Arylsulfatase from Streptomyces griseorubiginosus S980-14

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A new arylsulfatase designated Es-1, which desulfated toposide 4'-sulfate and p-nitrophenol sulfate, was isolated from Streptomyces griseorubiginosus S980-14 and purified to protein homogeneity by ammonium sulfate fractionation, ion exchange column chromatography, and chromatofocusing. The enzyme was active in monomeric form with an approximate molecular weight of 45,000, had a pI value of 4.95, and required calcium for full activity. At an optimum reaction pH of 8.5, iodoacetate, mercuric chloride, and EDTA severely inhibited the activity of Es-1 arylsulfatase.

In the preceding paper,13 screening of bacteria, yeasts, fungi, and actinomycetes for an arylsulfatase that specifically desulfated toposide 4'-sulfate for tumor target therapy was reported to result in the isolation of Streptomyces griseorubiginosus S980-14 and Streptomyces sp. T109-3 as new arylsulfatase-producing streptomycetes. To date, several arylsulfatases such as those from Aerobacter aerogenes (type I), Helix pomatia (type II), limpets (type II), and Aspergillus oryzae (type I) are known in the literature, but show no desulfating activity toward toposide 4'-sulfate at all.14 Under such circumstances, it was indispensable to enzymologically characterize arylsulfatases of Streptomyces griseorubiginosus S980-14 and Streptomyces sp. T109-3 (designated Es-1 and Es-2 enzymes, respectively), particularly for objective evaluation of these enzymes as the enzyme component of antitumor antibody-enzyme conjugates in comparison with known arylsulfatases. Since arylsulfatases in this series of studies are intended to be used in human therapy, it was also essential that the final enzyme preparations were at least highly homogeneous for avoidance of eventual adverse effects. As the two arylsulfatase-producing streptomycetes hydrolyzed p-nitrophenyl sulfate and toposide 4'-sulfate, the current authors assumed that sulfated cellulose affinity column chromatography was the first choice for efficient and rapid isolation and purification of both enzymes. Contrary to their expectation, however, a preliminary test clearly showed that sulfated cellulose exhibited no affinity for Es-1 enzyme, while Es-2 enzyme was well adsorbed. Realizing that the two arylsulfatases were distinct in substrate recognition and binding, the current authors attempted to purify them to protein homogeneity so that the two enzymes in pure form might be enzymologically characterized and objectively evaluated for potential use in human therapy without clinical troubles.

This paper describes the isolation, purification, and enzymological characterization of Es-1 arylsulfatase. Characterization of Es-2 enzyme and the detailed comparison of the streptomycete arylsulfatases with known enzymes will be reported in the following paper.

Materials and Methods

Substrates and chromatographic materials. Arylsulfates (p-nitrophenyl sulfate Product No. N3877; 4-acetylphényl sulfate A5013; 4-methylumbelliferyl sulfate 57513; and p-nitrocatechol sulfate N7251), gluco-sulfates (β-glucose 6-sulfate G3899; and N-acetylglucosamine 3-sulfate A7786), cerebrosides sulfate (sulfatides S1006), sterol sulfates (androsterone sulfate A7758; and estrone 3-sulfate E3881), and chondroitin sulfates (chondroitin sulfate A or chondroitin 4-sulfate C8529; and chondroitin sulfate C or chondroitin 6-sulfate C4384) were purchased from Sigma Chemical Co. Etoposide 4'-sulfate (BMY-29070) and 2',3'-di-O-acetyltetradecyl sulfate 4'-sulfate (BMY-28986) (for their chemical structures refer to Fig. 1 in ref. 1) were synthesized as previously described. 15 Butyl-Toyopearl 650C and 650M, Cellulose GC-700-m, and DEAE-cellulose were obtained from Tosoh Co., Seikagaku Kogyo Co., Ltd., and Wako Pure Chemical Industries, respectively. A protein marker kit which consisted of horse myoglobin, chymotrypsinogen, and egg and bovine serum albumins was obtained from Serva Feinbiochemical. PBS 94 and Polybuffer 74 for chromatofocusing were purchased from Pharmacia LKB Biotechnology Co.

Streptomyces and cultivation. Streptomyces griseorubiginosus S980-14 was shake-cultivated at 28°C for 2 days in a 500-ml Erlenmeyer flask containing 100 ml of GY seed medium (1% glucose and 1% yeast extract). One milliliter of the seed culture was added to 100 ml of production medium (1% t-xylene, 1% yeast extract, 0.5% malt extract, and 0.1% CaCO3), pH 7.2 and cultivated for 4 days at 28°C.

