Extracellular Localization of Lysozyme in Rachitic Rat Cartilage (37145)

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(Introduced by Alan S. Josephson)

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Lysozyme (muramidase) is a low molecular weight, cationic protein which has been identified in human costal and hyaline articular cartilage (1), bovine and canine scapulae (2), mouse femur (3), and chick embryonic skeleton (4). Lysozyme appears to be a component of the extracellular matrix of cartilage, where presumably its cationic nature leads it to interact ionically with proteoglycans. Since cartilage contains no known substrate for the muramidase activity of lysozyme (5), it has been suggested that lysozyme may regulate the catabolism of proteoglycans or participate in the process of mineral deposition (4, 6).

In many tissues, mineralization is associated with extracellular, membrane-bounded structures which have been identified by electron microscopy and have been termed "matrix vesicles" (7). These vesicles contain enzymatic activities which may play a role in calcification (8). Since both lysozyme and the vesicles are extracellular in cartilage, the present study was undertaken to determine whether cartilage lysozyme is associated with the vesicles.

Cartilage from the proximal tibial epiphyses of rachitic rats was chosen for this study because this tissue is easily obtained in large amounts (relative to the size of the animal) and because it contains large numbers of matrix vesicles which can be easily fractionated from other components of the cartilage. The presence of lysozyme in cartilage from rachitic rats was demonstrated. The lysozyme content of rachitic cartilage was found to be comparable to that of normal tissue. Independent, direct evidence of the extracellular nature of cartilage lysozyme was obtained.

Methods. Male rats of the CD strain (Charles River), initial weight 50 g, were maintained for 4 wk on a rachitogenic diet (9). The diet was deficient in phosphate and vitamin D but was supplemented with calcium. Tap water was given *ad libitum*. The animals were sacrificed by cervical dislocation and the proximal epiphyseal cartilages of both tibias were freed of surrounding tissue under a dissecting microscope. At the time of sacrifice, the animals usually exhibited severe skeletal rickets upon gross and histologic examination of the proximal tibial epiphyses. Neurologic signs of the disease were never present.

The technique for digestion and fractionation of the tissue was modified from that previously described for bovine cartilage (8). The specimens were incubated overnight at room temperature in 5 vol of buffer of the following composition: 0.12 *M* NaCl, 0.01 *M* KCl, 0.02 *M* TES buffer [*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid], adjusted to pH 7.4 with NaOH, 100 units/ml each of penicillin and streptomycin, and 1.7 mg/ml collagenase (Worthington, CLS grade). By progressive differential centrifugation (8), six pellets of material (fractions 1 through 6) and a final supernatant (fraction 7) were obtained. Electron microscopic studies, to be reported in detail elsewhere, show that fraction 1 (600 g min) consists of hydroxyapatite crystals, fractions 2 and 3 (20,000 to 145,000 g min) contain intact cellular elements, fraction 4 (307,000 g min) contains nuclei, mitochondria, and lysosomes, and fractions 5 and 6 (1.08 to 6.0 $\times$ 10^6 g min) contain the matrix vesicles which are believed to play a role in calcification.

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The substrate for measurement of lysozyme activity was freeze-dried *M. lysodeikticus* (Worthington) suspended in 0.063 *M* phosphate buffer (pH 6.3) at a concentration of 40 mg/100 ml. Upon addition of lysozyme, the rate of decrease of the apparent absorbance of the bacterial suspension at 645 nm was measured in a spectrophotometer equipped with Gilford electronics. Standards for the assay were prepared from purified human lysozyme isolated from the urine of patients with acute monomyelocytic leukemia and kindly supplied by Dr. E. Osserman. The method of least squares was used to determine the slope of the straight line best approximating the decrease of apparent absorbance as a function of time. The relationship between slope and lysozyme activity was linear over the concentration range from 4.7 to 23.4 μg lysozyme/ml ($r = 0.996$). This relationship was used to measure the lysozyme activity of the cartilage extracts; results are expressed as human lysozyme equivalents.

