J. Microbiol. Biotechnol. (2008), **18**(6), 1076–1080

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Expression, Purification, and Characterization of C-Terminal Amidated Glucagon in *Streptomyces lividans*

Qi, Xiaoqiang[1], Rong Jiang[1], Cheng Yao[1], Ren Zhang[1,2], and Yuan Li[1]*

[1]*Ministry of Health Key Laboratory of Biotechnology of Antibiotics, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Tiantan, Beijing 100050, China*
[2]*School of Biological Sciences, University of Wollongong, NSW 2522, Australia*

Received: June 18, 2007 / Accepted: September 5, 2007

Glucagon, a peptide hormone produced by alpha-cells of Langerhans islets, is a physiological antagonist of insulin and stimulator of its secretion. In order to improve its bioactivity, we modified its structure at the C-terminus by amidation catalyzed by a recombinant amidase in bacterial cells. The human gene coding for glucagon-gly was PCR amplified using three overlapping primers and cloned together with a rat α-amidase gene in plasmid pMGA. Both genes were expressed under control of the strong constitutive promoter of *aph* and secretion signal *melC1* in *Streptomyces lividans*. With Phenyl-Sepharose 6 FF, Q-Sepharose FF, SP-Sepharose FF chromatographies and HPLC, the peptide was purified to about 93.4% purity. The molecular mass of the peptide is 3.494 kDa as analyzed by MALDI TOF, which agrees with the theoretical mass value of the C-terminal amidated glucagon. The N-terminal sequence of the peptide was also determined, confirming its identity with human glucagon at the N-terminal part. ELISA showed that the purified peptide amide is bioactive in reacting with glucagon antibodies.

Keywords: Glucagon, amide, purification, *Streptomyces lividans*

Glucagon is a peptide consisting of 29 amino acids, which can increase the blood glucose level and is involved in carbohydrate and lipid metabolism in the human body [20]. It was first discovered by Kimbell and Murlin [14] and then purified and crystallized by Staub *et al.* [21]. Glucagon has been expressed in various microbes including yeast and *E. coli* [13, 18]. The recombinant glucagon produced by Novo Nordisk has been approved to be used in clinical treatment of the symptom of low blood glucose.

**Corresponding author*
Phone: 86-10-63153320; Fax: 86-10-63017303;
E-mail: yuanwli@263.net

C-Terminal amidated peptides, such as calcitonin, are often found to be more biologically active than their free carboxyl counterparts [11, 19]. Krstenansky *et al.* [15] reported that [Phe^{10} Phe^{13}] glucagon amide was more active than [Phe^{10} Phe^{13}] glucagon. Removal of the C-terminal negative charge of peptides by replacement with an amide improved the helix dipole and stabilized an enhanced proportion of the helix [22]. Unson *et al.* [24] synthesized various peptide antagonists of glucagon to confirm that an amphipathic helix in the residue 19–29 region of glucagon is important for receptor binding. It implies that a change such as amidation that enhances such a structure may increase the binding. Hence, we attempted to amidate glucagon at its C-terminus using a recombinant DNA technique to improve the biological activity of this peptide.

α-Amidating enzyme (α-AE, α-amidase) is a post-translation processing enzyme converting the C-terminal glycine-extended substrates to C-terminal amidated products. A gene coding for the rat α-amidase has been cloned and functionally expressed in *Streptomyces lividans* in our laboratory [27]. In this study, we expressed a glucagonsgly (glycine) gene together with the α-AE gene in *S. lividans*, which was chosen since it has been used by many as a very efficient bacterial host to produce recombinant proteins [1, 3–7, 10, 16, 25]. The recombinant glucagon was isolated, confirmed to be amidated at the C-terminus, and shown to be bioactive.

Materials and Methods

Strains, Plasmids, and Enzymes

E. coli (DH5α, Stratagene) and plasmid pUC18 were used for initial cloning, whereas *S. lividans* TK24 and pMSA (containing the gene encoding α-AE, constructed in our laboratory) were used as host and vector for protein expression. Restriction enzymes, Tag-Plus DNA polymerase, and T4 DNA ligase were purchased from Promega or Takara Biotechnology.

