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Purification of horse immunoglobulin isotypes based on differential elution properties of isotypes from protein A and protein G columns

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Abstract

Elution properties of horse immunoglobulin isotypes from protein A and protein G columns were examined. IgGa and IgGb isotypes were bound to protein A and protein G columns and were eluted by adjusting the pH of the elution buffer from 8.0 to 2.0. IgGc bound to protein G column but not to protein A column while IgG(T) bound to both columns. IgM and IgA apparently appeared not to bind to either column. New methods for purification of serum isotypes were developed using protein A and protein G columns as well as formerly established methods. Using these methods, it was possible to obtain purified isotypes for establishment of immunological assays for practical clinical use.

Keywords: Isotypes; Immunoglobulins

1. Introduction

Through establishment of methods for immunological research of equine infectious diseases, we have found that it is necessary to isolate immunoglobulin isotypes from horse sera. Also, establishment of a system for the quantitation of immunoglobulin isotypes is required for practical clinical use. Horse sera normally contain appreciable quantities of IgGa, IgGb, IgG(T) and IgM, and lesser amounts of IgGc, IgA and IgE [1,8]. However, their purification by physicochemical methods has proven to be difficult, particularly IgGa, IgGb, IgGc and IgA. Biochemical properties of these proteins, particularly IgGa and IgGb, are very similar and methods for their isolation have only recently been developed [10–12]. Although we previously succeeded in fractionating IgGa and IgGb based on small differences in agar-gel electrophoretic mobility, it was difficult to recover highly purified IgGb [12]. Furthermore, affinity chromatography using monoclonal antibody (MAb) coupled beads is also not a definite method for their purification. Additionally, recovery of large amounts of purified IgGc and IgA has also proven to be laborious.

The binding of immunoglobulins of various mammalian species to Staphylococcal protein A and Streptococcal protein G has been extensively studied [3,5,9,10]. In the present study, we could determine the elution properties of horse immunoglobulin

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isotypes such as IgGa, IgGb, IgGc, IgG(T), IgM and IgA, from protein A and protein G columns by varying the pH of the elution buffer. We succeeded in purification of isotypes using a combination of column chromatography and formerly established methods.

2. Experimental methods

2.1. Source of immunoglobulin isotypes

Sera were collected from 10 thoroughbred horses maintained in our institute including three males and seven females ranging in age from 3 to 10 years old.

2.2. Protein A and protein G column chromatography

A 1-ml aliquot of serum sample that was previously diluted 1:10 in phosphate-buffered saline (PBS), was applied to a column (25 mm in diameter and 100 mm in length) packed with Protein A Sepharose CL-4B or Protein G Sepharose 4B (Pharmacia Biotechnology AB, Uppsala, Sweden) previously equilibrated in PBS. Initial flow-through (FT) fractions were concentrated to 1 ml. The columns were then washed extensively with PBS. Bound proteins were eluted in disodium hydrogenphosphate–citric acid buffer solutions with pH levels ranging from 8.0 to 2.0. The buffer solutions formed a pH gradient by adjusting the ratios of 200 m*M* disodium hydrogenphosphate–100 m*M* citric acid from 100:0 (pH 8.0) to 0:100 (pH 2.0). Chromatography was completed within 30 min and was controlled using a FPLC system (Pharmacia Biotechnology AB) that included LCC-500 Plus controller, P-500 pumps, dual path monitor UV-2 UV detector and REC 102 recorder. pH levels of each fraction (2 ml each) were measured using a Ø50 pH meter (Beckman Instruments, Fullerton, CA, USA). Eluted fractions were neutralized with 0.5 *M* NaOH that included 0.08% phenol red as the indicator of pH. Chromatography was conducted at room temperature.

2.3. Molecular sieve chromatography

Isotypes were separated by molecular sieve chromatography using Sephacryl S300 (Pharmacia Biotechnology). The gel was packed in two columns (25 mm in diameter and 750 mm in length) and equilibrated in 100 m*M* tris(hydroxymethyl) aminomethane–HCl buffer (Tris–HCl, pH 8.0) that was supplemented with 1 *M* NaCl. Samples (5 ml) were applied to these columns at a flow-rate of 2.5 ml/min and fractionated in 5 ml. Chromatography was controlled by the same system for protein A and protein G column chromatography.

