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CONTINUOUS FORWARD AND BACK EXTRACTION OF LYSOZYME FROM EGG WHITE USING REVERSE MICELLES

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CONTINUOUS FORWARD AND BACK EXTRACTION OF LYSOZYME FROM EGG WHITE USING REVERSE MICELLES

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ABSTRACT

Gentle mixing characteristics in a Graesser contactor can avoid the formation of stable emulsions, and this is one advantage of this contactor when used with reversed micellar extractions. However, this characteristic of the contactor is a disadvantage for back extraction using the conventional method (extremes in pH and ionic strength) because it gives a low extraction yield and requires a larger contactor. In order to develop an integrated process for continuous protein separation using reverse micelles, this work focused on assessing the novel technique of back extraction using a counterionic surfactant in a mixer-settler. It was found that the process gave a high extraction yield (95–100%) within an extraction time of only 5 min. Lower mixing speeds (200 rpm) and pHs (6) resulted in the highest sedimentation rate in the organic phase. Unfortunately, the cloudiness of the aqueous phase and the slow rate of lysozyme transfer into the aqueous phase in the settler were potential drawbacks of the system. However, the use of centrifugal

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force was found to be an important factor in enhancing the rate of back extraction when using a counterionic surfactant. Based on previous work of ours on forward extraction in a Graesser contactor, the continuous separation and recovery of proteins using reverse micelles seems feasible.

INTRODUCTION

In downstream separation in biotechnology, liquid-liquid extraction of proteins from fermentation broth using reverse micelles is being seriously considered because it has the potential to separate, concentrate, and purify continuously and can be easily scaled up. The use of many liquid-liquid contactors with reverse micellar systems has been evaluated in the literature, such as mixer-settlers (1), centrifugal contactors (2), membranes (3,4,5), liquid membranes (6,7), spray columns (8,9), packed columns (10), and rotating disc contactors (11,12). However, most of these contactor designs suffer from a variety of serious drawbacks such as emulsion formation due to high shear, formation of stagnant droplets with poor mass-transfer performance, low interfacial mass-transfer areas, and low membrane fluxes due to slow pore diffusion. Ultimately, all these problems led to poor mass-transfer performance and low volumetric efficiencies. Another appropriate contactor design that has been used in other process industries in the past, but only sparingly in biotechnology (13,14), and has the potential to ameliorate many of the problems mentioned is the Graesser ("Raining Bucket") contactor.

The Graesser contactor consists of a single rotor operating on a horizontal axis in a cylindrical shell. The rotor is comprised of a series of circular discs between which are mounted a sequence of cylindrical buckets around the axis that are partly open in the direction of mixing. The two liquid phases flow countercurrently through an annular gap between the edge of the rotor and the inside of the shell, whereas the interface is maintained at the centreline of the vessel. The rotor is turned fairly slowly (2–8 rpm) so that the buckets continuously fill with each phase and then discharge it in the form of a sheet/large drops into a continuous medium of the other phase where it eventually merges with the mother phase at the interface. Therefore, the unit has the unusual feature of dispersing each liquid phase into the other giving a two-way upward and downward raining action. The fact that the drops that are eventually formed by the natural breakup of the stream leaving the buckets are relatively large, means they tend to be oscillating, thus promoting interfacial renewal and good mass transfer (15). In addition, the gentle rotation avoids the formation of emulsions.

The mass-transfer characteristics of lysozyme during forward extraction using reverse micelles in the Graesser contactor has been investigated in a previ-

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ous study (16). The concentration profiles of lysozyme in both the aqueous and organic phases showed that significant axial mixing occurred in the contactor. In addition, the extraction of lysozyme in the Graesser contactor can be described by the diffusion model. The optimal conditions for steady-state mass transfer for extraction were found to be a low rotor speed and a high aqueous-organic phase flow rate ratio, and rotor speed had more effect on mass-transfer performance than aqueous flow rate, or total throughput.

Most of the contactors studied for reverse micellar extraction have looked at forward extraction (6,8,10,12), whereas only two studies have evaluated back extraction at a large scale using a centrifugal contactor (17), and a packed column (9). In addition, only a few papers have examined continuous forward and back extraction (1,17,9).

