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Secretory Production of Human Leptin in *Bacillus subtilis*

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Abstract Human leptin is identified as a 16 kDa (146 amino acids) protein secreted from adipocytes which influences body weight homeostasis. In order to produce active leptin, the human *obese* gene coding for leptin was expressed in *Bacillus subtilis* WB600 strain which is deficient in six extracellular proteases. The recombinant leptin was produced in a culture supernatant, and in a culture supernatant, it was contained as high as 48% for total proteins. After simple purification steps, which consisted of ammonium sulfate precipitation and anion-exchange column chromatography, 2.3 mg of leptin with a purity greater than 95% was obtained from the 0.5 l culture with the recovery yield of 38.3%. The purified leptin showed the correct folding structure with one disulfide bond.

Key words: Leptin, *Bacillus subtilis*, heterologous gene expression

Obesity is a major risk factor for the development of chronic diseases such as hypertension, type II diabetes and hyperlipidermia. Human leptin, the product of *obese* gene, is a 16 kDa (146 amino acids) protein secreted from adipocytes circulate in the bloodstream which has recently been identified as one of the factors controlling satiety [2, 27]. Administration of recombinant leptin to *ob/ob* mice, which are deficient in the production of leptin, caused a reduction in both food intake and weight loss [2, 18]. Further detailed investigation of the function of leptin or its use as a potential therapeutic intervention in obesity depends on the availability of a highly pure, safe, and biologically active product. Although several groups have already made reports on the mass production of human leptin in *Escherichia coli* [5, 8, 22], it has been known that leptin refolded from inclusion bodies was remarkably less potent than that secreted from eukaryotic cells. Furthermore, the biological potency of leptin varied considerably depending on the refolding methods employed [13, 26]. However, the recombinant leptin which was secretorily produced in *E. coli* and animal cell showed a similar biological potency to native leptin [8, 16, 19].

Since the discovery of the method of transforming *Bacillus subtilis* with plasmid DNA [4], *B. subtilis* has become an attractive alternative to *E. coli* as a host for the expression of cloned genes because it has several advantages over *E. coli*. The greatest advantage is its ability to secrete proteins into the culture medium and accumulate them to a high level in a relatively pure state [6, 10, 25]. Secretion of proteins may circumvent the formation of inactive inclusion bodies, which occurs during the overexpression of foreign genes in *E. coli* [14, 15]. Also, the secreted proteins usually remain in a biologically active form [12, 17]. Furthermore, *B. subtilis* is not a human pathogen but it is a GRAS strain [11].

In this paper, we describe the secretory production of human leptin in a six proteases-deficient *B. subtilis* strain. We also explain simple procedures for the purification of leptin and demonstrate that human leptin secreted from *B. subtilis* formed the correct folding structure.

Materials and Metnods

Bacterial Strains and Plasmids and Cultivation

The bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* XL1-Blue was used as a host strain for cloning and maintenance of plasmids. *B. subtilis* WB600, which is a six extracellular proteases-deficient strain [24], was used as a host for expressing the human *obese* gene. A λ phage construct containing the cDNA of human *obese* gene was kindly provided by Dr. J. Friedman [27]. Both pBR322 and pUP43SACSP were used for the construction of *E. coli-B. subtilis* shuttle vector. In addition, pUP43SACSP contains the *Bacillus* origin of replication, strong P43 promoter and levansucrase signal sequence (SacB) inserted at the downstream of the promoter [23].

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Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Reference
Strains		
E. coli XL1-Blue	*SupE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac F(proAB⁺ lacI^q lacZΔM15 Tn10(tet^r))*	Stratagene[a]
B. subtilis WB600	*TrpC2 ΔnprE ΔaprE Δepr Δbpr Δmpr ΔnprB*	[24]
Plasmids		
pBR322	4361 bp, Tc^r, Ap^r	New England Biolabs[b]
pUP43SACSP	4162 bp, Km^r, P43 promoter, levansucrase signal peptide	[23]
pBRWB	6.4 kb, *E. coli-B. subtilis* shuttle vector, Ap^r, Km^r	This study
pBRWBOb	6.9 kb, *obese* gene fused to levansucrase signal peptide, Ap^r, Km^r	This study

[a]Stratagene Cloning Systems., La Jolla, CA.
[b]New England Biolabs, Inc., Berverly, MA.

