Purification and characterization of the short-chain alkylsulphatase of coryneform B1a

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Using a combination of streptomycin sulphate precipitation, and DEAE-cellulose and butyl-agarose chromatography, an alkylsulphatase active towards short-chain alkyl sulphates has been purified approx. 70-fold from extracts of coryneform B1a grown on butyl-1-sulphate. The enzyme protein is dimeric with a subunit molecular mass of 77.6 kDa, has an isoelectric point of pI 7.2, and converts butyl-1-sulphate stoichiometrically into butan-1-ol and inorganic sulphate. Stoichiometric incorporation of ^{18}O from H_2^{18}O into sulphate during the reaction showed that enzymic hydrolysis occurred at the O-S bond of the C-O-S ester linkage. The enzyme was active on C_3 – C_7 linear primary alkyl sulphates but not on higher ($\text{C}_{8,9}$) or lower ($\text{C}_{1,2}$) homologues,

although the latter pair were competitive inhibitors. The specificity constant ($k_{\text{cat.}}/K_m$) was highest for pentyl sulphate (K_m 1.89 ± 0.38 mM; $k_{\text{cat.}}$ 6.86 ± 0.52 s $^{-1}$) and decreased for higher and lower homologues. No activity was detected towards C_3 – C_9 racemic alkyl-2-sulphates, D- or L-enantiomers of butyl-2-sulphate, the symmetrical secondary alkyl sulphates pentyl-3-sulphate, heptyl-4-sulphate, nonyl-5-sulphate, C_1 – C_8 alkane sulphonates, choline sulphate, or butyric acid-4-sulphate; none of these compounds (except the symmetrical esters and butyric acid-4-sulphate, which were not tested) was demonstrably inhibitory. The enzyme was compared with other alkylsulphatases in terms of substrate specificity and mode of action.

INTRODUCTION

Long-chain primary alkyl sulphates are important components of many synthetic surfactants used in household detergents and personal hygiene products (Shore and Berger, 1976). Consequently significant quantities of long-chain alkyl sulphates are discharged to the environment during their manufacture and use, and the subsequent disposal of wastes. Alkyl sulphates are among the most environmentally acceptable of synthetic surfactants because they are readily biodegraded by environmental and sewage bacteria. Biodegradation is initiated by alkylsulphatase enzymes (see Dodgson et al., 1982; Dodgson and White, 1983; White and Russell, 1993) which hydrolyse inorganic sulphate from its ester linkage with alcohols, the latter being readily assimilated through 'normal' metabolic pathways (Lijmbach and Brinkhuis, 1973; Thomas and White, 1989). Bacteria possessing these enzymes are widely distributed in the environment (Anderson et al., 1988; White et al., 1989) and they often contain multiple enzymes. For example, the river-bank isolate *Pseudomonas* C12B (Payne and Feisal, 1963) produces, under appropriate conditions, two enzymes (designated P1 and P2) active on primary alkyl sulphates ($\text{RCH}_2\text{-OSO}_3^-$) and three enzymes (S1–S3) acting on secondary alkyl sulphates [R'CH(R'')-OSO_3^-]. Four of these enzymes have been purified to homogeneity and characterized in some detail (Bartholomew et al., 1978; Cloves et al., 1980; Shaw et al., 1980; Bateman et al., 1986). Although these enzymes differ from each other in several respects, a common feature is that their substrate specificities do not extend to compounds with alkyl chain-lengths shorter than C_5 . This lack of activity toward shorter-chain homologues does not reflect an absence of such compounds in the environment. On the contrary, short-chain alkyl sulphates enter the environment from a variety of natural and industrial sources. Thus for example, methyl, ethyl and propyl sulphates occur naturally in avian eggs (Yagi, 1964); C_1 – C_5 primary alcohols are converted into their

respective sulphate esters when incubated with rat liver (Vestermarck and Bostrom, 1959) or toad liver extracts (Scully et al., 1970); butyric-acid-4-sulphate is the end-product of SDS metabolism in mammals and fish (Denner et al., 1969); and choline sulphate is a storage form of sulphur in fungi (Dodgson et al., 1982). Industrial sources of short-chain alkyl sulphates include coal fly-ash (Lee et al., 1980) and, for methyl sulphate, spent liquors from methylation reactions using dimethyl sulphate (Ghisalba and Kuenzi, 1983).

