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Electrostatic interaction-induced inclusion body formation of glucagon-like peptide-1 fused with ubiquitin and cationic tag

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ABSTRACT

In an attempt to produce glucagon-like peptide-1 (GLP-1) using recombinant *Escherichia coli*, ubiquitin (Ub) as a fusion partner was fused to GLP-1 with the 6-lysine tag (K6) for simple purification. Despite the high solubility of ubiquitin, the fusion protein K6UbGLP-1 was expressed mainly as insoluble inclusion bodies in *E. coli*. In order to elucidate this phenomenon, various N- and C-terminal truncates and GLP-1 mutants of K6UbGLP-1 were constructed and analyzed for their characteristics by various biochemical and biophysical methods. The experiment results obtained in this study clearly demonstrated that the insoluble aggregation of K6UbGLP-1 was attributed to the electrostatic interaction between the N-terminal 6-lysine tag and the C-terminal GLP-1 before the completion of folding which might be one of the reasons for protein misfolding frequently observed in many foreign proteins introduced with charged amino acid residues such as the His tag and the protease recognition sites. The application of a cation exchanger for neutralizing the positive charge of the 6-lysine tag in solid-phase refolding of K6UbGLP-1 successfully suppressed the electrostatic interaction-driven aggregation even at a high protein concentration, resulting in properly folded K6UbGLP-1 for GLP-1 production.

Introduction

Even though recombinant *Escherichia coli* has been used extensively for the production of heterologous proteins for the biotechnology industry, many proteins fail to fold into their native conformation which usually results in the accumulation of insoluble aggregates known as inclusion body [1]. A number of studies suggest that the inclusion body formation is attributed to improper intra- and/or inter-molecular interactions in folding intermediates of nascent polypeptides that are overexpressed in *E. coli* [2,3]. Other possible mechanisms for inclusion body formation have been proposed: the aggregation of native proteins of limited solubility and the aggregation of the unfolded state [4].

Fusion of an affinity tag such as His tag either at the N- or C-terminus of a target protein gene has been widely used for simple purification using affinity chromatography [5]. Several studies have shown that the fusion of an affinity tag results in positive effects such as increased protein stability and solubility, improved purification and crystallization [5,6]. In other cases, the introduction of an affinity tag or a protease cleavage site to a target protein negatively affected the function and structure of the product protein [6,7]. It has been frequently reported that fusion of the His tag leads to protein misfolding, resulting in inclusion body formation or loss of biological function of the target protein [8,9]. However, the exact mechanism by which the His tag fusion causes this misfolding is not clearly understood.

Ubiquitin is a highly conserved 76 amino acids protein in eukaryotes. It functions as a chaperone in ribosome biogenesis and as a marker for targeting proteins to proteasomes [10]. A number of studies have reported that the fusion of ubiquitin to the N-terminal of a target protein gene improved both the quality and quantity of the fusion proteins expressed in recombinant *E. coli* [11]. Two-dimensional NMR studies showed that the energetics of the ubiquitin folding pathway is highly favored and the protein completes a major folding phase in 10 ms [12]. Although the inner core of ubiquitin is highly hydrophobic, its outer surface is quite hydrophilic [13]. These unique properties of ubiquitin presumably promote proper folding of the fused protein, and prevent formation of inclusion body in recombinant *E. coli* [11]. In a general ubiquitin fusion system, six consecutive histidine residues known as the His tag were fused at the N-terminal of ubiquitin for purification *via* nickel affinity chromatography. In our previous study, for production of glucagon-like peptide-1 (GLP-1) that is a pharmaceutical peptide for treating the type 2 diabetes mellitus, ubiquitin along with the 6-lysine tag as a purification tag were fused with GLP-1 of 31 residues at its N-terminal, resulting in the fusion protein K6UbGLP-1 [14]. Despite the high solubility of ubiquitin and the

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relatively low molecular weight of the attached protein (GLP-1), K6UbGLP-1 was found mainly in the insoluble fraction as inclusion bodies in *E. coli*.

In this study, various truncates and mutants of K6UbGLP-1 were constructed to decipher the reason for the inclusion body formation of K6UbGLP-1 in *E. coli*. Through various *in vivo* and *in vitro* analyses, the reason turned out to be the electrostatic interaction between the N-terminal 6-lysine tag and the C-terminal GLP-1. Furthermore, this study has shown that neutralization of the positive charge in the 6-lysine tag was able to suppress the electrostatic interaction-driven aggregation in a refolding process and thus allowed K6UbGLP-1 to fold into a proper conformation.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α is used for genetic manipulation. All plasmids constructed in this study were transformed into *E. coli* BL21 (DE3) for protein expression. The recombinant *E. coli* BL21(DE3) cells harboring each expression plasmid were cultured at 37 °C in a 500-ml baffled flask (Nalgene, NY, USA) containing Luria–Bertani medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) supplemented with 50 mg/L kanamycin. Expression of each gene was induced at the logarithmic growth phase (OD_{600} = 0.5–1.0) by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cell growth was monitored by measuring the optical density of the culture broth at 600 nm using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan).

