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Isolation and Preparation of Antisera to Ovine IgG Heavy Chain

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ÖZET

Koyun IgG'si jel-filtrasyon, iyon değişimi ve afinite kromatografisi kullanılarak izole edilmiştir. İzole edilen proteinler SDS-Page jel elektroforezi yöntemi ile ayrıştırılmıştır. Jel'de 52-56kD bölgesindeki proteinler elektroelüsyon yöntemi ile saflaştırılmış ve bu proteinlerle tavşan immünize edilmiştir. Tavşandan elde edilen antiserum Western Immuloblotting yöntemi ile analiz edilmiştir. Bu antiserum koyun serumunda, 54kD moleküler ağırlığındaki bir protein ile reaksiyon vermiştir. 54kD, IgG ağır zincirinin molekül ağırlığına eşdeğerdir.

Anahtar Kelimeler : IgG, koyun, izolasym,antiserum

SUMMARY

Isolation and Preparation of Antisera to Ovine IgG Heavy Chain.

Ovine immunoglobilin G was isolated from sheep serum using gel filtration, Jon-exchange and Gamma Bind-G affinity chromatography. The isolate was electrophoresed using SDS-Page gel electrophorersis.The proteinss in the region between 52-56kD were elecroeluted and rabbit was immunized. Obtained antiserum was analysed using Western Immunoblotting and reacted with a protein in sheep serum in the region 54kD which is identical to IgG heavy chain.

Key Words: IgG, ovine, isolation, antiserum.

INTRODUCTION

IgG is a monomer but higher polymers have also been reported (1, 2, 3, 4). In ruminants there are two well defined IgG subclasses IgG_1 and IgG_1 but IgG, a is also reported in sheep (5, 6). These two subclasses differ not only in their physicochemical properties, but also in some of their biological functions. Thus, antibodies belonging to the IgG_1 subcllass are able to fix heterologous complement and possess heterocytotropic activity, whereas those belonging to the IgG_2 subclass are unable to fix heterologous complament and possess homocytotropic activity (7, 8). In contrast, both subclasses participate in antibody-dependent target cell lysis mediated by leucocytes (9).

Antisera prepared against intact IgG, IgM and IgA molecules may cause cross-reaction between these immunoglobulins because of the similarities in the light chains. Antisera prepared in this way need intensive absorption. The cross-reactivity can be avoided either by preparing monoclonal or polyclonal antiserum specific for the heavy chain of immunoglobulins (10).

The aim of our study was to isolate ovine IgG and prepare an antiserum against its heavy chain. IgG was isolated from sheep serum, using gel filtration, ion-exchange and Gamme Bind-G affinity using gel filtration, ion-exchange and Gamma Bind-G affinity chromatography. The isolate was then run on SDS-Page Electroelution was performed and rabbit was immunized. The antiserum was analysed by Western Immunoblotting.

MATERIALS AND METHODS

Isolation of IgG

Two hundred mililitres of sheep serum was precipitated with 50% saturated ammonium sulphate solution. The serum samples were then applied to G-

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200 (pharmacia) gel filtration chromatography as described by before (11). Second peak on G-200 chromatography was collected, concentrated and equilibrated with 0.002 molar (M) phosphate buffer. The samples were then applied to a DEAE-cellulose (Whatman DE-52) ion-exchange column and stepwise elutions were performed with 0.002M, 0.01M, 0.02M, 0.03M, 0.04M and 0.05M phosphate buffer pH 7.4 fraction volumes ranging from 10 to 30ml were collected separately, concentrated to 10ml, dialysed in PBS and filtered through 0.2 micron microfilter. It was then applied to Gamme Bind-G prepack (BDH-England) wich was used for the purification of ovine IgG as it contains Streptococcal protein G. It was assumed that ovine IgG would bind to protein G. Elutions were performed with 0.5M ammonium acetate pH 3.0. The eluates were dialysed in PBS, concentrated to 2ml and analysed by Immunoelectrophoresis using rabbit anti-ovine whole serum and SDS-Page electrophoresis.

SDS-Page

SDS-Page electrophoresis was performed as described before (12). The eluates from Gamma Bind-G prepack were mixed with sample buffer 0.5M 25% (v / v) Tris pH 6.8 1% (w / v) sodium dodecyl sulphate and 2.5% (v / v) B-2 mercaptoethanol. The mixture was then heated to 95/100˚C for 5 minutes. Samples were loaded on 7.5% acyrlamide gel (Bio-Rad England). Prestained SDS-6H molecular weight markers (sigma chemicals Ltd-England) were run at each and of the gel. Four gels were run in total. Electrophoresis was performed with running buffer containing 9 g Tris, 37.5 g glycine, and 3 g sodium dodecyl sulphate in 3 litres of distilledwater. After electrophoresis these gels were used for the electroelution to purify ovine IgG heavy chain. Fractions from G-200 chromatography, semipurified ovine IgM, eluent from Gamma Bind-G prepack and SDS-6H molecular weight markers (sigma) were also run on one half of another 7.5% acrylamide gel and the sequence repeated on the other half. Electrophoresis was performed as described before. Proteins in the gel were transferred to a nitrocelluose membrane in blotting buffer containing 9 g Tris, 45 g glycine and 600 ml methanol in 3 litress of distilled water. The first half of the membrane was stained with 1% (v / v) Amido black staining and the specificity of rabbit anti-ovine IgG heavy chain.

