Utami *et al*. (2023) Vol. 11 No. 2: 87-95

**Chimica et Natura Acta**

p-ISSN: 2355-0864 e-ISSN: 2541-2574

Homepage: http://jurnal.unpad.ac.id/jcena

# Review Article: Effect of Co-Expression Chaperones on the Expression of Intracellular Recombinant Proteins in *Escherichia coli*

Diandra Firdiani Utami[1]*, Mamlikatu Ilmi Azizah[1], Sriwidodo Sriwidodo[2], Ryan Adibagus Haryanto[3], Riyona Desvy Pratiwi[4] and Iman Permana Maksum[1]*

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jl. Raya Bandung-Sumedang km 21, Jatinangor, 45363, West Java, Indonesia.
[2]Department of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, Universitas Padjadjaran, Jl. Raya Bandung-Sumedang km 21, Jatinangor, 45363, West Java, Indonesia.
[3]PT. Bio Farma, Bandung, Jl. Pasteur No. 28, Bandung 40161, West Java, Indonesia
[4]Indonesian Institute of Sciences, Jl. Raya Bogor Km. 46, Cibinong, Bogor 16911, West Java, Indonesia
*Corresponding author: iman.permana@unpad.ac.id, diandra17001@mail.unpad.ac.id

DOI: https://doi.org/10.24198/cna.v11.n2.46480

**Abstract:** *Escherichia coli* is widely used as a host for expressing recombinant proteins due to its well-studied genetics, fast growth, relatively low production costs, and high rate of protein expression. However, despite the high rate of protein expression, the availability of chaperone proteins was often insufficient, resulting in the formation of inclusion bodies due to errors in protein folding. These inclusion bodies can cause the protein to become inactive, and proper protein folding is crucial for maintaining the structure and function of proteins in living organisms. To overcome this limitation, chaperones have been developed as a strategy to help prevent protein folding errors and increase the recovery of soluble protein. In this review, we summarize several experiments related to co-expressing chaperones to enhance the expression of recombinant proteins in *E. coli.*

**Keywords:** *escherichia coli*, recombinant proteins, chaperones, inclusion bodies, protein folding

***Abstrak*:** *Escherichia coli merupakan bakteri yang paling banyak digunakan sebagai inang pada ekspresi protein rekombinan. Penggunaan Escherichia coli sebagai inang memiliki kelebihan diantaranya, genetikanya telah dipelajari dengan baik, pertumbuhannya cepat, biaya produksi relatif murah, dan laju ekspresi protein yang tinggi. Namun, tingginya laju ekspresi protein pada Escherichia coli tidak diimbangi dengan ketersediaan protein chaperon sehingga terbentuklah badan inklusi yang disebabkan oleh kesalahan pelipatan protein. Terbentuknya badan inklusi dapat menyebabkan protein menjadi tidak aktif. Salah satu strategi yang dikembangkan untuk mengatasi keterbatasan tersebut adalah penggunaan chaperon yang berfungsi untuk membantu mencegah terjadinya kesalahan pelipatan protein serta meningkatkan perolehan protein terlarut. Dalam artikel review ini, akan dipaparkan gambaran umum mengenai pengaruh ko-ekspresi chaperon (GroEL-ES dan Trigger Factor) dalam ekspresi protein rekombinan di Escherichia coli. Artikel ini diharapkan dapat menjadi referensi untuk penggunaan ko-ekspresi chaperon pada ekspresi protein rekombinan secara di Escherichia coli.*

***Keywords:*** *badan Inklusi, Chaperon, Escherichia coli, Ko-Ekspresi*

## INTRODUCTION

Recombinant proteins are proteins produced using recombinant DNA technology, commonly referred to as genetic engineering. Recombinant DNA technology is defined as the transfer of genetic material from one organism to another with the aim of making genes easier to engineer, study, and obtain products that match the desired characteristics and in large quantities (Maksum *et al*. 2017). *Escherichia coli* is a gram-negative bacterium that is usually used as a host cell for recombinant protein expression (Maksum *et al*. 2019). This is because *E. coli* has advantages such as fast growth, a cheap growth medium, and well-known genetics (Chae *et al*. 2017; Maksum *et al*. 2017). Protein production in *E. coli* can be achieved using two approaches: extracellular and intracellular (Sriwidodo *et al*. 2017; Maksum *et al*. 2017; Silaban *et al*. 2019). Su *et al*. (2006) stated that the level of extracellular protein expression was lower than intracellular protein expression (Wang et al., 2017). Therefore, intracellular protein expression can be used to increase recombinant protein expression. However, intracellular protein expression in *E. coli* has limitations, namely, it often leads to the

formation of inclusion bodies. The formation of inclusion bodies can be caused by protein folding errors and the lack of chaperones, which cause the protein to fail to form the original conformation in the reduced cytoplasmic environment and have the potential to be degraded by proteases (Chaudhuri & Paul 2006).

Several strategies can be used to overcome these problems, such as using addition of MBP protein fusion protein, refolding with freeze thawing method, glutathione redox system, and chaperon co-expression. Chaperones can be defined as proteins that monitor non-native conformations, stabilize the protein, and aid the folding process but are not part of the final structure of the original protein (Ellis *et al*. 1987). Various heterologous proteins expressed in *E. coli* using chaperone co-expression have been shown to increase the soluble fraction and decrease inclusion bodies. Therefore, a protein expression system with the help of chaperones was chosen to aid in proper protein folding and prevent the formation of inclusion bodies, increasing the efficiency of protein folding (Chaudhuri & Paul 2006). In this review article, we summarize the effect of chaperone co-expression on recombinant protein expression in *E. coli.*

## RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technology is a technique used to express a gene in different organisms by utilizing the central dogma system (Gellissen 2005). It involves transferring genetic material from one organism to another that has a different evolutionary level. The transfer aims to make it easier to engineer genes to produce the desired product in large quantities, which facilitates further study or commercial production (Maksum *et al*. 2017). The main components of recombinant DNA technology are genes or target DNA, vectors, and host cells. The target DNA is inserted into the vector using restriction endonuclease and ligase enzymes. Vectors that already contain target DNA are then transformed into host cells for expression (Gellissen 2005).

