Isolation and characterization of the pig endometrial arylsulphatase A

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1. The pig endometrial arylsulphatase A was purified 3322-fold to a specific activity of $150\mu\text{mol/min per mg}$. The purification involved $(\text{NH}_4)_2\text{SO}_4$ fractionation, chromatography on concanavalin A–Sephadex and DEAE–Sephadex, gel filtrations on Sephadex G-200 at pH 7.4 and 5, and a new preparative gel-electrophoresis technique. 2. The homogeneous enzyme is a glycoprotein containing 20% carbohydrate. The purified enzyme has M_r about 120 000 and it contains subunits of M_r 63 000. 3. The pig endometrial arylsulphatase A shows many properties in common with those of arylsulphatases A purified from other sources. The similarities include their low isoelectric points, the anomalous time–activity relationships, multi-pH optima, inhibition by SO_3^{2-} , SO_4^{2-} , phosphate ions, metal ions and nucleoside phosphates, pH- and ionic-strength-dependent polymerization and amino acid composition.

Arylsulphatases (arylsulphate sulphonydrolases, EC 3.1.6.1) are distinct enzymes capable of hydrolysing arylsulphate ester linkages. Arylsulphatase A is an acid glycoprotein and B is a basic glycoprotein. Arylsulphatases have been purified from various tissues, mammalian semen, testes and urine (see Farooqui & Srivastava, 1979). Among the reproductive processes, sulphated polysaccharides participate in ovulation (Gerbauer *et al.*, 1978) and in the regulation and expansion of the cumulus oophorus (Eppig, 1981). The sulphated glycolipid, namely seminolipid, which is a substrate for arylsulphatase A (Yamato *et al.*, 1974), is a constituent of the mammalian sperm membranes (Yung-hans *et al.*, 1974). Sterol sulphate present on the sperm plasma membrane is considered to be a membrane stabilizer (Lalumière *et al.*, 1975). Yanagimachi *et al.* (1972) suggested that the negative charge on the sperm plasma membrane might be due to sulphate groups. Since on incubation in the uterus the net negative surface charge on spermatozoa is decreased (Vaidya *et al.*, 1971), it appears that arylsulphatases may play some role in this process. As capacitation induces physicochemical changes on the sperm membranes, it seems likely that the endometrial arylsulphatases may participate in this process. The increase in arylsulphatase activity along with other lysosomal hydrolases in endometria of hormonally treated ovariectomized rabbits

may be involved in the capacitation and the implantation properties of the uterus (Rahi & Srivastava, 1981).

As this enzyme may play an important role in the reproductive process, we describe in the present paper the isolation, characterization and comparison of the pig endometrial arylsulphatase with the enzyme purified from other tissues. A new preparative electrophoresis technique is also described.

Materials and methods

Fresh pig uteri were obtained from the local abattoir. The following materials were obtained from the indicated sources. *p*-Nitrocatechol sulphate, *p*-nitrophenol, *p*-acetylphenyl sulphate, oestradiol 3-sulphate, oestrone 3-sulphate, heparin sulphate, chondroitin sulphate, D-glucose 6-sulphate, dihydroisandrosterone sulphate, acrylamide, *NN'*-methylenebisacrylamide, Coomassie Brilliant Blue G, nucleoside phosphates and concanavalin A were from Sigma Chemical Co., St. Louis, MO, U.S.A. The molecular-weight determination kit for gel filtration was from Pierce Chemical Co., Rockford, IL, U.S.A. The standard protein kit for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, Sephadex G-200, Blue Dextran 2000 and concanavalin A–Sephadex were purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. The Ampholine carrier ampholytes were obtained from LKB Instruments, Rockville, MD, U.S.A.

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Gelatin was from W. H. Curtin Co., Dallas, TX, U.S.A. Rhodizonate (dipotassium salt) was from Eastman Chemical Co., Rochester, NY, U.S.A. Ascorbic acid 2-sulphate was kindly provided by Dr G. Rebel (Faculté de Medecine, Institut de Chimie Biologie, Strasbourg, France). Wheat-germ agglutinin was from Boehringer, Mannheim, Germany. *Ulex europaeus* agglutinin I, *Ricinus communis* agglutinin 120 and soya-bean agglutinin were from Miles Laboratories, Kankakee, IL, U.S.A. Hyamine 2389 and Triton X-100 were purchased from Rohm and Haas Co., Philadelphia, PA, U.S.A. Bovine brain phosphodiesterase was kindly provided by Dr. H. W. Jarrett (of this Department). All other chemicals used were of analytical grade.

Assay of enzyme activity

Arylsulphatase activity was measured by following the release of *p*-nitrocatechol from *p*-nitrocatechol sulphate by incubating the enzyme with 10mM substrate in 0.25M-sodium acetate buffer, pH 5.5, as described by Farooqui & Srivastava (1979). One unit of enzyme is defined as the amount liberating 1 μ mol of *p*-nitrocatechol/min. The substrate specificity was assayed by the method of Dodgson (1961).

Cyclic AMP phosphodiesterase assay

The arylsulphatase A cyclic AMP phosphodiesterase activity was assayed with 1mM-cyclic AMP and 1 μ g of the homogeneous arylsulphatase A in a total volume of 0.1ml. The resulting AMP was converted into ATP and measured as described by Anderson *et al.* (1980).

