Comparison between Assembly Structures of sHSP20.8 and sHSP19.9 Small Heat Shock Proteins

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Small heat shock protein (sHSP) is the assembly of a number of identical subunits. sHSP19.9 and sHSP20.8 occur in the silkworm, *Bombyx mori*, and each of their subunits has a single cysteine residue: cysteine-123 in sHSP19.9 and cysteine-43 in sHSP20.8. The assembly structures of these sHSPs were examined by SDS-polyacrylamide gel electrophoresis, gel filtration chromatography, analytical ultracentrifugation, and chemical modification of cysteine residue. In both sHSPs, some sulfhydryl (SH) groups remained free, others formed inter-subunit disulfide bonds. The size distribution of assembly was invariant independently of protein concentration. SH groups were easily modified with a fluorescent alkyl halide: *N*-(iodoacet-aminoethyl)-1 naphthylamine-5'-sulfonic acid. This modification of a SH group never induced drastic changes in size distribution of subunit assembly besides suppressive activity of thermal aggregation of a protein. It was suggested that, between sHSP19.9 and sHSP20.8, there is difference in contents of SH groups, but only a little difference in assembly structures.

Key words: small heat shock protein, cysteine, *Bombyx mori*, subunit.

INTRODUCTION

Small heat shock protein (sHSP) is a family of proteins induced by heat treatment. sHSP occurs ubiquitously in a variety of organisms and, a variety of speculations about its physiological roles have been suggested. sHSP is the family of polypeptides having an α-crystallin domain which characterizes the lenticular α-crystallin. sHSP is a highly oligomeric assembly composed of a number of identical polypeptides (AUGUSTEYN, 2006; SUN and MACRAE, 2005). Structural details of sHSP however have not yet been clearly understood. We reported that the silkworm, *Bombyx mori*, has at least six genes encoding the following sHSPs: sHSP19.9, sHSP20.1, sHSP20.4, sHSP20.8, sHSP21.4, and sHSP23.7 (ASO et al., 2006; SAKANO et al., 2006a, b). The numerals in the names indicate molecular size (in kDa) from their deduced amino acid sequences. Based on various criteria including phylogeny, the *B. mori* sHSPs are classified into two groups: sHSP21.4 and others (SAKANO et al., 2006a). In consideration for difference in the number of cysteine (Cys) residues and their positions, the sHSPs are also grouped into the four types (Fig. 1). sHSP20.4 and sHSP21.4 in type I have no Cys residues. sHSP23.7 in type II has three Cys residues in a polypeptide: Cys-26, Cys-28, and Cys-201. Cys-26 and Cys-28 seemingly contribute to some oxidoreduction reactions. Each of sHSP20.8, sHSP19.9, and sHSP20.1 has a single Cys residue in a polypeptide. The Cys residue of sHSP20.8 in type III is the *N*-terminal side of α-crystallin domain. The amino acid sequence of sHSP20.8 is highly homologous to that of αCP25 sHSP from the Indianmeal moth, *Plodia interpunctella*; sHSP20.8 has 82.4% of identical residues, and its Cys-43 is overlapped with Cys-44 of αCP25 (SHIRK et al., 1998; SAKANO et al., 2006a). On the other hand, the Cys residues of sHSP19.9 and sHSP20.1 in type IV are the *C*-terminal side of the α-crystallin domain; Cys-123 of sHSP19.9 is overlapped with Cys-120 of sHSP20.1 (SAKANO et al., 2006a). In this context, we have been interested in the significance of Cys residues among the four types. Especially, the difference in positions of Cys residues between sHSPs in types III and IV. We had constructed the overproduction systems of the recombinant sHSP 20.8 and sHSP19.9 in *Escherichia coli* cells. In this study, we compared the assembly structure of sHSP 20.8 with that of sHSP19.9, taking into the difference in Cys residues consideration.