PCR and Cloning of Glucagon-gly Gene

According to the sequence of the human glucagon-gly gene [26], three primers were synthesized: P1, 5'AAACTGCAGCACTCTCAGGGTACTTTCACTTCTGACTACT (PstI restriction site underlined); P2, 5'TGAACGAAGTCCTGAGCACGACGGGAGTCCAGGTATTTGGAGTAGTCAG; and P3, 5'TTCTAGATTATTAGCCAGTGTTCATCAGCCACTGAACGAAGTCCTGA (XbaI restriction site underlined). Using P1 and P3 as primers and P2 as template, the gene encoding glucagon-gly was PCR assembled. After ligation of the PstI/XbaI double-digested PCR product and pUC18, the recombinant plasmid was transformed in *E. coli*. With the correct sequence being confirmed, the glucagon-gly gene was excised out and ligated into pMSA double-digested with PstI/XbaI. The resultant plasmid pMGA was then introduced into *S. lividans* by polyethylene glycol (PEG)-mediated protoplast transformation [14]. The tranformants were selected on R2 agar (each liter contains 103 g sucrose, 10 g glucose, 1 g yeast extract, 0.1 g casein acid hydrolysate, 5 g lab lemco powder, 10.12 g $MgCl_2 \cdot 6H_2O$, 0.25 g K_2SO_4, 0.05 g KH_2PO_4, 15 g agar, and 0.2% (v/v) trace element solution in 25 mM TES buffer, pH 7.2) plates supplemented with 50 μg/ml thiostrepton.

Cultivation of *S. lividans* Harboring pMGA

A preculture of *S. lividans* harboring pMGA was grown in 50 ml CM medium [glucose 1%, casein enzymatic hydrolysate 2%, Bacto-yeast extract 1%, and Trace element solution 1% (v/v), pH 7.2] at 28°C, 220 rpm, for 48 h. About 30 glass beads (D=3 mm) were added into the shake flask to improve the condition of cell growth. Five ml of this preculture was then inoculated into 50 ml of fresh CM medium, and after 72 h growth, the supernatant was harvested by centrifugation at 3,000 rpm for 20 min.

Purification of C-Terminal Amidated Glucagon

The supernatant was adjusted to 1 M $(NH_4)_2\ SO_4$ in 50 mM phosphate buffer (pH 7.0) and loaded on a Phenyl Sepharose 6 FF column (Amersham-Pharmacia Biotech) pre-equilibrated with 1 M $(NH_4)_2\ SO_4$ in 50 mM phosphate buffer (pH 7.0). The column was then washed with 2 volumes of the same buffer and eluted by a linear gradient of $(NH_4)_2SO_4$ (1 → 0 M) in 50 mM phosphate buffer (pH 7.0) with a flow rate at 0.6 ml/min. The biologically active fractions detected by ELISA assay were collected and ultrafiltered (1,000 NMWL, Minitan). The sample solution was then adjusted to be in 20 mM phosphate buffer (pH 6.5) and passed through a pre-equilibrated Q Sepharose FF column (Amersham-Pharmacia Biotech) at a flow rate of 0.2 ml/min. All elutions collected were loaded on a SP Sepharose FF column (Amersham-Pharmacia Biotech) pre-equilibrated with 20 mM phosphate buffer (pH 6.5) and eluted by a linear gradient (NaCl 0 → 1 M in 20 mM phosphate buffer, pH 6.5) with a flow rate at 0.5 ml/min. The biologically active fractions detected by ELISA were pooled and ultrafiltered. The sample was finally purified through a C18 column (reversed phase Inertsil ODS-2, 4.5×150 mm) on an HPLC (Shimadzu-10A) monitored at 215 nm and eluted by 10% acetonitrile-0.05% TFA. The purified product was lyophilized and kept at −20°C.

