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Oestrogen Sulfotransferase: Isolation of a High Specific Activity Species from Bovine Placenta

Stephen S. Moore[A], *E. O. P. Thompson and Allan R. Nash*[B]

Oncology Research Centre, Prince of Wales Hospital, Randwick, N.S.W., 2031, and School of Biochemistry, University of N.S.W., Kensington, N.S.W., 2033.
[A] Present address: Dept of Anatomy, University of Cambridge, Cambridge, CB2 3DY, United Kingdom.
[B] To whom all correspondence should be addressed.

Abstract

During the course of a study of the control of expression of steroid-binding proteins in human mammary cancer oestrogen sulfotransferase was isolated from bovine placenta. By a combination of salt precipitation and ion-exchange and gel-permeation chromatography two forms of the enzyme were isolated. The forms, which apparently differ only in charge, have specific activities 100–300 times greater than has previously been reported for the enzyme. Partial peptide sequences of these enzymes are presented.

Introduction

Steroid sulfotransferases are a class of enzymes that conjugate steroids by transfer to them of the sulfate moiety of adenosine 3′-phosphate 5′-phosphosulfate (PAPS). Specifically, oestrogen sulfotransferase (EC 2.8.2.4) transfers the sulfate from PAPS to the 3-OH group of oestrone. Although the enzyme is present in all human foetal tissues except brain (Wengle 1966) it has been detected in the adult only in the liver, adrenals and jejunal mucosa (Bostrom and Wengle 1967), endometrium (Pack *et al.* 1979), placenta (Tseng *et al.* 1985) and some breast tumours (Adams 1964). In breast tumours the activity of the enzyme appears to correlate with the concentration of oestrogen receptors (ER) and it has been proposed that enzyme activity may constitute an independent prognostic variable in breast cancer (Adams *et al.* 1979; Tseng *et al.* 1983).

The role of oestrogen sulfotransferase (EST) in breast cancer has not been defined but in human endometrium its induction is under the control of progesterone (Pewnim *et al.* 1980). In turn, transcription of the progesterone receptor gene is controlled by ER (Leavitt *et al.* 1978) and it appears likely that the feedback loop is completed when oestrogen is removed on sulfurylation by EST (Tseng and Gurpide 1974). Because of the apparent inter-dependence of the receptors and EST, at least in endometrial tissue, and as part of a larger program of study of the control of expression of steroid-binding proteins in breast cancer, EST was purified from bovine placenta as the first step towards the acquisition of the human gene sequence. Although the enzyme had been purified from this source previously (Adams and Low 1974) no reliable amino acid sequence data were available and there was a requirement for such data for nucleotide probe construction.

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Materials and Methods

All common chemicals and solvents were AR grade and all HPLC solvents were chromatography grade.

Enzyme activity was determined by measuring the rate of incorporation of radioactivity into either oestrone sulfate or oestradiol sulfate using, respectively, [^{35}S] PAPS and the method of Adams and Poulos (1967) or [^{3}H] oestradiol and the method of Clarke *et al.* (1982). Incubation time was 15 min. Specific activity was determined as nanomoles of oestrone sulfate or oestradiol sulfate formed/mg protein/min (nmol mg^{-1} min^{-1}) after the protein concentration had been determined by the method of Lowry *et al.* (1951). Total activities of column eluates were determined on aliquots that were appropriately diluted to yield comparable relative activities. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was carried out by the method of Laemmli (1970). Discontinuous polyacrylamide gel electrophoresis was carried out by the method of Davis (1964) and all gels were stained with silver by the method of Morrissey (1981).

