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Purification, Properties and Cellular Localization of the Stereospecific CS2 Secondary Alkylsulphohydrolase of *Comamonas terrigena*

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The availability of homogeneous samples of the potassium salts of L- and D-octan-2-yl sulphate has enabled the separation of the optically stereospecific CS1 and CS2 secondary alkylsulphohydrolases from extracts of cells of *Comamonas terrigena*. The CS2 enzyme was purified to homogeneity, and an initial study was made of its general properties, specificity, cellular localization and relationship to the CS1 enzyme. The CS2 enzyme has a molecular weight of approx. 250000 and a subunit size of approx. 58000, indicating that the molecule is a tetramer. Under the experimental conditions used the enzyme appears to be specific for (+)-secondary alkyl sulphate esters with the sulphate group at C-2 and with a chain length of at least six carbons. Enzyme activity towards racemic C-2 sulphates increases with increasing chain length up to C_{10}, and there is some indirect evidence to suggest that activity declines when that chain length is exceeded. Other indirect evidence confirms that the CS1 enzyme exhibits similar specificity, except that only (−)-isomers can serve as substrates. Both enzymes are present in broth-grown stationary-phase cells of *C. terrigena* in approximately equal amounts.

The biodegradation of primary and secondary alkyl sulphate detergents by micro-organisms appears to involve the enzymic removal of the ester sulphate group by primary or secondary alkylsulphohydrolase enzymes, followed by the oxidation of the liberated primary or secondary alcohols (Williams & Payne, 1964; Payne *et al.*, 1967; Lijmbach & Brickjuis, 1973). Studies on these alkylsulphohydrolase enzymes in the Cardiff laboratories have concentrated on those present in two soil micro-organisms, *Pseudomonas* C12B and *Comamonas terrigena* (Dodgson *et al.*, 1974; Fitzgerald *et al.*, 1975; Bartholomew *et al.*, 1977; Matcham *et al.*, 1977). The former organism can produce five such enzymes, two of which use primary alkyl sulphates as substrates and the remaining three act on secondary alkyl sulphates. In contrast, *C. terrigena* appears to be unable to produce primary alkylsulphohydrolases, but two secondary alkylsulphohydrolases are always present in cells grown in nutrient broth to the stationary phase. Earlier studies (Fitzgerald *et al.*, 1975) on these two enzymes suggested that one of them (designated enzyme CS2) is present in greater amounts than the other (designated enzyme CS1).

The present paper describes the purification of the CS2 enzyme and presents further information about its general properties, specificity, cellular localization and relationship to the CS1 enzyme.

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Materials and Methods

Unless stated otherwise, all chemicals were the purest available from the Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. or BDH Chemicals, Poole, Dorset, U.K.

Preparation of secondary alkyl sulphates

Secondary alkyl sulphates were prepared by sulphation of the parent alcohol with pyridine/SO_3 reagent as described by Matcham & Dodgson (1977). In a few cases (designated in the text) they were prepared by the H_2SO_4 or chlorosulphonic acid methods described by Dodgson *et al.* (1974).

Purification of the CS2 alkylsulphohydrolase

The CS2 enzyme was isolated from extracts of cells of *C. terrigena* grown to the stationary phase (see Fitzgerald *et al.*, 1975) in cultures of either 350 litres or 35 litres.

Cultures of 350 *litres.* The organism was grown at 30°C in 350 litres of nutrient broth in a 400-litre capacity New Brunswick Fermentor aerated at 1.8 m^3/h and agitated at 100 rev./min. Cells were harvested in a Sharples centrifuge (flow rate 90

litres/h, 4h duration), commencing 6h after reaching the stationary phase. The wet cells (yield, 1.2kg) were resuspended in 5mM-Tris/HCl buffer, pH7.5 (1g/ml) and ruptured by continuous passage through a Manton–Gaulin laboratory homogenizer operating at 60MPa (type 15M8 TBA, Manton–Gaulin Manufacturing Co., Everett, MA, U.S.A.). The broken-cell suspension (2.5 litres) was freeze-dried and the residual powder stored at −20°C until required.

