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Full Length Research Paper

Quantitative detection of fusion protein rIFN-β-HSA by a sandwich ELISA

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A method that allowed detection and quantification of recombinant fusion protein rIFN-β-HSA in complex mixtures and fermentation media was described. The method was based on a double antibody sandwich ELISA, which was developed by using an anti-IFN-β monoclonal antibody as capture antibody and an HRP-labeled anti- human serum albumin (HSA) monoclonal antibody as detection antibody. The practical working range was estimated to be 21.01 ng/ml to 672.50 ng/ml and the limit of detection was 13.88 ng/ml. Recoveries ranged from 91.9 to 110.4%, while the intra and inter-assay precisions were <4.86 and <8.08%, respectively.

Key words: ELISA, rIFN-β, human serum albumin (HSA), *Pichia pastoris*, fermentation.

INTRODUCTION

Interferons (IFNs) are a family of multifunctional cytokines, which possess a wide range of biological activities such as antiviral, antiproliferative, immune-regulatory and growth inhibition (Isaacs et al., 1957; Keeffe et al., 1997; Ringenberg et al., 1986; Rissanen et al., 1980; Sen et al., 1992). As a member of interferon family, interferon-β (INF-β) had been applied to treat a wide variety of diseases, ranging from multiple sclerosis to viral infections (Ogasawara et al., 2007; Patrucco et al., 2010; Zeniya et al., 2010). However, because of the short half-life of INF-β, frequent injection was required to obtain a clinical response. Human serum albumin (HSA) is the most abundant protein in blood plasma and has a long serum half-life of approximately 19 days (Peters et al., 1996). The preclinical studies displayed that the fusion protein of INF-β and HSA retains beneficial activities of the therapeutically active protein with prolonged half-life and reduced clearance. Compared with INF-β alone, rIFN-β-HSA showed a 140-fold decrease in clearance rate and a 5-6 fold increase in terminal half-life (Sung et al., 2003). Though rIFN-β-HSA appeared lower in *in vitro* activity than INF-β, the remarkably improved pharmacokinetic profile may have priority over bioactivity. The reduced clearance and the less frequent dosing regimen make it a promising therapeutic substitutor of INF-β for INF-β therapy in multiple sclerosis (MS), cancer, viral infections and immune-based disorders.

Pichia pastoris is an ideal expression system for high-level production of heterologous protein (Cereghino and Cregg, 2000; Gurkan and Ellar, 2005; Kobayashi et al., 2000). It is very suitable to produce HSA fusion protein (Huang et al., 2007). This expression system was used to express fusion protein rIFN-β-HSA, derived from the joining of human IFN-β to the N-terminus of HSA, and the rIFN-β-HSA product showed biological activity of IFN-β (Zhang et al., 2009b). For online quantitative monitor of the expression level of this fusion protein, a reliable assay will be needed. However, the available methods were not suitable to quantify this fusion protein molecule. In this research, a sandwich ELISA method for quantification of rIFN-β-HSA in fermentation medium was established.

MATERIALS AND METHODS

Chemicals and antibodies

ELISA microplates (96-well) were purchased from Corning-Costar Co. (Corning-Costar, NY, USA), urine microalbumin kits were purchased from Mind Bioengineering (Shanghai, China), ELISA

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reader (Multiscan MK3) was purchased from Thermo Fisher Scientific (New Hampshire, USA) and TMB substrate reagent set was purchased from Tiangen Biotech (Beijing, China) Mouse anti-human IFN-β Mab was purchased from R&D (Shanghai, China). HRP conjugated human serum albumin mouse mab (ab24438) was purchased from Abcam (London, UK).

rIFN-β-HSA preparation

The gene encoding IFN-β and HSA fusion proteins were amplified using a fusion polymerase chain reaction (PCR) method and subcloned into *P. pastoris* expression plasmid pPIC9K (Invitrogen, Beijing, China). The resulting rIFN-β-HSA expression vector was tranfected into *P. pastoris* GS115 by electroporation. The highest expressing stable cell line identified by immune staining screening was expanded.

