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Production of heterologous proteins using *S. cerevisiae* expression system

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In the present work, yeast expression vectors bearing either thioredoxin-encoding gene fused with Abeta peptide (*trx-ab*) or its mutant sequence or glucose dehydrogenase gene fused with Abeta (*GDH-ab*) under the control of galactose-inducible CYC1-GAL promoter were constructed. After a successful transformation of yeast *Saccharomyces cerevisiae* strains, 3PMR-1, 21PMR and α'1 the eukaryotic producents of the hybrid proteins were isolated. Analysis of stability of heterologous expression plasmids indicated a 90–95% maintenance of auxotrophic markers and only 50% of that of *trx-ab* or *GDH-ab* genes. Optimization of cultivation allowed a synthesis of ~20 μg of thioredoxin-Abeta and GDH-Abeta proteins from 1 g of wet yeast biomass.

Key words: *Saccharomyces cerevisiae*, heterologous expression system, hybrid proteins, Abeta peptide, thioredoxin, glucose dehydrogenase

INTRODUCTION

Recombinant DNA expression constitutes a major approach in gene function studies that naturally complement genetic and genomic research. Well-regulated expression systems provide an invaluable tool for investigating the cellular roles of novel genes either in their original cellular environment or in specialized host organisms [1]. These systems can be utilized to observe the biological effects of the controlled expression (or lack of it) of a given DNA sequence. Very often they also provide the means to produce and purify a desired gene product, opening the way to a comprehensive analysis and manufacture of proteins of biotechnological interest [2].

Traditionally, the expression and purification of heterologous proteins is accomplished using prokaryotic systems such as *Escherichia coli* or *Bacillus subtilis*. However, when the expression of eukaryotic proteins is desired, bacterial systems often fail because of a limited capacity to perform multi-step post-translational modifications such as protein *N*-glycosylation, phosphorylation and acetylation. Moreover, the production of bacterial toxins and their derivatives is highly desirable; however, attempts at heterologous expression using the traditional *E. coli* expression system are often problematic due to the formation of inclusion bodies that severely limit the final yields of a biologically active product [3].

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Unicellular eukaryotes such as the yeasts *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Kluyveromyces lactis* and *Schizosaccharomyces pombe* have become important systems in biotechnology for heterologous protein expression for both academic and industrial purposes. Yeasts combine the ability to grow on a simple medium at a very high cell density and secrete heterologous proteins. Moreover, they perform many of eukaryotic post-transductional modifications such as protein folding, proteolytic processing, disulfide bond formation and glycosylation [4].

S. cerevisiae has been widely employed as a host organism for expression of heterologous proteins, using regulated systems able to provide an accurate control of gene expression in the functional analysis, and the timely recombinant protein synthesis during fermentative production [2, 5]. For this purpose, yeast-derived promoters are used: *MET3* negatively regulated by the amino acid methionine, *PHO5* negatively regulated by inorganic phosphate, *CUP1* activated by Cu^{2+} ions, both *GAL1* and *GAL10* activated by galactose while repressed by glucose [2, 6].

In the present work, the expression of hybrid proteins consisting of Abeta40 peptide fused to bacterial thioredoxin (trx) or pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (GDH) was analysed in yeast *S. cerevisiae*. An aggregated form of β-amyloid peptide (Abeta), both 40 and 42 amino acid long peptides, is the principal component of amyloid in the core of plaques, which is characteristic of Alzheimer's

disease [7]. Thioredoxin, a product of the *E. coli trxA* gene, is 12 kDa protein acting as antioxidant by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange [8]. The PQQ-dependent dimeric glucose dehydrogenase from *Acinetobacter calcoaceticus* oxidizes a wide range of mono- and disaccharides to the corresponding lactones and is able to donate electrons to various artificial electron acceptors; also, it is a potential biocatalyst for an accurate monitoring of glucose in blood samples [9]. An attractive feature of Abeta peptide to form the β-sheet fibrils was on the target in creating novel hybrid proteins able to form self-assembled nanostructures. The selection of the fused-partner is based on a possible practical application of hybrids in various areas such as biotechnology, molecular electronics, medicine, etc. The cloning, expression and purification of trx-ab and GDH-ab hybrid proteins have been previously performed using prokaryotic cells (data not published) but have never been studied in a eukaryotic system. Given that the origin of the individual partners of the fused derivatives is both prokaryotic and eukaryotic, the expression peculiarities of these hybrids in eukaryotic organisms have a certain value to be studied.

