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Research Article

# Screening Molecular Chaperones Similar to Small Heat Shock Proteins in *Schizosaccharomyces pombe*

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**Abstract** To screen molecular chaperones similar to small heat shock proteins (sHsps), but without α-crystalline domain, heat-stable proteins from *Schizosaccharomyces pombe* were analyzed by 2-dimensional electrophoresis and matrix assisted laser desorption/ionization time-of-flight mass spectrometry. Sixteen proteins were identified, and four recombinant proteins, including cofilin, NTF2, pyridoxin biosynthesis protein (Snz1) and Wos2 that has an α-crystalline domain, were purified. Among these proteins, only Snz1 showed the anti-aggregation activity against thermal denaturation of citrate synthase. However, pre-heating of NTF2 and Wos2 at 70°C for 30 min, efficiently prevented thermal aggregation of citrate synthase. These results indicate that Snz1 and NTF2 possess molecular chaperone activity similar to sHsps, even though there is no α-crystalline domain in their sequences.

**Key words** α-Crystalline domain, Chaperone, Cofilin, NTF2, *Schizosaccharomyces pombe*, Small heat-shock proteins, Snz1, Wos2

Protein quality control systems are composed of molecular chaperones, folding catalysis, and proteases, which are all essential for cells [1, 2]. Molecular chaperones assist in maintaining the correct conformation and assembly of other polypeptides, but are not components of these assembled structures [1]. Chaperones are now known to participate in a variety of cellular functions and are widespread in organisms, tissues, and cells. It is noted that failure of chaperone activity leads to a negative impact, possibly serious, on one or more cellular function, which may lead to grave diseases and cell death [2].

Molecular chaperones function to prevent aggregation (anti-aggregation activity) and to assist correct refolding (refolding activity) of unfolded polypeptides or of partially unfolded polypeptides caused by heat or other triggering factors. Chaperones are also involved in cellular regulation of cell cycle regulation, transcription, and aging [3-5].

Most of the chaperones are heat shock proteins (Hsps), the expression of which is increased by heat shock. Chaperones, found in all organisms, are classified into families according to molecular weight of the polypeptide and amino acid sequence homology. These families are Hsp100, Hsp90, Hsp70, Hsp60, Hsp40/DnaJ, and small heat shock proteins (sHsps) [1, 2, 5-11].

The sHsps are oligomeric proteins composed of smaller subunits (less than 35 kDa) and with an α-crystalline domain, composed of about 100 amino acid residues. The sHsps show anti-aggregation activity, which prevents the precipitation of denatured proteins. The anti-aggregation activity of sHsps is known to be related with the formation of an oligomeric state in an ATP-independent manner [1, 9, 10].

*Schizosaccharomyces pombe* has various molecular chaperones including Hsp90, Hsp70, and sHsps. There is one gene, swo1, for Hsp90 and five genes, Sks2, Bip, Ssa1, Ssa2, and Pcc1, for Hsp70 in *S. pombe* [4, 11-13]. Up until now, three sHsps of the yeast have been identified. Two of these, Hsp16 and Hsp20, are characterized as members of the Hsp20/α-crystalline family, while the other, Hsp9, possess homology to Hsp12 of *Saccharomyces cerevisiae* [3, 14, 15]. These Hsps possess similarity with the known molecular Hsps from diverse organisms. For instance, Sks2 was identical with Ssb1p and Ssb2p of *S. cerevisiae*, both of which are

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known to be cytoplasmic Hsp70s responsible for protein synthesis by non-organelle-localized ribosomes [12]. In addition, Hsp60 possesses extensive sequence similarity with *S. cerevisiae* mitochondria Hsp60 and other properties strongly suggest that Hsp60 plays a similar role in *S. pombe* [13]. Hsp9 has homology to Hsp12 of *S. cerevisiae* and Hsp26 of *Drosophila melanogaster* [15]. Hsp9 has dual functions in stress adaptation and stress-induced G2-M checkpoint regulation via Cdc25 inactivation [3].

Recently, chaperone activity has been reported for some proteins which were known to have other biological functions. Thioredoxin (Trx) from *Escherichia coli* has been shown to promote the folding of proteins independent of their redox activity both by directly promoting protein folding and by enhancing the refolding activity of other molecular chaperones [16]. As described above, neither the redox state of the Trxs nor the active site cysteines or other amino acids, which are imperative for redox function, are required for chaperone activity [17, 18]. In addition, Trx promotes renaturation of citrate synthase (CS), while it does not protect CS against thermal denaturation [18, 19]. Tsa1 from *S. cerevisiae*, which is known to be a peroxidase using thioredoxin as an electron donor also exhibited strong chaperone activity. Yeast Tsa1 without an α-crystalline domain showed similar chaperone properties to sHsps [20].

