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CHAPERONE CO-EXPRESSION OF INDUSTRIALLY IMPORTANT ENZYMES

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Abstract

Overproduction of recombinant proteins particularly the industrial important enzymes continues to be an inevitable step in their biochemical and structural characterization. Along with other methods, chaperones highly contribute to the improvements in protein overexpression. Due to its versatility, molecular chaperones play a great role to inhibit protein denaturation, suppress the accumulation of disabled protein in the cell and assist in refolding and degradation of denatured proteins. This review focuses on recent advances utilizing chaperones in promoting and regulating protein folding among industrial enzymes; amylases, lipases, proteases and catalases. This review emphasizes the function and role of molecular chaperones which facilitate the production of recombinant enzymes. We presented the effect of chaperone and co-chaperone expression for the soluble expression of recombinant enzymes which would be advantageous for the developments and industrial applications of these enzymes in the future.

1. INTRODUCTION

It has long been a challenge in biotechnology to produce enzymes that are used commercially in various industries such as pharmaceuticals, food & beverage, livestock, chemical production, pharmaceuticals, biofuel, and consumer products. In order to fulfill the high demand, most of the enzymes are produced by using an efficient expression system, for example, *E. coli* or other heterologous hosts, which lead to high productivity. Even though developments of genetic engineering and protein chemistry have created versatile enzymes based on the rational modification to match specific requirements in industries, however, the productivity of these recombinant enzymes in heterologous host itself is said to be problematic. There are few possibilities that production protein in heterologous host will face two main problems; (1) foreign gene is difficult to be expressed or will be produced in a small amount (2) recombinant protein will be expressed as a less soluble protein, which hinders the effort to produce the target protein in large scale.

Production of heterologous protein in bacteria cells also often leads to the formation of inclusion bodies or misfolding as a result of target protein cannot reach and maintain their native state [1,2]. Misfolding often leads to aggregation and insolubility of proteins [3]. The yield of these recombinant proteins is low, which impedes both a detailed characterization of the protein structure and functionalities as well as future industrial applications. In most cases, proteins turn into inclusion bodies will require other steps of solubilization and refolding process to regain biological activity [4,5].

Many studies have documented the use of molecular chaperone proteins to promote the soluble expression of heterogeneous proteins [6,7,8]. One of the most considerably used approaches to improve the yield of soluble protein is by

co-expression of molecular chaperones in *E. coli* system [3,5]. Molecular chaperones are proteins that assist in many biological processes such as preventing the cells from unfavourable environmental conditions, assisting folding of the newly synthesized polypeptide, promoting solubility and refolding of stable protein aggregates, and permitting the development of new protein functions and phenotypic traits [9]. All these functions demonstrate chaperones as the ideal candidate for biotechnological applications [10,11]. This review will complement the existing reviews by focusing on the role and how molecular chaperone expression improves the solubility of industrial enzymes, particularly amylases, lipases, proteases and catalases. Due to the widespread use of these enzymes in various industrial processes, therefore, the increase in enzyme production, thermostability, and activity influenced by chaperone expression will have a direct impact on the process performance, economy and suitability in industrial applications.

2. CHAPERONES

A chaperone acts like a protein machine, which binds to non-native proteins to process the refolding/unfolding of their substrates. There are three well-documented chaperone systems and successfully used in co-expression recombinant enzymes: trigger factor (TF), GroEL system (Hsp60), and DnaK (Hsp70) [12,13,14]. Two of the latter are the major chaperone systems, composed of GroEL and GroES (for Hsp60) while DnaK and its co-chaperones DnaJ and GrpE (for Hsp70). Depending on the mechanism of action (Figure 1), chaperones can be divided into two major categories, folder and holder chaperones.