MATERIALS AND METHODS

The yeast expression plasmid pBK containing the *S. cerevisiae* K2 preprotoxin gene under the control of the inducible CYC-GAL1 promoter, as well as *URA*3 and *leu*2-d auxotrophic markers [10] and bacterial plasmids pET3a-abeta40 (donation of Dr. A. Olofsson), pET3a-abetacys2,39 bearing the double cys mutant of Abeta40 (S. Bruzyte, unpublished) and pTAbEcoI containing a GDH-abeta sequence (kindly provided by Dr. V. Časaitė, unpublished) were used for constructing the recombinant plasmids pBK-trx-ab, pBK-trx-abcys2,39 and pBK-GDH-ab, respectively.

General procedures for the construction and analysis of recombinant DNAs were performed as described by Sambrook et al. [11]. All restriction enzymes (*Sma*I, *Bam*HI, *Pst*I), T4 DNA ligase, bacterial alkaline phosphatase, the Klenow fragment, Pfu DNA polymerase and DNA size marker (GeneRuler™ DNA Ladder mix) were obtained from Fermentas (Lithuania) and used according to the manufacturer's recommendations.

For expression of the hybrid proteins, *S. cerevisiae* strains 3PMR-1 (*MAT*α *ura3-52*); 21PMR (*MAT*α *leu2 ura3-52*) and α'1 (*MAT*α *leu2*) were used [12]. Media for the propagation of *S. cerevisiae* yeast as well as standard genetic techniques were described in Ausubel et al. [13]. The *E. coli* strain DH5α (*F⁻ (ϕ80dΔ(lacZ)M15) recA1 endA1 gyrA96 thi1 hsdR17* ($r_k^- m_k^+$) *supE44 relA1 deoR Δ(lacZYA-argF) U169)* was used for plasmid isolation and maintenance [14].

Transformation of *S. cerevisiae* strains was performed using the LiAc/PEG method [15], and the transformants were selected by complementation of *URA3* or *LEU*2 auxotrophy followed by isolation of a plasmid of interest and PCR analysis. The transformation of *E. coli* was carried out using the calcium chloride method [11, 13].

Protein production was analysed after cultivation of clones under appropriate conditions. Yeast cells were harvested by centrifugation (3000 g) at 4 °C for 10 min, the biomass was (1 g) resuspended in 2 ml of A buffer (for trx-ab: 50 mM Tris-HCl, pH 8.5, 100 mM NaCl, 10% glycerol; for GDH-ab: 50 mM Tris-HCl, pH 8.5, 10% glycerol) and grinded using liquid nitrogen. The cell extract was cleared by centrifugation (11000 g) at 4 °C for 15 min. Purification of the fused trx-ab protein was performed by metalochelate separation on a HiTrap HP column. GDH-ab was purified by an ion-exchange chromatography on a CM-cellulose column according to the manufacturer's instruction (GE Healthcare). The concentration and purity of proteins were estimated from 15% SDS-PAGE data after visualization using Coomassie Brilliant Blue [13].

RESULTS AND DISCUSSION

The principal advantages of *S. cerevisiae* yeast in expressing recombinant proteins were used to study the expression peculiarities of the fused bacterial thioredoxin or glucose dehydrogenase genes with the Abeta peptide sequence.

Basing on *S. cerevisiae* expression system pBK prepared by using *Sma*I and *Bam*HI restriction endonucleases, the recombinant plasmids pBK-trx-ab and pBK-trx-abcys2, 39 were obtained by inserting 580 bp DNA fragments of *trx-ab* and *trx-abcys2,39* amplified by PCR (templates: pET3a-abeta40 and pET3a-abetacys2,39 and oligonucleotide primers: 4036F and 4612R) (Table), digested with *Xba*I restriction endonuclease and blunted with the Klenow enzyme with a subsequent *Bam*HI restriction [11] (Fig. 1. A). The *GDH-ab* fragment was PCR-amplified using pTAbEcoI plasmid as a template (primers: 3021F and 3021R) (Table) for the construction of pBK-GDH-ab. The fragment was digested with *Bam*HI and *Pst*I and inserted into the pBK vector linearized by the same enzymes (Fig 1. B). It is important to note that in all these constructs the expression of fused genes is regulated by a galactose-inducible CYC-GAL1 promoter.

