Release and Recovery of Porcine Pepsin and Bovine Chymosin from Reverse Micelles: A New Technique Based on Isopropyl Alcohol Addition

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After complete solubilization by the direct method, porcine pepsin was not released from AOT in isooctane reverse micelles even under aqueous-phase conditions which would not ordinarily allow uptake. Similarly, bovine chymosin, once forward-transferred at a pH below its isoelectric point, was not back-transferred into an aqueous contact phase buffered at a pH value above its isoelectric point. These results show that there is significant hysteresis in the forward- and backward-transfer processes and further imply that kinetics, and not equilibrium, control uptake or release processes for these enzymes. The addition of 10–15% isopropyl alcohol to the aqueous phase increases the rate of protein release dramatically and allows for nearly complete back-transfer of porcine pepsin and 70% back-transfer of bovine chymosin. IPA addition does not destroy the functional integrity of the system since forward transfer of bovine chymosin still occurs at pH values below (but not above) the pI of the protein.

Introduction

Aggregates of surfactant molecules are spontaneously generated in organic solvents as a result of molecular self-assembly (Kertes and Gutmann, 1975; Ruckenstein and Nagarajan, 1980). These aggregates can solubilize water in their polar cores giving rise to water-in-oil microemulsions, also commonly referred to as reverse micelles (Luisi and Straub, 1984). The discovery that proteins can be solubilized within these reverse micelles in active form (Martinek et al., 1977; Menger and Yamada, 1979; Douzou et al., 1979; Wolf and Luisi, 1979; Luisi et al., 1979) has opened up two practically important avenues of research (Luisi and Laane, 1986). One is directed toward enzymatic biosynthesis of various commercially useful products in reverse micelle media and constitutes the research area of micellar enzymology (Luisi, 1985; Martinek et al., 1986; Shield et al., 1986; Luisi et al., 1988; Madhusudhan Rao et al., 1990). The other is directed toward the use of reverse micelles as a novel extraction medium for the purification of proteins and enzymes (Kadam, 1986).

For some applications, reverse micelle systems may offer a promising alternative to chromatography for the large-scale purification of proteins. In theory, these systems are much easier to scale up than chromatographic separation processes and can allow for continuous separation processes for proteins which are analogous to the liquid–liquid extraction methods extensively used in the chemical processing industry for small molecules.

In order to use the reverse micelle media for protein separations, the micelles should exhibit two characteristic features. First, they should be capable of solubilizing proteins selectively. This protein uptake into reverse micelles is also referred to as the forward-transfer process. Second, it should be possible to release the protein from the reverse micelles so that a quantitative recovery of purified protein can be achieved. This is referred to as back-transfer.

The forward transfer of proteins into reverse micelles has been demonstrated for a number of proteins in various reverse micelle systems. A range of transfer efficiencies have been found including 100% efficiency or complete transfer into reverse micelles, for a number of cases. The studies show that the forward transfer is generally controlled by the size and charge of the proteins involved and the size and charge character of the reverse micelle system. For example, protein uptake data in reverse micelle systems involving AOT show that if the aqueous-phase pH is above the pI of the protein (thus the protein has net negative charge), the forward transfer into anionic AOT reverse micelles is completely inhibited. For pH values below the pI, the protein has a net positive charge and its uptake readily occurs. This is consistent with notions of equilibrium behavior according to which cationic proteins (pI above the pH of solution) interact attractively with the anionic AOT reverse micelle while the anionic proteins are repelled. Further, high ionic strengths in the aqueous phase tend to reduce the protein uptake as one may expect, because of the weakening of the electrostatic attractions between the reverse micelle and the oppositely charged protein (Goklen and Hatton, 1985a,b, 1987; Wolbert et al., 1989).

