Short Communications

The Kinetics of Degradation of Chondroitin Sulphates and Hyaluronic Acid by Chondroitinase from *Proteus vulgaris*

By C. F. THURSTON

Department of Microbiology, Queen Elizabeth College, Campden Hill Road, London W8 7AH, U.K.

and T. E. HARDINGHAM and HELEN MUIR

Division of Biochemistry, Kennedy Institute of Rheumatology, Bute Gardens, Hammersmith, London W6 7DW, U.K.

(Received 17 October 1974)

K_m and V_{max} were determined for the degradation by chondroitinase of chondroitin 4-sulphate, chondroitin 4-sulphate-proteoglycan, chondroitin 6-sulphate, dermatan sulphate and hyaluronic acid. Degradation of chondroitin 4-sulphate was inhibited by hyaluronic acid but not by keratan sulphate. The results are discussed with regard to the use of chondroitinase as a selective reagent for the degradation of tissue glycosaminoglycans.

Chondroitinase (chondroitin ABC lyase, EC 4.2.2.4) has been purified from *Proteus vulgaris* (N.C.T.C. 4636) by Yamagata *et al.* (1968). They showed that this enzyme degraded chondroitin sulphates, chondroitin and hyaluronic acid by an elimination reaction yielding $\Delta 4,5$ -unsaturated disaccharides. The enzyme has been used increasingly, as a specific reagent for the degradation of chondroitin sulphate in cartilage (e.g. Hascall *et al.*, 1972; Kempson *et al.*, 1973). Recent work has demonstrated the close involvement of hyaluronic acid and chondroitin sulphate in cartilage (Hardingham & Muir, 1972, 1973, 1974). The results presented here allow comparison of the relative susceptibilities of chondroitin sulphates and hyaluronic acid as substrates for chondroitinase. The importance of interference from hyaluronic acid and keratan sulphate in chondroitin sulphate degradation by chondroitinase is also assessed.

Materials

The sources of compounds used as substrates were as follows: sodium hyaluronate from human umbilical cords (BDH Chemicals Ltd., Poole, Dorset, U.K.); dermatan sulphate from pig skin (' β heparin'; Hoffmann-La Roche, Basle, Switzerland); chondroitin 6-sulphate from shark cartilage (galactosamine/sulphur molar ratio 1:1.17; 91.2% 6-sulphate; Seikagaku Kogyo Co. Ltd., Tokyo, Japan). Keratan sulphate-I was a reference standard supplied by M. B. Mathews, J. A. Cifonelli and L. Rodén, Department of Pediatrics, University of Chicago, Chicago, Ill., U.S.A. Chondroitin 4-sulphate was obtained from pig laryngeal cartilage (Hardingham & Muir, 1970). It contained negligible amounts of keratan

sulphate, since the galactosamine/glucosamine molar ratio was greater than 100:1. Chondroitin 4-sulphate proteoglycan was extracted from pig cartilage essentially as described by Tsiganos & Muir (1969). Aggregates (Hascall & Sajdera, 1969) are not extracted under these conditions (T. E. Hardingham & H. Muir, unpublished results). The preparation contained no more than 4% of keratan sulphate as shown by galactosamine/glucosamine molar ratios. Crystalline bovine plasma albumin was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. All other chemicals were analytical reagent grade.

Source of enzyme. Chondroitinase was extracted from *Proteus vulgaris* (N.C.T.C. 4636) and purified, essentially as described by Yamagata *et al.* (1968). Purified chondroitinase was stored at -20°C as a (frozen) solution containing 1 mg of protein/ml in 0.02 M-Tris-HCl buffer at pH 7.2. On electrophoresis in polyacrylamide gels by the method of Ornstein & Davis (1961), a single protein band was detected with Amido Black, and the enzyme was estimated to be not less than 95% pure. The above stock solution was diluted 500-fold for use in the experiments described. The diluent was 0.05 M-Tris-acetate, pH 8.0, containing 200 mg of bovine plasma albumin/litre.

Methods

Protein determination. The method of Lowry *et al.* (1951) was used. Dry crystalline bovine plasma albumin was used to make standards.

Determination of substrates. Chondroitin sulphates, dermatan sulphate and hyaluronic acid concentrations were expressed as millimolar equivalents of disaccharide calculated from uronic acid analysis and as mol/litre. Keratan sulphate concentration

Table 1. *Kinetic parameters of chondroitinase-catalysed degradation of chondroitin sulphates, dermatan sulphate and hyaluronic acid*

Assays were performed with 0.04 μg of purified chondroitinase and derivation of V_{max} and K_m values was by a least-squares fit to a rectangular hyperbola (see under 'Methods').

