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L-Asparaginase Release from *Escherichia coli* Cells with Aqueous Two-Phase Micellar Systems

Minjun Qin and Fengsheng Zhao*

School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200030, P. R. China, E-mail: fszhao@mail.sjtu.edu.cn

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Abstract

A method was proposed to release and separate L-asparaginase (EC 3.5.1.1) from *Escherichia coli* ATCC 11303 cells with aqueous two-phase micellar systems. The systems were composed of K_2HPO_4 and Triton X-100. The method combines enzyme release with enzyme purification. The influence of Triton X-100 concentration, K_2HPO_4 concentration, and pH on the release and partition of L-asparaginase was investigated. Experimental results showed that *E. coli* cells treated with 9.4% (w/v) K_2HPO_4 and 15% (w/v) Triton X-100 at 25°C for 15–20 h released nearly 80% of the enzyme. Most of the released enzyme was partitioned to the bottom phase (phosphate-rich phase). The effects of Triton X-100 concentration, K_2HPO_4 concentration, and pH on cloud point were also studied. Electron micrography indicated that the chemical treatment altered the inner structure of *E. coli* cells significantly.

Index Entries: L-Asparaginase; enzyme release; *Escherichia coli*; aqueous two-phase micellar system; dipotassium hydrogen phosphate; Triton X-100.

Introduction

L-Asparaginase (EC 3.5.1.1) can catalyze the hydrolysis of L-asparaginase to L-aspartic acid and ammonia. It is effective for the cure of leukemia, especially for puerile acute lymphocytic leukemia. L-Asparaginase is an intracellular enzyme, which is commonly obtained from microorganisms. The traditional process to purify L-asparaginase from submerged cultures of microorganism cells includes the preparation of cellular acetone powder and the extraction of enzyme-rich solution *(1,2)*. That method uses a large amount of acetone, produces cell fragments, and releases many cellular

*Author to whom all correspondence and reprint requests should be addressed.

components. The produced fragments and released substances (such as RNA and DNA) may create difficulties in succeeding with a purification process.

If the cells of microorganisms are treated with membrane-active compounds, the permeability of the cells may be changed and intracellular substances can be released. The cells keep integrating and high recovery results *(3,4)*.

Aqueous two-phase systems have been applied widely in purification of enzymes. The systems consist of incompatible components, such as polymer/polymer, polymer/salt, or surfactant/polymer. Process integration of enzyme release and enzyme purification by aqueous two-phase systems has been studied. β-Galactosidase in recombinant *Escherichia coli* cells was released by a 13% polyethylene glycol (PEG) 6000–10% phosphate system containing glycine *(5)*. Release yield attained 15%. The enzyme was concentrated in PEG phase. The release course lasted at least 10 h.

In recent years, many scientists have become interested in utilizing aqueous two-phase micellar systems to separate or concentrate proteins and other biologic materials *(6–8)*. Micelles are self-assembling aggregations of surfactant molecules. When surfactant molecules dissolve in water beyond a definite concentration known as critical micelle concentration (CMC), they spontaneously aggregate to form micelles. If nonionic surfactant solution is heated, it can separate into a micelle-rich phase in equilibrium with a water-rich phase. The temperature above which phase separation occurs is known as cloud point (Cp). Above Cp, the shape and size of micelles can be manipulated by changing solution conditions, such as the concentration of the nonionic surfactant. Aqueous two-phase micellar systems can provide a gentle and friendly environment for biologic materials.

Surfactants are often used to solubilize membrane proteins *(9)*. Aqueous two-phase systems formed with nonionic surfactants and hydrophilic polymers were applied as fast and efficient separation methods for isolation of membrane proteins. Membrane proteins were partitioned strongly to the micelle phase, while hydrophilic proteins were partitioned to the polymer phase *(8)*. An amphiphilic fusion protein endoglucanase I_{core}-hydrophobin I was purified with an aqueous two-phase micelle extraction system, which was formed by mixing nonionic surfactant Triton X-114 or Triton X-100 with hydroxypropyl starch polymers. After the primary recovery step, fusion protein was back-extracted into a water phase. Total recovery of the two separation steps was 90% with a volume reduction of six times *(10)*.

