An alternative approach to the synthesis of a recombinant galanin-like peptide (GALP)[1]

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GALP is a novel galanin-like peptide that has been isolated from porcine hypothalamus in our Research Division. In the present study, a large quantity of porcine GALP (pGALP) was synthesized with a high degree of purity using recombinant DNA technology. A basic fibroblast growth factor (bFGF) mutein, CS23, was used as a fusion partner and pGALP was released by chemical cleavage with cyanogen bromide (BrCN). Using various criteria such as HPLC, SDS–PAGE, amino acid analysis, amino acid sequence, relative-molecular-mass measurement by liquid secondary-ion mass spectrometry, as well as biological activity, the purified molecule was shown to be pGALP and was found to have the same biological activity as a chemically synthesized standard. The pGALP obtained here will be useful for elucidation of its physiological role *in vivo*. Furthermore, CS23ML76, a newly prepared bFGF mutein, was found to be more appropriate than CS23 as a fusion partner in this procedure.

Introduction

GALP[2] was recently isolated from porcine hypothalamus, as an endogenous ligand for the orphan receptor, GALR2,[3,4] in our Research Division. The cDNA structure of this peptide, and of the corresponding human and rat peptides (pGALP, hGALP and rGALP), indicates that a 60-amino acid mature peptide (Fig. 1) is produced from the respective preproprotein. The amino acid sequence of GALP-(9–21) is identical to that of galanin-(1–13).[5] Whereas galanin has a high affinity for both GALR1 and GALR2, GALP has a high affinity for GALR2 but a low affinity for GALR1.[2] The physiological significance of GALP is not yet known and study of its physiological function has only just begun. Recently, the distribution of the GALP neuron and GALP-containing fibers in the rat hypothalamus was determined by immunohistochemical studies and *in situ* hybridization.[6]

The GALP neurons are located specifically in the Arc (arcuate hypothalamic nucleus) and co-express leptin receptors. Therefore, GALP neurons may be directly regulated by leptin and participate in the regulation of feeding behavior and/or reproductive functions. To further investigate the function and role of GALP *in vivo*, we needed to prepare a large quantity of it.

Previously we described a novel system[7] for the preparation of recombinant orphan ligand peptides, apelin,[8] prolactin-releasing peptides[9] and GALPs.[10] This system utilizes CS23, a bFGF mutein,[11] as a fusion partner, and site-specific cleavage using a cyanylation reaction[12] with specific removal of the unwanted initiator methionine residue at the N-terminus using a transamination and scission reaction sequence.[13,14] Since N-terminal amino acid analysis showed that about 60% of the rGALP–CS23 fusion protein expressed in *E. coli* cells had the N-terminal methionine, rGALP was prepared in two steps as follows. The first step was the cleavage of rGALP and

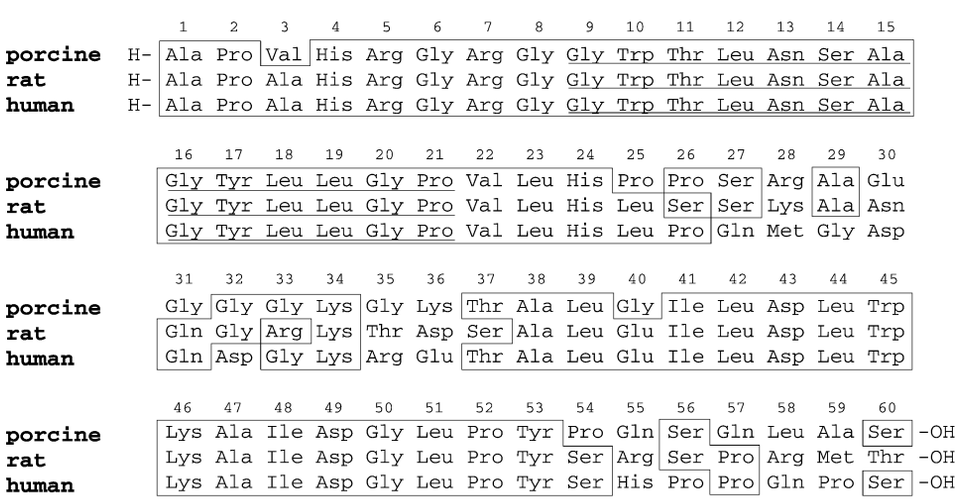


Fig. 1 Amino acid sequences of porcine, rat and human GALP. Underlined sequences are completely identical to those of galanin-(1–13).

methionylated rGALP (Met-rGALP) from the fusion protein, and the second step was the specific removal of the N-terminal methionine. Although the above system consists of an efficient chemical method for removing the methionine, we could not use this approach with rGALP because rGALP–CS23 and Met-rGALP–CS23 were not easily separable by chromatography. Therefore we prepared rGALP and removed the methionine with aminopeptidase as we previously described for the production of recombinant human interleukin 2 and human growth hormone.[15] However, this enzymatic method was too expensive for the large scale preparation needed here.

