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Original article

Purification of an ovine, androgen-dependent epididymal protein. Evidence for a strong amino acid sequence homology with serum albumin

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Summary — An ovine, testosterone-dependent protein was purified from an extract of epididymides of orchidectomized-, testosterone-implanted rams by ethylene glycol precipitation, anion exchange chromatography, preparative non-denaturing PAGE at alkaline pH and gel filtration. The protein which had previously been named ovine prealbumin-epididymis-specific protein (oPES), migrated as a single band ahead of ovine serum albumin (oSA). A single component, with an apparent MW of 60 kDa, lower than that of oSA, was also observed in SDS–PAGE. oPES was cleaved after lysyl residues using endoproteinase Lys-C and the hydrolysate was fractionated in 2 steps by reverse-phase HPLC. Six oligopeptides were recovered and sequenced. They all displayed complete identity with regions of bovine serum albumin scattered in the two-third N-terminal part. However, in 2 of them, there was no complete identity with homologous parts of oSA. This indicates that oPES and oSA are probably encoded by different genes.

ram / epididymis / androgen-dependent protein / serum albumin

Résumé — Purification d'une protéine ovine épididymaire, dépendant des androgènes. Mise en évidence d'une forte homologie de séquence avec la sérum-albumine. *Une protéine ovine, dépendante de la testostérone, a été purifiée, à partir d'un extrait d'épididymes de béliers orchidectomisés porteurs d'implants de testostérone, par précipitation au polyéthylène glycol, chromatographie d'échange d'anions, électrophorèse préparative en gel de polyacrylamide à pH alcalin en milieu non dénaturant, et filtration sur gel. La protéine donnait une seule bande migrant en avant de la sérum albumine ovine (oSA), d'où le nom qui lui a été donné antérieurement : ovine pré-albumine épididyme spécifique (oPES). Un seul constituant, de masse moléculaire apparente 60 kDa, inférieure à celle de l'oSA, fut aussi observé en électrophorèse sur gel de polyacrylamide en présence de SDS. L'oPES fut clivée après les résidus lysyle en utilisant l'endoprotéinase Lys-C, et l'hydrolysat fractionné en 2 étapes par HPLC en phase inverse. Six oligopeptides ont été obtenus et séquencés. Ils présentent tous une identité complète avec des régions de la molécule de sérum-albumine bovine situées dans les 2/3 N-terminaux. Cependant, pour 2 d'entre eux, il n'y a pas identité complète avec les parties homologues de l'oSA. Ceci indique que l'oPES et l'oSA sont probablement codées par des gènes différents.*

bélier / épididyme / protéine dépendante des androgènes / sérum-albumine

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INTRODUCTION

During their transit through the epididymis, spermatozoa undergo specific physical, biochemical and morphological changes resulting in the appearance of the flagellar motility and zona pellucida receptors on the plasma membrane of the head (Fournier-Delpech and Thibault, 1991). These changes occur together with variations in the contents of the epididymal lumen due to the synthesis, metabolism, absorption and secretion of compounds such as steroids, carnitine, or proteins (Cooper, 1986). Androgen-dependent, epididymal secretory proteins play a role in sperm maturation (Cuasnicu *et al,* 1984; Fournier-Delpech *et al,* 1985). In the ram, one of these proteins, previously referred to as ovine prealbumin-epididymis-specific protein (oPES) has been found on the plasma membrane covering the acrosomal cap of epididymal spermatozoa but not of testicular spermatozoa (Fournier-Delpech *et al,* 1985). This paper reports a procedure for an efficient preparation of oPES and its partial characterization from amino acid sequence data.

MATERIAL AND METHODS

Animals, chemicals and material

Animals were adult Ile-de-France rams. Testosterone was from Roussel-Uclaf (Paris, France), polyethylene glycol (PEG) from Prolabo (Paris, France), Ultrogel ACA44 from IBF (Villeneuve-La-Garenne, France), DEAE–Sephacel from Pharmacia (Uppsala, Sweden), acrylamide from Tebu (Le Perray-en-Yvelines, France), endoproteinase Lys-C from Boehringer (Mannheim, Germany), bovine serum albumin (BSA) from Sigma (St Louis, USA). Reverse-phase HPLC was performed with a Millipore-Waters system (Milford, USA). The 477A–120A sequencer and its reagents were from Applied Biosystems (Foster City, USA). The Speed-Vac concentrator was from Savant (Hicksville, USA).

