COMMUNICATION

Bio-organic synthesis of a newly isolated peptide metastin, metastasis suppressor gene *KiSS-1* processing product †

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Metastin, a newly isolated peptide ligand of orphan receptor hOT7T175, was synthesized by a bio-organic technique combining recombinant DNA technology with a cysteine-specific cleavage reaction.

KiSS-1 is a tumor metastasis suppressor gene isolated by subtractive hybridization of metastatic and nonmetastatic human melanomas.[1] The *KiSS-1* gene has a 435 bp open reading frame, however its gene product has not been well characterized.[2] Recently, we found that the *KiSS-1* gene encodes a precursor protein for a novel peptide, metastin, which is the endogenous ligand of an orphan G-protein-coupled receptor named hOT7T175.[3] In the search for endogenous ligands of the hOT7T175 receptor, we found that human placental extract possessed potent activity that induced a robust increase in the intracellular calcium ion concentration of hOT7T175-expressing Chinese hamster ovary (CHO) cells. This activity was purified and assigned to a C-terminally amidated peptide of 54 amino acid residues (Fig. 1) that was generated by the processing of the *KiSS-1* gene product. To investigate the biological activity of this peptide, we developed a procedure for its synthesis. The details of the biological activities of this peptide, including its receptor-binding affinity, calcium-mobilizing activity, cell migration inhibitory activity, cell invasion inhibitory activity, and tumor metastasis suppressing activity, were described elsewhere.[3]

Advances in DNA technology have facilitated the preparation of many biologically active proteins. However, the low molecular weight, biologically active peptides often fail to accumulate in the cells since they are rapidly degraded by host proteases.[4] Small peptides have usually been synthesized by gene fusion systems. To obtain the desired product from the fusion protein, it is important to design a method for site-specific cleavage. There are two major ways to prepare peptides by specific cleavage of fusion proteins: either enzymatic or chemical methods. Unlike chemical methods, enzymatic cleavage is affected by structural factors. Proteases are often inefficient when the cleavage site is buried in the fusion protein. Therefore, the cleavage site must be carefully engineered to be structurally accessible to the enzyme. In contrast, chemical methods are not affected by steric factors since chemical cleavage reactions can proceed under denatured conditions. Moreover, the reagents are easily separated from the reaction mixture by dialysis or a desalting step.

Previously, we reported an efficient chemical method to obtain small peptides.[5–10] The procedure of the method was as follows. The peptides were expressed in the form of fusion proteins with the basic fibroblast growth factor mutein (CS23). The fusion proteins were easily purified by heparin affinity chromatography since CS23 has a high affinity for heparin.[11] The proteins were cleaved at the cysteine residues of the junction site by cyanylation, and then exposure to alkaline pH. This method permits the preparation of the C-terminal amide form by treatment of the cyanylated fusion protein with aqueous ammonia, while the C-terminal acid form can be obtained by treatment with aqueous sodium hydroxide. Here, we describe the synthesis of metastin and its acid form by a combination of recombinant DNA technology and a cysteine-specific cleavage reaction.

Scheme 1 shows the strategy for the preparation of metastin. To obtain metastin, we constructed a metastin-CS23 expression vector in which metastin was fused at the N-terminus of CS23 with a cysteine residue as a linker. The vector was transfected into *Escherichia coli* (*E. coli*), and transformant *E. coli* MM294 (DE3)/pTFCKiSS-1 was obtained. The transformant was cultivated and the cells were collected by centrifugation. The cell-free extract was obtained by sonication and then the metastin-CS23 fusion protein was purified by heparin affinity column chromatography. The SH groups of the metastin-CS23 fusion protein were converted to SCN groups with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (DMAP-CN). Specific cleavage of the cyanylated protein was accomplished in 3 M ammonia containing 6 M urea at 25 °C, followed by passage through a column of Sephadex G-25. The resulting crude product was purified by chromatography on SP-5PW and C4P-50, and purified metastin was obtained. To confirm the structural identity of the purified metastin, protein chemical analysis was performed. The N-terminal amino acid sequence and the amino acid composition analysis were all in good agreement with those predicted from the corresponding DNA sequence. The C-terminal residue of the peptide could not be detected by hydrazinolysis. This result indicated that the C-terminal residue was in the amide form. Moreover, the C-terminal acid form of metastin was obtained by treatment with 0.05 M NaOH after the cyanylation reaction. The gel filtration profile, sodium dodecyl sulfate–polyacrylamide gel electrophoresis and reversed-phase HPLC (Fig. 2) of the purified metastin showed high homogeneity. It also gave the

† Electronic supplementary information (ESI) available: SDS-PAGE and mass spectrum of metastin. See http://www.rsc.org/suppdata/p1/b1/b104948h/

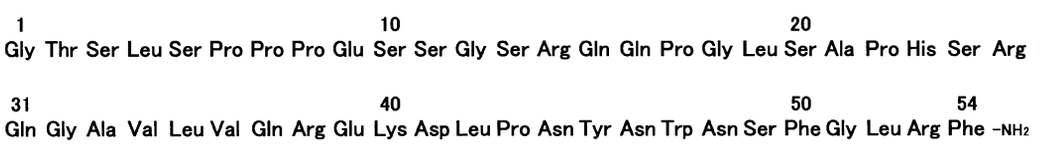

Fig. 1 Amino acid sequence of metastin.

