Scientific paper

Bacterial Growth Conditions Affect Quality of GFP Expressed Inside Inclusion Bodies

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Abstract

As it was shown in our previous studies, significant amount of proteins trapped inside bacterial inclusion bodies (IBs) can be properly folded. Properly folded, functional proteins can be recovered from such IBs with extraction in mild, non-denaturing conditions. Such IBs were designated non-classical inclusion bodies (ncIBs). They are easy and practical resource for extraction of active proteins.

In the present study, factors influencing the quality of the target protein inside IBs were studied. Green fluorescent protein (GFP) was used as the model protein, as its proper folding (activity) can be easily monitored by fluorescence. Various growth conditions for bacterial cultivation were tested in order to increase accumulation of active (fluorescent) GFP inside ncIBs.

Temperature and induction regime were recognized as very important factors affecting the growth of the bacteria *Escherichia coli*, as well as recombinant protein yield and protein folding. Decreasing the growth temperature resulted in higher final biomass production and slower bacterial metabolism; therefore the percentage of correctly folded target protein inside the cell was higher. In addition, the induction regime also influenced protein folding and immediate induction was found to be more suitable for production of GFP.

Our studies confirm that optimization of bacterial growth conditions at all levels is very important for the production of high quality recombinant protein.

Keywords: Inclusion bodies; GFP; temperature; induction regime; fluorescence

1. Introduction

Expression of heterologous genes in *Escherichia coli* is a simple, fast and cheap way to produce large amount of target proteins. However, even though understanding of protein production is rapidly expanding, producing biologically active proteins in bacterial cells remains a challenge. Because of the simple protein folding machinery and the lack of posttranslational modifications, heterologous proteins often fail to fold into their native conformation and deposit into aggregates called inclusion bodies (IBs).[1,2]

Until recently, IBs have been described as insoluble and inactive deposits of misfolded proteins.[3] With the increasing number of reports about the presence of correctly folded protein precursors inside IBs,[4] this view is slowly changing. The presence of native-like structures has been confirmed using FT-IR spectroscopy[5–8] and in some cases biological activity has also been reported.[7–13]

Isolation of target protein from IBs usually involves denaturation / renaturation steps that are time consuming and must be optimized for each protein individually. Therefore a number of strategies for optimization of soluble protein production in *Escherichia coli* have been described. Choosing an appropriate host strain, lowering growth temperature, co-expressing chaperones or employing other process alternations as well as protein modifications, are standard approaches to increase solubility of the target protein in the cytoplasm.[1,14,15]

However, in our group, a different approach was tested. By lowering the growth temperature, formation of IBs containing a large amount of correctly folded granulocyte colony stimulating factor (G-CSF) was achieved.[7] Since isolation of the protein from such non-classical

IBs (ncIBs) can be performed under non-denaturing conditions, which essentially simplifies the production process, it would be very interesting to determine whether this strategy could be expanded to a broader range of proteins.

Green fluorescent protein (GFP) was the protein of choice for studying the formation and solubility (extractability) of ncIBs. GFP is a well-known protein containing a fluorophore, composed of three successive amino acids (Ser^{65}, Tyr^{66} and Gly^{67}), inside a barrel-like structure, when correctly folded. In preliminary GFP expression experiments, fluorescent IBs were observed inside *E. coli* cells. The goal of this study was to optimize the biosynthesis process in order to define the conditions where IBs with the highest proportion of correctly folded, fluorescent GFP are formed. Such fluorescent (active) GFP can be extracted from IBs under mild, non-denaturing conditions, and remains fluorescent during the isolation process without renaturation.

2. Experimental

2. 1. Expression System

Green fluorescent protein (GFP) from plasmid p-GFP (Clontech) was subcloned into a pET19b plasmid between the restriction sites XhoI and NcoI. The gene is under the control of the IPTG inducible T7 promoter. Plasmid pET19b-GFP was then transformed into the *E. coli* BL21(DE3) production strain.

2. 2. Culture Preparation

Initial bacterial inoculums were prepared in a Luria–Bertani (LB) medium supplemented with 100 mg/l ampicillin and 2.5 g/l glucose. In all cases the inoculums were incubated for 16 hours at 25 °C and 160 rpm in the linear shaker. Afterwards they were transferred to the GYSP production medium[13] at a ratio of 1:20. Production cultures were incubated at 160 rpm at temperatures of 25 °C, 30 °C, 37 °C and 42 °C, respectively (Kühner linear shaker). Two induction regimes were studied. For immediate induction, the inducer (isopropyl β-D-1-thiogalactopyranoside – IPTG) was added to the production medium together with the inoculum, while, for late induction, IPTG was added to the bacterial culture in the early exponential phase (OD600 2–3) (Agilent 8453 spectrophotometer; Agilent technologies). IPTG was added to a final concentration of 0.4 mM. At the same time, culture without the addition of an inducer was prepared. The cultures were incubated until they reached the stationary phase.

