J Ind Microbiol Biotechnol (2014) 41:1667–1675
DOI 10.1007/s10295-014-1500-x

SIMB
Society for Industrial Microbiology and Biotechnology

The selective roles of chaperone systems on over-expression of human-like collagen in recombinant *Escherichia coli*

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Received: 12 June 2014 / Accepted: 12 August 2014 / Published online: 29 August 2014

Abstract Human-like collagen (HLC) is a novel biomedical material with promising applications. Usually, insoluble HLC was formed due to over-expression. In order to improve the production of soluble HLC, the effective chaperone proteins and their mediation roles on HLC were clarified. Trigger factor (TF) pathway with low specificity and high binding affinity to nascent chains could increase soluble HLC expression; GroEL-GroES could increase the expression level of HLC by assisting the correct folding of HLC and increase mRNA level of the gene coding for HLC by enhancing mRNA stability. DnaK chaperone system did not work positively on soluble HLC due to the unbalanced ratio of DnaK:DnaJ:GrpE, especially too high GrpE significantly inhibited DnaK-mediated refolding. The production of soluble HLC with co-expression of exogenous TF and GroEL-GroES was increased by 35.3 % in comparison with the highest value 0.26 g/L reported previously.

Keywords Chaperone · Co-expression · *Escherichia coli* · Over-expression · *pts*G deletion · RT-qPCR

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Introduction

Escherichia coli have been widely used as a host for protein expression due to its clear gene background, fast propagation, easy cultivation and utilization of multiple carbon substrates [1]. Collagen is a major structural protein in the extracellular matrix of animals, mainly exists in skeleton, tendon, cartilage, skin, and other ligament tissues, and plays important roles in numerous approaches to the human tissue engineering for medical applications [2]. Recently, collagen application is rapidly rising in cosmetics and pharmaceutical industries [3]. Human-like collagen (HLC, China patent number: ZL01106757.8) is a recombinant water-soluble collagen with a high-molecular-mass, which is expressed by recombinant *E. coli* BL21 containing human-like collagen cDNA [4]. In comparison to the collagen extracted from animal tissues with the conventional extraction methods, HLC possesses several advantages, such as low immunogenicity, easily modifiable, and no virus risk [5]. Currently, HLC is used as a novel hemostatic material and a novel scaffolding biomaterial for artificial bones, artificial skin, and blood-vessel scaffolds [6, 7].

Due to the promising future of HLC, producing it efficiently at a large scale was very important to meet the need of market demands. Fed-batch cultivation has been proved to be the most effective means of maximizing biomass and the target production [8]. Unfortunately, insoluble HLC was frequently formed due to over-expression. It is well known that the over-expression of exogenous proteins usually results in heavy metabolic burden to cell and insoluble inclusion body due to protein misfolding [9]. Chaperones, a set of conserved protein families, could assist the true folding of nascent polypeptide chains to reach the native steric state and provide a quality control system to refold the misfolded and aggregated proteins [10]. The ribosome-associated trigger factor, the DnaK system (with co-chaperones

Table 1 List of strains and plasmid

Strains and plasmids	Description	Source
Strains		
BL21 3.7 Δ*pts*G	*pts*G gene deletion from the chromosome, kanamycin resistance	Constructed and preserved in our laboratory
BL21 3.7 pTf16 Δ*pts*G	Carrying plasmid pTf16	This study
BL21 3.7 pG-Tf2 Δ*pts*G	Carrying plasmid pG-Tf2	This study
BL21 3.7 pGro7 Δ*pts*G	Carrying plasmid pGro7	This study
BL21 3.7 pKJE7 Δ*pts*G	Carrying plasmid pKJE7	This study
BL21 3.7 pG-KJE8 Δ*pts*G	Carrying plasmid pG-KJE8	This study
Plasmids		
pTf16	Carrying chaperone *tig* gene, which contained a chloramphenicol resistance gene (Cm^r) and an origin of replication derived from pACYC, *ara*B promoter	Purchased from Takara
pG-Tf2	Carrying chaperones *tig* and *gro*EL-*gro*ES, which contained a chloramphenicol resistance gene (Cm^r) and an origin of replication derived from pACYC, *Pzt-1* promoter	Purchased from Takara
pGro7	Carrying chaperone *gro*EL-*gro*ES, which contained a chloramphenicol resistance gene (Cm^r) and an origin of replication derived from pACYC, *ara*B promoter	Purchased from Takara
pKJE7	Carrying chaperone *dna*K-*dna*J-*grp*E, which contained a chloramphenicol resistance gene (Cm^r) and an origin of replication derived from pACYC, *ara*B promoter	Purchased from Takara
pG-KJE8	Carrying chaperones *dna*K-*dna*J-*grp*E and *gro*EL-*gro*ES, which contained a chloramphenicol resistance gene (Cm^r) and an origin of replication derived from pACYC, *ara*B or *Pzt-1* promoter	Purchased from Takara

DnaJ and GrpE, Hsp70/Hsp40) and the GroEL system (with co-chaperone GroES, Hsp60) were considered to be the most abundant and important chaperones for de novo folding of *E. coli* proteins [11]. In order to facilitate the application of these chaperone systems, some commercial plasmids have been developed. Takara's chaperone plasmid set consists of five different types of chaperone-team plasmids which are designed to enable efficient expression of multiple molecular chaperones [12].

