Enzymic Degradation of Keratosulphates

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(Received 10 March 1970)

1. A multienzyme system capable of degrading keratosulphates to yield galactose, *N*-acetylglucosamine and sulphate was found in the liver extract of a marine gastropod, *Charonia lampas*. 2. During the degradation, neither oligosaccharides nor sulphated sugars were produced. 3. It is suggested that the degradation could be attributed to the concerted action of β -galactosidase, β -*N*-acetylglucosaminidase and a sulphatase (sulphohydrolase), tentatively designated keratosulphatase. 4. Two forms of keratosulphatase (I and II) were separated by DEAE-Sephadex column chromatography. Both forms could release all the sulphate from keratosulphates and neither appeared to be identical with glycosulphatase or chondrosulphatase, both of which are also present in *Charonia lampas*. 5. β -Galactosidase and β -*N*-acetylglucosaminidase could degrade keratopolysulphate to a greater extent in the presence of keratosulphatase than in its absence. 6. It is suggested that keratosulphate was first desulphated by the action of keratosulphatase, and the desulphated polymer was then degraded to galactose and *N*-acetylglucosamine by the action of β -galactosidase and β -*N*-acetylglucosaminidase. 7. β -Galactosidase alone released a small amount of galactose from shark cartilage keratopolysulphate, but β -*N*-acetylglucosaminidase alone did not release *N*-acetylglucosamine. This indicates that unsulphated galactose residues occupy all the non-reducing terminal positions in keratopolysulphate chains.

Keratosulphates are sulphated polysaccharides composed of approximately equimolar amounts of D-galactose and *N*-acetyl-D-glucosamine and are widely distributed in connective tissues. However, apart from brief preliminary reports (Rosen, Hoffmann & Meyer, 1960; Nishida & Egami, 1969; Greiling, Stuhlsatz & Kisters, 1969), enzymes systems that are capable of degrading these polymers have not been described, and attempts to hydrolyse them with testicular hyaluronidase, emulsin, β -galactosidase and β -*N*-acetylglucosaminidases have been unsuccessful (Meyer, Linker, Davidson & Weissmann, 1953).

Liver extracts of the marine gastropod, *Charonia lampas*, are known to contain a variety of sulphatases (Takahashi & Egami, 1961) and glycosidases (Muramatsu & Egami, 1967; Fukuda, Muramatsu & Egami, 1969) and such extracts have now been shown to be capable of degrading keratosulphate preparations. Both glycosidic bonds and sulphate ester bonds are hydrolysed as a result of the concerted action of a galactosidase, a hexosaminidase and two sulphatases.

MATERIALS AND METHODS

Materials. Glucose 6-*O*-sulphate (potassium salt), galactose 6-*O*-sulphate (brucine salt), chondroitin 4-sulphate

(sodium salt) and chondroitinase ABC (Yamagata, Saito, Habuchi & Suzuki, 1968) were purchased from Seikagaku Kogyo Ltd., Tokyo, Japan. Keratopolysulphate (S, 8.1%, galactose 27.7%, glucuronolactone 5.1%, as chondroitin sulphate about 15%) from shark cartilage (Furuhashi, 1961) and horatin sulphate (S, 2.7%) from *C. lampas* (Inoue, 1965) were kindly supplied by Dr T. Furuhashi, Seikagaku Kogyo Ltd., Tokyo, Japan, and Dr S. Inoue of this Department respectively. *o*-Nitrophenyl β -D-galactoside was synthesized by Minoru Fukuda of our laboratory and phenyl *N*-acetyl- β -D-glucosaminide was purchased from Nakarai Chemicals Ltd., Tokyo, Japan. Bovine cornea was kindly supplied by Teikoku Zoki Ltd., Tokyo, Japan.

