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Aspartyl aminopeptidase of *Schizosaccharomyces pombe* has a molecular chaperone function

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To screen chaperone proteins from *Schizosaccharomyce pombe* (*S. pombe*), we prepared recombinant citrate synthase of the fission yeast as a substrate of anti-aggregation assay. Purified recombinant citrate synthase showed citrate synthase activity and was suitable for the substrate of chaperone assay. Several heat stable proteins including aspartyl aminopeptidase (AAP) for candidates of chaperone were screened from the supernatant fraction of heat-treated crude extract of *S. pombe*. The purified AAP migrated as a single band of 47 kDa on SDS-polyacrylamide gel electrophoresis. The native size of AAP was estimated as 200 kDa by a HPLC gel permeation chromatography. This enzyme can remove the aspartyl residue at N-terminus of angiotensin I. In addition, AAP showed the heat stability and protected the aggregation of citrate synthase caused by thermal denaturation. This study showed that *S. pombe* AAP is a moonlight protein that has aspartyl aminopeptidase and chaperone activities. [BMB reports 2009; 42(12): 812-816]

INTRODUCTION

Chaperones protect cells from damage caused by protein denaturation under environmental stress such as heat shock. Chaperones also function in proper folding of proteins during or after translation and targeting to cell organelles such as mitochondria (1, 2). Many heat shock proteins show chaperone activity. Small heat shock proteins having a molecular size between 10 kDa and 40 kDa show the chaperone activity protecting irreversible aggregation of protein by heating (3, 4). They can reversibly bind to partially unfolded polypeptide chains. Small heat shock proteins, generally, have an α-crystallin domain and are in various oligomeric states (5-7).

Recently, chaperone activity was reported for some proteins which were known to have other biological functions. Thioredoxin from *E. coli* have 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 (8). Tsa1 from *Saccharomyces cerevisiae* (*S. cerevisiae*) also showed strong chaperone activity, which is known to a peroxidase using thioredoxin as an electron donor (9). In addition, some amiopeptidases including methionine aminopeptidase II from *Xenopus* ovary, Hsp 31 form *E. coli* and aminopeptidase A from CHO cells showed chaperone activity (10-12). There was no α-crystalline domain in thioredoxin, Tsa1, and those aminopeptidases. Methionine aminopeptidase II from *Xenopus* ovary acts as a molecular chaperone for sarcoplasmic reticulum calcium ATPase that is required for correct folding of the methionine aminopeptidase II (10). C-terminal domain of aminopeptidase A from a Chinese hamster ovary also acts as an intramolecular chaperone required for correct folding of N-terminal domain and this correct folding provides the activity of the monozinc aminopeptidase (11). On the other hand, *E. coli* Hsp31 chaperone protein is an aminopeptidase of broad specificity. The aminopeptidase cleavage preference of Hsp31 is Ala > Lys > Arg > His (12).

It is well known that many kinds of chaperones are equipped in the cell. Thirty-two genes among 2,449 genes in *S. pombe* show Homology to heat shock proteins. There were a few functional studies of the fission yeast chaperone such as calnexin (13), cdc37 (14), and Hsp90/Git10 (15), Rsp1p (16), ASF1/CIA1 (17). In our lab, for screening of molecular chaperones without an α-crystalline domain from *S. pombe*, heat stable proteins were screened from the fission yeast and examined their anti-aggregation activity using the yeast citrate synthase. We report that *S. pombe* citrate synthase was suitable for substrate of chaperone assay. In addition, we showed several heat stable proteins with MALDI-TOF and recombinant aspartyl aminopetidase, one of the heat-stable proteins, showed both a molecular chaperone activity and aspartyl aminopeptidase activity.

RESULTS AND DISCUSSION

S. pombe citrate synthase as a substrate of chaperone assay

In chaperone assay, citrate synthase has been used as a model protein substrate to determine the anti-aggregation function of various molecular chaperones (18). To use *S. pombe* citrate

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synthase (Q10306, NP_593718, SPAC6C3.04) as a protein substrate for chaperone assay, we prepared the mature form of citrate synthase using an *E. coli* expression system. The recombinant citrate synthase were separated using DEAE-Sephacel ion exchange chromatography. The citrate synthase activity was eluted at 0.2 M NaCl concentration from a conventional DEAE-Sephacel column as shown in Fig. 1A. The major fractions showing the enzyme activity contained almost one protein band estimated at 49 kDa on the SDS-PAGE gel (Fig. 1B).

