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SELECTIVE PRECIPITATION OF WATER-SOLUBLE PROTEINS USING DESIGNED POLYELECTROLYTE

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ABSTRACT

By using lysozyme, hemoglobin (bovine), and pepsin as the model proteins and copolymer F1110 containing reactive functional groups of amido, sulfonate, and carboxylic acid groups as the flocculating agents, selective precipitation of proteins was performed by using two chemical processes. The experiment shows that the surface charge of copolymer F1110 is closely related to the pH value in solution. The flocculation behaviors of proteins with F1110 can be controlled completely by adjusting pH value in solution, which realizes the selective separation of mixed proteins. In addition, the selective precipitation of the proteins is promoted by a "modified flocculation" process, in two steps with a combination of F1110 and polyacrylamide. The characterization of modified flocculation is closely related to the property of the protein. These studies show a great prospect for selectively separating proteins from a mixture solution using designed polyelectrolytes containing reactive functional groups.

Key Words: Water-soluble protein; Polyelectrolyte; Flocculation; Selective precipitation

INTRODUCTION

It is well known that the removal and recovery of proteins in downstream processing is always limited by the extraction conditions or other separation methods. An alternative approach is to flocculate the cell debris using polymer flocculating agents and then recover bio-products from the fermentation broth (1,2). Protein separation arises during polyelectrolyte coacervation as a consequence of selective protein–polyelectrolyte complexation (3,4), which seems to be a very attractive and practical approach. This phenomenon may afford the possibility of large-scale protein purification in a manner that is fast, efficient, and inexpensive compared to traditional protein separation methods such as large-scale chromatography, filtration, or centrifugation (5).

Usually, the polyelectrolytes undergo fast and reversible changes in microstructure triggered by small changes of medium property (pH, temperature, ionic strength). These properties of the polyelectrolytes are exploited for the development of new protein purification techniques: affinity precipitation, affinity partitioning, and temperature-induced elution in dye-affinity chromatography (6). The studies on stability and efficiency of the interaction of protein and polyelectrolyte have been performed as a function of polymer molecular weight (7), and polyelectrolyte adsorption is used to modify the surface of chromatographic packings in order to make them more suitable for protein separation (8). The formation of protein–polyelectrolyte complexes is known to be driven by electrostatic interaction, and electrostatic parameters such as protein surface charge density and polymer linear charge density (9). Coacervation is found to occur when the polyelectrolyte–micelle complex that follows is at least about 45 nm. An increase in molecular weight of polyelectrolyte reduces the micelle charge required for coacervation and also increases coacervate volume fraction (10). In addition, the solution chemistry can influence both the formation and the stability of the protein–polyelectrolyte complexes. The efficiency and selectivity of the process are the two key factors that affect the applicability of polyelectrolyte coacervation to protein separation (4). In a mixed polymer system, there exists competitive adsorption relevant to the flocculation of proteins (11). The composition of a flocculating agent plays an important role in controlling the selective flocculation behavior (12). In previous studies, Nath demonstrated that little proteins could be precipitated through a random copolyelectrolyte (13). The studies on lysozyme flocculation with anionic

copolymer containing reactive functional groups show that complexation behavior of lysozyme and anionic copolymer can be controlled easily through adjustment of pH value in solution (14). In addition, the method of modified flocculation had been performed to investigate the flocculation behavior of lysozyme with a combination of anionic copolymer and polyacrylamide (15). These studies provide an alternative approach to control the flocculation behavior of protein.

One of the big problems we must consider is how to protect bio-product from denaturalization while extracting it from a mixture. Strong changes of pH value and ionic strength in solution could incur irreversible structural changes of bio-products. However, the denaturalization of protein incurred by polyelectrolyte is reversible and temporary. The objective of this paper is to take advantage of the mild chemistry of polyelectrolyte in solution to perform selective separation of three model proteins: pepsin, lysozyme, and hemoglobin (bovine), which were selected on the basis of their different isoelectric points (pIs) and molecular weights. Most important is to control the flocculation behavior of protein by designing polyelectrolyte containing specified functional groups based on the samples. A process for selective flocculation of proteins is proposed as shown in Fig. 1. The studies are focused on the interaction between protein and a synthetic copolymer F1110 containing reactive functional groups—especially, the possibility and efficiency of selectively precipitating proteins from the mixture by controlling the aqueous pH, and using modified flocculation process. It is necessary to do more studies on the effect of flocculating agent on selective separation of mixture proteins and how to isolate protein from protein–polyelectrolyte complexes efficiently in future work.

