Methods 55 (2011) 58–64

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Leishmania cell-free protein expression system

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ARTICLE INFO

Article history:
Available online 16 June 2011

Keywords:
Cell-free protein expression
Leishmania tarentolae
Overlap extension PCR
High throughput protein expression
Rab GTPase

ABSTRACT

Cell-free protein expression is an important tool for a rapid production, engineering and labeling of recombinant proteins. However the complex protocols for preparation of eukaryotic cell-free protein expression systems result in high manufacturing costs and limit their utility. Recently we reported a novel cell-free expression system based on the lysate of a fermentable protozoan *Leishmania tarentolae*. Herein we describe a protocol for high throughput protein expression using *Leishmania* cell-free lysate. The protocol combines PCR-based synthesis and engineering of translation templates with a combined transcription–translation system. The protocol is adapted to multiwell plate format and allows translation of large protein libraries. In the presented example we translate *in vitro* and isolate a nearly complete complement of mammalian Rab GTPases. Further applications and developments of the system are discussed.

1. Introduction

New advances in Life Sciences and Biotechnology rely upon our ability to replicate the building blocks of life *in vitro*, perform their modifications and then use them in medical and biotechnological applications. Much biotechnological progress in the last 40 years has focused upon developing more efficient analysis and synthesis technologies for both DNA and proteins. However while orders of magnitude reduction in costs for DNA sequencing and synthesis has been achieved during the last decade, the throughput and cost of technologies for protein production and engineering have changed comparatively little.

Cell-free protein expression is a rapid and potentially high throughput methodology for the conversion of genetic information into protein-mediated biochemical activities. It holds the promise to cover the technological gap between DNA and protein technologies and provide the platform for broad application of synthetic biology principles in the Life Sciences [1].

Although the first cell-free protein expression systems date back to the discovery of the genetic code, their use has not become as widespread as *in vivo* protein expression. This is largely due to the combination of two factors. Firstly the cheapest, most productive and scalable *Escherichia coli*-based cell-free system typically underperforms in folding of multidomain proteins comparing to eukaryotic systems [2–4]. In the same time eukaryotic cell-free systems such as Wheat Germ Extract (WGE), Rabbit Reticulocyte Lysate (RRL) and Insect Cell Extract (ICE) are much more complex in preparation and correspondingly more expensive. Nevertheless, multiple reports confirm that WGE is suitable for the production of proteins for structural and biochemical analysis including the construction of expressed human proteome [5–8]. Despite these successes, preparation of high quality wheat-based extract requires extensive optimizations [9] and the quality of the lysates available commercially vary widely. To our knowledge all reports of preparative expression in WGE use the lysate produced by Cell Free Sciences (Ehima, Japan) [10].

Although the WGE system has numerous advantages the long and environmentally influenced life cycle of the host, complex and largely manual preparation of the extract and laborious plant genetics present obstacles to the dissemination and development of this system. In an effort to develop a cell-free system based on a eukaryotic host amenable to rapid genetic modification, rapid and scalable cultivation, and straightforward lysate preparation, we recently focused our attention on a unicellular flagellate *Leishmania tarentolae* [11,12]. This organism is amenable to large scale fermentation, can be easily genetically modified and provides a source of translational active lysate that was used to express dozens of proto- and metazoan proteins [13]. In the course of method development we devised a strategy for suppression of endogenous mRNA with antisense DNA oligonucleotide targeting the splice leader sequences presenting in all *Leishmania* mRNAs. Further we developed a series of novel translation initiation assemblies based on polymeric RNA sequences that initiate protein production in all cell-free systems tested (i.e., not only in *Leishmania* based system). We termed these leaders Species Independent Translation initiation Sequences (SITS) and used them to express proteins in *L. tarentolae* extract [12]. In this article we provide a detailed description for preparation of *L. tarentolae* extract (LTE) and its use for protein expression and purification, using both PCR gener-

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1046-2023/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2011.06.006

ated as well as plasmid-based templates. We demonstrate expression of a nearly full compliment of human Rab GTPases and a heteromeric GTPase modifying enzyme farnesyl transferase. Finally, we discuss the future directions of LTE developments in relation to other cell-free systems.

