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Expression, Purification, and C-terminal Site-Specific PEGylation of Cysteine-Mutated Glucagon-Like Peptide-1

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Abstract Glucagon-like peptide-1 (GLP-1) is attracting increasing interest on account of its prominent benefits in type 2 diabetes. However, its clinical application is limited because of short biological half-life. This study was designed to produce a C-terminal site-specific PEGylated analog of cysteine-mutated GLP-1 (cGLP-1) to prolong its action. The gene of cGLP-1 was inserted into pET32a to construct a thioredoxinA fusion protein. After expression in BL21 (DE3) strain, the fusion protein was purified with Ni-affinity chromatography and then was PEGylated with methoxy-polyethylene glycol-maleimide ($mPEG_{10K}$-MAL). The PEGylated fusion protein was purified with anion exchange chromatography and then was cleaved by enterokinase. The digested product was further purified with reverse-phase chromatography. Finally, 8.7 mg $mPEG_{10K}$–cGLP-1 with a purity of up to 98% was obtained from the original 500 ml culture. The circular dichroism spectra indicated that $mPEG_{10K}$–cGLP-1 maintained the secondary structure of native GLP-1. As compared with that of native GLP-1, the plasma glucose lowering activity of $mPEG_{10K}$–cGLP-1 was significantly extended. These results suggest that our method will be useful in obtaining a large quantity of $mPEG_{10K}$–cGLP-1 for further study and $mPEG_{10K}$–cGLP-1 might find a role in the therapy of type 2 diabetes through C-terminal site-specific PEGylation.

Keywords Glucagon-like peptide-1 · Incretin · PEGylation · Diabetes · Enterokinase

Introduction

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted from enteroendocrine L-cells of the intestine in response to orally ingested nutrients [1]. This peptide potently stimulates insulin secretion in a glucose-dependent manner [2]. It not only decreases blood sugar levels in diabetes mellitus patients, but also stimulates proinsulin gene expression,

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and inhibits gastric emptying, gastric acid release, glucagon secretion, and food intake [3, 4]. All these properties have made GLP-1 an attractive treatment for type 2 diabetes [5]. However, endogenous as well as exogeneous GLP-1 is extremely rapidly inactivated in human bodies, and the biological half-life of GLP-1 in the plasma can last only about 5 min by subcutaneous injection [6, 7]. This property of GLP-1 limited its clinical application. Therefore, many researchers have been pursuing the GLP-1 with enhanced plasma stability and longer biological half-life [8–10].

In this study, we attempted to extend GLP-1 action through addition of a PEG chain to the C terminus of GLP-1. This site was chosen as it is far distant from the N terminus of GLP-1 which is most critical for intact biological activity. In most of the cases reported previously, it was essential for the peptides to be purified to homogeneity before the chemical modification, and then the modified peptide needed to be isolated from the reaction product again [11, 12]. Such a downstream processing is laborious and time-consuming. In this paper, a novel method with a simplified process was developed to synthesize and purify the C-terminal PEGylated analog of the cysteine-mutated GLP-1. The chemical and biological characteristics of this site-specific PEGylated peptide were also preliminarily studied.

Materials and Methods

Materials

Escherichia coli DH5α used for plasmid amplification and *E. coli* BL21 (DE3) used for the expression of the fusion protein were purchased from Tiangen Biotech Co., Ltd. (Beijing, China). Plasmid pET32a, a vector for producing fusion protein with thioredoxinA (TrxA), was obtained from Novagen (Darmstadt, Germany). Ni-Sepharose 6 Fast Flow, Q-Sepharose Fast Flow, and Source 30 RPC were purchased from Amersham-Pharmacia (Uppsala, Sweden). The monomethoxy polyethylene glycol-maleimide ($mPEG_{10K}$-MAL) was obtained from Jenkem Co., Ltd. (Beijing, China). Enterokinase and GLP-1 were ordered from Sigma (St. Louis, MO). Kunming mice (male, 10 weeks old) were purchased from the comparative medical center of Yangzhou University (Jiangsu, China). All other chemical reagents were obtained from standard commercial sources and were of analytical grade.

