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Research Article

Effects of different cytoplasmic chaperones on outer membrane solubility in *Escherichia coli*; rOMP25 modeling

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ABSTRACT

In this study, for the first time in the literature, is investigated the effects of different chaperones on outer transmembrane membrane protein solubility by modeling the expression of the recombinant Omp25 outer membrane protein, which is one of the main antigens against brucellosis and known to form inclusion bodies in the cytoplasm.

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INTRODUCTION

Proteins, which make up about 50 percent of the outer membrane of Gram-negative bacteria, have many metabolic functions such as substance exchange, nutrient uptake, protein secretion and signal transduction, enzymatic activity [1]. Although six outer membrane protein families have been defined according to their structural properties, most of the outer membrane proteins are transmembrane with a β-barrel region consisting of a self-closing antiparallel layer and stood together by hydrogen bonds between the first β-strand and a conserved C-terminal β-strand [2]. Because of outer transmembrane proteins antigenic properties, high, pure and well-characterized production of recombinant outer membrane proteins is extremely important for the pharmaceutical industry as vaccine candidates [3,4].

Bacterial expression systems for recombinant protein production are preferred because of their ability to grow rapidly and at high density in cheap substrates [5]. In recent years, there are many bacterial hosts optimized for recombinant protein production, while *E. coli* remains the most preferred host [3]. On the other hand, the most preferred cellular compartment due to its advantages is the cytoplasm and this compartment has the highest efficiency [4]. However, the disadvantage of releasing into the cytoplasm, which is frequently encountered especially in outer membrane protein production, is the formation of inclusion bodies. Inclusion body formation during protein folding emerges from the hydrophobic aggregation of the unfolded states. Recombinant outer membrane proteins produced as inclusion bodies in *E. coli* are inactive, aggregated, insoluble, generally unnatural structures with intra and molecular disulfide bonds [6]. To overcome the inclusion body by providing the natural three-dimensional structure of proteins, many strategies have been developed including expression with the improving of new strains from *E. coli* BL21 (DE3), the removal of signal

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sequence, a lowering of protein synthesis rate, a growing bacterial culture at low temperatures and using of the fusion partners [6,7].

Bacterial chaperones provide the conformational processing of cell polypeptides by ensuring correct folding and preventing aggregation [8-11]. Bacterial chaperones are divided into cytoplasmic and periplasmic according to their area of responsibility. GroEL, DnaK, GroES, Tig, DnaJ and GrpE are important cytoplasmic chaperones with well-defined coordinated activity as well as independent functions identified so far [12,13]. In the literature, there are several studies to enhance soluble expression by co-expression with the PG-tf2, ptf16, pGro7, pKJE7, and pG-KJE8 plasmids containing different cytoplasmic chaperons at the recombinant production of types of proteins like different enzymes, transcription and growth factors and various antibodies [12-15].

As potential immunogenic and protective antigens for brucellosis, outer membrane proteins (OMPs) of *Brucella* spp. are important candidates to produce recombinant vaccines against brucellosis [16]. Omp25 of *Brucella abortus* is a transmembrane protein located on the outer membrane which covalently linked to the peptidoglycan layer [17]. Based on topology estimates, the Omp25 carries eight strands of β barrel area, but it has loops exposed to a smaller surface than other OMPs. The characteristic β-barrel of Omp25 is comprised of amphipathic β-strands (with alternating hydrophobic amino acid residues) ensuring their solubility during secretion through the periplasm [18]. Omp25 is one of the major virulence factors and antigens for brucellosis disease [19]. A previous study has shown that Omp25 expressed in *E. coli* resulted in inclusion bodies [20].

With the presented study, the effects of plasmids containing different molecular cytoplasmic chaperones on the soluble expression level of outer membrane protein by modeling rOmp2 will be evaluated for the first time in the literature. In addition to the use of different cytoplasmic chaperones, the effect of different temperatures and inducer concentrations on co-expression will also be determined.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), HiMedia (Mumbai, India), Merck (Darmstadt, Germany), Biorad (Hercules, CA, USA), Isolab (Wertheim, Germany), Thermo-Scientific (Massachusetts, ABD) unless otherwise specified. BL21 Star™ *E. coli* competent cells were obtained by commercial purchase of the Champion™ pET Directional TOPO® Expression Kit of Invitrogen (California, USA). The chaperone plasmid set containing **pG-KJE8**, **pGro7, pKJE7**, **pG-Tf2**, **pTf16** plasmids was purchased from TaKaRa Bio (Otsu, Japan).

