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Development of Extraction System by using Thermo-Responsive Polymer for Bovine Serum Albumin Recovery

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Abstract: Thermo-responsive polymers, Dehypon®LS54 and Berol®370 were paired randomly with starch derivatives, K4484®dextrin and N-Lite®D maltodextrin for develop thermoseparating ATPS. The developed systems are DehyponLS54/K4484, DehyponLS54/N-Lite.D and Berol370/K4484. All systems were tested with partitioning of BSA as model protein. Among the tested systems, DehyponLS54/K4484 was found as most suitable system for BSA recovery. A small electrochemical potential effect was observed in DehyponLS54/K4484 dextrin system in which the change of pH (pH 8 to 6) had slightly influence the partitioning behavior of BSA. At both pH, BSA was observed had high affinity to starch enriched phase and it caused a low yield value of BSA after thermoseparation step. However, recovery of BSA in water phase at pH 6 was higher 40% compared to pH 8. The addition of Na_2SO_4 has lowered the cloud point of Dehypon®LS54.

Key words: ATPS, thermo-responsive polymers, protein extraction

INTRODUCTION

Aqueous polymer two phase system (ATPS) is obtained by mixing of two aqueous polymer solutions over its critical concentration. Due to high water content in the system, ATPS could offer a suitable environment for biomaterial separation. Similar to traditional Liquid Liquid Extraction (LLE) system (organic-aqueous solution), the basis of separation in ATPS is selective distribution of target substances between the two phases. Recently, application of thermo-responsive copolymers in ATPS for separation of biomolecules has been increasing. Compared to traditional ATPS polymer-polymer system (e.g., PEG-Dextran), thermoseparating ATPS is more interesting because one phase of aqueous polymer in equilibrium with a water phase can be simply achieved by heating up the temperature of the polymer solution above the Cloud Point (CP) with most of target protein are recovered in water phase (Persson *et al*., 2000). Moreover, the copolymer can be recovered after temperature-induced separation in which enable the reuse of the copolymer in another ATPS (Persson *et al*., 2000). In addition, most of these polymers is inert and unaffected the activity of biomolecules (Kumar *et al*., 2007).

Therefore, initial study was carried out in order to develop an economic ATPS extraction system by using thermo-responsive copolymers that are able to buy in local market. All the polymers used in this study are able to obtain in bulk locally, except for Berol®370 (which was obtained from Akzo Nobel, Singapore). For this preliminary study, Bovine Serum Albumin (BSA) is selected as the model protein and partitioning behavior of BSA in the developed system was studied.

MATERIALS AND METHODS

Chemicals: K4484®dextrin (base: tapioca) and N-Lite®D maltodextrin (base: waxy maize) were supplied by National Stach Food Innovation (Malaysia). The thermo-responsive polymers Dehypon®LS54 was obtained from Cognis Oleochemicals (Malaysia) and Berol®370 was a gift by Akzo Nobel (Singapore). Bovine Serum Albumin (BSA) was purchased from Sigma-Aldrich (Malaysia).

Aqueous Two Phase System (ATPS): The primary phase extraction systems were prepared with total mass of 2.5 g by weighing up appropriate amounts of a 100% (w/w) stock solution of polymer and a 30% (w/w) stock solution of starch derivative in test tubes. Tris-HCl buffer pH 8 and sodium phosphate buffer pH 6 were used with concentration 10 mM in the prepared system. Stock solution of concentrated BSA was added into the system, giving the final composition between 40-50% of system's total weight. The prepared systems were mixed using vortex. The separation into two-phases was assisted by centrifugation at 4000 rpm for 2 min.

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Temperature-induced separation (Thermoseparation): The top polymer phase of the primary phase systems was removed and isolated in separate test tube. 0.2 M of Na_2SO_4 was added into polymer phase in order to decrease the Cloud Point Temperature (CPT). Temperature-induced separation was performed at 32°C. A new two phase system is formed in which it consists of polymer-enriched top phase and water-enriched in the bottom phase.

Determination of protein content: The total protein content was determined according to Bradford method using Amresco Bradford Reagent (US). The absorbance was measured at 595 and 465 nm. The value at 595 nm (A595 nm) was subtract with value at 465 nm (A465 nm). System without protein was prepared as blank.

Calculations

Partition coefficient: The partitioning of BSA between light phase and bottom phase is described by partition coefficient, K:

$$K = \frac{C_L}{C_H} \quad (1)$$

where, C_L and C_H are the concentration of BSA in the light phase and heavy phase, respectively.

Recovery after thermoseparation step: Recovery of BSA in water phase (heavy phase) after thermoseparation step (subscript T) is calculated according to Eq. 2.

$$R_{H,T} = \frac{100}{1 + (R_V . K_T)} \quad (2)$$

where, R_V is volume ratio of light phase to heavy phase. K_T is the partition coefficient of BSA after thermoseparation step.

