Journal of Peptide Science
J. Pept. Sci. 2008; **14**: 588–595
Published online 12 November 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.942

JOURNAL OF PEPTIDE SCIENCE

Preparation and characterization of a novel exendin-4 human serum albumin fusion protein expressed in *Pichia pastoris*

YAN-SHAN HUANG,[a] ZHI CHEN,[b] YI-QIONG CHEN,[c] GUO-CHANG MA,[c] JIAN-FENG SHAN,[c] WEI LIU[a] and LIN-FU ZHOU[d]*

[a] Department of Cell Biology, School of Medicine, Zhejiang University, Hangzhou 310058, China
[b] Institute of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, China
[c] Hangzhou Jiuyuan Gene Engineering Co. Ltd., Hangzhou 310018, China
[d] Medical Biotechnology Laboratory, School of Medicine, Zhejiang University, Hangzhou 310058, China

Received 14 May 2007; *Revised 17 August 2007*; *Accepted 24 August 2007*

Abstract: A novel recombinant exendin-4 human serum albumin fusion protein (rEx-4/HSA) expressed in *Pichia pastoris* was prepared and characterized. Ex-4 is a 39-amino acid peptide isolated from the salivary gland of the lizard *Heloderma suspectum* and is thought to be a novel therapeutic agent for type 2 diabetes. But to gain a continued effect, the peptide has to be injected twice a day owing to its short plasma half-life ($t_{1/2} = 2.4$ h). To extend the half-life of Ex-4 molecule *in vivo*, we designed a genetically engineered Ex-4/HSA fusion protein. Between Ex-4 and HSA, a peptide linker GGGGS was inserted and the fusion protein was expressed in methylotrophic yeast *P. pastoris* with native HSA secretion signal sequence. The recombinant protein was secreted correctly and was obtained with high purity (typically >98%) by a three-step purification procedure. cAMP assay demonstrated that the fusion protein had a bioactivity similar to Ex-4 for interaction with GLP-1 receptors *in vitro*. Results from oral glucose tolerance test indicated that rEx-4/HSA could effectively improve glucose tolerance in diabetic db/db mice. Pharmacokinetics studies in cynomologus monkeys also showed that rEx-4/HSA had a much longer plasma half-life. Therefore, rEx-4/HSA fusion protein could potentially be used as a new recombinant biodrug for type 2 diabetes therapy. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: exendin-4; human serum albumin; fusion protein; *Pichia past*oris; preparation; characterization; diabetes

INTRODUCTION

Exendin-4 (Ex-4) is a 39-amino acid peptide isolated from the salivary gland of the lizard *Heloderma suspectum*. It shows 53% amino acid homology to the mammalian glucagon-like peptide-1 (GLP-1) and is a potent GLP-1 receptor (GLP-1R) agonist [1]. Ex-4 enhances glucose-dependent insulin secretion, suppresses inappropriately elevated glucagon secretion, and slows down gastric emptying. It also promotes β-cell proliferation and neogenesis *in vitro* and in animal models. In human subjects with type 2 diabetes, Ex-4 has a significant effect on fasting and postprandial glycemia and HbA1c levels [2–5]. In preclinical studies, synthetic Ex-4 (exenatide), the first clinically available incretin mimetic, has a 20- to 30-fold longer half-life and 5500-fold greater potency in lowering plasma glucose than GLP-1 [6,7]. In clinical trials, exenatide was injected subcutaneously (sc) twice-daily in human diabetic subjects, which resulted in statistically significant reductions in mean A1c, body weight, fasting plasma glucose, and postprandial plasma glucose excursions [8,9]. Because of its short half-life, amelioration in postprandial glycemia with exenatide twice-daily was prominent at breakfast and dinner, the meals before which exenatide is typically injected. To gain better effect, some kind of longer-half-life incretin mimetic formulations have been developed in clinical trials, which have exhibited better postprandial glycemic control effects compared to pure exenatide therapy [10,11].

Human serum albumin (HSA) fusion technology is based on the fact that fusing the heterogeneous proteins with full-length HSA molecule extends their circulatory half-life *in vivo* while still retaining their biological and therapeutic properties [12–14]. A recombinant human GLP-1-albumin protein (Albugon) has been developed with reduced EC_{50} compared to Ex-4, which exhibits glucose homeostasis *in vivo* [15].

In this study, by genetically fusing the nucleotide sequence encoding Ex-4 in-frame with the open reading frame of full-length HSA, we designed a novel rEx-4/HSA fusion protein that can be expected to be a more potent, long-acting therapeutic agent for type 2 diabetes.

*Correspondence to: Lin-Fu Zhou, Medical Biotechnology Laboratory, School of Medicine, Zhejiang University, 388 Yuhangtang Road, Hangzhou 310058, China; e-mail: 239zlf@zju.edu.cn

MATERIALS AND METHODS

Construction of the pPIC9-Ex-4/HSA Recombinant Expression Plasmid

Synthesized Ex-4 open reading frames (1–39) with HSA secretion signal peptide (Table 1) was cloned into plasmid pUC57 and amplified by PCR using primer 1 and primer 2 (Table 1) (Reaction 1). cDNA of full-lenth HSA molecule was amplified from pBlueHSA plasmid (constructed in our laboratory) with primer 3 and primer 4 (Table 1) (Reaction 2). These two PCR products were purified with a gel extraction kit (Watson Biotechnologies, Inc., China). Primer 2 and primer 3 contained the related complementary sequences for encoding the *C*-terminal amino acids of Ex-4, polypeptide linker GGGGS, and *N*-terminal amino acids of HSA (Table 1). To create the nucleotide sequence encoding the fusion protein of Ex-4-GGGGS-HSA, the overlap PCR was performed with two PCR products of the Reaction 1 and Reaction 2 as templates by using primer 1 and primer 4, annealing at 60 °C and amplified for 30 cycles. The final amplified product was digested with *Bam*H I and *Eco*R I restriction endonulease (New England Biolabs, Inc., USA) and then inserted into pPIC9 plasmid (Invitrogen Inc., USA) at *Bam*H I and *Eco*R I sites to obtain the recombinant expression plasmid pPIC9-Ex-4/HSA (Figure 1).

