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Application of molecular chaperone to increase the expression of soluble human-like collagen in *Escherichia coli*

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ABSTRACT

Human-like collagen (HLC) is a soluble recombinant protein expressed in *Escherichia coli* BL21, but the over-expression of recombinant proteins in host cells often leads to misfolding and aggregation. In order to increase percent of soluble HLC in total HLC, chaperone was introduced. G*ro*EL system cooperated with *Gro*ES was found to be beneficial for the enhancement of HLC solubility, and electrophoresis results showed that chaperone was coordinately co-overproduced with recombinant human-like collagen to optimize *de novo* folding. When 2.0 g/L of arabinose was added at the start of cultivation, the production of soluble HLC was increased by 55% in *Escherichia coli* BL21 3.7 pGro7 compared to its parent strain without carrying chaperone plasmid.

KEYWORDS

Chaperone;
Human-like collagen;
Co-expression;
Escherichia coli;
*pts*G gene.

INTRODUCTION

Escherichia coli has been widely used as a host for protein expression due to its simple components and well-studied background[1]. Collagen is a group of naturally occurring proteins found in animals, mainly existing in skeleton, tendon, cartilage, skin, and other ligament tissue, especially in the flesh and connective tissues of vertebrates, and plays important roles in numerous approaches to the human tissue engineering for medical applications[2]. Recently, collagen is rapidly expanding in cosmetics and pharmaceutical industries[3]. Human-like collagen (HLC, China patent number: ZL01106757.8), is a water-soluble protein with a molecular weight of 90 kDa expressed by recombinant *Escherichia coli* BL21 containing human-like collagen cDNA which transcribed from the mRNA coding for human collagen[4]. HLC contains a tri-helix structure similar to type I collagen which consists of one α1 and two α_2 chains, HLC comprises three identical modified chains[5]. Compared 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[6]. Currently, HLC is used as a novel hemostatic material and a novel scaffolding biomaterial for artificial bones, skin tissues, and blood-vessel tissues[7-9].

It is well known that the over-expression of heterogeneous proteins usually result in heavy metabolic burden to cell and form insoluble inclusion body due to protein misfolding. During the high cell density fermentation, insoluble HLC was formed because of over-

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expression. Chaperones could assist the true folding of nascent polypeptide chains to the native state and provide a quality control system that can refold the misfolded and aggregated proteins[10,11]. It has been considered that the ribosome-associated Trigger Factor, the DnaK system (with co-chaperones DnaJ and GrpE), and the GroEL system (with co-chaperone GroES) can help the nascent polypeptide chains to fold truly in *E. coli*,[12-16]. However, not all of these three chaperone systems were effective to a specific protein biosynthesis system, and a specific protein biosynthesis system would like to choose special chaperone systems to mediate the true folding of the target proteins *in vivo*[10].

To assess the full potential of molecular chaperones for the production of soluble recombinant proteins, the intrinsic functions of chaperone on HLC were investigated by co-expression HLC and chaperones, and the effects of chaperone proteins on cell growth were also taken into consideration.

MATERIALS AND METHODS

Strains and plasmids

The strains and plasmids used in this work were described in TABLE 1. Recombinant *E. coli* BL21 3.7[17] and its *pts*G mutant[18] were used as parent strains to construct strains BL21 3.7 pGro7 and BL21 3.7 pGro7 *Δpts*G through translation technology.

This chaperone plasmid carries an origin of replication derived from pACYC and a chloramphenicol resistance gene (Cm^r). The chaperone genes are located downstream of the *araB* promoter. The genes expressing the target protein HLC and the chaperone GroEL-GroES which are located in different plasmids can be induced separately (Figure 1).

Medium and seed culture preparation

The fermentation medium (TABLE 2) has been optimized by Guo et al[19] and proved to be suitable for HLC expression in recombinant *E. coli* BL21 3.7.

Seeds were reactivated by streaking on LB plates from the frozen glycerol stock and grew 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. Luria-Bertani (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 in middle and late logarithmic growth phase, the cultivation temperature was raised to 42 °C and kept for 3-4 h to induce HLC expression, and then lowered to 39 °C for further induction for 3-4 h. All experiments were performed in triplicate.

TABLE 1 : List of strains and plasmid

Strains and plasmids	Description	source
BL21 3.7	Carrying human-like collagen II cDNA, kanamycin resistance (Kmr), and induced at high temperature	Preserved and used in our laboratory
BL21 3.7 *Δpts*G	*pts*G gene deletion from the chromosome, kanamycin resistance	Constructed and preserved in our laboratory
BL21 3.7 pGro7 & BL21 3.7 pGro7 *Δpts*G	Carrying plasmid pGro7 (chaperone GroEL-GroES) which contains a chloramphenicol resistance gene (Cmr) and an origin of replication derived from pACYC, *ara*C promoter	This study Plasmid pGro7 was purchased from Takara.