In view of the occurrence of these compounds in the environment, the inactivity of the known alkylsulphatases towards short-chain esters has raised questions about the biodegradability of such compounds and the metabolic pathways involved. A variety of soil, sewage and aquatic samples have been used as sources for the isolation of bacteria with ability to metabolize methyl sulphate, ethyl sulphate, propyl/butyl-1-sulphates (White et al., 1987) and propyl-2-sulphate (Crescenzi et al., 1985). The shortest primary ester, methyl sulphate, has recently been shown to be readily metabolized in *Agrobacterium* and *Hyphomicrobium* spp. via a pathway initiated not by sulphatases but by monooxygenation (Davies et al., 1990; Higgins et al., 1993). The shortest secondary alkyl sulphate (propyl-2-sulphate) also undergoes initial oxidation before sulphate liberation (Crescenzi et al., 1984, 1985). Propyl-1-sulphate and butyl-1-sulphate were degraded by a coryneform sp. designated strain B1a (White et al., 1987). This organism was subsequently shown to grow on all members of the homologous primary alkyl sulphates from C_3 to C_7 (inclusive) but not on shorter or longer homologues (White and Matts, 1992). Similar growth on the corresponding C_3 – C_7 alcohols, together with gel-zymographic evidence of a single sulphatase enzyme with activity towards C_3 – C_7 alkyl-1-sulphates, led to the conclusion that degradation of these esters was initiated by a novel alkylsulphatase with substrate specificity different from those investigated hitherto. The present paper describes the purification and characterization of this novel short-chain alkyl-

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sulphatase from strain B1a, and provides a comparison of its properties with those of other alkylsulphatases.

EXPERIMENTAL

Except where otherwise stated, all chemicals were of analytical grade supplied by Merck or Sigma Chemical Co., both of Poole, Dorset, U.K.

Alkyl sulphates

Potassium methyl sulphate was prepared from dimethyl sulphate (Fluka, Glossop, U.K.) as described elsewhere (Davies et al., 1990). Other primary alkyl sulphates (C_2 – C_9) were synthesized as their potassium salts by the method of Lloyd et al. (1961). Butyl-1-sulphate prepared in this way yielded the following analysis (% by weight): potassium content, by flame photometry, found 19.7 (theoretical, 20.3); sulphate content, by gravimetric analysis as barium sulphate after acid hydrolysis of the ester, 61.8 (62.7); carbon, by combustion analysis, 24.8 (25.0); hydrogen, by combustion analysis, 4.61 (4.68). Infra-red (i.r.) spectroscopy in Nujol mulls using a Perkin–Elmer 257 grating spectrometer revealed strong absorption bands at 1210–1260 cm^{-1} corresponding to S–O stretching vibrations, and at 770–810 cm^{-1} , corresponding to the O–S bond of the C–O–S linkage (Lloyd et al., 1961).

Large-scale preparation of butyl-1-sulphate

Butyl-1-sulphate was required in large quantities (1.6 kg) as growth substrate for the organism [(0.2%, w/v) in two 400-litre fermentor batches]. The original method of Lloyd et al. (1961), which typically yielded 8 g of ester from 0.1 mol of butan-1-ol, was thus scaled up 50-fold and repeated four times. Although the volumes of reactants (sulphuric acid and butan-1-ol) were increased 50-fold, this factor was not appropriate to all liquid volumes in the original synthesis. The following modified method was therefore developed. Butan-1-ol (5 mol, 458 ml) was added cautiously, with constant stirring, to sulphuric acid (6 mol, 320 ml) continuously cooled to $< -10^\circ\text{C}$. The temperature of the reaction mixture was allowed to rise to ambient over a 2-h-long period, with constant stirring. The mixture was then added to ice-cold saturated $\text{Ba}(\text{OH})_2$ solution (2 litres) with vigorous stirring, adding more $\text{Ba}(\text{OH})_2$ if necessary to ensure a final pH between 7.0 and 8.0. Precipitated BaSO_4 and undissolved $\text{Ba}(\text{OH})_2$ were removed by centrifugation (5140 g_{av} , 15 min). Carbon dioxide was bubbled through the clear supernatant until all excess barium ions had been precipitated as barium carbonate and the precipitate was again removed by centrifugation. The supernatant was concentrated *in vacuo* to 200–300 ml using a Büchi Rotavapor R-10 10-litre-capacity rotary evaporator, operating at 40–50 $^\circ\text{C}$. The concentrate was then applied to a Dowex HCR-2W column (H^+ form, 8 cm $\times$ 35 cm column) and eluted with distilled water. The eluant was neutralized immediately with KOH (5 M), to ensure a final pH of between 6.0 and 7.0. The neutralized eluant was concentrated (as described above) and dried *in vacuo*. The dry solid was recrystallized from a minimal amount of boiling methanol. Chemical analysis of the final product gave results very close to expected theoretical values. Yields were 380–500 g per preparation, i.e. 40–50%, compared with around 30% for the small-scale preparation.

Organism coryneform B1a

The isolation of coryneform B1a (White et al., 1987) and conditions for its routine maintenance and culture (White and Matts, 1992) have been described elsewhere.