However, not all of these chaperone teams could effectively work to a specific biosynthesis system of proteins [13, 14]. To assess the potentially positive role of molecular chaperones on the production of soluble recombinant collagen, co-expression conditions of exogenous chaperone proteins and the target protein were optimized, the intrinsic mediation mechanisms of chaperone on HLC were investigated, and the effects of chaperone proteins on cell growth were also taken into consideration.

Experimental and methods

Strains and plasmids

The strains and plasmids used in this work were described in Table 1. Recombinant *E. coli* BL21 3.7 carrying a plasmid, which contained a kanamycin resistance gene and allowed high temperature induction, was used as a parent strain [15] to construct the *pts*G mutant strains. The recombinant *E. coli* BL21 3.7 Δ*pts*G was constructed using the one-step inactivation method, which was used to prepare the competent cells for transforming chaperone plasmid pGro7, pTf16, pG-Tf2, pKJE7 and pG-KJE8 respectively.

These chaperone plasmids carried an origin of replication derived from pACYC and a chloramphenicol resistance gene (Cm^r). The genes coding for chaperone proteins were situated downstream of either *ara*B or *Pzt-1* (tet) promoter. The genes coding for HLC and chaperone proteins located in different plasmids and allowed to be induced separately [10, 16].

Preparation of culture medium and seeds

A complex medium called fermentation medium has been optimized by Guo et al. [17] on the base of glucose enriched Luria–Bertani (LB) medium and proved to be suitable for HLC expression in recombinant *E. coli* BL21 3.7. The fermentation medium (per liter) contained 12.0 g of Glucose, 11.8 g of Yeast extract, 8.8 g of K_2HPO_4, 3.4 g of NaH_2PO_4, 5.6 g of $(NH_4)_2SO_4$, 2.5 g of $MgSO_4{\cdot}7H_2O$, 0.8 g of EDTA, 0.8 mL of trace element. Moreover, the

trace element solution contained (per liter) 6 g of Fe(III) citrate, 1.5 g of $MnCl_2{\cdot}4H_2O$, 0.8 g of $Zn(CH_3COO)_2{\cdot}2H_2O$, 0.3 g of H_3BO_3, 0.25 g of $Na_2MoO_4{\cdot}2H_2O$, 0.25 g of $CoCl_2{\cdot}6H_2O$, 0.15 g of $CuCl_2{\cdot}2H_2O$, and 0.84 g of (ethylenedinitrilo) tetraaceticacid disodium salt$\cdot 2H_2O$ [18]. All solutions were prepared with deionized water.

Seeds were reactivated by streaking on LB plates from the frozen glycerol stock and growing overnight. Primary and secondary seed cultures were incubated in a 250 mL flask containing 50 mL LB medium in a shaker at 34 °C and 220 rpm for 12 h. LB medium, consisting of 5 g/L of yeast extract, 10 g/L of peptone and 10 g/L of NaCl, was used for the seed culture.

Batch culture

The seed cultures were inoculated into flasks containing 50 mL fermentation medium with the inoculation size of 8 %. When the strains growth was at the middle and late logarithmic growth phase, the cultivation temperature was increased to 42 °C to induce HLC expression for 3–4 h, and then lowered to 39 °C for further induction for 3–4 h. All experiments were performed in triplicate.

Analysis methods

Cell density was measured at 600 nm with a spectrophotometer. The components in the supernatant, such as glucose, acetate, were measured using BioProfile analyzer 300A. The levels of HLC were determined by hydroproline colorimetry [19]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the co-expression of recombinant human-like collagen and chaperone proteins. The detailed procedures of preparing protein samples in supernatant and debris were as described [20].

