J. Chem. Tech. Biotechnol. 1995, 62, 295–300

Complexation Behavior of Proteins with Polyelectrolytes and Random Acrylic Polyampholytes using Turbidimetric Titration

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(Received 12 May 1994; revised version received 2 August 1994; accepted 31 August 1994)

Abstract: The complexation behavior of proteins with dilute solutions of a polyelectrolyte (polyacrylic acid) and a random acrylic polyampholyte composed of acrylic acid, dimethylaminoethyl methacrylate and methyl methacrylate was experimentally investigated using turbidimetric titration. The random polyampholyte had a number-average molecular weight of 70,000 and a polydispersity index of only 1·3. Polyampholyte–polyampholyte interaction (self-aggregation) and polyampholyte–protein complexation behavior was studied as a function of pH (3–9) and polymer dosage (50–400, 5000 mg polymer per g protein). Large increases in turbidity (> 500%) were observed for protein–polyampholyte mixtures (compared with polyampholyte alone). However, protein analysis of the supernatant and precipitate after centrifugation revealed that only about 10% of the protein precipitated with the random polyampholyte while 90% of the protein remained in the equilibrium liquid. This implies that a very small degree of protein–polymer interaction can lead to *unusually large* increases in turbidity. Experiments with a single polyelectrolyte (polyacrylic acid) and oppositely-charged protein showed the *opposite* trend with 90% precipitation of protein. Hence, great care needs to be taken in interpretation of turbidimetric titration data.

Key words: turbidimetric titration, turbidimetry, turbidity, proteins, polyampholytes, polyelectrolytes, precipitation, complexation, protein–polymer interactions.

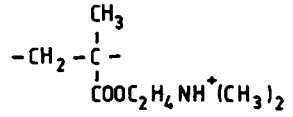
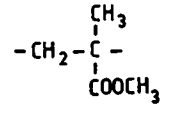
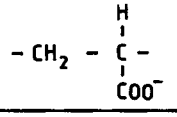
1 INTRODUCTION

Biochemical engineering today is trying to address several challenging problems in biotechnology at the process-scale.[1–5] Areas where large-scale process techniques are needed include protein refolding and protein purification. Often, the protein that needs to be separated is in a dilute aqueous solution along with a complex mixture of other bioproducts. To solve these problems, the selection or even synthesis of novel compounds that interact with proteins and improve protein separation and recovery would be necessary. Synthetic polymers and surfactants of varying chemical compositions, molecular weights and affinity ligands are increasingly being investigated for this purpose.[2,4]

Synthetic polyelectrolytes can interact strongly with proteins of opposite charge and form complexes; when the complexation yields insoluble products, this interaction has been employed for protein separation.[6–8] Precipitation with polyelectrolytes requires inexpensive chemicals in small amounts—the amount of polyelectrolyte required to neutralize the protein charge. Thus, the method is suitable for large-scale processing and for dealing with dilute solutions. A variety of polyelectrolytes has been tried out, but the same cannot be said of polyampholytes, which are polyelectrolytes capable of acquiring both positive and negative charges. In this work, the interaction of proteins with dilute solutions of (a) polyelectrolyte (polyacrylic acid) and (b) random polyampholyte of dimethylaminoethyl methacrylate, methyl methacrylate and acrylic acid has been investigated by turbidimetric titration. The chemical structure of these residues is shown in Table 1. The polyampholyte had a number-average molecular weight

J. Chem. Tech. Biotechnol. 0268-2575/95/$09.00

TABLE 1
Chemical Structure of the Polyampholyte Residues

Residue	*Structure*
Protonated dimethylaminoethyl methacrylate	
Methyl methacrylate	
Dissociated acrylic acid	

