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A biocompatible surfactant, methyl ester sulphonate (MES), as a precipitating ligand for protein purification

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ABSTRACT

A biocompatible surfactant, methyl ester sulphonate (MES), was evaluated in protein surfactant precipitation. The amount of lysozyme precipitated by MES was found to be a strong function of both the molar ratio of surfactant to protein (R_p), and pH. Precipitation increased proportionally with an increase in R_p up to an optimum of 16, where full precipitation was achieved, while with pH variations electrostatic interactions were found to be the main driver of precipitation. The precipitate was recoverable by solvent extraction (54–56% activity recovery by acetone, ethanol, 50% acetone/ethanol, and methanol), and counter-ionic surfactant, TOMAC (83.3% activity recovery at $R_r = 1.5$). Importantly, the structural integrity of the lysozyme recovered, either through solvent extraction or counter-ionic surfactant, was maintained, and this was confirmed by CD spectra and deconvolution. Precipitation with MES was compared to a conventional surfactant, AOT; in contrast with AOT, MES did not resolubilise the precipitate or cause structural transformations at higher R_ps. MES exhibited better selectivity than AOT since it had minimal hydrophobic interactions in the absence of electrostatic interactions between the surfactant's anionic group, and the protein's positively charged groups (pH > pI). Hence, along with its economic benefits, and environmental features, this work has highlighted the potential of using MES in surfactant precipitation.

1. Introduction

Consumer demand for specialty biotechnology products has driven the need for a more cost effective biomanufacturing process. In addition, downstream processing (DSP) of biomolecules is a major challenge which contributes up to 70% of the overall production cost [1,2]. Although conventional purification methods (e.g. chromatography) are known to have a high degree of purification, they also have many drawbacks, e.g., high costs, long processing times, tedious, complex and non-reproducible operation [3]. Therefore, the current trend in DSP is to look for alternative purification methods which are cheaper and simpler than conventional methods, but with comparable performance. In this context, new approaches like liquid-liquid extraction-based methods, namely reverse-micellar extraction, and aqueous two-phase have been proposed. However, there are also several disadvantages with these techniques, such as the degree of product loss, high cost, and complexity/unpredictability of the system [4–7].

Meanwhile, the potential of surfactant precipitation for protein recovery has been extensively reported recently [4,6,8,9–11,12]. Its simplicity, scalability, relative ease of use, high selectivity, not to mention its combined concentration, and purification advantages, has made it appealing in DSP. Work on surfactant precipitation based on the use of conventional surfactants has been substantial [8,9,11,13]. The most important finding was that the charge of the ionic surfactant's head group is central to precipitation, and this explains why conventional ionic surfactants such as: anionic (AOT) and their cationic counterparts (TOMAC, DODMAC, DTAB and CTAB) have been successfully applied.

However, the detergent production industry is focussing on the production of a biobased surfactant due to raised concerns about environmental sustainability [14]. One example of such a detergent is methyl ester sulphonate (MES), a palm oil-derived surfactant [15,16]. The two main features of MES that appeal to the detergent industry are its higher detergency, and environmentally friendly structure. Thus, the overall consumption cost is reduced signifi-

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Notation

a_f	Lysozyme activity in the final phase after recovery
a_i	Lysozyme activity in the initial phase before precipitation
AOT	Sodium bis-(2-ethylhexyl) sulfosuccinate
C_e	Concentration of lysozyme at equilibrium after formation of the precipitate Concentration of lysozyme at equilibrium after formation of the precipitate
C_f	Concentration of lysozyme in the final phase
C_i	Concentration of lysozyme in the initial phase
C_w	Concentration of lysozyme in the wash fraction
CD	Circular dichroism
CMC	Critical micelle concentration
COV	Coefficient of variance
CTAB	Cetyl trimethylammonium bromide
DODMAC	Dimethyl dioctadecylammonium chloride
DMSO	Dimethyl sulfoxide
DSP	Downstream processing
DTAB	Dodecyl trimethylammonium bromide
MES	Methyl ester sulphonate
pI	Isoelectric point
R_p	Molar ratio of surfactant to protein in precipitation stage
R_r	Molar ratio of counter-ionic surfactant to precipitating surfactant in recovery stage
TOMAC	Trioctyl methylammonium chloride
V_e	Volume of the equilibrium fraction after precipitation
V_f	Volume of the final phase after recovery
V_i	Volume of the initial phase before precipitation
V_w	Volume of the washing fraction

cantly as MES has higher detergency at a lower dose in comparison with linear alkyl benzene sulphonate [17]. From an environmental point of view, MES is also 'greener' than conventional surfactants because it is carbon neutral, results in lower organic effluents due to its lower dosage during washing, and exhibits a higher biodegradability [18]. In addition, MES has shown good compatibility with protease, and has no adverse effects on enzyme activity [19]. Hence, coupled with its anionic properties, it appears that MES might be appropriate to use in surfactant precipitation.

The first aim of this study was to examine the feasibility of using MES in surfactant precipitation. Secondly, to assess its performance compared to the conventional surfactant, AOT. Lysozyme was used as a model protein in a buffer system, and its performance in both the precipitation and recovery stages was examined. The influence of various parameters such as: the molar ratio of surfactant to protein (R_p), pH, and type of organic solvent on the process, as well as the possibility of using a counter-ionic surfactant to recover the lysozyme were examined. Finally, the structural integrity of the target protein was also analysed after recovery.

2. Materials and methods

2.1. Materials

Lysozyme from chicken egg white (EC 3.2.1.17, Mucopeptide N-acetylmuramylhydrolase, pI = 11.0, MW = 14.3 kDa) was from Sigma (Missouri, USA). Anionic surfactants (see Fig. 1): sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) (MW = 444.56 g/mol) were obtained from Sigma (UK), and, methyl ester sulfonate (MES), MIZULAN FL-80 (MW = 372–400 g/mol) was obtained from the LION group (Malaysia). *Micrococcus lysodeikticus* (ATCC No. 4698) for determination of lysozyme activity was purchased from Sigma.

2.2. Preparation of lysozyme solution

0.1 g of lysozyme powder was dissolved in 10 mL of 20 mM potassium dihydrogen phosphate/sodium phosphate dibasic, pH 6.23 to act as the stock solution. 1 mL of the stock solution was then diluted to a volume of 10 mL in a 15 mL tube to make up a 1 g/L solution that was used as a starting solution for the precipitation experiments.

2.3. Preparation of anionic surfactant, AOT phase

AOT was prepared by dissolving it in distilled water with a concentration range of 1.7–26.6 mM. When adding AOT to the protein sample, the final concentration ranged from 0.15 to 2.42 mM, and therefore AOT was present as a monomer as the critical micelle concentration (CMC) of AOT in water is 2.5 mM in 20 mM phosphate buffer; 4.1 mM in water at 25 °C [20], and increases to 6.7 mM with lysozyme prepared in buffer (between 0.03–0.2 mol%).

