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Fed-batch cultivation for the expression of Affibody® molecules with minimized phosphogluconoylation

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Abstract

Recombinant proteins produced in *Escherichia coli* are susceptible to phosphogluconoylation, a partial post-translational modification first described by Geoghegan *et al.* in 1999. The modification causes an additional mass of 258 Da and its dephosphorylation results in an excess mass of 178 Da. Homogeneous protein products are desirable and therefore the fraction of modified proteins is unwelcome. The aim of this study was to minimize the abundance of the modification by altering the conditions during fed-batch cultivation. In order to decrease the cell content of the precursor glucose-6-phosphate the carbon source was altered from glucose to glycerol. The influence of the specific growth rate on the modification was also evaluated. Data obtained from several fed-batch and continuous cultivations show that the modified proteins are less abundant on the model protein when glycerol is used as substrate, and when the specific growth rate is high (above 0.30 h^{-1}).

Keywords

Phosphogluconoylation, *Escherichia coli*, fed-batch, chemostat, Affibody® molecule

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Rickard Frost

Populärvetenskaplig sammanfattning

Storskalig produktion av proteiner sker ofta i bakteriekulturer. Bakterierna är genetiskt modifierade så att de i hög grad producerar ett specifikt protein. Då produkten har bildats befinner den sig vanligtvis inuti cellen och kan därför utsättas för olika modifieringar. En sådan modifiering är fosfoglukonylering, denna förekommer endast ibland och då endast på en del av proteinprodukten. Enligt en föreslagen mekanism, är det ett intermediat i nukleotidsyntesen som spontant kopplas till proteinet. Genom att analysera proteinerna med avseende på dess massa kan de modifierade proteinerna detekteras. En extra massa på 258 Da motsvarar fosfoglukonylerade proteiner, och en massökning med 178 Da motsvarar defosforylerade sådana.

I detta examensarbete undersöks om andelen fosfoglukonylerade proteiner kunde minskas genom att ändra betingelserna under odlingen av bakterierna. Substratet som tillförs under odlingen ändras från glukos till glycerol för att minska halten av reaktant i cellen. Även modifieringens beroende av bakteriernas tillväxthastighet undersöks. Som modellprotein används en Affibody® molekyl vilken är selekterad att binda HER2, en receptor som överuttrycks på vissa bröstcancerceller. Resultaten visar att andelen modifierat modellprotein minskar när glycerol används som substrat och odlingen sker vid en hög specifik tillväxthastighet.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet, januari 2006

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1. Introduction

1.1 Affibody AB

Affibody AB is a biotech company located in Stockholm, Sweden. It was founded in 1998 by scientists of the Royal Institute of Technology and Karolinska Institutet. The core of the company is the Affibody® molecule, a small protein that is mutated to gain affinity and specificity against any target molecule. These proteins are applicable in many different areas including proteomics, separomics, medical imaging, therapeutic apheresis and biopharmaceuticals. Because of this versatility the company has many focuses and several ongoing projects.

1.2 Aim

The aim of this master's degree project is to minimize the fraction of phosphogluconoylated and gluconoylated Affibody® molecules by altering the conditions during cultivation. A sidetrack of the project is to improve the elucidated cultivation protocol for production.

2. Theory and background

2.1 Affibody® molecule Z_{HER2}

The Affibody® molecule contains only 58 amino acids and has a molecular weight of 7 kDa. It originates from one of the IgG-binding domains of *Staphylococcus aureus* Protein A [1]. By randomizing 13 of the amino acids in the binding site an affinity ligand library has been created that contains billions of variants, all with different properties. Yet this is only a small fraction of the theoretical number of possible variants. The protein structure consists of three alpha helices which make the molecule robust and tolerant to extreme pH and elevated temperature [1, 2]. Compared to antibodies, the main difference besides the mentioned physical properties is its small size. The molecular weight of only 7 kDa, compared to 150 kDa for antibodies, makes it possible to synthesize Affibody® molecules chemically.

Selection of Affibody® ligands against a target is primarily performed by phage display [2,3]. When candidate ligands have been identified by the screening process these can be further matured by conserving some of the mutated amino acids

and re-randomize the others, before proceeding with another screening cycle. To decide which amino acids to conserve and which to re-randomize, the candidate ligands from the primary selection are sequenced. Similarities between the candidate ligands in the mutated positions are conserved and the remaining positions are further mutated. After the selection process, the Affibody® molecules can be multimerized to further increase the affinity to the target molecule. Affinity tags, e. g. poly His or ABD, can be attached to the selected ligands to facilitate purification [2].

A matured Affibody® molecule selected to bind Human epidermal growth factor receptor 2 (HER2) is designated Z_{HER2}. HER2 is overexpressed in 25-30 % of breast cancer tumors and these are the most serious cases. Today there is one biopharmaceutical (Herceptin™) on the market targeting HER2. The active pharmaceutical ingredient (API) in Herceptin is the monoclonal antibody trastuzumab which binds HER2 [4]. The Affibody® molecule ABD-$(Z_{HER2})_2$ is used as a model protein in this degree project.

2.2 Phosphogluconoylation

In 1999 Geoghegan *et al.* [5] and Yan *et al.* [6-7] described a novel post-translational modification on the N-terminus of histidine-tagged proteins. The modification causes an extra mass of 178 Da or 258 Da of the model fusion protein. They concluded that the additional mass is located on the His-tag and that it is independent of the fusion partner. The nature of the +258 Da modifications was found to be a phosphogluconoylation of the amino group on the N-terminal glycine and that its dephosphorylation results in an extra mass of 178 Da. Of the two forms of the modification, gluconoylation is the most common. Gluconoylated proteins occur in up to 20 % of the protein product in Geoghegan's studies, compared to 2 % phosphogluconoylated proteins. There are no known enzymatic mechanisms that catalyze the addition of phosphogluconoyl or gluconoyl groups but the modification can be explained in a different way. The suggested route is that the acylating agent 6-phosphoglucono-1,5-lactone, that is formed in the pentose phosphate pathway from glucose-6-phosphate by glucose-6-phosphate dehydrogenase, is coupled to the N-terminal amino group by a non enzymatic reaction (figure 1) [5-7].

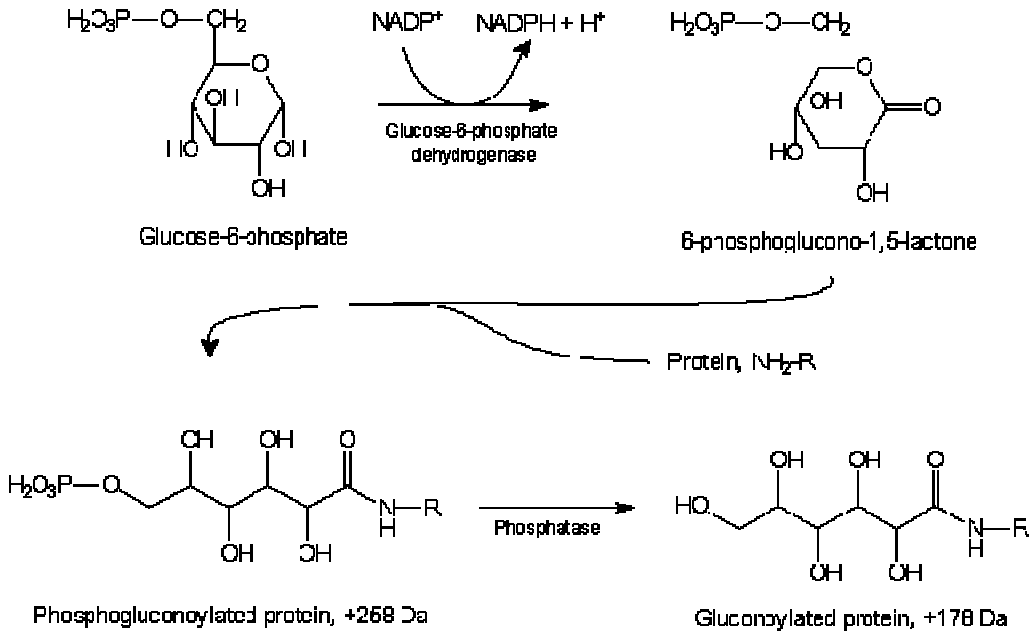

Figure 1: Proposed chemical route to phosphogluconoylated (+258 Da) and gluconoylated (+178 Da) proteins. Adapted from Geoghegan *et al.* [5].

