COMMUNICATION

Synthesis of a novel peptide, galanin-like peptide (GALP), by a combination of recombinant DNA technology and chemical cleavage reactions

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Galanin-like peptide, a newly isolated peptide ligand for the GALR2 receptor, was synthesized by a combination of recombinant DNA technology and chemical cleavage reactions.

GALP is a novel galanin-like peptide that has been isolated from porcine hypothalamus in our research division.[1] The cDNA of GALP encoded the precursor proteins with 115 amino acid residues in rats, 116 residues in humans, and 120 residues in pigs. GALP, with 60 amino acid residues (Fig. 1), is produced as a mature peptide from these precursor proteins. The amino acid sequence of GALP-(9–21) is identical to that of galanin-(1–13).[2] Whereas galanin has a high affinity for both GALR1 and GALR2 receptors, GALP has a high affinity for GALR2 receptor and a lower affinity for GALR1 receptor. The physiological significance of GALP is not yet known, and the study to elucidate the physiological function of GALP has only just begun. Tests on the function and role of GALP *in vivo* need a large amount of peptides. Production processes with high yields should be established to advance the research on GALP. Previously we have reported a novel process for the production of a recombinant peptide which involves the expression of a fusion protein using CS23, a basic fibroblast growth factor mutein[3] as a fusion partner,[4,5] and chemical cleavage of the fusion protein after cyanylation. In the previous studies, we obtained insulinotropin[4] and prolactin-releasing peptides[5] using this process. CS23 is a superior fusion partner as it has heparin affinity that enables the fusion protein to be purified easily and efficiently. We prepared rat and porcine GALPs using this technology with some improvements.

Scheme 1 shows the strategy for the preparation of rat GALP (rGALP). We constructed an rGALP–CS23 expression vector in which rGALP was fused at the amino-terminal of CS23 with cysteine as a linker, as reported previously. The expression plasmid was transfected into *Escherichia coli* MM294(DE3) to obtain the fusion protein expressing cells, *E. coli* MM294(DE3)/pTFRGAL. The expression of the fusion protein was induced with isopropyl-β-D-thiogalactopyranoside (IPTG). Next, the cells were collected by centrifugation and disrupted by sonication. The fusion protein was obtained as inclusion bodies. Since the N-terminal amino acid analysis shows that about 60% of the fusion protein expressed in *E. coli* has an additional methionine at the amino-terminus, the preparation of rGALP was performed in two steps. The first step was the cleavage of rGALP and methionylated rGALP (Met-rGALP) from the fusion protein, and the second step was the specific removal of the additional methionine at the amino-terminus. Although we reported an efficient method for removing the additional methionine at the amino-terminus of recombinant proteins,[6,7] we could not apply the method in this case because rGALP and Met-rGALP could not be separated

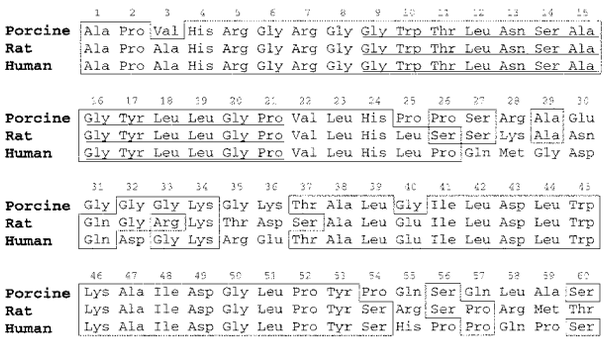

Fig. 1 Amino acid sequences of porcine, rat, and human GALP. Underlined sequences are identical to that of galanin-(1–13).

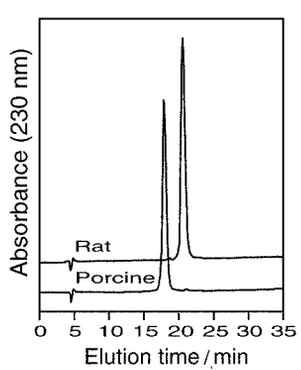

Fig. 2 HPLC profiles of the purified pGALP and rGALP. Purified pGALP and rGALP were analyzed by RP-HPLC using a C4P-50 column.

efficiently by chromatography. We removed the methionine with aminopeptidase as we previously reported in our description of the production of recombinant human interleukin 2.[8]

After the SH groups of the fusion protein were converted to SCN groups with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (DMAP-CN), rGALP and Met-rGALP were cleaved by treatment of the modified fusion protein with alkaline pH. The mixture of rat GALP and Met-rGALP were purified by cation-exchange chromatography on an SP-Toyopearl 650M column, followed by reversed-phase high-performance liquid chromatography (RP-HPLC) with a C4P-50 column. Next, the additional methionine at the amino-terminus of the purified Met-rGALP was removed with *Streptomyces griseus* aminopeptidase,[9] and the rGALP was purified by RP-HPLC on a C4P-50 column. Purified rGALP was shown to be of high purity from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and RP-HPLC (Fig. 2). The results of

