Acta Veterinaria (Beograd), Vol. 56, No. 2-3, 111-120, 2006.

DOI: 10.2298/AVB0603111S UDK 619:612.017:597.851

DATA ON THE ISOLATION OF IMMUNOGLOBULIN FROM THE SERUM OF THE GREEN FROG (*Rana esculenta*)

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(Received 17. December 2005)

Even though the green frog (Rana esculenta) is often used as an experimental model for further studies of physiological laws, little is still known about its serum proteins and their role in immunity. Its serum proteins have been studied quite extensively, as when they are taken up into the organism of another animal they represent antigens themselves.

In this work, an attempt was made to isolate some frog serum proteins and to investigate the electrophoretic qualities of the isolated components (electrophoresis on agar gel and immunoelectrophoresis).

IgG was isolated using the same procedure applied for human sera and one of the components found in the beta globulin zone was isolated from frog serum by the same procedure. Immunoelectrophoretic analysis, carried out with the full antiserum of a rabbit, obtained by immunisation of the rabbit with the frog serum, showed that the isolated component was pure.

The obtained results confirm the fact that there are no slow gamma globulins in the frog serum and point out that this procedure, used for isolating human IgG class, is also suitable for isolating one protein component from the serum of the frog, which has the electrophoretic speed of beta globulin and which may represent one category of frog immunoglobulins.

Key words: IgG, immune response, phylogenetic antibody structure.

INTRODUCTION

It is known that there are five classes of immunoglobulins in the serum of man, whose basic biological functions are that of antibodies. Comparative studies of the immunoglobulin systems of vertebrates have shown that not all vertebrates have the same number of immunoglobulin classes (Marchalonis *et al*., 2006), and it was confirmed that lower vertebrates (sharks) have only one class of

immunoglobulin (Hohman *et al*., 1995; Bernstein *et al*., 1996; Bernstein *et al*., 1996; Shen *et al*., 1996; Marchalonis *et al*., 1998; Marchalonis *et al*., 2001; Adelman *et al*., 2004). Research has shown that certain parts of the structures of immunoglobulin molecules of lower vertebrates and man are nevertheless similar (Nikolić *et al,,* 1969; Heremans, 1970).

Knowledge of the evolution of the structure and function of antibodies of vertebrates is of great significance because of the better theoretical understanding of the evolution of immunity itself, and, on the other hand, in the case need arises for specific antibody types, one should have the practical know-how of when and by which method they can best be isolated. There is ample information about the evolution of antibodies, from lower vertebrates to mammals. A significant contribution to the understanding of the evolution of the structure of these molecules was reported domestically in 1969 (Nikolić *et al*., 1969), before major works in this field were conducted by the great medical centres (Heremans, 1970; Diener and Marchalonis, 1970). There is data showing that some amphibian representatives were the first vertebrates in whose serums two classes of immunoglobulins were discovered: IgG and IgM classes (Marchalonis and Edelman, 1966).

When it comes to the green frog (*Rana esculenta*), it was established that it has a large genome and that there are seasonal variations in its total serum proteinaemia (Perišić and Stošić, 1972). Through a comparative study of the serum immunoglobulin systems of vertebrates, it has been shown that certain parts of the molecules of immunoglobulins of lower vertebrates and man are similar. In more recent works, which deal specifically with the evolution of immunoglobulins (Marchalonis *et al*., 2002), there is talk of the partial resemblance of the structure of immunoglobulin heavy chain genes of jawed vertebrates (Schluter *et al*., 1997), as well as of the cellular receptors of human T lymphocytes and their similarity to the receptors in mice (Liang *et al*., 1998). The possibilities of cross-examination of autoantibodies in human heart transplantation (Marchalonis *et al*., 1996), of autoantibodies on T cellular receptors in HIV-infected individuals (Marchalonis *et al*., 1997), and of human immunoglobular genetic organisation (Marchalonis and Schluter, 1998) have been studied in detal. These studies confirm the supposition that parts of the structure of immunoglobulins of lower and higher vertebrates are comparable. According to the results of these works, it can be assumed that procedures, which are used for isolating immunoglobulins from the serum of mammals and man, can be applied to isolate immunoglobulins of other vertebrates, as well. In addition, the immunisation of lower vertebrates with serum proteins of higher vertebrates produces antibodies, with the help of which one would be able to register the similarity of proteins of animal species which are not philogenetically distant.

