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Full Length Research Paper

Expression and characterization of recombinant human serum albumin fusion protein with C-peptide

Hai-Lin Yang[1]*, Yan-Yu Wei[2], Li-Ping Zhou[3] and Jing-Jing Kong[1]

[1]The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, PR China.
[2]Wuxi Biohermes Biotech Co., LTD, Wuxi 214092, PR China.
[3]School of Medicine and Pharmaceutics, Jiangnan University, Wuxi 214092, PR China.

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C-peptide (CP), connecting the A and B chains in proinsulin, has been considered to possess physiological effects in diabetes. In order to prolong the half-life of CP *in vivo*, a long acting CP analog [human serum albumin (HSA-CP)] was obtained by direct gene fusion of a single-chain CP to HSA and expressed in host *Pichia pastoris* GS115. After 72 h of growth on methanol, the recombinant HSA-CP concentration reached a level of 145 mg/L, representing 70% of total proteins in culture supernatant. The recombinant fusion protein was purified by ultra filtration, Q-sepharose fast flow column and Superdex 75 size-exclusion column. It was specifically recognized by the anti-human HSA antibody and CP antibody in Western blotting assay. *In vitro*, the recombinant HSA-CP can stimulate HEK293 cell proliferation. In Zucker diabetic fatty (ZDF) rats, 12 weeks recombinant HSA-CP treatment could prevent the accumulation of glomerular extracellular matrix, which leads to mesangial expansion and glomerular hypertrophy. The terminal biological half-time of the protein was 3.38±1.87 h after single administration of 500 nmol/kg of HSA-CP in Wister rats. The pharmacokinetic analysis of the protein indicate its promising application in clinical medicine.

Key words: C-peptide, human serum albumin, recombinant fusion protein, *Pichia pastoris*, bioactivity, biological half-time.

INTRODUCTION

The C-peptide (CP) is cleaved from proinsulin, stored in secretory granules, and eventually released into the bloodstream in amounts equimolar with those of insulin (Steiner et al., 1967). During the past decade, numerous studies have demonstrated that CP, although not influencing blood sugar control (Johansson et al., 2004), might play a role in a broad range of molecular and physiological activities, indicating that it is a hormonally active peptide. CP replacement in insulin dependent diabetes mellitus (IDDM, type 1 diabetes) patients and animal models of type 1 diabetes was accompanied by augmented blood flow in several tissues (Ido et al., 1997; Johansson et al., 2003), diminished glomerular hyper filtration, reduction of urinary albumin excretion (Samnegard et al., 2004) and prevention of glomerular hypertrophy and mesangial matrix expansion (Samnegard et al., 2005). Nociceptive sensory neuropathy (Kamiya et al., 2004), nodal and paranodal degeneration (Sima et al., 2004), cognitive dysfunction and hippocampal apoptosis (Sima and Li, 2005) can likewise be prevented by CP administration. Moreover, CP may have beneficial effects on cavernosal smooth muscle relaxation (Li et al., 2004) and reduce the risk of infection in diabetes mellitus (Langer et al., 2002). The biological half-life of CP in the plasma can last only about 20 min in rats by subcutaneous injection. It is possible to obtain more stable forms of a protein using gene fusion technology which can be used to express the target protein fused with human serum albumin (HSA). Fusion proteins with HSA can have long circulating life of albumin also with their biological and therapeutic properties (Giam et at., 2002). Many researchers have

*Corresponding author. E-mail: bioprocessor@yahoo.cn. or 19891996@sina.com. Tel/Fax: 0086 0510 85918119.

pursued insulin with longer biological half-time (Duttaroy et al., 2005).

Here, a new fusion protein including proinsulin CP and HSA was constructed and expressed in *Pichia pastoris*, aiming to prolong the biological half-time of the protein and maintain CP bioactivity simultaneously.

MATERIALS AND METHODS

Strains, plasmids and regents

The *Escherichia coli* DH5α and *E. coli* XL-1 BLUE, used as the host for cloning the HSA cDNA, CP cDNA and HSA-CP cDNA were kindly provided by Prof. Li from Key laboratory of industrial Biotechnology, Ministry of education, Jiangnan University, China. The *P. pastoris* host strain GS115 and pPIC9K used for expressed were purchased from Invitrogen Co. (Beijing, China). The plasmid pBlue-HSA and T-CP were constructed in our laboratory.

