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Production of C-Terminal Amidated Recombinant Salmon Calcitonin in *Streptomyces lividans*

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Abstract

Salmon calcitonin (sCT) is one of the many bioactive peptides that require C-terminal amidation for full biologic activity. To produce fully bioactive sCT in large scale, we constructed *Streptomyces lividans* [pMSA], an engineering *Streptomyces* strain. In the expression vector, glycine-extended sCT, the substrate for amidation, and rat α-amidating enzyme cDNA were cloned under the control of the strong constitutive promoter from the *Streptomyces fradiae aph* gene in pIJ680. Both were expressed in a secretory manner by the recombinant strain using the expression and secretion signals of *melC1*. Extracellularly expressed recombinant sCT was purified to near homogeneity and characterized by enzyme immunoassay, followed by direct amino-terminal sequencing. High-performance liquid chromatography, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry, and bioassay in vivo demonstrated purified product to be equivalent to synthetic standard. Thus, the engineered *Streptomyces* strain can produce bioactive, C-terminal amidated recombinant sCT in the culture supernatant directly. The ease of the recombinant process, as well as its potential for scale-up, makes it adaptable to production demands for sCT, and it may be applied to other bioactive peptides that need C-terminal amidation.

Index Entries: Salmon calcitonin; α-amidating enzyme; *Streptomyces lividans*.

Introduction

Calcitonin (CT) is a 32 amino acid peptide hormone produced from the C-cells of the thyroid gland in mammals and the ultimobranchial gland in nonmammalian vertebrates. Although CTs from different species are not highly conserved at the amino acid level, they share the basic structural

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features including a single disulfide bridge between the cysteine residues in positions 1 and 7, an amphipathic α-helix, and the C-terminal prolinamide; the last two features are essential for biologic activity *(1)*. As an endogenous regulator of calcium homeostasis, CT's main physiologic action is to inhibit osteoclastic bone resorption, and it is also involved in the central nervous system (e.g., it has analgesic effects). Salmon CT (sCT), which is more potent and longer lasting than human CT, has been used widely for the treatment of osteoporosis, Paget's disease, hypercalcemic shock, and chronic pain in terminal cancer patients.

Presently used sCT is produced by conventional peptide synthesis. Although bioactive recombinant sCT can be produced in eukaryotic expression systems *(2,3)*, they may not produce enough quantities to be cost competitive with chemical synthesis. The prokaryotic expression systems lack the necessary enzymatic machinery for production of C-terminal amides and require multiple-step processing to amidate the precursor peptide (sCT-Gly or sCT-Ala) in vitro *(4,5)*. α-Amidating enzyme (α-AE) is the posttranslational processing enzyme responsible for the conversion of glycine-extended substrates to the biologically active, C-terminal amidated products. The cloning, expression, and purification of soluble, secreted rat 75-kDa α-AE from recombinant mammalian cells has been reported *(6)*. The secretorily expressed rat 75-kDa α-AE in *Streptomyces lividans*, a prokaryotic expression system, has been shown to be bioactive *(7)*.

Streptomyces is one of the most important industrial microorganisms for production of many valuable compounds, such as antibiotics and industrial enzymes. *S. lividans* has been used extensively as an expression host for a wide variety of genes from diverse sources over the last decade *(8)*. Some eukaryotic proteins can be secreted in large amounts with biologic activity in *S. lividans (9,10)*. Such excellent secretion capacity for large amounts of active proteins makes streptomycetes interesting hosts for the secretory production of various recombinant proteins. Here, we report a recombinant process for the direct production of C-terminal amidated sCT in *S. lividans*.

Materials and Methods

Enzymes and sCT

Restriction endonucleases and other DNA-modifying enzymes were obtained from Promega. The oligonucleotides were ordered from Sangon (Shanghai, China). Thiostrepton was a gift from Bristol-Myers Squibb. Salmon DNA and sCT were obtained from Sigma. The sCT enzyme immunoassay kit was purchased from Peninsula.

