Enzyme-linked immunosorbent assay for angiotensin-converting enzyme in rat testes

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An enzyme-linked immunosorbent assay (ELISA) was developed to determine the concentration of angiotensin-converting enzyme (ACE) in the testes of rats. ACE was isolated and purified from the testes by affinity chromatography using the specific ACE inhibitor lisinopril bound to Sepharose CL-4B. Polyclonal antibodies to the purified ACE protein were produced in rabbits. A competitive antigen capture ELISA was developed by using antibody-coated polystyrene tubes as the solid phase and alkaline phosphatase covalently bound to purified ACE as the detector. This paper describes in detail the procedures for preparing the affinity gel and column, isolating and purifying ACE protein, producing and isolating the antibodies, production of the alkaline phosphatase-ACE conjugate, and the development of the ELISA. The assay was tested by determining the amount of ACE protein in rat testis as affected by zinc deficiency, a condition known to reduce ACE activity in this tissue. Preliminary results showed a direct correlation between the reduction in ACE activity and ACE protein in the Zn-deficient rats.

Keywords: ACE; ELISA; testis; rat

Introduction

Angiotensin-converting enzyme (EC 3.4.15.1, ACE) is a zinc-containing peptidyldipeptide hydrolase that catalyzes the cleavage of C-terminal dipeptides from oligopeptide substrates as angiotensin I and bradykinin. Presently it is thought that there are two isozymes; the somatic type with a molecular mass of 160 kD and the testicular type at approximately 110 kD. Recent studies have shown that the testis enzyme in the mouse and human is identical to the C-terminal half of the somatic type.[1,2] Studies by Howard et al.[3] suggest that transcription of testis ACE in the mouse is initiated at a unique site within the somatic ACE gene.

ACE may participate in numerous physiological functions, but studies have been directed largely to the area of blood-pressure regulation. However, there are large amounts of ACE activity in numerous tissues that may be unrelated to this function. The testis, for example, has a very high ACE

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activity that increases progressively as the animal matures and is considered to be under androgen regulation; somatic ACE is not.

The function of ACE in the testis is unknown, but its activity is responsive to the nutritional Zn status of the animal.[4,5] In previous studies we showed that testicular ACE activity was not fully expressed in the testes of rats fed a Zn-deficient diet before puberty.[6] Somatic ACE of the particulate type found in lung and epididymis was not affected by the deficiency; lung ACE activity tends to increase under this condition.[5]

Our next objective was to determine if the failure of expression of testicular ACE activity was caused by a reduction in the amount of ACE protein. Because there were no established procedures for measuring ACE protein concentration in rat testes, we developed an enzyme-linked immunosorbent assay (ELISA) for that purpose. This required the isolation and purification of ACE, which was accomplished with affinity chromatography using lisinopril (N^{α}-[(S)-1-carboxy-3-phenylproyl] L-lysyl-L-proline), a specific active site inhibitor of ACE, as the affinity ligand. The purified ACE was used to produce antibodies in rabbits, conjugated to alkaline phosphatase to be used as the colorimetric detector in the ELISA, and used to prepare a reference standard for the ELISA. The complete procedures for isolation and purification of ACE and the development of the ELISA are described in this report.

Methods and materials

Preparation of lisinopril affinity-gel

The lisinopril affinity-gel used to isolate and purify ACE from rat testes was made in our laboratory by modifying the procedures of Bull, Thornberry, and Cordes[7] and Hooper and Turner[8] as modified by Schullek and Wilson.[9] The entire procedure is outlined below. Unless specified, all chemicals of highest purity were purchased from Sigma Chemical Company, St. Louis, MO USA.

Fifty milliliters of packed Sepharose CL-4B (Pharmacia, Uppsala, Sweden) were suspended in 50 mL of 70% 1,4-butanediol diglycidyl ether. Fifty milliliters of 0.6 mol/L NaOH containing 2 mg/mL $NaBH_4$ were added and the mixture slowly stirred at room temperature (23 ± 2° C) for 8 hours. The resulting epoxy-activated Sepharose CL-4B was poured into a fritted disc glass funnel attached to a vacuum flask and washed with 5 L of distilled-deionized water. The gel was aspirated free of excess liquid, then 15 g of gel were added to 150 mL of 90 mmol/L 6-[N-(p-amino-benzoyl)] aminocaproic acid (Aldrich Chemical Co., Milwaukee, WI USA) in 0.1 mol/L $NaHCO_3$, pH 10.5. The mixture was incubated with gentle agitation for 48 hours in a 45° C water bath.

