Biochemical and Biophysical Research Communications **267,** 362–367 (2000)
doi:10.1006/bbrc.1999.1961, available online at http://www.idealibrary.com on IDEAL®

Production of Recombinant Salmon Calcitonin by Amidation of Precursor Peptide Using Enzymatic Transacylation and Photolysis *in Vitro*

Dou Hong, Zhuang Mingqiang, Li Min, Chen Changqing, and Mao Jifang[1]
Department of Biochemistry and Molecular Biology, Second Military Medical University, Shanghai 200433, China

Received November 5, 1999

The C terminal amidation is required for full biological activity of salmon calcitonin (sCT). We constructed BL21(DE3)/pGEX-sCT-Ala, an engineering *Escherichia coli* strain. The soluble fusion protein of GST-sCT-Ala expressed from BL21(DE3)/pGEX-sCT-Ala was purified by affinity chromatography after high density, high expression culture and sonication of bacteria. Following S-sulfonation of the fusion protein, the 33 alanine-extended peptides were released from the fusion protein by cyanogen bromide. The S-sulfonated precursor peptide was transacylated by CPD-Y, o-PNGA as a nucleophile, to produce photosensitive SO_3^--sCT-o-PNGA. After photolysis and folding, the biological activity of sCT was assayed as standard.

Calcitonin (CT) consisting of 32 amino acid residues is secreted by the parafollicular cells ("C" cells) of the thyroid, an endogenous regulator of calcium homeostasis, acting principally on bone. In nonmammalian vertebrates, CT is produced by ultimobranchial body. The therapeutic use of CT is involved in hypercalcemia, Paget's disease of bone, high-bone-turnover osteoporosis, vitamin-D intoxication, bone metastases, and chronic pain associated with bone disease.

C-terminal amidation is one crux of production of CT. *In vitro,* chemical synthesis of C-terminal amide of CT has been accomplished by one of the following procedures: (a) solid-phase synthesis using an acid-labile attachment (anchoring) group based on benzhydrylamine (or benzylamine), ammomolysis, catalytic transfer hydrogenolysis or a photocleavable anchor group; (b) liquid-phase peptide synthesis employing polyethylene glycol derivatized with an acid-cleavable or photocleavable anchoring group; (c) enzymatic transacylation using either ammonia or amino acid α-amides as nucleophiles. But waste disposal of chemical synthesis can bring on a lot of questions; sometimes the synthetic cost of long peptides is high and the efficiency is poor.

Long peptides can be produced by biological engineering, but prokaryotic expression systems lack the necessary enzymatic machinery for production of C-terminal amides. Amidation of enzyme catalysis by peptidylglycine α-amidating monooxygenase (PAM) and α-amidating enzyme (α-AE) has been used for C-terminal amidation [1, 2], however the preparation of PAM and α-AE by gene engineering or extraction are time-cost and expensive. Eukaryotic expression systems that are capable of *in vivo* modification may not produce sufficient quantities of calcitonin to be cost competitively with conventional peptide synthesis [3]. To overcome above-mentioned shortcomings, a recombinant process of prokaryotic expression systems combining CPD-Y (carboxypeptidase Y) transacylation with photolysis *in vitro* was developed in this paper.

MATERIALS AND METHODS

Materials

Vector DNA of pGEX-4T-3 and beads of Glutathione Sepharose 4B were purchased from Pharmacia Biotech. *Xho*1, *Bam*H1, ligase, T_4 polynucleotide kinase and vector DNA of pGEM-7(z^+) were obtained from Promega. All the oligonucleotides were ordered from Sangon (Shanghai). Most of the chemicals were from Sigma Chemical Co.

Synthetic Salmon Calcitonin cDNA and Plasmid Construction

The eight oligonucleotides, 20–45 bases in length, were synthesized by ABI-391 DNA synthesis instrument and were designed to use prokaryote preference codes in order to afford high expression of recombinant sCT in *E. coli* host cells. The overlapping oligonucleotides had the following sequences: F1, 5′-GATCCGACATCGAAGGTCGTTGCTCTAACCTGTCTACTTGC-3′; F2, 5′-GTTCTGGGTAAACTGTCTCAG-3′; F3, 5′-GAACTGCACAAACTGCAGACTTACCCGCGTACTAAC-3′; F4, 5′-ACTGGTTCTGGTACTCCGGCTTGATAAC-3′; F5, 5′-GTTAGAGCAACGACCTTCGATGTCG-3′; F6, 5′-TTTGTGCAGTTCCTGAGACAGTTTACCCAGAACGCAAGTAGACAG-3′; F7, 5′-ACCAGAAC-

[1] To whom correspondence should be addressed. Fax: 86-21-65491311. E-mail: jifang@hotmail.com.

