Purification and Properties of the Enzyme Chondroitinase*

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The enzymatic degradation of chondroitin sulfuric acid can be catalyzed by purified bovine testicular hyaluronidase (1, 2) and by an enzyme preparation from Flavobacterium heparinum (3). Dodgson and Lloyd (4) obtained a chondroitinase from Proteus vulgaris that converted chondroitin sulfuryric acid to the disaccharide, N-acetylgalactosaminyl N-acetylmuramidase. Previous work from this laboratory (5) has confirmed and extended the observations of Dodgson and Lloyd. In these studies two disaccharides were isolated from the reaction and identified as N-acetylmuramyl N-acetylgalactosaminyl N-acetylmuramidase sulfate (5). Studies on the purification and properties of the Proteus vulgaris chondroitinase are presented in this report.

EXPERIMENTAL

Materials and Analytical Procedures—The potassium salt of chondroitin sulfate was prepared according to the procedure of Elhinder and Schubert (6) with the use of crude chondroitin sulfuric acid purchased from General Biochemicals, Inc., as the starting material. The purified preparation had an optical rotation of $[\alpha]_D^{25} = -21.4^\circ$ (c = 2.2; water) and an elemental analysis of N, 2.7 per cent; C, 13.75 per cent; H, 6.80 per cent; S, 5.21 per cent; and K, 13.75 per cent. Galactosamine-HCl was prepared by acid hydrolysis of crude chondroitin sulfuric acid and purified by a large scale modification of the chromatographic method of Gardel (7). This material had an optical rotation of $[\alpha]_D^{25} = 95.5^\circ$ (c = 2; water) and an elemental analysis of N, 6.56 per cent; C, 33.7 per cent; H, 6.80 per cent. Acetylglactosamine was prepared by the method of Roseman and Ludoweig (8) and had a melting point of 184-185°C. The 2,4-dinitrophenol derivative had a melting point of 184-185°C (corrected). Heparin (123 units per mg.) was obtained from the Southern California Gland Company, Los Angeles, California. A generous gift of blood group substance derived from hog gastric mucosa was supplied by Dr. H. Baer, Tulane University.

N-Acetylglactosamine determinations were run with crystalline N-acetylglactosamine as the standard in accordance with the procedure of Reissig et al. (9). Paper chromatography was carried out by the descending technique with butanol-acetic acid-water (44:16:40) as the solvent mixture which proved to be suitable for separation of the products formed during chondroitin sulfate depolymerization. The papers were developed by the method of Partridge (10) for N-acetylhexosamines. Duplicate papers were dipped in an Azure A stain for identification of sulfated compounds.

In all cases the enzymatic activity was stopped by immersing aliquots of the reaction mixtures in a 65°C water bath for 5 minutes. Preliminary experiments showed that no increase in N-acetyl galactosamine color occurred when the enzyme preparation was incubated with chondroitin sulfuric acid under these conditions. Other experimental conditions are described in the legends of the tables and in the text.

RESULTS

Purification of Enzyme—Two grams of lyophilized P. vulgaris which had been grown for 48 hours with forced aeration on nutrient broth containing 0.1 per cent chondroitin sulfuric acid (5) were ground in the frozen state in a mortar with 3.5 gm. of powdered glass and 4 ml. of 0.01 M phosphate buffer, pH 7.0. The resulting mass was extracted for 1 hour with 60 ml. of cold 0.01 M phosphate buffer, pH 7.0. The suspension was centrifuged at 18,000 × g for 1 hour at 4°C and the supernatant fluid retained.