Yield after thermoseparation step: The normalized yield of BSA in water phase (heavy phase) after thermoseparation step (subscript T) is defined in Eq. 3:

$$Y_T = \frac{C_{H,T} \times V_{H,T}}{C_0 \times V_0} \quad (3)$$

where, C and V are the concentration of BSA and volumes of the phases, respectively. Subscript of H,T refers to the heavy phase (water phase) after thermoseparation step. C_0 is the concentration of BSA solution and V_0 is the volume of BSA solution that added in the beginning of extraction (primary phase separation).

RESULTS AND DISCUSSION

Developing thermoseparating ATPS: Phase separation of two polymer phases can be explained by the interaction between two unlike polymers molecules. They are repulsive in character that the molecules prefer to be surrounded by their own kind instead of being mixed. In this case the system will have its energetically most favorable state when the two polymers are separated (Zaslavsky, 1994). The result of mixing solutions of two polymers is therefore incompatibility and there arises one phase which contains the one polymer and the other phase with second polymer.

As for this study, thermo-responsive polymer is selected as first phase component. Meanwhile, polymer with higher molecular weight which is starch derivative has become the second phase component. Some properties of polymers and starch derivatives that are used in this study are shown in Table 1.

Aqueous two phase systems was attempted to develop by investigating different combinations of the selected polymers and starch derivatives (Table 2). The mixture was centrifuged at 4000 rpm for 2 min and allowed to separate at 25°C. About 10 mM buffer was added into the systems. For primary phase separation, light phase would be polymer-enriched phase and heavy phase is starch-enriched phase. After thermoseparation, a new ATPS will be formed. As for systems that contain Dehypon®LS54, polymer will enrich the top phase and water-enriched phase is at the bottom due to low density of Dehypon®LS54 compared to water. In contrast, systems that contain Berol®370, a new ATPS with water-enriched phase on the top and polymer-enriched phase at the bottom will be obtained. The compatibility of Berol®370

Table 1: Several chemical properties of phase components

Commercial name	Chemical name	Chemical family component	MW	Cloud point
First phase component				
Dehypon®LS54	Ethylene oxide propylene oxide		2600*	30°C
Berol®370	Polyethylene polypropylene glycol ether		1400**	32°C
Second phase component				
K4484		Dextrin	>10000	
N-Lite®D		Maltodextrin	>10000	

All data of the polymers were obtain from MSDS. *Ferro *et al.* (2009), **Misra *et al.* (2001)

Table 2: Compatibility of different studied polymers

Systems	Formation of two-phases
10% Dehypon/10% K4484	Yes
10% Dehypon/5% N.LiteD	Yes
10% Berol/10% K4484	No
35% Berol/5% K4484	Yes

Table 3: Approximate time of phase separation for two different polymer two-phase system

Phase system	Approximate time
Dehypon-K4484 dextrin	5-60 min
Berol-K4484 dextrin	60 min-overnight

and K4484®dextrin were tested at two different polymer concentrations. As initial step, total concentrations of polymers in the systems were chosen arbitrarily in order to test the compatibility of polymer combinations and to obtain a system with volume ratio near to 1.0. Therefore, there is possibility that two phases can be formed at different polymer concentrations. A better understanding could be achieved by construct phase diagram of the systems in future study.

The results show that phase separation in Dehypon/K4484 system was obtained at relatively low polymer concentration (10%) compared to Berol-K4484 dextrin system (35%). This phenomenon explained that Dehypon®LS54 has lower compatibility with K4484®dextrin compared to Berol®370 (Table 3).

Furthermore, time of phase separation was also different between the two systems, Dehypon/K4484 and Berol/K4484. After centrifuge at 4000 rpm for 2 min, it was found that Dehypon/K4484 system formed in shorten time compared to Berol/K4484 system at experiment temperature, 25°C. This could be due to the lower Molecular Weight (MW) of Berol®370 compared to Dehypon®LS54 whereby two polymers will separate more completely if higher MW is used (Persson *et al*., 2000).

Salt addition: effect on Cloud Point Temperature (CPT): Cloud point diagram of Dehypon®LS54 was shown in Fig. 1. Above the curve, the solution will separate into aqueous copolymer-enriched phase and water-rich phase.

Below the curve, the copolymer solution will be in homogenous phase. According to the curve, Cloud Point Temperature (CPT) for Dehypon is approximately 30-33°C at 15-35% Dehypon concentration. However, the CPT is decreased to 24°C at 25-35% dehypon concentration when 0.2 M Na_2SO_4 was added into the polymer solution. This can be explained by the fact that the salt decreased the solubility of the copolymer (Zaslavsky, 1994). The reduced of CPT using salt was an advantage in which it can help extraction of heat-sensitive protein in future study.

