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Expression of human c-reactive protein in different systems and its purification from *Leishmania tarentolae*

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A B S T R A C T

With its homo-pentameric structure and calcium-dependent specificity for phosphocholine (PCh), human c-reactive protein (CRP) is produced by the liver and secreted in elevated quantities in response to inflammation. CRP is widely accepted as a cardiac marker, e.g. in point-of-care diagnostics, however, its heterologous expression has proven difficult. Here, we demonstrate the expression of CRP in different *Escherichia coli* strains as well as by *in vitro* transcription/translation. Although expression in these systems was straightforward, most of the protein that accumulated was insoluble. We therefore expanded our study to include the expression of CRP in two eukaryotic hosts, namely the yeast *Kluyveromyces lactis* and the protozoon *Leishmania tarentolae*. Both expression systems are optimized for secretion of recombinant proteins and here allowed successful expression of soluble CRP. We also demonstrate the purification of recombinant CRP from *Leishmania* growth medium; the purification of protein expressed from *K. lactis* was not successful. Functional and intact CRP pentamer is known to interact with PCh in Ca^{2+}-dependent manner. In this report we verify the binding specificity of recombinant CRP from *L. tarentolae* (2 μg/mL culture medium) for PCh.

Introduction

C-reactive protein (CRP)[1] is a ubiquitous plasma protein found in both vertebrates and invertebrates. CRP was originally discovered by Tillet and Francis [1] and further studied by Abernethy and Avery [2]. In response to acute inflammation the liver secrets increased amounts of CRP into serum and CRP is involved in functions associated with host defence [3,4]. CRP levels below 10 mg/L are considered normal [5]. In disease states, for example upon acute inflammation after bacterial infection, CRP levels increase to a maximum of 400 mg/L within 48 h [6]. Structurally, CRP is composed of five identical discoid, planar and non-covalently arranged ~23-kDa subunits [7,8] each forming a Ca^{2+}-dependent phosphocholine (PCh)-binding site to interact with PCh which is present on the surface of *Streptococcus pneumoniae* cells, for example [9]. This model was verified by site-directed mutagenesis, demonstrating that an F66A mutant of CRP does not bind PCh [10].

CRP was proposed as a good bio-marker for chronic and acute inflammation [11,12]. It has been purified from human sera (e.g. malignant ascites or pleural fluids) by affinity chromatography on PCh-Sepharose, followed by DEAE-cellulose ion exchange chromatography [13] and calcium-dependent affinity chromatography, then followed by calcium-dependent gel filtration [14], barium sulfate and preparative agarose electrophoresis [15], or immunoaffinity chromatography using mouse anti-CRP monoclonal antibody followed by ion exchange on DEAE-sephacel [16]. Using clinical samples, CRP has been purified in levels ranging from 22 to 342 μg/mL [17].

Several heterologous protein expression systems have been developed over the years including *Escherichia coli* [18], yeasts [19], insect cells [20] and mammalian cells [21]. Additionally, the *in vitro* expression of proteins is now feasible in many cases [22]. However, identifying the most suitable expression host for a given protein remains a challenging and time-consuming task, and the *a priori* prediction of the most successful expression system for the protein of interest is normally impossible. Recombinant CRP has previously been expressed in mammalian cells [10], as well as in *E. coli* and insect cells and purified after secretion into the culture medium [8,23]. Here we report the expression of CRP *in vitro*, in the yeast *Kluyveromyces lactis* and in the parasitic protozoan *Leishmania tarentolae*, guided by the following considerations: The cell-free transcription/translation system is compatible with

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[1] *Abbreviations used:* CRP, c-reactive protein; PCh, phosphocholine

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microliter-scale reactions and in contrast to expression in intact cells protein synthesis can be executed within a few hours. Proteins expressed in *K. lactis* are accessible to the eukaryotic protein folding and glycosylation machinery that *E. coli* does not have, establishing it as a vital alternative to the bacterial expression system. Furthermore, *K. lactis* has been used successfully for protein production at an industrial scale where high cell densities can be achieved. Recently, the recombinant expression of human proteins in *L. tarentolae* has attracted much attention because it may serve as an alternative to mammalian expression systems. *Leishmania* cells are easy to handle and the oligosaccharide structures of proteins produced in this organism resemble those of mammalian cells [24,25]. In this report, we demonstrate that mature, i.e. N-terminally processed CRP encompassing amino acids 19–224 [26,27] can be expressed *in vitro*, in *E. coli*, in *K. lactis* and *L. tarentolae*. We have found that CRP is soluble only in the eukaryotic systems and can be purified as secreted protein from the supernatant of *L. tarentolae* culture medium.

