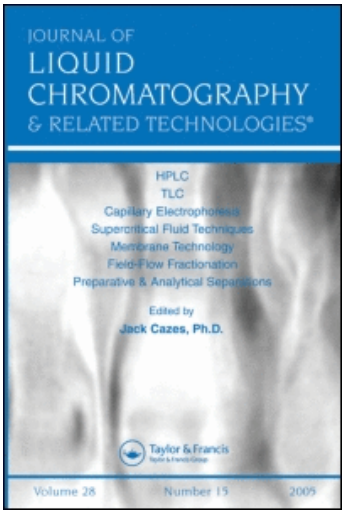

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PURIFICATION OF PEG-PROTEIN CONJUGATES BY CENTRIFUGAL PRECIPITATION CHROMATOGRAPHY

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ABSTRACT

Preparative scale purification of PEG-protein conjugates by centrifugal precipitation chromatography is discussed. By utilizing the transport of ammonium sulfate across a membrane and the centrifugal force, an ammonium sulfate gradient is formed and used for differential precipitation of PEG-protein conjugates. Native proteins can be separated from PEG-protein conjugates by using this technique.

INTRODUCTION

PEG-protein conjugates have long been used in several areas of biotechnology. By conjugation of PEG (polyethylene glycol) to protein, some proteins' properties, for example hydrophobicity, change significantly.[1] The increase of hydrophobicity benefits the usage of protein in some applications, especially in drug delivery where PEG conjugation increases the circulation time of protein, increase solubility, and reduces proteins' antigenicity.[2,3,4] Apart from drug deliv-

ery, PEG-protein conjugates are also applied for purification of proteins in affinity partitioning in aqueous two-phase systems.[5,6]

Since PEG-protein conjugates have been widely used in several applications, the conjugation reaction between PEG and protein has been extensively discussed in the literature.[1,7] In principle, the conjugation reaction between PEG and protein can be performed by mixing activated PEG and protein under the proper conditions. Unless the protein is very small, unreacted PEG and some small by-product can be separated from unreacted protein and PEG-protein conjugates by utilizing membrane separation or gel filtration. While the conjugation reaction and the separation of unreacted PEG from unreacted protein and PEG-protein conjugates is straightforward, the separation of unreacted protein from PEG-protein conjugates is troublesome, especially for preparative scale separation. Because molecular weights and the charges of PEG-protein conjugates and that of proteins are very similar, the separations by size and charge differences are difficult to achieve. In fact, Gotoh et al.[8] showed that the retention time in gel filtration of native silk fibroin and PEG-silk fibroin conjugates overlapped, while attempts to purify PEG-protein conjugates by ion-exhange chromatography[9,10] and isoelectric focusing[11] failed to separate highly-modified PEG-protein conjugates.

Since therapeutic efficacy of modified protein depends on the extent of PEGylation,[9] a better purification technique for PEG-protein conjugates is needed. Recently, Robert and Harris[12] have successfully shown that capillary electrophoresis could be used for the separation of PEG-lysozyme conjugates. Unfortunately, capillary electrophoresis is of analytical in nature and, thus, very difficult to scale up.

Although Delgado et al.[13] and Sookkumnerd et al.[14] have shown that counter-current distribution with polymer aqueous two-phase systems can be used for preparative scale purification of PEG-protein conjugates, the technique is troublesome and also difficult to scale up. In this article we, thus, investigate the possibility of using centrifugal precipitation chromatography as a technique to purify PEG-protein conjugates in preparative-scale. Centrifugal precipitation chromatography is the new protein purification technique, which utilizes the differences in protein solubility, invented by Ito.[15]

Briefly, the transport of ammonium sulfate across a membrane is used to form an ammonium sulfate gradient. Upon applying the centrifugal force, the gradient can readily be used for differential precipitation of PEG-protein. When PEG-protein is loaded into a gradient under the centrifugation, PEG-protein precipitates according to their solubility. Then, the gradient is gradually decreased and shifted toward the outlet. Because of the moving of the gradients, PEG-protein moves according to their solubility. With this strategy, mixture of PEG-protein conjugates can be purified.

In the experiment, PEG-lysozyme was first synthesized by mixing PEG-p-nitrophenyl carbonate (PEG-NPC) with lysozyme. By varying the amount of PEG and lysozyme, different degrees of modification PEG-lysozyme conjugates were synthesized. The average number of PEG conjugating to lysozyme of the conjugates was analyzed by using fluorescamine and fluorescence spectrophotometer. Then, a mixture of PEG-protein conjugates was loaded into thr centrifugal precipitation chromatograph. The eluted PEG-protein conjugates were detected using the UV spectrophotometer at 280 nm and SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

EXPERIMENTAL

Materials

PEG 5000-p-nitrophenyl carbonate (PEG 5000-NPC) was purchased from Shearwater Polymer. Other chemicals were of analytical grade and were purchased from Sigma or Fisher. The centrifugal precipitation chromatography used in the experiment was the same type as described in Ito.[15]

Synthesis and Characterization of PEG-Lysozyme Conjugates

PEG-lysozyme was synthesized from PEG-NPC based on Veronese et al.[16] The temperature of reaction was set to 4°C and the reaction time was approximately 48 hours. The number of PEG conjugated to lysozyme was analyzed by using fluorescamine and fluorescence spectrophotometer according to Karr et al.[17] PEG-lysozyme was then lyophilized and kept at below 0°C.

