Purification and Characterization of Leech Hyaluronic Acid-endo-β-glucuronidase*

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In 1960, Linker, Meyer, and Hoffman (1) reported that a crude leech preparation hydrolyzes the glucuronide linkages of hyaluronic acid, unlike bacterial and testes hyaluronidases, which act on glucosaminide linkages.

This paper deals with the first systematic attempt to purify the leech β-glucuronidase that specifically hydrolyzes hyaluronic acid. Purified preparations of the enzyme have indeed been prepared, a unit of enzyme activity has been tentatively defined, and a study has been made of a number of properties of the enzyme. This enzyme differs greatly from existing β-glucuronidases, not only with respect to substrate specificity but also with regard to its sensitivity to inhibitors.

EXPERIMENTAL PROCEDURE AND RESULTS

Purification of Enzyme—Essentially, the process requires an ammonium sulfate fractionation followed by column chromatography on DEAE-cellulose.

In a representative purification, 7.8 g of the fresh extremities of 45 live leeches were homogenized in 170 ml of cold distilled water and were centrifuged at 600 × g for 10 minutes. The supernatant solution was collected and pooled with one subsequently obtained by extracting the tissue pellet with 80 ml of cold distilled water. The pooled supernatants represent Step I material. Its protein content; activity; and specific activity appear in Table I along with data from subsequent Step II and III solutions.

Enzymically inert material was precipitated from a previously chilled Step I solution by adding solid ammonium sulfate to 40% saturation. This mixture was stirred for 10 minutes in an ice-water bath and centrifuged. The enzyme was now precipitated from the cold 40% ammonium sulfate supernatant by adjusting the concentration of the salt to 80% with solid ammonium sulfate and stirring for 10 minutes. The precipitate was separated by centrifugation (750 × g for 20 minutes). Additional protein impurities were leached out of the precipitate by suspending it in 20 ml of 70% ammonium sulfate, pH 7.0, and centrifuging.

An enzyme-rich fraction was prepared by collecting the pro-

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‡ The same products reported by Linker (1) with crude extracts were found by paper chromatography of an enzyme digest containing hyaluronate and the purified enzyme. Moreover, the liberation of terminal reducing glucuronic acid groups as a function of time was established in another study (8).
Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Yield</th>
<th>Specific activity*</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Supernatant of homogenate</td>
<td>250.0</td>
<td>431.0</td>
<td>10,500</td>
<td>100</td>
<td>24.4</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>Dialyzed solution</td>
<td>22.5</td>
<td>31.5</td>
<td>4,700</td>
<td>45</td>
<td>140.0</td>
<td>6.1</td>
</tr>
<tr>
<td>III</td>
<td>Best eluate from DEAE-cellulose</td>
<td>7.5</td>
<td>0.39</td>
<td>870</td>
<td>8.3</td>
<td>2,230.0</td>
<td>01.5</td>
</tr>
<tr>
<td></td>
<td>(Fraction 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fractions 8, 9, and 10</td>
<td>22.5</td>
<td>1.45</td>
<td>2,145</td>
<td>20.4</td>
<td>1,480.0</td>
<td>61</td>
</tr>
</tbody>
</table>

* Specific activity = activity (units)/protein (mg).

Properties of Enzyme—The optimal pH of this enzyme was found to be 6.0 based on experiments (Fig. 2A) with 0.06 N citrate buffer solution (pH 2.85 to 6.6) and 0.06 N Veronal buffer solution (pH 7.1 to 7.5).

The optimal temperature of this enzyme is 38° (Fig. 2B). With regard to substrate concentration, 0.5 mg of substrate (hyaluronate) is enough to saturate 5.4 units of the enzyme under the present conditions. A straight line relationship between enzyme activity and reaction time was observed up to 40 minutes (Fig. 3).

The leech enzyme hydrolyzed partially depolymerized streptococcal hyaluronic acid in addition to hyaluronic acid. It had no effect on phenolphthalein glucosiduronic acid, chondroitin sulfate B, chondroitin sulfate C, N-acetyl chondroitin, heparitin

* Specific activity = activity (units)/protein (mg).

substances are eluted from DEAE-cellulose by pH 6.0 glycyl-glycine buffer solution, and none remain in the pH 5.0 acetate buffer eluate. Therefore, this control is not required with Step III preparations.

