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Substrate Specificity and other Properties of the Inducible S3 Secondary Alkylsulphohydrolase Purified from the Detergent-degrading Bacterium *Pseudomonas* C12B

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The inducible S3 secondary alkylsulphohydrolase of the soil bacterium *Pseudomonas* C12B was purified to homogeneity (683-fold from cell-free extracts by a combination of column chromatography on DEAE-cellulose, Sephadex G-100 and Blue Sepharose CL-6B. The enzyme has a molecular weight in the region of 40000–46000, and is active over a broad range of pH from 5 to 9, with maximum activity at pH 8.2. The preferred substrates of the enzyme are the symmetrical secondary alkylsulphate esters such as heptan-4-yl sulphate and nonan-5-yl sulphate and the asymmetric secondary octyl and nonyl sulphate esters with the sulphate group attached to C-3 or C-4. However, for each asymmetric ester, the L-isomer is much more readily hydrolysed than the D-isomer. This specificity is interpreted in terms of a three-point attachment of the substrate to the enzyme's active site. The alkyl chains on either side of the esterified carbon atom are bound in two separate sites, one of which can only accommodate alkyl chains of limited size. The third site binds the sulphate group. Enzymic hydrolysis of this group is accompanied by complete inversion of configuration at the asymmetric carbon atom. The implied cleavage of the C–O bond of the C–O–S ester linkage was confirmed by ^{18}O-incorporation studies.

Biodegradation of primary and secondary alkyl sulphate detergents by soil micro-organisms requires the initial removal of the sulphate group by primary and secondary alkylsulphohydrolases, and this is followed by the oxidation of the liberated alcohol (Williams & Payne, 1964; Payne *et al.*, 1967; Lijmbach & Brinkhuis, 1973). Efforts in these laboratories have focused on the alkylsulphohydrolases present in two soil micro-organisms, *Pseudomonas* C12B and *Comamonas terrigena*. Of these, the former is much the more versatile in its ability to produce various alkylsulphohydrolases. When grown on nutrient broth, it produces one primary and two secondary alkylsulphohydrolases, designated P1, S1 and S2. Enzymes S1 and S2 are specific for the D- and L-isomers respectively of C-2 secondary alkyl sulphate esters. When the broth is supplemented with primary alkyl sulphates, an additional primary enzyme (P2) is produced (Cloves *et al.*, 1980*a*), and the presence in the broth of Oronite (a commercial detergent containing a mixture of C_{10}–C_{20} secondary alkyl sulphates) or a mixture of secondary alkyl sulphates and secondary alcohols of appropriate chain length but all with the substitution at C-2 leads to the induced synthesis of both enzyme P2 and a further secondary enzyme, S3 (Dodgson *et al.*, 1974). In contrast, *C. terrigena* produces only two secondary alkylsulphohydrolases, CS1 and CS2, which in terms of substrate stereospecificity are analogous to *Pseudomonas* enzymes S2 and S1 respectively (Matcham *et al.*, 1977*a*). The organism cannot hydrolyse primary alkyl sulphate esters, nor have any further alkylsulphohydrolases, either primary or secondary, been induced experimentally.

Of these seven alkylsulphohydrolases, the CS2 secondary alkylsulphohydrolase of *C. terrigena* and the analogous S1 enzyme from *Pseudomonas* C12B, together with the inducible primary enzyme (P2), have all been purified to homogeneity and studied in some detail (Matcham *et al.*, 1977*b*; Bartholomew *et al.*, 1978; Cloves *et al.*, 1980*b*). However, preliminary experiments have suggested that, at least in terms of substrate specificity, the S3 enzyme may be the most intriguing of all. The preferred substrates of relatively crude preparations of enzyme S3 are the

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symmetrical or near-symmetrical alkyl sulphate esters such as heptan-4-yl, octan-4-yl and nonan-5-yl sulphates, which are hydrolysed to completion. However, if the $-OSO_3^-$ grouping is further removed from the centre of the carbon chain, the enzyme begins to show a preference for L- as opposed to D-isomers (Matcham *et al.*, 1977*a*). Since substrates for the S1 and S2 enzymes are restricted to C-2 alkyl sulphate esters, the apparently less stringent requirements of the S3 enzyme and its affinity for the more symmetrical substrates become of paramount importance to the organism if it is to degrade a mixed commercial detergent such as Oronite.

The present paper describes the purification of the S3 enzyme and provides further information on its general properties, substrate specificity, mechanism of action and relationship to the S1 and S2 enzymes.

Experimental

Materials

Primary and racemic secondary alcohols were purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A., Koch–Light Laboratories, Colnbrook, Bucks., U.K., and Fluka through Fluorochem, Glossop, Derbyshire, U.K. L-Octan-2-ol was resolved from the racemic alcohol as described by Matcham & Dodgson (1977).

Primary alkyl sulphate esters (kindly supplied by Mrs. J. M. Cloves of this Department) and 'tetradecan-2-yl sulphate' and 'decan-5-yl sulphate' were prepared by treatment of parent alcohol with H_2SO_4 in accordance with the method of Dodgson *et al.* (1974). Secondary alkyl sulphate esters prepared in this way are known to be heterogeneous in terms of the position of the sulphate group along the alkyl chain (Matcham & Dodgson, 1977). Quotation marks have therefore been used to draw attention to this point. Racemic and optically pure isomers of other secondary alkyl sulphate esters were prepared either by the use of pyridine–SO_3 complex (Matcham & Dodgson, 1977) or by a new method involving sulphation of sodium alkoxides by triethylamine–SO_3 complex as described in the Appendix (White *et al.*, 1980).

