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Characterization of Bovine Atrial Angiotensin-Converting Enzyme

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Abstract—Bovine atrial angiotensin-converting enzyme (ACE) was purified to electrophoretic homogeneity. The purification procedure included ion-exchange chromatography on DEAE-Toyopearl 650M, affinity chromatography on lisinopril-agarose and gel filtration on Sephadex G-100. The bovine atrial ACE exhibited similar sensitivities to inhibition by lisinopril and captopril as lung ACE (the K_i values for the atrial and lung enzymes differed insignificantly). However, the kinetic parameters of hydrolysis of some synthetic tripeptide substrates (FA-Phe-Gly-Gly, FA-Phe-Phe-Arg, Cbz-Phe-His-Leu, Hip-His-Leu) catalyzed by bovine atrial and lung ACE varied to a greater extent. The enzymes were also characterized by some differences in activation by chloride, nitrate, and sulfate anions. These data support the hypothesis of tissue specificity of ACEs.

Key words: angiotensin converting enzyme, tissue specificity, bovine atrium, catalytic properties, tissue specificity

Angiotensin converting enzyme, a zinc-dependent hydrolase (ACE; peptidyl-dipeptidase A, EC 3.4.15.1), is involved in the regulation of blood pressure [1]. It links the kallikrein–kinin and renin–angiotensin (RAS) systems. Bradykinin and angiotensin I are the main substrates of ACE. Bradykinin, the main component of the kallikrein–kinin system, is a local arteriolar vasodilator. It also stimulates the synthesis of prostaglandins, which exert vasodilating, diuretic, and natriuretic effects [2, 3]. Angiotensin II, the product of ACE-dependent proteolytic conversion of angiotensin I, is a potent vasoconstrictor. Besides potent constrictor effect on the arterial blood vessels, it also regulates water and salt balance, myocardial contractility, and the biosynthesis of noradrenaline and other physiologically active compounds [3].

The major proportion of angiotensin I and bradykinin is degraded in lung [4], and initially ACE was considered as a component of some generalized system regulating blood homeostasis. Now this notion is significantly extended. Besides generalized blood RAS, local RASs also exist. The components of this system including ACE and angiotensin II have been found in brain, kidneys, eyes, heart, etc. [1, 5, 6]. Some experimental data suggest the existence of tissue-specific variations of ACE. For example, Strittmatter et al. studied the physicochemical characteristics of ACE isolated from rat brain and lung [7]. They found differences in molecular masses of brain and lung ACE (165 and 175 kD, respectively), which can be explained by different glycosylation degree. The enzymes also demonstrated differences in substrate specificity. In contrast to the lung enzyme, brain ACE was able to hydrolyze substance K and bombesin. Both enzymes catalyzed the hydrolysis of substance P but at different bonds. Lung ACE preferentially cleaved a tripeptide from this substrate, whereas substance P hydrolysis by brain ACE yielded both di- and tripeptide [7, 8]. These differences were not found in pig ACE isolated from brain and kidneys [9]. Study of ACE inhibition in plasma and homogenates of various human organs (aorta, kidneys, brain, and heart) by captopril, zophenopril, elanapril, ramipril, lisinopril, fozinopril [10, 11] revealed different efficacy of these drugs with respect to ACE of various tissues. For example, captopril was the most effective inhibitor of ACE in aorta, heart, lung, and kidney homogenates whereas fozinopril and elanapril were the most potent inhibitors of plasma and brain ACE, respectively.

RAS (and ACE as its component) plays special role in the heart because angiotensin II is the most active transmitter of cardiovascular remodeling [12]. During the last decade alternative pathways of angiotensin II formation including tonin, cathepsin G, and chymase have also been recognized [13]. Nevertheless, ACE inhibitors are

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still considered as the first choice drugs for medical treatment of hypertension; they are also effective in combined therapy of myocardial infarction and other cardiovascular diseases [2, 14, 15]. Among various possibilities, clear cardiovascular effects may reflect the existence of some characteristic features of cardiac ACE. It was shown that human heart ACE (in contrast to ACE from other organs) is characterized by one unique property: it can cleave atriopeptin II (natriuretic factor) and its C-terminal analog [16, 17]. The catalytic constant for heart ACE is 30 times higher than that for human kidney ACE during angiotensin I cleavage [18]. These data suggest tissue specificity of ACE and a possibility of local (tissue specific) correction of ACE-dependent pathological states in certain organs.

Thus, the major goal of the present study was to analyze tissue-specific properties of bovine atrial ACE. We focused our attention on analysis of such basic properties of this enzyme as catalytic and inhibitory specificity and activation by anions rather than search for some unique features (like cleavage of atriopeptin II by human heart ACE). ACEs from bovine lung and human heart were used as reference enzymes.

