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Journal of Chromatography B, 852 (2007) 167–173
JOURNAL OF CHROMATOGRAPHY B
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Aqueous micellar two-phase system composed of hyamine-type hydrophobically modified ethylene oxide and application for cytochrome P450 BM-3 separation

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Received 18 October 2006; accepted 8 January 2007
Available online 13 January 2007

Abstract

The hydrophobically modified ethylene oxide polymer, HM-EO, was modified with an alkyl halide to prepare a hyamine-type HM-EO, named N-Me-HM-EO, which could be used for forming N-Me-HM-EO/buffer aqueous micellar two-phase system. The critical micelle concentration of N-Me-HM-EO solution and the phase diagrams of N-Me-HM-EO/buffer systems were determined. By using this novel aqueous micellar two-phase system, the separation of cytochrome P450 BM-3 from cell extract was explored. The partitioning behavior of P450 BM-3 in N-Me-HM-EO/buffer systems was measured. The influences of some factors such as total proteins concentration, pH, temperature and salt concentration, on the partitioning coefficients of P450 BM-3 were investigated. Since the micellar aggregates in the N-Me-HM-EO enriched phase were positively charged, it was possible to conduct the proteins with different charges to top or bottom phases by adjusting pH and salt concentration in the system. A separation scheme consisting of two consecutive aqueous two-phase extraction steps was proposed: the first extraction with N-Me-HM-EO/buffer system at pH 8.0, and the second extraction in the same system at pH 6.0. The recovery of P450 BM-3 was 73.3% with the purification factor of 2.5. The results indicated that the aqueous micellar two-phase system composed of hyamine modified polysoap has a promising application for selective separation of biomolecules depending on the enhanced electrostatic interactions between micelles and proteins.
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Keywords: Aqueous micellar two-phase system; Hydrophobically modified ethylene oxide; Electrostatic interaction; Micellar aggregate; Cytochrome P450 BM-3

1. Introduction

The separation of a desired protein from fermentation broth containing a variety of biomolecules represents an important challenge encountered in today's biotechnology industry. One of the considerable approaches for addressing this challenge is the use of aqueous micellar two-phase system for liquid–liquid extraction process. Compared to the traditional aqueous two-phase systems including polymer/polymer or polymer/salt, aqueous micellar two-phase system can provide some unique features such as binary surfactant–water system, the self-assembling and labile nature of micelles, simultaneously offering hydrophobic and hydrophilic environments, and easily combining affinity, electrostatic and excluded-volume interactions in the systems, etc. [1–3].

Aqueous nonionic micellar two-phase system has been explored and widely applied to partition some hydrophobic proteins and small organic molecules. The attention was mainly paid to investigate the physicochemical properties of systems and enhance the partition selectivity by varying solution conditions. Recently, new aqueous micellar polymeric system containing hydrophobically modified polymer (HM-P) was developed [4,5]. Compared to the aqueous micellar two-phase system composed of nonionic surfactant with relative low molecular weight, new systems composed of macromolecular surfactant (named polysoap) display low critical solution temperature (LCST) in aqueous solution. Above the LCST, two phases can be formed, where one phase is polymer-depleted (micelle-depleted) and the other is polymer-enriched (micelle-enriched). In addition, the

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1570-0232/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2007.01.008

hydrophobically modified polymer usually exhibits the micellar aggregates, which entangled with each other to form a network [6,7].

