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Protein refolding using aqueous two-phase systems

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Abstract

We present a novel refolding technique in which aqueous two-phase systems are used to both dissociate protein aggregates (inclusion bodies) and to refold the protein in only one operation. Three denaturant salts (sodium thiocyanate, calcium chloride, and lithium bromide) were used as phase-forming salts with poly(ethylene glycol) (PEG). We have tested the technique by dissolving carbonic anhydrase II aggregates and refolding the denatured protein in PEG–NaCl–NaSCN systems.

1. Introduction

The expression of eukariotic proteins in a foreign host is frequently accompanied by the formation of aggregates or inclusion bodies of inactive proteins which accumulate within the cell [1–3]. Although inclusion bodies have been found mainly in *Escherichia coli* they have also been found in other bacteria [4–9], and even in insect cells [10].

Inclusion bodies are roughly spherical amorphous aggregates of protein. It is generally accepted that these protein aggregates are held together by hydrophobic, ionic, and covalent (disulfide bonds) forces. Studies performed on the temperature dependence of protein association suggest that hydrophobic forces play a dominant role in maintaining the proteins in the aggregates. This last observation also suggests that chaotropic salts can be used to dissolve the aggregates. Indeed, inclusion bodies are solubilized by chaotropic agents such as urea, guanidine hydrochloride (GuClH), detergents, and salts. Urea and GuClH are the most effective and commonly used solubilizing agents. Chaotropic salts at a concentration of 1 *M* are also effective in solubilizing protein aggregates [11]. If the aggregate is held together by covalent bonds such as disulfide bonds, it may be necessary to add β-mercaptoethanol [12]. However, the addition of thiol reagents is not necessary even though intermolecular and intramolecular disulfide bonds exist in the inclusion bodies if the solubilization of the aggregates is carried out at high temperatures [13].

The formation of inclusion bodies provides a convenient and clean alternative for protein purification. Under ordinary circumstances only a small amount of impurities contaminates the inclusion bodies formed by the eukariotic protein. Thus by separating the inclusion bodies from the cell debris by centrifugation [1] or ultrafiltration [14], it is possible to obtain a highly pure protein in a single purification step. After the inclusion bodies have been isolated, they are suspended in a buffer and exposed to denaturing conditions to solubilize the protein. The denaturing agent is then diluted (either by dialysis or by adding an appropriate buffer) to refold the protein. Unfortunately, during the refolding process the proteins re-aggregate. Thus only a fraction of the active protein is recovered [15].

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Several routes have been explored to prevent the re-aggregation of the proteins and thus improve the recovery of the active protein. Hagen *et al.* have used reverse micelles to decrease the local density of proteins in order to minimize protein association [16,17]. Although this approach is attractive, some hydrophobic proteins do not fold correctly because they interact with the hydrophobic moieties of the micelles [17]. Finally, Cleland and Wang [18] have found that by adding a cosolvent like poly(ethylene glycol), PEG, to the refolding mixture the formation of aggregates is minimized and the recovery of the active protein is enhanced. Cleland and Wang have successfully used PEG (3 to 30 g/l) for the recovery of carbonic anhydrase II. This approach is especially appealing because it seems independent of the kind of protein. However, this protocol produces a highly dilute protein solution contaminated by large amounts of denaturant. Clearly improvements to this technique are highly desirable to make it more commercially attractive.

In this paper we report preliminary results of a novel refolding technique in which aqueous two-phase systems are used to both dissociate the inclusion bodies and refold the protein in one operation. The aqueous two-phase system is a PEG–salt system chosen so that the proteins are conformationally stable in the PEG phase and are conformationally unstable in the salt phase. Therefore, the inclusion bodies dissociate in one phase and the denatured protein refolds in the other phase. The dissociation–refolding process continues until equilibrium is reached.

2. Experimental

2.1. Materials

PEG 6000 and carbonic anhydrase II (Lot # 30H9490) were obtained from Sigma (St. Louis, MO, USA). All the other chemicals were of analytical-reagent grade.

2.2. Preparation of the phase systems

Four-gram phase systems were prepared by mixing the polymer with nanopure water and salts in the appropriate amount and stirring for several minutes [19]. The pH was adjusted by adding either phosphate or Tris buffers at a concentration of 50 m*M*. Duplicates were prepared for each phase system. Most of the systems considered in this work are in the one-phase region at room temperature. Therefore, the systems are driven into the two-phase region by increasing the temperature. Three alternative techniques were used to determine the phase diagrams: protocols 1, 2 and 3.

