Purification and some properties of the D-lactate-2-sulphatase of *Pseudomonas syringae* GG

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A soil bacterium grown on propan-2-yl sulphate as sole source of carbon and sulphur yielded extracts containing an enzyme capable of liberating sulphate from racemic lactate-2-sulphate. The enzyme was purified to homogeneity by a combination of streptomycin sulphate precipitation of nucleic acids, batch treatment with DEAE-cellulose, and chromatography on columns of DEAE-cellulose, Sephacryl S-300 and butyl-agarose. The protein was monomeric with an M_r of 55 000–60 000. The enzyme activity was specific for D-lactate-2-sulphate (K_m 6.6 nM; maximal specific activity 14.3 $\mu\text{mol/min}$ per mg of protein) and showed no activity towards the L-isomer. The products of the enzyme's action were inorganic sulphate and D-lactate which were released in equimolar amounts and stoichiometrically with the amount of ester hydrolysed. No L-lactate was formed. Retention of configuration implied cleavage of the O–S bond of the C–O–S ester link and this was confirmed by ^{18}O -incorporation experiments in which ^{18}O from ^{18}O -enriched water in the incubation medium was incorporated exclusively and quantitatively into inorganic sulphate. Only two other esters (serine-*O*-sulphate and *p*-nitrophenyl sulphate) of a total of 29 compounds tested were substrates for the enzyme. D-Lactate, L-lactate-2-sulphate and the substrate analogues glycollate-2-sulphate and butyrate-2-sulphate were significantly inhibitory.

Studies in these laboratories (see Dodgson *et al.*, 1982; Dodgson & White, 1983, for summaries) have established that the biodegradation of primary and secondary alkyl sulphate surfactants is initiated by primary and secondary alkylsulphatase enzymes, some of the latter being stereospecific. However, these enzymes are apparently unable to deal with alkyl sulphates with alkyl chains containing fewer than five carbon atoms. It is known that short-chain alkyl sulphates do exist in nature. For example, a number of these esters are present in the allantois of the developing chick egg, the major component being propan-2-yl sulphate (Yagi, 1964, 1966). Furthermore, the end-products of the metabolism of alkyl sulphate surfactants in mammals and fish are short-chain sulphated compounds, e.g. butyric acid-4-sulphate is the major excretory product formed from the metabolism of sodium dodecyl sulphate in the rat (Denner *et al.*, 1969). Industry also makes its contribution to short-chain alkyl sulphates in the environment, and methyl sulphate has been identified as a significant component of the air-borne

particulate matter emitted from coal-burning power stations (Lee *et al.*, 1980).

As part of a programme to investigate the ways in which these short-chain alkyl sulphates undergo biodegradation, a soil bacterium has been isolated which can grow on potassium propan-2-yl sulphate as the sole source of carbon, energy and sulphur. No sulphatase enzyme capable of using propan-2-yl sulphate as substrate can be detected. However, a stereospecific enzyme that is able to remove the ester sulphate group from D-lactate-2-sulphate is produced by the bacterium. The present paper describes the purification and some properties of this new type of sulphatase.

Materials and methods

Unless stated otherwise, all chemicals were the purest available from Sigma or BDH. 2-Monochloropropionic acid, a gift from Professor H. Slater, University of Wales Institute of Science and Technology, Cardiff, U.K., was converted to the potassium salt by neutralization with KOH.

Chemical analysis

Potassium contents of potassium alkyl sulphate esters were measured by flame photometry in an EEL flame photometer (Evans Electroselenium, Halstead, Essex, U.K.) with KCl as standard. Ester sulphate contents were measured gravimetrically by conversion to BaSO₄, as described previously (White *et al.*, 1980). Elemental analyses for C and H contents were performed using a Technicon CHN Autoanalyser standardized with acetanilide.

