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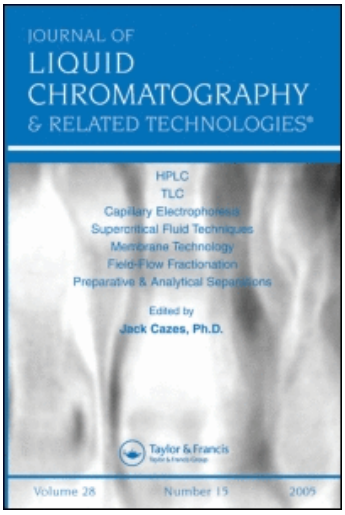


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# SEPARATION AND PURIFICATION OF N-DOMAIN OF BOVINE LUNG ANGIOTENSIN I-CONVERTING ENZYME BY SIZE EXCLUSION CHROMATOGRAPHY

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# SEPARATION AND PURIFICATION OF N-DOMAIN OF BOVINE LUNG ANGIOTENSIN I-CONVERTING ENZYME BY SIZE EXCLUSION CHROMATOGRAPHY

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## ABSTRACT

Bovine lung angiotensin I-converting enzyme is a monomer with two active sites, in its two (N and C) homologous domains. A process is described for the preparative isolation of the ACE N-domain to either the ACE soluble monomer or ACE aggregated one. After exposure to moderate heat for 7 hours and in presence of a protease, ACE N-domain was obtained as a whole, and only fragments of the C-domain. N-domain purified was separated by Sephacryl S-300 HR chromatography, and a recovery of 80% was obtained. Molecular mass was estimated to be 100 kDa by sodium dodecyl sulfate gel electrophoresis. After purifying and partially sequencing this domain, we have investigated some catalytic properties and the inhibition by captopril.

## INTRODUCTION

Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) plays an important role in blood pressure regulation. It is a dipeptidyl carboxypeptidase which converts angiotensin I into the potent vasopressor peptide angiotensin II and



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inactivates the vasodepressor peptide bradykinin.[1] ACE is an unusual zinc-metallopeptidase in that it is activated by chloride and lacks a narrow in vitro substrate specificity.[2]

Inhibition of ACE is a widely used approach in the treatment of hypertension. The first available competitive inhibitors of ACE were the naturally occurring peptides in snake venom. Clinical studies using the nonapeptide teprotide, the most efficient of these snake venom peptides in vivo, demonstrated the potential of ACE inhibitors as antihypertensive drugs. A lot of highly potent inhibitors of ACE which can be taken orally have subsequently been developed during the past two decades.[3, 4]

The molecular cloning and sequencing of the complementary DNA for human ACE revealed that ACE (somatic isozyme of ACE) is a glycoprotein that consists of a single polypeptide chain containing two homologous domains called the N and C domains, each bearing a potential catalytic site.[5, 6]

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ACE has been identified in many tissues and is specially abundant in lung. This enzyme is an integral membrane protein anchored to the plasma membrane via its hydrophobic C-terminus.[5] A soluble form of bovine lung ACE is obtained by tryptic treatment and using a Sephacryl S-300 HR chromatography.[7]

In this work we have described the preparative isolation of N-domain bovine ACE by thermal treatment in presence of a protease. The results revealed that a recovery of 80% was obtained and an apparent molecular mass of approximately 100 kDa, was obtained. After purifying and partially sequencing of this domain, we have investigated some catalytic properties and the inhibition by captopril.

## EXPERIMENTAL

### Materials

Standard liquid chromatographic materials, Superdex H200 10/30 for FPLC were purchased from Pharmacia. Trypsin, kallikrein, and subtilisin from *bovine pancreas*, *porcine pancreas* and *bacilus subtilis*, respectively, were obtained from Boehringer Mannheim. Captopril, furanacryloyl-L-phenylalanilglycylglicine, and N-(2-hydroxyethyl) piperazine-N'-(2-ethane-sulfonic acid) (Hepes) were purchased from Sigma; other chemicals were bought from either Sigma or Merck.

