was the separation of the polymer from water. Benzoyl dextran with a lower degree of substitution could not be separated from water by the same salt. The temperature does not affect the separation procedure at a range of 21–70°C.

The amount of recovered polymer from the benzoyl dextran-enriched bottom phase and the amount of contaminating proteins in the recovered polymer depended on the dilution of benzoyl dextran. Table 4 shows the effect of dilution on the recovery of benzoyl dextran and the amount of contaminating protein in the recovered polymer. The bottom phase from the primary Ucon–benzoyl dextran aqueous two-phase system contained about 13% benzoyl dextran. When the dilution ratio was increased (more water was added to the polymer), the volume ratio between the water top phase and the benzoyl dextran bottom phase was increased. The amount of recovered polymer was decreased with increasing dilution ratio, and the amount of contaminating proteins in the recovered polymer was decreased with increasing dilution ratio. Therefore, the recovery of benzoyl dextran was decreased with increasing polymer purity. We still need to find a suitable method to get both a higher recovery and a more purified polymer.

The use of aqueous two-phase systems for large-scale purifications is still relatively limited. The PEG–salt systems, which are based on inexpensive chemicals, are used for purification of industrial enzymes. For large-scale application of a phase system based on two polymers it will be necessary to develop methods for polymer recycling. With thermoseparating EO–PO copolymers it is possible to recycle one of the phase-forming polymers [5–7]. Use of hydrophobically modified dextrans opens the possibility of recycling also the second polymer. Separation systems can be designed, e.g. for large-scale protein purification, where the recycling of the phase components will allow drastic reduction in the costs of purification. Benzoyl dextran is a relatively expensive polymer, but the principles described in this work should have application for hydrophobically modified starch or cellulose polymers.

Table 4
Effect of dilution on the recovery of benzoyl dextran and the amount of contaminating protein in recovered polymer solution

Benzoyl dextran concentration after addition of salt to 1 *M* (%)	V_t/V_b^a	Recovery of BzDx (%)	Protein in recovered BzDx solution (%)
8.0	1.3	67.1	65.2
7.0	1.7	53.3	67.9
6.0	2.7	50.9	58.8
5.0	4.2	41.6	56.5
4.0	5.2	48.2	–
3.0	9.1	36.9	30.9
2.0	18.4	32.0	17.5

Bottom phase from a primary Ucon–benzoyl dextran aqueous two-phase system containing about 13% benzoyl dextran.
[a] V_t is water-enriched phase and V_b benzoyl dextran-enriched phase.

4. Conclusions

A new type of aqueous two-phase system can be formed by Ucon 50-HB-5100 and a hydrophobically modified dextran (benzoyl dextran). This two-phase system can be used to purify enzymes from cell homogenate. After extraction of enzyme to the top phase and separation from the bottom phase, the Ucon polymer was separated from target enzyme by addition of salt and increasing the temperature. The target enzymes were obtained in the water phase and the Ucon polymer was recovered from the new polymer phase. The benzoyl dextran phase from the primary system was diluted to a lower polymer concentration. By addition of salt a benzoyl dextran phase could be separated from a water–salt phase.

Acknowledgments

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