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Purification and Properties of the S1 Secondary Alkylsulphohydrolase of the Detergent-Degrading Micro-organism, *Pseudomonas* C12B

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The S1 secondary alkylsulphohydrolase of the detergent-degrading micro-organism, *Pseudomonas* C12B, was separated from other alkylsulphohydrolases and purified to homogeneity. Under the experimental conditions used the enzyme completely hydrolysed D-octan-2-yl sulphate (D-1-methylheptyl sulphate), but showed no activity towards the corresponding L-isomer. Additional evidence has been obtained to indicate that it is probably optically stereospecific for D-secondary alkyl sulphate esters with the ester sulphate group at C-2 and with a chain length of at least seven carbon atoms. Enzyme activity towards racemic samples of heptan-2-yl sulphate (1-methylhexyl sulphate), octan-2-yl sulphate and decan-2-yl sulphate (1-methylnonyl sulphate) increased with increasing chain length. L-Octan-2-yl sulphate is a competitive inhibitor of the enzyme, as are certain primary alkyl sulphates and primary alkanesulphonates. Inhibition by each of the last two types of compounds is characteristic of the behaviour of an homologous series. Inhibition increases with increasing chain length and plots of $\log K_I$ values against the number of carbon atoms in each alkyl chain show the expected linear relationship. A crude preparation of the S2 secondary alkylsulphohydrolase was used to show that this particular enzyme hydrolyses L-octan-2-yl sulphate, but is probably inactive towards the corresponding D-isomer. The similarity of the S1 and S2 enzymes to the CS2 and CS1 enzymes respectively of *Comamonas terrigena* was established, and some comments have been made on the possible roles of these and other alkylsulphohydrolases in the biodegradation of detergents.

Pseudomonas C12B is a detergent-degrading micro-organism that was isolated from soil near the outfall of a sewage-disposal plant in Georgia, U.S.A. When grown in nutrient broth the organism produces two secondary alkylsulphohydrolase enzymes (designated S1 and S2) and a primary alkylsulphohydrolase (designated P1). Addition of sodium dodecyl sulphate or many other primary alkyl sulphate esters to the nutrient broth leads to the appearance in the micro-organism of an additional primary alkylsulphohydrolase (designated P2), whereas the presence of Oronite (a commercial detergent consisting of a C_{10}–C_{20} mixture of secondary alkyl sulphate esters) leads to the additional appearance of a third (S3) secondary alkylsulphohydrolase (Dodgson *et al.*, 1974). The indications are that, collectively, these five enzymes are probably responsible for the initial attack on primary and secondary alkyl sulphate detergents (cf. Payne *et al.*, 1965; Williams *et al.*, 1966; Matcham *et al.*, 1977*a*).

Parallel studies have also been made on a second micro-organism, a strain of *Comamonas terrigena*, which produces two secondary alkylsulphohydrolases when grown on nutrient broth (Fitzgerald *et al.*, 1975). Both enzymes appear to be specific for compounds in which the ester sulphate group is at C-2 of the alkyl chain, but one of them (designated CS1) is specific for the (−)-optical stereoisomers, whereas the other (CS2) is specific for (+)-optical stereoisomers (Matcham *et al.*, 1977*a*,*b*; Matcham & Dodgson, 1977).

The present paper describes the separation and purification of the S1 secondary alkylsulphohydrolase of *Pseudomonas* C12B and establishes its specificity as being similar to that of the *C. terrigena* CS2 enzyme. A brief report on this specificity has already been made (Matcham *et al.*, 1977*a*).

Experimental

Materials

Primary and racemic secondary alcohols were from Aldrich Chemical Co. Inc., Milwaukee, WI, U.S.A. D- and L-Octan-2-ols were prepared from racemic octan-2-ol by the procedure of Kenyon (1922) as modified by Matcham & Dodgson (1977). With one exception, sulphate esters of these various alcohols were prepared by the pyridine/SO_3 procedure of Matcham & Dodgson (1977) and were crystallized as potassium salts. The exception, racemic 'potassium

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decan-5-yl (1-pentylpentyl) sulphate', was prepared from racemic decan-5-ol by sulphation with H_2SO_4 as described by Dodgson *et al.* (1974). This particular method of preparation is now known to lead to the production of other isomers (Matcham & Dodgson, 1977) and the product in this particular case probably consists largely of decan-2-yl (1-methylnonyl) sulphate. It is, however, a useful substrate for detecting secondary alkylsulphohydrolases on gel zymograms (Dodgson *et al.*, 1974) and for rough assays of crude preparations containing both S1 and S2 secondary alkylsulphohydrolases. Potassium salts of primary alkyl sulphates were kindly donated by Mrs. J. M. Cloves (of this Department); the sodium salts of alkanesulphonates were from Cambrian Chemicals, Croydon, U.K. All other chemicals used were the purest available from BDH Chemicals, Poole, Dorset, U.K., or Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.