In previous work of ours (16), continuous back extraction using conventional methodology (extremes in pH and ionic strength) was evaluated in the Graesser contactor. Although extraction was achieved, long contact times were required, and this still resulted in a low extraction yield. In order to develop an integrated process for protein separation, this work focused on assessing an alternative back-extraction technique using counterionic surfactant (18) in a mixer-settler. The operating parameters investigated were rotor speed, agitation time, and retention time. Finally, the continuous forward and back extraction of lysozyme was carried out in a Graesser contactor (forward extraction) and the mixer-settler (back extraction).

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MATERIALS AND METHODS

Bis(2-ethylhexyl)sulfosuccinate sodium salt (AOT) and 2,2,4-trimethylpentane (isooctane) were purchased from Sigma, and Trioctylmethylammonium chloride (TOMAC) was obtained from Aldrich. Lysozyme (pI = 11) was extracted from hen egg white, which was separated from the yolk of fresh eggs, and all other chemicals were purchased from Sigma and were of analytical grade.

Forward Extraction in a Graesser Contactor

The Graesser contactor employed in this study was the same as in previous work (16), as shown in Fig. 1. The procedure for the forward extraction using AOT/isooctane in the Graesser contactor is described elsewhere (16), and the optimal operating conditions were found to be a rotor speed of 5 rpm and an aqueous-organic phase flow rate of 60:20 mL/min. The AOT concentration used was 40 mM.

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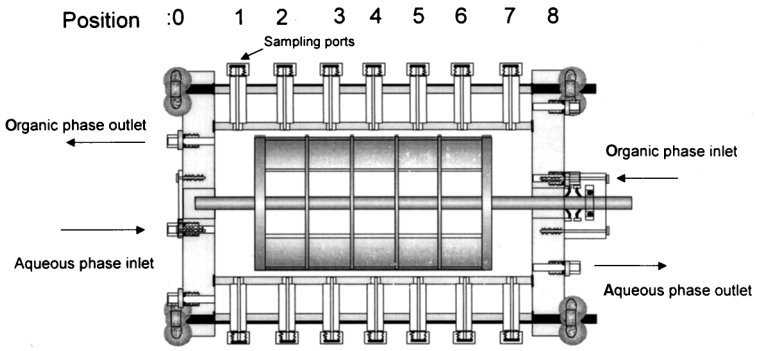

Figure 1. Schematic diagram of a Graesser contactor.

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Back Extraction in a Mixer-Settler by Adding TOMAC

Batch Operation

The mixer, as shown in Figure 2, was a stirred glass beaker (7.5 × 11 cm) with four baffles. Stirring was carried out using a turbine impeller of 4 cm diameter. 80 mL of buffer solution was added to the vessel followed by 3.75 mL of 1 M TOMAC solution. 80 mL of the organic phase of lysozyme encapsulated in reversed micelles (2.3–2.4 g/L) was then poured into the vessel, which should dilute the TOMAC solution to 40 mM, and the agitator started immediately at the required speed and time. After mixing, the mixture was poured into another 400 mL beaker (7.5 × 11 cm). The separation rate was measured by recording the height of the interface in each phase over time. The effect of agitation time, rotor speed, and pH of the buffer solution was studied using the overall mass-transfer coefficient, extraction yield, and separation rate as indicators. All samples were centrifuged at 12,000 rpm for 2 min.

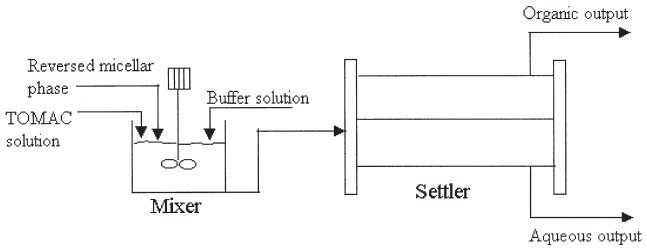

Figure 2. Schematic diagram of the mixer-settler setup used.

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Continuous Operation

This operation began in the same way as the batch system, but after 5 min a continuous feed was started. The input streams consisted of buffer solution, reverse micellar phase (40 mM AOT), and TOMAC solution (in a 20:20:1 ratio), and total flow rates of 20 and 40 mL/min were used in this study. After finding the optimum conditions for the mixer, the output stream was connected to a settler, which was a cylindrical vessel (9.2 × 20 cm). A pH 6 buffer solution was used as the aqueous phase, and the rotor speed was fixed at 200 rpm. All samples were centrifuged at 12,000 rpm for 2 min, whereas water content of the samples was measured using Karl-Fisher titration (18).