Since 1999 it has been shown that not only N-terminally His-tagged proteins are subjected to this modification, although several cases of such gluconoylations have been reported [8, 9]. At Affibody AB, the extra mass of 178 Da or 258 Da has been noticed at both His_6-tagged and ABD-tagged proteins, and the extent of the modification is sometimes even larger than reported by Geoghegan *et al*. The modified proteins give an unwanted heterogeneity in the protein product and it is also possible that the additional structure could stimulate an immune response when introduced in a eukaryotic species. These are the main reasons why the modification is undesirable and should be minimized.

2.3 Project background

With the outlined chemical route for phosphogluconoylation of proteins as a starting point (figure 1), the basic idea behind this project was formed. If the concentration of the precursor glucose-6-phosphate could be minimized in the cell, maybe the fraction of modified proteins could be reduced. In the metabolism of glucose, glucose-6-phosphate is first generated before further degradation in the glycolysis. If glucose is used as substrate during cultivation the cell content of glucose-6-phosphate is high and the protein product could be modified to a large extent. By altering the substrate from glucose to glycerol, the cell metabolism is changed. In glycerol catabolism the molecule is first converted to glyceraldehyde-3-

phosphate. This substance enters the glycolysis at a later stage than glucose and thereby the formation of glucose-6-phosphate is avoided. Through gluconeogenesis, the cell is still able to produce glucose-6-phosphate which is necessary for nucleotide synthesis. The hypothesis was that the abundance of the precursor needed for gluconoylation of proteins would be lower when glycerol is used as carbon source instead of glucose, and hence the fraction of modified proteins would be decreased.

2.4 *Escherichia coli* and expression system T7

Today *Escherichia coli* is the most commonly used microorganism for production of recombinant proteins, both in research and development and in the biotech industry [10]. The main reason for this is that *E. coli* is a well characterized organism. The drawbacks with *E. coli* are that it can not produce proteins with multiple disulfide bonds or proteins that require certain post-translational modifications for activity e.g. glycosylations. Most strains of *E. coli* are also incapable of secreting proteins which makes it necessary to purify the protein from the whole intracellular proteome or from the periplasm [11, 12].

To produce a high titer of the recombinant protein it is necessary to have a strong expression system. One such expression system originates from bacteriophage T7, first described by Studier and Moffatt in 1986. The bacteriophage T7 RNA polymerase, which is encoded by T7 *gene 1*, is highly specific for its own promoters and only a small amount of the polymerase is enough for an efficient transcription. By placing the gene of interest downstream of the T7 promoter, the protein product can accumulate to more than 50 % of total cell protein in a few hours if T7 RNA polymerase is present [13]. In *E. coli* BL21(DE3) a chromosomal *gene 1* is placed under control of the inducible *lac*UV5 promoter and on the plasmid a *lac* operator is centered 15 base pairs downstream of the T7 promoter. Until induction, both the *lac* operator on the multicopy vectors and the chromosomal *lac*UV5 promoter are repressed by lac repressor provided by a *lacI* gene on the vectors. In this way the basal expression of the target genes is very low. The *lac*UV5 promoter is induced with the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) [14].

2.5 Cultivation techniques

Three major modes of process operation exists, batch, fed-batch and continuous cultivation (chemostat). In batch cultivations, the growth medium with all

nutrients is present from the start, and the cells grow at the maximum specific growth rate (μ_{max}) until the depletion of some compound in the medium or until some byproduct becomes inhibiting. The most dominant technique for bioproduction is batch cultivation [10]. Fed-batch cultivations usually start with batch cultivation until the growth is inhibited by the lack of substrate, and then substrate is continuously added during the remaining cultivation time. This technique prolongs the exponential growth phase and the culture reaches a higher cell density. In continuous cultures, complete medium is added at a constant feed rate, and at the same rate fermentation broth is withdrawn. The cultivation volume remains constant, and eventually the continuous cultivation reaches a steady state where all parameters are constant [15].

In continuous cultivations the total cultivation time is considerably longer than in fed-batch cultivations. With this follows a larger number of generations and a higher probability for mutations. Mutations in the T7 promoter that reduces protein expression are beneficial for the producing cells, and could easily be established in the cultivation. By sequencing the promoter region, any mutation could be detected. The number of generations is calculated according to equation 1 below.

$$N = \frac{t_{\mu}}{t_{g,\mu}} \qquad \text{Eq. 1}$$

In equation 1, N is the number of generations, t_{μ} is the cultivation time for one specific growth rate and $t_{g,\mu}$ the generation time for the same specific growth rate (μ). The latter parameter, $t_{g,\mu}$, was derived from the following equation (Eq. 2) [15].

$$2 = e^{\mu \cdot t_{g,\mu}} \qquad \text{Eq. 2}$$

3. Materials and methods

3.1 Bacterial strain and plasmid

The plasmid construct pAY770 codes for the Affibody® molecule ABD-$(Z_{HER2})_2$ (figure 2). This construct has previously been transformed into *E. coli* strain BL21(DE3) at Affibody AB. During this project pAY770 was used in most cultivations, but the constructs pAY694, pAY773, pAY1116, pAY1151 and pAY1152

were used as well. Besides the Affibody® molecule, the constructs also contains genes for kanamycin resistance and lac-repressor protein.

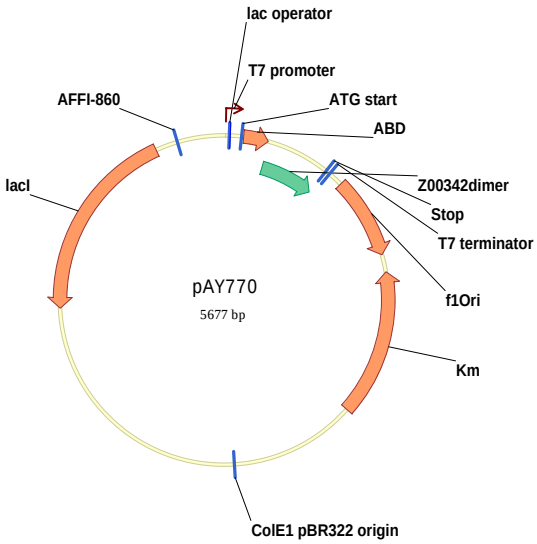

Figure 2: Plasmid map of the construct pAY770.