2.4. Preparation of anionic surfactant, MES phase

MES flakes were dissolved in distilled water to make up the same concentration range as AOT (1.7–26.6 mM) in order to facilitate the performance comparison of both surfactants.

2.5. Preparation of counter-ionic surfactant, TOMAC phase

TOMAC was dissolved in ethanol (due to its low solubility in distilled water) at various concentration ranges, from 11.18 to 33.54 mM, to make up the different molar ratios of TOMAC to MES used in the recovery (1–3). Its final concentration in solution ranged from 1.02 to 3.05 mM.

2.6. Determination of MES critical micelle concentration

The CMC of MES was determined by using the conductivity method. Gradual addition of the MES solution into a 20 mM phosphate buffer was made, and the conductivity measured; the conductivity was then plotted as a function of MES concentration. The coefficient of variance (COV) of the measurement was <5%.

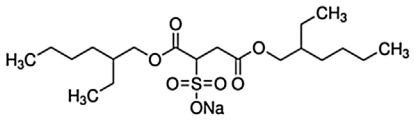
2.7. Precipitation procedures

2.7.1. Precipitation of lysozyme with AOT/MES

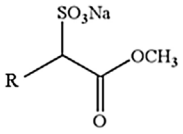
10 mL (1 g/L) of lysozyme solution was contacted with 1 mL of surfactant solution (AOT or MES), followed by 10 s of vortex mixing; an insoluble complex formed immediately. The sample was then centrifuged at 7500*g* for 10 min at 4 °C to separate the precipitate from solution, and the supernatant collected and analysed for protein content and enzyme activity. The amount of protein left in solution indicated the amount of protein precipitated by mass balance. The surfactant-protein precipitate was collected and the protein recovered with either solvent or counter-ionic surfactant.

2.7.2. Effect of pH on the precipitation of protein

To study the effect of pH on lysozyme precipitation by MES, lysozyme was initially dissolved (1 g/L) in a pH-specified 20 mM phosphate buffer ranging from pH 6 to 12. Lysozyme solution at all the pHs was used as the negative control for each pH examined. 1 mL of MES solution was later added to 10 mL of these single-protein solutions, to result in an R_p of 16. The pH study was carried out beyond the active pH range of lysozyme (at 6–9, as specified by product manufacturer, Sigma) up to 12, so as to evaluate the



i) Sodium bis-(2-ethylhexyl) sulphosuccinate (AOT) (MW=444.56 g/mol)



ii) Methyl ester sulphonates (MES) (MW=372-400 g/mol)

Fig. 1. Chemical structure of AOT and MES. Position of linear alkyl chain: in AOT, the hydrophilic group is flanked by a double hexyl chain with an ethyl group branched at position 2. In contrast, in MES the anionic head group is tailed by a single linear alkyl chain, with a 14–16 carbon chain length.

effect of electrostatic interactions between MES and the lysozyme molecule, which has a pI of about 11.

2.8. Recovery procedures

All the recovery studies were performed with optimally (R_p = 16) precipitated lysozyme. The protein was recovered from the precipitate by means of both organic solvent addition, and counter-ionic surfactant (TOMAC). Apart from determining overall protein/activity recovery, protein recovery efficiency and activity recovery efficiency were also determined to examine the effectiveness of the recovery of lysozyme from the precipitate. The efficiency of protein recovery was calculated from:

$$\text{Protein Recovery Efficiency}(\%) = \frac{C_f \cdot V_f}{C_i \cdot V_i - C_e \cdot V_e - C_w \cdot V_w} \times 100\% \quad (1)$$

where C_f, C_i, C_e and C_w refer to the concentration of lysozyme in the final phase, in the initial phase, at equilibrium after formation of the precipitate, and in the wash fraction, respectively. Meanwhile, V_f, V_i, V_e and V_w refer to the volume of the final phase, initial phase, equilibrium fraction after precipitation, and the washing fraction, respectively. The COV of the measurement was ±16.4%, and the efficiency of activity recovery was calculated as:

$$\text{Activity Recovery Efficiency}(\%) = \frac{a_f \cdot V_f}{a_i \cdot V_i - a_e \cdot V_e - a_w \cdot V_w} \times 100\% \quad (2)$$

where a_f, a_i, a_e and a_w refer to lysozyme activity in the final phase, in the initial phase, in the equilibrium fraction after precipitation, and in the washing fraction, respectively. The COV of this measurement was ±24.5%.

2.8.1. Recovery of lysozyme from a lysozyme-MES complex by solvent

The precipitate was washed with 10 mL of phosphate buffer, followed by 10 s of vortex mixing, and eventually centrifuged at 7500*g* for 10 min. The supernatant (wash fraction) was collected and further analysed for protein concentration and activity measurement. 10 mL of solvent was then added to the pellet, vortexed for 10 s, and then subjected to a second centrifugation. The effect of 6 solvents, namely; acetone, ethanol, acetone-ethanol (50% v/v), methanol, chloroform and dimethyl sulfoxide on recovery was examined. After addition of the solvent, the surfactant (MES) dissolves into the organic phase, while the lysozyme remains as a precipitate. The supernatant (solvent) was then removed and the surfactant-free precipitate dissolved in a fresh phosphate buffer at pH 6.2 before being analysed. Filtration through a 0.45 μm membrane was used to remove any undissolved precipitate.

2.8.2. Recovery of lysozyme from a lysozyme-MES complex by counter-ionic surfactant

The cationic surfactant, TOMAC, was used for counter-ionic surfactant recovery of lysozyme from the insoluble lysozyme-MES complex. After the addition of 10 mL of fresh phosphate buffer to the recovered precipitate, 1 mL of TOMAC-containing ethanol with various concentrations (11.18–33.54 mM) was added to the final phase; TOMAC was pre-dissolved in ethanol as it was not soluble in water. The mixture was later vortexed for 10 s to promote interactions between the anionic surfactant, MES, and its cationic counterpart, TOMAC, to form a MES-TOMAC dimer complex, while leaving lysozyme in solution. The sample was then subjected to centrifugation (7500*g*, 10 min) to remove the complex, and the supernatant was then analysed for protein concentration and activity.

2.9. Analytical techniques

2.9.1. Protein assay

The protein concentration in the initial lysozyme solution, aqueous phase after precipitation, wash fraction of the insoluble complex, and the final phase after recovery were determined by measuring their absorbance at 280 nm. The spectrophotometer was calibrated with 20 mM phosphate buffer (pH 6.23) used in the precipitation experiment. The protein concentration was determined from a standard curve of lysozyme, with concentrations ranging from 0 to 1.0 g/L (R^2 = 0.99). The lysozyme standards were also prepared in the same phosphate buffer. The COV of the measurement was ±15%.

