Collagenolytic Enzymes and Acid-Proteinase Activity in Granuloma Tissue

Inflammed tissue has a high cathepsin activity. Among cathepsins, cathepsin D and collagenolytic cathepsin are closely related with the destruction of connective tissue[1,2]. It is assumed that collagen fibres are first attacked by extracellular neutral collagenase and the subsequent digestion of the fragmented material is completed intracellularly in the digestive vacuoles with cathepsins[3]. Collagenolytic cathepsin alone is able to break down native acid-soluble and insoluble collagen at an acid pH[4]. An important substrate of cathepsin D are glycoproteins of connective tissue. Collagen is not degraded by this enzyme[5].

In our investigation, we studied the activity of acid proteinase (mainly cathepsin D) and collagenolytic cathepsin in granuloma tissue of rats. The granuloma was induced by s.c. administration of turpentine. Moreover we assessed the peptidase acting on a synthetic collagenase substrate 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (Pz-peptide). Pz-peptidase activity is also closely correlated with collagen degradation and metabolism[6,7]. The development of different enzyme activities was investigated in relation to the age of the granuloma.

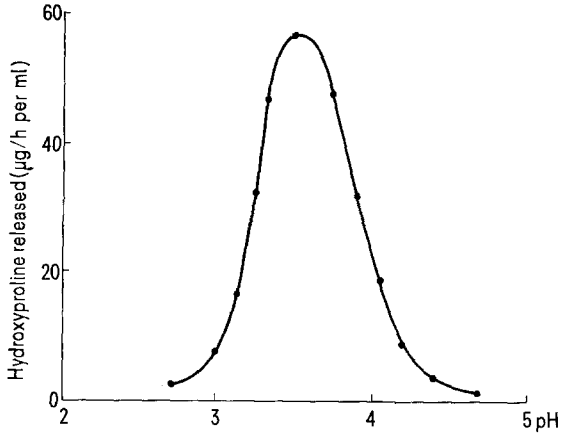
Materials and methods. Granuloma was induced by s.c. injection of 0.3 ml turpentine in Wistar rats, weighing 140–150 g. The tissue was removed on the required day, washed with cold isotonic KCl, blotted and weighed. A homogenate was prepared by initially grinding the tissue with cleaned sea-sand in a mortar and then adding 5 volume of cold isotonic KCl. Each extract was stirred in the cold for 2 h. and then centrifuged at 12,000 × *g* for 15 min. The Pz-peptidase activity was investigated in the extracts centrifuged additionally at 100,000 × *g*.

Acid proteinase (cathepsin D) and collagenolytic cathepsin were assayed by ETHERINGTON's method[4]. Acid proteinase was determined with hemoglobin (Serva) as a substrate at pH 3.5. The trichloroacetic acid soluble tyrosine was determined by the method of HANLEY et al.[8].

The collagenolytic cathepsin was assayed with insoluble Achilles tendon collagen (Worthington Biochemical Corp., Freehold, N.J. USA) at pH 3.5. The amount of degraded collagen was calculated from the concentration of hydroxyproline estimated by STEGEMANN's method[9].

Pz-peptidase activity was assayed with Pz-peptid (Fluka, Switzerland) at pH 7.4 by the method of WÜNSCH and HEIDRICH[10]. The activity is expressed in munits (1 unit of enzyme activity is defined as the quantity required to liberate 1 µmole of the product Pz-Pro-Leu per min). Protein in the extracts was determined by the method of LOWRY et al.[11] and bovine serum albumin was used as the standard.

The pH-activity curve for the digestion of insoluble collagen was determined using the 7-day-old granuloma tissue extract which was partly purified by isoelectric precipitation at pH 5 and dialyzed against water. 0.2 *M* sodium



pH dependence of the collagenolytic cathepsin activity.

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Relationship of enzyme activities with age of granuloma tissue

	Age of granuloma tissue (days)				
	3	7	10	14	23
Acid-proteinase activity (cathepsin D) (µg tyrosine/mg protein/h)	136.0 ± 9.76	169.6 ± 16.37	473.1 ± 30.58	320.8 ± 31.92	239.9 ± 31.5
Collagenolytic cathepsin (µg hydroxyproline/mg protein/h)	7.9 ± 1.33	11.0 ± 1.22	20.9 ± 1.66	24.1 ± 2.19	17.0 ± 1.94
PZ-peptidase activity	0.44 ± 0.11	0.52 ± 0.05	0.51 ± 0.05	0.82 ± 0.12	0.77 ± 0.05

5 animals (2-month-old) were used in each group. Enzyme determinations were made on the tissue from each animal, 3rd–23th day. All the results are presented as means, together with confidence limits of the 5% level of probability.

formate buffers containing 10 m*M* cysteine were used throughout. The pH values were determined from duplicate assay mixtures that had been incubated at 37 °C.

