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Application of the Aqueous Two-phase Thermoseparating Systems of Dehypon® LS 54-the Waxy Maize Starch for Protein Extraction

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Abstract: A thermo-separating aqueous two-phase system composed of Dehypon® LS 54, a polymeric surfactant and the waxy maize starch (amylopectin starch), has been used for partitioning of cutinase as a model protein. The phase diagram obtained for this novel polymer-polymer two-phase system shows two-phases with high polymer concentration. The waxy maize starch is enriched in the bottom phase while the copolymer of Ethylene Oxide (EO) and Propylene Oxide (PO) is found in the upper phase. Since, this copolymer (Dehypon® LS 54) is the thermo-reactive, the upper phase can be removed and heated above the copolymer's cloud-point resulting in the formation of a new two-phase system with a lower water phase containing the target protein and an upper copolymer-rich phase. Present results show that the systems were formed by the waxy maize starch and a copolymer Dehypon® LS 54 may be considered as an interesting alternative to be used in protein purification due to their low cost, in addition, they offer a viable solution to problems of polymer removal and recycling.

Key words: Polymeric surfactant, ATPS, Dehypon® LS 54, waxy maize starch, temperature-induced, protein partitioning

INTRODUCTION

An Aqueous Two-Phase System (ATPS) is formed when two structurally different polymers are mixed above a critical concentration in water (Walter and Johansson, 1994). The formed of two-phases are each enriched in one polymer, but the main component in both phases is water. Usually the water content is 80-95% and thus, aqueous two-phase systems constitute a mild method for separation of biomaterials. Bioseparation by using two-phase systems is a fast and simple technique and is relatively easy to scale up.

The most commonly used two-polymer system is composed of polyethylene glycol (PEG) and dextran. Since dextran is a rather expensive polymer, much research effort has been devoted on finding cost-effective alternatives. Polymer types other than PEG are also studied. The examples are thermo-separating EOPO copolymer and hydrophobic modified copolymer EOPO called HM-EOPO (Johansson *et al*., 1996; Persson *et al*., 1999). These polymers consist of Ethylene Oxide (EO) and Propylene Oxide (PO) units. The EOPO copolymers display a Lower Critical Solution Temperature (LCST) in water (Walter *et al*., 1985).

In the first extraction step, a thermo-polymer/polymer system is used and will form the two-phase, followed by a second extraction step where the recovered thermo-polymer-rich phase from the first step is heated to a temperature above the Cloud Point (CP). This will give rise to the formation of new two-phases, one polymer rich bottom phase and one almost pure water phase on top. The idea is that in the first extraction step the target protein is recovered in the thermo-polymer-rich phase while the contaminants are collected in the polymer-rich phase (Fig. 1). For the second extraction step it has been shown that almost all proteins are partitioned exclusively to the aqueous phase (Persson *et al*., 2000). Thus, the aqueous phase including the target protein can be processed further downstream while the concentrated polymer phase can be recycled.

As reported in previous studies, the major drawback of the most common polymer/polymer system which is composed of dextran and PEG is that the system is expensive for scaling up. Therefore, this problem may be overcome by the usage of alternative polymer such as starch derivatives, maltodextrin and cellulose derivatives (Bolognese *et al*., 2005).

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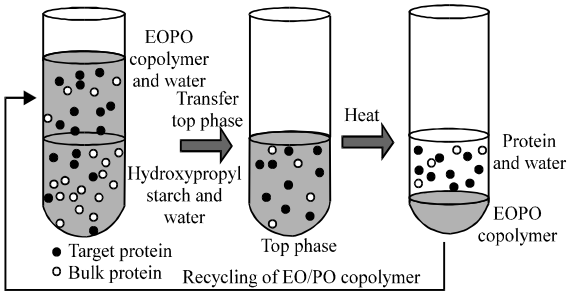

Fig. 1: Purification of a target protein in an aqueous two-phase system with recycling of EOPO copolymer by thermo-separation. Black dots symbolize the target protein and white dots the bulks proteins. The target protein is recovered in the top EOPO phase in the primary phase system where after the top phase is isolated and heated over the cloud point of the copolymer. After thermo-separation the target protein is recovered in the water rich top phase and the bottom phase copolymer enriched phase is free of protein. The copolymer phase is recycled and used in a new extraction

