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Native signal peptide of human ERp57 disulfide isomerase mediates secretion of active native recombinant ERp57 protein in yeast *Saccharomyces cerevisiae*

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ABSTRACT

Human ERp57 protein is disulfide isomerase, facilitating proper folding of glycoprotein precursors in the concert with ER lectin chaperones calreticulin and calnexin. Growing amount of data also associates ERp57 with many different functions in subcellular locations outside the ER. Analysis of protein functions requires substantial amounts of correctly folded, biologically active protein, and in this study we introduce yeast *Saccharomyces cerevisiae* as a perfect host for production of human ERp57. Our data suggest that native signal peptide of human ERp57 protein is recognized and correctly processed in the yeast cells, which leads to protein secretion. Secreted recombinant ERp57 protein possesses native amino acid sequence and is biologically active. Moreover, secretion allows simple one-step purification of recombinant ERp57 protein with the yields reaching up to 10 mg/L.

Introduction

Human thiol-disulfide oxidoreductase ERp57 is a member of the PDI (protein disulfide isomerase) family, which currently consists of 19 proteins [1]. PDI is the most studied protein of this family, but ERp57 has attracted plenty of attention recently due to its newly discovered functions and involvement in important human diseases (reviewed in [2–4]). Besides the ER, where ERp57 mainly acts as an essential molecular component in folding and quality control machinery of newly synthesized glycoproteins [5] and is required for the assembly of the major histocompatibility complex (MHC)[1] class I [6], ERp57 is also found on the cell surface, in the nucleus, cytoplasm and mitochondria, where vast array of functions are assigned to this protein, including regulation of gene expression through STAT3 [7,8]; membrane receptor for the steroid hormone 1,25(OH)2D3 [3]; interaction with the all-trans retinoic acid receptor α [9]; mediation of platelet aggregation, hemostasis, and thrombosis [10,11]; regulation of mTORC1 complex assembly and signaling [12] and other functions which are well summarized in the recent review [4]. Involvement of ERp57 in human diseases, such as Alzheimer's, Parkinson's, cancer or prion (PrP^{SC}) disorders attracts special interest. This protein appears to act as a protective agent in Alzheimer's [13] and prion [14] diseases, and as a harmful molecule in Parkinson's disease [15] and cancer [16,17]. In the light of these data, high quality recombinant human ERp57 has potential applications in fundamental, applied and therapeutic studies.

Currently, *Escherichia coli* is the host of choice for the production of recombinant human ERp57 protein. To facilitate purification, ERp57 coding sequence usually is fused with GST [18–20] or histidine [21–23] tags, and generation of tag-free protein requires additional tag removal step. Only in few cases *E. coli*-synthesized human ERp57 protein was purified without help of any tags [24]. Recombinant human ERp57 was also produced in insect cells [22,25] and using in vitro translation [26]. To the best of our knowledge, this work is the first to report yeast *Saccharomyces cerevisiae* as a host for production of human ERp57 protein. In our previous study [27], we inserted different human ER chaperones into the yeast cell to facilitate maturation of recombinant Measles virus hemagglutinin. Even though considerable amount of recombinant human ERp57 was found to be localized in the yeast ER [27], subsequent studies revealed that protein was also secreted outside the yeast cell.

Here we report how this discovery allows simple cost-effective purification of large amounts of active human ERp57. Our studies revealed that yeast-derived human ERp57 chaperone is correctly processed in the yeast cell, i.e. native signal sequence of ERp57 is cleaved exactly at the same position as in human cells [28], and possesses intact ER retention signal (QEDL sequence at the C-terminus). Moreover, mass spectrometry results of the purified human ERp57 isomerase indicated that recombinant protein has no yeast-derived modifications. Finally, insulin turbidity test showed that yeast-produced ERp57 possesses thiol-dependent reductase

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[1] Abbreviations used: MHC, major histocompatibility complex; BPTI, bovine pancreatic trypsin inhibitor.

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activity, which slightly exceeds that of the commercially available *E. coli*-produced protein. In conclusion, yeast *S. cerevisiae* is an excellent host for production of native recombinant human thiol-disulfide oxidoreductase ERp57.