2.4. DEAE ion-exchange chromatography

DEAE-Sephacel (Pharmacia Biotechnology) was used for purification of IgGab, IgG(T) and IgA. Gel columns (50 mm in diameter and 300 mm in length) and samples were equilibrated in 5 m*M* Tris–HCl (pH 8.0) before use. Bound proteins were eluted stepwise from 10 m*M* Tris–HCl, 20 m*M* Tris–HCl, 40 m*M* Tris–HCl, 40 m*M* Tris–HCl+40 m*M* NaCl and 40 m*M* Tris–HCl+80 m*M* NaCl at room temperature.

2.5. Preparative block electrophoresis

The starch block electrophoresis was used to separate IgG(T) and IgA isotypes. Potato starch (Kanto Chemicals, Tokyo, Japan) that was washed and equilibrated with 60 m*M* barbital buffer (μ= 0.15, pH 8.6) was packed into a tray (10 mm in depth, 50 mm in width and 300 mm in length) [13]. The sample that was previously dialyzed to the same buffer was poured into a ditch, 10 mm in length. The proteins were separated for 18 h with constant current at 10 mA at 4°C. Starch blocks were cut into 10-mm widths and proteins were eluted in saline solution.

2.6. Immunoglobulin determination

2.6.1. Enzyme-linked immunosorbent assay (ELISA)

A sandwich ELISA was used for detection of immunoglobulin isotypes in column purified frac-

tions. Microplates for ELISA (Maxisorp, Nunc, Roskilde, Denmark) were coated with monoclonal antibody (MAb) to horse IgGa, IgGb, IgGc, IgG(T), IgM or IgA [12]. For detection of IgGa, IgGb, IgGc, IgG(T), IgM or IgA, fractions were diluted 1:100, 1:100, 1:10, 1:1000, 1:10 and 1:10, respectively. Plates containing diluted fractions, that were prepared in duplicate, were then incubated for 60 min at 37°C. Horse-radish peroxidase (HRPO) conjugated MAb corresponding to the first antibody was added to the plates and incubated under the same conditions. HRPO was detected by incubation with 3,3′,5,5′-tetramethylbenzidine (TMBlue, Moss, Pasadena, MD, USA) and colorimetric reactions were stopped by addition of 1 *N* sulfuric acid (Kanto Chemicals). The optical density (OD) of wells was measured at 450 nm using an ELISA Reader (Corona Electric, Hitachi, Ibaraki, Japan).

For checking the contamination of isotypes in each purified material, isotypes were semi-quantified by sandwich ELISA. Concentrations of isotypes to make a standard curve were adjusted to 20, 40, 60, 80 and 100 ng/ml. Protein concentration was determined by dye binding assay using a Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA). Bovine serum IgG (Bio-Rad) was used as protein standard. Concentrations of purified isotypes were adjusted to 1000 ng/ml and reacted on the ELISA system for every isotype.

2.6.2. Immunoelectrophoresis

Finally purified isotypes were identified by immunoelectrophoresis using IEF plates for the Paragon electrophoresis system (Beckman Instruments). Polyclonal goat or rabbit antisera to each isotype was purchased from Bethyl Lab. (Montgomery, TX, USA) or Nordic Imm. Lab. (Tilburg, Netherlands).

2.6.3. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE)

The molecular weight (M_w) of the finally purified isotypes and heavy (H) and light (L) chains of them were estimated by SDS–PAGE. Purified isotypes under reducing and non-reducing conditions were electrophoretically separated in a 10% polyacrylamide gel purchased from ATTO Corporation (PAGEL GEL, Tokyo) at 20 mA constant current/cm. Tris–HCl buffer containing 0.1% SDS was used for electrophoresis. For separation of isotypes to H- and L-chains in reducing condition, 2-mercaptoethanol was used in the sample buffer. MultiMark multi-colored standard molecular weight markers were purchased from Novel Experimental Technology (San Diego, CA, USA). After the electrophoresis, gels were stained in Coomassie brilliant blue R250 (0.1% w/v) in 30% methanol–10% acetic acid.