2. Materials and methods

2.1. Preparation of supplemented lysate of L. tarentolae

Proteins were produced via a cell-free expression system based on lysate derived from the eukaryotic parasite *L. tarentolae*. Cultures (2 × 50 ml in tissue culture flasks, 74 rpm inclined agitation, 26.5 °C) were maintained in exponential growth (doubling time 6–7 h) in the concentration range 0.1–1.5 × 10^8 cells/ml, via suitable dilutions every 2–3 days. Cells were diluted 1 in 100 in PBS and counted using a haemocytometer. For lysate production, maintenance cultures were expanded successively over two nights in baffled 5 l flasks (26.5 °C, 74 rpm agitation) in 1 l of TB medium (Tryptone 12 g/l, Yeast extract 24 g/l, Glycerol 8 ml/l, Glucose 1 g/l, KH_2PO_4 2.31 g/l, K_2HPO_4 2.54 g/l). The typical expansion of maintenance culture was (2 × 50 ml; day 0) to (1 × 1 l; day 1), and finally to (10 × 1 l; day 2, lysate production), with actual dilutions adjusted to keep all cultures within the cell concentration range 0.1–1.5 × 10^8 cells/ml based on routine cell counting. The final culture step (typically 10 × 1 l) was harvested at 1.0–1.3 × 10^8 cells/ml. Harvested cells were pelleted at 2500*g* and resuspended in Sucrose Elution Buffer (SEB; 45 mM Hepes-KOH pH 7.6, 250 mM Sucrose, 100 mM KOAc, 3 mM $Mg(OAc)_2$) to 1.0 × 10^{10} cells/ml. Typical cell pellet was loose, and required careful decantation to avoid cell loss. The cells were washed twice in SEB and then disrupted using a nitrogen cavitation device (70 bar N_2, 45 min equilibration at 4 °C). The lysate was subjected to sequential 10,000*g* and 30,000*g* centrifugation, with approximately 2/3 of the supernatant carefully recovered each time via pipette. The debris pellets were generally firm for both 10,000*g* and 30,000*g* spin, but in some cases a small unclarified zone appeared over the pellet during the 30,000*g* spin. It was necessary to avoid this unclarified zone during supernatant withdrawal for good lysate activity. Sucrose was removed from the 30,000*g* supernatant by gel filtration on PD-10 Superdex 25 column (GE Healthcare) into fresh elution buffer (EB; 45 mM Hepes-KOH pH 7.6, 100 mM KOAc, 3 mM $Mg(OAc)_2$). The lysate was then supplemented with a feeding solution containing 5× the final transcription/translation reaction concentrations of necessary cofactors and enzymes. (6 mM ATP, 0.68 mM GTP, 22.5 mM $Mg(OAc)_2$, 1.25 mM spermidine, 10 mM DTT, 200 mM creatine phosphate, 100 mM Hepes-KOH pH 7.6, 5% (v/v) PEG 3000, 5.25× protease inhibitor (Complete™ EDTA-free, Roche), 0.68 mM of each amino acid, 2.5 mM rNTP mix (ATP, GTP, UTP and CTP), 0.05 mM anti-splice leader DNA oligonucleotide (CAATAAAGTACAGAAACTGATACTTATATAGCGTT), 0.5 mg/ml T7 RNA polymerase, 200 U/ml creatine phosphokinase). Feed solution supplementation prior to lysate freezing was 40 μl feeding solution added per 100 μl gel filtered lysate. To give the correct final reaction concentration of additives an additional dilution of 60 μl volume per 200 μl final reaction was required after lysate thawing for reactions, with the extra volume enabling final additions (typically DNA template plus milliQ water). The supplemented lysate was aliquoted, snap-frozen in liquid nitrogen and stored at −80 °C. Optical density of gel filtered lysate (prior to supplementation) measured at 260 nm was in a range between 120–230 optical units.

Magnesium concentration affected lysate activity, although the optimum was fairly broad as determined with GFP reporter. Hence, magnesium optimization did not appear necessary for routine lysate preparation in the LTE system. Typical optimum (final reaction) magnesium concentration was 5–7 mM, as measured using unsupplemented lysate that was supplemented at the point of reaction with feeding solutions prepared as above but with altered magnesium content. Taken from a typical lysate (2 h batch, GFP reporter, highest activity = 1.0), activities were (3 mM $Mg(OAc)_2$; 0.40, 4 mM; 0.81, 5 mM; 1.0, 6 mM; 1.0, 7 mM; 0.95, 8 mM; 0.86). For the typical final reaction mix as suggested above (after lysate thawing and final dilution), 1.5 mM $Mg(OAc)_2$ is contributed by the gel filtered lysate itself (via the EB buffer), with an additional 4.5 mM $Mg(OAc)_2$ from the feeding solution, to give 6 mM total. Fine tuning of the reaction magnesium concentration is possible by altering the $Mg(OAc)_2$ concentration in the 5× feeding solution (as above), or by adding extra $Mg(OAc)_2$ in the final reaction make up. The summary of compositions of stock solutions is provided in the Table 1.

2.2. Template preparation by overlap extension PCR

Overlap extension PCR (OE-PCR) was used to assemble templates for *in vitro* translation. The library of templates coding for human Rab proteins was assembled combining one universal SITS-containing fragment with 58 Rab encoding variable fragments. The universal fragment containing T7 promoter and a coupled SITS was synthesised in 200 μl of reaction mixture consisting of Phusion polymerase HF buffer (NEB), 0.2 mM each of four dNTPs, 0.5 ng/μl of template plasmid 1751 [13], 500 nM of primers 511 (CTACAACACGACCCTCTCCG) and 9152 (CGAGATCAGCTTCTGCT

Table 1
Composition of stock solutions used for assembling *L. tarentolae* based coupled transcription–translation reaction.

