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Partitioning of Recombinant Human Interleukin-2 in a Poly(ethylene glycol)-Dextran Aqueous Two-Phase System

LEE, IN YOUNG AND SUN BOK LEE[1]*

Genetic Engineering Research Institute, KIST,
P.O. Box 17, Taedok Science Town, Taejon 305-606, Korea
[1]Department of Chemical Engineering, Pohang Institute of
Science and Technology, P.O. Box 125, Pohang 790-600, Korea

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The partitioning of recombinant human interleukin-2(rhIL-2) in PEG 8000-dextran 38800 aqueous two-phase system has been investigated using three different sources of rhIL-2. In the case of pure rhIL-2, the solubility in a PEG-dextran two-phase system was low and most of rhIL-2 was partitioned into the bottom phase. For the recovery of rhIL-2 from insoluble protein aggregates, the inclusion bodies of recombinant *E. coli* were solubilized by the treatment with sodium dodecyl sulfate (SDS). The addition of SDS significantly enhanced not only the solubility of rhIL-2 but also the partitioning of rhIL-2 to the top phase. When the ratio of SDS to rhIL-2 was 2.0, the partition coefficient(K) and the recovery yield(Y) at the top phase were 4.5 and 88%, respectively, at pH 6.8. In order to reduce the recovery steps further, SDS was directly added to the intact recombinant *E. coli* cells and then partitioned into the PEG/dextran aqueous two-phase system. The observed partition coefficient ($K \cong 3.0$) and recovery yield ($Y \geq 80\%$) of this method were comparable to the rhIL-2 recovery from insoluble protein aggregates. The results obtained in this work indicate that PEG-dextran two-phase partitioning might provide a simple way for the recovery and partial purification of recombinant proteins which are produced as inclusion bodies.

Since the cDNA of human interleukin-2 gene has been successfully cloned and expressed in *E. coli* at Genetic Engineering Research Institute, KIST (11), studies on the fermentation conditions (15) and purification processes (20) have been carried out to produce the recombinant human interleukin-2(rhIL-2) in a large scale. The previous recovery and purification steps of rhIL-2 involved cell disruption, extraction with urea, solubilization of insoluble aggregates with sodium dodecyl sulfate (SDS), and purification using chromatographic methods (20). The recovery yield of rhIL-2 prior to the chromatographic processing step was less than 50% primarily due to the repeated use of centrifugation, washing and resuspension at each step.

Recently, on the other hand, a great attention has been paid to the application of the aqueous two-phase system to the recovery and purification of proteins (1, 5, 7-10, 12, 13) due to its advantage of easy operation, simplicity for scale-up, and stable maintenance of labile biomolecules. However, its application to the recovery of recombinant insoluble proteins has not yet been reported. Many eucaryotic proteins produced in procaryotic microorganisms, including rhIL-2, are frequently formed as aggregated inclusion bodies (4, 18, 19). In general, it is prerequisite to solubilize such inclusion body proteins for the purification, and therefore SDS has often been used as a solubilizing agent. Therefore, it is conceivable to apply the aqueous two-phase system for the recovery of such recombinant proteins after solubilization.

In this study, we have made an effort to improve the recovery yield of rhIL-2 from the recombinant cells by applying the aqueous two-phase system. We have also

*Corresponding author
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investigated the effect of SDS on the partitioning of proteins in an aqueous two-phase system. In order to reduce the purification steps further, the recombinant cells were directly treated with SDS and then applied to the PEG-dextran aqueous two-phase system. The results obtained in this work indicate that it would be possible to use the aqueous two-phase system to recover and purify other recombinant proteins produced as inclusion bodies via simple one-step processing.

MATERIALS AND METHODS

Isolation and Solubilization of rhIL-2

Escherichia coli M5248[pNKM21] was used for the production of rhIL-2. Structure and properties of the plasmid pNKM21 and culture conditions of recombinant *E. coli* cells were described elsewhere in detail (11, 15).

