THE ISOLATION AND SOME ENZYMATIC PROPERTIES OF DES-ARGINYLLEUCINE-LYSOZYME

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The use of the exopeptidases as sequence determinants for peptides and proteins is well documented (Fraenkel-Conrat et al.,1955). The exopeptidases have also been employed in studies which attempted to relate enzyme structure and function (Potts et al.,1964; Jollès and Jollès,1966). This report describes the effects of the carboxypeptidases (Cpases) on hen's egg-white lysozyme (N-acetylmuramide glycanohydrolase: EC 3.2.1.17). A des-arginyl-leucine-lysozyme was isolated which exhibited different activity from the native molecule, under conditions which affect the ionic state of proteins.

MATERIALS AND METHODS

Hen's egg-white lysozyme (lot 641 and 6JA) was obtained from Worthington. Cpase A-DFP (lot 46B-0401) and Cpase B-DFP (lot 86B-1560) were purchased from Sigma.

Enzymatic digestion: Approximately 500 mg of lysozyme was dissolved in 25 ml 0.1 M Tris buffer (pH 8.0; 0.1 M NaCl). The solution was equilibrated at 25° for 30 minutes, and then 5 ml of a Cpase A solution (1 mg enzyme/ml) was added. After allowing the reaction mixture to stand at 25° for 12 hours, 5 ml of a Cpase B solution (0.8 mg enzyme/ml) was introduced. The reaction was allowed to proceed at 25° for an additional 12 hours, and then dialyzed against 0.001 N HCl for 2 hours.

Chromatography: The dialyzed reaction mixture was chromatographed on a

2 x 40-cm Bio-Rex 70 column. The chromatogram was developed with the aid of a Technicon autograd which was employed as a four-stage gradient device. The first three stages each contained 150 ml of 0.05 M sodium phosphate buffer, pH 7.16, while the fourth stage contained 150 ml of 1 M sodium phosphate buffer, pH 7.22. Fractions (5 ml) were collected at a flow rate of 40 ml/hour, and analyzed for protein content by measuring the optical density at 280 mμ. The desired fractions were pooled, exhaustively dialyzed against water, and then lyophilized.

Estimation of liberated amino acids: Aliquots (0.1 ml) of the reaction mixture were removed at various times, added to 1 ml of 0.2 N sodium acetate buffer (pH 2.2), and chromatographed on a Beckman-Spinco model 120 C amino acid analyzer.

Lysozyme activity: The lysozyme activity of the digestion mixture was measured using M. lysodeikticus cell walls, in 0.1 M sodium phosphate buffer, pH 6.8 (Shugar,1952). The enzymatic activity of des-arginylleucine-lysozyme was compared to that of native lysozyme by employing: (a) the method of Shugar (1952); (b) the assay procedure described by Jollès (1962); and (c) the method of Sharon (1967). The substrate used in the latter assay, tetra-(N-acetylglucosamine), was isolated as described by Rupley (1964). The effects of ionic strength and pH on the activities of des-arginylleucine-lysozyme and native lysozyme were carried out according to the methods of Sela and Steiner (1963).

Amino acid analyses: The proteins were hydrolyzed in constant-boiling HCl in sealed and evacuated ampules for 72 hours at 110°. Analyses were performed with a Beckman-Spinco model 120 C amino acid analyzer. The cyanate method of Stark and Smyth (1963) was used for amino terminal end-group analyses.

RESULTS AND DISCUSSION

It is known that hen's egg-white lysozyme contains a -cys-arg-leu carboxyl

terminus (Jollès,1964; Canfield and Liu,1965). Cpase A treatment of lysozyme resulted in the release of leucine (Table I). The release of other amino acids was not observed, and the lysozyme activity of the digest was unaltered. Attempts to separate a des-leucine-lysozyme from the native protein, by means of ion-exchange chromatography, were unsuccessful. Treatment of lysozyme with Cpase A, followed by the addition of Cpase B, resulted in the release of leucine and arginine. The release of other amino acids was not observed, and the activity of the digest decreased to 75% of the original activity (Table I).

Table I. The enzymatic activity of Cpase treated lysozyme, and the amino acids released by this treatment.

Time of digestion (hours)		Amino acids released[a]		Enzymatic activity[b]
Cpase A	Cpase B	Leucine	Arginine	(%)
0	0	0	0	100[d]
6	0	0.39	0	99
12	0	0.53	0	102
18	6	0.60	0.52	79
24	12	0.67	0.59	75

[a] Expressed as residues amino acid/mole lysozyme.