DEAE-cellulose (DE-52; Whatman Biochemicals, Maidstone, Kent, U.K.), Sephadex G-100 and Blue Sepharose CL-6B (Pharmacia, Uppsala, Sweden) were washed and equilibrated in accordance with the manufacturers' instructions before use.

The protein standards used for determination of molecular weight by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were obtained from Boehringer Mannheim, Lewes, Sussex, U.K. The standards for determinations by gel-filtration chromatography and polyacrylamide-gel electrophoresis under non-denaturing conditions were gifts from Dr. J. Kay of this Department, with the exception of cytochrome *c* (Boehringer Mannheim).

Water enriched with ^{18}O (62.5 atoms % excess) was purchased from British Oxygen Co. (Prochem), London S.W.19, U.K.

All other chemicals were the purest available from BDH Chemicals, Poole, Dorset, U.K., or Sigma (London) Chemical Co., Poole, Dorset, U.K.

Growth of Pseudomonas C12B

The organism, originally isolated from soil near a sewage outfall in Athens, GA, U.S.A., by Payne & Feisal (1963), was maintained on nutrient agar slopes. For the present work, cells were grown in a 400-litre-capacity New Brunswick Fermentor containing 350 litres of nutrient broth supplemented with 1.0mM-'tetradecan-2-yl sulphate' and 1.0mM-tetradecan-2-ol, a medium that is known to induce synthesis of the S3 enzyme (Dodgson *et al.*, 1974). The culture medium was aerated at 1.8 m^3 of air/h and agitated at 100rev./min. Cells were harvested at the stationary phase of growth by using a Sharples centrifuge and the wet cells were resuspended in 5mM-Tris/HCl buffer, pH 7.5 (1g of cells/ml), before continuous passage through a Manton–Gaulin laboratory homogenizer operating at 60MPa (type 158; Manton–Gaulin Manufacturing Co., Everett, MA, U.S.A.). The resulting suspension was freeze-dried and stored at −20°C until required. The yield was 87g.

Assay of alkylsulphohydrolase activity

Liberation of inorganic sulphate from nonan-5-yl sulphate was used for the routine assay of the S3 enzyme. Preliminary experiments with crude cell extracts indicated suitable assay conditions to be 15mM substrate concentration in 0.1M-Tris/HCl buffer, pH 7.5. Enzyme (suitably diluted) and substrates in a total volume of 0.2ml were incubated at 30°C for 5–20min, appropriate control incubations being run simultaneously. Liberated sulphate was subsequently determined by the $BaCl_2$/gelatin method of Dodgson (1961). A unit of enzyme activity is defined as that amount of enzyme which catalyses the release of 1μmol of SO_4^{2-} ions/min. When complete reaction progress curves were required, or when accurate initial rates of hydrolyses were needed for kinetic analysis, the volume of the incubation mixture was suitably increased and 0.2ml portions were withdrawn for sulphate determination at appropriate intervals. The concentrations of secondary alkyl sulphate substrates and of primary alkyl sulphate inhibitors were kept below their respective critical micelle concentrations. Values of critical micelle concentrations not obtainable from the literature were determined by the method of Carey & Small (1969).

Determination of protein and nucleic acid

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, or, for very dilute solutions, by the spectrophotometric method of Waddell (1956). Nucleic acid was determined as a percentage of total u.v.-absorbing material by the method of Layne (1957).

Polyacrylamide-gel electrophoresis

Gel electrophoresis was performed routinely at each stage of the purification under the non-denaturing conditions described by Payne *et al.* (1974). S3 alkylsulphohydrolase activity was detected by incubation of gels at 30°C in a solution containing 15mM-potassium nonan-5-yl sulphate and 0.1M-Tris/HCl buffer, pH 7.5. Enzymic hydrolysis of the ester results in the formation in the gel of a white band consisting of insoluble alcohol droplets. Protein bands were stained with Brilliant Blue G-250 (Diezel *et al.*, 1972) and located by using a Gilford 250 photometer fitted with a gel-scanning attachment, operated at 650nm (Gilford Instruments, Teddington, Middlesex, U.K.).

Enzyme purification

All operations in the enzyme purification were performed at 0–4°C.

Stage 1. Freeze-dried broken cells (20g) were suspended in 160ml of 10mM-Tris/HCl buffer, pH 7.5. The rupture of the cells was completed by three passages through a chilled French pressure cell operating at 136MPa. Debris was removed by centrifugation at $30000 g_{av.}$ for 50min (MSE High Speed 18, 8 × 50ml fixed-angle rotor) and the supernatant was dialysed overnight against two changes of 2 litres of the suspending buffer.

Stage 2. Nucleic acid was precipitated from the stage 1 supernatant by slow addition of 3g of streptomycin sulphate/100ml of stirred solution. The resulting suspension was left for 90min before subjection to centrifuging and dialysis as described in stage 1. The precipitate of nucleic acid that appeared during dialysis was removed by further centrifugation.

Stage 3. The stage 2 material (147ml) was applied to a column (4.2cm × 34cm) of DEAE-cellulose (DE-52) previously equilibrated with 10mM-Tris/HCl buffer, pH 7.5. The sample was washed into the column with 0.5 litre of this buffer and eluted with a linear gradient of NaCl (0–0.15M in 2 litres) in buffer. Fractions (12.7ml) were collected automatically, and assayed for S3 sulphohydrolase activity and protein. A typical elution profile is shown in Fig. 1(*a*). Fractions 60–82 inclusive were pooled and dialysed as above.

