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CHARACTERISTICS AND SEPARATION OF LYSOZYME FROM HEN EGG WHITE

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ABSTRACT

Lysozyme is a small protein (14kDa) that occurs in almost all body fluids, and tissues of animal organisms. It exhibits bacteriolytic activity due to its ability of breaking bacterial cell walls. The demand of this enzyme has increased because of its diverse uses in pharmacy or food industry. Different methods for isolation have been proposed. Most of them are used in laboratory practice to obtain the pure enzyme of high activity, however only some of these methods are feasible on a commercial scale. The most useful method of lysozyme extraction are chromatography tecnhniques. Other methods, such as crystallization, aqueous two-phase extraction or membrane filtration, have also been reported. In this work, we studied a two-step protocol for the lysozyme extraction from chicken egg white, based on a protein precipitation coupled with chromatography. Three precipitation agents (polyethilenglycol, ammonium sulfate and sodium chloride) and four types of chromatography (cation-exchange chromatography, hydrophobic interaction chromatography, gel filtration chromatography and affinity chromatography) were assayed to determine the efficiency of each methodology. The most efficient methodology was the combination of protein precipitation using 3% polyethilenglycol and ion exchange chromatography, obtaining lysozyme of great purity and the activity was as high as 72U/ml.

RESUMEN

La lisozima es una proteína de bajo peso molecular (14kDa) que aparece en prácticamente todos los fluidos y tejidos de los organismos animales. Presenta actividad bacteriolítica debido a su capacidad para romper la pared bacteriana. Esta enzima ha cobrado un gran interés comercial por sus diversas aplicaciones en la industria farmacéutica y/o agroalimentaria. Se han propuesto diferentes métodos para aislar dicha proteína, la mayoría de ellos están planteados para la obtención de proteína pura de alta actividad a escala de laboratorio. Sólo algunos de ellos son factibles a escala comercial. El método más útil para aislar la lisozima son las técnicas de cromatografía; aunque también existen trabajos sobre otros métodos de purificación como la cristalización, filtración usando membranas o la extracción líquido-líquido. En este trabajo se ha estudiado un protocolo de purificación de lisozima en dos pasos, basado en una precipitación de proteínas seguida de una separación por cromatografía líquida. Se van a probar tres agentes de precipitación (polietilenglicol, sulfato de amonio y cloruro de sodio) y cuatro tipos de cromatografía líquida diferentes (de intercambio iónico, de interacción hidrofóbica, de filtración en gel y de afinidad) con el objetivo de determinar la eficiencia de cada metodología. El método más eficaz de los estudiados fue la cromatografía de intercambio iónico combinada con una previa precipitación de proteínas con polietilenglicol al 3%, obteniéndose una lisozima pura con una actividad de hasta 72U/ml.

1. - Introduction

The biotechnology industry has been increasing for the last years, due to the advances in genetic engineering and synthetic biology, and the augmented demand of the by-products of these processes. The development of the separation and purification techniques and methodologies has played a key role, since the purity of proteins is a pre-requisite for its application in industry or in general researching.

Lysozyme is one of these greatly demanded bioproducts. It has been widely used in several fields, such as clinical medicine, pharmacology, general researching and food industry. The commercial lysozyme is mostly separated from chicken egg white -which is the richest, most economic and abundant source of the protein- by a mix of techniques, including direct crystallization, chromatography or ultrafiltration. However, the occurrence of a large number of interferential proteins makes the purification and separation from chicken egg white challenging. In order to cover the great demand of this enzyme, it is necessary to establish a robust protocol of purification.

Taking into account these facts, the objectives of this work are:

1. - Making a literature review about the reported methods of the egg white lysozyme isolation.

2. - Analyzing different methods for lysozyme extraction and comparing the efficiency of each of them.

3. - Establishing an extraction protocol for the enzyme from the chicken egg white, by using the knowledge obtained.

2. - Literature review

2.1. - Lysozyme

Lysozyme was discovered by Alexander Fleming on 1922. He reported the occurrence of a bacteriolytic agent in tears and other human fluids. The name of lysozyme comes from its ability of breaking bacterial walls that causes them to lyse. Lysozyme occurs not only in tears and human fluids but in several sources, such as plants and egg white. This molecule protects the organisms from bacterial infection and, through the years, it has become a very commercially valuable enzyme.

2.1.1. - Characteristics of lysozyme

Lysozyme is a relatively small enzyme that catalyses the hydrolysis of 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in the peptidoglycan contained in cell walls. Lysozyme from chicken egg white (CEW) is a polypeptide of 129 aminoacids, having a molecular weight of 14 kDa, strongly basic protein with an isoelectric point (pI) of 10-11 [1]. Some other chemical properties of lysozyme are shown in Table 1.

Table 1. *Properties of lysozyme*

PROPERTY	VALUE
Molecular Weight	14,4 kDa
Nº of subunits	1
Aminoacids	129
Pi	10,7
Disulfide bonds	4
% Carbohydrate	0
$E^{1\%}$ 280 nm	26,4
Thermal D at 93ºC (Time to destroy 90% of activity)	110 minutes
Assay of enzyme activity	Bacterial lysis by turbidity

It is a very stable and compact enzyme, due to four disulfide bonds present in the polypeptide chain of the protein (***Fig.1)***. At least two of the disulfide bonds must be intact to maintain its enzymatic activity, and are also responsible for the thermal stability of the enzyme. Similar high stability is seen in acidic solution pH 3.0-4,0. However, thiol compounds rapidly inactivate lysozyme [1]. This protein is a molecule consisting of two domains separated by a helix-loop-helix motif, which has been found to play a key role in its antimicrobial function [1].

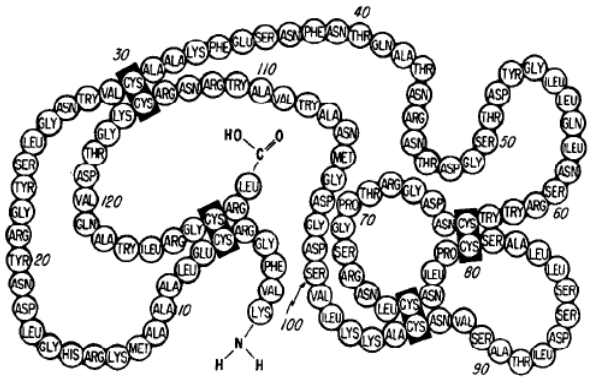

Fig. 1. *The structure of CEW lysozyme, indicating the positions of the four disulfide bonds. (The disulfide bonds of Egg White Lysozyme, Robert E. Canfield and Anne K. Liu. J. Biol. Chem. 1965, 240:1997-2002).*

Lysozyme is found mainly as a monomer, but it has been reported to exist also as a dimer in denaturation conditions of pH, concentration and temperature [3]. Moreover, it has been proved that, during storage, lysozyme intermolecular disulfide bond exchange can occur, leading to the formation of dimeric form [2]. In its monomeric form, this enzyme exhibits high bacteriolytic activity against gram-positive bacteria, such as Lactic Acid Bacteria. By contrast, it has limited effect against gram-negative bacteria and has no effect against eukaryotic cell walls.

However, it has been reported that in some conditions (i.e. partially unfolded lysozyme or reduction of the disulfide bonds), the action of the lysozyme could be augmented, and it could act against both of them [1]. Recent studies show that the chemical and thermal modification of lysozyme increases its antimicrobial properties towards Gram-negative bacteria with no loss of activity against Gram-positive bacteria [3]. For instances, *Mecitoğlu, Ç. et al* [7] demonstrates that, when it is combined with EDTA, the outer membranes of Gram-negative bacteria are destabilized, and the antimicrobial spectrum of lysozyme increases significantly.

2.1.2. - Egg white bioactive compounds

The egg white is the richest and most economic source of lysozyme. In the hen egg white, lysozyme accounts for 3,5% of the total egg white proteins. The activity of egg white lysozyme is affected by numerous factors such as management system of hens, feed modification and egg storage [3]. Hen egg white represents an essential ingredient, which has been used for many years by the food industry because of its excellent technological properties [2]. The egg white proteins are shown in Table 2, indicating the percentage of each protein in the egg white, their molecular weight (M. Wt), their isoelecctric point (pI) and some remarkable characteristics. The knowledge about the composition of the egg white is very important for establishing the purification protocol.

