DEPOLYMERISATION AND DESULPHATION OF CHONDROITIN SULPHATE BY ENZYMES FROM EMBRYONIC CHICK CARTILAGE

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1. Introduction

Previous studies on the degradation of the chondroitin sulphate proteoglycan in cartilage have indicated that the process is initiated extracellularly by limited proteolytic cleavage of the molecule [1]. Partially degraded proteoglycan may subsequently become incorporated into the chondrocytes for further, intracellular, breakdown [2,3]. However, little is known about the enzymes involved in the intracellular degradation of chondroitin sulphate in cartilage. Indirect evidence for the occurrence of chondrosulphatase(s) has been obtained in experiments with embryonic chick cartilage [2]and with rat costal cartilage [3], respectively, in organ culture. Attempts to demonstrate the presence of an endopolysaccharidase, acting on the chondroitin sulphate chain, have been unsuccessful [4].

The present investigation was undertaken in order to establish more conclusively the occurrence in the chondrocyte of chondroitin sulphate-degrading enzymes. The results demonstrate the presence of an endopolysaccharidase and a sulphatase, in a lysosomal fraction from this cell. The activity of each enzyme was tested and verified by use of radioactively labelled chondroitin sulphate, and/or oligosaccharides thereof, as substrates.

2. Materials

2.1. *Substrates*

Epiphyseal cartilage of tibias and femurs from fifty, 13-day old White Leghorn chick embryos was dissected free from adhering tissue, and kept in ice-cold Eagle's MEM [5]. The cartilage was divided into thin slices with a scalpel. The sampling medium was sucked off before the addition of 10 ml of incubation medium.

(a) ^{14}C-Labelled polysaccharide: The incubation medium was Eagle's MEM, lacking glucose and supplemented with baby calf serum (10%, v/v), fructose (4 mg/ml) and serine (0.35 mM). 1 mCi of [^{14}C]glucose (225 mCi/mmol, uniformly labelled; Radiochemical Centre, Amersham, U.K.) was added to the medium.

(b) ^{35}S-Labelled polysaccharide: The incubation medium was Eagle's MEM (including glucose), supplemented with baby calf serum (10%, v/v) and serine (0.35 mM); 700 μCi of carrier-free inorganic [^{35}S]sulphate (Radiochemical Centre) was added to the medium.

All the cultures were incubated at 37°C for 5 hr. After inactivation by heating at 100°C for 10 min each culture was dialysed at 4°C against several changes of water, containing 0.1% (w/v) of glucose (^{14}C-labelled polysaccharide) or 0.2 M Na_2SO_4 (^{35}S-labelled polysaccharide), respectively. After a final dialysis against water, the dialysates were lyophilized, resuspended in 10 ml of 0.1 M sodium acetate buffer, pH 5.5–0.01 M EDTA–0.01 M cysteine-HCl and digested with 10 mg of crystalline papain at 60°C for 20 hr. The liberated polysaccharide was recovered by repeated precipitation with cetylpyridinium chloride at an ionic strength of I = 0.3 [6]. The polysaccharide was converted to its sodium salt, precipitated with ethanol and dissolved in water. The resulting ^{14}C- and ^{35}S-labelled products showed spe-

cific activities of 35000 cpm/μg uronic acid and 90000 cpm/μg uronic acid, respectively.

The isolated polysaccharide was identified as chondroitin sulphate by eletrophoresis according to the procedures of Wessler ([7,8]; see Methods) and by its susceptibility towards treatment with Chondroitinase AC [9].

^{35}S-Oligosaccharides were prepared by incubation of the corresponding polysaccharide (0.05 mg of uronic acid/ml), with testicular hyaluronidase (Hyalas, AB Leo, Hälsingborg, Sweden). The digestion was performed for 12 hr at 37°C in 0.15 M NaCl–0.1 M sodium acetate buffer, pH 5.0, with 200 U of enzyme per 0.1 mg of uronic acid. After heating at 100°C for 5 min the digests were concentrated and were then desalted by passage through a 1 X 120 cm column of Sephadex G-10. The oligosaccharides were lyophilized and redissolved in water. Analysis of the products by chromatography on a calibrated column (1 X 200 cm) of Sephadex G-25 [10] showed a mixture of mainly hexa- and tetrasaccharides.