Transformation of Chaperone Plasmids and Omp25 Plasmid into *E. Coli* Bl21 (De3) Cells

The blunt-ended Omp25 amplicon obtained with appropriate primers from genomic DNA isolation of *B. abortus* biovar type 3 strain has been cloned in the fusion pET102 expression vector. This vector contains thioredoxin which increases translation yield and protein solubility and 6xHis tag for protein purification [19, 21]. An efficient method for constructing a system for co-expression of target proteins and chaperones includes two steps: transformation of chaperone plasmid to host and then transformation of expression plasmid for the target protein. At the first stage chaperone plasmids were separately transformed BL21 (DE3) chemically competent *E. coli* cells, then pET102 with Omp25 expression plasmid was also transformed into the cells including chaperone expression plasmids.

Coexpression of the Omp25 Gene with Chaperone Encoding Plasmids

To perform co-expression, the transformants were inoculated into 100 mL of LB medium containing related antibiotics for plasmid selection (100 μg/mL ampicillin and 20 μg/mL chloramphenicol) and inducer according to the type of chaperone plasmid (0.5-4 mg/mL L-arabinose and 1-10 ng/mL tetracycline concentration range). Only L-arabinose for pG-KJE8 plasmid; tetracycline and L-arabinose for the pGro7, pKJE7 and pTf16 plasmids; only tetracycline for pG-Tf2 plasmid were used the induction of chaperone expression and then the culture was shaken in a rotatory shaker at 37°C and 200 rpm. When the OD600 reached 0.6, the cultures were induced by IPTG addition. After induction, cells were shaken at 30°C 200 rpm at the stated time. In addition, the inducer of chaperone plasmids was tested in four different concentrations. Optimization studies including temperature (25 and 30 °C) and inducer concentration (0.5 and 1 mM IPTG) were carried out to obtain soluble protein at the highest yield. After expression period, samples were taken, centrifuged, resuspended in lysis buffer (pH 7.8) and disrupted on ice by sonication. All protein fractions were separated from the cell lysate by centrifugation at high speed 20 min., 4°C, 14000 g. Soluble and inclusion body fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

SDS-Page and Western Blot Analyses

After expression, all protein fractions were analyzed by SDS-PAGE and stained with Coomassie blue defined by Laemmli [22]. For Western blot analyses, the separated proteins in SDS–polyacrylamide gels were transferred to polyvinylidene difluoride membranes using transfer buffer (48 mM Tris base, 39 mM glycine, 1.3mM SDS, 20 % methanol) and subsequently probed with anti-His antibody in conjunction with IgG-HRP. Western blot analysis was carried out using His-probe Antibody (H-3): sc-8036 (SantaCruz, CA, USA) and Goat anti-Mouse IgG (H+L) (Invitrogen,

USA). Western blots were performed according to the manufacturer's instruction (Biorad, USA). Protein quantification assays were performed with Bradford Dye reagent (Biorad, USA).

Purification of rOMP25

The purification process of cultures which contains different chaperone plasmids and pET102 with Omp25 was conducted in two different ways both native and denaturing conditions. Cell pellet was resuspended in 10 mL of binding buffer (for native conditions) or lysis buffer (for denaturing conditions), and sonicated on ice (3 min, 50% pulse and 50% amplitude). The homogenate was then centrifuged at 15,000 g for 15 min at 4 °C, and the supernatant was loaded onto centrifuge tubes (with 1 mL of Ni–NTA agarose) and 2 mL binding buffer added. The protein was collected from the column with elution buffer which contains 50 mM NaH_2PO_4, 500 mM imidazole, 500 mM NaCl (pH 7.4). For denaturing conditions, elusion buffers contain Triton x-100, 500 mM NaCl, 8 M urea and 500 mM imidazole (pH 7.4), respectively. The proteins of the separated fractions both native and denaturing conditions were analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

The effect of parameters of chaperone plasmids, temperature, and inducer concentration on the recombinant outer membrane protein solubility to be produced was evaluated.