Component	Unsupplemented lysate	5× Feed solution	Supplemented lysate	Final reaction mixture
Gel filtered lysate	100% v/v	n/a	71% v/v	50% v/v
ATP	n/a	6 mM	1.71 mM	1.2 mM
GTP	n/a	0.68 mM	0.19 mM	0.13 mM
rNTP mix (ATP, GTP, CTP, UTP)	n/a	2.5 mM each	0.71 mM each	0.5 mM each
Spermidine	n/a	1.25 mM	0.36 mM	0.25 mM
DTT	n/a	10 mM	2.86 mM	2 mM
Creatine phosphokinase	n/a	200 U/ml	57 U/ml	40 U/ml
Creatine phosphate	n/a	200 mM	57.1 mM	40 mM
Mg(OAc)2	3mM	22.5 mM	8.57 mM	6 mM
HepesKOH pH7.6	45 mM	100 mM	60.7 mM	42.5 mM
KOAc	100 mM	n/a	71.4 mM	50 mM
PEG3000	n/a	5% v/v	1.43% v/v	1% v/v
Antisense oligonucleotide	n/a	50 μM	14.3 μM	10 μM
Protease inhibitor	n/a	5.25×	1.50×	1.05×
AA mix	n/a	0.68 mM each	0.19 mM each	0.14 mM each
T7 RNA polymerase	n/a	0.5 mg/ml	0.14 mg/ml	0.1 mg/ml

CCATGGTTTCACTTACGTGT), each and 20 U/ml of Phusion polymerase (NEB). Variable fragments coding for GFP fusions with Rab GTPases were synthesised in reaction mixtures of 15 μl formulated as above except forward 3510 (ATGGAGCAGAAGCTGATCTCGGAGGA GGACCTGGTGAGCAAGGGCGAGGAGC) and reverse 8581 (TGTGAAATTTGTGATGCTATTGC) primers were added at 300 nM concentration each and GFP-Rab GTPase plasmids were used as a template. The PCR reaction mixtures for both universal and variable fragments were subjected to thermal cycling as follows: initial denaturation 2 min at 98 °C, followed by 35 repeats of denaturation for 10 s at 98 °C, annealing for 30 s at 50 °C and elongation at 72 °C for 30 s for each 1 kb of the synthesised product. The final elongation step was performed for 5 min at 72 °C. The T7–SITS PCR product was than resolved on a 1% TAE agarose gel containing 0.01% (v/v) of SYBR™ safe dye and visualized on Safe Imager™ (Invitrogen). The DNA band corresponding to the universal fragment was excised and isolated with an SV Wizard PCR purification kit (Promega). In order to streamline the procedure the Rab coding fragments were not gel purified but instead subjected to Exonuclease I degradation of single strand oligonucleotides. 0.2 U of the Exonuclease I (NEB) was added to the PCR reaction mixture and DNA hydrolysis was carried out for 40 min at 37 °C followed by nuclease inactivation at 85 °C for 20 min. Overlap extension reaction was set up in 15 μl and contained 5 nM of gel-purified universal fragment, 1/15 (v/v) of nuclease treated crude PCR mixture of variable fragments, 0.2 mM of each of dNTPs, 20 U/ml of Taq polymerase (Fisher Biotech, Australia) in 10 mM Tris–HCl, 50 mM KOAc, 0.1% (v/v) Triton X100 and 1.5 mM $MgCl_2$, pH 8.6 at 20 °C. OE-PCR was performed under the following regime: 3 min at 95 °C, 8 cycles of 30 s denaturation at 95 °C, 3 min annealing at 45 °C and 2 min elongation per each 1 kb of the amplified sequence at 74 °C, followed by elongation for 5 min at 74 °C. The full-length OE-PCR DNA product was further amplified using flanking primers 511 and 8581. For this step, 45 μl of master mixture containing 500 nM of each of flanking primers and all other components of the reaction as described above (except template DNA) was added to each 15 μl of OE PCR mixture. The reaction was subjected to thermal cycling with 2 min initial denaturation at 94 °C, 25 repeats of denaturation at 94 °C for 30 s, annealing for 30 s at 45 °C and 1 min elongation per each of 1 kb at 74 °C. 5 min elongation step was applied after the final cycle.

