PURIFICATION PROCESS FOR HEAT SHOCK PROTEINS USING AQUEOUS TWO-PHASE SYSTEM AND PEG FRACTIONAL PRECIPITATION

RYOICHI KUBOI*[1], TETSUHIRO HASEGAWA,
KOJI YANO AND ISAO KOMASAWA
Department of Chemical Engineering, Osaka University, Toyonaka, Osaka 560

Key Words: Heat Shock Protein, Aqueous Two-Phase System, PEG Fractional Precipitation, Surface Property, Biospecific Affinity

A simple and effective purification process for heat shock proteins (HSPs), in which PEG fractional precipitation was combined with an aqueous two-phase system (ATPS), was successfully developed based upon the proteins' molecular surface properties. Both GroEL and GroES, typical HSPs from *E.coli*, were selectively partitioned to the PEG (top) phase of the ATPS. GroEL and GroES were selectively and stepwisely concentrated from the above PEG phase by PEG fractional precipitation. In addition, the purification of GroEL and GroES as a complex by using biospecific affinity with adenosine triphosphate (ATP) was achieved. GroEL and GroES can be purified either as individual native molecules or as a 1:1 complexed state by the control of ATP addition.

Introduction

Molecular chaperones, which belong to the group of heat shock proteins (HSPs), are considered to facilitate correct protein folding (Fisher, 1991; Martin *et al.*, 1991). GroEL and GroES obtained from *E. coli* are typical representatives of HSPs. It has been known that adenosine triphosphate (ATP) promotes association of GroEL and GroES (Viitanen *et al.*, 1990). However, details of the molecular mechanisms which assist protein refolding remain unknown. GroEL and GroES have been purified using multi-step separation methods such as the combination of ion exchange chromatography, gel exclusion chromatography, dialysis and salting-out effect (precipitation) (Chandrasekhar *et al.*, 1986; Hendrix, 1979). These methods seem to be unable to provide these materials in large quantities.

Aqueous two-phase systems (ATPS) are formed by spontaneous phase separation of different water-soluble polymers such as polyethylene glycol (PEG) and dextran (Dex). It has been known that the influence of PEG / Dex two-phase systems on the structure and stability of protein is minimal (Albertsson, 1986; Kuboi *et al.*, 1994). The surface properties of GroEL and GroES have been characterized using ATPS, and effective ATPS for selective partitioning of them to the PEG (top) phase have been also designed based on the differences in their surface properties as previously described (Yano *et al.*, 1994). Furthermore, cultivation of *E. coli* cells in ATPS media has also been investigated, and it has been shown possible to cultivate *E. coli* cells, and to produce intracellular enzymes in ATPS media (Kuboi *et al.*, 1995).

Several water-soluble nonionic polymers such as PEG, Dex and polyvinyl pyrrolidone have also been used for the precipitation of proteins. Among them, PEG is considered to be the most suitable protein precipitant because its solutions are less viscous and cause virtually no protein denaturation at room temperature. On the other hand, some precipitants such as ammonium sulfate, ethanol and other organic precipitants tend to cause denaturation unless conditions, especially temperature, are rigidly controlled (Harrison, 1994; Horio, 1994; Ingham, 1990).

In the present work, the design of a simple and effective purification process for GroEL and GroES has been attempted on the basis of the previous work using ATPS (Yano *et al.*, 1994). PEG fractional precipitation has been combined with ATPS to achieve efficiently the separation of GroEL and GroES. Separation of GroEL and GroES as an ATP-mediated complex by addition of ATP has also been investigated.

1. Materials and Methods

1.1 Materials

E. coli (OW10/pND5) strain was used for the production of heat shock proteins, GroEL and GroES. The details of this microorganism and plasmid have been described elsewhere (Miyake *et al*, 1993). Tryptone and yeast extract, which consisted of Luria Bertani (LB) broth medium, were supplied by Difco Laboratories (Detroit, USA). Ampicillin, tetracycline and sodium chlo-

* Received on June 5, 1995. Correspondence concerning this article should be addressed to R. Kuboi.