Citrate synthase activity was measured by DTNB reduction with CoASH generated by enzymatic reaction (Fig. 1C). There was a dose-dependent increase in the activity of the recombinant citrate synthase protein (data not shown). The specific activity of the purified citrate synthase was 2.6 μmol/min/mg. To investigate the suitability of recombinant citrate synthase as a substrate for chaperone assay, a light scattering at 360 nm caused by aggregation of the protein was monitored using a

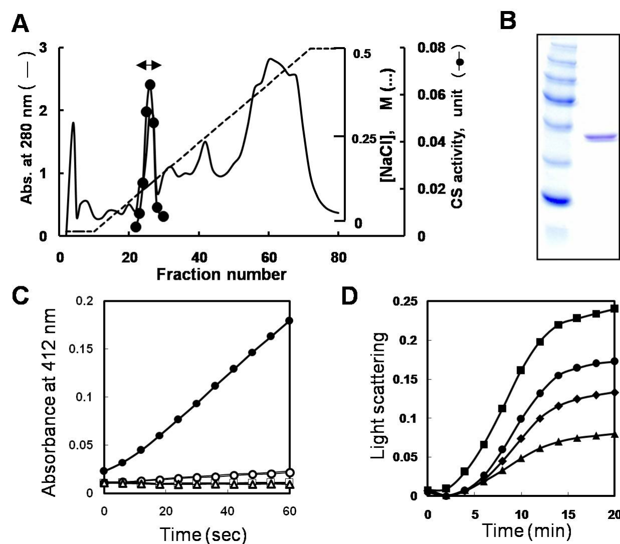

Fig. 1. Purification of *S. pombe* citrate synthase expressed in *E. coli* by a DEAE-Sephacel column chromatography and the measurement of citrate synthase activity. (A) Crude extract of *E. coli* expressed with citrate synthase was applied to a DEAE-Sephacel column equilibrated with 20 mM Tris-HCl (pH 7.4). Proteins were eluted at a flow rate of 2.5 ml/min by a linear NaCl gradient from 0 to 0.5 M. Citrate synthase activity was assayed by monitoring an increase of absorbance at 412 nm. (B) The purified citrate synthase was analyzed 12.5% reducing SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Molecular size standards are 140, 100, 70, 50, 24, and 20 kDa. (C) The citrate synthase reaction mixture contained 10 mM oxaloacetate, 10 mM DTNB, 10 mM acetyl-CoA, and 1.6 μM (●), 3.2 μM (■) and 4 μM (▲) citrate synthase in 50 mM HEPES (pH 7.0). The reaction was started by the addition of oxaloacetate and monitored a change of absorbance at 412 nm. (D) Thermal aggregation of citrate synthase was monitored by light scattering at 360 nm at 43°C. The assay mixture contained 0.5 μM (▲), 0.8 μM (◆), 1 μM (●) and 1.5 μM (■) of citrate synthase in 50 mM HEPES (pH 7.0).

spectrophotometer equipped with a thermostatic cell holder. As shown in Fig. 1D, the dose dependent aggregation of the citrate synthase at 43°C was observed. The *S. pombe* citrate synthase was a suitable substrate as a chaperone activity. According to those results, 1 μM of citrate synthase was used to measure the chaperone activity.

Screening of heat-stable proteins and preparation of recombinant aspartyl aminopeptidase of *S. pombe*

To find out the *S. pombe* heat-stable protein, crude extract of *S. pombe* was heat-treated at 70°C for 30 min. After centrifugation for 30 min, proteins in the supernatant were separated by a conventional DEAE-Sephacel and then HPLC gel filtration columns. The fractions showed the chaperone activity were pooled and separated on SDS-PAGE. Coomassie Blue stained protein bands were analyzed by MALDI-TOF Mass spectroscopy. Some proteins including heat shock protein 70 Homolog precursor (Hsp 70, NP_592867.1, SPAC1F5.06), alcohol dehydrogenase I (NP_588244, SPCC13B11.01), thioredoxin peroxidase (NP_588430, SPCC576.03c) and putative aspartyl aminopeptidase (AAP, O36014, NP_594745, SPAC4-F10.02) were well matched with *S. pombe* proteins (data not shown). In *E. coli*, heat stable proteins including chaperone Hsp70 (Dnak), histidine-binding periplasmic protein (HBP) and hypothetical protein YjgF were reported (19). AAP screened as a heat-stable protein showed sequence Homology to aspartyl aminopeptidase purified from rabbit brain cytosol and *S. cerevisiae*. Amino acid sequence of *S. pombe* AAP showed 50.2% identity to that of *S. cerevisiae* aspartyl aminopeptidase (YHR113W) and 46.9% identity to *Homo sapiens* aspartyl aminopeptidase (Q9ULA0). It was reported that some aminopeptidase including *Xenopus* methionine aminopeptidase II, *E. coli* Hsp 31, and Chinese hamster aminopeptidase A showed chaperone activity (10-12). There is no α-crystallin domain in those AAPs. To investigate the heat-stability and chaperone activity of *S. pombe* AAP, cDNA of AAP was cloned and overexpressed in *E. coli*. The recombinant AAP were separated using an anion-exchange chromatography. The AAP protein was eluted at 0.25 M NaCl concentration from a DEAE-Sephacel column (Fig. 2A). Proteins in fractions containing AAP were applied phenyl hydrophobic interaction chromatography. The recombinant AAP was not bound to the hydrophobic column (Fig. 2B). To get a high purity AAP protein, we loaded the fractions that eluted from phenyl column to gel filtration chromatography (Fig. 2C). The protein eluted gel filtration column showed a single band estimated as 47 kDa on the SDS-PAGE gel (Fig. 2D).