Purification of oPES

Epididymides were collected from 15 orchidectomized-, testosterone-implanted rams (Fournier-Delpech *et al,* 1981) and freed from most connective envelopes on ice. The following operations were performed at room temperature in the presence of sodium azide. The tissues were minced and homogenized in a 3 mM Tris–HCl buffer, pH 8.8, containing 0.2% sodium azide, referred to below as TH (3 ml per g tissue). After filtration on gauze and centrifugation (30 000 *g,* 10 min), aliquots of the supernatant were treated with solid PEG 4000 (5, 10, 15, 20, 25 or 30 g added to 100 ml). A further centrifugation as above gave 6 supernatants (S5 to S30) which were analyzed by PAGE (7.5% acrylamide, Tris–glycine buffer, pH 8.3) under non-denaturing conditions, to localize oPES after $ZnCl_2$ (Wang *et al,* 1989) and Coomassie blue staining. Most oPES was in S25; it was precipitated by addition of solid PEG-6000 (30 g to 100 ml). After centrifugation as above, precipitate (P + 30) was dissolved in a minimum volume of TH, 0.1% Tween 20, and chromatographed on a DEAE–Sephacel column (3 x 5 cm) equilibrated in TH with recording at 280 nm. The proteins eluted in 0.33 M NaCl were concentrated and desalted by dialysis against PEG-6000 and submitted to preparative non-denaturing PAGE after 90 min pre-electrophoresis, then stained as above. oPES and oSA were eluted, concentrated by dialysis against PEG as above, passed through an ACA44 column (1 x 90 cm) in water. After protein determination (Lowry *et al,* 1951), each recovered fraction was divided in 300-µg aliquots and freeze-dried. The purity of each protein was assessed by SDS–PAGE, after 2-mercapto-ethanol reduction and 3 min boiling, in 15% acrylamide and silver staining (Morrissey, 1981).

Preparation and sequencing of internal oligopeptides

Direct sequencing of oPES (*ca* 1 nmol) was attempted 3 times without success. The protein (*ca* 1 mg) was digested by endoproteinase Lys-C in 100 µl 0.1M ammonium bicarbonate buffer, pH 8.8. The reaction was performed overnight at 37 °C with an enzyme to substrate ratio of 1/100 (w/w). After Speed-Vac drying, the digest was

dissolved in 25 mM ammonium acetate, pH 6.7, (solvent A_1) and injected on a Nucleosil C18 reverse-phase column (5-µm beads, 10-nm pores, 250 x 4.6 mm; SFCC, France) equilibrated in solvent A_1 and kept at 40 °C. The elution was achieved by a linear gradient from solvent A_1 to solvent B_1 (20% 100 mM ammonium acetate, pH 6.7; 80% CH_3CN) in 60 min at a flow rate of 1 ml/min with absorbance recording at 214 nm. The 6 main fractions were collected, Speed-Vac dried, dissolved in 0.1% trifluoroacetic acid (TFA) and rechromatographed as above, with elution by a linear gradient from solvent A_2 (0.11% TFA) to solvent B_2 (80% CH_3CN, 0.1% TFA) in 30 min. After Speed-Vac drying, each recovered peptide was automatically sequenced.

RESULTS AND DISCUSSION

oPES preparation

oPES was shown by non-denaturing PAGE to be mainly present in fraction S25 (fig 1), which contained 45% of the soluble proteins of the initial epididymal extract. Precipitate (P + 30), obtained by PEG-6000 treatment of S25, contained 11% of the whole protein extract, mainly oSA and oPES.

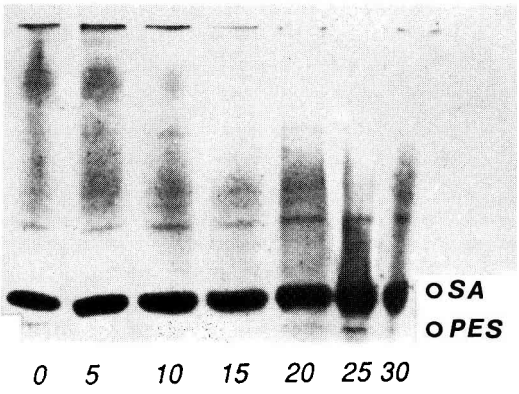

Fig 1. Non-denaturating PAGE of the soluble epididymis extract (0) and its PEG precipitation supernatants 5–30. The figures refer to the PEG concentrations.