Host cells used in recombinant DNA technology consist of various types, including bacteria, yeast, insect cells, plant cells, and mammalian cells. Host selection is determined based on the expression level and the need for post-translational modification of the target protein (Demain & Vaishnav 2009). Selecting the expression system is important in the production of recombinant protein because it affects the protein acquisition and ease of the purification process. In the production of recombinant proteins, the strategy of the expression system used and the target location of the expressed protein are also taken into account. The bacterial host has a cell membrane consisting of an outer and inner membrane; therefore, the location of the expressed protein can be in the cytoplasm, periplasm, or medium. The intracellular and extracellular expression systems are commonly used (Choi *et al*. 2006). The intracellular protein expression system occurs in the host cytoplasm, while the extracellular expression directs protein expression to the culture medium by adding signal peptides. The level of protein expression using intracellular method has a higher rate than extracellular (Choi & Lee 2004; Su *et al*. 2006).

## *Escherichia coli* EXPRESSION SYSTEM

*Escherichia coli* is a Gram-negative, rod-shaped, with a cell wall made of peptidoglycan, and is facultative anaerobic bacterium (Reece *et al*. 2014). These bacteria are commonly used in recombinant protein work. *E. coli* has a simple regulatory mechanism in expressing structural genes. Structural genes are grouped on one chromosome and expressed together. The expression system in *E. coli* uses an operon system where structural genes will be transcribed into polycistronic mRNA, which will then be translated into proteins. In addition to structural genes, in one operon, there is also a single regulator and promoter gene. This single promoter plays a role in the initiation of transcription and regulates the expression of genes on the operon (Nelson & Cox 2013).

The operon systems in *E. coli* that are commonly used in the production of recombinant proteins include the lac operon, which is negatively regulated, and the RHA operon, which is positively regulated (Baneyx 1999; Wegerer *et al*. 2008). The use of appropriate promoters in the expression of recombinant proteins will have a crucial impact on the expression process. The criteria for a good promoter include having a low basal expression level, so that the gene is tightly regulated, being easily transferred to other *E. coli* strains, having induction that can be done simply, and having an inducer that is not one of the culture media materials (Terpe 2006).

## INCLUSION BODY

In protein production through recombinant DNA technology, heterologous protein expression can occur at a high rate. In the host *E. coli*, the expression of excess protein can cause the formation of aggregations and insoluble solids called inclusion bodies (Jungbauer & Kaar 2007). Inclusion bodies are formed from folded, partially folded, and misfolded protein aggregates. They usually occur due to the rapid expression rate, but the chaperone activity fails to form the original conformation in a formation-reduced environment, leading to the potential for degradation by proteases. The formation of inclusion bodies is also caused by intermolecular protein interactions, both ionic and hydrophobic. Therefore, the proteins in inclusion bodies are generally inactive (Singh & Panda 2005).

The conformation of the inclusion body structure varies, and its formation is influenced by certain amino acid sequences. Classical inclusion bodies are

those that are resistant to protease degradation, while non-classical ones are known to have activities similar to native forms (Upadhyay *et al*. 2012; Maksum *et al*. 2019). It is proven that inclusion bodies produced at low temperatures have good activity but are prone to degradation. Meanwhile, when grown at high temperatures, their stability is greatly increased even though their activity is decreased or lost. At high temperatures (42ºC), the energy to form intermolecular interactions is achieved, so the formation of inclusion bodies tends to occur, although activity is lost and production tendency is low. The low temperature (18ºC) causes the protein to fold to the native shape slowly, blocking aggregate-forming sites and preventing aggregate formation (Strandberg & Enfors 1991; de Groot & Ventura 2006). The tight-sheet structure is responsible for the stability of the inclusion body. However, the strong hydrophobic/hydrophilic interaction on the helix is also one of the influencing factors (Upadhyay *et al*. 2012)

.

## THE ROLE OF CHAPERONE IN PROTEIN FOLDING

Proteins are linear polymers synthesized by ribosomes from activated amino acids. The product of this biosynthesis is a polypeptide chain that has a three-dimensional structure, characteristics, and functions. Protein folding is a crucial process because incorrect folding can disrupt the function of the protein. In protein folding in cells, proteins called chaperones help fold and maintain the functional state of the protein chaperon (Walter & Buchner, 2002). Chaperones can be found in all cell compartments where folding or conformational rearrangement of proteins occurs. Molecular chaperones have several roles: they act as holdases to help stabilize the conformation of non-native proteins, as foldases to assist in the folding process to the native state, or as unfoldases to open proteins that fail to fold properly or extract proteins from their aggregates (Hoffman *et al*., 2010).