Growth of Pseudomonas C12B

The organism was grown at 30°C in 350 litres of nutrient broth in a 400-litre-capacity New Brunswick Fermentor aerated with 1.8 m^3 of air/h and agitated at 100 rev./min. Cells were harvested at the stationary phase of growth by using a Sharples centrifuge (flow rate 90 litre/h) and the wet cells (approx. 1–1.5 kg) were resuspended in 5 mM-Tris/HCl buffer, pH 7.5 (1 g of cells/ml), and ruptured by continuous passage through a Manton-Gaulin laboratory homogenizer operating at 60 MPa (type 15M8, Manton-Gaulin Manufacturing Co., Everett, MA, U.S.A.). The broken-cell suspension (2.5 litre) was freeze-dried and stored at −20°C until required.

Assay of sulphohydrolase activity

Enzymically liberated SO_4^{2-} was determined by the $BaCl_2$/gelatin method of Dodgson (1961) as modified by Thomas & Tudball (1967). Enzyme and substrate were incubated at 31°C in the presence of 0.1 M-Tris/HCl, pH 7.1, for between 5 and 20 min, depending on circumstances. Appropriate control determinations were made. A unit of S1 enzyme activity is defined as 1 μmol of SO_4^{2-} liberated/min at 31°C when 7.5 mM-potassium D-octan-2-yl (D-1-methylheptyl) sulphate is used as substrate. A similar unit is defined for the S2 enzyme activity, except that 7.5 mM-potassium L-octan-2-yl sulphate is used as substrate. Evidence has previously been presented to relate these two isomers to D- and L-glyceraldehyde respectively (Bartholomew *et al.*, 1977).

Determination of protein and nucleic acid

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard. Nucleic acid was determined as a percentage of total u.v.-absorbing material (A_{280}/A_{260} ratio) by the method of Layne (1957).

Polyacrylamide-gel electrophoresis

The conditions used for the electrophoretic separation and location of primary alkylsulphohydrolase and secondary alkylsulphohydrolase on gels were as described by Payne *et al.* (1974).

Purification of the S1 secondary alkylsulphohydrolase

Stage 1. Freeze-dried broken cells (37.5 g) were resuspended in 500 ml of ice-cold 0.01 M-Tris/HCl, pH 7.8, and the whole was passed three times through a chilled French press (continuous-flow attachment) at a pressure of 126 MPa. The suspension was centrifuged for 1 h at 2°C and 35000$g_{av.}$ in the no. 69180 angle head on an MSE High Speed 18 centrifuge and the supernatant was dialysed for 18 h at 2°C against several changes (total of 15 litres) of 0.01 M-Tris/HCl, pH 7.8. At this and later stages, samples were withdrawn for protein and nucleic acid determinations, enzyme assays and gel electrophoresis. In early experiments enzyme activity was assayed with 'potassium decan-5-yl sulphate', but during the final purification procedure potassium D- and L-octan-2-yl sulphates were used. All subsequent stages were carried out at 2°C.

Stage 2. The supernatant (350 ml) was treated with 50 ml of aq. 5% streptomycin sulphate, which was added slowly with gentle stirring. After standing for 90 min the precipitated nucleic acid was removed by centrifuging as before for 1 h. The clear supernatant was then dialysed for 24 h against several changes (total of 15 litres) of 0.01 M-Tris/HCl buffer, pH 7.8.

Stage 3. Sufficient solid $(NH_4)_2SO_4$ was added to the dialysed supernatant over a period of 1 h to give 55% saturation, the solution being gently stirred and the pH maintained at 7.5 with dilute aq. NH_3. After keeping for a further 1 h, the precipitated protein was removed by centrifuging for 2 h as before and the clear supernatant was dialysed for 40 h against several changes (total of 45 litres) of the usual buffer. Much of the P1 enzyme was removed at this stage.

Stage 4. Preliminary experiments showed that addition of further $(NH_4)_2SO_4$ (at various pH values) failed to precipitate significant amounts of the enzyme. Accordingly, the dialysed enzyme solution (635 ml) was concentrated to approx. 210 ml in several dialysis sacs by treatment with several changes of Aquacide II-A (Calbiochem, Bishop's Stortford, Herts., U.K.). The concentrated solution was dialysed in fresh dialysis sacs for a few hours against several changes of the usual buffer.