For PCR synthesis of templates coding for N-terminally tagged subunits of mammalian FTase we used the above described approach. Briefly, the sequences of FTase α subunit (accession number: NM_012847.1) N-terminally truncated by 29 amino acids and β subunit (accession number: NM_172034.2) were amplified from plasmid vectors using primers 9339 (TCCGGTTCGGGCT CCGGTGGACCAGCACAGCAGCCGCAGGAA), 2483 (CCAGAAGTCAG ATGCTCAAGG) and 8751 (TCCGGTTCGGGCTCCGGTGGAGC TTCTTCGAGTTCCTTCACC), 2483 respectively. The PCR product coding for SITS–EGFP sequence under control of T7 promoter was amplified from plasmid 1751 using primers 8593 (CTACAACAC-GACCCTCTCC) and 8590 (ATGGAGCAGAAGCTGATCTCGG AGGAGGACCTGATGGAACACAACGGATCAGC). All PCR fragments were gel-purified and full-length templates were synthesized by OE-PCR.

2.3. Cell-free protein translation

To avoid pipetting of multiple components of cell-free translation system in each experiment, we formulated a lysate mixture containing all chemicals and enzymes necessary for optimal protein translation as described in section 2.2. In this way the transcription–translation reactions set-up only requires mixing of the desired DNA template with the supplemented lysate followed by adjustment with water to the final volume. The typical reaction mixture of 200 μl contained 140 μl (70% v/v) of supplemented lysate, 20 μl (10% v/v) of crude PCR mixture or 20 nM of purified DNA as a template and was adjusted with mQ water to its final volume. The reactions were allowed to proceed for 2 h at 27 °C with no shaking. For analytical scale translation of GFP-tagged encoding genes, 20 μl of the reaction mixtures were transferred into 384-well white plates and GFP fluorescence was recorded every 5 min using 480 nm (BP20) excitation and 540 nm (BP40) emission filters in a Synergy 4 plate reader (BioTek).

2.4. Isolation and analysis of in vitro synthesized proteins

For affinity purification, GFP-tagged proteins were expressed in 200 or 1000 μl reaction volumes in standard 1.5 ml plastic tube. Upon completion of the reaction, the mixtures were supplemented with Triton X100 to 0.2% (v/v), NaCl was adjusted to 150 mM and 20 μl or 50 μl of 50% slurry of GFP-Cap affinity matrix (Jena Bioscience, Germany) was added to 200 μl and 1000 μl reactions respectively. After 20 min of intensive agitation at ambient temperature the beads were separated from cellular lysate by centrifugation at 2000*g* for 5 min. The bead containing pellet was washed 5 times with 300 μl of 20 mM Tris–HCl pH 7.6 buffered solution of 500 mM NaCl. To elute captured proteins, the washed beads were either incubated with 20 μl of SDS loading buffer for 30 s at 98 °C (for 200 μl reaction mixture) or with 45 μl of 0.1 M Gly–HCl pH 2.2 for 30 s at ambient temperature (for 1000 μl reaction mixture). In the latter case the eluate was neutralized with 4.5 μl of 1 M Tris base. Eluted proteins were resolved on SDS–PAGE gel and visualized by Coomassie staining.

2.5. In vitro synthesis and activity analysis of mammalian FTase

N-terminally GFP-tagged α and β subunits of mammalian FTase were co-translated in 70 μl of LTE translation mixture primed with desalted templates at 10 nM final concentration of each template. Progress of the translation reaction was monitored by following GFP fluorescence intensity as described above. The synthesized proteins were isolated from the reaction mixture by adding 20 μl 50% slurry of GFP-Cap beads. The beads were washed three times with 300 μl of prenylation buffer (25 mM Hepes-KOH pH 8.0, 100 mM NaCl, 2 mM DTT, 2 mM $MgCl_2$, 2 μM $ZnCl_2$) and resuspended in 40 μl of prenylation buffer. Prenylation activity retained on the beads was measured by SDS–PAGE-based prenyltransferase assay [14] that monitors incorporation of the fluorescent farnesyl pyrophosphate (FPP) analog NBD-geranyl pyrophosphate (NBD-GPP) into the protein substrate. The resulting fluorescent protein is resolved on an SDS–PAGE gel and visualized by fluorescent scanning. The prenylation reactions were performed by mixing 20 μl of washed FTase-coated beads with NBD-GPP and mCherry-KRas as a protein substrate at 25 μM and 5 μM concentrations respectively. To confirm the specificity of NBD-labeled lipid incorporation into mCherry-KRas, the control reactions were supplemented with 125 μM of unlabeled FPP. FPP acts as a competitor of NBD-GPP and prevents incorporation of the fluorescent analog into the protein substrate. The prenylation reaction was allowed to develop at 25 °C for 15 min and was terminated by the addition of 20 μl of hot 2× SDS–PAGE sample buffer. The sample was resolved on 4–12% PAGE gel and fluorescent bands corresponding to the prenylated protein were visualized by fluorescent scanning using a Typhoon Trio (GE Healthcare) scanner at 488 nm excitation and 520 nm emission collection. To confirm the integrity and the apparent molecular weight of translated mammalian FTase, GFP-tagged alpha subunit, beta subunit or both subunits were translated in 50 μl of LTE system, isolated on GFP-Cap beads and analyzed on PAGE gel as described above.