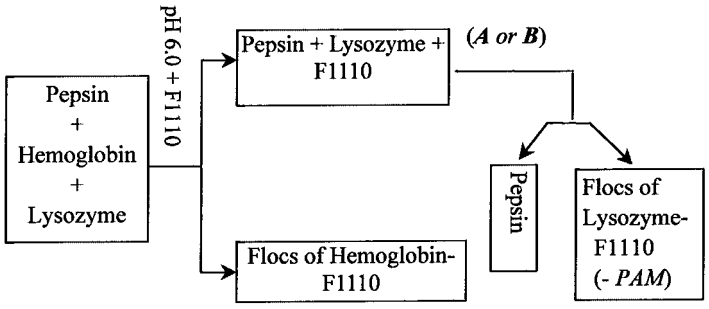

Figure 1. Schematic of selective flocculation of proteins using copolymer F1110: (***A***) the aqueous pH was adjusted to 9.30, the flocs are complexes of lysozyme and F1110; (***B***) PAM was added to the solution of proteins and F1110, then the flocs of lysozyme–F1110–PAM were produced.

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EXPERIMENTAL SECTION

Materials

The model protein, hemoglobin (bovine), was purchased from Sigma Company (USA), and lysozyme and pepsin from BeAo BioChemical Company, Shanghai, China.

Neutral polyacrylamide (nPAM, M_W: 300–500 million) was purchased from Tianjing Chemical Industrial Factory, China. The cationic polyacrylamide (cPAM) is a kind of derivative of nPAM prepared as described by Ma (16).

The anionic copolymer F1110 was prepared according to the method described by Yu (14,15). The radical polymerization initiated by $(NH_4)_2S_2O_8$–Na_2SO_3 (w/w = 1/1) was carried out at 70–80°C. All the solutions of chemicals were bubbled continuously for 30 min with nitrogen before reaction began in order to remove oxygen. Desirable material ratios of three monomers (acrylamide, sodium sulfonate, and acrylic acid) were mixed to synthesize the copolymer F1110 in 200 mL deionized water with 200 mg of the initiator $(NH_4)_2S_2O_8$–Na_2SO_3. A condenser was placed at the top to prevent monomer loss by evaporation, and nitrogen gas was bubbled continuously through the solution to prevent contamination by oxygen from the temperature inlet during reaction. Acetone was added to the reaction solution and the products were precipitated at room temperature. The anionic copolymer F1110 was dried at 70–80°C in vacuum for 10 hr. Its molecular weight is 1.2×10^4 by a Dynamic Light Scattering Photometer DLS-700 and a Difference Refractometer RM-102 (Japanese Otsuka Electronics), and its composition was established by elemental analyses of S, N and C as $-SO_3Na/-NH_2/-COOH$ = 1:5.77:6.81.

The flocculant stock solutions were approximately 2 g/L for F1110, and 1 g/L for nPAM and cPAM, respectively.

Flocculation Test

Precipitation of Proteins with F1110

The precipitation tests were carried out in a 50 mL glass beaker with magnetic stirring. An amount of 0.3 mg of copolymer F1110 was mixed with 10 mL of aqueous solution containing 3 mg protein, with stirring for 15 min. The filtrate was collected after the solution was centrifuged at 2500–3500 rpm. The flocculation was studied at the desired pH values. Protein solutions of desired pH were prepared by adding 0.1 *M* HCl prior to adding F1110 in solution.

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Precipitation of Proteins Using Modified Flocculation Process

As Yu (15) described, protein is first interacted with F1110 for forming fine "modified protein" particles, then polyacrylamide (nPAM or cPAM) is added into the modified protein solution for forming large flocs to precipitate. In this work, 150 μL (0.3 mg) copolymer F1110 was first mixed with 10 mL protein solution and stirred for 5 min, then followed by the addition of a given amount of nPAM or cPAM. Strong agitation ensures that the nPAM or cPAM disperses homogeneously in the initial period. Ten minutes later, the mixture was centrifuged to obtain the supernatant layer for determining the content of the residual protein.

Measurement

The optical density (OD) of protein concentration in the supernatant layer was measured by UV–Vis spectroscopy at 280 nm for lysozyme, 405 nm for hemoglobin and 271 nm for pepsin, respectively, with a 7542 UV–Vis spectrometer (Shanghai, China). The flocculation efficiency of proteins was determined by referring the OD values of protein solution in clear supernatant layer to that of the protein originally present in the initial aqueous solution.