To prevent heterogeneity at the N-terminus of the expressed pGALP fusion protein, we aimed for improvement in the preparation of peptides. The lack of a methionine residue within pGALP (Fig. 1) made it possible to cleave pGALP from the fusion protein in a one-step chemical cleavage reaction with BrCN (Scheme 1).[16,17] In this study we describe such a novel and useful procedure for the efficient, large-scale preparation of recombinant pGALP. Furthermore, the CS23ML76 improved mutein of CS23 as a fusion partner is presented.

Experimental

Construction of expression plasmids

All expression plasmids were constructed using plasmid pTF, a derivative of pTB960-7 described previously.[12] The cDNA fragment of pGALP was prepared by annealing and ligation of synthetic oligonucleotides, and was then subsequently ligated to pTF to obtain the pGALP–CS23 expression vector, pTFPGAL. The methionine codon at position 76 of CS23 in pTFPGAL was converted into a leucine codon using the QuickChange™ Site-Directed Mutagenesis kit (Stratagene) to obtain the pGALP–CS23ML76 expression vector, pTMLPGAL. The DNA sequences of the constructed plasmids were verified using an Applied Biosystems 377 sequencer.

Expression of fusion proteins

E. coli MM294 (DE3)[18] was used as the host strain. It carries the T7 RNA polymerase gene under control of the *lac* UV5 promoter in its chromosome and can transcribe genes from a T7 promoter in the presence of IPTG.[12,19] For large-scale preparation, transformants were cultured at 37 °C in a 50 L fermenter containing 20 L of modified M9 medium[19] with an agitation rate of 200 rpm. After growth for 6 hours, IPTG (50 μM) was added and incubation was continued for an additional 4–5 hours. The cells were collected by centrifugation and stored at −80 °C until use.

Purification of fusion proteins

Frozen cells (200 g wet weight) were sonicated in 600 mL of 50 mM phosphate (Na_2HPO_4–KH_2PO_4) buffer (pH 7.0) containing 150 mM NaCl, 0.1 mM EDTA and 0.1 mM *p*-amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF). After centrifugation, the fusion proteins were present in high purity as an insoluble pellet. Further purification was not necessary before cleavage of the fusion proteins.

Purification of pGALP

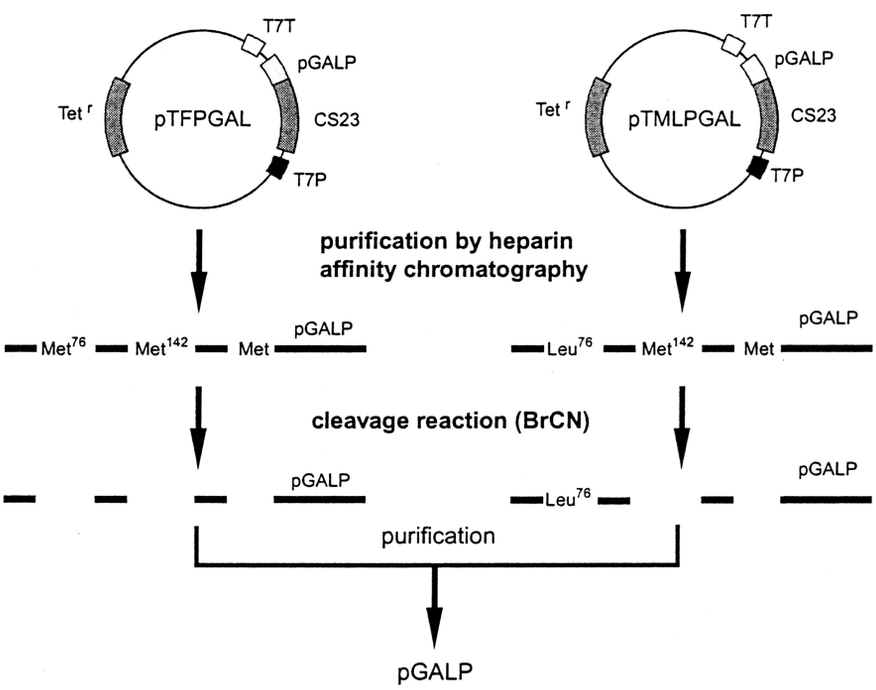
The pellet containing CS23–pGALP was dissolved in 70% aq. formic acid. After the addition of cyanogen bromide (BrCN, Sigma, MO, USA) with a 5-fold molar excess of methionine, the reaction mixture was incubated at 4 °C for 8–16 hours. This mixture was dialyzed against 50 mM Tris-HCl (pH 7.5), then applied to a TSK gel CM-5PW column (7.5 mm × 7.5 cm, Tosoh Corporation, Tokyo, Japan) equilibrated with 50 mM sodium acetate (pH 6.0) and the peptide was eluted with a linear gradient of NaCl (0–0.5 M). The eluate was applied to a C4P-50 column (4.6 mm × 25 cm, Asahipak, Tokyo, Japan) equilibrated with 0.1% TFA, and pGALP was eluted with a linear gradient of acetonitrile (36%) and lyophilized (70 mg). Preparation of pGALP from CS23ML76–pGALP was performed using the same procedure.