3. Results

3.1. Elution profiles of isotypes from protein A columns

Elution profiles of proteins from protein A column are shown in Fig. 1. A large quantity of protein was eluted in flow-through (FT) fractions. After washing the column with PBS, one small peak of OD at 280 nm was observed when proteins were eluted by pH gradient. Elution profiles of isotypes from protein A column are shown in Fig. 2. IgGa was detected only in fractions 21 through 30. IgGb was detected in fractions 18 to 27, while a small peak of OD in ELISA was observed in the initial FT fraction. IgG(T) was observed in fractions 18 to 27, while a small peak was observed in the FT. IgM, IgA and IgGc were detected only in the FT fraction. Elution

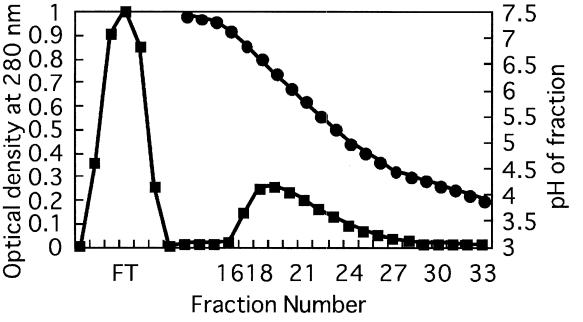

Fig. 1. Elution profile of horse serum proteins from protein A column. ■ and ● indicate optical density at 280 nm and pH of eluted fractions. FT means flow-through fractions. Proteins were eluted in buffer formed with a linear gradient mixture of 200 m*M* disodium hydrogenphosphate, pH 8.0 and 100 m*M* citric acid buffer, pH 2.0.

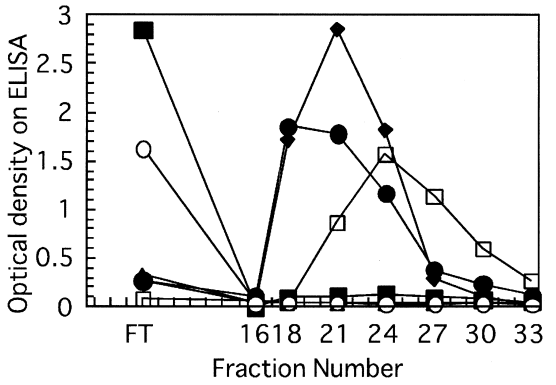

Fig. 2. Elution profile of horse immunoglobulin isotypes from protein A column. Data indicates optical density at 450 nm on the ELISA for detection of isotypes. □, IgGa; ●, IgGb; ▲, IgGc; ♦, IgG(T); ■, IgM; ○, IgA.

profiles of the other nine horse sera showed similar results.

3.2. Elution profiles of isotypes from protein G column

Elution profiles of proteins from protein G column are shown in Fig. 3. A large quantity of protein was eluted in flow-through (FT) fractions. After washing the column with PBS, two small peaks of OD at 280 nm were observed when proteins were eluted by pH gradient. Elution profiles of isotypes from the protein G column are shown in Fig. 4. IgGa was detected in fractions 40 through 50. IgGb was detected in

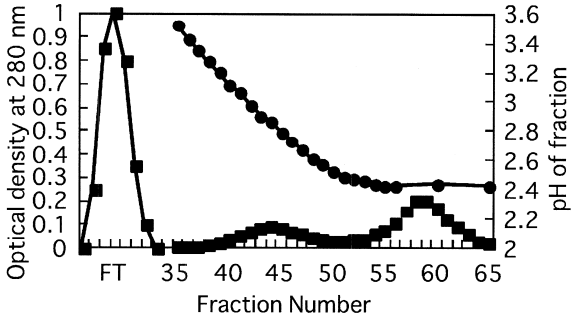

Fig. 3. Elution profile of horse serum proteins from protein G column. ■ and ● indicate optical density at 280 nm and pH of eluted fractions. FT means flow-through fractions. Proteins were eluted in buffer formed with linear gradient mixture of 200 m*M* disodium hydrogenphosphate, pH 8.0 and 100 m*M* citric acid buffer, pH 2.0.