Cells (20-40 mg) harvested from the culture broth were resuspended with 2.5 ml of 50 mM Tris-HCl (pH 8.3) and treated with lysozyme (200 μg/ml) for 30 min at 25℃. The cells were disrupted with a sonicator(Sonics and Materials Inc.) by 1 min, five times bursts using a microprobe. One ml of lysate was centrifuged at 5000 g for 10 min. The pellet harvested was washed with 1 ml of the Tris buffer. After centrifugation at 5000 g for 10 min, the pellets resuspended in the distilled water were solubilized by adding an appropriate amount of SDS and kept standing overnight at room temperature. After centrifugation at 10,000 g for 10 min, the supernatant was used as a sample solution for aqueous two-phase partitioning experiments.

Preparation of PEG-dextran Aqueous Two-phase System

Aqueous two-phase systems were prepared by mixing an appropriate amount of 20% (w/w) dextran 38800(Sigma) and 30% (w/w) poly(ethylene glycol) 8000(Sigma) with buffer solution. Final buffer concentration was adjusted to 10 mM with 0.2 M of the following buffer solutions: glycine-HCl (pH 2.1-2.6), sodium acetate (pH 3.4-4.3), sodium phosphate (pH 5.8-6.8), Tris-HCl (pH 7.2-8.8), and glycine-NaOH (pH 9.2-11.5). Afterwards, the samples were applied to the aqueous two-phase systems and the mixtures were equilibrated at room temperature for more than 3 h.

Analytical Methods

The PEG concentration was measured by the following method (6). Five ml of 0.5 M perchloric acid was added to 1 ml of PEG solution. After standing for 15 min the mixture was centrifuged at 40,000 g for 10 min. Then 1.0 ml of 5% barium chloride solution and 0.4 ml of 0.1 M iodine solution were added to 4.0 ml of supernatant. After standing for 15 min, the absorbance was measured at 535 nm with a spectrophotometer. The SDS concentration (0.2-2.0 μg/ml) was measured by the MBAS method (2).

Protein was assayed by the Lowry method (17) or the dye-binding assay method of Bradford (3). Protein solution containing rhIL-2 was taken from both top and bottom phase in the aqueous two-phase system. The partitioning coefficient(K) of protein was estimated using the following equation: $K = C_t/C_b$ where C_t and C_b are the equilibrium protein concentration in the top and the bottom phase, respectively. The content of rhIL-2 was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (14). SDS-PAGE was carried out using 14% polyacrylamide gel and the protein band corresponding to rhIL-2 (MW 15.0 kd) was identified using a standard sample of purified rhIL-2 and marker proteins of known size. The fraction of rhIL-2 to the total protein was determined by scanning the SDS-PAGE gel using a densitometer (Sebia, France).

RESULTS AND DISCUSSION

Partitioning of Pure rhIL-2

The standard sample of recombinant human interleukin-2(rhIL-2) used in this work contains a significant amount of SDS although the protein-base purity of rhIL-2 is more than 99.9%. In order to select the suitable aqueous two-phase system for the partitioning of proteins in the presence of SDS, various aqueous two-phase systems have been tested: PEG (1450, 3350, and 8000) as a top-phase material, and dextran (38800 and 500000) or the salt (such as potassium phosphate, sodium phosphate, and ammonium sulfate) as a bottom-phase material. In PEG/salt two-phase systems, especially in the case of a PEG/potassium phosphate system, proteins precipitated at the interface in the presence of SDS due to the interaction between salt and SDS. As a consequence, PEG 8000 and dextran 38800 were chosen as top and bottom phase constructing materials respectively, and used for subsequent experiments.

Before observing the effect of SDS on the partitioning of rhIL-2, we investigated the partitioning of SDS-free pure rhIL-2. To remove the SDS from rhIL-2/SDS complex, chloroform and acetone were tested as extractants of SDS. The mixture of acetone and 0.1 N HCl (6:1) was found to be very effective for the extraction of SDS. By extracting twice with this mixture, SDS associated with rhIL-2 was completely removed with the recovery yield of rhIL-2 higher than 90%.