Biological assay of pGALP

The biological activity of pGALP was determined using a receptor-binding assay with both CHO cells expressing rat GALR1 and rat GALR2.[2]

Amino acid analysis

The amino acid compositions of the peptides were determined following hydrolysis with 6 M HCl and 4% thioglycolic acid at



Scheme 1 Strategy for the preparation of porcine GALP.

110 °C for 24 and 48 hours using a Beckman model 6300E amino acid analyzer. The carboxy-terminal amino acid of the peptides was cleaved by hydrazinolysis[20] and analyzed on the amino acid analyzer. The amino-terminal sequence was determined by the gas-phase protein sequencer (Model 477A, Applied Biosystems, Inc., Foster City, CA, USA).

Relative-molecular-mass measurement

Relative-molecular-mass measurements were made on a matrix-assisted laser desorption ionization mass spectrometer (Kompact MALD III, Kratas).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis

SDS–PAGE was performed according to the method of Laemmli[21] using a Multigel 15/25 (Daiichi Kagaku Co. Ltd., Tokyo, Japan) under reducing and non-reducing conditions. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250. To check the partial N-terminal amino acid sequences of the fusion proteins, the proteins after SDS–PAGE were electroblotted onto a ProBlott membrane (Applied Biosystems, Inc., Foster City, CA). After the proteins were stained with Coomassie Brilliant Blue R-250, the bands corresponding to the fusion proteins were eluted and sequenced with the gas-phase protein sequencer described above.

Results and discussion

We recently described a novel system for the production of rGALP in large quantity and with a high degree of purity.[7,10] hGALP could also be prepared by this system. Although rat and human GALPs have a methionine in the molecule, the lack of a methionine residue within pGALP made it possible to develop an alternative strategy for the production of pGALP without the problem of heterogeneity at the N-terminal methionine. This approach relies on cleavage of pGALP from the fusion protein with BrCN.

Scheme 1 shows the strategy for the preparation of pGALP. We constructed a CS23–pGALP expression vector in which the pGALP gene was fused to the 3′ end of the CS23 gene with a methionine codon as a linker. A transformant of *E. coli* MM294 (DE3)[17] with pTFPGAL was cultured and the cells were collected by centrifugation. SDS–PAGE analysis showed that the fusion protein was the predominant product in the total cell lysate and existed as an insoluble form in *E. coli* (data not shown). pGALP was then cleaved from the CS23–pGALP fusion after solubilization in formate by a one-step chemical cleavage reaction with BrCN and purified by chromatography on CM-5PW and C4P-50.

Purified pGALP was shown to be of high purity by SDS–PAGE (Fig. 2A) and HPLC (Fig. 2B). To confirm the structural identity of the purified pGALP, protein chemical analysis was performed. The N-terminal amino acid sequence, amino acid composition analysis, the C-terminal amino acid (data not shown), and relative-molecular-mass measurement by liquid secondary-ion mass spectrometry (MH^+, *m/z* observed: 6205.1 Da *vs.* theoretical: 6205.19 Da) were all in good agreement with those predicted from the corresponding cDNA sequence. The biological activity of purified pGALP was determined using a receptor-binding assay[2] with CHO cells expressing GALR2 or GALR1 receptors, respectively. The purified pGALP showed the same affinity as chemically synthesized pGALP (Fig. 3).