Two kinds of hydrophobically modified polymers have been investigated. One is the end-capped HM-P such as hydrophobically modified random copolymer of ethylene oxide (EO) and propylene oxide (PO), HM-EOPO, with myristyl groups ($C_{14}H_{29}$) at both ends [8]. The polymer-enriched phase formed with HM-EOPO/water was not too concentrated, in favor of efficiently partitioning proteins to the polymeric phase [4,9]. Other is the comb-like HM-P such as a hydrophobically modified ethylene oxide polymer, HM-EO. The main feature of HM-EO copolymer is that the alkyl chains grafted onto the hydrophilic backbone containing poly(oxyethylene) chains interspersed by aliphatic tertiary amines [10]. HM-EO is a kind of cationic polysoap [11,12]. Therefore, in the HM-EO/water micellar two-phase system, the dominant interactions between micelles and proteins were shown to be electrostatic interaction rather than the hydrophobic and excluded-volume interactions [6,13]. However, the electrostatic interaction between HM-EO and proteins was quite weak because the net charge of micellar aggregates was particularly relevant to the hydrolytic reaction of tertiary amines, pH and ionic strength of two-phase systems. In order to enhance the efficiency of protein partitioning with the electrostatic interaction to the desired phase, it is necessary to increase the charges on the micelles formed by HM-EO. Addition of ionic surfactants such as sodium dodecyl sulfate (SDS) and tetradecyltrimethylammonium chloride is one of optional methods to strengthen the net-charge number of mixed micelles [14,15]. However, it was found that the dissociative surfactant with low molecular weight usually results in the denaturation of biomolecules [6].

In the present work, HM-EO will be modified with alkyl halide to prepare a hyamine-type HM-EO, named N-Me-HM-EO, and a new kind of aqueous N-Me-HM-EO/buffer micellar two-phase system will be introduced to enhance the electrostatic selectivity of protein partitioning. Similar to the HM-EO/water aqueous two-phase system, the hyamine-type HM-EO/water system also exhibits phase separation with the bottom micelle-rich phase and top micelle-poor phase at relative low temperature. The purpose of present work is to demonstrate the possibility of N-Me-HM-EO/buffer systems for separating target protein direct from cell extract. The purification of cytochrome P450 BM-3 will be used as the example. Cytochrome P450 BM-3 (CYP102) is a water-soluble, NADPH-dependent fatty acid hydroxylase, from *Bacillus megaterium* [16]. The high catalysis activity makes P450 monooxygenase system a suitable candidate for biotechnological applications [17,18].

2. Materials and methods

HM-EO polymer was purchased from Akzo Nobel Surface Chemistry AB (Stenungsund, Sweden) with the molecular weight of 56,000. N-Me-HM-EO was prepared using the nucleophilic substituted reaction with HM-EO solution and excessive alkyl halide (CH_3Br) under low temperature, gentle stirring and ultrasonic treatment. All polymers used were purified by repeating phase separation of aqueous solution for 10 times and then freeze-dried to remove the solvents. All other chemicals were of analytical grade.

Cytochrome P450 BM-3 fermentation broth was obtained by aerating cultivation of *E. coli.* DH5α pT-USC1 BM-3 at 37 °C with KLF2000 fermentor (Bioengineering, Switzerland) in 2 l Lurin–Bertani (LB) medium with 100 mg ml^{-1} ampicillin and 0.1 mg ml^{-1} $FeCl_3$. The initial pH of fermentation was controlled at 7.5. When the OD absorbance at 578 nm reached 1.0, the expression of P450 BM-3 was induced by the temperature shifting from 37 to 42 °C, and kept for 5 h. Then the cells were harvested by the centrifugation (Sorvall Super T21, DUPONT, USA) at 8000 rpm for 10 min. For cell disruption, the suspension was treated for 5 min by an ultrasonic disrupter JY92-II (Ningbo Scientz Biotechnology Co., Ltd., China) with the output level 200 W and duty cycle 50% in the presence of 0.1 mM EDTA (pH 7.5) and 0.1 mM PMSF. The supernatant of cell extract was obtained by the centrifugation (Sorvall Super T21, DUPONT) at 10,000 rpm for 20 min. Finally, the supernatant was diluted with the appropriate buffer until the total protein concentration reached 10 mg ml^{-1} for the experiments of aqueous two-phase extraction.