RESULTS AND DISCUSSION

Selective Precipitation of Proteins with Adjusting pH in Solution

The polyelectrolyte–protein interaction is determined by electrostatic interaction. Therefore, it is expected to be sensitive to pH and ionic strength. In this study, an attempt was made to study the effect of pH on selective precipitation of protein from mixture with the flocculant containing reactive functional groups. On the basis of its composition, F1110 contains three kinds of functional groups: $-SO_3Na$, $-NH_2$ and $-COOH$. While decreasing the pH of F1110 solution to less than 2.5, it shows a turbidity point at pH 2.50 due to F1110 precipitation from water solution. This suggests that the charge density on the surface of F1110 is closely related to the aqueous pH values. It is expected to change aqueous pH to control the interaction of protein and F1110.

Figures 2–4 show the optical density of hemoglobin solution as function of the dosage of F1110 with different aqueous pH. From Fig. 2, the optical density of hemoglobin solution was not nearly influenced with increase in dosage of F1110 at pH 3.9 and 9.3. However, the optical density of hemoglobin solution decreases to the lowest point at a dosage of 60 μg F1110/mg hemoglobin at pH

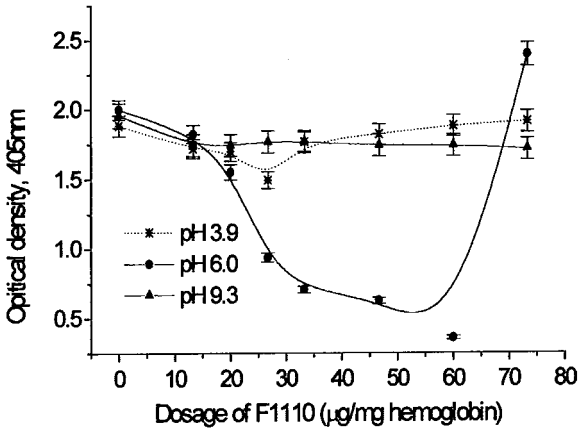

Figure 2. Flocculation of hemoglobin at different pH. Effect of F1110 dosage on optical density of hemoglobin solution (405 nm).

6.0, corresponding to around 89% removal of hemoglobin from water solution. This is usually called as Optimal Flocculation Dosage (OFD).

It is evident that such behavior of flocculation can be attributed mainly to the electrostatic interaction between the flocculating agent and protein molecules, because the aqueous pH determines the ionization state of the carboxylic groups. As an anionic copolymer with carboxylic groups, the surface of F1110 has a higher electrical density at pH 6.0 than at pH 3.9. Thus, the interaction between hemoglobin and F1110 is beneficial at higher pH value—around 89% 1of hemoglobin was removed from aqueous solution with a dosage of 60 μg/mg hemoglobin at pH 6.0 in contrast to no removal of hemoglobin at pH 3.9. However, increasing the aqueous pH to 9.30 brought a negative effect of pH on flocculation of hemoglobin, which may arise from a decreasing interaction between hemoglobin and F1110. Because electrical density on the surface of F1110 cannot be increased further up to pH 9.3 and higher pH value may reduce the charge on the surface of hemoglobin molecule.

Figures 3 and 4 show that the percentage of protein flocculation (e.g., of lysozyme and pepsin) is related to their different pIs. The flocculation of lysozyme does not occur at the aqueous pH 5.5 (Fig. 3). However, the lysozyme shows the similar flocculation trends at pH 9.3 and 10.3, and around 95% lysozyme can precipitate from solution at the optimal dosage of 67 μg/mg lysozyme. Evidently, the electrostatic interaction is still dominant in the interaction between lysozyme and F1110. The surface of the lysozme molecules is positively charged when the aqueous pH is less than the pI (11.0) of lysozyme, which suggests that the negatively charged F1110 has stronger bonding ability to

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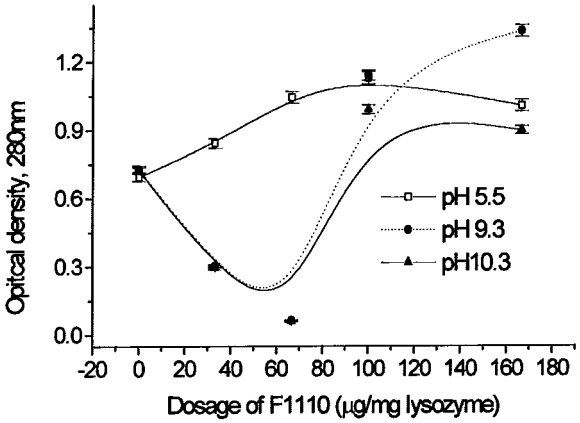

Figure 3. Flocculation of lysozyme at different pH. Effect of F1110 dosage on optical density of lysozyme solution (280 nm).

lysozyme in contrast to hemoglobin (pI = 6.90). This brings about higher flocculation efficiency of lysozyme than that of hemoglobin in alkaline solution.