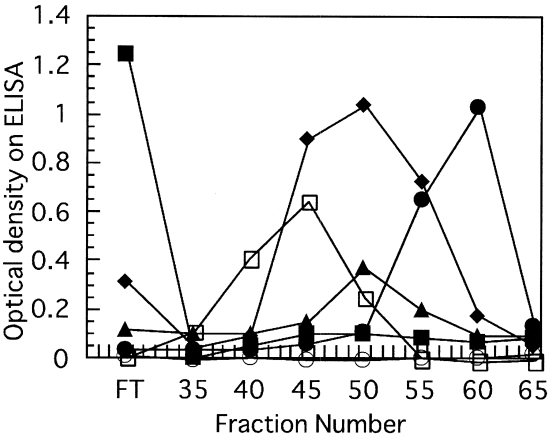

Fig. 4. Elution profile of horse immunoglobulin isotypes from protein G column. Data indicates optical density at 450 nm on the ELISA for detection of isotypes. □, IgGa; ●, IgGb; ▲, IgGc; ♦, IgG(T); ■, IgM; ○, IgA.

fractions 55 to 65, while a small peak of OD was found in the FT fraction. IgGc was detected in fractions 45 through 60, while a small peak of OD in ELISA was detected in the FT fraction. IgG(T) were detected in fractions 45 to 60, while a small peak of OD was detected in the FT fraction. IgM and IgA were detected only in the FT fraction. Elution profiles of the other nine horse sera showed similar results.

3.3. Newly established methods for purification of isotypes using protein A and protein G affinity chromatography

New methods using protein A and protein G columns, as well as formerly established methods were developed for purification of horse immunoglobulin isotypes. A schematic diagram outlining the methods is shown in Fig. 5.

Macroglobulins were precipitated from pooled horse serum by addition of calcium chloride (Kanto Chemicals) and dextran sulfate (Pharmacia Finechemicals AB) to final concentrations of 0.1 *M* and 0.2%. The supernatant was subsequently dialyzed to 5 m*M* Tris–HCl (pH 8.0). IgGab were recovered from the supernatant by DEAE-Sephacel column chromatography. The FT fraction, that contained IgGab, was dialyzed to PBS and applied to a protein G column. IgGa and IgGb were then eluted separ-

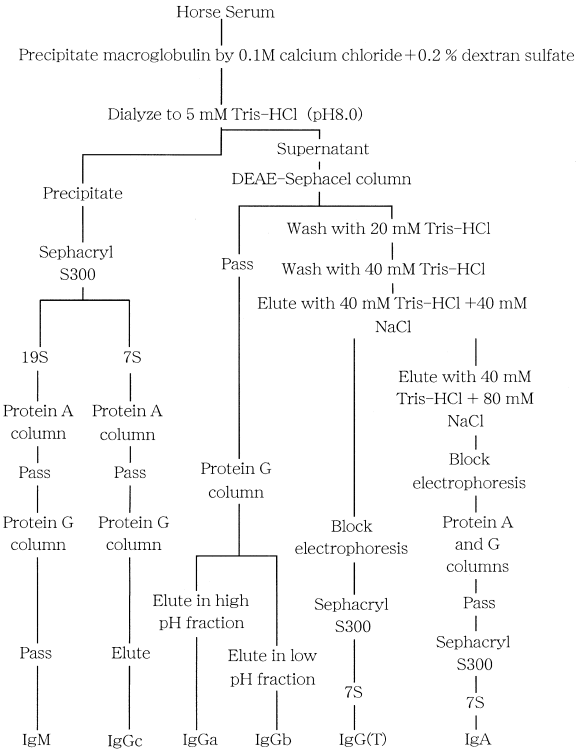

Fig. 5. Scheme of methods for purification of horse immunoglobulin isotypes by protein A and protein G column chromatography and other methods.

ately by gradient pH elution. Observation of OD values of the recovered fractions at 280 nm demonstrated two peaks. Purified IgGa was in fractions corresponding to the first half of the first peak while IgGb was recovered in fractions of the latter half of the second peak.

Precipitated euglobulin was dissolved in PBS and separated by molecular sieve chromatography. Pooled 19 S fractions were applied to protein A and protein G columns to remove contaminating isotypes. The recovered FT fraction contained purified IgM. Similarly, 7 S fractions were pooled and serially applied to protein A and protein G columns. Fractions that were subsequently eluted from the protein G column in 100 m*M* citric acid contained purified IgGc. The DEAE-Sephacel column containing bound immunoglobulins was then serially washed with 20 m*M* and 40 m*M* Tris–HCl. IgG(T)-rich fractions were collected by elution in 40 m*M* Tris–HCl supplemented with 40 m*M* NaCl. IgA-rich fractions were then eluted in 40 m*M* Tris–HCl supplemented with 80 m*M* NaCl. As these fractions were contaminated with IgA or IgG(T), they were further separated by starch block electrophoresis. Separated IgA was further passed through the protein G and A columns for absorption of IgG(T). Finally, these were passed through a Sephacryl S300 column, and the resultant 7 S fractions contained purified IgA and IgG(T), respectively.

