To conclude, it can be said that although LPST leads to breakdown of a significant part of the phosphoinositides (15-30%) and to the formation of considerable quantities of diacylglycerol (20%), platelet aggregation does not take place. This suggests that activation of the phosphoinositide cycle is probably not a necessary and sufficient condition for the induction of platelet aggregation.

LITERATURE CITED

1. N. V. Vasil'ev, N. B. Lutsyuk, G. K. Palii, and O. V. Smirnova, Biochemistry and Immunology of Microbial Polysaccharides [in Russian], Tomsk (1984).
2. A. N. Kuznetsov, The Spin Probe Method [in Russian], Moscow (1976).
3. S. Cockcroft, M. M. Barrowman, and B. D. Gomperts, FEBS Lett., 181, 259 (1985).
4. J. M. Gerrard, Meth. Enzymol., 86, 642 (1982).
5. R. I. Grove and S. D. Schimmel, Biochim. Biophys. Acta, 711, 272 (1982).
6. M. Lagarde, B. Drouot, M. Guichardant, et al., Biochim. Biophys Acta, 833, 52 (1985).
7. M. S. Liu, T. Onji, and N. E. Snelgrove, Biochim. Biophys. Acta, 710, 248 (1982).
8. M. S. Liu, Life Sci., 33, 1995 (1983).
9. T. J. Rink and T. J. Hallam, Trends Biochem. Sci., 9, 215 (1984).
10. P. M. Rossoff and L. C. Cantley, J. Biol. Chem., 260, 9209 (1985).
11. F. Rossi, B. V. Della, M. Grzeskowiak, et al., FEBS Lett., 181, 253 (1985).
12. G. F. Springer, J. C. Adye, A. Bezkorovainy, et al., J. Infect. Dis., 128, Suppl. S202 (1973).

COMPARATIVE STUDY OF THE PROPERTIES OF NATIVE AND MODIFIED COLLAGENASE PREPARATIONS

A. V. Maksimenko, O. Yu. Konovalova,*
A. D. Petrov, O. G. Arkhipova,
and V. P. Torchilin

UDC 615.355:577.152.344].07

KEY WORDS: collagenase; aldehyde-dextran; hyaluronidase; modification; catalytic activity and stability; distribution among organs.

Elimination of metabolic disturbances requires an increase not only in the selectivity, but also in the many-sideness of action of the therapeutic agent on the pathological focus [9]. Potential remedies for this purpose are preparations with combined action obtained by the chemical modification of enzymes [2]. The simultaneous presence of several types of therapeutic activity in these derivatives ensures strengthening and prolongation of their action on an affected organ or tissue.

In fibrosis collagen and proteoglycan synthesis is activated, with the consequent formation of connective tissue [4]. Collagenase, hyaluronidase, and elastase are its natural depolymerizing agents. It was shown previously that hyaluronidase, stabilized with dextran [3], has a marked inhibitory action on the development of fibrosis in the lungs in experimental silicosis [1]. It can be tentatively suggested that the use of preparations with a series of depolymerizing activities (collagenase, hyaluronidase, elastase, etc.) will be effective in the treatment of fibrotic processes. Thus the synthesis of derivatives with dual enzyme activity (collagenase and hyaluronidase, for example) may be very useful for the treatment of fibrosis.

*Deceased.

Laboratory of Engineering Enzymology, Institute of Experimental Cardiology, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow.

(Presented by Academician of the Academy of Medical Sciences of the USSR V. N. Smirnov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 105, No. 3, pp. 294-297, March, 1988. Original article submitted December 26, 1986.

0007-4888/88/0003-0342$12.50

TABLE 1. Catalytic Activity of Collagenase and Hyaluronidase Preparations

Preparation	Preserved catalytic activity, % of initial		Specific activity, IU				Protein content in preparation, %
			per mg protein		per mg of preparation		
	collagenase	hyaluronidase	collagenase	hyaluronidase	collagenase	hyaluronidase	
Native collagenase (collalytin)	100	—	140	—	140	—	100
Collagenase-aldehyde-dextran	56	—	78	—	18	—	23
Native hyaluronidase (lidase)	—	100	—	3,56	—	3,56	100
Collagenase-aldehyde-dextran-hyaluronidase	41	39	57	1,47	18	0,42	30

