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Ludger H. Poppenborg[a]; Aristidis A. Brillis[a]; David C. Stuckey[a]
[a] DEPARTMENT OF CHEMICAL ENGINEERING AND CHEMICAL TECHNOLOGY, IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY AND MEDICINE, LONDON, UK

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The Kinetic Separation of Protein Mixtures Using Reverse Micelles

LUDGER H. POPPENBORG, ARISTIDIS A. BRILLIS, and DAVID C. STUCKEY*

DEPARTMENT OF CHEMICAL ENGINEERING AND CHEMICAL TECHNOLOGY
IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY AND MEDICINE
PRINCE CONSORT ROAD, LONDON SW7 2BY, UK

ABSTRACT

Commercial interest in cost-effective methods that can separate, concentrate, and purify proteins continuously, and be easily scaled-up, has increased markedly in recent years. Liquid–liquid extraction using reverse micellar organic solvents has been found to have this potential. The overall objective of this work was to investigate the kinetic separation of protein mixtures using reverse micelles. The kinetics of simultaneous forward extraction of lysozyme, cytochrome *c*, and ribonuclease A were investigated in a stirred cell (Lewis cell). In addition, a Graesser contactor was used for the simultaneous extraction of lysozyme and cytochrome *c* from a buffer solution as well as from diluted hen egg white to which cytochrome *c* had been added. The Lewis cell experiments showed that the mutual effect on the overall mass transfer coefficient, k_0, of the three simultaneously extracted proteins at pH 5.5 was significant, and in some cases quite large. The presence of cytochrome *c* or ribonuclease A helped to prevent lysozyme precipitation at the interface, while the addition of cytochrome *c* altered the pH dependency pattern of lysozyme, and the presence of lysozyme reduced the k_0-value of cytochrome *c*. The kinetic separation of lysozyme and cytochrome *c* was possible with a Graesser contactor, and maximized at low rotor speed (2–3 rpm), low temperature (4°C), and a pH close to the pI of both proteins (pH 10); after 30 minutes about 80% of the lysozyme and only 10% of the cytochrome *c* were extracted into the reverse micellar phase. The extraction rate measured in the Graesser contactor differed from that measured in the Lewis cell, and this observation indicates that different steps of the reverse micellar transfer mechanism are controlling the transfer. Using hen egg white (the natural source of lysozyme), the kinetics of lysozyme and added cytochrome *c* were different from those with buffer solutions; cytochrome *c* ex-

* To whom correspondence should be addressed. Telephone: +44 171 594 5591. FAX: +44 171 594 5629. E-mail: d.stuckey@ic.ac.uk

traction was faster, making kinetic separation more difficult. In addition, a stable emulsion formed with hen egg white, which is not desirable in an industrial application since it results in an additional separation step.

Key Words. Extraction kinetics; Reverse micelles; Protein mixtures; Graesser contactor; Hen egg white

INTRODUCTION

Recent advances in biotechnology have resulted in the production of an increasing variety of proteins using both recombinant microorganisms and more traditional animal cell cultures. Hence, commercial interest in cost-effective methods that can separate, concentrate, and purify proteins continuously, and can be easily scaled-up, has increased markedly in recent years. Liquid–liquid extraction by means of reverse micellar organic solvents has been found to have this potential (9).

Reverse micelles are thermodynamically stable nanometer-sized water droplets within an organic solvent stabilized by a monolayer of surfactant molecules, and can solubilize organics such as amino acids and proteins. These proteins can be reextracted into an aqueous phase either under certain conditions of pH and ionic strength, or using a variety of alternative backward transfer techniques (5, 7, 13, 20, 24, 28), including the addition of a counterionic surfactant (10). However, a number of questions still need to be answered in order for this technique to be scaled up to a commercial process; two key questions relate to selectivity and contactor design.

Potentially, the selective extraction of a protein from a complex mixture can be achieved by adjusting the optimum pH and ionic strength depending on the chosen surfactant and solvent. Nevertheless, the specific separation of proteins with similar molecular weights and pIs from complex fermentation broths has only recently been attempted (10). Affinity reverse micelles have been developed (2, 19) but they substantially increase the costs of this technique. The kinetics of reverse micellar extraction of a number of single proteins have been studied in a stirred (Lewis) cell with a flat interface (18, 22), and it has generally been accepted that forward protein extraction is controlled by diffusion in the stagnant aqueous film. However, while the kinetic separation of a protein mixture from either a buffer or broth has never been attempted, it seems that this should be possible and could provide a new technique for separating similar proteins.

Most work on reverse micelles has shown that the intense mixing required during extraction leads to emulsification and makes phase separation very difficult. Therefore, a contactor design that reduces or eliminates emulsion formation without markedly reducing the rate of extraction is needed. A Graesser "raining bucket" contactor (3, 25, 27) is a low shear design potentially capa-

ble of operating in a continuous mode for both forward and backward extraction using reverse micelles. The only use of such a contactor in biotechnology is that of Coimbra et al. (6) who used it with an aqueous two-phase system to extract proteins from whey.

