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Expression and purification of human placenta lactogen in *Escherichia coli*

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Abstract

There are many growth factors secreted by placenta including growth hormone, placenta lactogen (PL), prolactin, follicle stimulating hormone, luteinizing hormone, thyroid stimulating hormone, and chorionic gonadotropin. For a systematic study of how these growth factors work together to result in the various biological functions and future clinical applications, it is needed to produce enough quantities of each protein. In this paper, we report the cloning of human PL (hPL) and expression by *Escherichia coli (E. coli)*. Four kinds of expression vectors containing the hPL gene were transformed into several kinds of suitable host strains and grown at 37 and/or 30 °C. Determination of the yield of recombinant hPL by SDS–PAGE reveals that among the various conditions, pQE30-PL in *E. coli* strain M15[pREP4] expressed the largest amount of recombinant hPL at 37 °C. However, the expressed recombinant hPL was accumulated in inclusion body forms. The inclusion bodies were solubilized in 8 M urea and purified by a His_6 tagged affinity column under denaturing condition and the final yield of hPL was determined to be 48 mg/L. Intra-chain disulfide bonds could be formed either by oxidation in the refolding buffer or by air oxidation in the presence of urea. The biological activity was examined by the fact that hPL could stimulate erythroid maturation by the formation of hemoglobin in K-562 cells in the presence of erythropoietin. Initial optimization studies resulted in the production of 282.4 mg/L of hPL.

Keywords: Human placental lactogen; Molecular cloning; Protein expression

Placenta is an endocrine organ and its extraction mix has been used as precious Chinese medicine [1]. It is known that placenta secretes various immune proteins, thromboplastin, hormones, and many trace substances [2]. These substances collectively adjust body endocrine secretion functions and promote metabolic activities. Many growth factors are secreted by placenta including growth hormone (GH)[1], placenta lactogen (PL), prolactin (Prl), follicle stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH), and chorionic gonadotropin (CG).

These placental hormones are important. For example, LH, FSH, and CG are also excreted by pituitary and are termed gonadotropins, which help the gonad cells to provide the hormonal milieu and essential factors for germ cell development and intra-testicular cell–cell interactions. These functions are maintained and modulated by other gonadotropins such as GH and Prl [3,4]. On the other hand, the detailed functions of hGH, hPL, and hPrl during pregnancy are still a matter of debate, but they may have significant influence on the carbohydrate and lipid metabolism of

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[1] *Abbreviations used:* GH, growth hormone; PL, placenta lactogen; Prl, prolactin; FSH, follicle stimulating hormone; LH, luteinizing hormone; TSH, thyroid stimulating hormone; CG, chorionic gonadotropin; hPL, human PL; hGHR, human growth hormone receptor; hGHbp, human growth hormone binding protein; PRLR, prolactin receptor; PCR, polymerase chain reaction; DAF, 2,7-diaminoflourene; SDS–PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NTA, nitrilotriacetic acid.

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mother and fetus [5,6]. PL may also stimulate development and cell division of mammary gland epithelial cells [5].

hGH, hPL, and hPrl also regulate a wide variety of physiological processes, including growth and differentiation of muscle, bone, and cartilage cells [7,8]. HGH binds to both the human growth hormone receptor (hGHR) and its soluble binding protein (hGHbp) at their extracellular portion. HGH also binds to the prolactin receptor (PRLR). In contrast, hPL and hPrl bind only to PRLR [9–12]. After binding, both the hGHR-substrate and PRLR-substrate complexes form dimers, which activate intracellular signal transduction pathways involving receptor-associated JAK kinases and phosphorylation-dependent STAT factors [13,14]. The detailed binding strengths of hGHR and hPRLR with hGH, hPrl, and hPL and their relative significance have not been documented.

The gene locus for hGH encompasses five highly related genes, termed GH-N, PL-L, PL-A, GH-V, and PL-B. Despite their extremely high nucleotide homology, these genes are expressed in an organ-specific manner. While GH-N is the only transcriptionally active gene in the pituitary, the other four genes, i.e., GH-V, PL-A:B, and PL-L, are expressed in the syncytiotrophoblastic villous layer of the placenta. Apart from the major transcripts encoding for 22 kDa proteins, multiple alternative splicing products for GH-V, PL-A, PL-B, and PL-L genes can be observed in placental tissue [14–16].

Recently, we have been studying the effects of placenta extracts on wound healing and anti-aging [17–20]. In the mean time, we have realized that there still are needs for better understanding of the structure–function relationships and mutual interactions of the related protein growth factors and receptors. In an attempt to evaluate systematically the effects of each protein growth factor in the placenta extract, we have set out to produce enough quantity of each protein. This paper reports the cloning of hPL from a cDNA library and the expression of hPL in *Escherichia coli* for further biological studies and clinical use. Several factors, such as the choice of promoter, host strain, temperature, concentration of inducers, and post-induction time, have been evaluated to examine their influence on protein expression. [21–25].

It is noted that hPrl and several other protein growth factor genes have been amplified by RT-PCR individually [16,26]. However, cloning from cDNA library may provide the advantage of simultaneous production of several proteins together. On the other hand, the expression of hPL has not been reported by either means.

Materials and methods

Materials

Human placenta cDNA library was purchased from Clontech (Palo Alto, CA, USA). Sheep polyclonal antibody against human placental lactogen was purchased from The Binding Site (San Diego, CA, USA). Rabbit anti-sheep IgG was purchased from Chemicon (Temecula, CA, USA). Ni^{2+}–Nitrilotriacetic acid (Ni–NTA) agarose was purchased from Qiagen (Valencia, CA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany) without any further purification. The buffer solutions used in this report were all filtered through a 0.45-μm filter.