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Continuous Forward and Back Extraction

The forward extraction of lysozyme from hen egg white was performed in the Graesser contactor by using optimum conditions, as described above. The output of the reversed micellar phase for the first 40 min was collected in a 1-liter conical flask in order to allow a mild emulsion to separate. The extracted protein in the reverse micellar phase was then back extracted in the mixer by adding TOMAC under optimal conditions. Finally, the outlet from the settler was sampled from 100 min onwards. The extraction yield in both the forward and back extraction steps was then examined over time at steady state.

Turbidity Measurement

The turbidity or cloudiness of the aqueous phase after mixing was measured with a spectrophotometer (Shimadzu UV2101) at 660 nm.

Overall Mass-Transfer Coefficient

The overall volumetric back extraction mass-transfer coefficient ($k_b a$) was calculated from an equation based on the two-film theory (19):

$$\ln((C^0_{rm} - (1 + m)C_{aq})/C^0_{rm}) = -(1 + m)(k_b a)t \quad (1)$$

where C^0_{aq} and C^0_{rm} are the initial protein concentrations in the aqueous and organic phases (g/l), respectively; C_{aq} and C_{rm} are the protein concentrations in the aqueous and reverse micellar phases (g/l), respectively; m ($= C^*_{rm}/C^*_{aq}$) is the partition coefficient of protein between both phases at equilibrium and was found

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to be 15.5 (16); k_b is the overall mass-transfer coefficient for back extraction; a is the specific surface area (m^2/m^3); and, t is the time (s).

Phase Separation Rate

The phase separation rate was calculated based on gravity settling. By assuming an exponential settling curve, the correlation between time and height was given by Eq. (2) (20).

$$t = \frac{1}{K_{1B}} \ln\left(\frac{h_o}{h}\right) + \frac{1}{K_{2B}} (h_o - h) \tag{2}$$

where h_o = initial height of each phase (mm).
h = height of each phase at time t (mm).
K_{1B} = constant in reciprocal relation for batch settler (1/s).
K_{2B} = constant in reciprocal relation for batch settler (m/s).

A multiple linear regression was then used to determine the K_{1B} and K_{2B}.

RESULTS AND DISCUSSION

Batch Operation

Effect of Mixing Speed and Agitation Time

Figure 3 shows the effect of agitation speed and time on the extraction yield. It is obvious that the extraction rate at high mixing speeds was faster than at low speed, as indicated by the volumetric overall mass-transfer coefficient for back extraction ($k_b a$) in Table 1. The extraction rates for all samples reached steady state within 5 min, consistent with the results of back extraction in test tubes (18). Therefore, 5 min of agitation should be suitable for back extraction in the mixer.

For the phase separation rate, it was found that the rate after high-speed mixing was slower than after low-speed mixing in both the aqueous and organic phases, as indicated by the phase boundary heights of sedimentation over time in Fig. 4. The figure indicates that the coalescence rate in the aqueous phase was greater than the sedimentation rate in the organic phase, resulting in a separation time of about 20 min in the aqueous phase and 1200 min in the organic phase. The profiles were assumed to be exponential, and hence the separation rate was determined from Eq. (2), and the results are shown in Table 1. At high speed, the smaller droplets and the presence of surfactants make the separation time in the organic phase longer because both AOT and TOMAC strongly partition into the organic phase. The surfactants should strongly retard the coalescence rate of

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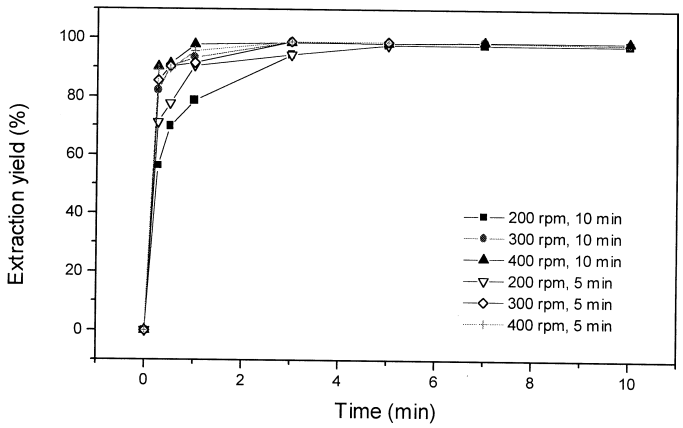

Figure 3. Effect of rotor speed and agitation time on extraction yield with 40 mM AOT.

two-phase liquid mixtures, causing the formation of stable emulsions (21). These results are in contrast with the forward extraction of lysozyme using AOT reverse micelles by mixing with a magnetic stirrer bar at 750 rpm, where separation under gravity was achieved within 10–15 min (22). This indicated that the mixture of AOT-TOMAC had the potential to form stable emulsions. Moreover, cloudiness in the aqueous phase, which is caused by an oil in water microemulsion, was encountered after phase separation. In contrast, this was not found in the test tube samples of the previous study (18) because centrifugation was used for phase separation.