0006-291X/00 $35.00

CAGTGTTAGTACGCGGGTAAGTCTGCAG 3′; F8, 5′-TCGAGTTAT-CAAGCCGGAGT-3′.

The synthetic cDNA fragment contained cohesive end of *Bam*H1 at 5′ terminal, cohesive end of *Xho*1 at 3′ terminal, and extra-sequence for encoding Met^{-1} and Ala^{+33}. The 20 μl mixture of 20 pmol each oligonucleotide was phosphorylated with 2 μl T_4 kinase (10 U/μl) and 2 μl 10× ligase buffer, then was incubated 30 min at 37°C. The mixture was heated to 95°C for 2 min to inactivate kinase and allowed to anneal over 35 min. For ligation reaction, 10 μl annealing mixture, 2 μl 10× ligase buffer, 2 μl ligase and 6 μl pGEM7z(+) cloning vector digested by *Bam*H1 and *Xho*1, were incubated at 14°C overnight. The reaction mixture was transformed into JM109, clones with desired recombinant plasmid were selected by α-complementation. After sequencing of insert DNA in pGEM7z(+) by ABI PRISM 310 Genegic Analyzer, the sCT cDNA was cloned into vector pGEX-4T-3 again, and then the recombinant pGEX-sCT plasmid was transformed into BL21 (DE3).

Expression and Affinity Purification of Recombinant Fusion Substrate

Fed-batch cultivation was carried out with 5 L fermentor (NBS BioFlo 3000 type) according to high-density culture of recombinant human calcitonin analogue [4]. 3 L production medium supplemented with 2% glycerol was used as a start-up medium. During the batch fermentation, the temperature and pH were maintained at 37°C and 7.0, respectively, and the dissolved oxygen level was controlled to 30% of the air saturation value by controlling the agitation speed. After the 2% glycerol had been consumed, an 80% glycerol solution was fed to promote transcription. The fed-batch cultivation was continued until cell growth had ceased.

The cells was pelleted at 7000 rpm for 15 min at 4°C and frozen prior to lysis. 150 g frozen cells was thawed and resuspended in 1.5 L PBS, disrupted by sonication (Sonic material, turn 20%, output 80%). 20% Triton X-100 was added to final concentration of 1%, mixed gently for 30 min to aid solubilization of the fusion protein, then centrifuged at 15,000*g* for 20 min at 4°C. The supernatant was transferred to 40 ml glutathione Sepharose 4B equilibrated with 1× PBS, incubated with gently agitation at room temperature for 30min. The mixture was centrifuged at 500*g* for 5 min to sediment the matrix. The sedimented matrix was packed into column (5 × 20 cm) and washed with PBS until the A_{280} returned to baseline. The fusion protein (GST-SO_3^--sCT-Ala) was eluted with 5 mM reduced Glutathione in 50 mM Tris–HCl, pH 8.0. The yield of eluted fusion protein (about 1.92 g) was calculated on Bradford protein assay. The column profile was assessed by SDS–PAGE stained by Coomassie blue, gel was scanned using a Bio-Rad densitometer to determine the percent of recombinant protein in total protein. Eluted fusion protein was concentrated by ammonium sulfate precipitation at 55% saturation at room temperature. Precipitates were stored at −20°C.

Chemical Modification and Cleavage of Fusion Protein

S-sulfonation. Purified GST-SO_3^--sCT-Ala was redissolved in water at 10 mg/ml protein concentration. Complete sulfonation of the Cysteine residues was accomplished by adding 5.2 mg of sodium sulfite and 2.5 mg of sodium tetrathionate/mg fusion protein and 0.1 ml of 1 M Tris–HCl (pH 8.0)/ml of solution and stirring the solution for 12 h in the dark at room temperature [5]. The salts were removed by G-25 column (8 × 60 cm).

