Maintenance of Mouse Bone Collagenase Activity in the Presence of Serum Protein by Addition of Trypsin[1] (36293)

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Serum has been shown to be a potent inhibitor of animal collagenases obtained from various tissues (1–4). Using both human skin collagenase and tadpole collagenase, Eisen, *et al.* (4) demonstrated that the only component in human serum which significantly inhibited the enzyme action was the alpha globulin fraction. Since both alpha-1 and alpha-2 globulins have been identified in human serum as protease inhibitors, the effect of alpha-1-antitrypsin and alpha-2 M globulin were tested and were both found to be potent inhibitors of collagenase activity (4). In the present experiments, we have found that the addition of trypsin prevents the inhibition of mouse bone collagenase by serum and that serum in which trypsin inhibitor has been removed also fails to inhibit collagenase activity. The data suggest that the inhibitor of collagenase activity in horse serum may be the same as the inhibitor of trypsin activity.

Materials and Methods. Mouse bone collagenase. Crude mouse bone collagenase was prepared from the tissue culture media of 5-day-old mouse tibiae (5) and further purified by $(NH_4)_2SO_4$ precipitation and molecular sieve filtration (6). The purified collagenase had no general protease activity, using casein and acid-denatured hemoglobin as substrates (6).

Collagenase assay. Collagenase activity was assayed by the method of Lapiere and Gross (7), using purified, reconstituted collagen gels labeled with ^{3}H-proline and hydroxyproline prepared from rat skin (8), and by viscometry, using purified, acid-soluble rat skin collagen as the substrate. Incubation of the collagen gels was carried out for 4 hr at 37°. Collagen gels incubated with crystalline trypsin served as controls. Viscometry was carried out at 25° in a reaction mixture consisting of 0.4 ml of 0.25% solution of collagen, to which was added in the appropriate experiments, 0.1 ml of trypsin solution (100 μg) and 0.1 ml of mouse bone collagenase solution (20 μg as protein). The total volume in each of the experiments was made up to 1.0 ml with 0.2 *M* Tris–HCl buffer, $CaCl_2$ (pH 7.6), 0.005 *M*.

Preparation of serum free of trypsin inhibitor. Insoluble trypsin was prepared by crosslinking trypsin to agarose resin by the cyanogen bromide procedure (9). After activation of the matrix at pH 11, trypsin (Worthington Chemical Co.) was bound to Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) (9). The insolubilized trypsin bound to Sepharose was poured into a 1 × 10 cm column and washed as described (9) and equilibrated with 0.05 *M* Tris–HCl buffer (pH 7.6), 0.005 *M* $CaCl_2$. One milliliter of horse serum was mixed with an equal volume of 0.2 *M* Tris–HCl buffer (pH 7.6), 0.005 *M* $CaCl_2$ and passed through the column.

Results and Discussion. In Table I, Expt. 1, is shown the effects of various concentrations of horse serum on the activity of partially purified mouse bone collagenase. The enzyme activity was completely inhibited by horse serum at a dilution of 1:100 in the reaction mixture which was equivalent to approximately 180 μg of serum protein. When 25 μg of trypsin alone was added to the

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TABLE I. The Effect of Trypsin on Collagenase Activity.

The reaction mixture consisted of 100 μl of a 0.2% collagen gel containing approximately 3000 cpm (^{3}H-proline and ^{3}H-hydroxyproline labeled neutral soluble rat skin collagen: 5×10^4 dpm/mg of collagen), 25 μl of purified mouse bone collagenase solution (5 μg as protein) and other substances in the incubations as noted. The mixture was made up to a total volume of 250 μl with 0.05 *M* Tris–HCl buffer (pH 7.6), containing 5 m*M* $CaCl_2$. The incubation was carried out for 4 hr at 37°. 25 μg of crystalline trypsin incubated with similar collagen gels served as controls.

Expt.	Serum dilution in the reaction mixture	Added (μg)		Activity (cpm)	Collagenase activity (%)	
		Trypsin	Trypsin inhibitor		Inhibition	Recovery
1	No serum	0	0	2491	—	100
	1:10	0	0	0	100	0
	1:100	0	0	0	100	0
	1:1000	0	0	947	62	38
2	No serum	25	0	561	77	23
	1:10	50	0	1099	56	44
	1:10	25	0	2356	~5	~95
	1:10	5	0	79	97	3
	1:100	25	0	553	78	22
	1:100	5	0	2533	0	100
	1:100	2.5	0	2453	~1	~99
3	No serum	25	50	2195	12	88
	1:10	50	50	1174	53	47
	1:10	25	50	1472	41	59
	1:100	5	50	2270	9	91

reaction mixture without the addition of serum, a marked loss of collagenase activity was observed (Table I, Expt. 2). This was probably due to the enzymatic hydrolysis of collagenase by trypsin. With the addition of varying amounts of trypsin to a reaction mixture containing serum, however, collagenase activity was recovered to various degrees, depending on the ratio of trypsin to serum added (Table I, Expt. 2).