Spectroscopic analysis

I.r. spectra of samples incorporated either in Nujol mulls between NaCl plates, or in KBr disks, were obtained by using a Perkin-Elmer Infracord 137 Spectrometer. For n.m.r. spectroscopy, samples (15–25 mg) were dissolved in ²H₂O (0.5 ml, 99.8 atom% excess) and spectra were determined with a Perkin-Elmer R32 spectrometer operated at 60 MHz.

Preparation of potassium lactate-2-sulphates

Lithium lactate (0.1 mol, racemic or L-isomer) was slowly added to stirred conc. H₂SO₄ (1 mol, cooled in an ice/salt bath) care being taken to ensure the temperature remained below 20°C. After addition, stirring was continued for 3 h at room temperature. The reaction mixture was poured into 1 litre of ice-cold water containing 100 g of Ba(OH)₂, and more solid Ba(OH)₂ was added until the pH was greater than 7. Precipitated BaSO₄ and undissolved Ba(OH)₂ were removed by centrifuging and excess Ba(OH)₂ was precipitated as BaCO₃ by passing CO₂ through the ice-cold solution. After centrifuging, the supernatant was concentrated (*in vacuo* at 40–45°C) to about 100 ml. The concentrate was passed through a Dowex 50 column (H⁺ form; 3 cm × 18 cm) and the eluate that emerged at pH 1–2 was collected and neutralized with KOH. After concentrating to 50 ml as before, the solution was applied to a Dowex 1 column (HCO₃⁻ form; 3 cm × 27 cm) which was then eluted with a linear gradient of NH₄HCO₃ (1.0–1.5 M) and then continuously with 1.5 M-NH₄HCO₃. Fractions (10 ml) were tested for the presence of the sulphate ester group (acid hydrolysis plus BaCl₂) and the positive fractions were pooled. Sufficient Dowex 50 (H⁺ form) was added to make the solution acidic (about pH 3) and the whole was then de-gassed *in vacuo*, neutralized with KOH and freeze-dried. The product was recrystallized from water/methanol (9:1, v/v). The yield of racemic lactate-2-sulphate was 34.3% and the chemical analysis was: C, 14.17% found (14.63% theoretical); H, 1.52% (1.63%); SO₄, 37.6% (39.0%); K⁺, 31.6% (31.7%). For L-lactate-2-sulphate the yield was 39.1% and the chemical ana-

lysis; C, 14.96%; H, 1.58%; SO₄, 39.4%; K⁺, 32.4% (theoretical values as for racemic compound). I.r. spectroscopy revealed strong absorption bands at 1200–1260 cm⁻¹ and 770–820 cm⁻¹ typical of covalent sulphate esters, and at 1600 cm⁻¹, characteristic of the carboxylate group. The n.m.r. spectrum of lactate-2-sulphate (racemic or L-isomer) contained a doublet [δ (p.p.m.) (²H₂O) 1.40 (3H, d, *J* 5 Hz, CH₃CH)] shifted slightly downfield from the corresponding signal in the lactic acid spectrum (δ = 1.28). The signal for the C-2 proton of lactate [δ (p.p.m.) (²H₂O) 4.00 (1H, q, *J* 4 Hz, CH₃CH)] was also shifted downfield in the sulphate ester and was partly obscured by the ¹H²HO resonance.

D-Lactate-2-sulphate was prepared in a similar manner except that the solution emerging from the Dowex 50 column was neutralized (KOH) and freeze-dried, and the residue was extracted with methanol. The white solid remaining was washed with diethyl ether, dried at 60°C, and then recrystallized as before. Adoption of this procedure enabled expensive unchanged D-lactate to be recovered from the methanol extracts. The yield of D-lactate-2-sulphate was 29.8% and the results of chemical and spectroscopic analyses were virtually identical to those for the racemic compound.

