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Purification and Characterization of ram Epididymal Angiotensin Converting Enzyme

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Abstract

Angiotensin converting enzyme (ACE) from ram epididymis was purified to homogeneity. The purification procedure consists of ion exchange chromatography on DEAE Sephadex A-50, gel filtration on Sephacryl S-300 and biogel hydroxyl apatite chromatography. The purified ACE showed single band on native PAGE, reducing SDS-PAGE and non reducing SDS-PAGE. The molecular mass of purified ACE was determined as 102 k Da by SDS-PAGE. The K_m ,V_{max}, K_{cat} and K_{cat} / K_m values of purified epididymal ACE were 1.9mM, 10μ moles/min/mg , 29420 min^{-1} and 15.48 x 10^6 M^{-1} min^{-1} for Hip-His-Leu respectively. Arginyl, glutamyl , tyrosyl and Iysyl groups are functional residues of epididymal ACE. EDTA and 1,10-phenanthroline completely inhibited epididymal ACE activity at 57μ M concentration. In presence of cobalt epididymal ACE activity increased to two folds.

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Introduction

Angiotensin converting enzyme (ACE, 3.4.15.1) is a zinc metalloprotease catalyzes hydrolysis of dipeptides from carboxy terminus of several peptide substrates. [1] Endothelial ACE also known as somatic ACE (sACE) plays major role in blood pressure regulation by generating angiotensin II and hydrolyzing bradykinin [2] and it is purified from several organs of many animal species [3-5].

Somatic enzyme consist of two homologous C and N domains each of which contains HEXXH as active site motif .[6] Further ions like chloride and cobalt influences peptide bond hydrolysis by ACE [7,8]. Using site directed mutagenesis an arginyl residue at position 1098 is found to be critical for the chloride dependent hydrolytic activity of human C- domain. [9]ACE is also found in high concentrations in male reproductive organs and it is known as germinal ACE (gACE). [10]. Even though ACE production in male sex organs is dependent on sexual maturation, the epididymal ACE was found to be more sensitive to the influence of hormones than testicular ACE. [11] Further an intrinsic angiotensin II generating system is reported in rat epididymis [12] and in monkey epididymis [13].

Germinal ACE is isolated from man, rat, canine testis [14-16] and ram epididymal fluid. [17] However ACE is not isolated from epididymis of any animal species. Recently crystallization of testicular ACE and its crystal structure with lisinopril are reported. [18,19] Crystal structure of testicular angiotensin converting enzyme – lisinopril complex indicates HEXXH motif in active site, involvement of arginyl residues in chloride binding and tyrosyl residues in substrate binding [19]. Chemical modification studies of rabbit pulmonary ACE have shown that lysyl residues are required for enzyme activity. [20] No information is available on functional residues of epididymal ACE at active site, involved in chloride binding and substrate binding from any animal species. In this paper we report purification, molecular properties, kinetic parameters and functional residues of angiotensin converting enzyme from ram epididymis.

Materials and Methods

Hippuryl-L-Histidyl-L-Leucine (HHL), hippuric acid, ammonium sulfate, acrylamide, N,N'-methylene bis acrylamide, N-N-N'-N''-tetra methyl ethylene diamine (TEMED), sodium dodecyl sulfate (SDS), tris (hydroxy methyl) amino methane (TRIS), ammonium persulfate, β-mercaptoethanol, Coomassie brilliant blue R-250 (Brilliant blue R), high molecular weight standard mixture for SDS-PAGE (molecular weight range 30-200 kDa) ,N-(2- hydroxyethyl)-piperazine-N-2-ethene sulfonic acid (HEPES), 2-(N-morpholino)-ethanesulfonic acid (MES), EDTA, 1,10-phenanthroline,Ninhydrin,1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide, N-acetyl imidazole, trinitrobenzene sulfonicacid (TNBS), 1-Fluoro-2,4-dinitrobenzene (FDNB) and bovine serum albumin were obtained from Sigma Chemical company, St. Louis, MO, USA. DEAE Sephadex A-50 and Sephacryl S-300 superfine were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio-Gel HTP (Hydroxyl apatite) was purchased from Bio-Rad Laboratories, Richmond, CA, USA. Bromophenol Blue was from BDH Chemicals Limited, Poole, England. Diethyl pyro carbonate (DEPC) and acetic anhydride were obtained from Polyscience, PA, USA. All other chemicals were of analytical grade.