3.2 Optical density (OD)

The optical density was measured with a CO8000 Cell Density meter (WPA, Cambridge, UK) at a wavelength of 600 nm (OD_{600}). Prior to measurement the samples were diluted with 0.9 % NaCl to the linear range of measurement between 0.1 and 1.0.

3.3 Working cell bank (WCB)

A tube containing 2 ml of 30 g/l tryptic soy broth (TSB) (Merck, Darmstadt, Germany), 5 g/l yeast extract (Merck) and 2 mg/l kanamycin (Amresco, Solon, OH) was inoculated with seed from transformed *E .coli* BL21(DE3). The tube was incubated in an Infors Multitron incubator (Infors AG, Bottmingen-Basel, Switzerland) for three hours (37°C, 170 rpm) and then the culture was transferred to a 300 ml Tunair® shake flask (Sigma) with 50 ml of the same growth medium. Two hours later, 20 ml of the culture was transferred to another sterile Tune-air shake flask. All measurements of the cell density were done with cells from this new shake flask. The two cultures were grown under identical conditions, and therefore the assumption was made that they grow with the same specific growth rate. When the optical cell density reached 1, the untouched culture was put on ice and 13 ml of cold 50 % glycerol (Merck) was added to obtain a final glycerol concentration of 15 %.

The culture was aliqouted 25 times 1 ml in 1.5 ml tubes and put in a Heto Ultra Freeze -80°C freezer (Heto Holten AS, Alleroed, Denmark).

3.4 Determination of lag phase and generation time

One tube from the WCB was thawed and 50 µl were used as inoculum for 5 ml defined growth medium (appendix A) in a cultivation tube. The cultivation was put on a shake board in 37°C overnight. From this culture a certain volume, 10-100 µl, was inoculated into 5 ml of the growth medium which was going to be analyzed. The inoculation volume was dependent on the properties of the growth medium. These new cultures were incubated (37°C, 175 rpm) for approximately three hours or until OD_{600} reached 1. If OD_{600} reached 1, the cultures were diluted 10 000 times into 5 ml growth medium. Otherwise the dilution was adjusted in a way that the same final cell concentration was obtained. 350 µl of every culture and corresponding blank were transferred to a 100-well plate and analyzed by a Bioscreen C turbidometer (Labsystems, Helsinki, Finland). Each sample was analyzed in five replicates. The instrument measured OD_{600} every 5 min during 16 h, and every measurement was preceded by four minutes of agitation. From the obtained raw data, the lag phase and the generation time of every cultivation could be determined. When the generation time was determined, the maximum specific growth rate of the cultivation could easily be calculated.

3.5 Sampling

An OD1 sample is a sample of biomass corresponding to 1 ml cell suspension with OD 1. A sample was prepared by diluting a specific volume of fermentation broth (V_{OD1}) (equation 3) with 0.9 % NaCl and centrifuging in a Heraeus Biofuge Fresco (Kendro Laboratory Products, Langenselbolg, Germany) for 10 minutes (16 060 ×g, 13 000 rpm) in 4°C. The supernatant was discarded and the cell pellet was frozen in -20°C.

$$V_{OD1} = \frac{1000}{OD} \ (\mu l) \qquad \text{Eq. 3}$$

3.6 Screening of growth media

A Tunair® shake flask with 100 ml defined growth medium and 100 µl kanamycin was inoculated with 100 µl culture from the WCB. The shake flask was incubated overnight (37°C, 175 rpm). New shake flasks, all with 100 ml of a unique growth medium, were preheated and inoculated with the overnight culture to a final OD_{600} of 0.1. The growth media Terrific broth, 2×YT, TSB + YE and ModNS85 with either glucose (40 g/l) or glycerol (20 g/l) were used (appendix A). The cultures were grown for 6.5 h and were induced with 50 µl 1M IPTG (0.5 mM) 4 h before harvest. At harvest each culture was sampled by taking out two OD1 samples. The cultivations were evaluated with respect to protein expression and phosphogluconoylation of the product.

3.7 Fermentation

All fermentations were carried out in a GRETA multifermentor (Belach Bioteknik AB, Solna, Sweden). The multifermentor has six parallel 1 l fermentors (figure 3). Besides from the fermentor unit, the system consists of software for regulation and surveillance and a cleaning in place (CIP) unit. Each fermentor is equipped with sensors for temperature, pH, dissolved oxygen (DO), optical density and broth level. These signals together with the parameters stirrer speed, feed rate and sum base are continuously logged throughout the cultivations.

Figure 3: GRETA multifermentor with CIP-unit and growth medium vessel (Belach Bioteknik AB).

3.7.1. Fed-batch

An inoculum was prepared prior to cultivation by adding 1 ml WCB to 100 ml defined medium with 2 mg/l kanamycin. The culture was incubated (37°C, 175 rpm) for approximately 8 h. The fermentors were washed by the CIP unit and sterilized *in situ*. Growth medium was prepared and 600 ml was automatically filled into each fermentor by the GRETA system. After filling, 0.5 ml Breox antifoam agent was aseptically added to each fermentor. The pH-electrodes were calibrated at 25°C, with an external pH measurement of the growth medium as reference. After heating the medium to the cultivation temperature (37°C) a two-point calibration was done on the DO-electrodes. All oxygen was depleted from the medium by replacing the aeration with nitrogen (N_2) gas and when the DO signals had stabilized they were set to zero percent and the aeration was turned back on. After a few minutes the maximum level of oxygen was dissolved in the medium and the signals were set as 100 percent. The desired profiles for temperature, stirrer speed and feed rate then were programmed into the control program.

The feed rate of substrate was calculated according to equation 4, which is derived from a mass balance with the assumption of constant cell yield on the substrate and insignificant substrate utilization due to maintenance [11, 16].

$$F(t) = \frac{\mu}{Y_{x/s}} \cdot \frac{X(t_0)V(t_0)}{S_F} e^{\mu(t-t_0)} \qquad \text{Eq. 4}$$

In equation 4, F is the feed flow rate (lh^{-1}), μ is the specific growth rate (h^{-1}), X is the cell concentration ($g(DCW)l^{-1}$), V is the total volume (l), S_F is the substrate concentration in the feed (gl^{-1}) and t_0 is the time when the feeding is started. $Y_{X/S}$ is the cell yield on the used carbon substrate and equals 0.5 $g(DCW)g^{-1}$ and 0.45 $g(DCW)g^{-1}$ for glucose and glycerol respectively. $X(t_0)$ was calculated from the cell density immediately after inoculation (X), the previously determined maximum specific growth rate (μ_{max}) and the time of batch cultivation prior to feed start (t_0) according to equation 5 [16].

$$X(t_0) = X \cdot e^{\mu_{\max} t_0} \qquad \text{Eq. 5}$$

The fermentors were inoculated with 6-20 ml cell culture depending on the OD of the inoculum. Feeding started after three hours and the cultivations were fed

with an exponential feed, either throughout the cultivation or until induction. In the latter case the feed was kept constant after induction. The cultivations were induced with 0.5 mM IPTG (Acros Organics, Geel, Belgium) four hours prior to harvest. At harvest the cultivations were sampled with OD1-samples.

3.7.2. Chemostat

The preparations were the same as for fed-batch cultivations until inoculation. After the opening batch phase, the cultivation was fed with complete growth medium including carbon substrate with an external P4 pump (W-M Alitea AB, Stockholm, Sweden). The pump had previously been calibrated with water. To keep the cultivation volume constant, another external P4 pump was used to remove fermentation broth from the surface. By feeding medium with a certain dilution rate until steady state, the specific growth rate was accurately controlled.