Enzyme Purification

Oestrogen sulfotransferase was purified by modifications to the method described by Adams and Low (1974). Cotyledons from bovine placenta were collected at a local abattoir and snap frozen in liquid nitrogen for later storage at −70°C. All subsequent operations were carried out at 4°C. Typically, tissue (500 g) was homogenized in two volumes of 10 mM phosphate buffered saline, pH 7·4, containing EDTA (1 mM), dithiothreitol (DTT, 1 mM) and 10 mg l^{-1} Trasylol (Sigma Chemical Co.). The homogenate was filtered through muslin, centrifuged for 1 h at 19 000 g and the supernatant filtered through muslin to remove lipid. Solid ammonium sulfate was added slowly to the supernatant, with vigorous stirring, to 55% saturation. The suspension was stirred for 30 min and the precipitate removed after centrifugation at 7000 g for 15 min. Additional ammonium sulfate was added to the supernatant to 75% saturation, the suspension stirred for a further 30 min and the precipitate recovered by centrifugation at 7000 g for 15 min. The precipitate was dissolved in 100 ml of the homogenization buffer and dialysed extensively against 20 mM phosphate buffer, pH 7·8, containing EDTA (1 mM) and DTT (1 mM).

Dialysed material from the salt precipitation step was centrifuged at 100 000 g for 30 min and the supernatant applied to a 26 mm × 350 mm column of DEAE–Sepharose CL-6B (Pharmacia) equilibrated in 20 mM phosphate buffer, pH 7·8, containing EDTA (1 mM) and DTT (0·1 mM). After elution of the unbound proteins with loading buffer the bound proteins were eluted with a phosphate gradient (20 mM to 100 mM) at a flow rate of 0·5 ml min^{-1}.

Tubes containing the bulk of the EST activity were concentrated to 3–5 ml on an Amicon PM 30 membrane (Amicon, Australia) in an ultrafiltration unit, and applied to a 26 mm × 950 mm column of Sephacryl S300 (Pharmacia) equilibrated in 10 mM phosphate–500 mM NaCl buffer, pH 7·8, containing EDTA (2 mM) and DTT (1 mM). The column was developed at 0·3 ml min^{-1} and fractions containing the peak of EST activity were pooled. This material was dialysed against 5 mM ammonium bicarbonate–1 mM EDTA–0·1 mM DTT and applied to a 16 mm × 100 mm column of DEAE–Sepharose CL-6B equilibrated in the same buffer. The bound protein was eluted with a 200 ml linear gradient of ammonium bicarbonate, pH 8·1 (5 mM to 500 mM).

Peptide Purification and Sequencing

All protein was reduced and alkylated prior to tryptic digestion. S-carboxymethylation followed the general method of Perham (1978). Tryptic digestion of the S-carboxymethylated protein was carried out by incubating 10 mg of EST with 0·1 mg of trypsin treated with L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK) to abolish chymotryptic activity (TPCK–trypsin, Sigma Chemical Co.) in 1% ammonium bicarbonate for 4 h at 37°C. The peptides were recovered by freeze-drying, re-dissolved in water and freeze-dried a second time.

Peptides were purified using a Waters modular HPLC system (Millipore Pty Ltd). The crude peptide mix was applied to a Protein Pak DEAE 5PW column (Millipore Pty Ltd) equilibrated in 5 mM ammonium bicarbonate. Bound peptides were eluted using a linear gradient of bicarbonate (5 mM to 500 mM) over 20 min at 1 ml min^{-1}. Peptides were monitored by their bond absorbance at 230 nm while the characteristic fluorescence of tryptophan was routinely monitored by using a Waters 420 fluorescence detector (Millipore Pty Ltd) with an excitation filter at 280 nm and emission filter at 360 nm. Individual fractions from the ion-exchange separation were re-run on a uBondapak C18 column (Millipore Pty Ltd). The fraction of interest was applied to the column, which had been equilibrated

in 0·07% trifluoroaacetic acid in water. Bound peptides were eluted with increasing concentrations of acetonitrile. Gradients were optimized for the separation of each peptide mix.

A Model 720A gas phase sequencer (Applied Biosystems) was used to determine peptide sequences. Analysis of the PTH-amino acids generated by the sequencer was carried out using the Waters HPLC equipped with an Applied Biosystems PTH (C18) column. The methodology essentially was that described by Hunkapiller (1985). Briefly, samples were applied to the column in methanol–acetonitrile and eluted with a gradient from 8% to 42% acetonitrile in acetate buffer made 5% w.r.t. tetrahydrofuran. The elution times of PTH-histidine and PTH-arginine were optimized by manipulation of the acetate buffer concentration between 20 mM and 30 mM. Similarly, elution times of PTH-aspartic acid and PTH-glutamic acid were optimized by manipulation of the pH of the acetate buffer between pH 3·8 and pH 4·2.

