Lysozyme – bioactive component of the hyperimmune egg PC2: characterization, purification and antimicrobial activity

Lizozimul – component bioactiv al oului hiperimun PC2: caracterizare, purificare și activitate antimicrobiană

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Abstract

Lysozyme obtained from PC2 hyperimmune egg whites, originating from hens immunized with a complex of bacterial and fungal antigens, was purified by ion exchange chromatography on Amberlite FPC 3500 resin. The purity of lysozyme was analysed by polyacrylamide gel electrophoresis in denaturing system (SDS-PAGE). Based on the migration pattern of the molecular marker, the presence of a single band with a molecular mass of 14.1 kDa was found. The agar gel immunodiffusion test (AGID) showed the presence of lysozyme which was tested in dilutions from 1/2 to 1/32 as compared to standard lysozyme (Sigma) and that obtained from eggs from chickens free of specific germs (SPF) and conventional (CV). The immunological identity between the standard lysozyme and the PC2 lysozyme was established by the AGID test. The PC2 lysozyme showed agglutination reactions with flaky clumps in the presence of *Micrococcus lysodeikticus* cultures and with granular clumps in the presence of *Staphylococcus aureus* cultures. The antimicrobial activity of lysozyme was intense against Gram-positive bacteria and less intense against Gram-negatives. The concentration of lysozyme was assessed by the lysoplate method against a *Micrococcus lysodeikticus* culture. Mean values ($\bar{x}$ ± ds) of purified lysozyme were obtained, ranging between 12.5 mg/mL and 14,0 mg/mL. Lythic units (mg/mL) were determined in immunologically active products containing lysozyme (gels, emulsions, solutions, powders). The mean values ($\bar{x}$ ± ds) were between 49.0±0.34 and 60.5±0.84. This study suggests that PC2 lysozyme exhibits an immunologic activity with an important role in the mechanisms of non-specific defence of organisms.

Rezumat

Lizozimul obținut din albuș de ouă hiperimune PC2, provenind de la găini imunizate cu un complex de antigene bacteriene și fungice, a fost purificat prin cromatografie de schimb ionic pe Amberlite FPC 3500. Puritatea lizozimului a fost analizată prin electroforeză în gel de poliacrilamidă în sistem denaturant (SDS-PAGE). Pe baza modelului de migrare a marker-ului molecular s-a constatat prezența unei singure benzi cu masa moleculară de 14,1 kDa. Prin testul de imunodifuzie în gel de agar (IDGA) s-a evidențiat prezența lizozimului care s-a testat în diluții de la 1/2 la 1/32 comparativ cu lizozimul standard (Sigma) și cu cel obținut din ouă de la găini libere de germeni specifici (SPF) și convenționale (CV). S-a stabilit identitatea imunologică între lizozimul standard și lizozimul PC2 prin testul IDGA. Lizozimul PC2 a evidențiat reacții de aglutinare cu aspect de flocoane față de cultura de *Micrococcus lysodeikticus* și cu aspect granular față de cultura de *Staphylococcus aureus*. Activitatea antimicrobiană a lizozimului a fost intensă asupra bacteriilor Gram pozitive și mai puțin intensă asupra celor Gram negative. Concentrația lizozimului s-a pus în evidență prin testul de determinare a lizei în placă față de cultura de *Micrococcus lysodeikticus*. S-au obținut valori medii ale lizozimului purificat cuprinse între 12,5 mg/ml și 14 mg/ml. S-au determinat unitățile litice (mg/ml) în produse imunologic active care conțin lizozim (geluri, emulsii, soluții, pulberi). Valorile medii ($\bar{x}$±ds) sunt cuprinse între 49,0±0,34 și 60,5±0,84. Acest studiu sugerează că lizozimul PC2 prezintă activitate imunologică având un rol important în mecanismele de apărare nespecifică a organismelor.

Introduction

Lysozyme is a glycoside hydrolase with an important role in the antibacterial defence mechanism. Its main action is the lysis of polysaccharides in the bacterial cell wall by cleaving the β-1,4 glycosidic bond between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG). For these reasons it is also called N-acetylmuramide glycanhydrolase or muramidase. Its bacteriostatic, bacteriolytic and bactericidal activity has been demonstrated, especially against Gram-positive bacteria, among them being a large number of pathogens present in food [7].

