

Research Paper

Development of a defined medium for heterologous expression in *Leishmania tarentolae*

Claudia Fritsche¹, Mandy Sitz¹, Marco Wolf² and Hans-Dieter Pohl¹

¹ Department of Medical Engineering and Biotechnology, University of Applied Science Jena, Carl-Zeiss-Promenade 2, 07745 Jena, Germany

² fzmb GmbH Research Centre of Medical Technology and Biotechnology, Blumenstraße 70, 99092 Erfurt, Germany

A defined medium is essential for studying nutritional requirements of microorganisms and for selective supplementation of necessary substances. Hemin is an essential ingredient for growth of *Leishmania tarentolae*, but tends to precipitate in aqueous solutions without further stabilization. These aggregates disturb the measurement of the optical density or the cell density and the following downstream processing. Therefore, we were looking for stabilizing substances and established a PEG-hemin-solution, which avoided flocculation and allowed the cultivation of *L. tarentolae* in a medium, which we termed SFP(II) medium. With addition of RNA from *Saccharomyces cerevisiae* to SFP(II) medium the SFP(III) medium was established. In this medium, the specific cell division rate was increased (0.103 h^{-1}) and stable for longer periods of time. The evaluation of the SFP(III) medium was done in shaker flasks by successful expression and segregation of the SAG2 protein, one of the main surface antigens of *Toxoplasma gondii*. With establishment and evaluation of this defined medium, the status of the *Leishmania tarentolae* expression system as an alternative to commonly used cell cultures is supported.

Keywords: *L. tarentolae* / Defined medium / Hemin stabilization / SAG2-expression

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Introduction

Leishmania tarentolae (*L. tarentolae*) is a protozoan of the family *Trypanosomatidae* and a new eukaryotic expression system for the production of recombinant proteins [1–3]. Beside the animal-like N-glycosylation pattern, the main advantages of the *L. tarentolae* expression system are the higher specific growth rate in comparison to mammalian cells and the cultivation in low cost media. The growth behavior of *L. tarentolae* in complex nutrient media had been studied detailed [4].

Current investigations have been directed towards defined media, which open up new possibilities for the determination of nutritional requirements, because the concentrations of all ingredients are known. In con-

trast, complex media contain a cocktail of mostly unknown substances, such as several carbon and energy sources and a directed and selective elimination of individual substances is impossible. Therefore, the design of fed batch strategies is difficult, since the limiting substances are unknown.

Different defined media have been published in the past, e.g. by Merlen *et al.* [5], McCarthy-Burke *et al.* [6] or Trager [7]. These authors did not mention that hemin tends to precipitate in defined media, as our studies have shown. Only Chang *et al.* [8] and Gaughan and Krassner [9] noted hemin precipitations in the culture media and in aqueous solutions. These precipitations will affect measurement of optical density or cell density and can furthermore influence the downstream processing.

The development of a complete synthetic medium can not be done, because the *Leishmania* species requires heme compounds in the form of blood, serum or hemin as essential supplemented ingredient for growth [10].

Correspondence: Claudia Fritsche, University of Applied Science Jena, Department of Medical Engineering and Biotechnology, Carl-Zeiss-Promenade 2, D-07745 Jena, Germany

E-mail: Claudia.Fritsche@fh-jena.de

Phone: +49-3641-205676

Fax: +49-3641-205601

Hemin is the chlorinated derivative of the heme-molecule and is traditionally produced from bovine or pork blood. Therefore, we focused our investigations to find substances or methods, which could avoid hemin precipitations, and enable the cultivation of *L. tarentolae* in the defined media to high cell densities.

For evaluation of the defined medium, the heterologous expression of the surface antigen SAG2 was examined. SAG2 is one of the main antigens found in *Toxoplasma gondii*, a parasite causing the widespread disease Toxoplasmosis, which is dangerous to pregnant woman and immunodeficient patients. In Europe, about 30% of the AIDS patients die from Toxoplasmosis [11]. SAG2 is an important attachment factor for the invasion of the parasite into the host cell [12].

The SAG2 protein was used for detection of toxoplasmosis by different analytical methods [13–15]. Recombinant expression of SAG2 was established previously in *Escherichia coli* [14, 16], in *Pichia pastoris* [17] and by using the baculovirus-based production with insect cells [15]. It was clearly pointed out that the correct folding of the SAG2 protein is crucial for antigenity and immunogenicity.

