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Effect of pH, ion type and ionic strength on partitioning of proteins in reverse micelle systems

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Abstract

Two proteins with different physico-chemical properties have been partitioned in reversed micelle systems: thaumatin and ribonuclease A. The organic phase was formed by sulphosuccinic acid bis(2-ethylhexyl) ester, sodium salt, in isooctane and the aqueous phase contained either KCl, KBr, $MgCl_2$ or NaCl. Aqueous phase pH was varied between 2 and 13 and ionic strength between 0.1 and 2.8. Small changes in pH (around the p*I*) were found to influence the solubilization of ribonuclease A but for thaumatin the pH change necessary to affect partition was much greater as a consequence of the difference in net charge (titration curves) of these protein molecules as pH changes. The type of ions present in the system was also a determining factor for partition, the larger ions (K^+) produced more electrostatic screening and hence less protein solubilization than the smaller ions (Na^+). With changes in ionic strength surface hydrophobicity was an important factor affecting solubilization of thaumatin.

1. Introduction

The extraction and purification of proteins using reversed micelle systems has been the subject of extensive study in recent years [1–6]. Reversed micelle systems have great potential for the liquid–liquid extraction of biomolecules under moderate conditions. Protein partitioning is dependent upon many factors which are directly related to the interaction between properties inherent to the system itself and those pertaining to the protein under investigation. Fundamental studies of the factors determining selective separation of biomolecules are necessary to establish correlations between the physico-chemical properties of the proteins and the reversed micelle system. These factors include pH, ionic strength and type of ions present in the system, type of surfactant and organic solvent used and physico-chemical properties of the proteins such as isoelectric point, hydrophobicity, size, charge density and charge distribution.

Reversed micelle systems formed from sulphosuccinic acid bis(2-ethylhexyl) ester, sodium salt (AOT), isooctane and water were used in this work. AOT is an anionic surfactant which will form micelles in non-polar solvents without the need for a cosurfactant. In reversed micelle systems the polar head groups of the surfactant molecules are directed towards the interior of the micelle and form a polar core which can solubilize water; the lipophilic chains are exposed to the solvent. Inside the micelles is a pool of water and it has been demonstrated that water in this pool behaves differently from normal water especially at low concentrations ($w_0 =$

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$[H_2O]/[AOT] < 10$). The solubilities of ions and proteins in the water pool can be greater than normal. Water content of a micellar system and phase ratio both have a strong effect on protein solubilization and subsequent function.

In AOT–isooctane systems the micelles are spherical, nanometre sized particles (with diameters ranging from 10 to 200 Å) which are thermodynamically stable. The molar water-to-surfactant ratio (w_0) is used to characterize micelle size.

Increasing ionic strength can affect micelle size, since charged surfactant headgroups repel each other and larger micelles are formed at lower ionic strengths. Also at higher ionic strengths there is a shielding effect between the charged surfactant head groups and the protein, making solubilization in the micelles more electrostatically unfavourable.

The aqueous phase pH in which the protein is dissolved directly affects the charge on the protein: at pH values below its isoelectric point (p*I*) a protein will have a net positive charge and at pH above the p*I* it will have a net negative charge. The net charge of a protein and the distribution of those charges on the protein molecules will affect the interaction with the surfactant headgroups and hence the transfer of the protein to the reversed micelles. The aim of this paper is to investigate the effects of pH, salt type and ionic strength on the partitioning of proteins in AOT–isooctane reversed micelle systems.

2. Materials and methods

2.1. Proteins

Ribonuclease A (type III-A from bovine pancreas) was purchased from Sigma, thaumatin (100% pure Talin, human-food grade) was obtained from Four-F Nutrition, Northallerton, UK.

2.2. Chemicals

AOT was obtained from Sigma and spectrophotometric-grade isooctane (2,2,4-trimethylpentane) from Aldrich. Both were used as supplied. All other chemicals used were of analytical-reagent grade.

2.3. Partition experiments

Protein transfer experiments were done in 25-ml stoppered flasks at room temperature (*ca.* 21°C). The organic phase was composed of 50 m*M* AOT in isooctane. The aqueous phase consisted of NaCl or KCl at different concentrations and pH values. The pH was adjusted by the addition of concentrated acid or base, buffers were not used because over such a wide range of pH values several different buffers would have been necessary involving the addition of a mixture of ions to the system. The pH given is the aqueous phase pH measured after phase equilibrium. Protein was dissolved in the aqueous phase at a concentration of 0.25 mg/ml. Equal volumes of organic and aqueous solutions were mixed and stirred for 5 min which was found to be ample time for equilibration. Phase separation was aided by centrifugation at 2000 rpm (*ca.* 450 *g*) for 5 min. Samples from both phases were then assayed for protein and water content.

