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Journal of Chromatography B, 786 (2003) 109–115
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Recombinant expression and purification of Ssa1p (Hsp70) from *Saccharomyces cerevisiae* using *Pichia pastoris*

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Abstract

Heat shock proteins with a molecular mass of 70 000 (Hsp70s) are a ubiquitous class of ATP-dependent molecular chaperones involved in the folding of cellular proteins. Sequencing the entire genome of *Saccharomyces cerevisiae* revealed 14 different genes for Hsp70 proteins in different cellular compartments. Among these 14 Hsp70s, the subclass of Ssa (Ssa1p–Ssa4p) is abundant and essential in the cytosol. Since high yield expression of cytoplasmic Ssa1p is inefficient in *Saccharomyces cerevisiae* and recombinant expression in *E. coli* yields low protein levels, we chose *Pichia pastoris* as the recombinant expression system. In *Pichia pastoris*, expression levels of Ssa1p are high and Ssa1p is soluble and correctly folded. Also, we present a new protocol for purification of Ssa1p. Previously described purifications include ATP-agarose chromatography leading to Ssa1p partially complexed with ATP. Our optimized purification protocol follows the CiPP strategy (capture, intermediate purification, polishing) avoiding ATP-agarose chromatography, which allows detailed studies on the ATP-dependent Hsp70 functions. We obtained Ssa1p in high purity and 400 times higher quantity compared to previous studies.

Keywords: Purification; Ssa1p; Hsp70; ATPase

1. Introduction

Prokaryotes and eukaryotes respond to a sudden change of temperature and other adverse environmental conditions with increased expression of a set of proteins collectively referred to as heat shock proteins. The expression of these proteins contributes to the repair of cells under stress conditions [1,2]. One of the most abundant families of heat shock proteins are the Hsp70s. Proteins of the Hsp70 class have a molecular mass of 70 000. They consist of a highly conserved N-terminal ATPase domain (M_r = 44 000), a less well conserved peptide-binding domain (M_r = 18 000) and a C-terminal variable domain (M_r = 10 000) of unknown function [3,4]. Hsp70s function in a diverse set of processes, including protein folding, protein activation, protein translocation across membranes and heat shock response regulation [5,6].

Strikingly, all eukaryotic cells contain a set of different Hsp70s to carry out this variety of functions. In the yeast *Saccharomyces cerevisiae* sequencing the entire genome predicted 14 different Hsp70s. Nine of the 14 yeast Hsp70 proteins are located in the cytosol and can be divided into the subfamilies Ssa, Ssb, Sse and Ssz (Ss denotes stress seventy related; a, b, e and z indicate the subfamily). The Ssa subfamily comprises four members (Ssa1p–

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1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(02)00724-9

Ssa4p). Expression of at least one Ssa is essential for viability making Ssas the only essential cytosolic family [7]. Ssa1p–4p have a molecular mass of 70 000 and a sequence identity of 80% among each other. At 23 °C, Ssa1p is expressed at high levels, but it is induced approximately 10-fold after a temperature shift to 37 °C. In contrast, Ssa2p is expressed at a high level at all temperatures. Ssa3p and Ssa4p are expressed at extremely low levels at 23 °C, but the amounts of Ssa3p and Ssa4p increase dramatically upon elevated temperature [8].

Hsp70 proteins are involved in the folding of polypeptide chains in an ATP-dependent manner. Cycles of substrate binding and release are coupled to ATP-binding, hydrolysis and nucleotide exchange [9,10]. In its ATP-bound form, eukaryotic Hsp70 has a low affinity for substrate proteins, whereas in the ADP-bound form, it exhibits a high substrate binding affinity. Therefore, ATP-hydrolysis converts Hsp70 from the ''fast-binding, fast-release'' to the ''slow-binding, slow-release'' form, stabilizing the interaction between Hsp70 and its substrate [9,11–13]. The Ssa1p ATPase is similar to those of other Hsp70s studied, with a K_m for ATP of 0.2 μM and a k_{cat} of 0.04 min^{-1} at physiological potassium concentrations [14]. This ATPase activity is regulated by a set of cochaperones [15]. In yeast, the cochaperones Ydj-1 and Sis1, which belong to the Hsp40 family, stimulate the ATPase activity of Ssa1p approximately 10-fold [16–18].