The polymers used in this study were amylopectin rich Waxy maize starch and the polymeric surfactant named Dehypon® LS 54. Dehypon® LS 54 is a block copolymer composed of Ethylene Oxide (EO)/Propylene Oxide (PO) with approximately 5 moles EO and 4 moles PO, which is also known as fatty alcohol polyglycol ether is a commercial surfactant that available from Cognis Corporation. The Waxy maize starch formed the heavy phase due to its higher density compared with the Dehypon® LS 54 phase. Recombinant *E.coli* cutinase-$(WP)_4$ was selected as model protein and partitioned in Dehypon® LS 54/waxy maize starch. In this work we also constructed and predicted the phase diagram for Dehypon® LS 54/waxy maize starch.

MATERIALS AND METHODS

Chemicals: The copolymer Dehypon® LS 54 (approx. 5 moles EO and 4 moles PO) (M_n = 2600) was purchased from Cognis Oleochemicals (Malaysia). The density of copolymer Dehypon® LS 54 at 25°C is determined by density meter DMA 4500 Anton Paar (Austria). The Waxy maize starch (10^6-10^7 g mol^{-1}) was purchased from Sigma Aldrich Company (Malaysia). The Waxy maize starch is composed of 93-95% amylopectin and 5-7% amylose. All other chemicals were analytical grade and water was distilled.

Protein: *Escherichia coli* strain expressing recombinant cutinase with a genetic engineering approach at N-terminal and sequences encoding a $(WP)_4$ hydrophobic tag at the C-terminal was obtained from the earlier study by Zulaikha *et al.* (2008). The purpose of $(WP)_4$ hydrophobic tag is to increase the partitioning to the EOPO (copolymer) rich phase.

Phase diagram determination: The systems were prepared by stock solution, 60% (w/w) Dehypon® LS 54 and 8% (w/w) the waxy maize starch in Tris-HCl buffer (50 mM; pH 8.0). Known masses of these solutions and water were weighted into a test tube to have the desired initial overall composition. Aqueous two-phase systems were prepared with a final mass of 10 g. All samples were prepared in 15 mL graduated plastic tubes. After the phase systems had been mixed thoroughly in a closed test tube by a vortex mixer, phase separation was speeded up by centrifugation at 4500 rpm for 10 min in a Eppendorf 5804 Centrifuge. Then the phase systems were left undistributed for at least 24 h at 25.0°C in a regulator water bath Protech 631D (Malaysia), whose temperature was controlled to within ±0.1°C. The top and bottom phases were isolated and diluted, top phase were diluted six times and bottom phase were diluted ten times. First, the concentration of waxy maize starch was determined in both phases by polarimetry using a digital polarimeter POLAX-2L, Atago (Tokyo, Japan) by making a polarimetric standard curve for Amylopectin starch. The presence of Dehypon® LS 54 had no effect on the optical rotation of waxy maize starch. The concentration of Dehypon® LS 54 in both phases was determined by measuring refractive index with a refractometer from Carl Zeiss (Ober-kochen/Württ, Germany) and by subtracting the refractive index contribution of waxy maize starch. The water contents were obtained by subtractions of copolymer and starch compositions. A few points in the phase diagram, around the critical point, were determined by titration of the two-phase system with water until the formation of a one phase system (Albertsson, 1986).

Preparation of the aqueous two-phase systems: All polymer concentration was calculated as % weight/weight (w/w). The waxy maize starch (amylopectin starch) were dissolved in water and added from stock solution 15%. The copolymer Dehypon® LS 54 was added as pure substances. Tris-HCl buffer pH 8.0 was added from 50 mM stock solution to maintain a constant pH. Aqueous two-phase systems were prepared with a final mass of 5 g. All samples were prepared in 15 mL graduated test tubes (which were calibrated for further accuracy). All experiments were performed in duplicate and the experimental data are average values. The additions of cutinase, from pre-made stock solution, were based on

volume to give final concentration of 1 mg mL^{-1}. Water was then added to give final weight of 5 g. After thorough gentle mixing of the system components, the phase separation was enhanced by centrifugation 10 min at 4500 rpm. Then the phase systems were left undistributed for at least 24 h at 25.0°C in a regulator water bath Protech (Malaysia), whose temperature was controlled to within ±0.1°C. All systems were made in duplicate and blank systems devoid of protein were also prepared. The volume of the top and bottom phases was estimated. Then the top and bottom phases were separated and diluted appropriately for the determination of protein content.

