Appl Biochem Biotechnol (2011) 163:80–89
DOI 10.1007/s12010-010-9018-9

# Expression and Secretion of a CB4-1 scFv–GFP Fusion Protein by Fission Yeast

**Julia Maria Naumann · Gabriele Küttner · Matthias Bureik**

Received: 13 April 2010 / Accepted: 17 June 2010 /
Published online: 10 July 2010


**Abstract** There is a rapidly growing demand for fluorescent single-chain Fv (scFv) antibody fragments for many applications. Yeasts have developed into attractive hosts for recombinant production of these functionalized proteins because they provide several advantages over prokaryotes and higher eukaryotes as expression systems, e.g., being capable of high-level secretion of heterologous proteins. In this study, we report *Schizosaccharomyces pombe* as a new host organism for secretory production of scFv-green fluorescent protein (GFP) fusions and compare it with previously described yeast expression systems. We cloned a plasmid for the expression and secretion of the anti-p24 (human immunodeficiency virus 1) CB4-1 scFv fused to GFP. After expression of the scFv–GFP fused to an N-terminal Cpy1 secretion signal sequence, fluorescence microscopy of living yeast cells indicated that the heterologous protein entered the secretory pathway. Western blot analysis of cell-free culture supernatants confirmed that the scFv–GFP was efficiently secreted with yields up to 5 mg/L. In addition, fluorescence measurements of culture supernatants demonstrated that the GFP moiety of the scFv–GFP protein is fully functional after secretion. Our data suggest that *S. pombe* has the potential for being used as alternative expression host in recombinant antibody fragment production by ensuring efficient protein processing and secretion.

**Keywords** Fission yeast (*Schizosaccharomyces pombe*) · Heterologous protein production · Secretion · Cpy1 · Recombinant antibodies · Single-chain Fv (scFv) fragments · CB4-1 · Green fluorescent protein (GFP) · Functionalized scFv

## Introduction

Fluorescent single-chain Fv (scFv) antibody fragments have a variety of applications, and in several experimental situations, they display a number of distinct advantages in

J. M. Naumann · M. Bureik (✉)
PomBioTech GmbH, Campus Building A1-1, 66123, Saarbrücken, Germany
e-mail: matthias.bureik@pombiotech.com

G. Küttner
Institute of Biochemistry, Medical Department, Humboldt University, Monbijoustr. 2, 10117 Berlin, Germany

comparison with conventional antibodies. Since its fluorescence property is preserved in chimeric fusion proteins, GFP has become a very popular reporter tag for specific scFv antibody fragments. There are many reports describing the expression of scFv–GFP fusions, which can be used for tumor assessment, drug screening, and for studying intracellular localization and dynamics of gene products in both *in vitro* and *in vivo* biological systems [1–13]. Although many applications make use of fluorescently labeled antibody fragments for immunological detection purposes, direct conjugation of scFvs with autofluorescent GFP can also be used for visualization and quantification.

Typically, scFv–GFP fusion proteins are expressed in *Escherichia coli* [2, 3, 5, 8, 9, 12], but several problems can occur that may render the production technically difficult and expensive. For instance, in many cases it is not possible to reproduce complicated posttranslational modifications of eukaryotic proteins after expression in prokaryotes. Also, expression in the reducing environment of the cytoplasm may not be universally possible for all scFvs because many scFv fragments are only correctly folded if intramolecular disulfide bonds are properly formed, which usually is not the case after cytoplasmic expression [14]. A strong expression in bacteria tends to lead to insoluble inclusion bodies, which require resolubilization and additional processing to retrieve functional proteins. Additionally, the yields of 100 to 200 μg/L culture of recombinant scFv–GFP proteins expressed in *E. coli* were rather low in the published cases as compared with other recombinant antibodies or antibody fragments [15]. In conclusion, it is often difficult to produce scFv–GFP fusion proteins in *E. coli* in sufficient quantities in an economic manner using the published procedures.

Mammalian and insect cells have also been used for the expression of scFv–GFP fusion proteins [4, 7, 13]. Eukaryotic expression systems are expected to be better suited for efficient folding of this kind of complex proteins due to the presence of eukaryotic chaperones that are missing in *E. coli*. Animal cells allow the production of eukaryotic proteins in their natural steric structures and with typical posttranslational modifications, but these cells are more difficult to handle than microorganisms and their culture is costly.