Large-scale culture of coryneform B1a

Studies reported elsewhere (White and Matts, 1992) showed that butyl-1-sulphatase activity in strain B1a was induced by growth on butyl-1-sulphate. Preliminary studies of growth versus activity in a 14-litre batch-fermentor showed that maximum yield of alkylsulphatase activity was obtained from cells grown on 10 mM butyl-1-sulphate in basal salts medium and harvested during the late exponential phase of growth. This protocol was followed in the large-scale culture of coryneform B1a with some modification to accommodate the increase in scale. Cells were grown at 30 $^\circ\text{C}$ in 400 litres of butyl-1-sulphate/basal salts medium containing (g/l): potassium butyl-1-sulphate, 2.0; K_2HPO_4 , 7.0; KH_2PO_4 , 3.0; NH_4Cl , 0.5; NaCl, 0.5; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.15. Anti-Foam A Concentrate (Sigma, 50 mg/l) was added to control excess foaming noted in small-scale trials. The fermentor (New Brunswick) was aerated at a rate of 1.0 m^3/h and stirred at 40 rev./min. This relatively low agitation rate was necessary to preserve as much as possible of the semi-mycelial morphology of the coryneform cells, because growth was hampered if this structure was compromised. Cells were grown to late exponential phase (54 h) and then harvested using a Sharples continuous-flow centrifuge operating at a flow rate of 60 l/h. The wet cells (645 g) were freeze-dried and stored at -70°C .

Alkylsulphatase assay

Butyl-1-sulphate was routinely employed as substrate for the assay of alkylsulphatase activity. For preliminary experiments, and during enzyme purification, substrate (10 mM final concentration) was incubated for 5 min with enzyme preparations at 30 $^\circ\text{C}$ in the presence of 100 mM Tris/HCl, pH 7.5. Samples (at least three) were removed at intervals and concentrations of enzymically liberated sulphate ions were measured by the BaCl_2 /gelatin method (Dodgson, 1961) as modified by Thomas and Tudball (1967). Initial velocities were measured over the linear part of the reaction curves and included at least three time-points. A unit of enzyme activity is defined as the amount of enzyme that liberates 1 μmol of SO_4^{2-} /min from butyl-1-sulphate under these conditions. For kinetic analyses, initial velocities were always determined in duplicate and data were fitted to the Michaelis–Menten equation using the method of Wilkinson (1961).

Stoichiometry of the enzymic reaction

Butyl-1-sulphate (1.25 mM final concn.) and pure enzyme (50 μg) were incubated at 30 $^\circ\text{C}$ in 0.1 M Tris/HCl, pH 7.5 (total volume 2 ml). At intervals, aliquots (1 μl) were removed and injected directly into a gas chromatograph to measure butan-1-ol concentrations. Simultaneously, aliquots were removed, mixed with 50 μl of trichloroacetic acid solution (15%, w/v) and analysed in the normal way for inorganic sulphate content by the method of Dodgson (1961).

Determination of butan-1-ol

Concentrations of butan-1-ol released from butyl-1-sulphate during incubation with purified alkylsulphatase were determined by g.c. analysis. Samples of incubation mixtures (1 μl) were injected directly on to a 2 m $\times$ 2 mm internal diameter glass column packed with Carbowax 20M (5% adsorbed on 60/80 Carbowax B; Jones Chromatography, Llanbradach, Wales). The column was installed in a Perkin–Elmer 8310 Gas Chromatograph fitted with a flame-ionization detector. The oven temperature was 100 $^\circ\text{C}$ and the carrier gas was N_2 . The system was calibrated with standard amounts of authentic butan-1-ol.

Nucleic acid and protein assays

Nucleic acid was determined by the method of Layne (1957). Protein was routinely determined by the Coomassie Brilliant Blue method of Bradford (1976) with BSA (fraction V) as standard, or in the case of column fractions, by measuring absorbance of samples at 280 nm.

Non-denaturing PAGE

Native gel-electrophoresis was carried out by the method of Davis (1964), using anodic discontinuous-buffer electrophoresis in 7% (w/v) polyacrylamide resolving gels (12 cm × 14 cm × 0.15 cm) with 3% (w/v) stacking gels, in an LKB 2001 Vertical Electrophoresis unit (LKB Instruments, Croydon, Surrey, U.K.). Sample wells contained protein samples (100 µl) mixed with 25 µl of 20% (v/v) glycerol in water containing 0.4% (w/v) Bromophenol Blue. Proteins were concentrated in the stacking gel at 60 mA and separated at 40 mA while maintaining the temperature of the gel at 10 °C. Gels were stained with Coomassie Blue.

¹⁸O Incorporation

The position of cleavage of the ester sulphate bond by the alkylsulphatase of coryneform B1a was determined by measuring the extent of incorporation of ¹⁸O from H₂¹⁸O into inorganic sulphate during enzymic hydrolysis of butyl-1-sulphate. A solution of butyl-1-sulphate (200 µl, 80 mM in 100 mM Tris/HCl, pH 7.5) was freeze-dried and then redissolved in an equivalent volume of H₂¹⁸O (98 atom% excess; Amersham International, Amersham, Bucks., U.K.). Pure alkylsulphatase from coryneform B1a (200 µl containing 10 µg of protein, 0.03 unit) was added, and the whole was incubated for 6 h at 30 °C. Hot saturated BaCl₂ solution (200 µl) was added to terminate the reaction and to precipitate inorganic sulphate as BaSO₄. The precipitate was recovered by centrifugation and washed sequentially with water, acetone and ether (3 × 1 ml of each). The washed BaSO₄ was then mixed with finely ground KBr before being dried overnight at 100 °C. The dried material was pressed into a disc and its i.r. spectrum determined (Perkin-Elmer 257 grating i.r. spectrophotometer) in order to assess ¹⁸O incorporation from the relative absorptions at 970 and 990 cm⁻¹ (see Spencer, 1959). A control sample was prepared in an identical manner except that normal water was used in place of H₂¹⁸O to reconstitute the dried substrate.

