Angiotensin-Converting Enzyme in Pericardial Fluid: Comparative Study with Serum Activity

Roseli Aparecida da Silva Gomes[1], Lívia das Graças Vieito Lombardi Teodoro[1], Isabel Cristina Rezende Lopes[1], Patrícia Alessandra Bersanetti[2], Adriana Karaoglanovic Carmona[2], Valdemar Hial[1]

Universidade Federal do Triângulo Mineiro, Uberaba, MG[1], Universidade Federal de São Paulo, São Paulo, SP[2] - Brazil

Summary

Background: The characterization of an angiotensin-converting enzyme (ACE) in human pericardial fluid is relevant, considering its role in the angiotensin II release and thus, the role of the pericardium in cardiovascular homeostasis.

Objective: To isolate and characterize an ACE from human pericardial fluid and to compare the angiotensin I converting activities of the pericardial fluid with that of the serum in patients submitted to cardiovascular surgery.

Methods: The enzyme from human pericardial fluid was purified through chromatographic steps and characterized by polyacrylamide gel electrophoresis (SDS-PAGE), hydrolysis of angiotensin I, bradykinin, Hip-His-Leu and synthetic substrates with internal fluorescence suppression. Lisinopril was used as inhibitor. The ACE activity was measured in blood and pericardial fluid samples of 23 patients submitted to cardiovascular surgery.

Results: The purified ACE (MM = 140 kDa), releases angiotensin II, hydrolyses bradykinin and the Hip-His-Leu substrate. The kinetic parameters k_{cat},(s-1) and k_{cat}/K_m (μM-1. s-1) were, respectively: Hip-His-Leu (1.14 and 7 x 10 -4) ; Abz-YRK(Dnp)P-OH (2.60 and 0.77), Abz-LFK(Dnp)-OH (2.77 and 0.36) and Abz-SDK(Dnp)P-OH (1.92 and 0.19). The angiotensin I converting activities (mean ± SD) in the pericardial fluid and in blood, were, respectively: 3.16 ± 0.90 mU x mg –1x min-1 and 0.33 ± 0.11 mU x mg –1x min-1. The difference was significant between the two fluids.

Conclusion: An ACE that bears great similarity with the somatic enzyme was isolated from human pericardial fluid. The angiotensin I converting activity is higher in the pericardial fluid when compared to the serum activity. These data are important evidence of the role of the pericardial fluid in the metabolism of active peptides. (Arq Bras Cardiol 2008;91(3):156-161)

Key words: Peptidil dipeptidase A; pericardiocentesis; lisinopril; comparative study.

Introduction

Studies with pericardial fluid have aimed chiefly at establishing the diagnosis of pericarditis, of pericardial effusion and have also been used for *post-mortem* diagnosis[1-3]. High levels of vasoactive and cardioprotective substances have been described in human pericardial fluid[4,5]. This fluid is in a strategic position to receive information concerning the pathologies that affect this space and the heart itself. Our interest regarding the biochemical study of the pericardial fluid aims at some enzymes, those that can function as local markers of physiopathological alterations. The present study was initiated with an isoform of the angiotensin-converting enzyme (ACE).

The angiotensin-converting enzyme (ACE) (EC 3.4.15.1) cleaves the angiotensin I, forming angiotensin II[6] and hydrolyses bradykinin and kalidin, generating inactive fragments[7]. ACE is a membrane protein, described in arterial endothelium, epithelial and neuroepithelial tissues[8-10]. Several ACE isoforms are present in mammal tissues. A somatic isoform (150 – 180 kDa), expressed in endothelial, epithelial and neuroepithelial cells has 2 homologous domains (N- and C- domains), each with its own catalytic site[11,12]. Another isoform with a lower molecular mass (90 – 110 kDa), found exclusively in male germinative cells, contain a single active site that corresponds to the C-domain of the somatic form[13].

Soluble forms of the enzyme are found in several body fluids, such as blood, amniotic fluid, semen, cerebrospinal fluid and urine[14-16]. A form constituted only of the N-domain was described in the ileal fluid[17]. Despite the high degree of homology, the C- and N-domains of the ACE differ in substrate specificity, inhibition profiles and chloride dependence[18,19]. The two active sites cleave angiotensin I, substance P and bradykinin with similar efficiency[19]. However, the natural tetrapeptide N-Acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP)[20] and angiotensin 1-7 (Asp-Arg-Val-Tyr-Ile-His-Pro)[21] are more specific for the catalytic N-domain. Peptides with internal fluorescence suppression [FRET (Fluorescence resonance energy transfer) peptides)], which contain sequences that

Mailing Address: Roseli Aparecida da Silva Gomes •
Praça Manoel Terra, 330 – Abadia 38015-050, Uberaba, MG - Brazil
E-mail: bioq@dcb.uftm.edu.br
Manuscript received November 26, 2007; revised manuscript received February 14, 2008; accepted February 15, 2008.

are susceptible to hydrolysis by ACE have been used for the characterization of N- and C-domains of the enzyme[22-24].

