Immunological characterization and verification of recombinant streptococcal protein G

HUCHENG ZHANG[1], WEIFENG LIANG[2], HAITAO FAN[1], JUN YANG[1], GUOWEI YANG[1], XIAOJIE WANG[1], LIANG CHEN[1] and TAO LIANG[3]

[1]Department of Biological Engineering, Beijing Polytechnics, Beijing 100176;
[2]Beijing Buchang Pharm Biopharmaceutical R&D Center, Beijing 100080;
[3]Department of Life Sciences, Tianjin Normal University, Tianjin 300384, P.R. China

Received October 9, 2014; Accepted June 17, 2015

DOI: 10.3892/mmr.2015.4162

Abstract. Streptococcal protein G (SPG), which is an antigen of the *Streptococcus* sp. cell wall, is important due to its high specificity to immunoglobulin (Ig), as compared with staphylococcal protein A. However, the cost of commercial recombinant (r)SPG has so far hindered further research into the application of rSPG. Efficient high cell density fermentation of genetically modified *Escherichia coli* and purification technology have previously been studied as a means to obtain rSPG. In the present study, the immunological characterization of purified rSPG was compared with commercial SPG via western blot analysis. The results of the present study demonstrated that the IgG-binding capacity of purified rSPG was markedly higher, as compared with commercial SPG. Furthermore, purified rSPG cross-linked with Q Sepharose® Fast Flow exhibited excellent affinity with IgG in murine serum. In order to obtain relatively pure and accurate rSPG, the purified rSPG was identified by Nanoflow Liquid Chromatography-Mass Spectrometry (MS)/MS spectrum. The results indicated that the two peptide fragments of purified rSPG corresponded to the *Streptococcus* sp. GX7805 protein G as listed in the National Center for Biotechnology Information database. The method described in the present study offers a novel practical method for the verification of rSPG in relatively pure form, in order to purify IgG or carry out immunolabeling processes.

Correspondence to: Dr Hucheng Zhang, Department of Biological Engineering, Beijing Polytechnics, 9 Liangshuihe 1st Street, Yizhuang, Beijing 100176, P.R. China
E-mail: zhanghchbj@163.com

Key words: immunology characterization, verification, recombinant streptococcal protein G, liquid chromatography tandem mass spectrometry

Introduction

Immunolabelled techniques (ILTs) are dependent on the binding of a specific antibody to a labeling material, such as enzymes (1), fluorescein dyes (2) and other compounds (3). When used to detect antibodies in animal species, the binding complex usually consists of the labeling molecule bound to a specific antibody targeting the immunoglobulin (Ig) of the species of interest (4-7). However, when conducting a serological test for a specific disease in various animal species including humans, a more appropriate technique is to use a single conjugate targeting all the tested species. This is carried out using *Staphylococcus aureus* protein A and streptococcus protein G (SPG) as ILT conjugates (8).

Protein A was originally detected in the cell wall of the bacterium *Staphylococcus aureus* (9), and exhibits a unique affinity for the IgG of numerous mammalian species (10), and for the IgM and IgA of certain species via interaction with the heavy chain (11). Numerous labeling molecules may be coupled to protein A without affecting its Ig-binding ability. Since its discovery, protein A has been widely used in various immunology and molecular biology fields (11). To increase the specificity of protein A for IgG for use in both research and bioprocessing, genetically engineered protein A can be expressed by *Escherichia (E.) coli* or yeast (12-15).

SPG is an Ig-binding protein, which was initially detected in group C and G *Streptococcus* (16), the gene structure and protein-binding properties of which have previously been described (17). Similar to protein A, SPG is able to bind to the Fc fragment of IgGs from numerous mammalian species over a wide range of pH (4.0-8.0). Furthermore, SPG has a broader range of reactivity than protein A, and SPG is more appropriate for the purification and isolation of IgG due to its binding specificities to all four subclasses of human IgG (14). SPG is a 65 kDa (group G protein G) or 58 kDa (group C protein G) cell surface protein that may be used to purify antibodies IgG1, IgG2, IgG3 and IgG4 by binding to the Fc region (14). Although the tertiary structures of protein A and SPG are very similar, their amino acid compositions differ significantly, resulting in different binding characteristics. SPG may be used for the purification of mammalian monoclonal and polyclonal IgGs, which do not bind well to

protein A. As compared with protein A, SPG has a greater affinity to most mammalian IgGs, particularly for certain subclasses of IgG, including human IgG3, mouse IgG1, and rat IgG2a (18). However, unlike protein A, SPG does not bind to human IgM, IgD, or IgA (12).