Materials and methods

Plasmid constructs

Expression vectors pRSETBH6-CRP (*in vitro* and *E. coli*), pKLAC1-CRP (*K. lactis*), pKLAC1-CRP-Avi (*K. lactis*) and pLEXSY-sat2-CRP (*L. tarentolae*) were generated by modifying vectors pRSETBH6 [28], pKLAC1 (New England Biolabs, Frankfurt am Main, Germany) and pLEXSY-sat2 (Jena Bioscience, Jena, Germany). The cDNA encoding CRP protein (amino acids 19–224) without its native secretory signal sequence (amino acids 1–18) was amplified by PCR using the I.M.A.G.E. full-length cDNA clone IRAUp969B0787D (imaGenes, Berlin, Germany) as template. The following forward and reverse PCR primers were used: (1) S-5′-CCATGGGTCGACCCAGACAGACATGTCGAGGAAG-3′ and R-5′-TAATTAGCGGCCGCTCAGGGCCACAGCTGGGGTTTG-3′ for cloning into the *Not*I and *Sal*I sites of the vector pRSETBH6 to result in plasmid pRSETBH6-CRP; (2) S-5′-CCGCTCGAGAAAAGACAGACAGACATGTCGAGGAAGG-3′ and R-5′-GAAGATCTTCAGGGCCACAGCTGGGGTTTG-3′ for cloning into the *Xho*I and *Bgl*II sites of the vector pKLAC1 to result in plasmid pKLAC1-CRP; (3) for fusion PCR cloning: S-5′-CCGCTCGAGAAAAGACAGACAGACATGTCGAGGAAGG-3′ (for first and second PCR) and R-1st-5′-TCTGAGCTTCGAAGATGTCGTTCAGTCCCCGCCGGGCCACAGCTGGGGTTTG-3′ (for first PCR on template IRAUp969B0787D) and R-2nd-5′-GGAGATCTTCATTCGTGCCATTCGATTTTCTGAGCTTCGAAGATGTCG-3′ (for PCR using the first PCR product as template); the fusion PCR amplicon was cloned into the *Xho*I and *Bgl*II sites of pKLAC1 to produce plasmid pKLAC1-CRP-Avi; (4) S-5′-TATCATGTCGACGCTGGCGCCCAGACAGACATGTCGAGGAAG-3′ and R-5′-AAGAATGCTAGCGGGCCACAGCTGGGGTT-TG-3′ for cloning into the *Sal*I and *Nhe*I sites of the vector pLEXSY-sat2 to result in plasmid pLEXSY-sat2-CRP.

Protein expression

For *in vitro* transcription/translation pRSETBH6-CRP plasmid template encoding for Avi-6xHis-CRP fusion protein was purified using the NucleoSpin Plasmid Miniprep Kit (Macherey & Nagel, Düren, Germany). *In vitro* expression was carried out using *E. coli* extracts generated according to the instructions of the European Molecular Biology Laboratory (www.embl.de/pepcore/pepcore_services/protein_expression/ecoli/cellfree_expressio_systems/) or using the commercially available RTS 100 *E. coli* HY Kit (Roche, Mannheim, Germany). Fifty-microliter reactions were set up in 1.5-mL plastic tubes. The reactions were incubated for up to 6 h at 30 °C. Samples (10–20 μL) were separated by SDS–PAGE either as crude extracts or as insoluble and soluble fractions after ultracentrifugation, followed by western transfer and immunological detection.

For protein expression in *E. coli*, pRSETBH6-CRP plasmid template was transformed into five different expression strains: BL21 (DE3) (New England Biolabs – NEB, Frankfurt am Main, Germany), BL21 (DE3) pLysS (Agilent Technologies, Waldbronn, Germany), BL21 (DE3) CodonPlus-RIL (Agilent Technologies), BL21 Star (DE3) pRARE and Rosetta-gami (Merck, Darmstadt, Germany) cells. The expression strain BL21 Star (DE3) pRARE was generated by isolating plasmid pRARE from Rosetta (DE3) pRARE cells and transformation into *E. coli* BL21 Star (DE3) (Invitrogen, Karlsruhe, Germany). Expression of the protein was induced in LB medium (2 mL in 24-deep-well plates) at 30 °C by 1 mM isopropyl thio-β-D-galactoside (IPTG) for 4 h followed by cell harvesting from 1 mL of culture and sonication in 100 μL lysis buffer (20 mM sodium phosphate buffer, pH7.3, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM benzamidin, 10 μg/mL aprotonin and 10 μg/mL leupeptin). Cell extracts were ultracentrifuged and 20 μL of the insoluble pellet and soluble supernatant fractions were separated by SDS–PAGE followed by western transfer and immunological detection. pKLAC1-CRP and pKLAC1-CPR-Avi plasmid templates, encoding for CRP and CRP-Avi fusion protein with N-terminal *K. lactis*-specific signal peptide