The restriction endonucleases, DNA polymerase with high-fidelity, and T4 ligase were purchased from Takara Co. (Dalian, China). Plasmid purification kit was purchased from Shanghai Sangon Co. (Shanghai, China). Human embryonic kidney 293 cell lines were purchased from Cell bank of Chinese academy of sciences (Shanghai, China). HSA polyclonal antibody was purchased from the Chengdu biological products, CP antibodies and CP standard were purchased from the Jingmei Co.(Nanjing, China).

Construction of fusion gene human serum albumin-CP (HSA-CP)

The CP gene was cloned from plasmid T-CP by polymerase chain reaction (PCR). The two PCR primers were designed as follows: Pr1 (5' GAAACCCTAGGCTTAGAAGCTGAGGACTTGCAAGTTGGTC 3') with *Saul* I site (underline) and Pr2 (5' GTTGCGGCC*GC*TTATTGAAGAGAACCTTCCAAAGCCA 3') with *Not* I site (underline). The PCR product was purified with gel extraction and digested with *Saul* I and *Not* I, and then it was cloned into the plasmid pBlue-HAS at restriction sites *Saul* I and *Not* I. The recombinant plasmid pBlue-HSA-CP was transformed into *E. coli* XL-1 BLUE and selected on lysogeny broth (LB) agar plates with 100 μg/ml ampicillin. The insertion was confirmed by restriction analysis and sequenced by Shanghai Sangon Co.

Construction of expression vector pPIC9K/ human serum albumin-CP (HSA-CP)

The plasmid pBlue-HSA-CP was digested with *EcoR* I and *Not* I, and then the insert was ligated to corresponding site of the expression vector pPIC9K. The constructed plasmid pPIC9K/ HSA-CP was transformed into *E. coil* DH5α strain and selected on LB agar plates with 100 μg/ml ampicillin. The expression plasmid pPIC9K/ HSA-CP was confirmed by both *Eco*R I and *Not* I digestion, and sent to Shanghai Sangon Co., for sequencing.

Transformation and selection of recombinant *P. pastoris* with high expression capacity of fusion protein

The resulting recombinant expression vector pPIC9K/ HSA-CP was digested with *Saul* I. Based on the procedure described in the operation manual of *Pichia* expression, the digested product was transformed into the *P. pastoris* GS115 under the condition of 40 μF, 150 Ω and 1.5 kV, which was spread on MD agar plates (1 mol/l sorbitol, 1.34% YNB, 0.004% histidine, 4×10^{-5} % biotin, in w/v, pH 7.0) with 4.0 mg/ml G418 and incubated 48–72 h at 30°C.

The colonies were inoculated into flask containing 100 ml of BMGY medium (100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5} % biotin, 2% peptone, 1% yeast extract and 2% glycerol, w/v) and incubated under constant shaking at 30°C until A_{600} =20–30. The cells were collected by centrifugation (8000 rpm, 10 min). The cell pellet was re-suspended in 25 ml BMMY medium (BMGY with 2.0% methanol instead of glycerol) and the culture broth was transferred into a 250 ml flask. The cultures were incubated under constant shaking at 30°C for 72 h, with an additional 0.5 ml methanol every 24 h. The culture was harvested by centrifugation and the supernatant was collected. The concentration of the recombinant HSA-CP was measured by the kit of trace urinary albumin (MinDian Co., Shanghai). The clone with highest secreting level was selected based upon the concentration of HSA.

Purification of human serum albumin-CP (HSA-CP) from *P. pastoris* culture supernatant

The culture method of *P. pastoris* GS115 is the same as described in the operation manual of *Pichia* expression. After 72 h, the supernatant was harvested by centrifugation (8000 rpm, 15 min) and concentrated to 50 times by ultra filtration. The concentrated supernatant was loaded into a 2.6×15 cm Q-Sepharose fast flow column pre-equilibrated with buffer A (20 mM PB, pH 6.0). The column was washed with buffer A and the protein was eluted with a linear gradient from 0 to 1.0 M NaCl in 20 mM PB, at pH 6.0. The eluted protein was analyzed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing HSA-CP were further concentrated by PEG 20,000 and loaded into a 1.6×90 cm Superdex 75 size-exclusion column pre-equilibrated with buffer B [20 mM phosphate-buffered saline (PBS) and 2.5 mM ethylene diamine tetraacetic acid (EDTA), pH 7.2]. The resulting solutions containing the targeted protein were analyzed on 12% SDS–PAGE and the concentration of recombinant HSA-CP was determined by the kit of trace urinary albumin.

