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Research Article

Plant Heat Shock Proteins Are More Effective in Enhancing Recombinant Alcohol Dehydrogenase Activity than Bacterial Ones *In Vitro*

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Background: Recombinant proteins produced in the cell factories are used in biological research, pharmaceutical production, and biochemical and agricultural applications. Molecular chaperones, such as heat shock proteins (Hsps), are co-expressed with recombinant proteins to enhance their yield, stability, and activity. When *Escherichia coli* (*E. coli*) is used as a cell factory, *E. coli* Hsps are the frequently used co-expression partners.

Objectives: We examined if there are differences in the molecular chaperone activities of plant and bacterial Hsps on recombinant protein activity. We compared the effects of the Hsps from carrot (*Daucus carota*) and *E. coli* on enhancing the recombinant alcohol dehydrogenase (ADH) activity and solubility under normal and heat conditions *in vitro*.

Materials and Methods: His-tagged carrot Hsps (DcHsp17.7 and DcHsp70), *E. coli* Hsps (IbpA, IbpB, and DnaK), and ADH from a thermophile *Geobacillus stearothermophilus* were individually cloned in a pET11a or a pET26b vector, introduced into *E. coli* BL21(DE3), and expressed by isopropyl β-D-1-thiogalactopyranoside treatment (0.5 mM, 16 °C , 20 h). The recombinant proteins were purified using Ni-NTA affinity chromatography and resolved in SDS-PAGE (17%). The recombinant ADH was treated with the individual Hsps or in combination, and the enzyme activity was examined by measuring the NADH product levels at $O.D._{340}$.

Results: The recombinant ADH was expressed at high levels in *E. coli* and very thermotolerant when the purified enzyme reacted (up to 70 °C). All five Hsps enhanced the ADH activity under normal and heat conditions *in vitro*, compared to the control. DcHsp17.7 and DcHsp70 were the most effective for improving the enzyme activity by up to 13.0- and 11.6-fold, respectively, followed by IbpA (8.4-fold), DnaK (6.5-fold), and IbpB (3.4-fold), at 37 °C . Combined incubation of DcHsp17.7-DcHsp70 and DcHsp17.7-DnaK further enhanced the ADH activity by 13.8 and 14.2-fold, respectively. DcHsp70 effectively enhanced ADH's solubility at 37 °C *in vitro*.

Conclusion: Our results suggest that plant Hsps can enhance recombinant protein activity, such as ADH, more effectively than their bacterial counterparts. Identifying effective molecular chaperones in the bacterial and eukaryotic domains will help enhance the production of recombinant proteins in *E. coli*.

Keywords: *Daucus carota*, Enzyme activity, *Escherichia coli*, Molecular chaperone, Recombinant protein

1. Background

Heat shock proteins (Hsps) function as molecular chaperones to maintain protein homeostasis by stabilizing proteins, protecting them from denaturation, and folding nascent and partially unfolded proteins under normal and stress conditions (1). They are also

induced during thermal or chemical induction of recombinant proteins in *Escherichia coli* (*E. coli*) (2). The mRNA levels of the bacterial Hsps, such as IbpA/B [small(s)Hsp], GroEL (Hsp60), DnaK (Hsp70), and ClpB (Hsp100), and other proteins, such as Lon and OmpT proteases, increased during recombinant protein production. However, proper folding and post-translational modification of recombinant proteins can be challenging because most are heterologous, so the bacterial cells do not have the information to process them properly. The sudden recombinant protein accumulation can easily exceed the capacity of the bacterial molecular chaperone system, resulting in reduced yield and the formation of protein aggregates and inclusion bodies (3).

E. coli, like other organisms*,* produces different classes of Hsps with distinctive structural and functional characteristics (4). For example, the two sHsps in *E. coli*, IbpA and IbpB, bind to unfolded proteins to prevent irreversible aggregation (5). The DnaK and GroEL complexes are central ATP-dependent refolding machinery for unfolded proteins. ClpB functions in protein disaggregation and proteolysis, reducing aggregated protein levels (6). Furthermore, Hsps of different classes collaborate to process denatured and aggregated proteins. For example, sHsps bind to denatured proteins and present them to the DnaK complexes for proper refolding (7). ClpB disaggregates aggregated proteins and provides them to the DnaK complex for subsequent refolding (8).

Efforts have been made to enhance recombinant protein production in *E. coli* by overproducing Hsps. Bacterial Hsps, mainly IbpA/B, GroEL, and DnaK, have been overexpressed individually or in combination to increase the yield, folding, and stability of various recombinant proteins (9). However, in some cases, the adverse effects of Hsp co-expression were also reported (10). For example, due to its unique barrel-shaped structure, GroEL only accommodates proteins that can fit in the cavity, so its overexpression was not helpful, especially for some large proteins (11). The beneficial effect of DnaK overexpression was observed on a case-by-case basis. Although many studies reported beneficial effects of DnaK on recombinant protein production, sometimes its overexpression resulted in no enhancement in the yield and solubility (12). Furthermore, recombinant protein yield decreased in the presence of overexpressed DnaK, possibly due to its proteolytic function presenting severely unfolded substrates to proteolytic complexes, such as a Lon protease (13). To solve this problem, we recently proposed selecting useful heterologous Hsps that effectively enhance recombinant protein production without adverse side effects (9).

2. Objectives

In this study, the molecular chaperone functions of bacterial Hsps, IbpA, IbpB, and DnaK, and eukaryotic plant Hsps, DcHsp17.7 and DcHsp70 from carrot (*Daucus carota* L.), were compared *in vitro* using ADH as a model. Bacterial Hsps are frequently chosen to be the co-expression partners of recombinant proteins. However, the two carrot Hsps also improved the yield and solubility of the recombinant ADH (14) and human insulin (15) in *E. coli*. The results of this study provide important information for selecting effective Hsps that can promote recombinant protein production in *E. coli*.

3. Materials and Methods

3.1. Cloning of Recombinant ADH and Hsp Coding Genes

An *ADH* gene from a thermophile *Geobacillus stearothermophilus* (GenBank accession no. D90421. 1) was cloned into a pET11a expression vector as previously described (14). The cloned gene was designed to contain a 6-His tag at the N-terminal domain of the protein for purification. The recombinant ADH was expressed in the transgenic *E. coli,* constitutively expressing the carrot DcHsp70. Briefly, the transgenic *E. coli* contained a *DcHsp70* gene inserted in the bacterial genome (in the middle of *yddE* pseudogene, GenBank accession no. NC_012971.2) by Red/ET mediated homologous recombination. The gene expression was driven by the constitutive Lpp (lipoprotein) gene promoter (GenBank accession no. NC_000913.2). This transgenic *E. coli* cell line expressing DcHsp70 was developed to enhance the yield of recombinant proteins, such as ADH (14) and human insulin (15). The *DcHsp70* gene inserted in the *E. coli* genome does not contain affinity tags, so the heterologously expressed DcHsp70 is not purified by affinity chromatography.