Standard assay conditions. Unless otherwise specified, Es-1 arylsulfatase activity was assayed with p-nitrophenol sulfate (reference substrate) and is expressed by the amount of p-nitrophenol liberated. A reaction mixture consisted of 50 μl of 1.0 mg/ml p-nitrophenyl sulfate in 50 mM Tris/HCl buffer, pH 7.5, 50 μl of an enzyme solution, and 100 μl of 50 mM Tris/HCl buffer, pH 7.5. After incubation for 30 min at 37°C, the assay reaction was stopped by addition of 50 μl of 0.2 N NaOH. The liberated p-nitrophenol was measured at 405 nm in a Beckman DU-70 spectrophotometer, followed by absorbance-to-mole conversion using the ε value of p-nitrophenol (17,800 at 405 nm in 0.1 N NaOH). One unit of the enzyme was defined as the amount of enzyme that liberated 1 nmol of p-nitrophenol per min at 37°C.

1 New Arylsulfatases from Streptomyces (II).

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**Streptomyces griseorubiginosus Arysulfatase**

**Determination of substrate specificity.** 4-Acetylphenyl sulfate (20,500 at 323 nm), 4-methylumbelliferyl sulfate (ε 18,400 at 362 nm), and p-nitroacetophenol sulfate (ε 12,400 at 510 nm) were incubated under conditions similar to the standard assay conditions. Two tosopside derivatives (tosopside 4'-sulfate and 2',3',4'-di-O-acetyl-etosopside 4'-sulfate) were assayed by incubating a mixture of 100 μl of 1.0 mg/ml tosopside substrate in 50 mM Tris/HCl buffer, pH 7.5, 50 μl of enzyme solution, and 50 μl of 50 mM Tris/HCl buffer, pH 7.5, for 30 min at 37°C. Thirty microliters of the reaction solution was spotted on a silica gel 60 F_{254} TLC plate (E. Merck, Darmstadt) and developed with a solvent system of chloroform and MeOH (10:1). The amount of the tosopside liberated was monitored by spectrophotometry at 254 nm using a Shimadzu CS-910 Dual-Wavelength TLC scanner. Sulfatides, mitomycin C sulfate (4- or 7-sulfate), glucose 6-sulfate, and N-acetyl-glucosamine 3-sulfate were tested by a slight modification of the method of Lloyd. Susceptibility of chondroitin sulfates A and C, and chondroitin sulfate, and estrone 3-sulfate to Es-1 was examined by detection of free sulfuryl ion using the method of Sandberg and Jenkins.

**Protein assay.** Protein contents of enzyme solutions and column chromatographic fractions were measured with a Bio-Rad protein assay kit (No. 500-0006, Bio-Rad Laboratories) and/or by spectrophotometry at 280 nm using bovine serum albumin as the reference standard.

**Molecular weight estimation.** The Cellulofine GC 700-m Es-1 arylsulfatase preparation (Table I) was subjected to gel filtration on TSK-Gel Toyopearl HW-55F (2.5 x 70 cm); No. 7457, Tosoh Co.) for molecular weight estimation. The void volume of the TSK-Gel Toyopearl HW-55F column was measured with Blue Dextran 2000. The elution peaks of myoglobin, chymotrypsinogen, albumins (markers), and Es-1 were spectrophotometrically monitored at 280 nm.

**Electrophoresis.** SDS PAGE was done at room temperature by the method of Laemmli using 12.5% polyacrylamide gel containing 1% SDS and bromophenol blue as a marker. After electrophoresis, the SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue R or with a silver stain kit (2D-Silver Stain II “DAICHI”, Daiichi Pure Chemical Co., Ltd.). For standardization, an SDS-PAGE low range standard kit (No. 161-0304, Bio-Rad Laboratories Co.) which was composed of molecular phosphorylase B (mol. wt. 97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (16,000) was used.

