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Toxicity of α-synuclein and Aβ40 in *Saccharomyces cerevisiae*

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Abstract

Saccharomyces cerevisiae expression and secretion systems bearing glucose dehydrogenase gene fused with Aβ40 or SNCA sequences were generated and applied to study toxicity of amyloid-β and α-synuclein in yeast. Analysis of transformant growth demonstrated that cytoplasm-localized Aβ40 peptide exhibits higher toxicity to the yeast cells than α-synuclein. Arrangement of Aβ-bearing hybrid to the secretory pathway reduced the toxic effect for host cells.

Keywords: Amyloid-β, α-synuclein, glucose dehydrogenase, toxicity, yeast

Introduction

Accumulation of amyloid-β (*Aβ*) or α-synuclein (*SNCA*) proteins and formation of oligomeric structures in the brain are associated with Alzheimer's (AD), Parkinson's (PD) and other neurodegenerative diseases causing human mental disabilities and mobility limitations (L. CREWS & al. [1]).

Amyloid-β is 39-42 residue peptide produced by proteolysis from a large transmembrane glycoprotein carrying out the nutritional and protective functions. *Aβ* N - terminal domain form complexes with metal ions, affecting kinetics of protein accumulation, morphology and toxicity. Central and C-terminal domains of the protein promote aggregation of *Aβ* peptide (N. FOX & al. [2]; W. KIM & al. [3]). Although *Aβ* accumulation and aggregation clearly plays a role in AD, recent studies indicate that insoluble fibrils themselves may not be the toxic species (W. KIM & al. [4]). However, soluble oligomeric forms of *Aβ* are neurotoxic *in vivo* and *in vitro* (G. BITAN & al. [5]).

α-Synuclein is an abundant, presynaptic 140 residue protein containing seven imperfect N-terminal repeats, presumed to function in vesicle binding. The middle portion is a hydrophobic domain important in the aggregation of α-synuclein (P. ZABROCKI & al. [6]). Although the precise physiological role of this protein is not fully understood, it has been suggested that *SNCA* is involved in the modulation of neurotransmitter release, ER/Golgi trafficking (M. VILAR & al. [7]). In mammalian cells, α-synuclein has been found in the nucleus, cytosol, associated with membranes and, in diseased brains, in large cytoplasmic inclusions. α-Synuclein oligomers are found to be the major cause of cellular toxicity (B. CAUGHEY & al. [8]). On the other hand, cytotoxicity of *SNCA* has been correlated to a dosage effect by protein overexpression resulting in supernumerary copies of the *sncA* gene (M. VILAR & al. [7]).

In order to investigate the mechanisms of action of these proteins, to characterize aggregation processes and the factors regulating them, extensive studies were carried out (L. CREWS & al. [1]). Single proteins or assembled in the form of hybrid constructs are

expressed in prokariotic as well as eukariotic systems, expression and purification methods optimized, features of fibril formation and possibilities of practical application are being analyzed (L. CREWS & al. [1]; P. ZABROCKI & al. [6]).

Different model systems have been developed to study the pathophysiology of neurodegenerative diseases; however, there is no model displaying all the hallmarks associated with AD or PD. Apart from transgenic rodents with stable or transient expression of particular proteins engaged in neurodegenerative diseases, less complex systems and organisms are in use, e. g. mammalian cell lines, *Drosophila melanogaster* and *Caenorhabditis elegants* (ZABROCKI & al. [6]). Most recently, humanized yeast cells were shown to recapitulate several fundamental aspects related to PD and the pathogenicity of α-synuclein as well as related to AD and processing of amyloid precursor protein (T. OUTEIRO & al. [9]). Given the strong conservation of protein folding, membrane trafficking and controlling factors between yeast and higher eukaryotes, *Saccharomyces cerevisiae* were used to uncover and establish basic aspects of both normal and abnormal α-synuclein biology (T. OUTEIRO & al. [9]).

In the present work *S. cerevisiae* expression and secretion systems were established and applied to study Aβ40 and α-synuclein toxicity by analysing stability of recombinant plasmids and dynamics of yeast growth.