It is expected that certain proteins that are already known to possess other biological functions or that are whose roles are still unknown have possible have molecular chaperone activity. In this study, in order to find new molecular chaperones in *S. pombe*, chosen as a simple eukaryotic model cell system, heat-stable proteins were screened using 2-dimensional electrophoresis (2DE) and matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The chaperone activity of the recombinant proteins for identifying screened heat-stable proteins was examined.

## MATERIALS AND METHODS

**Yeast strains and preparation of crude extracts of *S. pombe* and heat treatment.** The *S. pombe* strain used in this study was diploid SP286 ($h^+h^+$*ade6-M210/ade6-M216, ura4-D18/ura4-D18, leu1-32/leu1-32*) (Bioneer, Daejeon, Korea). *S. pombe* cells were grown in YE medium (0.5% yeast extract, 3% glucose) with additional required supplements at 30°C until stationary phase ($OD_{600}$ = 8) was reached and then they were harvested by centrifugation. The pellet was suspended in 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid and 0.2 mM phenylmethylsulfonyl fluoride and disrupted using 0.5-mm diameter glass beads in a mini-bead beater (Biospec Products, Bartlesville, OK, USA). The supernatant obtained by centrifugation was used as the crude extracts for screening of heat-stable proteins.

Crude extracts (1 mg/mL) of *S. pombe* were heated for 20 min at 45°C and 70°C in a water bath and aggregated proteins were removed by centrifugation. Proteins in the supernatant were precipitated by addition of trichloroacetic acid (10% w/v as final concentration). The precipitate was washed three times with cold acetone and dried in air. The dried protein precipitate was dissolved in lysis buffer comprised of 9 M Urea, 4% CHAPS, and 40 mM Tris-HCl buffer (pH 7.0). The concentration of protein in the lysis buffer was determined using the Bradford method.

**Two-dimensional SDS-PAGE.** To the protein sample dissolved in lysis buffer, rehydration buffer (8 M urea, 2% CHAPS, 0.5% immobilized pH gradient [IPG] buffer, 20 mM dithiothreitol [DTT], and 0.005% bromophenol blue) was added and applied onto an IPG strip (pH 4~7, 13 cm; Amersham Biosciences, Freiburg, Germany). Isoelectric focusing was performed at 20°C using IPG phore. The focused IPG strip was subjected to reduction and alkylation, and then the proteins were separated by 15% SDS-PAGE [21]. The gels were stained with Coomassie Blue or silver.

**In gel tryptic digestion of protein spots.** Excised spots from a 2D-PAGE gel were washed with distilled water, 50% acetonitrile (ACN), and 100 mM ammonium bicarbonate (ABC, pH 8.5). After 45 min of reduction with a reducing agent (10 mM DTT in a 100 mM ABC, pH 8.5), each piece of the gel was alkylated with 55 mM iodoacetamide in 100 mM ABC (pH 8.5) for 30 min with subsequent drying in a Speed Vac (Thermo Savant, NY, USA). For digestion of gel pieces, 40 μL of trypsin solution (50 mM ABC in 5 mM $CaCl_2$ with 12.5 ng trypsin) was added and incubated for 45 min at 4°C [22].

**MALDI-TOF mass spectrometer and database searches.** Mass analysis was performed using a Voyager DE-STR (Applied Biosystems, Foster City, CA, USA) at Equipment management at the maintenance center, Chonnam National University (Gwangju, Korea). Peptides were co-crystallized 1 : 1 (v/v) with matrices consisting of saturated α-cyano-4-hydroxycinnamic acid prepared in 50% (v/v) ACN/1% (v/v) trifluoroacetic acid. All MALDI spectra were externally calibrated using angiotensin I (1,296.79 Da), adrenocorticotropic hormone (ACTH clip 1~17, 2,093.01 Da), and ACTH clip 18~39 (2,465.20 Da) as external standards. Mono-isotopic peptide masses were assigned and used for database searches. Database searches were performed on the National Center for Biotechnology Information (NCBI) non redundant database using the MASCOT software package (Matrix Sciences, London, UK) and were confirmed manually [23].

