Original Study Article
DOI: https://doi.org/10.17816/ecogen676918 EDN: HGNCCR

Optimization of Conditions for the Production of Hsp70 Chaperones in *Saccharomyces cerevisiae* Cells

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ABSTRACT

BACKGROUND: Molecular chaperones regulate the proper folding of proteins in the cell. Members of the Hsp70 family, including the Ssa1 protein, are molecular chaperones that prevent protein aggregation, promote their proper folding and degradation, and are the most common among the various chaperones, highly conserved, and present in a variety of organisms.

AIM: The aim of the work was to optimize methods for the production, extraction and purification of Ssa1 protein from cells of *Saccharomyces cerevisiae*.

MATERIALS AND METHODS: The *SSA1-4* gene sequences were cloned into a vector under the control of the *TEF1* promoter and fused with a sequence encoding His_6-tag. Yeast strains with different genetic backgrounds were transformed with the obtained constructs, and the production of Ssa1-4 proteins was assessed under different cultivation conditions. Affinity and ion-exchange chromatography were used to purify the Ssa1 protein. Fluorescence microscopy was used to confirm the localization of recombinant Ssa proteins fused with TagRFP-T in the cytosol.

RESULTS AND CONCLUSIONS: Methods for the production, extraction and purification of Ssa1 protein from yeast cells have been optimized. The same approach can be further used to purify other Hsp70 proteins and adapted to obtain various proteins from eukaryotic cells.

Keywords: chaperones; heat shock proteins; *Saccharomyces cerevisiae*; yeast; prion; [*PSI*$^+$]; Hsp70; Ssa.

To cite this article
Matveenko AG, Tsvetkov AA, Rogoza TM, Barbitoff YuA, Zhouravleva GA. Optimization of Conditions for the Production of Hsp70 Chaperones in *Saccharomyces cerevisiae* Cells. *Ecological genetics*. 2025;23(2):191–201. DOI: 10.17816/ecogen676918 EDN: HGNCCR

Submitted: 07.03.2025 **Accepted:** 16.04.2025 **Published online:** 30.06.2025

Оригинальное исследование
DOI: https://doi.org/10.17816/ecogen676918 EDN: HGNCCR

Оптимизация условий для продукции шаперонов Hsp70 в клетках *Saccharomyces cerevisiae*

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АННОТАЦИЯ

Обоснование. Молекулярные шапероны регулируют правильную укладку белков в клетке. Члены семейства Hsp70, включая белок Ssa1, — это молекулярные шапероны, которые предотвращают агрегацию белков, способствуют их правильному сворачиванию и деградации, они являются наиболее распространенными среди различных шаперонов, высококонсервативными и присутствуют в различных организмах.

Цель — оптимизация методов продукции, выделения и очистки белка Ssa1 из клеток *Saccharomyces cerevisiae*.

Материалы и методы. Последовательности генов *SSA1-4* были клонированы в вектор под контролем промотора гена *TEF1* и слиты с последовательностью, кодирующей His_6-тэг. Штаммы дрожжей с различным генетическим фоном трансформировали полученными конструкциями и оценивали продукцию белков Ssa1-4 при различных условиях культивирования. Для очистки белка Ssa1 использовали методы аффинной и ионообменной хроматографии. Для подтверждения локализации рекомбинантных белков Ssa, слитых с TagRFP-T, в цитоплазме применяли флуоресцентную микроскопию.

Результаты и заключение. Оптимизированы методы продукции, выделения и очистки белка Ssa1 из дрожжевых клеток. Этот же подход может быть в дальнейшем использован для очистки других белков семейства Hsp70 и адаптирован для получения различных белков из эукариотических клеток.

Ключевые слова: шапероны; белки теплового шока; *Saccharomyces cerevisiae*; дрожжи; прион; [*PSI*$^+$]; Hsp70; Ssa.

Как цитировать

Матвеенко А.Г., Цветков А.А., Рогоза Т.М., Барбитов Ю.А., Журавлева Г.А. Оптимизация условий для продукции шаперонов Hsp70 в клетках *Saccharomyces cerevisiae* // Экологическая генетика. 2025. Т. 23. № 2. С. 191–201. DOI: 10.17816/ecogen676918 EDN: HGNCCR

Рукопись получена: 07.03.2025 **Рукопись одобрена:** 16.04.2025 **Опубликована online:** 30.06.2025

BACKGROUND

Molecular chaperones are polypeptides responsible for the correct folding of proteins in the cell. During their lifetime, cells constantly adapt to changing conditions. Among different chaperons the members of the Hsp70 (70-kDa heat-shock protein) family are the most abundant and highly conserved among different organisms (reviewed in [1]). The Hsp70 family proteins are molecular chaperones that prevent protein aggregation, promote their proper folding and degradation, thereby being key factors in the ability of cells to maintain proteostasis. Thus, given the involvement of these proteins in a variety of cellular processes, it is not surprising that they are considered as targets for the treatment of various human diseases, such as cancer and neurodegenerative diseases.