All polymer concentrations were defined as the percentage of weight to weight (%, w/w). The determination method of HM-EO was established previously [19]. In general, the same mass of acetone was added to the sample, then the mixture was diluted several times to a concentration of less than 1% (w/w). 0.5 g of the diluted sample and 5.0 ml of Coomassie assay reagent were added to a 10 ml test tube, and then the mixture was thoroughly mixed. After 30 min, the amount of polymer was determined by the absorbance at 620 nm. The measurement was carried out in triplicate, and the average values were given. No interference of the salts used was observed.

For the partitioning experiments, the enzyme sample was added into appropriate N-Me-HM-EO/buffer solution to form the system with total weight of 5 g. After mixing, the solution was centrifuged at 500 rpm for 3 min and put in a water bath of 10 °C for over 12 h. The samples of top and bottom phases were taken for the assays of enzyme and total protein. The partitioning coefficients of enzyme and total protein, K_e and K_p, were defined as the ratio of the concentration of top phase to that of bottom phase. The partitioning coefficients were measured in triplicate for two samples, and the average values were given. In general, the variation of assays was less than 10%, and the recovery rate of measurement was more then 95%.

The activity of P450 BM-3 was measured by NADPH consumption at 340 nm based on an extinction coefficient of 6.66 ml mol^{-1} cm^{-1}, and the activity unit (U ml^{-1}) was expressed as the micromole of NADPH consumed per minute and millilitre of enzyme solution. The measurement was performed with a spectrophotometer, Ultrospec 3300 pro (Amersham Biosciences, Uppsala, Sweden). Total protein concentrations were determined by Lowry method [20]. In order to observe protein distribution, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS–PAGE) and isoelectrofocusing electrophoresis (IEF) were used to analyze

the samples of cell extract supernatant and purification process [21,22]. The gel concentrations of SDS–PAGE and IEF were 10% (v/v) and 5% (v/v), respectively. The calibration markers were obtained from Amersham Biosciences.

The critical micelle concentration (CMC) of N-Me-HM-EO was determined with fluorescence probing. In the present work pyrene was selected as fluorescence probe due to the ability to form excimers and low solubility in water as published [23]. The solubility of pyrene in water is greatly increased in the presence of micelles. The emission spectrum of pyrene molecules shows several vibronic peaks, and the ratio I_1/I_3 of the intensities of the first and the third vibronic peaks is a sensitive indicator of polarity of pyrene microenvironment [24,25]. When the concentrations of polymer surfactants reach the CMC, the I_1/I_3 value decreases drastically and the CMC of the copolymer can be obtained.

3. Results and discussion

The chemical structure of N-Me-HM-EO is shown in Fig. 1 and compared to HM-EO. Unlike the micelles present in the HM-EO/buffer system, the micelles presented in the N-Me-HM-EO/buffer system have more positive net charge after hyamine modification of HM-EO. Two kinds of micelle structures are illustrated in Fig. 2a and b. The micelles in the micelle-rich phase are larger and more abundant than those in the micelle-poor phase. The numerical difference of positively charged micelles between the top phase and the bottom phase could possibly lead to the electrostatic potential difference between two phases. A schematic representation of micellar aqueous two-phase system composed of the polysoaps, HM-EO or N-Me-HM-EO, is shown in Fig. 2c. The localization of more charges to specific micelle is expected to influence the partitioning behavior of charged proteins and to enhance the partitioning selectivity of the desired proteins.

3.1. Phase diagrams of N-Me-HM-EO/buffer systems

The phase diagrams of N-Me-HM-EO/buffer systems with different NaCl concentrations are shown in Fig. 3. Above the coexistence temperature, the N-Me-HM-EO/buffer solution could separate into two phases. The bottom phase was enriched with the polymer and the top phase was depleted of polymer. Unlike the thermo-separating systems such as HM-EOPO/buffer system, the LCSTs of N-Me-HM-EO/buffer systems tested in the present work were below zero, which greatly depended on