The overall objective of this work was to investigate the kinetic separation of protein mixtures using reverse micelles. In order to accomplish this, the extraction kinetics from buffer of three proteins, both singly and in admixture, were determined in a defined area Lewis cell. A binary mixture was then separated in a Graesser contactor in order to assess the influence of hydrodynamics and system parameters (pH, rotor speed, temperature) on kinetic separation. Finally, the same mixture was extracted out of egg white to determine the effect of real systems on kinetic separation. The proteins chosen for this work had very similar molecular weights and pIs in order to determine whether this type of challenging separation was possible.

MATERIALS AND METHODS

Bis(2-ethylhexyl)sulfosuccinate sodium salt (AOT) and 2,2,4-trimethylpentane (isooctane) were obtained from Sigma (Poole, UK). KCl, KH_2PO_4, K_2HPO_4, K_2CO_3, and $KHCO_3$ were Anal-R grade from Merck (Dorset, UK). Trioctylmethylammonium chloride (TOMAC), lysozyme (hen egg white; pI 11; 14,300 Da), cytochrome *c* (horse heart; pI 10.6; 12,348 Da), and ribonuclease A type IIA (bovine pancreas; pI 7.8; 13,700 Da) were purchased from Sigma. Hen egg white was separated from fresh eggs. All other chemicals were purchased from Sigma and were of analytical grade.

Forward Extraction Procedure

The reverse micellar phase consisted of 50 mM AOT in isooctane. Aqueous solutions for forward transfer consisted of 50 mM KCl and 50 mM K-phosphate or K-carbonate buffer made up to the required pH, containing 1.0 g/L cytochrome *c*, 1.5 g/L lysozyme, or 2.1 g/L ribonuclease A. Hen egg white was diluted 15-fold in the same buffer adjusted to pH 9, and 1 g/L cytochrome *c* was added. Forward extraction was performed by contacting equal volumes of aqueous phase and reverse micellar phase in a Lewis cell or a Graesser contactor. All solutions were adjusted to the desired temperature prior to contacting, and were all phase equilibrated except for those used for hen egg white experiments.

Protein Assay

The concentration of each protein in the aqueous phase was determined using an HPLC system (Shimadzu, UK) fitted with a Zorbax 300SB C8 re-

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versed phase chromatography column (Jones, UK). The peak areas at 280 nm were used to calculate the protein concentrations. Protein concentrations in reverse micellar phase samples were measured after backextraction with a slightly altered method described by Jarudilokkul et al. (10). An aqueous solution (500 μL) consisting of 50 mM KCl and 50 mM potassium phosphate buffer (pH 8) and 500 μL of 60 mM TOMAC in isooctane was added to 500 μL of an organic phase sample in a 1.5-mL test tube and immediately rotary inverted at 50 rpm for 10 minutes. Phase separation was achieved by centrifuging the tubes for 2 minutes at 12,000 rpm. It was assumed that 95% of cytochrome *c* and lysozyme and 90% of ribonuclease A in the reverse micellar phase sample had always been back-extracted into the aqueous phase (11). The coefficient of variation for the entire backextraction process was ±2%.

Kinetic Studies

Overall Mass Transfer Coefficients in a Stirred Cell with a Flat Interface (Lewis cell)

Protein solutions containing only lysozyme, cytochrome *c*, or ribonuclease A, all possible binary mixtures, and a ternary mixture were used as the aqueous phase in the work. Experiments were conducted in a temperature-controlled Lewis cell (14) with 128 mL total volume and an interfacial area of 19.6 cm^2. The impeller speeds were set to result in laminar flow ($R_e = 1000$) in both phases. Samples (500 μL) were taken from the aqueous as well as the reverse micellar phase over time. Y in Eq. (1) (with initial protein concentration in the aqueous phase c°_{aq}, protein concentration in the reverse micellar phase c_{rm}, which was corrected by the corresponding backextraction yields, surface area A, phase volume V, phase distribution coefficient m_{ft}) was plotted against the extraction time (18). Only the protein concentrations of samples which gave a straight line in this plot were used for further calculations (usually the first five to eight samples, $R^2 > 0.95$). After linear fitting, the slope gave the overall mass transfer coefficient (k_0). All experiments were done in duplicate. For pH-dependence experiments the coefficient of variation for determination of k_0 was ±10%.

$$Y = \ln[\{c^\circ_{aq} - (1 + 1/m_{ft})c_{rm}\}/c^\circ_{aq}]/\{(A/V)\cdot(1 + 1/m_{ft})\} \quad (1)$$

