Engineering *Saccharomyces Cerevisiae* for the Production and Secretion of Affibody Molecules.

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Abstract

Background

Affibody molecules are synthetic peptides with a variety of therapeutic and diagnostic applications. To date, Affibody molecules have mainly been produced by the bacterial production host *Escherichia coli*. There is an interest in exploring alternative production hosts to address if improvements in terms of yield, ease of production and if purification advantages can be identified. In this study, we evaluated the feasibility of *Saccharomyces cerevisiae* as a production chassis for this group of proteins.

Results

We examined the production of three different Affibody molecules in *S. cerevisiae* and found that these Affibody molecules were partially degraded. An albumin-binding domain, which may be attached to the Affibody molecules to increase their half-life, showed to be a substrate for several *S. cerevisiae* proteases. We tested the removal of three vacuolar proteases, proteinase A, proteinase B and carboxypeptidase Y. Removal of one of these, proteinase A, resulted in intact secretion of one of the targeted Affibody molecules. Removal of either or both two additional proteases, carboxypeptidase Y and proteinase B, resulted in intact secretion of the two remaining Affibody molecules. The produced Affibody molecules were verified to bind human HER3 as potently as the corresponding molecules produced in *E. coli* in an *in vitro* surface-plasmon resonance binding assay. Finally, we performed a fed-batch fermentation with one of the engineered protease-deficient *S. cerevisiae* strains and achieved a protein titer of 530 mg Affibody molecule/L.

Conclusion

This study shows that engineered *S. cerevisiae* has a great potential as a production host for recombinant Affibody molecules, reaching high yields and for proteins where endotoxin removal could be challenging, the use of *S. cerevisiae* obviates the need for endotoxin removal from protein produced in *E. coli*.

Background

Affibody molecules are synthetic peptides that can be designed to bind with high affinity to other proteins. The part of the protein exhibiting binding properties originates from the B domain of the immunoglobulin-binding region of the staphylococcal protein A (SPA) [1]. Nilsson *et al.* isolated the B domain of SPA and increased its stability, which led to the creation of the synthetic Z domain [2]. By randomizing 13 surface-exposed residues the specificity and affinity of the Z domain can be altered. Over the years, several Z domain variants have been generated with affinity to diagnostically relevant substrates, such as cancer markers [3]–[5]. A specific example of this is HER3. Elevated HER3 expression is associated with malignant cancer in ovarian, prostate, gastric, bladder, lung, melanoma and colorectal tissue [6]. Current applications for Affibody molecules are mostly within diagnostics and therapeutics where the main

advantage of Affibody molecules compared to alternatives, like antibodies, is their small size which allows them to penetrate tissue more easily [7, 8].

Affibody molecules can be designed in a range of configurations, either containing solely Z-domains or Z-domains together with other peptide sequences. Affibody molecules composed of only the Z domain exhibit a relatively short half-life in the human body. Due to their small size, 6.7 kDa for a single Z domain, Affibody molecules are taken up by the kidneys and degraded [9]. To prolong the half-life of the Affibody molecules an albumin binding domain (ABD) can be added. The ABD originates from the GA3 module of Streptococcal protein G and binds to serum albumin. Serum albumin, with a size of 67 kDa, is above the glomerular filtration barrier of the kidneys and adding ABD to Affibody molecules thus increases both their half-life and efficacy [5][10]. Different configurations of the Z domain and the ABD domain, like the presence of multiple Z domains or an alternative localization of the ABD within the molecule, have shown to be effective in a range applications [7, 10, 11].

Currently, Affibody molecules are produced in the bacterial production host *Escherichia coli*. Bacteria are popular hosts for recombinant production due to rapid growth and high yields of the recombinant proteins [12]. However, recombinant protein production in *E. coli* lead to contamination with lipopolysaccharides from the bacterial cell wall, most of which exhibit endotoxin properties and can be difficult to remove during product purification [13, 14]. It is therefore of interest to explore other established and competitive production hosts like yeasts that do not have toxic components in their cell wall.

Yeasts are eukaryotic unicellular organisms, which secrete recombinant proteins and have been implemented as industrial production hosts for several pharmaceutical proteins [15, 16]. The most widely used yeast species for the industrial production of recombinant pharmaceutical proteins are *Saccharomyces cerevisiae* and *Pichia pastoris* [16]. *P. pastoris* has been used as production host of a fusion construct of human serum albumin and a HER2 Affibody molecule however *S. cerevisiae* has not been tested as a Affibody molecule production host yet [17]. *S. cerevisiae* is particularly known for its industrial production of insulin, human serum albumin, and hepatitis vaccines [18].

In this study, we tested an engineered *S. cerevisiae* strain for the production and secretion of three Affibody molecules that bind to the cancer marker protein HER3 [6]. Initially, we found that *S. cerevisiae* degraded the produced Affibody molecules. We were able to identify several proteases responsible for the degradation and upon removal of these proteases, all three Affibody molecules were secreted in an intact state. We verified high-affinity HER3 binding by the secreted Affibody molecules and performed a fed-batch cultivation where a high final titer was reached; thus demonstrating that *S. cerevisiae* is a competitive host for Affibody molecule production.

Materials And Methods

Strains and plasmids.

