# SUBUNIT STRUCTURE OF TESTICULAR HYALURONIDASE

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## 1. Introduction

Testicular hyaluronidase (hyaluronate glycan hydrolase EC 3.2.1.35), its isolation and characterisation has been reported by several authors [1–6]. However the composition and properties of the preparations described seem strikingly dissimilar. The most obvious explanation that these must have been different molecular forms [7] of the enzyme cannot however fully account for some grave discrepancies observed. Marked variations in the molecular weights [1–3, 5, 6], two N-terminal amino acids detected in otherwise homogeneous preparation [8], rapid inactivation of the enzyme when in low concentrations and the non-linear dependence of the specific activity on the concentration [9] would rather imply a quaternary structure interpretation than any other.

We report in this paper that the molecular form of the enzyme we had isolated and described [7] has a quaternary structure and consists of four subunits with molecular weight of approximately 14,000 each.

## 2. Materials and methods

Testicular hyaluronidase was prepared from a commercial Reanal sample of the enzyme according to a procedure described earlier [7]. Molecular forms of the enzyme were isolated. Quaternary structure studies were carried out with hyaluronidase E. In line with the recommendations of the Commission of the Biochemical Nomenclature [10] we suggest it to be named testicular hyaluronidase-1. The enzyme was not contaminated with proteases or other glycosidases, and was homogeneous according to gel filtration, sedimentation and polyacrilamide electrophoresis. Its specific activity by the turbidimetric method was 22,000 T.R.U. per mg protein [11].

The succinylated enzyme was prepared by portionwise addition of dry succinic anhydride (final concentration 3%) to 1% aqueous enzyme; pH was maintained constant by addition of dry tris-(hydroxymethyl)-aminomethane. The solution was kept for 2 hrs at room temperature and then 12 hrs at 4°. Low molecular weight contaminants were removed by gel filtration through Sephadex G-25. Amino groups in the native and succinylated enzyme were determined according to Moore and Stein [12], thiol groups were titrated with *p*-chlormercuribenzoate after Boyer [13]. Succinylation was found to result in acylation of about 61.5% of free amino groups and 57.5% of thiol groups.

Electrophoresis was carried out as described by Reisfield [14] in 7.5% polyacrylamide gel at pH 4.3 (4 mA per tube, 4 hr, 4–6°). The proteins were detected by staining with 0.3% solution of Coomassie brilliant blue and washing with 7% acetic acid. In bioassay electrophoresis the gels were cut in small slices, proteins were eluted with 0.1 M phosphate buffer pH 5.3 and their activity subsequently assayed.

Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out according to Weber and Osborn [15] with 10% acrylamide gels, 0.1 M sodium phosphate buffer pH 7.0 and 0.1% SDS. The samples were prepared as follows: i. Incubation of 1 mg/ml enzyme in β-alanine buffer, pH 4.3, containing 8 M urea and 0.1% mercaptoethanol (6 hrs, 37°); ii. Incubation of 2 mg/ml enzyme for 2 to 48 hrs in 0.01 M sodium

a

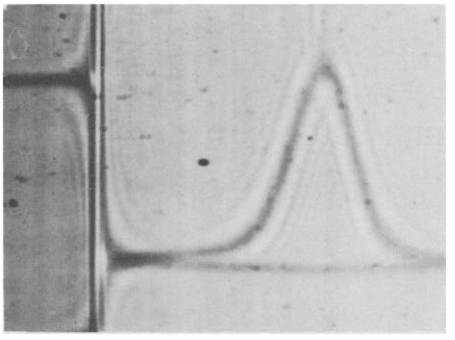

Fig. 1. Sedimentation of hyaluronidase-1 at 42040 r.p.m., 20°. a) The enzyme (3.98 mg/ml) in 0.05 M glycine-HCl buffer, pH 5.7.

phosphate buffer, pH 7.0, containing 0.1, 1 or 2% SDS and 1% mercaptoethanol (20°, 37°); iii. Incubation under conditions in ii, but in the presence of 4 M urea.

