African Journal of Biotechnology Vol. 11(81), pp. 14685-14695, 9 October, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.2258
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Recombinant production of the human complement factor 5a in *Escherichia coli*

Henrik Nausch[1], Jana Huckauf[1], Roswitha Koslowski[2], Udo Meyer[2], Inge Broer[1]* and Heike Mikschofsky[1]

[1]Agricultural and Environmental Faculty, University of Rostock, Agrobiotechnology, Justus-von-Liebig Weg 8, 18059 Rostock/Germany.
[2]Bioserv GmbH, Dr. Lorenz-Weg 1. 18059 Rostock / Germany.

Accepted 6 September, 2012

Up to now, the human complement factor 5a (C5a) has only been produced in small quantities in *Escherichia coli* in a soluble, bioactive conformation, which is not suitable for commercial production systems. This stems from the extremely high instability of C5a, as well as its aggregation-prone nature. Therefore, we analyzed several different methods for optimizing the solubility and biological activity of C5a produced by *E. coli*. The solubility of C5a was efficiently improved by expressing it as a glutathione-S-transferase (GST) fusion protein and, to a lesser extent, by lowering the cultivation temperature. Neither reducing the inductor concentration (isopropylthio-β-galactoside, IPTG) of the T7*lac* promotor nor the concomitant overexpression of endogenous chaperones was effective. However, the biological activity of the protein was improved by the overexpression of chaperones together with cultivation at 22°C, while fusion to GST slightly reduced its activity. Consequently, low cultivation temperature and the overexpression of chaperones seem to be the optimal strategy for expression of appropriate amounts of soluble and functional C5a. These findings should be the basis for the transfer to large-scale fermentation. Using C5a as an example, we showed that strain engineering in combination with specific cultivation conditions improve the production of difficult-to-express proteins in appropriate amounts and in a functional conformation facilitating the commercial manufacturing under good manufacturing practices (GMP) conditions.

Key words: Complement factor 5a (C5a), Origami 2, BL21, periplasm, cytoplasm, chaperones, Glutathione-*S*-Transferase (GST), temperature.

INTRODUCTION

Sepsis is one of the top 10 causes of death worldwide (Dombrovskiy et al., 2007; Parrish et al., 2008). Despite medical treatments in intensive care the mortality of sepsis patients units remains high, exceeding 70% (Cohen, 2009; Riedemann et al., 2003). The human complement factor 5a (C5a) as potent mediator of the innate immune response to infection, has been characterized as a 'key' mediator of sepsis and septic organ dysfunction (Czermak et al., 1999; Huber-Lang et al., 2001; Niederbichler et al., 2006; Riedemann et al., 2002; Rittirsch et al., 2008). Though not directly interacting with bacteria, C5a is a chemoattractant with pleiotropic functions which is involved in the recruitment and activation of broad range of inflammatory cells (Guo and Ward, 2005; Klos et al., 2009).

Inhibition of C5a mediated responses by blockade of C5a (Czermak et al., 1999; Flierl et al., 2009; Huber-Lang et al., 2001; Huber-Lang et al., 2002) or the C5a-receptor (Crane and Buller, 2007; Riedemann et al., 2002; Rittirsch

*Corresponding author. E-mail: inge.broer@uni-rostock.de. Tel: 0049 0381/4983080. Fax: 0049 0381/4983082.

Abbreviations: SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **PVDF,** polyvinylidene fluoride; **BSA,** bovine serum albumin; **TMB,** tetramethylbenzidine; **RBL,** rat basophilic leukemia; **ELISA,** enzyme-linked immunosorbent assay; **TSP,** total soluble proteins.

et al., 2008) has been shown to prevent multiple organ failure. Therefore, interception of C5a by antibody-based medications might be a worthwhile strategy - among others - to ameliorate the fatal outcome in septic patients and improve survival rates (Parrish et al., 2008; Ricklin and Lambris, 2007; Ward, 2008; Ward and Gao, 2009). The development of these biotherapeutics requires the efficient production of large quantities of soluble, functionally active C5a, which can be used for antibody production.

Recombinant methods for the generation of human proteins in bacteria, especially *Escherichia coli*, have been studied intensively over the last decade. Currently, 30% of approved biotherapeutics are produced in *E. coli* (Ferrer-Miralles et al., 2009). Therefore, there is a large body of knowledge about the basic molecular genetics and microbial physiology of *E. coli* that can be efficiently translated into practical applications at the process level (Schumann and Ferreira, 2004).

Most human proteins accumulate in inclusion bodies when expressed in *E. coli*, which is a major constraint of the production platform (de Marco et al., 2007). This is also true for C5a, and several attempts to produce C5a in a soluble conformation have failed (Bautsch et al., 1992; Franke et al., 1988; Mandecki et al., 1986; Mollison et al., 1987). A recombinant form of C5a was recovered from inclusion bodies by denaturation and refolding. However, the isolation of proteins from inclusion bodies is laborious and cost-intensive, impeding large-scale industrial production. Furthermore, resolubilization procedures may not fully restore the folding of the protein, thereby reducing its effective function. Consequently, further investigations to identify economically efficient expression systems of C5a are critical.

For the commercial production of protein therapeutics, the fermentation usually starts in a laboratory-scale bioreactor to identify suitable growth and protein expression conditions. These conditions are then used to establish optimal parameters for the final manufacturing scale in order to reach high productivity (Huang et al., 2012). Thus, using C5a as a model, we used a screening process to identify central parameters for the expression of difficult-to-express proteins. In order to obtain large quantities of C5a in the desired soluble conformation, the impact of a broad spectrum of expression approaches as well as different combinations of approaches was analyzed.

First, we investigated the expression of C5a in an oxidizing environment, the cytoplasm of the *E. coli* strain Origami 2. Second, C5a was targeted to the periplasm of *E. coli* BL21, a strain that is commonly used for the overexpression of recombinant proteins. In addition, we promoted transgene expression through the concomitant overexpression of peri- and cytoplasmic chaperones, respectively. Further, C5a was fused to Glutathione S-Transferase (GST) from *Schistosoma japonicum*, as a representative of solubility enhancing tags. These strategies were combined with the adaptation of the expression conditions, such as lowering the cultivation temperature and decreasing the inductor concentration.

In addition to the N-terminal fusion tags (His-tag or GST-His-tag), which can be removed by proteolytic digestion, a C-terminal histidine residue was fused to C5a in all constructs for the following reasons: (i) C5a is *in vivo* subjected to a serum peptidase, which inactivates C5a by the removal of the C-terminal arginine residue (Mollison et al., 1989). Since a sequence of at least five charged amino acids at the C-terminus protects the recombinant protein efficiently from degradation (Novagen pET system manual), the C-terminal His-tag may protect recombinant C5a from proteolytic degradation as observed for unmodified C5a expressed in *E. coli* (Mollison et al., 1987). (ii) According to the manufacturer (Novagen pET system manual), the two His-tags improve the downstream purification efficiency in order to gain pure recombinant protein, free of endotoxin and other bacterial contaminations, required for GMP compliance. (iii) His-tags enable the site-specific PEGylation (Cong et al., 2012) and the C-terminus of C5a would be most likely amenable for PEGylation, since - being responsible for receptor activation - it is exposed to the surface of the protein (Mollison et al., 1989). PEGylation is a clinically proven strategy to increase the circulation half-life of protein-based medicines (Cong et al., 2012). Since the efficacy of protein-based agents can be compromised by their rapid clearance from the blood circulatory system (Cong et al., 2012), PEGylated C5a may be more suitable for the production anti-C5a-antibodies. Nonetheless, the His-tag might interfere with the functionality of the C-terminal receptor binding (Mollison et al., 1989). However, this disadvantage might be compensated by a second immunodominant binding domain located between amino acids 20 to 40 that has proven to be sufficient to accomplish receptor binding (Johnson et al., 1987; Kola et al., 1999).

