ENZYME PURIFICATION: lysozyme from hen egg

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INTRODUCTION

Hen egg has a lot of proteins in which it is found the lysozyme (EC 3.2.1.17, peptidoglican N-acetil-muramil hydrolase), an enzyme with antibacterial function [1] that was described for the first time in 1965 [2] and with several applications in pharmacological and alimentary industries [3, 4, 5] as long as it´s easy and cheap to extract. It also belongs to the innate defense system.

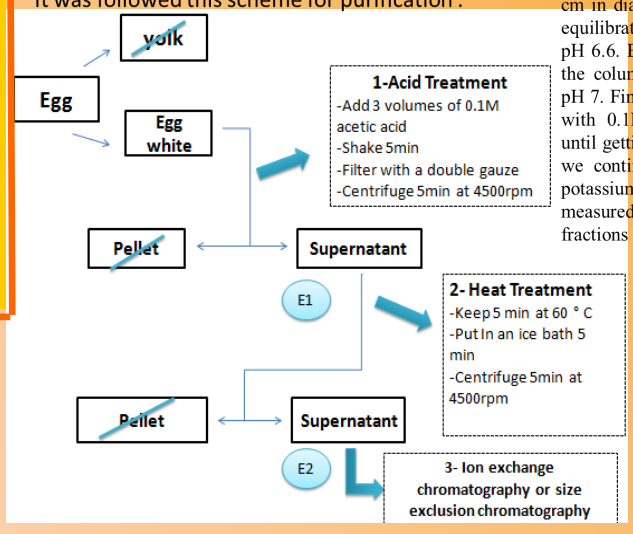
Lysozyme catalyzes the hydrolysis of the β 1-4 glycosidic bond in the bacterial cell wall. This was discovered by Alexander Fleming in 1922 [6], and it is mainly showed in *Micrococcus lysodeikticus.*

The objective of this report is to propose an isolation and purification method for the hen egg lysozyme.

METHOD

Purification of lysozyme from chicken egg

It was followed this scheme for purification :



Ion exchange chromatography

It is assembled the column chromatography, about 3 cm in diameter, with Amberlita CG-50. It must be equilibrated with 0.1M potassium phosphate buffer, pH 6.6. Before loading E2 sample, you must clean the column with 1M potassium phosphate buffer, pH 7. Finally, it is collected 10 ml fractions, eluting with 0.1M potassium phosphate buffer, pH 6.6, until getting an absorbance at 280 nm of zero. Next we continue collecting 3mL fractions with 0.6M potassium phosphate buffer pH 6.6. Absorbance is measured to 280nm for each tube, to select the fractions that show the highest absorbance.

Size exclusion chromatography

The chromatography will be carried out in 0.1M acetic acid. 1mL of E2 is added in the column and 2mL fractions are collected until a volume 20% higher than total volume of the column had eluded. Absorbance is measured to 280nm for each tube, to selecting the fractions that show the highest absorbance.

Enzyme assay

An aggregate of *M. lysodeikticus* walls was used to measure the velocity with the enzyme break these walls. First, 3mL of the suspension are pipetted in the spectrophotometer cuvette and check the absorbance at 450nm. Next, 0.3mL of the chromatography fraction are added in the cuvette and it is measured its absorbance at 450nm every 0.5min during 3min.

Samples that show highest activity are gathered as E3.

~ Turbidity should be linear in that time, if it is not, it must be performed the appropriate dilutions.

To quantify the enzymatic activity we used this equation:

Equation 1:

$$Actividad\ \left(\frac{UAL}{mL}\right) = (\Delta Abs_{450nm}/min) \cdot \frac{1UAL}{(-0.001/min)} \cdot \frac{1}{0.3mL} \cdot Factor\ dilución$$

Determination of protein concentration

First of all a standard curve with BSA 0.2mg/ml must be built. It was carried out by Bradford method, with dilutions of E1, E2 and E3 in a final volume of 0.3mL and 2.7mL of Bradford. Absorbance at 595nm are measured.

Electrophoresis

An electrophoresis in denaturing conditions (PAGE-SDS) is carried out, according to the method of Laemmli.

It is picked: 414µg of E1, 255µg E2, 30 µL of 1/20 dilution of the first peak of absorbance in the ion exchange chromatography (tube 3) and 4.92µg of E3. Then, loading buffer in a concentration of 2X is added to each dilution. Finally, the system is connected at 25mA for an hour. Later gel is put in a container with Coomassie blue to stain it. At the end it must be cleaned two times with off-colour solution and it was rested there some days.

