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Research paper

Preparation and characterization of a potent, long-lasting recombinant human serum albumin-interferon-α2b fusion protein expressed in *Pichia pastoris* ☆

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Abstract

A long-lasting recombinant human serum albumin-interferon-α2b fusion protein (rHSA/IFNα2b) was prepared and its structure and biological activities were studied. rHSA/IFNα2b was expressed in methylotrophic yeast *Pichia pastoris* with HSA's natural signal peptide and purified by dye affinity chromatography, hydrophobic interaction chromatography, ion exchange chromatography and Sephadex G25. Purity of the prepared rHSA/IFNα2b was greater than 97% analyzed by non-reduced SDS–PAGE and RP-HPLC. Structure and biological activities of the prepared rHSA/IFNα2b were characterized by physical, chemical and biological methods. Its p*I* was 5.3 and showed a single band on IEF gel. Molecular weight determined by MALDI-TOF was 86004.3 ± 29.2. Amino-terminal and carboxyl-terminal amino acid sequences were identical to predicted sequence. Its specific activity *in vitro* was $6.3 \pm 0.8 \times 10^5$ IU/mg fusion protein, retaining about 1.4% of that of unmodified rIFNα on a molar basis. After administered in monkeys, significant increases of 2′,5′-oligoadenylate synthetase activity relative to IFN-α were maintained for 14 days in serum and the rHSA/IFNα2b showed more potent biological activity than IFN-α on a molar basis. Therefore, markedly improved *in vivo* biological activity of rHSA/IFNα2b could exhibit more potent antiviral activity than IFNα2b in future clinical trials.

Keywords: Bioactivity; Characterization; Peptide mapping; *Pichia pastoris*; Purification; Recombinant human serum albumin-interferon-α2b

1. Introduction

Interferons (IFNs) are a family of soluble glycoproteins produced by different cells and exhibit antiviral, antitumor and immunomodulation functions. Interferons can be subdivided into two subfamilies: type I and type II that are related to the type of producing cells and receptors [1,2]. Many human cells produce type I interferons when infected by virus within several hours. Among type I interferons, IFNα exhibits the strongest antiviral activity and has been used to treat chronic hepatitis B and C infections [3]. The main shortcoming of interferon therapy is that its administration route is by subcutaneous injection only and therefore has a limited clinical application. Moreover, the half-life of IFN-α by this administration route is very short (4–16 h) which could impair the efficacy and bioavailability. In order to maintain its therapeutic effect, patients have to accept frequent injections (three times per week normally) for at least a half year. Patient compliance in these long-term dosing regimens is difficult to maintain. Therefore, development of an interferon with more conve-

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nient administration or with a longer half-life *in vivo* is a key problem to overcome for many researchers. Currently, there are two types of longer half-life products in the market: Pegasys and PEG-Intron. Pegasys is IFN-α2a modified with branched 40 kDa polyethylene glycol (PEG) and has a $t_{1/2}$ of 77 h in the human body [4]; PEG-Intron is IFN-α2b modified with a linear 12 kDa PEG molecule, and has a $t_{1/2}$ in humans of approximately 35 h [5]. Both of them needed to be injected only once a week, an improvement appreciated by the doctors and patients in clinical treatments. To avoid complicated chemical modification procedures, some researchers fused interferon-α2b with human serum albumin to obtain the same goal through the genetic recombinant expression technology [6]. Since human serum albumin has a long half-life of 19 days in human bodies, the half-life of the interferon could be prolonged effectively after fusion, thus fusion protein can reach a better therapeutic effect and even can be superior to those produced by the PEG-modifying method, especially in terms of homogeneity. *Pichia pastoris* expression system is very suitable to produce rHSA/IFNα2b for many reasons. Here we report the purification and characterization of rHSA/IFNα2b, a biologically active recombinant protein of IFNα2b fused to HSA, and compare the relative biological activities of rHSA/IFNα2b and IFNα *in vitro* and *in vivo*.