With the emergence of novel pathogens worldwide, more antibodies will be required in order to detect the pathogens of various animals. Purified recombinant SPG (rSPG) has an important role in the purification of pathogen-targeting antibodies, and for the detection of specific pathogenic antigens via the conjugation of antibodies with sensitive immunolabelled molecules. Furthermore, the use of purified rSPG conjugates may be vital for the diagnosis of certain diseases, such as autoimmune diseases. Since IgG3 is overexpressed in numerous autoimmune diseases and represents ≥45% of autoimmune antibodies, rSPG may prove useful for the study and diagnosis of autoimmunity. Due to these various properties, SPG has more commercial value than protein A.

The cost of commercial rSPG has so far hindered further research into the application of rSPG. Previous studies have described high cell density fermentation and purification of rSPG in *E. coli* (15,19,20). The present study aimed to promote the accuracy of synthesized rSPG, and decrease its price by developing an optimal fermentation and purification method.

Materials and methods

Fermentation and purification of rSPG. In order to obtain large amounts of highly purified rSPG, high cell density fermentation of genetically modified *E. coli* BL21, and purification technology were employed according to the method of a previous study by our group (20). Cell densities (optical density, 600 nm), inoculation, concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG; Takara Bio Inc., Otsu, Japan), dissolved oxygen, pH, and induction time were optimized. The expressed rSPG was then purified using a Ni-NTA column, Sephadex G-25 desalting step, and DEAE-FF ion-exchange chromatography. The protein content of the column elution was monitored by measuring absorbance at a wavelength of 280 nm. The purity of rSPG was subsequently determined by 12% SDS-PAGE, and the concentration of rSPG was determined using a Bradford assay normalized to bovine serum albumin (BSA).

Functional assessment of rSPG by western blotting and rSPG-Q Sepharose® Fast Flow. The purified rSPG and the commercial SPG (Beijing Dapbiotech Company, Beijing, China) were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc. Hercules, CA, USA), as described in a previous study by our group (20).

In order to compare the function of the purified rSPG with that of the commercial SPG, the two were immobilized using activated mercapto group Q Sepharose® Fast Flow (GE Healthcare Bio-Sciences, Pittsburg, PA, USA). A total of 1 mg purified rSPG dissolved in 2 ml Buffer L (0.2 mol/l Tris-HCl buffer, 2 mmol/l EDTA, pH 8.0) was mixed with 0.2 g activated mercapto group Q Sepharose® Fast Flow at 4˚C for 16 h, in order to form rSPG-Q Sepharose® Fast Flow. The rSPG-Q Sepharose® Fast Flow was placed into a column (6.6 mm x 32 mm; Omnifit® Solvent Plus Column; Diba Industries, Inc., Danbury, CT, USA) and equilibrated with Buffer L to wash off the unreacted rSPG, prior to further washing with Buffer M (0.01 mol/l phosphate-buffered saline, 0.137 mol/l NaCl, 2.7 mmol/l KCl, pH 7.4). A total of 50 ml mouse serum solution was loaded into the column, and the elution was monitored at 280 nm using a spectrophotometer (Shanghai Jinda Biochemical Instruments Co., Ltd., Shanghai, China). The flow-through fraction was subsequently collected, and the column was eluted using Buffer W (0.1 mol/l sodium citrate, pH 3.0). Using the Beer-Lambert law, the concentration of IgG (mg/ml) in the elution was calculated. Briefly, the amount of IgG bound to the column was determined by subtracting the amount of protein in the flow-through fraction from the total amount of protein in the sample prior to loading. The resulting value was used to calculate the IgG-binding rate of the rSPG-Q Sepharose® Fast Flow with the following equation:

$$\text{IgG-binding rate} = \frac{(At - Ap) \times 10^{-3}}{\frac{(1\ mg - Ar) \times 10^{-3}}{42{,}600\ g/mol} \times 160{,}000\ g/mol} \times 100\ \%$$