## CHAPERONE GroE

GroE protein is a molecular chaperone derived from *E. coli* bacteria. The groEL and groES genes encode 57 kDa and 10 kDa proteins, respectively, and both are required for the survival of *E. coli* (Grallert & Buchner 2001). A characteristic of GroEL is its quaternary structure, which resembles a barrel that is open at both ends. GroEL consists of three domains: equatorial, apical, and intermediate, as shown in Figure 1. The equatorial domain is composed of the middle part of the barrel, which binds and hydrolyzes ATP and mediates all contacts between the two rings. The apical domain is located on the outer periphery of the barrel and serves to bind protein substrates and GroES co-chaperones. The equatorial and apical domains are linked by intermediate domains that serve as hinges allowing for the rearrangement of large structures during the GroE functional cycle. The GroEL ring forms a cavity that serves as a folding compartment for the polypeptide substrate (Xu *et al*. 1997). GroES is a co-chaperone of GroEL, which has a dome-shaped ring structure and consists of seven subunits. An important feature of GroES is a mobile loop, a 16 amino acid stretch that mediates binding to GroEL and is dependent on the presence of ADP and ATP bound to the equatorial domain of GroEL (Klein & Georgopaulus 2001). GroEL and GroES are bacterial chaperonins that function together as a complex. GroEL acts as a folding cage, providing a protected environment for substrate proteins, while GroES acts as a lid to regulate the entry and release of substrates. Co-expression of GroEL and GroES ensures the proper assembly and function of the chaperonin complex, allowing efficient folding of proteins.

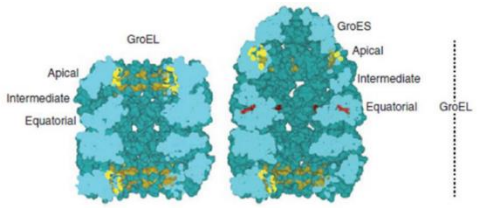


**Figure 1.** Crystallographyc models of unliganded GroEL and an asymmetric GroEL-GroES-ADP$_7$ complex. Image adapted from Ref. (Horwich 2011).

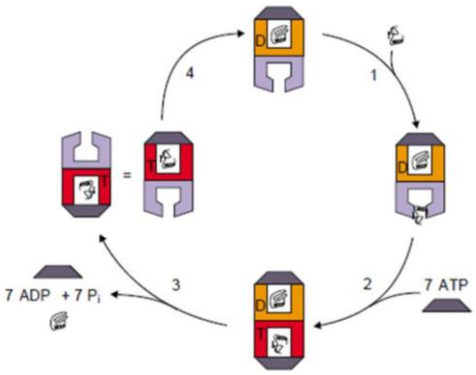


**Figure 2.** GroE chaperone cycle. Upper part: cis ring; bottom: ring trans. Polypeptide substrate capture (1), binding ATP and GroES (2), the release of GroES and folded polypeptide (cis ring) (3), hydrolysis of trans ring ATP, readily binding new polypeptides. Image adapted from Ref. (Walter & Buchner 2002).

The GroE chaperon cycle (Figure 2) consists of four steps. First, hydrophobic polypeptides are prevented from aggregating by binding to GroEL. The acceptor ring (purple) is nucleotide-free and therefore has a high affinity for the polypeptide. Second, the binding of ATP (T) and GroES to the

ring induces structural changes in GroEL (red ring in the figure). The affinity for the bound polypeptide decreases and it is released into the closed cavity where it begins to fold. Third, after ATP binds to the trans ring (red), GroES is released from the cis ring (orange), and the substrate polypeptide is released. Fourth, subsequent hydrolysis of ATP induces a second conformational change in GroEL (upper ring, orange) which allows the trans ring (lower ring, purple) to bind to the polypeptide and initiate a new cycle (Walter & Buchner 2002).

The trigger factor is an ATP-independent chaperone found in bacteria. The Trigger Factor has a molecular weight of 48 kDa based on sequence analysis (Valent *et al.* 1995). The Trigger Factor consists of three domains, namely, the PPIase domain, a C-terminal domain, and an N-terminal domain. As for the linker, the orange Arm1 and Arm2 are shown in Figure 3. The C-terminal domain is the central part with two protruding arms (Arm1 and Arm2). The PPIase domain is shaped like a dragon's "head" (residues 150-245 AA), and the N-terminal Trigger Factor domain (residues 1-149 AA) is associated with the part of the bacterial Hsp33 chaperone, forming a dragon "tail" with a "GFRxGxxP" motif at residues 43-50 AA, which links the Trigger Factor with ribosomes. The N-terminal domain is associated with the PPIase domain (residues 150-245 AA), which forms the "head" of the dragon, via a linker (residues 112-149 AA). The C-terminal domain (residues 246-432 AA) is the main modulator of the Trigger Factor (Hoffmann *et al.* 2010).

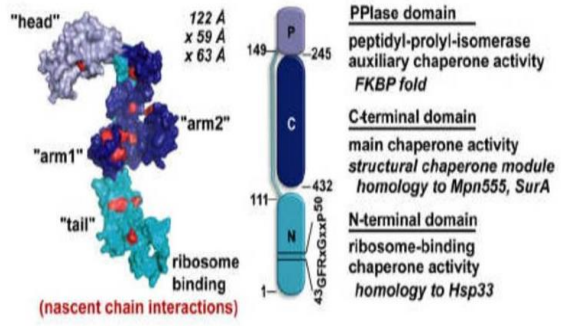


**Figure 3.** The structure and organization of the three domains of the Trigger Factor. Image Adapted from Ref. (Hoffman *et al*. 2010).

A previous study by Singhal *et al*. (2013) conducted molecular dynamics simulations to reveal the flexibility of the trigger factor. The results show that the flexibility and surface ability of the trigger factor allow it to interact with substrates of various sizes and compositions. Trigger factors are known to bind to proteins that do not fold properly or proteins that are partially folded. Trigger factors also interact with proteins that fold and stabilize the structure of proteins that have folded. Trigger factors that interact with partially folded protein structures can protect against aggregation and therefore can help proteins to form their native structures. Thus, the trigger factor not only has a role before the folding occurs but also when the protein folding takes place to form a native protein structure (Singhal *et al.* 2013).