RESULTS

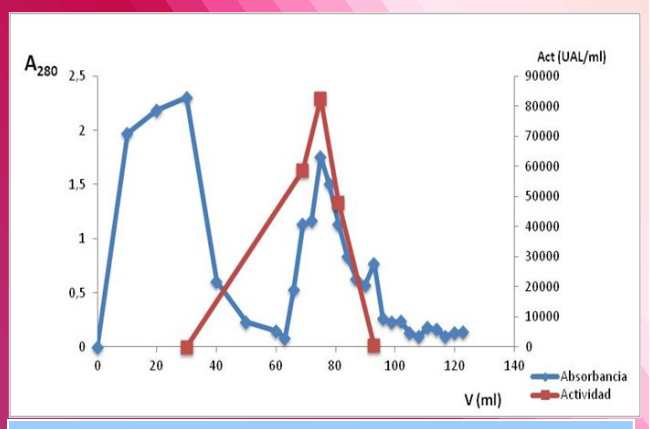


Figure 1. Ion exchange chromatography representation. ♦ Absorbance at 280nm. Washing step (0-60ml) with 0.1M potassium phosphate buffer, pH 6.6.; Elution step (60-123ml) with 0.6M potassium phosphate buffer, pH 6.6. ■ Activity representation. Peaks match but in tube 3 there isn't any activity.

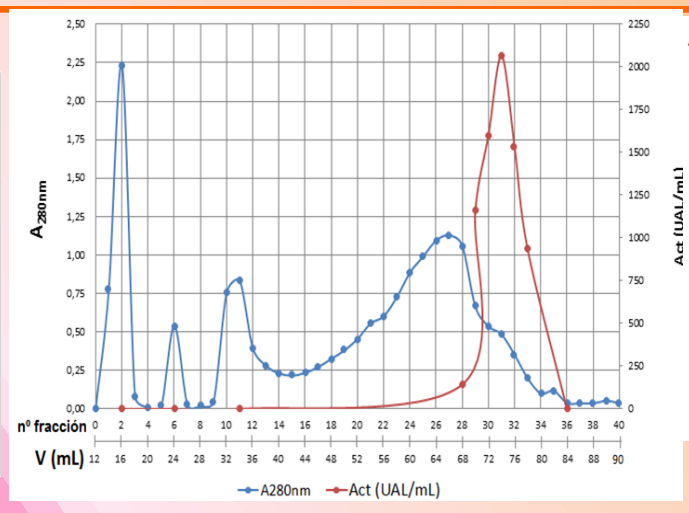


Figure 2. Size exclusion chromatography with Sephadex G-75 representation. It is carried out with 0.1M acetic acid.
●Absorbance at 280nm. ● Activity representation.
The chromatogram shows more peaks because this technique separates proteins according to their molecular mass, so we can see more proteins.

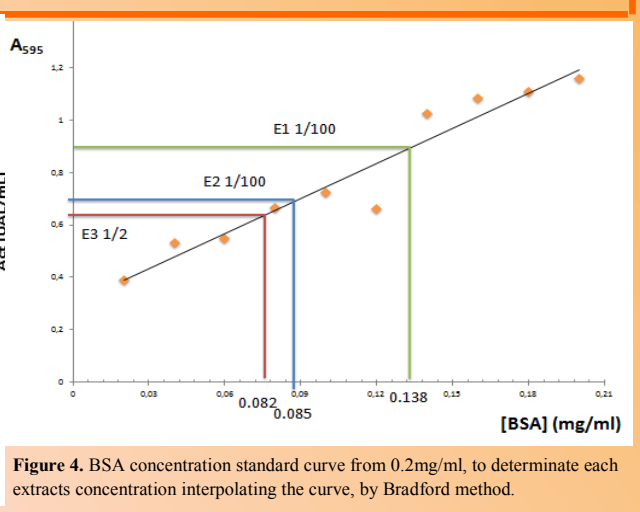


Figure 4. BSA concentration standard curve from 0.2mg/ml, to determinate each extracts concentration interpolating the curve, by Bradford method.