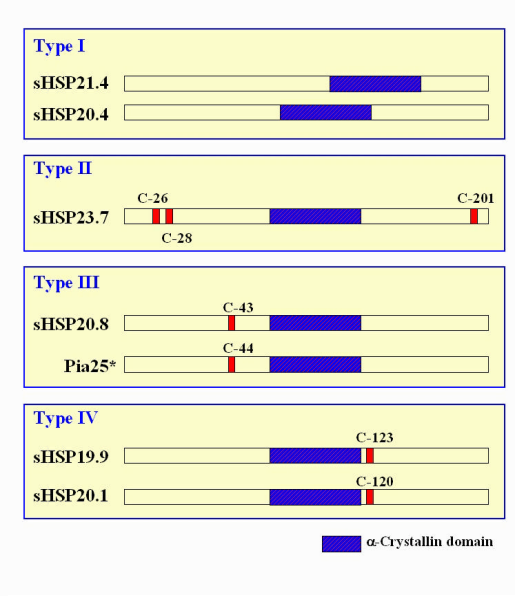

Fig. 1. Tentative classification of the *B. mori* sHSPs into the four types based on presence of Cys residues and their positions, or their absence. Pia25: *P. interpunctella* αCP25.

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MATERIALS AND METHODS

Reagents and proteins: Sephacryl S-300 and Sephadex G-25 were from GE Healthcare Life Sciences (Tokyo, Japan). Ni-NTA resin was from Novagen (Madison, WI, USA). *N*-(Iodoacetaminoethyl)-1 naphthylamine-5'-sulfonic acid (INS) and citrate synthase (CS) were from Research Organics Inc. (Cleveland, Ohio, USA) and Sigma Chemical Co. (Tokyo, Japan), respectively. Unless otherwise noted, the buffers used were 50 mM sodium phosphate buffer, pH 8, containing 0.1 M NaCl (buffer A), and 50 mM sodium phosphate buffer, pH 8, containing 0.3 M NaCl (buffer B).

Each of sHSP19.9 and sHSP20.8 genes was subcloned into a pET28a expression vector (Novagen), and the resulting plasmids were introduced into the (DE3) *E. coli* cells (Invitrogen, Tokyo, Japan). sHSP19.9 and sHSP20.8 were then overproduced as recombinant proteins having *N*-terminal His-tags (Sakano *et al*.; details will be described elsewhere). The cells were grown up at 37˚C on Luria-Bertani (LB) medium until mid-log phase. After induction with 1 mM isopropyl 1-thio-β-D-galactoside, the cells were further cultured for 3 h and collected from 100 ml of LB medium by centrifugation. The resulting cell pellet was suspended at 4^{O}C in 10 ml of buffer A and lysed by sonication. Insoluble substance were removed by centrifugation. From the resulting supernatant, the proteins were suspended in 80% saturation of ammonium sulfate and kept at 4^{O}C overnight. The resulting suspension was centrifuged. The resulting precipitate was redissolved with 1 ml of buffer A and was filtered through a Sephacryl S-300 (2 x 70 cm) pre-equilibrated with the same buffer. The fractions containing sHSP were collected and added to the suspension of Ni-NTA resin pre-equilibrated with buffer B containing 10 mM imidazole. After incubation for 1 h at room temperature, the suspension was poured into a syringe and washed with buffer B containing 20 mM imidazole. sHSP was eluted with buffer B containing 250 mM imidazole. sHSP solution thus prepared was dialyzed overnight against buffer A and stored at 4^{O}C until use. By this procedures, 4.5 mg and 3.1 mg of sHSP20.8 and sHSP19.9, respectively, were purified from 100 ml of LB medium.

Chemical modification: The chemical modification of Cys residue of sHSP was done using INS.
sHSP solution (1 mg/ml) was added to solid INS. The resulting suspension was incubated at room temperature for 30 min in a light-shielded tube. sHSP19.9-S-acetamino-1 naphythylamine-5'-sulfonic acid (sHSP 19.9-ANAS) and sHSP20.8-ANAS were thereafter isolated from reaction mixtures by gel filtration on a Sephadex G-25 column.