SDS-Tricine-PAGE and Western Blot Analyses of Purified C-Terminal Amidated Glucagon

The purified peptide was diluted in Laemmli buffer [9] containing 5% 2-mercaptoethanol, boiled for 5 min, and loaded onto 15% SDS-Tricine-PAGE gel in Tris-tricine buffer [11] and peptide bands were visualized with Coomassie Brilliant Blue R-250 after electrophoresis. For Western blotting, the purified peptide was transferred to a nitrocellulose blotting membrane using a Trans-blot SD Semi-dry Transfer Cell (Bio-Rad) [19]. The membrane was blocked with 5% dried milk in TBS+0.1% v/v Tween 20 (TBST) overnight at 4°C and then incubated for 2 h at room temperature with 5% dried milk in TBST containing a 1/500 dilution of rabbit anti-human glucagon antibody (Zhongshan Jinqiao). It was washed with TBST and then incubated for 2 h in 5% dried milk in TBST containing a 1/1,000 dilution of goat anti-rabbit IgG (H+L) antibody alkaline phosphatase (AP) conjugate (Zhongshan Jinqiao). Following incubation with the secondary antibody, the blot was washed and the protein visualized using NBT/BCIP (Promega) substrate for AP.

ELISA of C-Terminal Amidated Glucagon

One hundred μl of the sample was coated onto a 96-well immunoplate (Nunc) at 4°C for 8–12 h. To each well, 200 μl of 3% BSA in PBS buffer (KH_2PO_4 0.024%, Na_2HPO_4 0.363%, KCl 0.02%, and NaCl 0.8%, pH 7.4) was added and the plate was kept at 37°C for 2 h, followed by washing 2 times with PBS buffer and blotted dry. Then, 100 μl of rabbit anti-human glucagon antibody (1:1,000 dilution with PBS) was added to each well and kept at 37°C for 2 h. The plate was washed 3 times with PBST (PBS+0.1% Tween 20) and then washed 3 times again with PBS. After adding the goat anti-rabbit IgG (H+L) antibody conjugated with SA-HRP (1:5,000 dilution with PBS) to each well and keeping at 37°C for 1 h, the plate was washed again as above. One hundred μl of TMB solution (3,3',5,5-tetramethyl benzidine dihydrochloride) was added to each well and the reaction took place at room temperature for 1 h (solution should turn blue in color) before being stopped by the addition of 100 μl of 2 N HCl. The absorbance at 450 nm was recorded as measurement of the reaction.

Analysis of Molecular Mass and N-Terminal Amino Acid Sequencing

Molecular mass of the C-terminal amidated glucagon was analyzed by MALDI-TOF REFLEX III, BRUKER.

The N-terminal amino acid residues of the purified C-terminal amidated glucagon were identified by automated Edman degradation sequencing using Applied Biosystems Procise 491 (Applied Biosystems).

RESULTS

Sequence Confirmation of the Glucagon-gly Gene and its Expression

The nucleotide sequence of the PCR-amplified glucagon-gly gene was found to be identical to the published glucagon encoding sequence [26].

In the expression plasmid pMGA, the genes encoding glucagon-glycine *α-AE* were strung together, controlled by the strong constitutive *aph* promoter and the secretion signal *melC1*. After pMGA was transformed in *S. lividans* TK24, the C-terminal amidated glucagons were produced and secreted out of the cultivated *S. lividans* [pMGA]

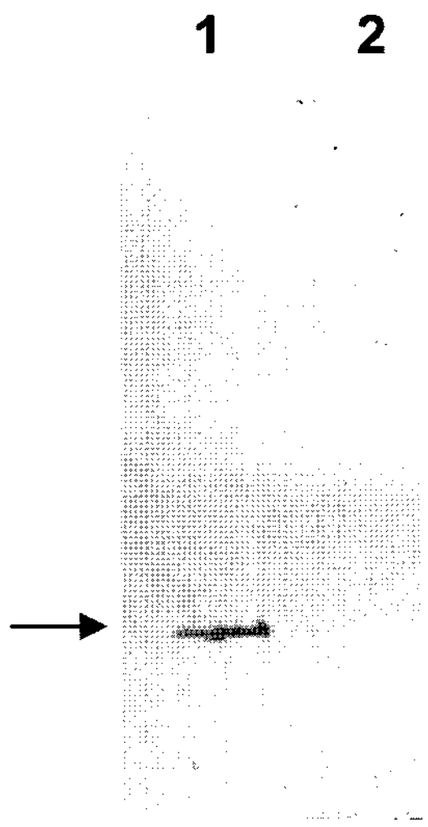

Fig. 1. Western blot analysis of the supernatants of *S. lividans* [pMGA] (lane 1) and *S. lividans* TK24 (lane 2).

cells. Analysis by SDS-Tricine-PAGE and ELISA of the fermentation supernatants of different culture times (24, 48, 72, and 96 h) indicated that the amount of the peptide produced achieved a maximum at 72 h (data not shown). Western blot analysis of the supernatant samples showed a positively reacted band at molecular mass ~3.5 kDa (Fig. 1).

