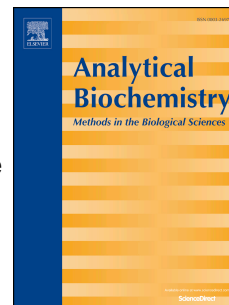


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Purification of equine IgG3 by lectin affinity and an interaction analysis via microscale thermophoresis

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Short title: Affinity purification of equine IgG3

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Abstract

The availability of purified antibodies is a prerequisite for many applications and the appropriate choice(s) for antibody-purification is crucial. Numerous methods have been developed for the purification of antibodies from different sources with affinity chromatography-based methods being the most extensively utilized. These methods are based on high specificity, easy reversibility and biological interactions between two molecules (e.g., between receptor and ligand or antibody and antigen). However, no simple techniques have yet been described to characterize and purify subclasses of immunoglobulins (Ig) from some animals of biotechnology importance such as equines, which are frequently used to produce biotherapeutic antibodies. The sera of these animals present a large number of Ig classes that have a greater complexity than other animals. The implementation of an effective protocol to purify the desired antibody class/subclasses requires meticulous planning to achieve yields at a high purity. The IgG3 subclass of equine-Ig has recently been used as antigen in a new diagnostic test for allergic responses to horse sera-based therapies. Here, we defined a simple method using Jacalin lectin immobilized on Sepharose beads to prepare highly pure equine IgG3 antibodies with a determination of the affinity constants for Jacalin lectin and horse IgG3.

Keywords: Jacalin-Sepharose, affinity chromatography, equine IgG₃, dissociation constant

1. Introduction

Antibodies are important for basic research and in industrial processes from serving as biomarkers for a broad range of diseases, to developing immunodiagnosics for the detection of foodborne pathogens, adulterants, toxins, and residues in food samples as well as environmental analysis/monitoring, to creating drug targeting elements in immunotherapeutics, to methods for the isolation of a range of proteins and other molecules of interest to immunoprophylaxis for treating acute conditions [1-11]. While a variety of simple and fast antibody purification strategies have been developed for a wide range of classes of immunoglobulins (Igs) from different animal species [12], almost nothing has been described for purifying the therapeutic Igs prepared from horse serum that serves as important immunotherapeutics and immunoprophylaxis.

Quintessential passive antibody therapies prepared from horse serum are antivenoms, which are indicated as the primary treatment for snake envenoming [13]. These immunobiological compounds contain polyclonal antibodies raised against the one or more venoms proteins principally obtained from horses. Other sources include sheep, goats and also the donkey, based on a recent evaluation. Intravenous administration of antivenom to patients with snake envenoming prevents or reverses clinical effects by the binding of the antibodies to circulating snake toxins, which neutralizes their activity and promotes their elimination [14]. The most severe side effect of antivenom treatment is an immediate, classical IgE-mediated hypersensitivity reaction that can be fatal and affects 1-40% of the recipients worldwide depending on the preparation [15-24].

Traditionally, at a commercial scale for therapeutic applications, horse IgG (hoIgG) is concentrated by ammonium sulfate induced precipitation [25] or by caprylic acid-based fractionation [26]. However, for the clear majority of Igs used at a research scale, in-house purifications are performed that utilize different techniques like Protein-A, G, A/G, and L affinity chromatography. The use of thiophilic chromatography has found an increasingly wider range of application, especially in the case of industrial-scale purifications where Protein A is the most preferred ligand of choice [27, 28]. Conversely, the efficiency of recovering a representative pool of antibodies can vary by methods (e.g. for human Igs, Protein A binds preferentially to IgM and IgG isotypes, but not to the IgG3 subclass) and the phenotypic characteristic of the Igs from the different animal species (presence or not of the determined carbohydrate). This is of interest for diseases models in which the Ig acts as a marker, as well as when the Ig may be associated with the protective immunity as the antivenoms.

The hoIgG class of immunoglobulins comprises a mixture of seven subclasses (IgG1>IgG3>IgG4>IgG7>IgG2=IgG5>IgG6) that are present at different relative concentrations with IgG3 being the second most abundant Ig in the sera [29]. This subclass is the key anti-venom protective Ig, but also the allergenic compound responsible for observed hypersensitivities to antivenom sera therapy [30-32]. The eqIgG antibodies are known to have important roles in protection against several diseases such as equine influenza virus [33] and equine Herpesvirus [34]. Yet, the structure of the subclasses is poorly characterized. For hoIgG3, it was determined that it possesses O-linked as well as N-linked glycan attached to the heavy chain [29].