After production, bacterial cells were collected by centrifugation at 2700 g for 5 min, washed with 50 mM Tris/HCl, containing 30 mM NaCl (pH 7). The bacterial pellet was stored for further analysis.

2. 3. Inclusion Bodies Isolation

The bacterial pellet was resuspended in pure water and cells were disrupted with a high-pressure Emulsiflex® – C5 (Avestin) homogenizer at an operating pressure of 75–100 MPa. The supernatant containing the soluble protein fraction (SN1) was stored for analysis. The pellet of IBs (P1) was washed twice with pure water and used for further analysis.

2. 4. Extraction of Correctly Folded GFP from IBs

Isolated IBs were resuspended with solubilizing buffer (40 mM Tris/HCl with 0.2% N-lauroyl sarcosine, pH 8.0).[7,13] The suspension was incubated for 24 hours at 20 °C (Kühner shaker) and later centrifuged at 4400g for 15 minutes. N-lauroyl sarcosine was removed from the sample during a one-hour incubation using a Dovex 1 × 4–50 ion exchange resin (Sigma). The supernatant (solubilized target protein from IBs; SN2) and pellet (insoluble fraction of IBs; P2), as well as the supernatant SN1 and pellet P1 (chapter 3.4 Inclusion bodies isolation), were analyzed by SDS-PAGE.

2. 5. IMAC Isolation of GFP

The protein prepared at 25 °C was isolated from the soluble cell fraction (SN 1) as well as from the solubilized IBs (SN 2). Both samples were incubated at 60 °C for 30 minutes in order to denature and remove thermally unstable proteins. Nucleic acids were removed by precipitation with 0.1% polyethyleneimine. The samples were then centrifuged (15 minutes, 17.600 g) and filtered through a PVDF membrane (0.22 μm; Millipore). These samples were used for chromatographic separation.

Chromatographic separations were carried out on Knauer HPLC system equipped with two HPLC pumps (Knauer), a variable UV-Vis wavelength monitor (Knauer) and a Foxy Jr. (Teledyne ISCO) fraction collector.

GFP was isolated on 10 ml column HR 10/100 (Amersham) packed with Chelating Sepharose Fast Flow medium (Amersham Biosciences) previously charged with Cu^{++}-ions. Samples were dissolved in a 100 mM K-phosphate buffer and applied to the column. A 100 mM K-phosphate buffer with 100 mM NaCl, pH 8.3, was used for washing the column and GFP was eluted with 100 mM imidazole in a 100 mM K-phosphate buffer with 100 mM NaCl, pH 8.3. After isolation, the buffer was changed using Amicon Ultra-15 filter device (Millipore). The protein was prepared in pure water for CD analysis, while phosphate buffered saline (PBS buffer) was used for protein storage. Protein concentrations were determined by the Bradford procedure.[16]

An in-house GFP standard was used for GFP determination, CD analysis, fluorimetic analysis, and densito-

metrical determination of GFP concentration on SDS-PAGE gels. This in-house standard was isolated from the recombinant yeast *Pichia pastoris*[17] and purified as described above. The in-house GFP standard was compared to the commercially available GFP (Clonetech) in terms of purity and fluorescent characteristics and found to be suitable as an in-house standard.[17]

2. 6. SDS-PAGE Analysis

Whole bacterial cells, as well as samples after homogenization (SN1, P1) and samples after protein extraction (SN2, P2), were analyzed by SDS-PAGE. Nu-PAGE Bis-Tris gels (4–12%, Invitrogen) were used and stained with Colloidal Blue (Invitrogen). The calibration curve was prepared on each gel individually, with three samples of different quantities of a pure protein. Quantity of the target protein was determined densitometrically using a ProExpress Imaging System (Perkin Elmer) with Total-Lab 100 software. Samples from several independent experiments were analyzed.