We found that Triton X-100 and K_2HPO_4 could release L-asparaginase quite well. A solution of 2% Triton X-100 and 13% K_2HPO_4 could release 70% of L-asparaginase from *E. coli* ATCC 11303 cells *(11)*.

It is known that PEG and phosphate can form aqueous two-phase systems. The Triton X-100 molecule consists of a PEG chain linked with a

hydrophobic group by ether bond, so Triton X-100 could act as a substitute for PEG to form an aqueous two-phase system with phosphate for release of L-asparaginase. A mixture of surfactant/phosphate/water will separate into a micelle-rich phase (top phase) and a salt-rich phase (bottom phase) above CMC.

We studied the release and partition of L-asparaginase with the proposed aqueous two-phase systems and report the results in this article.

Materials and Methods

Culture of Microorganism and Cell Count

E. coli ATCC 11303 was used as a source of L-asparaginase. The strain was incubated in Erlenmeyer flasks. Then the fermentation broth was harvested. The number of *E. coli* cells in the fermentation broth was determined. The detailed procedure is described in ref. *11*.

Release of Enzyme

The fermentation broth was centrifuged at 4°C and 12,000*g* for 5 min. The cell pellet was washed with distilled water and centrifuged again. Obtained cells were resuspended in distilled water to give a certain cell concentration.

Triton X-100 (Shanghai Chemical) was used without further purification. Five milliliters of the cell suspension was placed in a 100-mL plastic centrifuge tube. Fifteen milliliters of aqueous solution of K_2HPO_4 and/or Triton X-100 was added, and the release process was initiated. The solution was agitated at 230 rpm on a rotary shaker for 14–15 h. The reported concentrations of K_2HPO_4, Triton X-100, and *E. coli* cells corresponded to those in the final agitated mixture.

The treated mixture was centrifuged for 10 min (12,000*g*). If the concentrations of K_2HPO_4 and Triton X-100 were suitable, the supernatant separated into two phases. The micelle-rich phase corresponds to the top phase and the phosphate-rich phase to the bottom phase. The volumes of obtained phases were measured. Some samples were withdrawn from the two phases respectively for assay of enzyme. Release yield of the enzyme was calculated as the ratio of the amount of enzyme in both phases after release to the amount of enzyme in cells before release. The phase ratio is identified as the ratio of top-phase volume to bottom-phase volume. The partition coefficient of enzyme is defined as the ratio of the equilibrium concentration of enzyme in the top phase to that in the bottom phase.

Assay of L-Asparaginase

L-Asparaginase was assayed as described in ref. *11*. Triton X-100 interferes with protein determination severely, so we did not determine proteins.

Measurement of Cp

The measurement of Cp temperatures was carried out at room temperature (5–10°C). A sample (3 to 4 mL) of surfactant solution was prepared in a graduated tube at the concentration of the two-phase region. The tube was immersed in a water bath, and then the temperature was increased stably. Cp was determined visually as the temperature at which the solution began to become turbid.

Determination of Phase Diagram

A phase diagram of the aqueous two-phase micellar system was determined by titration method. Five milliliters of 66% (w/w) Triton X-100 solution was placed in a 250-mL Erlenmeyer flask and titrated by 47% (w/w) K_2HPO_4 solution until the solution in the flask became turbid (two-phase region was reached). The point corresponding to the solution composition was close to the boundary between the two-phase and one-phase regions. Another 5 mL of Triton X-100 solution with lower concentration than 66% (w/w) was placed in a 250-mL Erlenmeyer flask and titrated by 47% (w/w) K_2HPO_4 again to obtain another point. The titration was repeated several times. Each time a lower concentration of Triton X-100 was employed. This procedure determined the left half of the phase diagram (the half close to the Triton X-100 concentration axis). Titration of 47% (w/w) K_2HPO_4 by 66% (w/w) Triton X-100 solution determined the other half (the half close to the K_2HPO_4 concentration axis). Two half curves were linked to form a whole phase diagram.

Electron Microscopy

E. coli cells were washed twice with 0.1 *M* phosphate buffer (pH 7.0) and then fixed with 2.5% glutaraldehyde. The cells were washed again several times and postfixed with 1% OsO_4 fixative. The fixed cells were washed three times with phosphate buffer.

