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Surfactant precipitation and polar solvent recovery of α-chymotrypsin and ribonuclease-A

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Abstract

The purification of two enzymes, α-chymotrypsin and ribonuclease-A, was studied using sodium di-(2-ethylhexyl) sulfosuccinate (AOT) as precipitating ligand. The enzymes formed a water insoluble complex upon contact with AOT and precipitated from the aqueous solution. The molar ratio between AOT and the targeted enzyme, required to obtain a 100% precipitation from an aqueous solution, was 7 for α-chymotrypsin and 13 for ribonuclease-A. Acetone was then used to recover the enzymes from the protein-AOT insoluble complex. The enzyme precipitated as white solid, while the surfactant remained in the acetone phase. The recovered enzyme was free of surfactant and retained its original enzymatic activity. The selectivity of this precipitation method was a strong function of the isoelectric point (p*I*) of proteins indicating that the ionic interactions between oppositely charged protein and AOT is the driving force for precipitation. Proteins with p*I* values within one pH unit, cannot be selectively separated using this method.

Keywords: AOT; Enzyme activity; Bioseparation; Filtration; Precipitation; Protein recovery

1. Introduction

Di-(2-ethylhexyl) sulfosuccinate surfactant often referred to as AOT, has been used for the purification of biomolecules by reverse micellar extraction mechanism [1–6]. Upon contact of an aqueous phase containing electrolytes with an organic phase containing AOT, a reverse micellar structure is formed in the organic phase. In this structure, the surfactant molecules have their organic chains facing the organic solvent and their polar heads facing a core water pool. Proteins and other charged biomolecules are extracted into the water pool of the reverse micelles due to the electrostatic interactions [2]. The biomolecules entrapped inside the reverse micelles remain in an aqueous environment inside the organic phase [7]. This aqueous environment is believed to provide stability to proteins, thus preserving their biological properties [8]. It was reported that the maximum enzymatic activity is observed when the size of a reverse micelle is equal to the enzyme size [9]. Proteins, once extracted into a reverse micellar phase, are recovered by contacting the protein-containing organic phase with a fresh aqueous phase with pH and salt concentration adjustment. However, adjustment of pH and ionic strength alone was found to be insufficient back-extract the protein from the reverse micelles [10]. Addition of ethanol [11], isopropanol [12], ethyl acetate [13,14], and counter surfactants [3] have been attempted to improve the back extraction efficiency.

One limitation for the reverse micellar extraction of proteins has been the formation of white precipitate at the aqueous–organic interface [15–18]. This white precipitate was believed to be due to an insufficient hydrophobicity of the protein–surfactant complex to be extracted into the organic phase [8,19,20]. Upon mixing of a protein-containing aqueous phase and a surfactant containing organic phase, the charged surfactant head groups form an ion-paired complex with the oppositely charged proteins. Additional surfactant molecules wrap the protein–surfactant complex to yield hydrophobic surface, so that the protein can be solubilized into the organic phase. An insufficient hydrophobicity of protein–surfactant complex was believed to result in the formation of protein–surfactant precipitate at the aqueous–organic interface. This was a limiting factor for protein solubilization [17]. Development of new reverse micellar systems was suggested to minimize the amount of protein lost as a precipitate at the aqueous–organic interface [21].

More recently, it was reported that the presence of reverse micelles and of an organic phase is not necessary for the

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purification of proteins using AOT [22]. In that work, it was proved that the lysozyme precipitated at the aqueous–organic interface during the reverse micellar extraction process is indeed an insoluble lysozyme–AOT complex, and that the lysozyme can be recovered from this complex by dissolving it in a polar organic solvent. The recovered lysozyme was found to be free of surfactant and it retained original enzymatic activity. Purification of lysozyme from hen egg white using this method showed that proteins with different isoelectric (p*I*) values can be selectively precipitated with AOT by controlling the pH of the initial protein solution [23]. In this work, we study the precipitation and recovery of α-chymotrypsin and ribonuclease-A using AOT as the precipitation ligand and acetone as the polar recovery solvent. These two proteins have similar p*I* values and are commonly purified from bovine pancrease. The precipitation and recovery of these two proteins is compared with the results obtained using the reverse micellar extraction method. The precipitation method can be used to recover both proteins but not to selectively separate them due to the proximity of their p*I* values.