For molecular weight determinations two calibration curves were plotted using reference proteins (fig. 3 A, B). The latter were subjected to the same denaturating treatments or succinylation as hyaluronidase.

Sedimentation studies were carried out at 20° using a Spinco E analytical ultracentrifuge with the schlieren optics. Sedimentation velocities were measured in synthetic boundary cells at 42,040 r.p.m. in the concentration range of 3.10 – 6.92 mg protein per ml of 0.05 M glycine-HCl buffer, pH 5.7, containing 0.05 M NaCl. Diffusion measurements were carried out at 10,000 r.p.m.

Sedimentation studies of the enzyme at different pH's were carried out using 1–1.5% aqueous solution of the enzyme buffered before experiments with equal volumes of 0.05 M glycine-HCl or glycine-NaOH of appropriate pH, measured exactly with a glass electrode. Sedimentation studies of succinylated hyaluronidase at pH 8.05–10.0 were done at 59,780 r.p.m. at 20°.

### 3. Results and discussion

Sedimentation and polyacrylamide electrophoresis show freshly prepared hyaluronidase-I to be homogeneous (fig. 1a, 2a). However, storage, repeated lyophilization or pH change resulted in a marked activity

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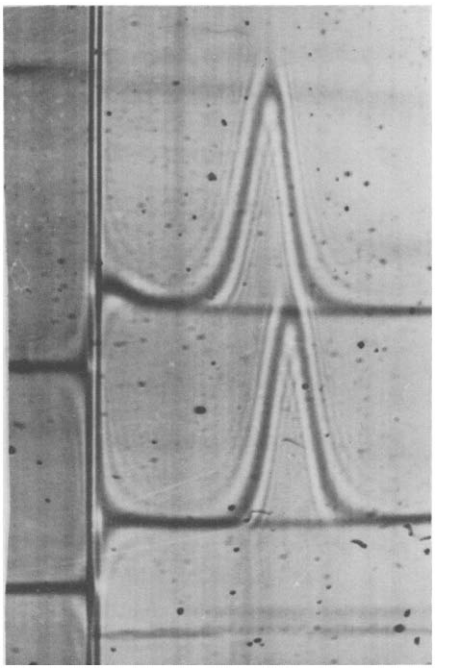

Fig. 1b) Influence of pH on sedimentation. Upper curve, the enzyme (6.62 mg/ml) in 0.05 M glycine-HCl, pH 2.3: lower curve, the enzyme in 0.05 M glycine-NaOH, pH 11.3. The pictures were taken at 24 min (a) and 16 min (b) after the beginning of sedimentation.

decrease. On electrophoresis, samples of deteriorated activity showed in addition to the initial enzymatically active band some other bands of higher mobility which were enzymatically inactive (fig. 2b). These inactive proteins apparently of lower molecular weight than the enzyme could have originated from it on its dissociation into subunits. To work out optimal conditions for dissociation we tested several denaturating agents checking the course of dissociation by polyacrylamide gel electrophoresis. The results are presented in fig. 2. In conditions c and d dissociation followed by a random aggregation occurs. A 24 hr incubation at 20° in the presence of 1% SDS (fig. 2e) gave proteins of 14,000 and 60,000 mol. wt. (as calculated from calibration plots) (fig. 3A); a 48 hr incubation with 1% SDS at 37° or incubation with 2% SDS, or preincubation with 4 M urea followed by SDS-treatment, resulted in 14,000 mol. wt. protein only (fig. 2f).

