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High expression of green fluorescent protein in *Pichia pastoris* leads to formation of fluorescent particles

Ana Lenassi Zupan[a,*], Sonja Trobec[a], Vladka Gaberc-Porekar[a], Viktor Menart[a,b]

[a] *National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia*
[b] *Lek Pharmaceuticals, d.d., Verovškova 57, SI-1000 Ljubljana, Slovenia*

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Abstract

Wild type gene for green fluorescent protein (GFP) was stably integrated into the *Pichia pastoris* genome and yielded an expression level of over 40% of total cellular protein. The high cytoplasmic concentration of fluorescent (properly folded and processed) GFP caused the formation of fluorescent spherical structures, which could be observed by fluorescence or confocal microscopy after controlled permeabilization of the yeast cells with 0.2% N-lauroyl sarcosine (NLS). Fluorescent GFP particles were also isolated after removal of the cell wall and found to be quite resistant to 0.2% N-lauroyl sarcosine. SDS-PAGE analysis of the isolated fluorescent particles revealed the presence of an 80 kDa protein (alcohol oxidase) and GFP (30%). We conclude that GFP is able to enter spontaneously into the peroxisomes and is inserted into densely packed layers of alcohol oxidase. Consequently, the formation of similar fluorescent particles can also be expected in other organisms when using high-level expression systems. As GFP is widely used in fusion with other proteins as a reporter for protein localization and for many other applications in biotechnology, care must be taken to avoid false interpretations of targeting or trafficking mechanisms inside the cells. In addition, when whole cells or cytoplasmic fractions are used for the quantitative determination of GFP levels, incorrect and misleading values of GFP could be obtained due to the formation of fluorescent particles containing material inside which is not available for fluorescence measurements.

Keywords: *Pichia pastoris*; GFP; Fluorescent particles; Peroxisomes; Alcohol oxidase

1. Introduction

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has become one of the most widely used reporter proteins in recent years. The major advantage of GFP as a reporter compared to other fluorescent proteins is that its fluorescence is stable, species-independent, allows simple detection under UV light and can be monitored non-invasively in living cells (Kain et al., 1995). Moreover, its fluorophore is gene encoded which has allowed cloning of *GFP* cDNA (Prasher et al., 1992) and expression of GFP protein in many heterologous host organisms (Chalfie et al., 1994; Yang et al., 1996) in the native, fluorescent form.

Fused to the N- or C-terminus of proteins, GFP can be used to establish their intracellular location and arrangement, to determine the level of gene expression

* Corresponding author. Tel.: +386-1-4760271; fax: +386-1-4760300.
E-mail address: ana.lenassi@ki.si (A. Lenassi Zupan).

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based on GFP fluorescence and to study transport and secretory processes in the cell.

Normally GFP fluorescence is measured in whole cells, with fluorescence intensities normalized to cell densities, or in cell lysates, and the protein level is usually determined from this fluorescence. These measurements are based on the assumption that GFP exists in a completely soluble form (Scholz et al., 2000; Sarramegna et al., 2002).

In *Pichia pastoris*, which is capable of accomplishing post-translational modifications resulting in proper folding of numerous foreign proteins, there are only a few reported cases of expression of proteins in the form of insoluble particles (Sreekrishna et al., 1988; Weik et al., 1998; Nakakubo et al., 2000; Wang et al., 2002) that are composed of incorrectly folded proteins and have no biological activity.

A few cases of targeting GFP to different organelles in *P. pastoris* have been reported. GFP targeted either to peroxisomes via the PTS1 (Monosov et al., 1996; Wiemer et al., 1996) or PTS2 leader sequence (Wiemer et al., 1996), or to the ER and Golgi structures via Sec13 fusion protein (Bevis et al., 2002) has been observed under confocal microscopy as intense fluorescent spots inside these organelles.