2. Materials and methods

2.1. rHSA/IFNα2b expression and purification

rHSA/IFNα2b is a recombinant fusion protein composed of human albumin genetically fused at its C-terminus to the N-terminus of IFNα2b, a protein linker Gly-Gly-Gly-Gly-Ser was inserted between them. rHSA/IFNα2b was produced using a yeast host system (*P. pastoris*) engineered to express the rHSA/IFNα2b fusion protein in a similar manner as previously described [7]. The pPIC9 vector (Invitrogen, USA) was used and HSA signal peptide instead of original α factor signal peptide to direct secretion of rHSA/IFNα2b. Recipes and fermentation procedures were all followed from the supplier's protocol and described briefly as follows. Fermentation of the rHSA/IFNα2b transformant of *P. pastoris* was carried out using a Biostat C 15 L fermenter (Sartorius AG). After 2 days of growth at 30 °C, rHSA/IFNα2b was induced by methanol for about 50 h. Fermentation broth containing the secreted rHSA/IFNα2b protein was collected by centrifugation (5000 rpm). The supernatant was diluted by 1 mol/L sodium chloride in 10 mmol/L sodium phosphate (pH 6.0) to 4-fold and applied on a Blue Sepharose Fast Flow (XK 50/20, Amersham Bioscience). The column was washed by 1 L of 1 mol/L sodium chloride in 10 mmol/L sodium phosphate; pH 6.0. rHSA/IFNα2b was eluted with 2 mol/L sodium chloride in 10 mmol/L sodium phosphate, pH 6.0, at a flow rate of 180 ml/h. The fractions containing the rHSA/IFNα2b were collected and ammonium sulfate was added to a final concentration of 0.5 mol/L. The rHSA/IFNα2b solution containing 0.5 mol/L ammonium sulfate was applied on a Phenyl Sepharose HP column (XK 50/20, Amersham Bioscience). The column was equilibrated and washed with 0.5 mol/L ammonium sulfate in 10 mmol/L sodium phosphate, pH 7.0. rHSA/IFNα2b was eluted with a linear gradient of 0.5–0 mol/L ammonium sulfate in 10 mmol/L sodium phosphate, pH 7.0, over 15 min period at a flow rate of 180 ml/h. The fractions containing the rHSA/IFNα2b were further purified by Q Sepharose Fast Flow column (XK 50/20, Amersham Bioscience). The column was equilibrated and washed with 20 mmol/L sodium chloride in 10 mmol/L sodium phosphate, pH 6.0, rHSA/IFNα2b was eluted with a linear gradient of 0.02–0.3 mol/L sodium chloride in 10 mmol/L sodium phosphate, pH 6.0, over 20 min period at a flow rate of 180 ml/h. Fractions containing rHSA/IFNα2b were pooled and applied on a Sephadex G25 (medium) gel filtration column (XK 50/70, Amersham Bioscience). rHSA/IFNα2b was eluted with 10 mmol/L sodium phosphate, pH 7.4, at a flow rate of 120 ml/h. Fractions containing rHSA/IFNα2b were pooled and purity was determined with non-reduced SDS–PAGE and RP-HPLC.

2.2. SDS–PAGE

Purified fusion protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 8% acrylamide gel and 5% condensing gel in the Mini-Protein II electrophoresis unit (Bio-Rad), which was stained with 0.25% Coomassie brilliant blue R-250 (Aldrich, USA). Running and staining procedures were all followed from the supplier's protocol.

2.3. Isoelectric focusing (IEF)

The samples were analyzed on Pharmacia MultiphorII horizontal electrophoresis system (Amersham Bioscience). Two microgram of samples from three different batches each was added and the assay was carried out followed with the supplier's protocol using pH 3.5–10 ampholine (Amersham Bioscience).

2.4. Western blot

Western blot was carried out according to the reported method with mouse anti-human IFN-α monoclonal antibody (Serotec Co.) and goat anti-human serum albumin monoclonal antibody (Serotec Co.) in two separate experiments [8].

2.5. Terminal analysis

Amino-terminal (N-terminal) and carboxyl-terminal (C-terminal) amino acid sequence analysis was carried out on Applied Biosystems Model 491N sequencer and 491A sequencer separately.

