HYALURONIDASE CHEMICALLY BOUND TO AGAROSE

W.H. STIMSON and A. SERAFINI-FRACASSINI
Department of Biochemistry, University of St. Andrews, St. Andrews, Fife, Scotland

Received 24 July 1971

1. Introduction

The extensive use of hyaluronidase (EC 3.2.1.35) in the structural analysis of proteoglycans always entails laborious procedures to remove the contaminating enzyme on completion of the reaction.

There is a wide range of literature available on the chemical attachment of enzymes to water-insoluble support materials [1–4]. This paper presents the application of the 2-amino-4,6-dichloro-*s*-triazine method [5] for the coupling of hyaluronidase to an agarose support. Although the enzymic activity of the insolubilised enzyme was tested using hyaluronic acid, its ability to degrade cartilage proteoglycan was also shown.

2. Materials and methods

2.1. *Materials*

Ovine testicular hyaluronidase (1640 I.U./mg) was obtained from Seravac Laboratories and hyaluronic acid (human umbilical cord) from the Sigma Chemical Company. Biogels A-50 m, 100–200 mesh, (approximately 2% agarose) and A-1.5 m, 100–200 mesh, (approximately 8% agarose) were supplied by Bio-Rad Laboratories. Cyanuric chloride was obtained from British Drug Houses Limited. Analar grade reagents were used wherever possible.

The cartilage proteoglycan was prepared from bovine nasal septa by the procedure of Malawista and Schubert [6] and fractionated by high-speed centrifugation [7].

2.2. *Methods*

Hyaluronidase was examined for proteolytic activity [8] and none was detectable at pH 5.6.

2-Amino-4,6-dichloro-*s*-triazine was prepared from cyanuric chloride by a modification [5] of the method of Thurston et al. [9].

Hyaluronidase was coupled to agarose (Biogel A-50m), using the triazine derivative, according to the procedure of Kay and Lilly [5]. The product was washed with 5 M NaCl until no more enzyme could be solubilized. The amount of enzyme bound to the water-insoluble support was calculated from the difference in E_{280} between the hyaluronidase added and that present in the washings.

The bound enzyme was assayed, by comparison with hyaluronidase of known activity, using the turbidity reduction method of Dorfman [10] at pH 5.6. The colorimetric procedure for reducing end-groups, employing ferricyanide [11], was used in the determination of the pH-activity curve. Assays were carried out at 37° in 0.05 M citrate-phosphate buffer made to 0.2 M by the addition of NaCl.

Cartilage proteoglycan (20 mg) was digested by 4 mg of bound enzyme for 24 hr, using the conditions specified above at pH 5.6.

A column (1.5 cm × 100 cm) packed with Biogel A-1.5 m and equilibrated with 0.2 M phosphate buffer pH 6.8 was used for gel filtration. The flow rate was adjusted to 9.5 ml/hr, 20 mg samples were loaded and 2.5 ml fractions collected. The fractions were tested for hexuronic acid by the carbazole method [12] and for protein content by absorption at 280 nm.

Sedimentations were carried out in a Spinco Model E ultracentrifuge at 59,780 rev/min and 20°. Samples

North-Holland Publishing Company – Amsterdam

were analysed in 0.2 M phosphate buffer pH 6.8. Sedimentation coefficients were calculated as described by Schachman [13].

3. Results and discussion

The weight of hyaluronidase bound to the agarose was found to be 127 mg/g (63% of available enzyme). From the turbidimetric assay, the bound enzyme was shown to have an activity of 252 I.U./mg (i.e. the enzyme retained approximately 15% of its original activity). The pH-activity curve (fig. 1) was obtained by measuring the initial rates of hydrolysis of hyaluronic acid by the bound enzyme, using the reducing group method. The results are expressed as a percentage of the maximum activity, found to be at pH 5.6. This value is within the range of pH-optima reported for the enzyme in the free state [14].

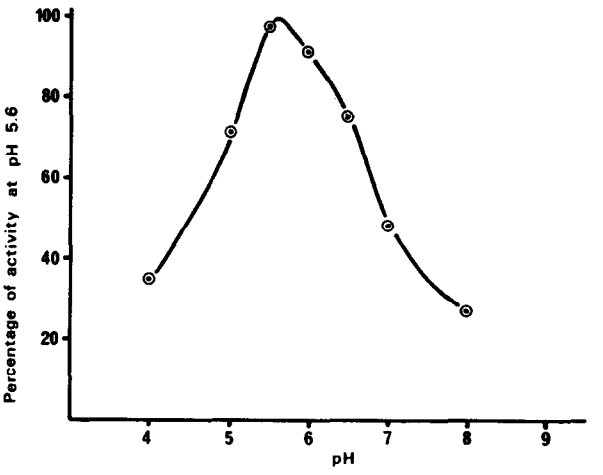

Fig. 1. Effect of pH on the initial rate of hydrolysis of hyaluronic acid by agarose-bound hyaluronidase.

