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Functional and proteomic comparison of different techniques to produce equine anti-tetanus immunoglobulin F(ab′)2 fragments

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ABSTRACT

Tetanus is still a major cause of human deaths in several developing countries. In particular, the neonatal form remains a significant public health problem. According to the World Health Organization, administration of tetanus toxoid is recommended for neonatal tetanus patients. Furthermore, tetanus antitoxin or anti-tetanus immunoglobulin (Ig) are used for mild case or intensive care. This paper discusses a novel purification technique for improving equine anti-tetanus Ig production. First, equine plasma dealt with two steps salting out with ammonium sulfate; second, ultrafiltration concentration liquid purified by one successive protein G based affinity chromatography steps; finally, the purified F(ab′)2 fragments was characterized using biochemical and proteomic methods and shown to be pure and homogeneous. Compared with the original technique product, specific activity increased by 80% (about 90,000 IU/g) and recovery of F(ab′)2 is approximately equal 75%. Furthermore, Proteomic profiling of total technique process is demonstrated by nano-HPLC-MS and bioinformatics analysis. New technique to produce equine anti-tetanus immunoglobulin F(ab′)2 fragments from crude plasma in high quality and yield. And it also could be used for industrial amplification.

1. Introduction

According to the World Health Organization [1], In 2013 it caused about 59,000 deaths-down from 356,000 in 1990. Tetanus – in particular, the neonatal form – remains a significant public health problem in non-industrialized countries with 59,000 newborns worldwide dying in 2008 as a result of neonatal tetanus. Vaccination is the best way to protect against tetanus. Furthermore, passive immunization should be supplemented by other health programs (medical practices) [2]. There are different types of products available for passive immunization: human and animal (mainly equine) polyclonal immunoglobulins or antibody fragments. In view of the high costs and limited access to human immunoglobulin (Ig), purified equine Ig products (antiserum or antitoxin) are also used in developing countries.

The main manufacturing process procedures in equine Ig products are including: equine are immune to antigen; plasmapheresis [3]; and antibody digestion used pepsin; refined; purification; vial filling procedure. After the enzymatic digestion, several refined or purification steps are usually required to ensure a high purity and efficacy of the products with minimal side effects. These process could be performed by protein precipitation by salt-out [4] or caprylic acid [5,6], thermo coagulation [7], colloid particle adsorption, chromatograph (ion-exchange [8,9]; hydrophobic interaction [10]; affinity [11]; immunoaffinity [12]), ultrafiltration, or a combination of these methodologies. To reduce allergic reactions to other equine proteins in this setting, equine Ig is exposed to peptic digestion and the resultant F(ab′)2 fragments, selectively purified, provide equivalent efficacy with excellent tolerability [2,13,14]. The process flow line is that any equine Ig purification technique will have to be simple and economical yet safe and effective in meeting purity requirements.

In worldwide view, the Present and the Future of China's Production-Capacity is super-power in antiserum products fields. The annual about 100 million vials are made. The variety of products have anti-tetanus, anti-venom (Viper; agkistrodon acutus; *Bungarus multicinctus*; CoBra), anti-rabies, anti-botulinum toxin (A; B; E), anti-diphtheria, anti-anthrax, anti-emphysematous gangrene (Williams; Edema; Dissolved; Pus), etc. However, many manufacturers are following ammonium sulphate induced two-steps salting-out procedure precipitation or caprylic acid-based fractionation. So then, specific activity of products were relatively low. When patients used these products, side

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effects of allergic reaction could be happen. According to *The pharmacopoeia of the People's Republic of China*, tetanus antitoxin's key quality control parameter: The recipe is for prevention ≥ 45,000 IU/g; the recipe is for treatment ≥ 55,000 IU/g.

In this paper, we describes an innovative method of purifying equine anti-tetanus Ig F(ab′)2 fragments by using a combination of two-steps salting-out procedure precipitation and protein G based affinity chromatography which could be a linearly scalable option of industrial-scale production F(ab′)2 fragments, while retains the existing production process conditions.

2. Materials and methods

2.1. Materials

Horse crude plasma and equine tetanus antitoxin product (Lot 2016002) were obtained from Jiangxi institute of biological products (Ji'an, PR China). Pepsin from porcine stomach was Active Pharmaceutical Ingredients grade (specific activity 12,440 U/g protein) and purchased from Chongqing Aoli Biopharmaceutical Co., Ltd. (Chongqing, PR China). Sodium sulfate (25 kg/b, Lot 20160805), NaCl (500 g/b, Lot 20141209), $Na_2HPO_4{\cdot}12H_2O$ (500 g/b, Lot 1607201), $NaH_2PO_4{\cdot}2H_2O$ (500 g/b, Lot 1006241), Glycine (500 g/b, F20111017), HCl (500 ml/b, Lot 150325), NaOH (500 g/b, Lot 151102), Ethanol (2.5 l/b, Lot 16051302), Tris base (100 g/b, C1215014) were Pharmaceutical grade or AR grade and purchased from domestic reagent. Ezfast Protein G 4 Fast Flow (1 × 5 ml) was graciously provided by Bestchrom Biosciences Co., Ltd. (Shanghai PR China). HiTrap Protein G HP (5 × 5 ml) and Protein G Sepharose 4 Fast Flow (25 ml) purchased from GE healthcare. The Laboratory Mouse purchased from hnsja Laboratory Animal Technology Co., Ltd. (Changsha, PR China). Biological Standards of tetanus antitoxin and tetanus toxin purchased from Chinese National Institutes for Food and Drug Control.