The present work is the study of an ACE isoform, isolated for the first time from human pericardial fluid. We also compared the ACE activities in pericardial fluid and sera of individuals submitted to cardiovascular surgery in the present study.

Methods

Reagents

Angiotensin I, angiotensin II, lisinopril, molecular mass patterns, bradykinin, o-phthalaldehyde, Hip-His-Leu, Mes (2-[N-morpholino] ethanesulfonic Acid), HEPES ((N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid))]) were acquired from Sigma. The substrates with internal fluorescence suppression (FRET peptides), Abz-YRK(Dnp)P-OH, Abz-LFK(Dnp)-OH and *Abz-SDK(Dnp)P-OH* (Abz = o-aminobenzoic acid; Dnp = 2,4-dinitrophenol) were synthesized at the Department of Biophysics of the Universidade Federal de São Paulo, São Paulo, SP, Brazil[22-24]. Other reagents were obtained from Merck.

Obtaining the human pericardial fluid for the purification of the angiotensin-converting enzyme (ACE)

The pericardial fluid was collected up to 12 hours after death, from individuals without cardiac disease, during the necropsy performed at the Anatomopathological Department of the Universidade Federal do Triângulo Mineiro (UFTM), Uberaba, MG, Brazil. All samples used in the study were free of contamination with blood.

Obtaining the human pericardial fluid and blood samples to measure the ACE activity

Pericardial fluid and venous blood samples were collected from 23 patients submitted to cardiovascular surgery at Hospital de Clínicas of Universidade Federal do Triângulo Mineiro (UFTM), Uberaba, MG, Brazil. Pericardial fluid samples were obtained from the pericardial cavity by aspiration through a syringe, during the opening of the pericardial membrane for the heart surgery. The blood was obtained by venipuncture of the arm.

The pericardial fluid and serum samples were centrifuged (1,000*g*, 15 minutes) and stored at -20°C. The pericardial fluid samples were free of contamination with blood. All patients gave their informed consent to participate in the study. All procedures were approved by the Ethics Committee in Research of UFTM (CEP/UFTM) and are in accordance with the Declaration of Helsinki.

Purification of ACE from pericardial fluid

Pericardial fluid (65 ml), free of visible contamination with blood or tissue and containing 1,550 mg of protein, was centrifuged at 3500 rpm, at 4°C. The supernatant was submitted to ultrafiltration (Amicon, YM-100) for a final volume of 20 ml containing 780 mg of protein. The concentrate was used for the purification of the angiotensin-converting enzyme, after dialysis against a 0.2 M sodium phosphate buffer, pH 7.0. The protocol of purification included 3 chromatographic steps: 1) gel filtration through a Superdex 75 HR 10/30 column, Pharmacia, balanced and eluted with a 0.05 M sodium phosphate buffer, containing 0.5M NaCl, pH of 6.5 and flow of 1 ml per second. Aliquots of 0.5 ml were applied to the column and 1 ml fractions were collected; 2) affinity chromatography in a blue agarose (Cibacron®) column, 0.9 x 30 cm, (Bio Rad), balanced and eluted with a 0.2 M sodium phosphate buffer, pH 7.0 and flow of 0.5 ml/min. Aliquots of 2.0 ml were applied and fractions of 1 ml were collected; and 3) ion-exchange chromatography in a Mono Q 5/5 HR column, Pharmacia, balanced with a 0.05 M sodium phosphate buffer, pH 6.5 and eluted by a NaCl gradient (0 to 0.5 M) using a 0.05 M sodium phosphate buffer, pH 6.5 (buffer A) and 0.05 M sodium phosphate buffer, pH 6.5, containing 0.5 M NaCl (buffer B).

The flow was maintained at 1ml/min. Aliquots of 0.5 ml were collected. All chromatographic steps were monitored at 280 nm in a UV/VIS spectrophotometer (Pharmacia). The enzymatic activity of the fractions obtained at the chromatographic steps was tested using the Hip-His-Leu substrate, according to Friedland and Silverstein[25]. The presence of other proteins in the preparations obtained after each chromatographic step was verified through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE -7.5%) under reducing conditions, as described by Laemmli[26] (Bio-Rad system). Aliquots of the enzymatic preparations containing 2 to 10 μg of protein were applied to the gel. The gels were stained with ammoniacal silver[27]. Beta-amylase (200 kDa), bovine albumin (65 kDa), carbonic anhydrase (29 kDa) and lactalbumin (14 kDa) standards (all acquired from Sigma) were used to measure the molecular mass. The molecular mass was estimated through graphic interpolation.

