European Journal of Pharmaceutics and Biopharmaceutics 72 (2009) 412–417

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Preparation and PEGylation of exendin-4 peptide secreted from yeast *Pichia pastoris*

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ARTICLE INFO

Article history:
Received 11 September 2008
23 January 2009
Accepted in revised form 1 February 2009
Available online 7 February 2009

Keywords:
Exendin-4
Pichia pastoris
PEGylation
Bioactivity
Histological

ABSTRACT

Exendin-4, a peptide analogue of glucagon-like peptide (GLP-1), has been developed for treatment of type 2 diabetes. Herein, the secretive exendin-4 peptide, expressed by methanol induction in *Pichia pastoris*, was purified to near homogeneity by Ni–NTA agarose chromatography. 103.6 mg of protein was obtained from 1 L of the supernatant and its purity was 96.1%. Subsequently, the PEGylated exendin-4 was prepared. The bioactivity of exendin-4 was determined by examining the glucose-lowering and insulin-releasing ability in plasma. Then, a safety evaluation was performed by histological examination of the main organs (liver, kidney and pancreas). PEGylated exendin-4 displayed glucose-lowering and insulin-stimulating action *in vivo* without obvious damage to the above organs. The results suggest that the *P. pastoris* expression could be used to produce large quantities of exendin-4, and PEGylation is a useful tool to maintain and enhance bioactivity of the peptide.

1. Introduction

Exendin-4 is a 39 amino acid peptide (53% structural homology to GLP-1) and was first isolated from the salivary secretions of Gila monster lizard (*Heloderma suspectum*) [1]. It shares many of the glucoregulatory actions with GLP-1 [2,3] and has aroused great attention for its potential for the treatment of diabetes. Clinical and nonclinical studies have shown that exendin-4 has several beneficial anti-diabetic actions that include glucose-dependent enhancement of insulin secretion, glucose-dependent suppression of inappropriately high glucagon secretion, slowing of gastric emptying, reduction of food intake and body weight, and an increase in β-cell mass [2–7].

For many years exendin-4 has been synthesized chemically at high cost and is thus unsuitable for mass production. It, therefore, needs to be produced in quantity by genetic recombinant technology. Exendin-4 has been successfully expressed in *E. coli* system, and it proved to exhibit glucose-lowering action *in vivo* [8,9]. However, few studies on expression of exendin-4 in yeast system have been reported. For large-scale production, previously we had constructed a recombinant *Pichia pastoris* that can produce exendin-4 constitutively and secrete it into the broth [10]. In addition, the exendin-4 short circulating half-life means that high doses must be administered frequently, which limit its clinical application. Short circulating life of this peptide probably is a major factor in its brief action also. The short half-life of exendin-4 is due to its rapid inactivation and clearance under physiological conditions by proteolytic enzymes such as dipeptidyl peptidase-IV (DPP-IV) and neutral endo-peptidase (NEP) 24.11 [11]. Until now, the main approach that has been used to improve the therapeutic level of exendin-4 is development of enzyme-resistant analogues by substituting one or two amino acids of the peptide [12]. Design of exendin-4 analogues showed a number of promising results. However, it still remains difficult to optimize conditions such as control of enzyme resistance and biological potency *in vivo* [13]. Protein modification is one of the convenient alternative ways to improve therapeutic profiles of native proteins. Several authors reported that PEGylated GLP-1 could prolong its half-life *in vivo* and improved its bioactivity [11,14,15]. However, the roles of PEGylation had not previously been evaluated in exendin-4.

In this study, we reported the preparation of exendin-4 peptide expressed in *P. pastoris* and investigated bioactivity and safety of PEGylated exendin-4 conjugates *in vivo*.

2. Materials and methods

2.1. Materials

All cell culture reagents were purchased from ShengGong (Shanghai, China) except yeast extract (YE), which was obtained from Omega Scientific. Methoxy polyethylene succinimidyl propionate 5000 ($mPEG_{5k}$-SPA) was purchased from Sigma. Glucose assay kit and insulin enzyme-linked-immunosorbent assay kit were acquired from DingGuo (Beijing, China). Other chemicals were of analytical grade and obtained from local commercial resources.