Bacterial Strains, Plasmids, and Growth Conditions

Escherichia coli JM109 and *S. lividans* TK54 were used as hosts for cloning the genes and expression, respectively. Subcloning of DNA fragments was done in pUC18/19. Plasmid pIJ680 *(11)* was used as basic vector for

expression of amidated sCT. This plasmid contains the thiostrepton-resistant gene *tsr* and *aph* gene, which encodes the enzyme aminoglycoside 3'-phosphotransferase.

E. coli strains harboring recombinant plasmids were grown at 37°C (300 rpm) in Luria broth in the presence of ampicillin (50 µg/mL). *S. lividans* TK54 was grown at 28°C with continuous shaking at 300 rpm in yeast extract malt extract medium (YEME) *(11)* plus 34% sucrose for plasmid isolation and protoplast preparation. Thiostrepton (5 µg/mL) was added when necessary. Protoplast formation and subsequent transformation of *S. lividans* were carried out as described by Hopwood et al. *(11)*. Expression studies were done using casein medium, consisting of 3% D(+)-glucose, 1% yeast extract, 2% casein enzymatic hydrolysate, and 1% trace element solution (v/v) (0.02% $ZnSO_4 \cdot 7H_2O$, 0.1% $FeSO_4 \cdot 7H_2O$, 0.0025% $CuCl_2 \cdot H_2O$, 0.00056% H_3BO_3, 0.1% $MnSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot H_2O$, 0.0019% $[NH_4]_6 Mo_7O_{24} \cdot 4H_2O$). Primary cultures were incubated for 48 h at 28°C and 300 rpm in shake flasks. For monitoring recombinant protein expression and secretion, 5-mL primary cultures were inoculated to 500-mL shake flasks containing 50 mL of casein medium and grown at 28°C and 300 rpm for 4 d. For production of recombinant sCT, the recombinant strain was cultured for 72 h in a 7-L fermentor (NBS Bioflo III) at 28°C. Casein medium (3.5 L) was used in the fermentor, and the dissolved oxygen level was controlled to >40% of the air saturation value by controlling the agitation speed. The pH was monitored during the fermentation.

Construction of Expression Plasmid pMSA

DNA manipulations were carried out using standard techniques *(12)*. According to the known sCT sequence in salmon DNA *(13)*, the primer sets P1/P2 (P1, 5'-TGCACTGCAGCAACCTCAGCACCTGT-3', P2, 5'-GC GGATCCTACTAGCCCGGCGTACCACTTCCCG-3') were used to amplify sCT-Gly coding sequence. According to the known *melC1* sequence originated from *Streptomyces antibioticus (14)*, the primer sets P3/P4 (P3, 5'-GCTGATCACGTCAGTTTTCG-3'; P4, 5'-TGCACTGCAGGCGCGGGC GGC GGGAAG-3') were designed to amplify the sequence of the expression and secretion signals of *melC1*. To obtain a direct in-frame fusion between the *melC1* signal sequence and sCT-Gly coding sequence, the serine codon TCC of sCT-Gly was altered to AGC in primer P1, and the last alanine codon GCG of *melC1* signal sequence was altered to GCC in primer P4 to get a *Pst*I site (underlined) in both primers. In primer P2, two stop codons (TAG) and a *BamH*I site (underlined) were added after glycine codon GGC. A *Bcl*I site (underlined) upstream of the promoter of *melC1* was included in P3.

As shown in Fig. 1, the amplified sCT-Gly coding fragment and the fragment of *melC1* expression and secretion signals were cloned separately within *BamH*I and *Pst*I of plasmid pUC19. The sequences were confirmed by DNA sequence analysis. Then the *Pst*I/*BamH*I fragment of sCT-Gly and the *EcoR*I/*Pst*I fragment of *melC1* were ligated with *EcoR*I/*BamH*I-cut pUC19, resulting in pUC19-mel/sCT. A *Sac*I/*Xba*I fragment of