Enzymatic activity measurement
Angiotensin I hydrolysis

Angiotensin I (80 nmols) in a 0.1 M sodium phosphate buffer, pH of 7.5 (350 μl), containing 0.3 M NaCl, was incubated with ACE purified from pericardial fluid (4 ng in 10 μl) at 37°C. At 10 and 30 minutes of incubation, the aliquots were submitted to chromatography in an octadecyl silane column (C-18), balanced with acetonitrile:deionized water (1:5), containing 0.1% of Trifluoroacetic acid (TFA), coupled to a high-efficiency liquid chromatography (HPLC) system (Shimadzu). The elution was carried out using a gradient consisting of acetonitrile:deionized water (1:5), containing 0.1% TFA (buffer A) and acetonitrile containing 0.1% TFA (buffer B). The peptides were monitored at 214 nm through a UV/VIS spectrophotometer and identified through the retention time using angiotensin I and angiotensin II as standards. The peak that corresponded to the angiotensin II standard was tested using a biological assay in isolated rat uterus[28]. The biological activity was estimated using solutions with known concentrations of angiotensin I and angiotensin II as standards. The assays that used animals were developed according to the directives established by the "Guide for Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC) and the Ethical Principles in Animal Experimentation of the Brazilian College of Animal Experimentation (COBEA).

Bradykinin hydrolysis

Bradykinin (BK, 5 nmols) was incubated with the converting enzyme purified from pericardial fluid (4 ng), in a 0.2 M phosphate buffer, pH 7.5 containing 0.3 M NaCl, for a final volume of 300 µl, at 37°C, during 10 minutes and tested using a biological assay in isolated rat uterus[28]. A BK standard (5 nmols) was incubated under the same conditions, in the absence of the enzyme and used as control. A BK dose-response curve was used to quantitatively assess the biological activity of the aforementioned incubation mixes. The disappearance of the biological activity of the BK was considered as kininasic activity. Inhibition assays were performed using Na_2EDTA (40 mM) and lisinopril (2.5 µM).

Kinetic studies

Hydrolysis of the hip-his-leu substrate

The establishment of the optimal pH for the hydrolysis of the Hip-His-Leu substrate was performed using the following buffers: 0.2 M glycine-HCl (pH 3.0 to 4.0), 0.2 M sodium acetate (pH 4.5 to 5.5), 0.2 M sodium phosphate (pH 6.0 to 7.5), 0.2 M Tris-HCl (pH 8.0 to 8.5) and 0.2 M glycine-NaOH (pH 9.0), containing 0.3 M NaCl, for a final volume of 490 µL, and the purified enzyme (4 ng). The assay was carried out as proposed by Friedland and Silverstein[25]. The kinetic parameters (k_{cat} and K_m) for the hydrolysis of the Hip-His-Leu substrate (0.25 – 3 mM) were determined using the purified enzyme (4 ng) in a 0.1 M potassium phosphate buffer, pH 8.3, containing 0.3 M NaCl, for a final volume of 490 µl. The mix was incubated at 37°C for 15 minutes. The reaction was interrupted by the addition of 1.2 ml of 0.34 M NaOH, followed by the addition of 200 µL of 3M HCl, and the His-Leu dipeptide was quantified fluorimetrically (Exc.365 nm; Em.495 nm) through the formation of a fluorescent product with 100 µl of a solution of 150 mM o-phthalaldehyde. The results were expressed as nmol x ml-1 x min-1. Lisinopril (2.5 µM) was used for the inhibition experiments.

Hydrolysis of Abz-YRK(Dnp)P-OH, Abz-LFK(Dnp)-OH and Abz-SDK(Dnp)P-OH

The assays with substrates that had internal fluorescence suppression were carried out as previously described[22-24]. The optimal pH was determined using aliquots (4 ng) of the purified enzyme and a buffer solution containing: 25 mM of glycine, 25 mM of acetic acid, 25 mM of Mes and 75 mM of Tris in the presence of 100 mM of NaCl and 10 µM of $ZnCl_2$ in a pH range of 5.0 to 9.5, for a final volume of 1 ml, at 37°C. The kinetic parameters were determined at 37°C, in 0.1 M Tris-HCl buffer (pH 7.0) containing NaCl (0.05 M), $ZnCl_2$ (10 µM) and the substrates Abz-YRK(Dnp)P-OH, Abz-LFK(Dnp)-OH and *Abz-SDK(Dnp)P-OH*. The enzymatic activity was continuously monitored through a fluorometer (Hitachi F-2000) and the fluorescence was measured at 420 nm (λ_{em}) after stimulation at 320 nm (λ_{exc}). The concentration of the enzyme to determine the initial velocity of the reaction was chosen so that the hydrolysis was not higher than 5% of the used substrate. The fluorescence variation was converted in micromoles of hydrolyzed substrate per minute, based on a calibration curve obtained after the complete hydrolysis of each peptide. The values of k_{cat}/K_m were calculated based on the kinetic parameters k_{cat} and K_m, obtained through the analysis of linear regression of the data with the GraFit program[29]. The standard deviations for k_{cat} and K_m were lower than 7%. The hydrolysis inhibition was carried out using lisinopril (2.5 µM).