In studying high-level intracellular expression of unmodified GFP in *P. pastoris*, we observed the formation of similar fluorescent particles. The appearance of fluorescent particles containing significant amounts of GFP in the solid phase raises interesting questions and we have studied their nature, location and mechanism of formation. The occurrence of fluorescent particles containing significant amount of GFP could lead to erroneous interpretation of experimental results.

2. Materials and methods

2.1. Strains, cloning, and transformation

Unmodified GFP protein was expressed in *P. pastoris* GS115 strain (Invitrogen), genotype *his4*, and GFP with C-terminal -SKL aminoacid tag (GFP–SKL) was expressed in *P. pastoris* X-33 strain (Invitrogen), wild type. Constructs were prepared as followed. Unmodified *GFP* gene from jellyfish *A. victoria* was provided on the pGFP plasmid (Clontech). Plasmid pPIC3 (Invitrogen), designed for intracellular protein expression using strong *AOX1* promoter for expression in *P. pastoris*, and pGFP were both digested with BamHI and EcoRI enzymes. Linearized pPIC3 vector was dephosphorylated and ligated with *GFP* gene insert. Prior to transformation the resulting plasmid pPIC3-GFP was linearized with BglII enzyme. A fragment containing the expression cassette was isolated from gel and purified on column with Qiagen Gel Extraction Kit. Yeast cells were transformed by spheroplasting according to the protocol from the *Pichia* Expression Kit (Invitrogen). Transformants were selected on regeneration plates based on complementation of histidine auxotrophy. Transformants containing functional cassettes with multiple copies of *GFP* gene stably inserted into the genome were selected visually, based on the GFP fluorescence on minimal methanol (MM) plates after illumination with a hand-held UV-lamp (Bioblock Scientific VL-6LC, 6 W, 365 nm/254 nm) at 365 nm. Fluorescent colonies were replica plated on minimal methanol and minimal glucose (MD) plates to determine methanol utilizing (Mut) phenotype. The best producing clone had Mut^S phenotype.

To prepare the GFP–SKL construct, the *GFP* gene was PCR amplified from pGFP plasmid with 5′GC**G AAT TC**A TGA GTA AAG GAG A′3 N-terminal (EcoRI restriction site in bold) and 5′GC**T CTA GA**T TAC AGC TTA GAT TTG TAT AGT TCA TCC′3 C-terminal primer (XbaI restriction site in bold). The PCR product was isolated from gel and purified on column with Qiagen Gel Extraction Kit. GFP–SKL PCR product and pPIC6BAmp vector were both digested with XbaI and EcoRI enzymes. Linearized vector was dephosphorylated and ligated with insert to yield pPIC6BAmp-GFP–SKL plasmid. Prior to transformation the resulting plasmid was linearized with BglII enzyme. A fragment containing the expression cassette was isolated from gel and purified on column with Qiagen Gel Extraction Kit. Yeast cells were transformed by electroporation according to the protocol from the *Pichia* Expression Kit (Invitrogen). Dominant selection of transformants was performed on YPDS-Bla regeneration and selection plates, based on resistance to the antibiotic blasticidin (0.3 mg/ml). Transformants with multiple copies of GFP–SKL expression cassettes stably inserted into genome were selected as described above.

2.2. Media composition and growth conditions

Cells were grown in YPD medium (yeast extract 1%; peptone 2%; D-glucose 2%) in 0.5 l or 1 l Erlenmeyer shake flasks at 30 °C and 180 rpm in a rotary shaker (Kambič Laboratorijska oprema, Slovenia). Selection of yeast transformants was performed on regeneration (RDB) plates (2% agarose; yeast nitrogen base without amino acids 1.34%; biotin 4×10^{-5}%; 1 M sorbitol; glucose 2%; L-glutamic acid, L-iso-leucine, L-leucine, L-methionine, 5×10^{-3}% each; 1.5% agar) when selecting for *P. pastoris* GS115 transformants and on YPDS-Bla plates (YPD medium with addition of 2% agar, 1 M sorbitol and 0.3 mg/ml blasticidin) when selecting for *P. pastoris* X-33 transformants. Expression of GFP protein in shake flasks was accomplished in two phases: for the growth phase we used a buffered minimal glycerol (BMG) medium (yeast nitrogen base without amino acids (Difco) 1.34%; biotin 4×10^{-5}%; glycerol 1%; 100 mM potassium phosphate buffer, pH 6.0). For the induction phase we used buffered minimal methanol (BMM) or BMM with sorbitol (BMMS) medium (the same composition as BMG, only 1% glycerol was replaced by 0.5% of methanol and, in the case of BMMS, 1 M sorbitol was also added).