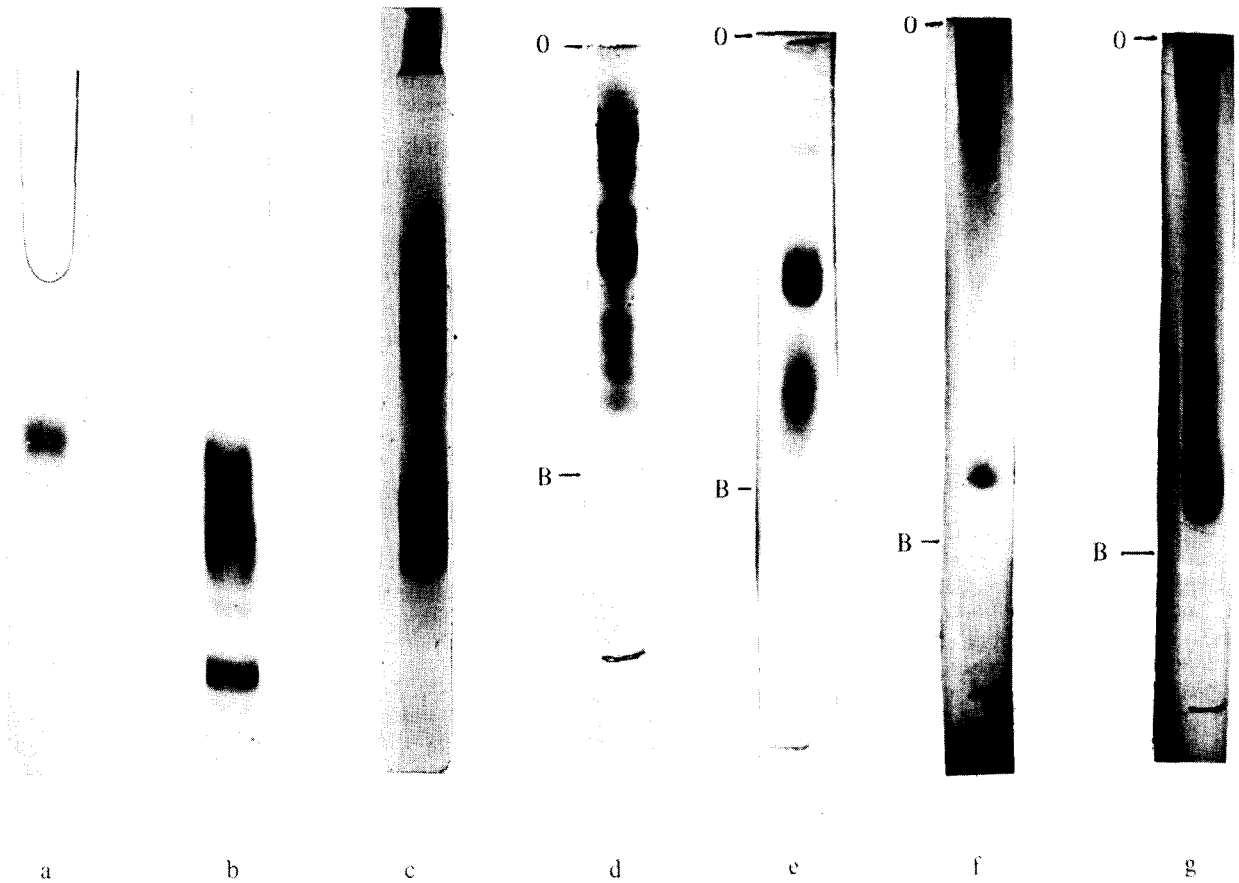


Fig. 2. Disc polyacrylamide gel electrophoresis of hyaluronidase-1. Details are given in Materials and methods. a. Freshly prepared enzyme. b. The enzyme stored for 10 days at 4°. c. The same enzyme after treatment with 8 M urea and 0.1% mercaptoethanol. SDS polyacrylamide gel electrophoresis of the enzyme treated with d. 0.1% SDS and 0.1% mercaptoethanol for 12 hrs at 37°. e. 1% SDS and 1% mercaptoethanol for 24 hrs at 20°. f. 1% SDS and 1% mercaptoethanol for 48 hrs at 37°. g. The succinylated enzyme incubated with 1% SDS, 1% mercaptoethanol for 12 hrs at 20°. O. is the origin, B is the position of the bromphenol blue marker.

An electrophoretic study of succinylated enzyme gave very much the same results except that the enzyme dissociated even more easily. Thus in the conditions of fig. 2g succinylated hyaluronidase gave two bands, a strong band of higher mobility, corresponding to about 14,000 mol. wt. and slowly moving minor band of about 28,000 mol. wt.