Hsp70 proteins act together with co-chaperons, such as members of the Hsp40 family, which contribute to the specificity of Hsp70 interactions. They also require the nucleotide exchange factors (NEFs) as their activity is ATP-dependent [2]. Both of these types of auxiliary factors are usually represented by multiple proteins in different organisms. The Hsp70 proteins of the yeast *Saccharomyces cerevisiae* are divided into seven subfamilies: four canonical-type Hsp70 chaperones (Ssa, Ssb, Kar, and Ssc) and three atypical Hsp70 proteins (Sse, Ssz, and Lhs) that play a regulatory role by modulating the activity of canonical Hsp70 partners [3]. All Hsp70 proteins contain two functional domains: NBD (Nucleotide Binding Domain) at the N-terminus and SBD (Substrate Binding Domain) at the C-terminus which are connected by a short interdomain linker [1]. The Ssa (Stress Seventy sub-family A) subfamily includes four main members (Ssa1, Ssa2, Ssa3 and Ssa4). While none of the four corresponding *SSA* genes is essential, at least one of them must be present for viability [4, 5]. The four members of the *SSA* subfamily arose through duplications and are highly conserved. Despite sequence similarity the corresponding proteins may be functionally distinct and act in different ways. While *SSA2* is expressed constitutively at high levels, the *SSA3-4* are expressed only during cellular stress, and *SSA1* is constitutively expressed at moderate levels, but is also upregulated during stress [6, 7]. Ssa proteins also differ in their stability, with Ssa4 being the most stable (half-life greater than 100 h) and Ssa3 having a half-life of 11.0 h [8].

Several functional differences between Ssa proteins were found when characterizing their effects on yeast prions. Yeast possesses nearly 20 naturally occurring prions, the most well studied of which are [*PSI*$^+$], [*PIN*$^+$], and [*URE3*] (reviewed in [9]). Propagation of yeast prions is dependent on the cellular chaperone machinery, essentially, the Hsp104/Hsp70/Hsp40 system. Hsp104 is a molecular disaggregase capable of breaking apart prion aggregates thus promoting their division and inheritance. However, in order to perform its function, Hsp104 has to be recruited by the Hsp70-Ssa, which in turn binds prion aggregates in complex with Hsp40 co-chaperones (reviewed in [10]).

Despite sequence similarities, Ssa proteins differ in some of their effects on prion propagation. For example, opposing effects on the curing of [*URE3*] have been reported for nearly identical Ssa1 and Ssa2, although, these effects were not reproducible in other strains [11, 12]. Similarly, opposing effects on [*PSI*$^+$] were occasionally observed for Ssa3 and Ssa4 [13, 14]. The *SSA1-21* (L483W) mutation was first described by its dominant [*PSI*$^+$]-curing phenotype. Even though it does not affect propagation of [*URE3*], introduction of the same mutation into the *SSA2* gene severely destabilizes [*URE3*] [15]. Finally, Ssa proteins demonstrate various effects on prion propagation when they are expressed from the only copy of *SSA* gene from constitutive promoter of the *SSA2* gene, particularly, Ssa1 and Ssa2, but not Ssa3, or Ssa4 are able to maintain the [*URE3*]. While all four Ssa are able to maintain [*PSI*$^+$], Ssa4 specifically weakened its propagation [16].

The role of the Ssa proteins is not limited to chaperone activity and prion propagation. Particularly, Ssa proteins participate in the translocation of proteins into the mitochondria and endoplasmic reticulum [17, 18]. Additionally, Ssa1/2 proteins function in cell cycle progression and DNA-damage response as they were shown to act in G2/M checkpoint regulation after UV irradiation. Ssa1/2 were found to take part in a complex with DNA damage checkpoint protein Rad9 which either includes the effector kinase Rad53, or does not, which affects the Rad9 phosphorylation. Interestingly, Ssa3 or Ssa4 can also be recruited to the Rad9 complex, but unable to substitute for the Ssa1/Ssa2 function [19].

Given the importance of the Hsp70 proteins for the prion propagation, as well as other cellular processes, we aimed at studying their properties *in vitro*. As the Ssa proteins are stable and abundant in yeast cells, these chaperones can be extracted directly from yeast, as shown for Ssa1 in earlier studies. Various approaches can be used for the purification of the Ssa proteins from yeast cells. For example, Ssa1/2 proteins were extracted from complexes with Rad9 and Rad53 after co-precipitation with heparin-affinity chromatography of hemagglutinin-tagged Rad9 [19]. Another approach utilized ATP-dependence of the Hsp70 chaperones by employing ATP-affinity purification, followed by anion exchange chromatography [20]. Immunoprecipitation was also used for the Ssa1/2 proteins [17]. All the aforementioned approaches are poorly suited for distinguishing nearly identical Ssa1 and Ssa2 proteins, which can be overcome by producing a tagged protein. In case of polyhistidine-tagged proteins, affinity purification using chelating sepharose was successfully used [21].

We aimed to use a similar approach with Ni-NTA agarose for the extraction of His_6-tagged proteins. In this work, we developed and optimized the techniques allowing for the extraction and purification of various Hsp70 proteins from yeast cells which required optimization of conditions for their production.

MATERIALS AND METHODS

Strains and media

Escherichia coli strain DH5α [22] was used for the plasmid selection, maintenance and amplification. *S. cerevisiae* strains used in this work are listed in Table 1. Standard methods of cultivation and manipulation of yeast and bacteria were used throughout this work [23, 24]. Yeast strains were cultivated at 30 °C in standard solid and liquid media: YEPD (rich media), or either of the two types of selective media were used. SC (synthetic complete) media has 6.7 g/L yeast nitrogen base (YNB) with ammonium sulfate (Invitrogen) as its core component. Alternatively, synthetic minimal media (Min) was used, which contains of 0.5 g of $MgSO_4 \cdot 7H_2O$, 3.5 g of $(NH_4)_2SO_4$, 0.1 g of K_2HPO_4, 0.9 g of KH_2PO_4, 0.2 mg of thiamine, 0.002 mg of biotin, and 0.5 mg of β-alanine per litre [25]. Both SC and minimal media contained all supplements required to compensate for all the auxotrophies of all strains, specifically, adenine, L-tryptophane, L-leucine, L-lysine, L-histidine, L-methionine, and L-threonine at concentrations described in [23]. The uracil was omitted, which was necessary for maintaining *URA3* plasmids.