2. 7. Circular Dichroism – CD Analysis

CD spectra were recorded on a Chirascan spectropolarimeter (Applied Photophysics) with temperature controller (MTCA series, Melcor). The far-UV spectra (190–250 nm) were recorded at 22 °C in a 1 mm path length cuvette with 300 µl of GFP solution in pure water (concentration 0.13 mg/ml). Six spectra with a resolution of 0.5 nm were averaged. Pure water blank spectra, obtained under the same conditions, were subtracted. The secondary structure of GFP from far-UV CD spectra was determined with SOMCD[18] (improved k2D algorithm[19]) software accessible via the internet.

2. 8. Observing the Fluorescence

Fluorescence of whole bacterial cells, isolated IBs (P1), and the soluble fraction (SN1) were observed with UV light (365 nm) using VL-6LC UV light lamp (6 W; 254 and 365 UV light tubes). Tubes with whole cells, supernatant, and IBs were placed in front of a black surface and photographed with an OLYMPUS SP-500 UZ digital camera.

2. 9. Fluorimetric Measurements

Emission spectra of GFP were taken on a Fluorimeter Quantsmaster C-61 (Photon Technology International) and analyzed with the software program Felix version 1.4. The excitation wavelength was 395 nm, while emission spectra were recorded from 420 to 600 nm. Fluorescent spectra of the GFP in-house standard and of the GFP from the soluble cell fraction (SN1), IBs, solubilized IBs (SN2), and in the insoluble fraction of IBs (P2), were measured. The spectra of purified GFP used for CD spectral analyses were also measured.

The percentage of fluorescent GFP was determined in each fraction. We anticipated that fluorescent GFP would be correctly folded. The concentration of correctly folded GFP in the GFP in-house standard is known, and therefore the fluorescence of the known concentration of the protein was determined. The fluorescent spectra of unknown GFP concentrations were measured, compared to the in-house GFP standard, and the concentration of fluorescent GFP in our samples was estimated. Total GFP concentration was determined by densitometry analysis of SDSPAGE gels. Fluorescent spectra of GFP isolated from the *E. coli* cytoplasm and IBs were also measured.

3. Results

3. 1. Biomass Production and GFP Accumulation

Two induction regimes (immediate induction and late induction at OD600 2) at four different growth temperatures (25, 30, 37, 42 °C) were tested and compared to non-induced cultures. For these studies several independent bacterial cultivations were prepared (15 for each given condition). It was found that growth temperature as well as the induction regime, affected cell growth (quantity of biomass produced) and also accumulation of GFP in the bacterial cells.

At lower temperatures higher biomass production was observed as up to 50% more biomass per liter produced at 25 °C than at 37 °C and 42 °C (Fig. 1). Statistical analysis (Student T-test) showed that significantly higher biomass production was observed for immediate induction at all tested temperatures (Figure 1).

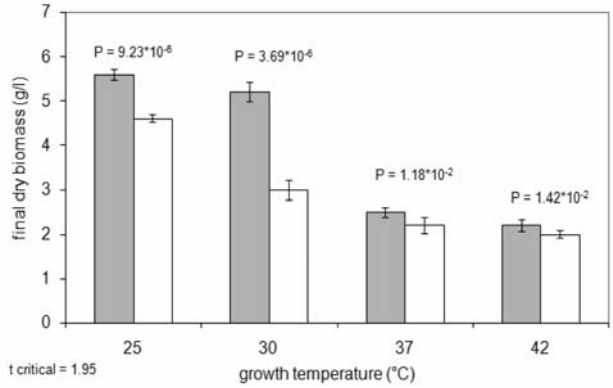

Figure 1: Final dry bacterial biomass production at different growth temperatures. At immediate induction (grey bars) more biomass per litre was produced than at delayed induction (white bars). Statistical analysis (T-test; P value) of 15 independent replicas showed that at all temperatures, the differences between the immediate and late induction regime regarding final biomass were significant.

GFP protein yield (regarding total *E. coli* proteins) was higher with the delayed induction regime at all tested temperatures (Fig. 2).

