COMMUNICATION

Synthesis of a newly isolated APJ receptor peptide ligand using recombinant DNA technology and chemical cleavage reactions

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Received (in Cambridge, UK) 10th January 2000, Accepted 7th March 2000
Published on the Web 29th March 2000

Apelin, a newly isolated peptide ligand of the APJ receptor, was prepared using recombinant DNA technology and chemical cleavage reactions.

Apelin,[1] an endogenous ligand of the APJ receptor,[2] was recently isolated in our Research Division from bovine stomach extracts by measuring the increase in the extracellular acidification rate of Chinese hamster ovary (CHO) cells expressing the APJ receptor. The cDNA of this peptide, and corresponding human, mouse and rat peptides encoded preproproteins with 77 amino acid residues. *N*-Terminal sequence analysis and gel filtration studies of this peptide indicated that 36 amino acid residues (Fig. 1) are produced as the mature peptide from the preproprotein. The physiological significance of apelin is not yet known and the study of its physiological function has only just begun. Recently, Choe *et al*. reported that the APJ receptor supported the efficient entry of human immunodeficiency virus (HIV) as a coreceptor with CD4.[3] Therefore, apelin may facilitate the discovery of candidates for therapeutic or preventative agents against HIV infection *via* the APJ receptor.

Recombinant DNA technology has made it possible to prepare many biologically active proteins in *Escherichia coli* (*E. coli*.). However, several problems exist in the production of recombinant polypeptides. One is the difficulty associated with the production of small molecular weight biologically active peptides,[4] and another is the addition of a methionine residue corresponding to the initiation codon to the *N*-terminal amino acid.[5] The synthesis of small peptides has often been unsuccessful since they are very sensitive to endogenous proteases.[4] Bacterial gene fusion systems have been used to overcome protease degradation problems. To obtain a high yield of the desired product, it is important to design a site-specific cleavage from the fusion partner. Moreover, fusion partners are available to aid the purification of heterologous proteins from *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.[5] The *N*-terminal methionine may affect the biological activity or antigenicity[6] of the protein if it was used for therapeutic purposes, so it is necessary to remove the residue as completely as possible.

In this regard, we previously reported two efficient methods to obtain recombinant peptides and proteins: 1. a cysteine site-specific cleavage reaction of the fusion proteins;[7–10] 2. removal of an additional *N*-terminal methionine residue after transamination.[11,12] The procedures of these two methods are as follows.

The peptides were expressed in the form of fusion proteins with basic fibroblast growth factor mutein (CS23), which were purified by heparin affinity chromatography. The SH groups of the denatured fusion proteins were converted to SCN groups with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (DMAP-CN), and then exposure to alkaline pH resulted in cleavage at the amino groups of the modified cysteine residues to yield the desired peptides.

An additional methionine residue of the recombinant protein was converted into an oxoacyl form with glyoxylic acid, copper sulfate and pyridine, and then cleaved from the rest of the protein with 3,4-diaminobenzoic acid in 1 M AcOH and 2 M HCOONa to obtain the non-methionylated recombinant protein. These two methods are very useful in the preparation of human apelin. Here we describe the synthesis of human apelin using recombinant DNA technology and chemical cleavage reactions.