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doi:10.1016/j.ejpb.2009.02.001

2.2. Expression of exendin-4 peptide

Pichia pastoris GS115 was transformed with plasmid pPIC 9 containing the gene encoding exendin-4, which was obtained from GenBank database (Accession No. AAB22006). The primary structure of exendin-4 was HGEGTFTSDLSKQMEEEQVRLFIEWLKNGGPSSGAPPPS. The vector was pPIC9k-His6 (construct based on plasmid pPIC9k, Invitrogen, by our laboratory which contained six consecutive CAT codons encoding six consecutive histidines). The details of the vector construction and transformation were described elsewhere [10]. Recipes and fermentation procedures were described briefly as follows. Fermentation of the pPIC9k/exendin-4 transformant was carried out using a 5-L fermentor. After 48 h of growth at 28 °C, the transformant *P. pastoris* was induced by methanol for 72 h. The culture was centrifuged at 8000*g* for 20 min to collect the supernatant, which was used as the source of crude protein.

2.3. Purification of the exendin-4 by Ni–NTA affinity chromatography

For the purification of exendin-4, the crude protein in the supernatant was firstly subjected to ammonium sulfate precipitation (20–65% saturation). His-tagged exendin-4 was purified by Ni–NTA affinity chromatography according to manufacturer's instructions. In brief, the Ni–NTA resin slurry was washed with distilled water and equilibrated with the binding buffer (50 mM Tris–Cl, pH 8.0) containing 10 mM imidazole. The solution containing soluble proteins was loaded onto the column and the flow-through fraction was collected. Weaked bound proteins were washed from the resin by the washing buffer (50 mM Tris–Cl, pH 8.0, 0.5 M NaCl) containing 20 mM imidazole. Bound proteins were eluted with the eluting buffer (50 mM Tris–Cl, pH 8.0, 0.5 M NaCl) in step-gradient (increasing concentration of imidazole from 40 to 500 mM).

2.4. Ultrafiltration

Fifty milliliters of solution containing soluble proteins were transferred to the centriplus centrifugal filter device with a cut-off of 2 kDa. The device was centrifuged at 5000*g* at 4 °C until 5–10 ml of retentate was left. The concentrated retentate was ultrafiltered twice more after being diluted with Tris–Cl buffer.

2.5. Tricine SDS–PAGE, Western blotting and HPLC assays

Tricine SDS–PAGE was carried out with the supplier's protocol (Amersham Bioscience). The separated proteins were stained with Coomassie Brilliant Blue R-250 or electroblotted to nitrocellulose membrane for Western blotting. After blocking with 3% (v/v) non-fat milk in 0.05% Tween/PBS (PBST), the membrane was washed three times with PBST for 10 min and incubated further with a 1:2000 dilution of polyclonal rat anti-exendin-4 antibody for 1 h, followed by washing as described above. The membrane was incubated with a 1:1000 dilution of HRP-conjugated goat anti-rat IgG as the second antibody, washed with the same procedure as above. Immunoreactivity was detected with diaminobenzidine (DAB) as a chromomeric substrate. Protein concentration was determined by the method of Bradford [16] using bovine serum albumin as a standard. HPLC assay was carried out on a 250 mm × 4.6 mm C_{18} column. The column was eluted with the linear gradient of acetonitrile (90%–0%) in 0.1% trifluoroacetic acid for 30 min at 0.5 ml/min.

2.6. Electrospray ionization mass spectrometry (ESI-MS)

Molecular weight was obtained from electrospray ionization mass spectrometry (ESI-MS) with a four-sector tandem mass spectrometer (Tokyo, Japan) equipped with an ESI ion source (Analytica of Branford, Branford, CT). The purified exendin-4 was dissolved in an equal volume of a mixture of acetonitrile/0.2% TFA in water/2-methoxyethanol/2-propanol and was delivered at the flow rate of 1 μl/min.

2.7. Preparation of PEGylated exendin-4

The activated PEG_{5k} was prepared according the Pernille's method [14]. The positional isomers of PEGylated exendin-4 conjugates were prepared according to the procedures described by Wolfgang et al. [17]. In brief, exendin-4 (500 μl, 1 mg/ml) in various 50 mM/L buffer solutions was reacted with 2 mole excess of PEG_{5k} (pH adjusted from 7.0 to 9.0 with 50 mM Tris–Cl buffer) at room temperature for 1 h. The unbound free PEG_{5k} was removed by size exclusion chromatography on a Sephadex G-25 column (1 × 50 cm, Tris–Cl, pH 7.5). Fractions were collected and assayed for protein using Bradford reagent. Fractions testing positive for protein were pooled and concentrated by freeze drying.