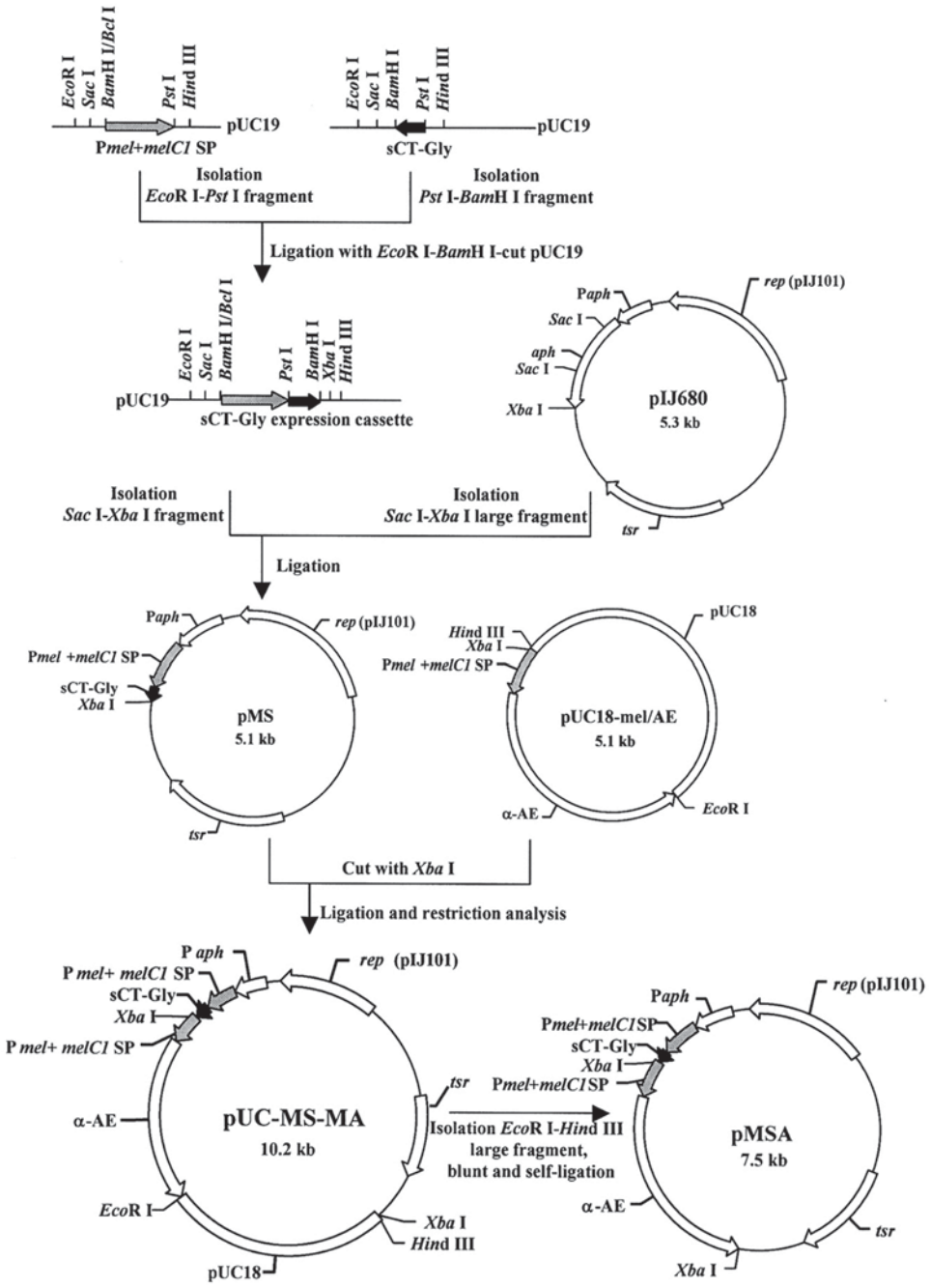

Fig. 1. Schematic of expression plasmid pMSA construction.

pUC19-mel/sCT containing expression cassette of sCT-Gly was transferred downstream of the strong constitutive promoter from the *Streptomyces fradiae aph* gene in the streptomycete multicopy plasmid pIJ680, resulting in plasmid pMS.

The same fragment of *melC1* expression and secretion signals was used to construct the expression cassette of α-AE cDNA in pUC18-mel/AE *(7)*, in which α-AE cDNA was fused in frame with *melC1* signal sequence. After *Xba*I digestion, pUC18-mel/AE was ligated with the same cut pMS. The resulting plasmid pUC-MS-MA, in which the α-AE expression cassette was cloned downstream of the sCT-Gly expression cassette, was identified by restriction analysis. Then the large *Eco*RI/*Hin*dIII fragment of pUC-MSA was blunted with Klenow fragment polymerase and self-ligated, yielding the expression vector pMSA (Fig. 1).