Yeast expression systems combine some of the advantages of mammalian and bacterial cultures in providing a eukaryotic cell system that can be handled in a similar way as bacteria, allowing for economic production of proteins, and several yeast species have been described as well suited expression systems for scFv–GFP fusions [1, 6, 10, 11]. Apart from protein production as such, secretion of the recombinantly expressed proteins is generally advantageous since protein purification from a culture supernatant is considerably more convenient than from a total cell lysate. Moreover, secretion may also reduce deleterious effects that intracellular overexpressed heterologous proteins may have on the host cell. Finally, by secretory production of a naturally exported foreign protein, the product is often identical or similar to its naturally occurring counterpart because the protein enters the secretory pathway in the host cells and undergoes appropriate processing such as formation of disulfide bonds and glycosylation.

Fission yeast *Schizosaccharomyces pombe* is considered to be closer to higher eukaryotes in various properties, such as regulation of cell cycle, transcription, chromosomal organization, and RNA splicing than other yeasts [16–20], and some posttranslational modifications of proteins produced in *S. pombe* are apparently similar to those in mammalian cells [20–23]. Furthermore, the quality control mechanism of glycoprotein folding seems to be similar in *S. pombe* and mammalian cells, while the respective mechanism in *Saccharomyces cerevisiae* is probably substantially different [24]. Thus, the use of *S. pombe* as a host for expression of secreted mammalian proteins may be likely to provide a protein that is close to its native form. For these reasons, *S. pombe* has emerged to be an attractive host for the expression and secretion of heterologous proteins that are therapeutic and commercially relevant [25].

It was the aim of this study to investigate whether *S. pombe* is also generally adapted as expression system for secretory production of scFv–GFP fusion proteins and to compare its production range with that of the established production systems for exported scFv–GFP. As a model antibody, the CB4-1 scFv fragment which recognizes the envelope protein p24 of the human immunodeficiency virus 1 (HIV-1) was employed. The CB4-1 scFv was N-terminally fused to the Cpy1 secretion signal sequence [26] and C-terminally fused to GFP. Expression of CB4-1 in *E. coli* has been reported but faced some difficulties that are reflected by a production yield of 1–3 mg/L [27]. Both its extensive characterization and the set of problems with expression in *E. coli* make this scFv fragment an interesting albeit presumably challenging model for the production of a secreted antibody fragment in fission yeast. We show here that as expected, the scFv–GFP fusion proteins entered the secretory pathway of the yeast cell; fluorescence microscopy as well as SDS-PAGE and Western analysis of cell-free culture supernatants confirmed that the scFv–GFP was efficiently secreted into the culture medium. In addition to these results, fluorescence measurements allowed the quantification of fluorescent scFv–GFP proteins in culture supernatants and demonstrated that the predominant fraction of secreted scFv–GFP is functional with respect to GFP fluorescence. Taken together, our data indicate that the fission yeast *S. pombe* is a promising new tool for the efficient secretory expression of scFv–GFP fusions.

## Materials and Methods

### Substrates, Chemicals, and Reagents

Chemicals and biochemicals used were obtained from Roth (Karlsruhe, Germany) and were of analytical grade. The molecular weight standard was from New England Biolabs (Frankfurt, Germany).

### Construction of Fission Yeast Strain JMN6

#### *CB4-1 scFv cDNA and Vectors*

General DNA manipulation methods were performed using standard techniques [28]. The fission yeast vector pREP42GFPC [29] allows expression of proteins with a carboxyterminal fusion to GFP. The expression of fusion proteins is regulated by the T4 mutated form of *nmt1* promoter [30]. For construction of pJMN6, we PCR-amplified the CB4-1 scFv cDNA to introduce a 5′-terminal *Nde*I site and a Strep-tag II [31] as well as a *Bam*HI site on the 3′-terminus. The resulting PCR product was cloned into a pCAD1 [32] vector that contained a Cpy1 secretion sequence [26] to yield pJMN1. Plasmid pJMN1 was used as template to amplify the Cpy1-scFv fragment without Strep-tag and stop codon, resp., but with flanking *Ase*I and *Bam*HI sites. The Cpy1-scFv was subcloned into pGEM-T vector II (Promega; Mannheim, Germany) and then cloned into pREP42GFPC using *Ase*I and *Bam*HI to yield pJMN6. Sequencing of the Cpy1-scFv cDNA revealed no alterations compared with the original sequence [27].

