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Production of human leptin in *Bacillus subtilis*

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Abstract

Human leptin is a 16 kDa (146 amino acids) protein secreted from adipocytes and influences body weight homeostasis. In this report, active human leptin was successfully produced in the culture medium of *Bacillus subtilis*. After simple purification steps consisting of ammonium sulfate precipitation and anion-exchange column chromatography, 2.3 mg of leptin with a purity of greater than 95% was obtained from the 0.5 L culture with the recovery yield of 54.9%. The purified leptin showed the correct folding structure with one disulfide bond.

Introduction

Human leptin, the product of *obese* gene, is a 16 kDa (146 amino acid) protein secreted from adipocytes into bloodstream and has recently been identified as one of the factors controlling satiety.[1,4,7] The decrease of the food intake, energy expenditure and body weight was observed when leptin was administrated to the *ob/ob* mutant mouse producing a defective leptin. The functions of leptin in regulating appetite and metabolism are currently under intense investigation. Therefore, development of efficient production for a highly pure and biologically active leptin are demanded. *Bacillus 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*.[2] The greatest advantage is its ability to secrete proteins directly into the culture medium and accumulate them to a high level in a relatively pure state and in biologically active form. In this report, we describe the secretory production of human leptin in six protease-deficient *Bacillus subtilis*. We also describe simple procedures for purification of leptin and demonstrate that human leptin secreted from *Bacillus subtilis* formed the correct folding structure.

Materials and methods

Bacterial strains, plasmids and cultivation. *E. coli* XL1-Blue was used as a host strains for cloning and maintenance of plasmids. *B. subtilis* WB600 which is six estracellular protease-deficient strain[6] was used as a host for the expression of human *obese* gene. A phage construct containing the cDNA of human *obese* gene was provided by Dr. J. Friedman.[7] pBR322 and pUP43SACSP were used for the construction of *E. coli-B. subtilis* shuttle vector

(pBRWB). pUP43SACSP contains the *Bacillus* origin of replication and strong promoter (P43) and levansucrase signal peptide (SacB) was inserted at the downstream from the promoter.[5] For the expression of human *obese* gene in *B. subtilis*, mature *obese* gene was cloned into pBRWB (Fig. 1). *E. coli* and *Bacillus subtilis* were cultivated in Luria-Bertani medium (tryptone, 10 g/L; yease extract, 5 g/L; NaCl, 5 g/L) at 37°C.

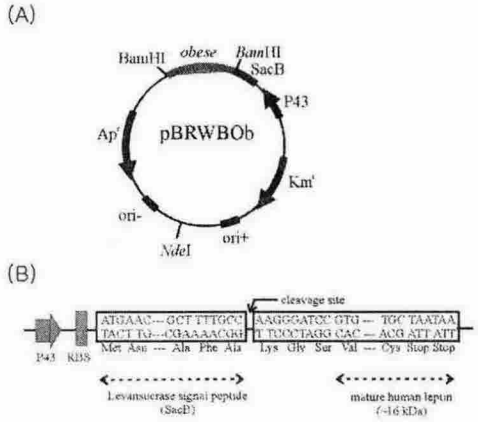

Fig. 1. Construction of plasmids. (A) Schematic diagram of plasmid construction. (B) Nuclotide and amino acid sequence at the fusion site.

Pruification of leptin. *B. subtilis* WB600 harboring pBRWBOb was grown in 0.5 L LB medium supplemented with kanamycine at 37℃ and the culture supernatant was collected by centrifugation at 3500 xg for 10 min. Proteins in the supernatant solution were precipitated by adding ammonium sulfate to 60% saturation. The precipitate was then dissolved in phosphate-buffered saline (PBS) buffer and dialyzed against the same buffer in a dialysis membrane (MWCO 3,500) for 32 hr with four buffer exchanges. Finally, the dialyzate were purified by anion-exchange column chromatography. The dialyzates solution was loaded onto a anion-exchange column (Bio-Scale Q2 column) preequilibrated with 50 mM Tris-HCl (pH 7.5), and then eluted by the linear gradient of NaCl (0 ~ 1.0 M in the same buffer) at 90 mL/h.