In the equation, At, Ap, and Ar represent the total amount of protein prior to loading, the amount of protein in the flow-through fraction, and the amount of rSPG in the flow-through fraction, respectively. The IgG-binding rate of commercial SPG-Q Sepharose® Fast Flow was determined in a similar manner. The antibodies eluted from the purified rSPG and the commercial SPG-Q Sepharose® Fast Flow were monitored for purity by 12% SDS-PAGE.

Identification of rSPG by Nanoflow Liquid Chromatography (LC)-Mass Spectrometry (MS)/MS. The purified rSPG was recovered from the SDS-PAGE using a Mini Protein Gel Extraction kit (Beijing Biolab Company, Beijing, China) and dissolved in deionized water. The sample was then reduced with dithiothreitol at a final concentration of 10 mmol/l at 56˚C for 1 h. The sample was subsequently alkylated with iodoacetamide at a final concentration of 55 mmol/l, at room temperature for 45 min. The sample was subsequently diluted with 25 mmol/l ammonium bicarbonate and digested in trypsin (12.5 μg/ml) overnight at 37˚C. Formic acid (FA; 0.1%) was added in order to terminate the reaction, and the supernatant was used for subsequent experimentation.

Nanoflow LC-MS/MS was performed by coupling a Dionex UltiMate 3000 Nano LC system (Thermo Fisher Scientific, Inc., Waltham, MA, USA) to a Q Exactive Mass Spectrometer system (Thermo Fisher Scientific, Inc.). A total of 10 μl sample acidified with 0.1% FA was delivered to a Trap column (Acclaim PepMap Protein and Peptide Column C18, 75 μm x 20 mm, 3 μm, 100 Å; Thermo Fisher Scientific, Inc.). The column was pre-equilibrated with mobile phase A (aqueous solution supplemented with 0.1% FA) and then eluted by a gradient of mobile phase B (acetonitrile solution supplemented with 0.1% FA) from 5-80% over 56 min (5-8% for 6 min, 8-30% for 34 min, 30-60% for 5 min, 60-80% for 3 min and 80% for 8 min), followed by 5% B at a flow rate of 0.4 μl/min.

The peptides were subsequently transferred to a Q Exactive Mass Spectrometer system using a C18 Venusil BP house-packed analytical column (4.6 mm x 150 mm, 5 μm,

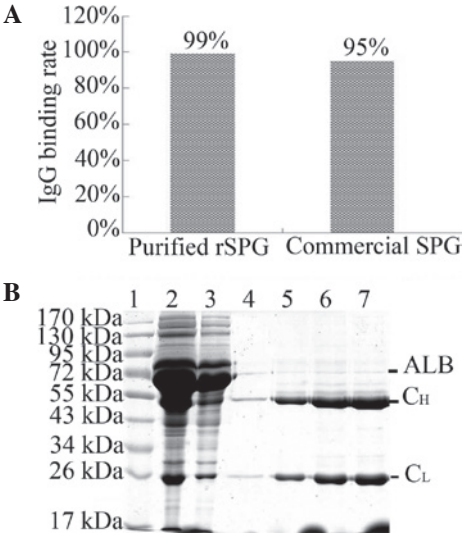

Figure 1. Functional comparison of the immunoglobulin (Ig)G binding ability of the purified recombinant streptococcal protein G (rSPG) and the commercial SPG. (A) IgG-binding rate comparison of the purified rSPG to that of the commercial SPG. (B) Binding of mouse IgG purified from mouse serum with purified rSPG- and commercial SPG-Q Sepharose® Fast Flow. Lane 1, Protein molecular weight markers; lane 2, mouse serum prior to column loading; lane 3, flow-through fraction of mouse serum; lane 4 and 6, 5, and 15 μl purified mouse IgG eluted from commercial SPG-Q Sepharose® Fast Flow; lane 5 and 7, 5, and 15 μl purified mouse IgG eluted from purified rSPG-Q Sepharose® Fast Flow. ALB, mouse serum albumin; C_H, heavy chain; C_L, light chain.