Three Hsps from *E. coli*, *IbpA*, *IbpB*, and *DnaK* (Genbank accession nos. 948200, 948192, and 944750, respectively) and two carrot Hsps, *DcHsp17.7* and

DcHsp70 (Genbank accession nos. X53851 and X60088, respectively), genes were individually cloned in a pET11a expression vector, except for the *DcHsp70* gene in a pET26b vector as previously described (16). The DNA constructs were designed to contain a 6-His tag for purification at the N-terminal end of the Hsps. The recombinant vector maps are shown in **Supplementary Figure 1**. Each recombinant plasmid was independently introduced into *E. coli* BL21(DE3).

3.2. Recombinant Protein Induction and Purification

The induction and purification of recombinant ADH and Hsps were performed as previously described (14). Briefly, the recombinant proteins were induced by the 0.5 mM Isopropyl β-D-thiogalactoside (IPTG; Biobasic, Canada) treatment at 16 °C for 20 h and extracted by sonication. The 6-His-tagged recombinant proteins (ADH, IbpA, IbpB, DnaK, DcHsp17.7, and DcHsp70) were purified from individually cloned cells using Ni-NTA His-Bind resin (Merk, Germany). Eluted proteins were quantified using a Bradford reagent (Merk, USA).

3.3. SDS-PAGE Analysis

An equal volume (20 μL) of the purified proteins was resolved in SDS-PAGE analysis (17% resolving gel, 5% stacking gel) and stained using Coomassie Blue R-250 (Dyne Bio, Korea). The protein band intensity was quantified using the Chemi Doc MP Imaging system (Biorad, USA).

3.4. Recombinant ADH Activity In Vitro

The enzyme activity was measured using the modified protocol described previously (17). Purified ADH (120 nM) was treated with purified Hsps (DcHsp17.7, IbpA, IbpB, DcHsp70, and DnaK; up to 1200 nM) in PBS buffer (pH 7.4) either individually or in combination. Bovine serum albumin (BSA, 120 nM) was used as a control. After incubation for 30 min at the indicated temperatures (37 ~ 60 °C), ethanol and NAD^+ were added at the final concentration of 10% and 5 mM, respectively. The amount of the reaction product NADH was measured using spectrophotometry at 340 nm. The error bar represents the standard deviation of at least three repeats.

3.5. Protein Solubility Assay

The overall procedure was performed using the modified protocol described previously (18). Purified ADH and Hsps were added to PBS buffer (pH 7.4) at a final concentration of 120 nM and up to 1200 nM, respectively, followed by incubation at 50 °C for 90 min. Samples were centrifuged at 37,800 x *g* (25 °C, 1 h). The precipitated pellet was washed with 500 μL ice-cold PBS and dissolved in 20 μL PBS buffer. BSA (240 nM) was used as a control. As described above, soluble and insoluble protein fractions were resolved in SDS-PAGE (17% resolving gel, 5% stacking gel). The experiment was repeated at least three times, and the most representative images were shown. The error bar represents the standard deviation of at least three repeats.

3.6. Statistical Analyses

The expression levels of the recombinant ADH, the recombinant sHsps (IbpA, IbpB, and DcHsp17.7), and Hsp70s (DcHsp70 and DnaK) were statistically processed using a *t*-test (ns: non-significance, *$p < 0.05$, and ****$p < 0.0001$). The recombinant ADH activities at elevated temperatures and in the presence of various Hsps were statistically processed using ANOVA ($p < 0.05$). Error bars show the standard deviation of the means.

4. Results

4.1. Purification of the Recombinant ADH and Hsps

The recombinant ADH was obtained from the transgenic *E. coli* for the present study. The level of the purified ADH was 2.9-fold higher when produced in the transgenic *E. coli* than that produced in the control cells **(Fig. 1A)**. We confirmed that the purified ADH was active and thermotolerant (**Fig. 1B**). When the incubation temperature was raised, the enzyme was relatively stable up to 70 °C, followed by a dramatic decrease in activity at 80 ~ 90 °C. The recombinant sHsps and Hsp70s were also expressed and purified. The levels of IPTG-driven recombinant Hsps are various among the Hsps. The purified IbpA level was approximately 10% of those of IbpB and DcHsp17.7 (**Fig. 2A**). The purified DcHsp70 level was 60% of that of DnaK (**Fig. 2B**).

4.2. Enhanced Recombinant ADH Activity by Hsps In Vitro

The *in vitro* assay allowed us to compare the molecular chaperone activities of eukaryotic and prokaryotic Hsps in enhancing ADH activity. Purified ADH