2. Materials and methods

2.1. Materials

Sodium di-(2-ethylhexyl) sulfosuccinate (AOT), at 99% purity, was purchased from Sigma (Oakville, Ont.). The enzymes, α-chymotrypsin Type II (EC 3.4.21.1; p*I* 8.5; 25,000 Da) and ribonuclease-A Type II-A (EC 3.1.27.5; p*I* 7.8; 13,700 Da), both from bovine pancreas, and the substrates used for the enzyme activity assays, benzoyl-L-tyrosine ethyl ester (BTEE) for α-chymotrypsin and cytidine 2′:3′-cyclic monophosphate for ribonuclease, were also purchased from Sigma (Oakville, Ont.). All other chemicals were of analytical grade and were purchased from Fisher Scientific (Montreal, Que.). All experiments were carried out using deionized water, prepared by passing distilled water through ion-exchange columns, type Easy pure RF Compact Ultrapure Water System, Barnstead Thermoline.

2.2. Formation of the enzyme–AOT complex and recovery

An aqueous solution containing 0.1–1 g/l enzyme was prepared. No pH or salt concentration adjustment was made to the aqueous phase. As the p*I*s of the enzymes were higher than the pH of deionized water, the enzymes had an overall positive surface charge and reacted with the negatively charged head group of AOT. An aqueous solution containing 5 g/l AOT was prepared. A volume of 0.1–0.8 ml of the AOT solution was then directly added to 20 ml of enzyme-containing aqueous solution. In order to avoid the formation of micelles in the aqueous phase, the concentration of AOT in the mixture was always less than its critical micellar concentration, which was reported to be between 2.5 and 4.1 mM at 25 °C [24]. Upon the addition of the AOT, an instant formation of an insoluble enzyme–surfactant complex was observed. This mixture was vortexed for 5 s, centrifuged and washed with distilled water. The precipitate was then collected for the enzyme recovery process.

Acetone was added to the collected enzyme–AOT precipitate to dissociate the enzymes from the surfactant. After all the solids were dissolved in the solvent phase, a small amount of NaCl solution (normally less than 10 μl of 0.1 M NaCl) was added to balance the charges of the dissociated enzyme and surfactant. The surfactant-free enzyme precipitated out from the acetone phase while the surfactant remained in the acetone solution. The recovered solid enzyme was washed with acetone to further remove any residual surfactant. The qualitative and quantitative analyses of the final product were then carried out by dissolving the recovered-solid enzyme into a fresh aqueous phase.

All experiments were carried out in triplicate samples and the standard deviation of the measurements is indicated in the tables and figures.

2.3. Protein assay

The concentration of protein in aqueous phase was determined by using an Agilent High Performance Liquid Chromatography 1100 (HPLC) with a Zorbax 300SB-C8 (4.6 mm ID × 150 mm length) column. The mobile phase consisted of two solvents with different polarities: solvent A containing 5% acetonitrile and 0.1% triflouric acid in water, and solvent B containing 5% water and 0.085% triflouric acid in acetonitrile. The mobile phase was set at an initial composition of 90% solvent A and 10% solvent B with a solvent gradient of 40% solvent A and 60% solvent B in 20 min. In order to avoid any impurities that could damage the column, HPLC grade solvents were used to prepare the mobile phase, and the samples were filtered before being injected to the column. The flow rate through the column was set at 1.0 ml/min, and the temperature of the column was set at 25 °C. The sample volume was set at 5 μl, and the wavelength of the detector was set at 210 nm.