2.2. *Enzyme preparation*

Epiphyseal cartilage of 13-day old chick embryos was prepared as described in the previous section. The epiphyses from approximately 30 embryos were homogenized in 5 ml of ice-cold 0.25 M sucrose–0.01 M Tris–HCl, pH 7.4 with Dounce A (6 strokes) and Dounce B (6 strokes) glass homogenizers. The homogenate was centrifuged at 4°C at 1000 *g* for 10 min; the supernatant was decanted and recentrifuged at 20000 *g* for 20 min. The precipitate was suspended in 2 ml of 0.15 M NaCl (for incubation with ^{35}S-labelled substrate) or in 2 ml of 0.3 M NaCl (for incubations with ^{14}C-labelled substrate), by gentle homogenization in the Dounce B homogenizer. The preparations were freezed and thawed three times and were then stored at −20°C until used. They contained acid hydrolases (acid phosphatase, β-*N*-acetylhexosaminidase and β-glucuronidase; demonstrated by use of conventional synthetic substrates), as expected for a lysosomal preparation [11].

3. Methods

3.1. *Analytical methods*

Uronic acid was determined according to Bitter and Muir [12]. Protein was determined according to Lowry et al. [13].

Radioactivity was measured either with a Beckman Model LS 250 liquid scintillation counter, with Insta-Gel (Packard Instruments) as scintillation medium, or in a Packard Model 7201 Radiochromatogram scanner.

Electrophoresis of glycosaminoglycans was performed on strips of cellulose acetate in 0.1 M barium acetate, pH 6.6 (2.7 V/cm for 6 hr) [7]or in 0.1 M hydrochloric acid, pH 1.2 (1.9 V/cm for 2 hr) [8].

High-voltage paper electrophoresis was performed on Whatman 3 MM paper in 0.08 M pyridine–0.05 M acetic acid, pH 5.3 or in 6% (v/v) formic acid, pH 1.7, at 70 V/cm for 30 min. Inorganic [^{35}S]sulphate was used as a standard.

Gel chromatography was performed on columns of Sephadex G-100 (1 X 100 cm) or G-25 (0.9 X 160 cm or 1 X 200 cm) (Pharmacia Fine Chemicals, Uppsala, Sweden), with 1.0 M sodium chloride–0.02% (w/v) sodium azide as eluant. Effluent fractions were desalted on a 1 X 120 cm column of Sephadex G-10, equilibrated with 10% (v/v) ethanol.

3.2 *Incubation with lysosomal enzymes*

Polysaccharide or oligosaccharide substrates were incubated with the enzyme preparation at 37°C for 14–16 hr. To 0.2 ml aliquots of enzyme preparation (approximately 0.2 mg of protein), were added 0.2 ml of substrate solution (corresponding to 50 μg of uronic acid or less) and 0.2 ml of incubation buffer; the buffers were 0.15 M sodium acetate, pH 3.6 to 5.0; 0.15 M sodium citrate–phosphate, pH 2.3 to 4.5; or 0.15 M sodium citrate–hydrochloric acid, pH 1.5 to 4.0. Occasionally, the final composition of the incution mixture was varied with respect to the concentration of sodium chloride (see Results section). Controls were obtained by heating the enzyme preparation at 100°C for 10 min before the addition of sub-