Cleavage. Urea and HCl were added to the collection to final concentrations at 8 M and 50 mM, respectively. Cyanogen bromide was mixed with the clarified solution of peptide at 20 mg CNBr/mg GST-SO_3^--sCT-Ala while stirring at room temperature. Aliquots of the reaction were removed at each half-hour and analyzed for SO_3^--sCT-Ala liberation by RP-HPLC using Alltech prosphere C_{18} column (4.6 × 150 mm, 5 μM particle size, 300 A pore size).

Purification of SO_3^--sCT-Ala. The cleaved protein solution was diluted with 4 volume of water and applied to column of S-Sepharose Fast Flow resin (6 × 30 cm), which had been equilibrated in 2 M urea, 10 mM HCl (solution A) at the rate of 10 ml/min. After the sample was applied, the column was washed with solution A until the A_{280} returned to base line, the SO_3^--sCT-Ala was eluted with solution A containing 100 mM NaCl. The peptide was applied to a Waters delta-Pak C_{18} column (5 × 30 cm) equilibrated in 0.1% trifluoroacetic acid (TFA) at 80 ml/min. The peptide was eluted with a gradient of 38% to 49% acetonitrile in 0.1% TFA in 11 min. The SO_3^--sCT-Ala was eluted as a single peak and evaporated under vacuum.

Synthesis of 2-Nitrophenylglycinamide Hydrobromide (o-PNGA)

2-Nitrophenylacetic acid (36.2 g, 0.2 mol) was suspended in thionyl chloride 30 ml (0.4 mol), C_4Cl 12 ml and stirred for 0.5 h at 65°C. NBS (N-bromosuccinimide) 50 g (0.28 mol), C_4Cl 50 ml and 7 drops 48% HBr were added to the mixture, then stirred for 2.5 h at room temperature. The mixture was evaporated to dryness, suspended in dry ether, and the precipitated N-hydroxysuccinimide was filtered off, then the ether solution was evaporated to dryness and cold concentrated aqueous ammonia (200 mL) was added. The α-bromo-(2-nitrophenyl)acetamide was collected by filtration and recrystallized from water:ethanol (4:1, v/v) [Yield: 36 g (70%). ^{1}H NMR ($CDCl_3$, 90 MHz) m.p. 126–129°C. δ ppm: 7.4–8.1 4H, 6.1 1H, 5.7–5.9 1H, 6.4–6.7 1H]. α-Bromo-(2-nitrophenyl)acetamide (4.0 g, 0.02 mol) was dissolved in DMSO (4 ml) and treated with gaseous NH_3 for 2 h, the reaction mixture was lyophilized, and o-PNGA was recrystallized from absolute ethanol [Yield: 2.6 g (37%). ^{1}H NMR (DMSO-d6 250 MHz) m.p. 116–120°C. δ ppm: 5.4 1H, 4–4.5 2H, 7.5–8.0 3H, 8.2–8.3 1H, 8.5–8.7 2H].

Transacylation of SO_3^--sCT-Ala

Mixture of 1.0 μmol CPD-Y, 1 mmol SO_3^--sCT-Ala, 0.2 M o-PNGA, 5 mM EDTA pH 6.5 was incubated at 30°C for 2 h. Products were purified by HPLC (Alltech prosphere C_{18} 4.6 × 150 mm, 5 μM particle size, 300 A pore size) using a linear gradient from solution A (0.1% TFA/H_2O) to 80% solution B (0.1% TFA/acetonitrile) over 30 min.

Photolysis

Mixture of 1.0 mM SO_3^--sCT-o-PNGA, 60 mM $NaHSO_3$ in 50% methanol (purged with Argon) pH 9.5 was irradiated for 4–6 h, using 400 W medium-pressure mercury lamp at distance 10 cm. A 40% copper sulfate solution was employed to filter out wavelengths below 340 nm. Products of photolysis were purified by RP-HPLC column (Alltech prosphere C_{18} 4.6 × 150 mm, 5 μM particle size, 300 A pore size) using a linear gradient as above.

Ring Closure

Mixture of 0.1 mM Tris–HCl, pH 8.0, 0.5 mM cysteine and 1 mg/ml SO_3^--sCT-NH_2 was incubated at 30°C for 5 min [6]. Products were purified by RP-HPLC as above.

Molecular Weight (MW) Analysis

ESI-MS analysis of MW was performed by Shanghai Institute of Organic Chemistry, C.A.S.

