A novel system for the preparation of orphan receptor ligand peptides[1]

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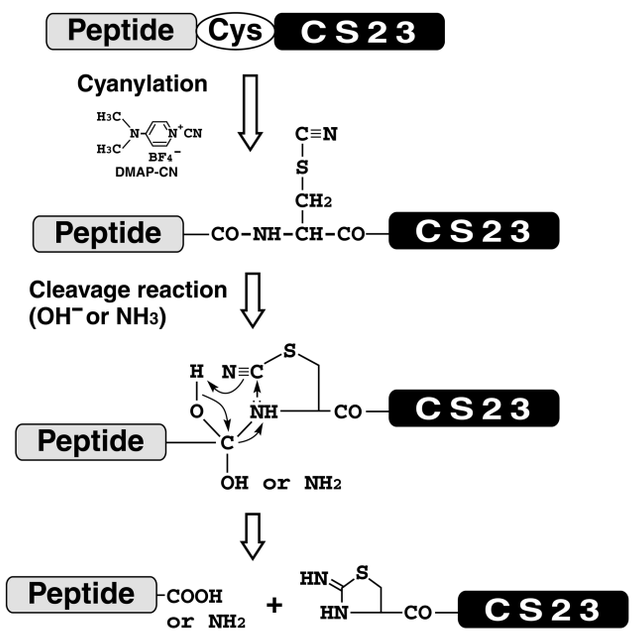
A new system is established for the preparation of biologically active recombinant peptides. The system consists of two newly developed chemical methods in combination with recombinant DNA technology. One method is a cleavage reaction by cyanylation from a recombinant fusion protein. The other is a scission reaction of an N-terminal extra-methionine by transamination from a recombinant peptide. This system is successfully established for the synthesis of three orphan receptor ligand peptides; apelin, prolactin-releasing peptide, and galanin-like peptide, isolated in our Research Division. The results show that this system is very versatile and might be useful in the preparation of biologically active peptides.

Introduction

Recombinant DNA technology provides an alternative to chemical synthesis in the production of peptides. However, several problems still remain in the production of recombinant polypeptides. One difficulty lies in the production of peptides of small relative molecular mass that are biologically active.[2–6] Another is in the addition of an unwanted methionine residue, corresponding to the initiation codon (ATG), to the N-terminal amino acid.[7] Also, many biologically active peptides have α-amides at their C-termini, which are generally essential for full biological activity.[8–12] However, microorganisms cannot produce this type of peptide because they lack the ability for the post translational modification.

The attempted synthesis of small peptides has often been unsuccessful due to their sensitivity to endogenous proteases, but bacterial gene fusion systems have been used to overcome these protease degradation problems.[2] In order to obtain high yields of the desired product, it is important to design a fusion protein with site-specific cleavage from the fusion partner. Moreover, fusion partners are available that aid in the purification of heterologous proteins from *Escherichia coli* (*E. coli*). Recombinant proteins produced in *E. coli* often possess an additional methionine at the N-terminus since the removal of the methionine residue is dependent on the specificity of the *E. coli* methionine aminopeptidase.[7] The extra methionine may affect the biological activity and antigenicity of the protein[13] if it is used for therapeutic purposes, so it is necessary to remove the residue as completely as possible.

We previously reported two efficient methods for obtaining recombinant peptides and proteins: a cysteine site-specific cleavage reaction of the fusion protein (Scheme 1);[14] and removal of an additional N-terminal methionine residue after transamination (Scheme 2).[15,16] In the first method peptides are expressed in the form of fusion proteins with basic fibroblast growth factor mutein (CS23) as a fusion partner, which is easily purified by heparin affinity chromatography.[17] The SH groups of the denatured fusion proteins are then converted to SCN groups with 1-cyano-4-(dimethylamino)pyridinium tetrafluoroborate (DMAP-CN), and subsequent exposure to alkaline conditions results in cleavage at the amino group of the modified cysteine residue at the junction site, to yield the desired peptides.[14] Interestingly enough, this method can yield the C-terminal α-amide by treatment of the cyanylated protein with ammonia,[18] while the C-terminal α-acid can be obtained by treatment with sodium or potassium hydroxide. Cyanogen bromide is generally used as a site-specific cleavage reagent of the fusion proteins.[2,4,6] However, this reagent cannot be applied to the preparation of methionine-containing peptides and cannot yield the C-terminal α-amide essential for the activity in many peptides.



Scheme 1 Schematic representation of the cyanylation and cleavage reactions.

In the second method the additional methionine residue of the recombinant protein is converted into an oxoacyl form with glyoxylic acid, copper sulfate and pyridine, then cleaved from the rest of the protein with 3,4-diaminobenzoic acid in 1 M acetic acid and 2 M sodium formate to obtain the non-methionylated recombinant protein.[15,16]

