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Novel hyaluronidase from Streptomyces

Two distinct types of hyaluronidase (hyaluronate lyase, EC 4.2.99.1) have hitherto been known to occur in nature, one being a glycohydrolase from animal origin (*e.g.* testis, sperm, skin, snake venom and leech) and the other an eliminase from bacterial sources (*e.g. Streptococcus hemolyticus, Staphylococcus aureus* and *Clostridium welchii*).

Our screening experiment carried out with soil sample resulted in one Streptomyces strain producing and secrete a constitutive hyaluronidase into culture medium. Subsequent studies on this enzyme clearly indicate that the enzyme is distinct from the known hyaluronidase in its substrate specificity and mechanism of action. In this communication, we wish to report the purification and properties of this enzyme.

The enzyme activity was measured by a modification of the turbidimetric method of TOLKSDORF AND SCHWENK[1]. The activity was also followed by an increase in reducing value[2].

The strain (*Streptomyces hyalurolyticus* nov. sp.) was grown on starch–peptone–meat extract medium (3% soluble starch, 0.5% polypeptone, 0.5% meat extract, 0.15% yeast extract, 0.8% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, initial pH 7.2) by shaking culture at 30° for 72 h. The cells were removed by centrifugation. Ethanol was added, with stirring, to the supernatant at a final concentration of 59% and the resultant precipitates were discarded. To the supernatant was added, with stirring, ethanol at a final concentration of 77%. The solution was allowed to stand at room temperature for about 30 min and the precipitates were collected by centrifugation. The yield of

TABLE I

PURIFICATION OF HYALURONIDASE FROM *S. hyalurolyticus*

Steps 3 and 4: The enzyme solution (in water) was filtered by the resins (previously washed with water) for decolorization. Steps 5–7: The columns of Duolite ES-80, DEAE-cellulose, and CM-cellulose were equilibrated with 0.05 M acetate buffer (pH 4.0), 5 mM phosphate buffer (pH 8.0), and 5 mM acetate buffer (pH 4.1), respectively. In each case, the enzyme solution was dialyzed against the same buffer used for conditioning and applied on the column, which was then eluted stepwise with 0.5 M acetate buffer (pH 6.0), 0.05 M phosphate buffer (pH 7.0) and 0.05 M acetate buffer (pH 5.0), respectively. A turbidity reducing unit was defined as the quantity of enzyme which causes in 30 min a reduction in the turbidity to that given by half the initial substrate concentration. Protein was estimated by the method of LOWRY *et al.*[9], using L-tyrosine as a standard.

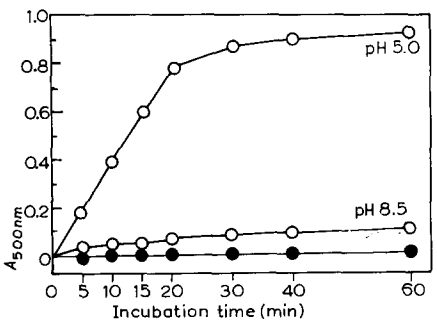
Step	*Total activity (turbidity reducing units)*	*Total protein (mg/ tyrosine)*	*Specific activity (enzyme units/mg protein)*
1. Culture fluid	400 000	31 000	13
2. Alcohol fractionation	287 500	4 900	59
3. Duolite A-2	250 000	1,300	192
4. Duolite ES-33	238 000	620	384
5. Duolite ES-80	151 200	18.2	8 308
6. DEAE-cellulose	114 800	6.56	17 500
7. CM-cellulose	95 600	1.5	63 733

this crude preparation was 115 g (dry weight) from a 20-l culture medium. Further purification of the enzyme included chromatography, in succession, on Duolite A-2, Duolite ES-33, Duolite ES-80, DEAE-cellulose and CM-cellulose. The results are summarized in Table I. An enzyme with the specific activity of 63 700 turbidity reducing units/mg tyrosine (which is about 5000-fold greater than the starting culture fluid) was thus obtained in a yield of 24%.

The purified enzyme was quite stable against pH as well as temperature; *i.e.* no significant loss of the activity was observed after incubation at 37° for 24 h at the pH range between 4.0 and 10.0. Even at 75°, the enzyme was not inactivated for at least 30 min. At 90°, however, the activity was entirely lost within 20 min. The optimal pH and temperature of the enzyme activity were shown to be pH 5.0 and 60°, respectively.

