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The Presence of Cathepsin B in Cartilage

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Lucy, Dingle & Fell (1961) showed that the autolytic degradation of embryonic chick cartilage in the presence of excess of vitamin A or under hypotonic conditions was due to an endogenous intracellular proteolytic enzyme. The release of chondromucoprotein from cartilage was ascribed to the action of a lysosomal protease on the protein-polysaccharide complex of the matrix (Dingle, 1961; Fell & Dingle, 1963). A similar chondrolytic enzyme maximally active at pH 5 was shown by Ali (1964) to be present in rabbit ear cartilage and in ox and human articular cartilage. It released up to 50% of the total cartilage chondromucopeptide within 24 hr. but little or no hydroxyproline. As this enzyme was inhibited by arginine, arginine analogues and to some extent by iodoacetamide, Ali (1964) postulated the presence of a cathepsin B-like enzyme in rabbit ear cartilage and ascribed the autolysis of the matrix at pH 5 to its action on the protein part of chondromucoprotein.

A study was therefore undertaken to determine whether particular synthetic substrates that are known to be specific for certain cathepsins (Fruton, 1960) are hydrolysed by this cartilage enzyme. In preliminary experiments, the characterization of this enzyme was attempted by incubating a cartilage extract with specific synthetic substrates and estimating their hydrolysis. These experiments were unsuccessful because of the difficulty in obtaining either a reproducible extract or a satisfactory homogenate of cartilage. The following

alternative procedure was adopted to facilitate the study of cartilage enzymes.

Young rabbit ear cartilage was dissected free of skin and adhering tissue, rinsed with 0.9% NaCl, cut up into 7.5 mm.² pieces and weighed into 0.3 g. portions. Blocks of frozen cartilage were then sectioned (18 μ) in a cryostat with the knife temperature at -35° and the cabinet temperature at -20° . Frozen cartilage sections were collected quantitatively into wide-mouthed bottles through a funnel made from a celluloid sheet. Cartilage sections (0.3 g.) were incubated with 50 mm-benzoyl-L-arginine amide in 5 ml. of universal buffer (Ellis, 1961) at 37° for 24 hr. Penicillin (5 000 units) and streptomycin (5000 units) were included to prevent bacterial contamination. Duplicate samples (0.2 ml.) were removed from the supernatant fluid of the reaction mixture and the ammonia liberated was titrated by the Conway micro-diffusion technique as described by Schwert, Neurath, Kaufman & Snoke (1948), with ammonium sulphate as the standard. On completion of the 24 hr. incubation, the cartilage sections were centrifuged (1000 g for 10 min.) and the release of hexuronic acid into the supernatant fluid was estimated by a modification (Bitter & Ewins, 1961) of the carbazole method of Dische (1947).

It was found that optimum hydrolysis of benzoyl-L-arginine amide by rabbit-ear-cartilage sections was at pH 5 in the presence of 10 mm-cysteine, and

Fig. 1. Effect of cysteine and iodoacetamide on the hydrolysis of benzoyl-L-arginine amide by cartilage cathepsin. Rabbit-ear-cartilage sections (0.3g.) were incubated with benzoyl-L-arginine amide in 5ml. of pH5 buffer at 37° for 24hr. Samples (0.2ml.) were withdrawn at intervals for estimation of the amount of ammonia released after incubation in the presence of: buffer alone (●); 10mM-cysteine (○); 10mM-cysteine and 25mM-iodoacetamide (Δ); 25mM-iodoacetamide (▲).

this activity was destroyed completely by preheating the cartilage sections in buffer at 80° for 15min. Fig. 1 shows the dependence of the cartilage cathepsin activity on SH reagents. The enzyme hydrolysing benzoyl-L-arginine amide at pH5 appears to be in a partially active state in cartilage and requires the presence of 10mM-cysteine for complete activation. Iodoacetamide inhibited 66% of the cysteine-activated enzyme activity and inactivates completely the cartilage enzyme in the absence of cysteine. The hydrolysis of benzoyl-L-arginine amide in the presence of cysteine was proportional to the amount of cartilage (range 0.1–0.4g.). Cartilage sections or benzoyl-L-arginine amide incubated on their own at pH5 for 24hr. did not liberate any ammonia. The presence of 0.1M-arginine inhibited the enzymic hydrolysis of benzoyl-L-arginine amide completely.

After incubation of the cartilage sections with and without the synthetic substrate for 24hr., the supernatant fluid was removed by centrifugation and its hexuronic acid content estimated as an indication of autolysis of cartilage. It was found that 0.3g. of cartilage sections incubated without the synthetic substrate released 357 μg. of hexuronic acid/ml. of incubation buffer at pH5 in the absence of cysteine. This release of hexuronic acid increased to 450 μg./ml. in the presence of 10mM-cysteine. Preheating the cartilage sections in buffer at 80° for 15min. limited the release to 90 μg. of hexuronic

acid/ml. The release of hexuronic acid in the cysteine-activated system was inhibited 36% in the presence of 50mM-benzoyl-L-arginine amide.

The amount of hexuronic acid released from cartilage sections in the presence of 50mM-benzoyl-L-arginine amide increased from 255 μg./ml. to 290 μg./ml. when 10mM-cysteine was included. The addition of 25mM-iodoacetamide to the cysteine-activated system in the presence of 50mM-benzoyl-L-arginine amide produced a 72% inhibition of hexuronic acid release from cartilage sections. Iodoacetamide in the absence of cysteine inhibited the release of hexuronic acid by approx. 85%. These values for the release of hexuronic acid from cartilage sections in the presence of the synthetic substrate are proportional to the release of ammonia from benzoyl-L-arginine amide in the same experiment (Fig. 1) and may indicate that the same enzyme is responsible for the two activities at pH5. It is possible that the synthetic substrate is competing for the enzyme with the normal protein substrate in the matrix, as benzoyl-L-arginine amide undergoes simultaneous hydrolysis while inhibiting the autolytic release of hexuronic acid from cartilage sections presumably by the same enzyme.

These experiments demonstrate the presence of an enzyme resembling cathepsin B in rabbit ear cartilage. Some of the properties found for cathepsin B in cartilage sections such as pH optimum, activation by cysteine and inhibition by arginine and iodoacetamide are shared by the enzyme responsible for the autolytic degradation of rabbit ear cartilage at pH5 (Ali, 1964). Further experiments are required to see whether other known synthetic substrates for cathepsins are hydrolysed by cartilage enzymes. It should eventually be possible to show whether only cathepsin B is responsible for the autolytic release of chondromucopolysaccharide from cartilage at pH5 or whether it acts in conjunction with other cathepsins or enzymes.

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