Functional and structural characterization of HspB1/Hsp27 from Chinese hamster ovary cells

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Small heat shock proteins (sHsps) endow cells with stress tolerance. sHsps bind to partially folded or denatured proteins, thereby preventing irreversible aggregation or promoting correct substrate folding [1,2]. Overall amino acid sequence homology between sHsps is considerably lower compared to other chaperones. Their common feature is the α-crystallin domain which is named after the α-crystallin in vertebrate lenses [3]. The N-terminal region is highly variable, and the C-terminal extension is partially conserved with the consensus IXI motif [4]. Most sHsps take large oligomeric structures composed of 12–36 subunits [5–7]. The sHsp from Methanocaldococcus jannaschii (MjHsp16.5) forms a spherical 24-mer oligomer with a diameter of 12 nm (PDB-ID: 1SHS) [7]. The sHsp of Sulfolobus tokodaii strain 7 (StHsp14.0) forms a similar oligomer composed of 24 subunits (PDB-ID: 3VQK) [8]. On the contrary, the sHsp from wheat (wHsp16.9) forms a double-ring-shaped oligomer consisting of 12 subunits (PDB-ID: 1GME) [6]. We have determined the crystal structure of a sHsp from the fission yeast, Schizosaccharomyces pombe, SpHsp16.0. SpHsp16.0 forms a hexadecameric oligomer structure in which

Abbreviations
sHsps, small heat shock proteins; CHO, Chinese hamster ovary; CgHspB1, HspB1 from CHO cells; CgHspB1WT, wild-type CgHspB1; CgHspB1S15D, CgHspB1 with S15D mutation; IPMDH, isopropyl malate dehydrogenase from Thermus thermophilus HB8; CS, porcine heart citrate synthase; SEC-MALS, size exclusion chromatography–multiangle light scattering; SAXS, small-angle X-ray scattering.
eight dimers of SpHsp16.0 form an elongated sphere with 422 symmetry (PDB-ID: 3W1Z) [9].

There exist 10 genes encoding sHsps in mammalian genomes [10]. They differ slightly in monomeric molecular weight, stress inducibility, oligomeric structure, chaperone activity, and tissue distribution [11–13]. HspB1/Hsp27 is almost ubiquitously expressed in all human tissues [11,13] and is involved in the regulation of many vital functions. HspB1 seems to be responsible for regulation and stabilization of the cytoskeleton [14,15], possesses anti-apoptotic activity [16,17], and protects the cell against oxidative stress [18,19]. Mammalian sHsps, which reflect the homeothermic status of mammals, are regulated by phosphorylation. Extracellular stresses induce phosphorylation two or three serine residues.

The molecular architecture of HspB1/Hsp27 is controversial. Analytical ultracentrifugation analysis showed that the mean molecular mass is 730 kDa [20]. On the contrary, HspB1/Hsp27 in the nonphosphorylated state was reported to form 24-mers by gel-filtration chromatography studies [21]. Lelj-Garolla et al. [22] showed that HspB1/Hsp27 exists in the equilibrium state of monomers/dimers, tetramers, 12-mers, and 16-mers based on sedimentation velocity analysis. The same group has shown that oligomerization of HspB1/Hsp27 increases with the temperature elevation from 10 to 40 °C. The largest oligomers at 10 °C were 8–12-mers, whereas oligomers as large as 22–30-mers were observed at 40 °C [23]. This observation contradicts the general knowledge that the large oligomeric structures of sHsps disassemble to smaller oligomers at the high temperature [24,25]. The analysis by size exclusion chromatography showed that the wild-type HspB1/Hsp27 eluted as a broad peak with an average molecular mass of approximately 590 kDa [26]. The molecular mass decreased by introducing phosphorylation mimic mutations. Chaperone activity is also increased by mutations. Therefore, it is reasonable to think that the dissociation of oligomers is correlated with molecular chaperone activity.

The crystal structure of the human HspB1 α-crystallin domain has been reported [27]. Unexpectedly, the HspB1 fragment does not form the typical β7/β7 dimers but rather hexamers by an asymmetric contact between the β4 and the β7 strands from the adjacent α-crystallin domain.

