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Development of an efficient expression system for human chaperone BiP in *Pichia pastoris*: production optimization and functional validation

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Abstract

Background Human BiP, or GRP78, is a molecular chaperone mainly found in the endoplasmic reticulum (ER). However, a growing amount of data also associates BiP with many distinct functions in subcellular locations outside the ER. Notably, several diseases have been BiP-related, so the protein could potentially be used for therapeutic purposes. This study aimed to optimize a high cell-density fermentation process for the production of recombinant human BiP (rhBiP) in yeast *Pichia pastoris* in a mineral medium.

Results *P. pastoris* cells successfully synthesized and secreted full-length rhBiP protein in a complex growth medium. However, secreted rhBiP titer was considerably lower when *P. pastoris* was cultivated in a defined mineral basal salt medium (BSM). During rhBiP synthesis optimization in shake flasks, it was found that the addition of reducing compounds (DTT or TCEP) to mineral BSM medium is essential for high-yield rhBiP production. Furthermore, rhBiP secretion in the BSM medium was significantly increased by feeding yeast with an additional carbon source. The addition of 2 mM DTT and 0.5-1.0% of glucose/glycerol to the BSM medium increased rhBiP titer ~ 8 times in the shake flasks. Glucose/methanol mixture feeding with added 2 mM DTT before induction was applied in high-density *P. pastoris* fermentation in bioreactor. Oxygen-limited fermentation strategy allowed to achieve ~ 70 mg/L rhBiP in BSM medium. Hydrophobic interaction and anion exchange chromatography were used for rhBiP protein purification. Approximately 45 mg rhBiP was purified from 1 L growth medium, and according to SDS-PAGE, ~ 90% purity was reached. According to data presented in this study, rhBiP protein derived from *P. pastoris* is a full-length polypeptide that has ATPase activity. In addition, we show that *P. pastoris*-derived rhBiP effectively inhibits neurodegenerative disease-related amyloid beta 1–42 ($A\beta_{42}$) peptide and alpha-synuclein (α-Syn) protein aggregation in vitro.

Conclusions A scalable bioprocess to produce rhBiP in *P. pastoris* was developed, providing a high yield of biologically active protein in a chemically defined mineral medium. It opens a source of rhBiP to accelerate further therapeutic applications of this important protein.

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Keywords BiP, *Pichia pastoris*, Secretion, Fermentation, DTT, Mineral medium, Mixed feeding

Background

Human BiP (immunoglobulin binding protein), also known as 78 kDa glucose-regulated protein (GRP78), is a molecular chaperone mainly located in the ER lumen. BiP has been referred to as the master regulator of the ER because it plays a crucial role in processes such as protein synthesis, folding, assembly, and translocation across the ER [1]. BiP protein is highly conserved among eukaryotes, including mammals, and widely expressed among all human tissue types [2]. Notably, several diseases have been BiP-related, including rheumatoid arthritis, neurodegenerative disorders, autoimmune inflammation, and tissue damage; therefore, it could potentially be used for the detection and/or treatment of these serious diseases [3–5].

It is a multifunctional and well-studied protein; however, new functions and features are still being identified. Recently, it was shown that highly conservative cysteines undergo posttranslational modification in both yeast and mammalian cells [6–8]. In mice, a conserved cysteine pair mediated by NPGPx forms an intramolecular disulfide bond under oxidizing conditions. Disulfide bond formation enhances BiP chaperone activity and facilitates the refolding of misfolded proteins [8]. Cysteine oxidation is also reported in BiP homolog in yeast (kar2). Yeast protein has only one cysteine, which is glutathionylated or sulfenylated during ER-derived oxidative stress. These modifications decouple BiP ATPase and peptide binding activities, turning BiP from an ATP-dependent foldase into an ATP-independent holdase [6, 7].

The primary BiP localization is in the ER; however, under cell stress conditions, this protein can be actively translocated to other cellular locations and secreted outside the cell. Consequently, BiP is found in several human fluids, including serum, synovial fluid, and oviductal fluid [9–11]. Extracellular BiP has properties that are quite distinct from those of protein inside the cell. For instance, extracellular BiP acts as an immunomodulator and has an anti-inflammatory property [12]. The potential application of BiP as a therapeutic agent was shown in animal models of autoimmune disease, including collagen-induced arthritis, a murine disease that resembles human rheumatoid arthritis [13]. Also, the I/IIA RAGULA (Rheumatoid Arthritis Regulator) clinical trial showed BiP safety and some efficiency for the treatment of rheumatoid arthritis in humans [14]. Moreover, in addition to the anti-inflammatory effect, BiP also shows anti-osteoclast activity, thus extending the applicability of this protein for the treatment of inflammatory disorders associated with bone loss [15]. In summary, the demand for recombinant human BiP protein for research and clinical trials is growing, and efficient production technology is needed.

The methylotrophic yeast *Pichia pastoris* (Komagataella phaffi) is one of the most popular recombinant protein expression hosts (review article [16]). Although the success of this expression system is highly protein-dependent, and some proteins achieved the desired production levels only after thorough process optimization, this expression system is highly flexible and has many benefits. *P. pastoris* performs proper protein folding in the ER and can secrete recombinant proteins into the growth medium. These yeasts grow in a simple salt medium within wide ranges of pH and temperature values; therefore, various fermentation strategies could be applied [17]. Despite the existence of multiple promoters, the highest production levels are achieved by using the classic alcohol oxidase AOX1 promoter. Each expression system has its own advantages, and it may be a challenge to choose one that is the most suitable for the production of a specific protein. For a long time, the debate over yeast expression systems has focused on which is preferable for recombinant protein expression: *P. pastoris* or *S. cerevisiae* [18]. Despite some notable exceptions, accumulated evidence, and multiple case studies indicate that *P. pastoris* is often the preferred option over *S. cerevisiae* for producing secreted recombinant proteins. Among the numerous advantages of *P. pastoris*, several stand out: limited endogenous protein secretion, high recombinant protein yield, rapid growth to high cell density in defined media, superior thermo- and osmo-tolerance compared to *S. cerevisiae*, efficient protein folding, and genetic stability [18–20].

In our previous studies, we reported that human ER chaperones (calreticulin, ERp57, and BiP) were correctly processed and secreted in yeast *Saccharomyces cerevisiae* [21–23]. Calreticulin and ERp57 (not published) were also expressed in methylotrophic yeast *P. pastoris* under the control of the AOX1 promoter. The amounts of both recombinant proteins in *P. pastoris* were approximately threefold higher compared to *S. cerevisiae*. It is reasonable because *P. pastoris* is Crabtree-negative and, under respiratory conditions, does not produce toxic ethanol, which results in higher biomass formation and consequently, more recombinant protein [24, 25]. Moreover, strategies for high-cell-density cultivation of *P. pastoris* in a bioreactor are well-developed and include fermentation processes in a chemically defined mineral media. Such media allow easier development of controllable recombinant protein production for therapeutic purposes. In the present study, we optimized the high-cell-density fermentation process of rhBiP protein production in *P.*

pastoris in a defined basal salt medium (BSM) in the bioreactor. A several-fold higher rhBiP yield was achieved using a synthetic BSM medium compared to previously reported rhBiP yields in YPD rich medium.

In addition to production optimization, this study also aimed to provide functional validation of the rhBiP product. ATPase activity is an intrinsic property of BiP, while anti-aggregation activities are at the core of the primary chaperone function of the protein. Evaluation of the biological activities of the purified rhBiP suggested that it may serve as a protective agent against protein aggregation and neurodegeneration. Misfolding of amyloidogenic proteins, such as $A\beta_{42}$ and alpha-synuclein, is the initial step in the formation of cytotoxic fibrils. Accumulation of such aggregates is linked with several neurodegenerative disorders, including Alzheimer's and Parkinson's diseases [26]. Since BiP is important for correct protein folding, it may have the potential to prevent the onset of these amyloid-related disorders. Here, we report promising results on the inhibition of $A\beta_{42}$ peptide and α-Syn protein aggregation by rhBiP in vitro.