The mixture was placed in a fritted disc glass funnel and washed with 200 mL of 0.1 mol/L $NaHCO_3$ and then with 500 mL of water. Excess water was aspirated before the gel was transferred into a beaker with 150 mL of 1 mol/L glycine, pH 10.0, and incubated at 37° C for 6 hours with gentle agitation. The gel was allowed to settle and the supernatant aspirated. The gel was washed twice with 250 mL of water and 150 mL of 1,4-dioxane (Aldrich Chemical Co.) were added.

Six hundred and ninety mg of N-hydroxysuccinimide (Aldrich Chemical Co.) dissolved in 30 mL of 1,4-dioxane were then added and the mixture was gently stirred for 15 min at room temperature. Then 15 g of 1,3-dicyclohexylcarbodiimide (Aldrich Chemical Co.) were added and the mixture was allowed to incubate for an additional 70 min at room temperature with gentle stirring.

The activated gel was returned to the fritted disc funnel and successively washed with 100 mL of 1,4-dioxane, 200 mL of methanol (Fischer Chemicals, Pittsburgh, PA USA), 500 mL of water, and 200 mL of 0.1 mol/L $NaHCO_3$, pH 10.5. The gel was then added to 60 mL of 2.2 mmol/L lisinopril (a gift from Merck, Sharpe and Dohme Research Laboratories, Rahway, NJ USA) in 0.1 mol/L $NaHCO_3$ and incubated at 4° C with gentle stirring for 48 hours. The gel was then washed in the fritted disc funnel with 200 mL of 0.1 mol/L $NaHCO_3$ and aspirated free of liquid. The tip of the funnel was blocked and 30 mL of 1.0 mol/L glycine was added. This mixture was incubated at room temperature for 4 hours with frequent stirring with a glass rod. The gel was successively washed with 400 mL of water, 150 mL of 0.5 mol/L NaCl, and 100 mL of 50 mmol/L HEPES containing 0.4 mol/L NaCl, pH 7.0.

The finished affinity-gel was suspended in 50 mL of the last wash solution and poured into a 0.9 cm O.D. glass column (Bio-Rad, Richmond, CA USA). At a flow

rate of 0.5 mL/min, the gel was packed to a height of 11 cm. The column could be stored at room temperature for long periods of time without losing binding capacity.

ACE protein isolation

The procedure for the isolation and purification of ACE from rat testes was similar to that used by Schellek and Wilson[9] for bovine kidney and lung, but was modified somewhat as described below. The capsules were removed from 10 g of fresh or frozen testes and the tissue minced with scissors. It was homogenized in 50 mL of ice-cold homogenizing buffer (0.4 mol/L NaCl and 50 mmol/L HEPES, pH 7.0). Homogenization was done with a Tissumizer equipped with an SDT-182EN probe (Tekmar, Cincinnati, OH USA) at 16,000 rpm for 2 min at 4° C. The homogenate was centrifuged at 38,000*g* for 40 min at 4° C and the supernatant discarded. The pellet was washed twice by suspending it in 50 mL of homogenizing buffer with short bursts of the Tissumizer.

After the last wash, the pellet was resuspended in 500 mL of solubilizing buffer (0.4 mol/L NaCl, 10 μmol/L $ZnCl_2$, 0.5% Triton-X-100 in 50 mmol/L HEPES, pH 7.0) and stirred for 2 hours at 4° C. This mixture was centrifuged at 38,000*g* for 40 min at 4° C and the supernatant filtered through Whatman filter paper #541 (Hillsboro, OR USA). The supernatant was then added to the lisinopril affinity-gel column at a flow rate of 0.25 mL/min at 4° C. The column was equilibrated at 4° C for at least 1 hr before use. After all of the supernatant had been pumped through the column (about 33 hr), the column was washed successively with 70 mL of solubilizing buffer, 35 mL of 0.5 mol/L NaCl, 70 mL of solubilizing buffer, and 15 mL of 10 mmol/L Na_2HPO_4, pH 7.0. The column and pump were moved into the laboratory and allowed to equilibrate at room temperature for 30 min. The effluent line was connected to a fraction collector and ACE protein was eluted with 30 mL of 50 mmol/L $Na_2B_4O_7$, pH 9.5, at 23° C.[10]