Two previous attempts to purify ACE from bovine atria [19, 20] failed to produce homogenous enzyme. The isolated enzyme differed from ACE in some physicochemical properties; it was active only with Cbz-Phe-His-Leu as substrate and was inhibited by captopril but not by another inhibitor, D-Cys-L-Pro. Thus, it was concluded that the isolated enzyme could be a new dipeptidyl-carboxyhydrolase. We also compared ACE isolated from bovine atria with that enzyme.

MATERIALS AND METHODS

Chemicals. The following chemicals were used in this study: N^{α}-3-(2-furyl)-acryloyl-L-phenylalanyl-glycyl-glycine (FA-Phe-Gly-Gly), phosphoramidon (N-(α-rhamnopyranosyl-oxyhydroxyphosphonyl)-L-leucyl-L-tryptophan), lisinopril (N-(S-1-carboxy-3-phenylpropyl)-L-lysyl-L-proline), captopril ((2S)-1-(3-mercapto-2-methylpropionyl)-L-proline) from Sigma (USA); hippuryl-L-histidyl-L-leucine (Hip-His-Leu), N^{α}-carbobenzoxy-L-phenylalanyl-L-histidyl-L-leucine (Cbz-Phe-His-Leu), thiorphan ((DL-3-mercapto-2-benzyl-propanoyl)-glycine) from Serva (Germany). N^{α}-3-(2-furyl)-acryloyl-L-phenylalanyl-L-phenylalanyl-L-arginine (FA-Phe-Phe-Arg) was synthesized as described elsewhere [21].

Enzyme purification. Bovine atrial tissue (400 g) was washed with 500 ml of 0.05 M phosphate buffer, pH 7.5, containing 0.15 M NaCl and 1 μM $ZnCl_2$ to remove other proteins. The washed tissue was homogenized (at 4°C) twice in the same buffer (400 ml) at 8000 rpm for 45 sec using an RT-1 homogenizer (Russia). The resulting homogenate was centrifuged in a Beckman J-21 centrifuge (USA) at 14,000 rpm for 35 min. The supernatant was decanted and discarded. The pellet was resuspended in 400 ml of the same buffer and the entire procedure was repeated twice. The resulting pellet after the third decanting was used for the subsequent purification.

Extraction. The pellet (250 g) was resuspended in 1 liter of 10 mM phosphate buffer, pH 7.5, containing 1% Triton X-100 (v/v), 1 μM $ZnCl_2$, and homogenized twice at 8,000 rpm for 1 min. After overnight incubation at 4°C with slow stirring, the homogenate was centrifuged in the Beckman J-21 centrifuge at 14,000 rpm for 1 h. The pellet was discarded and the supernatant was used for subsequent purification.

Ion-exchange chromatography. The supernatant from the previous step (1 liter) was applied to a DEAE-Toyopearl 650M column (30 × 520 mm) equilibrated with 10 mM phosphate buffer, pH 7.5, containing 0.1% Triton X-100 and 1 μM $ZnCl_2$. The application rate was 90 ml/h. The column was washed twice with the starting buffer and the enzyme was eluted by a linear NaCl gradient (from 0 to 0.2 M) in 300 ml of the starting buffer. Fractions containing enzymatic activity were pooled for subsequent purification step.

Affinity chromatography was carried out using a lisinopril-agarose column as described [22].

Gel filtration. The resultant enzyme preparation was concentrated to 5 ml using Amicon YM-30 (USA) membrane filtration. The concentrated preparation was applied (at flow rate 10 ml/h) to a Sephadex G-100 column (10 × 500 mm) equilibrated with 10 mM phosphate buffer, pH 7.5, containing 0.2 M NaCl and 1 μM $ZnCl_2$. Fractions containing active enzyme were stored at 4°C after addition of 10% glycerol (v/v).

Protein concentration was determined by the Lowry method [23].

Electrophoresis of ACE preparations was carried out by the Laemmli method in 7.5% polyacrylamide gel in the presence of 0.1% SDS and β-mercaptoethanol [24].

ACE activity during purification was determined by initial rates of hydrolysis of 50 μM Cbz-Phe-His-Leu in 50 mM Hepes buffer, pH 7.5, 0.15 M NaCl, 1 μM $ZnCl_2$, at 25°C. The accumulation of product, His-Leu, was determined by the reaction with *ortho*-phthalaldehyde using a Hitachi MPF-4 (Japan) spectrofluorimeter [25]. Products of enzymatic hydrolysis of Cbz-Phe-His-Leu catalyzed by the isolated enzyme were identified by thin-layer chromatography on Silufol UV 250 in ethanol–water (7 : 3). The development was carried out using 0.3% ninhydrin followed by subsequent heating until the appearance of spots. Initial substrate, histidine, leucine, and histidyl-leucine were used as standards. One unit of enzyme activity corresponds to the amount of ACE that catalyzes the hydrolysis of 1 μmole of substrate per min at 25°C. Specific activity was expressed as units per mg protein.