Construction of expression plasmids

The oligonucleotides used in this study are listed in Table 2. The 6-lysine tagged ubiquitin (K6Ub) gene was amplified from pAP-K6UbGLP-1 using the PCR primers of K6Ub-NdeI-F and Ub-BamHI-R, and was then inserted into a pAP vector in order to construct plasmid pAPK6Ub. To generate plasmid pAPUbGLP-1, the UbGLP-1 gene was amplified from pAPK6UbGLP-1 with the PCR primers of Ub-NdeI-F and GLP1-BamHI-R, and then cloned into the pAP vector. The schematic structures of K6UbGLP-1, K6Ub and UbGLP-1 are shown in Fig. 1A. The primers R6Ub-NdeI-F, H6Ub-NdeI-F, D6Ub-NdeI-F and S6Ub-NdeI-F were used to construct plasmids pAPR6UbGLP-1, pAPH6UbGLP-1, pAPD6UbGLP-1 and pAPS6UbGLP-1, respectively, with the same procedure as done for pAPUbGLP-1. The methods for synthesis of the GLP-1QN gene and for construction of pAPK6UbGLP-1QN were the same as those used for pAPK6UbGLP-1 [14] except for the two overlapping oligonucleotides of GLP-1QN-F and GLP-1QN-R. The amino acid sequences of GLP-1QN were shown in Fig 1B.

SDS–PAGE and Western blotting analysis

For sample preparation, protein concentrations were determined using the protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The samples were analyzed by SDS–PAGE with 14% or 16% T. Proteins were visualized by staining the gels with Coomassie brilliant blue R-250. The dried SDS–PAGE gels were analyzed by densitometry software (TotalLab 1.01, Nonlinear Dynamics Ltd., Newcastle, UK). The expression of K6UbGLP-1 was confirmed by Western blot analysis with a mouse monoclonal antibody against ubiquitin (Sigma, MO, USA) and a HRP-conjugated goat anti-mouse IgG secondary antibody (Bio-Rad, Hercules, CA). The bands of K6UbGLP-1 which specifically reacted with the antibodies were visualized by the Opti-4CN™ substrate and a detection kit (Bio-Rad, Hercules, CA).

In vivo solubility

A recombinant *E. coli* BL21(DE3) strain harboring each plasmid was grown at 37 °C and induced by addition of 1.0 mM IPTG for gene expression. After a 4-h induction, an appropriate volume of the culture broth was harvested by centrifugation at 13,000 rpm for 1 min, and the cell pellet was resuspended in 50 mM sodium phosphate buffer (pH 7.0), followed by cell disruption using an ultrasonic processor (Cole-Parmer, Niles, IL). The total cell lysate was separated into the soluble and the insoluble fractions by centrifugation at 14,000 rpm for 20 min, and then followed by SDS–PAGE analysis.

Electrostatic binding to cation exchanger

ÄKTA FPLC (GE Healthcare, Piscataway, NJ), an automated chromatography system, was used to analyze the electrostatic binding of K6UbGLP-1 and K6Ub on a cation exchanger. Two milliliters of the protein sample were injected into a HiTrap SP FF column (1 mL) packed with SP Sepharose Fast Flow (GE Healthcare, Piscataway, NJ) as a cation exchanger. The 50 mM sodium phosphate buffer (pH 7.0) was fed at a flow rate of 1 mL/min. After washing with 4 column volumes of the buffer, stepwise gradient of the buffer containing 1 M NaCl was applied to elute the proteins bound to the cation exchanger.

Table 1
Bacterial strains and plasmids used in this study.

Strain, plasmid	Relevant characteristics or construction	Source
Strains		
E. coli DH5α	F^- (Φ80Δ*lac*ZΔM15) *rel*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17(rK^-, mK+) *sup*E44 Δ(lacZYA-argF) U169	Invitrogen
E. coli BL21(DE3)	F^- *ompT hsd*SB(rB^-mB^-) *gal dcm* (DE3)	Novagen
Plasmids		
pUC18K6Ub	2.9 kb, Amp^r, 6-lysine tag and ubiquitin	AP technology
pAP	5.4 kb, Kan^r, *tac* promoter	AP technology
pAPK6UbGLP-1	5.8 kb, Kan^r, 6-lysine tagged ubiquitin fused to glucagon-like peptide-1, *tac* promoter	[14]
pAPK6UbGLP-1QN	5.8 kb, Kan^r, 6-lysine tagged ubiquitin fused to glucagon-like peptide-1QN, *tac* promoter	This study
pAPK6Ub	5.7 kb, Kan^r, 6-lysine tagged ubiquitin, *tac* promoter	This study
pAPUbGLP-1	5.8 kb, Kan^r, ubiquitin fused to glucagon-like peptide-1, *tac* promoter	This study
pAPR6UbGLP-1	5.8 kb, Kan^r, 6-arginine tagged ubiquitin fused to glucagons-like peptide-1, *tac* promoter	This study
pAPH6UbGLP-1	5.8 kb, Kan^r, 6-histidine tagged ubiquitin fused to glucagons-like peptide-1, *tac* promoter	This study
pAPD6UbGLP-1	5.8 kb, Kan^r, 6-aspartate tagged ubiquitin fused to glucagons-like peptide-1, *tac* promoter	This study
pAPS6UbGLP-1	5.8 kb, Kan^r, 6-serine tagged ubiquitin fused to glucagons-like peptide-1, *tac* promoter	This study