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### Isolation of ACE Monomer

Membrane-bound converting enzyme that had been eluted from lisinopril/Epoxy-activated Sepharose 6B affinity column (1 mg/mL) was digested with bovine pancreatic trypsin and separated by Superdex 200 HR 10/30 HPLC as described elsewhere.[7]

### Measurement of Protein Concentration

Monomer concentration was determined spectrophotometrically at 280 nm in a Beckman DU-7400 spectrophotometer and using the bovine-lung-enzyme absorbance coefficient 2.1 $10^5$ $M^{-1}$ $cm^{-1}$.[8] N-domain ACE concentration was measured according to the Lowry method.[9]

### Determination of the N-Terminal Amino Acid Sequence

The determination of the amino acid sequence of the N-domain in its purified form was done in the Biology Research Center (CSIC) from Madrid.

### Enzyme Assays, Kinetics and Inhibition Studies

Monomer and N-domain ACE assays were performed spectrophotometrically at 25°C by the method of Holmquist et al.,[10] using furanacryloyl-L-phenylalanilglycyl-glicine (FAPGG) as the substrate. Reaction mixtures contained 100 mM substrate in 50 mM Hepes, 300 mM NaCl, 10 mM zinc acetate, and 0.7 mg/mL to 2 mg/mL of enzyme at pH 7.5. Absorbance measurements at 334 nm were carried out in a Beckman DU-70 spectrophotometer with the cells maintained at 25°C. One unit of activity is defined as $\Delta A_{334}$ of $1min^{-1}$. The specific activity for monomer and N-domain was 24-26 $min^{-1}$ $mg^{-1}$ and 12-14 $min^{-1}$ $mg^{-1}$, respectively.

Kinetic constants for FAPGG hydrolysis at pH 7.5, 0.3 M NaCl, [E]=9-10 x $10^{-9}$ M, were determined from Lineaweaver-Burk plots of initial rates at 10-fold below and above $K_m$. Chloride dependence was estimated under first-order conditions with 0.1 mM FAPGG ($[S]<<K_m$) at pH 7.5 and [E]=9-10 x $10^{-9}$ M over the $[Cl^-]$ range 0-400 mM, and the apparent activator constant ($K_a$) determined from plots 1/v vs $1/[Cl^-]$. The enzyme used in these studies was first washed extensively with $Cl^-$ free buffer (50 mM Hepes, 10 μM $Zn(AcO)_2$ at pH 7.5) in a Centricon 30 microconcentrator (Amicon).

Inhibition by the tight-binding inhibitor captopril on the hydrolysis of the ACE monomer and N-domain was determined by preincubating the enzyme for

30 minutes at 25°C before addition of the substrate. A range of inhibitor concentrations (0 to 0.4 mM) was used. Apparent inhibitor constant ($K_I$) was determined by the graphical method of Dixon, with the following equation.[11]

$$v = \frac{V}{1 + \frac{K_m}{s}(1 + i/K_I)}$$

**Protein Electrophoresis Under Denaturing Conditions.**

Polyacrilamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) of the enzyme was performed on 7% or 6% gels according to Laemmli[12] using a Bio-Rad Protean II electrophoresis apparatus. Coomassie Brilliant Blue R-250 was used for staining the protein bands. Commercial protein size markers were used for calibration (Pharmacia).

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**Molecular Mass Determinations**

Analytical high performance liquid chromatography (HPLC) was carried out on a Beckman apparatus. Effluents were monitored with a diode array detector (DU168). The FPLC column was Superdex 200 HR 10/30 from Pharmacia. The column was calibrated with tiroglobuline (669 kDa), ferritin (440kDa), alcohol deshidrogenase (150 kDa), bovine serum albumin (67 kDa), ribonuclease (13,7 kDa), and Blue Dextran (2000 kDa) from Sigma. The column was equilibrated with 10 mM Hepes, 0.1 M NaCl, 10 mM $ZnCl_2$ at pH 7.

## RESULTS AND DISCUSSION

**Purification of ACE N-Domain**

N-domain bovine ACE was purified from ACE monomer, as described elsewhere,[7] and by thermal treatment in the presence of a protease. Our procedure is based on the findings of Deddish et al.[13] for the purification of the N-domain from somatic ACE of human kidney. N-domain was obtained using a Sephacryl S-300 chromatography.