Stage 5. The solution (100 ml portions) was applied to a DEAE-cellulose (DE-52) column (17 cm × 4.5 cm)

that had been equilibrated with 0.01M-Tris/HCl, pH7.8, and was washed in with 330ml of the same buffer. A linear gradient of the buffer was prepared between 450ml of 0.01M- and 450ml of 0.5M-Tris/HCl, pH7.8, and the flow rate was adjusted to 150ml/h. Protein was monitored at 282nm by the LKB Uvicord. Fractions (10ml) were collected and samples were taken from alternate fractions and assayed for both S1 and S2 secondary alkylsulphohydrolase activities and examined for the presence of the P1 primary alkylsulphohydrolase on polyacrylamide gel zymograms. S1 and S2 activities emerged at the leading edge of a large protein peak (Fig. 1*a*). Fractions 69–76, which contained no P1 activity, were pooled and dialysed against 0.01M-Tris/HCl, pH7.8, for 16h. The dialysed solution (90ml) was concentrated by membrane filtration (Diaflo UM10) to less than 10ml.

Stage 6. The concentrated solution was run into a Sephadex G-100 column (90cm×5cm) that had been equilibrated with the usual Tris buffer, and the column was eluted with the same buffer at a flow rate

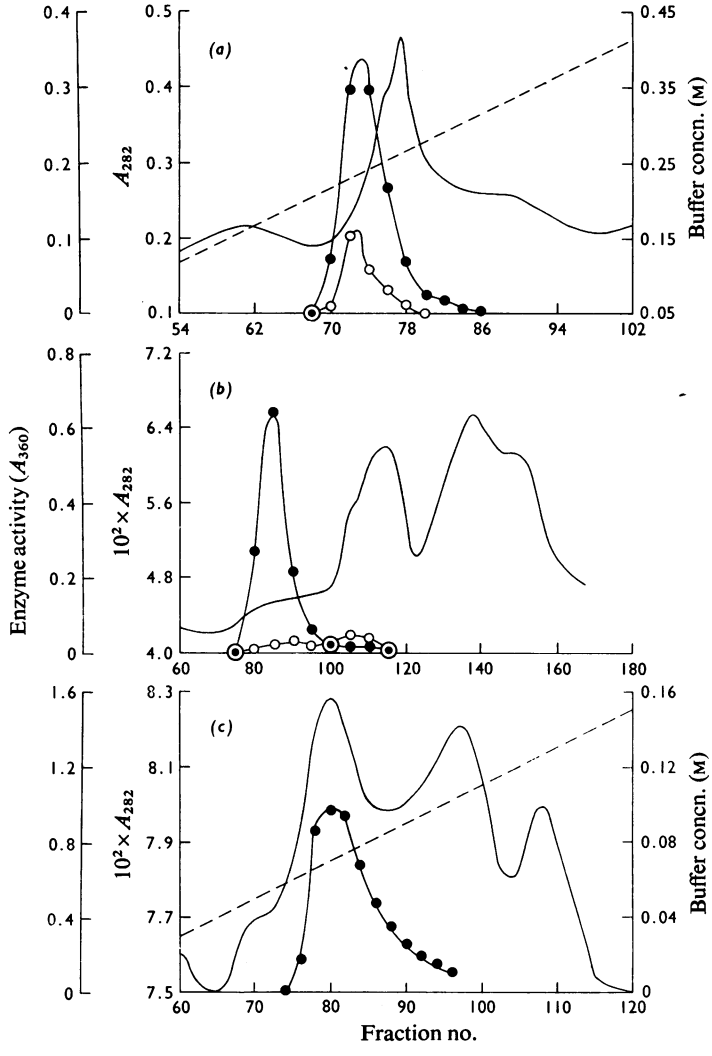

Fig. 1. *Elution profiles for S1 and S2 alkylsulphohydrolases from DEAE-cellulose and Sephadex G-100*
(*a*) Elution from DEAE-cellulose (DE-52) at Stage 5 of the purification procedure (10ml fractions collected); (*b*) elution from Sephadex G-100 at Stage 6 (6ml fractions); (*c*) elution from DE-52 at Stage 7 (3.5ml fractions). See the text for details. ●, S1 enzyme activity, ○, S2 enzyme activity. ——, Protein elution; ----, buffer gradient.

of 20ml/h. Fractions (6ml) were collected and every alternate one was assayed for S1 and S2 enzyme activities. Fractions 75–99 (Fig. 1*b*) contained the bulk of the S1 activity (small amounts of S2 were also present) and were pooled.