Purification of Recombinant sCT

The fermentation broth was harvested at 3000 rpm for 15 min at 4°C and decolored by resin absorbents Diaion® HP-20 (Supelco). Then the sample was adjusted to 1 *M* $(NH_4)_2SO_4$, 50 m*M* phosphate-buffered saline (PBS) (pH 7.0) and loaded onto a Phenyl Sepharose™ 6 Fast Flow column equilibrated in 1 *M* $(NH_4)_2SO_4$, 50 m*M* PBS (pH 7.0). Proteins were eluted from this column by applying a linear gradient of $(NH_4)_2SO_4$ from 1 to 0 *M* in 50 m*M* PBS (pH 7.0). Fractions containing sCT detected by enzyme immunoassay (EIA) were pooled, ultrafiltered (1000 nominal molecular weight limits, low-binding regenerated cellulose; Millipore) using a Minitan™ system (Millipore), and lyophilized. The lyophilized proteins were dissolved in 5 mL of 10% acetic acid and applied to Superdex 30 prep-grade column using ÄKTAexplorer 100 (Amersham) and eluted with the same buffer. The fractions containing sCT activity were lyophilized and dissolved in 1 mL of water and then applied to a Supelcosil™ LC-308 C_{14} column (1 × 25 cm, 5 µm, 300 Å; Supelco) equilibrated in 0.05% trifluoroacetic acid (TFA). The proteins were eluted with a gradient of 20–50% acetonitrile in 0.05% TFA at 2 mL/min. The peak with the same retention time as the standard sCT was collected and lyophilized. Peptide purity was analyzed on a Supelcosil L-308 C_{14} column (4.6 × 50 mm, 5 µm, 300 Å) using 5–50% CH_3CN in 0.05% TFA gradient over 30 min.

N-terminal Amino Acid Sequence Analysis

The N-terminal amino acid sequence of recombinant sCT was determined using an ABI Procise 491 protein-sequencing system.

Molecular Weight Analysis

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed by the Institute of Chemistry, Chinese Academy of Sciences.

Enzyme Immunoassay

In vitro immunologic activity of recombinant sCT was performed with the peptide enzyme immunoassay kit (Peninsula) as described by the manufacturer.

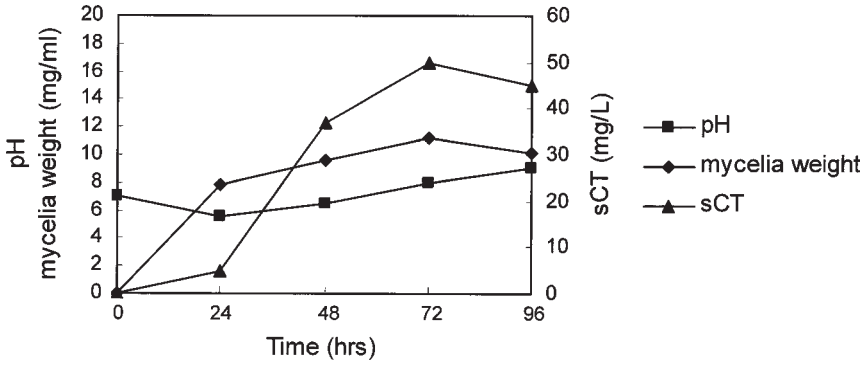

Fig. 2. Profile of sCT expressed by *S. lividans* [pMSA] in shake flasks.

Bioassay

The biologic activity of recombinant sCT was demonstrated in vivo with rat hypercalcemic assay as described in the British Pharmacopoeia and performed by the Department of Pharmacology, Institute of Basic Medicine, Chinese Academy of Medical Sciences.