SDS-PAGE and Western blotting assays

Analysis was performed with 12% SDS–PAGE. For Western blotting, proteins in the gel were transferred onto nitrocellulose membranes, and stained with Ponceau S to confirm equivalent transfer of samples. Membranes were blocked overnight at 4°C with Tris buffered saline (TBS; 10 mM Tris–HCl, 150 mM NaCl, pH 7.5) containing 3% skimmed milk. Thereafter, the membranes were incubated overnight at 4°C with the anti-human CP rabbit monoclonal antibody and the anti-HSA rabbit monoclonal antibody at a dilution of 1:100. The membranes were washed three times (5 min each) with TBS-T (TBS with 0.05% Tween 20) and incubated for 2 h at 37°C with the goat anti-rabbit IgG (HRP) (at dilution of 1:5000). Immunoreactivity was detected with diaminobenzidine (DAB) as a chromogenic substrate.

Bioactivity of human serum albumin-CP (HSA-CP) *in vivo* and *in vitro*

The study included three groups of Zucker diabetic fatty (ZDF) rats: (1) HSA-CP, 250 nmol/kg, sc, once daily; (2) HSA-CP, 500 nmol/kg, sc, once daily; (3) non-treated diabetic group (control group). After 12 weeks, ratio of kidney weight/body weight (KW/BW), glomerular volume [V(glom)], extracellular matrix area

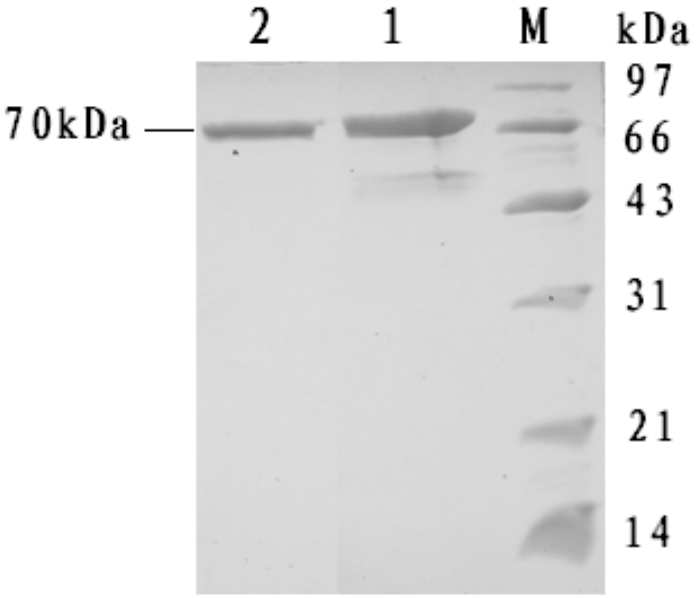

Figure 1. SDS-PAGE of recombinant HSA-CP secreted by *P. pastoris* GS115.**1**, purified HSA-CP; **M**, protein markers; **2,** culture supernatants of recombinant *P. pastoris* GS115 (pPIC9K/HSA-CP) after induction for 72 h.

fraction of glomerular cross-section [S(ECM)/S(glom)] and glomerular basement membrane thickness (GBMT) were measured.

The stably transfected HEK293 cells were seeded (2000 cells/well) in sterile 96-well plates in 100 µl Dulbecco's modified eagle's medium (DMEM) with 10% fetal calf serum (FCS) and incubated for 24 h at 37℃. HSA-CP and CP (control group without CP and HSA-CP) were added at different final concentrations (0.3，1，3，10，15，30，50，100，300 nM) in 100 µl DMEM plus 10% FCS. After incubation for 48 hours at 37℃, the cells were fixed in 50% trichloroacetate (TCA). The fixed cells were stained with 100 µl 0.4% sulphorhodamine B (SRB) in 1% acetic acid. Finally, the SRB dye was dissolved in 10 mM unbuffered Tris base (pH 10.5) for 10 min and measured at A_{540} in a microplate reader. The experiments were repeated at least three times.

The biological half-time of human serum albumin-CP (HSA-CP) in *vivo*

HSA-CP (500 nmo/kgl) was injected subcutaneously in Wister rats to study the metabolism characteristics. The plasma drug concentration was determined by immunoradiometric assay (IRMA).

