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Collagenolytic Activity of Mammalian Pancreas.* (29254)

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Several descriptions of the collagenolytic activity of the pancreas have been published. Ziffren and Hosie(1) originally claimed that pancreatic juice could solubilize collagen from Achilles tendon, but Straumford and Hummel(2) found that this activity was completely suppressed by soybean trypsin inhibitor. Banga and Balo(3) reported that pancreas contained a collagen solubilizing enzyme which attacked the "mucoprotein" cement maintaining the integrity of collagen bundles. These workers(4) later characterized this enzyme as "collagen mucoproteinase" and indicated that the products of the action of this enzyme were not dialyzable. Collagen mucoproteinase apparently does not attack peptide bonds, but rather attacks ester bonds.

We reported that saline extracts of whole pancreas could both inhibit the fiber forming ability and decrease the viscosity of solutions of salt soluble collagen in the presence of excess soybean trypsin inhibitor(5). This activity could be partially purified by extraction of the pancreas at pH 8.6(6). In the presence of soybean trypsin inhibitor, these extracts could release ninhydrin reacting materials from salt soluble collagen, and could reduce the intrinsic viscosity of these collagen solutions from 14dl/g to about 6dl/g while the optical rotation of collagen was decreased from −365 to −260°(6). This activity was not dependent upon the presence of calcium and was maximal at pH 5.5(6).

This paper describes the results of some critical experiments concerning the nature of this collagenolytic activity of pancreas.

Materials and methods. Salt soluble collagen was prepared from acetic acid extracts of calf skin as we have described previously (6). This material was purified by repeated solubilization in 0.5 M acetate (pH 5.5) and precipitation by dialysis against water. The final product had an intrinsic viscosity of 14.3dl/g and an optical rotation of −370° and contained 18.6% nitrogen(7) and 13.6% hydroxyproline(8).

Precipitated collagen was prepared from clarified acetic acid (0.5 M) extracts of bovine Achilles tendon by addition of $CaCl_2$ to a concentration of 0.5 M. The resulting precipitate was washed extensively with $CaCl_2$ and then with water.

Insoluble collagen was prepared by washing minced Achilles tendon in a Waring blendor for 3 minutes with 0.5 M acetate buffer pH 5.5 at 4°C. The tendon suspension was allowed to stand overnight in the cold. After centrifugation at 24,000 × *g*, the residue was then washed repeatedly with water and dried *in vacuo*.

Crude pancreatic collagenolytic activity was prepared by extracting 100 *g* of whole, defatted and desiccated pancreas (Bridase) in the cold in a Lourdes Omni-mixer for 5 minutes with 1500 ml of 0.1 M carbonate buffer, pH 8.6. After centrifugation, the clear extract was dialyzed and lyophilized.

Crystalline trypsin and soybean trypsin inhibitor were obtained from the Worthington Co. (New Jersey).

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TABLE I. Percent of Various Collagens Solubilized and Rendered Dialyzable (in Terms of μmoles of Hydroxyproline) by Trypsin and Alkaline of the Whole Pancreas.

Substrate	Enzyme system	% solubilization	% dialyzable†
Soluble collagen (1 mg/ml)	Control	—	0
	Trypsin (1 mg/ml)	—	4
	*Extract (.3 mg/ml)	—	53
Precipitated collagen (25 mg/ml)	Control	5	10
	Trypsin (5 mg/ml)	6	15
	*Extract (5 mg/ml)	12	58
Insoluble collagen (25 mg/ml)	Control	0	0
	Trypsin (5 mg/ml)	0	0
	*Extract (5 mg/ml)	1.3	51

* Extracts were mixed with equivalent amounts of soybean trypsin inhibitor prior to incubation with substrate.

† % of that which was soluble which could be dialyzed.

Solutions of soluble collagen were prepared by suspending 15 mg of salt soluble collagen in 10 ml of 0.5 M acetate buffer in the cold using a Potter homogenizer. These suspensions were allowed to stand overnight, and after centrifugation, were diluted with buffer to contain 1.25 mg/ml of collagen as judged from solution viscosity and optical rotation determinations.

Suspensions of either precipitated or insoluble collagen were prepared in a similar manner using a Potter homogenizer, with 125 mg of collagen per 4 ml of 0.5 M acetate buffer pH 5.5.