Comparing the results in Figs. 2 and 3 with those of Fig. 4, it is found that little pepsin was flocculated with increase in the dosage of F1110 at pH 3.81, and increasing pH brings little changes in pepsin flocculation (results not shown). The pI of pepsin is 1.0, which suggests that it is negatively charged when the pH is higher than 1.0. Thus, the interaction between F1110 and pepsin is not favored at pH values up to the pI of the enzyme.

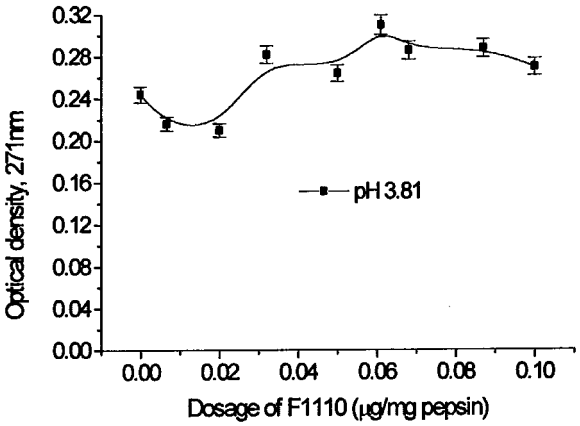

Figure 4. Effect of F1110 dosage on flocculation of pepsin at pH 3.81.

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Therefore, proteins with initial different pI show different flocculation behavior with F1110, and the flocculation behavior of proteins with F1110 can be controlled by adjusting the pH in solution to change the electric charge on the surface of F1110. On the basis of the above results, the mixture containing lysozyme, hemoglobin, and pepsin can be separated by using flocculating agent F1110 by adjusting the aqueous pH values, as the method *A* shown in Fig. 1. First, around 89% of hemoglobin can be flocculated by addition of F1110 at neutral pH in solution. Secondly, increasing aqueous pH up to 9.30, around 95% of lysozyme can be precipitated from the mixture, and finally pepsin is collected from the residual solution.

Modified Flocculation of Proteins by a Combination of F1110 and PAM

In some cases, it was expected to separate bio-products from mixture without changing solution environment for protecting bio-products against being damaged. Generally speaking, higher flocculation efficiency can be achieved by using higher molecular weight polyelectrolytes. However, flocculation of water-soluble protein does not always occur with polyelectrolyte (12). Some of the reasons can be that the proteins have low molecular weight, and the interaction between protein and polyelectrolyte is not strong enough to form large flocs to precipitate. As it was shown once, the nPAM with molecular weight of 300–500 million could not remove any lysozyme from protein water solution (15). Therefore, the flocculation behavior of protein is closely related to the polyelectrolyte structure and the affinity of functional groups in polyelectrolyte to proteins. The results shown in Fig. 3 indicate that F1110 cannot form large flocs with lysozyme and just make the protein solution opaque at pH 5.5, which displays some fine particles formed in the protein solution. The fine particles are the complexes of lysozyme and F1110 due to electrostatic attraction. The particles can be regarded as "modified lysozyme" because the reactive functional groups of F1110 attach to the surface of lysozyme molecules after the latter interact with F1110. Figure 5 shows that the flocculation efficiency of lysozyme is highly promoted with dosing PAM (cPAM or nPAM) into the solution of modified lysozyme. Almost all lysozyme can be removed with a dosage of 100 μg cPAM in the mixture. In contrast to cPAM, about 78% of lysozyme can be removed with a dosage of 100 μg nPAM. The differences between cPAM and nPAM may be attributed to the electrostatic interaction between modified lysozyme and cPAM, and "bridging" through hydrogen bonding between modified lysozyme and nPAM (15). Higher charge density on the surface of cPAM helps to enhance the interaction of modified lysozyme and cPAM.