Centrifugal Precipitation Chromatography of PEG-Protein Conjugates

15 mg of PEG-lysozyme, which was composed of 2.7 PEG per lysozyme, was mixed with 10 mg of PEG-lysozyme, which was composed of 1.46 PEG per lysozyme, and 5 mg of native lysozyme. The mixture had the average of 1.84 PEG per lysozyme and was dissolved in 1 mL of 50 mM potassium phosphate buffer, pH 7.0. The salt channel of the centrifugal precipitation chromatograph was eluted out with 40% saturation ammonium sulfate solution at 1 mL/min, while the water channel was eluted out with 50 mM potassium phosphate buffer, pH 7.0 at 0.06 mL/min. After 10 minutes of the elution, the mixture of PEG-lysozyme was injected into the water channel. Then, the fed ammonium sulfate concentration of the salt channel was kept at 40% saturation for 4 hours before it was gradually decreased to 0% saturation in 30 hours in order to elute the sample out.

The eluent from the water channel was continuously detected by the UV spectrophotometer at 280 nm and later collected by the fraction collector every

20 minutes. The fractionated samples were desalted by ultrafiltration and characterized by SDS-PAGE (sodium dodecyl sulfate gel electrophoresis).

RESULTS AND DISCUSSIONS

The chromatogram of PEG-lysozyme mixture from centrifugal precipitation chromatography is shown in Figure 1. As seen in Figure 1, PEG-lysozyme conjugate is fractionated to four fractions. Since PEG probably reduces the solubility of protein, it is reasonable to deduct that the first peak is lysozyme, the second peak is PEG-lysozyme, the third peak is $(PEG)_2$-lysozyme, and the last peak is composed of heavier PEG-lysozyme conjugate. This observation is proven by SDS-PAGE as shown in Figure 2. It should be noted that PEG-lysozyme conjugate, especially the heavier ones, seems to be heavier than its actual value (using molecular weight marker as a standard). We believe that the binding of PEG to lysozyme obstructs the binding of SDS to lysozyme, causing

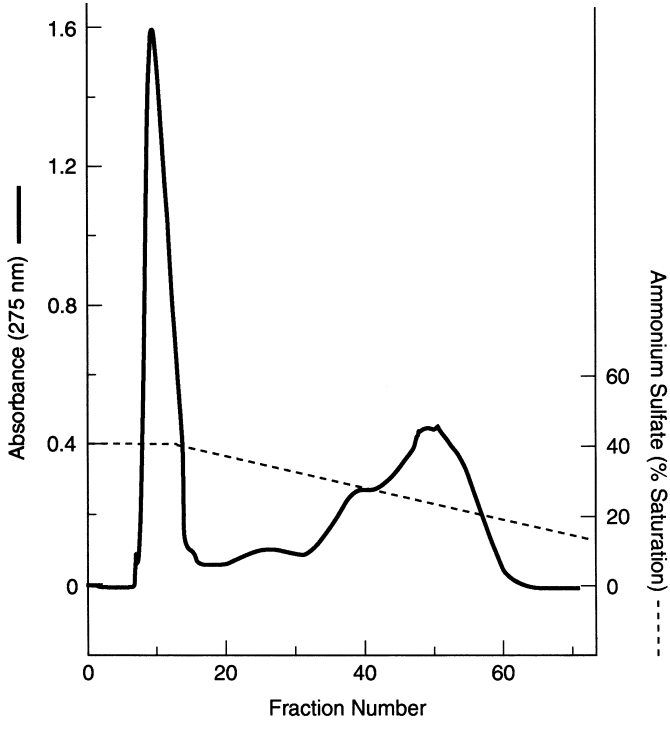

Figure 1. Chromatogram of PEG-Lysozyme mixture from centrifugal precipitation chromatography.

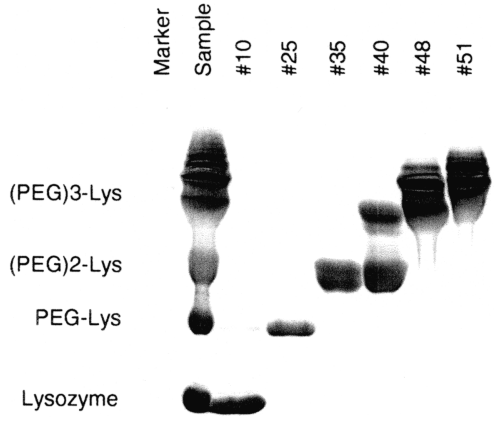

Figure 2. SDS-PAGE of fractionated PEG-lysozyme from centrifugal precipitation chromatography.

the PEG-lysozyme-SDS complex to have less charge per mass than the native proteins. Therefore, when running SDS-PAGE, the apparent molecular weight of PEG-lysozyme is heavier than its actual molecular weight.

As seen from Figure 1 and 2, native lysozyme and PEG-lysozyme can be purified readily in preparative scale by centrifugal precipitation chromatography. However, there is the difficulty in purifying heavier PEG-lysozyme. $(PEG)_3$-lysozyme appears to be eluted out with $(PEG)_4$-lysozyme and heavier (PEG)-lysozyme. By applying the more appropriate ammonium sulfate gradient, it should be possible to purify heavier (PEG)-lysozyme. Part of the concurrent research in our group is to optimize the ammonium sulfate gradient, which is suitable for this separation.

CONCLUSIONS

We have shown in this article that native protein and PEG-protein conjugates have different solubility in ammonium sulfate solution. For PEG-protein conjugates, the dependence of number of PEG conjugating to protein on its solubility is also illustrated. We have used these differences to fractionate the mixture of PEG-protein conjugates and protein by using centrifugal precipitation chromatography. From the experiment, we have shown that it is possible to separate protein from PEG-protein conjugates and also to separate light PEG-protein conjugates from the heavier ones. Although there is partial resolution in separating heavier PEG-protein conjugates, work on the optimization of

ammonium sulfate gradient in order to achieve the better separation is under progressed and will be reported in the future.

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