Cloning and Construction of Recombinant Plasmid

The GLP-1 gene was designed with an enterokinase site at the N terminus as well as a termination codon at the C terminus, by using the preferential codons of *E. coli*. The gene of GLP-1 was synthesized by Genebase (Shanghai, China) and cloned into the cloning vector pMD-18T (Takara). Two primers were designed and synthesized to obtain the gene of cysteine-mutated GLP-1 (cGLP-1) from the cloning vector pMD-18T-GLP-1. These primers were as the followings: Primer 1 (5'-CGTGGTACCGACGATGACGATAAAC-3'), Primer 2 (5'-AAGCTTTTAGCAACGGCCTTTCACCAGCCAAGC-3'). The amplification conditions were 30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C with La-taq DNA polymerase. The amplification products were purified from agarose gel by using gel extraction and purification kit and then ligated into the pET32a plasmid at the *Kpn*I and *Hind*III sites. *E. coli* DH5α was transformed with this recombinant plasmid and selected on Luria-Bertani (LB) broth containing ampicillin (100 μg/ml). The plasmid DNA was sequenced and those plasmid constructed correctly was transformed into BL21 (DE3) to produce the fusion protein (TrxA–cGLP-1).

Expression of TrxA–cGLP-1 Fusion Protein

A 20 ml, overnight culture of BL21 (DE3), containing the TrxA–cGLP-1 expression construct, was grown in LB medium containing 100 μg/ml ampicillin. Five hundred milliliters of fermentation medium (10 g/L Tryptone; 20 g/L yeast extract; 20 ml/L glycerol; and salts as Na_2HPO_4 12.8 g/L, KH_2PO_4 3.4 g/L, NH_4Cl 2.7 g/L, and Na_2SO_4 0.7 g/L; pH 6.8) was incubated with the overnight culture media (at a 1:100 inoculant-to-media ratio) in shake flasks (50 ml per flask). Cells were grown at 37 °C and IPTG was added to a final concentration of 0.8 mM when the OD_{600} reached 0.6. The cells were grown at 25 °C for a further 8 h and harvested by centrifugation at 8,000 rpm for 5 min at 4 °C.

Purification and Characterization of TrxA–cGLP-1 Fusion Protein

The cell pellets were washed by resuspension in 150 ml of wash buffer (20 mM Tris–HCl, pH 8.0) followed by centrifugation at 8,000 rpm for 5 min at 4 °C. The cell pellets were resuspended in 120 ml of lysis buffer (20 mM Tris–HCl, 1 mM dithiothreitol, 20 mM imidazole, and 100 mM NaCl; pH 8.0) and disrupted by sonication. The supernatant of cell lysate was loaded onto 10 ml Ni-Sepharose 6 Fast Flow resin column which had been equilibrated with buffer A (0.02 M Tris–HCl, 0.50 M NaCl, 1 mM dithiothreitol, and 0.02 M imidazole; pH 8.0) previously. The column was thoroughly washed with five column volumes of buffer B (0.02 M Tris–HCl, 0.50 M NaCl, 1 mM dithiothreitol, and 0.05 M imidazole; pH 8.0) before eluted with buffer C (0.02 M Tris–HCl, 0.50 M NaCl, 1 mM dithiothreitol, and 0.50 M imidazole, pH 8.0). The eluted fraction was pooled and dialyzed before analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and characterized by western blotting using the mouse anti-His antibody.

Preparation and Purification of the Site-Specific PEGylated TrxA–cGLP-1 Fusion Protein

The TrxA–cGLP-1 fusion protein was incubated for 2 h at 4 °C in a pH 8.0 solution containing 20 mM Tris–HCl and a fivefold molar excess of $mPEG_{10K}$-MAL. An additional threefold molar excess of $mPEG_{10K}$-MAL was then added to the reaction and the mixture was incubated for another 1 h. The PEGylation reaction was terminated by diluting the mixture 20-fold with a pH 8.0 solution containing 20 mM Tris–HCl and 1 mM dithiothreitol.

The reaction product was applied onto Q-Sepharose Fast Flow resin column which was pre-equilibrated with 20 mM Tris–HCl, pH 8.0. After loading the sample, the column was washed with five column volumes of 20 mM Tris–HCl, pH 8.0 and the PEGylated TrxA–cGLP-1 fusion protein was eluted with a salt gradient of NaCl (100–400 mM) in 20 mM Tris–HCl, pH 8.0. The effluent was fractionally collected and analyzed by 15% SDS-PAGE. The gel was stained with a solution containing 5% barium chloride, 0.01 M iodine and 0.01 M potassium iodide. After scanning, the gel was restained with Coomassie brilliant blue R-250.