Results and discussion

Expression of rhBiP in yeast *P. pastoris*

In our previous work, we expressed rhBiP protein in *S. cerevisiae*. The rhBiP titer in shake flasks reached about 15 mg per 1 L of complex YEPD yeast growth medium. Herein, we expressed rhBiP in *Pichia pastoris* under the control of methanol-inducible *AOX1* promoter using the same native human BiP secretion signal. SDS-PAGE analysis showed that *P. pastoris* cells secreted rhBiP into growth media (Fig. 1). We screened and selected a multicopy Mut^s phenotype *P. pastoris* clone BPp10 showing the highest rhBiP secretion into YEPM medium. The secretion titer of rhBiP in *P. pastoris* was similar to that of *S. cerevisiae* and reached about 11.8 ± 1.6 mg per 1 L YEPM medium after 42 h induction. Also, rhBiP was the main protein in the media, constituting approx. 50–60% of all secreted protein.

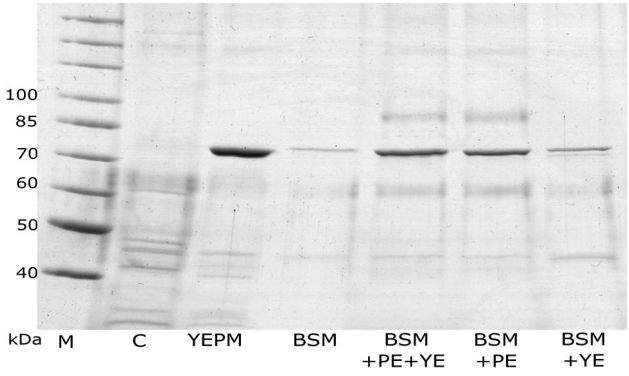

Fig. 1 SDS-PAGE analysis of rhBiP secretion into different growth media in *P. pastoris*. Lanes were loaded with samples of rhBiP-producing clone ppBiP10 cultivated in the shake flasks with indicated culture media: YEPM– complex media; BSM– basal salt medium; BSM + YE + PE– basal salt media supplemented with yeast extract and soy peptone; BSM + YE– basal salt media supplemented with yeast extract; BSM + PE– basal salt media supplemented with soy peptone. C lane– YEPM culture media from *P. pastoris* transformed with control pPIC3.5 K vector. All culture growth media were concentrated 10x and 7.5 µl (obtained from 75 µl of culture medium) was loaded onto the lane. M lane – unstained protein ladder (ThermoScientific, cat. no. 26614)

In recombinant protein production for biopharmaceutical purposes, *P. pastoris* is cultivated in a stirred tank bioreactor using fed-batch fermentation mode. Large-scale fermentation usually employs chemically defined basal salt medium (BSM) because of its low cost, better batch-to-batch consistency, and easier downstream processing. We cultivated clone BPp10 in a 5 L bioreactor Biostat A plus in BSM medium using a protocol adopted from Tolner [27]. Previously, we achieved a 1.5 g/l secretion yield of recombinant human calreticulin using these conditions [22], but this protocol was unsuitable for rhBiP production because the yield was much lower compared to the shake flask, and severe degradation of rhBiP was observed. The adjustments of fermentation conditions, such as temperature during induction, methanol feeding rate, pH, or BSM salt concentration, did not increase the yield of rhBiP in the mineral medium. The low rhBiP yield in the BSM medium could be due to a lower secretion rate and/or rhBiP instability in the mineral medium.

Optimization of rhBiP production in shake flasks

Optimization of fermentation conditions in a bioreactor is a time-consuming and expensive process; therefore, we decided to optimize rhBiP secretion in BSM medium in shake flasks. All shake flask experiments were carried out by accumulating yeast biomass in a complex YEPG medium before induction. After 18 h of yeast growth, the cultures were centrifuged, and the yeast cells were suspended in various induction media with 0.5% methanol. The mineral BSM medium is composed of common salts (potassium sulfate, magnesium sulfate, ammonium sulfate, calcium sulfate, sodium hexametaphosphate) and carbon source (glycerol, methanol). Yeast *P. pastoris* grow very well in this medium and can achieve very high cell densities in a short time. However, specific recombinant proteins may be unstable in this high-salt medium. They can aggregate and precipitate or be sensitive to yeast proteases. Meanwhile, soy peptone and yeast extract, the main YEPM medium components, are nutrient-rich complex materials that may positively affect the stability of a recombinant protein. Therefore, in the first experiment, we investigated whether soy peptone and yeast extract can increase the amount of rhBiP in the mineral medium and which of these components is more important for rhBiP secretion. We performed an experiment in shake flasks, in which yeast biomass was grown in

complex YEPG medium, and recombinant protein synthesis was induced by suspending yeasts in BSM medium supplemented with 2% soy peptone (PE), 1% yeast extract (YE), or combination of these substances (see methods) after centrifugation. Analysis of cell culture supernatants after 42 h induction by SDS-PAGE showed that rhBiP titer in BSM medium without additives was about fivefold lower compared to YEPM (Fig. 1). Addition of PE/YE or PE alone to BSM medium increased the amount of secreted rhBiP to 7–8 mg/L, which is fourfold higher compared to BSM, but still 30% lower than in YEPM medium. Meanwhile, the effect of yeast extract on rhBiP secretion was insignificant. The results suggested that soy peptone has some specific components which may stabilize rhBiP or enhance its secretion into BSM media.

Soy peptone and yeast extract are complex materials rich in carbohydrates, minerals, vitamins, amino acids, and peptides [28]. We decided to screen which substances present in soy peptone could increase rhBiP secretion in the mineral medium. Soy peptone is rich in amino acids, which are building blocks of proteins, so naturally, it implies that the addition of amino acids could increase rhBiP titer. Also, there are many examples in the literature of recombinant protein titer increments when amino acids were added to growth media [29, 30]. We tested all 20 amino acids at a concentration of 4 mM for their impact on rhBiP secretion, and, surprisingly, it was cysteine alone that increased the amount of rhBiP (Additional file 1: Fig. S1).

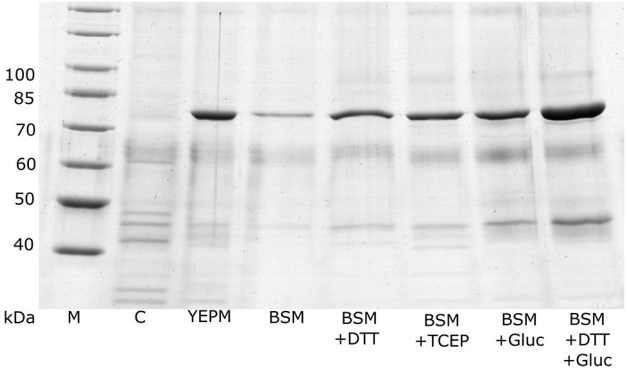

Fig. 2 Selection of optimal culture medium for rhBiP secretion in shake flask cultures of *P. pastoris*. SDS-PAGE was performed with following culture media samples on lanes: C- *P. pastoris* transformed with control pPIC3.5K vector in complex YEPM culture media; rhBiP-producing clone ppBiP10 cultivated in BSM – basal salt medium; BSM + DTT – basal salt medium supplemented with 2mM DTT; BSM + TCEP - basal salt medium supplemented with 2 mM TCEP; BSM + Gluc - basal salt medium supplemented with 0,75% (m/V) glucose; BSM + DTT + Gucose - basal salt medium supplemented with 2 mM DTT and 0.75% (m/V) glucose. All cultures (YEPM and BSM media with or without additives) contained the same 0.5% amount of the inducer methanol, as described in Methods. All culture growth media were concentrated 10x, and 7.5 µl (obtained from 75 µl of culture medium) was loaded onto the lane. M lane – unstained protein ladder (ThermoScientific, cat. no. 26614)

Human BiP has only two cysteines, so it is unlikely that yeast cells might lack cysteine for recombinant BiP synthesis. However, reductive cysteine property could be responsible for increased rhBiP stability and titer in the BSM medium. To test this hypothesis, we designed an experiment where BSM induction medium was supplemented with two reducing agents: dithiothreitol (DTT) or Tris(2-carboxyethyl) phosphine (TCEP) at concentrations of 2 mM. These reducing agents differ in size and ability to cross biological membranes. DTT is a small molecule that can easily cross a biological membrane, while TCEP is generally impermeable to cell membranes [31]. SDS-PAGE analysis showed that both reducing agents improved rhBiP secretion into BSM medium (Fig. 2). According to data obtained from densitometric analysis, rhBiP titer in BSM medium supplemented with reducing agents was approx. 8 mg/L and was similar to the titer achieved in mineral media supplemented with soy peptone. The fact that cell membrane-impermeable TCEP was also effective implies that reducing agents probably stabilize rhBiP outside the cell instead of enhancing protein synthesis or secretion.