Determination of Cloud-Point Temperature (CPT): The cloud-point temperatures were taken in a regulator water bath by immersing the co-polymer solution in a capped glass tube. The CPT was carried out by making an aqueous solution of the copolymer Dehypon® LS 54 at different concentration (from 0 to 70% w/w) in water solution and gradually raising the temperature 1°C min^{-1}, until the first sign of clouding appear (Johansson *et al*., 1996, 1998). All cloud-point measurements were performed three times.

Temperature-induced phase separation: The top phase of the primary phase system which containing the copolymer Dehypon® LS 54 was removed and isolated in a separate vessel. The thermo-separation was performed in 15 mL graduated capped test tubes and placed in water bath. The temperature of this phase was increased above the copolymer cloud point. To obtain a macroscopic separation of the copolymer and water phases in reasonable time the temperature must be raised 5-10°C higher than the cloud point (Berggren *et al*., 1995). The top phase was thermo-separated by increasing the temperature to 36°C for all night to obtain clearly a new two phase. The volume of the top and bottom phases of the new systems was estimated. Then the top and bottom phases were separated and diluted appropriately for the determination of protein concentration and the partition coefficient was calculated. The percent protein recovery (R) in the water phase was calculated according to:

$$R\,(\%) = \frac{C_{water} \times V_{water}}{C_o \times V_o} \quad (1)$$

where, C_{water} and V_{water} are the protein concentration and the water phase volume in the water-rich phase after thermo-separation and C_o and V_o are the initial protein concentration and the system volume, respectively.

Protein partitioning: The partitioning of the molecules in two-phase systems is described by the partition coefficient K. It is defined as the concentration of the target molecule in the top phase, C_T, divided by the concentration in the bottom phase, C_B: $K = C_T / C_B$. The total protein content was determined by according to Bradford using Coomassie Brilliant Blue G. The absorption was measured at 595 nm. Bovine serum albumin was used for standard (Bradford, 1976). The spectrophotometer used was Genesys 10uv from ThermoSpectronic (Madison, USA). For the determination of protein concentration, the top phase and bottom phase were diluted 10 times in Tris-HCl buffer pH 8.0. The yield (%) of the target protein in the top phase of the primary system (Y_p) and in the water-rich phase after thermo-separation (Y_t) are calculated as:

$$Y_p = \frac{100}{1 + \frac{V_t}{V_b} \times K} \quad (2)$$

$$Y_t = \frac{100}{1 + \frac{V_{copolymer}}{V_{water}} \times \frac{1}{K}} \quad (3)$$

where, V_t and V_b are the volumes of the top and the bottom phase and $V_{copolymer}$ and V_{water} are the copolymer-rich phase volume and the water-rich phase volume respectively (Albertsson, 1986; Kepka *et al*., 2003).

RESULTS AND DISCUSSION

Phase diagram for the primary phase system: The phase diagram determined for Dehypon® LS 54, the waxy maize starch (amylopectin starch) and water (Fig. 2) is similar to

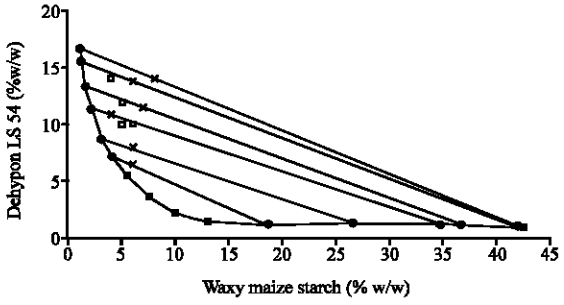

Fig. 2: The phase diagram with binodial and tie-lines for the Dehypon® LS 54 and Waxy maize starch at room temperature (approximately 25°C). Experimental values determined by analyzed two-phase systems (●) and by titration (■) is shown. Phase compositions used for diagram (×) determination. Phase compositions used in the primary extraction step (▫)