Table 2. *Egg white bioactive compounds*

Protein	Amount (%)	M. Wt (KDa)	pI	Characteristics
Ovoalbumin	54	45	4,5	
Ovotransferrin	12-13	77,7	6,0	Binds iron and other metal ions
Ovomucoid	11	28	4,1	Inhibits serine proteinases
Lysozyme	3,4-3,5	14,3	10,7	Lysis of bacterial cell walls
Ovomucin	1,5-3,5	220-270000	4,5-5,0	Interacts with lysozyme
G2 ovoglobulin	1,0	47	4,9-5,3	
G2 ovoglobulin	1,0	50	4,8	
Ovoflavoprotein	0,8	32	4,0	Binds riboflavin
Ovostatin	0,5	760-900	4,5-4,7	
Cystatin	0,05	12	5,1	Inhbitis cysteine proteinases
Avidin	0,05	68,3	10,0	Binds biotin
Thiamine-binding protein	-	38	-	Binds thiamine
Glutamyl aminopeptidase	-	320	4,2	
Minor glycoprotein 1	-	52	5,7	
Minor glycoprotein 2	-	52	5,7	

Hen egg white possesses many biologically active proteins that could offer a better valorisation for hen egg white: **lysozyme**, **ovalbumin**, **ovotransferrin** and **ovomucin,** which represent the major egg white proteins [3]. **Ovalbumin**, which is the major constituent of egg white proteins, is used in food industry due to its foaming and gelling properties. Moreover, it has high nutritional values and unique importance in immunological studies as well as animal cell culture and development of antibody [4]. **Ovotransferrin** is known for antimicrobial activity which is associated with its iron binding property [5]. **Ovomucin**, a glycoprotein in egg white, is responsible for the thick gel characteristics of liquid egg white. Besides its excellent foaming and emulsion capacities, it possesses anti-viral, anti-bacterial, anti-tumour and other bioactivities. In non-reducing conditions, it forms complexes with itself, reaching a molecular weight of 5000-8000 kDa [6]. Therefore, isolation and purification of valuable egg white components appears to be promising due to their potential uses.

2.1.3. - Lysozyme applications

Its antibacterial activity against gram-positive bacteria has a practical application in the **food processing industry** and **pharmaceutical industry**, as well as in **medicine**. Also, lysozyme may be used as cell disrupting agent of bacterial intercellular products, which is an important application in **general research** (i.e. in protein purification kits) [1, 3].

Lysozyme is one of the most used biopreservative for: meat, fish and their products; milk and dairy products; fruit and vegetables in antimicrobial packaging. The commercial lysozymes are quite pure. Nevertheless, for its application in food industry, the use of cheaper partially lysozyme may be economically more feasible. In the cheese industry, lysozyme is used in order to destroy vegetative forms of *Clostridium,* especially *C. tyrobutyricum,* which might causes butyric fermentation during cheese maturation and produces holes, crevices and undesirable flavours and odours. In the wine industry, the lysozyme is widely used to avoid the malolactic fermentation, since it destroys malolactic fermentation-causing bacteria. Moreover, lysozyme is alcohol-resistant and does not cause any effect against wine yeast [1].

The pharmaceutical industry uses this enzyme as a carrier, taking advantage of its low molecular-weight, which allows its rapid elimination from the blood by glomerular filtration. Lysozyme has been employed as a renal-specific carrier for targeting drugs to proximal tubular cells [9]. It also has been used in the manufacture of adjuvant drugs for antibiotics and analgesics in viral and bacterial infections (brochopulmonary diseases, dental caries, nasal tissue protection), in the treatment of leukaemia and neoplastic diseases [1, 3].

2.2. - Methods of isolation

Numerous methods are used in laboratory practice to separate lysozyme from egg white, but only some of them have been used in industry. A group of methods to separate lysozyme includes its direct crystallization from egg white, chromatographic techniques, or membrane techniques (especially ultrafiltration) [3].

2.2.1. - Crystallization and precipitation

The crystallization method was first reported by *Alderton et al* [8] on 1946. It is a classical laboratory and commercial procedure of lysozyme separation from the egg white based on direct enzyme crystallization with 5% NaCl, at a pH of 10-11. The crystallization starts when crystalline lysozyme is added to the egg white at these conditions. Nowadays, either the direct crystallization technique or protein precipitation, coupled with chromatography, is commonly used to obtain pure lysozyme in an industrial scale [12].

2.2.2. - Membrane separation: Ultrafiltration

Taking into account the physicochemical properties of lysozyme, especially its low molecular weight, it seems feasible to use membrane techniques to separate the enzyme from the egg white. On the other hand, lysozyme ability to bind ovomucin and the other negatively charged CEW proteins reduces its diffusion through the membrane. [8].

Different types of membranes, such as 30kDa ultrfiltration (UF) membrane [10, 11], 50kDa polysulfone membranes [9], hollow-fibre polysulfone membranes [12], 30kDa MWCO PES (biomax) or cellulose membranes [8] have been used so far to separate lysozyme from the

CEW. Those studies offered a rich knowledge about the process and allowed the improvement of the CEW lysozyme membrane separation. First studies reported that only a small amount of lysozyme (2%-4%) could pass through the UF membrane, obtaining a low lysozyme yield [10, 11]. A later study reported that lysozyme transmission is strongly affected by the pH solution and salt concentration [9]. Under optimized conditions, high lysozyme transmission could be achieved [12]. A new mode of ultrafiltration operation (Carrier Phase Ultrafiltration, **CPUF**) has been proposed to ensure the optimized conditions throughout an entire operation. In the lastest studies, with the use of a *Biomax* 30kDa membrane coupled with the CPUF operation mode, the lysozyme transmission was more than 80% and the purity obtained was greater than 94% [8].

Ultrafiltration could be an effective and useful tool for the fractionation of proteins from real biological solutions, and it may be easier to scale-up in comparison to chromatography and electrophoresis.

2.2.3. - Chromatography extraction

On protein purification, separation is usually accomplished by **liquid chromatography**. In chromatography extraction, the protein separation is dependent on their biological and physico-chemical properties, such as molecular size, net charge, biospecific characteristics and hydrophobicity [17]. There are two mechanisms for chromatography:

- Adsorption, such as ion exchange chromatography.

- Nonadsorption, such as gel filtration chromatography.

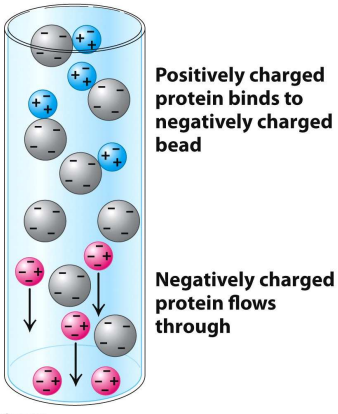

Fig. 2. *Cation exchange chromatography diagram (Biochemistry, Seventh Edition. H. Freeman, 2012).*

Purification of CEW proteins were mostly performed on liquid chromatography because of the absence of protein denaturation and its high selectivity. In this case, the chromatography is usually preceded by a protein precipitation step, due to the occurrence of some large proteins, such as ovomucin, which are responsible of the viscosity of the egg white. These large proteins may cause an obstruction in the chromatography column; therefore it is suitable to eliminate them. This method has been used on an industrial scale, in which lysozyme is extracted by a combination of chromatography and salting out precipitation techniques [4].

a) Cation-exchange chromatography

In cation-exchange chromatography, positively charged molecules are attracted to a negatively charged solid support (***Fig. 2***). The attraction to the solid support depends either on the pH value of the buffer, which determines the charge of the molecules, and on its ionic strength. When the ionic strength is higher than the matrix strength, the molecules in the mix will be more attracted by the ions on the solution, and they will break the bond to the matrix.

Ion-exchange chromatography has been widely used in order to separate the lysozyme from the rest of CEW proteins [13, 14, and 15]. As shown in the table below, it is clear that the isoelectric point of lysozyme is separated from practically all the rest of the proteins in the CEW by more than two units of pH (***Table 3)***. This fact suggest that at a pH between 6,5-10, the lysozyme will uniquely possess a net positive charge, therefore the use of a cation exchange chromatography will separate the lysozyme from the rest of the CEW proteins.

Table 3. *CEW proteins and their isoelectric point (pI) values*

Protein	Ovalbumin	Ovotransferrin	Ovomucoid	Lysozyme	Ovomucin
pI	4,5	6,0	4,1	10,7	4,5-5,0

As a protein responsible for the viscous nature of egg white, **ovomucin** is usually isolated first. Ovomucin could be diluted with three volumes of water at pH 6; however the resulting solution also contained lysozyme and ovoalbumin [14]. A new two-step precipitation method using 100mM NaCl and 500mM NaCl solutions [14] or polyethilenglycol (PEG) precipitation [15] are other options to isolate ovomucin. Moreover, some authors proposed to hydrolase ovomucin to increase its solubility [13].