BSM medium supplemented with reducing agents improved rhBiP secretion; however, rhBiP titer in complex YEPM medium was still ~ 30% higher. Soy peptone contains high concentrations of naturally occurring carbohydrates, which can serve as an additional carbon source and alleviate the metabolic stress experienced by yeast during growth on methanol. Thus, we conducted an experiment in which BSM media were supplemented with two *AOX* promoter-repressing (glucose and glycerol) and two nonrepressing (mannitol and sorbitol) carbon sources. Four concentrations were used: 0.5%, 0.75%, 1,0%, and 2,0% (m/V). Also, 2 mM DTT and 0.5% methanol were added to the growth medium before induction. SDS-PAGE analysis of growth media after 42 h inductions showed that glucose or glycerol addition at a 0,5% – 1.0% concentration increased rhBiP titers to approx. 17 mg/L, whereas 2% of these carbon sources completely repressed rhBiP synthesis (Additional file 1: Fig. S2). Mixed fed with sorbitol or mannitol also increased rhBiP protein secretion, but final titers were slightly lower in comparison with glucose/glycerol (Additional file 1: Fig. S2). The results of rhBiP secretion experiments (secretion titers and wet cell weights) in different *P. pastoris* culture media compositions are provided in Additional file 1: Table S1. Finally, the best mineral culture medium for rhBiP secretion was defined as BSM with 2 mM DTT and 0.75% glucose (Fig. 2; Table 1).

Mixed-feeds using methanol and a multi-carbon source is a common strategy to enhance recombinant protein production in *P. pastoris* [32–34]. Various co-substrates such as glycerol, glucose, sorbitol, mannitol, and lactate have been shown to enhance the expression

Table 1 Optimization of rhBiP secretion in the mineral culture medium in shake flasks

Growth media	rhBiP yield (titer), mg/L after 42 h induction	WCW, g/L after 42 h induction	Induction medium components
YEPM	11.8 ± 1.6	65.7 ± 3.7	2% soy peptone, 1% yeast extract, 0.5% methanol
BSM	2.2 ± 1.0	53.2 ± 6.9	¼ BSM salts, 100 mM potassium phosphate, 0.5% methanol
BSM + 2 mM DTT	7.7 ± 2.2	56.0 ± 1,4	¼ BSM salts, 100 mM potassium phosphate, 2 mM DTT, 0.5% methanol
BSM + 2 mM TCEP	8.8 ± 1.3	56.5 ± 3.5	¼ BSM salts, 100 mM potassium phosphate, 2 mM TCEP, 0.5% methanol
BSM + 0.75% Glucose	7.9 ± 3.4	74.5 ± 3.3	¼ BSM salts, 100 mM potassium phosphate, 0.75% glucose, 0.5% methanol
BSM + 2 mM DTT + 0,75% Glucose	17.2 ± 0.7	72.4 ± 2.3	¼ BSM salts, 100 mM potassium phosphate, 2 mM DTT, 0.75% glucose, 0.5% methanol

of recombinant proteins in the yeast *P. pastoris* [32, 35–37]. A higher yield of recombinant protein in mixed-fed *P. pastoris* cultures is explained by reduced metabolic burden caused by the overexpression of foreign protein [33, 34]. Co-substrate feeding strategy during induction is more applicable when methanol assimilation capacity is limited (Mut^S phenotype) because reduced carbon metabolism may interfere with recombinant protein production. However, to our knowledge, there is only one case in which reducing agents have increased the yield of a recombinant secreted protein in yeast [38]. In that work, the production of recombinant human serum albumin and porcine follicle-stimulating hormone fusion protein (HSA-pFSHβ) was enhanced by supplementing the culture medium with N-acetyl-L-cysteine. The authors claim that the mechanism of the beneficial effect NAC has on the secretion of HSA-pFSHβ is associated with its thiol property and improved intracellular GSH content [39]. A positive effect of reducing agents on recombinant protein secretion was also demonstrated in Chinese hamster ovary cells that secreted recombinant human erythropoietin [40]. Authors of that study suggested that thiol-reducing agents may alter the redox state of the ER from an oxidative state to a slightly reduced state, thereby facilitating protein folding and enhancing the secretion rate of proteins that would otherwise be retained in the cell. In our case, the fact that the cell membrane-impermeable TCEP also improved rhBiP secretion suggests that rhBiP may be retained in the yeast cell membrane or the periplasmic space via disulfide bridges. Recently, it has been shown that BiP oligomerizes upon interaction with negatively charged phospholipids and membrane incorporation forming intermolecular disulfide bonds between N-terminus and C-terminus domains [41]. Our results align well with a model of BiP oligomerization proposed by the authors of that work. Dores-Silva et al. suggested the presence of antiparallel oligomers of rhBiP after incorporation into the lipid bilayer via formation of disulfide bonds (the model is illustrated in Fig. 3 of their publication [41]). The addition of a reducing agent likely disrupts intermolecular disulfide bonds within oligomers, releasing monomeric rhBiP from the cell surface membranes to the yeast culture medium and increasing its secretion titer as observed in this study. Remarkably, the ability of native BiP to associate with membranes via disulfide bonds may enable its cell surface localization, despite lacking a transmembrane domain, where it functions as a receptor or co-receptor for soluble ligands, aids in cancer survival, and potentially serves as a receptor for viruses like SARS-CoV-2 (reviewed in [41]). Although the mechanism for the enhancement of rhBiP secretion by reducing agents requires further research, the scope of our work is restricted to enhancing secreted protein yield and establishing an efficient production system. We also tried to express other recombinant proteins, such as human ERp57, bovine serum albumin (BSA), and human calreticulin (CRT) in a mineral medium with DTT and glucose. Interestingly, reducing conditions in the mineral medium also increased the secretion of ERp57 protein. Meanwhile, CRT yield remained unchanged, and BSA decreased (not shown). ERp57, like the BiP protein, is poorly secreted in a mineral medium and contains active sulfhydryl groups, which can also be responsible for protein retention in yeast cells.