Determination of enzyme molecular mass

The native molecular mass of the pure enzyme was estimated by gel-exclusion chromatography on a Sephadex G-200 column (2.5 cm × 63.0 cm) equilibrated in 100 mM Tris/HCl, pH 7.5. Standards (2.0 mg each in 5 ml of column buffer) used were Blue Dextran, bovine catalase (240 kDa), pig heart lactate dehydrogenase (145.9 kDa), hen ovotransferrin (76–78 kDa), hen ovalbumin (45 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), equine myoglobin (17 kDa), equine cytochrome *c* (12.3 kDa) and potassium dichromate (294 Da).

Subunit molecular masses were determined by SDS/PAGE under denaturing conditions according to the method of Laemmli (1979). Denaturation was achieved by incubating protein standards and purified alkylsulphatase fractions in sample buffer [60 mM Tris/HCl, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% (w/v) Bromophenol Blue] at 100 °C for 1 min. Standards used were

rabbit muscle phosphorylase B (92.5 kDa), hen ovotransferrin (76–78 kDa), hen ovalbumin (45 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), equine myoglobin (17.2 kDa) and equine cytochrome *c* (12.3 kDa).

Determination of isoelectric point

The isoelectric point of the alkylsulphatase of coryneform B1a was determined using a modification of the method of Caswell and Gacesa (1986), developed to determine multiple forms of alginate lyase in crude bacterial extracts. The technique employs a substrate-overlay to locate the enzyme in the focusing gel by activity-staining. The extreme insolubility of BaSO₄ has previously been exploited to indicate the location of alkylsulphatase enzymes in polyacrylamide gels (Cloves et al., 1980). The following method therefore uses a butyl-1-sulphate substrate overlay containing barium ions for detection of alkylsulphatase activity. Ultra-thin (0.41 mm) polyacrylamide gels (100 mm × 125 mm) were cast on support film (Gelbond, Bio-Rad Laboratories) between glass plates (separated by 0.41 mm plastic spacers) using 10 ml of a solution containing 5% (w/v) Electran-grade acrylamide, 0.17% (w/v) Electran-grade *N,N'*-methylenebisacrylamide, 20% (v/v) glycerol, 0.5 ml of Pharmalyte carrier ampholytes (pH range 3–10; Pharmacia Ltd, Milton Keynes, U.K.), 10 µl of *N,N,N',N'*-tetramethylethylenediamine and 0.038% (w/v) freshly prepared ammonium persulphate. Isoelectric focusing was performed using LKB apparatus using an anodic wick (Whatman 3MM paper) dampened with aspartic acid (0.04 M) and a cathodic wick dampened with NaOH (0.5 M) and saturated with 2% (v/v) Pharmalyte carrier ampholytes, pH range 8.0–10.5. Gels were pre-focused at 2.0 W constant power for 30 min before sample application. Samples (5–10 µl) of crude extract or standard (Pharmacia) were applied directly to 1 mm × 10 mm wells cut 1 cm from the cathodic or anodic pole of the gel. Proteins were focused for 2–3 h at 4.5 W constant power, or 2 kV maximum voltage. Agarose gel-substrate overlays were prepared by dissolving 1% (w/v) agarose (Sigma, Grade II), 20 mM butyl-1-sulphate and 20 mM barium acetate in boiling 100 mM Tris/HCl, pH 7.5. Gels were cast between warm glass plates (50 °C) on to Gelbond (Gelbond for Agarose, Bio-Rad Laboratories).

The polyacrylamide gel and substrate overlay were sandwiched together and incubated in a humid atmosphere for 4–5 h to allow full desulphation of butyl-1-sulphate substrate. After incubation, the agarose gel overlay was dried slowly at 50 °C, and the polyacrylamide gel stained for protein using the method of Blakesley and Boezi (1977), and then dried slowly at 50 °C.

Purification of the alkylsulphatase of coryneform B1a

Throughout the following procedures, solutions, reagents and columns containing enzyme were maintained at 0–4 °C.

Stage 1: Preparation of crude extract

Freeze-dried cells (3 g) were resuspended in ice-cold 100 mM Tris/HCl, pH 7.5, (35 ml) and stirred for 10 min. The cell suspension was then passed three times through a chilled (30 min in ice-water) French pressure cell operating at 120 MPa. The disrupted cell suspension was centrifuged (31000 *g*_{av.}, 4 °C; Sorvall RC-5B centrifuge, SS34 rotor) for 30 min to remove unbroken cells and cell debris. The universal proteinase inhibitor, α₂-macroglobulin (Boehringer-Mannheim, Darmstadt, Germany), was added to the extract to a final concentration of 0.5 mg/ml, and the whole mixture stirred for 5 min on ice.