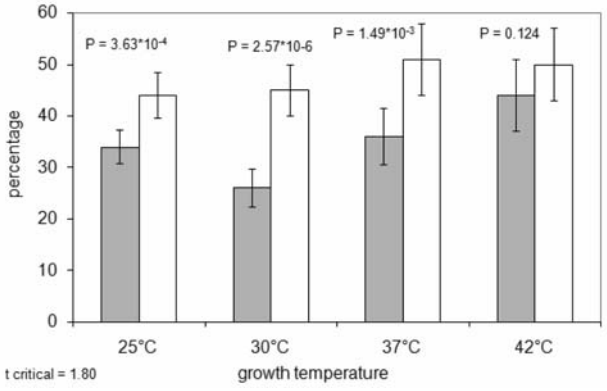

Figure 2: Percentage of GFP accumulated in bacterial cells compared to total cell proteins. At all tested temperatures the accumulation level of GFP was higher under conditions of delayed induction (white bars); (grey bars – immediate induction). Statistical analysis (T-test; P value) of 15 independent replicas showed that at all temperatures, the differences between immediate and late induction regime regarding final GFP accumulation were significant.

3. 2. Fluorescence of the Cells and Cell Fractions

During biomass harvesting we noticed that the cultures grown at 25 °C were fluorescent (green), due to GFP accumulated in the cells. When inspected under UV light (Bioblock Scientific; 365 nm), the cultures prepared at 25 °C, regardless of the induction regime, showed high fluorescence, while cultures prepared at 30 °C were slightly less fluorescent (Fig. 3).

Cultures prepared at higher temperatures showed no fluorescence. As expected, the negative controls (cultures that were not induced and did not produce GFP) were also non-fluorescent.

After homogenization and centrifugation the soluble cell fractions (soluble proteins) were separated from the IBs. Upon inspection under UV light it was found that IBs produced at 25 °C (regardless of induction regime) were highly fluorescent. IBs produced at 30 °C with immediate induction also showed some fluorescence, while IBs prepared at 30 °C with delayed induction were barely fluorescent (Fig. 3). IBs prepared at higher temperatures showed no fluorescence.

Protein in the soluble cell fraction was also fluorescent. As in the insoluble fraction, soluble GFP prepared at 25 °C also showed higher fluorescence than GFP prepared at 30 °C. Higher fluorescence of GFP was also observed with immediate induction at both temperatures.

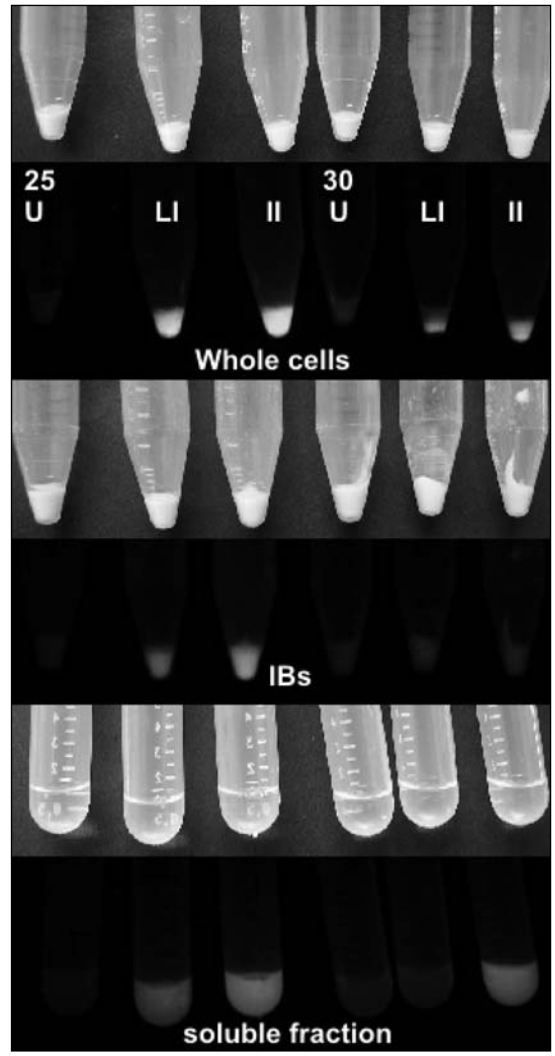

Figure 3: Fluorescence of the cells, IBs and soluble cell fraction, observed under UV light. The quality of the protein produced in the bacterial cell is dependent on bacterial growth conditions. At lower growth temperatures, more fluorescent GFP is produced inside bacteria. GFP is dispersed between the soluble (cytoplasm) and insoluble (IBs) cell fraction. More fluorescent protein inside IBs can be observed when bacterial cells are grown at low temperatures and at immediate induction.
25 – bacterial cells were grown at 25 °C;
30 – bacterial cells were grown at 30 °C
U – uninduced culture; no inducer (IPTG) was added
LI –late induction; IPTG was added to bacterial culture in the early exponential phase (OD600 2–3)
II – immediate induction – IPTG was added to the production medium together with the inoculum