Cloning of placental growth factors

HGH, hPL, hPrl, hCG, hLH, and hFSH are cloned by polymerase chain reactions (PCRs) from human placenta cDNA library (Clontech). The primers were designed according to the mRNA sequences found in National Center for Biotechnology Information (NCBI). Primers used to generate recombinant PL are GGGAATTCCATATG GTCCAAACCG with *Nde*I site and CGACGTCGAC CTACTAGAAGCCACA with *Sal*I site for pET-12a (Novagen); CATGCCATGGTCCAAACCGTTCCGTTA TC with *Nco*I site and TCCCCCGGGCACCTAGAA GCCACAGCTG with *Sma*I site for pPW500; CGC GGATCCGTCCAAACCGTTCCGTTATC with *Bam*HI site and CCCAAGCTTCACCTAGAAGCCACAGCTG with *Hin*dIII site for pQE30 (Qiagen), and pTrcHisA (Invitrogen). The restriction enzyme sites were underlined. Each PCR was performed in a final volume of 50 μl with the following reagent concentrations: 0.5 μM of each primer, 0.6 mM of dNTPs, and 5 U Taq in appropriate buffer. After initial denaturation, 30 cycles of amplification were performed with the respective standardized elongation; denaturation temperatures and times 68–72 °C for 2 min and 94 °C for 1 min, and with the calculated annealing temperatures for each primer pair, i.e., 52–65 °C for 1 min. Each PCR product was purified from agarose gel and cloned to the TA cloning vector, or digested with proper restriction enzymes and then subcloned into the same sites of pUC19. The recombinant plasmids were examined by suitable restriction enzyme and gel electrophoresis using 0.8% agarose gel to check the fragment size.

hPL and hPrl expression and purification

Table 1 shows the vectors, expressed construct, expected MW, promoters, and purchasing sources of the present work. The DNA fragments coding for hPL and hPrl were subcloned, in-frame downstream of the histidine tag under *T5* promoter-*lac* operator control using pQE30 expression vector in *E. coli* strain M15 containing the plasmid pREP4. The transformed cells were cultured in LB medium in the presence of ampicillin (50 μg/ml) and kanamycin (25 μg/ml). The cultures were induced with 1 mM IPTG and were further grown for 5 h. Different expression vectors were used in our experiment: pET-12a purchased from Novagen contains the *T7* promoter, pPW500 (from Professor S. D. Lin) has the *Trc* (*Trp-lac*) promoter, pQE30 and pTrcHisA are commercial expression plasmids purchased from Qiagen and Invitrogen, respectively. pQE30 contains phage *T5*

Table 1
The vectors, expressed construct, expected MW, promoters, and purchasing sources of the present work

Vector	pET-12a	pPW500	pQE30	pTrcHisA
Expressed construct	Untagged hPL	Untagged hPL	6× his_hPL	6× his_hPL
Expected MW	22 kDa	23 kDa	23 kDa	26 kDa
Promoter	*T7*	*Trc*	*T5*	*Trc*
From	Novagen	Prof. S.D. Lin	Qiagen	Invitrogen

Table 2
The vector/*E. coli* strain combinations tested

Vector	*E. coli* strain				
	BL21(DE3) (none)	M15[pREP4] (kanamycin)	SG13009[pREP4] (kanamycin)	Origami(DE3) (kanamycin, tetracyclin)	BL21trxB(DE3) (kanamycin)
pET-12a (ampicillin)	Low	N/A	N/A	N/A	N/A
pPW500 (ampicillin)	Low	N/A	N/A	N/A	N/A
pQE30 (ampicillin)	High	149	138	82	124
pTrcHisA (ampicillin)	High	15	24	4	2

The hPL protein yield of each combination is shown in mg/L. Antibiotic resistance is shown in the parenthesis.

promoter and pTrcHisA contains the *Trc* promoter. Both of the expression products from pQE30 and pTrcHisA consist of six histidine residues in series for purification purpose. The different expression vectors were individually transformed into suitable host strains, including JM109, BL21(DE3), TOP10, M15[pREP4], and SG13009[pREP4]. These cultures were individually grown in 5 ml of LB medium (with appropriate antibiotics, Table 2) with vigorous shaking at 37 and/or 30 °C. Expression was confirmed by SDS–PAGE and Western blot.

The recombinant hPL was expressed in the form of inclusion bodies. These inclusion bodies were solubilized in 8 M urea and purified by a His-tag affinity chromatography column on Ni^{2+}–nitrilotriacetate (NTA) resin. The purified product was analyzed by SDS–PAGE and confirmed by Western blotting. Recombinant hPL aggregated in inclusion bodies was denatured and refolded after purification. About 5 μg of purified recombinant hPL was added in 1 ml of refolding buffer for 48 h at 4 °C. The protein was precipitated in 5% trichloroacetic acid and washed with acetone. Purified recombinant hPL was also air oxidized by incubation at 4 °C and ambient temperature. The purified recombinant hPL treated with DTT was taken as the control. The formations of intra-chain disulfide bonds for recombinant hPL after oxidation were examined by SDS–PAGE in reducing and non-reducing conditions. This method was based on the fact that intra-chain disulfide bond formation between cysteine residues condenses the protein monomer in non-reducing conditions and increases the electrophoretic mobility.

hPL biological activity assay

Human erythroleukemia K-562 cells are erythroid precursor cells and hPL could stimulate, in a dose-dependent fashion, the erythroid maturation in K-562 cells in the presence of erythropoietin and result in enhanced formation of erythrocyte-like particle and hemoglobin. Hemoglobin possesses a pseudo-peroxidase activity and catalyzes the reaction of 2,7-diaminofluorene (DAF) with hydrogen peroxide to form the blue-colored fluorene blue ($A_{max} = 610$ nm) in the K-562 cells. Thus, in the presence of DAF and H_2O_2, the amount of hemoglobin could be estimated by counting the number of blue color-stained K-562 cells using a flow cytometer. The numbers of the blue color-stained K-562 cells resulted by treating the normal K-562 cells with commercial and recombinant hPL and negative control could be used to assay the hPL biological activity.