Oxygen-limited mixed-feed fermentation strategy for rhBiP production in bioreactor

The popularity of the *P. pastoris* expression system could be associated with the ability to reach very high cell densities and the usage of very strong methanol-inducible *AOX1* promoter. Various fermentation strategies have been developed and successfully applied for recombinant protein production in yeast *P. pastoris* (μ-stat, DO-stat, methanol-stat, biomass-stat) [17]. In this work, rhBiP production in high-density *P. pastoris* bioreactor fermentation was performed by feeding oxygen-limited glucose/methanol mixture with the addition of 2 mM DTT before induction. Yeast was cultivated in 5 L bioreactor Biostat A plus in 1 L BSM medium (Methods). High-cell-density fermentation was divided into 3 phases: S1 – glycerol batch, S2 – glycerol fed batch, and S3 – recombinant protein induction (Additional file 1: Table S2). Phases

S1 and S2 in all experiments were the same. Briefly, the glycerol batch phase lasted about 26 h, and subsequent glycerol fed batch was maintained for 4 h. Cell density reached ~ 150 g/L WCW during phase S1, and additional feeding with glycerol led to ~ 200 g/L WCW. Previously, we successfully produced rhCRT in methanol limited fed-batch fermentation [22]; therefore, we decided to test this feeding strategy for rhBiP production. However, we used an aqueous methanol/glucose solution instead of feeding the yeast with methanol alone. In addition, 2 mM DTT was injected before the induction phase in all experiments. The glucose/methanol mixture feeding rate was adjusted to 6 ml/h/l for all substrate-limited fermentation experiments. We tested various methanol/glucose ratios (25%/12.5%, 12.5%/6.25%, 12.5%/12.5%, 10%/20% V/m) for rhBiP induction; however, the final rhBiP titers in most cases were very low and severe degradation was observed. The highest rhBiP titer (~ 10 mg/L) obtained using 12.5%/6.25% methanol/glucose mixture was lower than the quantity obtained in the shake flask (~ 17 mg/L) despite cell density being several times higher. Based on these results, we speculated that the constitutive synthesis of rhBiP could be harmful to yeast cells when they grow in mineral media. In flasks experiments, rhBiP synthesis was induced by injecting substrate mixture once every 24 h, and under these conditions, rhBiP maintained stability up to 72 h. Thus, we decided to mimic shake flasks and apply the same feeding strategy in the bioreactor. Similarly to shake flasks, rhBiP expression was induced by injecting methanol and glucose mixture to the final concentration of 0.5% (v/v) and 1.0% (m/v), respectively. Oxygen supply to the bioreactor was limited by reducing the stirring speed to 400–600 RPM and turning off oxygen enrichment. Under these conditions, the dissolved oxygen level in cell culture sharply drops to zero immediately after substrate mixture injection. After 6–7 h of fermentation, both substrates were consumed, and as a result, a sudden increase in dissolved oxygen was observed. We determined that methanol/glucose mixture injection at 0,5%/1.0% every 8 h is optimal for rhBiP production in a bioreactor. This induction strategy was successful, and according to SDS-PAGE analysis, after 48 h of induction, rhBiP titer reached ~ 70 mg/l (Fig. 3). Although this amount is fourfold higher than that obtained in shake flasks, the efficiency of rhBiP secretion is similar, because the density of yeast biomass in the bioreactor at the beginning of induction was also about fourfold higher. However, we were unable to continue the induction for longer than 48 h because of product degradation and a decrease in the yield of rhBiP (Fig. 3).

Purification of rhBiP protein from a fermentation growth medium

High-level secretion of rhBiP protein to BSM yeast culture media facilitates purification procedure. Previously, we used affinity ATP agarose chromatography to purify rhBiP from *S. cerevisiae* growth media. According to SDS-PAGE, a one-step purification procedure allowed for achieving purity greater than 95%. However, for larger-scale purification of rhBiP, affinity chromatography is not economically feasible due to the high cost of the resin and ATP ligand. Therefore, we used hydrophobic interactions, gel filtration, and ion exchange chromatography,

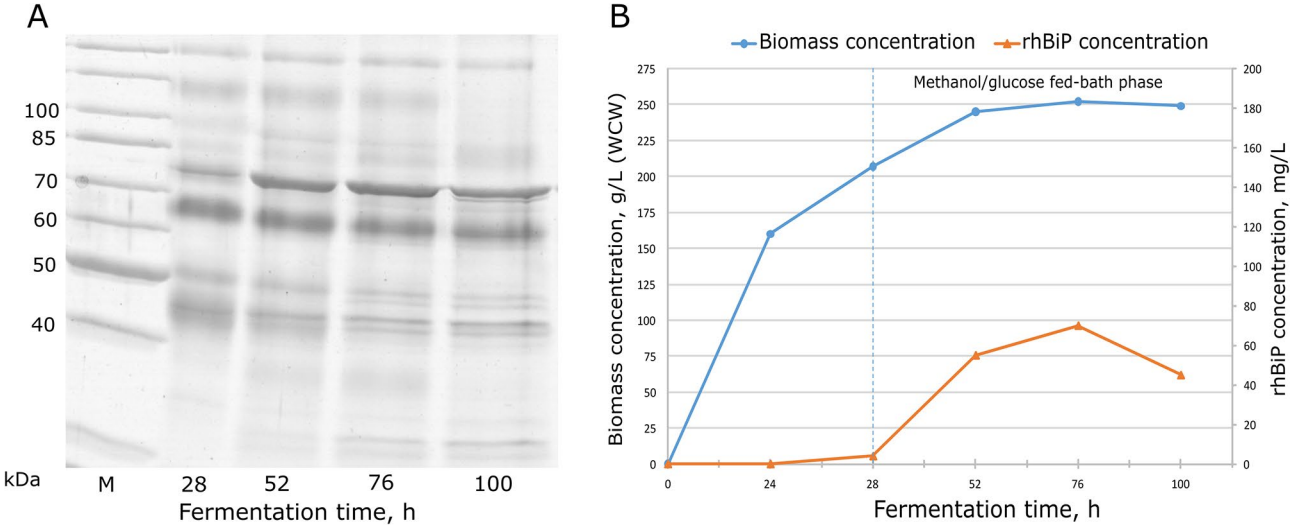

Fig. 3 Secretion of rhBiP in high-cell-density fermentation of *P. pastoris* in a bioreactor. (**A**) SDS-PAGE analysis of culture supernatant during fermentation. 35 µl of *P. pastoris* clone ppBiP10 culture media, taken at different time points, was loaded onto each lane. (**B**) Time-course profile of rhBiP secretion in high-density *P. pastoris* culture during fermentation. Circles represent wet cell weight (WCW), and triangles show the concentration of the secreted rhBiP in the culture medium. 0–24 h indicates glycerol batch phase, 24–25 h - glycerol fed-batch phase, 25–28 h - transition phase, and 28–100 h - methanol/glucose fed-batch phase. Only the amount of intact secreted rhBiP protein form was calculated

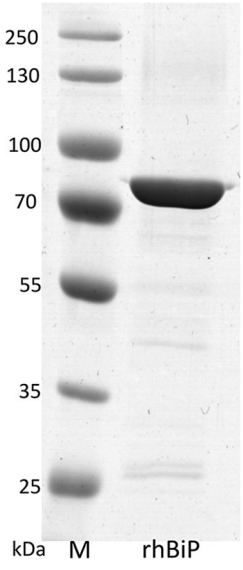

Fig. 4 SDS-PAGE analysis of rhBiP purified from *P. pastoris* culture medium. 5 µg of purified rhBiP was loaded on the gel. M - prestained molecular ladder (ThermoScientific, cat. no. 26618)

Table 2 The purification process of rhBiP from *P. pastoris* culture medium

Purification step	Sample Volume, ml	rhBiP concentration, mg/ml	rhBiP quantity, mg	Purity according to SDS-PAGE, %	Recovery, %
Supernatant	1050	0.0666	70	28	100
Phenyl chromatography	120	0.49	58.8	64	84
Buffer exchange with sephadex G25	220	0.267	58.8	64	84
First sepharose Q	25	2.068	51.7	81	73.9
Second sepharose Q	30	1.51	45.3	90	64.7
Buffer exchange with sephadex G25	45	1	45	90	64

which are widely used in industry for recombinant protein purifications.