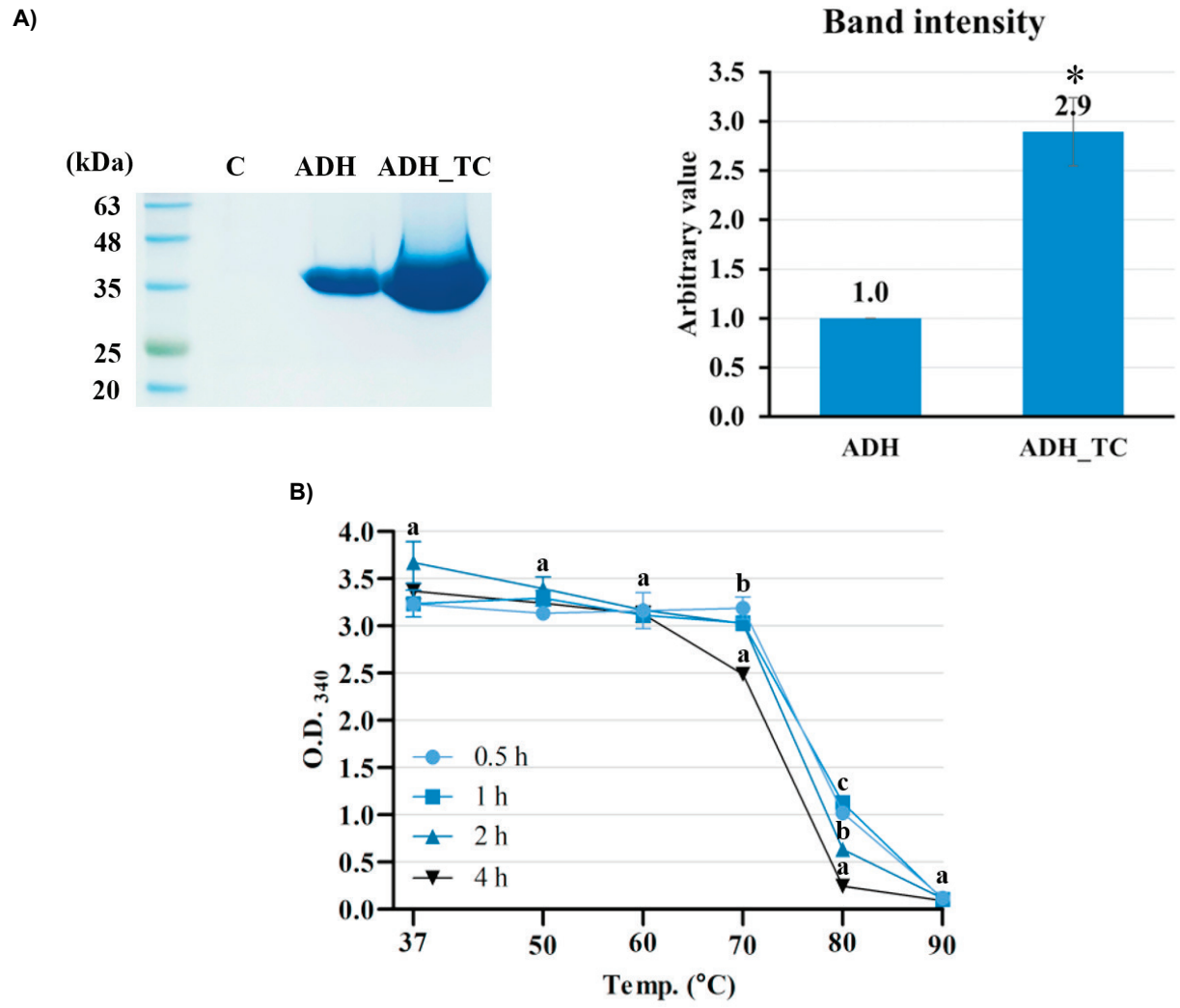

Figure 1. Thermotolerance of the recombinant ADH. A) Purified recombinant ADH. C: *E. coli* BL21; ADH: Recombinant ADH expressed in *E. coli* BL21; ADH_TC: Recombinant ADH expressed in transgenic *E. coli* BL21 constitutively expressing DcHsp70. **B)** Recombinant ADH activity at elevated temperatures. It was incubated at the indicated temperatures (37 ~ 60 °C) for up to 4 h, and the amount of the reaction product NADH was measured using spectrophotometry at 340 nm. The recombinant ADH levels were compared using a *t*-test (*$p < 0.05$). The recombinant ADH activities were compared using ANOVA ($p < 0.05$).

was incubated with the five Hsps individually or in combination at normal and elevated temperatures, and the enzyme activity was measured. The ADH level was kept minimal (120 nM) to examine the maximum enhancement of the enzyme activity achieved by Hsps. In all temperature ranges examined, all five Hsps enhanced the ADH activity, compared to that in the absence of Hsps. At 37 °C, the co-incubation with DcHsp17.7 was the most effective in enhancing the recombinant ADH activity **(Fig. 3A)**. Compared to ADH alone, DcHsp17.7 increased the enzyme activity by 13.0-fold, followed by DcHsp70 (11.6-fold), IbpA (8.4-fold), DnaK (6.5-fold), and IbpB (3.4-fold). Incubation with BSA did not alter the ADH activity. The enzyme activity decreased in heat conditions. At 50 °C, the incubation with DcHsp70 resulted in a 10.4-fold increase in the ADH activity, followed by DcHsp17.7 (9.4-fold), IbpA (6.7-fold), DnaK (5.6-fold), and IbpB treatments (3.0-fold; **Fig. 3B**). As the temperature further increased, the extent to which Hsp

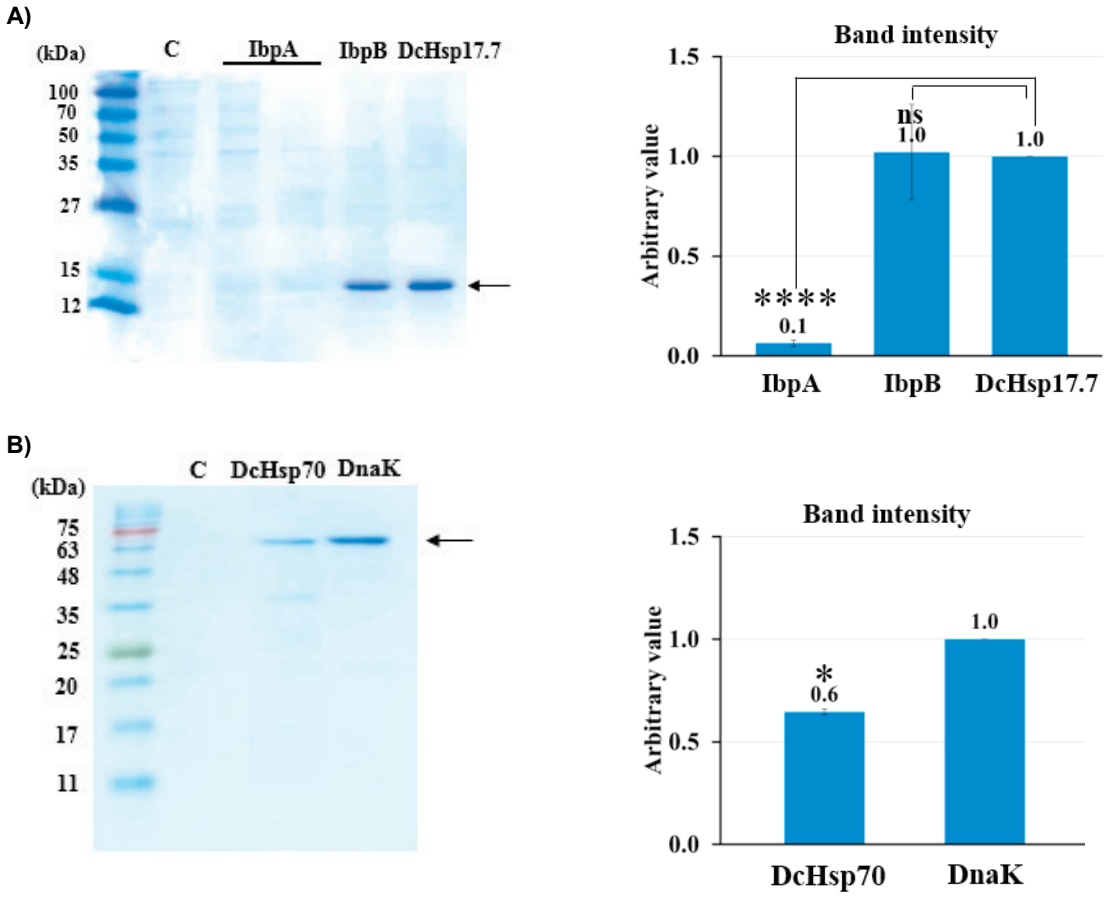

Figure 2. Purified recombinant Hsps. A) Purified sHsps, DcHsp17.7, IbpA, and IbpB. **B)** Purified Hsp70s, DcHsp70 and DnaK. The statistical comparison was performed using a *t*-test (ns: non-significance, *p < 0.05, and ****p < 0.0001).

improved the ADH activity decreased. At 55 and 60 °C, the incubation with the individual Hsps increased the enzyme activity by less than 5-fold (**Supplementary Fig. 2A, 2B**).