3. Results and discussion

3.1. PCR-based synthesis of translation templates

As a test set we chose a nearly full compliment of mouse Rab GTPases. Rab proteins comprise the largest group in the Ras GTPase superfamily and play key roles in the regulation of vesicular transport in eukaryotic cells [15]. Despite their structural similarity, Rab proteins share as little as 30% sequence identity with each other [16]. Conservation of protein fold and basic biochemical activity but high divergence of the sequences of 60 mammalian Rabs make this family a convenient test set for the analysis of expression and folding in various expression systems. We used OE-PCR to assemble a library of Rab encoding templates fused to a poly(A) SITS as a translation initiator. To achieve this we combined a universal DNA fragment (coding for the SITS leader under control of the T7 polymerase promoter) with 58 fragments coding for different GFP-tagged Rab encoding genes (Fig. 1A). The fragments for OE-PCR were obtained by PCR and required gel purification in order to remove remaining primers prior to the overlap extension step, significantly limiting the throughput of the approach [17]. In order to develop a more rapid protocol, we replaced the gel-purification with a treatment by single strand specific DNA exonuclease to hydrolyze the remaining primers. The universal fragment was gel purified as it did not limit the method's throughput and improved the yield of OE-PCR reaction (Fig. 1B). Prepared DNA fragments were subjected to OE-PCR resulting in 55 full-length products synthesised with sufficient yields (Fig. 1C). Out of the initially chosen set of 58 Rab GTPases, construction of one gene (Rab19) failed during first PCR and two genes (Rab9a and Rab12) on the stage of OE-PCR.

3.2. Parallelized protein translation in L. tarentolae lysate

To produce Rab GTPase in the LTE system we used linear templates generated by OE-PCR in a form of crude PCR mixture. Despite the fact that the efficiency of OE-PCR varies for different templates, addition of 10% v/v of typical PCR reaction results in 10 nM final concentration of the template, which is close to the saturating template concentration [13]. Addition of more then 10% v/v of crude PCR mixture inhibits translation reaction and therefore desalting and concentration of PCR template is needed to achieve maximal productivity in case of inefficient OE-PCR reactions. Productivity of the coupled transcription–translation reaction as measured with the fluorescence of GFP reporter was found to be efficient between 20 °C and 28 °C with the optimum at 27 °C. For parallelized translation of GFP-RabGTPases fusions, cell-free translation reaction was primed with crude PCR mixture and incubated at 27 °C for 2 h, which resulted in protein yield between 10 and 30 μg/ml for different Rabs (Fig. 2).