Egg white derived lysozyme has special properties and is considered a good preservative for food [5, 11]. The modified lysozyme molecule is used in many fields, for example to prevent infections, acting as a natural antibiotic or as a nonspecific stimulator of the immune system [9].

In the pharmaceutical industry, the use of lysozyme in various formulations (creams, eye drops, gels, etc.) has expanded [5]. Chicken egg white is the most important source of lysozyme (3.5%). The egg white lysozyme is a polypeptide with 129 amino acids having a molecular mass of 14 kDa [6]. The isoelectric point is between 10-11. Lysozyme was the first sequenced protein, whose three-dimensional structure was completely analysed. It is a molecule consisting of two domains linked by an α-helix [2].

Lysozyme is present in secretions, all body fluids and tissues of the human and animal body. It has also been isolated from plants, bacteria and bacteriophages being a very stable enzyme [8]. It is a key factor in the egg's natural defence against bacterial aggression. The lytic action of lysozyme on the cell wall of *Micrococcus lysodeikticus* is one of the methods used to evaluate enzymatic activity [7]. Due to the use of the enzyme in various fields, its purification studies have become essential. Various methods such as crystallization and precipitation [4], membrane filtration [6], affinity chromatography [11], ion exchange chromatography [1] and ultrafiltration [5] are used in lysozyme purification processes. The research aimed to purify, characterize and determine the concentration of lysozyme in egg white hyperimmune PC2 and immunologically active products containing this protein.

1. MATERIALS AND METHODS

The study was conducted in the Research & Development Department of Romvac Company S.A.

1.1. PC2 hyperimmune eggs

Were obtained according to the methodology described by Chiurciu et al. (2014) and originate from clinically healthy Rhode Island Red hens, 20–23 weeks old, immunized with bacterial and fungal antigens [3,10].

1.2. Eggs from SPF (specific pathogen free) and conventional (CV) chickens

Were obtained from the company's farms.

1.3. The *Micrococcus lysodeikticus*

ATCC 4698 (Sigma Aldrich) strain was cultivated on BHI medium enriched with 8% foetal serum. The obtained culture was washed three times with phosphate buffer pH = 6.2 by centrifugation at 3400 x g for 20 minutes. The optical density of the suspension was determined on a SpectraMax 190 spectrophotometer (Molecular Devices LLC) and adjusted to an OD value of 2.4.

1.4. Lysozyme from chicken egg white lyophilized powder (Sigma Aldrich)

Was used as a standard in all the performed experiments.

1.5. Anti-lysozyme serum

Was prepared "in house" by immunizing rabbits with standard lysozyme. Three inoculations were performed with 5 mg/mL emulsified lysozyme in Montanide ISA 70 adjuvant (SEPPIC). The first inoculation was made intradermally with 2 ml antigen administered in 3-4 points on the sides of the body. The second inoculation was performed

21 days after the first administration by the same route and the same dose similar to the first inoculation. The third was done 14 days after the second administration.

1.6. Separation of lysozyme from PC2 hyperimmune egg white by ion exchange chromatography.

Lysozyme was prepared according to the method of Abeyrathne et al. (2014) with some modifications. Purification was performed by cation exchange chromatography using Amberlite FPC 3500 (Acros Organics) as cation exchange resin. The egg white was diluted with an equal volume of deionized water (1:1) calculating 0.5 g Amberlite (styrene-divinylbenzene, total exchange capacity >2.6 mEq/g) per 10 ml of egg white; the mixture was homogenized for 12 h at 4 °C using a low speed magnetic stirrer (Stuart US152). The solution was centrifuged at 3400 x g for 20 minutes at 4 °C; the amberlite was collected and washed several times with deionized water and then with glycine buffer - 0.1 M NaOH, pH = 9.3. The lysozyme was eluted with glycine buffer - 0.1 M NaOH, pH = 9.3 containing 0.5 M NaCl. The eluate was desalted by ultrafiltration on a 30 kDa cassette (Millipore) and lyophilized using 150 DKS Zirbus Sublimator.

1.7. The agar gel immunodiffusion test (AGID)

Was performed in 90 mm diameter Petri dishes using 1% Noble agar gel prepared in borate buffer pH = 8.6. Seven 6 mm diameter wells were punched out, one central and six peripherals. In the central well 40 µL anti-lysozyme serum were dispensed, and in the peripheral wells 40 µL of purified lysozyme undiluted and in binary dilutions from 1/2 to 1/2048. Standard lysozyme (20 mg/mL) was used as a control. The reactions were read after 24 hours by visualizing the precipitation lines.

