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Hydrolysis of Polysaccharide Sulphate Esters by a Sulphatase Preparation from *Charonia lampas*

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The large marine gastropod, *Charonia lampas* (*Tritonia sauliae*), is known to be a source of glucosulphatase (Soda & Hattori, 1933), arylsulphatase (Soda & Egami, 1933), chondrosulphatase (Soda & Egami, 1938), myrosulphatase (Ishimoto & Yamashina, 1949), cellulose polysulphatase (Takahashi & Egami, 1960) and steroid sulphatase (N. Takahashi & F. Egami, unpublished observation).

Takahashi (1960*a, b, c*) has shown that cellulose polysulphate, containing glucose residues sulphated at positions 6 and 2 and/or 3, can be degraded to glucose by the combined action of cellulose polysulphatase, a polysaccharase and glucosulphatase, all of which are present in crude extracts of the liver of *Charonia*. It was suggested that cellulose polysulphate was first partially desulphated by cellulose polysulphatase, ester sulphate bonds at positions 2 and 3 being preferentially attacked. Subsequently the polysaccharide chain of the partially desulphated product was degraded by the polysaccharase to yield glucose and glucose monosulphates (principally the 6-*O*-sulphate) and, finally, the glucose monosulphate residues were desulphated by glucosulphatase, an enzyme which preferentially attacks glucose 6-*O*-sulphate.

The present work describes the activity of a cellulose polysulphatase preparation, from which the associated polysaccharase has been removed,

towards a number of polysaccharide sulphate esters, and provides additional support for the suggestions of Takahashi (1960*a, b, c*).

MATERIALS AND METHODS

Enzyme preparation. The cellulose polysulphatase of *Charonia* liver was concentrated and separated from associated polysaccharases, by a method based on the procedures described by Takahashi (1960*a, b*). All operations were carried out at 0°. The fresh liver of *Charonia* (20 g.) was macerated in 100 ml. of 0.1M-sodium acetate-acetic acid buffer, pH 6.0. The suspension was centrifuged, and sufficient acriflavine was added to the clear supernatant to give a final concentration of 1% (w/v). Precipitated material was separated by centrifuging and chilled ethanol was added (with stirring) to the clear supernatant until the concentration of ethanol was 70% (v/v). The resulting precipitate was separated by centrifuging and dissolved in 50 ml. of water. The solution was dialysed overnight and insoluble material was removed by centrifuging. Sufficient M-sodium acetate-acetic acid buffer, pH 5.2, was added to the clear supernatant to give a final buffer concentration of 0.01M and 8.0 ml. of the solution (containing 37 mg. of protein, as determined by the method of Lowry, Rosebrough, Farr & Randall, 1951) was passed through a column (0.7 cm.³ × 30 cm.) of carboxymethylcellulose (approx. 3 g. of adsorbent, buffered with 0.01M-sodium acetate-acetic acid buffer, pH 5.2). The column was then washed with 100 ml. of the 0.01M-acetate buffer before applying, from a 250 ml. mixing chamber, a buffer gradient increasing from 0.01M, pH 5.2, to 0.5M, pH 6.0. The flow-rate was 2 ml./hr. and the eluate was collected in 5 ml. fractions. The enzyme was eluted within the range 0.1–0.2M-buffer (cf. Takahashi, 1960*b*) and the appropriate fractions were pooled and dialysed against water before freeze-drying. The final preparation contained darylsulphat-

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Table 1. *Relative rates of hydrolysis of polysaccharide sulphates by a partially purified preparation of the cellulose polysulphatase of Charonia lampas*

Experimental conditions are given in the text.