Two previously constructed *S. cerevisiae* strains were used in this study, CEN.PK 530.1C [*MAT α URA3 HIS3 LAU2 TRP1 SUC2 MAL2-8^c tpi1(41-707)*] (AAC) and B184k. In a previous study, AAC was evolved for improved protein production by UV-mutagenesis which led to the construction of strain B184k [19]. B184k was shown to be an effective host for the production of several recombinant proteins in combination with the CPOT plasmid [19]. The CPOT plasmid contains the *Schizosaccharomyces pombe* gene *POT1* which partially complements the *TPI1* deficiency. The recombinant protein expression cassette contains the native *TPI1* promoter and terminator and an α -leader sequence. The empty CPOT is a CPOT plasmid without the recombinant protein gene. The previously constructed pNatAmyCPOT was cut by restriction digest with enzymes XhoI and KpnI to remove only the α -amylase gene. Genes for Z_{HER3_1}-ABD, Z_{HER3_1}-Z_{HER3_1}-ABD and Z_{HER3_1}-ABD-Z_{HER3_1} were synthesized from GenScript. The Affibody molecule genes were codon optimized for expression in *S. cerevisiae* but without repetitive DNA sequences to reduce the risk of homologous recombination within the ORFs. The three Affibody molecule genes were amplified with homologous overhangs of the α -leader and the CPOT backbone using primers presented in Table S1. The backbone and Affibody genes were assembled by Gibson assembly and the final constructs verified by sequencing using primer #7 that binds in the α -leader sequence. All primers for the construction of the plasmids can be found in Table S1. After the plasmid construction, the newly assembled CPOT plasmids, pNatZACPOT, pNatZZACPOT and pNatZAZCPOT were used to transform AAC and B184k. B184k still contained a kanamycin resistance marker in the *TPI1* gene. In AAC, the marker had been removed previously. The marker has two flanking *loxP* sites. pSH66 from the Euroscarf deletion marker set was used to remove the kanamycin resistance marker [48]. pSH66 contains an expression cassette for Cre recombinase under control of the *GAL1* promoter and a nourseothricin resistance gene. After the strains had been transformed with the pSH66 plasmid with selection for nourseothricin, the positive transformants were streaked out on solid media with YPGal with nourseothricin to activate Cre recombinase expression. Removal of the kanamycin marker was confirmed by absence of growth on solid media with G418. *PEP4*, *PRC1* and *PRB1* were deleted by using plasmids pECAS9-gRNA-kanMX-tPEP4, pECAS9-gRNA-kanMX-tPRC1, pECAS9-gRNA-kanMX-tPRB1, which contain both a *cas9* gene and a gRNA expression cassette [49]. The plasmids pECAS9-gRNA-kanMX-tPEP4, pECAS9-gRNA-kanMX-tPRC1 and pECAS9-gRNA-kanMX-tPRB1 were constructed using pECAS9-gRNA-kanMX-tHFD1 as template [49]. First, the backbone of pECAS9-gRNA-kanMX was obtained by linearizing pECAS9-gRNA-kanMX-tHFD1 by digestion with MunI and EcoRI. The 'left' fragment was constructed with primer #14 in combination with either #9 (*PEP4*), #11 (*PRC1*) or #13 (*PRB1*) and the 'right' fragment was constructed with primer #15 in combination with either #8 (*PEP4*), #10 (*PRC1*) or #12 (*PRB1*). The correct assembly of the plasmids was confirmed by sequencing using primer #16. The genes were deleted in B184 pNatZACPOT, B184 pNatZZACPOT and B184 pNatZAZCPOT. The genomic deletions were verified using forward primers #17 and #18 with reverse primer #19 for *PEP4*, forward primers #20, #21 and reverse primer #22 for *PRC1*, and forward primers #23, #24 and reverse primer #25 for *PRB1*. The CRISPR plasmids were removed by subsequent cultivation in liquid YPD confirmed by absence of growth on solid media with G418. All the primers are presented in Table S1 and repair oligos for CRISPR in Table S2. *E. coli* DH5 α was used for plasmid amplification. The transformation protocol used for *E. coli* was according to a known protocol [50]. The strains and plasmids used and constructed in this study are shown in Table 2 and Table 3.

Table 2
Yeast strains used in this study

Strain	Genotype	Reference
AAC	CEN.PK113-7D (<i>MATa URA3 HIS3 LEU2 TRP1 MAL2-8c SUC2 tpi1(41-707)::loxP-loxP</i>)	[51]
B184k	UV-mutant with AACk as background (<i>tpi1(41-707)::loxP-KanMX4-loxP</i>)	[19]
B184	UV-mutant with AACk as background (<i>tpi1(41-707)::loxP-loxP</i>)	
B184 <i>pep4Δ</i>	B184 <i>pep4Δ</i>	This study
B184 <i>prc1Δ</i>	B184 <i>prc1Δ</i>	This study
B184 <i>prb1Δ</i>	B184 <i>prb1Δ</i>	This study
B184 <i>pep4Δ prc1Δ</i>	B184 <i>pep4Δ prc1Δ</i>	This study
B184 <i>pep4Δ prb1Δ</i>	B184 <i>pep4Δ prb1Δ</i>	This study

Table 3
Plasmids used in this study

Plasmid	Description	Reference
pECAS9-gRNA-kanMX-tHFD1	2μ vector with kanMX marker expressing eCas9 under the <i>TEF1</i> promoter and CYC1 terminator and the gRNA targeting HDF1 under the SNR52 promoter	[49]
pECAS9-gRNA-kanMX-tPEP4	2μ vector with kanMX marker expressing eCas9 under the <i>TEF1</i> promoter and CYC1 terminator and the gRNA targeting PEP4 under the SNR52 promoter	This study
pECAS9-gRNA-kanMX-tPRC1	2μ vector with kanMX marker expressing eCas9 under the <i>TEF1</i> promoter and CYC1 terminator and the gRNA targeting PRC1 under the SNR52 promoter	This study
pECAS9-gRNA-kanMX-tPRB1	2μ vector with kanMX marker expressing eCas9 under the <i>TEF1</i> promoter and CYC1 terminator and the gRNA targeting PRB1 under the SNR52 promoter	This study
pNatAmyCPOT	2μ vector with cassette expressing POT1 gene from <i>S. pombe</i> and an expression cassette with α-leader sequence and α-amylase gene under native TPI1 promoter and terminator	[21]
pCPOT	2μ vector with cassette expressing POT1 gene from <i>S. pombe</i> and an expression cassette with native TPI1 promoter and terminator without recombinant protein gene	[21]
pNatZACPOT	2μ vector with cassette expressing POT1 gene from <i>S. pombe</i> and an expression cassette with α-leader sequence and Z _{HER3_1} -ABD gene under native TPI1 promoter and terminator	This study
pNatZZACPOT	2μ vector with cassette expressing POT1 gene from <i>S. pombe</i> and an expression cassette with α-leader sequence and Z _{HER3_1} -Z _{HER3_1} -ABD gene under native TPI1 promoter and terminator	This study
pNatZAZCPOT	2μ vector with cassette expressing POT1 gene from <i>S. pombe</i> and an expression cassette with α-leader sequence and Z _{HER3_1} -ABD-Z _{HER3_1} gene under native TPI1 promoter and terminator	This study

Media and culture conditions

The media used for *S. cerevisiae* strain construction were YPD, YPGal, YPE and YPEG. The experiments were always performed at 30°C and for liquid cultures at 220 rpm. YPD medium contained 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose and was used for regular cultures. YPGal media contained 10 g/L yeast extract, 20 g/L peptone, and 20 g/L galactose and was used for induction of the Cre recombinase gene on pSH66. For the selection of the kanMX marker on the CRISPR plasmid, 200 mg/L G418 was added to the YPD medium. For the selection of pSH66-containing cells, 100 mg/L nourseothricin sulfate was added to YPD and YPGal medium. The YPE medium contained 10 g/L yeast extract, 20 g/L peptone, 20 g/L absolute ethanol and was solely used as a solid medium. For liquid cultivations, 30 g/L glycerol was added, and the medium referred to as YPEG. Both YPE and YPEG were only used for *S. cerevisiae* strains without CPOT plasmids since those are unable to ferment glucose as

the sole carbon source [52]. To solidify the media 20 g/L agar (Merck Millipore) was added. The protein expression and physiological experiments were performed in SD2xSCAA media at 30°C and 220 rpm. SD-2XSCAA medium contained 20 g/L glucose, 6.9 g/L yeast nitrogen base without amino acids, 190 mg/L Arg, 400 mg/L Asp, 1260 mg/L Glu, 130 mg/L Gly, 140 mg/L His, 290 mg/L Ile, 400 mg/L Leu, 440 mg/L Lys, 108 mg/L Met, 200 mg/L Phe, 220 mg/L Thr, 40 mg/L Trp, 52 mg/L Tyr, 380 mg/L Val, 1 g/L BSA, 5.4 g/L Na₂HPO₄, and 8.56 g/L NaH₂PO₄·H₂O with a pH of 6.4. Cells for protein production experiments were grown at 30°C at 220 rpm in aerated 24-well plates CR1224 (Bioscreen) with a volume of 2.5 mL and a start OD₆₀₀ of 0.1 or in 14 mL-cultivation tubes with a volume of 2 mL. For the binding assay experiment, a volume of 200 mL of SD2xSCAA, inoculated with an overnight culture to an OD of 0.1, was cultivated for 96 h at 30°C at 220 rpm. The supernatant was stored at -80°C before being sent to Affibody AB on dry ice. The *E. coli* cells were grown in Luria-Bertani (LB) media at 37°C and 200 rpm. Selection media contained 80 mg/L ampicillin.