#### *Strains and Media*

The preparation of media and basic manipulation methods of *S. pombe* were carried out as described [33]. Fission yeast strain NCYC2036 [34] with the genotype $h^-$ *ura4.dl18* was used for transformation by pJMN6. Transformation was done by the lithium acetate method

[35] to yield the new strain JMN6. Transformed cells were plated on Edinburgh minimal media (EMM) with 5 μM thiamine and incubated at 30°C. Cells were grown in EMM with supplements as necessary. Permanent cultures were stored in double concentrated yeast extract with supplements (YES) and 25% glycerol (vol/vol). For biomass production, cells from permanent cultures were streaked on EMM dishes containing 5 μM thiamine and grown for 3 days at 30°C. Cell material from a plate was transferred to a 10-mL EMM preculture in the absence of thiamine. All subsequent cultures were also done in the absence of thiamine to keep the *nmt1* promoter in an active state. For main cultures, 100 mL EMM were inoculated with a 10-mL preculture. Usually, the final cell density was around $5\times 10^7$ cells/mL. Cells were generally centrifuged at 5,000×*g* at room temperature for 5 min. For production of secreted scFv–GFP protein, cells from 100 mL cultures were harvested after 24-h incubation, resuspended in 15 mL EMM containing 100 g/L glucose, and incubated as before for further 48 h. While this work was under way, the fission yeast strain JMN6 was also used in an analysis of protein secretion from single cells [36].

### Fluorescence Microscopy

Cells from 100 mL cultures were harvested after 24- and 72-h incubation, washed twice in PBS (pH 7.4) and finally resuspended in 10 mL PBS. Fluorescence microscopic images were obtained with a cooled charge-coupled device camera as image detector attached to a DM LB HC fluorescence microscope (Leica; Wetzlar, Germany) with standard GFP settings (excitation wavelength, 488 nm; emission filter, 515 to 550 nm). Data collection was controlled by the analySIS image acquisition and processing program (Olympus Soft Imaging Solutions; Muenster, Germany).

### SDS-PAGE and Western Blot Analysis

SDS-PAGE and Western blot analysis were performed using standard techniques [28]. Proteins from culture supernatants were separated by SDS-PAGE (12%) and electroblotted to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). A monoclonal mouse anti-GFP antibody (at a dilution of 1:1,000) obtained from Roche (Mannheim, Germany) and a secondary peroxidase coupled rabbit anti-mouse antibody (1:1,000; Acris; Herford, Germany) were used for immunologic detection. Visualization was done using the ECL Advance Western blot detection kit from Amersham (Piscataway, NJ, USA).

### Quantification of Secreted scFv–GFP Protein

For scFv–GFP quantification, 12 mL of culture supernatants were concentrated by ultrafiltration with centrifugal filter devices (Amicon Ultra 10K NMWL; Millipore, Carrigtwohill, Ireland). Supernatant concentrated by the factor of eight was used for further analysis. All experiments were done in triplicate.

#### *Quantification by Western Blot Analysis*

For estimation of the total amount of scFv–GFP in culture supernatant, concentrated supernatant proteins as well as purified eGFP (BioVision, Mountain View, CA, USA) in known concentrations were separated by SDS-PAGE and Western blotted as described above. After visualization, GFP concentration in culture supernatants was compared with that of the references.

*Quantification by Fluorescence Measurement*

For quantification of the functionally secreted scFv–GFP, the fluorescence of volume-reduced culture supernatants in microtiterplates was measured by a Tecan Genios reader (Tecan, Männedorf, Switzerland; excitation and emission wavelength 485 and 535 nm, respectively). Dilutions of pure eGFP in culture supernatant of the parental strain NCYC2036 were used for calibration. For the calculation of the scFV–GFP concentration from GFP calibration line, the measured fluorescence values were normalized to NCYC2036 background fluorescence; furthermore, the approximately double size of the fusion protein compared with GFP had to be taken into account.

## Results

### Expression of scFv–GFP Fusion Proteins in Fission Yeast

Fission yeast strain NCYC2036 was transformed using pJMN6 as described in materials and methods to yield strain JMN6 (*h⁻ ura4.dl18/pJMN6*). After 3 days, colonies grown on selective media were tested for the presence of Cpy1-scFv DNA by PCR. In order to analyze the subcellular localization of the scFv–GFP protein, strain JMN6 was grown in the absence of thiamine to induce the *nmt1* promoter, and cells from this strain as well as from the parental strain NCYC2036 were examined by fluorescence microscopy (Fig. 1). The fluorescence pattern of the fusion protein was characteristic for a protein that has been translocated to the ER lumen and, hence, entered the secretion pathway [26, 37, 38]; intracellular GFP fluorescence was predominantly detected in the ER and Golgi system as well as at the cell surface, but also in other intracellular regions, suggesting that a large fraction of the scFv–GFP accumulates in the secretion pathway (Fig. 1 a). After prolonged cultivation for 72 h, the overall appearance of the cells was similar, but the scFv–GFP protein was also found in large spots with intense fluorescence that were often distinctly visible on the cell poles (Fig. 1 b). In comparison, cells of the parental strain NCYC2036 hardly displayed any fluorescence at all (Fig. 1 c, d).

