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Short communication

Enzyme-responsive artificial chaperone system with amphiphilic amylose primer

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ABSTRACT

An enzyme-responsive artificial chaperone system which employs an amphiphilic amylose primer (dodecyl maltopentaose, C12-MP) as a surfactant and phosphorylase b was designed to enable protein refolding. Effective refolding of carbonic anhydrase B after both heat denaturation (70 °C for 10 min) and guanidine hydrochloride (6 M) denaturation was observed by controlled association between the protein molecules and the C12-MP primer micelle through an enzymatic reaction.

In the post-genome era, effective refolding (Swietnicki, 2006; Choe et al., 2006), stabilization and preservation of recombinant proteins (Shuellekens, 2002) are of great importance for analysis of protein structure and function. In general, protein refolding from inclusion bodies (protein aggregates) is achieved by solubilization of the proteins with denaturants followed by their removal to enable refolding. However, in many cases, re-aggregation occurs during the refolding process and the yield of native protein is generally not high. One way to improve the renaturation and refolding of proteins is to use artificial molecular chaperone systems (Yoshimoto et al., 2003; Lanckriet and Middelberg, 2004; Lu et al., 2005; Yazdanparast and Khodarahmi, 2007; Nomura et al., 2003; Morimoto et al., 2005; Ikeda et al., 2006; Rozema and Gellman, 1996a,b; Daugherty et al., 1998). Rozema and Gellman (1996a,b) have reported a two-step refolding system that works like molecular chaperones in living systems (Rozema and Gellman, 1996a,b; Daugherty et al., 1998). It includes inhibition of protein aggregation by the formation of complexes between denatured proteins and surfactants through hydrophobic interactions followed by protein refolding via the removal of the surfactants by the addition of cyclodextrin. Linear dextran (Sundari et al., 1999) and cycloamylose (Machida et al., 2000) have also been used as alternatives to cyclodextrin. The process of surfactant removal is important for the effective refolding of proteins. In this report, we suggest a novel protein refolding system employing an enzymatic-responsive oligosaccharide surfactant.

Amylose can be enzymatically synthesized in the presence of phosphorylase and α-D-glucose-1-phosphate (G1P) (Whelan and Bailey, 1954). The elongation reaction of the saccharide chain proceeds from the non-reducing 4-OH terminus of the (α,1 → 4)-glucan chain. Therefore, the reducing terminus can be modified (Ziegast and Pfannemueller, 1987; Kobayashi et al., 1996; Akiyoshi et al., 2002). In our previous report (Morimoto et al., 2007), we synthesized an amylose primer substituted with alkyl groups. The amphiphilic amylose primers formed micelles in water and dissociated with an increase in the hydrophilicity brought about through enzymatic polymerization. Using this property, the micelle-to-vesicle transition of mixed lipid/primer systems can be controlled. This enables a reconstitution of the trans-membrane protein to a liposome.

We report here a new artificial chaperon system in which alkylated amylose primer (dodecyl maltopentaose, C12-MP) micelles prevent the aggregation of denatured protein by the complexation and refolding of the native form followed by the removal of C12-MP micelles from the protein complex by enzymatic polymerization of the C12-MP primer. This is the first report of the use of enzymatic-responsive artificial molecular chaperones for protein refolding.

Fig. 1 shows the enzymatic polymerization scheme of C12-MP primers. It is reported that the critical micelle concentration (CMC) of C12-MP was ~0.5 mM, and the spherical shape C12-MP micelle (1.0 mM) was the optimum concentration for enzymatic polymerization (Morimoto et al., 2007). Chaperone like activity