Protocol 1

The phase systems were prepared as indicated above. They were placed in a controlled-temperature water bath and the temperature of the water bath increased in intervals of 2 or 3°C. The systems remained in the water bath at each temperature for about 30 min to allow stabilization. The temperature at which the systems become cloudy was recorded as the cloud point temperature. The cloud point temperature indicates the onset of the liquid–liquid phase transition.

Protocol 2

The phase systems were prepared as indicated above. They were placed into a stirred cell of a temperature-controlled spectrometer and the absorbance at 450 nm was measured as a function of temperature. First, we increased the temperature until a sharp increase in the absorbance was observed indicating that the system had become cloudy. The temperature at which the systems became cloudy was recorded as the cloud point temperature (forward experiments). Second, we decreased the temperature until the absorbance observed at room temperature was recovered. The temperature at which the system became transparent was recorded as the cloud point temperature (backward experiments). The cloud point temperature obtained by the backward and forward experiments agreed within 1°C.

Protocol 3

The phase diagrams were determined as indicated by Albertsson [20]. A salt or PEG solution of known concentration was titrated with PEG or salt, respectively, until the solution turned turbid. The composition of the mixture was followed by weight.

2.3. Partition experiments

For the partition experiments 0.5 or 1 ml of a 2.5 mg/ml protein stock solution was added to the phase systems replacing an equal amount of buffer. The systems were stirred for several minutes and placed into a water bath to induce the phase transition. After complete phase separation (between 5 to 12 h), samples from top and bottom phase were taken carefully with a micropipette. After dilution, the absorbance at 280 nm was measured using quartz cuvettes in a double-beam Hitachi spectrophotometer (Model U-2000). The calculated extinction coefficient for the native protein was 1.90 mg/ml, while the extinction coefficient for the denatured protein was 1.65 mg/ml. The enzymatic activity was measured by recording the increase of absorbance at 348 nm due to the hydrolysis of *p*-nitrophenylacetate [15]. The activity of the enzyme was determined as the slope of the absorbance at 348 nm *versus* time plot. Since *p*-nitrophenylacetate hydrolyses even in the absence of the protein a blank was discounted to account for the changes in the reactant. The enzymatic activity of the fresh enzyme was 1.9 units/mg of protein at 20°C.

2.4. Protein denaturation

Carbonic anhydrase II was denatured by incubating 25 mg of the enzyme for 24 h in 1 ml of 5 *M* guanidine hydrochloride. A volume of 0.1 ml of this solution diluted to 10 ml with 5 *M* guanidine hydrochloride was used as our denatured protein. This solution was diluted with 25 ml of Tris buffer pH 7.5 to a final concentration of 0.174 *M* guanidine hydrochloride to induce the formation of aggregates [18]. These aggregates were centrifuged at 3000 *g* for 20 min and the precipitate was used in the partition experiments. These protein aggregates resemble inclusion bodies.

3. Results and discussion

3.1. Phase system selection

Our first objective was to find a PEG–salt system such that the phase-forming salt was able to dissolve the aggregates. Non-chaotropic salts such as phosphate, sulphate or citrate which form two liquid phases with PEG at room temperature cannot dissolve the inclusion bodies. Therefore, only chaotropic salts needed to be considered. However, we found very difficult to obtain two phases by mixing chaotropic salts with PEG even at high temperatures. Fortunately, we also found that by replacing a fraction of the chaotropic salt with NaCl the phase transition temperature decreased to acceptable limits. To induce phase separation at high temperatures may be beneficial for this project because the dissociation of the aggregates is generally facilitated at high temperatures. The refolding process in the PEG-rich phase will not be adversely affected at high temperatures because PEG increases the protein melting temperature (the temperature at which the protein unfolds).

LiBr, $CaCl_2$, and NaSCN were tested as phase-forming salts with PEG at several temperatures. Our studies show that the addition of small amounts of NaCl to NaSCN–PEG, $CaCl_2$–PEG and LiBr–PEG systems generally facilitates the phase separation. Fig. 1 shows the transition temperature *versus* the amount of NaCl replaced by NaSCN, LiBr or $CaCl_2$ for a total salt concentration of 15% (w/w) and a polymer concentration of 20% (w/w) (in this Figure and in all subsequent ones the lines are given solely to guide the reader's eye). LiBr and $CaCl_2$ form two liquid phases with PEG at rather high temperatures. The cloud point temperature for PEG–NaCl–LiBr and PEG–NaCl–Ca_2Cl sys-

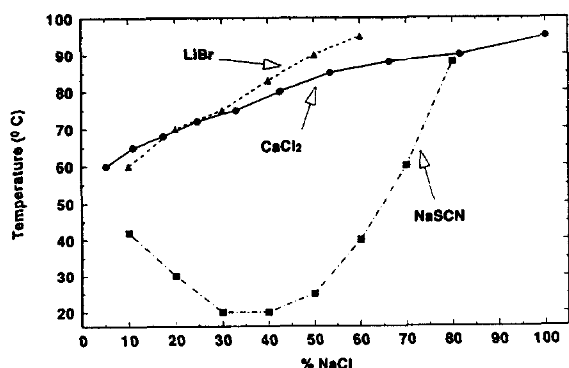

Fig. 1. Cloud point temperature *versus* % of NaCl replaced by LiBr, CaCl or NaSCN. Total salt concentration 15% (w/w). Total PEG concentration 20% (w/w).