Preparation of alkyl sulphates

Primary alkyl sulphates and racemic 'decan-5-yl sulphate' (containing other decyl sulphate isomers; see Matcham & Dodgson, 1977) were prepared from the parent alcohols by the method of Dodgson *et al.* (1974). Potassium propan-2-yl sulphate was prepared by the method of Lloyd *et al.* (1961) scaled up 50-fold. Other secondary alkyl sulphates were prepared by the method of White *et al.* (1980). Most values for C and H contents were within 2% and 0.2% of theoretical values, respectively. Percentage contents of SO₄ and K⁺ were within 2% and 1% of expected values. I.r. spectra contained strong absorption bands at 1200–1260 and 770–810 cm⁻¹ typically associated with sulphate esters (Lloyd *et al.*, 1961). N.m.r. spectra of alkyl sulphate esters conformed to the patterns described previously (White *et al.*, 1980).

Growth of *Pseudomonas syringae* GG

The bacterium (the isolation of which will be described elsewhere) was grown on a large scale (400 litre New Brunswick Fermentor) in 350 litres of basal salts (Fitzgerald & Payne, 1972) supplemented with potassium propan-2-yl sulphate (0.3%). The fermentor was aerated with 1.0 m³ of air/h and was agitated at 60 rev./min. Cells were harvested after 20 h (stationary phase) with a Sharples centrifuge (flow rate 90 litres/h) and the wet cells (approx. 1 kg) were resuspended in water, freeze-dried and stored at -20°C until required.

Assay of sulphatase activity

Enzymically liberated SO_4^{2-} ions were measured by the BaCl_2 /gelatin method of Dodgson (1961) as modified by Thomas & Tudball (1967). Enzyme and substrate were routinely incubated at 25°C in the presence of 0.1M-Tris/HCl buffer, pH 7.5, and appropriate control determinations were made. A unit of enzyme activity is defined as the amount of enzyme that liberates $1\mu\text{mol}$ of SO_4^{2-} /min at 25°C when 10mM-D-lactate-2-sulphate is employed as substrate.

For assays performed during enzyme purification, incubation periods varied depending upon circumstances. For kinetic work, preliminary experiments with pure enzyme indicated that sulphate release was linear with time over a 5min period provided that the extent of hydrolysis did not exceed 15% of the substrate. The activity of the pure enzyme was proportional to protein concentration up to at least $32\mu\text{g/ml}$ of incubation mixture.

Determination of protein and nucleic acid

Protein contents of column fractions were estimated by measuring A_{280} . Protein concentrations in pooled fractions at each stage of purification were determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard. Nucleic acid was determined by the method of Layne (1957).

Determination of lactate

In order to establish the reaction stoichiometry and the configuration of the lactic acid liberated as a result of enzyme action, D- and L-lactic acids were assayed using either D- or L-lactate dehydrogenase and the method described in the Sigma Technical Bulletin no. 826-UV.

Polyacrylamide-gel electrophoresis

Conditions used for separating and locating primary and secondary alkylsulphatases on gels were as described by Payne *et al.* (1974). Sodium dodecyl sulphate was the substrate for the detection of the primary enzymes and 'decan-5-yl sulphate' for the secondaries. Staining of gels for lactate-2-sulphatase activity was carried out by incubating gels with a solution containing 20mM-potassium lactate-2-sulphate and 20mM-barium acetate in 0.1M-Tris/HCl, pH 7.5. Enzyme activity revealed itself as a white band of precipitated BaSO_4 .

Staining of gels for ability to dechlorinate potassium DL-2-chloropropionate (the chloro-analogue of DL-lactate 2-sulphate) was achieved by using the AgNO_3 precipitation method of Weightman & Slater (1980) except that the incubation buffer was 0.1M-Tris/ H_2SO_4 , pH 7.5, and the substrate con-

centration was 20mM. This procedure reveals dehalogenase activity as a band of insoluble AgCl .

Staining for protein was according to the directions of Diezel *et al.* (1972) except that Page Blue G90 was substituted for Coomassie Brilliant Blue G250.