Yeast transformation was performed as described [26].

Fluorescence microscopy

Yeast cells from liquid cultures grown to mid-log phase (OD_{600} = 0.4–0.6) were analyzed using Zeiss Axioscope A1 wide-field fluorescence microscope equipped with a Zeiss AxioCam 506 Color camera. Images were acquired using Zeiss Zen software.

Plasmids

Plasmids used in this work are listed in Table 2.

The pTEF-His_6-SSA2 plasmid was generated by amplification of the *SSA2* gene fragment from the pDM64 plasmid with the Ssa2-BamH1(-1)-F and Ssa2-BcuI-R primers. The amplified PCR fragment was digested with the BamHI and BcuI restriction enzymes and ligated into the backbone

Table 1. Yeast strains used in this work

Strain	Genotype	Reference
74-D694	*MAT*a *ade1-14 his3-Δ200 ura3-52 leu2-3,112 trp1-289* [*psi*$^-$] [*PIN*$^+$]	[27]
OT56	74-D694 [*PSI*$^+$]^S [*PIN*$^+$]	[28]
prb1Δ0-P-74-D694	74-D694 *prb1Δ0* [*PSI*$^+$] [*PIN*$^+$]	[29]
prb1Δ0-2-74-D694	74-D694 *prb1Δ0* [*psi*$^-$] [*pin*$^-$]	[30]
yAO066	74-D694 *prb1Δ::HIS3MX* [*psi*$^-$] [*PIN*$^+$]	[31]
yAO121	74-D694 *pep4Δ::HIS3MX* [*psi*$^-$] [*PIN*$^+$]	[31]
P2.1.1-yAO121	74-D694 *pep4Δ::HIS3MX* [*PSI*$^+$]^S [*PIN*$^+$]	[30]
2-yAO121	74-D694 *pep4Δ::HIS3MX* [*psi*$^-$] [*pin*$^-$]	[30]
BY4742	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	[32]
prb1Δ-BY4741	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 prb1::KanMX*	[29]

Table 2. Plasmids used in this work

Plasmid	Description	Reference
pRS316	*AmpR, CEN, URA3*	[33]
pTEF-SSA1	*AmpR, CEN, URA3,* P_{TEF1}-His_6-Xpress-SSA1	[34]
pTEF-SSA1021	*AmpR, CEN, URA3,* P_{TEF1}-His_6-Xpress-SSA1-21	[10]
pDM64	*AmpR, CEN, HIS3,* P_{SSA2}-SSA2	[11]
pTEF-SSA3	*AmpR, CEN, URA3,* P_{TEF1}-SSA3	[13]
pTEF-SSA4	*AmpR, CEN, URA3,* P_{TEF1}-SSA4	[13]
pTEF-His_6-SSA2	*AmpR, CEN, URA3,* P_{TEF1}-His_6-Xpress-SSA2	This work
pTEF-His_6-SSA3	*AmpR, CEN, URA3,* P_{TEF1}-His_6-Xpress-SSA3	This work
pTEF-His_6-SSA4	*AmpR, CEN, URA3,* P_{TEF1}-His_6-Xpress-SSA4	This work
pIM35	*AmpR, CEN, URA3,* P_{MET17}-yTagRFP-T	[35]
pTEF-His_6-yTagRFP-T-SSA1	*AmpR, CEN, URA3,* P_{TEF1}-His_6-Xpress-yTagRFP-T-SSA1	This work
pTEF-His_6-yTagRFP-T-SSA1-21	*AmpR, CEN, URA3,* P_{TEF1}-His_6-Xpress-yTagRFP-T-SSA1-21	This work
pTEF-His_6-yTagRFP-T-SSA2	*AmpR, CEN, URA3,* P_{TEF1}-His_6-Xpress-yTagRFP-T-SSA2	This work
pTEF-His_6-yTagRFP-T-SSA3	*AmpR, CEN, URA3,* P_{TEF1}-His_6-Xpress-yTagRFP-T-SSA3	This work
pTEF-His_6-yTagRFP-T-SSA4	*AmpR, CEN, URA3,* P_{TEF1}-His_6-Xpress-yTagRFP-T-SSA4	This work

of the pTEF-SSA1 plasmid digested with the same enzymes. For the construction of the pTEF-His_6-SSA4 plasmid the same approach was used; for the amplification of *SSA4* Ssa4-BamHI(-1)-F and Ssa4-BcuI-R primers and pTEF-SSA4 plasmid were used. pTEF-His_6-SSA3 was obtained as follows: the *SSA3* gene fragment was first amplified using Ssa3-BamHI(–1)-F and T7 20-mer primers from pTEF-SSA3. The BamHI-EcoRI fragment of the amplified product was inserted into pTEF-SSA1 in place of the *SSA1*-containing fragment resulting in pTEF-His_6-SSA3ΔC. Finally, the XbaI-XbaI fragment of pTEF-SSA3 was replaced by analogous fragment from pTEF-His_6-SSA3ΔC containing His_6-tag sequence, yielding pTEF-His_6-SSA3. The pTEF-His_6-yTagRFP-T-SSA1 was constructed as follows: first, a fragment containing the *yTagRFP-T* gene was generated by amplification of a fragment from the pIM35 plasmid with the pBlueScr-SK and yTR-T-ns-R-NheI primers (Table 3). The amplified PCR fragment was then digested with the NheI and BcuI restriction enzymes and ligated into the backbone of the pTEF-SSA1 plasmid digested with NheI. For the construction of the pTEF-His_6-yTagRFP-T-SSA1-21 pTEF-His_6-yTagRFP-T-SSA2, pTEF-His_6-yTagRFP-T-SSA3 and pTEF-His_6-yTagRFP-T-SSA4 plasmids the same approach was used.