In this study, we expressed and characterized HspB1/Hsp27 from Chinese hamster ovary (CHO) cells. According to the scientific name of the Chinese hamster, Cricetulus griseus, it is referred to as HspB1 from CHO cells (CgHspB1) hereafter. CHO cells are mostly used for industrial production of therapeutic proteins. Proteostasis in CHO cells should be important for the production of therapeutic proteins. However, there have been only a few reports on chaperones, including HspB1/Hsp27 in CHO cells. One of the advantages of CHO cells compared with other mammalian cells is its robustness. HspB1/Hsp27 is also known to play a role in the inhibition of apoptosis and actin cytoskeletal remodeling. Thus, it may take an essential role in the robustness of CHO cells.

We have firstly performed structural and functional characterization of HspB1/Hsp27 from CHO cell. The results will give the insights not only to the functional mechanism of HspB1/Hsp27 but also to the proteostasis and robustness of CHO cell.

### Materials and methods

#### Cloning, expression, and purification

The full-length gene for CgHspB1 was amplified from total cDNA of CHO cells using the primers 5’-GGA TAT CCA TAT GAC CGA GCG CCG CG-3’ and 5’-GAA TTC CTA CTT GCC TGC TAA GTC TTC CGA CTT C-3’. The amplified DNA fragment was digested with Nde I and EcoR I and inserted into the Nde I/EcoR I site of pET23b. Then, the constructed plasmid, pET23b-wild-type CgHspB1 (CgHspB1WT), was used for the production of CgHspB1WT in *Escherichia coli* BL21 Star (DE3). The plasmid for the production of the phosphorylated mimic CgHspB1, CgHspB1 with S15D mutation (CgHspB1S15D), was made through site-directed mutagenesis with the primers 5’-GCT GCT GCG GAG CCC CGA CTG GGA and 5’-CCC GGA ATG GTT CCC AGT CGG GGC TCC GCA GCA GC-3’. The amplified DNA fragment was digested with Nde I and inserted into the Nde I/EcoR I site of pET23b.

*C. griseus* were cultured in TS medium [29], and *Escherichia coli* BL21 (DE3) cells transformed with pET23b-CgHspB1WT were grown at 37 °C in Luria–Bertani medium containing 100 µg·mL⁻¹ ampicillin for 24 h. The cells were harvested by centrifugation at 5000 g for 10 min at 4 °C.

The harvested cells were suspended in buffer A (50 mM Tris/HCl, pH 8.0) and disrupted by sonication, and the suspension of disrupted cells was centrifuged at 24 000 g for 30 min at 4 °C. The supernatant was applied to a TOYOPEARL DEAE-650 anion exchange column (Tosoh, Tokyo, Japan) equilibrated with buffer A. Proteins were eluted with a linear gradient of 0–400 mM NaCl in buffer A. Fractions containing CgHspB1 were pooled and dialyzed with buffer A overnight. The dialyzed protein solution was applied to a RESOURCE Q column (GE Healthcare Bio-Sciences, Buckinghamshire, UK) equilibrated with buffer A. Proteins were eluted with a linear gradient of 0–500 mM NaCl in buffer A. Fractions containing CgHspB1 were pooled, concentrated by ultrafiltration.
(Amicon Ultra, Merck Millipore, Billerica, CA, USA), and then applied to a HiLoad 26/60 Superdex 200 pg size exclusion column (GE Healthcare Bio-Sciences) equilibrated with buffer B (50 mM Tris/HCl pH 7.5, 0.1 mM EDTA, 150 mM NaCl).

Isopropyl malate dehydrogenase from *Thermus thermophilus* HB8 (IPMDH) was expressed in *E. coli* and purified as described previously [29].

**Protein aggregation measurements**

The thermal aggregation of porcine heart citrate synthase (CS) was monitored by measuring light scattering at 500 nm with a spectrophotometer (FP-6500; JASCO, Tokyo, Japan) at 45 °C as described previously [30]. Native CS (50 nm, monomer) was incubated in TK buffer (50 mM Tris/HCl, pH 7.5, 100 mM KCl, and 25 mM MgCl2) with or without CgHspB1WT or CgHspB1S15D. The assay buffer was preincubated at 45 °C and continuously stirred throughout the measurement.