Twenty-five 0.5 mL fractions were collected and analyzed for protein content according to procedures of Sigma Chemical Co. using bicinchoninic acid, and for ACE activity by Reeves and O'Dell.[5,11] Fractions containing ACE activity were pooled (3.5 mL) and concentrated to 1 mL in a Centricon filtering apparatus (Amicon, Beverly, MD USA) with 10 kD weight cut-off. To discourage protein binding to the filters, each was treated overnight with a 5% solution of Tween-20 in phosphate buffered saline (PBS, 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 per liter of water, pH 7.2) and rinsed with PBS before use. The $Na_2B_4O_7$ in the concentrated ACE fraction was removed by diluting the sample with 10 mL of PBS and re-concentrating it to 1 mL. The resulting fraction was analyzed for protein concentration and used for the production of antibodies, for conjugation to alkaline phosphatase, and as a standard for the ELISA. The ACE fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Bio-Rad on a 7% gel to determine purity and the molecular mass of the isolated ACE protein.

When ACE was to be used for the ELISA standards, the procedure for collecting ACE as it eluted from the column was different. To use ACE protein as a standard, the precise concentration must be known. Because ACE protein avidly binds to glass or plastic, the amount in solution can be greatly reduced with time. Therefore, to prevent ACE binding to the collecting tubes, each tube was soaked in a 5% Tween-20 solution overnight and allowed to air dry. Then exactly 50 μg bovine serum albumin (BSA) in 0.01 mL PBS was added to each tube before the ACE protein fractions were collected. Total protein concentration was determined by a micro-bicinochoninic acid procedure (Micro-BCA, Pierce, Rockford, IL USA) to minimize the volume of sample used. The amount of known BSA present in each tube was subtracted from the total protein determined. The ACE protein collected in this manner was pooled and lyophilized (Labconco Corp., Kansas City, MO USA). The residue was dissolved in 0.1% BSA to obtain a final concentration of 10 μg ACE protein/mL.

Antibody production

Polyclonal antibodies to ACE protein were produced in rabbits according to the procedures of Harlow and Lane.[12] One mL of purified ACE protein (600 μg/mL) was emulsified with 1.0 mL of Freund's complete adjuvant and injected intradermally on the upper back of two New Zealand white rabbits (Hazelton Research Products, Denver, PA USA). Each rabbit received 15 injections of 0.05 mL each. After 4 weeks, 5 mL of blood were taken from an ear vein and the serum collected. IgG was isolated with a Pierce ImmunoPure IgG Purification Kit (Pierce, Rockford, IL

USA) and a Bio-Dot (Bio-Rad Laboratories) titration plate was used to determine the titer to ACE. Booster injections of ACE were made in Freund's incomplete adjuvant to give a secondary immune response. After 2 weeks, the ACE antibody titer was found to be much higher than the first and serum was collected for IgG isolation.

Preparation of antibody coated tubes

The rabbit anti-rat testicular ACE IgG was mixed with 0.1 mol/L $NaCO_3$, 0.02% NaN_3, pH 9.6, at 1 μg/mL. One mL was incubated in disposable polystyrene tubes (12 × 75 mm) at 37° C for 3 hours. The coated tubes were stored, with the antibody solution, at 4° C until used. The coated tubes could be stored in this manner for up to 2 months without serious deterioration.

