DEMONSTRATION OF COLLAGENASE ACTIVITY IN RAT LIVER HOMOGENATE

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Summary: Collagenase activity became detectable in rat liver homogenate by washing liver tissue repeatedly with buffered saline before homogenization. This enzyme activity was inhibited by adding minute quantities of serum. These data suggest that collagenase is active *in situ* in the liver, but is made inactive during the homogenization by forming a complex with contaminating serum factors.

In a previous communication we reported that collagenase activity was demonstrable in Kupffer cells isolated from the rat liver (1). However, there have been no other reports which describe collagenase activity in liver homogenate.

Eisen et al. found an inhibitory effect of human serum *in vitro* on human skin and tadpole collagenases (2). Subsequently Abe and Nagai could activate latent collagenase in rheumatoid synovial fluid by treatment with 3M NaSCN and suggested that the latent collagenase was an enzyme-inhibitor complex (3). We are obtaining evidence that collagenase is present in rat liver homogenate as a masked form, which can be activated by the NaSCN treatment (4). The activated collagenase is inhibited by adding human serum but not by the serum pretreated with NaSCN (4). These observations lead to a suggestion that collagenase in the liver is active *in situ*, but is converted into an inactive form during the homogenization procedure by binding to serum inhibitors.

This paper describes the demonstration of collagenase activity in the homogenate prepared from the rat liver tissue which had been washed beforehand repeatedly with buffered saline.

Materials and Methods

Preparations of the enzyme: Male Wistar rats weighing about 150 g were

used. Under light anesthesia with sodium pentobarbital i.p., the liver was excised. The liver was cut with a pair of scissors into small pieces and the pieces of 1-2 cubic mm were collected by use of an appropriate sieve. After having been washed vigorously with 10 volumes of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.9 % NaCl on a magnetic stirrer for 15 min at room temperature, the tissue pieces were collected on filter paper. A portion of the pieces was taken for the preparation of homogenate and the remainder was washed similarly. These procedures were successively repeated.

The washing was also performed at 4°C or 37°C and with Tyrode solution or the buffered saline containing either NaSCN (0.25-3.0 M) or trypsin (0.002-0.08 %).

The liver pieces were homogenized with 5 volumes of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.4 M NaCl, 5 mM $CaCl_2$ and 0.1 % Triton X-100 and were used for the enzyme assay.

In some experiments the homogenate was preincubated at 37°C for 30 min with a quarter volume of saline containing various concentrations of rat serum.

Collagenase assay and its identification: Collagenase activity of the sample was assayed by measuring the soluble peptides liberated from ^{14}C-labeled reconstituted collagen fibrils (1) and its specificity was confirmed by analysing the reaction products of collagen digested with the enzyme sample of homogenate which had been purified by affinity chromatography as previously reported (1).

Protein determination of the samples: The amount of protein in the sample was determined by Lowry's method (5).

Results

Collagenase activity and effect of washing: Although collagenase activity was negligible in initial homogenate of the liver, it became clearly detectable when the liver pieces had been washed more than 3 times prior to the homogenization. The activity increased progressively with an increase in

number of wash, reaching a peak value at the 6 times wash. Further repetition of wash failed to increase, but rather caused a decrease in the enzyme activity (Table 1).

Table 1 Effect of washing of liver pieces on collagenase activity of resultant homogenate.

Number of wash	Collagenase activity (cpm/mg prot. of homogenate)	Number of wash	Collagenase activity (cpm/mg prot. of homogenate)
0	trace	6	61.2
1	trace	7	54.3
2	1.7	8	31.2
3	35.6	9	22.1
4	47.7	10	16.3
5	53.8	11	12.6

The liver pieces were washed with buffered saline. The assays were done in duplicate.

Similar increase in the enzyme activity was observed when washing was performed at 4°C and 37°C, or when the buffered saline was replaced by Tyrode solution. Inclusion of NaSCN or trypsin in the buffered saline produced no favourable influence on the enzyme activity, but rather deleterious effect on the tissue pieces at their high concentrations.