1.8. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis was performed using the Laemmli method on an OmniPAGE Electroblotter (Cleaver Scientific Ltd.). Lysozyme samples were diluted to a final protein concentration of 2 mg/mL using Laemmli buffer with 2-mercaptoethanol and bromophenol blue (Sigma Aldrich).

After incubating the samples for 10 minutes at 96 ºC, 5 µL of each sample was added to the 10% migration gel and 4% concentration gel.

A protein VI marker (AppliChem), containing a mixture of 12 proteins with molecular weights of 10 to 245 kDa, was used. Electrophoresis was performed at 90 mV and 185 mA for 90 minutes; staining was performed with Comassie Brilliant Blue (Sigma Aldrich).

1.9. Rapid agglutination reaction (RAR).

The test was performed in glass plates with 12 wells of 16 mm in diameter and 1.5 mm depth (Marienfeld). The *Micrococcus lysodeikticus* culture was used as a positive control.

To perform the test the following were used: inactivated bacterial cultures (*Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Salmonella typhimurium*), standard lysozyme, as a positive control, and lysozyme from SPF egg whites, as negative control.

1.10. Lysoplate diffusion method

Six ml of *Micrococcus lysodeikticus* suspension was warmed to 56 °C and mixed with 6 ml of 2% Noble agar prepared in PBS pH = 6.2. The mixture was poured into 90 mm diameter Petri dishes and left at room temperature to solidify.

Five mm diameter wells were punched in the gel at a distance of 1 cm. The standard lysozyme was dispensed in the central well and the lysozyme samples under assessment in the lateral wells; the reaction was read after 24 hours. The diameters of the lysis areas were read in mm, using a digital calliper with electronic display (Digital Calliper). Lysozyme concentration values were assessed against a standard curve and expressed in mg/mL.

2. RESULTS AND DISCUSSIONS

Purification of PC2 lysozyme from hyperimmune egg whites, SPF and CV, was performed by ion exchange chromatography using Amberlite FPC 3500. Using this method, lysozyme can be obtained in considerable amounts and with a high degree of purity. The analysed samples are presented in Fig. 1.

By the SDS-PAGE technique, based on the migration pattern of the molecular marker, the presence of a single band was found, which demonstrates the purity of the tested lysozyme. Calculation of the molecular masses showed that the lysozyme obtained from egg whites laid by SPF, PC2 and CV hens is pure and has the same molecular mass (14.1 kDa).

The results are presented in Fig. 1, Table 1 and Fig. 2.