Finally, protein and peptides were hydrolysed *in vacuo* in 6 M HCl containing 0·1% phenol and 0·05% mercaptoethanol, for 24, 48 and 72 h. Analysis of the hydrolysates was carried out on a Beckman model 121M amino acid analyser. Values for serine and threonine were determined by extrapolation to zero time. Valine and isoleucine values were taken as those determined after hydrolysis for 72 hours.

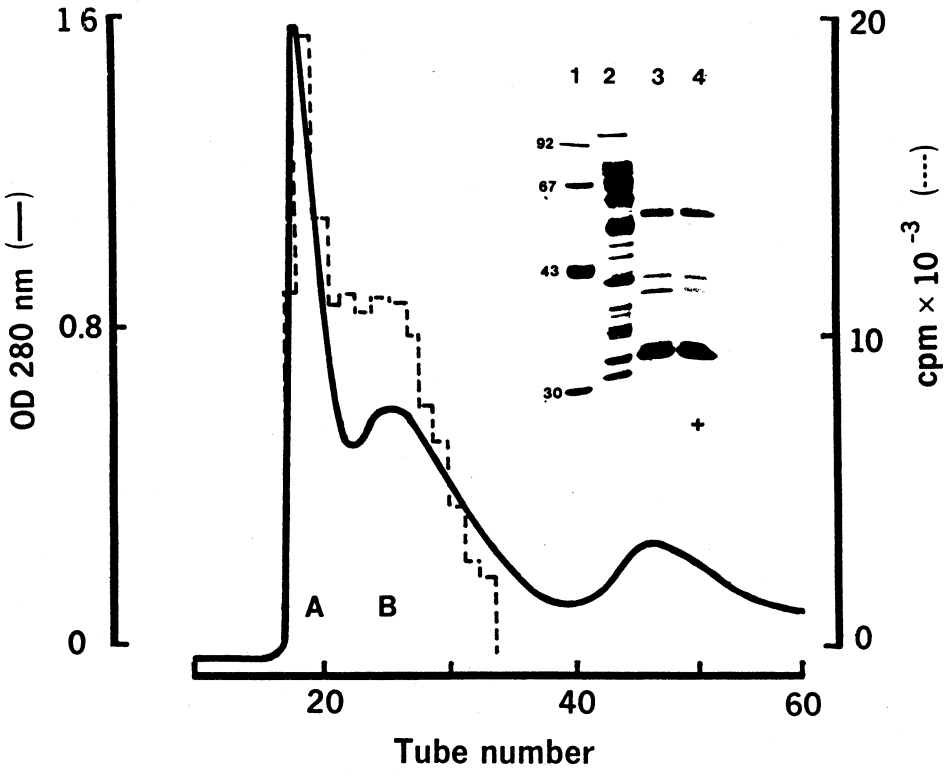

Fig. 1. Ion-exchange chromatography of a bovine placental extract on DEAE-Sepharose CL-6B. Material precipitating between 55% and 75% saturation with ammonium sulfate was applied to the column (26 mm × 350 mm) of DEAE-Sepharose CL-6B equilibrated in 20 mM phosphate buffer, pH 7·8, containing dithiothreitol (0·1 mM) and EDTA (1 mM). Unbound material was eluted with loading buffer, then a 500 ml linear gradient (20 mM to 100 mM) of phosphate was applied. Flow rate was 0·5 ml min^{-1} and fractions of 9 ml were collected. Enzyme activity and optical density at 280 nm were determined for each fraction. The peak tubes of enzyme activity (18–20 and 21–27) were pooled separately for subsequent operations. Material from these tubes was subjected to SDS-PAGE. Lane 1 contained markers with the molecular weights ($\times 10^{-3}$) indicated; lanes 2–4 contained material from tubes 19, 23 and 24, respectively.