Aminopeptidase activity of AAP

To confirm the aspartyl aminopeptidase activity of recombinant AAP, angiotensin I was used as a substrate and the degradation of angiotensin I with AAP was analyzed by MALDI-TOF Mass spectroscopy (20). After reaction with recombinant AAP, the relative peak height of the product peptide with a mass of

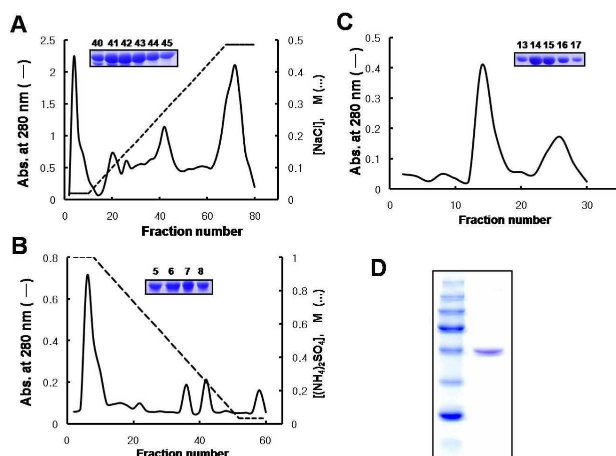

Fig. 2. Purification of aspartyl aminopeptidase expressed in *E. coli*. by conventional DEAE-Sephacel chromatography (A) and HPLC using TSK Phenyl 5-PW (B) and gel filtration (C) columns. (A) Crude extract of *E. coli* expressed with AAP was applied to DEAE-Sephacel column that had been equilibrated with 20 mM Tris-HCl (pH 7.4). The proteins were eluted at a flow rate of 2.5 ml/min by a linear NaCl gradient from 0 to 0.5 M. (B) Protein sample was applied to a TSK Phenyl 5-PW that had been equilibrated with 20 mM HEPES-NaOH (pH 7.0) and 1 M $(NH_4)_2SO_4$. The proteins were eluted at a flow rate of 1 ml/min by decreasing $(NH_4)_2SO_4$ gradient from 1 to 0 M. (C) Protein sample was applied to a HPLC gel filtration column that had been equilibrated with 100 mM sodium phosphate buffer (pH 7.0) and 0.15 M NaCl. The proteins were eluted at a flow rate of 0.5 ml/min. (Inset of A, B, and C) The 15 μl of each fraction was separated by 12.5% SDS-PAGE and then visualized with Coomasie Brilliant Blue R-250. (D) The purified AAP protein was separated 12.5% reducing SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Molecular size standards are 140, 100, 70, 50, 24 and 20 kDa.

1181.4 corresponding to the peptide removed N-terminal Asp residue was increased and angiotensin I peak (m/z = 1296) was dramatically decreased (Fig. 3). These data showed the recombinant AAP removed aspartic acid residue at N-terminus. For *S. cerevisiae* AAP, the loss of aspartic acid at the N-terminus of angiotensin I was observed by the same method during incubation of the substrate with the AAP (20). This result suggests that AAP has the aspartyl aminopeptidase activity.

