IN VITRO AND *IN VIVO* STUDIES OF NATIVE AND DEXTRAN-MODIFIED HYALURONIDASE

A. V. Maksimenko, O. Yu. Konovalova,
V. P. Berdichevskii, O. G. Arkhipova,
and V. P. Torchilin

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Enzume preparations have justified their use as effective therapeutic agents [8]. However, their widespread use in medical practice is delayed partly by the low stability of native enzymes under physiological conditions [2]. This limitation can be overcome by modification of enzymes, which can substantially enhance their stability [1]. That this is a productive approach is shown by the successful use of stabilized streptokinase in clinical practice [3, 5]. Native hyaluronidase, marketed in the USSR under the name "lidase," is used to improve tissue permeability in connection with subcutaneous injection [3]. Production of long-acting modified hyaluronidase derivatives would not only shorten the course of treatment and reduce its cost, but would also extend the scope for use of the preparation to other diseases, cardiovascular for example, or in conjunction with surgical operations [7, 11]. As a modifying matrix, stabilizing the enzymes, we chose the partially oxidized natural polymer dextran, which has already been used successfully for this purpose [5].

The aim of this investigation was to obtain and study the properties of preparations of hyaluronidase, native and modified by aldehyde dextran, *in vitro* and *in vivo*.

EXPERIMENTAL METHOD

Covalent binding of hyaluronidase to dextran with mol. wt. of 40,000 daltons (from Sigma, USA), modified by partial periodate oxidation [6], was carried out in 0.1 M phosphate buffer solution, pH 8.7, containing 0.15 M NaCl, at 4°C for 24 h. Commercial preparations of hyaluronidase (from Reanal, Hungry) and lidase (Leningrad Medical Preparations Factory) were used. Modified enzyme derivatives, after incubation and reduction with sodium borhydride [6], were isolated by ultrafiltration on an Amicon (USA) apparatus with XM-100 filter. The protein concentration in the preparation was determined by Bradford's method. Catalytic activity of the enzyme derivatives was estimated viscosimetrically [10] in 0.1 M phosphate buffer solution with 0.15 M NaCl, pH 5.45. For this purpose the rate of change of viscosity of a 0.06% solution of hyaluronic acid (from Serva, West Germany) was determined in an Ostwald B-688 viscosimeter (USA) at 34°C, using the scheme suggested previously to calculate enzyme activity [4]. The thermostability of the various hyaluronidase derivatives was determined from the residual catalytic activity after incubation in 0.1 M phosphate buffer solution, pH 7.4, at 48°C.

The distribution of preparations of native hyaluronidase with radioactivity of $(4.49 \pm 0.08)\cdot10^6$ cpm and of modified hyaluronidase with radioactivity of $(2.84 \pm 0.08)\cdot10^6$ cpm, labeled with ^{125}I, among the organs of inbred mice (BALB/c) was investigated after injection of 50 µl of a solution of the preparation in 0.1 M phosphate buffer, pH 7.4, into the caudal vein of a mouse. The results were calculated in percentages of the injected dose per weight of the organ (approximate mean weights were taken to be: liver 0.9 g, lungs 0.18 g, blood 1.5 g) after sacrifice of the mice at the appropriate time after the injection. Radioactivity of the samples was measured on a Compugamma counter (from LKB, Sweden).

Each value given is the average of six experimental determinations.