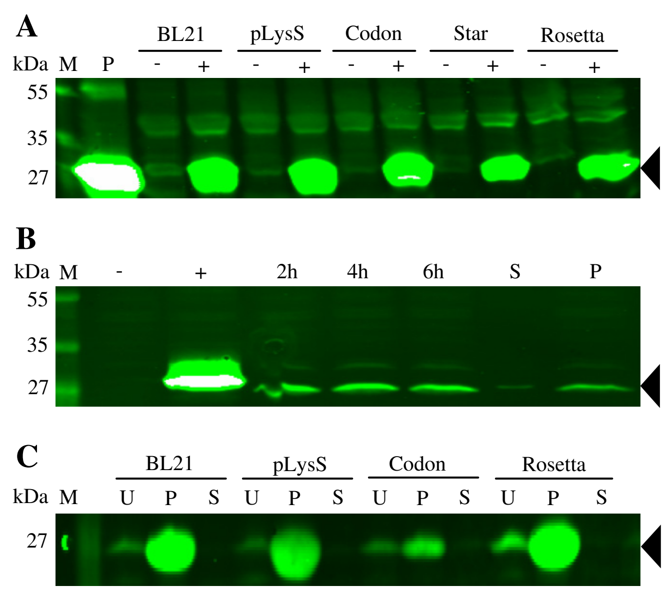

Fig. 1. CRP expressed *in vitro* and in *E. coli*. Avi-6xHis-CRP fusion protein-containing samples were separated by SDS–PAGE followed by western transfer and immunological detection of the 6xHis tag moiety at 800 nm by means of the Odyssey Infrared Imaging System (Li-COR). (A) Avi-6xHis-CRP protein was expressed *in vitro* using a commercial transcription/translation kit as positive control (P) and in-house-made transcription/translation lysates derived from the *E. coli* strains BL21 (DE3) (‘BL21’), BL21 (DE3) pLysS (‘pLysS’), BL21 (DE3) CodonPlus-RIL (‘Codon’), BL21 Star (DE3) pRARE (‘Star’), and Rosetta-gami (‘Rosetta’). Lysates containing expression plasmids are labeled ‘ + ’; negative controls without plasmid DNA are labeled ‘–’. (B) Avi-6xHis-CRP fusion protein was expressed *in vitro* using a commercial transcription/translation kit. Plasmid-free and 6xHis-GFP fusion protein (27 kDa)-expressing translation extracts were used as negative (‘–’) and positive controls (‘ + ’). Avi-6xHis-CRP fusion protein-expressing translation extracts were analyzed at different time points (2, 4 and 6 h). Supernatant (S) and pellet (P) fractions of the translation extract incubated for 6 h was used for analysis after ultracentrifugation. (C) Protein extracts obtained from Avi-6xHis-CRP fusion protein-expressing *E. coli* strains BL21 (DE3) (‘BL21’), BL21 (DE3) pLysS (‘pLysS’), BL21 (DE3) CodonPlus-RIL (‘Codon’), and Rosetta-gami (‘Rosetta’) were analyzed. Un-induced crude extracts (U) as well as pellet (P) and supernatant (S) fractions of cells induced for 6 h and then disrupted were used for the analysis after ultracentrifugation. Black arrowheads indicate positions expected for the protein Avi-6xHis-CRP (26 kDa). M, molecular mass marker (kDa).

for protein secretion, were used for protein expression and secretion into medium by means of the *K. lactis* Protein Expression Kit (NEB) as described in the manufacturer's instructions. After 2, 3, 4, 5 and 6 days of incubation, respectively, 20 µL of galactose-induced CRP or CRP-Avi fusion protein-containing expression media derived from different cell lines were used for dot blot and immunological detection or SDS–PAGE separation (of ultracentrifuged samples resulting in insoluble and soluble fractions) followed by immunological detection. Depending on the experiment, protein expression was carried out using standard or baffled Erlenmeyer flasks. *In vitro* biotinylation was performed using 40 µL of CRP- or CRP-Avi-containing medium and the RTS AviTag Biotinylation Kit following the instructions given by the manufacturer (Roche). pLEXSY-sat2-CRP plasmid template, encoding for CRP-6xHis fusion protein with N-terminal signal peptide for protein secretion, was used for protein expression in *L. tarentolae* and secretion into medium by means of the LEXSYcon2 Expression Kit (Jena Bioscience). Tissue culture flasks (25 cm^2) were used for this experiment. After 5 days under static or dynamic (50 rpm) conditions, 20 µL of CRP-6xHis-containing cell-free medium was used for SDS–PAGE, followed by western transfer and immunological detection. Protein expression was scaled up using a 150 cm^2 tissue culture flask in a culture volume of 50 mL. Proteins were concentrated (final volume 2 mL) by centrifugation of the expression medium through an Amicon Ultra-15 (10 K) centrifugal device (Millipore, Schwalbach/Ts., Germany). Protein purification was carried out using 1 mL of concentrated protein samples.