*[1] Tel: 06-850-6271, Fax: 06-850-6273

ride were obtained from Wako Pure Chemical Industries, Ltd. (Wako). Polyethylene glycol (PEG) 1540, 4K, 6K (average molecular weight : 1.5k, 3k, 7.5k dalton, respectively) and dextran (Dex) 100K-200K (molecular weight : 100k-200k dalton) were supplied by Wako. Nonionic detergent Triton X-405 was supplied by Sigma Chemical Co.. Potassium phosphate (KPi) was prepared to obtain the required pH by mixing KH_2PO_4 and K_2HPO_4 as supplied by Wako. Adenosine-5'-triphosphoric acid disodium salt (ATP) was supplied by Oriental Yeast Co., Ltd. Other chemicals were of analytical grade.

1.2 Methods

1) Production of HSPs *E. coli* cells were grown at 303 K in LB broth medium as described previously (Yano *et al.*, 1994). After heat treatment at 315 K for 45 minutes, the cells were centrifuged at 10,000 rpm for 10 min at 273 K and suspended in 50 mM Tris-HCl buffer (pH 8.0) / 10 mM $MgSO_4$. Intracellular products including GroEL and GroES were released by the ultrasonic method. The extract, including intracellular products, was centrifuged at 10,000 rpm for 10 min at 298 K and the resultant supernatant was used as the initial solution (crude extracts) for the following separation steps of GroEL and GroES. The total protein concentration in this solution was typically 1.5 - 4.5 mg/m*l*.

2) Partition of HSPs The aqueous two-phase systems (ATPS) used were PEG / Dex systems. The preparation methods of ATPS and the analytical methods employed in the ATPS in respect of surface properties such as surface net hydrophobicity (*HFS*), local hydrophobicity ($\Delta \ln K_{pr}$), molecular weight (M.W.) and isoelectric point (pI) were described elsewhere (Kuboi *et al.*, 1994; Yano *et al.*, 1994). In this case, the ATPS contained 20%(w/w) the initial solution containing GroEL and GroES, and so the ATPS contained 10 mM Tris-HCl buffer / 2 mM $MgSO_4$, and the value of pH was 8.0.

3) PEG fractional precipitation The PEG (top) phase in PEG/Dex two-phase system, to which GroEL and GroES were partitioned selectively, was used for the starting solution in PEG fractional precipitation experiments. PEG was added to the starting solution (volume = 0.6 - 1.6 *l*), which was thoroughly mixed using a magnetic stirrer and centrifuged at 5,000 rpm for 10 min at 298 K. Most of the supernatant was then removed and the remaining suspended material was centrifuged again in a smaller centrifuge tube (volume = 10 m*l*) under the same conditions. In this case, the precipitate was obtained not as a pellet, but as a concentrated solution of proteins (bottom phase). It is known that precipitates in PEG fractionation often appear as viscous fluids which contain concentrated protein and PEG (Atha and Ingham, 1981; Horio, 1994). Further PEG was added to the resulting supernatants, which were considered now to be the top phase for successional fractionation. These procedures were repeated until the protein concentration in the top phase was sufficiently low (about 0.1 mg/m*l* : determination limit). The process scheme, consisting of ATPS and PEG fractional precipitation, is shown in Fig.1.

