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A SIMPLE, ONE-STEP CHROMATOGRAPHIC PROCEDURE FOR THE PURIFICATION OF LYSOZYME

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Summary

A rapid method for the purification of lysozyme (mucopeptide *N*-acetylmuramoylhydrolase, EC 3.2.1.17) from hen egg-white has been devised. It was that gel filtration chromatography on agarose columns can be used selectively to purify lysozyme, due to the fact that this protein interacts with the agarose matrix and elutes later than the corresponding total volume for the column. Thus, lysozyme is directly obtained in a relatively pure form and with a high specific activity. In principle, this simple method can be used to prepare lysozymes from other sources.

Introduction

Lysozyme (mucopeptide *N*-acetylmuramoylhydrolase, EC 3.2.1.17) from hen egg-white is relatively easy to purify, requiring several steps for a high degree of purity. Direct precipitation by addition of 5% NaCl to an egg-white solution buffered at pH 9.5 was used for bulk preparation of lysozyme [1]; however, in order to obtain a less contaminated enzyme preparation it is necessary to perform at least ion-exchange and a gel filtration chromatography [2,3]. The precipitation method also has the disadvantage that the enzyme is not stable in the buffer (pH 9.5) for long periods [4,5]. The use of affinity chromatography columns is a better selective method for purifying lysozyme, but requires the often lengthy preparation of the affinity matrices [6—8]. Dispersed chitin has been used as affinity adsorbent [9—13] for lysozyme, although it is gradually digested by the chitinase activity of the lysozyme molecule.

Recently, we observed than hen egg-white lysozyme interacts specifically with agarose [14], which may be due to the resemblance of the agarose structure with substrate mucopolysaccharides such at chitin and bacterial cell wall

components. Since the elution volume of lysozyme on agarose columns is far greater than the total volumes of the corresponding columns, contamination with other proteins is unlikely.

Materials

Hen egg-white and turtle (*Testudo graeca*) egg-white were obtained from fresh laid eggs. Eggs from the insect *Ceratitis capitata* were collected each 24 h from the insect culture [15] and stored at −20° C until the quantity required was obtained. Hen egg-white lysozyme, horse serum albumin and bacterial cell walls from *Micrococcus lysodeikticus* were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Biogel A, 0.5 M was from Bio-Rad Laboratories (Richmond, U.S.A.). All other chemicals were reagent grade.

Methods

Preparation of hen egg-white lysozyme. 25 ml of hen egg-white were diluted with 50 ml of 0.05 M potassium phosphate (pH 6.3) and 10 ml applied to a Biogel A, 0.5 mM column (30 × 2.6 cm) equilibrated and eluted with the same buffer. The eluate was continuously monitored at 280 nm on a LKB Uvicord II absorptiometer and lysozyme activity was measured in each 3-ml fraction.

Preparation of turtle egg-white lysozyme. 3.5 ml of egg-white from a *T. graeca* egg were diluted by three volumes of 0.05 M potassium phosphate buffer (pH 6.3) and 6 ml applied to a Biogel A, 0.5 mM column (30 × 2.6 cm) equilibrated with the same buffer. The column was then washed with the equilibration buffer until minimum absorbance at 280 nm was registered and lysozyme displaced from the column with 0.5 M potassium phosphate (pH 6.3). The eluate was monitored as above.

Preparation of lysozyme from eggs of the insect C. capitata. Homogenization, extraction and heat treatment of the insect eggs were as previously reported [16]. The clear solution obtained was applied to a Biogel A, 0.5 mM column (30 × 2.6 cm) equilibrated with 0.05 M potassium phosphate (pH 6.3). The elution of the agarose column was performed and monitored as above.

Enzyme assays.s Lysozyme activity was determined by its lytic activity towards *M. lysodeikticus* cell walls at pH 6.3 and 0.1 M ionic strength according to the Sigma Chemical Co. (St. Louis, U.S.A.) assay procedure. One unit of lysozyme activity is defined as the decrease of 0.001 absorbance units per min at 450 nm in those assay conditions.

Protein determination. Protein estimation was carried out according to the method of Lowry et al. [17] using horse serum albumin as standard.

Polyacrylamide gel electrophoresis. Purity of lysozyme was checked on polyacrylamide gel electrophoresis performed according to Panyim and Chalkley [18], using Amido Black dye protein stain.

Amino acid analyses. Samples were hydrolyzed at 108° C for different times in 5.7 M HCl containing 0.1% (w/v) phenol in evacuated, sealed tubes. Hydrolysates were analyzed on a Durrum amino acid analyzer (model D 500).

Results and Discussion

Lysozyme from hen egg-white has been purified approx. 74-fold with 100% yield (Table I) by a simple and rapid procedure, making use of its retention on agarose matrices previously reported [14]. The procedure consists of chromatography on an agarose column (Fig. 1). The last peak eluted from the column corresponds to lysozyme activity and the volume was greater than the total column volume (V_t); thus, it is unlikely that it contains contaminants. Comparative patterns of polyacrylamide gels electrophoresis of hen egg-white lysozyme obtained by this method and commercial hen egg-white lysozyme are shown in Fig. 2. Amino acid analyses of both preparations also agree.