SDS–PAGE, Coomassie staining and western blot

Protein samples were separated in 12% SDS–polyacrylamide gels using the Mighty Small II system (Hoefer, Massachusetts, USA) and analyzed by: (i) immunological detection after western transfer or (ii) Coomassie staining. For (i) immunological detection of the 6xHis- or Avi-moiety, proteins were visualized at 800 nm after SDS–PAGE and western transfer to nitrocellulose membranes by means of the Odyssey Infrared Imaging System (Li-COR, Bad Homburg, Germany). A monoclonal mouse antibody directed against the 6xHis epitope (Merck) and an IRDye800CW conjugated goat anti-mouse secondary antibody (Li-COR) were used for detection of the 6xHis moiety. The biotinylated Avi-tag moiety was detected using IRDye800CW-conjugated streptavidin (Li-COR). For CRP detection a monoclonal mouse antibody directed against CRP (Santa Cruz Biotechnology, Heidelberg, Germany) and an IRDye800CW-conjugated goat anti-mouse secondary antibody (Li-COR) were used. All incubations were performed at room temperature and antibodies were diluted 1:10,000. For (ii) Coomassie visualization of proteins, SDS–polyacrylamide protein gels were stained and destained in standard solutions. Documentation of the Coomassie stained protein gels was carried out at 700 nm using the Odyssey Infrared Imaging System (Li-COR).

Purification of CRP

One milliliter of concentrated CRP-6xHis fusion protein derived from the *Leishmania* expression system (see above) was used for protein purification using either a 1-mL HisTrap HP column (GE Healthcare, Munich, Germany) connected to the Äkta-Purifier FPLC system and washing buffer supplemented with 40 mM imidazole, or a PCh-conjugated agarose column according to the instructions of the manufacturer (Thermo Fisher Scientific, Bonn, Germany).

Analysis of purified CRP by mass spectrometry

The SDS–PAGE separated and Coomassie-stained protein bands were de-colored using a mixture of 40% [v/v] acetonitrile and 60%