The cells were dehydrated by ethanol solutions with increasing concentration. They were washed in a series of ethanol solutions of 40, 60, 80, 95, and 100% concentration, respectively, and then were embedded in epoxy resin Model 618. Thin sections were cut with an ultramicrotome Model LKB-V. The sections were stained with uranium acetate and lead citrate successively. The inner structures of *E. coli* cells were observed and photographed with a transmission electron microscope Model JEM-100CXII (JEOL, Japan).

Results and Discussion

Effects of Triton X-100 Concentration, K_2HPO_4 Concentration, and pH on Release and Partition of Enzyme

The release courses of L-asparaginase with K_2HPO_4 or Triton X-100 alone and with aqueous two-phase micellar system composed of K_2HPO_4

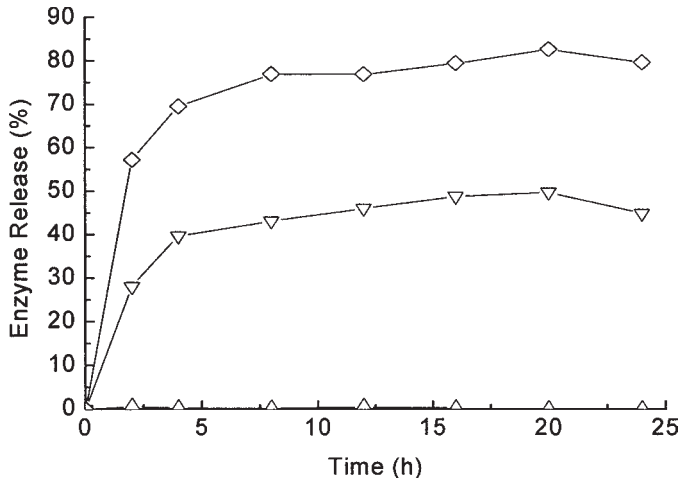

Fig. 1. Time course of L-asparaginase release in three solutions: (△) 9.4% (w/v) K_2HPO_4; (▽) 15% (w/v) Triton X-100; (◇) 9.4% (w/v) K_2HPO_4 and 15% (w/v) Triton X-100. Samples were treated at 25°C and cell concentration was 3.2×10^8 cells/mL.

and Triton X-100 were determined (Fig. 1). It was indicated that each medium affected differently the release of enzyme. When K_2HPO_4 was used alone, the release of L-asparaginase was very low (near 0). Triton X-100 solution was much more effective. The aqueous two-phase micellar system consisting of Triton X-100 and K_2HPO_4 was the most effective, with a yield of 82.5%. Most of the L-asparaginase was released in the first 4 h. Then, the release rate decreased. The release course lasted about 20 h. Some treatments were made to evaluate the effects of Triton X-100, K_2HPO_4 and pH on the release of enzyme.

Triton X-100 concentration affected enzyme release; Fig. 2 shows the results. In the solution of 6.3% K_2HPO_4 and 5% Triton X-100, only one phase existed. When Triton X-100 concentration increased, the aqueous solution separated into two phases. The top phase (surfactant-rich phase) growing up and the bottom phase (phosphate-rich phase) descending down led to the increase in phase ratio. The release of enzyme increased at first, then decreased. The highest release yield was obtained at 25% Triton X-100 concentration. The partition coefficient decreased with increasing Triton X-100 concentration, but it was always lower than 0.2.

The concentration of K_2HPO_4 also affected enzyme release; Fig. 3 shows the results. Two phases could be formed except at quite low K_2HPO_4 concentration (3.13%) with 15% Triton X-100. With a rise in K_2HPO_4 concentration, phase ratio descended while partition coefficient rose. The release yield of L-asparaginase ascended to a top level at 9.38% K_2HPO_4 concentration, then descended rapidly.