In order to examine the partition coefficient of pure rhIL-2, the SDS-free rhIL-2 solution was applied to the

Table 1. Partition coefficient of rhIL-2 at different composition of PEG and dextran

PEG 8000(w%)	dextran 38800(w%)	K
12	10	<0.01
8	8	0.03
6	7	0.07
5	6	0.12

aqueous two-phase systems constructed by PEG 8000 and dextran 38800 with different compositions. As shown in Table 1, most of rhIL-2 was partitioned into dextran-rich bottom phase (i.e. $K \ll 1$). This might have resulted from the observed lower solubility of SDS-free rhIL-2 in PEG-based aqueous two-phase systems. In view that most high molecular-weight proteins as well as cell debris tend to partition to the bottom phase (1), it would be desirable to partition rhIL-2 to the top phase for obtaining a high degree of recovery. In order to examine the partitioning of rhIL-2/SDS complex in aqueous two-phase system, the effect of SDS—a solubilizing agent for the inclusion bodies—on the partitioning of proteins was investigated.

Effect of SDS on Partitioning of BSA and rhIL-2

Prior to investigating the effect of SDS on rhIL-2 partitioning in PEG/dextran two-phase system, we examined the partitioning of bovine serum albumin(BSA) in the presence of SDS. Fig. 1 shows the results for separate partitioning experiments of BSA and SDS in PEG 8000 (5%)-dextran 38800 (7%) aqueous two-phase system. While BSA was partitioned mainly to the dextran-rich bottom phase, SDS was partitioned to the top phase with a partition coefficient of 1.8-1.9 all over the range of pH. In view that the partition coefficient of SDS was greater than that of BSA, the addition of SDS to BSA solution was expected to promote the partitioning of BSA to the top phase. In the next experiment the effect of SDS on BSA partitioning was examined by changing the amount of SDS at pH 5.8. As shown in Fig. 2, both the partition coefficient and the recovery yield of BSA at the top phase were increased along with increasing the ratio of SDS to BSA, which indicates that SDS greatly affects the partitioning of proteins in the aqueous two-phase system.

In the next experiment, the effect of SDS on the partitioning of rhIL-2 was examined. In this experiment, SDS was added to the suspension of insoluble proteins aggregates(12.5 mg/ml) which were obtained from the lysate of recombinant *E. coli* following the methods described in Materials and Methods. After standing the mixtures overnight to solubilize the insoluble proteins, the mixtures containing the final protein concentration of about

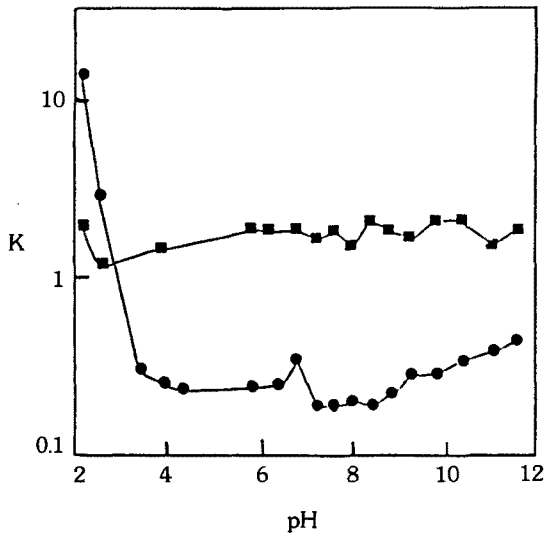

Fig. 1. Partition coefficients of bovine serum albumin (●) and sodium dodecyl sulfate (■) in the PEG 8000 (5%)-dextran 38800 (7%) aqueous two-phase system at the various pH.
Buffers used for adjustment of pH were described in the text.