This observation leads to the use of Jacalin lectin for the affinity purification of hoIgG3 from the horse sera. Lectins have proven to be useful affinity reagents for the identification, enrichment, isolation, and characterization of a number of glycoconjugates that can provide a glycosylation fingerprint for the discrimination of various glycoproteins. Here, we show the application of Jacalin-Sepharose lectin-based affinity purification as a simple and suitable methodology that could be a viable option for the large-scale production of eqIgG3, which is a key antigen in allergenic antivenom sera therapy. In addition, it was demonstrated that the lectin jacalin presents a high affinity (0.43 nM or $K_d = 2.3 \text{ nM} \pm 0.3 \text{ nM}$) for the hoIgG3.

2. Materials and methods

2.1. Materials

Reagents for solid phase peptide synthesis were from Calbiochem-Merck (Darmstadt, Germany) except 3,3'-diaminodipropylamin (DADPA) and N-benzyloxycarbonyl-L-tyrosine (N-cbz-L-Tyr), which were obtained from Fluke AG (Switzerland) and Acros Organics (USA), respectively. Sepharose CL-4B and Superdex 200 prep were purchased from GE Healthcare Life Science (SP, Brazil) and the silver stain kit from Bio-Rad Laboratories, CA, USA. Gel filtration standard kit proteins and α -D-galactose were obtained from Sigma-Aldrich (St Louis, MO, USA). Fluorescent dyes (NT-647, NT-532 or NT-488) and Monolith NT™ Protein Labeling Kits were from NanoTemper Technologies GmbH (Germany). Microwell plates for ELISA were from Nunc, New York, USA. Horse serum was supplied by INCQS-FIOCRUZ (Rio de Janeiro, Brazil). Rabbit anti-human-IgG and goat anti-human IgE labeled with alkaline phosphatase or HRP were purchased from KPL (Kirkegaard & Perri Laboratories, USA).

2.2. Animals and serum sampling

Horse and donkey serum was supplied Vital Brazil Institute (Niterói, RJ, Brazil) and the National Institute of Quality Control (INCQS) FIOCRUZ (Rio de Janeiro, Brazil) provided mule serum. Human serum that served as the IgE antibodies source was collected from four selected patients, who had been victims of one or more snake bites and who had presented with a severe anaphylactic reaction when treated with antivenom (polyvalent anti-bothropic sera). The allergic response was confirmed by clinical history and positive response to hoIg and characterized by measuring specific IgE reactivity [35].

Twenty sera from healthy individuals who had never received any therapeutic injection products produced in horses were used as a control. Informed consent was obtained from all patients and volunteers. Our study was approved by the Ethics Committee of the Santa Casa de Misericórdia of Rio de Janeiro Hospital (028/10) and by the FIOCRUZ-Fernandes Figueira Institute (0071/10).

2.3. Preparation of jacalin-Sepharose

The jacalin lectin was purified using a polyacrylate DEAE-HPLC (Shimadzu, Kyoto, Japan) column [36] and then conjugated to Sepharose CL4B beads with carbodiimide according to the protocol described by the manufacturing to generate an affinity lectin column (GE Healthcare).

2.4. Jacalin based affinity chromatography procedure

For affinity purification of immunoglobulins, 4 ml of serum from each animal (donkey, mule, and horse) was mixed with an equal volume of 2x Tris-buffer (350 mM Tris-HCl, pH 7.4, containing 300 mM NaCl) and filtered (0.45 μ m). Next, the filtrate was passed through a Jacalin-Sepharose CL4B-column (3 cm x 1 cm, i.d.) that was previously equilibrated with Tris-buffer (175 mM Tris-HCl, pH 7.4, containing 150mM NaCl). After washing with $\geq$ 25 ml of equilibrating buffer, adsorbed proteins were eluted with 5 ml of 0.8 M α -D-Galactose prepared in Tris-buffer at a flow rate of 1 ml/min and room temperature (25 °C). The protein content of the fractions was monitored by the absorbance change at 280 nm on a NanoDrop 2000 spectrophotometer (Thermo Scientific) and the concentration estimated using the extinction coefficient (E) 0.1% at 280 nm, 1 cm pathway = 1.36. Alternatively, total protein concentration was measured using the Thermo Scientific Pierce Protein Assay. A bovine serum albumin (BSA) solution of 0–0.2 mg/mL was used as a standard. For downstream applications, pooled protein peak fractions were dialyzed against Tris-buffer to remove α -D-Galactose.

