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Review

Purification and assay methods for angiotensin-converting enzyme

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Abstract

Angiotensin-converting enzyme (ACE; EN 3.4.15.1) is a peptidyl dipeptide hydrolase that removes the carboxyl terminal His-Leu from angiotensin I to produce the octapeptide angiotensin II. In addition, ACE inactivates bradykinin, a vasodilator peptide/mediator of inflammation, as well as substance P, enkephalins and endorphins. Because of the importance of ACE and its active site-directed inhibitors in the pathogenesis and treatment of cardiovascular disorders such as hypertension and heart failure, ACE purification and assay are of clinical and commercial, as well as scientific interest. This review summarizes the historical development of ACE purification and assay methods and presents some innovative high-performance liquid chromatography-based techniques developed in our own laboratory for high yield and efficient purification and sensitive and specific assay of ACE.

Keywords: Reviews; Enzymes; Angiotensin-converting enzyme

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1. Introduction

Angiotensin-converting enzyme (ACE; EN 3.4.15.1) is a high-molecular-mass ($M_r \approx 150\,000$) glycosylated integral membrane protein located on the luminal surface of the cell membrane. ACE is a dipeptidyl carboxypeptidase that plays a major role in cardiovascular homeostasis by catalyzing the conversion of the decapeptide angiotensin I (ANG I) to the octapeptide angiotensin II (ANG II), a potent pressor hormone [1], and the hydrolysis of bradykinin, a mediator of inflammation and vasodilator/natriuretic peptide which is also a potent stimulator of vasodilator prostaglandin and nitric oxide syn-

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thesis [2–4]. ACE also inactivates other peptides such as substance P, enkephalins and endorphins. Site-directed inhibitors of ACE are effective in the treatment of systemic hypertension and congestive heart failure [4]. ACE is found in a large variety of cells, tissues and biological fluids, including plasma, semen, proximal renal tubular cells, intestinal epithelial cells, stimulated macrophages, brain, testis, lung, vascular endothelium, and the medial and adventitial layers of blood vessel walls [1,4–7]. ACE associated with vascular endothelium has received the most intensive study both in vivo and in vitro, in part because of its contribution to the maintenance of blood pressure in normal subjects and to the pathogenesis of systemic hypertension in animal models and man. Although ACE is an abundant membrane protein, it has proven difficult to isolate and purify.

ACE is a zinc metalloenzyme and is related to other zinc metalloenzymes such as thermolysin, neutral endopeptidase and collagenase [8]. The nucleotide sequence of ACE mRNA has been determined by cloning the DNA complementary to human vascular endothelial cell and mouse kidney ACE mRNA [9,10]. The amino acid sequences deduced from these complementary (c)DNAs reveal a high degree (83%) of homology between the human and murine enzymes. ACE is the product of a single gene which has been localized to the q23 portion of human chromosome 17. The human cDNA consists of 4024 nucleotides which code for 1306 amino acids. ACE is a dimeric glycoprotein with a M_r of 146 000, with 17 glycosylation sites, and consists of a bilobed extracellular domain which includes the amino terminus, a transmembrane spanning region and a short intracellular domain which includes the carboxyl terminus (Fig. 1). There is a high degree of internal homology between two large domains of the extracellular region, each of which contains a Zn^{2+} binding region and a catalytic site, suggesting that the ACE gene may represent the duplicated product of a precursor gene [8–12].

The dimeric form of ACE, with two active sites and two Zn^{2+} atoms per molecule, is expressed in all organs except the testis. Mutagenesis studies have shown that each of these sites is catalytically competent and can hydrolyze ANG I to ANG II [11,13]. However, the active site closer to the carboxyl terminus of ACE appears to convert ANG I to ANG II more efficiently than the other active site [11]. The testicular form of the enzyme consists of only one arm of the dimeric protein attached to the membrane and the intracellular carboxyl terminus (Fig. 1) [11,14]. It therefore has only a single catalytic site. Plasma ACE, the circulating form of the enzyme, is derived by cleavage from the cell membrane and lacks the transmembrane and intracellular domains but contains the two catalytic sites [15]. Whether plasma ACE is derived by specific proteolytic pro-

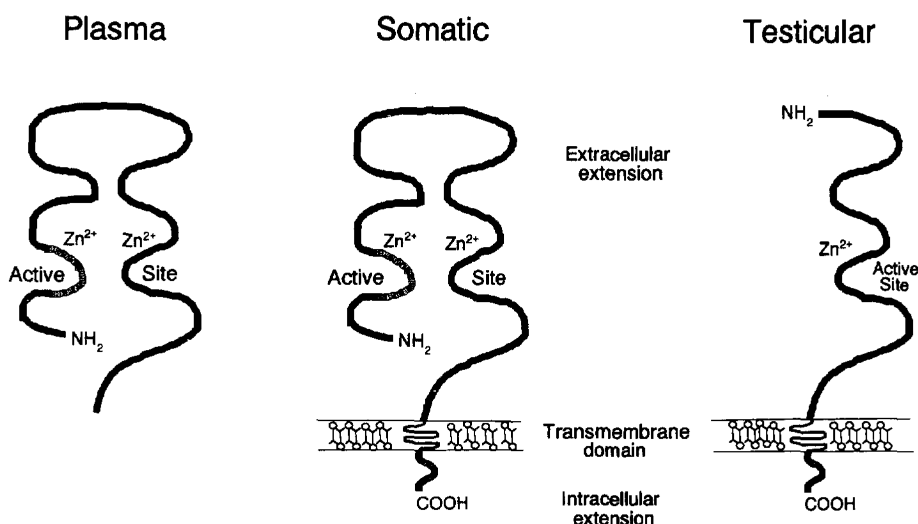

Fig. 1. Structure and conformation of angiotensin-converting enzyme derived from plasma, somatic cell origin or testis, showing the active catalytic sites, zinc dependency and the amino (NH_2) and carboxyl-terminal (COOH) ends. Reproduced from [8] with permission.

cessing from cells in specific tissues or from senescent or dying endothelial cells is not known [8]. Further, it is not clear whether plasma ACE is an accurate reflection of ACE activity on the cell membrane and whether it is regulated in the same manner as tissue ACE.

Many important questions remain concerning the expression of the ACE gene and the posttranslational processing of the large and complex protein. Further, detailed structural characterization of ACE, including crystallographic examination of the molecule and mapping of its active site, has not yet been performed due to the difficulty in purifying large amounts of the enzyme to homogeneity. This review will summarize published methods for purification and assay of ACE, with emphasis on recent procedures developed in our laboratory that will facilitate studies of the role of ACE in normal circulatory homeostasis and in the pathogenesis of cardiovascular disease.

2. Purification

ACE was first described in horse plasma by Skeggs et al. in 1956 [16]. The enzyme received little attention until a decade later, when Ng and Vane [17] and Oparil, Haber et al. [18–20] demonstrated that the biologically important conversion of circulating ANG I to ANG II was catalyzed by ACE in the vascular bed of lung and other organs. In order to characterize in detail this catalytic process and to develop biologically active inhibitors of ACE for investigative and clinical use, it was necessary to obtain purified enzyme.