150 Å; Agela Technologies) for identification. Conditions for the initial ionization in the positive ionization mode included electrospray voltages at 1.8 kV and a temperature of 360˚C. Intact peptides were detected in the Orbitrap at a first full scanning resolution of 70,000 from 350-2,000 Da. Tandem MS (MS/MS) was performed using helium as the collision gas at 11.0 pounds per square inch (psi). Peptides were selected for MS/MS using high-energy collisional dissociation operating mode with a normalized collision energy setting of 28%. Second full-scan m/z spectra were automatically selected depending on the level of the first parent ion mass with a scanning resolution of 17,500. Automatic gain control was used to prevent overfilling of the Orbitrap; $1x10^4$ ions were accumulated in the ion trap to generate CID spectra. Data were analyzed with Mascot (version 2.3.0; Matrix Science Inc., Boston, MA, USA).

MS data analysis of the purified rSPG. The MS/MS data were first searched using Mascot with the following parameters: Type of search, MS/MS ion search; enzyme-trypsin, fixed modifications-carbamidomethyl; variable modifications, Gln->pyro-Glu (N-term Q); mass values: monoisotopic, protein mass: unrestricted, peptide mass tolerance ± 15 ppm, fragment mass tolerance ± 20 mmu, and maximum missed cleavages: 1. The samples were further analyzed using an algorithm with the DAT files generated by Mascot. Positive identification was determined if a minimum of 8 amino acids matched with minimum probabilities of 95% at the protein level, and 95% at the corresponding peptide level, and $P<0.05$ was considered to indicate a statistically significant result. Finally, all potential MS/MS spectral matches were subjected to manual inspection