The pH was measured using an accupHast reference/pH probe purchased from Fisher Scientific 13-620-116 (Montreal, Que.) filled with saturated silver chloride solution. An OAKTON pH/mV Benchtop Meter model WD-35616-00 was used to monitor pH measurements.

2.4. Enzyme activity assay

The enzyme activity assay was performed with a Cary Varian 1/3 UV/visible spectrophotometer (Varian Techtron Pty. Ltd., Vic., Australia). The enzyme activity of α-chymotrypsin in aqueous solution was determined at 25 °C according to Hummel [25] by measuring the increase in absorbance, at 256 nm, resulting from the hydrolysis of BTEE. One unit hydrolyzes 1 μmol of BTEE per minute at

pH = 7.8 and 25 °C under the specified conditions. A substrate solution of 0.00107 M BTEE in 50% w/w methanol (63 ml absolute methanol added to 50 ml reagent grade water) and a solution of 0.08 M Tris–HCl buffer, pH = 7.8 containing 0.1 M calcium chloride were prepared. The temperature of the UV cells was maintained at 25 ± 0.1 °C, and the wavelength was set at 256 nm. The solutions were pipetted into sample cuvettes as follows: 1.5 ml of 0.08 M Tris–HCl buffer, pH = 7.8 containing 0.1 M $CaCl_2$ and 1.4 ml of 0.00107 M BTEE and placed in to the UV cells for about 2 min to achieve temperature equilibrium. A 100 μl volume of Tris–HCl buffer solution was pipetted into the reference cuvette and 100 μl of enzyme solution was added to the sample cuvette, and immediately after, the increase in the absorbance was monitored as a function of time for 4 min. From the linear portion of the curve ΔA_{256}/min is calculated and

$$\text{Units/mg} = \frac{\Delta A_{256/\text{min}} \times 1000}{964^* \times \text{mg/ml in the reaction mixture}} \quad (1)$$

(* extinction coefficient of BTEE at 256 nm)

The enzyme activity of ribonuclease-A was measured following the method described by Crook et al. [26]. A substrate solution of 0.1 mg/ml cytidine 2′:3′-cyclic monophosphate in 0.025 M phosphate buffer, pH = 7.5, was prepared. A 2.8 ml volume of substrate solution was pipetted into each of the two cuvettes, and the spectrophotometer reading was set to 0 at 286 nm. A 200 μl volume of sample was added to one cuvette and 200 μl of buffer to the other, and the contents were mixed by inverting the cuvettes several times. The change in absorbance at 286 nm was recorded over the time for the two cuvettes. The difference in the absorbance for the initial rate was determined by extrapolation to time zero. The results of this assay were expressed in units of moles substrate converted to product per unit enzyme per unit time. This was calculated from the kinetic data by dividing the initial rate by the ribonuclease-A concentration and multiplying by a constant as follows:

$$\begin{aligned} &1 \text{ unit activity of ribonuclease A} \\ &= \frac{\text{mol S} \rightarrow \text{P}}{\text{mol RNase} \times \Delta t} \\ &= \frac{C}{\text{conc. RNase (mg/ml)}} \frac{\Delta \text{OD}_{286}}{\Delta t} \end{aligned} \quad (2)$$

where C was measured to be equal to 8 for the aqueous assay using 200 μl sample.

3. Results and discussion

3.1. Precipitation and recovery of α-chymotrypsin

As the concentration of α-chymotrypsin increased from 0.004 to 0.04 mmol/l, the natural pH of the initial aqueous solution without pH or salt concentration adjustment changed from 4.5 to 3.6. The error in the pH measurement was within 0.2 pH units. The α-chymotrypsin has an overall surface charge of +7 at a pH = 4.5 [27].