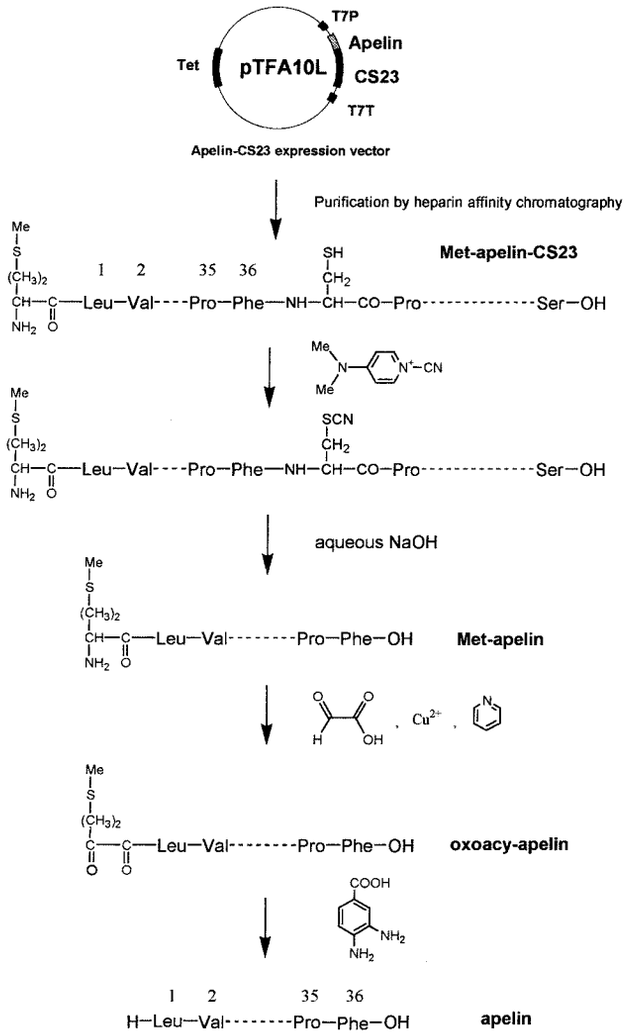
Scheme 1 shows the strategy for the preparation of human apelin. To obtain human apelin, we constructed a human apelin-CS23 expression vector in which human apelin was fused at the *N*-terminus of CS23 with cysteine residue as a linker. The vector was transfected into *E. coli*, and transformant *E. coli* MM294 (DE3)/pTFA10L was obtained. The transformant was cultivated and the cells were collected by centrifugation. The fusion protein was extracted with guanidine hydrochloride (Gu·HCl) and then refolded using L-arginine.[13,14] After renaturation, the fusion protein was purified by heparin affinity column chromatography. The obtained protein had 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 in 0.05 M NaOH containing 6 M urea at 0 °C and then passed through a column of Sephadex G-25. The resulting crude product was purified by chromatography on SP-5PW and ODS-

	1								10										20
human	H – Leu	Val	Gln	Pro	Arg	Gly	Ser	Arg	Asn	Gly	Pro	Gly	Pro	Trp	Gln	Gly	Gly	Arg	Arg	Lys
bovine	H – Leu	Val	Gln	Pro	Arg	Gly	Pro	Arg	Ser	Gly	Pro	Gly	Pro	Trp	Gln	Gly	Gly	Arg	Arg	Lys
mouse/rat	H – Leu	Val	Lys	Pro	Arg	Thr	Ser	Arg	Thr	Gly	Pro	Gly	Ala	Trp	Gln	Gly	Gly	Arg	Arg	Lys

21									30						36	
Phe	Arg	Arg	Gln	Arg	Pro	Arg	Leu	Ser	His	Lys	Gly	Pro	Met	Pro	Phe	–OH
Phe	Arg	Arg	Gln	Arg	Pro	Arg	Leu	Ser	His	Lys	Gly	Pro	Met	Pro	Phe	–OH
Phe	Arg	Arg	Gln	Arg	Pro	Arg	Leu	Ser	His	Lys	Gly	Pro	Met	Pro	Phe	–OH

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

DOI: 10.1039/b000152j



Scheme 1 Strategy for the preparation of human apelin. T7P, T7 promoter; T7T, T7 transcription terminator; Tet, tetracycline resistance gene.

120T, and purified methionylated human apelin (Met-apelin) was obtained.

In a previous paper, we reported the chemical cleavage of an additional *N*-terminal methionine from recombinant methionylated human growth hormone.[11] Recently, we reported an improved method for the scission reaction of recombinant methionylated proteins[12] and applied the new scission conditions to the removal of the *N*-terminal methionine residue from Met-apelin.

To obtain human apelin, we optimized the transamination and scission reactions. The best conditions were: 6 mM $CuSO_4$, 0.5 M glyoxylic acid and 10% pyridine for the former and 40 mM 3,4-diaminobenzoic acid, 1 M AcOH and 2 M HCOONa for the latter. Met-apelin was converted into an oxoacyl form with glyoxylic acid, copper sulfate and pyridine, and then passed through a column of Sephadex G-25. The resulting product was cleaved with 3,4-diaminobenzoic acid in 1 M AcOH and 2 M HCOONa. The reaction product was purified by chromatography on Sephadex G-25, SP-5PW and ODS-120T to give the purified non-methionylated human apelin. To confirm the structural identity of the purified human apelin, protein chemical analysis was performed. The *N*-terminal amino acid sequence, the *C*-terminal amino acid and the amino acid analysis were all in good agreement with those predicted from the corresponding DNA sequence. The gel filtration profile, sodium dodecylsulfate-polyacrylamide gel electrophoresis and reversed-phase HPLC (Fig. 2) of the purified human apelin showed high homogeneity. The obtained peptide showed a retention time identical to that of a chemically synthesized standard (Fig. 2) and it gave the expected result upon molecular