For a typical experiment, the K562 cells (10^5) were cultured in 0.5 ml RPMI 1640 medium supplemented with 10% fetal bovine serum, 1×10^{-4} M α-thioglycerol, 0.25 U human erythropoietin, penicillin, streptomycin, and various concentrations of recombinant hPL. To each of several sterile 17 × 100 mm polypropylene tubes was dispensed 0.5 ml of the cultured solution and allowed to stand at 37 °C in the presence of 5% CO_2 for 3 days. The DAF reagent solution was freshly prepared and consisted of 100 μl of DAF solution stock (0.01 g DAF in 1 ml 90% acetic acid, stored in the dark at 4 °C), 10 ml of 200 mM Tris–HCl buffer (pH 7.0), and 100 μl of 30% H_2O_2. To perform the hPL activity assay, 100 μl of the DAF reagent solution was added into each tube and mixed thoroughly and allowed stand for 3 min. A flow cytometer was used to count the blue color-stained cells in 10^4 increments.

High cell density fermentation

In high cell density fermentation, a 5-L fermentor with a 1.2 L initial working volume was used for batch and fed-batch mode experiments. The temperature was 37 °C and the pH was maintained at 7.0 adjusted with a 25% ammonia solution and a 2 N HCl solution. The air was supplied at a rate of 2 L/min and the agitation rate was controlled between 500 and 900 rpm to maintain a dissolved oxygen

concentration at 30% air saturation. *E. coli* cells were grown for 6 h in batch mode and then nutrient feeding was initiated to maintain the specific growth rate at 0.15 h^{-1} for the desired cell optical density induced with 2 mM IPTG. The expression of recombinant hPL was checked by SDS–PAGE.

For a typical experiment, a single colony of *E. coli* M15[pREP4] containing plasmid pQE30-PL was used to inoculate 5 ml LB medium. The culture was grown overnight at 37 °C. A total of 180 ml LB medium was inoculated with 1.8 ml of the overnight culture and incubated at 37 °C for 1.6 h. The cells were harvested by centrifugation at 8000 rpm for 15 min at 4 °C and re-suspended in 20 ml of batch medium. Batch medium consisted of 5.3 g/L KH_2PO_4, 23.3 g/L K_2HPO_4, 2.3 g/L NH_4Cl, 2 g/L NaCl, 10 g/L yeast extract, 0.12 g/L $CaCl_2 \cdot 2H_2O$, 0.12 g/L $FeSO_4 \cdot 7H_2O$, 10 g/L glucose, 0.48 g/L $MgSO_4 \cdot 7H_2O$, 0.016 g/L thiamine–HCl, and 0.0001% (v/v) antifoam A. Stock solutions of $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, glucose, $MgSO_4 \cdot 7H_2O$, and thiamine–HCl were prepared first, filtered with a 0.22-μm nylon filter, and aseptically added after sterilization prior to inoculation. The resuspended culture was used to inoculate a 1.2 L batch medium solution, which was sterilized inside the fermentor. Fermentations were carried out in a 5-L working volume using a BioFlo III (New Brunswick Scientific) interfaced with Advanced Fermentation Software version 3.0 (New Brunswick Scientific) for data acquisition and experimental control. Ammonium hydroxide [25% (v/v)] was used as a pH control agent and as a nitrogen source. The pH was measured with a Mettler-Toledo pH electrode (Mettler-Toledo GmbH, Germany).

Oxygen concentration was measured with an O_2-sensor-12/320 electrode (Mettler-Toledo GmbH, Germany). The feeding rate was controlled to maintain a specific growth rate of 0.15 h^{-1} in the exponential growth phase until the glucose was consumed. Samples were taken at 1-h intervals and the optical density was read at 600 nm to estimate the dry cell weight. Briefly, a 1 ml sample was centrifuged at 13,000*g* for 5 min. The cell pellet was weighed and the supernatant was stored at −20 °C for further analysis. Glucose concentration existing in the culture was also read using YSI 2700 SELECT Bioprocess Monitoring (Yellow Springs Instruments, Yellow Springs, OH, USA).

Gel electrophoresis and densitometry

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli's method with minor modifications as previously described. Samples (typically 10–20 μg) for SDS–PAGE were preheated at 100 °C for 10 min in a loading buffer [50 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 100 mM dithiothreitol, and 0.1% bromphenol blue]. The samples were run for about 2 h at 100 V and stained using Coomassie brilliant blue R-250. Densitometric analysis of SDS–PAGE experiments was performed using a Molecular Dynamics densitometer for data acquisition and the Image Quant software for integration and data analysis.