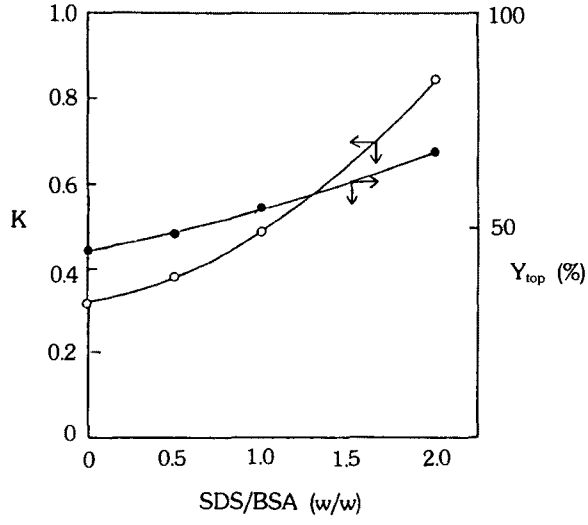

Fig. 2. Effects of sodium dodecyl sulfate (SDS) on the partitioning and recovery yield of bovine serum albumin (BSA) in PEG 8000(5%)-dextran 38800 (7%) aqueous two-phase system at pH 5.8.
Changes of partition coefficient (○) and recovery yield(●) at the top phase are shown as a function of the ratio of SDS to total protein (w/w).

3.0 mg/ml were partitioned into PEG/dextran aqueous two-phase system.

Fig. 3 shows the results of the partitioning of rhIL-2 in the presence of SDS. The partition coefficient of

the SDS-treated rhIL-2 was remarkably increased as compared to that of rhIL-2 without SDS, showing higher values with the increasing ratio of SDS to protein at the acidic to neutral condition. When the ratio of SDS to the insoluble protein was 2.0, for example, the partition coefficient of rhIL-2 at pH 6.8 was 4.5 and the recovery yield at the top phase reached 88%. Partition coefficient of rhIL-2 was even higher than that of pure SDS (1.8-1.9). Increased partitioning of the rhIL-2 to the top phase seemed to result from the enhancement of mobility by binding of SDS to the protein in addition to the increase in the solubility of rhIL-2 by SDS. The partition coefficient of rhIL-2 at the alkaline pH was similar irrespective of the ratio of SDS to protein. This might be due to the reduction of the electrostatic interaction between rhIL-2 and SDS as the net charge of both rhIL-2 and SDS becomes negative at the pH values higher than the isoelectric point of rhIL-2(pI=7.7) (16).

Results of SDS-PAGE of proteins at the top and bottom phases show that high molecular-weight *E. coli* proteins were mainly partitioned to the bottom phase (Fig. 4). From densitometric scanning of the gel, it was found that the rhIL-2 content of the total insoluble protein

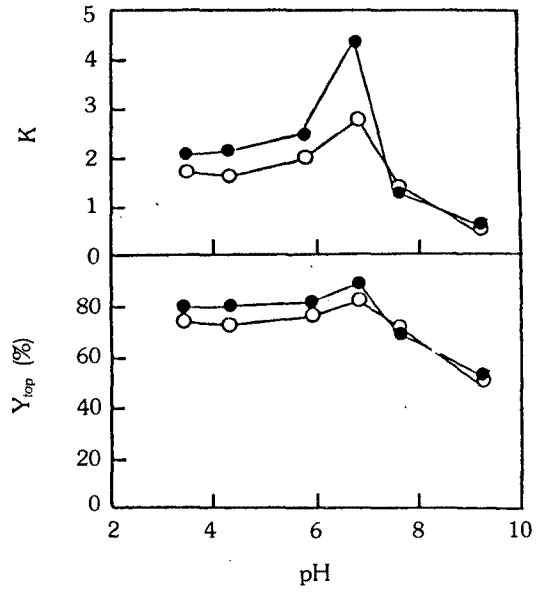

Fig. 3. Effect of SDS on the partitioning of rhIL-2 in PEG 8000 (5%)-dextran 38800 (7%) aqueous two-phase system after solubilization of insoluble proteins.

(○), SDS/total protein(w/w)=1.0; (●) SDS/total protein (w/w)=2.0.