Determination of protein

Protein was determined by the method of Bradford (1976), with bovine serum albumin as the standard. The chromatographic profiles were based on A_{280} .

Procedure for purification of arylsulphatase A from pig endometrium

Unless otherwise stated, all operations were performed at 0–4°C.

Preparation of endometrium extracts. For each preparation five to ten fresh pig endometria were scraped with a razor blade in 50mM-Tris/HCl buffer, pH 7.4, containing 0.5M-NaCl, 0.2% Hyamine 2389 and 0.2% Triton X-100, and homogenized in 1–2 litres of the same buffer in a Waring blender for 2min. The homogenate was stirred for 1h, and then centrifuged at 17300g for 30min. The supernatant was gradually mixed with 2vol. of cold 95% (v/v) ethanol (–20°C), and left for 5–12h at –20°C. The precipitated protein, collected by centrifugation at 10400g for 30min, was dried under vacuum. The combined ethanol-dried endo-

metrial extracts from 32 animals were stirred at room temperature for 1h, with 1600ml of 20mM-Tris/HCl buffer, pH 7.4, containing 0.25M-NaCl. The mixture was centrifuged at 30900g for 30min, and the supernatant was retained. The pellet was resuspended in the same buffer, stirred once again for 1h and centrifuged as above. The two supernatants were combined and subjected to the $(\text{NH}_4)_2\text{SO}_4$ fractionation.

Ammonium sulphate fractionation. The supernatant was made 60% saturated by addition of solid $(\text{NH}_4)_2\text{SO}_4$ with constant mechanical stirring. After standing overnight at 4°C, it was centrifuged at 30900g for 30min. The pellet was dissolved in 250ml of cold water and dialysed against the same until free from sulphate. Finally the solution was dialysed against 10vol. of 20mM-Tris/HCl buffer, pH 7.4, containing 1M-NaCl, 1mM-MnCl₂ and 1mM-CaCl₂ for 18h at 4°C.

Concanavalin A–Sephadex chromatography. The dialysed solution was subjected to a concanavalin A–Sephadex column chromatography as described by Farooqui & Srivastava (1979). The fractions containing arylsulphatase were pooled, concentrated to 50ml on an Amicon cell (UM 10) and dialysed against 10vol. of 20mM-Tris/HCl buffer, pH 7.4, for 18h at 4°C. The precipitate that appeared during the dialysis was removed by centrifugation at 27000g for 30min. The clear supernatant was subjected to DEAE-Sephadex 4B chromatography.

DEAE-Sephadex 4B chromatography. The above solution was applied to a column (2cm $\times$ 24cm) of DEAE-Sephadex 4B equilibrated with 20mM-Tris/HCl buffer, pH 7.4. The column was eluted first with the buffer, and fractions (4.5ml each) containing arylsulphatase B were pooled. The elution was continued with a linear gradient of 0–0.5M-NaCl in the same buffer. Fractions with arylsulphatase A activity were pooled and concentrated to 5ml by ultrafiltration.

Gel filtration on Sephadex G-200 at pH 7.4. The concentrated DEAE-Sephadex fraction was applied to a Sephadex G-200 column (2.5cm $\times$ 90cm), pre-equilibrated with 20mM-Tris/HCl buffer, pH 7.4, containing 0.15M-NaCl. The column was eluted with the buffer at 12ml/h. The active fractions were pooled and concentrated by 5ml by ultrafiltration.

Gel filtration on Sephadex G-200 at pH 5.0. The above sample, dialysed against 25mM-sodium acetate, pH 5.0, containing 0.15M-NaCl, was applied to the same Sephadex G-200 column pre-equilibrated and eluted with the above buffer. Fractions having maximum arylsulphatase A activity were pooled and dialysed against 5mM-Tris/glycine buffer, pH 8.3. This preparation was concentrated and used for preparative gel electrophoresis.

Preparative gel electrophoresis. The preparative

electrophoresis apparatus was designed by us (Fig. 1). Matching 'male' and 'female' ground-glass fittings are depicted on each of the apparatus sections.

The primary electrophoretic column *A* is a cooling-jacketed glass column (2.5 cm × 30 cm). The column gel between the angled vent (*d*) and the upper vent (*e*) was prepared as follows. A large rubber stopper was inserted in the upper column end just below the three upper vents. The column was then inverted and the gel solution was poured into the column between the stopper and a few centimetres below the angled bubble vent. After complete polymerization, the stacking gel was applied immediately below the air-bubble vent (*d*). The column was then inserted into one end of the U-shaped buffer vessel. The other buffer reservoir was attached to the other end of the U-shaped vessel, and the buffer was added until the fluid level in it was above the bottom of the gel in the column. Trapped air bubbles were removed through the three sample vents and then the vents were closed.

Fig. 1. Apparatus used for the preparative gel electrophoresis

Key: *a*, inlet for cooling liquid; *b*, outlet for cooling liquid; *c*, cooling mantle; *d*, vent for air bubble; *e* and *f*, sample-collecting vents; *g*, inlet for elution buffer; *h*, stacking gel; *k*, running gel; *m*, polyacrylamide blocking gel; *n*, dialysis tubing; *A*, electrophoretic column; *B*, lower buffer vessel; *C* and *D*, buffer reservoir beakers.