Purification, N-Terminal Sequence, and Molecular Mass of the Recombinant Glucagon

Using the purification procedures mentioned above, purified peptide was obtained with a yield of 24.2 mg per liter of culture. The purity of the recovered peptide was 93.4% as analyzed by HPLC. The purified protein was subjected to SDS-Tricine-PAGE and visualized with Coomassie Brilliant Blue R-250 (Fig. 2).

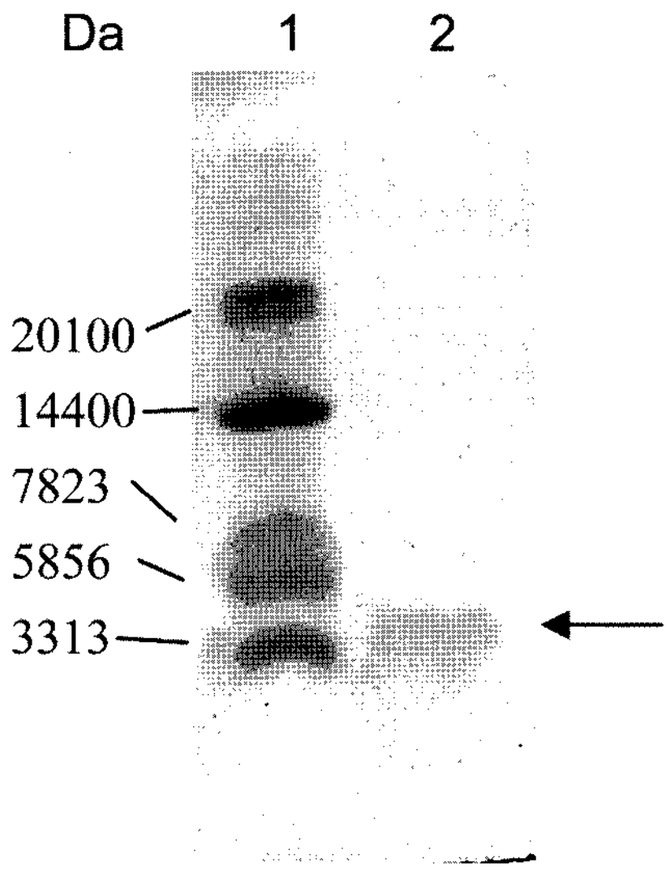

Fig. 2. SDS-Tricine-PAGE analysis of the purified recombinant glucagon amide.
Lane 1, molecular mass makers; lane 2, purified amidation glucagon.

The first fourteen N-terminal amino acid residues of the recombinant peptide were determined as HSQGTFTSDYSKYL by the Edman degradation method. This result unambiguously identified the recombinant peptide as the human glucagon and confirmed that no N-terminal degradation occurred in the process of heterogeneous protein expression in *S. lividans* TK24.

The molecular mass of the recombinant peptide was confirmed to be 3.494 kDa using MALDI-TOF (Fig. 3), which matches the theoretical mass value of C-terminal amidated glucagon.