Controls—Hyaluronic acid itself gives a low value for reducing sugar which, however, is increased by prolonged heating in alkali. Consequently, this value must be subtracted from the reducing glucose values of the samples analyzed. Corrections are also required for the reducing power of Step I and Step II material before incubation with substrate. However, all these reducing

![Fig. 1. Pattern of chromatography of leech enzyme on DEAE-cellulose column (1.5 x 50 cm). ——, Enzyme activity in units per fraction; · · · · ·, amount of protein in micrograms per fraction. The arrows indicate the position of change in buffer systems as explained in the text.](http://www.jbc.org/)

![Fig. 2. A, effect of pH on the activity of leech enzyme. A mixture of 0.4 ml of 0.06 N buffer solution, 0.1 ml of hyaluronic acid solution* (0.5 mg of potassium hyaluronate), and 3.0 units of enzyme in 0.1 ml was incubated for 30 minutes at 38°. B, effect of temperature on the activity of leech enzyme. A mixture of 0.5 ml of 0.00 N citrate buffer solution (pH 5.7), 200 μg of hyaluronate solution in 0.1 ml of water, and 0.05 ml of enzyme (1.5 units) was incubated for 60 minutes at the specified temperatures.](http://www.jbc.org/)
sulfate, heparin tetrasaccharide, Type III capsular polysaccharide, and oxidized starch.

Mercuric ions at a concentration of $10^{-5} \text{ M}$ completely inhibited the leech enzyme. The substances in 0.001 to 0.0001 M solution which tested negatively as inhibitors were potassium hydrogen succinate, succinylmethylcarbinol, gum arabic, potassium dihydrogen phosphate, EDTA, potassium cyanide, sodium fluoride, magnesium sulfate, ethanol, acetone, chloroform, oxalate, tartrate, $p$-hydroxymercuribenzoate, and diphenyl glucosiduronic acid.

**DISCUSSION**

*Specificity of Leech Enzyme*—The present findings confirm the ability of the leech enzyme to hydrolyze hyaluronic acid and its oligosaccharides and extends the list of mucopolysaccharides and glucosiduronic acids which are not substrates. It is clear that the nonglucuronic acid hexose unit in the mucopolysaccharide must be N-acetylglucosamine lacking a sulfate group. Glucose units, alternating with glucuronic acid as in Type III capsular polysaccharide or randomly located as in oxidized starch, do not take the place of N-acetylglucosamine. Neither does N-acetylgalactosamine which alternates with glucuronic acid in N-acetyl chondroitin. Accordingly, the specificity of the leech enzyme for hyaluronate would now appear to be unique and exclusive for the endoglucuronide linkage. Furthermore, simple $O$-glucosiduronic acids such as those of phenolphthalein and of $p$-hydroxydiphenyl are not hydrolyzed at all.

*Endo-$\beta$-glucuronidase versus Existing $\beta$-Gluconoridases*—Excluding substrate specificity, certain properties of the leech enzyme are those expected from $\beta$-glucuronidases (6). Others are not.

For example, the thermal stability, solubility in ammonium sulfate solutions, insensitivity to organic solvents, and acid pH optimum are the usual properties noted in many $\beta$-glucuronidase preparations. The differences are the insensitivity of the leech enzyme to inhibition by succinylmethylcarbinol, glucuronic acid, and competing substrate, diphenylglucosiduronic acid, as well as its much greater sensitivity to mercuric ions. The temperature maximum at 38° is of interest. This value is lower than ones which characterize the $\beta$-glucuronidase tissues of warm-blooded animals.

The crude leech homogenate does have the ability to hydrolyze phenolphthalein glucosiduronic acid, but this activity is removed in the ammonium sulfate fractionation.

**SUMMARY**

An enzyme has been prepared from medicinal leeches which hydrolyzes glucuronide linkages of hyaluronic acid and its oligosaccharides exclusively. For this reason, this enzyme has been named hyaluronic acid-endo-$\beta$-glucuronidase. The enzyme is stable at temperatures below 37° and dissolves in ammonium sulfate at 50% but not 70% saturation. It is adsorbed on diethylaminoethyl cellulose and is eluted preferentially with acetate buffer solution (pH 5.6). The enzyme unit is tentatively defined as equal to the reducing power of glucuronic acid (glucose equivalent) liberated per hour from hyaluronic acid at 38° and at pH 6.0. A pH optimum at 6.0 and an optimal temperature at 38° are characteristics of the preparation. The enzyme is completely inhibited by $10^{-5} \text{ M}$ mercuric ions. A comparison is made of the properties of this particular enzyme with those characterizing the classical $\beta$-glucuronidases.

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**REFERENCES**

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