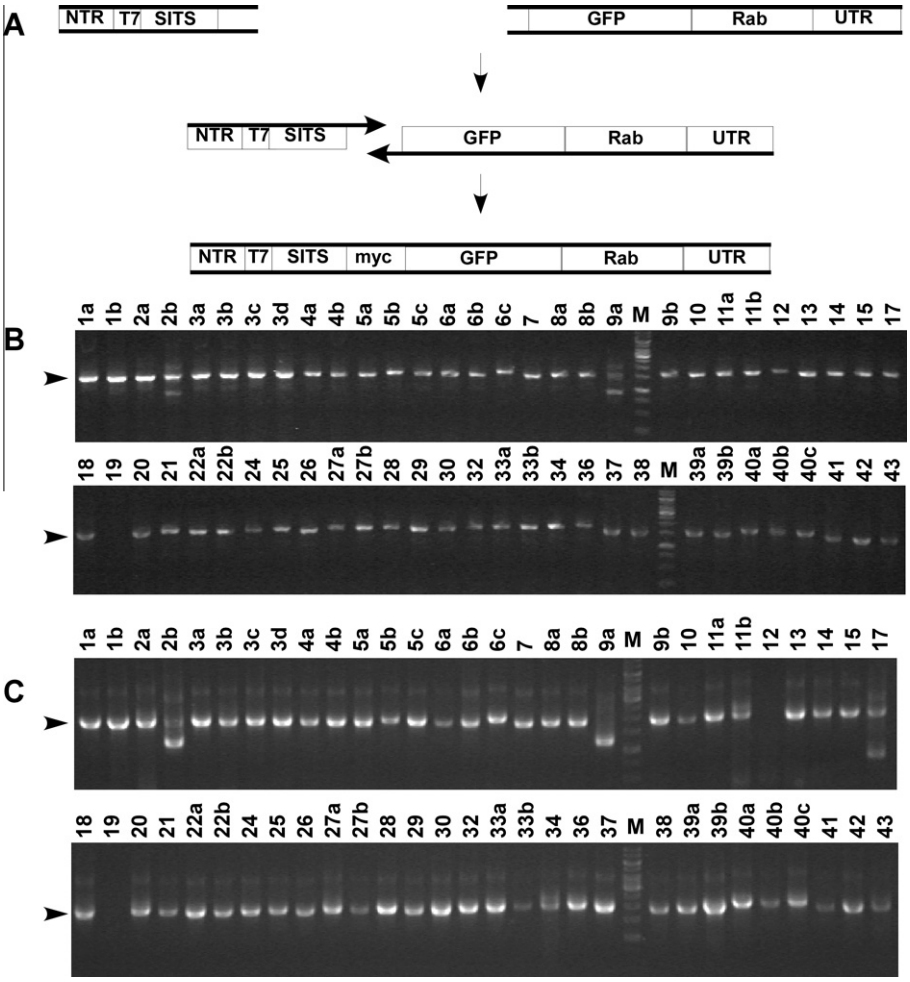

Fig. 1. Synthesis of Rab GTPase encoding templates by overlap extension PCR. (A) Schematic representation of OE-PCR mediated assembly of Rab-coding templates. NTR – non transcribed region, T7 – T7 promoter, UTR – untranslated region. (B) PCR fragments coding for GFP-Rab fusions resolved on agarose gel. Lanes are designated correspondingly to Rab protein number. M – 1 kb DNA ladder (Gene Ruler, Fermentas). Arrowheads indicate the expected position of specific products. (C) Products of OE-PCR reaction resolved on agarose gel and annotated as in B.

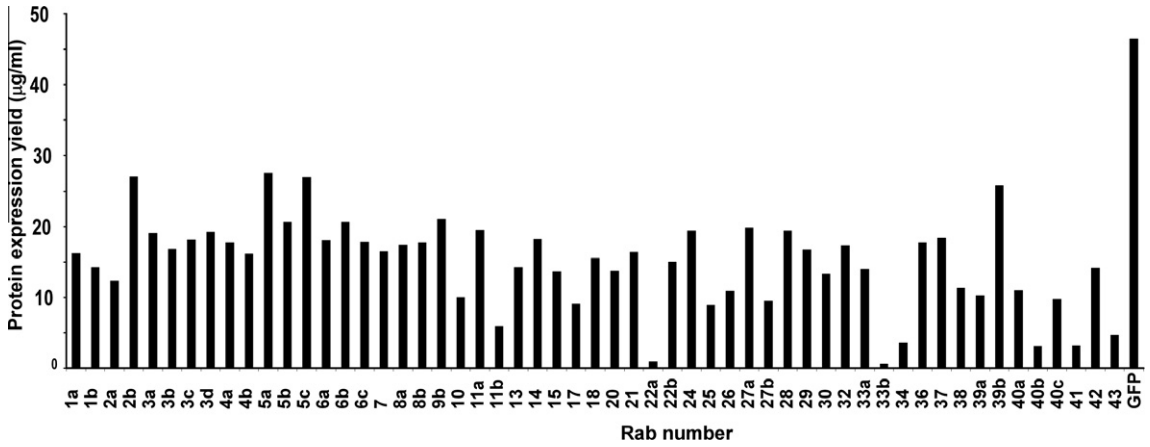

Fig. 2. Expression yield of GFP-tagged Rab proteins expressed in LTE. Translation reactions were programmed with crude PCR mixtures. The linearized plasmid coding for SITS–EGFP assembly was used as a positive control (GFP). The reactions were allowed to progress for 3 h at 26 °C after which the 20 μl aliquots were measured in a fluorescence plate reader. The yield of Rab expression was estimated based on GFP fluorescence after background subtraction.

3.3. Analysis of GFP-tagged Rab protein library

Addition of GFP domain to the N-terminus of Rab proteins enables real time monitoring of translation reaction progress that is reflected by GFP fluorescence. Total yields of GFP-tagged Rabs calculated on the fluorescence of the GFP domain are given in Fig. 2. The GFP-domain was also used as an affinity tag to isolate the proteins on the GFP-affinity matrix known as GFP-Cap. The isolated proteins were resolved on SDS–PAGE and analyzed by Coomassie staining. As can be seen in Fig. 3 all 55 full-length templates could be isolated albeit with different yields. As judged by Coomassie-stained SDS–PAGE gels the amounts of the isolated proteins were in a good agreement with yields estimated from total fluorescence. This indicates that most of the proteins were present in solution in non-aggregated form and could efficiently bind to the affinity resin. Interestingly, in several cases the product presented as a stack of closely migrating bands. We attribute this fact to either electrophoresis artefact or the presence of closely migrating background bands as anti-myc western blot analysis shows single specific bands for all Rabs with the exception of Rab15 (data not shown). Double bands of Rab15 is not a unique feature of *Leishmania* translation system since the same pattern was observed for the protein translated in Wheat Germ Extract (data not shown). The aberrant 30 kDa low molecular weight product observed in case of GFP-Rab2b likely represents a spuriously amplified and translated SITS–GFP assembly (Fig. 1C).