Although the preparation of pure pGALP was achieved from the CS23–pGALP fusion protein, the purification efficiency after BrCN cleavage was poor due to the presence of fragments from CS23 after BrCN cleavage, as judged by SDS–PAGE (Fig. 4A) and CM-5PW HPLC (Fig. 5A). CS23 itself has two methionine residues, at positions 76 and 142. To improve the purification efficiency after cleavage (Scheme 1), leucine was

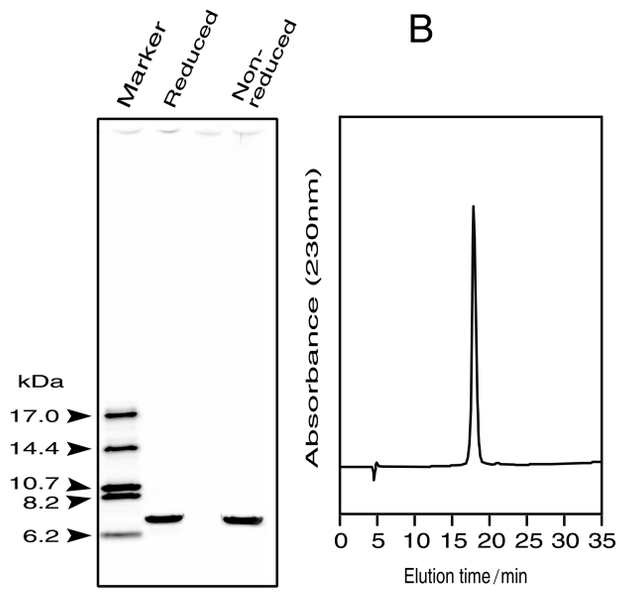


Fig. 2 Analyses of purified pGALP. (A) SDS–PAGE analysis. SDS–PAGE was carried out with Multigel 15/25 under reducing and non-reducing conditions. (B) HPLC profiles of the purified pGALP. Purified pGALP was analyzed by RP-HPLC using a C4P-50 column.

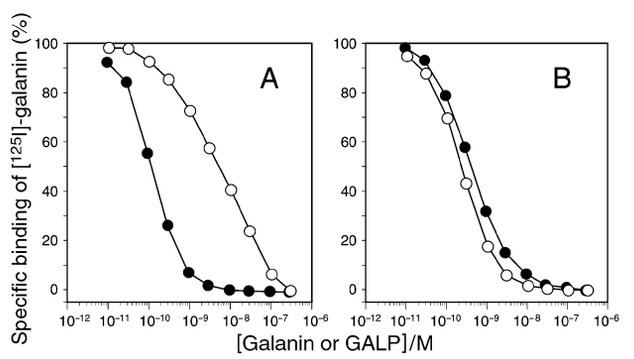


Fig. 3 Biological activity of purified pGALP. ^{125}I-Galanin-binding assay with CHO cells expressing GALR1 (A) or GALR2 (B). ○; the purified pGALP, ●; synthetic rat galanin.

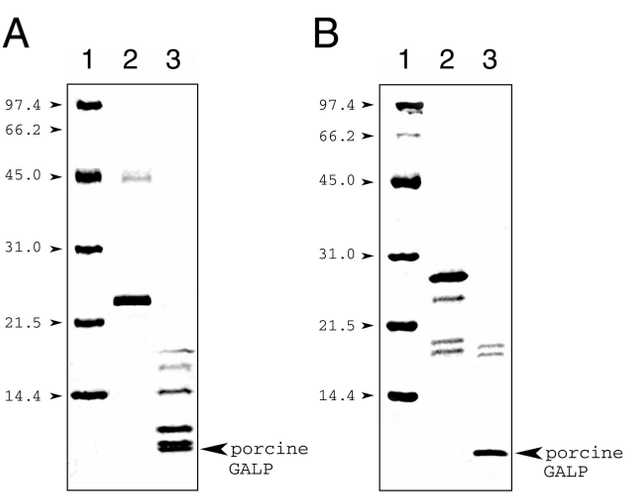


Fig. 4 SDS–PAGE analyses of the fragments from the fusion partners caused by BrCN cleavage. (A) Analysis of the fragments from CS23–pGALP under reducing conditions. Lane 1; MW marker, Lane 2; fragments before BrCN cleavage, Lane 3; fragments after BrCN cleavage. (B) Analysis of the fragments from CS23ML76–pGALP under reducing conditions. Lane 1; MW marker, Lane 2; fragments before BrCN cleavage, Lane 3; fragments after BrCN cleavage.