Supernatant from the culture was filtered through a 0.45 μm pore-size filter and concentrated by ultra-filtration. The concentrated solution was loaded onto a 25 ml Blue Sepharose 6 Fast Flow affinity column (Amersham Pharmacia Biotech, Sweden) pre-equilibrated with buffer 1 (0.02 mol/l PB,0.1 mol/l NaCl, pH 7.2). The column was first washed with buffer 1 at the rate of 5 ml/min, and then was eluted with buffer 2 (0.02 mol/l PB, 2 mol/l NaCl, 50% ethylene glycol, pH 7.2). The fractions containing rIFN-β-HSA were further loaded onto an immobilized metal ion affinity chromatography (IMAC) resin containing nickel (Ni^{2+}-IMAC) pre-equilibrated with buffer 3 (0.02 mol/l PBS, 0.15 mol/l NaCl, pH 7.2). Ni^{2+}-IMAC column was then washed with buffer 3 at the rate of 2 ml/min. The protein was further eluted step-gradient with buffer 4 (0.02 mol/l PBS, 0.15 mol/l NaCl, 0.05% imidazole, and pH 7.2). The resulting elutes that contained the targeted proteins were loaded onto DEAE Sepharose Fast Flow pre-equilibrated with buffer 5 (0.02 mol/l PBS, 0.05 mol/l NaCl, pH 7.2). After that, the column was washed with buffer 5 at the rate of 2 ml/min. The fusion protein was eluted step-gradient with buffer 6 (0.02 mol/l PBS, 0. 5 mol/l NaCl, pH 7.2). The fractions containing a single protein bond at 89 kDa were joined together, extensively dialyzed and freeze-dried. Protein microsequencing confirmed that the NH_2 terminal sequence corresponds to IFN-β. The obtained protein power was regarded as the standard of rIFN-β-HSA and was stored at –80°C.

Sandwich ELISA procedure

ELISA microplates (96-well) were pre-incubated with 100 μl/well coding solution [with mouse anti-human IFN-β Mab diluted in coating buffer (0.05 M carbonate buffer, Ph 9.6)]. Plates were sealed and incubated over night at 4°C. Following coating, the plates were washed three times with 200 μl/well PBST and then blocked with 250 μl/well blocking buffer (with 1%BSA (w/v) and 0.01% sodium mercurothiolate in PBS) for 2 h at 37°C. After washing three times, standards, controls or samples were added in triplicate (100 μl) and incubated at 37°C for 2 h. Subsequent to washing three times, 100 μl/well detector antibody (HRP conjugated human serum albumin mouse mab) diluted in PBST were added and incubated for 2 h at 37°C. Following washing, 100 μl TMB substrate reagent set (Tiangen Biotech, Beijing, China) was added to each well. After incubation for 10 min at room temperature in the dark, the reaction was stopped by the addition of 50 μl/well 2 M H_2SO_4. The absorbance was measured at 450 nm using an ELISA plate reader.

Optimization

On the basis of the procedure described earlier, the optimal antigen and antibody concentration, sample incubating time and detecting antibody exposure time were determined.

The capture antibody was diluted with coating buffer in a concentration range from 1 to 6 μg/ml. The detector antibody was diluted with PBST at dilutions of 1:20,000 and 1:30,000. The rIFN-β-HSA standard was twofold diluted from 1/20 to 1/20480. The absorbance at 450 nm was measured and the resulting OD for each concentration tested was then plotted against the rIFN-β-HSA concentration. Least-squares regression was used to determine the relationship between measured absorbance and rIFN-β-HSA concentration. The conditions that gave the least squares regression which supported a simple linear dependence of OD on rIFN-β-HSA concentration and lowest detection concentration were chosen as the optimal working conditions.

The optimal sample incubating time was determined by incubating the sample for 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 min. The optimal detecting antibody exposure time was determined by stopping the reaction after exposure for 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 min.

ELISA validation

Sensitivity

The limit of detection (LOD) was estimated as the average of ten zero samples plus two standard deviations. The limit of quantification (LOQ) was estimated as the rIFN-β-HSA concentration in the lowest working range.