In the present study, the SAG2 gene used was a truncated form. The GPI-anchor, the signal peptide and the transmembrane domain of the native protein were removed and a hexa-histidine-tag (His₆-tag) was fused. The resulting molecular weight was calculated to about 19 kDa [after calculation using the protein database ExPASy, Swiss Institute of Bioinformatics, www.expasy.org, 2007].

Materials and methods

Strains and cultivation

Two different strains were used for cultivation. The first strain was the *L. tarentolae* laboratory strain p10 (wild type strain (WT), Jena Bioscience, Germany). The second strain was the recombinant strain *L. tarentolae* p10 pF4SPSAG2sat#05 (fzmb, Germany) with the chromosomal ssu integrated gene for SAG2 with His₆-tag and the resistance-gene for Nourseothricin (Jena Bioscience, Germany). For cultivation of the recombinant strain, 100 mg l⁻¹ Nourseothricin was added to the medium. Both strains were maintained as static suspension cultures in the YE-medium at 26 °C as described elsewhere [4]. For inoculation of the defined media, late logarithmic phase growing agitated cultures (containing the YE-medium) were used and the cells were washed once with 0.9% NaCl (centrifuged at 2000 × g, 20 °C, 5 min). Minimal cell density was approximately

2 × 10⁷ cells ml⁻¹. Growth was monitored as described before [4].

Preparation of the defined media

The medium C of Trager [7] was prepared from sterile stock solutions of amino acids, vitamins, trace elements, salts and nucleotides as described. Ingredients were obtained from SIGMA, Carl Roth or Fluka (Germany) in analytical grade. Prior to filter sterilization, the pH was adjusted to 6.7 with 2 M KOH. Glucose was added to 3 g l⁻¹, Penicillin-Streptomycin to 50 unit's ml⁻¹ and hemin to 5 mg l⁻¹ as described before [4].

The medium, which we termed SFP(I) (Sitz-Fritsche-Pohl(I) medium), is based on the medium C of Trager [7]. Additionally, BSA (bovine serum albumin, Serva, Germany) was added to 0.528 g l⁻¹. In the SFP(II) medium BSA and hemin were replaced by a PEG-hemin-solution, prepared by dissolving of 2 g l⁻¹ hemin in 50% triethanolamine and 10% PEG1000 before autoclaving. The medium SFP(III) was prepared as the medium SFP(II) but supplemented with RNA from *Saccharomyces cerevisiae* (*S. cerevisiae*) (Fluka, Germany) to a final concentration of 77 mg l⁻¹ from a sterile stock solution of 4 g l⁻¹.

Stabilization of hemin

Different substances and stabilization methods were tested, summarized in Table 1 and Table 2. The hemin precipitation was evaluated by eye or the two-phase-extraction method [4, 18].

SAG2-expression studies

L. tarentolae was cultivated in SFP(III) medium, supplemented with 100 mg l⁻¹ nourseothricin. For cultivation, 2 × 500 ml shaker flasks (filled with 100 ml medium) were used and the inoculation was done time shifted. During cultivation, 1 ml medium supernatant was precipitated with 10% (w/v) trichloroacetic acid (TCA), washed once with acetone and resuspended in 10 µl SDS-PAGE-sample buffer. The preparation of the cell homogenate of 1 ml sample and the SDS-PA-Gel-electrophoresis with 12.5% polyacrylamide gels under reducing conditions were carried out as described previously [4]. Western blotting was performed using 0.2 µm nitrocellulose membranes (Kisker Biotech, Germany), a current of 280 mA and a transfer buffer with 39 mM Glycin (Carl Roth), 48 mM TRIS (Merck), 0.037% (w/v) SDS (Merck), 20% (v/v) Methanol (Merck). Blocking was done with 1% (w/v) BSA (Sigma-Aldrich) in TBST-buffer (50 mM TRIS, 150 mM NaCl (Carl Roth), 0.1% (v/v) Tween^R 20 (Carl Roth), pH 7.5). For detection, the primary antibody was an anti-His₆-antibody (Mouse IgG1)

(0.2 µg ml⁻¹, (Roche Diagnostics, Germany) in TBS-buffer (50 mM TRIS, 150 mM NaCl, pH 7.5) with 1% (w/v) BSA. The secondary antibody was an anti-Mouse IgG-conjugated with alkaline phosphatase (produced in goat, Sigma-Aldrich, Germany), diluted 1:3 × 10⁴ in TBS-buffer with 1% (w/v) BSA. Visualization of the reaction was done with the BCIP/NBT Liquid Substrate System (Sigma-Aldrich, Germany).