Kerato[³⁵S]sulphate was prepared as follows. Slices of fresh bovine cornea (9.8 g wet wt.) were incubated with 3 mCi of H₂³⁵SO₄ (3.8 × 10⁸ c.p.m.) in 60 ml of Krebs-Ringer phosphate* medium with shaking at 37°C for 20 h. Mucopolysaccharides were prepared by the procedure of Mathews & Cifonelli (1965) with the exception that pronase-P (170 mg) was used instead of papain for the digestion of tissue. The crude mucopolysaccharide preparation was digested with chondroitinase ABC (1 unit) (Yamagata *et al.* 1968) in 0.1 M-tris-HCl buffer, pH 8.0,

* Krebs-Ringer phosphate medium: NaCl 560 mg, KCl 525 mg, KH₂PO₄ 135 mg, MgCl₂ 24 mg, water 97 ml, 0.1 M-sodium phosphate buffer (pH 7.4) 20 ml, 1.21 M-glucose 1 ml, 1.22% CaCl₂ 3 ml.

at 37°C for about 20 h and the undegraded keratosulphate was separated by chromatography on a column (1 cm × 48 cm) of Dowex 1 (X2; 200–400 mesh), the fraction eluted between 2 M-NaCl and 4 M-NaCl being pooled and dialysed against water. The yield was 22 μmol as galactose (determined by the anthrone method) with the specific radioactivity 2.61×10^4 c.p.m./μmol of galactose. The purified material gave negative results with the carbazole reaction for uronic acids.

Chondroitin [^{35}S]sulphate was prepared by the procedure of Yamagata *et al.* (1968) by using chicken tibio-femoral epiphyseal cartilage (4.3 g wet wt.) and 3 mCi (3.8×10^8 c.p.m.) of $\text{H}_2^{35}\text{SO}_4$. The yield was 35 μmol as glucuronic acid (determined by the carbazole method) with a specific radioactivity of 2.7×10^5 c.p.m./μmol of glucuronic acid. The purified material gave negative results with the anthrone reaction for hexoses.

Preparation of enzyme extracts. All procedures were carried out below 5°C. *C. lampas* liver (250 g) was homogenized with 1 litre of water for 5 min and the whole allowed to stand for several hours. After centrifuging for 30 min at 4500g, the supernatant was adjusted to 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ and centrifuged for 30 min at 7500g. The supernatant was then adjusted to 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ and centrifuged for 30 min at 7500g. The precipitate was dissolved in 350 ml of 0.05 M-sodium acetate buffer, pH 5.0, dialysed overnight against 0.01 M-sodium acetate buffer, pH 5.0, and centrifuged for 30 min at 7500g to remove the precipitate that formed. The supernatant (400 ml) was used as the crude enzyme preparation for many experiments and formed the basis for further purification studies.

Enzymic liberation of reducing sugars. The reaction mixture consisted of 20 μl of 0.2 M-sodium acetate buffer, pH 5.0, containing 150 μg of sodium keratopolysulphate and 10 μl of enzyme solution. After incubation for 2 h at 37°C liberated reducing power was measured by the method of Park & Johnson (1949) or of Somogyi (1952) in the presence of phosphate ion, which inhibits keratosulphatase activity. β -Galactosidase and β -N-acetylglucosaminidase were assayed as described by Fukuda *et al.* (1969).

Enzymic liberation of inorganic sulphate. Method 1. The incubation mixture consisted of 0.1 ml of enzyme solution and 0.1 ml of 0.2 M-sodium acetate buffer, pH 5.8, containing 1 mg of sodium keratopolysulphate. After incubation for 4 h at 37°C, 0.2 ml of 10% (w/v) cetylpyridinium bromide in 0.3 M-HCl solution was added and the precipitate formed was removed by centrifugation. A portion (0.2 ml) of the supernatant was mixed with 0.4 ml of BaCl_2 -gelatin solution (Dodgson, 1961) and the resulting turbidity (due to BaSO_4) was measured from the extinction at 500 nm.

Method 2. The incubation mixture consisted of 10 μl of enzyme solution and 10 μl of 0.2 M-sodium acetate buffer, pH 5.8, containing ^{35}S -labelled keratosulphate (700 c.p.m., 26 nmol as galactose). After incubation for 2 h at 37°C, 5 μl of the reaction mixture was applied to Toyo no. 51A filter paper together with about 0.03 μmol of unlabelled Na_2SO_4 as an internal marker. Electrophoresis was carried out in 0.05 M-ammonium acetate-acetic acid buffer, pH 5.0, for 25 min at a potential gradient of 35 V/cm. The area corresponding to SO_4^{2-} ion was located by means of BaCl_2 -rhodizonate reagent

(Schneider & Lewbart, 1956), cut out and its radioactivity counted in a low-background gas-flow counter.