To confirm the successful secretion of the expressed scFv–GFP fusion protein, cells of strain JMN6 as well as from the parental strain NCYC2036 were grown in the absence of thiamine as described in "Materials and Methods"; proteins from culture supernatants were concentrated by ultrafiltration and examined by Western blot analysis using an anti-GFP antibody (Fig. 2). As expected, scFv–GFP could be detected in the culture supernatant of JMN6, and its apparent molecular weight is in good agreement with the calculated mass of 55 kDa. No scFv–GFP could be detected in the growth medium of NCYC2036 cells (control). These data show that *S. pombe* cells of strain JMN6 are not only able to express the scFv–GFP but also to secrete it efficiently into growth medium.

### Quantification of the Secreted scFv–GFP Protein

Comparison with several GFP standard samples of known concentrations in Western blot analysis allowed a rough estimation of the total amount of secreted scFv–GFP protein to be in the range of approximately 5 mg protein per liter of culture medium (data not shown). In addition to immunological detection, the cell supernatants were also analyzed by fluorescence measurements in order to determine the amount of functional GFP. For this purpose, strain JMN6 was used for the production of the scFv–GFP fusion proteins as

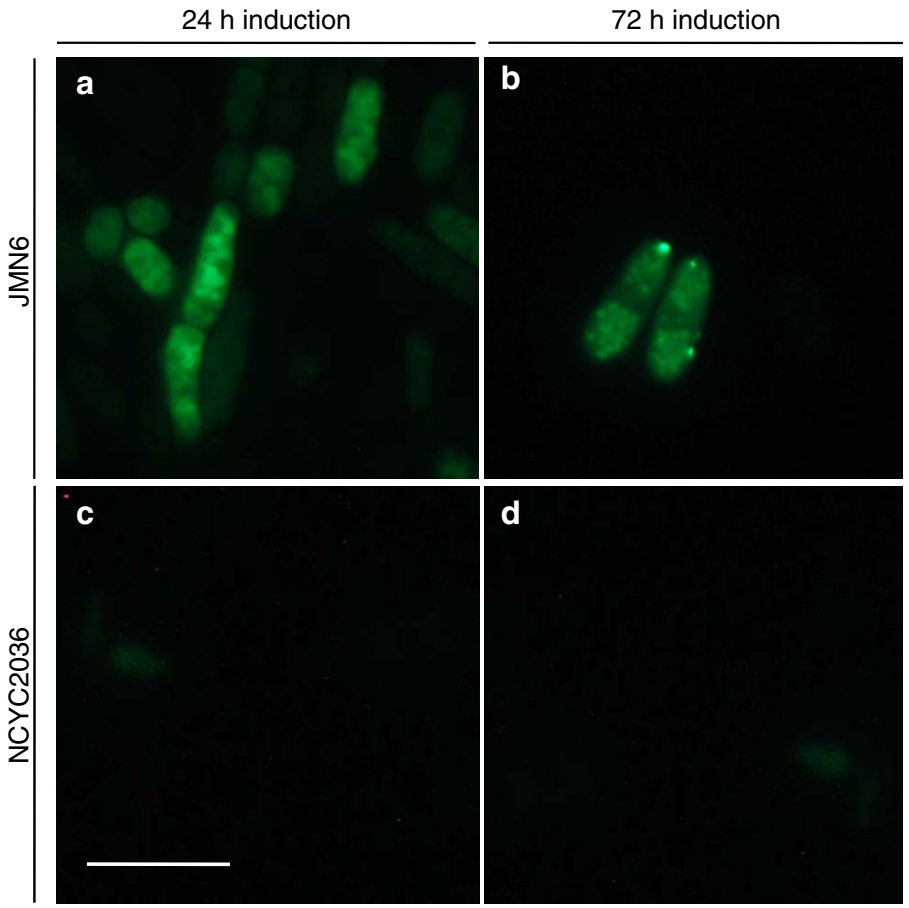


**Fig. 1** Expression of the scFv–GFP fusion protein in *S. pombe*. Fluorescence microscopy of fission yeast cells after *in vivo* expression of scFv–GFP fused to the amino-terminal secretion signal Cpy1. Secretion strain JMN6 (*a*, *b*) and the parental strain NCYC2036 (*c*, *d*) used as reference were grown for 24 h (*a*, *c*) and 72 h (*b*, *d*), resp., under induced conditions in the absence of thiamine (*bar*, 10 μm)