Purification and Characterization of the Site-Specific PEGylated cGLP-1

The site-specific PEGylated TrxA–cGLP-1 fusion protein was incubated for 6 h at 16 °C in a pH 7.6 solution containing 20 mM Tris–HCl, 100 mM NaCl and enterokinase (with the ratio of 0.3 mg PEGylated TrxA–cGLP-1 to 1 IU enterokinase). The cleavage product was applied onto Ni-Sepharose 6 Fast Flow resin column which was pre-equilibrated with

20 mM Tris–HCl and 100 mM NaCl, pH 8.0. The effluent was further purified by Source 30 RPC resin column (30 μm, 300×10 mm I.D.) with an acetonitrile gradient of 30% to 40% over ten column volumes. The effluent from the reverse-phase column was fractionally collected and lyophilized.

The purified $mPEG_{10K}$–cGLP-1 was separated on 15% SDS-PAGE, and then the loaded gel was soaked in the fixation solution (10% acetic acid, 45% methanol, and 5% barium chloride) for 2 min at room temperature. The gel was washed by distilled water for two times and then was incubated in the staining solution (10% acetic acid, 45% methanol, 0.25% Coomassie brilliant blue R-250, 0.01 M iodine, and 0.01 M potassium iodide) for 5 min. The gel was washed with distilled water for three times to remove residuary staining solution and then was soaked in the destaining solution (10% acetic acid and 45% methanol) for 60 min.

The purity of the $mPEG_{10K}$–cGLP-1 was determined by reverse-phase HPLC with an YMC-Pack C4 reverse-phase column (5 μm, 250×4.6 mm I.D.). A linear gradient of mobile phase 10–60% B in 30 min (mobile phase A: water with 0.1% TFA and mobile phase B: acetonitrile with 0.1% TFA) at a flow rate of 1 ml/min, and ultraviolet (UV) detection at 210 nm was utilized.

The circular dichroism (CD) measurements were performed using a Jasco J-715 spectropolarimeter over a range of wavelength of 190–260 nm, under constant N_2 purging according to the manufacturer's instructions, using GLP-1 and $mPEG_{10K}$–cGLP-1 at a concentration of 0.15 mg/ml in 10 mM sodium phosphate buffer pH 7.2, in quartz cuvettes of 0.1 mm path length. CD spectra were analyzed using K2D program.

Assay of Biological Activity

Effects of $mPEG_{10K}$–cGLP-1 on plasma glucose were determined using 10-week-old male Kunming mice. The animals were housed individually in an air-conditioned room at 22±2 °C with a 12 h light/12 h dark cycle. After food was withdrawn for 18 h, these 48 mice were divided into eight groups randomly according to the body weight. The saline used for this experiment was 0.9% NaCl. For the first four groups, one group was intraperitoneally (i.p.) injected with saline only (as negative control) and the other three groups were i.p. injected with glucose (18 mmol/kg body weight) in combination with saline (as positive control), GLP-1, or $mPEG_{10K}$–cGLP-1 (10 nmol/kg body weight) at 0 min.

For the other four groups, one group was also i.p. injected with saline only (as negative control). The other three groups received i.p. administration of saline (as positive control), GLP-1, or $mPEG_{10K}$–cGLP-1 (10 nmol/kg body weight) firstly, and then glucose (18 mmol/kg body weight) was i.p. injected alone after 120 min.

Retro-orbital blood samples were collected at 0, 15, 30, 45, and 60 min after i.p. administration of glucose. The serum glucose was measured by the glucose oxidase method [13]. All data were expressed as means±SD and analyzed by using Student's *t* test.