Assay of glycosulphatase. The incubation mixture consisted of 0.1 ml of enzyme solution and 0.1 ml of 0.2 M-sodium acetate buffer, pH 5.7, containing 2 μmol of potassium glucose 6-O-sulphate. After incubation for 1 h at 37°C, 0.2 ml of 0.3 M-HCl solution and 0.4 ml of BaCl_2 -gelatin solution were added, and turbidity was measured from the extinction at 360 or 500 nm.

Assay of chondrosulphatase. The incubation mixture consisted of 10 μl of enzyme solution and 10 μl of 0.2 M-sodium acetate buffer, pH 5.0, containing ^{35}S -labelled chondroitin sulphate (3300 c.p.m., 12 nmol as glucuronic acid). After incubation for several hours at 37°C, inorganic [^{35}S]sulphate was measured by the procedure described in assay method 2 described above.

Enzyme unit. One enzyme unit is defined as the amount of enzyme required to release 1 μmol of *o*-nitrophenol (β -galactosidase), phenol (β -N-acetylhexosaminidase) or inorganic sulphate (sulphatase) from the substrate per min under the appropriate assay conditions. Specific activity is expressed as units per mg of protein.

Other methods of analysis. In following the degradation of keratosulphates, paper chromatography of reaction products was carried out on Toyo no. 50 filter paper with the descending solvent [*n*-butanol-ethyl acetate-water (10:3:5, by vol.)] technique. Sugars were detected with alkaline AgNO_3 (Trevelyan, Procter & Harrison, 1950).

N-Acetylhexosamine was determined by the modified Morgan-Elson reaction (Reissig, Strominger & Leloir, 1955), hexose by the anthrone reaction (Trevelyan & Harrison, 1952) or by the phenol-sulphuric acid reaction (Hodge & Hofreiter, 1962), and uronic acid by the carbazole method (Dische, 1947).

The protein content of the enzyme solution was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as a standard.

RESULTS

Preliminary experiments showed that the crude liver extracts were capable of liberating reducing substances and inorganic SO_4^{2-} ions from keratopolysulphate. Apparent maximum enzyme activities were obtained at pH 5.0 for the former activity and at pH 6.0 for the latter (Fig. 1). The activities were dependent on both pH and the nature of the buffer.

Release of reducing material from keratopolysulphate by crude enzyme preparation. Keratopolysulphate (10 mg) was dissolved in 0.2 ml of 1.0 M-sodium acetate buffer, pH 5.0, and to this solution 0.2 ml of crude enzyme preparation was added. The whole was incubated at 37°C in the presence of toluene and at 24 h intervals a portion (10 μl) of the incubation mixture was removed for the measurement of reducing power, and a further 0.2 ml of enzyme solution was added to the reaction mixture. In the control experiment the enzyme and the substrate were incubated separately, and mixed only immediately before assaying for reducing

Fig. 1. (a) Effect of pH on the release of reducing material from keratopolysulphate by crude enzyme preparation. Experimental conditions were those described in the Materials and Methods section except that the buffers were: O, 0.2M-sodium acetate buffer, pH 3.5-6.0; Δ, 0.2M-sodium citrate buffer, pH 4.3-5.7. Incubation was for 2 h at 37°C. (b) Effect of pH on sulphate liberation from keratopolysulphate by crude enzyme preparation. Experimental conditions were the same as described in method 1 except that the buffers were: ▲, 0.1M-tris-maleate buffer, pH 6.0-7.0; ●, 0.1M-sodium acetate buffer, pH 4.5-5.8. Incubation was for 4 h at 37°C.

power. The values obtained with the control experiments were about 70% of those obtained in the test experiments.

As shown in Fig. 2 (a), the release of reducing power tends towards a limiting value at which time 17% of the total sugar present in keratopolysulphate has been released. At this point about 26% of the total sulphate present in keratopolysulphate was also released. In the presence of 0.1M- NaH_2PO_4 , which inhibits keratosulphatase activity, less reducing sugar was eventually released.