Graesser Contactor Studies

Binary protein mixtures of lysozyme and cytochrome *c* were used in experiments with a single compartment Graesser contactor due to their similar pIs. The Graesser contactor used for the experiments (Fig. 1) had a total volume of 450 mL, with a Perspex rotor made of two 91 mm discs 2.54 cm apart, and was

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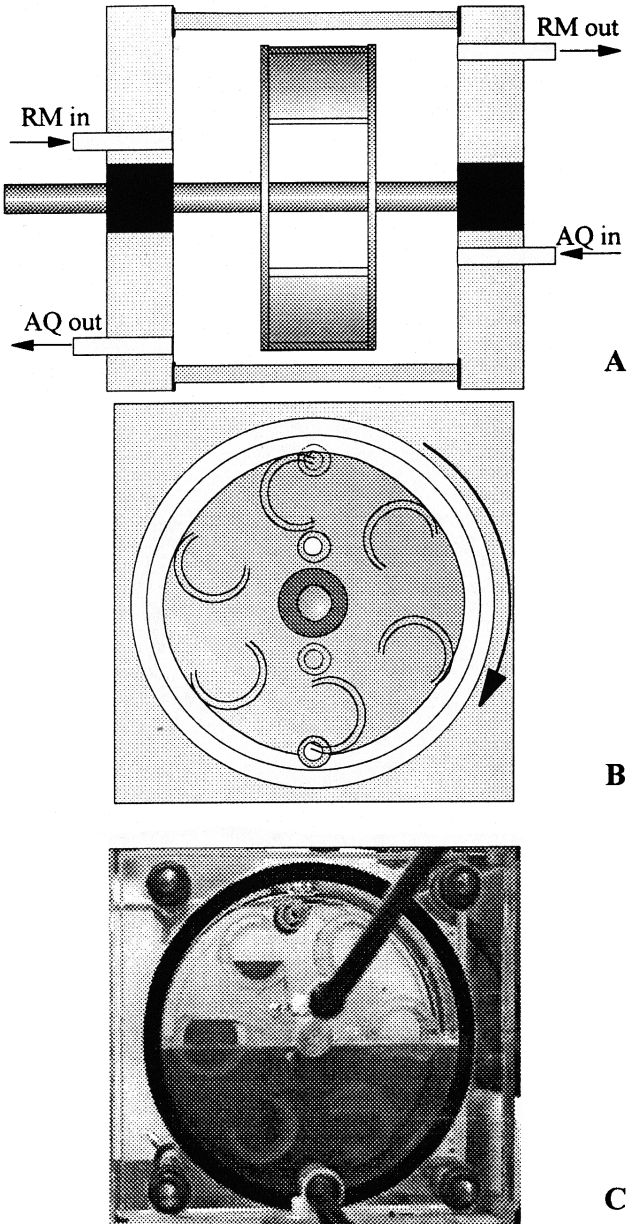

FIG. 1 Schematic of a single compartment Graesser contactor. Side view (A), front view (B), and photograph of the contactor filled with aqueous protein solution (bottom phase) and reverse micellar phase about 3 seconds after mixing started (C).

driven with a speed-controlled electrical motor. The contactor was provided with solvent inlet (near axis) and outlet ports (near top or bottom) for both phases, and peristaltic pumps were used to circulate both phases independently. The contactor was submerged in a temperature-controlled water bath.

RESULTS AND DISCUSSION

Forward Extraction in a Lewis Cell

Individual Mass Transfer Kinetics of Proteins: Influence of Additional Proteins

Equilibrium experiments in 1.5 mL test tubes revealed that the optimum conditions for reverse micellar extraction with 50 mM AOT in isooctane were pH 5–10.5 and 50–500 mM KCl for lysozyme, pH 5–10 and 50–250 mM KCl for cytochrome *c*, and pH 4.5–6.5 and 25–100 mM KCl for ribonuclease A, respectively (11). Within these ranges 86–97% of each protein was extracted, and hence it was reasonable to assume a similar mass transfer driving force from the aqueous to the reverse micellar phase for all three proteins at pH 5.5 and 50 mM KCl. We investigated the influence of additional proteins on the overall mass transfer coefficient, k_0, of individual proteins from a mixture of proteins in 50 mM buffer (pH 5.5) and 50 mM KCl into a 50 mM AOT–isooctane reverse micellar phase. Both the rate at which particular proteins appeared in the organic phase (Fig. 2; positive scale) as well as the rate at which these disappeared from the aqueous phase (Fig. 2; negative scale) were deter-