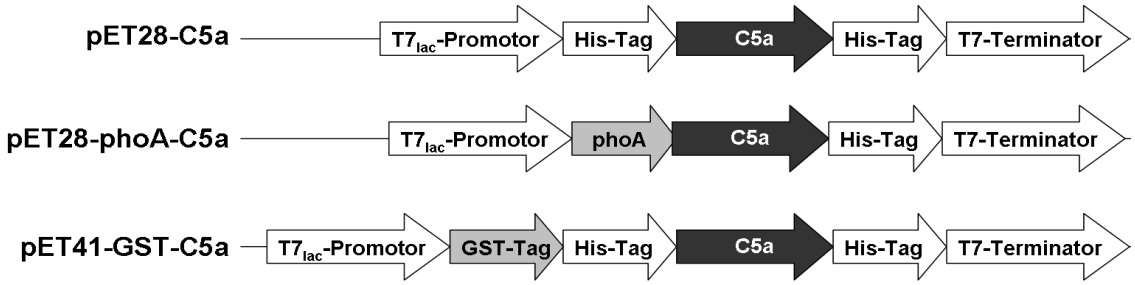
Although the experiments were done under laboratory-scale conditions, the basic findings may be relevant for large-scale fermentation of C5a under commercial good manufacturing practice (cGMP).

MATERIALS AND METHODS

Construction of the expression plasmid and overexpression strains

Based on the amino acid (aa) sequence of the human complement factor C5a, a cleavage product of the precursor protein C5 (Acc. No.: P01031|678aa – 751aa), a synthetic C5a coding sequence was designed with the optimal codon usage for *E. coli* (Figure 1b). The C5a cassette was integrated in frame into the vector pET28a (Novagen) by *Eco*RI/*Xho*I and into the vector pET41a by *Mun*I/*Xho*I digestion. The pET28a vector carries an N-terminal His-Tag/thrombin/T7-Tag and a C-terminal His-Tag. The pET41a vector contains an N-terminal 220 aa GST-fusion as well as a His-Tag/thrombin element and a second C-terminal His-Tag. To target the human proteins to the periplasm, the upstream N-terminal

A



B

```
 M   T   L   Q   K   K   I   E   E   I   A   A   K   Y    14
ATG ACC CTG CAG AAA AAA ATT GAA GAA ATT GCG GCG AAA TAT   42

 K   H   S   V   V   K   K   C   C   Y   D   G   A   C    28
AAA CAT AGC GTG GTG AAA AAA TGC TGC TAT GAT GGC GCG TGC   84

 V   N   N   D   E   T   C   E   Q   R   A   A   R   I    42
GTG AAC AAC GAT GAA ACC TGC GAA CAG CGC GCG GCG CGC ATT  126

 S   L   G   P   R   C   I   K   A   F   T   E   C   C    56
AGC CTG GGC CCG CGC TGC ATT AAA GCG TTT ACC GAA TGC TGC  168

 V   V   A   S   Q   L   R   A   N   I   S   H   K   D    70
GTG GTG GCG AGC CAG CTG CGC GCG AAC ATT AGC CAT AAA GAT  210

 M   Q   L   G   R   L   *                               76
ATG CAG CTG GGC CGC CTG TAA                             231
```

Figure 1. (A) Schematic representations of the constructs used to express the *C5a* gene. Both vector backbones harbor a kanamycin resistance gene and the pBR322 origin of replication. GST™-Tag: glutathione *S*-transferase (220 aa) from *Schistosoma japonicum*; His-Tag: 6 histidine residues; T7-Tag®: the first 11 aa of the gene *10* protein from the T7-phage; phoA: periplasmic targeting signal from the *E. coli* alkaline phosphatase; Thrombin: 6 aa comprising the thrombin protease cleavage site. C5a (76 aa) synthetic, codon-optimized gene sequences based upon the mature protein sequences; (B) Synthetic, codon-optimized coding region of the human C5 split-product C5a.

coding portion of the pET28a vector was substituted by a codon-optimized synthetic sequence for the 21 aa signal peptide of the alkaline phosphatase (phoA) from *E. coli* (Acc.-No.: AAA83893) using *Nco*I/*Eco*RI digestion. All constructs were expressed under the control of the inducible T7*lac* promotor and terminator. The constructs are shown in Figure 1a.

The helper plasmids pBB540 and pBB5542, encoding the cytoplasmic chaperones DnaK/DnaJ/GrpE and GroEL/GroES, were generously provided by the workgroup of Prof. Dr. Bernd Bukau (de Marco et al., 2007). The vector pTUM4.1, expressing the periplasmic chaperones DsbA, DsbC, SurA and FkpA, was shared by the workgroup of Prof. Dr. Arne Skerra (Schlapschy et al., 2006). The combinations of different expression vectors and *E. coli* strains used in this work are listed in Table 1.

Competent BL21 (DE3) and Origami 2 (DE3) cells (Novagen) were first transformed with the pET-vectors expressing the various

Table 1. Recombinant strains used in this work.

Host strain	Recombinant plasmids	Fusion Tag
Origami 2	pET28-C5a	n- & c-terminal His Tag T7-Tag
L21	pET28-C5a	n- & c-terminal His Tag T7-Tag
BL21	pET28-phoA-C5a	c-terminal His-Tag
BL21	pET28-C5a pBB540/pBB542	n- & c-terminal His Tag T7-Tag
BL21	pET28-phoA-C5a pTUM4.1	c-terminal His-Tag
BL21	pET41-GST-C5a	n-terminal GST-Tag n- & c-terminal His-Tag

target genes by electroporation. The resulting recombinant strains were subsequently transformed using the helper plasmids pBB540/pBB542 or pTUM4.1. Cells were always grown in the presence of appropriate antibiotics to ensure the maintenance of all plasmids.

Protein expression

Single colonies from the transformed cells were used to inoculate a 5 ml pre-culture of LB medium supplemented with the appropriate antibiotics. Liquid cultures were grown at 37°C with shaking at 220 rpm overnight. A 1 ml aliquot of the pre-culture was then used to inoculate 100 ml of antibiotic-containing LB medium. The main cultures were incubated at 37°C with shaking at 220 rpm until they reached an OD600 of 0.5. For protein expression at 37°C, 1 mM isopropylthio-β-galactoside (IPTG) was added immediately, and cells were incubated at 37°C for 4 h. For protein expression at 22°C, the cultivation temperature was changed from 37 to 22°C, and cells were allowed to acclimatise to the new temperature for 30 min before 1 mM IPTG was added; the cells were then further grown at 22°C overnight. Overnight cell cultures were pelleted, the medium was removed, and the cell pellet was resuspended in the same amount of fresh medium supplemented with 200 µg/ml chloramphenicol. After a 2 h incubation of the culture at 22°C, the cultures were centrifuged and the pellet was stored at -28°C until harvest.