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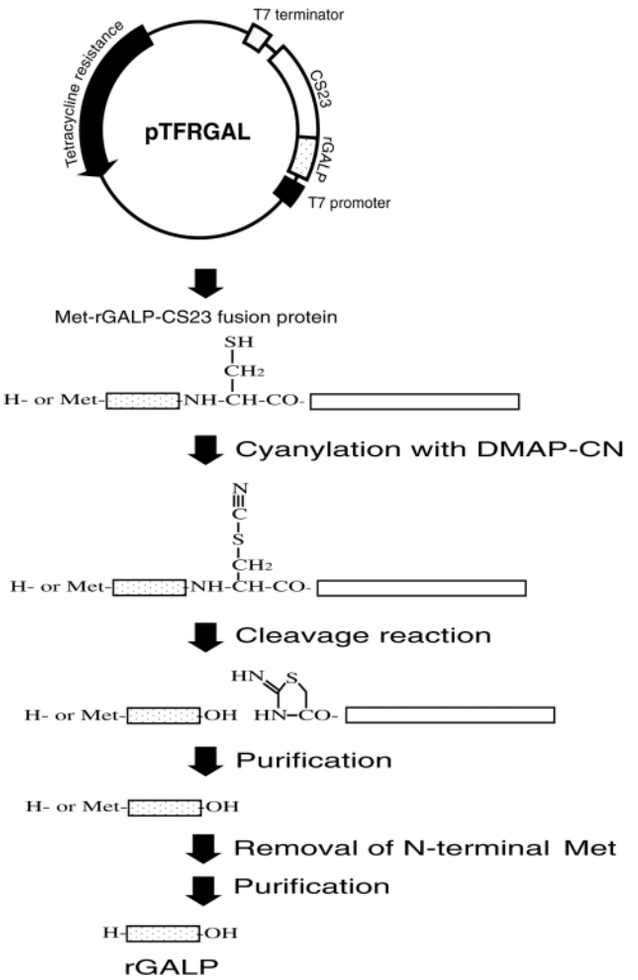


Scheme 1 The strategy for preparation of rat GALP.

amino acid composition, N-terminal amino acid sequence, and C-terminal amino acid analyses agreed with theoretical values.

To avoid the additional methionine at the N-terminus, as observed in the preparation of rGALP, we selected another method in the case of porcine GALP (pGALP). The lack of a methionine residue within pGALP made it possible to cleave pGALP from fusion proteins with cyanogen bromide (BrCN).[10] We fused pGALP at the carboxy-terminal of CS23 with methionine as a linker, CS23-pGALP, as shown in Scheme 2. Porcine GALP was cleaved from CS23-pGALP with BrCN and purified in the same manner as rGALP-CS23. Purified pGALP was shown to be of high purity from SDS-PAGE and RP-HPLC (Fig. 2), and the analytical data of amino acid composition, N-terminal amino acid sequence and C-terminal amino acid analyses, and molecular weight measurement by liquid secondary ion mass spectrometry agreed with theoretical values (MH^+, *m*/*z* observed: 6205.1 *vs*. theoretical: 6205.1). The biological activity of purified pGALP was determined using receptor binding assay with Chinese hamster ovary (CHO) cells expressing GALR2 or GALR1 receptors,[1] respectively. The purified GALP showed the same affinity as chemically synthesized pGALP.[1]

Although we obtained pure pGALP preparation from CS23-pGALP fusion protein, the purification efficiency after BrCN cleavage was not as good, due to the presence of fragments from CS23 caused by the BrCN cleavage. CS23 itself has two methionine residues at positions 76 and 142. To improve the purification efficiency after cleavage, leucine was substituted for Met^{76} by site-directed mutagenesis to obtain CS23ML76. As the substitution of Met^{76} by leucine did not alter the affinity to heparin, CS23ML76 can also be purified by heparin affinity chromatography. Porcine GALP was fused at the carboxy-terminal of CS23ML76 with methionine as a linker in the same manner as described for CS23. The purification efficiency after cleavage of CS23ML76-pGALP by BrCN was greatly improved, as shown in Fig. 3. In the process of recombinant peptide synthesis using BrCN cleavage reaction, CS23ML76 is more appropriate than CS23.