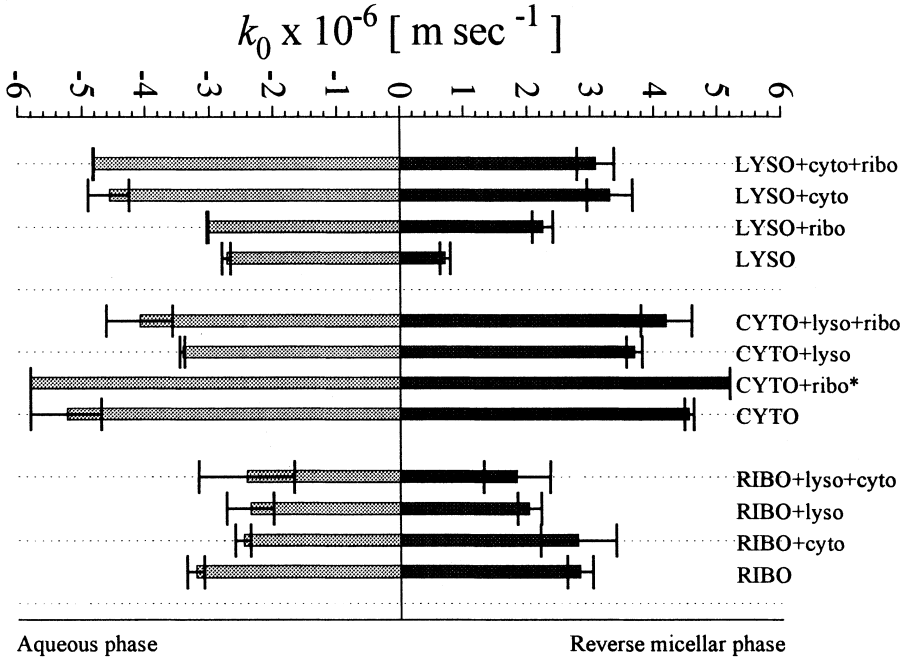

FIG. 2 Average k_0-values for the forward extraction of individual proteins from an aqueous protein mixture into a reverse micellar phase at pH 5.5 and 4°C. LYSO or lyso = lysozyme; CYTO or cyto = cytochrome *c*; RIBO or ribo = ribonuclease A. Capitals = individual protein; lower case = additional proteins;* = one measurement.

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mined. For lysozyme, in all cases, the value based on the protein concentration in the reverse micelle phase resulted in a smaller k_0 (at the 95% confidence interval) than that based on aqueous phase concentrations, and this indicated that there was lysozyme precipitation at the interface. Most studies in the literature have ignored the problem of protein precipitation at the interface by using only aqueous phase concentrations to calculate k_0. However, in our study the difference between k_0^{AQ} and k_0^{RM} is a measure of the rate of protein precipitation at the interface, and this useful insight may enable system parameters to be changed to minimize precipitation. It was also interesting to note that despite the precipitation, the slope of the mass transfer line, and hence the k_0, did not change much over time until the end of the extraction, implying that the distribution coefficient was constant despite protein accumulation at the interface.

The k_0-values for ribonuclease A were the least influenced by the presence of other proteins. Nevertheless, addition of both cytochrome *c* and lysozyme resulted in a small but significant reduction in the aqueous phase k_0, while only lysozyme reduced the RM phase k_0, presumably due to the precipitation of ribonuclease A at the interface. The addition of both proteins together did not seem to have any additional effect. The reasons for this reduction in mass transfer must have to do with both protein properties, e.g., hydrophobicity, and competition for reverse micelles at the interface which is related to the physical method of extraction, e.g., hydrophobic, electrostatic, or ion exchange (23). This is an extremely interesting question, but beyond the scope of this preliminary study.

The forward extraction of lysozyme (based on the aqueous phase) was slightly, but statistically significantly, increased if ribonuclease A was present. Interestingly, the k_0 based on the RM concentrations was a lot lower, indicating significant precipitation at the interface, and this was seen visually. Cytochrome *c* was found to have a significant influence on the mass transfer of lysozyme, increasing it substantially. The presence of both cytochrome *c* and ribonuclease A also seemed to have a beneficial effect on reducing the precipitation of lysozyme at the interface, possibly by forming a dimer which prevented it from denaturing at the interface. Once again, the addition of both proteins did not seem to result in significantly different behavior to the single proteins.

The k_0-value of cytochrome *c* (aqueous phase) was not affected by the presence of ribonuclease A, although again, the presence of another protein seemed to reduce the amount of precipitation of cytochrome *c* at the interface. Lysozyme reduced the mass transfer substantially, and with both proteins added the transfer seemed to be a net result of the two. Overall, the effect of most added proteins on the individual k_0-values was found to be statistically significant, and either reduced or enhanced mass transfer. The addition of two proteins seemed to result in a combined effect in most cases.