Western blot analysis

Following the separation of proteins by SDS–PAGE, the gel, nitrocellulose paper, and 3MM paper were soaked in a transfer buffer containing 48 mM Tris–HCl, 39 mM glycine, 0.037% SDS, and 20% methanol at pH 8.3 for 30 min. The protein was then electrotransferred to a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) at 90 mA for 1 h in a semi-dry transfer cell (Bio-Rad) containing a transfer buffer. The blot membrane was then immersed in 5% skimmed milk in PBS for 1 h at room temperature while shaking gently. After three times washing with PBS for 5 min, the membrane was incubated with a primary goat polyclonal antibody against human Hp [1:5000 dilution in PBS washing buffer containing 1% (w/v) skimmed milk and 0.05% Tween 20 for 1 h] at room temperature and washed three times for 5 min. The membrane was then incubated with 1:10,000 diluted rabbit anti-goat IgG conjugated with horseradish peroxidase in washing buffer for 1 h. In addition, the membrane was washed two times with washing buffer and further washed one time with PBS. Finally, the membrane was developed according to ECL Western Blotting Detection Kit (Amersham Biosciences, Little Chalfont, UK) and exposed to X-ray film with 10–30 s exposures.

Results and discussion

Human placental lactogen is a single polypeptide hormone of 22 kDa with no carbohydrate residue. It is composed of 191 amino acids with two intramolecular disulfide bonds, one between residues 53 and 164 and the other between 182 and 189. The mature human placental lactogen is derived from a precursor of 25 kDa that has a 26 amino acid signal sequence cleaved from its amino-terminal end [2,11].

Cloning of placental growth factors

We have cloned the following growth factors in our laboratory, including hGH, hPL, hPrl, hLH, and hFSH. The restriction enzyme fragment sizes were checked by the 0.8% agarose gel and show that these genes are appropriate for cloning (data not shown).

Protein expression

To examine further the specificity of these proteins, we need to be able to routinely produce milligram quantities of native proteins for biological and kinetic studies. *E. coli* expression system has been used to produce many mammalian proteins. While *E. coli* has some disadvantages, including the absence of intracellular sorting and packaging mechanisms, it does have many advantages over

mammalian expressions. These advantages include ease of maintaining bacterial cultures, high-level expression of recombinant proteins, and the simplicity of *E. coli* genetics. Among these placental growth factors, hGH, hPL, and hPrl are not glycosylated and it is suitable to use *E. coli* system for protein expression and optimization. We shall discuss the expression of hPL below.

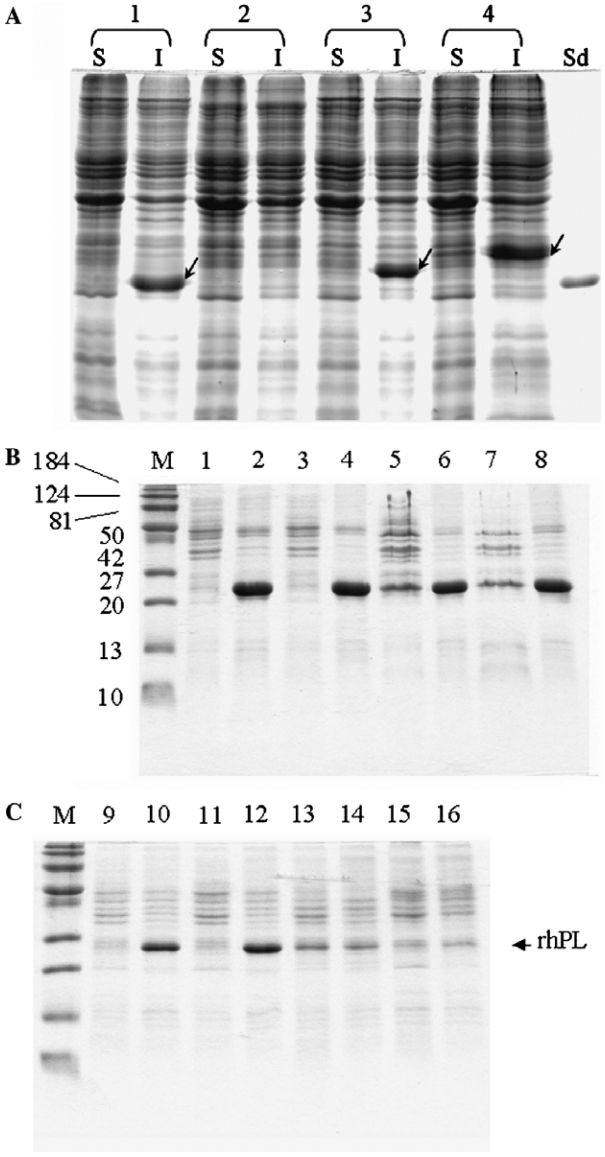

Fig. 1. Recombinant hPL analysis by 15% SDS–PAGE with Coomassie blue staining. (A) Expression with different promoters in host cell BL21(DE3) at 37 °C and OD 0.8. Arrows represented the induced recombinant hPL. Lanes 1–4: pEt-12a-PL, pPW500-PL, pQE30-PL, and pTrcHisA-PL, respectively. Sd: commercial standard hPL, S: soluble fraction, I: insoluble fraction. (B) Expression in different host *E. coli* at 37 °C. M: protein molecular markers of known mass (kDa), Lanes 1: pQE30-PL M15[pREP4] supernatant, 2: pQE30-PL M15[pREP4] precipitate, 3: pQE30-PL SG13009[pREP4] supernatant, 4: pQE30-PL SG13009[pREP4] precipitate, 5: pQE30-PL Origami(DE3) supernatant, 6: pQE30-PL Origami(DE3) precipitate, 7: pQE30-PL BL21trxB(DE3) supernatant, 8: pQE30-PL BL21trxB(DE3) precipitate. (C) Lanes 9: pTrcHisA-PL M15[pREP4] supernatant, 10: pTrcHisA-PL M15[pREP4] precipitate, 11: pTrcHisA-PL SG13009[pREP4] supernatant, 12: pTrcHisA-PL SG13009[pREP4] precipitate, 13: pTrcHisA-PL Origami(DE3) supernatant, 14: pTrcHisA-PL Origami(DE3) precipitate, 15: pTrcHisA-PL BL21trxB(DE3) supernatant, 16: pTrcHisA-PL BL21trxB(DE3) precipitate.