Analysis methods

Cell density was measured at 600 nm using a spectrophotometer. The cell concentration was measured by the dry cell mass (DCW): 50 mL the broth samples were centrifuged (10 min, 10000 rpm), and then washed three times with distilled water and dried to a constant mass in a 105 °C oven. The byproducts were measured using BioProfile analyzer 300A. The concentration of HLC levels were determined by hydroproline colorimetry[16]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the co-expression of recombinant human-like collagen and chaperone proteins.

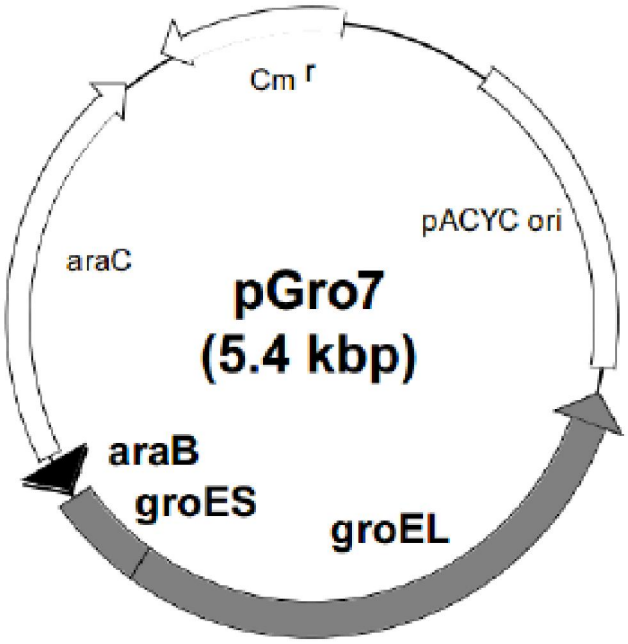


Figure 1 : Chaperone pGro7 plasmid set

TABLE 2 : The compositions of the fermentation medium

Components	Fermentation medium (g/L)
Glucose	12
Yeast extract*	11.8
K_2HPO_4	8.8
NaH_2PO_4	3.4
$(NH_4)_2SO_4$	5.6
$MgSO_4$	2.5
EDTA	0.8
Trace element**	0.8
Antifoam	0.1

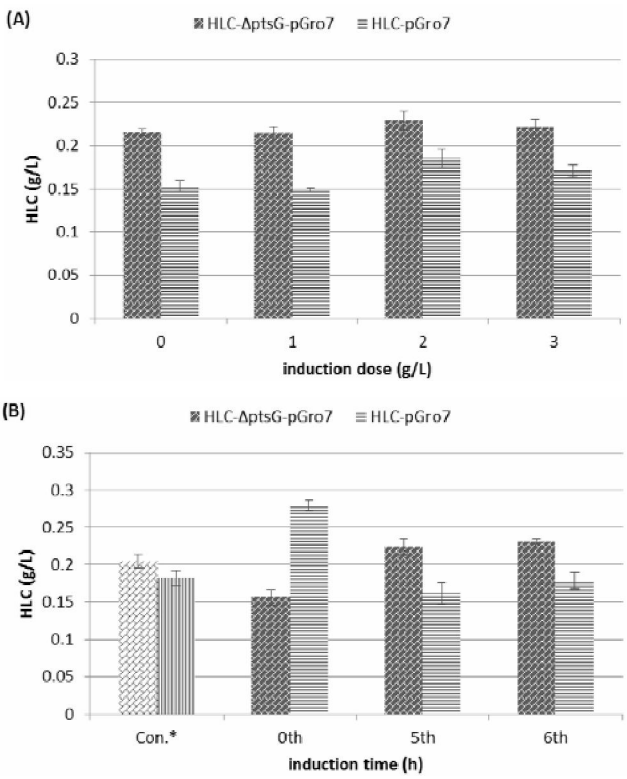
*** The yeast extract (Type LP0021) was manufactured by Oxoid, Basingstoke, Hampshire, England, UK; **The trace metal solution contained (per liter) 6 g of Fe(III) citrate, 1.5 g of $MnCl_2 \cdot 4H_2O$, 0.8 g of $Zn(CH_3COO)_2 \cdot 2H_2O$, 0.3 g of H_3BO_3, 0.25 g of $Na_2MoO_4 \cdot 2H_2O$, 0.25 g of $CoCl_2 \cdot 6H_2O$, 0.15 g of $CuCl_2 \cdot 2H_2O$, and 0.84 g of (ethylenedinitrilo) tetraaceticacid disodium salt$\cdot 2H_2O$**