2.4. Assays

Protein in both phases was assayed by absorbance at 280 and 310 nm in a Pharmacia Ultrospec III spectrophotometer, standard curves were prepared for each phase with each protein. Water was measured in some organic phases by Karl Fischer titration using a Mettler DL37 KF coulometer.

3. Results and discussion

The molecular masses of thaumatin and ribonuclease A are 22 200 and 13 500, respectively, and their p*I* values are *ca.* 11.5 and 7.8. These two proteins have fairly low molecular masses and should be accommodated into the reversed micelles without an appreciable increase in micelle size and their p*I* values are quite different so the effect of protein charge over a large pH range can be studied. The

surface hydrophobicities of these proteins have been measured, thaumatin has a very high surface hydrophobicity [7], and ribonuclease A is a hydrophilic protein.

3.1. Effect of pH

Fig. 1 shows the effect of the pH of the aqueous phase on the solubilization of thaumatin in reversed micelle systems composed of AOT, isooctane and 0.1 *M* KCl or KBr. All experiments were carried out with initial protein concentration of 0.25 mg/ml. The protein shows similar behaviour with both salts. At pH values 2 or 3 units below its p*I* most thaumatin (*ca.* 100%) partitions to the reversed micelles, at pH values around the p*I* there is a drop in the amount solubilized and at pH values above the p*I* little protein is in the reversed micelle phase. There are slight differences in the two curves; with KCl the pH values at which a drop in solubilization occurs are higher than those for KBr.

At pH values above their p*I* proteins have a net negative charge and so do not interact with, but are repelled by, the negatively charged head groups of the surfactant in the reversed micelles. Conversely, at pH values below their p*I* proteins have a net positive charge and are able to interact with the surfactant and become solubilized in the reversed micelles as in Fig. 1.

The change from net negative charge to net positive charge that occurs at pH values close to the p*I* of the protein depends on the titration curve and charge distribution of the protein. For example, the titration curve for thaumatin is very flat between pH 5 and 9.5 indicating that there is little change in the net charge of the protein molecules in this pH range [7] and hence in Fig. 1 the curve for thaumatin is not as steep as those for other proteins such as ribonuclease A (Fig. 2) where the net charge is much more sensitive to changes in pH.

Fig. 2 shows the solubilization of ribonuclease A in reversed micelles with three different salts; KCl, KBr and $MgCl_2$ over a range of pH values. With all three salts there is an apparent drop in % solubilization at the lowest pH values. This has been attributed to denaturation of the protein or hydrolysis of the AOT at low pH but is now thought to be due to aggregation of AOT and protein at the interface. We have measured the absorbance spectrum of AOT between 190 and 950 nm over a range of pH values from 2 to 12. An absorbance peak occurred at 232 nm in all the samples and no significant differences in the absorbance spectra were seen indicating that hydrolysis of AOT is not occurring in this range of pH values. Lye [8] has found AOT–protein precipitates when working with proteins in

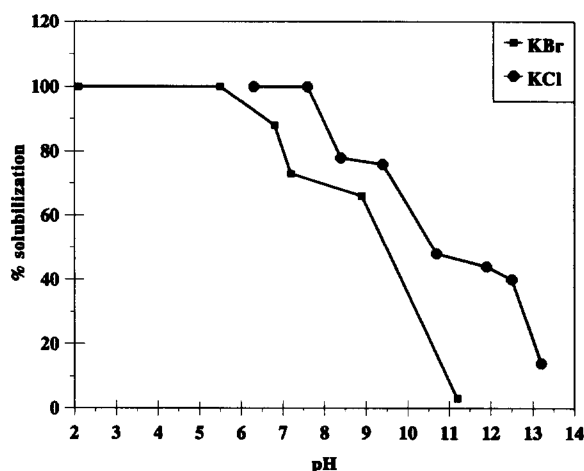

Fig. 1. Effect of pH on the solubilization of thaumatin in the reversed micelle phase; 50 m*M* AOT in isooctane, 0.1 *M* KBr or KCl as aqueous phase, 0.25 mg/ml protein.

