LYSOZYME OF THE HUMAN PAROTID GLAND SECRETION: ITS PURIFICATION AND PHYSICOCHEMICAL PROPERTIES[1]

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SUMMARY

Lysozyme was isolated from human parotid saliva (HPL) and chromatographically purified. The amino acid composition of the purified HPL was similar to other human lysozymes. The minimum molecular weight calculated from the amino acid composition compared favorably with values reported from other lysozymes (1). The molecular weight determined by analytical ultracentrifugation was in the range from 15,000 to 17,000, depending on the value used for the partial specific volume in the calculation. The specific activity of HPL was 2.5 times greater than that of hen's egg white lysozyme (HEL). Accordingly, the true concentration of the enzyme in the native parotid gland secretion is about 0.5 mg per 100 ml and thus lower than that based on enzyme assay using HEL as the standard of reference.

Although lysozyme-like activity has been demonstrated in human parotid saliva (2,3), isolation of the enzyme has not been reported. Human parotid saliva has been resolved into 6 to 8 protein bands by paper electrophoresis, but no evidence of lysozyme activity could be found (4). The present paper describes the isolation and purification of human parotid saliva lysozyme (HPL) and presents some of its physiocochemical properties.

MATERIALS AND METHODS

Lysozyme was assayed by measuring the rate of lysis of Micrococcus lysodeikticus cells according to the method of Shugar (5). Hen's egg white

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lysozyme[2] (HEL) was used as the standard, and enzyme activities were expressed as HEL equivalents. Ultraviolet light absorption in the eluates of chromatographic separations was monitored at 280 mμ. Protein concentration was calculated assuming the molar absorptivity (ε) for HEL (ε = 35,000 at 280 mμ) as applicable to HPL.

Specific activity was the HPL activity expressed in HEL equivalents per mg of protein. The dry weight of protein used in calculating specific activity of the purified enzyme was obtained from the amino acid analysis, after correction for water of hydrolysis.

Amino acid analyses were performed on a Beckman-Spinco Model 120B amino acid analyzer following hydrolysis of protein samples for 22 hours in 6 N HCl, under vacuum, at 110° C. The molecular weight of the purified HPL was determined in a Beckman-Spinco Model E analytical ultracentrifuge according to the method of Yphantis (6).

Experimental - Gel Filtration Studies

Parotid saliva was collected from male donors by indirect cannulation of the right parotid gland, using sour lemon drops to stimulate the secretion. In one set of experiments, native pooled saliva at pH 4.5 was applied to a G-25 Sephadex column (4 x 140 cm) and eluted with glass-distilled water. In another set of experiments, a heat-treated saliva filtrate was applied on a G-25 Sephadex column having the same dimensions and eluted in the same manner as in the preceding experiments. Gel filtration experiments were performed at room temperature.

Step 1 - Preparation of Heat-Treated Saliva Filtrate

The pH of a pooled parotid saliva sample (300 ml) was adjusted to 4.5 by the addition of glacial acetic acid, heated to 91° C in a double boiler and held at this temperature for two minutes. Coagulated protein was separated by high speed centrifugation at 18,800 g for 30 minutes at 0° C., and the

[2]Crystallized lysozyme from Armour Pharmaceutical Company.

supernatant was passed through a fine sintered glass filter.

Step 2 - Adsorption of Lysozyme on Bio-Rex 70

The filtrate from the preceding step was brought to pH 6.7 by the addition of 12.5 N sodium hydroxide. Four ml of Bio-Rex 70 resin, previously equilibrated with 0.2 M phosphate buffer (pH 6.7), was added to the saliva filtrate and stirred for 30 minutes at room temperature. The supernatant was routinely examined for residual lysozyme activity.

Step 3 - Bio-Rex 70 Column Chromatography

The 4 ml of Bio-Rex 70 carrying lysozyme was quantitatively transferred to the top of a Bio-Rex 70 column (0.9 x 20 cm) previously equilibrated with 0.2 M phosphate buffer (pH 6.7). Elution was accomplished with the same buffer, at room temperature.

