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HUMAN TESTICULAR ANGIOTENSIN-CONVERTING ENZYME IS A MIXTURE OF TWO MOLECULAR WEIGHT FORMS. ONLY ONE IS SIMILAR TO THE SEMINAL PLASMA ENZYME

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Two molecular weight (M_r) forms of angiotensin-converting enzyme are present in human testis. Both the high M_r 140,000 form and the low M_r 90,000 form are catalytically similar but immunologically distinct. After isoelectric focusing, the profile of sialylated M_r 140,000 isozymes resembled that of seminal plasma converting enzyme, whereas the nonsialylated M_r 90,000 isozymes were distinct. These data suggest that the M_r 140,000 testicular converting enzyme may be a source of converting enzyme in seminal plasma.

Angiotensin-converting enzyme (ACE, EC 3.4.15.1) from most animal tissue is a single polypeptide chain glycoprotein with molecular weight (M_r) 140,000 (1,2) on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). However, the catalytically and immunologically similar ACE-like enzyme from rabbit testis, referred to by the more general term dipeptidyl carboxypeptidase, is structurally different having an M_r of only 100,000 (3). The unique structure of the testicular enzyme is emphasized by the observation that ACE from rabbit seminal plasma (4), and other rabbit tissues are the high M_r forms (5-7). Since all previously reported ACE from human tissue, including prostate (8) and seminal plasma (9), are high M_r forms, we sought to determine if human testis contained ACE or an ACE-like enzyme with a unique molecular structure. We report here that human testis

Abbreviations: ACE angiotensin-converting enzyme
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
CHAPS 3-[(3-chloroamidopropyl)-dimethylammonio]-1-propane sulfonate
HHL hippuryl-L-histidyl-L-leucine; IgG immunoglobulin G
CELIA competitive enzyme-linked immunoassay

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contains both M_r 140,000 and M_r 90,000 forms of the enzyme in approximately a 1:4 ratio. The high M_r form resembles ACE from seminal plasma.

METHODS

Purification of ACE. Testes were obtained either from cadavers or patients undergoing orchiectomy for prostatic carcinoma, and stored frozen at -20°C until needed. The epididymis, capsule, and other associated tissue were removed from the thawed testes. A 20% w/v homogenate was prepared from 25 g rete testes in 0.02 M potassium phosphate buffer, pH 8.3, containing 1 mM phenylmethylsulfonyl fluoride by homogenizing small pieces of tissue for 3 x 30 s bursts at high speed in a Waring blender with cooling in ice between bursts. The homogenate was centrifuged at 9,000 x g for 2 h and the supernate was separated from the pellet. The pellet was homogenized as above to produce a 20% w/v suspension. Triton X-100 was added to a final concentration of 0.1% and the suspension was shaken at 25°C for 1.5 h. The mixture was centrifuged at 9,000 x g for 2 h and the pellet discarded. The extract was dialyzed against 12 l of 0.02 M potassium phosphate, 0.15 M sodium chloride, containing 0.05% Triton X-100 for 24 h at 5°C with one change of buffer. An inhibitor-ligand affinity gel was prepared by a modification of the procedure of El-Dorry et al. (3) by replacing Sepharose CL-4B with Sepharose-6B as the solid support and coupling with ligand, N-(1-carboxy-3-phenylpropyl)-L-lysyl-L-proline (Merck L-154,826, a gift from Dr. D. M. Gross, Merck Institute, West Point, PA) for three days at 55°C in 0.5 M potassium carbonate, pH 11. The dialyzed extracted ACE sample was applied at 45 ml/h to a 1.6 x 30 cm column of affinity gel at 25°C and washed in with 3-4 bed volumes of dialysis buffer at 90 ml/h. The column was eluted with 2 bed volumes 5 mM potassium phosphate, pH 6.8, containing 92 µM captopril (SQ-14,225, a gift from Dr. D. W. Cushman, Squibb Institute, New Brunswick, NJ) and 0.1% CHAPS (Calbiochem-Behring 220201). Active samples were pooled and concentrated in an Amicon cell with a YM-30 membrane. The concentrate (5-10 ml) was applied at 30 ml/h to a 2.6 x 88 cm column of Ultrogel ACA-34 (LKB) previously equilibrated with 0.02 M potassium phosphate, pH 8.3, containing 0.025% CHAPS, 0.15 M sodium chloride, 0.02% sodium azide, and eluted isocratically. Other tissue ACE were prepared similarly.