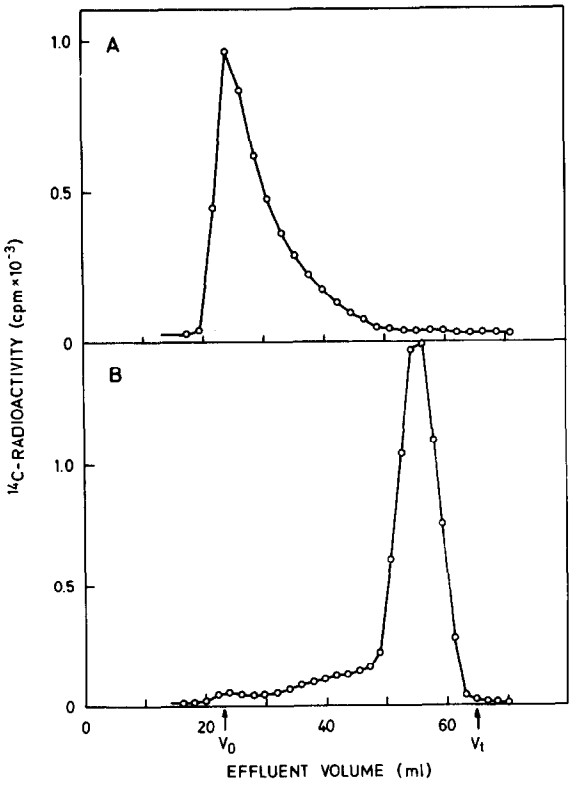

Fig. 1. Chromatography on Sephadex G-100 (1 × 100 cm) of ^{14}C-labelled polysaccharide, incubated with lysosomal enzyme preparation at pH 2.3 (0.05 M sodium citrate–phosphate buffer–0.15 M NaCl). (A) heat-inactivated enzyme; (B) intact enzyme. In some experiments with intact enzyme a larger proportion of the radioactivity appeared at the void volume of the column, well separated from the retarded degradation products. The significance of this finding is unclear at present.

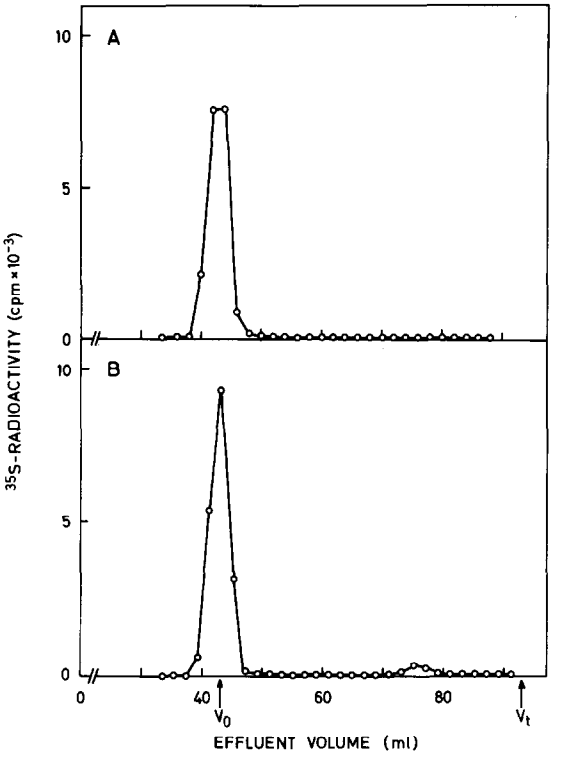

Fig. 2. Chromatography on Sephadex G-25 (0.9 × 160 cm) of ^{35}S-labelled polysaccharide, incubated with lysosomal enzyme preparation at pH 5.0 (0.05 M acatate buffer–0.05 M NaCl). (A) heat-inactivated enzyme; (B) intact enzyme.

strate. The incubations were terminated by heat-inactivation under the same conditions.