In addition to the effect of protein and surfactant charge, the size of the protein molecule also seems to influence uptake. Larger proteins appear to be more difficult to transfer into reverse micelles, and therefore the optimum pH (i.e., the pH for 50% transfer) is displaced relative to the isoelectric point. A large cationic protein needs to be transferred at a pH lower than its isoelectric point while a small protein can be transferred at a pH very close to its isoelectric point. An empirical relation between the pI and the molecular weight of the protein and the pH value at which maximum transfer efficiency is realized has been developed by Wolbert et al. (1989) on the basis of the experimental data of Goklen (1986) for AOT reverse micelles:

$$|\mathrm{pH(optimum)} - \mathrm{p}I| = (+0.12 \times 10^{-3})M_w - 1.07$$

where M_w refers to the molecular weight of the protein. In this case, the optimum pH is lower than the pI of the

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protein since the reverse micelle is negatively charged. Similarly, the forward-transfer data obtained by Wolbert et al. (1989) for 19 proteins in the cationic TOMAC reverse micelles have been used to obtain the following empirical correlation:

$$|\mathrm{pH(optimum)} - \mathrm{p}I| = (+0.11 \times 10^{-3})M_w - 0.97$$

In this case, the optimum pH is larger than the pI of the protein since the reverse micelle is positively charged. These empirical rules can help one design an appropriate separation system.

In contrast to the large number of studies investigating forward transfer, release and recovery of proteins from reverse micelles have received little attention in the literature. Most of the available studies tacitly assume that conditions which normally prevent protein uptake in the forward-transfer experiments would promote their release in the back-transfer experiments. However, in most reported attempts to release the solubilized proteins from the reverse micelles, low yields were obtained. Menger and Yamada (1979) were unable to recover any chymotrypsin from AOT in heptane reverse micelles. Similar findings regarding low recoveries have been reported by others (Goklen and Hatton, 1985a,b; Shaffer, 1988; Luisi et al., 1979; Rahaman et al., 1988). The major exception to this is the work of Dekker et al. (1986, 1987, 1989) studying the extraction of α-amylase using TOMAC/isooctane reverse micelles. These investigators were able to recover essentially 100% of the α-amylase in their system. The difference in recovery reported in these studies versus the other studies referred to above may be due to the fact that the TOMAC system has a completely different chemical composition than the more widely used AOT systems.

Conditions of high salt and high pH (above the pI of the protein), for which protein solubilization by anionic AOT reverse micelles does not take place in uptake experiments, often seem to fail to completely release the proteins that are already solubilized within the reverse micelles. The fact that uptake is nearly complete and easy to accomplish for many proteins, yet recovery is incomplete in at least some cases, suggests that there is a hysteresis in the uptake and release processes. This implies that the kinetics of protein release are significant and may effect the quantitative release of the solubilized protein.

In this study we show that, once solubilized, porcine pepsin and bovine chymosin cannot be released from AOT/isooctane systems even under conditions that ordinarily prevent uptake. Back-transfer from the organic to the aqueous phase does not take place in 0.01–1 M KCl solution or at pH values well above the protein isoelectric point. We also show that release can be promoted by changing the composition of the overall system by the addition of 10–15% (v/v) isopropyl alcohol (IPA) to the aqueous phase. Addition of IPA at this level allowed for full release of pepsin and 70% recovery of chymosin. Furthermore, in the presence of this concentration of IPA, the reverse micelles retained their functional integrity and forward transfer remained possible. Both uptake and release of proteins by reverse micelles were found to occur rapidly in the presence of IPA.

Materials and Methods

Chemicals. The experiments described below employed the anionic surfactant AOT and the solvent isooctane; both were obtained from Aldrich Chemical Co. Bovine (calf) chymosin (EC 3.4.23.4) and porcine pepsin (EC 3.4.23.1) were obtained from Sigma Chemical Co. Pepsin was essentially pure protein; however, the chymosin sample contained only 40% total protein. The total protein content was made up of around 19% bovine pepsin and 81% chymosin. The chymosin was used in the experiments without further purification. Potassium phosphate, sodium phosphate, sodium sulfate, potassium chloride, and sodium chloride, all used in the buffer solutions, were obtained from Sigma Chemical Co.

Chromatography. Gel permeation chromatography was used to determine the concentration of the proteins. A 300 mm × 7.5 mm Bio-Sil TSK125 column (Bio-Rad) was used on a Waters 840 HPLC system equipped with a λ Max 481 UV detector, a Waters 710 automatic sample injector, and Model 510 pumps. A 75 mm × 7.5 mm Bio-Sil SEC guard column was used ahead of the separation column. The mobile phase consisted of 0.002 M $NaHPO_4$, 0.005 M Na_2SO_4, and 0.1 M KCl and was pumped through the column at 1 mL/min. Standard protein samples were used to calibrate the detector.