An atypical elution of (P + 30) proteins by TH from DEAE–Sephacel was observed: the proteins were not retained by the ion-exchanger. This agrees with other observations on atypical behaviour of proteins in gel filtration due to PEG (Arakawa, 1985). After different attempts to eliminate PEG, we found that the addition of Tween 20 to the dissolved (P + 30) allowed protein retention on the column and elution of oPES together with oSA by 0.33 M NaCl, as previously observed (Fournier-Delpech *et al,* 1988, 1990). For further preparations, (P + 30) in TH, 0.1% Tween 20, was loaded on the ion-exchanger equilibrated in TH containing 0.20 M NaCl, washed with this solution and oPES was eluted by 0.33 M NaCl in TH (fig 2) and purified by electrophoresis as indicated in the *Methods* section, together with oSA.

oPES electrophoretic behaviour

On PAGE purified oPES migrated as a single band, faster than oSA, in the prealbumin region (fig 2). From SDS–PAGE, it displayed, and apparent MW of *ca* 60 kDa, lower than that of oSA (fig 3). In both systems a single component was observed.

Sequence data

From direct sequencing it appeared that oPES was probably N-terminally blocked since no PTH-amino acid was released on any Edman cycle. In order to generate a limited number of oligopeptides, native oPES was enzymatically digested. Indeed, most globular proteins undergo limited superficial proteolysis if they have not been previously denatured. Endoproteinase Lys-C, which cleaves peptide chains after lysyl

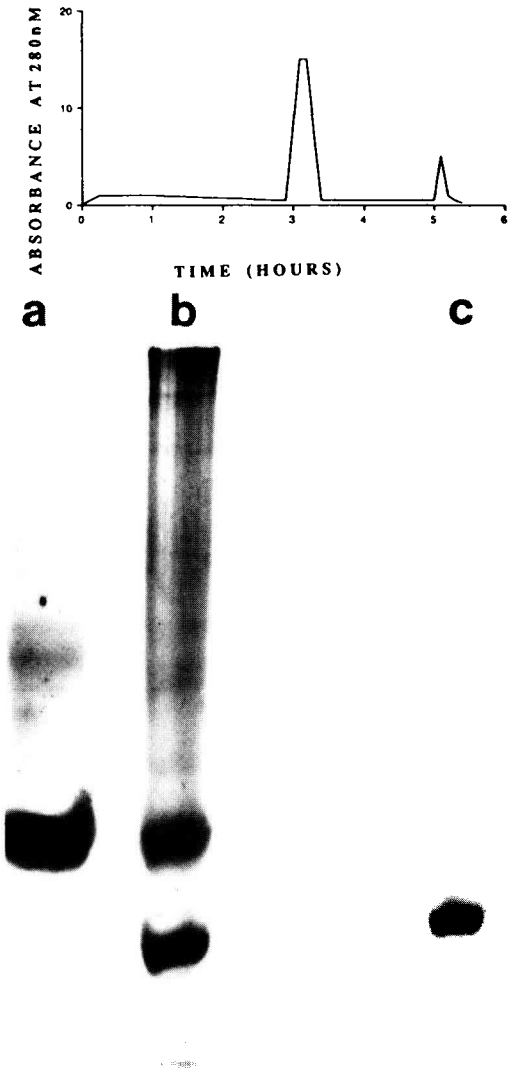

Fig 2. Purification of oPES by DEAE–Sephacel column chromatography and preparative electrophoresis. Top : Elution profile of (P + 30) from DEAE–Sephacel. First peak: proteins eluted by 0.2 M NaCl. Second peak: proteins eluted by 0.33 M NaCl. Bottom :P 280 280 Non-denaturating PAGE of proteins eluted by 0.2 (a) and 0.33 (b) M NaCl.c, oPES obtained by preparative electrophoresis from the second fraction (0.33 M NaCl). The main spot in (a) is oSA.

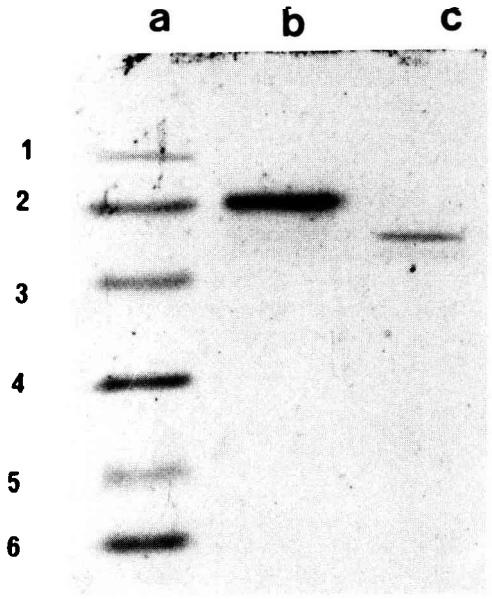

Fig 3. SDS–PAGE of electrophoretically purified oSA (b) and oPES (c). a, mw markers: 96 (1), 67 (2 and bSA), 43 (3), 30 (4), 20.1 (5) and 14.4 kDa (6). Silver staining.