The newly created heterologous expression plasmids were introduced into *S. cerevisiae* 3PMR-1 *ura*3-52, 21PMR *ura*3-52 *leu*2 and α'1 *leu*2 strains, and the transformants were selected for uracil or leucine auxotrophy. The screening of transformants by leucine marker was found to lead to an internal plasmids' instability and the formation of different phenotypic groups depending on the morphology of the colonies. This effect had been observed previously and depended on the defective leucine gene *leu*2-d action [16]. Additionally, we have found that the presence of *Abeta* sequence in combination with *leu*2-d decreases the efficiency of transformation, and yeast colonies appear only after 10–14 days in the case of selection for leucine auxotrophy, in contrast to 3–5 days for a regular transformation. We have determined that the stability of the new constructs, monitored by maintaining the *URA*⁺ or *LEU*⁺ phenotype, reaches 90–96%, while the stability by Abeta is only approximately 50%. The data

Table. **Sequences of oligonucleotide primers**

Name	Sequence (5' to 3')
4036F	CACAACGGTTTCCCTCTAGA
4612R	TCCTTTCGGGCTTTGTTAGC
3021F	GCATGGATCCAAATGAATAAACATTTATTG
3021R	GCATCTGCAGCACTTCACAGGTCAAGC
EclNcoF	CGCCATGGAGCTCGACGCTGAATTCCGTCACG
SnaBR	GCCGGATCCTCTACGTAAACAACACCACCAACCATC