RNA extraction and cDNA synthesis

E. coli BL21 3.7 Δ*pts*G (parent strain) and all transformants with initial introduction of chaperone plasmids were cultivated in the fermentation medium at 34 °C and in a shaker at 220 rpm, then temperature was raised to 42 °C to induce HLC gene expression and until OD_{600} reached approximately 0.70. Afterwards, cells were harvested by centrifugation at 12,000 rpm for 2 min. Total RNA was extracted using the RNAprep Pure Cell/Bacteria Kit (Tiangen Biotech Co. Ltd., Beijing, China) in accordance with the manufacturer's instructions, and quantified spectrophotometrically (NanoDrop ND-1000, Thermo Fisher scientific Inc.). A total of 5 μL of RNA was used immediately in reverse transcription reactions using PrimeScript™ RT Master Mix Perfect Real Time (TaKaRa Biotechnology Co., Ltd., Dalian, China). The substrates for cDNA synthesis in 10 μL of total volume were as follows: 2 μL of 5× PrimeScript RT Master Mix, 5 μL of total RNA and 3 μL of RNase free ddH_2O. The reverse transcription reaction was performed at 37 °C for 15 min followed by 85 °C for 5 s for inactivation of reverse-transcriptase. For each sample, a no amplification control (containing RNA but not reverse transcriptase) was also prepared. The obtained undiluted cDNA was used in qPCR immediately, or stored at −20 °C until use.

qPCR amplification

To analyze the level of mRNA, the primer extension assay was performed. The q-PCR reactions were carried out in a 96-well plate in CFX96 Real-time PCR Detection System (Bio-Rad Laboratories Inc., USA) using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc., USA). Reactions was performed in 15 μL of reaction volume containing 1.2 μL of cDNA, 0.6 μL of each primer (10 μM), 7.5 μL of 2 × iQ™ SYBR® Green Supermix, and 5.1 μL of nuclease free water. Thermocycling was performed in the CFX96 Real-time PCR Detection System. The q-PCR cycling conditions consisted of an initial cycle of 3 min at 95 °C (the antibody-mediated hot-start iTaq™ DNA polymerase activation), followed by 39 cycles of 95 °C for 5 s (denaturation), 60 °C for 30 s (annealing/extension), and a final melting curve analysis using the defaulted program of CFX96 manager. Each plate included samples for no amplification control as well as samples for no template control (ddH_2O instead of cDNA). All samples were conducted in triplicate.

The $2^{-\Delta\Delta CT}$ (Livak) method was used for data analysis [21], and *ihf*B was used as an endogenous control gene.

Results and discussion

The co-expression conditions of exogenous molecular chaperones and HLC

The influence of the dose of chaperone inducers on the production of soluble HLC

All transformants were cultivated in the fermentation medium at 34 °C for 5 h and then induced by L-arabinose and/or tetracycline for the expression of molecular chaperones. The cultivation temperature was raised to 42 °C to induce HLC expression at the 6th hour. Cell concentration and HLC concentration were used as the evaluation parameters to determine the dose of chaperone inducers. In Fig. 1a, 2.0 g/L of L-arabinose and/or 10 mg/L of tetracycline leaded to a little higher production of soluble HLC than the other

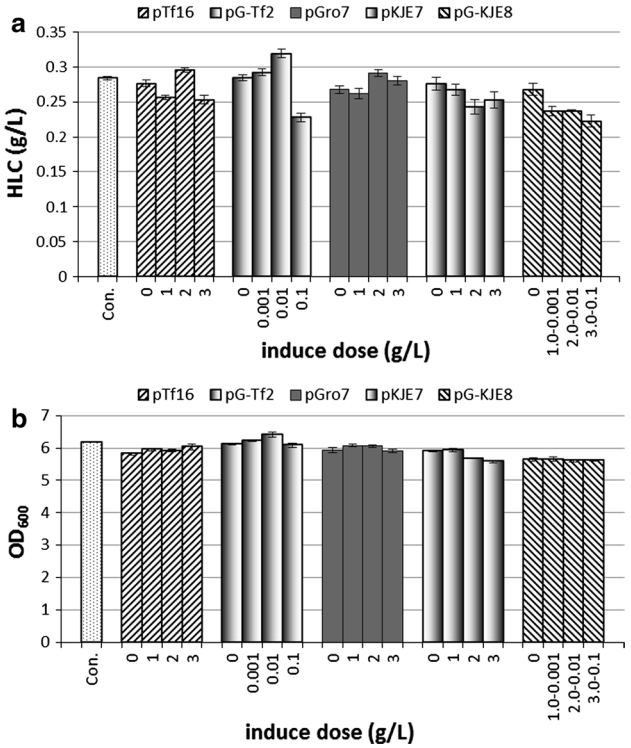

Fig. 1 The HLC production and cell density of recombinant *E. coli* at different inducer doses. The chaperone plasmids were induced at 5th hour and the high temperature induction for synthesizing HLC were performed at 6th hour. The HLC levels and cell densities were compared in Plot **a** and Plot **b** respectively. The control strain did not carry chaperone plasmid