**Enzymological characterization.** The optimal reaction pH for Es-1 was determined in 50 mM (final concentration) sodium acetate (pH 4.0-5.5), Tris/maleate (pH 5.5-8.0), Tris/HCl (pH 7.5-9.0), and glycine/NaOH (pH 9.0-10.0) buffers. The pH stability was examined by incubating the enzyme at 37°C for 30 min in 50 mM (final concentration) sodium acetate (pH 4.0-5.5), Tris/maleate (pH 5.5-8.0), Tris/HCl (pH 7.5-9.0), and glycine/NaOH (pH 9.0-10.0) buffers. After the reaction mixtures were diluted with the same volume of 0.5 M Tris/HCl buffer, pH 7.5, the remaining enzymatic activities were measured under the standard assay conditions. The optimal reaction temperature was determined by relative comparison of initial reaction velocities at the indicated temperatures. The thermostability profile of the enzyme was obtained by incubating the standard assay mixtures for 30 min at the indicated temperatures, followed by analysis of the remaining arylsulfatase activities.

**Kinetic study.** Fifty microliters (1 unit) of the Cellulofine GC 700-m Es-1 solution was diluted with 350 μl of 0.5 M MOPS [3-(N-morpholino)propanesulfonic acid] buffer, pH 7.4, in the presence or absence of 1 mM calcium chloride, and then mixed with 100 μl of p-nitrophenol sulfate solution as substrate from 3.7 to 17.μM in 0.5 M MOPS buffer, pH 7.4. After incubation at 37°C for 3 min, the p-nitrophenol released was measured by spectrophotometry at 405 nm. The Michaelis constant (K_m) was measured by the Lineweaver-Burk, Eadie-Hofstee, Scatchard, Hanes-Woolf, and direct linear plots and the non-linear regression, Wilkinson, and Bannister methods using initial reaction velocity data (0-3 min) and a personal computer program designated DHIDS.3,4

**EDTA treatment and reactivation.** For complete depletion of metal from the active enzyme, 900 μl of the Cellulofine GC 700-m Es-1 preparation was mixed with 100 μl of 10 mM EDTA and incubated at 4°C for 30 min. The mixture was dialyzed twice for 6 h each in 1000 ml of 10 mM Tris/HCl buffer, pH 7.5, containing 1 mM EDTA, and then three times for 6 h each in 1000 ml of 10 mM Tris/HCl buffer, pH 7.5, containing no EDTA.

Complete absence of the desulfation activity in the EDTA-treated Es-1 preparation was confirmed under the standard assay conditions.

**Results**

**Isolation and purification of Es-1**

All isolation and purification steps were done at 4°C in a cold room, using 50 mM Tris/HCl buffer, pH 7.5, unless otherwise specified. As described above, a preliminary test showed that Es-1 was not adsorbed on sulfated cellulose in spite of desulfation activity, suggesting that the enzyme recognized and bound to the tosopside (aryl) portion of tosopside 4'-sulfate. This was one of the critical differences of Es-1 from the other streptomycete arylsulfatase (Es-2; see the subsequent paper). Accordingly, for purification to protein homogeneity, Es-1 required more steps than Es-2.

Broth filtrate (4800 ml) of Streptomyces griseorubiginosus S980-14 was condensed to 460 ml by ultrafiltration with a UF-module (M.W. > 5000, Asahi Kasei Co.). The total desulfation activity after condensation was 3934 units. Fine powder of ammonium sulfate to 30% saturation was slowly added at 4°C to the enzyme concentrate under agitation, while the pH of the solution was maintained in a pH range of 7.0-7.5 with 4N NH_4OH. After stirring for 30 min, the solution was left overnight at 4°C. Precipitates were removed by centrifugation at 13,000 rpm for 15 min and the supernatant was put on a Butyl-Toyopearl 650C chromatographic column (1.5 x 20 cm; #07473, Tosoh Co.) that had been equilibrated with 50 mM Tris/HCl buffer, pH 7.5, containing ammonium sulfate at 30% saturation. After the column was washed with the 30%-saturated ammonium sulfate-containing buffer, the enzyme was eluted with 50 mM Tris/HCl buffer, pH 7.5, containing no ammonium sulfate (Fig. 1). Combined active fractions (28 ml in total) were put on a DEAE-cellulose chromatographic column (2.5 x 25 cm) which had been equilibrated with 50 mM Tris/HCl buffer, pH 7.5. After the column was washed with 180 ml of the same buffer, the enzyme activity was recovered by a linearly increasing concentration gradient of the same buffer from 0.05 to 0.4 M. By the same procedure as detailed above for the first Butyl-Toyopearl 650C column chromatography, finely powdered ammonium sulfate to 30% saturation was added to the combined active fractions (252 ml) and the Es-1 supernatant was adsorbed on the second Butyl-Toyopearl 650M column. After washed with 50 mM Tris/HCl buffer, pH 7.5, containing 30%-saturated ammonium sulfate, the column was eluted by a linearly decreasing concentration gradient of ammonium sulfate from 30 to 10% in 50 mM Tris/HCl buffer, pH 7.5. Collected active fractions (72 ml) were dialyzed against 5 liters of 25 mM imidazole/HCl buffer, pH 7.4, and then concentrated to 14 ml by ultrafiltration with a UHP-43 module and a UK-10 ultrafilter (Toyoo Roishi Kaisha, Ltd.). The enzyme concentrate was put on a PBE 94 chromatofocusing column (1.5 x 18 cm, Pharmacia LKB Bio-technology Co.) that had been equilibrated with 25 mM imidazole/HCl buffer, pH 7.4. After the column was rinsed, the enzyme was eluted by a linearly decreasing pH gradient of Polybuffer 74/HCl buffer from 7.0 to 4.0, as illustrated in Fig. 2. Each eluate fraction was buffered with 0.5 M Tris/HCl buffer, pH 7.5, immediately after elution. Active fractions were gathered (15 ml) and concentrated to 4.5 ml.
by UK-10 ultrafiltration. For removal of Polybuffer 74 and protein impurities, the Es-1 concentrate was passed through a Cellulofine GC 700-m chromatographic column (2.5 × 70 cm, Seikagaku Kogyo Co.) that had been buffered with 0.2 M Tris/HCl, pH 7.4. Active fractions were combined (25 ml) and condensed to about 5 ml by UK-10 ultrafiltration.