The formation of the protein–AOT complex was expressed in terms of the precipitation efficiency written as:

$$\text{Precipitation (\%)} = \frac{C_{\text{P}^0} V_0 - C_{\text{P}^e} V_{\text{T}}}{C_{\text{P}^0} V_0} \times 100 \quad (3)$$

where C_{P^0} and C_{P^e} indicate the concentration of protein in the aqueous phase before and after the addition of AOT, respectively. The symbols V_0 and V_{T} indicate the initial volume of the protein solution and the total volume of protein–AOT aqueous phase mixture, respectively.

Fig. 1 shows the precipitation efficiency, as defined by Eq. (3), plotted as a function of the molar ratio, R, between the AOT and the α-chymotrypsin initially presented in the aqueous solution. The equilibrium pH after the formation of the insoluble α-chymotrypsin–AOT complex is also presented in this figure. As shown in Fig. 1, the precipitation efficiency increased as R increased. At $R = 7$, a complete precipitation of α-chymotrypsin was obtained. When R was less than 7, the concentration of AOT remaining in the solution was below the detection limit of the HPLC, indicating that all the ligand added to the solution formed lysozyme–ligand complex and was removed from the aqueous phase. When R was greater 7, as all protein was removed, excess ligand was detected in the aqueous phase. As the percent precipitation increased to 100%, the pH of the mixture increased to a value of about 5. Note that the percent precipitation line, shown in the figure, can be written as $y = 7x$. This indicates that the α-chymotrypsin and the α-chymotrypsin–AOT complex intermediates containing less than 7 mol of AOT per mole of protein are soluble in water. The compound containing 7 mol of AOT per mole of α-chymotrypsin is insoluble in water, and thus precipitates out of the aqueous phase. For example, if 3.5 mol of AOT are added to the protein solution, not all protein share the AOTs equally, but it is 50% of the protein that get all the AOT, at a 7–1 mol ratio between α-chymotrypsin and AOT, and precipitate. The other 50% of protein molecules remain in aqueous solution free of AOT.

In comparison, the minimum amount of AOT required to extract α-chymotrypsin into an AOT reverse micellar phase was 57 mol of AOT per mole of protein [18]. Furthermore, the present method has the additional advantage over the reverse micellar extraction that it does not require the contact with an organic phase containing reverse micelles.

The α-chymotrypsin was recovered as a pure solid by dissolving protein–AOT complex in acetone. The percent overall recovery of the α-chymotrypsin, based on the enzyme in the original aqueous solution, was 37 ± 18%. A mass loss in the protein was expected during washing. Due to the small amounts of precipitate collected, any loss affects significantly the overall percent recovery. The maximum overall recovery was of the order of 55%.