Enzyme-labeled ACE

ACE labeled with alkaline phosphatase was used as the detector. ACE was conjugated to alkaline phosphatase with methods described by Engvall and Perlmann.[13] Alkaline phosphatase (Sigma type VII-S, Sigma Chemical Co.) in 3.2 mol/L ammonium sulfate (2,800 U/mg protein) was used. Thirty-six microliters of this suspension containing 0.3 mg protein were centrifuged at 4° C for 10 min at 1000*g* and the supernatant discarded. The pellet was suspended in 0.1 mL of purified ACE protein (200 μg/0.1 mL) and transferred to dialysis tubing (cellulose ester membrane, 15 kD cut-off, 0.44 mL/cm, Spectrum Medical Industries, Inc., Los Angeles, CA USA) and dialyzed overnight at 4° C against PBS. Glutaraldehyde (Grade II, 25% solution) was added to a final concentration of 0.4% and the mixture incubated at room temperature for 2 hours. The conjugate preparation was then diluted to 1.0 mL with PBS and dialyzed overnight at 4° C against PBS. The ACE-alkaline phosphatase conjugate (ACEAPC) was diluted to 10 mL with 5% BSA in TBM (50 mmol/L Tris-HCl containing 1 mmol/L $MgCl_2$ and 0.02% NaN_3, pH 8.0) and stored at 4° C.

ELISA procedure

A competitive antigen capture ELISA was used to detect ACE protein concentration in rat testes. We ran the ELISA at a concentration of the detector, ACEAPC, that would occupy approximately 50% of the binding sites on the antibody coated tubes.

Determination of ACEAPC concentration. Antibody-coated tubes were washed three times with 1.0 mL each of 1% BSA solution containing 20 mmol/L Tris-HCl, 50 mmol/L NaCl and 0.05% Tween-20, pH 7.5, to remove any unbound IgG protein. Serial dilutions of ACEAPC were made from 1:10 to 1:160. One mL of each dilution was incubated overnight in the antibody-coated tubes. The solution was aspirated and the tubes washed two times with the above buffer. One mL of alkaline phosphatase substrate solution (p-nitrophenylphosphate, 1 mg/mL in 50 mmol/L $NaHCO_3$) was added and the tubes incubated for 40 min at room temperature. The absorbance was read at 400 nm. In the example shown in *Figure 1,* 50% binding capacity was attained at a 1:40 dilution of ACEAPC.

ELISA protocol. Antibody-coated tubes were prepared as described above. The tubes were placed in an ice-water bath and 100 μL samples containing either unknown amounts of ACE or ACE standards were added (*Table 1*). ACE standard dilutions ranged from 1–1000 ng in 0.1% BSA in water. Nine-tenths mL of working ACEAPC (1:40 dilution) solution was then added to each of the assay tubes. Tubes to assess total binding contained 0.1 mL of 1% BSA, ACEAPC, and no sample or standard. Blank tubes contained 0.1 mL of 1% BSA solution and no ACEAPC or sample. All tubes were incubated, for 18–24 hr at 4° C. After incubation, the solution was aspirated and the tubes washed twice to remove the unbound protein. One mL of alkaline phosphatase substrate solution was added and the tubes incubated for 40 min at room temperature. The absorbance was determined at 400 nm. The ratios of absorbance at each concentration of standard ACE (B) to maximal absorbance (tubes containing no ACE protein, B_o) were plotted against the log of ACE concentration in the standard tubes (*Figure 1*). This curve was linearized by log-logit transformation and used to calculate the amount of ACE protein in the unknown samples.