Inhibition of collagenase activity by serum: The homogenate from the liver pieces washed 6 times was preincubated with saline containing various concentrations of rat serum. The collagenase activities in these homogenates were shown in Table 2.

Addition of serum resulted in a dose-related inhibition of the enzyme activity; the serum diluted to 1:2000 in the reaction mixture exhibited a significant effect of inhibition.

Table 2 Inhibition of collagenase activity in rat liver homogenate by rat serum.

% of serum in saline		Collagenase activity (cpm/mg prot. of homogenate)	% inhibition
0	(no serum)	61.2	
0.1	(1:5000)	62.1	0
0.25	(1:2000)	43.3	29.2
0.50	(1:1000)	30.8	49.6
1.00	(1: 500)	15.3	75.0
5.00	(1: 100)	2.1	96.5

The homogenate from liver pieces washed 6 times was used as the enzyme specimen. The homogenate was preincubated at 37°C for 30 min with a quarter volume of saline containing various concentrations of rat serum. Final dilution of serum in the reaction mixture was shown in parentheses.

Disc electrophoresis of the reaction products of collagen: The products of collagen reacted with the purified enzyme from homogenate were displayed on a disc electrophoresis. A representative result with the enzyme from the homogenate of liver pieces washed 6 times is illustrated in Fig 1.

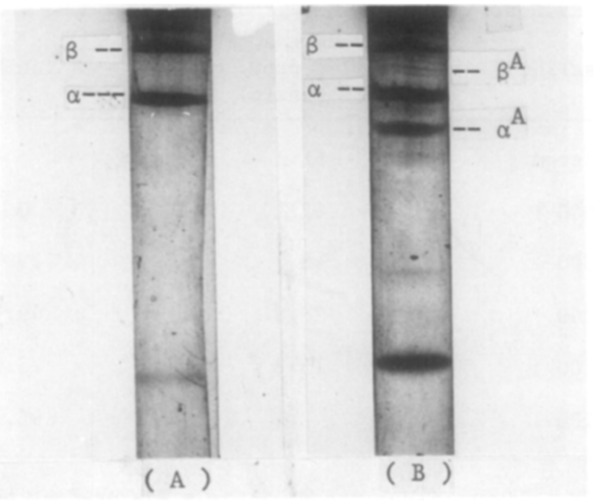
The electrophoretic pattern indicates the presence of α^A, β^A and smaller fragments in addition to α,β and a fraction of high molecular weight which are present in the control.

Discussion

The present study was performed in an attempt to test the view that collagenase is present in the liver as an active form, but is converted into an inactive form in vitro by forming a complex with serum inhibitors.

To remove contaminating serum, the liver was cut into small pieces and washed serially with various kinds of solution. The addition of NaSCN or trypsin to the washing medium, which was expected to denature or to trap serum inhibitors for collagenase, produced no better result on the enzyme activity, but rather deleterious effect on the tissue pieces at their high

Fig 1 Disc electrophoretic pattern of the digested products of collagen.



Collagen was digested with the purified enzyme specimen of homogenate from the liver pieces washed 6 times. The reaction products were denatured and displayed on a disc electrophoresis.
(A) control (vehicle of the enzyme specimen was used)
(B) enzyme treated

concentrations. These findings suggest that in the present experiment the enzyme activity became detectable only when the cells in the liver were maintained intact throughout the procedure and that simple mechanical removal of serum inhibitory materials from the tissue appears a major factor in the development of the enzyme activity in the subsequent homogenate. This inference was also supported by the finding that the serial washing was similarly effective irrespective of the temperature.

Addition of minute quantities of serum produced a dose-related inhibition of the enzyme activity in the homogenate from the washed liver. It is evident from the data of Table 2 that contamination in homogenate with endogenous serum in the liver should cause an inhibition of more than 95 % of the enzyme activity. In view of the presence of nonspecific proteolytic reaction in our assay system, it is implied that the contamination should lead to nearly 100 % inhibition of the enzyme activity when homogenate of the whole organ was employed for the assay.

In conclusion, it is definitely shown that collagenase activity is demonstrable in liver homogenate when due care is taken for the preparations.

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