The major problems associated with ACE purification from tissue are the separation of ACE from other proteolytic enzymes that hydrolyze ACE, its substrate, ANG I, and its product, ANG II, (angiotensinases), and removal of the intact enzyme from the cell membrane. In one of the first published procedures for ACE purification, Hollemans et al. homogenized lungs at 60°C to inactivate angiotensinases, centrifuged the tissue suspensions and used the supernatant as the source of ACE [21]. Other early procedures used low temperature homogenization followed by differential centrifugation to concentrate ACE in the high speed pellet, the angiotensinases remaining in the supernatant [6]. Isotonic sucrose in the presence of Mg^{2+}, followed by differential centrifugation to concentrate ACE in the 25 000 g pellet and further centrifugation of this pellet on a discontinuous density gradient gave a more active preparation [22]. Use of ammonium sulfate fractionation of lung homogenates yielded a preparation "almost free of angiotensinase" and was incorporated into most subsequent purification procedures [23]. Because there was no standard assay for ACE, specific activity of ACE and n-fold purification from the starting material could not be calculated in these early procedures.

Cushman and Cheung advanced the field by developing a novel spectrophotometric assay procedure (see Section 3 which employed the artificial substrate hippuryl histidyl leucine (HHL) to standardize and quantitate ACE activity/n-fold purification [24]. These investigators utilized multi-step chromatographic procedures to obtain, from rabbit lung, a preparation of ACE that appeared to be homogenous on disc-gel electrophoresis and was purified 285-fold compared to the starting material [25] (Table 1). Table 2 summarizes the level (n-fold)

Table 1
Purification of angiotensin-converting enzyme from rabbit lung

Fractionation step	Total protein (mg)	Total activity[a] (units)	Specific activity (units/mg)	Activity recovered (%)	Purification (n-fold)	Activity ratio (HHL/AI)
Extract	9700	750	0.08	100	1.0	(27)[b]
Acidification	6100	710	0.12	96	1.5	20
$CaHPO_4$ gel	300	360	1.2	48	15	20
DEAE-cellulose	34	260	7.5	34	97	19
BioGel P-300	8	190	22	25	285	18

[a] A unit of angiotensin-converting enzyme hydrolyses one μmole of HHL per minute, as assayed spectrophotometrically.
[b] Hydrolysis of AI was assayed fluorometrically; this assay could not be accurately employed with crude extract.
Reproduced from [25] with permission.

Table 2
Highly purified preparations of ACE

Tissue	Species	Total purification (*n*-fold)	Specific activity (units/mg)	Substrate	References
Lung	Rabbit	740,1300	60–90	Hip-His-Leu	[25–27] [28–30]
	Hog	1 400–1 500	10–14	Hip-Gly-Gly	[31–33]
	Rat	330	18	Hip-His-Leu	[34]
	Dog	1 800	25	Hip-Lis-Leu	[35]
	Bovine	2 200	29	Z-Phe-Lis-Leu	[36]
		2 300	14	Hip-His-Leu	[37]
	Guinea Pig	740[a]	17	Hip-His-Leu	[38]
	Baboon	1 800	10	Hip-His-Leu	[39]
	Human	6 600–34 000[a]	10–75	Hip-His-Leu	[40–42]
		-	17	Hip-Gly-Gly	[43]
Kidney	Hog	-	28–31	Hip-Gly-Gly	[43–45]
	Bovine	1 600	31	Z-Phe-His-Leu	[46]
	Human	-	12	Hip-Gly-Gly	[43]
Plasma	Human	101 000	40	Hip-His-Leu	[39–45,45–47]
Serum	Rabbit	60 000	91	Hip-His-Leu	[48]
Semen	Human	430	0.7	Z-Phe-His-Leu	[49]
Prostate	Human	420	19	Hip-His-Leu	[50]

[a] Purification calculated from specific activity of crude extract reported by others.
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of purification and specific activity of ACE purified from various tissues derived from several species using multi-step chromatographic procedures, principally gel filtration and ion-exchange chromatography. In each case, ACE activity was quantitated using the tripeptide artificial substrate indicated and the enzyme appeared to be purified to near homogeneity.

The next major advance in ACE purification was the use of affinity chromatography, which because of its high selectivity made possible the preparation of highly purified ACE in a single chromatographic step, greatly simplifying the purification procedure. Partial purification of ACE has been achieved with affinity chromatography employing several types of ligands. The artificial substrate HHL coupled through its carboxyl terminus afforded a 50-fold purification [51]. Matrices employing the active site-directed ACE inhibitors S-benzyl-L-cysteinyl-L-proline and L-cysteinyl-L-proline provided up to 3300-fold purification from plasma, and have been incorporated into a preparative procedure for human plasma ACE [52,53]. Immobilized anti-ACE antibodies have also been used for immunoabsorbent chromatography [48]. ACE from human serum was purified to homogeneity using immobilized antibodies raised against electrophoretically homogenous ACE from baboon lung [39], but the low capacity and denaturing elution conditions needed for this method limited its preparative utility. Active site-directed ACE inhibitors are currently the most commonly used ligands for affinity chromatography in the purification of ACE. Harris et al. prepared agarose gels containing L-Cys-L-Pro and L-Cys (Bzl)-L-Pro immobilized through their amino groups for use in the affinity purification of ACE [52]. These affinity gels showed good selectivity for ACE but their capacities were so low that they did not provide a practical method for high yield purification. El-Dorry et al. purified testicular ACE with an agarose gel containing N-α-(1-(S)-carboxy-3-phenylpropyl)-L-lysyl-L-proline, a ligand with high affinity for ACE [54]. While this procedure yielded highly purified ACE, the yield was so low that it proved not to be satisfactory on a preparative scale. Pantoliano advanced the field by demonstrating that the binding capacity of affinity resins for ACE is strongly dependent on the distance between the support matrix (agarose) and the immobilized ligand [55] (Table 3). These investigators described affinity chromatography of rabbit lung and testicular ACE employing N^{α}-[(S)-1-carboxy-5-aminopentyl]-L-

Table 3
Effect of affinity ligand space length on resin binding capacity

Spacer group-ligand	Spacer length (A)[a]	Amount of immobilized ligand (μmol/ml of resin)[b]	Binding capacity (mg/ml of resin)[c]
$-OCH_2CHOHCH_2O(CH_2)_4OCH_2CHOHCH_2$-CA-D-Phe-Gly	14	3.5	0.02[d]
$-OCH_2CHOHCH_2O(CH_2)_4OCH_2CHOHCH_2NHC_6H_4CONH(CH_2)_5CO$-CA-L-Phe-Gly	28	2.5	>7.0[d]
$-OCH_2CONH(CH_2)_2NHCO(CH_2)_2CO$-CA-Ala-Pro	10	0.3	<0.01[e]
$-OCH_2CHOHCH_2O(CH_2)_4OCH_2CHOHCH_2$-CA-Ala-Pro	14	ND	<0.02[f]
$-OCH_2CHOHCH_2O(CH_2)_4OCH_2CHOHCH_2NH(CH_2)_5CO$-CA-Ala-Pro	22	3.3	<0.02[f]
$-OCH_2CHOHCH_2O(CH_2)_4OCH_2CHOHCH_2NHC_6H_4CONH(CH_2)_5CO$-CA-Ala-Pro	28	2.5	>7.0[g]
$-OCH_2CHOHCH_2O(CH_2)_4OCH_2CHOHCH_2NHC_6H_4CONH(CH_2)_5COO$	28	none	<0.001[g]