Dialysis membranes (cut off molecular weight 10-12 kDa) were from Spectraphor Arthur H.Thomas Co, Philadelphia, PA, USA. Immersible CX-10 PLGC filters (10,000 NMWL) and centriprep-30 membrane concentrators (molecular weight cut off 30 kDa) were obtained from Millipore/Amicon Company, Beverly, MD, USA.

Enzyme assay: ACE activity was measured with Hippuryl-L-Histidyl-L-Leucine (HHL; Sigma Chemical Company, USA) as substrate by a method modified from Cushman and Cheung. [21] The reaction mixture (0.175 ml) contained 0.1 ml of 5 mM HHL dissolved in 0.2 M phosphate buffer pH 8.3 containing 0.6 M NaCl, tissue extract (enzyme) and distilled water. The tissue extract and column elutes in a volume of 5-15 μl were used. After 30 minutes incubation at 37°C the reaction was stopped by addition of 0.175 ml of 1 M HCl. Hippuric acid was extracted with ethyl acetate, dried and resuspended in ethanol and measured spectrophotometrically at 228 nm [21]. One unit of ACE was defined as the amount of enzyme catalyzing the release of 1 nmole of hippuric acid from HHL per minute at 37°C.

Purification of ACE

All purification steps were carried out at 4°C.

Crude extract preparation: From mature sheep testes epididymis was separated, cut into small pieces and washed with 0.1 M phosphate buffer pH 8.3 containing 0.05 M NaCl. Sliced tissue (50 gm) was homogenized in 500 ml of 0.1 M phosphate buffer pH 8.3 containing 0.05 M NaCl. Homogenization was done in Waring Blender at maximum speed six times for 30 seconds with one minute cooling interval. The homogenate was centrifuged at 20,000 g for 20 minutes. Supernatant was collected and dialyzed overnight against 20 volumes of extraction buffer and designated as crude extract.

Ammonium sulfate precipitation: To the dialyzed crude extract (545 ml) 113.99 gm of solid ammonium sulfate was added to 35% saturation with continuous stirring and solution

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was left for 4 hours. Then it was centrifuged at 20,000 g for 20 minutes and the supernatant was collected (548 ml). To this 175.82 gm solid ammonium sulfate was added to 80% saturation with continuous stirring and the solution was left for 4 hours. The precipitate was collected by centrifugation at 20,000 g for 20 minutes and dissolved in 0.1 M phosphate buffer pH 8.3 containing 0.05 M NaCl. Later it was dialyzed against same buffer for overnight and concentrated by millipore immersible CX-10 filters and designated as 35-80% ammonium sulfate fraction.

DEAE Sephadex A-50 ion exchange chromatography:

The ammonium sulfate fraction (984 mg) was dialysed against 0.005 M phosphate buffer pH 8.3 and loaded on to a column of DEAE Sephadex A-50 (1.5 X 28.3 cm, bed volume: 50 ml) equilibrated with 0.005 M phosphate buffer pH 8.3 at a flow rate of 12 ml/hour. Then the column was washed with 3 bed volumes of equilibration buffer and washings containing inactive proteins were discarded. The column was eluted with a linear gradient system of phosphate ion formed between 250 ml of 0.005 M phosphate buffer pH 8.3 in the mixing chamber and 250 ml of 0.05 M phosphate buffer pH 8.3 in the reservoir and 10 ml fractions were collected. The active fractions (tube numbers 13-20, Fig. 1) were pooled, concentrated by Amicon Centreprep-30 membrane and designated as DEAE Sephadex A-50 fraction.

Sephacryl S-300 gel chromatography: The concentrated DEAE Sephadex A-50 fraction (26.5 ml) was dialyzed against one litre of 0.1 M phosphate buffer pH 8.3 containing 0.15 M NaCl for 8 hours. Batch wise gel chromatography using 1 ml of dialyzed DEAE Sephadex-A-50 fraction (9 mg protein) was done. The one ml fraction was allowed to flow through column of Sephacryl S-300 (1.4 X 78 cm, bed volume:120 ml) equilibrated with 0.1 M phosphate buffer pH 8.3 containing 0.15 M NaCl at flow rate of 12 ml/hour and four ml fractions were collected. The active fractions (tube numbers 19-21, Fig. 2) were pooled.