Materials and methods

Materials

Enzymes and kits for DNA manipulations were purchased from ThermoScientific. Primers were purchased from IDT. Mouse monoclonal antibodies against human ERp57 protein were purchased from Santa Cruz Biotechnology (cat. No. sc-23886).

Plasmids, strains, media, yeast transformation and cultivation

All DNA manipulations were performed according to standard procedures [29]. Recombinant plasmids were amplified in *E. coli* DH5αF' cells. The yeast *S. cerevisiae* strain AH22 MATa leu2 his4 was used for expression experiments. Transformation of *S. cerevisiae* cells was performed by conventional methods [29]. The selection of transformants resistant to formaldehyde was carried out on YEPD (yeast extract 1%, peptone 2%, dextrose 2%) agar supplemented with 4 mM formaldehyde. *S. cerevisiae* transformants were grown in YEPD medium supplemented with 4 mM formaldehyde.

Protein expression and purification

Yeast cells carrying human *PDIA3* gene were grown for 36 h in YEPD medium. Cells were separated from the medium by centrifugation at 2000*g* for 10 min. Yeast growth medium was further prefiltered through qualitative filter paper (VWR, cat. No. 516-0812) with subsequent microfiltration through 1.6 μM (SartoriusStedim Biotech, cat. No. FT-3-1101-047), 0.45 μM (SartoriusStedim Biotech, cat. No. 15406-47) and 0.2 μM (SartoriusStedim Biotech, cat. No. 15407-47-MIN) filters. After microfiltration, proteins were concentrated and transferred into the binding buffer (20 mM Tris–HCl, pH 8.0) through tangential ultrafiltration using cassettes with 50 kDa cut-off membranes (SartoriusStedim Biotech, cat. No. VF20P3). Further, proteins were incubated for 30 min. with heparin Sepharose (GE Healthcare, cat. No. 17-0998-01) in batch format. Unbound proteins were removed, while bound proteins were eluted with step NaCl (150 mM – 250 mM – 350 mM) gradient. Elution fractions were analyzed by SDS–PAGE and all showed more than 90% pure human ERp57 protein. All fractions were pooled and dialyzed against 20 mM Tris–HCl, pH 8.0, 100 mM NaCl buffer.

Insulin turbidity assay was performed as described previously [19,30]. The assay mixture was prepared in a cuvette by addition of 50 μL insulin (Sigma–Aldrich, cat. No. I2643) (1 mg/mL in 100 mM potassium acetate pH 7.5, 2 mM EDTA) plus tested protein and water to give a final volume of 60 μL. The reaction was started by pipetting 2 μL dithiothreitol (10 mM) in a cuvette. The cuvette was then thoroughly mixed and placed in the spectrophotometer (Tecan's Infinite M200). The measurements were performed at 650 nm using 60-s recordings. Assays lasting up to 60 min were not mixed further. The onset of aggregation was defined as the time where OD650 had reached the value of 0.025. The enzyme concentration at which this occurred was plotted against the onset of aggregation in order to obtain a concentration-dependent activity curve for the reductase activity of each oxidoreductase. *E. coli* thioredoxin was purchased from Sigma–Aldrich (cat. No. T0910) and *E. coli*-produced recombinant human ERp57 was purchased from Nordic BioSite (cat. No. PAT-80438-1).

Preparation of crude yeast lysates, SDS–PAGE and Western blotting were performed exactly as described previously [27].

Other methods

N-terminal sequencing of yeast secreted human ERp57 protein by Edman degradation was performed by AltaBioscience. The molecular mass of protein was measured by electrospray mass spectrometry using Agilent Q-TOF 6520 mass spectrometer. Protein concentrations were determined by Roti-Nanoquant Protein-assay (Carl Roth Gmbh., cat. No. K880).

Densitometric analysis of SDS–PAGE gels scanned with Image-Sanner III (GE Healthcare) was performed with ImageQuant TL (GE Healthcare) software using default settings.

Precipitation of proteins from yeast growth medium for SDS–PAGE analysis was performed based on a defined methanol–chloroform–water mixture as described earlier [31].