Results and discussion. The pH dependence of the activity of collagenolytic cathepsin is illustrated in the Figure. A sharp maximum of activity is observed at pH 3.5. This result is in keeping with the finding of ANDERSON[12], who found the optimum activity near pH 3.5 in rat liver extract, and also with the findings of ETHERINGTON[4], who found the same optimum pH in liver extract and in extracts from the involuting uterus[13]. BAZIN and DELAUNAY investigated collagenolytic cathepsin in turpentine granuloma with very labile acid soluble collagen as substrate and located maximal activity at pH 4.6, with smaller peaks at pH 3.2 and 5.6. We could not find any activity at pH 4.6 when using insoluble collagen as substrate. The collagenolytic activity of the granuloma extracts was strongly inhibited by iodoacetamide and *p*-chloromercuribenzoate.

The activities of the enzymes investigated in relation to the age of the granuloma are summarized in the Table. The activity of cathepsin D reached maximum values in 10-day-old granuloma, while activities of collagenolytic cathepsin and Pz-peptidase have their maximum in 14-day-old granuloma. It is interesting that both Pz-peptidase and collagenolytic cathepsin, which are closely correlated with the degradation of collagen, reach their maximum at the same time.

The collagen concentration in turpentine granuloma declines slowly after 7 days, which is associated with the absorbtion of the granuloma tissue[14]. It is probable that high activities of collagenolytic enzymes in this stage reflect the increased degradation of the collagen component of granuloma tissue.

Zusammenfassung. Die Kathepsin D-Aktivität des kollagenolytischen Kathepsins und der Pz-Peptidase wurden während der Bildung des mittels s.c. Injektion von Terpentinol hervorgerufenen Granuloms bei Ratten verfolgt. Die Kathepsin D-Aktivität erreichte am 10. Tag ihren Höhepunkt, diejenige des kollagenolytischen Kathepsins und der Pz-Peptidase erst am 14. Tag. In dieser Phase ist die Kollagenkonzentration herabgesetzt, während sich eine ausgeprägte Resorption des Granuloms zeigt.

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Proteinase Inhibitor Activity in Connective Tissues

Many proteinase inhibitors have now been identified in tissues and body fluids of many species[1]. Naturally occurring proteinase inhibitors generally are polypeptides or glycoproteins of varying molecular weights and can be anionic or cationic. In man, at least 7 proteinase inhibitors have been found in blood[2]. We have recently observed that cartilage contains a cationic trypsin inhibitor[3], and now report that other connective tissues contain a similar inhibitor.

The method for the demonstration of the trypsin inhibitor was as follows: weighed aliquots of fresh diced tissue were stirred in a ratio of 1:10 (w/v) overnight at room temperature in 3 *M* guanidinium chloride (GuCl) buffered with 0.02 *M* N-morpholinoethane sulfonic acid, pH 6. The extract was decanted, dialyzed against deionized water, and any precipitate which formed was removed by centrifugation. The clear supernate was lyophilized and redissolved in distilled water to a volume 1/10th that of the original extract and used to assay for the presence of trypsin inhibitor. Plates containing denatured fibrinogen dissolved in agar yield a cloudy semi-opaque gel when the agar solidifies. If trypsin is placed into a well in the gel, it diffuses into the agar, digests the fibrinogen, and leaves a clear zone whose radius is exponentially related to the trypsin concentration[4]. If a solution containing a trypsin inhibitor is placed into wells opposite those containing trypsin the inhibitor also diffuses into the agar. At the interface between enzyme and inhibitor the circular ring of clearing is interrupted and a straight line of inhibition forms[5].

10 µl of a 40 µg/ml trypsin solution were placed into one well and 10 µl of the test sample into opposite wells to test for trypsin inhibition (Figure 1). This method gives a semi-quantitative estimation of inhibitory activity.

To determine relative charge, an electrophoretic sandwich technique similar to that described for the detection of small amounts of lysozyme was used[6,7]. A cellulose acetate membrane was soaked in barbital

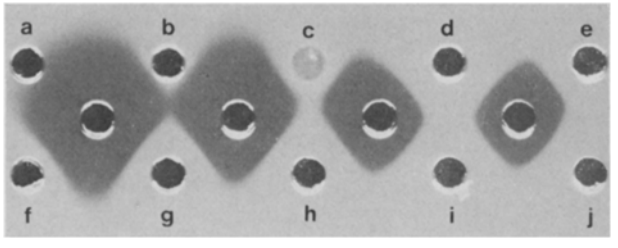

Fig. 1. Agar diffusion method for the detection of trypsin inhibitor. The central row of wells contains trypsin (40 µg/ml). Well a) contains 0.15 *M* NaCl; well b) dialyzed epiphyseal cartilage extract; well c) dialyzed (concentrated 1:10) epiphyseal cartilage extract; well d) dialyzed (concentrated 1:10) articular cartilage extract; well e) dialyzed (concentrated 1:10) aorta extract; wells f) to j) Trasylol[8] solutions containing 1, 5, 25, 100 and 250 µg/ml respectively. In the Table an inhibitory activity equivalent to that of 100 to 250 µg/ml Trasylol was designated as ++++ and on inhibitory activity equivalent to that of 5 to 25 µg/ml Trasylol was designated as +++.

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