For the construction of shuttle vector, pBRWB, pUP43SACSP was digested with *Bam*HI and *Nde*I, and was ligated with pBR322 which was digested with the same restriction enzymes (Fig. 1A). For the expression of human *obese* gene in *B. subtilis*, mature *obese* gene was cloned into pBRWB. The *obese* gene from the λ phage construct was first cloned into pUC19 at the *Eco*RI site (pUCOb) [9]. The mature *obese* gene was cloned by PCR as follows: The forward primer 5' GCGGATCCGTGCCCATCCAAAAA GTCC 3' was designed to contain a *Bam*HI site (underlined) upstream of the first amino acid (Val) of mature human leptin. The reverse primer 5' GCGGATCCTTATTAGC ACCCAGGGCTGAGG 3' was designed to contain a *Bam*HI site (underlined) and tandem TAA stop codons. PCR was performed using a PCR Thermal Cycler MP TP3000 (Takara Shuzo Co. Shiga, Japan) and a High fidelity PCR system (Boehringer Mannheim, Mannheim, Germany). The PCR product was digested with *Bam*HI and it was cloned into pBRWB at the *Bam*HI site (pBRWBOb). In this construction, three amino acids of Lys-Gly-Ser were fused to N-terminal of mature human leptin and the first amino acid residue of fused protein, Lys, directly followed the C-terminal of levansucrase signal peptide, Ala-Phe-Ala at residues - 3 to - 1 relative to the cleavage site (Fig. 1B). The pBRWB and pBRWBOb were transformed into *B. subtilis* WB600 as described by Dubnau *et al.* [3]. *E. coli* and *B. subtilis* strains were cultivated in Luria-Bertani (LB) medium (tryptone, 10 g/l; yease extract, 5 g/l; NaCl, 5 g/l) at 37°C. Ampicillin (Ap, 50 mg/l) and kanamycin (Km, 25 mg/l) were supplemented for the cultivation of recombinant *E. coli* and *B. subtilis*, respectively. All DNA manipulations including restriction digestion, ligation and agarose gel electrophoresis were carried out as described by Sambrook *et al.* [20].

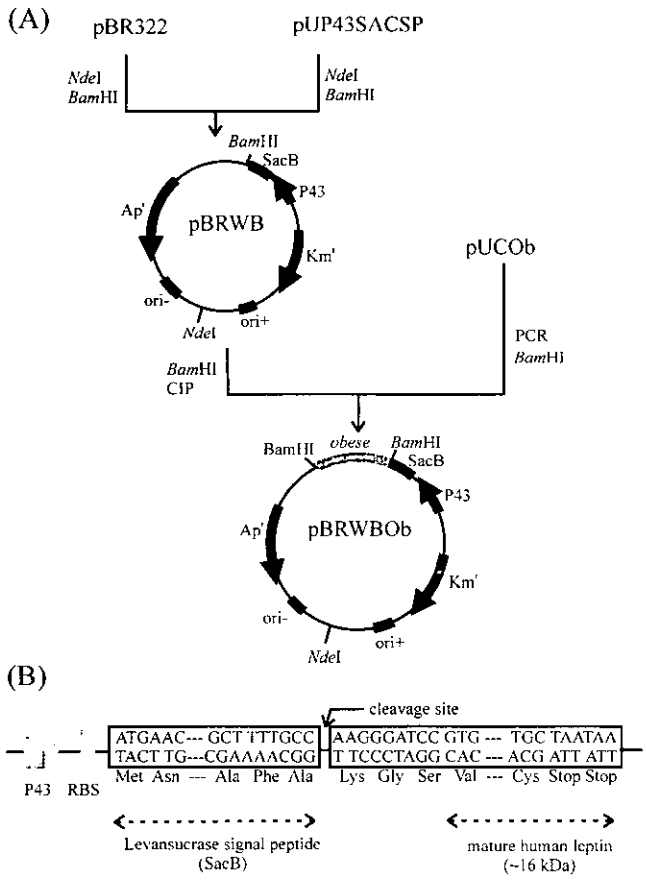

Fig. 1. Construcation of plasmids.
(A) Schematic diagram of plasmid construction. Abbreviations: ori+, origin of replication for *B. subtilis*; ori-, origin of replication for *E. coli*; Ap^r, β-lactamase gene; Km^r, kanamycin resistance gene; P43, P43 *Bacillus* promoter; SacB, levansucrase signal peptide. (B) Nucleotide and amino acid sequence at the fusion site. Three amino acid residues are fused to N-terminal of mature leptin and tandem repeat of stop codons follows the human *obese* gene.