Two Steps Transformation

The six different transformation reactions were established for each chaperone plasmids and pET102 with Omp25. All of them were separately transformed into *E. coli* BL21(DE3) competent cells according to the relevant transformation procedure [23]. Then, it was incubated for 17 h at 37 °C on LB agar containing 20 μg/mL chloramphenicol and 100 μg/mL ampicillin, and the colonies selected and verified the colony PCR reaction (data not shown). After that, the pET102 with Omp25 plasmid isolation was conducted from the competent cell using the ZymoPURE Plasmid Miniprep Kit (The amount of plasmid DNA; 110 ng/μL; A260/A280 ratio; 1.9) [24]. The transformation of pET102 with Omp25 into the five different competent cells containing five chaperone plasmids was performed according to the transformation procedure.

Determination of Different Chaperone Plasmids, Inducer Concentration and Temperature Effect on Co-Expression of Romp25

CO-expression at 30°C with different inducer concentrations

Expression of the Omp25 in *E. coli* BL21 (DE3) was induced using 1 mM IPTG with chaperone plasmids for 5h at 30 °C. Total weight of rOmp25 is around 41 kDa, which is the 25 kDa outer membrane protein together with the 13 kDa thioredoxin protein and the His-tag of 3 kDa.

Since the concentration amount of L-arabinose inducer of pGro7, pKJE7 and pTf16 chaperone plasmids is recommended in the range of 0.5-4 mg/mL, 4 different concentrations were tried (0.5 mg/mL-1 mg/mL-2 mg/mL- 4 mg/mL) and the best result was obtained with the use of 1 or 2 mg/mL L-arabinose and the results are presented detail in Figure 1 (b-c-d). The inducer concentration of pG-Tf2 chaperone plasmid tetracycline was recommended in the range of 1-10 ng/mL, 4 different concentrations were tried (1 ng/ml- 2 ng/ml- 5 ng/mL -10 ng/ml) and the best result in chaperone expression 5-10 ng/mL was obtained using tetracycline and the results are shown in detail in Figure 1(e). The inducers of the pG-KJE8 chaperone plasmid are L-arabinose and tetracycline. L-arabinose concentration was kept at 1 mg/mL and 4 different tetracycline concentrations were tried. The best chaperone expression was observed in the use of 5 and 10 ng/mL tetracycline with SDS-PAGE results and chaperone expression profiles are shown in detail in Figure 1(f).

As in Figure 1 (e) shown; surprisingly, expression of rOmp25 protein in pellet reduced with the co-expression of pG-Tf2 plasmid. Especially, when the pG-Tf2 plasmid was induced with 10 ng/mL tetracycline, rOmp25 protein expression decreased compared to 5 ng/mL tetracycline. In Figure 1(d), in the presence of 2 mg/mL L-arabinose inducing agent, chaperone protein expression was significantly increased under co-expression conditions with pTf16 plasmid, while Omp25 expression decreased. When pKJE7 plasmid with 1 and 2 mg/mL L-arabinose inducers was stimulated, the expression levels of both chaperone proteins and Omp25 protein were similar in Figure 1(c). In Figure 1(b), while induction of pGro7 plasmid with 1 or 2 mg/mL L-arabinose did not change the level of chaperone expression, Omp25 expression induced by 2 mg/mL L-arabinose was higher than 1 mg/mL. Finally, when pG-KJE8 plasmid was stimulated with 5 ng/mL tetracycline and 1 mg/mL arabinose inducers, rOmp25 expression was higher than 10 ng/mL tetracycline and 1 mg/mL arabinose (Figure 1f). The co-expression culture with pG-KJE8 chaperone plasmid increased rOmp25 amount in the pellet. However, rOmp25 was not observed in the supernatant in SDS-PAGE analysis because of expression trials with molecular chaperones for 5 hours at 30 °C, but it was detected in the pellet.

CO-expression at 25°C with 1MM IPTG

Lowering the temperature which is one of the strategies to increase protein solubility, was accomplished by inducing expression during 21 hours at 25 °C. Expression was induced with 1 mM IPTG for 21 hours at 25 °C. Chaperone inducer concentrations were chosen according to 30 °C expression studies and optimum concentrations were determined as 1 mg/mL for L-arabinose and 5 mg/mL for tetracycline (Figure 2).