**Size exclusion chromatography**

Size exclusion chromatography was performed with a gel-filtration column (SB-804HQ; Showa Denko, Tokyo, Japan) using an HPLC system, PU-1580i, connected to a MD1515 multiwavelength detector (JASCO) as described previously [31]. CgHspB1WT or CgHspB1S15D was diluted to the specified concentrations (as monomer) in buffer B. A 100-µL aliquot of diluted CgHspB1WT or CgHspB1S15D was heated at the specified temperature for 30 min and then loaded onto a column heated at the same temperature and eluted with buffer B with or without 20% ethylene glycol at a flow rate of 1.0 mL·min⁻¹. The proteins are monitored by the absorbance at 215 nm. To examine the reversibility of the dissociation, CgHspB1WT or CgHspB1S15D preheated at 45 °C for 30 min was analyzed by gel filtration at room temperature after cooling at 25 °C for 30 min.

**Size exclusion chromatography–multiangle light scattering**

The purified CgHspB1 was analyzed by size exclusion chromatography–multiangle light scattering (SEC-MALS) on a TSKgel G3000XL column (Tosoh) connected to a multiangle light-scattering detector (MINI DAWN; Wyatt Technology, Santa Barbara, CA, USA) and a differential refractive index detector (Shodex RI-101; Showa Denko) with an HPLC system, PU-980i (JASCO), as described previously [31]. A 100-µL aliquot of sample was injected into the column and eluted with buffer B at 1.0 mL·min⁻¹. The molecular weight and protein concentration were determined according to the instructional manual (Wyatt Technology).

**SAXS measurements**

Small-angle X-ray scattering (SAXS) was performed on a laboratory system (NANO-Viewer system; Rigaku, Tokyo, Japan). The two-dimensional scattering data were measured using a two-dimensional detector (PILATUS 100K; Detris, Baden, Switzerland), and the data were circularly averaged to one-dimensional data. The sample detector distance was set to 791 mm, which was calibrated with silver behenate. The scattering intensity, I(Q), was measured for scattering vectors (Q = 4π sinθ/λ) ranging from 0.012 to 0.2 Å⁻¹. The temperature was maintained at 25 °C or 55 °C. The innermost part of I(Q) was fitted under the Guinier approximation [32] to the equation I(Q) = I(0)exp {−Rg²Q²/3}, where I(0) and Rg are the forward scattering intensity (Q = 0) and the radius of gyration, respectively. A series of diluted samples were measured to extrapolate C/I (0) and Rg to zero protein concentration. The sample concentration ranged from 0.56 to 9.93 mg·mL⁻¹. The low-resolution model was constructed from the SAXS data at 25 °C by DAMMIF [33] without symmetrical constraints. Ten independent models were averaged by DAMAVER [34]. Figures of the low-resolution model were prepared using the PYMOL program [35].

**Results**

We amplified full-length cDNA for HspB1/Hsp27 from total cDNA of CHO cells using PCR. The amino acid sequence of CHO HspB1/Hsp27 (CgHspB1) was almost identical to those of other mammals (Fig. 1). Among three putative phosphorylation sites, Ser 15 and Ser 82 of human HspB1 are conserved in CgHspB1. It is known that murine and human HspB1 has only one cysteine residue, and the dimeric unit is connected by a disulfide bond [36,37]. Although the disulfide bond is not indispensable for dimer formation, it is thought to be related to the regulation of HspB1 by oxidative stress. The cysteine residue is also conserved in CgHspB1.

Wild-type CgHspB1 was expressed in *E. coli* and purified to homogeneity. First, we examined the chaperone activity of CgHspB1WT. CgHspB1WT protected CS from thermal aggregation at 45 °C (Fig. 2). Near-complete suppression of aggregation was attained by the addition of 24 excess molar CgHspB1WT.