Firstly, phenyl sepharose hydrophobic interaction chromatography allowed the capture of rhBiP protein and removal of some impurities (Additional file 1: Fig. S3a). Fractions containing rhBiP were combined, and buffer exchange was performed by Sephadex G-25 size exclusion chromatography. Human BiP is an acidic protein with theoretical pI 5.1; therefore, this protein strongly binds to Q-Sepharose FF ion-exchange resin even at low pH. The linear elution gradient from 20 to 250 mM NaCl allowed the separation of lower molecular weight proteins, constituting the most significant impurities left after hydrophobic interaction chromatography (Additional file 1: Fig. S3b). Selected fractions with rhBiP were combined and 2x diluted with a 20 mM TRIS buffer (pH = 7.5). The diluted sample was loaded on a sepharose Q HP column for the final polishing step. The same elution gradient was applied as in the first ion exchange chromatography (Additional file 1: Fig. S3c). Fractions containing rhBiP protein were pooled, and buffer was exchanged for storage buffer (20 mM TRIS, 300 mM NaCl, pH 7,5) using Sephadex G25 column. The concentration of the purified protein was determined by measuring sample absorbance at 280 nm (Methods). The yield obtained from 1.05 L BSM growth medium was approx. 45 mg, which corresponds to a final ~ 64% recovery. The purity of the rhBiP analyzed by SDS-PAGE was ~ 90% (Fig. 4 and Table 2).

Characterization of rhBiP derived from *P. pastoris*

In a previous study, we showed that yeast *S. cerevisiae* synthesizes and secretes a full-length (19–654 aa) human BiP/GRP78 protein that does not have any yeast-derived modifications. We also showed that rhBiP synthesized by *S. cerevisiae* (ScrhBiP) possesses HSP70 protein family-inherent ATPase activity and that most is in the active monomeric form. Mass spectrometry data and N-terminal sequencing by Edman degradation of *P. pastoris*-derived rhBiP (PprhBiP) also confirmed that recombinant polypeptide corresponds to the theoretically predicted mass of mature human BiP (19–654 aa) with correctly processed N-terminal end (Fig. 5). Measured ATP hydrolysis rate of PprhBiP was similar to that of ScrhBiP and ~ 3 times higher than bacteria-derived rhBiP (Fig. 6). In contrast to ScrhBiP, analysis of PprhBiP by native PAGE

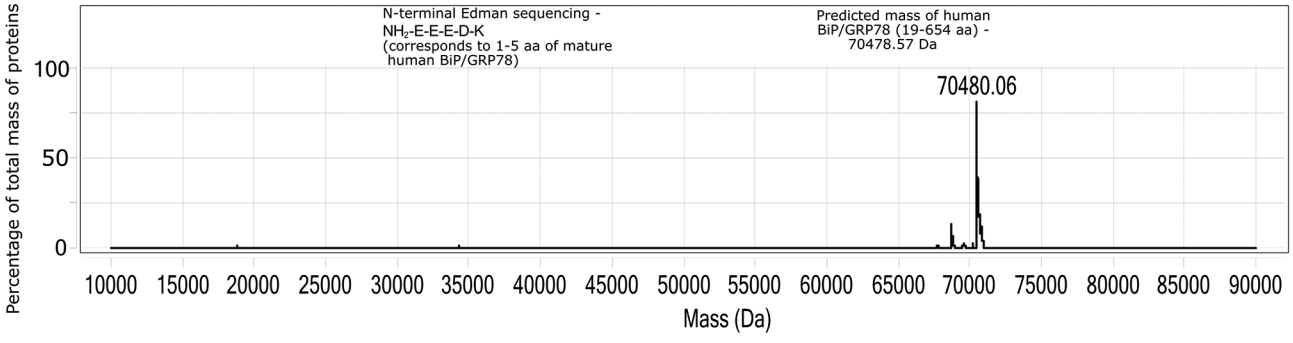

Fig. 5 ESI-MS and N-terminal sequencing of rhBiP from *P. pastoris*

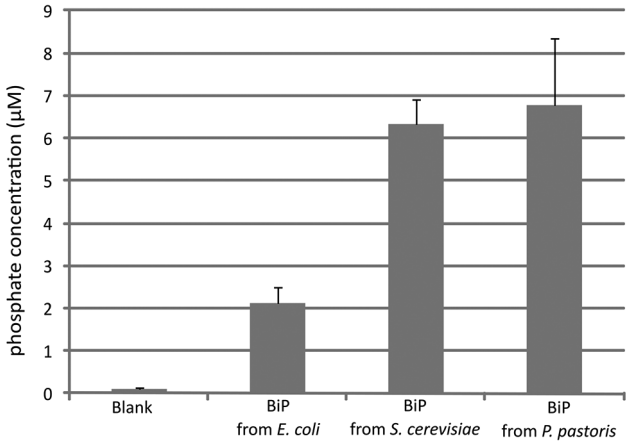

Fig. 6 ATPase activity of *P. pastoris*-secreted human BiP. The amount of released phosphate by 1 μg of either bacterial or yeast-derived rhBiP was determined after incubation at 25 °C for 75 min. with a non-radioactive procedure. Values are the mean of three separate experiments with the SD error bar

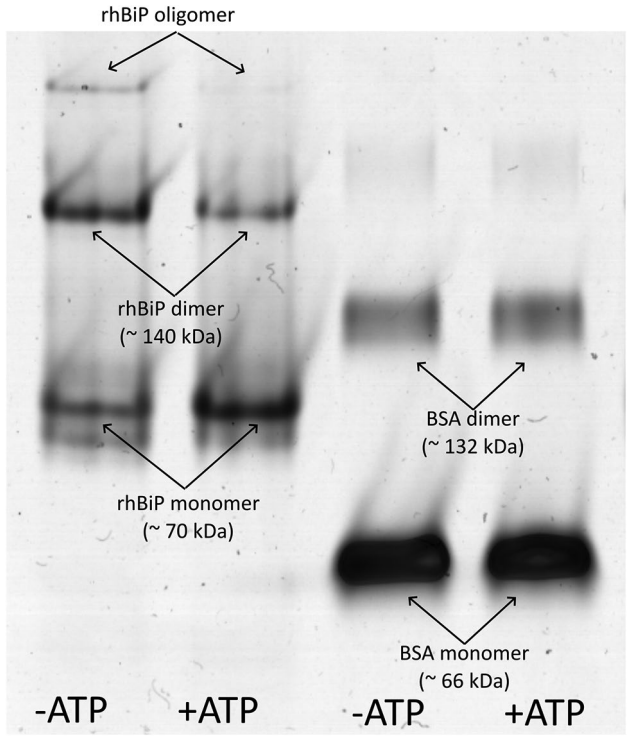

Fig. 7 Monomerization of rhBiP after exposure to ATP. Purified rhBiP derived from *P. pastoris* culture medium was incubated with 5mM of ATP for 15 min at 30 °C. 5 μg of protein was loaded on gel and analyzed by native PAGE. Commercial BSA was used as a control and molecular weight marker

revealed that most of the purified protein is in dimeric and oligomeric forms (Fig. 7, lane 1). This is not surprising because ATP-agarose affinity chromatography was used during ScrhBiP purification, and it is well known that ATP binding to BiP protein promotes its monomerization [42, 43]. Meanwhile, PprhBiP was purified by hydrophobic interaction and ion exchange chromatography and did not interact with ATP. Incubation of PprhBiP with 5 mM ATP for 15 min significantly reduced oligomers and dimers while increasing the monomeric form (Fig. 7, lane 2). Correct folding of PprhBiP was evaluated by partial proteolysis of BiP protein, as described previously for ScrhBiP [21]. As in the previous study, the addition of ADP protected the 44-kDa fragment, whereas ATP protected 60- and 44-kDa fragments from digestion by proteinase K, demonstrating the correct folding of PprhBiP (Additional file 1: Fig. S4).