Results

Salt-precipitated material from bovine placenta could be separated into three components by the initial ion-exchange chromatography step (Fig. 1). Enzyme activity was associated with material from the first two peaks, with the highest proportion of total activity residing under the second peak. When re-chromatographed on Sephacryl S300, material from peak A could be resolved partially into multiple components with elution volumes consistent with molecular weights in the range 70 000–100 000 (Fig. 2). When the peak tube of enzyme

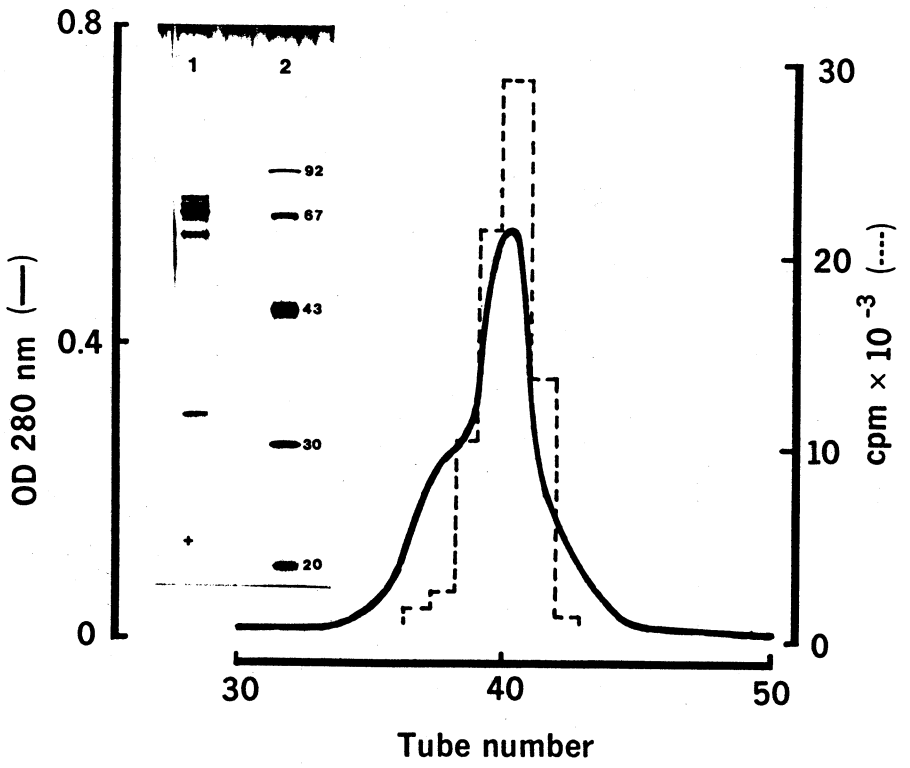

Fig. 2. Gel-permeation chromatography of peak A from Fig. 1. Material from tubes 18–20 (Fig. 1) was applied to a column (26 mm × 950 mm) of Sephacryl S300 equilibrated in 10 mM phosphate buffer, pH 7·8, which contained NaCl (500 mM), dithiothreitol (0·1 mM) and EDTA (1 mM). The column was developed at a flow rate of 0·2 ml min^{-1} and fractions of 6 ml were collected. Enzyme activity and optical density at 280 nm were determined for each fraction. Material from tube 41 was subjected to SDS–PAGE. Lane 1 contained this material; lane 2 contained markers with the molecular weights ($\times 10^{-3}$) indicated.

activity was analysed by SDS–PAGE this composition was confirmed but there appeared an additional component with an apparent molecular weight of 36 000. Material from this tube had a specific activity of 11 nmol mg^{-1} min^{-1} and no further purification of it was attempted. Similar gel-permeation chromatography of material from peak B of Fig. 1 yielded a single UV absorbing peak with an elution volume consistent with a molecular weight of approximately 70 000 (Fig. 3). The peak of enzyme activity was not coincident with the UV-absorbing peak being displaced one tube. Analysis of this material by SDS–PAGE electrophoresis revealed the presence of a major 36 000 dalton protein species but no significant 70 000 dalton species. The peak tube of enzyme activity from this column could be resolved into two peaks of activity by a second DEAE–Sepharose step (Fig. 4). On a non-denaturing gradient gel this material migrated with an apparent molecular weight