SDS-Page electroelution

The technique was used to purify ovine IgG heavy chain. It was assumed that the heavy chain of IgG would separate in the region of 52-56 kD in SDS-Page electrophoresis, the bands between 52-56kD were cut into small pieces and put into electroelution tubes. Electroelution was performed in a Bio-Rad (mini cell) electroeluter. Proteins were eluted from the gels in electroelution buffer containing 3 g Tris base, 14.4 g Glycine in 1 litre of distilled water, at 45mA for 6 hours. The eluates were dialysed overnight in PBS and filtered through 0.2 micron microfilter.

Immunization of rabbit

Electroeluted proteins were emulsified in Freund's complete adjuvant. New Zealand white rabbit weighing 2-3kg was immunized with 1ml of emulsion administered at two intramuscular and one subcutaneous sites. Subsequent immunizations were made with 1ml emulsion of antigen in Freund's incomplete adjuvant by the same route, at fortnightly intervals over a 3 month period. The antiserum was absorbed with affinity purified ovine IgM (Yılmaz, Ilgaz and Morgan submitted for publication) rendering them monospecific.

Immunoblotting

Immunoblotting was performed as described before (13). Briefly, the nitrocellulose membrane containing fractions from G-200 chromatography, semi-purified ovine IgM and eluent from Gamma Bind-G prepack was incubated with the absorbed rabbit anti-ovine IgG heavy chain at a dilution of 1:00 for 2 hours at room temperature, after blocking the remaining binding sites with 5% (w / v) dried fat free skimmed milk (marvel) in PBS. After washing with PBS containing 0.05% Tween-20 (v / v), the membrane was incubated with peroxidase

conjugated donkey anti-rabbit immunoglobulins (Amersham) at a dilution of 1:1000 for 2 hours. It was then washed again in PBS Tween-20. Fnally, the colour was developed with 1mg/ml 3.3'-diaminobenzidien (sigma) in PBS containing 0.01% hydrogen peroxide.

RESULTS AND DISCUSSION

IgG_1 is the major immunoglobulin of both serum and colostrum and its concentration in serum and in early lactation is 18.02mg/ml and 5.05mg/ml, respectively. The concentration of IgG_2 in serum is 6.74mg/ml and in early lactation 0.15 (14). On immunoelectrophoresis the mobility of IgG_1 is slower than IgG_1 and the separate spur produced by IgG_1 is characteristic of bovine IgG_1 and IgG_2 subclases (5, 15, 16). This was also observed in our study when the final preparation from Gamma Bind-G was run on immunoelectrophoresis. The difference in electrophoresis behaviour correlates with their elution pattern on ion-exchange chromatography. IgG_2 is eluted with low ionic strength and this is the basis of the separation of these subclasses (15, 16,17).

One problem associated with the preparation of ruminant immunoglobulins by ion-exchange chromatography is the isolation of pure IgG_1, free of IgG_2. In contrast to IgG_1, the preparation of IgG_2 by gel filtration and ion- exchange chromatography is more satisfactory (15, 17). Protein A and protein G (gamma bind-G) have also been used to purify IgG. The binding of IgG to protein A and protein G has been reported for a number of species (11, 18, 19).

In the present study, it was attempted to purify ovine IgG from G-200, ion-exchange and Gamma Bind-G prepack. The aim of the work was not to purify subclasses of IgG. The aim was to isolate the heavy chain of IgG. G-200 gel filtration, ion-exchange and Gamma Bind-G affinity chromatography resulted in a preparation in which ovine IgG was present. This was confirmed by immunoelectrophoresis and Western Immunoblotting. Only IgG bands were seen on immunoelectrophoresis using rabbit anti-ovine whole serum. On Western Immunoblotting, a faint band on the region of 68,62 and 40 were detected when stained when stained with Amido black staining. Two strong staining were also detected in the region of 22 and 54kD which are identical to light chain and heavy chain of IgG (5, 15). The faint bands seen in the region of 68 and 62 kDs might be associated with contamination of albumin and IgA. In order to avoid this contamination the final preparation from Gamma Bind-G was run on SDS-Page and the heavy chain of IgG in the region of 52-56kd were electroeluted from four gels in total. However, heavy chain of IgG can also be separated using proteolytic enzymes (20). Consequently, electroelution product from SDS-Page was used to immunize rabbit.