The trigger factor does not have a single binding site, but uses the entire surface to form a cavity to bind to the substrate. Trigger factors can also bind to substrates with diverse compositions (polar and nonpolar) which are marked on the surface residue of the Trigger Factor capable of interacting with hydrophobic substrates and hydrophilic (Hoffmann *et al*. 2010). The trigger factor is the only known chaperone in bacteria that can associate with ribosomes. The L23 ribosomal protein is the main binding site for the trigger factor. Therefore, trigger factors can directly exit the growing polypeptide chain on the ribosome before entering the cytosol. As a result, trigger factors have the potential to interact with most of the early polypeptides during synthesis and then associate with newly synthesized polypeptide chains and limit access to downstream factors, such as the chaperones DnaK and GroEL which cooperate with the Trigger Factor in de novo folding of cytosolic proteins (Hoffmann *et al.* 2010).

## CO-EXPRESSION OF CHAPERONE

Nishihara *et al*. (2000) constructed several expression plasmids to assist the co-expression of the Trigger Factor chaperone in recombinant protein folding in *E. coli* (Figure 4).

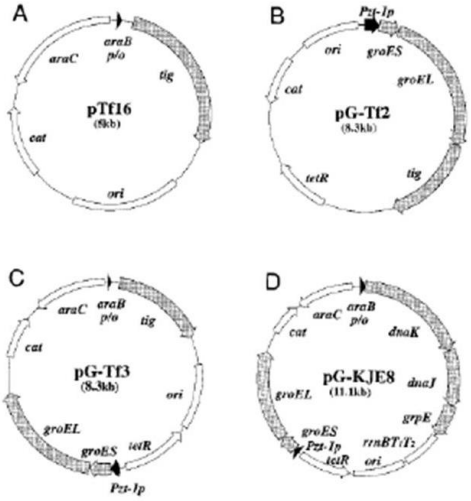


**Figure 4.** Expression plasmid structures of Trigger Factor and other constructed chaperons, including pTf16 (A), pG-Tf2 (B), pG-Tf3 (C), and PG-KJE8 (D). ori, replication origin of pACYC184; paint, chloramphenicol acetyltransferase gene; araB p/o, araB promoters; araC, the araC repressor gene; Pzt-1p, Pzt-1p promoter; tetR, the tetR repressor gene;

tig, the gene encoding TF. Image Adapted from Ref. (Nishihara *et al*., 2000).

The tig gene encoding TF was placed under the control of either the L-arabinose-induced araB promoter or the tetracycline-induced Pzt-1p promoter. All plasmids carried the chloramphenicol resistance gene (cat) for selection during *E. coli* growth (Nishihara *et al*. 2000).

In a previous study, Nishihara *et al*. (1998) investigated the role of the GroEL/ES and DnaK-DnaJ-GrpE chaperones on the Cryj2 protein in *E. coli*. The results had little effect on cell growth under the conditions used. Therefore, Nishihara *et al*. (2000) investigated the effect of Trigger Factor co-expression, either alone or in combination with GroEL/ES, on three mammalian proteins (rat endostatin, human ORP150, and lysozyme) to prevent the formation of protein aggregates.

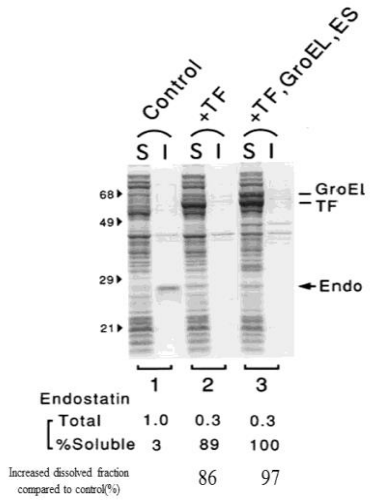


**Figure 5.** Results of SDS-PAGE analysis Effect of co-expression of TF and other chaperones on rat endostatin production. E. coli cells strain BL21 containing the expression plasmid pTB01#8 endostatin and the expression plasmid chaperon pTf16 (lane 1 and 2), pG-Tf2 (lane 3), or PG-KJE8 (lane 4) were grown at 37°C in LB medium. liquid with or without L-arabinose and/or tetracycline and cells infected with CE6 phage (2×109 PFU/ml) to induce endostatin in the mid-log phase. After 1 h of incubation, cells were harvested, sonicated, and separated into a soluble fraction (S) and an insoluble fraction (I). Image Adapted from Ref. (Nishihara *et al*., 2000).

The expression of rat endostatin protein is shown in Figure 5. Lane 1 represents the control, and most of the expressed endostatin was present in the insoluble fraction. In lane 2, there was an 86% increase in the amount of endostatin expressed when the Trigger Factor was co-expressed (before endostatin production), with a corresponding gain in the soluble fraction compared to the control. This indicates that co-expression of the Trigger Factor can effectively prevent the aggregation of recombinant proteins, although the amount produced is still reduced. Co-expression of Trigger Factor with GroEL-GroES (lane 3) provides the most significant increase in the amount of soluble endostatin at 97%, which is consistent with the results obtained with Trigger Factor co-expression alone (Nishihara *et al*. 2000). These findings are consistent with the description by Hoffman *et al*. (2010) that the Trigger Factor, as an upstream chaperone, can assist newly synthesized polypeptide chains in folding into their native structure with an efficiency of up to 70%. Polypeptide chains that remain unfolded in the cytosol can be assisted by downstream chaperones such as GroEL-GroES to achieve correct folding with an efficiency of up to 15% (Hoffman *et al*. 2010).

