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Large-Scale Purification of Protease Produced by *Bacillus* sp. from *Meju* by Consecutive Polyethylene Glycol/Potassium Phosphate Buffer Aqueous Two-Phase System

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Abstract Protease produced from *Bacillus* sp. FSE-68 was isolated from *Meju*, a Korean fermented soybean starter, and was purified by a two-consecutive aqueous two-phase system. The change of partition coefficient (K) in the polyethylene glycol (PEG)/potassium phosphate buffer (PPB) aqueous two-phase system was measured at different pHs (6.0–9.2), PPB concentrations (8–12%), and temperatures (4 and 20°C). As the PPB concentration in the aqueous two-phase system increased, the protease concentration in the top phase (PEG-rich phase) increased, thereby enhancing the partition coefficient. The minimum partition coefficient of the protease was achieved at pH 7.0, whereas that of the total protein was at pH 6.0. The biggest difference in partition coefficients of total protein and protease occurred at pH 6.0. It was interesting to note that the partition coefficient of protease decreased as the temperature increased. The optimum condition of the primary aqueous two-phase extraction of *Bacillus* sp. FSE-68 was pH 6.0, 14% (w/w) PPB, and 16% (w/w) PEG at 4°C, and the crude enzyme concentration in this system was 50% (w/w). The protease, which was concentrated in the top phase, was further mixed with 15% (w/w) PPB (pH 7.0) in the ratio of 1:1 at 20°C to elute the bottom phase (PPB-rich phase). Using these steps, the purification fold achieved was 9.2 with a 44.7% yield.

Key words: Two-phase extraction, *Bacillus* protease, *Meju*

The advancement of biotechnology in a field of biological science augments the production of biological materials. The efficiency level for the production of these materials is considered to be low and often requires costly methods to purify these materials [17]. Such a circumstance is indeed disadvantageous to industries that rely strongly on these biological materials [11]. Generally, various methods of precipitation, centrifugation, ultrafiltration, and chromatography are applied in industry [23]. Although precipitation is a common method used, it has low selectivity. As for centrifugation and ultrafiltration, materials separated according to size and density have some limitations for their utilization. Chromatography divides materials according to the differences in their particle size, charge, and affinity, but it is a costly process with low yied. Recently, aqueous two-phase systems have been utilized to separate biological materials of low concentration [16, 18], and an example of this is the use of polyethylene glycol (PEG) mixed with potassium phosphate buffer at an appropriate pH and temperature.

This aqueous two-phase system has different physical and chemical environments between the top and bottom phases, a low osmotic pressure, and high water activity, and it is sensitive enough for purification of biological materials [14, 17]. Aqueous two-phase systems have previously been used for separating plant and animal cells, cell organelles [1], enzymes [8, 9], nucleotides, and other biological materials [5]. The important advantage of this aqueous two-phase system is that it does not require any expensive equipments like HPLC, and that it can rapidly purify materials [12, 13, 24].

The purpose of the present research was to examine the effects of varying the potassium phosphate buffer concentration and pH in an aqueous two-phase system, and to identify the optimal composition of an aqueous two-phase system and pH. The reason for this was to separate *Bacillus* sp. FSE-68 protease on a large scale for the purpose of purification.

MATERIALS AND METHODS

Chemicals

Polyethylene glycol 8,000 (PEG 8000) was supplied by Sigma Co. (St. Louis, U.S.A.), and mono- and dibasic

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potassium phosphates (K_2HPO_4 and KH_2PO_4) were purchased from Kanto Chemical (Japan). All other chemicals used were of analytical grade.

Identification of Protease-Producing Bacterium

Bacterium to produce protease was isolated from *Meju*, and identified using the Bergey's Manual of Systematic Bacteriology [21] and The Prokaryote [2] according to its morphological and physiological characteristics.

Culture Media

TSB broth prepared with 17 g tryptone, 3.0 g soytone, 2.5 g dextrose, 5.0 g NaCl, and 2.5 g K_2HPO_4 in 1 l distilled water was used for pre-culture. For the main enzyme-producing culture media, 10 g beef extract, 10 g defatted soybean flour, 5 g NaCl, and 10 g potato starch mixed in 1 l distilled water, adjusted to pH 7.0 with NaOH, was used.

