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RADIOIMMUNOASSAY FOR PIG ANGIOTENSIN I CONVERTING ENZYME: A COMPARISON OF IMMUNOLOGIC WITH ENZYMATIC ACTIVITY

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SUMMARY Angiotensin I converting enzyme in body fluids and extracts of various pig tissues was measured by radioimmunoassay and by enzymic assay. Based on the ratios of enzymic to immunologic activity, the extracts could be separated into two groups. One group, with ratios around 4 U/mg, included urine and extracts from the adrenal, choroid plexus, epididymis, gall bladder, heart, liver, retina, spleen, and testis. The other group, with ratios around 12 U/mg, contained serum and extracts from lung and kidney. Explanations are offered for why one group had a lower enzymic to immunologic ratio than the other.

Angiotensin I converting enzyme or kininase II (EC 3.4.15.1) is a dipeptide hydrolase that inactivates bradykinin by cleaving Phe-Arg from its carboxyl terminus and that activates angiotensin I by converting it to angiotensin II with the release of His-Leu (1,2). Since bradykinin and angiotensin have functions in physiologic and pathologic conditions, much attention has been focused on these peptides and the enzymes that metabolize them. Antibodies to converting enzyme have been prepared and their ability to inhibit converting enzyme activity has been studied (3,4,5). Purified converting enzyme antibodies labeled with microperoxidase (6) or fluorescein isothiocyanate (7,8,9) have been used to localize converting enzyme on cells and/or in tissue slices.

A converting enzyme radioimmunoassay could extend our knowledge of this enzyme by making it possible to detect forms of the enzyme, such as a pro-enzyme or enzyme-inhibitor complexes, that may have little or no enzymic activity but are nonetheless immunologically active. In this paper, we

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report the use of a radioimmunoassay to measure converting enzyme in body fluids and crude extracts of various pig tissues and compare the results obtained with those from an enzymic assay.

MATERIALS AND METHODS

DEAE Sephadex A-50 and Sephadex G-200 and G-10 were purchased from Pharmacia Fine Chemicals, Piscataway, NJ; $Na^{125}I$ from New England Nuclear, Boston, MA; bovine serum albumin (fraction V), and HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) from Sigma Chemical Co., St. Louis, MO; and hippurylglycylglycine from Pfaltz and Bauer, Inc., Stamford, CN; SQ 20881, a competitive inhibitor of converting enzyme, was a gift from Dr. Z. Horowitz of the Squibb Institute of Medical Research, Princeton, NJ. All other reagents were analytical grade and obtained from commercial sources.

Tissues were excised from young male pigs weighing approximately 8 kg that had been killed with an overdose of sodium pentobarbital. Each tissue was rinsed with cold phosphate buffered saline (PBS)[2], pH 7.4, and then homogenized in 0.1 M Tris buffer, pH 7.4 (4 ml per g of tissue) with a Polytron homogenizer Model PT 10-35 equipped with a PT 10 generator. The homogenates were stored overnight at 5°C and then centrifuged at 100,000 x g for 2 hr. The supernatants were dialyzed at 5°C against 0.05 M HEPES buffer, pH 7.5, that contained 0.1 M NaCl, aliquoted, and then stored at -80°C. The protein concentrations of the samples were estimated by measuring their absorption at 280 nm.

Tissue extracts (0.035-1.69 mg of protein) were preincubated for 10 min at room temperature in 0.1 M Tris buffer, pH 7.4, that contained 0.1 M NaCl and 10^{-3} M $CoCl_2$ either with or without SQ 20881 (10^{-4} M). Enzyme reactions were initiated with the addition of hippuryl-glycylglycine (10^{-3} M) substrate and incubated at 37°. The reactions were stopped by the addition of an equal volume of 3% sulfosalicylic acid and the precipitated proteins removed by centrifugation. The supernatants were filtered through 0.45 μ Millipore filters, and their glycylglycine and glycine contents were determined with a Beckman 121 Automatic Amino Acid Analyzer. Glycylglycine and glycine were resolved on a Beckman Custom Spherical Resin Type PA 35 column (0.9 x 5.0 cm) eluted with 0.2 M citrate buffer, pH 3.25. Converting enzyme activity was expressed in nanomoles of glycylglycine, corrected for loss due to its hydrolysis to glycine by dipeptidases in the tissue extracts and body fluids. This correction was made using data obtained when SQ 20881 was included in the reaction mixtures. 1 U of converting enzyme activity = 1 μmol of glycylglycine released from Hip-Gly-Gly per min at 37°C. Reaction rates were linear over the time periods studied.