Seminal plasma was separated from human sperm by centrifugation at 150 x g for 0.5 h at 5°C. Soluble ACE from seminal and blood plasma were prepared by batch adsorption of the samples to the affinity gel at a ratio of 20 ml plasma per g gel for 16 h at 25°C. Elution was done as described above in a sintered glass funnel. The sample was concentrated and applied to a 1.6 x 40 cm column of hydroxylapatite (Bio-Rad HTP) previously equilibrated with 5 mM potassium phosphate, pH 6.8. Under these conditions contaminants were adsorbed while ACE passed through the column as an unbound fraction.

Assay. Samples were assayed for ACE activity by a modification of the Cushman and Cheung procedure with hippuryl-L-histidyl-L-leucine (HHL) as substrate as previously described (10). Those eluted from the affinity gel had 1 mM N-ethylmaleimide added to the assay buffer to destroy captopril and uncover cryptic ACE activity. A unit (U) is defined as the amount of ACE that cleaves 1 µmol of HHL substrate per minute at 37°C.

Antiserum. Monospecific antiserum was prepared, and the IgG fraction isolated as previously described (10,11).

Kinetics. Michaelis constants (K_m) were calculated from individual data points on the velocity against HHL substrate concentration curves by the direct linear plotting method of Eisenthal and Cornish-Bowden (12).

Electrophoresis. SDS-PAGE was performed in 7% polyacrylamide gels according to Weber and Osborn (13) and staining was done with Coomassie Brilliant Blue R-250 (10).

Isoelectric Focusing. Focusing was done on 1% agarose gels followed by southern blotting to nitrocellulose and immunofixation as previously described (14).

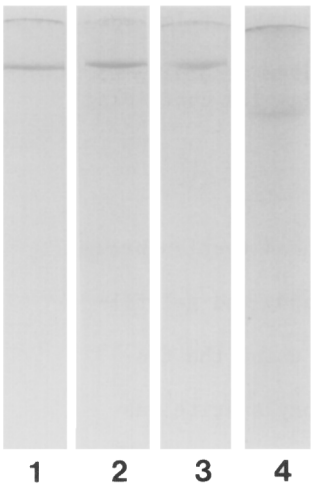
Enzyme Immunoassay. Competitive enzyme-linked immunoassay (CELIA) was performed as previously described (11).

Protein. Determination of protein concentration was done according to Lowry et al. (15) with bovine serum albumin as standard. Samples containing Triton X-100 were assayed by the Bradford method (16).

RESULTS AND DISCUSSION

Membrane-bound ACE was purified to homogeneity from human testis through a combination of detergent extraction, affinity chromatography and gel filtration. The soluble ACE from seminal plasma was purified using the two-step chromatographic protocol whereby ion-exchange on hydroxylapatite was substituted for gel filtration to facilitate removal of contaminants present at higher levels after affinity chromatography of the soluble ACE. Testicular ACE was obtained in two forms, as assessed by SDS-PAGE (Fig. 1), a M_r 90,000 form with a specific activity of 55 U/mg and a M_r 140,000 form with a specific activity of 40 U/mg. The M_r and specific activity of seminal plasma ACE were identical to those of the high M_r testicular ACE, and similar to previously purified ACE from human tissue (10, 17-22). Approximately 20% of the total testicular ACE was the high M_r form based upon an analysis of the area under the peaks from the Ultrogel ACA-34 column.