Culture Conditions

Bacillus sp. FSE-68 was activated in a test-tube with 10 ml pre-culture media for 18 h in a rotary shaker (150 rpm) at 37°C. Subsequently, the pre-culture media were inoculated into 500 ml of the enzyme-producing media and incubated in a rotary shaker (200 rpm) at 37°C for 45 h.

Crude Protease Preparation

Cultured enzyme-producing media were centrifuged for 20 min at 3,000×g. Cells and other insoluble materials were discarded. The supernatant was used as the source for crude enzyme.

Protease Activity Assay

Jensen's method [10] was used for the protease assay. A total volume of 3 ml substrate consisting of 1% Hammersten casein and 0.1 M phosphate buffer (pH 7.0) was preheated in a 37°C water-bath and hydrolyzed by adding 0.1 ml enzyme solution. After 15 min incubation at 37°C, the reaction was terminated by adding 5 ml TCA solution prepared with 1.8% trichloroacteic acid, 1.8% sodium acetate, and 1.98% acetic acid. This mixture was filtered through Whatman No. 5 filter paper. The absorbance of the filtrate was measured at 275 nm. A unit of protease activity was defined as the activity of 1 mg tyrosine per 1 ml enzyme solution per min.

Protein Assay

Protein concentration was measured by the Bradford method [4], using bovine serum albumin as a standard. The samples of top and bottom phases were diluted 10-fold with distilled water before adding the dye to eliminate the interference of PEG 8000 and potassium phosphate on the protein assay.

Preparation of Aqueous Two-Phase System

An appropriate amount of PEG 8000 and potassium phosphate was added to crude protease solution. After moderate mixing, the aqueous two-phase solution was centrifuged for 10 min at 3,000 ×g in order to accelerate phase separation. The pH of the aqueous two-phase system was adjusted by using the appropriate ratio of K_2HPO_4 and KH_2PO_4.

Calculation of Partition Coefficient (K)

Partition coefficient, K, is calculated as the ratio of protease concentrations in the top (PEG-rich) and the bottom (PPB-rich) phase as follows:

$$K = [\text{material in top phase}]/[\text{material in bottom phase}]$$

$K_{protein}$ and $K_{protease}$ represent the partition coefficients of protein and protease, respectively.

Purification by Aqueous Two-Phase Extraction and Back Extraction

The purification process of FSE-68 protease by the aqueous two-phase system is illustrated in Fig. 1. In the primary extraction, FSE-68 protease was selectively partitioned to

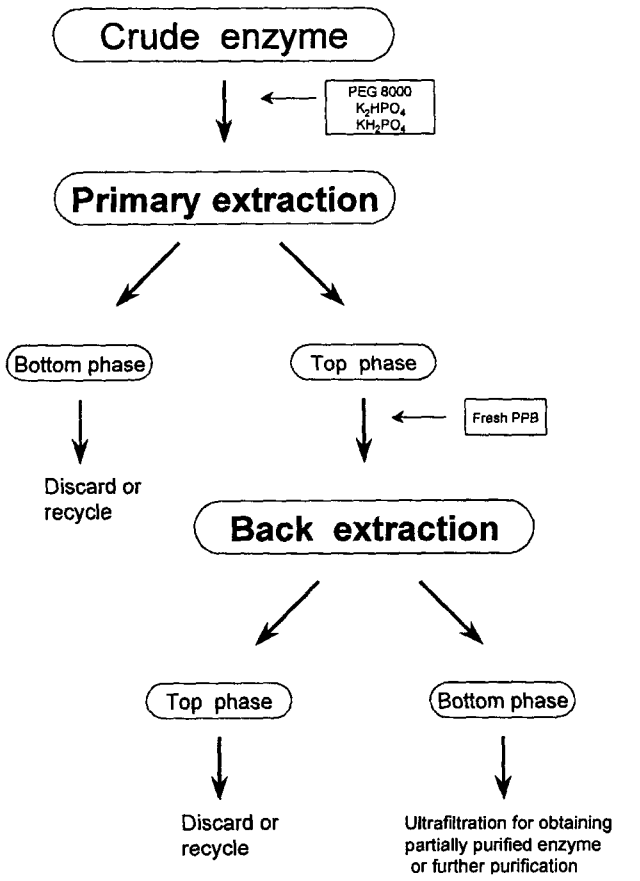

Fig. 1. Schematic diagram for the isolation of protease from *Bacillus* sp. FSE-68.

the top phase. In the back extraction, the bottom phase was replaced by fresh phosphate buffer solution and the protease was transferred from the PEG-rich top phase of the first extraction to the phosphate-rich bottom phase. The back extraction step facilitates further treatment of protease since protease is separated from the PEG-rich top phase.