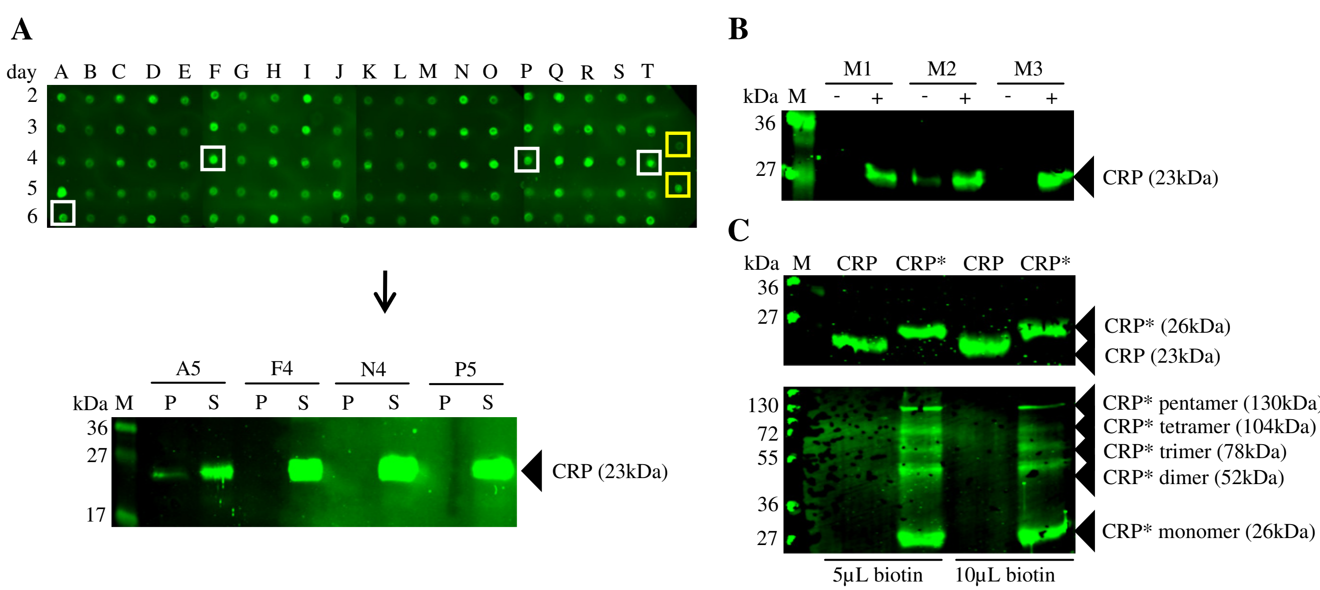

Fig. 2. CRP secreted by *K. lactis*. CRP (23 kDa) and CPR-Avi (26 kDa) proteins were immunologically detected in expression media at 800 nm using the Odyssey Infrared Imaging System (Li-COR). (A) Twenty different, randomly selected *K. lactis* transformants (upper panel, A–T) were used and protein expression was induced by galactose. After 2, 3, 4, 5 and 6 days of expression, respectively (upper panel, day 2–6), 20 µL of CRP- (and CRP-Avi-, not shown) containing medium was used for dot blot and immunological detection. This experiment allowed the identification of the strongest CRP (and CRP-Avi) signals and the corresponding cell lines (upper panel, white squares). Commercial CRP (10 and 100 ng) was used as a positive control (upper panel, yellow squares). Insoluble (P) and soluble (S) fractions of protein samples derived from cell lines A5, F4, N4 and P5 were separated by SDS–PAGE and analyzed by western blot after ultracentrifugation (lower panel). (B) Initially, standard growth medium as described by the manufacturer (M1) was used for CRP expression. This medium was supplemented with 2 mM $CaCl_2$ (M2), or 2 mM $CaCl_2$ plus 100 mM NaCl (M3). Protein expression was carried out in standard ('−') or baffled ('+') Erlenmeyer flasks. (C) After SDS–PAGE and western transfer both, CRP (CRP) and CRP-Avi (CRP*) proteins were immunologically detected using anti-CRP first antibody and an IRDye-conjugated secondary antibody (upper panel). The biotinylated Avi moiety of the CRP-Avi protein was detected using IRDye-conjugated streptavidin (lower panel). Biotinylation of the Avi moiety was carried out in 50 µL reaction volume containing 5 or 10 µL of commercial biotinylation mix. M, molecular mass marker (kDa).

[v/v] 50 mM NH_4HCO_3. The gel pieces were dried by vacuum centrifugation and incubated for 24 h with trypsin (approximately 20 μL; 30 ng/μL). Proteolytic peptides were extracted by repetitive incubation with acetonitrile, 5% [v/v] formic acid and again acetonitrile. The combined supernatants were lyophilized and resolved in a small volume of 0.1% [v/v] TFA. α-Cyano-4-hydroxy cinnamic acid (15 mg/mL, dissolved in 70% [v/v] acetonitrile) served as the matrix. A microflex MALDI-TOF (Bruker–Daltonik, Bremen, Germany) was used in the reflector mode.

Results

Expression of CRP in multiple expression systems

Complementary DNA encoding human CRP (amino acids 19–224, without N-terminal secretory signal sequence) was cloned into the vector pRSETBH6 for expression *in vitro* and in *E. coli*. For CRP expression in *K. lactis* and *L. tarentolae* the cDNA was cloned into vectors pKLAC1 and pLEXSY-sat2, respectively, downstream of the secretory signal sequences. The resulting sequence-verified expression vectors encode for the following proteins: Avi-6xHis-CRP (pRSETBH6-CRP for expression *in vitro* and in *E. coli*), CRP (pKLAC1-CRP for expression in *K. lactis*), CRP-Avi (pKLAC1-CRP-Avi for expression in *K. lactis*), and CRP-6xHis (pLEXSY-sat2-CRP for expression in *L. tarentolae*). The Avi (GLNDIFEAQKIEWHE) and 6xHis tags both allow immunological detection and purification of fusion proteins by affinity chromatography using streptavidin- (Avi-tag) or Ni^{2+}-coated (6xHis-tag) resins. Avi-tagged proteins were biotinylated using BirA ligase [29] which covalently attaches biotin to a centrally located lysine residue within the Avi-tag.

In vitro expression of the Avi-6xHis-CRP fusion protein was successful using both, a commercial transcription/translation kit (Roche) as well as our own cell-free expression lysates isolated from different *E. coli* strains (Fig. 1A). After 2, 4 and 6 h of *in vitro* synthesis, respectively, the insoluble pellet and soluble supernatant of ultracentrifuged protein samples were used for immunological detection of the Avi-6xHis-CRP fusion protein through the 6xHis moiety. The *in vitro* synthesised protein accumulated primarily, if not exclusively, in the insoluble fraction (Fig. 1B). We therefore tested *in vivo* expression in five *E. coli* hosts and found it to be successful (Fig. 1C). Again, however, the protein mainly accumulated in the insoluble fraction (Fig. 1B and C).