Purification of SAG2

Cells were cultivated in the SFP(III) medium, supplemented with nourseothricin (100 mg l⁻¹), and harvested after 29 h (end of phase 2). Medium supernatant was obtained by centrifugation (3000 × g, 5 min, 20 °C) and imidazole (Merck) was added to 5 mM. 70 ml were loaded onto a 1 ml HisTrapTM FF crude column with Ni SepharoseTM Fast Flow (GE Healthcare), using a flow rate of 1 ml min⁻¹. Column equilibration was done

with 5 × column volume (CV) of equilibration buffer (20 mM sodium phosphate, 500 mM NaCl and 5 mM imidazole). After loading, the washing step followed over 15 × CV with equilibration buffer and then a stepwise elution over 10 × CV with the elution buffer (20 mM sodium phosphate, 500 mM NaCl and 300 mM imidazole).

Results

Problems with defined media or solving the “hemin precipitations”

Various defined media, e.g. after Merlen *et al.* [5], McCarthy-Burke *et al.* [6], Trager [7], and the Medium 199 with Hank's salts (Invitrogen, Germany) have been examined for their ability to support growth of the new promising eukaryotic expression system *L. tarentolae*.

Table 1. Stabilization of hemin with different substances. The preparation was divided into three methods: “A” indicates hemin stock solutions, “B” the addition of the substance to the medium after dissolving in water and “C” the mixture of the substance stock solution with the hemin stock solution (2.5 g l⁻¹ hemin in 50% triethanolamine) prior to the addition to the medium. Evaluation was done visually with the classification of the precipitation to (––) strong, (–) weak and (+) no precipitation. Evaluations by “extract” were done using the two-phase-extraction method and calculation of the loss of hemin in per cent. Abbreviations: DMSO – Dimethylsulfoxide, PVA – Polyvinyl alcohol, CD – Hydroxypropyl-β-cyclodextrin, BSA – Bovine serum albumin, PEG – Polyethylene glycol.

| Substance | Type of preparation | Final substance concentration in cultivation media | Evaluation | |
|---------------------|---------------------|---|------------|-------|
| | | | Type | Scale |
| Hemin-Arginine [19] | A* | 9 mg l ⁻¹ | visual | – – |
| DMSO [20] | A** | – | visual | – – |
| PVA+ CD [21] | A*** | PVA: 90 mg l ⁻¹ CD: 98 mg l ⁻¹ | visual | – – |
| CD [22] | A**** | 98 mg l ⁻¹ | visual | – – |
| PVA [23] | B | 1 g l ⁻¹ | visual | – – |
| | | 100 mg l ⁻¹ | | |
| DMSO [20] | B | 1%; 10% (v/v) | visual | – – |
| Imidazole [24] | B | 3.4 × 10 ³ mg l ⁻¹ | visual | – – |
| Niacin amide [24] | B | 6.1 × 10 ³ mg l ⁻¹ | visual | – – |
| HEPES [24] | B | 23.8 × 10 ³ mg l ⁻¹ | visual | – – |
| BSA [25] | B | 15 mg l ⁻¹ | visual | – – |
| | | 528 mg l ⁻¹ | | + |
| PEG 400 | B | 0.1%; 1% (v/v) | visual | – – |
| Silicon | B | 0.05%; 0.5% (v/v) | visual | – – |
| BSA [25] | B | 528 mg l ⁻¹ | extract | 8.5% |
| CD [22] | B | 0.1% (w/w) | extract | 67% |
| PEG 20000 | C | 4% (w/w) | extract | 70% |
| | | 8% (w/w) | | 54% |
| PEG 1000 | B | 0.1% (w/w) | visual | – – |
| | | 1% (w/v) | | – |

A* Hemin and arginine were mixed in a molar ratio of 1:3, dried over night and dissolved in 50% triethanolamine; final hemin concentration of 2.5 g l⁻¹.

