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Operation conditions of enzyme refolding by chaperonin and recycle system using ultrafiltration

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Abstract

To clarify the efficient refolding conditions of enzymes using chaperonin GroE from *Escherichia coli*, the effects of various factors on the chaperonin-mediated refolding of enzymes from 4M guanidine hydrochloride (GdnHCl) were investigated. Three enzymes, *Bacillus subtilis* α-amylase, bovine deoxyribonuclease I (DNase I) and yeast enolase were used as the model systems. In all enzymes, the maximum recovery of activities (90–150% with respect to the enzyme activities before denaturation) was attained in the presence of 2mM ATP and 2–5-fold molar excess of GroE 21-mer or GroEL 14-mer over enzyme molecules. Since the recovery of enzyme activity by GroEL is close to that by GroE, GroEL as well as GroE are applicable for the refolding systems of these enzymes. GroE significantly enhanced the recovery of enzyme activities at around 25–40 °C, pH 6–9 and up to rather high final GdnHCl and enzyme concentrations. Therefore, the chaperonin efficiently mediates the enzyme refolding under wide operation conditions. To test the reusability of chaperonins in enzyme refolding, GroEL was separated from refolded enzymes by ultrafiltration and recycled. GroEL repeatedly mediated the refolding of enzymes by choosing appropriate membranes. Therefore, protein refolding processes based on chaperonins are promising. © 1997 Elsevier Science S.A.

Keywords: Chaperonin; GroE; Protein refolding

1. Introduction

The aggregation of accumulating recombinant protein, namely inclusion body formation has been often observed in heterogeneous gene expression in many eukaryotic and prokaryotic cells such as *Escherichia coli*, *Bacillus*, baculovirus, *Saccharomyces* and monkey COS-7 cells. Since (i) the productivity of the inclusion body is high, (ii) the inclusion body is stable, easy to recover and >90% in mass pure protein, and (iii) methods for separating the inclusion body have been well established, the inclusion body strategy is advantageous. However, a low refolding yield of the useful bioactive products from recovered inclusion body often precludes the inclusion body strategy. Therefore, it is necessary to establish high efficiency renaturation processes of the inclusion body.

Recently, it has become clear that the so-called "molecular chaperons" play important roles in the folding of newly synthesized polypeptides in vivo [1]. Among the molecular chaperons, DnaK, DnaJ, GrpE and GroE systems of *E. coli* have been investigated actively. Especially, GroE is classified as "chaperonin" and the information on its structure and function has been accumulated [1–10]. GroE has proved to have the ability to mediate the protein folding in an adenosine triphosphate (ATP)-dependent manner. GroE is known to be an oligomeric protein complex composed of two types of subunits, GroEL (molecular weight 57 000) and GroES (molecular weight 10 000). The resulting complex GroE is a 21-mer with a molecular weight of 868 000, and its three-dimensional structure was reported [11,12]. Moreover, there are some reports on the application of molecular chaperons to the refolding of inclusion bodies [13,14].

We studied the enzyme refolding properties of GroE and GroEL under various conditions to clarify the efficient refolding conditions using chaperonin. This information is important to develop industrial enzyme refolding systems based on chaperonins. *Bacillus subtilis* α-amylase, bovine deoxyribonuclease I (DNase I) and yeast enolase were used as the model enzymes. Enzymes from various organisms and different subunit structures were chosen to elucidate a more general

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tendency. GroE was overproduced in *E. coli* and affinity purified using an immobilized casein column. The enzymes were denatured by 4M guanidine hydrochloride (GdnHCl) and refolded in the absence and presence of GroE or GroEL under various conditions. In the application of chaperonins to the industrial refolding system, they should be recycled. Therefore, an ultrafiltration system was used for separation of the GroEL 14-mer with an extremely large molecular weight from the refolded enzymes, and the effect of repeated use on the refolding ability of GroEL was investigated.

2. Materials and methods

2.1. Materials

GdnHCl (finest grade), casein and corn starch were obtained from Wako Pure Chemical Industries (Osaka, Japan). *Bacillus subtilis* α-amylase was obtained from Seikagaku Corporation (Tokyo, Japan). DNA from calf thymus and bovine DNase I were obtained from Worthington Biochemical Corporation (Freehold, NJ, USA). Yeast enolase and ATP were obtained from Oriental Yeast Corporation (Tokyo, Japan). 2-phosphoglyceric acid (2-PGA) and bovine serum albumin (BSA) were obtained from Sigma Chemical Company (St. Louis, MO, USA) and Nacalai Tesque (Kyoto, Japan), respectively. Enolase is a dimeric protein with a subunit molecular weight of 47 000. α-amylase and DNase I are monomeric proteins with molecular weights of 45 000–55 000 and 31 000, respectively. All other chemicals used were of reagent grade.