2.6. Molecular weight assay

Molecular weight was obtained from MALDI-TOF mass spectrometry using a BIFLEX mass spectrometer (Bruker Bremen, Germany). Samples were prepared by mixing a 1 μL of aliquot with 2 μL of the matrix solution, a saturated solution of alpha-cyano-4-hydroxycinnamic acid in 50% water/ACN with 0.3% TFA. Ionization was accomplished with the 337 nm beam from a nitrogen laser with a repetition rate of 3 Hz and 1.5 m flying tube and 20 kV acceleration voltage was used.

2.7. Trypsin digestion

Trypsin digestion was performed as previously described [9]. Samples were dissolved with 100 μl of l00 mmol/L NH_4HCO_3 (pH 8.3) buffer to 1 μg/μl, 2.5 μl (1 μg/μl) of trypsin (treated with TPCK, Sigma) was added and incubated at 37 °C for 4 h. Then another 2.5 μl trypsin was added and continued to incubate for 18 h. After the digestion was finished, it was cooled down to room temperature and 50% acetic acid was added to stop the reaction.

2.8. Enzymatic peptide mapping

Analysis of Trypsin digestion mixture by RP-HPLC was performed on a 300 Å, 4.6 mm × 150 mm C_{18} column (Vydac Co.) using 1100 HPLC system (Agilent Co.). The flow rate was 0.5 ml/min and the gradient conditions were used as follows: solvent A was water/TFA: 99.9/0.1(v/v) and solvent B was water/TFA/ACN: 9.9/0.1/90(v/v/v), the gradient started with 0% solvent A mobile phase, which was linearly increased to 90% solvent B in 65 min. Elution was monitored at 214 nm. Twenty microliters of digestion mixture was injected.

2.9. Mass spectrometric peptide mapping

Mass peptide mapping was carried out on LCQ Deca XP LC/MS (Thermo Co.), C_{18} reversed-phase column (RP-300,ABI), 2.1 mm × 30 mm. Flow rate was 0.2 ml/min and the gradient conditions were used as follows: solvent A was water/TFA/ACN: 98/0.02/2(v/v/v) and solvent B was water/TFA/ACN: 2/0.02/98(v/v/v). The gradient started with 0% solvent B mobile phase, kept for 2 min, then was linearly increased to 100% solvent B in 90 min. Scan range was 40-2000DAL. Results were analyzed by the system attached software.

2.10. Circular dichroism (CD) spectroscopy analysis

CD spectra were recorded using a JASCO J-715 automatic recording spectropolarimeter (JASCO). The mixture of human serum albumin and interferon-α2b (with molar ratio of 1:1, 0.5 mg/ml of total protein concentration) and rHSA/IFNα2b fusion protein (0.5 mg/ml) was determined, respectively.

2.11. In vitro antiviral activity assay

Antiviral activity of rHSA/IFNα2b was tested on WISH cell line (American Type Culture Collection) that had been exposed to encephalomyocarditis virus (EMCV). The assay was performed as previously described [10] with some modification. Briefly, WISH cells were seeded 2.5×10^4–3.5×10^4/100 μl/well in flat-bottomed 96-well plates and incubated in 5% CO_2, 37 °C for 5 h. Serial dilutions of rHSA/IFNα2b or IFN-α standard (7500 IU/ampoule, National Institute for the Control of Pharmaceutical and Biological Product, NICPBP, China) were added to the wells. After 24 h of incubation, optimal concentrations of the viruses were added. After additional 24 h of incubation, cells were stained with 0.5% crystal violet in 20% ethanol for 20 min at room temperature. Scoring was accomplished by extraction of stained cells with 50% ethanol/1% acetic acid and absorbance was determined at 580 nm in a Bio-Rad 680 ELISA microplate reader.