Stage 4. A column (2.2cm × 80cm) of Sephadex G-100 (superfine grade) was equilibrated with 0.1M-Tris/HCl buffer, pH 7.5, and a 1.5cm band of DEAE-cellulose suspended in the same buffer was layered on the gel bed. The dialysed material from stage 3 (292ml) was applied to this column, the DEAE-cellulose layer serving to concentrate the protein sample into a sharp band (Miller *et al.*, 1976). This band (which was visible, owing to the presence of pigmented protein) was washed into the gel bed with buffer containing 0.3M-NaCl. Elution was continued with 300ml of salt-free buffer. Fractions (3.7ml) were collected automatically and assayed, and fractions 46–52 (see Fig. 1*b*) were pooled for the next stage.

Stage 5. The pooled fractions were dialysed against two 1-litre changes of 10mM-piperazine/HCl buffer, pH 5.5, and applied to a column (2cm × 11.5cm) of Blue Sepharose CL-6B previously equilibrated with the same buffer. The column was eluted with 180ml of this buffer followed by 120ml of Tris/glycine buffer, pH 8.9 (3g of Tris and 2.6g of glycine per litre). Fractions (8.8ml) were collected automatically and assayed for protein content and S3 enzyme activity. The active fractions (21–25 inclusive; see Fig. 1*c*) were pooled and dialysed against two 1-litre changes of 10mM-Tris/HCl buffer, pH 7.5.

Stage 6. Gel-filtration chromatography was repeated as described for stage 4, except that 4ml fractions were collected and numbers 44–50 pooled. This material, stored at 0–4°C, constitutes the final preparation of enzyme used in the experiments described below.

Estimation of molecular weight

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by the method of Weber & Osborn (1969). A loading of 10μg of pure enzyme was used, and cytochrome *c* (mol.wt. 11700), pepsin (pig stomach mucosa, mol.wt. 35000), albumin (bovine serum, mol.wt. 68000) and ovalbumin (hens-egg, mol.wt. 43000) were employed as standards. The molecular weight of the native enzyme was estimated by gel filtration of a sample of pure enzyme (10μg) on a 1.4cm × 48cm column of Sephadex G-100 equilibrated in 0.1M-Tris/HCl buffer, pH 7.5. The column was calibrated with $K_2Cr_2O_7$ (mol.wt. 294), cytochrome *c*, soya-bean trypsin inhibitor (mol.wt. 22000), pepsinogen (mol.wt. 40000), bovine serum albumin and Blue Dextran (mol.wt. 2×10^6). Electrophoresis in polyacrylamide gels of various concentrations (3, 6, 7, 8, 9, 10, 11 and 12%) was also used to estimate the molecular weight of the intact enzyme (Hedrick & Smith, 1968). Gels were each run with soya-bean trypsin inhibitor, pepsinogen and bovine serum albumin as standards, and with 6μg of the pure enzyme. Protein bands were located with Brilliant Blue G-250 as described by Diezel *et al.* (1972).

Isotope-incorporation experiments

Incorporation of ^{18}O from solvent into one or other of the products of the reaction catalysed by the S3 enzyme was examined. A solution containing 18μmol of potassium L-octan-2-yl sulphate, 0.3 ml of $H_2{}^{18}O$ (62.5 atoms % excess), 0.3 ml of 0.2 M-Tris/HCl buffer, pH 8.0, and 0.6 ml of purified S3 enzyme (activity towards nonan-5-yl sulphate, 3.8 units/ml) was incubated at 30°C for 3 h. Liberated octan-2-ol was extracted into diethyl ether (2 × 1.2 ml) and analysed for ^{18}O content by g.l.c.-mass spectrometry as previously described (Bartholomew *et al.*, 1977). The SO_4^{2-} ions remaining in the aqueous layer after ether extraction were precipitated as $BaSO_4$ and analysed for ^{18}O content by a modification (Tudball & Thomas, 1972) of the i.r. method devised originally by Spencer (1959).

Polarimetry

The stereochemistry of catalysis by the S3 enzyme was established by measuring the optical rotation of the alcohol released during the hydrolysis of L-octan-2-yl sulphate. The potassium salt of the ester (100μmol) was dissolved in 9 ml of 50 mM-Tris/HCl buffer, pH 8.0, and mixed with 1 ml of purified S3 enzyme (0.75 unit/ml when assayed with nonan-5-yl sulphate). The solution was incubated at 30°C for 24 h, by which time 65% of the substrate had been hydrolysed. The liberated alcohol was extracted into diethyl ether (3 × 10 ml), and the ether extracts were pooled and evaporated in a stream of air. The residue of octan-2-ol was dissolved in 1.2 ml of ethanol and the optical rotation at 546 nm was determined in an NPL type 243 automatic polarimeter (Thorn Automation, Nottingham, U.K.) with a 1 cm path length. To allow calculation of specific rotations, the exact concentration of octan-2-ol in the solution was determined by g.l.c. analysis.

Results

Enzyme purification

The purification procedure for the S3 enzyme is summarized in Table 1. During the development of the procedure several other approaches were tested, and abandoned for various reasons. These included precipitation of protein with $(NH_4)_2SO_4$ or by adjustment of the pH, and hydrophobic chromatography.