2.2. Plasmid construction

λ 649 from the Kohara clone bank was digested with *Eco*RI and *Hind*III. The 8.5×10^3 base fragment encoding both the *groEL* and *groES* genes was recovered and ligated with the plasmid pUC118 [15]. This plasmid was digested with *Sma*I and religated after removing the 2.9×10^3 base fragment. The resulting plasmid pGroE containing the 5.6×10^3 base fragment encoding both the *groEL* and *groES* genes was used for overproduction of the chaperonin.

2.3. Overproduction and purification of GroE

E. coli DH5α [16] cultures harboring the plasmid pGroE were grown overnight in Luria–Bertain (LB) medium (Difco yeast extract 5 g l^{-1}, Difco tryptone 10 g l^{-1} and NaCl 5 g l^{-1}) containing 100 mg l^{-1} ampicillin at 30 °C using 2 l Erlenmeyer flasks. Induction was accomplished by raising the temperature to 42 °C. After 3 h, cells were harvested by centrifugation at $5\,000 \times g$ for 20 min and resuspended in 50mM Tris-HCl, pH 8.0, 0.15M NaCl, 0.05% Tween 20 (TST buffer). The sonicated cell suspensions were centrifuged at $10\,000 \times g$ for 30 min. Chaperonin GroE was affinity purified from the supernatant by using casein–cellulofine which was prepared by coupling casein onto formyl-cellulofine (approximately 20 mg casein per ml gel) according to the manufacturer's instruction (Chisso Corporation, Tokyo, Japan). Bound GroE was eluted from casein–Cellulofine with 100mM ATP. GroEL and GroES were separated by gel filtration chromatography on a Sephacryl S-300 (Pharmacia Biotech) column equilibrated with TST buffer. GroEL and GroES samples were dialyzed against 50mM Tris-HCl pH 7.8, containing 10mM $MgCl_2$, 20mM KCl and 2mM dithiothreitol. The concentrations of GroEL and GroES were determined by the method of Bradford [17] using BSA as a standard. Purity of the proteins was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Activity measurements of α-amylase, DNase I and enolase

The activity of α-amylase was measured at 30 °C using corn starch as a substrate [18]. 20 μl of enzyme solution was mixed with 0.98 ml of substrate solution (20mM phosphate, pH 7.0, 0.5% corn starch) for 5 min. The amount of reducing fragments liberated was measured by adding 1% 3,5-dinitrosalicylic acid reagent (1.0 ml), heating in boiling water for 10 min and measuring the absorbance at 546 nm.

The activity of DNase I was measured at 37 °C using DNA as a substrate [19]. 10 μl of enzyme solution was mixed with 40 μl of assay buffer (10mM Tris-HCl, pH 7.5, 10mM NaCl, 3mM $MgCl_2$) and 50 μl of calf thymus DNA solution (2.5 mg per ml assay buffer). The reaction mixture was incubated at 37 °C for 10 min. After stopping the reaction by the addition of 25 μl of cold 1M perchloric acid and subsequent centrifugation at 12 000 rpm for 10 min, the samples were diluted 20 times with distilled water, and the absorbance at 260 nm was measured.

The activity of enolase was measured at 25 °C using 2-PGA as a substrate [20]. 40 μl of enzyme solution was mixed with 0.96 ml of substrate solution (50mM Tris-HCl, pH 7.8, 1mM 2-PGA, 1mM $MgCl_2$). The activity was determined from the increase in the absorbance at 240 nm as a function of time.