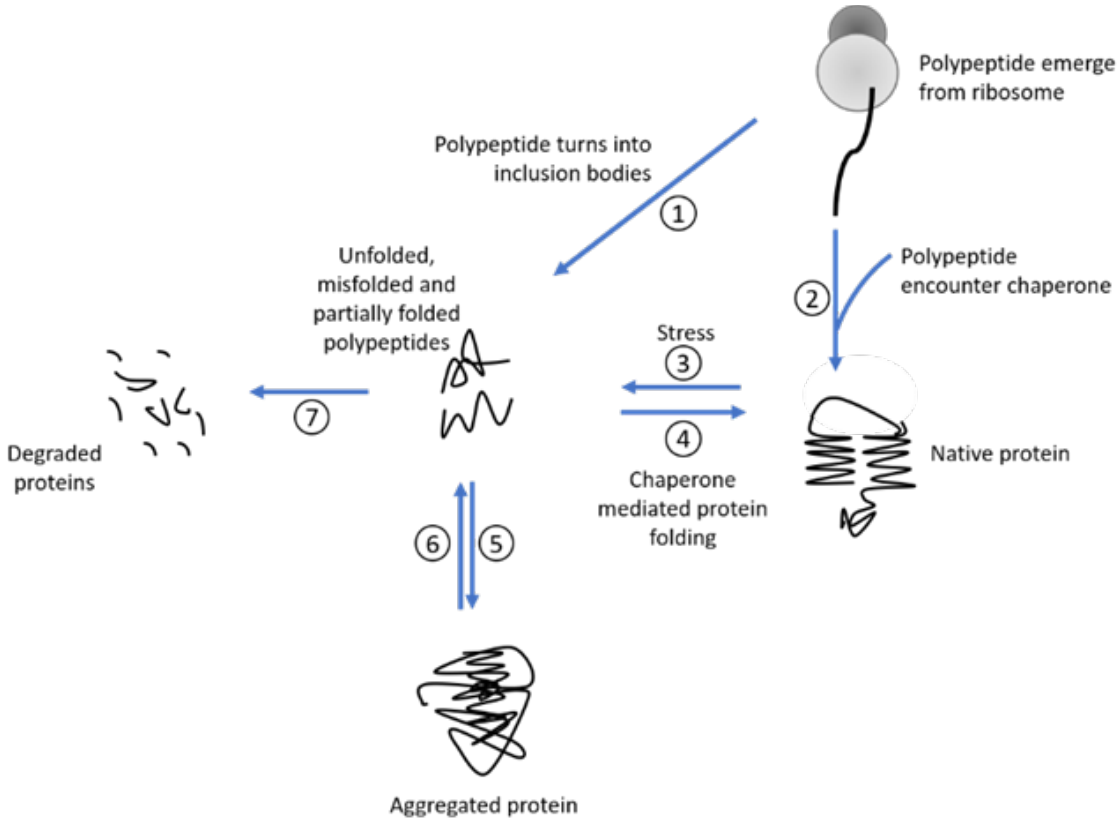

Figure 1: Nascent polypeptides interact with TF chaperone as come out from ribosome. Many recombinant polypeptides are prone to misfolding upon expression and expressed as unfolded or misfolded proteins. ① Polypeptides that are accompanied by chaperones upon leaving the ribosome promote folding to a native ②or partially folded polypeptides in stress condition ③. The latter possibly re-captured by DnaK and/or TF to repeat this folding cycle until it reaches its native state, then interact with GroEL-GroES, DnaK or Hsp90 ④ to complete folding to its native conformation or undergo aggregation ⑤. Aggregation can be protected if the target protein interacts with small heat shocks protein, Hsp70/DnaK, Hsp90 or CIp/Hsp100. CIp will cooperate with Hsp70 family for an effective disaggregation of unfolded proteins ⑥, followed again by their transfer to the DnaK/DnaJ/GrpE machinery for completion of folding. Those unfolded, misfolded and non-native proteins will be delivered to compartmentalized protease in the presence of CIp/Hsp100 for protein degradation ⑦.

The folder chaperones are ATP-dependent. For example, GroEL and DnaK chaperone systems actively participate in the folding and activation of proteins caused by the inactivation of certain stress factors. These folder chaperones aid in correcting 3D conformation, restoring the function, and biological properties of the target substrates [15,16]. A model has been proposed in which GroEL (Hsp60 family) and DnaK (Hsp70 family) work successfully in protein folding in *E. coli* [17,18]. GroEL firstly allows nonnative substrates smaller than 60 kDa to bind to the interior of its rings through hydrophobic interactions. Before binding of cofactor GroES to main domain GroEL, ATP binds to the substrate which drives the conformational changes of the substrate. As a result, GroES binds to the complex and encloses the substrate. Once ATP is hydrolyzed, ADP dissociated from the cis-ring of GroEL and finally substrate is released. For substrates that are unfolded, they will be rebound rapidly, while folded substrates, it no longer contains expose hydrophobic residues necessary for binding [19].

Holder chaperones, for example Ibp and ClpB, nevertheless, focus to prevent protein aggregation by forming stable complexes to the misfolded proteins. Additionally, the holder chaperones cannot actively restore the native protein conformation [20,21].

Unlike enzymes with their specific substrate to the active sites, chaperones operate on a wide range of substrates. The chaperones can prevent non-productive hydrophobic interactions, thus facilitate the conformational processing of polypeptides into their correct tertiary conformation [1,12]. For example, the accumulation of damaged proteins increases due to adverse environmental conditions. Therefore, the presence of these misfolded polypeptides induces the protein quality control system, which consists of chaperones and proteases to refold proteins or remove them from a cell by degradation [7].