In aqueous solution, pH affects electric charges of K_2HPO_4 and proteins (including enzyme). Thus, pH affects their solubilities, as well as the release of enzyme and proteins. The effect of pH on enzyme release is

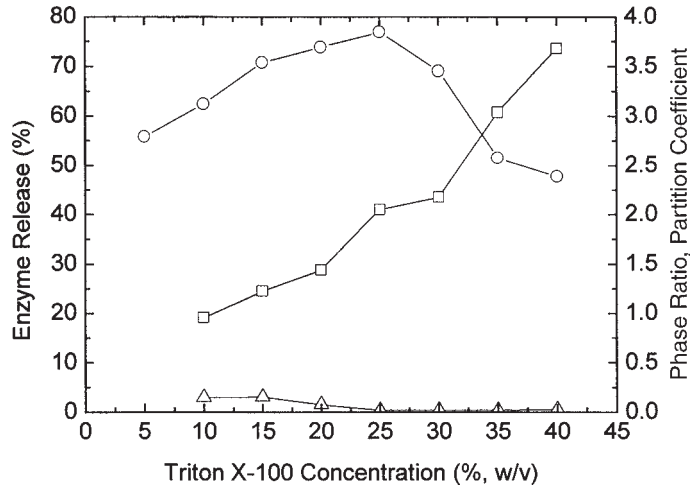

Fig. 2. Effect of Triton X-100 on release of L-asparaginase from *E. coli* ATCC 11303 cells: (○) enzyme release yield; (□) phase ratio; (△) partition coefficient. Samples were treated for 14 h at 25°C with Triton X-100, 6.3% (w/v) K_2HPO_4 and cell concentration was 3.2×10^8 cells/mL.

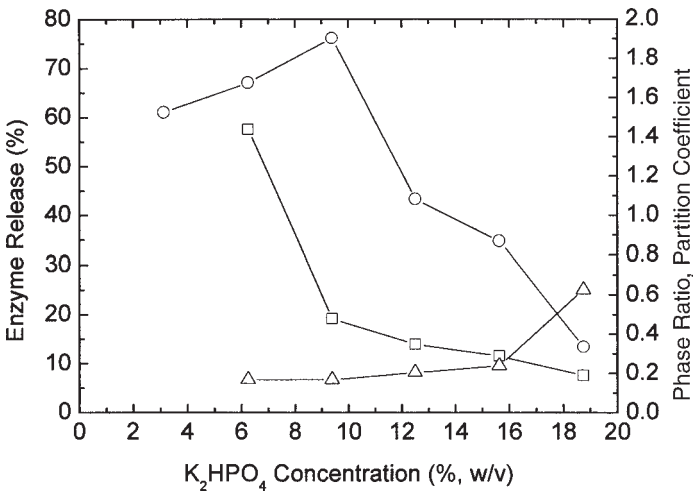

Fig. 3. Effect of K_2HPO_4 on release of L-asparaginase from *E. coli* ATCC 11303 cells: (○) enzyme release yield; (□) phase ratio; (△) partition coefficient. Samples were treated for 15 h at 25°C with K_2HPO_4, 15% (w/v) Triton X-100 and cell concentration was 3.2×10^8 cells/mL.

shown in Fig. 4. When the pH increased from 6.0 to 12.0, phase ratio only varied slightly, and release yield rose to a top point and then dropped. An increase in pH caused a decrease in partition coefficient. The pH range 8.5–9.5 was beneficial to the release of enzyme. pH values beyond the range would cause the enzyme release to drop severely.

The natural pH value of the aqueous two-phase micellar system composed of 15% Triton X-100 and 9.4% K_2HPO_4 is 8.7. At that pH, the release