2.2. Salting out step

Crude horse plasma diluted by water for injection until plasma protein level get to 2%. Solution adjusted pH to 2.90–3.50 by 1 mol/l HCl. 1 ml Solution added pepsin 3–10 activity units. Controlled temperature at 30 ± 1 °C and digested 1.0–1.5 h. Then we obtained the digestive solution.

The digestive solution added by ammonium sulfate until final concentration of ammonium sulfate level get to 15% (w/v), Solution adjusted pH to 5.20–5.60 by 1 mol/l NaOH. Controlled temperature at 58 ± 1 °C and kept warm 0.5 h. By filter pressing, we obtained the first Salting out solution.

Solution adjusted pH to 7.20–7.40 by 1 mol/l NaOH. The first Salting out solution added by ammonium sulfate until final concentration of ammonium sulfate level get to 35% (w/v), by filter pressing, we obtained the second Salting out protein precipitation.

The second Salting out protein precipitation diluted by water for injection until plasma protein level get to 2%. And mixed an alum solution of $KAl(SO_4)_2{\cdot}12H_2O$, Solution adjusted pH to neutral pH 7.70–7.90. Stirred and adsorbed 0.5 h. By filter pressing, we obtained the adsorbed solution.

After adsorbed solution ultra-filtered, we obtained the first Salting out step solution (Protein level 117 g/l, Titer 5800 IU/ml, Specific activity 49,573 IU/g).

2.3. Affinity chromatography step

The first Salting out step solution diluted by 20 mM PB binding buffer (pH 7.10–7.30) until plasma protein level get to 20 mg/ml. Wash the column with 3–5 column volumes of binding buffer at 5 ml/min. Apply the sample, by pumping it onto the column at 5 ml/min. and then wash the column with 3–4 column volumes of binding buffer at 5 ml/min. Elute with 4–5 column volumes of 0.1 M Gly-HCl elution buffer (pH 2.70–3.00). column regeneration with 3–5 column volumes of binding buffer at 5 ml/min. Eluted solution use 1 M Tris base adjust pH to 7.0. GE Unicorn 7.0 software (GE Healthcare) was used to analyze all data and generate new compare imagines.

Experiment of loading quantity of sample: 8 mg protein/1 ml chromatography medium; 12 mg protein/1 ml chromatography medium; 16 mg protein/1 ml chromatography medium.

Experiment of different media: GE HiTrap Protein G HP; GE Protein G Protein G Sepharose 4 Fast Flow; Bestchrom Ezfast Protein G 4 Fast Flow.

Experiment of medium magnification: GE HiTrap Protein G HP (15–25 ml); GE Protein G Protein G Sepharose 4 Fast Flow (25 ml).

2.4. Quality qualification

2.4.1. Total protein content

The mirco Kjeldahl's method was used to quantitative determinate total protein content.

2.4.2. Antitoxin titers assay

Antitoxin titers in products determinate by gold standard in vivo mouse neutralization test. The potency of equine tetanus antitoxin manufactured for human use by licensees in the PR China is tested according to pharmacopoeia of PR China (2015), Part 3, General rule 3508, General rule Page 123. As outlined in the regulation, a comparative toxin-antitoxin neutralization test is conducted using a standard antitoxin and a standard toxin. The ability of the antitoxin in the test sample to neutralize the standard toxin, as defined by prevention of tetanic paralysis or death of the mouse injected with the toxin-antitoxin mixture, is compared to that of the standard antitoxin.

2.4.3. Sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE)

Non-reducing SDS-PAGE was used to separate proteins according to their molecular weight [15] using a 5.0% stacking gel and 8.0% separating gel. Bio-Rad Mini-Protean Tetra Cell and PowerPac HV Power Supply were used for SDS-PAGE measurements. (Bio-Rad, Hercules, California). All samples were diluted protein content about 1 mg/ml. Protein content were test by a simple and rapid absorption UV spectra method (A280nm). Thermo Nanodrop2000 were used for rapid protein content measurements (Thermo, Wilmington, Delaware USA). The diluted samples (10 µl per lane) were applied and subjected to electrophoresis at 60 V for approximately 45 min in stacking gel and approximately 80 min in separating gel. A mixture of broad-ranged marker proteins of known molecular weights was also run with the protein samples (NO. C610011, Sangon Biotech, Shanghai, PR China). Protein bands were visualized using Coomassie blue staining and scanned with a UMAX POWERLOOK 2100XL USB scanner (UMAX, Dallas, TX, USA) with MagicScan software. PDQuest 8.0 software (Bio-Rad) was used to analyze the images.

2.4.4. Mass spectrometry

Trypsin was added in a ratio of 1:100 (w/w) in 25 mM ammonium bicarbonate, and peptide digest collected the following day. Peptide digests were resuspended in 100 µl of 1% TFA and sonicated in a water bath for 1 min.

The peptide mixture were prepared Q-Exactive Hybrid Quadru-pole-Orbitrap mass spectrometer via EASY-nLC1000 UHPLC system (Thermo Fisher Scientific, MA, USA) coupled in C-18 reverse phase pre-column (Acclaim™ PepMap™ 100 C18 LC columns, 2 cm × 100 µm, 3 µm; Thermo Fisher) using 0.1% TFA/H_2O as mobile phase and then separated on a nano-HPLC C-18 column (EASY-Spray™ C18 column, 12 cm × 150 µm, 1.9 µm; Thermo Fisher) with a gradient of A phase 0.1% TFA/H_2O and B phase 0.1% TFA/ACN, 0–16 min 95%A + 5%B; 16–51 min 90%A + 10%B; 51–71 min 78%A + 22%B; 71–72 min