There are many options to carry out the cation exchange chromatography: many matrixes are available on the market. In example, *Wu, J. et al* [14] used in their work a *High-Prep 16/10 column of SP Sepharose* purchased from *GE Healthcare BioSciences*; whereas *Guèrin-Dubiard et al* [13] used a *S Ceramic Hyper DF* (cation exchanger) purchased from *Biosepra.*

b) Affinity chromatography

Affinity chromatography is one of the most effective methods for the purification of biological macromolecules. Compared with other techniques for lysozyme purification, the affinity chromatography seems to be advantageous due to the specific selection. However, in the industrial production, affinity chromatography is not equally employed as the other techniques. It may be because of the high costs and the low stability of classical affinity ligands (i.e. antibodies). These disadvantages limit the application of affinity chromatography in industrial production of lysozyme. In order to expand the application of affinity chromatography in industrial production of lysozyme, novel ligands are required for developing low-cost, stable and repeatable affinity columns [16].

The first adsorbent used to bind lysozyme was **chitin,** but nowadays new materials have been developed, such as glucochitin or chitosan. It has been reported that Sephadex G75, commonly used for size exclusion chromatography, binds lysozyme in a pH depedent manner [18]. Two different and new systems of affinity ligands for lysozyme purification have been developed in recent studies, in which affinity molecules have been bound to a matrix in order to obtain an affinity column. On first studies, *Denizli, A. et al.* [17] prepared a dye-affinity magnetic adsorbent, using magnetic poly-(2-hydroxyethyl methacrylate), or mPHEMA, beads. Later, *Liu, F. et al.* [16] developed a highly efficient and low-cost affinity purification strategy for lysozyme, immobilizing Tris on macroporous silica spheres.

The aim of those new studies is to prepare an affinity adsorbent for an efficient and less expensive separation of lysozyme from CEW. Thus, the adoption of new affinity ligands could make lysozyme affinity extraction much more convenient and cost effective.

2.2.4. -Aqueous two-phase extraction

The aqueous two-phase extraction could be another potential method for the extraction of CEW lysozyme. This system is suitable for continuous large-scale purification of biomolecules and allows the use of traditional liquid-liquid extraction equipment. The two-phase extraction of lysozyme by PEG/salt system has been investigated in order to determine the possibility of using the aqueous two-phase system (ATPS) for partioning of lysozyme from the egg white. If the correct salt is selected, this method could be an efficient an inexpensive lysozyme purification system [19]. It offers many advantages, such as low processing time, low energy consumption, biocompatible environmental and the relative ease of it scaling-up. However, the recovery of the enzyme is complicated and requires back extraction into a salt-rich phase, ultrafiltration or ion-exchange chromatography. Later studies demonstrated that protein separation in ATPS can be simplified and more efficient if thermoseparating polymers, such as ehylene oxide-propylene oxide (EOPO) random copolymers, are used instead of PEG [20].

3. - Materials and methods

3.1. - Protocol design

The diagram below explains the two-step protocol for the lysozyme extraction: a protein precipitation step followed by a chromatography step. The first step was performed to remove some unwanted proteins present in the egg white and may cause any problem during the chromatography. These proteins have a high molecular weight and are able to polymerize with themselves, forming a huge net that traps the rest of proteins and causes the viscosity of the egg white.

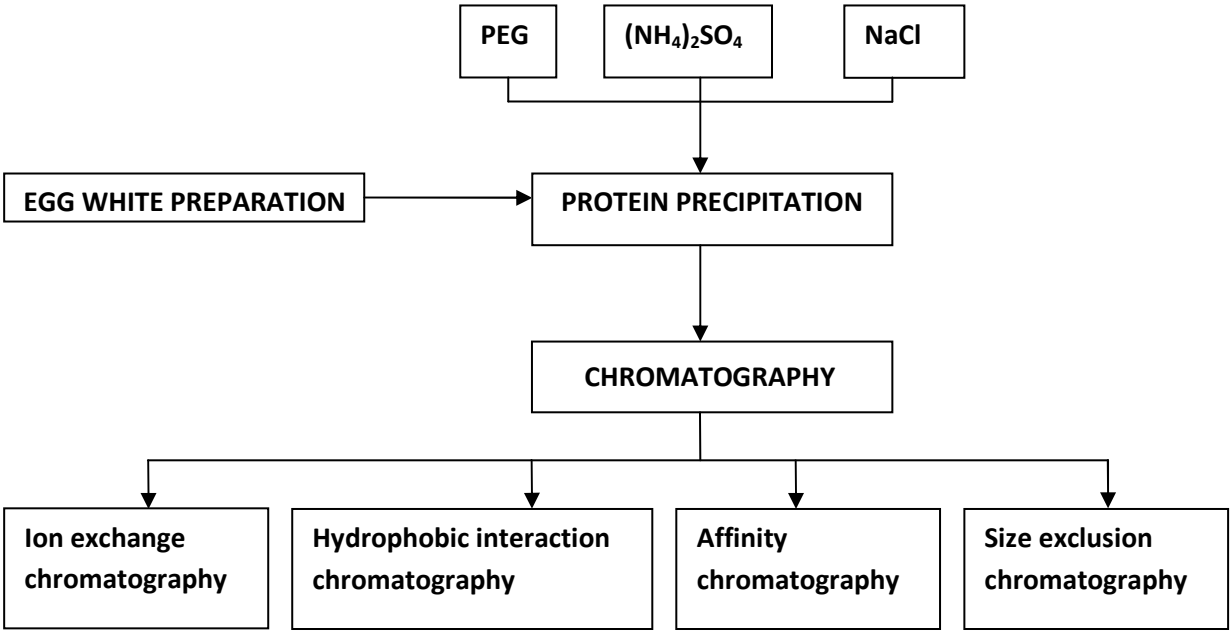

Fig. 1. *Diagram of the lysozyme extraction procedure.*

Three different precipitation agents and four kinds of chromatography were performed. After each step, the efficiency of the process was evaluated in order to decide, at the end, which protocol was the most suitable for the extraction of the lysozyme, with the available resources.

3.2. - Egg white preparation

Fresh eggs were purchased from the supermarket in Ankara, Turkey. The egg white was separated from the yolk, filtered and diluted 2-fold into 50mM Tris-Base solution. The pH was adjusted to 6’0 while stirring

3.3. - Protein precipitation

The PEG600 (*Bioshop*), Ammonium Sulfate (*Merck*) and Sodium Chloride (*Fischer Scientific*) were weighted and added in solid form to reach the final concentration shown in the table below.

Table 4. *Concentration of the precipitant agents*

	PEG	NaCl	$(NH_4)_2SO_4$
Experiment 1	3%	100mM	100mM
Experiment 2	6%	500mM	250mM
Experiment 3	9%	1M	500mM

The resulting solutions were kept on ice, shacked for one hour. After these steps, the egg white was shared out into Falcon tubes for centrifugation, and the tubes were centrifuged. Both supernatant and pellet were stored at -20ºC.

3.4. - Chromatography

All the chromatographies were carried out in a *BioRad* column device. The sample was injected into the column through a loop with 90ml of volume. Chromatography protocols, sample preparation and buffer composition are presented according to the mode of separation. After elution, all the fractions were kept at -80ºC and then freeze-dried.

3.3.1 -Ion-exchange chromatography

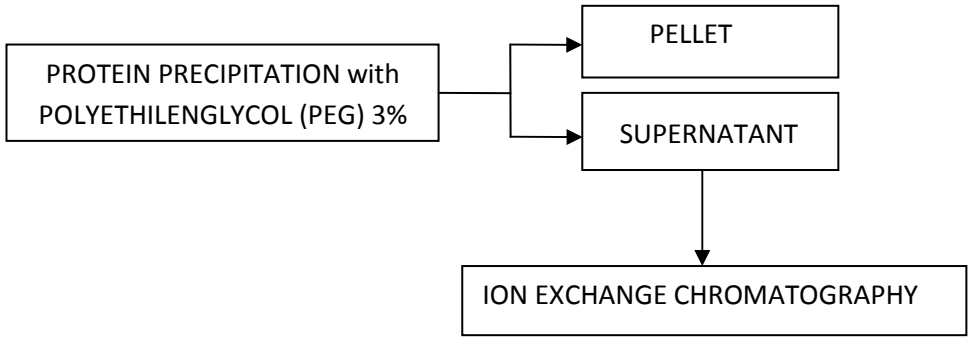

Fig. 2. *Diagram of the ion-exchange chromatography purification procedure.*

As shown in the diagram above, the supernatant resulting of the CEW protein precipitation using PEG 3% was injected into a column filled with 5 ml of S-Sepharose (GE Healthcare). The binding buffer composition was 50mM Tris solution (pH 8). The bound lysozyme was eluted with 1M NaCl in 50mM Tris buffer solution (pH 8). The flow rate maintained throughout the process was 1ml/min.