Materials and Methods

Escherichia coli strain, plasmids and DNA manipulations

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)* D. WOODCOCK & al. [10]) was used for cloning experiments, plasmid isolation and maintenance. Transformation of *E. coli* was carried out using calcium chloride method or electroporation (F. AUSUBEL & al. [11]).

General procedures for the construction and analysis of recombinant DNAs were performed according to SAMBROOK & al [12]. All restriction enzymes (*Sac*I, *Bam*HI, *Pst*I), T4 DNA ligase, bacterial alkaline phosphatase, Pfu DNA polymerase and DNA size marker (GeneRuler™ DNA Ladder mix) were obtained from UAB Fermentas (Vilnius, Lithuania) and used following the manufacturer's recommendations.

Yeast expression plasmids pBK-GDH and pBK-GDHab (Figure 1), bearing *Acinetobacter calcoaceticus* glucose dehydrogenase gene (*gdh*) alone (GenBank accession number GC657400.1) or fused with human amyloid-β (*aβ40*) sequence (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV), were constructed as follows. First, coding sequence of *Aβ40* was amplified by PCR from the plasmid pET3a-trx-abeta40 (kindly provided by dr. A. Olofsson, Umea University) using primers EclNcoF 5'-CGCCATGGAGCTCGACGCTGAATTCCGTCACG-3' and SnabR 5'-GCCGGATCCTCTACGTAAACAACACCACCAACCATC-3'. For generation of in-frame fused *gdh:aβ*40 gene the obtained DNA fragment was cut by *Eco105I* and ligated into the *Eco47III* restriction site of pAI3-PT15 plasmid (Patent No. WO2004099399) resulting in pTAbEcoI. Then the *GDH:Aβ40* (1.65 kb) sequence was amplified by PCR from pTAbEcoI and *GDH* (1.5 kb) - from pAI3-PT15, respectively. The following primers were used: GDH_{Fw} 5'-CCCAGGTCTCAAATGAATAAACATTTATTG-3' and $GDHab_{Rw}$ 5'-AACACGGTCTCAGCGCTCTGAGCTTTATATG-3'. Amplified DNA fragments were cut by *BamHI* and *PstI* and cloned into *BamHI – PstI* sites of plasmid pBK (E. SERVIENE & al. [13]), downstream the GAL-CYC1 promoter.

The yeast secretion plasmid pYEsec1-GDHab (Figure 1) was obtained by the insertion of a *GDH:Aβ40* sequence into pYEsec1 plasmid (C. BALDARI & al. [14]), downstream the

GAL-CYC1 promoter and in frame with *Kluyveromyces lactis* toxin signal sequence. For this purpose, *GDH:Aβ40* was PCR-amplified from bacterial plasmid pTAbEcoI using previously described reverse oligonucleotide and the following forward primer 5'-GCATGGATCCAATAAACATTTATTGGC-3', cut by *BamHI* and *PstI* restriction enzymes and ligated to the receiving vector pYEsec1.

