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Stabilization of Free and Immobilized Enzymes Using Hyperthermophilic Chaperonin

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Chaperonins suppress the denaturation of proteins and promote protein folding *in vivo*. Because hyperthermophilic chaperonins are expected to be used as a stabilizer for proteins, the effects of a group II chaperonin from a hyperthermophilic archaeum, *Thermococcus* strain KS-1 (*T.* KS-1 cpn), on the stabilization of mesophilic and thermophilic free enzymes and an enzyme co-immobilized with *T.* KS-1 cpn were studied. *T.* KS-1 cpn prevented the thermal inactivation of yeast alcohol dehydrogenase (ADH), jack bean urease, and *Thermus flavus* malate dehydrogenase (MDH) at high temperatures. *T.* KS-1 cpn also improved the long-term stability of ADH at lower temperatures. Moreover, the residual ADH activity of ADH co-entrapped with *T.* KS-1 cpn was improved and maintained at a higher level than that of the entrapped ADH without chaperonin. *T.* KS-1 cpn is useful for the stabilization of free and immobilized enzymes and applicable to various fields of biotechnology.

[**Key words:** archaea, chaperonin, heat stabilization, hyperthermophile, immobilized enzyme]

The chaperonin family, a group of molecular chaperones, plays a central role in protein folding *in vivo* (1, 2). Hyperthermophilic chaperonins belong to group II chaperonins, which are found in archaea and eukaryotic cytosol. Group II chaperonins form cylindrical structures made up of two stacked rotationally symmetrical rings consisting of eight or nine subunits, and have no co-chaperonin such as the *Escherichia coli* GroES. Hyperthermophilic group II chaperonins have one to three distinct subunits, α, β and γ. In previous studies, several thermophilic group II chaperonins, namely *Sulfolobus solfataricus* chaperonin (3) and recombinant *Methanococcus thermolithotrophicus* chaperonin expressed in *E. coli* (4), have been found to promote the refolding of chemically denatured proteins in an ATP-dependent manner. Recombinant group II chaperonin α and β subunit homo-oligomers from a hyperthermophilic archaeum, *Thermococcus* strain KS-1 expressed in *E. coli* have been found to form cylindrical structures composed of two stacked 8-fold rotational symmetric rings of each subunit. *T.* KS-1 chaperonin (*T.* KS-1 cpn) α and β homo-oligomers exhibited ATPase activity, and facilitated the refolding of chemically denatured proteins in the presence of ATP (5–8). Furthermore, *T.* KS-1 α and β cpn monomers facilitated the refolding of chemically denatured enzymes even in the absence of ATP (8).

Thermophilic and hyperthermophilic chaperonins are expected to be used for stabilizing proteins because they are thermostable and suppress the thermal inactivation of enzymes. In previous studies, a thermophilic group I chaperonin from *Bacillus* strain MS stabilized enzymes at 30°C and 80°C (9). Mesophilic group I chaperonins, such as GroEL/ES from *E. coli*, stabilized several proteins in a range of temperatures from 4°C to 48°C (10–12). Among hyperthermophilic group II chaperonins, *S. solfataricus* chaperonin (13) and recombinant *Thermococcus kodakaraensis* KOD1 (formerly *Pyrococcus* sp. KOD1) chaperonin expressed in *E. coli* (14, 15) stabilized enzymes at 50°C.

When enzymes are used in biotechnological processes, they are often immobilized onto insoluble support materials. The advantage of immobilization is not only the fact that enzyme is reusable but it is also capable of stabilizing enzymes. However, because immobilization causes a structural change of enzymes, the activity of immobilized enzymes is generally lower than that of free enzymes. Moreover, despite the increase in their stabilities, immobilized enzymes are gradually inactivated. Therefore, it is desirable that enzymes are immobilized without loss of activity, and that immobilized enzymes maintain their activities for a longer period. The co-immobilization of chaperonin has some advantages compared with small-molecule stabilizing agents, the chemical modification of enzymes and protein engineering such as amino acid replacement. One advantage is the ease of preparation of chaperonin co-immobilized enzymes because chaperonins and target enzymes can be immobilized using the same method simultaneously. Another is that many enzymes can be simultaneously immobilized because of the low specificity of chaperonin to substrate proteins. Hence, the co-immobilization of *T.* KS-1 cpn is expected to stabilize immobilized enzymes.