Molecular biology techniques

S. cerevisiae strains were transformed using the LiAc/SS carrier method [53]. 1000 ng of DNA was used for the transformation of plasmids and an additional 1 to 2 µg repair fragment if required. To verify deletions or test for the presence of the CPOT plasmids, colony PCR using SapphireAmp fast PCR mix (TaKaRa Bio) was performed. For DNA construction, Phusion High Fidelity DNA polymerase (Thermo Scientific) or Herculase II Fusion DNA polymerase (Aligent) was used. Restriction digestion was done using FastDigest (Thermo Scientific) products. All techniques were used according to the manufacturers' protocols unless stated otherwise.

Growth profiler experiments

Three independent transformation colonies per strain were grown for 24 h in 1 mL SD2xSCAA media in a 14 mL-cultivation tube. Those precultures were used to inoculate the main cultures of the growth experiment in technical triplicates with a starting OD of 0.01. The *S. cerevisiae* strains were cultivated for 96 h in 250 µL SD2xSCAA media at 30°C and 1200 rpm in 96-well plates (Enzyscreen CR1496d). The growth curves were measured using a Growth Profiler 960 (Enzyscreen).

Protease activity experiments

For the incubation experiments, supernatant of cultivations were used. The initial cultivation was a 24 h-cultivation, if not mentioned otherwise, of a single colony in 2 mL of SD2xSCAA media. After the incubation, the culture was harvested by centrifugation at 6000 rpm for 3 min. The supernatant was kept on ice or frozen at -20°C. The incubation was performed in 1.5-mL Eppendorf tubes with 200 µL volume of supernatant. As positive controls, standards of purified ZHER3_1-ABD (1.97 mg/mL), ZHER3_1-ZHER3_1-ABD (0.77 mg/mL) and ZHER3_1-ABD-ZHER3_1 (1.34 mg/mL) provided by Affibody AB were used with a concentration of 0.01 g/L or stated otherwise. The experiments with the protease inhibitors were done by adding the protease inhibitor before adding the Affibody standard at the concentration suggested by the supplier. We used the Halt Protease Inhibitor Cocktail EDTA-free (100x) (Thermo Fisher) and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (final concentration 1 mM),

aprotinin (final concentration 6.5 µg/mL), bestatin hydrochloride (final concentration 50 µM), leupeptin (final concentration 20 µM), E-64 (final concentration 15 µM), and pepstatin A (final concentration 10 µM). All these chemicals were purchased from Merck.

SDS-PAGE and western blotting

The samples and controls were loaded and separated with reducing SDS-PAGE. Stain free 4-20% gels were used (Bio-rad). The proteins were transferred to 0.45-micron PVDF membranes (Bio-rad) using the Trans-Blot Turbo transfer system (Bio-rad). The blot was blocked using the Western blocker solution (Sigma Aldrich) and incubated in with either anti-Z-domain (2.87 mg/mL) (1:1000) or anti-ABD (1 mg/mL) (3:1000) antibodies, both obtained from Affibody AB followed by incubation with either anti-mouse antibody (1:5000) for anti-Z-domain or anti-rabbit (1:5000) antibody for anti-ABD antibodies, respectively. Both secondary antibodies were HRP-conjugated and visualized by using West Pico Plus HRP substrate (Thermo Fischer) and measured with a ChemidoC XRS image analyzer (Bio-Rad). As positive controls, standards of purified Z_{HER3_1}-ABD (1.97 mg/mL), Z_{HER3_1}-Z_{HER3_1}-ABD (0.77 mg/mL) and Z_{HER3_1}-ABD-Z_{HER3_1} (1.34 mg/mL) provided by Affibody AB were used with a concentration of 0.01 g/L or stated otherwise.

Mass spectrometry analysis

Intact mass analysis was performed on an Agilent 1200 LC system equipped with a C8 RP-HPLC column (Poroshell 300SB-C8, 5µm, 2.1x75mm, Agilent) and coupled to a high-resolution Q-TOF (Bruker Maxis Impact). Buffer A was 0,1% formic acid in 10% acetonitrile, buffer B was 0,1% formic acid in 90% acetonitrile and the column temperature was 80°C. Proteins were eluted from the LC column by using a linear gradient of solvent B from 5–75% over 5 min at a flow rate of 0.3 mL/min. The MS analysis was performed with an ESI source and in positive mode. MS was set in a MS only mode. The following MS parameters were used: mass range 500-4500, capillary 4500 V, nebulizer 1.2 Bar, drying gas 8.0 L/min and temperature 200°C. The MS data were deconvoluted in Compass DataAnalysis (Bruker, version 4.4). The compound spectra were first smoothed using the Savitzky Golay algorithm with a smoothing width of 0.2 Da and 3 cycles. Then the spectrum baseline was subtracted with 0.8 flatness. Afterwards, the raw spectra were deconvoluted using the MaxEnt algorithm. For the native proteins, the spectrums were deconvoluted within the mass range of 1.000-25.000 and a resolving power of 1250. The observed masses were compared to theoretical masses of different fragment sequences with GPMaw version 12.50 with a precision in Da at 0.5. Only fragment sequences with a mass accuracy ≤2 Da were reported.

Binding kinetics analysis

Surface plasmon resonance (SPR) analysis by Biacore 8K (Cytiva, Marlborough, MA) was performed at 25°C in a run buffer of HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20, Cytiva) and with 15 mM HCl as regeneration solution. Recombinant human ErbB3/Her3 Fc (R&D Systems, Minneapolis, MN) was immobilized by standard amine coupling (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS)) on a CM5 sensor chip (Cytiva, Marlborough, MA) at ~ 1000 RU. The coated chip was preconditioned by three regeneration

rounds to stabilize surfaces prior to injection of analyte. Binding of Affibody molecules to HER3 was analyzed by single cycle kinetics via injection of analyte at five different concentrations of purified Affibody molecule (1.25, 2.5, 5, 10 and 20 nM) over immobilized HER3/Fc. The experiment was performed in duplicates. Biacore Insight Evaluation Software was used to process, analyze and fit data.