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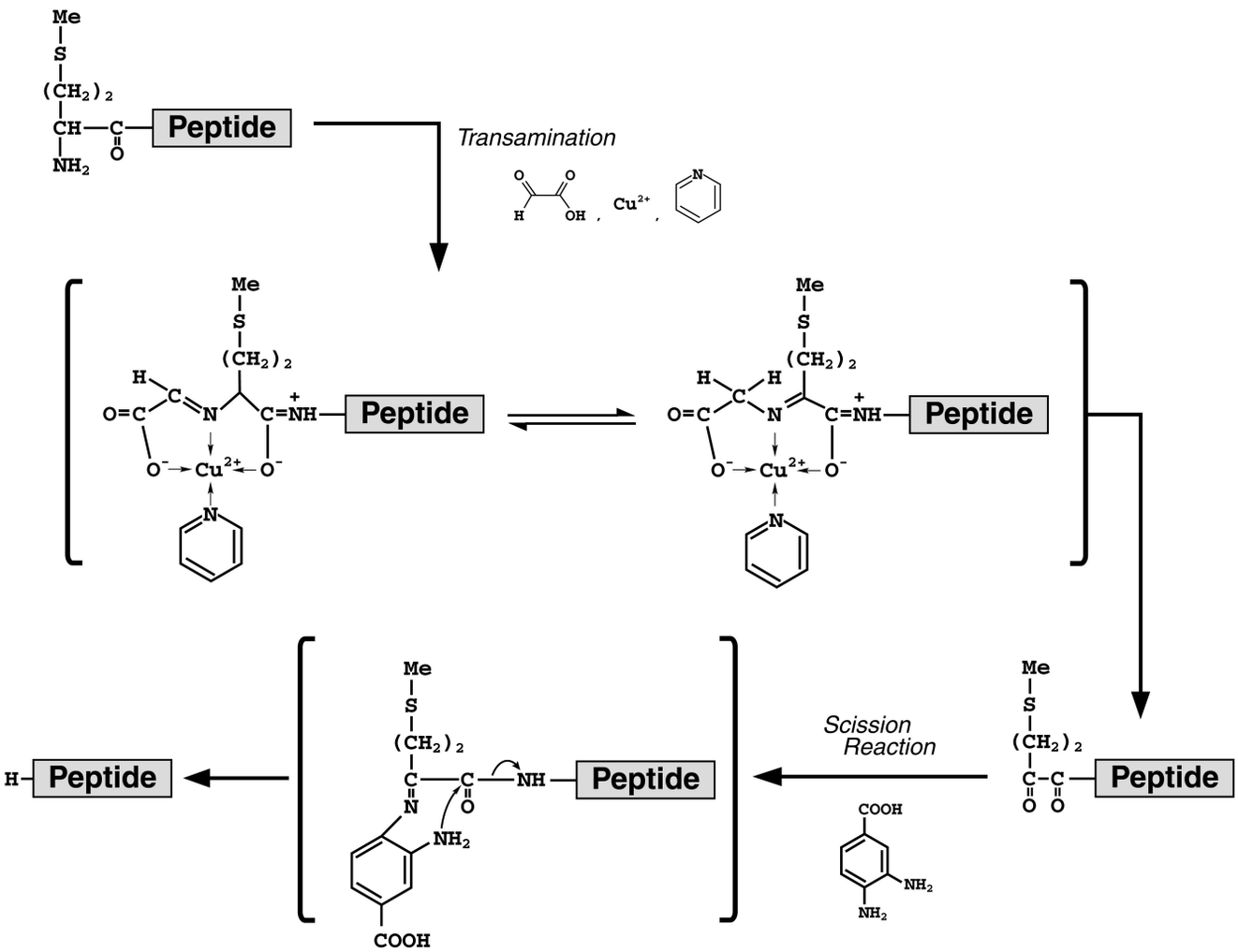


Scheme 2 Schematic representation of the transamination and scission reactions.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
human	H-	Leu	Val	Gln	Pro	Arg	Gly	Ser	Arg	Asn	Gly	Pro	Gly	Pro	Trp	Gln
bovine	H-	Leu	Val	Gln	Pro	Arg	Gly	Pro	Arg	Ser	Gly	Pro	Gly	Pro	Trp	Gln
mouse/rat	H-	Leu	Val	Lys	Pro	Arg	Thr	Ser	Arg	Thr	Gly	Pro	Gly	Ala	Trp	Gln

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
human	Gly	Gly	Arg	Arg	Lys	Phe	Arg	Arg	Gln	Arg	Pro	Arg	Leu	Ser	His
bovine	Gly	Gly	Arg	Arg	Lys	Phe	Arg	Arg	Gln	Arg	Pro	Arg	Leu	Ser	His
mouse/rat	Gly	Gly	Arg	Arg	Lys	Phe	Arg	Arg	Gln	Arg	Pro	Arg	Leu	Ser	His

	31	32	33	34	35	36	
human	Lys	Gly	Pro	Met	Pro	Phe	-OH
bovine	Lys	Gly	Pro	Met	Pro	Phe	-OH
mouse/rat	Lys	Gly	Pro	Met	Pro	Phe	-OH

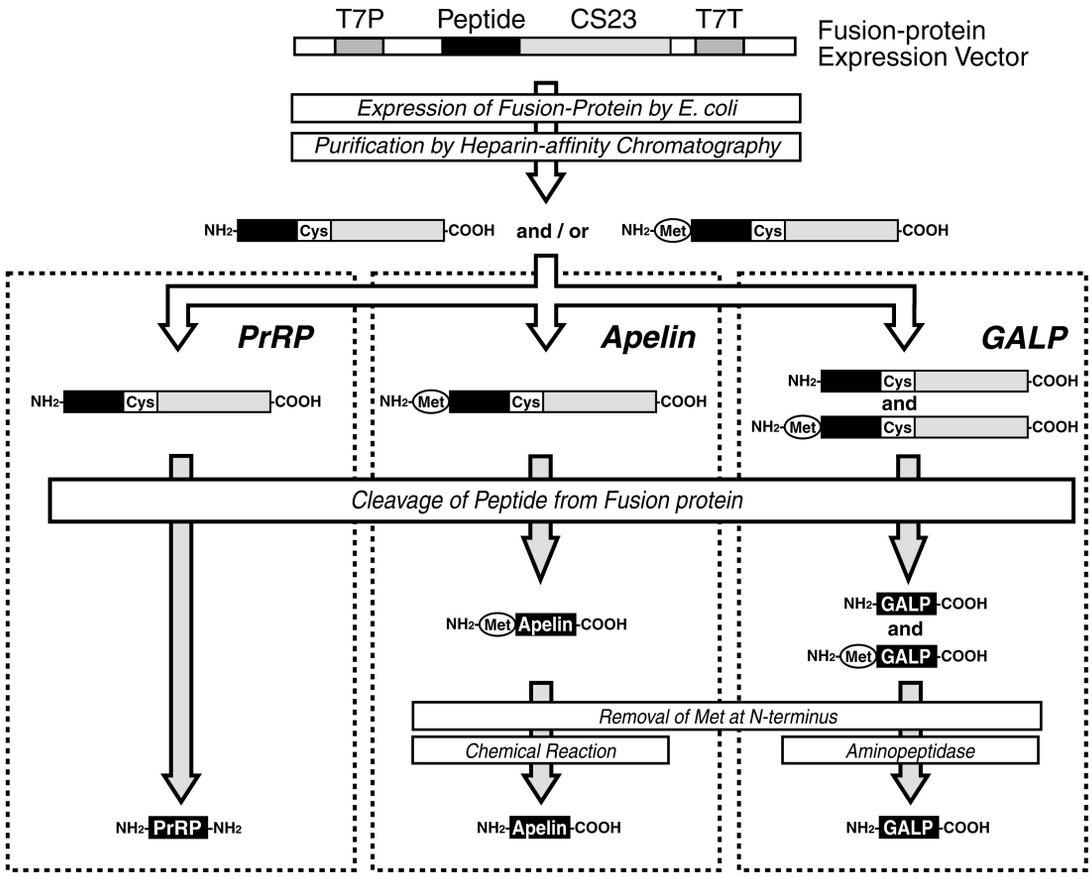
Fig. 1 Amino acid sequences of human, bovine, mouse and rat apelin.