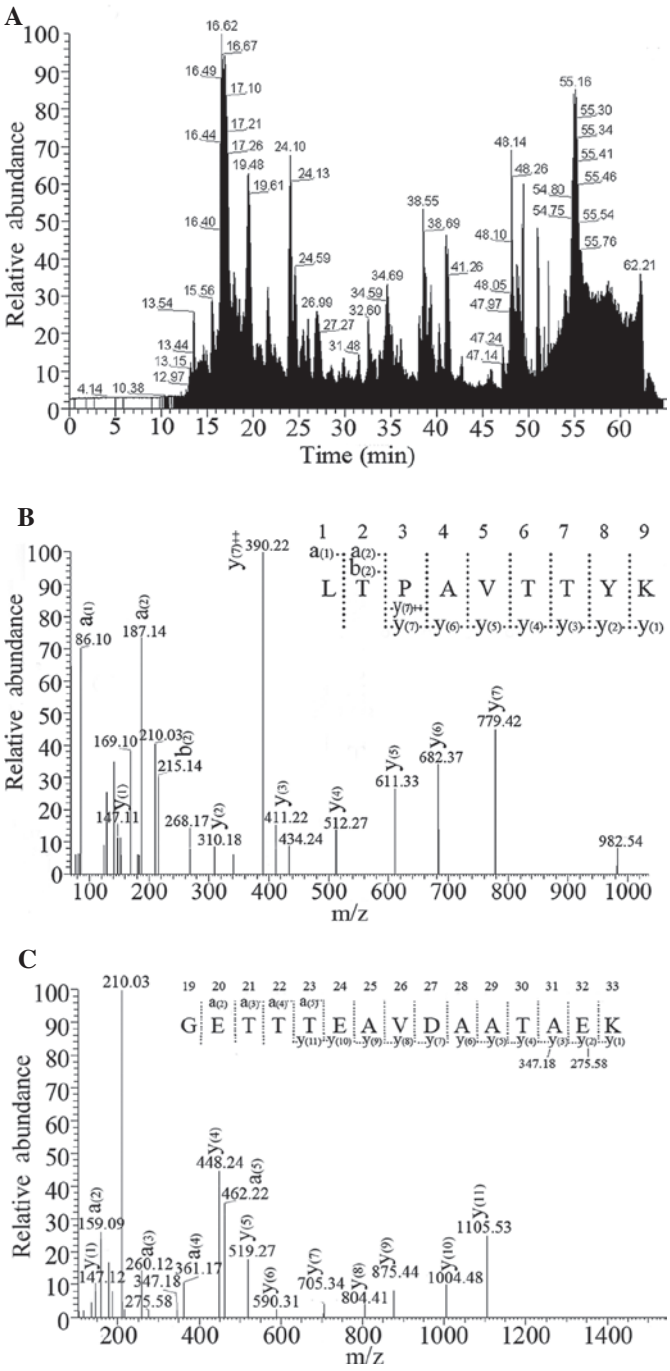

Figure 2. Identification of purified recombinant streptococcal protein G (SPG) by Liquid Chromatography-Mass Spectrometry (MS)/MS. (A) Total ion current mass peptide map of the purified rSPG. (B) Peptide 1-9 spectra obtained during the analysis of the purified rSPG, the full-scan product ion spectra of the 1-9 fragment ions in the rSPG indicates that the amino acid sequence is LTPAVTTYK. (C) Peptide 19-33 spectra obtained during the analysis of the purified rSPG, the full-scan product ion spectra of the 19-33 fragment ions in the rSPG indicates that the amino acid sequence is GETTTEAVDAATAEK.

as a final validation step prior to acceptance as valid peptide identification.

Results and Discussion

Optimization of the expression conditions of rSPG was first carried out for a shake-flask culture. The preliminary results suggested that the levels of rSPG increased over 4 h

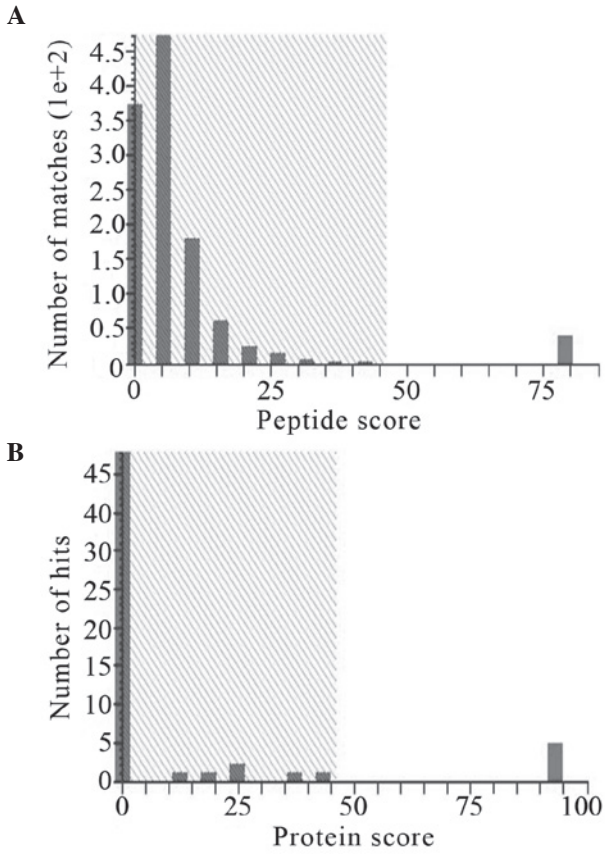

Figure 3. Mascot score histogram of the purified recombinant streptococcal protein G. (A) Peptide score distribution. (B) Protein score distribution.

of induction (20). In order to further increase the rSPG yield, various parameters, including inoculation, dissolved oxygen, pH, and IPTG, were optimized for the fermentation carried out in a fermenter (2).

rSPG purification. The highest expression levels of rSPG obtained were >20% of the total expressed protein. The rSPG was isolated and purified using affinity Ni-NTA chromatography and Anion exchange DEAE-FF chromatography (data not shown). A final yield of 15 ml of 0.45 mg/ml rSPG was obtained, indicating a yield of 87.2% for rSPG (20).