Vector effect in recombinant hPL expression

The cDNA fragment of hPL was amplified by polymerase chain reaction and cloned into expression vectors including pET-12a (*T7* promoter), pPW500 (*Trc* promoter), pQE30 (*T5* promoter), and pTrcHisA (*Trc* promoter). These constructed plasmids were then sequenced and transfected into BL21 (DE3). It is known that the expression of recombinant protein with these expression vectors is regulated by *lac* operon. Plasmid pET-12a containing a *T7* promoter is well known. T7 is a commonly used phage promoter to initiate mRNA expression of recombinant protein through *T7* RNA polymerase. Both plasmids pPW500 and pTrcHisA contain *Trc* (*Trp-lac*) promoter. Plasmid pQE30 contains *T5* promoter which was also a phage promoter but can be driven by either *T5* or *E. coli* RNA polymerase. Moreover, 2 lac operators located beyond the *T5* promoter can enhance the binding efficiency with lac repressor to promote a tighter regulation of transcription.

The expressed hPL with different promoters was analyzed by SDS–PAGE (Fig. 1A). Strains BL21(DE3) containing pQE30-PL, pTrcHisA-PL, and pET-12a-PL expressed larger quantities of recombinant PL deposited in inclusion bodies as could be confirmed with Western blotting (data not shown). Among these experiments, pQE30-PL exerted the largest yield. Interestingly, although both pPW500-PL and pTrcHisA-PL contain the same promoter, the expression of recombinant PL in pTrcHisA-PL was substantially higher than pPW500.

Host effect in recombinant hPL expression

To improve the expression yield, M15[pREP4], SG13009[pREP4], Origami(DE3), and BL21trxB(DE3)

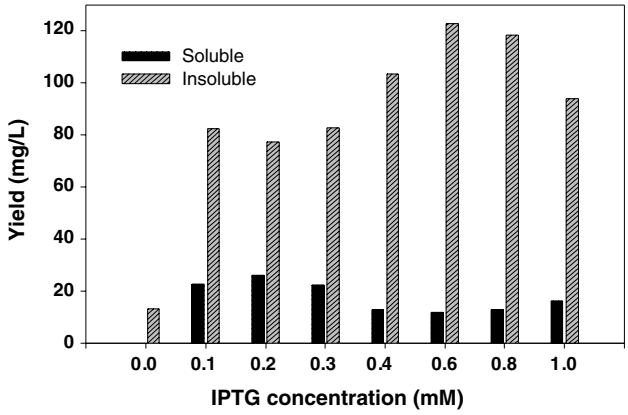

Fig. 2. Expression of recombinant hPL at various concentrations of IPTG with strain Origami(DE3) containing plasmid pQE30-PL, cultured the same way as that of Fig. 1. Proteins were analyzed by 15% SDS–PAGE (data not shown) and quantitated using a Molecular Dynamics densitometer and the Image Quant software for integration and analysis.

were separately infected with pQE30-PL to investigate the host effect in hPL expression. In addition, JM109, BL21(DE3), and Top10 were also studied (data not shown). All the used hosts contain lacI gene in their chromosomes for producing repressors, except M15[pREP4] and SG13009[pREP4] whose lacI gene was located on the plasmid pREP4. Among these tested hosts, M15[pREP4] showed the largest yield of recombinant PL, as inclusion bodies (Figs. 1B and C). However, some hosts expressed smaller amounts of recombinant PL in soluble form in addition to larger amounts of inclusion body forms, e.g., Origami(DE3) and BL21trxB(DE3). This might result from the fact that Origami(DE3) has both the characteristics of thioredoxin-reductase-mutant ($trxB^-$) and glutathione-reductase- mutant (gor^-) in its genotype properties, whereas BL21trxB(DE3) possesses $trxB^-$ property but not that of gor^-. Both characteristics can facilitate the formation of disulfide linkages in recombinant protein indirectly and make it soluble. The yield of soluble protein in Origami(DE3) was slightly higher than BL21trxB(DE3).

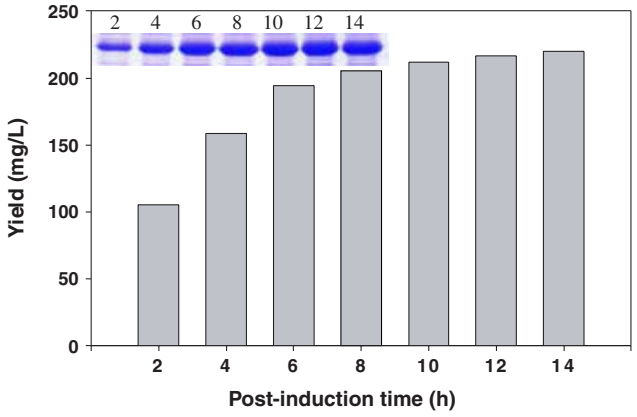

Fig. 3. Effect of post-induction time on the expression of recombinant hPL with strain M15[pREP4] transfected with plasmid pQE30-PL and expression initiated with 1 mM of IPTG. Proteins were analyzed by 15% SDS–PAGE.

