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A novel method for the large-scale production of PG-CNP37, a C-type natriuretic peptide analogue

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ABSTRACT

Achondroplasia is the most common form of human dwarfism caused by a mutation in the fibroblast growth factor receptor 3 (FGFR3), resulting in abnormal endochondral bone formation. C-type natriuretic peptide (CNP) is a potent stimulator of endochondral bone growth and represents a potential therapy for achondroplasia. We have developed a novel, simple and cost effective method to produce a CNP analogue, PG-CNP37, at a large scale from *Escherichia coli*. A PG-CNP37 fusion protein was overexpressed as inclusion bodies in *E. coli*, which were purified then cleaved by formic acid to release the PG-CNP37 peptide. Approximately 0.5 g of 95% pure, soluble and active PG-CNP37 peptide was produced from 1 L of culture using this method and may represent a viable means for large-scale production of other therapeutic peptides.

1. Introduction

C-type natriuretic peptide (CNP) has recently been shown to be a potent stimulator of endochondral bone growth and may represent a potential therapy for achondroplasia, the most common form of human dwarfism (Yasoda et al., 1998, 2004; Olney et al., 2006; Pejchalova et al., 2007; Yasoda and Nakao, 2010). In humans, CNP is normally produced from the natriuretic peptide precursor C gene as a single chain 126-amino acid pre-pro polypeptide (Sudoh et al., 1990). Removal of the signal peptide yields pro-CNP, and further endoprotease cleavage generates an active 53-amino acid peptide (CNP53), which is secreted and cleaved again by an unknown enzyme to produce the mature 22-amino acid peptide (CNP22) (Wu et al., 2003). We have demonstrated that several neutral endopeptidase resistant CNP variants had a relative increased half life in normal mice while improving bone growth activity in FGFR3-related chondrodysplasia ex vivo and in vivo mouse models (United States Patent Application 20100297021).

Large-scale chemical synthesis of large peptides is difficult and is associated with higher impurity profiles and high cost. Recombinant technology using bacterial cultures such as *Escherichia coli* is currently the method of choice for the large-scale manufacture of large peptides. One of the challenges to bacterial production of peptides such as PG-CNP37 includes rapid degradation by proteases. However, this problem can be overcome by expressing the peptide with a large protein fusion partner, which protects the peptide from proteolysis (Jenny et al., 2003). Chemical or enzymatic cleavage steps must then be added to the manufacturing process to release the final peptide product from the fusion partner.

Protease cleavage is often less practical and more costly than chemical cleavage at the manufacturing scale, because of supply, consistency and narrow pH range restrictions. Efficiency of proteases is also affected by parameters such as solubility and conformation of the substrate. Moreover, one must then demonstrate removal of the protease from the final drug substance, making this an even less practical approach for large-scale peptide production (Crimmins et al., 2005; Fong et al., 2010). Chemical cleavage has its own set of problems, most notably that many chemicals used to cleave peptide bonds are potentially dangerous and less specific, often incurring other unwanted modifications. For example, cyanogen bromide (CNBr) cleaves proteins on the C-terminal side of unoxidized methionine residues (Crimmins et al., 2005); however, it is acutely toxic by inhalation, physical contact, or ingestion. Hydroxylamine (NH_2OH) cleaves proteins on the C-terminal side of asparagine–glycine peptide bonds (Hu et al., 2008) but may explode on heating. Chemical cleavage procedures often yield long peptides because they target amino acid residues and dipeptide linkages that are present in relatively low frequencies (Crimmins et al., 2005). Acid-labile aspartate–proline (Asp–Pro) dipeptide sites can be engineered to facilitate cleavage of the peptide from the fusion protein (Li et al., 2006; Vidovic et al., 2009) and several reports have described the susceptibility of the Asp–Pro site to formic acid cleavage (Li et al., 2006, 2007a,b; Vidovic et al., 2009).

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Packaging of recombinantly expressed proteins into inclusion bodies is ideal for large scale production as it reduces the likelihood of proteolysis, vastly improves downstream purification and is often associated with very high specific productivity yields, where the recombinant proteins sometimes are making up more than 30% of the total cellular proteins (Singh and Panda, 2005). Production of insoluble recombinant peptides is also advantageous for peptides that may be toxic to the host cell in their soluble form (Li et al., 2006; Vidovic et al., 2009).

In this paper, we present a simple and scalable high yield production and purification method for producing an active 39-amino acid CNP analogue, PG-CNP37. This production method yields 0.5 g of 95% pure PG-CNP37 per liter of reactor culture using standard ion-exchange chromatography. In addition, the method for producing PG-CNP37 described here is more economical than existing methods and can be applied to the large-scale production of other therapeutic peptides without internal Asp–Pro peptide bonds.

2. Experimental procedures

2.1. Cloning fusion protein TAFm-PG-CNP37 and its expression in E. coli

The TAFm-PG-CNP37 fusion protein-encoding DNA fragment was synthesized and cloned into the expression vector pJexpress401 (DNA2.0). The CNP fusion protein expression plasmid (pJex-TAFm-PG-CNP37) was transformed into *E. coli* BL21 (DE3) (Agilent) and plated on LB agar plates containing 50 μg/mL kanamycin and incubated overnight at 37 °C. A single colony was chosen for culture in 4 mL LB medium containing 50 μg/mL kanamycin at 37 °C. When an A_{600} of bacterial culture reached 0.6, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the cell media and incubated at 37 °C for 3 h. The bacterial cells were harvested by centrifugation at 4000 rpm for 10 min and the cell pellets were stored at −80 °C. Cell pellets were subsequently lysed (B-PER II Bacterial Extraction Reagent; Thermo Fisher: 0.4 mL per 4 mL of bacterial culture, and Benzonase Nuclease; Novagen: 0.025 U/mL) at room temperature for 10 min. Following centrifugation, the total cell lysate, supernatant and pellet were assayed for expression and solubility by SDS-PAGE and Western Blot.

