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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.

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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

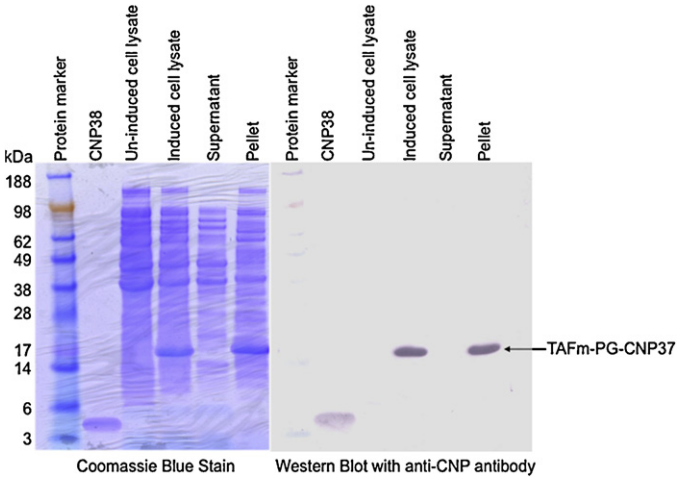

Fig. 1. PG-CNP37 fusion protein is expressed in *Escherichia coli* as inclusion bodies. The expression of PG-CNP37 fusion protein (TAFm-PG-CNP37) was induced in BL21 (DE3) cells with 1 mM IPTG at 37 °C for 3 h in a shake flask. The cell lysates, soluble supernatant and insoluble pellet were analyzed with SDS-PAGE (reduced condition) and western blot with chemically synthetic CNP38 as control.

is included as a specificity control for the NPR-B receptor. Each concentration was performed in duplicate and the data was normalized to cell number. Cells were lysed for the CatchPoint cGMP Detection Assay according to manufacture's protocol (Molecular Devices).

3. Results

3.1. PG-CNP37 fusion protein is expressed in E. coli as inclusion bodies

A mutated histone fold domain of human transcription factor TAF12 (TAFm) was used as fusion partner to express PG-CNP37 fusion protein as inclusion bodies: all aspartic acid residues (D) in TAF12 were changed to glutamic acid (E) to prevent nonspecific formic acid cleavage; one cysteine (C) in TAF12 was changed to Alanine (A) to prevent nonspecific disulfide bond formation (Vidovic et al., 2009; amino acid sequence shown in Section 2.1). The TAFm-PG-CNP37 was expressed in *E. coli* induced with 1 mM IPTG at 37 °C for 3 h in a shake flask. The majority of TAFm-PG-CNP37 fusion protein was detected in the IPTG induced cell lysate and in the lysate pellet, with a molecular weight around 16 kDa (Fig. 1) indicating that TAFm-PG-CNP37 was expressed as inclusion bodies. Western blot analysis with anti-CNP antibody confirmed the expression of the PG-CNP37 fusion proteins.

3.2. PG-CNP37 is liberated from TAFm-PG-CNP37 inclusion bodies by formic acid cleavage

An acid-labile Asp–Pro bond was engineered in the TAFm-PG-CNP37 polypeptide between the fusion partner TAFm and PG-CNP37. Formic acid cleavage conditions were optimized based on time, temperature and formic acid content to prevent nonspecific cleavage of other peptide bonds. Optimal acid cleavage conditions were obtained with 2% formic acid at 55 °C for 20 h. Using these conditions, the majority of TAFm-PG-CNP37 was cleaved and analysis of SDS-PAGE showed a band of a size expected for PG-CNP37. LC/MS results confirmed that the pH 7 neutralized soluble fraction was primarily PG-CNP37, while the insoluble fraction primarily consisted of TAFm and non-hydrolyzed TAFm-PG-CNP37 (date not shown). Further time and temperature optimization was performed on TAFm-PG-CNP37 inclusion bodies treated with 2% formic acid at 42 °C, 55 °C or 70 °C for 6–24 h.

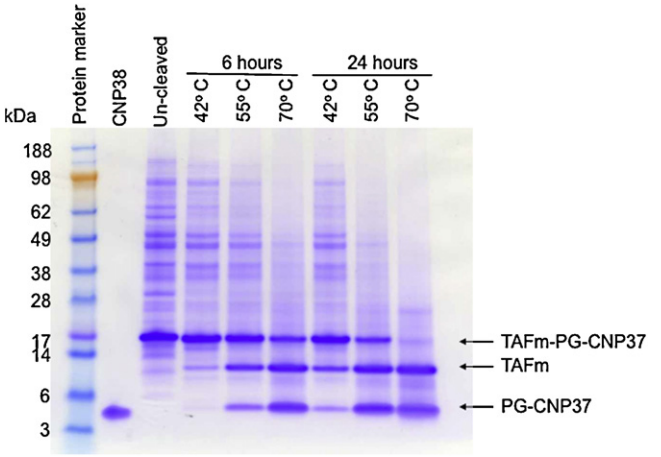

Fig. 2. Formic acid cleavage of TAFm-PG-CNP37 inclusion bodies. The TAFm-PG-CNP37 inclusion bodies were cleaved with 2% formic acid at 42 °C, 55 °C or 70 °C for 6–24 h. The formic acid cleavage products were neutralized and assayed by SDS-PAGE (reduced condition).