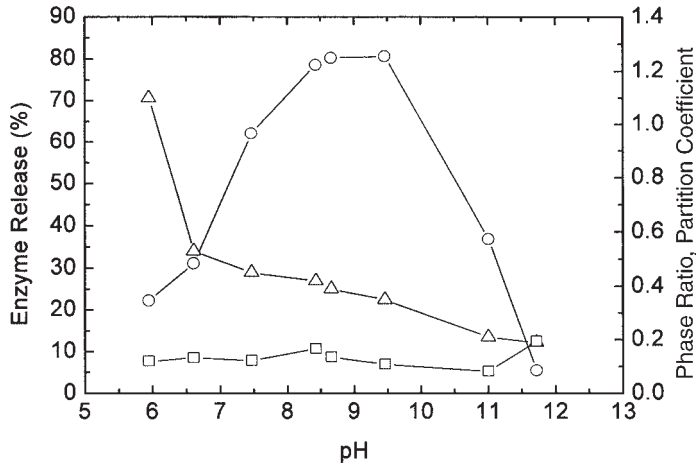

Fig. 4. Effect of pH on release of L-asparaginase from *E. coli* ATCC 11303 cells: (○) enzyme release yield; (□) phase ratio; (△) partition coefficient. Samples were treated for 15 h at 25°C with 9.4% (w/v) K_2HPO_4, 15% (w/v) Triton X-100 and cell concentration was 3.2×10^8 cells/mL.

yield of enzyme is satisfactorily high. Thus, the natural pH of the micellar system could be used easily.

L-Asparaginase released from *E. coli* cells by the aqueous two-phase micellar systems tended strongly to enter into the bottom phase (phosphate-rich phase), while little enzyme was partitioned to the top phase (surfactant-rich phase).

In our study, the release yield of L-asparaginase was affected by many factors. In general, the concentrations of Triton X-100 and K_2HPO_4 had a more important influence. The envelope architecture of *E. coli* cells shows that the outer membrane has an asymmetric structure. The outer layer is lipopolysaccharide and the inner layer is glycerophospholipids. The Triton X-100 molecule has a similar structure of hydrophilic "head" and hydrophobic "tail" to phospholipids, which constitutes the skeleton of cell membrane. Thus, the micelle composed of Triton X-100 molecules might insert into the two-layer structure of membrane and cause the release of intracellular materials. As already stated, the effect of Triton X-100 with phosphate on enzyme release is much better than Triton X-100 used alone. Perhaps phosphate anions act on the lipopolysaccharide in cell membrane more easily, which leads Triton X-100 to enter into membrane deeply.

Effects of Surfactant Concentration, K_2HPO_4 Concentration, and pH on Cp

Cp is one of the unique characteristic features of nonionic surfactant. Above Cp temperature, surfactant solution originally in a homogeneous phase can divide into two phases. One is the surfactant-rich phase and the other is the aqueous phase. The latter contains surfactant, the concentra-

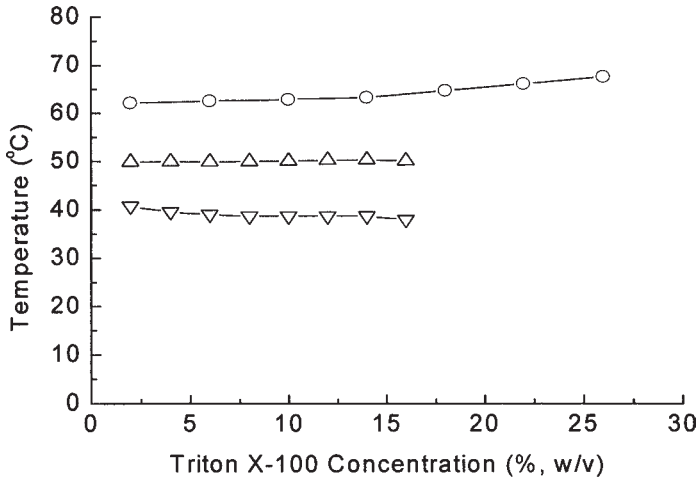

Fig. 5. Cp vs concentration of Triton X-100: (○) no K_2HPO_4; (△) 2.64% (w/v) K_2HPO_4; (▽) 5% (w/v) K_2HPO_4.

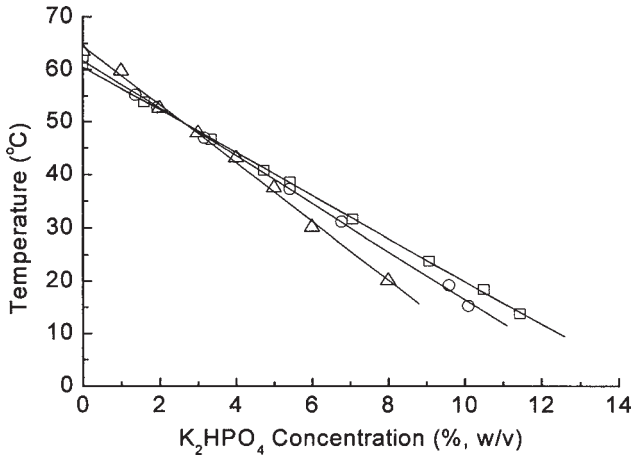

Fig. 6. Cp vs concentration of K_2HPO_4: (□) 5% (w/v) Triton X-100; (○) 10% (w/v) Triton X-100; (△) 15% (w/v) Triton X-100.

tion of which is near CMC. The phenomenon is reversible: when cooled, the single phase is obtained again.