Cultures of 35 litres. Two sterile 17.5-litre nutrient-broth cultures, contained in suitably adapted 20-litre carboys, were maintained at 30°C and aerated and agitated by a stream of sterile air. Providing no contamination was apparent after 36h, each culture was inoculated with 130ml of a mid-exponential phase nutrient-broth culture of *C. terrigena*, and growth continued until 8h after reaching the stationary phase. Cells were then harvested in a Sharples centrifuge (flow rate, 15 litres/h; bowl speed, 30000rev./min) and resuspended for immediate use.

Stage 1: *preparation of dialysed cell extract.* Either 30g of freeze-dried powder or the wet cells from a 35-litre culture were resuspended in 400ml of 50mM-sodium phosphate buffer, pH6.0, and stirred for 2h at 4°C. Cell rupture and solubilization of material was achieved by passage three times through a chilled French pressure cell (with rapid-fill attachment) operating at 126MPa. During and after this treatment the volume was increased to 560ml. Any remaining whole cells and insoluble cell debris were removed by centrifuging for 3h at $96000g_{av.}$ (Beckmann L2-65B, type 42 rotor, 4°C), and the clear supernatant (approx. 400ml) was dialysed overnight against 50mM-sodium phosphate buffer, pH6.0 (10 litres) at 4°C.

Stage 2: *removal of nucleic acid.* The dialysed cell extract was washed through a column (8cm×30cm) of pre-equilibrated DEAE-cellulose (DE-52; Whatman Biochemicals, Maidstone, Kent, U.K.) at 4°C with 50mM-sodium phosphate buffer, pH6.0. The eluate was monitored for u.v.-absorbing material by collecting 50ml samples and measuring A_{280} and A_{260} in a Hilger Uvispeck spectrophotometer. After discarding the void volume (600ml), the nucleic acid-free protein fraction (600–700ml) was collected until the A_{280} had decreased or nucleic acid began to emerge from the column.

Stage 3: *fractionation with* $(NH_4)_2SO_4$. The pH of the eluate was adjusted to 6.5 and finely-ground solid $(NH_4)_2SO_4$ was added to the ice-cold stirred solution until 55% saturation was achieved (350g/l). The pH was maintained at 6.5 by the addition of solid Tris. The mixture was stirred for 1h, then insoluble material was removed by centrifuging for 1h at 4°C (MSE High-Speed 18 centrifuge, 6×25ml rotor, $23000g_{av.}$) and the concentration of $(NH_4)_2SO_4$ in the supernatant was increased to 65% saturation (81g/l of original nucleic acid-free solution). The mixture was stirred for a further 1h at 2°C and precipitated protein was collected by centrifuging as above. Percentage saturations were calculated from the nomograph of Dixon (1953) without correction for temperature.

Stage 4: *gel filtration on Sephadex G-100.* The protein fraction precipitating between 55 and 65% saturation with $(NH_4)_2SO_4$ was resuspended in 20ml (or less) of 0.1M-Tris/HCl, pH7.5, and passed through a column (4.5cm×80cm) of Sephadex G-100 at 4°C. The column was eluted with 50mM-Tris/HCl, pH8.8, at a flow rate of 30ml/h and 11ml fractions were collected. The alkylsulphohydrolase-containing fractions were eluted immediately after the void volume and were pooled for further purification.

Stage 5: *fractionation on DEAE-cellulose.* A column (1.25cm×11cm) of DEAE-cellulose (DE-52) was pre-equilibrated with 50mM-Tris/HCl, pH8.8, at 4°C. The stage-4 enzyme was applied to the column and washed in with a further 10ml of the same buffer. The column was then eluted with a concentration gradient of 0.1–0.25M-Tris/HCl, pH8.80, in 400ml and 5ml fractions were collected.