Precision and accuracy

The accuracy of rIFN-β-HSA sandwich ELISA was determined by performing recovery studies of six replicates of quality control samples at levels 42.03, 147.11 and 336.25 ng/ml by rIFN-β-HSA sandwich ELISA. The mean values of recovery and the coefficient variations (CV) were calculated.

Intra-assay precision was determined by testing quality control samples containing 42.03, 147.11 and 336.25 ng/ml rIFN-β-HSA. These samples were repeated six times in an intra-assay run and the coefficient variations (CV) were calculated. Inter-assay precision was determined by testing the same quality control samples in triplicate analyses on 6 separate days.

Specificity

rIFN-β and rHSA were assayed in this sandwich ELISA to evaluate their specificity. They were diluted to the same concentration as rIFN-β-HSA (336.25, 147.11 and 42.03 ng/ml separately) in PBST and assayed according to the procedure of rIFN-β-HSA sandwich ELISA.

Parallelism

1:10 and 1:100 dilutions of blank fermentation medium or blank mouse plasma spiked with rIFN-β-HSA standard were analyzed in 2-fold dilutions ranging from 5.25 to 5380 ng/ml. The standard curves were made according to rIFN-β-HSA sandwich ELISA procedure. All the samples were tested three times.

Stability

The stability of the ELISA kits were evaluated by prolonged storage at 4°C for 1 to 7 days or storage at –30°C for 15 days and incubated at 37°C for 1 to 7 days. Samples were assayed three

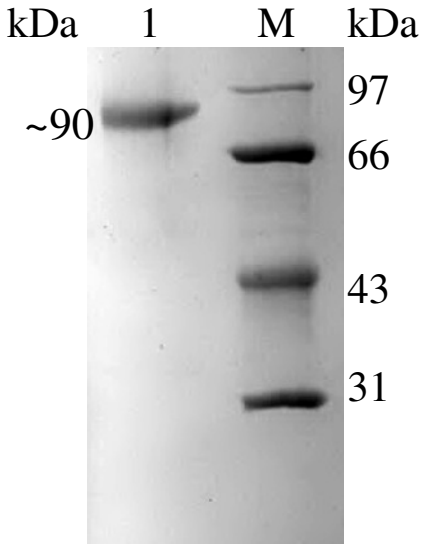

Figure 1. SDS-PAGE analysis of prepared recombinant IFN-*β*-HSA. The rIFN-*β*-HSA (90 kDa) expressed in *Pichia* was purified and analyzed on 12% acrylamide gels. Proteins were detected by means of Coomassie brilliant blue. M; marker proteins; Lane 1; purified protein used as standard protein.

times.

RESULTS

Standard rIFN-*β*-HSA preparation

Supernatant containing the interest protein of rIFN-*β*-HSA from the culture was filtered with a 0.45 µm cellulose membrane, and concentrated 50 times by ultrafiltration with a 10 KD cutoff filter. The concentrated product was further purified with Blue Sepharose Fast Flow column, an immobilized metal ion affinity chromatography (IMAC) resin containing nickel (Ni^{2+}-IMAC) and DEAE Sepharose Fast Flow. At the end, rIFN-β-HSA fusion protein with more than 95% purity was obtained. The molecular weight of this recombinant protein was 89 kDa (Figure 1). This was regarded as rIFN-*β*-HSA standard used in this study.

Optimization

During the development of the ELISA, the following parameters were investigated: concentration of IFN-*β* antibodies for coating, time of coating, concentration of HRP conjugated HSA mab and the incubation time of the sample. During the development phase, all ELISA plates were processed manually and all steps were performed in 0.01 mol/l PBS, pH 7.4 buffer. This buffer is better than Tris buffer and other concentration PBS buffer according to previous research. The analyses indicated the following optimal conditions which gave a better linear range: the coating time for the coating antibody was overnight, the incubation time for the sample was 160 min, the incubation time for detecting antibody was 60 min, the concentration for the capture antibody was 3 µg/ml and the detecting antibody was 1:30000-diluted.