These two methods are very useful for the preparation of biologically active peptides and we describe here the system for the synthesis of three orphan receptor ligand peptides: apelin,[19] prolactin-releasing peptide (PrRP)[20] and galanine-like peptide (GALP).[21] These three peptides were discovered in our Research Division as endogenous ligands for the orphan receptors APJ,[22] GPR10[20,23,24] and GALR2,[25,26] respectively.

Results and discussion

Preparation of apelin

Apelin was recently isolated from bovine stomach extracts in our Research Division.[19] The cDNA structure of this peptide, and the corresponding human, mouse and rat peptides, indicate that 36 amino acid residues (Fig. 1) are produced as a mature peptide from a preproprotein of 77 amino acid residues. The physiological significance of apelin is not yet known and study of its physiological function has only just begun. Recently, it was reported that APJ supported the efficient entry of human immunodeficiency virus (HIV) as a coreceptor with CD4.[27] Therefore, apelin may facilitate the discovery of candidates for therapeutic or preventive agents against HIV infection *via* APJ. Scheme 3 shows the strategy for the preparation of human apelin.[28] To obtain human apelin, we constructed a human apelin-CS23 expression vector in which the human



Scheme 3 Strategies for the preparation of apelin, PrRPs and rat GALP. T7P, T7 promoter; T7T, T7 terminator.

apelin gene was fused to the 5′ end of the CS23 gene with a cysteine codon as a linker. The transformant *E. coli* MM294 (DE3)[29]/pTFA10L was cultivated and the cells were collected by centrifugation. In sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis, the fusion protein was the main product in the total cell lysate and existed as a soluble form in *E. coli* cells (data not shown). Although the fusion protein was not degraded in the cells, it was easily degraded by endogenous proteases at the N-terminal 22 amino acid residue during sonication (data not shown). Therefore, the fusion protein was extracted directly from the cells with guanidine hydrochloride (Gu·HCl) and then refolded using L-arginine.[30,31] After renaturation, the fusion protein was easily purified using heparin affinity column chromatography. Using N-terminal amino acid sequence analysis (data not shown), the protein obtained was found to be human apelin-CS23, with an additional methionine residue at the N-terminus. The SH groups of the methionylated human apelin-CS23 (Met-apelin-CS23) fusion protein were converted to SCN groups by DMAP-CN. Specific cleavage of the modified protein was accomplished by resuspension in 0.05 M NaOH containing 6 M urea at 0 °C, and then passing it through a column of Sephadex G-25. The resulting crude product was purified by chromatography on SP-5PW and ODS-120T, and methionylated human apelin (Met-apelin) was obtained (Fig. 2A, B). The biological activity of the purified Met-apelin was found to be of the same order as a chemically synthesized standard (data not shown). Next, to obtain non-methionylated human apelin, we optimized the transamination and scission reactions. Met-apelin was converted into an oxoacyl form with glyoxylic acid, copper sulfate and pyridine, and passed through a column of Sephadex G-25. The resulting product was cleaved with 3,4-diaminobenzoic acid in 1 M acetic acid and 2 M sodium formate, to give the non-methionylated human apelin (apelin). The purified apelin migrated as a single band on SDS-PAGE (Fig. 2A) and showed a retention time identical to that of a

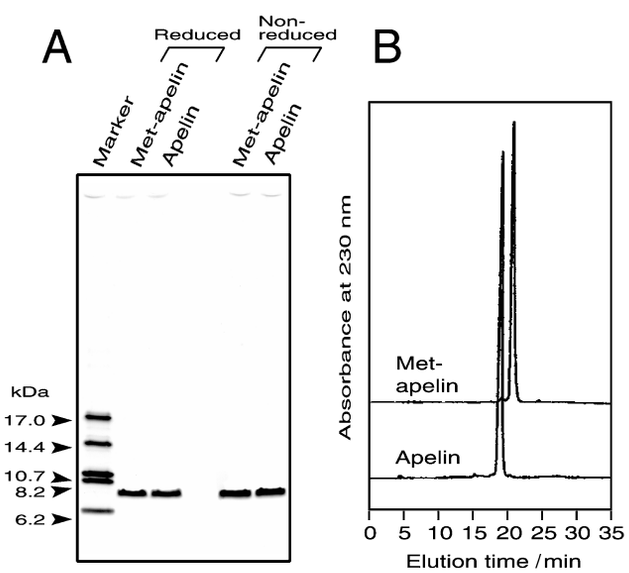


Fig. 2 Purification of apelin. (A) SDS–PAGE analysis. SDS–PAGE was carried out with Multigel 15/25 under reducing and non-reducing conditions. (B) HPLC profiles of the purified Met-apelin and apelin. Purified Met-apelin and apelin were analyzed by RP-HPLC using a C4P-50 column (Shodex, 4.6 mm × 25 cm) with a linear gradient of 12% to 22.4% acetonitrile in 0.1% TFA for 30 min at a flow rate of 0.8 ml min^{-1}. The retention times of Met-apelin and apelin are 21.1 and 19.2 min, respectively.

chemically synthesized standard by reversed-phase HPLC (Fig. 2B). Apelin showed the electrophoretic mobilities of an approximately 7 kDa species under both non-reducing and reducing conditions, although the predicted molecular size is only 4.2 kDa. This discrepancy may be due to the characteristic low mobility of apelin in an SDS–polyacrylamide gel due to its primary structure. To confirm the structural identity of the

Table 1 Amino acid compositions of apelin, bPrRP and rGALP

Amino acids	Residues per molecule (values predicted from cDNA sequences)		
	Apelin	bPrRP	rGALP
Asx	1.0 (1)	2.0 (2)	5.0 (5)
Thr	0.0 (0)	0.9 (1)	2.9 (3)
Ser	1.8 (2)	1.7 (2)	5.5 (6)
Glx	3.0 (3)	2.0 (2)	2.3 (2)
Pro	5.7 (6)	3.2 (3)	4.0 (4)
Gly	5.6 (6)	2.9 (3)	7.4 (7)
Ala	0.0 (0)	3.0 (3)	6.0 (6)
Val	1.0 (1)	1.0 (1)	1.3 (1)
Met	1.0 (1)	1.0 (1)	1.0 (1)
Ile	0.0 (0)	3.0 (3)	1.9 (2)
Leu	2.0 (2)	0.0 (0)	9.2 (9)
Tyr	0.0 (0)	1.0 (1)	1.4 (2)
Phe	1.8 (2)	1.0 (1)	0.0 (0)
His	1.0 (1)	1.9 (2)	2.6 (2)
Lys	1.8 (2)	0.0 (0)	3.1 (3)
Arg	7.2 (8)	4.8 (5)	5.0 (5)
Trp	0.9 (1)	0.9 (1)	N.D.(2)[a]

[a] N.D.: not determined.

purified apelin, protein chemical analysis was performed. The N-terminal amino acid sequence, the C-terminal amino acid (data not shown), the amino acid composition analysis (Table 1) and relative molecular mass measurement by liquid secondary-ion mass spectrometry (MH^+, *m*/*z* observed: 4196.4 Da *vs.* theoretical 4196.9 Da) were all in good agreement with those data predicted from the corresponding cDNA sequence. The purified human apelin thus obtained was subjected to a biological assay using a Cytosensor.[19] It was demonstrated that the purified apelin and a chemically synthesized standard both equally increased the extracellular acidification rate of Chinese hamster ovary (CHO) cells expressing APJ (Fig. 3A). Also, the obtained apelin showed the same ability as a chemically synthesized standard in suppressing cAMP production in the same cells (Fig. 3B).