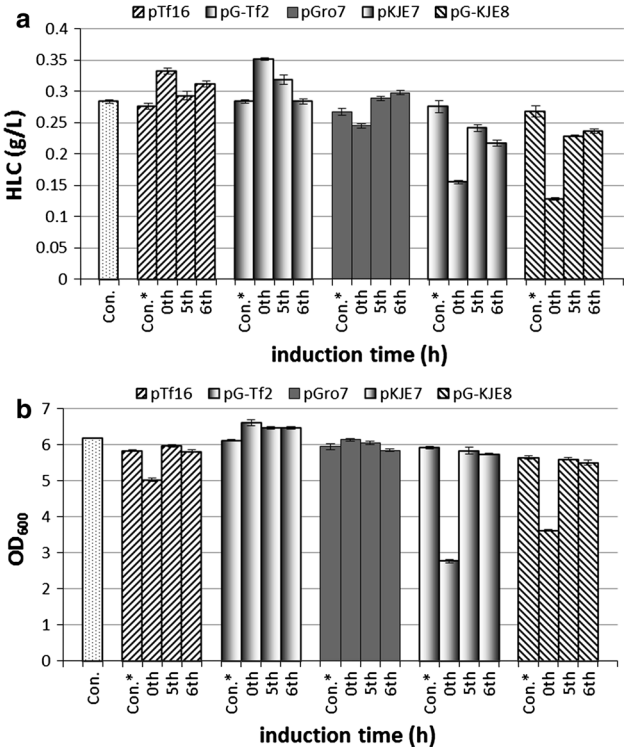

Fig. 2 The HLC production and cell density of recombinant *E. coli* at different induction time. The chaperone plasmids were induced by 2.0 g/L of L-arabinose and/or 10 mg/L of tetracycline at 0th, 5th and 6th hour individually; and the high temperature induction were performed at 6th hour to synthesize HLC. The HLC levels and cell densities were compared in Plot **a** and Plot **b** respectively. The control strain did not carry chaperone plasmid. "Con.*" meant that the transformants carried chaperone plasmids but the chaperone plasmids were not induced to express

inducer dosages in the strains carrying pGro7, pTf16 and pG-Tf2. However, chaperone teams of pKJE7 and pG-KJE8 did not show any help for improving the soluble HLC production. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and q-PCR were used to identify the expression of these chaperone proteins at the translation and transcription levels (data not shown here). The reason of this phenomenon might be that the induction time was not suitable for the accumulation of molecular chaperone to play positive roles in the expression of soluble HLC. Fortunately, these five chaperone plasmids did not bring in significant negative burdens on cell growth (Fig. 1b). The size of these five Takara's chaperone plasmids was ranged from 5 to 11.1 kb, which size might not affect cell growth significantly. Compared with the HLC production of the control strain, the abilities of producing soluble HLC of all transformants carrying chaperone plasmids were very close when without the expression of exogenous chaperone proteins (Fig. 1a). This result further verified that these five plasmids did not impact an extra big burden on recombinant *E. coli* BL21 3.7 *Δpts*G. By light of the above analysis, the inducer dose of 2.0 g/L of L-arabinose and/or 10 mg/L of tetracycline were chosen to further study.

The influence of the chaperone induction time on the production of soluble HLC

All strains were cultivated at 34 °C and 220 rpm with the fermentation medium, the chaperone plasmids were induced by 2.0 g/L of L-arabinose and/or 10 mg/L of tetracycline at 0th, 5th and 6th hour to activate the expression of chaperone plasmids respectively. And the cultivation temperature was raised to 42 °C for the induction of HLC at the 6th hour. As shown in Fig. 2, the initial induction of plasmid pTf16 and pG-Tf2 (induced at the 0th hour) with the over-expression of trigger factor (TF) and GroEL system was benefit for improving the production of soluble HLC, while the induction of chaperone teams of pKJE7 and pG-KJE8 still impaired the formation of soluble HLC, and the initial induction of plasmid pGro7, pKJE7 and pG-KJE8 caused the lowest production of soluble HLC in comparison with the other induction time. Nishihara et al. [16] reported that over-expression of TF alone was sufficient to prevent aggregation of endostatin, over-expression