3. 3. GFP Extraction from IBs

For protein extraction from IBs under non-denaturing conditions with 0.2% N-lauroyl sarcosine we used the procedure previously described by Jevsevar et al.[7] and Peternel et al.[13] Using 0.2% N-lauroyl sarcosine more than 85% of GFP from IBs prepared at 25 °C and 30 °C with immediate induction could be extracted. A high per-

Table 1: Percentage of GFP extracted from IBs under non-denaturing conditions using 0.2% N-lauroyl sarcosine

Cultivation temperature(°C)	GFP extracted from IBs (%) immediate induction	delayed induction
25	85 ± 1	83 ± 4
30	85 ± 1	72 ± 2
37	78 ± 4	75 ± 3
42	76 ± 2	60 ± 2

centage of target proteins were also extracted from IBs prepared under different conditions (Table 1).

3. 4. IMAC Isolation of GFP

Using pre-chromatographic steps as described under Materials and Methods and IMAC as the sole chromatographic step, more than 95% pure GFP protein was obtained from the *E. coli* soluble cell fraction, while GFP prepared from the solubilized IBs contained somewhat more impurities (85% pure protein). These purity data were obtained by densitomeric analysis of an SDS-PAGE gel, applying 1–1,1 microgram of GFP per well (Figure 4).

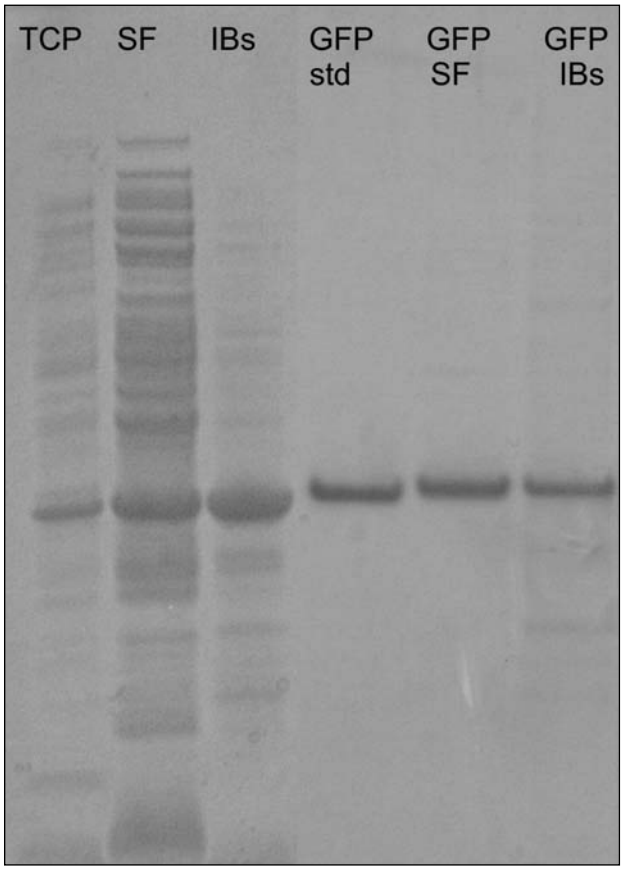

Figure 4: SDS-PAGE analysis of samples before and after protein isolation. GFP isolated from the soluble cell fraction is more than 95% pure, while GFP isolated from the solubilized IBs contained somewhat more impurities (85% pure protein).
TCP – total cell proteins (bacteria *E. coli* producing GFP)
SF – soluble cell proteins (soluble fraction)
IBs – proteins from inclusion bodies
GFP std. – in-house GFP standard isolated from *P. pastoris*
GFP SF – GFP isolated from soluble cell fraction of *E. coli*
GFP IBs – GFP isolated from *E. coli* inclusion bodies

3. 5. Fluorimetric Measurements

Spectra of the GFP in-house standard in 10 mM TRIS/HCl buffer (pH 8), *E. coli* supernatant (soluble GFP), fluorescent IBs, and GFP dissolved from IBs with 0.2% N-lauroyl sarcosine show the same emission peak at 506 nm, therefore we assume that N-lauroyl sarcosine did not affect the internal structure of GFP.

Fluorimetric measurements show that under immediate induction at 25 °C around 12% of all GFP produced in the cell is fluorescent, 7% of which is the soluble form, with the remaining 5% of the active protein being trapped inside IBs. Under the conditions of delayed induction, less of the protein produced is in a fluorescent form (around 9%), and the percentage of active protein in IBs (2.5%) is noticeably lower than in the soluble fraction (6%).

