Hyaluronidase Activity in Lysosomes of Bone Tissue

By G. VAES

Laboratoire de Chimie Physiologique, University of Louvain, Belgium

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1. The distribution pattern of hyaluronidase in subcellular fractions of bone-tissue homogenates is closely similar to that reported by Vaes & Jacques (1965*b*) for the other acid hydrolases of this tissue. The highest specific activity of hyaluronidase is also found in the light-mitochondrial fraction. 2. In cytoplasmic extracts of bone, about 60% of the activity of hyaluronidase is latent, and is unmasked by a number of treatments (digitonin, low osmotic pressure, freezing and thawing, Waring Blender) that unmask the lysosomal β -glucuronidase in a closely parallel manner. Low concentrations of Triton X-100 render a larger proportion of β -glucuronidase than of hyaluronidase accessible to external substrates, but release the same proportion of both enzymes in unsedimentable form. 3. These results support the concept of an association of hyaluronidase with lysosomes in bone.

Hyaluronidase activity, optimum about pH 3.7, has been demonstrated in cytoplasmic extracts of bone tissue and a method for its quantitative assay has been described (Vaes & Jacques, 1965*a*). In preliminary studies (Vaes, 1964), this activity was found to be partly bound to cytoplasmic particles and to occur in partly latent form; this suggested the possibility of its association with lysosomes. The investigations described in the present paper provide additional evidence in support of this conclusion.

EXPERIMENTAL

Fractions were prepared from homogenates of bone (calvaria) from infant rats up to 7 days of age and assayed for hyaluronidase and other enzymes as described by Vaes & Jacques (1965*a,b*). Hyaluronidase was assayed at 37° in an incubation medium containing potassium hyaluronate (0.2–0.3%), acetate buffer, pH 3.7 (0.1 M), and NaCl (0.15 M); 1 unit of activity was defined as the amount of enzyme that under the conditions of the test reduces the viscosity of the substrate to half its value in 20 min. The reaction rate remained constant for at least 20 min. if the enzyme activity was lower than 0.25 unit. Heating the enzyme preparation at 60° for 15 min. resulted in a complete loss of its capacity to decrease the viscosity of the substrate; in the absence of enzyme, the viscosity remained constant for up to 12 hr. (Vaes & Jacques, 1965*a*).

Total release of hyaluronidase was ensured by pretreating the tissue preparations with 0.1% Triton X-100 for 15 min. at 0° before starting the assay. Latency studies were performed on cytoplasmic extracts (E) as reported by Vaes (1965), with some modifications that are described in the text below. It was verified that free or soluble hyaluronidase was neither activated nor inhibited at all by Triton X-100 in the concentrations used.

RESULTS

As illustrated by the results of Fig. 1, the distribution of hyaluronidase between subcellular fractions of bone-tissue homogenates is similar to that of the lysosomal acid hydrolases previously studied in this tissue (Vaes & Jacques, 1965*b*); the highest relative specific activity of hyaluronidase was found in the light-mitochondrial fraction (L). Fractions N, M, L, P and S contained respectively 7.3, 28.1, 28.2, 6.6 and 25.4% of the total hyaluronidase activity of the sum of the nuclear fraction and cytoplasmic extract (E+N) (recovery 95.6%). A higher proportion of protein is present in the N fraction (60%) than in previous experiments ($41.5 \pm 11.2\%$, average of 7 determinations $\pm$ s.d.; Vaes & Jacques, 1965*b*). This seems to be due mostly to the use of infant rats that were, on the average, a few days older than in the previous work. The high degree of variability observed in the protein content of the N fraction reflects the difficulty encountered in the homogenization of the extracellular fibrous calcified matrix of the tissue, whose remnants sediment with this fraction; the filtration of the N fraction on gauze removes a considerable amount of protein (in the form of gross fragments presumably originating mostly from the matrix) but little enzyme activity (Vaes & Jacques, 1965*a*).

Hyaluronidase also displays an important degree of latency in cytoplasmic extracts: its free activity (see Vaes, 1965), measured at pH 3.7, was $42.8 \pm 5.8\%$ of its total activity (average of 8 determinations $\pm$ s.d.). Partial or full activation of hyaluronidase could be achieved by several of the

Fig. 1. Distribution patterns of enzymes in subcellular fractions of bone-tissue homogenates: (a) hyaluronidase; (b) β -galactosidase; (c) β -glucuronidase; (d) alkaline phenylphosphatase; (e) cytochrome oxidase. The results, presented in the manner used by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955), are the means of two experiments. The ordinate shows the mean relative specific activity of the fractions. On the abscissa, the fractions are represented by their relative protein content, in the order in which they are isolated (see Vaes & Jacques, 1965b), i.e. from left to right: N ('nuclear'), M ('heavy-mitochondrial'), L ('light-mitochondrial'), P ('microsomal') and S (final supernatant).

treatments that release the other acid hydrolases in bone cytoplasmic extracts (Vaes, 1965). As shown in Fig. 2, hyaluronidase was unmasked to essentially the same extent as β -glucuronidase after exposure of the extract to 0.083 M-sucrose, to increasing concentrations of digitonin, to two cycles of freezing and thawing or to the action of a Waring Blendor for increasing lengths of time. On the other hand, increasing concentrations of Triton X-100, up to 0.05–0.06%, released less hyaluronidase than β -glucuronidase (Fig. 2 and Table 1), although both enzyme activities were totally released with higher concentrations of the detergent.

