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ORIGINAL PAPER

Thermotolerance and molecular chaperone function of the small heat shock protein HSP20 from hyperthermophilic archaeon, *Sulfolobus solfataricus P2*

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Abstract Small heat shock proteins are ubiquitous in all three domains (Archaea, Bacteria and Eukarya) and possess molecular chaperone activity by binding to unfolded polypeptides and preventing aggregation of proteins in vitro. The functions of a small heat shock protein (S.so-HSP20) from the hyperthermophilic archaeon, *Sulfolobus solfataricus P2* have not been described. In the present study, we used real-time polymerase chain reaction analysis to measure mRNA expression of S.so-HSP20 in *S. solfataricus P2* and found that it was induced by temperatures that were substantially lower (60°C) or higher (80°C) than the optimal temperature for *S. solfataricus P2* (75°C). The expression of S.so-HSP20 mRNA was also up-regulated by cold shock (4°C). *Escherichia coli* cells expressing S.so-HSP20 showed greater thermotolerance in response to temperature shock (50°C, 4°C). By assaying enzyme activities, S.so-HSP20 was found to promote the proper folding of thermo-denatured citrate synthase and insulin B chain. These results suggest that S.so-HSP20 promotes thermotolerance and engages in chaperone-like activity during the stress response.

Keywords sHSP · S.so-HSP20 · *Sulfolobus solfataricus P2*

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Introduction

Archaea, one of the three major groups of life forms, are a highly diverse and abundant group that includes many "extremophiles" that thrive in harsh environments, such as hot springs, salt lakes, and submarine volcanic habitats (Madigan et al. 2000; Eckburg et al. 2003). Current knowledge on stress genes in archaea is considerably more limited than that for bacteria and eukaryotes. The stress response in archaea was first studied in 1980, but it was not until 1991 that an archaeal stress gene (hsp70) was first cloned and sequenced (Macario et al. 1991; Klumpp and Baumeister 1998). Since then, several studies have focused on stress genes and their expression in archaea (Macario et al. 1999), but many aspects of the archaeal stress response are yet to be studied. *Sulfolobus solfataricus P2* is a very widely studied crenarchaeal organism that grows well in the low pH, high sulfur environment of volcanic hot springs. It is used as a model organism in archaeal research to study processes such as DNA replication, cell cycle, chromosomal integration, and RNA processing (She et al. 2001; Nicholas et al. 2004). The optimum temperature for *S. solfataricus P2* is about 75°C, although it can live between 55°C and 90°C and in a pH range of 0.9–5.8, with its optimum pH being 2–3. The genome of *S. solfataricus P2* was sequenced in 2001. It consists of a single circular chromosome of 2,992,245 base pairs, with 3,032 genes encoding 2,977 proteins (Ehrnsperger et al. 1997; Jakob et al. 1993; chang et al. 1996). Heat shock proteins (HSPs) are ubiquitously expressed cellular proteins that form a major, conserved protein family. Some HSPs are expressed constitutively under physiological conditions (Lindquist and Craig 1998; Haslbeck et al. 2005), while others are induced in response to chemical or physical stressors such as heat, heavy metals, oxidative stress, ethanol (Charles et al. 1996; Laszlo and Li 1993; Sun et al. 2007).

Small heat shock proteins (sHSPs) are a family of stress-inducible molecular chaperones that range in size from 12 to 43 kDa (Arrigo 1998; Ehrnsperger et al. 1998; MacRae 2000; Van Montfort et al. 2001) and that form oligomers consisting 9 to 50 subunits (Boston et al. 1996; Waters et al. 1996; She et al. 2001). The ability of sHSPs to form oligomers contributes to their thermal stability and ability to avoid denaturation in response to high temperatures. sHSPs have chaperone-like activity in vitro and protect organisms from various stresses. At denaturing temperatures, sHSPs can prevent the aggregation of proteins by binding to, and forming a stable complex with, folding intermediates of their substrate proteins. In some cases, sHSPs can also promote renaturation of unfolded polypeptides (Brock et al. 1972; Esposito et al. 1998). In this study, we subcloned the coding sequence of the small heat shock protein, HSP20 (SSO2427) from the thermoacidophilic archaeon *S. solfataricus P2* into a bacterial expression vector and produced recombinant protein for structural and functional analysis. To investigate the level of HSP20 mRNA in *S. solfataricus P2*, we measured its mRNA expression profile in response to variations in culture temperature, including cold shock. *Escherichia coli* transformed with HSP20 was protected from a thermal stress of 50°C and cold stress of 4°C. Purified S.so-HSP20 behaved as a molecular chaperone by inhibiting the thermal aggregation of citrate synthase and insulin B chain. These results indicate that HSP20 plays an important role in the response to thermal stress.