Transformation of *Pichia Pastories* and Screening for His^+ Mut^+ Transformants

The identified recombinant plasmid construct pPIC9-Ex-4/HSA was linearized with *Sal I* and integrated in *P. pastories* genome by electroporation using a GENE-PULSER II (Bio-Rad Laboratories, USA) according to the manufacturer's instruction (Invitrogen). Four days later, several colonies were picked up and cultivated in BMGY medium (10 ml), and then transferred to 50 ml BMMY medium for the recombinant protein expression (30 °C, 250 rpm). High-level expression colonies were analyzed by SDS-PAGE.

Expression and Purification of rEx-4/HSA

Recipes and fermentation procedure were performed following the manufacturer's protocol (Invitrogen Inc., USA) and are briefly as follows: Fermentation of the rEx-4/HSA transformant of *P. pastoris* was carried out using a Biostat C 15L fermenter (Sartorius AG, Germany). Briefly, after 1 day of growth at 30 °C, rEx-4/HSA was induced by methanol (~0.5%, v/v) for about 50 h. The fermentation broth containing the secreted rEx-4/HSA protein was collected by centrifugation (7000 rpm, 15 min). The supernatant was diluted 4 times with 0.5 M sodium chloride in 20 mM sodium phosphate (pH 6.5) and applied on a Blue Sepharose Fast Flow column (XK 50/20, Amersham Bioscience, USA). The column was washed with 1 l of 1 mol/l sodium chloride in 20 mM sodium phosphate (pH 6.5). rEx-4/HSA was eluted with 2 mol/l sodium chloride in 20 mM sodium phosphate (pH 6.5) at a flow rate of 180 ml/h. Then the fractions containing rEx-4/HSA were collected and ammonium sulfate was added to 0.5 M, followed by application on a Phenyl Sepharose FF column (XK 50/20, Amersham Bioscience, USA) which was pre-equilibrated by 0.5 M ammonium sulfate with 10 mM sodium phosphate (pH 7.0). rEx-4/HSA was eluted with a linear gradient of 0.5–0 mol/l ammonium sulfate in 10 mM sodium phosphate (pH 7.0), over a 15 min period at a flow rate of 180 ml/h. Fractions containing rEx-4/HSA were pooled and applied on a Sephadex G25 (medium) gel filtration column (XK 50/70, Amersham Bioscience, USA). rEx-4/HSA was eluted with 10 mM sodium phosphate (pH 7.4) at a flow rate of 120 ml/h. Fractions containing rEx-4/HSA were pooled and the purity was determined with nonreduced SDS-PAGE and RP-HPLC. *N*-terminal amino acid sequences of rEx-4/HSA were determined using an ABI 477A protein sequencer (Perkin-Elmer, USA).

SDS-PAGE

The purified fusion protein was analyzed by SDS-PAGE with 10% acrylamide gel and 5% condensing gel, and was stained with 0.25% Coomassie Brilliant Blue R-250 (Aldrich, USA).

Mass Spectrometry of rEx-4/HSA Fusion Protein

Molecular weight of rEx-4/HSA was determined by MALDI-TOF mass spectrometry using a BIFLEX mass spectrometer (Bruker Bremen, Germany). Samples were

Table 1 Oligonucleotides used in the generation of exendin-4-GGGGS-HSA (All oligonucleotides and primers were synthesized by Sagon Biotechnologies, Inc., Shanghai, China)

Oligonucleotides	Sequences
Nucleotide sequence encoding Ex-4 with HSA signal peptide	5′-GGATCCAAACG<u>ATGGCTATCCCAAAGTGGGTTACTTTCATCTCCTTGCTT TTCTTGTTTTCTTCCGCTTACTCCAGAGGTGTTTTCCGTAGACAC</u>GGTGAGGG TACTTTCACTTCTGACTTGTCTAAGCAAATGGAGGAGGAGGCTGTTAGATTG TTCATTGAGTGGTTGAAGAACGGTGGTCCATCTTCTGGTGCTCCACCACCATC TGGAGGAGGAGGATCT-3′
Primer 1	5′-CCG*GGATCC*AAACGATGGCTATCCC-3′
Primer 2	5′-GCAACCTCAGACTTGTGAGCATCAGATCCACCTCCACCAGATGG-3′
Primer 3	5′-CCATCTGGTGGAGGTGGATCTGATGCTCACAAGTCTGAGGTTGC-3′
Primer 4	5′-CGC*GAATTC*TTATAAGCCTAAGGCA-3′

Sequences encoding the HSA signal peptide are underlined, while *BamH* I and *EcoR* I sites are in italic.