2.12. In vivo biological activity assay

To compare the *in vivo* biological activity of rHSA/IFNα2b and IFNα2b in monkeys, 15 cynomolgus monkeys (3–4 years old, 4.2–4.8 kg), eight females and seven males, were obtained from Animal Center of the Chinese Academy of Military Medical Sciences. Dosing volume for all animals was 0.5 ml/kg. rHSA/IFNα2b was prepared at a concentration of 1.0 mg/ml in a buffered glycine (2.3%, pH 7.4) solution, which was diluted by saline to suitable concentration before administration. Animals were randomized into five groups, three cynomolgus monkeys per group. Three groups were given a single dose of 0.36, 1.0 and 3.6 pmol/kg of rHSA/IFNα2b by subcutaneous injection (s.c.), respectively. One group was given a single dose (s.c.) of 0.36 pmol/kg of IFNα2b as positive control group and one group was given a single dose (s.c.) of 0.5 ml/kg of saline as blank control group. The activity of serum 2′,5′-OAS was assayed before the injection and 1, 2, 4, 8, 10 and 14 days after the injection. Measurement of 2′,5′-OAS activity was performed using a 2′,5′-oligoadenyl-5′-triphosphate radioimmunoassay kit (Eiken Chemical Co., Tokyo, Japan) according to the manufacturer's instructions.

3. Results

3.1. Protein expression and purification

After induction by methanol for 50 h, rHSA/IFNα2b concentration in broth was about 0.25 g/L which was detected by SDS–PAGE (Fig. 1). Blue Sepharose Fast Flow was used to capture protein from broth because the fusion protein contains albumin which can bind to Cibacron Blue specifically. Fractions pooled from Blue Sepharose Fast Flow contain 2 mol/L sodium chloride, which is suitable for hydrophobic interaction chromatography at

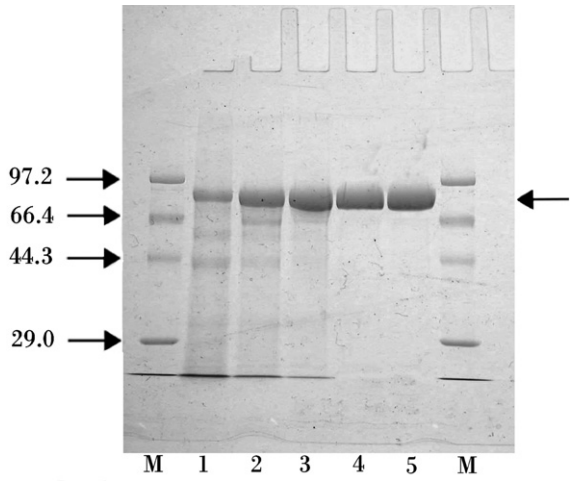

Fig. 1. Non-reduced SDS–PAGE analysis of purified rHSA/IFNα2b. Lanes 1–4: broth supernatant, fraction of Blue Sepharose FF, fraction of Phenyl Sepharose FF, fraction of Q Sepharose FF. The arrow indicates the fusion protein. M is protein molecular weight marker (low) (Takara Company).

high conductivity. rHSA/IFNα2b was eluted from the Phenyl Sepharose HP column by 10 mmol/L sodium phosphate and can be applied to Q Sepharose Fast Flow column directly. The results of the purification are shown in Table 1. About 64 mg rHSA/IFNα2b proteins were purified from 1 liter cell-free broth, about 22% total protein recovery. rHSA/IFNα2b purity was analyzed by non-reduced SDS–PAGE (Fig. 1) and RP-HPLC and was greater than 97% (data not shown).

3.2. Physical and chemical characterization of rHSA/IFNα2b

In SDS–PAGE (Fig. 1), rHSA/IFNα2b had an apparent molecular size of 86K. As shown in Fig. 2, rHSA/IFNα2b showed a single band on the IEF gel and the p*I* of three successive batches of products were all 5.3, near the theoretical value (p*I* 5.6) calculated from primary amino acid sequences.

Western blot results showed that in the two hybridizations with mouse anti-IFN-α McAb and goat anti-HSA McAb, respectively, there appeared to be corresponding positive bands at theoretical positions (data not shown).