At higher temperatures, the percentage of fluorescent protein is lower, however the ratio of fluorescent protein found in the soluble and in the aggregated form remains more or less the same – there is always more fluorescent protein in the soluble fraction.

Fluorescence of whole IBs in suspension was also measured. Results show that whole IBs are only slightly fluorescent. Surprisingly, the fluorescence of whole IBs was almost two-fold lower than predicted from our results of fluorescence of solubilized IBs. The spectra of the pure GFP isolated from the *E. coli* cytoplasm (soluble protein) and from IBs were compa-

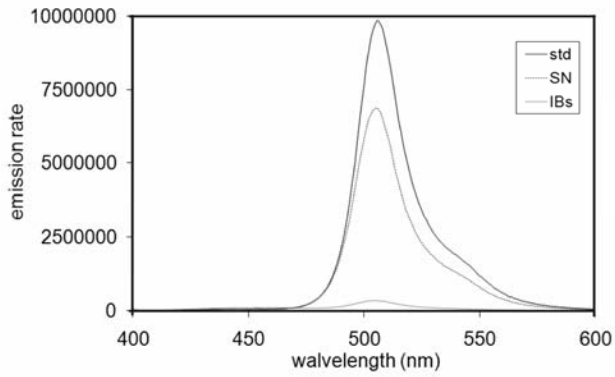

Figure 5: Fluorescence of various preparations of purified GFP. GFP standard from *P. pastoris* is more fluorescent than GFP isolated from *E. coli* cytoplasm. Fluorescence of the GFP isolated from IBs is even lower.

std – GFP in-house standard
SN – GFP isolated from soluble cell fraction – cytoplasm (supernatant)
IBs – GFP isolated from IBs

red to the spectra of the in-house GFP standard isolated from *P. pastoris*. As seen in Figure 5, the GFP standard from *P. pastoris* is more fluorescent than the GFP isolated from the *E. coli* cytoplasm. Fluorescence of the GFP isolated from IBs is even lower, almost 30 times lower than the fluorescence of the GFP standard, and 20 times lower than that of GFP isolated from the cytoplasm.

3. 6. CD Analysis

The far UV CD spectra of in-house GFP standard (from *P. pastoris*) and GFP from the soluble cell fraction of recombinant *E. coli* are very similar (Figure 6). The slight difference between the spectra can probably be assigned to some impurities present in the GFP isolated from the *E. coli* cytoplasm. The secondary structure content recovered with the SOMCD program[20] is: 19% α-helices, 47% β-sheet, 16% β-turn and 18% random. These results are very similar to the data previously published by Visser et al.[21] The only difference is the higher amount of non-organized structure at the expense of β-sheets. As described by several authors,[22–24] these results are not quite consistent and can depend on the choice of proteins in the standard protein database.

The spectra of GFP extracted from IBs have a greater portion of α-helices and random structure on behalf of

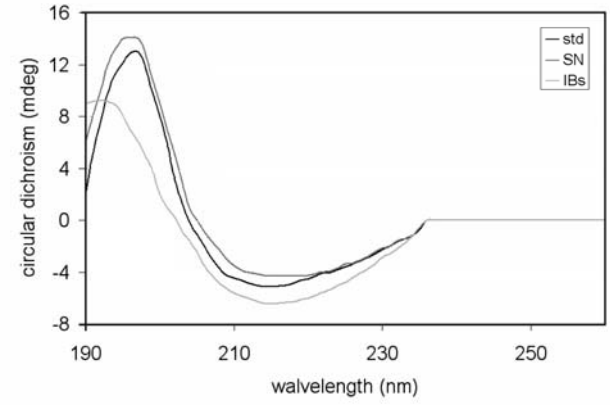

Figure 6: CD spectral analysis of pure GFP isolated from *E. coli* cytoplasm (SN; dark grey line) and IBs (light grey line), compared to in-house GFP standard isolated from *P. pastoris* (std – black line). The slight difference between the spectra of GFP standard and GFP isolated from *E. coli* cytoplasm can probably be assigned to some impurities present in the GFP isolated from *E. coli* cytoplasm. Difference in the spectra of GFP isolated from IBs can probably be assigned to the improper or incomplete folding of the protein inside the IBs.