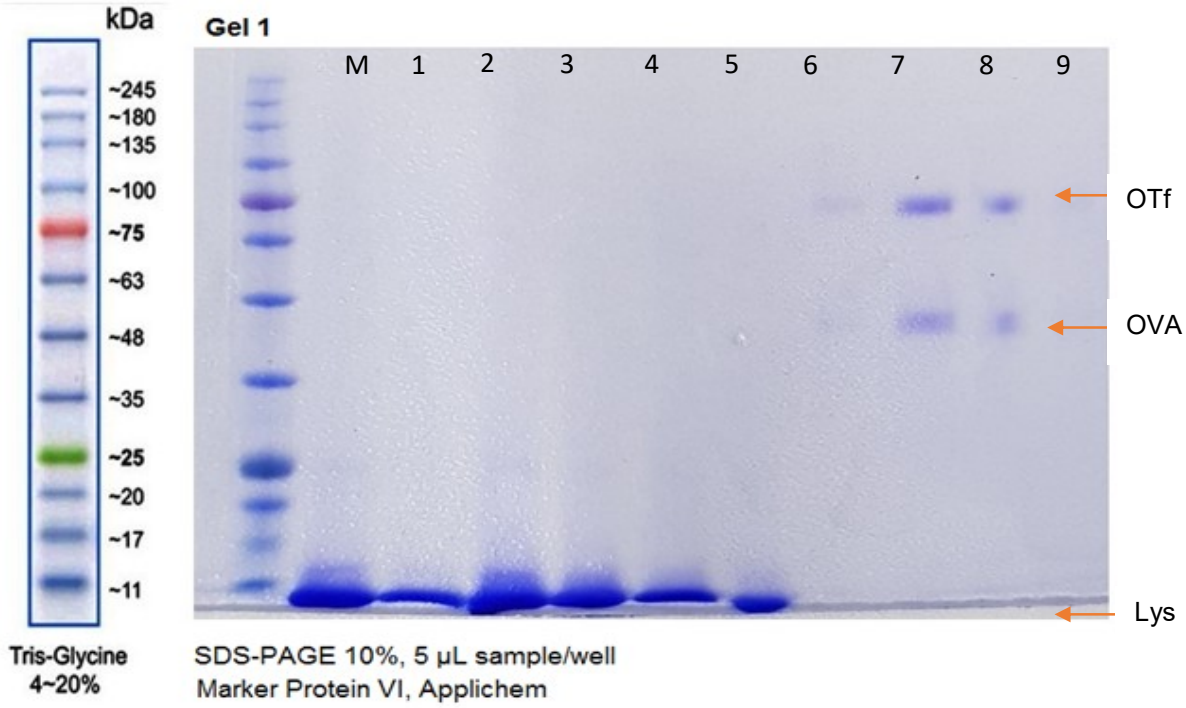

Fig. 1. SDS-PAGE - Lysozyme purity test (Lys) obtained by ion exchange chromatography (Amberlite FPC 3500) from PC2, CV and SPF egg whites: M – protein marker; 1 - standard lysozyme (20 mg/mL), dilution 1/20; 2 - SPF lysozyme (10 mg/mL), dilution 1/10; 3 - PC2 lysozyme lot I (10 mg/mL), dilution 1/10; 4 - PC2 lysozyme lot II (10 mg/mL), dilution 1/10; 5 - PC2 lysozyme lot III (10 mg/mL), dilution 1/10; 6 - CV lysozyme (10 mg/mL), dilution 1/10; 7,8,9 - ovotransferrin (OTf) and ovalbumin (OVA) fractions from PC2 egg white after lysozyme separation

Table 1.
Calculation of molecular weights (M) of lysozyme in PC2, CV and SPF egg whites

Gel 1	dp	Rf	Log M	MW	M (kDa)
1 lysozyme standard 20 mg/mL	62	0.9841	4.150967	14157	14.15
2 SPF lysozyme 10 mg/mL	62	0.9841	4.150967	14157	14.15
3 PC2 lysozyme lot I 10 mg/mL	47	0.7460	4.404502	25381	25.38
	62	0.9841	4.150967	14157	14.15
4 PC2 lysozyme lot II 10 mg/mL	62	0.9841	4.150967	14157	14.15
5 PC2 lysozyme lot III 10 mg/mL	62	0.9841	4.150967	14157	14.15
6 CV lysozyme 10 mg/mL	62	0.9841	4.150967	14157	14.15
7 OTf	20	0.3175	4.860866	72588	72.58
OVA	33	0.5238	4.641136	43766	43.76
8 OTf	20	0.3175	4.860866	72588	72.58
OVA	33	0.5238	4.641136	43766	43.76
9- OTf	20	0.3175	4.860866	72588	72.58
OVA	33	0.5238	4.641136	43766	43.76

After isolation of lysozyme from the egg white, the remained major fractions were: ovotransferrin (M = 72.58 kDa) and ovalbumin (M = 43.76 kDa). They are shown in Fig. 1 and Table 1 (fractions 7, 8 and 9) and can be separated sequentially using precipitation and heat treatment techniques.

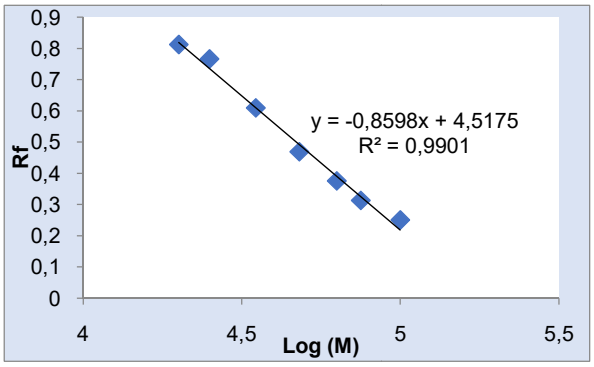

Fig. 2. Calculation of the molecular weights of the SPF, PC2 and CV lysozyme lots as well as the protein fractions remaining in the egg white after isolation of lysozyme (OTf and OVA)

The AGID test revealed the presence of PC2, SPF and CV lysozyme diluted from 1/2 to 1/32 as compared to a standard lysozyme.