Because *Rana esculenta* is often used in experiments, as a representative of the family of ranids, and is easily obtainable, serves in the substantiation of various biological laws. In view of the current knowledge about its immunoglobulins there are still great uncertanties about how many categories of immunoglobulins *Rana esculenta* has and to determine a way in which we could

isolate a protein component from the serum of the immunized animal, which would correspond to a certain category of immunoglobulin in man.

MATERIAL AND METHODS

Protein components were isolated from 55 green frog serums of both sexes during two seasons: (n=29) in spring and (n=26) in autumn. The initial serum volumes from which the preparations were isolated were from 4.5 mL to 17 mL.

1. Determination of the total frog serum protein concentration and of the isolated frog serum protein fraction was carried out using the turbidimetric method (Mejbaum-Katzenellenbogen, 1955).

2. Preparation of the serum proteins was carried out using the rivanol/ ammonium sulphate procedure, in the same way as applied in isolating one part of class G immunoglobulin in man and in some mammals (Nikolić *et al*., 1969). We obtained one protein component, an isolated green frog serum protein fraction, on which determination of protein concentration by turbidimetric method, according to the procedure described by Mejbaum-Katzenellenbogen, was also carried out.

3. The isolated green frog serum protein fraction was analysed through:

a) electrophoresis on filter paper;

b) high-voltage electrophoresis on agar gel (Wieme, 1965);

c) immunoelectrophoresis (Backhausz, 1967), whose first stage also involved electrophoresis on agar gel, according to Wieme, was carried out using rabbit-anti-frog antiserum (Perišić and Stošić, 1972).

4. In order to obtain rabbit-anti-frog antiserum, immunisation of the rabbit (*Oryctolagus cuniculus*) was carried out. Each rabbit received 30 mg of frog serum protein via a single subcutaneous injection, with complete *Freund*'s adjuvant divided into two equal subcutaneously given doses with an interval of one week between injections. Two weeks after the final injection, the rabbits were given 4.4 mg of frog serum protein absorbed subcutaneously on allum gel. The rabbit serum, obtained from the blood (the animals were bled by puncturing the *v. angularis auriculae*), three weeks after the last dose of antigen was administered. This represented the rabbit-anti-frog antiserum and was subsequently used for immunoelectrophoretic analysis (Perišić and Stošić, 1972).

RESULTS

During spring, the total frog serum protein concentration is relatively low and ranges from 8 to 22.5 g/L, while the average value for this group of animals amounts to 17.9 g/L. The average value of the total frog serum protein concentration for this species of frog during the autumn is twice as large, and totals 39.7 g/L (Table 1).

Investigation of the frog serum protein fraction by electrophoresis on filter paper obtained the lowest number of protein fractions i.e. only five. Out of which the fastest fraction was the most intensely coloured, so it is possible that,

according to these characteristics, it corresponds to human serum albumins (Figure 1).

Table 1. Concentrations of total green frog serum proteins and concentration of green frog serum protein fraction from two series of animals, in spring and autumn

Frog Series	Season	n	Volume (ml)		Protein Concentration (g/l)	
			Serum	Preparation	Serum	Preparation
I	Spring	11	7.2	0.75	17.9	3.42
II	Autumn	9	17	0.5	39.7	0.74

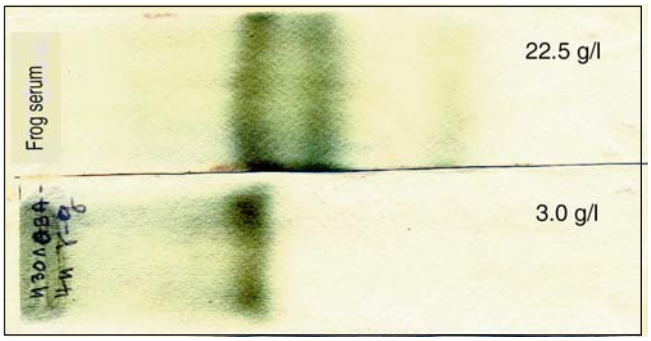

Figure 1. Investigation of frog serum protein fraction by electrophoresis on filtration paper: above – full frog serum; below – frog serum protein immunoglobulin fraction

On the electrophoresogram carried out via high-voltage electrophoresis on agar gel (Figure 2) more protein fractions (eight) were isolated. In Figure 2, full frog serum is compared with the serum of a healthy man.

