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Model process for separation based on unfolding and refolding of chymotrypsin inhibitor 2 in thermoseparating polymer two-phase systems

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Abstract

For the design of a new separation process based on unfolding and refolding of protein, the partitioning behaviour of proteins was studied in thermoseparating polymer two-phase systems with varying pH and temperature. Chymotrypsin inhibitor 2 (CI2), which unfolds reversibly in a simple two-state manner, was partitioned in an aqueous two-phase system (ATPS) composed of a random copolymer of ethylene oxide and propylene oxide (Breox) and dextran T-500. Between 25 and 50°C, the partition coefficients of CI2 in Breox–dextran T-500 systems remain constant at neutral pH. However, there is a drastic increase at pH values below 1.7, 2.1, and 2.7 at 25, 40 and 50°C, respectively. The partitioning behavior of CI2 was also investigated in thermoseparating water–Breox systems at 55–60°C, where CI2 was partitioned to the polymer-rich phase at pH values below 2.4. These results on the CI2 partitioning can be explained by the conformational difference between the folded and the unfolded states of the protein, where the unfolded CI2 with a more hydrophobic surface is partitioned to the relatively hydrophobic Breox phase in both systems. A separation process is presented based on the partitioning behavior of unfolded and refolded CI2 by control of pH and temperature in thermoseparating polymer two-phase systems. The target protein can be recovered through (i) selective separation in Breox–dextran systems, (ii) refolding in Breox phase, and (iii) thermoseparation of primary Breox phase. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Aqueous two-phase systems (ATPS), which are spontaneously formed in solutions of two non-compatible polymers (e.g. dextran and poly(ethylene glycol) (PEG)) or a polymer (e.g. PEG) and a high concentration of salt (e.g. phosphate), have widespread use in biochemistry and biotechnology for purification of biological products [1,2]. Recently, the use of thermoseparating polymers in aqueous two-phase systems has been introduced [3–10]. When such polymers are heated above a critical temperature, i.e. the cloud point, the solubility of the polymer will decrease and a system composed of water and a polymer phase is formed. This makes it possible to perform temperature-induced phase separation whereby a target protein can be separated from the polymer and recovered in a clean water phase. Such smart polymer systems may improve the

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protein purification process, due to the removal of polymer from the final product and the recycling of its polymer [11]. For practical applications, the protein separation process using such thermoseparating polymer systems has already been applied with success [12].

Natural polymers, such as proteins, also undergo conformational changes in response to solvent conditions such as temperature, pH, and denaturants [13–17]. It is attractive to investigate the unfolding and refolding of proteins in stimuli-responsive ATPS from operational and economical viewpoints based on their response to various external conditions. The surface properties of protein (i.e. surface net hydrophobicities) are affected by solvent conditions [13–17]. A process for protein recovery may be based on unfolding and refolding in thermoseparating polymer two-phase systems. The more hydrophobic unfolded protein is selectively recovered and refolded in the hydrophobic polymer phase, while the more hydrophilic refolded protein is partitioned to the hydrophilic phase.

The objective of this paper is to study the basic principle for a separation process for proteins based on the unfolding and refolding in thermoseparating polymer two-phase systems (Fig. 1). The unfolded target protein is recovered in the top phase after partitioning in a Breox–dextran system and refolded by changing the environmental conditions in this phase. The target protein is recovered from the water phase after thermoseparating the water–Breox systems. CI2 was selected due to its simple two-state folding behavior. The partitioning behavior of CI2 was investigated in Breox–dextran and water–Breox systems under various operational conditions, e.g. pH and temperature. The refolding of CI2, induced by changing the operational condition, was investigated. Based on these results, a process for sepa-