3.4.2. -Hydrophobic interaction chromatography

The supernatant resulting of the egg white protein precipitation using ammonium sulfate ($(NH_4)_2SO_4$) 500mM was injected into a column filled with 5ml of Octyl Sepharose (GE Healthcare). The binding buffer composition was 1,7M, 500mM or 2M Ammonium Sulfate 100mM Sodium Phosphate (pH 7); the elution buffer composition was 100mM Sodium Phosphate (pH 7).

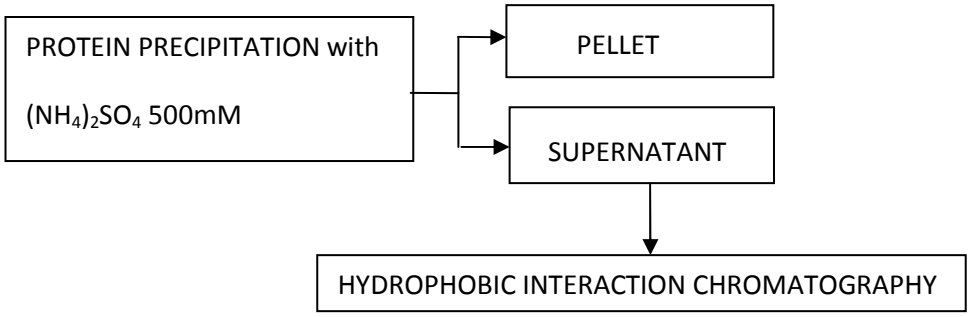

Fig. 3. *Diagram of the hydrophobic interaction chromatography extracion procedure.*

3.4.3. -Gel filtration

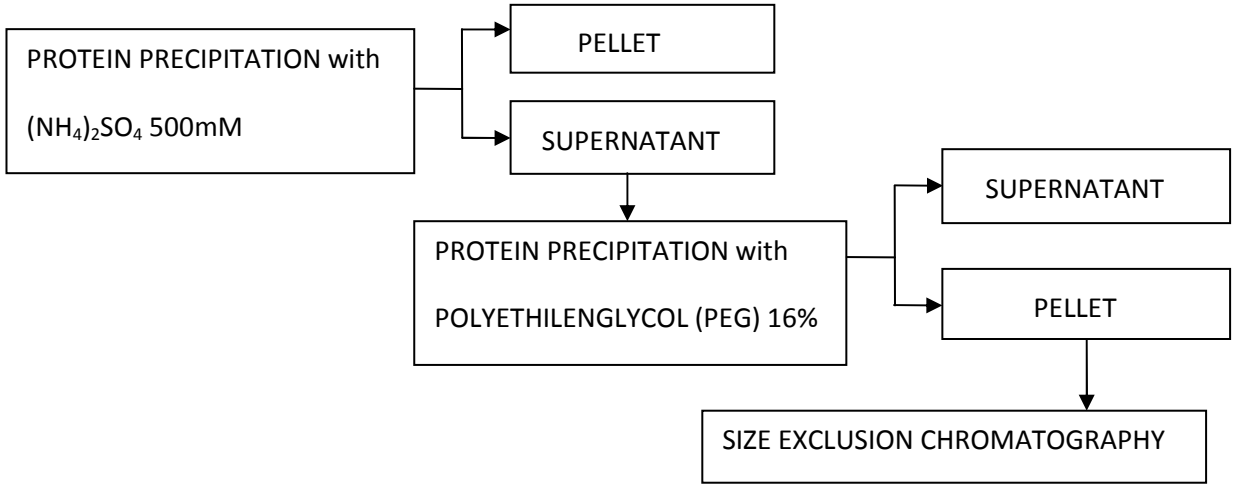

Fig. 4. *Diagram of the size exclusion chromatography extraction procedure.*

After 500mM $(NH_4)_2SO_4$ protein precipitation, a second precipitation was carried out, using 16% PEG. The pellet was collected and redisolved with Tween 20 (Bioshop) and glycerol (Bioshop). Ultrasonication was used to help the homogenization. The resulting solution was injected into a column filled with Sephadex G-50. Sephadex G-50 is supplied as a dry powder and must be allowed to swell in excess buffer before its use. The matrix has to take up the full surface of the column. The swelling conditions vary depending on the chosen volume, as is described in Table 4.

Table 5. *Swelling conditions*

Medium	Approx. bed volume (ml/1 g medium)	Swelling time (h), 20°C	Swelling time (h), 90°C
Sephadex G-10	2–3	3	1
Sephadex G-25 (all grades)	4–6	3	1
Sephadex G-50 Fine	9–11	3	1

The resulting slurry has to stay overnight at room temperature. Then, it has to be poured into the column and let to settle down. The buffer composition was 1M NaCl 50mM Tris (pH 8).

3.4.4. –Affinity chromatography

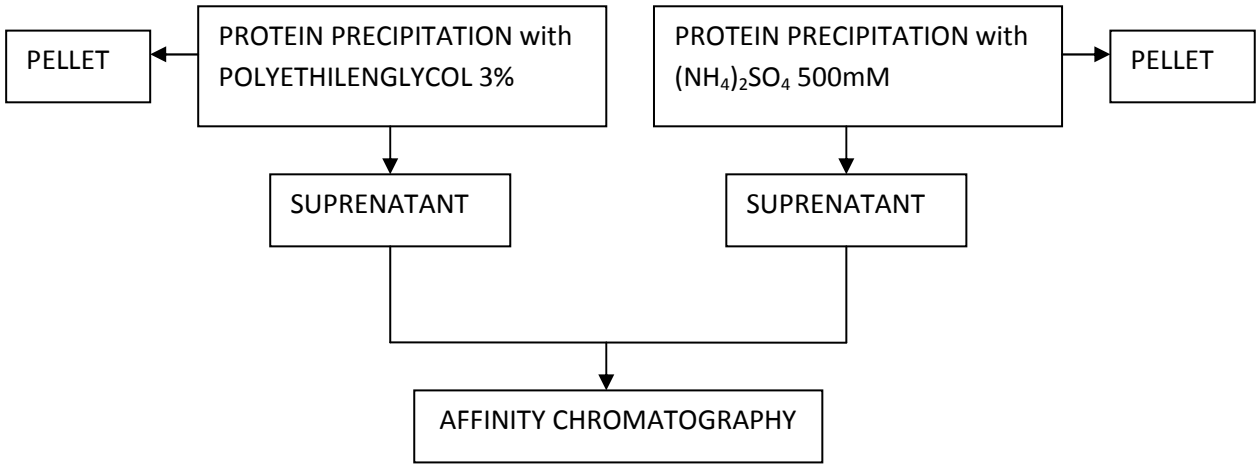

Fig. 5. *Diagram of the affinity chromatography extraction procedure.*

The supernatant fractions resulting from 3% polyethilenglycol precipitation and from 500mM ammonium sulfate precipitation were injected into the column filled with Sephadex-G50. The binding buffer composition was 50mM Tris solution (pH 8). The bound lysozyme was eluted with 1M NaCl 50mM Tris buffer solution (pH 8).

3.5. - Electrophoresis

SDS-PAGE was carried out using 12% and 14% gels, in Tris-Glycin-SDS buffer, at a constant voltage mode of 100V for 3h. The samples loaded in the electrophoresis were those obtained from the protein precipitation process (both supernatant and pellet) and the series of fractions obtained from the chromatography process. After the electrophoresis, the gels stood 30 minutes into the fixation solution (25% Isopropanol, 10% acetic acid) and was stained with 0,005% Coomasie Brilliant Blue. The gel images were analyzed by *Image J program.*

3.6. - Activity

The purified lysozyme activity was assayed in both Gram-negative (*Escherichia coli)* and Gram-positive (*Staphylococcus epidermidis)* bacteria. A colony of each microorganism was inoculated into Luria-Bertani (LB) liquid medium and let to grow overnight at 37ºC. The day after, the suspension was centrifuged and the bacterial pellet was resuspended into new LB liquid medium to obtain a solution with an O.D600 of 0,5 – which is approximately equivalent to $2x10^8$ bacterial cells/ml. Lysozyme ativity was assayed in flat bottomed 96 well microplates. Each well was filled with 150µl of bacterial suspension and 50µl of the lysozyme fraction. Each sample was loaded in triplicate. Bacterial lysis was assessed by spectrophotometric turbidity measurement performed at 600 nm wavelength. The blank was composed uniquely by 150µl of bacteria. Measurements were done at t=0, t=30 min, t=60 min, t=90 min and t=1110 min, having a total of five measurements.