2.8. Biological activity assay

The effects of exendin-4 peptide on plasma glucose concentration and insulin level were examined using 6-week-old male Wistar rats. Animals were housed five per cage (three repetitions) at 25 °C environmental conditions with free access to food and water. They were allowed 1 week to adapt to their environment before the experiment. Food was withdrawn for 24 h before intraperitoneal injection of glucose (20 mmol/kg body weight) alone or in combination with exendin-4 (10 nmol/kg body weight). Test solutions were administered in a final volume of 1 ml/kg body weight [18]. Blood samples were collected at 15, 30, 60 till 240 min post-injection from the eye socket with a capillary into chilled heparin Eppendorf tubes. Blood samples were centrifuged and plasma samples were stored at −20 °C before determination.

Plasma glucose and insulin level were assayed by a glucose oxidase procedure with glucose assaying kit and insulin enzyme-linked immunosorbent assay kit (DingGuo, Beijing), respectively. Results were expressed as mean ± SE, and were analyzed by one-way analysis of variance (ANOVA) by SPSS 11.0 software. Differences were considered significant at $P < 0.05$ or $P < 0.01$.

2.9. Histological evaluation

In order to determine the possible toxic effects of exendin-4 expressed by *P. pastoris* in rats, histological sections of liver, kidney and pancreas were analyzed 24 h after injection. The organs were removed and fixed in 10% formaldehyde for histological processing. The tissues were embedded in paraffin, cut into 3–5 μm sections, stained with hematoxilin-eosin (HE) and processed further for light microscopy.

3. Results

3.1. Expression, detection and purification of exendin-4 peptide

The results of expression and detection of exendin-4 peptide are shown in Fig. 1. The culture supernatant was analyzed by Tricine SDS–PAGE, and a prominent band corresponding to approximately 4.3 kDa was shown following Coomassie Brilliant blue staining (Fig. 1A). The yield of the recombinant protein was about 150 mg/L (Table 1). Western blot analysis showed a single major band in clones, but no band in the control, indicating high reagent specificity for the expressed protein (Fig. 1B).

To purify soluble exendin-4 from *P. pastoris*, the culture supernatant was firstly desalted with a desalting column (G-25). The

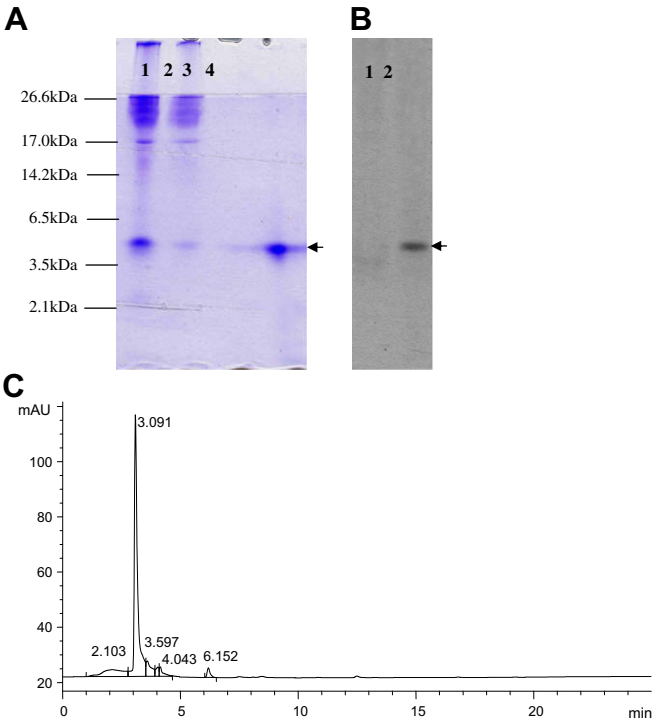

Fig. 1. Expression, detection and purification of exendin-4. (A) Tricine SDS–PAGE analysis of the expression and purification of exendin-4 peptide. Lane 1, broth supernatant; lane 2, flow-through fraction from the Ni-affinity column; lane 3, exendin-4 eluted from the Ni-affinity column with 100 mM imidazole; lane 4, target protein eluted from the Ni-affinity column with 250 mM imidazole. The position of the target proteins induced by methanol is indicated by arrow. (B) Western blotting of expressed protein in *P. pastoris*. Lane 1, water (blank); lane 2, expressed protein of recombinant GS115/pPIC9/exendin-4 after 72 h. (C) The purity analysis by HPLC. The target peak was at 3.09 min.