RESULTS AND DISCUSSION

The influence of the molecular chaperone on HLC syntheses

The influence of the chaperone inducer dose on HLC production

In Figure 2 (A), strains were cultivated for 5 h in the fermentation medium at 34 °C and then induced using arabinose for the expression of chaperone. The cultivation temperature was raised to 42 °C to induce HLC expression at the 6th hour. The data in Figure 2 (A) showed that the HLC concentrations of BL21 3.7 pGro7 and BL21 3.7 pGro7 *Δpts*G were a little higher than that of others when the induce dose was 2.0 g/L, and *pts*G gene deletion was beneficial for HLC synthesis. The reason of this phenomenon might be that the induction time was not suitable for molecular chaperone to play positive roles in true folding of soluble HLC. The induction dose of 2.0 g/L was chosen to further study.



***The control strains did not carry plasmid pGro7**

Figure 2 : The HLC production by recombinant E. coli at different inducer doses and induction time

The influence of the chaperone induction time on HLC production

In Figure 2 (B), strains were cultivated at 34 °C and 220 rpm in the fermentation medium and induced by 2.0 g/L of inducer at 0th, 5th and 6th hour to promote chaperone expression respectively. And the cultivation temperature was raised to 42 °C for the induction of HLC at the 6th hour. From the data in Figure 2 (B), the conclusion could be drawn that the optimal induction time was the 0th hour (initial induction) for recombinant *E.coli* BL21 3.7 pGro7, the initial induction brought in

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the sufficient synthesis of chaperone proteins, which could assist the true folding of soluble HLC. However, the production of soluble HLC in recombinant *E. coli* BL21 3.7 pGro7 *Δpts*G strain reduced at initial induction model and it was not improved significantly when induced at 5th and 6th hour. Our previous research has shown that the deletion of *pst*G gene could improve cell growth ability and decrease toxic acetate accumulation[18]. In order to explain this conflict, further analysis was done at following part.

The function mechanism of chaperone system GroEL/GroES

The strains were cultivated in the fermentation medium at 34 °C and 220 rpm. Chaperone genes were induced by 2.0 g/L of arabinose from the start of cultivation. After 6 h cultivation the temperature was raised to 42 °C for the induction of HLC expression and lasted for 3 hours, and then the temperature was reduced to 39 °C to yield HLC. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed to analyze the ingredients of soluble proteins and insoluble proteins in recombinant *E.coli* BL21 3.7, BL21 3.7 *Δpts*G, BL21 3.7 pGro7 and BL21 3.7 pGro7 *Δpts*G (Figure 3). It was obviously that GroEL achieved high level expression, which ensured GroEL-GroES system to play a crucial role in nascent peptide folding.

The hydrophobic and soluble proteins were analyzed in the cell debris and supernatant respectively. In the supernatant (Figure 3), the concentration of HLC with co-expression pGro7 was increased in the strain BL21 3.7 but decreased in the strain BL21 3.7 *Δpts*G. In the debris, the higher the soluble HLC in supernatant was, the lower the hydrophobic HLC in cell debris became. And with co-expression pGro7, hydrophobic HLC in cell debris was decreased significantly in all strains.

From Figure 3, GroEL/GroES seemed to increase the percent of soluble HLC instead of increase the production of total HLC. F. Ulrich Hartl[20] has demonstrated that GroES is a single-ring heptamer that binds to GroEL in the presence of ATP or ADP. The nascent polypeptide chain binding to an asymmetric GroEL-GroES complex might lead to local unfolding[21]. ATP binding then triggered a conformational rearrangement of the GroEL apical domains to facilitate the binding of GroES (forming the *cis* complex), and produced rigid-body movements in the apical domains to release the polypeptide into the sequestered space for triggering folding. At the same time, ADP and GroES dissociated from the opposite (*trans*) GroEL ring, allowing the release of polypeptide that had been enclosed in the former *cis* complex. Folding continued seamlessly in the encapsulated space of the *cis* ADP complex, for the time needed to hydrolyze the seven ATP molecules in the newly formed *cis* complex (~10 s). Importantly, the affinity of GroEL for GroES was weakened in this complex relative to the ATP complex, thus priming the *cis* complex for release of its ligands. The released polypeptide either had reached the native state or was still in a non-native state, and the non-native polypeptide could bind to another GroEL molecule for a further attempt at folding (Figure 4).