Bioassay Analysis

The assessment of the biological activity of the recombinant sCT was standard of rat hypercalcemic assay *in vivo* comparing with potencies of a standard sCT that had an international reference

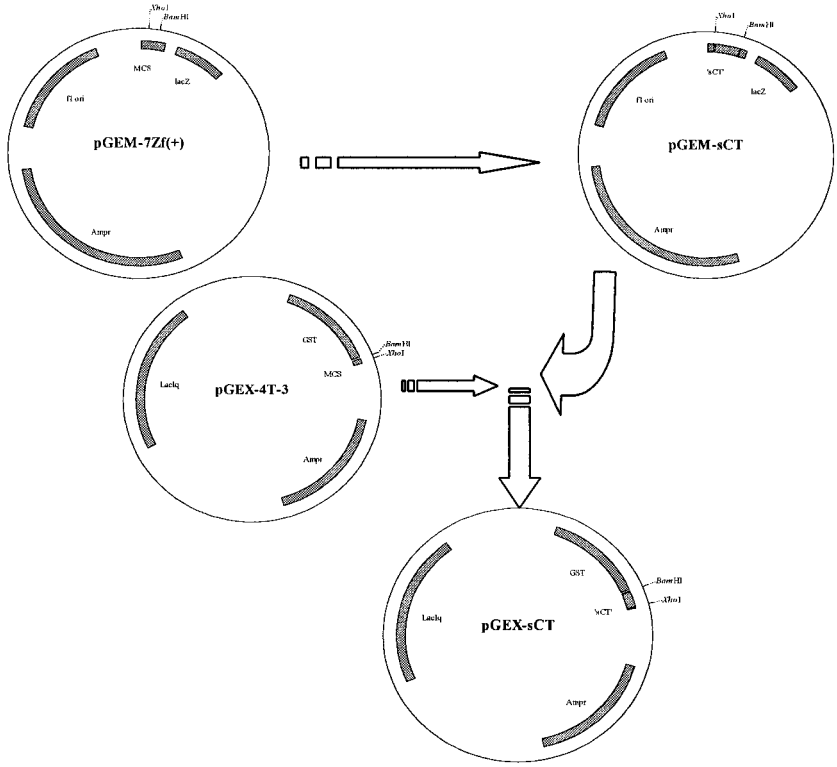


FIG. 1. Synthesis cDNA fragment. Synthetic salmon calcitonin cDNA fragment with *Bam*H1 and *Xho*1 ends was ligated into MCS of pGEM-7z(+) via the *Bam*H1 and *Xho*1, after being selected by α-complementation, the identified cDNA fragment was ligated in-frame with glutathione-*S*-transferase(GST) gene of the pGEX-4T-3 expression vector. The nucleotide acid sequence was the same as designed. The amino acid sequence was MCSNLSTCVLGKLSQELHKLQTYPRTNTGSGTPA with a Met for CNBr cleavage and an Ala for transacylation of CPD-Y.

value of 4000 IU/mg as described in Chinese Pharmacopoeia. The assay employed 3 different concentration of recombinant sCT at 6, 10, 20 mU per 60 g body mass and 10 rats/group. Serum calcium levels were determined by ultraviolet spectrophotometer.

RESULTS

Precursor Peptide Production

A synthesis sCT cDNA fragment ligated by oligonucleotides was cloned into pGEM-7z(+). ABI PRISM 310 Genetic Analyzer identified the sequence of positive clone. The correct cDNA fragment was cloned into pGEX-4T-3, then transformed into *E. coli* strain BL21(DE3) (see Fig. 1). The sCT cDNA fragment included a Methionine code at 5′ terminal to facilitate CNBr release, and an Alanine code at 3′ terminal for transacylation of CPD-Y. Alanine code at 3′ terminal for transacylation of CPD-Y.

Recombinant *E. coli* BL21(DE3)/pGEX-sCT was grown by technique of high density and high expression culture, the cells were harvested after induced 2 h by 0.3 mM IPTG, wet weight of cells was 150 g/L. The soluble fusion protein was separated by Glutathione Sepharose 4B affinity chromatography after sonication.

Densitometric scanning of the stained gel demonstrated GST-sCT-Ala fusion protein accounted for 20–30% in soluble cytoplasmic protein. The fast migrating band in the gel was probably a fusion protein truncated at Cys^{7} of sCT as report [2] (see Fig. 2).

