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Short Communication

Time-course and degradation rate of membrane scaffold protein (MSP1D1) during recombinant production

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ABSTRACT

Membrane scaffold proteins (MSPs) are synthetic derivatives of apolipoprotein A-I, a major protein component of human high-density lipoprotein complexes. The most common among these is the variant MSP1D1, which has been in the focus of research on membrane mimetics in the past. As such, the amphipathic MSP1D1 has the ability to self-assemble in the presence of synthetic phospholipids into discoidal nanoparticles, so called nanodiscs. The recombinant production of MSP is exclusively reported using a standard laboratory expression system of the pET family. However, strong variations in both yield and achieved concentration as well as complications related to unspecific degradation are commonly reported. In addition, the time-course of recombinant protein as well as specific protein yields have not yet been quantified conclusively. In this study, the time-course of MSP1D1 concentration was investigated in a standard pET expression system in terms of quantification of production and degradation rates in comparison to a reference protein (eGFP).

1. Introduction

Membrane scaffold protein 1D1 (MSP1D1) is a synthetic derivate of apolipoprotein A-I, which is the major protein element of human high-density lipoproteins [1]. The group of Stephen Sligar (University of Illinois, USA) synthesized the gene expressing the protein and provided the required bacterial translation sequences, optimized codon usage and minimized secondary structures of the corresponding mRNA [2,3]. The amphipathic, synthetic protein has the ability to self-assemble in the presence of synthetic phospholipids into discoidal nanoparticles, so called nanodiscs [2,3]. According to that, nanodiscs are self-organizing model membranes, which are soluble and stable in aqueous solutions and preserve the general state of the phospholipid bilayer architecture [4]. Nanodiscs technology has been developed to overcome the limitations of membrane mimicking systems for studying membrane proteins, applications in biotechnology and medicine [5–7]. Applications for nanodiscs are based on the feature of solubilization of a membrane protein in native conformation or use as a model membrane system (applications are reviewed in more detail in [7]). The microbial production of MSP1D1 is exclusively reported using a standard plasmid-based expression system of the pET family (pET28a) under the control of a T7 promoter, which was established during early research on MSP proteins by the group of Stephen Sligar at the University of Illinois, USA [2,3,5,8]. This expression system is well-established for recombinant protein expression in general because of the high yield of recombinant protein which can represent up to 50% of the total cell protein [9,10]. Even though this expression system is a standard for production of MSP on a laboratory scale, strong variations in yield, achieved concentration as well as complications related to unspecific degradation are commonly reported [3,11,8,12]. Additionally, relevant efficiency parameters such as specific production rates as well as specific yields are rarely stated in literature. Existing reports differ strongly from each other, both in terms of values and units, and therefore fail to provide a conclusive view on the efficiency of this expression system for MSP1D1 production.

Reported concentrations all rely on the pET28a plasmid system in *E. coli* BL21(DE3). Membrane scaffold proteins are first published in 2002 by Bayburt et al., and production of these proteins at a level of about 250 mg per liter of culture were reported [3]. In 2011, a yield of purified membrane scaffold protein between 100 and 200 mg/l of culture was published [11]. A few years later, 2013 Inagaki et al. published a concentration of purified MSP1D1 of ~ 6.5 mg/ml of 6.5–7.0 g wet cell pellet per 1 L culture [8]. In the same year researchers of the University of Connecticut, claimed that 0.5 L cells gave an average of 12–13 mg of membrane scaffold protein [12].