RESULTS AND DISCUSSION

Construction of expression vector pPIC9K/ human serum albumin-CP (HSA-CP)

To improve the expression of HSA-CP, the codon usage of the gene encoding for CP was optimized according to *P. pastoris* codon bias and reduced repetitive AT and GC content. The artificial CP gene was fused to the HSA cDNA in the same reading frame. The fused gene HSA-CP was inserted into expression vector pPIC9k to construct recombinant plasmid pPIC9K/HSA-CP. The expression plasmid pPIC9K/HSA-CP was confirmed by agarose electrophoresis and DNA sequencing (data was not shown).

Expression and purification of fusion protein human serum albumin-CP (HSA-CP)

The recombinant expression vector pPIC9K/HSA-CP was linearized with *Sal* I, and the digested product was transformed into *P. pastoris* GS115. The concentration of secreted recombinant HSA-CP reached a peak level of 145 mg/L at 72 h. The molecular weight of the protein was approximately 70 kDa as determined by SDS–PAGE (Figure 1). Western blotting analysis showed that the protein was recognized specifically by goat anti-CP antibody and goat anti-HSA antibody, indicating that the expressed heterogeneous protein was recombinant HSA-CP (Figure 2).

The secreted HSA-CP obtained from the culture broth was first purified by ultra filtration and Q-sepharose fast flow chromatography with most of the HSA-CP tightly binding to the column. The purified protein solution was concentrated with PEG 20,000 and further purified with a Superdex 75 size exclusion column. HSA-CP purity

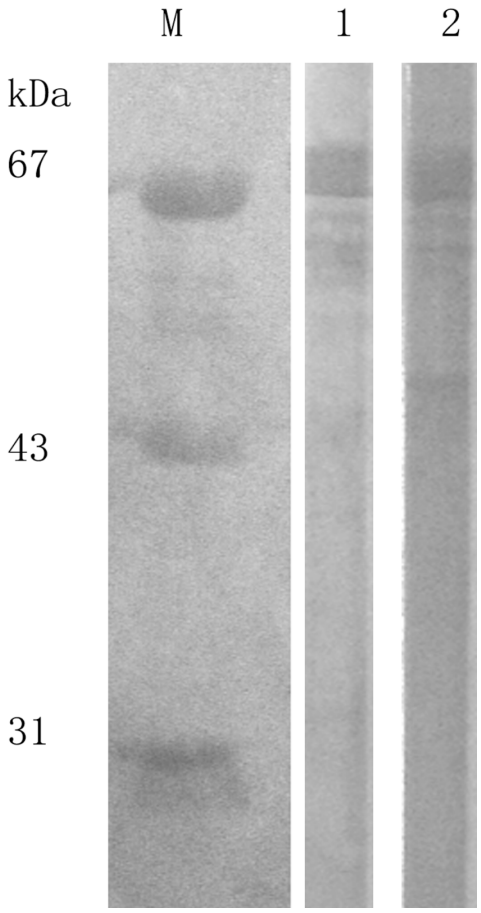

Figure 2. Western blot analysis of recombinant HSA-CP expressed in *P. pastoris* GS115. **M**, protein markers; **1**, HSA-CP Western blot with anti-human serum albumin rabbit monoclonal antibody; **2,** HSA-CP Western blot with anti-human CP rabbit monoclonal antibody.

eventually reached over 90%, as revealed by SDS–PAGE (Figure 1). As a result, 46 mg of pure HSA-CP protein was obtained out of 1 L of the culture supernatant and the purification yield of the protein reached 20.5%.

Bioactivity of human serum albumin-CP (HSA-CP) *in vitro*

The effect of HSA-CP and CP on in HEK293 cell growth was investigated by SRB assay and the results are shown in Figure 3. With the concentration increasing from 0.3 to 300 nM/L, CP and HSA-CP had effects on HEK293 cell proliferation, as compared with the control (without CP and HSA-CP). The optimal addition concentrations of HSA-CP and CP were 15 nM/L and 3 nM/L, respectively.

The effects of human serum albumin-CP (HSA-CP) in the treatment of rats with diabetic nephropathy

In ZDF rats, 12 weeks recombinant HSA-CP treatment (250 and 500 nmol/kg, sc, once daily) prevented the accumulation of glomerular extracellular matrix, which leads to mesangial expansion and glomerular hypertr-ophy but there was no prevention effect on whole kidney hypertrophy (Table 1).