4. - Results and discussion

4.1. - Protein precipitation

Three different precipitation agents were assayed in order to determine the one which offers the most efficient precipitation of high molecular weight, with minimal loss of lysozyme in the pellet. The results of the protein precipitation were analyzed by using SDS.PAGE. Both supernatant fraction and pellet for each precipitation agent have been analyzed. The quantification of protein bands in each lane were performed by using *Image J* program, as explained before. The results are shown in the following figures and tables.

4.1.1. - Polyethilenglycol 600 (PEG600)

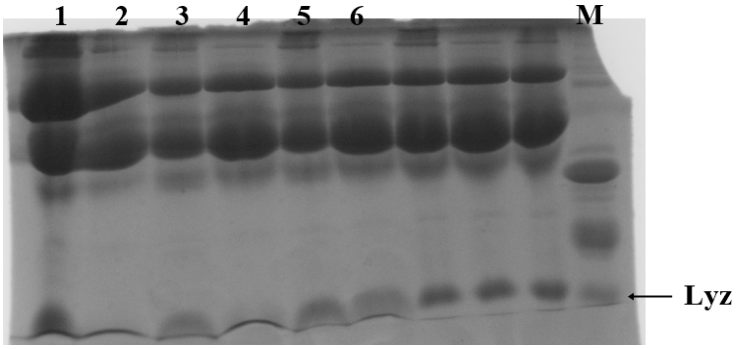

Fig. 8. *SDS-PAGE pattern of fractions obtained by PEG precipitation. M: marker; 1: 9% PEG, pellet; 2: 9% PEG supernatant; 3: 6% PEG pellet; 4: 6% PEG supernatant; 5: 3% PEG pellet; 6: 3% PEG, supernatant. The remaining three patterns do not belong to PEG precipitation. One band appears around 14kDa in each fraction, which is supposed to be lysozyme.*

Table 6 shows the results obtained from the analysis of the gel (Fig. 8.) with the *Image J* program. The data are an estimation of the concentration of proteins in each band of the lane profiling. As it has been explained before, the *Image J* program converts the picture in a map of points, which draws a series of peaks that correspond to each band of protein. The area of the peak, corresponding to the intensity of grey in the band, is directly proportional to the concentration of the protein in the band. Thus, we estimated that, in 3% PEG supernatant precipitation, 43,8% of the total amount of lysozyme was retained in the supernatant, whereas the percentage of lysozyme in the 6% PEG and 9% PEG supernatant fraction are only 16,2% and 3,3% of the total amount of lysozyme, respectively.

In light of these results, **3% PEG** was found to be the most suitable for the clarification of the egg white by means of protein precipitation. Even though massive amount of lysozyme was lost in the pellet, 3% PEG precipitates more than 50% of the unwanted proteins, in turn offers convenient consistency for the following chromatography step.

Table 6. *Estimation of the concentration of lysozyme in relation to the total concentration of proteins after the precipitation with PEG. S: Supernatant; P: Precipitate; Lys: Lysozyme; No lys: Rest of proteins; Prot: Proteins.*

BAND ANALYSIS						
	9% PEG P	9% PEG S	6% PEG P	6% PEG S	3% PEG P	3% PEG S
Lysozyme	1979,3	68,1	580,4	112,3	962,9	751,7
No lysozyme	10180,2	6104,1	6523,9	6237,8	6047,1	6174
Total Prot.	12159,5	6172,2	7104,3	6350,1	7010	6925,7
%Lysozyme	**16,3**	**1,1**	**8,2**	**1,8**	**13,7**	**10,9**

GLOBAL SUMMARY			
Total Lyz	2047,4	692,7	1714,6
Total no Lyz	16284,3	12761,7	12221,1
Total Prot	18331,7	13454,4	13935,7
% Lyz P	96,7%	83,8%	56,2%
% Lyz S	**3,3%**	**16,2%**	**43,8%**
% No Lyz P	**62,5%**	**51,1%**	**49,5%**
% No Lyz S	37,5%	48,9%	50,5%
% Prots P	66,3%	52,8%	50,3%
% Prots S	33,7%	47,2%	49,7%

4.1.2. -Ammonium Sulfate ($(NH_4)_2SO_4$)

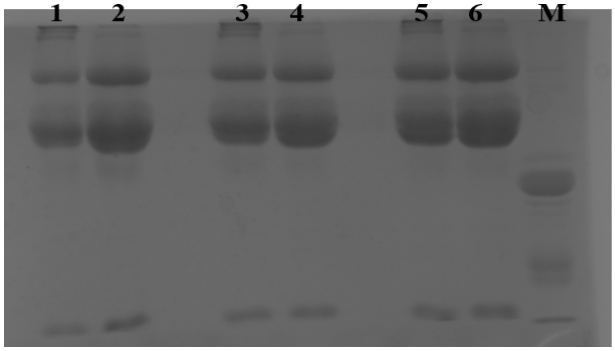

Fig. 9. *SDS-PAGE pattern of fractions obtained by NH4(SO4)2 precipitation. M: marker; 1: 500mM NH4(SO4)2, suprenatant; 2: 500mM NH4(SO4)2 pellet; 3: 250mM NH4(SO4)2 suprenatant; 4: 250mM NH4(SO4)2 pellet; 5: 100mM NH4(SO4)2 suprenatant; 6: 100mM NH4(SO4)2, pellet. One band appears around 14kDa, which is supposed to be lysozyme.*

As we can see in Table 7, where the data of the estimated concentration of protein in each band are shown, the concentration of **500mM of $(NH_4)_2SO_4$** appears to be the most efficient of the three concentrations assayed. The estimated percentage of lysozyme in the 500mM $(NH_4)_2SO_4$ supernatant fraction is 67,1%, higher than in the other supernatant fractions, where lysozyme accounts 54,8% (100Mm fraction) and 60,8% (250mM fraction). However, the precipitation of unwanted proteins seems to be more efficient in the **250 mM fraction**, since the percentage of precipitated no-lysozyme proteins is equal to 48,7%, whereas in the 500mM

fraction is only the 38,9%. Even so, the concentration chosen for the following protein precipitation was 500mM, since the aim is to obtain as much protein as possible at the end of the experiment.

Table 7. *Estimation of the concentration of lysozyme in relation to the total concentration of proteins after the precipitation with ammonium sulfate. S: Supernatant; P: Precipitate; Lys: lysozyme; No lys: Rest of proteins; Prot: Proteins.*

BAND ANALYSIS						
	100mM S	100mM P	250 mM S	250mM P	500 mM S	500mM P
Lysozyme	260	214,7	266	171,5	373,6	183
No lysozyme	2741	2470,5	2360	2244,1	2773	1768
Total	3001	2685,2	2626	2415,6	3146,6	1951
%Lysozyme	**8,65**	**7,99**	**10,14**	**7,1**	**11,87**	**9,37**

GLOBAL SUMMARY			
Lys	474,7	437,47	556,6
No Lys	5211,5	4604,1	4541
Total	5686,2	5041,61	5097,6
LyS P	45,2%	39,2%	32,9%
Lys S	**54,8%**	**60,8%**	**67,1%**
No Lys P	**47,4%**	**48,7%**	**38,9%**
No Lys S	52,6%	51,3%	61,1%
Prot S	52,8%	52,1%	61,7%
Prot P	47,2%	47,9%	38,3%

4.1.3. -Sodium Chloride (NaCl)

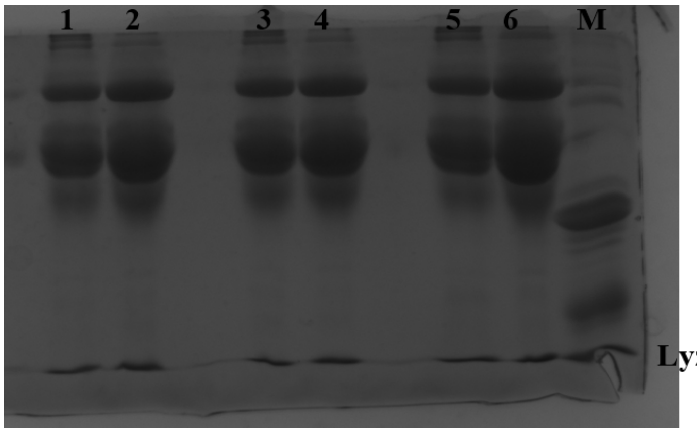

Fig. 10. *SDS-PAGE pattern of fractions obtained by NaCl precipitation. M: marker; 1: 1M NaCl, suprenatant; 2: 1M NaCl pellet; 3: 500mM NaCl suprenatant; 4: 500mM NaCl pellet; 5: 100mM NaCl suprenatant; 6: 100mM NaCl pellet. One band appears around 14kDa, which is supposed to be lysozyme.*

The results of the precipitation with NaCl are shown in Table 8. In this case, the concentration of 1M NaCl appears to be the most suitable for obtaining the lysozyme, accounting 61,5% of the total lysozyme in the supernatant fraction. The precipitation with 500mM NaCl and

100mM gave a percentage of lysozyme in the supernatant equal to 54,9% and 55,1% respectively.

