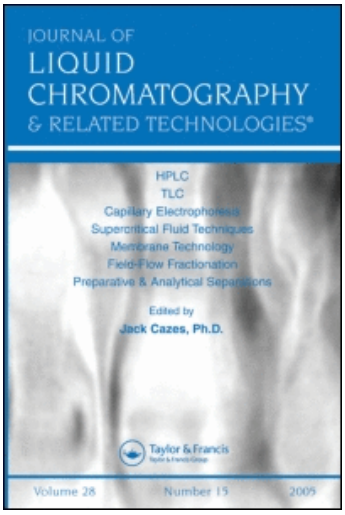

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PURIFICATION OF PEG-PROTEIN CONJUGATES BY COUNTERCURRENT DISTRIBUTION IN AQUEOUS TWO-PHASE SYSTEMS

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ABSTRACT

Purification of PEG-protein conjugates by countercurrent distribution (CCD) in aqueous two-phase systems is reported. By using CCD in 19.36% PEG 3400/14.68% potassium phosphate, we can separate PEG-lysozyme conjugates, which contains an average number of 1.14 PEG per lysozyme, into three peaks with 55 number of transfers. Further analysis of the samples reveals that these three peaks mainly represent unmodified lysozyme, $(PEG)_1$-lysozyme and $(PEG)_2$-lysozyme.

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' antigenic-

ity.[2,3,4] Apart from drug delivery, 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 condition. 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 protein 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 were overlapped, while attempts to purify PEG-protein conjugates by ion-exchange 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] the better purification technique for PEG-protein conjugates is needed.

In addition, because of the lack of techniques to separate PEG-protein conjugates from unreacted protein (and also, for example $(PEG)_1$-protein from $(PEG)_2$-protein, etc.), the number of PEG conjugated to protein is generally reported in the literature as the average number of PEG in PEG-protein conjugates; hence, the differences in the distribution of PEG-protein conjugates is neglected.

In this article, the possibility of using countercurrent distribution (CCD) in aqueous two-phase systems as a technique to purify PEG-protein conjugates in preparative-scale is investigated. Countercurrent distribution in aqueous two-phase systems has been proven to be highly selective for closely related proteins,[12] which have only slight differences in partitioning behavior. Since it was suggested that the partition coefficient of $(PEG)_1$-protein should be different from unmodified protein, $(PEG)_2$-protein, $(PEG)_3$-protein and etc.,[13] the prospect of using countercurrent distribution for preparative-scale purification of PEG-protein conjugates is very promising. Recently, a similar approach has been conducted by Delgado et al.,[14] in which they selected PEG/dextran as a phase system.

In the experiment, PEG-lysozyme conjugates were first synthesized by mixing PEG-p-nitrophenyl carbonate (PEG-NPC) with lysozyme. By mixing

the appropriate amount of PEG-NPC and lysozyme, desired 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-lysozyme conjugates was subjected to countercurrent distribution in 19.36% PEG 3400/14.68% potassium phosphate. After 55 number of transfers, the contents in each tube were diluted and unloaded. The absorbance at 280 nm of the samples in each tube was measured by an UV-VIS spectrophotometer. The average number of PEG conjugating to lysozyme in selected tubes was then analyzed with fluorescamine and fluorescence spectrophotometer.

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.

Synthesis and Characterization of PEG-Lysozyme Conjugates

PEG-lysozyme conjugates were synthesized from PEG-NPC based on Veronese et al.[15] 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,[16] PEG-lysozyme conjugates were then lyophilized and kept at below 0°C.

Countercurrent Distribution of PEG-Protein Conjugates

The phase system of 19.36% PEG 3400/14.68% potassium phosphate (pH 7.0) was formed by mixing appropriate amounts of PEG 3400 (in solid form), 22% potassium phosphate buffer, pH 7.0, and water for 3 hours. The phase system was left to settle for 24 hours at room temperature in a separating funnel. After that, the top and bottom phases were separated.

PEG-lysozyme conjugates were dissolved in the separated top phase. After it is dissolved, 0.5 mL of the top phase, containing PEG-lysozyme conjugates, was transferred to the first tube, in which 0.5 mL of the fresh bottom phase was already in the tube. The phases were mixed and left to settle. Then, the top phase in the first tube was transferred to the fresh bottom phase in the second tube, mixed, and left to settle. At the same time, the fresh top phase was

mixed with the bottom phase in the first tube and then left to settle. This process was continued 55 times. The total number of transfers was thus 55.