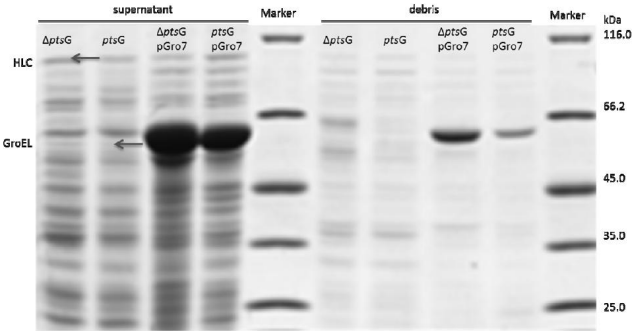


Figure 3 : The results of SDS-PAGE with the initial induction of chaperone plasmid

According to Figure 2 and Figure 3, with the initial expression chaperone proteins, *E.coli* BL21 3.7 pGro7 produced 0.28 g/L of soluble HLC that was 55% higher than that of its parent strain; the *E.coli* BL21 3.7 pGro7 *Δpts*G produced 0.16 g/L of soluble HLC that was 20% lower than that of its parent strain. The reason for this phenomenon was that the over-expression of the chaperone plasmid might compete with the HLC expression in the strain BL21 3.7 *Δpts*G (Figure 4). The strain BL21 3.7 *Δpts*G partially blocked the PTS glucose transport system[18]. Therefore, glucose uptake rate in *E.coli* BL21 3.7 was higher than in *E.coli* BL21 3.7 *Δpts*G. A low glucose uptake rate might be result in the competition of carbon source distribution. Further, the deletion of *pts*G increased GroEL expression significantly (Figure 4), and the production of GroEL in *E.coli* BL21 3.7 *Δpts*G is 1.56 times as much as that of *E.coli* BL21 3.7. And then HLC production was

decreased in *pts*G mutant strain carrying chaperone plasmid at initial induction model.

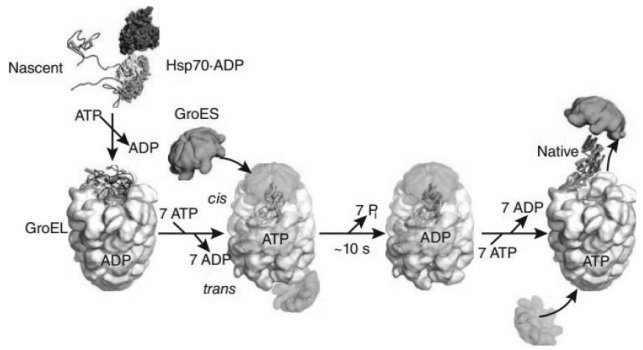
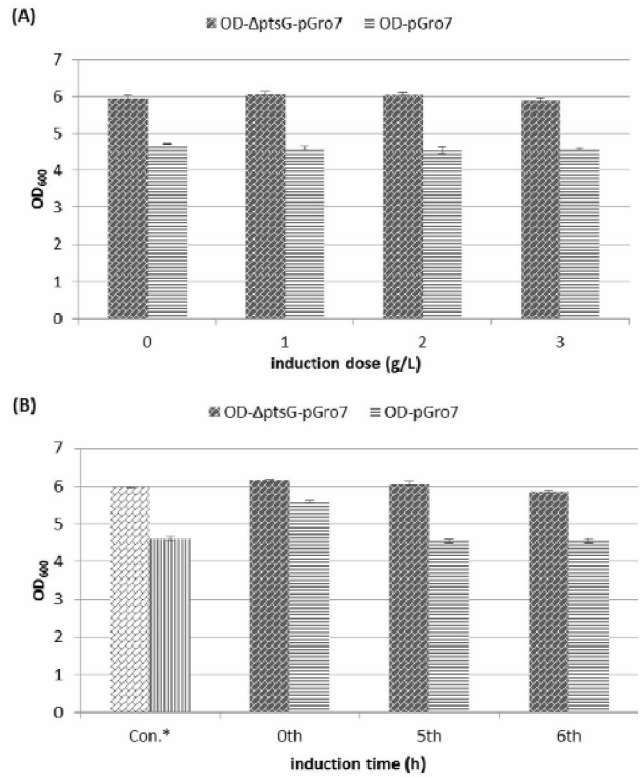


Figure 4 : Folding of the nascent proteins in the GroEL-GroES chaperonin cage



***The control strains did not carry plasmid pGro7**

Figure 5 : The influence of induction dose and induction time for the cell density

The influence of the chaperone on cell growth

The effects of inducer dose and induction time of chaperone on cell growth of these four strains were investigated respectively. The data in Figure 5 (A) showed that the cell density (OD_{600}) of *E.coli* BL21 3.7 *Δpts*G was a slightly higher than that of the others when the inducer dose was 2.0 g/L. And cell density (OD_{600}) of *E.coli* BL21 3.7 pGro7 was 1.22 times as much as that of the parent strain without carrying pGro7 when the chaperone plasmid was introduced by 2.0 g/L of arabinose at the initial cultivation (Figure 5 (B)). This phenomenon illustrated that the expression of chaperone plasmid had limited ability to improve the cells viability, but it would not add metabolic burden to cells.