Table 8. *Estimation of the concentration of lysozyme in relation to the total concentration of proteins after the precipitation with NaCl. S: supernatant; P: precipitate; Lys: Lysozyme; No lys: Rest of proteins; Prot: Proteins.*

BAND ANALYSIS						
	1 M P	**1 M S**	**500 mM P**	**500mM S**	**100mM P**	**100mM S**
Lysozyme	180,9	288,7	249,4	303,3	174,1	213,1
No lysozyme	3852,7	4962,2	4509,8	4530,6	3681,3	5587,3
Total	4033,6	5250,9	4759,2	4833,9	3855,4	5800,4
%Lysozyme	**4,5**	**5,5**	**5,2**	**6,3**	**4,5**	**5,5**
GLOBAL SUMMARY						
Total Lyz	469,6		552,7		387,2	
Total no Lyz	8814,9		9040,4		9268,6	
Total Prot	9284,5		9593,1		9655,8	
% Lyz P	38,5&		45,1%		44,9%	
% Lyz S	**61,5%**		**54,9%**		**55,1%**	
% No Lyz P	**43,7%**		**49,9%**		**39,7%**	
% No Lyz S	56,3%		50,1%		60,3%	
% Prots P	43,4%		49,6%		39,9%	
% Prots S	56,6%		50,4%		60,1%	

4.1.4. –Summary of protein precipitation experiments

Table 9. *Summary of protein precipitation.*

	3% PEG	**500mM AS**	**500mM NaCl**
% Lysozyme related to the total proteins in the sample	10,9%	9,4%	6,3%
% Lysozyme related to the total lysozyme (pellet + supernatant)	43,8%	67,1%	54,9%
% Unwanted Proteins on the pellet	49,5%	38,9%	49,9%

As it is shown in Table 9, the most effective concentrations for each precipitation agents are:
- 3% for PEG: 43,8% of the total lysozyme is present in the supernatant fraction, which represents 10,9% in relation to the rest of proteins, and it precipitates 49,5% of unwanted proteins.
- 500mM for ammonium sulfate: The supernatant fraction contains 67,1% of the total lysozyme, which represents 9,4% of lysozyme in relation to the rest of proteins. The pellet contains 38,9% of unwanted proteins.
-1M for NaCl: The percentage of lysozyme in the supernatant is equal to 54,9%, which represents 6,3% of lysozyme in relation to the rest of proteins. The pellet contains 49,9% of the unwanted proteins.

4.2. – Chromatography

4.2.1. -Ion exchange chromatography

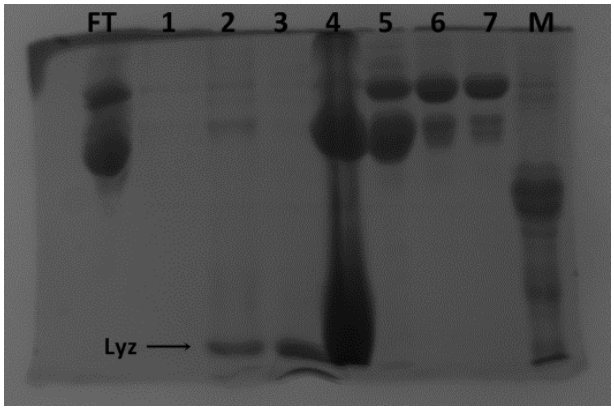

Fig. 11. *SDS-PAGE pattern of fractions obtained by 3% PEG precipitation and ion exchange chromatography. M: Marker; FT: flow-through; 1-7: fractions from 11-5 (in order of elution); 3,2: fractions 9,10. In those fractions, one band around 14kDa appears which is supposed to be lysozyme.*

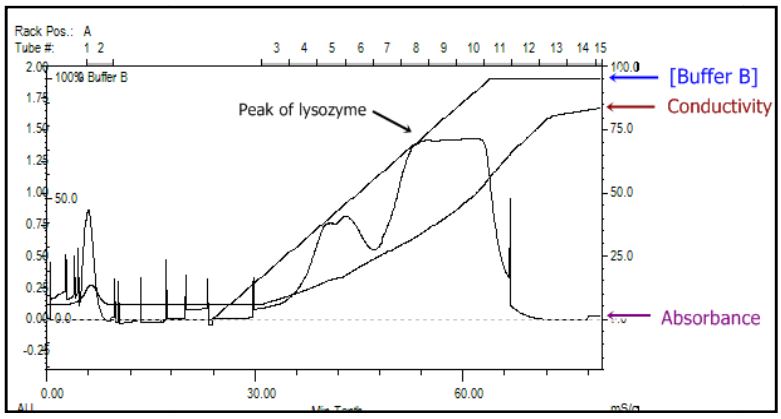

Fig. 12. *Chromatogram of the cation exchange chromatography.*

The supernatant obtained after the protein precipitation step was injected into the cation exchange column. The pH inside the column has a value around 8, therefore the egg white proteins, except lysozyme and avidin (Table 2), will be of negative charge. The only protein we expect that these protein will bind to the matrix; the rest of proteins are expected to pass through the column (FT). For the elution of the lysozyme, 1M NaCl was used. As shown in the chromatogram (Fig. 12), the increase of buffer containing NaCl (named buffer B) implies an increase of the conductivity. When the conductivity started to rise, the fractions started to be collected. Fractions number 1 to 4 were discarded; fractions number 5 to 11 and the flow-through were loaded on the gel (Fig. 11), obtaining lysozyme in fraction 9 and 10.

4.2.2. -Hydrophobic interaction chromatography

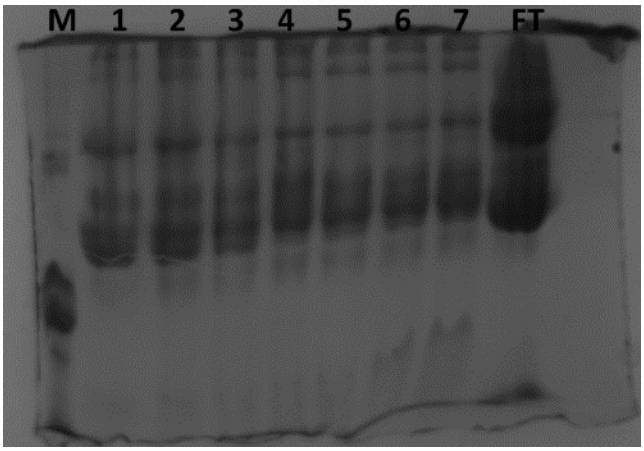

Fig. 13*. SDS-PAGE pattern of fractions obtained by (NH4)2SO4 precipitation and hydrophobic interaction chromatography. M: Marker; 1-7: fractions 2-8 (in order of elution); FT: flow-through. No clear lysozyme band can be appreciated.*

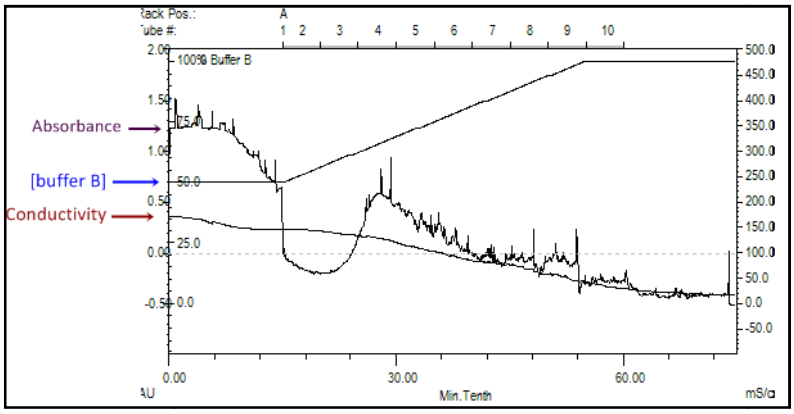

Fig. 14. *Chromatogram of hydrophobic interaction chromatography. No clear peak of lysozyme can be appreciated in the chromatogram.*

Sulfate salts -such ammonium sulfate- are cosmotropic agents, known for their ability to contribute to the stability of intermolecular interaction in proteins. Cosmotropes are used to prevent protein aggregation at protein extraction. The high amount of this salt after the protein precipitation offers the possibility of using the supernatant on hydrophobic interaction chromatography. This type of chromatography takes the advantage of the hydrophobicity of proteins, promoting its separation on the basis of hydrophobic interactions between the hydrophobic matrix and non-polar regions on the surface of proteins. Thus, the supernatant obtained from the precipitation with 500mM ammonium sulfate was injected into the octyl sepharose column. As the adsorption increases with high salt concentration in the mobile phase, the equilibration buffer contains around 1,5M ammonium sulfate, and the salt concentration will decrease in order to elute the different fractions. Hydrophobic interactions are stronger at a pH closer to the pI of the protein, so that the sample was adjusted to a pH

value of 7. Lysozyme was expected to be retained longer than the rest of proteins. The fraction collection started when the absorbance increased and the conductivity decreased (Fig.14). However, no pure lysozyme was obtained with this method at these conditions (Fig. 13).