A family of charged isomers for each of the major forms was revealed upon isoelectric focusing in agarose followed by southern blotting and immunofixation (Fig. 2). This technique was employed to enhance sensitivity for minor charged isomers which were not revealed by Coomassie Blue staining of pressed and dried agarose. The focused pattern for M_r 140,000 testicular ACE resembled that of seminal plasma ACE more closely than it resembled those from other tissues. The complex multibanded pattern, with pI values ranging from 4.4 to 4.55, contained less sialic acid than human plasma ACE. Upon neuraminidase treatment, a closely spaced doublet of major bands associated with additional minor bands was generated from M_r 140,000 ACE from either testis or seminal plasma. This desialylated pattern, with an average pI of 4.6, was similar for all high M_r ACE. Testicular ACE M_r 90,000 was resolved into two bands (pI 4.7, 4.75) which were refractive to neuraminidase treatment, and thus appeared not to be sialylated.



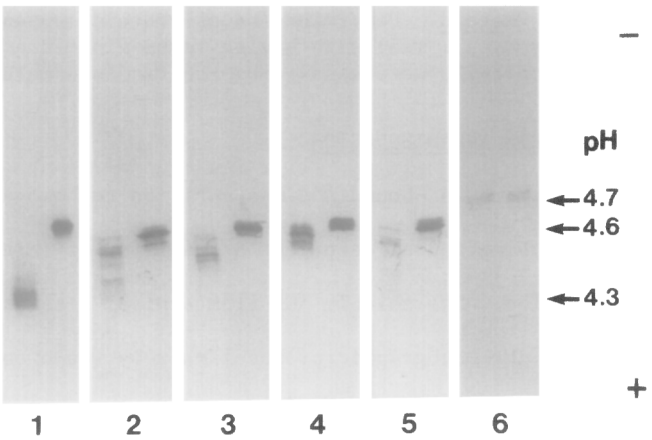


Figure 1 Purified angiotensin-converting enzymes (2-5 μg) separated by SDS-PAGE on 7% gels are from the following tissues: 1, blood plasma; 2, seminal plasma; 3, testis (M_r 140,000); 4, testis (M_r 90,000).

Figure 2 Immunofixation of angiotensin-converting enzyme transferred to nitrocellulose by southern blotting after agarose isoelectric focusing (Servalyte T 4-9). Samples are from the following tissues: 1, blood plasma; 2, seminal plasma; 3, lung; 4, kidney; 5, testis (M_r 140,000); 6, testis (M_r 90,000). Before (left) and after (right) neuraminidase.

To assess the catalytic and immunological relationship between these ACE isozymes, Michaelis constants, the effect of specific inhibitors, and the effect of anti-human plasma ACE antibodies were compared. All isozymes released dipeptides from angiotensin-1 and bradykinin. The Michaelis constants with HHL as substrate over a range of 1-10 mM were similar for all isozymes, being 1.7 mM for blood plasma ACE, 1.6 mM for seminal plasma ACE, 1.7 mM for M_r 140,000 testicular ACE and 1.85 mM for M_r 90,000 testicular ACE. These results agreed well with those obtained for ACE from other human and animal tissues (3,5,6,17,19,21). All ACE isozymes were completely inhibited by 10^{-4} M EDTA, 10^{-6} M captopril, 10^{-6} M Merck L-154,826, and when assayed in buffers devoid of chloride ion. Thus, each isozyme exhibits those properties accepted to be characteristic of true ACE (23).