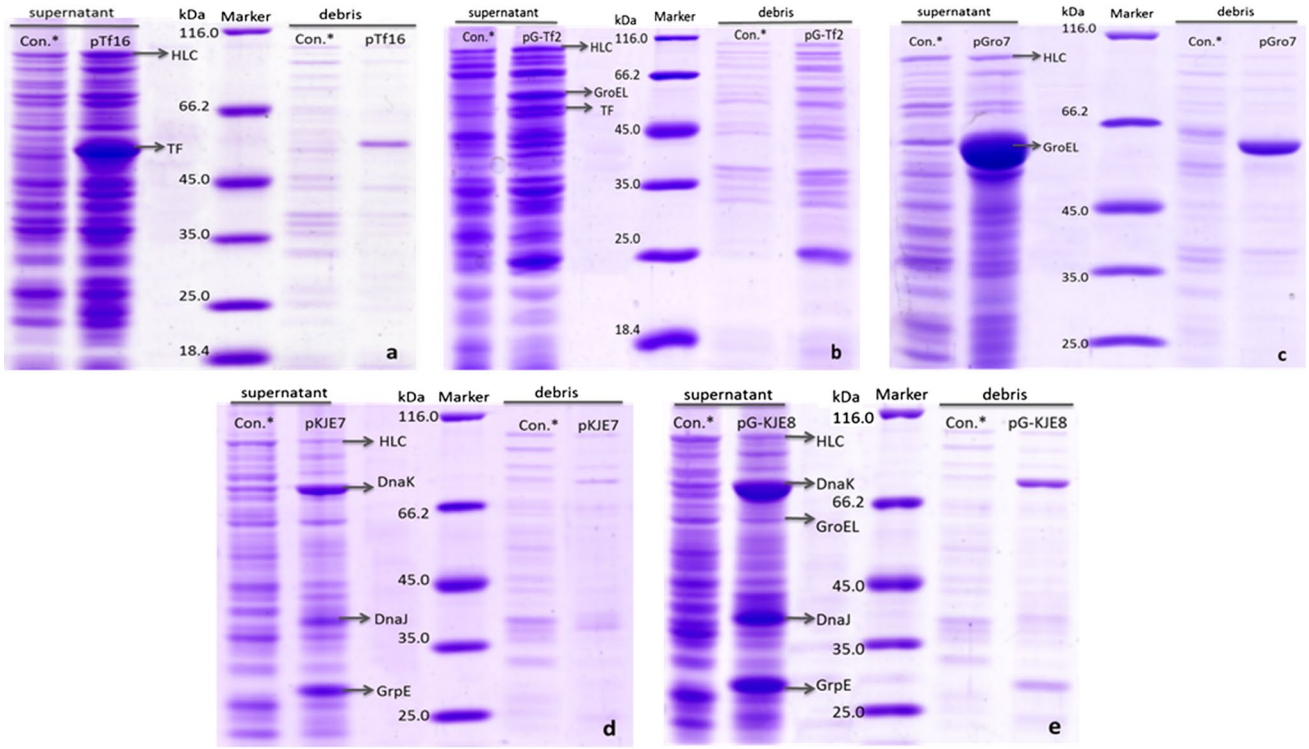

Fig. 3 The results of SDS-PAGE with the initial induction of chaperone plasmids. The plasmids pTf16 (**a**), pG-Tf2 (**b**), pGro7 (**c**), pKJE7 (**d**) and pG-KJE8 (**e**) were induced at the start of cultivation, the high temperature induction were performed at 6th hour to synthesize HLC. Lysates of soluble (supernatant) and hydrophobic (debris) proteins from cells were separated by SDS-PAGE and stained by Coomassie brilliant blue. "Con.*" meant that the transformants carried corresponding chaperone plasmids but the chaperone plasmids were not induced to express

of TF together with GroEL-GroES was more effective for ORP150 and lysozyme, co-expression of the DnaK-DnaJ-GrpE chaperones was effective for endostatin and ORP150. That was to say, these chaperone proteins were selective to play roles in assisting the correct folding of proteins.

As for cell growth, the over-expression of chaperone teams did not affect cell growth significantly except for the initial induction of plasmid pKJE7 and pG-KJE8. Plasmid pKJE7 contained the genes coding for GrpE, DnaK and DnaJ, and plasmid pG-KJE8 expressed all chaperone proteins expressed by plasmid pKJE7 and GroEL-GroES system. The initial induction might result in too much of DnaK and GrpE, then cell growth was impaired, finally biomass decreased. It has been reported that the over-expression of DnaK and GrpE results in a defect in cell division and growth, although DnaJ over-expression does not [22], and GrpE over-expression on cell morphology is related to the cooperation of DnaK.

As for HLC, over-expression of TF and/or GroEL-GroES could enhance the production of soluble HLC; while the DnaK system seemed to not work positively on HLC, the reasons would be analyzed in next part. As mentioned above, the optimum co-expression of exogenous chaperone proteins and target protein was to induce chaperone plasmids from the start of cultivation with 2.0 g/L of L-arabinose and/or 10 mg/L of tetracycline, and the production of soluble HLC was increased by 23.7 % in comparison to the control strain. Compared with the highest value 0.26 g/L reported by Guo et al. [23], the production of soluble HLC was increased by 35.3 %.