4.3. Combined Treatment of a SHsp and an Hsp70 Further Enhanced the Recombinant ADH Activity.

We examined if the combined treatment of a sHsp and an Hsp70 can further enhance the ADH activity *in vitro*, compared to the individual Hsp treatment. Our results showed the synergistic effects of the combined treatment of two different classes of Hsps on the enzyme activity. The co-treatment with DcHsp17.7 and either DcHsp70 or DnaK were the most effective in enhancing the ADH activity **(Fig. 4A)**, compared to the other Hsp combinations, in a concentration-dependent manner. The DcHsp17.7-DcHsp70 and DcHsp17.7-DnaK co-treatments increased the enzyme activity by approximately 14-fold, followed by the IbpA-DcHsp70 and IbpA-DnaK combinations (10.3- and 11.6-fold, respectively), compared to ADH alone. The combined treatments of IbpB-DcHsp70 and IbpB-DnaK were the least effective in enhancing ADH activity (less than a 3-fold increase). It is worth noting that the two IbpB combinations resulted in the maximum $O.D._{340}$ of 0.7, which was much lower than the individual treatments of DcHsp70 (max. $O.D._{340}$ of 2.5) and DnaK (max. $O.D._{340}$ of 1.5), and similar to the IbpB single treatment ($O.D._{340}$ of 0.7; **Fig. 3A**). It appears that IbpB inhibited the molecular chaperone function of the two Hsp70s on ADH *in vitro*.

At 50 °C, a more rapid increase in the ADH activity was observed in the presence of DcHsp17.7-DcHsp70 and DcHsp17.7-DnaK, compared to those at 37 °C

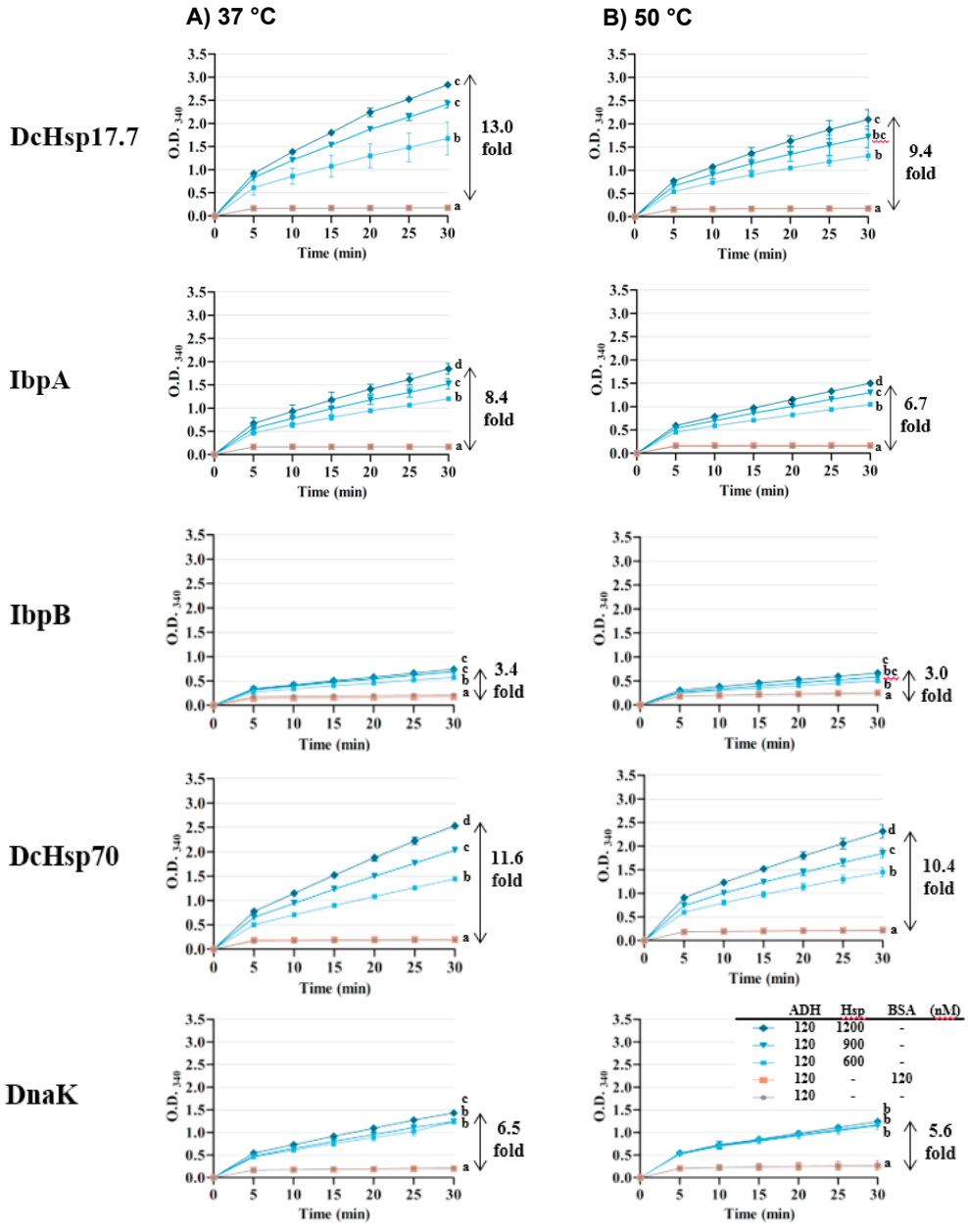

Figure 3. Enhanced recombinant ADH activity by Hsp *in vitro*. Purified recombinant ADH (120 nM) was incubated with Hsps, DcHsp17.7, IbpA, IbpB, DcHsp70, and DnaK (up to 1200 nM) at **A)** 37 °C and **B)** 50 °C. The enzyme activity was analyzed by measuring the amount of product NADH at $O.D._{340}$. The statistical comparison was performed using ANOVA ($p < 0.05$).

(**Fig. 4B**). At this elevated temperature, the maximum enzyme activity remained similar to that at 37 °C in all Hsp combinations examined. A further increase in incubation temperature to 55 °C resulted in dramatic decreases in the enzyme activity (**Supplementary Fig. 3**). The maximum $O.D._{340}$ was below 0.5 in all the conditions examined. Our results show that DcHsp17.7 is more effective in exerting a high level of synergy with both eukaryotic and prokaryotic Hsp70s in promoting ADH activity, compared to the bacterial IbpA and IbpB.