Two continuous cultivations were carried out in this project. The first was constantly induced by the presence of 0.1 mM IPTG (Acros Organics) in the feed. During this cultivation the specific growth rates 0.10, 0.25, 0.30, 0.35 and 0.40 h^{-1} were set. In the second continuous culture, a part of the culture was transferred to another reactor where it was induced. This time the IPTG concentration was 0.5 mM. After the transfer, four hours of fed-batch cultivation began. The culture was fed exponentially with a feed corresponding to the same specific growth rate as in the chemostat. Afterwards the chemostat was filled up with growth medium to the cultivation volume and the feed was set on a new dilution rate. This process was repeated for the growth rates 0.10, 0.20, 0.30 and 0.40. Each time the transferred culture was diluted with an appropriate volume of growth medium for the cultures to reach approximately the same OD at harvest.

Both continuous cultivations were sampled with OD1-samples and two 5 ml samples of the fermentation broth at all specific growth rates. The latter samples were centrifuged in a Heraeus Multifuge 3S-R (Kendro Laboratory Products) (3000 ×g, 5 min) and the pellet and supernatant were separated and frozen in -20°C.

3.8 Expression analysis

The expression level of the recombinant protein was analyzed by SDS-PAGE. The OD1-samples to be analyzed were thawed and treated with 200 µl CelLytic™ (Sigma). The samples were vortexed and put on a shaker board for about 15 minutes

to lyze the cells. The suspensions were then centrifuged for 10 minutes, 16 060 ×g (13 000 rpm) at room temperature using a Heraeus Biofuge Fresco. Due to the good solubility of the protein the supernatants were transferred to new Eppendorf tubes and the pellets were discarded. A mixture of 66 µl NuPAGE® LDS Sample Buffer (4x) (Invitrogen, Carlsbad, CA) and 26 µl 0.5M DL-1,4-dithiothreitole (DTT) (Acros Organics) was prepared and added to each supernatant. After mixing, the samples were heated to 70°C for 15 minutes. 29 µl of each sample were loaded on a 10-well 4-12 % Bis-Tris NuPAGE® gel (Invitrogen) together with 4 µl of MultiMark (Invitrogen) and known amounts of reference protein. The gel was run for 35 min with the voltage 200 V using an XCell Surelock Electrophoresis Cell (Invitrogen) with NuPAGE® MES-buffer (Invitrogen). The gel was stained for 1 h under agitation in room temperature, with a comassie staining solution containing PhastGel™ Blue R (GE Healthcare, Uppsala, Sweden), ethanol (30 %) and acetic acid (10 %). Next, the solution was exchanged for a destaining solution containing ethanol (30 %) and acetic acid (10 %) and the gel was destained for approximately 1.5 h. Finally the gel was placed in a conservation solution containing glycerol (5 %) and acetic acid (10 %) for a minimum of 20 minutes. The gel then was scanned and dried overnight using DryEase® Mini-Cellophane (Invitrogen). From the references, the amount of protein loaded on the gel could be estimated and the total protein concentration calculated.

3.9 Protein purification and concentration

In case the expression level of the recombinant protein was too low for further analysis, it was purified by affinity chromatography. Because of the ABD-tag on the model protein the purification was performed with HSA-Sepharose columns (Amersham Biosciences, HSA coupled at Affibody AB). Cell pellets from 5 ml fermentation broth samples were thawed and dissolved in 10 ml 20 mM Tris-HCl, pH 8.0. The suspensions were sonicated by a Vibra Cell VC750 (Sonics & Materials Inc., Newtown, CT) for 2 x 2 min, pulses of 2 s with 40 % amplitude and 0.5 s pauses in between, to lyze the cells. To separate the cell debris from the soluble proteins the samples were centrifuged for 30 min (26 000 ×g, 4°C) in an Avanti™ HP-20 XPI centrifuge (Beckman Coulter Inc., Fullerton, CA). The supernatants then were filtered through a 0.45 µm filter and 8 ml were transferred to 14 ml cultivation tubes. 400 µl of 20 x TST was added to each tube. All prepared samples were placed in the sample rack of BioRobot 3000 (Qiagen, Hilden, Germany). The BioRobot then equilibrated

the columns with TST buffer pH 8.0, loaded the samples, washed with 5 mM NH_4Ac pH 5.5 and eluted with 8 ml 0.5 mM HAc pH 2.8. After purification, the samples were freeze dried by a Christ freeze drier (Martin Christ, Osterode, Germany) in vials, 4 ml in each. At the time for further analysis by LC/MS the protein was dissolved in 100 µl Buffer A (MilliQ / 0.1 % TFA). In this way the protein was concentrated 40 times.

3.10 Protein analysis

To find out whether or not the protein was phosphogluconoylated, its mass was accurately determined by an LC/MS analysis. Samples with low expression levels were first purified by HSA-affinity chromatography as described above. All other samples were prepared according to the following. OD1-samples were treated with 150 µl CelLytic (Sigma) and put on a shaker board for 15 min. The suspension of lyzed cells were centrifuged in a Heraeus Biofuge Fresco for 10 min (16 060 ×g, 13 000 rpm) at room temperature. The supernatants were transferred to new Eppendorf tubes and filtered through a 0.45 µm filter. After filtration the samples were analyzed by liquid chromatography with online mass spectrophotometry (LC/MS) (Agilent Technologies, Waldbronn, Germany). A narrow-bore Zorbax® 300SB-C8 column (Agilent Technologies) was used to separate the proteins. The mobile phase was a combination of Buffer A (MilliQ / 0.1 % TFA) and Buffer B (ACN / 0.1 % TFA). Initially, at the time of injection, the mobile phase consisted of 80 % Buffer A and 20 % Buffer B. During separation this ratio was changed through a linear gradient until 30 % Buffer A and 70 % Buffer B were eluted.

3.11 Sequencing

In the constantly induced chemostat, the number of generations was calculated to about 50 according to equation 1. To detect if the low expression levels could be due to a mutation in the promoter region this region was sequenced.

Plasmids were purified from pellets of 5 ml samples using a QIAprep® Spin miniprep kit (Qiagen). After the purification the concentration of the samples were determined with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., Rockland, DE).

Approximately 200 ng DNA-template were mixed with 1 µl BigDye-terminatormix (Applied Biosystems, Foster city, CA), 5 pmol primer (Thermo Electron, Ulm, Germany) and 7 µl Cycle sequencing buffer (6.5 mM $MgCl_2$, 26 mM

Tris-HCl, pH 9.0). The proceeding PCR consisted of 30 temperature cycles (denaturation: 96°C, 30 s; annealing: 50°C, 15 s; elongation: 60°C, 4 min) and was performed in a Mastercycler epgradient S (Eppendorf AG, Hamburg, Germany).

