Protein extraction using reversed micelles

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Abstract. Proteins can be extracted from an aqueous phase into reversed
micelles. This transfer is governed by electrostatic interactions, for protein
and surfactant have to bear opposite charges. The presence of high salt
concentrations diminishes the attractive interactions and can lead to
expulsion of the solubilized proteins. This is used to recover extracted
proteins.
Larger proteins require a larger number of charged residues on their
surface in order to be transferred into reversed micelles, so the larger the
protein, the further the pH of maximal transfer is removed from the
isoelectric point.
The transfer profiles can be manipulated by micellar size and charge
density at the interface. When the charge density at the interface is
modified by variation of the type of head group of the cosurfactant, shifts
of transfer profiles to higher or lower pH values are observed.
Variation of the number and length of the tails of quaternary ammonium
surfactants revealed that of the 16 surfactants tested, only didecyldimethyl
ammonium chloride and trioctylmethylammonium chloride enabled transfer.
There was no relation between the water content of the organic phase and
the transfer properties of the surfactants tested.
Because for application not only forward transfer is important, an
alternative method for back transfer was tested. Exposing an enzyme
containing reversed micellar solution to a temperature increase led to
expulsion of aqueous phase and enzyme, yielding a highly concentrated
enzyme preparation.

INTRODUCTION

With the new methods that have become available for the large scale production of
enzymes, a need has developed for new simple methods for protein purification. The
observation that, via their aqueous interior, reversed micelles can transfer proteins from
one aqueous phase to a second aqueous phase (ref. 1) prompted investigations into the
applicability of such systems for protein purification. In order to be suitable as a
purification step, the extraction process must be selective, and scale up must be possible.

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Our research has focussed on both aspects: the study of factors that determine protein transfer into reversed micelles, and the scaling up of the process. This article will focus on the first aspect, but a novel method of desolubilization will be discussed also. In the reversed micellar extraction process, selectivity can be introduced either in the forward or in the back transfer. We have mainly investigated factors that influence forward transfer. Transfer of a protein from the aqueous phase into reversed micelles was found to depend on the pH of the aqueous solution (ref. 1-4).

As a typical example of our studies, an aqueous phase consisting of an ethylene diamine buffer containing α-amylase is extracted with 8 mM trioctylmethylammonium chloride (TOMAC) in isooctane for 2 min. The organic phase is supplemented with octanol (0.1%) and Rewopal HV5 (2mM) (a non-ionic surfactant) as cosurfactant. After phase separation part of the organic phase is removed and contacted with an equal volume of a second aqueous phase consisting of 0.5 M phosphate buffer, pH 6.9. After phase separation the enzyme activity in the second aqueous phase can be measured. With this system, transfer of α-amylase is observed at a pH around 10, that is 5 pH-units above its isoelectric point. For AOT (dioctyl sulphosuccinate) reversed micelles, protein transfer is observed at and below the isoelectric point (ref. 5). Whereas with TOMAC reversed micelles narrow transfer profiles were observed, with AOT reversed micelles, Göklen (ref. 6) generally found wide transfer profiles for small proteins, and narrow profiles for larger ones. These observations could be interpreted as follows: Transfer requires in most cases adaptation of the micelle size to the size of the protein, for protein filled reversed micelles are larger than empty micelles. The energy required for this enlargement is derived from electrostatic interactions between surfactant and charged groups on the protein. The larger the protein, the more charge is required, so the further away from the isoelectric point is transfer observed. Such a relationship was confirmed for 19 proteins in TOMAC reversed micelles, (Fig. 1) and a similar correlation for AOT and protein size could be derived from extraction data of Göklen as presented in ref. 6 (ref. 7).
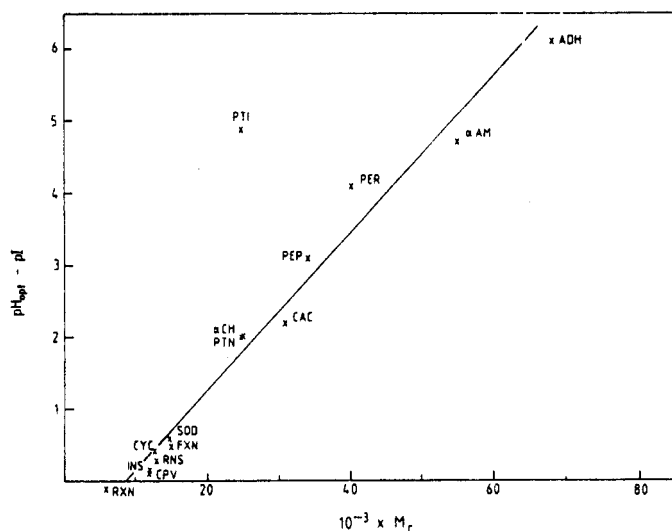