The CS1 enzyme, which was carried through to this stage, was washed through in the loading buffer and collected. The CS2 enzyme was eluted at a buffer concentration of approx. 0.2M. The active fractions were pooled, concentrated to approx. 5ml in a Diaflo concentrator (50ml cell, UM10 membrane, 120kPa for 4h) and stored at −20°C in 0.5ml portions, without loss of activity.

Measurement of alkylsulphohydrolase activity

For all assays involving resolved octan-2-yl sulphates as assay substrates, four identical 200μl incubation samples containing suitably diluted enzyme solution, 7.5mM resolved substrate [prepared with pyridine/SO_3 as described by Matcham & Dodgson (1977)] and 100mM-Tris/maleate, pH7.5, were mixed and placed on ice. Three of these incubation mixtures were transferred to a 31°C-water bath for 5, 10 and 15min respectively and then replaced on ice. In experiments with crude enzyme 50μl of 15% (w/v) trichloroacetic acid solution was added to precipitate protein and, after brief centrifugation, a 200μl portion was removed from the supernatant for estimation of liberated SO_4^{2-} ions by the $BaCl_2$/gelatin method (method B) of Dodgson (1961) as modified by Thomas & Tudball (1967). With pure enzyme, the 200μl samples were mixed directly with 1.3ml of 4% (w/v) trichloroacetic acid solution, and liberated SO_4^{2-} ions were determined without prior removal of protein. The measurement of actual rates of hydrolysis described here is particularly important if the substrate is a potential inhibitor, as in this case (possible detergent properties). A unit of enzyme activity is defined as the liberation of 1μmol of SO_4^{2-}/min.

In all cases suitable control determinations were carried out in which enzyme and substrate were incubated separately and only mixed immediately before the addition of trichloroacetic acid.

In experiments designed to test the relative ability of racemic or symmetrical secondary alkylsulphates to act as substrates for the CS2 enzyme or for the CS1/CS2 enzyme complement of dialysed crude cell extracts, the following incubation procedure was adopted. For each potential substrate, 2.5ml of a solution, containing the racemic or symmetrical alkyl sulphates at 4mM concentration (i.e. 10μmol), 0.3M-Tris/maleate buffer, pH7.5, and pure CS2 enzyme (0.48 to 48 units depending on the substrate) or dialysed cell extract (approx. 0.4 unit of each enzyme present), was incubated at 31°C. At intervals, (up to 90min for the CS2 enzyme; up to 45min for cell extract) 200μl samples were withdrawn and assayed appropriately as described above, in order to obtain the initial rate of hydrolysis and (in the case of the CS2 enzyme) the extent of hydrolysis at the completion of the reaction.

Analytical procedures

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

Polyacrylamide-gel electrophoresis was carried out under non-denaturing conditions by the method of Payne & Painter (1971), except that gels were run at pH8.9 and 20°C. Protein bands were stained with Coomassie Brilliant Blue R250 as described by Meyer & Lamberts (1965). Secondary alkylsulphohydrolase activity was located on the gels by the method of Payne *et al.* (1974). SDS*/polyacrylamide-gel electrophoresis was performed by the method of Weber & Osborn (1969). Cytochrome *c*, ovalbumin (hen egg), catalase (ox liver), albumin (bovine serum) (all Boehringer–Mannheim, Lewes, Sussex, U.K.) and pepsin (hog stomach mucosa) were used as standards.

The molecular weight of the intact enzyme was determined by gel filtration on Sephadex G-200 and CL Sepharose 6B. In each case, a 1ml sample was applied to the column (1.6cm×99cm) and eluted with 0.1M-Tris/maleate, pH7.5. Lysozyme (egg white), trypsin inhibitor (soya bean) (both BDH), catalase (ox liver), alcohol dehydrogenase (horse liver) (both Miles Laboratories, Slough, Bucks., U.K.), D-amino acid oxidase (hog kidney), aldolase (rabbit muscle) (both Boehringer–Mannheim) and apoferritin (horse spleen, Calbiochem, San Diego, CA, U.S.A.) were used as standards.