All ammonium sulfate precipitations were carried out by the slow (about 30 minutes) addition of the dry salt to the cold, stirred enzyme preparations. The fraction precipitating between 20 to 80 per cent saturation was dissolved in 60 ml. of 0.001 M phosphate buffer, pH 7.0. After the addition of 0.05 part of 1.0 M manganous chloride, the solution was dialyzed against distilled water until the dialysate showed less than 3 μg. of ammonium per ml. as determined by nesslerization. The dialyzed enzyme preparation was clarified by centrifugation and again fractionated with ammonium sulfate. The protein fraction that precipitated between 40 to 60 per cent saturation contained the chondroitinase activity and was stable for at least 6 months when stored at -15°C.

Calcium phosphate gel, washed four times with distilled water before use, was added to 60 ml. of the (40 to 60 per cent saturation) fraction to a gel to protein ratio of 0.3 and the protein was allowed to adsorb on the gel for 10 minutes in the cold. After centrifugation the gel was resuspended in 60 ml. of 0.01 M phosphate buffer, pH 8.0, for 20 minutes. The mixture was centrifuged and the supernatant fluid was discarded. Elution of chondroitinase from the gel was carried out with 30 ml. of 2 M sodium acetate solution pH 7.6 for 12 hours in the cold with occasional stirring. The gel eluates were dialyzed for 4 hours against 6 l. of distilled water which was changed hourly. The clear solution was stored at -15°C in 2-ml. aliquots. A summary of this procedure given in Table I shows that the enzyme was purified approximately 42-fold. The purified chondroitinase was not entirely free from chondroacetylase and was capable of

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TABLE I

Comparison of activity of crude and purified enzyme toward chondroitin sulfate

<table>
<thead>
<tr>
<th></th>
<th>Crude extract</th>
<th>40 to 60% (NH₄)₂SO₄</th>
<th>Gel eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units of activity/ml enzyme</td>
<td>224</td>
<td>132</td>
<td>173</td>
</tr>
<tr>
<td>Volume</td>
<td>60</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Total units</td>
<td>13,440</td>
<td>7,920</td>
<td>5,190</td>
</tr>
<tr>
<td>Mg. protein/ml</td>
<td>22</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Units/mg. protein</td>
<td>10.2</td>
<td>16.5</td>
<td>432</td>
</tr>
</tbody>
</table>

* A unit of activity is defined as the micrograms of N-acetylgalactosamine equivalents liberated per minute.

degrading both hyaluronic acid and chondroitin sulfuric acid. There was little difference in enzyme activity against chondroitin sulfuric acid when acetate, tris(hydroxymethyl)aminomethane, or tris(hydroxymethyl)aminomethane-maleate buffers were used. A slightly slower rate was found with phosphate buffer.

Effect of pH and Specificity of Enzyme—Fig. 1 shows the effect of pH on the rate of degradation of chondroitin sulfate and hyaluronate by the purified chondroitinase preparation. The optimum rate against chondroitin sulfate was approximately pH 8.0 and against hyaluronate was about 6.7 to 6.8. The relative rates of degradation of the two substrates at various pH values are also shown. At the optimum pH for both substrates, chondroitin sulfate was degraded approximately 6 times faster than hyaluronate. This ratio of activity between the two substrates remained constant throughout the purification procedure. Therefore, the question of a separate enzyme for the two activities or a single enzyme that degrades both hyaluronate and chondroitin sulfate has not been resolved. If a single enzyme acts on both polymers, then it can be surmised that the difference in pH optima for the two polysaccharides might possibly be due to the maintenance of the substrates in the proper ionic state for enzyme action, rather than a pH effect on the enzyme itself. The enzyme preparation was not able to degrade blood group substance from hog gastric mucosa or heparin as measured by the Somogyi-Nelson determination for reducing sugars (11).

Effect of Substrate and Enzyme Concentration—the rate of chondroitin sulfate acid degradation was studied as a function of chondroitin sulfate acid (Fig. 2) and enzyme (Fig. 3) concentration. Although it is not shown in Fig. 2, increasing the chondroitin sulfate acid concentration above 12 mg. per ml. to 40 mg. per ml. did not show an increase in rate of depolymerization, nor was any inhibition of enzyme activity observed at this high substrate concentration. From Fig. 3, note that increasing the concentration of enzyme produces an increase in reaction rate as measured by the production of N-acetylgalactosamine equivalents from chondroitin sulfate acid.