Materials and methods

Culturing *S. solfataricus P2* at different temperature and cold-shock treatment

For the cultivation of *S. solfataricus P2*, a standard culture medium (yeast extract, 2.0 g; KH_2PO_4, 3.10 g; $(NH_4)_2SO_4$, 2.50 g; $MgSO_4 \times 7H_2O$, 0.20 g; $CaCl_2 \times 2H_2O$, 0.25 g; Distilled water, 1,000.00 ml) was used. The pH of the medium was adjusted to 3.5 at room temperature with 10 N H_2SO_4 prior to autoclaving. *S. solfataricus P2* was cultured at different temperatures (60°C, 75°C, 80°C). The cells were harvested during the log growth phase at an OD_{600}= 0.6. For cold-shock treatment, *S. solfataricus P2* was exposed to 4°C for 2 h after culturing at 75°C.

RNA extraction

All samples were homogenized in Trizol Reagent (Invitrogen, CA, USA), and total RNA was prepared according to the manufacturer's instructions. Total RNA was quantified on a Genova UV/visible spectrophotometer at 260 nm.

Measurement of S.so-HSP20 expression by quantitative real-time PCR

Complementary DNA was synthesized from 2 μg of total RNA from each sample using random decamer primers and Murine Molony Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA) in a 25-μl reaction. The expression pattern of S.so-HSP20 was analyzed in various samples using gene-specific primers HSP20RT-F, HSP20RT-R (Table 1) and the MiniOpticon TM System (Bio-Rad, USA). Each polymerase chain reaction (PCR) reaction mix contained 12.5 μl of Supermix, 10 μM primer HSP20RT-F, 10 M primer HSP20RT-R, and 1 μl RT-products. PCR products were detected using the iQ SYBR Green Supermix kit (Bio-Rad). A standard curve was constructed using tenfold serial dilutions (1:10^4, 1: 10^5, 1: 10^6, 1: 10^7, and 1: 10^8) of purified plasmids subcloned with S.so-HSP20 together with a non-template control. A standard curve for NusG (Samson et al. 2008, 2011) was constructed using a purified plasmid subcloned with the NusG gene fragment that had been amplified with primers NusGF and NusGR (Table 1). This plasmid was then diluted in a tenfold series (1:10^5, 1:10^6, 1:10^7, 1:10^8, and 1:10^9). All samples were analyzed in triplicate. Statistical significance was performed using the two-tailed paired Student's *t* test. S.so-HSP20 gene expression profiles obtained from real-time PCR were normalized with the NusG transcript to correct for differences in the starting amount of RNA and in the efficiency of the reverse transcription reactions. Melting curve analysis of the PCR reaction was performed to assess specificity.

Genomic DNA extraction and plasmid purification

S. solfataricus P2 was cultured at 75°C for 2 days using a standard culture medium. Cells were collected from 30 ml of culture by centrifugation at 4,200×g for 10 min. *S. solfataricus P2* genomic DNA and Plasmids (Table 2) were extracted and purified using EZ-10 Spin Column Genome DNA isolation Kit, EZ-10 Spin Column Plasmid Mini-

Table 1 Primers used in this work

Primer	Sequence(5′-3′)
HSP20F	CGggatccATGCCCAAGAGGGAAGAAAAGGATA
HSP20R	CCGctcgagTTACTCAACTTTTATGTCAACACCA
NusGF	GTGGTTGGAGGGCAAGAAATAA
NusG	GGGACCAGAAATTACTTCTACTACATC
HSP20RT-F	CAGACGGTGTGCCGAAAATAG
HSP20RT-R	TAGCCTTAGCCGCCTTCTCG

Lowercase letters indicate restriction endonuclease recognition sequences (BamHI, XhoI)