Fig. 3 shows the results for the optimal working dilutions for PC2 lysozyme, which was 1/4, and for SPF and CV lysozyme, which was ½

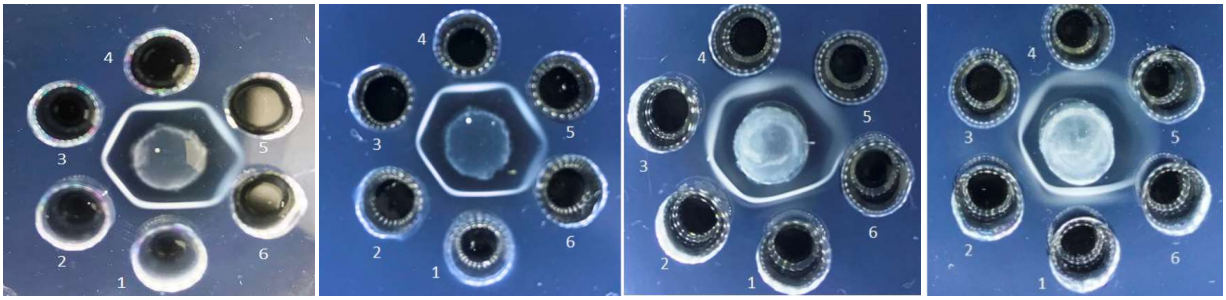

Fig. 3. AGID test: (a) - standard lysozyme; (b) - PC2 egg white lysozyme; (c) - SPF egg white lysozyme, (d) - CV egg white lysozyme; well 1 - integral lysozyme; wells 2 to 6 - lysozyme dilutions from 1/2 to 1/32; central well - anti-lysozyme serum

The PC2 lysozyme was comparatively tested to a standard lysozyme by the AGID test. The results indicate the identity between standard lysozyme (wells 3 and 5) and PC2 lysozyme (wells 2 and 6). The test was performed using anti-lysozyme rabbit serum. The precipitation line between the two samples shows continuity demonstrating their immunological identity (Fig. 4).

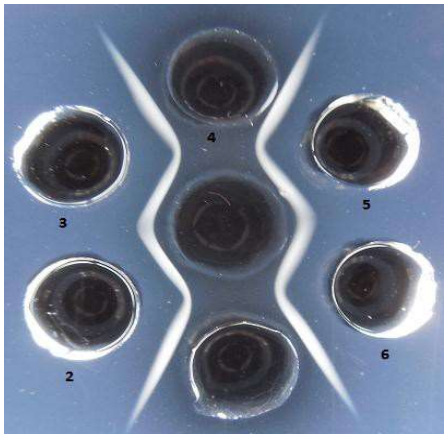

Fig. 4. AGID test: establishing the identity between standard lysozyme (wells 3 and 5) and PC2 lysozyme (wells 2 and 6); in 1,4 wells and centrally distributed anti-lysozyme rabbit serum

Fig. 5a shows the flake-like agglutination between the PC2 lysozyme and the culture of *Micrococcus lysodeikticus*. The agglutination between PC2 lysozyme and the *Staphylococcus aureus* culture showed a granular appearance (Fig. 5b). The negative control with homogeneous appearance was represented by the culture of *Micrococcus lysodeikticus* and the 0.15M NaCl solution (Fig. 5c).