All plasmid constructions were verified by sequencing in the Resource Center "Development of Molecular and Cell Technologies" of Saint Petersburg State University.

Protein analysis

Cells for protein extraction were grown in liquid media at 30°C with shaking at 200 rpm until reaching OD_{600} = 0.6–0.8 or stationary phase (OD_{600} = 2). The modified alkaline lysis method [36, 37] was employed for the analysis of protein amount with SDS-PAGE. Briefly, cells were pelleted and washed twice in water. Cell pellets were incubated for 5 min with 2 M lithium acetate (LiAc) on ice. LiAc-treated cells were then centrifuged, the supernatant was discarded, and the cells were resuspended in 0.4 M NaOH and placed on ice for 5 min. After centrifugation pellets were resuspended in Laemmli buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 5 min, and cleared by centrifugation before separation by SDS-PAGE. After electrophoresis proteins were eluted onto PVDF membrane by semi-dry transfer and visualized with Western blot hybridization [24]. Antibodies used are present in Table 4. The antibodies used to detect the Ssa proteins (anti-Hsp70) were IgM, hence, required compatible secondary antibodies; anti-mouse IgG H&L were used for this purpose. Immunoblots were detected using ECL Select Western Blotting Detection Reagent (GE Healthcare) and GeneGnome hardware and software (Syngene).

Purification of recombinant proteins

For the purification of the His_6-Ssa proteins at least 500 ml of yeast cells from liquid culture were first harvested by centrifugation. The pelleted cells were then washed and frozen in –80°C. Total protein lysate was extracted in non-denaturing conditions using glass beads technique [38, 39]. The lysis buffer used was 20 mM TrisHCl, 500 mM NaCl, 10% glycerol and 10% protease inhibitors cocktail (Sigma #P8215-5ML). The crude lysate was clarified by centrifugation at 4°C at 800 g for 10 minutes. The supernatant was then loaded into a 5 ml HiTrap HP Ni-NTA agarose column (GE Healthcare). The NGC chromatography system (BIO-RAD) was used for the purification procedure. The buffer for the column wash was the same as the lysis buffer, but without protease inhibitors and containing 10 mM imidazole. The elution buffer contained 250 mM imidazole. The elution was performed by increasing a proportion of elution buffer to the wash buffer

Table 3. Primers used in this work

Primer	Sequence (5' → 3')
Ssa2-BamH1(-1)-F	atattggatcctatgtctaaagctgtcggtattgat
Ssa2-BcuI-R	gaccactagtcttaatcaacttcttcgacagttggacc
Ssa3-BamHI(-1)-F	aacaggatcctatgtctagagcagttggtattg
T7 20-mer	taatacgactcactataggg
Ssa4-BamHI(-1)-F	aacaggatcctatgtcaaaagctgttggtattg
Ssa4-BcuI-R	gtactagtctaatcaacctcttcaaccgttg
pBlueScr-SK	tctagaactagtggatc
yTR-T-ns-R-NheI	ccagctagccttatacaattcgtccataccgt

Table 4. Antibodies used in this work

Name	Dilution	Antigene	Reference
Anti-His_6	1:4000	His_6-tag	GE Healthcare, #27471001
Peroxidase-labeled anti-mouse	1:10000	mouse IgG	GE Healthcare, #NIF 825
Anti-Hsp70 [2A4]	1:3750	human Hsp70 437-479 fragment	Abcam, #ab5442
Peroxidase-labeled goat anti-mouse IgG H&L	1:10000	mouse IgG H&L	Abcam, #ab205719

creating a linear gradient of imidazole concentrations. Fractions were collected using an automated fraction collector (BIO-RAD) and checked by SDS-PAGE. Fractions with the highest content of the Ssa protein were then concentrated using AmiCon Ultra15 centrifugal concentrators with a 3 kDa molecular weight cutoff (Millipore). The buffer was simultaneously changed to the Ssa storage buffer (20 mM TrisHCl, pH 7.6, 50 mM KCl, 5 mM BME, 10% glycerol). Additional purification was performed with anion exchange chromatography using HiTrap Q HP 5 ml column (GE Healthcare). The start buffer used was 20 mM TrisHCl, pH 7.6, 100 mM KCl, 6 mM BME, 5% glycerol; the elution buffer was the same, except the concentration of KCl was 500 mM.

RESULTS AND CONCLUSION

Selection of a *S. cerevisiae* strain optimal for producing the chaperone Ssa1

Various strains are known to differ in the abundance of molecular chaperones [40] making the choice of strain for the production of the Hsp70 chaperones to be critical. Several factors may affect the chaperone abundance; among them is the genetic background which depends on the strain ancestry. Another factor is the constant presence of the heritable misfolded proteins, such as prions, as their aggregates are known to sequester some cytosolic chaperones, including Hsp70s, thus affecting their overall abundance either directly or by compensatory feedback mechanisms (reviewed in [10]). The strain of choice also should demonstrate rapid growth and high stability of the protein of interest. We selected strains with either BY4741/BY4742 or 74-D694 genetic background, which ascend either to the reference S288C strain or Peterhof genetic lineage, respectively. We tested strains of both origins with deletions of vacuolar proteases (*prb1Δ* and *pep4Δ*). We also tested variants containing [*PSI*$^+$] and [*PIN*$^+$] prions in different combinations.