In order to investigate Ssa1p, which has the highest similarity to other eukaryotic cytosolic Hsp70s, we employed a high level expression system to ease investigations on the purified protein. We chose the *Pichia pastoris* expression system for Ssa1p, since overexpression in *Saccharomyces cerevisiae* and recombinant expression in *E. coli* have been shown to be inefficient (Ref. [16] and data not shown). *Pichia pastoris* is closer related to higher eukaryotes than *Saccharomyces cerevisiae* concerning protein processing, protein folding and post-translational modification. Since hyperglycosylation, which occurs in *Saccharomyces cerevisiae*, is not observed, the glycomoiety resembles the glycoprotein structure of higher eukaryotes more closely [19,20]. *Pichia pastoris* is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source [21]. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxidase. In addition to formaldehyde, hydrogen peroxide is generated [22]. To avoid hydrogen peroxide toxicity, methanol metabolism takes place within the peroxisome, a specialized cell organelle which separates toxic by-products from the rest of the cell. Since alcohol oxidase has a poor affinity for O_2, *Pichia pastoris* upregulates the enzyme in the presence of methanol as the sole carbon source [22]. The involved promoter (P_{AOX1}) is used to control Ssa1p expression in *Pichia pastoris* and yields a high expression in the presence of methanol.

Further, we developed a three-step chromatographic purification protocol, which allows to obtain Ssa1p in high quantities and in an ATP-free form essential for analyzing ATP-dependent Hsp70 functions.

2. Experimental

2.1. Chemicals

All buffer substances were from ICN Biochemicals (Costa Mesa, USA). Protease inhibitors (Complete tablets without EDTA) were from Roche Diagnostics (Mannheim, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany).

2.2. Equipment

Cells were lysed using a cell disruption system from Constant Systems (Warwick, UK) and an ultrasonicator B-12 from Branson (Sonic Power Company, USA). All chromatography steps were performed by FPLC (Amersham Biosciences, Uppsala, Sweden) using DEAE–Sephacel, butyl-Sepharose and a prepacked Superdex 200 prep grade (Amersham Biosciences, Uppsala, Sweden). Ssa1p was concentrated by ultrafiltration with a YM30 membrane (Millipore, Bedford, MA, USA).

2.3. Yeast strains and media

The *Saccharomyces cerevisiae* strain MW141 [16] was used for the expression of Ssa1p in *Sac-*

charomyces cerevisiae. The *Pichia pastoris* strain GS 115 (Invitrogen, Groningen, The Netherlands) was used for the expression of Ssa1p in *Pichia pastoris*. GS 115 was grown in dextrose histidine medium (MDH) (1.34% (w/v) yeast nitrogen base, 0.4% (w/v) L-histidine, 2% (w/v) glucose, 4×10^{-5}% biotin). Expression of Ssa1p was induced with methanol in synthetic histidine medium (MMH) (1.34% (w/v) yeast nitrogen base, 0.4% (w/v) L-histidine, 4×10^{-5}% biotin, 0.5% (v/v) methanol).

Preparation of yeast genomic DNA from strain W303 was performed as described [23].

2.4. Construction of the Ssa1 expression plasmid

PCR primers were designed to amplify *SSA1* using yeast genomic DNA (strain W303) as a template with the appropriate restriction sites (*Xho*I and *Bam*HI) linked to the 5′ and 3′ end primers, respectively. The PCR product was gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and digested for 2 h with the restriction enzymes *Xho*I and *Bam*HI (Roche, Mannheim, Germany). The pPICZB vector (Invitrogen, Groningen, The Netherlands) containing a zeocin resistance was amplified in the *E. coli* strain DH10B. After isolation the vector was digested with the restriction enzymes *Xho*I and *Bam*HI over night and finally dephosphorylated using calf intestinal phosphatase (Roche, Mannheim, Germany) for 2 h at 37 °C. The digested *SSA1* PCR product and the pPICZB vector were ligated for 16 h at 4 °C using T4 ligase (Promega, Madison, WI, USA). The sequence identity to Ssa1 was confirmed by DNA sequencing.