2.5. Enzyme refolding experiments

Enzyme (α-amylase, DNase I and enolase) solutions were mixed with an equal volume of 8M GdnHCl. The unfolded enzymes in 4M GdnHCl (7.5 μl) were subsequently diluted into the refolding buffer (50mM acetate pH 5.0, phosphate pH 6.0–7.0 or Tris-HCl, pH 7.8–10 buffer containing 10mM $MgCl_2$, 20mM KCl and 2mM dithiothreitol). ATP (final concentration 2mM) and desired amounts of GroE 21-mer or GroEL 14-mer were added to the refolding mixture where indicated. Desired amounts of GdnHCl were added to the refolding mixture to adjust the final GdnHCl concentration where indicated. The final volume of refolding mixture was 500 μl. The activities of refolded enzymes were measured,

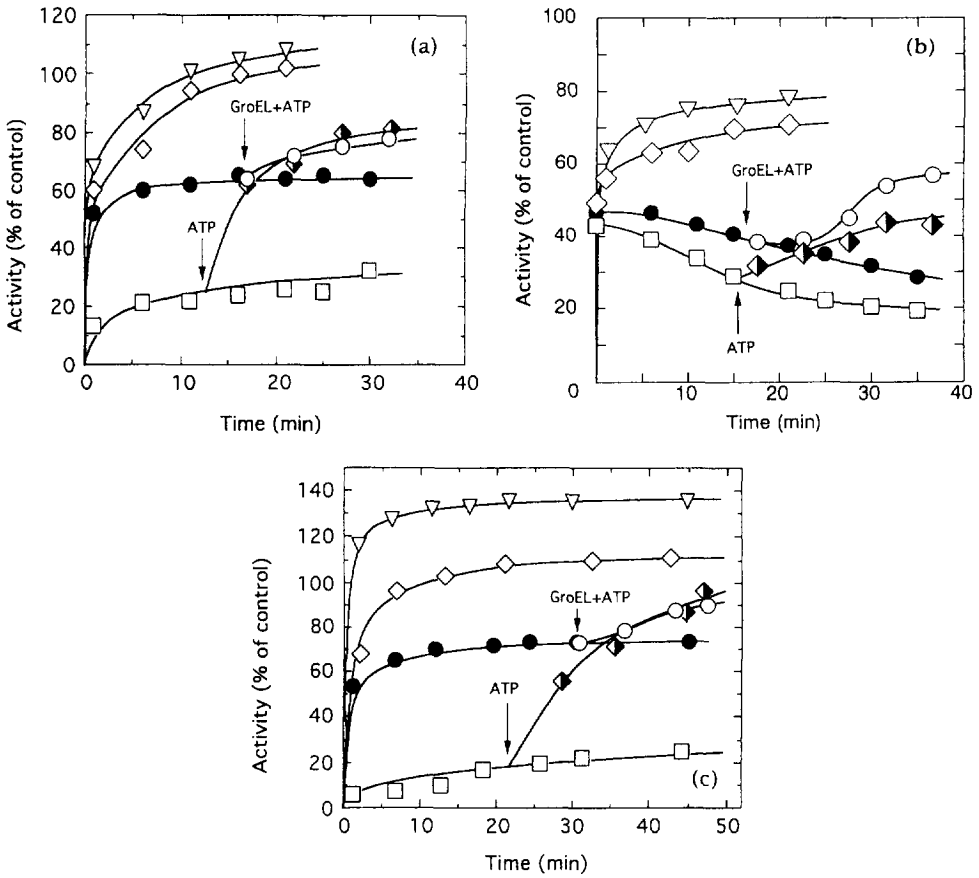

Fig. 1. Refolding of (a) α-amylase, (b) DNase I and (c) enolase in the absence and presence of the same molar amount of GroE 21-mer or GroEL 14-mer at 37 °C and pH 7.8. The final concentrations of enzymes and GdnHCl were 0.2μM and 60mM, respectively. (●) Refolding in the absence of chaperonin; (▽) refolding in the presence of GroE and 2mM ATP; (◇) refolding in the presence of GroEL and 2mM ATP; (□) refolding in the presence of GroEL and in the absence of ATP; (○) refolding by the addition of GroEL and 2mM ATP (at the time indicated by the arrow) after the enzyme activities reached to the plateau in the absence of chaperonin; (◑) refolding of enzymes trapped by GroEL by the addition of 2mM ATP (at the time indicated by the arrow).

and the refolding efficiency was evaluated as the percentage of specific activities of refolded enzymes relative to those of enzymes before denaturation under identical conditions.