Fed-batch bioreactor cultivations

For the Bioreactor experiments, the Na_2HPO_4 and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ were replaced by KH_2PO_4 with a concentration of 2 g/L in the SD2xSCAA media. The batch fermentations were performed in 1-L bioreactors (DasGip) with a start OD_{600} of 0.01 in 500 mL of SD2xSCAA media. The conditions in the reactors were controlled and maintained at a pH of 6.0 by 4 M KOH, agitation at 600 rpm, temperature at 30°C degrees and airflow of 30 L/h. The batch experiments were performed in biological quadruplicates. After all the glucose was consumed, culture broth was removed until a start volume of 250 mL. The conditions of the fed-batch were set to a pH of 6.0, maintained by 4 M KOH and 3 M HCl, agitation was set to 600 rpm, the temperature was set to 30°C degrees, a constant airflow of 18 L/h and an exponentially increasing feed rate of 0.05 h^{-1} . When the dissolved oxygen in the bioreactor would decrease below 30% the airflow and agitation would increase. The airflow would increase to 48 L/h and agitation would increase to 1000 rpm. When both the agitation and airflow would be at their maximum levels the feeding was changed to a pulse feed controlled by the dissolved oxygen level. The feed rate would stop if the dissolved oxygen was below 25% which resulted in a pulsed feeding rate around 8 mL/h. Two types of media were used for the fed-batch experiments. The first medium was the low-glucose feed with 200 g/L, 69 g/L yeast nitrogen base without amino acids, 50 g/L casamino acids (Formedium), 1 g/L BSA, and 20 g/L KH_2PO_4 . After adding an average of 320 mL of the low glucose feed the low-glucose feed was replaced by a high-glucose feed with the same composition as the low-glucose feed except for a glucose concentration of 400 g/L. The fermentation was ended after addition of 230 mL of the high-glucose feed. The fed-batch experiments were conducted in biological triplicates.

Affibody molecule quantification by Bio-Layer Interferometry (BLI).

The broth samples were centrifugated for 20 min at 4000 rpm to remove the biomass and the supernatant was stored at -80°C. After thawing some precipitation appeared in the samples. To redissolve the precipitation the samples were 1:1 diluted in 1 NaCl in two duplicates per fermenter per sample point and transported on dry ice. Upon arrival the samples were thawed and either not diluted or diluted 1:3.75, 1:7.5 in sample diluent (1x PBS, 0.1% BSA, 0.02% Tween 20) based on the expected concentration. 225 μL of the pre-dilution was mixed with either 75 μL sample diluent (unspiked) or 75 μL of 100 $\mu\text{g/mL}$ $\text{Z}_{\text{HER3}_1^-}$ ABD- Z_{HER3_1} (produced by *E. coli*) (spiked) was added resulting in a 1:5, 1:10 or 1:1.33 final dilution. 200 μL of the spiked and unspiked sample was moved to a black flat bottomed 96 well plate (Greiner, #655209) together with 200 μL of the samples for the standard curve, 200 μL sample diluent (reference well) and 200 μL cultivation medium (negative control). High precision streptavidin (SAX) biosensors (ForteBio, #18-0037) which were previously loaded offline (Technical Note #10, ForteBio) with 50 $\mu\text{g/mL}$ biotinylated HSA (Recombunin Prime, Albumedix; Thermo Fisher #A39259) (MCR 1:1) were placed for 10

minutes in sample diluent before the measurements. The data was acquired using an Octet HTX system and Octet Data Acquisition software (ForteBio, ver. 12). The measurement settings were as follows: 8 channel read head, 5.0 Hz acquisition rate, quantitation step: 120 s, 400 rpm, plate temperature 30°C. The quantity was determined based on the six-point standard curve with Z_{HER3_1}-ABD-Z_{HER3_1} produced by *E. coli* (100 µg/mL-3.13 µg/mL). Data Analysis HT (ForteBio, ver. 12) was used to process the data. The reference well signal was subtracted from the samples and standard curve, and results with 70-143% spike recovery were included in the calculations. The assay was run twice for each sample.

Results

The albumin-binding domain is degraded by *S. cerevisiae*.

We first tested whether Affibody molecules could be produced and secreted by *S. cerevisiae*. We included three different Affibody molecules in this study. These three molecules have different configurations of either one or two Z_{HER3_1} domains, the Z domain variant that binds to HER3, and one albumin binding domain (ABD) (Figure 1). *S. cerevisiae* strain B184k, previously evolved for high levels of protein secretion, was used as the host [19, 20]. Genes encoding the three different Affibody molecules shown in Figure 1 were cloned into the backbone of a CPOT plasmid, thus generating the three plasmids pNatZACPOT (Z_{HER3_1}-ABD), pNatZZACPOT (Z_{HER3_1}-Z_{HER3_1}-ABD), and pNatZAZCPOT (Z_{HER3_1}-ABD-Z_{HER3_1}) [21]. The CPOT is a recombinant protein expression plasmid for use in *S. cerevisiae* *tpi1*Δ strains. The CPOT plasmid contains the *POT1* gene encoding triose phosphate isomerase from *Schizosaccharomyces pombe* which complements partly the removal of the *TPI1* gene and restores the ability to grow on glucose as a carbon source. The partial complementation will lead to a high abundance of the plasmid in the cell, which is combined with the expression regulation of the recombinant gene by the native *TPI1* promoter and terminator [21]. The combination of the CPOT expression system and B184 has shown effective for high-level production of several recombinant proteins [19, 20]. The plasmids pNatZACPOT, pNatZZACPOT and pNatZAZCPOT were used to transform B184k and the positive transformants were grown for 48 h in liquid SD2xSCAA media [22]. The supernatant was analyzed by a reducing SDS-PAGE followed by western blot using antibodies against both the ABD and the Z domain (Figure 2A, 2B and 2C). Although all three Affibody molecules were secreted the bands did not appear with the expected sizes on the blot. We expected a 12 kDa band for Z_{HER3_1}-ABD and 18.9 kDa for Z_{HER3_1}-ABD-Z_{HER3_1} and Z_{HER3_1}-Z_{HER3_1}-ABD. The supernatant derived from the Z_{HER3_1}-ABD-Z_{HER3_1} expressing strain showed three separate smaller bands on the anti-Z-domain blot with sizes compared to the Z_{HER3_1}-ABD standard around 12 kDa instead of one intact band seen in the Affibody standard around 18.9 kDa (Figure 2B). The supernatant of the Z_{HER3_1}-ABD expressing strain showed two bands instead of one on the anti-Z-domain blot which appear around the expected size of 12 kDa (Figure 2B). The anti-Z-domain blot for Z_{HER3_1}-Z_{HER3_1}-ABD showed an apparently intact band around the correct size of 18.9 kDa (Figure 2B). Finally, the ABD domain showed none or little signal on the anti-ABD blot for any of the Affibody molecules (Figure 2C). The absence of bands on the anti-ABD blots indicated an absence of intact ABD in the secreted Affibody molecules.

We hypothesized that the secreted molecules were degraded by *S. cerevisiae* proteases leading to fragmentation (Figure 2B and 2C). We also checked if the presence of BSA might have an influence. Since the albumin-binding domain binds to serum albumin [5], and one of the components of the SD2xSCAA media that was used for cultivation was bovine serum albumin (BSA), we wanted to exclude any BSA related interference. We tested our hypotheses by incubating one Affibody molecule standard for the Z_{HER3_1}-ABD in four different solutions. The solutions were fresh sterile medium, spent supernatant, demineralized water with 0.1% BSA and demineralized water. The spent supernatant is the supernatant of cultures in which yeast has been cultivated and removed by centrifugation. If the absence of the intact ABD is due to proteolytic activity, we expect to see degradation by incubating the Affibody molecule standard in the spent supernatant. The spent supernatant for this experiment was derived from a 24-h culture of B184k producing α -amylase, from which the cells had been removed by centrifugation. After the incubation, the proteins were separated by reducing SDS-PAGE and analysed by western blotting against the ABD (Figure 2D and 2E). The western blot shows intact bands for the fresh sterile medium, demineralized water with 0.1% BSA, and demineralized water. For the spent supernatant, the stained SDS-PAGE shows two bands instead of one and there is no band visible at the western blot against the ABD, which indicates degradation of the Affibody molecule in the spent supernatant.