residues, was employed. HPLC fractionation of the whole digest gave 6 main fractions, A–F (fig 4). Fraction F did not contain enough material for further analysis. The 5 remaining fractions were purified by chromatography in another solvent system. Fraction C gave 2 pure peptides, C1 and C2, while the other 4 fractions gave each a single pure peptide. They were called A, B, D and E (fig 5 a, b, c, d). In figure 5 the peaks with retention times below 10 min correspond to the injection, while those appearing between 10–15 min are artefactual (the corresponding fractions do not contain any peptide). The 6 peptides were sequenced. A search in data banks showed that they were identical to regions of bSA amino acid sequence (Brown and Shockley, 1982) and are released in accordance with the specificity of endoproteinase Lys-C (fig 6). As usual when se-

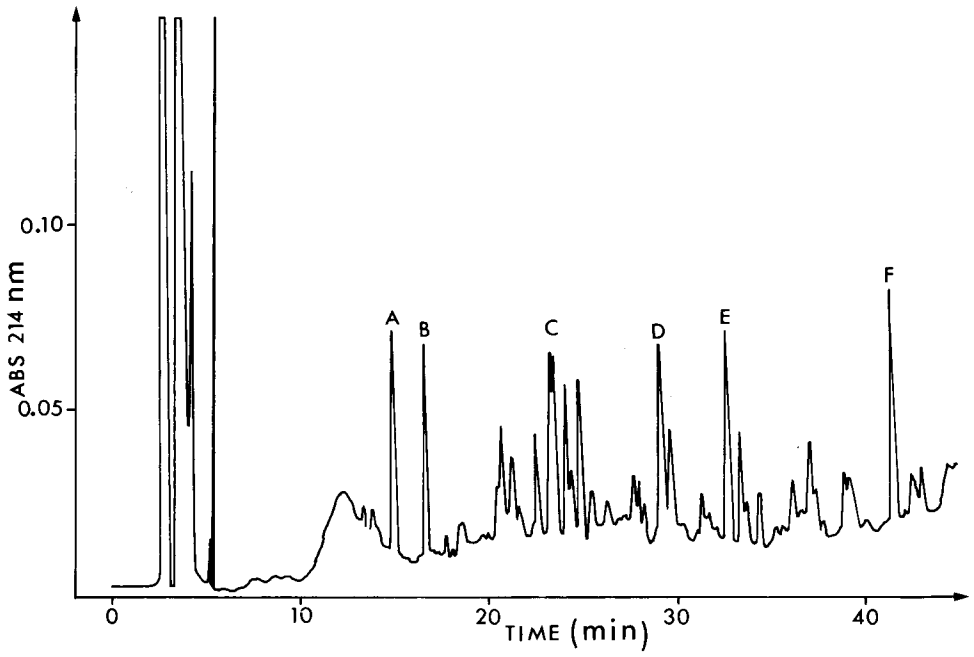

Fig 4. Reverse-phase HPLC of the endoproteinase Lys-C digest of oPES. Column: Nucleosil (SFCC, France; C18, 5-µm beads, 10-nm pores, 250 x 4.6 mm). Linear gradient from 25 mM ammonium acetate pH 6.7 to 85% solvent B_1 (20% 100 mM ammonium acetate pH 6.7, 80% CH_3CN) in 60 min. Column temperature: 40 °C. Flow rate: 1 ml/min. Fractions A,B,C,D and E were collected, dried and rechromatographed (fig 5).

quencing the C-terminus of peptides, apart from D, the last (peptides A, C1, C2 and E) or last 2 (peptide B) residue(s) could not be identified. The 6 peptides are located in region 37–410 (numbering from the initiator Met) of bSA. They were also searched for in the recently published sequence of oSA, deduced from that of its cDNA, which is > 90% identical to that of bSA (Brown *et al,* 1989). Indeed, peptides C1, C2, D and E were found in homologous positions, each beginning after a lysine and having lysine as C-terminal amino acid. However, for peptide A, an His (oPES) replaced an Asn (oSA). Furthermore, the specificity of the endoproteinase Lys-C did not fit with the C-terminal Gln. For peptide B, 3 differences occurred between oPES or bSA and oSA: Glu → Asp twice, Val → Thr (oPES → oSA).