of 70000 and a polydispersity index of 1·3. This is in contrast to previous studies in which the polyelectrolyte polydispersity indices varied from 3 to 10.[8,9] Turbidimetry of complexes of synthetic polyampholytes has been performed by several investigators.[10–15] Compared with turbidimetric studies on polyelectrolyte systems (where the interaction is only between polyelectrolyte and oppositely-charged protein), the turbidimetric titration of polyampholyte systems presents additional complications because the turbidity increases can be caused by intermolecular protein–polymer interactions or intermolecular polymer–polymer interactions. Recently, Nath *et al.*[14] reported the results of experiments performed to differentiate between these two alternatives and showed that the turbidity increases that take place are indeed a result of protein–polymer interactions. In the present work, turbidimetric titration experiments have been carried out for interaction of random polymers (0·025–0·15, 5·0 mg cm^{-3}) with ribonuclease A from bovine pancreas (pI 8·8; 0·25–1 mg cm^{-3}) and soybean trypsin inhibitor protein (pI 4·5; 0·25–1 mg cm^{-3}) at various polymer dosages (50–400, 5000 mg polymer per g protein).

Finally, synthetic polyampholytes behave like proteins in terms of isoelectric point, solubility and titration curve behavior. Thus, they can be considered attractive models and information obtained from studying them will contribute toward an understanding of biological systems.[10,11,16]

2 EXPERIMENTAL

2.1 Polymer synthesis

Random acrylic polyampholyte was synthesized through free-radical polymerization of methyl acrylate, dimethylaminoethyl methacrylate and methyl methacrylate. To avoid side reactions, such as acid–base reactions and Michael addition,[17] which decrease the yield of polymerization, methyl acrylate was used instead of acrylic acid. After polymerization, the methyl acrylate residues were hydrolyzed to acrylic acid residues with a stoichiometric amount of potassium hydroxide at 75°C. The methyl acrylate residues were hydrolyzed in 30 min, but the methyl methacrylate residues were not hydrolyzed.[17] The molar ratio of basic, neutral and acidic residues (B:N:A) was determined from an elemental analysis. The polyampholyte used in this work had an actual molar B:N:A residue ratio of 0·97:0·83:1·0, a number-average molecular weight of 69000, a polydispersity index of 1·3 and an isoelectric point of 6·4.[14]

2.2 Molecular weight

Molecular weight and molecular weight distribution were determined by gel permeation chromatography using a series of Waters ultrastyragel columns in a Hewlett-Packard 1090 HPLC apparatus connected to a refractometer. The mobile phase was tetrahydrofuran at a flow rate of 1 cm^3 min^{-1}. Narrow poly(methyl methacrylate) standards were used for calibration. The logarithm of the molecular weight of the standards varied linearly with retention time.[14]

2.3 Isoelectric point

The isoelectric point of the polymer was determined by equilibrating neutral polymer powder with deionized water and measuring the pH of the resulting suspension. The values of the isoelectric pH determined in this way are reliable[18] because they are measured in the absence of salt which may interact with the polyampholyte and change its isoelectric point. The pI thus found was in good agreement with that obtained by the titration curve method as described by Merle *et al.*[19] and by use of solubility–pH data.[20]

2.4 Turbidimetric titration

Turbidity was measured spectrophotometrically as the optical density at 420 nm. Turbidimetric titrations of polymer and protein–polymer mixtures were carried out in 10 mM citric acid–phosphate buffer. The pH was varied between 3 and 9 by adding one drop (33 mm^3) of KOH of varying concentration (0–0·70 M in steps of 0·05 M) to each cuvette containing the polymer or protein–polymer solution. For each of the 15 cuvettes, the pH and the optical density at 420 nm were measured after a period of 30 min. This time period was fixed after observing the kinetics of the turbidity increase with time—the increase in turbidity was rapid during the first few minutes, then slowed down and reached a steady state by 30 min.

Soybean trypsin inhibitor solutions were filtered through a 0·22 μm Millipore filter to obtain a clear solution. Thirty minutes after polymer–protein contacting, the suspension was centrifuged for 20 min at 13 000 rpm. In this work, all values of polymer and protein concentrations and dosages refer to those obtained *after* mixing.

2.5 Materials

Soybean trypsin inhibitor (catalog number T 9128) and ribonuclease A from bovine pancreas (catalog number R 5503) was purchased from Sigma Chemicals. Solid potassium hydroxide was bought from Mallinckrodt Inc. while polyacrylic acid (25% solids in water; average molecular weight 90 000) was purchased from Polysciences Inc.