Results. Four ml samples of buffer containing 5 mg of soluble collagen were mixed with: a) 1 ml of buffer alone; b) 1 ml of buffer containing 5 mg of trypsin; c) 1 ml of buffer containing 1.5 mg of pancreas extract and 1.5 mg of soybean trypsin inhibitor, all in the cold (14°C). These solutions were allowed to stand overnight at 14°C. Aliquots were removed for acid hydrolysis and hydroxyproline determination(8), while the remainder of the solution was dialyzed against 800 volumes of 0.5 M acetate buffer for 48 hours with stirring at 4°C. After dialysis, aliquots of each sample were analyzed for hydroxyproline and after suitable correction for volume changes, the percent of the hydroxyproline lost *via* dialysis from each sample was calculated.

Similar mixtures of 4 ml samples of buffer containing either 125 mg of precipitated collagen or 125 mg of insoluble collagen were prepared with: a) buffer alone as a control; b) with 25 mg of trypsin or c) with 25 mg of pancreas extract and soybean trypsin inhibitor. These mixtures were allowed to stand for 12 hours at 14°C and were then resuspended, centrifuged clear and aliquots removed for hydroxyproline determination. In this fashion the percent of insoluble collagen solubilized was determined. The supernatants were then subjected to dialysis at 4°C for 48 hours against 800 volumes of buffer. The amount of hydroxyproline remaining was then determined and the % of the solubilized collagen which was dialyzable was calculated.

The results, along with final concentrations of each reactant, are presented in Table I. These results indicate that about half of the hydroxyproline content of the soluble collagen was rendered dialyzable by pancreas extract in the presence of soybean trypsin inhibitor, while only about 4% of the collagen was rendered dialyzable by trypsin alone. Further, pancreas extract solubilized considerably more of each kind of insoluble collagen than did trypsin. About half of that hydroxyproline which had been solubilized by pancreas was also dialyzable. Although only 4.3% of the insoluble collagen was solubilized at 14°C, this still represents about ⅔ of a μmole of hydroxyproline per ml of solution, and this amount was quite sufficient for analysis.

Two 20 ml solutions of soluble collagen in buffer were made up to contain 1 mg/ml of substrate, 0.125 mg of pancreas extract and 0.125 mg/ml of soybean trypsin inhibitor.

TABLE II. Kinetics of Pancreatic Collagenolytic Activity Analyzed upon a Solution of Salt Soluble Collagen.*

Incubation time, min	μmoles/ml ninhydrin	"Reduced" viscosity
0	.00	44
5	.18	—
10	.27	32
15	.28	—
20	.28	26
25	.28	—
30	.28	24

* .125 mg of enzyme prep/ml of acetate buffer .5M pH 5.5 containing 1.0 mg/ml of salt soluble collagen at 20°C assayed with respect both to release of ninhydrin reacting material and reduced viscosity of solution at various times after incubation.

1) Optical density equivalent to that produced by various amounts of glycine with ninhydrin.

2) Specific viscosity/conc in g % at 1 mg/ml.

These solutions were incubated at 20°C and pH 5.5 for 0 to 30 minutes. One ml aliquots of one of these solutions was removed every 5 minutes and mixed with an equal volume of isopropanol. The clear supernatant from this mixture was analyzed with respect to its concentration of ninhydrin reacting material by the method of Rosen(9) and the data expressed in terms of similar amounts of blue color produced by glycine (glycine equivalents). The reduced viscosity of the other solution was determined about every 10 minutes.

The kinetics of peptide bond hydrolysis and reduction of viscosity of the substrate were determined during the incubation of pancreas extract with soluble collagen in the presence of soybean trypsin inhibitor. The results are presented in Table II and demonstrate that within 15 minutes at 20°C the release of isopropanol soluble terminal amino acids from soluble collagen was complete. The major proportion of the reduction of solution viscosity had also occurred by this time.

The ability of pancreas extract (0.125 mg/ml) in the presence of soybean trypsin inhibitor (0.125 mg/ml) to either release ninhydrin reacting materials from soluble collagen (1 mg/ml) or to reduce the viscosity of these solutions at 20°C and pH 5.5 was neither increased by addition of up to 0.5 M $CaCl_2$ nor inhibited by 10^{-3} M EDTA or by 10^{-3} M p-chloromercurobenzoate.