Western blot analysis

The frozen bacterial pellet was resuspended in 5 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and 1 mg/ml lysozyme was added to the suspension. The suspension was incubated on ice for 2 h with nutation, followed by a sonication in a water bath for 5 min. The cell debris was separated from the supernatant by centrifugation. Sodium dodecyl sulfate (SDS) sample buffer was added to both fractions, and a defined volume was boiled and loaded on a 15% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes for 2 h at 1 mA/cm^2 for Western blot analysis. The subsequent steps were performed at room temperature. Membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline containing 0.1% Tween-20 (PBST) overnight. Membranes were incubated with a 1:1,000 dilution (in PBST) of a mouse monoclonal anti-C5a antibody (Dianvova, Hamburg / Germany) for 2 h. Following a triple washing step with phosphate-buffered saline with Tween 20 (PBST), the membranes were incubated with a 1:10,000 dilution of a peroxidase-conjugated goat monoclonal anti-mouse-IgG antibody (Dianova, Hamburg / Germany) for 2 h. After 5 washes with PBS, the probed proteins were visualized on Biomax Light film (Kodak, Sigma-Aldrich, Taufkirchen / Germany) with homemade chemiluminescent substrate (ECL solution I: 100 mM Tris (pH 8.5), 2.5 mM luminol, 400 µM p-coumaric acid; ECL solution II: 100 mM Tris (pH 8.5), 5.4 mM H_2O_2. ECL solutions were mixed immediately before use).

Enzyme-linked immunosorbent assay (ELISA)

The cell lysates, supernatant and cell debris fraction used for the Western blot assay were also used in Bradford and enzyme-linked immunosorbent assays. The protein concentration was measured by the Bradford method (1976) using a Pierce reagent with bovine serum albumin (BSA) as the standard (Thermo Scientific, Bonn / Germany). The quantification of recombinant C5a was conducted with a commercial C5a-ELISA-Kit (Human Complement Component C5a DuoSet ELISA; R&D Systems, Cat.: DY2037) according to the manufacturer's instructions. In brief, 96-well plates were coated with a mouse anti-human C5a-specific antibody at a final concentration of 1 µg/ml at room temperature (RT) overnight. Following 5 washes with 1xPBS containing 0.05% Tween-20, the plates were incubated with 100 µl leaf extract (at a suitable dilution) at RT for 2 h. After another washing step, the plates were incubated with the corresponding biotinylated detection antibody at RT for 1 h. After a washing step, the plates were hybridized with streptavidin conjugated to horseradish-peroxidase at RT for 30 min. Finally, the plate was incubated with the substrate tetramethylbenzidine (TMB) at RT for 15 min in the dark. The reaction was stopped with 250 mM sulfuric acid. Extinction was measured at 450 nm in Synergy HT multi-detection reader (Bio-Tek, Bad Friedrichshall / Germany).

Determination of the biological activity of recombinant C5a

A rat basophilic leukemia (RBL) cell-line transfected with human C5aR was used, which was developed by Ali et al. (1993). The bioassay for lysosomal enzyme secretion was conducted as described by Goldstein and Weissmann (1974). The RBL-C5aR-cell

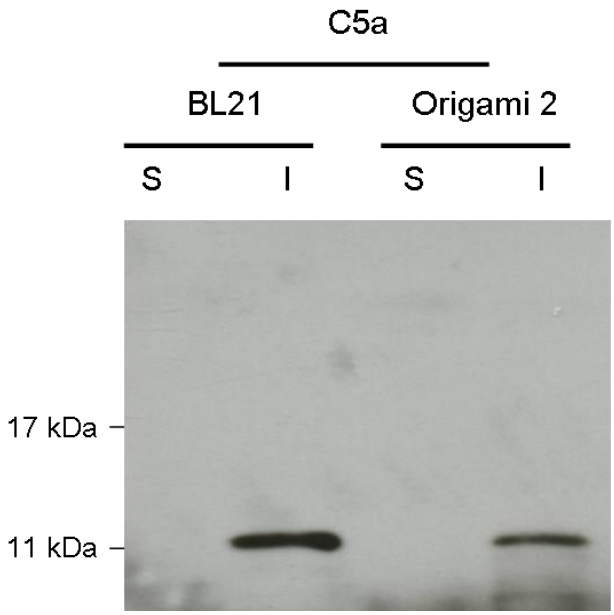


Figure 2. Western blot analysis of soluble (S) and insoluble (I) fractions of C5a expressed in the *E. coli* strains BL21 and Origami 2.

line was incubated with recombinant 'antigenic' C5a. The amount of antigenic C5a was calculated on the basis of the C5a-specific ELISA, which detect the number of antigenic C5a per ml regardless the different molecular weight of the recombinant C5a or GST-C5a, respectively. Therefore, the concentration was determined as ng C5a equivalents per ml. Biologically active C5a induces the release of the enzyme N-acetyl-ß-D-glucosaminidase, which removes 4,5 p-nitrophenolate from the substrate 4-nitrophenyl-N-ß-D-glucosamide. The photometric determination of the resulting concentration of nitrophenolate is used to calculate the concentration of biologically active recombinant protein. The concentration of recombinant antigenic protein that induced half-maximal enzyme release was defined as the EC_{50}-value.

RESULTS

Comparison of *E. coli* (DE3) strains BL21 and Origami 2 as production systems

The pET28C5a plasmid was introduced into the *E. coli* strains BL21 ($lon^-/ompT^-$) and Origami 2 ($trxB^-/gor^-$). To determine the effect of the oxidizing cytoplasm of the Origami 2 strain on the establishment of the disulfide bonds in C5a, the expression patterns in the 2 strains were compared by Western blot. When the genes were expressed by induction with 1 mM IPTG at 37°C for 4 h, the recombinant protein accumulated only in the insoluble fraction of the cell lysate in both strains (Figure 2, lanes I). However, the total amount of C5a produced was significantly higher in the BL21 strain.

Because Origami did not lead to any improvement of protein solubility, we decided to conduct further investigations on the *E. coli* BL21 strain due to its vigorous growth and higher expression level.

Expression in the cytoplasm and periplasmic space of *E. coli* BL21

To combine the superior growth kinetics of *E. coli* BL21 with an oxidizing environment to ensure the correct folding of the intramolecular disulfide bonds, C5a was targeted to the periplasm. This translocation was accomplished by replacing the N-terminal His- and T7-Tag in the corresponding pET28-C5a vector with the signal peptide from the endogenous alkaline phosphatase from *E. coli* (*phoA*). As shown in Figure 3 (periplasm), this strategy had a negative effect on the expression of C5a. Neither soluble nor insoluble C5a was found in the whole cell extracts.