Isotope-incorporation experiments

Incorporation of ^{18}O from water into one or other of the products of the reaction catalysed by the enzyme was examined. A reaction mixture containing $25\mu\text{mol}$ of D-lactate-2-sulphate dissolved in $250\mu\text{l}$ of H_2^{18}O (70 atom% excess), pure D-lactate-2-sulphatase solution ($125\mu\text{l}$, 0.053 units) and 0.4M-Tris/HCl, pH 7.5 ($125\mu\text{l}$), was incubated at 25°C for 6h. Under these conditions, the extent of hydrolysis was in excess of 90%. Reaction was stopped by addition of $100\mu\text{l}$ of 10M-HCl and liberated inorganic sulphate was precipitated immediately by adding $200\mu\text{l}$ of a hot saturated solution of BaCl_2 . The precipitate was separated by centrifuging and set aside. The supernatant was extracted with diethyl ether ($10 \times 1\text{ml}$) and the pooled extracts containing enzymically liberated lactic acid were evaporated to dryness in a stream of N_2 . The residue was redissolved in methanol and analysed in a Finnegan 4500 gas chromatograph/mass spectrometer to determine the extent of ^{18}O incorporation from water that had occurred during hydrolysis. The mass spectrum obtained by chemical ionization was identical with that of standard un-enriched lactic acid. The main peak at m/z 91 corresponded to $(M+H)$, where M is the molecular ion.

The precipitated BaSO_4 was washed (distilled water, $3 \times 1\text{ml}$; methanol, $3 \times 1\text{ml}$; diethyl ether, $4 \times 1\text{ml}$), mixed with finely ground KBr (150mg) and oven-dried at 90°C . The mixture was compressed into a disc and its i.r. spectrum in the range $800\text{--}1400\text{cm}^{-1}$ was recorded. The ^{18}O enrichment of the BaSO_4 was calculated from absorptions at 970cm^{-1} and 990cm^{-1} according to the method of Spencer (1959).

Purification of D-lactate-2-sulphatase

Stage 1. Freeze-dried cells (2g) of the bacterium were resuspended in 30ml of ice-cold 10mM-Tris/HCl buffer, pH 7.5, and stirred mechanically for 30min at 2°C . The cell suspension was then passed four times through a chilled French press at a pressure of 120MPa. Cell debris was removed by centrifuging for 1h at 4°C and $31000g_{\text{av}}$ (SS34 rotor, Sorvall RC-5B centrifuge) and the clear supernatant was dialysed against the resuspension buffer (3×2 litres) for a total of 18h.

Stage 2. The protein concentration of the dialysed extract (31ml) was adjusted to 10mg/ml with buffer and sufficient streptomycin sulphate

solution (5%, w/v) was added to the stirred solution to bring the concentration of the streptomycin sulphate also to 10mg/ml. Stirring was continued for 30min, when precipitated nucleic acid was removed by centrifuging as before and the clear supernatant was dialysed against 10mM-Tris/HCl buffer (4 × 5 litres) for 18h at 4°C.

Stage 3. The cell extract from stage 2 (approx. 120ml) was treated with a settled suspension of DEAE-cellulose (DE52, 25ml) in 10mM-Tris/HCl buffer, pH 7.5. The mixture was left for 15min at 2°C with occasional stirring and then centrifuged for 15min at 10000*g*_{av.} and 4°C. The supernatant was decanted and the pellet was resuspended in 10mM-Tris/HCl buffer (25ml, pH 7.5) and stirred for 10min. After centrifuging, the supernatant was discarded and the pellet was washed with 0.2M-NaCl (2 × 25ml) and 0.5M-NaCl (2 × 25ml) with intermediate centrifuging. The 0.5M-NaCl eluates were pooled and dialysed against 50mM-Tris/HCl buffer, pH 7.5 (3 × 5 litres) for 18h.