Figure 5 shows the relationship between Cp and Triton X-100 concentration. When no K_2HPO_4 was in the solution, the Cp increased with a rise in Triton X-100 concentration. However, in the presence of 2.64% (w/v) K_2HPO_4, the Cp was nearly constant (49.80). In 5% (w/v) K_2HPO_4 solution, the Cp dropped slowly with an increase in Triton X-100 concentration.

Figure 6 shows that Cp declined linearly with a rise in K_2HPO_4 concentration. Triton X-100 concentration also affected Cp. The higher the Triton X-100 concentration, the more rapidly Cp decreased with an increase in K_2HPO_4. Interestingly, all of the straight lines pass through a common point

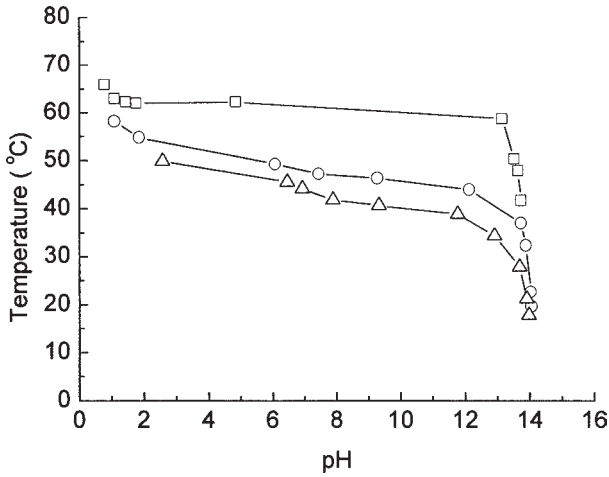

Fig. 7. Cp vs pH: (□) 10% (w/v) Triton X-100; (○) 10% (w/v) Triton X-100 and 3% (w/v) K_2HPO_4; (△) 10% (w/v) Triton X-100 and 5% (w/v) K_2HPO_4.

(2.64%, 49.80°C). This means that at 2.64% K_2HPO_4 solution, Cp is always 49.80°C, whatever the concentration of Triton X-100 is. This finding is concordant with the results in Fig. 5.

The effect of pH on Cp is shown in Fig. 7. In the pH range of 1.0–13.5, the effect of pH is not evident. At a pH beyond the range, Cp changed rapidly. The Cp of the solution without K_2HPO_4 was higher than that with K_2HPO_4, but the slopes of the curves obtained from the solutions containing K_2HPO_4 were more oblique.

Phase Diagram of Aqueous Two-Phase Micellar System

The phase diagram of the aqueous two-phase micellar system is shown in Fig. 8. A common phase diagram of the aqueous two-phase system has a smooth curve. However, the phase diagram of an aqueous two-phase micellar system consisting of Triton X-100 and K_2HPO_4 is different. It is nearly composed of two straight lines. The point of intersection is the critical point. The right half of the phase diagram is horizontal and closed with a horizontal axis, so the bottom phase only contains a little Triton X-100 (lower than 0.5%). The low content of Triton X-100 in the bottom phase (rich in enzyme) is beneficial to the success of purification.

Electron Micrography of E. coli Cells

Some chemical treatments could alter the structure of *E. coli* cells and enable intracellular components to be released. This alteration in cell structure can be observed with electron microscopy. Figure 9A is an electron micrograph of untreated *E. coli* cells. The cytoplasm is dispersed evenly in the cells. A periplasmic space can be seen clearly. After treated with 0.01 *M* Tris-HCl buffer, the membrane and wall of the cells were not harmed (Fig. 9B). Treatment did not change the cell shape evidently.