**Plasmid construction and bacterial expression.** The genes for screened heat-stable proteins including Snz1, Wos2, NTF2, and cofilin were cloned by PCR using *S. pombe* cDNA as a template. Primers for the cloning of heat-stable protein genes contained an *Nde*I site (underlined) in the forward primer and *BamH*I or *Eco*RI site (underlined) in the reverse primer: Snz-forward, 5′-AAA CAT ATG TCT

GCC GAA ATT AAG-3′ and Snz-reverse, 5′-AAA GGA TCC TTA CCA CCC GCG AGT-3′; Wos2-forward, 5′-AAA CAT ATG AGT TTA AAT ACA CAA-3′ and Wos2-reverse, 5′-AAA GGA TCC TTA CTC TTT CTT TTC GTT-3′; NTF2-forward, 5′-AAA CAT ATG GCT GAC TAT AAT-3′ and NTF2-reverse, 5′-AAA GAA TTC TCA ACC ATA GTT-3′; Cofilin-forward, 5′-AAA CAT ATG TCT TTT TCA GGT GTC-3′ and Cofilin-reverse, 5′-AAA GGA TCC TTA CTT ACG AGT AAC-3′. The PCR reaction mixture contained 100 ng of *S. pombe* cDNA, 20 pmole of both forward and reverse primers, and 5× Taq-PCR Mix (Genotech Ltd., Daejeon, Korea). The purified PCR product was cloned into pGEM-T Easy TA cloning vector (Promega, Madison, WI, USA). The sequence of genes was confirmed by Solgent (Daejeon, Korea).

The PCR products containing genes of heat-stable proteins were expressed using pET17b system in *E. coli* BL21(DE3) with induction by 0.4 mM isopropyl β-D-1-thiogalactopyranoside. The overexpressed *E. coli* cells were harvested by centrifugation and stored at −20°C until used.

**Purification of recombinant heat-stable protein.** The harvested cells (5 g) were suspended in buffer (20 mM Tris-HCl [pH 7.6], 0.01 mM PMSF), disrupted using a Branson sonifier 250 (Branson, Danbury, CT, USA). Crude extracts were applied onto a DEAE-Sephacel anion exchange column pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.6) and then eluted with 0.0~0.5 M NaCl linear gradient. The fractions containing recombinant protein were pooled, precipitated with ammonium sulfate (70% saturation), and resuspended in buffer A (20 mM HEPES-NaOH [pH 7.0] containing 1 M ammonium sulfate) for phenyl column chromatography. After centrifugation, the supernatant was applied onto a TSK Phenyl-5PW HPLC hydrophobic interaction column (TOSCH, Tokyo, Japan). Proteins were eluted with a 1.0~0.0 M inverse gradient of ammonium sulfate at a flow rate of 1 mL/min. Fractions containing protein were pooled and dialyzed against 20 mM HEPES-NaOH (pH 7.0). The purified heat-stable proteins were stored at −70°C until use.

**Thermo-stability of recombinant proteins.** The purified recombinant protein solution was incubated for 30 min at 40°C, 50°C, 60°C, 70°C, or 80°C. After cooling in ice, the respective samples were centrifuged for 20 min. Subsequently, the amount of remaining protein in the supernatant was measured by the Bradford method [21].

**Chaperone activity assay.** Chaperone activity was determined as anti-aggregation activity using CS. Light scattering of a reaction mixture containing 1 μM CS, and purified recombinant protein in 50 mM Tris-HCl (pH 8.0), was monitored at 360 nm with a spectrophotometer (U2800 UV-Vis Double Beam Spectrophotometer; Histachi, Tokyo, Japan) for 20 min at 43°C [23].

## RESULTS

**Screening of heat-stable proteins from *S. pombe* by 2DE analysis.** For the screening of heat-stable proteins as candidates as being molecular chaperones without an α-