Results and Discussion

Construction of the Recombinant Plasmid

The native GLP-1 can be rapidly degraded by DPP-IV which can remove a dipeptide from the N terminus of GLP-1 [6]. It has been reported that the alanine at the penultimate

position of the N terminus of GLP-1 played an important role in this degradation process and analogs of GLP-1 with D-Ala or glycine at this position could exhibit enhanced stability [14, 15]. Therefore, we replaced this alanine with a glycine to increase the stability of cGLP-1 (Fig. 1). In order to realize site-specific PEGylation, a cysteine was added to the C terminus of cGLP-1 (Fig. 1). Using a custom designed primer pair, the desired fragment of cGLP-1 gene was amplified by PCR. The sequence of cGLP-1 gene was correct as shown by sequence analysis. The cGLP-1 gene was cloned into pET32a, where it was fused with TrxA and under the control of T7 promoter from the vector (Fig. 1). The expression vector pET32a was chosen because it provides TrxA sequence and His-tag to the expressed peptides, which should facilitate their soluble expression and downstream purification, and this vector has been used successfully for the expression and purification of many proteins and peptides [16, 17].

Expression, Purification, and Characterization of TrxA–cGLP-1 Fusion Protein

In order to avoid the formation of inclusion body, the culture was incubated at 37 °C to early log-phase and then was induced at a lower temperature (25 °C). With the optimized culture condition, the cGLP-1 gene could be efficiently expressed in the form of TrxA–cGLP-1 (Fig. 2a). After disruption and centrifugation, most of the TrxA–cGLP-1 was localized in the soluble fraction. By Ni-ion metal affinity chromatography separation, the fusion protein got a purity of greater than 95% (Fig. 2a). Since there was a His-tag in the sequence of the fusion protein, these results were further confirmed by western blotting using mouse anti-His antibody (Fig. 2b).

Preparation and Purification of the Site-Specific PEGylated TrxA–cGLP-1 Fusion Protein

Cysteine is regarded as an ideal site for the site-specific PEGylation due to the high chemoselectivity of maleimide to thiol group and protein containing cysteine allows for straightforward site-specific attachment by mPEG-maleimide. The TrxA–cGLP-1 fusion protein was PEGylated according to the methods mentioned in the references [18–20]. After optimization, the conditions used in our experiment yielded significant amounts of

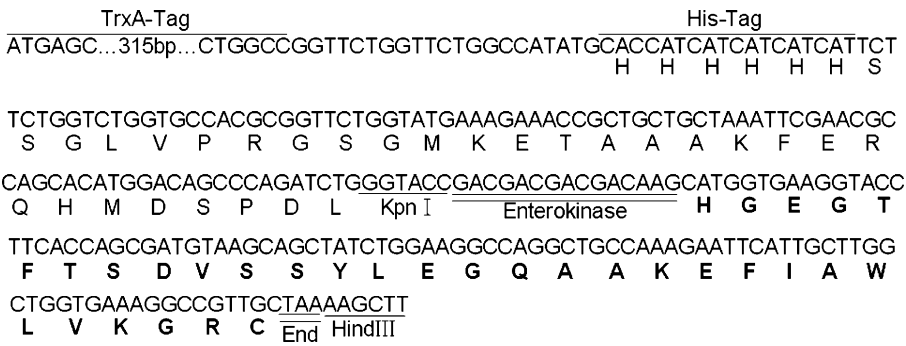

Fig. 1 Partial pET32a and complete cGLP-1 nucleotide sequence and predicted amino acid sequence. Amino acid sequence is indicated by the *one-letter code* written below the second nucleotide of each codon. The *Kpn* I and *Hind* III site are *underlined*. The enterokinase site and termination codon are *double underlined*

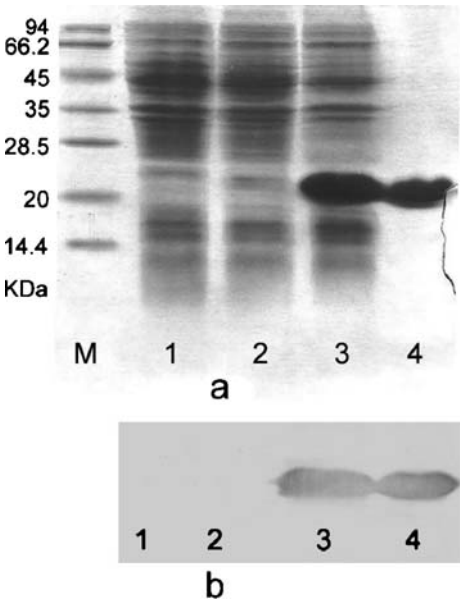

Fig. 2 Expression and purification of the TrxA–cGLP-1 fusion protein. **a** SDS-PAGE analysis of the expression and purification. *M* protein molecular weight marker, *lane 1* total protein of the *E. coli* BL21 (DE3), *lane 2* total protein of the *E. coli* BL21 (DE3) containing pET32a–cGLP-1, before induction, *lane 3* total protein of the *E. coli* BL21 (DE3) containing pET32a–cGLP-1 inducted with IPTG, *lane 4* the TrxA–cGLP-1 fusion protein. **b** Western blot analysis of the expression and purification. Protein samples were separated on 15% SDS-PAGE, transferred to a nitrocellulose membrane and probed with mouse anti-His antibody. The arrangement of the lanes was the same with **a**