BSA partitioning in dehypon-K4884 dextrin system: Figure 2 shows the aqueous two phase system consists of 10% dehypon and 10% K4484 with 10 mM buffer was used for partitioning of model protein, BSA. Isoelectric point, pI of BSA is 5.3 and the net charge of BSA at pH 8.0 and 6.0 is -17 and -2, respectively (Persson *et al*., 2000). In primary phase separation, majority of BSA was

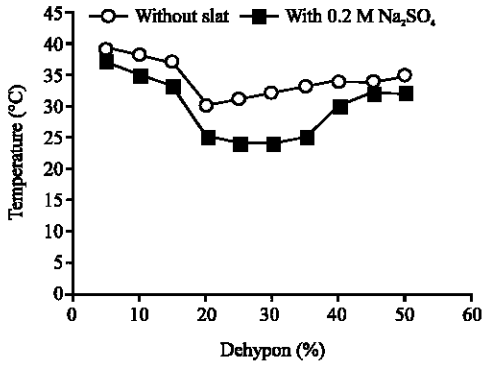

Fig. 1: Cloud point diagram for Dehypon®LS54. The diagram was determined for pure copolymer in water solution

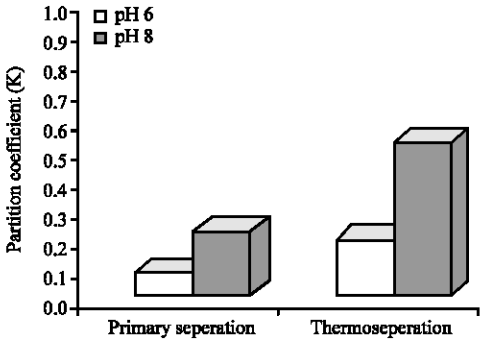

Fig. 2: Partitioning of BSA in 10% Dehypon/10% K4484 dextrin system at pH 6 and 8

preferably partitioned into starch phase with highest partition coefficient obtained is 0.08 for pH 6 and 0.22 for pH 8. As non-ionic polymer, Dehypon®LS54 offers no charged in the system, therefore it is uninfluenced the partition behaviour of BSA directly. Thus, it is suggest that partition behavior of BSA is depends on the electrochemical potential occurred based on pH system and net charge of studied protein. In earlier study, negative charge protein generally had higher affinity into hydrophobic polymer phase. At pH 8, BSA displayed more negative charge compared to pH 6. Therefore, K value obtained at pH 8 is higher compared to pH 6. Same pattern is obtained in thermoseparation step. This is because at negative charge, more BSA will partition into hydrophobic phase, which is polymer phase.

BSA recovery after temperature-induced phase separation: Recovery of BSA after thermoseparation step is shown in Fig. 3. In thermoseparation step, the recovery is determined by refer to the amount of BSA that is separated into hydrophilic water phase. It was found that system with pH 6 gave higher recovery of BSA compared

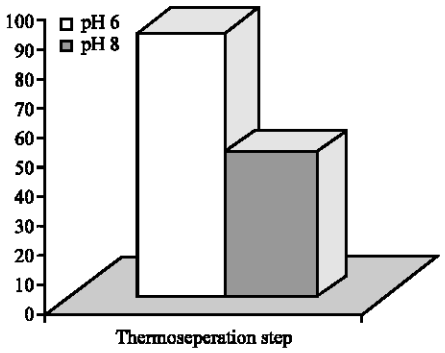

Fig. 3: BSA recovery in 10% Dehypon/10% maltodextrin system at pH 6 (sodium phosphate buffer) and pH 8 (Tris HCl buffer)

to pH 8 (Fig. 3). This is possible be caused by the net charge of BSA which is more positive in pH 6 compared to pH 8. As positively charged protein, BSA will have high affinity into hydrophilic water phase. Furthermore, the addition of hydrophilic salt, Na_2SO_4 during thermoseparation step could also influence the affinity of BSA into water phase.

However, yield of BSA after thermoseparation step is 3.7% for pH 6 and 1.2% for pH 8. A low yield of BSA is due to majority of BSA added into the system had extracted into starch phase compared to polymer phase in primary step separation. Therefore, it suggests that BSA can't be directed into polymer phase by depending on electrochemical potential factor only. Therefore, another factor (such as addition of salt or detergent) should be taken into account to enhance more protein partition into polymer phase in primary separation step in future study.

CONCLUSION

Thermo responsive copolymer, Dehypon®LS54 and starch derivative, K4484®dextrin are the suitable pair as phase components for develops economic thermoseparating aqueous two phase system since both polymers can be obtained locally. In primary phase separation, majority of BSA was partitioned into starch-enriched phase. Low affinity of BSA into polymer phase in primary separation has lowered BSA yield value in thermoseparation step. Although, the yield is low, this system has shown the ability to recover BSA from polymer phase (of primary phase separation) about 90% in water phase (after thermoseparation step). Since, there is no additional salt was added in primary phase separation, it was initiated that only electrochemical potential factor occurred in the studied systems. Majority of BSA was scarcely directed into polymer-enriched phase with the presence of electrical chemical factor, only. Thus, it is suggests that electrochemical potential factor had slightly affected the partitioning behaviour of BSA. The addition of hydrophilic Na_2SO_4 in thermoseparation step was lowered the cloud point of Dehypon®LS54 and also enhanced the partitioning of BSA into water phase (in thermoseparation step).

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