Protein homogeneity of this Cellulofine GC 700-m Es-1 ariysulfatase preparation was checked by SDS-PAGE (Fig. 3), apparently showing that no protein impurity was present. Table I lists the yield of ariysulfatase at each isolation and purification step. As a whole, Es-1 (the Cellulofine GC 700-m Es-1 ariysulfatase preparation) was successfully purified 800-fold in 4.4% yield from the broth.
filtrate of *Streptomyces griseorubiginosus* S980-14.

**Enzymological characteristics**

The isoelectric point of Es-1 was read to be 4.95 from Fig. 2. SDS-PAGE showed that Es-1 had an approximate molecular weight of 45,000 (Fig. 3). For analysis of the possible polymeric composition, the enzyme preparation was tested by 1% mercaptoethanol-SDS–PAGE, but only one protein band was detected at a molecular weight of 45,000 (data not shown). Consequently, Es-1 was concluded to be a monomer having a molecular weight of 45,000. Es-1 was stable at temperatures below 40°C (Fig. 4a). At higher temperatures, the enzyme was rapidly inactivated, with no activity at 70°C. The effects of incubation temperature on the reaction velocity are presented in Fig. 4b, indicating that Es-1 had an optimal reaction temperature of 37°C.

The pH stability profile of Es-1 (Fig. 5a) discloses that the enzyme was most stable in the range of pH 8–9.5 and became more labile in the acidic range than in the alkaline range. As seen in Fig. 5b, Es-1 arylsulfatase was the most active at pH 8.5 in Tris/HCl buffer. It is important to note that the pH profile of this enzyme, although very narrow, is suitable for use in animals. Effects of divalent metal ions on Es-1 (Table II) demonstrate that the tested model ions other than calcium were found to be inhibitory to a more or less significant extent, while only calcium had no adverse effect on the enzyme activity. These results suggest that Es-1 might require calcium ion for the enzymatic activity, and that metal ions other than calcium might expel calcium from the enzyme protein, leading to marked inhibition of the desulfation activity [this may explain why little enzyme activity was produced in fermentation media containing tap water (Yamamoto et al.)].