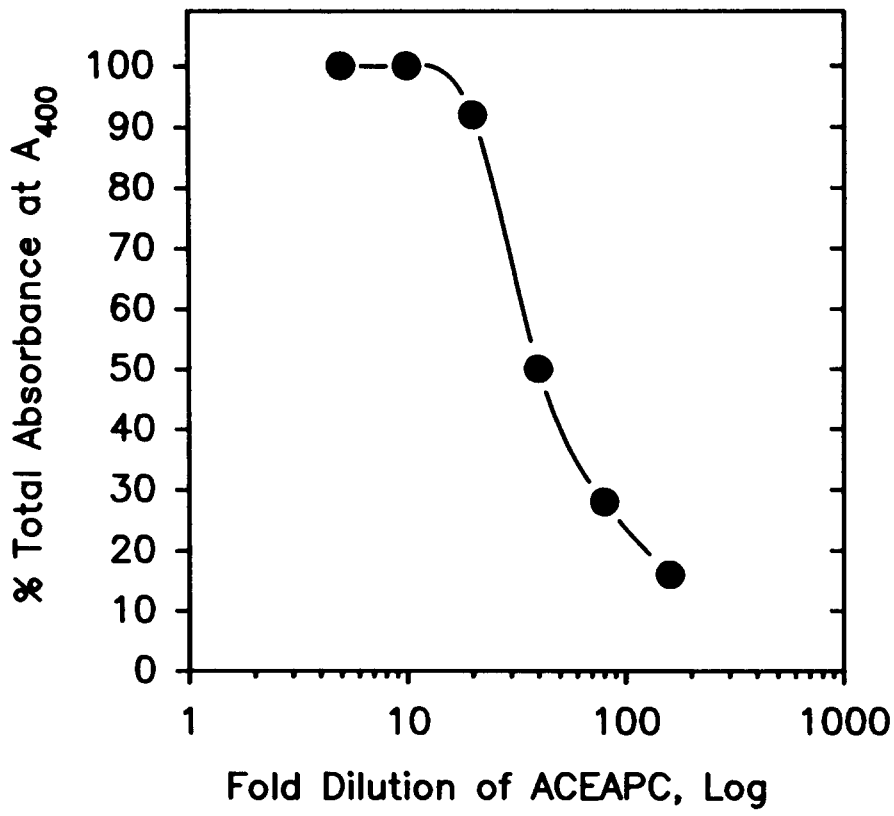

Figure 1 ACE-alkaline phosphatase conjugate binding to ACE antibody coated tubes. ACE protein was conjugated to alkaline phosphatase and used as the detector in the ACE ELISA. This graph shows that a dilution of 1:40 of the conjugate was required to attain approximately 50% binding to ACE antibodies coated onto polystyrene tubes.

Table 1 Recommended protocol for ACE ELISA

Tube #	Description	0.1% BSA µL	ACE Std. µL	ACE Std. Concentration ng/mL	ACEAPC* (1:40) µL
1,2	Blank†	100	0		900
3,4	Total Bound	100	0		900
	ACE, ng				
5,6	1	99	1	1,000	900
7,8	4	96	4	1,000	900
9,10	8	92	8	1,000	900
11,12	16	98.4	1.6	10,000	900
13,14	32	96.8	3.2	10,000	900
15,16	64	93.6	6.4	10,000	900
17,18	128	87.2	12.8	10,000	900
19,20	256	74.4	25.6	10,000	900
21,21	512	48.8	51.2	10,000	900
22,23	1000	0	100	10,000	900
25,26	Unknown #1	95	5		900
27,28	Unknown #2	95	5		900
29,30	Unknown #3	etc.			

*A 1:40 dilution of ACEAPC was made with 0.1% TBM.
†Blank tubes received 900 µL of 0.1% TBM without ACEAPC.

Results and discussion

The 0.5 mL fractions collected from the affinity column were analyzed for protein content and ACE activity. An example of the results of these analyses

is shown graphically in *Figure 2*. There was only one protein peak, and it coincided with ACE activity. Fractions with the greatest amount of ACE protein were routinely collected and concentrated. Recoveries of 20–35 μg of Triton-X solubilized ACE protein were obtained per gram of rat testis. This corresponds to approximately 6 μg recovered per grams of human testis by Ehlers et al.[1] using a similar method.

To verify that we had purified ACE to a single protein, a 7% SDS-PAGE was run on the concentrate. *Figure 3* shows that only one major protein was isolated, which indicates that the ACE fraction was essentially pure. The average molecular mass of this protein was estimated to be 112 kD. However, there was a very diffuse band at a lower molecular mass, which, by densitometry scan analysis, constituted only about 15% of the total area for both bands. The estimated average molecular mass of this diffuse band was 100 kD. By using anion exchange chromatography with a NaCl gradient, Vanha-Perttula et al.[14] also found two proteins in rat testes with ACE activity. The area of their minor peak was about 19% of total activity.

Our findings, however, are somewhat at variance with those of Ehlers et al.[1] for human testis. Using the Triton-X solubilization technique, they found two proteins with ACE activity: one with a molecular mass of 110 kD, similar to the rat, and the other 165 kD, both in equimolar concentration. Upon further treatment of the residue from the Triton-X preparation with trypsin, additional ACE was released. The overall ratio of large to small ACE in the human testis was 2:1. Our methods should not have been able to discriminate between the two ACE proteins if they are both present in rat testis, but in none of the isolations were we able to detect the heavier isozyme.