Extraction Protocol. ***Forward Transfer.*** Preliminary experiments showed that acetate and phosphate buffers containing 0.07–0.1 M KCl form clear two-phase systems with 250 mM AOT in isooctane. In a typical forward-extraction experiment, 1 mg/mL of chymosin powder was dissolved into a 0.1 M KCl in 0.01 M acetate buffer, pH 4.0, and then the solution was contacted with an equal volume of 250 mM AOT in isooctane solution. The mixture was shaken in a vortex mixer and then separated at 3000 rpm in a bench-top centrifuge at room temperature. Two clear phases were obtained, with a small amount of cloudy material collected at the interface. The lower phase of this system was sampled (25–50 μL) and assayed for chymosin and bovine pepsin by HPLC as described above. A similar procedure was used for uptake experiments with IPA/buffer mixtures.

Solubilization. Pepsin was solubilized within reverse micelles by direct contact of an acetate buffer (0.01 M; pH 4.1–4.2; no KCl) containing the proteins with a (dry) AOT/isooctane solution. Up to 200 μL of pepsin could be solubilized in 1.0 mL of AOT/isooctane solution still forming only a single phase. Chymosin could not be solubilized by this technique because of limited solubility in low ionic strength solutions, but it was easily solubilized using forward transfer as described above.

Back-Transfer. Back-transfer experiments were conducted by contacting the organic phase (with included protein) with various buffers, usually containing some KCl. When the KCl concentration exceeded 0.05 M in the final mixture (based on the aqueous phase volume), two clear phases were formed. Sodium phosphate buffer (pH 6.3) and potassium acetate (pH 4.1) were used in different experiments. The lower phase was assayed as before.

In some of the back-transfer experiments, IPA was added to the system, either by adding it to the aqueous phase before contact with the organic phase or by admixing it to the system after contact of the aqueous and organic phases. In other experiments (described under Results), the two phases were contacted by carefully layering one over the other in a 4-mL capped vial and stirring the lower phase slowly with a small magnetic stirrer.

Results

Pepsin Transfer. Pepsin is a highly anionic protein with an isoelectric point below 2.0. At a pH of 4.1, as used in the solubilization and release experiments, the protein carries a net negative charge of between 15 and 20 and therefore one would expect that it could not be solubilized into anionic AOT in isooctane reverse micelles. This was

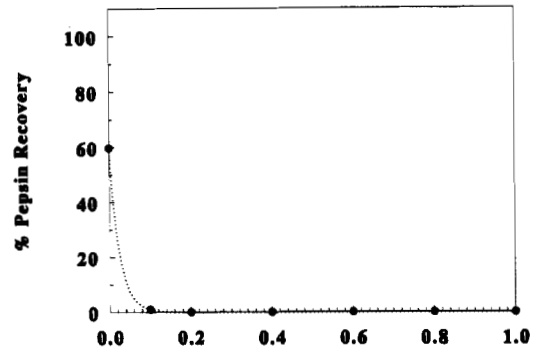

Figure 1. Percent recovery of pepsin solubilized in AOT in the isooctane reverse micelle system as a function of the salt concentration in the extraction buffer. The recovery was determined by taking the entire lower phase (which was cloudy at low salt concentrations) and assaying for the protein. The original upper phase contained 20% (v/v) 5 mg/mL pepsin solution in 0.01 M acetate buffer, at pH 4.1, solubilized in 0.25 M AOT in isooctane solution. The original lower extractant phase was a 0.01 M acetate buffer at pH 4.1 with varying concentrations of KCl as indicated in the figure. The percent recovery is with reference to the total amount of pepsin initially solubilized within the upper phase. The extraction experiment was carried out by intensely mixing the upper and the lower phases and then causing a phase separation by centrifuging. The lower phase was assayed for the related protein. The points are experimental data, and the dotted line is a smooth connecting curve.