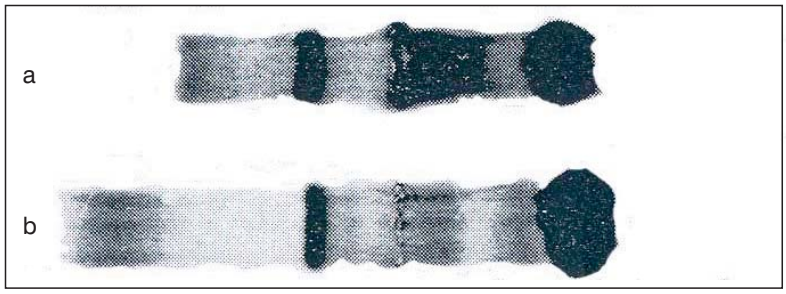

Figure 2. High-voltage electrophoresogram on agar gel
a. Full green frog serum
b. Normal human serum

It is noticeable that the frog serum proteins are relatively heterogeneous. This is, above all, related to most of the electrophoretic fractions, which are not only heterogeneous but are also slower than human serum albumins. In this zone, which corresponds' to alpha 2 and beta 1 globulin region of human serum proteins, are the frog serum fractions that are sharply separated and which are, judging from the intensity of the colour, of high concentration. Not a single fraction in this zone corresponds to human gamma globulin.

The frog serum protein fraction was isolated from the frog serum in the following way (Figure 3): 3.5 ml 4 g/L of rivanol was added to 1 mL of serum; after centrifugation, the dye was removed from the supernatant by animal charcoal and after adjustment to pH 7, ammonium sulphate was added *in substantiam* (264 mg to 1 mL of supernatant). The sediment was dissolved in distilled water, 2% kaolin was added (20 mg to 1 mL). After centrifugation the sediment was discarded and the supernatant was further analysed. Just a single protein component remained in the supernatant, which corresponds to one part of IgG class of mammals.

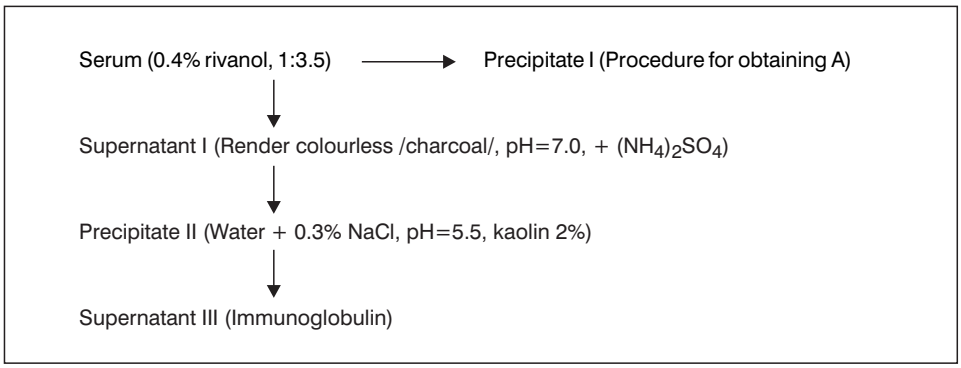

Figure 3. Procedure for isolating human serum immunoglobulin of IgG class

The isolated frog serum protein fraction had the following characteristics: high-voltage electrophoresis on agar gel (Figure 4) indicated one fraction in the slowest zone of frog serum proteins, which corresponds to the beta 2 globulin region of human proteins. By electrophoretic analysis of this type no other frog serum protein fractions could be seen in the preparation.

Because immunoelectrophoretic analysis is a delicate procedure, we applied it to check out the cleanliness and immunochemical homogeneity of the isolated preparation. Analysis showed (Figure 5) that within the beta 2 globulin zone only one sharply defined line of precipitation was present. From this line separated two lines of significantly weaker intensity and sharpness towards the cathode: one from the upper and the other from the lower side of the main line of precipitation. This indicates that some green frog immunoglobulins were obtained and the isolated preparation was immunochemically heterogeneous.

