PURIFICATION OF HEN EGG WHITE LYSOZYME

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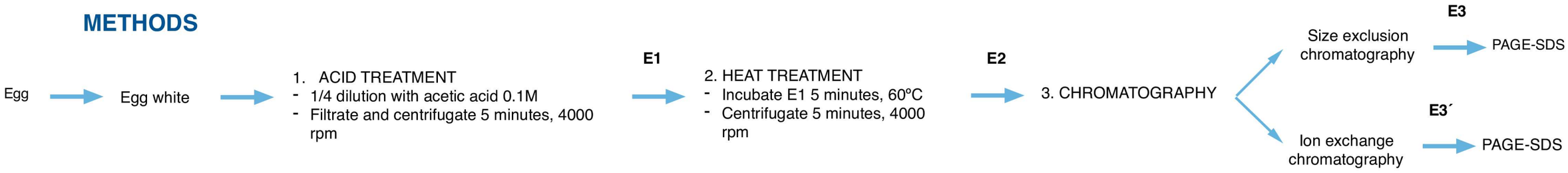
INTRODUCTION

Lysozyme (EC 3.2.1.17; muramidase) is a globular protein with only one polipeptidic cadena of 14.3 KDa and four disulfuric bridges. It is found in human secretions and it has ß(1-4) glucosaminidase activity on N-acetylglucosamine and N-acetylmuramic polymers that constitute the bacteria´s cell walls. Due to its wide distribution, it has been considered that this enzyme belongs to a primitive and inespecific defense system. The bactericidal properties of this enzyme turn it into a perfect mechanism for food preservation, as it prevents the growth of certain bacterias on the food.
Lysozyme was the first enzyme to be sequenced, the first enzyme we could obtained a 3D model by x-ray crystallography of, and the first one we could proposed a detailed catalytic mechanism for. It is a basic protein (pI=10.5-11.0) with a small molecular weight (14.4 KDa) which offers a grate resistance to acid pH.

Thanks to all this information a lysozyme-hen egg white purification process was done. The crude egg white extract was submitted to an acid treatment (acetic acid 0.1M) followed by a thermal treatment (60ºC). The resulting supernatant was passed through a cation exchanger Amberlite CG-50 and the bound lysozyme was eluted with 0.6M potassium phosphate, pH 6.6. These chromatography results were compared with Sephadex G-75 size exclusion ones. Acetic acid 0.1M was used as eluyent. To verify all the steps results, activity and protein content tests, as well as polyacrylamide gel electrophoresis were carried out. The purified protein coming from ion-exchange chromatography showed almost a single band on polyacrylamide gel electrophoresis. In conclusion, a lysozyme purification method with several improvements was proposed.

METHODS



After the isolation of lysozyme, several analyses were carried out:

- E3 and E3´were dialysed in phosphate buffer and concentrated in polyethilenglycol.
- Enzymatic assay: the absorbance of aliquots of each extract (E1,E2,E3,E3') mixed with *Micrococcus lysodeikticus* walls was measured at 450nm. Enzymatic activity in UAL/min was calculated using the following equation:

$$Actividad\ \left(\frac{\text{UAL}}{\text{ml}}\right) = \left(\frac{Abs\ 450\ nm}{min}\right)\left(\frac{1\ \text{UAL}}{-\left(0.001/_{\text{min}}\right)}\right)\left(\frac{1}{0.3\ \text{ml}}\right) * \text{Factor dilución}$$

- Colorimetric assay to determine protein concentration of each extract was done following the Bradford method [13].
- SDS-PAGE electrophoresis were run for extracts E3 and E3´ by the Laemmli method [14] in order to determine lysozyme molecular weight and the degree of purity achieve.

RESULTS

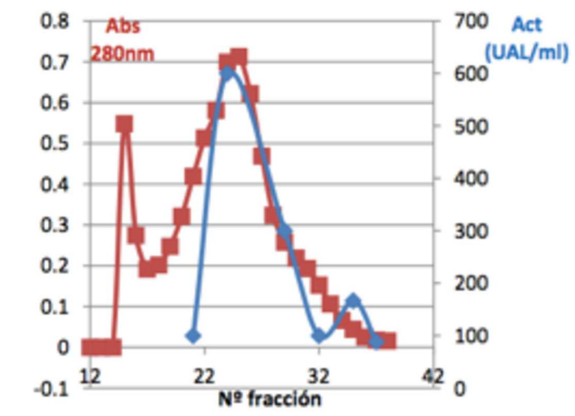


Fig. 1. Elution profile of the size exclusion chromatography (red) and lysozyme enzymatic activity profile (blue). Lysozyme was recollected in fractions 23 to 29 obtaining extract E3´ (12ml). First peak corresponds to contaminant proteins with higher molecular weights than lysozyme. It was supposed that the second peak corresponds to lysozyme.