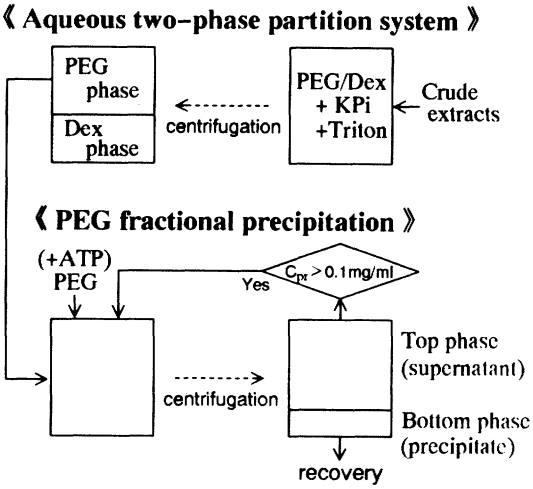


Fig. 1 Process scheme consisting of ATPS and PEG fractional precipitation

4) Protein determination Total soluble protein concentration was determined by the Bradford method (Bradford, 1976). The content of each protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The transmittance of this gel was measured by using a Dual-Wavelength Flying-Spot Scanner CS-9000 (Shimadzu Corp.).

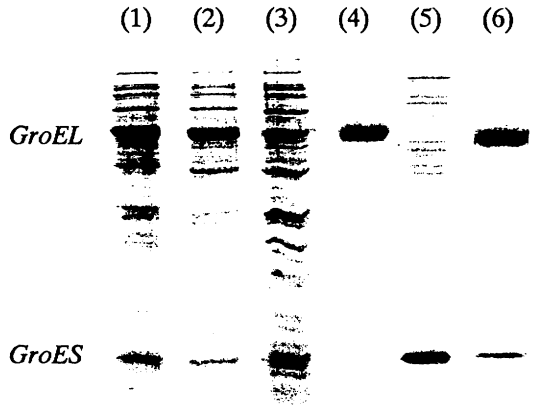
2. Results and Discussion

2.1 Separation of GroEL and GroES using aqueous two-phase system

The surface properties of GroEL, GroES and total soluble proteins in terms of surface net hydrophobicity *(HFS)* and local hydrophobicity ($\Delta \ln K_{pr}$) have been characterized by using ATPS. Effective separation systems for GroEL and GroES were designed based on the differences in their surface properties (Yano *et al.*, 1994). In these, System 1 (PEG4K(9%) / Dex100K-200K(9%) + 10 mM KPi + 1 mM Triton X-405) and System 2 (PEG6K(9%) / Dex100K-200K(9%)), GroEL and GroES were found to be partitioned selectively into the PEG (top) phase, though the recovery of GroES to the PEG phase was not high, especially in the latter system. Since the recovered PEG phase from System 1 included both GroEL and GroES with moderately good yield, it was used for the starting feed solution for the following PEG precipitation step. The results of SDS-PAGE, which reflect partition behavior in System 1, were shown in **Fig. 2** (Lanes 1, 2 and 3). In the case of System 1, the volume ratio of PEG phase to Dex phase is about two to one and the partition coefficients of GroEL, GroES and the other proteins are about 5, 1.5 and 0.5, respectively, so the recoveries of GroEL, GroES and the other proteins to the PEG phase are about 90%, 75% and 50%, respectively.

2.2 Purification and concentration of GroEL and GroES by PEG fractional precipitation

When further PEG was added to the starting feed



Lane

(1) Crude extracts
(2) PEG phase in System 1
(3) Dex phase in System 1
(4) GroEL condensed phase (fraction (i) in Fig.3)
(5) GroES condensed phase (fraction (ii) in Fig.3)
(6) Complex condensed phase (fraction (iii) in Fig.5)

Fig. 2 Results of SDS-PAGE for purification of GroEL and GroES

solution (PEG phase) in the above System 1, a small volume of a concentrated solution of proteins was formed as a bottom phase. In most previous experiments on PEG fractional precipitation, concentration and activities of proteins in supernatants were mainly analyzed (Atha and Ingham, 1981; Mahadevan and Hall, 1992). In the present work, the protein concentrated solution (bottom phase) was analyzed as well as the supernatant (top phase).