described in "Materials and Methods", and the parental strain NCYC2036 was used as a control (Fig. 3). In this way, the average concentration of the scFv–GFP fusion protein in JMN6 culture supernatants was determined to be 4.5±1.2 mg/L, while the supernatant from the parental strain only showed low background fluorescence. Thus, the corresponding scFv–GFP protein amount is similar to the whole amount of secreted scFv–GFP proteins. These data demonstrate that scFv–GFP is efficiently expressed and secreted by *S. pombe* strain JMN6 and indicate that the predominant fraction of the secreted fusion protein is functional in terms of GFP fluorescence.

## Discussion

ScFv–GFP fusion proteins combine immunological and autofluorescent properties and thus have many potential applications in diagnostics and analytics. It was the aim of the present

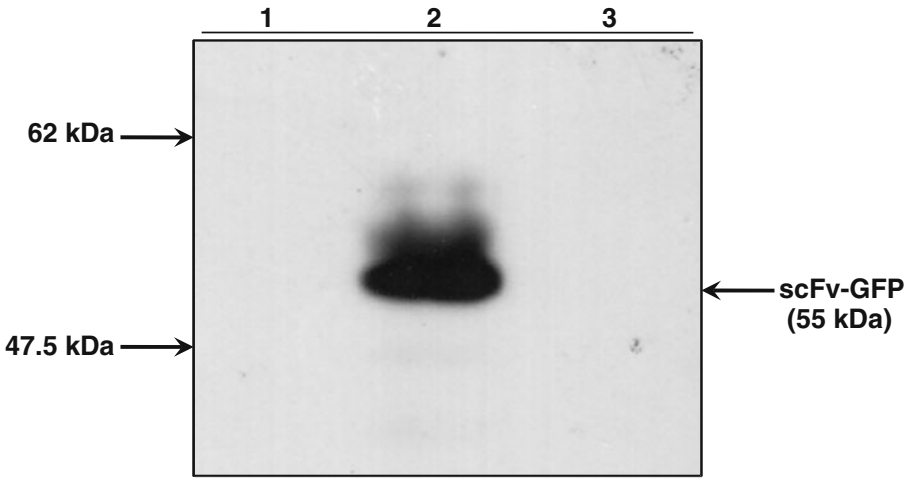


**Fig. 2** Cpy1-driven scFv–GFP secretion in fission yeast. Western blot analysis of concentrated cell-free culture supernatants probed with a monoclonal anti-GFP antibody was performed as described in "Materials and Methods". *Lane 1* prestained marker protein standard, *lane 2* supernatant of strain JMN6, *lane 3* supernatant of the parental strain NCYC2036 (negative control). The position and calculated mass of the scFv–GFP fusion protein is indicated

study to investigate the general adaptation of *S. pombe* as an expression system for secretory production of scFv–GFP fusion proteins and to compare the production range with that of the established production systems. The well-characterized anti-p24 (HIV-1) CB4-1 scFv [27] was used as model antibody. GFP was C-terminal fused to this scFv, and as N-terminal export signal, Cpy1 was used, which is known to function well for the secretion of GFP in *S. pombe* [26].

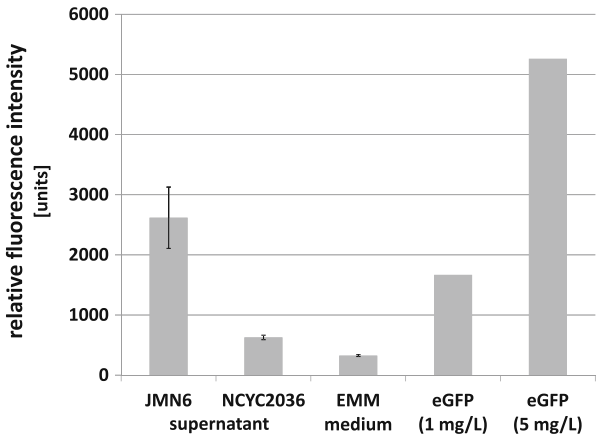


**Fig. 3** Quantification of the fluorescence of secreted scFv–GFP fusion proteins. Fluorescence measurements (excitation wavelength, 488 nm; emission filter, 515 to 550 nm) of concentrated cell-free culture supernatants of scFv–GFP secreting *S. pombe* strain JMN6 and its parental strain NCYC2036 (negative control) as well as EMM culture medium and dilutions of pure eGFP (1 and 5 mg/L) in NCYC2036 supernatant. From these data, the scFv–GFP concentration was calculated to be 4.5±1.2 mg/L