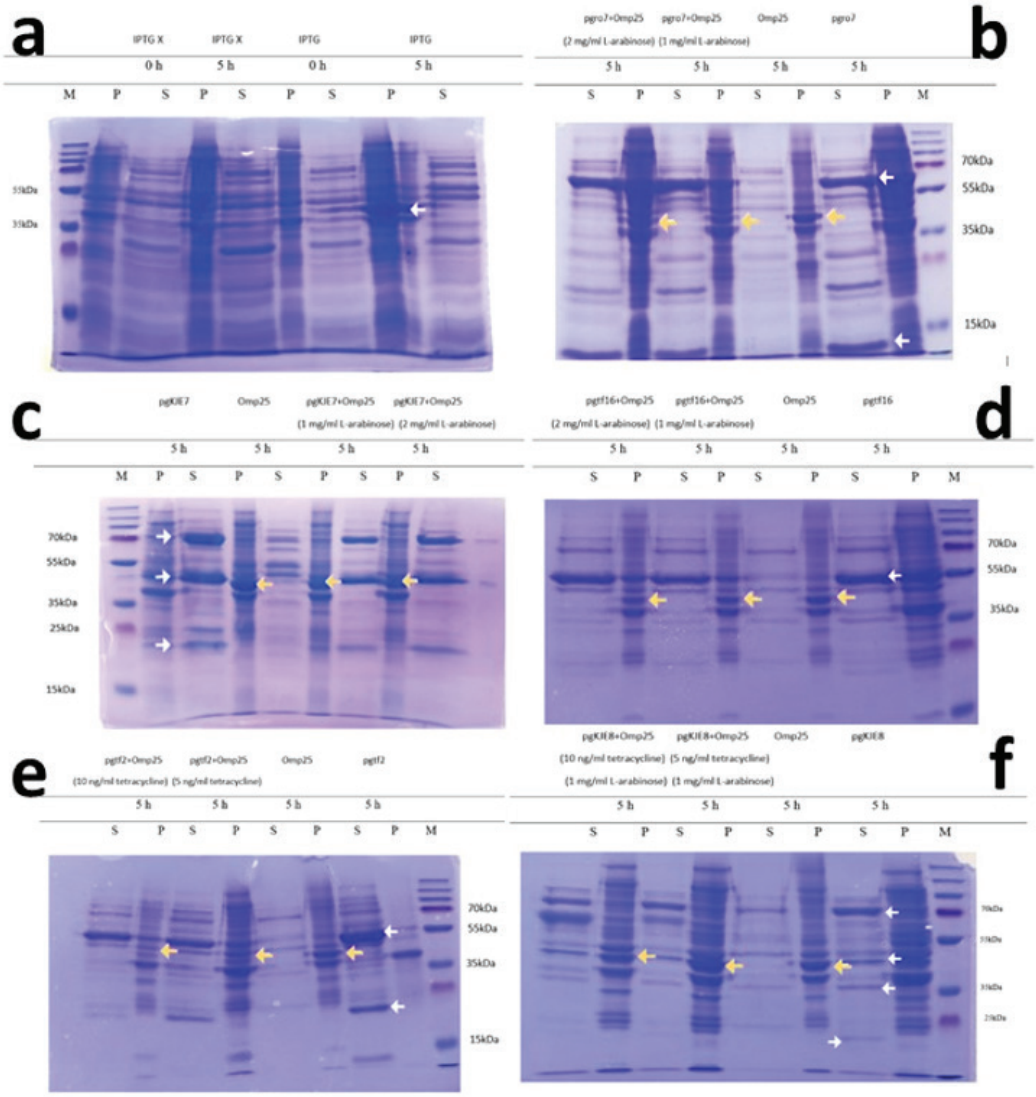

Figure 1. The expression profile of *E. coli* BL21 (DE3) harboring the pET102 with Omp25 is shown in **Figure 1(a).** Cell expression profiles containing other five chaperone plasmids (including pGro7, pKJE7, pTf16, pGKJE8, pG-Tf2 separately) and pET102 with Omp25 together provided separately in different cultures, respectively in **Figure 1 (b-c-d-e-f).** Chaperones plasmids and their chaperones with molecular sizes: i) pGro7; GroEL (60 kDa) and GroES (10 kDa), ii) pKJE7; DnaK (70 kDa), DnaJ (40 kDa) and GrpE (22kDa), iii) pTf16; Tig (56 kDa), iv) pG-KJE8; DnaK, DnaJ, GrpE, GroEL, groES and Tig, v) pG-Tf2: GroEL, GroES, and Tig. Thick bands observed in the supernatant are the induced chaperone plasmids which are in different molecular weights. While the white arrows indicate the different types of chaperones, the yellow arrows show Omp25 plasmid.