Results and Discussion

Identification of Protease Producing Bacterium

The protease-producing bacterium isolated from *Meju* was rod-shaped, gram-positive, catalase-positive, and motile. Therefore, it was classified as a *Bacillus* species. The physiological characteristics of this bacterium are shown in Table 1. According to Bergey's Manual of Systematic Bacteriology [21] and The Prokaryote [2], the bacterium was identified as *Bacillus* sp. FSE-68 of *Bacillus subtilis*.

Effects of PPB Concentration on the Partition Coefficient

The polymer concentration was maintained at 16% (w/w) and the PPB concentration was increased from 8% (w/w) to 14% (w/w) at pH 7.0 in this system. Figures 2 and 3 represent the changes of $K_{protein}$ and $K_{protease}$, respectively, in increasing PPB concentration. Since $K_{protein}$ was below 1.0 in all PPB concentrations tested, it is assumed that the protein was mainly in the bottom phase of the PEG/PPB aqueous two-phase system. $K_{protein}$ became progressively greater in value from 0.19 to 0.79 with an increasing PPB concentration. $K_{protease}$ also increased by the increment of PPB concentration from 0.36 to 2.25, but it should be pointed out that the increment of $K_{protease}$ was much greater than the $K_{protein}$ increment.

In the PEG/PPB aqueous two-phase system, the top phase is the PEG-rich phase, while the bottom phase is the PPB-rich phase. Protein solubility in the top phase was determined by hydrophobic interaction between the ethylene group of PEG and the hydrophobic residue of protein, while that in the bottom phase was determined by a salting-out effect with the presence of salts [3, 15]. Decreasing the protein solubility in the bottom phase by increment of PPB concentration increased the partition coefficient of both total protein and protease. Because the

Table 1. Physiological and biochemical characteristics of the protease-producing bacterium and *B. subtilis*.

Characteristics	The strain from *Meju*	*B. subtilis*[a]
Shape of cell	Rod	Rod
Gram staining	+	+
Spore formation	+	+
Catalase	+	+
Anaerobic growth	-	-
Voges-Proskauer test	+	+
Methyl-red test	+	D[b]
Acid from glucose	+	+
Gas from glucose	-	-
Acid from mannitol	+	+
Hydrolysis of casein	+	+
Hydrolysis of starch	+	+
Nitrate reduced to nitrite	+	+
Growth in 4% NaCl	+	+
Growth in 7% NaCl	+	+
Growth at 50°C	+	D[b]
Growth at 65°C	-	-

[a]Bergey's Manual of Systematic Bacteriology, Vol. 2.
[b]11 - 89% of strains are positive.

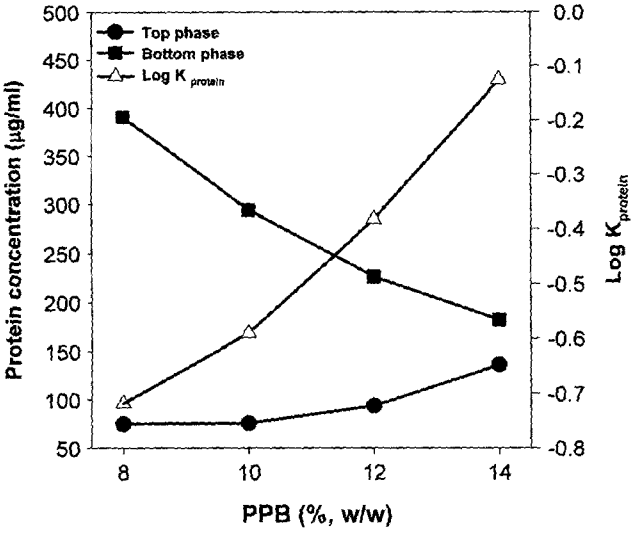

Fig. 2. The effect of potassium phosphate buffer concentration on the partition of total protein.
Concentration of PEG was 16% (w/w) at pH 7.0.