Standard curve of detecting rIFN-*β*-HSA fusion protein

rIFN-*β*-HSA sandwich ELISA standard curve was based on 11 calibration standard obtained by a 2-fold dilution of rIFN-*β*-HSA standard ranging from 5.25 to 5380 ng/ml (Figure 2). Six consecutive standard points in the range of 21.02 to 672.50 ng/ml showed good linear correlation coefficient (R^2 = 0.9928). This range was defined as the working range of the assay.

ELISA validation

Sensitivity

The LOD was estimated as the average of ten zero samples plus two standard deviations. In this study, the

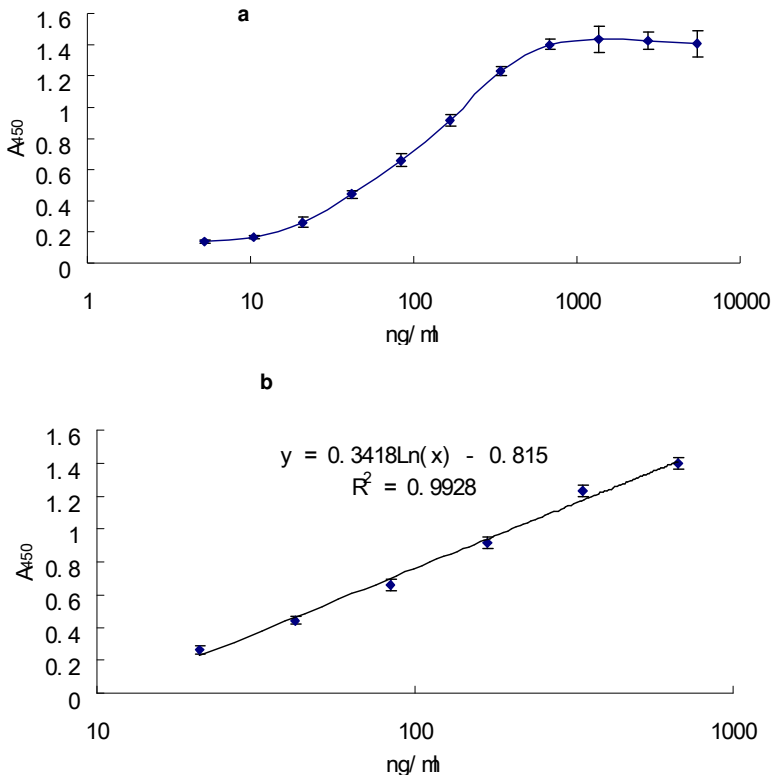

Figure 2. The standard curves obtained in rIFN-β-HSA sandwich ELISA. a) The standard curve of rIFN-β-HSA fusion protein by double sandwich ELSIA. Standard curve was prepared by PBST spiked with rIFN-β-HSA ranging from 5.25 to 5380 ng/ml with 2-fold sequential dilution. The graph shows the average values of triplicates in one experiment. b) The linear range in standard curve of rIFN-β-HSA sandwich ELSIA with the linear regression. Results were expressed as the mean±SD for 3 independent measurements. Error bars represent standard deviations of triplicate samples.

LOD was 13.88 ng/ml. The LOQ was estimated as the rIFN-β-HSA concentration in the lowest working range. The LOQ was 21.01 ng/ml in 1:10 and 1:100 dilution of the fermentation broth and 1:100 dilution of blank mouse plasma.

Precision and accuracy

Intra-assay precision was determined by studies of triplicate of quality control samples at levels of 42.03, 147.11 and 336.25 ng/ml. The coefficient of variation (CV) of intra-assay ranged from 2.79 to 9.74% in 1:100 dilution of the fermentation broth. Inter-assay precision was determined by studies of the same quality control samples in six times analyses on 6 separate days. The CV of inter-assay ranged from 2.46 to 8.89% in 1:100 dilution of the fermentation broth (Table 1).

Fermentation broth spiked with rIFN-β-HSA standard at levels of 336.25, 147.11, 42.03 ng/ml were analyzed with sandwich ELISA. Recovery was measured as the ratio of rIFN-β-HSA concentration predicted by the calibration curve and the theoretical concentration of rIFN-β-HSA of the sample. The percentage recovery in all samples was within 80 to 120% and ranged from 91.30 to 108.44%.