If the primer was biotinylated the product was purified with a Magnatrix™ 8000 (Magnetic Biosolutions, Stockholm, Sweden), otherwise it was precipitated by ethanol. The precipitation was performed by mixing 20 µl PCR product with 10 µl 3 M NaAc (pH 5.2) and 70 µl 95 % ethanol. The solution was incubated for 30 minutes at room temperature and then centrifuged in a Heraeus Biofuge Fresco for 20 minutes (13 000 rpm, 4°C). After discarding the supernatant the pellet was washed with 500 µl 70 % ethanol and centrifuged for an additional 5 min as above. The pellet was dried by evaporation and then dissolved in 20 µl MilliQ H_2O. After the precipitation or the Magnatrix purification, the DNA was sequenced by an ABI 3100 Genetic Analyzer (Applied Biosystems). The obtained DNA sequences were aligned with the known reference by the software Sequencher v 4.0.5 (Gene Codes Corporation, Ann Arbor, MI), and the promoter regions were compared.

3.12 Improvement of cultivation protocol

To facilitate the set up before fermentation, a trial with combined substrate and alkali feed was made. Different solutions of 50 % glycerol and 25 % NH_3 were prepared and used as one single feed during fed-batch cultivations. All other aspects in the test cultivations were the same. Six different ratios of alkali and substrate (ml_{alkali} / $ml_{substrate}$) were tested, these were: 0.1; 0.14; 0.18; 0.20; 0.25 and 0.30. To further minimize the time between cultivations, all additives to one batch (5 l) of the growth medium ModNS85 were mixed and frozen before use.

3.13 Glycerol analysis

The content of glycerol in the fermentation broth was determined with an EnzyPlus glycerol kit (Diffchamb, Västra Frölunda, Sweden). Supernatants from 5 ml samples were thawed and filtered through 0.45 µm filters prior to analysis. A standard solution of 0.40 gl^{-1} glycerol was made and used as control. Absorbance measurements were performed on a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc.).

4. Results

4.1 Effect of glycerol concentration on cell growth

Lag phases and generation times were analyzed in both defined and complex growth media with different concentrations of glycerol. Glycerol concentrations in the range of 1 – 30 gl^{-1} did not considerably change these growth parameters (figure 4), although there was a trend of an increased lag phase with a higher glycerol concentration for defined growth medium. For the complex growth medium the trend was reversed. With no respect of glycerol concentration the average lag phase was 510 min and the generation time was 79 min for the defined growth medium. For the complex growth medium the average lag phase and generation time were calculated to 440 min and 31 min, respectively.

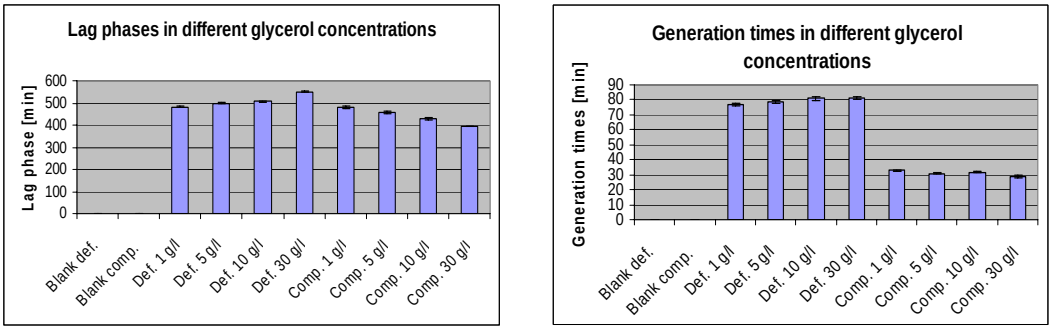

Figure 4: Lag time and generation time dependence of glycerol concentration in defined (Def) and complex (Comp) growth medium.

4.2 Screening of growth media

A set of fed-batch cultivations was carried out with a defined growth medium (appendix A), which all resulted in a low protein expression. Because of this, and the fact that the generation time is considerably longer in defined growth media, only complex growth media was included in the following screening.

The screened growth media, Terrific broth, 2×YT, TSB + YE and ModNS85 with either glucose or glycerol, were analyzed with respect to lag phase, generation time, protein expression and phosphogluconoylation. The growth parameters are presented in table 1 below.

Table 1: Lag times and generation times of *E. coli* in different growth media.

Medium	Lag phase (min)	Generation time (min)
Terrific broth	300	27
2×YT	250	28
TSB + YE	240	26
ModNS85 + glucose	350	34
ModNS85 + glycerol	300	32

All screening cultivations had a high expression level of the recombinant protein, as shown in figure 5. LC/MS analysis showed that the cultivations with 2×YT and ModNS85 with glucose both resulted in gluconoylated proteins (+178 Da). None of the other growth media had any modification of the recombinant protein in these cultivations.

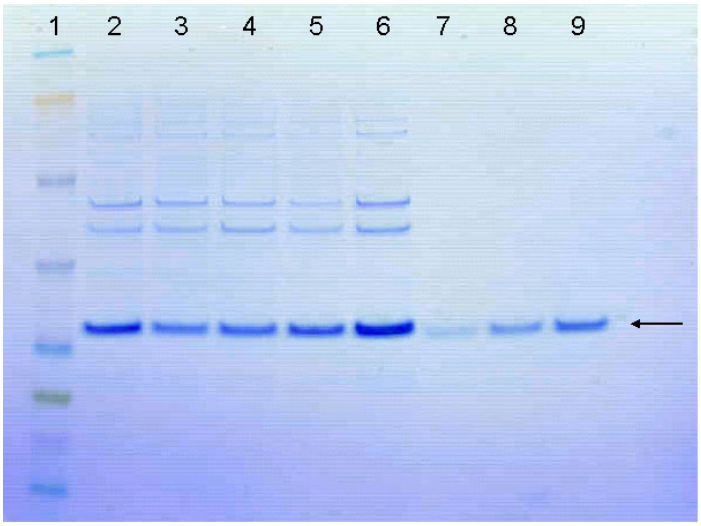

Figure 5: Expression analysis of ABD-$(Z_{HER2})_2$ (arrow) in screening cultivations. MultiMark size marker was run in lane 1. In lanes 2-6 proteins produced in cultivations with the following growth media were run: ModNS85 + Glucose (2), ModNS85 + Glycerol (3), Terrific Broth (4), 2×YT (5), TSB + YE (6). Lane 7, 8 and 9 contains 0.5, 1.0 and 1.5 µg of a reference protein (19 kDa) respectively.

The growth medium ModNS85 with glycerol was chosen to be used in future cultivations. Due to its high content of phosphate it should be possible to reach high cell densities.

4.3 Phosphogluconoylation and protein expression

A comparison between glucose and glycerol was made. It consisted of three cultivations of both substrates, all with different specific growth rates. The cultivations were analyzed with respect to phosphogluconoylation and expression level every hour after induction until harvest. Expression of the recombinant protein increased the first three hours after induction and then was kept at the same level until

harvest (data not shown). The results from the mass spectrophotometric analysis are presented in table 2 below.

Table 2: Comparison between glucose and glycerol as carbon source and specific growth rate, with respect to modification of the protein product.

Carbon source	Specific growth rate (h^{-1})	Additional mass (Da)	Time after induction		
			2 h	3 h	4 h
			Abundance (%)		
Glucose	0.25	+ 178	-	-	15
		+ 258	6.9	6.5	8.0
	0.30	+ 178	5.7	11	12
		+ 258	5.6	-	-
	0.35	+ 178	-	10	10
		+ 258	4.4	-	-
Glycerol	0.25	+ 178	4.8	9.4	11
		+ 258	-	-	6.6
	0.30	+ 178	-	5.6	-
		+ 258	-	-	-
	0.35	+ 178	-	-	-
		+ 258	-	-	-

The results indicate that the post translational modification is less abundant if glycerol is used as substrate and when the specific growth rate is high (above 0.3 h^{-1}). Figure 6 shows an example of components found in a mass spectrophotometric analysis of the Affibody® molecule ABD-$(Z_{HER2})_2$.