The mediation roles of different chaperone systems on the formation of soluble HLC

The influence of molecular chaperones on the HLC distributions in supernatant and debris

The strains were cultivated in the fermentation medium at 34 °C and 220 rpm. Chaperone genes were induced by 2.0 g/L L-arabinose and/or 10 mg/L tetracycline from the start of cultivation. After cultivation for 6 h, the temperature was raised to 42 °C for the induction of HLC synthesis and lasted for 3 h, and then the temperature was reduced to 39 °C to yield HLC. SDS-PAGE was performed to analyze the ingredients of soluble proteins and insoluble proteins in recombinant BL21 3.7 Δ*pts*G and its transformants carrying Takara's chaperone plasmids (Fig. 3). Although Takara's chaperone plasmids were not induced to express,

the heat shock proteins, such as *dnaK*, *groEL* and *tig* existing in *E. coli* were expressed at a low level due to high temperature induction. As reported, DnaK, GroEL and TF were abundant in eubacteria [24]. As shown in Fig. 3, the addition of arabinose and/or tetracycline from the start of cultivation leaded to most chaperone proteins to achieve high level expression, which ensured chaperone co-expression system to impact the expression of the other cytosolic proteins. Since the molecular mass of GroES was about 10 kDa, GroES of cell lysates did not show in our SDS-PAGE gels.

The soluble and insoluble proteins were analyzed in the supernatant and cell debris, respectively. Consistent with the results shown in Fig. 2, the concentration of soluble HLC with co-expressing pTf16 or pG-Tf2 was increased apparently, but it decreased with the initial induction of pGro7, pKJE7 or pG-KJE8 in the supernatant (Fig. 4a). In accordance with the design of Takara's chaperone plasmids, both pTf16 and pG-Tf2 contained the genes coding for TF, and pG-Tf2 expressed GroEL extra; GroEL system and DnaK system were expressed by pGro7 and pKJE7 individually, and both were co-expressed by pG-KJE8. In eubacteria, trigger factor (TF) could bind to the ribosome, and it was the first chaperone to interact with newly synthesized polypeptides and assisted their folding [25]. Additionally, in Figs. 3a and 4, the high level expression of TF resulted in not only an obvious increase of the soluble HLC production but also a decrease of the concentration of insoluble HLC in debris, and the total production of HLC was increased significantly under the help of TF. It was recognized that TF interacted with small polypeptides; as for HLC with larger molecular mass, several molecules of TF might associate with each chain [26, 27]. In Gram-negative bacteria, TF could function at several levels, protecting nascent chains from digestion by proteases, preventing misfolding by delaying folding until translation completed, and cooperating with other chaperones to facilitate proteolysis of aggregate-prone conformations [28].

Surprisingly, when TF cooperated with GroEL-GroES complex (pG-Tf2), the soluble HLC concentration was increased without significantly decreasing the insoluble HLC concentration by co-expressing HLC and chaperone proteins (Figs. 3b, 4). As reported, GroEL-GroES chaperone team was a double-ring complex with central cavity of GroEL and its lid-like co-chaperonin GroES, it served as a foldase to interact with partially folded polypeptides and assist in additional folding [29]. GroEL-GroES might not be able to undo previous aggregation [30], and GroEL did not bind to nascent chains and would like to receive an appreciable fraction of its substrates after their interaction with TF [31]. So the insoluble HLC formed by false aggregation from upstream could not be converted to soluble HLC under the help of GroEL-GroES if the fast

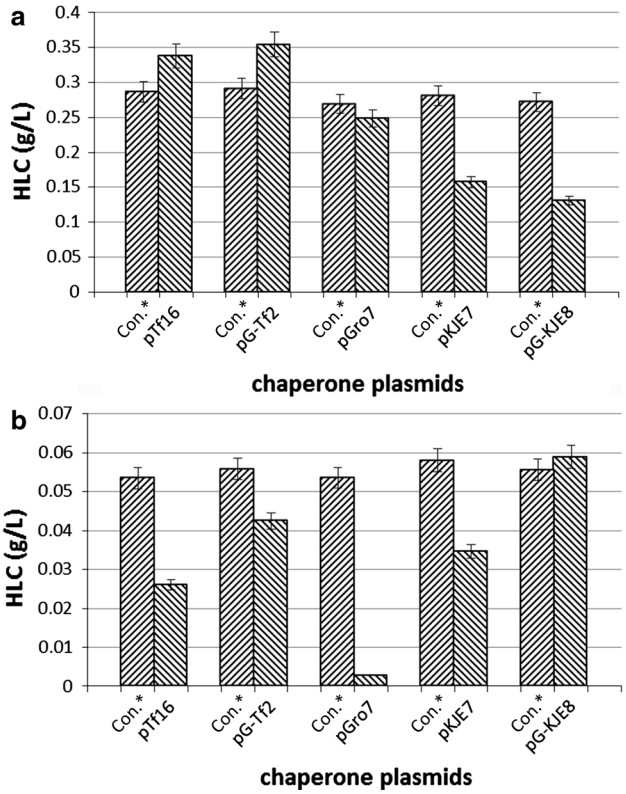

Fig. 4 The concentrations of HLC calculated from gel imaging analysis system under the initial induction of chaperone plasmids. The productions of HLC were analyzed in the cell supernatant (**a**) and debris (**b**) respectively. "Con.*" meant that the transformants carried corresponding chaperone plasmids but the chaperone plasmids were not induced to express

over-expression of HLC antagonized the assistant folding of TF. As for the non-aggregated HLC, GroEL-GroES mediated protein folding through multiple rounds of binding, encapsulation, and release of substrate protein [32]. As the consequence, the transformant carrying pG-Tf2 plasmid could produce more soluble HLC than the transformant carrying pTf16 plasmid, TF and GroEL-GroES played synergistic roles in vivo.