DOI: 10.1002/psc

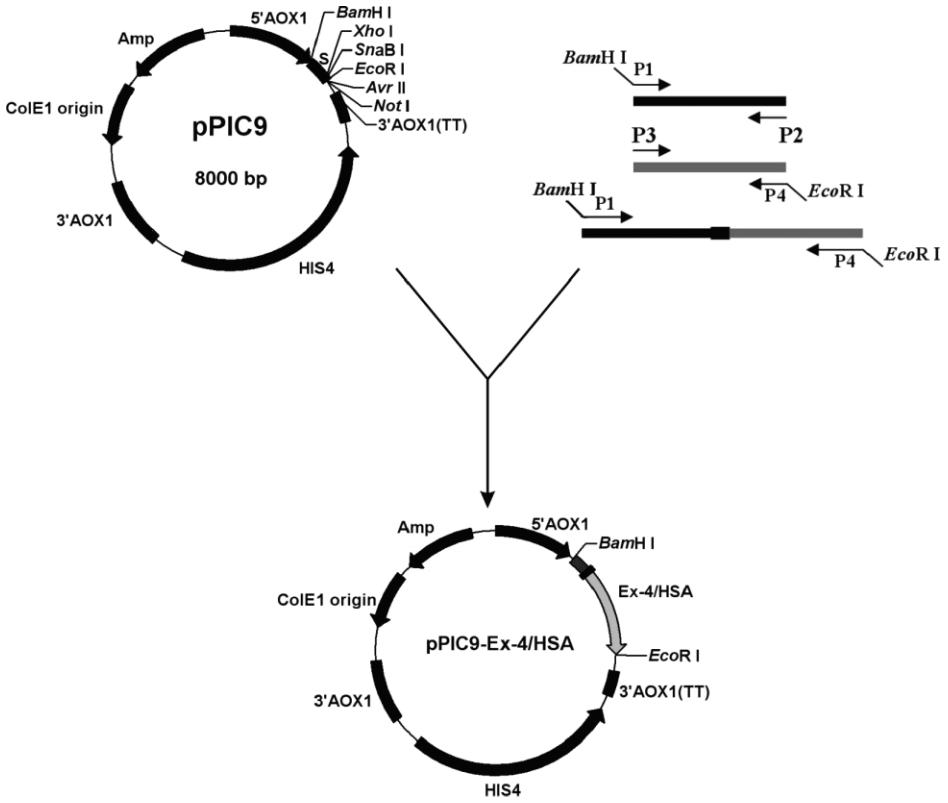

Figure 1 Construction of recombinant expression plasmid of pPIC9-Ex-4/HSA. The nucleotide sequence encoding Ex-4/HSA was amplified by two-step overlap PCR and included a *Bam*H I, *Eco*R I site at its 5′ and 3′ ends, respectively. A polypeptide linker GGGGS was inserted between Ex-4 and HSA.

prepared by mixing 1 µl aliquot with 2 µl of the matrix solution, a saturated solution of alpha-cyano-4-hydroxycinnamic acid in 50% of water/ACN with 0.3% TFA. Ionization was accomplished by using the 337 nm beam from a nitrogen laser with a repetition rate of 3 Hz. A 1.5 m flying tube and 20 kV acceleration voltage were used.

In vitro Bioactivity

Baby hamster kidney (BHK) cells stably transfected with human GLP-1R (BHK-GLP-1R) were generated and propagated in a medium containing 0.05 mg/ml G418 as described previously [16]. Before analysis, BHK-GLP-1R cells were grown to 70–80% confluence in 24-well plates in the absence of G418. The cells were then pretreated with 100 µmol/l of 3-isobutyl-1-methylxanthine (IBMX, Sigma, USA) and incubated for 10 min. rEx-4/HSA, HSA and Ex-4 (American Peptide Company Inc., USA) were diluted with PBS and added to the 24-well plate with serial concentrations. All tests were carried out in triplicate and terminated by addition of ice-cold ethanol. Cell extracts were collected and stored at −80 °C until assayed. For cAMP determinations, supernatant of ethanol extracts were lyophilized, and cAMP levels were measured using a cAMP radioimmunoassay kit (Yamasa, Japan).

In vivo Bioactivity: Oral Glucose Tolerance Test(OGTT)

To investigate the biological potency of rEx-4/HSA *in vivo*, OGTT was carried out in C57BL/6J db/db mice (female, 8–12 week old, purchased from Model Animal Research Center of Nanjing University, China). All animals were housed under a 12 h light/12 h dark cycle and were fasted overnight prior to the experiment. Animal experiments were compliant with the Principles of Laboratory Animal Care (Zhejiang University). A single dose of rEx-4/HSA (0.3, 1 and 3 mg/kg) or HSA (3 mg/kg) was injected intraperioneally. Glucose (1.5 g/kg) was given orally through a gavage needle. Blood samples were drawn from the tail vein at 0.5, 10, 15, 30, 60, 90, and 120 min after glucose administration, and blood glucose levels were measured using a Glucometer (Elite blood glucose meter; Bayer, Canada).

OGTT test was carried out at 0.5, 12, 24, and 36 h post-rEx-4/HSA administration to evaluate he prolonged glucose-lowering action of the fusion protein.