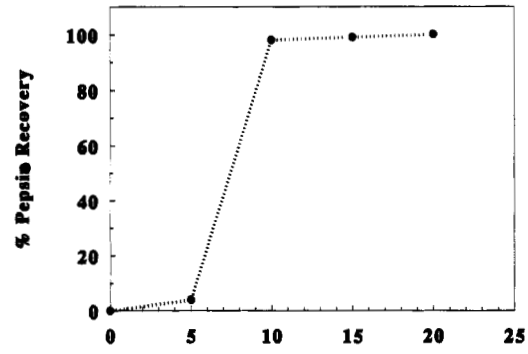

Figure 2. Percent recovery of pepsin from AOT in the isooctane reverse micelle system as a function of the amount of isopropyl alcohol added to the extraction buffer. The upper phase was identical to that described in Figure 1. The lower extractant phase was a 0.01 M acetate buffer at pH 4.1 and containing 0.1 M KCl and varying amounts of isopropyl alcohol as indicated in the figure. The extraction experiment was carried out as described in Figure 1. The points are experimental data, and the dotted line is a connecting curve.

found to be the case in attempts to forward-extract pepsin from a pH 4.1 acetate buffer containing 0.1 M KCl. In the same buffer, bovine chymosin (pI = 4.6) is 100% removed from the aqueous phase (Shaffer, 1988).

Pepsin can be solubilized into AOT reverse micelles by adding subsaturating amounts of the aqueous protein solution (no KCl) to an AOT in isooctane solution. We were able to add 20% (v/v) of a 5 mg/mL pepsin solution in 0.01 M potassium acetate buffer (containing no KCl salt) to a 0.25 M AOT in isooctane solution and obtain a clear and homogeneous single-phase system. Activity measurements (after release; see below) showed that the solubilized pepsin retained nearly 100% of its activity for several days.

The contact of this reverse micelle phase containing presolubilized pepsin with an equal volume of 0.01 M potassium acetate/0.1 M KCl, pH 4.1, buffer did not promote pepsin release, even though forward extraction from the same buffer was not possible as noted above. To explore this phenomenon further, the solubilized pepsin solution was contacted with aqueous phases containing different levels of KCl. When the aqueous buffer contained no added salt, its contact with the reverse micelle phase containing solubilized pepsin resulted in a cloudy lower phase into which about 60% of the protein was released. However, although increasing the salt concentration resulted in a clear lower phase, it did not promote the release of any pepsin (Figure 1).

Similarly, a higher pH (6.3), which should increasingly disfavor the solubilized state of pepsin, did not promote protein release. Indeed, no protein whatsoever was recovered from contact of the reverse micelle phase containing the solubilized pepsin with a phosphate buffer at pH of 6.3, containing 0.1 M KCl (data not shown). Thus, the pepsin release experiments clearly show that the equilibrium notions of high pH and high ionic strength promoting the protein release are not operative for some reason.

A profound change in the extent of pepsin release was observed on the addition of isopropyl alcohol to the system (Figure 2). When 5–10% IPA (v/v) was added to the extraction buffer, containing 0.1 M KCl, there was a dramatic increase in pepsin release from the AOT reverse micelles, the release being at about 10% IPA. From the volume of the phases it appeared that the IPA was mainly in the aqueous phase at these conditions, but addition of IPA exceeding about 20% (v/v) caused a reduction in the volume of the upper reverse micelle phase indicating solubilization of some isooctane into the lower aqueous phase. Between 10 and 15% IPA addition was determined to be optimal for pepsin release, yet not detrimental to protein stability at room temperatures. The released pepsin retained essentially all its activity at these concentrations of IPA as determined by a comparison of milk clotting activity and quantitative chromatograms.