The protein sample and tracking dye were layered on the top of the stacking gel. After the protein had been electrophoresed into the running gel, the electrophoresis was continued until the tracking dye reached to within a few centimetres from the bottom of the gel. Then the electrophoresis column (*A*) was removed, inverted and re-inserted into the other end of the U-shaped vessel, and the lower system was again filled with the buffer up to the lower gel surface. The air bubbles were removed and more buffer was added to the lower buffer reservoir. On the upper end of the gel, a buffer reservoir (*D*), with its mouth blocked with 20% polyacrylamide gel (*m*) and covered with a dialysis membrane (*n*), was inserted. The electrodes were re-attached with the positive electrode now being on the upper end of the column, and the electrophoresis was continued. As proteins were eluted into the buffer on the upper surface of the gel, a continuous buffer flow was maintained across the gel surface via the inlet for the elution buffer (*g*). The two sample vents (*e* and *f*) removed the proteins in solution to a fraction collector.

The above apparatus was used for the final purification of arylsulphatase A. The standard alkaline-discontinuous-buffer gel-electrophoresis system described by Brewer & Ashworth (1969) was applied, with a 7.5% running gel and a 5% stacking gel. The gels were pre-electrophoresed for 4 h to remove ammonium persulphate. The sample contained 10% (w/v) sucrose and 2 µg of Bromophenol Blue in a total volume of 1 ml. The voltage and current applied were 330 V and 12 mA at the beginning of the experiment and 550 V and 9 mA before elution of the proteins. The elution and electrophoresis buffer was 5 mM-Tris/glycine, pH 8.3. The elution started after 11 h when the tracking dye reached near the surface of the gel. The elution was continued for 20 h at the rate of 95 ml/h, and 5.5 ml fractions were collected. Since the position of the arylsulphatase A as the first emerging protein band had already been determined from the results of the analytical disc-gel electrophoresis, the first 110 fractions, with high arylsulphatase activity, were combined and concentrated to 5.6 ml. The concentrated sample, dialysed against 20 mM-Tris/HCl buffer, pH 7.4, containing 5% (v/v) glycerol was divided in small tubes and kept frozen (−20°C) for further experiments.

pH optima

The pH optima of arylsulphatase A were determined by performing the assay in 0.1 M-glycine/HCl buffer, pH 3–3.5, 0.2 M-sodium acetate buffer, pH 4–6.5, and 0.05 M-Tris/HCl buffer, pH 6.5–9, at a final substrate concentration of 8 mM-nitrocatechol sulphate.

Determination of kinetic parameters

The substrate optimum was assessed by varying the nitrocatechol sulphate concentration from 0.25 to 20 mM in 0.25 M-sodium acetate buffer, pH 5.5. $V_{\max}$, K_m and K'_m were calculated from the linear plots of the reciprocal of the reaction velocity versus the reciprocal of the substrate concentration (Lineweaver & Burk, 1934). Inhibition constants (K_i) were determined by using the relation:

$$K'_m = K_m(1 + [I]/K_i)$$

where K'_m is the K_m in the presence of a defined concentration of the inhibitor [I]. In most cases, results from a control and two different inhibitor concentrations were used to determine the inhibition constant.

The time-enzyme-activity relationship was determined by a discontinuous method. The tubes containing the reaction mixture were removed at appropriately spaced time intervals and the reaction was immediately stopped with 2.5 ml of 1.0 M-NaOH.

Effect of inhibitors and activators

The compounds under consideration were mixed with sodium acetate buffer, and the pH was checked and if necessary adjusted to pH 5.5 with 0.5 M-acetic acid or 0.4 M-NaOH. The enzyme (0.2–0.5 μ g of protein) was added to the mixture and kept at 37°C for 15 min. The reaction was started by the addition of 0.2 ml of 20 mM-nitrocatechol sulphate to give a final volume of 0.4 ml and a final concentration of 0.25 M-sodium acetate. The reaction was stopped after 5 min with 2.5 ml of 1 M-NaOH. The results were compared with the control containing all the components except the compound under study.

Isoelectric focusing of arylsulphatase A

The isoelectric focusing was performed on an LKB electrofocusing column as described by Yang & Srivastava (1976). Isoelectric focusing was performed at 5°C at 2–3 W for 48 h with a starting voltage of 500 V. At the end, fractions (1.8 ml) were collected at a rate of 30 ml/h. The pH and arylsulphatase activity of each fraction were measured.

Analytical gel electrophoresis

Analytical alkaline gel electrophoresis was performed in 7.5% polyacrylamide gels at pH 7.2 and pH 8.3 for 3 h at a current density of 1.5 mA/tube by the method of Brewer & Ashworth (1969). Bromophenol Blue was used as the tracking dye. The gels were fixed and stained with 0.04% Coomassie Brilliant Blue G-250 dye in 3.5% (v/v) HClO_4 and destained with water (Reisner *et al.*, 1975). For activity determinations unstained gels were cut into

25 mm segments and incubated in the assay system for arylsulphatase for 30–120 min.

Determination of relative molecular mass

The M_r of the enzyme was determined by gel filtration on a Sephadex G-200 column (48 cm $\times$ 2 cm) as described by Yang & Srivastava (1976). The enzyme was assumed to be a globular protein for derivation of the M_r .