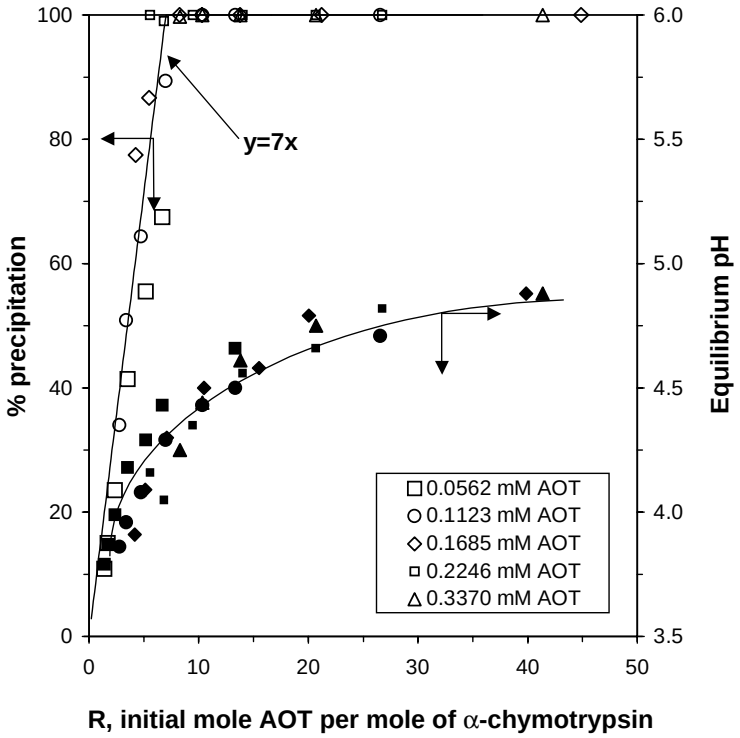

Fig. 1. Percent precipitation of α-chymotrypsin as a function of the molar ratio *R* between AOT and protein: initial aqueous solution, 0.1–1.0 g/l α-chymotrypsin, no salt added, no pH adjustment.

The recovered α-chymotrypsin retained its original activity of 43 ± 5 units/mg protein. Affinity chromatography and ion exchange column are commonly used to purify α-chymotrypsin from pancreas [28]. A 45% recovery was obtained after three-step chromatographic procedure [29]. The percent recovery of α-chymotrypsin using an AOT reverse micellar phase has not been reported in literature.

Fig. 2 shows the precipitation efficiency when NaCl was added to the initial protein solution. A decrease in the percent precipitation is observed as the salt concentration increased in the initial α-chymotrypsin solution. For example, at $R = 7$, the percent precipitation decreased from 100% to less than 80% when the salt concentration increased from 0 to 0.05 M NaCl. A further decrease in the percent precipitation was observed, and at 0.3 M NaCl, the percent precipitation was about 30%, independent on the molar ratio *R*.

3.2. Precipitation and recovery of ribonuclease-A

As the concentration of ribonuclease-A increased from 0.012 to 0.071 mmol/l, the natural pH of the initial ribonuclease-A aqueous solution decreased from 4.5 to 4.0. In this pH range, the ribonuclease-A has an overall surface charge from +13 to +10 [27]. Fig. 3 shows the precipitation of ribonuclease-A upon the addition of AOT. Similar to the α-chymotrypsin, the percent precipitation of ribonuclease-A increased as the molar ratio of AOT to protein increased. The slope of the precipitation line in Fig. 3 is 13, indicating that the solubility protein–AOT complex is a compound containing 13 mol of AOT per mol of ribonuclease-A, and no other intermediates are formed. In comparison, for α-chymotrypsin, the molar ratio of AOT to protein, required for a complete precipitation of the protein, was 7 (Fig. 1).

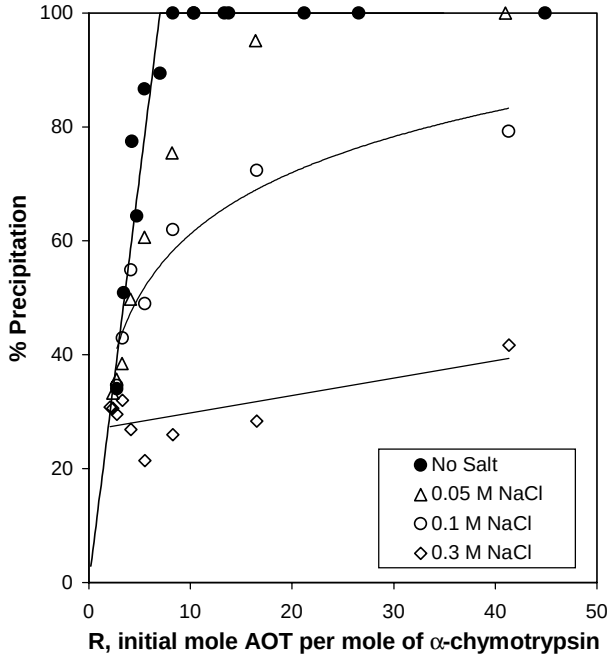

Fig. 2. Effect of salt on the percent precipitation of α-chymotrypsin: initial aqueous solution, 0.1–1.0 g/l α-chymotrypsin, 0.1685 mM AOT, no pH adjustment.