2.5. Electrophoresis

Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% polyacrylamide gels in Laemmli buffers [37] under reductive conditions. Electrophoresis was performed for 90 min at 100 V using 10% polyacrylamide gels. Detection was performed with Coomassie blue G-250 or silver staining. Low Molecular weight marker (97.400–6.500 kDa) was loaded in the first well of gel to estimate molecular weight in the experimental samples.

2.6. Gel-filtration chromatography

An amount of 100-300 µg (50 µl) of the pooled affinity purified hoIgG₃ (peak 1) was injected onto a Superdex 200 HR10/30 (30 mm x 10mm i.d, GE Healthcare) FPLC column previously equilibrated in 8 mM phosphate buffer (pH 7.4) containing 3 mM KCl, 140 mM NaCl and 1.5 mM KH₂PO₄. Proteins were fractionated at a flow-rate of 0.5 ml/min over a 56 min run at 25 °C on an AKTA Pure (GE Healthcare) FPLC system.

For molecular mass characterization, the column was calibrated in the same buffer with blue dextran (K_{av} 0.07), ferritin (Mr 440.00; K_{av} 0.24), aldolase (Mr 158.000; K_{av} 0.37), conalbumin (Mr 75.000; K_{av} 0.45), ovalbumin (Mr 44.000; K_{av} 0.52), carbonic anhydrase (Mr 29.000; K_{av} 0.58) and ribonuclease (Mr 13.700; K_{av} 0.67).

2.7. MicroScale Thermophoresis (MST)

Purified IgG₃ was labeled with the fluorescent dye NT-647 using Monolith NT™ Protein Labeling Kits (cysteine-reactive) and mixed at 1:1 molar ratio with non-labeled protein. For the interaction experiments of fluorescent hoIgG₃-Jacalin, the concentration of labeled hoIgG₃ was maintained constant, while the concentration of the non-labeled Jacalin (y-axis) varied from 0.07 to 200 nM. The assay was performed in Tris-buffer (50 mM Tris containing 150 mM NaCl, 10 mM MgCl₂ and 0.05 % Tween 20, pH 7.6) and after a short incubation time, the samples were loaded into MST NT.115 standard glass capillaries. The analysis was performed as described above but in PBS. The *K_d* data was calculated using the NanoTemper software package.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The ELISA was conducted as described previously [35]. Briefly, an ELISA 96-well plate was coated with 80 µl of the chromatographically purified horse, donkey and mule IgG₃ protein prepared in coating buffer (Na₂CO₃–NaHCO₃, pH 9.6) overnight at 8 °C. After

each incubation step, the plate was washed three times using PBS-T washing buffer (PBS with 0.1% Tween-20, pH 7.2) and blocked (200 μ l) with 2% defatted milk by a 2h incubation at 37 °C. A pool of sera from allergic patients (n=4) and healthy individuals (n=20) were diluted (1:10; 1:60; 1:300 and 1:1200) in coating buffer and 100 μ l was applied to the immunosorbent plates. After a 2h incubation at 37 °C, plates were washed several times with PBS-T followed by a 2h incubation with 100 μ l goat anti-human IgE conjugated to HRP (1:2.000 dilutions at blocking buffer). After a final series of washes, plates were developed 15 min with OPD substrate containing 0.02% H₂O₂. Signal development was stopped with 50 μ l of stop solution (2M H₂SO₄). Absorbance values at 405 nm were measured using an ELX800 Microplate Reader (Bio-Tek Instruments Inc) within 2h of the addition of Stop Solution.

3. Results and discussion

3.1. Purification and characterization of the eqIgG₃ proteins

Immunoglobulins are glycoproteins and can, therefore, can be purified using affinity chromatography based on immobilized lectin adsorbents [38]. There are several examples in the literature using affinity chromatography for purifying classes and subclasses of Igs from various species, including human, mice [38], dog [39] and rabbit [40].