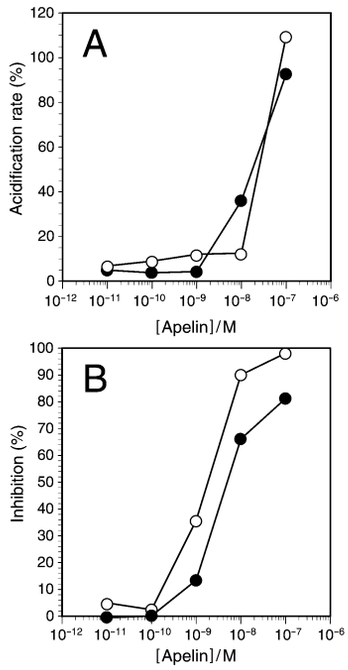


Fig. 3 Biological activities of purified apelin. (A) Promotion of extracellular acidification rate. (B) Suppression of cAMP production in CHO cells expressing APJ. ○; chemically synthesized standard, ●; purified apelin.

Preparation of PrRPs

PrRP was also recently isolated from bovine hypothalamic tissue extract in our Research Division.[20] The cDNA structure of this peptide, and the corresponding human and rat peptides (bPrRP, hPrRP and rPrRP), indicated that 31 amino acid residues with a C-terminal amide group (Fig. 4) are produced as mature peptides from their preproproteins. The biologically active peptide is mainly expressed in the rat hypothalamus and promotes prolactin secretion from rat anterior pituitary cells. Scheme 3 shows the strategies for preparation of PrRPs.[32] To obtain PrRPs, we constructed PrRPs-CS23 expression vectors in which the PrRP genes were fused to the 5′ end of the CS23 gene, with a cysteine codon as a linker. The transformants, *E. coli* MM294 (DE3)/pTB960–10 for bPrRP, *E. coli* MM294 (DE3)/pTB960–11 for rPrRP and *E. coli* MM294 (DE3)/pTB960–12 for hPrRP, were cultivated and the cells were collected by centrifugation. In SDS–PAGE analysis, the fusion proteins were the main product in the total cell lysates and were obtained in soluble forms (data not shown). The N-terminal amino acid sequences of these main bands blotted to poly (vinylidene difluoride) (PVDF) membranes were in good agreement with the sequences of PrRPs, and N-terminal methionines were not found in the PrRP products (data not shown). On the other hand, in the case of apelin, the fusion protein was obtained as its methionylated form. Therefore, the removal of the N-terminal methionine in *E. coli* may vary depending on the amino acid sequences of the peptides. After sonication, the resulting bPrRP-CS23 fusion protein was easily purified by heparin affinity chromatography from the crude supernatant. The SH group of the fusion protein was converted to an SCN group by DMAP-CN, and the modified protein was cleaved at the cysteine residue of the junction site by treatment with ammonia. To obtain the desired peptides, which were both the C-terminal α-acid forms and α-amide forms, we optimized the cleavage reaction and found improved conditions (α-acid form: 0.05 M NaOH, at 0 °C for 15 min, α-amide form: 3 M ammonia, at 25 °C for 15 min), compared with the previously reported conditions.[18] The amide form of bPrRP was purified by chromatography on SP-5PW and C4P-50. We also obtained the acid form of bPrRP by NaOH hydrolysis, and the amide forms of hPrRP and rPrRP in essentially the same manner as bPrRP. Analyses by gel filtration, SDS-PAGE (Fig. 5A) and reversed-phase HPLC (Fig. 5B) of the purified PrRPs showed high homogeneity. To confirm the structural identity of the purified bPrRP, protein chemical analysis was performed. The N-terminal amino acid sequence, the C-terminal amino acid (data not shown), the amino acid composition analysis (Table 1), and molecular-mass measurement by liquid secondary-ion mass spectrometry (MH^+, *m*/*z* observed: 3577.1 Da *vs.* theoretical: 3577.1 Da) were all in good agreement with the predicted data from the corresponding cDNA sequence. The purified bPrRP showed full biological activity in binding to its receptor, expression in CHO cells (Fig. 6A) and releasing arachidonic acid metabolite (AA release) from those same cells (Fig. 6B). The C-terminal acid form obtained by NaOH hydrolysis showed few of these activities (Fig. 6A, B), indicating that the C-terminal amide structure is very important for expressing biological activity. The formation of α-amides in mammalian cells usually proceeds by means of the C-terminal α-amidating enzyme.[33] Precursor protein is processed by processing enzyme to yield the glycine extended peptide. This glycine is the amide donor and is then converted to the α-amide. However, this post translational modification cannot be expressed in microorganisms, which lack the necessary enzymatic machinery for production of C-terminal α-amides. Therefore the amide forms of the peptides are not produced directly by recombinant DNA technology. Although this amidating enzyme system has been applied to the preparation of recombinant α-amidated peptide,[34] this process requires the purified glycine-extended precursor peptides and the enzyme

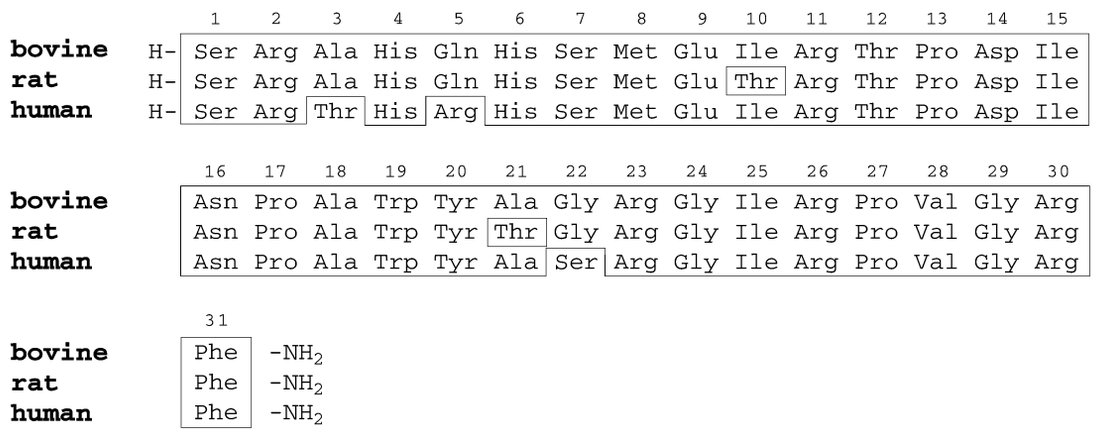


Fig. 4 Amino acid sequences of bovine, rat and human PrRPs.