The subunit M_r was estimated by using a 10% polyacrylamide gel and a discontinuous buffer system as described by King & Laemmli (1971). The homogeneous enzyme preparation and the standards from the Pharmacia Low Molecular Weight Calibration Kit (phosphorylase *b*, M_r 94 000; bovine serum albumin, M_r 67 000; ovalbumin, M_r 43 000; carbonic anhydrase, M_r 30 000; trypsin inhibitor, M_r 20 100; α -lactalbumin, M_r 14 400) were treated with 2.5% (w/v) sodium dodecyl sulphate and 5% (v/v) 2-mercaptoethanol at 100°C for 5 min. The protein bands were detected as for the analytical gel electrophoresis. The M_r of arylsulphatase A subunits was determined from the calibration curve established by plotting the logarithm of subunit M_r against the mobility of the standard proteins.

Low-speed sedimentation-equilibrium experiments were performed in the Beckman model E analytical ultracentrifuge, with the scanning absorbance optical system at 280 nm. Two different experiments were performed, with protein at initial concentrations of 0.36 and 0.5 mg/ml and rotor speeds of 4009 and 5270 rev./min respectively at 20°C. One sample was thoroughly dialysed against sodium acetate/NaCl buffer, pH 5.0 and *I* 0.1, and the other sample was dialysed against Tris/HCl/NaCl buffer, pH 7.4 and *I* 0.1; these were run simultaneously. The path-length of the ultracentrifuge cell was 12 mm. The partial specific volume of the protein was assumed to be 0.73 ml/g (Nichol & Roy, 1964). The M_r of arylsulphatase A was determined from the slope of a graph of $\log c$ versus r^2 by using the equation (Yphantis, 1964):

$$M_r = \frac{4.606 RT}{(1 - \bar{v}\rho)\omega^2} \cdot \frac{d(\log c)}{d(r^2)}$$

Amino acid and carbohydrate analysis

The amino acid analysis was performed on a Beckman model 119CL amino acid analyser. Hydrolysates were prepared by heating 50 μ g of the purified arylsulphatase A in 6 M-HCl *in vacuo* at 110°C for 24 h. The hydrolysate was evaporated to dryness under N_2 , dissolved in 0.5 ml of 0.2 M-sodium citrate buffer, pH 2.2, and analysed. The total neutral-sugar content of arylsulphatase A was determined by the anthrone/ H_2SO_4 reaction, with glucose as the standard (Ashwell, 1957).

Arylsulphatase A binding to concanavalin A and other lectins

Portions (50–100 µg) of concanavalin A, wheat-germ agglutinin, *Ulex europaeus* agglutinin I, *Ricinus communis* agglutinin 120 and soya-bean agglutinin were incubated separately with 50 µg of the purified arylsulphatase A at 4°C or 37°C for 1–12 h in 2.5 ml of 20 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl, 1 mM-CaCl₂ and 1 mM-MnCl₂. The formation of a lectin–enzyme complex was judged from the precipitate formed on centrifugation at 30900 g (Farooqui & Srivastava, 1981). If the above conditions did not result in the formation of the enzyme–lectin complex, the reaction mixture was divided in two, and the pH of one half was adjusted to 5.5 and to the other more lectins were added. The formation of enzyme–lectin complex was investigated in these preparations too.

Dispersal of cumulus oophorus by purified pig endometrial arylsulphatase A

This experiment was performed as described by Farooqui & Srivastava (1979). Freshly ovulated ova from superovulated rabbits were incubated with various amounts of purified arylsulphatase A. The dispersal of the cumulus matrix with time was observed at room temperature.

Results

Purification of pig endometrial arylsulphatase A

The results of arylsulphatase A purification are summarized in Table 1. The enzyme was purified 3322-fold to a specific activity of approx. 150 units (µmol/min)/mg and 23% overall recovery. The arylsulphatase activity in the initial homogenates from five different samples was 4.12 ± 1.2 units/g dry wt. of endometrium. The yield of arylsulphatase A was 5.88 mg/kg dry wt. of the endometrium.

Approx. 90% of the contaminant remaining from the previous steps was removed by the affinity chromatography on a concanavalin A–Sephacryl column. The retained enzyme could be eluted with 0.1–0.4 M-methyl α -D-mannoside. Further

purification on DEAE-Sephacryl chromatography indicated the presence of two forms of arylsulphatase, A and B. The enzyme in the washing of the column with the buffer alone was designated the B form, whereas the A form was eluted with 0.2 M-NaCl. In the pig endometrium, the B-form/A-form arylsulphatase ratio for three different preparations was 1.75 ± 1.0 . Because of the pH-dependent polymerization of arylsulphatase A, gel filtration on Sephadex G-200 at pH 7.4 and pH 5.0 (Figs. 2a and 2b) was used to remove most of the impurities. The gel electrophoresis showed only one contaminant. The final step, i.e. the preparative gel electrophoresis, resulted in a homogeneous enzyme preparation, as judged by disc-gel electrophoresis at pH 7.2 and 8.3, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and sedimentation-equilibrium centrifugation, with a recovery of 60% from the previous step. This step removed the impurities, which could not be separated by Sephacryl S-200 gel filtration, SP (sulphopropyl)-Sephacryl chromatography and a second DEAE-Sephacryl 4B column chromatography.