Pruification of Leptin

Recombinant *B. subtilis* WB600 harboring pBRWBOb was grown in a 0.5 l LB medium supplemented with kanamycin (25 mg/l) at 37°C. The culture supernatant was collected at an early stationary phase by centrifugation at 3,500 ×g and 4°C for 10 min. Proteins in the supernatant solution were precipitated by adding ammonium sulfate to

60% (w/v) of saturation. The solution was left overnight and the precipitate formed was collected by centrifugation (10,000 ×g, 4°C, 30 min). The precipitate was then dissolved in phosphate-buffered saline (PBS) buffer (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH 7.4) and dialyzed against the same buffer in a dialysis membrane (MWCO 3,500, Spectrum Lab. Inc., Laguna Hills, CA) for 32 h with four buffer exchanges. Finally, leptin was purified through anion-exchange column chromatography (BioLogic HR system, Bio-Rad, Hercules, CA). The dialzed protein solution was loaded onto an anion-exchange column (Bio-Scale Q2 column, Bio-Rad) preequilibrated with 50 mM Tris-HCl (pH 7.5), and it was eluted with the linear gradient of NaCl (0–1.0 M in the same buffer) at 90 ml/h. The protein concentration of each fraction was monitored by using the UV detector (Bio-Rad). NaCl in leptin fraction was removed by dialysis (MWCO 3,500, Spectrum Lab. Inc.) against 1 l PBS buffer for 24 h with three buffer exchanges.

Analysis of Oxidation State

To determine the presence of a putative disulfide bond between the two cysteines in leptin, the purified leptin was treated with 5 mM of dithiothreitol (DTT) for 1 h at room temperature and it was lyophilized in a Speed-Vac Concentrator (Savant Co., New York, NY). The lyophilized sample was resuspended in SDS-PAGE sample buffer without reducing agent (12.5 mM Tris-HCl, pH 6.8, 2.5% SDS, 10% glycerol, 0.05% bromophenol blue). The control sample was prepared by the same method mentioned without DTT treatment. The cysteines were blocked by adding 3 µl of 0.5 M iodoacetamide. Both protein samples were analyzed by SDS-PAGE.

Analytical Methods

Protein samples were analyzed by electrophoresis on a 15% (w/v) sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) as described by Laemmli [13]. The protein bands on the SDS-PAGE gels were quantified by densitometry (ImagerMaster™, Pharmacia Biotech, Uppsala, Sweden). The amount of soluble protein was determined by Bio-Rad protein assay kit (Bio-Rad) using bovine serum albumin (BSA) as a standard. The purity of leptin was analyzed by SDS-PAGE and human leptin ELISA kit (Diagnostic Systems Laboratory Inc., Webster, TX). The molecular mass of the purified leptin was measured by matrix-assisted laser desorption/ionization mass spectrometry (MALDI/TOF/MS, PerSeptive Biosystems, Framingham, MA). To determine the N-terminal amino acid sequence of produced proteins, the proteins on SDS-PAGE gel were electroblotted onto a PVDF membrane (Bio-Rad Lab., Hercules, CA). The leptin band on the PVDF membrane was excised and sequenced by the gas phase sequencer (Model 476A, Applied Biosystems Inc., Foster, CA).

RESULTS AND DISCUSSION

Expression of Human Leptin in *B. subtilis* WB600

For the expression of human obese gene in *B. subtilis*, human *obese* gene was subcloned into *E. coli-B. subtilis* shuttle vector pBRWB at the *Bam*HI site to make pBRWBOb (see MATERIALS AND METHODS). In this construction, three additional amino acids (Lys-Gly-Ser) derived from the vector are supposed to be connected to the N-terminal of mature human leptin, that is, if the precise processing did take place during secretion (Fig. 1B). For the efficient and stable production of leptin, *B. subtilis* WB600, the six proteases-negative strain, was used. After cultivation of *B. subtilis* WB600 harboring pBRWBOb in LB medium for 12 h, the proteins in culture supernatant were concentrated by acetone precipitation [21]. The putative human leptin produced from *B. subtilis* WB600 harboring pBRWBOb was analyzed on SDS-PAGE (Fig. 2). Densitometer scanning of the commasie-stained SDS-PAGE gel indicated that the secreted leptin accounts for 48% of the total soluble protein in the culture medium. To confirm the correct processing of the signal peptide, the N-terminal amino acid sequences of leptin were analyzed. The determined sequence was Lys-Gly-Ser-Val-Pro-Ile-Gln-Lys-Val-Gln-Asp-Asp, which is consistent with the N-terminal amino acid sequence of leptin and additional amino acids derived from the vector. These results indicate that the levansucrase signal peptide had been cleaved off and properly processed.