Results and discussion

Construction of yeast vector for expression of human ERp57

Human ERp57 coding gene (*PDIA3*, Acc. No. U42068) was cloned under constitutive yeast *PGK1* promoter in pFDC vector, yielding pFDC-hERp57 plasmid (Fig. 1), as it was described previously [27]. Briefly, human *PDIA3* gene was cloned from human adult liver cDNA library (Clontech) using primers that generate *XbaI* restriction sites on both ends of the gene, allowing restriction cloning into the *Xba*I site of pFDC vector between yeast *PGK1* promoter and terminator.

Human ERp57 protein expression, purification and characterization

Yeast expression vector pFDC-hERp57 was transformed into the *S. cerevisiae* strain AH22. Yeast cells harboring human *PDIA3* gene were grown in YEPD medium and secreted native recombinant human ERp57 protein was purified to 90% purity as described above (Fig. 2). According to data obtained from densitometric analysis of

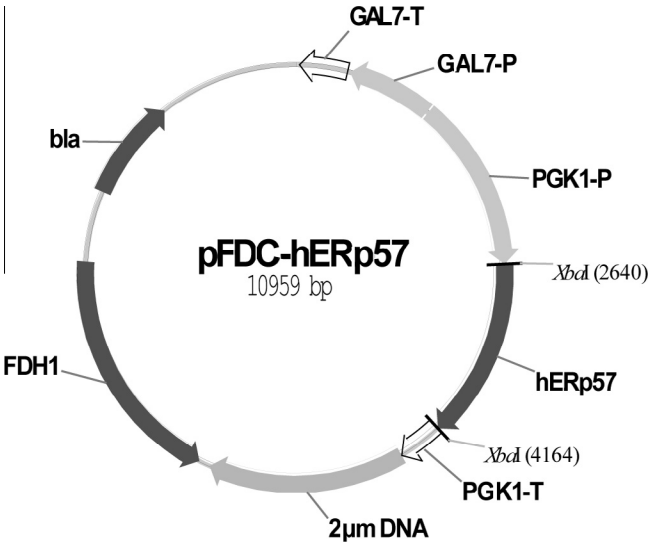

Fig. 1. Yeast expression vector pFDC-hERp57. pFDC vector contains one inducible expression cassette under control of the *Saccharomyces cerevisiae* GAL7 promoter with corresponding transcription terminator and the other cassette under control of the constitutive *S. cerevisiae* promoter PGK1 with corresponding transcription terminator. 2 μm DNA, 1.74 kb fragment of yeast 2 μm DNA. PGK-P, *PGK1* gene promoter (- 1 to - 541 bp); PGK-T, *PGK1* gene transcription terminator (371 bp). GAL7-P, *GAL7* gene promoter (- 1 to - 716 nt); GAL7-T, GAL7 gene transcription terminator (381 bp); FDH1, *FDH1* gene of *Candida maltosa* conferring resistance to formaldehyde; bla – beta lactamase gene conferring resistance to ampicillin; hERp57 – human ERp57 encoding gene (*PDIA3*, Acc. No. U42068).

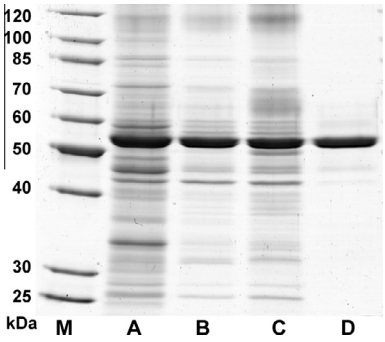

Fig. 2. Purification of secreted recombinant human ERp57 protein from yeast culture medium. M – unstained protein ladder (ThermoScientific, cat. No. 26614). A – crude yeast growth medium (20x concentrated), B – yeast growth medium after microfiltration (20x concentrated), C – 20x concentrated proteins from yeast growth medium in binding buffer after tangential ultrafiltration; D – purified yeast-derived recombinant human ERp57 protein (2 μg).