Specificity

rIFN-β-HSA sandwich ELISA showed no cross-reactivity with rIFN-β and rHSA which contains the similar structure with rIFN-β-HSA at concentrations of up to 336.25, 147.11 and 42.03 ng/ml, respectively. The OD_{450} values of rIFN-β and rHSA showed no remarkable difference from the blank sample's value (Figure 3.).

Parallelism

1:10 and 1:100 dilutions of blank fermentation medium or blank mouse plasma spiked with rIFN-β-HSA standard were analyzed in 2-fold dilutions ranging from 5.25 to 5380 ng/ml. The standard curves were made according to rIFN-β-HSA sandwich ELISA procedure. All the

Table 1. Precision of rIFN-β-HSA sandwich ELISA [a].

Spiking concentration (ng/ml)	Inter-assay CV (%)	Intra-assay CV (%)
336.25	8.89	9.74
147.11	5.41	6.27
42.03	2.46	2.79

[a] Samples were repeated three times in an intra-assay run and tested in six analyses on 6 days in an inter-assay run.

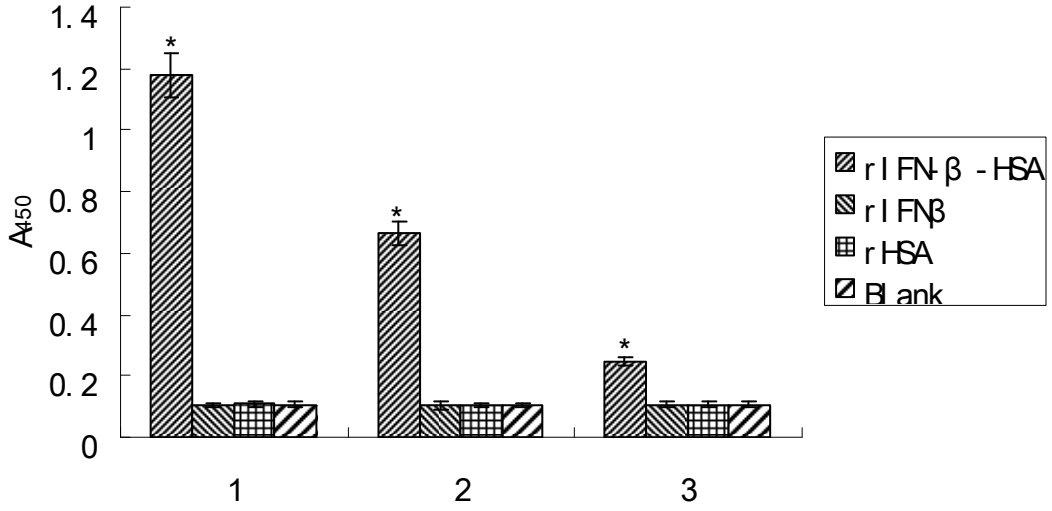

Figure 3. Specificity of the established ELISA method. Different concentrations of rIFN-β-HSA, rIFN-β and rHSA were measured by the sandwich ELISA. The OD measured showed that there was no cross reaction with rIFN-β and rHSA. 1 to 3 here represents 336.25, 147.11 and 42.03 ng/ml respectively. All values were mean±SD of triplicate wells. *p ≤ 0.05, compared with the other bars.

samples were tested for three times.

Parallelism between the calibrating standard curve and dilution curves of the fermentation broth and mouse plasma spiked with rIFN-β-HSA were observed. As shown in Figure 4a, the calibration curve prepared by 1:10 and 1:100 dilutions of fermentation broth coincided with standard curve, and this suggested the absence of effection from the fermentation broth. The calibration curve prepared by 1:10 dilution of blank mouse plasma only had a linear correlation coefficient of R^2 = 0.93, which indicated that mouse plasma contained the substance which could affect the ELISA reaction. When the mouse plasma was further diluted to 1:100 dilution, the effect caused by mouse plasma was reduced. The calibrating curve was lower but parallel with standard curve and had a linear correlation coefficient of R^2 = 0.97 in the working range (Figure 4).