mono-PEGylated TrxA–cGLP-1 (Fig. 3). After SDS-PAGE, protein could be stained by Coomassie brilliant blue R-250 but not iodine. On the contrary, PEG could be stained by iodine but not Coomassie brilliant blue R-250 [21]. Therefore, the SDS-PAGE gel was firstly stained with iodine to visualize the PEGylated product and then stained with Coomassie brilliant blue R-250 to visualize the protein. The PEGylated TrxA–cGLP-1 was separated from the native fusion protein by Q-Sepharose column chromatography and SDS-PAGE analysis of the eluted fractions indicated that the early eluting peak was the PEGylated TrxA–cGLP-1.

Purification and Characterization of the Site-Specific PEGylated cGLP-1

As there is an enterokinase site at the N terminus of the cGLP-1, the purified mono-PEGylated TrxA–cGLP-1 can be cleaved by enterokinase. With the optimized conditions, almost all of the $mPEG_{10K}$–cGLP-1 could be released from the fusion protein (Fig. 3). Even though cGLP-1 is a peptide and should be stained with Coomassie brilliant blue R-250, the $mPEG_{10K}$–cGLP-1 obtained in our experiment can only be visualized by iodine but not Coomassie brilliant blue R-250 (Fig. 3). One reason for this may be that the cGLP-1 represents only a very small proportion of $mPEG_{10K}$–cGLP-1. The cleavage product was applied onto Ni-ion metal affinity chromatography column in order to isolate $mPEG_{10K}$–cGLP-1 from its fusion partner. The fusion partner can be combined with the resin because there is a His-tag in its sequence. With this single-step purification, the fusion partner was removed from the $mPEG_{10K}$–cGLP-1 completely (Fig. 3). Since $mPEG_{10K}$–cGLP-1 could not be combined with the resin, it was localized in the effluent.

The effluent from the Ni-ion metal affinity chromatography column was applied onto the Source 30 RPC resin column for further purification. With the optimized elution conditions, there was only one main peak in the elution profile and this result indicated that $mPEG_{10K}$–cGLP-1 could be polished, desalted, and concentrated simultaneously through this single step. The fraction of $mPEG_{10K}$–cGLP-1 was pooled and lyophilized before next analysis.