Electrophoresis and gel filtration chromatography: SDS-polyacrylamide gel electrophoresis was done using 4% stacking and 10% separating gels by the method according to Laemmli (Laemmli, 1970) in the presence or absence of 2-mercaptoethanol. Protein marker protein was Protein Marker, Broad Range (2-212 kDa) (New England Biolabs, Inc.). Gel filtration chromatography analysis was done on a Sephacryl S-300 column (2 x 70 cm) equilibrated with buffer A at room temperature. Sample solution (1 ml) was loaded on to the column, and 1 ml of aliquot was collected.

Spectroscopic analysis and analytical ultracentrifugation: The fluorescence spectrum was monitored at 30^{O}C with a Shimadzu RF-5000 spectrofluorometer. The intensity of fluorescence ascribed to tryptophan residues was measured at 340 nm upon excitation at 280 nm. The fluorescence spectra of sHSP20.8-ANAS and sHSP19.9-ANAS were measured upon excitation at 340 nm.

Sedimentation experiments were done with a Beckman XL analytical ultracentrifuge and an An-60 Ti rotor at 35,000 rpm and 30^{O}C by sedimentation velocity method. Time- and distance-dependent changes in absorbance at 280 nm were analyzed using the SEDFIT software (Schuck, 2003; Brown and Schuck 2006).

Suppressive activity of thermal aggregation: The suppression of thermal aggregation citrate synthase (0.028 mg/ml buffer A) at 60^{O}C was examined by measuring the intensity of light scattering at right angle. The intensity was monitored using a Shimadzu RF-5000 spectrofluorometer, setting both the excitation and emission wavelengths at 465 nm.

RESULTS AND DISCUSSION

sHSP19.9 and sHSP20.8 overproduced as N-terminally His-tagged recombinant proteins were prepared with relatively high yield: several milligrams of sHSP from 100 ml of LB medium. Large amounts of proteins were thus available for the characterization of sHSPs. Polypeptide composition of sHSP was examined by SDS-PAGE under reducing and non-reducing conditions. As shown in Fig. 2, although several minor bands were detected, each of sHSP19.9 and sHSP20.8 was stained as one major band: B-IV and B-III in Fig. 2, respectively. Comparing with the relative mobilities of marker polypeptides, the molecular sizes of sHSP19.9 and 20.8 were evaluated to be 26.6 and 28.9 kDa, respectively. Theoretical values of molecular weights of sHSP19.9 and 20.8 based on deduced their amino acid residues were calculated to be 22,054 and 25,777 Da, respectively. The difference between these size values is therefore ranged from 3 to 5 kDa. However, using