The main function of BiP/GRP78 protein is to facilitate the transport and folding of newly synthesized proteins inside ER. In addition, BiP chaperone binds to unfolded proteins and restricts their folding or aggregation. This so-called "holdase" activity of BiP has been shown in vitro by suppressing aggregation of amyloid beta 1–42 ($A\beta_{42}$) [44] and human amylin [45] peptides. Herein, we investigated the ability of the PprhBiP protein to inhibit aggregation of $A\beta_{42}$ peptide and alpha-synuclein (α-Syn) protein in vitro using a thioflavin-T (ThT) fluorescence assay (described in the Methods section). As shown in Fig. 8A 1 μM of rhBiP protein is sufficient to inhibit aggregation of 1 μM $A\beta_{42}$, while lower than 0.2 μM concentrations had only a small influence on the aggregation kinetics. In the case of $A\beta_{42}$, this result is in agreement with the previously described study, where $A\beta_{42}$ aggregation was inhibited by *E. coli*-derived BiP [44]. However, inhibition of α-Syn protein aggregation by rhBiP was much more efficient, with almost total suppression at the molar ratio of 1:20 (5 μM of rhBiP was enough to inhibit aggregation of 100 μM α-Syn, as shown in Fig. 8B). This reveals rhBiP as a powerful potential agent against α-Syn protein aggregation, which is associated with the pathology of several neurodegenerative diseases, including Parkinson's disease [46, 47]. To the best of our knowledge, inhibition of α-Syn protein aggregation by rhBiP was not reported previously, although cumulative evidence points towards a crucial role for protein quality control (PQC) systems, including the molecular chaperones, in modulating α-Syn aggregation and toxicity [48]. Molecular chaperones have been extensively implicated as protective agents against protein aggregation and neurodegeneration [49]. Our data suggests that rhBiP might be used for the development of new therapeutic strategies against neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases. We recently found that rhBiP and other recombinant molecular chaperones may be delivered to the brain by a non-invasive method using packaging into exosomes (Čiplys and Slibinskas 2023, European patent application unpublished). Taken together, our results provide a promising tool for the generation of good quality, biologically active rhBiP protein. Its potential therapeutic applications require further studies.

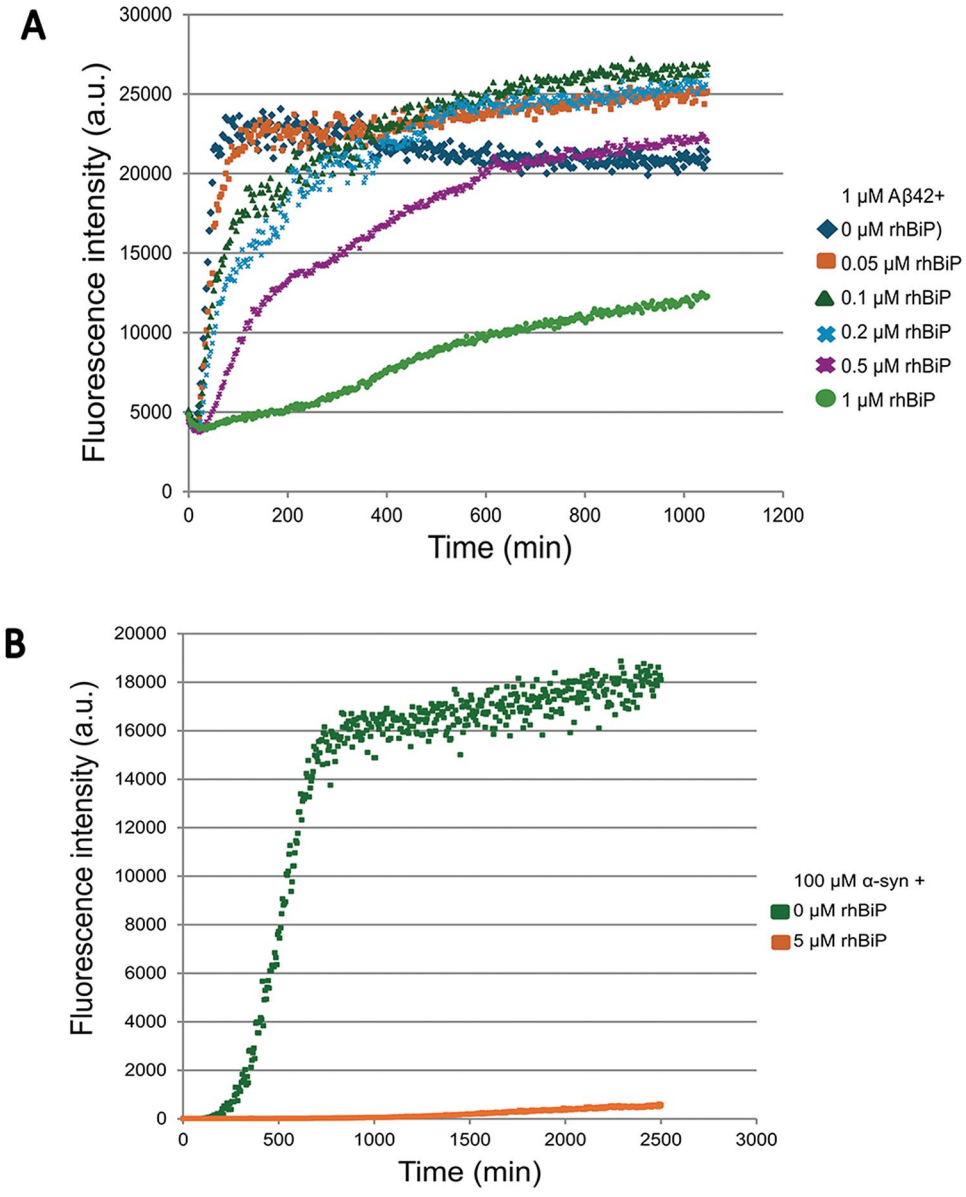

Fig. 8 Inhibition of aggregation of $A\beta_{42}$ (**A**) and α-Syn (**B**) by rhBiP derived from *P. pastoris*. (**A**) Aggregation kinetics of the $A\beta_{42}$ peptide in solution at different rhBiP concentrations. (**B**) Aggregation kinetics of the alpha-synuclein in solution at presence of 5 μM rhBiP or without chaperone. All aggregation measurements were carried out using the ThT assay as described in Methods

Comparison of rhBiP production in *P. pastoris* and *S. cerevisiae*

In our previously published study, we concluded that yeast *S. cerevisiae* is an excellent host for producing rhBiP protein [21]. Indeed, purified rhBiP from both *P. pastoris* and *S. cerevisiae* is almost identical in regard to molecular weight (see Fig. 5 in this work and Fig. 5 in [21]) and ATPase activity (Fig. 6). There is no difference in glycosylation (sometimes presented as a drawback of *S. cerevisiae*) or other noticeable differences in the quality of purified protein between the two yeast species. Therefore, it is worth comparing details of rhBiP production and discussing what advantages this work brings over previous studies.

We started this study on rhBiP expression in the complex growth medium in the shake flasks with insufficient secretion titers of ~ 10 mg/L and an expensive production method. The purification procedure included tangential ultrafiltration of culture medium supernatant and subsequent affinity ATP agarose chromatography [21], which is not economically feasible. We aimed to increase rhBiP yield and make the production less expensive, fully controllable, and scalable. The first step was to transfer yeast cultivation to a fully synthetic mineral medium which is

desirable for production technology due to its multiple advantages over complex growth medium. In this case, it was a serious bottleneck, which was eliminated in an unusual way, as described in the Results above. Further, the rhBiP biosynthesis process in a bioreactor was developed based on a combination of techniques specific to the work with *P. pastoris*. The question is whether similar improvements could be made to secreted rhBiP expression in *S. cerevisiae*? We do not know because the same conditions are not transferable between the species. There are different carbon sources and inducers, as well as different growth parameters specific to *P. pastoris* or *S. cerevisiae*. High-cell-density fermentation processes for *S. cerevisiae* are not as well developed as those for *P. pastoris*. Moreover, there may be additional issues specific to *S. cerevisiae*, such as stability of the expression plasmid at the high cell density [50] and slower biomass accumulation [51] compared to *P. pastoris*. Considering the notable sensitivity of the secreted rhBiP to various culture medium conditions, possible optimization of its production in *S. cerevisiae* seems no less complex task than in *P. pastoris*. Therefore, we can only compare rhBiP production in the two yeast species based on available data.