Table 1. Effect of Agitation Time, Rotor Speed, and pH on Back Extraction in the Mixer

Condition	Agitation Speed (rpm)	$k_b a$, 1/s $\times 10^{-3}$	K_{1B}, 1/s (AQ) $\times 10^{-3}$	K_{1B}, 1/s (OR) $\times 10^{-4}$	Absorbance at 600 nm
pH 9,$^{10\ min}$	200	30.7	352.1	15.0	—
	300	56.7	41.9	1.4	—
	400	78.5	35.6	0.4	—
pH 9,$^{5\ min}$	200	44.5	361.0	59.0	0.38
	300	55.4	193.7	44.0	0.49
	400	69.9	68.6	3.0	0.50
pH 6,$^{5\ min}$	200	16.6	847.4	1.0	0.18
	300	18.6	239.2	0.2	0.32
	400	63.0	78.5	0.06	0.38

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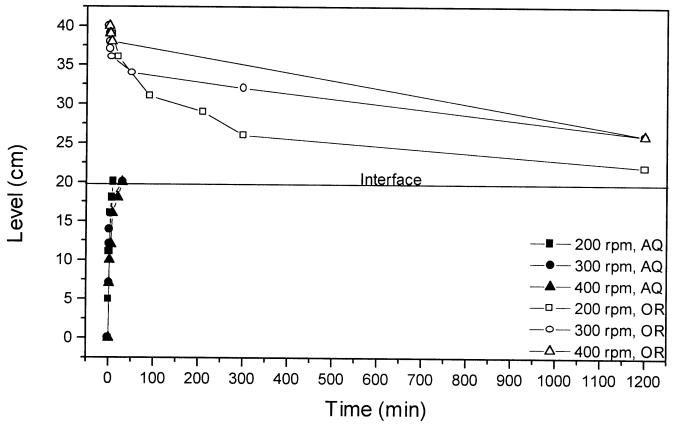

Figure 4. Separation time for each phase after a mixing time of 10 min at pH 9.

Effect of pH

Because the pH of the aqueous phase could affect the solubilization or formation of micelles in the aqueous phase (23), an acidic pH (pH 6) and basic pH (pH 9) were studied in order to reduce the turbidity of the aqueous phase. At the same agitation speed, the aqueous absorbance at 600 nm was used as an indicator of turbidity. It was found that at pH 6 samples were less turbid than at pH 9, as shown in Table 1. Moreover, the pH of the aqueous phase also affected the $k_b a$, (see Table 1), and the back extraction rates at pH 6 were slower than at pH 9. However, the extraction yields reached steady state within 5 min despite the slower rate of back extraction at pH 6. These results might be explained by the behavior of the phase diagram, which could change at different pHs resulting in the solubility of the surfactant complex altering, and this could also cause the formation of vesicles or micelles in the aqueous phase. In addition, at pH 6 the net positive charge on the protein surface should be higher than at pH 9, resulting in more electrostatic interactions between the surfactant and lysozyme and thus hindering the transfer of protein at low pHs.

The separation time for the aqueous phase was considerably faster than the organic phase at both pH 6 and 9, as shown in Figs. 5 and 6. The sedimentation and coalescence profiles were also assumed to be exponential, and the separation rates were determined as shown in Table 1. These results indicate that a more stable emulsion was created at pH 6 in the organic phase, which may also hinder the back-extraction rate as mentioned above. From these results, the optimal conditions for the mixer were determined to be a speed of 200 rpm and a mixing time of 5 min in pH 6 buffer solution.