In this study, the time-course of MSP1D1 concentration was

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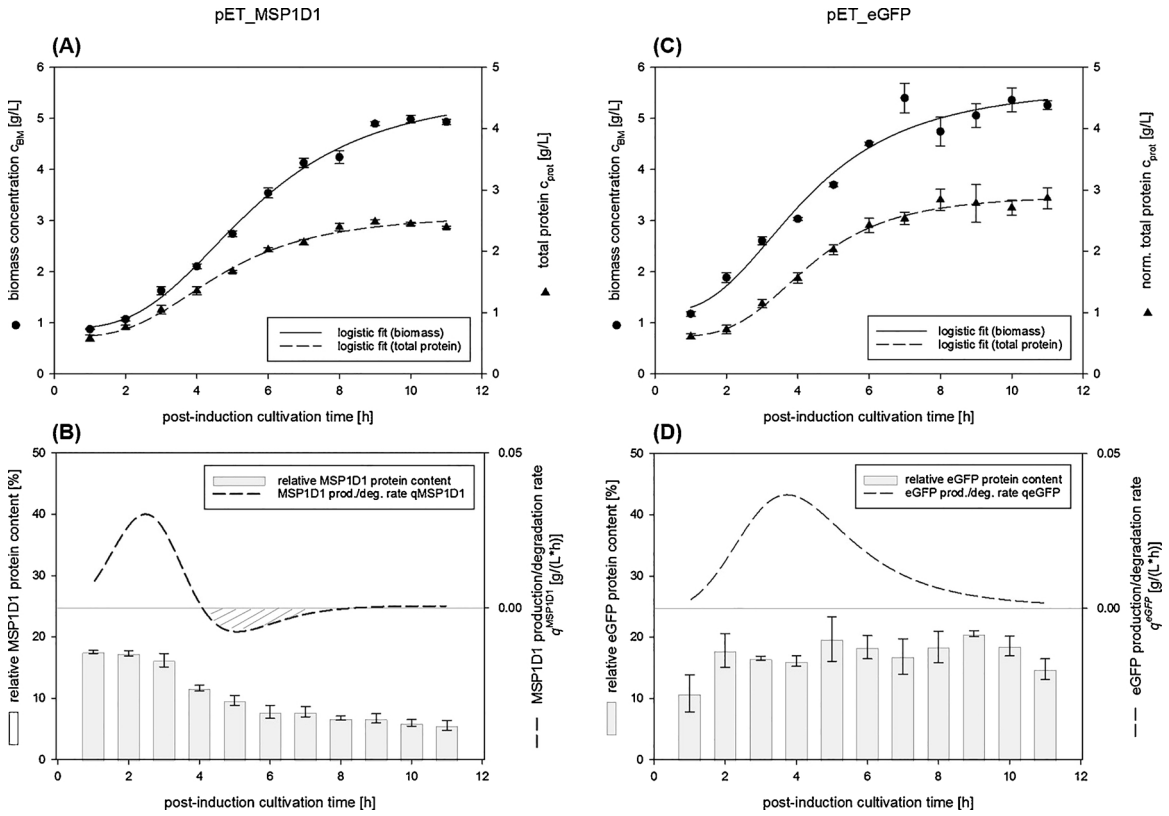

Fig. 1. Time-course post induction of biomass concentration, total protein concentration, relative protein content and protein production/degradation rate for MSP1D1 and eGFP as reference. (A) biomass and total protein for MSP1D1, (B) production/degradation rate and relative MSP1D1 protein content, (C) biomass and total protein for eGFP, (D) production/degradation rate and relative eGFP protein content. Fits for production/degradation rates (B) and (D) are calculated from logistic 4-parameter fits of biomass and protein concentration (A) and (C). Shaded area in B indicates negative values of the absolute production rate, indicating MSP1D1 degradation is higher than production rate leading to the deduction that MSP1D1 is actively degraded and removed from the system.

investigated in a standard pET expression system in comparison to a reference protein. For this purpose, relevant efficiency parameters including specific protein content, specific production rates and observed degradation rate are presented.

2. Material and methods

2.1. Materials

Chemicals used were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Bio-Rad Laboratories, Inc. (Hercules, USA) and Merck KGaA (Darmstadt, Germany). The membrane scaffold protein 1D1, as lyophilized powder, was purchased from Sigma-Aldrich (Taufkirchen, Germany). The expression plasmid pMSP1D1 was a gift from Stephen Sligar, University of Illinois, Urbana, USA (Addgene plasmid # 20061) [2]. The eGFP standard was purified by IMAC with Ni-NTA columns (HisTrap HP, 5 × 5 ml nickel column; GE Healthcare Life Sciences; Chicago, United States of America) using a chromatography system (ÄKTA start chromatography system; GE Healthcare Life Sciences, Chicago, United States of America).

2.2. Methods

2.2.1. Genetic works, bacterial strain and growth conditions

The eGFP gene (obtained from the pJOE4056.2 plasmid [13,14]) was used to replace the gene for MSP1D1 in the pMSP1D1 plasmid (obtained from [2]) under the control of a T7 promoter. All cultivations and expressions were performed in *E. coli* BL21Gold(DE3). For the overnight culture the required amount of TB medium [5] was supplemented with 50 µg/ml kanamycin, and cultivation was performed in baffled shaking flask. Then 50 µl of a glycerol stock of the strain was added. Incubation was performed at 37 °C and 120 rpm overnight in a shaker incubator (Newbrunswick™/Innova® 44, Eppendorf AG, Hamburg, Germany).