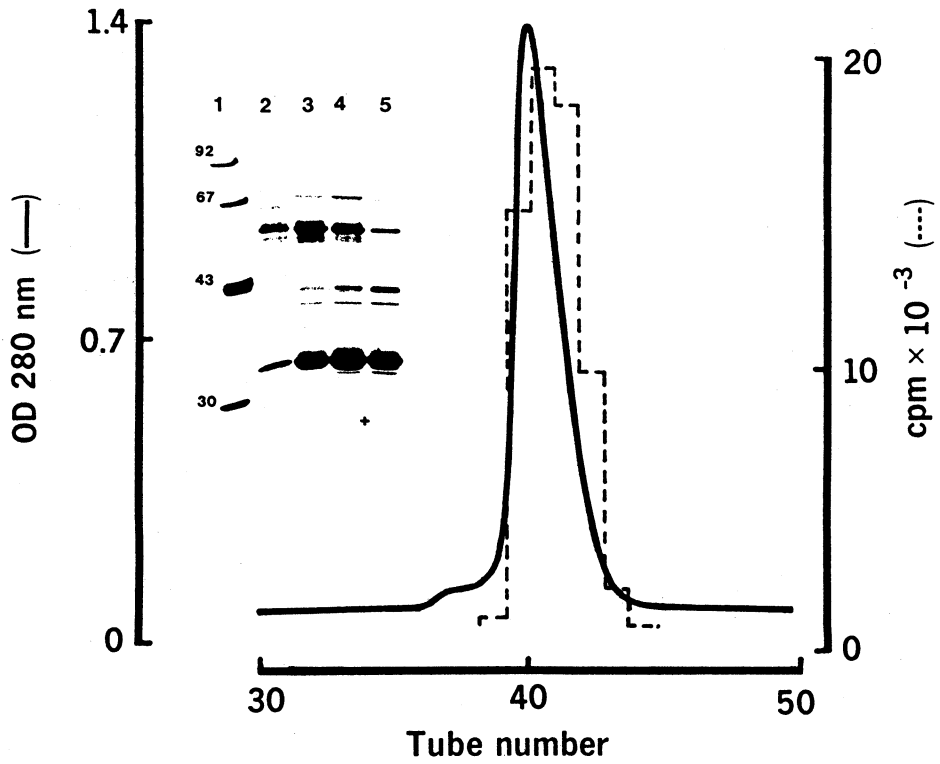

Fig. 3. Gel-permeation chromatography of peak B from Fig. 1. Material from tubes 21–27 (Fig. 1) was applied to a column (26 × 950 mm) of Sephacryl S300 equilibrated in 10 mM phosphate buffer, pH 7·8, which contained NaCl (500 mM), dithiothreitol (0·1 mM) and EDTA (1 mM). The column was developed at a flow rate of 0·2 ml min^{-1} and fractions of 6 ml were collected. Enzyme activity and optical density at 280 nm were determined for each fraction. Material from the peak tubes of enzyme activity was subjected to SDS–PAGE. Lane 1 contained markers with the molecular weights ($\times 10^{-3}$) indicated, while lanes 2–5 contained material from tubes 40–43 respectively. The contents of tubes 41–43 were pooled for further purification.

Table 1. Partial amino acid sequences from bovine oestrogen sulfotransferase
Peptides prefixed A and B were derived from material under peaks A and B respectively of Fig. 4. Peptide A.8.1 yielded no identifiable residue beyond position 13 although the analysis contained a further three residues, including lysine. Repeated attempts to obtain an amino-terminal sequence for the intact protein were unsuccessful; the protein appears to have a blocked *N*-terminal

Peptide		Sequence
A.1.1	B.3.2	K G D V G D W K
A.3.4	B.2.4	N N P S T N Y T T L P D E V Q N Q K
A.5.4	B.5.2	K F I E Q F H N V E E F E A R P D D L V I V T Y P K
A.7.2	B.6.2	F I E Q F H N V E E F E A R P D D L V I V T Y P K
A.7.1	B.7.2	S W W E K
A.8.1	–	F M D G E V P Y G E W F E . . .
–	B.1.11	L G G I P M Y K
–	B.3.4	F D M H Y E Q Q M K
–	B.2.5	L L P V E F W E K