2.5. Expression of Ssa1 in GS 115

The *Pichia pastoris* strain GS 115 was transformed with pPICZB-Ssa1 using the lithium acetate method according to the manufacturers protocol (Invitrogen). Transformants were selected on MDH_{Zeo} plates. For large scale expression of Ssa1p, a pre-culture of GS 115 pPICZB-Ssa1 was grown in dextrose histidine medium supplemented with 25 μg zeocin per ml (MDH_{Zeo}) at 30 °C. Expression of Ssa1p was induced by a shift to MMH at an optical density (600 nm) of 1.0. After 12 h of incubation at 30 °C, cells were harvested (1000 *g*, 15 min, 4 °C) and the cell pellet resuspended and washed with 50 ml Standard buffer I (20 m*M* Hepes pH 7, 10 m*M* KCl, 5 m*M* $MgCl_2$, 50 m*M* NaCl, 5 m*M* β-mercaptoethanol, 5% glycerol). The cell pellet was frozen in liquid nitrogen and stored at −20 °C.

2.6. Cell disruption

After thawing, the cell pellets were resuspended with 50 ml of Standard buffer I. Cells were lysed with a cell disruption system in the presence of protease inhibitors to inhibit proteolytic cleavage. To shear DNA, the lysate was ultrasonicated (3×15 s on ice). Finally, insoluble and soluble material was separated by centrifugation (40 000 *g*, 45 min, 4 °C).

2.7. Chromatographic purification

Purification of Ssa1p from *Saccharomyces cerevisiae* was performed as described elsewhere [16]. Purification of Ssa1p from *Pichia pastoris* was performed as described in the following. All purification steps were carried out at a flow-rate of 1.0 ml/min at 4 °C. First, a 70-ml DEAE–Sephacel column (Amersham Biosciences, Uppsala, Sweden) was equilibrated with Standard buffer I. After loading the cell lysate, bound Ssa1p was eluted with a linear gradient from 50 m*M* to 1 *M* NaCl. The Ssa1p containing fractions were saturated with 40% ammonium sulfate. After centrifugation, the supernatant was applied on a 10-ml butyl-Sepharose column (Amersham Biosciences). The column was equilibrated with Standard buffer I saturated with 40% ammonium sulfate. Ssa1p was eluted with a gradient from 40 to 0% ammonium sulfate. As a final purification step, size exclusion chromatography was performed using a 120-ml Superdex 200 HiLoad (Amersham Biosciences) in Standard Buffer II (20 m*M* Hepes pH 7, 200 m*M* KCl, 5 m*M* $MgCl_2$, 5 m*M* β-mercaptoethanol, 2.5% glycerol). After every purification step the presence of Ssa1p was confirmed on 12.5% SDS–polyacrylamide gels. The SDS–PAGE was performed as described [24]. The gels were either silver stained according to Heukeshoven and Dernick [25] or Coomassie stained as described by Fairbanks et al. [26]. The identity of Ssa1p was determined by Western blotting using a polyclonal

Ssa antibody provided by E. Craig (University of Wisconsin, Madison, WI, USA) and mass spectroscopy. The protein was stored in 40 m*M* Hepes pH 7.5, 150 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* DTT at −80 °C.

2.8. Mass spectroscopy

Ssa1p was identified by mass spectroscopy (MALDI-TOF) using a Biflex-III equipped with a SCOUT 26 ion source (Bruker Daltonik, Bremen, Germany). Ssa1p was in-gel digested with trypsin (Roche, Mannheim, Germany) and the resulting peptide-mix was prepared for MALDI-TOF–MS as described [27]. The acceleration voltage was set to 20 kV, and the reflection voltage to 21 kV. Data aquisition was obtained by the XACQ software Version 4.0. Post analysis data processing was performed with the XMASS software, Version 5.1.

The sequences of the MALDI-TOF–MS fingerprint spectra were analyzed with ExPASy (http://www.expasy.ch/tools/peptident.html). For the ExPASy searches, the following parameters were used: monoisotopic masses, static carbamidomethylation of cysteines (+57), a mass tolerance of ±1, one missed cleavage site was allowed and trypsin was chosen as the specific protease.