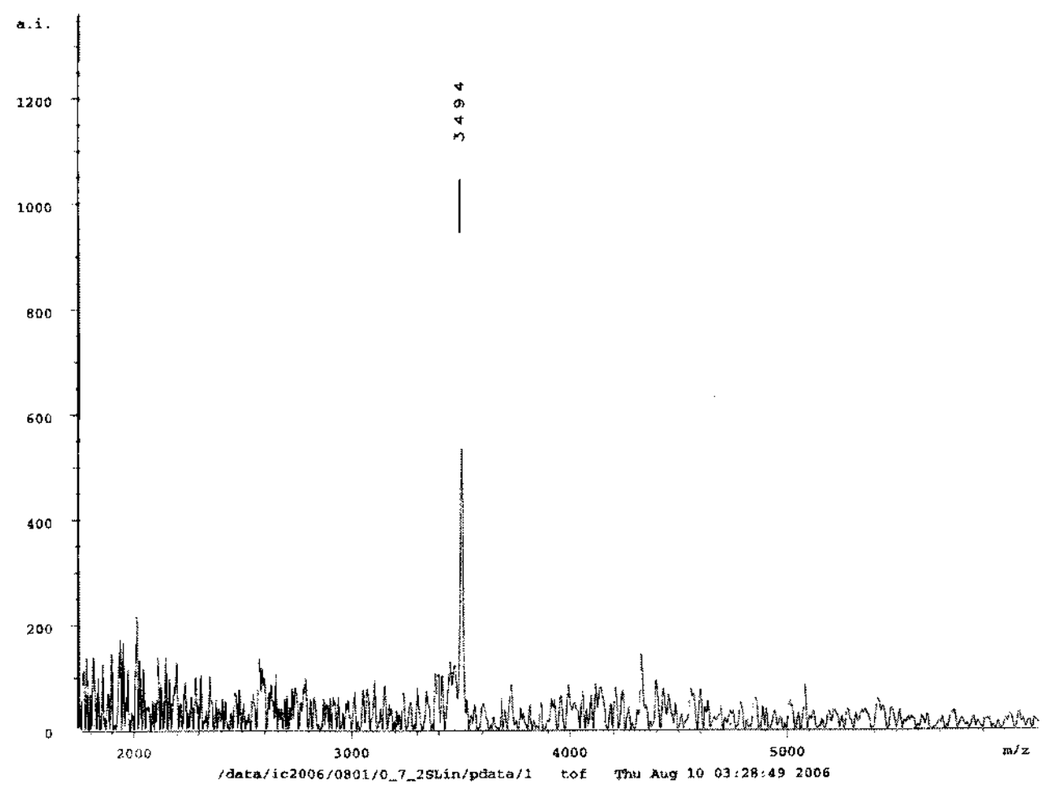

Fig. 3. MALDI-TOF analysis of the molecular mass of the purified recombinant glucagon with C-terminal amide.

Immunologic Activity of the C-Terminal Amidated Glucagon

ELISA result indicated that the recombinant C-terminal amidated glucagon had the binding activity with the glucagon antibody. With increasing concentrations (up to

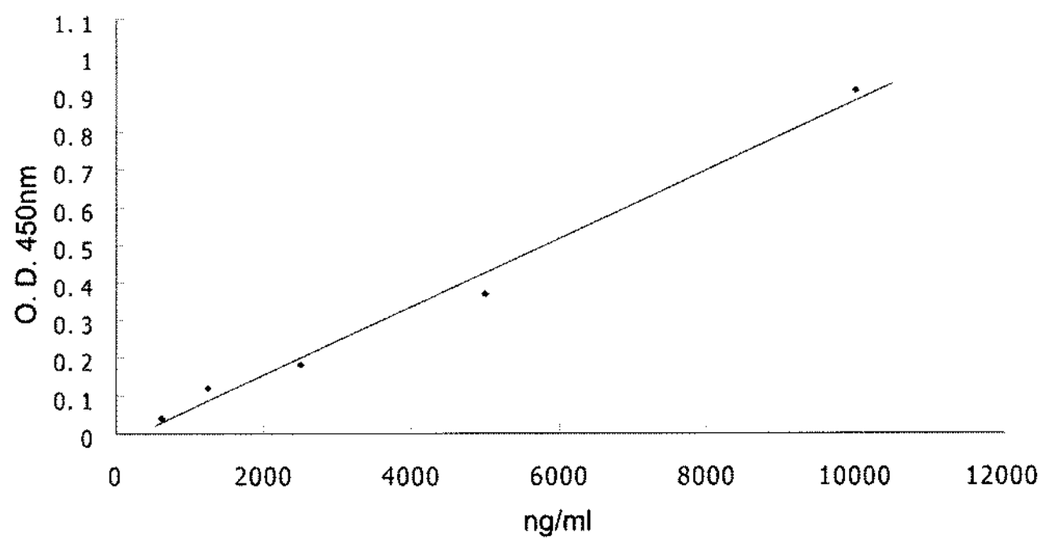

Fig. 4. ELISA analysis showing dose-response of binding between the purified recombinant glucagon amide and glucagon antibody.