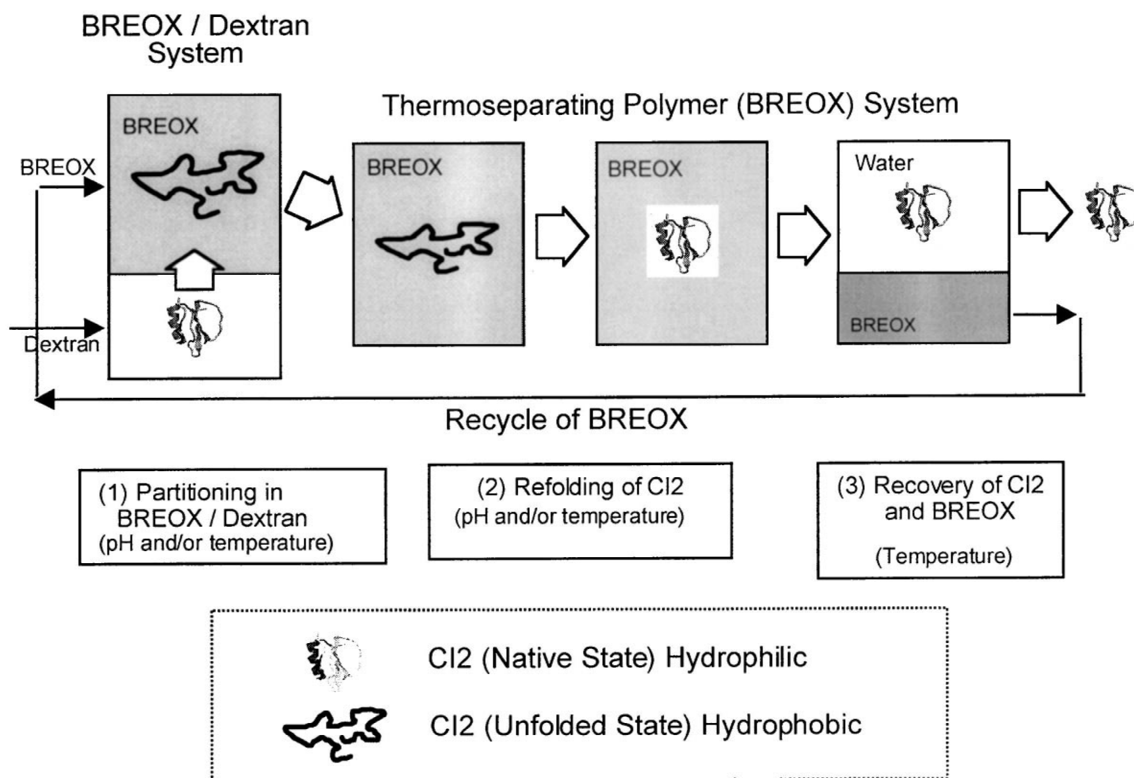

Fig. 1. Scheme for a separation process based on protein unfolding in a thermoseparating Breox–dextran system. The target protein is unfolded in the primary phase system. The unfolded protein is partitioned to the Breox phase. The Breox phase is isolated and the target protein is refolded. After thermoseparation, the refolded target protein is recovered in the water phase.

ration of the target protein by unfolding and refolding in thermoseparating polymer two-phase systems is presented.

2. Experimental

2.1. Materials

The random copolymer Breox PAG 50A 1000 (M_r 3900, Breox) with 50% ethylene oxide and 50% propylene oxide was used as top phase polymer and was obtained from International Specialty Chemicals (Southampton, UK). As a bottom phase polymer, dextran T-500, molecular mass, M_r 500,000, obtained from Amersham Pharmacia Biotech (Uppsala, Sweden), was used. The chymotrypsin inhibitor 2 (CI2) was produced, expressed, and purified as previously described [18]. Chemicals of analytical grade were obtained from KEBO Lab (Stockholm, Sweden).

2.2. Partitioning of CI2 in aqueous two-phase systems

Polymer stock solutions of 60% (w/w) Breox and 30% (w/w) dextran were prepared. The basic aqueous two-phase systems (ATPS) were 6% (w/w) Breox–6% (w/w) dextran T-500 systems and water–20% (w/w) Breox systems. The total weights of Breox–dextran and water–Breox systems were 3 and 5 g, respectively. All polymer concentrations were calculated as weight/weight percent. The ATPS were buffered with 20 m*M* sodium phosphate (pH 7.4). When performing the pH change experiments, the pH (0.75–4.3) was adjusted by the addition of concentrated acid, phosphoric acid for the unfolding and hydrochloric acid for the refolding experiments. All the phase system components were weighed in and mixed before CI2 was added (final concentration; 19 μ*M*). The phase separation was performed at 25, 40, and 50°C for Breox–dextran systems and at 55 and 60°C for the water–Breox systems by allowing the ATPS to stand in a water bath for 1.5 h. The temperature for phase separation of the Breox–dextran system was selected so that the temperatures were under the cloud point temperature of the Breox copolymer (50°C). The temperature for each system was selected based on previous studies [3–5] showing the phase diagram of water–Breox systems in order to prevent the formation of a third polymer phase in Breox–dextran systems.

2.3. Analysis

The conformational change of CI2 was monitored by measuring fluorescence intensity with an excitation wavelength of 280 nm and an emission wavelength of 356 nm [19]. All fluorescence experiments were performed using a Perkin-Elmer LS5B (Norwalk, CT, USA) luminescence spectrometer. The concentration of CI2 was determined from the absorbance at the wavelength of 280 nm. All the measurements on protein concentration of CI2 were performed using a Shimadzu Spectrophotometer UV-2010 (Kyoto, Japan).