As in Figure 2(a) shown, in the co-expression study with plasmid pG-Tf2, the total amount of soluble protein increased significantly at 25 °C compared to 30 °C. Omp25 expression in the pellet decreased with co-expression of pG-Tf2 compared to other chaperone plasmids. The amount of soluble protein in the presence of PTf16, pKJE7 and pGro7 plasmids increased significantly at 25 °C. Expression of rOmp25 was increased in both the pellet Figure 2(a-b-c). In Figure 2(d), while the expression of soluble protein in the presence of plasmid pG-KJE8 increased again at 25 ° C, it was observed that rOmp25 expression in the pellet decreased.

CO-expression at 25°C with 0.5 MM IPTG

Reducing the concentration of inducer, another strategy of increasing soluble protein expression, was also evaluated by reducing the amount of IPTG in the study. For this purpose, expression was induced for 21 hours at 25 °C using 0.5 mM IPTG. However, trials on co-expression with pG-Tf2 weren't carried out because of lower expression. After all,

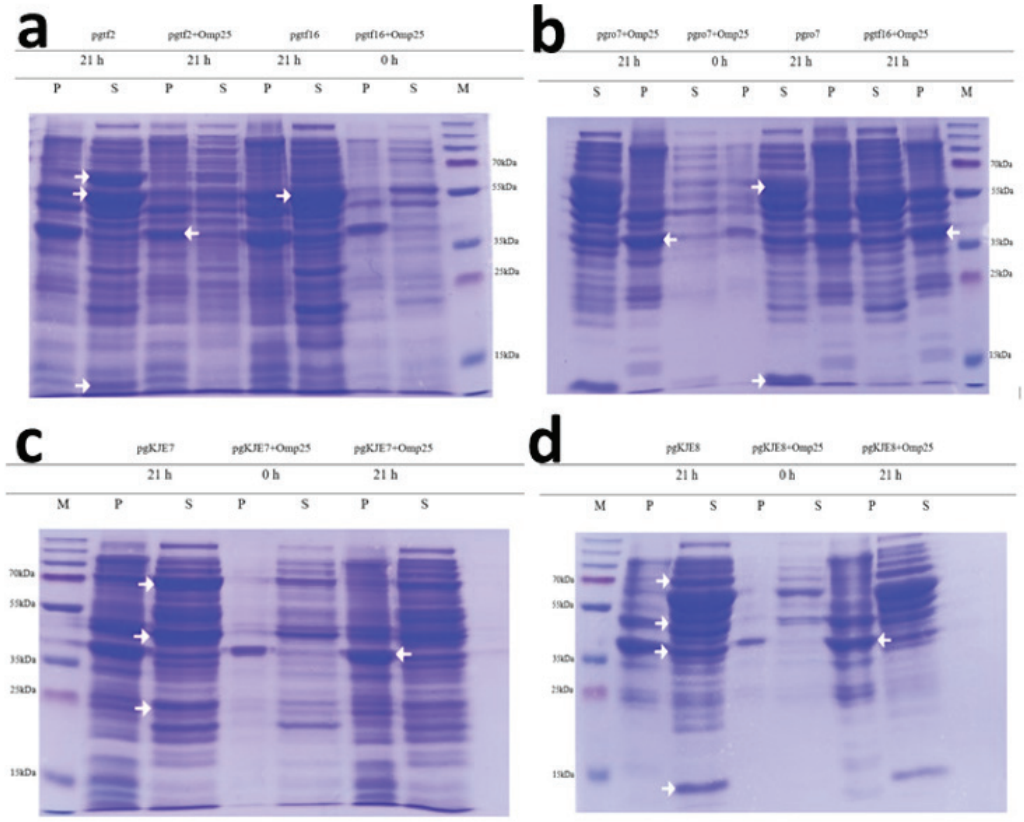

Figure 2. The expression profile of *E. coli* BL21 (DE3) containing the pET102 with Omp25 plasmid with different chaperon plasmids at 25°C by 1 mM IPTG. (a); The culture expression profile containing the pGTf2 and pTf16 chaperon plasmids with Omp25 plasmid. Thick bands observed in the supernatant are the induced GroEL, GroES and tig chaperones, (b); The culture expression profile containing the pTf16 and pGro7 chaperone plasmids with Omp25 plasmid. Thick bands observed in the supernatant are the induced tig, groEL and groES chaperones, (c); The culture expression profile containing pKJE7 chaperone plasmid with Omp25 plasmid. The thick bands observed in the supernatant are the induced dnaK, dnaJ and grpE chaperone, (d); The culture expression profile containing pGKJE8 chaperone plasmid with Omp25 plasmid. The thick bands observed in the supernatant are the induced dnaK, dnaJ, grpE, groEL, groES, tig chaperone (The arrows in the right direction show the chaperones, while the left arrow indicates the expressed Omp25).

SDS-PAGE analyzes are seen in Figure 3. As a result, as expected, the amount of protein in the pellet decreased to 1 mg / ml, but no significant difference was observed in the amount of protein in the supernatant.

In Figure 3, the better results were obtained in co-expression performed with pKJE7 and pG-KJE8 plasmids rather than co-expression with other plasmids. However, for all plasmids, the total amount of protein decreased with 0.5 mM IPTG induction compared to the presence of 1 mM inducing agent.

Western Blot

The presence of the 41 kDa Omp25 fusion protein expressed in BL21 (DE3) *E. coli* host was confirmed using the Western Blot analysis (Figure 4). In western blot, while Omp25 was detected in the expression pellet at 25 °C, it was not detected in the supernatants. Western blot analysis was also applied on the elution after purification but the presence of Omp25 could not be confirmed again. Commercial recombinant Omp25 (rOmp25) protein was used as positive control (The commercial rOmp25 expected protein weight is 27.8 kDa.)

Purification of rOMP25

The his-tag regions were added to the C-terminal of the recombinant Omp25 to enable purification to be accomplished by affinity chromatography. The purification was performed under natural and denaturing conditions from the culture obtained because of the expression induced at 25 °C of the cell containing all chaperone plasmids and pET-102 with Omp25. While the protein band of the expected size (41 kDa) wasn't obtained in the purification process carried out under natural conditions (Figure 5) but it was detected in the purification process under denaturing conditions.