Strains of *S. cerevisiae* specified in Table 1 were transformed with pTEF-SSA1 or pTEF-SSA1-21 plasmids. Transformants were selected at the solid SC-Ura media, grown in the same liquid media and tested by SDS-PAGE followed by immunoblotting. The liquid cultures were typically grown to mid- or late-log phase, in which the majority of cells are actively growing and dividing, however, several Ssa proteins are known to increase their abundance during transition to the stationary phase [4]. We therefore additionally assessed the protein levels in three strains grown to the stationary phase. For the detection of Ssa1 two types of antibodies were used: anti-Hsp70 and anti-His$_6$ (Fig. 1). The anti-Hsp70 antibodies we used are able to recognize all four Ssa proteins, thus, we were able to detect not only the overproduced His$_6$-Ssa1 protein, but also the Ssa proteins produced from their respective endogenous loci. As a result, we could

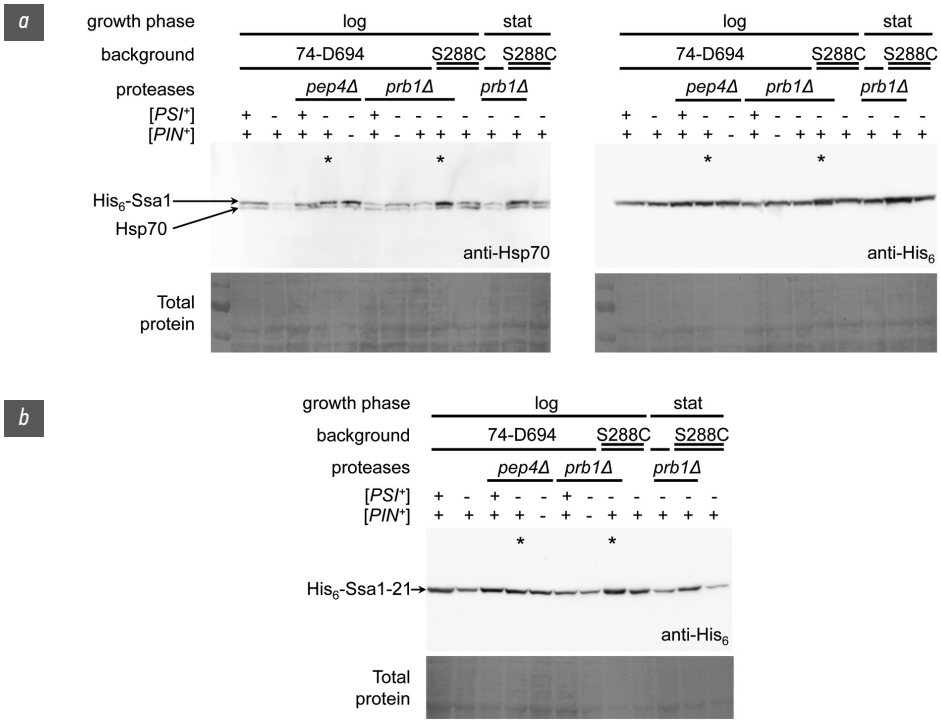

Fig. 1. Comparison of Ssa1 production levels in different yeast strains by immunoblotting. pTEF-SSA1 (*a*) or pTEF-SSA1-21 (*b*) plasmids were used for transformation. Total protein was visualized using Coomassie R250 membrane staining. log, yeast culture in logarithmic (OD_{600} = 0.7–1.0), stat, in stationary (OD_{600} = 2) growth phase. Anti-His6 and anti-Hsp70 antibodies were used. Strains selected for further analysis are marked with asterisks. The order of the strains in panel A is as follows (from left to right): OT56, 74-D694, P2.1.1-yA0121, yA0121, 2-yA0121, prb1Δ0-P-74-D694, prb1Δ0-2-74-D694, yA0066, prb1Δ-BY4741, BY4742, yA0066 (stat), prb1Δ-BY4741(stat), BY4742(stat). The order of the strains in panel B is as follows (from left to right): OT56, 74-D694, P2.1.1-yA0121, yA0121, 2-yA0121, prb1Δ0-P-74-D694, prb1Δ0-2-74-D694, prb1Δ-BY4741, BY4742, yA0066 (stat), prb1Δ-BY4741(stat), BY4742(stat).

observe two bands on the western blot, the upper corresponding to the heavier His_6-Ssa1, and the lower representing the mix of native Ssa1-4 proteins. This allowed us to compare the abundance of the overproduced protein to the natively produced Ssa. We also used another approach, in which we compared the relative amounts of the overproduced protein using total protein load as a reference. Comparison of different strains in different growth phases allowed us to select the prb1Δ-BY4741 and yAO121 strains growth to the logarithmic phase for further analysis.