Fig. 1
The difference between the pH where maximal solubilization occurs (pH_{opt}) and the isoelectric point (pI) as a function of M_r for trioctylmethylammonium chloride reversed micelles (from ref. 7).

Luisi *et al.* (ref. 2) already reported that the type of ions in solution has a large effect on the transfer behaviour of proteins. Leodidis and Hatton (ref. 8) have studied this in more detail. These results indicate that micellar size is affected by the counter ion, and that a larger micelle might facilitate transfer.

Not only does the amount of protein transferred depend on the size of the molecules and their charge density, also a good correlation between % of transfer and symmetry of charge distribution as calculated by Barlow and Thornton (ref. 9) was observed (Fig. 2). Increasing the TOMAC concentration leads to increased extraction percentages (ref. 1,10). A relation between surfactant concentration and amount of protein transferred was also observed for AOT (ref. 4,11).
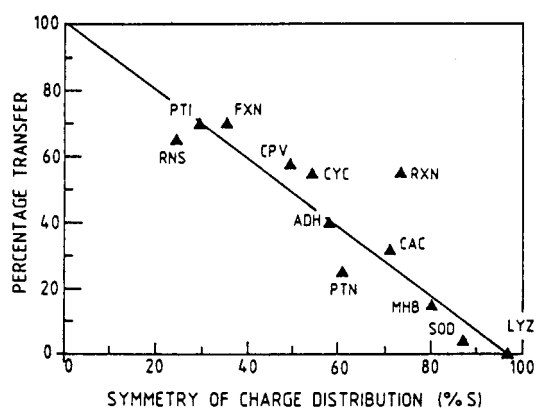
Fig. 2 The percentage of protein solubilized in trioctylmethylammonium chloride reversed micelles as a function of the symmetry of charge distribution over these proteins as given in ref. 9 (from ref. 7).
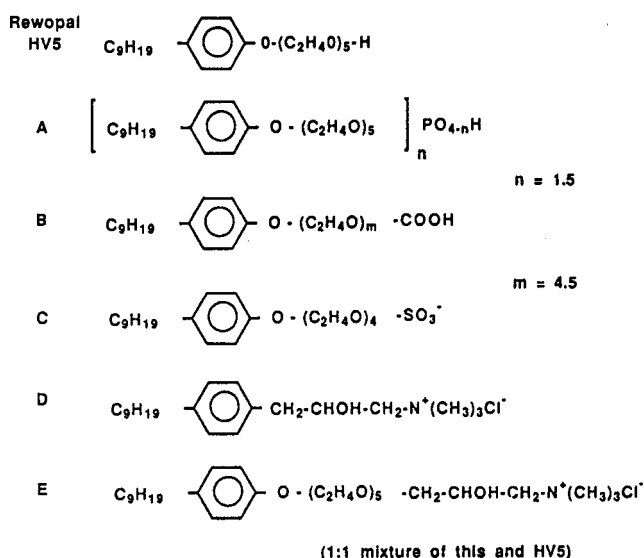

Fig. 3. Structures of surface active compounds used as cosurfactants for the extraction of α-amylase.