2.2.2. Cultivation and expression of MSP1D1 and eGFP

The overnight culture was diluted with TB medium to an OD_{600nm} of 0.1 for starting the cultivation and the particular antibiotic kanamycin with a concentration of 50 µg/ml was added. The cultivation was performed at 37 °C and 120 rpm in an incubator shaker (Newbrunswick™/Innova® 44; Eppendorf AG, Hamburg, Germany). Induction was started between an OD_{600nm} of 1.2–1.3 with 1 mM of sterile filtered isopropyl-β-D-thiogalactopyranosid (IPTG) and the temperature was decreased to 30 °C. The induction was carried out over 11 h.

2.2.3. Sampling, replicates and sample analysis

All experiments were performed in triplicate as biologically independent experiments and data is shown as average values ± standard deviation. Every hour after the induction a sample was taken and analyzed regarding the OD_{600nm} and biomass. The OD_{600nm} was determined using a spectrophotometer (UV-3100 PC; VWR GmbH, Darmstadt, Germany). The cell pellet was harvested via centrifugation at 4 °C and 10,000 × *g* for 10 min (Microcentrifuge 5430R; Eppendorf AG, Hamburg, Germany). Then a chemical cell disruption was carried out for the intracellular located target proteins (BugBuster Master Mix; Merck KGaA, Darmstadt, Germany). For determination of protein concentration a colorimetric Bradford assay [15] was used (Roti® Quant; Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The determination of protein sizes and quantification of proteins was performed using SDS-PAGE with 12% polyacrylamide gels (TGX Stain-Free™ FastCast™ Acrylamide Solutions; Bio-Rad Laboratories, Inc., Hercules, USA) and coomassie staining (Roti®-Blue quick; Carl Roth GmbH & Co. KG;

Karlsruhe, Germany). To account for variations in extraction efficiency and to enable comparison of datasets, total protein concentration was calculated and 2 µg of total protein (as determined by Bradford assay [15]) was added to each lane. The gels were scanned using a gel documentation system (Quantum ST5; Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany). The digitalized images were processed with ImageJ [16] software as described by Májek et al. [17].

3. Results and discussion

The comparison of the time-course of biomass concentration, total protein concentration, relative protein content and protein production/degradation rate of the both proteins, MSP1D1 and eGFP, is shown in Fig. 1A–D. The presented time-course includes data post induction (t = 0) at an optical density (OD_{600nm}) of 1.2, representing a biomass concentration of approximately 0.4 g/L. The time-course of biomass growth, represented by biomass concentration c_{BM} [g/L], is comparable for both strains, with a maximum concentration of 5.06 g/L for the protein MSP1D1 and 5.37 g/L for eGFP, respectively. Similar growth behavior is furthermore supported by comparing maximum specific growth rates μ_{max} [1/h], which show similar values of 0.36 1/h for MSP1D1, and 0.37 1/h for eGFP, respectively (see Table 1). After ~8 h post induction, biomass growth was significantly decreasing during transition to the stationary phase, which consequently resulted in less increase in total protein concentration c_{prot} [g/L] for both of the investigated proteins (Fig. 1A and C). This is furthermore reflected by a decrease in specific protein production rates for MSP1D1 and eGFP (see also following section, Fig. 1B and D).

Furthermore, the maximum specific protein content [$g_{Protein}/g_{BM}$] per biomass yielded values of 0.68 g/g for MSP1D1 and 0.54 g/g for eGFP, respectively. Average values were in a comparable range, with values of 0.58 ± 0.07 g/g for MSP1D1 and 0.51 ± 0.04 g/g for eGFP, respectively. This is in accordance with average total specific protein contents reported in *E. coli,* with values of approximately 0.5 g/g [18]. It should be noted however, that maximum values of specific protein content were achieved at low biomass and protein concentrations. Therefore, these maximum values are more susceptible to measurement errors, which may explain the higher value for MSP1D1 of 0.68 g/g. Upon quantification of recombinant protein content [g/L] of both produced proteins, it was shown that the maximum amount of recombinant MSP1D1 of 0.17 g/L was in fact only one third of the maximum amount of 0.51 g/L for eGFP. This is furthermore supported by average values of 0.15 g/L for MSP1D1 and 0.36 g/L for eGFP. The time-course of the relative content of recombinant protein is shown in Fig. 1B and D. In the first three hours post induction, the relative protein content is comparable at levels of approximately between 15 and 20 %. Thereafter, the relative content of MSP1D1 is dramatically decreased to values below 10 %, with the last monitored value of 5.6 % at 11 h post induction. In comparison, the relative protein content of eGFP consistently stays above 15 % throughout the induction phase (see also Table 1).