[b] Assay media contained M. lysodeikticus cells (300 µg/ml) and lysozyme (6 µg/ml) in 0.1 M sodium phosphate buffer, pH 6.8.

[c] Cpase B was added 12 hours after the introduction of Cpase A.

[d] A lysozyme control, in which buffer was substituted for the Cpase solutions, showed (over a 24 hour period) no decrease in lysozyme activity.

Preliminary experiments demonstrated that 4 additions of Cpase A (0.5 mg/addition), over a 48 hour period, to 5 ml of a lysozyme solution (8.3 mg/ml) resulted in a stoichiometric release of leucine. Lysozyme activity of the digest was not altered. Upon the addition of Cpase B (0.48 mg), a stoichiometric amount of arginine was observed in 6 hours. The lysozyme activity decreased to 56% of the original.

Chromatography of the Cpase A and B treated lysozyme resulted in two

protein components (labeled as A, and B in Figure 1). Analytical ion-exchange chromatography demonstrated that these components were chromatographically homogeneous.

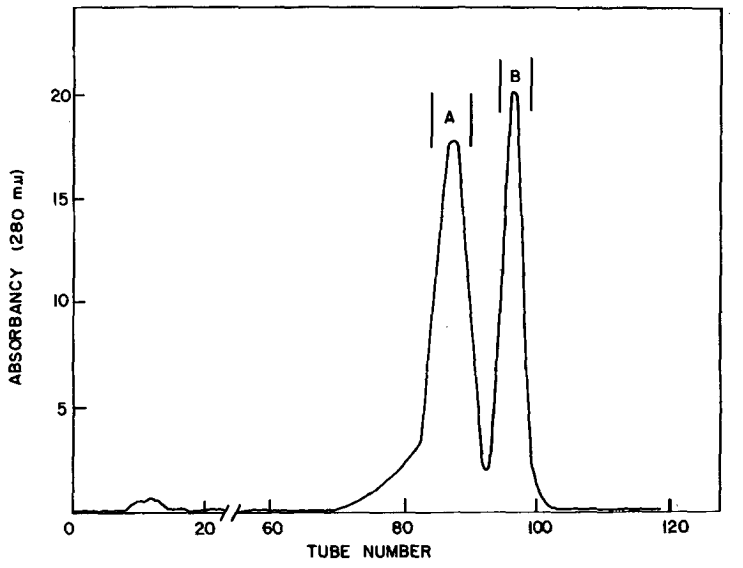

Figure 1: Chromatography of Cpase A and B treated lysozyme (500 mg). The vertical lines represent those fractions which were pooled, dialyzed and lyophilized.

Component B possessed an amino acid composition similar to native lysozyme (Table II). Component A differed from native lysozyme in that it contained one less residue of both leucine and arginine per mole protein. The enzymatic activities of component B and native lysozyme were indistinguishable; on the other hand, component A was 60% as active as the native enzyme (Table II).

The N-terminal amino acid of native lysozyme, and of components A and B was lysine. The yields of lysine for the three proteins ranged from 0.8 to 0.9 residues per mole protein. No other amino acids, except for small amounts of glycine and alanine (0.05 to 0.1 residues/mole protein) were noted. It is concluded that component A was a lysozyme derivative which lacked the arg-leu carboxyl terminus (des-arginylleucine-lysozyme). It is believed that component B was a mixture of two proteins; namely, native lysozyme (as the major constituent) and des-leucine-lysozyme.

Table II. The amino acid analyses and enzymatic activities of components A and B, and of native lysozyme.

Component	Leucine[a]	Arginine[a]	Enzymatic activity (%)[b]
Theory	8	11	100
Native	7.98	10.90	100
A	6.93	9.62	60
B	7.99	10.73	98

[a] The values are averages of 4 analyses, and are expressed as residues per mole protein. Analyses were calculated on the basis of 12 alanine residues per mole protein. The yields of all other amino acids were, within experimental error, similar to the previously reported values (Jollès,1964).

[b] Assays performed according to the procedure of Shugar (1952).