A similar chaperone co-expression experiment was also carried out by Nishihara *et al*. (2000) for the production of human lysozyme protein. The results are seen in Figure 6. Most of the lysozyme produced in control cells was insoluble, but it was partially converted to the soluble form on Trigger Factor co-expression with an increase of 34% (lane 2) compared to control, although there was a slight decrease in yield gain (compared to lanes 1 and 2). GroEL-GroES co-expression was less effective in dissolving lysozyme (lane 3), but the results of Trigger Factor co-expression with *GroEL*-GroES showed more effective results with an increase in the soluble fraction of 74%, and most of the soluble lysozyme was obtained without a decrease in yield (lane 4). It was found that the co-expression

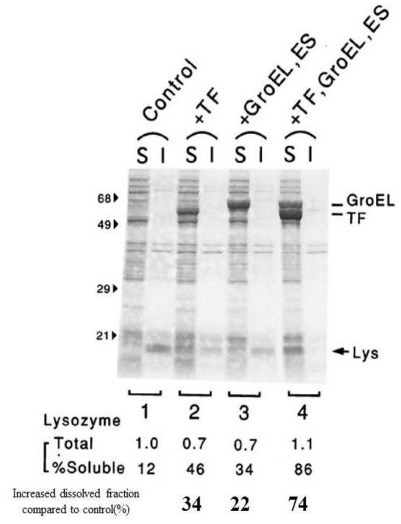


**Figure 6.** Results of SDS-PAGE analysis Effect of coexpression of TF and other chaperones on human lysozyme protein production. E. coli cells of the JM109 strain contained the pLY-46 lysozyme expression plasmid and the TF expression plasmid or another chaperon grown on RM media and 1 mM IPTG was added to induce lysozyme in the mid-log phase. After 4 h of incubation, cells were harvested, sonicated, and separated into a soluble fraction (S) and an insoluble fraction (I). Image Adapted from Ref. (Nishihara *et al*., 2000).

of Trigger Factor with GroEL-GroES was effective in preventing lysozyme aggregation under the conditions used (Nishihara *et al*. 2000).

Shen *et al*. (2011) conducted an experiment to express the recombinant lipase LIP-948 protein co-expressed with chaperones from five different plasmids in *E. coli*. They first used a combination of GroEL/ES chaperones and DnaK-DnaJ-GrpE (pG-KJE8), followed by co-expression using GroEL/ES (pGro7), trigger factor (pTf16), pKJE7 (DnaK-DnaJ-GrpE), and a combination of trigger factor with GroEL/ES (pG-Tf2). The results showed that co-expression using pTf16 (encodes trigger factor) and pGro7 (encodes GroEL/ES) decreased the number of soluble LIP-948 (0.8-0.9% of total soluble protein) compared to expression without a chaperone (1.7% of total soluble protein) and activity specificity also decreased. However, soluble LIP-948 was enhanced when co-expressed with the chaperon plasmids pKJE7, pG-Tf2, and pG-KJE8. The expression of soluble LIP-948 was significantly increased (11.4% of the total soluble protein) when co-expressed with pG-Tf2 (encodes GroEL/ES and trigger factor), indicating that chaperone proteins cooperate in LIP-948 formation which dissolves. LIP-948 was also efficiently expressed in a soluble form when co-expressed with pG-KJE8 (encodes GroEL/ES and DnaK-DnaJ-GrpE), 18.9% of the total soluble protein by yield). Specific activity and total dissolved protein were 109.77 U/mg and 11.8 mg/mL, respectively (Shen *et al*. 2011).

Šiurkus & Neubauer (2011) found that co-expressing the chaperones GroEL/ES with Ribonuclease Inhibitor (RI) increased protein activity compared to when no chaperones were co-expressed (Šiurkus & Neubauer 2011). In another study, Alibolandi *et al*. (2010) discovered that co-expression of trigger factor (TF) alone, carried by pTf16, was most effective in increasing the production of soluble human basic fibroblast growth factor (hbFGF) and reducing the amount of insoluble hbFGF compared to co-expression with a combination of chaperones TF and GroEL-GroES. The latter resulted in a decrease in both soluble and insoluble hbFGF. Overexpressing TF and similar DnaK-DnaJ-GrpE chaperones can also affect the solubilization of rhbFGF, reducing the formation of protein aggregates (inclusion bodies) (Alibolandi *et al*. 2010).

Haacke *et al*. (2009) conducted an experiment to evaluate the impact of chaperone over-expression on the production of soluble recombinant protein kinases in *E. coli.* One approach to enhance soluble protein expression involves the over-expression of molecular chaperones, such as GroEL/ES, DnaK/DnaJ/GrpE, and Trigger Factor (TF). The formation of soluble aggregates can be attributed to the overexpression of chaperones, which can bind to unfolded or misfolded proteins, preventing their aggregation into insoluble inclusion bodies. However, these chaperones can also interact with partially folded or misfolded proteins, leading to the formation of soluble aggregates that lack proper structure and function. Haacke's findings indicate that the increased yields of soluble protein kinases obtained through chaperone over-expression are mainly due to these soluble aggregates rather than correctly folded, functional proteins (Haacke *et al*. 2009).