10,000 ng/ml) of C-terminal amidated glucagon, dose-response assays of the recombinant C-terminal amidated glucagon binding to glucagon antibody were carried out. As the concentration of C-terminal amidated glucagon increased, the binding activity measured as OD_{450} value increased significantly and linearly (Fig. 4).

DISCUSSION

Glucagon is a 29-residue polypeptide interacting with the hepatic receptor *via* a cAMP-mediated pathway to stimulate glucose production and release. It has been reported that insulin deficiency causes impairment of glucose utilization, and glucagon is the primary mediator of the overproduction of glucose and ketone bodies in diabetes [8, 23]. The renewed interest in the role of glucagon in diabetes and maintenance of normal blood glucose level has stimulated research aimed at understanding the mechanism of glucagon action and synthesis of glucagon analogs as potential therapeutics. Structure-function studies of glucagon analogs showed that the introduction of a C-terminal amide did increase the proportion of the α-helix and decrease the β-sheet structure, which may enhance its receptor binding activity [22]. Some peptides such as calcitonin are in amide forms *in vivo* at their C-termini to assume full bioactivities.

Through genetic engineering, we have been able to produce large quantities of C-terminal amidated glucagon in bacterial cells. Since it was secreted into the culture medium, the purification for this recombinant peptide was relatively easy. With correct N-terminal amino acid residues and the full molecular mass confirmed, this bacterial cell produced peptide is believed to be identical to the C-terminal amidated human glucagon. The amidation process seemed very efficient since no significant amount of glucagon-gly was detected. It is also significant that this amidated peptide showed good reactivity to the glucagon antibody. Works are in progress to test its *in vivo* activities and further improve the purification procedures.

Compared with chemical synthesis, the expression of recombinant polypeptides in microbes such as *E. coli* and *Streptomyces* is more convenient and cost efficient, especially in large-scale productions. By coexpressing the α-amidating enzyme, we have successfully produced C-terminal amidated calcitonin previously [16] and the glucagon reported in this study, demonstrating the capability and effectiveness of producing modified biopeptides using biotechnology. *S. lividans* has been the of choice host in both cases because of its excellent secretion capacity for recombinant proteins.

Our technique should be generally useful for C-terminal amidation of other recombinant peptides when such modification is desired.

Acknowledgment

This work was supported by a project grant under the Chinese National 863 Program for High Technologies (grant No. 2004AA215132).

REFERENCES

1. Anne, J. and L. Van Mellaert. 1993. *Streptomyces lividans* as host for heterologous protein production. *FEMS Microbiol. Lett.* **114:** 121–128.
2. Ausubel, F. M. 1995. *Short Protocols in Molecular Biology*, 3rd Ed. John Wiley & Sons, Inc.
3. Bender, E., K. P. Koller and J. W. Engels. 1990. Secretory synthesis of human interleukin-2 by *Streptomyces lividans*. *Gene.* **86:** 227–232.
4. Binnie, C., D. Jenish, D. Cossar, A. Szabo, D. Trudeau, P. Krygsman, L. T. Malek, and D. I. H. Stewart. 1997. Expression and characterization of soluble human erythropoietin receptor made in *Streptomyces lividans* 66. *Protein Expr. Purif.* **11:** 271–278.
5. Binnie, C., J. D. Cossar, and D. I. H. Stewart. 1997. Heterologous biopharmaceutical protein expression in *Streptomyces*. *TIBTECH* **15:** 315–320.
6. Brawner, M. E. 1994. Advances in heterologous gene expression by *Streptomyces*. *Curr. Opin. Biotechnol.* **5:** 475–481.
7. Brawner, M., G. Poste, M. Rosenberg, and J. Westpheling. 1991. *Streptomyces*: A host for heterologous gene expression. *Curr. Opin. Biotechnol.* **2:** 674–681.
8. Chiasson, J. L. and A. D. Cherrington. 1983. *In*: *Glucagon and Liver Glucose Output in Vivo* (*Glucagon I, Handbook of Experimental Pharmacology*), pp. 361–382. Springer-Verlag, Berlin, Germany.
9. Choi, N. S., K. H. Yoo, K. S. Yoon, K. T. Chang, P. J. Maeng, and S. H. Kim. 2005. Identification of recombinant subtilisins. *J. Microbiol. Biotechnol.* **15:** 35–39.
10. Fornwald, J. A., M. J. Donovan, R. Gerber, J. Keller, D. P. Taylor, E. J. Arcuri, and M. E. Brawner. 1993. Soluble forms of the human T cell receptor CD4 are efficiently expressed by *Streptomyces lividans*. *Biotechnology* **11:** 1031–1036.
11. Hong, B., B. Wu, and Y. Li. 2003. Production of C-terminal amidated recombinant salmon calcitonin in *Streptomyces lividans*. *Appl. Biochem. Biotechnol.* **110:** 113–123.
12. Ishizaki, J., M. Tamaki, M. Shin, H. Tsuzuki, K. Yoshikawa, H. Teraoka, and N. Yoshida. 1992. Production of recombinant human glucagon in the form of a fusion protein in *Escherichia coli* recovery of glucagon by sequence-specific digestion. *Appl. Microbiol. Biotechnol.* **36:** 483–486.
13. Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood. 2000. *Practical Streptomyces Genetics*. Crowes, Norwich, England.
14. Kimball, C. P. and J. R. Murlin. 1923. Aqueous extracts of pancreas III. Some precipitation reactions of insulin. *J. Biochem.* **58:** 337–346.
15. Krstenansky, J. L., D. Trivedi, D. Johnson, and V. J. Hruby. 1986. Conformational considerations in the design of a glucagon analogue with increased receptor binding and adenylate cyclase potencies. *J. Am. Chem. Soc.* **108:** 1696–1698.