Stability

The ELISA assay plate would be influenced by prolonged storage at 4℃. There was no significant change after storage at 4℃ for 7 days. There was a slight loss of concordance correlation coefficients when samples were stored at –30℃ for 15 days and incubated at 37℃ for 3 days (equals to 4℃ for 3 months). Thus, it was concluded that the ELISA assay plate could be stored at 4℃ for 3 months.

DISCUSSION

The potential of rIFN-β for treatment of many diseases was restricted by its short half-life time and resources (Fierlbeck et al., 1996; Salmon et al., 1996). A fusion protein of rINF-β-HSA, derived from the joining of human IFN-β to the N-terminus of HSA, had been developed for long-term activity (Sung et al., 2003), and high product expression had been obtained with *P. pastoris* expression system (Zhang et al., 2009b). Though much progress had been done in developing rINF-β-HSA as a new therapeutic molecule, there is no available method suitable to quantify this new molecule.

In this study, a novel assay specifically for the quantification of rINF-β-HSA was established. The

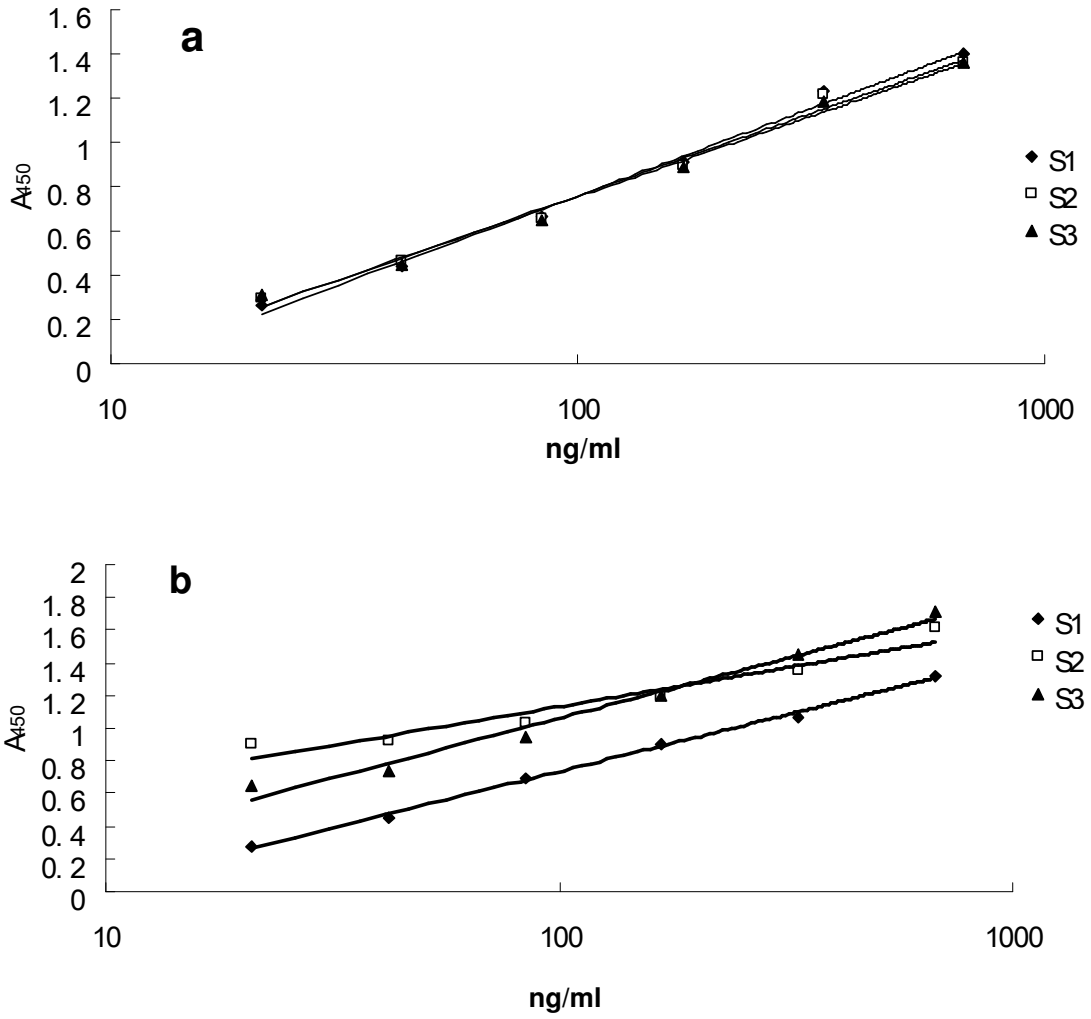

Figure 4. Parallelism of the rIFN-β-HSA ELISA. a) Parallelism of standard curve and calibrant for rIFN-β-HSA between calibrant buffer and diluted fermentation curves prepared by PBST (♦S1), 1:10 (□S2) and 1:100 (▲S3) dilution of fermentation medium spiked with rIFN-β-HSA standard ranging from 5.25 to 5380 ng/ml on medium. b) Parallelism of standard curve and calibrant for rIFN-β-HSA between calibration buffer and diluted curves prepared by PBST (♦S1), 1:10 (□S2) and 1:100 (▲S3) dilution of mouse plasma spiked with rIFN-β-HSA standard ranging from 5.25 to 5380 ng/ml on medium. The lines show the independent values obtained from the triplicate runs.

method was based on a sandwich ELISA, in which the capture antibody was aimed at INF-β and the detecting antibody targeted the HSA. In this way, only the intact fusion protein which harbors both the epitope of HSA and INF-β can be targeted. This method allows the accurate, precise and specific quantification of INF-β-HSA in complex biological mixtures. The practical working range of the assay was 21.01 to 672.50 ng/ml with the following parameters: linearity (21.01 to 672.50 ng/ml, r^2 = 0.9928); accuracy (91.9 to 110%); recovery intra-assay precision (%CV, 4.86%); inter assay precision (%CV, 8.08%); detection limit (13.88 ng/ml) and quantification limit (21.01 ng/ml).