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To apply hyperthermophilic chaperonins to biotechnological processes, we studied the heat stabilization effect of enzymes using a group II chaperonin from a hyperthermophilic archaeum, *Thermococcus* strain KS-1. Furthermore, the stability of a *T.* KS-1 cpn co-immobilized enzyme by an entrapping method was examined.

MATERIALS AND METHODS

Materials Yeast alcohol dehydrogenase (ADH: EC 1.1.1.1, 37 kDa, tetramer) and ATP were purchased from Oriental Yeast (Tokyo). *Thermus flavus* malate dehydrogenase (MDH: EC 1.1.1.37, 35 kDa, dimer) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Jack bean urease (EC 3.5.1.5, 60 kDa, trimer) was purchased from Wako Pure Chemical Industries (Osaka). Bovine liver glutamate dehydrogenase (GlDH: EC 1.4.1.3, 56 kDa, hexamer) was purchased from Roche Diagnostics (Basel, Switzerland). The other materials were purchased from Nakalai Tesque (Kyoto).

Expression and purification of chaperonin *T.* KS-1 cpn α and β subunits (*T.* KS-1 α and β cpns) were overexpressed as described previously (8). Cells were harvested by centrifugation at 5000×*g* for 10 min at 4°C, resuspended in 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)–NaOH buffer (pH 7.5), disrupted by sonication, and recentrifuged at 30,000×*g* for 60 min at 4°C. Magnesium chloride, dithiothreitol (DTT) and glycerol were added to the recovered supernatant at final concentrations of 25 mM, 1 mM, and 5%, respectively, and the supernatant was heated at 70°C for 30 min to remove most *E. coli* proteins. After removing denatured proteins by centrifugation at 30,000×*g* for 60 min at 4°C, the extract was dialyzed against 50 mM HEPES–NaOH buffer (pH 7.5) containing 25 mM $MgCl_2$. The concentrations of *T.* KS-1 α and β cpns were determined by the method of Bradford using bovine serum albumin (BSA) as a standard (16). The purity of the chaperonins was determined by 12.5% SDS–PAGE and monomeric and oligomeric forms of the chaperonins were confirmed by 6% native PAGE. The percentage of *T.* KS-1 hexadecamer (16-mer) in the total *T.* KS-1 cpn was quantified by NIH imaging (ver. 1.62).

Stabilization of free enzymes ADH, urease or MDH was mixed with incubation buffer (50 mM Tris–HCl buffer (pH 7.8) containing 300 mM KCl, 50 mM $MgCl_2$, 100 mM guanidine hydrochloride and 83.3 μM DTT) containing *T.* KS-1 cpn pre-incubated at 50°C, 30°C or 4°C (ADH), 65°C (urease), and 85°C (MDH). The enzyme solutions were incubated at the same temperatures. The stabilization effect was evaluated as the residual activity which is the relative activity of incubated enzymes relative to untreated enzymes.

Enzyme assay The activities of ADH, urease and MDH were measured by the following methods. An aliquot of ADH solution was added to 100 mM glycine–KOH buffer (pH 9.5) containing 2 mM NAD^+ and 100 mM ethanol. The ADH activity was determined by measuring the absorbance at 340 nm as a function of time at 25°C. Urease activity was determined by measuring NADH oxidation rates using NADH-dependent GlDH and ammonia produced by urease (17). An aliquot of urease solution was added to 50 mM Tris–HCl buffer (pH 7.8) containing 0.81 mM 2-oxoglutarate, 0.24 mM NADH, 10 mM urea and 3.0 U/ml GlDH. The urease activity was determined by measuring the absorbance at 340 nm as a function of time at 25°C. An aliquot of MDH solution was added to 90 mM Tris–HCl buffer (pH 7.4) containing 0.5 mM oxalacetate and 0.25 mM NADH. The MDH activity was determined by measuring the absorbance at 340 nm as a function of time at 25°C. Detailed experimental conditions are described in the figure legends.