The size of the proteins also determines the efficiency of folding. Small size proteins could be efficiently folded faster, while multi-domain proteins may need assistance during folding and demand the function of certain chaperones and various cofactors to facilitate the process. For instance, the GroEL chaperone consists of two heptameric rings and forms two back-to-back cavities with the hydrophobic parts in the apical domains and hydrophilic sites inside the cavities. The hydrophobic sites of the GroEL interact with the hydrophobic residues of nascent proteins, trap the appropriate size of proteins into the cavity [22,23], and increase the solubility of the proteins. Bacterial proteins are initially synthesized in the cytoplasm before transported to the cellular envelope or extracellular space. This process requires the assistance of chaperone proteins [24]. However, a well-studied and successful secretion strategy for one protein does not always necessarily apply the same promising production for another protein [25].

3. CHAPERONE EXPRESSION OF INDUSTRIAL ENZYMES

3.1. α-amylase

α-amylases are widely produced by animals, plants, and microorganisms [26-29]. They have attracted great attention, because of their imperative role in biotechnological applications especially in baking, starch saccharification, biofuel production, detergent production and the pharmaceutical industry [30-32].

A novel thermostable α-amylase isolated from *Geobacillus* sp. GS33 was overexpressed in *E. coli* BL21 (λDE) in the presence of combination chaperones DnaK-DnaJ-GrpE (pKJE7) [29] resulted in marked stabilization and accumulation of the target enzyme. The specific activity was found among the highest compared to the expression of α-amylase without a chaperone expression system [33]. Some studies showed that it is necessary to overexpress both the GroEL-GroES and DnaK-DnaJ-GrpE. The overexpression with only a single molecular chaperone will cause a defect in cell septation and the formation of cell filaments, which are harmful to the bacterial [34,35]. On the other hand, soluble expression of α-amylase from *Pyrococcus furiosus* (PFA) was enhanced with co-expression of chaperonin or small heat shock protein in *E. coli* BL21 (DE3) [9]. The chaperones (alone or in pairs) and recombinant PFA were cloned into plasmid pCDFDuet-1 that has a higher copy number resulted in the highest supernatant enzyme activity. Co-expression of prefoldin and small heat shock protein (sHSP60) showed similar levels of soluble PFA as in prefoldin alone, which indicates the co-expression with only prefoldin was adequate for promoting the soluble expression of PFA. Previous studies showed prefoldin works efficiently to increase tolerance of *E. coli* host to various environmental stresses, such as the presence of an organic solvent [36] or high temperature [37]. This is due to the ability of prefoldins to protect the aggregation of diverse heterogenous proteins [38]. Synergistic roles of different combination of chaperones in assisting folding and increasing soluble recombinant protein has also been extensively discussed [18,39,40].

In contrast to overexpression in *E. coli*, *B. subtilis* is also an attractive host to overexpress amylases, which is frequently used in food industries because it is considered as a generally recognized as safe (GRAS) organism [41]. It has a naturally high secretory capacity and exports proteins directly into the extracellular medium. But, in many cases, overexpression in *B. subtilis* can cause jamming of the membrane because of the shortage of Sec pathway components. Chen and co-workers cloned the AmyL and AmyS into vector pMA5 by overexpressing these two genes into *B. subtilis* 1A751 in the presence of prsA and partial

dnaK operon (GrpE-DnaK-DnaJ) and resulted in the highest α-amylase activity of both AmyL and AmyS [42]. Intracellular molecular chaperones such as DnaK helps in mediating protein folding and minimizing aggregation prior to protein secretion. Another role of DnaK is to facilitate pre-proteins with a resistance against intracellular protease which improves the heterologous protein secretion in *B. subtilis* [7,18].