Influence of chlorides on catalytic activity

The influence of chloride ions (0 – 500 nM) on the catalytic activity of the enzyme isolated from human pericardial fluid was investigated using the substrate Abz- YRK(Dnp)P-OH (10 µM) at 37°C in 1 ml of 50 mM HEPES buffer (pH 7.6) containing $ZnCl_2$ (10 µM) and 4 ng of the enzyme. The hydrolysis was fluorimetrically monitored as previously described[22].

Angiotensin converting activity in serum and pericardial fluid

The angiotensin I-converting activities in serum and pericardial fluid were measured using Abz-YRK(Dnp)P-OH (10 µM) – substrate for the N and C-domains of the enzyme at 37°C in a 0.1 M Tris-HCl buffer (pH 7,6) containing 0.05 M NaCl and 10 µM $ZnCl_2$. One hundred µL of the pericardial fluid and serum samples were added to the substrate solution for a final volume of 2.0 ml and the enzymatic activity was monitored fluorimetrically as previously described[22]. A fluorescence curve was obtained using the peptide standards before and after total hydrolysis. The enzymatic activity was expressed as milliunit per milligram of protein per minute of incubation (mU × mg-1 × min-1). The measurements were made in duplicate. The hydrolytic activity of the enzyme was totally inhibited by 2.5 µM lisinopril. The data were analyzed through non-paired Student's t test with a level of significance set at 0.05. The protein concentrations in serum and pericardial fluid were measured by the biuret method[30]. A standard protein solution was used in the same conditions of the samples and the results were expressed as mg x ml-1.

Results

Pericardial fluid ACE purification

An angiotensin-converting enzyme was purified up to homogeneity from human pericardial fluid using YM-100 membrane filtration to eliminate proteins with molecular masses < 100 kDa followed by three chromatographic phases and among them, an affinity chromatography phase (Cibacron® blue agarose) with the objective of removing the residual albumin still present in the concentrate. The enzyme was purified around 300 times. The protocol used resulted in 200 µg of purified protein, with specific activity of 3,975 nmol His-Leu x mg-1 x min. The SDS-PAGE yielded a single protein band under reducing conditions, with apparent molecular mass of 140 kDa (Figure 1).

The purified enzyme converts angiotensin I into angiotensin II as demonstrated by the reverse-phase chromatographic analysis (C18-CLAE) (Figure 2) and by the biological activity on the isolated rat uterus (Figure 3).

The kininasic activity of the enzyme was demonstrated

through the disappearance of the biological activity of bradykinin on the isolated rat uterus, after incubation with the purified enzyme (Figure 4). All the hydrolytic activities shown by the enzyme were totally inhibited by lisinopril.

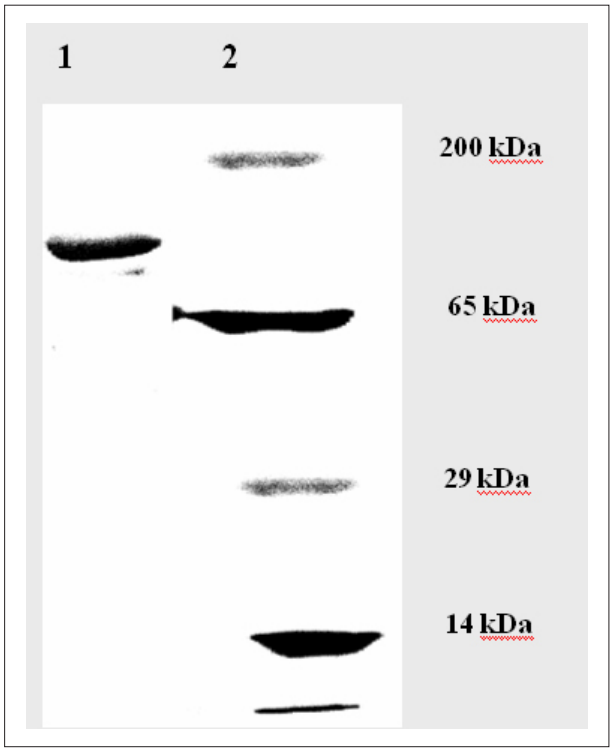

Figure 1 - Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis, SDS-PAGE (7.5% of cross-binding), under reducing conditions, of ACE isolated from human pericardial fluid. Slot 1 – ACE from pericardial fluid (5 µg); Slot 2 – molecular mass standards (beta-amylase = 200 kDa; bovine albumin = 65 kDa; carbonic anhydrase = 29 kDa; lactalbumin = 14 kDa)