In this study, we have generated a Jacalin lection-Sepharose affinity column to purify the IgG₃ subclass from equine sera with high recovery and to apparent homogeneity. Following the passage of equine sera through the Jacalin-Sepharose column, along with greater than 10 column volumes of wash, a single peak was observed when the bound material was desorbed with 0.8M galactose. Equivalent profiles were obtained for sera from either horse, donkey or mule (**Fig 1**). The yield of the recovered protein eqIgG₃ (hoIgG₃, doIgG₃, and muIgG₃) was approximately 1.6-2.13 % of the total equine protein sera applied to the affinity matrix that correlated to 2.56-3.07 mg IgG₃/column using 4 ml of sera (30-40 mg/ml) applied. This quantification was supported by measurements of the total bound and unbound proteins in relation to the affinity chromatography.

No data has been reported in the literature related to the total serum Ig concentration in horses, nor on the percentage of individual subclasses, which precludes comparisons of our results. However, it estimated that the total Ig concentration in human sera is approximately 13 mg/ml. Thus, the value of 0.64-0.76 mg/ml of eq-IgG₃ purified would be reasonable since hoIgG₃ is the second most prevalent Ig subclass in horse sera [29].

The eqIgG3 purified proteins, when analyzed by HPLC-gel filtration, showed a difference in their patterns depending on the animal (**Fig. 2**). A molecular mass of 192 kDa was deduced for hoIgG₃ (± 11.5 kDa) and doIgG₃ (± 7.7 kDa), while a mass of 151.4 ± 9.1 kDa was observed for muIgG3. Under the reducing conditions of SDS-PAGE, the hoIgG3 and doIgG₃ presented bands with an apparent molecular weight of 65 and 25 kDa for the heavy and light chain of Ig, respectively. For muIgG3, the heavy band appeared at 50 kDa and the light chain at 25 kDa (**Fig. 3**). This significative difference in the molecular mass can be attributed to the genetic background of the equines. The mule, *Equus mulus*, is the offspring (F1 hybrid) of two equine species: a male donkey and a female horse and they differ in the number of chromosomes [41,42].

3.2. The investigation by ELISA if all three equine IgG3 express the same anaphylactic epitope

To confirm that the all purified proteins belong to the IgG3 subclass, 96 wells microtiter plates were sensitized with the purified Ig and reacted with serum from patients with known allergies to horse serum therapy using an alkaline phosphatase conjugated to anti-human IgE as the secondary. The results, presented in **figure 4**, show a robust reactivity for all three species eqIgG3 to the IgE antibodies in the human sera at the lowest dilution of 1:10. Under the same conditions, no reactivity was observed with serum from healthy subjects. These results demonstrated the strict specificity of the reaction. The presence of a major IgE epitope at hoIgG3 was previously demonstrated by our group in antigenic mapping experiments using a peptide microarray assay [30] and two immunological assays demonstrating the IgE reaction specificity [31,34].

3.3. Characterization of the interaction hoIgG3-Jacalin

We further investigated the utility of the Jacalin-Sepharose resin by determining the binding affinity of jacalin lectin for the hoIgG3 to better understand the observed selectivity and specificity of this ligand/binding partner. There are many ways to measure this interaction. Here, we have applied microscale thermophoresis (MST) to measure the dissociation constant (K_d) in solution and consequently determine their affinity ($1/K_d$). This methodology is based on the movement of molecules in a temperature gradient and has the advantage of performing fast measurements applicable to molecular interactions in solutions without requiring immobilization of one of the binding partners to a surface [43].

Figure 5 highlights the measured K_d values for hoIgG3.Jacalin using MST. A K_d of 2.3 nM $\pm$ 0.3 nM (0.43 nM affinity) was determined by the interaction hoIgG3-Jacalin in solution. This high value for K_d was surprising since the association constant of free pNP-sugars and O-glycosylated peptides for Jacalin by frontal affinity chromatography ranged 10^5 - 10^6 M⁻¹ [44]. The discrepancy in the results most likely relates to the use of different techniques as well as the size and composition of the molecular entities with a distinct conformational organization and number/molecule of oligosaccharides ligands, which may interfere in the determination of the constants. Therefore, more studies are needed to clarify the difference in values.

Originally it was reported that Jacalin primarily bound to the Gal β 1-3GalNAc α -(Core1) structure [45,46] and then that it had a wider binding specificity [47,48]. Still, more recently, it was demonstrated that among the Gal/GalNAc reactive lectins, Jacalin remains as a prime candidate for a serving as a tool to specifically select O-glycans. However, Jacalin does not bind to O-glycans with any C6-substituent at the reducing end GalNAc [44].