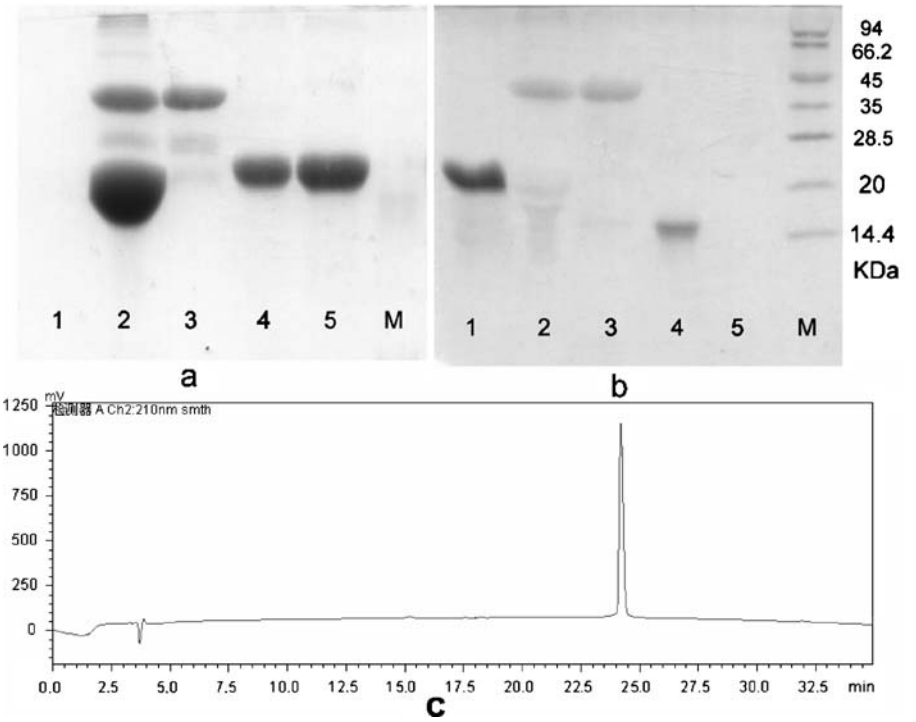

Fig. 3 Purification and cleavage of the PEGylated TrxA–cGLP-1 fusion protein. **a** Fifteen percent SDS-PAGE gel stained with 5% barium chloride, 0.01 M iodine, and 0.01 M potassium iodide. *Lane 1* the TrxA–cGLP-1 fusion protein, *lane 2* the PEGylation product, *lane 3* the PEGylated TrxA–cGLP-1 fusion protein after purification, *lane 4* the cleavage product of the PEGylated TrxA–cGLP-1 fusion protein, *lane 5* the $mPEG_{10K}$–cGLP-1 after purification, *M* protein molecular weight marker. **b** The same gel restained with Coomassie brilliant blue R-250. **c** The analytical reverse-phase HPLC profile of $mPEG_{10K}$–cGLP-1. A linear gradient of mobile phase 10–60% B in 30 min (mobile phase A: water with 0.1% TFA and mobile phase B: acetonitrile with 0.1% TFA) at a flow rate of 1 ml/min, and ultraviolet (UV) detection at 210 nm was utilized

Fifteen percent SDS-PAGE was used to characterize $mPEG_{10K}$–cGLP-1. To determine the apparent molecular weight of $mPEG_{10K}$–cGLP-1, an altered staining method was used in our experiment, as described in the "Materials and Methods". This method could visualize PEG and protein simultaneously and was of great importance to the characterization of PEGylated proteins and peptides. Comparison of the $mPEG_{10K}$–cGLP-1 band with a standard protein marker revealed an apparent molecular weight of 24 KDa for $mPEG_{10K}$–cGLP-1 (Fig. 4, lane 1), and this apparent higher mass is a typical observation when analyzing PEGylated proteins and peptides by SDS-PAGE [22, 23]. Because $mPEG_{10K}$–cGLP-1 is the conjugation of $mPEG_{10K}$-MAL and cGLP-1, it should migrate a little slower than $mPEG_{10K}$-MAL in the same gel. The result obtained in our experiment was in good agreement with this speculation (Fig. 4). The SDS-PAGE analysis showed that there was no impurity in the sample. However, some impurities, small peptides especially, cannot be detected by this method. Therefore, analytical reverse-phase HPLC was also utilized in our experiment to determine the purity of $mPEG_{10K}$–cGLP-1. According to the elution profile of analytical reverse-phase HPLC, the purity of $mPEG_{10K}$–cGLP-1 was up to 98% (Fig. 3).

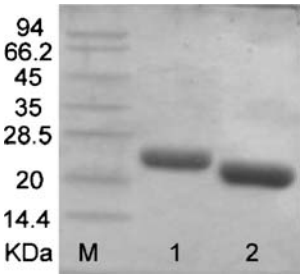

Fig. 4 Comparison of $mPEG_{10K}$–cGLP-1 with $mPEG_{10K}$-MAL on 15% SDS-PAGE gel. The gel was stained with iodine and Coomassie brilliant blue R-250 simultaneously as mentioned in the "Materials and Methods". *M* protein molecular weight marker, *lane 1* $mPEG_{10K}$–cGLP-1, *lane 2* $mPEG_{10K}$-MAL

Spectroscopic techniques are sensitive to the structural changes in proteins and peptides. To investigate the possible conformation changes, the secondary structure of $mPEG_{10K}$–cGLP-1 was determined by far-UV CD spectra and compared with that of native GLP-1. In 10 mM sodium phosphate buffer and at pH 7.2, the CD spectrum of the $mPEG_{10K}$–cGLP-1 was found to be very similar to that of native GLP-1 (Fig. 5). This result suggested that $mPEG_{10K}$–cGLP-1 maintained the secondary structure of native GLP-1. By using K2D program, the α-helix content for $mPEG_{10K}$–cGLP-1 and native GLP-1 was both calculated to be 30% and this value was roughly similar to that (about 35%) reported previously by others [24].