Stabilization of chaperonin co-immobilized ADH An entrapping method, which is one of the immobilization strategies, is routinely used for the preparation of several immobilized enzymes. Entrapment using calcium alginate gel is used for several enzymes (18, 19). In this study, ADH and *T.* KS-1 cpn were co-entrapped in calcium alginate gel. Sodium alginate (5%) was dissolved by heating using an autoclave (105°C, 1 min). After cooling down to room temperature, ADH and *T.* KS-1 cpn or BSA were added to the sodium alginate solution. This mixture was dropped into ice-cold buffer A (50 mM Tris–HCl buffer (pH 7.0) containing 1.0 M $CaCl_2$). Four types of gel beads were prepared: entrapped ADH (ADH-alone) gel beads, ADH co-entrapped with *T.* KS-1 α cpn (ADH-*T.* KS-1 α cpn) gel beads, ADH co-entrapped with *T.* KS-1 β cpn (ADH-*T.* KS-1 β cpn) gel beads and ADH co-entrapped with BSA (ADH-BSA) gel beads. These gel beads were stored at 4°C in 50 mM Tris–HCl buffer (pH 7.0) until the measurement of entrapped ADH activity. ADH activity was measured by the following method. Entrapped ADH (0.2 g wet gel) was added to 100 mM glycine–KOH buffer (pH 9.5) containing 2 mM NAD^+ and 100 mM ethanol. The total reaction volume was 1.5 ml. After 5 min of stirring, the entrapped ADH activity was determined from the increase in the absorbance at 340 nm as a function of time at 25°C by measuring the absorbance at 340 nm of the reaction solution at intervals of 5 min to 15 min. The stabilization effect was evaluated as the residual activity compared to the entrapped ADH activity stored for 1 d after preparation.

RESULTS

Stabilization of free enzymes at high temperatures Hyperthermophilic chaperonins are expected to increase the thermal stability of enzymes because of their high thermostability. Figure 1 shows the thermal inactivation of ADH at 50°C in the presence and absence of *T.* KS-1 cpn. After a 120-min incubation, ADH activity decreased in the absence of *T.* KS-1 cpn. A 5-fold excess concentration of *T.* KS-1

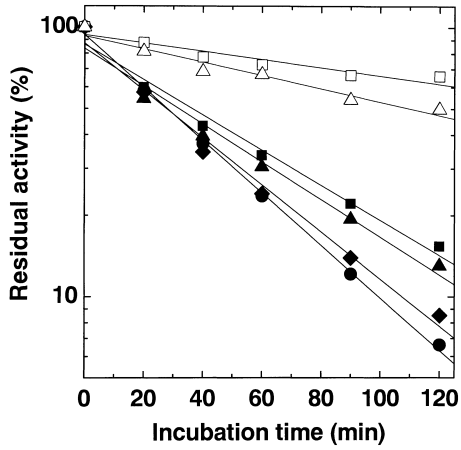

FIG. 1. Thermal inactivation of yeast ADH at 50°C and pH 7.8. The final concentrations of ADH, BSA and *T.* KS-1 cpn are 50 nM, 250 nM and 250 nM, respectively. The concentration of *T.* KS-1 cpn was calculated by assuming that all subunits formed a 16-mer. The residual activity was calculated as the percentage of incubated enzyme activity relative to untreated enzyme activity. Closed and open symbols indicate the incubation in the absence and presence of 5 mM ATP, respectively. Circles, Incubation in the absence of *T.* KS-1 cpn and BSA; diamonds, incubation in the presence of BSA; squares, incubation in the presence of *T.* KS-1 α cpn; triangles, incubation in the presence of *T.* KS-1 β cpn.