Stage 4. The extract from stage 3 was applied to a DE52 column (4.3cm × 45cm) that had been equilibrated with 50mM-Tris/HCl buffer, pH 7.5. The column was washed with 100ml of the same buffer and then eluted with a linear gradient of NaCl (0.2–0.5M) in the Tris/HCl buffer (total volume, 1500ml). Flow rate was adjusted to 75ml/h and 10ml fractions were collected and monitored for protein and enzyme activity. Fractions with the highest specific enzyme activity were pooled (fractions 95–104, see Fig. 1*a*), concentrated (Millipore CX 10 immersible membrane) to 10ml and dialysed for 18h against 0.1M-Tris/HCl buffer, pH 7.5 (3 × 5 litres).

Stage 5. The stage 4 enzyme was applied to a Sephacryl S-300 column (2.5cm × 80cm) that had been equilibrated with 0.1M-Tris/HCl, pH 7.5. The column was eluted with the same buffer at a flow rate of 5ml/h and 6ml fractions were collected.

Stage 6. Stage 5 fractions containing the highest specific activity (fractions 34–38, see Fig. 1*b*) were pooled and applied to a butyl-agarose column

(1.6cm × 40cm) that had been equilibrated with 10mM-Tris/HCl, pH 7.5. The column was washed with the buffer (20ml) followed by a linear gradient of NaCl (0–0.015M) in 800ml of buffer. Fractions containing enzyme as a single protein band on gel electrophoretograms were combined (fractions 59–64, see Fig. 1*c*).

Results

Purification of lactate-2-sulphatase

Table 1 provides details of the activities and recoveries of the enzyme during a typical purification procedure. Problems were encountered when attempts were made to scale-up the purification procedure in that the final preparation was contaminated with a minor protein component. Multiple preparations on a 2g (dried cells) scale were consistently satisfactory. Tests for primary and secondary alkylsulphatases in polyacrylamide gels of the crude cell extract and the final preparation (using sodium dodecyl sulphate and 'decan-5-yl sulphate' as the respective substrates) were negative.

Determination of M_r

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis gave a single band corresponding to an M_r of 55000. Carbonic anhydrase (bovine erythrocytes), albumin (chicken egg), albumin (bovine serum), phosphorylase *b* (rabbit muscle), β -galactosidase (*Escherichia coli*) and myosin (rabbit muscle) were the standard proteins.

The M_r of the purified native enzyme was determined by the electrophoretic method of Zwann (1967) using ferritin (horse spleen), catalase (bovine liver), aldolase (rabbit muscle), albumin (chicken egg), albumin (bovine serum) and chymotrypsin (bovine pancreas) as standard proteins. The native M_r was 59800, suggesting a monomeric structure for the enzyme. Gel filtration on Sephacryl S-300 with the same set of standards gave an M_r of 89000. The reason for this discrepancy is not

Table 1. *Purification of D-lactate-2-sulphatase from Pseudomonas syringae GG*
See the text for experimental details and for definition of the unit of enzyme activity.

Purification stage	Volume (ml)	Nucleic acid (%)	Protein (mg)	Enzyme activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
1. Cell extracts	31	17	1088	48.9	0.045	100	1.0
2. Streptomycin sulphate	123	1.75	745	80.2	0.106	164	2.4
3. DEAE-cellulose batch treatment	52		250	47.4	0.190	94	4.3
4. DEAE-cellulose column	10		9.2	30.3	3.3	62	73
5. Sephacryl S-300	30		5.3	24.4	4.6	50	101
6. Butyl-agarose	47		1.03	11.3	10.9	23	243

Fig. 1. Elution profiles for D-lactate-2-sulphatase from DEAE-cellulose, Sephacryl S-300 and butyl-agarose (a) Elution from DEAE-cellulose (DE52) at stage 4 of the purification; (b) elution from Sephacryl S-300 at stage 5; (c) elution from butyl-agarose at stage 6. See the text for details. $\circ$, Protein; $\bullet$, D-lactate-2-sulphatase activity; ---, NaCl concentration gradient.

known, but problems encountered with gel filtration columns during the purification had indicated that some sort of aggregation might be occurring.