3.4. Analysis of the purified isotypes by immunoelectrophoresis and SDS–PAGE

Only one band was observed between purified isotype and anti-whole horse serum, the same as monospecific antiserum to each isotype by immunoelectrophoresis. Their patterns are shown in Fig. 6. In SDS–PAGE analysis on finally purified isotypes, one to two bands that were suggested to be purified isotypes were observed in each lane under non-reducing conditions as shown in Fig. 7. Under reducing conditions, two bands that were suggested to be the H-chain and L-chain of each isotype were observed in each lane as shown in Fig. 8. In lane 6 in which purified IgM was separated, four bands were observed. Of them, two were suggested to be H-chain and L-chain from the estimated molecular weight. The remaining two bands were suggested to be insufficiently reduced IgM. Their molecular weights are summarized in Table 1.

3.5. Purity of final products determined by ELISA analysis

As shown in Table 2, 1000 ng/ml of purified IgGa contained 40 ng/ml of IgG(T), while the other four isotypes were not detected by the ELISA; 1000 ng/ml of purified IgGb contained 40 ng/ml of IgM, while the other four isotypes were not detected; 1000 ng/ml of purified IgGc contained 6 ng/ml of IgGb, while the other four isotypes were not detected. Purified IgG(T) did not contain detectable amounts of the other five isotypes; 1000 ng/ml of purified IgM contained 4 ng/ml of IgGb and 26 ng/ml of IgA, while the other three isotypes were not detected. Purified IgA did not contain detectable amounts of the other five isotypes.

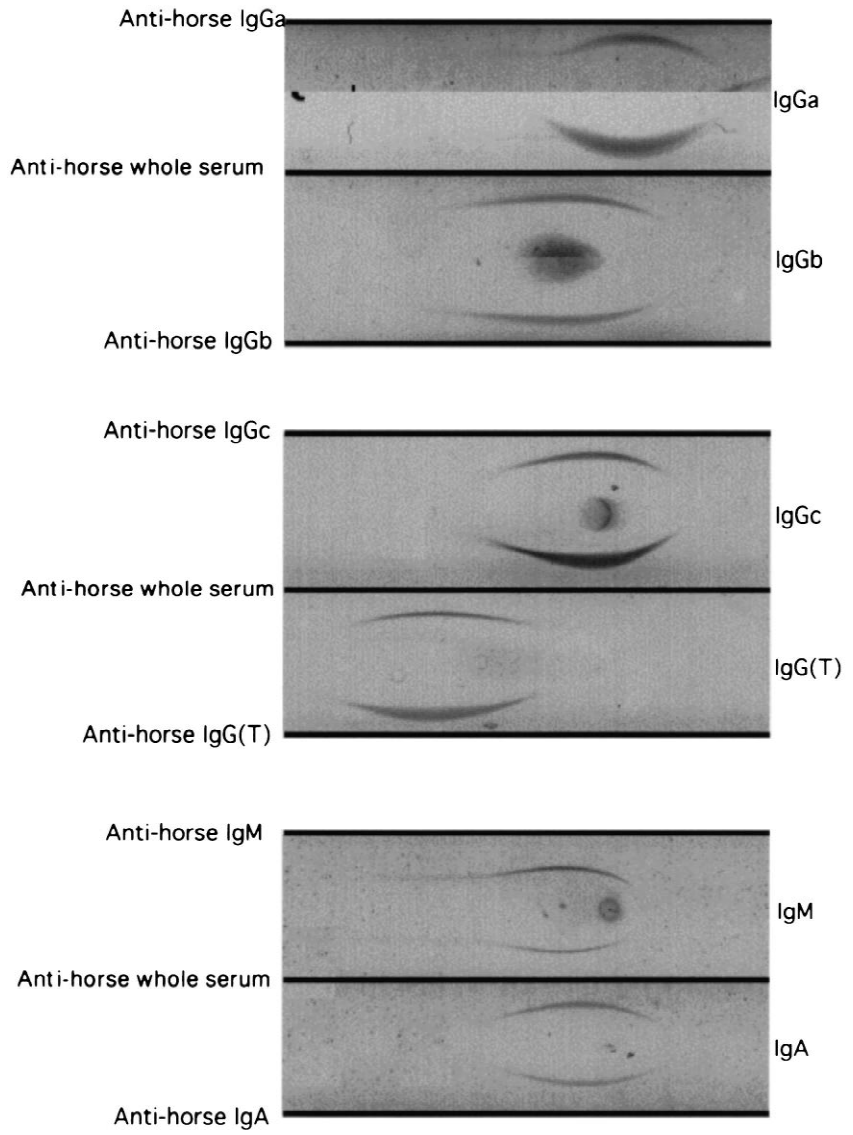

Fig. 6. Immunoelectrophoretic pattern of horse immunoglobulin isotypes which were purified by the methods using protein A and protein G columns and others.