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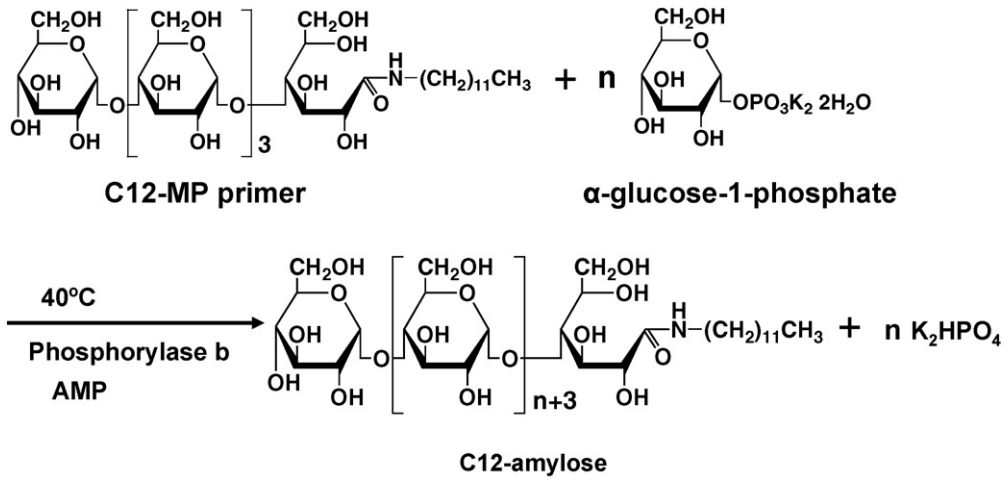

Fig. 1. Enzymatic polymerization of C12-MP primer.

in the heat denaturation process (heat shock protein-like activity) was investigated using carbonic anhydrase B, CAB (molecular weight 30,000) as a model protein. CAB is a monomeric metalloenzyme, and its secondary structure consists primarily of β-sheets (Eriksson et al., 1988). When the solution of CAB (0.06 mg/mL) was heated at 70 °C for 10 min, a precipitate was formed. The activity of CAB was completely lost and was not recovered even after lowering the solution to room temperature. Heat denatured CAB easily aggregated and formed a precipitate. In the presence of C12-MP primer micelle (2.0 mM), the precipitation was repressed and a clear solution was obtained after heating the CAB (0.06 mg/mL) in 50 mM Tris–sulfate buffer (pH 7.5) at 70 °C for 10 min because of the complexation of heat denatured protein and C12-MP primer micelle. To the complex of CAB and C12-MP micelle solution, a mixture of α-D-glucose-1-phosphate (G1P, 200 mM), phosphorylase b (0.52 μM) and adenosine 5′-monophosphate sodium salt (AMP, 20 mM) in Tris–sulfate buffer was added (1:1, v/v) and enzymatic polymerization of C12-MP primer was initiated at 40 °C. The recovery of enzyme activity of CAB was evaluated by hydrolysis rate of *p*-nitrophenylacetate (Pocker and Stone, 1967). The results were shown in Fig. 2. The enzyme activity was not recovered at all in the absence of phosphorylase b. By the addition of phosphorylase b, the recovery of CAB enzyme activity started at 40 min. After 180 min, more than 70% of the enzyme activity of CAB was recovered.

The polymerization of C12-MP primer (1.0 mM) under similar refolding conditions without CAB was monitored by the production of orthophosphate in the reaction buffer (Fiske and Subbarow, 1925). After 120 min, an additional six sugar units on average was introduced into the C12-MP primer (Fig. 2). The hydrophilicity of the C12-MP primer increased with increasing the degree of polymerization and the C12-amylose loses its activity as a surfactant. Surfactants interact with the hydrophobic surface of the denatured protein and inhibit its aggregation (Horowitz, 1993). The recovery of enzyme activity was induced by the release of C12-amylose from the protein complex.

When the concentrations of CAB increased from 0.06 mg/mL to 0.2 mg/mL or 0.6 mg/mL, C12-MP (2.0 mM) micelle also effectively inhibited the aggregation after heating at 70 °C for 10 min. Then the solution was added to enzymatic polymerization mixture (0.26 μM of phosphorylase b). After 3 h of enzymatic polymerization, the enzyme recovery was 69.5 ± 1.6% (0.2 mg/mL) and 72.8 ± 4.6 % (0.6 mg/mL), respectively. These recovery rates are comparable to that at 0.06 mg/mL of CAB. Next, we invesitaged the effect of the concentration of phosphorylase b enzyme (0.13 μM, 0.26 μM, and 0.52 μM) for the recovery of CAB enzyme activity from the complex of C12-MP (2.0 mM) and CAB (0.06 mg/mL). The onsets of the recovery of the enzyme activity were shortened with increasing