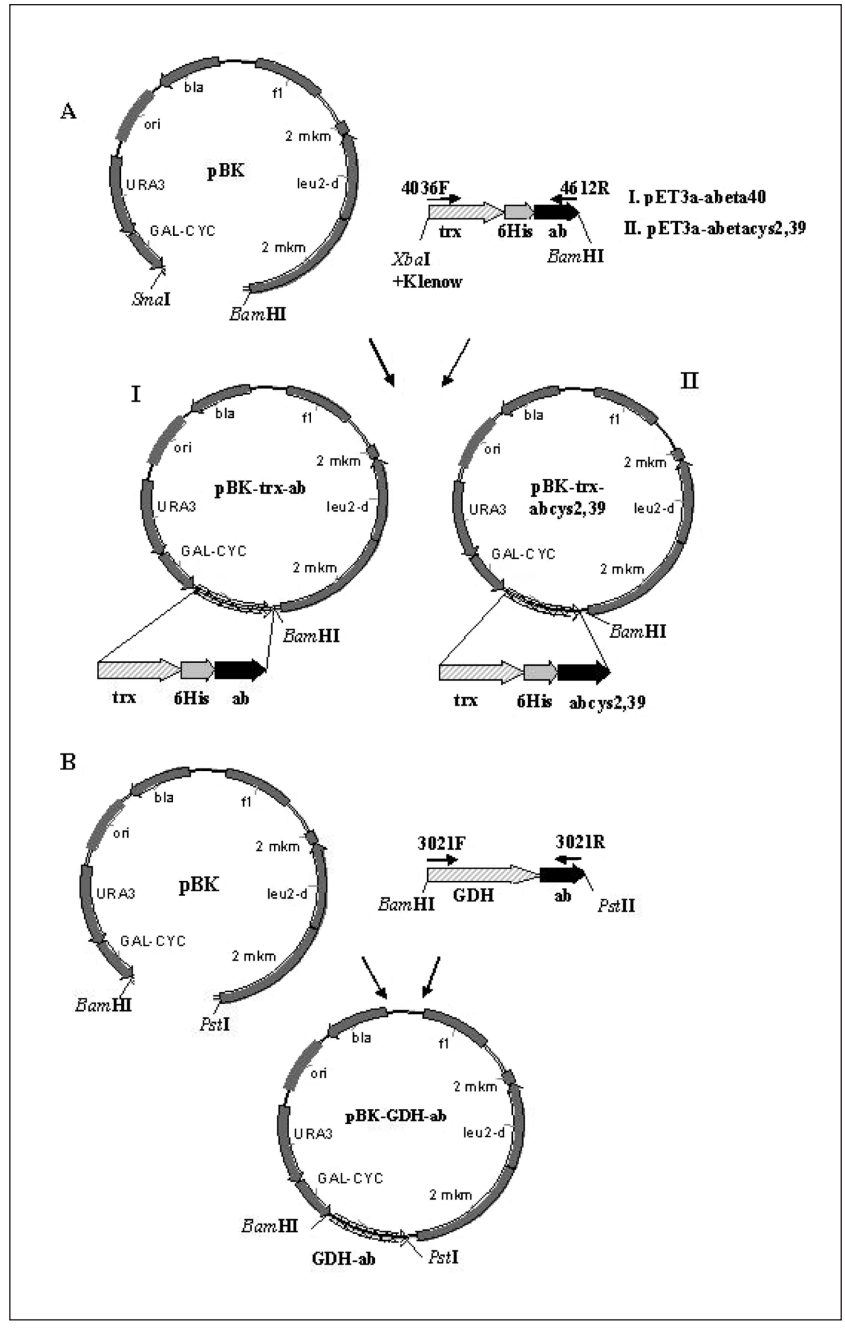

Fig. 1. Construction scheme of plasmids pBK-trx-ab, pBK-trx-abcys2,39 (A) and pBK-GDH-ab (B).
URA3, leu2-d – genetic markers; 2 mkm – sequence originated from 2 μ plasmid of *S. cerevisiae; bla* – gene for β-lactamase; ori – pMB1 replication origin; GAL-CYC1 – galactose inducible promoter; *trx* – thioredoxin gene; 6His – hexahistidine linker; *ab* – Abeta polypeptide sequence; *abcys2,39* – Abeta with cysteine in 2 and 39 positions; *GDH-ab* – glucose dehydrogenase gene fused with Abeta; *Sma*I, *BamH*I, *Pst*I – restriction endonuclease sites

were confirmed by PCR analysis. It is possible that the toxicity of Abeta peptide influences the yeast survival. In order to analyse the accuracy of the fused sequence, the recombinant plasmids were maintained in yeast, isolated and verified by PCR analysis. The primer pairs EclNcoF/SnaBR and 3021F/3021R were used for the analysis of the *trx-ab* and *GDH-ab* genes containing plasmids, respectivelly (Table).

In the next step of this work, we investigated the growth conditions of persisting yeast transformants in order to achieve the maximal production of the hybrid proteins. Cultures were grown in liquid YEPD (2% glucose), YEPG (3% galactose) or minimal media supplemented with galactose or glucose by changing induction time and cultivation temperature. We have found that only the 21PMR strain bearing the recombinant constructs of interest possesses a satisfactory transformation efficiency and is able to produce a detectable amount of the hybrid protein. The trx-ab and GDH-ab proteins accumulate during the growth of 21PMR [pBK-trx-ab] and 21PMR [pBK-GDH-ab] transformants at 18 °C in the minimal medium containing galactose as a carbon source and supplemented with uracil. The highest level of both hybrid proteins was achieved only after culturing yeasts for 96 h. However, the clone 21PMR [pBK-trx-abcys2,39] produced a detectable amount of the fused protein trx-abcys2,39 after the growth in YEPG medium at 18 °C for 96 h. In all the other cases, shortening the cultivation time (24 h, 48 h or 72 h) led to the accumulation of an insufficient amount of biomass or the protein started to degrade after a too long incubation (120 h). An increase of induction temperature from 18 °C to 23 °C or more resulted in a barely detectable amount of expressed soluble proteins. The differences

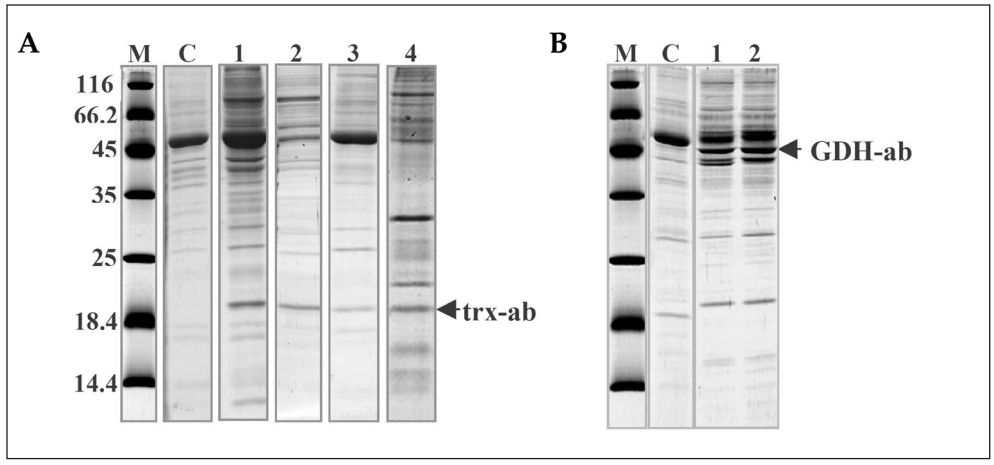

Fig. 2. SDS-PAGE electrophoresis of hybrid proteins.