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Individual Mass Transfer Kinetics of Proteins: Influence of pH

Lysozyme and cytochrome *c* have similar molecular weights and pIs, and this results in a very similar pH dependency of their phase distribution (9). To test whether their extraction kinetics had a similar pH dependency, and if they influenced each others' extraction, the individual and simultaneous forward extraction mass transfer kinetics of lysozyme and cytochrome *c* were investigated at 4 different pHs. The results shown in Fig. 3 reveal that the extraction of cytochrome *c* by itself was very slow at pHs >7, whereas lysozyme extraction was fastest in the pH range between 7 and 9, and substantially faster than that of cytochrome *c*. At pH 5.5 and pH 10 the k_0-value of lysozyme dropped noticeably. Only at pH 5.5 was the k_0 of lysozyme lower than that of cytochrome *c*, but this was primarily because lysozyme by itself tended to precipitate at the interface. The addition of cytochrome *c* altered the pH depen-

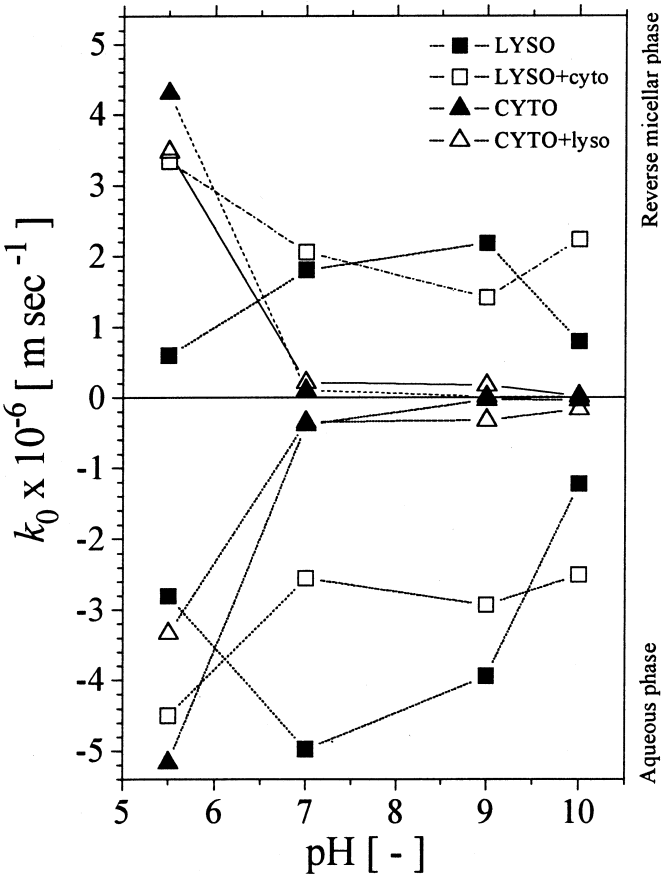

FIG. 3 k_0-values for the forward extraction of individual proteins from an aqueous protein mixture into a reverse micellar phase at different pHs and 4°C. LYSO or lyso = lysozyme; CYTO or cyto = cytochrome *c*; Capitals = individual protein; lower case = additional proteins.

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dency pattern of lysozyme considerably. In this case lysozyme mass transfer was fastest at pH 5.5, and was similar or lower at pH 7 and 9. The presence of lysozyme, however, had little influence on the k_0-value of cytochrome *c* over the whole pH range. The influence of pH on the forward transfer kinetics of cytochrome *c* and lysozyme manifests itself in the way that it affects the phase distribution of these proteins. The forward extraction kinetics of cytochrome *c* depend strongly on pH within the 5.5–10 pH range, whereas its phase equilibrium is more or less independent of pH within this range. Similarly, lysozyme extraction kinetics are also more pH dependent than their phase equilibrium, but less than cytochrome *c*.

It seems reasonable to assume that lysozyme extraction is governed mainly by hydrophobic interactions with the surfactant AOT, whereas cytochrome *c* extraction depends more strongly on electrostatic interactions which are low at a pH close to the pI of the protein, and therefore forward extraction slows down as the pH increases. However, a recent hypothesis that extraction is based on ion exchange (23) may need to be investigated in more depth.

Forward Extraction in a Graesser Contactor

Unfortunately, k_0-values for the forward extraction of lysozyme or cytochrome *c* from binary mixtures in a Graesser contactor could not be determined due to the complexity of the interface and an inability to measure its area accurately. Hence the progress of forward extraction was monitored and calculated using

$$\text{Protein } [\%] = \frac{\text{protein remaining in the aqueous phase}}{\text{initial protein in the aqueous phase}} \times 100 \qquad (2)$$

Influence of pH on Mass Transfer

At pH 5.5, a pH much lower than the pI of both proteins, lysozyme and cytochrome *c* were extracted at the same rate (Fig. 4). The extraction rates for both proteins decreased with increasing pH; however, cytochrome *c* extraction was affected much more. At pH 10, close to the pI of both proteins, cytochrome *c* was extracted much slower than lysozyme. About 80% of the lysozyme was extracted after 30 minutes compared to only 10% of the cytochrome *c*. Interestingly, the effect that pH changes had on the extraction of cytochrome *c* are different from the observations in the Lewis cell. In the Lewis cell a relatively fast cytochrome *c* transfer was observed only at pH 5.5, with equally low transfer rates for pH 7–10, whereas the transfer rate at pH 5.5 and 7 in a Graesser were alike; moreover, the transfer rate decreased gradually as the pH approached the pI. This observation could indicate that different steps of the reverse micellar transfer mechanism (8) in the Graesser contactor and the Lewis cell are controlling the transfer of cytochrome *c*, allowing it to