Results

Secretory Expression of sCT by S. lividans *TK54*

sCT is synthesized in the salmon ultimobranchial gland as a preprocalcitonin, which is then processed to sCT-Gly as the substrate for α-AE. Thus, for the amidation of recombinant sCT, a glycine codon was added before stop codons as the thirty-third amino acid of sCT coding sequence by polymerase chain reaction. For secretory expression of sCT-Gly and α-AE, the expression and secretion signals of *melC1* (the promoter, the ribosome-binding site, and the signal peptide) were used to construct the expression cassette of sCT-Gly and α-AE. The expression plasmid pMSA, in which the two expression cassettes were strung together, may also use the *aph* promoter to direct the expression of sCT-Gly and α-AE. *S. lividans* TK54 transformed with pMSA, named *S. lividans* [pMSA], was used to produce C-terminal amidated sCT in a secretory manner. The α-amidating activity of α-AE produced by recombinant *S. lividans* was recently identified in our laboratory *(7)* with the amidation of a synthetic tripeptide dansyl-Tyr-Val-Gly followed by high-performance liquid chromatography (HPLC) separation and fluorescence detection. The highest activity of α-AE was detected in the culture supernatant of 48 h. The secretion of sCT within culture supernatants was detected by EIA (including amidated and unamidated). In shake-flask culture, the expression level of sCT was highest at 72 h and decreased after prolonged growth in stationary phase (Fig. 2). The intracellular recombinant sCT was almost not detectable by EIA assay after 24 h. Similar to the flask culture, the pH of the culture in the 7-L fermentor was slowly decreased to about pH 5.0 at 48 h and then increased

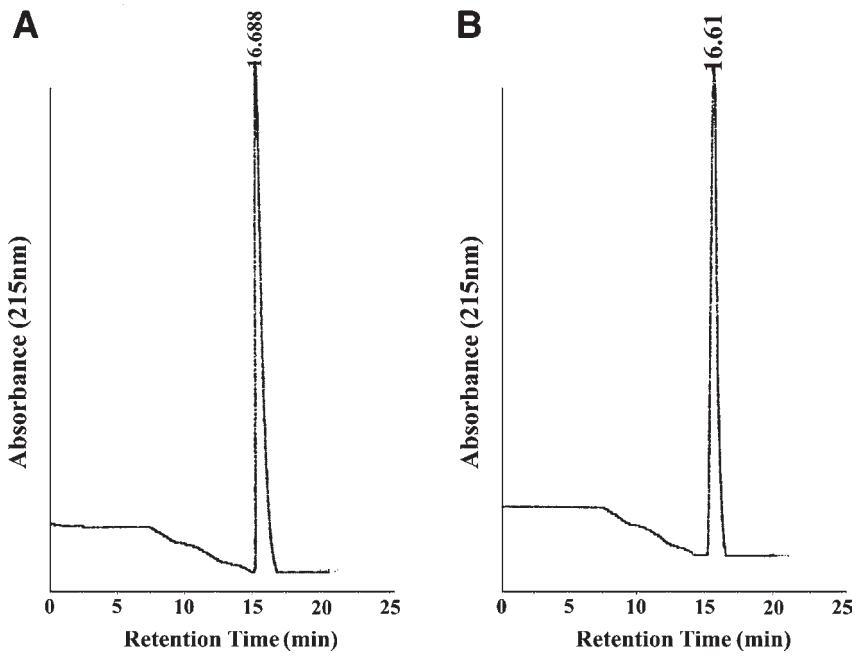

Fig. 3. RP-HPLC analysis of **(A)** sCT standard sample, with a retention time of 16.688 min, and **(B)** purified recombinant sCT, with a retention time of 16.61 min.

to pH 7.5–8.0 at 72 h. The expression level of recombinant sCT in the fermentor was confirmed to be >30 mg/L.