4. Discussion

The present investigation describes elution properties of horse serum immunoglobulin isotypes from protein A and protein G columns shown in Table 3. It also describes newly established methods for purification of isotypes from horse serum. Isotypes of horse immunoglobulin have been purified using many methods including ion-exchange chromatography, isoelectrofocusing, and others such as affinity chromatography using MAb. However, it is difficult to recover isotypes of high purity and high yield for research of immunological properties of isotypes or for establishment of quantitative methods.

Although IgGab can be purified quite easily using methods such as DEAE ion-exchange chromatography, separation of IgGa and IgGb was very difficult. We have succeeded in recovering purified

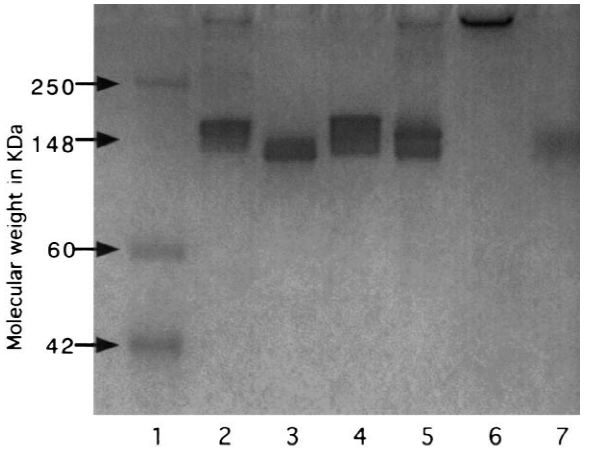

Fig. 7. SDS–PAGE analysis of non-reduced isotypes. Lane 1, molecular weight markers in kDa; lane 2, IgGa; lane 3, IgGb; lane 4, IgGc; lane 5, IgG(T); lane 6, IgM; lane 7, IgA. Finally purified isotypes were analysed.

IgGb by affinity chromatography using anti-IgGa and anti-IgGb antibody that were prepared by immunization of precipitin lines recovered from IEP [12]. In the present study, IgGa and IgGb could be easily and effectively separated by gradient pH elution from protein G columns. A practical method for purification of isotypes using gradient pH elution from protein A and protein G columns have never been reported, although it is known that different isotypes

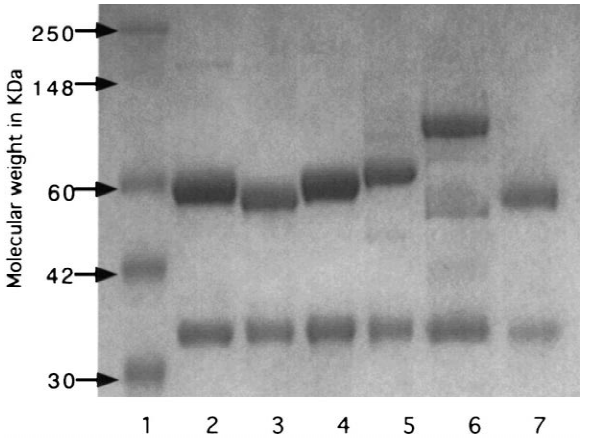

Fig. 8. SDS–PAGE analysis of reduced isotypes. Lane 1, molecular weight markers in kDa; lane 2, IgGa; lane 3, IgGb; lane 4, IgGc; lane 5, IgG(T); lane 6, IgM; lane 7, IgA. Finally purified isotypes were analysed.