Nucleic acid concentration in crude cell extracts was successfully decreased from 25% to 2.8%, without significant loss of enzyme activity, by treatment with streptomycin sulphate. This simple method has thus proved to be of particular value in removing nucleic acid during the purification of alkylsulphohydrolases from *Pseudomonas* C12B (Bartholomew *et al.*, 1978; Cloves *et al.*, 1980*b*). Elution of the enzyme with a salt gradient from DEAE-cellulose (Fig. 1*a*) achieved a 55-fold purification with small loss of activity. After gel filtration on Sephadex G-100 (Fig. 1*b*), the sample was adsorbed at low pH on Blue Sepharose CL-6B. The activity was released from the gel by raising the pH of the eluent buffer (Fig. 1*c*). Experiments with DEAE-cellulose and SP-cellulose eliminated the possibility of the phenomenon being a simple ion-exchange. The recovery of S3 enzyme from Blue Sepharose, which was relatively low compared to that achieved with the other purification steps, was not merely the result of discarding side-fractions. The reason for the less-than-quantitative recovery is unknown. It has been proposed (Thompson *et al.*, 1975) that affinity for Cibacron Blue F3G-A (the affinity ligand of Blue Sepharose CL-6B) indicates the presence in an enzyme of the so-called 'di-nucleotide-binding fold,' which is a region of super-secondary structure found in enzymes with nucleotide cofactors, and in some without (e.g. the bacterial extracellular proteinase subtilisin). With the S3 alkylsulphohydrolase, the further possibility

Table 1. *Purification of the S3 secondary alkylsulphohydrolase from Pseudomonas C12B*
See the text for definition of the unit of activity and for experimental details.

Purification stage	Protein (mg)	S3 enzyme activity (units)	S3 enzyme activity (% yield)	S3 specific activity (units/mg)	Purification
1. Cell-free extract	3016	548.2	100	0.182	1
2. Streptomycin sulphate	2234	524.1	96	0.235	1.3
3. DEAE-cellulose	35.33	462.5	84	13.09	72
4. Sephadex G-100	12.09	356.1	65	29.56	162
5. Blue Sepharose CL-6B	3.71	151.9	28	41.00	225
6. Sephadex G-100	0.92	114.2	21	124.3	683

exists that interaction may occur between the enzyme and the complex arenesulphonate groups of the affinity ligand. In this context it is noteworthy that the enzyme, when bound at low pH, can also be eluted by solutions of its substrate, nonan-5-yl sulphate.

At this stage, polyacrylamide-gel electrophoresis revealed the presence of two protein bands, only one of which possessed S3 alkylsulphohydrolase activity. Re-chromatography on Sephadex G-100 gave a peak of enzyme activity coincident with an identical peak of protein. Polyacrylamide-gel electrophoresis of this material gave a single major protein band with impurities collectively accounting for less than 5% of the total stained protein. The protein band was coincident with the S3 alkylsulphohydrolase activity revealed by incubation with nonan-5-yl sulphate. Furthermore, incubation in 'decan-5-yl sulphate' (prepared by using H_2SO_4 and containing decan-2-yl sulphate; see Matcham & Dodgson, 1977) showed the absence of the other secondary alkylsulphohydrolases (S1 and S2) of *Pseudomonas* C12B. The purified enzyme was also devoid of primary alkylsulphohydrolase activity. The final preparation, containing 4.1 units/ml, at a specific activity of 124.3 units/mg of protein, represented an overall purification from the crude extract of 683-fold.

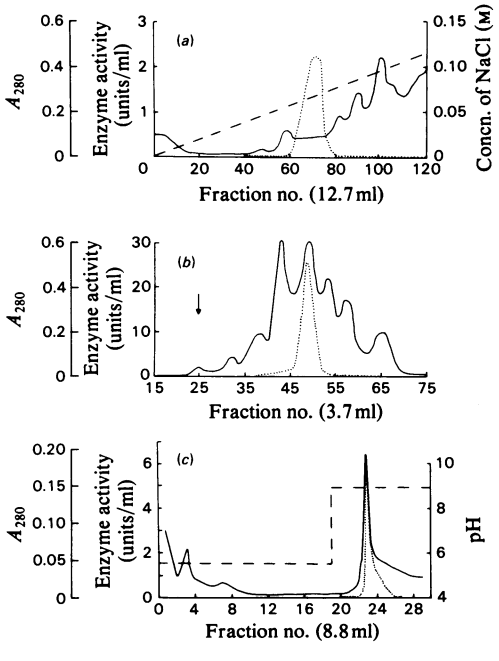

Fig. 1. *Elution profiles for S3 alkylsulphohydrolase from DEAE-cellulose, Sephadex G-100 and Blue Sepharose CL-6B*

(*a*) Elution from DEAE-cellulose (DE-52) at stage 3 of the purification procedure; (*b*) elution from Sephadex G-100 at stage 4 (arrow indicates the void volume); (*c*) elution from Blue Sepharose CL-6B. See the text for details. ·····, S3 enzyme activity; ——, protein; – – – –, eluent concentration or pH.

Molecular-weight determinations

The molecular weight of the S3 alkylsulphohydrolase was 40000 as measured by Sephadex G-100 chromatography, and 46000 by electrophoresis in various concentrations of polyacrylamide gel under non-denaturing conditions. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed an intense band corresponding to a molecular weight of 13000, with two contaminating bands corresponding to molecular weights of 17000 and 49000. Interpretation of these results must take into account the fact that the S3 alkylsulphohydrolase is a detergent-degrading enzyme and, as such, its interaction with the primary alkyl sulphate, sodium dodecyl sulphate, may be abnormal. Because anomalous behaviour of this type could lead to unreliable estimates of molecular weight, the subunit structure of the S3 enzyme remains uncertain.