Concomitant expression of cytoplasmic and periplasmic chaperones

The effect of the concomitant overexpression of a set of cytoplasmic chaperones (DnaK, DnaJ, GrpE, GroES, and GroEL) and key periplasmic folding factors (DsbA, DsbC, SurA, and FkpA) was investigated. The corresponding plasmids were kindly provided by Prof. Dr. Bernd Bukau and Prof. Dr. Arne Skerra.

The concomitant expression of the cytoplasmic chaperones had no effect on the expression of C5a in the cytoplasm (Figure 3; cytoplasm + C-chaperones); its accumulation in inclusion bodies remained unchanged. However, the direction of the protein into the periplasm, in combination with the coexpression of periplasmic folding factors, improved the yield of insoluble C5a without affecting the soluble fraction (Figre 3; periplasm + P-chaperones).

Expression of C5a as a GST fusion protein

To fuse GST to C5a, the plasmid pET41-GST-C5a was constructed. The GST tag had a beneficial impact on the expression pattern of C5a under standard cultivation conditions at 37°C (Figure 3; cytoplasm + GST). A significant solubilization of C5a was achieved, and the amount of C5a in the inclusion bodies was decreased.

Influence of optimized cultivation conditions

A well-known approach to limit the *in vivo* aggregation of recombinant proteins is cultivation at a reduced growth temperature. The lowering of the cultivation temperature from 37°C, which represents the optimal growth temperature for *E. coli*, 20 to 22°C, has been successful in many cases (de Marco et al., 2007).

However, in case of C5a, the reduction of the cultivation

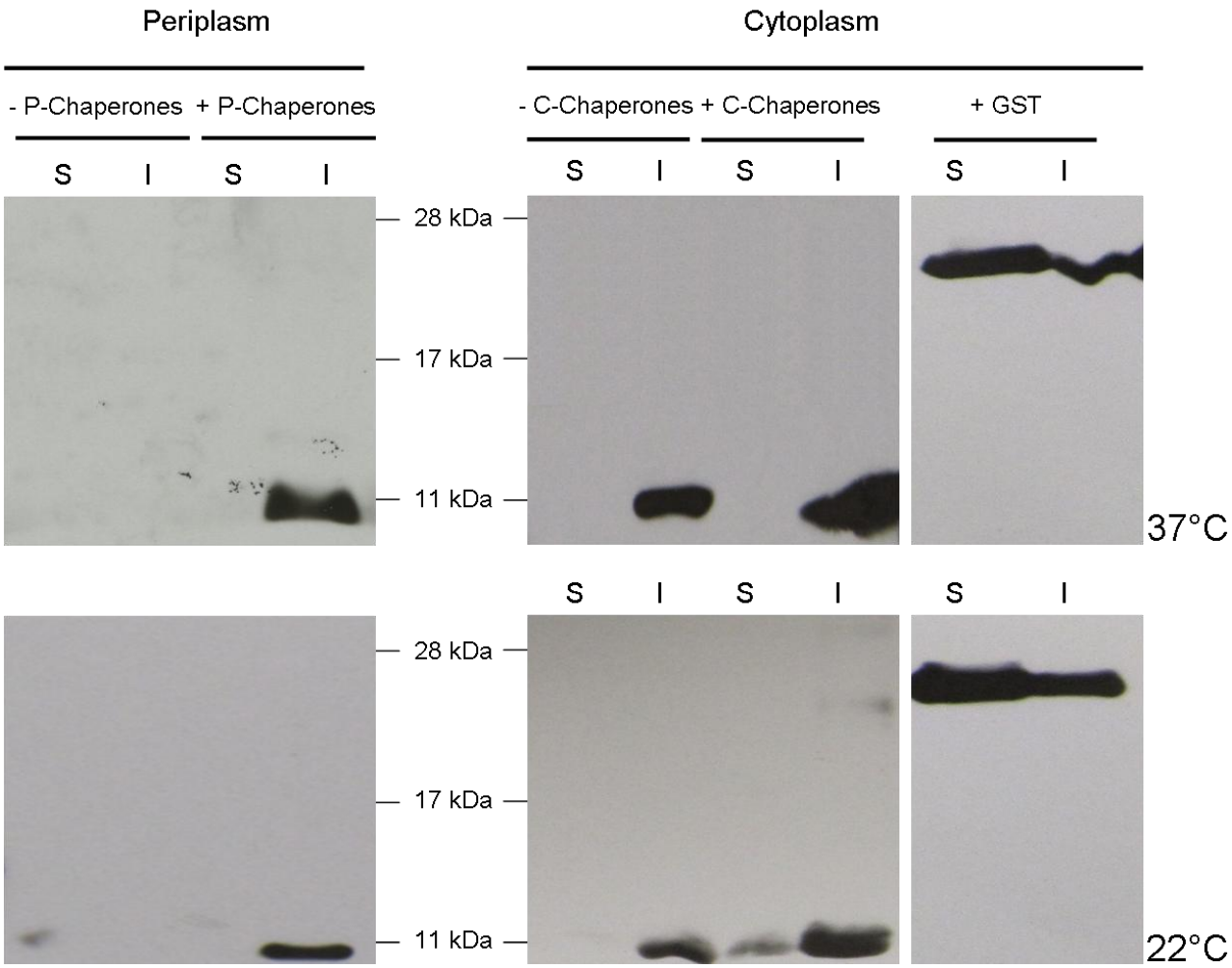


Figure 3. Western blot analysis of soluble (S) and insoluble (I) fractions of C5a expressed in the cyto- and periplasm of *E. coli* BL21 with and without the concomitant overexpression of the endogenous cytoplasmic chaperones DnaK, DnaJ, GrpE, GroES, and GroEL (C-Chaperones) or the periplasmic chaperones DsbA, DsbC, SurA, and FkpA (P-Chaperones) at 37°C and 22°C, respectively. Additionally, C5a was expressed as a GST fusion in the cytoplasm.

temperature had no significant effect on any combination of the different C5a expression strategies tested. A slight impact on the strain with improved chaperone status (Figure 3; Cytoplasm + C-Chaperones) was observed. At 22°C, a reproducible but weak signal was detected in the soluble fraction, while no such signal was detected at 37°C. Nevertheless, the total amount of C5a was reduced when cells were grown at 22°C.

A further adaptation of the biosynthesis of the foreign protein to normal physiological expression rates can be achieved by reducing the inductor concentrations. Because the expression of all recombinant proteins is completely induced at concentrations above 0.4 mM IPTG, overexpression was stimulated with 0.1 mM instead of the conventionally used 1 mM IPTG, as proposed by de Marco (de Marco et al. 2007). However, reducing the inductor concentrations did not have any effect on the expression of human C5a (data not shown).

Quantification and evaluation of the biological activity of *E. coli* derived human C5a

To measure the expression levels of soluble recombinant C5a, the fraction of total soluble proteins (TSP) was extracted from pellets of the cell culture by lysozyme digestion and centrifugation. The different fractions of the cell lysate were tested by corresponding C5a-specific ELISA, which detects the concentration of the recombinant antigenic protein regardless of the different molecular weight of C5a and GST-C5a, respectively. The ELISA data served as basis for the calculation of the concentration of recombinant antigenic protein - which was used in the bioassay - so that the difference in molecular weight had no impact. Therefore, the concentration was determined as ng C5a equivalents per ml. For the bioassay, a RBL cell line transfected with human C5aR was used. Upon binding of biological active

Table 2. Quantification of soluble antigenic C5a and GST-C5a, respectively, by ELISA and the evaluation of its biological activity. The commercial recombinant C5a (B&D Systems) has an EC_{50} value of 200.94 ng C5a equivalents /ml.