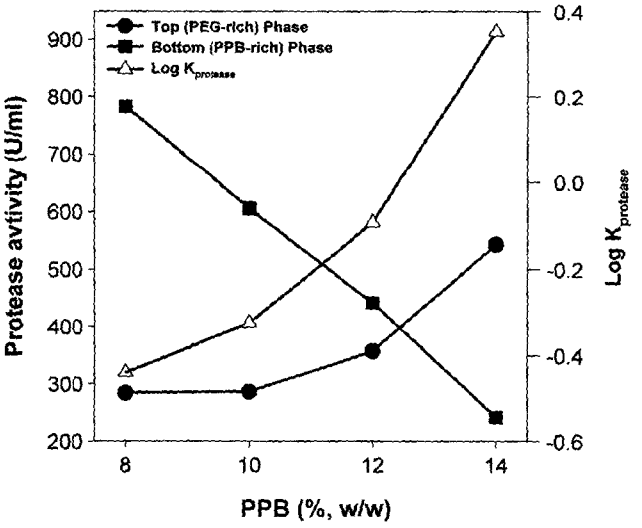

Fig. 3. The effect of potassium phosphate buffer concentration on the partition of protease.
Concentration of PEG was 16% (w/w) at pH 7.0.

partition coefficient of protease was larger than that of total protein at the same PPB concentration, it can be stated that protease may be concentrated and separated from the rest of the proteins.

Effects of pH on the Partition Coefficient

PEG and PPB concentrations were maintained at 16% (w/w) and 12% (w/w), respectively. Figures 4 and 5 show the changes of $K_{protein}$ and $K_{protease}$ at different pHs (6.0-9.2). As pH rose from 6.0 to 9.2, $K_{protein}$ increased linearly from 0.31 to 1.19. However, protease had a minimum partition coefficient at around pH 7.0. Any change of pH subsequently altered the ratio of K_2HPO_4 and KH_2PO_4 effects on H^+ concentration, along with the phase boundary electric potential difference and composition between the top and bottom phase [7].

Since phosphate ion in the PEG/PPB aqueous two-phase system is distributed more in the bottom than in the top phase, the bottom phase is generally more negatively charged than the top phase. Decrease in the pH condition results in more positively-charged protein, therefore, most proteins move toward the bottom phase. Also, low pH creates a large ratio of KH_2PO_4 in the aqueous-two phase system and, furthermore, KH_2PO_4 with low solubility [25] can greatly influence the partition coefficient. The increment of $K_{protease}$ at pH 6.0 resulted in the salting-out effect of KH_2PO_4 and it was postulated that protease possessed lower solubility than most of other proteins in a crude enzyme preparation.

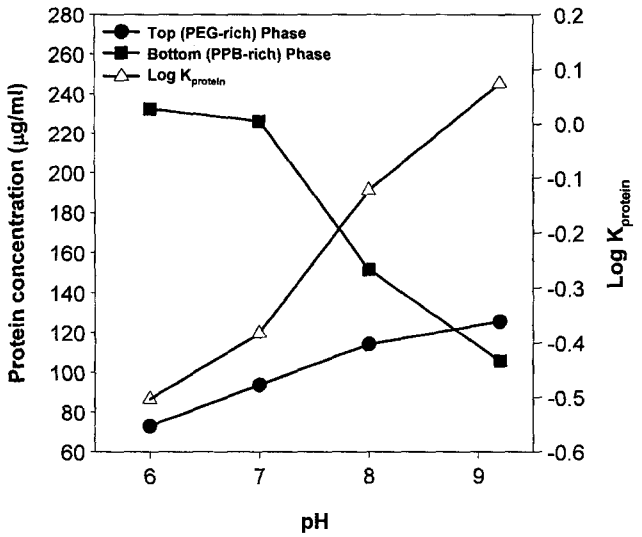

Fig. 4. The effect of potassium phosphate buffer pH on the partition of total protein.
The concentration of PEG and PPB was 16% (w/w) and 12% (w/w), respectively.