SDS–PAGE gels, secreted human ERp57 protein constitutes for approx. 30% of all yeast secreted protein (Fig. 2 lane A), subsequent microfiltration increases its purity to approx. 50% (Fig. 2 lane B) and one-step affinity chromatography using heparin Sepharose is enough to reach over 90% purity (Fig. 2 lane D). Yields obtained were approx. 9 mg from 1 L culture medium with purification efficiency reaching up to 90% (Table 1). In summary, secretion of human ERp57 into the yeast growth medium allows simple and cost-effective purification of native recombinant protein.

N-terminal sequencing by Edman degradation was performed for identification and characterization of purified secreted protein. The first five N-terminal amino acids of the recombinant protein were NH_2-SDVLE, which corresponds the N-terminal sequence of mature human ERp57 protein after signal cleavage [28]. These results indicate, that native ER signal sequence of human ERp57 protein is recognized and correctly processed in yeast cells, and this allows translocation of recombinant protein into the ER [27] following unexpected secretion outside the yeast cell. *S. cerevisiae* alpha-mating factor signal sequence is usually employed for secretion of recombinant proteins in yeast, since native secretion signals are rarely effective [32–34], even though usage of native secretion signals offers several advantages. First of all, it simplifies the cloning of the gene, and, most importantly, it allows secretion of recombinant protein without any additional amino acids. In contrast, some additional amino acids are usually introduced into recombinant product when using non-native signal sequences [35].

Mass spectrometry results of yeast-derived purified human ERp57 protein showed the mass of 54265.55 Daltons, which exactly corresponds to theoretically predicted mass of mature human ERp57 (25–505 aa) (Fig. 3). It indicates two things: (a) recombinant human ERp57 protein is exactly the same polypeptide as mature human ERp57 (including predicted ER retention signal QEDL on the C terminus of the protein) and (b) it has no yeast-derived modifications – a very important characteristic for recombinant proteins. Moreover, mass spectroscopy analysis revealed that the protein was highly pure (Fig. 3).

The presence of intact QEDL sequence in recombinant human ERp57, which usually serves as ER retention signal in human cells, raises question about reasons of secretion of human protein by yeast cells and about retention of proteins in the ER in general. As already mentioned, in some cases ERp57 was found on the surface of the human cells, and this suggests several important functions for the protein [4]. Secretion of human ERp57 protein by *S. cerevisiae* cells could be explained by yeast preference for the HDEL rather than QDEL signal for the retrieval of ER-residing proteins [36], but it is not the reason in this case, because replacement of QEDL with the HDEL sequence did not suppress the secretion of ERp57 (our unpublished data). Also, overload of the yeast ER retrieval machinery can be omitted as the reason for secretion of human ERp57, because overexpression of yeast KAR2 protein with native HDEL ER retrieval sequence using the same pFDC vector did not lead to the secretion of this protein (our unpublished data). Moreover, human PDI, which is homologue of human ERp57 and yeast PDI proteins and contains KDEL ER retrieval sequence, was also

Table 1
Summary of purification of *secreted human ERp57* recombinantly produced in *S. cerevisiae*.

Step	Amount of ERp57 (mg)	Purity of ERp57 (%)	Yield (%)	Volume (mL)
Crude yeast growth medium	10–12	30	100	1000
Prefiltration (filter paper)	10–12	30	100	1000
Microfiltration (1.6 μM)	10–11	40	90–100	1000
Microfiltration (0.2 μM)	10–11	50	90–100	1000
Ultrafiltration	9–9.5	40	75–90	50
Heparin Sepharose	9	95	75–90	10

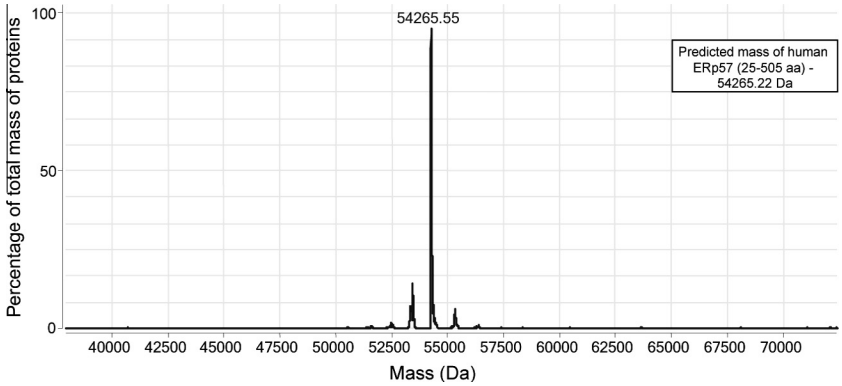

Fig. 3. ESI-MS of recombinant human ERp57 purified from *S. cerevisiae*.