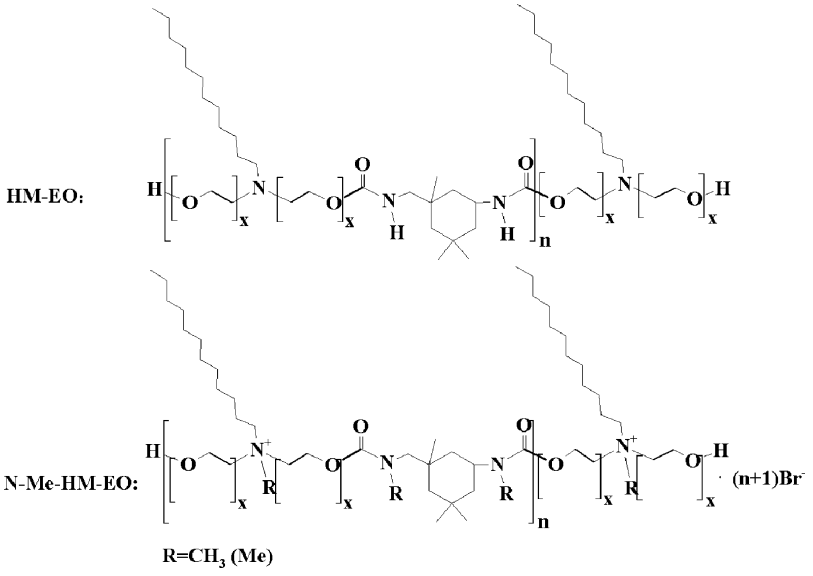

Fig. 1. The chemical structures of HM-EO and N-Me-HM-EO.

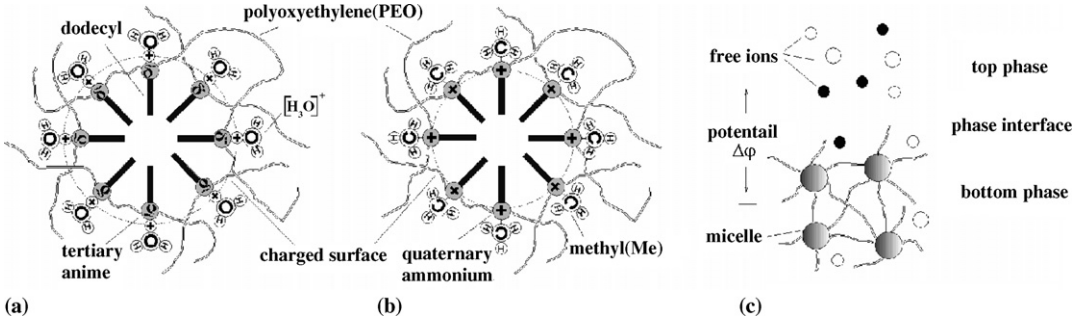

Fig. 2. The structures of polysoap micelles and network of micellar aggregates in aqueous two-phase system: (a) hydrophobic microdomain (micelle) of HM-EO, (b) hydrophobic microdomain of N-Me-HM-EO and (c) network of charged micellar aggregates.

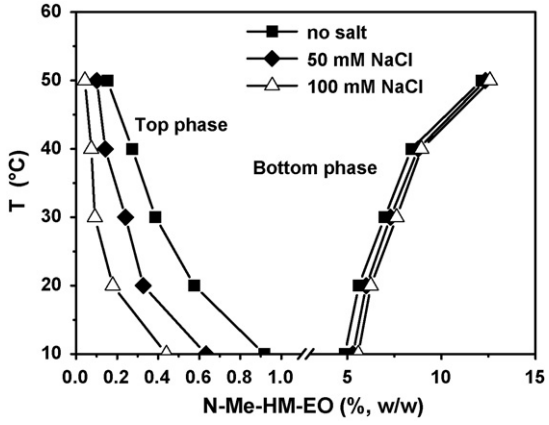

Fig. 3. Phase diagrams of aqueous micellar two-phase systems at different salt concentrations. The conductivity of barbiturate buffer of pH 9.0 was 2.0 mS cm^{-1} at 25 °C. The total concentration of N-Me-HM-EO in each system was 2.5% (w/w).