* Abbreviation: SDS, sodium dodecyl sulphate.

Results

Enzyme purification

A typical purification analysis is shown in Table 1. The potassium salts of L- and D-octan-2-yl sulphates were used to measure CS1 and CS2 alkylsulphohydrolase activities respectively. The optical stereospecificity of each enzyme is apparent after their separation on the final ion-exchange column and has already been demonstrated (see Matcham & Dodgson, 1977; Matcham *et al.*, 1977).

The very high amounts of nucleic acid present in cell extracts of *C. terrigena* were efficiently removed by the first DE-52 column and, after fractionation with $(NH_4)_2SO_4$, two distinct but not separate enzyme peaks were eluted from Sephadex G-100 (Fig. 1*a*) and then pooled. On gradient elution from the final DE-52 column (Fig. 1*b*), peaks of protein (A_{280}) and CS2 enzyme activity, which corresponded exactly, were detected. The CS2 preparation obtained by this procedure was homogeneous by SDS/polyacrylamide-gel electrophoresis and produced a single

Table 1. *Purification of the CS2 secondary alkylsulphohydrolase of Comamonas terrigena*

Starting point for this particular preparation was 30g of freeze-dried stationary-phase broth-grown cells. The CS1 and CS2 enzyme activities were assayed with L- or D-octan-2-yl sulphate respectively, as described in the text. See the text for definition of enzyme units.

Purification stage	Nucleic acid (%)	Protein (mg)	CS1 enzyme activity (Units)	CS1 enzyme activity (% yield)	CS2 enzyme activity (Units)	CS2 enzyme activity (% yield)	Specific CS2 enzyme activity (units/mg)	CS2 enzyme purification
1	22.5	6610	1280	100	1185	100	0.179	1
2	0	2710	1110	87	1084	92	0.4	2.25
3	—	769	592	47	951	80	1.24	6.9
4	—	17.5	96	7.5	874	74	50	279
5								
50mM peak	—	0.43	11.5	1	—	—	—	—
200mM peak	—	0.57	—	—	435	37	768	4290

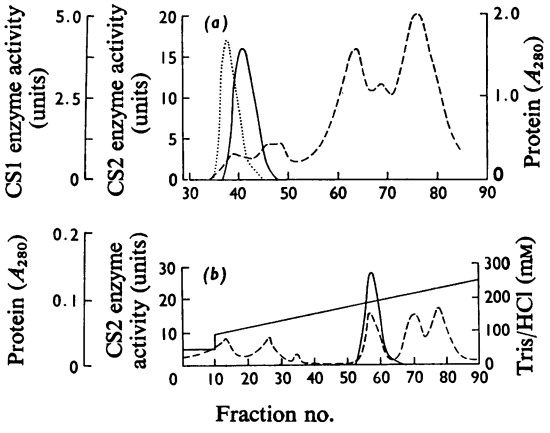

Fig. 1. *Elution of secondary alkylsulphohydrolase activity and protein from Sephadex G-100 and DEAE-cellulose chromatography columns at stages 4 and 5 of purification*

(*a*) Sephadex G-100 column size, 4.5cm×80cm; elution buffer, 50mM-Tris/HCl, pH8.8; flow rate, 30ml/h; 11ml fractions were collected. ——, CS2 enzyme activity; ····, CS1 enzyme activity; ----, protein (A_{280}). See the text for further details. (*b*) DEAE-cellulose DE-52 column size, 1.25cm×11cm; elution buffer, 0.1–0.25M-Tris/HCl buffer, pH8.8 in 400ml; 5ml fractions were collected. ——, CS2 enzyme activity and buffer gradient; ----, protein (A_{280}). See the text for further details.