Substrate	Molecular weight	Product	$10^{-3} \times$ Molar extinction coefficient of product at 232nm	V_{max} (nmol/min)	K_m (μM)	K_m (mm equivalents of disaccharide)
Chondroitin 4-sulphate	14000	* $\Delta\text{di-4S}$	5.1	4.7	0.95	0.028
Chondroitin 4-sulphate proteoglycan	—	* $\Delta\text{di-4S}$	5.1	4.3	—	0.031
Chondroitin 6-sulphate	33000	† $\Delta\text{di-6S}$	5.5	2.8	0.40	0.028
Dermatan sulphate	15000	* $\Delta\text{di-4S}$	5.1	1.6	0.32	0.010
(chondroitin sulphate B)						
Hyaluronic acid	322000	‡($\Delta\text{di-0S}$)	5.7	1.2	0.51	0.42

* 2-Acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyransyluronic acid)-4-*O*-sulpho-D-galactose.

† 2-Acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyransyluronic acid)-6-*O*-sulpho-D-galactose.

‡ The product from hyaluronic acid is supposedly 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyransyluronic acid)-D-glucose. The molar extinction coefficient for this compound has not been determined and the value used was that for 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enepyransyluronic acid)-D-galactose, from Yamagata *et al.* (1968).

was expressed as millimolar equivalents of disaccharide calculated from galactose analysis. Uronic acid was determined by the method of Bitter & Muir (1962) and galactose by the method of Trevelyan & Harrison (1952), automated procedures being used for both determinations (Heinegård, 1973). Dermatan sulphate could have been underestimated by up to 17% (Bitter & Muir, 1962), as no correction was made for the presence of iduronic acid in the place of glucuronic acid in its composition. Molar concentrations of chondroitin sulphates and dermatan sulphate were calculated by using molecular weights estimated by elution volume from a Sephadex G-200 column (Wasteson, 1969). Molar concentration of hyaluronic acid was calculated from a viscometric determination of its molecular weight (Rodén *et al.*, 1972) (Table 1).

Chondroitinase assay. A continuous spectrophotometric assay has been previously described (Thurston, 1974). For the data reported here a double-beam spectrophotometer with scale expansion (Unicam Instruments Ltd., Cambridge, U.K.) was used such that a full-scale deflexion on a 10mV potentiometric recorder represented an increase in extinction of 0.1. The assay mixture contained, in a final volume of 1.0ml, 50 μmol of Tris-acetate buffer, pH 8.0, 200 μg of bovine plasma albumin, various amounts of a substrate and 0.04 μg of purified chondroitinase, which was added after the other components had been allowed to equilibrate to 37°C. The reference cuvette contained the same components, except the enzyme. The presence of plasma albumin was necessary as otherwise purified chondroitinase rapidly loses activity at the concentration used in this assay (C. F. Thurston, unpublished work). The rate of product formation was calculated from the initial rate of increase of extinction at 232nm, by using the molar

extinction coefficients of the reaction products published by Yamagata *et al.* (1968) and which are shown in Table 1. Reaction rate is expressed as nmol of $\Delta\text{4,5}$ -unsaturated disaccharide produced per min at 37°C and pH 8.0. At least two enzyme assays were performed for each concentration of each substrate tested.

Estimation of kinetic parameters. Data for reaction velocity versus substrate concentration were fitted to a rectangular hyperbola by computer, by using a numerical method of least squares. Initial estimates of V_{max} and K_m for each substrate, which were required for this procedure, were derived by linear regression of the reciprocal of reaction velocity on the reciprocal of substrate concentration. The difference between initial and best estimates was 4–12%.

Results and discussion

Fig. 1 shows that, for the range of concentrations where measurements could be made, chondroitin 4-sulphate was the substrate most rapidly degraded by chondroitinase. Degradation of chondroitin 4-sulphate proteoglycan was essentially the same. This suggests that, when chondroitin 4-sulphate chains are attached to the proteoglycan protein core, they are initially no less accessible to the enzyme. By comparison V_{max} for chondroitin 6-sulphate was significantly lower (Table 1). The decreased efficiency of catalysis may have resulted from the different position of the sulphate group in this substrate and/or from the higher degree of sulphation of this particular sample (galactosamine/sulphur molar ratio 1:1.17). Catalysis was even less efficient with dermatan sulphate from pig skin where about 92% of uronic acid residues are reported to be iduronic acid (Obrink,

Fig. 1. Substrate saturation curves for chondroitinase-catalysed degradation of chondroitin sulphates

The assay procedure is described in the text. The product formed from each substrate is given in Table 1. The curves drawn are computed hyperbolic lines of best fit and the points are means of duplicate experimental determinations for: ●, chondroitin 4-sulphate; ○, chondroitin 4-sulphate proteoglycan; ▲, chondroitin 6-sulphate; ▽, dermatan sulphate.