In conclusion, the affinity chromatography using Jacalin lectin served as an excellent method for the biorecognition and purification, with high selectivity IgG3, from equine sera. The relatively high affinity of the eqIgG3 arising from a special organization of oligosaccharides ligands may offer a suitable approach for economical production of high-quality eqIgG3. This method could be also valuable for the development of other produced therapeutic dependent of eqIgG₃ response as for example rabies toxin, tetanus toxins, botulinic toxin and insect toxins.

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Conflict of Interest

The authors declare no conflict of interest.

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Abbreviations

(doIgG3) donkey immunoglobulin G3
 (EDTA) ethylenediaminetetraacetic acid
 (eqIgG3) equid IgG3
 (hoIgG3) horse immunoglobulin G3
 (HPLC) high-performance liquid chromatography
 (huIgE) human immunoglobulin E
 (Ig) immunoglobulin
 (IgE) immunoglobulin E
 (IgG) immunoglobulin G
 (MST) microscale thermophoresis
 (muIgG3) mule immunoglobulin G3
 (PAGE) polyacrylamide gel electrophoresis
 (PBS-NP40) phosphate buffer pH 7.3 containing 150 mM NaCl and 0.1% Nonidet P-40
 (PVDF) poly (vinylidene difluoride)
 (SDS) sodium dodecyl sulfate.

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Legends to figures

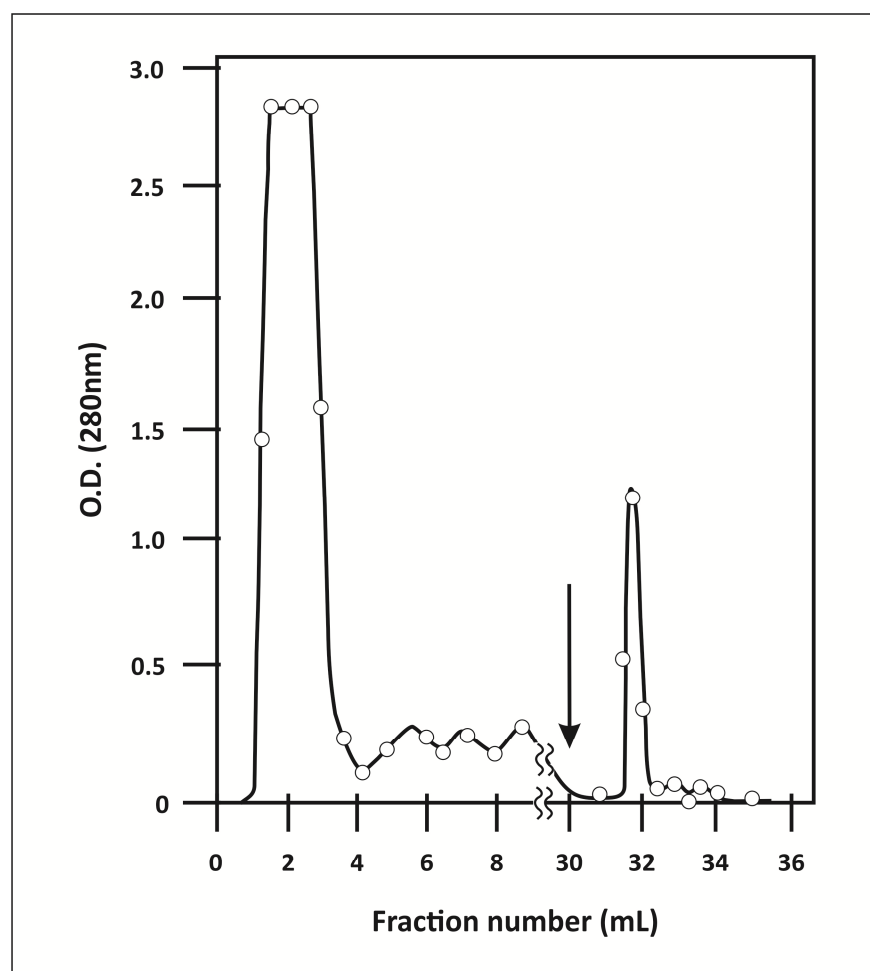
Figure 1: Purification of hoIgG3 by affinity chromatography. Four ml of horse serum diluted in 4 ml of 350 mM Tris–HCl, 300mM NaCl (pH 7.5) was filtered and applied on a Jacalin-Sepharose column (3 cm x 1 cm, i.d.). After washing with ≥ 25 ml of equilibrating buffer (175 mM Tris–HCl containing 150mM NaCl, pH 7.5), the retained proteins were eluted with 0.8 M α -D-Galactose prepared in Tris-buffer (arrow indicates the point of application).