Pharmacokinetic Studies

A pharmacokinetic study of the fusion protein was performed in cynomologus monkeys; six cynomolgus monkeys (3–4 years old, 4.2–4.8 kg), three females and

DOI: 10.1002/psc

three males, were purchased from the Animal Center of the Chinese Academy of Military Medical Sciences. Animal experiments were compliant with the Principles of Laboratory Animal Care (Zhejiang University). Three monkeys per group were injected intravenously (iv) and sc with 4 mg/kg of fusion protein diluted in PBS. Blood samples were collected predose and at 0.5, 1, 4, 8, 12, 24, 48, 72, 96, 120, 144, 192, 240, 288, and 336 h postdose into tubes containing EDTA. Plasma concentrations of rEx-4/HSA were determined using an ultrasensitive Ex-4 RIA Kit (Phoenix pharmaceuticals, Inc., USA). Samples with a protein concentration beyond the calibration range were diluted by addition of blank monkey plasma. Pharmacokinetic parameters were acquired by the 3p87 software package (version 1.1 PK software, Chinese Pharmacological Association).

RESULTS

Construction, Expression, Purification, and Characterization of rEx-4/HSA Fusion Protein

To circumvent the short biological half-life of Ex-4 due to renal clearance, we designed a novel rEx-4/HSA fusion protein which was expected to exhibit longer half-life owing to HSA's long circulation half-life of 19 days in humans.

In order to retain the bioactivity of Ex-4, HSA was fused at the *C*-terminus of Ex-4 and a 5-aa linker peptide (GGGGS) was inserted between them. HSA signal peptide was used for the correct secretion. The identified recombinant yeast expression strain was cultured and induced by methanol for 50 h. rEx-4/HSA samples were analyzed by SDS-PAGE and the

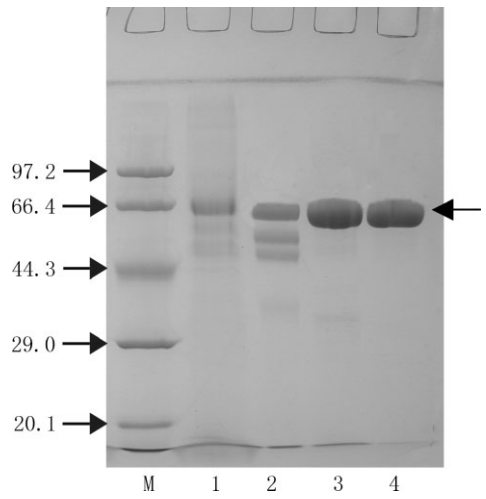

Figure 2 Reduced SDS-PAGE analysis of expression and purification of rEx-4/HSA fusion protein. Molecular weight standards (lane M), supernatant of fermentation (lane 1), fraction pooled from Blue SepharoseFF elution (lane 2), fraction pooled from Source PHE (lane 3), fraction pooled from Sephadex G-25 (lane 4). The right arrow indicates rEx-4/HSA.

Table 2 A summary of the purification of rEx-4/HSA fusion protein

Purification Step	Total protein (mg)	Purity (%)	Yield (%)
Fermentation of broth supernatant	~1365	71.5	100
Blue Sepharose FF	878	83.2	64.3
Phenyl Sepharose FF	528	93.4	38.7
Sephadex G25	475	98.2	34.8

Approximately 6.5 L supernatant of the fermentation broth obtained from the 15 L fermenter and rEx-4/HSA concentration was estimated by nonreduced SDS-PAGE.

expressed fusion protein concentration in the broth was about 0.21 g/l (Figure 2). A Blue Sepharose Fast Flow column was used to capture protein from the broth because rEx-4/HSA contains albumin that binds to Cibacron Blue specifically. Fractions pooled from Blue Sepharose Fast Flow contain 2 mol/l NaCl, which made it suitable for hydrophobic interaction chromatography for high conductivity. The results of the purification are shown in Table 2. About 73 mg rEx-4/HSA proteins was obtained from 1 l of cell-free broth, with about 35% total protein recovery. Nonreduced SDS-PAGE (Figure 2) and RP-HPLC demonstrated that rEx-4/HSA purity was more than 98%. The *N*-terminal sequence was NH2-HGEGTFTSDL, consistent with that of Ex-4 completely, and it also confirmed our correct processing of the *N*-terminus.

To further determine the exact molecular weight of rEx-4/HSA recombinant protein, rEx-4/HSA was analyzed by MALDI-TOF mass spectrometry. As shown in Figure 3, the fusion protein showed a molecular weight of 71 023 Da, very closed to the theoretical molecular mass deduced from its amino acid sequence (70 952.5 Da).

In vitro Bioactivity: Cyclic AMP Generation

GLP-1R belongs to the G-protein coupled-receptor family, which is coupled to the adenylate cyclase system. In response to the GLP-1 molecule or its analogs (e.g. Ex-4) binding, GLP-1R directly stimulates adenylate cyclase, leading to a rise of intracellular cAMP. So the change of cAMP concentration in BHK-GLP-1R cells can reflect the *in vitro* bioactivity of GLP-1, Ex-4, or rEx-4/HSA. As shown in the representative experiments reported in Figure 4, rEx-4/HSA produced a dose-dependent stimulation of cAMP accumulation in BHK-GLP-1R cells, although it was not as effective as Ex-4 in activating the GLP-1R (Figure 4: Ex-4

DOI: 10.1002/psc

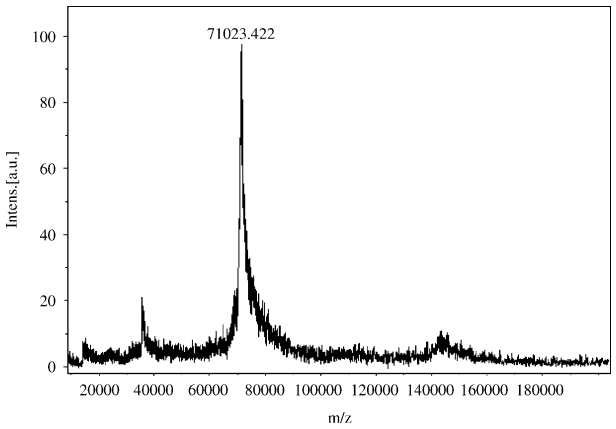

Figure 3 Molecular weight determination of rEx-4/HSA by mass spectrometry.