Amicon Centreprep-30 membranes were used to concentrate active fractions of the batches and designated as Sephacryl S-300 fraction (volume 5 ml).

Biogel HTP-Hydroxyl apatite chromatography:

Sephacryl S-300 fractions (56.6 mg protein) was dialysed against 500 ml of 0.001 M phosphate buffer pH 6.8 for ten hours. This fraction was allowed to flow through Biogel HTP hydroxyl apatite column (1.4X 13 cm, bed volume: 20 ml) equilibrated with 0.001 M phosphate buffer pH 6.8 at a flow rate of 5 ml/hour. Then the column was eluted with a linear gradient system of phosphate ion formed between 100 ml of 0.001 M phosphate buffer pH 6.8 in the mixing chamber and 100 ml of 0.1 M phosphate buffer pH 6.8 in the reservoir. Four ml fractions were collected at a flow rate of 5 ml/hour. The active fractions (tube numbers from 15-19, Fig. 3) were pooled and concentrated by Amicon centreprep-30 membranes. The concentrate was dialysed against 20 volumes of 0.1 M phosphate buffer pH 8.3 containing 0.05 M NaCl and used for further studies.

Protein estimation: Protein content of tissue extract and column eluates was measured by the method of [22] using bovine serum albumin as standard.

Electrophoresis: Native polyacrylamide gel electrophoresis (PAGE) with 30 µl sample containing 6.3 µg protein was performed according to method. [23] Sample was diluted in non denaturing buffer (0.6 M Tris-HCl pH 6.8, 10 % glycerol and 0.025% bromophenol blue). The electrophoresis was performed with 0.02 M Tris, 0.192 M glycine pH 8.3 using 7.5 % gel and a current of 180-200 volts for 4-5 hours. Coomassie Brilliant Blue R-250 was used to stain protein (0.25% in methanol, acetic acid and water in 5:1:5 ratios).

Step	Volume (ml)	Protein Content (mg/ml)	Total protein (mg)	Total activity Units	Specific activity	Folds purification	Yield (%)
Crude extract	545	4.05	2208	52970	23.99	1	100
Ammonium sulphate	50	19.68	984	48216	49.00	2.04	91.02
DEAE Sephadex A-50	26.50	9.06	240	36720	153	6.378	69.32
Sephacryl S-300	5.0	11.32	56.60	26249	463.76	19.33	49.55
Hydroxyl apatite	12.74	0.2109	2.687	17485	6507.2	271.25	33.0

Table – 1 Purification of angiotensin converting enzyme from ram epididymis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Non-reducing SDS-PAGE: It was performed according to the method [23] using 7.5 % gel, 0.25 M Tris, 0.192 M glycine, 0.1% SDS (w/v) at pH 8.3. The samples were treated with SDS-non reducing buffer containing 0.6 M Tris-HCl pH 6.8, 1% SDS, 5% glycerol and 0.012 % bromophenol blue and boiled for five minutes at 90°C. The electrophoresis was carried out for 4-5 hours at voltage of 180-200 V. The protein bands are stained as described above.

SDS-reducing PAGE: Molecular weight of purified ACE was determined by SDS-reducing PAGE which was performed as described above in presence of β-mercaptoethanol.

Carbohydrate estimation: The carbohydrate content of purified ACE was determined by phenol-sulfuric acid method. [24]

Kinetic studies

Michaelis constant (K_m) and maximum velocity (V_{max}) of purified ram epididymal ACE with Hip-His- Leu as substrate were determined from Direct linear plotting method of Eisenthal and Cornish Bowden [25] at different substrate concentrations ranging from 0.5 to 10 mM in phosphate buffer pH 8.3 containing 0.6M NaCl. K_m and V_{max} values are given by the points where dashed lines inter sect abscissa and ordinate respectively. The purified ACE protein 1.05 µg was used in assay. Catalytic constant (K_{cat}) was calculated from V/(E) where (E) is the concentration of enzyme in assay based on the molecular mass obtained and V is the initial velocity [26]. Then hydrolytic coefficient K_{cat} / K_m was calculated.