Release of sulphate from keratopolysulphate by crude enzyme preparation. Keratopolysulphate (8mg) was dissolved in 150 μl of 0.5M-sodium acetate buffer, pH 5.8, and incubated with 50 μl of enzyme preparation at 37°C in the presence of toluene. At 24h intervals a portion (10-40 μl) of the reaction mixture was removed for measurement of inorganic SO_4^{2-} ions, and a further 40 μl of enzyme solution was added to the reaction mixture. Appropriate control determinations were made and were always less than 20% of the test determinations.

As shown in Fig. 2 (b), the release of sulphate was virtually complete after 8 days. At this time about 68% of the total reducing power had been liberated. Thus more reducing power was produced at pH 6.0 (optimum pH for sulphate liberation over short

incubation periods, see Fig. 1) over this long incubation period than was produced at pH 5.0 (optimum pH for increase in reducing power over short incubation periods, see Fig. 1). This seems to indicate that removal of sulphate is a prerequisite for the extensive degradation of the polymer chain.

Identification of degradation products. A previously incubated reaction mixture (at pH 5.0, about 17% reducing power released; see Fig. 2a) was applied to a column (1.5cm $\times$ 98cm) of Sephadex G-50, superfine grade, that had been equilibrated with 0.1M-sodium chloride, and the column was then eluted with the same solution (Fig. 3). Two peaks only were found, corresponding in position to undegraded keratopolysulphate and galactose. The latter fraction was rechromatographed on Sephadex G-15 (1.5cm $\times$ 86cm column) and a single peak again emerged corresponding in position to galactose (this peak is hereafter referred to as the monosaccharide fraction).

Paper chromatography showed that galactose and *N*-acetylglucosamine were both present in the monosaccharide fraction. No oligosaccharides could be detected in the reaction mixture even when a very short incubation time (4h) was used. The molar ratio of hexose to *N*-acetylhexosamine in the monosaccharide fraction was 2.4:1.0.

These results suggest that keratopolysulphate

Fig. 2. (a) Release of reducing power from keratopolysulphate by crude enzyme preparation. Δ , In the presence of 0.1 M NaH_2PO_4 ; $\circ$, in the absence of phosphate. Experimental conditions are described in the text. The results are expressed as a percentage of the total reducing power liberated from keratopolysulphate by acid hydrolysis. (b) Release of inorganic sulphate from keratopolysulphate by crude enzyme preparation. Experimental conditions are described in the text. The results are expressed as a percentage of the total sulphate present in the keratopolysulphate.

was hydrolysed by β -galactosidase and β -N-acetylglucosaminidase, which were acting as exoglycosidases.

Comparison of heat-stability of related enzymes in the crude extract. The results of heat-stability experiments on the β -galactosidase, β -N-acetylglucosaminidase, glycosulphatase and keratosulphatase activities of the crude extract are shown in Fig. 4. Keratosulphatase exhibited the lowest stability to temperature. The curves also suggest that glycosulphatase and keratosulphatase are different enzymes.

Effects of other carbohydrate sulphates on keratosulphatase activity. Keratosulphatase activity was assessed by measuring the liberation of [³⁵S]sulphate from ³⁵S-labelled keratosulphate. As shown in

Fig. 3. (a) Gel filtration of enzymic digests on Sephadex G-50. The digest was applied to a column (1.5 cm $\times$ 98 cm) equilibrated with 0.1 M NaCl, and eluted with the same solution. Fractions (2.5 ml) were collected and hexose was measured by the extinction at 490 nm after the phenol- H_2SO_4 reaction. (b) Gel filtration of the later peak material shown in Fig. 3 (a) on Sephadex G-15. The sample was applied to a column (1.5 cm $\times$ 86 cm) equilibrated with 0.1 M NaCl, and eluted with the same solution. Fractions (2 ml) were collected. The position of galactose is indicated by the arrow.