Inducer concentration effect in expression of recombinant hPL

Since Origami(DE3) containing plasmid pQE30-PL produced some soluble, expressed recombinant hPL, the effect of the added IPTG inducer concentrations was examined to see if the ratios of the amounts of soluble to insoluble expressed recombinant proteins could be increased at 30 and 37 °C. The results showed that the experiments performed at 37 °C gave better protein yield but the ratio of soluble protein to insoluble form did not improve substantially. Fig. 2 shows the hPL yields at various added [ITPG] at 37 °C. It is observed that 0.1 mM of IPTG was enough to achieve high yield of hPL.

Post-induction-time effect in expression of recombinant hPL

Since the yield of the soluble form of recombinant hPL could not be raised substantially, M15[pREP4] containing plasmid pQE30-PL, the strain with the highest total yield (i.e., the sum of soluble and insoluble forms) was chosen for further studies. The post-induction-time assay was used to determine the optimal time to achieve the highest PL yield. The yield of recombinant PL could reach 200 mg/L after 6 h of IPTG induction (Fig. 3).

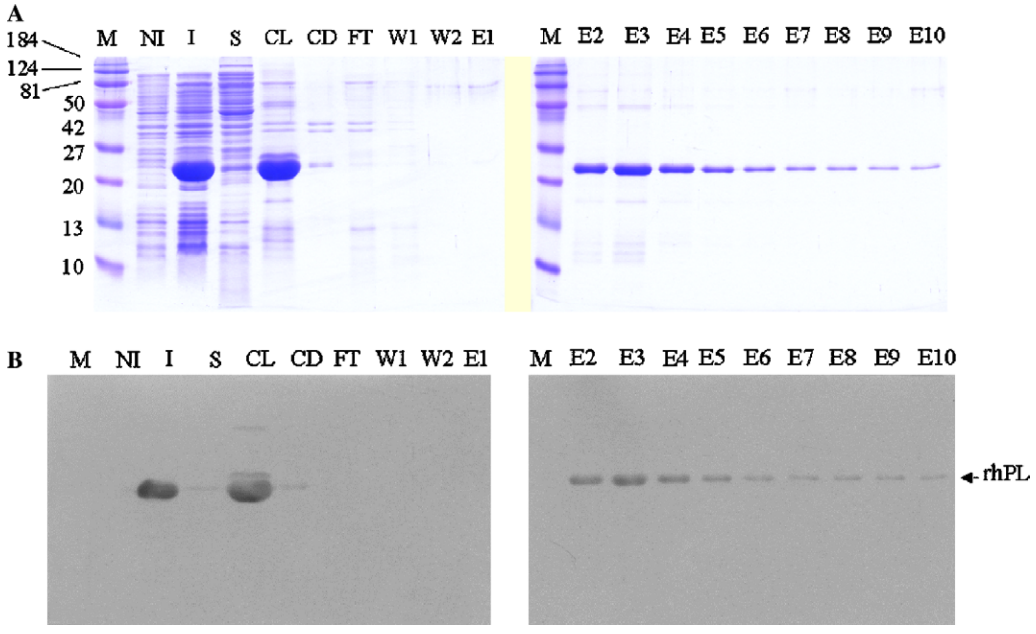

Fig. 4. (A) Purification of recombinant human placental lactogen under denaturing condition. Proteins are analyzed by 15% SDS–PAGE with Coomassie blue staining, M: protein molecular markers of known mass (kDa), NI: non-induced control, I: induced control, S: supernatant, CL: clear lysate, CD: cellular debris, FT: flow-through, W1&W2: wash, E1–E10: eluates. (B) Western blot analysis of the purified product, M: protein molecular markers of known mass (kDa), NI: non-induced control, I: induced control, S: supernatant, CL: clear lysate, CD: cellular debris, FT: flow-through, W1&W2: wash, E1–E10: eluates.

Purification of recombinant hPL

The expressed recombinant hPL in M15[pREP4] containing plasmid pQE30-PL was synthesized as a fusion protein with a 6×His tag at the N terminus. Hence, recombinant PL could be purified by immobilized metal affinity chromatography column using Ni^{2+}–nitrilotriacetic acid (NTA) resin and eluted at a lower pH. The pKa of the histidine residue is approximately 6.0, at an acidic pH 4.5–5.3, the recombinant PL will be protonated and would not bind to nickel ions and would elude from the Ni–NTA column.

The inclusion bodies were solubilized in a buffer containing 8 M urea at pH 8.0. The supernatant containing the recombinant PL was applied onto the NTA column after removal of the cellular debris by centrifugation. Non-specific binding protein was removed at pH 6.3 and the 6× His tagged recombinant hPL was eluted at pH 4.5. The pooled fractions containing recombinant hPL were analyzed by 15% SDS–PAGE and confirmed by Western blot (Fig. 4B). SDS–PAGE analysis revealed that most impurities were high-molecular weight proteins which bind weakly to the affinity column. The purity of the recombinant PL was approximately 96% as analyzed by SDS–PAGE, indicating an efficient purification process. The overall purification yield was ca. 50 mg/L.