Selection of optimal conditions for incubation of yeast culture for the production of His_6-Ssa1

As members of a heat shock protein group, some of the Ssa proteins, including Ssa1, are known to be produced in increased amounts during heat shock and other stress conditions [4]. To find out whether growth at elevated temperatures affects the amount of the overproduced Ssa1, we incubated the transformants of the selected strains at different temperatures (30°C, 37°C and 42°C). Western blot hybridization was then performed with antibodies to Hsp70. We found that the His_6-Ssa1 and His_6-Ssa1-21 proteins were most actively produced in the prb1Δ-BY4741 strain at 30°C (Fig. 2, *a*). Elevated temperatures indeed increased the production of native Hsp70s, however, this did not affect the production of His_6-tagged proteins regulated by the constitutive *TEF1* promoter. Our observations confirm that the temperature-dependent production of the Hsp70 mostly relies on their transcriptional regulation, while the expression of the *SSA1* gene under control of non-native constitutive promoter remains unaffected by increasing temperature.

The composition of the media for cell culture growth, specifically, shortage of rich carbon or nitrogen sources may also promote stress reactions in cells. We thus decided to compare the efficiency of cultivation and protein production in YNB-based SC medium and in salt-based minimal (Min) medium (both without uracil). The level of protein production in the minimal salt-based medium was slightly lower, but in the lysates of yeast grown in SC-Ura and visualized using antibodies to Hsp70, an additional band was occasionally observed (Fig. 2, *b*). Presence of a band corresponding to a protein with lower molecular weight which is bound by anti-Hsp70 antibodies may point to the proteolysis of Hsp70 occuring during growth in SC medium. Due to this, and also in order to optimize costs, it was decided to continue further work with Hsp70 in a salt-based minimal medium without uracil.

Production of chaperones Ssa2, Ssa3 and Ssa4

Having selected the optimal conditions for the production of the Ssa1 and Ssa1–21 chaperones, we checked whether they were also suitable for production of Ssa2, Ssa3, and

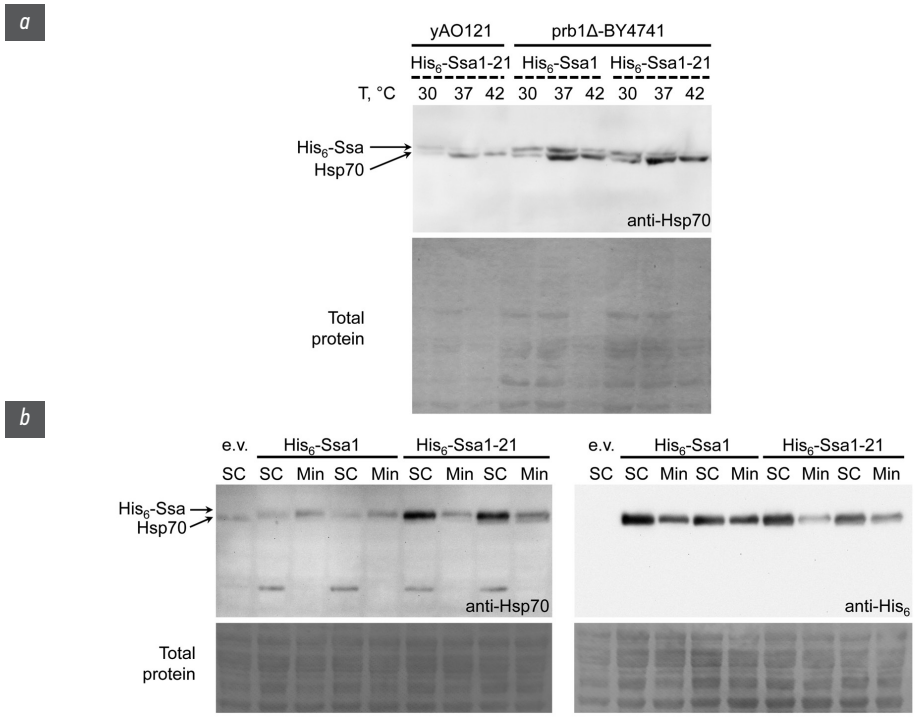

Fig. 2. Comparison of Ssa1 production levels in the selected strains under different conditions: *a*, yAO121 and prb1Δ-BY4741 transformants from fig. 1 were first grown in 30°C to mid-log phase (OD_{600} = 0.4–0.6), and then incubated at the indicated temperatures for 4 hours: *b*, Strain prb1Δ-BY4741 was transformed with pTEF-SSA1, pTEF-SSA1-21, or pRS316 (e.v., empty vector) plasmids; the selected transformants were grown at SC-Ura (SC) or Min-Ura (Min) media at 30°C to the late-log phase. Two independent transformants are shown in each case. Shown are results of western blot analysis. Total protein was visualized using Coomassie R250 membrane staining.

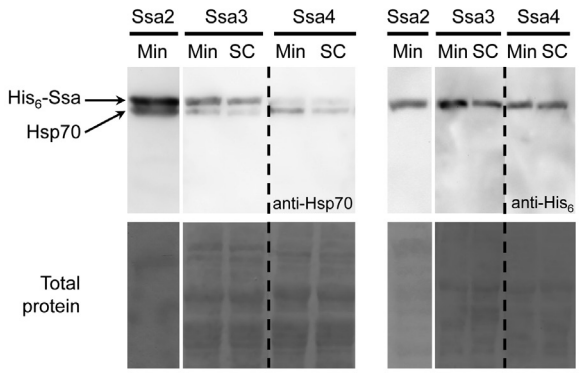

Fig. 3. Selection of a medium for the production of Ssa2, Ssa3 and Ssa 4. Strain prb1Δ-BY4741 was transformed with pTEF-His_6-SSA2, pTEF-His_6-SSA3 and pTEF-His_6-SSA4; selected transformants were grown in SC-Ura (SC) or Min-Ura (Min) media at 30°C until OD_{600} = 0.8–1.1, and then subjected to protein extraction and western blotting.