The effect of IPA addition on the rate of release of pepsin from the reverse micelle phase was also examined. A total of 2 mL of the reverse micelle phase [containing 20% (v/v) 5 mg/mL pepsin in 0.01 M acetate buffer] described above was carefully overlaid on 1.7 mL of a 0.01 M acetate/0.1 M KCl buffer in a glass vial. Only the bottom aqueous phase was gently stirred without disturbing the interface. At the beginning of the experiment (t = 0), 300 μL of IPA was injected into the lower aqueous phase so that the phase volumes of the upper reverse micelle phase and the lower aqueous stripping solution phase were equal; 50-μL samples were taken periodically from the lower phase and assayed for pepsin content. The results (Figure 3) indicate that pepsin release began immediately and that by 160 min almost 90% of the pepsin had been released from the reverse micelle phase. An empirical first-order rate constant describing the rate of pepsin transfer across the interface was determined to be 4.2×10^{-4} cm/s on the basis of this data. No release of pepsin was observed over the same time period if no IPA was added.

Chymosin Transfer. To determine whether or not the reverse micelle structure was destroyed by the addition of IPA, a forward-transfer experiment was carried out. An aqueous protein solution buffered with 0.01 M potassium acetate and containing 0.1 M KCl (pH 4.2) containing 2.4 mg/mL of a mixture of bovine chymosin and bovine pepsin (pepsin occurs as an impurity in the bovine chymosin preparation at about 19 wt %) and 15% (v/v) IPA was

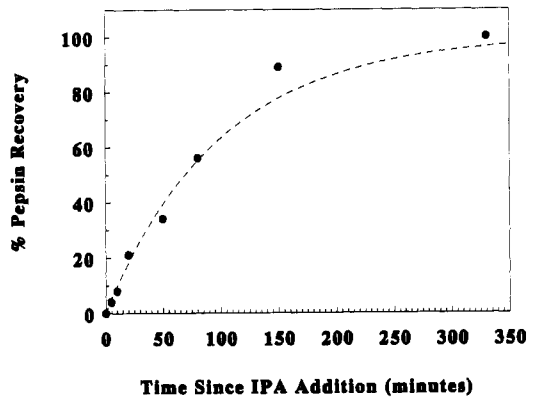

Figure 3. Percent recovery of pepsin from AOT in the isooctane reverse micelle system as a function of time when the extractant buffer contained 15% (v/v) isopropyl alcohol. The original upper phase was as described in Figure 1. The lower extractant phase was a 0.01 M acetate buffer at pH 4.1 and having a salt concentration of 0.1 M KCl and 15% (v/v) isopropyl alcohol. The release experiment was carried out by contacting the upper and lower phases in a 5-mL-capacity tube. The lower phase was gently stirred while the upper phase and the interface remained practically undisturbed. The points are experimental data, and the line is calculated assuming a first-order release process for the rate constant specified in the text.

contacted with an equal volume of 250 mM AOT in isooctane. On the basis of the notions of equilibrium discussed earlier, one would expect that chymosin (p*I* of 4.6) would be forward-transferred into the reverse micelles, while pepsin (p*I* less than 2) will be excluded by the reverse micelle and hence retained in the aqueous phase. The chromatograms obtained by liquid chromatography for the original aqueous phase and the aqueous phase following the contact with the reverse micellar phase are shown in panels a and b of Figure 4, respectively. One can see the peaks for both chymosin and the pepsin impurity in Figure 4a. Figure 4b shows that essentially 100% of the chymosin was forward-transferred into the reverse micelle phase while all of the pepsin was retained in the aqueous phase. This result is quantitatively similar to the result obtained if no IPA is used; however, there is no cloudiness observed at the interface when IPA is present.

The forward-transfer rate of chymosin was measured in the same system in which the backward-transfer rate of pepsin was measured. In this case, the AOT/isooctane solution was overlaid on an aqueous protein solution containing 0.1 M KCl and acetate buffer at pH 4.1. Both solutions were initially clear; after contact the lower phase grew cloudy within a few minutes and then gradually cleared again over a long period of time roughly corresponding to the time to complete the forward-transfer process (data not shown).

The chymosin present in the reverse micelle phase can be recovered by contacting with an aqueous stripping solution at a pH of 6.3, by the addition of 10–15% IPA to the stripping solution as in the case of pepsin back-transfer. Figure 4c shows the chromatogram of the aqueous stripping solution where this was done. The chymosin peak corresponds to a 68% recovery. Phase volume measurements showing no changes in the relative volumes of the aqueous and the reverse micelle phases are consistent with the idea that the IPA remains virtually in the aqueous phase at the conditions employed; however, no direct measurements of phase compositions for their IPA content have yet been carried out.