3 RESULTS AND DISCUSSION

3.1 Precipitation of pure polymer

First, polyampholyte–polyampholyte interaction (self-aggregation) was studied turbidimetrically as a function of pH and polymer concentration. Figure 1 represents turbidity–pH curves at various polymer concentrations (0·0025–0·015% for random acrylic polyampholyte). The pure polymer self-aggregates around its isoelectric point for a range of pH values; its solubility increases with pH on either side of the pI and the extent of aggregation increases with increase in polymer concentration (Fig. 1). The polymer titrations were performed by altering the pH from low to high values (i.e. 3 to 9) using KOH solutions. Thus, the random polyampholyte starts precipitating at pH 5·5 and goes back into solution at pH 7·5 in 10 mM citric acid–phosphate buffer (Fig. 1). The value of the isoelectric point for the random polymer was determined to be 6·4. The values of the critical pH (pH of onset of precipitation) in 10 mM citric acid–phosphate buffer were found to be about 5·5 and 7·5 for the random polyampholyte. The critical pH increased with increasing ionic strength but was unaffected by polymer concentration at constant ionic strength for neutral block polyampholyte (data not shown). Any apparent change in the critical pH with polymer concentration for random polyampholyte might be in part just a loss of sensitivity at low polymer concentrations. Thus, the critical pH may be determined by the critical net charge,[8,9] which is independent of the polymer concentration.

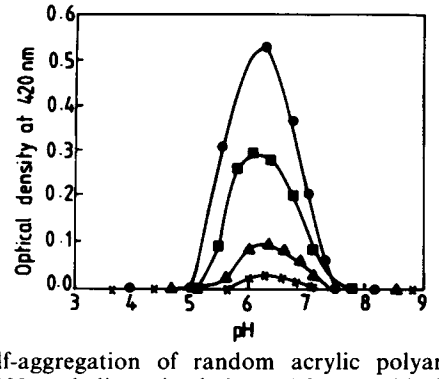


Fig. 1. Self-aggregation of random acrylic polyampholyte (M_n = 69 000; polydispersity index = 1·3; pI = 6·3) in 10 mM citric acid–phosphate buffer as a function of pH and polymer concentration (●, 0·15 mg cm^{-3}; ■, 0·1 mg cm^{-3}; ▲, 0·05 mg cm^{-3}; ×, 0·025 mg cm^{-3}) studied using turbidimetric titration.

3.2 Turbidimetry experiments on polymer–protein mixtures

Figure 2 shows the experimental turbidity–pH profiles for pure polymer and polymer–protein mixtures. Random polyampholyte (0·1 mg cm^{-3}) was tested with the proteins ribonuclease A and soybean trypsin inhibitor. The polymer dosage was 200 mg polymer per g protein. The polymer–protein mixtures exhibit a higher optical density at 420 nm than pure polymer—greater than 500% increases in the turbidity were observed for polymer–protein mixtures (compared with pure polymer) (Fig. 2). Similar results were obtained at a dosage of 50 mg polymer per g protein (0·025 mg cm^{-3} polyampholyte, 0·5 mg cm^{-3} protein) (Fig. 3). The critical pH of the polyampholyte–soybean trypsin inhibitor mixture was found to be lower than that of pure random polymer (4·5 compared with 5·5) (Figs 2 and 3). In other words, the critical pH shifts toward the pI of the protein. Further, the critical pH is not dependent on the polymer dosage (50–200 mg polymer per g protein) in the dilute regime, as seen from Figs 2 and 3. The turbidity increase and the shift in critical pH are two possible measures of the extent of polymer–protein interaction. Note that the increase in turbidity with polymer concentration may mean the formation of more particles, the formation of larger

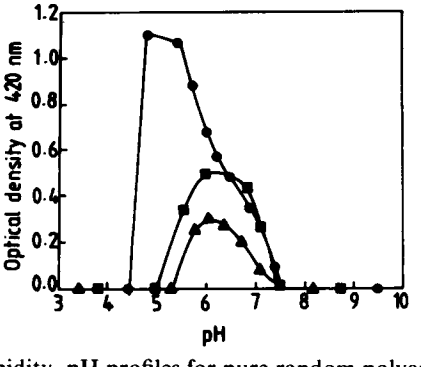


Fig. 2. Turbidity–pH profiles for pure random polyampholyte (0·1 mg cm^{-3}) (▲); polyampholyte + ribonuclease A (■); polyampholyte + ribonuclease A + soybean trypsin inhibitor (●) in 10 mM citric acid–phosphate buffer. The protein concentrations were 0·5 mg cm^{-3} resulting in a dosage of 200 mg polymer per g protein. All values of polymer and protein concentrations and dosages refer to those obtained after mixing.