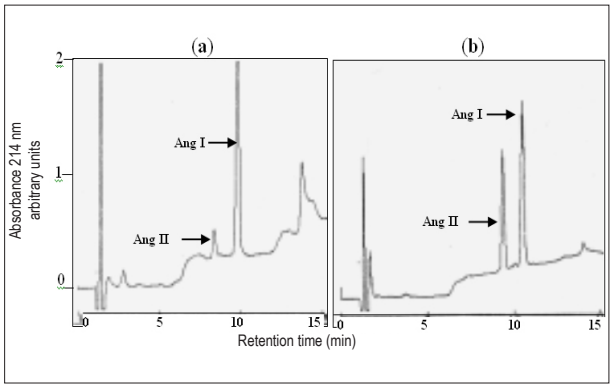

Figure 2 - High-efficiency liquid chromatography - HELC (Reverse Phase – C18) of the incubation mixture of ACE of human pericardial fluid and of Angiotensin-I (80 mmols) in 0.1 M sodium phosphate buffer, pH 7.5, containing 0.3 M NaCl, during 10 minutes (a) and 30 minutes (b); The mixture was applied to a LiChrospher column RP-18 (4.6 x 125 mm, Merck), equilibrated with 20% acetonitrile in water, containing 0.1% of trifluoracetic acid (0.1%) (solvent A) and eluted with 100% acetonitrile containing 0.1% of trifluoracetic acid (0.1%) (solvent B) and flow = 1 ml/min. A 0% to 80% linear gradient of buffer B was carried out in 25 minutes; ***Ang I = Angiotensin I; Ang II = Angiotensin II.***

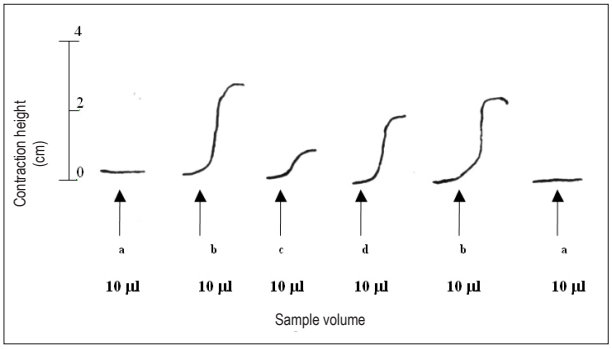

Figure 3 – Biological assay in isolated rat uterus. Angiotensin II formation after incubation with ACE of human pericardial fluid with angiotensin I. (a) angiotensin I (80 nmols in 350 µl of 0.1 M sodium phosphate buffer, pH 7.5, containing 0.3 M NaCl, incubated for 10 minutes, at 37oC); (b) angiotensin II (80 nmols – in 350 µl of 0.1 M sodium phosphate buffer, pH 7.5, incubated for 10 minutes, at 37oC); (c) angiotensin I (80 nmols) and ACE ECA from human pericardial fluid (4 ng) in 350 µl 0.1 M sodium phosphate buffer, pH 7.5, at 37oC, incubated for 10 minutes; (d) angiotensin I (80 nmols) and ACE from human pericardial fluid (4 ng) in 350 µl 0.1 M sodium phosphate buffer, pH 7.5, at 37oC, incubated for 30 minutes.

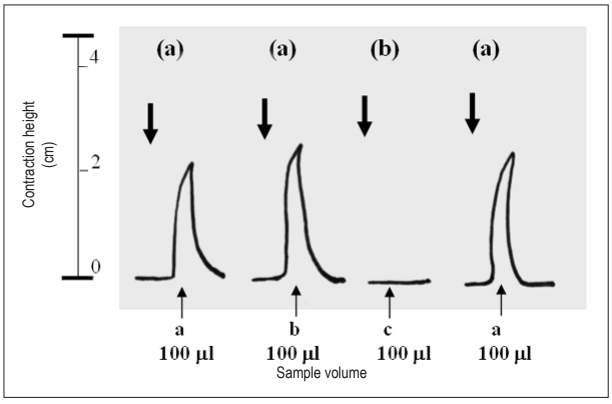

Figure 4 – Biological assay in isolated rat uterus. ACE kininasic activity of human pericardial fluid using bradykinin as substrate. (a) Bradykinin pattern (BK) – 5 nmols in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.3 M NaCl; (b) Control – BK - 5 nmols in 0.2 M sodium phosphate buffer, pH 7.5, containing 0.3 M NaCl, incubated at 37oC for 10 minutes; (c) Test – BK - 5 nmols in 0.2 M sodium phosphate buffer, pH 7.5, incubated at 37oC with ACE from pericardial fluid (4 ng) for 10 minutes.