Table 1, keratopolysulphate, depending on its concentration, apparently inhibited keratosulphatase. On the other hand, glucose 6-O-sulphate and galactose 6-O-sulphate had practically no inhibitory effects. These results also suggested that keratosulphatase was different from glycosulphatase. Keratosulphatase activity was only slightly inhibited by chondroitin 4-sulphate; moreover, inhibition was independent of the chondroitin 4-sulphate concentration in the concentration range tested, indicating that the effect is of a different nature from the inhibition by keratopolysulphate. It also suggests that chondroitin 4-sulphate is not a substrate for keratosulphatase.

DEAE-Sephadex chromatography of chondrosulphatase and keratosulphatase. Fig. 5 shows the behaviour of the crude enzyme extract on DEAE-Sephadex A-50 column chromatography. Keratosulphatase activities were separated into two fractions (I and II). The elution pattern for chondrosulphatase was rather different, with the exception of the overlapping peaks designated as fraction I and it could not be excluded that the

Fig. 4. Heat stability of sulphatases and glycosidases in the crude enzyme preparation. The preparation was dialysed overnight against 0.01 M-sodium acetate buffer, pH 5.0. It was heated for 3 min at the required temperature in a water bath and then cooled in ice. Residual activity is expressed as percentage of the activity in the untreated control. ●, Keratosulphatase; ▲, glycosulphatase; □, β-galactosidase; ■, β-N-acetylglucosaminidase.

Table 1. *Effects of some carbohydrate sulphates on the enzymic liberation of sulphate from keratosulphate*

The concentration of kerato[³⁵S]sulphate was 1.3 mM. Activities were measured by assay method 2.

Sugar sulphate	Concentration (mM)	Relative activity† (%)
None	—	100
Glucose 6- <i>O</i> -sulphate	1	113
	10	85
Galactose 6- <i>O</i> -sulphate	1	80
	10	97
Chondroitin 4-sulphate*	1	71
	10	70
Keratopolysulphate*	1	81
	10	43

* Mol of chondroitin 4-sulphate and keratopolysulphate are the mol of uronic acid and hexose contained respectively.

† The values were determined in duplicate and may be subject to an experimental error of about 10%.

keratosulphatase of this fraction had a dual specificity.

Partial purification of keratosulphatase. Crude enzyme solution (400 ml) was applied to a column

Fig. 5. DEAE-Sephadex A-50 column chromatography of crude enzyme preparation. Crude enzyme solution (420 ml from 250 g of liver), after dialysis against 0.01 M-tris-HCl buffer, pH 8.0, was applied to a column (3 cm × 60 cm) of DEAE-Sephadex A-50 equilibrated with 0.01 M-tris-HCl buffer, pH 8.0. The column was developed by linear gradient elution with 2 litres of 0.01 M-tris-HCl buffer, pH 8.0, in the mixing flask and 2 litres of the same buffer containing 0.5 M-NaCl in the reservoir. Fractions (20 ml) were collected. ●, Keratosulphatase; ▲, chondrosulphatase.

(5 cm × 30 cm) of CM-Sephadex C-50 equilibrated with 0.01 M-sodium acetate buffer, pH 5.0, and washed with 2 litres of the same buffer containing 0.05 M-sodium chloride. Elution was carried out with the same buffer containing 0.25 M-sodium chloride. The major active fractions were pooled and concentrated to 45 ml by dialysis against polyethyleneglycol. This was dialysed against 0.02 M-tris-HCl buffer, pH 8.0, and applied to a column (2 cm × 18 cm) of DEAE-Sephadex A-50, equilibrated with 0.02 M-tris-HCl buffer, pH 8.0. The column was developed by linear gradient elution with 200 ml of 0.02 M-tris-HCl buffer, pH 8.0, in the mixing flask and 200 ml of the same buffer containing 0.5 M-sodium chloride in the reservoir. Fractions (5 ml) were collected (Fig. 6).

On DEAE-Sephadex column chromatography, keratosulphatase activity was separated into two components (I and II). The early fraction (I), which passed through without adsorption, was free from glycosulphatase activity and β-N-acetylglucosaminidase activity. In fraction II keratosulphatase activity overlapped with β-galactosidase activity, β-N-acetylglucosaminidase activity and glycosulphatase activity. Each fraction with keratosulphatase activity was pooled and concentrated to 1 ml by pressure dialysis. Keratosulphatase preparations I and II were stored at -20°C in the presence of 0.1 M-sodium chloride without appreciable loss of activity for several weeks.