Affibody molecules are degraded by aspartyl protease(s).

Proteolytic activity can drastically decrease recombinant protein yield; however, this can often at least partially be prohibited by the identification and removal of the responsible proteases [23]. First we wanted to check if the degradation was a characteristic of the engineered yeast strain B184k, since a potential solution for the degradation would be to switch to another strain. We checked the supernatant of the parental strain of B184k, AAC[19]. The influence of expression of the recombinant protein on the degradation was also tested by the expression of α -amylase by AAC. Both B184k and AAC were cultivated with either pNatAmyCPOT or an empty CPOT plasmid. The Affibody standard of Z_{HER3_1}-ABD-Z_{HER3_1} was incubated in spent medium from those cultivations and the mixture with the proteins was separated by reducing SDS-PAGE (Supplementary Figure 1). We included supernatant after 24, 48, 72, and 96 h of cultivation and tested the presence of a protease inhibitor cocktail to confirm that the degradation was due to proteases. Degradation of the Affibody molecules was also observed in spent medium of AAC (Figure S1). In the supernatant containing protease inhibitor cocktail, we did not observe degradation, confirming that the fragmentation of the Affibody molecules was a result of proteolytic activity. We observed a similar degradation pattern as for B184k. Degradation, however, seemed slightly elevated in supernatant derived from strains with recombinant α -amylase production, which could potentially indicate an induction of protease expression or activity upon recombinant protein production. Since the degradation was observed also in the parental strain of B184k we concluded that the degradation is not a characteristic of B184k so therefore we continued with B184k as the production host.

The next step was to identify the responsible proteases for the degradation. Protease inhibitors mostly block the activity of a specific class of proteases. Therefore, a mixture of different protease inhibitors were combined in protease inhibitor cocktails to ensure inhibitions of all sort of proteases. Since in the

experiment with AAC (Figure S1) a protease inhibitor cocktail showed effective against the degradation we tested the isolated inhibitors from that specific cocktail. The spent medium of B184k expressing α -amylase was incubated overnight with Affibody molecule standards and the individual components of the protease inhibitor cocktail. The mixture was subsequently analyzed by a reducing SDS-PAGE. The results of the SDS-PAGE show that the Affibody molecules were degraded in the presence of AEBSF, aprotinin, bestatin, E-64 or leupeptin (Figure 3). Only incubation with pepstatin A prohibited degradation and resulted in Affibody molecules with the correct size (Figure 3). Both the Affibody molecules Z_{HER3_1} - Z_{HER3_1} -ABD and Z_{HER3_1} -ABD- Z_{HER3_1} clearly showed the absence of proteolytic processing on the SDS-PAGE in the presence of pepstatin A. For Z_{HER3_1} -ABD the difference was less explicit which could be a result of the small size difference between the intact and degraded molecule. Pepstatin A inhibits aspartyl proteases, indicating that the protease(s) responsible for Affibody molecule processing are of this type.

The removal of several proteases results in the secretion of intact Affibody molecules.

One of the major proteases in *S. cerevisiae* and a proven target for improving recombinant protein production yield is a vacuolar aspartyl protease, proteinase A [23–25]. We removed the *PEP4* gene encoding proteinase A in B184 using CRISPR/Cas9-based gene deletion and expressed all three Affibody molecules in B184 *pep4* Δ . Z_{HER3_1} -ABD- Z_{HER3_1} produced by this strain was intact and showed a signal on western blots using antibodies against either the Z-domain or ABD (Figure 4), indicating that proteinase A was indeed responsible for its cleavage in B184. In contrast, Z_{HER3_1} -ABD and Z_{HER3_1} - Z_{HER3_1} -ABD, the western blot against the Z-domain shows small shift of the bands upon removal of *PEP4* but there is no band visible on the blot against the ABD (Figure 4A). We suspected that (an)other protease(s) was involved in cleaving the ABD domain of Z_{HER3_1} -ABD and Z_{HER3_1} - Z_{HER3_1} -ABD in B184 *pep4* Δ .

We therefore removed two additional proteases in B184 *pep4* Δ in the hope of producing intact Z_{HER3_1} -ABD and Z_{HER3_1} - Z_{HER3_1} -ABD, namely carboxypeptidase Y and proteinase B. Carboxypeptidase Y is a serine-based exopeptidase active in the vacuole and proteinase B, a serine-based endopeptidase also located to the vacuole. Proteinase B, together with proteinase A, plays an essential role in the activation of several vacuolar proteases including itself and carboxypeptidase Y. The *PRC1* gene encoding carboxypeptidase Y and the *PRB1* gene encoding proteinase B were removed in B184 and B184 *pep4* Δ (*pep4* Δ *prc1* Δ and *pep4* Δ *prb1* Δ) and a triple deletion strain (*pep4* Δ *prc1* Δ *prb1* Δ) was constructed. The single, the two double and the triple deletion strains were grown for 48 h in an aerated 24-well plate and the spent supernatant was analyzed for the presence of intact Affibody molecules. In the B184 *pep4* Δ *prc1* Δ strain we observed intact Z_{HER3_1} -ABD and Z_{HER3_1} - Z_{HER3_1} -ABD (Figure 5A and 5B). Interestingly, the removal of solely *PEP4* during cultivation for 48 h instead of 96 h, resulted in very weak bands for the ABD for both Z_{HER3_1} -ABD and Z_{HER3_1} - Z_{HER3_1} -ABD on the blot against the ABD. This indicates that the degradation in B184 *pep4* Δ could be time dependent. The single removal of either *PRC1* and *PRB1* did not result in a band on the western blot using antibody against the ABD. However, the combination of *pep4* Δ (proteinase A) with either *prb1* Δ (proteinase B) or *prc1* Δ (carboxypeptidase Y) resulted in the production of intact Z_{HER3_1} -ABD as well as intact Z_{HER3_1} -ABD- Z_{HER3_1} . The deletion of all

three proteases (*pep4Δprc1Δprb1Δ*) resulted in titers comparable to the two double deletion strains (Figure 5A and 5B).

Proteases fulfill a crucial role in the degradation of macromolecules in the vacuole and have even been reported to benefit aging [26, 27]. Therefore, the removal of one to three major proteases can have an impact on vacuolar homeostasis and cellular growth. We monitored the growth profiles of the protease-deficient strains in aerated 96-well plates. The protease deficiency leads to a slight reduction in the later stages of the growth, from 50 h to 96 h, but overall growth of the deletion strains, even the triple deletion strain, was similar to B184 with the proteases intact. This was observed for both Z_{HER3_1}-ABD and Z_{HER3_1}-Z_{HER3_1}-ABD (Figure 5C and 5D).