[a] Lengths were calculated by using the extended conformation of space-filling models. An additional 14 A must be added to obtain the overall length (spacer+ligand) of the ligand chain.
[b] Measured by amino acid analysis after hydrolysis (ND, not determined).
[c] Binding capacity is defined as the amount of enzyme removed from solution after it reaches equilibrium with a given volume of affinity resin (normalized per milliliter of resin). For the resins containing immobilized CA-L-Phe-Gly, this process is completely reversible. It is only partially reversible, however, for resins containing immobilized CA-Ala-Pro.
[d] pH 6.0 (MES), 0.5 *M* NaCl, 0.1 m*M* $ZnCl_2$.
[e] pH 7.0 (HEPES), 0.1 *M* NaCl.
[f] pH 6.5 (MES), 0.1 *M* NaCl, 0.1 m*M* $ZnCl_2$
[g] pH 7.0 (HEPES), 0.5 *M* NaCl.
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phenylalanylglycine and N^α-[(S)-1-carboxy-5-aminopentyl]-DL-alanyl-L-proline. They observed that the use of very long (28 Å) spacers for these ligands, twice the length of the epoxide space employed with lisinopril, affords at least a 350-fold improvement in binding capacity, from which they inferred that the active sites may be deeply recessed in the ACE molecule (Table 4).

Most purification schemes for ACE in current use employ affinity chromatography with lisinopril, N^α-[(S)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline, a potent active site-directed ACE inhibitor, as the stationary ligand [56]. Affinity chromatography utilizing lisinopril as the stationary ligand has yielded electrophoretically homogenous ACE directly from crude homogenates of rabbit lung tissue, a 1000-fold purification [56]. Also, it afforded a 100 000-fold enrichment of human plasma ACE in a single step (Table 5). While this procedure can be used to prepare highly purified ACE, its yield is low and it is not practical for preparative use. The affinity of ACE for the Sepharose-space-lisinopril matrix ($K_i = 1 \cdot 10^{-5}$ *M*) is weak compared to its affinity for free lisinopril ($K_i = 1 \cdot 10^{-10}$ *M*), so the recovery of

Table 4
Affinity purification of pulmonary ACE on Sepharose-28-CA-L-Phe-Gly

Step	Vol (ml)	Total units	Protein (mg)[a]	Sp. act. (units/mg)	Purification (*n*-fold)	Yield (%)	Time (days)
(1) detergent solubilization[b]	4050	6480	28 700	0.23	1	100	1
(2) ammonium sulfate fractionation	111	3230	5 770	0.56	2.4	50	3
(3) affinity chromatography[c]	1.6	2190	26.8	82	356	34	2

[a] Protein was determined by $A_{280}^{0.1\%} = 1.58$.
[b] See text for details of individual steps.
[c] Affinity chromatography was performed as described for Fig. 1. Eluted ACE was pooled and concentrated with a collodion bag apparatus (Schleicher and Schuell, Keene, NH, USA).
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Table 5
Purification of angiotensin-converting enzyme by affinity chromatgraphy

Step	Volume (ml)	Protein (mg)	[Protein] (mg/ml)	Enzyme (nmol)	[Enzyme] (n*M*)	Specific Activity (s^{-1})	Purification (*n*-fold)	Recovery overall (%)
Rabbit lung								
Rabbit lung homogenate	14 000	500 000	36	4 600	330	0.38	1	100
Membrane fraction	6 800	180 000	26	1 610	240	0.37	i	35
Affinity chromatography	12.5	43	3.4	240	19 000	230	607	5.2
Sephadex G-200, peak 1	68	44	0.64	190	2 800	178	469	4.2
Sephadex G-200, peak 2	80.6	11	0.14	69	860	258	682	1.5
Supernatant, 25 000 *g*	7 700	100 000	13	1 100	140	0.45	1.2	24
Affinity chromatography	10.6	54	5.02	210	20 000	160	425	4.6
Sephadex G-200, peak 1	80.5	48	0.60	160	2 000	137	360	3.5
Sephadex G-200, peak 2	79	10.4	0.13	53	670	210	550	1.15
Human Plasma								
Crude human plasma	6 000	460 000	76	18	3	0.0016	1	100
Affinity chromatography	4.26	0.162	0.038	0.703	165	179	110 000	4

Specific activity is expressed as apparent k_{cat} in s^{-1}, derived assuming all protein as M_r=130 000. Enzyme concentration was determined from first-order rate constants with furanacryloyl-L-phenylalanylglycylglycine as substrate using the catalytic constants: k_{cat}=317 s^{-1} and K_m=300 μM. The recovery of protein in the gel filtration step of the rabbit lung membrane fraction is not quantitative, reflecting an experimental error.
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ACE from tissue extracts is low. Furthermore, because of its large size, steric hindrance may greatly limit access of the enzyme to the immobilized inhibitor in the affinity column. A subsequent procedure utilized affinity chromatography with lisinopril as the stationary ligand combined with elution of ACE with a second active site-directed inhibitor, captopril, rather than with free lisinopril [57]. Captopril was chosen as the eluent because its inhibitory potency could be quenched by alkylation of its sulfhydryl group with N-ethylmaleimide, which was added to the buffer when samples from the affinity column were assayed. ACE has been purified to apparent homogeneity from several human tissues, including lung, kidney, testis, blood plasma and semen, by this method [57] (Table 6). Each preparation appeared homogenous by sodium dodecyl sulfate–polyacrylamide (SDS–PAGE) on 7% gels and by immunoelectrophoresis on 1% agarose gel followed by protein blotting to nitrocellulose and immunofixation.

Recognizing the limitations of affinity chromatography in the large scale purification of ACE, our laboratory developed a multi-step chromatography procedure for their purpose [58]. This method took advantage of the rapidity and high resolution of high-performance liquid chromatography (HPLC). We purified ACE from human lung using a five-step procedure consisting of ammonium sulfate precipitation, ion-exchange chromatography on DEAE Sephadex A-50, gel permeation on Sephadex G-200, chromatofocusing on a polybuffer exchange (PBE 94) column and HPLC gel permeation on a Bio-Sil TSK-250 column [58] (Table 7) and compared this method to an affinity chromatographic procedure [59]. Both methods yielded a pure protein of molecular mass ≈150 000 and a specific activity of ≈40 units/mg; the yield from the five-step purification procedure was 20-fold greater than that from affinity chromatography. ACE purified by the two methods and subjected to gel electrophoresis on a 7% SDS–PAGE revealed a single band of M_r 150 000 (Fig. 2 and Fig. 3). A single band was also found on Western blot for both products (Fig. 4). Thus, the novel five-step chromatographic procedure is advantageous for preparative use in the structural characterization of ACE because of its higher yield.