Table 1
Molecular weight of horse immunoglobulin isotypes

Isotypes	Molecular weights (Da)		
	H-chain	L-chain	Whole molecule
IgGa	56,000	34,000	170,000
IgGb	54,000	34,000	135,000
IgGc	57,000	34,000	180,000
IgG(T)	60,000	34,000	159,000
IgM	54,000	34,000	ND
IgA	56,000	34,000	148,000

can be eluted from protein A column by stepwise pH elution [4,5,10,14]. Separation of IgGa and IgGb has also been done by Sheoran et al. using gradient pH elution from these columns. They, however, could not find a clear difference in behavior of IgGa and IgGb from these columns, as IEP instead of ELISA was used for detection of isotypes.

Because similar physicochemical properties are shared between IgG(T), IgA and IgGc, purification of IgGc was also very difficult. The present investigation outlined easy methods for the purification of IgGc by protein A and protein G column chromatography after precipitation as euglobulin for preliminary concentration of IgGc, based on observations that IgGc bound to protein G but not to protein A. Also, IgGc has not been previously characterized as a type of euglobulin.

IgM and IgA molecules displayed extremely low affinity to protein A and protein G columns. IgM was therefore purified by molecular sieve chromatography following precipitation as euglobulin.

Protein A and protein G columns were ineffective in the purification of IgG(T). After crude IgG(T) was added to the protein G column, we tried to wash out contaminated IgM and IgA from the columns. They were also eluted from the column with IgG(T).

Binding of immunoglobulin isotypes of various mammalian species to protein A and protein G has been widely studied for their purification. Those of equine isotypes to protein A and protein G were first examined by Björck and Kronvall [2] who showed that IgGab and IgGc bound to protein A and protein G. Fernandes et al. [6] also showed that IgGab was bound to protein A, while Goudswaard et al. [7] described general binding and elution properties of horse IgGab, IgGc and IgG(T) from protein A. Sheoran et al. [10], although, reported that only IgGa

Table 2
Quantitative analysis of finally purified isotypes by enzyme linked immunosorbent assay

ELISA for quantitation of isotypes	Concentration of isotypes in purified samples (ng/ml)					
	IgGa	IgGb	IgGc	IgG(T)	IgM	IgA
IgGa	1000	ND[a]	ND	40	ND	ND
IgGb	ND	1000	ND	ND	40	ND
IgGc	ND	6	1000	ND	ND	ND
IgG(T)	ND	ND	ND	1000	ND	ND
IgM	20	4	ND	ND	1000	26
IgA	ND	ND	ND	ND	ND	1000

[a] ND, not detected.

bound to protein A while IgGa, IgGb, IgGc and IgG(T) bound to protein G. Our results showed that IgGa and IgG(T) have eluted from protein A column, while a small amount of them were passed through the column. There was a possibility that these bound isotypes did not elute under their pH conditions. On the other hand, although Björerck and Kronvall [2] and Goudswaard et al. [7] showed that IgGc demonstrated low affinity to protein A, IgGc was not found to bind protein A in this study in agreement with the report by Sheoran et al. [10]. The reasons for this difference could not be determined as the specificity of polyclonal antibodies for identification of IgGc in previous reports was uncertain. Binding properties of IgM and IgA in this study were consistent with a previous report by Goudswaard et al. [7] describing the properties of immunoglobulin isotypes of other mammalian species.

The molecular weights of whole molecule, H- and L-chain of each IgGa, IgGb and IgG(T) have been already determined by Sheoran et al. [10] by SDS–PAGE analysis. Almost the same molecular weights with their results were shown about three isotypes. The M_w of the whole molecule of IgGc and IgA were 180,000 and 148,000 Da. The M_w of H-chain of IgGc, IgM and IgA were 57,000, 54,000 and 56,000 Da. All isotypes showed a common L-chain M_w of 34,000 Da. Molecular weight in reducing and non-reducing conditions was also shown to be different as reported by Sheoran et al. [10].

Table 3
Binding and elution properties of horse immunoglobulin isotypes from protein A and protein G columns

Isotypes	Gels	
	Protein A	Protein G
IgGa	Bound	Bound
	Eluted from pH 7.5 to 5.5	Eluted from pH 5.5 to 4.0
IgGb	Bound	Bound
	Eluted from pH 3.0 to 2.5	Eluted from pH 2.5 to 2.4
IgGc	Did not bind	Bound
		Eluted from pH 2.7 to 2.5
IgG(T)	Bound	Bound
	Eluted from pH 3.0 to 2.5	Eluted from pH 3.0 to 2.6
IgM	Did not bind	Did not bind
IgA	Did not bind	Did not bind

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