Table 1
A summary of the purification of recombinant rHSA/IFNα2b fusion protein

Step	Total protein (mg)	Purity (%)	Yield (%)
Fermentation broth supernatant	~2130*	68	100
Blue Sepharose FF	1414	82	66.3
Phenyl Sepharose HP	898	93.4	42.1
Q Sepharose FF	603	97.8	28.3
Sephadex G25	542	98.2	25.4

* Approximately 8.5 L fermentation broth supernatant obtained from the 15 L fermenter and rHSA/IFNα2b concentration was estimated by non-reduced SDS–PAGE.

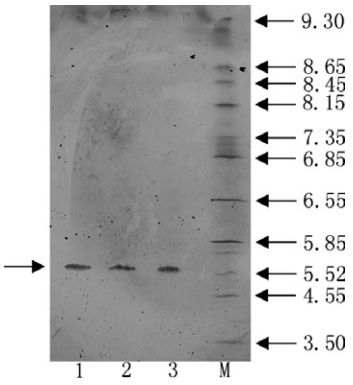

Fig. 2. IEF Analysis of rHSA/IFNα2b. Lanes 1–3: three successive batches of rHSA/IFNα2b (20030401, 20030402 and 20030501), lane M: Amersham Biosciences p*I* marker, 3.5–9.3. The arrow indicates the fusion protein.

Sequence of the N-terminal was as follows: NH_2-DAHKSEV AHRFKDLG, which was consistent with that of human serum albumin completely. The sequence of C-terminal three amino acids was SKE-COOH, completely consistent with that of IFNα2b.

Molecular weights of three batches of samples detected by MALDI-TOF mass spectrometry were 86020.6, 86021.7 and 85970.6, respectively, with an average value of 86004.3 ± 29.2.

According to the protein sequence, there should be 83 fragments if rhHSA/IFNα2b was degraded completely by trypsin. However we got more peaks by peptide mapping because of incomplete enzyme digestion and non-specific digestion due to the large molecular weight. The peptide maps of three batches of samples were basically identical (data not shown). Results of mass peptide mapping are shown in Fig. 3, 75% of the theoretical peptide fragments could be found.

Fig. 4 shows the CD spectra of rHSA/IFNα2b and the HSA-IFNα2b mixture. The CD spectra show that there are similar peaks at wavelengths 209 and 224 nm of rhHSA/IFNα2b fusion proteins and mixture (mixed at a molar ratio of 1:1).

3.3. In vitro and in vivo biological activity

As shown in the representative experiments reported in Fig. 5, rHSA/IFNα2b had considerable activity on WISH cells, with specific bioactivities of three successive batches at 6.6×10^5, 6.9×10^5 and 5.4×10^5 IU/mg, respectively, by average $6.3 \pm 0.8 \times 10^5$ IU/mg. rHSA/IFNα2b retain about 1.4% of IFNα (2.0×10^8 IU/mg) bioactivity on a molar basis.

Fig. 6 shows the activity–time curves of serum 2′,5′-OAS in cynomolgus monkeys with different dosage of rHSA/IFNα2b or single dose of IFNα2b by subcutaneous injection. There was an obvious dose–effect relationship of