2.9.2. Lysozyme activity assay

The activity of lysozyme was determined based on the enzymatic lysis rate of a *Micrococcus lysodeikticus* cell suspension by lysozyme [21]. The cell suspension was prepared in 20 mM phosphate buffer (pH 6.23) with an absorbance at 450 nm of between 0.6–0.7 versus a buffer blank. $2\frac{1}{2}$ mL of the substrate was loaded into the two reference Cells A and B, and 100 μL of buffer and lysozyme-containing sample were added to the substrate in cells A and B, respectively. After immediate mixing by inversion, the decrease in the substrate turbidity at 450 nm was recorded for 5 min. The rate (A450 nm/min) was determined from the linear curve of the graph of absorbance (A450 nm) as a function of time. The linear portion chosen was over at least a one minute interval.

The specific activity was calculated as:

$$\text{Specific Activity}(\text{units/mg}) = \frac{\Delta A_{450\text{nm}}/\text{min}}{\text{mg lysozyme in reaction mixture}} \left(\frac{1\text{U}}{0.001}\right) \quad (3)$$

The COV of the measurement was ±15%.

2.9.3. Determination of the MES concentration

MES concentration was determined using a cationic dye, methylene blue, which forms a complex with any anionic surfactant [22,23]. 1 mL of 0.007% of a methylene blue solution prepared in 0.1% sodium sulphate solution was added to 5 mL of chloroform and mixed. 100 μL of sample was then added to the initial mixture and vortex mixed for 20 s. The bottom phase (chloroform) was separated from the water phase and analysed for MES concentration by spectrophotometer at a wavelength of 650 nm. The concentration of MES was determined based on a linear standard curve with concentrations ranging from 0 to 1 mM. The COV of the measurement was ±10%.

2.9.4. Determination of circular dichroism

The concentration of the protein sample in solution was diluted to 0.1 g/L in 20 mM potassium phosphate buffer, or immediately analysed if the original sample was below this concentration prior to the CD measurement. The sample was scanned over a far-UV wavelength range of 190 to 260 nm at 20 °C in a thermostated cell holder. The scan speed was set at 10 nm/min with path length of 1 mm, step resolution of 0.5 nm and a bandwidth of 1 nm. The phosphate buffer used was set as the baseline for the measurement to determine the spectra of the protein. The signal was presented as molar ellipticities [θ] based on a mean molecular mass per residue of 129 Da. The spectra were analyzed by using deconvolution software (CDNN program version 2.1), where the software calculates the secondary structure of the protein by comparing it with a base set of 13 known protein structures. The COV of the measurement was ±5%.

3. Results

3.1. Effect of R_p on the precipitation level

Precipitation of protein by MES and AOT was examined, and their performances compared. Both surfactants were prepared in the concentration range such that an R_p of between 2.38 and 38 was possible. Fig. 2 shows the remaining fraction of protein, and activity in solution, after precipitation as a function of the molar ratio between MES and lysozyme (R_p). As the R_p was increased from 2.38 to 16, the precipitation percentage increased as indicated by the drop in the fraction of protein and activity (74.5%–0%). The fraction of activity also reduced accordingly from 71% (R_p = 2.38) to 0% (R_p = 16), at which point full precipitation of lysozyme was achieved. This complete level of precipitation for MES was found to be maintained up to an R_p of 38. It was also clear (Fig. 2) that the activity was in accordance with the remaining mass in solution after precipitation over the range of all the R_ps.

In contrast, in the case of AOT precipitation (Fig. 2), the ascending trend of precipitation as a function of R_p was only observed until its optimum R_p (16), before the level of precipitation reduced to about 87.5% (R_p = 29) and eventually to 33.4% (R_p = 38). The activity of lysozyme in the remaining solution was also found not to correspond to the mass of protein: 2.8% (R_p = 29) and 16.4% (R_p = 38), for respective protein fractions of 2.8% and 66.6%. This finding was in agreement with the work of Cheng and Stuckey [11].

Fig. 3 shows the binding isotherm at equilibrium for both MES and AOT. It can be observed that beyond the point of optimum binding for complete precipitation (R_p = 16), rather than remain as free surfactant, both MES and AOT anions continue to bind with the protein-surfactant complex. However, in this work it can be seen that the excess binding of MES (R_p > 16) to the complex was more pronounced in comparison with AOT.

In contrast, the concentration of AOT anions detected in the supernatant were significant after exceeding the optimum R_p (Fig. 3), indicating that there was an unfavourable interaction between AOT and the neutral complex beyond the optimum R_p (16). Nevertheless, interestingly, the redissolution effect was pronounced in AOT precipitation, specifically at R_p = 28 onwards, although its binding ratio on the complex was significantly lower.

3.2. Effect of R_p on the structure of lysozyme

CD was used to monitor any structural changes in the residual lysozyme in the supernatant after precipitation. Fig. 4 shows the CD spectra of lysozyme at R_p = 2.4 to R_p = 9.5 in conjunction with its native form. In general, lysozyme has a negative band in the far-UV range of 200–240 nm, with a shoulder at about 222 nm, and the strongest intensity at 208 [24], while 30.9 ± 0.4% α-helix, 24.0 ± 0.1% β-sheet, 18.5 ± 0.1% β-turn and 26.7 ± 0.4% random coil were calculated to be present in native lysozyme through the CDNN deconvolution software. It was apparent that there were no significant changes in the secondary structure of the soluble lysozyme as the R_p was increased from 2.4 to 9.5 (Fig. 4). As a control, the CD spectra (Fig. 4e) including the deconvolution result (Table 1) of the residual lysozyme in the supernatant after precipitation with AOT at R_p = 39 is also presented, which clearly indicates the significant structural changes that occurred (statistically different at the 95% confidence interval). These results are consistent with the findings of previous researchers [6]; the unfolding pattern observed was the loss of the α-helical structure and an increase in β-sheet and random coil fractions.

3.3. Effect of pH on the precipitation of protein

Fig. 5 shows the fraction of the mass, and lysozyme's activity in the remaining solution after precipitation, as a function of the pH of the solution. Full precipitation was obtained at pH 6.23 and the level of precipitation decreased with increasing pH up to 12, as indicated by the increase in the remaining fraction, from 21% (pH 8.2) to almost 100% (pH 12).

Furthermore, in this study determination of the equilibrium MES concentration in the supernatant after precipitation was also carried out in order to gain a better understanding of the interaction (Fig. 5). From Fig. 5 it can be seen that there was an increase in the concentration of free MES in solution with increasing pH, and this result was in accordance with the amount of precipitated lysozyme. CD and deconvolution analysis of the non-precipitated lysozyme over the pH range examined was also performed to check on their structural integrity (Fig. 7 and Table 2), and only a slightly altered CD profile of the lysozyme at pH 12 was observed (statistically different than the native at the 95% confidence interval).