Previously, Zhang in the laboratory had published an ELISA method for quantitative detection of INF-β-HSA (Zhang et al., 2009a) but the purity of INF-β-HSA at that time was lower than 90%. When compared with the quantification limit (51.88 ng/ml) in that research, this assay gave much lower quantification limit. In addition, in this assay, the possibility of using this ELISA in mouse plasma medium was checked.

From the parallelism data, it can be seen that this method can be well used in the fermentation medium, and there was little effect from the medium. It is important to mention that this medium was from the cells transfected with empty pPIC9k plasmid; this will avoid the non-anticipated substance from the plasmid. Thus, this method can be well used in the research on fermentation,

for example, to modify the industrial technology, the purification process and so on. This method seems not suitable for pharmacodynamic and pharmacokinetics analysis. There are some substances which can affect the ELISA. It is reasonable since HSA is a fundamental protein in the blood and there are huge amounts of it. This affection can be reduced by further dilution with PBST, but the dilution will substract the sensitivity of the method. Though this method gives a good linearity of the calibration line when the serum is diluted 1:100 by PBST, and the detection limit can basically fit with the functional concentration of INF-*β*-HSA, it stills need to be modified. This modification can be done by replacing the antibody with the one which contains higher affinity to INF-*β*-HSA. Also, the sensitivity can be improved by the substitution of more sensitive detection system, for example, biotin-avidin labeling system, enzyme-linked radioactive assay, etc.

In summary, an ELISA for measuring the concentration of INF-*β*-HSA in fermentation medium was described. This assay specifically measured INF-*β*-HSA. This method produced low backgrounds and is suitable for monitoring the quantification in research and industrial settings. The method represents a marked improvement over published assays for the detection of INF-*β*-HSA due to standardized reagents, an absolute calibration and validation for measuring INF-*β*-HSA in multiple circumstances.

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