The expression of the scFv–GFP protein in the new fission yeast strain was first confirmed by fluorescence microscopy of whole cells. As shown in Fig. 1, strain JMN6 expressed the scFv–GFP under the appropriate culture conditions. The observed fluorescence pattern inside living scFv–GFP-expressing yeast cells was characteristic for a protein that enters the secretory pathway in *S. pombe* [26, 37, 38]. More specifically, GFP fluorescence was detectable at the outer cell surface as well as peri-nuclearly and in a vesicular pattern, suggesting that GFP was transported to the endoplasmic reticulum, the Golgi apparatus, and secretory vesicles, compartments that are part of the secretory pathway. However, the scFv–GFP proteins seemed to accumulate in the cells with prolonged cultivation.

To further confirm that scFv–GFP is indeed secreted into the medium and not retained within the yeast cells, culture supernatants of JMN6 were subjected to Western analysis. As shown in Fig. 2, the secreted scFv–GFP was readily detected and appeared to have the expected size of approximately 55 kDa. Based on direct comparison of the amount of scFv–GFP secreted by JMN6 with GFP standard samples of known concentration that had been loaded on the same immunoblot, it was estimated that Cpy1-driven scFv–GFP secretion yielded approximately 5 mg protein per liter of culture medium (data not shown). Thus, expression of the Cpy1-scFv–GFP construct resulted in high yields of extracellular scFv–GFP. This rather efficient scFv–GFP secretion was also confirmed by the observation that GFP fluorescence was detectable in cell-free concentrated culture supernatants (Fig. 3). Furthermore, this study describes for the first time a correlation between GFP fluorescence results and immunological detection for the quantification of the fraction of fluorescent (i.e., presumably correctly folded) GFP of the total amount of secreted GFP protein in *S. pombe*. On the basis of a GFP calibration line, the average concentration of the scFv–GFP fusion protein in JMN6 culture supernatants was determined to be 4.5±1.2 mg/L. These results show that within variation limits, at least the predominant fraction of the secreted scFv–GFP proteins is functional.

*S. pombe* has repeatedly been used for high-level heterologous protein production and has also been reported to be a suitable host for the secretion of different types of recombinant proteins, including proteins of human origin [25, 39] and others, with protein secretion levels up to 115 mg/L culture medium [40–43]. In contrast, the secretion of GFP or GFP fusion proteins with recombinant fission yeast was so far never reported to exceed final protein levels of 5 to 10 mg/L [26, 37, 38, 44]. Our data reported in this study also fit into this range. Literature data on scFv–GFP secretion efficiency in other host systems are very heterogeneous, and a direct comparison is difficult, which is partly due to the fact that secretion efficiency depends on the type of scFv under study and on the nature of the fusion construct. Overall, the reported yields of secreted scFv–GFP fusion proteins recombinantly expressed by insect and mammalian cells were in the range of 100 μg/L to 3 mg/L [13]. Somewhat higher values could in some instances be obtained with yeasts: For *S. cerevisiae*, yields between 4 μg and 4 mg of scFv–GFP per liter of culture medium were reported [6]. The group of Markus Deckert developed a *Pichia pastoris*-based secretory production system for an anti-A33 (colorectal carcinoma antigen) A33scFv::GFP fusion protein and reached yields up to 12 mg/L [10, 11]. For *S. pombe*, there is one report on the successful production of a biologically functional scFv–GFP fusion protein, but in that case, the fusion protein was not secreted [1]. With about 5 mg/L, the total yield of secreted scFv–GFP protein observed in this study is higher than all previously published yields for scFv–GFP fusion protein production in *S. cerevisiae*, but lower than that reported for *P. pastoris*. However, an optimization of the fission yeast-based scFv–GFP production and secretion may well be possible. For instance, increased secretion levels might be reached by

engineering host cells that provide optimized protein expression, folding, and secretion. Wentz and Shusta already showed enhanced secretion of several single-chain antibody fragments from *S. cerevisiae* by such an approach [45]. Moreover, it is known that proteolysis occurring in the culture medium can sometimes be a serious problem for production of secreted heterologous proteins. One promising approach to overcome this difficulty is the creation of appropriate protease-deficient mutants. For example, the production of protease-sensitive human growth hormone or human transferrin was found to be significantly enhanced in *S. pombe* strains that lack single or multiple proteases [46–49]. In addition, it has been previously reported that modification of the growth medium conditions clearly improved the secretion efficiency [37, 50]. Finally, the scFv–GFP expression construct itself could be optimized in terms of secretion signal sequence, codon usage, and promoter.