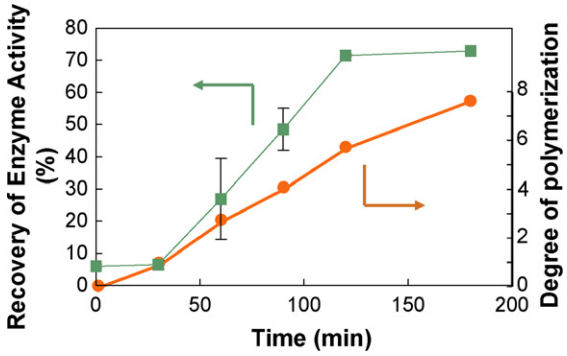

Fig. 2. Recovery of enzyme activity from heat-denatured CAB (0.03 mg/mL)–C12-MP (1.0 mM) complex by enzymatic polymerization of C12-MP using phosphorylase b (0.26 μM) and the degree of polymerization of C12-MP in the same condition. The enzyme activity of CAB was measured that a dry acetonitrile solution (50 μL) of 50 mM *p*-nitrophenylacetate was added to 450 μL of the sample solution (50 mM Tris–sulfate buffer, pH 7.5). After 5 s of mixing, an increase in the *p*-nitrophenolate concentration was monitored by the absorbance at 400 nm as a function of time. The percent recovery of enzyme activity was calculated on the basis of the initial velocity of the activity of native CAB. The protein concentration of native CAB was determined by absorbance at 280 nm with a coefficient of 1.83 mg protein/mL cm.

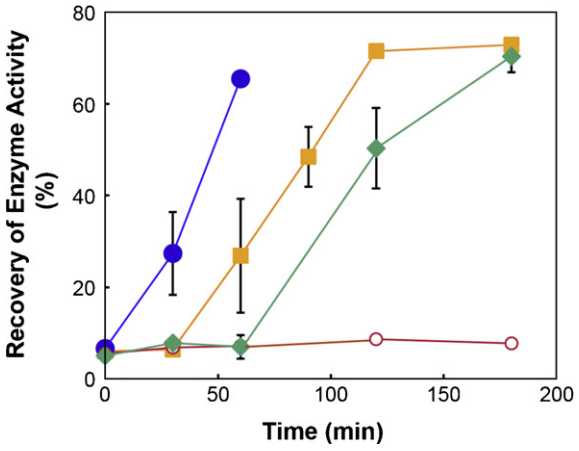

Fig. 3. Recovery of enzyme activity from heat-denatured CAB (0.03 mg/mL)–C12-MP (1.0 mM) complex by enzymatic polymerization of C12-MP with various concentration of phosphorylase b: (●) 0.52 μM, (■) 0.26 μM, (♦) 0.13 μM, and (○) 0 μM.

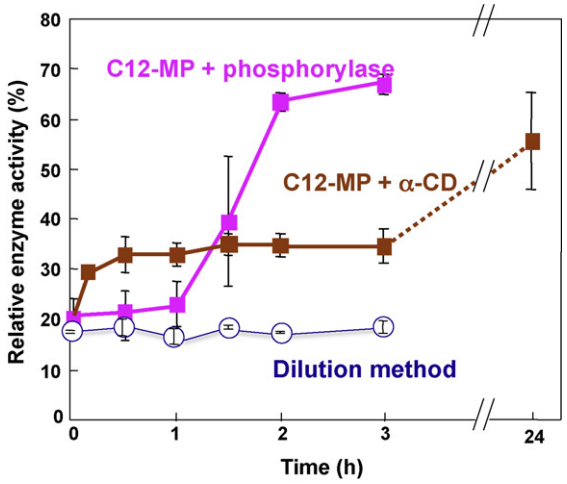

Fig. 4. Comparison of CAB renaturation rate between enzymatic polymerization and cyclodextrin addition to CAB–C12-MP primer complex as a function of time.

the concentrations of phosphorylase b due to the faster elongation reaction of sugar surfactant (Fig. 3). These results indicate that the recovery of CAB enzyme activity was triggered by enzymatic polymerization of C12-MP primer and the rate determine step of the recovery is probably a process of the release of the surfactants from the complex.