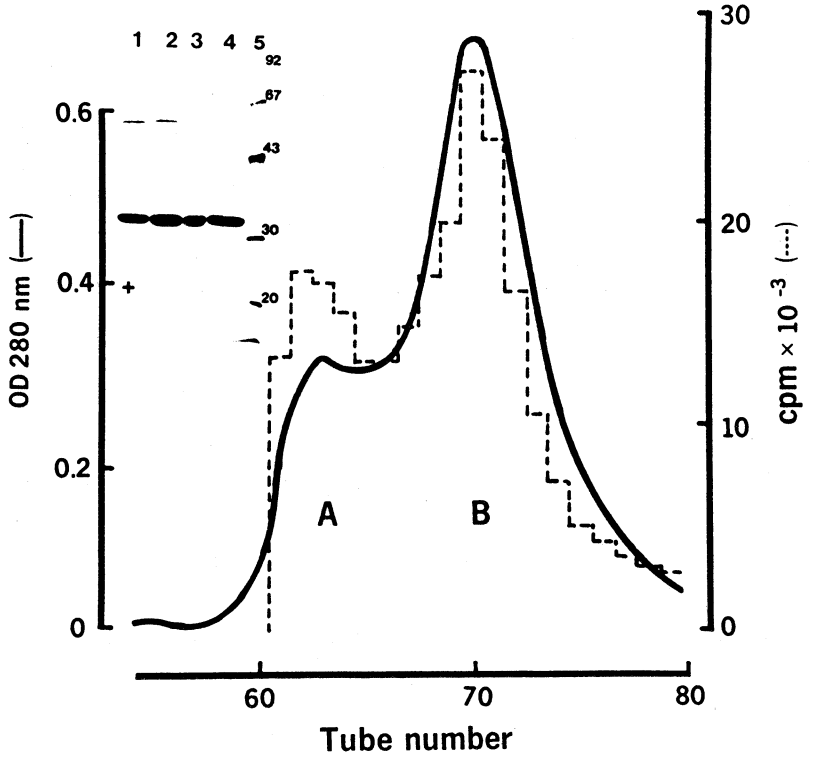

Fig. 4. Final ion-exchange chromatography step in the purification of EST. Material from tubes 41–43 (Fig. 3) was dialysed against 5 mM ammonium bicarbonate containing dithiothreitol (0·1 mM) and EDTA (1 mM), then applied to a column (16 mm × 100 mm) of DEAE-Sepharose CL-6B equilibrated in the bicarbonate buffer. Bound proteins were eluted with a 200 ml linear bicarbonate gradient (5 mM to 500 mM) at a flow rate of 0·1 ml min^{-1} and fractions of 2 ml were collected. Enzyme activity and optical density at 280 nm were determined for each fraction. Material from both peaks was subjected to SDS-PAGE. Lane 5 contained markers with the molecular weights ($\times 10^{-3}$) indicated; lanes 1–4 contained material from tubes 62, 66, 70 and 72, respectively.

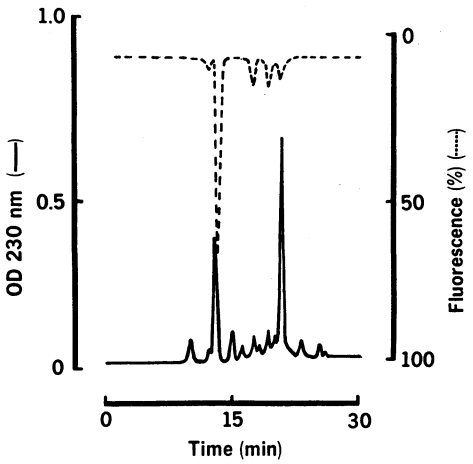

Fig. 5. Purification of tryptic peptides by reverse-phase chromatography. Tryptic peptides from a digest of EST were partially purified by passage over a Waters Protein Pak DEAE 5PW column. Single components from this separation were applied to a 4·6 mm × 150 mm Waters uBondapak C18 column equilibrated in 0·07% aqueous TFA–acetonitrile. Baseline separation of individual peptides (see Table 1) was achieved by application of an acetonitrile gradient (24% to 48%) over 30 min. Here, peptides B.3.2, containing tryptophan and with a retention time of 13 min, and B.5.2, with a retention time of 22 min, were separated.