Aproximately 250 µg of ACE monomer in 200 µL of 10 mM Hepes, 0.3 M NaCl 10 µM $ZnCl_2$, pH 7 were incubated at 44ºC for the exposure periods of: 1/2, 1, 2, and 7 hours. At each time the sample was incubated for 45 minutes at 37°C with the protease tripsin, subtilisin, or kallikrein (1/1000 w/w with respect to ACE) and 20 µL were injected in a HPLC. Figure 1 shows profiles of the Superdex 200 HR 10/30 elution at the above mentioned incubated times.

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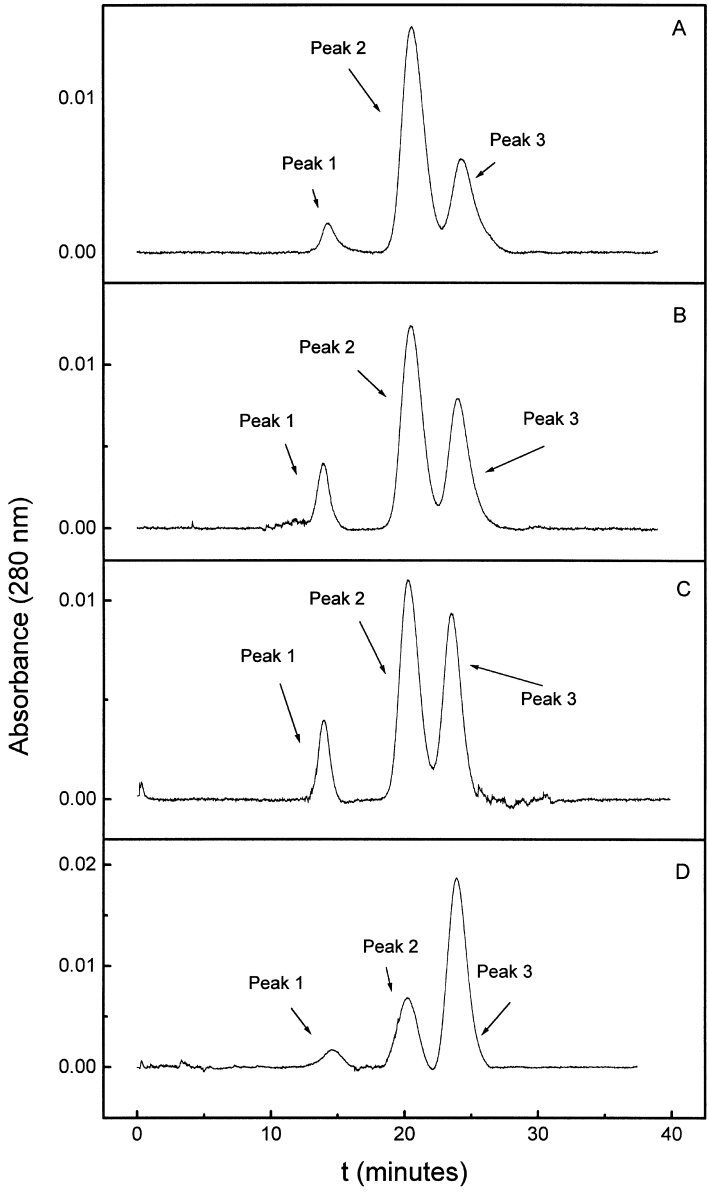


**Figure 1**. HPLC chromatograms of bovine lung angiotensin converting enzyme using the protease kallikrein (1/1000 w/w with respect to ACE). The elution profiles correspond at the injection of 20 µL of ACE (1.25 mg/mL) incubated for 45 minutes at 37ºC with the protease. Previously, the sample was incubated at 44°C for (A) ½ (B) 1, (C) 2 and (D) 7 hours. The chromatography was performed on Superdex S-200 10/30 column equilibrated with 10 mM Hepes, 0.3 M NaCl, 10 µM $ZnCl_2$ pH 7, at a flow rate of 0.5 mL/min at room temperature.