General properties and substrate specificity of the S3 enzyme

The effect of pH on the hydrolysis of heptan-4-yl sulphate and nonan-5-yl sulphate (both 15mM) by the pure S3 enzyme is shown in Fig. 2. Maximum activity occurred at pH 8.2, but the enzyme was capable of acting efficiently over a broad range of pH from 5 to 9. The general form of the pH–activity

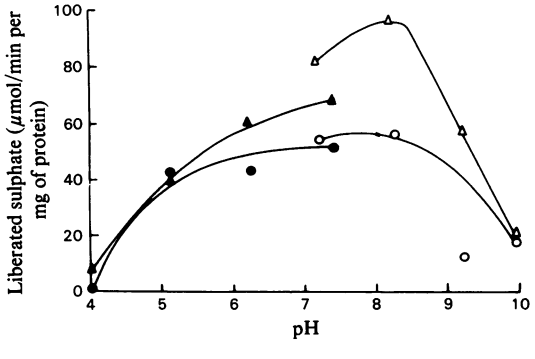

Fig. 2. *pH–enzyme activity curve for the S3 enzyme*

Activity was assayed by the normal procedure (15mM substrate concentration) except for changes in the buffers used. See the text for details. ▲, Nonan-5-yl sulphate in 0.1M-citric acid/KOH buffer; △, nonan-5-yl sulphate in 0.1M-Tris/HCl buffer; ●, heptan-4-yl sulphate in 0.1M-citric acid/KOH buffer; ○, heptan-4-yl sulphate in 0.1M-Tris/HCl buffer.

curve was unchanged by using 8mM or 40mM concentrations of heptan-4-yl sulphate as substrate.

Enzyme activity–substrate concentration curves for the hydrolysis of the symmetrical alkyl sulphate esters, pentan-3-yl sulphate, heptan-4-yl sulphate and nonan-5-yl sulphate (examples of which are shown in Fig. 3), conformed to the Michaelis–Menten pattern, except that with nonan-5-yl sulphate enzyme inhibition occurred at substrate concentrations above 15mM. Values of K_m and $V_{max.}$ obtained from plots of [S]/v versus [S] are collected in Table 2. K_m values increased markedly as the length of the carbon chain decreased. Changes in $V_{max.}$ were less regular, with the values for C_9 and C_7 substrates being equal, and 20 times that for C_5 substrate. The pure enzyme was also capable of releasing inorganic sulphate from undecan-6-yl sulphate. However, accurate kinetic values were unobtainable because residual substrate interfered with the $BaCl_2$/gelatin assay method.

In addition to these symmetrical compounds, a number of asymmetric esters were tested for their ability to serve as substrates for the S3 enzyme. Fig. 4 shows the reaction progress curves for several secondary alkyl sulphate esters, each at 3mM

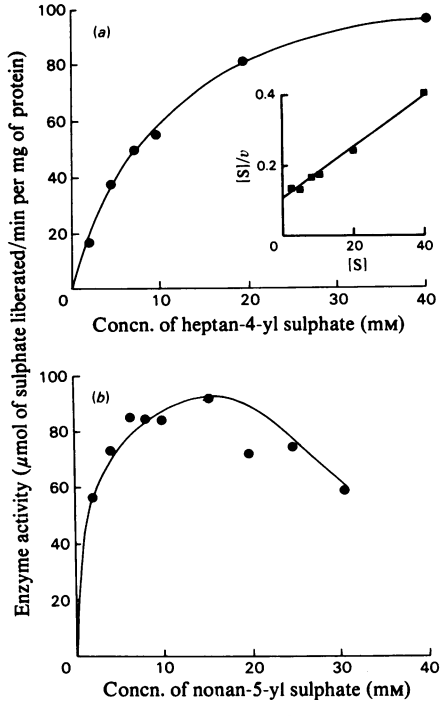

Fig. 3. *S3 enzyme activity–substrate concentration curves for the hydrolysis of symmetrical substrates*
(*a*) Heptan-4-yl sulphate. Inset: plot of [S]/v versus [S] where [S] is substrate concentration and v is the enzyme activity, both expressed in the units quoted on the major axes; (*b*) nonan-5-yl sulphate. See the text for experimental details.

Table 2. *Kinetic constants for hydrolysis of symmetrical secondary alkyl sulphate esters by the S3 enzyme*
Experiments were performed in 0.1M-Tris/HCl buffer, pH 7.5. See the text for further details.

Substrate	K_m (mM)	$V_{max.}$ (μmol/min per mg of protein)
Pentan-3-yl sulphate	40	7.4
Heptan-4-yl sulphate	15	169
Nonan-5-yl sulphate	1.5	169