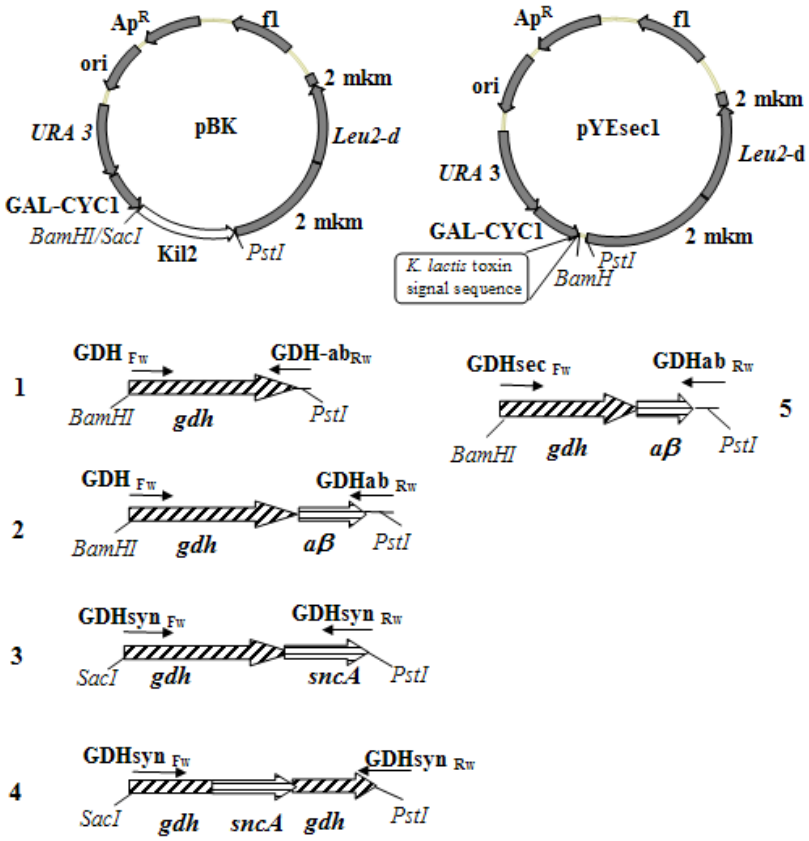

Figure 1. Principal scheme of pBK-GDH (1), pBK-GDHab (2), pBK-GDHsynEco (3), pBK-GDHsynPvu (4) and secretion pYEsec1-GDHab (5) plasmids.
*URA*3, *Leu*2-d – genetic markers; 2mkm – sequence originated from 2μ plasmid of *S. cerevisiae*; ApR – gene for β-lactamase; ori – pMB1 replication origin; GAL-CYC1 – galactose inducible promoter; f1 - f1 bacteriophage replication origin; Kil2 - *S. cerevisiae* K2 preprotoxin gene; *gdh* – glucose dehydrogenase gene; *sncA* – α-synuclein gene; aβ – amyloid-β (Aβ40) coding sequence; Fw and Rw – marked forward and revers primers; *BamHI, SacI, PstI* – restriction endonuclease sites.

Yeast recombinant plasmids pBK-GDHsynEco and pBK-GDHsynPvu (Figure 1), expressing *Acinetobacter calcoaceticus* glucose dehydrogenase and human α-synuclein (EMBL-Bank accession number AY049786.1) hybrid (*GDH:SNCA*), were obtained as follows. First, the *SNCA* gene was amlified from the plasmid pRK17 (R. HODARA & al. [15]) using SynF (5'-ATAGATATCGATGTATTCATGAAAGGACTTTC-3') and SynR (5'-TTTCGATATCTTAGCCGGCTTCAGGTTCGTAG-3') primers. Generated DNA fragment was digested by *Eco321* and ligated into *PvuII* (for the construction of plasmid pTsynPvu) or *Eco47III* (for the construction of pTsynEco) restriction sites of pAI3-PT15 plasmid. Then the PCR reactions were carried out using as a template plasmids pTsynEco and pTsynPvu and following primers: $GDHsyn_{Fw}$ 5'- GCATGAGCTCAAATGAATAAACATTTATTG-3' and $GDHsyn_{Rw}$ 5'- GCATCTGCAGCACTTCACAGGTCAAGC-3'. The resulting DNA products (2.0 kb) were digested by *SacI* and *PstI* enzymes and ligated to vector pBK, previously prepared by abovementioned restriction enzymes and dephosphorylation.

Plasmid DNAs were isolated using alkaline lysis method (H. BIRNBOIM & al. [16]) and performing column purification according to manufacturer recommendations (UAB Fermentas, Vilnius, Lithuania).

Yeast strains and culture conditions

For the expression of *GDH*, *GDH:Aβ40* and *GDH:SNCA* constructs *S. cerevisiae* strain 21PMR (MATα *leu2ura3-52*) was used (kindly provided by dr. T. Jokantaite). Media for propagation of *S. cerevisiae* yeast as well as standard genetic techniques have been described in F. AUSUBEL & al. [11]. Transformation of *S. cerevisiae* strains was performed following LiAc/PEG method (R. GIETZ & al. [17]) and transformants selected by complementation of *URA3* auxotrophy.