3.4. Recovery of lysozyme from a lysozyme-MES complex

3.4.1. The use of solvent in lysozyme recovery

Fig. 6 shows the performance of the solvents in terms of overall protein recovery (%), protein recovery efficiency (%), overall activity recovery (%) and activity recovery efficiency (%). Basically, comparable performances were obtained for all the solvents tested for both protein and activity recovery. Overall activity recoveries of about 54–56% were obtained for acetone, ethanol, acetone-ethanol (50% v/v) and methanol. An analysis of variance (ANOVA) showed that the effect of the type of solvent on the protein recovery, protein recovery efficiency, activity recovery, and activity recovery efficiency was not significant.

For the CD analysis of the recovered lysozyme, only minor changes in the α-helix content of the lysozyme sample recovered by acetone and ethanol were detected, as indicated by the relative percentage difference shown (Table 3), and through a "Student *t*-test analysis". The α-helix content of the acetone and

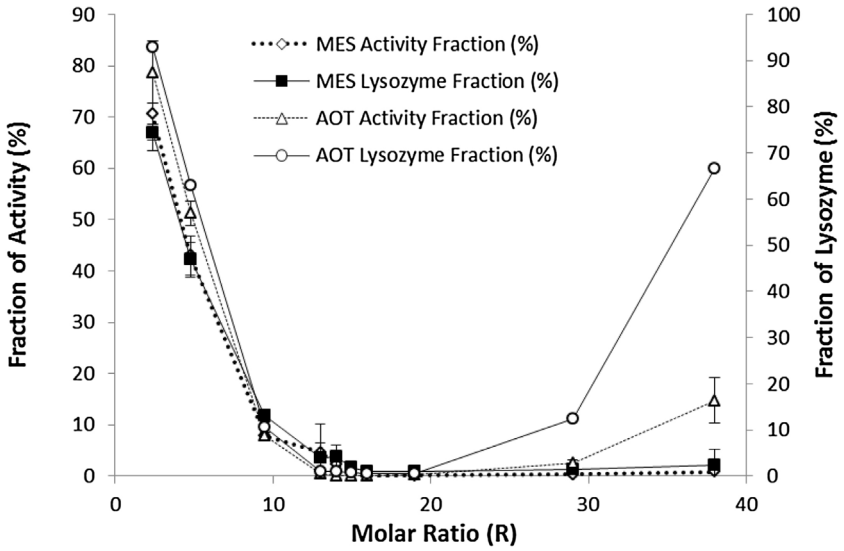


Fig. 2. Amount of residual lysozyme and its activity in solution after precipitation with AOT and MES. The error bars highlight the standard deviation for each point.

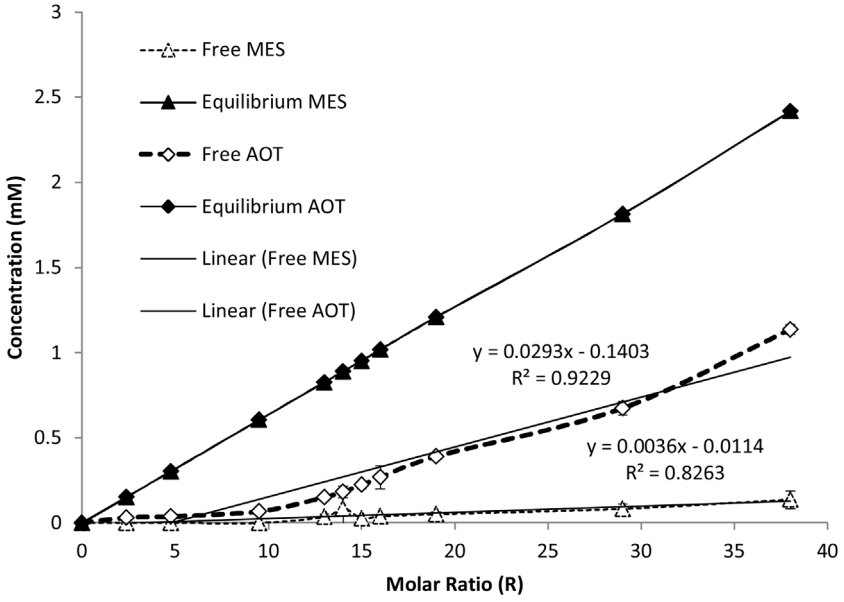


Fig. 3. Free and equilibrium concentrations of AOT and MES in lysozyme solution at pH 6.2 as a function of R_p.

Table 1
Secondary structure content of the non-precipitated lysozyme at R_p = 39 with AOT, and its relative difference (%) with the native lysozyme structure.

Secondary Structure (%)	Sample/R_p		
	Native	R_p = 39 (AOT)	Relative Change in Structure (%)[a]
α-helix	30.9 ± 0.4	19.5 ± 1.7	−36.9
β-sheet	24.0 ± 0.1	30.0 ± 2.1	+25.0
β-turn	18.5 ± 0.1	16.6 ± 0.2	−10.3
Coil	26.7 ± 0.4	34.0 ± 0.2	+27.3
Average Difference (%)[b]			24.9

[a] The difference in the percentages of secondary structure between the protein sample and the native state, determined by computational analysis [25]. In general, it is accepted that there is a major deviation from the native state when the difference (%) is larger than 10 [26].
[b] The average difference for all of the secondary structure.

ethanol-recovered lysozyme increased slightly (33.6% and 32.9%, respectively) compared to the native form (30.9%) (statistically different – 95% confidence). Meanwhile the random coil fraction in acetone-recovered lysozyme reduced slightly to 24% from 26.7% for a relative difference of 10.2% (statistically different with 95% confidence). Nevertheless, based on the activity results and CD profile presented, it is clear that the major conformation of the recovered lysozyme was still retained.