Binding capacity of purified rSPG and commercial SPG. To detect the IgG-binding ability of the purified rSPG, and to compare the binding ability of rSPG with that of the commercial SPG, each of the proteins were immobilized onto PVDF membranes at a concentration of 15 μg. Although both the purified rSPG and commercial SPG bound efficiently to goat IgG, the IgG-binding capacity of the purified rSPG was higher, as compared with that of the commercial SPG (20). The IgG-binding rate of the purified rSPG and the commercial SPG-Q Sepharose® Fast Flow were 99 and 95% (Fig. 1A), respectively, indicating that both the purified rSPG and the commercial SPG have similar IgG-binding abilities. Monitoring of the mouse serum and its flow-through fraction from both the columns via the reduction of SDS-PAGE indicated that the majority of serum IgGs were bound by the SPG-Q Sepharose® Fast Flow (Fig. 1B). When the same volume of mouse serum was loaded into both the SPG-Q Sepharose® Fast Flow columns, the purified rSPG-Q Sepharose® Fast Flow bound more IgG, as compared with the commercial-Q Sepharose® Fast Flow. These results support the hypothesis that purified rSPG is more efficient than commercial SPG at binding IgG.

Identification of purified rSPG by LC-MS/MS. During the early stages of the mass spectrometry analysis, both the electrospray ionization (ESI) and atmospheric pressure chemical ionization sources were evaluated. ESI was shown to offer higher protein sensitivity, and was therefore chosen as the ion source for the present study. Since the positive ion mode produced higher intensity signals than the negative ion mode, the positive ion mode was selected for the analysis of rSPG. The total ion current (TIC) chromatogram represents the sum of the intensities of the entire range of masses detected at each analysis point. The purified rSPG TIC chromatographic peak demonstrates the necessity of well distribution to achieve the ideal separation effect (Fig. 2A). Each peptide ion in the mass peptide map acted as the parent ion, and was collided by inert gas with high velocity in order to cause a collision-induced dissociation in the mass spectrometer chamber. The polypeptide chain was broken in the amide bond forming various ions, including a, b, c, and x, y, z ions.

The spectra in Fig. 2B and C were obtained from the precursor at 18.5 min and 19.2 min, respectively. The 1-9 and 19-33 fragment ions spectra indicated that the amino acid sequence was LTPAVTTYK and GETTTEAVDAATAEK, respectively. The two ion spectra indicated that the two peptide fragments matched the protein G from *Streptococcus* sp. GX7805 as listed in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/protein/CAA68489.1).

The ionic score was l g (P), where P is the probability that the observed match is a random event. In the present study, individual ion scores >46 indicated that the peptide or protein sequence was identical or had extensive homology ($P<0.05$), as compared with the NCBI protein, and rSPG exhibited a peptide score of ~80 (Fig. 3A) and a protein score of ~95 (Fig. 3B).

The purified rSPG in the present study was successfully identified using the LC-MS/MS techniques described above, and was matched to protein G from *Streptococcus* sp. GX7805 as listed in the NCBI database.

In conclusion, the methods described in the present study may prove useful for the overexpression of rSPG in *E. coli*, and the purification of rSPG. In addition, the method may provide an appropriate choice for large-scale commercial production of rSPG. However, further research is required in order to investigate the stability and sensitivity of the antibody and purified rSPG labeling molecule-conjugated complex.

Acknowledgements

The authors thank Professor Xiulan Xin for her valuable instructions and suggestions on the thesis as well as her careful reading of the manuscript. The present study was supported by a grant from the Program for Research Team in Beijing Polytechnic.