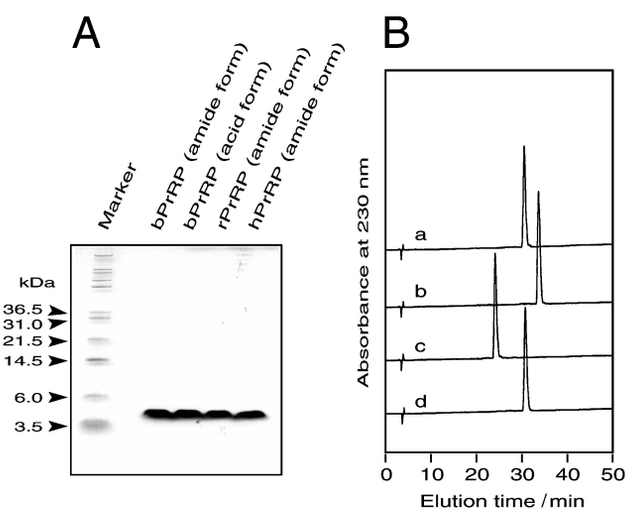


Fig. 5 Purification of PrRPs. (A) SDS–PAGE analysis. SDS–PAGE was carried out with Peptide–PAGE mini (TEFCO) under reducing conditions. (B) HPLC profiles of the purified PrRPs. Purified PrRPs were analyzed by RP-HPLC using an ODP-50 column (Shodex, 4.6 mm × 15 cm) with a linear gradient of 16% to 48% acetonitrile in 0.1% TFA for 50 min after equilibration with 16% acetonitrile in 0.1% TFA for 10 min at a flow rate of 0.5 ml min^{-1}. The retention times of PrRPs are shown for a; bPrRP (amide form, 31.0 min), b; bPrRP (acid form, 34.5 min), c; rPrRP (amide form, 25.0 min), d; hPrRP (amide form, 31.5 min).

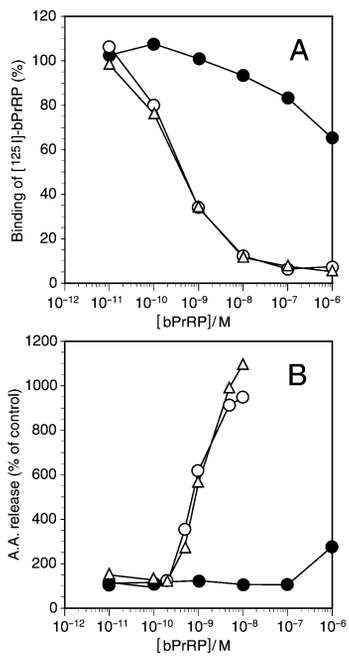


Fig. 6 Biological activities of the purified bPrRP. (A) Binding assay with CHO cells expressing GPR10. ○; bPrRP (amide form), ●; bPrRP (acid form amide form), △; chemically synthesized standard. (B) Arachidonic acid metabolite release assay. Symbols are the same as shown in (A).

system is not easily available. Other enzymatic methods for the preparation of recombinant α-amidated peptide using carboxypeptidase Y have been reported.[35,36] In this study, our recombinant and chemical cleavage methods make it possible to produce both the C-terminal acid and amide forms. This should be useful for checking to see whether the structures of C-termini are essential for the biological activities of peptides. Compared with standard enzymatic methods, we found our process to be simpler and more productive. Moreover, our method provides a way to obtain peptides with a C-terminal alkylamide for cleavage from the recombinant fusion protein using an alkylamine instead of ammonia.[18] This type of product has not been obtained by the above enzymatic procedures.

Preparation of GALP

GALP was also recently isolated from porcine hypothalamus in our Research Division.[21] The cDNA structure of this peptide, and the corresponding human and rat peptides (pGALP, hGALP and rGALP), indicate that 60 amino acid residues (Fig. 7) are produced as mature peptides from the respective preproproteins. The amino acid sequence of GALP-(9–21) is identical to that of galanin-(1–13).[37] Whereas galanin has a high affinity for both GALR1 and GALR2, however, GALP has a high affinity for GALR2 but a low affinity for GALR1. The physiological significance of GALP is not yet known, and the study to elucidate the physiological function of GALP has only just begun. Scheme 3 shows the strategy for the preparation of rGALP.[38] We obtained rGALP essentially in the same manner as described above by constructing an rGALP-CS23 expression vector in which the rGALP gene was fused to the 5′ end of the CS23 gene with a cysteine codon as a linker. The transformant *E. coli* MM294 (DE3)/pTFRGAL was cultivated and the cells were collected by centrifugation. The fusion protein, which was obtained in an insoluble form, was solubilized with Gu·HCl and then refolded using L-arginine. After renaturation, the fusion protein was purified by heparin affinity column chromatography. N-Terminal amino acid analysis showed that about 60% of the rGALP-CS23 fusion protein expressed in *E. coli* cells had an additional methionine at the N-terminus (data not shown). The first step was then cleavage of rGALP and methionylated rGALP (Met-rGALP) from the fusion protein using a cyanylation reaction, and the second step was the specific removal of the additional methionine at the N-terminus. Although we applied an efficient