ACE from the human lung has also been purified to apparent homogeneity using HPLC following trypsin treatment of the detergent-extract [60]. A

Table 6
Purification of human angiotensin-converting enzymes

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (n-fold)	Yield (%)
Blood plasma (4 liters)					
Outdated plasma	261 000	78	0.0003	1	100
Affinity gel	1.4	41	29	96 600	52
Hydroxylapatite	0.7	28	40	133 000	36
Lung (150 g)					
Homogenate	8 760	50.5	0.0057	1	100
Triton extract	1 990	42	0.021	3.7	83
Affinity gel	1.64	32.8	20	3 500	65
Ultrogel AcA-34	0.53	21.7	40.6	7 120	43
Kidney (50 g)[a]					
Ultrogel AcA-34, M_r 140 000	1.3	52	41	1 640	35
Testis (100 g)[b]					
Ultrogel AcA-34, M_r 140 000	0.05	2	40		30[c]
Ultrogel AcA-34, M_r 90 000	0.14	7.8	55		
Seminal plasma (40 ml) Hydroxylapatite	0.3	12	40	1 000	24

[a] Triton-extracted.
[b] Trypsin-extracted.
[c] Total yield of both M_r forms.
Reproduced from [57] with permission.

1750-fold purification was achieved with a 26% yield. The specific activity of the enzyme was 105 units/mg protein with the substrate HHL at 37°C, and the K_m value for HHL was 1.9 mM. Thus, chromatographic procedures based on HPLC appear to be superior to affinity chromatography for the purification of ACE on a preparative scale.

Efficient and complete removal of intact ACE

Table 7
Purification of angiotensin-converting enzyme from human lung by chromatography and gel permeation HPLC

Step	Protein concentration (mg/ml)	Volume (ml)	Total protein (mg)	Specific activity (units/mg)	Total activity (units)	Purification (n-fold)	Yield (%)	
Detergent solubilization	40	250	10 000	0.06	600	1	100	
Ammonium sulfate	29	147	4 260	0.12	510	2	85	
DEAE-A50	27	45	1 200	0.27	324	4.5	54	
G-200, form I	48	4	190	0.86	163	14.3	27	47[b]
G-200, form II	43	3	130	0.93	120	15.5	20	
Chromatofocusing, form I	11	1.6	17.6	9	158	150	26	
								44[b]
Chromatofocusing, form II	11	1.4	15.4	7	108	117	18	
HPLC, form I	1.1	1.5	1.7	47	78	783	13	
						≈700[a]		22[b]
HPLC, form II	1.2	1.2	1.4	36	52	600	9	

[a] Specific activity ratio.
[b] Total activity ratio.
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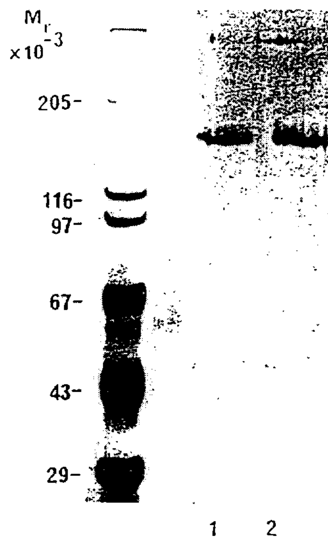

Fig. 2. SDS–PAGE of purified angiotensin-converting enzyme. Angiotensin-converting enzyme (2–4 μg) purified by the HPLC gel filtration method was subjected to gel electrophoresis on 7% SDS–PAGE. The M_r 150 000 form (monomer) was run in lane 1 and the M_r 300 000 form (dimer) in lane 2. Molecular mass markers were: myosin (M_r 200 000); β-galactosidase (16 250); phosphorlase *b* (97 400); serum albumin (66 200); ovalbumin (45 000); carbonic anhydrase (31 000). Reproduced from [58] with permission.

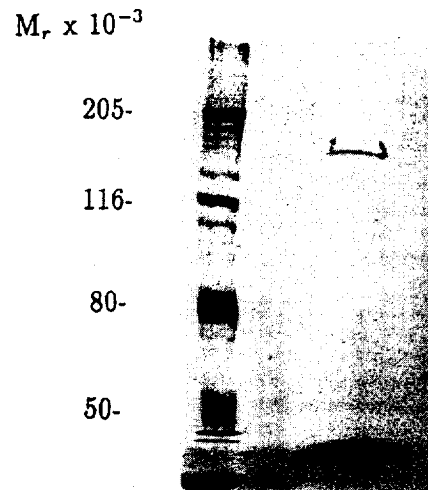

Fig. 3. Angiotensin-converting enzyme (2–4 μg) purified by Sepharose-28 (nm)-lisinopril affinity chromatography was subjected to gel electrophoresis on 7% SDS–PAGE. The silver-stained band has an estimated M_r of 150 000. Molecular mass markers were: myosin (M_r 200 000); β-galactosidase (116 250); serum albumin (66 200); ovalbumin (45 000). Reproduced from [58] with permission.

from the cell membrane has been one of the most difficult technical problems in ACE purification. Detergent extraction is effective in efficiently removing ACE from membranes with its membrane binding sequence intact but has several inherent problems. (1) Some detergents, such as Triton X-100, are nondialyzable and difficult to remove from the purified enzyme. (2) Detergents interfere with assay procedures for ACE, making quantitation of the enzyme and calculation of yields from purification procedures difficult. (3) Detergent-extracted membrane-bound ACE aggregates on gel filtration chromatography. Because of these difficulties, many investigators prefer to remove ACE from membranes with trypsin digestion. Trypsin-extracted and soluble ACE do not aggregate on gel filtration chromatography but lack the membrane binding sequence. In order to determine whether trypsin extraction alters the structural and catalytic properties of ACE, Lanzillo et al. compared detergent-extracted and trypsin-extracted ACE by SDS–PAGE electrophoresis and isoelectric focusing [57]. The two preparations were indistinguishable, indicating that the membrane binding sequence of ACE contributes minimally to the charge and size of the molecule. Further, the catalytic and kinetic properties, assessed by interactions

Fig. 4. Western blot analysis of purified angiotensin-converting enzyme. Left lane: angiotensin-converting enzyme (3–5 μg) purified by the five-step chromatographic method. This was a mixture of the M_r 300 000 and 150 000 forms. Right lane: angiotensin-converting enzyme (3–5 μg) purified by Sepharose-28 (nm)-lisinopril affinity chromatography. Reproduced from [58] with permission.

with substrates, inhibitors and antibodies, were the same for ACE from all organs tested prepared by either detergent or trypsin extraction. Thus, either trypsin or detergent extraction can be used in the purification of ACE without altering the molecule.

3. Assay

The earliest assays of ACE activity were based on physiological responses, either contractile [61,62] or hemodynamic [63], to ANG II generated by ACE from its native substrate, ANG I. Radiochromatographic, colorimetric and radioimmunoassay procedures for ACE activity employing either ANG I or bradykinin as substrate followed [64–66]. These procedures were cumbersome and suffered from interference by other peptidases that degrade both substrate and products of ACE. The development of simple chemical and radiometric methods for assay of ACE activity utilizing selective amino-substituted tripeptide substrates greatly facilitated both ACE purification and studies of the role of ACE in cardiovascular homeostasis [24].

3.1. Biological assays

Biological assays of ACE activity are based on the principle that ANG II, the natural product of ACE, but not its substrate, ANG I, is a potent constrictor of smooth muscle. Helmer [67] used a spiral cut strip of rabbit aorta (which responds to ANG II but not ANG I) to assess ACE activity in human plasma. Huggins et al. used this bioassay to follow their purification of lung and plasma ACE [23]. Bumpus et al. measured ACE activity by the increase in oxytocic activity in incubation mixtures of tissue homogenates and ANG I [68]. Andersen developed an ACE bioassay based on the contraction of the isolated rat uterus [61]. Lyoke using the time required for conversion of ANG I to ANG II in rat serum in an attempt to quantitate ACE activity [63].