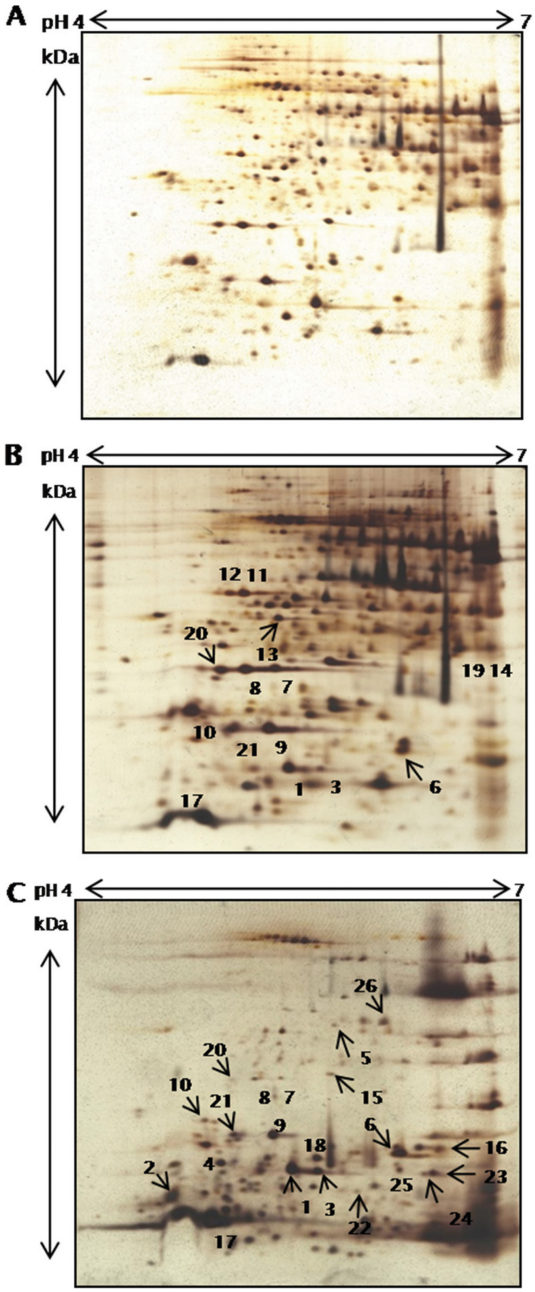


**Fig. 1.** Representative 2-dimensional electrophoresis gels of *Schizosaccharomyces pombe* crude extracts with or without heat-treatment. Heat treated crude extracts of *S. pombe* were separated by isoelectrofocusing using an IPG strip (pH 4~7), followed by 15% SDS-PAGE and visualized with silver staining. Crude extracts; 50 μg proteins (A), 100 mg proteins heated at 45°C (B) and 70°C (C) for 30 min. Numbers and arrows indicate position of spots identified by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (Table 1).

crystalline domain, crude extracts of *S. pombe* were heated and the remaining proteins in the supernatant fraction were analyzed by 2DE and MALDI-TOF mass spectrometry. First of all, we applied heat treatment and conducted 2DE more than 5 times to know the change of protein patterns. In addition, for preparation of MALDI-TOF mass spectrometry analysis, we carried out heat treatment and 2DE at least 3 times more on another day.

As shown in Fig. 1A, more than 40 protein spots in the molecular size range of 25 kDa to 45 kDa were observed after silver staining of 2DE gel for crude extracts of *S. pombe*. The image of protein spots on the 2DE gel for soluble proteins after heat-treatment at 45°C was similar to that of crude extracts without heating, as can be seen in Fig. 1B. The number of spots for soluble proteins was significantly decreased by heat-treatment at 70°C for 20 min (Fig. 1C). The protein spots were decreased in the molecular size range of 25 kDa to 45 kDa by heat treatment. An increase in the intensity of some spots with low molecular size was observed and new spots appeared on 2DE gel by heat-treatment at 70°C. It seemed that some spots were enriched, whereas others were generated by proteolysis during heat-treatment.

**Identification of heat-stable proteins of *S. pombe*.** Protein spots on the 2DE gel were analyzed by MALDI-TOF mass spectrometry after in-gel digestion with trypsin for identification. Protein spots existing on both the gel for crude extracts and the gel for supernatant fraction after heat-treatment at 70°C having molecular size 15~30 kDa were focused for screening of chaperones similar to sHsps. Sixteen proteins were identified, as shown Table 1. There were a sHsp, Hsp16, and p23 (Wos2) that was reported as co-chaperone of Hsp90 from *S. cerevisiae* [24]. Both Hsp16 and Wos2 have an α-crystalline domain [14]. In addition, three antioxidant enzymes, SOD, TPx, and PMP20 were identified. Our previous study showed that TPx and PMP20 in *S. pombe* have thermal stability and chaperone activity [21] and Trx also has a chaperone activity in *E. coli* [16]. Hsp16 and transthyretin were not expressed in the *E. coli* expression system pET17b. In addition, ADH, Cwf14, and Mmf1 were produced as insoluble proteins.

**Expression and purification of the identified heat-stable proteins.** sHsps are known to have a molecular mass between 15 and 30 kDa. Among the sixteen heat-stable proteins, four proteins, including cofilin (19115653), NTF2 (nuclear transport factor Nxt2/162312440), Snz1 (probable pyridoxin biosynthesis PDX1-like protein/19115894), and Wos2 (p23 homolog/19115498), all have a molecular mass between 15 and 30 kDa and were not known to have chaperone activity, were selected as candidates for chaperones with properties similar to sHsps. These candidates were successfully expressed in the *E. coli* expression system and purified. The purified proteins were separated under reducing and non-reducing conditions by SDS-PAGE. As shown in Fig. 2A, Snz1, Wos2, cofilin, and NTF2 showed single bands with an estimated size of 31.4 kDa, 20 kDa, 15 kDa, and 10 kDa, respectively on a reducing SDS-PAGE. All four proteins exhibited single bands of the same molecular size on non-reducing SDS-PAGE. There were no inter-chain disulfide bonds in these four recombinant proteins.