2.9. ATPase assay

ATPase assays were performed according to Kornberg et al. [28]. Five μ*M* Ssa1p were incubated in 40 m*M* Hepes pH 7.5, 150 m*M* KCl, 5 m*M* $MgCl_2$ at 37 °C. The ATPase reaction was initiated by addition of the respective ATP concentrations containing [α-^{32}P]ATP (Hartmann Analytic, Braunschweig, Germany). For steady-state hydrolysis measurements, a final ATP concentration of 500 μ*M* was used. In the case of single turnover experiments, the protein to ATP ratio was kept constant at 1:0.8. A sample contained 0.1 μCi of [α-^{32}P]ATP. ATP hydrolysis was stopped at individual time-points by the addition of 24 m*M* EDTA and the samples were spotted on PEI-cellulose plates (Merck). Thin-layer chromatography was performed in 0.5 *M* LiCl and 2 *M* formic acid. Plates were dried under red light and the ATP to ADP ratio was quantified with a Typhoon 9200 PhosphorImager (Amersham Biosciences). Hydrolysis rates were corrected for uncatalysed, spontaneous ATP hydrolysis.

3. Results and discussion

Pichia pastoris can be genetically manipulated as easy as *E. coli* or *Saccharomyces cerevisiae* [29]. Further, *Pichia pastoris* is faster, easier, and less expensive to use than other eukaryotic expression systems such as insect cells (baculovirus system) or mammalian tissue culture. This makes *Pichia pastoris* a perfect expression system for eukaryotic proteins, yielding 10–100-fold higher heterologous protein expression levels compared to *Saccharomyces cerevisiae* [29,30]. We made use of *Pichia pastoris* to express Ssa1p from *Saccharomyces cerevisiae*, since other expression systems such as *E. coli* or *Saccharomyces cerevisiae* have been shown to be inefficient for Ssa1p (Ref. [16] and data not shown). The *Pichia pastoris* system yields high expression levels of Ssa1p (Fig. 1). Maximum expression was obtained 12 h after induction in medium containing methanol and therefore we harvested the cells at this timepoint.

We developed a three-step purification protocol to purify Ssa1p to homogeneity yielding high quantities. The first step is an anion-exchange chromatography (Fig. 2A), which allows to separate Ssa1p from metabolites and proteolytic enzymes. The second step, an ammonium sulfate precipitation, prepares Ssa1p for the hydrophobic interaction chromatography using butyl-Sepharose (Fig. 2B). The most efficient purification step is the final size-exclusion chromatography with separation of Ssa1p from most

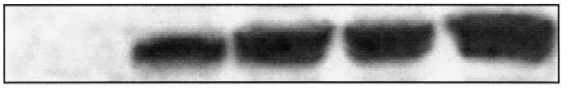

Fig. 1. Expression kinetics of *Saccharomyces cerevisiae* Ssa1p in *Pichia pastoris*. At different time points after induction in MMH medium, equal amounts of *Pichia pastoris* lysate were separated on a 12.5% SDS–PAGE and immunoblotted with Ssa1 antibody. Lanes: (1) before induction, (2) 3 h after induction, (3) 6 h after induction, (4) 9 h after induction, (5) 12 h after induction.