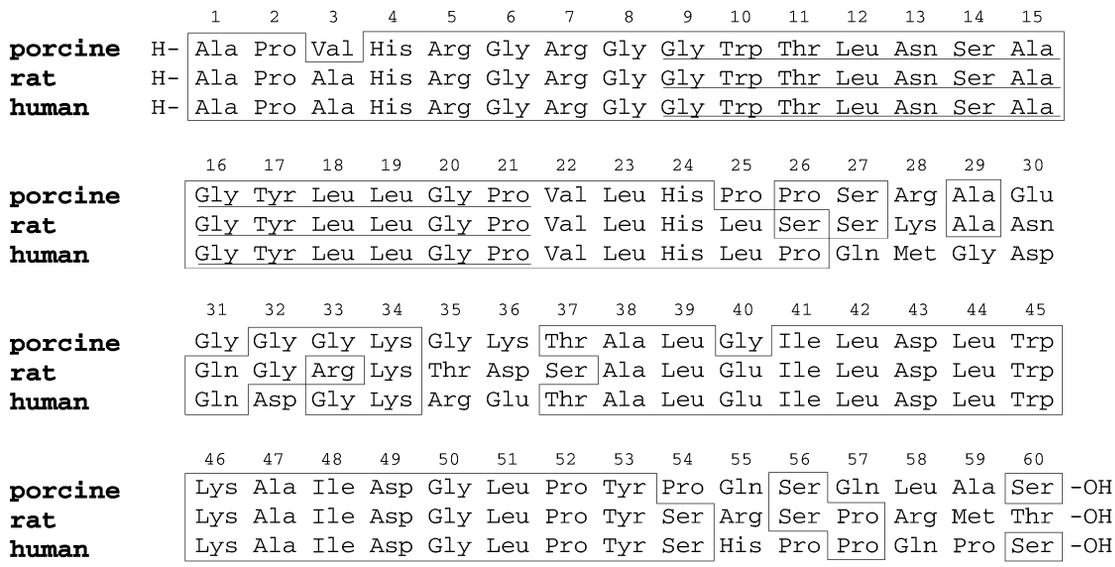


Fig. 7 Amino acid sequences of porcine, rat and human GALP. Underlined sequences are completely identical to those of galanin-(1–13).

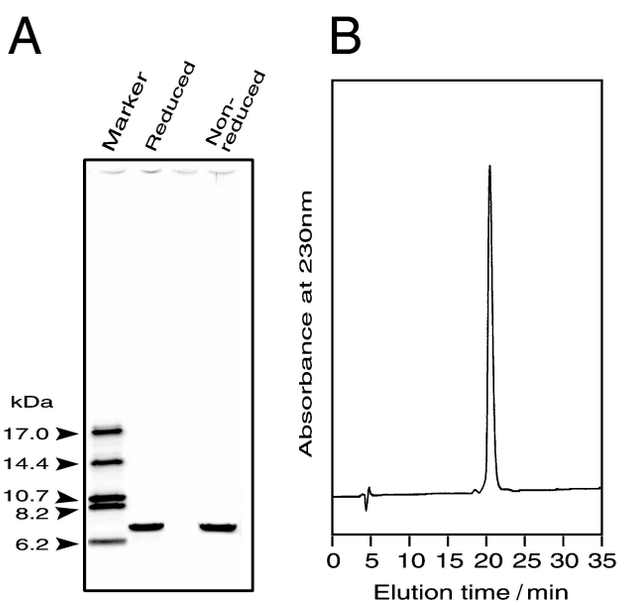


Fig. 8 Purification of GALPs. (A) SDS–PAGE analysis. SDS–PAGE was carried out with Multigel 15/25 under reducing and non-reducing conditions. (B) HPLC profiles of the purified GALPs. Purified rGALP was analyzed by RP-HPLC using a C4P-50 column (Shodex, 4.6 mm × 25 cm) with a linear gradient of 26.4% to 34.4% acetonitrile in 0.1% TFA for 30 min at a flow rate of 0.8 ml min^{-1}. The retention time of rGALP is 20.5 min.

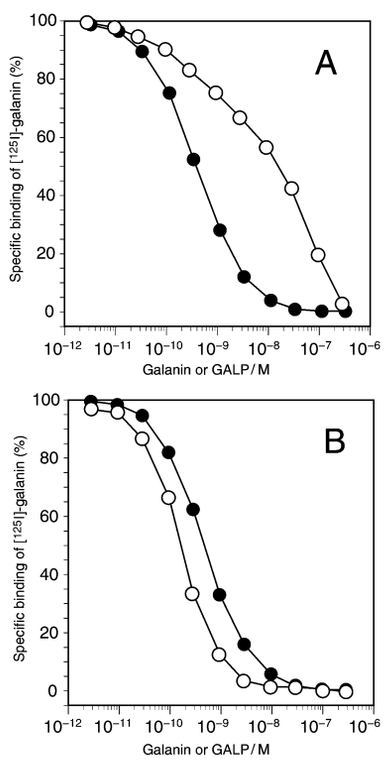


Fig. 9 Biological activity of purified rGALP. ^{125}I-Galanin-binding assay with CHO cells expressing GALR1 (A) or GALR2 (B). ○; the purified rGALP, ●; rat galanin.

chemical method for removing the additional methionine in the case of apelin, we could not use the method in this case because rGALP and Met-rGALP could not be separated efficiently by chromatography (data not shown). Thus, we applied an enzymatic method using aminopeptidase for the removal of the additional methionine residue as we previously reported in our description of the production of recombinant interleukin 2 and human growth hormone.[39] After the SH groups of the fusion protein were converted to SCN groups with DMAP-CN, rGALP and Met-rGALP were cleaved by NaOH hydrolysis. After the cleavage reaction, rGALP and Met-rGALP were purified by successive chromatography on SP-Toyopearl 650M and C4P-50. Next, the additional methionine at the N-terminus of the purified Met-rGALP was removed with amino peptidase,[40] and the rGALP was purified by reversed-phase HPLC on C4P-N50. Human GALP could also be prepared in the same manner. Purified rGALP was shown to be of high purity by SDS-PAGE (Fig. 8A) and HPLC profile (Fig. 8B). To confirm the structural identity of the purified rGALP, protein chemical analysis was performed. The N-terminal amino acid sequence, the C-terminal amino acid (data not shown), the amino acid composition analysis (Table 1), and relative molecular mass measurement by liquid secondary-ion mass spectrometry (MH^+, *m/z* observed: 6502.9 Da *vs.* theoretical: 6502.5 Da) were all in good agreement with data predicted from the corresponding cDNA sequence. The biological activity of purified rGALP was determined using a receptor binding assay[21] with CHO cells expressing GALR2 or GALR1, respectively. The purified rGALP showed the same high affinity for GALR2 and low affinity for GALR1, compared with chemically synthesized rat galanin (Fig. 9A, B).