Effect of Temperature—The optimum temperature for chondroitinase activity was found to be approximately 38°C (Fig. 4). In these experiments the reaction mixtures were preincubated at the respective temperatures for 5 minutes before the addition of 0.1 ml. of enzyme solution. Above this optimum temperature there is a very rapid drop in enzyme activity, only 50 per cent of total activity remaining at 43°C. These results differ from those obtained by Korn (3) who used acetone powder extracts from F. heparinum. The preparation reported by Korn had an optimum temperature of 45°C for chondroitin sulfuric acid degradation, whereas the chondroitinase from P. vulgaris has little or no activity at this temperature.
TABLE II

Effect of ions on chondroitinase activity

Reaction mixture contained 0.08 ml. of enzyme (32 μg. of protein), 4 mg. of chondroitin sulfate, 0.25 ml. of tris(hydroxymethyl)aminomethane-HCl (pH 8.0; 0.1 M), and the additions on the table in a total volume of 1.0 ml. Reaction time was for 20 minutes at room temperature.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>N-Acetylgalactosamine equivalents produced</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>CuSO₄</td>
<td>10⁻³</td>
<td>0.05</td>
<td>79.2</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>5 × 10⁻⁴</td>
<td>0.08</td>
<td>66.7</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>10⁻³</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>10⁻³</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>10⁻⁴</td>
<td>0.05</td>
<td>79.2</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>10⁻⁵</td>
<td>0.24</td>
<td>0</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>10⁻⁴</td>
<td>0.17</td>
<td>29.2</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>10⁻⁴</td>
<td>0.16</td>
<td>33.3</td>
</tr>
</tbody>
</table>

TABLE III

Inhibition of chondroitinase action by heparin

Reaction mixture contained 0.1 ml. of the purified enzyme (23 μg. of protein), 0.25 ml. of tris(hydroxymethyl)aminomethane-HCl buffer pH 8.0; 0.1 m, and the indicated concentrations of chondroitin sulfate and heparin in a total volume of 1.0 ml. Incubated for 25 minutes at room temperature.

<table>
<thead>
<tr>
<th>Chondroitin sulfate</th>
<th>Heparin</th>
<th>N-Acetylgalactosamine equivalents produced</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>mg.</td>
<td>mg./ml.</td>
<td>%</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0.44</td>
<td>61.4</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0.17</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0.11</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.08</td>
<td>81.8</td>
</tr>
</tbody>
</table>

Inhibition of Chondroitinase by Heparin—When equivalent amounts of chondroitin sulfate and heparin were incubated with the purified enzyme, approximately an 80 per cent inhibition in the production of N-acetylgalactosamine equivalents was observed (Table III). Decreasing the heparin concentration or increasing the chondroitin sulfate concentration lowered the inhibition. These results again point to the differences in the enzymatic properties between the chondroitinase of P. vulgaris and the preparations which Korn (3) obtained from F. heparinum. The latter preparations depolymerize heparin in addition to chondroitin sulfate and hyaluronic acid.

Effect of Ions on Chondroitinase Activity—A study was made to determine if the enzyme had a requirement for ions or whether certain ions elicited an inhibition of enzymatic activity. The results of these experiments are presented in Table II. Zinc and copper were found to inhibit the rate of depolymerization of chondroitin sulfate by the enzyme as measured by N-acetylgalactosamine equivalents production. From the fact that ZnSO₄ and ZnCl₂ inhibited identically at the same molar concentrations, it can be surmised that the sulfate ion, per se, does not account for the observed inhibitions with cupric and zinc salts. At present it is not clear whether these inhibitions by Zn⁺⁺ and Cu⁺⁺ are effects on the enzyme or on the mucopolysaccharide, a molecule which has great avidity for ion binding. The following compounds at 10⁻³ M were found to produce no measurable effect on the rate of the reaction: NH₂Cl, MgCl₂, Na₂SO₄, NaF, Na₂AsO₄. Ethylenediaminetetraacetate at 0.1 mg. per ml. had no effect on the rate of reaction.