earlier determined phase diagrams for $EO_{50}PO_{50}$ and waxy barley starch systems. Both systems are composed of a thermo-separating EOPO copolymer and a low cost starch polymer. The phase diagram for Dehypon® LS 54/waxy maize starch shows that the binodial has been shifted towards lower polymer concentration compared with the Waxy barley starch system. This is accordance with the higher molecular mass of waxy maize starch (1000-10.000 kg mol^{-1}) relative to the Waxy barley starch (500 kg mol^{-1}). Thus, one advantage of using Waxy maize starch is the reduction in consumption of both polymers in a large-scale process (Kepka *et al*., 2003). The phase diagram was determined without neither biomass nor salts. However, their presence could be expected to displace the binodial towards lower polymer concentration (Albertsson, 1986). The tie lines for each mixture were constructed by plotting the best line that could fit the points of initial composition, of the top and the bottom phases. The compositions of the phases for the Dehypon® LS 54-waxy maize starch-water system at 25°C are shown in Table 1.

Partitioning of protein in dehypon® LS 54/waxy maize primary phase system: Protein partitioning can be affected by altering the polymer concentration in the system. It has previously been shown that with increasing polymer concentrations, i.e., increased tie-line length, a more extreme partitioning towards one of the two phases will be observed (Albertsson, 1986; Zaslavsky, 1995; Forciniti *et al*., 1991). However, in this case a slightly stronger partitioning to the Dehypon® LS 54 phase was observed with decreased tie-line length (Table 2). The partition coefficient values (K) higher than 1 were observed for all the ATPSs assayed, thus indicating that the partition equilibrium is displaced to the Dehypon® LS 54-enriched phase. A higher yield in the top phase was achieved for protein in the Dehypon® LS 54/waxy maize starch system when tie-line length was increased (Table 2). The enhancement of yield was due to the increased partition coefficient, but also to the increased volume ratio (Kepka *et al*., 2003).

Cloud-point diagram: The cloud-point diagram for the binary system of Dehypon® LS 54 in water solution is shown in Fig. 3. The curve shape is very typical of this kind of copolymer. The critical point was 30°C at Dehypon® LS 54 concentration between 3 and 10% (w/w).

It should be noted that the presence of salts and a second polymer will affect the cloud-point and, therefore, this diagram should be used as a guideline only (Hatti-Kaul, 2000).

Protein partitioning in temperature-induced two-phase systems: Generally, water solution of thermo-separating copolymer will, upon heating, form a turbid solution. At the cloud-point the copolymer will form macroscopic aqueous droplets which are dispersed in the water phase

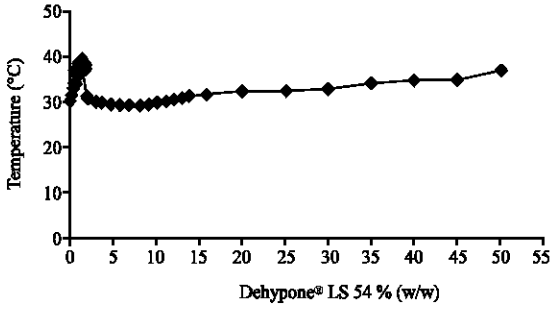

Fig. 3: Cloud-point diagram for the system Dehypon® LS 54/water

Table 1: Composition of the phases in the waxy maize starch-Dehypon® LS 54-water system at 25°C

Total system			Top phase			Bottom phase		
Waxy maize	Dehypon LS 54	H_2O	Waxy maize	Dehypon LS 54	H_2O	Waxy maize	Dehypon LS 54	H_2O
(%w/w)								
8.00	14.00	78.00	1.05	16.64	82.31	42.30	0.84	56.86
6.00	14.00	80.00	1.19	15.57	83.24	41.82	0.89	57.29
7.00	11.50	81.50	1.62	13.29	85.09	36.61	1.13	62.26
4.00	11.00	85.00	2.18	11.36	86.46	34.74	1.20	64.06
6.00	8.00	86.00	3.14	8.76	88.10	26.52	1.32	72.16
6.00	6.50	87.50	4.11	7.20	88.69	18.53	1.27	80.20

Table 2: Partitioning (K), yield (Y), volume ratio (V_t/V_b) and tie-line length in aqueous two-phase systems with different composition of Dehypon® LS 54 and Waxy maize starch. All systems contains 50 mM Tris-HCl buffer pH 8.0