### Table I. Summary of Purification of Es-1 Arylsulfatase

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total act. (U)</th>
<th>Spec. act. (U/mg)</th>
<th>Rel. sp. act. (x)</th>
<th>Recovery (%)</th>
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<tbody>
<tr>
<td>Broth filtrate</td>
<td>4800</td>
<td>3110</td>
<td>4420</td>
<td>1.4</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td>UF-module concentrate</td>
<td>460</td>
<td>2640</td>
<td>3934</td>
<td>1.5</td>
<td>1.1</td>
<td>89.0</td>
</tr>
<tr>
<td>1st Butyl-Toyopearl 650C</td>
<td>28</td>
<td>217.6</td>
<td>2330</td>
<td>10.7</td>
<td>7.5</td>
<td>52.7</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>252</td>
<td>42.0</td>
<td>1136</td>
<td>27.0</td>
<td>19.0</td>
<td>25.7</td>
</tr>
<tr>
<td>2nd Butyl-Toyopearl 650C</td>
<td>14</td>
<td>2.9</td>
<td>376</td>
<td>129.5</td>
<td>91.2</td>
<td>8.5</td>
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<tr>
<td>Chromatofocusing</td>
<td>4.5</td>
<td>0.39</td>
<td>270</td>
<td>691.8</td>
<td>487.2</td>
<td>6.1</td>
</tr>
<tr>
<td>Cellulofine GC 700-m</td>
<td>5</td>
<td>0.17</td>
<td>195</td>
<td>1144.7</td>
<td>806.1</td>
<td>4.4</td>
</tr>
</tbody>
</table>

**Fig. 4.** Thermostability and Temperature Dependence of Es-1 Arylsulfatase.

Enzyme activity was expressed in percentage relative to the control activity observed at 4°C.
Fig. 5. pH Stability and Dependence of Es-1 Arysulfatase. See the text for details.

Table II. Inhibitory Effects of 1 mM Metal Ions on Es-1 Arysulfatase

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>43</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>34</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>61</td>
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<tr>
<td>BaCl₂</td>
<td>21</td>
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<tr>
<td>CaCl₂</td>
<td>47</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>64</td>
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<tr>
<td>FeCl₂</td>
<td>48</td>
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<tr>
<td>CaCl₂</td>
<td>0</td>
</tr>
<tr>
<td>HgCl₂</td>
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<tr>
<td>AlCl₃</td>
<td>43</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>51</td>
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</tbody>
</table>

Table IV. Effects of 1 mM Inhibitors on Es-1 Arysulfatase

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<tr>
<th>Inhibitor</th>
<th>Percent inhibition</th>
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<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>83</td>
</tr>
<tr>
<td>1,3-Phenanthrolone</td>
<td>51</td>
</tr>
<tr>
<td>Cysteine</td>
<td>41</td>
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<tr>
<td>Citrate</td>
<td>72</td>
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<tr>
<td>p-Chloromercuribenzoate</td>
<td>69</td>
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<tr>
<td>N-Ethylmaleimide</td>
<td>44</td>
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<tr>
<td>Iodoacetate</td>
<td>100</td>
</tr>
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<td>2-Mercaptoethanol</td>
<td>4</td>
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<tr>
<td>Dithiothreitol</td>
<td>37</td>
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<tr>
<td>Imidazole</td>
<td>0</td>
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<tr>
<td>p-(Aminophenyl)methanesulfonyl fluoride HCl</td>
<td>22</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>100</td>
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</table>

Table III. Reactivating Effects of 1 mM Metal Ions on EDTA-treated Es-1 Arysulfatase

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Percent reactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>0</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>0</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>0</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0</td>
</tr>
<tr>
<td>BaCl₂</td>
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<td>CaCl₂</td>
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<td>FeCl₂</td>
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<td>CaCl₂</td>
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<tr>
<td>AlCl₃</td>
<td>0</td>
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<tr>
<td>FeCl₃</td>
<td>0</td>
</tr>
</tbody>
</table>

Table V. Inhibitory Effects of 5 mM Acid Radicals on Es-1 Arysulfatase

<table>
<thead>
<tr>
<th>Acid radical</th>
<th>Percent inhibition</th>
</tr>
</thead>
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<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>70</td>
</tr>
<tr>
<td>NaCl</td>
<td>78</td>
</tr>
<tr>
<td>KCl</td>
<td>41</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>63</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>65</td>
</tr>
<tr>
<td>K₃SO₄</td>
<td>48</td>
</tr>
</tbody>
</table>

Essential role of calcium as cofactor

For examination of the above-described conjectures, the Es-1 preparation was demetalized by repeated dialysis in 10 mM Tris/HCl buffer, pH 7.5, containing 1 mM EDTA. As expected, the dialyzed enzyme preparation completely lost the desulfation activity, which clearly showed that some metal was essential for desulfation activity. Subsequently, the enzyme-reactivating effects of 1 mM metal ions on the EDTA-inactivated enzyme preparation were compared (Table III). The EDTA-inactivated Es-1 preparation was reactivated to the original level of enzyme activity with calcium chloride only, while the other metal ions showed no reactivating effect at all. As a result, it is evident that Es-1 specifically required calcium as a cofactor for full enzymic activity.