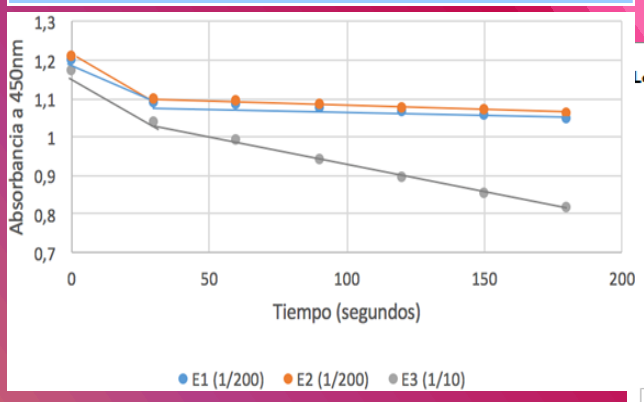


Figure 3: Representation of absorbance at 450nm versus time, for the different extracts. With the slope of the lines and **equation 1** we obtain the activity of each extracts (**chart 1**).

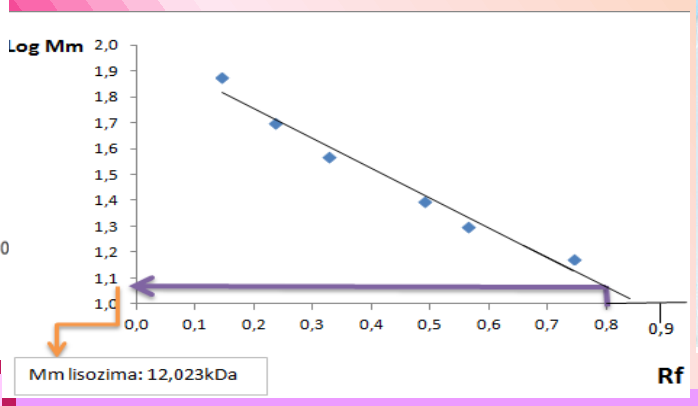


Figure 6. Standard curve to calculate the molecular mass of the samples charged in the electrophoresis. It is showed the logarithm of molecular mass of the pattern used, in front

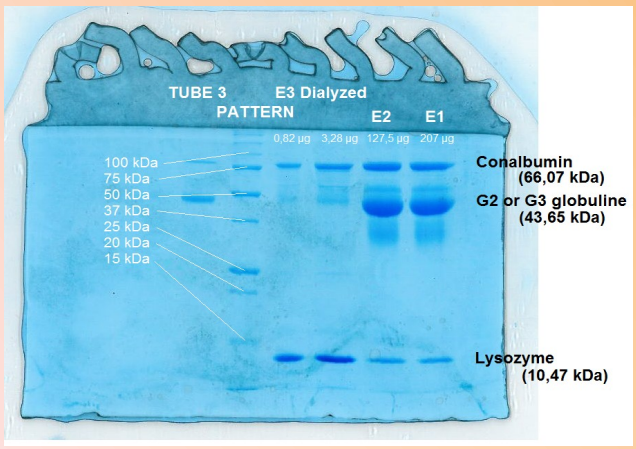


Figure 5. SDS-PAGE electrophoresis by preparing a 15% acrylamide gel. There is no difference between E1 and E2, so we conclude that the heat treatment is irrelevant in order to purify the lysozyme. There are proteins like G2 or G3 globulin (43,65kDa) and conalbumin (66,07 kDa) apart from the lysozyme (10.47 kDa) [6]. It is seen that in E3 still remain some protein traces that we identify with conalbumin, not just lysozyme, so the purification isn´t complete.

Chart 1: Activity of each extract

Sample	Dilution	Activity (UAL/mL)
E1	1/100	11000
E2	1/100	11500
E3	1/20	8266.67

Chart 2: Purification table

ETAPE	Total volume (ml)	Total protein (mg)	Total activity (UAL)	Specific activity (mg/ml)	Throwput (%)	Purification (nº of times)
E1	15	202,5	165000	814,81	100	1
E2	12,8	108,8	147200	1352,94	89	1,66
E3	17	2,788	140533,39	50406,5	85,17	61,863

CONCLUSION

-It is decided to remove heat treatment due to be ineffective.
-We propose the following method of purification:
·acid treatment to suspend the white egg.
·Two chromatographies: size exclusión and ion exchange, both in acid conditions.
·Activity determination by an essay with bacterial walls.
·Proteins concentration determination with the aid of a BSA standard curve.

REFERENCES

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