Jhamb *et al*. (2012) investigated the effects of process conditions and chaperone co-expression on cell growth and production of xylanase. The choice of process conditions, such as temperature, inducer concentration, and cultivation time, plays a crucial role in protein expression and solubility. Firstly, temperature has a significant impact on the folding and stability of recombinant proteins. Lowering the temperature during protein expression can enhance solubility by reducing the rate of protein synthesis, allowing more time for proper folding. Inducer concentration is another critical parameter that affects protein solubility. High inducer concentrations can lead to rapid protein synthesis, increasing the likelihood of misfolding and inclusion body formation. Optimizing the inducer concentration can help achieve higher levels of soluble protein expression. Lastly, the cultivation time should be carefully considered as prolonged cultivation can lead to protein degradation or cell lysis, compromising protein yield and solubility. Co-expression of Hsp70 chaperones with recombinant proteins in *E. coli* has been shown to enhance protein solubility by assisting in correct folding and preventing aggregation. Similarly, co-expression of chaperones like GroEL-GroES, DnaK-DnaJ, and trigger factor (TF) has also been reported to improve the production of soluble recombinant proteins in *E. coli.* The production of soluble recombinant proteins in E. coli is a complex process influenced by various factors. Optimization of process conditions, including temperature, inducer concentration, and cultivation time, can significantly enhance protein solubility. Moreover, co-expression of molecular chaperones, such as Hsp70, GroEL-GroES, DnaK-DnaJ, and TF, can effectively improve the solubility and yield of recombinant proteins (Jhamb *et al*. 2012).

Ahn & Jung (2023) discussed about improved recombinant protein production using Heat Shock Proteins in *Escherichia coli*. Heat shock proteins are a family of molecular chaperones that play a critical role in cellular protection against various stresses, including heat, oxidative stress, and protein misfolding. They facilitate protein folding, prevent aggregation, and assist in refolding denatured proteins. The utilization of Hsps (GroEL/ES, DnaK/J/GrpE, IbpA/B) can improve the production of recombinant proteins by enhancing folding efficiency and minimizing the formation of inclusion bodies. The co-expression of sHsps with the target protein can significantly reduce the formation of

inclusion bodies and improve the quality of recombinant protein production (Ahn & Jung 2023).

In another study, Mamipour *et al*. (2017) provided a comprehensive description of strategies to enhance the expression of soluble recombinant proteins with proper folding. These strategies include the use of chemical chaperones, gene protein optimization, signal peptide sequences, and molecular chaperones. Molecular chaperones, whether located in the cytoplasm or periplasm, prove more effective in producing recombinant proteins with the correct folding. Cytoplasmic chaperones such as Hsp100 (Clp), Hsp90, Hsp70 (DnaK/DnaJ/GrpE), Hsp60 (GroEL/GroES), and small Hsps interact with aggregated and misfolded recombinant proteins, facilitating their unfolding and ultimately enabling them to acquire the appropriate folding. HSP60, HSP70, and their co-chaperones utilize ATP to solubilize and refold aggregated recombinant proteins. On the other hand, periplasmic chaperones, located in the periplasmic space, promote correct folding by facilitating the formation of disulfide bonds, resulting in the production of soluble recombinant proteins. The efficiency of the folding process mediated by periplasmic chaperones depends on the specific type of recombinant protein. Certain factors, such as increasing the levels of specific periplasmic chaperones like FkpA and SurA, aid in the slow cis-trans isomerization of peptidylprolyl bonds during the folding of target proteins. Additionally, proteins like DsbA and DsbC assist in the formation of disulfide bridges. These activities play crucial roles in overcoming the production of misfolded proteins (Mamipour *et al*. 2017).

Maksum *et al*. (2020) expressed intein-prothrombin-2Ti, pH in *E. coli* using the carrier plasmid pG-KJE8 to encode the chaperone gene. Co-expression of GroEL/ES, DnaK-DnaJ-GrpE, and a combination of both was found to be more effective in preventing inclusion bodies. The method involved isolating pTWIN1-prothrombin-2Ti, pH and pG-KJE8 from *Escherichia coli* TOP10 and DH5α. Then, *E. coli* ER2566 was transformed using pG-KJE8 and pTWIN-prothrombin-2Ti, pH. Chaperone co-expression was carried out by inducing DnaK-DnaJ-GrpE and GroEL/ES with L-arabinose or tetracycline, followed by induction with IPTG and cell culture growth at 22°C for 6 hours before harvesting. The results were characterized using SDS-PAGE and UV-Vis spectrophotometer at 280nm. The presence of chaperones helped in the folding of intein-prothrombin-2Ti, pH, as judged by the analysis of protein absorbance in the resulting soluble fraction with a spectrophotometer at 280 nm.

The results of the SDS-PAGE analysis shown in Figure 7 demonstrate that (A) without co-expression of the chaperone, prothrombin-2Ti,pH combined with intein resulted in a molecular weight of 63 KDa expressed in the insoluble fraction. The possible cause is that the high-level expression of the protein is not matched by adequate folding, resulting in the production of many insoluble fractions. (B) Co-expression of the chaperones GroEL/GroES + DnaK-DnaJ-GrpE shows a band from intein-prothrombin-2Ti, pH in the soluble fraction compared to no chaperone. This indicates that the chaperones can assist in folding to produce more soluble fractions. (C) Co-expression of the DnaK-DnaJ-GrpE chaperone shows a band of intein-prothrombin-2Ti, pH, but it is still thicker. This may be because DnaK-DnaJ-GrpE does not provide enough folding space, causing interference during the folding of surrounding macromolecules. The hsp, including DnaK, play a role in assisting the folding and degradation of unstable proteins. (D) Co-expression of the GroEL/GroES chaperone results in a higher production of soluble prothrombin-2Ti, pH compared to when induced by DnaK-DnaJ-GrpE or its combination. This may be due to the role of GroEL in providing a folding space for the protein, allowing intein-prothrombin-2Ti, pH to fold without any disturbance. Therefore, from the analysis of the results of this study, co-expression of chaperones can aid in the solubilization of intein-prothrombin-2 (Maksum *et al*. 2020).