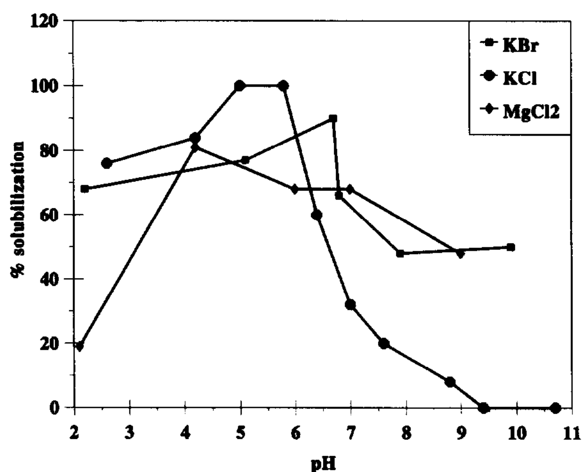

Fig. 2. Effect of pH on the solubilization of ribonuclease A in the reversed micelle phase. Conditions as for Fig. 1.

AOT–isooctane systems. Göklen and Hatton [9] also observed lower solubilities for some proteins at low pH and attributed this to possible protein denaturation. Matzke *et al.* [4] obtained similar results for chymotrypsin using AOT as surfactant; they tested for denaturation using circular dichroism and fluorescence absorption and found no evidence of gross conformational changes in protein structure or changes in the spectra over the pH range 2 to 12. Using organic-phase atomic absorption Matzke *et al.* [4] determined the amount of AOT in the organic phase. As AOT is the major source of sodium in the organic phase, any significant changes could be attributed to a change in the amount of AOT in the organic phase. It was found that at pH 4 the sodium ion concentration is much lower for the chymotrypsin-containing sample than for the protein-free sample. In the intermediate pH range (6–10) the concentrations are similar and at pH 11 the protein-containing sample again has a lower sodium ion concentration. These results suggest that less AOT is present in the organic phase at low and high pH for the samples containing protein, which indicates that AOT may be aggregating at the interface.

Fig. 2 shows that with $MgCl_2$ at low pH values (below 4) the decrease in transfer of ribonuclease is larger than with the other salts indicating that the ions present in the system may also influence the degree of AOT–protein precipitation.

3.2. Effect of ionic strength and salt type

To study the influence of aqueous phase ionic strength (I) on protein transfer to the organic phase each protein was partitioned in reversed micelle systems with KCl, KBr and NaCl of ionic strengths varying from 0.1 to 1.0.

Fig. 3 shows the effect of ionic strength of KCl, KBr and NaCl on thaumatin solubilization into the reversed micelle phase (50 m*M* AOT in isooctane). In Fig. 3 the curves show a very sharp drop in solubilization at ionic strengths between 0.2 and 0.4 with KCl, between 0.1 and 0.3 with KBr and between 0.4 and 0.7 with NaCl. As the ionic strength increases the ions form an electrostatic shield around the micelles so the protein molecules cannot interact with them and hence the percentage of protein in the organic phase decreases. At higher ionic strengths the screening effect decreases charged surfactant headgroup repulsions resulting in a decrease in water solubilization and a lower w_0. For example, in a system with NaCl and thaumatin the w_0 value decreases from 48 with 0.1 *M* salt to 18 with 1.0 *M* NaCl. Hence the resulting micelles are smaller than at low ionic strengths and this can lead to the protein being excluded on the basis of size as ionic strength is increased.

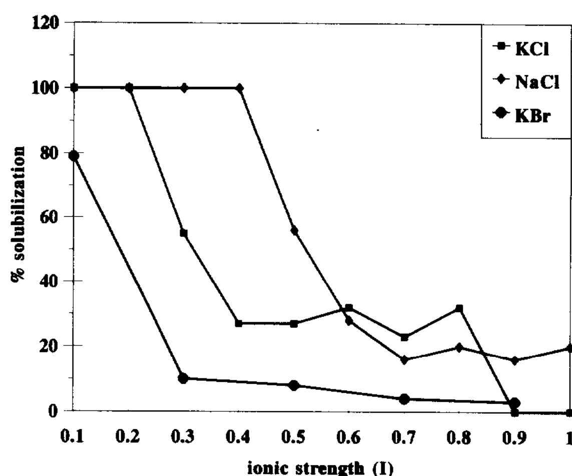

Fig. 3. Effect of ionic strength of aqueous phase on the solubilization of thaumatin into the reversed micelle phase; 50 m*M* AOT in isooctane, KCl, KBr and NaCl as aqueous phase, pH 7.