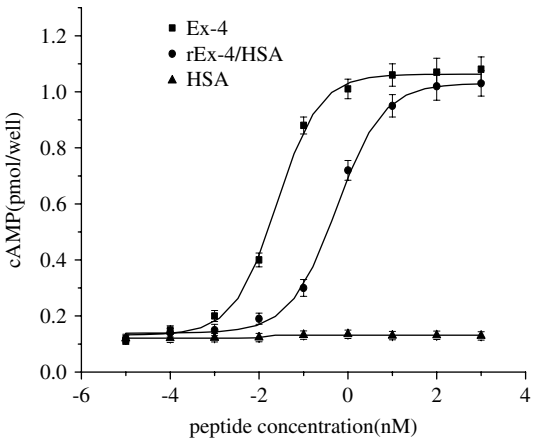

Figure 4 Ex-4/HSA bioactivity determined by cyclic AMP generation in BHK. Ex-4/HSA exhibits similar efficacy but lower potency than Ex-4 at the BHK-GLP-1R *in vitro*. A stable BHK cell line expressing the rat GLP-1R was treated with increasing concentration of Ex-4 standard or rEx-4/HSA. cAMP levels were measured by radioimmunoassay and used to calculate total cAMP content per well. ● ●:Ex-4; ■ ■:rEx-4/HAS; ▲ ▲:HSA.

standard $EC_{50} = 0.024 \pm 0.008$ nM, rEx-4/HSA $EC_{50} = 0.47 \pm 0.05$ nM, respectively).

In vivo Glucose-lowering Effect of rEx-4/HSA

To determine *in vivo* bioactivity of rEx-4/HSA, we carried out OGTT in diabetic db/db mice by a single injection of rEx-4/HSA. As shown in Figure 5(a–d), rEx-4/HSA effectively improved glucose tolerance in diabetic db/db mice compared with the control group. There was an obvious dose–effect relationship of serum glucose concentration after a single dose of 0.3, 1, and 3 mg/kg rEx-4/HSA. Postdose time-course observation indicated that the glucose-lowering effect of rEx-4/HSA lasted quite a long time but decreased gradually. OGTT

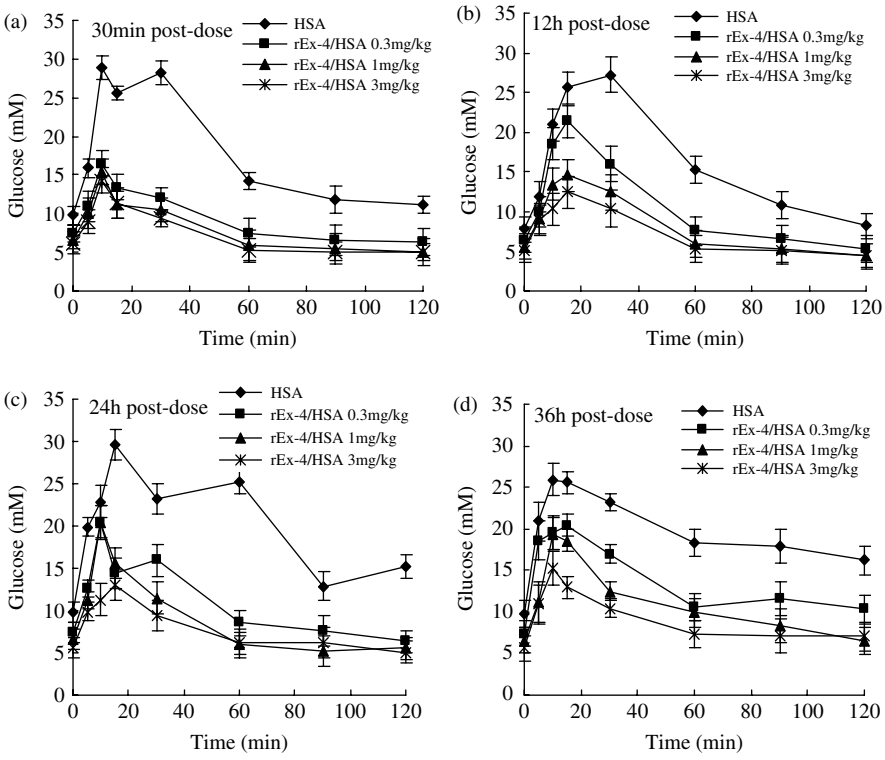

Figure 5 Ex-4/HSA lowering the blood glucose in db/db mice by oral glucose tolerance test (OGTT). Single dose of Ex-4/has (0.3, 1, and 3 mg/kg) or HSA (3 mg/kg) were injected intraperioneally in mice. OGTT were carried out at various times postdose to evaluate the duration of Ex-4/HSA action (a): 0.5 h; (b): 12 h; (c): 24 h; (d): 36 h. Values are expressed as means ± SE; $n = 5$ mice/group.

DOI: 10.1002/psc

carried out at 36 h postdose demonstrated that rEx-4/HSA still exhibited potent glucose-lowering activity *in vivo*.