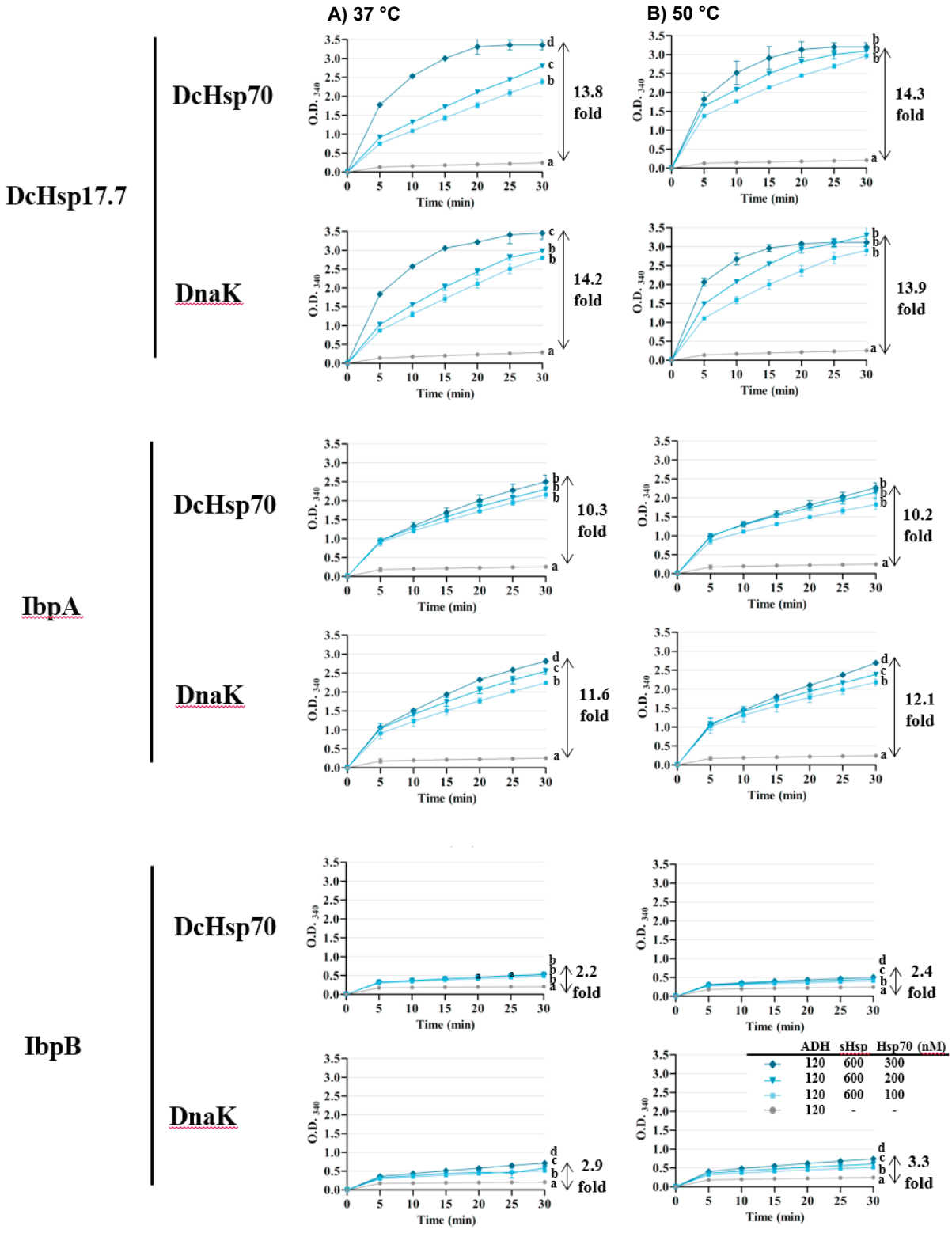

Figure 4. Enhanced recombinant ADH activity by combined treatment of two different classes of Hsps *in vitro*. Purified recombinant ADH (120 nM) was co-incubated with a sHsp (600 nM) and a Hsp70 (up to 300 nM) in the combinations of DcHsp17.7-DcHsp70, DcHsp17.7-DnaK, IbpA-DcHsp70, IbpA-DnaK, IbpB-DcHsp70, and IbpB-DnaK at **A)** 37 °C and **B)** 50 °C. The enzyme activity was analyzed by measuring the amount of product NADH at $O.D._{340}$. The statistical comparison was performed using ANOVA ($p < 0.05$).