Then, we examined the temperature- and concentration-dependent conformational change of CgHspB1WT using size exclusion chromatography on an HPLC system (Fig. 3). CgHspB1WT exists as a large oligomer similar to other sHsps. From the retention time, the molecular weight is estimated to be larger than 100 kDa. The CgHspB1WT oligomer decreased at the elevated temperature, and the oligomer reappeared when the...
temperature was shifted to the room temperature (Fig. 3A). Curiously, peaks for the dissociated small oligomers or monomers were not observed at the elevated temperatures (Fig. 3B–D). When the concentration was 6 µM, the oligomer peak completely disappeared, and only trace broad peaks for smaller oligomers appeared. The oligomer dissociation was also dependent on concentration. Almost no change was observed when the concentration was 30 µM (Fig. 3D). To examine the conformation of CgHspB1WT at the elevated temperature, size exclusion chromatography was performed with a buffer containing 20% ethylene glycol (Fig. 4) [31]. As ethylene glycol reduces hydrophobic interactions, nonspecific interactions between CgHspB1WT and the column resin should be reduced. Under these conditions, we observed the peak for CgHspB1WT at a position for small oligomers. Since the retention time of the peak corresponds to that for the polymers with the molecular weights of several ten kDa, they seem to be dimers. The results suggest that CgHspB1WT dissociates into dimers at elevated temperature and the hydrophobic surface is exposed.

The mixtures of CgHspB1 and client proteins were analyzed with size exclusion chromatography (Fig. 5). CgHspB1WT (30 µM) and CS appeared as separate peaks at room temperature. At 55 °C, the peak for CgHspB1WT remained, but the peak for CS disappeared.

**Fig. 1.** Amino acid sequence alignment of Chinese hamster HspB1 with various sHsps. Amino acid sequence alignment of human HspB1 (HUMAN_HSPB1, P04792), Chinese hamster HspB1 (Cg-HSPB1), mouse HspB1 (MOUSE_HSPB1, P14602), human HspB2 (HUMAN_HSPB2, Q16082), human HspB3 (HUMAN_HSPB3, Q12988), human HspB4 (HUMAN_HSPB4, P02489), and human HspB5 (HUMAN_HSPB5, P02511) is shown. The three phosphorylated Ser residues in Human HspB1 are marked by ‘+’. The Cys residue that forms inter-subunit disulfide bond is marked by ‘#’. 