Table 1
Summary of exendin-4 purification.

Purification step	Total protein[a] (mg)	Product[b] (mg)	Yield (%)
Culture supernatant	414.6	150.7	100
Ni–NTA affinity chromatography	196.3	103.6	68.8

[a] Total protein concentration was determined by Bradford protein assay using bovine serum as a standard.
[b] The amount of protein of interest was determined by quantifying the amount in each gel lane by densitometer (Totallab V 1.11 software).

ammonium sulfate precipitated protein was dissolved in the binding buffer (50 mM Tris–Cl, pH 8.0, 0.3 M NaCl), and bound to Ni–NTA resin through His-tag. Proteins were eluted by stepwise increasing concentrations of imidazole. Exendin-4 was finally eluted with the buffer containing 250 mM imidazole, and little target protein was detected in the pass-through fraction, indicating that the peptide (exendin-4) was bound to the nickel column. The purity analyzed by HPLC was 96.1%, which was performed on a 250 mm × 4.6 mm C_{18} column. The column was eluted with the linear gradient of acetonitrile (90% to 0%) in 0.1% trifluoroacetic acid for 40 min at 0.5 ml/min. The target peak was at 3.09 min (Fig. 1C). The final yield of exendin-4 was 68.8% (Table 1). Tricine SDS–PAGE showed that the apparent molecular weight of the target protein was about 4.3 kDa, which was in agreement with the calculated molecular weight by ESI-MS (4355.1 Da) (Fig. 2).

3.2. Preparation of PEGylated exendin-4 conjugates

The conjugation of exendin-4 with PEG_{5k} resulted in two main peaks in the size exclusion chromatogram, as shown in Fig. 3. It

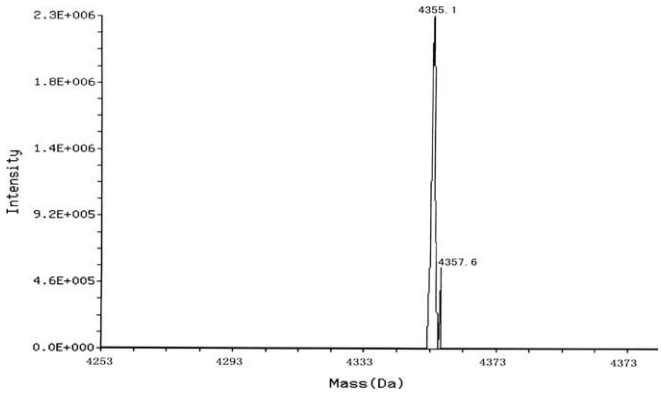

Fig. 2. ESI mass spectrum of the purified exendin-4. The molecular weight (4355.1 Da) of purified peptide, which was calculated by deconvolution.

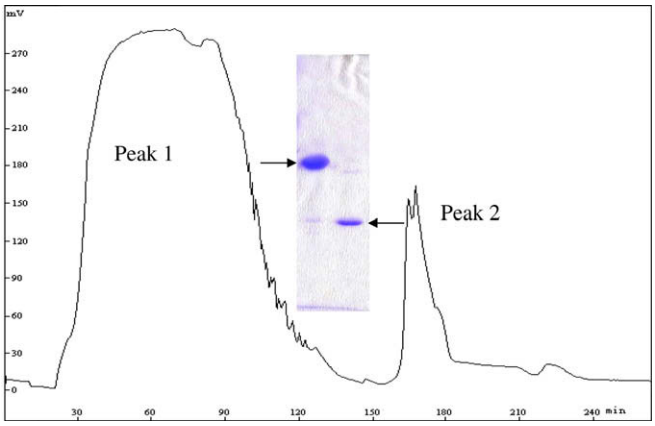

Fig. 3. Preparation of PEGylated exendin-4 conjugates. After reaction according to Section 2, the PEGylated exendin-4 and non-PEGylated peptide were separated by size exclusion chromatography. Peak 1, PEGylated exendin-4; peak 2, non-PEGylated peptide. The inset shows the Tricine SDS–PAGE of peak 1 and peak 2.