expressed using the same pFDC vector, and in this case secretion of recombinant protein was not observed (our unpublished data). These experiments indicate that retention of ER luminal proteins is complicated and still unsolved mechanism, which does not strictly depend on HDEL/KDEL retrieval mechanism. Our finding, secretion of human ERp57 by yeast cells, could serve as a convenient model for studying this phenomenon.

As it was mentioned, human ERp57 protein was expressed previously in *S. cerevisiae* and found inside the cell [27]. Here we have shown that this protein is also secreted. To determine the efficiency of secretion of human ERp57 protein driven by its native signal sequence, we compared amounts of intracellular and secreted ERp57 protein. SDS–PAGE analysis of crude lysates harboring pFDC-hERp57 plasmid did not reveal clear additional band of recombinant human ERp57 compared to control cells carrying pFDC vector (Fig. 4A, lanes pFDC and pFDC-hERp57). However, quantitative Western blot using antibodies against human ERp57 protein (Fig. 4B) showed considerable intracellular expression of ERp57 constituting for ~1.3% of total cell protein. Evaluation of amount according to cell biomass produced from 1 L of culture suggested that approx. 80% of ERp57 was expressed internally (approx. 50 mg), whereas up to 20% was secreted into the culture medium (approx. 10 mg). Similar yeast-expressed amounts and ratio of secreted to intracellular recombinant protein were previously reported for bovine pancreatic trypsin inhibitor (BPTI), as intracellularly accumulated BPTI constituted 1.2–1.4% of total cell protein [37]. Secretion efficiency varies markedly among proteins and can be particularly low for overexpressed heterologous proteins [38–43]. Therefore, secretion efficiency of human ERp57 in yeast cells can be defined as moderate and is sufficient for generation of large amounts of purified protein. Moreover, there are a many ways to improve secretion efficiency of successfully expressed recombinant protein; however, this was beyond the scope of this study.

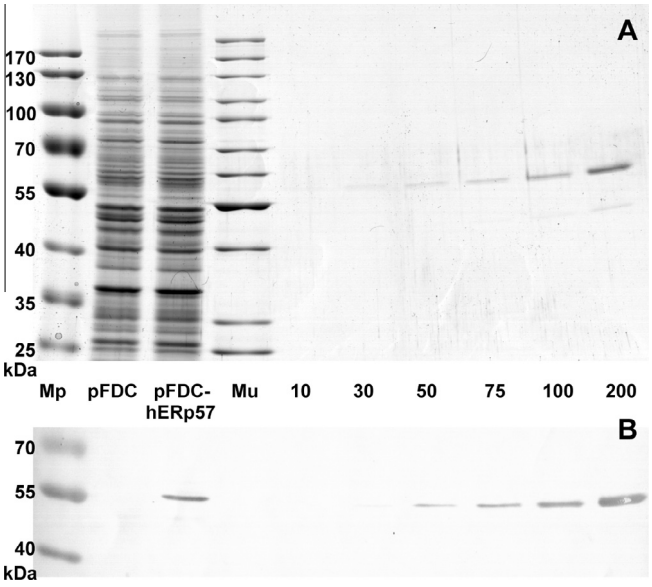

Fig. 4. Evaluation of amount of intracellularly accumulated human ERp57 protein in yeast *S. cerevisiae* cells. (A) SDS–PAGE of crude yeast lysates and indicated amounts of purified ERp57; (B) Western blot using monoclonal antibodies against human ERp57. Mp – prestained protein ladder (ThermoScientific, cat. No. 26618), Mu – unstained protein ladder (ThermoScientific, cat. No. 26614). pFDC and pFDC-hERp57 – crude lysates (10 μg of whole cell protein in each lane) of yeast cells transformed with pFDC vector and pFDC-hERp57 plasmid, respectively. 10, 30, 50, 75, 100, 200 – amounts in nanograms of purified secreted human ERp57 protein loaded on gel.