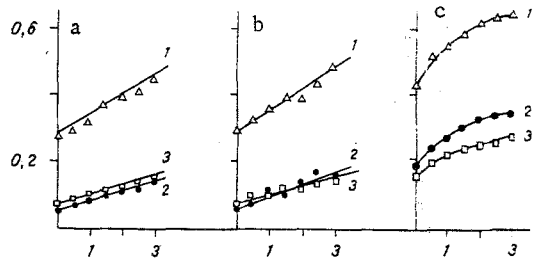

Fig. 1. Determination of catalytic activity of preparations of native collagenase (1), collagenase modified by aldehyde dextran (2), and collagenase cross-linked through aldehyde-dextran with hyaluronidase with respect to lysis of type I collagen (a) or tissue of a human sclerotic plaque (b, c) at pH 7.5 and 37°C. Abscissa, time (in h); ordinate, optical density of solution at 420 nm (a, b) or at 280 nm (c). Preparations used in equal protein concentrations (by Bradford's method). The 420 nm degradation products were stained with trinitrobenzoic acid, 280 nm products were recorded by change in optical density of solution.

In the investigation described below a collagenase preparation conjugated through aldehyde-dextran with hyaluronidase was obtained and some of its properties were determined.

EXPERIMENTAL METHOD

Collagenase was conjugated with hyaluronidase by successive modification of the collagenase with dextran with mol. wt. of 40 kilodaltons (from Sigma, USA) after preliminary periodate oxidation [5] in a solution of 0.1 M phosphate buffer (pH 8.3), containing 0.2 M NaCl, at 4°C for 3-5 h. In the second stage, a solution of hyaluronidase in the above-mentioned buffer was added to the incubation mixture, and incubation of the mixture then continued with stirring for 24 h at 4°C. The ratio of the enzymes by weight was 1:1. The Schiff bases formed in the course of the binding reaction were than reduced with sodium borhydride [5] and the resulting derivatives were isolated either by gel chromatography on a K 9/60 column (Pharmacia, Sweden) with Sephadex G-100, or by ultrafiltration on an Amicon (USA) apparatus with an XM-100 filter. The incubation mixture was treated with sodium borhydride, after which the aldehyde dextran-modified collagenase was isolated by gel-filtration or ultrafiltration (XM-100) methods. The ratio of enzyme to carrier was 1:4 by weight. The following enzyme preparations were used: collagenase isolated from hog pancreas (collalytin) was obtained from the Laboratory of Organ Preparations, All-Union Research Institute of the Meat Industry (Moscow), hyaluronidase (lidase) was obtained from the Leningrad Medical Preparations Factory. The protein concentration in the preparations was determined by Bradford's method. Catalytic activity of the collagenase derivatives was measured as lysis of insoluble type I collagen (C-9879, from Sigma), washed beforehand in a solution of the same buffer, or of a piece of aorta from a man dying suddenly from myocardial infarction, and taken 1-3 h after death. Standard quantities of these substrates were placed on the semipermeable grid of a tube [2] above the washing solution (15 ml) of 0.05 M Tris-buffer, containing 0.2 M NaCl and 0.01 M $CaCl_2$. The preparations of native and modified collagenase (pH 7.5) at

TABLE 2. Radioactivity (in % of initial dose) of Samples of Preparations of Native and Modified Collagenases in Organs of Mice after Intravenous Injection (M ± m)