Figure 1 Elution of the coryneform B1a alkylsulphatase from butyl-agarose

●, Enzyme activity; solid line, protein concentration; broken line, NaCl concentration. Active fractions (64–70, 4 ml each) showing a single band on non-denaturing polyacrylamide gels were combined, dialysed, and used for enzyme characterization within 24 h of completion of the final purification step.

Stage 2: Removal of nucleic acid

The cell-free extract from stage 1 (30 ml) was treated with sufficient streptomycin sulphate (5%, w/v) to give a final concentration of 1 mg of antibiotic/mg of protein. After stirring for 30 min at 4 °C, precipitated nucleic acid was removed by centrifugation as before. The clear supernatant was dialysed overnight against 100 mM Tris/HCl, pH 7.5 (3 × 5 litres).

Stage 3: DEAE-cellulose (DE 52) column chromatography

The nucleic acid-free extract from stage 2 (35 ml) was applied to a DE 52 cellulose column (1.5 cm × 30 cm) that had been pre-equilibrated with column buffer (100 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol). The column was washed with column buffer (50 ml) followed by a linear gradient of NaCl (0–0.8 M, total volume 200 ml) in column buffer. The flow rate was adjusted to 5 ml/h and 2 ml fractions were collected automatically and assayed for protein and enzyme activity. Fractions with the highest specific activity (fractions 43–47) were combined and dialysed for 2 h (2 × 3 litres column buffer).

Stage 4: Hydrophobic column (butyl-agarose) chromatography

The pooled fractions from stage 3 (10 ml) were applied to a butyl-agarose column (1 cm × 15 cm) that had been pre-equilibrated with column buffer (see stage 3). The column was washed with column buffer (20 ml) followed by a linear gradient of NaCl (0–0.2 M, total volume 400 ml) in column buffer. The flow rate was adjusted to 12 ml/h and fractions (4 ml) were collected automatically and assayed for protein and activity as before. Fractions showing a single protein band on polyacrylamide gels (fractions 64–70, Figure 1) were combined and dialysed for 1 h (2 × 5 litres column buffer).

RESULTS

Enzyme stability

Preliminary studies showed that the short-chain alkylsulphatase activity in crude cell extracts (0.1 M Tris/HCl, pH 7.5) retained

> 90 % activity during storage at 4 °C for about 4 days but thereafter activity decreased rapidly (70–80 % loss in 12 days). Stability was not altered significantly by changing the storage temperature (–20 °C or +25 °C) or by using glass or plastic containers, or by changing pH in the range 6.5–9.5. Dialysis of extracts, or addition of glycerol or thiols (2-mercaptoethanol, dithiothreitol) accelerated the inactivation (90 % loss in 3 days). In contrast, inclusion of the protease inhibitor α_2 -macroglobulin (0.5 mg/ml) produced marked stabilization of the enzyme activity (0 % and 55 % loss after 12 days and 41 days respectively). This inclusion was made in all subsequent experiments involving purification of enzyme from crude extracts.

Enzyme purification

Table 1 shows a typical purification record for the alkylsulphatase of coryneform sp. B1a. The purification procedure was repeated several times and the purification factor achieved from crude extract to final preparation was always in the range 65- to 76-fold with recoveries of 62–68 %. Final specific activities were 3.24 ± 0.23 units/mg (mean $\pm$ S.D., eight preparations).

Preliminary experiments showed that treatment of crude extracts with 0.5 mg of streptomycin sulphate/mg of protein increased enzyme activity to 120–200 % of the starting value, and simultaneously decreased total protein to 70 % of the initial amount, but with virtually no precipitation of DNA. When the streptomycin sulphate concentration was increased to 1.0 mg/mg of protein (as used in the routine purifications), there was no further change in enzyme activity or protein concentration but a marked precipitation of DNA (> 90 % precipitated). Thus the increase in enzyme activity upon treatment with streptomycin sulphate was attributed to removal of proteinaceous inhibitor(s) rather than removal of DNA.

α_2 -Macroglobulin was separated from the alkylsulphatase by DEAE-cellulose chromatography. Non-denaturing PAGE at various stages of purification (Figure 2) confirmed that the butyl-agarose step (Figure 1) was particularly effective (see also Table 1). The faster-moving diffuse band at this stage was not irradiated either by repetition of butyl-agarose chromatography with a 0.1–0.2 M NaCl gradient, or by gel-exclusion chromatography. The final preparation appeared as a closely spaced triplet of protein bands on PAGE. Multiple bands were evident only after stage 3, at which point α_2 -macroglobulin was removed. These observations, together with the stabilization achieved by inclusion of α_2 -macroglobulin, indicate that the multiple bands may arise from deamidation of asparaginyl and glutaminyl residues as observed in other protein preparations (Robinson, 1974), and that deamidation may be associated with loss of activity.

Final pure fractions were still labile with half-lives of approx. 4–5 days. Therefore all subsequent experiments involving pure enzyme were performed only with freshly prepared material within 24 h of completion of the purification procedure.