The degree of purification of testicular ACE by the lisinopril affinity method was somewhat lower than that found by other investigators for other tissues. *Table 2* shows the steps in a typical purification and the increase in specific activity. The overall increase in purification was approximately 1090

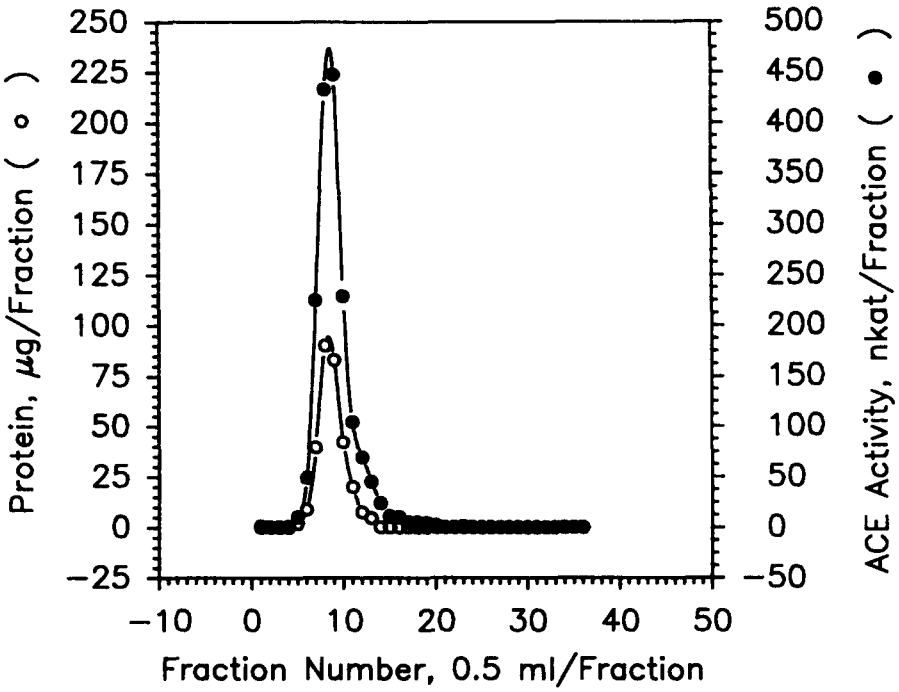

Figure 2 Separation of ACE on lisinopril-Sepharose CL-4B affinity column. ACE from the 38,000*g* supernatant of the Triton-X–solubilized testes was bound to the column and eluted with 50 mmol/L sodium borate, pH 9.5, at 23° C. The open circles show the protein peak and the closed triangles show the activity of ACE associated with the protein. At the peak, ACE activity was approximately 5400 nkat/mg protein. This compares to about 28 nkat/mg protein in the Triton-X–solubilized fraction.

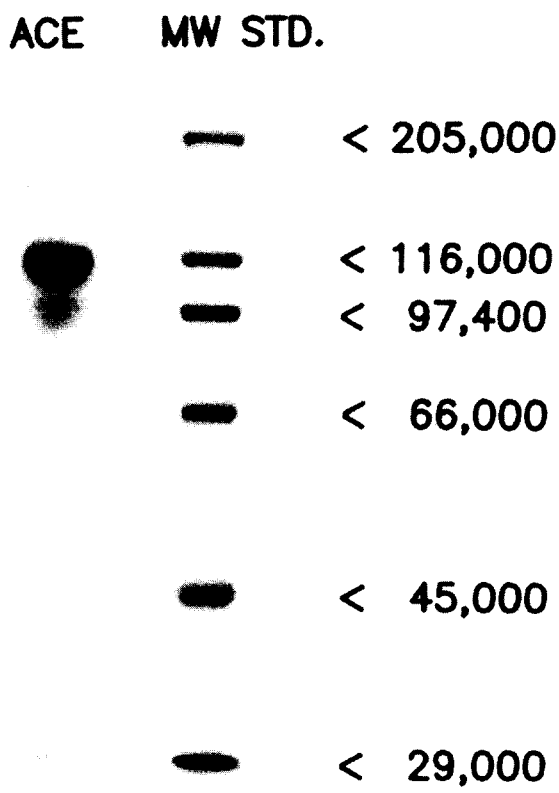

Figure 3 SDS-PAGE of rat testis ACE. ACE was isolated as described in the Methods section. Ten micrograms of the protein was compared to a total of 3 μg of standard molecular mass markers. The average molecular mass of the major ACE peak was approximately 112 kD, and the diffuse minor peak had an average molecular mass of 100 kD.