Besides the composition of the aqueous phase the properties of the micellar phase influence transfer. The effect of the sign of charge of the surfactant has already been mentioned. In our case, addition of the cosurfactant Rewopal HV5 to TOMAC reversed micelles (in a 1:4 ratio) resulted in the enlargement of the micelles, as judged by an increase in water content of the organic phase, and the broadening of the transfer profile (ref. 12). Shi *et al.* (ref. 13) observed that transfer in mixed micelles is not directly related to micelle size, but rather to the properties of the surfactants. To study the effect of the charge density at the interface on transfer, we employed a series of cosurfactants of the same structure as Rewopal, but with different charged groups attached to the head group (Fig. 3).

Fig. 4 shows that transfer profiles are shifted indeed. Attachement of a negatively charged moiety to the cosurfactant leads to a shift to higher pH values. This can be explained by partial neutralization of the positively charged TOMAC, leading to a decreased charge density in the interface. This is more pronounced for the sulphate than for the phosphate headgroup. Surprisingly, addition of a carboxylic acid moiety completely abolished transfer.
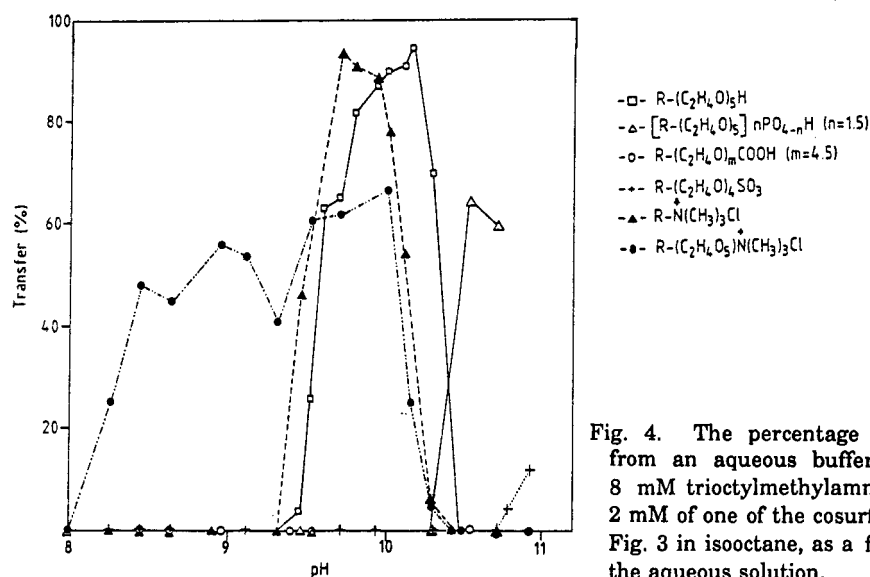

Fig. 4. The percentage of protein transferred from an aqueous buffer into a solution of 8 mM trioctylmethylammonium chloride and 2 mM of one of the cosurfactants shown in Fig. 3 in isooctane, as a function of the pH of the aqueous solution.

Initially it was thought that it might be caused by the inability to form reversed micelles, but when this was tested by measuring the capacity to solubilize water, the water content was virtually the same as for the standard reversed micellar medium. Introduction of positively charged groups into Rewopal resulted in shifts of the transfer profile to lower pH values. In the case of surfactant D the headgroup is slightly larger than the TOMAC head group, the water content is virtually the same, and the transfer profile is shifted by about 0.3 pH unit. For surfactant E, the headgroup is more bulky, reflected in a larger capacity to solubilize water, (about twice as much as in the standard system) and transfer is observed at much lower pH, and over a wider range. We presume that the higher charge density in the interface facilitates transfer.