A** 2.5 g l⁻¹ hemin were dissolved in DMSO.

A*** Solution 1: dissolve 2.8 mg hemin in 14 ml 96% ethanol (pH 9); solution 2: dissolve 50 mg PVA and 55.2 mg CD in 6 ml deionized water; combine solution 1 and 2; stir overnight; remove ethanol and water by vacuum distillation and lyophilization; dissolve solid residue in water; final hemin concentration of 1 g l⁻¹.

A**** Dissolve 2.8 mg hemin in 14 ml 96% ethanol (pH 9); dissolve 55.2 mg CD in 6 ml deionized water; mix both solutions; stir overnight; remove ethanol and water by vacuum distillation and lyophilization; dissolve solid residue in water; final hemin concentration of 1 g l⁻¹.

Table 2. Further experiments with PEG1000 for the stabilization of hemin. Different kinds of preparation were utilized. Hemin was used in a final concentration of 5 mg l⁻¹. Loss of hemin was determined by two-phase-extraction.

| Preparation | | | Evaluation | |
|-------------|--|---------------|---------------------------------------|-------------------|
| Type | Method | Sterilization | Final PEG1000 concentration (% (w/w)) | Loss of hemin (%) |
| A | PEG was diluted with water and added to the cultivation media | autoclaved | 1 | 45 |
| | | | 4 | 66.5 |
| | | | 8 | 36 |
| | | | 10 | 36 |
| | | | 20 | 31 |
| B | PEG-stock solution was mixed with hemin-stock solution, then added to the cultivation media | autoclaved | 4 | 43 |
| | | | 8 | 46 |
| | | | 10 | 30 |
| C | PEG-hemin-stock solution was prepared by dissolving of 2 g l ⁻¹ hemin in 50% triethanolamine and 10% or 30% (w/w) PEG | autoclaved | 0.025 | 27 |
| | | filtrated | 0.075 | 35 |
| | | autoclaved | 0.075 | 34 |
| D | PEG-hemin-stock solution was prepared by dissolving 1.25 g l ⁻¹ hemin in 50% triethanolamine and 10% or 30% (w/w) PEG | filtrated | 0.04 | 75 |
| | | | 0.12 | 86 |

During the growth experiments with the defined media, dark, brown precipitates of hemin could be observed. Those aggregates were present at all stages of cultivation. Due to this precipitation and the fact that *L. tarentolae* depends upon exogenous hemin supplementation, growth barely occurred. Sub-cultivation over more than two passages was not possible at all. In complex media such as BHI or YE-medium no hemin precipitation occurred (for media composition see [4]). Apparently, these media contain unknown components, which are capable of stabilizing hemin and preventing it from precipitation.

Therefore, literature was searched for promising substances. Several chemical compounds were found and tested, using the defined medium C composed by Trager [7]. According to Table 1, BSA gave the best result for stabilizing hemin in the defined medium. The hemin loss upon precipitation or degradation did not exceed to more than 8%. However, BSA is animal derived and therefore a risk for contamination of the recombinant product exists. Polyvinyl alcohol was reported to be an appropriate substitute for BSA [26], but it did not stabilize hemin at all. Therefore, some other synthetic polymers such as PEG (polyethylene glycol) or silicone were tested. Only PEG1000 (molecular weight of 1000) seemed to be a promising candidate and was studied in detail, as it can be seen in Table 2. The primary objective of those experiments was the reduction of the final concentration of PEG1000 in the cultivation media. This was achieved by a PEG-hemin stock solution, which was able to stabilize over 70% of the provided hemin and to reduce the loss of hemin to 27%, respectively.

Growth of *L. tarentolae* in the media SFP(I)-(III)

The stabilization of hemin facilitated the cultivation of *L. tarentolae* WT strain in a chemically defined medium. BSA was used as stabilizing agent in the SFP(I) medium. In the SFP(II) medium BSA was replaced by PEG-hemin.

