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Synthesis of neurosecretory protein GM composed of 88 amino acid residues by native chemical ligation

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ABSTRACT

Recently, we found a novel neuropeptide and named it neurosecretory protein GM (NPGM). Rat NPGM is composed of 88 amino acid residues and has an amidated C-terminus and an intramolecular disulfide bond. In our preliminary experiment, NPGM production using conventional solid-phase peptide synthesis was impossible due to its length and hydrophobicity. In the present study, NPGM was produced by native chemical ligation and a disulfide bond was formed by potassium ferricyanide.

We recently identified two novel genes, *neurosecretory protein GL* (*NPGL*) and *neurosecretory protein GM* (*NPGM*) in the avian and mammalian hypothalamus.[1] Mature NPGL and NPGM are small secretory proteins composed of 80–88 amino acid (aa) residues and an amidated C-terminus. Furthermore, they have 2 or 3 Cys residues that possibly form an intramolecular disulfide bond. In rat NPGM containing 3 Cys residues, we revealed that an intramolecular disulfide bond is formed between two N-terminal Cys residues based on the structural analysis using mammalian cultured cells (Chinese Hamster Ovary cells).[2] The structures of mature NPGL and NPGM are shown in Figure 1. In chicks, subcutaneous administration of NPGL caused an increase in body weight without affecting food intake.[1] However, the function of NPGM is still unknown due to its synthetic and handling difficulties.

As NPGL and NPGM are long, hydrophobic peptides, it is difficult to synthesize them using conventional solid-phase peptide synthesis (SPPS). After much trial and error, we recently succeeded in synthesizing rat NPGL using microwave-assisted SPPS.[3] However, in the same synthetic method, yield of rat NPGM was extremely low. In addition, we attempted to produce NPGM using CHO cells in the previous study and found that the yield was scarce and the C-terminus of NPGM was not amidated.[2]

Native chemical ligation (NCL)[4] has significantly improved the synthesis of longer peptides and challenging proteins.[5] In particular, NCL contributed to the synthesis of the 123 aa androgenic gland hormone precursor protein[6], 166 aa erythropoietin[7], and 203 aa HIV-1 protease.[8] With NCL, a peptide containing a thioester at the C-terminus and another peptide with a Cys residue at the N-terminus are ligated by a native peptide bond.[4] When the peptides are mixed under neutral conditions, the reaction is completed in a short time with a high yield.[4]

In the present study, we attempted the synthesis of NPGM using expressed protein ligation which is a semi-synthetic version of NCL.[9,10] Although NPGM has 3 Cys residues, Cys^{24}, Cys^{28}, and Cys^{80}, we set Cys^{28} as the ligation site. We will describe (1) production of the $NPGM^{1-27}$-thioester by Intein-mediated thioesterification, (2) microwave-assisted SPPS of $NPGM^{28-88}$, (3) NCL of the $NPGM^{1-27}$-thioester and $NPGM^{28-88}$, and (4) disulfide bond formation by potassium ferricyanide.

First, $NPGM^{1-27}$ was produced as C-terminal Intein-fusion protein by *Escherichia coli* expression system. We cloned $NPGM^{1-27}$ into the multiple cloning site of the pTXB1 vector (NEB), which also encodes Intein and the Chitin Binding Domain (CBD), for affinity purification (Fig. 2A). Using the expression plasmid, we transformed the *E. coli* BL21 (DE3) strain. *E. coli* that was transformed with the plasmid produces Met-$NPGM^{1-27}$-Intein-CBD, with a molecular mass of approximately 33 kDa (Met-$NPGM^{1-27}$ = 3 kDa, Intein-CBD = 30 kDa). The target protein was expressed in a soluble form (Fig. 2B). The recombinant protein was purified using a Chitin column with an affinity for CBD. Furthermore, the bound protein was reacted with sodium 2-mercaptoethanesulfonate (MESNa) on the column to cleave the Intein-CBD and form thioester at the C-terminus of the cleaved Met-$NPGM^{1-27}$. Although thioester