RESULTS AND DISCUSSION

Synthesis and Characterization of PEG-Lysozyme Conjugates

By mixing PEG-NPC and lysozyme under the basic condition, PEG-lysozyme conjugates were obtained. Following the procedure of Karr et al.,[16] the PEG-lysozyme conjugates were analyzed. It was found that PEG-lysozyme conjugates contained 1.14 PEG per one lysozyme.

Countercurrent Distribution of PEG-Lysozyme Conjugates in PEG 3400/Potassium Phosphate System

The chromatogram of countercurrent distribution of PEG-lysozyme conjugates was shown in Figure 1. As shown in Figure 1, three distinguished peaks were observed at tube number 6, 19, and 45. Assuming that the countercurrent distribution was ideally operated, these three distinguished peaks corresponding to the substance that had the partition coefficients of 0.122, 0.528, and 4.5, respectively. Since it was observed by others[5] that conjugating more PEG to protein increased the partition coefficient of PEG-protein, these three distinguished peaks probably represented unmodified lysozyme, $(PEG)_1$-lysozyme and $(PEG)_2$-lysozyme. Using fluorescamine and fluorescence spectrophotometer according to Karr et al.,[16] the number of PEG conjugating to lysozyme of these three peaks was 0.1468, 1.193, and 1.753, respectively. Therefore, within the experimental errors, these three peaks represented unmodified lysozyme, $(PEG)_1$-lysozyme and $(PEG)_2$-lysozyme.

As seen in Figure 1, $(PEG)_2$-lysozyme was partially purified from unmodified lysozyme and $(PEG)_1$-lysozyme. On the other hand, the peaks of unmodified lysozyme and $(PEG)_1$-lysozyme were slightly overlapped. Theoretically, the resolution of unmodified lysozyme and $(PEG)_1$-lysozyme can be improved by increasing the number of transfers. Because of the mild reaction condition, the highest number of PEG conjugating to lysozyme will be approximately 2; thus, no peaks of the heavier PEG-lysozyme conjugates (hence, $(PEG)_3$-lysozyme, $(PEG)_4$-lysozyme and etc.) were observed.

CONCLUSIONS

In this article, we have shown that it is possible to purify PEG-lysozyme conjugates by countercurrent distribution in aqueous two-phase systems. We have used countercurrent distribution on PEG-lysozyme conjugates, containing

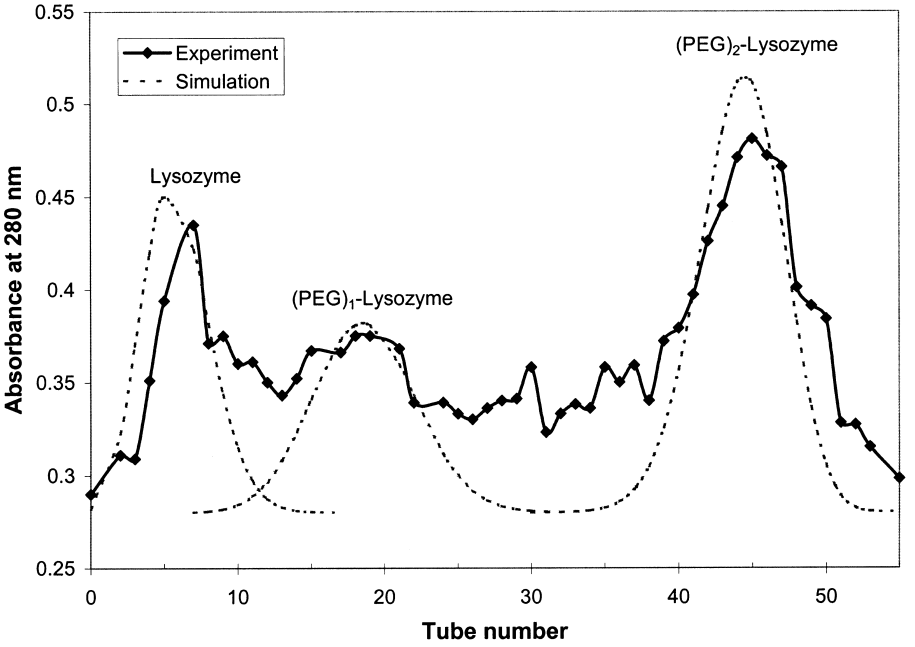

Figure 1. Countercurrent distribution (CCD) of PEG-lysozyme conjugates in 19.36% PEG 3400/14.68% potassium phosphate, pH 7.0, at 25°C. Three distinguished peaks were observed at tube number 6, 19, and 45. The average number of PEG conjugating to lysozyme of each peak was labeled. 3.52 mg of PEG-lysozyme conjugates, which contains an average of 1.14 PEG per lysozyme, was loaded into the countercurrent distribution. Solid line: experimental result; Dashed line: simulation results.