In contrast to testicular and bacterial hyaluronidase, the Streptomyces hyaluronidase was not inhibited by heparin, condroitin sulfate B and polyvinyl alcohol sulfate. Upon treatment of hyaluronic acid (prepared from human umbilical cord) with this enzyme, the reducing value was increased with a concomitant increase of the value of the Morgan–Elson reaction[3] for *N*-acethylhexosamine, indicating that the *N*-acetylglucosaminidic linkage was cleaved by this enzyme. Moreover, the ultraviolet light absorption at 232 nm was also increased, suggesting that elimination but not hydrolysis of hyaluronic acid by the enzyme occurs, as shown with bacterial hyaluronidase[4] and chondroitinases[5].

As far as examined, hyaluronic acid was the only mucopolysaccharide which could be degraded by this enzyme. Thus, various types of chondroitin sulfates (A, B, C, D[6], E), chondroitin (from squid skin), heparin, keratosulfate (from shark cartilage) and chitin were not attacked by this enzyme at all (Fig. 1). Also noted was the fact that the tetrasaccharide obtained from hyaluronic acid by testicular hyaluronidase



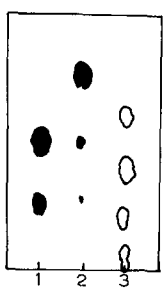


Fig. 1. Rate and extent of degradation of various mucopolysaccharides with Streptomyces hyaluronidase. Incubation was carried out with the enzyme solutions containing 20 turbidity reducing units/ml in 0.02 M acetate buffer (pH 5.0) and 0.02 M Tris–HCl buffer (pH 8.5) at 60°. Degradation of each substrate was assayed by the Nelson–Somogi method[2] with the following compounds as substrate: ○, hyaluronic acid; ●, chondroitin sulfates (A, B, C, D and E), chondroitin, keratosulfate, heparin and chitin at both pH values (5.0 and 8.5, respectively).

Fig. 2. Paper chromatogram showing the action of Streptomyces, bacterial (*S. aureus* Terajima), and testicular hyaluronidase on hyaluronic acid. Ultraviolet absorption spots were shown as closed symbols. The spots corresponding to saturated oligosaccharides could not be located by observation under ultraviolet lamp but could be visualized with $AgNO_3$ and were indicated by open symbols. 1, products of Streptomyces enzyme; 2, products of bacterial enzyme; 3, hydrolysate of testicular enzyme. Solvent: *n*-butyric acid–0.5 M NH_3 (5:3, v/v).

is not degraded by this enzyme. Such substrate specificity was proved to be clearly distinct from that of testicular hyaluronidase (which degrades chondroitin sulfates and chondroitin) and of bacterial hyaluronidase (which degrades chondroitin).

The reaction products were examined by paper chromatography using known $\Delta^{4,5}$-unsaturated disaccharides as a standard. As shown in Fig. 2, two ultraviolet-absorbing spots (to be referred to as X and Y) were observed which were distinct in their R_F values from the known unsaturated disaccharides. Each spot was eluted from the paper with water for further characterization. The molar ratio, terminal *N*-acetylglucosamine[3] to total glucosamine[7] and to total glucuronic acid[8] were 1.00: 2.08:1.98 for the upper compound (X) and 1.00:2.90:2.97 for the lower compound (Y). The data indicated that the compounds are unsaturated tetrasaccharide (X) and hexasaccharide (Y), respectively, with $\Delta^{4,5}$-unsaturated glucuronic acid residue at the nonreducing end. Neither X nor Y was further degraded by re-treatment with the Streptomyces hyaluronidase, showing that the compounds are end products. Upon treatment of X and Y with bacterial hyaluronidase (from *St. aureus* Terajima) followed by paper chromatography, a single ultraviolet-absorbing product appeared in each case on the chromatogram which coincided with nonsulfated $\Delta^{4,5}$-unsaturated disaccharide (*i.e.* 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose). The yields of the disaccharides thus obtained were 2.08 moles and 3.04 moles from 1 mole of X and Y, respectively. There was little doubt therefore that the products X and Y are $\Delta^{4,5}$-unsaturated tetrasaccharide and hexasaccharide, respectively.

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