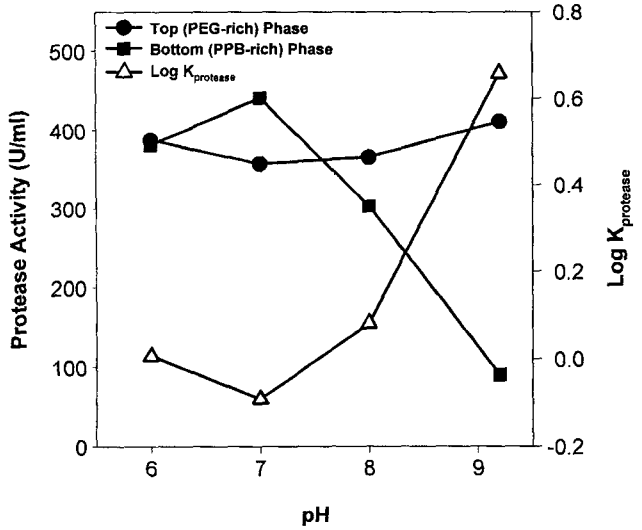

Fig. 5. Effect of potassium phosphate buffer pH on the partition of protease.
The concentration of PEG and PPB was 16% (w/w) and 12% (w/w), respectively.

Determination of the Optimum Condition for Aqueous Two-Phase Extraction

Our previous work indicated pH 6.0 as the optimum for protease purification. Increase of PPB concentration widened the $K_{protein}$ and $K_{protease}$ difference, thereby increasing the specific activity of protease. In the top phase, more protease was partitioned and concentrated, but the volume decreased by the increased PPB concentration, producing a low yield of protease. Thus, the testing of yield and monitoring of the change of specific activity by PPB concentration at pH 6.0 is very important to determine the optimum condition for aqueous two-phase extraction. Figure 6 shows the yield and change of specific activity by

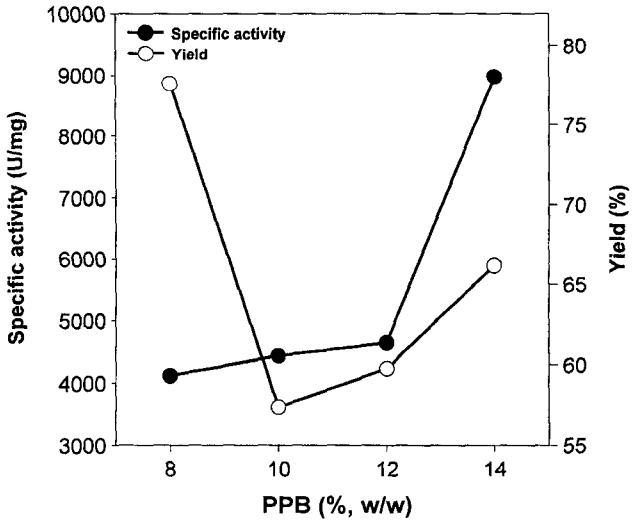

Fig. 6. The effect of PPB concentration on the specific activity and yield of *Bacillus* sp. FSE-68 protease in the top (PEG- rich) phase.

different PPB concentrations. Although 8% (w/w) PPB concentration yielded the best protease (77.6%), the specific activity increased only by 2.3 folds. The yield of protease in 14% (w/w) PPB concentration dropped to 66.1%, but the specific activity of protease increased by 5.0 folds. Due to low solubility of KH_2PO_4, concentrations above 14% (w/w) PPB were not applied in this PEG/PPB aqueous two-phase system. Our experiment demonstrated that 14% (w/w) PPB at pH 6.0 was the best optimal condition for protease purification.