was carried out on a Sephadex G-25 column (1 × 50 cm) pre-equilibrated with Tris–Cl buffer (pH 7.5) at 0.2 ml/min. These collected fractions were subjected to Tricine SDS–PAGE and their identities were assigned by determining the molecular mass. In this way, peak 1 was regarded as the PEGylated exendin-4 and was prepared to be used in the following experiment.

3.3. Biological activity

To investigate the biological potency of PEGylated exendin-4 *in vivo*, equivalent doses (10 nmol/kg body) of the peptides were administered to rats by i.p. injection. Blood glucose reduced significantly after the administration of PEGylated exendin-4 compared with that of native exendin-4 or saline (control) (Fig. 4). There was no obvious difference between the glucose-lowering capabilities of native exendin-4 group and control group, only slight difference appeared at 15 min. By comparing the two groups, we found that the plasma glucose concentration in PEGylated exendin-4 significantly reduced at 30 min ($P < 0.05$), then a more marked difference was appeared after 60 min ($P < 0.01$). The capabilities of glucose lowering by PEG/exendin-4 could extend to 240 min (Fig. 4A). The plasma insulin concentration in the PEGylated exendin-4-treated group was also obviously elevated ($P < 0.01$) at 30 min compared with that in the control group, and the ability of stimulate insulin secretion can continued to 240 min (Fig. 4B). The maximal increase in insulin in rats treated with PEGylated exendin-4 was 4.5 times higher than the control group after 30 min.

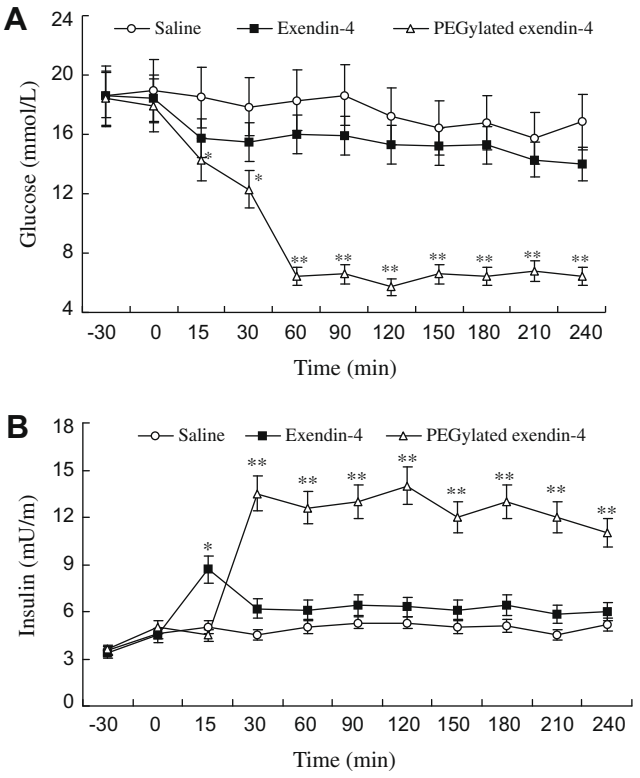

Fig. 4. Biological activity assay of peptides. The capabilities of glucose-lowering and insulin-releasing after intraperitoneal (i.p.) glucose alone (20 mmol/kg body) (control group), or glucose in combination with either native exendin-4 or PEGylated exendin-4 in rats. Values are mean ± SE ($n = 5$). Asterisk denotes statistically significant differences ($^*P < 0.05$; $^{**}P < 0.01$) between control and trial groups by one-way ANOVA.