4. Results and discussion

4.1. *Endopolysaccharidase*

^{14}C-Labelled chondroitin sulphate was incubated with the enzyme preparation at pH 2.3 and 0.15 M NaCl concentration. Analysis of the incubation products by gel chromatography on Sephadex G-100 revealed considerable depolymerisation as compared to a control treated with heat-inactivated enzyme (fig. 1). The molecular weight of the degraded ^{14}C-labelled chondroitin sulphate was approximately 2000, as estimated from the maximum of the elution profile [14]. Degradation was impeded in the absence of sodium chloride. The polysaccharidase was active in both citrate–phosphate and in acetate buffer, with an apparent optimum around pH 2.3 (maximum retardation of degradation products on gel chromatography). It is notable that the enzyme retained appreciable activity at pH 1.5. The chondroitin sulphate-degrading endoglycosidase of cartilage thus appears to differ with regard to pH-optimum from the analogous enzymes of other tissues [11]. At pH 4.5 (acetate buffer) no random depolymerisation was detectable; however, exoglycosidase activity was indicated by the appearance of radioactive material eluting as a monosaccharide on Sephadex G-25. No exoglycosidase activity was observed at pH 2.3, probably due to inhibition of β-glucuronidase and β-*N*-acetylgalactosaminidase.

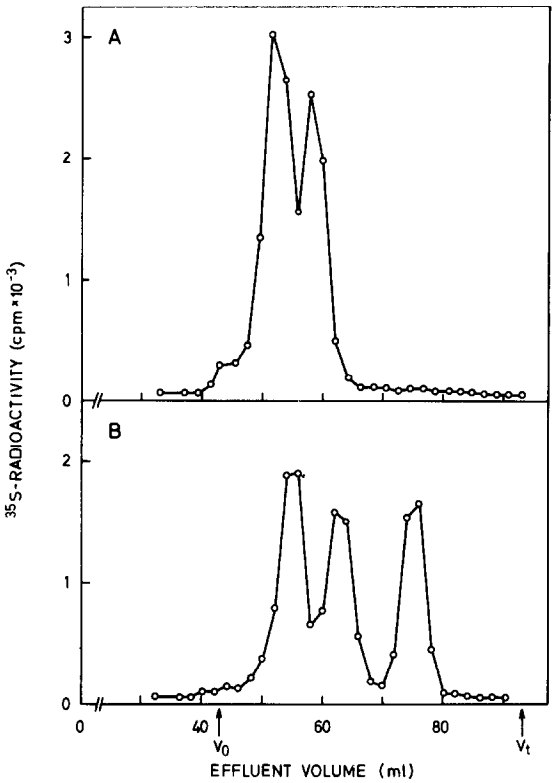

Fig. 3. Chromatography on Sephadex G-25 (0.9 × 160 cm) of ^{35}S-labelled oligo-saccharides, incubated with lysosomal enzyme preparation at pH 5.0 (0.05 M acetate buffer–0.05 M NaCl). (A) heat-inactivated enzyme; (B) intact enzyme.

4.2. *Chondrosulphatase*

Incubation of ^{35}S-labelled chondroitin sulphate with lysosomal fraction at pH 5.0 (acetate buffer) resulted in the release of a small amount of low-molecular labelled material (fig. 2), which migrated like inorganic sulphate on paper electrophoresis at pH 1.7 and at pH 5.3. The chondrosulphatase was inhibited by the presence of citrate–phosphate buffer. The effect of the enzyme was dramatically increased by using the corresponding oligosaccharides rather than the intact polymer as substrate (fig. 3); almost one third of the total sulphate was liberated during incubation of a chondroitin sulphate tetrahexasaccharide mixture. The enzyme (s) is thus different from that present in bovine aorta [15]; in this case the addition of hyaluronidase to incubation mixtures containing polymeric chondroitin [^{35}S]sulphate did not stimulate the release of sulphate.

The functional relationship between the various chondroitin sulphate-degrading enzymes in cartilage is not completely understood. However, the present findings are compatible with the idea that the intracellular breakdown of chondroitin sulphate is initiated by an endopolysaccharidase. The resulting oligosaccharides may then be degraded further by the concerted action of sulphatase(s) and exoglycosidases.

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