fold. This compares with 2100 fold for human lung tissue and 4400 fold for human kidney as prepared by Ehlers et al.[1]

The procedure for the ELISA was straightforward and rather easy to set up. *Figure 1* shows the dilution curve for binding of ACEAPC to the antibody-coated tubes. Fifty percent saturation of the antibody binding sites was obtained at 1:40 dilution of the conjugate. This dilution was approximately the same for all subsequent conjugations (data not shown). *Figure 4a* shows a plot of the ratio of absorbances at each concentration of ACE standard to the maximal absorbance without standard (B/B_o) versus the log of ACE concentration (ng). The curve was linearized by log-logit transformation (*Figure 4b*) and used to calculate the amount of ACE protein in the unknown samples.

A major problem encountered during the isolation and purification of ACE was the binding of the protein to the polypropylene collecting tubes. This resulted in the loss of protein and low recovery. When the isolated

Table 2 Purification of rat testicular ACE

Purification step	Specific activity (nkat/mg Protein)	Fold purification
1. Tissue homogenization	5	
2. Triton-X solubilization	28	5.6
3. Affinity Chromatography	5450	195
Overall		1090

fraction was to be used as the reference standard for the ELISA, we collected the ACE fractions into tubes previously treated with 5% Tween-20 and containing exactly 50 μg BSA/tube. The BSA acted as a blocking agent for binding sites remaining on the Tween-20–treated tubes. After analysis of total protein content, the amount of added BSA was subtracted from each tube. We found that this procedure provided a more precise concentration of ACE protein for use as the reference standard.

To test the newly developed ELISA, we determined the amount of ACE protein in rat testes as affected by Zn deficiency. Zinc deficiency has been shown to reduce the activity of testicular ACE in young rats by as much as 80%. Does this correlate with a change in ACE protein concentration? Three-week-old rats were divided into three groups and provided with three