Affinity-purified fusion protein was concentrated by ammonium sulfate precipitation and resuspended in water. Cysteine residues of the affinity-purified fusion

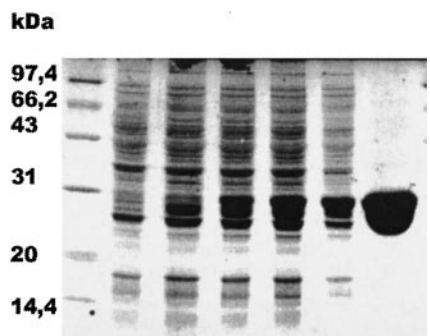


FIG. 2. SDS–PAGE analysis of the fermentation and affinity purification of the fusion protein. Lane 1, protein marker; lanes 2–6, induction 0–2 h; lane 7, fusion protein after purification (lanes 1–7 from left to right).

TABLE 1

The Yield of Purification Step for Recombinant sCT

Step	Component	g	Step yield (%)	Overall yield (%)
Fermentation cell lysis clarification	GST-sCT-Ala	1.92	100	100
Affinity purification	GST-sCT-Ala	0.768	40	40
Modification cleavage	SO_3^--sCT-Ala	0.084	60	24
Transpeptide purification	SO_3^--sCT-o-PNGA	0.060	70	16.8
Lysis purification	SO_3^--sCT-NH_2	0.054	90	15.1
Disulfide reformation purification	sCT-NH_2	0.048	90	13.6

Note. Recombinant *E. coli* cells (150 g) were lysed and clarified; the yield of GST-sCT-Ala was determined by Bradford protein assay; yield of the subsequent components, SO_3^--sCT-Ala, SO_3^--sCT-NH_2 and sCT, was determined by amino acid analysis.

protein were S-sulfonated. Following complete S-sulfonation of the Cysteine residues, the sulfonating salts were removed by gel filtration. In the presence of a 50 fold molar excess of CNBr over Methionine residues, cleavage went to completion within 5 h. The S-sulfonated sCT-Ala (SO_3^--sCT-Ala) in the cleavage mixture was purified by a single ion exchange chromatography (S-Sepharose) step. Most of other peptides in the cleavage reaction were retained on the ion exchange resin in the presence of 2 M urea/100 mM NaCl used to elute the SO_3^--sCT-Ala. The eluted peptide was desalted by reverse phase (RP) HPLC and lyophilized in preparation for enzymatic amidation. Recovery of the peptide substrate was greater than 24% (Table 1). The purity of the SO_3^--sCT-Ala was >99% (Fig. 3A) by RP-HPLC analysis (see Fig. 3A) and its MW was confirmed by ESI-MS (see Fig. 3B).

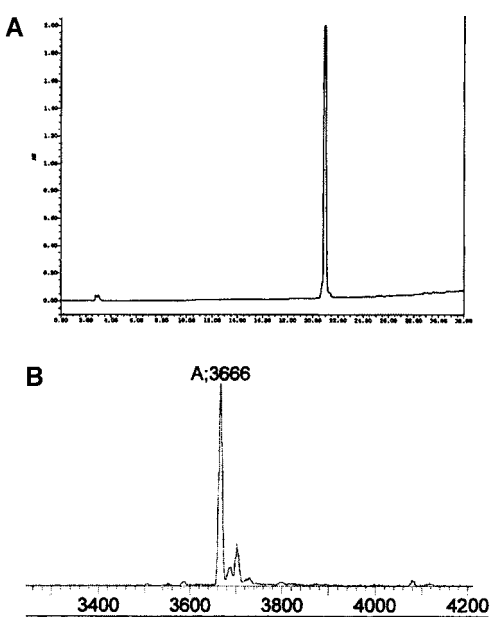


FIG. 3. (A) RP-HPLC analysis of the purified SO_3^--sCT-Ala prior to *in vitro* amidation. The assay was performed on Alltech prosphere C_{18} column. The retention time of peak was 20.9 min. (B) ESI-MS analysis of the purified SO_3^--sCT-Ala. The peptide sample gave the expected spectrum at *m/z* 3666.