**Thermo-stability of the purified recombinant proteins.** Thermo-stability of the recombinant proteins was examined. Snz1 and cofilin remained soluble up to 50°C, but precipitated after 30 min at 60°C (Fig. 2B). In contrast to NTF2, which was stable up to 70°C and precipitated at 80°C, Wos2 was

**Table 1.** Identification of *Schizosaccharomyces pombe* heat stable protein by MALDI-TOF analysis

| No. | Gene ID | Description | Theoretical | | Sequence coverage (%) | No. of the peptide |
|---|---|---|---|---|---|---|
| | | | MW (kDa) | pI | | |
| 1 | 19115653 | Cofilin | 15.724 | 5.60 | 71 | 5 |
| 2 | 162312440 | Nuclear transport factor Nxt2 (NTF2) | 14.077 | 4.80 | 42 | 3 |
| 3, 23, 24, 25 | 19113225 | Mmf1p | 17.760 | 9.41 | 13 | 5 |
| 4 | 19115498 | p23 homolog (Wos2) | 20.951 | 4.46 | 55 | 5 |
| 5, 26 | 19115894 | Probable pyridoxin biosynthesis PDX1-like protein (Snz1) | 31.403 | 5.92 | 22 | 4 |
| 6 | 19114075 | Cu, Zn-Superoxide dismutase | 16.017 | 5.80 | 30 | 4 |
| 7, 8, 20 | 19075930 | Thioredoxin peroxidase | 21.292 | 5.37 | 90 | 9 |
| 9, 21 | 19075206 | Peroxisomal membrane protein (PMP20) | 16.722 | 5.18 | 12 | 6 |
| 10 | 19075930 | Thioredoxin peroxidase (cut) | 21.292 | 5.37 | 26 | 7 |
| 11, 12 | 19114548 | Inorganic pyrophoshate | 32.675 | 5.20 | 55 | 5 |
| 13 | 2500128 | Thioredoxin reductase | 34.618 | 5.19 | 77 | 7 |
| 14, 19 | 19075524 | Triosephosphate isomerase (Tpi) | 27.096 | 6.60 | 37 | 9 |
| 15 | 65898 | Alcohol dehydrogenase | 37.428 | 6.46 | 28 | 11 |
| 16 | 19112757 | G10 protein | 17.093 | 9.12 | 30 | 4 |
| 17 | 19114764 | Thioredoxin (Trx1) | 11.165 | 4.86 | 42 | 3 |
| 18 | 19112883 | Heat shock protein 16 | 15.958 | 5.72 | 16 | 5 |
| 22 | 19075833 | Transthyretin-like protein | 13.521 | 6.25 | 25 | 4 |

MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight.

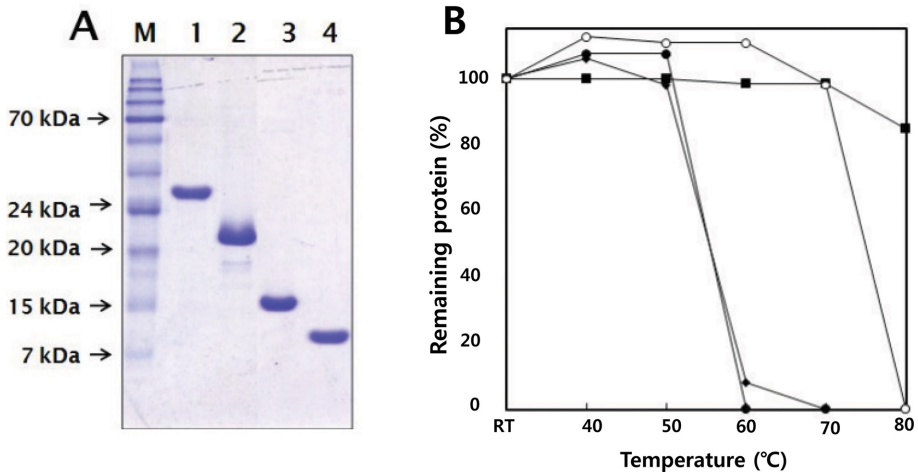


**Fig. 2.** SDS-PAGE analysis and thermo-stability of purified recombinant proteins. A, The protein samples were separated on 15% SDS-PAGE reducing gel and stained with Coomassie Brilliant Blue R-250. Positions of molecular size standards were indicated as kDa. M, size marker; lane 1, Snz1; lane 2, Wos2; lane 3, cofilin; lane 4, NTF2. B, Each protein was heat-treated at various temperatures for 30 min and the remaining protein in the supernatant was measured by the Bradford method. Data are representative of three trials and are expressed as the mean ± SD. ◆, Snz1; ■, Wos2; ●, cofilin; ○, NTF2; RT, room temperature.