Table 2 Strains and plasmids used in this work

Strain or plasmid	Relevant genotype or features	Reference
Strains		
Sulfolobus solfataricus p2	*Sulfolobus solfataricus DSMZ1617*	DSMZ
E.coli BL21(DE3)	*E.coli* B (DE3)[F⁻dcm ompT hsdS_B ($r_B^-m_B^-$)]	Studier et al. 1990
E.coli P	*E.coli* BL21(DE3) with pET-28a	This work
E.coli H	*E.coli* BL21(DE3) with pET-28a-HSP20	This work
E.coli HR	*E.coli* DH5α with pUCm-T-HSP-RT	This work
E.coli NusG	*E.coli* DH5α with pUCm-T-NusG-RT	This work
Plasmids		
pET-28a-HSP20	pET28a(+) with HSP20*	This work
pUCm-T-HSP-RT	pUCm-T with HSP-RT DNA	This work
pUCm-T-NusG	pUCm-T with NusG-RT DNA	This work

*HSP20, NCBI reference sequence: NP_343781.1 (She et al. 2001)

Preps Kit and EZ-10 Spin Column Gel Extraction Kit (BIO BASIC INC). All samples were prepared according to the manufacturer's instructions and DNA concentration was measured using a Genova UV/visible spectrophotometer at 260 nm.

Cloning, expression, and purification of recombinant S.so-HSP20

The open reading frame of S.so-HSP20 was amplified from the chromosomal DNA of *S. solfataricus P2*. The forward primer (HSP20F) contained a BamH I site (indicated lowercase, Table 1) and the reverse primer (HSP20R) contained a unique XhoI site (indicated lowercase, Table 1) with stop codon. The PCR product was digested with BamHI and XhoI, and ligated into the pET-28a vector (Novogen, USA) digested with BamHI and XhoI, generating pET-28a-S.so-HSP20, which encodes S.so-HSP20 with an N-terminal His6 extension. pET-28a-S.so-HSP20 was transformed into *E. coli* BL21 (DE3), and His6-S.so-HSP20 was induced by addition of isopropyl-β-D-Thiogalactoside (IPTG; 1-mM final concentration) to exponentially growing cultures of *E. coli* (pET -28a-S.so-HSP20) in Lauria-Bertani (LB) medium containing 50 μg/ml of kanamycin. His6-tagged protein was purified by Ni-NTA (Qiagen, USA) according to the manufacturer's instructions. S.so-HSP20 was purified to homogeneity, based on Coomassie blue staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Thermotolerance experiments with transformed *E. coli*

For thermotolerance experiments, *E. coli* (pET-28a-S.so-HSP20) was cultured as described above. IPTG (1 mM) was added to mid-log phase cultures (OD_{600}=0.8), and the cells were incubated at 30°C for 2 h. The cultures were incubated at 50°C for 0, 15, 30, 45, and 60 min and at 4°C for 1, 2, 3, and 4 d. The cultures were then diluted to 5.0×10^6 cells/ml and 50 μl samples were plated in triplicate on LB plates containing 50 μg/ml of kanamycin. The plates were incubated overnight at 37°C before scoring colony formation to determine the percentage of survivors. For each treatment, the proportion of surviving *E. coli* colonies was calculated by dividing the number of viable cells per plate in the treated samples versus untreated samples (no exposure to heat or cold shock). All samples were analyzed in triplicate. Statistical significance was performed using the two-tailed paired Student's *t* test.

Assay of molecular chaperone activity

Citrate synthase (Sigma, USA) was diluted with 40 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) KOH buffer at pH 7.5 to a final concentration of 150 nM in 1.0-ml cuvettes and heated at 43°C with 150 nM or 300 nM of purified S.so-HSP20 or at 45°C with 150 nM of purified S.so-HSP20. Aggregation of citrate synthase was monitored by measuring turbidity at 360 and 320 nm with a SPECTRA Max PLUS spectrophotometer every 5 and 10 min for 1 h. Bovine serum albumin (BSA) was used at 150 nM to evaluate non-specific protection of citrate synthase (Chen et al. 2007). Chaperone-like activity of S.so-HSP20 was determined by measuring its ability to prevent dithiothreitol (DTT)-induced aggregation of insulin B chain in 50 mM Tris–HCl (pH 7.4) buffer with 0.15 M NaCl for 25 min at 40°C. The DTT was used at a final concentration of 20 mM. The turbidity of insulin B chain was measured using a SPECTRA max *PLUS* spectrophotometer at 360 nm. The ratio of S.so-HSP20 to insulin concentration for the assay was 1:2.