Selection of high producing clones and determination of the Mut phenotype of selected transformants was done on minimal methanol plates: yeast nitrogen base without amino acids (Difco) 1.34%; biotin 4×10^{-5}%; methanol 0.5%; agar 1.5%, and on minimal glucose plates: yeast nitrogen base without amino acids (Difco) 1.34%; biotin 4×10^{-5}%; glucose 2%; agar 1.5%. All strains were stored deep-frozen at −80 °C in 15% glycerol.

2.3. Expression of GFP protein

A 100 ml shake flask containing 20 ml BMG growth medium was inoculated with one yeast colony from a YPD plate. Shake flasks were incubated in a rotary shaker at 30 °C and 180 rpm for 48 h. Cultures were pelleted for 5 min at 5000 rpm in a sterile 50 ml Falcon tube. The supernatant was removed and the pellet resuspended in induction BMM medium (approximately 50 ml) to produce a cell suspension with OD_{600} around 10. The culture was shaken in a 0.5 l shake flask under the same conditions as in the growth phase. The duration of the induction phase was 120 h. Every 24 h the culture was supplemented with 250 μl of 100% methanol. After 120 h the culture was centrifuged for 10 min at 5000 rpm and 4 °C, the pellet washed with 50 mM PBS buffer, pH 7.4 and stored at −20 °C.

The protein expression level was determined using SDS-PAGE according to Laemmli (1970). Samples were prepared as followed. Wet pellet of induced *P. pastoris* cells (200 mg) was washed once with PBS buffer and resuspended in 800 μl of PBS buffer, pH 7.4. One volume of glass beads (0.45–0.50 mm, B. Braun Melsungen, AG) was added to cell suspension. Disruption of more than 99% of cells was achieved by eight cycles of 1 min full-speed vortexing followed by 1 min cooling on ice. Level of disruption was determined by optical microscopy. Whole cell homogenate was centrifuged at 5000 rpm for 5 min to remove any unbroken cells and cell debris. Supernatants were centrifuged further at 12,000 rpm for 15 min. A 50 μl samples of whole cell homogenate, supernatant and cell pellet were mixed with 2× SDS sample buffer and thermally denaturated for 5 min at 95 °C.

To assess GFP concentration using fluorimetry or densitometry of SDS-PAGE and modified SDS-PAGE gels (without thermal denaturation), an in-house GFP standard was prepared from an induced culture of recombinant *P. pastoris*. GFP protein was purified according to our own procedure (unpublished results), compared to the commercially available GFP (Clontech) in terms of purity and fluorescent characteristics and found to be suitable as an in-house standard.

2.4. Treatment of yeast cells with 0.2% N-lauroyl sarcosine (NLS)

To visualize the presence of intracellular GFP fluorescent particles in the cytoplasm of *P. pastoris* the cells were permeabilized with 0.2% N-lauroyl sarcosine which removed the majority of soluble GFP. NLS is a mild solubilizing detergent, which does not denature proteins when used in low concentrations. A wet pellet of induced *P. pastoris* cells (200 mg) was resuspended in 800 μl of 0.2% NLS. After gentle shaking overnight at 30 °C, the culture was centrifuged at 5000 rpm for 5 min, washed once with PBS buffer and resuspended in 1600 μl of PBS buffer. The cell suspension was used for examination under the confocal microscope (PpGFP-NLS).