Although GroEL-GroES accompanied with TF was verified to assist the folding of HLC, the transformant carrying pGro7 plasmid only co-expressed GroEL-GroES from the initial cultivation did not increase the production of soluble HLC (Figs. 3c, 4). In the SDS-PAGE gel, the level of GroEL was too high, and the over-expression of this chaperone protein might compete with the HLC expression in the strain BL21 3.7 Δ*pts*G pGro7 (Fig. 3c). While a modest expression of GroEL by pGro7 and pG-Tf2 was benefit to soluble HLC synthesis (Figs. 2, 3b, 4). Interestingly, the expression levels of TF and GroEL were both decreased with their co-expression (Fig. 3a–c), which meant that over-expression TF or GroEL seemed to affect the production of

the other one. The over-expression regulation between TF and GroEL agreed to Endo's report [30].

As for DnaK chaperone system, the initial induction of pKJE7 plasmid caused the lowest biomass among all transformants, correspondingly, HLC production was close to the lowest value. In SDS-PAGE gels, both HLC and chaperone proteins were at a low level (Fig. 3d). While the induction time of plasmid pKJE7 was adjusted to 1 h earlier than the high temperature induction for expressing HLC, the production of soluble HLC was approached to that of the control without lowering biomass (Fig. 2). This result was reappeared by the transformant carrying pG-KJE8 plasmid (Fig. 2). It was well known that the DnaK-DnaJ chaperone system and TF were able to compensate for each other in vivo [33]. Just like TF, DnaK interacted with considerably shorter nascent polypeptide chains [27, 34], moreover, TF and DnaK shared potential binding sites present in nascent polypeptide substrates [31]. However, DnaK-DnaJ-GrpE chaperone system seemed to be invalid for improving the production of this specific soluble protein, HLC.

In DnaK-DnaJ-GrpE chaperone system, DnaK was one of the most abundant constitutively expressed and stress inducible chaperones in *E. coli* [35], it associated with its co-chaperones, DnaJ and GrpE to modulate its ATP exchange and ATPase activity [36]. As reported, DnaK chaperone system alone involved in the folding of newly synthesized proteins, transporting polypeptide chains through membranes, refolding of denatured and aggregated proteins, and controlling the regulatory proteins via the nucleotide-regulated binding and release cycles [24, 26]. In the DnaK chaperone system, the ratios of DnaK:DnaJ:GrpE could affect their selectivity on substrate. For example, protein ratio of 1:0.2:0.1 (DnaK:DnaJ:GrpE) were effective to prevent heat-shock induced protein aggregation in *E. coli* [24], and under in vivo conditions, an estimated chaperone ratio of DnaK:DnaJ:GrpE was 10:1:3 [37]. From the quantitative analysis of SDS-PAGE gels, the ratios of DnaK:DnaJ:GrpE were 1:0.6:0.9 and 1:0.4:0.7 in pKJE7 transformant and pG-KJE8 transformant respectively, which ratios might not suitable for DnaK chaperone system to play effectively positive roles on the synthesis of soluble HLC. Besides, the biomasses of these two transformants were lower than that of the others (Fig. 2). According to the report, over-expression of DnaJ did not impair cell division and growth, but over-expression of DnaK and GrpE did [22]. All these inferred that the expression level of GrpE could affect the function of DnaK-DnaJ-GrpE chaperone system.

Serving as a nucleotide exchange factor that promoted ADP-dissociation from the ATPase domain of DnaK, GrpE functioned cotranslationally and post-translationally to promote protein folding and disaggregation in cells [38] and negatively regulated the transcription of heat shock genes by direct interaction with σ^{32} [39]. Under physiological conditions, the synergistic action of the two co-chaperones DnaJ and GrpE controlled the steady-state distribution between the high-affinity state (ADP-bound state) and low-affinity state (ATP-bound state) of DnaK and provided the substrate proteins with an opportunity to be efficiently folded into their correct structure. Under GrpE over-expressing conditions, the balanced distribution of these states shifted towards the low-affinity state (ATP bound state or nucleotide-free state), which decreased the fraction of substrate bound to DnaK in the high-affinity state and then drastically decreased the folding yield [40]. In addition, an excess of GrpE appeared to be deleterious to cell division, then cell growth and colony formation were defected [40], but over-expression of GrpE did not affect the expression levels of DnaK (Fig. 3d, e) [41]. At the same time, over-expression of DnaK-DnaJ-GrpE chaperone system affected the expression of downstream GroEL, so the GroEL was at a low level in SDS-PAGE gel (Fig. 3e).