Purification and Characterization of Recombinant sCT

Recombinant sCT was isolated from the fermentation supernatant of *S. lividans* [pMSA] at 72 h. Decolored by resin HP-20, the culture supernatant was purified by the column chromatography of Phenyl Sepharose 6 Fast Flow, Superdex 30 prep-grade, and preparative reverse phase (RP)-HPLC. The recombinant sCT was determined by EIA within the purification procedure. The overall yield for the purification steps was about 15%. The identity and purity of purified sCT were confirmed by N-terminal sequencing, analytical RP-HPLC, and MALDI-TOF MS. The first 15 residues by N-terminal amino acid sequence analysis were consistent with the known sequence of sCT. HPLC analysis of the purified material had an identical retention time to the authentic standard and was >95% pure (Fig. 3). The amidated sCT was 41.6% of the total recombinant sCT, according to the HPLC analysis before the preparative RP-HPLC. The same mass spectrum as the authentic standard was obtained (Fig. 4), without the peak of sCT-Gly (mol wt = 3490), demonstrating effective removal of sCT-Gly by preparative RP-HPLC. That the recombinant sCT could be amidated at the C-terminal during culturing of *S. lividans* [pMSA] is therefore confirmed.

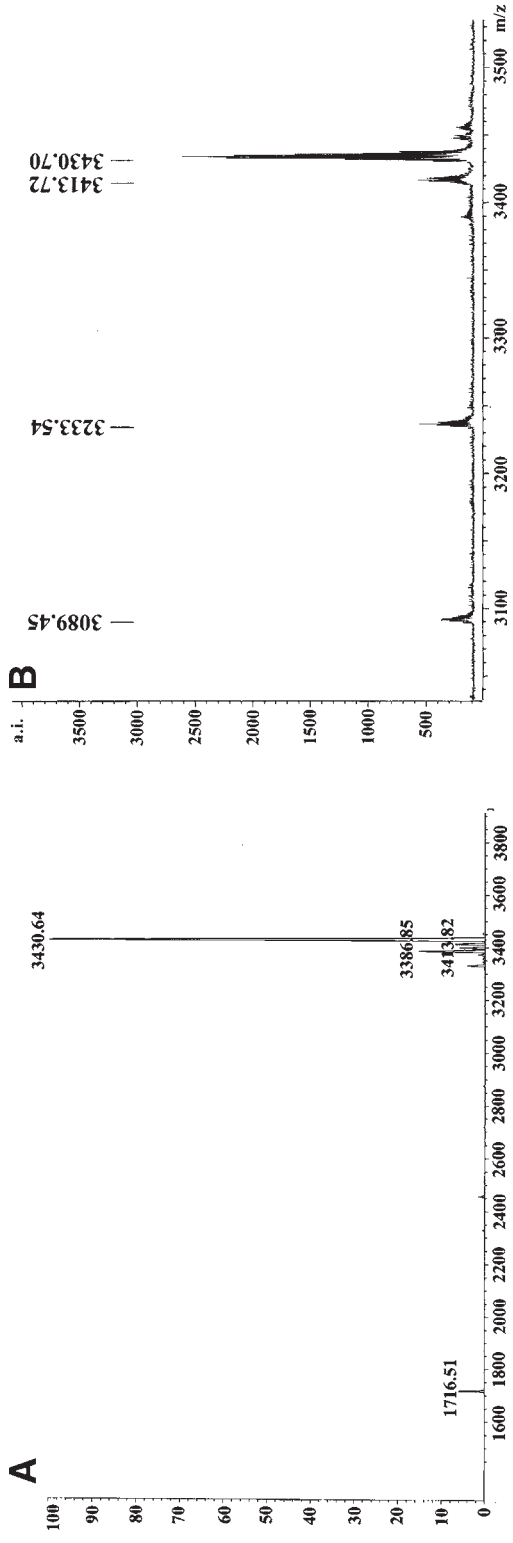

Fig. 4. MALDI-TOF MS analysis of **(A)** sCT standard sample, with a main peak of 3430.64 Daltons, and **(B)** purified recombinant sCT, with a main peak of 3430.70 Daltons.

Bioassay Analysis

The biologic activity assays of the recombinant sCT were carried out using a rat hypercalcemic assay in vivo according to the standard protocol (British Pharmacopoeia). The amidated sCT exhibits much higher bioactivity than does its immediate precursor, sCT-Gly. Statistical analysis results showed that the biologic activity of recombinant sCT is 4000 IU/mg, which is equivalent to authentic sCT standard.