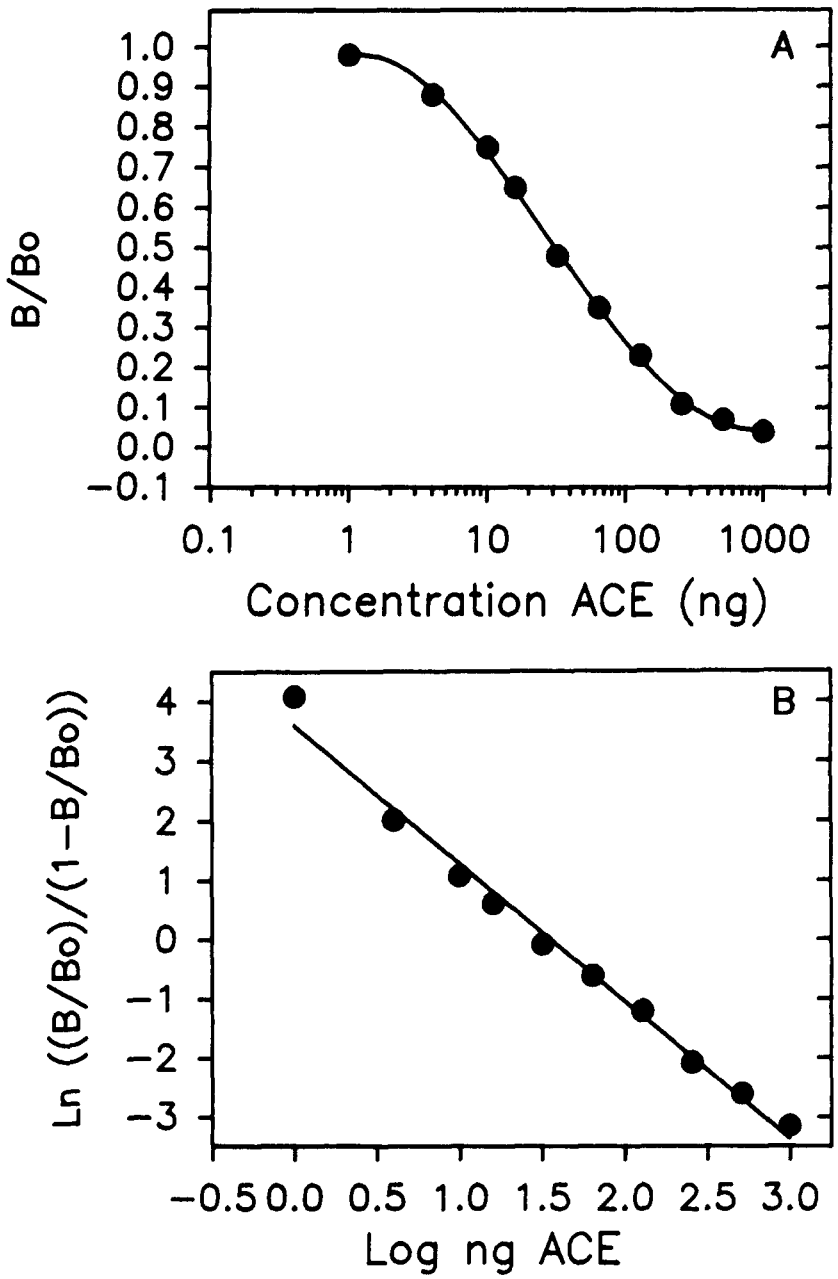

Figure 4 Representative standard curve for the ACE ELISA. Panel A shows a plot of a typical ACE standard curve used in the ELISA. Panel B is a log-logit plot of the same data. The latter was used to determine the amount of ACE protein in unknown samples.

Table 3 Effect of Zn deficiency on ACE activity and ACE protein concentration in rat testis

Treatment*	ACE activity (nkat/mg protein)	ACE protein (μg/g Testis)
+ZnAL†	30.5 ± 1.1[a]	31.0 ± 1.8[a]
−ZnAL	7.8 ± 1.7[b]	11.5 ± 1.0[b]
+ZnPF	24.7 ± 2.8[a]	27.0 ± 3.0[a]

*Treatments: +ZnAL, 40 μg Zn/kg diet, ad libitum; −ZnAL, <1 μg Zn/kg diet, ad libitum; +ZnAL, 40 μg Zn/kg diet, pair-fed to the −ZnAL group.
†Values represent the mean ± SEM of seven replicates per group. Means between treatment groups with different superscripts are significantly different ($P < 0.001$).

different feeding regimens. One group was fed ad libitum a Zn-adequate (40 mg Zn/kg) diet (+ZnAL); another group was fed ad libitum a diet deficient in Zn (<1 mg Zn/kg, −ZnAL); a third group was fed a Zn-adequate diet but the amount of food consumed by each rat was restricted to the amount consumed by a paired mate in the Zn-deficient group; commonly known as paired feeding (+ZnPF). Food intake depression is a consistent and reproducible occurrence in young Zn-deficient rats; therefore, this procedure was used to determine the effects of Zn deficiency alone.

After 4 weeks on their respective diets, the rats were anesthetized with sodium pentobarbital and the testes removed and treated as described for the isolation of ACE. Supernatants from the Triton-X–solubilized particulate fractions were analyzed for ACE activity and ACE protein concentrations. The results are shown in *Table 3*. Similar to previous findings,[5] Zn deficiency caused a significant decrease in ACE activity when compared with that in either control group. This corresponded closely with a reduction in the concentration of ACE protein. We have also demonstrated that ACE activity and ACE protein concentrations are restored to normal values when Zn-deficient rats are refed an adequate Zn diet for 2 weeks (data not shown). A more complete account of this study can be found in a report by Reeves and Rossow.[15] During two separate studies involving a total of 14 rats, the concentration of ACE in the testis of control rats as measured by ELISA, ranged from 20 to 60 μg/g of fresh tissue. This corresponds to a range of 20–35 μg of ACE protein per gram of tissue, recovered from the affinity column over the course of approximately 10 separate isolations.

In summary, this paper describes in detail the development of an ELISA for ACE protein in rat testis. It demonstrates the use of the assay to measure ACE protein in testis homogenate and describes the isolation and purification of ACE protein from this tissue.

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