BL21 strain	Soluble C5a		Insoluble C5a (inclusion bodies)	
	Portion of TSP (%)	Biological activity EC_{50} value of C5a (ng C5a equivalents/ml)	Portion of TSP (%)	Biological activity EC_{50} value of C5a (ng C5a equivalents/ml)
pET28- C5a	0.23	279.63	1.07	935.17
pET28- C5a +Chaperones	0.65	192.03	2.58	828.29
pET41-GST-C5a	1.41	508.15	1.21	1356.58

C5a to the receptor, the enzyme N-acetyl-ß-D-glucosaminidase is released, which is quantified by addition of its substrate. The concentration of recombinant protein that induces a half-maximal enzyme release (EC_{50}-value) was used to describe the biological activity. High concentrations of recombinant C5a necessary to induce the half-maximal response, correspond to low biological activity and *vice versa*.

The highest yield of soluble complement factor was measured in the GST-C5a fusion, in which it made up 1.41% of the TSP (Tab. 2). However, this chimeric protein had only limited biological activity in the corresponding assay, with an EC_{50}-value of 508.15 ng/ml. In contrast, C5a obtained by the BL21 strain with the additional chaperone expression showed the best performance. Its activity, 192.03 ng/ml (Table 2), was equivalent to that of commercial recombinant C5a (B&D Systems) at 200.94 ng/ml. The yield accounted for 0.65% of the TSP. For comparison, the BL21 strain that expressed C5a protein without any helper elements had the lowest yield of soluble C5a, at 0.23% of TSP. Its biological activity (279.63 ng/ml) was lower than that of the standard. Insoluble fractions were also analyzed by ELISA and bioassays, as shown in Table 2. Comparing the insoluble fractions of the three strains, similar tendencies were recorded as observed for the soluble fraction. Insoluble C5a expressed using the chaperone-assisted strategy showed the highest yield of aggregated recombinant protein (2.58%) and the highest biological activity (EC_{50} value of 828 ng/ml) of all insoluble fractions. The insoluble GST-C5a showed the worst functionality, with an EC_{50} value of 1356 ng/ml. Nevertheless, the biological activity of the inclusion bodies of the three strains was three to four times lower compared to the soluble counterpart. Even the insoluble fraction with the highest biological activity (828 ng/ml of the chaperone-assisted strain) was inferior compared to the soluble fraction with the worst biological activity (508 ng/ml of the GST-C5a expressing strain).

DISCUSSION

Solubilization of recombinant protein produced in *E. coli* is often a prerequisite for the biological activity that is necessary for therapeutic purposes. Therefore, an applicable and cost-efficient system for the production of C5a must focus on the development of a strategy to drive the expression in *E. coli* as a soluble and properly folded protein rather than an aggregated protein in the inclusion bodies.

Because previous attempts to express C5a in the cytoplasm of *E. coli* using individual solubilization strategies failed to develop a heterologous expression system for C5a that suits commercial demands (Bautsch et al., 1992; Franke et al., 1988; Mandecki et al., 1986; Mollison et al., 1987), we hypothesized that a combination of a broad range of strategies might solve this problem. In order to identify crucial parameters that might improve the production of C5a in large-scale cGMP fermentation and under laboratory conditions, we evaluated a combination of several approaches (Table 3). According to Mollsion et al. (1989), post-translational modifications are essential for the constitution of C5a and its biological activity. More specifically, the correct crosslinking of the 3 disulfide bridges that stabilize the four core α-helices of C5a are crucial for protein assembly, since reduced C5a and mutants with deleted Cys-residues have been shown to be biologically inactive (Gerard et al., 1979; Johnson et al., 1987; Mollison et al., 1989). Moreover, it has been recorded that an incorrect disulphide bridging was deleterious for the functionality of C5a and that particularly Cys_{27} is important for the constitution of the immunodominant conformation (Hennecke et al., 1997; Johnson et al., 1987). Consequently, we focused first on the expression of the recombinant factor in an oxidizing environment to enable disulfide bridge formation (de Marco, 2009). However, expression in the oxidizing cytoplasm of the *E. coli* Origami 2 did not lead to the solubilization of C5a (Table 3), and the expression of aggregated C5a was lower in Origami 2 than in the BL21 strain (Figure 2). This is in contrast to expression analysis of other human proteins that are present in the blood, such as the tissue plasminogen activator (Bessette et al., 1999), the serine protease inhibitor HF6478 (Lauber et al., 2001), the lutropin/choriogonadotropin receptor (Lobel et al., 2002) and antigen binding fragments (Venturi et al., 2002). Hence, the expression of eukaryotic proteins in *E. coli* depends on the protein produced. Because *E. coli*

Table 3. The evaluation of expression parameters on the solubilization of recombinant C5a in terms of yield (-: no soluble target protein; +: <10%; ++: 10 -25%; +++ 25-50%; ++++: >50% of total recombinant protein in the soluble fraction) and biological activity. n.i., not experimentally investigated.

Expression strategy	Description	Temperature (°C)	Yield of soluble C5a	Bioactivity of soluble C5a as EC_{50} value (ng C5a equivalents/ml)
1	Origami 2- cytoplasm	37	-	--
		22	-	n.i.
2	BL21 periplasm	37	-	--
		22	-	--
3	BL21 periplasm + P-Chaperones	37	-	--
		22	-	--
4	BL21- cytoplasm	37	-	n.i.
		22	+	250-500
5	BL21-cytoplasm + C-Chaperones	37	-	n.i.
		22	++	<250
6	BL21-cytoplasm + C-Chaperones	37	+++	n.i.
		22	++++	>500

Origami 2 also had inferior growth kinetics compared to the strain BL21, this strategy was not pursued further. The higher level of C5a expression in BL21 might be attributed to the deficiency in 2 major proteases that are known to degrade recombinant proteins (Novagen product manual). Additionally, in a comparative study of various *E. coli* strains, BL21 exhibited the lowest stress response to high synthesis rates of foreign protein (Seo et al., 2003). Because of these features, B strains are highly desirable in industry (Huang et al., 2012). 15 out of 43 approved biopharmaceuticals are produced in this strain (Waegeman and Soetaert, 2011). K12 derivates, such as Origami 2, are disfavoured because of the high acetate accumulation, which interferes with the growth of the bacteria (Waegeman and Soetaert, 2011). To combine the advantageous features of BL21 with those of an oxidizing environment, C5a was targeted to the periplasm in BL21 by fusion to the phoA signal peptide. This strategy has been found to improve the expression patterns of several proteins, such as the human plasma retinol-binding protein and the dendritic cell membrane receptor DC-SIGN (Schlapschy et al., 2006), the bacterial glycoamidase PNGase F (Loo et al., 2002) and the human granulocyte colony-stimulating factor (Jeong and Lee, 2001). However, phoA-fused C5a was not detectable in the periplasm. This is consistent with a report by Xu et al. (2008a), who did not find any PalB in the periplasm and attributed that effect to the inherent instability of the recombinant protein (Xu et al., 2008b). This might also be true for C5a because C5a is normally present in the human blood as inactive C5 precursor. After the proteolytic release of C5a, it is rapidly metabolized by serum peptidases in order to permit the appropriate regulation of the immune response (Martin et al., 1988). As a possible solution to this problem, it has been widely reported that the overexpression of periplasmic folding factors can interact with secreted proteins to enhance functional expression via improved translocation and/or polypeptide stabilization (Choi and Lee, 2004; Duguay and Silhavy, 2004; Kurokawa et al., 2000; Mogensen and Otzen, 2005; Schlapschy et al., 2006; Xu et al., 2008a). This effect has been confirmed for the periplasmic expression of C5a in our study as well. However, in the case of C5a, most of the protein was deposited in inclusion bodies, where it might be protected from degradation. Although no soluble C5a was detectable, it is possible that soluble C5a is produced but degraded so fast that it is not detectable. This would be consistent with the finding that C5a is extremely unstable in the human blood (Fernandez and Hugli, 1978). Nevertheless, this approach is unfeasible because it does not permit the isolation of soluble C5a.