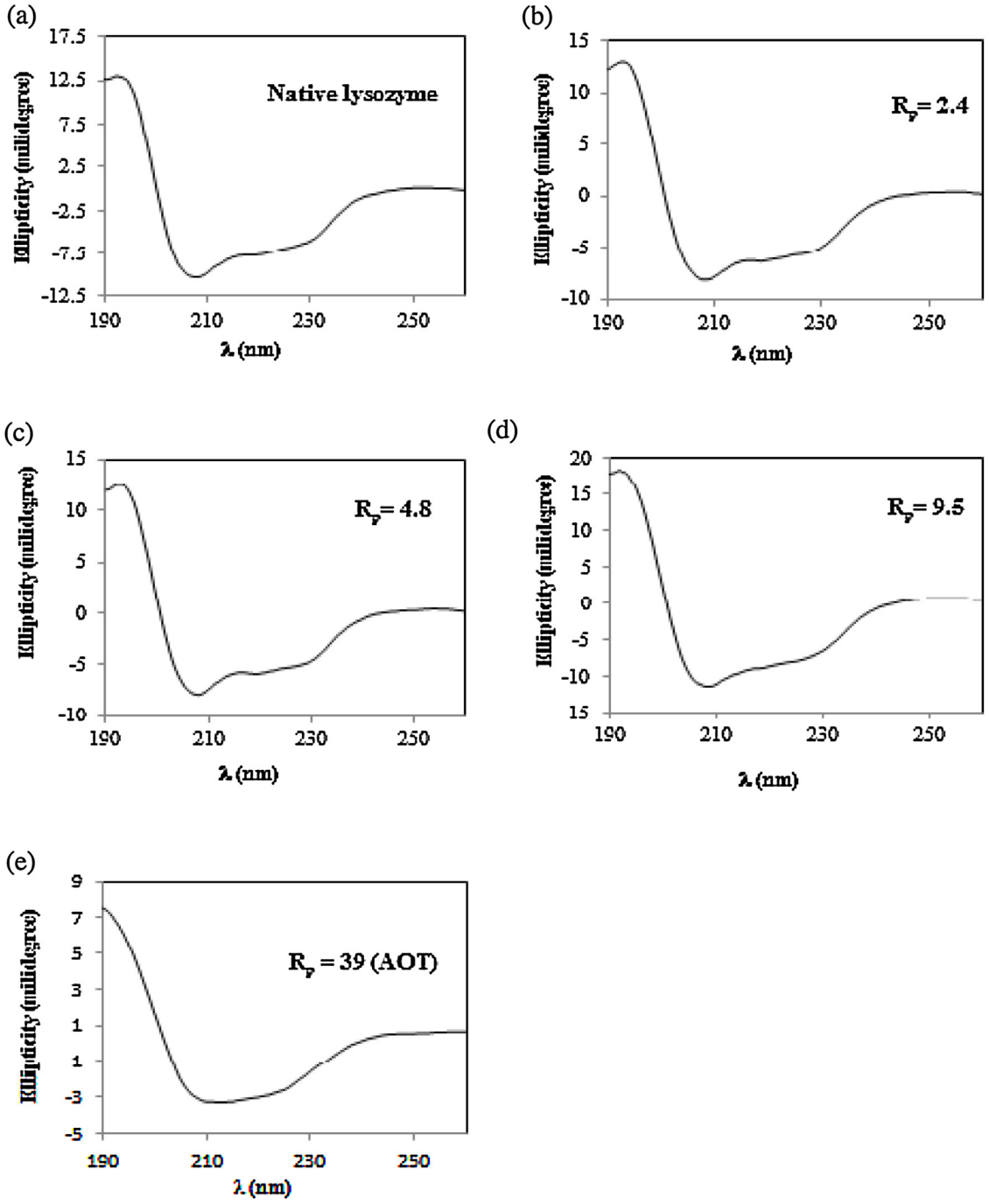


Fig. 4. Far-UV CD spectra of native lysozyme and the residual lysozyme in the supernatant from R_p = 2.4 to 9.5 (MES) and R_p = 39 (AOT), in 20 mM phosphate buffer, pH 6.23: (a) Native lysozyme, (b) R_p = 2.4, (c) R_p = 4.8, (d) R_p = 9.5, (e) R_p = 39 for AOT.

In addition, the results also highlighted that the overall protein/activity recovery and their recovery efficiency was comparable for all solvents, since the concentration of lysozyme in the supernatant after precipitation (C_e) and the washing fraction (C_w) was not significant. MES was able to completely precipitate the lysozyme in the sample, and there was no protein lost during the washing step (phosphate buffer) in the recovery stage. The recovery/extraction effect was achieved solely by the addition of an organic solvent to dissolve the surfactant (and hence remove it from the protein) while precipitating the target protein. Again, this pointed to the effectiveness of the precipitation method overall.

3.4.2. The use of a counter-ionic surfactant in lysozyme recovery

In order to recover lysozyme from the lysozyme-MES complex (precipitate), trioctyl-methyl ammonium chloride (TOMAC) was used. The precipitate recovered from the optimum R_p (16) was added to 20 mM phosphate buffer, pH 6.2, followed by the addition of 1 mL of TOMAC solution (dissolved in ethanol) to result in a molar ratio (R_r) of TOMAC to MES of between 0 and 3.0 (0–3.05 mM).

As illustrated in Fig. 7, it can be seen that there is an increasing trend of protein and activity recovery in conjunction with the recovery efficiency with an increment in R_r. In addition, the CD results, together with the deconvolution of the spectra obtained across the range of R_rs examined (Table 4), highlight the fact

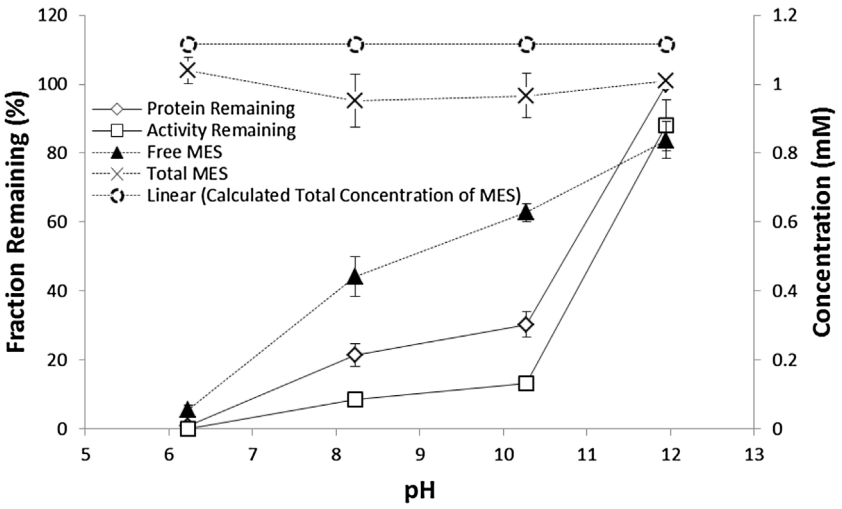


Fig. 5. Fraction of protein, activity remaining, total concentration of MES, and final concentration of free MES in the after-precipitation solution as a function of pH.

Table 2
Secondary structure of the non-precipitated lysozyme at pH 12, and its relative difference (%) with regards to its native lysozyme structure.

Secondary Structure (%)	Sample/R_p		
	Native	pH 12	Relative Change in Structure (%)[a]
α-helix	30.9 ± 0.4	26.3 ± 2.1	−14.9
β-sheet	24.0 ± 0.1	32.1 ± 2.2	+33.8
β-turn	18.5 ± 0.1	18.8 ± 0.2	+1.8
Coil	26.7 ± 0.4	22.7 ± 0.7	−15.0
Average Difference (%)[b]			16.4

[a] The difference in the percentages of secondary structure between the protein sample and the native state, determined by computational analysis [25]. In general, it is accepted that there is a major deviation from the native state when the difference (%) is larger than 10 [26].
[b] The average difference for all of the secondary structure.