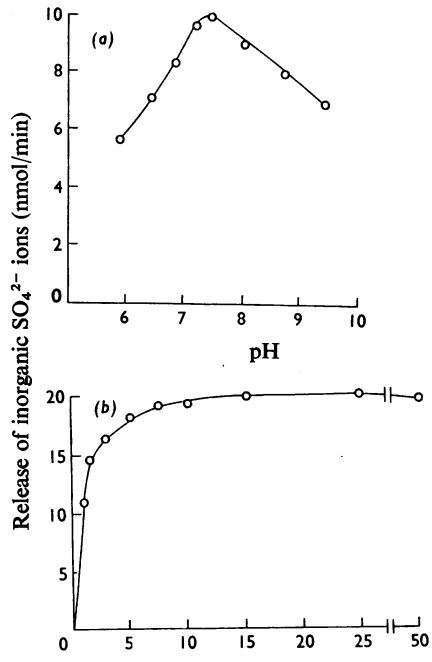

Fig. 2. *pH–activity and substrate–activity curves for the pure CS2 secondary alkylsulphohydrolase acting on potassium* D-*octan-2-yl sulphate*

In (*a*) the concentration of substrate in incubation mixtures was 15mM and the buffer was 0.1M-Tris/maleate. In (*b*) the buffer was 0.1M-Tris/maleate, pH7.5. Incubation temperature in both cases was 31°C.

active band when run under non-denaturing conditions. In the CS1 enzyme fraction, which washed straight through the final column, three inactive contaminating protein bands were also present. No attempts have yet been made to purify this enzyme fraction further.

Molecular-weight determinations

The molecular weight of the pure CS2 enzyme, determined by SDS/polyacrylamide-gel electrophoresis, was 57500. However, the intact mol.wt. was 250000 as measured by Sephadex G-200 chromatography and 249000 by CL Sepharose 6B chromatography, indicating a tetrameric structure. Although measurement of the molecular weight of the CS1 enzyme was not possible, its earlier elution from Sephadex G-100 suggests it is larger than the CS2 enzyme.

General properties of the CS2 enzyme

The effect of pH on the hydrolysis of potassium D-octan-2-yl sulphate by the pure CS2 enzyme is shown in Fig. 2(*a*). Each 200μl sample contained 0.01 unit of enzyme, 15mM-D-octan-2-yl sulphate and 0.1M-Tris/maleate buffer. Otherwise, the procedure followed the standard assay method. Maximum activity was detected at about pH7.5.

In order to measure the effect of concentration of D-octan-2-yl sulphate on the initial velocity of its hydrolysis by pure CS2 enzyme, 200μl samples were prepared containing 0.02 enzyme unit and 1–50mM substrate. In spite of its potential detergent nature, there was no inhibition by higher concentrations of D-octan-2-yl sulphate (Fig. 2*b*). The K_m value, calculated from the double-reciprocal plot of Lineweaver & Burk (1934), was approx. 1mM.

Inhibition by potassium L-*octan-2-yl sulphate*

The effect of 0, 2.0 and 4.0mM-potassium L-octan-2-yl sulphate on the hydrolysis of potassium D-octan-2-yl sulphate by the pure CS2 alkylsulphohydrolase was investigated at substrate concentrations of 1, 1.33, 2.0 and 4.0mM and enzyme concentration of 0.02 unit/200μl of incubation mixture. Incubation periods were varied up to 20min to facilitate accurate

measurement of the initial rate. Potassium L-octan-2-yl sulphate competitively inhibited the CS2 enzyme with a K_i value of 3.6mM as deduced from the double-reciprocal plot. The K_m for potassium D-octan-2-yl sulphate, calculated from the same plot in the absence of inhibitor, was 1.38mM.

Hydrolysis of other secondary alkyl sulphates

The significant inhibition of potassium D-octan-2-yl sulphate hydrolysis by the corresponding L-isomer eliminated any possibility of deriving meaningful kinetic constants for racemic substrates. Unfortunately, methods for preparing other resolved secondary alkyl sulphates have not yet been established, and it was therefore necessary to investigate the hydrolysis of unresolved alkyl sulphates under the arbitrary conditions defined in an earlier section. Because of the differences in the experimental conditions used in the two instances, the present studies also included some esters (heptan-4-yl, nonan-5-yl, and racemic octan-4-yl and -3-yl sulphate) that had previously been shown (Matcham & Dodgson, 1977) to be non-substrates for either the CS1 or CS2 enzymes.