2.1.1. TAFm-PG-CNP37 amino acid sequence

MVLTKKKLQELVREVAPNEQLEEEVEEMLLQIAEEFIESVVTAAAQLARHRKSSTLEVKEVQLHLERQWNMWIMGSSHHHHHHSSGLVPRGSHTGEEEEKHMDPGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC.

2.1.2. PG-CNP37 amino acid sequence

PGQEHPNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC.

2.2. SDS-PAGE and western blot detection of TAFm-PG-CNP37 expression

Total cell lysates, supernatant and re-suspended lysate pellet were separated by SDS-PAGE (Invitrogen; reduced NuPAGE 4–12% Bis–Tris Gel, MES SDS buffer). The gel was stained using 20 mL Imperial Protein Stain (Thermo Fisher) at room temperature for 1 h and de-stained with water. Western Blot analysis was performed by transferring the protein to a nitrocellulose membrane with an I-Blot (Invitrogen). The membrane was blocked with 5% non-fat milk in TBS-T buffer (TBS containing 0.05% Tween 20) and incubated with rabbit anti-CNP22 polyclonal antibody (BACHEM, 0.4 μg/mL) at room temperature for 2 h. The membrane was washed three times with TBS-T buffer and incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Promega, 0.2 μg/mL) at room temperature for 1 h. The membrane was washed three times with TBS buffer and the expressed proteins were visualized by adding 10 mL Western Blue® Stabilized Substrate (Promega).

2.3. Purification of TAFm-PG-CNP37 inclusion bodies and formic acid cleavage

The cell pellet was re-suspended in 25 mL (1/10 of cell culture media) of buffer (B-PER II Bacterial Extraction Reagent; Thermo Fisher Scientific Inc.), sonicated on ice for 10 min, centrifuged at 12,000 rpm for 20 min at 4 °C, and re-suspended in 25 mL 20× diluted B-PER II buffer. This was repeated 3–5 times until the supernatant became clear. One mL of re-suspended TAFm-PG-CNP37 inclusion bodies in 20× diluted B-PER II buffer were transferred to 1.5 mL tubes and centrifuged at 14,000 rpm for 15 min. The supernatants were discarded and the pellets were dissolved with 500 μL of 50%, 10% or 2% formic acid (Fisher) and incubated at various temperature from 25 ° to 70 °C for 6–48 h. The formic acid cleavage products were neutralized with 10 M NaOH or 0.5 M Tris-base to pH 7.0 and assayed by SDS-PAGE and LC/MS.

2.4. LC/MS assay

Quantitative assessment and identification of PG-CNP37 and other impurities was achieved by high-performance liquid chromatography (HPLC) coupled to an ultraviolet and an electrospray ionization-mass selective detector (Agilent). Peptides were eluted with an increasing gradient of acetonitrile, in the presence of water and 0.05% trifluoroacetic acid. The standard flow rate, column temperature and injection volume was 0.25 mL/min, 45 °C and 10 μL respectively, unless otherwise noted.

2.5. PG-CNP37 production and purification

BL21 (DE3) cells transformed with the pJexpress-TAFm-PG-CNP37 plasmid were grown in a 10 L fermenter at 37 °C and induced with 1 mM IPTG at 35–37 °C resulting in a TAFm-PG-CNP37 titer of approximately 8–10 g/L at a culture density of $A_{600} \geq 150$. The cell pellet was re-suspended in phosphate-buffered saline at an $A_{600} = 100$ and lysed by three passes through a high-pressure homogenizer. The resulting lysate was centrifuged at 6500 g for 10 min and the pellet fraction containing insoluble TAFm-PG-CNP37 inclusion bodies was re-suspended in phosphate-buffered saline and centrifuged at 6500 g for 10 min. The resulting inclusion body pellet was re-suspended in water, formic acid was added to a 2% final concentration and incubated at 55 °C for 20–24 h. The cleavage reaction was neutralized by adding Na_2HPO_4 to 25 mM final concentration followed by titrating with 50% (w/v) NaOH to pH 7. The neutralized reaction was centrifuged at 6500 g for 15 min. The supernatant was sterile-filtered and purified by anion-exchange chromatography (Fractogel TMAE Hi-CAP; EMD Biosciences) at pH 7.0–7.2. The flow-through fraction contained PG-CNP37 was further purified by cation-exchange chromatography (SP-Sepharose Fast Flow; GE Healthcare) with sodium phosphate buffer pH 7.0. Purified PG-CNP37 was concentrated and buffer exchanged by weak cation-exchange chromatography (CM-Sepharose Fast Flow, GE Healthcare) with elution mediated by weak acid to obtain peptide in acidified water.

2.6. Activity assay (in vitro cell-based assay)

Production of cGMP as readout of NPR-B activity was used. Mouse NIH3T3 fibroblasts (ATCC) expressing NPR-B endogenously were grown to subconfluency, pretreated with 0.75 mM phosphodiesterases inhibitor IBMX (Sigma) for 15 min and treated with increasing concentrations of CNP22 and PG-CNP37 for 15 min. ANP