Fig. 2 shows that TAFm-PG-CNP37 was cleaved completely at 70 °C for 24 h. Although the 70 °C/24 h cleavage gave the highest yield, nonspecific cleavage products were more abundant using this condition (Table 1). Since it was difficult to purify PG-CNP37 from these off-target peptides using standard chromatography techniques, the 55 °C/24 h cleavage condition was selected for PG-CNP37 production.

3.3. Purification and characterization of PG-CNP37

TAFm-PG-CNP37 was cleaved in 2% formic acid at 55 °C for 24 h. The cleavage reaction was neutralized and centrifuged. The non-hydrolyzed TAFm-PG-CNP37, TAFm and other insoluble protein impurities and peptides were precipitated while approximately 80% pure acid liberated PG-CNP37 remained in the supernatant with minimal recovery loss upon neutralization (Fig. 3a and b). The supernatant was further purified by passing the PG-CNP37, a cationic peptide (pI = 10), through an anion-exchange chromatography resin (Fractogel TMAE Hi-CAP column) designed to remove residual DNA, endotoxin and peptide impurities present after centrifugation. Cation-exchange chromatography with SP-Sepharose Fast Flow removed any remaining DNA, endotoxins and other peptide impurities. The purified PG-CNP37 was characterized by SDS-PAGE/Coomassie blue stain (Fig. 4a) and LC/MS assay (Fig. 4b). The overall yield of TAFm-PG-CNP37 inclusion bodies was approximately 8–10 g/L at a portion of 20% of dry cell weight. The final purification yield of PG-CNP37 peptide was approximately 0.5 g/L with a purity of 95%. Product related impurities included ~3% oxidized PG-CNP37 (Met34 sulfoxide), less than 1% succinimide intermediate and deamidated (Asn7 and/or Asn15) PG-CNP37, less than 1% PG-CNP37 Gly22-Cys23 cleavage and less than 0.5% non-product related impurities. Compared to native CNP (CNP22), PG-CNP37 demonstrated similar in vitro activity in cell-based cGMP stimulation assays using mouse NIH3T3 fibroblasts (Fig. 4c).

4. Discussion

We present a simple and cost effective method for producing an active 39-amino acid CNP analogue, PG-CNP37. The PG-CNP37 fusion protein is overexpressed in large scale as inclusion bodies in *E. coli* followed by formic acid cleavage to release the soluble PG-CNP37 peptide from its fusion partner. Following neutralization at pH 7, initial purification is accomplished by a simple centrifugation. This simple neutralization and centrifugation step can produce

Table 1
LC/MS analysis of formic acid cleavage of TAFm-PG-CNP37 inclusion bodies*.

Identification	MW (Da)	6 h at 42 °C	24 h at 42 °C	6 h at 55 °C	24 h at 55 °C	6 h at 70 °C	24 h at 70 °C
Asp29 Ring Cleavage	3142	0.1	1.2	2.0	15.3	12.0	26.0
Met34 sulfoxide	4118	0.0	0.7	0.9	8.3	5.9	21.8
PG-CNP37	4102	12.6	46.8	51.6	96.9	85.6	66.2
PG-CNP37 Dimer	8204	0.0	1.1	5.4	11.9	13.2	6.8
Asp29 succinimide	4084	0.3	1.0	2.3	10.7	7.5	21

* Mass spectrum quantification is based on area under the curve (AUC)/10^6 of extracted ion chromatogram (EIC).