The use of BSM medium in *P. pastoris* fermentation enabled to avoid ultrafiltration step as it was possible to directly load culture supernatant on the first chromatography column (see Methods). Next, the affinity chromatography step was replaced by common, inexpensive, and widely used chromatography techniques. This allows us to produce a higher amount of rhBiP with significantly less cost. Meanwhile, the development of a controllable production process and the use of synthetic mineral medium enables easier scale-up to much larger bioreactor volumes. Currently, all of this is an advantage of rhBiP production in *P. pastoris* over *S. cerevisiae* expression system, where the protein is still expressed in the shake flasks. It is not feasible to transfer improvements of rhBiP purification in *P. pastoris* to the current production process in *S. cerevisiae*. The complex growth medium in *S. cerevisiae* needs to be replaced with the mineral medium to enable direct loading of culture supernatant with rhBiP onto the first chromatography column; otherwise, this technique does not work. In contrast to *P. pastoris*, *S. cerevisiae* growth in the mineral medium is inefficient without various additives (vitamins, etc.) [52]. In summary, rhBiP production processes in *P. pastoris* and *S. cerevisiae* are significantly different and not interchangeable. Optimization of rhBiP production in *S. cerevisiae* would require an entirely independent development. A potential specific drawback of *P. pastoris* is the use of methanol in the production process, which may cause security concerns during further scale-up. On the other hand, both yeast species have several shared advantages for rhBiP production over bacterial expression systems. For example, yeasts possess a typical eukaryotic secretory pathway and can correctly process and secrete the mature rhBiP product to the culture medium, in contrast to the intracellular protein production in *E. coli* without the secretion pathway. A comparison of different expression systems for rhBiP production is provided in Table S3.

Conclusions

In the present study, we developed oxygen-limited mixed-feed fermentation process for efficient rhBiP production in high-cell-density bioreactor cultivation of *P. pastoris*. Optimization of culture conditions showed that the addition of reducing agents and using mixed glucose/methanol feeding considerably improves secreted rhBiP expression in the mineral medium. Glucose/methanol mixture feeding and 2 mM DTT addition increased rhBiP secretion titer to >70 mg/L in a synthetic BSM medium with a final yield of ~45 mg/L, which is several fold higher than previously reported rhBiP yields in rich YPD medium cultivations. Moreover, the development of a controllable protein production process in a defined mineral medium opens a source of high-quality rhBiP for possible future applications, including the development of new drug candidates. We also show that *P. pastoris*-derived rhBiP effectively suppresses aggregation of $A\beta_{42}$ and α-Syn peptides involved in the progression of neurodegenerative disorders.

Materials and methods

Plasmids, yeast strains, transformation, and selection of transformants

DNA manipulations were performed according to standard procedures [53] and bacterial recombinants were screened in *Escherichia coli* DH5αF′ cells. Plasmid pPIC3.5K-BiP for the inducible expression of full-length human GRP78/BiP protein was generated by subcloning of HSPA5 gene (GenBank accession no. AF216292) from the plasmid pFDC-BiP [21] into the XmaJI site of vector pPIC3.5K (Invitrogen) under control of *P. pastoris* AOX1 gene promoter. The cloned BiP gene coding sequence (beginning from start codon ATG and ending with STOP codon TAG) was verified by DNA sequencing and generated plasmid pPIC3.5K-BiP for the expression of full-length BiP protein precursor with the native N-terminal signal peptide was used for transformation of yeast *P. pastoris* strain GS115 (Δhis4). For a selection of multicopy strains, His^+ transformants were plated on YEPD media with different G418 concentrations – 0.5, 1, and 2 mg/ml. Strains with resistance to 0.5 and 1 mg/ml G418 were screened for secretion of BiP (no colonies grew on 2 mg/ml G418 concentration). Clone ppBiP10, with the most efficient secretion of BiP protein, was resistant to 1 mg/ml of G418. Scorer et al. [54] determined that *P.*

pastoris strains resistant to 1-1.75 mg/ml G418 concentrations have 2–5 copy numbers of integrated genes. The ppBiP10 strain was determined to be Mut^S phenotype and was chosen for further experiments.

Protein expression in shake-flask cultivations

P. pastoris transformants with multicopy integrations of expression vector pPIC3.5K-BiP were initially grown in YEPG medium containing glycerol as carbon source (yeast extract 1%, peptone 2%, glycerol 1%, biotin 2×10^{-5}%) for 24 h up to OD of 18–20, and culture medium was changed to YEPM (yeast extract 1%, peptone 2%, methanol 0.5%, biotin 2×10^{-5}%) or BSM media (potassium sulfate, 3.7 g/l; calcium sulfate, 0.23 g/l; magnesium sulfate heptahydrate, 2.9 g/l; ammonium sulfate, 2.3 g/l; potassium phosphate, 100 mM, pH 6.5; PTM solution, 1 ml/L; 0.5% methanol) for induction of BiP cDNA expression. Flasks were incubated in the shaker at 28 °C, 220 rpm for 48 h. To maintain protein expression 0.5% methanol was added every 24 h. Stock solutions (20% m/V) of additional carbon sources (glycerol, glucose, mannitol, and sorbitol) were sterilized by filtration. Induction media supplements or specific cultivation conditions are described in a results section. *P. pastoris* cell cultures were centrifuged at 10,000×g for 10 min at 4 °C. Supernatants were collected and stored on ice.

High cell density fed-batch fermentation of recombinant *P. pastoris*

For this study, the selected Mut^S phenotype *P. pastoris* GS115 transformant strain BPp10, which carried multiple copies of the human BiP/GRP78 gene under the control of the *AOX1* promoter, was used. A 50 ml culture for bioreactor inoculation was prepared in two stages. First, a 20-ml primary seed YEPD medium was prepared using 200 µL of the user seed lot, which was grown at 28 °C in an orbital shaker (New Brunswick Innova 40R) at 220 rpm for 24 h. This primary seed medium was used to inoculate a 50 ml secondary seed medium containing fermentation media to 0.1 OD, then was grown for 16–18 h to 4–6 OD under the same conditions to serve as inoculum for the bioreactor culture. Cultures were grown in flasks whose volumes were 5× the culture volume to permit adequate aeration. High-cell-density fed-batch cultivation was carried out in a 5 L BIOSTAT-A plus (Sartorius Stedim Biotech) bioreactor interfaced with MFCS/ DA software for data acquisition and control. A 50 ml inoculum, prepared as described above, was transferred to the bioreactor containing 0.95 L fermentation media. The fermentation media contained per liter: glycerol, 63 g; potassium sulfate, 4.6 g; calcium sulfate, 0.24 g; magnesium sulfate heptahydrate, 3.7 g; trace metal solution (PTM1), 4 ml; ammonium sulfate, 2.25 g; potassium hydroxide, 1.0 g; phosphoric acid, 5.6 g. The PTM1 solution containing: sodium iodide, 0.08 g; manganese sulfate monohydrate, 3.0 g; sodium molybdate dihydrate, 0.2 g; boric acid, 0.02 g; zinc chloride, 20.0 g; ferric sulfate heptahydrate, 65.0 g; cupric sulfate pentahydrate, 6.0 g; biotin, 0.2 g; and sulfuric acid, 5.0 ml. 0.2 ml of antifoam (Antifoam 204, SigmaAldrich) per 1 L media was added manually before inoculation to control foaming in the bioreactor. The Aeration rate of 1 vvm was constant throughout the entire process. During glycerol batch and fed-batch phases, the dissolved oxygen (DO) was controlled at 30% saturation using an automatic DO control by agitation cascade between 400 and 750 rpm and oxygen supplementation. When a maximum of 750 rpm was reached, pure oxygen was supplied through a gas blender to control dissolved oxygen at 30% saturation. The temperature throughout the entire process was kept at 28 °C, and pH at 5.0 was maintained with 28% (v/v) NH_4OH. After exhaustion of glycerol, indicated by an increase in the DO concentration, the glycerol-fed batch phase was started. During the glycerol-fed batch phase, pH was raised and maintained at 7.0. 50% glycerol (v/v) with PTM1 was fed at 20 ml/l/h for 1 h before being ramped-down to 0 ml/l/h at a uniform rate over a 3 h period. The ramping-down of glycerol marked the beginning of the transition phase, and 4 ml/l of methanol/glucose mixture (12.5%:25%) was added to the bioreactor to allow the cells to adjust to methanol. After the transition phase, rhBiP induction was started by the addition of methanol/glucose mixture at a constant 6 ml/h in the substrate-limiting experiments or by injecting methanol/glucose mixture to the final methanol and glucose concentration of 0.5% and 1.0%, respectively. Also, 2 mM DTT (1.3 mL of 1.5 M stock solution) was injected into the bioreactor before induction. The temperature during the rhBiP production phase was maintained at 28 °C and pH at 7.0. In the oxygen-limited experiment, the dissolved oxygen (DO) control was set up to 20%, and the stirrer agitation speed was restricted to 400–600 rpm. Pure oxygen supplementation was turned off.