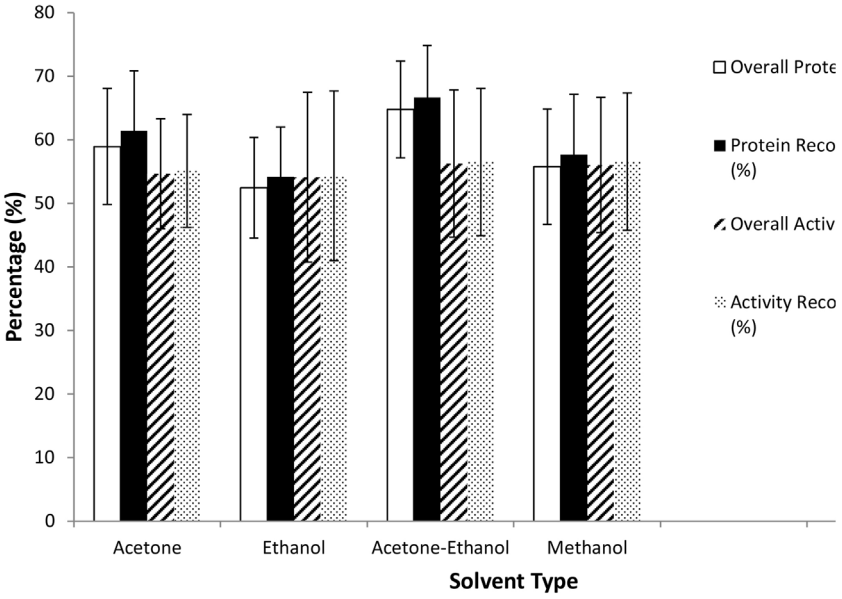


Fig. 6. The effect of organic solvent type on the recovery of lysozyme from precipitate. Error bars indicates the standard deviation of the measurement.

that there were no significant changes in the conformation of the lysozyme recovered, except for minor changes, i.e. reduction of α-helix and increment in β-sheet content, at lower R_rs, i.e. 1, 1.5, and 2 (statistically significant).

4. Discussion

Lysozyme was chosen as the model protein as it has been studied extensively, is relatively cheap, and was reportedly used by previous researchers [6,8] in studies on surfactant precipitation. Most

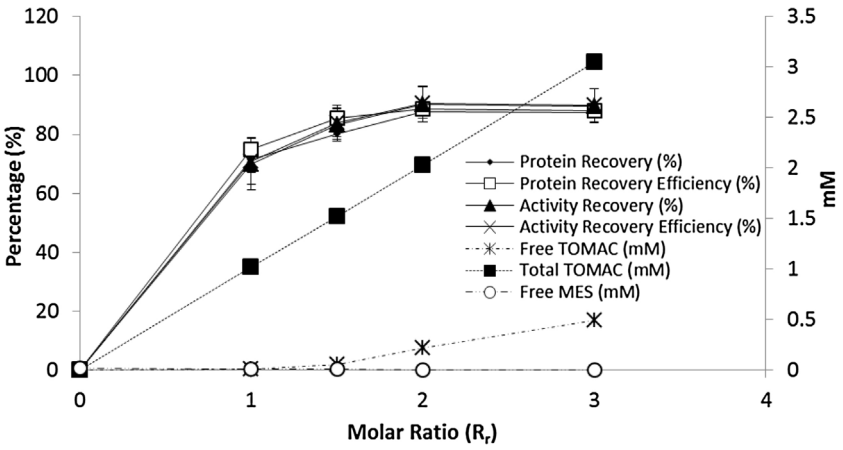


Fig. 7. Effect of the counter-ionic surfactant, TOMAC to MES molar ratio (R_r) on the recovery of lysozyme.

Table 3
Secondary structure content of the lysozyme recovered by various types of solvent.

Sample	Percentage of Secondary Structure (%)				Relative Change in Structure (%)[b]
	α-helix	β-sheet	β-turn	Coil	
Native	30.9 ± 0.4	24.0 ± 0.1	18.5 ± 0.1	26.7 ± 0.4	–
Acetone	33.6 ± 1.5[c]	23.5 ± 2.0	19.0 ± 0.1[c]	24.0 ± 0.7[d]	
Difference (%)[a]	8.8	−2.3	+2.4	−10.2	5.93
Ethanol	32.9 ± 2.1	23.1 ± 0.5[c]	18.8 ± 0.3	25.2 ± 1.9	
Difference (%)	+6.6	−3.6	+1.5	−5.7	4.35
Acetone-Ethanol	31.6 ± 0.1[c]	23.6 ± 0.5	18.6 ± 0.2	26.2 ± 0.8	
Difference (%)	+2.4	−1.8	+0.5	−1.9	1.65
Methanol	31.7 ± 1.0	22.7 ± 1.2	18.4 ± 0.1	27.0 ± 0.1	
Difference (%)	+2.6	−5.2	−0.3	+1.0	2.28

[a] The difference in the percentages of secondary structure between the protein sample and the native state, determined by computational analysis [25]. In general, it is accepted that there is a major deviation from the native state when the difference (%) is larger than 10 [26].
[b] The average difference for the four kinds of secondary structure.
[c] Data that is significantly different than the native (based on the "Student *t*-test"), but with a minor deviation (i.e. relative difference <10%).
[d] Data that shows a major deviation from the native (statistically different as based on the "Student *t*-test").

Table 4
Fractions of α-helix, β-sheet, β-turn, and random coil for the native and recovered lysozyme by counter-ionic surfactant – TOMAC, from R_r = 1.0 to 3.0, respectively.

Sample/R_r	Percentage of Secondary Structure (%)				Average Difference (%)[b]
	α-helix	β-sheet	β-turn	Coil	
Native	30.9 ± 0.4	24.0 ± 0.1	18.5 ± 0.1	26.7 ± 0.4	–
1.0	29.9 ± 0.2[c]	24.8 ± 0.2[c]	18.5 ± 0.1	26.6 ± 0.3	
Difference (%)[a]	−4.1	+3.3	−0.1	+0.3	1.95
1.5	29.6 ± 0.8[c]	24.9 ± 0.5[c]	18.4 ± 0.1	27.2 ± 0.4	
Difference (%)	−5.1	+3.7	−0.6	+2.5	2.98
2.0	28.8 ± 0.7[c]	25.8 ± 1.0[c]	18.4 ± 0.0	27.0 ± 0.2	
Difference (%)	−7.4	+7.1	−0.6	+1.7	4.2
2.5	30.3 ± 0.4	24.1 ± 0.3	18.4 ± 0.1	27.2 ± 0.3	
Difference (%)	2.7	+0.8	−0.5	+2.4	1.6
3.0	30.5 ± 1.1	24.6 ± 1.1	18.5 ± 0.1	26.4 ± 0.2	
Difference (%)	+2.1	+2.5	0.1	−0.3	1.25

[a] The difference in the percentages of secondary structure between the protein sample and the native state, determined by computational analysis [25]. In general, it is accepted that there is a major deviation from the native state when the difference (%) is larger than 10 [26].
[b] The average difference for the four kinds of secondary structure.
[c] Data that is significantly different than the native (based on the "Student *t*-test"), but with a minor deviation (i.e. relative difference <10%).

importantly, the high pI of lysozyme (about 11), and its pH stability range (6–9), made it very suitable for studying surfactant precipitation using anionic surfactants. One advantage of using AOT and MES in protein precipitation is their lack of interference in the spectroscopic measurement of proteins at A280 nm, i.e. their absorbance of about 0.0157 with 27 mM MES, and only 0.0010 at 2.7 mM. Hence, at the maximum concentration employed in this work, 2.42 mM, to make up a molar ratio of 38 (at the moment of MES addition), surfactant interference can be neglected. Hence, the use of an easy method like spectrophotometry for protein concentration determination is possible, rather than the more complicated method of

HPLC. Furthermore, MES and AOT did not interfere with the activity determination of lysozyme also.

