A Convenient Plate Assay for the Quantitation of Hyaluronidase in Hymenoptera Venoms

PAUL G. RICHMAN AND HAROLD BAER

Allergenic Products Branch, Division of Bacterial Products, Bureau of Biologics, FDA, 8800 Rockville Pike, Bethesda, Maryland 20205

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A simple plate assay for hyaluronidase activity in biological samples is described. Hyaluronic acid is incorporated into agarose gels and the enzyme is allowed to diffuse from punched wells. The undigested hyaluronic acid is then precipitated with cetylpyridinium chloride and the diameters of the clear circles are proportional to the logarithm of the enzyme concentration applied to the well. The assay was utilized to examine commercially available hymenoptera venoms, manufactured for use in allergy diagnosis and treatment, for their content of hyaluronidase as a measure of lot-to-lot consistency. The assay permits the analyses of a large number of samples with good reproducibility, without the need for any special instrumentation. Based on the quantity of purified hyaluronidase reported in honey bee venom (T. P. King, A. K. Sobotka, L. Kochoumian, and L. M. Lichtenstein, 1976, Arch. Biochem. Biophys. 172, 661–671) we estimate that the assay should detect 70 ng/ml of purified honey bee venom hyaluronidase.

Hyaluronidase catalyzes the breakdown of the acidic mucopolysaccharide, hyaluronic acid, to oligosaccharides (usually tetra- and disaccharides) (for a review, see (1)). Various methods for the assay of hyaluronidase have been described that rely on turbidity (2) or viscosity (3) reduction of the substrate, or the measurement of the increase in reducing power caused by the appearance of end products (4). In addition a sensitive spectrophotometric assay has been described that measures the decrease in dye-binding capacity of the undigested substrate (5).

The assay described in this paper was developed to monitor commercial extracts of hymenoptera venoms, all of which contain hyaluronidase (6,7), for lot-to-lot consistency. The principle of the assay involves radial diffusion of an enzyme sample through hyaluronic acid dispersed in agarose, hydrolyzing the substrate in the process. The undigested hyaluronic acid is then precipitated in the gel with cetylpyridinium chloride (8) causing the appearance of a clear circle that corresponds to the distance diffused by the enzyme. The diameter of the clear circle is proportional to the logarithm of the enzyme concentration originally present in the punched hole. The assay has the advantage of extreme simplicity and the ability to test a large number of samples without the need for sophisticated instrumentation. Although we developed and evaluated the assay for hyaluronidase in hymenoptera venoms it should be suitable for hyaluronidase determinations in other biological samples as well.

EXPERIMENTAL PROCEDURES

Materials. Hyaluronic acid (grade 1, human umbilical cord) and cetylpyridinium chloride were obtained from Sigma Chemical Company. Agarose (Type HSC) was a product of Litex, Denmark. Hyaluronidase standard was the Bureau of Biologics reference preparation of honey bee (Apis...
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melliferu) venom (obtained from Mr. Charles Mraz, Middlebury, Vermont) which contained 1 mg of venom protein/ml in 0.045 M citrate buffer (pH 4.8) containing 50% (v/v) glycerol. This enzyme preparation was considered to contain 1000 units/ml of hyaluronidase, by definition. Purified bovine testes hyaluronidase was obtained from Worthington Biochemical Company. Venom and venom protein preparations were manufactured by Hollister-Stier and Pharmacia AB and included honey bee (Apis melliferus), wasp (Polistes sp.), yellow jacket (Vespula sp.), white-faced hornet (Dolichovespula maculata), yellow hornet (Dolichovespula arenaria), and mixed vespid (yellow jacket, white-faced hornet, and yellow hornet). All other chemicals were the purest grades commercially available.

**Description of assay.** The plates consisted of 1 mg/ml hyaluronic acid dispersed in 1.5% (w/v) agarose and were buffered with 0.05 M sodium citrate (pH 5.3) containing 0.15 M NaCl and 0.02% (w/v) sodium azide (buffer A). A stock solution of 3% agarose in buffer A was stored (ambient temperature) in 9.5-ml aliquots. A stock solution of 2 mg/ml hyaluronic acid in buffer A was prepared by stirring the powder in buffer overnight (4°C) (stored at 4°C, and discarded after 1 week). A 9.5-ml aliquot of 3% agarose was melted in a boiling-water bath, cooled to 60°C, and added to 9.5 ml of 2 mg/ml hyaluronic acid in a 60°C bath with constant stirring. The solution was stirred for about 1 min to ensure complete mixing, and then rapidly poured into a 9 × 9-cm plastic petri dish on a level surface. After the gel had set, the plate was covered and incubated for 20 h at 37°C on a level surface. After incubation the gel was covered with 10% (w/v) cetylpyridinium chloride in water (ca. 10 ml) and as soon as the circles became distinct (ca. 10–20 min) their diameters were measured to the nearest 0.1 mm using a calipers and reading against a dark background.

The means for duplicate determinations of zone diameter in millimeters were plotted against the logarithm of the hyaluronidase activities (for the standard) in units per milliliter. A linear regression curve was computed for the standard and the concentration of hyaluronidase in the unknowns was calculated from the regression curve. In those experiments in which we evaluated the slopes of the dose–response curves of various venom types, a linear regression curve was also computed for the unknown venom.

The protein content of the venom preparations was determined as described (9).
using bovine serum albumin as the standard. The results agreed with the values supplied by the manufacturers.

RESULTS AND DISCUSSION

The results of a typical plate assay using the honey bee venom standard is shown in Fig. 1 and the data were plotted in Fig. 2. The correlation coefficients for the computed linear regression curves have ranged from 0.95 to 1.00 in more than 50 determinations. A time-course for zone development under the assay conditions (Fig. 3) shows that the increase in zone diameter is linear for at least 24 h.