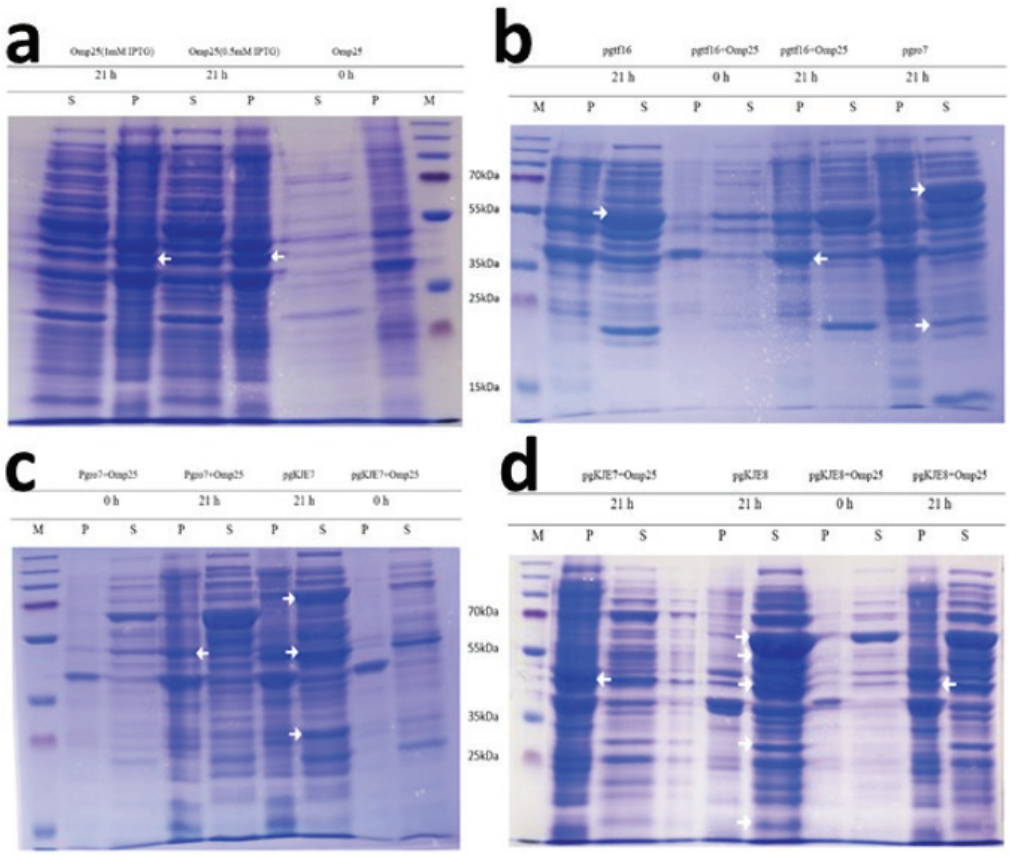

Figure 3. The expression profile of *E. coli* BL21 (DE3) containing the pET012 with Omp25 plasmid with different chaperon plasmids at 25°C by 0.5 mM IPTG. (a); The culture expression profile containing only Omp25 plasmid induced for 21 hours with 0.5mM and 1 Mm IPTG, (b); The culture expression profile containing the pTf16 and pGro7 chaperon plasmids with Omp25 plasmid. Thick bands observed in the supernatant are the induced groEL and groES chaperones, (c); The culture expression profile containing the pGro7 and pKJE7 chaperone plasmids with Omp25 plasmid. Thick bands observed in the supernatant are the induced dnaK, dnaJ, grpE, groEL and groES chaperones, (d); The culture expression profile containing pKJE7 and pGKJE8 chaperone plasmids with Omp25 plasmid. The thick bands observed in the supernatant are the induced dnaK, dnaJ, grpE, groEL, groES, tig chaperone. The arrows in the right direction show the chaperones, while the left arrow indicates the expressed Omp25.

Figure 4. Western blot analysis of rOmp25 after expression induced with different molecular chaperones at 25 °C (M: Marker, 1: commercial rOmp25, 2: pTf16-Omp25 pellet, 3: pGro7-Omp25, 4: pGTf2-Omp25, 5: pGKJE8-Omp25 pellet, 6: pTf16-Omp25 pellet).