Stage 7. The pooled fractions (4.6mg of protein) were re-fractionated on a DE-52 column as described for Stage 5 except that 3.5ml fractions were collected and the flow rate was 50ml/h. S1 activity was distributed over fractions 74–100 (Fig. 1*c*), but only fractions 74–78 contained a single protein as evidenced by gel electrophoresis. Fractions 79–84 all gave two protein bands on polyacrylamide gels and fractions 85–90 gave three bands. Fractions 74–78 were pooled and dialysed against 0.01M-Tris/HCl buffer, pH7.8, and 16.5ml of pure enzyme solution (specific activity 566 units/mg of protein) was recovered and stored at −10°C in 3ml portions in glass vessels. Fractions 78–84 were also pooled, dialysed and stored similarly. This 'less pure' enzyme (specific activity approx. 180 units/mg of protein) was used for some preliminary experiments.

Separation of the S2 alkylsulphohydrolase by gel electrophoresis

The Stage-3 enzyme extract was subjected to polyacrylamide-gel electrophoresis, 0.045 unit of activity being placed on each of eight gels. Electrophoresis was continued until marker dye was more than two-thirds of the way down the gel. One of the gels was used to locate the S2 enzyme (Payne *et al.*, 1974) and the appropriate section cut out from the remaining gels. The sections were placed (in pairs) in glass tubes of similar size to those used for electrophoresis, but with a restriction about 1cm from the bottom so as to form a chamber of about 0.3ml capacity. The sections were sealed in with fresh gel and the bottom end of the tubes closed with dialysis tubing. Tubes were then filled with the usual running buffer and the enzyme caused to migrate out of the sections and into the cavity by continued electrophoresis for about 3h (400V, current approx. 1mA per tube) in the cold-room (2°C). The enzyme was recovered from the lower chamber (anode) by syringe (total of 1.3ml recovered, containing 0.05 unit of activity).

Results

Enzyme purification

Table 1 provides details of the activities and recoveries of the S1 alkylsulphohydrolase during the purification procedure. In developing the final purification procedure many other possible approaches were made, but were abandoned for one reason or another. They included CM-cellulose chromatography, hydrophobic chromatography, precipitation of unwanted protein with sodium dodecyl sulphate, and alternative methods for removing nucleic acid.

The removal of the other secondary alkylsulphohydrolase (S2) appeared to be associated with its apparent relative instability. Table 1 shows the steady decrease in S2 activity, considerable losses occurring at each purification stage. The enzyme could not be detected in fractions emerging from the final DE-52 column. The primary alkylsulphohydrolase (P1) present in cell extracts persisted until Stage 5, when it was finally eliminated on the DE-52 column. The results also indicate that the S1 and S2 enzymes are present in broth-grown *Pseudomonas* C12B cells in approx. equal amounts; furthermore, that of almost 8g of protein present in cell extracts the S1 enzyme is represented by less than 1mg. Both S1 and S2 are known to be localized in the periplasmic region of the cell (Fitzgerald & Laslie, 1975).

Specificity of the S1 and S2 enzymes

During preliminary purification studies on the S1 enzyme 'potassium decan-5-yl sulphate', prepared by

Table 1. *Purification of the S1 secondary alkylsulphohydrolase of Pseudomonas C12B*

Starting point for this particular preparation was 37.5g of freeze-dried stationary phase broth-grown cells. The S1 and S2 enzyme activities were assayed with D- or L-octan-2-yl sulphate respectively. See the text for definition of enzyme units.

Purification stage	Protein (mg)	Enzyme S1 activity (units)	Enzyme S1 activity (yield)	Enzyme S2 activity (units)	Enzyme S2 activity (yield)	Enzyme S1 specific activity	Enzyme S1 purification
1	7840	855	100	1017	100	0.11	1
2	3772	1134	133	648	65	0.30	2.7
3	1143	639	75	251	25	0.56	5.1
4	1143	535	63	151	15	0.47	4.3
5*	104	396	46	78	8	3.8	35
6*	10	303	35	20	2	30.3	275
7*	0.074	42	4.9	0	0	566	5150

* Values corrected to volume at Stage 4.