Chaperone function of AAP

Chaperone proteins show resistance against denaturation by heating. The heat-stability of AAP was examined. AAP was not precipitated by heat treatment for 30 min at 60°C. Only less than 10% of AAP protein was precipitated by heating at 70°C. Those data showed AAP had a feature of heat stable protein. To examine the chaperone activity of AAP, the ability to inhibit the thermal aggregation of the citrate synthase was investigated. AAP efficiently suppressed the thermal aggregation of citrate synthase at 43°C. Incubation of citrate synthase with increasing amounts of AAP resulted in a concomitant decrease in citrate synthase aggregation, and at a subunit molar ratio of

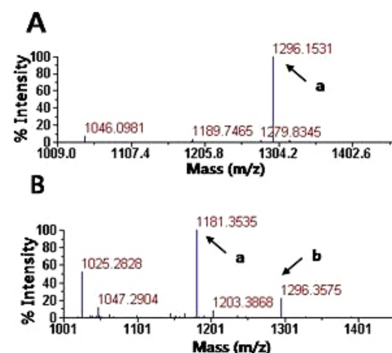

Fig. 3. Aspartyl aminopeptidase activity of *S. pombe* AAP. Reaction mixture containing HEPES-NaOH (pH 7.0), 0.05 mM angiotensin I, and *S. pombe* AAP (B) or not (A) was incubated at 37°C for min. The reaction was stopped by the addition of acetic acid and was analyzed by MALDI-TOP mass spectroscopy. Letters of a and b indicate the peak of angiotensin I and the product removed Asp residue from angiotensin I by AAP.

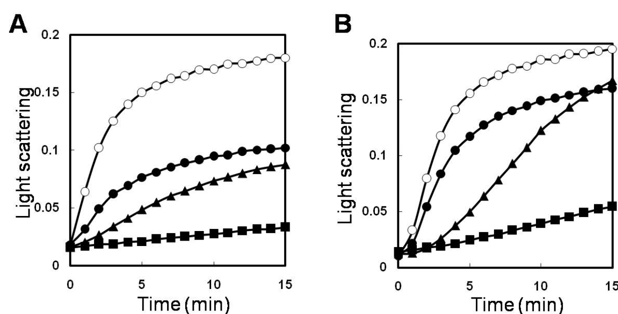

Fig. 4. The molecular chaperone function of AAP. Chaperone activity of AAP (A) and pre-heated AAP (B) was measured to protect the thermal aggregation of citrate synthase in 50 mM HEPES-NaOH (pH 7.0) at 43°C. Substrate aggregation was monitored by measuring the light scattering at 360 nm. Chaperone activity was measured in the absence (○) or presence of AAP at ratios of AAP to citrate synthase of 1 : 1(●), 1 : 2(▲), 1 : 4(■).

AAP to citrate synthase of 4 : 1, citrate synthase aggregation could be completely inhibited (Fig. 4A). The AAP chaperone activity was about 30- and 6-fold stronger than that of HSP16.5 of *Methanococcus jannaschi* (21) and α-crystallin, respectively. Moreover, it has been reported that a 16-fold molar excess of goat tublin and 30 moles of HSP16 in *Caenorhabditis elegans* are required to protect one mole of CS (22). Therefore, the AAP chaperone activity was about 4- and 8-fold stronger than that of goat tublin and HSP16, respectively. However, compared with the yeast Tsa1, a powerful chaperone, this AAP was shown as a half chaperone activity of Tsa1 (9).

Anti-aggregation activity of molecular chaperones such as small heat shock proteins are increased by heat treatment (23). To test whether the molecular chaperone activity of AAP was changed by heat-treatment, the protein was pre-treated at 60°C

for 30 min. The chaperone activity of AAP was not increased by heat treatment. Heat-treated AAP even showed less activity compared to the native protein (Fig. 4B). Those results showed that the characteristics of the chaperones of AAP were different from those of general small heat shock proteins.

It was reported that the oligomeric state of small heat shock proteins was important to anti-aggregation activity of the chaperones. To investigate the native oligomeric state of AAP and the influence of heat treatment, protein samples were analyzed by HPLC gel filtration chromatography. AAP without treatment of heat was estimated at approximately 200 kDa and it seems to be that AAP was a tetrameric protein. *S. pombe* AAP was smaller than the AAP from *Saccharomyces cerevisiae*. The budding yeast AAP is comprised of 12 identical subunits with a size of 56 kDa (24). When AAP was heat-treated at 50 or 60°C for 30 min, elution profiles from a gel permeation column didn't show any change. However, at 70°C, AAP showed high molecular weight peaks (data not shown). The change in chaperone activity and oligomeric state was not observed for *S. pombe* AAP. Therefore AAP is a molecular chaperone with a oligomeric state distinct from that of the general small heat shock proteins.

In conclusion, we confirmed the aspartyl aminopeptidase activity of *S. pombe* AAP and found out its molecular chaperone properties which differ from small heat shock proteins in conserved domain and oligomeric states.