2.6. Repeated enzyme refolding experiments in an ultrafiltration system

The unfolded DNase I and enolase in 4M GdnHCl (7.5 μl) were subsequently diluted into the refolding buffer containing desired amounts of GroEL 14-mer and 2mM ATP. The final volume of refolding mixture was 500 μl. After refolding reaction for 20 min, the refolding mixture was ultrafiltrated to separate the refolded enzyme from GroEL using YM100 for DNase I (molecular weight cut off > 100 kDa) and XM300 for enolase (molecular weight cut off > 300 kDa) (Amicon Inc., Beverly, MA, USA) until the volume was reduced to 250 μl. Then, 250 μl of the refolding buffer with 2mM ATP and 3.75 μl of unfolded enzyme were added, and the refolding reaction of the next cycle was started. The refolding mixture was filtrated again until the volume was reduced to 250 μl. This refolding cycle was repeated 10 times. The enzyme activities of the filtrate were measured. As the control experiments, repeated refolding without GroEL and ATP was performed. In addition, filtration experiments of native enzymes dissolved in the refolding buffer (concentration 0.2μM) were performed. The refolding efficiency was evaluated as the percentage of the activity of refolded enzyme recovered in the filtrate relative to those of native enzyme in the filtrate.

3. Results

3.1. Refolding of enzymes in the presence of GroE or GroEL

Fig. 1 compares the refolding profile of α-amylase, DNase I and enolase in the absence and presence of GroE or GroEL and ATP. α-amylase and enolase spontaneously recovered approximately 60–70% of their initial activity after denaturation in 4M GdnHCl and subsequent refolding. DNase I reduced its activity during refolding process, because of the heat inactivation. On the other hand, in the presence of GroE or GroEL and 2mM ATP, the enzyme activity was significantly improved. Addition of other proteins such as BSA, ovalbumin and egg white lysozyme or polyethylene glycol with various molecular weights did not attain similar recovery

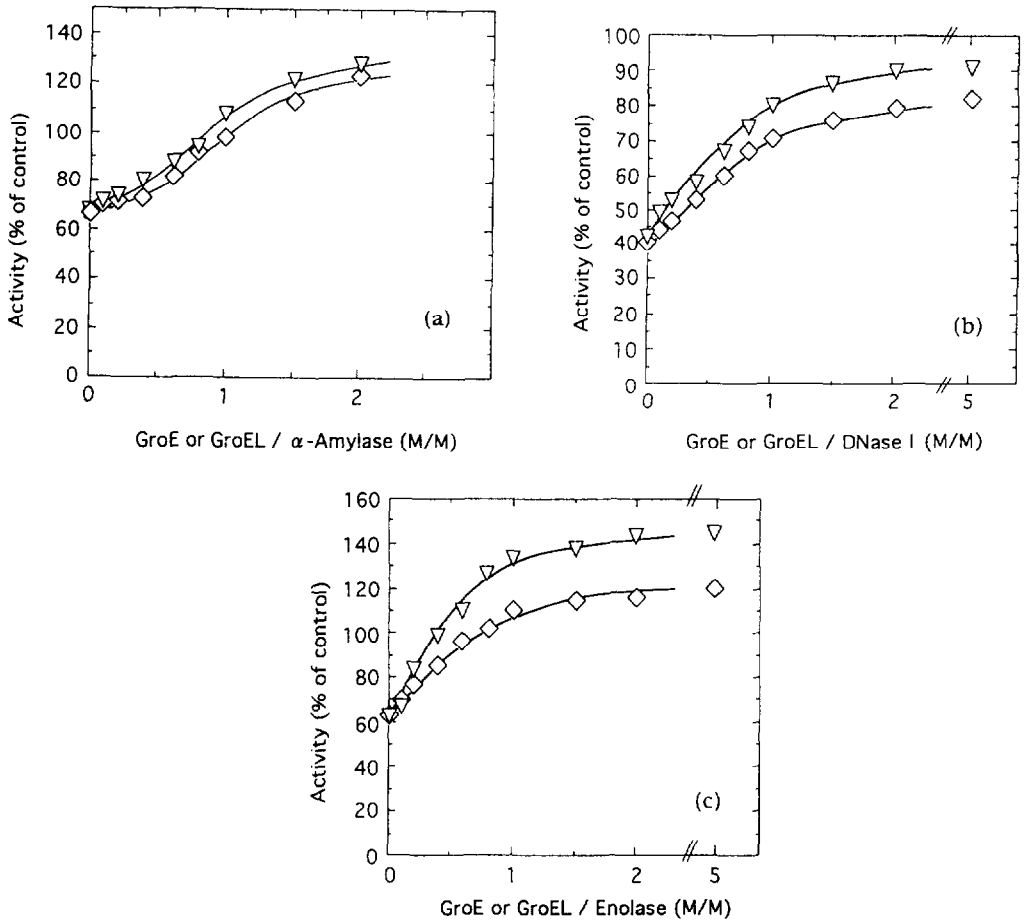

Fig. 2. Effect of the amount of added GroE 21-mer (▽) or GroEL 14-mer (◇) on the recovery of enzyme activities of (a) α-amylase, (b) DNase I and (c) enolase in the presence of 2mM ATP after 30 min refolding at 37 °C and pH 7.8. The final concentrations of enzymes and GdnHCl were 0.2μM and 60mM, respectively.

of activities (data not shown), indicating the efficient enhancement of protein refolding by the chaperonin.