Chemical modification studies
Modification of arginyl residues: Arginyl groups of purified epididymal ACE were modified by treatment with ninhydrin. [27] The purified protein (105.45µg) was treated with 890.50µg of ninhydrin (5mM) in a volume of 1ml (100 µ moles) of borate buffer pH 9 at 4 ^{0}C. At definite time intervals 0.2 ml of aliquots were withdrawn and dialyzed against 0.1M phosphate buffer pH 8.3 containing 0.05M NaCl for 8 hrs at 4^{0}C. The dialyzed aliquots were assayed for residual ACE activity Carboxyl groups modification:

Carboxyl groups of purified ACE were modified by treatment with cyclohexyl morpholinoethyl carbodiimide. [20] The purified ACE protein (210.9 µg) was treated with 16.04 mg of cyclohexyl morpholinoethyl carbodi imide (20mM) in a volume 0f 2ml 0.05M MES buffer containing 10^{-4} M Zn^{2+}, pH 6.0 at 4^0 C. After definite time intervals aliquots were with drawn dialyzed and assayed for residual ACE activity as described above.

Modification of tyrosyl residues:

Hydroxy phenyl groups of purified ACE were modified by treatment with N-acetyl imidazole [20] and 1- fluoro -2,4-dinitrobenzene , FDNB [28]. The purified ACE protein (105.45 µg) was treated with 550.5µg of N-acetyl imidazole (5mM) in a volume of 1ml 0.05M HEPES buffer pH 7.5 and with 558.3 µg FDNB (3mM) in a volume of 1ml of 0.1M borate buffer pH 8.5 separately at 4°C . After definite time intervals aliquots were withdrawn, dialyzed and assayed for residual activity as described earthier.

Modification of lysyl groups: Free (ε) amino groups of lysine of purified ACE were modified by treatment with TNBS, DEPC and acetic anhydride [20]. The purified ACE protein 105.45 µg was treated with 1.466 mg of TNBS (5mM) and with 810.5 µg of DEPC (5mM) in a volume of 1 ml of 0.05M HEPES buffer pH 8.5 separately at 4^0C.After definite time intervals aliquots were with drawn dialyzed and assayer for residual activity as described earlier.

To study effect of acetic anhydride 105.45 µg of purified ACE protein was treated with 204.18 µg of acetic anhydride (2mM) in a volume of 1ml of 0.05 M HEPES buffer pH 7.5 at 4^0 C . Aliquots were with drawn after definite time intervals and processed as above. Controls without chemical modifiers were run simultaneously in all cases.

Modifier	Concentration	Time in hours	Residual Enzyme Activity
Ninhydrin	5mM	0.50	35.0
		1.50	0.0
Cyclohexylmorpholino ethyl carbodi imide	20mM	0.50	66.28
		1.50	50.26
N-Acetyl imidazole	5mM	0.50	25.0
		1.50	0.0
FDNB	3mM	0.50	55.0
		1.50	8.30
TNBS	5mM	0.50	33.47
		0.50	14.2
DEPC	5mM	0.50	10.50
		1.50	0.0
Acetic anhydride	2mM	0.50	2.40
		1.00	0.00

Table 2.Chemical Modification of ram epididymal angiotensin converting enzyme

Effect of EDTA and 1,10 – phenanthroline

The purified ACE protein (1.05 μg) was treated with a final concentration of 0.057mM EDTA and 1,10- phenanthroline [21]. 10 μl each of EDTA and 1.10- phenanthroline were included in the assay mixture. After 30 minutes incubation at 37°C residual ACE activity was determined.

Effect of cobalt nitrate

To study effect of Cobalt nitrate 1.05 μg of purified ACE protein was treated with various concentrations of cobalt nitrate ranging from 0.1μM to 650mM in a volume of 10μl [21].10μl of each of cobalt nitrate was included in the assay mixture. After 30 minutes incubation at 37°C ACE activity was measured.

Results

The results of purification of ram epididymal ACE are summarized in Table-1. Each step of purification was assessed by non reducing SDS-PAGE and measurement of ACE activity (Fig. 4). Ram epididymal ACE was isolated with specific activity of 6507 units per mg protein and there was 271 times enrichment

in ACE activity. The ammonium sulphate precipitation removed large amount of protein with little loss of ACE activity. Ion exchange chromatography with DEAE Sephadex A-50 yielded a single peak between 17-23 mM phosphate ion concentration (Fig. 1). The hydroxyl apatite chromatography enhanced ACE activity by 14 times with only slight loss of ACE activity. A single peak of ACE activity was eluted between 31-39 mM phosphate ion concentration (Fig. 3).