2.5. Isolation of fluorescent particles

Yeast cells were lysed enzymatically using Lyticase (Sigma). Prior to disruption, all culture samples were subjected to three freeze/thaw cycles in liquid nitrogen. Two hundred milligram of induced culture pellet were resuspended in 1600 μl of 50 mM PBS buffer and 40 μl lyticase (100 U/μl) added. The cell suspension was incubated at room temperature for several hours. Spheroplast formation was monitored under the optical microscope. Spheroplasts were lysed with the addition of 0.2% NLS and short incubation at 37 °C.

The cell lysate was centrifuged at 5000 rpm for 1 min to remove any unbroken cells and cell debris. The supernatant was centrifuged further at 12,000 rpm for 5 min. The final pellet, containing fluorescent particles, was further examined by fluorescence and confocal microscopy (GFP particles).

For protein analysis with SDS-PAGE 30 μl volume of supernatant and isolated GFP particles was mixed with 10 μl of 4× SDS sample buffer and thermally denaturated for 5 min at 95 °C. In addition to the conventional method, a modified procedure with 0.2% SDS in the sample buffer and without thermal denaturation was also employed. This procedure enabled GFP fluorescence to be observed on SDS-PAGE gels.

2.6. Fluorescence measurements

Fluorescence was measured using a Quantamaster C-61 fluorimeter (Photon Technology International, South Brunswick, NJ, USA) with excitation at 395 nm and maximum emission at 507 nm. The amount of GFP protein was calculated on the basis of the in-house standard GFP calibration curve in the range of 0.05–1 μM. PBS buffer (50 mM, pH 7.5) was used for dilution. The protein concentration of the standard solution was determined according to Bradford procedure using a BSA calibration curve.

2.7. Fluorescence and confocal microscopy

Samples of permeabilized yeast cells and isolated particles, prepared as described above were directly examined under an Axiovert 135 fluorescence microscope (Zeiss, Germany) with Plan-NEOFLUAR objective 100×/1.30 (oil); α/0.17 and pictures were taken using a CCD Axiovision camera and Axiovision 3.0 software (Zeiss, Germany).

Fluorescent images of whole untreated cells, permeabilized cells and isolated particles were collected using an LSM 510 confocal microscope (Zeiss, Germany) with an oil immersion objective (63× and numerical aperture 1.4). GFP was excited by argon laser at 488 nm and the fluorescence collected through an LP 505 filter. Pixel time in Fig. 2A was 1.60 and 2.56 μs in Fig. 2B and C.

3. Results

3.1. Intracellular expression of GFP in yeast P. pastoris

Wild type GFP was expressed in the cytoplasm of yeast *P. pastoris*. To achieve maximal expression level we isolated *P. pastoris*-GFP transformants with multi-copy gene insertions based on simple visual selection of colonies under UV light. In this way, a transformant was isolated with a GFP level of over 40% of total cellular protein (Fig. 1) (Trobec et al., 2000). To our knowledge this is the highest expression level of GFP reported in methylotrophic yeasts.

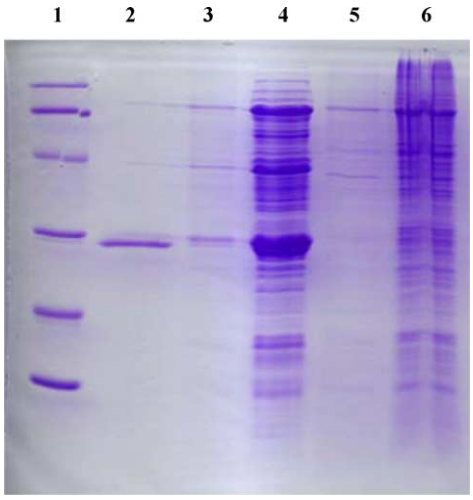

Fig. 1. SDS-PAGE analysis showing high-level expression of soluble GFP inside *P. pastoris* cells. *Legend*: lane 1: LMW standard (BioRad); lane 2: GFP in-house standard; lane 3: whole cell homogenate; lane 4: supernatant; and lanes 5 and 6: cell pellet.