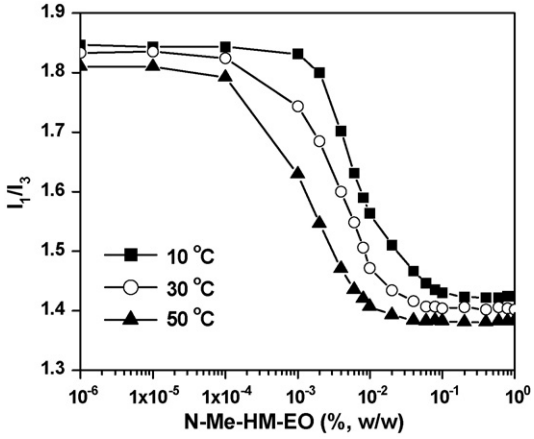

Fig. 4. Effect of temperature on the CMC of N-Me-HM-EO solutions. The conductivity of barbiturate buffer of pH 7.0 was 20.1 mS cm^{-1}. The total concentration of N-Me-HM-EO in each system was 2.5% (w/w).

the high molecular weight of N-Me-HM-EO. Furthermore, in the 2.5% (w/w) N-Me-HM-EO/buffer system, the two-phase area increased with the increase of salt concentration under same pH condition. The salt-out effect was considered as the main reason.

3.2. Critical micelle concentration of N-Me-HM-EO

As shown in Figs. 1 and 2, the molecular structure of N-Me-HM-EO indicates that the copolymer is a kind of polysoaps. The association of long alkyl chain grafted onto quaternary ammonium in the backbone has great contribution to form the micellar aggregates. The I_1/I_3 ratios measured by fluorescence probe method at different polymer concentrations are shown in Fig. 4. From the sharp drop of I_1/I_3 curves, the critical micelle concentration for N-Me-HM-EO could be determined approximately between 0.001 and 0.01% (w/w). With the increase of temperature, the CMC of N-Me-HM-EO solution decreased. It could be assumed that higher phase-separating temperature is in favor of micelle formation, which possibly lead to changing the charges of the polysoap micellar surface and then affect the partitioning behavior of target protein. However, high temperature could also affect the enzyme activity of P450 BM-3. So the suitable phase-separation temperature was selected as 10 °C in the present work.

3.3. Partitioning of P450 BM-3 and bulk proteins

The partitioning behavior of P450 BM-3 and bulk proteins of cell extract supernate in N-Me-HM-EO/buffer systems was investigated in order to optimize the separation conditions for P450 BM-3. The polymer concentration of all systems was selected as 2.5% (w/w) with the phase volume ratio of about 1:1. The influences of some important factors such as the addition of cell extract supernate, pH and buffer concentration, on the partition of P450 BM-3 and total protein were investigated. The results are shown in the following sections.

3.4. Effect of addition amount of cell extract supernate on the partitioning

The addition of cell extract supernate was characterized by the total protein concentration in the systems. The influences of total protein concentration on the partitioning of P450 BM-3 in N-Me-HM-EO/buffer systems at pH 6.0 and 8.0 are shown in Fig. 5a and b, respectively. It could be found that P450 BM-3