Thermal stability

Purified enzyme, in buffer at pH 7.5, was preincubated for 5 min at various temperatures from 15

to 50°C in the absence of substrate. Subsequently samples of the enzyme were assayed in the usual way at 25°C. The enzyme was stable to preincubation at temperatures up to 25°C but at higher temperatures there was a steady decrease in activity until at 50°C no activity remained. When enzyme was assayed at different temperatures without pre-

incubation, there was a marked (3-fold) increase in activity from 15 to 25°C and a maximum at 35°C beyond which thermal inactivation caused a decrease in activity.

Substrate specificity

Initial experiments with dialysed crude cell extracts showed that the enzymic reaction ceased when approx. 50% of the racemic lactate-2-sulphate preparation had been hydrolysed. Addition of further cell extract (so that the enzyme concen-

Fig. 2. Reaction progress curves for the enzymic hydrolysis of racemic and D-lactate-2-sulphate

(a) ○, Enzymic release of SO_4^{2-} from a solution containing 4 mM-DL-lactate-2-sulphate and 0.12 unit of enzyme/ml in 0.1 M-Tris/HCl, pH 7.5. At the arrow, the incubation mixture was further supplemented so that either (□) the enzyme concentration was raised to 0.18 unit/ml, or (Δ) the total substrate concentration was raised to 6 mM. (b) Hydrolysis products released from 2 mM-D-lactate-2-sulphate in a solution containing 0.12 units of enzyme/ml of 0.1 M-Tris/HCl, pH 7.5. ●, Inorganic sulphate; ■, D-lactate; ▲, L-lactate.

tration was doubled) at this point did not cause any further hydrolysis of substrate. On the other hand, addition of further racemic substrate (so that the original concentration was doubled) gave a further hydrolysis equivalent to about 42% of the additional substrate. These findings were confirmed in similar experiments with pure enzyme (Fig. 2a). This suggested that the enzyme was hydrolysing only one of the optical stereoisomers present in the racemic substrate. Other experiments in which either the D- or the L-isomer was incubated with the purified enzyme showed that only the D-form could serve as a substrate. Time-course experiments in which D-lactate-2-sulphate was completely hydrolysed by the pure enzyme showed that SO_4^{2-} ions and D-lactate were released at the same rate (Fig. 2b) and no L-lactate was produced.

A wide variety of other sulphate esters was tested as possible substrates for the enzyme. Potential substrates were present at a final concentration of 20 mM and the pH (7.5) was optimal for the normal substrate. Of the 29 compounds tested, only two served as substrates (albeit very poor ones) for the enzyme. D-Serine-O-sulphate was hydrolysed at a rate equivalent to 9% of that of D-lactate-2-sulphate, whereas *p*-nitrophenyl sulphate was hydrolysed at a rate equivalent to 6%. The sulphate esters of L-lactate, methanol, ethanol, propanol, propan-2-ol, butanol, butan-2-ol, pentanol, pentan-2-ol, pentan-3-ol, hexanol, hexan-2-ol, heptanol, heptan-2-ol, heptan-4-ol, octanol, octan-2-ol, decanol, decan-5-ol, 2-hydroxybutyrate, 2-hydroxyisobutyrate, butoxyethanol and phenoxyethanol, the 6-sulphate esters of glucosamine and *N*-acetylglucosamine, and the 3-sulphate esters of glucosamine and *N*-acetylgalactosamine were not substrates for lactate-2-sulphatase. When gels containing pure D-lactate-2-sulphatase were incubated with potassium DL-2-chloropropionate (the chloro-analogue of DL-lactate-2-sulphate) no white bands were observed, indicating the absence of dehalogenase activity.