When expressing GFP in *Escherichia coli* DH5α [pGFP] we observed that, after centrifugation of whole cells, the majority of highly fluorescent cells sedimented to the bottom of centrifuge tube while non-fluorescent cells remained in the top layer. Fluorescent cells sedimented more rapidly due to the presence of GFP in the form of inclusion bodies (unpublished results). We decided to explore if the situation is similar with *P. pastoris*, especially in the case of high expression of GFP.

3.2. Fluorescent GFP particles in untreated and permeabilized yeast cells as observed by confocal microscopy

P. pastoris cells expressing GFP intracellularly were observed under the confocal microscope. Even in untreated cells more fluorescent regions can be observed (Fig. 2A). To achieve better resolution of these intracellular structures cells were treated with 0.2% NLS. This resulted in partial removal of the cell membranes

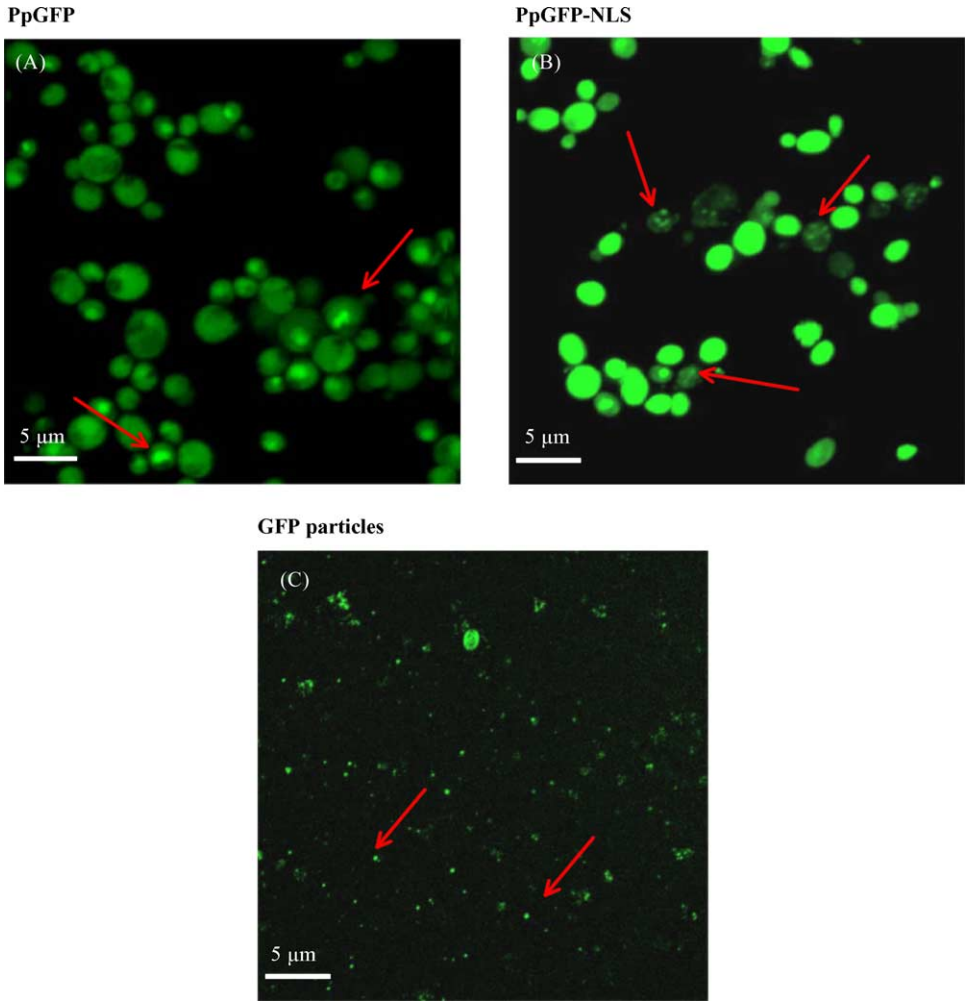

Fig. 2. Confocal microscopy of (A) *P. pastoris* cells with high expression of GFP (PpGFP), (B) *P. pastoris* cells expressing GFP, treated with 0.2% NLS (PpGFP-NLS), and (C) GFP particles isolated from permeabilized *P. pastoris* GFP cells (GFP particles). Pixel time in (A) is 1.60 μs and in (B) and (C) is 2.56 μs. The *P. pastoris* cells in (B) shows the same fluorescence as in (A) if the pixel time was lowered to 1.60 μs. Samples were prepared as described in Section 2.

leading to permeabilization and allowing soluble cytosolic GFP to diffuse into the surrounding buffer.

Fluorescent structures appeared as fluorescent particles less than 0.5 μm in diameter (Fig. 2B). Five to ten particles were observed per cell. The majority of cells contained such fluorescent particles, although in some cells these structures appeared only as dimmed forms probably due to incompletely removed soluble GFP. Based on these results we considered the two most probable hypotheses on the location and nature of the observed fluorescent particles.

(a) Formation of fluorescent particles in the cytoplasm composed of unfolded proteins but also containing trapped GFP. This could be possible as a consequence of exceeding the solubility of GFP in the yeast cytoplasm due to the very high expression levels.
(b) Transfer of GFP to certain cell organelles.