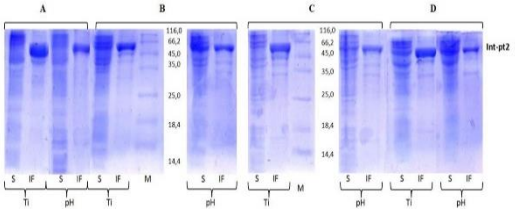


**Figure 7.** SDS-PAGE Electrophoregram of intein-pretrombin-2Ti, pH expression with and without chaperone co-expression. A) without co-expression; B) GroEL/ES + DnaK-DnaJ-GrpE co-expression; C) DnaK-DnaJ-GrpE co-expression; and D) GroEL/ES co-expression. M, marker protein; S, soluble; IF, insoluble fraction; Ti, pTWIN1-prethrombin-2Ti; pH, pTWIN1-prethrombin-2pH; int-pt2: intein-prethrombin-2Ti, pH. Expression was conducted in 37°C for 3 hours (before induction) and 22°C for 6 hours (after induction). Image adapted from Ref. (Maksum *et al*., 2020) with free permission from Jurnal Kimia Valensi.

## CONCLUSION

In conclusion, based on this review, it can be stated that there is no perfect chaperone co-expression system that can work efficiently for all recombinant proteins due to the diverse issues encountered in each protein. Nonetheless, the co-expression of chaperones has been found to enhance soluble protein production and decrease the formation of inclusion bodies. This study provides valuable insights that can help scientists develop new strategies to overcome inclusion bodies in the

intracellular expression of recombinant proteins, particularly through the use of co-expressed chaperones.

**ACKNOWLEDGEMENT**

This work was supported by grant No.116/IV/KS/11/2022 from the National Innovation Research Agency, Ministry of Technology Research and Higher Education and Academic Leadership Grant No.1549/UN6.3.1/PT.00/2023 from Universitas Padjadjaran.

**REFERENCES**

Ahn, Y. J. & Jung, M. (2023). Improved recombinant protein production using heat shock proteins in Escherichia coli. *Biocatalysis and Agricultural Biotechnology*, **50**, 102736.

Alibolandi, M., Mirzahoseini, H., Nehi, F.M. & Tabatabaian, G. (2010). Improving recombinant protein solubility in *Escherichia coli*: Identification of best chaperone combination which assists folding of human basic fib*r*oblast growth factor. *African Journal of Biotechnology*. **9(47)**: 8100-8109.

Baneyx, F. (1999). Recombinant protein expression in *Escherichia coli*. *Current Opinion in Biotechnology*. **10(5)**: 411-421.

Grallert, H. & Buchner, J. (2001). A structural view of the GroE chaperone cycle. *Journal Of Structural Biology*. **135(2)**: 95-103.

Chae, Y.K., Kim, S.H. & Markley, J.L. (2017). Relationship between recombinant protein expression and host metabolome as determined by two-dimensional NMR spectroscopy. *PLoS ONE*. **12(5)**: 1–12.

Chaudhuri, T. K. & Paul, S. (2006). Protein-misfolding diseases and chaperone-based therapeutic approaches. *The FEBS Journal*. **273(7):** 1331-1349.

Choi, J.H., Keum, K.C. & Lee, S.Y. (2006). Production of recombinant proteins by high cell density culture of *Escherichia coli*. *Chemical Engineering Science*. **61(3)**: 876–885.

Choi, J.H. & Lee, S.Y. (2004). Secretory and extracellular production of recombinant proteins using *Escherichia coli*. *Applied Microbiology and Biotechnology*. **64(5):** 625–635.

de Groot, N. S. & Ventura, S. (2006). Effect of temperature on protein quality in bacterial inclusion bodies. *FEBS Letters*. **580(27)**: 6471-6476.

Demain, A. L. & Vaishnav, P. (2009). Production of recombinant proteins by microbes and higher organisms. *Biotechnology Advances*. **27(3)**: 297-306.

Ellis, J. (1987). Proteins as molecular chaperones. *Nature*. **328(6129)**: 378-379.

Gellissen. (2005). Production of Recombinant Proteins Novel Microbial Eukaryotic Expression Systems. Wiley-VCH. Germany.

Haacke, A., Fendrich, G., Ramage, P. & Geiser, M. (2009). Chaperone over-expression in *Escherichia coli*: Apparent increased yields of soluble recombinant protein kinases are due mainly to soluble aggregates. *Protein Expression and Purification*. **64(2)**: 185–193.

Hoffmann, A., Bukau, B. & Kramer, G. (2010). Structure and function of the molecular chaperone trigger factor. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*. **1803(6)**: 650-661.

Horwich, A. L. (2011). Protein folding in the cell: an inside story. *Nature Medicine*. **17(10)**: 1211-1216.

Jhamb, K. & Sahoo, D. K. (2012). Production of soluble recombinant proteins in *Escherichia coli*: Effects of process conditions and chaperone co-expression on cell growth and production of xylanase. *Bioresource Technology*. **123**, 135–143.

Jungbauer, A. & Kaar, W. (2007). Current status of technical protein refolding. *Journal of Biotechnology*. **128(3)**: 587–596.

Klein, G. & Georgopoulos, C. (2001). Identification of important amino acid residues that modulate binding of escherichia coli groel to its various Co-chaperones. Genetics. **158(2)**: 507–517.

Maksum, I.P., Utama, E., Sriwidodo. & Subroto T. (2017). Extracellular secretion of recombinant human epidermal growth factor by using trimethylamine N-Oxide reductase a (TORA) signal peptide in *Escherichia coli* BL21 (DE3). *J Pharm Sci Res*. **9(6)**: 1007–16.