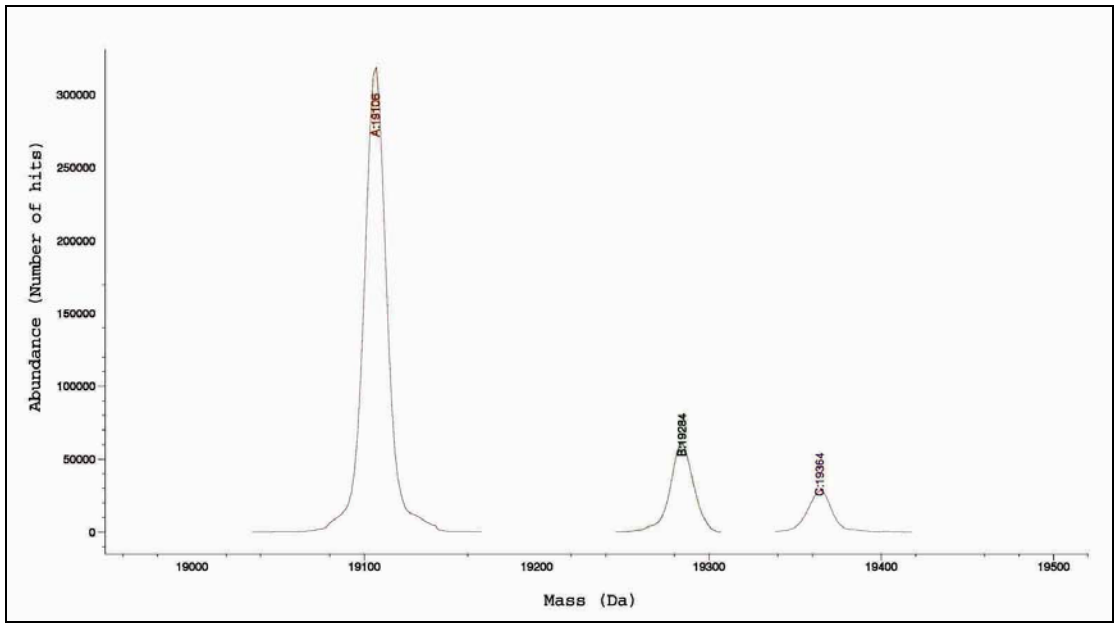

Figure 6: Example of spectrogram obtained in a mass spectrophotometric analysis of ABD-$(Z_{HER2})_2$ (Mw = 19106 Da). Both gluconoylated (+178 Da) and phosphogluconoylated (+258 Da) proteins are present.

Further investigation of the influence of specific growth rate on phosphogluconoylation was performed by two continuous cultivations. In the constantly induced chemostat the expression level was low and the protein had to be purified with affinity chromatography according to section 3.7. All specific growth rates gave gluconoylated proteins, although to different extent. The abundance of the gluconoylated proteins was 10 to 19 %. Phosphogluconoylated proteins only occurred in one sample, when the specific growth rate was set to 0.35 h^{-1}. The continuous cultivation with subsequent fed-batch cultivations gave a higher expression of the recombinant protein. This time only $\mu = 0.10\ h^{-1}$ gave modified proteins, both +178 and +258 Da. Due to insufficient oxygen transfer, the fed-batch cultivation with $\mu = 0.40\ h^{-1}$ did not grow and no results were obtained from this sample.

The latter chemostat and the first fed-batch cultivations indicate that the modification of the protein disappear if the specific growth rate is high. These results were followed up with six new fed-batch cultivations, three with $\mu = 0.20\ h^{-1}$ and three with $\mu = 0.30\ h^{-1}$. Both gluconoylated and phosphogluconoylated proteins were abundant in two of the cultivations with $\mu = 0.20\ h^{-1}$, the third cultivation was cancelled due to an error in the pH-regulation. When μ was set to 0.30 h^{-1} a part of the proteins were still gluconoylated but no phosphogluconoylation was detected in the analysis. The results are presented in table 3.

Table 3: Comparison of the specific growth rates 0.20 h^{-1} and 0.30 h^{-1} with respect to gluconoylation (+178 Da) and phosphogluconoylation (+258 Da).

Specific growth rate (h^{-1})	Bioreactor (BR)	Additional mass (Da)	Abundance (%)
0.20	BR 1	+178	Cancelled
		+258	Cancelled
	BR 2	+178	10
		+258	4.5
	BR 3	+178	10
		+258	6.1
0.30	BR 4	+178	8.0
		+258	-
	BR 5	+178	12
		+258	-
	BR 6	+178	8.7
		+258	-

Again it seems that the modification of the proteins is less abundant at a high specific growth rate, especially the phosphorylated form. These results are valid for the trial cultivations for combined alkali and substrate feed as well. Here the +258 Da

modification was absent in all cultures and the +178 Da modification present in about half of the cultures which could be analyzed. The specific growth rate was set to 0.35 h^{-1}.

Final cultivations were performed according to the elucidated cultivation protocol. In these cultivations, six different Affibody® molecules (table 4) were produced in order to test different proteins inclination to gain the modification.

Table 4: Constructs cultivated in the final cultivations and their expression levels. GA001 and GA002 are Affibody® molecules selected to bind HSA.

Bioreactor	Construct	Affibody® molecule	Expression level
BR 1	pAY694	His_6-$(Z_{HER2})_2$-Cys	Low
BR 2	pAY770	ABD-$(Z_{HER2})_2$	High
BR 3	pAY773	$(Z_{HER2})_2$	High
BR 4	pAY1116	ABP-$(Z_{HER2})_2$	High
BR 5	pAY1151	GA002-$(Z_{HER2})_2$	Cancelled
BR 6	pAY1152	GA001-$(Z_{HER2})_2$	Low

Only the cultivations with a high expression level gave interpretable data in the LC/MS analysis. The Affibody® molecules in these cultivations had no gluconoylation or phosphogluconoylation.

The elucidated cultivation protocol was applied in a set of production cultivations by an Affibody AB coworker. Five different His_6-tagged proteins were produced. Protein from four cultivations gave interpretable data when analyzed by LC/MS. In all analyzed cultivations gluconoylated proteins were present. The extents of the modification were in the range 7.8 – 21 %. Phosphogluconoylated proteins were abundant in one of the cultivations (5.1 %).

4.4 Sequencing

Samples from the constantly induced chemostat were used to sequence the promoter region of the recombinant protein. This analysis showed that no mutation had occurred and therefore the low expression levels must have another explanation.

4.5 Improvement of cultivation protocol

In total, twelve test cultivations were carried out to determine a suitable mixture of NH_3 and glycerol for combined feed. Of the six different solutions that were tested, the one with the ratio 0.14 (V_{NH3} / $V_{glycerol}$) kept pH closest to seven and was chosen to be used in the final cultivations. NH_3 stored in temporary vessels are

not to be used in the mixture, it is important that the concentration is 25 %. A constant feed after induction was applied to prevent the pH from rising when the cell growth declines and the protein production begins. In spite of promising results in the test cultivations, pH dropped almost one unit approximately at the time of induction in the final cultivations (figure 7).