Determination of plasmid stability and growth rate of transformants

At various stages of growth, samples were diluted with sterile water and plated in triplicate onto selective and nonselective medium. Plates were incubated at 30 ^{o}C for 3-4 days. The percentage of the population still maintaining the plasmid was estimated by dividing the average number of colonies growing on selective plates against the average number of colonies growing without selective pressure. In order to analyse the accuracy of the fused sequences DNA from 50 colonies were isolated and verified by PCR analysis.

The cell growth was monitored every 24h by spectrophotometrically measuring OD_{600}. Yeast transformants were cultured at 18 ^{o}C for 24-96 h in liquid synthetic (SC-gal) or rich (YEPG) media with galactose as the carbon source. Experiments were repeated at least 3 times and the results of measured cell density averaged providing standard deviation.

Results and discussion

To study the toxicity of α-synuclein or Aβ40 polipeptide, both proteins were expressed in the well-defined yeast model system. High copy number yeast expression plasmids pBK-GDHsynEco, pBK-GDHsynPvu, pYEsec1-GDHab and pBK-GDHab were constructed (for details see Materials and Methods; Figure 1) and transformed into *S. cerevisiae* 21PMR strain. α-synuclein or Aβ40-encoding sequences were linked to glucose dehydrogenase gene. In case of pBK-GDHsynEco plasmid *sncA* was fused to 3‘ termini of *gdh*, while in pBK-GDHsynPvu it was inserted into the glucose dehydrogenase gene. For *GDH:Aβ40* we choose two constructs with intracellular (pBK-GDHab) and extracellular (pYEsec1-GDHab) synthesis of hybrid protein. In all cases expression of hybrids was controlled by GAL-CYC1 promoter, inducible by galactose.

Selected yeast transformants were grown in different liquid media (YEPG and minimal SC-gal) under the inducing conditions by varying cultivation time (24-96 h) and temperature (18-30 ^{o}C). Manipulations with growth were carried out in order to obtain the highest possible level of hybrid derivatives and to test toxic effect of α-synuclein and Aβ40. It was observed that highest hybrid production is obtained by the cultivation of transformants at 18 ^{o}C (S. ČEPONONYTĖ & al. [18]; S. PILEVIČIENĖ & al. [19]; E. SERVIENĖ & al. [20]). Stability of recombinant plasmids and integrity of fusion sequences were evaluated by genetic tests and PCR analysis. It was determined that stability of new constructs monitored by maintaining the URA^{+} or LEU^{+} phenotype reaches 90-96%, while the maintenance of *GDH:Aβ40* or *GDH:SNCA*, as confirmed by PCR analysis, was lower (Figure 2). It was found that stability of both pBK-GDHsynPvu and pBK-GDHsynEco plasmids decreases while growing cultures either in liquid minimal or YEPG media. After cultivation for 2 days in YEPG medium intact $GDH:SNCA_{Eco}$ fusion was detected only in 72% of transformants and $GDH:SNCA_{Pvu}$ – in 64%. In case of SC-gal medium transformants show somewhat greater stability: after growing for 4 days, the first plasmid shows 88%, while the second - 68% stability. Intracellular GDH-

Aβ40 hybrid protein-producing yeasts cultivated in both rich and minimal media possessed low (~50%) plasmid stability (Figure 2). Yeast producing secreting variant pYEsec1-GDHAb were more stable - about 66% of the culture maintain *GDH:Aβ*40 gene. For comparison, stability of pBK-GDH construct containing a single glucose dehydrogenase gene was 90-94% under all conditions tested.