In the absence of ATP, GroEL caused significant (enolase and α-amylase) or slight (DNase I) reduction in recovery of enzyme activity. In all cases, upon delayed addition of ATP to the reaction mixture, the recovery of activity increased significantly. The chaperonin activity of GroEL against refolded enzymes was further investigated. A slow increase in the recovery of enzyme activity was observed in all enzymes by the addition of GroEL and ATP after the plateau of enzyme activity was reached in the absence of chaperonin. These results indicate that GroEL binds enzymes with some non-native conformational states and convert them to native conformation.

Fig. 2 shows the effect of the amounts of GroE or GroEL on the recovery of activities of α-amylase, DNase I and enolase in the presence of ATP. In all cases, the recovery of enzyme activity increased with increasing amount of added chaperonin and saturated. The maximum activity of 90–150% was attained in the presence of 2–5-fold molar excess of GroE 21-mer or GroEL 14-mer over enzyme molecules. The recoveries of the activities of α-amylase and enolase were much higher than 100%. This is probably because the purchased enzyme samples contained a certain amount of denatured enzymes.

3.2. *Effects of temperature, pH, final GdnHCl and enzyme concentrations on the recovery of enzyme activities in the presence of GroE*

To know the efficient refolding conditions by chaperonins, the effects of various factors on the recovery of enzyme activity were further investigated. Fig. 3 shows the effects of temperature, pH and final GdnHCl concentration on the recovery of enzyme activity of α-amylase in the absence and presence of GroE and ATP, respectively. The recovery of enzyme activity was significantly enhanced by GroE over wide temperature and pH ranges, and up to a rather high final GdnHCl concentration. The highest recovery of enzyme activity was attained at around 37 °C (Fig. 3(a)), pH 7 (Fig. 3(b)) and below the final GdnHCl concentration of approximately 100mM (Fig. 3(c)). In the refolding of DNase I and enolase in the presence of GroE, a similar tendency was observed. GroE significantly enhanced the recovery of activities of these enzymes at around 25–40 °C, pH 6–9 and up to a rather high final GdnHCl concentration.