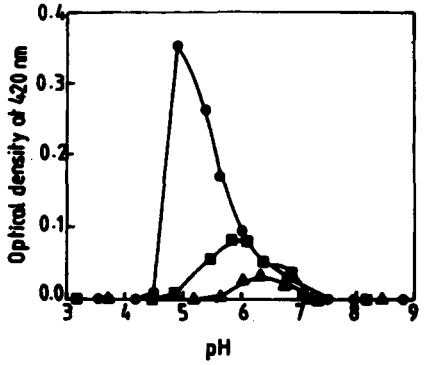


Fig. 3. Turbidity–pH profiles for pure random polyampholyte (0·025 mg cm^{-3}) (▲); polyampholyte + ribonuclease A (■); polyampholyte + ribonuclease A + soybean trypsin inhibitor (●) in 10 mM citric acid–phosphate buffer. The protein concentrations were 0·5 mg cm^{-3}, leading to a dosage of 50 mg polymer per g protein.

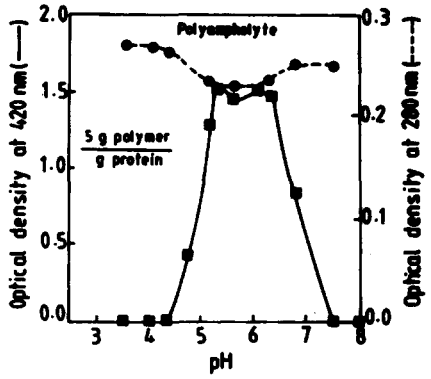


Fig. 4. Optical density at 420 nm (bold line) and absorbance at 280 nm (dashed line) of the supernatant after centrifugation for random polyampholyte–ribonuclease A (0·5 mg cm^{-3}) mixtures in 10 mM citric acid–phosphate buffer. The dosage measured 5 g polymer per g protein. After centrifugation, the optical density (420 nm) of the supernatant had a value of zero.

particles, or both. No aggregation was observed for pure protein solutions—their optical density at 420 nm vs pH yielded flat lines.

3.3 Determination of precipitated protein

It was now of importance to quantitatively determine the percentage of protein that actually precipitated and caused the large turbidity increases in the experiments shown in Figs 2 and 3. After 30 mins of polymer–protein contacting, the suspension was centrifuged at 13 000 rpm for 20 mins and the absorbance of the supernatant was measured at 280 nm. These readings showed the opposite trend from the absorbance values of the dissolved precipitate as expected from material balance. In all cases, the supernatant optical density at 420 nm was remeasured after centrifugation and was found to have a value of zero. The protein concentration was 0·5 mg cm^{-3} (after mixing with polymer) in all cases.

First, turbidity and absorbance measurements were carried out on random polyampholyte–soybean trypsin inhibitor mixtures at a dosage of 200 mg polyampholyte per g protein. Despite turbidity readings of close to 1·2 (the polyampholyte alone gave a maximum turbidity reading of 0·3) (Fig. 2), greater than 90% of the protein was found in the supernatant. Similar results were obtained for random polyampholyte–ribonuclease A mixtures at a dosage of 200 mg polymer per g protein (the maximum optical density reading for the mixture being 0·5 (Fig. 2)). It was therefore decided to increase the polyampholyte dosage to 5 g polyampholyte per g ribonuclease A. This corresponds to a molar ratio of polyampholyte to protein of 1:1. The results of this experiment are plotted in Fig. 4. The maximum turbidity reading now was about 1·5, but even at this increased dosage level, only about 10% of the ribonuclease A precipitated while about 90% of the protein was found in the supernatant (Fig. 4). Finally, experiments per-