We are then faced with a surprising result: in 6 small regions, oPES appears to be identical with bSA, while 2 of them are clearly different from the corresponding regions of oSA (fig 6). However, oPES differs from bSA in electrophoretic behaviour: its apparent MW in SDS–PAGE is lower than that of oSA which is the same as bSA; it migrates faster in non-denaturing PAGE. Furthermore, anti-oPES serum saturated with bSA reacts with oPES (Fournier-Delpech *et al,* 1988). Could it be that, in the ram, both a gene encoding bSA, which could be postranslationally modified (*eg* by

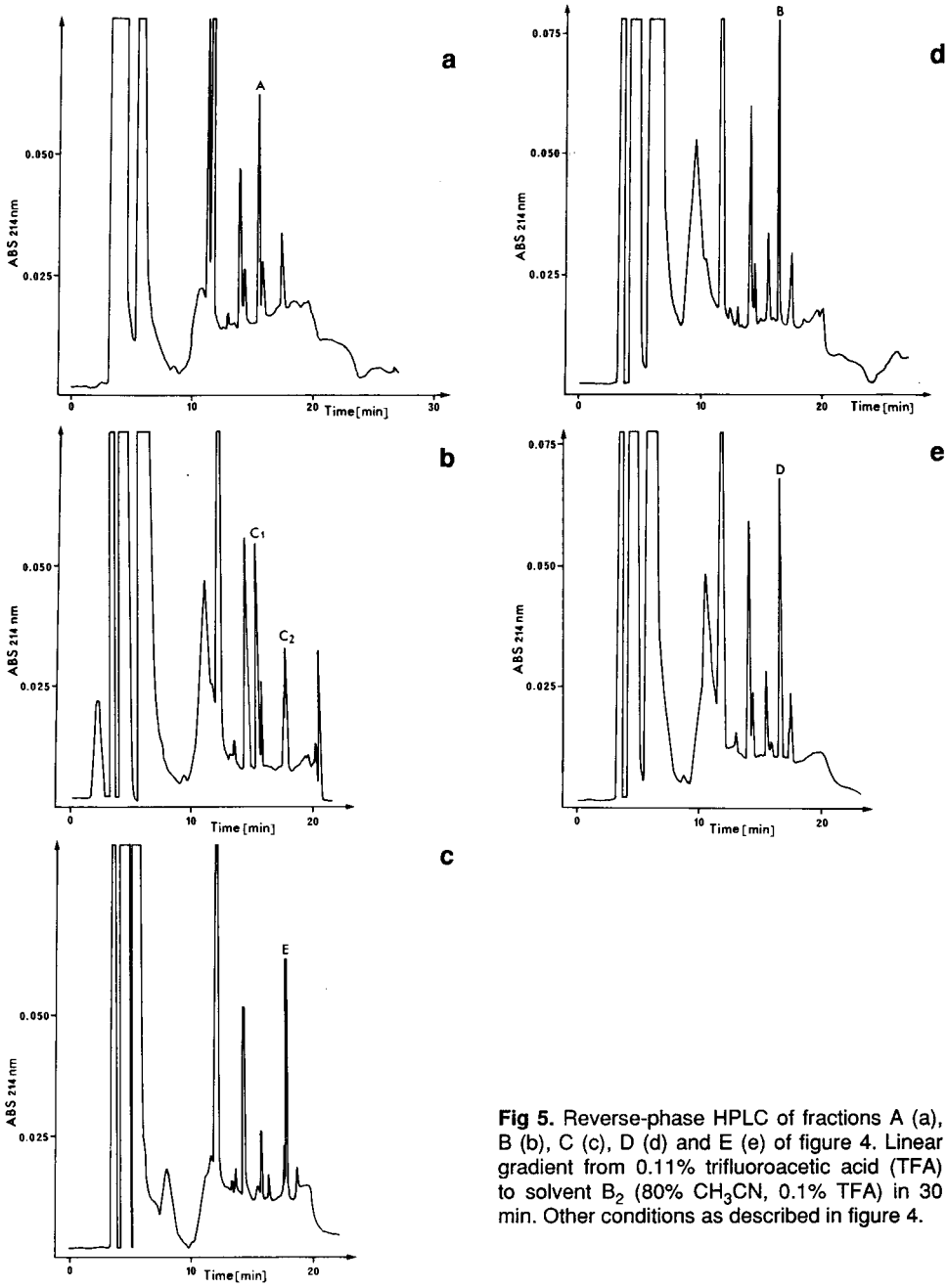

Fig 5. Reverse-phase HPLC of fractions A (a), B (b), C (c), D (d) and E (e) of figure 4. Linear gradient from 0.11% trifluoroacetic acid (TFA) to solvent B_2 (80% CH_3CN, 0.1% TFA) in 30 min. Other conditions as described in figure 4.