β-sheets (33% α-helices, 27% β-sheet, 8% β-turn and 32% random). That can also be partially assigned to the presence of impurities in the sample, but above all to the improper or incomplete folding of the protein inside the IBs.

4. Discussion

In the present study, GFP was used as a model protein for exploring characteristics of non-classical inclusion bodies (ncIBs), which are classified as IBs containing a significant portion of correctly folded target proteins that can be extracted under non-denaturing conditions[7] and have some interesting new properties.[13] Therefore, they are interesting for biotechnology, especially for the production of biopharmaceuticals.

For high protein production yields, the whole bioprocess should be optimized. However, the quality of the final product is very important and it is not enough to consider and check product quality only during the last manufacturing steps. The whole production process should be optimized in order to get as much and as active protein as possible already in the first production phase, during biosynthesis. Therefore, understanding the variability of the bioprocess and its critical points enables us to predict the consequence of changes in the process. Process planning with quality designed or built into the process/product,[25] has become known as Quality by Design.[26]

The importance of bioprocess optimization can also be observed from our studies. Though some authors report that temperature does not affect the final biomass production, this was not the case in our study. At higher temperatures, lower biomass production was observed, which is rather unexpected for an enterobacterium. According to the literature data, bacterial culture enters the stationary phase when the medium runs short of oxygen.[27] At higher temperatures less oxygen is soluble in the medium, therefore we believe that the level of oxygen can limit the final biomass production.

Final dry biomass production is also affected by the induction regime. A statistically significant difference between the immediate and late induction regime was observed at all tested temperatures.

Several authors have already reported that lowering the cultivation temperature leads to an increased level of correctly folded and thus fluorescent GFP inside the IBs[28–30] and this was also the case in our study. At lower growth temperatures metabolism is slower, which is probably the reason for slower translation and folding process. Slower aggregation of proteins could lead to higher yields of correctly folded proteins, since there is enough time to permit folding.[28]

Furthermore, our results show that not only the temperature but also the induction regime have a great impact on the folding of the proteins. While the amount of fluorescent protein in the soluble cell fraction varies only slightly, the amount of fluorescent protein in the IBs depends strongly on the induction regime. The amount of correctly folded protein inside IBs was significantly lower with late induction at all temperatures tested. Under conditions of late induction a higher percentage of GFP is formed in the bacterial cell in a shorter time. The translation

and protein folding are faster; therefore the lower percentage of correctly folded and fluorescent protein in the cell is not unexpected.

Based on the results of final dry biomass production and the amount of total GFP accumulation in bacterial cells we might speculate that, with regard to the induction regime, the addition of inducer to the medium during the early exponential phase (late induction) has a huge impact on the bacterial culture. Instead of growing, bacterial cells switch their metabolism over to the producing target protein. Thus more target protein but less biomass is produced in the cells than at immediate induction.

Surprisingly, the fluorescence of whole IBs was almost two-fold lower than predicted from the results on the fluorescence of solubilized IBs. Such a low fluorescence of the whole IBs is probably the consequence of the mechanism of IBs formation, where correctly folded proteins are trapped inside the IBs, while misfolded proteins are "glued" on the surface of the IB. As previously described by Garcia-Fruitos et al.,[30] correctly folded proteins are preferentially found at the core of the aggregates, while the surface is poor in functional protein, when observing under fluorescent microscope. Since misfolded proteins are also selectively removed from the IBs' surface during bacterial growth this could further enrich the core of the IBs with native-like protein precursors.[30] The presence of native GFP in the aggregates was further confirmed with FT-IR spectroscopy analysis, where high amount of properly folded GFP was detected inside ncIBs prepared at lower temperatures.[8] The induction of proper folding by the extraction process could not be entirely excluded. However, since large amount of the protein is already in its native conformation inside ncIBs, it is more likely, that during extraction oxidation of GFP fluorophore occurs and thus the fluorescence increases. Spontaneous oxidation of disulphide bonds by air oxygen during the extraction process of GCS-F was previously described by Jevsevar et al.[7]

Since more biomass is produced under conditions of immediate induction and there is 50% more of the correctly folded protein inside the IBs than under the conditions of late induction, immediate induction of the recombinant *E. coli* culture seems to be more suitable for GFP production. Results show that from one litre of liquid culture prepared at 25 °C at immediate induction, more than 52 mg of fluorescent GFP could be isolated, while with late induction only around 42 mg of fluorescent GFP could be obtained. The difference is even more obvious when temperatures are raised to 30 °C. At immediate induction approximately 13 mg of fluorescent protein can be obtained from 1 litre of liquid culture, while at delayed induction just about 8 mg of the fluorescent GFP can be acquired.