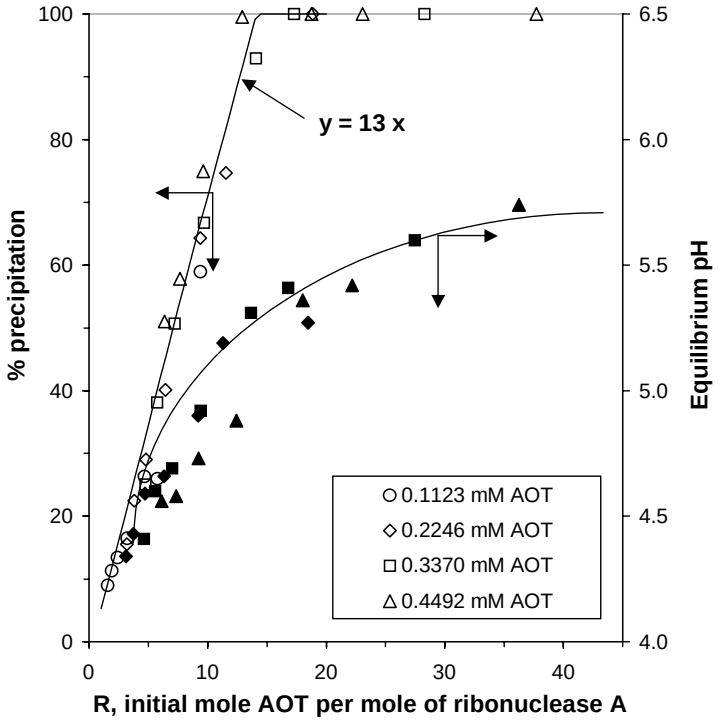

Fig. 3. Percent precipitation of ribonuclease-A as a function of the molar ratio R between AOT and protein: initial aqueous solution, 0.1–1.0 g/l ribonuclease-A, no salt added, no pH adjustment.

Even though the molecular weight of the ribonuclease-A is about half of that of α-chymotrypsin, the number of moles of AOT required to precipitate a mole of ribonuclease-A is about twice as many as that required to precipitate 1 mol of α-chymotrypsin. This result clearly indicates that the formation of protein–AOT insoluble complex is a function of the surface charge of the protein and not of molecular weight. The minimum amount of AOT required to extract ribonuclease-A in to an AOT reverse micellar phase was 55 mol of AOT per mole of protein [17].

The equilibrium pH of the protein solution after the precipitation is also presented in Fig. 3. As the percent precipitation increased to 100%, the pH of the aqueous solution reached a value of 5.7.

The percent recovery of solid ribonuclease-A obtained from the insoluble complex was found to be between 50 and 100%, with most samples showing around 84% recovery. The recovered ribonuclease retained its original enzymatic activity. The enzyme activities measured before and after the process were 41 ± 7 and 42 ± 5 units/mol enzyme, respectively. The percentage of recovery of protein depends largely on the mass lost during washing. In comparison, the percent recovery of Ribonuclease A from an AOT reverse micellar phase was 70% or less [30], representing a 38% of the initial protein in the aqueous phase before the contact with AOT [13].

An increase in the salt concentration in the initial protein solution resulted in a decrease in the percentage removal of protein at a given value of R. Fig. 4 shows the percent precipitation of ribonuclease-A as NaCl was added to the initial protein solution. As shown in this figure, at $R = 22$, the percentage removal of ribonuclease decreased to about 95 and 40% when the salt concentration was set at 0.1 and