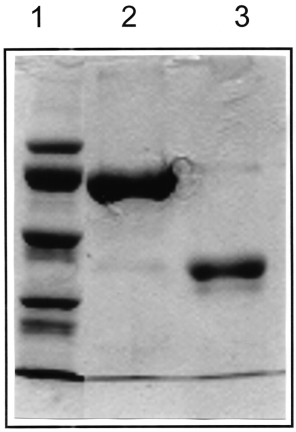


**Figure 2**. Slab gel electrophoresis of the ACE N-domain: *lane 2* contained 20 μg of the ACE monomeric (peak 2 in figure 1); lane *3* contained 20 μg of N-domain (peak 3 in Figure 1). Lane 1 *c*ontained the following five protein molecular mass standards: myosin (212 kDa); $\alpha_2$-macroglobulin (170 kDa); β-galactosidase (116 kDa); transferrin (76 kDa); and glutamate deshydrogenase (53 kDa). Samples were electrophoresed in a 6% polyacrylamide gel under reducing conditions and were stained with Coomassie brilliant blue R-250.

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In the profiles several peaks were obtained with ACE activity: peak 1, near the void volume corresponding to aggregated angiotensin-converting enzyme as described elsewhere,[7] peak 2 with an apparent molecular mass of 300 kDa, corresponding to soluble ACE monomer, and finally, peak 3, with molecular mass of 200 kDa. As the incubation time at 44ºC rises the peak 3 is major, indicating that ACE monomer (peak 2) is converted in a specie with less molecular mass and with ACE activity. If the incubation time is higher than 7 hours, the yield corresponding to peak 3 scarcely changes.

The results of electrophoresis in SDS of chromatographed peaks are shown in Figure 2. Only one band is seen in all cases, the apparent molecular mass of the fractions corresponding to peaks 2 and 3 are 160 kDa and 100 kDa, respectively. The apparent molecular mass corresponding to peak 3 was similar to that obtained by Deddish et al.[13] for N-domain purified from ileal fluid, indicating that this peak could be referred to N-domain of bovine lung ACE.

The N-terminal amino acid sequence of N-domain from bovine lung enzyme (peak 3) was analyzed and determined up to 12 residues as follows: ($NH_2$)-Glu-Leu-Asp-Pro-Ala-Leu-Glu-Pro-Gly-Asn-Phe-Pro. A single sequence was obtained showing that the sample was homogeneous. Figure 3

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Bovine lung*** | ***($NH_2$)*** | ***Glu*** | ***Leu*** | ***Asp*** | ***Pro*** | ***Ala*** | ***Leu*** | ***Glu*** | ***Pro*** | ***Gly*** | ***Asn*** | ***Phe*** | ***Pro*** |
| ***Human lung*** | | ($NH_2$) | X | X | Pro | Gly | Leu | Glu | Pro | Gly | X | Phe | Ser |
| ***Rabbit lung*** | ($NH_2$) | Thr | Leu | Asp | Pro | Gly | Leu | Leu | Pro | Gly | Asp | Phe | Ala |
| ***Pig kidney*** | | ($NH_2$) | Leu | Asp | Ser | Ala | Leu | Glu | Pro | Gly | Cys | Phe | Thr |
| ***Bovine kidney*** | ($NH_2$) | Glu | Leu | Asp | Pro | Ala | Leu | Glu | Pro | Gly | Asn | Phe | Pro |
| ***Mouse kidney*** | | ($NH_2$) | Leu | Asp | Pro | Gly | Leu | Glu | Pro | Gly | Asn | Phe | Ser |
| ***Human kidney*** | | ($NH_2$) | Leu | X | Pro | Ala | Arg | Pro | | | | | |

**Figure 3**. Comparison of N-terminal amino acid sequence of bovine lung angiotensin converting enzyme with those reported for human,[19] rabbit lung[15] and pig,[16] bovine,[17] mouse,[17] and human kidney[18] enzymes. Identical amino acid residues are boxed.