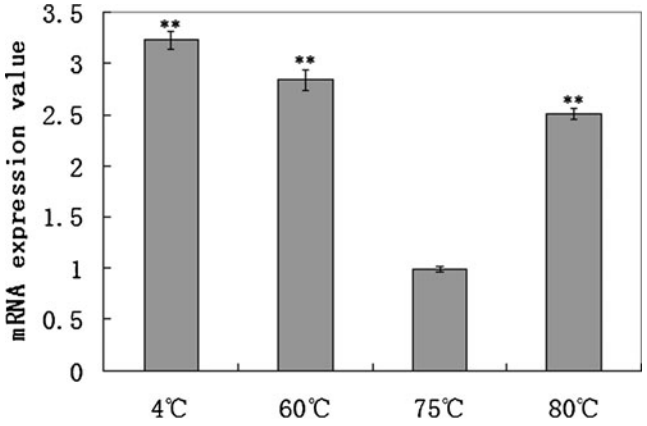

Fig. 1 S.so-HSP20 mRNA expression in *S. solfataricus P2* analyzed by real-time PCR. The level of S.so-HSP20 mRNA was measured in cells cultured at the indicated growth temperature or cold shock at 4°C for 2 h, and normalized against NusG (Internal control) levels. The mRNA expression value at 75°C was set to 1 and the other values were normalized against this. Data are representative of three trials and were expressed as the mean±SEM. Statistical significance was performed using the two-tailed paired Student's *t* test. (**$p<0.01$)

Results

S.so-HSP20 mRNA expression in *S. solfataricus P2* at different growth temperatures and under cold-shock conditions

The expression of S.so-HSP20 mRNA in *S. solfataricus P2* was measured at different culture temperatures (Fig. 1). S.so-HSP20 mRNA expression was strongly increased at culture temperatures far from the optimal growth temperature of 75°C. The increases in S.so-HSP20 mRNA expression at temperatures that were lower (60°C) or higher (80°C) than the optimum culture temperature (75°C) were 2.83- and 2.5-fold, respectively. S.so-HSP20 mRNA expression also increased 3.23-fold in response to cold-shock treatment (4°C).

Survival after thermal stress in *E. coli* expressing S.so-HSP20

To determine whether S.so-HSP20 could enhance the thermotolerance of *E. coli*, the effect of heat stress on the growth of *E. coli* cells transformed with S.so-HSP20 was examined. *E. coli* expressing S.so-HSP20 was more resistant to heat stress than cells containing only the pET-28a vector. As shown in Fig. 2, after exposure to 50°C, the survival of cells transformed with pET-28a only dropped to about 1.5% at 1 h, whereas that of the cells with pET-28a-S.so-HSP20 was 37.9%. Transformation with S.so-HSP20 also protected *E. coli* from exposure to cold (4°C), with survival recorded as 18.8% after 3-days cold exposure, compared to just 1.2% in *E. coli* transformed with pET-28a only. These results show that expression of S.so-HSP20 in *E. coli* cells increases cellular thermotolerance.

S.so-HSP20 exhibits chaperone activity in vitro

A key characteristic of molecular chaperones is their ability to suppress the aggregation of non-native proteins. To test whether S.so-HSP20 exhibits chaperone activity, we performed thermal unfolding assays using citrate synthase and insulin B chain as model substrates (Figs. 3 and 4). Purified S.so-HSP20 effectively protected citrate synthase against heat-induced denaturation. At concentrations of 150 or 300 nM, S.so-HSP20 reduced heat-induced turbidity resulting from incubation at 43°C for 60 min by 48.1% and