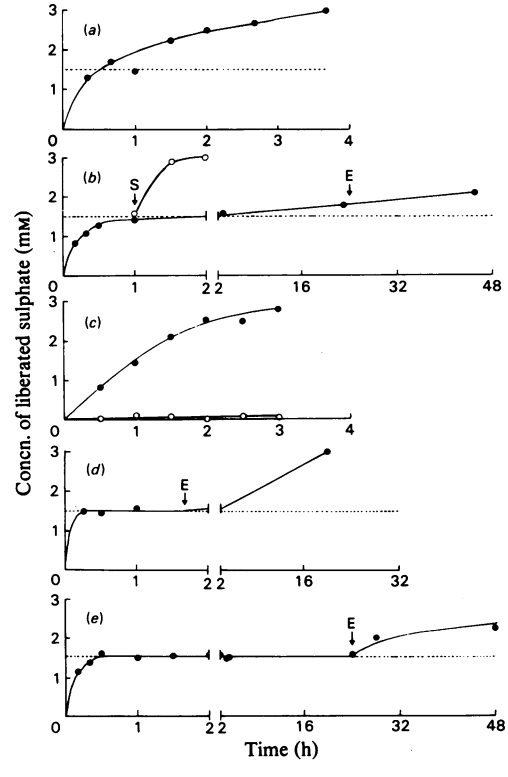

Fig. 4. *Reaction progress curves for the hydrolysis of asymmetric alkyl sulphate esters catalysed by the S3 enzyme*
Incubation mixtures (1.0–1.2ml) contained 0.1M-Tris/HCl buffer, pH 7.5, and: (*a*) 3mM-DL-octan-4-yl sulphate; (*b*) ●, 3mM-DL-octan-3-yl sulphate; O, 3mM-DL-octan-3-yl sulphate, raised at 'S' by a further 3mM; (*c*) ●, 3mM-L-octan-2-yl sulphate; O, 3mM-D-octan-2-yl sulphate; (*d*) 3mM-DL-nonan-4-yl sulphate; (*e*) 3mM-DL-nonan-3-yl sulphate. The final enzyme concentration was 0.075 unit/ml, except for (*c*), for which it was 0.375 unit/ml. At points marked 'E', the total enzyme concentration was doubled to 0.15 unit/ml by the addition of a small volume of concentrated enzyme solution.

concentration. The near-symmetrical DL-octan-4-yl sulphate was a good substrate, and the racemic mixture was hydrolysed to completion by the S3 enzyme (Fig. 4*a*), implying roughly comparable activities towards both D- and L-isomers. However, with DL-octan-3-yl sulphate (Fig. 4*b*) 50% of the ester was hydrolysed rapidly and the remainder at a much lower relative rate. The premature termination of hydrolysis at 50% was not due to inactivation or inhibition of the enzyme, as addition of a further quantity of substrate at the end point of the initial reaction ('S' in Fig. 4*b*) resulted in a further release of sulphate, equivalent to one-half of the amount of racemic ester added. On the other hand, addition of fresh enzyme (at 'E' in Fig. 4*b*) to a 24h incubation mixture, in which hydrolysis had already progressed to 50%, failed to produce more than a slight acceleration of sulphate release (i.e. no more than the doubling expected for a doubling of enzyme concentration). These observations suggested that 50% of the DL-octan-3-yl sulphate can serve as a good substrate for the S3 enzyme, the remaining 50% being much inferior. By analogy with the established specificities of the S1 and S2 secondary alkylsulphohydrolases for D- and L-isomers of C-2 esters, the most obvious explanation for this phenomenon is that the S3 enzyme exhibits a preference for one enantiomer over the other. Unfortunately, resolved D- and L-isomers of octan-3-yl sulphate were not available to test this explanation. However, resolved isomers of octan-2-yl sulphate were readily available, and these were tested for their susceptibility to hydrolysis by the S3 enzyme (Fig. 4*c*). When D-octan-2-yl sulphate was treated with the enzyme, no sulphate was liberated, whereas the L-isomer was completely hydrolysed, albeit at a relatively low rate.

Both DL-nonan-4-yl sulphate and DL-nonan-3-yl sulphate were also hydrolysed in the presence of the pure S3 alkylsulphohydrolase in two distinct phases: an initial rapid hydrolysis to 50% followed by a several-hundred-fold slower hydrolysis of the remainder (Figs. 4*d* and 4*e*).

None of the primary alkyl sulphate esters from C_2 to C_{10} was hydrolysed by the purified S3 enzyme.

Inhibition by primary alkyl sulphate esters

By using nonan-5-yl sulphate as substrate in the concentration range 1–16mM to avoid the complication of inhibition by excess substrate, each member of the homologous series of primary alkyl sulphate esters (potassium salts) from ethyl sulphate (C_2) to decyl sulphate (C_{10}) was tested for its ability to inhibit the S3 enzyme. The higher members of this series (C_8–C_{10}) produced non-competitive inhibitory effects in which decrease of $V_{max.}$ in the presence of inhibitors was not reversed by raising the substrate concentration. That the inhibition was also closely dependent on chain length is reflected in the estimated K_I values for octyl sulphate, nonyl sulphate and decyl sulphate of 43, 4.8 and 2.4mM respectively. The first of these values must be viewed with some caution, since inhibition by the K^+ counter-ion becomes a significant factor at the concentrations of potassium octyl sulphate required to produce a measurable effect.

Of the remaining primary esters from C_2 to C_7, only butyl sulphate (C_4) showed any significant inhibition. In this case the inhibition was essentially competitive, with K_I 4mM. Potassium salts of propyl sulphate (C_3) and pentyl sulphate (C_5) showed a slight tendency to inhibit, but, again, the contribution of K^+ ions was probably significant.

Polarimetry and incorporation of ^{18}O during enzymic hydrolysis

As mentioned above, the L-octan-2-yl sulphate was hydrolysed by the S3 enzyme, thus affording an opportunity to examine the stereochemistry of the enzymic hydrolysis. The specific rotation of the octan-2-ol recovered from incubation mixtures containing S3 enzyme and L-octan-2-yl sulphate was $+9.6 \pm 1.6°$ (*c* 3mg/ml in ethanol). The specific rotation, $[\alpha]^{20}_{546}$, of a standard solution of D-octan-2-ol under equivalent conditions was $+11.2 \pm 1.2°$. Allowing for experimental error, these results show that the hydrolysis proceeds with complete Walden inversion of configuration at C-2 of this particular alkyl sulphate ester. Such an inversion implies that the C–O, as opposed to the S–O, bond of the C–O–S ester linkage is being broken during enzymic hydrolysis. Hydrolysis in a solution enriched with $H_2{}^{18}O$ should then lead to incorporation of ^{18}O exclusively into the alcohol. The results of such an experiment with L-octan-2-yl sulphate are shown in Table 3. The close agreement of the experimental results with values predicted for C–O cleavage add strong support to the conclusions drawn from polarimetry measurements, namely that the S3 enzyme catalyses a C–O bond cleavage with a concomitant inversion of configuration of the carbon atom.