A study of the effects of ionic strength on enzymatic activity (Figure 2) yielded results for the native protein which were similar to those reported by

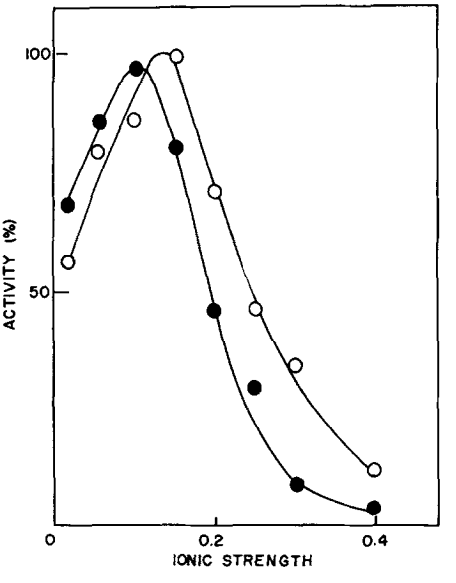

Figure 2: Enzymatic activities as functions of ionic strength. Assay media contained 0.0075 M Tris and 0.0075 M malonic acid, adjusted with NaOH to pH 6.0 and with NaCl to the indicated ionic strength (Sela and Steiner,1963). The rate constant for the activity of lysozyme at an ionic strength of 0.15 was considered to be 100% (Sharon,1967).
○ -- lysozyme; ● -- des-arginylleucine-lysozyme.

Sela and Steiner (1963). At low ionic strength, des-arginylleucine-lysozyme was slightly more active than the native molecule, whereas at high ionic strength des-arginylleucine-lysozyme was shown to be significantly less active.

A study of the effects of pH on lysozyme activity revealed that, between pH 5.5-8 and at an ionic strength of 0.2, des-arginylleucine-lysozyme was less active than was the native molecule (Figure 3). On the other hand, at an ionic strength of 0.1 and between pH 6-8, des-arginylleucine-lysozyme was more active (10-20%) than was lysozyme. It was also noted that, at an ionic strength of 0.1, the activity curves showed broader pH optima than those depicted in Figure 3.

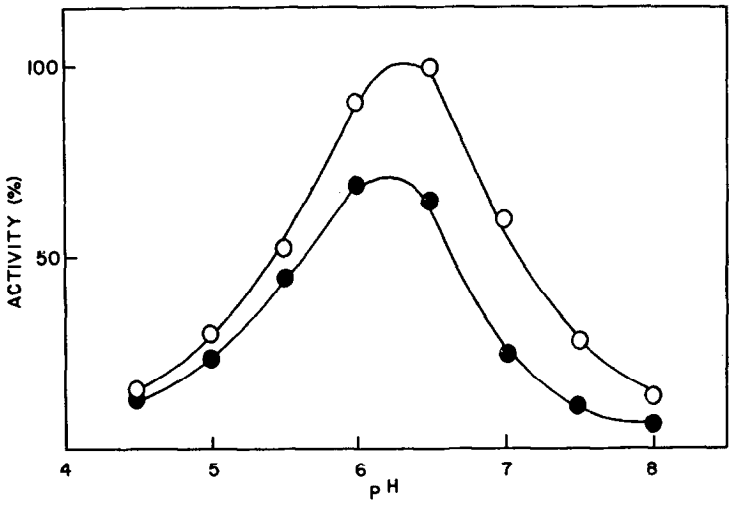

Figure 3: Enzymatic activities as functions of pH. Assay media contained 0.025 M Tris and 0.025 M malonic acid, adjusted with NaOH to the desired pH and with NaCl to an ionic strength of 0.2. The rate constant for the activity of lysozyme at pH 6.5 was considered to be 100% (Sharon,1967). O -- lysozyme; ● -- des-arginylleucine-lysozyme.

In experiments employing tetra-(N-acetylglucosamine) as the substrate (assay media contained citrate-phosphate buffer; pH 5.8; Sharon,1967), des-arginylleucine-lysozyme was 15-20% less active than the native molecule. Using the assay procedure of Jollès (1962), no significant difference in activities was noted. Under the conditions employed in the latter procedure

(M/15 phosphate buffer; 0.1% NaCl; pH 6.2), it would be expected, on the basis of the pH and ionic strength results, that the activities should be similar.

Previous reviews have reported that the action of the Cpases on lysozyme resulted in the release of leucine and arginine (Jollès,1964; Jollès,1967). Furthermore, it was reported that the lysozyme activity of these digests was unaltered. We have observed that, under certain conditions of ionic strength and pH, the enzymatic activity of a chromatographically homogeneous des-arginylleucine-lysozyme differed from that of native lysozyme. These activity differences may reflect altered conformational properties arising from variations in the mechanisms by which neutral salts affect the organized structures of the two proteins.

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