3.4. Histological assays

Microscopic analyses of kidney, liver and pancreas were performed in untreated rats and native exendin-4 or modified exendin-4-injected rats. Fig. 5 shows histological sections of organs from different groups of animals. The liver, kidney and pancreas were normal and no histopathology appeared in the saline group (control). In trial groups, it was observed that native exendin-4 caused a slight acute toxicity, producing some histopathological damages (fatty degeneration) to kidney and liver after administration (Fig. 5). The same characteristics were encountered in PEGylated exendin-4-treated rats. However, the damage was reversible. In pancreas, no damage was observed after native exendin-4 or PEGylated exendin-4 injection.

4. Discussion

An efficient protein production system is critical for obtaining large amounts of recombinant protein for study. Previously, the production of exendin-4 was mainly by chemical synthesis which necessitated a high cost. Fortunately, bioengineering methods of producing exendin-4 and analogues have appeared. Yi et al. [8] and Yin et al. [9] cloned the exendin-4 gene and obtained stable expression in *E. coli*. However, the *E. coli*-expressed exendin-4 existed initially in inclusion bodies and bioactivity was realized only after renaturation, which made the purification process complicated and led to a low yield. Furthermore, clinical application of bacterially produced products may be affected by the possible presence of endotoxins that sometimes contaminate protein preparations expressed by *E. coli*. We have several reasons to select *P. pastoris* as an alternative recombinant expression host for exendin-4 synthesis. *P. pastoris* has the advantages of large production, genetically stable expression strains, the potential to secrete recombinant proteins into culture medium, and simple inexpensive culture conditions [19]. In addition, *P. pastoris* expression systems have the ability to promote protein folding, posttranslational modifications, and more simplified protein processing procedures compared to other prokaryotic expression systems [20]. In this study, the expression level of recombinant peptide exendin-4 was estimated at about 150 mg/L culture, which was higher than *E. coli* system. Furthermore, recombinant exendin-4 synthesized by *P. pastoris* was effectively purified from the culture medium through one step by a His-tag in this study. The whole purification process was simple and easy. These properties make *P. pastoris* a superior system to the *E. coli* for the preparation of exendin-4.

The chemical modification of therapeutic peptides and proteins with polyethylene glycol (PEG), known as PEGylation, is a useful tool to increase the therapeutic potential [21]. Biological activity of native exendin-4 and PEGylated exendin-4 was evaluated in terms of glucose-lowering and insulin-releasing capabilities *in vivo*. As shown in Fig. 4, native peptide exendin-4 did not effectively stabilize the blood glucose level against the glucose challenge because of its short biological life-time as reported previously [22]. The results suggested that the duration of native peptide exendin-4 to reduce plasma glucose to a certain level was at 15 min, while the activity of PEGylated peptide appeared at 30 min and can extend to 240 min (Fig. 4A). This indicates that the PEGylated exendin-4 has a longer duration of action and a greater potency than the native peptide. Also, in the native exendin-4 group, the level of insulin in plasma did not significantly change. This is one of the most important hormonal responses and is linked to glucose-lowering responses. In contrast, PEGylated exendin-4 displayed prominent stimulating insulin secretion action *in vivo*. These results are consistent with those reported in previous GLP-1 studies[14,15,23]. Furthermore, we observed that the capabilities of stimulating insulin secretion by native exendin-4 were only maintained up to 15 min, while PEGylated exendin-4 was up to 240 min (Fig. 4B). So, the half-life of PEGylated peptide in plasma is longer than the intact peptide. There are two main reasons for this. Primarily, PEGylation enhance the proteolytic stability of exendin-4. Another reason is that the PEGylated exendin-4 has higher molecular weight than nonmodified exendin-4, which can reduce the glomerular filtration *in vivo*. In our opinion, we should not rule out the possibility that the modificated exendin-4 has higher receptor binding ability, which needs further investigation.

In order to determine the safety of exendin-4, we evaluated the histological properties after 24 h by administering the compounds by i.p. injection to rats. The findings show that there were no obvious observable signs of toxicity in rats administered with exendin-4 or PEGylated exendin-4 treated animals, except a slight histopathological damage in kidney and liver. Moreover, the damage was associated with acute toxicity that was reversible. In agreement with the histopathological examination, we found that the rat behavioral traits were normal at the 24 h post-injection (data not shown). The results indicated that the recombinant exendin-4 from *P. pastoris* did not do any obvious harm to the animals. These results demonstrate that PEGylated exendin-4 analogue is resistant to degradation. In addition, its enhanced biological potencies highlight its potential for new glucose-lowering agents.