Table 3. *Incorporation of ^{18}O into the reaction products of the S3 secondary alkylsulphohydrolase of Pseudomonas C12B*

	^{18}O (atoms % excess)	
	L-Octan-2-ol	SO_4^{2-}
Calculated for C–O split	15.6	0
Calculated for S–O split	0	3.9
Found	15.9	0

Discussion

The purification procedure described in the present paper has enabled the S3 secondary alkylsulphohydrolase to be purified 683-fold from crude cell extracts. Interestingly this value, like that obtained for the purification to homogeneity of the P2 inducible primary alkylsulphohydrolase of *Pseudomonas* C12B (237-fold), is approximately an order of magnitude lower than the purification factors of 5150 and 4290 achieved for the constitutive enzymes S1 (from *Pseudomonas* C12B; Bartholomew *et al.*, 1978) and CS2 (from *C. terrigena*; Matcham *et al.*, 1977*b*) respectively. The values presumably reflect the high activities attained within the cell for inducible as opposed to constitutive enzymes.

This similarity of S3 and P2 alkylsulphohydrolases does not, however, extend to their general properties. The S3 enzyme (mol.wt. 40000–46000) is much smaller than the P2 enzyme (mol.wt. 162000; Cloves *et al.*, 1980*b*) and P1 and S1 enzymes (both in excess of mol.wt. 250000; J. M. Cloves, unpublished work). The relative insensitivity of the activity of the S3 enzyme to pH over a broad range (Fig. 3) is reminiscent of other secondary alkylsulphohydrolases (Bartholomew *et al.*, 1978; Matcham *et al.*, 1977*b*), and contrasts with the behaviour of the P2 enzyme, which has a narrower pH–activity profile with a sharp fall in activity above pH 8.7 (Cloves *et al.*, 1980*b*).

Purification of the S3 alkylsulphohydrolase has allowed some progress to be made towards an understanding of its substrate specificity. The preferred substrates of the pure enzyme appear to be the symmetrical alkyl sulphate esters such as heptan-4-yl and nonan-5-yl sulphates, and the asymmetrical octyl sulphates and nonyl sulphates with substitution at C-3 or C-4. However, when the more asymmetric substrates are used, hydrolysis of the racemic compounds occurs on two distinct time scales. One-half of the racemic mixture is hydrolysed relatively quickly, and the remainder at a much lower rate. The controlling factor in determining the relative rates of the two stages appears to be the remoteness of the $-OSO_3^-$ group from the centre of the carbon chain. Thus the near-symmetric ester DL-octan-4-yl sulphate, in which the alkyl chains on either side of the esterified carbon atom differ by only one carbon atom, is hydrolysed to completion, although there is a suggestion of a discontinuity at 50% hydrolysis (Fig. 4*a*). DL-Nonan-4-yl sulphate and DL-octan-3-yl sulphate, with alkyl chains differing by two and three atoms respectively, are each hydrolysed in two stages, with the later stage at least two orders of magnitude slower than the first. DL-Nonan-3-yl sulphate (alkyl chains differing by four carbon atoms) exhibits rates differing by three orders of magnitude, and finally only 50% of DL-octan-2-yl sulphate (alkyl chains differing by five carbon atoms) is hydrolysed at all. In this last case, experiments with pure isomers show that L-octan-2-yl sulphate is the substrate for the enzyme, the D-isomer being unaffected. By analogy, it is presumably the L-isomer of each of the other asymmetric esters that undergoes rapid hydrolysis, and the D-isomer that is less favoured. Unfortunately, current unavailability of the resolved enantiomers precludes direct experimental confirmation.

Attempts at interpretation of these findings in terms of a detailed mechanism would be premature at the present time. Nevertheless a tentative scheme can usefully be formulated as a basis for future work. As pointed out previously (Matcham *et al.*, 1977*b*), a prerequisite for optical stereospecificity is a three-point interaction between enzyme and substrate. The total inactivity of the S3 alkylsulphohydrolase towards primary alkyl sulphates, together with its high activity towards symmetrical secondary esters, strongly suggest that two alkyl chains, one on either side of the esterified carbon atom of the symmetrical alkyl sulphates, must be bound to the enzyme for hydrolysis to occur. These two sites then constitute two of the points of interaction. The third must surely be the sulphate group itself. In the asymmetric esters, the alkyl chains attached to the $>CH-OSO_3^-$ group are of different lengths. Of the two possible enantiomers, only the L-form appears to bind well and with suitable orientation of the sulphate group to allow for its efficient detachment from the carbon chain. The D-isomer either cannot bind or, if it does so, then suitable juxtaposition of the sulphate group is not achieved. This in turn implies that the active site of the enzyme is asymmetric insofar as the hydrophobic sites for binding the alkyl chains are different, possibly one accommodating short chains, the other binding longer chains. The enzyme–substrate complexes for a symmetric, and the L-isomer of an asymmetric, alkyl sulphate might then be visualized as shown in Figs. 5(*a*) and 5(*b*) respectively. If the D-isomer binds with its alkyl chains in the same orientation as the L-isomer, the resulting complex would be inactive because the sulphate group does not occupy the site at which catalytic hydrolysis occurs (Fig. 5*c*). Proper positioning of the sulphate group can only be achieved by rotating the molecule, thereby reversing the positions of the alkyl chains (Fig. 5*d*). Because the preferences of the alkyl-chain-binding sites are then not satisfied, this arrangement would not be particularly favourable, and only weak activity might be expected. The possibility of hydrolysis of D-isomers via a complex in which the sulphate group and only one alkyl chain are in preferred sites (Fig. 5*e*) is denied by the fact that primary alkyl sulphate esters (which would bind