	Total system composition (%w/w)					
No.	Waxy maize	Dehypon LS 54	Volume ratio V_t/V_b	Partition coefficient (K)	Yield % (Y_p)	Tie-line length
I	5.0	12.0	1.52	1.56	29.66	37.04
II	6.0	10.0	1.19	1.89	30.78	34.11
III	5.0	10.5	1.24	2.85	22.06	34.10
IV	4.0	14.5	1.55	1.16	35.74	43.20

Table 3: Partitioning (K), yield (Y) and volume ratio (V_t/V_b) in temperature-induced two-phase systems with different composition of Dehypon® LS 54 and Waxy maize starch. Letters I, II, III and IV represent the different total system composition according to Table 2

Total system composition (%w/w)	Volume ratio V_t/V_b	Partition coefficient (K)	Yield (%) (Y_t)
I	0.47	0.79	72.90
II	0.67	0.92	61.98
III	0.50	0.39	83.68
IV	0.39	0.42	85.96

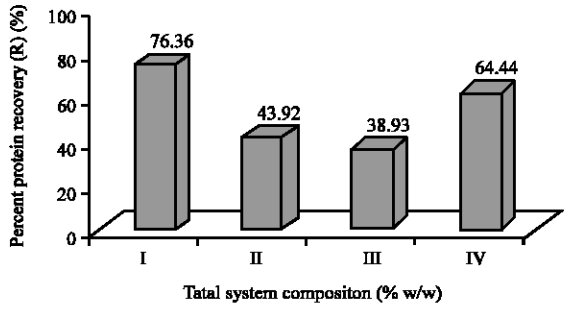

Fig. 4: Percent protein recoveries (R) for cutinase in the water enriched phase of a temperature-induced two-phase systems obtained after heating the top phase of a Dehypon® LS 54/ Waxy maize systems with different compositions (I, II, III and IV) on x-axis represent the different total system composition according to Table 2

and at higher temperature the copolymer droplets will aggregate and form one top aqueous copolymer phase in equilibrium with bottom water phase (Kepka *et al*., 2003). However, in this study the top phase enriched with EOPO and the water phase at the bottom phase. This might be explained by lower density of Dehypon® LS 54, which is 0.933-0.938 g cm^{-3}, as compared to the density of water. As a consequence, the target protein will be recovered in the bottom phase. The high affinity of the protein for the lower water phase is due to both the strong interaction between charged groups in the protein and the polar water molecule and the excluded volume effect in the copolymer-enriched phase. When separated, the bottom phase is composed of approximately more than 50% of water and the upper phase is copolymer Dehypon® LS 54-enriched. The protein partition coefficient (Table 3) shows that proteins were more evenly partitioned in the bottom phase (water-enriched phase). The percent protein recoveries (R) in the bottom water phase after thermo-separation is shown in Fig. 4. A significant R values between 60 and 80% is observed in systems with total composition 12% (w/w) Dehypon® LS 54.5% (w/w) waxy maize starch and 14.4% (w/w) Dehypon® LS 54.4% (w/w) waxy maize starch, respectively.

CONCLUSIONS

In this study, the separation properties of a novel two-phase system formed by Dehypon® LS 54 and the waxy maize starch were evaluated. The Dehypon® LS 54/waxy maize starch system was found to have similar characteristics with the earlier $EO_{50}PO_{50}$/waxy barley starch system. Top phase has a high EOPO concentration, while bottom is waxy maize starch-enriched. The tie line of the phase diagram are practically parallel, thus allowing us to determined the top and bottom compositions for any given total polymer composition. A temperature increase of this phase, above the polymer cloud point, results in a second temperature induced two-phase system with a copolymer-enriched upper phase and a water-enriched lower phase, containing most of initial protein. The copolymer Dehypon® LS 54 is also advantageous because of the low cloud point, which reduces the risk for denaturation in the temperature-induced phase separation. No addition of chemicals was required in the thermo-separation step resulting in that the chemical consumption was reduced. Finally, we conclude that Dehypon® LS 54/waxy maize starch system may be considered as an interesting alternative for protein purification due to the low cost of the phase-forming polymers (suitable for large-scaling), the waxy maize starch biodegradability and their possibility of forming temperature-induced systems which will let both the protein in water-buffer medium were recovered and the copolymer recycled.

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