The growth of *L. tarentolae* under agitated conditions in the different media was analyzed by calculation of the specific cell division rate ν in h⁻¹ from the cell density (N) using the following equation:

$$\frac{dN}{dt} = \nu * N.$$

Specific cell division rates of $\nu = 0.089 \pm 0.007$ h⁻¹ (four independent measurements) could be achieved in the SFP(I) and (II) media, see Fig. 1. In comparison to complex media (e.g. $\nu = 0.103$ h⁻¹ for YE-medium [4]) ν is smaller. After 13–18 h of cultivation, first limitations of media components occurred and ν decreased drastically. A tendency for declining could also be observed for the yield coefficient $Y_{N/S}$, which is calculated from the produced cell density divided by the consumed glucose concentration in a defined time interval and volume. After a total cultivation time of 24 h, multiple limitations occurred in the media and growth was nearly succumbed.

Stabilization of hemin by BSA or PEG1000 was very successful in all examined shaker flasks, because no precipitation occurred.

Interesting was furthermore, if the medium contained all essential components for long-term growth. For clarification, cells were cultivated in the SFP(I) medium and passed into new medium at the end of expo-

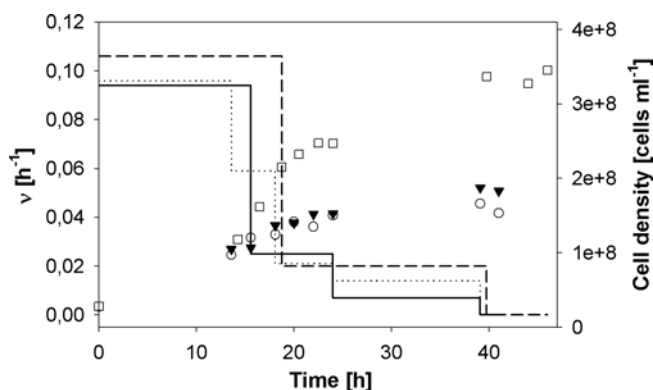


Figure 1. Plotting of the cell density (symbols) and v (lines) of *L. tarentolae* WT in the media SFP(I) (\circ , solid line), SFP(II) (∇ , dotted line) and SFP(III) (\square , broken line). The experiment was done using 250 ml flasks with baffles, filled with 30 ml, under agitated conditions. v was plotted with assumed transition intervals.

ponential growth, 3 times in a series. In total, 4 generations could be achieved and the specific growth rate μ (calculated from OD [4]) was constant in the range of 0.070–0.077 h^{-1} . This indicated, that the growth was not based on accumulated substances of the pre-culture (YE-medium).

Furthermore, we tried to increase the cell density in the media by appropriate supplements. It turned out, that addition of RNA from *S. cerevisiae* had positive effects on the growth of *L. tarentolae*. Using the SFP(III) medium, the first growth phase was extended and the specific cell division rate was raised by 15% ($v = 0.103 \pm 0.007 h^{-1}$, four independent measurements). A significantly higher cell density could be measured ($3.1 \times 10^8 \pm 0.9 \times 10^8$ cells ml^{-1}) in comparison to media without RNA ($1.6 \times 10^8 \pm 0.5 \times 10^8$ cells ml^{-1}). A plus of one generation could be obtained by the addition of RNA, resulting in 3.4 generations in total (for comparison: 2.3 generations without RNA addition).

SAG2-expression studies

For evaluation of the SFP(III) medium, the expression of SAG2 protein was examined by SDS-PAGE in combination with the growth behavior of the heterologous strain.

The SDS-PA-gels demonstrated, that the SFP(III) medium was suitable for constitutive expression and secretion of the SAG2 protein (Fig. 2). The intensity of the SAG2 band in the cell homogenate (after sonification) was <1.7 lane-purity (in %). The growth was different to the behavior of the wild type strain, as μ (calculated from the OD) and v kinetics in Fig. 3 indicate. Four different growth phases were observed, which were also detected in complex media, such as BHI or YE.