1972) whereas only glucuronic acid is present in the chondroitin sulphates. Hyaluronic acid was the least good substrate tested. This is not fully shown by the value for V_{max} (Table 1) because this compound gave strong substrate inhibition. Maximum observed reaction velocity was 0.8 nmol/min at a substrate concentration of 0.713 mM equivalents of disaccharide. Increasing the substrate concentration to 1.78 mM equivalents decreased the reaction velocity to 0.2 nmol/min (i.e. by 75%).

Interpretation of K_m values is made uncertain because of the differences in chain length of the substrates tested. If chondroitinase acts exclusively as an exoenzyme removing disaccharide units from free ends of polymer chains, K_m values in terms of molar concentration of substrate may be compared (except for chondroitin 4-sulphate proteoglycan in which there are many glycosaminoglycan chains per molecule). Such comparison would suggest a lower affinity of chondroitinase for chondroitin 4-sulphate than for the other substrates tested (Table 1). Alternatively, if internal (endo) cleavages are also catalysed by the enzyme, K_m values in terms of concentration of sus-

ceptible bonds is better expressed as millimolar equivalents of the disaccharide units. This would indicate a significantly higher affinity for dermatan sulphate than for the chondroitin sulphates and a relatively very low affinity for hyaluronic acid (Table 1).

To test whether keratan sulphate or hyaluronic acid inhibited chondroitin 4-sulphate degradation, assays were performed with chondroitin 4-sulphate at 0.029 mM equivalents of uronic acid, which is about half-saturating concentration for chondroitinase activity. Keratan sulphate was included in assays at concentrations of 0.357–0.018 mM equivalents of galactose without producing measurable inhibition. At concentrations of 0.178, 0.089 and 0.018 mM equivalents of disaccharide, hyaluronic acid inhibited digestion by 65, 38 and 6% respectively (compared with the calculated sum of the rates for the digestion of both components of each mixture).

We conclude that chondroitinase can be used to degrade chondroitin sulphate from cartilage or cornea without inhibition by, or alteration of, attendant keratan sulphate. Hyaluronic acid is present in cartilage in very small amounts as part of proteoglycan aggregates (Hardingham & Muir, 1974), in which form it is resistant to digestion with chondroitinase (Gregory & Hascall, 1972). For these reasons it is unlikely to interfere with the digestion of chondroitin sulphate in cartilage. It may not be justified, however, to extend this conclusion to other tissues, such as skin, where the proportion of hyaluronic acid relative to chondroitin sulphate is much higher.

We are most grateful to Dr. M. J. Bazin and Dr. P. T. Saunders for their help and advice with the computing, and to the Arthritis and Rheumatism Council for general support for T. E. H. and H. M.

- Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334
 Gregory, J. D. & Hascall, V. C. (1972) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **31**, 433
 Hardingham, T. E. & Muir, H. (1970) *FEBS Lett.* **9**, 145–148
 Hardingham, T. E. & Muir, H. (1972) *Biochim. Biophys. Acta* **279**, 401–405
 Hardingham, T. E. & Muir, H. (1973) *Biochem. Soc. Trans.* **1**, 282–284
 Hardingham, T. E. & Muir, H. (1974) *Biochem. J.* **139**, 565–581
 Hascall, V. C. & Sajdera, S. W. (1969) *J. Biol. Chem.* **244**, 2384–2396
 Hascall, V. C., Riolo, R. Z., Hayward, J. & Reynolds, C. C. (1972) *J. Biol. Chem.* **247**, 4521–4528
 Heinegård, D. (1973) *Chim. Scripta* **4**, 199–201
 Kempson, G. E., Muir, H., Pollard, C. & Tuke, M. (1973) *Biochim. Biophys. Acta* **297**, 456–472
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

- Obrink, B. (1972) *Biochim. Biophys. Acta* **254**, 354–361
- Ornstein, L. & Davis, B. J. (1961) *Disc Electrophoresis*, Distillation Products Industries Preprint
- Rodén, L., Baker, J. R., Cifonelli, J. A. & Mathews, M. B. (1972) *Methods Enzymol.* **28**, 73–140.
- Thurston, C. F. (1974) *J. Gen. Microbiol.* **80**, 515–522
- Trevelyan, W. E. & Harrison, J. S. (1952) *Biochem. J.* **50**, 298–303
- Tsiganos, C. P. & Muir, H. (1969) *Biochem. J.* **113**, 879–884
- Wasteson, A. (1969) *Biochim. Biophys. Acta* **177**, 152–154
- Yamagata, T., Saito, H., Habuchi, O. & Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1523–1535