To test the heat stability of the active principle isolated from rachitic epiphyseal cartilage, aliquots of a representative supernatant fraction (fraction 7) rich in lysozyme activity (*vide infra*), were mixed with equal volumes of either 0.9% NaCl, 0.2 *M* acetate buffer (pH 3.7), or 0.2 *M* glycine buffer (pH 10.4). The specimens were heated at 100° for 1 min and were retested for activity in comparison to an unheated control of similar dilution.

Previous work with freeze-powdered human costal cartilage indicated that 1 *M* NaCl was an effective reagent for extraction of lysozyme from cartilage (1). A sample of rachitic epiphyseal cartilage, wet weight 240 mg, was subjected to four sequential 1-hr extractions at room temperature in 1.0 ml of 1 *M* NaCl, after which the residue was digested overnight with collagenase as described above. The fractions were dialyzed against normal saline before assay for both lysozyme and for alkaline phosphatase (10).

Results. The relative lysozyme contents of the seven fractions prepared by centrifugation of the collagenase digest of pooled tibial epiphyses from 9 rachitic rats is shown in Fig. 1. If the lysozyme content of the unfractionated digest is taken as 100%, then 80.5% of the total activity was recovered in the supernatant (fraction 7). Trace amounts of activity were noted in the cellular fractions (2 and 3); no activity appeared in the vesicular fractions (5 and 6). In contrast, the relative specific activity of alkaline phosphatase was greatest in fractions 5 and 6; relatively small amounts appeared in the supernatant. Quantitative determination of the total lysozyme content of 3 pools of rachitic epiphyseal cartilage yielded values of 0.096, 0.109, and 0.078 μg lysozyme/mg wet cartilage. For comparison, the total lysozyme content of pooled tibial cartilage from a group of normal rats was determined to be 0.104 μg lysozyme/mg wet weight.

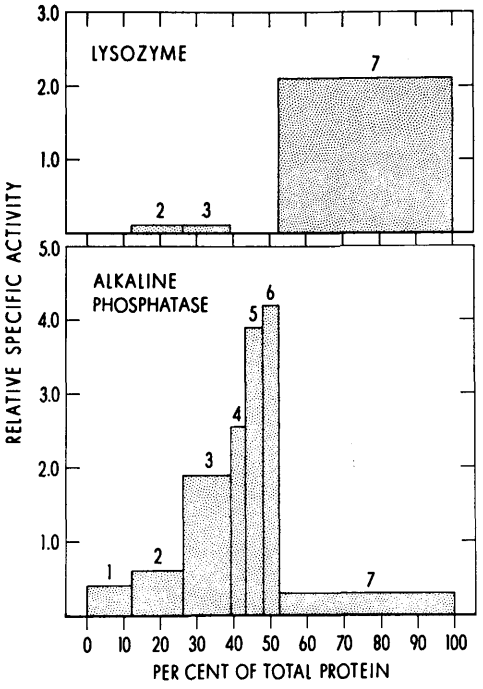

FIGURE 1.

The active principle in a representative sample of fraction 7 was tested for heat stability as described above. A specimen heated in 0.9% NaCl lost only 1.8% activity. When heated in alkaline and acid buffers, the same specimen lost 79.8 and 25.6% of its activity, respectively. Relative heat stability in acid but not in alkali is a well-known characteristic of many animal lysozymes (11).

When rachitic cartilage was extracted with sequential changes of 1 *M* NaCl, 72.8% of the lysozyme was eluted in the first extract (Table I). The remaining lysozyme was recovered in increments in 2 subsequent incubations. The residual cartilage, digested with collagenase, contained no detectable lysozyme activity. Parallel collagenase digestion of another aliquot of the same cartilage indicated that extraction with 1 *M* NaCl yielded 82% of the total detectable lysozyme. In contrast, the 1 *M* NaCl extracts contained only 19.6% of the total alkaline phosphatase activity; the remainder was retained in the cartilage until collagenase digestion. By both differential centrifugation and sequential extraction with molar sodium chloride, lysozyme and alkaline phosphatase activities were clearly shown to be dissociated.