Transacylation of SO_3^--sCT-Ala

70% SO_3^--SCT-Ala was conversed into SO_3^--sCT-o-PNGA, which be catalyzed by CPD-Y at 10:1 mass rate of SO_3^--sCT-Ala to CPD-Y. The reaction was initiated by addition of CPD-Y at 30°C. A time course of transacylation was shown as Fig. 4A. The products of transacylation were purified by HPLC. MW of SO_3^--sCT-o-PNGA was analyzed by ESI-MS (see Fig. 4B).

Photolysis, Folding, and Characterization

In the experiment, light wavelength was longer than 340 nm. Time course of photolysis was shown as Fig. 5. The yield of photolysis reaction was >90%. After desalinization and lyophilization, the SO_3^--sCT-NH_2 was folded in 3–5 min and purified by HPLC. The purity of final recombinant sCT was analyzed by HPLC (see Fig. 6A), and its MW was confirmed by ESI-MS (see Fig. 6B) as expected.

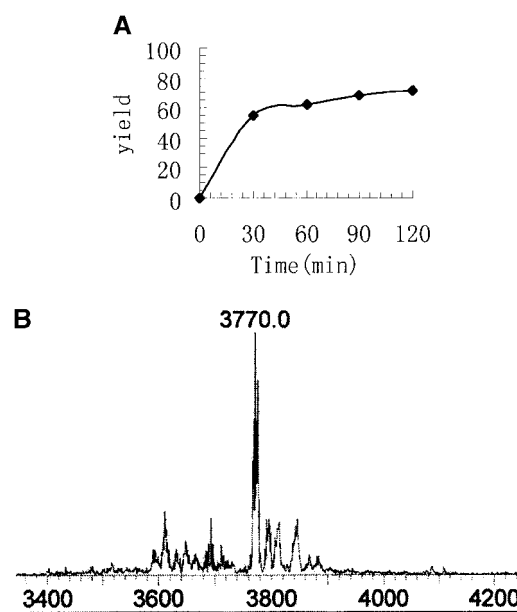


FIG. 4. (A) Time course of transacylation of SO_3^--sCT-Ala peptide by CPD-Y. (B) ESI-MS analysis of SO_3^--sCT-o-PNGA. Its MW was 3770.

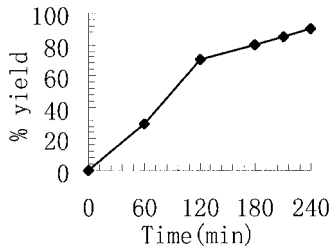
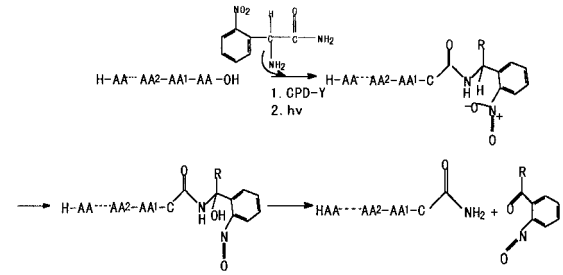


FIG. 5. Time course of photolysis of SO_3^--sCT-o-PNGA. The products was SO_3^--sCT-NH_2.



SCHEME 1

Assay of Biological Activity

The biological activity of recombinant sCT was assayed by hypercalcemic assay *in vivo* performed by Shanghai Institute of Pharmaceutical Quantity. Statistical analyses of the bioassay data demonstrated that above recombinant sCT had equivalent biological activity of sCT as commercial products (4000 IU/mg).

DISCUSSION

Since the discovery of CT by Coppin in 1961, CT has been identified in several animal species, sCT and eel CT (eCT) have biological activity 20–40 times greater than human CT (hCT) and porcine CT (pCT). sCT and eCT are considerably more potent than hCT and pCT in therapeutic use. The amphipathic α-helix and a carboxyl terminal prolinamide seem to be unequivocally required by all species CT for biological activity [7]. The bioactivity of amidated CT is 1600 times greater than CT without amidation, the amidation may confer upon this hormone structure of configuration which is important for its bioactivity and also possible to increase the resistance of CT to enzymatic degradation [8, 9]. Human or rat CT with no disulfide bond is virtually no activity, but the roll of disulfide bond in sCT is not so important for sCT biological activity [8].