We applied this system to the refolding of chemically denatured CAB, which is a model system of renaturation from the aggregated protein such as inclusion bodies. Guanidine hydrochloride was added to the CAB solution (37.5 mg/mL) at a final concentration of 6 M, and denatured for 16 h at room temperature. The denatured solution was diluted 1000 times by buffer solutions in the absence and in the presence of C12-MP primer micelle solution (1.3 mM). In the absence of primers, only ~15 % of denatured CAB refolded and ~85 % aggregated and precipitated. After the dilution with the primer micelle solution, 20% of enzyme activity was recovered and no precipitation was observed. The aggregation of protein upon refolding was prevented in the presence of micelles due to the complexation. To the complex solution, G1P (500 mM), phosphorylase b (1.3 μM) and AMP (50 mM) were added (1:4, v/v) and enzymatic polymerization of C12-MP primer was initiated. Fig. 4 shows the enzyme recovery of chemically denatured CAB as a function of polymerization time after the addition of phosphorylase b. The recovery of CAB enzyme activity started after 60 min and reached ~70 % after 3 h. The activity recovery rate and yield was almost similar to that of the heat denatured system.

Rozema and Gellman (1996a,b) reported that denatured protein complexed with surfactants refolds by removal of the surfactants from the protein in the presence of cyclodextrin (Rozema and Gellman, 1996a,b; Daugherty et al., 1998). We examined the effect of cyclodextrin in our system. To the complex of C12-MP (1.0 mM) and chemically denatured CAB (0.03 mg/mL), α-cyclodextrin (10 mM) was added and the recovery of enzyme activity was followed. Approximately 30% of the enzyme activity was obtained at 2 h and 50% at 24 h after the addition of α-cyclodextrin as shown in Fig. 4. The slow rate of enzyme recovery in the presence of α-cyclodextrin may be difficult to apply to unstable proteins which are more prone to denaturing. The enzyme-responsive system showed a higher efficiency than cyclodextrin as the stripping agent of surfactant.

In conclusion, denatured CAB was effectively refolded in a two-steps process as shown in Fig. 5. First, an intermediate state of CAB interacted with the C12-MP micelle and formed the complex before CAB could aggregate. Then, enzymatic polymerization of the complex solution was achieved by the addition of phosphorylase b

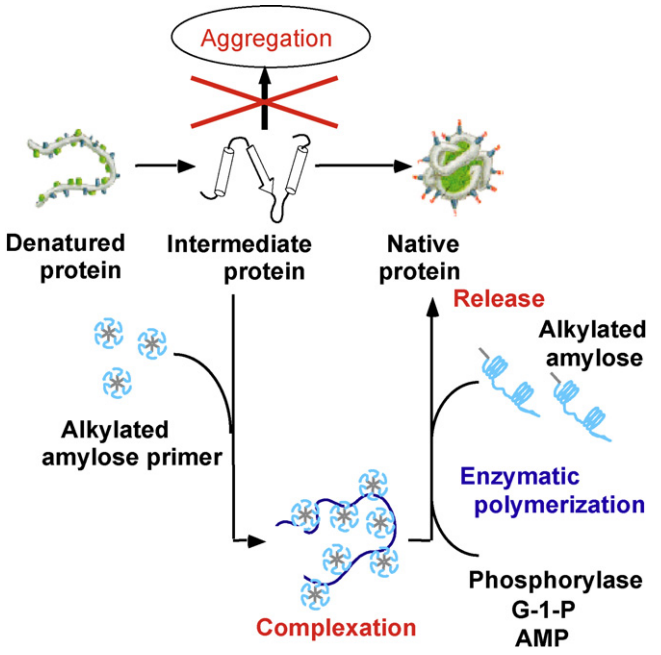

Fig. 5. Schematic illustration of artificial chaperone system by enzymatic polymerization of C12-MP primer.

and the substrate. While increasing the degree of polymerization, the polymerized C12-amylose decreased the hydrophobicity and could be removed from the intermediate CAB. At the same time, the CAB which was refolded gradually maintained enzyme actvity. This enzyme-responsive system which can act as an artificial chaperone provides a new option for effective protein refolding.

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