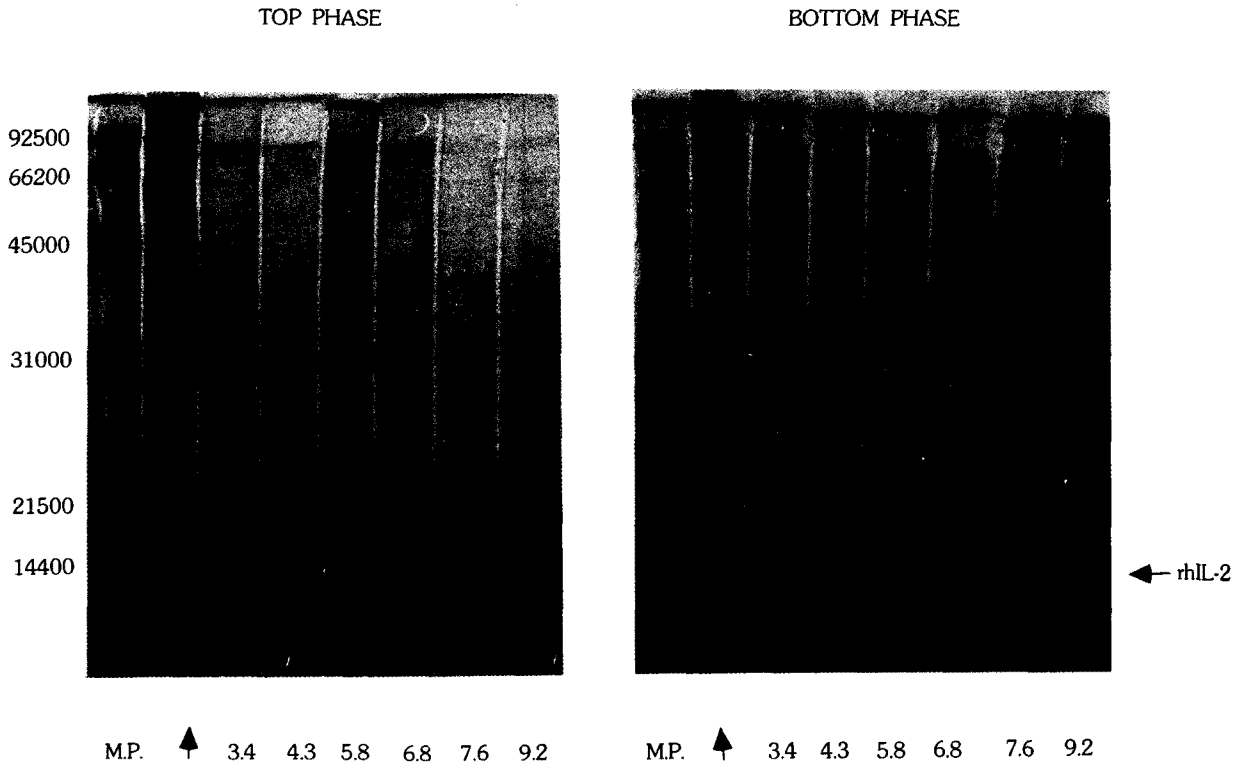

Fig. 4. Analysis of partitioning of rhIL-2 by SDS polyacrylamide gel electrophoresis.

The numbers in the ordinate and abscissa represent the molecular weight of marker proteins and pH of partitioning experiment, respectively (M.P.; marker proteins, upward arrows; control samples before application to the aqueous two-phase partitioning).

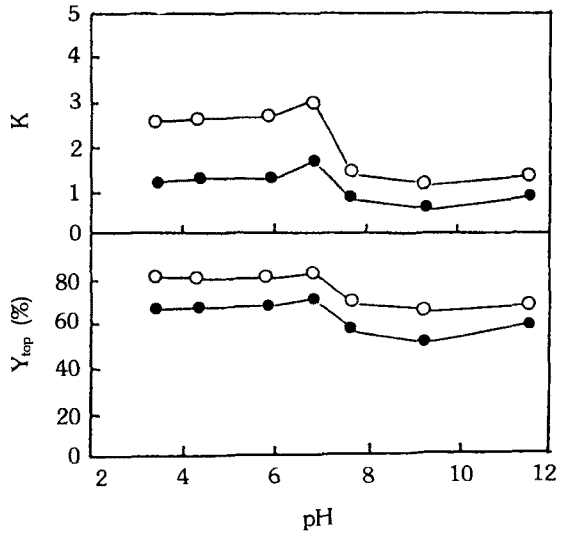

Fig. 5. Effect of SDS on the partitioning of rhIL-2 and total intracellular proteins in PEG 8000 (5%)-dextran 38800 (7%) aqueous two-phase system.
(○), rhIL-2; (●), total intracellular proteins.