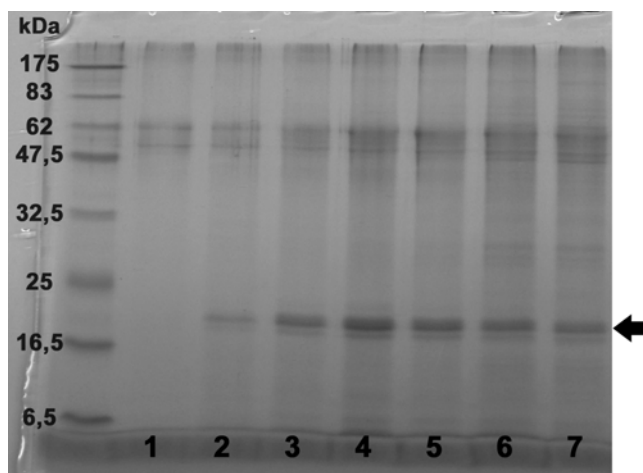


Figure 2. SDS-PA-gel shows the expression of SAG2 by *L. tarentolae*, cultivated in the SFP(III) medium. Samples of one shaker flask are shown representatively. Lane 1) start of cultivation; 2) after 6 h; 3) after 22.75 h; 4) 27 h; 5) 30 h; 6) 46.5 h and 7) 52.5 h of cultivation. The main SAG2 band is marked by an arrow, underneath the thin “shadow” band is visible. The expression levels of SAG2 were determined as intensity of the SAG2 band in the SDS-PAGE in comparison to the total intensity of the lane (lane%).

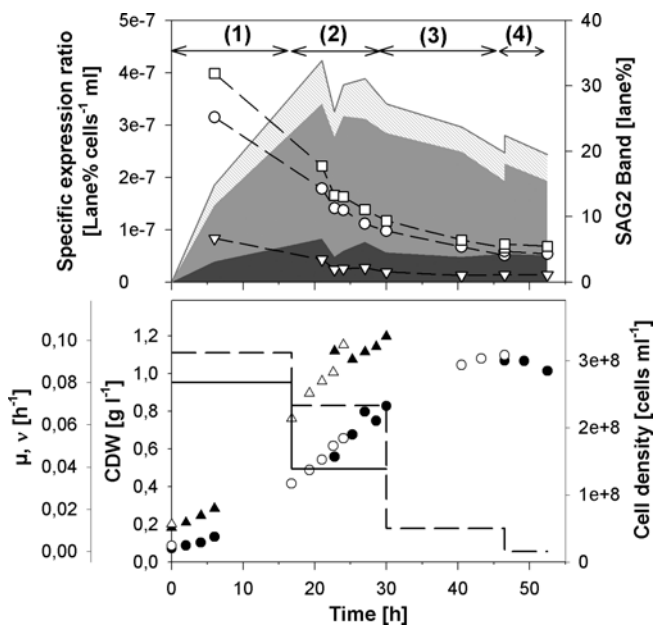


Figure 3. Expression of SAG2 by cultivation of *L. tarentolae* in the SFP(III) medium. Upper graphic: Plotting of the intensity of the main SAG2 band (mid grey area), of the SAG2 “shadow” band (dark grey area) and of the total intensity of the SAG2 bands (light grey area). Furthermore, plotting of the specific ratio of SAG2-expression (calculated by dividing the intensity of SAG2 band by the actual cell density) of the main (∇), the “shadow” (\square) and the total (\circ) SAG2-band. Graphic below: Plotting of μ (—), v (---), the cell densities (\circ , \bullet) and the cell dry weight (CDW) (∇ , \blacktriangledown) of the two shaker flasks. Curves were plotted with assumed transition intervals. Numbers and arrows indicate different growth phases.

At the end of phase 1, the cell density was about 1.2×10^8 cells ml^{-1} (0.8 g l^{-1} cell dry weight, calculated from OD). The highest SAG2 concentration was estimated at the end of phase 2. Afterwards, the product concentration continuously decreased with increasing cultivation time to a plateau concentration of about 20 lane%. Maximal cell densities of 3×10^8 cells ml^{-1} (2 g l^{-1} cell dry weight) were obtained in the shaker flasks.

Interestingly, the specific expression ratio, calculated from the intensity of the SAG2 band divided by the actual cell density, was decreasing dependent to growth, as it can be seen in Fig. 3. The values at 6 h cultivation time were possibly inaccurate because of intensities at the detection limit of Coomassie Brilliant Blue, but a tendency was seen.

During expression, a faint, “shadow” band was visible underneath the main SAG2 band in an approximately constant intensity and was detected in western blotting using anti-His₆-antibodies. Probably, a basal protease activity occurred, which was independent from the morphological status of the cells. Therefore, a well-timed harvest of the medium supernatant is essential.