Organ, system	Preparation	Time of determination after injection, h					
		1/12	1/2	1	4	24	48
Blood	Native collagenase	22,45±1,14	7,5±0,30	7,2±0,99	2,35±0,35	0,48±0,04	0,23±0,06
	Collagenase-aldehyde-dextran	30,27±1,2	10,26±0,53	6,87±0,21	3,41±0,45	0,25±0,06	0,04±0,02
	Collagenase-aldehyde-dextran-hyaluronidase	35,1±2,07	13,46±1,05	12,76±0,83	6,43±0,42	1,22±0,11	0,45±0,08
Lungs	Native collagenase	1,24±0,15	0,49±0,09	0,54±0,02	0,24±0,02	0,02±0,01	0,009±0,003
	Collagenase-aldehyde-dextran	2,02±0,31	0,76±0,13	0,63±0,15	0,25±0,07	0,02±0,005	0,02±0,01
	Collagenase-aldehyde-dextran-hyaluronidase	1,73±0,42	0,86±0,16	1,17±0,23	0,45±0,07	0,07±0,01	0,03±0,01
Liver	Native collagenase	6,46±0,35	3,03±0,16	2,72±0,35	0,88±0,12	0,2±0,01	0,14±0,03
	Collagenase-aldehyde-dextran	11,61±0,25	12,77±0,2	10,85±0,33	8,28±0,26	5,29±0,16	4,16±0,08
	Collagenase-aldehyde-dextran-hyaluronidase	9,58±0,68	8,78±0,41	8,12±0,4	5,61±0,26	2,82±0,08	1,94±0,13
Spleen	Native collagenase	0,44±0,06	0,28±0,04	0,25±0,06	0,09±0,02	0,02±0,01	0,004±0,001
	Collagenase-aldehyde-dextran	0,52±0,08	0,33±0,01	0,25±0,03	0,19±0,03	0,02±0,002	0,01±0,003
	Collagenase-aldehyde-dextran-hyaluronidase	0,36±0,02	0,35±0,02	0,31±0,03	0,18±0,03	0,03±0,01	0,008±0,003
Kidneys	Native collagenase	13,12±1,06	6,03±0,29	3,71±0,28	1,07±0,06	0,17±0,01	0,18±0,03
	Collagenase-aldehyde-dextran	10,24±0,38	6,13±0,24	3,51±0,11	1,32±0,06	0,2±0,006	0,16±0,009
	Collagenase-aldehyde-dextran-hyaluronidase	12,9±0,65	5,94±0,21	4,35±0,29	1,6±0,04	0,35±0,01	0,21±0,03
Heart	Native collagenase	0,46±0,04	0,19±0,01	0,15±0,03	0,06±0,01	0,008±0,003	0,005±0,004
	Collagenase-aldehyde-dextran	0,47±0,05	0,19±0,01	0,14±0,01	0,08±0,005	0,002±0,0004	0,001±0,0008
	Collagenase-aldehyde-dextran-hyaluronidase	0,57±0,05	0,26±0,02	0,34±0,06	1,15±0,03	0,02±0,01	0,002±0,001
Total	Native collagenase	44,17	17,52	14,57	4,69	0,89	0,57
	Collagenase-aldehyde-dextran	55,13	30,44	22,25	13,53	5,78	4,39
	Collagenase-aldehyde-dextran-hyaluronidase	60,2	29,7	27,05	14,42	4,51	2,64

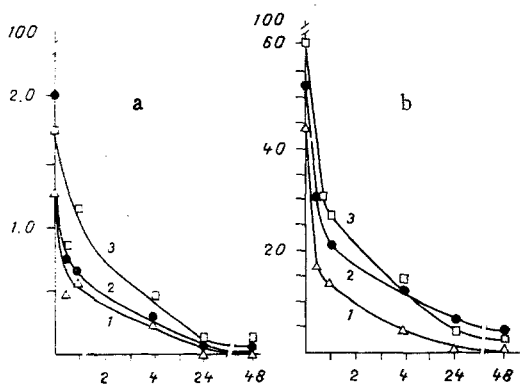

Fig. 2. Thermoinactivation curves of preparations of native collagenase (1), collagenase modified with aldehyde-dextran (2), and collagenase conjugated through it with hyaluronidase (3), at 50°C. Abscissa, time (in h); ordinate, relative catalytic (collagenolytic) activity (in %). 0.05 M Tris-buffer with 0.2 M NaCl and 0.01 M $CaCl_2$ (pH 7.5).

37°C were applied in equal quantities according to Bradford's method to the same grid. The course of lysis was monitored spectrophotometrically: as the increase in optical density [2] of the washing solution (280 nm) or by titrating amino groups [7] of fragments of the lysed substrate, which had passed into solution, with trinitrobenzoic acid (values of the background reaction of the control experiment were correspondingly subtracted). Hyaluronidase activity was estimated viscosimetrically, as described previously [3]. The thermostability

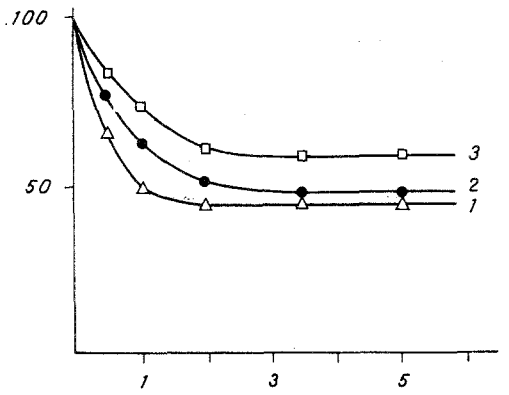

Fig. 3. Changes in radioactivity of samples (in % of initial) of preparations of native collagenase (1), collagenase modified by aldehyde-dextran (2), and then with hyaluronidase (3), in lung tissues (a) of mice and in all organs tested, taken together (b), after intravenous injection. Abscissa, time (in h); ordinate, relative radioactivity of samples (in %).

of the collagenase derivatives was determined from the value of residual catalytic activity after incubation of the enzyme in a solution of 0.05 M Tris-buffer, containing 0.2 M NaCl and 0.01 M $CaCl_2$ (pH 7.5), at 50°C.