3. Results and discussion

3.1. Partitioning behaviour of CI2 in Breox–dextran systems at various pH values

The partitioning behavior of CI2 was investigated in Breox–dextran aqueous two-phase systems at different pH. The effect of pH on the partitioning behaviors of CI2 was investigated in 6% (w/w) Breox–6% (w/w) dextran T-500 systems at 25°C. The partition coefficients of CI2, K_{CI2}, at various pH are summarized in Table 1. The K_{CI2} value showed very little change (1.4 to 2.1) in the pH range from 7.4 to 2.0. A sharp increase of the partition coefficient was observed at a pH between 2.0 and 1.2.

In aqueous polymer two-phase systems, the partition coefficient of biomolecules has been found empirically to depend upon (i) electrostatic, (ii) hydrophobic, and (iii) ligand effects [20]. The electrostatic effect on the partitioning has been kept constant since a high concentration of dominating salt (100 m*M* NaCl) was used over the whole pH range [22]. No ligand-bound polymers were used in this experiment. The surface net charge of CI2, Z_{CI2} [21] was calculated (Table 1). The Z_{CI2} values increased with decreasing pH value from 7.4 to 2.0, where the partition coefficients of CI2 were not changed. The above results indicate only a minor

Table 1
Partition coefficient and net charge of CI2 in Breox (6%)–dextran T-500 (6%) aqueous two-phase systems within a pH range from neutral to acidic. Salt composition: 10 mM sodium phosphate and 100 mM NaCl. Temperature: 25°C

pH	Partition coefficient of CI2 (K_{CI2})	Net charge[a] of CI2 (Z_{CI2})
7.3	1.4	−0.2
6.0	1.4	0.2
3.7	1.4	8.5
2.9	1.7	10.3
2.2	2.1	10.9
2.0	2.1	10.9
1.2	3.2	11.0
1.0	5.6	11.0
~0.6	8.2	11.0
~0.5	13.3	11.0

[a] Net charge of CI2 was calculated according to Cameselle et al. [21] based on the amino acid sequence of CI2.

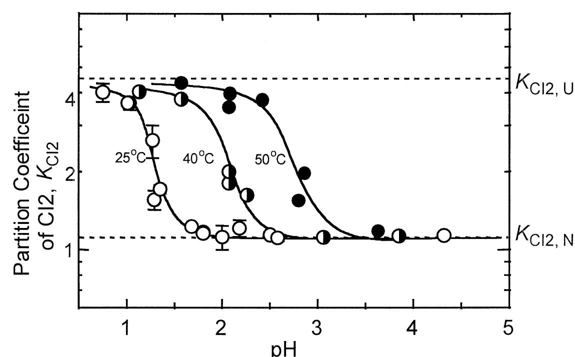

Fig. 2. Effect of pH on partition coefficient (K) of CI2 in Breox–dextran systems at temperatures of 25°C (○), 40°C (◑), and 50°C (●). $K_{CI2, N}$ and $K_{CI2, U}$ indicate K-values for native and unfolded CI2, respectively.

electrostatic effect on the CI2 partitioning in the two-phase systems used here. However, the K_{CI2} value increased below pH 2.0 although the Z_{CI2} values at +11.0 remain constant in this range. This suggests that other effects are responsible for the change in partitioning behaviour. One such possible effect is acid-induced unfolding. The folding and unfolding of CI2 conforms to a simple two-state model under equilibrium conditions [23]. The unfolded state becomes more stable than the native state below pH 1.7 [24]. Hydrophobic amino acids, buried inside the protein molecules in a native state are exposed to the protein surface making the protein surface more hydrophobic in the unfolded state [25,26]. For several proteins, unfolding has been shown to increase partitioning to the more hydrophobic PEG phase in a poly(ethylene glycol)–dextran aqueous two-phase system [13,14,27]. This also appears to hold for CI2. The increase of CI2 partition coefficient shows that the conformation of CI2 was changed to the unfolded state at the lower pH range and favoured partition to the relatively hydrophobic Breox phase in the Breox–dextran two-phase system.

