

Production of Lipocortin-1₁₋₁₈₅ Using A Recombinant of *Escherichia coli*.

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The aim of the present study was to optimize culture condition for the expression of lipocortin-1₁₋₁₈₅ in a recombinant of *Escherichia coli* using batch system. Plasmid (pHT22) carrying lipocortin-1₁₋₁₈₅ gene was well maintained in the recombinant with the addition of ampicillin as a selection pressure. Optimum temperature was 28°C for seed culture and 40°C for main culture and the optimum pH was 7.0. The production of Lipocortin-1₁₋₁₈₅ was closely associated with cell growth and related to plasmid amplification.

Key words: Lipocortin-1₁₋₁₈₅, *E. coli*, pHT22, optimization, yield.

Lipocortins induced by glucocorticoids show inhibitory activity against phospholipaseA2 (2). Lipocortin-1 (LC-1) consisting of 347 amino acids was discovered from lung, kidney, bone marrow, intestine, spleen, thymus, leukocytes, brain and macrophages, in which it plays as a second messenger in glucocorticoid related anti-inflammatory pathway (2, 11). Several fragments of LC-1 (LC-1₁₋₃₄₇, LC-1₁₋₁₈₈ and LC-1₁₃₋₁₈₈) were also identified to show different physiological activities, where the activity was determined by the structural characters of N-terminus region in the protein (2). cDNA of full sequence of LC-1₁₋₃₄₇ (PT2) was prepared from human placental mRNA and the gene for LC-1₁₋₁₈₅ fragment was also cloned (pHT22), both of them were expressed in *E. coli* HB101 (5).

The aim of this study was to evaluate optimum culture conditions for the over-expression of LC-1₁₋₁₈₅ in the recombinant of *E. coli* using batch system. The relations between production of LC-1₁₋₁₈₅ and cell growth were investigated with different culture temperature and pH.

Materials and Methods

Recombinant plasmid and microbial strain

Recombinant plasmid (pHT22) containing LC-1₁₋₁₈₅ gene carried replication origin derived from pUC19, *Trc* promoter, strong transcription terminator, and

amp^r. The plasmid was transformed into *E. coli* HB 101 (5) (F⁺, *leuB6*, *proA2*, *recA13*, *thi-1*, *ara-14*, *lacY* 1, *galK2*, *xyl-5*, *mtL-1*, *rpsL20*, *lambda*, *supE44*, *hsdS20*, rB⁺, mB⁺).

Media and culture conditions.

Rich medium consisted of tryptone 1%, yeast extract 0.5%, NaCl 1%, KH₂PO₄ 0.113%, MgSO₄ · 7H₂O 0.0232%, CaCl₂ · 2H₂O 0.0011%, FeSO₄ · 7H₂O 0.0007%, MnSO₄ · 4H₂O 0.0002%, ZnSO₄ · 7H₂O 0.0002%, CuSO₄ · 5H₂O 0.0004%, CoCl₂ · 6H₂O 0.0004%, EDTA · Na₂ · 2H₂O 0.0394%, and 1.5% of agar for solid culture.

A single colony developed on the rich agar medium containing ampicillin (0.01%) was inoculated into 20 ml of the rich liquid medium contained in a 250 ml baffled flask. After cultivation at 28°C for 18~22 h in a rotary shaking incubator (150 rpm), the culture was inoculated again into 100ml of the rich liquid medium contained in a 500 ml baffled flask for the seed culture of main cultivation.

The seed culture was inoculated into main cultures with an inoculum of 5% (vol/vol). Main cultures were carried out using a 2 L glass jar reactor (Korea Fermentor Co., KF-2L) with a working volume of 1,350 ml. Culture pH was controlled at 7.0 by the addition of 2 N HCl and 2 N NaOH using a two-way pH controller. Aeration and agitation were maintained at 1 vvm (volume per volume per minute) and 450 rpm, respectively. IPTG (final 0.1 mM) was added to the main culture

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at 2 h after inoculation.

Analytical methods

Microbial growth was evaluated with dried cell weight after drying at 100°C for 15–18 h. For the counting of viable cells, culture samples were diluted immediately with the fresh rich medium and plated on the rich agar plates. Cells developed after one day incubation at 28°C were counted as colony forming units (CFU). Total cell numbers were measured with a haemocytometer under a phase-contrast microscope. Cell morphology was observed with a phase-contrast microscope.