Pharmacokinetics of rEx-4/HSA

Plasma concentrations of rEx-4/HSA (mean ± SE) from the pharmacokinetic study in monkeys are presented in Figure 6, and results of pharmacokinetic analysis are presented in Table 3. The terminal half-life of rEx-4/HSA in monkeys was about 57 h after iv administration and 77 h after sc administration. rEx-4/HSA was absorbed slowly in the monkeys, reaching a C_{max} of 16 953 ng/ml about 28 h after sc injection of 4 mg/kg.

Table 3 Mean pharmacokinetic parameter values after single-dose iv or sc administration with 4.0 mg/kg of rEx-4/HSA in monkeys

Parameter	4 mg/kg iv	4 mg/kg sc
AUC (ng.h/ml)	2 068 159.12	1 797 587.5
CL (ml/h/kg)	2.1	NA
CL/F (ml/h/kg)	NA	2.0
C_{max} (ng/ml)	NA	16 953.27
$t_{1/2}$ (h)	56.7	77.4
T_{max} (h)	NA	28.6
V_Z (ml/kg)	102	NA
V_Z/F (ml/kg)	NA	184
Bioavailability (F)%	100%	86.92%

Abbreviations: $t_{1/2}$, terminal half-life; T_{max}, time of maximum concentration; C_{max}, maximum concentration; AUC, area under the curve; CL/F(s), clearance over bioavailability; V/F, volume of distribution using the terminal phase.
NA, not applicable.

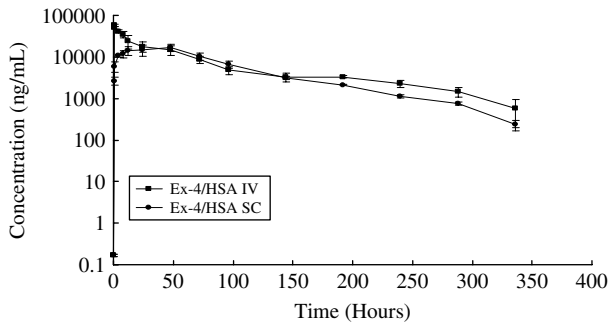

Figure 6 Pharmacokinetics study of rEx-4/HSA. Three monkeys per group were injected intravenously (iv) and subcutaneously (sc) with 4.0 mg/kg of rEx-4/HSA. Blood was collected predose and at 0.5, 1, 4, 8, 12, 24, 48, 72, 96, 120, 144, 192, 240, 288, and 336 h postdose into tubes containing EDTA.

DISCUSSION

Exenatide, a synthetic Ex-4, is the first clinically available incretin mimetic for type 2 diabetes therapy [9,17]. Because of its small molecular weight, exenatide can be cleared quickly in the kidney, with a half-life of only 2.4 h [18]. Serum concentration of exenatide fluctuates greatly and cannot stimulate the GLP-1R persistently even when it is injected twice a day. Some recent clinical trials have proved that long-lasting incretin mimetic formulations can offer better therapeutic efficacy. Exenatide LAR, a long-acting release formulation of exenatide administrated once-weekly, was observed to cause fourfold greater reduction in fasting glucose and twice A1c reduction when compared with exenatide twice-daily in type 2 diabetes patients. In addition, exenatide LAR provides postprandial glycemic control with all meals [10]. All these improvements may be attributed to the continuous therapeutic concentration of exenatide. Many pharmaceutical approaches have been made to develop even longer-acting incretin mimetics that retain the ability to activate GLP-1Rs continuously, such as sustained-release microspheres [10,19], PEG modification [20], fatty acid chain modification [21], and albumin binding [22]. Liraglutide is a fatty acylated human DPP-IV-resistant GLP-1 analog that binds to albumin and exhibits a $t_{1/2}$ of 11–15 h after parenteral administration in humans [11,23]. CJC-1131, is a DPP-IV-resistant GLP-1 analog modified with a reactive chemical linker that forms a covalent bond with a single amino acid residue within human serum albumin. Treatment with 0.4 mg/kg of CJC-1131 in db/db diabetic mice reduces the increase in mean blood glucose values up to 12 h postdose [22].

Since human serum albumin has a long half-life of 19 days in the human body, albumin fusion is a novel strategy for improving the pharmacokinetics of small proteins or peptides [12]. Comparing to other methods, the production of long-lasting albumin fusion can avoid complicated chemical modification or formulation process and therefore is easy and economical. Some albumin fusion proteins have been successfully generated, such as some hormones (human growth hormone, insulin) [13,24] and cytokines (interferon-α, interferon-β, IL-2) [14,25,26]. Pharmacokinetic properties and therapeutic efficacy of these molecules have been testified to be efficiently improved. Albugon, a recombinant GLP-1/albumin fusion protein, also exhibits the full spectrum of biological activities with reduced bioactivity *in vitro* [15].

Since Ex-4 exhibits a more potent effect on lowering glucose than GLP-1 [6,7], recombinant Ex-4/albumin fusion protein is expected to have higher efficacy as a novel long-acting therapeutic agent in type 2 diabetes treatments. Protokaryotic expression system, such as *E.coli*, is not suitable to express Ex-4/albumin fusion

DOI: 10.1002/psc

protein because of complicated molecular structure of albumin (17 disulfide bridges). *P. pastoris* eukaryotic expression system has many advantages in protein folding and post-translational modifications. It is usually used to produce large molecules such as albumin fusion recombinant proteins. In addition, the bioprocess cost of *P. pastoris* system is much lower than other eukaryotic expression systems [27].