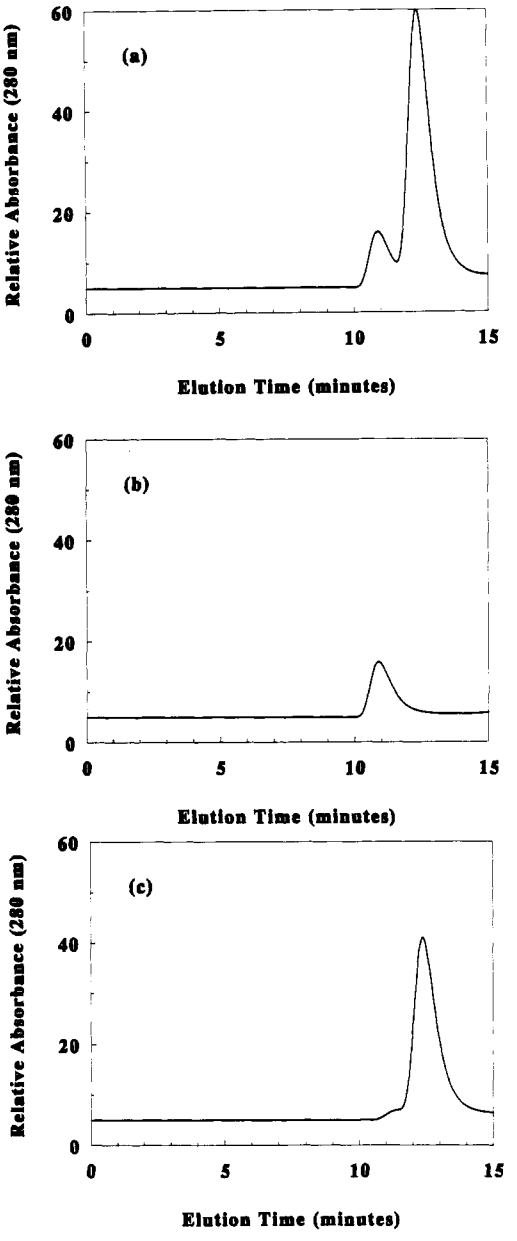

Figure 4. (a) GPC chromatogram of Sigma rennin. The first peak has been identified as a 19% contamination of bovine pepsin (which is similar in composition to porcine pepsin). The second peak is bovine chymosin. (b) GPC chromatogram of the same aqueous solution after contact with 250 mM AOT in the isooctane reverse micelle phase. Only the pepsin remains in the aqueous phase. (c) GPC chromatogram of the aqueous stripping solution after contacting with the above reverse micelle phase. The stripping solution is a pH 6.3 buffer containing 0.1 M KCl and 15% (v/v) IPA. The recovery of pepsin was 94% and that of chymosin was 68%.

Discussion

Three important observations were made in this study. First, pepsin cannot be released from an organic AOT/isooctane phase by contacting the solution with an aqueous phase at pH 4.1 even though no pepsin is forward-extracted from the same buffer. This is particularly striking with pepsin because it has a low isoelectric point and a high net charge at the conditions of the experiment. According to conventional arguments, pepsin should be rapidly ejected from reverse micelles under these conditions if equilibrium were rapidly attained. The inability to back-transfer protein also explains low recovery yields previously

observed with chymosin and other acid proteases (Shaffer, 1988). Furthermore, both chymosin and pepsin are of similar molecular weight and three-dimensional structure and they have radically different isoelectric points; therefore, the size of the proteins is not an issue here, and the failure to eject the proteins does not appear to depend on the ionic nature of the protein.

The second observation relates to the effect of IPA on protein release. Addition of IPA to the aqueous extraction phase promotes a rapid and nearly complete release of both pepsin and chymosin from the micellar phase. This is true whether IPA is admixed with the system or is added to the aqueous phase before contact with the reverse micelle/protein phase. Rapid release is achieved only when greater than 5% IPA is used. Assays and material balances show that the pepsin is still essentially 100% active after release in this way even when it has been retained in the micellar phase for several days. IPA addition promotes chymosin release from the organic phase in the same way and greatly improves the yield of protein as compared to adding a KCl solution alone. Close examination of Shaffer's results show that the only chymosin recovery found without IPA addition was the chymosin collected at the interface of the extraction system. No recovery was obtained from the clear organic phase.