Additionally, we were interested in identifying the cut site of the endopeptidase proteinase A in the ABD. It is known that proteinase A has a broad and variable activity for different ligands and seems to favor cut sites between adjacent hydrophobic residues [28, 29]. We incubated the Z_{HER3_1}-ABD-Z_{HER3_1} standard in spent supernatant for 24 h and analysed the mixture with mass spectrometry. The total ion chromatogram (TIC) showed four peaks, two large peaks and two smaller ones (Figure 6A). The peptides within the two larger peaks were analysed. In the first peak, several fragments of Z_{HER3_1}-ABD-Z_{HER3_1} were identified where either the N- or C-terminus was intact and the other terminus was located within the ABD indicating a cut site of an endopeptidase (Figure 6B). The peptides in the fourth peak were fragments which had the N-terminus in the ABD and the C-terminus in the second Z_{HER3_1} sequence (Figure 6B). Based on these fragments it seems that proteinase A cuts at several sites within the ABD and interestingly the C-terminal Z_{HER3_1} domain was also not intact.

Z_{HER3_1}-ABD-Z_{HER3_1} molecule produced by B184 *pep4Δ* shows similar binding kinetics compared to the molecule produced by *E. coli*.

We next aimed to test whether the engineered *S. cerevisiae* strain is a competitive host for the Affibody molecule production with regards to product quality. The Z_{HER3_1}-ABD-Z_{HER3_1} molecule produced and secreted by B184 *pep4Δ* was tested in a surface plasmon resonance binding assay with the substrate of Z_{HER3_1}, HER3. The Z_{HER3_1}-ABD-Z_{HER3_1} Affibody has two binding sites for HER3; therefore, the bivalent binding kinetics were determined and presented as average value of duplicates in Table 1. The equilibrium dissociation constant of the first site has a value in the nano molar range which indicates high affinity. The values of the Z_{HER3_1}-ABD-Z_{HER3_1} molecules secreted by *E. coli* and *S. cerevisiae* show comparable kinetics which shows that the molecules produced by *S. cerevisiae* are fully functional (Table 1 & Figure S2).

Table 1. Bivalent binding kinetics assay parameters of ZHER3_1-ABD-ZHER3_1 produced by B184 *pep4Δ* and by the original host *E. coli*.

Production host	k_a1 (1/Ms)	k_a2 (1/RUs)	k_d1 (1/s)	k_d2 (1/s)	K_D1 (M)	K_D2 (M)
<i>E. coli</i>	5.21×10^5	1.23×10^{-3}	1.43×10^{-2}	2.29×10^{-5}	2.75×10^{-8}	1.85×10^{-2}
<i>S. cerevisiae</i>	3.83×10^5	1.71×10^{-3}	1.79×10^{-2}	2.76×10^{-5}	4.68×10^{-8}	1.62×10^{-2}

k_a1 = association rate constant of the first site; k_a2 = association rate constant of the second site; k_d1 = dissociation rate constant of the first site; k_d2 = dissociation rate constant of the second site; K_D1 = equilibrium dissociation constant for the first site, K_D2 = equilibrium dissociation constant for the second site.

B184 *pep4Δ* produces a high titer of Z_{HER3_1}-ABD-Z_{HER3_1} in a fed-batch bioreactor.

For *S. cerevisiae* to be suitable host for Affibody molecule production it must be able to produce competitive titers of Affibody molecules. So next, we wanted to assess the productivity of *S. cerevisiae* in bioreactors. We decided to use B184 *pep4Δ* producing Z_{HER3_1}-ABD-Z_{HER3_1} as a proof of concept. To exclude any major impact of the deletion of *pep4Δ* on cellular growth of B184 while producing Z_{HER3_1}-ABD-Z_{HER3_1} we tested B184 and B184 *pep4Δ* in a micro-cultivation experiment in an aerated 96-well plate (Figure S3). Deletion of *pep4Δ* conferred only a minor impact on growth (Figure S3). After this confirmation the productivity for Z_{HER3_1}-ABD-Z_{HER3_1} of B184 *pep4Δ* was tested in a bioreactor experiment. The cultivation set-up was a batch fermentation followed by fed-batch fermentation. During the batch phase, a specific growth rate of 0.31 h⁻¹ was measured (Figure S4) which is the same growth rate as for B184 carrying an intact *PEP4* gene and producing α-amylase which is confirmation that B184 *pep4Δ* does not suffer from a growth impact [20]. The feeding of a low-glucose feed into the bioreactors was started after 36 h and was switched to a high-glucose feed after 130 h for the remaining duration of the fermentation until 180 h. At the end of the fed-batch cultivation, the biomass concentration exceeded 100 g/L (Figure 7A). We also observed an increasing trend of Affibody molecule titer with duration of the experiment especially after the switch to the high-glucose feed (Figure 7A). During the cultivation, minor production of byproducts was observed. Ethanol and glycerol were present in the medium after the batch phase but were rapidly consumed after the feed was started (Figure 7B). At the end of the fed-batch, residual glycerol increased again and reached a final concentration of 10 g/L (Figure 7B). We determined the exact quantity of Z_{HER3_1}-ABD-Z_{HER3_1} at three timepoints using a BLI based method. The results show a final titer of 530 mg/L (Figure 7C).

Discussion

In this study we examined *S. cerevisiae* as a host for Affibody molecule production. Currently, the molecules are mainly produced in *E. coli*. In the current study, we present engineered protease-deficient *S. cerevisiae* as a suitable alternative.

Our results show that all three Affibody molecules were produced and secreted by *S. cerevisiae* after the removal of only proteinase A or with the additional removal of proteinase B or carboxypeptidase Y. Removal of only proteinase A was sufficient for the production of intact Z_{HER3_1}-ABD-Z_{HER3_1} and a small amount of both Z_{HER3_1}-ABD and Z_{HER3_1}-Z_{HER3_1}-ABD in the case of a shorter cultivation of 48 h instead of 96 h. We expect that longer duration of the experiment would lead to more degradation. The additional removal of carboxypeptidase Y and/or proteinase B is required for the intact production of Z_{HER3_1}-ABD and Z_{HER3_1}-Z_{HER3_1}-ABD, respectively. The ABD is in Z_{HER3_1}-ABD-Z_{HER3_1} flanked by the two Z_{HER3_1} domains, which potentially protects the ABD from exopeptidase activity from proteases like carboxypeptidase Y. This would explain why we observe intact Z_{HER3_1}-ABD and Z_{HER3_1}-Z_{HER3_1}-ABD upon removal of carboxypeptidase Y in proteinase A deficient B184 *pep4Δ*. Additionally, we confirmed that the removal of carboxypeptidase Y does not further improve the production of Z_{HER3_1}-ABD-Z_{HER3_1} in proteinase A-deficient B184 *pep4Δ* (Figure S5). Interestingly enough there is also production of intact Z_{HER3_1}-ABD and Z_{HER3_1}-Z_{HER3_1}-ABD upon removal of proteinase B, which is an endopeptidase, in B184 *pep4Δ* and the additional deletion of proteinase B in B184 *pep4Δprc1Δ* (lacking proteinase A and carboxypeptidase Y) or carboxypeptidase Y in B184 *pep4Δprb1Δ* (lacking proteinase A and proteinase B) does not influence the titer of intact Z_{HER3_1}-ABD and Z_{HER3_1}-Z_{HER3_1}-ABD. This observation can be explained the following.