To evaluate the reproducibility of the assay, a single lot of honey bee venom was tested in 32 replicates, using six different plates that were tested on different days. The activity for each sample was calculated from the standard curve obtained from reference enzyme that was tested on the same plate. The arithmetic mean for the hyaluronidase activities obtained was 78 units/ml with a standard deviation of 16, indicating a precision of about 20%.

The effect of variation of pH on the assay results was examined. Plates were prepared as described under Experimental Procedures except the pH's of the buffers were 4.3, 4.8, 5.3, or 5.8. Samples of the reference honey bee venom were serially diluted in buffers at each of the four pH values.
Samples at each pH were assayed on plates prepared at the four pH values. The data (not shown) demonstrated that the pH value of the enzyme sample (in this range) does not alter the assay result. Furthermore, there was no difference in the results obtained on plates prepared at pH 4.8 to 5.8. The plates prepared at pH 4.3 did not show any significant enzymatic activity.

The dose–response curves for venoms from wasp, white-faced hornet, yellow hornet, yellow jacket, honey bee, and mixed vespid (using a twofold serial dilution series) were compared to those of our reference honey bee venom preparation. The results, presented in Table 1, indicate that the slopes are the same for all of the venom types.

Since the slopes of the dose–response curves for all of the commercially available venoms were the same as the slopes obtained with our reference enzyme preparation, we examined a large number of lots of the individual venoms for their hyaluronidase activities using single concentrations (100 μg venom protein/ml) rather than complete curves. The results are shown in Fig. 4. There was no difference in the distribution of values obtained with venoms from the two commercial suppliers. The white-faced hornet venoms gave a much wider distribution of values in different lots than any of the other venoms. It is not clear from the limited amount of data whether this represents natural variation in the hyaluronidase content of the venom, or variation as a consequence of the manufacturing process. One sample of wasp venom gave a value (236 units/ml) that was reproducibly higher than that of the other samples.

Based on the data given by King et al. (10), we estimate that honey bee venom contains about 4.7 mg of purified hyaluronidase/g of venom protein. This number is based on the specific activity given for

Fig. 2. Dose–response curve for the reference honey bee venom. The data for the enzyme standard, obtained from the plate in Fig. 1, was plotted using a semilogarithmic scale. The experimental points are the mean values of duplicate determinations with the ranges indicated. The line is a computed linear regression curve for zone diameter versus logarithm of hyaluronidase activity.

Fig. 3. Time course of zone development. Reference honey bee venom at 250 μg/ml was assayed as described under Experimental Procedures for the indicated times. The experimental points are the arithmetic means of six replicates with the indicated standard deviations. The line is a computed linear regression curve (correlation coefficient = 0.99).
TABLE I
COMPARISON OF SLOPES OF DOSE–RESPONSE CURVES FOR DIFFERENT HYMENOPTERA VENOMS

<table>
<thead>
<tr>
<th>Venom</th>
<th>Slope</th>
<th>Determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honey bee</td>
<td>4.6</td>
<td>2</td>
</tr>
<tr>
<td>White-faced hornet</td>
<td>5.8</td>
<td>2</td>
</tr>
<tr>
<td>Yellow jacket</td>
<td>5.0</td>
<td>2</td>
</tr>
<tr>
<td>Yellow hornet</td>
<td>5.7</td>
<td>5</td>
</tr>
<tr>
<td>Wasp</td>
<td>5.4</td>
<td>5</td>
</tr>
<tr>
<td>Mixed vespid</td>
<td>5.1</td>
<td>2</td>
</tr>
<tr>
<td>Reference honey bee</td>
<td>5.0 ±0.7</td>
<td>19</td>
</tr>
</tbody>
</table>

Note. The indicated venoms were assayed as described under Experimental Procedures. The slopes are the arithmetic means for the indicated number of determinations. They were computed from linear regression curves for zone diameter (in mm) versus the logarithm of the dilution factors. The standard deviation is given for the reference honey bee venom.

Using this number we calculate that the assay described in this paper should be able to detect approximately 70 ng/ml of purified honey bee venom hyaluronidase. A preliminary experiment in which the reference enzyme preparation was diluted as much as 100,000-fold and the plates were incubated for 48 h indicated that the assay can be made at least 100-fold more sensitive. At this increased sensitivity the assay would have the ability to detect as little as 0.0003 National Formulary units, based on the reported specific activity for the reference honey bee venom (11). This sensitivity is 7-fold less than the most sensitive hyaluronidase assay (5).

The assay as described measures a rate of diffusion, not a rate of reaction, and conditions that change the rate of diffusion of the enzyme should interfere. Of course if an interfering substance inhibits the enzymatic reaction sufficiently, no clear circle would be observed. Purified bovine testes hyaluronidase gave a dose–response curve in the assay with a slope identical to that obtained with honey bee venom and a mixing experiment (honey bee venom with bovine hyaluronidase) demonstrated the absence of inhibitors in honey bee venom for hyaluronidase (data not shown). It should be noted that the commercial extracts utilized in these studies contain mannitol (both companies) and human serum albumin (Pharmacia AB) as additives and these substances did not alter the slopes of the dose–response curves (Table 1).

![Fig. 4. Hyaluronidase activities of commercially available hymenoptera venoms. The indicated venoms were tested for hyaluronidase activity as described under Experimental Procedures at a concentration of 100 μg venom protein/ml. Each experimental point represents the activity obtained with a different lot of venom.](image-url)
ACKNOWLEDGMENTS

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REFERENCES