Additionally, purification of SAG2 was examined by metal chelate chromatography using the fused His₆-tag. The resulting chromatogram is shown in Fig. 4. The different process steps were analysed in SDS-PAGE and western blotting, seen in Fig. 5. The purification by the HisTrapTM column was very effective since all SAG2 protein was bound to the column. The resulting purity was about 80 lane%. Once more, a main SAG2 band and a faint “shadow” band were detected. Surprisingly, a faint band was visible in the western blot at about the

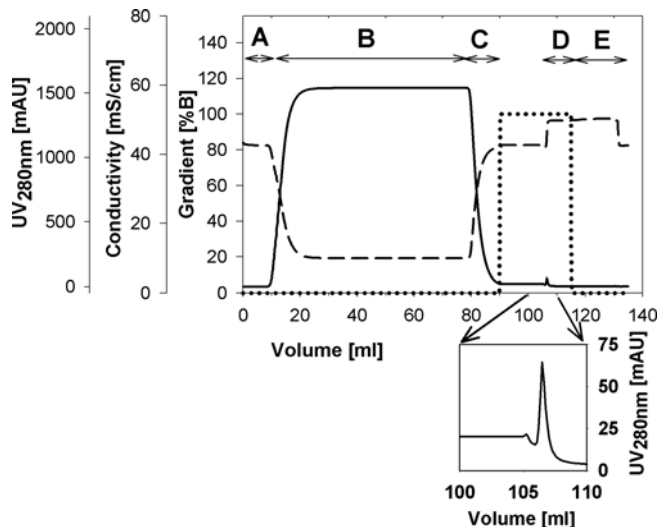


Figure 4. Chromatogram of the SAG2 purification with the HisTrapTM FF crude column, showing the UV line (solid line), the conductivity (broken line) and the gradient of the pump (dotted line). The process steps were performed at a flow rate of 1 ml min^{-1} and marked with A) equilibration, B) loading, C) washing, D) elution and E) re-equilibration. The elution peak is shown expanded underneath. Equilibration was done with 20 mM sodiumphosphate, 500 mM NaCl and 5 mM imidazole. Elution was done with a higher concentration of imidazole (300 mM).

double molecular weight of SAG2. This was not studied in detail, because it was not detected before.

Determination of the final concentration of SAG2 was not possible, because the determination of the protein concentration after Bradford was disturbed by imidazole in the elution buffer. The detection of SAG2 was done using the fused His₆-tag because no anti-SAG2-antibody is commercially available at this time.

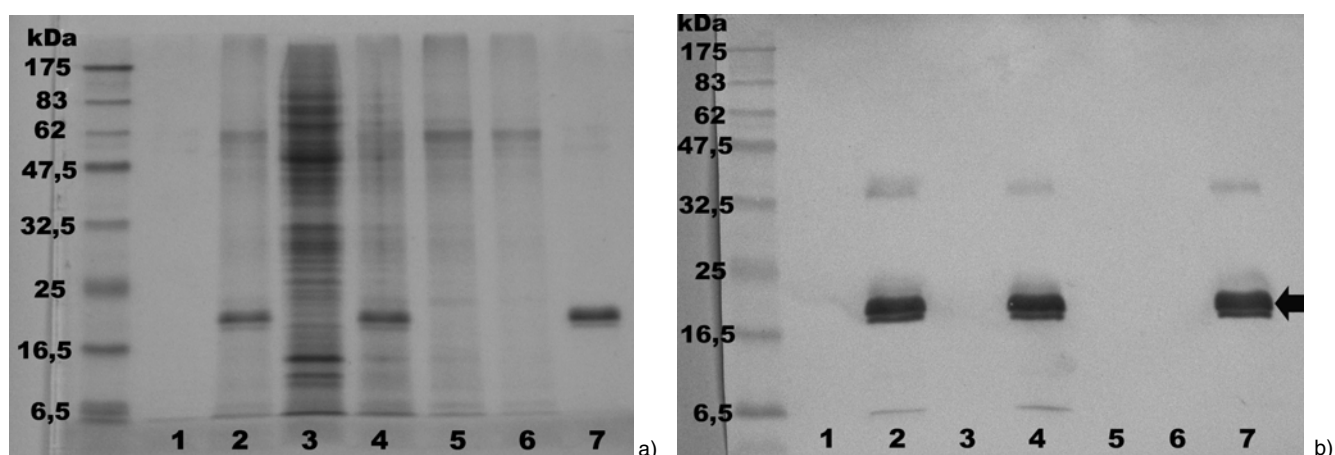


Figure 5. Expression of SAG2 by *L. tarentolae* in SFP(III) medium and purification by metal chelate chromatography. Samples for analysis were 100 times concentrated by TCA precipitation, except the cell homogenate sample. The SDS-PAGE is shown in the left picture, western blot in the right one. An arrow marks the SAG2 band. The following samples were loaded: 1) SFP(III) medium at start of the cultivation; 2) medium supernatant at harvest; 3) cell homogenate and further the samples of the HisTrapTM column: 4) loading; 5) flow through; 6) washing; 7) elution of SAG2.