Purification of recombinant human BiP protein from fermentation medium

The supernatant containing the recombinant human BiP was collected for protein purification after centrifugation at 10,000 g at 4 °C for 30 min. 20 mM TRIS pH 7.0 and 1.6 M ammonium sulfate was slowly added to the supernatant and allowed to mix for 1 h at 4 °C. Next, the sample was loaded on a Phenyl Sepharose FastFlow (Cytiva, cat. no. 17096503) resin column, pre-equilibrated with 10 column volumes (CV) of binding buffer (1.6 M ammonium sulfate, 20 mM Tris-HCl pH 7.0). After extensive washing with binding buffer, bounded proteins were eluted with a linear gradient (ammonium sulfate 1.6–0 M). HIC purification fractions were analyzed using

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing rhBiP were pooled, and the buffer was exchanged to 20 mM Tris-HCl, 100 mM NaCl, and pH 7.5 using the Sephadex G25 (Cytiva, cat. no. 17003302) column. Further, proteins were loaded onto the column packed with Q Sepharose FastFlow resin (Cytiva, cat. no. 17051010) equilibrated in the same buffer. The column was washed with five volumes of binding buffer, and bound proteins were eluted with a NaCl concentration gradient (100–500 mM). Elution fraction containing recombinant protein was pooled, diluted 2 times with 20 mM Tris-HCl pH 7.5, and loaded on HiTrap Q HP column (Cytiva, cat. no. 17115301). The column was washed with five volumes of binding buffer, and bound proteins were eluted with a NaCl concentration gradient (100–500 mM). Elution fractions containing purified recombinant protein were pooled, and buffer was exchanged for BiP storage buffer (20 mM Tris-HCl, 250 mM NaCl, pH 7,5) using a Sephadex G25 column. Protein was stored frozen at – 70 °C.

ATPase assay

A non-radioactive ATPase assay was performed as described previously [21]. Reactions were performed in 50 µl volumes as follows: 1 µg of recombinant BiP protein (or equal volume of buffer for negative control) with 20 mM KCl and 20 µM ATP in ATPase buffer (50 mM HEPES, pH 6.8, 50 mM NaCl, 2 mM $MgCl_2$) was incubated at 25 °C for 75 min. The concentration of the phosphate liberated from ATP was measured by spectrophotometer (TECAN Infinite 200, wavelength 620 nm) using Malachite Green Phosphate Assay Kit (Cayman Chemical, cat. no. 10009325) according to manufacturers' recommendations.

Inhibition of aggregation of $A\beta_{42}$

To study the aggregation-inhibiting effect of rhBiP, the fibril formation kinetics of $A\beta_{42}$ and α-Syn were tracked using a thioflavin-T (ThT) fluorescence assay. ThT binding to amyloid fibrils causes a significant increase in the dye's fluorescence emission intensity, which can be used to determine the relative abundance of aggregates within the sample [55]. The $A\beta_{42}$ peptide was synthesized and purified according to the methodology described in the previous article [56]. Freshly purified (within 30 min after gel filtration) monomeric $A\beta_{42}$ solution was supplemented with 20 µM thioflavin-T (ThT, an amyloid-specific dye) and diluted using buffer A (20 mM sodium phosphate pH 7.0) containing 20 µM ThT to reach $A\beta_{42}$ concentrations of 2 µM. For the inhibition experiments, 2 µM, 1 µM, 0.4 µM, 0.2 µM, 0.1 µM solutions of rhBiP in buffer A containing 20 µM ThT were prepared. Monomeric 2 µM $A\beta_{42}$ solution was mixed with these rhBiP solutions (or with buffer A as a control) in a 1:1 ratio. Each sample was divided into three 100 µL aliquots into wells of a 96-well non-binding plate (cat. No 3881, Fisher Scientific). Kinetics of aggregation were followed under quiescent conditions at a constant 37°C temperature using a ClarioStar Plus plate reader (BMG Labtech). The intensity of ThT fluorescence was measured through the bottom of the plate every 3 min using 440 nm excitation and 480 nm emission wavelengths.

Inhibition of aggregation of alpha-synuclein

Alpha-synuclein was synthesized and purified according to the methodology described in the previous article [56]. The protein was further purified from oligomers and buffer exchanged to PBS (pH 7.4) using size-exclusion chromatography. The resulting protein was concentrated to 600 µM and stored at -20°C. The alpha-synuclein and rhBiP stock solutions (or PBS for the control) were mixed and diluted with PBS and ThT (10 mM) solutions to a final reaction mixture containing 100 µM alpha-synuclein, 5 µM rhBiP and 100 µM ThT. The sample was divided into three 100 µL aliquots into wells of a 96-well non-binding plate (each well contained a 3 mm glass bead), and kinetics of aggregation were followed under constant 600 RPM orbital agitation and 37°C temperature. The intensity of ThT was measured every 10 min.

Other methods and materials

Purified rhBiP concentrations were determined by measuring absorption at 280 nm wavelength with NanoDrop 2000. The used extinction coefficient and molecular weight were 28.02 $M^{-1}cm^{-1}$ and 70.48 kDa.

Amounts of secreted BiP in the culture medium were compared to a range of known quantities of BiP in a linear dynamic range ($R^2 > 0.99$) of Coomassie-stained protein bands in SDS-PAGE gels. To determine BiP purity in crude yeast culture media, total secreted yeast proteins were concentrated by precipitation and yeast protein impurities were evaluated by SDS-PAGE.

Densitometric analysis of SDS-PAGE gels and Western blots, scanned with ImageScanner III (GE Healthcare), was performed with ImageQuant TL (GE Healthcare) software using default settings.

The precipitation of proteins from yeast growth medium for SDS-PAGE analysis was performed using a defined methanol-chloroform-water mixture, as described earlier [57].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02679-z.

Supplementary Material 1

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Author contributions
E.Ž. performed experiments, collected and analysed data, and wrote a manuscript. E.Č. conceived of the study, performed experiments, collected and analysed data, and revised manuscript. A.S. purified amyloid-beta peptide and alpha-synuclein. M.Ž. measured kinetics of aggregation inhibition and analysed data. R.S. was involved in planning of the study, analysed, interpreted data, and drafted the manuscript. All authors read and approved the final manuscript.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests
A part of the work presented in this paper was filed as international patent application.

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