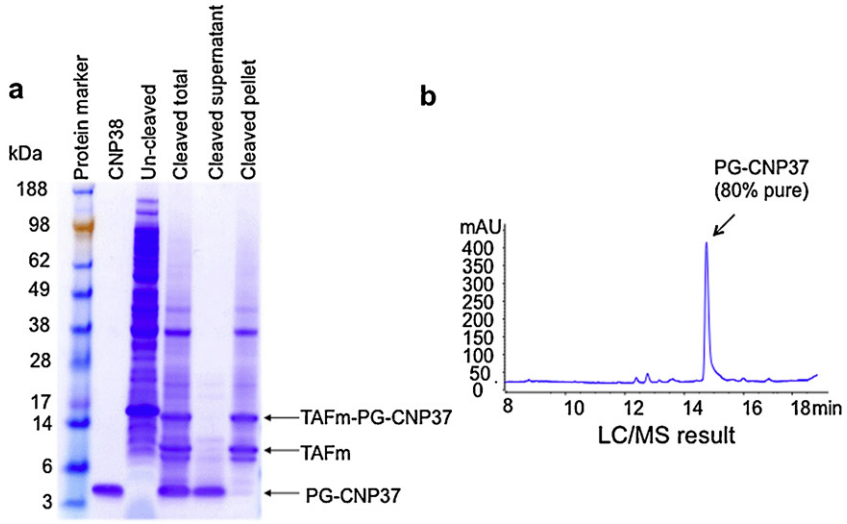

Fig. 3. Formic acid cleavage of TAFm-PG-CNP37 inclusion bodies and pH neutralization. The TAFm-PG-CNP37 inclusion bodies were cleaved with 2% formic acid at 55 °C for 24 h. The formic acid cleavage reaction was neutralized and centrifuged. The supernatant from cleaved TAFm-PG-CNP37 after pH neutralization and centrifugation was assayed by SDS-PAGE (reduced condition) (a) and LC/MS (b).

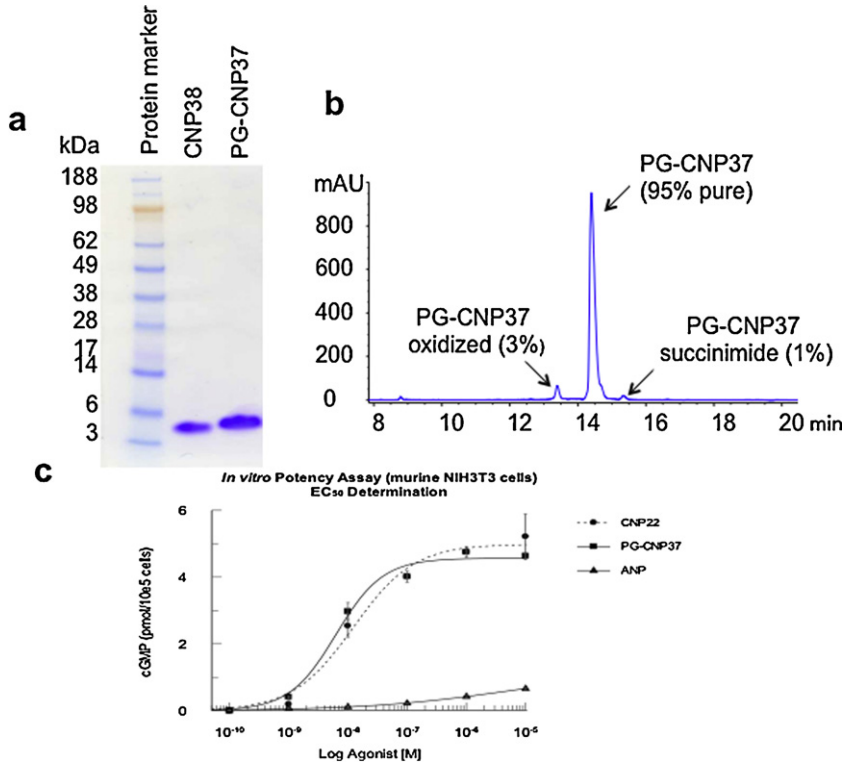

Fig. 4. Characterization of purified PG-CNP37 peptide. The purified PG-CNP37 peptide was characterized by SDS-PAGE/Coomassie blue stain (unreduced condition) (a), LC/MS (b) and in vitro potency assay [cGMP stimulation assay in murine NIH3T3 fibroblasts (c)].