1 DAHKSEVAHR FKDLGEENFK ALVLIAFAQY LQQCPFEDHV
KLVNEVTEFA KTCVADESAE
61 NCDKSLHTLF GDKLCTVATL RETYGEMADC CAKQEPERNE
CFLQHKDDNP NLPRLVRPEV
121 DVMCTAFHDN EETFLKKYLY EIARRHPYFY APELLFFAKR
YKAAFTECCQ AADKAACLLP
181 KLDELRDEGK ASSAKQRLKC ASLQKFGERA FKAWAVARLS
QRFPKAEFAE VSKLVTDLTK
241 VHTECCHGDL LECADDRADL AKYICENQDS ISSKLKECCE
KPLLEKSHCI AEVENDEMPA
301 DLPSLAADFV ESKDVCKNYA EAKDVFLGMF LYEYARRHPD
YSVVLLLRLA KTYETTLEKC
361 CAAADPHECY AKVFDEFKPL VEEPQNLIKQ NCELFEQLGE
YKFQNALLVR YTKKVPQVST
421 PTLVEVSRNL GKVGSKCCKH PEAKRMPCAE DYLSVVLNQL
CVLHEKTPVS DRVTKCCTES
481 LVNRRPCFSA LEVDETYVPK EFNAETFTFH ADICTLSEKE
RQIKKQTALV ELVKHKPKAT
541 KEQLKAVMDD FAAFVEKCCK ADDKETCFAE EGKKLVAASQ
AALGLGGGGS CDLPQTHSLG
601 SRRTLMLLAQ MRRISLFSCL KDRHDFGFPQ EEFGNQFQKA
ETIPVLHEMI QQIFNLFSTK
661 DSSAAWDETL LDKFYTELYQ QLNDLEACVI QGVGVTETPL
MKEDSILAVR KYFQRITLYL
721 KEKKYSPCAW EVVRAEIMRS FSLSTNLQES LRSKE

Fig. 3. Result of mass spectrometric peptide mapping of rHSA/IFNα2b (blackened sequences are the detected peptide, **GGGGS** is protein linker).

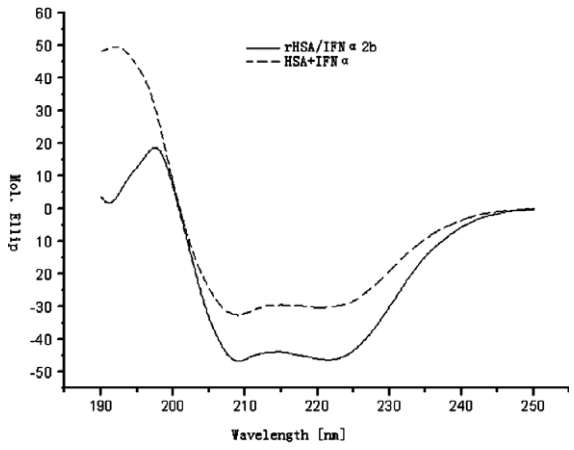

Fig. 4. Circular dichroism spectra of rHSA/IFNα2b and HSA + IFNα2b mixture. The mixture of human serum albumin and interferon-α2b (with molar ratio of 1:1, 0.5 mg/ml of total protein concentration) and rHSA/IFNα2b fusion protein (0.5 mg/ml) was, respectively, determined.

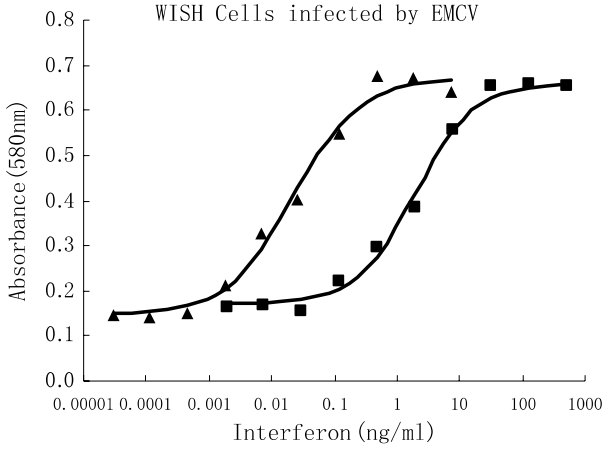

Fig. 5. Antiviral activity of rHSA/IFNα2b. The protective effect of rHSA/IFNα (square symbols) and IFN-α (triangle symbols) at the concentrations indicated was evaluated in antiviral assays using bovine WISH cells challenged with EMCV as described under Section 2.