The unreliability of bioassay techniques for ACE activity is reflected in the reports of Carlini et al. [69] and Gross and Turrian [70] that ANG I stimulates smooth muscle contraction in the isolated guinea pig ileum. Several other groups of investigators found that ANG I was vasoactive in the perfused hindquarters of rat [69], toad [69,71] and rabbit [70]. It is now known that ANG I lacks receptors and biological activity. The apparent biological activity of ANG I in these early reports reflects the difficulty of blocking ACE, with consequent rapid conversion of ANG I to ANG II in vivo. This problem, plus losses of both ANG I and ANG II due to hydrolysis by angiotensinases present in all tissues and the inconvenience and low throughput of biological assay methods, have limited their use. Biological methods of measuring ACE activity have been completely replaced by more rapid, reproducible chemical methods.

3.2. ACE assays using ANG I as substrate

Both ^{3}H and ^{14}C-labeled ANG I have been synthesized and used to measure ACE activity. The first radiochemical assay for ACE activity measured the amount of ^{14}C-Leu^{10} released from ANG I [72]. Commercially available [^{3}H-Leu^{10}]-ANG I was also used by Oparil et al. to quantitate ACE activity by high voltage paper electrophoresis [18]. Alternatively, unlabeled ANG I has been used as substrate in assays of ACE activity. Hollemans et al. used selective radioimmunoassays for ANG I and II to measure ACE activity in lung tissue [21], while Dorer et al. developed an automated ninhydrin method that measures the chloride ion-dependent increase in ninhydrin-reactive material upon incubation ACE with synthetic ANG I [73].

Use of the native substrate, ANG I, for quantitation of ACE activity has received renewed attention in recent years because of a need to differentiate ACE from other ANG II-forming pathways and to elucidate the mechanism of action of the ACE inhibitors. Multiple ANG II-forming pathways, including enzymes which convert angiotensinogen to ANG II (i.e. tissue plasminogen activator, tonin, cathepsin G) and enzymes which convert ANG I to ANG II (i.e. ACE, chymase, tonin, cathepsin G) have been described [74,75]. Particular attention has been given to the alternative intracardiac ANG II-generating enzyme, "chymostatin-sensitive angiotensin generating enzyme" (heart chymase), which has been isolated from human heart [76]. It is insensitive to ACE inhibition and has higher specificity and catalytic activity for conversion of ANG I to ANG II

than ACE [76,77]. Urata et al. have reported that heart chymase represents approximately 90% of the ANG II-forming capacity in tissue extracts from human myocardium [78] and Dell'Italia et al. have reported similar findings for dog heart, suggesting that ACE is not the major ANG II-forming enzyme in the left ventricle of larger mammals in vitro [79]. Thus, heart chymase provides an active alternative ANG II-forming mechanism that becomes increasingly important in the setting of ACE inhibitor therapy, which results in increased ANG I levels. In contrast to the in vitro situation, ANG II formation across the coronary vascular bed of humans and dogs in vivo is largely mediated by ACE [75,80].

In order to compare the in vivo and in vitro conversion of ANG I to ANG II by ACE and heart chymase, it is necessary to use ANG I rather than HHL or one of the other tripeptide artificial substrates that are routinely used to measure ACE activity because the latter are not cleaved by chymase and the K_m of ACE for HHL is greater than that for ANG I. Thus, use of different substrates for the two enzymes would likely skew the results. Our laboratory recently assessed the relative roles of ACE and chymase in the formation of ANG II in the dog heart in vitro and in vivo. In vitro ACE and chymase activity measurements were made on samples of homogenized left ventricular tissue that had been thoroughly washed, subjected to repeated centrifugation and treated with 0.6% Triton X-100 to release the enzymes from membranes [75]. Aliquots of tissue extracts were preincubated with enzyme inhibitor solutions that contained: for chymase activity assay, 2 m*M* EDTA, 100 μM captopril (a selective ACE inhibitor), 1 m*M* *o*-phenanthroline and 20 μM aprotonin; for ACE activity assay, 1 m*M* *o*-phenanthroline, 20 μM aprotonin and 100 μM chymostatin (a selective chymase inhibitor). Samples were then incubated with ANG I in a phosphate buffer (pH=8.2) solution containing 300 m*M* NaCl, 10^{-4} *M* ZnCl (omitted from chymase activity assays) and 0.01% Triton X-100. Generated ANG II was separated using a reversed-phase Alltima 5 micron phenyl-HPLC column with an eluent consisting of 20% acetonitrile in 0.1 *M* ammonium phosphate buffer, pH 4.9, and quantitated using a UV detector, as recently described in our laboratory [81]. ACE activity was defined as captopril inhibitable ANG II formation and chymase-like activity, as chymostatin inhibitable ANG II formation. A representative chromatogram of the in vitro formation of ANG II in extracts of dog heart is shown in Fig. 5. Incubation with captopril had a minimal effect on the ANG II peak height; in contrast, incubation with chymostatin produced a significant decrease in the ANG II peak height. ANG II formation from chymase-like activity was significantly greater than ANG II formation from ACE activity: 84.4% of ANG II formation was chymostatin inhibitable, while only 6.2% was captopril inhibitable.

In the same study, ANG II formation across the coronary vascular bed was determined by infusing ANG I into the left anterior descending coronary artery and measuring ANG II by radioimmunoassay

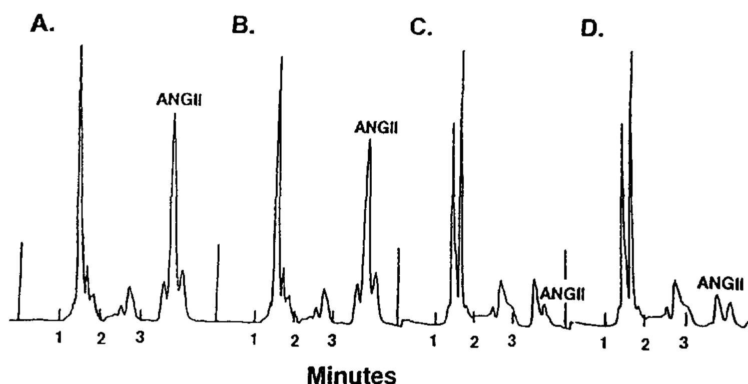

Fig. 5. HPLC chromatogram of ANG II at baseline (A), and during incubation with 10^{-3} *M* captopril (B), chymostatin (C) and chymostatin and captopril (D). Reproduced from [79] with permission.

in the coronary sinus and proximal aorta. ACE-mediated generation of ANG II, assessed by measuring ANG II in coronary sinus and proximal aorta during co-administration of captopril and ANG I, accounted for 60% of ANG II production across the coronary vascular bed. In contrast, co-infusion of chymostatin with ANG I did not alter coronary sinus ANG II levels, suggesting that chymase contributed little to the intracoronary generation of ANG II in vivo. Further study, including application of sensitive and specific assays for ACE, chymase and other ANG II-generating enzymes to an examination of ANG II generation in cardiac interstitial fluid, is needed for a full understanding of ANG II formation in the healthy heart and in various disease states.