Determination of the Optimum Condition for Aqueous Two-Phase Back Extraction

As the PEG-protease mixture in the top phase was unable to separate from each other, the utilization and further purification of the protease extracted in the top phase was difficult. After the aqueous two-phase extraction, various methods were examined to remove the phase-forming polymer, PEG in this system, and to purify the target product from impurities. These included back extraction (by an aqueous two-phase system) [7], ultrafiltration (UF) [6], and gel chromatography [19]. Among them, back extraction by the aqueous two-phase system has been known to be the most favorable one. As mentioned earlier, since protease has minimum $K_{protease}$ at pH 7.0 and is partitioned mostly at the bottom phase, the back-extraction condition was tested at pH 7.0. In Fig. 5, the lower the PPB concentration, the more protease was partitioned to the bottom phase. However, the volume of the bottom phase decreased as the PPB concentration was lowered. As a result, it was difficult to predict whether the yield of protease increased or not. According to Walter *et al.* [24], temperature was another factor determining the partition coefficient. Protein was dissolved in both top and bottom phases by different mechanisms [21], and protein solubility of the bottom phase increased at high temperature. Yields of protease as a function of PPB concentration and temperature are shown in Fig. 7. The yield of protease at 20°C increased up to 15% PPB, which was higher than that at 4°C showing about 13.8% at 12% PPB and 8.8% at 15% PPB, respectively. However as the PPB concentration increased to 18%, the yield of protease decreased, and the difference between at 4°C and 20°C remained only at about 6.6%.

Protease was efficiently purified from the top phase when the top phase was mixed with 15% (w/w) PPB in a ratio of 1:1. The purification fold increased to 9.2 by the back-extraction step and its yield was 44.7%. Table 2 summarizes the overall purification folds and yields of protease during the present aqueous two-phase separation process.

In our study, we have demonstrated a purification process of protease from *Bacillus* sp. FSE-68 using an aqueous two-phase system. The high yield with high purification fold of the protease suggests that such method may be more cost-effective than the conventional means of chromatography, ultrafiltration, or centrifugation. Furthermore, industries are strongly urged to consider application of an aqueous two-phase system to purifying biological materials.

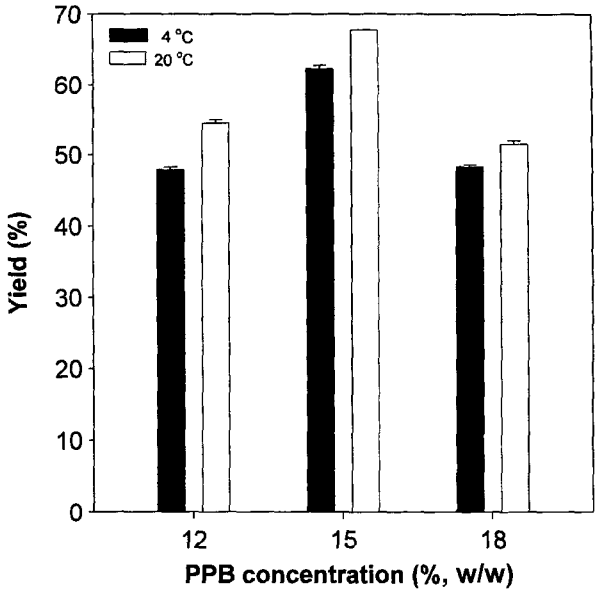

Fig. 7. Comparison of the protease yields with different PPB concentrations and temperatures in the bottom (PPB-rich) phase.

Table 2. Summary of purification of the protease from *Bacillus* sp. FSE-68.

Step	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Crude enzyme	183,890	103.3	1,780	1.0	100.0
Primary extraction	121,553	13.6	8,938	5.0	66.1
Back extraction	82,201	5.0	16,440	9.2	44.7

Acknowledgments

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REFERENCE

1. Albertsson, P. A. 1986. *Partition of Cell Particles and Macromolecules*, 3rd Ed., Wiley Interscience, New York U.S.A.