Conclusions

In the present study, apelin, PrRPs and rGALP were obtained in large quantities and with a high degree of purity by a new system using recombinant DNA technology and specific chemical cleavage reactions. The productivity of this system is not dependent on the size of peptide, although the solid-phase peptide synthesis is still problematic for the preparation of relatively high-molecular-mass peptides, such as GALP, chemically. The key points of this system are three major advantages over standard methods. First is the introduction of a cysteine residue at the junction of the fusion protein to give reliable site-specific cleavage using a cyanylation and cleavage reaction sequence. Moreover, this reaction made it possible to produce both the C-terminal α-acid form and α-amide form, which are generally essential for full biological activity. Second, the conversion of recombinant methionylated peptide into its non-methionylated form was achieved using a transamination and scission reaction sequence. Third, CS23 was used as a fusion partner because heparin affinity chromatography made it easy to purify the fusion protein in high yield. Finally, we successfully established the novel system for the preparation of biologically active peptide, consisting of utilization of CS23 as a fusion partner, site-specific cleavage using a cyanylation reaction, and specific removal of the methionine residue at the N-terminus. The specificity and simplicity of the present system make it both versatile and convenient for the preparation of biologically active peptides. The peptides obtained here might be very useful for clarification of their physiological role *in vivo*.

Experimental

Glyoxylic acid monohydrate, copper(II) sulfate pentahydrate, pyridine and 3,4-diaminobenzoic acid were all obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1-Cyano-4-(dimethylamino)pyridinium tetrafluoroborate (DMAP-CN) and cyanogen bromide (BrCN) were obtained from Sigma (St. Louis, USA).

Construction of expression plasmids

All expression plasmids were constructed using pTF plasmid, a derivative of pTB960-7, as described previously.[14] The cDNA fragments of human apelin, PrRPs (bovine, rat and human) and GALPs (porcine, human and rat) were prepared by the annealing of synthetic oligonucleotides and insertion into the cloning site of pTF plasmid.

Expression of fusion proteins

E. coli MM294 (DE3)[29] was used as the host cell, which carries the T7 RNA polymerase gene under control of the lac UV5 promoter in its chromosome and can strongly express fusion genes under the control of the T7 promoter in the presence of IPTG as described previously.[14,41] For large-scale preparation, transformants were cultivated at 30 °C in a 500 l fermenter containing 250 l of modified M9 medium[41] with an agitation rate of 450 rpm. After cultivation for 5 hours, IPTG (420 μM) was added and cultivation was continued for an additional 4 hours. The cells were collected by centrifugation and stored at −80 °C until further use.

Purification of fusion proteins

All fusion proteins reported here were prepared in essentially the same manner as described previously.[14] The protocols with some improvements are described below.

Frozen cells (300 g wet weight) were sonicated with 900 ml of 50 mM phosphate (Na_2HPO_4–KH_2PO_4) buffer (pH 6.0) containing 10 mM EDTA and 0.1 mM *p*-amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF). After centrifugation of total cell lysate, the supernatant was pooled and the precipitate was re-extracted twice in the same manner. In the case of PrRPs,[33] the fusion proteins were expressed in a soluble form. Therefore, the three extracts were combined and adjusted to pH 6.0 and applied to an AF Heparin Toyopearl 650M column (3.0 × 50 cm, Tosoh Corporation, Tokyo, Japan) and equilibrated with 50 mM phosphate buffer (pH 6.0). The column was washed with the same buffer and the fusion proteins were eluted with a linear gradient of NaCl (0–2 M). The eluate was desalted and concentrated using a Diaflo YM10 membrane (Amicon Corp). In the case of apelin,[28] the fusion protein was extracted directly from the cells to avoid degradation with 7 M Gu·HCl, and then refolded using L-arginine as described previously.[30,31] In the case of GALPs,[38] the fusion proteins were expressed in an insoluble form. Thus, the insoluble fusion protein obtained after sonication was solubilized with 7 M Gu·HCl and then refolded using L-arginine as described previously.[30,31]

Cleavage of fusion proteins with cyanylating reagents

The fusion protein solutions were dissolved in 6 M urea followed by addition of 0.1 M acetic acid. After the addition of 2.4 mM DMAP-CN in a 5-fold excess over the total thiol concentration, the reaction mixtures were incubated at 25 °C for 15 min. These mixtures were then applied to a Sephadex G-25 column (4.6 × 50 cm, Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated with 50 mM phosphate buffer (pH 6.0) and the cyanylated fusion proteins were eluted with the same buffer. The main fraction was concentrated, and mixed with 6 M urea. To obtain the desired peptides from the cyanylated fusion proteins in high yield, we optimized the cleavage reactions, and found the best conditions as follows. For the preparation of the acid form, the cyanylated fusion protein solutions were added to 0.05 M NaOH, and incubated at 0 °C for 15 min. For the preparation of the amide form, the cyanylated fusion protein solutions were added to 3 M ammonia, and incubated at 25 °C for 15 min.

Purification of Met-apelin

The cleaved fusion protein mixture was applied to a Sephadex G-25 column (4.6 × 50 cm, Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated with 50 mM phosphate buffer (pH 6.0) and eluted with the same buffer. The main fraction was applied to a SP-5PW column (2.15 × 30 cm, Tosoh Corporation, Tokyo, Japan), equilibrated with 50 mM phosphate buffer (pH 6.0) containing 3 M urea, and eluted at 35 min with a linear gradient of 0.3 M to 0.4 M NaCl in the same buffer for 50 min after a gradient elution of 0 M to 0.3 M NaCl in the same buffer for 10 min at the flow rate of 6 ml min^{-1}. The desired fraction was applied to an ODS-120T column (2.15 × 30 cm, Tosoh Corporation, Tokyo, Japan) and equilibrated with 16% acetonitrile in 0.1% TFA. The Met-apelin was eluted at 40 min with a linear gradient of 16% to 32% acetonitrile in 0.1% TFA for 60 min at a flow rate of 6 ml min^{-1} and the eluate was collected and lyophilized.

Transamination of Met-apelin

500 mg of glyoxylic acid monohydrate were added to 0.3 ml of 0.2 M $CuSO_4$ and 1 ml of pyridine and the total volume was adjusted to 2 ml with distilled water. Then 8 ml of aq. Met-apelin (protein concentration: 6.25 mg ml^{-1}) containing 3 M urea were then added and incubated at 25 °C for 1 hour. The reaction mixture was applied to a Sephadex G-25 column (2.5 × 60 cm, Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated with 10 mM phosphate buffer (pH 6.0) containing 2.5 M urea, and the sample was eluted with the same buffer. The main fraction was pooled (50 ml).

