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Preparation of soluble isotopically labeled human growth hormone produced in *Escherichia coli*

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ABSTRACT

Isotopically labeled proteins have been used as internal standards for mass spectrometry (MS)-based absolute protein quantification. Although this approach can provide highly accurate analyses of proteins of interest within a complex mixture, one of the major limitations of this method is the difficulty in preparing uniformly labeled standards. Human growth hormone (hGH) is one of the most important hormones that circulate throughout the body, and its measurement is primarily of interest in the diagnosis and treatment of growth disorders. In order to provide a useful internal standard for MS-based hGH measurement, we describe an efficient strategy to produce a potentially valuable, stable isotope-labeled hGH with high purity and yield. The strategy involves the following steps: solubilization of hGH under labeling conditions, detection of stable isotope incorporation, large-scale purification, analysis of the labeled protein, and assessment of the labeling efficiency. We show that the yield of soluble hGH under selective isotopic labeling conditions can be greatly increased by optimizing protein expression and extraction. Our efficient method for generating isotopically labeled hGH does not influence the structural integrity of hGH. Finally, we assessed the efficiency of stable isotope labeling at the intact protein level, and the result was further verified by amino acid analysis. These results clearly indicate that our labeling approach allows an almost complete incorporation of $^{13}C_6{}^{15}N_4$-arginine into the hGH expressed in *E.coli* without detectable isotope scrambling.

1. Introduction

Human growth hormone (hGH) is produced in the pituitary gland and is considered to be one of the most important hormones circulating in the human body because of its various biological functions [1,2]. Therefore, the accurate measurement of hGH is critical for diagnosing the deficiency or excess of this hormone and for the treatment of its related diseases [3–5]. Although immunoassay methods using antibodies against hGH have been extensively employed to measure hGH levels in biological fluids [6], substantial progress has been made in the development of analytical methods for accurate protein quantitation. In particular, mass spectrometry (MS) –based protein analysis has rapidly evolved as one of the most powerful tools for targeted protein quantitation. To determine the exact amount of a specific protein in a complex biological sample, isotopically labeled synthetic peptides have been used as internal standards at known concentrations to measure the corresponding targets [7–10]. However, this approach often requires consideration of the efficiency of proteolytic digestion of the target proteins following addition of the standard peptide. Recently, intact proteins with stable isotope labeling that exhibit the same chemical properties as their unlabeled counterparts during pre-analytical treatments have been applied for absolute protein quantification [11,12]. Although this approach shows high potential for routine use in protein quantification, it is limited to the production of recombinant proteins that can be efficiently purified following the incorporation of a given stable isotope. High-level production of hGH in *Escherichia coli* (*E. coli*) has proven difficult because of the formation of protein aggregates referred to as inclusion bodies that often occur during the production of many other recombinant pro-

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teins [13–18]. High concentrations of chemical denaturants such as urea or guanidine hydrochloride are commonly used to solubilize inclusion bodies, and the solubilized proteins are then refolded by the removal of such agents. However, the refolding conditions must be optimized and the overall recovery of biologically active protein is often low [13,19]. Recently, we reported a useful approach that enables the efficient production of hGH expressed in *E. coli* by minimizing the formation of inclusion bodies and other protein aggregates [20]. The approach consists of lowering the induction temperature and optimizing protein extraction conditions in order to significantly improve the solubility of hGH.

Here, we demonstrate that despite the aggregation tendency of recombinant hGH, we improved protein solubility by amino acid-selective isotope labeling that resulted in the highly efficient production of soluble, isotopically labeled hGH in *E. coli*. The efficient labeling described in this study did not alter the structural integrity of hGH. Moreover, using MS-based analytical methods, we assessed the isotopic labeling efficiency of the recombinant hGH. The results suggest that the efficient production of uniformly isotopic labeled hGH presented in this study is useful for preparing an internal standard for MS-based absolute quantification.

2. Materials and methods

2.1. Materials

The following unlabeled amino acids were purchased from Sigma-Aldrich (St. Louis, MO, USA): L-alanine, L-Arginine monohydrochloride, L-glutamic acid, L-glycine, L-histidine monohydro chloride monohydrate, L-cysteine hydrochloride, L-tyrosine, L-valine, L-glutamine, L-leucine, L-asparagine, L-threonine, L-tryptophan, L-lysine monohydrochloride, L-serine, L-phenylalanine, L-aspartic acid, L-proline, L-isoleucine, and L-methionine. The labeled $^{13}C_6{}^{15}N_4$-arginine was obtained from Cambridge Isotope Laboratories (Andover, MA, USA).

2.2. Gene cloning of hGH

The cDNA encoding amino acids 1–191 of hGH (NCBI Reference: NM_000515.3: 141–719) was synthesized from BIONEER Co. (Daejeon, Korea). NheI (GCTAGC) and XhoI (CTCGAG) restriction enzyme cleavage site were inserted at the 5′- and 3′-ends of the cDNA sequence, respectively. The synthesized cDNA was ligated to the pET-28a vector (Novagen, Madison, WI, USA) by using the restriction enzymes, leading to the construction of the His-hGH expression vector that expressed the recombinant hGH with a 6×-histidine tag and thrombin cleavage site at the N-terminus. The nucleotide sequences of the inserts were confirmed by automatic sequencing.