The relatively low percentage of fluorescent protein in the soluble fraction could be a consequence of aggregation of the proteins in the soluble cell fraction,[14;31] but most probably this is not the only reason. Based on spectroscopy studies[8] we believe that there is more correctly folded GFP produced inside *E. coli* than can be observed from fluorescence studies.

The correctly folded GFP is composed of eleven β-sheets, which form a barrel-like structure with an α-helix bearing the fluorophore in the centre. The fluorophore is formed post-translationally with rapid cyclization between Ser^{65} and Gly^{67} followed by very slow (several hours) oxidation of Tyr.[66] It is generally accepted that correct GFP folding is a prerequisite for intense fluorescence. The matching of CD spectra of an in-house GFP standard and GFP isolated from *E. coli* is good evidence that fluorescent GFP isolated from the soluble fraction of *E. coli* is correctly folded. However, a comparison of fluorescence measurements per unit mass or the use of an in-house GFP standard fluorescence with known protein concentration as a standard for fluorimetric assessment of correctly folded protein inside the cell fractions reveals that GFP isolated from *E. coli* cytoplasm shows lower fluorescence than the GFP standard isolated from *P. pastoris*. This is a clear indication that not all molecules in the GFP from the *E. coli* soluble fraction are oxidized, which can probably be assigned to the reductive environment inside the bacterial cell. In such a reductive environment oxidation is difficult, which slows down the formation of the fluorescent centre of the GFP molecule. Although affinity chromatography used for GFP isolation is based on an interaction of surface histidine residues with the copper-charged matrix, the differences in surface structure are not big enough to allow discrimination among forms with a correctly oxidized interior and forms with incompletely oxidized fluorophore.

Our results show, that with process optimization, a higher proportion of correctly folded protein can be produced in the cytoplasm as well as in the ncIBs. Non-classical IBs are an interesting source of correctly folded proteins. The spectroscopy analysis of both GFP and G-CSF IBs show high amount of correctly folded protein inside ncIBs,[7,8] therefore we believe that that the amount of properly folded GFP inside ncIBs is higher than detected by fluorimetry studies. However, in the case of GFP, fluorescence was not an optimal method to measure correct folding. Our results show that not all the correctly folded protein molecules have been fully oxidized and possess an active fluorophore. Since non-classical IBs containing properly folded proteins extractable from such ncIBs under non-denaturing conditions have already been described for at least two other proteins (G-CSF and Tumour necrosis factor alpha – TNFα)[7,8,13] we believe this could be a broader occurrence.

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Povzetek

Kot smo pokazali v naših predhodnih raziskavah, je velik delež proteinov, ki se ujamejo v notranjosti inkluzijskih teles (IT), lahko pravilno zvitih. Pravilno zvite proteine lahko iz IT pridobimo z ekstrakcijo v blagih, nedenaturirajočih raztopinah. Takšna IT smo poimenovali neklasična inkluzijska telesa (nkIT). So preprost in praktičen vir za pridobivanje aktivnih proteinov.

V tej raziskavi smo preučevali dejavnike, ki vplivajo na kakovost tarčnih proteinov znotraj IT. Za modelni protein smo izbrali zeleno fluorescentni protein (GFP), saj je mogoče njegovo pravilno zvitje (aktivnost) preprosto spremljati z merjenjem fluorescence. Z namenom pridobivanja večje količine aktivnega (fluorescentnega) proteina GFP znotraj IT, smo preizkušali različne pogoje za rast bakterijske kulture.

Ugotovili smo, da sta temperatura in način indukcije zelo pomembna dejavnika, ki vplivata tako na rast bakterije *Escherichia coli*, kot tudi na količino in kvaliteto proizvedenega rekombinantnega proteina. Z zniževanjem temperature gojenja se poveča količina končne biomase v gojišču in upočasni metabolizem bakterij. Zato se poveča delež pravilno zvitih tarčnih proteinov v notranjosti bakterijskih celic. Poleg temperature ima na pravilno zvitje proteinov v celici močan vpliv tudi način indukcije. Ugotovili smo, da je za pridobivanje pravilno zvitega proteina GFP bolj primerna takojšnja indukcija.

Naše raziskave potrjujejo, da je optimizacija pogojev za rast bakterijske kulture je zelo pomembna za proizvodnjo visoko kakovostnih rekombinantnih proteinov.