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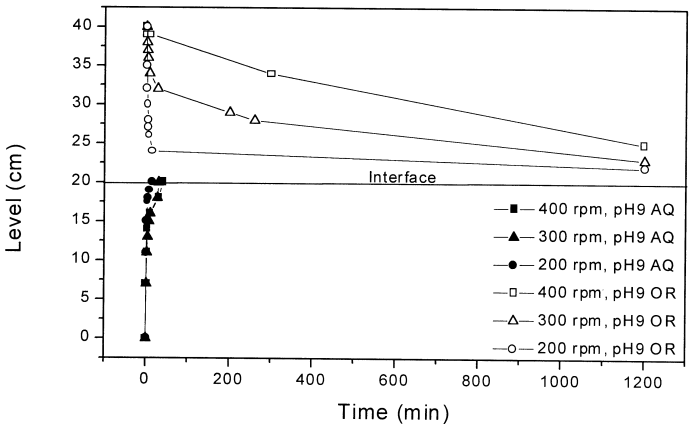

Figure 5. Separation time for each phase after a mixing time of 5 min at pH 9.

Continuous Operation

In order to study the effect of residence time (RT) and mixing on the extraction performance of the mixer, the same flow rates of the reverse micellar (RM) and aqueous (AQ) phases were investigated of 10 and 20 mL/min, which gave an RT of 8 and 4 min, respectively. Figure 7 shows the continuous performance of the mixer. After starting continuous operation (after 5 min), the performance as indicated by the extraction yield under all conditions was quite steady and gave a yield of 95–100%. Thus, a total flow rate of 40 mL/min (retention time of 4 min) for both phases should be used for this operation.

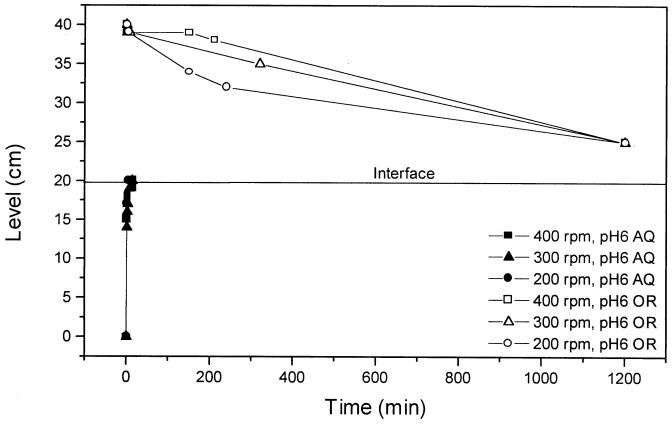

Figure 6. Separation time for each phase after a mixing time of 5 min at pH 6.

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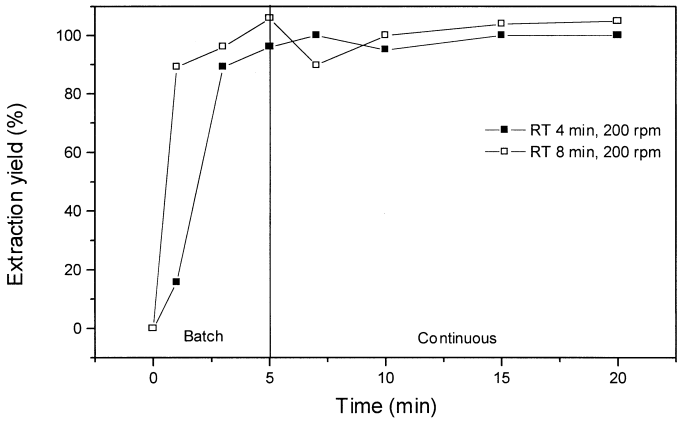

Figure 7. Performance of the mixer during continuous operation.

For the settler study, a cylindrical vessel was used that provided a retention time for each phase of about 30 min at an inlet flow rate of 40 mL/min. The coalescence rate was faster than the sedimentation rate, which was consistent with the study above. Therefore, there was no dispersion band, which usually occurs in a gravity settler. However, the organic phase contained a stable emulsion or dense-packed zone, whereas the outlet aqueous phase was clear (absorbance at 600 nm = 0.18). Figure 8 shows the operating results of the mixer-settler during continuous operation. The samples were taken from the aqueous phase outlet of the settler, and the extraction yields were in the range 62–66%. The low extraction yield

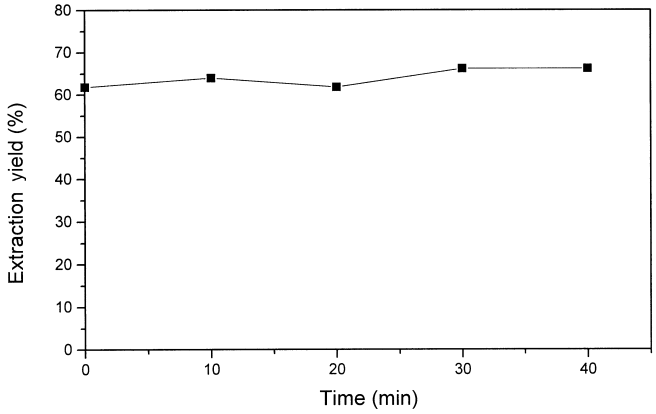

Figure 8. Continuous extraction yield from the mixer.