About 2.7 mg of homogenous epididymal ACE was obtained from 50 gm of ram epididymis. The enzyme purity was confirmed by three different methods. Only one single band was obtained on native PAGE (Fig. 5), non-reducing SDS-PAGE (Fig. 4) and reducing SDS-PAGE (Fig. 6). The molecular mass of this homogenous enzyme was estimated as 102 kDa from the position of this band and those of the molecular weight markers by SDS-PAGE (Fig. 6). The carbohydrate content of ram epididymal ACE was estimated as 4.7 % of the weight.

Table -3. Kinetic Parameters of different ACE isoforms

ACE isoform	K_m (mM)	V_{max} μmol/min/mg	K_{cat} min^{-1}	K_{cat}/K_m M^{-1} min^{-1}
Ram epididymal ACE	1.9	10	29420	15.48×10^6
Ram epididymal Fluid ACE	2.65	163	--	---
Rabbit testicular ACE	2.60	22	18500	---
Chicken lung ACE	0.50	15.36nmol/min	515	990 m M^{-1} min^{-1}
Rabbit lung AC E (FAPGG Substrate)	0.30	--	19000	63×10^6
Porcine germinal ACE (Synthetic substrate)	0.68	1.0	33 s^{-1}	4.87×10^4 M^{-1} s^{-1}

Michaels constant (K_m) and V_{max} of purified epididymal ACE were 1.9 mM on and 10 µ moles/min/mg respectively (Fig.7). Based on molecular mass obtained K_{cat} was calculated as 29420 min^{-1}. The hydrolytic coefficient K_{cat} / K_m was15.48 x 10^6 min^{-1} M^{-1}. Data on effect of chemical modifiers on purified ACE activity are presented in Table-2. Arginyl groups modifier ninhydrin completely inactivated enzyme. But carboxyl group modifier cyclohexyl morpholino ethyl carbodi imide caused loss of half of the enzyme activity, Tyrosyl group modifier N-acetyl imidazole completely in activated enzyme (Fig.8A, 8B). Further ACE activity is not lost to same extent with FDNB. Enzyme activity was completely eliminated by lysyl modifiers acetic anhydride and diethyl pyrocarbonate (Fig.8B).TNBS another amino group modifier decreased enzyme activity significantly.
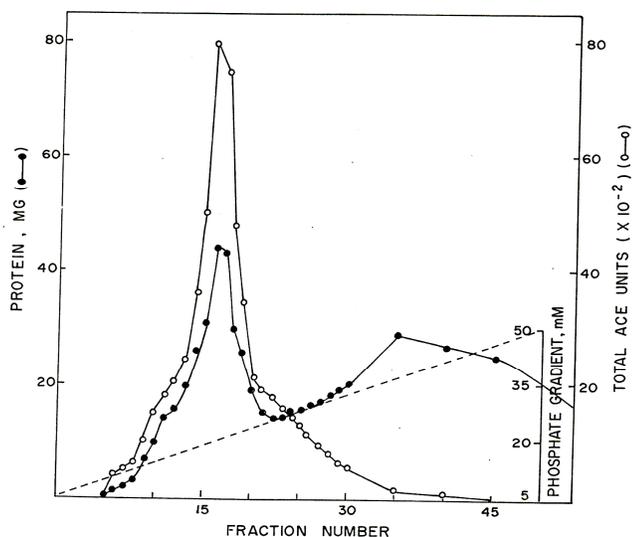
Figure 1. DEAE Sephadex A-50 ion exchange chromatography of ammonium sulfate fraction. Experimental details are given in text.

Both metal chelators EDTA and 1,10-phenanthroline inactivated enzyme. At concentration of 57 µM they completely inhibited ACE activity. Effect of cobalt nitrate on ACE activity is shown in Fig.9. In presence of cobalt nitrate initially ACE activity increased and reached maximum and decreased there after. Maximum activation (nearly two fold) was observed at 1mM and at concentration of 40 mM ACE activity was inhibited .