The purification of proteins under native conditions assumes the accumulation of a target protein in the soluble fraction. Using two different eukaryotic expression systems, *K. lactis* and *L. tarentolae*, soluble CRP was successfully expressed and secreted into the growth medium. In these experiments, the CRP encoding sequence was fused downstream from the secretory leader sequences encoded by the pKLAC1 (*K. lactis*) and pLEXSY-sat2 (*L. tarentolae*) vectors. Twenty different *K. lactis* clones were randomly selected and cell lines that expressed well were identified by dot blot and immunological analysis (Fig. 2A, upper panel). In addition, secreted CRP protein (and CRP-Avi fusion protein, not shown) was detected by western blot after SDS–PAGE (Fig. 2A, lower panel). We then used baffled Erlenmeyer flasks to grow the cells and observed that CRP signal intensity increased independently of co-factor $CaCl_2$ which was added to the culture medium and is known to play a role in the formation of CRP homo-pentamers (Fig. 2B). This result may be due to an increase in oxygen transfer in baffled Erlenmeyer flasks as compared to standard flasks where CRP fusion protein signal was low or absent (Fig. 2B). Next we tested the purification of biotinylated CRP-Avi fusion protein. To this end, secreted CRP and CRP-Avi were detected using anti-CRP antibody (Fig. 2C, upper panel). They were subjected to BirA-mediated biotinylation and subsequently analyzed using IRDye-conjugated streptavidin (Fig. 2C, lower panel). This figure shows that CRP-Avi protein forms homo-multimers. However, the purification of CRP or biotinylated CRP-Avi using either a PCh-conjugated agarose column or streptavidin particles was not possible (data not shown).

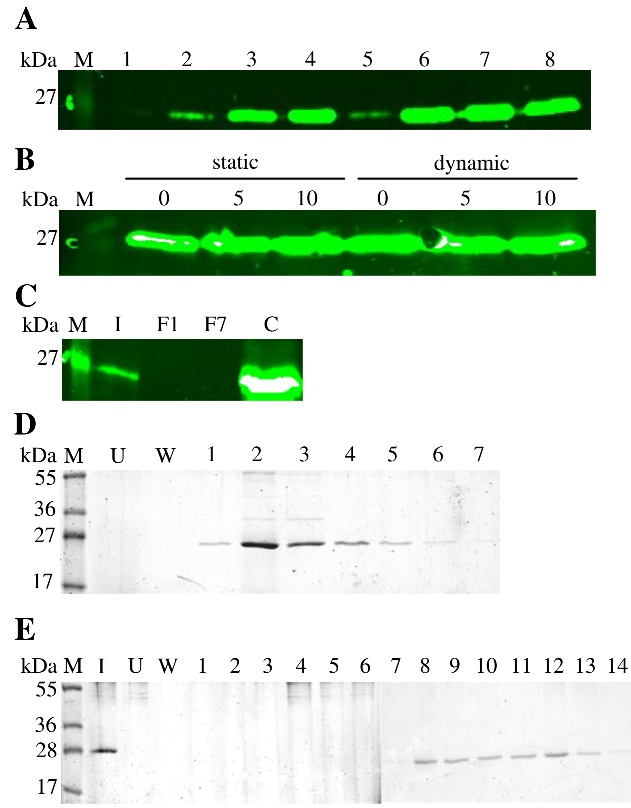

Fig. 3. Purification of CRP secreted by *L. tarentolae*. CPR-6xHis fusion protein (26 kDa) present in the culture medium was detected by western blot using the Odyssey Infrared Imaging System (at 800 nm). (A) Eight randomly selected *L. tarentolae* transformants (1–8) were used for protein expression analysis. Twenty microliter of cell-free medium from each cell line was used for immunological detection after 5 days of protein expression. (B) Initially, standard expression medium ('0') as described by the manufacturer was used for expression of CRP-6xHis. This medium was supplemented with 5 mM ('5') or 10 mM ('10') $CaCl_2$. Protein expression was carried out in 25-cm^2 tissue culture flasks under static ('static') or agitated ('dynamic') conditions. (C) The expression scale-up was carried out using a 150-cm^2 tissue culture flask and a culture volume of 50 mL. Protein was concentrated from cell-free medium by centrifugation through an Amicon Ultra-15 centrifugal device, resulting in a final volume of 2 mL. Twenty microliter of the input ('I'), flow through ('F1' and 'F7') and concentrate ('C') were analyzed by Western blot. (D and E) One milliliter of concentrated CRP-6xHis fusion protein was used for protein purification using a 1-mL HisTrap HP column coupled to an Äkta-Purifier FPLC system (D) or a PCh-conjugated agarose column (E). Twenty-microliter aliquots of the following fractions were analyzed by Coomassie staining after SDS–PAGE separation: 'I', input; 'U', unbound protein in flow-through solution; 'W', protein in wash solution; '1'–'7' ('14'), protein after elution. M, molecular mass marker (kDa).