4.2.3. -Size exclusion chromatography

The supernatant obtained from 500mM ammonium sulphate precipitation was then re-precipitated by using 16% PEG. The pellet obtained from this precipitation was resuspended and injected into the Sephadex column. Sephadex is a material that allows the separation of proteins on the basis of its molecular weight. The largest and heaviest proteins will pass through the column first, while the smallest will be retained onto cavities present on the matrix, and will be eluted later. For this kind of chromatography, uniquely one buffer solution is used during the process: 1M NaCl 50mM Tris (pH 8). The elution started when the absorbance rose (Fig. 16).

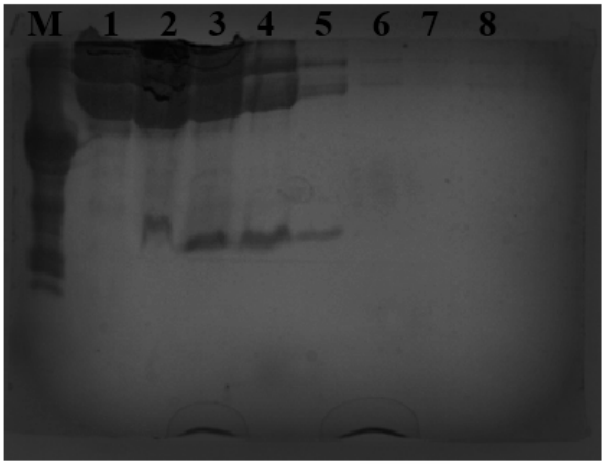

Fig. 15. *SDS-PAGE pattern of fractions obtained by size exclusion chromatography. M: Marker; 1-8: fractions. One band around 14kDa appears which is supposed to be lysozyme.*

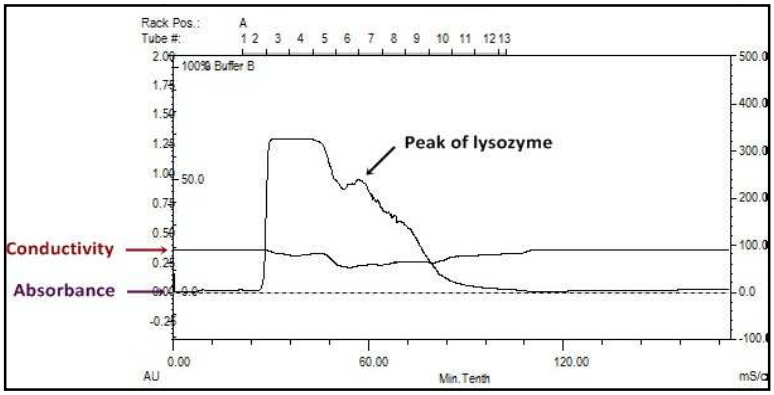

Fig. 16. *Chromatogram of the gel filtration chromatography.*

As lysozyme is the smallest protein in the egg white, it will be eluted at the end of the process. However, no pure lysozyme was obtained with this method at these conditions (Fig. 15).

4.2.4. -Affinity chromatography

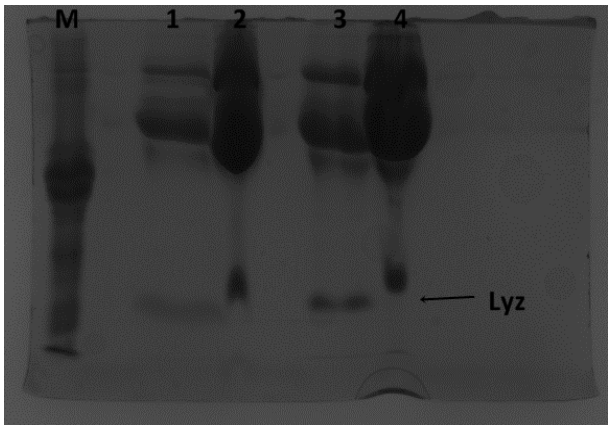

Fig. 17. *SDS-PAGE pattern of fractions obtained by precipitation with 3% PEG (1, 2) and ammonium sulfate (3, 4) coupled with affinity chromatography. M: Marker; 1: Eluted fraction; 2: Flow-through; 3: Eluted fraction; 4: Flow-through.*

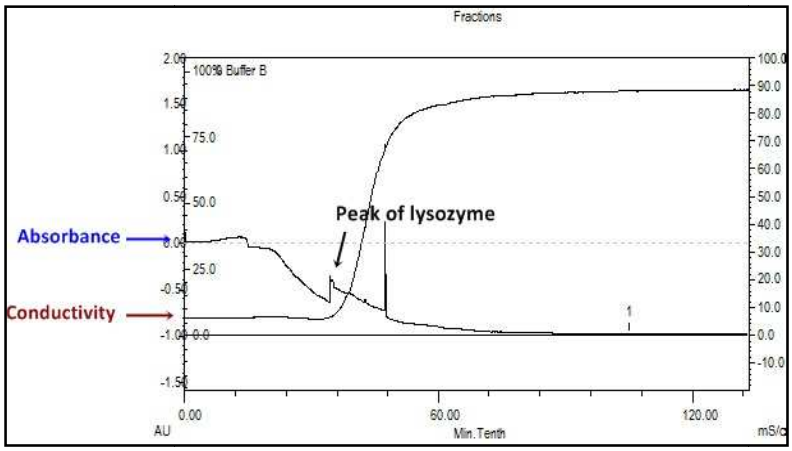

Fig. 18. *Chromatogram of the affinity chromatography coupled with PEG precipitation*.

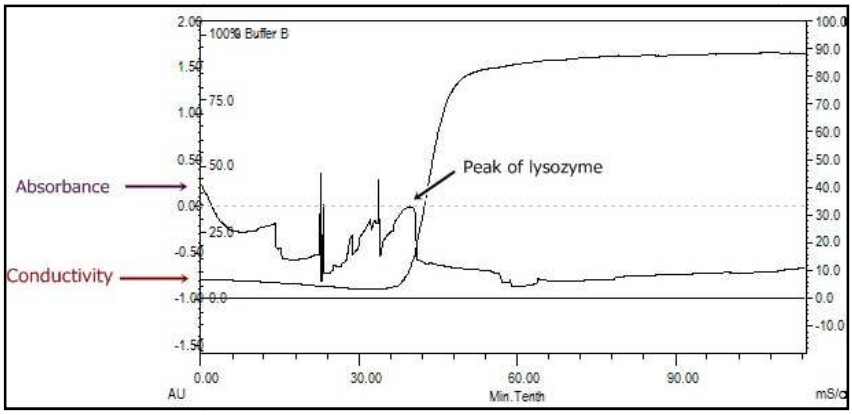

Fig. 19. *Chromatogram of the affinity chromatography coupled with ammonium sulfate precipitation.*

The supernatant obtained from 3% precipitation and the one obtained from ammonium sulfate precipitation were injected into the affinity column, using the same material as in gel filtration

chromatography: Sephadex. It has been reported that lysozyme binds selectively Sephadex in a pH dependant manner [18]. The pH value was adjusted to 8 before injecting the sample. Only lysozyme is expected to bind to the matrix (fractions 1 and 3), the other proteins are expected to pass through the column (fractions 2 and 4). 1M NaCl was used to elute the lysozyme. The flow-through and the elution fraction were collected in one tube each. The samples were then loaded on a SDS-PAGE gel to determine the purity of the lysozyme (Fig. 17). Both fractions contained a band, supposed to be lysozyme. No pure lysozyme was obtained in this method at these conditions.

4.2.5. –Summary of chromatography results

- Only the cation exchange chromatography allowed the extraction of pure lysozyme.