The concentration of glucose in culture supernatant was analyzed by the DNS method (9). Total soluble protein extracted from cells was determined by the Bradford method (1). The profiles of total protein were determined by SDS-PAGE (6). The SDS-PAGE gel bands were electrotransferred (35 V, 1 h) to nitrocellulose membrane. Monoclonal mouse anti-LC-1 antibody (Zymed Co.) was used as the primary antibody, and anti-mouse antibody conjugated with horse radish peroxidase was used as the secondary antibody.

The amount of LC-1₁₋₁₈₅ was determined as follows: the SDS-PAGE was scanned with a gel scanner connected to a computer (UltraScan XL Pharmacia Co.) then the area of corresponding band (20 kDa) was compared to that of total protein. The amount of LC-1₁₋₁₈₅ was calculated from the ratio of the band to the amount of total proteins.

Measurement of plasmid copy number was determined by comparisons of the band area of chromosome to that of plasmid developed on agarose gel: Chromosomal and plasmid DNA were isolated from cell by SDS method (7, 8). 3.2 µl of the SDS lysate (for chromosomal DNA) or 32 µl of the SDS lysate (for plasmid DNA) was loaded on agarose gel.

Electrophoresis was carried out in Tris-acetate buffer (40 mM Tris-acetate, 1 mM EDTA) at 5 V cm⁻¹ for about 2.5 h. DNA bands appeared on the gel were photographed over a trans-illuminator, using Polaroid film Type 665 (Polaroid Co.). The negative films of gel were scanned along the lanes using a double beam densitometer (UltraScan XL, Pharmacia Co.). The relative amounts of plasmid DNA and chromosomal DNA in the lysate were obtained from the peak areas of the responding bands in the photographic negatives. Mean plasmid copy number per genome equivalent of DNA was calculated from the molecular weight of plasmid and chromosomal DNA and the area of the peaks of the corresponding DNA. λ /HindIII fragments were used as a standard for DNA quant-

ization. Plasmid copy number was calculated as follow:

$$\text{Plasmid copynumber} = \frac{\text{moles of plasmids}}{\text{moles of chromosomes}}$$

Chemicals and reagents

Restriction enzymes were purchased from New England BioLabs. Enzymes and Penicillin G were obtained from Sigma Chemical Co..

Results and Discussion

The changes of cell growth and LC-1₁₋₁₈₅ expression at various temperature and pH. Cell growth and LC-1₁₋₁₈₅ productions in batch culture at different culture temperature are shown in Fig. 1. Although cell grew well in the cultures at 37°C, production of LC-1₁₋₁₈₅ was optimum when the culture temperature was shift from 28°C (for seed culture) to 40°C (for main culture).

Cell growth and LC-1₁₋₁₈₅ expression in batch cultures at different culture pH are shown in Fig. 2. LC-1₁₋₁₈₅ was not produced at all when culture pH was not adjusted. When culture time elapsed in the condition, the culture pH changed to acid condition (final pH 4.0). Both of cell growth and

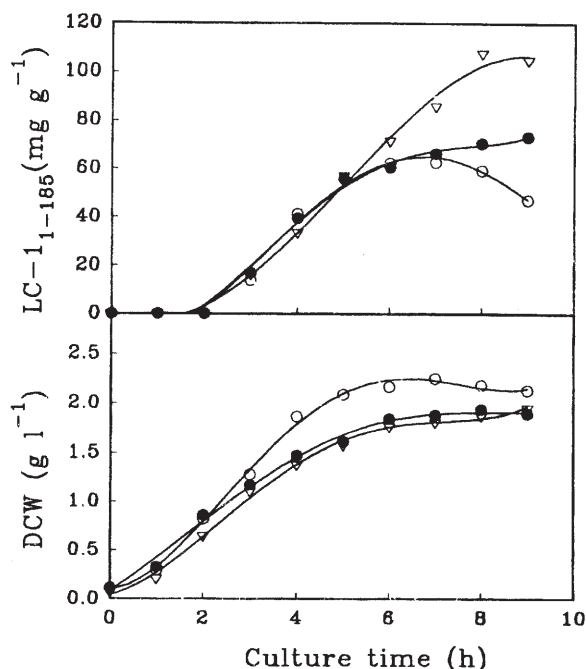


Fig. 1. Profile of growth and LC-1₁₋₁₈₅ production in batch cultures of *E. coli* HB101 (pHT22) on different temperature conditions. Symbols; ○, 37°C→37°C (seed medium → main culture); ●, 37°C→40°C; ▽, 28°C→40°C. 28°C for seed medium and 40°C for main culture were used as optimum temperature to express LC-1₁₋₁₈₅.