Table 1 – Kinetic parameters of ACE of the human pericardial fluid

Substrate	k_{cat} (s-1)	K_m (µM)	k_{cat}/K_m (µM-1x s-1)
Hip-His-Leu	1.14	1654	7 x 10^{-4}
Abz-YRK(Dnp)P-OH	2.60	3.4	0.77
Abz-LFK(Dnp)-OH	2.77	7.7	0.36
Abz-SDK(Dnp)P-OH	1.92	10.2	0.19

Assay's conditions: The hydrolyses of the substrates with fluorescence suppression by ACE of the human pericardial fluid were performed at 37°C, pH 7.6 (100 mM Tris-HCl buffer, containing $ZnCl_2$ 10 µM). The enzymatic activity was continuously followed by measuring fluorescence at 420 nm (λ_{em}) after stimulation at 320 nm (λ_{exc}). The kinetic parameters of purified ACE (k_{cat} and K_m), for the hydrolysis of the Hip-His-Leu substrate (0.25 – 3mM), were measured at pH 8.3 (0.1 M potassium phosphate buffer, containing 0.3 M NaCl), at 37°C, using 4 ng of the purified enzyme. The His-Leu dipeptide was quantified fluorimetrically (Exc.365 nm; Em. 495nm) after the addition of o-phthalaldehyde.

The effect of pH on the hydrolysis of the fluorescent substrate AbzYRK(Dnp)P-OH showed better hydrolytic activity at pH 7.65. The curve obtained is similar to the hydrolysis profile of the native enzyme for several substrates.

The effect of the concentration of NaCl on the hydrolysis of the substrate Abz-YRK(Dnp)P-OH was determined to assess the C-domain activation by the chloride ions. The maximum activity was observed with 100 mM NaCl and maintained at the same level up to 500 mM.

The angiotensin-converting activity in the patients' serum and pericardial fluid is shown in Table 2. The data analysis showed a significant difference between the two fluids. Regarding the protein content of the pericardial fluid, a ratio was maintained, which varied from 1/2 to 1/3 compared to the plasma. Therefore, the enzymatic activity was expressed as specific activity (mU x mg-1 x min-1).

Discussion and Conclusion

The present study isolated and characterized, for the first time, an angiotensin-converting enzyme from human pericardial fluid. The molecular mass (140 kDa) obtained through polyacrylamide gel electrophoresis for the isolated isoform, showed to be different when compared to literature data, where molecular masses of 90-110 and 150-180 kDa are described[12–14]. This difference could be due to the post-translational modifications, such as polypeptide chain cleavages and/or glycosylation in the isoform of the pericardial fluid.

Our data show that the enzyme hydrolyses the classic substrate for the angiotensin-I converting enzyme, Hip-His-Leu, cleaves the Phe-His bond in angiotensin I generating angiotensin II and has kininase activity, hydrolyzing bradykinin to inactive products.

These observations indicate that the enzyme isolated here bears great hydrolytic similarity to the somatic enzyme. With the objective of better characterizing the enzyme from human pericardial fluid, assays with synthetic substrates susceptible to hydrolysis by the C- and N-domains of ACE were used and the results obtained showed that the isolated isoform presents the two active sites, similar to the somatic ACE. The effect of the chloride ions on the catalytic activity showed an activation profile that was very similar to the native isoform[23]. Regarding the catalytic constants obtained for the purified enzyme using the several synthetic substrates, we observed data for $K_{m,}$ that were similar to those found for the recombinant form. However, the data obtained for k_{cat}/K_m showed that the enzyme of the pericardial fluid presented less catalytic efficiency than the recombinant form[23].

The inhibition of all hydrolytic activities that were tested using lisinopril reinforced the nature of the angiotensin-converting enzyme of the isoform isolated here.

Our findings are even more relevant considering that they concern human pericardial fluid, as it is known that *in vitro* studies performed in the myocardium of different species showed that ACE constitutes the main angiotensin-II forming pathway, with the human heart, as well as the hamster and dog hearts, being the exception to this rule. Therefore, great caution must be exercised when extending to the human heart, data collected from other species[31].

The endothelium has been considered the main source of ACE in the normal heart[32,33]. However, experimental studies have shown a strong ACE labeling in fibrosed visceral pericardium, in post-infarction myocardium as well as in the control group, where only the opening of the pericardial membrane was performed, suggesting that the fibrosed heart tissue can be a source of angiotensin-II[34].

The possibility of synthesis and secretion of macromolecules through the mesothelial layer of the pericardium can be supported by the data obtained by Ishihara *et al*[35], who observed intercellular junctions between adjacent mesothelial cells, which constitute a barrier to the free diffusion of macromolecules between the blood and the pericardial cavity[36].

Using the same substrate susceptible to the C-and N-domains of ACE, used to characterize the isoform that was isolated in this study, we performed a comparative study to determine the angiotensin-I converting activity in the pericardial fluid and plasma, showing that the activity in the pericardial fluid is higher than that in plasma.