Discussion

Since sheep epididymis has high ACE activity among all organs [29] a protocol for its purification is presented for the first time. To the best of our knowledge ACE is not purified from epididymis of any animal species. This five step protocol yielded a homogenous ACE with molecular mass of 102 kDa. Further single protein band obtained with three types of PAGE indicated that it is single polypeptide chain. Unlike porcine seminal plasma ACE which was composed of two identical subunits [30] ram epididymal ACE do not contain two subunits .The molecular mass of epididymal ACE was close to C-terminal domain of somatic ACE [31] and with in the range of 90-110 kDa reported for testicular ACE in rat, rabbit and man [14,15,32]. However it is different from canine testicular ACE which had molecular mass of 65-70 kDa. [16] The ram epididymal ACE had high molecular mass than 94 kDa ACE isolated from ram epididymal fluid [17] but it differs from high molecular mass 290 kDa human prostate ACE [33] and 182 kDa porcine seminal plasma ACE [30]. These differences suggest existence of tissue specific ACE isoforms in male mammals.
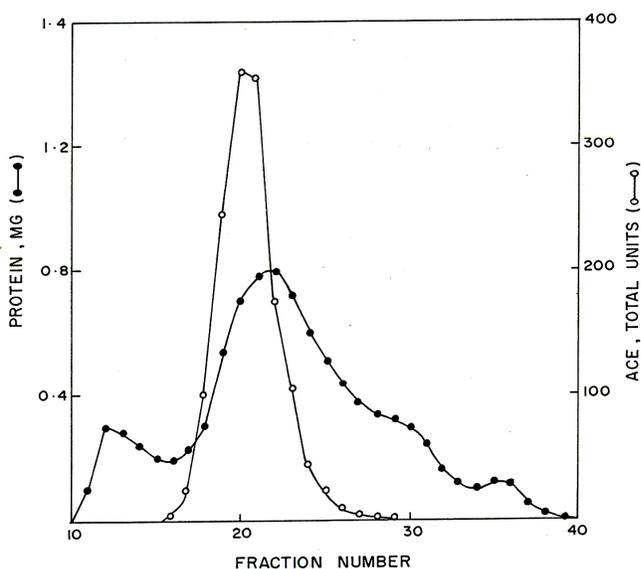

Figure 2. Sephacryl S-300 gel chromatography of DEAE Sephadex A-50 fraction. Experimental details are given in text.

The epididymal ACE is glycoprotein like other mammalian converting enzymes. Its carbohydrate content is less than rabbit testicular

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and pulmonary ACE [32,34] but similar to chicken lung ACE. [26] These differences are due to differential glycosylation of ACE in different organs or species [35].

Kinetic parameters of different ACE iso forms are summarized in Table- 3. The K_m and V_{max} values of ram epididymal ACE were similar to ram epididymal fluid ACE [17] and rabbit testicular ACE [32] but different from chicken lung ACE [26], rabbit lung ACE [36] and porcine germinal ACE [30].
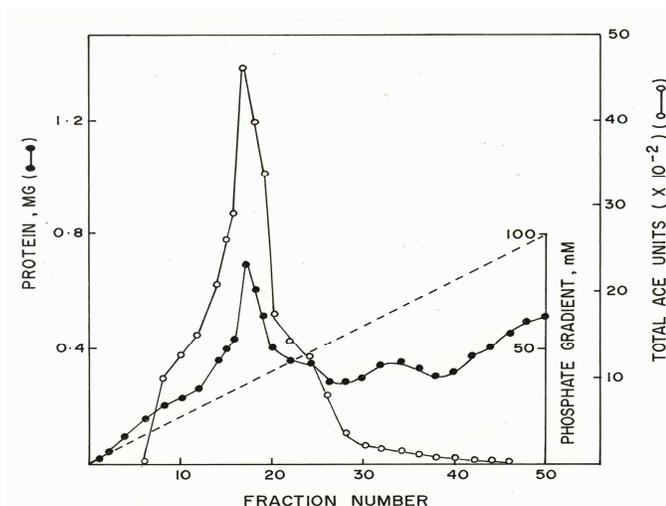

Figure 3. Biogel hydroxyl apatite chromatography of Sephacryl S-300 fraction. Experimental details are given in text.