The variations of (a) total protein concentration (C_{pr}) in the top and bottom phases, (b) content of GroEL and GroES in the bottom phase and that of the other proteins in the top phase, and (c) volume of the bottom phase per unit starting PEG solution (PEG phase) of 100 m*l* by addition of PEG4K, are shown in **Fig. 3**. In the case of Fig.3, the starting PEG solution contained 53% GroEL, 18% GroES and 29% other proteins and the total protein concentration of this solution was 0.8 mg/m*l*. It is evident that the protein concentration in the top phase is gradually decreased with increasing PEG concentration. Small additions of PEG yield GroEL concentrated solutions which have more than 90% purity. The maximum protein concentration in the bottom phase is one hundred times higher than the protein concentration of the starting PEG solution. With increasing PEG addition, GroEL was completely transferred to the bottom phase (precipitated), and then by further addition of PEG, the concentrated solution of proteins in which GroES is a main component was easily recovered. In comparison with GroEL, the resulting purity of GroES is not so high, because the surface properties of GroES are similar to those of other proteins as previously described (Yano *et al.*, 1994). On the other hand, the content of other proteins remaining in the top phase is gradually increased.

The molecular weights of GroEL and GroES have

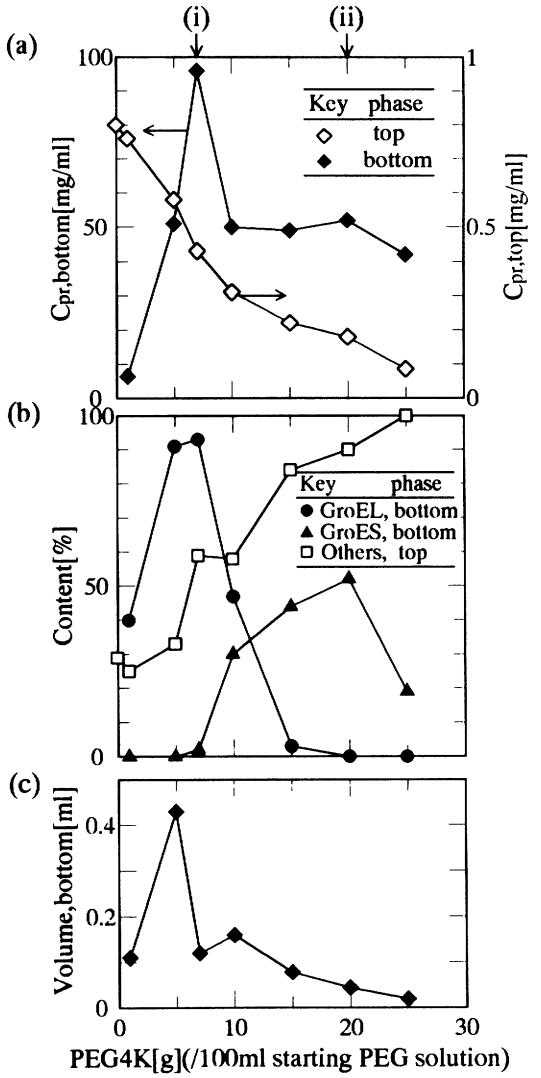


Fig. 3 Effects of addition of PEG4K on (a) total protein concentration in top and bottom phases, (b) content of GroEL and GroES in bottom phase and that of other proteins in top phase, (c) volume of bottom phase per unit starting PEG solution of 100 ml (fractions (i) and (ii) are samples of Lanes (4) and (5) in Fig.2, respectively)

been calculated from the amino acid sequences (GroEL: 57,259 × 14mer, GroES: 10,368 × 7mer) (Hemmingsen, 1988) or determined from SDS-PAGE (GroEL: 60,000 × 14mer, GroES: 15,000 × 7mer). GroEL is concentrated (precipitated) at a lower PEG concentration than GroES. The molecular weights of many other proteins can not be clearly defined, because the amino acid sequences and association state remain unknown. However, the larger protein tends to be concentrated at a lower PEG concentration than the smaller one, as judged from the results of SDS-PAGE. Each protein can be separated according to monomer molecular weight in this method. This may support the steric exclusion mechanism for the precipitation of proteins by PEG (Harrison, 1994; Mahadevan and Hall, 1992). It has also been demonstrated that the increment of *HFS* caused by addition of salts is dependent on the molecular weight of the protein, which is supported by the concept of salting-out as previously described (Yano *et al.*, 1994).