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Table 2. Effect of Centrifugal Force on the Extraction Yield and Water Content

	Centrifuge		No Centrifuge	
Sample	Extraction Yield (%)	H_2O Content/10 μl	Extraction Yield (%)	H_2O Content/10 μl
1	98.5	20.0	61.2	64.8
2	94.6	20.3	60.0	61.0
3	99.5	24.0	63.3	62.4
4	98.5	24.4	64.8	65.1
5	97.7	24.0	60.0	64.3

for back extraction was not encountered in the mixer experiment because these samples were centrifuged. The effect of centrifugal force should accelerate the rate of protein separation to the aqueous phase. Moreover, the presence of hydrophobic interactions between exposed hydrophobic amino acid residues and the surfactant tails due to the partial unfolding of lysozyme in the AOT system (24), may hinder the diffusion of the protein to the aqueous phase. Therefore, the protein concentration and the water content from the mixer samples that were not separated by centrifuge were examined and compared with the centrifuged samples, as shown in Table 2.

These results show that the centrifuge strongly influenced the extraction yield and water content. These results were confirmed by an examination of the protein concentration in the aqueous phase of the settler over time after stopping the feed from the mixer, as shown in Table 3. These results show that the protein in the organic phase was gradually released into the aqueous phase despite the fact

Table 3. Protein Concentration in the Aqueous Phase of the Settler

Time (h)	Extraction Yield (%)
1	64.0
2	74.7
3	80.2
4	80.8
5	82.8
6	86.7
7	88.2
8	89.5
9	90.5
18	98.0

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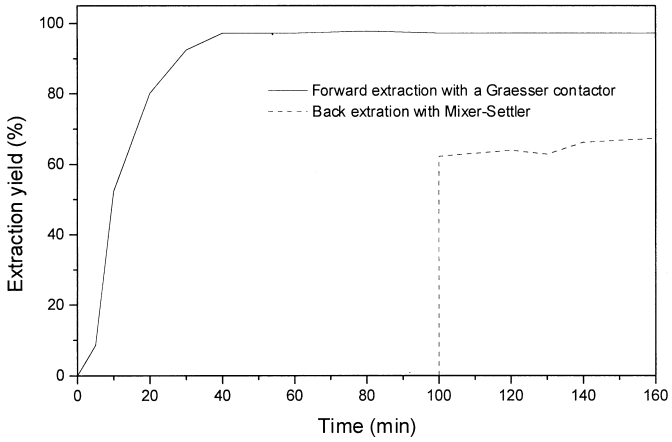

Figure 9. Continuous forward and back extraction of lysozyme from hen egg white.

that the two phases had clearly separated. Therefore, in order to achieve the same concentration as in the centrifuged sample, the settling time in the settler should be about 18 h, resulting in a very large settler (if operated in continuous mode).

Continuous Forward and Back Extraction

Figure 9 shows the results of the continuous forward and back extraction. The forward-extraction yield at steady state was in the range 93–98%, whereas the back-extraction yield was in the range 63–67%. However, as mentioned above, the mixer-settler may not be suitable for back extraction, and hence a centrifugal contactor may be a more viable alternative for this operation.

CONCLUSIONS

- Counterionic back extraction in the mixer-settler showed promising results with an extraction yield of 95–100% (after centrifugation), after an extraction time of 5 min. Lower mixing speeds (200 rpm) and lower pHs (6) resulted in the highest sedimentation rate in the organic phase, but had little effect on the coalescence rate in the aqueous phase.
- The cloudiness of the aqueous phase and the slow rate of lysozyme transfer into the aqueous phase were potential drawbacks of the system, which decreased the extraction yield to 63–67%. The effect of centrifugal force was found to be a vital factor in the back extraction by adding counterionic surfactant.

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- Continuous forward and back extraction of lysozyme using reverse micelles in a Graesser contactor and a centrifugal contactor, respectively, should be possible with high recovery.

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