2.3. Expression and purification of $^{13}C_6{}^{15}N_4$-arginine-labeled hGH

E. coli BL21 (DE3) cells transformed with the His-hGH expression vector were inoculated into 25 ml of Luria-Bertani medium (10 g Bacto tryptone, 5 g yeast extract, and 10 g NaCl per liter of solution) containing 50 μg/ml kanamycin and then incubated in the orbital shaker at 37 °C for 6 h. The cells were washed twice with M9 medium (0.2% glucose, 0.5 μg/ml thiamine, 1 mM magnesium sulfate and 0.1 mM calcium chloride) and inoculated into 1 l of M9 medium containing 50 μg/ml kanamycin. When the cells were grown to an OD_{600} of approximately 0.6 at 37 °C, 19 unlabeled amino acids and $^{13}C^{15}N$-labeled arginine (200 mg each) were added to the culture. Following incubation at 37 °C for 30 min and then on ice for 30 min, protein synthesis was induced with 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) in an orbital shaker at 16 °C for 16 h. The cells were harvested and His-hGH was purified using our previously described method [20]. Briefly, the harvested cells were suspended in 100 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.1% Triton X-100, 1 mg/ml lysozyme, 1 × protease inhibitor cocktail) and disrupted by sonication. The soluble supernatant was applied onto a Ni-NTA agarose column (Qiagen, Hilden, Germany) and eluted with elution buffer (1× PBS, pH 7.4, containing 150 mM NaCl, 200 mM imidazole, 0.1% TritonX-100, 10% glycerol). Fractions containing His-hGH were pooled, dialyzed against buffer containing 50 mM Tris-HCl (pH 8.0) and 10% glycerol, and further purified by anion-exchange chromatography using a Mono Q column (GE Healthcare, Little Chalfont, UK) with a linear gradient of 0–0.5 M NaCl. Fractions containing His-hGH were pooled and finally purified by gel-filtration using a HiLoad 26/30 Superdex 200 column (GE Healthcare) equilibrated with buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 10% glycerol. Purified proteins were dialyzed against storage buffer (10 mM Na_2HPO_4, pH 7.4, 0.5% glycine, 2.25% mannitol).

2.4. MADLI-TOF mass spectrometry

The bands containing stable isotope-labeled hGH were excised from SDS-polyacrylamide gels and washed with distilled water. Next, 50 mM ammonium bicarbonate/acetonitrile (1:1, v/v) was added to the gel and incubated for 15 min. The solution was discarded carefully, and gel pieces were covered with 100 μl of acetonitrile. Immediately after removing the acetonitrile, the gel pieces were incubated with 100 μl of 50 mM ammonium bicarbonate for 5 min and covered again with 100 μl of acetonitrile. The acetonitrile was removed and gel pieces were dried at 66 °C for 10 min in an oven. For reduction and alkylation, the gel pieces were incubated with a solution containing 10 mM dithiothreitol and 50 mM ammonium bicarbonate for 45 min at 56 °C and then covered with iodoacetamide/50 mM ammonium bicarbonate in the dark for 30 min. After drying the gel pieces, tryptic digestion was initiated by the addition of 20 ng/μl trypsin in 25 mM ammonium bicarbonate for 16 h. The resulting peptides were extracted with 0.1% trifluoroacetic acid/50% acetonitrile with sonication for 15 min and then further incubated at room temperature for 20 min. The resulting tryptic digested peptides were mixed with the same volume of MALDI matrix (10 mg/ml of α-cyano-4-hydroxycinnamic acid) on the MALDI MS plate, which was examined using an Autoflex III Smartbeam device (Bruker Daltonics, Billerica, MA, USA). Selected peptides were fragmented by applying a TOF/TOF lift mode equipped with a 355 nm SmartBeam laser. The spectra of the peptide MS and MS/MS were acquired over a range of m/z 500–2000 and m/z 40–1450, respectively. To analyze the intact protein, mass spectrometry was performed as described previously [20]. Briefly, the samples were mixed with MALDI matrix (α-cyano-4-hydroxycinnamic acid) and spotted directly onto a MALDI target plate. The samples were then analyzed on an Autoflex III Smartbeam device (Bruker Daltonics) in the positive ion mode. External calibration was performed using the ProteomMass peptide and protein MALDI/MS calibration kit (Sigma).

2.5. Characterization of $^{13}C_6{}^{15}N_4$-arginine-labeled hGH

Protein purity was determined by reversed-phase high-performance liquid chromatography (RP-HPLC) (Prominence, Shimadzu, Kyoto, Japan) equipped with a Kinetex C18 column (2.6 μm, 150 × 2.10 mm; Phenomenex, Torrance, CA, USA) [21]. Proteins were separated using a linear gradient of acetonitrile (28–100%) in 0.1% trifluoroacetic acid. The flow rate was 0.25 ml/min, and the column temperature was 40 °C. Protein secondary structure was analyzed by circular dichroism (CD) measurement using a J-815 CD spectrometer (Jasco, Oklahoma City, OK,