Maksum, I.P., Lestari, A., Fauzia, R.P., Rachman, S.D. & Soedjanaatmadja, U.M.S. (2019). *Escherichia coli* BL21(DE3) expression system using TorA signal peptide for Recombinant Human Albumin (rHA) secretion. International Journal of Research in Pharmaceutical Sciences. **10(4):** 19-24.

Maksum, I.P., Wildan, D.A.Y., Hasan, K. & Subroto, T. (2020). The effect of single Co-expression of The DnaK-DnaJ-GrpE and GroEL/ES chaperones and their combination on expression intein-pretrombin-2 in *Escherichia coli* ER2566. *Jurnal Kimia Valensi*. **6(1)**: 47–54.

Mamipour, M., Yousefi, M. & Hasanzadeh, M. (2017). An overview on molecular chaperones enhancing solubility of expressed recombinant proteins with correct folding. *International Journal of Biological Macromolecules*. **102**: 367–375.

Nelson, D. L., & Cox, M. M. Lehninger Principles of Biochemistry 6th Edition (2013).

Nishihara, K., Kanemori, M., Yanagi, H. & Yura, T. (2000). Overexpression of trigger factor prevents aggregation of recombinant proteins in *Escherichia coli*. *Applied and Environmental Microbiology*. **66(3)**: 884–889.

Nishihara, K., Kanemori, M., Kitagawa, M., Yanagi, H. & Yura, T. (1998). Chaperone coexpression plasmids: differential and synergistic roles of DnaK-DnaJ-GrpE and GroEL-GroES in assisting folding of an allergen of Japanese cedar pollen, Cryj2, in *Escherichia coli*. *Applied and Environmental Microbiology*. **64(5)**: 1694-1699.

Reece, J.B., Urry, L.A., Cain, M.L., Wasserman, S.A., Minorsky, P. & Jackson, R.B. (2014). *Campbell Biology*. Pearson. Boston.

Shen, J., Cui, C. & Lin, X. (2011). Effects of co-expression of molecular chaperones heterologous soluble expression of the cold-active lipase Lip-948. *Protein Expression and Purification*. **77**: 166-172.

Silaban, S., Gaffar, S., Simorangkir, M., Maksum, I. P. & Subroto, T. (2018). Effect of IPTG concentration on recombinant human prethrombin-2 expression in *Escherichia coli* BL21 (DE3) ArcticExpress. In *IOP Conference Series: Earth and Environmental Science*. **217(1)**: 012039.

Singh, S. M. & Panda, A. K. (2005). Solubilization and refolding of bacterial inclusion body proteins. *Journal Of Bioscience And Bioengineering*. **99(4)**: 303-310.

Singhal, K., Vreede, J., Mashaghi, A., Tans, S. J. & Bolhuis, P. G. (2013). Hydrophobic collapse of trigger factor monomer in solution. *PLoS One*. **8(4)**: e59683.

Šiurkus, J. & Neubauer, P. (2011). Heterologous production of activeribonuclease inhibitor in *Escherichia coli* by redox statecontrol and chaperonincoexpression. *Microbial Cell Factories*. **10**, 1–11.

Sriwidodo., Maksum, I.P., Riswanto, N., Rostinawati, T. & Subroto, T. (2017). Extracellular secretion recombinant of human epidermal growth factor (hEGF) using pectate lyase B (PelB) signal peptide in escherichia coli BL21 (DE3). *Int J Res Pharm Sci*. **8(1)**: 1–8.

Strandberg, L. & Enfors, S.O. (1991). Factors influencing inclusion body formation in the production of a fused protein in *Escherichia coli*. *Applied and Environmental Microbiology*. **57(6)**: 1669–1674.

Su, Z., Huang, Y., Zhou, Q., Wu, Z., Wu, X., Zheng, Q., Ding, C. & Li, X. (2006). high-level expression and purification of human epidermal growth factor with SUMO fusion in *Escherichia coli*. *Protein & Peptide Letters*. **13(8)**: 785–792

Terpe, K. (2006). Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Applied microbiology and biotechnology*. **72**, 211-222.

Upadhyay, A. K., Murmu, A., Singh, A. & Panda, A. K. (2012). Kinetics of inclusion body formation and its correlation with the characteristics of protein aggregates in Escherichia coli. *PloS One*. **7(3)**: e33951.

Valent, Q. A., Kendall, D. A., High, S., Kusters, R., Oudega, B. & Luirink, J. (1995). Early events in preprotein recognition in *E. coli*: interaction of SRP and trigger factor with nascent polypeptides. *The EMBO Journal*. **14(22)**: 5494-5505.

Walter, S. & Buchner, J. (2002). Molecular chaperones—cellular machines for protein folding. *Angewandte Chemie International Edition*. **41(7)**: 1098-1113.

Wang, Y., Van Oosterwijk, N., Ali, A. M., Adawy, A., Anindya, A. L., Dömling, A. S. & Groves, M. R. (2017). A systematic protein refolding screen method using the DGR approach reveals that time and secondary TSA are essential variables. *Scientific Reports*. **7(1)**: 9355.

Wegerer, A., Sun, T. & Altenbuchner, J. (2008). Optimization of an *E. coli* L-rhamnose-inducible expression vector: test of various genetic module combinations. *BMC Biotechnology*. **8(1)**: 1-12.

Xu, Z., Horwich, A.L., and Sigler, P.B. (1997). The Crystal Structure of the Asymmetric GroEL/ES-(ADP)7 Chaperonin Complex. *Nature*. **388**: 741-750.