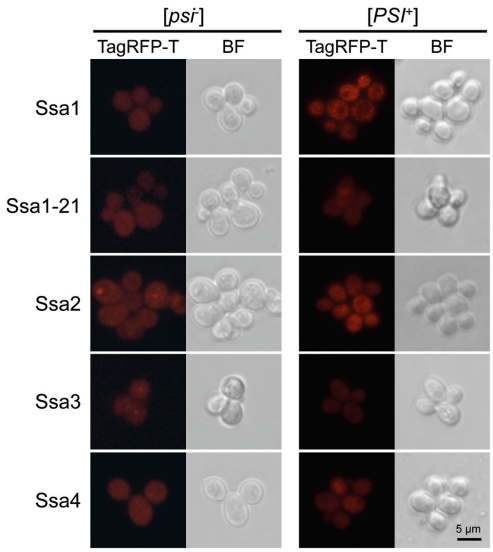

Fig. 4. The analysis of intracellular distribution of Ssa proteins. OT56 ([PSI^+]) and 74-D694 ([psi^-]) strains were transformed with a series of pTEF-His_6-yTagRFP-T-SSA plasmids. The resulting transformants were analyzed using fluorescence microscopy. BF, bright field.

Ssa4. We transformed the prb1Δ-BY4741 strain with the plasmids pTEF-His_6-SSA2, pTEF-His_6-SSA3, and pTEF-His_6-SSA4 and compared the protein production levels in SC-Ura and Min-Ura (Fig. 3). As in the case of Ssa1 and Ssa1-21, it was decided to cultivate the transformed yeast cells in a minimal medium without the addition of uracil. Both conditions appeared to be suitable for the chaperone production, confirming the choice of a minimal medium as optimal. It should be noted that the observed levels of Ssa4 were significantly lower than Ssa3 when detected by the anti-Hsp70 antibodies. No such difference was observed when anti-His_6 antibodies were used. It is likely that the affinity of the anti-Hsp70 antibodies we used was lower for the Ssa3 protein, even though the sequences of the fragments homologous to the implied epitope are nearly identical in all the Ssa proteins.

Thus, we were able to select the most suitable strain and conditions for the production of chaperones Ssa1, Ssa1-21, Ssa2, Ssa3 and Ssa4. The experiments also confirmed the suitability of the vector constructs we obtained for protein production in yeast cells.

Determination of the intracellular localization of Ssa chaperones using fluorescence microscopy

Addition of tags on the N-terminus can affect properties of a protein including its localization which in turn may result in decreasing yield of a purified protein. We checked whether tagged variants of the Ssa proteins retained their cytosolic localization. In order to visualize distribution of the Ssa proteins we constructed variants with red fluorescent protein gene *yTagRFP-T* inserted in-between the His_6-tag and reading frame of the respective gene. We transformed the OT56 ([PSI^+]) and 74-D694 ([psi^-]) strains with the resulting five plasmids and analyzed the cells of the transformants using fluorescence microscopy. In all cases we observed diffuse distribution in most of the cells (Fig. 4). Cells with heterogeneously distributed proteins forming clumps or multiple foci could also be observed, however, they were rare. Such cells could be found in both [PSI^+] and [psi^-] cells producing either of the Ssa protein variants studied. These results suggest that N-terminally tagged Ssa proteins are predominantly localized in cytosol, and thus can be purified from the fraction of soluble proteins.

Production and purification of the Ssa1 chaperone

We then attempted to purify the Ssa1 protein from cells grown at the selected conditions. Purified preparations of the His_6-Ssa1 protein was obtained using affinity chromatography (Fig. 5, *a*). Several additional proteins appeared to also bind the Ni-NTA agarose, with some of them of similar molecular weight to the His_6-Ssa1. Thus, some of the obtained fractions were tested by Western blot for the presence of the target protein. We confirmed that His_6-Ssa1 was present in the elution fractions as it binded both anti-Hsp70 and anti-His_6 antibodies (Fig. 5, *b*). The major co-purified protein, which was slightly less massive than Hsp70, reacted only to the anti-His_6 antibodies (Fig. 5, *b*).

Due to the insufficient purity of the obtained preparations, the fractions were concentrated, and the buffer was replaced, after which the samples were subjected to additional purification by anion exchange chromatography (Fig. 5, *c*). The protein amounts in the resulting fractions were then assessed using SDS-PAGE. This allowed us to obtain fractions with significantly reduced amounts of non-target protein. Overall, the combination of affinity and anion exchange chromatography proved to be effective for purification of the Ssa1 protein from yeast cells. The same approach may be further used for purification of Ssa2, Ssa3, and Ssa4 proteins, and, in principle, can be adapted for *in vitro* studies of other yeast proteins.