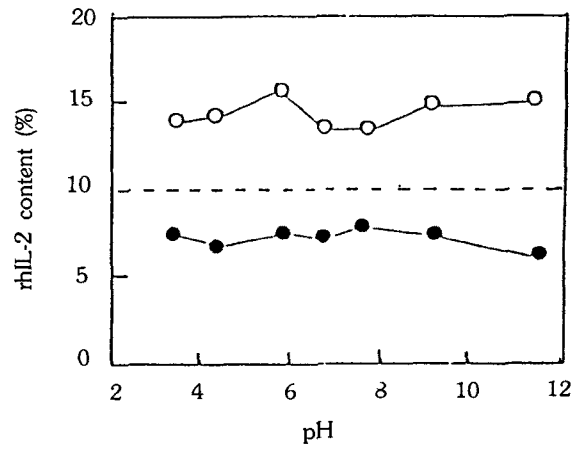

Fig. 6. Changes in rhIL-2 content after aqueous two-phase partitioning. Recombinant cells were treated with SDS and total lysates were applied to the PEG 8000-dextran 38800 aqueous two-phase system.
Dotted line indicates the rhIL-2 content of cell lysate before application to the aqueous two-phase partitioning. (○), rhIL-2 content at the top phase; (●) rhIL-2 content at the bottom phase.

at the top phase reached 30-36%, which corresponds to about 1.5-fold enrichment.

Single Step Recovery of rhIL-2 from Recombinant Cells

Previously, the solubilized rhIL-2 has been obtained by three-step processing: cell disruption, extraction with urea, and solubilization by SDS. Due to the repeated use of centrifugation, washing and resuspension at each step, less than 50% of initial rhIL-2 was recovered during this operation (20). Since a high dose of SDS could efficiently disrupt the cell wall, we further examined the feasibility of simultaneous cell disruption and solubilization of rhIL-2 with SDS in order to reduce the processing steps. Based on the results in the preceding section, twice the amount of SDS to the total intracellular proteins of recombinant *E. coli* cells was added directly to the recombinant cells harvested from the culture broth. By this simple treatment with SDS, we were able to obtain more than 90% of total rhIL-2 quantity in the cell. When the supernatant of cell lysates was added to the PEG/dextran aqueous two-phase system, rhIL-2 was partitioned to the PEG-rich top phase with the partition coefficient of 2.5 to 3.0 in the acidic to neutral region, and the recovery yield of rhIL-2 at the top phase was more than 80% (Fig. 5). In addition, the partition coefficient of rhIL-2 was found to be much higher than that of other proteins. Fig. 6 shows that the rhIL-2 content at the top phase is about 1.5-fold higher as compared to the initial rhIL-2 content (10.2%), indicating relatively specific influence of SDS on the rhIL-2 partitioning. Although the difference in partitioning of rhIL-2 and other proteins in the presence of SDS can not be explained in detail, it appears that in the presence of SDS lower molecular-weight proteins could be partitioned more effectively as compared to higher molecular-weight proteins.

In conclusion, by treating the recombinant cells with SDS directly and by partitioning the resulting cell lysate in PEG/dextran aqueous two phase, we were able to recover rhIL-2 via a single-step processing with a recovery yield of more than 80%. Also, the purification factor was 1.5-fold enhanced by applying this method. Since the appropriate selection of PEG-derivatives in the aqueous two-phase system can enhance the purification factor (5, 7, 10), further study on affinity partitioning could enable us to develop a more efficient method for the purification of recombinant proteins usually formed as inclusion bodies.

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