an average of 1.14 PEG per lysozyme. From CCD, we can separate PEG-lysozyme conjugates into three distinguished peaks. These three peaks represent unmodified lysozyme, $(PEG)_1$-lysozyme and $(PEG)_2$-lysozyme.

Although the countercurrent distribution is this article was done manually, the more practical purification approach can be performed on high speed countercurrent chromatography or thin-layer countercurrent distribution, which are now available commercially.

Using such equipment should overcome the difficulties in purifying PEG-protein conjugates and result in higher purity of individual PEG-protein conjugates in preparative scale.

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REFERENCES

1. A. Matsushima, Y. Kodera, M. Hiroto, H. Nishimura, Y. Inada, J. Molecular Catalysis B:Enzymatic, **2,** 1-17 (1996).

2. A. Abuchowski, T. van Es, N. C. Palczuk, F. F. Davis, J. Biol. Chem., **252 (11)**, 3578-3581 (1977).

3. S. Zalipsky, C. Gilon, A. Zilkha, Eur. Polym. J., **19 (2),** 1177-1183 (1983).

4. J. M. Harris, E. C. Struck, M. G. Case, S. Paley, M. Yalpani, J. M. van Alstine, D. E. Brooks, J. Polymer Sci., **22**, 341-352 (1984).

5. K. A. Sharp, M. Yalpani, S. J. Howard, D. E. Brooks, Anal. Biochem., **154,** 110-117 (1986).

6. L. Elling. M.-R. Kula, Biotech. Appl. Biochem**., 13**, 354-362 (1991).

7. J. M. Harris, M. Yalpani, "Polymer-Ligands Used in Affinity Partitioning and Their Synthesis," in **Partitioning in Aqueous Two-Phase Systems**, H. Walter, D. E. Brooks, D. Fisher, eds., Academic Press, Orlando, 1985.

8. Y. Gotoh, M. Tsukada, N. Minoura, Bioconjugate Chem., **4(6)**, 554-559 (1993).

9. R. Clark, K. Olson, G. Fuh, M. Marian, D. Mortenson, G. Teshima, S. Chang, H. Chu, V. Mukku, E. Canova-Davis, T. Somers, M. Cronin, M. Winkler, J. A. Wells, J. Biol. Chem., **271**, 21969-21977 (1996).

10. K. Olson, R. Gehant, V. Mukku, K. O'Connell, B. Tomlinson, K. Totpal, M. Winkler, "Preparation and Characterization of Poly(ethylene glycol)ylated Human Growth Hormane Antagonist," in **Poly(Ethylene Glycol) Chemistry and Biological Applications**, J. M. Harris, S. Zalipsky, eds., ACS Symposium Series 680, American Chemical Society; Washington, DC, 1997, pp. 170-181.

11. M. Kunitani, G. Dollinger, D. Johnson, L. Kresin, J. Chromatogr., **588**, 125-137.

12. G. Johansson, M. Andersson, H.-E. Akerlund, J. Chromatogr., **298**, 483-493 (1984).

13. D. E. Brooks, K. A. Sharp, D. Fisher, "Theoretical Aspects of Partitioning," in **Partitioning in Aqueous Two-Phase Systems**, by H. Walter, D. E. Brooks, D. Fisher, eds., Academic Press, Orlando (1985).

14. C. Delgado, G. E. Francis, D. Fisher, US Patent 5,349,052 (1999).

15. F. M. Velonese, R. Largajolli, E. Boccu, C. A. Benassi, O. Schiavon, Appl. Biochem. Biotech., **11**, 141-152 (1985).

16. L. J. Karr, D. L. Donnelly, A. Kozlowski, J. M. Harris, Methods in Enzymology, **228**, 377-390 (1994).

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