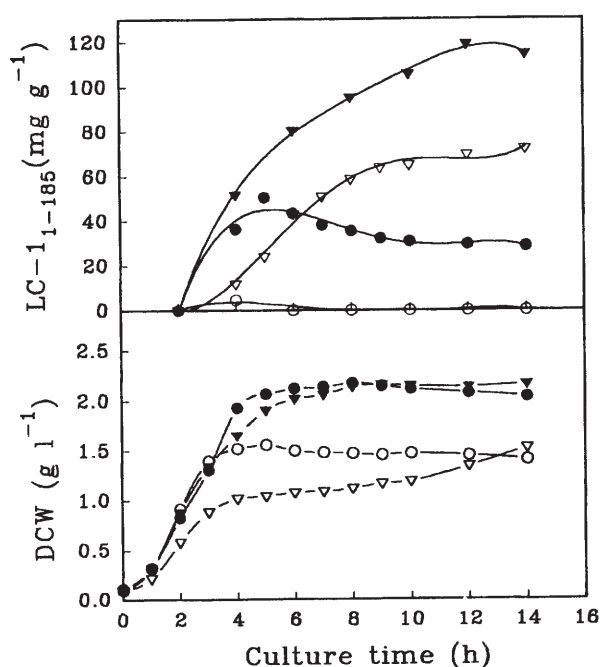


Fig. 2. Profile of growth and LC-1₁₋₁₈₅ production in batch cultures of *E. coli* HB101 (pHT22) at different pH. Symbols; ○, culture without pH adjustment; ●, culture at pH 6; ▽, culture at pH 8; ▼, culture at pH 7. pH 7 was determined as optimum pH to express LC-1₁₋₁₈₅.

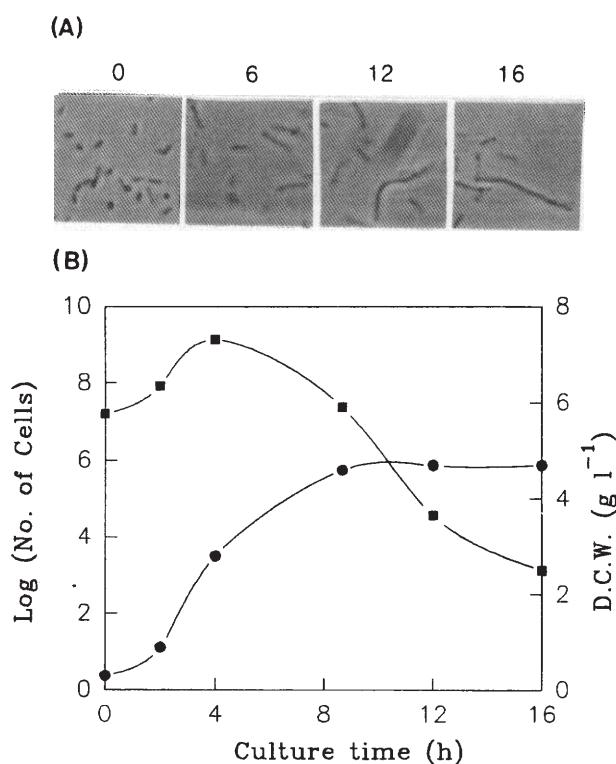


Fig. 3. Profile of cell growth and change of the cell morphology in the batch culture of *E. coli* HB101 (pHT22) on the optimum condition. (A) Morphology of *E. coli*; (B) Change of D.C.W. (●) and colony forming unit (■) on LB plate.

the production of LC-1₁₋₁₈₅ were optimum at pH 7. From the experiments it was clear that culture pH was a significant factor for the production of LC-1₁₋₁₈₅.

Batch culture kinetics

Dried cell weight, total cells, viable cells, and cell morphology in a batch culture are shown in Fig. 3. The pattern of total cell number was similar to the dried cell weight (data not shown), but viable cells were significantly reduced in the stationary phase. When the growth turned to stationary phase, cells changed to elongated or filamentous pseudomycelia. Production of LC-1₁₋₁₈₅ initiated with the addition of IPTG but the production did not last when glucose was completely consumed (data not shown). It was clear that production of LC-1₁₋₁₈₅ was closely associated to the cell growth. Profiles of SDS-PAGE and immunoblot of LC-1₁₋₁₈₅ produced during the batch culture are shown in Fig. 4.