The differences in the solubilization curves with the three different salts may be a consequence of the relative sizes of the K^+ and Na^+ ions. The atomic radii of these ions are 1.33 and 0.97 Å, respectively. In general the smaller ions (Na^+) produce less screening and hence allow more protein to interact with and transfer to the micelles. This is seen in the figures as a shift to higher ionic strengths of NaCl at which the percentage solubilization decreases. KBr gave very similar results to KCl indicating, as expected, that with AOT as surfactant (with negatively charged head groups) the cations have more influence than the anions.

Thaumatin has a very high surface hydrophobicity and this can influence the interactions between the protein and the surfactant molecules under certain conditions and hence determine partitioning behaviour. We have studied the behaviour of thaumatin at pH values near to and above its p*I* [10] with KCl and NaCl. With NaCl at high pH values (12) all of the protein is solubilized in the organic phase at ionic strengths between 0.3 and 0.8, this has been attributed to the high surface hydrophobicity of the thaumatin molecules which enables them to interact with the non-polar chains of the AOT. When comparing the behaviour of thaumatin in the systems with Na^+ and K^+ it appears that the order by which the ions promote hydrophobic interactions follows the Hofmeister or lyotropic series ($Mg^{2+} > Li^+ > Na^+ > K^+ > NH_4^+$).

Fig. 4 shows the partitioning of ribonuclease A in reversed micelles with an aqueous phase consisting of KCl, KBr, NaCl and $MgCl_2$ with ionic strengths from 0.1 to 2.7. $MgCl_2$ allowed protein transfer in the range of ionic strengths from 0.3 to 2.7, significantly higher than both KCl and NaCl. The Mg^{2+} ion has an atomic radius of 0.66 Å and is thus smaller than both K^+ and Na^+, this result supports the idea that smaller ions produce less screening of the micelles and thus allow more protein transfer.

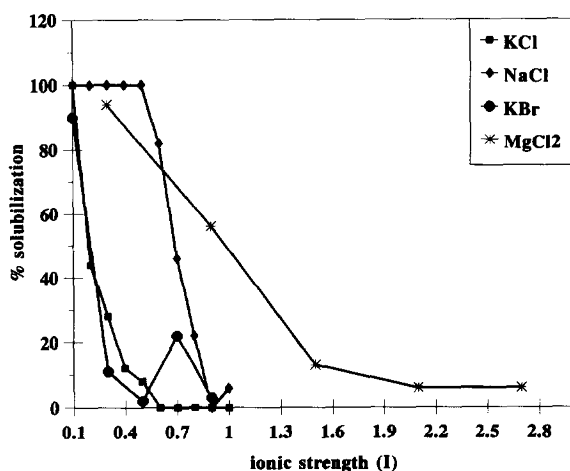

Fig. 4. Effect of ionic strength of aqueous phase on the solubilization of ribonuclease A into the reversed micelle phase. KCl, KBr, NaCl and $MgCl_2$ as aqueous phase, pH 5.

Marcozzi *et al.* [3] have studied the factors affecting the forward and backward transfer of α-chymotrypsin in AOT–isooctane systems. They used four salts, KCl, NaCl, LiCl and $CaCl_2$ and found that protein transfer occurred at lowest ionic strengths with KCl followed by $CaCl_2$, NaCl and LiCl. The atomic radii of K^+, Ca^{2+}, Na^+ and Li^+ are 1.33, 0.99, 0.97 and 0.68 Å, respectively, indicating that the size of the ions may also be important in this system.

4. Conclusions

Thaumatin and ribonuclease A have been partitioned in reversed micelle systems and the effects of pH, ionic strength and ion type have been investigated.

With changes in pH the protein solubilization depends on the p*I* and net charge (titration curve) of the protein. The type of ions in the system affect protein solubilization by causing electrostatic screening of the surfactant headgroups and thus hindering interactions between the protein and the surfactant. Larger ions such as K^+ cause more screening and hence less solubilization than smaller ions such as Na^+.

Increasing ionic strength decreases protein partition to the reversed micelle phase with proteins that do not have high surface hydrophobicities (*e.g.* ribonuclease A). In the case of hydrophobic proteins (*e.g.* thaumatin) the interaction of the protein with the surfactant and/or organic solvent is so strong that no effect of ionic strength is seen at some values of pH.

5. Acknowledgement

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