Another possible strategy to enhance the expression of soluble C5a is the use of chaperone proteins (de Marco, 2009). de Marco et al. (2009) expressed 64 different proteins together with chaperones and thereby enhanced the solubility of nearly 70% of these proteins (de Marco et al., 2007). Interestingly, for C5a, the solubilizing effect of chaperone co-expression was dependent on the cultivation conditions and only occurred at 22°C, the temperature at which soluble C5a reached up to 0.65% of TSP, corresponding to 3.8 mg C5a per liter of cell culture.

The positive effect of the temperature reduction was also observed for expression approaches without additional chaperones, but to a lesser extent (Table 3). The final yield of C5a averaged 0.23% of TSP when cultivated at 22°C. This effect might be explained by the fact that the aggregation reaction is favored at high temperature because of the strong temperature dependence of hydrophobic interactions (Kiefhaber et al., 1991). The higher yield might also be due to reduced degradation processes at lower temperatures. In addition, it has been hypothesized that recombinant protein might accumulate in inclusion bodies when the quantity of newly produced recombinant protein exceeds the number of available chaperones. In this case, the hydrophobic stretches of proteins cannot be bound by the chaperones, and folding into the native conformation is not possible (Hoffmann et al., 2004). Lowering the temperatures may slow the protein synthesis rate to better match the chaperone capacity (de Marco et al., 2007).

Nevertheless, further slowing of the protein synthesis rate by lowering the inductor concentration did not show any effect at all, although several other authors have reported that this approach increased the soluble protein expression (Baneyx and Mujacic, 2004). Hence, the protein synthesis rate cannot be the only factor that influences the aggregation of C5a in the presence of chaperones. Recombinant antigenic C5a derived from the strains with and without chaperone-assistance showed a similar biological activity compared to the commercial standard. Interestingly the C-terminal hexahistidine residue did not seem to interfere with the C-terminal receptor binding domain, which has been recorded to be sensitive to alterations (Mollison et al., 1989). The additional His-tag might even increase the overall activity, since it should according to the manufactures manual (Novagen pET system manual), prevent the C-terminal degradation, which is assumed to deactivate the protein in *E. coli* (Mollison et al., 1987). In addition, the likeliness of degradation was reduced in our experiment by the usage of a protease deficient strain. The usage of such a strain led to a remarkable increase of insoluble C5a (Franke et al., 1988; Mandecki et al., 1986). Moreover Franke et al. (1988) could prove by protein sequencing that the protein was still intact. Hence the low amounts of soluble cytoplasmic C5a observed are unlikely to result from proteolytic degradation.

However, it has to be mentioned that a synthetic decapeptide, representing definite regions of C5a, showed a biological activity (Ember et al., 1992). Therefore biological activity does not necessarily suggest that folding has occurred.

To overcome the intrinsic instability of C5a, the fusion to a partner with protein stabilizing properties was a reasonable strategy. This strategy is widely used, as described in the reviews of Fox et al. (2003) and Marblestone et al. (2006). Indeed, solubilization or stabilization of soluble C5a was achieved by its fusion to the highly soluble protein GST (Tables 3), which is known to protect the target protein from intracellular proteases (Terpe, 2003). More than 50% of total GST-C5a was soluble when produced at 22°C, corresponding to 1.23% of the TSP or 5.1 mg C5a per liter of cell culture. However, it must be noted that the fusion protein had an inferior biological activity, with an EC_{50} value of 508.15 ng/ml, compared to the soluble, unfused C5a. The fusion tag may either alter the conformation of C5a or affect the biological activity by sterical hindrance. The last one would be more likely since C5a did show a great tolerance to alterations if the core and C-terminal binding domain or the disulphide bridging remained unaffected (Kola et al., 1999; Mollison et al., 1989). However, this sterical hindrance might be a result of the specific combination of C5a and GST, since C5a binding domains proved to be functional when exposed on the surface phages (Hennecke et al., 1997). Nevertheless downstream processing to remove the GST tag would be required to prepare a biocatalyst. This was unexpected because, in most cases, tags do not interfere with the biological activity of the target protein, and fusion proteins are commonly used to immobilize biocatalysts on a matrix for the intended application (Terpe, 2003).

C5a aggregated in inclusion bodies yielded between 1.07 and 2.58% of TSP (Table 2). This is in line with the findings of previous researchers who purified 13 mg C5a per liter of cell culture, or 3% of TSP, recovered from inclusion bodies after de- and renaturation (Mandecki et al., 1986; Mollison et al., 1987). Interestingly, the insoluble fraction showed a low activity, suggesting that some aggregated C5a is correctly folded. This is in line with previous reports, which showed that (mis) folding and aggregation are 2 independent processes (Garcia-Fruitos and Villaverde, 2010). As was found for soluble C5a, the biological activity of the inclusion bodies was highest in the strain with engineered chaperones (EC_{50} value of 828 ng/ml). However, the insoluble GST-C5a fusion protein had the lowest activity (EC_{50} value of 1356 ng/ml) because only soluble proteins can be used as therapeutics (Vincentelli et al., 2011), the insoluble fraction would require solubilization via denaturation and refolding before use. In light of the small differences in the yield of soluble and insoluble C5a, this strategy does not seem to be feasible.

To our knowledge, this is the first time that active, soluble C5a has been produced in *E. coli* directly without laborious refolding processes. The combination of temperature reduction with the overexpression of endogenous chaperones seems to be a reasonable strategy for producing large quantities of soluble and bioactive recombinant C5a.

Conclusions

Using C5a as a model, we identified essential production

parameters, which are relevant for the production of difficult-to-express proteins, and are transferable to commercial fermentation systems under cGMP conditions. In line with previous reports (Platas et al., 2011), we proved that recombinant protein yield and quality are divergent features and need to be dealt with separately. The fusion of C5a with GST improved the solubility, but negatively affected the biological activity and made laborious tag removal procedures necessary since the fusion protein was not the product of interest. The concomitant expression of chaperones, the reduction of cultivation temperature or inductor concentration all had a positive impact on the biological activity. This positive effect was significantly improved by combining these parameters, which facilitates downstream processing.