| CgHspB1     | Mouse_HspB1   | Human_HspB1  | Human_HspB2 | Human_HspB3 | Human_HspB4 | Human_HspB5 |
|-------------|---------------|--------------|-------------|-------------|-------------|-------------|
| MTERVPFSLLRSQSPWEVRDFW--YPARSHISLQDPAGVPLPDDEWSQWFSAAWPGYVRP | MTERVPFSLLRSQSPWEVRDFW--YPARSHISLQDPAGVPLPDDEWSQWFSAAWPGYVRP | MTERVPFSLLRSQSPWEVRDFW--YPARSHISLQDPAGVPLPDDEWSQWFSAAWPGYVRP | MTERVPFSLLRSQSPWEVRDFW--YPARSHISLQDPAGVPLPDDEWSQWFSAAWPGYVRP | MTERVPFSLLRSQSPWEVRDFW--YPARSHISLQDPAGVPLPDDEWSQWFSAAWPGYVRP | MTERVPFSLLRSQSPWEVRDFW--YPARSHISLQDPAGVPLPDDEWSQWFSAAWPGYVRP | MTERVPFSLLRSQSPWEVRDFW--YPARSHISLQDPAGVPLPDDEWSQWFSAAWPGYVRP |
| LPAATAEGPAAVHLAPLAFRLNRQLSSGVEIRGTRADFWRSDLVDNHPAEEILT | LPAATAEGPAAVHLAPLAFRLNRQLSSGVEIRGTRADFWRSDLVDNHPAEEILT | LPAATAEGPAAVHLAPLAFRLNRQLSSGVEIRGTRADFWRSDLVDNHPAEEILT | LPAATAEGPAAVHLAPLAFRLNRQLSSGVEIRGTRADFWRSDLVDNHPAEEILT | LPAATAEGPAAVHLAPLAFRLNRQLSSGVEIRGTRADFWRSDLVDNHPAEEILT | LPAATAEGPAAVHLAPLAFRLNRQLSSGVEIRGTRADFWRSDLVDNHPAEEILT | LPAATAEGPAAVHLAPLAFRLNRQLSSGVEIRGTRADFWRSDLVDNHPAEEILT |
| RAAPA------GQSPVDESAETPREGSHFPQIFDLVFQPLPED | RAAPA------GQSPVDESAETPREGSHFPQIFDLVFQPLPED | RAAPA------GQSPVDESAETPREGSHFPQIFDLVFQPLPED | RAAPA------GQSPVDESAETPREGSHFPQIFDLVFQPLPED | RAAPA------GQSPVDESAETPREGSHFPQIFDLVFQPLPED | RAAPA------GQSPVDESAETPREGSHFPQIFDLVFQPLPED | RAAPA------GQSPVDESAETPREGSHFPQIFDLVFQPLPED |
| IQTEGGLLIALQHRTRMDHEGFRISFRTPQKLDPVEIKDDQLCHDGILVVEKP | IQTEGGLLIALQHRTRMDHEGFRISFRTPQKLDPVEIKDDQLCHDGILVVEKP | IQTEGGLLIALQHRTRMDHEGFRISFRTPQKLDPVEIKDDQLCHDGILVVEKP | IQTEGGLLIALQHRTRMDHEGFRISFRTPQKLDPVEIKDDQLCHDGILVVEKP | IQTEGGLLIALQHRTRMDHEGFRISFRTPQKLDPVEIKDDQLCHDGILVVEKP | IQTEGGLLIALQHRTRMDHEGFRISFRTPQKLDPVEIKDDQLCHDGILVVEKP | IQTEGGLLIALQHRTRMDHEGFRISFRTPQKLDPVEIKDDQLCHDGILVVEKP |
| VVQDVFQVHGRGHRERQDDHGLISREPHKRYIPADVLITTSSLSDDGVTNGRP | VVQDVFQVHGRGHRERQDDHGLISREPHKRYIPADVLITTSSLSDDGVTNGRP | VVQDVFQVHGRGHRERQDDHGLISREPHKRYIPADVLITTSSLSDDGVTNGRP | VVQDVFQVHGRGHRERQDDHGLISREPHKRYIPADVLITTSSLSDDGVTNGRP | VVQDVFQVHGRGHRERQDDHGLISREPHKRYIPADVLITTSSLSDDGVTNGRP | VVQDVFQVHGRGHRERQDDHGLISREPHKRYIPADVLITTSSLSDDGVTNGRP | VVQDVFQVHGRGHRERQDDHGLISREPHKRYIPADVLITTSSLSDDGVTNGRP |
| KTAT------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KTAT------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KTAT------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KTAT------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KTAT------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KTAT------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KTAT------QSEAETIVPTVEARQIGGQEQKSEQSGAK |
| KAVT------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KAVT------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KAVT------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KAVT------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KAVT------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KAVT------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KAVT------QSEAETIVPTVEARQIGGQEQKSEQSGAK |
| KLA------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KLA------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KLA------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KLA------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KLA------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KLA------QSEAETIVPTVEARQIGGQEQKSEQSGAK | KLA------QSEAETIVPTVEARQIGGQEQKSEQSGAK |
| GRHLDTENVYISLLPAPDPHEEEMEAIVE | GRHLDTENVYISLLPAPDPHEEEMEAIVE | GRHLDTENVYISLLPAPDPHEEEMEAIVE | GRHLDTENVYISLLPAPDPHEEEMEAIVE | GRHLDTENVYISLLPAPDPHEEEMEAIVE | GRHLDTENVYISLLPAPDPHEEEMEAIVE | GRHLDTENVYISLLPAPDPHEEEMEAIVE |
| VGTK---------- | VGTK---------- | VGTK---------- | VGTK---------- | VGTK---------- | VGTK---------- | VGTK---------- |
| QTGLDAHERAIPVSREEKTPSAPSS | QTGLDAHERAIPVSREEKTPSAPSS | QTGLDAHERAIPVSREEKTPSAPSS | QTGLDAHERAIPVSREEKTPSAPSS | QTGLDAHERAIPVSREEKTPSAPSS | QTGLDAHERAIPVSREEKTPSAPSS | QTGLDAHERAIPVSREEKTPSAPSS |
| QV------SGPERTPIPITKEKFAVTAAPFK | QV------SGPERTPIPITKEKFAVTAAPFK | QV------SGPERTPIPITKEKFAVTAAPFK | QV------SGPERTPIPITKEKFAVTAAPFK | QV------SGPERTPIPITKEKFAVTAAPFK | QV------SGPERTPIPITKEKFAVTAAPFK | QV------SGPERTPIPITKEKFAVTAAPFK |