3.3. Direct radioimmunoassay (RIA)

The availability of purified preparations of ACE and complete analysis of its amino acid sequence has made possible generation of specific antibodies to ACE and development of radioimmunoassays for ACE protein. Using polyclonal antibodies to human lung ACE raised in rabbits, Alhenc-Gelas et al. developed a sensitive, direct RIA for human ACE [82]. This method has the advantage that ACE inhibitors do not interfere with it, thus permitting enzyme content to be monitored in patients and experimental animals undergoing ACE inhibitor treatment. The expense and difficulty of generating high affinity, high titer antibodies, the difficulty of separating bound from free ligands and the possible reactivity of anti-ACE antibodies with catalytically inactive metabolites of ACE have limited the application of direct radioimmunoassays for ACE.

3.4. Immune isolation with quantitation by gel electrophoresis

Immobilized anti-ACE antibodies have also been used for analytical purposes to isolate ACE antigen from biological fluids or cell culture media, with subsequent quantitation of ACE by SDS–PAGE electrophoresis [83]. Our laboratory used anti-ACE immunoglobin G (IgG) produced in rabbits by intradermal injection of purified porcine lung ACE emulsified in buffer in 1:1 ratio with complete Freund's adjuvant to isolate ^{35}S-methionine-labeled ACE from cultured porcine pulmonary endothelial cells and from conditioned media derived from the cultures. Cell lysates or conditioned media were incubated with rabbit anti-ACE IgG and the soluble antigen–antibody complexes were adsorbed with goat anti-rabbit IgG immunobeads. Labeled ACE was solubilized by boiling the immunobeads in buffer containing 2% SDS. Affinity-purified ACE was then added to provide a visual marker of the electrophoretic position of ACE, the mixture was reduced by addition of β-mercaptoethanol and samples were analyzed by SDS–PAGE using 10% acrylamide according to the method of Laemmli [84] (Fig. 6). The gel was stained with Coomassie blue and sliced, solubilized with 2% periodic acid, and radioactivity determined by a scintillation counter. A single radiolabeled peak (M_r 150 000) with the same electrophoretic mobility as purified porcine lung ACE was observed (Fig. 7). Exposure of endothelial cells to hypoxia was associated with an increase in

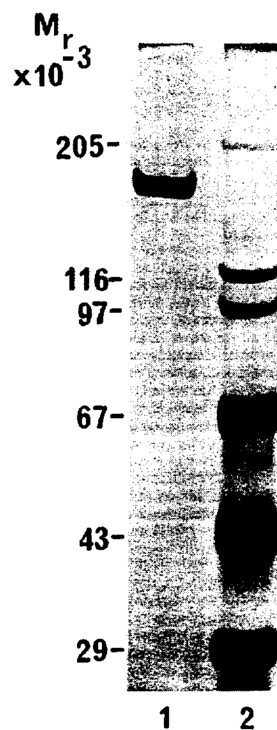

Fig. 6. SDS–PAGE of porcine lung-derived ACE eluted from lisinopril-Sepharose affinity column (lane 1). Major silver-stained band has an estimated M_r of 150 000. Lane 2: reference standards for M_r determinations. Reproduced from [83] with permission.

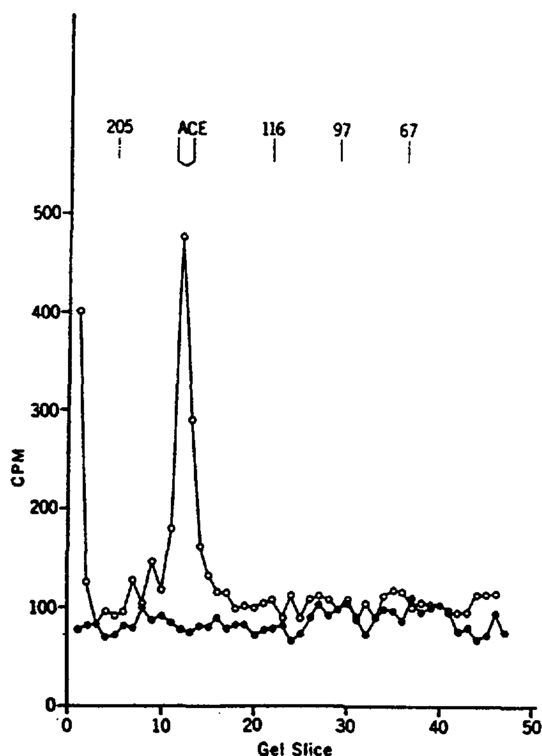

Fig. 7. Representative SDS–PAGE of [^{35}S]methionine-labeled ACE isolated from labeled porcine pulmonary artery endothelial cells using the immunobead method. Gels were sliced into 2 mm slices, solubilized and radioactivity determined. (○) Pattern obtained when rabbit anti-ACE immunoglobin G (IgG) was used as first antibody; (●) pattern obtained when nonimmune rabbit IgG was used as 1st antibody (control). Arrow, position of purified porcine lung-derived ACE as shown in lane 1, Fig. 6. Positions of SDS–PAGE M_r standards are indicated. Reproduced from [83] with permission.

ACE antigen synthesis without a concomitant change in ACE activity. This suggests that an inactive form of ACE is synthesized by cultured pulmonary artery endothelial cells under hypoxic conditions and points out the importance of assaying both ACE activity and antigen content when studying ACE gene regulation.

3.5. Enzyme inhibitor binding assay

The enzyme inhibitor binding assay is based on the specific binding of a labeled inhibitor to the active site of ACE, thus allowing quantitation of numbers of active sites. Thus, the inhibitor binding assay for ACE measures neither inactive ACE nor immunologically cross-reactive fragments of the ACE molecule, both of which are measured in the direct radioimmunoassay and the immune isolation/ gel electrophoresis procedures. Fyhrquist et al. developed a method for measuring the ACE content of tissue homogenates or serum by quantitating the binding of ^{125}I-labeled 351A, a *p*-hydroxybenzamidine derivative of lisinopril N-(1-carboxy-3-phenylpropyl)-L-lysyl-L-proline) [85,86]. 351A is a highly specific active site-directed inhibitor of ACE that interacts with the catalytically active Zn^{2+} in the active site; it can be displaced by other active site-directed ACE inhibitors and by Zn^{2+} chelators such as disodium EDTA and 8-OH quinoline, but not by other enzymes or components of plasma [85,86]. The sensitivity of the inhibitor binding assay is 0.1 unit (5.0 ng or 52 fmol of ACE) in routine assays (incubation of serum or membrane preparations for 4 h at 37°C) and can be increased by prolonging incubation time to 24 h [85,86]. Results of the inhibitor binding assay and the enzyme kinetic assay of Cushman and Cheung [24] as modified by Lieberman [87] for rat and human serum ACE are closely correlated [85,86]. Our laboratory demonstrated a positive correlation between ^{125}I-351A binding ACE activity, as assessed by the Cushman and Cheung technique [24], and tissue stores of ANG II in rats adapted to chronic hypoxia, suggesting that alterations in catalytically active ACE and ACE active sites are reflected in changes in local generation of ANG II [88]. The hypoxia-induced alterations in ACE were reversible after return to a normoxic environment. Nonspecific binding of ^{125}I-351A to organ homogenates, as assessed by addition of excess unlabeled lisinopril, is <10%.