ACKNOWLEDGEMENTS

The author appreciates the grant from the doctorate program of the University of Rostock. We would like to thank Prof. Dr. Arne Skerra, Prof. Dr. Bukau and Dr. Ario de Marco for providing the chaperone expressing helper plasmids used in this study.

REFERENCES

Ali H, Richardson RM, Tomhave ED, Didsbury JR, Snyderman R (1993). Differences in Phosphorylation of Formylpeptide and C5A Chemoattractant Receptors Correlate with Differences in Desensitization. J. Biol. Chem. 268:24247-24254.

Baneyx F, Mujacic M (2004). Recombinant protein folding and misfolding in Escherichia coli. Nature Biotechnol. 22:1399-1408

Bautsch W, Emde M, Kretzschmar T, Kohl J, Suckau D, Bittersuermann D (1992). Human C5A Anaphylatoxin - Gene Cloning and Expression in *Escherichia coli*. Immunobiol. 185:41-52.

Bessette PH, Aslund F, Beckwith J, Georgiou G (1999). Efficient folding of proteins with multiple disulfide bonds in the *Escherichia coli* cytoplasm. Proceedings of the National Academy of Science the United States of America 96:13703-13708.

Choi JH, Lee SY (2004). Secretory and extracellular production of recombinant proteins using *Escherichia coli*. Appl. Microbiol. Biotechnol. 64:625-635.

Cohen J (2009). Non-antibiotic strategies for sepsis. Clin. Microbiol. Infect. 15:302-307.

Cong YH, Pawlisz E, Bryant P, Balan S, Laurine E, Tommasi R, Singh R, Dubey S, Peciak K, Bird M, Sivasankar A, Swierkosz J, Muroni M, HeidelbergerS, Farys M, Khayrzad F, Edwards J, Badescu G, Hodgson I, Heise C, Somavarapu S, Liddell J, Powell K, Zloh M, Choi JW, Godwin A, Brocchini S (2012). Site-Specific PEGylation at Histidine Tags. Bioconjugate Chem. 23:248-263.

Crane JW, Buller KM (2007). Systemic blockade of complement C5a receptors reduces lipopolysacharride-induced responses in the paraventricular nucleus and the central amygdala. Neurosci. Lett. 424:10-15.

Czermak BJ, Sarma V, Pierson CL, Warner RL, Huber-Lang M, Bless NM, Schmal H, Friedl HP, Ward PA (1999). Protective effects of C5a blockade in sepsis. Nat. Med. 5:788-792.

de Marco A (2009). Strategies for successful recombinant expression of disulfide bond- dependent proteins in *Escherichia coli.* Microb. Cell Fact. 8(1):26.

de Marco A, Deuerling E, Mogk A, Tomoyasu T, Bukau B (2007). Chaperone-based procedure to increase yields of soluble recombinant proteins produced in *E. coli*. BMC Biotechnol. 7:32.

Dombrovskiy VY, Martin AA, Sunderram J, Paz HL (2007). Rapid increase in hospitalization and mortality rates for severe sepsis in the United States: A trend analysis from 1993 to 2003. Crit. Care Med. 35:1244-1250.

Duguay AR, Silhavy TJ (2004). Quality control in the bacterial periplasm. Biochimica et Biophysica Acta-Mol. Cell Res. 1694:121-134.

Ember JA, Sanderson SD, Taylor SM, Kawahara M, Hugli TE (1992). Biol. Activity Synthetic Analogs of C5A Anaphylatoxin. J. Immunol. 148:3165-3173.

Fernandez HN, Hugli TE (1978). Primary Structural-Analysis of Polypeptide Portion of Human C5A Anaphylatoxin - Polypeptide Sequence Determination and Assignment of Oligosaccharide Attachment Site in C5A. J. Biol. Chem. 253:6955-6964.

Ferrer-Miralles N, Domingo-Espin J, Corchero JL, Vazquez E, Villaverde A (2009). Microbial factories for recombinant pharmaceuticals. MicroB. Cell Fact. 8:17.

Flierl MA, Stahel PF, Rittirsch D, Huber-Lang M, Niederbichler AD, Hoesel LM, Touban BM, Morgan SJ, Smith WR, Ward PA, Ipaktchi K (2009). Inhibition of complement C5a prevents breakdown of the blood-brain barrier and pituitary dysfunction in experimental sepsis. Crit. Care 13:R12.

Fox JD, Routzahn KM, Bucher MH, Waugh DS (2003). Maltodextrin-binding proteins from diverse bacteria and archaea are potent solubility enhancers. Febs Lett. 537:53-57.

Franke AE, Andrews GC, Stimlergerard NP, Gerard CJ, Showell HJ (1988). Human C5A Anaphylatoxin - Gene Synthesis, Expression, and Recovery of Biologically-Active Material from *Escherichia coli*. Methods Enzymol. 162: 653-668.

Garcia-Fruitos E, Villaverde A (2010). Friendly production of bacterial inclusion bodies. Korean J. Chem. Eng. 27:385-389.

Gerard C, Chenoweth DE, Hugli TE (1979). Molecular Aspects of the Serum Chemotactic Factors. J. Reticuloendoth. Soc. 26:711-718.

Goldstein M, WeissmanG (1974). Generation of C5-Derived Lysosomal Enzyme-Releasing Activity (C5A) by Lysates of Leukocyte Lysosomes. J. Immunol. 113:1583-1588

Guo RF, Ward PA (2005). Role of C5A in inflammatory responses. Annu. Rev. Immunol. 23:821-852.

Hennecke M, Kola A, Baensch M, Wrede A, Klos A, Bautsch W, Kohl J (1997). A selection system to study C5a-C5a-receptor interactions: Phage display of a novel C5a anaphylatoxin, Fos-C5a(Ala27). Gene 184:263-272.

Hoffmann F, van den Heuvel J, Zidek N, Rinas U (2004). Minimizing inclusion body Escherichia coli at bench and pilot plant scale. Enzyme Microb. Technol. 34:235-241.

Huang CJ, Lin H, Yang XM (2012). Industrial production of recombinant therapeutics in *Escherichia coli* and its recent advancements. J. Industrial Microbiol. Biotechnol. 39:383-399.

Huber-Lang MS, Sarma JV, McGuire SR, Lu KT, Guo RF, Padgaonkar VA, Younkin EM, Laudes IJ, Riedemann NC, Younger JG, Ward PA (2001). Protective effects of anti-C5a peptide antibodies in experimental sepsis. Faseb J.15:568-570.

Huber-Lang MS, Younkin EM, Sarma JV, McGuire SR, Lu KT, Guo RF, Padgaonkar VA, Curnutte JT, Erickson R, Ward PA (2002). Complement-induced impairment of innate immunity during sepsis. J. Immunol. 169:3223-3231.

Jeong KJ, Lee SY (2001). Secretory production of human granulocyte colony-stimulating factor in *Escherichia coli*. Protein Expr. Purif, 23:311-318.

Johnson RJ, Tamerius JD, Chenoweth DE (1987). Identification of an antigenic epitope and receptor binding domain of human C5a. J. Immunol. 138:3856-3862.