The third finding is that 10–15% IPA addition does not destroy the functional integrity of the reverse micelle system. This distinguishes the strategy proposed here from those proposed in the studies of Eremin and Metelitsa (1989) and Woll et al. (1989), where the addition of a large amount of the organic solvent functionally destabilizes the reverse micelles and thus releases the solubilized protein. With 10% IPA in the aqueous phase, forward extraction of chymosin was observed at pH 4.1 but not at pH 6.3. This clearly indicates that transfer was still possible in the presence of IPA but only at a pH where the protein is positively charged. The bovine pepsin (which appears as a contaminant in the commercial chymosin) was not extracted at either pH: further proof that, in the presence of IPA, transfer is determined by the ionic nature of the protein and micelle system.

The appearance of a cloudy interface in systems without IPA and the lack of a cloudy interface when IPA is present as well as other behavior previously observed (Shaffer, 1988) suggest that without IPA some of the chymosin is not transferred completely into the upper phase while with IPA it is. This has implications for a mechanistic model of the process as proposed below.

Mechanism of Protein Uptake and Release. No study in the current literature offers a satisfactory explanation for why pepsin would be retained within the reverse micelle phase when the conditions for its residence are so unfavorable. The observations made in this study have implications for a new model of the mechanism of interfacial transfer of proteins between an organic reverse micelle phase and an aqueous phase.

The forward- and backward-transfer results show that the pepsin/AOT/isooctane system does not attain an equilibrium; therefore, a kinetic explanation appears to be required. A minimal model requires there to be two kinetic processes in series, one involving a fast equilibrium and one involving a slow equilibrium:

$$\text{AOT} + \text{protein} \rightleftharpoons \text{aqueous complex} \rightleftharpoons \text{solubilized protein}$$

In this case, the second equilibrium involving the transfer of the aqueous complex to the organic phase appears to be the slow process preceded by a rather rapid equilibrium to establish a "transferable" complex. Another way of looking at this is that there appears to be some mechanism involving AOT leaving the organic phase and going out to interact with protein molecules to form a complex in the aqueous phase. (This complex may be the reason for the cloudiness in the lower phase.) Once the complex is formed, it can mediate the transfer of protein into the organic phase in a second step. In the second step, the equilibrium lies far to the right so that the forward-transfer process is fast but the reverse of this step is slow.

The experimental facts suggest that without IPA the reverse step of the second process is very slow and virtually disallows transfer of protein back into the aqueous phase (at least when salt is present). IPA addition appears to increase the rate of the reverse process in this second step allowing equilibrium to be established. One may further speculate that the first step in the process involves the charge of the protein and the salt concentration. High salt or high pH values prevent complex formation and therefore disallow forward transfer. The backward-transfer process is rate-controlled, so it is little affected by salt or pH.

In view of the mechanism suggested above, IPA appears to work by enhancing the solubility of the AOT in the aqueous phase. The IPA-induced increase in the rate of the second major step facilitates the entry of the solubilized protein into the aqueous phase as an aqueous complex where decomplexation and the eventual release of the protein occur. This is supported by the observation that there is substantial clouding of the lower phase during the back-transfer experiment.

Summary

These experiments show clearly that the addition of isopropyl alcohol is a practical method for protein back-extraction from the AOT reverse micelles. For the first time, one can obtain 100% recovery of a solubilized protein from the reverse micelles, while at the same time retaining almost all of the activity. Forward-transfer experiments with chymosin show that extraction is possible with 15% IPA and thus provide definitive evidence to conclude that the addition of IPA at the levels employed does not affect the functional integrity of the reverse micelle system. The generality of this procedure for other reverse micelle formulations will be explored in the future.

Acknowledgment

A critical reading of an early version of the manuscript by Professor T. A. Hatton helped us realize that explicit experimental data would be useful to highlight one unique feature of our results, namely, that reverse micelles remain functionally intact even in the presence of the added IPA solvent.

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