Swine kidney converting enzyme (SKCE)[2] was purified as previously described (3). Antibody to the enzyme, elicited in a goat (7), was purified by chromatography on a DEAE-Sephadex A-50 column and by gel filtration on a Sephadex G-200 column (3). The purified antibody was dialyzed overnight at 5°C against 0.05 M sodium carbonate buffer, pH 9.5. Antibody coated tubes were prepared as described by Catt and Tregear (10). Polystyrene tubes, 12 x 75 mm (Falcon #2054), were coated at 4°C for 1 hr with antibody diluted in the carbonate buffer. The antibody solution was then aspirated and saved for future use. The tubes were washed once with cold PBS and then coated at 4°C with 5.0 ml of a 1% solution of bovine serum albumin in PBS. After 1 hr the albumin solution was poured off and stored. The tubes were washed twice

[2] Abbreviations used: PBS, phosphate buffered saline; SKCE, swine kidney converting enzyme.

with PBS and then aspirated. Antibody and albumin solutions contained 0.1% sodium azide to retard bacterial growth. SKCE was labeled with [^{125}I] according to the procedure of Freedlender et al. (11). Unreacted iodide was removed from the labeled enzyme on a column of Sephadex G-10 (0.8 x 20 cm). The specific activity of the radioactive enzyme, 0.5 Ci/μmol, was estimated by competitive displacement (12,13). The molecular weight of SKCE was taken to be 195,000 (3). The radioimmunoassay buffer was 0.05 M HEPES, pH 7.5, that contained 0.1 M NaCl. The final incubation volume (0.3 ml) was composed of 0.1 ml of buffer or a dilution of standard, i.e., purified swine kidney converting enzyme (SKCE), body fluid, or tissue extract (0.004-2.0 mg of protein) and 0.2 ml of [^{125}I]-labeled SKCE (10,000 cpm). After a 5-day incubation at 5°C, the tubes were aspirated, washed once with cold PBS, and counted in a gamma counter.

RESULTS

A typical standard curve, obtained when serial dilutions of purified unlabeled SKCE were incubated with constant amounts of [^{125}I]-labeled SKCE (10,000 cpm) and goat anti-SKCE serum, is shown in Fig. 1. In the absence of competing unlabeled antigen, 20-30% of the [^{125}I]-SKCE was specifically bound to the antibody coated tubes. Half of this binding could be inhibited by a concentration of unlabeled SKCE equaling 160 ng/ml, giving an apparent binding affinity constant of 1.2×10^{9} M^{-1}.

Immunologic estimates of converting enzyme concentrations in the crude pig tissue extracts and body fluids were made by comparing their inhibition of [^{125}I]-SKCE antibody binding to that obtained with standard, purified SKCE. Comparisons were made over the linear portion of the standard curve. The enzymatic activity in the extracts was assessed as described in the Methods section. Ratios of enzymatic to immunologic activity for each extract or fluid were calculated. The results obtained for one pig are shown in Table 1. Similar results were obtained when 0.05% Triton X-100 was included in the homogenization buffer (data not presented).

DISCUSSION

Based on the ratios of enzymic to immunologic activity, the pig tissue extracts and body fluids can be separated into 2 groups. One group, A, has a ratio of about 4.0 U/mg and includes urine and extracts from the adrenal, choroid plexus, epididymis, gall bladder, heart, liver, retina, spleen, and testis. The other group, B, contains lung, kidney, and serum. The ratios ob-