Molecular properties

Elution of the purified enzyme on Sephadex G-200 gel-exclusion medium yielded a molecular mass for the native protein of 148 kDa. Andrews (1964) estimated the errors associated with determination of molecular mass of globular proteins in the 10–150 kDa range using this method were ± 10 %. In the determination of molecular mass for the coryneform B1a sulphatase, the calibration showed good linearity and the elution volume for the sulphatase was reproducible. Thus using Andrews' estimate of error, the molecular mass of the native protein may be taken to be in the range 133–163 kDa. SDS/PAGE of

Table 1 Purification of alkylsulphatase from extracts of coryneform B1a

Stage	Fraction	Volume (ml)	[Protein] (mg/ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)	Purification (fold)
1	Cell extract	30	6.1	183	9.0	0.048	100	1
2	Streptomycin sulphate-treated	35	4.7	164	20.6	0.125	228	2.6
3	DEAE-cellulose pooled fractions	10	3.75	37.5	11.25	0.30	125	6.25
4	Butyl-agarose pooled fractions	48	0.035	1.7	5.9	3.5	65.5	73

Figure 2 Progressive steps in the purification of alkylsulphatase from coryneform B1a

A typical non-denaturing polyacrylamide gel electrophoretogram of samples from each stage (1–4) of the purification procedure. See text for details. Lane a, crude cell extract (stage 1); lane b, streptomycin sulphate-treated (stage 2); lane c, after DEAE-cellulose (stage 3); lane d, after butyl-agarose chromatography (stage 4); lane e, after repeat of stage 4; lane f, enlargement of band in lane e. Electrophoretic migration of proteins was from top (cathode) to bottom (anode) of the photograph.

denatured protein yielded a single band corresponding to 77.6 kDa. Collectively these values indicate that the native enzyme has a dimeric subunit structure. The isoelectric point of the pure enzyme determined by isoelectric focusing was 7.0–7.2.

Reaction catalysed

When incubated with butyl-1-sulphate, the purified enzyme liberated butan-1-ol and inorganic sulphate in amounts which were equimolar with each other throughout the time-course of the reaction and, at the end of the reaction, with the amount of substrate initially present. I.r. spectroscopy was used to determine the extent of incorporation of ^{18}O from H_2^{18}O into the oxygen atoms of the SO_4^{2-} ion during enzymic hydrolysis. Thus $\text{BaS}^{16}\text{O}_4$ precipitated after enzymic hydrolysis of butyl-1-sulphate in natural-abundance water gave a single sharp band at 990 cm^{-1} superimposed on the shoulder of a broad sulphate absorption band (Figure 3). In contrast, BaSO_4 produced after enzymic hydrolysis in ^{18}O -enriched water produced an additional peak at 970 cm^{-1} corresponding to $\text{BaS}^{16}\text{O}_3^{18}\text{O}$. Using the method of Spencer (1959), the percentage of $\text{BaS}^{16}\text{O}_3^{18}\text{O}$ in this barium sulphate sample was calculated as 48.9% using the formula $100\text{B}/(\text{A} + \text{B})$ where A and B were the peak heights of the 990 and 970 cm^{-1} absorptions respectively. The atom % excess of ^{18}O in the original water was 49%, thus indicating that the enzymic reaction proceeded with incorporation of one molecule of water into sulphate ion for every molecule of substrate hydrolysed.

Figure 3 Incorporation of ^{18}O from H_2^{18}O into inorganic sulphate during enzymic hydrolysis of butyl-1-sulphate

After enzymic hydrolysis of butyl-1-sulphate in (a) natural-abundance water, or (b) water enriched with a 49 atom % excess of H_2^{18}O , the liberated inorganic sulphate was precipitated with BaCl_2 (see text). Resulting barium sulphate samples were washed and dried, and i.r. spectra were determined in KBr discs. Atom % excess of ^{18}O in the inorganic sulphate was determined by comparison of absorption bands at 990 cm^{-1} ($\text{BaS}^{16}\text{O}_4$) and 970 cm^{-1} ($\text{BaS}^{16}\text{O}_3^{18}\text{O}$) by the method of Spencer (1959).

Catalytic characteristics

pH profile

The enzyme activity was maximal in the range 7.5–8.0, and almost constant (< 10% variation) across a broad range of pH from 7.0 to 8.5, but outside this range activity decreased to about 75% of maximum at pH 9 and 6. Changing the pH at which the sample was maintained (pH 6.5, 7.5, 8.5 and 9.5) had no effect on enzyme stability.

Table 2 Kinetic parameters for hydrolysis of linear primary alkyl sulphates by the alkylsulphatase from coryneform B1a

Values of k_{cat} were calculated from values of V_{max} using a molecular mass of 148 kDa. Primary kinetic constants, k_{cat} and K_m , are quoted $\pm$ S.E.M. from non-linear regression analysis. S.E.M. values for k_{cat}/K_m have been calculated assuming that the primary constants are statistically independent so that the squares of their coefficients of variation are additive.