It is important to note that exendin-4 molecule has three possible sites for PEGylation: the primary amino group of N-terminus (His^7) and two lysine residues (lys^{26} and lys^{34}) [11]. In this study, the PEGylated exendin-4 was prepared using a conventional nonspecific PEGylation method, not a site-specific PEGylation process. The nonspecific PEGylation process probably results in substantial

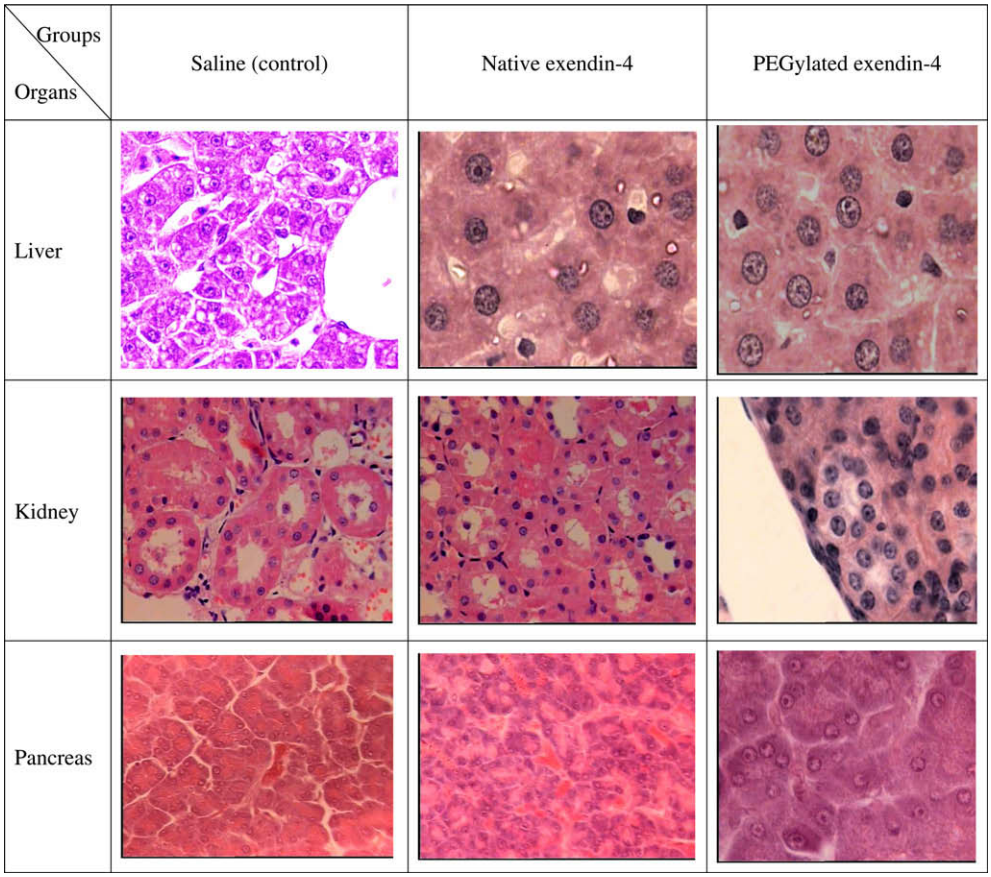

Fig. 5. Histological analysis of kidney, liver and pancreas of control group (A), native exendin-4 group (B) and PEGylated exendin-4 group (C) after injection.

loss of biological activity when PEG binds around the protein active sites [15]. In the next step, we will focus on the structure analysis and evaluation of the therapeutic efficacy of site-specific PEGylated peptides.

5. Conclusion

In this study, we have successively produced recombinant exendin-4 with a yield of 103.6 mg/L and with purity above 95% by using the *P. pastoris* expression system. PEG modification is a good method to improve therapeutic profiles of native peptides. The PEGylated exendin-4 has good stability and long half-life. In addition, their enhanced biological potencies highlight their potential as a new glucose-lowering agent.

Acknowledgements

This work was supported by China Postdoctoral Science Foundation (Grant No.: 20080440359) and the National Natural Science Foundation (Grant No.: 2006AA100311). We also thank Dr. Yan Wang for reading the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2009.02.001.

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