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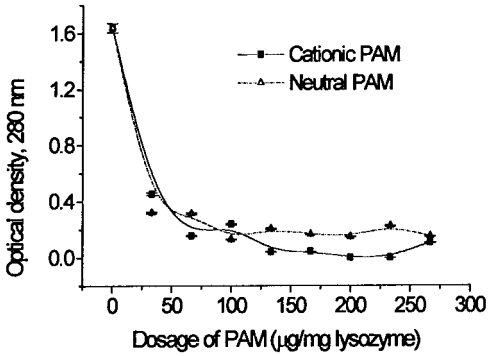

Figure 5. Modified flocculation of lysozyme with a combination of PAM and F1110. Optical density of lysozyme solution (280 nm) as a function of PAM dosage in 10 mL solution of 3 mg lysozyme and 0.3 mg F1110.

Under the same conditions, there is no flocculation for hemoglobin (Fig. 6) and pepsin (not shown in this text). According to Fig. 2, around 89% of hemoglobin is flocculated from its aqueous solution with the addition of 60 μg F1110/mg hemoglobin at pH 6.0. It was noted that the flocculation behavior is controlled by the ratio of hemoglobin to F1110 also. Increase in addition of F1110 will lead the protein solution to be opaque. If the mass ratio of hemoglobin to F1110 is less than around 17, according to Fig. 7, hemoglobin can only form fine particles with F1110. But the mass ratio of hemoglobin and F1110 is 10 in the system shown in Fig. 6, which suggests that large amount of fine particles of negatively charged "modified hemoglobin" are formed in the solution. Thus,

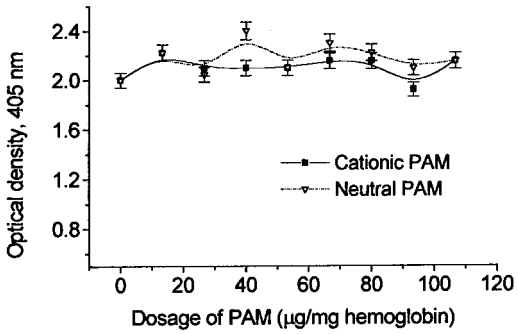

Figure 6. Modified flocculation of hemoglobin with a combination of PAM and F1110. Optical density of hemoglobin solution (405 nm) as a function of PAM dosage in 10 mL solution of 3 mg hemoglobin and 0.3 mg F1110.

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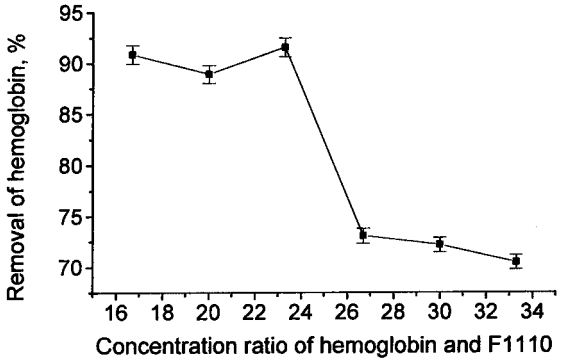

Figure 7. Effect of concentration ratio of protein and F1110 on removal of hemoglobin at the aqueous pH 6.0.

electrostatic repulsion among primary flocs (fine particles of modified hemoglobin) caused the primary fine particles to restablilize (17), and protein solution became opaque and no flocculation occurred.

Therefore, the mixed proteins can be separated selectively by a combination of F1110 and polyacrylamide as the method *B* shown in Fig. 1: first, F1110 is added to the mixture to remove hemoglobin from the mixture, and secondly the lysozyme can be separated from the aqueous mixture with addition of polyacrylamide. Finally, the residual solution contains only pepsin.

CONCLUSIONS

The surface charge density on the surface of F1110 containing functional groups is related closely to the aqueous pH by ionization of the functional groups of F1110, which affects strongly the interaction of protein molecules and F1110. The flocculation behaviors of proteins can be controlled by adjusting pH in solution. In addition, the flocculation behaviors of proteins are based on their isoelectric point. These results provide the possibility of selectively separating protein from a mixture.

In addition, the modified flocculation process shows that the reactive function groups of F1110 attached to protein can enhance the interaction between lysozyme and high molecular weight polyelectrolyte. The characterization of modified flocculation of protein is also concerned with properties of protein molecule and mass ratio of protein to flocculant, the differences among the three model proteins also provides the possibility of selectively separating them from a mixture by a combination of F1110 and PAM.

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