Related molecular mass

The results of determinations of the M_r of pig endometrium arylsulphatase A by gel filtration, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and sedimentation equilibrium are presented in Table 2. The linear plots of $\log A$ versus r^2 gave a calculated M_r of about 120 000 at pH 7.4 and 10.1. At pH 5.0 the M_r was more dependent on the protein concentration. Therefore the M_r and polymerization properties of pig endometrium arylsulphatase are similar to those of arylsulphatases A from other sources; although there are some differences, these may be more apparent than real. Thus the data in Table 2 indicate that the native molecule is a dimer consisting of two subunits each of M_r 63 000. The active enzyme can also exist as a tetramer (M_r 420 000) and probably a trimer (M_r 290 000), depending on the ionic strength, pH and protein concentration. The M_r values obtained for pig endometrium arylsulphatase A by sodium

Table 1. Summary of purification of pig endometrial arylsulphatase A
The definition of units and other details are given in the text.

Stage	Total protein (mg)	Activity (total units)	Specific activity (units/mg)	Yield (%)
Ethanol-dried endometrial extract	50817	2311	0.05	100
30900 g supernatant	7046	1494	0.21	64
0–60% satn.-(NH ₄) ₂ SO ₄ fraction	1622	1425	0.88	61
Concanavalin A–Sephacryl fraction	174	1256	7.22	54
DEAE-Sephacryl fraction	89	1362	19.80	59
Sephadex G-200 fraction, pH 7.4	25.4	1024	40.31	44
Sephadex G-200 fraction, pH 5.0	7.4	911	123.00	39
Preparative gel electrophoresis	3.6	549	149.52	23

Fig. 2. Sephadex G-200 gel filtration of arylsulphatase (a) at pH 7.4 and (b) at pH 5.0

(a) The concentrated DEAE-Sephacrose fraction was applied to a Sephadex G-200 column (2.5 cm $\times$ 90 cm) and eluted with 20 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl, at 12 ml/h, and 3.0 ml fractions were collected. (b) The fractions containing arylsulphatase from the above were pooled and rechromatographed on the same Sephadex G-200 column with 25 mM sodium acetate buffer, pH 5.0, containing 0.15 M-NaCl. Other details are given in the Materials and methods sections. $\blacktriangledown$, Arylsulphatase A; $\triangle$, A_{280} .

Table 2. Summary of data on the determination of the M_r of pig endometrium arylsulphatase A

Technique	pH	I	Concn. of protein	No. of expts.	M_r
Gel filtration	7.4	0.17	2.3–23 μ g/ml	3	140 000
	5.0	0.20	<6 μ g/ml	1	210 000
	5.0	0.20	>10 μ g/ml	2	420 000
Sedimentation equilibrium	7.4	0.10	0.5 mg/ml	1	120 000
	5.0	0.10	0.5 mg/ml	1	290 000
	7.4	0.10	0.32 mg/ml	1	120 000
	5.0	0.10	0.32 mg/ml	1	200 000
Sodium dodecyl sulphate/polacrylamide-gel electrophoresis	8.3	—	15 μ g	2	63 000

dodecyl sulphate/polyacrylamide-gel electrophoresis and gel filtration are in the range of those obtained for the same enzyme from different sources (Nicholls & Roy, 1971; Worwood *et al.*, 1973; Balasubramanian & Bachhawat, 1975; Shapira & Nadler, 1975; Waheed & Van Etten, 1979). However, the values obtained by the sedimentation-equilibrium method at pH 5.0 are more complex. For example, the value of 290 000 cannot be simply assigned either to a dimer or to a trimer. The discrepancy between the gel-filtration and the sedimentation results may be due to the high percentage (20%) of carbohydrate in this enzyme.

Amino acid and carbohydrate compositions

The partial amino acid composition of pig endometrial arylsulphatase A is presented in Table 3. These values are given as numbers of residues per

100 000 daltons of protein (Waheed & Van Etten, 1980), in order to compare them with the results obtained by other workers. The amino acid composition of pig endometrial arylsulphatase A is comparable with respect to several amino acids with that of other arylsulphatases A, especially the liver enzymes. Thus the pig enzyme also has a relatively high content of proline, glycine and leucine and low amounts of basic amino acids (Waheed & Van Etten, 1980).

The amino acid analysis of pig endometrium arylsulphatase A, like that of the rabbit liver enzyme (Waheed & Van Etten, 1980), also showed a so-called 'unusual peak' near the ascending edge of the histidine peak. This amino acid might be lysinoalanine (Waheed & Van Etten, 1980). No other arylsulphatase A is reported to show such an amino acid. The neutral sugar of pig endometrial arylsulphatase A, on the basis of two deter-

Table 3. *Partial amino acid composition of pig endometrial arylsulphatase A*

No corrections were made for the possible destruction during hydrolysis. N.D., Not determined.

Amino acid	Composition (residues/ 100 000 daltons)
Aspartic acid	72
Threonine	56
Serine	71
Glutamic acid	86
Proline	97
Glycine	115
Alanine	95
Half cystine	N.D.
Valine	52
Methionine	11
Isoleucine	14
Leucine	135
Tyrosine	23
Phenylalanine	47
Histidine	29
Lysine	14
Arginine	39
Tryptophan	N.D.

minations, was found to be $20 \pm 3\%$ by weight, a value that is not much different from the carbohydrate contents of the sheep brain enzyme (25%; Balasubramanian & Bacchawat, 1975) and the rabbit testis enzyme (20%; Farooqui & Srivastava, 1979). However, the value is much higher than the enzymes from rabbit liver (4.6%; Waheed & Van Etten, 1980) and ox liver (10%; Graham & Roy, 1973).