Lysozyme was prepared in 20 mM phosphate buffer (pH 6.23) because it was reported that maximal activity occurred at pH 6–9, with varying ionic strengths of 20–100 mM [27]. In this work, the salt concentration used in the phosphate buffer was kept at 20 mM as a minimum possible to prevent the salt ions from competing with MES and lysozyme in ionic interactions.

In the initial stage of precipitation ($R_p < 16$) there was insufficient amounts of MES to form a complex with all the lysozyme, and subsequently as more MES was added, the level of precipitation increased and finally approaching 100% at an R_p of 16. The optimum R_p for complete precipitation of lysozyme by MES was found to be very similar to AOT (Fig. 2), suggesting that 16 mol of MES had interacted sufficiently with 1 mol of lysozyme to precipitate it all out to form an insoluble complex.

Previous research has highlighted the fact that surfactant aggregates (micelles) were responsible for the redissolution of the protein precipitate back into solution [28,29]. During protein-surfactant interactions, as the concentration of surfactant increases, surfactant molecules nucleate on the protein chain in a 'pearl necklace' structure [30]. Normally, redissolution occurs when a critical concentration of surfactant molecules which aggregate on the complex is reached [29]. Thus, in a homologous surfactant, redissolution at higher surfactant concentrations (R) is more efficient with a more strongly associating surfactant [28,29]. As the surfactant aggregates (micelles) are mainly responsible for the solubilisation of the neutral surfactant-protein complex, a surfactant with a lower CMC is thought to have a better solubilizing effect than one with a higher CMC value.

MES has a straight hydrophobic chain, rather than a branched one as in AOT (Fig. 1). Moreover, AOT has a hydrophilic group (sulphate) positioned at the centre of the hydrophobic chain, rather than at the end of it like MES. Thus, AOT has two hydrophobic tails, each of about 6 carbon chain lengths, and an ethyl group that is branched at the second position. In contrast, in MES a single linear hydrophobic tail of about 12–14 carbons exist. Two tail surfactants generally have a higher CMC in aqueous solution compared to an analogous single tail surfactant since the linear non-branched and flexible hydrophobic tail is more efficient in forming micelles than a two tail surfactant. In addition, the branched hydrophobic chain of AOT sterically inhibits micellization which results in a high CMC value [31]. Hence, theoretically, as far as redissolution of the precipitate is concerned, MES is expected to act more effectively at lower concentrations than AOT. However, the results obtained in this work are not consistent with the above theory, in the sense that although MES has a lower CMC (determined to be around 0.2 mM), precipitate redissolution which occurs in AOT precipitation was not encountered for MES; this point will be discussed in more depth later in this paper.

In addition, beyond the optimum R_p (equilibrium concentration above the CMC), and in the presence of a lysozyme-MES complex in the aqueous phase, MES tended to bind with the complex to achieve thermodynamic equilibrium rather than forming micelles among themselves. The long linear hydrophobic chain in MES explained the greater adsorption of MES through cooperative binding on the neutral protein-surfactant complex through hydrophobic interactions. However, the "charging" of the complex with negative charges due to excessive MES binding was somehow insufficient to provide a repulsive electrostatic force to overcome the hydrophobic attraction that kept the complex together.

In this work it seems that the excess binding of MES (with a higher amount bound than AOT at similar R_ps) did not cause a loss in native structure which was responsible for the drop in precipitation encountered in the AOT-lysozyme system [11], and this continued in the precipitation region, even up to $R_p = 39$. Cheng and Stuckey [11] previously postulated that the structural integrity of lysozyme was a prerequisite for effective precipitation through the charge neutralisation mechanism driven by favourable electrostatic interactions between the AOT anionic group and ionic groups on the lysozyme surface. In fact, once the excessive binding of AOT on lysozyme occurs ($R_p > 23$), its native structure and activity is lost as AOT started to bind with the protein's non-polar surface groups and enter its hydrophobic intracavity, eventually resulting in less lysozyme being precipitated. Furthermore, taking into account that the highest concentration of AOT employed in the system (2.4 mM at $R_p = 38$) was far below its CMC, it is logical to presume that the redissolution of the neutral precipitate was a result of structural changes in lysozyme after interacting with the bulkier hydrophobic structure of branched AOT, instead of the charging effect of the complex by excessive binding.

In the study of pH effect, the further away the pH was from the pI of lysozyme (i.e. 11), the more likely is the formation of an ion pair between the anionic MES head group and the positively charged protein surface charge, and thus the greater the amount of precipitate obtained. In general, the overall net charge of a protein depends on the pH of the solution; protein will assume an overall net positive charge at a pH below its pI, a net neutral charge at its pI, and a net negative charge at a pH above this point [32]. It is reported that lysozyme has 18 cationic amino acid residues (6 lysyl including 1 N-terminal, 11 arginyl and 1 histidyl) [33]. In surfactant precipitation, specifically in the case of MES and lysozyme at a pH < pI, it is hypothesized that the anionic head group of the MES monomer will electrostatically interact with the positively charged groups on lysozyme molecules to produce a neutral insoluble MES-lysozyme complex that will eventually precipitate [8,11]. At a pH >pI there will be an electrostatic repulsion between the two similar negatively charged molecules of lysozyme and MES, thus preventing the formation of the neutrally charged complex.

One intriguing observation from our data was that most of the non-precipitated lysozyme's activity remained at pH 12 (88%), contrary to the AOT precipitation study reported by Cheng and Stuckey [11]. They previously reported that despite more than 50% of the lysozyme not precipitating, only 9% of its activity remained at an R_p of 16 and pH 12. It was claimed that hydrophobic interactions between AOT and the lysozyme took place at this pH (>70% bound AOT) which consequently caused a significant drop in its activity. However, it was apparent that for MES, even when mild (manual end-over-end mixing) or rigorous (≈10–20 s vortex) mixing was carried out, no significant binding was observed at pH 12.