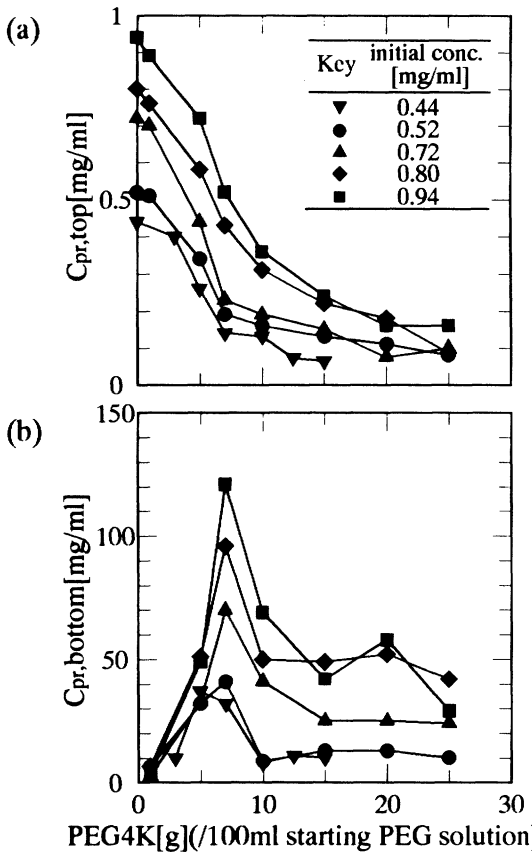


Fig. 4 Effects of addition of PEG4K on total protein concentration in (a) top and (b) bottom phases from various starting PEG solutions with different protein concentrations

GroEL and GroES can now be separated from the mixture and fractionated in the bottom phase by the control of PEG addition.

The effect of the total protein concentration in the starting PEG solution on their precipitation behavior is shown in **Fig. 4**. The data for 0.8 mg/m*l* are identical to those shown in Fig.3(a). The protein concentration of the bottom phase is also increased with increasing protein concentration in the starting PEG solution. Both the content of GroEL and GroES in the bottom phase and that of other proteins in the top phase, and the volume of bottom phase per unit starting PEG solutions show similar trends to those in Fig.3 (b and c) for all the protein concentrations in the starting PEG solution. Accordingly, a starting solution with high protein concentration is better for efficient separation than that with low protein concentration.

2.3 Purification of GroEL and GroES as their complex

Based on partitioning studies using ATPS, it was confirmed that GroEL and GroES form a 1:1 complex in the presence of ATP, as reported by Viitanen *et al.* (1990). The partition coefficients and surface properties (*HFS* and $\Delta \ln K_{pr}$) of the complex of GroEL and GroES changed from their respective values and indicated identical values (*i.e.* complex's value) when ATP was added. On the other hand, those of other proteins did not change.

Table 1 Surface properties of GroEL, GroES and their complex (values of M.W. given here were determined from SDS-PAGE)

	M.W. [dalton]	*HPS* [kJ·mol^{-1}]	$\Delta \ln K_{pr}$ [-]
GroEL	60k × 14mer	-360	1.9
GroES	15k × 7mer	-220	0.60
complex	60k × 14mer + 15k × 7mer	-380	0.40