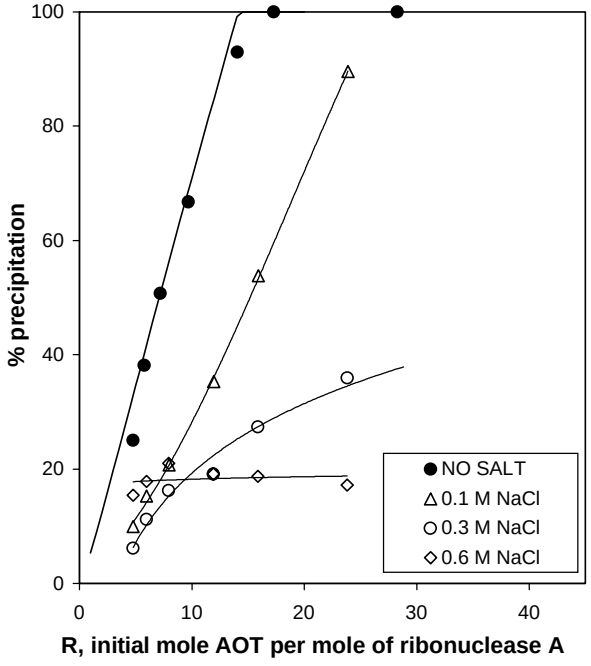

Fig. 4. Effect of salt on the percent precipitation of ribonuclease-A: initial aqueous solution, 0.1–1.0 g/l ribonuclease-A, 0.3370 mM AOT, no pH adjustment.

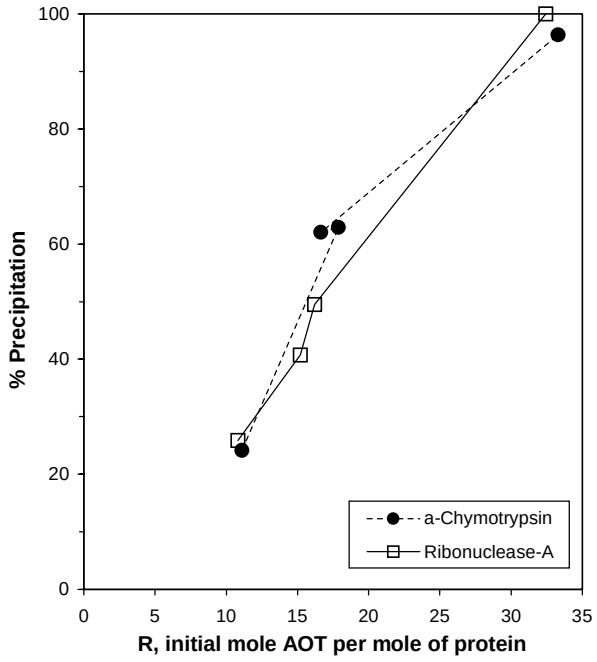

Fig. 5. Precipitation of α-chymotrypsin and ribonuclease-A from mixture: initial aqueous solution, 0.5 g/l α-chymotrypsin and 0.3 g/l ribonuclease-A, pH = 5.5 adjusted using HCl.

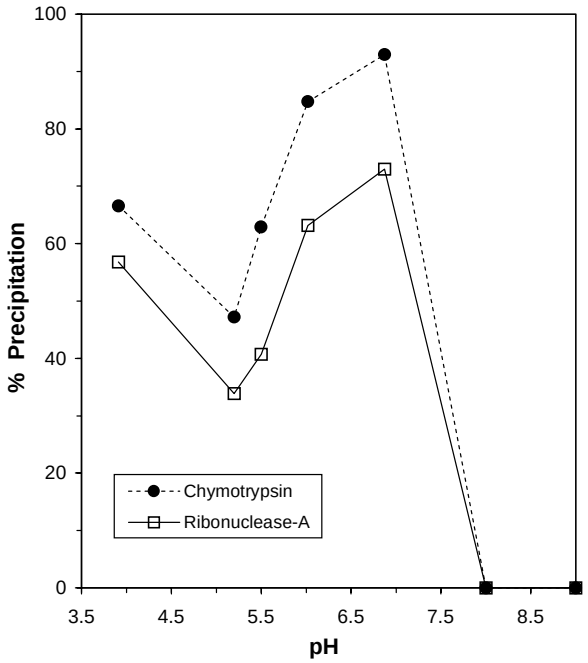

Fig. 6. Selectivity of precipitation of α-chymotrypsin and ribonuclease-A from mixture: 0.5 g/l α-chymotrypsin and 0.3 g/l ribonuclease, 0.3370 mM AOT, pH adjusted using HCl or NaOH.