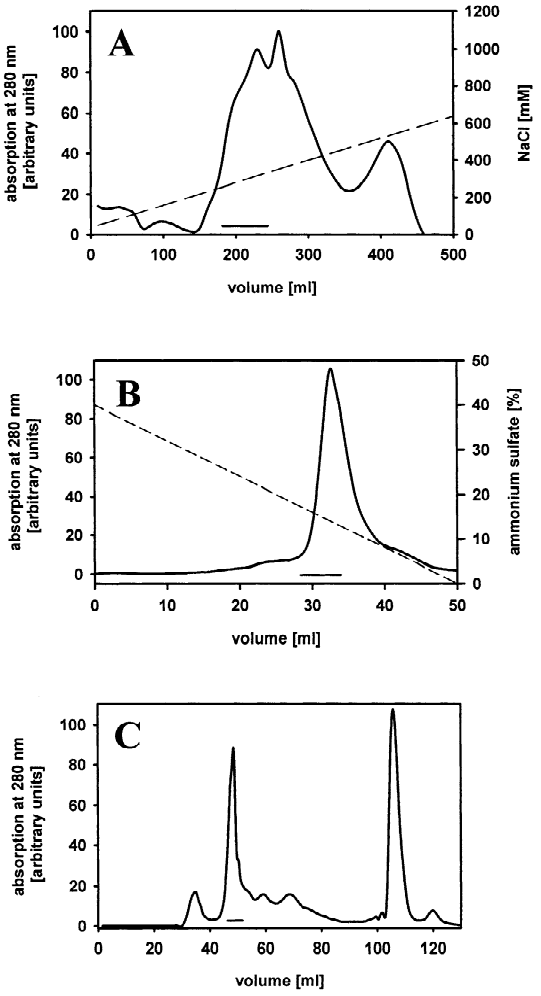

Fig. 2. Elution profiles of the chromatography experiments. The elution profile of the DEAE-Sephacel (weak anion-exchange) chromatography is shown in (A). The profile of the butyl-Sepharose is shown in (B). (C) Depicts the size exclusion chromatography (Superdex 200 pg). Solid lines correspond to eluting protein detected by measuring absorption at 280 nm. The dashed lines show the gradient concentrations. The short straight line corresponds to eluting Ssa1p.

of its impurities (Fig. 2C). The increasing purification yield can be seen in Fig. 3.

Our expression and purification protocol yields 200 mg Ssa1p from 9.6 g *Pichia pastoris* cells, which is about 400 times higher than previously obtained (Ref. [16] and data not shown). An important improvement is that no time- and protein-wasting dialysis step is necessary.

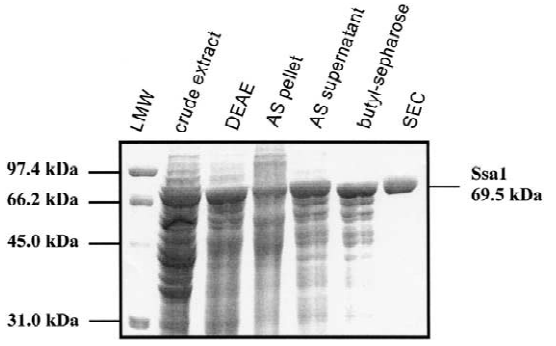

Fig. 3. Purification gel of the three-step chromatographic purification of Ssa1p (12.5% SDS–PAGE, Fairbanks staining). Lanes: (1) LMW, (2) crude extract, (3) combined Ssa1p fractions of the DEAE–Sephacel chromatography, (4) pellet of the 40% ammonium sulfate precipitation, (5) supernatant of the 40% ammonium sulfate precipitation, (6) combined Ssa1p fractions of the butyl-Sepharose chromatography, (7) purified Ssa1p after size-exclusion chromatography. LMW, low molecular mass marker.

The identity and purity of Ssa1p was shown by mass spectroscopy. Purified Ssa1p was in-gel digested with trypsin and the resulting peptides were analyzed by MALDI-TOF. The identification of Ssa1p was confirmed with ExPASy (data not shown).

To test for functionality of the purified Ssa1p, we performed ATPase assays (Fig. 4). It turned out, that Ssa1p exhibits the theoretical k_{cat} of 0.04 min^{-1} [14].

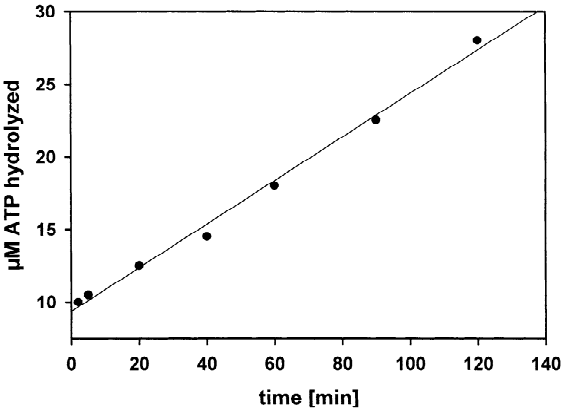

Fig. 4. Steady state ATPase assay with Ssa1p. The ATPase activity was determined using [α-^{32}P]ATP and thin-layer chromatography as described. The ADP to ATP ratio was determined with a PhosphorImager. The Ssa1p concentration in the assay was 5 µ*M*.