Fig. 6. DEAE-Sephadex A-50 column chromatography of partially purified (CM-Sephadex) extract. The procedures for chromatography are described in the text. ●, Keratosulphatase; △, glycosulphatase; □, β -galactosidase; ■, β -N-acetylglucosaminidase.

Table 2. *Partial purification of keratosulphatase*

Enzyme activities were measured by assay method 2 with kerato[^{35}S]sulphate as substrate.

Step	Volume (ml)	Protein (mg/ml)	Total activity (m-units)	Specific activity (m-units/mg of protein)
1. Crude enzyme	400	22	76	0.0086
2. CM-Sephadex column chromatography	46	20	153	0.166
3. DEAE-Sephadex column chromatography				
fraction I	3	1.7	1.24	0.24
fraction II	11	5.6	7.70	0.125

The overall purification steps and yields of keratosulphatase activity are summarized in Table 2. The enzyme activity of the crude homogenate is not recorded because it could not be determined accurately owing to the presence of endogenous sulphated mucopolysaccharide. By CM-Sephadex column chromatography, endogenous sulphated mucopolysaccharides were largely removed. It was probably the reason for the apparent increase in total activity on reaching step 2.

Fractions containing β -galactosidase activity (fractions 32–40) were free from keratosulphatase activity and β -N-acetylglucosaminidase activity, and fractions containing β -N-acetylglucosaminidase activity (fractions 64–80) were free from keratosulphatase and β -galactosidase activities. Those fractions were concentrated by pressure dialysis and were used in later studies as β -galactosidase and β -N-acetylglucosaminidase preparations respectively.

Properties of the partially purified keratosulphatase. Table 3 shows the effects of several

compounds on keratosulphatases I and II. Potassium dihydrogen phosphate, sodium fluoride and *p*-chloromercuribenzoate apparently inhibited both keratosulphatases I and II, as they do most other known *C. lampas* sulphatases.

The optimum pH values of keratosulphatases I and II were about 4.5 and 6.0 respectively.

Digestion of keratopolysulphate by β -galactosidase, β -N-acetylglucosaminidase or both. (1) Keratopolysulphate (1mg) containing 7000 c.p.m. of kerato[^{35}S]sulphate was dissolved in 20 μl of 1.0M-sodium acetate buffer, pH 5.0. To this solution 10 μl of β -galactosidase preparation (9m-units) was added. The reaction mixture was incubated at 37°C in the presence of toluene. At 24h intervals a portion of the incubation mixture was withdrawn for measurement of reducing power, and a further 10 μl of β -galactosidase preparation was added. β -Galactosidase liberated 6% of the sugar moiety present in keratopolysulphate in 125h. Inorganic sulphate was not liberated during this time.

(2) Keratopolysulphate (1mg) containing 7000

Table 3. *Effects of several compounds on partially purified keratosulphatases I and II*

Experimental conditions are those in assay method 2.

Compound	Concentration (mM)	Relative enzymic activity (%)	
		Keratosulphatase I	Keratosulphatase II
None	1	100	100
EDTA	1	87	97
MgCl ₂	1	67	93
Zinc acetate	1	29	95
MnCl ₂	1	35	93
NaF	1	23	21
KH ₂ PO ₄	10	0	24
PCMB* †	1	35	23
Iodoacetate	1	80	100
Cysteine	1	53	96

* Abbreviation: PCMB, *p*-chloromercuribenzoate.Table 4. *Extent of degradation of keratopolysulphate by β -galactosidase, β -N-acetylglucosaminidase or both*

In Tables 4 and 5 additions of enzyme are indicated as x m-units $\times y$, where x is the number of units per addition and y the number of additions. Multiple additions of enzyme were made because of the long incubation, which may cause enzyme inactivation. Substrates used in Expts. 1, 2 and 3 were sodium keratopolysulphate (1 mg) and kerato[³⁵S]sulphate (7000 c.p.m.), and that in Expt. 4 was keratopolysulphate (1 mg).