Discussion

Synthetic sCT is a therapeutic peptide widely used for treating osteoporosis. In addition, sCT produces analgesia with a mechanism different from opiate and is effective in patients with refractory pain such as the pain of advanced cancer *(15)*. However, one of the major disadvantages of sCT therapy is its high cost. The therapeutic use of the peptide has necessitated the search for suitable industrial microorganisms for large-scale production of recombinant sCT. Many bioactive peptides including sCT require an amidated C-terminus for full activity. Ray et al. *(4)* succeeded in producing amidated sCT by using recombinant α-AE secreted from Chinese hamster ovary cells for amidation of the glycine-extended sCT, which was produced by *E. coli* and purified. Hong et al. *(5)* reported the amidation of alanine-extended sCT produced by *E. coli* using enzymatic transacylation and photolysis in vitro. In these procedures, to avoid *E. coli* proteases, the sCT precursor was expressed as a fusion with glutathione-*S*-transferase; then a multistep process of purification of sCT-Gly or sCT-Ala, in vitro amidation of the substrate, and reformation of the intramolecular disulfide bond is necessary to produce full bioactive sCT.

Streptomyces are Gram-positive, spore-forming soil microorganisms, which are most often considered a vast reservoir of nature products, such as antimicrobial, antifungal, antitumor, and immunosuppressive compounds. They are also used widespread for industrial production of extracellular enzymes. The ability of *Streptomyces* to secrete eukaryotic proteins was first reviewed by Chang et al. *(16)*, who utilized an expression system in *S. lividans* harboring the stable, multicopy plasmid pIJ702 with additional transcriptional, translational, and secretory elements drawn from separate species of *Streptomyces*. The strong constitutive promoters including the *S. fradiae* aminoglycoside phosphotransferase (*aph*) gene promoter were utilized in combination with signal peptide from the *S. antibioticus* tyrosinase gene locus (*melC1*). Since then, many articles have been published on the expression of eukaryotic proteins in *Streptomyces* that have further illustrated the general utility and versatility of this genus as a host for expression of bioactive proteins *(17–22)*. *S. lividans* has some superiorities as a commercially viable host, such as an excellent capacity to secrete properly folded, bioactive proteins into the medium; a low level of endogenous protease activity; generally recognized as safe status, and the extensive fermentation experience gained from their long use in antibiotic production.

In our study, we coexpressed and secreted glycine-extended sCT and α-AE in *S. lividans* and purified the authentic sCT directly from culture supernatant. The strategy we used was to express sCT-Gly gene and rat α-AE cDNA simultaneously by *S. lividans* [pMSA], in which *melC1* expression and secretion signals were used to direct secretion of sCT precursor and α-AE separately. Although α-AE is a eukaryotic enzyme with glycosylation, it has been reported that glycosylation does not affect the activity of the amidating enzyme *(23)*. Furthermore, *Streptomyces* has the ability to produce enzymes that require ascorbate for full activity *(24)*. By adding copper to the medium, another mandatory factor for α-AE, recombinant *Streptomyces* may secrete active α-AE into the medium *(7)*. The α-AE produced by *S. lividans* with α-amidating activity coexisted with sCT-Gly in the culture supernatant, and the conversion of sCT-Gly into sCT might occur at this stage. According to the analysis results of RP-HPLC, MALDI-TOF MS, and bioassay in vivo, we can conclude that the recombinant sCT purified directly from the culture medium of *S. lividans* [pMSA] was amidated at the C-terminus, which has the same bioactivity as native sCT. Although the overall yield is similar with production of recombinant sCT in *E. coli (4,5)*, downstream purification is much more simplified in the present strategy because in vitro enzymatic or chemical amidation and renaturation are not necessary. With the well-developed techniques for *Streptomyces* culture in industrial facilities, production of amidated sCT might be optimized and scaled up to meet increasing demand.

In summary, the studies reported here demonstrated direct production of recombinant amidated sCT in *S. lividans* by coexpression of sCT-Gly and α-AE. It may be a feasible alternative to in vitro–catalyzed amidation of the precursor produced by *E. coli*. This procedure may also provide one of the potentially useful ways for C-amidation of other recombinant peptides, which require C-terminal amidation for full biologic activity. Furthermore, because the chemical synthetic cost increases dramatically with peptide length, this recombinant process may provide a more reasonable choice for the production of longer bioactive peptides.

Acknowledgments

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