Substrate alkyl-chain length	k_{cat} (s^{-1})	$10^3 \times K_m$ (M)	$10^{-3} \times k_{\text{cat}}/K_m$ ($\text{s}^{-1} \cdot \text{M}^{-1}$)
3	2.81 ± 0.20	7.98 ± 1.05	0.35 ± 0.05
4	9.00 ± 0.52	6.96 ± 0.97	1.29 ± 0.19
5	6.86 ± 0.52	1.89 ± 0.38	3.63 ± 0.78
6	9.10 ± 0.40	6.51 ± 0.54	1.40 ± 0.13
7	2.30 ± 0.20	7.69 ± 1.60	0.30 ± 0.07

Substrate specificity

A homologous series of primary alkyl sulphate esters (C_1 – C_9) was used to determine the substrate specificity of the purified enzyme. No activity was detected towards methyl sulphate, ethyl sulphate, octyl-1-sulphate or nonyl-1-sulphate at pH 7.5 and 30 °C, even when incubation times were extended to 1 h. Activity was detected, however, towards all primary alkyl sulphates with intervening chain lengths from C_3 to C_7 . Values of K_m and V_{max} for each substrate were determined by measuring linear initial velocities at several concentrations in the following ranges: C_3 , 0.625–10 mM; C_4 , 0.625–30 mM; C_5 , 0.625–30 mM; C_6 , 0.625–30 mM; C_7 , 1–35 mM. Data were fitted to the Michaelis–Menten equation to yield parameter values shown in Table 2.

None of the following compounds served as substrates: C_2 – C_9 alkyl-2-sulphates at a range of concentrations up to 20 mM; D- and L-enantiomers of butyl-2-sulphate; C_1 – C_8 primary alkane sulphonates (containing the $-\text{CH}_2-\text{SO}_3^-$ linkage): the symmetrical esters pentyl-3-sulphate, heptyl-4-sulphate and nonyl-5-sulphate; choline sulphate; or butyric acid-4-sulphate.

Inhibitors

Using 2 mM pentyl-1-sulphate (the optimum substrate), various compounds were tested as inhibitors. No inhibition was detected with C_1 – C_8 primary alkanesulphonates or C_3 – C_8 alkyl-2-sulphates, each tested at 2 and 4 mM. Choline sulphate was also not inhibitory. In contrast, the non-substrates methyl sulphate and ethyl sulphate were competitive inhibitors. Non-linear regression analysis of initial velocity data recorded at 0, 2 and 4 mM concentrations of inhibitor, for substrate concentrations in the range 1.25–10 mM, yielded K_i values of 5.3 mM and 3.9 mM for methyl and ethyl sulphates respectively.

DISCUSSION

Despite significant difficulties arising from enzyme instability, a purification protocol has been developed for the coryneform alkylsulphatase which has provided sufficient enzyme to allow determination of its characteristics for comparison with other alkylsulphatases. The reproducible purification factor of about 70-fold is similar to those achieved for other inducible bacterial alkylsulphatases (Cloves et al., 1980; Shaw et al., 1980; Bateman et al., 1986) and contrasts with values of a few thousand for non-inducible alkylsulphatases (Matcham et al., 1977; Bartholomew et al., 1978; Bateman et al., 1986). This observation is consistent with earlier indications (White and Matts, 1992) that the coryneform short-chain alkylsulphatase is inducible, and indicates that

this particular enzyme constitutes about 1.5% of the protein in cells grown on butyl-1-sulphate.

Hydrophobic-interaction chromatography with an increasing salt gradient (Figure 1) is unusual. In most applications, the initial salt concentration is kept high in order to sequester water, thus exposing hydrophobic patches in proteins and allowing them to associate with the hydrophobic stationary phase. Decreasing salt gradients then progressively diminish the protein–column interaction and allow sequential elution of proteins. In the present application, the enzyme must have a hydrophobic binding site which is already exposed without the need for high salt concentrations, because the enzyme readily binds substrates with alkyl chains up to C_7 . In this situation, butyl-agarose is used as an affinity-chromatography medium because the butyl chains on the agarose mimic substrate alkyl chains. It is, of course, quite common to use increasing salt gradients in affinity chromatography, and alkyl-agarose columns have been used in this way to purify several other alkylsulphatases (Cloves et al., 1980; Lillis et al., 1983; Crescenzi et al., 1984; Bateman et al., 1986).

The coryneform alkylsulphatase catalysed simple hydrolysis of the ester bond in alkyl sulphates with chain-lengths from C_3 to C_7 . This substrate specificity of the pure enzyme coincides exactly with the growth-substrate specificity of the organism and is entirely consistent with earlier gel-zymographic analysis of crude cell extracts (White and Matts, 1992). The chain-length specificity of the coryneform enzyme thus overlapped the lower end of the range of alkyl sulphate homologues accommodated by the corresponding P1 and P2 primary alkylsulphatases from *Pseudomonas* C12B for which minimum chain lengths are C_6 and C_4 respectively (Cloves et al., 1980; Bateman et al., 1986). Values of K_m in the area of overlap (C_6 – C_7) for all three enzymes fall within an order of magnitude. Despite these similarities, the coryneform alkylsulphatase was clearly distinguished from the P1 and P2 enzymes in that the first possesses a unique specificity for the C_3 – C_7 range whereas the others act on, and have increasing activity towards, longer-chain compounds.