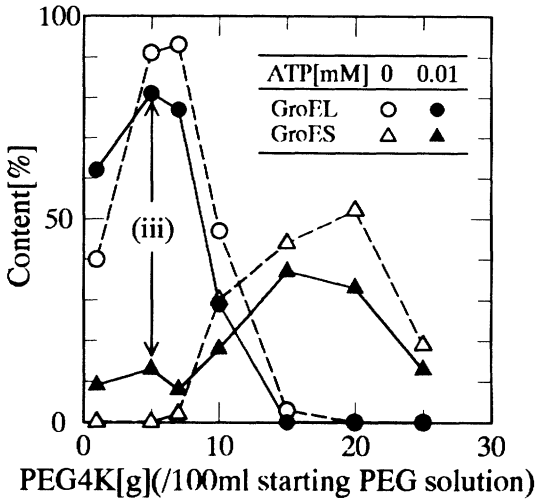


Fig. 5 Effect of addition of PEG4K on content of GroEL and GroES in bottom phase with and without ATP (fraction (iii) is sample of Lane (6) in Fig. 2)

Therefore, the complex of GroEL and GroES is formed by biospecific affinity in the presence of ATP. Surface properties of GroEL, GroES and their complex evaluated by using ATPS are shown in **Table 1**. The data for GroEL and GroES are cited from the previous work (Yano *et al.*, 1994). The local hydrophobicity ($\Delta \ln K_{pr}$) of the complex is considerably smaller than those of GroEL and GroES. This result suggests that each hydrophobic binding site of GroEL and GroES is combined by biospecific affinity in the presence of ATP. On the other hand, the surface net hydrophobicity (*HFS*) of the complex is similar to that of GroEL. This corresponds to the understanding that *HFS* is related to the accessible surface area, *i.e.* the molecular weight of protein (Yano *et al.*, 1994).

PEG fractional precipitation was then carried out in the presence of ATP to purify GroEL and GroES as a molecular complex. ATP was added to the starting solution (PEG phase in System 1) prior to PEG fractional precipitation. The effect of addition of PEG4K on the content of GroEL and GroES in the bottom phase with and without ATP is shown in **Fig. 5**. In the absence of ATP, GroES was not precipitated with GroEL, as expected. On the other hand, in the presence of ATP, GroES was precipitated with GroEL as a complexed state in the case of less than 10 g (/100 m*l* starting solution) of PEG4K added, and more than 60% of GroES was precipitated as a complexed state. The contents in Fig.5 were weight percent determined by quantitative scanning of SDS-PAGE analyses. If such estimates

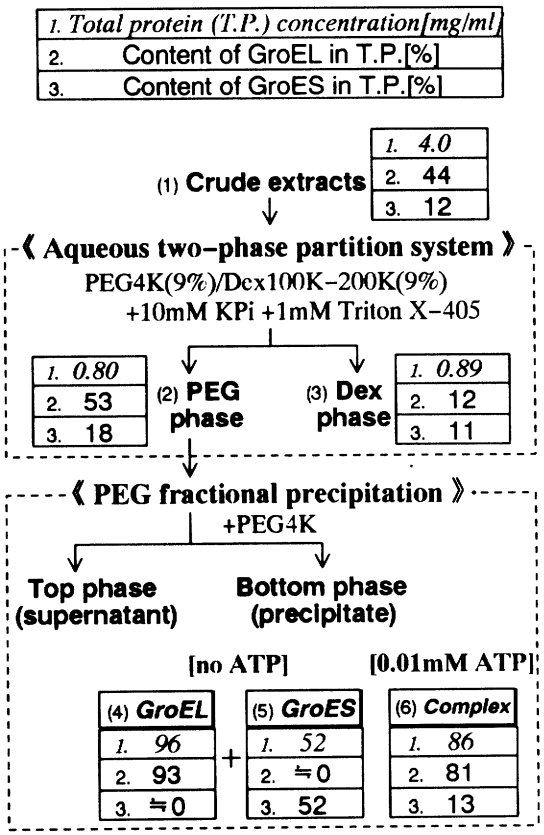


Fig. 6 Purification process for GroEL and GroES (fractions with numbers (1)-(6) correspond to Lanes (1)-(6) in Fig. 2, respectively)

are converted into moles of native protein by using their molecular weights in Table 1, the ratio of GroEL to GroES is approximately one to one. Therefore, GroEL and GroES can be purified as a 1:1 complexed state in the presence of ATP.