The results (Table 2) showed that CS2 enzyme activity towards racemic 2-yl sulphates increased with increasing chain length. In the case of racemic decan-2-yl sulphate, 0.48 unit of enzyme hydrolysed 5μmol of the substrate within 8min [i.e. the total amount of (+)-isomer in the substrate]. In contrast, no activity towards pentan-2-yl sulphate could be detected over 90min with 48 units of enzyme. No hydrolysis of racemic octan-3-yl and octan-4-yl sulphate or the symmetrical heptan-4-yl and nonan-5-yl sulphate could be detected under similar conditions, thus confirming previous observations (Matcham & Dodgson, 1977). Table 2 also presents the results of analogous experiments with dialysed crude cell extracts over incubation periods of up to 45 min. A similar increase in enzyme activity with increasing chain length again occurred and the esters not hydrolysed by pure CS2 enzyme were also not hydrolysed by the cell extract.

Hydrolysis of C_{12} and C_{14} secondary alkyl sulphates

It has not yet been possible to prepare homogeneous secondary alkyl sulphates of chain length greater than C_{10} by the pyridine/SO_3 method of Matcham & Dodgson (1977). However, the work of those authors would indicate strongly that sulphation of racemic decan-2-ol, dodecan-2-ol or tetradecan-2-ol with H_2SO_4 or chlorosulphonic acid would yield products containing significant amounts of racemic decan-2-yl sulphate, dodecan-2-yl sulphate or tetradecan-2-yl sulphate respectively. Unfortunately, a further complication to the study of the C_{12} and C_{14} esters as potential substrates lies in the inability to use them in association with the usual method for assaying liberated SO_4^{2-} ions. This arises from the relatively insoluble nature of their barium salts. A qualitative indication of the ability of the three heterogeneous esters to serve as substrates for the CS2 enzyme was obtained by subjecting the enzyme to polyacrylamide-gel electrophoresis (2 units/gel) as described by Payne *et al.* (1974) and incubating the gels at 31°C in 10mM solutions of 'decan-2-yl sulphate', 'dodecan-2-yl sulphate' or 'tetradecan-2-yl sulphate' in 0.1M-Tris/maleate buffer, pH7.5. Under these circumstances enzyme bands developed in the gels in 1, 15 and 240min respectively. Although too much emphasis should not be placed on these results, they do perhaps indicate that the activity of the CS2 enzyme declines with substrates of chain lengths greater than C_{10}. Confirmation of this must await the preparation of adequate substrates and the development of a new assay method.

Table 2. *Hydrolysis of racemic or symmetrical secondary alkyl sulphates by pure CS2 enzyme and by dialysed cell extracts*
Each incubation mixture contained 10μmol of substrate [equivalent to 5μmol of (+)-isomer for the racemic substrates]. See the text for other details and for definition of enzyme unit.

	Pure CS2 enzyme hydrolysis			Cell-extract hydrolysis	
Potential substrate	Initial rate (units/mg)	Relative rate	Extent at end of reaction (μmol)	Initial rate (units/mg)	Relative rate
Decan-2-yl sulphate	670	268	4.9	0.134	223
Nonan-5-yl sulphate	0	—	0	0	—
Octan-2-yl sulphate	240	96	5.0	0.059	98
Octan-3-yl sulphate	0	—	0	0	—
Octan-4-yl sulphate	0	—	0	0	—
Heptan-2-yl sulphate	16	6.4	5.0	0.0037	6.2
Heptan-4-yl sulphate	0	—	0	0	—
Hexan-2-yl sulphate	2.5	1	4.95	0.0006	1
Pentan-2-yl sulphate	0	—	0	0	—