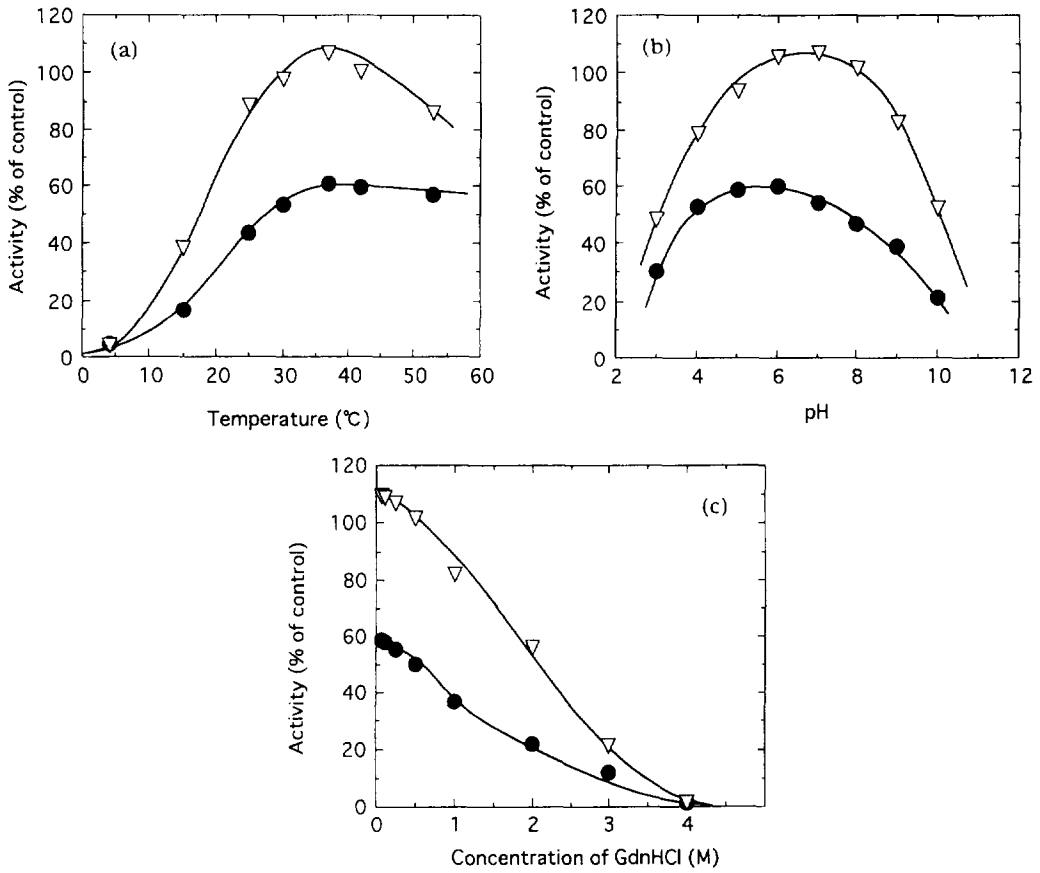

Fig. 3. Effects of (a) refolding temperature (pH 7.8, final GdnHCl concentration 60mM), (b) pH (37 °C, final GdnHCl concentration 60 mM) and (c) final GdnHCl concentration (pH 7.8, 37 °C) on the recovery of enzyme activities of α-amylase in the absence (●) or presence (▽) of the same molar amount of GroE 21-mer and 2mM ATP after 30 min refolding. The final concentration of enzyme was 0.2μM.

In addition to the environmental conditions examined above, protein refolding was known to be significantly affected by the final protein concentration. Therefore, the effect of final enzyme concentration on the recovery of enzyme activities of α-amylase, DNase I and enolase in the absence and presence of GroE and ATP was further examined (Fig. 4). In all cases, the recovery of enzyme activity was significantly enhanced by GroE up to a rather high final enzyme concentration.

3.3. Repeated enzyme refolding in the ultrafiltration system

For the separation of GroEL from refolded DNase I and enolase, YM100 and XM300 membranes were used, respectively. As the refolding conditions, pH 7.8, 37 °C and a final GdnHCl concentration of 60mM were chosen according to the information described above. Approximately 97% of native DNase I and 70% of native enolase were passed through the corresponding membrane. Fig. 5 shows the effect of repeated cycles on the recovery of enzyme activity, namely, the refolding ability of GroEL. In the refolding of DNase I, the same molar amount of GroEL was used, while in the refolding of enolase, both the same molar amount and a 3-fold molar excess of GroEL over enzyme molecules were used. The recovery of DNase I activity was similar during the repeated cycles (Fig. 5(a)), indicating the high durability of GroEL. Moreover, the reduction in the recovery of enolase activity during repeated cycles was small, when a 3-fold molar excess of GroEL over enolase molecules was used. On the other hand, the recovery of enolase activity decreased rapidly, when the same molar amount of GroEL was used (Fig. 5(b)). SDS-PAGE analysis shows the existence of GroEL in the filtrate of the XM300 membrane, while GroEL was not observed in the filtrate of YM100. Therefore, reduction in the recovery of enolase activity during repeated cycles is attributable to leakage of GroEL subunits.

4. Discussion

GroE and GroEL significantly improve the recovery of activities of α-amylase, DNase I and enolase during refolding in the presence of 2mM ATP (Figs. 1 and 2). Since the recoveries of enzyme activities by GroEL are close to those by GroE, the refolding of these enzymes is mainly facilitated by GroEL. In the folding of several other enzymes, GroES has also been reported to be obligatory [4,5,9]. Since all of the enzymes used in our study and these reports [4,5,9] spontaneously recovered a relatively high percentage of the enzyme activity (50–70%), a short time interaction with