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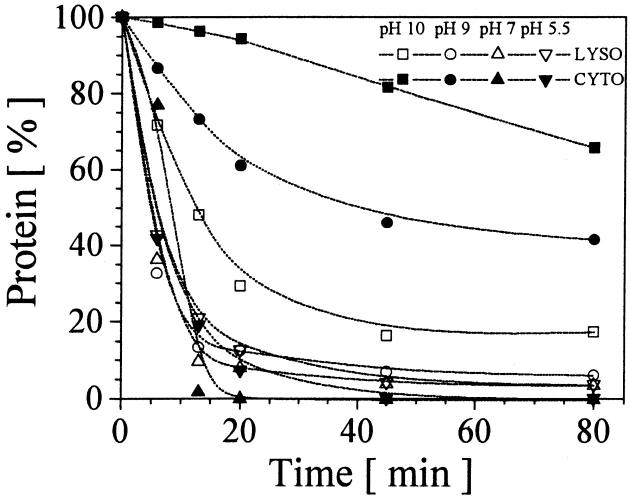

FIG. 4 Simultaneous forward extraction of lysozyme (LYSO) and cytochrome *c* (CYTO) in a single compartment Graesser contactor at different pHs, 3 rpm, and 4°C.

be extracted relatively quickly at pH 7. It is known that the drop rise and fall velocities are high in a Graesser contactor, thus a fast interfacial renewal is achieved, leading to good mass transfer characteristics (3, 6, 25, 27). It seems that the different drop and contactor hydrodynamics compared to the stagnant interface in the Lewis cell has led to very different mass transfer characteristics in the Graesser contactor for different proteins.

The pH dependency pattern of lysozyme transfer did not change as much, and this might be due to the different ways lysozyme (26) and cytochrome *c* (1) are extracted by the reverse micelles, with lysozyme interacting strongly with AOT molecules.

Influence of Mixing Speed on Mass Transfer

Figure 5 shows the forward extraction results obtained from a Graesser contactor operated at pH 10 and 4°C with different rotor speeds. For all rotor speeds lysozyme was extracted faster than cytochrome *c*; however, only for the slow rotor speeds of 2 or 3 rpm would a kinetic separation of the two proteins be possible. The relationship between the extraction rate and the rotor speed was not linear, and doubling the rotor speed from 3 to 6 rpm caused an approximate tenfold increase in the extraction rate of cytochrome *c* (based on percent protein extracted within the first 12 minutes). Since droplet size in a Graesser is related to rotor speed by $\text{RPM}^{-0.5}$ (3), the increase in droplet size (and mass transfer area) with rotor speed would not explain the tenfold increase in extraction rate. Hence it seems possible that the rapid interfacial renewal of the drops may displace any precipitated protein from the droplet in-

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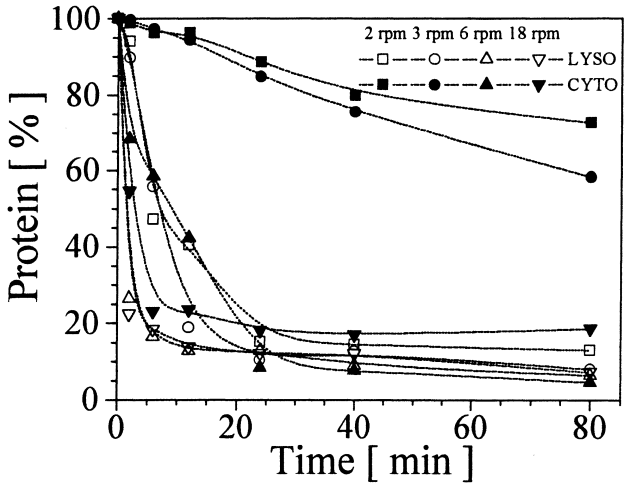

FIG. 5 Simultaneous forward extraction of lysozyme (LYSO) and cytochrome *c* (CYTO) in a Graesser contactor at different rotor speeds, pH 10, and 4°C.

terface, leading to rapidly enhanced rates of mass transfer at higher mixing speeds.