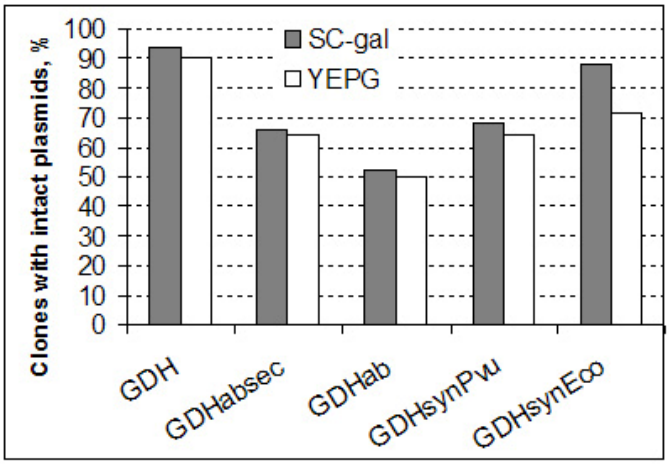

Figure 2. Maintenance of hybrid sequences in recombinant plasmids.

These data indicate that decreased stability of hybrid sequences possessing recombinant plasmids can be related to the toxic effect of α-synuclein or Aβ40. Expression of even slightly toxic gene products in microorganisms causes lost or structural rearrangement of plasmids (Y. TIERNY & al. [21]; M. ROMANOS & al. [22]). This can explain spontaneous generation of non-toxic constructs lacking *GDH:Aβ*40 or *GDH:SNCA* hybrid sequences, which during the time overcome in the yeast transformants population. Also one of the most common responses to toxicity within yeasts is reduced copy number of recombinant plasmid. However, when *Leu2-d* selection is used the drop in copy number that can occur without affecting growth is limited and other mechanisms such as structural rearrangement predominate (M. ROMANOS & al. [22]). Similar to our finding, plasmid mutation was observed during the expression of polyomavirus middle T antigen. Transformants grew very slowly and there was either plasmid loss or spontaneous generation of plasmids expressing a truncated antigen lacking the membrane-spanning domain and thus non-toxic (M. ROMANOS & al. [22]).

Given the reduced genetic stability of constructs bearing α-synuclein or Aβ40, the dynamics of cell growth (change of optical density of a culture during cultivation) has been analyzed. For comparison, control yeast strains – parental, 21PMR and 21PMR-[pBK-GDH], producing single glucose dehydrogenase protein - were investigated. In case of intracellular *GDH:Aβ40* construct (pBK-GDHab) the hybrid protein accumulates in the cytoplasm of yeast cell, while secretory variant (pYEsec1-GDHab) is directed out of cytoplasm (S. ČEPONONYTĖ & al. [18]; S. PILEVIČIENĖ & al. [19]). As shown in Figure 3 (A), 21PMR strain and its transformant expressing *GDH* alone and GDHsynPvu (α-synuclein inserted into glucose dehydrogenase sequence) reaches similar cell densities after 4 days of cultivation in minimal medium. Meanwhile, GDHsynEco-producing yeast slows down growth rate after 72 hours. This could be explained by the higher toxicity of the α-synuclein, produced in frame with *GDH*. Most likely, insertion of *sncA* sequence into the central part of *gdh* reduces toxic effect of the former peptide. ZABROCKI & collaborators [6] observed that C-terminally fused *SNCA:GFP* protein displayed similar growth curves comparing to strains transformed by empty vector and did not induce toxicity. This result differs from our observation that

toxicity of specific protein can be decreased or eliminated depending on the fusion means and hybrid-partner.

Cytoplasm-localized GDH-Aβ40 demonstrated the major influence on the growth of yeast cells. 21PMR-[pBK-GDHab] transformants are growing 2.5-3 times slower during 3-4 days of cultivation in synthetic minimal medium comparing to parental strain or control GDH-producing yeast transformants. On the other hand, elimination of *Aβ40* (in secretory *GDH:Aβ*40construct) minimizes the toxicity to the host cells. The growth dynamics is similar to that of the control strains (Figure 3 A).

Cultivation for 2 days in rich medium (YEPG) allowed to accumulate more biomass of all yeast transformants. Under these conditions some delay in growth of GDHsynEco-producing strain was observed after 24 h of cultivation (Figure 3 B), most likely because of toxic *GDH:SNCA*$_{Eco}$ hybrid production. However, these transformants yielded a similar growth phenotype comparing to control as well as GDHsynPvu and extracellular GDH-Aβ40 producing yeasts after 48 hours. The decreased stability of recombinant plasmids under non-selective conditions is the most likely reason of increased growth of tested yeast over a longer timescale. Meanwhile, 21PMR-[pBK-GDHab] transformants are unable to avoid intracellular *Aβ40* toxicity despite growing conditions.