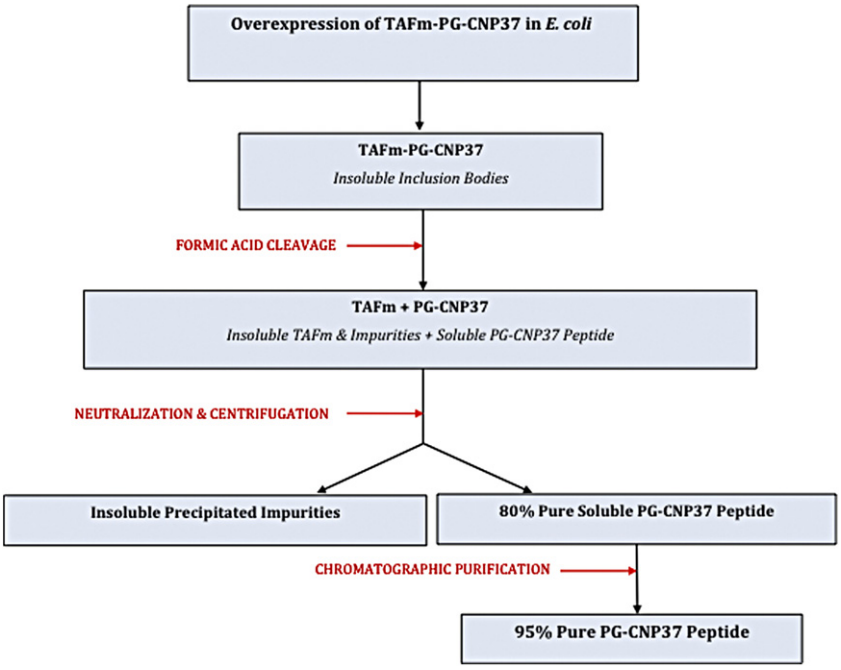

Fig. 5. Overall production strategy for PG-CNP37 peptide. Using *E. coli*, PG-CNP37 peptide is expressed with an insoluble fusion partner, mutated histone fold domain of the human transcription factor TAF12 (TAFm), as inclusion bodies followed by formic acid cleavage to release the soluble PG-CNP37 peptide. Initial purification is accomplished by a simple neutralization and centrifugation step. Final chromatographic purification results in PG-CNP37 peptide with high purity and activity.

80% pure PG-CNP37 peptide. Purification by standard ion-exchange chromatography results in a final PG-CNP37 peptide with 95% purity and less than 0.5% non-product related impurities (Fig. 5). This method to generate PG-CNP37 titers of approximately 0.5 g/L that are 95% pure represents a novel means of producing large quantities of a desired peptide.

With respect to PG-CNP37 production, this novel method offers several advantages to other methods. Chemical synthesis is the most common method to produce peptides. But large-scale chemical synthesis of large peptides (>30 amino acids) is difficult and associated with higher impurity profiles and high cost (Andersson et al., 2000). The CNP analogues were chemically synthesized by a contract company. The yields of CNP analogues were extremely low, which made the production cost really high. Additionally, the impurity profiles were high with lot-to-lot variation.

Another approach for the large-scale production of large peptides is to overexpress the target peptide with fusion tags in *E. coli*. The fusion tags can then be enzymatically or chemically removed to yield the peptide of interest. However, enzymatic cleavage is more costly and is often inefficient or unspecific (Li et al., 2006). The only published method we found to produce NT-proCNP peptide was one in which NT-proCNP was expressed in *E. coli* as fusion protein with thioredoxin followed by enterokinase cleavage. The method failed to produce NT-proCNP peptide due to newly identified protease cleavage sites in the NT-proCNP peptide (Liew et al., 2005). One peptide structurally related to CNP, brain natriuretic peptide (BNP), was produced using mini-intein as a fusion partner. The mini-intein mediated peptide-bond hydrolysis was triggered by pH shift. Although this method promises to produce peptides without the use of proteolytic or chemical reagents, the final yield was only 2.4 mg from 1 L of *E. coli* culture (Sun et al., 2005). Another related peptide, atrial natriuretic peptide (ANP), was produced by various fusion protein expression and cleavage methods. The final yields of purified ANP were all in the mg range from 1 L of *E. coli* culture (Ma et al., 1995; Baumanis et al., 1997; Wang et al., 2003). Chemical cleavages are more economical than chemical synthesis and protease cleavage methods, but toxicity (such as with CNBr), unspecific cleavage and unwanted modification could be issues when using chemical cleavage (Crimmins et al., 2005). Using the formic acid cleavage method we developed, all these issues were minimized and 0.5 g/L of 95% pure PG-CNP37 was achieved compared to mg levels (1.7 mg, 2.6 mg, 10 mg and 2 mg) when using published formic acid cleavage methods (Li et al., 2006, 2007a,b; Vidovic et al., 2009; Ramo et al., 2010).

Extracellular production of recombinant proteins using *E. coli* has several advantages over the intracellular production (protection against proteolytic activity, properly folded protein accumulation in the media, easy isolation and purification), but the secretion strategies are limited to pilot-scale studies as the insufficient secretory machinery of prokaryotes is an obstacle to industrial scale-up (Mergulhao et al., 2005; Yoon et al., 2010). PG-CNP37 is a peptide which possesses a single disulfide bond and does not require native state production. The inclusion bodies approach was thus selected here, since not only does it provide the same advantages as secretion systems (such as reducing the proteolysis and simplifying the downstream purification), but it is also often associated with high yield. For CNP-fusion inclusion bodies, the overall yield was approximately 8–10 g/L and making up 20% of the dry cell weight. Additionally, with this method, 80% purity of PG-CNP37 was achieved before chromatography with a simple neutralization and centrifugation step, making it easy and cost-effective for downstream purification at an industrial scale.

Other advantages to this method include the use of simple, well-understood genetics, rapid cell growth, inexpensive culture media, and a fermentation process that can be easily scaled up to produce industrial quantities of peptides. This method may also be applied to the large-scale production of other peptides without an internal Asp–Pro peptide bond.

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