The downstream processing used in this study is a novel one. In most of the cases reported previously, the peptide was always needed to be purified to homogeneity before chemical modification [11, 12]. In such a processing, it was essential for the peptides to be handled carefully, especially when there were cysteines in the sequence of the peptides, because they were not stable. At the beginning, we also tried such a downstream processing for many times. The aim peptide (cGLP-1) was firstly purified to homogeneity before the chemical modification. After PEGylation, the desired product ($mPEG_{10K}$–cGLP-1) was then separated from the reaction mixture. Such a downstream processing is laborious and time-consuming, because it needs five steps of chromatography. Moreover, cGLP-1 is not stable and needs to be handled carefully throughout the process. All these problems resulted in the extremely low final yield. At the end, we even could not get enough highly pure $mPEG_{10K}$–cGLP-1 for the quantitative determination.

Therefore, in this study, a novel method was attempted in order to facilitate the preparation of $mPEG_{10K}$–cGLP-1. With this novel method, as mentioned in the "Materials and Methods",

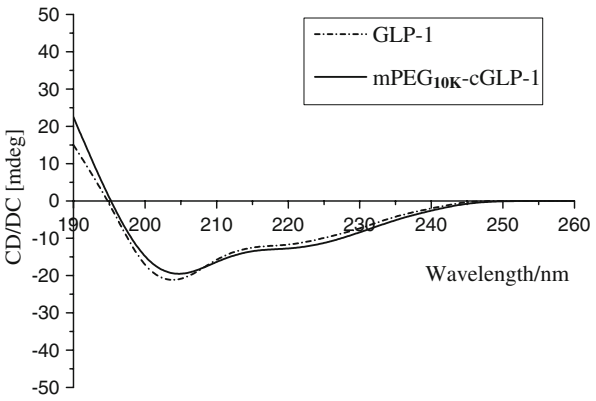

Fig. 5 Far-UV CD spectra of GLP-1 and $mPEG_{10K}$–cGLP-1 in 10 mM sodium phosphate buffer (pH 7.2)

Table 1 Summary of the purification steps[a].

Purification steps	Component	Content (mg)	Purity (%)	Yield (%)
Bacterial lysate clarification	TrxA–cGLP-1	572	ND[b]	100
Affinity chromatography	TrxA–cGLP-1	231	95[c]	40.4
Ion exchange chromatography	$mPEG_{10K}$–TrxA–cGLP-1	138	90[c]	24.1
Affinity chromatography	$mPEG_{10K}$–cGLP-1	13.9	90[d]	2.4
Reverse phase chromatography	$mPEG_{10K}$–cGLP-1	8.7	98[d]	1.5

[a] A total of 8.5 g wet weight of cells from 500 ml culture were lysed. The amounts of TrxA–cGLP-1 and $mPEG_{10K}$–TrxA–cGLP-1 were determined by Lowry protein assay, using bovine serum albumin as a standard; the amount of $mPEG_{10K}$–cGLP-1 was determined by RP-HPLC

[b] Not determined

[c] Determined by 15% SDS-PAGE

[d] Determined by RP-HPLC

the fusion protein was PEGylated firstly and then was cleaved by enterokinase. After cleavage, the PEGylated peptide could be separated easily from its fusion partner. There are many advantages for such a process. First, this approach reduced the complexity of downstream processing, and it needs only four steps of chromatography. Second, the aim peptide, cGLP-1 is not stable. However, the fusion protein, TrxA–cGLP-1 is much more stable and it needs not additional protection. Finally, using this novel method, 8.7 mg $mPEG_{10K}$–cGLP-1 was obtained from the original 500 ml culture (Table 1). This final yield was much higher than that of the traditional method.