3.2. Lipases

Lipases usually display exquisite chemoselectivity, regioselectivity and stereoselectivity, which make them so attractive to the organic chemist. Over the last few years, many microbial lipase genes had been isolated, sequenced, modified, and successfully expressed in homologous or heterologous hosts such as *E. coli*. Some of the cases ended up in lower expression of enzymes thus does not meet the requirements for large-scale industrial applications. Even though some of the lipase families depend solely on the chaperone that has been called "intramolecular chaperoning", which located adjacent to the lipase structural gene to assist in folding [43-45], a variety of recombinant lipases co-expressed with individual chaperones in *E. coli* has also been reported. Molecular chaperone assists in the folding, which increase the solubility and reduce the aggregation occurrence in recombinant proteins. This includes the one from eukaryotic proteins. *Candida antartica* lipase A (CalA) was highly expressed in the pColdIII vector with co-expression of pGro7 containing GroES-GroEL compared to the one without chaperone [46]. This indicates GroES/GroEL chaperone system gives such a strong impact on the correct folding of CalA. However, only a slight increase of CalA expression was observed when co-expressed with DnaK-DnaJ-GrpE. The size of the substrates plays a critical role when pairing with a chaperone. GroES/GroEL most preferred substrates are the proteins with molecular mass less than 60 kDa (protein < 60kDa) because of the volume of the GroES/GroEL cavity [47]. While DnaK-DnaJ system is mostly recognized the target proteins with molecular weight higher than 60 kDa [48].

Another lipase, Lip-948 demonstrated that molecular chaperones played synergistic roles in the formation of soluble proteins [49]. Even though co-expression with pTf16 (encode trigger factor) and pGro7 (encode GroES/GroEL) resulted in the lower amount of soluble Lip-948 compared to the expression without chaperones, the soluble Lip-948 was enhanced when co-expressed with ''chaperone team'' plasmids (pKJE7, pG-Tf2, pG-KJE8) suggested that molecular chaperones work together in producing of soluble protein. TF may interact with most polypeptides during the initial stage of ongoing synthesis. Newly formed nascent proteins in bacteria associated with the TF as soon as they exit the ribosome [50-53]. It associates with nascent chains, restricts the access of other chaperones such as DnaK and GroEL in the first place [6]. Then, it will be tagged by DnaJ and later recognized by DnaK and GrpE, the Hsp70 chaperone family which prevent the formation of inclusion bodies by reducing aggregation and promoting proteolysis of misfolded proteins. Unlike DnaK-DnaJ-GrpE system, the GroEL-GroES (Hsp60 chaperone family) does not have many interacting partners, and their active sites are hidden from the outer surface of the protein complex [28]. The GroEL-GroES shares the common property of binding to the non-native substrate and controls the transit between soluble and insoluble protein fractions [54].

3.3. Proteases

Proteases are considered as an alternative to chemicals and of commercial value due to their multiple applications in various industrial sectors. Proteases catalyse the cleavage of peptide bonds and serve as essential tools to degrade damaged proteins and peptides. In most cases, cloning, expression and purification of proteases often fail due to their catalytic functions which, in turn, cause toxicity in the *E. coli* heterologous host. For that reason, proteases have been considered the most challenging enzyme due to production of unregulated foreign proteases in *E. coli* host cells which, often resulting in the formation of inclusion bodies, non-expression, or cytotoxicity [55-56]. However, just like the other enzymes, the soluble form of proteases is the key to industrial applications. Otherwise, a larger reaction volume, longer incubation times and more restricted reaction conditions, which impose a negative impact on the feasibility of the process will be the cost of poorly soluble enzymes [57].

Schrodel and co-workers succeed in increasing the solubility of CLIPB14 protease up to 20% when co-expressed with GroELS chaperones while the co-expression with the ibpA/B chaperones was insignificant. No yield improvement of the soluble CLIPB14 was observed when it was co-expressed without chaperone activity, indicating the solubilizing effect induced by GroELS [58]. Binding ATP to GroEL will allow subsequent action of co-chaperonin GroES to cap one of the cavities and provides unceasing environment proteins to fold. The well-folded protein in its designed conformation will be released when ATP is hydrolyzed [59]. Even though co-expression of the native *E. coli* GroELS chaperonin is said to increase the solubility of proteins [60], the increased solubility of target proteins will not necessarily produce higher activities [61]. Besides GroEL, Hsp70 plays a role in response to the temperature effect. In the presence of Hsp70 system, the number of active proteins decreased less rapidly than in the absence of chaperones. It proves the contribution of the Hsp system in maintaining the active protein concentration, provided all the effects of temperature on the different reaction rates were included [62].

On the other hand, the chaperone activity of serine protease HtrA *Helicobacter pylori* serves as a crucial survival factor under stress conditions [63]. Meanwhile,

protease HtrA *Campylobacter jejuni* without CIpB is more sensitive to temperature stress [64], which showed the presence of chaperone in assisting the stability of target protein towards harsh environment. For proteases under subtilisin family, they are equipped with pro peptide sequence at the N-terminal that acts as a chaperone essential for correct folding of active enzyme [65].