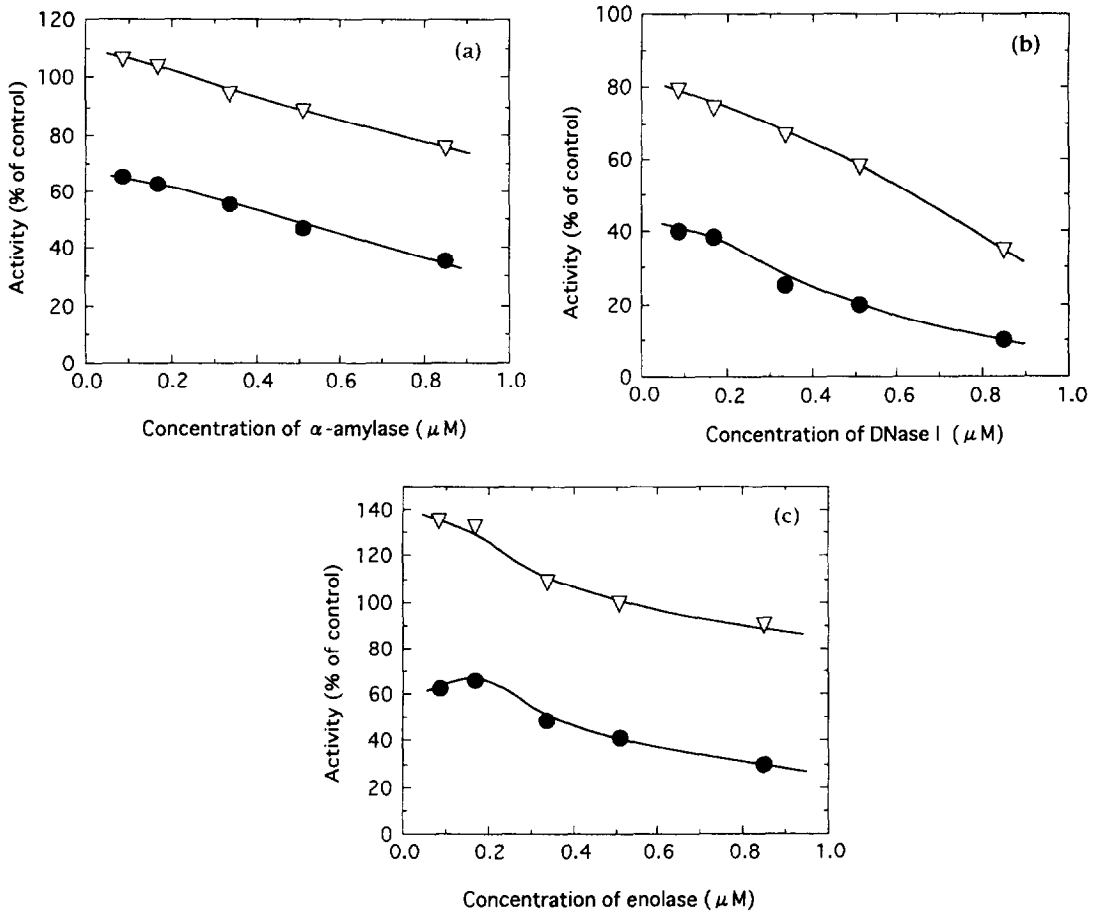

Fig. 4. Effect of final enzyme concentration on the recovery of enzyme activities of (a) α-amylase, (b) DNase I and (c) enolase in the absence (●) or presence (▽) of the same molar amount of GroE 21-mer and 2mM ATP after 30 min refolding at 37 °C and pH 7.8. The final GdnHCl concentration was 60mM.

GroEL is sufficient to obtain the conformational form that can then proceed spontaneously to the native state. Therefore, GroEL as well as GroE are applicable for the refolding systems of this type of enzymes.

To attain the maximum recovery of enzyme activity, 2–5-fold molar excess of GroE 21-mer or GroEL 14-mer over enzyme molecules are necessary (Fig. 2). 4–15-fold molar excess over folding enzyme has been reported to be necessary to attain the maximum recovery of enzyme activity [7,9,10]. The excess amount of GroE is necessary to achieve the efficient enzyme refolding, because the interaction between GroE and folding intermediates is probably not so strong. The different GroE concentration dependence of protein folding is attributable to the difference in the affinity of GroE for folding intermediates and the kinetics of aggregate formation of the folding intermediates.