The following results were obtained by ultracentrifugation. The sedimentation coefficients of the enzyme at different pH's were calculated from sedimentation profiles and reduced to normal conditions. They were 3.76 S (pH 5.7), 3.04 S (pH 2.3) and 2.06 S (pH 11.3). The observed decrease in sedimentation coefficient cannot be accounted for only by changes in the geometry of the molecule. The succinylated enzyme of 0.6 mg/ml concentration precipitated as a homogeneous protein of 2.57 S (pH 5.7). The ultracentrifuge pattern for it at higher pH, however, showed heterogeneity, two components being observed, 3.3 S and 2.1 S (pH 9.05), and 6.2 S and 4.3 S (pH 10.0). These data backed by gel electrophoresis of the samples after ultracentrifugation were indicative of a pH-dependent dissociation proceeding together with an aggregation.

The assumption that testicular hyaluronidase is composed of subunits would be helpful in explaining some inconsistencies reported in the literature. The lowest mol. wt. of the enzyme determined according

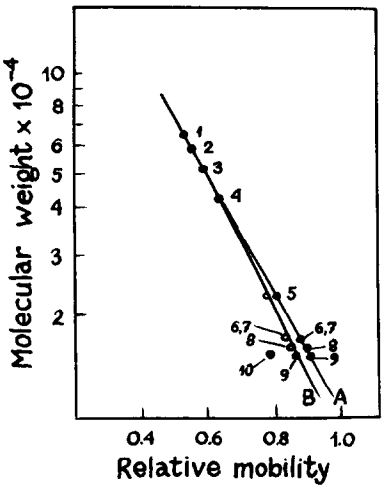


Fig. 3. The correlation between the molecular weight and electrophoretic mobility. 1, bovine serum albumin (67,000); 2, hyaluronidase (60 000); 3, glutamate dehydrogenase (53 000); 4, ovalbumin (43 000); 5, trypsin (23 000); 6, lysozyme (13 500); 7, hyaluronidase (14 000); 8, ribonuclease A (13 500); 9, 10 (chymotrypsin 2 chains 13 000, 11 000). Molecular weights of the reference proteins are from Weber [5]. A. Reference proteins; B. Succinylated proteins

to its diffusion and sedimentation velocities by Archibald's method is 11,000 and 14,000 [6]. Hahn [16] reported the sedimentation constants to be 4.6 S or 4.3 S (as derived from two different calculation procedures he used). Malgrem [6] on the other hand gave it as 3.6 S for a hyaluronidase preparation obtained as described by Tint [17], and 1.2 S for a preparation of Hogberg [18]. Brunish and Hogberg [1] for their part gave the mol. wt. of their enzyme preparation as 43,200 (by Archibald's method) but also observed the peak to be non-homogeneous and the mol. wt. to tend to decrease with the time. Mol. wt. of crude hyaluronidase determined by Borders and Raftery [2] by gel filtration was 126,000. It is notable that for the purified enzyme they gave the mol. wt. as 61,000.

We evaluated the mol. wt. according to Swedberg. Sedimentation ($S_{20,w}$) and diffusion ($D_{20,w}$) constants were determined by us as 4.17 S and $6.8 \times 10^{-7}$ $cm^2$ $sec^{-1}$ respectively, and were used as such in calculations. We determined the apparent partial specific volume of hyaluronidase to be of 0.72, using the pycnometric technique. This value is higher than that of Brunish (0.678) the difference however may be due to differences in the carbohydrate composition of the preparations. Brunish's preparation contained 10% of carbohydrates while our enzyme had 3.5%. Also Brunish remarked on the heterogeneity of his sample. The mol. wt. of hyaluronidase-1 was calculated by us to be about 55,000.

Thus our data apparently allow us to infer that hyaluronidase-1 has a quaternary structure and is composed of four subunits with mol. wt. 14,000 each. The similarity of the properties of hyaluronidase-1 and other molecular forms of testicular hyaluronidase [7] seems to indicate them to have a similar subunit structure.

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