Effect of time of incubation on enzyme activity

It is well known that arylsulphatase A from many sources has an anomalous time-activity relationship with nitrocatechol sulphate as the substrate. The enzyme purified from pig endometrium showed similar behaviour.

pH optima

The optima were at pH 5.5 and 5.1, which are in the usual range of pH optima for arylsulphatases A from other sources (Yang & Srivastava, 1976).

Isoelectric point

The isoelectric point of pig endometrial arylsulphatase A was found to be 4.38, which is the same as that for the rabbit endometrium enzyme (H. Rahi & P. N. Srivastava, unpublished work). The isoelectric point of the ox liver enzyme is pH 3.4 (Nichol & Roy, 1964). Most other arylsulphatases A, including those from human urine (Stevens *et al.*, 1973), liver (Neuwelt *et al.*, 1972) and rabbit testis (Yang & Srivastava, 1976), show a pI value of 4.6, which is very near to the pI value of the endometrial enzyme. The relatively high content of potential

acidic amino acids and the low content of basic amino acids (Table 3) account for the low isoelectric point of arylsulphatase A enzymes.

Immobilization of pig endometrial arylsulphatase A on lectins

To investigate the functional importance of the different carbohydrate moieties of the arylsulphatase A, attempts were made to compare the properties of the free and the lectin-bound enzyme. The formation of lectin immobilized enzyme was monitored as described in the Materials and methods section. Among five lectins studied, only concanavalin A, which is specific for α -D-Glc(GlcNAc) and α -D-mannose, was able to complex with the enzyme. Other lectins, namely wheat-germ agglutinin, which is specific for GlcNAc- β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc, *Ulex europaeus* agglutinin I, specific for fucose, *Ricinus communis* agglutinin 120, specific for β -D-galactose, and soya-bean agglutinin, specific for D-N-acetyl-galactosamine, did not complex with the arylsulphatase A at 4°C or 37°C in pH 7.4 or pH 5.5 buffer at a lectin/enzyme concentration ratio of 2:1 or higher. Similar results have been reported with respect to the binding of the arylsulphatase of human liver homogenate to different lectins immobilized on agarose (Fiddler *et al.*, 1979).

The binding of arylsulphatase A from different sources to concanavalin A has been used as a method for purification of the enzyme (Farooqui & Srivastava, 1979).

Recently a nearly pure preparation of arylsulphatase A from bull seminal plasma was immobilized on concanavalin A (Farooqui & Srivastava, 1981). The immobilized enzyme showed a lower pH optimum, less inhibition by metal ions, sulphate ions and phosphate ions, increased K_m values and increased thermal stability as compared with the free enzyme.

Substrate specificity of pig endometrial arylsulphatase A

The substrate specificity of pig endometrium arylsulphatase A was tested on various sulphate esters as well as on cyclic AMP (Uchida *et al.*, 1981) (Table 4). The rate of hydrolysis of all substrates tested was low compared with that of 4-nitrocatechol sulphate. The rates of hydrolysis of ascorbate 2-sulphate and other sulphate esters by pig endometrium arylsulphatase A were lower than the rates of hydrolysis of the same by enzymes prepared from other sources (Farooqui & Srivastava, 1979). Also, pig endometrial arylsulphatase A did not hydrolyse chondroitin sulphate and heparin sulphate. The hydrolysis of some of the sulphate esters listed in Table 4 was also assayed by the method of

Table 4. *Substrate specificity of pig endometrium arylsulphatase A*

The activity towards nitrocatechol sulphate was assayed by measuring nitrocatechol released. The activities towards other sulphate esters was measured by the method of Dodgson (1961); the cyclic AMP phosphodiesterase activity was assayed by using the firefly bioluminescence assay described by Anderson *et al.* (1980).

Substrate	pH	Specific activity (nmol/min per mg of protein)
Nitrocatechol sulphate	5.5	138 000
	4.5	102 120
Ascorbate 2-sulphate	4.5	8840
<i>p</i> -Acetylphenyl sulphate	4.5	212
Phenolphthalein disulphate	4.5	40
Oestrane 3-sulphate	4.5	29
Glucose 6-sulphate	4.5	9
Dehydroisoandrosterone sulphate	4.5	8
Oestradiol 3-sulphate	4.5	8
Chondroitin sulphate (mixed isomers)	4.5	Not detectable
Heparin sulphate	4.5	Not detectable
Cyclic AMP $\pm$ Mg ²⁺	4.5	25
	8.0	0

Waheed & Van Etten (1978). The results were comparable with those obtained by the method of Dodgson (1961). Therefore the low activities measured for some of the sulphate esters are not due to the lack of efficiency of the method used for measuring the sulphate released.

The pig endometrial arylsulphatase showed some cyclic AMP phosphodiesterase activity at pH 4.5, and did not require Mg²⁺ ions. At pH 8.0, which is optimal for many phosphodiesterases, pig endometrium arylsulphatase A did not show any phosphodiesterase activity. The activity at pH 4.5 was about one-third of the phosphodiesterase activity reported for ox liver arylsulphatase A (Uchida *et al.*, 1981).

Enzyme stability

The impure enzyme with a specific activity of less than 10 units/mg was stable at 4°C for more than 1 month. Freezing (−20°C) also had no significant effect on such samples. However, purified enzyme at 10 µg of protein/ml lost 60% of its activity in each freeze–thaw cycle. At concentrations of 90 µg of protein/ml, the preparation was nearly stable to freezing and thawing. However, if such a sample was assayed just after thawing, less than 30% of the activity was found. If it was assayed after equilibrium at room temperature, or after being kept for a few hours in an ice bucket, the normal activity was obtained. This might be due to a slow conformational change occurring during freezing and thawing.