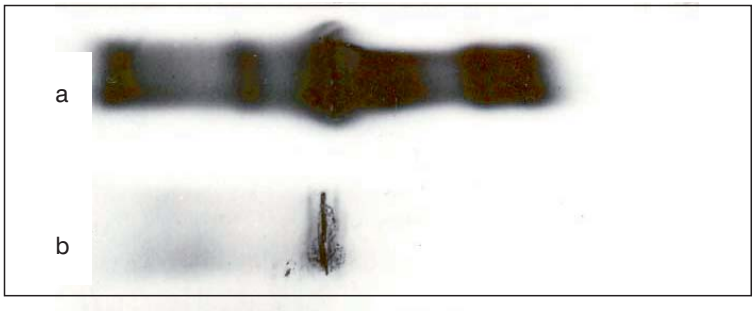

Figure 4. High-voltage electrophoresis on agar gel according to Wieme
a. Full frog serum proteins
b. Isolated frog serum immunoglobulin fraction

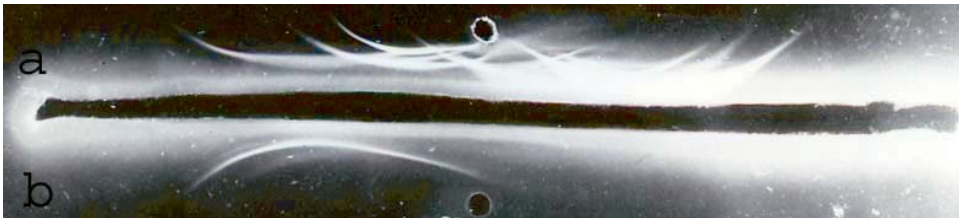

Figure 5. Immunoelectrophoretic analysis carried out on the rabbit-anti-frog antiserum
a. Full green frog serum
b. Frog serum immunoglobulin fraction
Anode is at the right.

To obtain such a clearly defined line of precipitation, the concentration of the preparation had to be high enough, because a preparation of 0.74 g/L did not provide a visible line of precipitation. Preparations of 3.00 g/L and 3.84 g/L provided clearly defined lines. The protein concentrations in the frog serum protein fraction, and in the frog serum immunoglobulin fraction, isolated according to the procedure for mammalian immunoglobulins were different in spring and in autumn (Table 1) and stood opposite to the concentration of total frog serum proteins. Table 1 illustrates that the concentration of proteins in the frog serum protein fraction was 4-5 times greater in spring than the total frog serum protein concentration in autumn. That, in effect, means that in spring we needed a considerably smaller quantity of serum in order to obtain frog serum immunoglobulins of optimal concentration for immunoelectrophoretic analysis.

DISCUSSION

Literature provides a great deal of information about serum proteins, structures, and antibody evolution in vertebrates: fish (sharks), amphibians,

reptiles, and birds. The impression is that in the last few years the main findings have been formulated in many works of *Marchalonis* and his collaborators (Schluter *et al*., 1997; Dehghanpisheh *et al*.; 1995, Liang *et al*., 1996).

The results of our research indicate that the obtained isolated protein fraction could represent the frog serum immunoglobulin of *Rana esculenta* in this region of Europe. It was established earlier that a category of serum albumins, of some representatives of the *Ranids*, moves with the same speed of beta globulin (Marchalonis *et al*., 1966) during electrophoresis. Until now no research of this type has been carried out *Rana esculenta*. The data we acquired do not show that, if the proteins we isolated, represent immunoglobulins, which are the specific antibodies of the immunised frog.

On the basis of the investigation we carried out, it can be stated that there are significant seasonal variations in the concentration of total serum frog proteins and that the individual serum fractions are heterogeneous. Serum fractions, which represent antigen capabilities in relation to the rabbit of other European territories, were described by Ramsland (2001), who gave an explanation for the immunogenetic determination of the structure of antibody-combinatorial loci in the evolution of species.

Immunoelectrophoretic analysis of the frog *Rana esculenta* serum proteins carried out under certain conditions (with full rabbit antiserum obtained through the immunisation of rabbits with green frog serum, both animals being from local habitats) showed that the isolated serum protein component was pure and immunochemically heterogeneous. Regard this matter there is scarse published data (Nikolić *et al*., 1969).

The obtained results confirm the fact that in the serum of frogs (*Amphiba anura*) from different territories, there is no slow gamma globulin fraction and point out that the procedure, which is used for isolating human IgG class, is also suitable for isolating one protein component from the serum of the green frog, which has the electrophoretic speed of beta globulin and which could represent one category of frog immunoglobulins. Research prior to ours on the frog *Rana catesbiana* (Marchalonis *et al*., 1966), and also supplemented by our data on the frog *Rana esculenta*, also represents a contribution to the understanding of origin, structure, and function of antibodies in philogenesis (Marchalonis *et al*., 1996, Marchalonis *et al*., 2001).

CONCLUSION

Our attempt at fractioning serum proteins of the green frog *Rana esculenta* indicates:

1. By the procedure used for isolating one part of G class immunoglobulin of man and some other mammals, an electrophoretically clean precipitate from the serum of the green frog (*Rana esculenta*) can be separated, which corresponds electrophoretically to beta 2 globulin in man.