Proteinase A, proteinase B and carboxypeptidase Y are all three vacuolar proteases and before vacuolar proteases reach their final destination the vacuole, they pass through the secretory pathway as inactive precursors with a prepro-signalpeptide [30]. The pre-signal peptide is removed after arrival in the ER and the pro-signal peptide during trafficking to or upon arrival in the vacuole [30]. Proteases can have multiple pro-signal peptides which prohibits the proteases to reach their active form. Proteinase A and B are responsible for the removal of pro-signal peptide(s) on other vacuolar proteases and are therefore a central part of the activation cascade of vacuolar proteases.

For maturation of our deletion targets proteinase A, proteinase B and carboxypeptidase Y, activity of both proteinase A and B is required to reach their activate form. The maturation of proteinase A starts with an auto activation step to the pseudo form upon entering the vacuole which is followed by removal of a second pro-signal peptide by proteinase B [31, 32]. The intermediate form of the protease with one pro-signal peptide and one already removed is called the pseudo-form. The exact activation trigger for the proteases remains unknown but has been linked to the acidic pH in the vacuole [33, 34]. Proteinase B has two pro-peptides, one at the N-terminus and another at the C-terminus. The removal of the N-terminal pro-peptide is autocatalytic and occurs in the Golgi apparatus and the C-terminal pro-peptide is removed by proteinase A [35]. Carboxypeptidase Y has an N-terminal pro-peptide which will be partly removed by proteinase A and the other part by proteinase B upon arrival in the vacuole.

As explained here, proteinase B catalyzes the activation of carboxypeptidase Y and based on our results we expect that proteinase B fulfills a role in the activation of proteinase A and carboxypeptidase Y rather than to recognize Affibody molecules as a substrate. We expect, based on our results, that in a

pep4Δprb1Δ mutant carboxypeptidase Y remains in an inactive pro-form, which limits the capacity of carboxypeptidase Y to degrade the ABD [36]. We, however, observe digestion of the ABD by strain B184 *pep4Δ*. It has been shown that in a *PEP4* deficient *S. cerevisiae* strain pro-proteases of both carboxypeptidase Y and proteinase B accumulate, alongside the reduction of their activity [37]. The combination of deleting proteinase A and proteinase B for recombinant protein production has been reported for several microbial hosts, including *S. cerevisiae* [25, 38, 39].

The proteases travel through the secretory pathway to reach their destination, the vacuole. The initiation of the activation should start in the proximity of the vacuole or upon transport into this organelle [40]. Proteinase A and B are trafficked directly from the Golgi apparatus to the vacuole, but carboxypeptidase Y travels through a multivesicular body intermediate [26]. Even though the proteases are targeted for the vacuole, residual secretion has been reported and linked to the glucose concentration in the media. In the experiments performed in this study 2% glucose concentration in the media was used which showed to be sufficient for extracellular activity of proteinase A [34]. This supports the observations of proteinase A activity in the spent supernatant.

Another interesting aspect is the propensity of the proteases for digesting ABD. Since potentially even pro-Prc1 and pseudo-Prc1 seem to digest ABD, it could well be that even partly active yeast proteases retain a high propensity for digesting the ABD as a defense mechanism against bacteria. Proteolytic activity targeting the ABD was previously found in bacterial hosts [41].

The mass spectrometry performed for the analysis of $Z_{\text{HER3}_1}\text{-ABD-}Z_{\text{HER3}_1}$ showed that the molecular weight of the molecule produced by *S. cerevisiae* consisted of an extra 700 Da compared to the molecule produced by *E. coli*. This is visible on several SDS-PAGEs and western blots (Figure 4, Figure 5, Figure S1 & Figure S5). In the construct expressed by *S. cerevisiae*, a Kex2 recognition site is located behind the α -leader sequence and is processed in the ER. Between the Kex2 site and the start of the protein, a linker peptide with the amino acid sequence Glu-Glu-Gly-Glu-Gly-Ser-Met was inserted, which exhibits a molecular weight of 737 Da and thus explains the additional mass of the produced $Z_{\text{HER3}_1}\text{-ABD-}Z_{\text{HER3}_1}$. Since the binding kinetics indicate no interference of this additional peptide, we did not include the removal of the linker peptide in the scope of this study, but for future applications its removal may be desirable.

As a final experiment to show the potential of *S. cerevisiae* as interesting production host for Affibody molecules, B184 *pep4Δ* producing $Z_{\text{HER3}_1}\text{-ABD-}Z_{\text{HER3}_1}$ was grown in fed-batch bioreactors. During the fed-batch cultures, buildup of residual glycerol was observed. The quantity of glycerol accumulating was variable between the reactors resulting in high variation between the samples. Glycerol is a by-product of fermentation to reoxidize NADH to counteract redox imbalances. However, we do not expect that to be the cause of the buildup [42]. During later stages of the fed-batch, the feed was controlled by the dissolved oxygen in the reactor leading to pulse feed to ensure respiratory growth. Besides, in the case of alcoholic fermentation, there should be buildup of ethanol as well [43]. Therefore, the oxygen shortage does not seem to be the source of the potential redox imbalance. Besides redox imbalance, another explanation

could be the expression platform used. *TPI1* deficiency, which is the deficiency that the CPOT plasmid complements, is known to result in the accumulation of high levels of glycerol [44]. During the later stages of the experiment, this partial complementation could become limiting leading to the accumulation of excess glycerol. Nevertheless, high biomass concentration and Affibody molecule titer were achieved during the fermentation. The titer that was reached was 0.530 g/L. For *E. coli* expression titers for different recombinant proteins are reported in the range from 0.25 g/L to 8.5 g/L [45–47]. The titer of Z_{HER3_1}-ABD-Z_{HER3_1} is within this range and can be the starting point for improvement by further engineering *S. cerevisiae* or by optimizing the cultivation method in a sequential study.

The results of this study indicate that *S. cerevisiae* shows potential as a host for Affibody production. Here, we tested only three different Affibody molecules, and for the future it would be interesting to expand the selection of Affibody molecules and potentially test more complex Affibody molecule based proteins like AffiMabs [7].

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Materials are available from the corresponding author upon reasonable request.

Competing interests.

The authors declare that they have no competing interests.

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Authors' Contributions

VG, VS, FD, AS and ME conceived and designed the study. VG performed most of the experiments. MTM helped with the fermentation experiments. SE performed the BLI experiment, RG performed the binding kinetics assay, ST performed the mass spec experiment and data analysis, VG, VS and MM have written or revised the manuscript. All the authors have approved the submitted manuscript.

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Figures

Figure 1

Figure 1

Schematic representation of domain configurations in the three Affibody molecules produced in yeast. The ZHER3_1 domain is the black peptide and the albumin binding domain (ABD) the colored peptide.