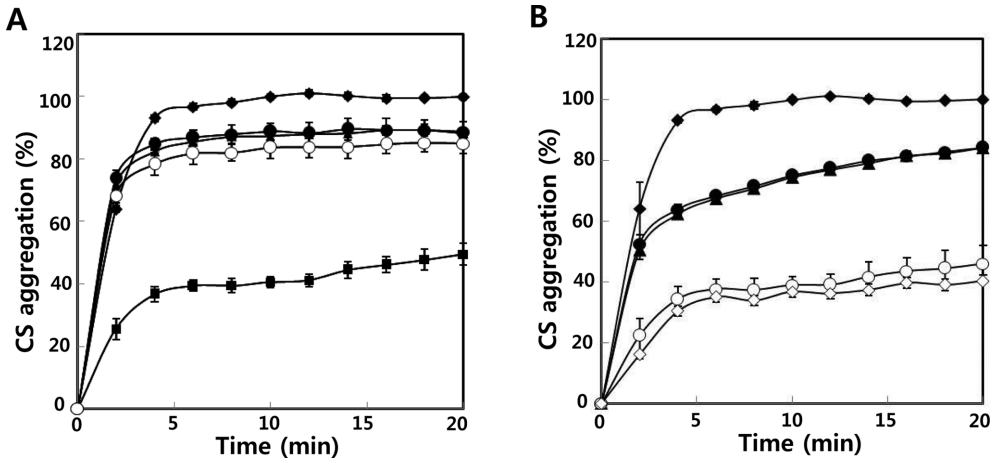


**Fig. 3.** Molecular chaperone function of the recombinant protein. Chaperone activity was measured as protection potency against thermal aggregation of 1 μM citrate synthase (CS) in 50 mM HEPES-NaOH buffer (pH 7.0) at 43°C in the absence or presence of the recombinant proteins by light scattering at 360 nm. Data are representative of three trials and were calculated as a percentage of the maximum aggregation of CS after 20 min for each trial and are expressed as the mean ± SD. A, The molecular chaperone functions of the recombinant proteins. CS only (◆) or in presence of the recombinant proteins at molar ratio of the recombinant proteins to CS of 1 : 8. ■, Snz1; ▲, Wos2; ●, cofilin; ○, NTF2. B, Concentration dependent anti-aggregation activities of Snz1 were measured at various molar ratios of protein to substrate, 1 : 1 (◆), 1 : 2 (▲), 1 : 4 (●), 1 : 8 (○), and 1 : 16 (◇).

stable up to 80°C, with no decrease in concentration of the protein by precipitation.

**Protection of CS from thermal aggregation.** To examine whether the recombinant proteins possess molecular chaperone activity, their effects on the thermal aggregation of CS of *S. pombe* were measured. As shown in Fig. 3A, increase in light scattering by aggregation of CS at 360 nm was observed at 43°C. Generally, irreversible aggregation occurs within 2 min of incubation at 43°C and reaches maximum levels after approximately 5 min. CS aggregation was not inhibited by addition of cofilin, NTF2, or Wos2 at the recombinant protein/CS subunit molar ratio of 8. Under these conditions, the three proteins did not show any anti-aggregation chaperone activity.

However, Snz1 reduced thermal aggregation of CS by 50% under subunit molar ratio of 8 : 1. Snz1 slowed down the CS aggregation significantly at lower molar ratios, 2 : 1 and 4 : 1. A further increase in anti-aggregation activity was not observed at a greater molar ratio of 16 : 1 (Fig. 3B).

**Effect of heat-treatment of the recombinant proteins on molecular chaperone activity.** Anti-aggregation activity of molecular chaperones, such as small Hsps, is generally increased by heat treatment [19]. To test whether the molecular chaperone activity of the recombinant protein was altered by heat-treatment, the protein was pre-treated to the highest temperature without precipitation of protein. As shown in Fig. 4A, no difference on CS aggregation was observed for cofilin pre-treated at 50°C for 30 min. A minor decrease in the chaperone activity of Snz1 was observed by heat-treatment at 50°C. On the other hand, NTF2 and Wos2 preheated at 70°C for 30 min efficiently prevented thermal aggregation of CS.