^{125}I-351A can also be used as an alternative to ^{125}I-ANG I as a ligand for localization and semiquantitative assessment of ACE density in tissues by radioautography [89,90]. For this application, it has the advantage of resistance to hydrolysis by angiotensinases and non-ACE enzymes that convert ANG I to ANG II.

More recently, a radioligand has been developed that recognizes both of the active sites of somatic ACE [91]. Ro 31-8472, the hydroxyl derivative of

the potent ACE inhibitor cilazaprilat, binds to twice the number of sites/mole of somatic tissue ACE as 351A. Whereas 351A, which has a bulky side chain, binds to only the carboxyl-terminal active site of ACE, Ro 31-8472, which has a more compact structure, binds to both active sites. This suggests that the amino-terminal active site, which is on the second arm of the enzyme protein, has physical conformational restraints [8]. This is consistent with the results of studies on the purification of ACE using affinity chromatography with biotin-spacer inhibitors, which have shown that one of the active sites of ACE is deeply recessed [92]. Studies by Perich et al. have shown a close correlation between the Hill coefficient and slope factor, with the latter varying inversely with the size of the side chain of ACE inhibitors [93]. The presence of two active sites with different conformational requirements suggests that there may be different endogenous substrates for each active site of ACE and that it may be possible to design ACE inhibitors which are specific for each of these sites [8].

3.6. ACE assays using synthetic substrates

Except for the special applications described above, ACE activity assays utilizing simple tripeptide artificial substrates have largely replaced assays which use the natural substrates ANG I and bradykinin. The artificial substrates are easily synthesized, inexpensive, not susceptible to hydrolysis by angiotensinases (endo- or aminopeptidases) and have K_ms for ACE comparable to or greater than the natural substrates [29] (Table 8). The dipeptides cleaved from the artificial substrates by ACE can be quantitated by spectrophotometric, fluorometric or radiochemical methods [94]. The most commonly utilized of these methods is the spectrophotometric assay of Cushman and Cheung, which uses the ACE-specific substrate HHL coupled with spectrophotometric assay of the reaction product hippuric acid (HA) [24]. HHL is hydrolyzed 20 times more rapidly than ANG I by the same amount of ACE; thus 1 HHL unit=0.05 ANG I unit of ACE activity [26]. Although highly selective for ACE, this procedure has low sensitivity and reproducibility due to incomplete inhibition of ACE at the termination of incubation and/or the presence of interfering substances that are extracted in the standard liquid–liquid extraction procedure with ethyl acetate, resulting in high blank values.

In order to increase the sensitivity of the Cushman Cheung assay, our laboratory recently developed a

Table 8
Effect of the COOH-terminal dipeptide sequence on binding of substrates and inhibitors to angiotensin-converting enzyme

Peptide Substrate or Competitive Inhibitor	Dissociation Constant (μM)		Cl^- Activation (n-fold)
	K_m	K_i	
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	30	10	+15
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	0.85[a]	0.85	+3[a]
<Glu-Lys-Trp-Ala-Pro		0.06	−20[b]
Hip-His-Leu	2200		+13
Hip-Phe-Arg	330	250	+3
Hip-Ala-Pro	30	7.0	−4
His-Leu		420	
Phe-Arg		120	
Ala-Pro		30	

[a] These values were determined by Dorer et al. [114].
[b] This approximate value is obtained from the rates of disappearance of inhibitory activity of BPP_{5a} upon incubation with angiotensin-converting enzyme in the presence and absence of chloride ion [26].
K_m and K_i values compared in this table were derived, unless otherwise indicated, from enzyme reation rates measured at pH 8.3 in the presence of 300 mM NaCl. Rates of hydrolysis of angiotensin I and the three hippuryl dipeptide substrates by ACE were assayed fluorometrically; results with Hip-His-Leu and Hip-Phe-Arg were confirmed using the spectrophotometric assay. Inhibition of ACE, for determination of K_i values, was measured with Hip-His-Leu as the substrate.
Reproduced from [29] with permission.

sensitive method for the accurate and reproducible quantitation of membrane-bound ACE activity in tissue, in which ACE is extracted with detergent and the reaction product is isolated from the reaction mixture by reversed-phase HPLC, thus eliminating interference from the detergent, unreacted substrate, and reaction byproducts [81]. ACE activity was determined in detergent-extracted canine hearts utilizing the synthetic ACE-specific substrate HHL, both in the presence and the absence of the site-specific inhibitor captopril. Tissue ACE activity was quantitated from the moles of HA formed, in time-fixed assays, utilizing HPLC separation of HA from HHL and UV spectrophotometry for quantitation of HA as in the standard Cushman and Cheung assay [24]. Separation of HA from HHL was performed by reversed-phase HPLC on a phenyl silica gel column with an eluent consisting of 20% acetonitrile in 0.1 *M* aqueous ammonium phosphate buffer, pH 6.8. After the standard liquid–liquid extraction procedure with ethyl acetate, HPLC analysis revealed the presence of unreacted substrate, HHL, in amounts comparable to the product of interest, HA, in the final assay; moreover, the amount of HA formed did not fall completely to zero in the presence of captopril (Fig. 8). ACE activity measured in midwall of the left ventricle with the HPLC-based assay was 1.24±0.18 munits/g (n=6), 4-fold the values obtained with the standard Cushman and Cheung assay (0.31±0.09 munits/g, n=6). Values for K_m (1.34±0.08 m*M*) and V_{max} ($36.8\pm11.5\times10^{-10}$ *M*/min) of ACE were in agreement with published results obtained using purified preparations of ACE (Fig. 9). Utilizing our HPLC method, the time dependence of HA generation was linear out to 30 min (Fig. 10). Application of HPLC to the standard Cushman and Cheung assay improved the sensitivity and specificity of the standard assay and enabled the use of much smaller amounts (~4 vs. ~400 mg) of tissue for ACE activity assay.

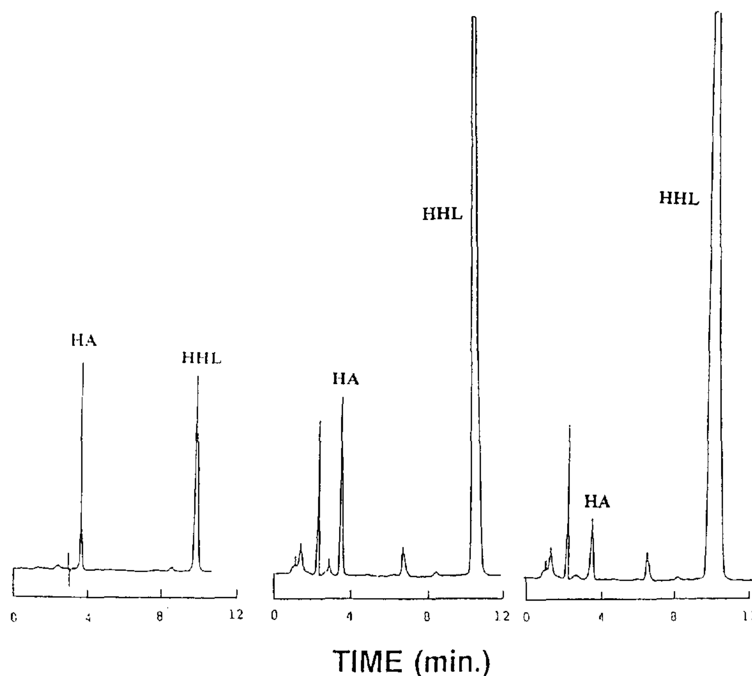

Fig. 8. HPLC chromatograms. Left panel: chromatogram from 0.01 μg hippuric acid and hippuryl histidyl leucine (HHL) standards. Middle panel: chromatogram from dog left ventricle assay (1 mg of midwall) showing hippuric acid peak and persistent substrate (HHL) peak following 40 μl HPLC injection from final reaction product after extraction. Right panel: chromatogram from dog left ventricle assay in presence of 0.1 m*M* captoptril following 90 μl HPLC injection from final product after extraction. Reproduced from [81] with permission.