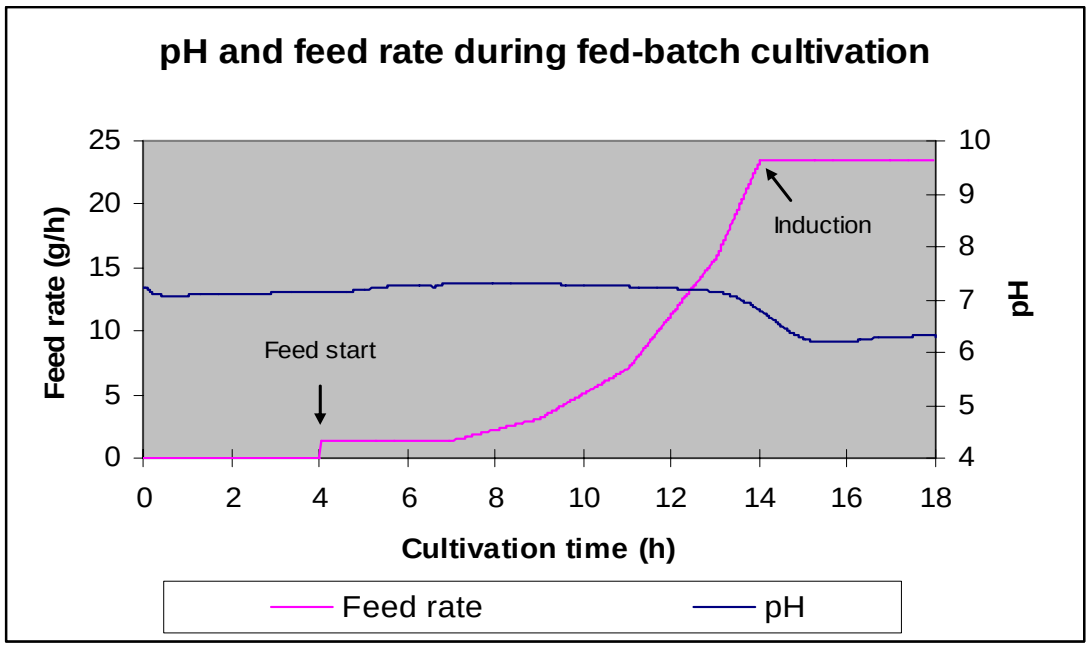

Figure 7: A plot of pH and feed rate values during a fed-batch cultivation with combined glycerol and NH_3 feed. After 13 h a drop in pH of about one unit occurs.

No precipitation was visible after thawing the mixture of additives to ModNS85. It is therefore possible to prepare such mixtures and store in -20°C until usage.

4.6 Glycerol analysis

The fermentation broth leaving the reactor in the constantly induced chemostat was analyzed to determine if all glycerol was consumed in the reactor. Samples from all growth rates during the cultivation were analyzed and the results were as expected all very low. The average glycerol concentration in the leaving fermentation broth was determined to be 0.2 g/l. In the feed into the chemostat the glycerol concentration was 5 g/l. The glycerol concentration of the standard solution (0.40 g/l) was determined to 0.38 g/l by the EnzyPlus analysis kit.

5. Discussion

The basic idea that the substitution of glucose with glycerol as substrate in cultivations could minimize the fraction of gluconoylated and phosphogluconoylated proteins was evaluated in this project. Previous studies at Affibody AB indicate that the specific growth rate also is an important variable in this aspect. These factors, glycerol as carbon source and the specific growth rate during cultivation, were tested in both fed-batch and continuous cultivations. First a growth medium was selected that gave a high expression level of the recombinant protein and contains little complex ingredients. A complex growth medium was chosen because the cultivation time has to be kept short to enable four cultivations per week. Of the screened growth media ModNS85 was chosen. It gave a good protein expression and contains only 10 g/l YE which is the only undefined component. The high content of phosphate gives the medium a high buffer capacity and L-methionine prevents incorporation of norleucine [17]. In the screening TSB + YE had the highest level of protein expression, but this growth medium has a high complexity and it contains glucose. This growth medium is currently used at Affibody AB and was included only for comparison.

When the substrates glucose and glycerol were compared in fed-batch cultivations, the results strengthened the presented theory. Previous studies at Affibody AB gave the indication that a high specific growth rate gives a high abundance of modified proteins. In this study, the cultivations showed a decreasing abundance of modified proteins at high growth rates. As it is preferable to apply a high specific growth rate to shorten the cultivation time, the obtained results are promising. The contradictive data were further investigated by continuous cultivations where the specific growth rate is accurately controlled. When the chemostat was constantly induced the expression level was low at all specific growth rates tested, and there was no correlation between the abundance of modified proteins and the specific growth rate. Maybe the cells can not produce the recombinant protein to the same extent when the protein is constitutively expressed, or when the concentration of IPTG was lowered to 0.10 mM. The chemostat with subsequent fed-batch cultivation gave higher expression levels, and again a relation between specific growth rate and modified proteins were seen. Results obtained in the latter cultivation, which is more trustworthy due to the similarity to the existing protocol, support the results from the

first fed-batch cultivation. Because of the high buffer capacity in the growth medium no pH-regulation was needed during the continuous cultivations. Additional fed-batch cultivations were carried out and the results all agreed with earlier data. In the final cultivation, when the specific growth rate was set to 0.40 h^{-1}, no gluconoylation or phosphogluconoylation could be detected in the protein product.

Geohegan et al. only analyzed His-tagged proteins and synthetic His-tags and concluded that the N-terminal sequence GSSHHHHHH- was more readily acylated than other sequences [5]. In this study the model protein ABD-$(Z_{HER2})_2$ have the N-terminal sequence GSSLAEAKV- which is often gluconoylated during protein production. The other constructs used in the final cultivations also have the N-terminal sequence GSS- and unfortunately the expression level of the His-tagged protein was low and no comparison could be made. A probable cause to the low expression levels of His_6-$(Z_{HER2})_2$-Cys and GA001-$(Z_{HER2})_2$ was the drop in pH of approximately one unit about four hours prior to harvest.

When the elucidated cultivation protocol was applied in the production of different His-tagged proteins, a part of the product was modified in all cultivations. This result is in agreement with the statement made by Geohegan *et al.* that proteins with the N-terminal sequence GSSHHHHHH- are more susceptible to the modification.

It is possible to carry out fed-batch cultivations with combined substrate and alkali feed, although the post induction feed have to be optimized to prevent the obtained drop in pH. In future cultivations the exponential feed can be prolonged to circumvent this problem. With combined feed less tubing is needed, and due to the high pH of the mixture (pH 11.5) several cultivations can be performed before sterilization of feed vessels and tubes.

If glycerol is used as a substrate during cultivations it is preferable to have a method to analyze the glycerol content in the fermentation broth. Preferably it should be a quick and reliable test that could be used during cultivation. It would then be possible to follow the decreasing concentration of glycerol during batch phase and to start the feed at a predicted time of when it reaches zero. There is a wide range of quick tests for glucose due to the heavy research on diabetes, but glycerol analysis is not as common. The analysis kit used in this project is usually used in the wine and juice industry. The kit works well but a measurement takes too long for it to be useful during fed-batch cultivations (approximately 30 min).

6. Conclusions

The data give clear indications of a lower abundance of phospho-gluconoylation and gluconoylation of the model protein when glycerol is used as substrate, and when a specific growth rate above 0.30 h^{-1} is applied. His_6-tagged proteins seem more susceptible to this modification.