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compares the N-terminal amino acid sequence of the bovine lung enzyme with those reported for other tissues and species.[14-19] This sequence was consistently homologous with those of enzymes from various tissues and species[14-19] being similar to that obtained to N-terminal from bovine lung.[14] However, it showed a striking difference from that reported for human kidney enzyme.[18] This analysis also shows that the fragment purified was probably the N-domain of ACE.

On the other hand, the profiles displayed in Figure 1 are non-dependent to the protease used: tripsin, subtilisin, or kallikrein. On increasing the amount of protease, the area of peak 3 was not increased with respect to that of peak 2. However, if the ratio of protease/enzyme is higher than 1/500, the chromatographic profile shows an additional peak (after peak 3) corresponding to the protease absorbance (results not shown). In all cases, the enzyme activity was 20 units/mg, 26 units/mg and 12 units/mg for the peak 1, 2, and 3, respectively.

Separation of the 160 kDa and 100 kDa forms of ACE (peaks 2 and 3 in Figure 1) was accomplished by gel filtration chromatography which yielded two distinct peaks of enzyme activity and protein absorbance. In addition, several low molecular mass peaks (<14 kDa) appeared that ACE activity is lacked. These peaks are not visualized in Figure 1 because the absorbance was too small.

Thus, we have conducted a thermal treatment in presence of a protease, and the use of size exclusion chromatography as the last purification step to

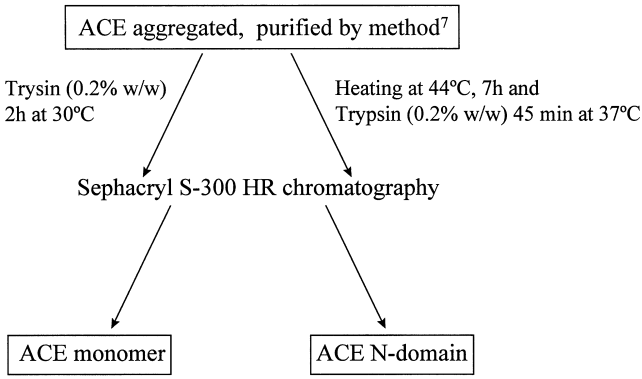


**Figure 4**. General steps for purification of both monomer and N-domain of ACE.

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obtain the ACE N-domain from ACE monomer. However, another method to obtain N-domain of ACE could be applied to perform the treatment above mentioned (using for example tripsin as protease) after protein elution by affinity chromatography. In the last case, we obtain the N-domain directly from bovine lung, this being the most convenient procedure likely to obtain only the N-domain.

Thus, the general procedure designed for purification of ACE[7] could be used to obtain either ACE monomer or N-domain in function of treatment with protease that we performed. A scheme of these two possibilities is displayed in Figure 4. For 1 kg of bovine lung, the procedure described enables us to obtain 12 mg of N-domain of ACE with a specific activity of 12 units $mg.^{-1}$ In this case we used a column of Sephacryl S-300 (1.6 x 70 cm) to separate the N-domain.

An elution profiles similar to those shown in Figure 1 was obtained for apo-ACE monomer but yields corresponding to peak 3 (N-domain) was lower that holo-ACE, indicating the dependence of Zn cofactor to its stability. This cofactor dependence to stability of apo-ACE may suggest that the tridimensional structure of enzyme without Zn exhibits slight changes with respect to that of holo-ACE

Deddish et al.[13] had showed that treatment of somatic ACE at 45°C for 2 h decreased its enzymatic activity by 70%. Moreover, subsequent treatment with tissue kallikrein does not decrease the activity further but reduces the molecular mass to about 100 kDa. We have studied that the form of bovine lung ACE

of 100 kDa was equally obtained in the presence of other protease (not only kallikrein) if the sample is previously heated at 44ºC for more than seven hours.

These results could be discussed suggesting that the heating for that time (more than 7 hours) could produce a partial denaturation of enzyme molecule that probably corresponds to C-domain. In this case, the C-domain will be accessible to cleavage by the protease. A similar but more physiological process, for example, as a consequence of the activation of the blood-borne protease, may be responsible for the release of N-domain in vivo, in human ileal fluid.