Effects of Inhibitors

The Es-1 preparation was assayed in the presence and absence of a variety of enzyme inhibitor compounds (Table IV). SH-inhibitors such as iodoacetic acid, mercurous
chloride, and \( p \)-chloromercuribenzoic acid (PCMB) and chelating agents such as EDTA and 1,10-phenanthroline severely inhibited Es-1. Known arylsulfates from \textit{Aerobacter aerogenes} and \textit{Aspergillus oryzae}, in contrast, are described as being resistant to EDTA, because no metal cofactor is necessary for enzyme activity.\(^7\) As type II arylsulfatases are known to be markedly inhibited by acid radicals such as phosphate, sulfate, and chloride, the inhibitory effects of acid radicals were examined on Es-1 arylsulfatase (Table V). Like known type II arylsulfatases, Es-1 arylsulfatase was also significantly inhibited by chloride, phosphate, and sulfate.\(^9\)

**Substrate specificity**

Table VI summarizes the desulfation rates of Es-1 on a variety of sulfate substrates. It was obviously valid to include this enzyme in the arylsulfatase family, as potent activities were observed on the key arylsulfatases, while the sugar sulfates were insusceptible. It is noteworthy, however, that commercially available types I and II arylsulfatases such as \textit{Aerobacter aerogenes} and \textit{Helix pomatia} arylsulfatases\(^7\) had no desulfation activity on the etoposide 4'-sulfates at all, while Es-1 efficiently desulfated them.

**Kinetic properties**

The EDTA-treated Es-1 arylsulfatase preparation was assayed in the presence and absence of 1 mM calcium chloride at varied concentrations of \( p \)-nitrophenyl sulfate from 3.7 to 17 \( \mu \)M. The \( K_m \) of the calcium-reactivated Es-1 preparation was calculated to be approximately 4 \( \mu \)M in the -45-degree-rotated Eadie–Hofstee space\(^5,6\) as well as by the other 7 methods (Fig. 6).

Similar kinetic parameters (\( K_m \) and \( V_{max} \)) were also obtained with the intact (i.e., EDTA-nontreated) Es-1 preparation, showing that the EDTA treatment induced no irreversible change in the enzyme through calcium depletion. Type I \textit{Aerobacter aerogenes} and \textit{Klebsiella aerogenes} arylsulfatases are reported to have \( K_m \)'s of 3.3 and 9 \( \mu \)M, respectively, for \( p \)-nitrophenyl sulfate.\(^7,10\) Consequently, Es-1 showed a 1000-fold higher affinity to \( p \)-nitrophenyl sulfate than the bacterial enzymes.

**Discussion**

Arylsulfatases exist in a wide variety of plants, animals,
and microorganisms, and have been classified as 2 types based on their subcellular distribution, chemical and physical properties, and enzymological characteristics.\textsuperscript{9–13)}

Generally speaking, type I arylsulfatases have neutral to alkaline pH optima, are not inhibited by $K_2SO_4$ and $K_2HPO_4$, preferentially hydrolyze synthetic phenolic substrates such as $p$-nitrophenyl sulfate and $p$-acetylphenyl sulfate. Type II arylsulfatases, in contrast, have acidic pH optima, are most often localized in lysosomes, are inhibited by $K_2SO_4$ and $K_2HPO_4$, and preferentially hydrolyze $p$-nitrocatechol sulfate.

The enzymological characteristics of Es-1 evidently showed that the enzyme belonged to neither of the two known types of arylsulfatases. In addition, the calcium requirement as cofactor has not been observed in hitherto known arylsulfatases, although ox liver and sea urchin arylsulfatases contain calcium.\textsuperscript{14,15)} Accordingly it seems reasonable to consider that Es-1 constitutes a third and new type of arylsulfatase.

In addition to the reactivity on etoposide 4'-sulfate, the very high affinity ($K_m$ 4 $\mu$M to $p$-nitrophenyl sulfate) and the reaction pH optimum of 5.5 of Es-1 are clearly advantageous over known arylsulfatases, particularly because this enzyme is intended to be used for site-specific and efficient desulfation of low concentrations of the least cytotoxic etoposide 4'-sulfate to highly cytotoxic etoposide at tumor sites of a host animal by target therapy using the monoclonal antitumor antibody-enzyme conjugate.

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References