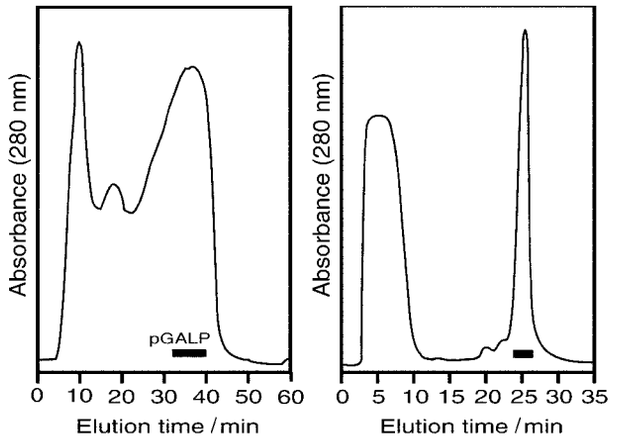
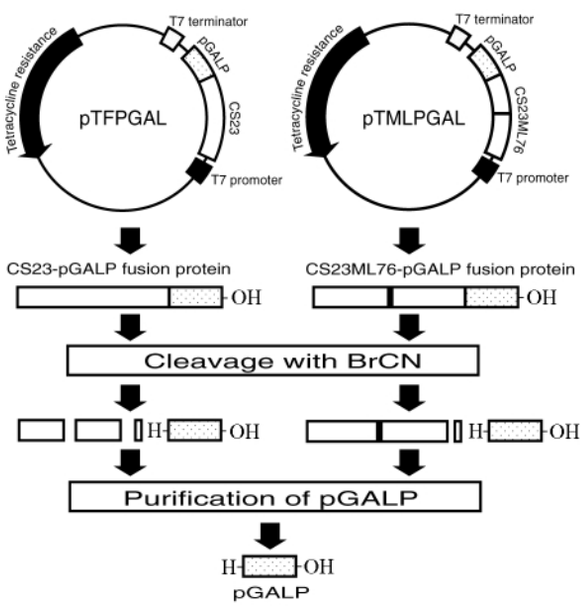


Fig. 3 Purification of pGALP after cleavage by BrCN. IE-HPLC profiles of pGALP cleaved from pGALP-CS23 (A) and pGALP-CS23ML76 (B), respectively.



Scheme 2 The strategy for preparation of porcine GALP.

We successfully obtained rat and porcine GALPs in large quantities and with high degrees of purity by a combination of recombinant DNA technology and chemical reaction. Human GALP that has a methionine residue in the molecule could also be prepared by cysteine specific cleavage of human GALP-CS23 fusion protein in the same manner as described for that of rGALP. The process for peptide preparation using the bFGF muteins CS23 and CS23ML76 as a fusion partner is confirmed as being useful for peptide preparation in large amounts. The use of this process should advance the research on the functions and roles of novel peptides.

Experimental

Construction of expression plasmids

All of the expression plasmids were constructed using pTF plasmid, a derivative of pTB960-7.[4] Rat GALP and porcine GALP were inserted into the amino- and carboxy-terminal of CS23, respectively. Methionine at position 76 in CS23 was converted into leucine using QuickChange™ Site-Directed Mutagenesis Kit (Stratagene).

Cleavage of rGALP from rGALP-CS23 fusion protein

Rat GALP-CS23 and methionylated rGALP-CS23 were *S*-cyanylated in 0.1 M acetic acid containing 8 M urea and 2.4 mM DMAP-CN for 15 min at room temperature. After dialysis against 0.05% acetic acid, cleavage of the modified fusion protein was accomplished in 0.06 M NaOH containing 6 M urea for 15 min at 0 °C.

Removal of an additional methionine at the amino-terminus of rGALP

Twenty milligrams of the mixture of rGALP and methionylated rGALP was digested with 50 mg of aminopeptidase SG (Takara) in 50 mM Tris-HCl (pH 7.5) containing 3 M guanidine hydrochloride for 24 h at room temperature. Rat GALP was purified on a C4P-50 column (4.6 mm id × 25 cm, Asahipak).

Purification of CS23-pGALP fusion protein

Inclusion bodies were extracted with 0.2 M Tris-HCl (pH 8) containing 7 M guanidine hydrochloride and centrifuged. Next, CS23-pGALP was refolded by diluting it with 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 0.1 M arginine hydrochloride, 4 M urea, 0.5 mM glutathione, oxidized form, and 1 mM glutathione, reduced form. After dialysis against 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 0.1 M arginine hydrochloride and 4 M urea, CS23-pGALP was purified by affinity chromatography on a heparin-5PW column (7.5 mm id × 7.5 cm, Tosoh).

Cleavage of CS-23-pGALP and purification of pGALP

Purified CS23-pGALP was dialyzed against distilled water, and the resulting precipitate was dissolved in 70% formic acid. After the addition of a 5-fold molar concentration of methionine, the reaction mixture was incubated at 4 °C for 8–16 h. The reaction mixture was then dialyzed against 50 mM Tris-HCl (pH 7.5) followed by chromatography on a TSKgel CM-5PW column (7.5 mm id × 7.5 cm, Tosoh). Porcine GALP was further purified on a C4P-50 column (4.6 mm id × 25 cm, Asahipak).

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