The aim of the preparation of antiserum to IgG heavy chain was to achive specificity in order to avoid the cross-reactivity with other immunoglobulins. The antiserum produced in rabbit was absorbed with purified ovine IgG for rendering them monospecific although the eluent from Gamma-Bind G prepack nad revealed only IgG lines on immunoelectrophoresis. The absorption was performed in case there is any light chain activity of the antiserum. The antiserum was analysed on Western Immunoblotting which is highly sensitive technique. This antiserum revealed one single band in G-200 fraction of sheep serum and Gamma Bind-G eluent, on immunoblotting in the region of 54kD which is identical to IgG heavy chain (5, 15, 17, 21). There was no staining in the region of ovine IgM and IgA (21).

This study shows that the proteins isolated from sheep serum using gel filtration, ion-exchange chromatography, Gamma Bind-G and SDS-Page gel electroelution are ovine IgG heavy chain. The antisera prepared to this preparation indicates that molecular weight of its heavy chain appears to be 54kD.

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REFERENCES

1. Kickofen B, Hammer DH, Scheel D: Isolation and characterization of G type immunoglobulin from bovine serum and colostrum, Hoppa-Seyler's J Physiol Chem 349:1755 (1968).
2. Sullivan AL, Prendergast RA, Antunes LJ, Silverstein AM, Tomasi TB: Characterization of the serum and secretory immune system of the cow and sheep J Immunol 103:334 (1969).
3. Butler JE: Physicohemical and immunochemical studies of bovine IgA and glycoprotein, Biochem Biophys Acta 251:435 (1971).
4. Butler JE: Immunoglobulins of the mammary secretions, In B.L. Larson and V. Smith (Eds), Press, New York. pp.217-255 (1974)
5. Butler JE: Bovine immunoglobulins, an augmented review, Vet Immunol and Immunopathol 4:43 (1983).
6. Curtain CC: A new immunoglobulin sub-class in the sheep. Immunology 16:373 (1968).
7. Esteves MB, Santanna OA, Dos-Santos Annes VC, Binaghi RA: Characterization and properties ot an anaphylactic antibody in sheep, J Immunol 112:722 (1974).
8. Yasmeen D: Antigen-specific cytophilic activity of sheep IgG_1 and IgG2 antibodies, Ajebak 59:297 (1981).
9. Grant CK, Adams EP, Nass M: Appearrance of cytolytic antibodies in sheep ymph following immunization with tumour cells, Aust J Exp Biol Med Sci 53:381 (1975).
10. Nielsen K, Duncan JR, Stemshorn B: Preparation of heavy chain specific antisera to bovine IgA, IgM, IgG_1 and IgG_2 . Vet Immunol Immunopathol 9:361 (1985).
11. Yılmaz H, Roe Jm, Morgan KL: Isolation and preparation of antisera to ovine IgE, Int Arch Allergy and Immunol 101:369 (1993).
12. Laemli UK: Cleavage of structural protein during the assembly of the head of bacteriophage T4, Nature 227:680 (1970).
13. Towbin H, Theophil S, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets, procedure and some applications, Proc Natl Acad Sci 76(9): 4350 (1979).
14. Cripps AW, Husband AJ, Scicchitano R, Sheldrake RF: Quantitaion of sheep IgG1, IgG2, IgA, IgM and albumin by radioimmunoassay, Vet Immunol Immunopathol 8:137 (1985).
15. Beh KJ: Production and characterization of monoclonal antibodies spesific for IgG subclasses, IgG1 or IgG2. Vet Immunol Immunopathol 14:187 (1987).
16. Duncan JR, Wilkie BN, Hiestand F, Winter AJ: The serum and secretory immunoglobulins of cattle: Charecterization and quantitaion. J Immunol 108:965 (1972).
17. Pervez K: The immune system of the water buffalo. PhD thesis. University of Bristol Dept. of. Veterinary Medicine. U.K. (1992).
18. Peng ZK, Simons FE, Becker AB: Differential binding properties of protein A and protein G for dog immunoglobulins, J Immunol Methods 145(1-2):255 (1991).
19. Langone JJ: Protein A of Staphylococcus aureus and related immunoglobulin receptors produced by streptococci and pneumococci, Adv Immunol 32:157 (1982).
20. Butler JE, Kennedy N: The differential enzyme susceptibility of bovine IgG1 and IgG2 to pepsin and papain. Biochim Biophys Acta 535:125 (1978).
21. Beh KJ: monoclonal antibodies against sheep immunoglobulin light chain IgM and IgA, Vet Immunol Immunopathol 18:19 (1988).