16. Lichenstein, H., M. E. Brawner, L. M. Miles, C. A. Meyers, P. R. Young, P. L. Simon, and T. Eckhardt. 1988. Secretion of interleukin-1 beta and *Escherichia coli* galactokinase by *Streptomyces lividans*. *J. Bacteriol.* **170:** 3924–3929.
17. Moody, A. J., F. Norris, and K. Norris. 1987. The secretion of glucagon by transformed yeast strains. *FEBS Lett.* **212:** 302–306.
18. Na, K. H., S. C. Kim, K. S. Seo, S. H. Lee, W. B. Kim, and K. C. Lee. 2005. Purification and characterization of recombinant human follicle stimulating hormone produced by Chinese hamster ovary cells. *J. Microbiol. Biotechnol.* **15:** 395–402.
19. Ray, M. V., P. Van Duyne, and A. H. Bertelsen. 1993. Production of recombinant salmon calcitonin by *in vitro* amidation of an *Escherichia coli* produced precursor peptide. *Biotechnology (NY)* **11:** 64–70.
20. Roman Esipov, S., N. Vasily Stepanenko, and I. Alexandr Gurevich. 2006. Production and purification of recombinant human glucagon overexpressed as intein fusion protein in *Escherichia coli*. *Protein Peptide Lett.* **13:** 343–347.
21. Staub, A., L. Sinn, and O. K. Behrens. 1955. Purification and crystallization of glucagon. *J. Biol. Chem.* **214:** 619–632.
22. Stewart, J. M., E. J. York, R. L. Baldwin, and K. R. Shoemaker. 1985. Nature of the charged-group effect on the stability of the C-peptide helix. *Proc. Natl. Acad. Sci. USA* **82:** 2349–2353.
23. Unger, R. H. 1976. Diabetes and the alpha cell. *Diabetes.* **25:** 136–151.
24. Unson, C. G., D. Andreu, M. Ellen Gurzenda, and R. B. Merrifield. 1987. Synthetic peptide antagonists of glucagons. *Proc. Natl. Acad. Sci. USA* **84:** 4083–4087.
25. Van Mellaert, L., C. Dillen, P. Proost, E. Sablon, R. DeLeys, A. Van Broekhoven, H. Heremans, J. Van Damme, H. Eyssen, and J. Anne. 1994. Efficient secretion of biologically active mouse tumor necrosis factor α by *Streptomyces lividans*. *Gene* **150:** 153–158.
26. White, J. W. and G. F. Saunders. 1986. Structure of the human glucagon gene. *Nucleic Acids Res.* **14:** 4719–4730.
27. Wu, B.Y. and Y. Li. 2000. Studies of cloning and expression of rat α-amidase gene in *Streptomyces lividans*. *Chin. J. Biotechnol.* **16:** 574–577.