Polysaccharide sulphate	S content of potassium salt (%)	Liberated SO ₄ ²⁻ (%)					
		2 hr.	3 hr.	4 hr.	6 hr.	10 hr.	18 hr.
Cellulose polysulphate	18.0	18.3	27.8	35.0	48.4	51.5	57.0
Cellulose sulphate (enzymically desulphated)	12.5	—	16.7	—	26.1	—	31.0
Cellulose sulphate (acid-hydrolysed)	6.8	—	26.4	—	57.3	—	98.0
Charonin sulphate (S-rich fraction)	16.2	16.1	28.6	34.5	49.0	59.0	80.0
Charonin sulphate (S-poor fraction)	7.6	8.7	—	12.0	—	13.0	—
Dextran polysulphate	17.0*	—	6.4	—	8.2	—	13.7
Chondroitin sulphate A	4.4*	—	—	9.8	—	12.8	—
Chondroitin sulphate (shark cartilage)	6.0*	4.3	—	8.3	—	10.0	10.4
Heparin	11.1*	1.4	—	2.0	—	2.2	—
Amylose polysulphate	15.8	—	—	—	—	—	0.5
Glycogen sulphate	10.7	1.2	—	1.6	—	2.9	—
Glucan sulphate (<i>Busycon</i>)	11.7	—	—	—	—	3.9	—
Jelly-layer of sea-urchin eggs	4.7	—	1.2	—	1.9	—	2.8
Polysaccharide sulphate of <i>Chondrus</i> sp.	9.8	—	0.9	—	1.2	—	1.9

* S content of Na salt.

ase (20-fold purification), glucosulphatase (160-fold purification) and cellulose polysulphatase (sixfold purification) but was now free from polysaccharases (see Takahashi, 1960b).

Polysaccharide sulphates. Potassium salts of the polysulphate esters of cellulose, amylose and glycogen were prepared by sulphation of the parent polysaccharides with chlorosulphonic acid in the presence of pyridine as described by Traube, Blaser & Lindemann (1932). The S content of these and other polysaccharide sulphates are recorded in Table 1.

Potassium cellulose sulphate (S content, 12.5%) was prepared by partial enzymic desulphation of the polysulphate (S content 18.0%) with the *Charonia* cellulose polysulphatase preparation as described by Takahashi (1960c). Potassium cellulose polysulphate (15 mg.) was incubated at 37° for 20 hr. with 5 ml. of the enzyme preparation and a few drops of chloroform, the whole being adjusted to pH 6.0 with 0.1 M-sodium acetate-acetic acid buffer, pH 6.0. The reaction mixture was then boiled and centrifuged and the clear supernatant thoroughly dialysed before freeze-drying.

Potassium cellulose sulphate (S content 6.8%) was prepared by hydrolysis of 300 mg. of potassium cellulose polysulphate with 30 ml. of 0.1 N-H₂SO₄ for 30 min. at 100°. The reaction mixture was then neutralized with BaCO₃, precipitated material was removed by centrifuging and the clear supernatant thoroughly dialysed before freeze-drying.

The potassium salts of charonin sulphate (S-rich fraction) and charonin sulphate (S-poor fraction) were prepared from the mucous gland of *Charonia* by the method of Egami *et al.* (1955).

Sodium dextranpolysulphate, a synthetic anticoagulant, was a gift from Dr Sasaki, Nagoya University (see Sasaki, Takemoto & Oka, 1957). Sodium chondroitin sulphate (of mammalian origin and consisting mainly of chondroitin sulphate A) was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Shark-cartilage sodium chondroitin sulphate was a gift from Seikagaku-Kenkyusho Ltd., Japan. The sodium salt of heparin was purchased from N. V. Organon-Oss, Holland. Glucan sulphate, extracted

from the odontophore of a marine snail, *Busycon caniculatum*, was kindly supplied by Dr M. W. Whitehouse, Oxford University (see Lash & Whitehouse, 1960). Fucan sulphate from the jelly-layer of sea-urchin (*Hemicentrotus pulcherrimus*) eggs was prepared by the method of Nakano & Ohashi (1953). Polysaccharide sulphate (composed mainly of sulphated galactose and fructose residues) from a seaweed (*Chondrus* sp.) was supplied by Dr T. Mori, University of Tokyo (see Mori & Tsuchiya, 1938).

Measurements of enzyme action. Cellulose-polysulphatase activity was followed by estimating liberated SO₄²⁻. The enzyme preparation (0.5 ml. of a solution of suitable concentration in water) was incubated at 37° with 0.5 ml. of 0.1 M-sodium acetate-acetic acid buffer, pH 6.0, containing 3–12 mg. (≡ about 500 μg. of S) of the polysaccharide sulphate and a few drops of chloroform. The incubated mixture was mixed with 2 ml. of an aqueous 1% (w/v) solution of acriflavine. Precipitated polysaccharide sulphate was removed by centrifuging and a portion (2 ml.) of the clear supernatant was withdrawn for determination of inorganic SO₄²⁻ as described by Dodgson & Spencer (1953). The results are given in Table 1. Routine assay of cellulose polysulphatase was made with potassium cellulose polysulphate (S content 18.0%) as substrate.