Finally, similar concentrations of pancreas extracts and soybean trypsin inhibitor at 20°C were *unable* to effect the proteolysis of denatured hemoglobin at pH 5.5.

Discussion and conclusions. It is possible that this collagenolytic activity of pancreas might be due to one or more of the following reasons: 1) contamination of the pancreas by bacterial collagenase; 2) "collagen mucoproteinase" activity; 3) thermal denaturation of the substrate leading to an attack by proteolytic enzymes upon the partially degraded collagen and thereby mimicking true collagenolytic rather than proteolytic activity and 4) the non-specific action of tissue cathepsins.

At 14°C and at pH 5.5, thermal denaturation of collagen is extremely unlikely(10). The susceptibility of the 3 collagen substrates used toward trypsin was indeed not greatly in excess of the activity demonstrated by the buffer alone which would not be the case for denatured collagen. Further, solutions of soluble collagen had intrinsic viscosities (14dl/g) and optical rotations (−360°) appropriate to native collagen(10). Therefore, it is extremely unlikely that these substrates were denatured to a significant degree.

Pancreas extract was able to solubilize both insoluble collagens and to convert about half of the soluble and solubilized collagens into a dialyzable form. Thus this pancreatic extract possessed the ability to hydrolyze peptide bonds. This property is not shared by pancreatic "collagen mucoproteinase"(4). Further evidence for peptide bond hydrolysis by these alkaline pancreas extracts was the release of isopropanol soluble ninhydrin reactive material from soluble collagen.

Collagenolytic activity of alkaline extracts of porcine did not require calcium ions, nor was it inhibited by EDTA: it therefore differs from the activity displayed by collagenase of bacterial origin(11). Pancreatic collagenase activity was also not inhibited by sulfhydryl binding agents such as para-dichloromercurobenzoate. This fact, coupled with the lack of hydrolysis of denatured hemoglobin at acid pH demonstrated by alkaline extract of the pancreas when mixed with soybean trypsin

inhibitor, suggests that the collagenolytic activity contained in this extract was not due to cathepsins(12).

Therefore the ability of alkaline extracts of porcine pancreas to hydrolyze the peptide backbone of collagen would *not* appear to derive from: 1) contamination with bacterial collagenase; 2) "collagen mucoproteinase" activity; 3) general proteolysis of denatured collagen or; 4) non-specific cathepsin activity. Whether pancreatic collagenolytic activity is produced by one enzyme or a system of enzymes (*i.e.*, one solubilizing, another proteolytically acting upon the solubilized product) such as has been found by Franklin and Wynn(13,14) in the liver lysosome is not yet known. Like "collagen mucoproteinase"(4), this latter liver enzyme system was found not to release either dialyzable hydroxyproline or ninhydrin reacting materials from collagen (14).

Summary. Alkaline extracts of the whole pancreas were found to be capable of rendering about one-half the hydroxyproline content of native soluble collagen into a dialyzable form. These extracts could also solubilize small amounts of dialyzable hydroxyproline from washed insoluble collagen. This apparent collagenolytic activity of pancreas extracts was not inhibited by EDTA, soybean trypsin inhibitor or mercurobenzoate. These fractions did *not* demonstrate any proteolytic activity upon denatured hemoglobin.

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Comparison of Anti-Granuloma, Thymolytic and Glucocorticoid Activities of Anti-Inflammatory Steroids. (29255)

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Since the demonstration of anti-rheumatic (1) and anti-inflammatory activities(2) of adrenocortical steroids, there have been numerous reports of the effectiveness of synthetic corticoids in suppressing inflammation in laboratory animals. These compounds were generally compared with cortisone or hydrocortisone for their relative activity in inhibiting artificially induced inflammation of several varieties and for such other corticoid effects as thymolysis, liver glycogen concentration and alterations in the numerical count of various white blood cells. The methods employed for assay of the steroids varied in each of the reporting laboratories, and the strain and species of animal, as well as the laboratory conditions, have not been uniform. Through necessity, comparison of relative activities of commercially available and experimental corticoids have been made using data or reports from several laboratories(3,4,5,6, 7). The present investigation was undertaken to establish the relative activities of most of the commercially available corticoids in well