This corroborates the hypothesis of a local-origin isoform, with hydrolytic properties that are similar to the plasma ACE, which does not exclude the possibility of the presence of other isoforms. The pericardial fluid is a plasma ultrafiltrate through the myocardium and it is very unlikely that the isoform isolated in our study has plasmatic origin, as the process of diffusion is limited to molecules with molecular masses < 40 kDa. It is more probable that it originates from cells inside the pericardium or the mesothelial layer.

Therefore, one cannot consider the pericardium a mere container that has a small amount of lubricating fluid inside.

The presence of the angiotensin-I converting enzyme in the pericardial fluid-myocardium interface constitutes important evidence of the role of the pericardium in the regulation of

Table 2 – Angiotensin-I converting activity and protein content in the patients' pericardial fluid and serum

	Pericardial Fluid		Serum	
	Protein mg x ml-1	ACE mU x mg –1x min-1	Protein mg x ml-1	ACE mU x mg –1x min-1
Mean (n = 23)	20.6#	3.16*	61.7	0.33
SD	5.6	0.90	8.3	0.11

*Assay's conditions: ACE activity in pericardial fluid and serum samples of patients submitted to cardiovascular surgery was measured using the Abz-YRK(Dnp)P-OH (10 μM) substrate at 37°C , in 0.1 M Tris-HCl buffer (pH 7.0) containing 0.05 M of NaCl and 10 μM of ZnCl, in a final volume of 2.0 ml. Abz = o-aminobenzoic acid; DNP = 2,4-Dinitrophenol. The assays were performed in duplicate. Protein content was measured by the biuret method. SD= standard deviation; *p < 0.05, #p < 0.05, compared with serum.*

the cardiovascular homeostasis. It is possible that part of the effects of the angiotensin-II on the heart is a consequence of its pericardial production.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Sources of Funding

This study was funded by CNPq.

Study Association

This article is part of the thesis of master submitted by Isabel Cristina Rezende Lopes, from Universidade Federal do Triângulo Mineiro.