Purification of Human Leptin Produced from *B. subtilis* WB600 (pBRWBOb)

Recombinant leptin was purified from 0.5 l culture supernatant obtained by flask culture of *B. subtilis* WB600 harboring pBRWBOb as described in Materials and

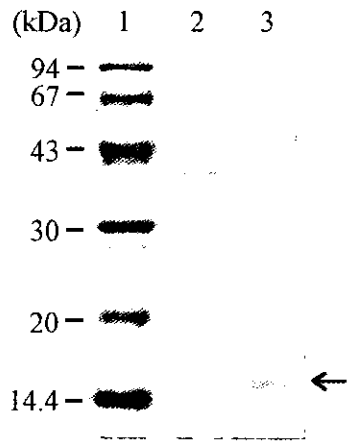

Fig. 2. Expression of human *obese* gene in *B. subtilis*.
Lane 1, molecular mass standards; lane 2, culture supernatant of *B. subtilis* WB600 (pBRWB); lane 3, culture supernatnat of *B. subtilis* WB600 (pBRWBOb). The arrow indicates the secreted leptin.

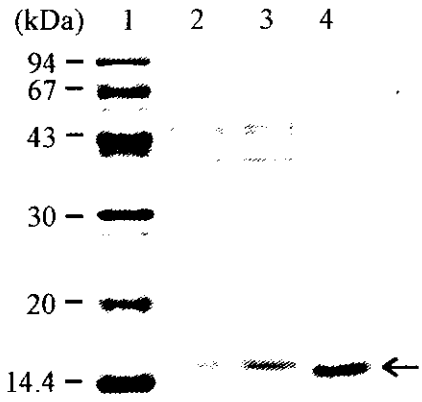

Fig. 3. Purification of human leptin from the culture supernatant of *B. subtilis* WB600 harboring pBRWBOb.
Lane 1, molecular mass standards; lane 2, culture supernatant; lane 3, ammonium sulfate precipitation; lane 4, Anion-exchange chromatography. The arrow indicates the purified leptin.

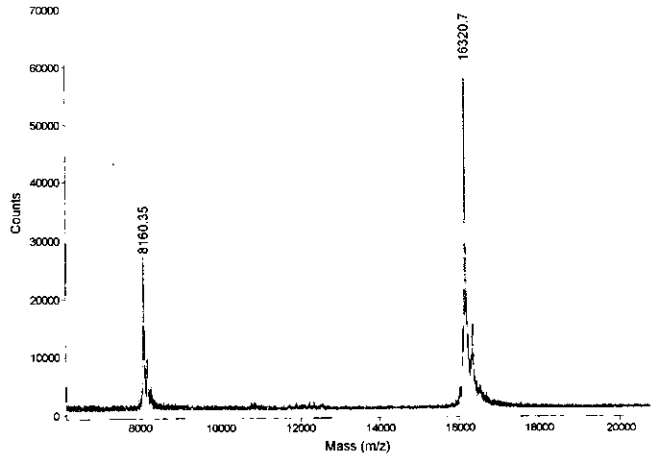

Fig. 4. Mass spectrum of purified human leptin.
The MALDI mass spectrum is dominated by a single component (leptin, second peak) with a measured molecular mass of 16320.7. The first peak (8160.35) represents the doubly protonated form of the protein arising from the MALDI mass spectronic process.

Methods. Figure 3 shows the SDS-PAGE gel patterns of each purification steps. After ion-exchange chromatography, the purity as determined by SDS-PAGE was greater than 95%. The results are summarized in Table 2. The final amount and the recovery yield of the purified leptin were 2.3 mg and 38.3%, respectively. The purity of leptin determined by ELISA kit was higher than 95%. Mass spectrum analysis of recombinant leptin showed that the MW of recombinant leptin was 16,320.7 Da, which is consistent with the size of human leptin plus the additional three amino acid residues (Fig. 4). The mass spectrum analysis also showed that the recombinant leptin was pure.