In order to understand the effect of temperature on induction of chaperone activity for NTF2 and Wos2 by preheating, the respective proteins were heated at various temperatures (up to 80°C). No effect on the anti-aggregation activity was observed for NTF2 pretreated at 40~60°C, however, NTF2 showed anti-aggregation activity by pretreatment at 70°C (Fig. 4B). In the same manner, Wos2 did not demonstrate chaperone activity by preheating up to 60°C, but the protein heated at 70°C or 80°C exhibited almost complete suppression of aggregation (Fig. 4C).

The effect of the concentration of NTF2 heated at 70°C on thermal aggregation of CS was examined. At molar ratios of 1 : 1 or 2 : 1, NTF2 had little effect on the thermal aggregation of CS, but it exhibited anti-aggregation activity at molar ratio of 4 : 1. The maximum effect of NTF2 on prevention of thermal aggregation of CS could be seen at molar ratio of 8 : 1. No further increase in anti-aggregation activity was observed at molar ratio of 16 : 1 (Fig. 4D). For preheated Wos2, thermal CS aggregation could almost be

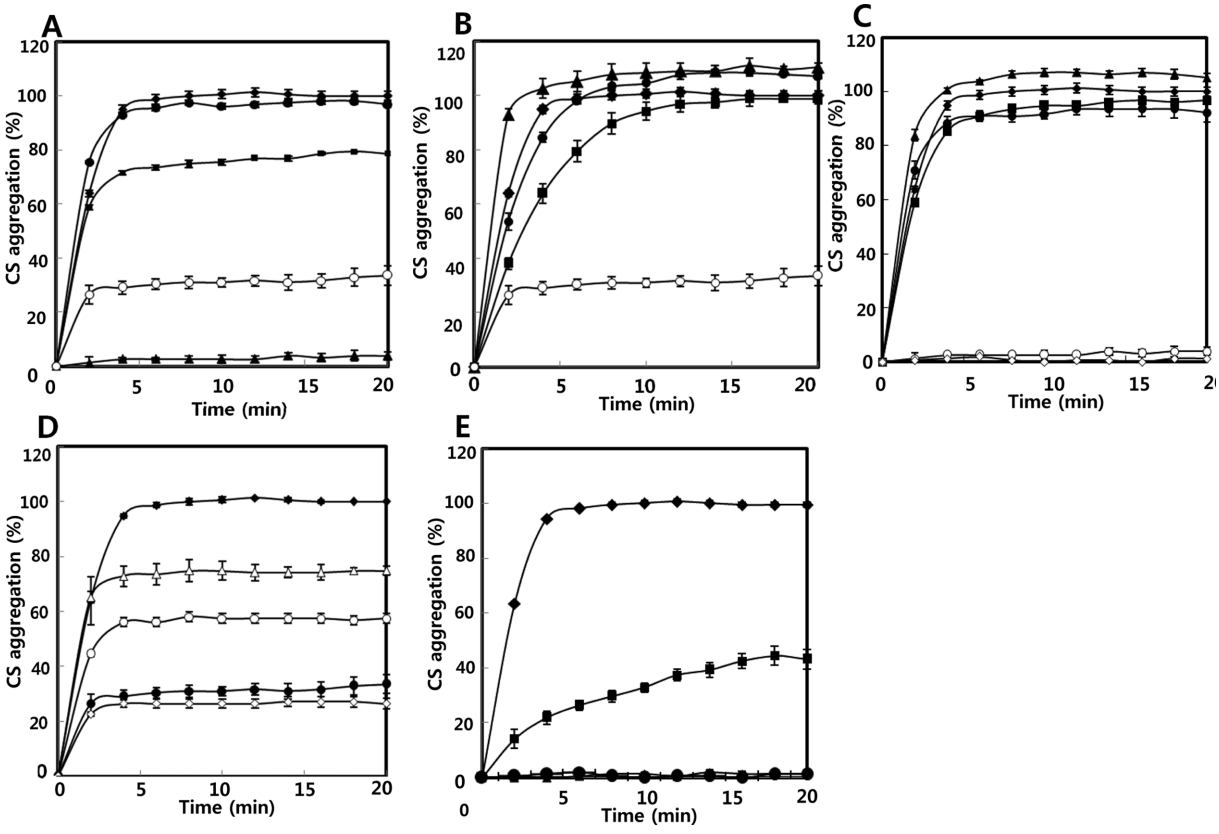


**Fig. 4.** Effect of the heat-treatment on molecular chaperone function of the recombinant proteins. Chaperone activity was measured as protection potency against thermal aggregation of 1 μM citrate synthase (CS) in 50 mM HEPES-NaOH buffer (pH 7.0) at 43°C in the absence or presence of the recombinant proteins by light scattering at 360 nm. Data are representative of three trials and were calculated as a percentage of the maximum aggregation of CS after 20 min for each trial and are expressed as the mean ± SD. A, Heat treatment was performed for 30 min. CS only (◆) or in presence of the heated recombinant proteins at molar ratio of the heated proteins to CS of 1 : 8. Snz1 heated at 50°C (■), Wos2 heated at 70°C (▲), cofilin heated at 50°C (●), and NTF2 heated at 80°C (○). B, C, Effect of the heat treated temperature on the molecular chaperone function of NTF2 (B) and Wos2 (C). Heat treatment was performed at various temperatures for 30 min. CS only (◆) or in presence of NTF2 or Wos2 at molar ratio of protein to CS of 1 : 8. Proteins were heated at 40°C (■), 50°C (▲), 60°C (●), 70°C (○), and 80°C (◇). D, E, Effect of the heat treated protein concentrations on the molecular chaperone function of NTF2 (D) and Wos2 (E). Heat treatment was performed at 80°C for 30 min. CS only (◆) or in presence of Wos2 at molar ratio of Wos2 to CS of 1 : 0.5 (■), 1 : 1 (▲), 1 : 2 (△), 1 : 4 (○), 1 : 8 (●), and 1 : 16 (◇).

completely suppressed at molar ratio of 1 : 1, as shown in Fig. 4E.

## DISCUSSION

2DE gel of proteins present in the supernatant fraction of *S. pombe* crude extracts heated at 45°C and 70°C for 20 min resulted in identification of sixteen proteins by MALDI-TOF mass spectrometry. Among these proteins, three antioxidant enzymes (SOD, TPx, and PMP20), two molecular chaperones (Hsp16 and Wos2), and Mmf1 are generally known to be thermally stable [21]. Wos2 and Hsp16 have an α-crystalline domain that is highly conserved in sHsp [14, 24]. Wos2 has been reported as a co-chaperone of Hsp90 [11, 14].