proteolysis, glycosylation) to give oPES, and a gene encoding oSA be present, taking into account that these genes originate from a common acestor? This is hard to believe since it implies that an identical evolution from a common precursor resulted

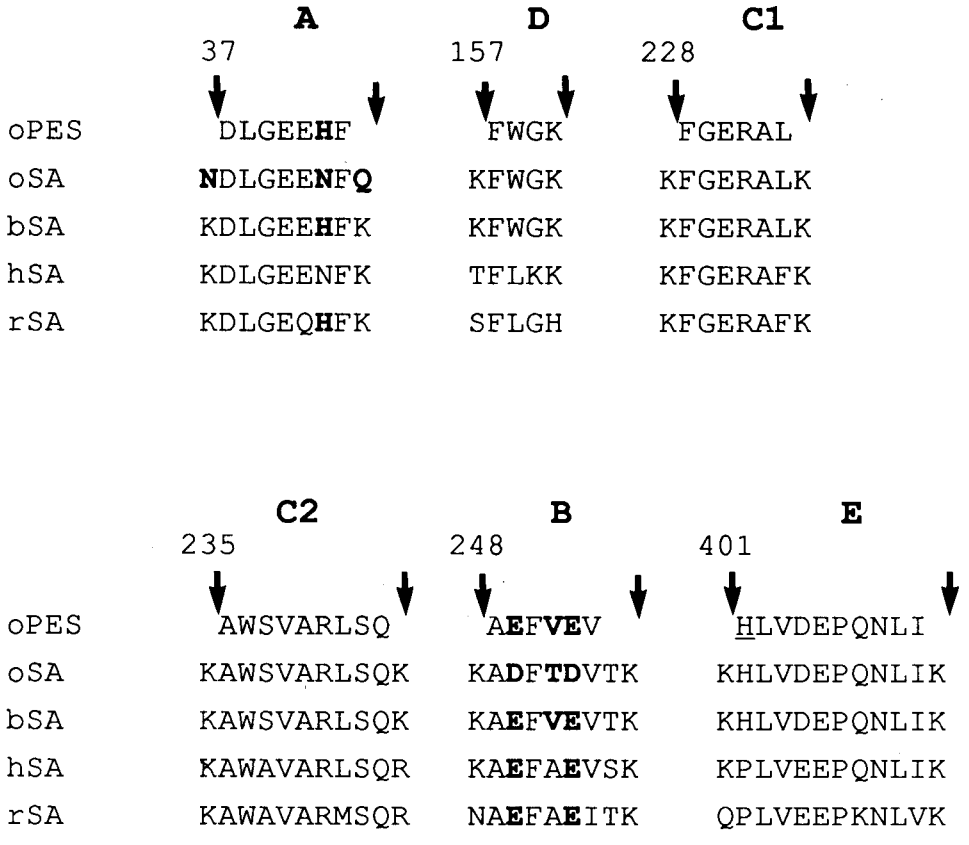

Fig 6. Comparison of the 6 peptides (A,B,C_1,C_2,D,E; fig 5) from oPES with the homologous regions of 4 known serum albumins. hSA, human; rSA, rat; oSA, ovine; bSA, bovine. For each peptide, first arrow: cleavage site in oPES; second arrow: most probable cleavage site in oPES. H, uncertain identification. Bold-faced: residues differing in oPES and oSA, and residues identical to those of oPES in bSA, hSA or rSA at the same positions.

in bSA in bovines and oPES in ovines. We are of the opinion that the genes encoding bSA, oSA and oPES are different and originate from a common precursor. The evolution from this precursor to oSA and oPES would have followed a different pathway and oPES is almost certainly different from bSA in parts of the molecule that have not been studied, and it is worth noting (fig 6) that several of the substitutions we observed between oPES and oSA do not exist when oPES is compared with rat or human serum albumin.

No relationship was found between oPES and "rat-PES", also called protein D-E (Fournier-Delpech *et al,* 1988) whose MW is *ca* 32 kDa and cDNA sequence is known (Brooks *et al,* 1986).

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