As an alternative method to change the system, the structure of the cationic surfactant was varied, i.e. both the length and the number of alkyl tails were varied. Sixteen different surfactants varying in composition between 1 to 3 tails, and 4 to 18 C-atoms in their tails, were tested for their ability to promote transfer in a system containing a minimal amount of octanol as cosurfactant, without Rewopal addition. Of those only 5 showed transfer, three of which for less than 15 %. Only $2C_{10}$ and $3C_8$ gave good transfer. An explanation for this observation was sought in the inability of the systems to form aqueous microdomains, but determination of the water content revealed no relation between protein transfer and the amount of solubilized water. $3C_8$ for example solubilizes only 3 water molecules per surfactant molecule, whereas $2C_{10}$ shows a ratio of 60 water per surfactant molecule. Some of the other surfactants were able to solubilize up to 80 water molecules per surfactant, but this was not accompanied by transfer. An alternative explanation might be that other types of microstructures are formed that are not large enough or cannot enlarge themselves so as to host proteins. Therefore we have to conclude that the presence of a positive charge in itself is not sufficient to promote transfer.

Similar observations have been made by Shi *et al.* (ref. 13) who studied protein transfer using reversed micelles composed of phosphatidylcholine and phosphatidylethanolamine and AOT combined with one of these phospholipids. Their results also indicated that it is not the water content of the system alone that promotes transfer, but rather interactions between protein and interface.

Jolivalt *et al.* (ref. 10) varied the organic solvent and therewith the size of the reversed micelles. Their results too led them to conclude that the water content of the system is not the main determinant for transfer.

BACK TRANSFER

Since the first reports on protein solubilization in reversed micelles, proteins have been recovered by contacting the reversed micellar solution with a buffer of different pH and/or high ionic strength. Hatton (ref. 4,14) has employed differences in ionic strength to introduce selectivity in the reextraction. Our research group has routinely used a high ionic strength to recover protein from the reversed micelles. Scaling up of the extraction and reextraction has been studied in a mixer settler unit (ref. 15). This system has been optimized with respect to minimal protein inactivation, by increasing the partition coefficient and the rate of mass transfer. Under these conditions it proved possible to recover 85% of active α-amylase, 17 fold concentrated (ref. 16).

Recently novel ways to recover protein have been suggested. Leser (ref. 17) used silica to reextract proteins from reversed micelles, and John *et al.* (ref. 18) used clathrate hydrate formation to desolubilize protein. Because it was observed that the mass transfer rate in the back extration was slow (ref. 19), we developed an alternative method based on the observation that the amount of solubilized water drops with increasing temperature (ref. 20). This decrease makes the reversed micelles too small to accommodate protein, so protein is ejected from the aqueous core. The feasibility of this method was investigated by using pilot scale centrifugal extractors, and compared to the extraction at high ionic strength that had been used till then. With the temperature jump method 75 % of active α-amylase, about

2000-fold concentrated, could be recovered, whereas the yield with the standard method using the same centrifugal process (under non-optimized conditions) was 30-50%, only tenfold concentrated. The forward extraction was carried out at 10 °C, the back extraction at 35 °C, using Westphalia Centrifugal extractors.

It is interesting to note that for the recovery of enzyme and product from a microemulsion Larsson *et al.* (ref. 21) have used a similar method, that is based on a phase transition caused by a change in temperature. In that case however additional buffer or organic solvent is added.

CONCLUSIONS

These results show that we begin to understand the various factors that govern protein solubilization in reversed micelles.

By varying the composition of the system, it is possible to obtain selective extraction. Hatton *et al.* (ref. 4,14) have shown that micellar extraction is a possible means for the separation of a protein mixture, and to selectively isolate one protein from a fermentor broth (ref. 22). Also introduction of a surface active affinity label can enhance the selectivity (ref. 23,24). Leser *et al.* (ref. 25) have shown that this method is feasible for the extraction of proteins from a solid meal. Dekker has shown that this method can be scaled up to a continuous process, and that α-amylase can be recovered at a high yield in an active form (ref. 26).