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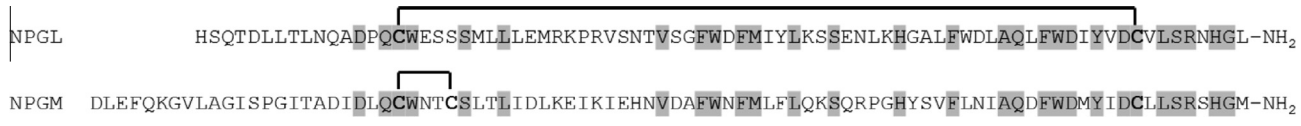

Figure 1. Structures of mature NPGL and NPGM in rat. Cys residues are indicated in bold and intramolecular disulfide bonds are shown by a solid line. Conserved amino acids are highlighted in gray.

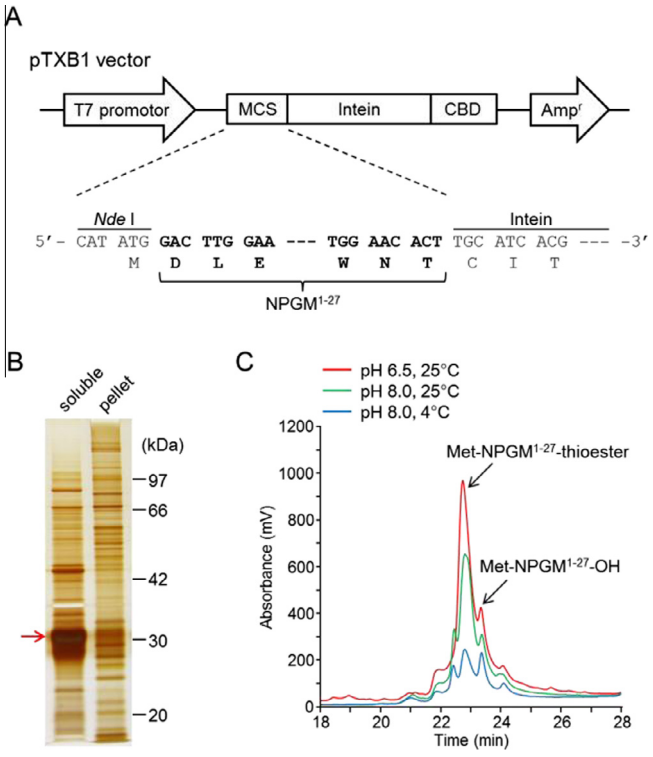

Figure 2. Production of NPGM$^{1-27}$-thioester. (A) Construction of the expression plasmid. (B) Expression of Met-NPGM$^{1-27}$-Intein-CBD. (C) RP-HPLC of Met-NPGM$^{1-27}$-thioester.