Oxidation State of Recombinant Leptin

Leptin contains 2 cysteine residues (Cys 96 and Cys 146) that form a disulfide bond which is necessary for the biological activity of human leptin [7]. To determine the existence of disulfide bond in purified leptin, the redox state was analyzed as described in Materials and Methods. When leptin was reduced by treating with 5 mM DTT, the position of the leptin band on the SDS-PAGE gel was shifted upward compared with that of the non-reduced leptin (Fig. 5). Therefore, it can be concluded that the two Cys residues in the purified leptin are correctly linked by disulfide bond, which is necessary for biological activity. The presence of three additional amino acid (Lys-Gly-Ser) fused to N-terminal of mature leptin may affect on the activity of leptin. However, Altmann *et al.* reported that the fusion of FLAG peptide (8 amino acid residues) to the N-terminal of mature leptin had no effect on the biological activity of leptin [1]. After purification, the purity of leptin was determined by using an ELISA analysis kit (Diagnostic Systems Lab.). This analysis showed that there was very good correlation between the activities of the purified leptin and standard leptin supplied by the ELISA kit (data not shown). Varnerin *et al.* showed that the quantification of the amount of leptin by a human leptin RIA kit was useful for making an estimation of specific activity [22]. It was shown that the RIA kit also showed a strong correlation (r^2=0.96) with an ELISA kit (Technical bulletin of Diagnostic Systems Laboratory Inc.). According to these several evidences, even though we did not determine the biological potency of purified leptin by injecting into *ob/ob* mouse, it can be concluded that the purified leptin possesses biologically activity.

Human leptin can be produced into periplasm of *E. coli*. There have been a couple of reports on the secretion of leptin in *E. coli* [8, 19]. However, the amount of leptin produced and the efficiency of secretion were not

Table 2. Protein recovery in a representative experiment.

Purification step	Vol (ml)	Conc. of protein[a] (μg/ml)	Amount of protein (mg)	Amount of leptin[b] (mg)	recovery (%)	Purity
Culture supernatnat	500	26.4	13.2	6.0	100	45.5
Ammonium sulfate precipitation	10	780	7.8	4.5	75.0	57.7
Anion-exchange column	8	301	2.4	2.3	38.3	95.8

[a]Concentration was determined by a Bio-Rad protein assay kit.
[b]As determined by an ELISA kit.

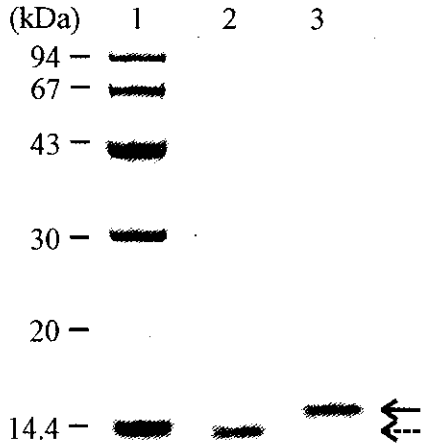

Fig. 5. Electrophoretic analysis of the redox state of leptin after treatment with a reducing agent (5 mM DTT).
Lane 1, molecular mass standard; lane 2, leptin not treated with DTT; lane 3, DTT-treated leptin. The dashed and solid arrows indicate the oxidized and reduced form of leptin, respectively.

satisfactory to be used in actual production system. Guisez *et al.* [8] used the OmpA signal peptide for the secretion of human leptin which was encoded by the modified sequence based on the *E. coli* preferable codon usage. The content of leptin secreted to the periplasm was 2-4 mg/ml/cells, and 2 mg of human leptin could be purified from 3 l culture. Rentsch *et al.* [19] used the PelB signal peptide for the secretion of mouse leptin, but the content was very low and only 1 mg of leptin could be purified from 1 l culture. In our result, 2.3 mg of leptin was purified from the 0.5 l culture (Table 2). Although the leptin were produced more in *E. coli* system, the final yield of leptin in *Bacillus* system after easy purification showed much higher level. This is one of the advantages for use of *Bacillus* sp. as a host.

In this report, we have described the secretory production of human leptin in a six proteases-deficient *B. subtilis* strain and its efficient purification. Overproduction of leptin by fed-batch culture of recombinant *B. subtilis* is the next obvious target, and is currently under investigation.

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