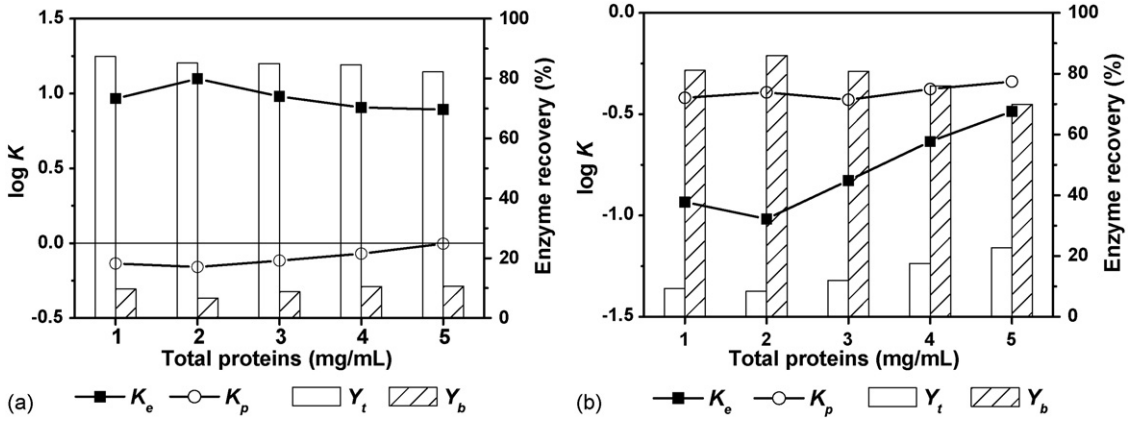

Fig. 5. Effect of amount of cell extract supernate on the partitioning behavior of P450 BM-3 in 2.5% (w/w) N-Me-HM-EO/10 mmol l^{-1} phosphate buffer systems at 10 °C: (a) pH 6.0 and (b) pH 8.0.

was concentrated in the top phase at pH 6.0, but the trend of partitioning behavior at pH 8.0 was almost the opposite to that at pH 6.0. Meanwhile, the total proteins were mainly partitioned to the bottom phase in the systems at both pH 6.0 and pH 8.0. With the increase of cell extract supernate in the system, the partition coefficients of P450 BM-3 (K_e) and total proteins (K_p) would tend to approach 1. The proteins have the ability to neutralize the charges on the surface of N-Me-HM-EO micelles. By increasing the total protein concentration from 1.0 to 5.0 mg ml^{-1} in the systems, the maximum value of K_e at pH 6.0 (for which target P450 BM-3 mainly exists in the top phase) and the minimum value of K_e at pH 8.0 (for which target P450 BM-3 mainly exists in the bottom phase) were obtained at the total protein of 2.0 mg ml^{-1}, i.e. the addition of cell extract supernate was 1.0 ml for 5 g system, while the enzyme yield reached relatively high level.

3.5. Effect of pH on the partitioning of P450 BM-3

The influences of pH on the partitioning coefficient of P450 BM-3 are shown in Fig. 6. The buffer used was 10 mmol l^{-1} barbiturate buffer with wide pH range from 2.6 to 12.0. Unlike cationic tertiary amine-type HM-EO whose positive net charge strongly depends on the solution pH, the hyamine-type HM-EO is always positively charged due to nearly complete ionization of hyamine at wide pH range. In addition, the positive charges of hyamine-type HM-EO were much abundant. Since the pH of solution is able to change the electrostatic feature of protein surface (negatively or positively charged), it was considered that positively charged micelles of N-Me-HM-EO could attract the negatively charged proteins to the polymer-rich phase (bottom phase), and simultaneously repulse the positively charged proteins to polymer-free phase (top phase). It was interesting to find that the partitioning coefficients of P450 BM-3 were 12.5 at pH 6.0 and 0.096 at pH 8.0, respectively. The isoelectric point (p*I*) of P450 BM-3 is close to 7.0. The change of electrostatic interactions between N-Me-HM-EO micelles and P450 BM-3 around pH 7.0 resulted in the reverse partitioning trends that P450 BM-3 shifted from one phase to another phase as shown in Fig. 6. The total proteins were mainly partitioned to the bottom phase

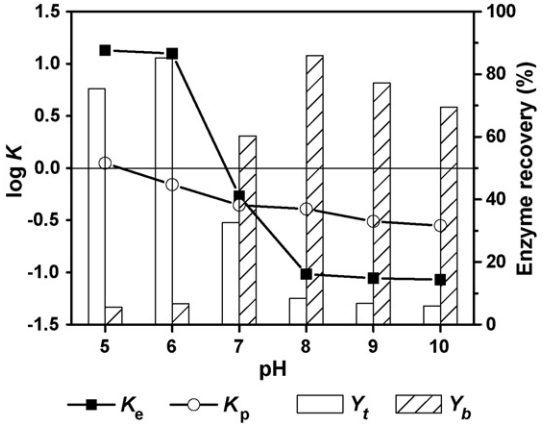

Fig. 6. Effect of pH on the partitioning behavior of P450 BM-3 in 2.5% (w/w) N-Me-HM-EO/10 mmol l^{-1} barbiturate buffer systems at 10 °C.