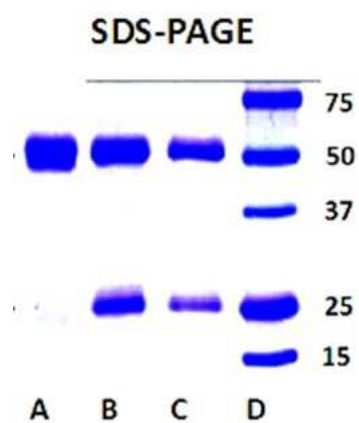
Figure 2: Gel filtration chromatography characterization of jacalin lectin purified equine IgG3. A Sephadex 200 HR10/30 (30 mm x 10 mm, i.d.) column was used for gel filtration chromatography at a flow-rate of 0.5 ml/min during 56 min at 25 °C in an FPLC system. The elution profile of IgG3 affinity purified from horse, donkey and mule sera were compared to a commercial kit of standards molecular weight proteins. The molecular mass of each Ig is indicated.

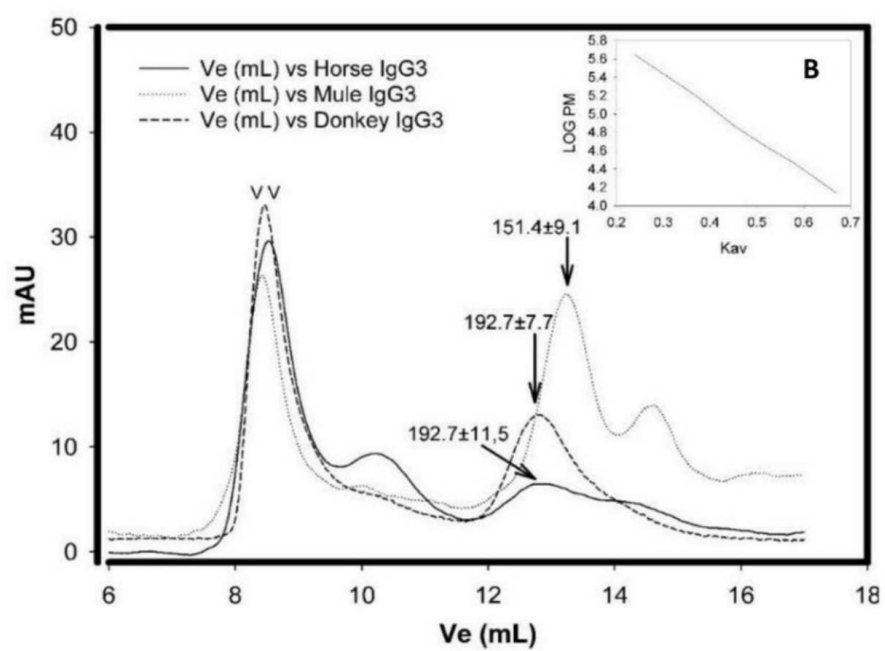
Figure 3: Characterization of the eqIgG3 by SDS-PAGE (10%) under reducing conditions. The samples (30 μ g) loaded into lanes A, B, and C were purified from the mule (mIgG3), donkey (dIgG3) and horse (hoIgG3) serum (30 μ g). The SDS-PAGE was revealed by Coomassie blue.