References

1. Burgess LJ, Reuter H, Carstens ME, Taljaard JJ, Doubell AF. Cytokine production in patients with tuberculous pericarditis. Int J Tuberc Lung Dis. 2002; 6: 439-46.
2. Meyers DG, Meyers RE, Prendergast TW. The usefulness of diagnostic tests on pericardial fluid. Chest. 1997; 111: 1213-21.
3. Reis VF, Gomes RAS, Almeida HO, Hial V. Study of the post-morten proteic content of human pericardial fluid in normal and cirrhotic individuals. Rev Med Minas Gerais. 1991; 1: 15-7.
4. Horkay F, Szokodi I, Selmeci L, Merkely B, Kekesi V, Vesisey T, et al. Presence of immunorective endothelin-1 and atrial natriuretic peptide in human pericardial fluid. Life Sci. 1998; 62: 267-74.
5. Mebazza A, Wetzel RC, Dodd-o JM, Redmond EM, Shah AM, Maeda K, et al. Potential paracrine role of the pericardium in the regulation of cardiac function. Cardiovasc Res. 1998; 40: 332-42.
6. Skeggs LT Jr, Khan JR, Shumway NP. The purification of hypertensin II. J Exp Med. 1954; 103: 301-7.
7. Yang HI, Erdös EG, Levin Y. A dipeptidyl carboxypeptidase that converts angiotensin I and inactivates bradykinin. Biochim Biophys Acta. 1970; 214: 373-6.
8. Hial V, Gimbrone MA Jr, Peyton MP, Wilcox GM, Pisano JJ. Angiotensin metabolism by cultured human vascular endothelial and smooth muscle cells. Microvasc Res. 1979; 17: 314-29.
9. Erdös EG, Skidgel RA. The angiotensin I-converting enzyme. Lab Invest. 1987; 56: 345-8.
10. Stevens BR, Phillips MI, Fernandez A. Ramipril inhibition of rabbit (Oryctolagus cuniculus) small intestinal brush border membrane angiotensin converting enzyme. Comp Biochem Physiol C. 1988; 91: 493-7.
11. Hubert C, Houot AM, Corvol P, Soubrier F. Structure of the angiotensin I-converting enzyme gene: two alternate promoters correspond to evolutionary steps of a duplicated gene. J Biol Chem. 1991; 266: 15377-83.
12. Kumar RS, Thekkumkara TJ, Sen GC. The mRNAs encoding the two angiotensin-converting isozymes are transcribed from the same gene by a tissue-specific choice of alternative transcription initiation sites. J Biol Chem. 1991; 266: 3854-62.
13. Ehlers MR, Fox EA, Strydon DJ, Riordan JF. Molecular cloning of human testicular angiotensin-converting enzyme: the testis isoenzyme is identical to the C-terminal half of endothelial angiotensin-converting enzyme. Proc Natl Acad Sci (USA). 1989; 86, 7741-5.
14. Oksanen V, Fyhrquist F, Somer H, Gronhagen-Riska C. Angiotensin converting enzyme in cerebrospinal fluid: a new assay. Neurology. 1985; 35: 1220-3.
15. Siems WE, Heder G, Hilse H, Baeger I, Engel S, Jenzsch KD. Angiotensin-converting enzyme and other peptidolytic enzymes in human semen and relations to its spermatologic parameters. Andrologia. 1991; 2: 185-9.
16. Casarini DE, Plavinik FL, Zanella MT, Marson O, Krieger JE, Hirata JY, et al. Angiotensin-converting enzymes from human urine of mild hypertensive untreated patients resemble the N-terminal fragment of human angiotensin I-converting enzyme. Int J Biochem Cell Biol. 2001; 33: 75-85.
17. Deddish PA, Wang J, Michel B. Naturally occurring active N-domain of human angiotensin I-converting enzyme. Proc Natl Acad Sci (USA). 1994; 91: 7807-11.
18. Ehlers MR, Chen YN, Riordan JF. Spontaneous solubilization of membrane-bound human testis angiotensin-converting enzyme expressed in Chinese hamster ovary cells. Proc Natl Acad Sci (USA). 1991; 88: 1009-13.
19. Jaspard E, Wei, Alhenc-Gelas F. Differences in the properties and enzymatic specificities of the two active sites of angiotensin I-converting enzyme (kininase II): studies with bradykinin and other natural peptides. J Biol Chem. 1993; 268: 9496-503.
20. Rousseau A, Michaud A, Chauvet MT, Lenfant M, Corvol P. The haemoregulatory peptide N-acetyl-ser-Asp-Lys-Pro is a natural and specific substrate of the N-terminal active site of human angiotensin-converting enzyme. J Biol Chem. 1995; 270: 3656-61.
21. Deddish PA, Marcic B, Jackman HL, Wang HZ, Skidgel RA, Erdös EG. N-domain-specific substrate and C-domain inhibitors of angiotensin-converting enzyme: angiotensin-(1-7) and keto-ACE. Hypertension. 1998; 31: 912-7.
22. Alves M F, Araujo MC, Juliano MA, Oliveira EM, Krieger JE, Casarini DE, et al. A continuous fluorescent assay for the determination of plasma and tissue angiotensin I-converting enzyme activity. Braz J Med Biol Res. 2005; 38: 861-8.
23. Araujo MC, Melo RL, Cesari MH, Juliano MA, Juliano L, Carmona AK. Peptidase specificity characterization of C- and N-terminal catalytic sites of angiotensin I-converting enzyme. Biochemistry. 2000; 39: 8519-25.
24. Bersanetti PA, Andrade MC, Casarini DE, Juliano MA, Nchinda AT, Sturrock ED. Positional-scanning combinatorial libraries of fluorescence resonance energy transfer peptides for defining substrate specificity of the angiotensin I-converting enzyme and development of selective C-domain substrates. Biochemistry. 2004; 43: 15729-36.
25. Friedland J, Silverstein E. A sensitive fluorimetric assay for serum angiotensin-converting enzyme. Am J Clin Pathol. 1976; 66: 416-24.
26. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227: 680-5.
27. Tuñon P, Johanson KE. Yet improved silver staining method for the detection of proteins in PAGE. J Biochem Biophis Methods. 1984; 9: 171-9.
28. University of Edinburg. Department of Pharmacology. Pharmacological experiments on isolated preparations. 2nd ed. Edinburg: Livingstone; 1970.
29. Leatherbarrow RJ. GraFit Version 3.0 Staines, UK: Erithacus Software Ltd; 1992.
30. Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the biuret reaction. J Biol Chem. 1949; 177: 751-66.
31. Resende MM, Mill, JG. Alternate angiotensin II-forming pathways and their importance in physiological or physiopathological conditions. Arq Bras Cardiol. 2002; 78: 432-8.
32. Sun Y, Weber KT. Angiotensin converting enzyme and myofibroblasts during tissue repair in the rat heart. J Mol Cell Cardiol. 1996; 28: 851-8.
33. Dostal DE, Rothblum KA, Chernin ML, Cooper GR, Baker KM. Intracardiac detection of angiotensinogen and renin: a localized renin-angiotensin system in neonatal rat heart. Am J Physiol. 1992; 263 (4 Pt 1): C838-50.
34. Yoneda T, Fujita M, Kihara Y HAsegawa K, Sawamura T, Tanaka T, et al. Pericardial fluid from patients with ischemic heart disease accelerates the growth of human vascular smooth muscle cells. Jpn Circ J. 2000; 64: 495-8.
35. Ishihara T, Ferrans VJ, Jones M, Boyce SW, Kawanami O, Roberts WC. Histologic and ultrastructural features of normal human parietal pericardium. Am J Cardiol. 1980; 46: 744-53.
36. Kluge T, Hovig T. The ultrastructure of human and rat pericardium. 2. Intercellular spaces and junctions. Acta Pathol Microbiol Scand. 1967; 71: 547-63.