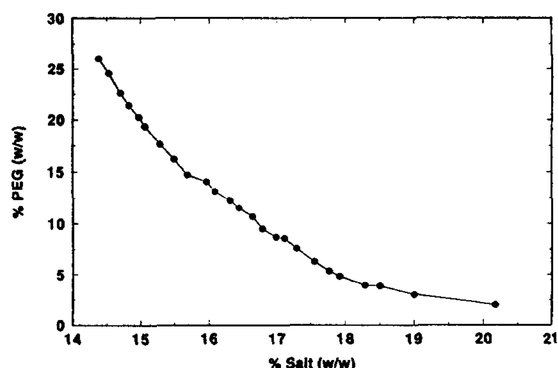

Fig. 2. Phase diagram for PEG–NaCl–NaSCN system at 20°C. The salt composition is 60% NaSCN and 40% NaCl.

tems increases with increasing substitution of NaCl by the chaotropic salt. NaSCN, on the contrary, forms two liquid phases at reasonable temperatures even if a large amount of NaCl is replaced by NaSCN. In addition, the mutual solubility of PEG–NaCl–NaSCN systems exhibits a minimum at about 25°C. Further experiments are needed to explain this phenomenon. In conclusion, NaSCN is the most promising salt from those tested by us since it is a very strong denaturant and forms two-liquid phases with PEG over a wide range of temperatures. Therefore all the following experiments were performed in PEG–NaCl–NaSCN systems.

In an attempt to understand the PEG–NaCl–NaSCN systems we determined phase diagrams and we studied the effect that the total concentration of PEG and the pH have on the cloud point temperature of these systems. Fig. 2 shows a phase diagram for NaSCN–NaCl–PEG at 20°C; 60% of the total salt is NaCl and 40% is NaSCN; the total salt concentration is 15% (w/w). This figure shows that the amount of salt in each phase is rather high. A high concentration of salt in the bottom phase is desirable because it will favor the dissolution of the aggregates. On the other hand, a high concentration of salt in the top phase is undesirable because it will inhibit the refolding of the protein. The total amount of salt and the proportion of NaSCN in the mixture have to be optimized in the partition experiments to allow the dissolution of the inclusion bodies in either the homogeneous systems or in the salt-rich phase and to facilitate the refolding of the protein in the PEG-rich phase. Fig. 3 shows the cloud point temperatures at two different PEG concentrations, 15 and 20% at a total salt concentration of 25%; 10% NaCl and 90% NaSCN. This figure shows that an increase in the PEG concentration favors the phase separation. Finally, in Fig. 4 we show the effect that pH has on the two-phase system. This figure is for a total salt concentration of 25%, with a salt composition of 10% NaCl, and 90% NaSCN at pH values 7.5 and 5.6. Clearly, a decrease in the pH favors the phase separation.

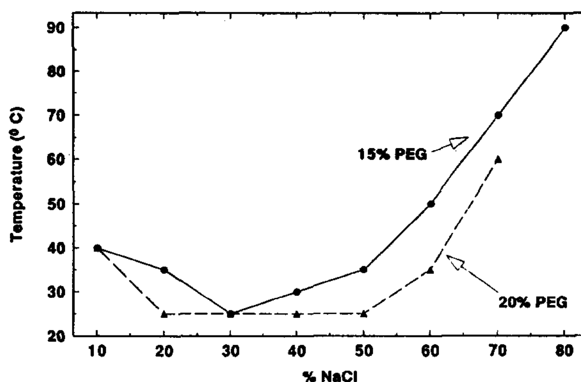

Fig. 3. Cloud point temperature *versus* % of NaCl replaced by NaSCN at two PEG concentrations. Total salt concentration 15% (w/w).