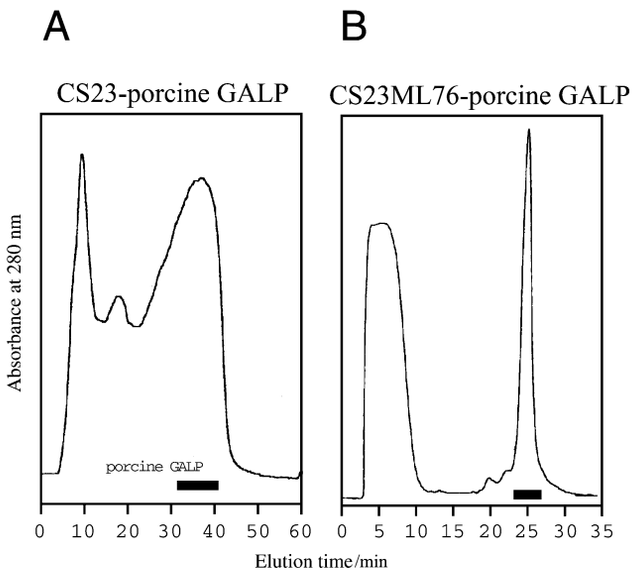


Fig. 5 Cation-exchange chromatography profiles of the fragments from the fusion partners caused by BrCN cleavage. (A) Profile of the fragments from CS23–pGALP. (B) Profile of the fragments from CS23ML76–pGALP. The fractions denoted by the bar correspond to pGALP.

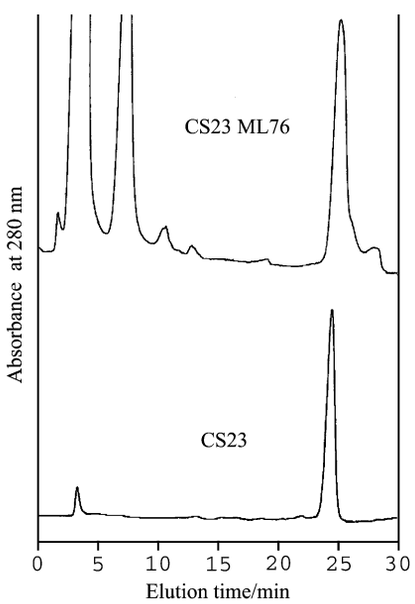


Fig. 6 Heparin-affinity of CS23ML76. CS23 and CS23ML76 were applied to a TSK gel Heparin-5PW column (7.5 cm × 75 mm) equilibrated with 20 mM Tris-HCl containing 1 mM EDTA and were eluted with a linear gradient of NaCl (0–2 M). CS23ML76 has the same affinity to heparin as does CS23.

substituted for Met^{76} in CS23 by site-directed mutagenesis to obtain CS23ML76. This mutation did not alter the affinity to heparin (Fig. 6), whereas substitution to alanine or valine eliminated the affinity (data not shown). Therefore CS23ML76 can also be purified by heparin affinity chromatography. CS23ML76–pGALP was prepared in the same manner as described above for CS23–pGALP. The purification efficiency after cleavage of CS23ML76–pGALP by BrCN was greatly improved as shown in Fig. 4B and Fig. 5B. For the purification of recombinant peptide using BrCN cleavage, CS23ML76 is more appropriate than CS23 as a fusion partner.

In conclusion, we have successfully prepared recombinant pGALP on a large scale by this novel procedure, which consists of a combination of recombinant DNA technology and specific chemical cleavage using BrCN. The key advantages of this procedure are as follows. First, introduction of the methionine residue at the junction of the fusion protein gave a reliable site-specific cleavage in high yield after chemical cleavage with BrCN. Moreover, this reaction successfully removes the methionine residue at the N-terminus of the recombinant peptide. Second, CS23ML76, a newly prepared bFGF mutein, was shown to be useful as a fusion partner. The data presented here show that gene-fusions using a bFGF mutein as a fusion partner and specific cleavage using BrCN provide a novel and useful procedure for the preparation of recombinant biologically active peptides.

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References

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