2.4 Combined process

The proposed process consisting of ATPS and PEG fractional precipitation is shown in **Fig. 6**. The results of SDS-PAGE for each solution are shown in Fig. 2. In this case, the initial solution (crude extracts) contained 44% GroEL, 12% GroES and 44% other proteins (Lane 1). The total protein concentration of the initial solution was 4.0 mg/m*l*. When the initial solution was partitioned in System 1 (PEG4K(9%) / Dex100K-200K(9%) + 10 mM KPi + 1 mM Triton X-405), GroEL and GroES were partitioned selectively into the PEG phase. The content of GroEL and GroES in the PEG phase was 53% and 18% of total soluble protein, respectively (Lane 2). On the other hand, the content in the Dex phase was comparatively low (Lane 3).

The PEG phase was used for the starting solution in the following PEG fractional precipitation. By addition of PEG4K, GroEL was concentrated (precipitated) in the bottom phase and thus purified (96 mg/m*l* total protein content, 93% of purity) (Lane 4, fraction (i) in Fig. 3). GroEL was recovered from the two bottom phase solutions with above 90% purity, as shown in Fig. 3 (b), and the recovery compared with the initial solution (crude extracts) was above 50 %. With an increase in the amount of PEG4K addition, GroES was also concentrated in the bottom phase (52 mg/m*l* total protein content, 52 % of purity) (Lane 5, fraction (ii) in Fig. 3). This content of GroES in the bottom phase was not especially high. However, GroES can be recovered without other protein contaminants by separating as a complex with GroEL in the presence of ATP (86 mg/m*l* total protein content, purity of GroEL: 81 % + GroES: 13 %) (Lane 6, fraction (iii) in Fig. 5). GroEL and GroES can be recovered from the bottom phase at above 50% and above 30 %, respectively, compared with crude extracts. GroEL, GroES and their complex in the protein concentrated solution form are found to keep their stability for several months in a refrigerator.

Conclusions

The design of a simple and effective purification process for GroEL and GroES from *E. coli* cells was presented with the following results.

1) System 1 (PEG4K (9 %) / Dex100K-200K (9 %) + 10mM KPi + 1mM Triton X-405) was selected as a suitable ATPS for the selective partitioning of GroEL and GroES to the PEG (top) phase based on their surface properties.

2) GroEL and GroES were fractionated from the recovered PEG phase in System 1 and concentrated in the bottom phase (precipitate) by further addition of PEG (PEG fractional precipitation).

3) GroEL and GroES were found to form a 1:1 complex by biospecific affinity in the presence of ATP. The complex was also concentrated by PEG fractional precipitation.

4) The combined purification process, in which PEG fractional precipitation is combined with ATPS (System 1), was found to be effective. GroEL and GroES were able to be purified and concentrated as both individual native molecules and as a 1:1 complexed state by the control of ATP addition.

Acknowledgement

This work was partly supported by a Grant-in-Aid for Developmental Research (B) (No.06555237). One of the authors (K.Y.) was supported by the fellowships of the Japan Society for the Promotion of Science for Japanese Junior Scientists.

Nomenclature

HF	=	hydrophobic factor of two-phase system	[mol·kJ^{-1}]
HFS	=	surface net hydrophobicity of protein	[kJ·mol^{-1}]
K_{pr}	=	partition coefficient of protein in PEG / Dex system	[-]
$\Delta \ln K_{pr}$	=	local hydrophobicity of protein (the increment of partition coefficient of protein caused by 1 mM Triton X-405)	[-]
C_{pr}	=	total soluble protein concentration	[mg·ml^{-1}]

Literature Cited

Albertsson,P.A., Partition of Cell Particles and Macromolecules, 3rd ed., p.112-146, John Wiley, New York, USA (1986)