- The hydrophobic interaction chromatography, affinity chromatography or gel filtration chromatography techniques yielded no pure lysozyme at these work conditions.

4.3. – Activity

Lysozyme exhibits high bacteriolytic activity against gram-positive bacteria, due to its ability to break the 1,4-β-linkages presents in the peptidoglycan layer, which is the component of cell walls in bacteria. However, it has low effect against gram-negative bacteria, because of the complex structure of the cell wall. When the lysozyme breaks the bacterial cell wall, the bacteria die and the turbidity decreases.

Normally, a bacterial suspension of *Micrococcus lisodeikticus* is used for the activity measurement of lysozyme [21]. In this work, we used *Escherichia coli* and *Staphylococcus epidermidis* to determine the activity of lysozyme in both gram-negative and gram-positive bacteria. The measurement of the lysozyme activity was done as explained before. The absorbance data, in relation to time, were represented on dispersion graphs. To calculate the activity, we used the following formula:

$$\frac{\Delta Absorbance \times dilution\ factor}{\min \times \varepsilon} = \frac{\mu M}{min} = \frac{\mu mol}{ml \times min} = {}^{U}/_{ml}$$

As it is reported in Table 9, the enzyme exhibits a lower activity against *E.coli* (gram negative bacteria) than against *S. epidermis* (gram positive bacteria). We have selected only the fractions containing pure lysozyme to make this analysis. Some samples (29_8 and 29_9) present low activity against *S. epidermis*. It might be caused by a type of denaturation during the storage, which made the protein to lose its activity.

Table 10. Activity values of different samples obtained from ion exchange chromatography.

	S. epidermis		***E.coli***	
IEXCH fractions	**Abs/t**	**activity (U/ml)**	**Abs/t**	**Activity (U/ml)**
27_8	0,006	48	0,002	16
27_9	0,001	8	0,0004	3,2
28_9	0,005	40	0,001	8
28_10	0,002	16	0,0004	3,2
29_8	0,0003	2,4	0,0006	4,8
29_9	0,0005	4	0,0006	4,8
30_8	0,009	**72**	0,004	32

CONCLUSIONS

1. The most effective precipitants are polietilenglycol and ammonium sulfate -at a concentration of 3% and 500mM, respectively- since these substances achieved a great depletion of high molecular weight proteins from the supernatant, providing its clarification to overcome the egg white viscosity problem.

2. Uniquely the ion exchange chromatography technique yielded lysozyme of high purity. At our work conditions, no pure lysozyme was obtained after the hydrophobic interaction, gel filtration or affinity chromatography.

3. The most advantageous method for lysozyme isolation from the egg white is a two-step protocol based on a protein precipitation with 3% PEG, followed by cation exchange chromatography.

CONCLUSIONES

1. Los agentes precipitantes más eficaces son polietilenglicol y sulfato de amonio -a una concentración del 3% y de 500mM, respectivamente- ya que consiguen una gran disminución en el sobrenadante de las proteínas de elevado peso molecular y, por tanto, la clarificación del mismo, solucionando así los problemas relacionados con la viscosidad de la clara de huevo.

2. Únicamente la cromatografía de intercambio iónico posibilitó la obtención de lisozima de alta pureza. En las condiciones de trabajo, no se obtuvo lisozima pura tras la cromatografía de interacción hidrofóbica, ni tras la de exclusión molecular ni tras la de afinidad.

3. El método más ventajoso para la extracción de lisozima a partir de la clara del huevo se basa en un protocolo en dos pasos que combina una precipitación de proteínas con polietilenglicol al 3%, seguida de una cromatografía de intercambio iónico.

6. –Bibliography

1. *Bioactive Egg Compounds* (ed. by Rainer Huopalahti, Rosina López-Fandiño, Marc Anton and Rüdiger Schade). Springer-Verlag Berlin Heidelberg 2007.

2. W. Carrillo, A. García-Ruiz, M. V. Moreno-Arribas, I. Recio. *Antibacterial Activity of Hen Egg White Lysozyme Modified by Heat and Enzymatic Treatments against Oenological Lactic Acid Bacteria and Acetic Acid Bacteria*. Journal of Food Protection, Vol. 77, No. 10, 2014, Pages 1732–1739.

3. R. Cegielska-Radziejewska, G. Leśnierowski, J. Kijowski. *Properties and applications of egg white lysozyme and its modified preparations- A review-* Pol. J. Food Nutr. Sci. 2008, Vol. 58, No. 1, pp. 5-10.

4. C. Guérin-Dubiard , M. Pasco, A. Hietanen, A. Quiros del Bosque, F. Nau, T. Croguennec. *Hen egg white fractionation by ion-exchange chromatography*. Journal of Chromatography A, 1090 (2005) 58–67.

5. D. A. Omana, J. Wang, J. Wu. *Co-extraction of egg white proteins using ion-exchange chromatography from ovomucin-removed egg whites*. Journal of Chromatography B, 878 (2010) 1771–1776.

6. D. Datta, S. Bhattacharjee, A.Nath, R. Das, C. Bhattacharjee, S. Datta. *Separation of ovalbumin from chicken egg white using two-stage ultrafiltration technique.* Separation and Purification Technology. Volume 66, Issue 2, 20 April 2009, Pages 353–361.

7. Ç. Mecitoğlu, A. Yemenicioğlu, A. Arslanoğlu, Z. S. Elmacı, Figen Korel, A. E. Çetin. *Incorporation of partially purified hen egg white lysozyme into zein films for antimicrobial food packaging.* Food Research International 39 (2006) 12–21.

8. W. H. Gordon Alderton, H. L. Fevold. *Isolation of lysozyme from egg white.* J. Biol. Chem. 1945, 157:43-58.

9. R. Haselberg, S. Harmsen, M.E.M. Dolman, G.J. de Jong, R.J. Kok, G.W. Somsen**.** Characterization of drug-lysozyme conjugates by sheathless capillary electrophoresis–time-of-flight mass spectrometry. Analytica Chimica Acta 698 (2011) 77– 83.

10. Y. Wan, J. Lu, Z. Cui. *Separation of lysozyme from chicken egg white using ultrafiltration.* Separation and Purification Technology 48 (2006) 133–142.

11. N. Ehsani, S. Parkkinen, M. Nystrom. *Fractionation of natural and model egg white protein solution with modified and unmodified polysulfone UF membranes*. J. Membr. Sci. 123 (1997) 105–119.

12. C.T. Chang, L.H. Chen, H.Y. Sung, M.D. Kao. *Studies on the purification of lysozyme from egg white by ultrafiltration*. J. Chin. Agric. Chem. Soc. 24 (1986) 86–93.

13. T.D. Durance. *Separation, purification and thermal stability of lysozyme and avidin from chicken egg white*. J.S. Sim, S. Nakai (Eds.), Egg Uses and Processing Technologies, New Developments, International Cab, Wallingford, 1994, pp. 77–93.

14. R. Ghosh, S. Sudarshana Silva, Z. Cui. *Lysozyme separation by hollow-fibre ultrafiltration*. Biochemical Engineering Journal 6 (2000) 19–24.

15. F. Geng, Q. Huang, X. Wu, G. Ren, Y. Shan, G. Jin, M. Ma**.** *Co-purification of chicken egg white proteins using polyethyleneglycol precipitation and anion-exchange chromatography*. Separation and Purification Technology 96 (2012) 75–80.

16. L. Quan, Q. Cao, Z. Li, N. Li, K. Li, F. Liu. *Highly efficient and low-cost purification of lysozyme: A novel tris(hydroxymethyl)aminomethane immobilized affinity column*. Journal of Chromatography B, 877 (2009) 594–598.

17. N. Başar, L. Uzun, A. Güner, A. Denizli. *Lysozyme purification with dye-affinity beads under magnetic field*. International Journal of Biological Macromolecules 41 (2007) 234–242.

18. R. Islam, J. Kite, A. S. Baker, A. Ching Jr., M. R. Islam. *Affinity purification of hen egg lysozyme using sephadex G75*. African Journal of Biotechnology Vol. 5 (20), pp. 1902-1908, 16 October 2006.

19. Chia-Kai Su, Been Huang Chiang. *Partitioning and purification of lysozyme from chicken egg white using aqueous two-phase system*. Process Biochemistry 41 (2006) 257–263

20. R. Dembczynski, W. Białas, K. Regulski, T. Jankowski. *Lysozyme extraction from hen egg white in an aqueous two-phase system composed of ethylene oxide–propylene oxide thermoseparating copolymer and potassium phosphate*. Process Biochemistry 45 (2010) 369–374.

21. Sigma Aldrich, product information. *Sigma quality control test procedure: Enzymatic Assay of LYSOZYME (EC 3.2.1.17*).