Kiefhaber T, Rudolph R, Kohler HH, Buchner J (1991). Protein Aggregation *in vitro* and *in vivo* - A Quantitative Model of the Kinetic Competition Between Folding and Aggregation. Bio-Technology 9:825-829.

Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, Kohl J (2009). The role of the anaphylatoxins in health and disease. Mol. Immunol. 46:2753-2766.

Kola A, Baensch M, Bautsch W, Klos A, Kohl J (1999). Analysis of the C5a anaphylatoxin core domain using a C5a phage library selected on differentiated U937 cells. Mol. Immunol. 36:145-152.

Kurokawa Y, Yanagi H, Yura T (2000). Overexpression of protein disulfide isomerase DsbC stabilizes multiple-disulfide-bonded recombinant protein produced and transported to the periplasm in *Escherichia coli.* Appl. Environ. Microbiol. 66:3960-3965.

Lauber T, Marx UC, Schulz A, Kreutzmann P, Rosch P, Hoffmann S (2001). Accurate disulfide formation in *Escherichia coli*: Overexpression and characterization of the first domain (HF6478) of the multiple Kazal-type inhibitor LEKTI. Protein Expr. Purif. 22:108-112.

Lobel L, Pollak S, Lustbader B, Klein J, Lustbader JW (2002). Bacterial expression of a natively folded extracellular domain fusion protein of the hFSH receptor in the cytoplasm of *Escherichia coli*. Protein Expr. Purif. 25:124-133.

Loo T, Patchett ML, Norris GE, Lott JS (2002). Using secretion to solve a solubility problem: High yield expression in *Escherichia coli* and purification of the bacterial glycoamidase PNGase F. Protein Expr. Purif. 24:90-98.

Mandecki W, Powell BS, Mollison KW, Carter GW, Fox JL (1986). High-Level Expression of a Gene Encoding the Human-Complement Factor C5A in *Escherichia coli*. Gene 43:131-138.

Marblestone JG, Edavettal SC, Lim Y, Lim P, Zuo X, Butt TR (2006). Comparison of SUMO fusion technol. with traditional gene fusion systems: Enhanced expression and solubility with SUMO. Protein Sci. 15:182-189.

Martin SE, Chenoweth DE, Engler RL, Roth DM, Longhurst JC (1988). C5A Decreases Regional Coronary Blood-Flow and Myocardial-Function in Pigs - Implications for A Granulocyte Mechanism. Circ. Res. 63:483-491.

Mogensen JE, Otzen DE (2005). Interactions between folding factors and bacterial outer membrane proteins. Mol. Microbiol. 57:326-346.

Mollison KW, Fey TA, Krause RA, Mandecki W, Fox JL, Carter GW (1987). High-Level C5A Gene-Expression and Recovery of Recombinant Human C5A from *Escherichia-coli*. Agents and Actions 21:366-370.

Mollison KW, Mandecki W, Zuiderweg ERP, Fayer L, Fey TA, Krause RA, Conway RG, Miller L, Edalji RP, Shallcross MA, Lane B, Fox JL, Greer J, Carter GW (1989). Identification of Receptor-Binding Residues in the Inflammatory Complement Protein-C5A by Site-Directed Mutagenesis. Proceedings of the National Academy of Science The United States of America 86:292-296.

Niederbichler AD, Hoesel LM, Westfall MV, Gao HW, Ipaktchi KR, Sun L, Zetoune FS, Su GL, Arbabi S, Sarma JV, Wang SC, Hemmila MR, Ward PA (2006). An essential role for complement C5a in the pathogenesis of septic cardiac dysfunction. J. Exp. Med. 203:53-61.

Parrish WR, Gallowitsch-Puerta M, Czura CJ, Tracey KJ (2008). Experimental Therapeutic Strategies for Severe Sepsis Mediators and Mechanisms. Neural Signaling: Opportunities for Novel Diagnostic Approaches and Therapies 1144:210-236.

Platas G, Rodriguez-Carmona E, Garcia-Fruitos E, Cano-Garrido O, Villaverde A (2011). Co-production of GroELS discriminates between intrinsic and thermally-induced recombinant protein aggregation during substrate quality control. Microbial Cell Factories 10.

Ricklin D, Lambris JD (2007). Complement-targeted therapeutics. Nat. Biotechnol. 25:1265-1275.

Riedemann NC, Guo RF, Hollmann TJ, Gao HW, Neff TA, Reuben JS, Speyer CL, Sarma JV, Wetsel RA, Zetoune FS, Ward PA (2003). Regulatory role of C5a in LPS-induced IL-6 production by neutrophils during sepsis. Faseb J. 17:370-+.

Riedemann NC, Guo RF, Neff TA, Laudes IJ, Keller KA, Sarma VJ, Markiewski MM, Mastellos D, Strey CW, Pierson CL, Lambris JD, Zetoune FS, Ward PA (2002). Increased C5a receptor expression in sepsis. J. Clin. Investig. 110:101-108.

Rittirsch D, Flierl MA, Nadeau BA, Day DE, Huber-Lang M, Mackay CR, Zetoune FS, Gerard NP, Cianflone K, Kohl J, Gerard C, Sarma JV, Award P (2008). Functional roles for C5a receptors in sepsis. Nat. Med. 14:551-557.

Schlapschy M, Grimm S, Skerra A (2006). A system for concomitant overexpression of four periplasmic folding catalysts to improve secretory protein production in *Escherichia coli*. Protein Eng. Des. Sel. 19:85-390.

Schumann W, Ferreira LCS (2004). Production of recombinant proteins in *Escherichia coli*. Genetics and Mol. Biol. 27:442-453.

Seo JH, Kang DG, Cha HJ (2003). Comparison of cellular stress levels and green-fluorescent-protein expression in several Escherichia coli strains. Biotechnol. Appl. Biochem. 37:103-107.

Terpe K (2003). Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. Appl. Microbiol. Biotechnol. 60:523-533.

Venturi M, Seifert C, Hunte C (2002). High level production of functional antibody Fab fragments in an oxidizing bacterial cytoplasm. J. Mol. Biol. pp. 315-318.

Vincentelli R, Cimino A, Geerlof A, Kubo A, Satou Y, Cambillau C (2011). High-throughput protein expression screening and purification in *Escherichia coli*. Methods 55:65-72.

Waegeman H, Soetaert W (2011). Increasing recombinant protein production in *Escherichia coli* through metabolic and genetic engineering. J. Ind. Microbiol. Biotechnol. 38:1891-1910.

Ward PA (2008). Role of the complement in experimental sepsis. J. Leukoc. Biol. 83:467-470.

Ward PA, Gao HW (2009). Sepsis, complement and the dysregulated inflammatory response. J. Cell. Mol. Med. 13:4154-4160.

Xu YL, Lewis D, Chou CP (2008a). Effect of folding factors in rescuing unstable heterologous lipase B to enhance its overexpression in the periplasm of *Escherichia coli*. Appl. Microbiol. Biotechnol. 79:1035-1044.

Xu YL, Yasin A, Wucherpfennig T, Chou CP (2008b). Enhancing functional expression of heterologous lipase in the periplasm of *Escherichia coli*. World J. Microbiol. Biotechnol. 24:2827-2835.