Polysaccharase activity was determined, under similar experimental conditions, by estimating reducing power by the method of Somogyi (1945) after deproteinization of the incubation mixture with zinc hydroxide gel.

DISCUSSION

Table 1 records the relative rates of hydrolysis of the various polysaccharide sulphates by the same concentration of enzyme. Glucan polysulphates with β-1→4-glycosidic linkages such as cellulose polysulphate and charonin sulphate (S-rich fraction) were rapidly desulphated by the enzyme preparation, justifying the name 'cellulose polysulphatase' proposed by Takahashi & Egami (1960), and suggesting that charonin sulphate, which is

present in considerable amounts in the mucous gland of *Charonia*, can be regarded as one of the natural substrates of the enzyme.

Concerning the effect of the position of sulphate bond, Takahashi (1960c) suggested that cellulose polysulphatase preferentially attacks ester bonds at the 2- and 3-positions, and glucosulphatase attacks preferentially 6-position ester bonds. The slow hydrolysis of enzymically desulphated cellulose sulphate may be explained by the relative resistance of 6-sulphate ester to the enzyme. The rapid hydrolysis of acid-hydrolysed cellulose sulphate cannot be yet explained. Possible explanations are that acid hydrolysis splits preferentially 6-ester bonds resistant to cellulose polysulphatase, or that the molecule has undergone some degree of depolymerization enabling it to be attacked by both cellulose polysulphatase and glucosulphatase.

The greater rate of hydrolysis of charonin sulphate (S-rich fraction) as compared with charonin sulphate (S-poor fraction) can be attributed to the difference in structure of the polysaccharide part. The former consists essentially of a β -glucosidic cellulose-like structure, whereas the latter possesses both α - and β -glucosidic bonds (Egami *et al.* 1955; Nakanishi, Takahashi & Egami, 1956).

Chondroitin sulphate A and shark-cartilage chondroitin sulphate were also hydrolysed by the enzyme preparation, although only at about one-fifth of the rate of cellulose polysulphate. However, the relative rates of hydrolysis of cellulose polysulphate and chondroitin sulphate has varied appreciably with different enzyme preparations (three different preparations gave ratios of cellulose polysulphate hydrolysis/shark chondroitin sulphate hydrolysis 1:0.03, 1:0.06 and 1:0.33). We suggest that a chondrosulphatase is responsible for the desulphation of chondroitin sulphate and that, whereas cellulose polysulphatase is a constitutive enzyme, chondrosulphatase is an induced enzyme whose concentration in the tissues depends upon the nutritional state of *Charonia*.

The stability of the glucan sulphate of *Busycon* towards the enzyme preparation was surprising. This sulphate is a natural product of an organism closely related to *Charonia* and is considered to have a cellulose-type of structure (M. W. Whitehouse, personal communication). A possible explanation is that sulphate is attached almost exclusively to the 6-position of the glucose residue in this compound.

Only dextran polysulphate of all the other substrates tested was appreciably hydrolysed by the enzyme preparation. It remains to be elucidated whether cellulose polysulphatase is the responsible enzyme or whether the enzyme preparation contains a second polysaccharide polysulphatase, specific for α -1 $\rightarrow$ 6-glucans.

The comparative stability of the remaining polysaccharide sulphates to the enzyme preparation does not exclude the possibility that these compounds might be hydrolysed by glucosulphatase after depolymerization of the polysaccharide chain.

SUMMARY

1. The enzymic desulphation of various synthetic and naturally occurring polysaccharide sulphates by a partially purified sulphatase preparation from a marine gastropod *Charonia lampas* has been studied.

2. Cellulose polysulphate and charonin sulphate were rapidly desulphated by the cellulose polysulphatase of the enzyme preparation. Heparin, amylose polysulphate, glycogen sulphate and polysaccharide sulphates from a seaweed (*Chondrus* sp.), from sea-urchin eggs and from the marine gastropod *Busycon* were hardly affected.

3. Chondroitin sulphate and dextran sulphate were slowly desulphated but hydrolysis of the former can probably be attributed to the presence of chondrosulphatase in the enzyme preparation.

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