Titration of catalytic activity with anti-plasma ACE antibodies gave identical titration curves for all ACE isozymes (Fig. 3). However, the M_r 90,000 enzyme was only partially homologous with the M_r 140,000 enzymes when

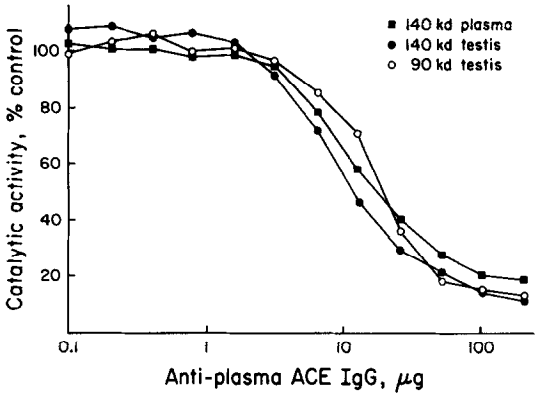


Figure 3 Titration of angiotensin-converting enzyme catalytic activity with anti-human plasma converting enzyme IgG. Converting enzyme samples (100 µl, 50 mU/ml) were incubated with IgG (25 µl) for 1 h at 37°C. Aliquots (100 µl) were added to buffered HHL substrate (400 µl) and assayed in triplicate for 2 h at 37°C.

compared by enzyme immunoassay (Fig. 4). These data indicate that anti-plasma ACE antibodies are equally reactive against epitopes on blood plasma ACE, seminal plasma ACE, and M_r 140,000 testicular ACE, but are either less reactive or react with fewer epitopes on M_r 90,000 testicular ACE. These immunological data are similar to those previously reported for ACE from rabbit tissue (3).

The amount of ACE in seminal plasma at 1,200 mU/ml (40-fold higher than the 30 mU/ml in blood plasma, and among the highest concentrations of ACE in human tissues) (24) with a specific activity of 35 mU/mg protein suggests a potential role for this enzyme beyond metabolism of angiotensin and bradykinin. It does not appear that the high M_r form in testis was a contaminant

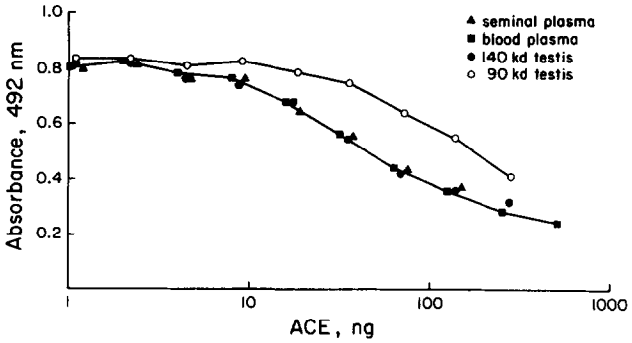


Figure 4 Competitive enzyme-linked immunoassay (CELIA). Each point is the mean of quadruplicate determinations.

from other tissues, particularly epididymis, since both the epididymis and capsule were discarded prior to homogenization. In addition the supernate after centrifugation of the homogenate, which would contain soluble ACE contaminants, was discarded also.

That human testis contains two ACE M_r forms is significant. Only the low M_r form is present in rabbit testis (3) and rat testis (JJ Lanzillo and Y Dasarathy, unpublished data). Our observation that the isoelectric profile of one form of human testicular ACE is similar to that of seminal plasma ACE suggests that one source of seminal plasma ACE may be the testis. The contribution to seminal plasma ACE from sperm remains unresolved. Azospermic samples of human semen contain substantial ACE (9). Nevertheless, sperm does contain ACE. Strittmatter and Snyder (25) localized ACE to spermatid heads of rat sperm, and Yotsumoto et al. (26) localized ACE to spermatids in swine testis and sperm. However, sperm ACE may be the low M_r form. A monoclonal antibody against rat lung ACE did not react with the sperm form in testis (25).

The function of ACE in the male reproductive system remains unknown. Bradykinin may play a role in sperm motility and spermiogenesis (27); yet neither bradykinin nor angiotensin are present in semen (9,28), although renin exists in seminal plasma (29) and in the uterus (9). Possibly ACE functions in fertilization since the uterus is readily stimulated by bradykinin (9). ACE in seminal plasma correlates with sperm motility and density (30). Thus, ACE may be an index of sperm quality. Further research will be required to elucidate the function of both testicular ACE and seminal plasma ACE. (This work was supported by NHLBI Grant HL 14456.)

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