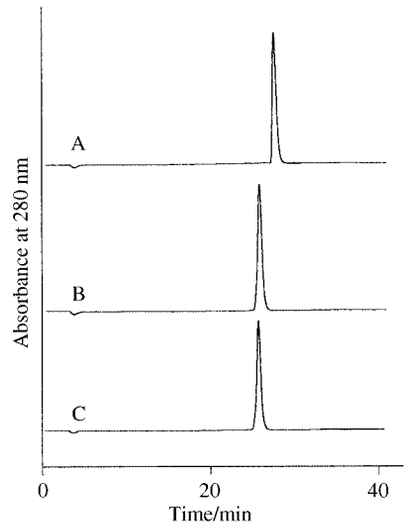


Fig. 2 HPLC profile of the purified Met-apelin and human apelin. (A) Met-apelin obtained from the fusion protein, (B) human apelin obtained from Met-apelin after transamination and (C) synthetic human apelin. Peptides (10 μg) were applied to a ODS-120T column (4.6 × 150 mm) and eluted with a linear gradient of acetonitrile (16%–32%) in the presence of 0.1% TFA.

weight measurement by liquid secondary ion mass spectrometry (MH^+, *m/z* observed: 4196.4 *vs.* theoretical: 4196.9). The purified human apelin was subjected to a biological assay using a Cytosensor,[15] showing that the human apelin and a chemically synthesized standard equally increased the extracellular acidification rate in CHO cells expressing APJ receptor.[1] Also, the human apelin showed the same ability as an authentic sample in suppressing the cAMP production in CHO cells expressing APJ receptor.[1]

Thus, using DNA technology and a chemical procedure, we obtained human apelin in high purity. Moreover, we could show that the new scission conditions for the removal of the additional methionine residue are applicable to the small molecular weight biologically active peptide. The obtained human apelin may be very helpful for the clarification of the biological significance and roles *in vivo*, and lead to new drug discoveries, especially an anti-HIV agent, in the future.

Experimental

Construction of the expression plasmid

Plasmid pTFC was derived from pTB960-7.[7] The fragment of apelin structural gene was prepared by the annealing of six synthetic oligonucleotides and inserted into the *Nde*I-*Ava*I cloning site of pTFC to obtain expression plasmid pTFA10L.

Expression of Met-apelin-CS23

Plasmid pTFA10L was introduced into *E. coli* MM294 (DE3) and the cells were cultivated. After cultivation, *E. coli* cells (500 g wet weight) were collected by centrifugation.

Purification of Met-apelin-CS23 fusion protein

E. coli cells (500 g wet weight) were suspended in 1000 ml of 20 mM Tris–HCl (pH 8.0) containing 7 M Gu·HCl. The suspension was stirred for 3 h and then centrifuged at 17.000 *g* for 60 min. The supernatant was diluted 50-fold with the refolding buffer and the final refolding solution consisted of 50 mM Tris·HCl, 1 mM dithiothreitol and 0.6 M L-arginine. After renaturation, the refolding solution was applied to an AF-Heparin Toyopearl 650 M (3.0 × 50 cm) (Tosoh) equilibrated with 50 mM phosphate buffer (pH 6.0) at a flow rate of 40 ml min^{-1}. After adsorption, the column was washed with the same buffer and the proteins were eluted with a linear gradient of NaCl (0–2 M). The desired fractions were collected and the eluate was concentrated using a Pellicon cassette system (Millipore).

Cleavage of Met-apelin-CS23 fusion protein with DMAP-CN

Urea (6 M) was dissolved in the fusion protein solution followed by addition of 0.1 M acetic acid. After the addition of 2.4 mM DMAP-CN, the reaction mixture was incubated at 25 °C for 15 min. This mixture was applied to a Sephadex G-25 column (4.6 × 50 cm) (Amersham Pharmacia Biotech) equilibrated with 50 mM phosphate buffer (pH 6.0) at a flow rate of 10 ml min^{-1} and the sample was eluted with the same buffer. The main fraction was concentrated, mixed with 6 M urea and 0.05 M NaOH, and incubated at 0 °C for 15 min.

Purification of Met-apelin

The reaction mixture was applied to a Sephadex G-25 column (4.6 × 50 cm) equilibrated with 50 mM phosphate buffer (pH 6.0) at a flow rate of 10 ml min^{-1} and the sample was eluted with the same buffer. The main fraction was pooled. The eluate was applied to a SP-5PW column (2.15 × 30 cm) (Tosoh) equilibrated with 50 mM phosphate buffer (pH 6.0) containing 3 M urea at a flow rate of 5 ml min^{-1}. The protein was eluted with a linear gradient of NaCl (0–1 M). The desired fraction was applied to an ODS-120T column (2.15 × 30 cm) (Tosoh) at a flow rate of 5 ml min^{-1} and eluted with a linear gradient of 10–40% acetonitrile in the presence of 0.1% TFA. The eluate was collected and lyophilized (50 mg).