Study of the distribution of the resulting enzyme preparations, labeled with ^{125}I, among the organs of inbred male (BALB/c) mice was undertaken by the scheme suggested previously [3]. The preparation, in a volume of 100 µl, was injected into the caudal vein of a mouse. After definite time intervals the animals were decapitated, blood was collected and the organs were removed, and the latter washed with physiological saline, gently dried, and used to determine the radioactivity of samples on a 1275 Minigamma Gamma Counter, LKB Wallac (Sweden). Each value is the mean of five experimental determinations.

EXPERIMENTAL RESULTS

The gel chromatogram of native collagenase consists of two closely situated peaks with mol. wt. of 20-30 kilodaltons. This is in full agreement with data in the literature [12]. Electrophoretic determination [6] showed that the collalytin preparation hydrolyzed collagen with the formation of two fragments, equivalent to about one-quarter and three-quarters of the original substrate, evidence of the truly collagenolytic action of the enzyme from a vertebrate source [8].

Modified collagenase derivatives, as shown by the results of gel chromatography and electrophoresis in 12% polyacrylamide gel in the presence of sodium dodecylsulfate, had a molecular weight of about 150 kilodaltons. Some parameters of the water-soluble enzyme preparations obtained are given in Table 1.

Since 60-70% of the protein is bound with a carrier, modification of the amino groups of the collagenase has no significant effect on its level of catalytic activity (Table 1), in agreement with data in the literature [10]. Lysis of collagen, like lysis of sclerotic plaque tissue from the aorta of men dying suddenly of myocardial infarction, takes place at at the same rate under the influence of native and modified preparations (Fig. 1). Modification of collagenase by aldehyde-dextran widens the pH-optimum of catalytic activity of the enzyme (pH_{opt} = 7.5) toward the acid side. Subsequent covalent addition of hyaluronidase (pH_{opt} 4.5-6.9) leads to mutual widening of the pH-optimum of action of both enzymes. As regards preservation of their collagenolytic activity, the modified preparations, incidentally, were more thermostable than the native enzyme (Fig. 2). The stabilizing action of modification in respect of hyaluronidase has been observed previously [3].

The possibility of intravenous injection of collagenase has been reported [11]. Data on the pattern of distribution of the enzyme preparations among organs of mice are given in Table 2. The fact will be noted that the two-enzyme complex accumulates in lung tissue (Fig. 3a). On the whole, modified collagenase is retained longer in the body than the native enzyme (Fig. 3b).

Thus covalent cross-linking of collagenase through aldehyde-dextran with hyaluronidase yields a preparation which may be promising for therapeutic use. In fact, as a result of this modification the catalytic activity of the enzymes remains substantially unchanged, their thermostability is increased, and the conditions for lysis of specific and biological substrates are widened. The distribution of the preparation among the organs of mice after intravenous injection indicates that it may be possible to use it to eradicate lung lesions.

LITERATURE CITED

1. O. G. Arkhipova, V. V. Yaglov, A. V. Maksimenko, et al., Byull. Éksp. Biol. Med., No. 2, 221 (1987).
2. A. V. Maksimenko and V. P. Torchilin, Vopr. Med. Khim., 31, No. 4, 12 (1985).
3. A. V. Maksimenko, O. Yu. Konovalova, V. R. Berdichevskii, et al., Byull. Éksp. Biol. Med., No. 11, 567 (1986).
4. V. P. Serov and A. B. Shekhter, Connective Tissue [in Russian], Moscow (1981).
5. V. P. Torchilin, I. L. Reizer, V. N. Smirnov, and E. I. Chazov, Bioorg. Khim., 2, 1252 (1976).
6. M. D. Bond, D. S. Auld, and R. R. Lobb, Anal. Biochem., 155, 315 (1986).
7. R. Fields, Biochem. J., 124, 581 (1971).
8. J. Gross and Y. Nagai, Proc. Natl. Acad. Sci. USA, 54, 1197 (1965).
9. A. V. Maksimenko and V. P. Torchilin, Thromb. Res., 38, 277 (1985).
10. I. Trocheris, P. Herry, V. Keil-Dlouha, and B. Keil, Biohim. Biophys. Acta, 615, 436 (1980).
11. K. -P. Volkl and R. Dierichs, Thromb. Res., 42, 11 (1986).
12. S. M. Wilhelm, T. Javed, and R. L. Miller, Collagen Rel. Res., 4, 129 (1984).