Step 4 - Isolation of Parotid Fluid Lysozyme

Fractions from the Bio-Rex 70 column containing lysozyme activity were pooled (total volume approximately 100 ml) and concentrated to a final volume of approximately 5 ml in a Diaflo[3] cell equipped with a UM-2 membrane at room temperature. The concentrate was quantitatively transferred to a dialysis bag (Nojax 23)[4], dialyzed against 0.2 M ammonium acetate (pH 6.6) at 4° C., and finally lyophylized.

RESULTS

Figure 1 shows G-25 Sephadex elution patterns of native parotid saliva and parotid saliva filtrate. The effect of the heat treatment is demonstrated by the elution pattern of the filtrate, wherein a drastic reduction in absorbance of the earlier peak(s) was noted. The high absorbance at 280 mμ noted for the last peak in both of the chromatograms was mainly due to the presence of uric acid which absorbs strongly at 290 mμ (7). In both experiments, fractions possessing lysozyme activity contained glyco-proteins,

[3]Diaflo ultrafiltration cells and membranes are products of the Amicon Corp.
[4]Product of Union Carbide Corporation, Food Products Division.

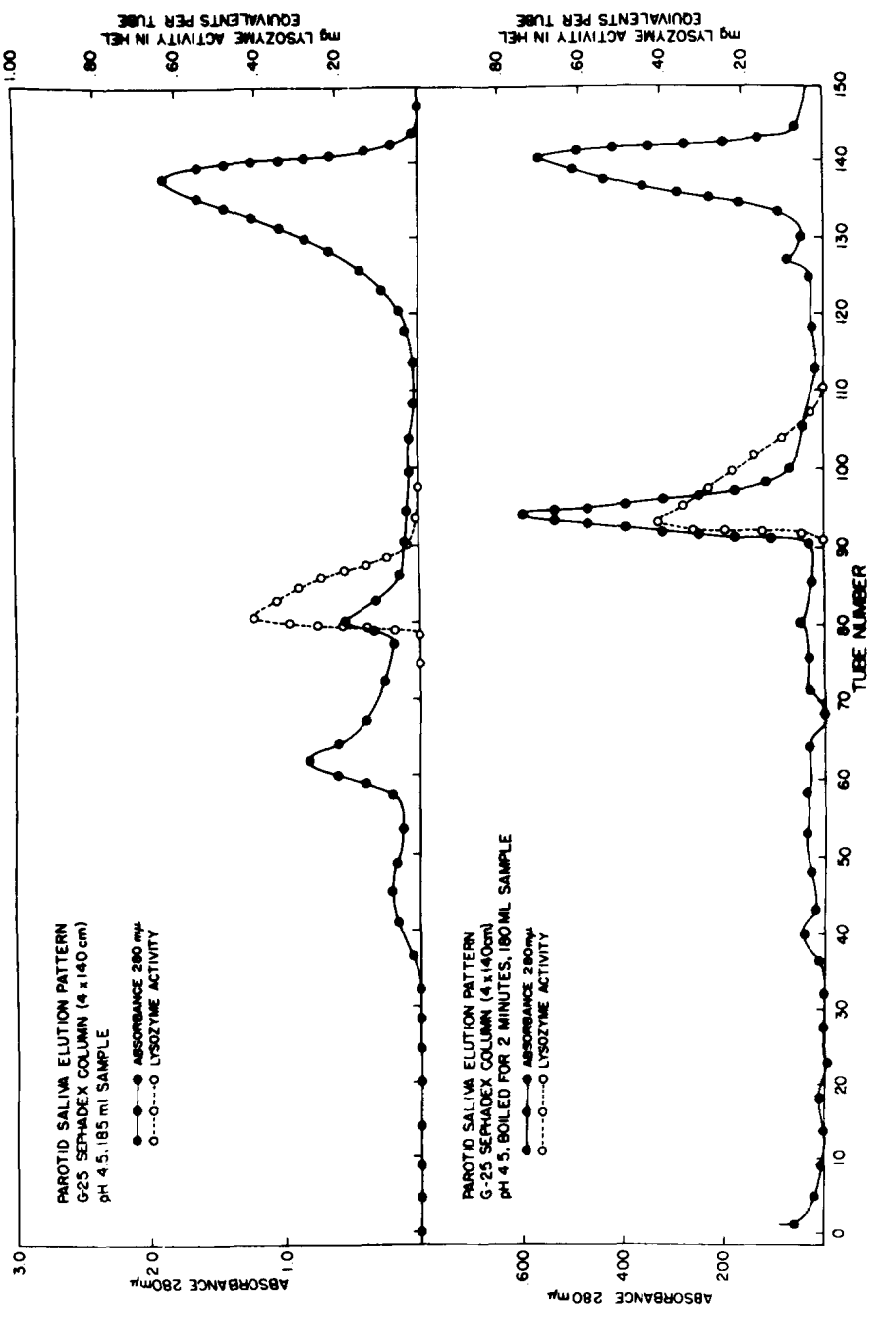

Fig. 1. G-25 gel filtration patterns of parotid saliva and parotid saliva filtrate at room temperature. Column dimensions were 4 x 140 cm and the eluant was glass distilled water. Note suppression of earlier peaks in the filtrate elution pattern due to heat treatment and removal of heat labile proteins. The last peak contained salt and uric acid. Volume collected, 12 ml per tube.