2. Isolated beta 2 globulin frog serum protein fraction is an immunogen for the rabbit and is immunochemically heterogeneous.

3. When there is a need to isolate a category of protein from the green frog serum, which is supposed to correspond to the green frog immunoglobulin, a greater quantity of preparation will be obtained if it is isolated from the serum of animals in spring.

4. We have proven that the green frog *Rana esculenta* has a seasonal variation in its total serum protein concentration, i.e. total proteinaemia is significantly higher in autumn. The proteins from the serum of this type of anura-amphibians are immunogens for the domestic rabbit, thus they create classes of antibodies of different high-voltage electrophoretic mobility.

ACKNOWLEDGEMENTS:
The authors wish to thank Professor Dr. Nenad Milić, Ph.D., for his help and suggestions in the course of this work, and Professor Maja Marković, Ph.D., who provided valuable assistance and support to this research.

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REFERENCES

1. *Adelman MK, Schluter SF, Marchalonis JJ*, 2004, The natural antibody repertoire of sharks and humans recognizes the potential universe of antigens, *Protein J*, 23, 103-18.
2. *Backhausz R*, 1967, *Immunodiffusion und Immunoelektrophorese*, Budapest: Akademia Kiado, 355.
3. *Bernstein RM, Schluter SF, Bernstein H, Marchalonis JJ*, 1996, Primordial emergence of the recombination activating gene 1 (RAG1): sequence of the complete shark gene indicates homology to microbial integrases, *Proc Natl Acad Sci USA.* 93, 9454-9.
4. *Berstein RM, Schluter SF, Shen S, Marchalonis JJ*, 1996, A new high molecular weight immunoglobulin class from the carcharhine shark:implications for the properties of the primordial immunoglobulin, *Proc Natl Acad Sci USA*, 93, 3289-93.
5. *Dehghanpisheh K, Huang D, Schluter SF, Watson RR, Marchalonis JJ*, 1995, Production of IgG autoantibodies to TCRs in mice infected with the retrovirus LP-BM5, *Int Immunol*, 7, 31-6.
6. *Diener E, Marchalonis J*, 1970, Cellular and Humoral Aspects of the primary immune response of the toad, Buffo marinus, *Immunol*, 18, 279-93.
7. *Heremans* J, 1970, Molecular variation in proteins. *Prot Biol-Fluids*, 17, 3-20.
8. *Hohman VS, Schluter SF, Marchalonis JJ*, 1995, Diversity of Ig light chain clusters in the sandbar shark (Carcharhinus plumbeus), *J Immunol,* 155, 3922-8.
9. *Liang B, Marchalonis JJ, Zhang Z, Watson RR*, 1996, Effects of vaccination against different T cell receptors on maintenance of immune function during murine retrovirus infection, *Cell Immunol*, 1996 172, 126-34.
10. *Liang B*, *Zhang Z*, *Inserra P*, *Jiang S*, *Lee J*, *Garza A*, et al., 1998, Injection of T-cell receptor peptide reduces immunosenescence in aged C57BL/6 mice, *Immunology*, 93, 462-8.
11. *Marchalonis JJ, Adelman MK, Robey IF, Schluter SF, Edmundson AB*, 2001, Exquisite specificity and peptide epitope recognition promiscuity, properties shared by antibodies from sharks to humans, *J Mol Recognit*, 14, 110-21.
12. *Marchalonis JJ*, *Adelman MK*, *Schluter SF*, *Ramsland PA*, 2006, The antibody repertoire in evolution: Chance, selection, and continuity, *Dev Comp Immunol*, 30, 223-47.