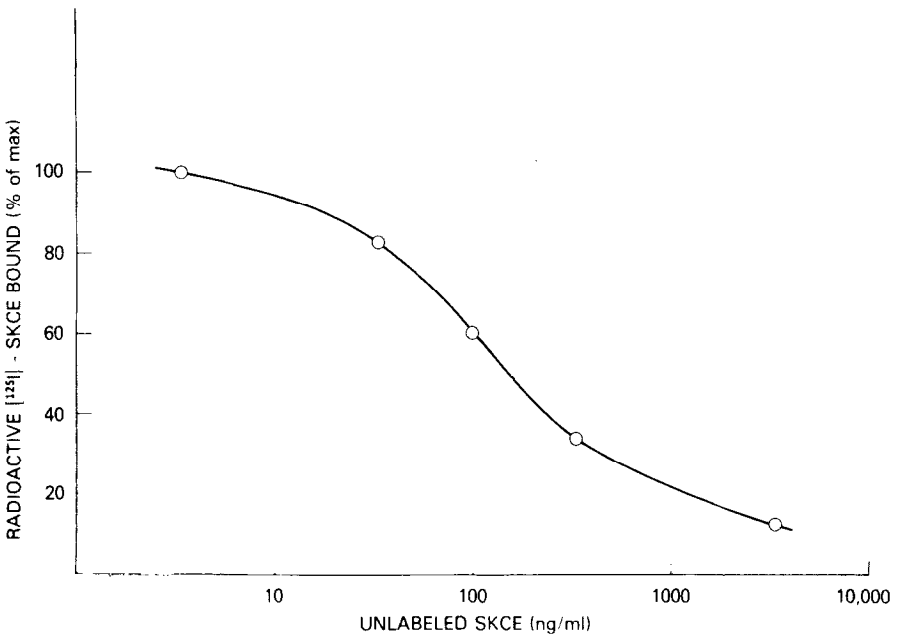

Fig. 1. Standard Curve for the Swine Kidney Converting Enzyme Radioimmunoassay Serial dilutions of purified unlabeled swine kidney converting enzyme were incubated with constant amounts of [^{125}I]-labeled SKCE (10,000 cpm) and goat anti-SKCE serum as described in the Materials and Methods section. The amounts of radioactivity bound to the tubes corrected for non-specific binding, expressed as a percentage of the amount of radioactivity specifically bound to the tubes in the absence of competing unlabeled antigen are plotted versus their respective concentration of unlabeled antigen.

tained for lung (10.4) and kidney (16.4)approach the specific activities of the pure enzymes, 18.3 and 27 U/mg protein, respectively (14,3). This result suggests a close similarity in structure between these converting enzymes.

Our results differ with those reported by Das and Soffer (5), who used a radioimmunoassay for rabbit lung converting enzyme. They found that serum and detergent extracts of rabbit lung, kidney, and brain had ratios of enzymic to immunologic activity ranging from 93 to 103 U/mg. These values are comparable to the specific activity of pure rabbit lung converting enzyme, 89 U/mg protein. Seminal plasma was an exception with a ratio of 324 U/mg. These investigators concluded that most extrapulmonary converting enzyme was indistinguishable from the lung enzyme. The differences between their results and ours could be

Table I. Comparison of immunologic and enzymatic activity of converting enzyme from pig tissues and fluids

Tissue	Radioimmunoassay (RIA) (μg CE/ml)[a]	Amino Acid Analyzer Assay (AAA) (mU/ml)[a]	Ratio AAA/RIA (U/mg CE)
Adrenal	1.36	5.9	4.3
Choroid Plexus	0.25	1.0	4.0
Epididymis	2.14	8.8	4.1
Gall Bladder	0.23	0.9	3.9
Heart	1.10	4.5	4.1
Kidney	11.34	185.5	16.4
Liver	1.27	4.0	3.2
Lung	5.58	58.2	10.4
Retina	0.59	2.6	4.4
Serum	9.48	114.2	12.0
Spleen	1.30	5.4	4.2
Testis	3.44	13.2	3.8
Urine	1.53	5.6	3.7

[a] Values are expressed per ml of sample.

attributed to one or more of the following: species differences, age and/or sex differences of the animals, the larger number of tissue extracts measured in the present study, and differences in the specificities of the converting enzyme antibodies employed.

There are several explanations for our obtaining lower ratios of enzymic to immunologic activity for group A extracts versus those in group B. Group A extracts could contain 1) a pro-converting enzyme that has little or no enzymic activity but complete immunologic activity, 2) an enzyme-inhibitor complex with full immunologic activity, 3) fragments of converting enzyme that are immunologically active but reduced in enzymic activity, 4) a converting enzyme that has been modified in a way that makes it less active immunologically but

does not change its enzymic activity, and 5) a converting enzyme with a specific activity around 4 U/mg protein.

Since radioimmunoassay is based only on immunologic reactivity, it can be used to monitor the purification of forms of converting enzyme that are reduced in enzymic activity but are nonetheless immunologically active. To test the feasibility of this approach to the investigation of converting enzyme, we have determined that concentrations of SQ 20881 that completely inhibit the enzymic activity of converting enzyme do not interfere with its ability to bind to antibody in our radioimmunoassay. With this approach we hope to be able to determine which, if any, of the five explanations listed above is correct. Radioimmunoassay may prove to be a useful tool not only in the study of converting enzyme but also in the study of any enzyme that will be immunologically active, even when it is not enzymatically active.

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