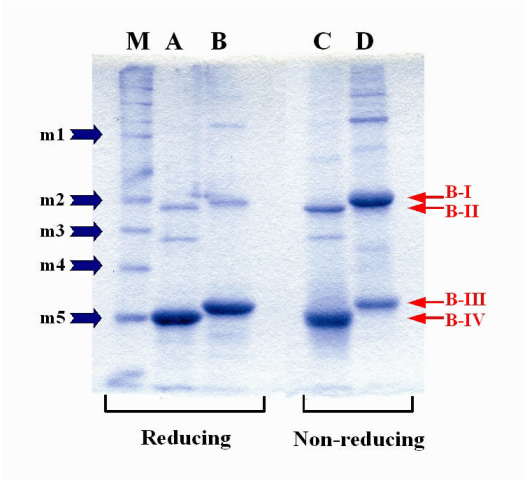

Fig. 2. SDS-polyacrylamide gel electrophoreses of sHSP19.9 and sHSP20.8. SDS-PAGE of sHSP19.9 was done under reducing (lane A) and non-reducing (lane C) conditions. SDS-PAGE of sHSP20.8 was also done under reducing (lane B) and non-reducing (lane D) conditions. The mixture of marker proteins was used under reducing conditions (lane M); m1, m2, m3, m4, and m5 stained bands correspond to the stained bands of rabbit muscle phosphorylase b (97.2 kDa), bovine serum albumin (66.4 kDa), *E. coli* maltose-binding protein (42.7 kDa), porcine muscle lactate dehydrogenase (36.5 kDa), and rabbit muscle triosephosphate isomerase (26.6 kDa), respectively. Red arrows indicate stained bands of sHSPs. B-I and B-III bands were from sHSP20.8 preparation. S-II and S-IV bands were from sHSP19.9 preparation.

anti-His-tag antibody both were immunologically confirmed to be His-tagged polypeptides (data not shown). The molecular sizes of sHSP19.9 and 20.8 were almost consistent with theoretical values by and mass spectroscopy (ZHANG et al., unpublished results). Based on these results, we confirmed that both the sHSPs are purified to homogeneity.

Results from SDS-PAGE under non-reducing conditions implied that both the preparations of sHSPs contain some disulfide bonds. The stained band (B-IV in Fig. 2) of sHSP19.9 became less dense than that under reducing conditions, and the second band (B-II in Fig. 2) became more clear at the position corresponding to be twice as large as molecular size of sHSP19.9. On the other hand, major part of sHSP20.8 polypeptide band was detected at the position corresponding to be twice as large as its molecular size (B-I in Fig. 2). Using anti-His-tag antibody, both the polypeptides at the positions of B-I and B-II bands (Fig. 2) were also confirmed to have a His-tag (data not shown). Comparing with the relative mobilities of marker polypeptides, the molecular sizes of polypeptides corresponding to B-II and B-I bands were evaluated to be 50.5 and 28.9 kDa, respectively. These values are two times as large as those from B-V and B-IV bands. Each of sHSP19.9 and sHSP20.8 is considered to have only a single Cys reside, based on deduced amino acid sequence (Fig. 1; SAKANO et al., 2006a). The results from the SDS-PAGE under non-reducing conditions therefore suggested that both the preparations of sHSP19.9 and sHSP20.8 contain a dimer linked with an inter-polypeptide disulfide bond and that the dimer content of sHSP19.9 is less than that of sHSP20.8. In other word, it was suggested that both the preparations are the mixtures of the dimer without any free Cys residues and the monomer with a free Cys residue and that the monomer-dimer ratio of sHSP19.9 is more than that of sHSP20.8.

It has been considered that sHSP form different structures composed of a number of identical subunits and that the structures are reversibly changeable. It was reported that the population of assembly structures is dependent on protein concentration in dissociation-association equilibrium of subunits (LIANG and LIU, 2005 & 2006). We examined the protein-concentration dependence of the structures of sHSP by gel filtration. The solution (1 ml) containing 1.8 mg/ml to 0.05 mg/ml of sHSP19.9 or sHSP20.8 was applied onto a Sephacryl S-300 column, developed, and fractionated. Proteins in eluate were measured by detecting fluorescence ascribed to Trp residues, because high sensitivity was required for wide concentration ranges of protein. As shown in Fig. 3, drastic changes in elution patterns of sHSP19.9 and sHSP20.8 were never detected. This result suggested that sHSP19.9 and sHSP20.8 are far from reversible dissociation-association equilibrium state.