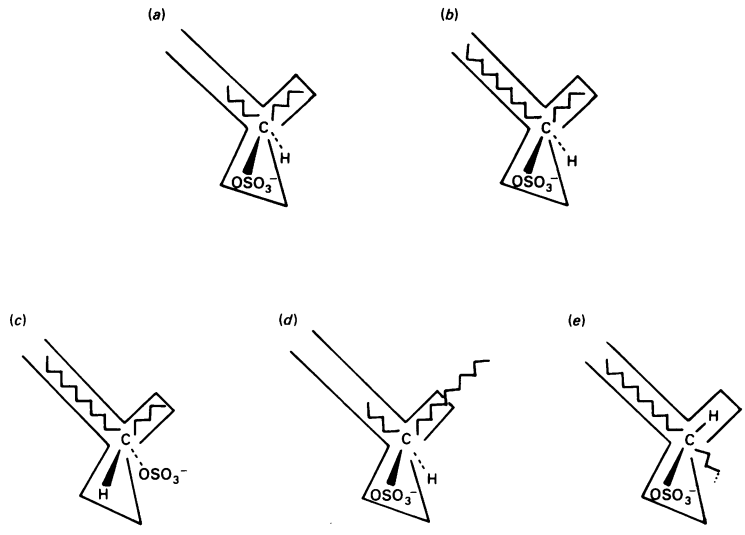

Fig. 5. *Proposed model of substrate binding to the S3 enzyme*

(*a*) Binding of a symmetrical alkyl sulphate ester with the two alkyl chains in separate sites and the sulphate group in the third site, leading to an active enzyme–substrate complex; (*b*) binding of the L-isomer of an asymmetric ester with the short and the long alkyl chains in their respective sites and the sulphate group suitably positioned in its site for hydrolysis to occur; (*c*) binding of the D-isomer with the preferred alignment of alkyl chains, but the sulphate group forced away from its site; (*d*) binding of the D-isomer after a rotation that, although bringing the sulphate group into the proper position, results in misalignment of the alkyl chains with their binding sites; (*e*) binding of the D-isomer with only one alkyl chain bound.

in an analogous way) are not hydrolysed. The absence of inhibition by C_2–C_7 primary alkyl sulphates with the exception of butyl sulphate may reflect strict chain-length requirements of the hydrophobic site with affinity for smaller alkyl chains. It is perhaps relevant that space-filling models show that the C_4 primary ester butyl sulphate closely resembles the CH_3–$[CH_2]_3$–CH–OSO_3^- group of the substrate, nonan-5-yl sulphate. The notion that the inhibition by C_8–C_{10} primary alkyl sulphates results from binding at the hydrophobic site with high affinity for longer alkyl chains is attractive, but must be tempered with the knowledge that at these chain lengths alkyl sulphates begin to develop detergent properties and as such almost certainly bind non-specifically to other parts of the protein. Indeed, this factor is probably responsible for the non-competitive kinetics observed, and may also be related to the substrate inhibition occurring in the presence of excess of nonan-5-yl sulphate (Fig. 3).

Clearly, further experimentation is necessary to verify this model for the substrate specificity of the S3 enzyme, and to elaborate the details. It is obvious from Fig. 4 that D-isomers of some secondary alkyl sulphate esters, however unsuitable, do serve as substrates, and must therefore be able to bind to the enzyme. Consequently the kinetics of hydrolyses performed with racemic compounds are necessarily complicated by the mutual competitive inhibition existing between each pair of enantiomers. Further experiments must therefore await the resolution of D- and L-isomers of the various asymmetric esters. An alternative approach to the problem, avoiding this diversion, would be the use of symmetrical alkyl sulphates with longer chain lengths (e.g. undecan-6-yl sulphate and tridecan-7-yl sulphate), which may help to define the chain-length requirements of the hydrophobic sites. At present, little progress can be made in this direction owing to the interference of these compounds with the $BaCl_2$/gelatin assay of liberated sulphate.

Regardless of the mechanism underlying the substrate specificity, some important practical considerations emerge from the present work. *Pseudomonas* C12B produces up to three secondary alkylsulphohydrolases. Of these enzymes, S2 and S3 have overlapping specificities because both hydrolyse L-enantiomers of C-2 alkyl sulphates. Consequently, if enzyme S2 is under study, absence of the S3 enzyme (or at least an allowance for its contribution) must be established by assaying for it specifically with nonan-5-yl sulphate. If S3 enzyme activity is being examined, nonan-5-yl sulphate is the substrate of choice, and the presence or absence of enzyme S2 can only be established by the polyacrylamide-gel-electrophoresis zymogram method.

Like other stereospecific secondary alkylsulphohydrolases, the S3 enzyme when acting on L-isomers

of secondary alkyl sulphates catalyses the cleavage of the C–O bond of the C–O–S ester linkage, with accompanying inversion of configuration at the asymmetric carbon atom. With the symmetrical esters the question of inversion of configuration does not arise, but presumably here, too, C–O cleavage occurs. This mechanism is proving to be a common feature of both primary (Cloves *et al.*, 1977) and secondary (Bartholomew *et al.*, 1977) alkylsulphohydrolases in the detergent-degrading bacteria, *Pseudomonas* C12B and *C. terrigena*, and contrasts sharply with the arylsulphohydrolases where, in all cases examined, S–O cleavage is the rule (Spencer, 1958, 1959; Cloves *et al.*, 1977). Such divergence in behaviour must surely arise from fundamental differences in the mechanism of action of the two types of sulphohydrolase, although what these differences may be must, for the time, remain a mystery.

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