showing that the latter survived heat treatment. The amino acid composition of the glycoproteins was consistent with previously published data (8).

Heat treatment did not result in a loss in overall lytic activity in the native secretion. As shown in Table I, the coagulation of the heat-labile proteins was responsible for the gain in specific activity in the filtrate (3.5 to 5.8).

TABLE I

Summary of Purification of Parotid Saliva Lysozyme

	Total Volume (ml)	Total Activity[1] (mg)	Total Protein (mg)	Specific Activity[4] x 10^3	Yield (%)
Native secretion	325	3.6	1020	3.5	100
Filtrate after heat treatment	325	3.6	617	5.8	100
Bio-Rex 70	150	3.6	1.2[2]	3000	100
Concentration, buffer exchange, freeze-drying	8.7	1.9	0.75[3]	2500	53

(1) Expressed as egg white lysozyme.
(2) Calculated using absorbance at 280 mμ and the molar absorptivity (ε) of egg white lysozyme.
(3) Dry weight of protein calculated from amino acid analysis, correction being made for water of hydrolysis.
(4) Specific Activity = $\frac{\text{Total Activity}}{\text{Total Protein}}$

A typical elution pattern from the Bio-Rex 70 column is shown in Fig. 2. The first peak(s) consisted of glycoproteins, while the fractions collected between 200 and 300 ml contained the lysozyme. The protein concentration calculated from enzymatic activity of the eluted fractions was markedly greater than that calculated from the absorbance at 280 mμ. Assuming equal molar absorptivities for HEL and HPL, it can be calculated that HPL is enzymatically 3 times more active. Had the specific activities of the two lysozymes been the same, the two protein concentration peaks would be superimposed.