The recovery of enzyme activities in the presence of GroE is significantly affected by the refolding conditions (Fig. 3). GroE efficiently mediates the enzyme refolding between 25–40 °C in all enzymes. The highest recovery of activities in the presence of GroE was achieved at around 37 °C, although the heat stability of enzymes is different (α-amylase > enolase > DNase I). The smaller extent of enhancement by GroE at 53 °C is probably attributable to the heat denaturation of GroE. The lower recovery of activity at low temperature is probably attributable to slower refolding reaction rates and lower chaperonin activity. Moreover, GroE efficiently mediates the enzyme refolding at pH 6–9 in all cases. The highest recovery of enzyme activities was attained at around pH 7–8, although the optimum pH for spontaneous refolding was different depending on enzymes. GroE mediates the refolding of enzymes up to a high final GdnHCl concentration. In some enzymes, the highest recovery of enzyme activities by spontaneous refolding was reported to be observed at a rather high final GdnHCl concentration [21]. GroE is probably effective even for the refolding of these enzymes under rather high final GdnHCl concentrations. Moreover, the recovery of activities was significantly enhanced by GroE even at relatively high enzyme concentrations in all cases (Fig. 4). GroE is effective to suppress the aggregation of folding intermediates during refolding and increase the recovery of bioactive products up to rather high final protein concentrations. These results indicate that chaperonin efficiently mediates enzyme refolding at around 25–40 °C, pH 6–9 and up to rather high final GdnHCl and enzyme concentrations.

As shown in Fig. 5, GroEL can be repeatedly used for refolding of DNase I and enolase in the ultrafiltration system

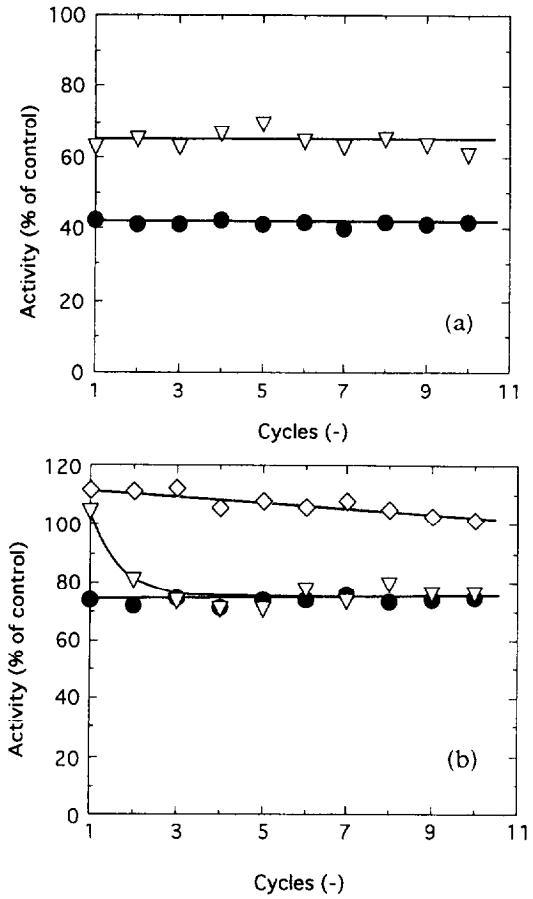

Fig. 5. Effect of repeated use of GroEL on the recovery of enzyme activities of (a) DNase I and (b) enolase in the ultrafiltration system using YM100 (molecular weight cut off > 100 kDa) and XM300 (molecular weight cut off > 300 kDa), respectively. (●) Refolding in the absence of chaperonin; (▽) refolding in the presence of the same molar amount of GroEL and 2mM ATP; (◇) refolding in the presence of a 3-fold molar excess of GroEL over enzyme molecules and 2mM ATP. In each cycle, enzymes were refolded for 20 min at 37 °C and pH 7.8. The final concentrations of enzymes and GdnHCl were 0.2 μM and 60 mM, respectively.

using a membrane with an appropriate molecular weight cut off, indicating the high durability of GroEL. This result also indicates the effectiveness of the refolding system using GroEL in combination with an ultrafiltration system. In the case of the membrane with a molecular weight cut off > 300 kDa, the dissociated subunits of GroEL probably pass through the membrane. Since a higher concentration of GroEL is favorable for the association of subunits, GroEL showed much a higher reusability for the refolding of enolase by using a 3-fold molar excess over enolase molecules. The chaperonins from thermophilic and hyperthermophilic bacteria could be more suitable for this system, because they form more stable complexes [22,23].

The above results indicate that the recovery of active enzymes was significantly enhanced by chaperonin GroE and GroEL under wide refolding conditions. Moreover, GroEL shows a high durability in repeated refolding of GdnHCl solubilized proteins. Therefore, the protein refolding processes based on molecular chaperons are promising.

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