13. *Marchalonis JJ, Adelman MK, Zeitler BJ, Sarazin PM, Jaqua PM, Schluter SF,* 2001, Evolutionary factors in the emergence of the combinatorial germline antibody repertoire, *Adv Exp Med Biol*, 484,13-30.
14. *Marchalonis JJ, Ampel NM, Schluter SF, Garza A, Lake DF, Galgiani JN, et al.,* 1997, Analysis of autoantibodies to T-cell receptors among HIV-infected individuals: epitope analysis and time course, *Clin Immunol Immunopathol*, 82, 174-89.
15. *Marchalonis JJ, Bernstein RM, Shen SX, Schluter SF*, 1996, Emergence of the immunoglobulin family: conservation in protein sequence and plasticity in gene organization, *Glycobiology*. 6, 657-63.
16. *Marchalonis J, Edelman GM*, 1966, Immunoglobulins in the primary immune response of the bullfrog, *Rana catesbiana*, *J Exp Med*, 124, 901-13.
17. *Marchalonis JJ, Jensen I, Schluter SF*, 2002, Structural, antigenic and evolutionary analyses of immunoglobulins and T cell receptors, *J Mol Recognit*, 15, 260-71.
18. *Marchalonis JJ, Kaymaz H, Schluter SF, Lake DF, Landsperger WJ, Suciu-Foca N*, 1996, Autoantibodies to T-cell receptor beta chains in human heart transplantation: epitope and spectrotype analyses and kinetics of response, *Exp Clin Immunogenet*, 13, 181-91.
19. *Marchalonis JJ, Schluter SF*, 1998, A stochastic model for the rapid emergence of specific vertebrate immunity incorporating horizontal transfer of systems enabling duplication and combinational diversification, *J Theor Biol*, 193, 429-44.
20. *Marchalonis JJ, Schluter SF, Bernstein RM, Hohman VS*, 1998, Antibodies of sharks: revolution and evolution, *Immunol Rev*, 166, 103-22.
21. *Mejbaum-Katzellenbogen W*, 1955, *Acta Bioch, Polonica*, 2, 279.
22. *Nikolić V, Nikolić B, Vukotić M*, 1969, Savremena shvatanja Gama globulinskog
sistema telesnih tečnosti, *Bilten transfuzije*, Suppl. 25, 1-81.
23. *Perišić M*, *Stošić S*, 1972, Serumski proteini žabe u svetlosti genske osnove sinteze proteina, *Medicinski podmladak*, 9-16.
24. *Ramsland PA, Kaushik A, Marchalonis JJ, Edmundson AB*, 2001, Incorporation of long CDR3s into V domains: implications for the structural evolution of the antibody-combining site, *Exp Clin Immunogenet*, 18, 176-98.
25. *Schluter SF*, *Adelman MK*, *Taneja V, David C, Yocum DE, Marchalonis JJ*, 2003, Natural autoantibodies to TCR public idiotopes: potential roles in immunomodulation, *Cell Mol Biol (Noisy-le-grand)*, 49, 193-207.
26. *Schluter SF, Bernstein RM, Marchalonis JJ*, 1997, Molecular origins and evolution of immunoglobulin heavy-chain genes of jawed vertebrates, *Immunol Today*, 18, 543-9.
27. *Shen SX, Bernstein RM, Schluter SF, Marchalonis JJ*, 1996, Heavy-chain variable regions in carcharhine sharks: development of a comprehensive model for the evolution of VH domains among the gnathanstomes, *Immunol Cell Biol*, 74, 357-64.
28. *Wieme RK*. 1965, *Agar gel electrophoresis*, New York: Elsevier, Public Comp.

Acta Veterinaria (Beograd), Vol. 56. No. 2-3, 111-120, 2006.
Stošić Svetlana *et al.*: Data on the isolation of immunoglobulin from the serum of the green frog (*Rana esculenta*)

PODACI O IZOLOVANJU IMUNOGLOBULINA IZ SERUMA ZELENE ŽABE (*Rana esculenta*)

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SADRŽAJ

Zelena žaba (*Rana esculenta*) se koristi kao eksperimentalna životinja za dokazivanje mnogih fizioloških zakona, ali se o proteinima seruma ove vrste i njihovoj ulozi u imunosti još uvek malo zna. U ovom radu je učinjen pokušaj da se postupkom, koji se primenjuje za izolovanje proteina seruma čoveka i drugih sisara, izoluju neki od proteina seruma žabe i da se ispitaju elektroforetske osobine izolovanih komponenti elektroforezom na gelu agara i imunoelektroforezom.

Postupkom koji je primenjen u našem radu kod čoveka se izoluje IgG, a iz seruma žabe je istim postupkom izolovana jedna komponenta koja se nalazi u zoni beta globulina. Imunoelektroforetska analiza, izvedena punim antiserumom kunića dobijenog imunizacijom kunića serumom žabe, pokazala je da je izolovana komponenta čista.

Dobijeni rezultati potvrđuju poznatu činjenicu da u serumu žabe nema sporih gama globulina i ukazuju da je postupak, koji se primenjuje za izolovanje ljudskih IgG pogodan i za izolovanje jedne komponente iz seruma žabe, koja ima brzinu beta globulina i koja može predstavljati kategoriju imunoglobulina žabe.