In summary, in this study, we approached the suitability of fission yeast as an expression host for the production of recombinant antibody fragments. To the best of our knowledge, we demonstrate here for the first time that fission yeast is capable of comparatively high-yield secretion of a scFv–GFP fusion protein into culture medium with the fluorescence marker of the exported protein being functionally active. These encouraging data support the notion that *S. pombe* could be a well-adapted expression system for production of other functionalized antibody fragments, for diagnostic or even therapeutic purposes, and should be examined more closely in this regard.

## References


1. Alting-Mees, M. A., Risseeuw, E. P., Liu, E., Desautels, M., Crosby, W. A., & Hemmingsen, S. M. (2006). *Methods in Molecular Biology, 313*, 97–105.
2. Cao, M., Cao, P., Yan, H., Ren, F., Lu, W., Hu, Y., et al. (2008). *Applied Microbiology and Biotechnology, 79*, 423–431.
3. Casey, J. L., Coley, A. M., Tilley, L. M., & Foley, M. (2000). *Protein Engineering, 13*, 445–452.
4. Didier, P., Weiss, E., Sibler, A. P., Philibert, P., Martineau, P., Bigot, J. Y., et al. (2008). *Biochemical and Biophysical Research Communications, 366*, 878–884.
5. Hink, M. A., Griep, R. A., Borst, J. W., van Hoek, A., Eppink, M. H., Schots, A., et al. (2000). *The Journal of Biological Chemistry, 275*, 17556–17560.
6. Huang, D., & Shusta, E. V. (2006). *Applied and Environmental Microbiology, 72*, 7748–7759.
7. Ishikiriyama, M., Nishina, T., Kato, T., Ueda, H., & Park, E. Y. (2009). *Journal of Bioscience and Bioengineering, 107*, 67–72.
8. Lu, M., Gong, X. G., Yu, H., & Li, J. Y. (2005). *Journal of Zhejiang University. Science B, 6*, 832–837.
9. Morino, K., Katsumi, H., Akahori, Y., Iba, Y., Shinohara, M., Ukai, Y., et al. (2001). *Journal of Immunological Methods, 257*, 175–184.
10. Panjideh, H., Coelho, V., Dernedde, J., Fuchs, H., Keilholz, U., Thiel, E., et al. (2008). *Bioprocess and Biosystems Engineering, 31*, 559–568.
11. Petrausch, U., Dernedde, J., Coelho, V., Panjideh, H., Frey, D., Fuchs, H., et al. (2007). *Protein Engineering, Design & Selection, 20*, 583–590.
12. Schwalbach, G., Sibler, A. P., Choulier, L., Deryckere, F., & Weiss, E. (2000). *Protein Expression and Purification, 18*, 121–132.
13. Peipp, M., Saul, D., Barbin, K., Bruenke, J., Zunino, S. J., Niederweis, M., et al. (2004). *Journal of Immunological Methods, 285*, 265–280.
14. Worn, A., & Pluckthun, A. (2001). *Journal of Molecular Biology, 305*, 989–1010.
15. Arbabi-Ghahroudi, M., Tanha, J., & MacKenzie, R. (2005). *Cancer and Metastasis Reviews, 24*, 501–519.
16. Brys, R., Nelles, L., van der Schueren, E., Silvestre, N., Huylebroeck, D., & Remacle, J. E. (1998). *DNA and Cell Biology, 17*, 349–358.
17. Jones, R. H., Moreno, S., Nurse, P., & Jones, N. C. (1988). *Cell, 53*, 659–667.
18. Kaufer, N. F., Simanis, V., & Nurse, P. (1985). *Nature, 318*, 78–80.
19. Remacle, J. E., Albrecht, G., Brys, R., Braus, G. H., & Huylebroeck, D. (1997). *The EMBO Journal, 16*, 5722–5729.