sulphation of decan-5-ol with H_2SO_4, was used as substrate. This material is heterogeneous and contains large amounts of decan-2-yl sulphate (Matcham & Dodgson, 1977) and, as will subsequently become clear, it is the (+)-decan-2-yl sulphate component of this impure material that serves as substrate for the S1 enzyme. During these preliminary studies it was not realized that the S1 and S2 alkylsulphohydrolases exhibited optical specificity, but, following the work of Matcham *et al.* (1977*b*) on the CS1 and CS2 secondary alkylsulphohydrolases of *C. terrigena*, the possibility arose that similar considerations might apply to the *Pseudomonas* C12B enzymes. This was confirmed by incubating (at 31°C) 2.4ml of a solution containing 0.3 unit of the S1 enzyme, 2.5mM-potassium D-octan-2-yl sulphate and 0.1M-Tris/HCl buffer, pH7.1. Samples (200μl) were withdrawn at various time intervals and liberated SO_4^{2-} was determined. The substrate was completely hydrolysed in 60min. No liberation of sulphate occurred when potassium L-octan-2-yl sulphate was substituted as potential substrate.

In a similar type of experiment, with only limited amounts of enzyme available, the S2 alkylsulphohydrolase (0.012 unit), which had been separated by gel electrophoresis, was incubated for 12h at 31°C with either L- or D-potassium octan-2-yl sulphate (3mM) in a total volume of 0.6ml of 0.1M-Tris/HCl, pH7.1. Liberated sulphate was measured at the end of the incubation period, when it was found that the L-isomer had been completely hydrolysed, whereas the D-isomer had lost less than 8% of its ester sulphate content. It is not clear whether this small amount of hydrolysis of the D-isomer represents traces of the S1 enzyme in the preparation or whether it reflects a feeble activity of the S2 enzyme towards the isomer. In any event, it is clear that the S2 enzyme is analogous to the CS1 enzyme of *C. terrigena* in hydrolysing L-substrates. Further specificity studies on the S2 enzyme must await the development of a more suitable purification procedure.

In further experiments the S1 enzyme was incubated with a number of different racemic or symmetrical secondary alkylsulphates to make a rough assessment of its overall specificity. Enzyme (0.04 unit) and potential substrate (3mM) were incubated together at 31°C for 1h in a total volume of 200μl of 0.1M-Tris/HCl, pH7.1, before determining liberated sulphate. The enzyme was active towards racemic potassium heptan-2-yl sulphate and racemic potassium decan-2-yl sulphate, both of which were hydrolysed 'completely' (i.e. to the extent of 50%) in 1h. No activity was detected towards the potassium salts of pentan-3-yl (1-ethylpropyl) sulphate, hexan-2-yl (1-methylpentyl) sulphate, heptan-4-yl (1-propylbutyl) sulphate, octan-3-yl (1-ethylhexyl) sulphate, octan-4-yl (1-propylpentyl) sulphate and nonan-5-yl (1-butylpentyl) sulphate. When the hydrolysis of the potassium salts of racemic heptan-2-yl (1-methylhexyl) sulphate, octan-2-yl sulphate and decan-2-yl sulphate was followed as a function of time, the relative rates of hydrolysis were respectively 1.9, 9.6 and 18.8nmol of SO_4^{2-} ions liberated/min. Not too much significance should be attached to these relative rates because, as will become clear in a later section, the enzyme is inhibited by L-octan-2-yl sulphate. Probably the L-forms of the other substrates will also serve as inhibitors and hydrolysis rates of racemic substrates under these circumstances can only be regarded as tentative.

Collectively, these various results indicate that, like the CS2 secondary alkylsulphohydrolase of *C. terrigena*, the S1 enzyme is additionally specific for the ester sulphate group in the C-2 position of the alkyl chain.

General properties of the S1 enzyme

The enzyme exhibited a broad shallow pH–activity

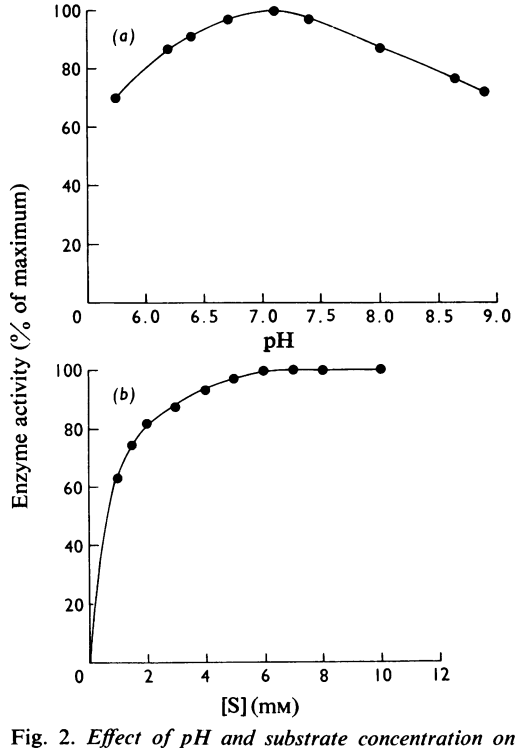

Fig. 2. *Effect of pH and substrate concentration on the activity of the S1 alkylsulphohydrolase*