Additional or alternative reasons regarding the higher stability of the N-ACE, may be referred to its higher carbohydrate content,[20] and differences in the three-dimensional structure of the two domains. As indicated above, although there is a high degree of identity in the amino acid sequences of the two active sites, there is evidence that the C-domain is more susceptible to denaturation than the N-domain.

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**Hydrolysis of FA-Phe-Gly-Gly**

The results of the hydrolysis of FAPGG by monomer and N-ACE from bovine lung are summarized in Table 1. It is of interest to note that $K_m$ values obtained by both monomer and N-ACE under the conditions used are approximately similar, whereas $k_{cat}$ for the ACE monomer is approximately 3-fold higher than that for the N-domain. These results are consistent with those shown by Wei et al.[21, 22] for human ACE and using Hip-His-Leu as substrate.

To determine the effect of $Cl^-$ concentration on FAPGG, hydrolysis was exposed to the enzymes at a $Cl^-$ concentration ranging from 0 to 400 mM. In these conditions the apparent activation constants are found to be 10.3 and 3.6 mM for monomer and ACE N-domain, respectively.

**Table 1**

**Kinetic Parameters for the ACE-Catalyzed Hydrolysis of FAPGG**

| | $K_M$ (μM) | $k_{cat}$ ($Min^{-1}$) | $K_{cat}/K_M$ ($Min^{-1} . \mu M^{-1}$) |
|---|---|---|---|
| Monomer | 78 | 6024 | 77 |
| N-domain | 100 | 1940 | 19 |

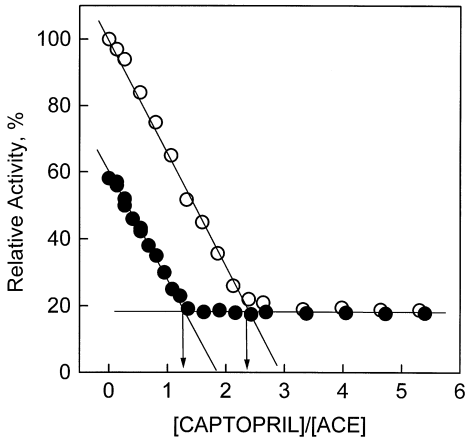


**Figure 5**. Inhibition by captopril of monomer (o) and ACE N-domain (•). It is shown the relative activity versus total captopril concentration to the total protein concentration ratio. The samples were incubated with captopril at 25°C for 30 minutes. The buffer used was 10 mM Hepes, 0.3M NaCl, 50 µM $ZnCl_2$ pH 7.5. The reaction was initiated by the substrate FAPGG. The intersection of the extrapolated linear portion of the curves show a stoichiometry of 2 and 1 mol of captopril per mol of protein to monomer and N-domain of ACE, respectively.

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## Inhibition by Captopril

To check the properties of N-domain obtained, we have tested captopril to inhibit the hydrolysis of FAPGG by homogenous N-domain and monomer of ACE. The results suggest that inhibitor inactivate the enzyme, indicating the existence of two active sites for the ACE monomer, and only one site for the N-domain (Figure 5). The inhibition constant obtained by Dixon method[11] were 22- and 5 nM, to both monomer and N-domain of ACE, respectively. Thus, the $K_i$ value of captopril to ACE N-domain for the inhibition of FAPGG hydrolysis is about 4-fold lower than that with ACE-monomer.

## CONCLUSIONS

Purified bovine lung ACE, contains two domains N and C, each containing a catalytic site. The N-ACE represents a low molecular mass active form of the enzyme, which can be found in ileal fluid. Also, the C-domain can be found physiologically in testis. In the present study, we have showed that after moderate heating and exposure to a protease, the monomer ACE releases active N-domain and only fragments of the C-domain. Thus, despite the similarity in

the amino acid sequence of the two domains, the C-domain is denaturated at 45°C while the N-domain is kept intact. On the basis of this denaturation the attack of several proteases is possible, indicating the non-specificity of the protease used to obtain the N-domain. We have described a purification procedure, which enables us to obtain 12 mg of N-domain with a molecular mass of 100 kDa and a specific activity of 12 units $mg^{-1}$, from 1 kg of bovine lung.

## ACKNOWLEDGMENTS

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