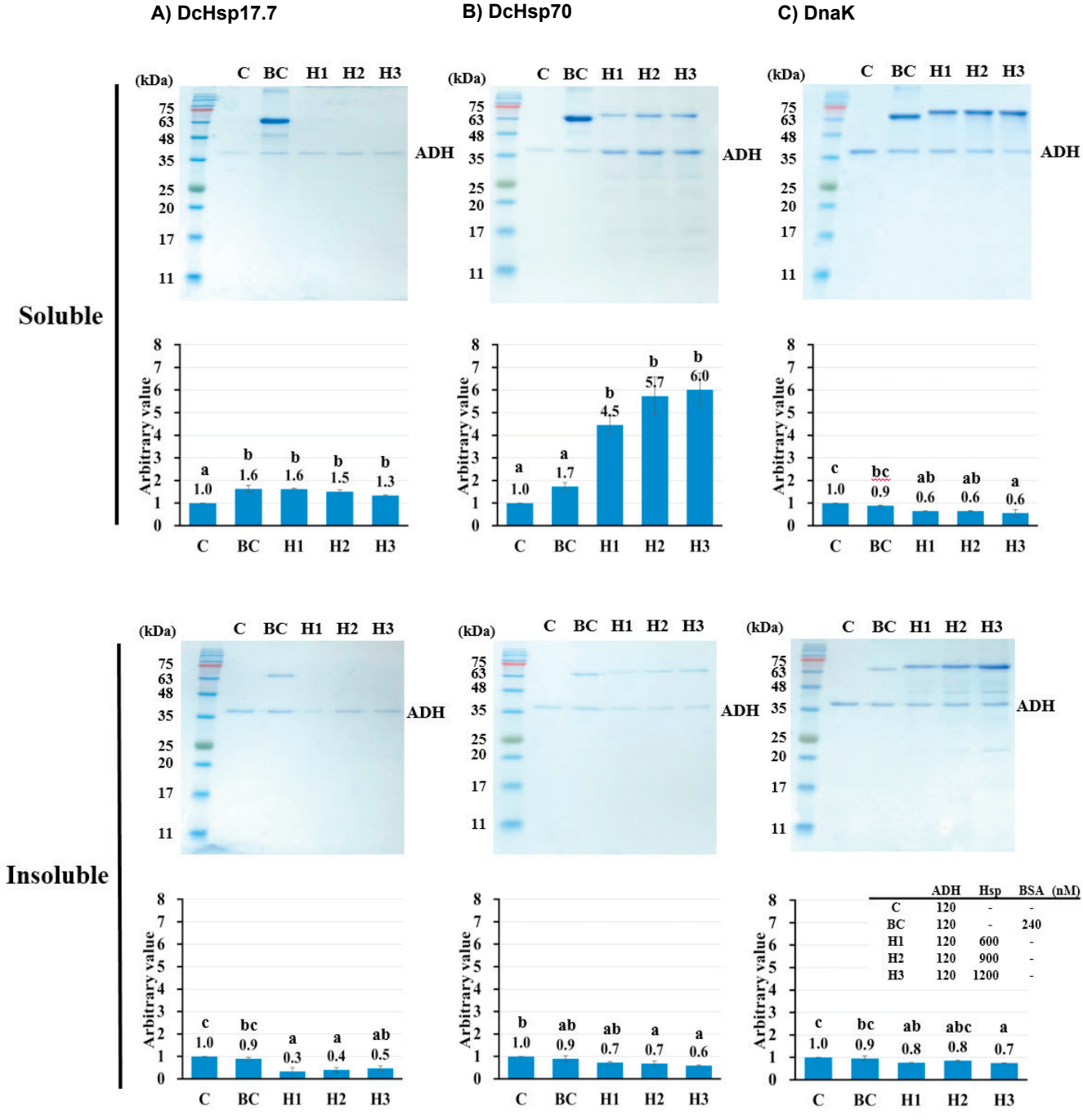

Figure 5. Recombinant ADH solubility in the presence of Hsps *in vitro*. Purified ADH (240 nM) was incubated with Hsps (600 ~ 1200 nM) [**A)** DcHsp17.7, **B)** DcHsp70, and **C)** DnaK] at 50 °C for 90 min, followed by centrifugation to obtain soluble and insoluble fractions. Proteins were resolved in SDS-PAGE (17%). C: ADH; BC: ADH treated with bovine serum albumin; H1~H3: ADH treated with increasing amounts of Hsps. The statistical comparison was performed using ANOVA ($p < 0.05$).

4.4. DcHsp70 Enhanced the Solubility of ADH under Heat Stress In Vitro

DcHsp17.7, DcHsp70, and DnaK, the ones that showed high enhancing effect on the ADH activity, were examined if they could enhance the solubility of ADH when exposed to a denaturing condition (50 °C for 90 min) *in vitro* **(Fig. 5)**. The purified ADH, DcHsp70, and DnaK were visible at the expected molecular weights in SDS-PAGE. Although the same molar amount was used in the experiment, DcHsp17.7 was not observed, possibly due to its relatively small size. The addition of DcHsp17.7 resulted in a moderate increase (up to 1.6-fold) in the soluble ADH levels and a decrease in the insoluble levels (**Fig. 5A**). DcHsp70 was the most effective in enhancing the enzyme solubility (up to 6.0-fold) and decreasing the insoluble fractions, compared to control (**Fig. 5B**). DnaK failed to increase the soluble levels of ADH *in vitro* (**Fig. 5C**).

5. Discussion

Recombinant proteins are used as biopharmaceuticals, antibodies, vaccines, and industrial enzymes (19). Alcohol dehydrogenase (ADH), found in all three domains of life, is a primary enzyme for processing alcohol (20). It is a redox catalyst in the oxidation of alcohols and reducing carbonyl compounds (21). Various ADHs have been recombinantly produced in *E. coli* and *S. cerevisiae* in the food, pharmaceutical, and fine chemical industries (22). For example, chiral synthons produced using ADHs synthesize chirally pure pharmaceutical agents (21). In ethanol fuel cells, ADH breaks down the fuel (23). Biocatalysts, such as enzymes, are environmentally friendly compared to conventional inorganic ones. In addition to its importance in the industry, ADH is a widely used model substrate for molecular studies (24). The optimum temperature range for most ADHs reported previously is 35 ~ 45 °C (25). However, the recombinant ADH examined in this study showed outstanding thermotolerance up to 70 °C, possibly because the gene was cloned from a thermophilic bacterium, *Geobacillus stearothermophilus*. Resistance to harsh conditions, such as high temperatures, is an important characteristic of a biocatalyst in organic synthesis (22). The thermotolerant ADH can be valuable in many industrial applications.

Previous *in vitro* studies have mainly focused on the molecular chaperone function of the sHsps on recombinant proteins. For example, the purified IbpA and IbpB maintained the citrate synthase activity under thermal stress (26). Furthermore, Hsps from eukaryotic organisms could protect the recombinant proteins from denaturing conditions *in vitro*. Hsp16 from *Caenorhabditis elegans* prevented citrate synthase from heat-induced aggregation (27). Xenopus Hsp30 reduced aggregation and inactivation of citrate synthase under thermal stress (28). Murine Hsp25 and yeast Hsp26 prevented thermal aggregation of various model substrates (29). The former was more effective for a-glucosidase, rhodanese, and insulin, but the latter was for citrate synthase, suggesting that their molecular chaperone activities depend on the type of substrate. Many plant Hsps have shown the molecular chaperone activities on model substrates *in vitro*. Pea (*Pisum sativum*) Hsp18.1 and Hsp17.7 prevented substrate aggregation from thermal stress (30). *Arabidopsis thaliana* Hsp17.6 promoted the reactivation of a chemically denatured citrate synthase and lactate dehydrogenase (31). Wheat (*Triticum aestivum*) cytosolic Hsp16.9 and Hsp17.8 prevented thermal aggregation of malate dehydrogenase (32). Barley (*Hordeum vulgare*) Hsp17.5 protected the SwaI restriction enzyme from thermal inactivation (33). These and our results show that eukaryotic, especially plant Hsps, can be useful in promoting recombinant protein activity and protecting them from aggregation. The heterologous expression of plant Hsps in *E. coli,* which expresses recombinant proteins, can be a useful strategy to enhance production efficiency.

In this study, DcHsp17.7 was more effective in enhancing the ADH activity than its bacterial counterparts, IbpA and IbpB. This difference may primarily be due to their sequence differences. DcHsp17.7 shows approximately 50% similarities to IbpA and IbpB in amino acid sequences (16). Mammalian Hsp20 showed much lower *in vitro* chaperone activity than the related αB-crystallin (34). The authors speculated that the shorter C-terminal extension in Hsp20 may be responsible for the low efficiency. These results suggest that detailed functional mechanisms of sHsps may vary. Notably, the sole treatment of IbpB resulted in the most minor effect on enhancing ADH activity. Furthermore, unlike DcHsp17.7 and IbpA, which showed synergistic effects with Hsp70s, IbpB failed to do so. Studies have shown that IbpA and IbpB are functionally different

and collaborate to process denatured substrates. For example, IbpA is first bound to heat-denatured luciferase (35). IbpB was then bound to the IbpA containing aggregates and facilitated subsequent disaggregation by the DnaK-ClpB system. IbpA reduced the size of the heat-denatured aggregates, while the presence of IbpB did not alter the aggregate size. A recent study reported that IbpA, but not IbpB, interacted with aggregating substrates (36). IbpB did not bind to luciferase but increased substrate refolding. It facilitated the dissociation of IbpA from IbpA-substrate aggregates and subsequent refolding by the DnaK complex. These and our results suggest that IbpB does not directly interact with Hsp70.