CONCLUSIONS

Chaperone *Gro*EL-*Gro*ES could effectively improve HLC expression in *E.coli* BL21 3.7 when the strains were induced by 2.0 g/L of arabinose from the initial cultivation. The results of the HLC production and OD_{600} showed that chaperones have greatly potential to improve the percent of soluble HLC without increasing cell metabolic burden.

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REFERENCES

[1] P.Bhandari, J.Gowrishankar; J.Bacterio; **l79(13)**, 4403–4406 **(1997)**.
[2] W.Friess; Eur.J.Pharm Biopharm., **45(2)**, 113–36 **(1998)**.
[3] F.Pati, B.Adhikari, S.Dhara; Bioresour.Technol., **101(10)**, 3737-3742 **(2010)**.
[4] D.D.Fan, Y.E.Luo, Y.Mi, X.X.Ma, L.A.Shang; Biotechnol Lett., **27(12)**, 865–870 **(2005)**.
[5] R.Xu, Y.E.Luo, D.D.Fan; Biotechnology and Applied Biochemistry., **59(5)**, 330–337 **(2012)**.
[6] G.V.Smirnova, O.N.Oktyabr'skill; [Translated version: Microbiology (USSR). 54:205–209.], **54**, 252-256 **(1985)**.
[7] C.H.Zhu, D.D.Fan, Z.G.Duan, W.J.Xue, L.A.Shang, F.L.Chen, Y.E.Luo; J.Biomed Mater Res A., **89(3)**, 829–840 **(2009)**.
[8] K.Hu, F.Z.Cui, Q.Lv, J.Ma, Q.L.Feng, L.Xu, D.D.Fan; J.Biomed Mater Res A., **84(2)**, 483–490 **(2008)**.

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[9] Y.Wang, F.Z.Cui, Y.Zhai, X.M.Wang, X.D.Kong, D.D.Fan; Mater.Sci.Eng., **26**, 635–638 **(2006)**.
[10] A.Marco, E.Deuerling, A.Mogk, T.Tomoyasu, B.Bukau; BMC Biotechnoly., **7**, 32 **(2007)**.
[11] K.Nishihara; Appl.Environ.Microbiol., **64**, 1694-1699 **(1998)**.
[12] E.Deuerling, B.Bukau; Crit Rev Biochem Mol Biol., **39(5-6)**, 261-277 **(2004)**.
[13] F.U.Hartl, M.Hayer-Hartl; Science, **295**, 1852-1858 **(2002)**.
[14] H.Schröder, T.Langer, F.U.Hartl, B.Bukau; DnaK, DnaJ, EMBO J, **12(11)**, 4137-4144 **(1993)**.
[15] J.C.Young, V.R.Agashe, K.Siegers, F.U.Hartl; Nat Rev.Mol.Cell.Biol., **5(10)**, 781-791 **(2004)**.
[16] J.Martin, A.L.Horwich, F.U.Hartl; Science, **258(5084)**, 995-8 **(1992)**.
[17] D.D.Fan, M.R.Duan, Y.Mi, J.R.Song, J.F.Xi, D.W.Wang, G.Z.Wang; J.Chem.Ind.Eng., (In Chinese), **53**, 752-754 **(2002)**.
[18] Y.E.Luo, T.Zhang, D.D.Fan, T.Z.Mu, W.J.Xue, J.F.Hui et al; Biotechnol Appl.Biochem, BAB-13-0094. (Revised), **(2013)**.
[19] L.Guo, Y.E.Luo, D.D.Fan, R.Xu, J.F.Hui, X.X.Ma, C.H.Zhu; African Journal of Microbiology Research., **6(17)**, 3856-3865 **(2012)**.
[20] F.U.Hartl, A.Bracher, M.Hayer-Hartl; **475**, 324-332 **(2011)**.
[21] F.U.Hartl, M.Hayer-Hartl; Nature Struct.Mol.Biol., **16**, 574-581 **(2009)**.