L. tarentolae has the potential to become a preferred host for the expression of human proteins because it is easy to handle and has posttranslational modification patterns resembling those of mammalian cells. Using this innovative expression system soluble CRP-6xHis fusion protein was successfully expressed and secreted into the culture medium as shown below. After transformation, we randomly selected eight different *L. tarentolae* clones and identified cell lines that expressed well by means of Western blot analysis of expressed proteins in the growth medium (Fig. 3A). A cell line that expressed well was used to test protein expression levels depending on two parameters, $CaCl_2$ concentration and static versus dynamic incubation, however, no significant differences were

Table 1
Mass spectrometry analysis of the secreted CRP-6xHis fusion protein. Peptide masses between 0.5 and 3 kDa are listed. The overall sequence coverage was more than 70%.

Calculated mass	Detected mass	AA sequence	AA positions
708.40	n.d.	AFVFPK	10–15
836.49	836.54	KAFVFPK	9–15*
865.38	n.d.	GAQTDMSR	1–8
993.47	993.5	**(GA)QTDMSRK**	1–9*
999.43	999.49	GTHHHHHH	213–220
1128.53	n.d.	ESDTSYVSLK	16–25
1136.56	1136.6	GYSIFSYATK	50–59
1292.65	1292.68	GYSIFSYATKR	50–60*
1392.7	1392.77	QDNEILIFWSK	61–71
1548.8	1548.83	RQDNEILIFWSK	60–71*
1817.92	1817.94	AFVFPKESDTSYVSLK	10–25*
1872.89	1872.92	AFTVCLHFYTELSSTR	34–49
1977.08	1977.09	ESDTSYVSLKAPLTKPLK	16–33*
2220.16	2200.18	YEVQGEVFTKPQLWPASLK	194–212
2532.37	2532.39	ALKYEVQGEVFTKPQLWPASLK	191–212*
2722.44	2722.46	APLTKPLKAFTVCLHFYTELSSTR	26–49*

Bold – the resulting N-terminus with two additional vector-derived masses in parentheses.
AA, amino acid; n.d., not detected.
* One trypsin miss-cleavage site.

observed (Fig. 3B). Therefore, dynamic incubation in a medium supplemented with 5 mM $CaCl_2$ was chosen for a 50-mL scale-up. Using bovine serum albumin (BSA) as the standard run on Coomassie stained SDS–polyacrylamide gel, the expression level of CRP-6xHis protein was estimated to have a concentration of ~2 μg per mL of *L. tarentolae* expression culture (not shown). The 50-mL scale-up was followed by up-concentration (Fig. 3C) and purification of the protein using a 1-mL HisTrap HP column connected to an Äkta-Purifier FPLC system (Fig. 3D) and a PCh-conjugated agarose column (Fig. 3E). The Coomassie-stained protein gels revealed less contamination by other proteins when CRP-6xHis fusion protein was purified using both columns. Any interaction or purification of CRP with PCh or PCh-coated particles (PCh-conjugated agarose column) is restricted to the intact CRP homopentamer.

Finally, the amino acid sequence of the secreted CRP-6xHis fusion protein was analyzed by mass spectrometry, and correct protein processing during protein expression and secretion was verified (Table 1). The amino acid sequence GAQTDMSRK was found at the N-terminus of the CRP-6xHis fusion protein with only two additional amino acids (GA) at the N-terminus required for the proper secretion of CRP.