In the case of Hsp70s, DcHsp70 was more effective than DnaK in enhancing ADH activity in the absence of ATP *in vitro*. Previously, the incubation with either DnaK or PfHsp70 (*Plasmodium falciparum*, a protozoan parasite causing malaria) enhanced malate dehydrogenase (MDH) activity in the absence of ATP *in vitro* (37). The authors speculated that the enhanced recombinant protein activity was likely due to preventing aggregation of MDH and not refolding the unfolded substrates by the Hsp70s. Some Hsp70s may be able to function in the absence of ATP, and their functions may differ depending on the availability of ATP. Furthermore, Hsp70 was treated with other classes of Hsps to protect recombinant proteins to a higher level. The combined treatment of bacterial Hsps, ClpB, DnaK, DnaJ, and GrpE with unfolded luciferase allowed 57% reactivation of the enzyme (38). It suppressed the enzyme aggregation, while the individual Hsp treatment resulted in less than 1% reactivation. The collaboration and synergistic effects of Hsps from different classes have been elucidated in cellular environments (2). *In vitro* assays can be useful to identify an Hsp combination optimized for expressing target recombinant proteins in *E. coli*.

This study shows that plant Hsps are effective molecular chaperones that can promote the activity and solubility of recombinant proteins. Plants express divergent and numerous Hsps, which can confer advanced protection from various stressors to the sessile organisms. For example, while *E. coli* has only two sHsps, IbpA and IbpB, plants produce more than 40 different sHsps targeted to various subcellular compartments, such as cytosol, endoplasmic reticulum, chloroplast, and mitochondria (39). In the recombinant protein production in *E. coli*, the co-expression of plant Hsps may be able to compensate and collaborate with the bacterial Hsp systems, enhancing recombinant protein production and bacterial cell growth during large-scale industrial fermentation.

6. Conclusion

The efficient recombinant protein production can greatly benefit a wide range of biological research and large-scale industrial production of valuable biopharmaceuticals and biocatalysts. Additional expression of molecular chaperons, such as Hsps, in the cell factories can significantly enhance recombinant protein production. Our results suggest that DcHsp17.7 and DcHsp70 are more effective in enhancing the ADH activity *in vitro* than the bacterial ones. Furthermore, the plant and bacterial Hsps could perform synergistic effects on further enhancing the enzyme activity. The DcHsp17.7-DcHsp70 and DcHsp17.7-DnaK combinations selected from this study will be co-expressed with ADH or other important recombinant proteins in *E. coli* to enhance protein production.

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References

1. Hu C, Yang J, Qi Z, Wu H, Wang B, Zou F, *et al*. Heat shock proteins: Biological functions, pathological roles, and therapeutic opportunities. *Med Comm*. 2022;**3**(3):e161. doi: 10.1002/mco2.161
2. Valdez-Cruz NA, Ramirez OT, Trujillo-Roldan MA. Molecular responses of *Escherichia coli* caused by heat stress and recombinant protein production during temperature induction. *Bioeng Bugs*. 2011;**2**(2):105-110. doi: 10.4161/bbug.2.2.14316
3. Rosano GL, Ceccarelli EA. Recombinant protein expression in *Escherichia coli*: advances and challenges. *Front Microbiol*. 2014;**5**:172. doi: 10.3389/fmicb.2014.00172
4. Richter K, Haslbeck M, Buchner J. The heat shock response: life on the verge of death. *Mol Cell*. 2010;**40**(2):253-66. doi: 10.1016/j.molcel.2010.10.006
5. Arsene F, Tomoyasu T, Bukau B. The heat shock response of *Escherichia coli*. *Int J Food Microbiol*. 2000;**55**(1-3):3-9. doi: 10.1016/s0168-1605(00)00206-3
6. Barnett ME, Zolkiewska A, Zolkiewski M. Structure and activity of ClpB from *Escherichia coli*. Role of the amino-and -carboxyl-terminal domains. *J Biol Chem*. 2000;**275**(48):37565-37571. doi: 10.1074/jbc.M005211200
7. Zwirowski S, Klosowska A, Obuchowski I, Nillegoda NB,