0.3 M NaCl, respectively. At 0.6 M NaCl, the percentage of ribonuclease precipitated was found to be around 20%, independent of the value of *R*.

3.3. Selectivity

The potential selectivity of the precipitation of α-chymotrypsin and ribonuclease-A using AOT was studied. An initial protein solution was prepared using 0.5 g/l (0.02 mM) α-chymotrypsin and 0.3 g/l (0.02 mM) ribonuclease-A in distilled water. The pH of the protein solution was adjusted to 5.5 using HCl, and no salt concentration adjustment was made in the initial protein solution. Fig. 5 shows the percent precipitation of each protein as a function of the initial mole ratio between AOT and the particular protein, *R*. These ratios were calculated from the values of the concentration of AOT and the concentration of each protein in the solution. Even though, values of $R = 7$ and 13 were sufficient to obtain 100% precipitation of α-chymotrypsin and ribonuclease-A in single protein solution, when in a mixture, the moles of AOT required to precipitate the enzymes increased. As shown in Fig. 5, at $R = 11$, only 24% of initial α-chymotrypsin precipitated. The precipitation efficiency of ribonuclease-A showed similar values as those of α-chymotrypsin at all range of *R* tested. At $R = 16$, the percent precipitation of α-chymotrypsin and ribonuclease-A was 60 and 50%, respectively, indicating that the AOT was distributed equally between the two proteins. In order to obtain the complete precipitation of two proteins, the molar ratio of 33 between AOT and the proteins was required.

The selectivity of the AOT precipitation for the two enzymes was studied as a function of pH. The initial concentration of 0.5 g/l (0.02 mM) α-chymotrypsin and 0.3 g/l (0.02 mM) ribonuclease-A in distilled water was prepared, and the pH of the protein solution was adjusted using HCl or NaOH. As shown in Fig. 6, the percent precipitation of α-chymotrypsin was slightly higher than that of ribonuclease-A. However, due to the close values of the p*I*'s of these two enzymes (7.8 for α-chymotrypsin and 8.5 for ribonuclease-A) the precipitation resulted in a product containing a composition of α-chymotrypsin and ribonuclease-A similar to that in the initial sample. For the case of proteins with different p*I*'s it has been reported a selective separation of 100% by surfactant precipitation [23]. In that work, lysozyme ($pI = 11$) was selectively precipitated, and the recovered lysozyme was free of other contaminant proteins, such as albumin ($pI = 5$) and ovotransferrin ($pI = 6$). To the best of our knowledge, the selectivity of either the reverse micellar extraction method or the surfactant precipitation method to separate proteins with similar p*I* values has not been previously reported in the literature.

4. Conclusions

The surfactant precipitation of two enzymes of industrial importance was successfully carried out using AOT. The stoichiometric ratios for the formation of insoluble protein–AOT complex were determined to be 7 for

α-chymotrypsin and 13 for ribonuclease-A, which is about 25% of the amount of AOT required for a reverse micellar extraction. In this study, both the α-chymotrypsin and ribonuclease-A were recovered by treating the insoluble solid AOT–enzyme complex with acetone. Although not done in this work, the nonvolatile AOT can be easily recovered from the acetone phase by simple distillation. Due to the close p*I* values of the two enzymes studied, their selective separation could not be achieved by the surfactant precipitation method with simple pH or salt concentration adjustment. The possibility of using an affinity ligand with the AOT to selectively precipitate the targeted protein is presently under study.

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