Comparing Ssa1p purified from *Saccharomyces cerevisiae,* using a conventional protocol including ATP-agarose, with Ssa1p purified from *Pichia pastoris* following our protocol, revealed no difference in steady state ATPase measurements. In these measurements, excess of ATP is used and therefore Ssa1p hydrolyses ATP with maximum velocity. However, in the case of single turnover ATPase measurements, a difference between the two Ssa1p batches existed. In such assays, about 80% of Ssa1p is saturated with ATP. Thus, a single hydrolysis step could be monitored. This allows, in comparison with the steady state measurement, to determine the rate-limiting step of the ATPase cycle. In the case of Ssa1p purified from *Saccharomyces cerevisiae* using ATP-agarose, data analysis was not possible, because the starting concentration of ATP remains unknown (Fig. 5). As can be seen in Fig. 5, Ssa1p purified from *Saccharomyces cerevisiae* has an A_{280}/A_{260} ratio of 0.9, which indicates that nucleotides are copurified. In the case of Ssa1p purified from *Pichia pastoris*, the A_{280}/A_{260} ratio has a value of 1.7, which shows that it was purified in a nucleotide-free form. Further, it was shown to be difficult to remove bound nucleotides from Hsp70s by dialysis or ammonium sulfate precipitation [31,32] and therefore it is of high importance to purify Ssa1p in a nucleotide-free form.

In summary, our expression system yields high quantities of purified Ssa1p in a nucleotide-free form, because our protocol does not employ ATP-agarose chromatography for purification purposes as described in former purification protocols. Therefore, analysis of ATP-dependent Hsp70 function can be performed without any interference and at high accuracy.

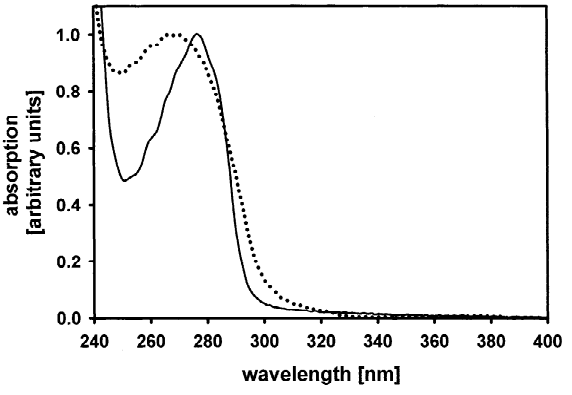

Fig. 5. UV spectra of Ssa1p purified from *Saccharomyces cerevisiae* and Ssa1p purified from *Pichia pastoris*. The UV spectrum of *Saccharomyces cerevisiae* Ssa1p purified from *Saccharomyces cerevisiae* (dotted line) has a maximum at 268 nm and the A_{280}/A_{260} ratio has a value of 0.9, which indicates that nucleotides are co-purified. The UV spectrum of *Saccharomyces cerevisiae* Ssa1p purified from *Pichia pastoris* (solid line) has a maximum at 279 nm and the A_{280}/A_{260} ratio has a value of 1.7, which indicates that it was purified in a nucleotide-free form.

Acknowledgements

We would like to thank Elizabeth Craig (University of Wisconsin, Madison, WI, USA) for the kind gift of Ssa antibody. We gratefully acknowledge the experimental help of Holger Grallert and thank Helmut Krause for performing the mass spectroscopy. We also would like to thank Thomas Scheibel and Stefan Walter for inspiring discussions and help with the manuscript. Harald Wegele was supported by fellowships of the Studienstiftung des deutschen Volkes and the Fonds der Chemischen Industrie.

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