Potassium D-octan-2-yl sulphate was the substrate and incubation was at 31°C in the presence of 0.1M-Tris/HCl buffer. Initial rates of hydrolysis were determined for each experimental point as described in the text. (*a*) pH–activity curve; (*b*) substrate concentration–activity curve.

profile (Fig. 2) when assayed against potassium D-octan-2-yl sulphate (7.5mM) in the presence of 0.1M-Tris/HCl buffer. Enzyme (6×10^{-3} unit) was incubated with substrate in a total volume of 200μl for 5, 10 and 15min periods at each pH value. A straight-line relationship between time of incubation and activity was obtained at each pH. Maximum activity was obtained at pH7.1.

The effect of increasing substrate concentration on the initial rates of the enzymic hydrolysis at pH7.1 is also shown in Fig. 2. With each concentration of substrate, enzyme activity was assayed at intervals of 5, 10 and 15min respectively. A straight-line time–activity relationship was obtained in each case at substrate concentrations of 1mM or greater. The K_m value, calculated by the double-reciprocal plot of Lineweaver & Burk (1934) and by the method of Eisenthal & Cornish-Bowden (1974), was 0.78mM.

With optimum conditions (pH7.1 and 7.5mM substrate), a straight-line relationship between enzyme activity and time was obtained over a period of 30min, beyond which activity began to decrease. Over a 20min incubation period, the enzyme concentration–activity relationship was linear.

Inhibition studies on the S1 enzyme

General inhibitors. The effects on the S1 enzyme of some compounds that have previously been shown to inhibit or activate several different types of sulphohydrolase are shown in Table 2. In all cases, the compounds were present in incubation mixtures at a final concentration of 20mM. The results are noteworthy in that relatively little effect was seen with any compound other than iodoacetamide. Dithiothreitol did not protect the enzyme against iodoacetamide inhibition.

Inhibition by L-octan-2-yl sulphate and other secondary alkylsulphates. The S1 secondary alkylsulphohydrolase of *Pseudomonas* C12B will be faced with racemic mixtures of alkylsulphates during detergent biodegradation, and therefore it was of considerable interest to know whether optical stereoisomers of normal substrates could serve as inhibitors. Apart from the obvious relevance to biodegradation, the absence of inhibition by such isomers would allow racemic mixtures to serve as substrates for enzymological studies, thus avoiding the need for the tedious and wasteful procedures for resolution of the secondary-alcohol starting materials. It was also of interest to know whether secondary alkyl sulphates with ester sulphate groups in positions other than C-2 of the alkyl chain would serve as inhibitors, particularly if such compounds were symmetric and hence without optical activity.

In these experiments, three concentrations of potassium D-octan-2-yl sulphate (1, 2 and 4mM) were used in association with the following concentrations of potential inhibitor: 2mM- and 4mM-L-octan-2-yl sulphate, 9mM-heptan-4-yl sulphate and -nonan-5-yl sulphate, 4.5mM-pentan-3-yl sulphate and 9mM- and 18mM-racemic octan-3-yl sulphate. Initial rates of hydrolysis were measured as usual. With the exception of pentan-3-yl sulphate, all the compounds tested served as competitive inhibitors (Table 3), with L-octan-2-yl sulphate being considerably more potent than the others. The K_i for that particular inhibitor (1.85mM) was more than twice the value of the K_m (0.78mM) for the D-isomer serving as normal substrate.

Inhibition by primary alkyl sulphates and primary alkanesulphonates. Similar inhibition experiments to those described in the previous section were carried out with primary alkyl sulphates and primary alkanesulphonates of increasing chain length. Because of the limitations inherent in the procedure for determining liberated SO_4^{2-} it was not possible to use alkyl sulphates or alkanesulphonates of chain length greater than C_{10} or C_9 respectively. This is due to their tendency to form insoluble barium salts under the conditions of the assay. Preliminary experiments showed that good recoveries of added SO_4^{2-} ions could be obtained with the compounds finally used. Both alkyl sulphates and alkanesulphonates acted as competitive inhibitors of the S1 enzyme; inhibition within each series increasing with increasing chain length. When $\log K_i$ values were plotted against the number of carbon atoms in the alkyl chain, a straight-line relationship between the two was obtained with each series (Fig. 3). At the lower chain length the alkyl sulphates were more effective inhibitors than the alkanesulphonates. However, the differences between the two became steadily less as chain length increased and extrapolation of the curve obtained for the alkanesulphonates would suggest that no differences would exist at C_{10} chain length.