A. Coomassie Brilliant Blue-stained gel of trx-ab and -trx-abcys2,39 fused proteins purified on a chelating column. Lane M – unstained protein molecular weight marker (UAB Fermentas), masses are shown in kDa; lane C – proteins of the untransformed 21PMR yeast strain purified on Ni-column; lane 1 – a single-step sample purification; His-tagged trx-ab eluted using 500 mM imidazole; lane 2 – gradient purification of trx-ab sample, elution at 250 mM imidazole; lane 3 – a single-step purification of trx-abcys2,39; lane 4 – a single-step purification of trx-ab in presence of 6 M urea

B. Coomassie Brilliant Blue-stained gel of GDH-ab fused protein purified on CM column. Lane M – protein molecular weight marker; lane C – proteins of the untransformed 21PMR yeast strain purified on CM-column; lanes 1, 2 – purification of GDH-ab by ion-exchange chromatography; samples eluted at 250 mM and 300 mM NaCl, respectively)

in the expression level were observed between clones after two or more passages on solid media. Only a freshly-transformed yeast culture yielded the highest amount of fused proteins (data not shown).

Single-step Ni-chelating chromatography was applied for purification of both trx-ab and trx-abcys2,39 proteins. The proteins were eluted with 50 mM Tris-HCl buffer, pH 8.5, containing 500 mM imidazole. A protein band migrating at 20 kDa, corresponding to the expected size of trx-ab, was detected after SDS electrophoresis (Fig. 2 A, lanes 1, 3). The gradient purification approach showed that the protein of interest could be eluted from the column at a 150–200 mM imidazole concentration (Fig. 2 A, lane 2). The maximal yield of the expressed protein reached approximately 20 μg from 1 g wet weight of yeast cells.

Purification of GDH-ab was performed on a CM ion-exchange column. It was demonstrated that 45 kDa hybrid protein-enriched fractions were eluted at a NaCl concentration of 250–300 mM. The level of the heterologous protein expressed in yeasts was similar to trx-ab and reached about 25–30 μg / 1 g wet yeast culture (Fig. 2 B, lanes 1, 2).

Interestingly, using both metalochelate separation and ion-exchange chromatography, an approximately 52 kDa protein (high density band on SDS-PAGE) was observed (Fig. 2 A, lanes 1, 3; B, lanes 1, 2). Purification of the hybrid protein showed an interaction between trx-ab (or GDH-ab) and the "leading" protein which can be distorted in the presence of 6 M urea (Fig. 2 A, lane 4). In this case, only the His-tagged trx-ab binds to the Ni-column. It is possible that the interaction partner might be involved in the regulation of transport, folding and / or processing of the hybrid proteins (in a particular case of an Abeta peptide-bearing construct) and requires additional experiments to be proven.

Thus, the expression and purification of both trx-ab and GDH-ab proteins have been successfully performed using yeast *S. cerevisiae*. The highest production output of the hybrid proteins was achieved by growing the yeast strains 21PMR [pBK-trx-ab] and 21PMR [pBK-GDH-ab] under inducing conditions of 18 °C for 96 h. The yield from 1 g of the yeast culture reached about 20–25 μg of the target proteins.

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HETEROLOGINIŲ BALTYMŲ PRODUKCIJA PANAUDOJANT *S. CEREVISIAE* EKSPRESIJOS SISTEMĄ

Santrauka

Šio darbo metu sukonstruoti mielių ekspresijos vektoriai pBK-trx-ab, pBK-trx-abcys2,39 ir pBK-GDH-ab, kuriuose hibridinių baltymų – tioredoksino, sujungto su Abeta polipeptidu bei jo mutantiniu vediniu, ir gliukozės dehidrogenazės, sujungtos su Abeta peptidu – raiška yra reguliuojama galaktoze indukuojamu CYC1-GAL promotoriumi. Atlikus sėkmingą *Saccharomyces cerevisiae* 3PMR-1, 21PMR ir α'1 mielių kamienų transformaciją rekombinantinėmis plazmidėmis, atrinkti optimalūs heterologinių baltymų producentai. Optimizavus kultūrų auginimo sąlygas, pasiekta ~20 μg tioredoksino-Abeta bei GDH-Abeta produkcija iš 1 g 21PMR[pBK-ab-trx] ir 21PMR[pBK-GDH-ab] mielių biomasės. Ištyrus sukonstruotas DNR plazmides, nustatytas didelis (90–95%) auksotrofinių žymenų stabilumas ir tai, kad tik apie 50% *trx-ab* arba *GDH-ab* genų išsilaikė mielėse.