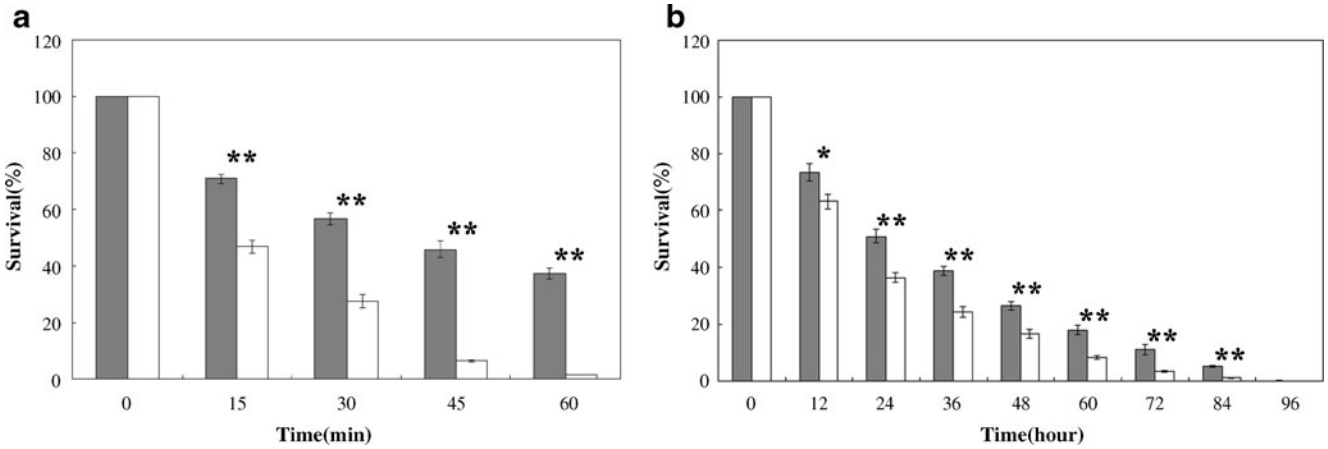

Fig. 2 Thermotolerance assays were performed at 50°C (**a**) and 4°C (**b**). *E. coli* P (with pET-28a) and *E. coli* H (with pET-28a-S.so-HSP20) were cultured as described and subjected to 50°C treatment for up to 60 min and 4°C treatment for up to 4 days. After heat or cold treatment, samples were taken at the time indicated, diluted, and immediately plated on LB plates with kanamycin. Survival of *E. coli* H cells (*black boxes*) and *E. coli* P cells (*open boxes*) are shown, and are expressed as a percentage of the survival obtained in LB plates. Data are representative of three trials and were expressed as the mean±SEM. Statistical significance was performed using the two-tailed paired Student's *t* test. (*$p<0.05$; **$p<0.01$)

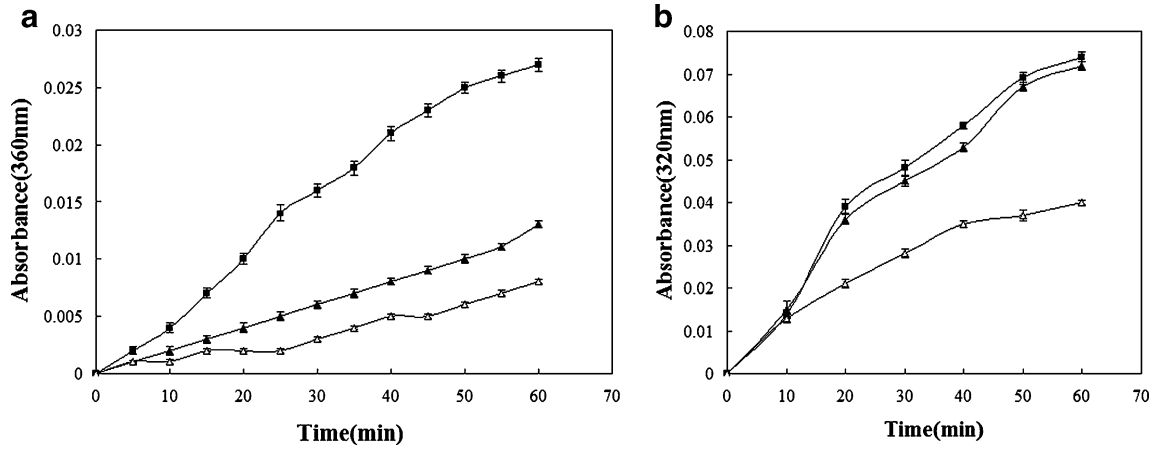

Fig. 3 S.so-HSP20 prevents heat-induced denaturation of citrate synthase. Purified S.so-HSP20 was incubated at 43°C (**a**) with 150 nM citrate synthase, and turbidity was measured at 360 nm. S. so-HSP20 concentrations were 0.0 nM (*black square*), 150 nM (*black triangle*), 300 nM (*open triangle*). S.so-HSP20 was also incubated at 45°C (**b**) with 150 nM citrate synthase, and turbidity was measured at 320 nm. The S.so-HSP20 concentration was 0.0 nM (*black triangle*) or 150 nM (*open triangle*). BSA at 150 nM (*black square*) was used as a control. Data are representative of three trials and were expressed as the mean±SEM