3.4. Medium scale protein expression and isolation

Changes in the volume of *in vitro* translation reactions from μl to ml scale were reported to lead to significant decrease of the productivity of some cell-free systems [18]. We analyzed productivity of the LTE system upon increase of the reaction volume from 20 μl to 1 ml. Although priming the translation reaction with crude PCR

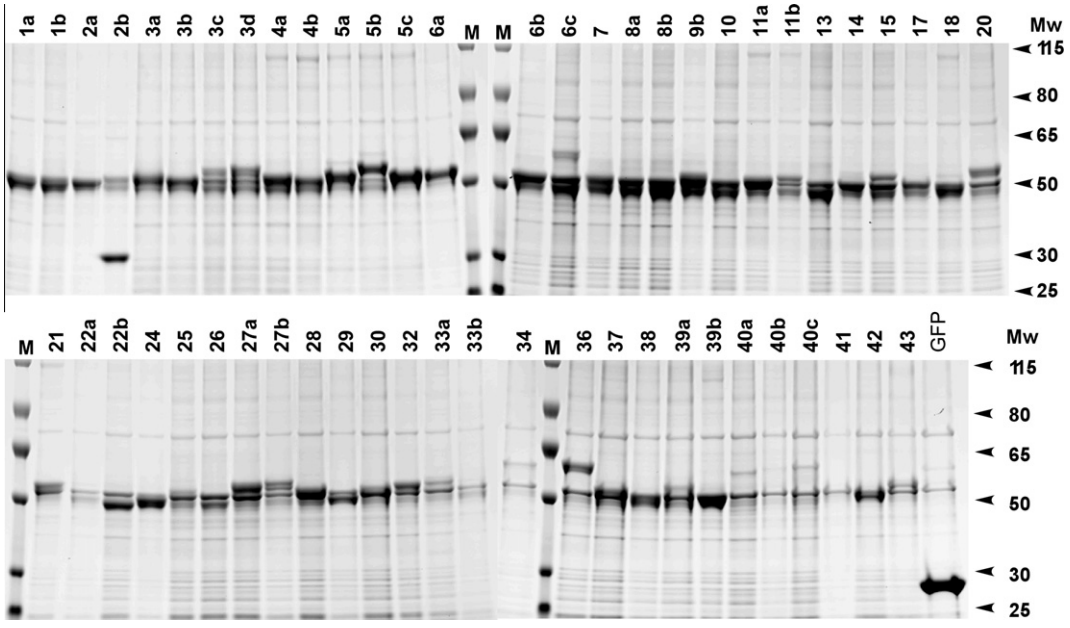

Fig. 3. SDS–PAGE analysis of GFP-tagged Rab proteins isolated from LTE reaction mixtures. GFP-tagged Rab proteins synthesised in 200 μl reaction mixture were isolated on GFP-Cap beads, resolved on 4–12% SDS–PAGE (Invitrogen) and stained with Coomassie stain. The lanes are numbered according to Rab number, GFP – reaction primed with GFP-coding plasmid template, M – molecular weight ladder (Page Ruler Plus Prestained, Fermentas).

mixture is ideal for parallelized production of small amounts of multiple proteins, the yield is lower than in reactions primed with circular DNA templates. Further time and cost associated with production of large amounts of PCR products reduces the benefits of PCR-based template preparation for medium and large scale protein expression. Therefore, we inserted selected Rab templates into the pUC vector and amplified them in *E. coli*. Three batch-mode LTE reactions containing 20 μl, 100 μl or 1000 μl of reaction mixture were primed with plasmids coding either GFP-Rab15 or GFP-Rab20 genes. The reactions were transferred into standard 1.5 ml plastic tubes, allowed to progress for 2 h without shaking and the final yield of GFP tagged product at the three reaction scales was measured. As judged by florescence yields all three reactions displayed similar expression yield suggesting that the system scaled linearly (data not shown). We chose 1 ml batch reaction scale to produce GFP-Rab15, GFP-Rab20 and GFP for medium-scale protein preparation. The GFP-tagged products were captured on a solid support using the GFP-Cap matrix as described above. The bound proteins were eluted by low pH buffer. The proteins were isolated in soluble form with 95% purity according to densitometry of Coomassie-stained PAGE (Fig. 4). The amounts of isolated proteins as measured by UV absorbance at 280 nm were 69 μg for GFP-Rab15, 87 μg for GFP-Rab20 and 54 μg for GFP.