The elution profile of the hyaluronidase digest of cartilage proteoglycan is shown in fig. 2. While the excluded fraction contained both hexuronic acid and protein, the retarded material consisted exclusively of polysaccharide. A similar pattern has been previously reported using soluble enzyme [15]. The retarded material (89.7% of the recovered hexuronic acid) showed high polydispersity both on gel filtration and analytical ultracentrifugation, and by comparison with the elution profiles of chymotrypsinogen and ovalbumin [16], a value of 23 Å was given for the Stokes radius of the maximum ordinate. Therefore, this fraction is composed of chondroitin sulphate degradation products. The product emerging at the void volume (10.3% of the recovered hexuronic acid) had a $S^0_{20,w}$ of 7.2 as compared with a value of 14.3 S for the original preparation. The data suggests that this material consists of the protein core of the proteoglycan attached to degraded chondroitin sulphate side chains and the keratan sulphate moiety, the latter being resistant to enzymic action [17].

These results show that the coupling of hyaluronidase to an agarose support does not impair its hydrolytic action on polymers of high molecular weight.

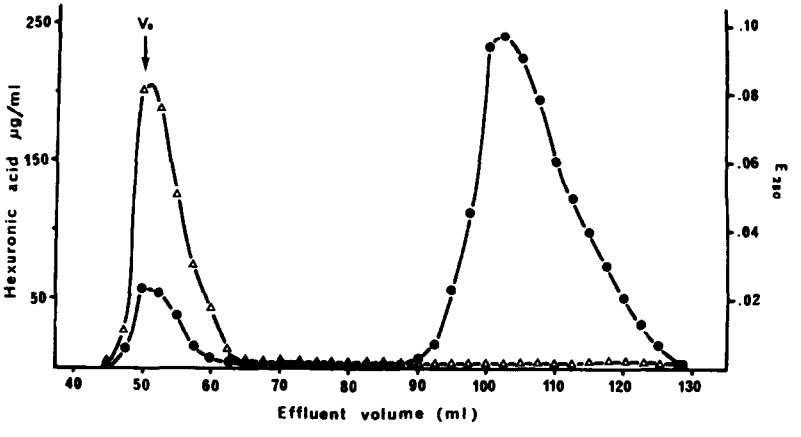

Fig. 2. Gel filtration on Biogel A-1.5 m of cartilage proteoglycan after digestion with agarose-bound hyaluronidase. △, Absorbance at 280 nm; ●, hexuronic acid; V_0, void volume.

Acknowledgements

This work was supported by grants from the Medical Research Council and the Wellcome Foundation Trust.

References

[1] I.H. Silman and E. Katchalski, Ann. Rev. Biochem. 35 (1966) 873.
[2] L. Goldstein and E. Katchalski, Z. Anal. Chem. 243 (1968) 375.
[3] G. Kay, Process Biochem. 3 (1968) 36.
[4] A.S. Lindsey, J. Macromol. Sci.; Rev. Macromol. Chem. C3 (1969) 1.
[5] G. Kay and M.D. Lilly, Biochim. Biophys. Acta 198 (1970) 276.
[6] I. Malawista and M. Schubert, J. Biol. Chem. 230 (1958) 535.
[7] B.R. Gerber, E.C. Franklin and M. Schubert, J. Biol. Chem. 235 (1960) 2870.
[8] M. Kunitz, J. Gen. Physiol. 30 (1947) 291.
[9] J.T. Thurston, F.C. Schaefer, J.R. Dudley and D. Holm-Hansen, J. Am. Chem. Soc. 73 (1951) 2981.
[10] A. Dorfman, in: Methods in Enzymology, eds. S.P. Colowick and N.O. Kaplan, Vol. 1 (Academic Press, New York, 1955) p. 166.
[11] M.M. Rapport, K. Meyer and A. Linker, J. Biol. Chem. 186 (1950) 615.
[12] T. Bitter and H. Muir, Anal. Biochem. 4 (1962) 330.
[13] H.K. Schachman, in: Methods in Enzymology, eds. S.P. Colowick and N.O. Kaplan, Vol. 4 (Academic Press, New York, 1957) p. 52.
[14] M. de Salegui, H. Plonska and W. Pigman, Arch. Biochem. Biophys. 121 (1967) 548.
[15] J.D. Gregory, T.C. Laurent and L. Roden, J. Biol. Chem. 239 (1964) 3312.
[16] T.C. Laurent and J. Killander, J. Chromatog. 14 (1964) 317.
[17] K. Meyer, A. Linker, E.A. Davidson and B. Weissmann, J. Biol. Chem. 205 (1953) 611.