70.4%, respectively (Fig. 3a). Similarly, heat-induced turbidity resulting from a 60-min incubation at 45°C was reduced by S.so-HSP20 by 38.3% (Fig. 3b). Heat-induced turbidity of the insulin B chain was reduced by S.so-HSP20 by 47% after 24 min at 40°C (Fig. 4). These results demonstrate that S.so-HSP20 exhibits chaperone activity in vitro.

Discussion

In the present study, we examined the thermotolerance and molecular chaperone properties of *S. solfataricus P2* sHSP, HSP20. The coding region of the HSP20 gene of the thermoacidophilic archaeon, *S. solfataricus P2*, was cloned into a plasmid vector and overexpressed in bacteria. The recombinant protein was used for structural and functional analysis. We analyzed mRNA expression of HSP20 in response to different culture temperatures. Many sHSPs are not constitutively expressed, and their expression is induced specifically in response to stress conditions, such as elevated temperatures. We found that the expression of S. so-HSP20 mRNA is markedly increased following culture of *S. solfataricus P2* at temperatures far from the optimal growth temperature. This result suggests that S.so-HSP20 mRNA expression is stress-induced, and may protect *S. solfataricus P2* from stress. Expression of S.so-HSP20 in *E. coli* also protected these cells from temperature stress including cold shock. *E.coli* HSPs have been suggested to play a major role in protecting cells from thermal killing. The function of S.so-HSP20 in *E. coli* may be to promote thermotolerance by enhancing protein synthesis in *E. coli* (including HSPs) or by protecting *E. coli* proteins from heat denaturation through its chaperone activity. We demonstrated chaperone activity for S.so-HSP20 of *S. solfataricus P2* at physiological and heat shock temperatures. The ability of S.so-HSP20 to protect against heat-induced aggregation of citrate synthase (Keisuke et al. 2001; Ding et al. 2008; Martin et al. 2008; Angelo et al. 2004) and insulin B chain (Azzoni et al. 2004; Satoru 2006) indicates that S.so-HSP20 possesses chaperone activity and the ability to recognize and bind unfolded proteins to prevent their aggregation in vitro. The relationship between cellular thermotolerance and S.so-HSP20 expression in an *E. coli* expression system has not previously been reported. The results of this study provide evidence that S.so-HSP20 can protect *E. coli* from thermostress and behave as a chaperone in vitro. Future studies will develop an S.so-HSP20 expression system in *Sacharomyses cerevisiae*, and will assess the ability of S.so-HSP20 to regulate apoptosis.

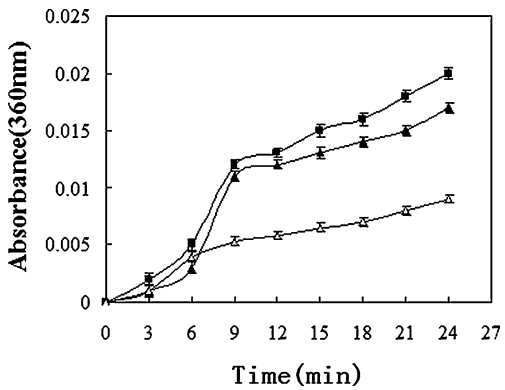

Fig. 4 S.so-HSP20 has chaperone-like activity. Chaperone-like activity of S.so-HSP20 was determined by measuring turbidity during DTT-induced aggregation of the insulin B chain (0.2 mg/ml) for 24 min at 40°C; insulin B chain alone (*black triangle*), insulin B chain in the presence of 0.2 mg/ml BSA (*black square*), or the insulin B chain in the presence of 0.1 mg/ml S.so-HSP20 (*open triangle*). Data are representative of three trials and were expressed as the mean±SEM

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