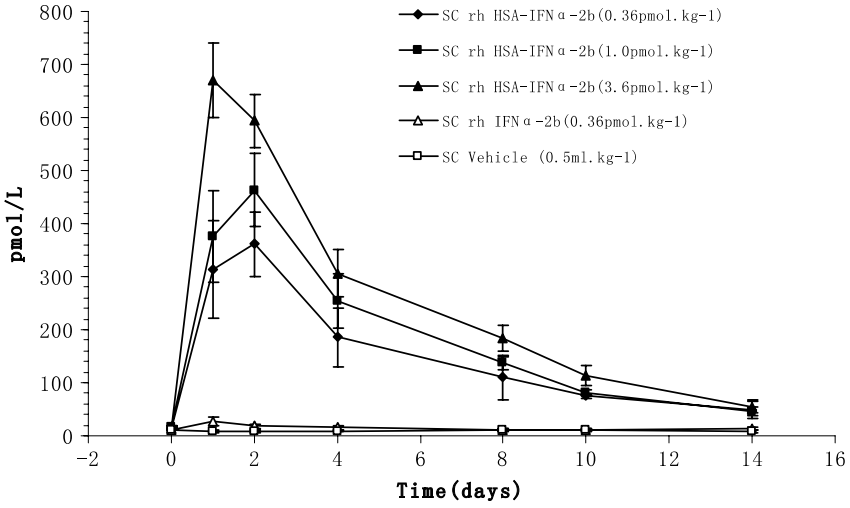

Fig. 6. Mean 2′5′-OAS activity level in serum after single-dose subcutaneous administration of rHSA/IFNα2b or single-dose subcutaneous administration of IFNα2b ($n = 3$/dose).

serum 2′,5′-OAS activity after single subcutaneous injection of 0.36, 1.0 and 3.6 pmol/kg rHSA/IFNα2b. The activity of serum 2′,5′-OAS reached a peak in 2 days after injection. 2′,5′-OAS activity could be detected for 14 days after injection in all the rHSA/IFNα2b groups. Whereas in the IFNα2b (0.36 pmol/kg) group, the 2′,5′-OAS activity was near baseline at day 6 which was markedly lower than those of all the rHSA/IFNα2b groups.

4. Discussion

An efficient protein production system is critical for obtaining large amounts of correctly folded recombinant protein for study. The *P. pastoris* expression system offers the potential for high yields of secreted recombinant proteins (up to g/L from the culture supernatant) [11]. In addition, the *P. pastoris* eukaryotic protein expression system has many advantages for recombinant protein production, because of its ability of protein folding, posttranslational modifications, and more simplified protein processing procedures compared to other protokaryotic expression systems [12]. Unfortunately, heterologous expression with this system often results in incomplete N-terminal processing [13]. To solve this problem, we selected HSA's natural signal peptide to replace α-factor signal peptide in pPIC-9 vector because HSA had been expressed correctly in yeast with its natural signal peptide followed by correct N-terminal processing [14]. The N-terminal sequencing results further proved our initial design.

But for recombinant proteins produced from *P. pastoris*, homogeneous structure and high biological activity are all important for therapeutic purposes. Elucidation of fusion protein structure and function is very difficult; so many physical, chemical and biological methods should be used to characterize recombinant proteins. Isoelectric focusing results had shown a single band and the p*I* of three successive batches of products had good consistency, demonstrating that the recombinant protein we prepared has little heterogeneity in terms of surface electric charge. Moreover, the results can partially prove that there are no isomers of acetylation or desamidation. This fact was further proved by the results of peptide mapping and MALDI-TOF-MS with good batch to batch consistency. The average molecular weight (86004.3 ± 29.2 Da) of three batches of proteins detected by MALDI-TOF had little difference compared to theoretical value (86040.063).

Mass peptide mapping is an important tool to analyze a protein's primary structures, including amino acid sequence, acetylation, deamidation and other posttranslational processing. For common small proteins, there is often high accordance between detected peptide fragments and theoretical sequence. But for rHSA/IFNα2b fusion protein, the coincidence rate was lower than those of small proteins because of its large molecular weight and incomplete enzyme digestion. The number of peptide fragments that we found accounted for 75% of the total theoretical sequence. For a fusion protein, in addition to the primary structure, it should be confirmed that the three-dimensional structures and bioactivities of the two proteins were not substantially affected by each other after fusion. CD spectroscopy was an important method to analyze the secondary structure of proteins. Because of the existence of intramolecular compensation after fusion, the spectra of rHSA/IFNα2b were not the same as those of rHSA/IFNα2b mixture, while the appearance of characteristic peaks indicated their secondary structures were similar, suggesting there was no significant change in the secondary structure after fusion of HSA and IFNα2b.