Atha,D.H. and K.C.Ingham; "Mechanism of Precipitation of Proteins by Polyethylene Glycols," *J.Biol.Chem.*, **256**, 12108- 12117 (1981)

Bradford,M.M.; "A Rapid and Sensitive Method for the Quantification of Micrograin Quantities of Protein Utilizing the Principle of Protein-Dye Binding," *Anal.Biochem.*, **72**, 248-254 (1976)

Chandrasekhar,G.N., K.Tilly, C.Woolford, R.Hendrix and C.Georgopoulos; "Purification and Properties of the groES Morphogenetic Protein of *Escherichia coli*," *J.Biol.Chem.*, **261**, 12414-12419 (1986)

Fisher,M.T.; "Promotion of the in Vitro Renaturation of Dodecameric Glutamine Synthetase from *Escherichia coli* in the Presence of GroEL (Chaperonin-60) and ATP," *Biochemistry*, **31**, 3955-3963 (1991)

Harrison,R.G., Protein Purification Process Engineering, p.115- 208, Marcel Dekker, New York, USA (1994)

Hemmingsen,S.M., C.Woolfold, S.M.van der Vies, K.Tilly, D.T.Dennis, C.P.Georgopoulos, R.W.Hendrix and R.J.Ellis; "Homologous Plant and Bacterial Proteins Chaperone Oligomeric Protein Assembly," *Nature*, **333**, 330-334 (1988)

Hendrix,R.W.; "Purification and Properties of groE, a Host Protein Involved in Bacteriophage Assembly," *J.Mol.Biol.*, **129**, 375-392 (1979)

Horio,T., Theory and Practice on Enzymes and Other Proteins, 2nd ed., p.93-117, Nankodo, Tokyo, Japan (1994)

Ingham,K.C.; "Precipitation of Proteins with Polyethylene Glycol," *Methods in Enzymology*, **182**, 301-306 (1990)

Kuboi,R., H.Umakoshi and I.Komasawa; "Extractive Cultivation of *Escherichia coli* Using Poly(ethylene glycol) / Phosphate Aqueous Two-Phase Systems to Produce Intracellular β– Galactosidase," *Biothecnol.Prog.*, **11**, 202-207 (1995)

Kuboi,R., K.Yano and I.Komasawa; "Evaluation of Surface Properties and Partitioning of Proteins in Aqueous Two-Phase Extraction Systems," *Solv.Extr.Res.Dev.Japan*, **1**, 42-52 (1994)

Mahadevan,H. and C.K.Hall; "Experimental Analysis of Protein Precipitation by Polyethylene Glycol and Comparison with Theory," *Fluid Phase Equilibria*, **78**, 297-321 (1992)

Martin,J., T.Langer, R.Botena, A.Schramel, A.L.Howich and F.- U.Hartl; "Chaperonin-mediated Protein Folding at the Surface of groEL through a 'Molten globule'-like Intermediate," *Nature*, **352**, 36-42 (1991)

Miyake,T., S.Araki and T.Tsuchido; "Synthesis and Sedimentation of a Subset of 15-kDa Heat Shock Proteins in *Escherichia coli* Cells Recovering from Sublethal Heat Stress," *Biosci.Biotech.Biochem.*, **57**, 578-583 (1993)

Viitanen,P.V., T.H.Lubben, J.Reed, P.Goloubinoff, D.P.O'Keefe and G.H.Lorimer; "Chaperonin-Facilitated Refolding of Ribulosebisphosphate Carboxylase and ATP Hydrolysis by Chaperonin 60 (groEL) are K^+ Dependent," *Biochemistry*, **29**, 5665-5671 (1990)

Yano,K., T.Hasegawa, R.Kuboi, I.Komasawa and T.Tsuchido; "Characterization of Surface Properties of Heat Shock Proteins for the Separation Using Aqueous Two-Phase Systems," *J.Chem.Eng.Japan*, **27**, 808-814 (1994)