**Fig. 1.** Amino acid sequence alignment of Chinese hamster HspB1 with various sHsps. Amino acid sequence alignment of human HspB1 (HUMAN_HSPB1, P04792), Chinese hamster HspB1 (Cg-HSPB1), mouse HspB1 (MOUSE_HSPB1, P14602), human HspB2 (HUMAN_HSPB2, Q16082), human HspB3 (HUMAN_HSPB3, Q12988), human HspB4 (HUMAN_HSPB4, P02489), and human HspB5 (HUMAN_HSPB5, P02511) is shown. The three phosphorylated Ser residues in Human HspB1 are marked by ‘+’. The Cys residue that forms inter-subunit disulfide bond is marked by ‘#’.
disappeared. The oligomer peaks stayed in the same position. It is reasonable to think that not the original large oligomer but the dissociated small oligomers interact with the denatured CS. Then, the heated mixture was cooled and analyzed at room temperature. The large complex of CgHspB1 and CS appeared. The same experiment was performed using a thermostable protein, IPMDH (Fig. 5). Since IPMDH does not denature at 55 °C, the presence of IPMDH did not affect CgHspB1.

The molecular mass of CgHspB1WT at room temperature was determined to be 384 kDa using SEC-MALS (Fig. 6A). As the deduced molecular mass of a subunit is 23.4 kDa, the oligomer is calculated to be a 16-mer. The oligomeric state of CgHspB1WT was also investigated with SAXS (Fig. 6B). $R_g$ at 25 °C was estimated to be 60.9 Å, and the molecular mass calculated from the $I(0)$ value was 361 kDa, which was almost the same as that calculated from the results of SEC-MALS. The low-resolution model of the oligomer of CgHspB1WT in solution was constructed from the SAXS data at 25 °C (Fig. 6C). The SAXS model was an oval sphere, which is similar to the crystal structure of SpHsp16.0 [9]. Curiously, dissociation of the oligomer was not observed by SAXS. At 55 °C, the $R_g$ value increased to 75.3 Å (Fig. 6B). The results partly coincide with the size exclusion chromatography results in which dissociation was not observed at high protein concentrations, because the SAXS experiment is performed at a high protein concentration.

Among the putative phosphorylation sites of HspB1, two are conserved in CgHspB1 (Fig. 1). Since Ser15 is

![Fig. 2. Effect of CgHspB1WT on thermal aggregation of CS. The thermal aggregation of CS from porcine heart was monitored by measuring light scattering at 500 nm with a spectrofluorometer at 45 °C. CS (50 nM, monomer) was incubated in the assay buffer with or without CgHspB1WT (150, 300, 600, and 1200 nM as monomers). The average values with the error bars of standard deviations from triplicate assays are plotted.](image)

![Fig. 3. Oligomer dissociation of CgHspB1 at elevated temperatures. CgHspB1WT (A. 6 µM; B. 12 µM; C. 18 µM; D. 30 µM as monomer) was incubated at the specified temperature for 30 min and then analyzed using size exclusion chromatography at the same temperature. CgHspB1WT heated to 55 °C was analyzed by gel filtration at room temperature after cooling at 25 °C for 30 min [25 °C(Re)].](image)
conserved only in HspB1, we focused Ser15 and examined the effect of phosphorylation by analyzing S15D mutant. Compared with the wild-type, the CgHspB1S15D oligomer is unstable (Fig. 7A). Even at the room temperature, CgHspB1S15D was partially dissociated into small oligomers, likely dimers (Fig. 7A,B). CgHspB1S15D showed a significantly high capacity to protect CS from thermal aggregation (Fig. 7C). An equimolar amount of CgHspB1S15D was sufficient to suppress the increase in light scattering induced by aggregation of CS.