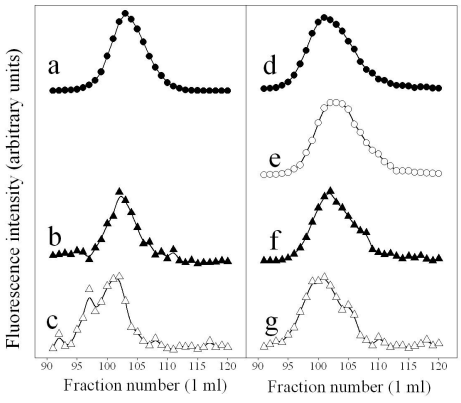

Fig. 3. Gel filtrations of sHSP19.9 (panel A) and sHSP20.8 (panel B). Sample solution (1 ml) containing 1.8 mg/ml (a), 0.1 mg/ml (b), or 0.05 mg/ml sHSP19.9 (c) was submitted to gel filtration at room temperature on a Sephacryl S-300 column and resolved. Protein in a fraction was detected by measuring its fluorescence intensity at 340 nm upon excitation at 280 nm. Sample solution (1 ml) containing 1.8 mg/ml (d), 1.0 mg/ml (e), 0.1 mg/ml (f), or 0.05 mg/ml sHSP20.8 (g) was also analyzed by the same method.

To confirm the occurrence of inter-polypeptide disulfide bond, sHSP19.9 and sHSP20.8 were subjected to the sulfhydryl (SH)-specific modification with fluorescent INS. SH groups were easily modified with a fluorescent alkyl halide: *N*-(iodoacet-aminoethyl)-1

naphthylamine-5'-sulfonic acid. The reaction mixtures were applied to the gel filtrations on a Sephacryl S-300 column. As shown in Fig. 4A and 4B, without any modifications, the elution positions of sHSP19.9 and sHSP20.8 were similar at fraction number 102-103. When measured fluorescence intensity at 470 nm, upon excitation at 340 nm, sHSP19.9-ANAS and sHSP20.8-ANAS were found to be eluted faster than intact sHSPs; their elution peaks were at around fraction number 97 (Fig. 4C and 4D). These changes in elution positions will be discussed later. Upon excitation at 340 nm, the emission maximum of INS fluorescence was at 434 nm. The red shift to 470 nm was detected after the chemical modifications of both sHSPs, indicating the attachment of INS to polypeptide. Effects of the modification were further inspected by fluorescence spectroscopy. Upon excitation at 280 nm, unmodified sHSP20.8 and sHSP19.9 showed typical fluorescence spectra ascribed to tryptophan residue(s) (Trp): emission maxima at 340 nm. Upon excitation at 280 nm, the emission maximum of sHSP19.9-ANAS was detected not at 340 nm, but at 470 nm, suggesting that the Trp fluorescence at 340 nm is absorbed by INS group emitting fluorescence at 470 nm. On the other hand, two emission maxima of sHSP20.8-ANAS were detected at 340 nm and 470 nm. This difference in fluorescence spectra between sHSP19.9-ANAS and sHSP20.8-ANAS might be due to the difference in positions of Cys and contents of free SH groups.

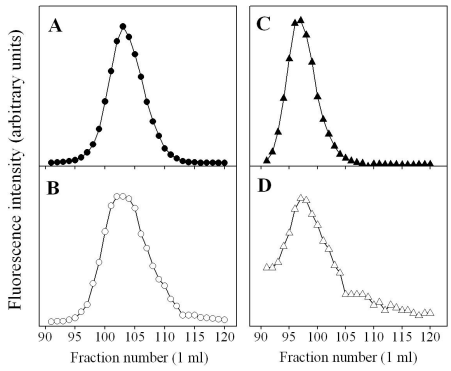

Fig. 4. Gel filtrations of sHSP19.9, sHSP20.8, sHSP19.9-ANAS, and sHSP20.8-ANAS. Gel filtration was done on a Sephacryl S-300 column at room temperature. Elution patterns of sHSP19.9 (panel A) and sHSP20.8 (panel B) were monitored by measuring intensity of fluorescence at 340 nm upon excitation at 280 nm. Elution patterns of sHSP19.9-ANAS (panel C) and sHSP20.8-ANAS (panel D) were monitored by measuring intensity of fluorescence at 470 nm upon excitation at 340 nm.