The appearance and average number of fluorescent particles suggested that mitochondria or peroxisomes could be the possible target for spontaneous GFP import.

Fluorescent images of GFP transported into the peroxisomes of *P. pastoris* cells using the targeting -SKL sequence attached to the C-terminus of GFP (Monosov et al., 1996, Wiemer et al., 1996) and the RL-X_5-H/QL sequence attached to the N-terminus of GFP (Wiemer et al., 1996) showed structures very similar to those observed in our case. Although the amino acid sequence of the wild type GFP used in our experiments contains no such or similar leading sequences, it is possible that transport into the peroxisomes is achieved with a sub-optimal leader sequence, aided by the high cytoplasmic concentration of GFP. As peroxisomes are the most abundant organelles in the methanol induced *P. pastoris* cells, occupying up to 80% of cell volume, we first checked these structures as the most probable target for spontaneous GFP import. Targeting to other organelles appears less probable because they cannot be isolated as particles resistant to the surfactant NLS used in our procedure.

3.3. Fluorescent particles isolated from yeast cells

Fluorescent GFP particles were isolated from lyticase treated *P. pastoris* cells. Cell debris was removed by low speed centrifugation followed by the GFP particles at a higher speed (12.500 rpm). This indicates that fluorescent GFP particles are not as compact as the inclusion bodies usually found in *E. coli*. Further, it appears that GPF particles are abundant, with properly folded GFP (Fig. 2C). Analysis of isolated GFP particles treated by sample buffer according to a modified SDS-PAGE procedure (Fig. 3A) revealed the presence of GFP, exhibiting the same molecular weight as GFP in the soluble fraction and as the GFP standard. The ratio of particulate to soluble GFP was determined from the modified SDS-PAGE (see Section 2). In *P. pastoris*, more than 95% of GFP is expressed in soluble form.

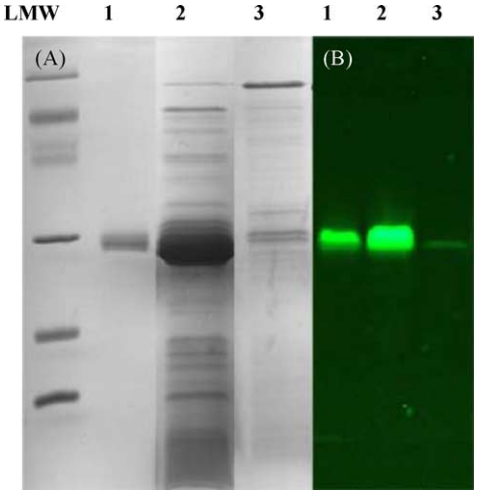

Fig. 3. Analysis of isolated GFP particles by SDS-PAGE (A) and modified SDS-PAGE (B) procedures (see Section 2). *Legend*: lane 1: GFP in-house standard, lane 2: *P. pastoris*-GFP supernatant; and lane 3: isolated fluorescent particles.