MATERIALS AND METHODS

Construction of *Escherichia coli* expression vectors

The DNA fragment corresponding to the mature form of *S. pombe* citrate synthase (NP_593718, GI:19115657) started from the 23rd amino acid to the last amino acid and aspartyl aminopeptidase (AAP, NP_594745, GI:19114630) were obtained by a polymerase chain reaction from the *S. pombe* cDNA. The forward and reverse primers for citrate synthase were 5'-CATATGTCCCGTCAATTAAACCCCCTT-3' and 5'-GGATCCTCATTTCGTTTCAACCATCTT-3', respectively (*Nde*I and *BamH* I sites are underlined). The forward and reverse primers for AAP were 5-CATATGACTGCCACTGCAAAAAGT-3 and 5'-GGATCCCTATTCATCAATAATGAT-3', respectively (*Nde*I and *BamH* I sites are underlined). PCR products were ligated into the Topo TA cloning vector pCR2.1 (Invitrogen, USA), yielding pCR2-CS and pCR2-AAP, respectively. Each recombinant plasmid was confirmed by amino acid sequence analysis (Korea Basic Science Institute in Gwangju). The *Nde*I-*BamH* I fragments from both plasmids were subsequently subcloned into the *E. coli* expression vector pET17b (Novagen, USA), thereby generating pET17b-CS and pET17b-AAP, respectively.

Preparation of recombinant proteins

To purify recombinant proteins, transformed *E. coli* BL21(DE3) cells transformed with pET17b-CS or pET17b-AAP were cultured in 5 L of Luria-Bertani broth, and protein expression was induced by culturing with 1 mM isopropyl-1-thio-D-galactoside (IPTG) for 4 hrs. *E. coli* cells were harvested by centrifugation, resuspended in 10 volume of 20 mM Tris-HCl (pH 7.5) buffer and then disrupted by ultrasonication. Cell debris was removed by centrifugation at 10,000 rpm. The crude extract was loaded onto a DEAE-Sephacel column preequilibriated with 20 mM Tris-HCl (pH 7.5) buffer. After washing with the same buffer, proteins were eluted with a linear gradient of 0 to 500 mM NaCl in 20 mM Tris-HCl (pH 7.5) buffer. Fractions eluted from the DEAE-Sephacel column were analyzed by SDS-PAGE.

The fraction containing AAP protein was precipitated by the addition of ammonium sulfate to be 75%. The precipitate obtained by centrifugation was dissolved in 50 mM HEPES buffer, pH 7.0 containing 1 M ammonium sulfate and the supernatant was applied to the TSK phenyl-5PW HPLC column preequilibriated with the same buffer containing 1 M ammonium sulfate. The protein was eluted with a linear gradient of 1 to 0 M ammonium sulfate in 0 mM HEPES buffer, pH 7.0. Major fractions containing AAP were pooled and concentrated. The purified recombinant proteins were stored at −70°C until use.

Thermal aggregation of citrate synthase

A reaction mixture containing protein samples in 50 mM HEPES (pH 7.0) was preincubated for 5 min at 43°C and then after the addition of citrate synthase to be 1 μM. A light scattering due to thermal aggregation of citrate synthase was monitored at 360 nm by a spectrophotometer equipped with a thermostatic cell holder (2).

Size exclusion chromatography

The native size of protein was determined by a HPLC gel filtration. The protein sample solution was injected into a gel filtration column (Bio-Sil SEC-250) using HPLC. The elution buffer contained 150 mM NaCl in 50 mM sodium phosphate buffer (pH 7.0) and the flow rate was 0.5 ml/min.

Citrate synthase activity assay

The citrate synthase activity was determined with the increase of absorbance at 412 nm caused by the reduction of DTNB by CoASH produced by enzymatic reaction. A reaction mixture of 480 μl containing 10 mM acetyl-CoA, 10 mM oxaloacetate, 1 mM DTNB in 50 mM HEPES buffer (pH 7.0) was preincubated at 25°C for 5 min and reaction was started by addition of citrate synthase (25).

Aspartyl aminopeptidase activity

Angiotensin I was used as a substrate for the detection of aspartyl aminopeptidase activity. Angiotensin I and its product generated by the enzymatic reaction were identified by MALDI-TOF mass spectroscopy. A reaction mixture containing 5 μg of AAP and angiotensin I in 20 mM HEPES (pH 7.0) was incubated for 1 min. The reaction was stopped by the addition of 50% acetic acid (20).

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