Discussion

Recombinant human CRP was successfully expressed in four different expression systems: in *E. coli*, *K. lactis*, *L. tarentolae* and *in vitro*. Our results show that CRP can be rapidly expressed *in vitro* and in *E. coli*, but CRP primarily accumulated in the insoluble fraction. This may be due to missing posttranslational modifications or the lack of a disulfide bond-favouring environment required for proper protein folding. The use of detergents or chaperones may support protein solubilization or folding. CRP does indeed contain two cysteine residues (amino acids 54 and 115) involved in the formation of intrachain or interchain disulfide bonds [30]. The use of the RTS 100 *E. coli* Disulfide Kit (Roche) providing chaperones and lysates in a redox buffer for maintaining the system under oxidizing conditions could enhance the yield of properly folded proteins with established disulfide bonds. However, this approach can be expensive, especially if protein expression is to be scaled up for preparative purposes. Different *E. coli* strains, including the Rosetta-gami strain (Merck) that supports disulfide bond formation were not useful for the production of soluble CRP. Further options for soluble CRP production that were not investigated in this report might include the secretion of CRP into the bacterial periplasmic space [31] or into the cultivation medium by co-expression of the *CRP* and *kil* genes [8]. Here, soluble CRP was obtained using two eukaryotic systems, *K. lactis* and *L. tarentolae*, by efficiently secreting protein into the culture medium. Small, almost undetectable amounts of CRP were found in the intracellular fractions (data not shown). C-terminally positioned 6xHis tags are not recommended for proteins expressed in *K. lactis* (see manufacturer's instructions), since C-terminal amino acids may be cleaved off by host carboxypeptidases. In our experiments, we expressed both untagged CRP and CRP with an Avi-tag at its C-terminus. All of the *K. lactis* transformants analyzed secreted CRP or CRP-Avi. However, even in the presence of Ca^{2+}, purification of CRP or biotinylated CRP-Avi using PCh-conjugated agarose column and streptavidin particles was not achieved. A possible reason could be non-human glycosylation patterns in *K. lactis* expressed proteins which may adversely affect the half-life or immunogenicity of a protein [19] or the purification of CRP expressed in this report. Glycosylation of human CRP (addition of e.g. glucose, galactose and mannose moieties) was found in some pathological conditions [17]. A possible explanation for the unsuccessful purification of CRP-Avi using streptavidin particles could be that the biotinylated Avi moiety of the fusion protein is not accessible for streptavidin. However, this interpretation is perhaps less realistic since *in vitro* biotinylation of the Avi-tag requires a BirA ligase-dependent reaction and, therefore, enzyme accessibility. The secretion of

Table 2
Purification of recombinant CRP from *L. tarentolae*.

Purification step	Volume (mL)	CRP concentration (mg/mL)[a]	Total CRP (mg)	Protein yield (%)
Culture supernatant[b]	50	0.002	0.100	–
Concentrated sample[c]	2	0.048	0.096	–
HisTrap input[d]	1	0.048	0.048	–
HisTrap eluate[e]	6	0.007	0.042	84
PCh input[f]	1	0.048	0.048	–
PCh eluate[g]	3	0.006	0.018	36

[a] CRP concentrations were estimated by Coomassie staining of protein gels after SDS–PAGE separation using bovine serum albumin as the standard.
[b] CRP was secreted by *L. tarentolae* into the medium (culture volume = 50 mL; final cell density = 8×10^7 cells/mL) in a 150 cm^2 tissue culture flask as described in Materials and methods.
[c] Fifty milliliter of CRP-containing cell-free culture medium was used to concentrate CRP in Amicon Ultra-15 (10 K) centrifugal devices (final volume after concentration was 2 mL).
[d] HisTrap HP column coupled to the Äkta-Purifier FPLC system was used for purification of 1 mL of concentrated CRP.
[e] Six CRP-containing 1-mL elution fractions were pooled after Äkta-Purifier FPLC purification.
[f] An PCh-conjugated agarose column was used for purification of concentrated CRP protein.
[g] Six CRP-containing 0.5-mL elution fractions were pooled after PCh-column purification.

6xHis-tagged CRP was successful in all *L. tarentolae* transformants tested here. Expression levels were not significantly affected by varying Ca^{2+} concentrations in the growth medium or cultivation conditions (static versus agitated). We were able to purify CRP-6xHis protein from *Leishmania* medium by using either a 1-mL HisTrap HP column (GE Healthcare) connected to an Äkta-Purifier FPLC system or a PCh-conjugated agarose column (Thermo Fisher Scientific). The latter purification method is restricted to intact pentameric CRP, indicating the expression of functional protein in *L. tarentolae*. A mass spectrometric analysis of recombinant CRP revealed amino acid sequences identical to those of native human CRP with the exception of two additional amino acids at the N-terminus derived from the expression system and required for the proper processing of secreted proteins.

The expression level achieved here for CRP, i.e. ~2 mg/L (Table 2), resembles that of other secreted proteins previously reported for *L. tarentolae* (0.1–5 mg/L [25]). Higher levels of CRP production were previously reported for *E. coli* in large-scale fermentations (27 mg/L [8]), Chinese hamster ovary (CHO) cells (5 mg/L, [10]), and baculovirus-infected insect cells (125 mg/L [23]). However, it may be possible to improve recombinant protein production in *Leishmania* by employing inducible expression technologies and by optimizing culture conditions. In fact, *L. tarentolae* can be grown at high cell densities ($\sim 1 \times 10^9$ cells/mL), and protein production of up to 5 mg/L has been achieved [25]. Since human CRP is glycosylated in some physiological conditions [17], the expression of recombinant CRP in eukaryotic cells may be more advantageous than expression in bacterial systems that lack post-translational modification pathways. Viral contamination which may occur in mammalian or insect cell expression hosts are not a major problem in *L. tarentolae* cells which can be grown in serum-free medium containing only hemin as a substance of animal origin [32]. Furthermore, mammalian and insect cells are much more difficult to handle than *L. tarentolae* cells.

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