The time-courses of production/degradation rates of the target proteins MSP1D1 and eGFP are shown in Fig. 1B and D. Comparable production rates are observed for both proteins in the first 3 h post induction, with maximum values of 0.030 g/h (MSP1D1) and 0.037 g/h (eGFP). In addition, maximum specific production rates per biomass are the same for both proteins with 0.025 g/g*h (Table 1). After reaching its maximum, the production rate rapidly decreases for MSP1D1 and reaches values below zero (shaded area) at approximately 4 h post induction, which indicates degradation of the target protein. However, it should be noted that degradation may be underestimated until 4 h for MSP1D1, because the shown absolute rate represents an overlay of production and degradation (Fig. 1). The production rate of the recombinant formed eGFP shows a different behavior. While the production rate also decreases after its maximum at 4 h, a retained production comparing to MSP1D1 is visible, and no negative values for the production rate are assumed. The mean cumulative production rate of 0.016 g/h for eGFP is four times higher than for MSP1D1 with 0.004 g/h (Table 1), however, it should be noted that for MSP1D1 these values represent an overlay of production and degradation.

To quantify the extent of specific MSP1D1 degradation, values are taken from the interval of degeneration between 4 and 9 h (Table 1). A maximum degradation rate of 0.008 g/h could be observed at approximately 5 h post induction, which is 27 % of the maximum observed production rate. Additionally, an average value of 0.004 g/h was detected, which is similar to observed average cumulative production rates (Table 1). This underlines the strong effect of degradation post induction for recombinant MSP1D1 production. From the data presented in this study, cultivation strategies can be deduced for optimized MSP1D1 production. With the described system, maximum MSP1D1 levels can be obtained by harvesting the cells at 4 h post induction before the degradation dominates the observed rate (Fig. 1). In addition, induction near the end of the exponential growth phase may be an option to increase MSP1D1 yield, which accounts for the early onset of degradation after induction. Furthermore, as a protein of human origin, incorrect or incomplete folding may occur during biosynthesis of MSP1D1 resulting in the observed strong bacterial proteolysis. Consequently, this may potentially be avoided by choosing a different expression system with lower overall expression rate compared to T7 promoter-based systems. One example for this is the rhamnose-inducible *rhaBAD* promotor expression system, which is characterized by a slow expression answer, a low level of basal transcription because the promotor is efficient, tightly regulated and well balanced [10,19].

Table 1
Average and maximum values of growth rate, protein content and relevant efficiency parameters for comparison of the recombinantly produced proteins MSP1D1 and eGFP. n.a. = not applicable.

	pET_MSP1D1		pET_eGFP	
	Maximum	Average value	Maximum	Average value
Specific growth rate µ [1/h]	0.36	0.17	0.37	0.14
Protein content [$g_{Protein}/g_{BM}$]	0.68	0.58 ± 0.07	0.54	0.51 ± 0.04
Recombinant protein content [g/L]	0.17	0.15 ± 0.02	0.51	0.36 ± 0.12
Relative target protein content [%]	17.57	10.28 ± 0.27	20.55	17.14 ± 1.05
Cumulative production rate				
q [g/h]	0.030	0.004	0.037	0.016
q spez. [g/(g*h)]	0.025	0.004	0.025	0.011
Degradation rate during degeneration interval [g/h]	0.008	0.004	n.a.	n.a.

4. Conclusion

The recombinant production of MSP is exclusively reported using a standard laboratory expression system of the pET family. Strong variations in both yield and achieved concentration as well as complications related to unspecific degradation are commonly reported for this system. It was hypothesized that MSP proteins are distinctly susceptible to proteolysis, and persistent post induction growth results in significant decrease of the MSP yield [5]. However, this hypothesis was never experimentally verified, and no conclusive quantitative data and efficiency parameters were available for this system up to now. In this study, eGFP was used as a reference protein in comparison to the time-course of recombinant protein MSP1D1. Calculated efficiency parameters reveal that the maximum overall and specific production rates are similar for both proteins, indicating that degradation is likely the reason for decreased yields during MSP1D1 expression. While virtually no degradation could be observed for eGFP, the degradation rate for

MSP reached values of up to 27 % of its maximum production rate. With its very high degradation rate as shown in this study, the production of MSP may be further applied as a reference system for recombinant production of human protein in the future.

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