The elimination half-life of human serum albumin-CP (HSA-CP) in rats

The pharmacokinetics of HSA-CP were investigated in Wister rats (n=6) by IRMA. After injection subcutaneously

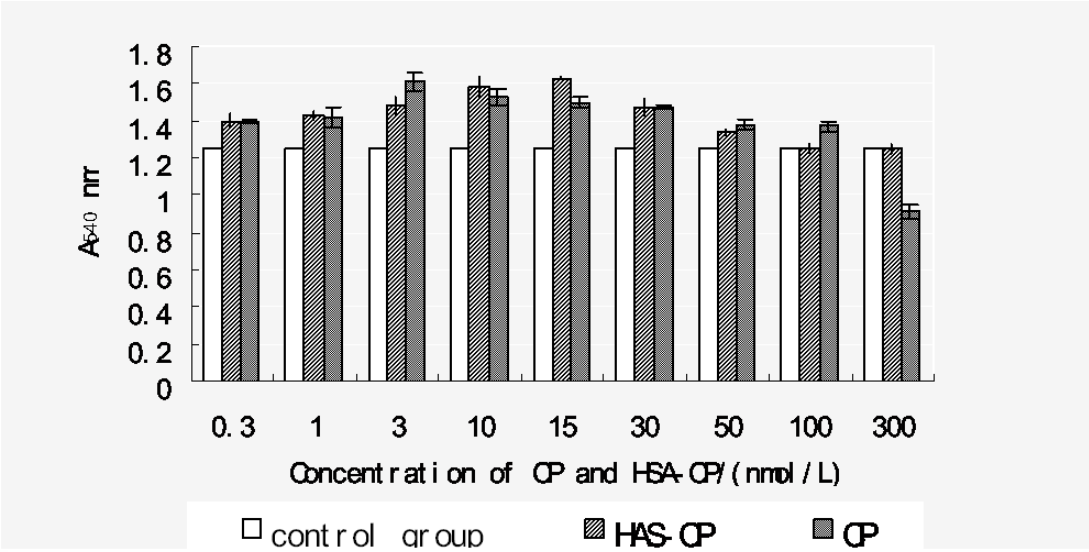

Figure 3.. HEK293 cell growth effects of 48 h exposure of various C-peptide and fusion protein of HSA-CP.

Table 1. The changes of kidney weight/body weight (KW/BW) and morphological analysis of kidney on rats.

Group	Dosage (nmol/kg)	Number	KW/BW (10^{-3})	V(glom) ($10^3 \mu m^3$)	S(ECM)/S (glom) (10^{-2})	GBMT (nm)
Control	—	8	4.6±0.7	621±58	32.7±1.0	181±29
HSA-CP	250	7	4.4±0.6	435±58**	26.7±1.0*	171±29
HSA-CP	500	9	4.1±0.5	349±60**	24.6±0.8**	179±32

*P<0.05 versus control group, **P<0.01 versus control group.

with 500 nmol/kg HSA-CP, C_{max} was achieved at 601±201 ng/mL. The elimination half-life ($t_{1/2}$) was calculated to be 3.38±1.87 h.

Production strategies for recombinant small peptides gave relatively low yields of the target peptide. In order to increase the amount of produced target peptide, one strategy is to synthesize a gene product with multiple copies of the target peptide (Li et al., 2003; Shen, 1984) and the other is gene fusion strategy. Proteins resulting from HSA gene fusions have the long circulating life of albumin while retaining their biological and therapeutic properties (Osborn et al., 2002; Sung et al., 2003). In this study, a new fusion protein including one CP mutant and one human albumin was properly designed. The strain efficiently expressing the fusion protein was successfully constructed with *P. pastoris* used as the host. With the expression system, HSA-CP could reach a maximum level of 145 mg/L at 72 h fermentation. The purity of the fusion protein reach 95% after purification with ultra filtration and columns in order. The recombinant HSA-CP protein obtained could stimulate HEK293 cell proliferation *in vitro*. The therapy of HSA-CP can prevent the accumulation of glomerular extracellular matrix. In addition, the biological half-time of the HSA-CP was about 10-fold longer than that of CP.

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Abbreviations

CP, C-peptide; **HSA-CP**, human serum albumin -CP;

ZDF, Zucker diabetic fatty; **KW/BW**, kidney weight/body weight; **GBMT**, glomerular basement membrane thickness; **FCS**, fetal calf serum; **SRB**, sulphorhodamine B; **IRMA**, immunoradiometric assay; **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **LB**, lysogeny broth; **PCR**, polymerase chain reaction; **V(glom)**, glomerular volume; **S(ECM)/S(glom)**, extracellular matrix area fraction of glomerular cross-section.

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