3.5. In vitro synthesis of active farnesyl-transferase heterodimer

In the next step we wanted to test whether the systems was suitable for expression of active multisubunit enzymes. As a test example we chose a heterodimeric farnesyl transferase (FTase) that catalyses covalent attachment of farnesyl isoprenoid to the cysteine in CaaX motif at the carboxyl terminus of a target protein [19]. Both FTase subunits were N-terminally tagged with EGFP as described above for RabGTPases using OE-PCR and co-expressed in LTE translation system. The GFP-tagged products then were separated from the translation mixture using GFP-Cap matrix and the transferase activity was assayed using its ability to incorporate NBD-labeled isoprenoid into the protein substrate mCherry-KRas following the previously described assay [20]. The isolated enzyme was active as demonstrated by the emergence of fluorescent NBD-geranyl modified mCherry-KRas directly in the gel (Fig. 5A). Covalent attachment of fluorescent isoprenoid was specific as the addition of non-labeled competitor efficiently abolished fluorescent labeling of kRas (Fig. 5A). In addition, the molecular weight and integrity of *in vitro* translated components of FTase were confirmed by SDS–PAGE analysis (Fig. 5B).

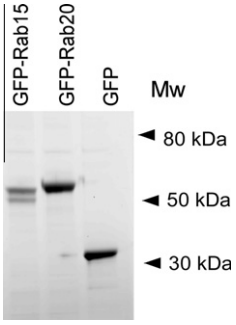

Fig. 4. PAGE analysis of the protein isolated from medium scale of LTE reaction mixture. Protein synthesis was programmed with circular DNA templates encoding GFP-Rab15, GFP-Rab20 and GFP alone. Upon reaction completion, the proteins were captured on GFP-Cap beads and eluted with low pH buffer. After pH neutralization, 5% of total volume of the eluates were resolved on 4–12% SDS–PAGE and stained by Coomassie blue.

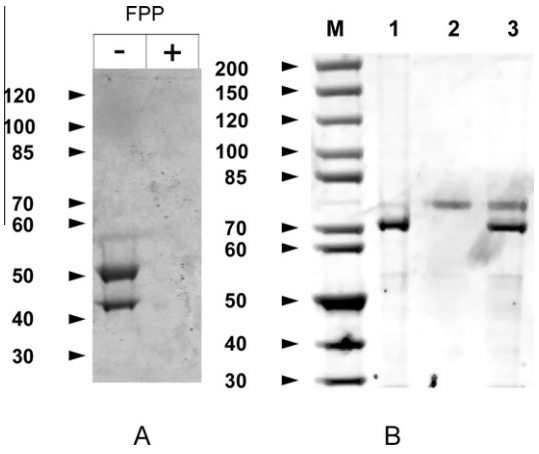

Fig. 5. *In vitro* synthesis and activity of mammalian farnesyl transferase. (A) SDS–PAGE-based fluorescent prenyltransferase activity assay. FTase was isolated from *in vitro* translation mixture and assayed for the ability to incorporate NBD-GPP into its cognate substrate mCherry-kRas. The prenylation reaction contained either NBD-GPP (lane FPP -) or NBD-GPP with 5-fold molar excess of nonfluorescent phosphoisoprenoid (lane FPP +). The products of prenylation reaction were resolved on 4–12% PAGE gel and visualized by fluorescent scanning. The lower band represents typical breakdown product of mCherry protein in the SDS–PAGE gel. (B) SDS–PAGE analysis of FTase α/β heterodimer synthesised in LTE system. Lane 1 – EGFP–FTase α, lane 2 – EGFP–FTase β, Lane 3 – co-expression of both templates simultaneously. M denotes molecular weight marker ladder. The translated products were isolated from the reaction mixture on GFP-Cap matrix and resolved on 4–12% PAGE gel followed by subsequent Coomassie staining.

4. Conclusions

The collection of cell-free protein expression systems has recently been expanded with the system based on a protozoan organism *L. tarentolae* [12]. While this system is in its infancy it has a number of potential advantages over other eukaryotic cell-free systems. Unlike all other sources of translational active eukaryotic lysates, *L. tarentolae* is a rapidly growing protozoan host that can be cultivated on a large scale. Since the translation of the endogenous mRNAs can be readily suppressed with anti-splice leader oligonucleotide, preparation of the extract is rapid and scalable. Here we describe a protocol for parallelized protein expression which combines PCR-based template synthesis protocol with one-tube transcription–translation reaction. Use of GFP as reporter and affinity tag allows direct monitoring of the expression yields and rapid purification of the resulting polypeptides. We expanded this approach to the expression of heterodimeric farnesyl transferase and demonstrated that the enzyme was catalytically active. The fact that all manipulation of the coding sequences could be carried out without cloning and that microgram amounts of >50 pure proteins were obtained indicates that the system is suitable for high throughput construction of expressed proteomes, construction and analysis of protein variants and production of multisubunit enzymes. The next most important step in ensuring dissemination of the system is improvement of the expression yields and the development of large scale lysate production protocols. The latter is particularly important prerequisite for the successful use of the system in structural biology applications.

Acknowledgments

This work was supported in part by grant ARC grant KA003363 to K.A. O.K was supported by a fellowship of the University of Queensland.

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