Cellular localization of CS1 and CS2 alkylsulphohydrolases

In the case of *Pseudomonas* C12B at least one of the primary alkylsulphohydrolases and two of the secondary alkylsulphohydrolases are released from cells that have been subjected to various cell-washing or osmotic-shock procedures (Fitzgerald & Laslie, 1975). This indicates an exocytoplasmic location for the enzymes. However, attempts (Fitzgerald *et al.*, 1975) to release secondary alkylsulphohydrolase activity towards 'potassium decan-5-yl sulphate' (heterogeneous preparation, see Matcham & Dodgson, 1977) from *C. terrigena* by similar treatments were unsuccessful.

Some release of the *C. terrigena* enzymes has now been achieved by modifying the composition of the cell-wash fluid. The experiments were carried out exactly as the corresponding ones described by Fitzgerald *et al.* (1975), except that resolved octan-2-yl sulphates were used to measure CS1 and CS2 enzyme activity and the cell-wash fluid consisted of 20% sucrose solution containing 10mM-EDTA, 30mM-Tris/HCl buffer, pH8.0, and 1.0mg of lysozyme/ml (i.e. increases in concentration amounting to 3-fold for the buffer, 100-fold for the EDTA and 2-fold for lysozyme, over those originally used). Two successive treatments of *C. terrigena* cells with the modified wash fluid released a total of 38% of the CS2 enzyme and 22% of the CS1 enzyme with relatively little release of protein (Table 3).

In contrast, cells washed once with wash fluid without the lysozyme component, released neither enzyme when subsequently subjected to osmotic shock.

Discussion

As well as providing a homogeneous preparation of the CS2 secondary alkylsulphohydrolase for further study, the purification procedure described here has provided additional information about the CS1 enzyme. On the basis of gel zymogram experiments, the former enzyme was previously believed to be the predominant of the two in *C. terrigena* cells (Fitzgerald *et al.*, 1975). However, the availability of resolved and homogeneous octan-2-yl sulphates for the study of the two enzymes has now enabled the demonstration of their presence in cell extracts in approximately equal amounts (see Table 1). The precise cellular localization of the enzymes is still not established with certainty, but their release from the cell in substantial quantities by cell-washing procedures suggests that, like the corresponding *Pseudomonas* C12B enzymes (see Matcham *et al.*, 1977), they have an exocytoplasmic localization. There is some evidence (B. Bartholomew, J. M. Cloves & K. S. Dodgson, unpublished results) that the alkylsulphohydrolase enzymes of *Pseudomonas* C12B have mol.wts. of the order of 60000. In contrast, the mol.wt. of the CS2 enzyme of *C. terrigena* is of the order of 250000, whereas that of the CS1 enzyme appears to be even greater. It is possible that differences in molecular sizes may be the most important factor in explaining why the *C. terrigena* enzymes are less readily released from cells by cell-washing procedures than those of *Pseudomonas* C12B.

The need for two enzymes in *C. terrigena* presumably stems from their respective optical stereospecificities. The CS1 enzyme will hydrolyse L-octan-2-yl sulphate but not the D-isomer, whereas the CS2 enzyme shows the reverse stereospecificity. Hence both enzymes would be necessary for the hydrolysis of a racemic substrate such as might be present in a detergent mixture. It is noteworthy that CS2 enzyme activity towards D-octan-2-yl sulphate is competitively inhibited by the corresponding L-isomer and, although not detailed in the present paper, the CS1 enzyme is similarly inhibited by the D-isomer. In both cases, however, the K_i values for the inhibitor isomers were two to three times greater than the K_m values for the substrate isomers, so that hydrolysis of a racemic substrate by the intact cell could proceed.

Table 3. *Effect of cell washing and osmotic shock on the release of the CS1 and CS2 secondary alkylsulphohydrolases from stationary-phase cells of Comamonas terrigena*

See the text for details and for definition of enzyme unit. The values given in parentheses represent percentages of the values for the untreated control.