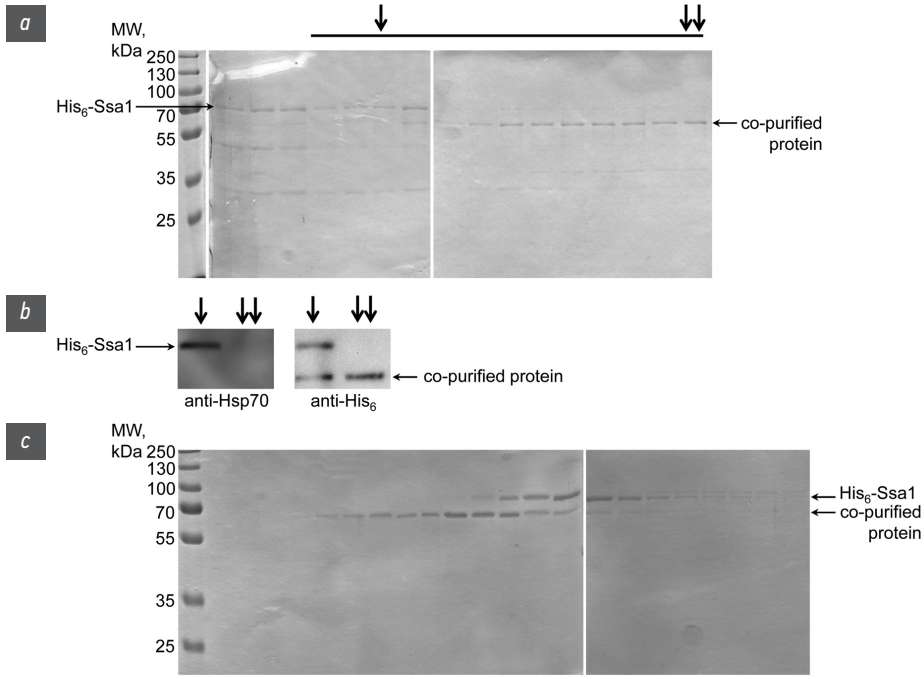

Fig. 5. Purification of the Ssa1 protein from yeast cell lysates: *a*, SDS-PAGE of affinity chromatography fractions of yeast lysates producing His_6-Ssa1 under non-denaturing conditions. Shown is Coomassie R250 staining of gels loaded with elution fractions. Fractions that were taken for subsequent concentration and purification by ion exchange chromatography are marked with a line, fractions marked with one and two arrows were used for the immunoblotting; *b*, Western blot of representative fractions (marked with one and two arrows at the panel A) after affinity purification; *c*, SDS-PAGE analysis of ion exchange chromatography fractions, stained with Coomassie R250.

ADDITIONAL INFO

Author contribution: A.G. Matveenko: planning and conducting experiments, writing the text of the manuscript; A.A. Tsvetkov: conducting experiments, figures preparation; T.M. Rogoza: conducting experiments; Yu.A. Barbitoff: research concept, discussion of results; G.A. Zhouravleva: discussion of results, writing the text of the manuscript. All authors have approved the publication version and also agreed to be responsible for all aspects of the each part of the work and ensured reliable consideration of the issues related to the accuracy and integrity.

Acknowledgments: We thank Anastasiia S. Mikhailichenko and Nina P. Trubitsina for help with experiments. This study utilised the equipment of the core facility center "Development of molecular and cellular technologies" of the scientific park of Saint Petersburg State University.

Funding sources: The work is supported by Russian Science Foundation grant No. 23-74-01121, except for the part of the work regarding fluorescence analysis, which is supported by Russian Science Foundation grant No. 23-14-00063. The authors acknowledge Saint-Petersburg State University for a research project 103972122 for supporting the maintenance of the collection of *S. cerevisiae* strains used in this work.

Disclosure of interests: The authors have no relationships, activities or interests for the last three years related with for-profit or not-for-profit third parties whose interests may be affected by the content of the article.

Statement of originality: The authors did not use previously published information (text, illustrations, data) to create this paper.

Data availability statement: All data obtained in the present study are available in the article.

Generative AI: Generative AI technologies were not used for this article creation.

Provenance and peer-review: This work was submitted to the journal on its own initiative and reviewed according to the standard procedure. Two external reviewers, and a member of the editorial board participated in the review.

ДОПОЛНИТЕЛЬНАЯ ИНФОРМАЦИЯ

Вклад авторов. А.Г. Матвеенко — планирование и проведение экспериментов, написание текста рукописи; А.А. Цветков — проведение экспериментов, подготовка рисунков; Т.М. Рогоза — проведение экспериментов; Ю.А. Барбитов — планирование и обсуждение экспериментов; Г.А. Журавлева — обсуждение экспериментов, написание текста рукописи. Все авторы одобрили рукопись (версию для публикации), а также согласились нести ответственность за все аспекты работы, гарантируя надлежащее рассмотрение и решение вопросов, связанных с точностью и добросовестностью любой ее части.

Благодарности. Выражаем благодарность Анастасии Сергеевне Михайличенко и Нине Павловне Трубициной за помощь в проведении экспериментов. Работа выполнена с использованием оборудования Ресурсного центра "Развитие молекулярных и клеточных технологий" Санкт-Петербургского государственного университета.

Источники финансирования. Работа поддержана грантом Российского научного фонда № 23-74-01121, за исключением части работы, касающейся флуоресцентного анализа, которая поддержана грантом Российского научного фонда № 23-14-00063, а также части работы по поддержанию коллекции штаммов *S. cerevisiae*, использованных в данной работе, которая выполнена при поддержке ФГБОУ ВО «Санкт-Петербургский государственный университет», шифр проекта 103972122.

Раскрытие интересов. Авторы заявляют об отсутствии отношений, деятельности и интересов за последние три года, связанных с третьими лицами (коммерческими и некоммерческими), интересы которых могут быть затронуты содержанием статьи.

Оригинальность. При создании настоящей работы авторы не использовали ранее опубликованные сведения (текст, иллюстрации, данные).
Генеративный искусственный интеллект. При создании настоящей статьи технологии генеративного искусственного интеллекта не использовали.
Рассмотрение и рецензирование. Настоящая работа подана в журнал в инициативном порядке и рассмотрена по обычной процедуре. В рецензировании участвовали два внешних рецензента и член редакционной коллегии.

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