Table 5. *K_i values for some inhibitors of arylsulphatase A*

The substrate was 4-nitrocatechol sulphate. Other experimental conditions are given in the Materials and methods section.

Inhibitor	K _i (M)
Na ₂ SO ₃	2.5×10^{-6}
Na ₂ SO ₄	7.4×10^{-4}
P _i	2.5×10^{-4}
ATP	5.3×10^{-3}
ADP	4×10^{-3}
AMP	17.6×10^{-3}
UMP	17.5×10^{-3}
Cyclic AMP	17.9×10^{-3}
Citrate	2.37×10^{-3}

Kinetic parameters

The values of K_m and V_{max} for nitrocatechol sulphate were 2.1 mM and 173 µmol/min per mg of protein respectively. Further experiments gave K_m values of 2 mM and 1.8 mM. The duration of the incubation and the incubation temperature influence the kinetic parameters of arylsulphatase A (Luijten *et al.*, 1978), and this makes exact comparison of these values with the literature values somewhat difficult. However, the values obtained in the present work are in the range of those obtained with other arylsulphatases A (Yang & Srivastava, 1976; Farooqui & Srivastava, 1979).

Effects of inhibitory substances

Our results support the findings of other workers (Bleszynski & Leznicki, 1967; Farooqui & Srivastava, 1979) that various cations had an inhibitory effect on the enzyme activity. This effect was most marked with Cu²⁺, Ag⁺ and Hg²⁺ (60–90% inhibition) and less with Co²⁺, Fe³⁺, Fe²⁺ and Zn²⁺ (40% inhibition). Ascorbate, semicarbazide and hydroxylamine were potent inhibitors. Urea at a concentration of 3.2 M rendered the enzyme completely inactive. As with the ox brain enzyme (Bleszynski & Leznicki, 1967), a low concentration (up to 20 mM) of Tris slightly activated pig endometrial arylsulphatase.

Nucleoside monophosphatases at substrate-level concentration (20 mM) displayed inhibitory characteristics on the enzyme activity. However, cyclic AMP, at 5 mM concentration, slightly activated the enzyme.

The K_i values for some other inhibitors are given in Table 5. All these compounds inhibit arylsulphatase A activity towards nitrocatechol sulphate competitively. In general, the K_i values of phosphate and nucleoside phosphates are higher for pig endometrial arylsulphatase as compared with the arylsulphatases from other sources (Roy, 1953; Lee & Van Etten, 1975; Yang & Srivastava, 1976; O'Fagain *et al.*, 1980).

Since EDTA has no effect on arylsulphatase A activity, it appears that the inhibition by citrate is not a chelating effect. On the other hand, as an arginine residue has been shown to be essential for the arylsulphatase activity of the human liver enzyme, and it has been proposed that arylsulphatase A acts by interaction of this arginine with the negatively charged sulphate moiety of its substrate (Hook *et al.*, 1973), the action of all the above inhibitors including citrate may be only due to their negative charge.

Effect of pig endometrial arylsulphatase A on the cumulus oophorus

The pure pig arylsulphatase A disperses the cumulus oophorus of rabbit ova. There was no visible effect on the corona radiata and the zona pellucida of the ovum. The time required for dispersal of the cumulus was dependent on the enzyme concentration. The cumulus in the control, without the enzyme, remained intact for the whole day. These results are similar to those obtained by Farooqui & Srivastava (1979).

Discussion

Compared with other homogeneous preparations, the specific activity of the purified pig endometrial arylsulphatase is much lower than that of the rabbit liver enzyme (Lee & Van Etten, 1975) but higher than that of the human urine enzyme (Stevens *et al.*, 1975). However, the specific activity of pig endometrial arylsulphatase A is comparable with the specific activity of the rabbit testis enzyme (Farooqui & Srivastava, 1979). The yield of enzyme per kg dry wt. of endometrium was 5.88 mg, which is lower than reported for rabbit testes (Farooqui & Srivastava, 1979), rabbit liver (Lee & Van Etten, 1975) and ox liver (Nichol & Roy, 1964).

The procedure used was relatively simple and the recovery in each step was high. For unknown reasons the total activity of the enzyme increased after it passed through the DEAE-Sepharose column. We could not detect any inhibitor in the concanavalin A-Sepharose fraction or an activator in the DEAE-Sepharose fraction. Similar activation of arylsulphatase of the sperm acrosome after passage through DEAE-cellulose was reported by Yang & Srivastava (1974). They showed that this activation was not due to the removal of a heat-stable inhibitor. The amount of B enzyme recovered from this step was about 2% of A form of arylsulphatase, which is a very low amount compared with other tissues (Hook *et al.*, 1973). This once again shows the considerable variation in the proportion of A and B forms of arylsulphatases in different tissues and from species to species (Roy, 1958; Hook *et al.*, 1973).

The removal of contaminating protein from the

Sephadex G-200 fraction was achieved by the preparative gel electrophoresis. Unlike other methods (Brewer & Ashworth, 1969; Gordon, 1969), in our system the electrophoresis takes place upwards. Thus, after the protein band is eluted from the gel, it migrates very slowly upward, and can be removed in a stream of buffer. In this way, the chance of the separated protein binding to the cellophan membrane is very low.