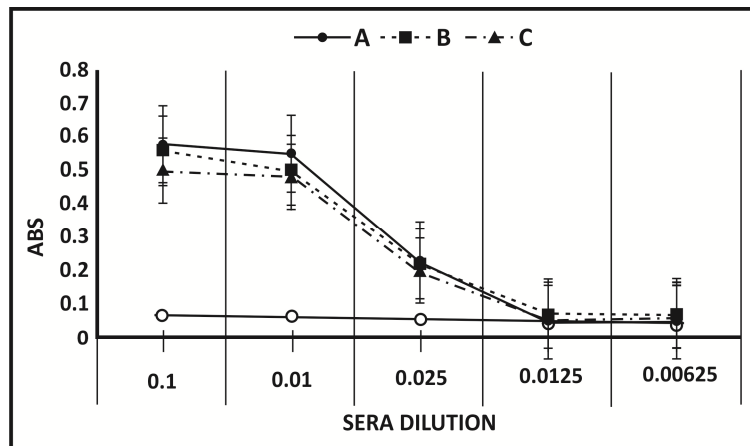
Figure 4: Enzyme Linked Immune Sorbent Assay (ELISA). Reactivity of three eqIgG3 [hoIgG3 (●), doIgG3 (▲) and muIgG3 (■)] adsorbed onto ELISA plates to allergenic patient sera (n=4) containing IgE anti-hoIgG3 and sera from healthy individuals sera (O, n=20) at different dilution. The results are the median ($\pm$ S.D.) of the optical density (405 nm) from three separate experiments.

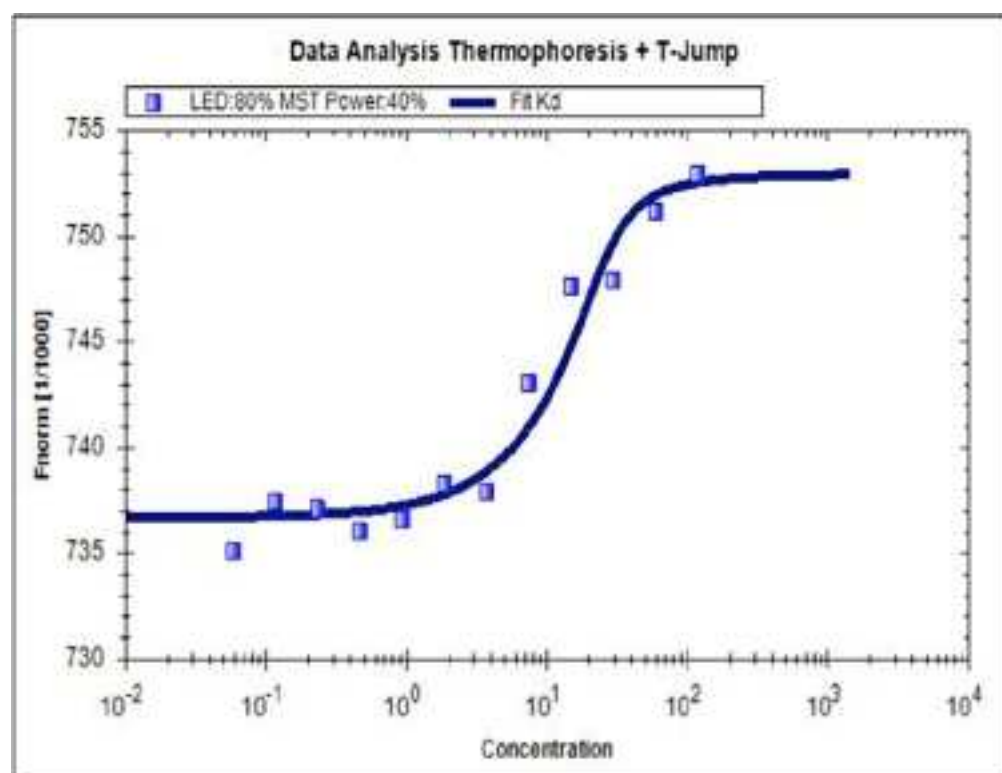
Figure 5: Determination of the K_d of the interaction hoIgG3-Jacalin by MicroScale Thermophoresis (MST). For the interaction experiments of hoIgG3-Jacalin, the quantity of NT.647 labeled hoIgG3 was maintained constant, while the concentration of the non-labeled Jacalin (y axis) varied from 0.07 to 200 nM. The assay was performed in PBS and after a short incubation time the samples were loaded into MST NT.115 standard glass capillaries. Concentrations on the y-axis were plotted in nM. A K_d of 2.3 nM $\pm$ 0.3 nM was calculated.











Highlights

Jacalin-Sepharose was used to purify IgG3 from equine sera

The eqIgG3 is the major allergenic compound of therapeutic sera

The interaction jacalin-hIgG3 is strong with an affinity of 0.43 nM