Complete loss of ACE activity on treatment with arginyl group modifier may be due to abolition of chloride binding sites [9]. Partial loss of ACE activity with carboxyl modifier suggest glutamate is part of active site HEXXH motif like testicular ACE [20]. Alternatively it may be due to involvement of other glutamate residues that are far away from active site in enzyme activity [19]. Total and 92% loss of enzyme activity by N-acetyl imidazole and FDNB respectively indicate that tyrosyl residues of epididymal ACE are involved in interaction with substrate [20]. Inactivation of epididymal ACE by lysyl group modifiers suggest that lysyl groups are essential for enzyme activity like rabbit lung ACE [20].

Inhibition of epididymal ACE activity by EDTA and 1,10 – phenanthroline indicates that it is a metalloprotease like other ACE iso forms [17,26]. Activation of epididymal ACE by cobalt was higher than rat epididymal ACE [7] and rabbit lung ACE [21]. This suggests that activation of ACE isoforms by cobalt varies among tissues and species.
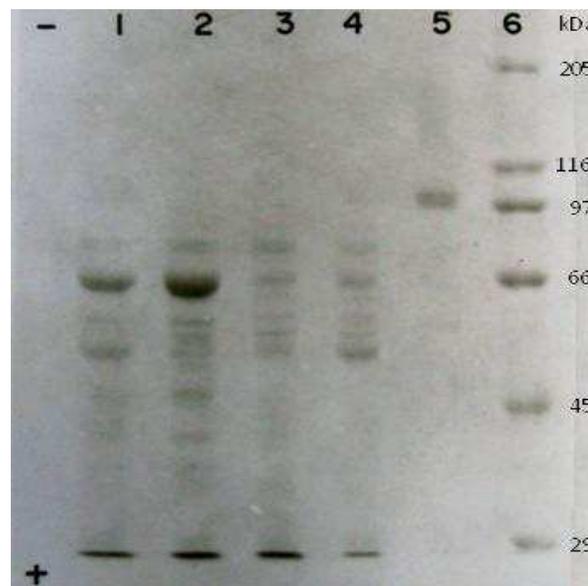

Figure 4. Non reducing SDS-PAGE of purified ram epididymal ACE, other purification fractions and molecular weight standard proteins. Lane 1 is crude extract, Lane 2 is ammonium sulfate fraction, Lane 3 is DEAE Sephadex A 50 fraction, Lane 4 is Sephacryl S-300 fraction, Lane 5 is purified ram epididymal ACE (Biogel hydroxyl apatite fraction). Lane 6 is molecular weight standard proteins. Myosin (205 kDa); , β-galactosidase (116 kDa); Phosphorylase b (97 kDa); Bovine albumin (66 kDa); Egg albumin (45 kDa); Carbonic anhydrase (29 kDa). Experimental details are given in text.

Unlike ram epididymal fluid ACE, purified epididymal ACE may not be derived from testicular ACE [17]. It may be part of intrinsic rennin-angiotensin system of

epididymal cells [12,13] and its exact role in male reproductive organs remains to be explored.

Conclusion

Our data suggest that an ACE isoform exist in sheep epididymis which may be part of epididymal rennin-angiotensin system. Further studies are needed to know ACE role in male reproductive system.

Figure – 5: Native PAGE of purified ram epididymal ACE. Experimental d

details are given in text.

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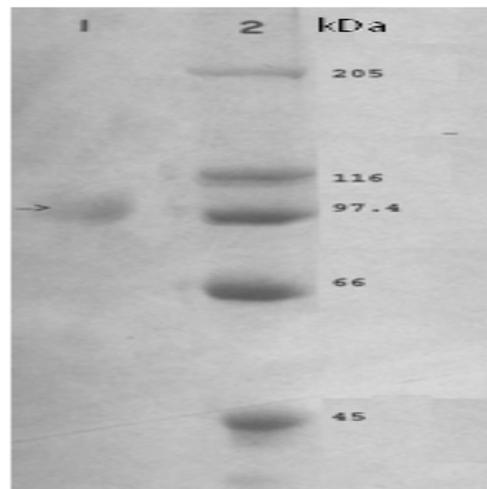
Figure 6. Reducing SDS-PAGE of purified ram epididymal ACE and molecular weight standard proteins. Lane 1 is purified ACE; Lane 2 is molecular weight marker containing myosin (205 kDa); β-galactosidase (116 kDa); Phosphorylase b (97.4 kDa);