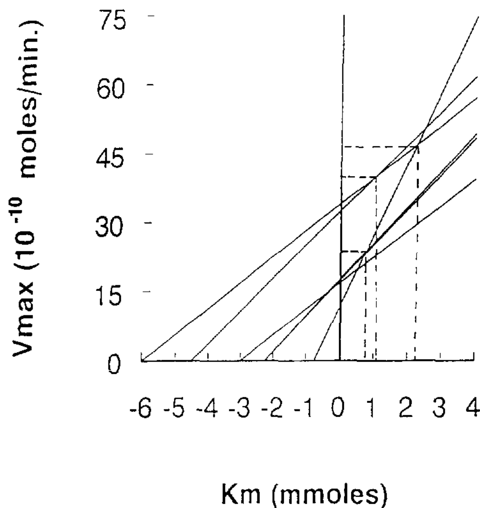

Fig. 9. Direct linear plot for canine lung ACE activity as determined by the HPLC assay. K_m and V_{max} were determined by averaging the corresponding values from the points of intersection between the dashed lines and the abscissa and ordinate, respectively. Reproduced from [81] with permission.

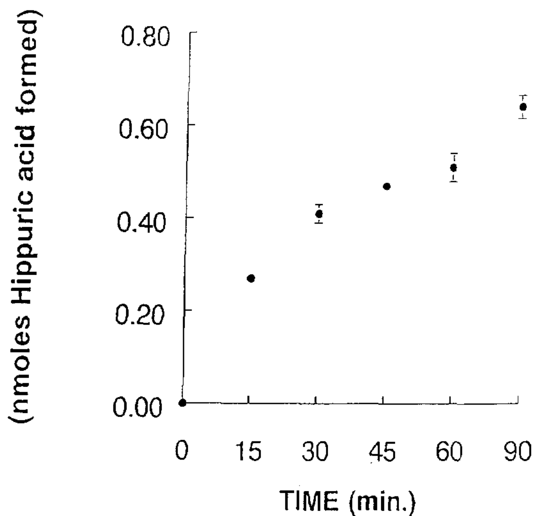

Fig. 10. Time dependence of HPLC based assay in canine lung in the presence of 1.5 m*M* HHL. Each point=mean of three determination. Data are expressed as means±SD. Reproduced from [81] with permission.

4. Biomedical applications

There is a large body of clinical data demonstrating the beneficial effects of ACE inhibitors in reducing mortality, rate of recurrent myocardial infarction, and subsequent development of heart failure in patients after myocardial infarction [95,96] and in patients with chronic congestive heart failure [97–99]. These beneficial effects of the ACE inhibitors are not fully accounted for by their antihypertensive action [100]. This clinical information, coupled with the evidence for the existence of a functional intracardiac renin–angiotensin system in experimental animals and humans, has led to the hypothesis that the intracardiac renin–angiotensin system is activated locally in response to increases in regional wall stress [4]. The continued presence of intracardiac ANG II appears to have important pathophysiologic consequences, i.e., myocyte hypertrophy, myocardial remodeling, fibrosis, vasoconstriction of the small coronary arteries, arrhythmogenesis and diastolic dysfunction mediated by effects on Ca^{2+} homeostasis (Fig. 11). Similar local effects of ANG II on cell hypertrophy and hyperplasia have been reported in blood vessels and kidney [4]. Specific assay methods that are sufficiently sensitive to permit accurate quantitation of ACE activity in small (mg) samples of heart tissue are needed for studies of the role of the tissue renin–angiotensin system in the pathogenesis of cardiovascular disease in animal models and humans, as well as in documenting the effects of ACE inhibitor and ANG II antagonist therapy on these processes. These sensitive assay procedures will be particularly useful in studying transgenic and knockout mouse models [101,102] and in examining human myocardial biopsy material.

Polymorphisms have been discovered in the ACE gene that are linked to blood pressure regulation in experimental animals [103,104] and left ventricular hypertrophy and cardiomyopathy in human subjects [105–108]. A deletion (D)–insertion (I) polymorphism of the human ACE gene that appears to affect the level of circulating ACE activity has also been identified [109,110]. This 287 base pair insertion–deletion in intron 16 of the human ACE gene accounts for 47% of the variability in plasma ACE activity levels in the population. The genotype (DD) with deletion of this 287 base pair sequence is associated with the highest circulating ACE levels, the highest incidence of left ventricular hypertrophy, and increased risk of myocardial infarction and of dilated and hypertrophic cardiomyopathy.

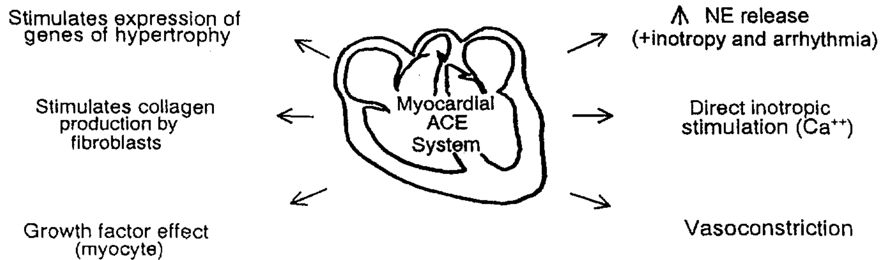

Fig. 11. Schematic representation of the pathophysiologic consequences of activation of the intracardiac renin–angiotensin system by increases in regional wall stress. Reproduced from [4] with permission.

The mechanism by which the DD genotype of the ACE gene affects cardiac hypertrophy and accelerated coronary artery disease is unknown, but is unrelated to hypertension since the ACE polymorphism does not appear to be linked to blood pressure in humans [110,111]. These findings are consistent with the clinical observations that: (1) ACE inhibitors appear to be superior to other antihypertensive agents in inducing regression of cardiac hypertrophy, independent of their antihypertensive efficacy [112] and (2) ACE inhibitors promote better survival in heart failure than do other vasodilators [113]. Even in the absence of clear delineation of a phenotype relating the DD genotype of the ACE gene to increased cardiovascular risk, the presence of this linkage may provide a useful clinical test for cardiovascular risk assessment. The availability of sensitive assays for ACE activity and content and of efficient methods for purifying the isoforms of ACE will be useful in clarifying the relationships between altered ACE genotype, phenotypic variations in ACE expression and the development of cardiovascular diseases.

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