3.2. Combined effect of pH and temperature on partitioning behaviour of CI2 in Breox–dextran systems

The effects of both temperature and pH on the partition coefficient of CI2 were investigated in 6% (w/w) Breox–6% (w/w) dextran T-500 systems. The effects of pH on the partition coefficient of CI2, K_{CI2}, at 25, 40, and 50°C are shown in Fig. 2. At 25°C, the K_{CI2} values were almost the same in the pH range 1.5 to 7.4. The K_{CI2} values increased significantly from 1.1 to 3.6 at pH below 1.5. At 40 and 50°C, a similar tendency could be observed where the minimum pH values for the sharp increase of the K_{CI2} values were less than 2.5 and 3.2, respectively. It was found that the partition coefficient of CI2 was increased at acidic pH and at every temperature tested here and that the critical pH value was increased with temperature. Thus, it is important to consider the combined effect of the different conditions on the protein conformation in order to control the partitioning behaviors of target proteins in aqueous two-phase systems using thermoseparating polymers. In the case of CI2, the pH value for the destabilization of its structure was 3.0 and 3.5 at the temperature of 64.0 and 73.8°C, respectively [28].

3.3. Comparison of CI2 partitioning with conformational change

The conformational change of CI2 monitored by intrinsic fluorescence [23] as well as the partitioning behaviors in Breox–dextran and water–Breox systems was investigated at various pH and temperatures. The results on the critical condition for the change of CI2 partitioning and those determined from the conformation analysis are summarized in

Fig. 3. The solid line in Fig. 3 shows the conformational transition of native to unfolded protein based on the partitioning behaviour of CI2. The dotted line in Fig. 3 shows the conformational transition of CI2 based on fluorescence data. The critical points determined from CI2 fluorescence were very similar to those determined from the CI2 partitioning, although the former curve was slightly shifted to higher pH at lower temperature. This is probably because no polymers are present in the fluorescence experiments. Polymers have a stabilizing effect on proteins, and PEG, which is similar to Breox used in the two-phase systems, is known to stabilize proteins [1,28–30]. Breox is therefore expected to shift the pH-induced unfolding transition to a lower pH at any given temperature.

3.4. CI2 partitioning in water–Breox systems

The partitioning behaviour of CI2 was investigated in water–Breox systems. This water–polymer phase system can be formed after thermoseparation of the Breox top phase from the primary Breox–dextran phase system (Fig. 1). In this step, the target protein can be recovered in a water phase, and the polymer can be recovered in a concentrated form and separated from protein.

The operational temperature to separate the phase systems was set to a value that is above the lower critical temperature (50°C) of the Breox copolymer. Fig. 4 shows pH dependence of the partition coefficient of CI2, K_{CI2}, in a thermoseparated water–Breox system at 55°C. A similar tendency in the pH dependence of the CI2 partitioning was obtained in this system as in the Breox–dextran systems (compare with Fig. 2). CI2 was partitioned strongly to the water phase at neutral pH values, and the protein was partitioned to the bottom Breox phase at acidic pH. The partition coefficient of CI2 was almost constant in the pH range 3.0 to 6.0. Further decrease in the pH from 3.0 to 1.0 caused a sharp decrease in partition coefficient (K_{CI2}=5.0 at pH 3.0 and K_{CI2}= 0.3 at pH 2.0). This is due to unfolding of CI2. Partitioning studies of CI2 were performed at 60°C, but formation of irreversible aggregates of CI2 was observed and the partition coefficients in water–Breox systems could not be determined at this temperature. Compared with the results on the conformational change of CI2 determined by fluorescence (Fig. 3), the critical pH value at 55°C was shifted to lower pH compared with the expected curve from the fluorescence data. The shift of the critical pH value is most probably due to the

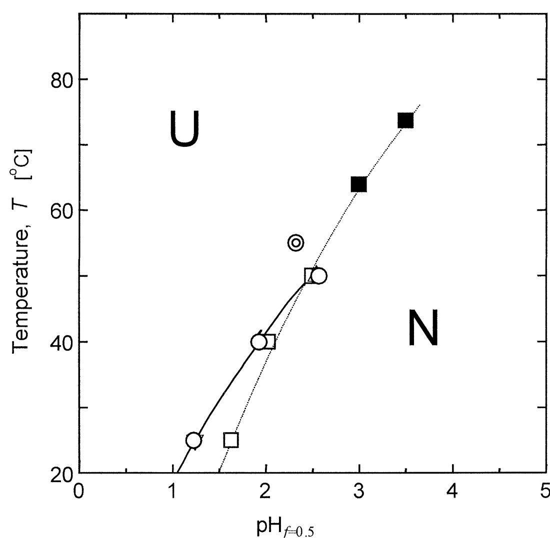

Fig. 3. Temperature and pH dependence for the conformational change of CI2. The midpoint value for the partition coefficient (K=2.3), see Fig. 1, and the conformational change measured by fluorescence was defined as $pH_{f=0.5}$. These values indicate the point where half the protein is in native (N) and half in unfolded (U) state. The partition coefficients in Breox–dextran systems are indicated by (○); partition coefficient in water–Breox systems (◎); intrinsic fluorescence in sodium phosphate (□); and intrinsic fluorescence data from Jackson et al. [19] (■).