Pirog A, Zietkiewicz S, *et al*. Hsp70 displaces small heat shock proteins from aggregates to initiate protein refolding. *EMBO J.* 2017;**36**(6):783-796. doi: 10.15252/embj.201593378
8. Hayashi S, Nakazaki Y, Kagii K, Imamura H, Watanabe YH. Fusion protein analysis reveals the precise regulation between Hsp70 and Hsp100 during protein disaggregation. *Sci Rep*. 2017;**7**(1):8648. doi: 10.1038/s41598-017-08917-8
9. Ahn YJ, Jung M. Improved recombinant protein production using heat shock proteins in *Escherichia coli*. *Biocatal Agric Biotechnol*. 2023;**50**(2023). doi: 10.1016/j.bcab.2023.102736
10. Martinez-Alonso M, Garcia-Fruitos E, Ferrer-Miralles N, Rinas U, Villaverde A. Side effects of chaperone gene co-expression in recombinant protein production. *Microb Cell Fact*. 2010;**9**:64. doi: 10.1186/1475-2859-9-64
11. Han KG, Lee SS, Kang C. Soluble expression of cloned phage K11 RNA polymerase gene in *Escherichia coli* at a low temperature. *Protein Expr Purif*. 1999;**16**(1):103-108. doi: 10.1006/prep.1999.1061
12. de Marco A, Deuerling E, Mogk A, Tomoyasu T, Bukau B. Chaperone-based procedure to increase yields of soluble recombinant proteins produced in *E. coli*. *BMC Biotechnol*. 2007;**7**:32. doi: 10.1186/1472-6750-7-32
13. Jubete Y, Maurizi MR, Gottesman S. Role of the heat shock protein DnaJ in the lon-dependent degradation of naturally unstable proteins. *J Biol Chem*. 1996;**271**(48):30798-30803. doi: 10.1074/jbc.271.48.30798
14. Kim M, Jang B, Ahn YJ. Enhanced Production of Recombinant Alcohol Dehydrogenase Using the Genetically Engineered *Escherichia coli* Strain that Heterologously Expresses Carrot Heat Shock Protein 70. *Curr Microbiol*. 2019;**76**(11):1338-1344. doi: 10.1007/s00284-019-01730-8
15. Jang B, Ahn YJ. Enhanced recombinant insulin production in transgenic *Escherichia coli* that heterologously expresses carrot heat shock protein 70. *Biocatal Agric Biotechnol*. 2019;**20**(2019). doi: 10.1016/j.bcab.2019.101180
16. Jung M, Ahn YJ. Growth-enhancing effect of bacterial and plant heat shock proteins in *Escherichia coli*. *Biocatal Agric Biotechnol*. 2022;**46**(2022). doi: 10.1016/j.bcab.2022.102545
17. Walker JRL. Spectrophotometric determination of enzyme activity: Alcohol dehydrogenase (ADH). Biochemical Education. 20: Wiley; 1992. **p**. 42-43
18. Mymrikov EV, Daake M, Richter B, Haslbeck M, Buchner J. The Chaperone Activity and Substrate Spectrum of Human Small Heat Shock Proteins. *J Biol Chem*. 2017;**292**(2):672-684. doi: 10.1074/jbc.M116.760413
19. Baeshen MN, Al-Hejin AM, Bora RS, Ahmed MM, Ramadan HA, Saini KS, *et al*. Production of Biopharmaceuticals in *E. coli*: Current Scenario and Future Perspectives. *J Microbiol Biotechnol*. 2015;**25**(7):953-962. doi: 10.4014/jmb.1412.12079
20. Edenberg HJ. The genetics of alcohol metabolism: role of alcohol dehydrogenase and aldehyde dehydrogenase variants. *Alcohol Res Health*. 2007;**30**(1):5-13
21. Machielsen R, Uria AR, Kengen SW, van der Oost J. Production and characterization of a thermostable alcohol dehydrogenase that belongs to the aldo-keto reductase superfamily. *Appl Environ Microbiol*. 2006;**72**(1):233-238. doi: 10.1128/AEM.72.1.233-238.2006
22. de Miranda AS, Milagre CDF, Hollmann F. Alcohol Dehydrogenases as Catalysts in Organic Synthesis. *Front. Catal*. 2022;**2**. doi: 10.3389/fctls.2022.900554
23. Ali MA, Pacheco JC, Iost RM, Rahman A, Hassan A, Cardoso DR, *et al*. Metal-free, low-cost, and high-performance membraneless ethanol fuel cell. *J. Power Sources*. 2022;**551**(15). doi: 10.1016/j.jpowsour.2022.232164
24. Mendoza JA, Weinberger KK, Swan MJ. The Hsp60 protein of helicobacter pylori displays chaperone activity under acidic conditions. *Biochem Biophys Rep*. 2017;**9**:95-99. doi: 10.1016/j.bbrep.2016.11.011
25. Zhao Q, Hou Y, Gong GH, Yu MA, Jiang L, Liao F. Characterization of alcohol dehydrogenase from permeabilized brewer's yeast cells immobilized on the derived attapulgite nanofibers. *Appl Biochem Biotechnol*. 2010;**160**(8):2287-2299. doi: 10.1007/s12010-009-8692-y
26. Kitagawa M, Miyakawa M, Matsumura Y, Tsuchido T. *Escherichia coli* small heat shock proteins, IbpA and IbpB, protect enzymes from inactivation by heat and oxidants. *Eur J Biochem*. 2002;**269**(12):2907-2917. doi: 10.1046/j.1432-1033.2002.02958.x
27. Leroux MR, Melki R, Gordon B, Batelier G, Candido EP. Structure-function studies on small heat shock protein oligomeric assembly and interaction with unfolded polypeptides. *J Biol Chem*. 1997;**272**(39):24646-24656. doi: 10.1074/jbc.272.39.24646
28. Fernando P, Heikkila JJ. Functional characterization of Xenopus small heat shock protein, Hsp30C: the carboxyl end is required for stability and chaperone activity. *Cell Stress Chaperon*. 2000;**5**(2):148-159. doi:10.1379/1466-1268(2000)005<0148:fcoxsh>2.0.co;2
29. Stromer T, Ehrnsperger M, Gaestel M, Buchner J. Analysis of the interaction of small heat shock proteins with unfolding proteins. *J Biol Chem*. 2003;**278**(20):18015-18021. doi: 10.1074/jbc.M301640200
30. Lee GJ, Pokala N, Vierling E. Structure and *in vitro* molecular chaperone activity of cytosolic small heat shock proteins from pea. *J Biol Chem*. 1995;**270**(18):10432-10438. doi: 10.1074/jbc.270.18.10432
31. Sun W, Bernard C, van de Cotte B, Van Montagu M, Verbruggen N. At-HSP17.6A, encoding a small heat-shock protein in Arabidopsis, can enhance osmotolerance upon overexpression. *Plant J*. 2001;**27**(5):407-415. doi: 10.1046/j.1365-313x.2001.01107.x
32. Basha E, Lee GJ, Demeler B, Vierling E. Chaperone activity of cytosolic small heat shock proteins from wheat. *Eur J Biochem*. 2004;**271**(8):1426-1436. doi: 10.1111/j.1432-1033.2004.04033.x
33. Reddy PS, Kavi Kishor PB, Seiler C, Kuhlmann M, Eschen-Lippold L, Lee J, *et al*. Unraveling regulation of the small heat shock proteins by the heat shock factor HvHsfB2c in barley: its implications in drought stress response and seed development. *PLoS One*. 2014;**9**(3):e89125. doi: 10.1371/journal.pone.0089125
34. van de Klundert FA, Smulders RH, Gijsen ML, Lindner RA, Jaenicke R, Carver JA, *et al*. The mammalian small heat-shock protein Hsp20 forms dimers and is a poor chaperone. *Eur J Biochem*. 1998;**258**(3):1014-1021. doi: 10.1046/j.1432-1327.1998.2581014.x
35. Ratajczak E, Zietkiewicz S, Liberek K. Distinct activities of *Escherichia coli* small heat shock proteins IbpA and IbpB promote efficient protein disaggregation. *J Mol Biol*. 2009;**386**(1):178-189. doi: 10.1016/j.jmb.2008.12.009
36. Obuchowski I, Pirog A, Stolarska M, Tomiczek B, Liberek K. Duplicate divergence of two bacterial small heat shock proteins

reduces the demand for Hsp70 in refolding of substrates. *PLoS Genet*. 2019;**15**(10):e1008479. doi: 10.1371/journal.pgen.1008479

37. Makhoba XH, Burger A, Coertzen D, Zininga T, Birkholtz LM, Shonhai A. Use of a Chimeric Hsp70 to Enhance the Quality of Recombinant *Plasmodium falciparum* S-Adenosylmethionine Decarboxylase Protein Produced in *Escherichia coli*. *PLoS One*. 2016;**11**(3):e0152626. doi: 10.1371/journal.pone.0152626
38. Zolkiewski M. ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation. A novel multi-chaperone system from *Escherichia coli*. *J Biol Chem*. 1999; **274**(40):28083-28086. doi: 10.1074/jbc.274.40.28083
39. Waters ER, Vierling E. Plant small heat shock proteins-evolutionary and functional diversity. *New Phytol*. 2020;**227** (1):24-37. doi: 10.1111/nph.16536