The content of GFP is about 30% in isolated fluorescent particles (see Fig. 3B). The major protein component is a protein with molecular weight around 80 kDa. The far most abudant peroxisomally located enzyme in methanol grown cells is alcohol oxidase, which is expressed up to 35% of total cellular proteins. Since the molecular weight of alcohol oxidase monomer is 80 kDa, the major protein component of the fluorescent particles corresponds most probably to this peroxisomal enzyme. We therefore conclude that fluorescent particles are remnants of peroxisomes, composed of insoluble alcohol oxidase clusters containing entrapped GFP.

3.4. Targeting of GFP–SKL to peroxisomes

The fusion protein GFP–SKL with C-terminal SKL peptide tag is known to enter peroxisomes. Transformed *P. pastoris* cells expressing GFP–SKL were examined by fluorescence microscopy and fluorescence was seen to be located in the peroxisomes, consistent with results obtained by Monosov et al. (1996). The GFP–SKL expression level was around 4% of total soluble protein, as determined by SDS-PAGE.

P. pastoris cells containing GFP–SKL were permeabilized using the same procedure as for cells expressing unmodified GFP. After extracting soluble GFP–SKL, similar fluorescent structures remaining in the cells were observed as shown for unmodified GFP in Fig. 2B. Also in this case the number of structures per cell is 5–10, and the size of them is also less then 0.5 μm in diameter.

3.5. Fluorescent particles were found at lower levels of GFP expression

We checked whether fluorescent particles are formed also at 10-fold lower GFP expression level (in this case GFP represents only around 4% of total proteins). Another carbon source sorbitol was used as it does not repress the AOX promoter completely and can be used to lower the expression level. *P. pastoris* culture prepared in modified medium was permeabilized using the same procedure as described above. Also at 4% GFP expression level fluorescent structures could be observed.

4. Conclusions

High-level expression (>40%) of fluorescent (functional) GFP in *P. pastoris* cytoplasm led to the formation of fluorescent GFP particles as revealed by fluorescence and confocal microscopy. However, also at 10-fold lower expression level of GFP (around 4% of total proteins) the particles were formed. Microscopy of permeabilized cells showed that the majority of cells contain 5–10 globular fluorescent structures. The nature of these structures was determined. Standard and modified SDS-PAGE analysis revealed two major protein components. We suppose that prevailing component (molecular weight 80 kDa) is alcohol oxidase, while the second most abundant protein (around 30%) was confirmed to be fluorescent (functional) GFP. Isolated fluorescent particles are resistant to washing with 0.2% N-lauroyl sarcosine, indicating their rather compact and stable structure. We conclude that unmodified GFP enters into the growing peroxisomes without known signal sequence during induction with methanol and is inserted into densely packed layers of alcohol oxidase. Consequently, the formation of similar fluorescent particles may also be expected in other organisms when using high-level expression systems. When expressing GFP fused to other proteins, or with attached oligopeptide tags, it is important to check carefully whether the fusion protein is soluble or whether some or all of it is in the form of fluorescent particles. In such cases the use of whole cells or cytoplasmic fraction only for the on-line quantitative determination of GFP levels can be incorrect and misleading as significant amount of GFP could occur in the form of compact particles with a small part of GFP not available for direct fluorescence measurements.

Special consideration is reasonable when GFP is used as a reporter for localization of in-fusion partner proteins. As already mentioned, according to our experiments, it is possible to expect that spherical fluorescent particles will be formed containing deposited insoluble material which could lead to false positive results.

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