Assay of Biological Activity

To investigate the glucose lowering activity of $mPEG_{10K}$–cGLP-1, glucose in combination with GLP-1 or $mPEG_{10K}$–cGLP-1 was administrated by i.p. injection simultaneously. The group got saline only was used as negative control and the group got glucose and saline simultaneously was regarded as positive control. Compared with the positive control, the blood glucose area under curve (AUC) was significantly lower following administration of GLP-1 or $mPEG_{10K}$–cGLP-1 (Fig. 6). Moreover, $mPEG_{10K}$–cGLP-1 was found to be more effective than the native GLP-1 with p value less than 0.05 (Fig. 6). This result may be

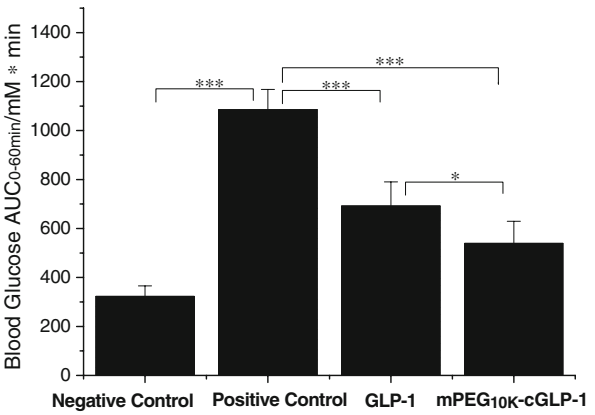

Fig. 6 Results of glucose lowering activity determination. Blood glucose AUC values for up to 60 min. ***$p<0.001$, *$p<0.05$ (means±SD, $n=8$)

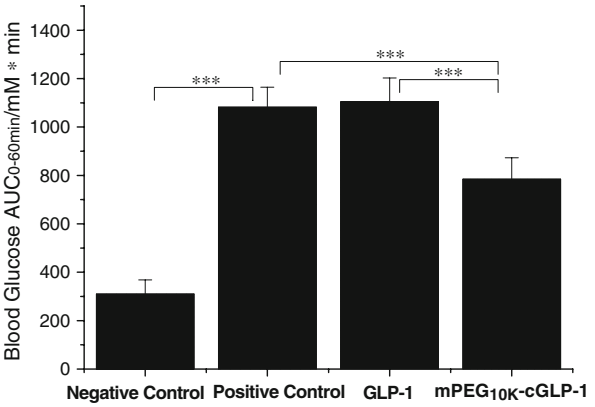

Fig. 7 Results of in vivo stability test. Blood glucose AUC values for up to 60 min. ***$p<0.001$ (means±SD, $n=8$)

caused by the improved stability of $mPEG_{10K}$–cGLP-1 over GLP-1. First, for $mPEG_{10K}$–cGLP-1, the conjugated PEG chain could improve the stability of cGLP-1, and this chain did not bind around the peptide's active sites, and therefore it had no prominent unfavorable effect on the biological activity of cGLP-1. On the other hand, for the native GLP-1, most of the molecules might have been degraded before they exhibited glucose lowering activity. Taken together, the in vivo glucose lowering activity of $mPEG_{10K}$–cGLP-1 was not only maintained but also enhanced as compared with that of native GLP-1 (Fig. 6).

In order to investigate the duration of glucose lowering activity of $mPEG_{10K}$–cGLP-1, saline, GLP-1, or $mPEG_{10K}$–cGLP-1 were administrated by i.p. injection firstly, and glucose was i.p. injected alone after 120 min. After such an interval, native GLP-1 had no effect on the blood glucose level as compared with the positive control (Fig. 7). However, the glucose lowering potency of $mPEG_{10K}$–cGLP-1 was found to be statistically significant as compared with the positive control or the native GLP-1 group, with p value less than 0.001 (Fig. 7). Because the activity of the increased early insulin stimulated by GLP-1 is unlikely to sustain more than 120 min [11], this result is more likely caused by the enhanced stability of $mPEG_{10K}$–cGLP-1. These results suggested that $mPEG_{10K}$–cGLP-1 obtained a significantly improved in vivo stability as compared with native GLP-1.

Conclusions

This paper described a novel and highly efficient method of producing large quantities of $mPEG_{10K}$–cGLP-1, and this method could facilitate further studies of $mPEG_{10K}$–cGLP-1 in the near future. As compared with that of native GLP-1, the glucose lowering activity of $mPEG_{10K}$–cGLP-1 was not only maintained but also enhanced and the in vivo stability of $mPEG_{10K}$–cGLP-1 was also significantly improved. These results indicated that $mPEG_{10K}$–cGLP-1 might find a role in the therapy of type 2 diabetes through C-terminal site-specific PEGylation.

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