Influence of Temperature on Mass Transfer

Measurements of the extraction rate over time in a Graesser contactor at 3 rpm and pH 10, and at different temperatures, are presented in Fig. 6. The extraction rates of lysozyme and cytochrome *c* differed most at low tempera-

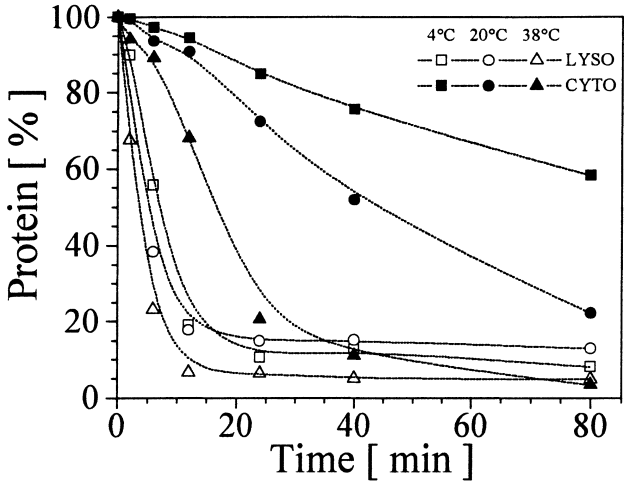

FIG. 6 Simultaneous forward extraction of lysozyme (LYSO) and cytochrome *c* (CYTO) in a Graesser contactor at different temperatures, pH 10, and 3 rpm.

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tures. With increasing temperature this difference became much less pronounced, and at 38°C cytochrome *c* extraction was almost as fast as for lysozyme at 4°C. The forward extraction equilibria of both proteins were very much the same for all the temperatures investigated, with an extraction yield of 92–98%, indicating that possible conformational changes did not affect phase distribution. Temperature influences a number of properties of the system, including both viscosity and interfacial tension, and this in turn could alter the holdup of the dispersed phase. Since droplet size, and hence mass transfer area, is a function of $(\text{holdup})^{0.8}$ and $(\text{surface tension/viscosity})^{0.5}$ (3), increasing temperature should increase mass transfer, but not by as much as appears from the results. One possible explanation for the stronger effect of increased temperature on the extraction kinetics of cytochrome *c* could be that hydrophobic interactions become more important than electrostatic interactions, although both should be weaker at higher temperatures. Hence it not clear at this point why temperature has such a strong influence on the kinetics of cytochrome *c* extraction.

Forward Extraction of Proteins from Diluted Hen Egg White in a Graesser Contactor

Kinetic Separation of Lysozyme from Hen Egg White

The selective reverse micellar extraction of proteins from simple buffer solutions is in many cases relatively easy to accomplish provided important properties such as the pI and molecular weight of the target protein, and also the other proteins present, are known. It has been demonstrated that under certain conditions selective extraction of a protein from more complex protein solutions, for instance, whole fermentation broth, is possible (12). However, the possibility of separating similar proteins based on their extraction kinetics appears to be a useful technique. Since the Graesser contactor performed well with an aqueous buffer solution for the kinetic separation with little tendency to form emulsions, we investigated the performance of the Graesser contactor for the reverse micellar extraction of lysozyme from hen egg white (17). Cytochrome *c* was added to diluted egg white to test whether the impurities in the hen egg white would influence the extraction kinetics and hence the ability to separate cytochrome *c* and lysozyme. We found that the extraction kinetics of lysozyme from egg white were very similar to the extraction from pure buffer under the same conditions of temperature, rotor speed, and pH (Fig. 7). Cytochrome *c* extraction, however, was found to be considerably faster compared to the buffer system. The kinematic viscosity of 15-fold diluted hen egg white was only slightly higher (1.60×10^{-6} m^2/s) than the buffer solution (1.45×10^{-6} m^2/s), but this should affect the extraction of all proteins present in the mixture. Hence, we think that a higher viscosity is unlikely to be re-

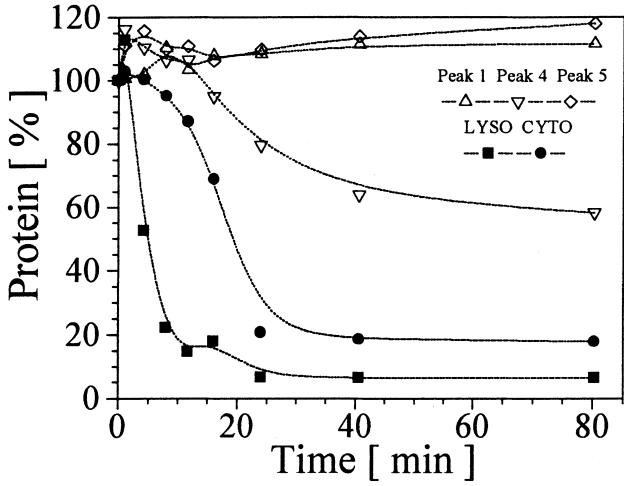

FIG. 7 Forward extraction of proteins from 15-fold diluted hen egg white with 1 g/L cytochrome *c* added in a Graesser contactor at 4°C, pH 9, and 3 rpm; LYSO = lysozyme; CYTO = cytochrome. Peaks 1, 4 and 5: see Fig. 8.