The α-factor secretion signal peptide of *Saccharomyces cerevisiae* has been widely used for commercial *P. pastoris* expression vectors [28]. Unfortunately, heterogonous expression with this system often causes incomplete *N*-terminal processing [28]. We have expressed rEx-4/HSA fusion proteins with α-factor signal peptide but it was inactive. *N*-terminus sequencing results indicated that the *N*-terminus of rEx-4/HSA secreted with α-factor signal peptide was heterogonous and the fusion protein structure was incorrect. To solve this problem, we selected the native signal peptide of HSA molecule to substitute for the α-factor peptide because HSA has been expressed correctly in *P. pastoris* with its native signal peptide [29]. We have successfully expressed rHSA/IFN fusion protein with the correct structure using a similar strategy in *P. pastoris* [30].

It has been observed in many cases that fusion with albumin can decrease the protein's bioactivity because of increased spatial blockade. Albugon, the GLP-1/albumin fusion protein with an unknown structure, has been reported to retain only 1% activity of Ex-4 *in vitro* [15]. We expressed rEx-4/HSA with a linker sequence GGGGS, and the activity of the product was improved up to about 5%. A longer linker peptide GGGGSGGGGS (named rEx-4L/HSA) could not improve the bioactivity further (rEx-4L/HSA $EC_{50} = 0.51 \pm 0.04$ nM). Results of OGTT at different time points postdose showed that rEx-4/HSA has an improved long-acting, glucose-lowering effect compared to CJC-1131 (36 h *vs* 12 h). The pharmacokinetic studies *in vivo* also confirmed that the half-life of rEx-4/HSA could be as long as 70–80 h, which is nearly 33 times longer than synthetic Ex-4 ($t_{1/2} = 2.4$ h) [18]. The half-life of rEx-4/HSA is also much longer than those prepared by PEG modification ($t_{1/2} = 33.4$ min) [20] and fatty acid chain modification ($t_{1/2} = 11–15$ h) [23].

In summary, we have presented here a novel rEx-4/HSA fusion protein expressed in *P. pastories* that retains the biological properties and exhibits longer glucose lowering effect. Pharmacokinetic data suggested that rEx-4/HSA can be administrated once a week. It could be a new potential drug candidate for type 2 diabetes therapy.

REFERENCES

1. Eng J, Kleinman WA, Singh I, Singh G, Raufman JP. Isolation and characterization of exendin-4: an exendin-3 analogue from Heloderma suspectum venom: further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. *J. Biol. Chem.* 1992; **267**: 7402–7405.
2. Schepp W, Schmidtler J, Riedel T, Dehne K, Schusdziarra V, Holst JJ, Eng J, Raufman JP, Classen M. Exendin-4 and exendin-(9–39)NH2: agonist and antagonist, respectively, at the rat parietal cell receptor for glucagon-like peptide-1-(7–36)NH_2. *Eur. J. Pharmacol.* 1994; **269**: 183–191.
3. Baron AD, Kim D, Weyer C. Novel peptides under development for the treatment of type 1 and type 2 diabetes mellitus. *Curr. Drug Targets Immune Endocr. Metabol. Disord.* 2002; **2**: 63–82.
4. Nauck MA. Glucagon-like peptide 1 (GLP-1) and incretin mimetics for the treatment of diabetes. *Pract. Diabetes Int.* 2005; **22**: 171–179.
5. Baggio LL, Drucker DJ. Biology of incretins: GLP-1 and GIP. *Gastroenterology* 2007; **132**: 2131–2157.
6. Parkes D, Jodka C, Smith P, Nayak S, Rinehart L, Gingerich R, Chen K, Young A. Pharmacokinetic actions of exendin-4 in the rat: comparison with glucagon-like peptide-1. *Drug Dev. Res.* 2001; **53**: 260–267.
7. Young AA, Gedulin BR, Bhavsar S, Bodkin N, Jodka C, Hansen B, Denaro M. Glucose-lowering and insulin-sensitizing actions of exendin-4: studies in obese diabetic (ob/ob, db/db) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (Macaca mulatta). *Diabetes* 1999; **48**: 1026–1034.
8. Nielsen LL, Young AA, Parkes DG. Pharmacology of exenatide (synthetic exendin-4): a potential therapeutic for improved glycemic control of type 2 diabetes. *Regul. Pept.* 2004; **117**: 77–88.
9. Fineman MS, Bicsak TA, Shen LZ, Taylor K, Gaines E, Varns A, Kim DW, Baron AD. Effect on glycemic control of synthetic exendin-4 (AC2993) additive to existing metformin and/or sulfonylurea treatment in patients with type 2 diabetes. *Diabetes Care* 2003; **27**: 2370–2377.
10. Kim D, MacConell L, Zhuang D, Kothare PA, Trautmann M, Fineman M, Taylor K. Effects of once-weekly dosing of a long-acting release formulation of exenatide on glucose control and body weight in subjects with type 2 diabetes. *Diabetes Care* 2007; **30**: 1487–1493.
11. Madsbad S, Schmitz O, Ranstam J, Jakobsen G, Matthews DR, NN2211-1310 International Study Group. Improved glycemic control with no weight increase in patients with type 2 diabetes after once-daily treatment with the long-acting glucagon-like peptide 1 analog liraglutide (NN2211): a 12-week, double-blind, randomized, controlled trial. *Diabetes Care* 2004; **27**: 1335–1342.
12. Chuang VT, Kragh-Hansen U, Otagiri M. Pharmaceutical strategies utilizing recombinant human serum albumin. *Pharm. Res.* 2002; **19**: 569–577.
13. Osborn BL, Sekut L, Corcoran M, Poortman C, Sturm B, Chen G, Mather D, Lin HL, Parry TJ. Albutropin: a growth hormone-albumin fusion with improved pharmacokinetics and pharmacodynamics in rats and monkeys. *Eur. J. Pharmacol.* 2002; **456**: 149–158.
14. Osborn BL, Olsen HS, Nardelli B, Murray JH, Zhou JX, Garcia A, Moody G, Zaritskaya LS, Sung C. Pharmacokinetic and pharmacodynamic studies of a human serum albumin-interferon-alpha fusion protein in cynomolgus monkeys. *J. Pharmacol. Exp. Ther.* 2002; **303**: 540–548.
15. Baggio LL, Huang Q, Brown TJ, Drucker DJ. A recombinant human glucagon-like peptide (GLP)-1–albumin protein (albugon) mimics peptidergic activation of GLP-1 receptor–dependent pathways coupled with satiety, gastrointestinal motility, and glucose homeostasis. *Diabetes* 2004; **53**: 2492–2500.
16. Li Y, Hansotia T, Yusta B, Ris F, Halban PA, Drucker DJ. Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis. *J. Biol. Chem.* 2003; **278**: 471–478.
17. Cvetković RS, Plosker GL. Exenatide: a review of its use in patients with type 2 diabetes mellitus (as an adjunct to metformin and/or a sulfonylurea). *Drugs* 2007; **67**: 935–954.
18. BYETTA prescribing information [article online], URL: http://pi.lilly.com/us/byetta-pi.pdf. [last accessed July 2007].