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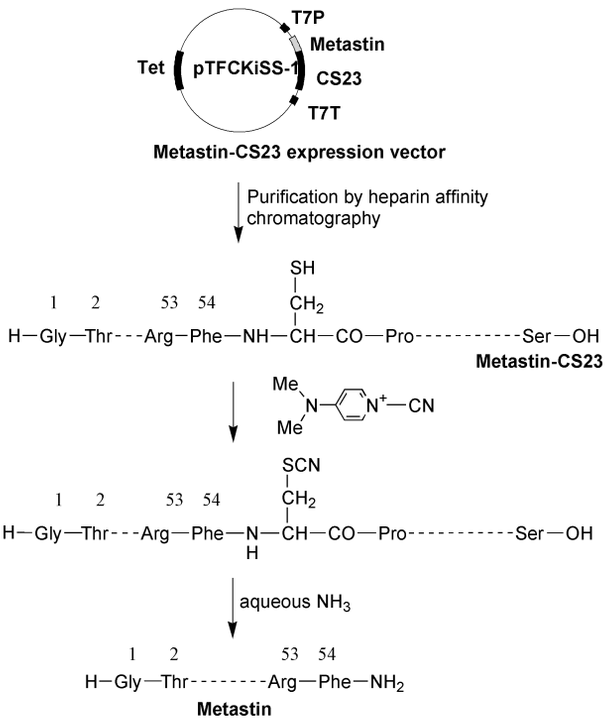

Scheme 1 Strategy for the preparation of metastin. T7P, T7 promoter; T7T, T7 transcription terminator; Tet, tetracycline resistance gene.

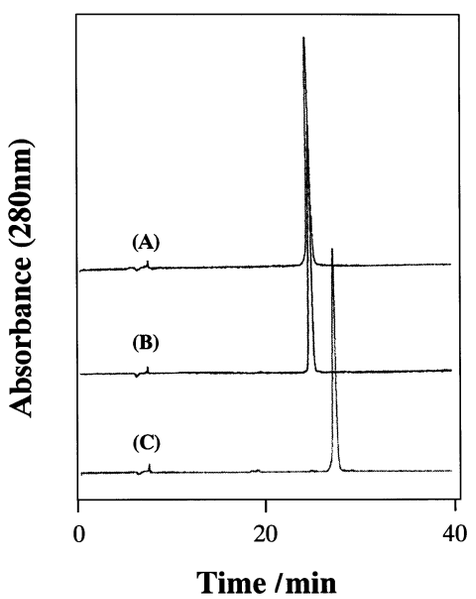

Fig. 2 HPLC profile of the purified metastin. (A) chemically synthesized metastin (amide form), (B) metastin (amide form) and (C) metastin (acid form) obtained from the fusion protein after cyanylation reaction. Peptides (10 μg) were applied to a C4P-50 column (4.6 × 250 mm) (Showa Denko, Japan) and eluted with a linear gradient of acetonitrile (20–36%) in the presence of 0.1% TFA.

expected result upon molecular weight (*M*) measurement by electrospray ionization mass spectrometry (observed: 5857.7 *vs.* theoretical: 5857.5). The metastin (amide form) thus obtained was subjected to biological assay, and the results indicated that the metastin (amide form) and a chemically synthesized standard (amide form) equally increased the calcium response in CHO cells expressing hOT7T175. In contrast, the C-terminal acid form could not increase the calcium response, indicating that the C-terminal amide structure is essential for biological activity.

Thus, we obtained metastin in high purity by bio-organic synthesis combining recombinant DNA technology with a cysteine-specific cleavage reaction. It is known that the synthesis of peptides of the size described here is still problematic using solid phase synthesis. In this paper, we have shown that our method is applicable to the preparation of biologically active cysteine-free peptides. The obtained peptide might be helpful to discover the therapeutic agents of metastatic melanoma.

Experimental

Construction of the expression plasmid

Plasmid pTFC was derived from pTB960-7.[5] The fragment of metastin structural gene was prepared by the annealing of six synthetic oligonucleotides and was inserted into the *Nde I-Ava I* cloning site of pTFC to obtain the expression plasmid pTFCKiSS-1.

Expression of metastin-CS23

Plasmid pTFCKiSS-1 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 metastin-CS23 fusion protein

E. coli cells (500 g wet weight) were suspended in 1500 ml of 20 mM EDTA (pH 6.0) and 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride and disrupted by sonication. The suspension was centrifuged at 17000 *g* for 60 min and the supernatant was pooled. The pellets were re-extracted in the same manner as described above, then centrifuged. The supernatants were combined and applied to an AF-Heparin Toyopearl 650M (11.3 × 13.5 cm) (Tosoh, Japan) equilibrated with 50 mM phosphate buffer (pH 6.0) at a flow rate of 50 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, USA).

Cleavage of metastin-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, Sweden) 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 3 M ammonia, and incubated at 25 °C for 15 min.

Purification of metastin

The reaction mixture was applied to a Sephadex G-25 column (4.6 × 50 cm) equilibrated with 50 mM 2-morpholinoethanesulfonic acid (MES) buffer (pH 4.5) at a flow rate of 10 ml min^{-1} and the sample was eluted with the same buffer. The main fractions were pooled. The eluate was applied to a SP-5PW column (5.5 × 30 cm) (Tosoh, Japan) equilibrated with 50 mM MES buffer (pH 4.5) containing 3 M urea at a flow rate of 35 ml min^{-1}. The protein was eluted with a linear gradient of NaCl (0–1 M). The desired fraction was applied to a C4P-90 column (5 × 50 cm) (Showa Denko, Japan) at a flow rate of 30 ml min^{-1} and eluted with a linear gradient of 16–40% acetonitrile in the presence of 0.1% TFA. The eluate was collected and lyophilized (300 mg).

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