In the light of these findings, it is proposed that the enzyme be referred to as D-lactate-2-sulphatase.

Position of bond cleavage

Retention of configuration at C-2 during enzymic hydrolysis of D-lactate-2-sulphate strongly implicated the O-S bond as the cleavage site in the C-O-S linkage. When enzymic hydrolysis of D-lactate-2-sulphate was performed in the presence of H_2^{18}O (35 atom% excess), there was no significant incorporation of ^{18}O above natural abundance into D-lactate. In contrast, the ^{18}O -enrichment of the SO_4^{2-} ions was 8.92 atom% excess, in close agreement with the theoretical value of 8.75% for O-S cleavage. This, and the failure to find any

Fig. 3. Product inhibition of D-lactate-2-sulphatase by D-lactate

D-Lactate-2-sulphatase was assayed in the presence of 0 (○), 6.25 mM (●), 12.5 mM (▲) and 25.0 mM (■) concentrations of D-lactate under the usual assay conditions. Liberated sulphate was assayed at 1 min intervals up to 10 min to provide accurate initial velocity measurements.

enrichment in the D-lactate co-product, proved conclusively that D-lactate-2-sulphatase acts upon the O-S bond of the C-O-S ester linkage.

Effect of pH and substrate concentration

Maximum enzyme activity towards 15 mM-D-lactate-2-sulphate was obtained at pH 7.5. Tris/HCl buffer (0.2 M) was used to cover the pH range 7.0–9.0 and acetic acid/NaOH buffer (0.2 M) to cover the range pH 6.0–7.5. The K_m value at pH 7.5 as determined by a double-reciprocal plot was 6.7 mM (maximal specific activity 14.3 $\mu\text{mol/min per mg}$ of protein) and by the direct linear plot (Eisenthal & Cornish-Bowden, 1974) was 6.6 mM (maximal specific activity 14.8 $\mu\text{mol/min per mg}$ of protein).

Effects of inhibitors

Potassium L-lactate-2-sulphate inhibited the enzyme in a non-competitive manner with $K_i = 16.3 \text{ mM}$ (Lineweaver-Burk plot). D-Lactic acid, a product of the enzyme's action on D-lactate-2-sulphate, also inhibited the enzyme but the double reciprocal plots were non-linear (Fig. 3). At low substrate concentrations, the curves were parallel and the inhibition resembled the uncompetitive type. At high substrate concentrations the curves converged. L-Lactic acid was not an inhibitor. Several other compounds, mainly sulphate

Table 2. Some inhibitors of D-lactate-2-sulphatase

Pure enzyme was incubated with D-lactate-2-sulphate (20 mM) and each potential inhibitor (20 mM unless stated otherwise) in the normal assay buffer for 4 min. Liberated SO_4^{2-} was determined as described in the text and activities are expressed as percentages of that observed in the absence of inhibitor.

Inhibitor	Residual activity (%)
D-Lactic acid	53
L-Lactate-2-sulphate (12 mM)	57
Glycollate-2-sulphate	58
Butyrate-2-sulphate	58
Propan-1-yl sulphate	78
Propan-2-yl sulphate	82
Butan-2-yl sulphate	75
Pentan-3-yl sulphate	90
Hexan-2-yl sulphate	87
D-Serine-O-sulphate	89
2-Chloropropionate	92

esters, were also tested as potential inhibitors of the enzyme (Table 2). Not surprisingly, D-lactic acid and L-lactate-2-sulphate were the best inhibitors. Glycollate-2-sulphate and butyrate-2-sulphate, which are carbon chain-length homologues of lactate-2-sulphate, were also inhibitory, but chloropropionate and other sulphate esters tested were weak inhibitors.