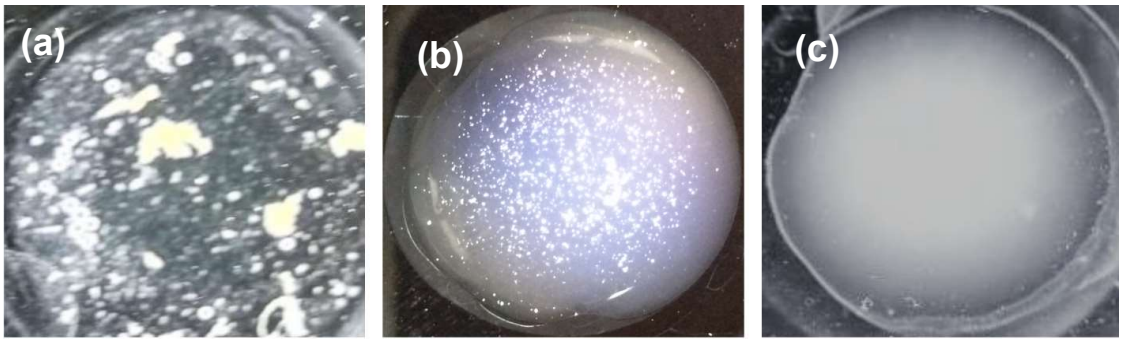

Fig. 5. Rapid agglutination reaction on the slide (RAR):
(a) - PC2 lysozyme and *Micrococcus lysodeikticus* culture (positive reaction, flaky clumps); (b) - PC2 lysozyme and culture of *Staphylococcus aureus* (positive reaction, granular clumps); (c) - *Micrococcus lysodeikticus* culture and 0,15M NaCl solution (negative reaction)

The appearance of the *Micrococcus lysodeikticus* culture on blood agar medium is shown in Fig. 6.

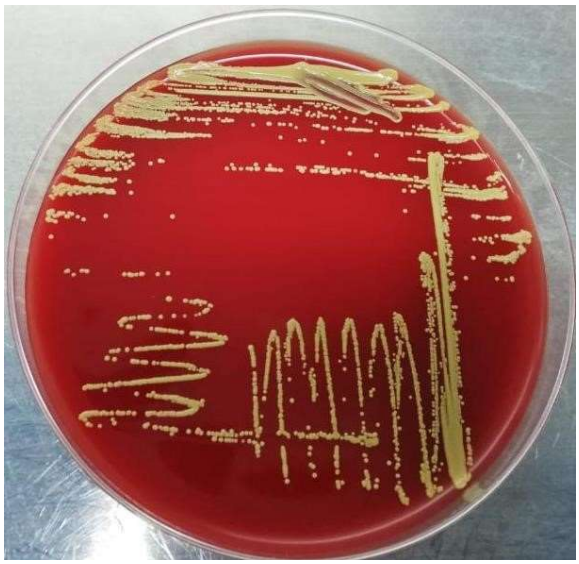

Fig. 6. A 24-hour culture of *Micrococcus lysodeikticus* on blood agar medium

The agglutinating activity of PC2 lysozyme was assessed against Gram-positive and Gram-negative bacteria. The presented results showed that lysozyme agglutinates Gram-positive bacteria, while Gram-negative bacteria are partially agglutinated (Table 2).

The mean values of SPF lysozyme concentration (lot I, n =30) were 4.4 mg/ml and 4.6 mg/ml for SPF lysozyme (lot II, n =30).

Table 2.
RAR - testing of the antimicrobial activity of PC2, CV and SPF lysozyme against Gram positive and Gram-negative bacteria

Bacterial species	PC2 Lysozyme	CV Lysozyme	SPF Lysozyme
Gram positive bacteria			
Micrococcus lysodeikticus	+++	+	-
Staphylococcus aureus	++	±	-
Staphylococcus epidermidis	++	-	-
Gram negative bacteria			
Pseudomonas aeruginosa	+	-	-
Escherichia coli	-	-	-
Klebsiella pneumoniae	-	-	-
Salmonella typhimurium	±	±	-

The lytic action of lysozyme on the culture of *Micrococcus lysodeikticus* is shown in Fig. 7. Lyophilized PC2 lysozyme was tested at concentrations of 20 mg/ml, 10 mg/ml, 5 mg/ml and 2.5 mg/ml to obtain lysozyme values of 60.5 mg/ml, 50.5 mg/ml, 37.0 mg/ml, and 25.5 mg/ml, respectively. Lysozyme from egg whites from two groups of CV hens (lot I - n = 30; lot II - n=30) showed average concentrations of 12.5 mg/ml and 14.0 mg/ml, respectively.