of approximately 70 000 (results not shown) but by SDS–PAGE as a species with an apparent molecular weight of 36 000. The specific activity of the material under peaks A and B in Fig. 4 were, respectively, $3{\cdot}3 \times 10^3$ and $2{\cdot}3 \times 10^3$ nmol mg^{-1} min^{-1}. These activities were 100 to 300 times higher than any previously reported.

Although these preparations contained a trace of 50 000–60 000 dalton contaminant they were considered sufficiently pure for analysis and sequencing. The amino acid compositions of the two peaks were closely similar with only minor variations in the content of serine and glycine in total compositions of, respectively, 299 and 294 residues.

Complex peptide mixtures were generated by tryptic digestion of the two peaks from Fig. 4. Two HPLC steps were required to obtain the baseline separations considered necessary for sequence analysis (Fig. 5). Peptides derived from peaks A and B (Fig. 4) were purified and sequenced, with the results shown in Table 1. Of those sequenced, most major peptides from peak A had an obvious counterpart in peak B and there were no differences in these sequences. The purification system used was selected because it enabled tryptophan-containing peptides to be identified and isolated. Such peptides were considered desirable to reduce the degeneracy of oligonucleotide probes derived from their sequence. Hence, not all peptides were sequenced and the 'pairs' for single peptides were not sought.

Discussion

Bovine tissue used in this study was derived from maternal placental tissue (cotyledons) obtained at slaughter from cows of unknown gestational stage. Although gestational-stage-dependent changes in placental EST concentration have been observed in the mouse (Hobkirk *et al.* 1983) such variations have not been recorded for the cow. In Fig. 1 the ratio of total protein under peak A to that under peak B varied from preparation to preparation. While this may reflect a temporal change in the concentration of either EST or the proteins under peak A, the phenomenon was not further explored. There is no evidence, from any species, that qualitative changes in EST occur during the course of pregnancy.

The methodology for the purification of bovine placental EST was established by Adams and Low (1974) and, with minor modifications, has been used by most later workers (e.g. Clarke 1982). In the current study high-efficiency chromatographic resins were used to effect the isolation of the enzyme and the purification was followed by silver staining of acrylamide gels (Morrissey 1981), a technique substantially more sensitive than those available to earlier workers. As a consequence, a species of EST of a purity adequate for micro-sequence analysis and with specific activity of 3×10^3 nmol mg^{-1} min^{-1} was isolated. This specific activity may be compared with those activities previously found for bovine placental EST, namely 10 nmol mg^{-1} min^{-1} (Adams and Low 1974) and 25 nmol mg^{-1} min^{-1} (Clarke *et al.* 1982). EST derived from human placenta (Tseng *et al.* 1985) and mouse placenta (Hobkirk *et al.* 1983) have specific activities in the pmol mg^{-1} min^{-1} range.

When subjected to gel-permeation chromatography or gradient gel electrophoresis under non-denaturing conditions, the enzyme isolated in this study behaved as a molecule with an apparent molecular weight of approximately 70 000. However, under the denaturing conditions used in SDS–PAGE an apparent molecular weight of around 36 000 was found. These data are consistent with an hypothesis that the active form of EST is a dimer of approximately 72 000 made up of identical, or nearly identical, subunits with approximate molecular weights of 36 000. Brooks *et al.* (1987) recently reported the isolation from porcine endometrium of a species of EST with an apparent molecular weight of 31 000.

The basis for the apparent charge differences on the 'isomers' in Fig. 4 is not known. The amino acid compositions of the two species are in close agreement and peptides so far sequenced, and common to both species, display no differences. Their separability on DEAE–Sepharose may be due to partial deamidation of a relatively fixed percentage of a single species during purification. The explanation for this phenomenon, and the full

structure of bovine EST, will become available when the sequence of the bovine cDNA is determined in the course of the current study.

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