sponsible for the observed effect. The Graesser contactor was almost totally filled with an emulsion after 80 minutes of operation, and only a small amount of clear top phase remained. Only after the contactor was left on the bench for around 2 days did the top phase and bottom phase become clear again, with a thin precipitate at the interface. Figure 8 shows that the added cytochrome *c*

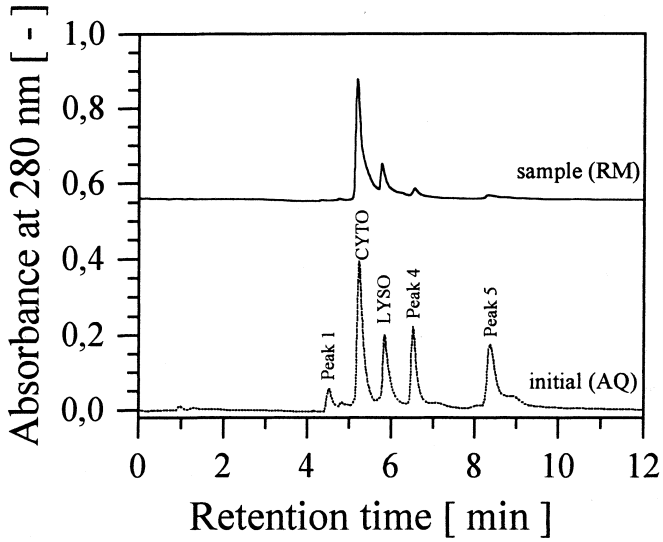

FIG. 8 HPLC chromatograms of the initial 15-fold diluted hen egg white used and the proteins found in the reverse micellar phase after 80 minutes of extraction time. AQ = aqueous phase sample; RM = reverse micellar phase sample; LYSO = lysozyme; CYTO = cytochrome.

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and the lysozyme were extracted with a high degree of purity. Only a few low strength impurities were found in the reverse micellar phase, and protein 4, which disappeared from the aqueous phase (Fig. 7) but did not appear in the reverse micellar phase, must have precipitated at the interface.

CONCLUSIONS

Extraction experiments in a Lewis cell showed that the mutual effect on the overall mass transfer coefficient, k_0, of simultaneously extracted lysozyme, cytochrome *c*, and ribonuclease A at pH 5.5 was statistically significant, and in some cases quite large. At present the reasons for this are unclear. However, it was found that the presence of cytochrome *c* or ribonuclease A helped to prevent lysozyme precipitation at the interface. Addition of cytochrome *c* altered the pH dependency pattern of lysozyme, and the presence of lysozyme reduced the k_0-value of cytochrome *c*.

The pH dependency pattern found for cytochrome *c* extraction in a Lewis cell compared to extraction in a Graesser contactor changed noticeably, whereas the pH dependency of lysozyme changed only marginally. This may have been caused by the different ways lysozyme and cytochrome *c* are extracted by reverse micelles and where they are physically located within the reverse micelle, with lysozyme interacting more strongly with the AOT molecules, and cytochrome c partitioning into the nonpolar tail region. Lysozyme extraction was slower than cytochrome *c* in a Lewis cell. Nevertheless, for all the experiments conducted in the Graesser contactor, lysozyme was extracted faster than cytochrome *c*. This indicates that different steps of the reverse micellar transfer mechanism in this contactor type and a Lewis cell are controlling the transfer of cytochrome *c*. Moreover, lysozyme extraction seems to be governed mainly by hydrophobic interactions with the surfactant AOT, whereas cytochrome *c* extraction seems to depend more strongly on electrostatic interactions. This interaction depends on the experimental conditions, so that at low ionic strength and a pH far from the pI, extraction is controlled by hydrophobic interactions (21). Nevertheless, it may be that the extraction is controlled more by ion exchange, and this should be looked at in more detail.

The use of a Graesser contactor to mix a reverse micellar phase and a buffer solution of lysozyme and cytochrome *c* allowed the kinetic separation of these proteins. This was achieved best at low mixing speed, low temperature, and a pH close to the pI of both proteins where the extraction rate of cytochrome *c* was much lower than of lysozyme. High mixing speeds caused more emulsification, but stable emulsions were not formed, and usually phase separation was achieved in less than an hour under quiescent conditions.

In general, the technique of separating lysozyme and cytochrome *c* kinetically can be transferred from a buffer system to the more realistic situation of

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using hen egg white as a natural source for lysozyme. The extraction kinetics of lysozyme were as fast as in a buffer system, but added cytochrome *c* was extracted faster, making the kinetic separation from this source more difficult. A slower rotor speed and an even lower temperature should help to optimize such a task. Another problem which needs to be studied in more detail is emulsion formation during extraction, which was more pronounced with hen egg white solution; consequently, phase separation took much longer.

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