20. Russell, P., & Nurse, P. (1986). *Cell, 45*, 781–782.
21. Chappell, T. G., & Warren, G. (1989). *The Journal of Cell Biology, 109*, 2693–2702.
22. Giga-Hama, Y., Tohda, H., Okada, H., Owada, M. K., Okayama, H., & Kumagai, H. (1994). *Biotechnology (New York), 12*, 400–404.
23. Moreno, S., Sanchez, Y., & Rodriguez, L. (1990). *The Biochemical Journal, 267*, 697–702.
24. Parodi, A. J. (1999). *Biochimica et Biophysica Acta, 1426*, 287–295.
25. Takegawa, K., Tohda, H., Sasaki, M., Idiris, A., Ohashi, T., Mukaiyama, H., et al. (2009). *Biotechnology and Applied Biochemistry, 53*, 227–235.
26. Kjærulff, S., & Jensen, M. R. (2005). *Biochemical and Biophysical Research Communications, 336*, 974–982.
27. Winkler, K., Kramer, A., Kuttner, G., Seifert, M., Scholz, C., Wessner, H., et al. (2000). *Journal of Immunology, 165*, 4505–4514.
28. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual* (2nd ed.). Cold Spring Harbor: Cold Spring Harbor Press.
29. Craven, R. A., Griffiths, D. J., Sheldrick, K. S., Randall, R. E., Hagan, I. M., & Carr, A. M. (1998). *Gene, 221*, 59–68.
30. Basi, G., Schmid, E., & Maundrell, K. (1993). *Gene, 123*, 131–136.
31. Schmidt, T. G., Koepke, J., Frank, R., & Skerra, A. (1996). *Journal of Molecular Biology, 255*, 753–766.
32. Dragan, C. A., Zearo, S., Hannemann, F., Bernhardt, R., & Bureik, M. (2005). *FEMS Yeast Research, 5*, 621–625.
33. Forsburg, S. L., & Rhind, N. (2006). *Yeast, 23*, 173–183.
34. Losson, R., & Lacroute, F. (1983). *Cell, 32*, 371–377.
35. Suga, M., & Hatakeyama, T. (2005). *Yeast, 22*, 799–804.
36. Kortmann, H., Kurth, F., Blank, L. M., Dittrich, P. S., & Schmid, A. (2009). *Lab on a Chip, 9*, 3047–3049.
37. Braspenning, J., Meschede, W., Marchini, A., Muller, M., Gissmann, L., & Tommasino, M. (1998). *Biochemical and Biophysical Research Communications, 245*, 166–171.
38. Eiden-Plach, A., Zagorc, T., Heintel, T., Carius, Y., Breinig, F., & Schmitt, M. J. (2004). *Applied and Environmental Microbiology, 70*, 961–966.
39. Giga-Hama, Y. (1997). Fission yeast *Schizosaccharomyces pombe*: An attractive host for heterologous protein production. In Y. Giga-Hama & H. Kumagai (Eds.), *Foreign gene expression in fission yeast Schizosaccharomyces pombe* (pp. 3–28). Berlin: Springer.
40. Heintel, T., Zagorc, T., & Schmitt, M. J. (2001). *Applied Microbiology and Biotechnology, 56*, 165–172.
41. Ciofalo, V., Barton, N., Kretz, K., Baird, J., Cook, M., & Shanahan, D. (2003). *Regulatory Toxicology and Pharmacology, 37*, 286–292.
42. Okada, H., Sekiya, T., Yokoyama, K., Tohda, H., Kumagai, H., & Morikawa, Y. (1998). *Applied Microbiology and Biotechnology, 49*, 301–308.
43. Okada, H., Tada, K., Sekiya, T., Yokoyama, K., Takahashi, A., Tohda, H., et al. (1998). *Applied and Environmental Microbiology, 64*, 555–563.
44. Kjærulff, S., Muller, S., & Jensen, M. R. (2005). *Biochemical and Biophysical Research Communications, 338*, 1853–1859.
45. Wentz, A. E., & Shusta, E. V. (2008). *Biotechnology Progress, 24*, 748–756.
46. Idiris, A., Bi, K., Tohda, H., Kumagai, H., & Giga-Hama, Y. (2006). *Yeast, 23*, 83–99.
47. Idiris, A., Tohda, H., Bi, K. W., Isoai, A., Kumagai, H., & Giga-Hama, Y. (2006). *Applied Microbiology and Biotechnology, 73*, 404–420.
48. Idiris, A., Tohda, H., Sasaki, M., Okada, K., Kumagai, H., Giga-Hama, Y., et al. (2010). *Applied Microbiology and Biotechnology, 85*, 667–677.
49. Mukaiyama, H., Tohda, H., & Takegawa, K. (2009). *Applied Microbiology and Biotechnology, 86*, 1135–1143.
50. Mukaiyama, H., Giga-Hama, Y., Tohda, H., & Takegawa, K. (2009). *Applied Microbiology and Biotechnology, 85*, 155–164.