Refolding of recombinant hPL

Non-reducing SDS–PAGE (in the absence of DTT) was used to distinguish between refolded and denatured forms of recombinant hPL because the formations of intra-chain disulfide linkages result in the condensation of the protein monomer and in the increased electrophoretic mobility of the protein in the absence of DTT. It can be seen from Fig. 5 that, the recombinant hPL was represented at 23 kDa in reducing conditions and 20 kDa in oxidizing conditions. Intra-chain disulfide bonds could be formed either by the refolding buffer or air oxidation in the presence of urea. Air oxidation of free SH groups did not lead to the disulfide-linked insoluble aggregates, and only the intra-chain disulfide bonds formed. Although recombinant PL contains four SH groups and random intramolecular pairing would pro-

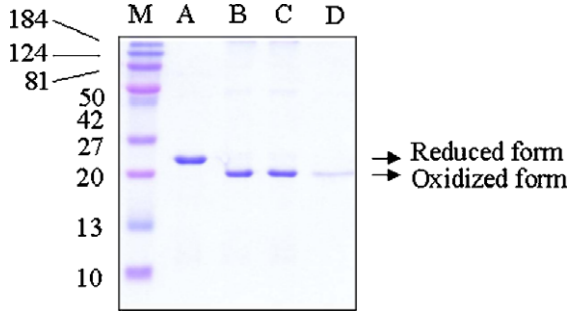

Fig. 5. Preliminary detection of small-scale refolding. Recombinant hPL was analyzed by 15% SDS–PAGE in reducing (lane A) and non-reducing conditions (lanes B–D), M: protein molecular markers of known mass (kDa), A: recombinant hPL in reducing conditions, B: air oxidation at ambient temperature, C: air oxidation at 4 °C, D: oxidation in refolding buffer at 4 °C.

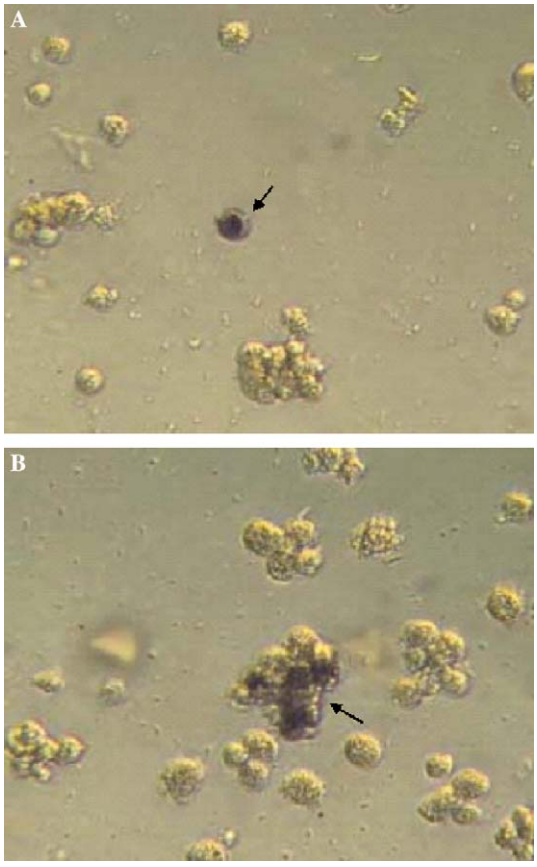

Fig. 6. Photographs of DAF stained colonies. K562 cells were treated with EPO and recombinant hPL, and followed by a DAF colorimetric staining. This photograph was with a 200× objective in culture dish and 2 types of morphology were observed. (A) Colony-forming units-erythroid (CFU-E) derived colony. (B) Pure erythroid burst. Arrows represented the erythroid colonies.

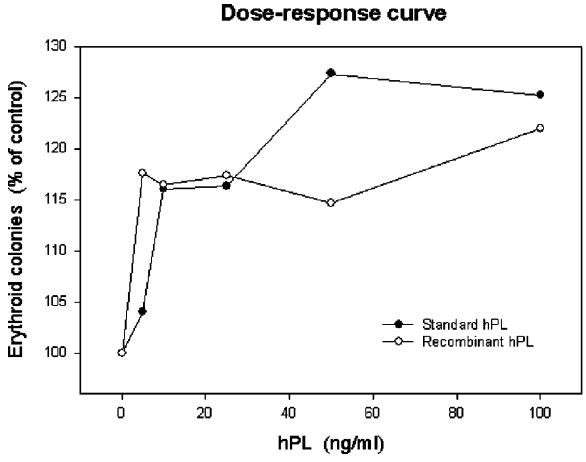

Fig. 7. Effects of standard human placental lactogen and recombinant human placental lactogen on erythroid colony formation from K562 cells. Control culture was identical to others except that they lacked hPL.

duce up to 10 different species, only one type of refolded recombinant hPL was detected in non-reducing conditions.

Biological activity assay

This assay was based on the pseudo-peroxidase activity of hemoglobin using 2,7-diaminofluorene as a hydrogen donor. In the presence of hydrogen peroxide, hemoglobin would catalyze the formation of fluorene blue which had a maximum absorption at 610 nm and resulted in blue color-stained K-562 cells (Fig. 6). These stained cells could be counted by a flow cytometer. Fig. 7 shows that both the recombinant and the commercial PL possessed the ability to enhance the production of hemoglobin, which proves that the recombinant hPL possesses appreciable in vitro biological activity similar to standard hPL.