When trypsin and serum were mixed and incubated at 37° for 1 hr before the addition of collagenase, the same results were found. When 50 μg of soybean trypsin inhibitor was added to each reaction mixture (Table I, Expt. 3), the marked decrease in collagenase activity seen on the addition of 25 μg of trypsin was prevented. When 25 μg of trypsin was added in the presence of serum and trypsin inhibitor, the inhibitory effect of serum was only partially prevented (Table I, Expt. 3). It was also found that mouse bone collagenase was not inhibited by soybean trypsin inhibitor. The effect of trypsin in preventing the inhibition of collagenase activity by serum was also confirmed by viscometry (Fig. 1).

The addition of heat-denatured trypsin or DFP-trypsin did not prevent the inhibition of collagenase activity by serum. It would therefore appear that active sites of trypsin are necessary to prevent the inhibition of collagenase activity by serum. Alpha-chymotrypsin, elastase and papain were equally as effective as trypsin in preventing the inhibition of collagenase activity by serum, but pepsin was found to be completely ineffective (Table II).

It was found that in the presence of 25 μl of serum, 35 μg of trypsin produced the optimal effect in preventing the inhibition of collagenase activity by serum (Table III). Since 80% of the trypsin used was determined to be enzymatically effective (judged by its reaction with soybean inhibitor), the optimal ratio of milligrams of trypsin:milliliter of

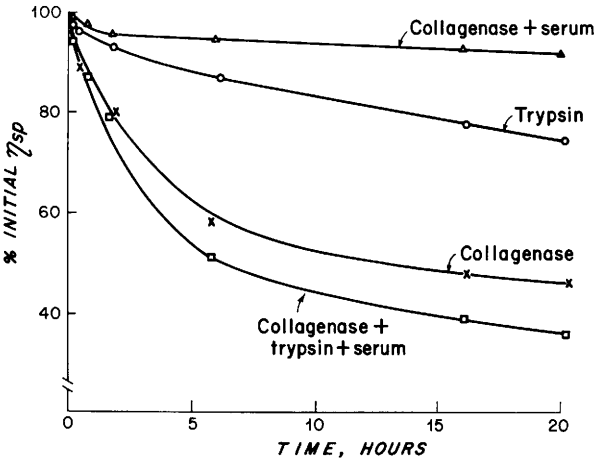

FIG. 1. The effect of mouse bone collagenase, serum and trypsin on the viscosity of collagen solutions: The inhibition of collagenase activity on solutions of collagen by serum and its reversal on the addition of trypsin is clearly shown; see text for experimental details.

serum was calculated to be 1.12. This is very close to the value reported by Dyce and Haverback (10) for the neutralization of crystalline trypsin by serum: 1.15 mg of trypsin neutralized by 1 ml of normal serum.

Horse serum, which was passed through the Sepharose 4B column to which trypsin had been covalently bound, was found to contain no trypsin inhibitor activity. It also failed to inhibit mouse bone collagenase activity. These data suggest that the major component in horse serum which inhibits collagenase activity may be identical with that which inhibits trypsin activity.

The use of trypsin in maintaining collagenase activity in the presence of serum inhibitors may prove useful in demonstrating the presence of collagenase activity in serum and in biological fluids, in general, which would otherwise be masked by the presence of inhibitors.

Summary. The addition of trypsin main-

TABLE II. Effect of Various Proteases on the Recovery of Collagenase Activity on the Presence of Serum Protein.

Added				Collagenase activity (%)	
Serum (μl)	Proteases (μg)		Activity (cpm)	Inhibition	Recovery
0			2348	—	100
25			0	100	0
25	Trypsin	25	1988	15	85
	Heat denatured trypsin	25	0	100	0
	DFP-trypsin	25	0	100	0
		50	0	100	0
	Elastase	25	2334	1	99
		50	2166	8	92
	Alpha-chymotrypsin	25	2179	7	93
		50	2409	0	103
	Papain	25	2169	7	93
		50	2286	3	97
	Pepsin	25	0	100	0
		50	0	100	0

TABLE III. Effect of Various Amounts of Trypsin on the Recovery of Collagenase Activity in the Presence of Serum Protein.

Serum added (μl)	Trypsin (μg)	Activity (cpm)	Inhibition (%)	Recovery (%)
0	0	2424	—	100
25	10	255	90	10
	20	1710	29	71
	30	2370	2	98
	40	2094	14	86
	50	18	99	1

tained mouse bone collagenase activity in the presence of a concentration of serum which otherwise completely inhibited collagenase activity. The stoichiometry of the reaction and the fact that serum prepared free of trypsin inhibitors failed to inhibit collagenase activity, suggests that the component in serum which inhibits collagenase activity may be identical with that which inhibits trypsin activity.

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