The inability of primary alkane sulphonates ($\text{R}-\text{CH}_2-\text{SO}_3$) to serve as either substrates or inhibitors indicated that the presence of the ester-oxygen in the alkyl sulphates ($\text{R}-\text{CH}_2-\text{O}-\text{SO}_3$) was critical for substrate binding to occur. Secondary alkyl-2-sulphates [$\text{R}-\text{CH}(\text{CH}_3)-\text{O}-\text{SO}_3$] were also neither substrates nor inhibitors, indicating a binding requirement for the $\text{R}-\text{CH}_2-\text{O}-\text{SO}_3$ moiety.

Values of the specificity constant (k_{cat}/K_m) increased about 3-fold per methylene unit from C_3 to C_5 (the best substrate). This rate of increase is very close to that observed for both P1 and P2 alkylsulphatases, albeit in higher chain-length ranges (Figure 4), and indicates an important hydrophobic contribution to the interaction between enzyme and these substrates. The specificity constant decreased progressively for the coryneform enzyme from C_5 to C_7 , contrasting markedly with P1 and P2 enzymes which showed progressive increase in $\log(k_{\text{cat}}/K_m)$ for chain lengths up to at least C_9 (Figure 4). Octyl-1-sulphate and nonyl-1-sulphate were neither substrates nor inhibitors despite possessing the requisite $-\text{CH}_2-\text{O}-\text{SO}_3$ moiety. Collectively these results suggested that the enzyme–substrate interaction involved hydrophobic contributions from the alkyl chain and that there is an upper limit (C_7) to the size of alkyl chain that can be accommodated. Space limitations in the active site would also account for lack of binding by choline-*O*-sulphate with its bulky $(\text{CH}_3)_3\text{N}$ substituent. Methyl and ethyl sulphates contain the requisite $-\text{CH}_2-\text{OSO}_3$ structure and indeed they were competitive inhibitors, indicating an ability to bind at the active site. Failure of these very short homologues to act as substrates indicated that

Figure 4 Comparison of specificity constants (k_{cat}/K_m) for the alkylsulphatase of coryneform B1a with those of the P1 and P2 alkylsulphatases of *Pseudomonas* C12B

Data for the coryneform enzyme (○) are derived from Table 2, using a molecular mass of 148 kDa. Kinetic and molecular-mass data for the P1 (●) and P2 (▲) enzymes are taken from Bateman et al. (1986) and Cloves et al. (1980) respectively.

catalytic hydrolysis only occurs when a significant length of alkyl chain ($\geq C_3$) occupies the active site.

Stoichiometric liberation of butan-1-ol and SO_4^{2-} from butyl-1-sulphate confirmed the true hydrolytic nature of the reaction catalysed. This is consistent with results of experiments with whole cells during which SO_4^{2-} was liberated stoichiometrically from butyl-1-sulphate (White and Matts, 1992); butan-1-ol was also detected in the medium but in less than stoichiometric amounts as a result of its further metabolism by the cells. Stoichiometric incorporation of ^{18}O from $H_2^{18}O$ into SO_4^{2-} during the degradation of butyl-1-sulphate by the pure enzyme showed that fission of the C-O-S ester linkage occurred at the O-S bond. This O-S cleavage mechanism is common to all known arylsulphatases (Dodgson et al., 1982), the P1 alkylsulphatase of *Pseudomonas* C12B (Cloves et al., 1977), the 2,4-dichlorophenoxyethyl sulphate sulphatase in *P. putida* (Lillis et al., 1983) and the D-lactate-2-sulphate sulphatase in *P. syringae* (Crescenzi et al., 1984). The C-O mode of fission is confined, so far, to the other long-chain primary alkylsulphatase (P2) and three secondary alkylsulphatases of *Pseudomonas* C12B, and a secondary long-chain alkylsulphatase in *Comamonas terrigena* (for review, see White and Russell, 1993).

Earlier claims for the existence of a sulphatase acting on methyl sulphate in *Hyphomicrobium* spp. (Ghisalba and Kuenzi, 1983; Ghisalba et al., 1985, 1986) have recently been challenged, and there is now good evidence that sulphate liberation from methyl sulphate occurred not via a sulphatase-mediated hydrolysis, but by mono-oxygenation to methandiol monosulphate and subsequent elimination of sulphate to yield SO_4^{2-} and formaldehyde (Davies et al., 1990; Higgins et al., 1993). Thus the metabolism of methyl sulphate reflects its nature as a C_1

compound, rather than as a sulphate ester. The mono-oxygenation pathway for the C_1 ester contrasts with the present study which has shown unequivocal involvement of a sulphatase in the biodegradation of C_3 – C_7 primary alkyl sulphates. It will now be interesting to complete the list by determining the initiating steps in the pathway for biodegradation of the C_2 compound, ethyl sulphate.

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