CPD-Y had been employed as catalysts for the formation of peptide bond in peptide synthesis and protein semisynthesis as follows [10, 11]:

$$X–Y–OH + H–Z–NH_2 \rightarrow –X–Z–NH_2 + H–Y–OH$$

(X, Y, Z = amino acid)

Unfortunately serine carboxypeptidase will not accept all amino acid amides as nucleophiles, thereby preventing production of peptide amides, which terminate in proline, glutamic or aspartic acid. To solve the problem that peptide has a C terminal of Pro-NH_2, Henriksen *et al.* [12, 13] designed an approach where a peptide precursor was subject to a CPD-Y catalyzed transacylation with a photocleavable nucleophile, and followed by photolysis to give the desired peptidamide (Scheme 1). This method was more efficient than NH_3 as a nucleophile in transacylation [4]. However, the substrate being chosen usually had not multiple −SH in molecule. It was possible that the oxidative properties of o-PNGA lead a covalent linkage to form between molecules. The difficulty was overcome by protecting −SH, such as S-sulfonation in our experiment. Meanwhile, the S-sulfonation prior to chemical cleavage temporarily

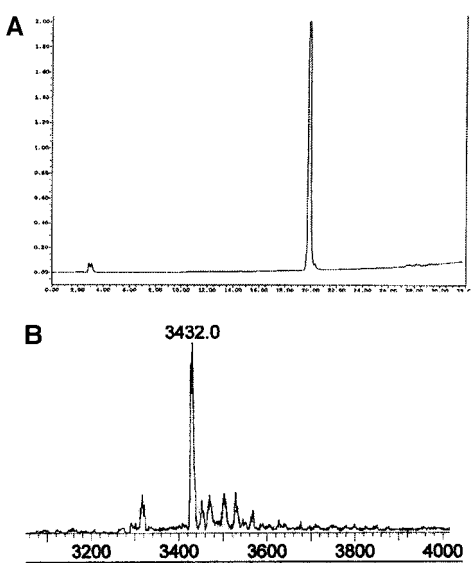
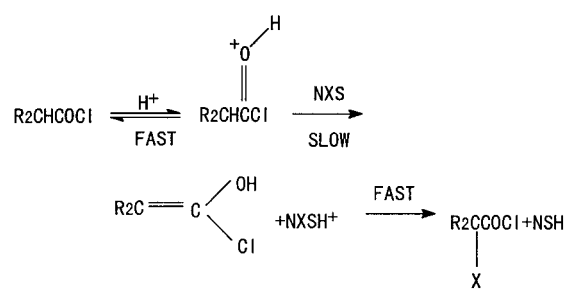
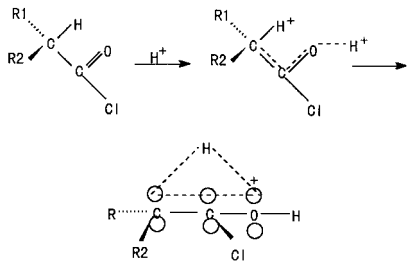


FIG. 6. (A) RP-HPLC analysis of the purified sCT. The assay was performed on Alltech prosphere C_{18} column. The retention time of peak was 20.5 min. (B) ESI-MS analysis of the purified sCT. The peptide sample gave the expected spectrum at *m*/*z* 3432.



SCHEME 2



SCHEME 3

disrupted the formation of the cys1-cys7 disulfide bond, which increased the efficiency of the CNBr release of the peptide from its fusion partner and solubility for the subsequent enzymatic amidation [14].

Our approach to synthesis of o-PNGA compared with other reports is time-saving and highly productive. The addition of a trace of mineral acid has a strong accelerative effect on the bromination of phenylacetyl chloride [15]. The mechanism could involve in the N-halosuccinimide acting as a base to remove α-hydrogen to form the enol intermediate in the slow step (Scheme 2). The enol would then be halogenated in a rapid step. Another possibility base on the steric and deuterium isotope study, which might explain the steric effect of the α-substituent as well as the dependence of the reaction rate on the nature and the concentration of the halogenation agent (Scheme 3). This mechanism proposed that the intermediate in the halogenation of acid chlorides is a cationic complex in which the C_α–H bond of the conjugate acid is highly ionized. The rate-determining step would be that of an unusual electrophilic displacement of a proton on the complex by NXS.

In photolysis, we used 40% copper sulfate sodium to filter out wavelength below 320 nm, which was harmful to aromatic amino acids like Tyr and Trp [16].