Analysis of pig endometrial arylsulphatase A by disc-gel and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated that the enzyme is homogeneous. The sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that the enzyme is composed of subunits of identical size with M_r about 63 000. Although this value and the M_r calculated from the gel-filtration data lie in the range of M_r values of arylsulphatases from other sources, the validity of these values is questionable owing to the high content (approx. 20%) of carbohydrate in this enzyme and its anomalous behaviour in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and in the gel-filtration column. Thus the value of 120 000 obtained by sedimentation equilibrium at pH 7.4 might be considered closer to the real M_r of the pig endometrial arylsulphatase A monomer.

A comparison of the properties of arylsulphatase A isolated from pig endometrium with arylsulphatases A purified from other mammalian sources shows that they have many in common. The similarities include their glycoprotein nature, low isoelectric point, anomalous time-activity relationships, multi-pH optima, inhibition by SO_3^{2-} , SO_4^{2-} , phosphate ions, metal ions and nucleoside phosphates, pH- and ionic-strength-dependent polymerization, and their amino acid compositions (high contents of proline and glycine).

Pig endometrial arylsulphatase A can hydrolyse sulphate esters other than 4-nitrocatechol sulphate (Table 4), but the rate of hydrolysis of such compounds is lower than that by enzymes from other sources (Farooqui & Srivastava, 1979). The inhibition of pig endometrial enzyme activity towards 4-nitrocatechol sulphate by phosphate and phosphate-containing compounds also was weaker than that observed with other arylsulphatases (Roy, 1953; Lee & Van Etten, 1975; Yang & Srivastava, 1976; O'Fagain *et al.*, 1980).

It was found that the pig endometrial enzyme, like the ox liver arylsulphatase A (Uchida *et al.*, 1981), showed some phosphodiesterase activity (Table 4). The K_i value for cyclic AMP with nitrocatechol sulphate as the substrate (Table 5) was much higher than the value obtained for the ox liver enzyme (9.0 mM) (Uchida *et al.*, 1981). Thus the phosphodiesterase activity of arylsulphatase A seems to be a general phenomenon, but the rate of activity might

be species-dependent. Uchida *et al.* (1981) also raised the possibility of a converse relationship between arylsulphatase A and phosphodiesterase activity. In this regard, no detectable sulphatase activity was found in ox brain phosphodiesterase towards nitro-catechol sulphate in the presence or in the absence of Mg^{2+} or Ca^{2+} and at pH 5.5 and 8.0 (results not shown).

Pig endometrium arylsulphatase A, like the rabbit testis enzyme (Farooqui & Srivastava, 1979), dispersed the cumulus oophorus of rabbit eggs. Thus this property of arylsulphatase A might be considered as a general one. The endometrial enzyme may play an important role in reproduction in some other ways. It is shown (Table 4) that the pig endometrium enzyme is able to hydrolyse steroid sulphates. A cyclic pattern in sterol sulphatase activity in the hamster reproductive tract also has been reported (Legault *et al.*, 1980). Therefore endometrium arylsulphatases may participate in reactions such as enzymic hydrolysis of oestrogen sulphate to oestrogen, a process that is important in controlling the concentration of the active hormone in this tissue (Holinka & Gurpide, 1980).

The role of purified pig endometrial arylsulphatase in capacitation remains to be fully explored. We designed an apparatus for the isoelectric focusing of whole spermatozoa for this purpose. Full details of these studies are not included in the present paper, but we found that washed ejaculated rabbit spermatozoa incubated with 12 units of the purified pig endometrial arylsulphatase for 90 min at pH 7.0 showed the same isoelectric-focusing profile as that of the untreated control. Thus both the treated and the untreated spermatozoa had a pI of 4.3. The net negative charge on spermatozoa may be ascribed to the presence of sialic acid. Yanagimachi *et al.* (1972) argued against this effect of sialic acid, as neuraminidase treatment of spermatozoa did not prevent the binding of colloidal iron hydroxide (positively charged). They suggested that the spermatozoal surface sialic acid may be so substituted as to be resistant to the neuraminidase treatment. It is probable that one of the hydroxy groups of sialic acid may be sulphated. In such event a combination of both arylsulphatases and neuraminidase may be required to nullify the surface negative charge. The presence of endometrial neuraminidase has been reported from this laboratory (Srivastava & Farooqui, 1980). Furthermore, the surface modification might involve an ordered sequential action of several lysosomal enzymes, as was observed for the effect on the zona pellucida, a sulphated sialoglycoprotein layer of the ovum (Farooqui & Srivastava, 1980). Another group of sulphated compounds, namely sterol sulphates, are considered to be the stabilizer of the spermatozoal membrane (Legault *et al.*, 1980). The above authors suggested that the desulphation

of the sterol sulphates by sterol sulphatases of the female reproductive tract may play a role in the subsequent destabilization of the acrosomal membranes. Kopf & Garbers (1980) have implicated yet another fucose sulphate-rich polymer isolated from the jelly coat of sea-urchin egg in the acrosome reaction. Finally, our studies do not show any direct effect of the purified pig endometrial arylsulphatase vis-à-vis the capacitation, the acrosome reaction of the spermatozoa or the nidation and implantation of the zygote. Nevertheless, the strong possibility exists that arylsulphatases in conjunction with the endometrial lysosomal hydrolases may play a significant role in above processes.

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