Wos2 is abundantly distributed in yeast cells and a drastic decrease in the expression was noted as cells entered into early stationary phase, indicating that its function is associated with cell proliferation. In addition, Wos2 is significantly homologous to human p23, which is one of the most abundant Hsp90-associated co-chaperone [24]. Also, Wos2 acts *in vivo* as a "first aid chaperone" that maintains proteins in a folding-competent state when environmental changes take place, until Hsp90 and other chaperones accumulate and initiate refolding [24]. Wos2 shares characteristics similar to other sHsps. In this study, purified recombinant Wos2 did not show anti-aggregation activity even at a high molar ratio of 1 : 16 and it was stable up to 80°C. By heat-treatment at 70°C or 80°C for 30 min, Wos2 induced chaperone activity similar to sHsps.

In this study, the results suggest that Snz1 is not only heat-stable, but also prevents thermal aggregation of CS. The thermal aggregation of CS was inhibited by 50% under subunit molar ratio of Snz1 : CS of 8 : 1. Snz1 significantly slowed down CS aggregation at lower molar ratios of 2 : 1 and 4 : 1 as well. A further increase in anti-aggregation activity was not observed at a molar ratio of 16 : 1.

However, unlike other sHsps, anti-aggregation activity of Snz1 was not increased by heat treatment. Snz1, a member of a highly conserved gene family, was first identified as a protein whose synthesis increased dramatically as yeast cells entered stationary phase [25]. Snz1 required production of pyridoxine and was reported to be involved in growth arrest and cellular response to nutrient limitation [25]. Also, of the highly conserved SNZ and SNO genes in *S. cerevisiae* only the protein encoded by Snz1 is required for vitamin B6 biosynthesis [26]. So far, there has been no citation about its molecular chaperone activity.

The properties of recombinant NTF2 were observed to be very similar to Wos2. There was no effect of NTF2 on thermal aggregation of CS at molar ratios up to 16 : 1. The heat-treated NTF2 at 70°C, however, efficiently prevented thermal aggregation of CS at a molar ratio of 8 : 1. NTF2 is a homodimer of approximately 14 kDa subunits [27]. NTF2 was originally identified as a factor required for efficient import of nuclear localization signal-containing proteins into the nucleus [28]. NTF2 binds to both RanGDP and FxFG repeat-containing nucleo-porins and localizes to the nuclear rim at steady state [29, 30].

In summary, we presented the first experimental evidence that three proteins are capable of preventing thermal aggregation of CS *in vitro*. These results strongly support their potential functions as molecular chaperones. Further detailed investigations on the behavior of the three proteins are required to obtain insight into the biological functions of molecular chaperones from *S. pombe*, including thermal aggregation of CS *in vivo*.

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