Analytical methods. The purity of leptin was analyzed by SDS-PAGE and human leptin ELISA kit. The molecular mass of the purified leptin was measured by matrix-assisted laser desorption/ionization mass spectrometry. To determine the N-terminal amino acid sequence of produced proteins, leptin band electroblotted onto the PVDF membrane was excised and sequenced by the gas phase sequencer. To determine the presence of a putative disulfide bond between the two cysteines in the leptin, the purified leptin was treated with 5 mM dithiothritol (DTT) for 1 h at room temperature and was lyophilized in a Speed-Vac Concentrator. The lyophilized sample was resuspended in SDS-PAGE sample buffer without reducing agent. The control sample was prepared by the same method without DTT treatment.

Results and discussion

Expression of human leptin in *B. subtilis* WB600. For the expression of human *obese* gene in *B. subtilis*, human *obese* gene[3,7] was subcloned into *E. coli-B. subtilis* shuttle vector, pBRWB at the *Bam*HI site (pBRWBOb). In this construct, three amino acids, 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. 1). For the efficient and stable production, the six protease-negative strain, *B. subtilis* WB600 was transformed with pBRWBOb. After cultivation of *B. subtilis* WB600 harboring pBRWBOb in LB medium for 12hr, the human leptin was produced in the culture supernatant (48% of the total soluble protein in the culture medium). To confirm the correct processing of the signal peptide, N-terminal amino acid sequence of leptin was analyzed. The determined sequence was Lys-Gly-Ser-Val-Pro-Ile-Gln-Lys-Val-Gln-Asp-Asp, and these results indicate that the levansucrase signal peptide had been cleaved and that the mature secreted protein had been properly processed.

Purification of human leptin. Recombinant leptin was purified from 0.5 L culture supernatant obtained by flask culture of *B. subtilis* WB600 harboring pBRWBOb with simple purification methods consisting of ammonium sulfate precipitation and anion-exchange column chromatography and the purification is summarized in Table 2. The final amount and the recovery yield of the purified leptin were 2.3 mg and 54.9%, 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 additional three amino acid residues (Fig. 2).

Table 1. Protein recovery in a representative experiment.

Purification step	Vol (mL)	Amount of protein (mg)	Amount of leptin (mg)	Recovery (%)	Purity
Culture supernatnat	500	13.2	6.4	100	48.5
Ammonium sulfate precipitation	10	7.8	4.5	73.3	57.4
Anion-exchange column	8	2.4	2.3	54.9	95.8

Oxidation state of recombinant leptin. 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 non-reduced leptin (Fig. 4). Therefore, it can be concluded that the two Cys residues in the purified leptin are correctly linked by disulfide bond and this result suggested that the purified leptin possessed potency equivalent to leptin produced by mammalian systems or natural source.

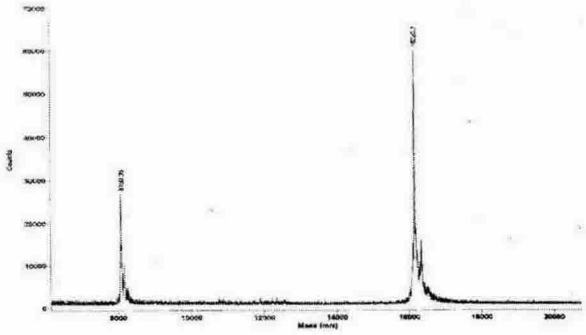

Fig. 4. Mass spectrum of purified human leptin.

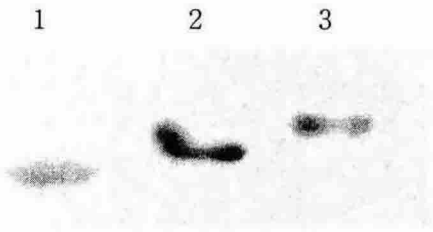

Fig. 5. Electrophoretic analysis of the redox state of leptin after treatment with 5 mM DTT. Lane 1, molecular mass standard; lane 2, leptin not treated with DTT; Lane 3, DTT-treated leptin.

Acknowledgements

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