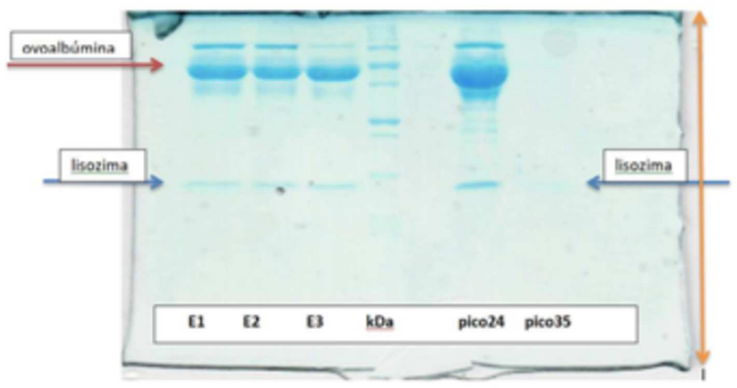


Fig. 5. SDS-PAGE electrophoresis were run in order to analyze the protein content in each extract of the different purification steps of hen egg white. Before running the gels, protein markers were used to create a calibration curve where we interpoled the Rf once we had separated the proteins, in order to know their molecular weights. 15 μl of each sample were put in the wells.
A)Sephadex G-75. E1 and E2 (dilution 1/20) have bands of higher molecular weights which belong to other proteins such as ovoalbumine (44.5KDa) or globulin (49KDa) and a band of 14.3 KDa which belongs to lysozyme. Ovoalbumine is present in all the samples, this means it was not possible to isolate lysozyme by this method.
B) Amberlite G-50. E1, E2 and E3 were diluted 1/20. The pattern of the wash fraction has bands of higher molecular weights, 50 and 60 KDa, that could correspond to ovoalbumine or conalbumine among others. The biggest band in the E3 pattern has a molecular weight of 12.023 KDa. It belongs to the lysozyme, which has been isolated quite well by this method.

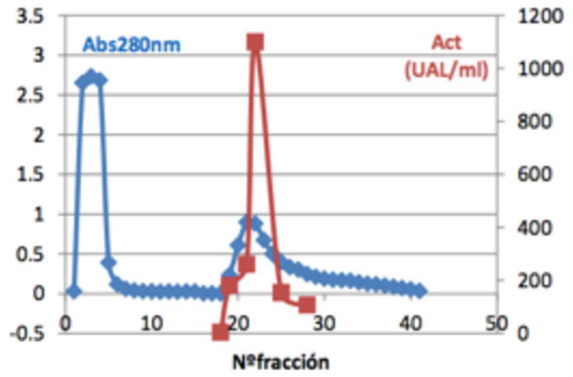


Fig. 2. Elution profile of the ion exchange chromatography (red) and lysozyme enzymatic activity profile (blue). Lysozyme was recollected in fractions 18 to 36 obtaining extract E3 (55ml). The second peak had other basic proteins from the egg white besides lysozyme.

TABLE I. Purification table of lysozyme. Size exclusion chromatography.

Stage	Total volume (ml)	Total Protein (mg)	Total activity (UAL)	Specific activity (UAL/mg)	Rendimiento %	Purification	Activity (UAL/ml)
E1	15.7	254.34	52332.81	205.76	100	1	3333.3
E2	15.5	300.7	51666.15	171.82	98.73	0,835	3333.3
E3´	12	95.7	81235.38	848.85	10.71	4.125	466.87

TABLE II. Purification table of lysozyme. Ion exchange chromatography.

Stage	Total volume (ml)	Total Protein (mg)	Total activity (UAL)	Specific activity (UAL/mg)	Rendimiento %	Purification	Activity (UAL/ml)
E1	15.5	11.2	173.6	946.43	100	1	10600
E2	12	14	168	697.62	71.33	0,7371	9766.66
E3	55	0,22	2.1	1893.9	13.95	2.001	416.66

CONCLUSIONS

- Ion exhange chromatography with Amberlite G-50 was more effective than size exclusion chromatography with Shepadex G-75.
- It was not possible to totally purify lysozyme with any of the two chromatographic methods, however, the Amberlite one allowed us to obtain isolated lysozyme without contaminants.
- The molecular weights obtained for lysozyme are 13.8KDa (size exclusion ch.) and 12.08KDa (ion exchange ch.). Both are near the real molecular weight which is 14.4 KDa.

The heat treatment could be eliminated and we could replace the acid treatment in order to achieve better results. Both chromatography's did not purify the lysozyme, so combining them would lead to better purification with less contaminants. Some other modifications could be done to improve the resolution of the process, such as increasing the length of the molecular exclusion column, changing the eluyents of ion exchange chromatography for a more powerful ones or reducing the pore´s size of the electrophoresis gel to identify lysozyme more easily.