References

1. Lequin RM: Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clin Chem 51: 2415-2418, 2005.
2. Singhrao SK, Müller CT, Gilbert SJ, Duance VC and Archer CW: An immunofluorescence method for postembedded tissue in the acrylic resin Technovit 9100 New using fluorescein isothiocyanate secondary detection. Microsc Res Tech 72: 501-506, 2009.
3. Harle DG, Baldo BA and Fisher MM: Immunoassays employing substituted ammonium compounds other than neuromuscular blocking drugs to increase the detection of IgE antibodies to these drugs. Mol Immunol 27: 1039-1045, 1990.
4. De Boer GF, Back W and Osterhaus ADME: An ELISA for detection of antibodies against influenza A nucleoprotein in humans and various animal species. Arch Virol 115: 47-61, 1990.
5. Ebel GD, Dupuis AP II, Nicholas D, Young D, Maffei J and Kramer LD: Detection by enzyme-linked immunosorbent assay of antibodies to West Nile virus in birds. Emerg Infect Dis 8: 979-982, 2002.
6. Seiler BM, Yoon KJ, Andreasen CB, Block SM, Marsden S and Blitvich BJ: Antibodies to influenza A virus (H1 and H3) in companion animals in Iowa, USA. Vet Rec 167: 705-707, 2010.
7. Ding S, Yin H, Xu X, Liu G, Jiang S, Wang W, Han X, Liu J, Niu G, Zhang X, *et al*: A cross-sectional survey of severe fever with thrombocytopenia syndrome virus infection of domestic animals in Laizhou city, Shandong Province, China. Jpn J Infect Dis 67: 1-4, 2014.
8. Sidorin EV and Solov'eva TF: IgG-binding proteins of bacteria. Biochemistry (Mosc) 76: 295-308, 2011.
9. Löfkvist T and Sjöquist J: Chemical and serological analysis of antigen preparations from *Staphylococcus aureus*. Acta Pathol Microbiol Scand 56: 295-304, 1962.
10. Forsgren A and Sjöquist J: 'Protein A' from S. aureus I. Pseudo-immune reaction with human gamma-globulin. J Immunol 97: 822-827, 1966.
11. Björk I, Petersson BA and Sjöquist J: Some physiochemical properties of protein A from *Staphylococcus aureus*. Eur J Biochem 29: 579-584, 1972.
12. Graille M, Stura EA, Corper AL, Sutton BJ, Taussig MJ, Charbonnier JB and Silverman GJ: Crystal structure of a *Staphylococcus aureus* protein A domain complexed with the Fab fragment of a human IgM antibody: Structural basis for recognition of B-cell receptors and superantigen activity. Proc Natl Acad Sci USA 97: 5399-5404, 2000.
13. Hao J, Xu L, He H, Du X and Jia L: High-level expression of Staphylococcal Protein A in *Pichia pastoris* and purification and characterization of the recombinant protein. Protein Expr Purif 90: 178-185, 2013.
14. Kmiecik S and Kolinski A: Folding pathway of the B1 domain of protein G explored by multiscale modeling. Biophys J 94: 726-736, 2008.
15. Yee L and Blanch HW: Recombinant protein expression in high cell density fed-batch cultures of *Escherichia coli*. Biotechnology (NY) 10: 1550-1556, 1992.
16. Björck L and Kronvall G: Purification and some properties of Streptococcus protein G, a novel IgG-binding reagent. J Immunol 133: 969-974, 1984.
17. Sjöbring U, Björck L and Kastern W: Streptococcal protein G, gene structure and protein binding properties. J Biol Chem 266: 399-405, 1991.
18. Aybay C: Differential binding characteristics of protein G and protein A for Fc fragments of papain-digested mouse IgG. Immunol Lett 85: 231-235, 2003.
19. Riesenberg D and Guthke R: High-cell-density cultivation of microorganisms. Appl Microbiol Biotechnol 51: 422-430, 1999.
20. Zhang HC, Yang J, Yang GW, Wang XJ and Fan HT: Production of recombinant protein G through high-density fermentation of engineered bacteria as well as purification. Mol Med Rep 12: 3132-3138, 2015.