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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.