Effect of temperature on chondroitinase activity

Reaction mixtures contained: 3.47 mg. of chondroitin sulfate; 0.25 ml. of tris(hydroxymethyl)aminomethane-HCl buffer (pH 8.0, 0.05 M); enzyme in amounts indicated; total volume 1.0 ml. Incubated 20 minutes at indicated water bath temperatures.

Inhibition of Chondroitinase by Heparin—When equivalent amounts of chondroitin sulfate and heparin were incubated with the purified enzyme, approximately an 80 per cent inhibition in the production of N-acetylgalactosamine equivalents was observed (Table III). Decreasing the heparin concentration or increasing the chondroitin sulfate concentration lowered the inhibition. These results again point to the differences in the enzymatic properties between the chondroitinase of P. vulgaris and the preparations which Korn (3) obtained from F. heparinum. The latter preparations depolymerize heparin in addition to chondroitin sulfate and hyaluronic acid.

Extent of Depolymerization of Chondroitin Sulfuric Acid and Product of Chondroitinase Action—The extent of degradation of chondroitin sulfuric acid by chondroitinase preparations was determined by prolonging the incubation time until a plateau was reached.
reached and maintained in N-acetylgalactosamine equivalents production. Recoveries of N-acetylgalactosamine equivalents in the order of 90 per cent of theoretical have been observed with the purified enzyme (Fig. 5). At the end of the incubation period, the mole ratio of glucuronic acid to reactive acetylhexosamine was 1:1.07. A similar experiment was run with testicular hyaluronidase to degrade chondroitin sulfate. A plateau was attained when acetylgalactosamine equivalents production was 45 per cent of theory and the mole ratio of glucuronic acid to reactive acetylhexosamines at the termination of the reaction was 2:1. These data confirm the production of a disaccharide as the end product of chondroitinase action (5) and the production of tetrasaccharides as the end product of the hyaluronidase catalyzed degradation of chondroitin sulfate (12). The final product of chondroitin sulfate depolymerization by the purified enzyme appears to be N-acetylc chondrosin sulfate. This was determined by cochromatographing aliquots of the chondroitin sulfate reaction mixture during the course of the reaction with N-acetylc chondrosin sulfate prepared as previously described (5). Similar results have been reported by Dodgson and Lloyd (4). In addition to N-acetylc chondrosin sulfate, small amounts of N-acetylc chondrosin were detected with some enzyme preparations at the completion of the reaction. In these experiments N-acetylc chondrosin was only detectable after 2 hours of incubation, whereas N-acetylc chondrosin sulfate was detected as early as 1 hour of incubation. Neither disaccharide was detected after 15 and 30 minutes of incubation of the reaction mixture. Instead compounds giving a positive reaction for acetylhexosamine and sulfate but possessing lower mobilities than the sulfated and nonsulfated disaccharides were detected. If it is assumed that these compounds appearing early in the reaction are oligosaccharides, then it could be postulated that chondroitinase is similar to hyaluronidase (13) in its action. That is, the enzyme apparently hydrolyzes random acetylhexosaminide bonds along the polymer chain. Chondroitinase differs from hyaluronidase in that the products of the reaction were found to be disaccharides and not tetrasaccharides. Whether or not chondroitinase acts by a transglycosylating mechanism as has been postulated for hyaluronidase, remains to be investigated (14).

SUMMARY

1. The enzyme, chondroitinase, has been purified approximately 45-fold from an extract of lyophilized Proteus vulgaris.

REFERENCES

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Purification and Properties of the Enzyme Chondroitinase
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