The GRETA system can be used in studies requiring continuous cultivations, for example studies of protein expression in constitutive expression systems.

Combined glycerol and NH_3 feed can be used in fed-batch cultivations. A volume ratio of 0.14 between NH_3 and glycerol keeps pH around seven during exponential growth. Approximately at the time of induction, after which the feed is kept constant, a drop in pH occurs. Further trial cultivations are needed to circumvent this problem.

It is possible to freeze all additives to the growth medium ModNS85, including glycerol, prior to usage.

With the glycerol analysis kit EnzyPlus (Diffchamb) it is possible to analyze the glycerol concentration in the fermentation broth and it is easy to use. A drawback is that a measurement of one sample takes approximately 30 minutes, which is too long for it to be useful during cultivation.

7. Acknowledgements

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8. Abbreviations

ABD	Albumin binding domain
ABP	Albumin binding protein
ACN	Acetonitrile
BR	Bioreactor
CIP	Cleaning in place
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DTT	DL-1,4-dithiothreitole
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-thiogalactopyranoside
HER2	Human epidermal growth receptor 2
HSA	Human serum albumin
LC/MS	Liquid chromatography / Mass spectrometry
OD	Optical density
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TFA	Trifluoric acid
TSB	Tryptic soy broth
WCB	Working cell bank
YE	Yeast extract

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Appendix A: Growth media

Terrific Broth	Tryptone	12	g/l
	Yeast extract	24	g/l
	K_2HPO_4	9.4	g/l
	KH_2PO_4	2.2	g/l
	Glycerol	10	g/l
2 x YT	Tryptone	16	g/l
	Yeast extract	10	g/l
	NaCl	5	g/l
ModNS85	K_2HPO_4	9	g/l
	$NaNH_4HPO_4{\cdot}4H_2O$	23	g/l
	Citric acid monohydrate	2	g/l
	L-methionine	0.75	g/l
	Yeast extract	10	g/l
	$MgSO_4{\cdot}7H_2O$	1.5	g/l
	Trace element solution	1	ml/l
	$FeCl_3{\cdot}6H_2O$	53	g/l
	$ZnSO_4{\cdot}7H_2O$	16	g/l
	$CoCl_2{\cdot}6H_2O$	4	g/l
	$CuSO_4{\cdot}5H_2O$	4	g/l
	H_3BO_3	4	g/l
	$MnSO_4{\cdot}H_2O$	20	g/l
TSB+YE	TSB	30	g/l
	Yeast extract	5	g/l
Defined medium	$(NH4)_2SO_4$	2.5	g/l
	K_2HPO_4	2	g/l
	KH_2PO_4	3	g/l
	$Na_3C_6H_5O_7{\cdot}2H_2O$	1.25	g/l
	Trace element solution (see ModNS85)	0.66	ml/l
	$CaCl_2{\cdot}2H_2O$	14	g/l
	Thiamine solution 3 %	2.3	ml/l
	$MgSO_4{\cdot}7H_2O$	1	g/l
	Vitamin solution	0.65	ml/l
	DL-pantothenic acid	0.5	g/l
	choline cloride	0.5	g/l
	folic acid	0.5	g/l
	myo-inositol	1	g/l
	nicotineamide	0.5	g/l
	pyroxidal hydrochloride	0.5	g/l
	riboflavine	0.05	g/l
	thiamine hydrochloride	0.5	g/l

Appendix B: Cultivations

Batch cultivations

050907	Tunair® 1	Tunair® 2	Tunair® 3	Tunair® 4	Tunair® 5
Construct:	pAY770	pAY770	pAY770	pAY770	pAY770
Purpose:	Screen different complex growth media.				
Comment:	The growth media Terrific broth, 2×YT, TSB + YE and ModNS85 with either glucose (40 g/l) or glycerol (20 g/l) were screened.				

Fed-batch cultivations

050824	BR 1	BR 2	BR 3	BR 4	BR 5	BR 6
Construct:	pAY770	pAY770	pAY770	pAY770	pAY770	pAY770
Purpose:	Perform fed-batch cultivations with defined growth media, test reproducibility.					
Comment:	Depletion of oxygen during night, cultivations were discarded.					

050830	BR 1	BR 2	BR 3	BR 4	BR 5	BR 6
Construct:	pAY770	pAY770	pAY770	pAY770	pAY770	pAY770
Purpose:	Perform fed-batch cultivations with defined growth media, test reproducibility.					
Comment:	Successful cultivations.					

050919	BR 1	BR 2	BR 3	BR 4	BR 5	BR 6
Construct:	pAY770	pAY770	pAY770	pAY770	pAY770	pAY770
Purpose:	Investigate the influence of the specific growth rate on phosphogluconoylation. Compare the substrates glucose and glycerol.					
Comment:	Depletion of oxygen during night, cultivations were discarded.					

050920	BR 1	BR 2	BR 3	BR 4	BR 5	BR 6
Construct:	pAY770	pAY770	pAY770	pAY770	pAY770	pAY770
Purpose:	Investigate the influence of the specific growth rate on phosphogluconoylation. Compare the substrates glucose and glycerol.					
Comment:	Successful cultivations.					

051108	BR 1	BR 2	BR 3	BR 4	BR 5	BR 6
Construct:	pAY770	pAY770	pAY770	pAY770	pAY770	pAY770
Purpose:	Investigate the influence of the specific growth rate on phosphogluconoylation.					
Comment:	Inoculation in cold fermentors. BR 1 cancelled, successful cultivations in BR 2 - BR 6.					

051115	BR 1	BR 2	BR 3	BR 4	BR 5	BR 6
Construct:	pAY770	pAY770	pAY770	pAY770	pAY770	pAY770

Purpose: Perform fed-batch cultivations with combined glycerol and NH_3 feed.
Comment: One of the tested mixtures kept the pH close to seven,
but the used NH_3 probably had a low concentration due to evaporation.

051123	BR 1	BR 2	BR 3	BR 4	BR 5	BR 6
Construct:	pAY770	pAY770	pAY770	pAY770	pAY770	pAY770

Purpose: Perform fed-batch cultivations with combined glycerol and NH_3 feed.
Comment: One of the tested mixtures kept the pH close to seven.

051205	BR 1	BR 2	BR 3	BR 4	BR 5	BR 6
Construct:	pAY694	pAY770	pAY773	pAY1116	pAY1151	pAY1152

Purpose: Perform fed-batch cultivations according to elucidated protocol.
Test different proteins susceptibility to phosphogluconoylation.
Comment: Automatic induction was not trigged. Dip in pH at end of cultivations.

051207	BR 1	BR 2	BR 3	BR 4	BR 5	BR 6
Construct:	pAY694	pAY770	pAY773	pAY1116	pAY1151	pAY1152

Purpose: Perform fed-batch cultivations according to elucidated protocol.
Comment: Dip in pH at end of cultivations.

Continuous cultivations

051004	BR 3
Construct:	pAY770

Purpose: Investigate the influence of the specific growth rate on phosphogluconoylation.
Comment: The cultivation was constantly induced.

051024	BR 3
Construct:	pAY770

Purpose: Investigate the influence of the specific growth rate on phosphogluconoylation.
Comment: Parts of steady state cultivation were transferred to new reactors.
Induction at start of subsequent fed-batch cultivations.