2. Balows, A. 1991. *The Prokaryotes*, 2nd Ed., Springer-Verlag, New York, U.S.A.
3. Baskir, J. N., T. A. Hantton, and U. W. Suter. 1989. Protein partitioning in two-phase aqueous polymer system. *Biotechnol. Bioeng.* **34:** 541-558.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
5. Cascone, O., B. A. Andrews, and J. A. Asenjo. 1991. Partitioning and purification of thaumatin in aqueous two-phase system. *Enzyme Microb. Technol.* **13:** 629-635.
6. Guan, Y., T. E. Treffry, and T. H. Lilley. 1994. *E. coli* penicillin acylase isolation by selective release, aqueous two-phase partitioning and ultrafiltration. *Bioseparation* **4:** 89-99.
7. Guan, Y., T. H. Lilley, T. E. Treffry, C. L. Zhow, and P. B. Wilkinson. 1996. Use of aqueous two-phase system in the purification of human interferon-α1 from recombinant *Escherichia coli*. *Enzyme Microb. Technol.* **19:** 446-455.
8. Han, J. H. and C. H. Lee. 1997. Effects of salts and poly(ethylene glycol)-palmitate on the partitioning of protein and *Bacillus subtilis* neutral protease in aqueous two-phase system. *Colloids and Surface B: Biointerface* **9:** 109-116.
9. Hustedt, H., K. H. Kroner, W. Stach, and M. R. Kula. 1978. Procedure for the simultaneous large-scale isolation of pullulanase and 1,4-α-glucan phosphorylase. *Biotechnol. Bioeng.* **20:** 1989-2005.
10. Jensen, D. E. 1972. Continuous production of extracellular protease by *Bacillus subtilis* in a two-stage fermenter. *Biotechnol. Bioeng.* **14:** 647-662.
11. Kelly, B. D. and T. A. Hatton. 1991. The fermentation/downstream processing interface. *Bioseparation* **1:** 303-349.
12. Kula, M. R. 1990. Trends and future prospects of aqueous two-phase extraction. *Bioseparation* **1:** 181-189.
13. Kula, M. R., H. Hustedt, K. H. Kroner, and H. Schtte. 1983. *New Development in Large Scale Protein Isolation Process*, Online Publication Ltd, Northwood.
14. Kula, M. R., K. H. Kroner, and H. Hustedt. 1982. Purification of enzyme by liquid-liquid extraction. *Adv. in Biochem. Engineer.* **24:** 73-118.
15. Lee, C. H. and S. I. Sandler. 1990. Vancomycin partitioning in aqueous two-phase system; effect of pH, salts, and an affinity ligand. *Biotechnol. Bioeng.* **35:** 408-416.
16. Lee, B. Y., C. H. Lee, and C. H. Lee. 1990. Effects of pH on the separation and purification of model protein using counter current distribution. *Kor. J. Food Sci. Technol.* **22:** 56-60.
17. Lee, S. M. 1989. The primary stages of protein recovery. *J. Biotechnol.* **11:** 103-118.
18. Lee, S. P., Y. M. Kim, and C. H. Lee. 1987. Purification of intercellular β-galactosidase from *Lactobacillus sporogenes* in an aqueous poly(ethylene glycol)-potassium phosphate two-phase system. *Kor. J. Appl. Microbiol. Bioeng.* **15:** 84-88.
19. Menge, U., M. Morr, U. Mayr, and M. R. Kula. 1983. Purification of human fibroblast interferon by extraction in aqueous two-phase system. *J. Appl. Biochem.* **5:** 75-90.
20. Murray, R. G. E., P. H. A. Sneath, D. Claus, and R. C. W. Berkeley. 1986. *Bergey's Manual of Systematic Bacteriology* Vol. 2, Williams Wilkins, London, U.K.
21. Schmidt, A. S., B. A. Andrews, and J. A. Asenjo. 1996. Correlations for the partition behavior of proteins in aqueous two-phase systems: Effect of overall protein concentration. *Biotechnol. Bioeng.* **50:** 617-626.
22. Schmidt, A. S., A. M. Ventom, and J. A. Asenjo. 1994. Partitioning and purification of α-Amylase in aqueous two-phase system. *Enzyme Microb. Technol.* **16:** 131-142.
23. Scope, R. K. 1987. *Protein Purification-Principles and Practice*, 2nd Ed., Spinger, New York, U.S.A.
24. Walter, H., D. E. Brooks, and D. Fisher. 1985. *Partitioning in Aqueous Two-Phase Systems*, Academic Press Inc., London, U.K.
25. Windholz, M. 1976. *The Merck Index,* 9th Ed., Merck & Co. Inc., U.S.A.