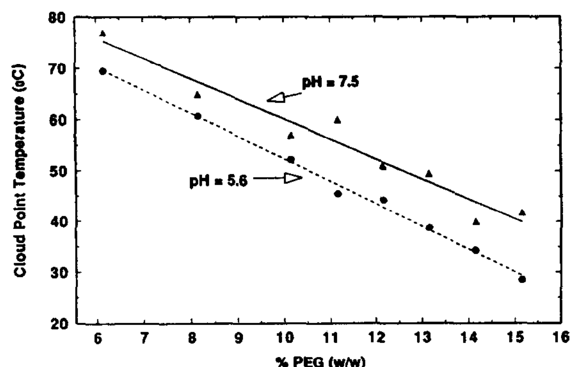

Fig. 4. Cloud point temperature *versus* PEG concentration in % (w/w) at two different pH values. Total salt concentration 25% (w/w). Salt composition is 10% NaCl and 90% NaSCN.

3.2. Protein partition experiments

Partition experiments were performed with the native protein and with the protein aggregates. Three phase systems containing 20% PEG and 15% salt with variable amounts of NaSCN were selected for our partition experiments. All experiments were performed at 40°C and the systems were buffered with Tris (pH = 7.0). Table 1 summarizes our results for the native protein. System A contains 50% NaSCN and 50% NaCl, System B contains 40% NaSCN and 60% NaCl, and system C contains 30% NaSCN and 70% NaCl. System A is at the onset of phase separation at room temperature while systems B and C are in the one-phase region at room temperature. The protein partition coefficient, $K = C_{top}/C_{bottom}$, was very high in all cases. The protein mass balance, however, did not close indicating some absorption of the protein at the interface. The interface of the system containing a high concentration of NaSCN becomes turbid and very broad when 1 ml of the stock protein solution is added but remains clear when 0.5 ml of the stock protein solution is added. This indicates that the composition of the phases depends on the amount of protein present in the system. Our findings suggest that the usual assumption that the phase systems are not altered by the addition of low protein concentrations is not valid for these systems.

Table 1
Partition coefficient and activity of carbonic anhydrase II in PEG–NaCl–NaSCN systems

System	Partition coefficient	Activity (units/mg)	
		Top	Bottom
A	1.94	0.016	0.0
B	1.26	1.39	0.0
C	3.58	0.239	0.0

Systems composition: 20% PEG and 15% salt; $t = 40°C$; pH 7.0. System A contains 50% NaSCN and 50% NaCl, system B contains 40% NaSCN and 60% NaCl and system C contains 30% NaSCN and 70% NaCl. Total protein concentration: 0.625 g/l.

The enzymatic activity was monitored before and after demixing of the phase systems. Our results are reported in the last columns of Table 1. We did not observe any enzymatic activity in the systems before demixing, in the salt-rich phases (bottom phases), and in the PEG-rich phase (top phase) of the system containing large amounts of NaSCN (System A). We did observe activity in the top phase of the systems containing relatively small amounts of NaSCN. These observations suggest that we can optimize the protocol by varying the relative amounts of non-chaotropic to chaotropic salts. In independent experiments we found that the activity of carbonic anhydrase II was completely lost in NaSCN concentrations as low as 5% (w/w). However, the activity was recovered by diluting the denaturating mixture with Tris buffer.

Finally, we used the PEG–NaCl–NaSCN systems described above (Systems A,B, and C) to dissolve the carbonic anhydrase aggregates and to refold the protein. First, we suspended the aggregates in different amounts of Tris buffer. The aggregates did not dissolve and no enzymatic activity was observed. The aggregates were then placed into the selected phase systems. The aggregates dissolved almost instantaneously when the systems were still in the one phase region and no enzymatic activity was detected. The systems were placed in a water

bath at 40°C to induce the phase separation. Samples from top and bottom phase were analyzed for protein content and enzymatic activity. No enzymatic activity was found in either phase of systems A and B. In the system containing 30% NaSCN no activity was recovered in the bottom phase but 0.386 units/mg were recovered in the top phase. We did not find any protein in the bottom phase, indicating that the protein accumulates at the interface.

4. Conclusions

It is evident that to efficiently produce a recombinant protein the active protein has to be recovered from the aggregates. A refolding process should provide: (a) high recovery of the biologically active protein; (b) easy separation of the product from misfolded polypeptides; and (c) highly concentrated protein (volume reduction) at the end of the refolding process. In addition, the refolding process should be easy to scale up and it should proceed at an acceptable rate. We believe that the combined dissociation–refolding technique that we are developing will fulfil these requirements.

Our results show that a new kind of two-phase system developed by us may be used for the recovery of active proteins from inclusion bodies. Still the protocol has to be optimized to increase the recovery of the active protein. Work is in progress in this direction. The use of a harmful salt like NaSCN may raise some concerns. However, the salt normally used in solubilizing inclusion bodies, guanidine hydrochloride, is as toxic as NaSCN. The ultimate challenge of our protocol will be to test it with inclusion bodies from eukariotic proteins engineered in *E. coli*.

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