Ray *et al.* demonstrated that α-AE showed low affinity to sCT precursor in modification reaction, which was happened as same as our experiment while using CPD-Y for transacylation to produce sCT, the mass rate was 10–20:1 of peptide substitute to enzyme under the condition described, preliminary work suggested that mass of peptide was 1000-fold greater than enzyme utilized in amidation reaction [17]. To overcome associated problem with use of CPD-Y in our researches, two approaches could be taken. One was to extend time of reaction. Another was to increase amount of CPD-Y, this would result in increase of cost during preparing recombinant sCT, but the low expenses in isolating CPD-Y from baker's yeast could make up the defect.

In summary, this method exhibited a valuable alternative in the preparation of sCT from prokaryotic expression systems such that the amidation of recombinant salmon calcitonion was produced by recombinant peptide precursor, which was modified by enzymatic transacylation and photolysis *in vitro.*

ACKNOWLEDGMENTS

We thank Professor Lu Deru for proposing this project and Lu Zhonghua, Xiu Zhaoyang, Sheng Guoxiang, and He Qiang for the aiding in the research.

REFERENCES

1. Suzuki, K., Shimoi, H., Iwasaki, Y, Kawahrra, T., Matsuura, Y., and Nishikawa, Y. (1990) *EMBO J.* **9,** 4259–4265.
2. Ray, M. V. L., Duyne, P. C., Bertelsen, A. H., Jackson-Mattews, D. E., Sturmer, A. M., Merkler, D. J., Consalvo, A. P., Young, S. D., Gilligan, J. P., and Shields, P. P. (1993) *Bio/Technology* **11,** 64–70.
3. Merli, S., De Falco, S., Verdoliva, A., Tortora, M., Villain, M., Silvi, P., Cassani, G., and Fassina, G. (1996) *Protein Expression Purif.* **7,** 347–354.
4. Hong, D., Jifang, M., *et al.* (1999) Preparation of Recombinant Human Calcitonin Analogue, in press.
5. Chan, W. W. C. (1968) *Biochemistry* **8,** 4247–4254.
6. Andreu, D., Alvericio, F., Sole, N. A., Munson, M. C., Ferrer; M., and Barany, G. (1994) *in* Methods in Molecular Biology: Peptide Synthesis Protocols (Pennington, M. W., and Dunn, B. M., Eds.), Vol. 35, pp. 91–169, Humana Press Inc., Totowa, NJ.
7. Becker, K. L., Nylen, E. S., Cohen, R., and Snider, R. H., Jr. (1996) *in* Principles of Bone Biology (Bilezikian, J. P., Raisz, L. G., and Rodan, G. A., Eds.), Chap. 34, pp. 471–494, Academic Press, San Diego, CA.
8. Epand, R. M., and Caulfield, M. P. (1990) *in* Comprehensive Medicinal Chemistry (Hansch, C., Sammes, P. G., and Tylor, J. B., Eds.), Vol. 3, pp. 1023–1045, Pergamon Press, Oxford.
9. Rittel, W., Maier, R., Brugger, B., Kamber, B., Rimker. B., and Steber, R. (1976) *Experiments* **32,** 246–248.
10. Widmer, F., Breddam, K., and Johansen, J. T. (1981) *Carlsberg Res. Commun.* **46,** 97–106.
11. Widmer, F., Breddam, K., and Johansen, J. T. (1981) *Carlsberg Res. Commun.* **46,** 121–128.
12. Henriksen, D. B., Breddam, K., Moller, J., and Buchardt, O. (1992) *J. Am. Chem. Soc.* **114,** 1876–1877.
13. Henriksen, D. B., Breddam, K., and Buchardt, O. (1993) *Int. J. Peptide Protein Res.* **41,** 169–180.
14. Gearing, D. P., Nicola, N. A., Metcalf, D., Foote, S., Wilson, T. A., Gough, N. M., and Williams, R. J. (1989) *Bio/Technology* **7,** 1157–1161.
15. Harpp, D. N., Bao, L. Q., Black, C, J., Gleason, J. G., and Smith, R. A. (1975) *J. Org. Chem.* **40,** 3420–3427.
16. Pillai, V. N. R., and Mutter, M. (1980) *J. Org. Chem.* **45,** 5364–5370.
17. Breddam,K., Eidmer, F., and Meldal, M. (1991) *J. Peptide Protein Res.* **37,** 153–160.