Discussion. These experiments indicate that lysozyme activity is present in the epiphyseal cartilage of rachitic rats. Lysozyme is a soluble component of the extracellular, proteoglycan-rich fraction of the tissue. By the techniques used here, it was associated neither with the lysosomal fraction nor with the membrane-bounded, alkaline phosphatase containing matrix vesicles of this tissue.

At least 5 different mammalian and avian species have been shown to possess cartilage lysozyme (1–4). Although studies of the lysozyme content of other rat tissues have been performed (12, 13), direct measurement of rat cartilage lysozyme has not been reported. In a prior study (1), strict criteria were applied to identification of the lytic principle isolated from human cartilage, including inactivation of lysozyme by specific antiserum.

TABLE I. Sequential Extraction of Lysozyme and Alkaline Phosphatase from Rachitic Rat Cartilage.

Extract	Lysozyme[a]	Alkaline phosphatase[a]
First NaCl	72.8	11.6
Second NaCl	23.6	4.3
Third NaCl	3.6	1.5
Fourth NaCl	0	1.2
Collagenase digest of residue	0	81.4

[a] Percentage of total activity per fraction.

Antiserum to rat lysozyme was not available for the present study, but it is reasonable to identify the lytic principle as lysozyme on the basis of its heat stability and characteristic elution profile in 1 *M* NaCl.

Indirect evidence of the extracellular, nonlysosomal nature of cartilage lysozyme was obtained in a prior study (1) on the basis of relative lysozyme and beta-glucuronidase activities of joint tissues differing widely in cellularity, *i.e.*, cartilage and synovium, and by the failure of streptolysin S to increase lysozyme extraction from cartilage. Direct evidence that lysozyme is extracellular in chick embryonic cartilage was obtained by Kuettner and coworkers (4) by immunofluorescent staining of tissue with specific antiserum and by study of lysozyme release from tissue during sequential enzymatic digestion. This group has also shown that lysozyme, identifiable in a whole cartilage extract from chick embryo, could not be detected in subcellular fractions, including a lysosome fraction, prepared by differential centrifugation of sonicated chick chondrocytes (6). The current study confirms this finding in a mammalian cartilage by direct analysis of a whole cartilage extract.

Proteoglycans have long been implicated in the process of mineral deposition in calcifying cartilage (14, 15), as have the matrix vesicles prepared in the current study (8). Lysozyme is strongly cationic and interacts with proteoglycans in solution (16) and in cartilage extracts (2). The lysozyme content of bovine scapular cartilage is highest in the hypertrophic zone, adjacent to the zone of calcification (2). This is also the area of greatest alkaline phosphatase activity (8). Mobilization of calcium from long bones by parathormone injection causes a loss of bone lysozyme (17). Bonucci has suggested that the relationship between the matrix vesicles and lysozyme should be investigated if this unusual protein is to be implicated in the calcification process (18). The current data suggest that if lysozyme does play a role in mineralization, its function is more likely to be related to its interaction with proteoglycans than to participation in apatite formation in the matrix vesicles.

Summary. Epiphyseal cartilage from rachitic rats was found to contain approximately 0.1 μg of lysozyme/mg of wet tissue. This lysozyme is not associated with the membrane-bounded, enzymatically active matrix vesicles which appear to be the locus for initial apatite crystal formation in calcifying cartilage. Lysozyme and alkaline phosphatase activity were found in different fractions of a preparation of digested cartilage subjected to differential centrifugation. Lysozyme, but not alkaline phosphatase, could be extracted from cartilage in appreciable amounts with 1 *M* NaCl. The possible role of lysozyme in calcification may relate to interaction with proteoglycans.

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