The use of outer membrane proteins with antigen properties as vaccine candidates is extremely important in preventive medicine, especially in protection from microbial infections [25,26,27]. Therefore, the production of these proteins in pure and high amounts is a critical step in the pharmaceutical industry. Bacterial expression systems such as *E. coli* are often used during the production process with recombinant DNA technology. However, one of the most serious limitations is the formation of an inclusion body, which is an aggregate, biologically inactive, insoluble. In this study, the effect of five different chaperone plasmids with different temperature and inducing concentration parameters on outer transmembrane protein solubility as rOmp25 modeling, which is one of the important outer membrane proteins in *B. abortus*, was investigated for the first time in the literature.

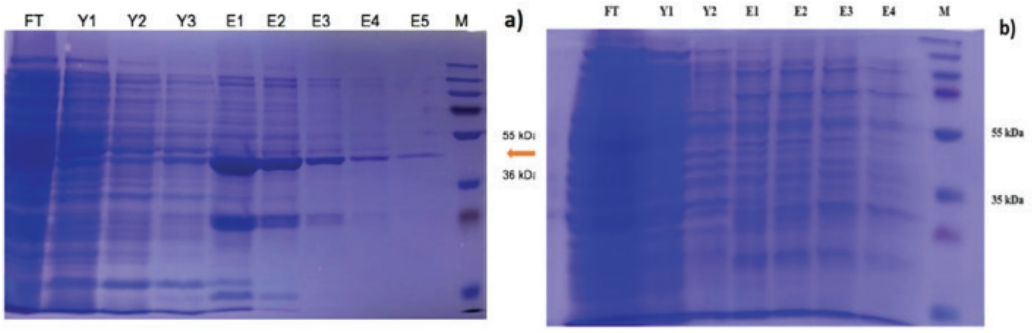

Figure 5. Purification image of the expression culture induced by the cell containing chaperone plasmid and Omp25 gene region at 25 °) In the denature conditions b) In the native conditions (FT: Flow through; Y1: Wash1; Y2: Wash 2; E1: Elution1; E2: Elution 2; E3: Elution 3; E4: Elution 4).