Protein expression optimization by fed-batch fermentation

To increase the yield of recombinant hPL, a series of fed-batch fermentation experiments were performed as described in "Materials and methods". Fig. 8 depicts the profiles of cell growth with pQE30-PL/M15[pREP4], nutrient medium feeding volume, glucose concentration, and yield of recombinant hPL. In the condition without IPTG induction, the maximum dry cell mass was estimated to be 42.6 g/L after 23 h of cultivation (Fig. 8A, Table 3). With IPTG-induction at $OD_{600}=60$ and $OD_{600}=80$, the respective maximum dry cell masses were 31.7 and 40.9 g/L (Table 3). The cell density of that induced at $OD_{600}=80$ was close to that of non-induced culture, but higher than that of induced at $OD_{600}=60$. Feeding of the nutrient medium was initiated while the glucose concentration was almost completely consumed in 6 h until the end of fermentation at a controlled specific growth rate of $0.15\,h^{-1}$ (Fig. 8B and C). Between 6 and 20 h, the fed glucose was

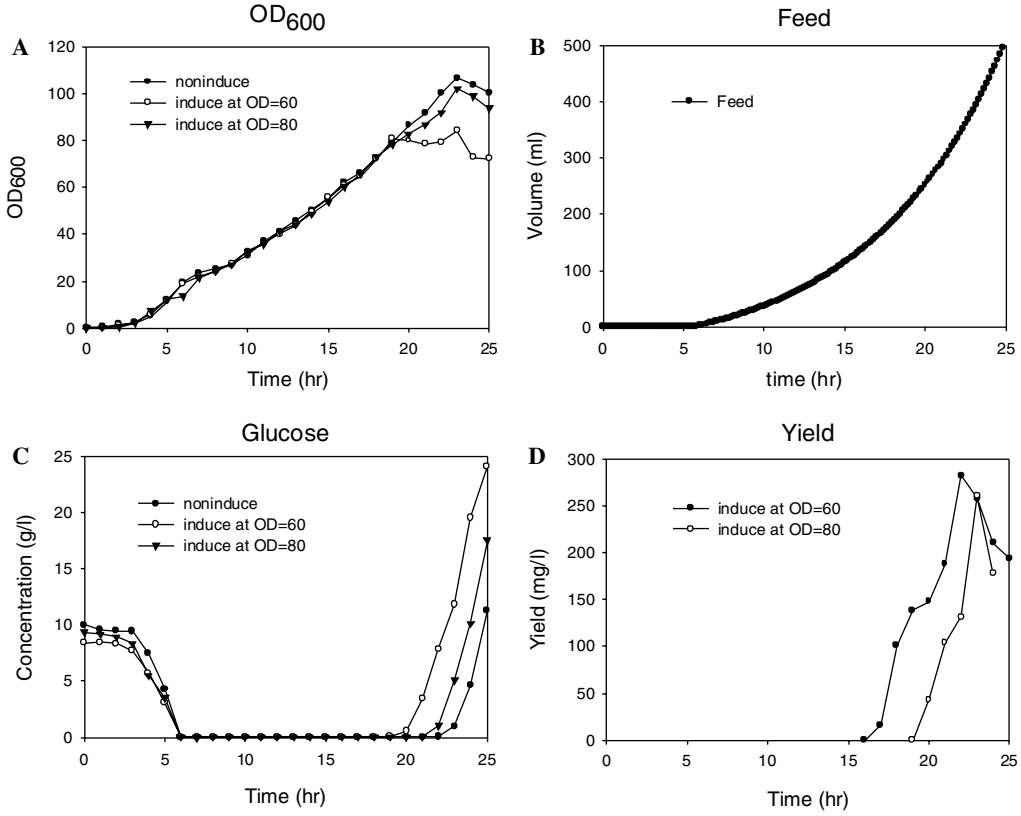

Fig. 8. Fed-batch fermentation of *E. coli* M15[pREP4] containing plasmid pQE30-PL. Fermentations were carried out in a 5-L working volume using a BioFlo III fermentor (New Brunswick Scientific) interfaced with Advanced Fermentation Software version 3.0 (New Brunswick Scientific) for data acquisition and experimental control. The relevant data were monitored and represented as: (A) optical density at 600 nm, (B) nutrient medium feed, (C) glucose concentration, and (D) PL yield.

Table 3
Summary of results of recombinant PL biosynthesis in fed-batch cultures

Time of IPTG induction	OD_{600}	Dry cell weight (g/L)	Maximum yield (mg/L)	Specific yield (mg/g of cells)	Volumetric yield (mg/L/h)
Non-induction	106.6 (23 h)	42.6	N/A	N/A	N/A
Induce at OD = 60	79.2 (22 h)	31.7	282.4	8.9	12.8
Induce at OD = 80	102.2 (23 h)	40.9	260.2	6.4	11.3

almost completely exhausted due to the cell growth. After 20-h of cultivation, the fed glucose started to accumulate rapidly, with a rate fastest for the $OD_{600} = 60$ induction case, and the slowest for the non-induced case. This suggests that cell growth rates reached a stationary stage and the glucose accumulation rates corroborate with those of cell density growth. However, high cell density did not produce high yield of recombinant protein. The specific yield of that induced at $OD_{600} = 60$ (i.e., 8.9 mg/g of cells) was higher than that of induced at $OD_{600} = 80$ (i.e., 6.4 mg/g of cells), and the maximum yield of the recombinant hPL in the high cell density fed-batch fermentation reached 282.4 mg/L (Fig. 8D).

Conclusion

We have successfully cloned and expressed hPL in *E. coli*, which could be used for further biological studies and clinical applications such as anti-aging, wound healing, and many other effects. The biological activity of the recombinant hPL was confirmed by an erythroid colony assay. Using the pQE30 expression vector and *E. coli* host strain M15[pREP4], and with proper glucose as carbon source for a fed-batch operation, a better recombinant hPL yield of 282.4 mg/L could be obtained.

Acknowledgments

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