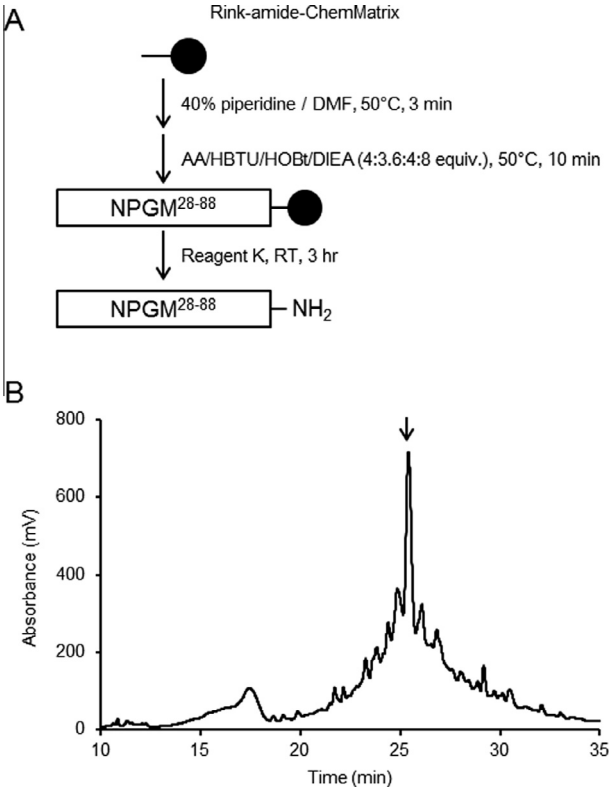

Figure 3. Microwave-assisted SPPS of NPGM$^{28-88}$. (A) Synthetic procedure of NPGM$^{28-88}$. (B) RP-HPLC of synthetic products.

formation is accelerated at a higher pH (>pH 8), the formed thioester is more stable under lower pH conditions (<pH 7).[11] To optimize the reaction rate and stability, thioester formation was carried out in a buffer containing 50 mM MESNa under three conditions: pH 8.0 and at 25 °C; pH 6.5 and at 25 °C; and pH 8.0 and at 4 °C. After overnight reaction, each elution was purified using reverse-phase high performance liquid chromatography (RP-HPLC) (Fig. 2C). As a result, the most suitable condition was pH 6.5 and temperature 25 °C, and the yield of Met-NPGM$^{1-27}$-thioester was 2.7 mg/L. Although we prepared peptide thioester by *E. coli* expression system in the present study, the yield may be increased by Fmoc chemistry using the sulfamylbutyryl resin.[12,13]

Second, NPGM$^{28-88}$ was synthesized by microwave-assisted SPPS using an automatic peptide synthesizer Syro *Wave* (Biotage). The synthetic procedure is shown in Figure 3A. ChemMatrix is a polyethylene glycol resin that is efficient for the synthesis of hydrophobic and highly structured peptides due to its amphiphilic and highly cross-linked matrix, which suppresses the aggregation of the synthetic products.[14,15] Rink Amide ChemMatrix resin was used to obtain the C-terminal amidated peptide. Fmoc deprotections were performed using 40% piperidine/DMF at 50 °C for 3 min. Amino acid couplings were performed using HBTU and HOBt at 50 °C for 10 min. The synthetic products were cleaved from the resin using the reagent K.[16] and purified using RP-HPLC (Fig. 3B). The yield was 2% in 50 µmol of synthetic scale. The yield could easily be increased by replacing HBTU with HATU.

Third, full length NPGM was produced by NCL of Met-NPGM$^{1-27}$-thioester and NPGM$^{28-88}$. Although NCL reaction progress just under the neutral condition, thiol is generally added as a catalyst. In particular, 4-mercaptophenylacetic acid (MPAA) is a highly effective catalyst for NCL, highly water soluble, and non-malodorous.[17] The reaction was performed in a buffer solution containing 8 M guanidine–HCl, 0.1 M Na_2HPO_4 at pH 7.0, 0.1 M MPAA, and 20 mM *tris*(2-carboxyethyl)phosphine. NCL is generally performed under conditions that include the peptide thioester and excess Cys-peptide. Although we attempted to dissolve NPGM$^{1-27}$-thioester and NPGM$^{28-88}$ at a concentration of 1 and 3 mM, respectively, the solubility was insufficient. Thus, each fragment was dissolved at a final concentration of 1 mM. The mixture was then nitrogen-substituted. The ligation reaction rate depends on an amino acid residue at the Xaa-Cys ligation site.[18] In the present case, Xaa is the Thr residue and the reaction rate was generally slow. In actuality, the reaction was completed within 20 h at 25 °C (Fig. 4). From the HPLC chromatogram, the reaction was completed with a high yield. However, the collection rate calculated from the weight of purified peptide was 30%. In the synthesis of 124-aa human secretory phospholipase A2 by NCL, the yield from the ligation reaction was 80–90% by HPLC, although there was an additional loss of 50% from the HPLC purification step.[18] The low yield of NPGM may be also caused by non-specific adsorption in the purification step due to its hydrophobicity.