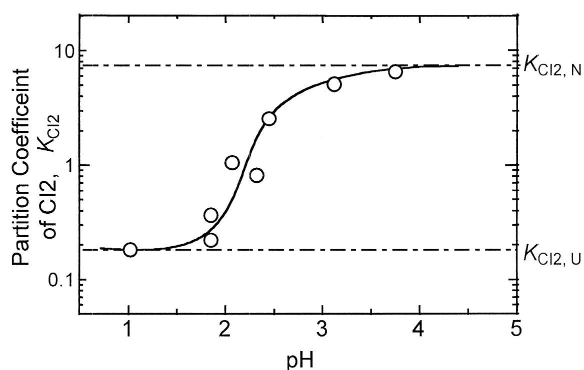

Fig. 4. Effect of pH on the partition coefficient of CI2 in a thermoseparated water–Breox system at a temperature of 55°C. Breox concentration: 20% (w/w). $K_{CI2, N}$ and $K_{CI2, U}$ indicate K-values for native and unfolded CI2, respectively.

stabilizing effect of Breox in a similar way as the effect of PEG on protein stability.

The partitioning of small biomolecules, such as amino acids and peptides, in water–Breox systems has already been investigated in a series of previous works, where some limitations in the practical usage of this system have also been discussed [5,6]. The most important limitation is the difficulty in partitioning large molecules because of the excluded volume effect of the polymer phase [6]. The molecular weight of CI2 (7.1 kDa) is relatively low. Therefore, CI2 may be partitioned to the polymer phase because of the relatively low excluded volume effect for this low molecular weight protein and the increase in the hydrophobicity of the protein surface due to unfolding.

Thus, unfolded CI2 can be partitioned to the polymer phase in water–Breox two-phase systems by controlling temperature and pH as operational parameters. Above pH 3.0, where the protein is in the native state, CI2 can be recovered in the water phase (Fig. 1).

3.5. Proposed bioprocess based on the conformational change of proteins using Breox–dextran systems and water–Breox systems

The possibility of a protein recovery process using thermoseparating polymer two-phase systems (Fig. 1) has been shown by the partitioning behaviour of CI2 in Breox–dextran and water–Breox systems and by control of conditions for unfolding of CI2. Here, we discuss the potentials of this bioprocess.

The first step (Fig. 1) is the selective separation of the target protein in the unfolded state. At this stage, the degree to which the polymer stabilizes the protein [28–30] must be taken into account (Fig. 3), as this is likely to vary from protein to protein [31]. The selective separation of the target protein from other proteins may be achieved by selective denaturation of the target protein, if such conditions can be found.

The second step is the refolding of unfolded protein in the recovered Breox-phase. Much research on protein folding has already been reported both from the theoretical and practical points of view. Some researchers have also reported that the refolding of other proteins, which are difficult to refold in isolation because of aggregation, can be enhanced by the addition of biological chaperones (i.e. GroEL and GroES) [15] or 'artificial chaperones', e.g. poly(ethylene oxide) polymers [28–30].

The third step is the recovery of refolded CI2 in the water phase of water–Breox phase systems in order to remove the polymer from the solution. The condition for the recovery of the CI2 in folded state in the water phase can be selected based on the partitioning behaviour of CI2 in water–Breox systems and the unfolding of CI2 (Fig. 3). The target protein CI2 was found to partition to the water phase at neutral pH at each temperature.

4. Conclusions

The possibility to design a separation process based on unfolding and refolding of target protein was investigated in thermoseparating polymer systems, including Breox–dextran and water–Breox two-phase systems, by using CI2 as a model protein. The following results were obtained. (i) The partitioning of CI2 in the two-phase systems could be controlled by changing pH and temperature. (ii) The partitioning behaviour of CI2 in Breox–dextran and water–Breox systems was due to conformational change between native and unfolded state. (iii) The unfolded CI2 could be refolded to the native form with high yield in Breox solution. Based on the above findings, a separation process is proposed including (1) selective partitioning based on CI2 unfolding, (2) refolding of CI2 in recovered top-phase, and (3) recovery of target protein in a water phase after thermoseparation. The above work applies to the behaviour of a small model protein; further investigation on larger proteins with more complex structure is needed.

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