Figure 2

Figure 2

SDS-PAGE and western blot analysis of supernatant of B184k expressing different Affibody molecules. (A) SDS-PAGE of supernatant after 48 h of cultivation of B184k producing three different Affibody molecules (cropped). The lane marked with ZA sup contains supernatant of B184k expressing ZHER3_1-ABD, the lane marked with ZZA sup contains supernatant of B184k expressing ZHER3_1-ZHER3_1-ABD, the lane marked with ZAZ sup contains supernatant of B184k expressing ZHER3_1-ABD-ZHER3_1, the

lane marked with ep contains supernatant of B184k with empty plasmid, the lane marked with ZA and st1 contains supernatant of ZHER3_1-ABD standard (12 kDa) dissolved in water, the lane marked with ZAZ and st2 contains supernatant of ZHER3_1-ABD-ZHER3_1 standard standard (18.9 kDa) dissolved in water. (B) Western blot against the Z-domain (cropped), the membrane was blotted with anti-Z-domain antibody (1:1000) followed by an anti-Mouse (1:5000) secondary antibody. (C) Western blot against the ABD (cropped), the membrane was blotted with anti-ABD antibody (3:1000) followed by an anti-Rabbit (1:5000) secondary antibody. (D) SDS-PAGE of supernatant of dissolved ZHER3_1-ABD in different solutions (cropped). The lane marked with media contains fresh SD2xSCAA media with (+) and without (-) ZHER3_1-ABD standard added, The lane marked with sup contains spent supernatant of a 24 h old culture of B184k in SD2xSCAA medium with (+) and without (-) ZHER3_1-ABD standard added, the lane marked with BSA contains water with 0.1% BSA with (+) and without (-) ZHER3_1-ABD standard added and the lane marked with H2O, + contains ZHER3_1-ABD standard in water. (E) Western blot against the ABD (cropped), the membrane was blotted with anti-ABD antibody (3:1000) followed by an anti-Rabbit (1:5000) secondary antibody.

Figure 3

Figure 3

Protease inhibitors essay with spent supernatant of B184 and Affibody molecule standards. SDS-PAGE of spent supernatant with Affibody molecules and addition of different protease inhibitors (cropped). The supernatant is derived from a cultivation of B184k producing α -amylase grown for 24 h at 30°C at 220 rpm. The Affibody molecules standards were incubated overnight at 30 °C at 220 rpm. The lane marked with pc contains the positive control with spent supernatant with respective Affibody molecule and the

complete protease inhibitor cocktail, the lane marked with nc contains the negative control with spent supernatant with respective Affibody molecule, the lane marked with st contains the standard of respective Affibody molecule in water, the lane marked with AEBSF contains spent supernatant with respective Affibody molecule with 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride 1 mM, the lane marked with aprotinin contains spent supernatant with respective Affibody molecule with aprotinin 6.5 µg/mL, the lane marked with bestatin contains spent supernatant with respective Affibody molecule with bestatin hydrochloride 50 µM, the lane marked with E-64 contains spent supernatant with respective Affibody molecule with E-64 15 µM, the lane marked with leupeptin contains spent supernatant with respective Affibody molecule with leupeptin 20 µM and the lane marked with pepstatin A contains spent supernatant with respective Affibody molecule with pepstatin A 10 µM.

Figure 4

Figure 4

Western blot analysis of the Affibody molecules produced in B184 and B184 *pep4Δ*. Western blot against the Z-domain (cropped) (A) and against ABD (cropped) (B) of the supernatant after 96 h of cultivation of B184 and B184 *pep4Δ* expressing the three Affibody molecule genes. For the western blot against Z-domain, the membrane was blotted with anti-Z-domain antibody (1:1000) followed by an anti-Mouse (1:5000) secondary antibody. For the Western blot against ABD, the membrane was blotted with anti-ABD

antibody (3:1000) followed by an anti-Rabbit (1:5000) secondary antibody. The lane marked with st1 contains the standard of ZHER3_1-ABD (10 mg/L) in water, the lane marked with st2 contains the standard of ZHER3_1-ABD-ZHER3_1 (10 mg/L) in water and the lane marked with em contains empty SD2xSCAA media.

Figure 5

Figure 5

Affibody molecule production and growth of B184 carrying deletions of three protease genes. (A)(B) Western blot analysis against the ABD (cropped). The samples are from a 48 h cultivation of B184, B184 pep4 Δ , B184 prc1 Δ , B184 prb1 Δ , B184 pep4 Δ prc1 Δ , B184 pep4 Δ prb1 Δ and B184 pep4 Δ prc1 Δ prb1 Δ expressing ZHER3_1-ABD (A) or ZHER3_1-ZHER3_1-ABD (B). For the Western blot against ABD, the membrane was blotted with anti-ABD antibody (3:1000) followed by an anti-Rabbit (1:5000) secondary antibody. The lane marked with st contains the standard of the respective Affibody molecule, the lane marked with em contains empty SD2xSCAA media and the lane marked with PL contains the protein ladder. (D) OD600 data from 96 h of growth in SD2xSCAA media of B184, B184 pep4 Δ , B184 prc1 Δ , B184 prb1 Δ , B184 pep4 Δ prc1 Δ , B184 pep4 Δ prb1 Δ and B184 pep4 Δ prc1 Δ prb1 Δ expressing ZHER3_1-ABD (C) or ZHER3_1-ZHER3_1-ABD (D). The graphs show averaged data based on biological triplicates with technical triplicates. The lighter bars show the standard deviation. The first 5 h were excluded from the graphs due to noise

Figure 6

Figure 6

Fragmentation of ZHER3_1-ABD-ZHER3_1 based on LC-MS analysis. The TIC chromatogram ZHER3_1-ABD-ZHER3_1 standard was incubated in spent supernatant of B184k while producing α -amylase. (A) Total Ion chromatogram (TIC) (B) Visualization of the ABD amino acid sequence and identified cut sites. The green marked sequence indicates the ABD. The blue arrows indicate the endings of detected fragments with intact C-terminus and a cut site in the ABD and the orange arrows indicate a detected

fragment with intact N-terminus and a cut site in the ABD. These fragments are based on the first peak of the TIC. The red and black arrows indicate beginning and endings of two additional detected fragments that were determined based on the fourth peak which had both beginning and ending within the ZHER3_1-ABD-ZHER3_1 molecule.

Figure 7

Figure 7

Fed-batch fermentation of B184 pep4 Δ expressing ZHER3_1-ABD-ZHER3_1. Experimental data from a fed-batch bioreactor experiment of B184 pep4 Δ expressing ZHER3_1-ABD-ZHER3_1. The cells were cultured in SD2xSCAA media and condensed feeding media. Details of the feeding media and strategy are presented in the materials and methods section. (A) Affibody concentration, cell dry weight (CDW) and glucose addition to the bioreactors. The quantity of the Affibody molecule concentration is based on western blot analysis against the albumin binding domain. All the data are based on biological triplicates and the average and standard deviations are shown. (B) Metabolic byproduct concentrations in the supernatant during the fed-batch cultivation. All the data are based on biological triplicates and the average and standard deviations are shown. (C) Affibody titers after the batch phase, after the low-glucose feed phase and at the end of the fed-batch fermentation. This quantification was done using BLI determination for ZHER3_1-ABD- ZHER3_1. The data are based on biological triplicates and technical duplicates and the average and standard deviations are shown.

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