Less MES was interacting with lysozyme as the pH increased due to the reduced electrostatic interactions between MES and lysozyme as a consequence of the reduced net positive charge of lysozyme. Moreover, at higher pHs (above the pI of about 11) a substantial amount of free MES (about 85%) could still be detected. This implied that the degree of hydrophobic interactions between lysozyme and MES at this point was not very significant compared to AOT precipitation [6], which explained why the activity of the non-precipitating lysozyme was preserved i.e. 88%, for MES precipitation. The slightly altered CD profile of the lysozyme at pH 12 is understood to be a result of the binding (15% of bound MES). However, in comparison to the AOT results where a major loss in conformation was observed [6], the degree of change caused by MES was relatively minor, and in fact most of its activity was retained. It is believed that with AOT, the dominant effect of the hydrophobic interactions at higher pH (>pI) might be due to the larger steric effect of its bulky branched double hydrophobic tail.

After scrutinizing the results, it was clear that the interaction between MES and the protein was mainly governed by electrostatic interactions that were required to bring both molecules closer to form a solid-like complex. However, if such a condition was not met, it seems that any interaction between them, including

the hydrophobic interactions which were apparently encountered in the AOT-lysozyme system, was not very favourable for MES-lysozyme. Thus, this study highlighted another advantage of MES over AOT in the sense that MES is more specific in interacting with lysozyme, and exhibited significantly less denaturing hydrophobic interactions. Basically, this demonstrates that MES is a more appealing choice for a surfactant ligand for protein precipitation than AOT.

The high protein recoveries obtained for acetone can be explained by the strong absorption of traces of acetone at 280 nm which caused an over-estimation of protein concentration in the final phase. Traces of acetone remained after the addition of phosphate buffer at pH 6.2 in the final stage, even though a precautionary step was taken to ensure its complete removal by air drying in the fume hood for 3 h, instead of the conventional 30 min–1 h period. The timeframe was also not increased further in order not to compromise the lysozyme stability factor. In addition, such circumstances were also encountered by other researchers [11], in which it was reported that a protein recovery as high as 157.3% was obtained for acetone recovery.

It was reported earlier that lysozyme was not denatured by acetone, alcohol, ether, chloroform or toluene, even when they have acted for a considerable time [34]. In fact, these solvents can be used for the preservation of lysozyme-containing materials. Thus, the discussion on the recovery of lysozyme by organic solvents was made based on lysozyme's solubility in organic solvents, rather than denaturation. The more the protein dissolves in them, the less the amount precipitated out, and the lower the recovery yield obtained. For instance, the solubility of lysozyme was >100 mg/mL and 0.25 mg/mL, in DMSO and chloroform, respectively, as compared to 0.01 mg/mL and 0.02 mg/mL, in methanol and ethanol, respectively [35]. This fact explains the generally comparable results of all of the solvents studied (i.e. 54–59%), except DMSO and chloroform where none of the lysozyme was recovered.

Essentially TOMAC is a quaternary ammonium salt, and thus possesses a permanent positive charge which can form salts with anions over a wide pH range. In principle, the ionic interactions between TOMAC and MES are stronger than between TOMAC and lysozyme, and this facilitates the release of lysozyme from the neutral complex into solution while leaving the simultaneously formed TOMAC-MES salt as a precipitate [11].

There is a direct relationship between the percentage of protein/activity recovery, and the R_r. Initially, at $R_r = 0$ (negative control), MES was released from the complex, possibly due to the stripping effect of ethanol. At an R_r below 2, there was also an insufficient amount of the counter-ionic surfactant, TOMAC to interact with MES from the MES-protein complex. At $R_r = 1.5$, near ideal/complete interaction between both surfactants occurred, indicated by the non-detection of either MES or TOMAC. In the final stage (beyond $R_r = 2.0$), most of the TOMAC added bound with the lysozyme-MES complex, but did not release the remaining precipitated lysozyme fraction. Moreover, based on the activity and CD results, it was shown that MES can perform as effectively as AOT as a precipitant without causing any denaturation of the target protein.

Previously, Cheng and Stuckey [6] claimed to have successfully recovered lysozyme from the lysozyme-AOT complex using the counter-ionic surfactant, TOMAC, where 100% recovery was obtained at an R_r of 2. However, they did not address the problem of TOMAC accumulation in solution after its addition up to $R_r = 3$ (final concentration of 3.07 mM). They only performed a methylene blue (cationic dye) assay on the recovered lysozyme solution which can only detect anionic surfactants (AOT and MES). In our work, a methyl orange (anionic dye) assay was performed to detect any remaining TOMAC in solution according to the method of Wang and Langley [36], with a slight modification in terms of volume. Hence, this allowed for a better monitoring of any carry-over of both surfactants in the recovered phase, and optimization of the recovery process based on counter-ionic surfactant. Cheng and Stuckey [6] previously reported the use of several cationic surfactants: TOMAC, DTAB and DODMAC to recover lysozyme from a lysozyme-AOT complex. TOMAC/DTAB were reported to have achieved full recovery, but for DODMAC, however, the highest recovery achieved was only about 80%; in addition to this, the recovery dropped as the concentration exceeded that point. The authors put this down to the effect of the DODMAC structure, which it is highly hydrophobic due to the presence of long alkyl chains.

In this work, it is clear that the optimum R_r was 1.5, where 83.3% of activity recovery was obtained with no carry-over of TOMAC. Nonetheless, a compromise on protein purity after protein precipitation depends on the downstream separations units that follow. Surfactant removal from protein samples can be very important; for example, in mass spectrometry the protein sample must be free from any detergent prior to analysis, because even at low concentrations it can contaminate instruments and interfere with column binding, elution and ionization.

However, surfactant carry-over should not be the bottleneck for surfactant precipitation because in order to remove the surfactant, a physical method can simply be employed after the counter-ionic recovery such as chromatography, addition of an adsorbent, or filtration. In this study, the lysozyme recovered at $R_r = 2$ (where 90% recovery was achieved) can be further purified by additional filtration to remove the TOMAC carried over.

5. Conclusions

Overall, it can be concluded that the precipitation level of lysozyme by MES was a strong function of R_p and pH. The optimum R_p was chosen to be 16 (100% precipitation) at a pH < pI. It was found that electrostatic interactions were the main driving force behind MES precipitation. The lysozyme precipitate was also successfully recovered using solvent – acetone, ethanol, 50% acetone/ethanol, or methanol (54–56% activity recoveries), or counter-ionic surfactant – TOMAC at $R_r = 1.5$ (83.3% protein recovery). Most significantly, the structural integrity of all the lysozymes recovered was preserved.

In contrast to AOT, MES caused no major structural transformations at higher surfactant concentrations, had minimal hydrophobic interactions with the protein molecules, and exhibits better selectivity than AOT. Hence, along with its economic benefits and environmental features, this work has highlighted the considerable potential of using MES as the precipitant in surfactant precipitation separation, and it can be scaled up easily at very low cost.

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