Generally, TF with low specificity and high binding affinity to nascent chains show a generally positive effect on soluble HLC, GroEL-GroES could assist the true folding of HLC and played synergistic roles in vivo combined with TF. DnaK-DnaJ-GrpE chaperone system did not work positively on the soluble HLC due to the unbalance ratio of DnaK-DnaJ-GrpE, especially too high GrpE significantly inhibited DnaK-mediated refolding.

The influence of molecular chaperones on the target protein at transcriptional level

As described above, these five chaperone systems impacted different effects on the HLC expression respectively. Currently, the effect of chaperone on protein was considered to assist the true folding of nascent polypeptide chains (TF and DnaK chaperone system) to the native state and provide a quality control system that refolds misfolded (GroEL chaperone system and DnaK chaperone system) and aggregated proteins (DnaK chaperone system) [10]. In order to explore the mechanism of chaperone for improving the expression of soluble HLC, the transcriptional level of the genes coding for HLC were analyzed using real-time fluorescence quantitative PCR. As shown in Fig. 5, compared with the control strain, the mRNA level of HLC gene were obviously increased by GroEL-GroES (pGro7), and slightly increased by TF (pTF16) as well as co-expression of GroEL-GroES and TF (pG-TF2). While the DnaK-DnaJ-GrpE (pKJE7) and the combination of DnaK-DnaJ-GrpE and GroEL-GroES (pG-KJE8) decreased the mRNA level of HLC gene (Fig. 5). Since all samples for real-time fluorescence quantitative PCR were prepared with initial induction of chaperone genes, as mentioned above, the initial inductions of pKJE7 and pG-KJE8 were not good for cell

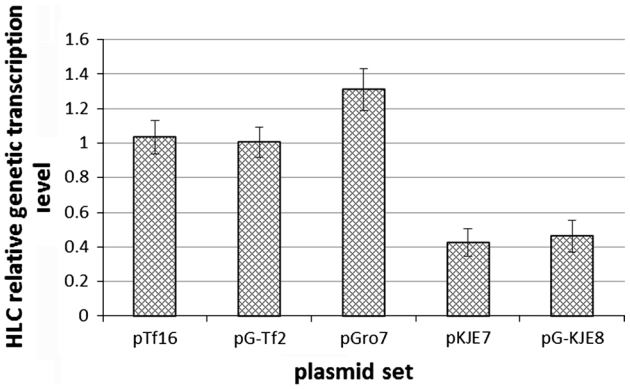

Fig. 5 Relative transcriptional levels of HLC gene. The relative transcriptional levels of the gene coding for HLC with co-expression of different chaperone plasmids were compared with the parent strain *E. coli* BL21 3.7 Δ*pts*G

growth and HLC synthesis (Figs. 3d, e, 4), this result was further confirmed at transcriptional level.

As for GroEL, it has been known to be an element to constitute an RNA-binding complex, which provides mRNA protection against RNaseE [42] due to its Hairpin structures at the 5′-end of mRNA [43]. A higher level of mRNA could be gained by enhancing the mRNA stability of target genes during high temperature induction; consequently, a higher protein production was achieved [29]. That was to say, some of the heat-shock proteins could protect mRNA from nuclease attack and assist protein folding.

Hyunjin et al. [29] reported that co-expression of *dna*K-*dna*J-*grp*E (pG-KJE6) or *gro*EL-*gro*ES (pGro7) in *E. coli* increased the mRNA stability and the level of gene expression further, even though the degree of stabilization was varied depending on the genes tested. Interestingly, the stabilization roles of different chaperone proteins varied with the researched genes, for example, endoxylanase mRNA was stabilized more by GroEL-GroES, but *gfpuv* mRNA showed a longer half-life in the presence of DnaK-DnaJ-GrpE [29].

In general, GroEL enhanced the mRNA stability and resulted in higher protein production, suggesting that the heat-shock proteins tested in this study function as a controller of gene expression on the level of mRNA as well as on protein synthesis.

Conclusions

As reported, co-expression of molecular chaperones could improve the expression level of soluble exogenous protein of *E. coli*, but these positive roles were highly protein-specific. TF could increase the production of soluble HLC with/without the cooperation of GroEL-GroES; GroEL-GroES enhanced the expression level by both assisting the correct folding and stabilizing mRNA, but this system seemed to be invalid in refolding the aggregated proteins. The functions of DnaK system could be impaired by the unbalance ratio of DnaK:DnaJ:GrpE, too much GrpE not only did harm to cell growth but also inhibited DnaK-mediated refolding.

Acknowledgments This study was financially supported by the National Natural Science Foundation of China [21176200]; the Scientific Research Program of Shaanxi Provincial Department of Education, China [2010JC21]; and Shaanxi Biochemical Engineering Key Discipline Program, China.

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