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TABLE 1. Content of Preparations (in %) of Native and Modified Hyaluronidase in Organs of Mice after Intravenous Injection (M ± m)

Preparation	Organ	Time after injection						
		5 min	30 min	1 h	4 h	1 day	2 days	3 days
Native hyaluronidase	Blood	29,0±0,9	17,2±0,4	16,8±0,7	4,5±0,2	2,0±0,2	2,0±0,2	1,6±0,4
	Liver	13,9±0,8	7,5±0,4	5,9±0,4	2,5±0,2	1,3±0,1	1,4±0,1	1,3±0,3
	Kidneys	35,5±0,5	9,4±0,5	5,5±0,5	1,5±0,1	0,7±0,1	0,7±0,1	0,8±0,2
	Lungs	1,6±0,2	1,3±0,1	1,3±0,1	0,5±0,1	0,2±0,1	0,2±0,1	0,1±0,1
	Heart	0,5±0,1	0,4±0,1	0,3±0,1	0,1	0,1	0,1	0,1
	Spleen	0,8±0,1	0,7±0,1	0,2±0,1	0,1	0,1	0,1	0,1
	Total	81,2±1,5	36,6±0,8	29,3±0,9	9,3±0,3	4,2±0,2	4,2±0,2	3,7±0,7
Modified hyaluronidase	Blood	39,1±5,1	23,9±0,7	19,3±1,4	6,0±0,8	1,5±0,1	1,2±0,3	0,7±0,1
	Liver	23,9±1,2	14,1±0,5	10,0±0,6	3,9±0,6	2,1±0,2	1,5±0,1	0,1±0,1
	Kidneys	19,6±0,9	8,6±0,4	4,7±0,3	1,5±0,2	0,7±0,2	0,6±0,1	0,4±0,1
	Lungs	2,7±0,2	1,3±0,1	1,5±0,2	0,4±0,1	0,2±0,1	0,4±0,1	0,4±0,1
	Heart	0,6±0,1	0,3±0,1	0,3±0,1	0,1	0,1	0,1	0,1
	Spleen	1,0±0,1	0,8±0,1	0,7±0,1	0,3±0,1	0,1	0,1	0,1
	Total	86,8±5,3	49,1±1,6	36,5±2,6	12,5±1,7	4,6±0,3	3,3±0,3	2,2±0,2

EXPERIMENTAL RESULTS

Optimization of the conditions for binding (pH 8.7, 4°C, incubation for 17 h) of hyaluronidase with aldehyde-dextran led to addition of 65-85% of protein relative to the original amount to the carrier. The residual catalytic activity of the modified enzyme derivative under these circumstances was 90-100% of the initial value. As a result of modification of the hyaluronidase, not only was its catalytic activity increased somewhat (by about 30%), but so also was its thermostability (by 1.3-1.5 times). According to the results of electrophoresis of the enzyme derivatives by Laemmli's method (in 12% polyacrylamide gel in the presence of sodium dodecylsulfate), the molecular weight of the hyaluronidase, covalently bound with aldehyde-dextran, was 150,000 daltons. The protein concentration (by weight) in this water-soluble preparation was 40%. Hyaluronidase preparations, produced in Hungary and in the USSR (lidase), behaved identically on modification. The specific activity of the native enzyme and of its modified derivative was very close: about 3.2 conventional units/mg protein or about 1.28 conventional units/mg of the preparation.

The study of the distribution of preparations of native and modified hyaluronidase among the organs of mice after intravenous injection showed (Table 1) that the pattern observed was on the whole the traditional one for polymer derivatives [9]. Both preparations accumulated in the heart and spleen comparatively little. Most uptake of the derivatives took place by the liver and kidneys. There was a comparatively increased concentration of modified hyaluronidase in the lungs. Previously the writers described appreciably accumulation of fluorescent-labeled aldehyde-dextran itself in the lungs when injected intravenously into mice, and this was confirmed by the results of histologic investigations. The mean half life of the preparations in the blood stream was increased for modified hyaluronidase (30 min) by comparison with the native enzyme (20 min).

As a result of chemical modification of hyaluronidase with aldehyde-dextran the catalytic activity and stability of the enzyme thus increased. According to data on the distribution of intravenously injected modified hyaluronidase among the organs of mice, this preparation may be effective for the treatment of lung diseases. To increase the dose of the active principle in the lungs, other methods of administration of the preparation are probably more promising, namely intraperitoneal injection or inhalation.

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