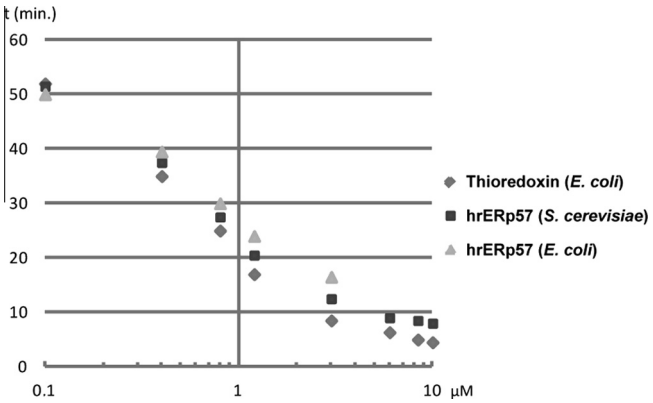

Fig. 5. Thiol-dependent catalytic activity of yeast-derived recombinant human ERp57 protein assayed using the insulin precipitation method. Various concentrations of *E. coli* thioredoxin (♦), purified *S. cerevisiae* secreted human recombinant ERp57 (■) and human recombinant ERp57 purified from *E.coli* (▲) were tested for their ability to catalyze the reduction of 130 mM insulin by 0.33 mM DTT. The onset of aggregation was defined as the time when the optical density at 650 nm had reached a value of 0.025 and was plotted against the concentration of catalyst used. Data are the average of three independent experiments. Error bars were too small to be visible. Note – activity of recombinant *E.coli*-derived human ERp57 at higher concentrations was not measured due to the absence of large amount of the protein.

Activity of yeast-derived recombinant human ERp57 protein was measured by insulin turbidity test that is often used to characterize protein disulfide isomerases [19,23,30,44]. Recombinant ERp57 exhibited thiol-dependent reductase activity which catalyzes the reduction of insulin disulfides by dithiothreitol (Fig. 5). Reductase activity of the protein was compared to *E. coli* thioredoxin and commercially available *E. coli*-derived recombinant human ERp57. As it is shown in Fig. 5, both recombinant human ERp57 proteins catalyzed the reduction of insulin in slower rate than thioredoxin, in agreement with the results of the previous studies [19,30]. Nevertheless, activity of yeast-secreted human ERp57 was slightly but reliably higher than that of *E. coli*-derived protein. This may be explained by the fact that yeast-secreted human ERp57 must undergo thorough protein quality control throughout the yeast secretion pathway, which allows secretion of correctly folded proteins only, meanwhile *E. coli*-synthesized ERp57 is purified by capturing all histidine tag-containing proteins independently of their folding state. In summary, our method for production of native recombinant human ERp57 yields active protein, thus enabling its application in various studies.

Conclusions

We introduced a novel method for production of recombinant human ERp57 protein. *S. cerevisiae* allows production of human thiol-disulfide oxidoreductase ERp57 in eukaryotic endoplasmic reticulum, where environment is well suited for maturation of such proteins. Here we demonstrate that the native signal of human ERp57 protein is correctly cleaved and drives secretion in yeast cells. Sequence of yeast-secreted human ERp57 fully corresponds to mature protein from human cells, and protein is free of yeast-derived modifications. Secretion of human ERp57 into the yeast culture medium not only allows efficient, simple and cost-effective one-step purification of the protein, but also ensures its higher activity compared to *E. coli*-produced ERp57. In conclusion, *S. cerevisiae* is the perfect host for production of human ERp57 protein and also could serve as a convenient model for studying the retention of ER luminal proteins.

Conflict of interest

A patent application has been filed for the technology disclosed in this publication.

Contributions

E.Č. designed research, analyzed data and wrote the manuscript. E.Ž. executed the experiments. R.S. was involved in protein purification experiments, also helped to analyze data and reviewed the manuscript.

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