Treatment	Fraction	Protein (mg)	Total enzyme activities (units) CS1	CS2
Untreated control	Cell extract	130.5	14.75 (100)	14.87 (100)
Sucrose/Tris/EDTA/ lysozyme wash	Cell extract	156.8	11.92 (81)	9.09 (61)
	First wash fluid	37.1*	1.80 (12)	2.55 (18)
	Second wash fluid	34.4*	1.48 (10)	3.01 (20)
Osmotic shock	Cell extract	127.1	14.85 (101)	14.80 (99)
	Wash fluid†	9.6	0	0
	Shock fluid	12.6	0	0

* Including the lysozyme, 25mg initially present in each wash fluid.
† The usual wash fluid minus lysozyme.

Apart from the optical stereospecificity difference, there appears to be a similarity in substrate specificity between the CS2 and CS1 enzymes when the rates and extents of hydrolyses of various racemic substrates by the former are compared with those established for crude cell extracts (both enzymes present). The pure CS2 enzyme hydrolysed the appropriate isomer [presumably the D(+) (see Bartholomew *et al.*, 1977)] present in racemic hexan-2-yl, heptan-2-yl, octan-2-yl and decan-2-yl sulphates, with activity increasing with increasing chain length (Table 2). It did not hydrolyse racemic pentan-2-yl sulphate, any of the symmetrical esters tested nor any of the racemic esters in which the sulphate group was not in the C-2 position. The crude cell extract, containing both enzymes, followed a similar pattern; not only was activity confined to those esters with the sulphate group in the C-2 position but the relative initial rates of hydrolysis were similar to those obtained with the CS2 enzyme. Again activity increased with increasing chain length. Although studies on resolved substrates will need to be made with homogeneous preparations of both enzymes, the use of racemic esters described here at least provides some indications of the specificities of these enzymes. Two other observations, not reported here, provide further support for some of the conclusions made above. First, 8mM-racemic octan-3-yl sulphate did not inhibit the hydrolysis of 2mM-racemic octan-2-yl sulphate by the pure CS2 enzyme, showing that the non-hydrolysis of the (+)-component of racemic octan-3-yl sulphate by the CS2 enzyme is not the result of strong inhibition by the (−)-isomer. This is also indicated by the failure of crude cell extract to hydrolyse this racemic substrate. Secondly, all those secondary alkyl sulphates that were not hydrolysed by *C. terrigena* enzymes are hydrolysed by the S3 enzyme of *Pseudomonas* C12B (Matcham *et al.*, 1977).

Finally some indication of the mechanism of action of the CS2 enzyme can be obtained from the observations described above. A prerequisite for optical stereospecificity is a three-point interaction between enzyme and substrate. Clearly, 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 sulphates nor (+)-secondary alkyl sulphates, in which the position of sulphation is C-3 or greater, are substrates for the CS2 enzyme. The third point of interaction must surely be through the sulphate group. The difference between the binding of the substrate and non-substrate isomers of octan-2-yl sulphate probably resides with the spatial orientation of the ester sulphate group. The fact that the K_m is less than half the K_i value for both the CS1 and CS2 enzyme/substrate/non-substrate combinations suggests that the sulphate group does contribute to enzyme–substrate interaction. Owing to the tetrahedral arrangement of the substituent groups around the asymmetric carbon of D-octan-2-yl sulphate, the fourth substituent, a proton, will be directed away from the surface of the enzyme. It seems likely that the introduction of the water molecule during the hydrolytic fission of this substrate will occur from this side. It has already been established that the mechanisms of action of the CS2 alkylsulphohydrolase involves the cleavage of the C–O bond of the C–O–S ester linkage and produces an inversion of configuration (Bartholomew *et al.*, 1977). It seems likely that the conversion of the high-affinity D-configuration of the substrate into the low-affinity L-configuration of the alcohol may contribute to the subsequent release of this reaction product. Although largely speculative, this postulated mechanism of action is consistent with the observations described here and elsewhere.

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