Discussion

The purification factor of 243-fold achieved for D-lactate-2-sulphatase is typical of other inducible bacterial alkylsulphatases that have been purified hitherto (Cloves *et al.*, 1980; Shaw *et al.*, 1980; Lillis *et al.*, 1983). Other similarities exist; for example, the enzyme is stereospecific, it exhibits an alkaline pH optimum, and it acts as a simple sulphatase releasing sulphate and D-lactate in stoichiometric amounts. However, there are also some significant differences. First, D-lactate-2-sulphatase catalyses fission of the O-S bond of the C-O-S ester linkage, whereas the simple alkylsulphatases of *Pseudomonas* C12B and *Comamonas terrigena* cleave at the C-O bond. In this respect the D-lactate-2-sulphatase is like the 2,4-dichlorophenoxyethyl sulphatase (Crag herbicide sulphatase) of *P. putida* (Lillis *et al.*, 1983), which is the only other alkylsulphatase known to act at the O-S bond. Secondly, D-lactate-2-sulphatase has a very narrow substrate specificity, failing to hydrolyse even glycollate-2-sulphate or butyrate-2-sulphate (the immediate homologues of D-lactate-2-sulphate), or any of a number of other simple primary or secondary alkyl sulphates. In contrast, the alkyl sulphatases of *Pseudomonas* C12B and *C.*

terrigena are characterized by their ability to accept a wide range of chain lengths above a minimum around C₅–C₆. Even the Crag herbicide sulphatase shows activity towards long chain alkyl sulphates, including octyl and dodecyl sulphates. The narrow specificity of D-lactate-2-sulphatase is thus unusual for this type of enzyme. The ability of serine-O-sulphate and *p*-nitrophenyl sulphate to serve as substrates was, at first, surprising. However, construction of space-filling models showed a close similarity in size and overall structure with D-lactate-2-sulphate. Moreover the propensity of aryl sulphates for the S–O mode of hydrolysis that this enzyme catalyses is well-established (Dodgson *et al.*, 1982). Absence of any dehalogenase activity associated with D-lactate-2-sulphatase is perhaps to be expected in view of the O–S bond-splitting action of the enzyme; only a C–OSO₃ splitting enzyme expelling the intact OSO₃ moiety of a sulphate ester might be expected to break a C–Cl bond.

Further light was thrown on the catalytic mechanism by the inhibition patterns produced by D-lactate, one of the products of enzymic hydrolysis. The uncompetitive type of inhibition observed at low substrate concentrations could arise from a mechanism involving formation of a sulphated-enzyme (E–SO₃) intermediate, and reversible departure of D-lactate prior to rate-determining hydrolysis of inorganic sulphate. High concentrations of free D-lactate would hinder further dissociation of D-lactate from the E–SO₃...D-lactate complex, thereby inhibiting (uncompetitively) subsequent release of sulphate. Such a mechanism would be compatible with O–S cleavage, and with the absence of inhibition by L-lactate which would have the wrong stereochemistry. The picture becomes further complicated at high concentrations of substrate, which alleviate the inhibitory effects of D-lactate (Fig. 3). A possible explanation of this observation may be that excess substrate and D-lactate now compete for binding to E–SO₃, but the E–SO₃...D-lactate-2-sulphate complex is still able to release sulphate. A more detailed analysis of the mechanism, to accommodate both this protective effect of excess substrate and the non-competitive inhibition by L-lactate-2-sulphate, must now await further experimentation.

P. syringae GG grows well on propan-2-yl sulphate, but its failure to produce a corresponding sulphatase eliminates the usual pathway for alkyl sulphate degradation in micro-organisms, namely initial desulphation followed by oxidation. However, the discovery of D-lactate-2-sulphatase in extracts of cells grown on propan-2-yl sulphate strongly suggests an alternative route requiring

initial oxidation of propan-2-yl sulphate to lactate-2-sulphate before desulphation to lactate. Work is currently in progress to establish the existence of this pathway, and it will be interesting to see how the stereochemistry of any oxidation steps compare with the stereospecificity of the D-lactate-2-sulphatase enzyme.

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