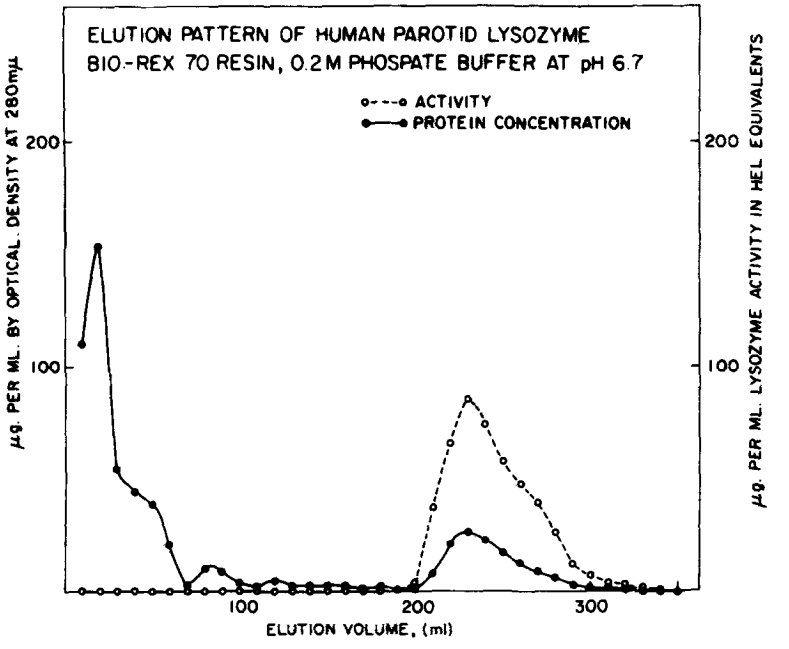

Fig. 2. Purification of parotid saliva lysozyme on Bio-Rex 70. The enzyme was taken up on Bio-Rex 70 resin from a saliva filtrate (see text) and eluted from a column of the same resin (0.9 x 20 cm) with 0.2 M phosphate buffer (pH 6.7) at room temperature. Protein concentration determined by ultraviolet light absorption at 280 mμ (—●—●—). Protein concentration calculated from enzymatic activity with hen's egg white lysozyme (HEL) as the standard (--o--o--).

Also, the specific activity was uniformly constant within the activity peak thus indicating chromatographic purity. In Table I, the last entry under specific activity was based on the dry weight of the protein (by amino acid analysis), and this value (2.5) is in good agreement with the value reported for (human) whole saliva (9).

The data from the amino acid analyses of purified HPL from two different lots of pooled saliva are shown in Table II. The Table also lists the amino acid compositions of HEL and other human lysozymes. The averaged values of amino acids from the two runs are in good agreement with those reported for other human lysozymes. Using the amino acid composition, the minimum molecular weight, based on one histidine residue per mole, was 14,900. Hen's egg

TABLE II

AMINO ACID COMPOSITION OF HUMAN PAROTID SALIVA LYSOZYME

	Amino Acid Residues Per Mole					
	HEL(*)	HPL_1(*)	HPL_2(*)	Average Value	Human Lysozymes(10)	HEL(11,12)
Lysine	6	6.2	5.1	5-6	5	6
Histidine	1	1.0	1.0	1	1	1
Arginine	11	13.9	11.3	13	11-12	11
Aspartic Acid	21	19.2	17.3	18	18 ±1	21
Threonine	7	6.0	4.7	5	6 ±1	7
Serine	9	7.2	6.4	7	6 ±1	10
Glutamic Acid	5	10.6	10.3	10	9 ±1	5
Proline	3	2.7	3.9	3	3	2
Glycine	12	13.0	12.4	13	12 ±1	12
Alanine	12	15.3	13.3	14	12 ±1	12
Half Cystine	10	6.9	5.2	6	6	8
Valine	6	8.2	6.9	7-8	7	6
Methionine	2	2.8	1.7	2	2	2
Isoleucine	6	4.8	4.1	4	5	6
Leucine	8	9.0	7.6	8	8	8
Tyrosine	3	6.3	6.6	6	5	3
Phenylalanine	3	2.1	2.3	2	2	3
Tryptophan	3	1.7	1.6	2	5 ±1	6

HEL = Hen's egg white lysozyme, HPL = Human parotid saliva lysozyme

(*) Calculated on the basis of one histidine residue per mole.

white lysozyme was also analyzed under identical conditions and the results appear in the first column of Table II.

The molecular weight of purified HPL was determined in a Beckman-Spinco Model E ultracentrifuge by the Yphantis method (6). The rotor speed was 37,020 rpm at 20° C. The plot of lnC versus r^2 was rectilinear indicating homogeneity of the purified enzyme in this system. Molecular weights determined by ultracentrifugation are highly dependent on the partial specific volume ($\bar{v}$). Two of several reported values of $\bar{v}$ for HEL were used in the present calculations. A $\bar{v}$ value was also calculated from the amino acid composition of human lysozymes based on the data in the fifth column of Table II. When the $\bar{v}$ used was 0.703 (13), the calculated molecular weight was 16,300. For a $\bar{v}$ of 0.688 (14), the calculated molecular weight was 15,500, and 17,000 for a $\bar{v}$ of 0.716 derived from the amino acid composition of human lysozymes (9).

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