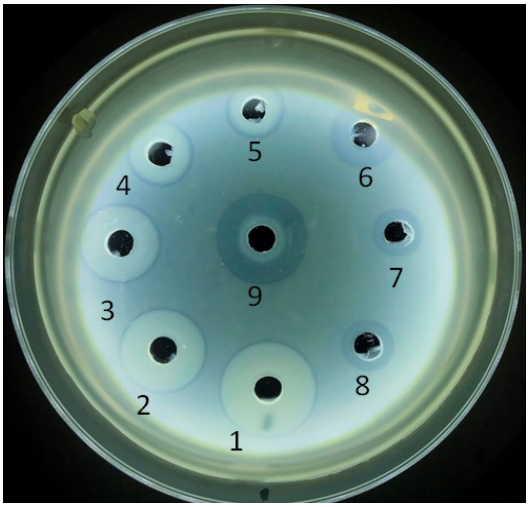

Fig. 7. Dosage of lysozyme by diffusimetric lysoplate method Well: 1 - PC2 lysozyme (20 mg/mL); 2 - PC2 lysozyme (10 mg/mL); 3 - PC2 lysozyme (5 mg/mL); 4 - PC2 lysozyme (2.5 mg/mL); 5 – CV lysozyme lot I; 6 – CV lysozyme lot II; 7 - SPF lysozyme SPF lot I; 8 - SPF lysozyme lot II; 9-standard lysozyme 20 mg/mL

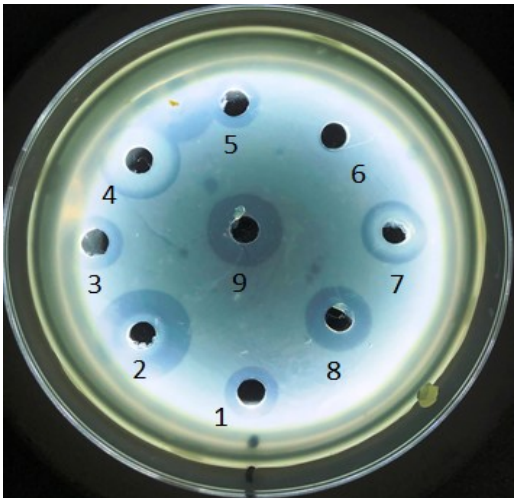

Fig. 8. Determination of PC2 lysozyme concentration in immunologically active products by the lysoplate method. Wells: 1 - Emulsion with lysozyme lot I; 2 - Egg white powder; 3 - Emulsion with lysozyme lot II; 4 - Lysozyme gel; 5 – SPF lysozyme; 6 - 0.15M NaCl solution; 7 - PC2 egg protein solution lot 32; 8 - PC2 egg protein solution lot 33; 9 - PC2 lysozyme

Fig. 8 shows the mean values ($\bar{x} \pm ds$) of lysis zone diameters (mm) and lysis units mg/ml ($\bar{x} \pm ds$) of PC2 lysozyme in some immunologically active products that contain only lysozyme (gel, emulsion) and PC2 hyperimmune egg proteins (solution, powder).

The diameter of the lysis zones ($\bar{x} \pm ds$) varied according to the amount of lysozyme added to the product preparation formula as follows: gel with lysozyme $\bar{x}$ = 16.6±0.27 mm and 55.5±0.31 mg ml lithic units; hyperimmune egg protein solution PC2 $\bar{x}$ = 14.5±0.34 mm and 50.0±0.70 mg/ml lysis units; emulsion with lysozyme $\bar{x}$ = 13.9±0.37 mm and 49.0±0.34 mg/ml lithic units; egg white powder $\bar{x}$ = 17.7±0.54 mm and 60.5±0.84 mg/ml lysis units. High values of PC2 lysozyme concentration were noticed.

Table 3.
Testing of the concentration of PC2 lysozyme in immunologically active products by the lysoplate method

Preparations	Lot no.	Lysis area diameter (mm) $\bar{x}$±ds	Lysis units (mg/ml) $\bar{x}$±ds
Gel with lysozyme	5	16,6±0,27	55,5±0,31
PC2 hyperimmune egg protein solution	33	14,5±0,34	50,0±0,70
Emulsion with lysozyme	4	13,9±0,37	49,0±0,34
PC2 hyperimmune egg white powder	6	17,7±0,54	60,5±0,84

The results obtained demonstrate that lysozyme can be isolated from egg white by ion exchange chromatography using Amberlite FPC 3500. The presented results are in agreement with the literature data on the purification and characterization of lysozyme in chicken egg white [1, 4, 9]. Purified PC2 lysozyme has the ability to lyse the cell membrane of Gram-positive bacteria due to the presence of the peptidoglycan layer (substrate for lysozyme). Gram-negative bacteria are less susceptible to the bacteriolytic action of this enzyme because they have a more complex cell wall structure [8].