Co-expression of five chaperone plasmids with Omp25 under different temperature and inducer concentration had no significant effect on Omp25 outer membrane protein solubility, which is known to form the inclusion body. However, it was clearly determined that the two chaperone plasmids have different effects on outer membrane protein production. Firstly, it was observed that rOmp25 in the pellet decreased in the present of pG-Tf2 plasmid containing GroEL, GroES, and Tig chaperones at 30 and 25 °C with 1 M IPTG concentration. The trigger factor in this plasmid, which is in the ribosomal exit site, directs the newly synthesized polypeptide chains, protects the growing polypeptide chains against misfolding and aggregation, allows it folding directly to the natural structure, and also is supported by additional chaperones such as DnaK and GroEL that do not bind to ribosomes. The interaction of TF occurs through hydrophobic interactions. In the same plasmid, GroEL and GroES chaperones interact with the hydrophobic regions of substrates of the unfolded or misfolded proteins. Since the intensity of hydrophobic interactions in polypeptide binding depends on the thermodynamic properties of the binding reaction, it is thought that the desired interaction between rOmp25 and these chaperones does not occur. Secondly, pG-KJE8 (DnaK, DnaJ, GrpE, GroEL, GroES, and Tig chaperones) plasmid increased rOmp25 in the pellet in the presence of 1 and 0.5 M inducer, respectively, at temperatures of 30 and 25 °C. The plasmid continued its effectiveness by decreasing the inducer concentration when the temperature decreases. Other plasmids in the study had no significant effect on rOmp25. Also, decreasing the expression temperature to 25 °C in the study increased the total amount of protein in the supernatant (1.7 mg/ml). Although there were thick bands in the region of about 41 kDa in the supernatant for each expression profile at this temperature, these could not be confirmed as rOmp25 by western blot analysis. Therefore, the thick bands of this size in SDS-PAGE are thought to be either another protein produced by *E. coli* or could not be confirmed by western blot analysis due to low rOmp25 production. However, the rOmp25 in the pellet was verified by Western Blot analysis. When inducer concentration was halved, the total amount of protein in the pellet decreased to 1 mg/ml, but there was no significant difference in the amount of protein in the supernatant and rOmp25 in the pellet remained stable compared to the concentration of 1 mM IPTG. Also, although the expected size (41 kDa) protein band could not be obtained in the purification process carried out under natural conditions, it was detected and confirmed in the purification process under denaturing conditions.

CONCLUSION

In the literature, it is seen that solubility enhancement trials with molecular chaperones differ in different types of recombinant proteins. However, there is no study related to the expression of outer membrane proteins. The cold-active lipase gene Lip-948 was transformed with pColdl plasmid into *E. coli* BL21 and expressed by SDS-PAGE analysis that the protein constitutes approximately 39% of the total protein and most of them are inclusion bodies. However, although co-expression with pTf16 and pGro7 chaperones decreased the amount of soluble LIP-948, soluble expression increased with co-expression with the chaperone plasmids pKJE7, pG-Tf2, pG-KJE8, respectively [28]. In another study, co-expression of 3A21 scFv (an antibody), with different cytoplasmic and periplasmic chaperones was investigated and was found the changing in percentages of antigen-binding activity in the cytoplasmic soluble fraction, the periplasmic fraction, and the extracellular medium, but there was no substantial distinction in the total activity [29]. Also, in the study performed by Nazari et al., it was shown that the expression of pG-KJE8 cytoplasmic chaperone plasmid together with humanized anti-EGFR scFv increased the amount of active soluble scFv, while pG-Tf2 containing tig chaperone had the least effect [15], and the results are like our results. Mirzahoseini et al.

examined that whereas the co-expression of periplasmic chaperones Skp and FkpA was extremely advantageous for the secretory production of scFvs by using *E. coli*, cytoplasmic chaperones and multiple-chaperone combinations weren't efficient [30]. Lastly, soluble expression of CD137L (an antibody) was improved significantly by co-expression with pG-Tf2 chaperone and the purified rCD137L yield was greater higher than the original level. There wasn't observed any significant effect among co-expression with other different chaperone plasmids [31]. Since both the proteolytic stability and solubility of a particular recombinant protein cannot be predicted, trials for soluble protein production are ongoing.

AUTHORSHIP CONTRIBUTIONS

Authors equally contributed to this work.

DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

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