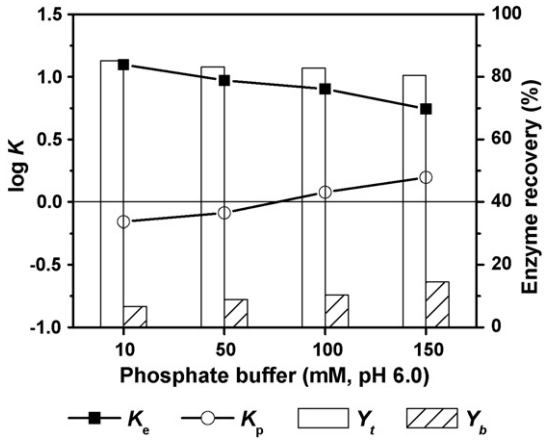

Fig. 7. Effect of buffer concentration on the partitioning behavior of P450 BM-3 in 2.5% (w/w) N-Me-HM-EO/buffer systems at 10 °C.

at the pH range tested. The higher or lower pH may be in favor of isolating P450 BM-3 from total proteins, but it would lead to the inevitable denaturation of P450 BM-3. Therefore, the optimized pHs, pH 6.0 and 8.0, were chosen for ensuing both the purification efficiency and enzyme yield.

3.6. Effect of buffer concentrations on the partitioning of P450 BM-3

A higher buffer concentration can result in better ability to avoid the unexpected alteration of pH in the system. However, the ionic strength would increase with the increase of buffer salts. As shown in Fig. 7, the partition coefficients of P450 BM-3 decreased as the concentrations of phosphate buffer increased from 10 to 150 mmol l^{-1}. Meanwhile, some amount of bulk protein partitioned to the top phase from the bottom phase. In order to ensure the high partition selectivity and low denaturation, 10 mmol l^{-1} phosphate buffer was considered as the appropriate condition.

3.7. Separation scheme for the recovery of P450 BM-3

Based on the results mentioned above, a scheme for the separation of P450 BM-3 from cell extract supernate was developed. This scheme mainly consists of two consecutive extraction steps as shown in Fig. 8. The results are summarized in Table 1.

The first extraction step was performed in 2.5% (w/w) N-Me-HM-EO/10 mmol l^{-1} phosphate buffer system at 10 °C at pH 8.0, and the phase volume ratio was about 1.0. The total system weight was 50 g, containing 10 g cell extract supernate with protein concentration of 10 mg ml^{-1}. After centrifuged at 500 rpm for 5 min, 86.3% of P450 BM-3 could be recovered in the polymer-rich bottom phase, with the purification factor of about 1.2.

After the first phase separation, the bottom polymer-rich phase containing the target protein was transferred into a dialyzed bag with the fractionation molecular weight of 5000. The solution was dialyzed in the 10 mmol l^{-1} phosphate buffer of pH 7.0 until the pH reached 7.0. Then the pH was changed to