Due to the antimicrobial properties of lysozyme, it can be used in the food industry (for preserving carcasses, cheeses, wine), in the pharmaceutical industry (lysozyme being included in various therapeutic preparations) and in medicine (in the form of aerosols, prophylactic for treating dental caries, for protection and repair of dystrophic and inflammatory lesions of the skin and soft tissues) [5, 7].

3. CONCLUSIONS

Lysozyme is a biologically active component of the PC2 hyperimmune egg

white that can be obtained in appreciable quantities and purified by chromatographic techniques. The specificity of lysozyme PC2 is that it reacts with the epitopes of antigens used to immunize the chickens. It has an important role in the defence of the organism, being considered part of the immune system.

Lysozyme cleaves the peptoglycan component of the bacterial cell wall, which loses its integrity and causes death of the cell. In addition, the hydrolysis products are able to increase the secretion of immunoglobulins, to activate the macrophages and to rapidly eliminate the pathogenic bacteria.

These data indicate that lysozyme may be a viable alternative to antibiotics and may be added to animal feed (Oliver et al., 2015). Due to its properties, lysozyme PC2 can be used in the food industry to extend the validity of products, as well as in biomedical applications as a preparation with immunotherapeutic potential.

The results of this research could be considered as a reference for other future studies.

4. FINANCING

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References

1. **Abeyrathne, E.D.N.S., Lee, H.Y., Ahn D.U.** (2014). Sequential separation of lysozyme, ovomucin, ovotransferrin and ovalbumin from egg white, *Poult. Sci.* **93** [4], 1001-1009.
2. **Al Awwaly, K.U., Manab A., Manik Eirry Sawitri, Eny Sri Widyastuti, Yuny Susanti Haniyah** (2016). Hen Egg White Lysozyme Extraction Using SiO_2: Effect of pH and Mineral on Antimicrobial Activity, *Int. J. Curr. Microbiol. App. Sci.* **5** [3], 432-436.
3. **Chiurciu Viorica, I.V. Pătrașcu, C. Chiruciu, Georgiana Topilescu.** Specific Chicken Immunoglobulins (IgY) for Prevention and Treatment and Human Bacterial Infections. European Biotechnology Congress Lecce 2015.
4. **Gemili S.E.S., Umdu, Nilgün Yaprak, Fatma Işık Üstok, F.Y.G., Yener, Çiğdem Mecitoğlu Güçbilmez, Sacide Alsoy Altinkaya, A., Yemenicioğlu** (2007). Partial Purification of Hen Egg White Lysozyme by Ethanol Precipitation Method and Determination of the Thermal Stability of Its Lyophilized Form. *Turk J Agric For*, **31**, 125-134.
5. **Leśnierowski, G., Renata Cegielska-Radziejewska.** (2012). Potential possibilities of production, modification and practical application of lysozyme, *Acta Sci. Pol. Technol. Aliment*. **11** [3], 223-230.
6. **Lu J., Y., Wan, Z., Cui** (2005). Fractionation of Lysozyme and Chicken Egg Albumin Using Ultrafiltration with 30-kDa Commercial Membranes. *Ind. Eng. Chem. Res.,* **44**, 7610-7616.
7. **Rainer Huopalahti, Rosina López-Fandiño, Marc Anton, Rüdiger Schade** (2007). Bioactive Egg Compounds. Egg white compounds, Lysozyme, Springer-Verlag Berlin Heidelberg
8. **Ramanauskiene Kristina, Asta Marija Inkeniene, A., Savickas, Ruta Masteikova, V., Brusokas** (2009) Analysis of the antimicrobial activity of propolis and lysozyme in semisolid emulsion systems. *Acta Pol. Pharm.,* **66** (6), 681-688.
9. **Shahmohammadi A.** (2018). Lysozyme separation from chicken egg white: a review. *Eur. Food Res. Technol.,* **244** (4), 577–593.
10. **Supeanu Teodora, Mădălina Tablică, Cristina Urducea, Ioana Alina Dimulescu, Lucica Sima, Viorica Chiurciu, Mariana Oporanu, C. Chiurciu** (2019). Hyperimmune egg: Preparation, characterization and alternative immunotherapeutic activity. *Vet. Drug*, **13** [1], 84-93.
11. **Susanto, E., Rosyidi, D., Lilik Eka Radiati, Manab, A.** (2014). Improved antibacterial spectrum of hen egg white lysozyme with thermal modified, *RRBS,* **8** [11], 437-442.