Discussion

We have performed functional and structural characterization of HspB1 from Chinese ovary cell (CgGspB1). CgHspB1 could suppress the thermal aggregation of CS. CgHspB1 exists as a large oligomer and exhibits temperature-dependent dissociation. The dissociation also depends on the concentrations. Curiously, almost no change was observed at high concentrations. SAXS experiments also showed that CgHspB1 remained as the large oligomer at high temperature.

The molecular architecture of HspB1 is controversial. Analytical ultracentrifugation showed that the mean molecular mass is 730 kDa [20], and gel-filtration chromatography studies indicated that HspB1 forms 24-mers in the nonphosphorylated state [21]. Our result coincides with the observation by Lelj-Garolla et al. [22] that HspB1 exists as an equilibrium mixture of monomers/dimers, tetramers, 12-mers, and 16-mers based on sedimentation velocity analysis. Analytical ultracentrifugation experiments with various HspB1 concentrations clearly demonstrate that the oligomeric size increases from 10 to 40 °C. These larger oligomers are in equilibrium with smaller species, and their association is reversible. Therefore, they are not nonspecific aggregates [22]. Although we have shown that CgHspB1 exists as 16-mer structure, it seems to be variable as there is a difference in the oligomeric structures between at the room temperature and the elevated temperature.

Rogalla et al. [21] demonstrated that both phosphorylated HspB1 and the phosphorylation mimic mutant showed significantly decreased chaperone activity in vitro. They concluded that large oligomers of sHsps are necessary for chaperone action. However, other studies have clearly shown that phosphorylation mimic mutations destabilize HspB1/Hsp27 oligomers and enhance chaperone activity [26].

We have shown that a single phosphorylation mimic at S15 significantly increased chaperone

![Fig. 4. Oligomer dissociation of CgHspB1 analyzed by gel filtration in the presence of 20% ethylene glycol. CgHspB1WT (6 µM) was analyzed by gel filtration using buffer containing 20% ethylene glycol.](image)

![Fig. 5. Interaction of CgHspB1 with client proteins. CgHspB1WT (30 µM) was incubated with CS (A) or IPMDH (B) at 25 and 55 °C for 30 min and then analyzed by size exclusion chromatography at the same temperature. The mixture incubated at 55 °C was cooled to 25 °C and analyzed at 25 °C (55 °C Re). (A) CgHspB1 + CS. +, complex of CgHspB1 and CS; *, CgHspB1 oligomer; #, CS. (B) CgHspB1 + IPMDH. * CgHspB1 oligomer; & IPMDH dimer; $, IPMDH monomer.](image)
activity and decreased oligomer stability. Even at the room temperature, CgHspB1S15D partially dissociated into small oligomers, likely dimers (Fig. 7A, B). The hydrophobic character of the dissociated dimers was clearly shown by the interaction with the gel-filtration column. The correlation between chaperone activity and oligomer dissociation is clearly shown by the comparison between CgHspB1WT and CgHspB1S15D. However, the idea is contradicting with the fact that dissociation of CgHspB1WT was
not observed at the relatively high concentration. The discrepancy can be explained as follows. CgHspB1 is in the dynamic equilibrium between large oligomers and small oligomers. In the large oligomeric conformation, the hydrophobic surface remains inside. The hydrophobic surfaces are exposed by dissociation of the oligomers to interact with unfolded polypeptides. At a high concentration, most of the CgHspB1 remains in the large oligomeric conformation. However, the large oligomers are not static. They exchange dimer units which interact with unfolded proteins. Thus, the hydrophobic surface of the large oligomers is occasionally exposed by releasing a dimer unit, which may induce the formation of various oligomeric structures.

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**Conflicts of interest**

The authors declare no conflict of interest.

**Author contributions**

MY conceived and designed the experiments. ES, MN, KA, YYY, and TO performed the experiments. YYY, MY wrote the manuscript.

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