Molecular size distribution of sHSP was examined by sedimentation velocity method using an analytical ultracentrifuge. Sedimentation was done at 30^{O}C and 35,000 rpm, monitoring absorbance changes at 280 nm, and data were analyzed by the SEDFIT software. As shown in Fig. 5A and 5B, the whole molecular size (18.7S) of sHSP19.9 and its distribution were quite similar to those of sHSP20.8, although some aggregate-like signals were slightly detected in the distribution of HSP20.8. As shown in Fig. 5C and 5D, the chemical modification with INS induced only a little changes in these size distributions; the molecular sizes of sHSP19.9-ANAS and sHSP20.8-ANAS were both evaluated to be 17.7S (Fig. 5). The modification of Cys residue with INS results in the conversion of SH to sulfonate and then increase in negative charge of a polypeptide. Therefore, slight decrease in value of sedimentation coefficient might be caused by changes in surface charge accompanied by the modification. In other words, increase in negative charge might alter the hydrodynamic properties of sHSP slightly. As described above, upon gel filtration, sHSP-ANAS was eluted earlier than sHSP. One of possible explanation is that a certain interaction of sHSP with Sephacryl resin is weakened by an increase in negative charge.

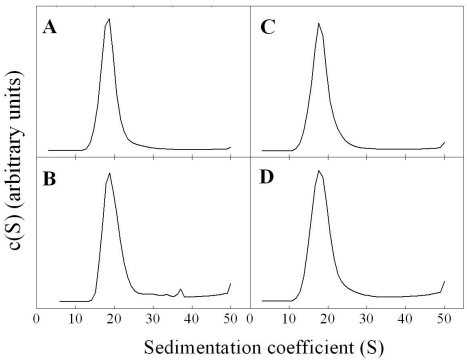

Fig. 5. Distribution of sedimentation coefficients of sHSPs by sedimentation velocity method. Sample solution containing sHSP19.9 (panel A), sHSP20.8 (panel B), sHSP19.9-ANAS (panel C), or sHSP20.8-ANAS (panel D) was centrifuged at 30^{O}C and 35,000 rpm. Time- and distance-dependent changes in absorbance at 280 nm were acquired and analyzed by the SEDFIT software.

Finally, we examined the effects of the modification on sHSP activity suppressing the aggregation of other polypeptides accompanied by their thermal denaturation. sHSP19.9-ANAS (Fig. 6A) and sHSP20.8-ANAS retained the suppressive activities. Based on these results, it was suggested that sHSP19.9 is similar to sHSP20.8 at least *in vitro*. Only difference was in disulfide bond contents. Sine a Cys residue of sHSP19.9 is close to C-terminal side of an α-crystallin domain, whereas that of sHSP20.8 is relatively far from *N*-terminal side of the domain. It was therefore plausible that some steric constraints are responsible for the

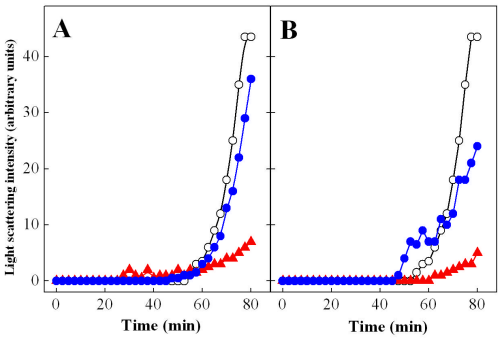

Fig. 6. Suppression of thermal aggregation of citrate synthase by sHSP19.9-ANAS (panel A) and sHSP20.8-ANAS (panel B). The aggregation of citrate synthase (0.028 mg/ml) was monitored at 60°C by measuring the intensity of light scattering at 465 nm and right angle. The solutions contained the following proteins: none (a and d); 0.003 mg/ml sHSP19.9-ANAS (b); 0.06 mg/ml sHSP19.9-ANAS (c); 0.003 mg/ml sHSP20.8-ANAS (e); 0.06 mg/ml

difference in the content. Further investigations are underway in our laboratory.

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