Table 2. *Effects of potential sulphohydrolase activators or inhibitors on S1 enzyme activity*

Activators/inhibitors (adjusted to pH7.1) were present at a final concentration of 20mM. Incubation was for 15min.

Activator/inhibitor	Enzyme activity (%)
None	100
NaCl	86
Na_2HPO_4/NaH_2PO_4	66
$NaNO_3$	67
NaF	91
KCl	91
$MgCl_2$	95
$MnCl_2$	83
$CoCl_2$	90
EDTA	90
Iodoacetamide	46
Dithiothreitol	92
Iodoacetamide+dithiothreitol	45

Table 3. *Competitive inhibition of S1 enzyme activity by various alkyl sulphates and alkanesulphonates*
See the text for details. Inhibitor constants were calculated from the double-reciprocal plot of Lineweaver & Burk (1934).

Inhibitor	Inhibitor concentration (mM)	Average K_I (mM)
Secondary alkyl sulphates		
L-Octan-2-yl sulphate	2.0, 4.0	1.85
Racemic octan-3-yl sulphate	9.0, 18.0	13.0
Pentan-3-yl sulphate	4.5	0
Heptan-4-yl sulphate	9.0	27.0
Nonan-5-yl sulphate	9.0	16.5
Primary alkyl sulphates		
Butyl sulphate	15.0, 30.0	23.0
Pentyl sulphate	15.0, 30.0	14.0
Hexyl sulphate	4.5, 9.0	6.0
Heptyl sulphate	4.5, 9.0	1.9
Octyl sulphate	4.5, 9.0	0.9
Nonyl sulphate	2.0, 4.0	0.4
Decyl sulphate	1.0, 2.0	0.14
Alkanesulphonates		
Hexanesulphonate	5.0, 10.0	29.0
Heptanesulphonate	4.0, 8.0	7.85
Octanesulphonate	4.0, 8.0	2.4
Nonanesulphonate	2.0, 4.0	0.58

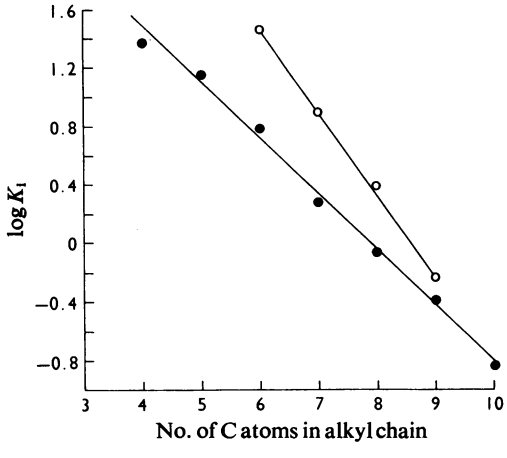

Fig. 3. *Relationship between log K_I and chain length of a series of alkyl sulphate and alkanesulphonate competitive inhibitors of the S1 alkylsulphohydrolase*
Potassium D-octan-2-yl sulphate was the substrate and incubation was at 31 °C in the presence of 0.1 M-Tris/HCl buffer, pH 7.1. ●, Primary alkyl sulphates; ○, primary alkanesulphonates.

Discussion

The development of a procedure for the purification of the S1 enzyme of *Pseudomonas* C12B has now enabled some preliminary work to be done on the specificity and general properties of the enzyme. This has revealed the enzyme to be analogous to the CS2 secondary alkylsulphohydrolase of *C. terrigena* in that it appears to be specific for D-isomers of secondary alkyl sulphates of which the ester sulphate group is in position C-2 of the alkyl chain. The competitive inhibition of the enzyme by L-octan-2-yl sulphate means that information concerning specificity, when only racemic forms of other potential substrates are available, must be regarded with caution. However, in common with the CS2 enzyme of *C. terrigena*, it seems probable that S1 activity increases with increasing chain length between C_7 and C_{10} (at least), although the CS2 enzyme was also able to hydrolyse hexan-2-yl sulphate slowly (Matcham *et al.*, 1977*b*). Further work on specificity must await the preparation of resolved forms of substrates and potential substrates, as well as the development of suitable assay procedures for use with substrates of chain length greater than C_{10}. Bartholomew *et al.* (1977) have already established the striking similarity between the S1 and CS2 secondary alkylsulphohydrolases, in terms of their mechanisms of action. Both catalyse the scission of the C–O bond of the C–O–S linkage and this scission is accompanied by inversion of configuration with the consequent production of the L-alcohol.