Discussion

We have demonstrated that *L. tarentolae* can be successfully cultivated in defined media. The precipitation of hemin can be avoided by addition of BSA or by using a PEG-hemin stock solution. PEG-hemin is advantageous over BSA, which may cause contamination as it is derived from bovine source. The precipitation of hemin is rarely described in the literature. Chang *et al.* [8] and Gaughan and Krassner [9] reported that hemin tends to precipitate in the culture media and forms aggregates in aqueous solutions, but they did not recognise this as disturbing. In the context of bioprocessing, these precipitations will disturb measuring of OD, may obstruct the capillary of cell counter devices or may interfere the downstream processing.

The SFP medium is based on the medium C reported by Trager [7]. This publication contains no statements according to growth rates or doubling times, only maximal cell densities of 5×10^7 cells ml⁻¹ are reported. For comparison, we achieved 1.6×10^8 cells ml⁻¹ with the SFP(II) medium, corresponding to a factor of 3.2. Static suspension cultures have been regularly used according to the literature. In contrast, we established agitated cultures, which provide higher oxygen input and allow higher growth rates [4].

Various other defined media were described. Melo *et al.* [27] noted only 3.5×10^7 cells ml⁻¹ for *L. tarentolae* in combination with $\nu = 0.008$ h⁻¹ (own calculation from values). The highest cell densities were reported by Merlen *et al.* [5] with 7.9×10^7 cells ml⁻¹ for *Leishmania* species that are human pathogenic.

The specific cell division rate ν is an additional important factor. In the media SFP(I) and (II), ν was about 0.089 h⁻¹ and was smaller than in complex media e.g. $\nu = 0.103$ h⁻¹ for the YE-medium [4]. The media were suitable for long-term growth and contained probably all essential substances. This was shown for the SFP(I)-medium with 3 passages into new medium.

The obtained specific cell division rates and specific growth rates were extremely high in comparison to literature data. McCarthy-Burke *et al.* [6] reported 0.077 h⁻¹ for *L. donovani* ($N_{\max} = 4 \times 10^7$ cells ml⁻¹) and ν was calculated to about 0.03 h⁻¹ for the medium of O'Daly *et al.* [28].

The supplementation of RNA and its growth supporting effect was very surprising. To our knowledge, RNA as additive was not used before in *Leishmania* cultivation, only the addition of single nucleobases, nucleotides or nucleosides have been reported [5, 7, 27, 28]. The addition of RNA resulted in an increased specific cell division rate ($\nu = 0.103$ h⁻¹), which was stable over a

longer time, and a total cell density of 3.1×10^8 cells ml⁻¹ could be measured. In comparison to the highest cell density reported by Merlen *et al.* [5], the cell density in our culture was 3.9 times higher.

The cell density in the established SFP(III) medium was still lower compared to the cell densities found in complex media, e.g. YE-medium supported the growth of *L. tarentolae* to 1×10^9 cells ml⁻¹ [4]. Further studies have to be done to understand the nutrient requirements of *L. tarentolae*, to find out essential components and to develop strategies for higher cell densities. More investigations are necessary e.g. by the use of methods of the design of experiments (DoE) to develop a medium with optimal concentrations of single substances.

The SFP(III) medium is utilizable for expression of a recombinant protein, as it has been shown for the successful expression and segregation of the SAG2 protein, which was evaluated by SDS-PAGE and by western blotting. Furthermore, successful purification of the SAG2 protein to about 80 lane% (protein purity) could be shown using metal chelate chromatography. Further work on the protein identity according to antigenity and immunogenity are necessary, because at this stage the SAG2 could only be detected by the His₆-tag and not by a specific antibody or anti-serum.

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