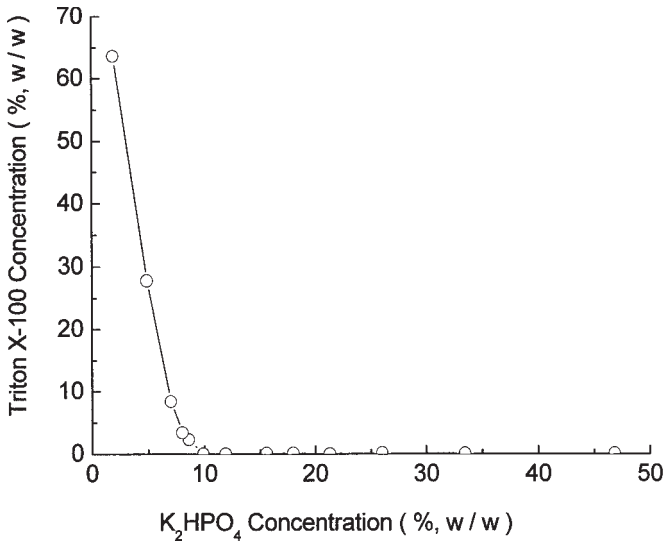

Fig. 8. Phase diagram of K_2HPO_4/Triton X-100 aqueous two-phase micellar system (30°C).

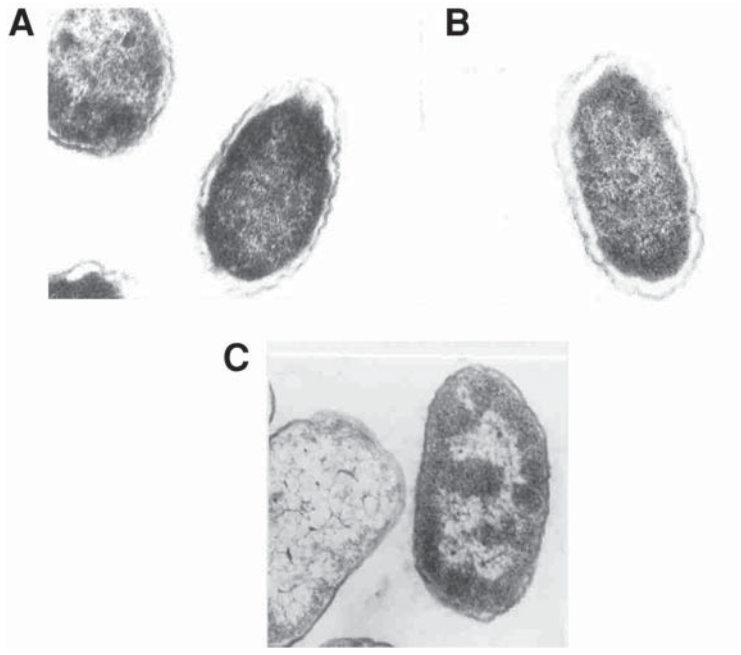

Fig. 9. Electron micrograph of *E. coli* ATCC 11303 cells (×50,000). *E. coli* cells were untreated **(A)**, or treated for 20 h at 25°C with 0.01 *M* Tris-HCl buffer (pH 7.0) **(B)** or 9.4% K_2HPO_4/35% Triton X-100 **(C)**.

The release of enzyme was minor (only 5%). Figure 9C is an electron micrograph of *E. coli* cells treated with 9.4% K_2HPO_4 and 35% Triton X-100. Cytoplasm swelled and entranced into the periplasmic space. Holes appeared in the center of the cells. After treatment, 70% of the enzyme was released while the cells maintained whole.

The advantages of release with two-phase systems are as follows: First, released enzyme is partitioned to the bottom phase. This bottom phase can be adjusted into less volume, so that concentrated enzyme solution can be obtained. Second, according to the phase diagram of the aqueous two-phase micelle system, only little surfactant (lower than 0.5%) remained in the bottom phase. If a one-phase system of Triton X-100 and K_2HPO_4 was used, 2% Triton X-100 would be needed *(11)*. As known, separating surfactant from protein solution is rather difficult.

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