Transamination of Met-apelin

To 0.5 g of glyoxylic acid monohydrate were added 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 Met-Apelin (protein content 6.25 mg ml^{-1}) aqueous solution containing 3 M urea was added and incubated for 1 h at 25 °C. The reaction mixture was applied to a Sephadex G-25 column (2.5 × 60 cm) equilibrated with 10 mM phosphate buffer (pH 6.0) containing 2.5 M urea at a flow rate of 4 ml min^{-1}, 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 for 4 days at 25 °C. After the incubation, the reaction mixture was applied to a Sephadex G-25 column (4.6 × 60 cm) equilibrated with 10 mM phosphate buffer (pH 6.0) containing 2.5 M urea at a flow rate of 10 ml min^{-1} and the sample was eluted with the same buffer. The pooled fraction was applied to a SP-5PW column (2.15 × 15 cm) equilibrated with 50 mM phosphate buffer (pH 6.0) containing 3 M urea at a flow rate of 5 ml min^{-1}. After adsorption, the protein was eluted with a linear gradient of NaCl (0–0.8 M). The desired fraction was applied to an ODS-120T column (2.15 × 30 cm) at a flow rate of 5 ml min^{-1} and eluted with a linear gradient of 15–35% acetonitrile in the presence of 0.1% TFA. The eluate was collected and lyophilized (15 mg).

Acknowledgements

We are grateful to Mr M. Hosoya for determining the biological activity of apelin.

Notes and references

1 K. Tatemoto, M. Hosoya, Y. Habata, R. Fujii, T. Kakegawa, M.-X. Zou, Y. Kawamata, S. Fukusumi, S. Hinuma, C. Kitada, T. Kurokawa, H. Onda and M. Fujino, *Biochem. Biophys. Res. Commun.*, 1998, **251**, 471.
2 B. F. O'Dowd, M. Heibber, A. Chan, H. H. Q. Heng, L.-C. Tsui, J. L. Kennedy, X. Shi, A. Petronis, S. R. George and T. Nguyen, *Gene*, 1993, **136**, 355.
3 H. Choe, M. Farzan, M. Konkel, K. Martin, Y. Sun, L. Marcon, M. Cayabyab, M. Berman, M. E. Dorf, N. Gerard, G. Gerard and J. Sodroski, *J. Virol.*, 1998, **72**, 6113.
4 S. Gottesman, *Methods Enzymol.*, 1990, **185**, 119.
5 A. Ben-Bassat, K. Bauer, S. Y. Chang, K. Myambo, A. Boosman and S. Chang, *J. Bacteriol.*, 1987, **169**, 751.
6 K. Glasbrenner, *J. Am. Med. Assoc.*, 1986, **255**, 581.
7 N. Koyama, M. Kuriyama, N. Amano and O. Nishimura, *J. Biotechnol.*, 1994, **32**, 273.
8 S. Nakagawa, Y. Tamakashi, T. Hamana, M. Kawase, S. Taketomi, Y. Ishibashi, O. Nishimura and T. Fukuda, *J. Am. Chem. Soc.*, 1994, **116**, 5513.
9 S. Nakagawa, Y. Tamakashi, Y. Ishibashi, M. Kawase, S. Taketomi, O. Nishimura and T. Fukuda, *Biochem. Biophys. Res. Commun.*, 1994, **200**, 1735.
10 O. Nishimura, T. Moriya, M. Suenaga, Y. Tanaka, T. Itoh, N. Koyama, R. Fujii, S. Hinuma, C. Kitada and M. Fujino, *Chem. Pharm. Bull.*, 1998, **46**, 1490.
11 O. Nishimura, M. Suenaga, H. Ohmae, S. Tsuji and M. Fujino, *Chem. Commun.*, 1998, 1135.
12 M. Suenaga, H. Ohmae, N. Okutani, T. Kurokawa, T. Asano, T. Yamada, O. Nishimura and M. Fujino, *J. Chem. Soc., Perkin Trans. 1*, 1999, 3727.
13 R. Rudolph, in *Modern Methods in Protein and Nucleic Acid Analysis*, ed. H. Tchesche, Walter de Gruyter, Berlin and New York, 1990, pp. 149–171.
14 M. Suenaga, H. Ohmae, S. Tsuji, T. Itoh and O. Nishimura, *Biotechnol. Appl. Biochem.*, 1998, **28**, 119.
15 H. M. McConnell, J. C. Owicki, J. W. Parce, D. L. Miller, G. T. Baxter, H. G. Wada and S. Pitchford, *Science*, 1992, **257**, 1906.