Conversion of the oxoacyl-apelin to apelin

The protein solution (50 ml) was mixed with 50 ml of 2 M AcOH, 4 M HCOONa, 2.5 M urea and 0.61 g of 3,4-diaminobenzoic acid, and incubated at 25 °C for 4 days. The reaction mixture was applied to a Sephadex G-25 column (2.5 × 60 cm, Amersham Pharmacia Biotech, Piscataway, NJ), equilibrated with 10 mM phosphate buffer (pH 6.0) containing 2.5 M urea, and the sample was eluted with the same buffer. The pooled fraction was applied to an SP-5PW column (2.15 × 15 cm, Tosoh Corporation, Tokyo, Japan) equilibrated with 50 mM phosphate buffer (pH 6.0) containing 3 M urea and the peptide was eluted at 35 min with a linear gradient of 0.3 M to 0.4 M NaCl in the same buffer for 50 min after a gradient elution of 0 M to 0.3 M NaCl in the same buffer for 10 min at a flow rate of 6 ml min^{-1}. The desired fraction was applied to an ODS-120T column (2.15 × 30 cm, Tosoh Corporation, Tokyo, Japan), equilibrated with 16% acetonitrile in 0.1% TFA, and eluted at 40 min with a linear gradient of 16% to 32% acetonitrile in 0.1% TFA for 60 min at a flow rate of 6 ml min^{-1}. The eluate was collected and lyophilized (15 mg).

Purification of PrRPs

The C-terminal amide form of bPrRP in the cleaved fusion protein mixture was purified by 3 steps of column chromatography as follows. The first step was gel filtration on a Sephadex G-25 column (4.6 × 60 cm, Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated with 50 mM phosphate buffer (pH 6.5). The fractions corresponding to the relative molecular mass of bPrRP were applied to an SP-5PW column (2.15 × 15 cm, Tosoh Corporation, Tokyo, Japan), equilibrated with the same buffer, and the peptide was eluted at 33 min with a linear gradient of 0.15 M to 0.35 M NaCl in the same buffer for 40 min after a gradient elution of 0 M to 0.15 M NaCl in the same buffer for 10 min at a flow rate of 6 ml min^{-1}. The final step was reversed-phase column chromatography on a C4P-50 (2.15 × 30 cm, Showdex, Showa denko, Tokyo, Japan) equilibrated with 16% acetonitrile in 0.1% TFA. The amide form of bPrRP was eluted at 33 min with a linear gradient of 16% to 32% acetonitrile in 0.1% TFA for 40 min at a flow rate of 6 ml min^{-1} and lyophilized (90 mg). The C-terminal acid form of bPrRP in the cleaved fusion protein mixture was purified with reversed-phase column chromatography on an ODS-120T (2.15 × 30 cm, Tosoh Corporation, Tokyo, Japan) equilibrated with 16% acetonitrile in 0.1% TFA. The peptide was eluted at 33 min with a linear gradient of 16% to 32% acetonitrile in 0.1% TFA for 40 min at a flow rate of 6 ml min^{-1} and lyophilized (76.5 mg). Both C-terminal amide forms of hPrRP and rPrRP were also obtained (13 mg and 94 mg, respectively) in the same manner as described above for bPrRP.

Purification of rGALP

The mixture of rGALP and Met-rGALP obtained after the cleavage reaction was applied to an SP-Toyopearl 650M column (3.2 × 12.6 cm, Tosoh Corporation, Japan) equilibrated with 50 mM phosphate buffer (pH 6.5) containing 3 M urea, and eluted at 19 min with a linear gradient of 0 M to 0.6 M NaCl in the same buffer for 50 min at a flow rate of 10 ml min^{-1}. The desired fraction was applied to a C4P-50 column (2.15 × 30 cm, Showdex) equilibrated with 0.1% TFA and eluted at 45 min with a linear gradient of 26.4% to 34.4% acetonitrile in 0.1% TFA for 50 min after a gradient elution of 0% to 26.4% acetonitrile in 0.1% TFA for 5 min at a flow rate of 5 ml min^{-1}. The eluate was collected and lyophilized (21 mg). Next, 20 mg of the mixture of rGALP and Met-rGALP was digested with 50 μg of aminopeptidase SG (Takara, Japan) in 50 mM Tris-HCl (pH 7.5) containing 3 M guanidine hydrochloride at 25 °C for 24 hours. The digested solution was then applied to a C4P-50 column (4.6 mm × 25 cm, Asahipak, Showa denko, Tokyo, Japan) and equilibrated with 0.1% TFA. The rGALP was eluted at 44 min with a linear gradient of 26.4% to 34.4% acetonitrile in 0.1% TFA for 50 min after a gradient elution of 0% to 26.4% acetonitrile in 0.1% TFA for 5 min at a flow rate of 5 ml min^{-1} and lyophilized (13 mg).

Biological assay of apelin

The extracellular acidification rate in the medium of CHO cells expressing human APJ receptor was measured using the Cytosensor. cAMP production in the same cells was also determined, as described previously.[19]

Biological assay of PrRPs

The biological activity of PrRPs was determined using a receptor-binding assay with CHO cells expressing bPrRP receptor. An arachidonic acid metabolite-release assay was also determined with the same cells, as described previously.[20]

Biological assay of GALPs

The biological activity of GALPs was determined using a receptor binding assay with both CHO cells expressing rat GALR1 and rat GALR2 receptors, as described previously.[21]

DNA sequence analysis

The DNA sequences of the constructed plasmids were determined by the Applied Biosystems 377 autosequencer (Foster City, CA).

Amino acid analysis

The amino acid compositions of the peptides were determined following hydrolysis with 6 M HCl and 4% thioglycolic acid at 110 °C for 24 and 48 hours by the Beckman model 6300E amino acid analyzer.

Amino-terminal sequence analysis

The amino-terminal sequence was determined by the gas-phase protein sequencer (model 477A, Applied Biosystems, Foster City, CA).

Carboxy-terminal amino acid analysis

The carboxy-terminal amino acid of the peptides was cleaved by hydrazinolysis,[42] and analyzed on a Beckman model 6300E amino acid analyzer.

Relative molecular mass measurement

The molecular mass of apelin and bPrRP was determined by liquid secondary-ion mass spectrometry using a JMS-HX110 double-focusing mass spectrometer (JEOL, Tokyo) equipped with a caesium ion source. The instrument was operated at an accelerating voltage of 10 kV, a mass resolution of 1 : 1000, and with 300 Hz filtering. The caesium ion gun was operated at 15 kV with a 2.2 A heater current. A mixture of glycerol and thioglycerol (1 : 1) was used as the sample matrix.

The mass of rGALP was determined by electrospray mass spectrometry using a ThermoFinnigan LCQ ion-trap mass spectrometer (ThermoQuest, San Jose, CA). Sample was dissolved in 50% acetonitrile in 1% AcOH.

SDS–PAGE

SDS–PAGE was performed according to the method of Laemmli[43] using Multigel 15/25 (Daiichi Kagaku Co., Ltd., Japan) and Peptide–PAGE mini (TEFCO) 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 sequenced by the gas-phase protein sequencer as described above.

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