The biological activity assay also gives important evidence for characterization of the correct structure of rHSA/IFNα2b. After protein fusion or chemical modification, specific activity *in vitro* usually decreases due to increasing stereo specific blockade resulting in more difficultly to bind to their receptors. From Table 2, we observe

Table 2
Relationship of molecular weight and *in vitro* bioactivity of different kind of IFN-α

Interfeorns	Mol. weight (kDa)	Specific bioactivity (IU/mg IFN-α)	Relative bioactivity (%) (calculated on molar basis)
IFN-α	19	2.0×10^8	100
PEG-Intron	31	7.0×10^7	35
PegaSys	59	1.4×10^7	7
rHSA/IFNα2b	86	3.3×10^6	1.4

PEG-Intron is modified with a linear 12-kDa PEG molecule; Pegasys is IFNα2a modified with branched 40-kDa PEG molecule. All the modified interferons' specific bioactivity was calculated by interferon's weight.

the relationship of molecular weight and *in vitro* bioactivity of different kinds of modified IFN-α. It is understandable that the specific activity of rHSA/IFNα2b *in vitro* was lower than those of PEG-IFNα2b (PEG-INTRON) because the molecular weight of HSA (67 kDa) was larger than that of PEG (12 or 40 kDa) which was used to modify interferon. However, after fusing with human serum albumin, the fusion protein was not easily degraded *in vivo* and had avoided renal clearance, therefore its half-life prolonged to about 80 h [6]. Thus, its biological activity even increased *in vivo*. It has been shown in rhG-CSF research [15] that *in vitro* bioactivity decreased with the increasing molecular weight of modifying molecules, whereas it had increased *in vivo*. Similar to PEG-IFNα, *in vivo* activity could not have been predicted by *in vitro* assays in previous research [4].

It is difficult to determine the biological activity of IFN *in vivo* directly and its activity *in vivo* can be evaluated by the activity of 2′, 5′-OAS. The production of 2′,5′-OAS was induced by IFNα, which was involved in the mechanism of antivirus activity of interferons [16,17]. Therefore, the expression level of 2′,5′-OAS is regarded as an important marker of the antiviral activity of IFNα and it can be used to evaluate the biological activity of IFNα and modified IFN *in vivo* [18]. 2′,5′-OAS activity had remarkably increased after single-dose injection of rHSA/IFNα2b and it had been maintained on the 14th day after the injection, compared to that of IFNα2b (only 2 days). Therefore, although the activity *in vitro* had reduced markedly after fusion, *in vivo* activity of rHSA/IFNα2b had notably increased compared to IFNα, suggesting that overall duration of contact is more important than the affinity of the interaction. So rHSA/IFNα2b could exhibit more potent antiviral activity than IFNα2b in future clinical trials. The current results demonstrate that the half life of rHSA/IFNα2b is even longer than that of pegylated-interferon, therefore even less frequency of clinical administration could be achieved. The reduced dosing frequency may improve patient compliance, improving the response to treatment. In addition, the less frequent dosing may alleviate toxicities associated with fluctuations of blood drug concentration. However, some adverse events associated with IFNαs including fatigue, depression and neutropenia [19] could be exacerbated due to prolongation of half life and increase of *in vivo* efficacy. In addition, the introduction of HSA could induce immunogenicity. All these need to be further evaluated.

In conclusion, the results demonstrate that human serum albumin-interferon-α2b fusion protein expressed in *P. pastoris* has a correct structure and is more potent than normal IFNα2b *in vivo* due to its long half-life. In addition, results from SDS–PAGE, IEF, peptide mapping, MALDI-TOF demonstrate that our preparation of rHSA/IFNα2b is of very high homogeneity.

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