Enzyme preparation	Incubation time (h)	Reducing sugar released (%)	SO ₄ ²⁻ released (%)
1. β -Galactosidase (9 m-units $\times$ 6)	125	6	0
2. β -N-Acetylglucosaminidase (4.9 m-units $\times$ 1)	24	0	0
3. β -Galactosidase (9 m-units $\times$ 3) β -N-Acetylglucosaminidase (9.7 m-units $\times$ 3)	48	16	0
4. β -Galactosidase (9 m-units $\times$ 3) β -N-Acetylglucosaminidase (4.8 m-units $\times$ 2) Keratosulphatase II (0.75 m-unit $\times$ 3)	96	65	

c.p.m. of kerato[³⁵S]sulphate was dissolved in 20 μ l of 1.0M-sodium acetate buffer, pH 5.0, and incubated at 37°C for 24h with 10 μ l of β -N-acetylglucosaminidase solution (4.9 m-units). Reducing material was not released.

(3) Keratopolysulphate (1mg in 1M-sodium acetate buffer, pH 5.0) containing 7000 c.p.m. of kerato[³⁵S]sulphate was incubated with 9.0 m-units of β -galactosidase and 9.7 m-units of β -N-acetylglucosaminidase. At several time-intervals, reducing power was measured and further portions of the enzymes were added. β -Galactosidase and β -N-acetylglucosaminidase collectively released 16% of reducing power from keratopolysulphate in 48h (Table 4). Inorganic sulphate was not liberated during this time. The digested material was applied to a column (1.2cm $\times$ 140cm) of Sephadex G-50. All the radioactivity was eluted at the position corresponding to high-molecular-weight polysaccharide and no radioactivity was detected that could be attributed to monosaccharides. This sug-

gested that β -galactosidase and β -N-acetylglucosaminidase were unable to hydrolyse the glycosidic bonds of sulphated sugars.

(4) Keratopolysulphate (1mg) was dissolved in 20 μ l of 1M-sodium acetate buffer, pH 5.8, and incubated at 37°C with 10 μ l of β -galactosidase (9 m-units), 5 μ l of β -N-acetylglucosaminidase (4.8 m-units) and 20 μ l of keratosulphatase II (0.75 m-unit). At 24h intervals liberated reducing material was measured and further portions of enzyme solution were added. In the presence of keratosulphatase II, β -galactosidase and β -N-acetylglucosaminidase released reducing materials equivalent to a degradation of 65% of the keratopolysulphate in 96h.

Release of sulphate from keratopolysulphate and other sulphated polysaccharides. (1) Keratopolysulphate (1mg) was incubated at 37°C with 37 μ units (50 μ l) of keratosulphatase I in 0.5M-sodium acetate buffer, pH 5.8. At 24h intervals additional 50 μ l portions of keratosulphatase I

Table 5. Release of sulphate from keratopolysulphate, keratosulphate and horatin sulphate by keratosulphatases I and II

Enzyme preparation	Substrate	Incubation time (h)	SO ₄ ²⁻ liberated (%)
1. I (0.037 m-unit × 4)	Keratopolysulphate (1 mg)	96	80
II (0.86 m-unit × 4)	Keratopolysulphate (1 mg)	96	100
2. I (0.01 m-unit × 4)	Kerato[³⁵ S]sulphate (7000 c.p.m.)	96	95
II (0.45 m-unit × 2)	Kerato[³⁵ S]sulphate (7000 c.p.m.)	76	100
3. I (0.021 m-unit × 2)	Horatin sulphate (1 mg)	48	<3
II (0.51 m-unit × 2)	Horatin sulphate (1 mg)	48	100

solution were added. In 96h, 80% of the total ester sulphate content of keratopolysulphate was released as inorganic SO₄²⁻ ion (Table 5). When keratosulphatase II was employed 100% of the total sulphate was released under the same experimental condition.

(2) Corneal kerato[³⁵S]sulphate (7000 c.p.m.) was incubated with 10 μ units of keratosulphatase I in 40 μ l of 0.5 M-sodium acetate buffer, pH 5.8. At 24 h intervals liberated inorganic sulphate was measured and further portions of enzyme solution were added. In 96h 95% of the total available sulphate was released. Keratosulphatase II could also release all the sulphate from kerato[³⁵S]sulphate under the same experimental condition.