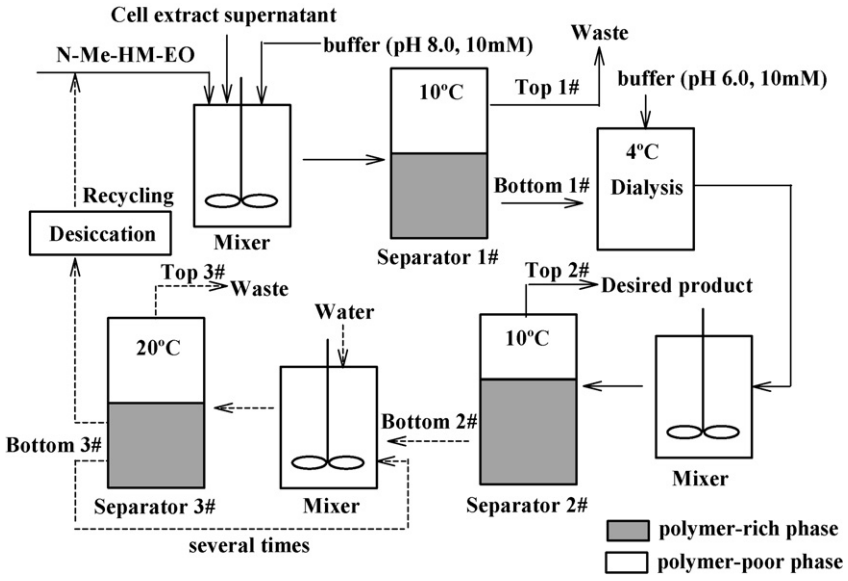

Fig. 8. Scheme for the separation of P450 BM-3 using N-Me-HM-EO/buffer systems.

Table 1
P450 BM-3 separation process with 2.5% (w/w) N-Me-HM-EO/10 mmol l^{-1} phosphate buffer systems at 10 °C

Purification step	Volume (ml)	Total protein concentration (mg ml^{-1})	Enzyme concentration (U ml^{-1})	Protein recovery (%)	Enzyme recovery (%)	Purification factor
Cell extract supernate	10	10.0	0.572	100	100	
Bottom phase of the first partitioning (pH 8.0)	25.5	2.78	0.194	70.8	86.3	1.22
Top phase of the second partitioning (pH 6.0)	19.0	1.52	0.221	28.9	73.3	2.54

6.0 using the same method. The solution in the dialyzed bag was transferred into the second separator followed by adding the 10 mmol l^{-1} phosphate buffer of pH 6.0 until the total system weight reached 50 g. Finally, the top phase containing target P450 BM-3 was collected with the centrifugation. After the second extraction step, the activity yield of P450 BM-3 reached 73.3%, with a purification factor of about 2.5.

The possible recycling of N-Me-HM-EO is also given in Fig. 8. Although the phase-forming polymer, N-Me-HM-EO, was much cheaper than most of the traditional phase-forming components such as dextran, the recycling use of HM-EO and N-Me-HM-EO could be achieved if necessary.

4. Conclusions

A novel aqueous micellar two-phase system containing hyamine-type polysoaps, N-Me-HM-EO, was introduced and used for the separation of intracellular enzyme P450 BM-3 from the cell extract. Compared with tertiary amine-type HM-EO/buffer system, the new system has two main advantages that are suitable for the purification of charged biomolecules. First, the cationic hyamine-type micelles are restricted by poly(ethylene oxide) chains, whose charge feature is independent of the pH of systems. Therefore, N-Me-HM-EO/buffer system could be considered as a kind of the electrostatic interaction-dependent systems. The charges of proteins strongly depend on the solution pH, so the proteins with different isoelectric points can be partitioned selectively to the desired phases. Secondly, lower LCST of N-Me-HM-EO with high molecular weight makes it possible to separate proteins at relatively low temperature.

The partition behavior of P450 BM-3 and bulk protein was investigated under varying separation conditions, including amount of cell extract addition, pH, buffer concentration, etc. The separation scheme with two consecutive extraction steps at different pHs was developed for the recovery of P450 BM-3 from cell extract supernate. A new purification strategy for the removal of bulk proteins over or below the isoelectric point of target protein was introduced relating to the electrostatic interaction-dependent system. After two separation steps, about 73.3% in activity of P450 BM-3 could be recovered with a purification factor of 2.5. It could be concluded that new aqueous micellar two-phase extraction is one of promising downstream processing methods for the separation of intracellular enzymes.

Acknowledgements

This work was supported by the grants from the Ministry of Science and Technology of China (National Basic Research Program of China, 2007CB707805) and the National Natural Science Foundation of China.

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