This rapid method yields 100% recovery of a highly pure, fully active lysozyme preparation. Obviously, this method, based on the specific interaction of agarose with the active center of lysozyme, provides only those molecules of enzyme that exhibit catalytic activity, since inactive molecules have been observed to elute before the V_t on agarose columns [14]. These facts and the simple and easy experimental performance of the method, as well as the ready repetition of the procedure, are obvious advantages.

The method can be extended to lysozymes from other sources. There appear to be no restrictions on the general use of this method, excepting those cases using sources of proteins with other proteins that could interact with agarose (for example, agarase, EC 3.2.1.81). We have also used the method for the isolation of lysozyme from the dipterous *C. capitata*, with a high ionic strength buffer, which was required for desorption of the *C. capitata* enzyme from the agarose column. This fact is, presumably, due to a stronger interaction between the insect lysozyme and the agarose matrix (compared to hen enzyme). The resultant enzyme from *C. capitata* gives a single band on polyacrylamide gel electrophoresis and an amino acid analysis that agrees well with those of the enzyme purified as previously reported [16]. Agarose columns have been also used in the purification of *T. graeca* egg-white lysozyme in a single step (Rodriguez, R. and Fernández-Sousa, J.M., unpublished). As in the case of the insect enzyme, it was also necessary to apply 0.5 M potassium phosphate in order to elute the turtle lysozyme from the column.

In general, this novel and rapid method for obtaining preparations of lysozyme seems to be widely applicable, with slight modifications pertinent to each source. Also, elution conditions for the agarose columns should be chosen in each case according to the strength of the lysozyme-agarose interaction.

TABLE I

PURIFICATION OF HEN EGG-WHITE LYSOZYME

Step	Volume (ml)	Total activity (units)	Activity/ml (units/ml)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Starting material	10	250 000	25 000	455	549	—	100
Lysozyme peak	96	250 000	2 604	6.1	40 783	74.1	100

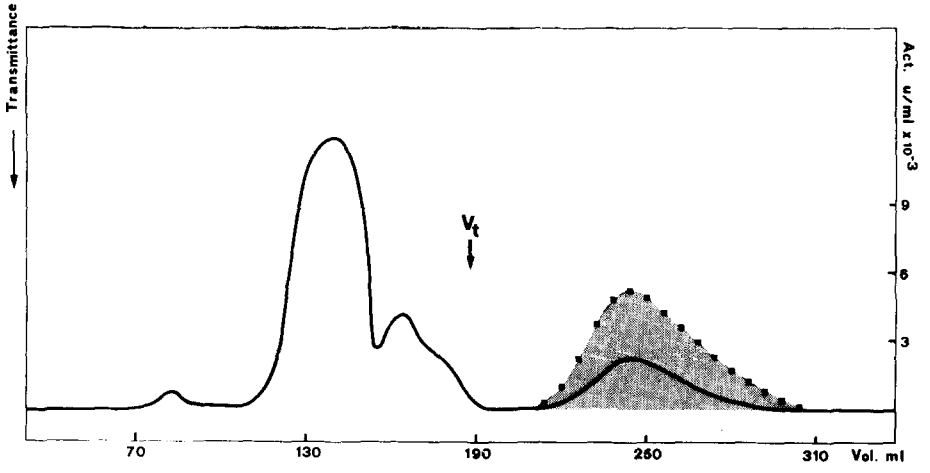

Fig. 1. Elution profile of a diluted (1 : 2, v/v) hen egg-white solution on a Biogel A, 0.5 mM column (30 X 2.6 cm) and developed as described in the text. Total protein was monitored at 280 nm through a LKB Uvicord II (———). Fraction of 3 ml were assayed for lysozyme activity (■). The shaded zone corresponds to the peak exhibiting lysozyme activity. Total volume (V_t) of the column is indicated by an arrow.

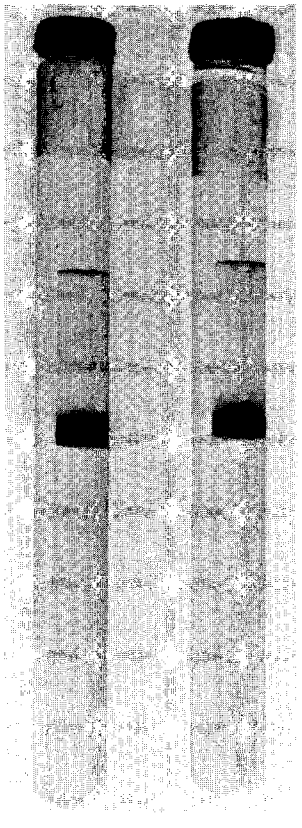

Fig. 2. Electrophoresis in polyacrylamide gels of hen egg-white lysozyme. Protein was stained with Amido Black. Direction of electrophoresis is from top to bottom. Left gel: Commercial lysozyme. Right gel: Lysozyme obtained by the procedure described in the text.

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