Several experiments were carried out in order to explore further the significance of the difference in behaviour of the two enzymes in the presence of Triton X-100. It was found that less activation of hyaluronidase (but not of the other acid hydrolases) takes place if the detergent is added together with the substrate mixture than if the extract is first preincubated for 15 min. at 0° with the same concentration of Triton X-100, and then added to the substrate mixture to start the assay. Thus the effectiveness of Triton X-100 in releasing the enzyme

Fig. 2. Correlation between free activities of hyaluronidase and β -glucuronidase in untreated cytoplasmic extracts ($\circ$) and in cytoplasmic extracts exposed to 0.083 M-sucrose ($\bullet$), treated with increasing amounts of digitonin (Δ) or of Triton X-100 ($\blacktriangle$), frozen-and-thawed twice ($\square$) or subjected to the action of a Waring Blendor for increasing lengths of time ($\blacksquare$). These treatments were done as described in Vaes (1965). The free activities were measured in 20 min. assays for hyaluronidase and in 2 hr. assays for β -glucuronidase; they are expressed as percentages of the corresponding total activities. The regression line and the correlation coefficient, $r=0.96$, were calculated on all values represented, except those given for the treatment with Triton X-100 ($\blacktriangle$).

Table 1. Influence of pretreatment of cytoplasmic extract with Triton X-100 or digitonin on free activities of hyaluronidase and of β -glucuronidase

Cytoplasmic extracts, containing the equivalent of 32 mg. of fresh calvarium tissue/ml., were preincubated for 15 min. at 0° in the presence of the detergent concentrations indicated. Appropriate amounts of these mixtures and of untreated cytoplasmic extract were then used for the measurement of free activity, as in Vaes (1965), with 20 min. assays for hyaluronidase and 2 hr. assays for β -glucuronidase. Total activities were measured on extracts pretreated with 1 mg. of Triton X-100/ml. Results are expressed as percentages of the corresponding total activities.

Addition to the cytoplasmic extract	Free activity (% of total)	
	Hyaluro-nidase	β -Glucuro-nidase
None	46	43
Digitonin (0.10 mg./ml.)	58	59
Digitonin (0.15 mg./ml.)	68	71
Digitonin (0.20 mg./ml.)	81	84
Digitonin (0.25 mg./ml.)	95	96
None	44	45
Triton X-100 (0.15 mg./ml.)	54	79
Triton X-100 (0.30 mg./ml.)	71	96
Triton X-100 (0.60 mg./ml.)	99	100
Triton X-100 (1.0 mg./ml.)	100	100

is inhibited by some component of the substrate mixture, presumably hyaluronate.

However, this effect does not explain the differences shown in Fig. 2, since the assays of free activity were performed on pretreated extracts in those experiments. As indicated by the results of Table 1, a difference occurs only with Triton X-100, and not with digitonin as activating agent.

The possibility was considered that the full effect of Triton X-100 manifests itself only when the preparations are brought to acid pH and 37°, at which time a selective protection of the lysosomes by hyaluronate could have taken place. However, this explanation was ruled out by the use of identical conditions for the assay of both enzymes. When the pretreated extracts were incubated for the same length of time with a hyaluronidase-substrate mixture containing 1.5 mM-phenolphthalein glucuronidate, β -glucuronidase still exhibited a higher free activity than hyaluronidase in extracts exposed to 0.01–0.03% Triton X-100. Full activation of both enzymes occurred with pretreatment at detergent concentrations of 0.05% or higher. Interestingly enough, β -glucuronidase displayed a higher free activity than hyaluronidase with the mixed substrate even when no pretreatment with Triton X-100 had been carried out. Thus exposure to the more acid hyaluronidase assay conditions suffices to release some β -glucuronidase activity, without similarly affecting hyaluronidase.

Fig. 3. Correlation between unsedimentable activities of hyaluronidase and β -glucuronidase in untreated cytoplasmic extracts (O) and in cytoplasmic extracts treated with increasing amounts of Triton X-100 (▲). The treatments were done as described in Vaes (1965). Samples of the preparations were centrifuged at 3000000g-min. and the supernatants were assayed for their total enzyme activities; these are given as percentages of the corresponding total activities measured on non-centrifuged samples. The correlation coefficient, r , was 0.94.

It was finally found that a much better correlation is obtained between hyaluronidase and β -glucuronidase if the effect of Triton X-100 is measured by the proportion of enzyme activity released in soluble non-sedimentable form (Fig. 3).

DISCUSSION

When considered in the context of what has been shown for eight other acid hydrolases present in homogenates of bone tissue (Vaes & Jacques, 1965b; Vaes, 1965), the experiments described in this paper suggest strongly that the hyaluronidase activity of bone is largely or totally associated with lysosome-like particles, as are the other acid hydrolases. Aronson & Davidson (1965) and Hutterer (1966) have demonstrated the lysosomal localization of hyaluronidase in liver cells. Whether this enzyme also belongs to lysosomes in the other tissues where it has been detected (Bollet, Bonner & Nance, 1963) remains to be checked.

The differential effect of Triton X-100 on the unmasking of hyaluronidase could be taken as evidence that this enzyme is preferentially located in particles that are more resistant to the action of the detergent. Bone lysosomes do indeed constitute a heterogeneous population (Vaes, 1965), as do those of many other tissues. However, a more likely explanation, suggested by the results of Fig. 3, is that the release of the lysosomal hydrolases in soluble form by Triton X-100 proceeds by way of an intermediate stage in which the particle membrane has become permeable to glucuronidate and to the substrates of most other hydrolases, while still retaining the enzymes and excluding the hyaluronate molecules.

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