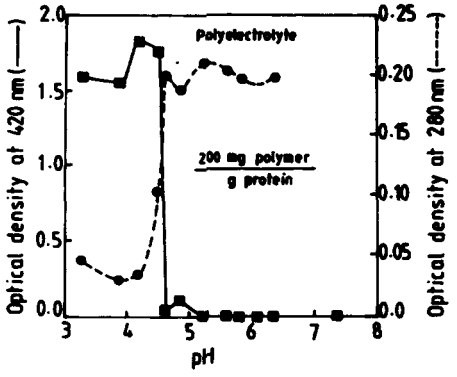


Fig. 5. Optical density at 420 nm (bold line) and absorbance at 280 nm (dashed line) of the supernatant after centrifugation for polyacrylic acid–ribonuclease A (0·5 mg cm^{-3}) mixtures. The polymer dosage measured 200 mg polymer per g protein. The optical density (420 nm) of the supernatant after centrifugation had a value of zero.

formed with a single polyelectrolyte (polyacrylic acid with an average molecular weight of 90 000) and oppositely-charged protein (ribonuclease A) at a dosage of 200 mg polyelectrolyte per g protein showed the opposite trend with about 90% of the protein in the precipitate and only 10% in the equilibrium liquid (Fig. 5).

Thus, despite the large turbidity increases (Figs 2 and 3), the polymer–protein complex formed may contain largely polyampholyte (polybase + polyacid + neutral residues) and only a little protein ($\approx$10%) when polyampholyte is used as the precipitating agent. This implies that even a very small extent (say on the order of 10% of the total protein present) of protein–polymer complexation can lead to *unusually large* (>500%) increases in turbidity. On the other hand, only about 10% of the protein stays in the supernatant, while 90% of the protein precipitates and is found in the polymer–protein complex when a polyelectrolyte such as polyacrylic acid is used for precipitation. Hence, the complexation behavior of proteins with random acrylic polyampholyte

can be radically different from that observed with oppositely-charged polyelectrolyte. Turbidimetric titration experiments should always be used in conjunction with centrifugation and A_{280} measurements on the supernatant and dissolved precipitate and/or total protein determination in the two phases. This will go a long way in ensuring that turbidimetric titration data are not erroneously interpreted.

4 CONCLUSIONS

A turbidimetric titration study of the interaction of proteins with a polyelectrolyte and a random acrylic polyampholyte (number-average molecular weight 70 000, polydispersity index 1·3) has been presented, embracing polyampholyte self-aggregation and polyelectrolyte and polyampholyte–protein complexation behavior as a function of pH and polymer dosage. Unlike the polyelectrolyte, the polyampholyte precipitated around its isoelectric point (even at low polymer concentrations (0·1 mg cm^{-3})) because of electrostatic attraction between the molecules. The following conclusions were arrived at in this study:

1. A critical pH, independent of the polymer concentration, characterized the onset of turbidity increase.
2. Large (>500%) turbidity increases were observed for both protein–polyelectrolyte and protein–polyampholyte mixtures (compared with pure polyampholyte) for various polymer dosages.
3. Use of random acrylic polyampholyte precipitated *only about 10%* of the protein while the remaining 90% of the protein stayed in the equilibrium liquid. Thus, a small degree of polymer–protein interaction can lead to unusually large increases in turbidity. The same results were obtained at various polymer dosages (50 mg–5000 mg polymer per g protein). On the other hand, use of polyelectrolyte precipitated *more than 90%* of the protein at *very low dosages* (200 mg polymer per g protein). Similar results were obtained with other acrylic polyampholyte systems.
4. Turbidimetric titration experiments should always be *supplemented* by centrifugation and A_{280} measurements on supernatant and dissolved precipitate and, in the case of crude extracts, with enzyme activity and total protein determinations in the two phases.
5. As far as protein precipitation is concerned, use of polyelectrolytes proves to be far more efficient than use of random polyampholytes.

ACKNOWLEDGEMENTS

The author thanks Prof. T. A. Hatton and Dr C. S. Patrickios for facilities and assistance and Mr S. K. Ahuja for help with polymer characterization and centrifugation experiments.

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