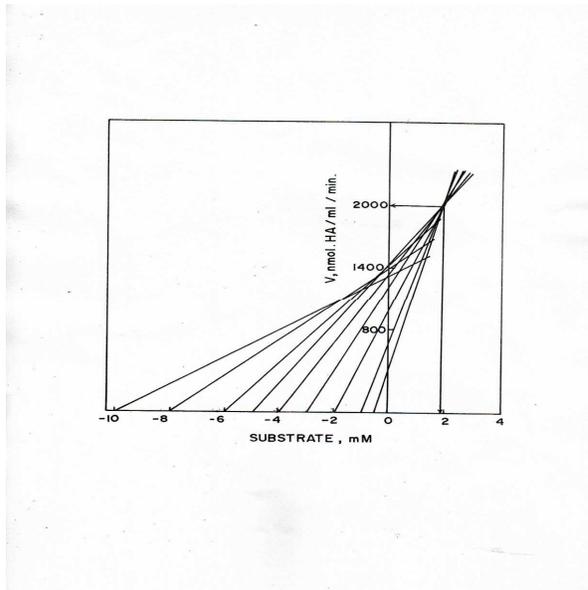bovine albumin (66 kDa); Egg albumin (45 kDa); Experimental details are given in text.
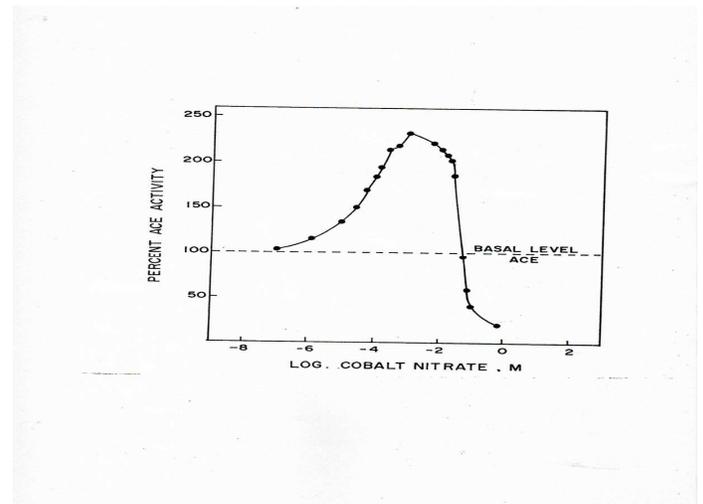
A: After 30 minutes B: After 90 minutes.

Figure 9. Effect of cobalt ion on epididymal ACE with HHL as substrate. Experimental details are given in text.

Figure 7. Direct linear plot of epididymal ACE with HHL as substrate Each line represents an observation of s and v and has intercepts - s and v on x and y axes respectively. Experimental details are given in text.
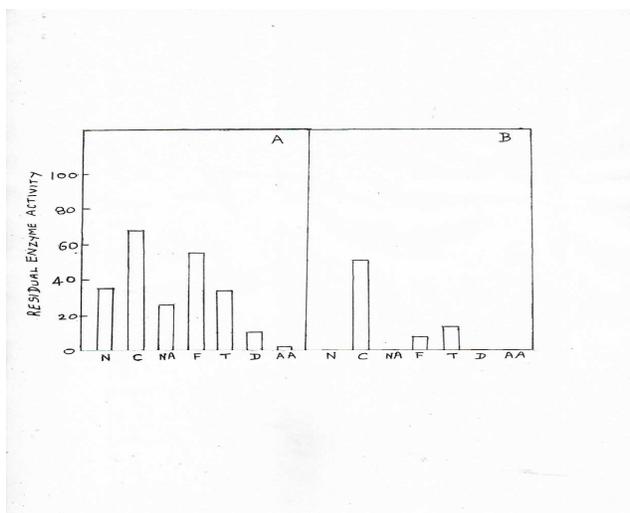

Figure 8. Ram epididymal ACE activity after treatment with chemical modifiers. Ninhydrin (N), cyclohexyl morpholino ethyl carbodiimide (C), N-acetyl imidazole (NA), FDNB (F), TNBS(T), DEPC(D) and acetic anhydride (AA).