(3) Horatin sulphate is a sulphated polysaccharide composed of fucose, mannose, glucose, galactose, glucosamine and galactosamine, which can be isolated from the liver of *C. lampas* (Inoue & Egami, 1963; Inoue, 1965). Horatin sulphate (2mg) was dissolved in 10 μ l of 1 M-sodium acetate buffer, to which 21 μ units (30 μ l) of keratosulphatase I were added. After incubation for 24h a further 30 μ l of keratosulphatase I was added and liberated inorganic sulphate was measured after a further 24h incubation period. Keratosulphatase I did not liberate sulphate from horatin sulphate. When keratosulphatase II was used, all the ester sulphate of horatin sulphate was released as inorganic SO₄²⁻ ions.

Shift of optimum pH for liberation of reducing power. When *o*-nitrophenyl β -D-galactoside was employed as a galactosidase substrate, both the crude liver extracts and the separated β -galactosidase fraction had optimum pH values of 4.0. The latter preparation also showed maximum activity towards keratopolysulphate at pH 4.0. However, the crude liver extract has optimum activity at pH 5.0 towards the polymeric substrate (see Fig. 1a). This apparent shift might be explained by the assumption that in crude extracts keratosulphatases (pH optimum at 6.0) first desulphated keratopolysulphate, and then glycosidases (pH optimum 4.0) hydrolysed the glycosidic bonds linking the desulphated sugars. Under such circum-

stances there might be an apparent shift in the pH optimum for release of reducing material from keratopolysulphate from pH 4.0 to pH 5.0.

DISCUSSION

Keratosulphates were digested by an extract of *Charonia lampas* liver to yield galactose, *N*-acetylglucosamine and inorganic sulphate. Oligosaccharides or sulphated monosaccharides were not produced. These observations suggest that the digestion was the result of the concerted action of β -galactosidase, β -*N*-acetylglucosaminidase and keratosulphatase and this was confirmed by examining the combined action of preparations of the individual enzymes. As β -galactosidase and β -*N*-acetylglucosaminidase, in the absence of keratosulphatase, liberated only a small amount of reducing material, it was concluded that keratosulphatase first liberated sulphate from keratosulphates, and that the glycosidases subsequently hydrolysed the glycosidic bonds (with exo-action) in the desulphated carbohydrate moiety.

It is noteworthy that several animal sulphatases such as cellulose polysulphatase (Takahashi, 1960), chondrosulphatase (Held & Buddecke, 1967) and keratosulphatase liberate sulphates from sulphated polysaccharides directly, unlike bacterial sulphatases (Dodgson & Lloyd, 1957; Yamagata *et al.* 1968), which liberate sulphate only after depolymerization.

Keratosulphatase appears to be different from glycosulphatase and probably from chondrosulphatase in *C. lampas*; however it remains to be elucidated if it is indeed a novel enzyme or an additional activity of a known sulphatase such as cellulose polysulphatase (Takahashi, 1960; Takahashi & Egami, 1961). Keratosulphatase II, which hydrolysed horatin sulphate, may be a novel polysaccharide sulphatase with a broader specificity.

β -Galactosidase free from keratosulphatase released a small amount of galactose from keratopolysulphates. This finding suggests that a small amount of galactose is present as a non-reducing side chain or more probably as a small block at the non-reducing terminus of a keratopolysulphate

chain, similar to the situation in corneal kerato-sulphate (Bhavanandan & Meyer, 1967). The combined action of β -galactosidase and β -N-acetylglucosaminidase, in the absence of kerato-sulphatase, released a further amount of reducing material, indicating that near the non-reducing terminus some galactose and N-acetylglucosamine residues are not sulphated.

These enzymes participating in the digestion of keratosulphates should provide useful tools for the structural analysis and for the medical and biological studies of these polymers.

The authors thank Professor K. S. Dodgson for the revision of the manuscript. Part of the expense of this work was defrayed by a grant from the Ministry of Education of Japan.

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