REFERENCES

1. P.L. Luisi, F.J. Bonner, A. Pellegrini, P. Wiget and R. Wolf, Helv. Chim. Acta 62, 740-753 (1979).
2. P. Meier, E. Imre, M. Fleschar and P.L. Luisi, Surfactants in solution, p. 999-1012, Plenum Press, New York (1984).
3. K. van 't Riet and M. Dekker, Proc. 3rd Eur. Congress on Biotechnology, Munich, III 541-544 (1984).
4. K.E. Göklen and T. A. Hatton, Proc. Int. Solvent Extraction Conf. Munich, III, p. 587-595, (1986).
5. K.E. Goklen and T.A. Hatton, Biotechnol. Progress 1, 69-74 (1985).
6. K.E. Göklen, Ph.D. Thesis, Mass. Inst. Technol., Cambridge, MA (1986).
7. R.B.G. Wolbert, R. Hilhorst, G. Voskuilen, H. Nachtegaal, M. Dekker, K. van 't Riet and B.H. Bijsterbosch, Eur. J. Biochem. 184, 627-633 (1989).
8. E.B. Leodidis and T.A. Hatton, Structure and Reactivity in Reverse Micelles, p. 270-302 Elsevier, Amsterdam (1989).
9. D.J. Barlow and J.M. Thornton, Biopolymers 25, 1717-1733 (1986).
10. C. Jolivalt, M. Minier and H. Renon, J. Colloid Interface Sci. 135, 85-96 (1990).
11. P.D.I. Fletcher and D. Parrot, J. Chem. Soc. Faraday Trans. I 84, 1131-1144 (1988).
12. M. Dekker, J.W.A. Baltussen, K. van 't Riet, B.H. Bijsterbosch, C. Laane and R. Hilhorst, Biocatalysis in organic media. Studies in Organic Chemistry 29, p. 285-288 Elsevier, Amsterdam (1987).
13. H. Shi, X. Lei, Z. Shen and J. Wang, Huagong Jinzhan 3, 28-32 (1988).
14. K.E. Göklen and T.A. Hatton, Sep. Sci. Technol. 22, 831-841 (1987).
15. M. Dekker, K. van 't Riet, S.R. Weijers, J.W.A. Baltussen, C. Laane, and B.H. Bijsterbosch, Chem. Eng. J. 33, B27-B33 (1986).

16. M. Dekker, K. van 't Riet, B.H. Bijsterbosch, R.B.G. Wolbert and R. Hilhorst, <u>AIChE J.</u> <u>35</u>, 321-324 (1989).
17. M. Leser, K. Mrkoci, and P.L. Luisi, in preparation.
18. J.B. Phillips, H. Nguyen and V. T. John, <u>Biotechnol. Progress</u> <u>7</u>, 43-48 (1991).
19. M. Dekker, K. van 't Riet, B.H. Bijsterbosch, P. Fijneman and R. Hilhorst, <u>Chem. Eng. Sci.</u> <u>45</u>, 2949-2957 (1990).
20. M. Dekker, K. van 't Riet, J.J. van der Pol, J.W.A. Baltussen, R. Hilhorst and Bijsterbosch, <u>Chem. Eng. J.</u>, in press (1991).
21. K.M. Larsson, U. Olsson, P. Adlercreutz and B. Mattiasson, <u>Biotechnol. Bioeng.</u> <u>35</u>, 135-141 (1990).
22. R.S. Rahaman, J.Y. Chee, J.M.S. Cabral and T.A. Hatton, <u>Biotechnol. Progress</u> <u>4</u>, 218-224 (1988).
23. J.M. Woll, A.S. Dillon, R.S. Rahaman and T.A. Hatton, <u>Protein Purification: Micro to Macro</u>, 117-130, A.R. Liss, New York (1987).
24. J.M. Woll, T.A. Hatton and M.L. Yarmush, <u>Biotechnol. Progress.</u> <u>5</u>, 57-62 (1989).
25. M.E. Leser, P.L. Luisi and S. Palmieri, <u>Biotechnol. Bioeng.</u> <u>34</u>, 1140- (1989).
26. M. Dekker, <u>Ph.D. Thesis</u>, Agricultural University, Wageningen, The Netherlands (1990).