Plasmid copy number varied from 170 to 2,300

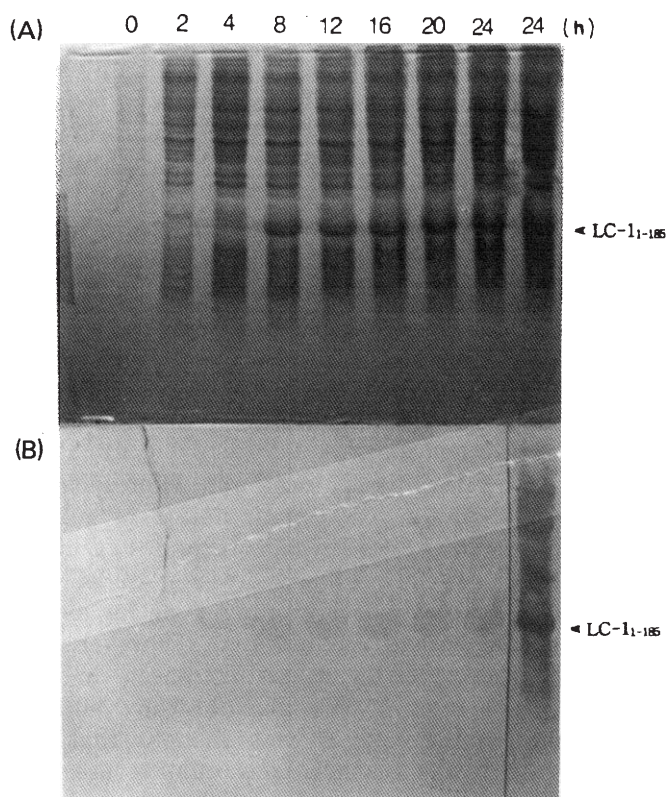


Fig. 4. Profiles of SDS-PAGE and immunoblot analysis. (A) SDS-PAGE remained after electrotransfer to nitrocellulose membrane; (B) Nitrocellulose membrane showing LC-1₁₋₁₈₅ signal. Primary antibody was monoclonal anti-LC-1 antibody and secondary antibody was anti-mouse antibody conjugated with horse radish peroxidase. Note that LC-1₁₋₁₈₅ signal appeared first at 4 h after the induction by IPTG addition at 2 h.

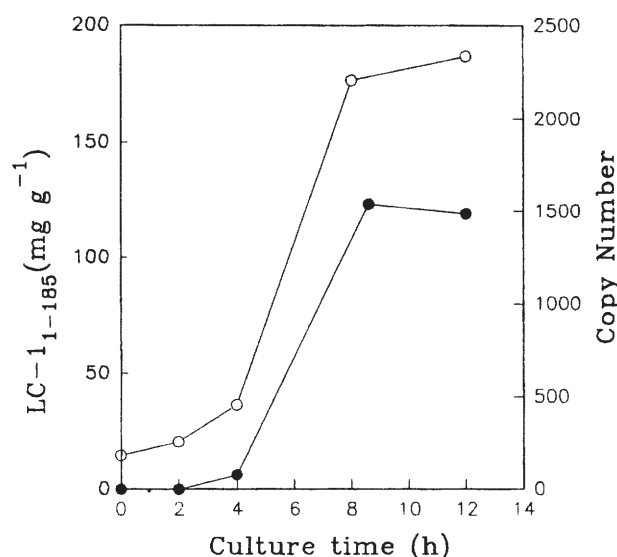


Fig. 5. Change of plasmid copy number (○) and LC-1₁₋₁₈₅ production (●) during the culture of *E. coli* HB101 (pHT22) at the optimum condition.

as the culture time elapsed (Fig. 5). More clearly the profile of LC-1₁₋₁₈₅ production followed the change of the plasmid copy number (Fig. 5). It was thought that the plasmid number was amplified rapidly by the runaway replication at 40°C, because the plasmid (pHT22) carries replication origin of pUC19 that forms multi-copy number when cultured at 40°C (3, 4, 10).

Fractions of plasmid harboring cells were reduced apparently in the culture without of ampicillin (1 at 0 h, 0.84 at 2 h, and 0.73 at 4 h). The determination of plasmid harboring fractions after 4 h were not accurate, because cells formed pseudomycelium. It implied again that plasmid pHT22 harbored in *E. coli* HB101 was very unstable when ampicillin was not added.

From the experiments, optimum expression of LC-1₁₋₁₈₅ was obtained by manipulation of culture temperature (28°C for seed culture and 40°C main culture) and pH (pH 7.0). Expression of LC-1₁₋₁₈₅ was related to plasmid copy number as far as the microbial cell growth maintained. However, the lipocortin production was not increased in stationary phase even if the plasmid copy number was stably maintained. It was thought that microbial growth and plasmid copy number are crit-

ical factors for the over-expression of LC-1₁₋₁₈₅.

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