DOI: 10.1002/psc

19. Gedulin BR, Smith P, Prickett KS, Tryon M, Barnhill S, Reynolds J, Nielsen LL, Parkes DG, Young AA. Dose-response for glycaemic and metabolic changes 28 days after single injection of long-acting release exenatide in diabetic fatty Zucker rats. *Diabetologia* 2005; **48**: 1380–1385.
20. Lee SH, Lee S, Youn YS, Na DH, Chae SY, Byun Y, Lee KC. Synthesis, characterization, and pharmacokinetic studies of PEGylated glucagon-like peptide-1. *Bioconjug. Chem.* 2005; **16**: 377–382.
21. Knudsen LB, Nielsen PF, Huusfeldt PO, Johansen NL, Madsen K, Pedersen FZ, Thogersen H, Wilken M, Agerso H. Potent derivatives of glucagon-like peptide-1 with pharmacokinetic properties suitable for once daily administration. *J. Med. Chem.* 2000; **43**: 1664–1669.
22. Kim JG, Baggio LL, Bridon DP, Castaigne JP, Robitaille MF, Jette L, Benquet. C, Drucker DJ. Development and characterization of a glucagon-like peptide 1-albumin conjugate. *Diabetes* 2003; **52**: 751–759.
23. Elbrond B, Jakobsen G, Larsen S, Agerso H, Jensen LB, Rolan P, Sturis J, Hatorp V, Zdravkovic M. Pharmacokinetics, pharmacodynamics, safety, and tolerability of a single-dose of NN2211, a long-acting glucagon-like peptide 1 derivative, in healthy male subjects. *Diabetes Care* 2002; **25**: 1398–1404.
24. Duttaroy A, Kanakaraj P, Osborn BL, Schneider H, Pickeral OK, Chen C, Zhang G, Kaithamana S, Singh M, Schulingkamp R, Crossan D, Bock J, Kaufman TE, Reavey P, Carey-Barber M, Krishnan SR, Garcia A, Murphy K, Siskind JK, McLean MA, Cheng S, Ruben S, Birse CE, Blondel O. Development of a long-acting insulin analog using albumin fusion technology. *Diabetes* 2005; **54**: 251–258.
25. Sung C, Nardelli B, LaFleur DW, Blatter E, Corcoran M, Olsen HS, Birse CE, Pickeral OK, Zhang J, Shah D, Moody G, Gentz S, Beebe L, Moore PA. An IFN-b-albumin fusion protein that displays improved pharmacokinetic and pharmacodynamic properties in nonhuman primates. *J. Interferon Cytokine Res.* 2003; **23**: 25–36.
26. Melder RJ, Osborn BL, Riccobene T, Kanakaraj P, Wei P, Chen G, Stolow D, Halpern WG, Migone TS, Wang Q, Grzegorzewski KJ, Gallant G. Pharmacokinetics and it *in vitro* and *it in vivo* anti-tumor response of an interleukin-2-human serum albumin fusion protein in mice. *Cancer Immunol. Immunother.* 2005; **54**: 535–547.
27. Daly R, Hearn MTW. Expression of heterogonous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. *J. Mol. Recognit.* 2005; **18**: 119–138.
28. Cereghino JL, Cregg JM. Heterogonous protein expression in the methylotrophic yeast Pichia pastoris. *FEMS Microbiol. Rev.* 2000; **24**: 45–66.
29. Hodgkins MA, Sudbery PE, Perry-Williams S, Goodey A. Secretion of human serum albumin from Hansenula polymorpha. *Yeast* 1990; **65**: 435.
30. Huang YS, Chen Z, Yang ZY, Wang TY, Zhou L, Wu JB, Zhou LF. Preparation and characterization of a potent, long-lasting recombinant human serum albumin-Interferon-α2b fusion protein expressed in *Pichia pastoris*. *Eur. J. Pharm. Biopharm.* 2007; **67**: 301–308.

DOI: 10.1002/psc