3.4. Catalases

Catalases are one of the most studied group of enzymes catalyse disproportion of hydrogen peroxide into di-oxygen and water using a heam co-factor [66]. One of its widely known applications is in the fabric industry; to remove excess hydrogen peroxide from fabric. Removing hydrogen peroxide is a crucial and economic step to improve dyeing cotton fabrics without having to use a big volume of water, longer process time or use of chemical products harmful to the environment. This enzyme is also used along with other enzymes such as glucose oxidases in the food processing industry [67], lipases in waste management and hydrocarbon degradation [68].

Applying co-expression with chaperone has successfully overexpressed a potent catalase in *E. coli* host [69]. For instance, a bio-active rice plant CAT-A fused to reduced thioredoxin (Trx) followed by co-expression of molecular chaperone. The effects of chaperones as individual systems or combination were examined on the solubilization and properties of CAT-A fusion protein. Overexpression with any of the chaperones system affects the production of CAT-A fusion proteins, which resulted in different induction temperatures. The synthesis of CAT-A fusion protein was failed at 37˚C but at a lower temperature of 20˚C, a soluble fraction of fusion protein was observed. Interestingly, it is only overexpressed in the presence of combination groEL-ES and TF or TF alone but not in the presence of a high level of GroEL-ES alone.

TF is essential for cell viability in the cold by promoting the refolding of cold-damaged proteins [70]. Unlike the major molecular chaperones in *E. coli*, it is induced at a low temperature of 20˚C. The process of maintenance or repair function which, part of protein synthesis may be particularly important to proceed very slowly at low temperatures. Additionally, TF which is ribosome-associated during translation seems to be more useful to produce solubilized CAT-A. About 46-47% of hydrophobic amino acid residues in CAT-A have made the role of TF becomes more obvious in protecting the primary structure of catalase. Molecular chaperones play an important role in folding alpha/beta structures enriched in hydrophobic and basic residues of the substrates [28]. TF protein is specific to prokaryotes. Ribosomal bound TF interacts directly with the nascent polypeptides chain that comes out from the ribosome and assists initial folding stages through different well-described mechanisms [71-74]. Many have documented the specific regulation on solubility of recombinant protein by using chaperones [71,75].

Hook and Harding co-expressed catalase in the presence of chaperone GroEL [76]. The GroEL protects catalase against thermal inactivation at 60˚C. Protection of catalase against thermal inactivation was conferred specifically by the molecular chaperones alpha crystallin and GroEL. In comparison, Hsp25 alone was unable to protect catalase against thermal inactivation at 60˚C or even at lower thermal stress of 55˚C. In this work, the protection of catalase over thermal stress by GroEL was conferred stoichiometrically, which influences the effect of chaperones in promoting refolding at low concentrations of protein. In an overcrowded environment of high protein concentrations; ~300 to 400 mg/ml, will lead to spontaneous folding that frequently error-prone and thus such protection of enzyme activity maybe lost [6]. Numerous studies have indicated that in high protein concentration, the availability of chaperones is limited to cope with the demands of high concentration in which, causes protein instability and lead to protein aggregation [77-78]. Thus, the addition of individual chaperones or chaperone sets may increase the viability to solubilize target proteins during overexpression [79]. In other cases, heat shock protein is highly linked to aerobic metabolism and oxidative stress in catalase. Heat shock induces oxidative stress activities, which trigger many imperative actions from antioxidant enzymes to play their role in heat-induced oxidative stress resistance [80]. In *E. coli*, the enzymes involved in oxidative stress defense were highly up-regulated under the elevated temperature of 47.5˚C and enhanced survival under thermal stress [81].

To fulfil the requirement of thermostable enzymes in the industry, CAT2 was overexpressed with a small heat shock protein, the Hsp17.6CII, which increases CAT2 activity and prevents it from thermal aggregation [82]. In the meantime, catalase-B, in which another catalase from the plant was successfully overexpressed as a soluble and functional enzyme in the presence of chaperone GroEL/ES. [83]. The soluble expression for both native and variant rice plant catalase in bacterial cytosol by applying chaperones system demonstrates that this method can be considered for the expression of other plant proteins in *E. coli*.

CONCLUSION

Chaperone co-expression has been shown successful in many cases, but there can be no 'right' answer to which chaperone systems fit any of the recombinant enzymes. Even though the selection of suitable chaperone(s) is still a trial-and-error process, some of the combinations or single chaperone systems work successfully to improve solubility and even improve the properties of the recombinant enzymes. In addition to that, recent advances in understanding the mechanisms and substrate specificities of the major chaperones and how the chaperone works would

give some reasonable options to the right chaperone(s) to be used for coproduction of the enzymes in this review.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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