The finding that the electrophoretic preparation of the S2 enzyme of *Pseudomonas* C12B will hydrolyse L-octan-2-yl sulphate, but not the D-isomer, suggests

that it is analogous to the CS1 enzyme of *C. terrigena*. Collectively, the S1 and S2 enzymes could presumably initiate the degradation of both D- and L-forms of C-2 secondary alkyl sulphates present in commercial detergent mixtures. The enzymes are present in cell extracts in roughly equal amounts. The degradation of other components of a complex secondary alkyl sulphate detergent such as Oronite may be initiated by the inducible S3 enzyme of *Pseudomonas* C12B, as indicated by Matcham *et al.* (1977*a*).

In common with the CS2 enzyme of *C. terrigena*, the S1 enzyme is inhibited competitively by the L-isomer of its normal substrate. Although this is clearly a disadvantage from the biodegradation point of view, the fact that the K_i of the L-isomer is more than twice the value of K_m for the D-substrate would allow the reaction to proceed, even if the S2 enzyme was not also present.

In commenting on the mechanism of action of the CS2 enzyme of *C. terrigena*, Matcham *et al.* (1977*b*) indicated the necessity for invoking a three-point interaction between enzyme and substrates as a prerequisite for optical stereospecificity. They further suggested that the C-3 to C-*n* region of the alkyl chain of secondary alkyl sulphate substrates affects the rate of hydrolysis and must be involved in substrate binding. Moreover, the C-1 methyl group must interact with a high degree of specificity as neither primary alkyl sulphate nor D-secondary alkyl sulphates in which the position of sulphation is C-3 or greater are substrates for the enzyme. The third point of interaction was held to be the sulphate group itself, with the difference between the binding of the substrate and non-substrate isomers of octan-2-yl sulphate probably residing with the spatial orientation of the ester sulphate group. Similar comments might be applied to the S1 enzyme, and the contribution of the alkyl chain in this case is further stressed by the results of the studies on the inhibition by primary alkyl sulphates and primary alkanesulphonates. With both types of compound the affinity of inhibitor for enzyme increases with increasing chain length, behaviour that is typical of a homologous series. At a chain length of C_6 the K_i for the alkanesulphonate is almost 5 times that for the corresponding alkyl sulphate, suggesting that the sulphate group is making a very positive contribution to the binding of inhibitor to enzyme. However, as each homologous series is ascended, this positive contribution decreases in significance until, at a chain length of C_9, it is completely overshadowed by the contribution of the alkyl chain (Table 3, Fig. 3). It will clearly be important to extend these observations to compounds of greater chain length when appropriate assay methods become available, although it is possible that complications may emerge, because of the increasing ability to form surfactant micelles under the conditions of the experiment.

It is debatable as to what extent it is valid to relate results between different classes of compounds containing alkyl chains. Nevertheless, attention might be drawn to the K_i values for the inhibition of the S1 enzyme by L-octan-2-yl sulphate and hexan-1-yl sulphate. In each case a C_6 alkyl chain might be regarded as being adjacent to the ester sulphate group. The fact that the K_i for L-octan-2-yl sulphate is less than one-third that for hexan-1-yl sulphate may provide some support for the view outlined above that the C-1 methyl group may be important in the binding of substrate and inhibitor isomer to the enzyme.

From the biodegradation point of view, the powerful inhibition of the S1 enzyme by primary alkyl sulphates and primary alkanesulphonates might indicate that *Pseudomonas* C12B would encounter problems in initiating the degradation of secondary alkyl sulphate detergents in effluents containing primary alkyl sulphate and primary alkanesulphonate detergents. However, the organism also produces two primary alklysulphohydrolases, P1 in the stationary phase of growth and P2 (induced by alkyl sulphates) in the exponential phase. Moreover, the organism is capable of growing on alkanesulphonates as C and S source (Fitzgerald *et al.*, 1977), although this property has not yet been studied in any detail. The same situation does not occur in *C. terrigena*, which cannot produce primary alkylsulphohydrolases, nor can it grow on alkanesulphonates (Fitzgerald *et al.*, 1975). Its ability to participate in biodegradation processes must therefore be very limited.

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