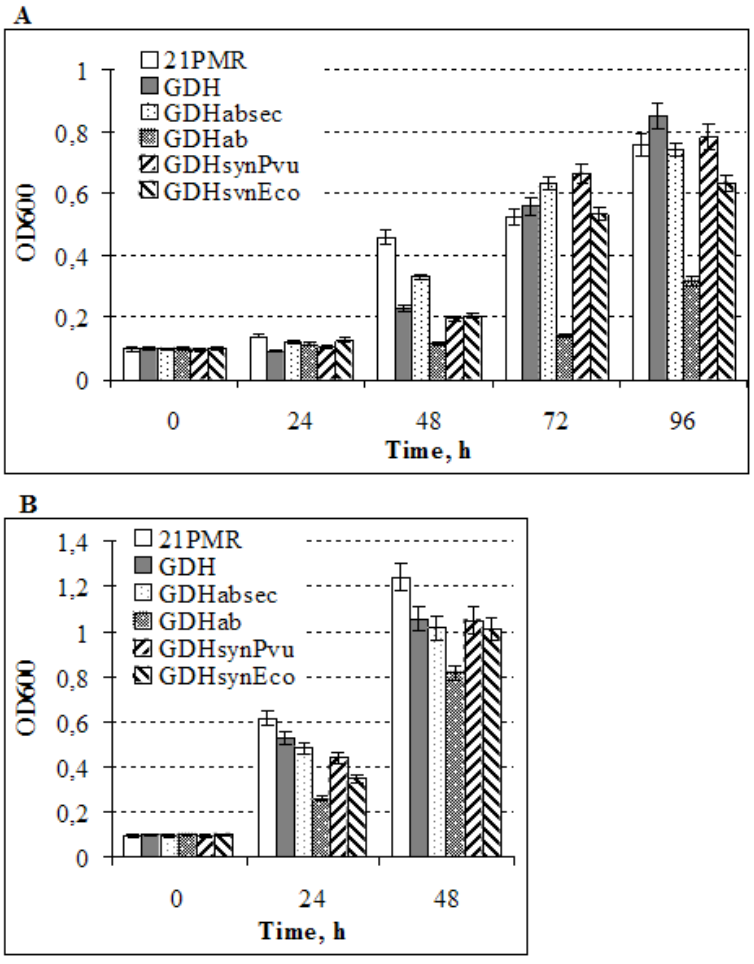

Figure 3. Growth of yeast transformants cultured in minimal SC-gal (A) and rich YEPG (B) media.

Generally, growth rate is inversely related to plasmid stability although the contrary has also been reported suggesting that each particular case should be evaluated individually (L. PALOMARES & al. [23]). KLEINMAN & collaborators [24] observed that reducing the

growth rate of the yeast culture led to a more rapid loss of the plasmid from the cells. In our case, Aβ40 toxicity determined transformants producing GDH-Aβ40 hybrid grow slower as well as stability of plasmid was decreased, too. It is established that reduced growth rate may increase the copy number of continuously replicating plasmid (L. PALOMARES & al. [23]). On the other hand, increase in copy number leads to plasmid instability (B. APOSTOL & al. [25]). Given these trends, toxicity of α-synuclein synthesized by yeast transformants influences growth of GDHsyn-producing strains not considerably. Our results indicate that almost unaffected growth of strains was possible due to accumulation of cells bearing plasmid(s) missing hybrid protein.

In summary, our results demonstrate that *Aβ40* peptide exhibit higher toxicity to the yeast cells comparing to that of α-synuclein. Decrease of internal stability of recombinant plasmids and/or affected growth rate of transformants were determined consequences of the toxicity of cloned peptides. Directing of amyloid-β-bearing hybrid to the secretory pathway reduced the toxic effect for yeast.

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