The concentration of active ACE molecules in preparations was determined by the method of non-stoichiometric titration [26] using the specific competitive inhibitor lisinopril. The enzyme (~10 nM) was incubated in 50 mM Hepes-buffer, pH 7.5, containing 0.15 M NaCl, 1 μM $ZnCl_2$, with various concentrations of the inhibitor (0-0.2 μM) for 2 h at 25°C until equilibrium. The enzymatic reaction was started by adding 50 μM Cbz-Phe-His-Leu and the remaining activity was determined as described above.

The number of active sites was calculated using plots $[I_0]/(1 - a)$ versus $1/a$ where $a = v_i/v_{0,}$ $[I_0]$ is inhibitor concentration in the sample, and v_i and v_0 are reaction rates in the presence and in the absence of inhibitor, respectively. The intersection on the ordinate axis corresponds to the concentration of active sites.

Determination of kinetic parameters for hydrolysis of substrates FA-Phe-Gly-Gly and FA-Phe-Phe-Arg. Kinetics of enzymatic hydrolysis of FA-Phe-Gly-Gly and FA-Phe-Phe-Arg was studied spectrophotometrically using a Shimadzu UV-265FW (Japan) spectrophotometer [21]. The enzyme concentration was 0.3 nM, and the substrate concentration was varied from 0.05 to 1 mM.

Determination of kinetic parameters for hydrolysis of substrates Hip-His-Leu and Cbz-Phe-His-Leu. The rate of enzymatic hydrolysis of Hip-His-Leu and Cbz-Phe-His-Leu was determined fluorometrically as described above. The enzyme concentration was 0.1 nM and substrate concentration was varied from 0.05 to 2 mM.

The data were analyzed using Lineweaver–Burk plots. Statistical treatment was carried out using standard programs employing the least square method. Experimental error did not exceed 10%.

Inhibition of ACE by chelating agents (EDTA and *ortho*-phenanthroline) and metalloproteinase inhibitors (lisinopril, captopril, phosphoramidon, and thiorphan) was determined by the decrease in enzymatic hydrolysis of Cbz-Phe-His-Leu in 50 mM Hepes-buffer, pH 7.5, containing 0.15 M NaCl and 1 μM $ZnCl_2$. The mixture of enzyme and inhibitor was preincubated at 25°C for 2 h until equilibrium (chelating agents did not require incubation). The reaction was initiated by addition of the substrate. After incubation for 30 min, the reaction was stopped, and the fluorescence of the product was determined. Concentrations of inhibitors and chelating agents was varied within ranges of 0.05 nM-0.1 mM and 1 μM-1 mM, respectively. Efficacy of phosphoramidon and thiorphan inhibition was evaluated by IC_{50} values (concentration required for 50% inhibition of enzyme activity). Inhibition constants (K_i) for lisinopril and captopril were determined as described earlier [21]. Substrate concentrations in the reaction mixture were varied from 5 μM to 0.1 mM. The enzyme concentration was 1 nM.

The effects of anions on ACE activity were evaluated by measuring the initial rates of hydrolysis of Cbz-Phe-His-Leu in the presence of various concentrations of chloride, sulfate, and nitrate anions (from 0 to 1 M) in the reaction medium. The anions were added as sodium salts in 0.05 M Hepes buffer, pH 7.5, containing 1 μM $Zn(CH_3COO)_2$, which was prepared using deionized water.

The effect of zinc ions on activity of native enzyme was evaluated by measuring the initial rates of hydrolysis of Cbz-Phe-His-Leu in 0.05 M Hepes buffer, pH 7.5, containing 0.15 M NaCl and varying concentrations of zinc ion (from 0.1 μM to 1 mM). All solutions were prepared with deionized water.

RESULTS AND DISCUSSION

In agreement with previous studies [19], we failed to detect ACE activity in bovine heart ventricles, and thus only bovine atria were used as the source of ACE. ACE was extracted from membranes at rather high Triton X-100 concentration (1%) because preliminary experiments with different Triton X-100 concentrations (0.05-3%, v/v) revealed that the presence of 1% Triton X-100 provided maximal extraction of atrial ACE activity. The ACE content is low in the heart. For example, it is 30 times less than ACE content in bovine lung [27]. Due to high quantities of other proteins in the Triton X-100 extracts, the major proportion of atrial ACE did not bind to the affinity sorbent. Thus, we had to introduce an additional step, ion-exchange chromatography on DEAE-Toyopearl 650M, which removed the main part of the other proteins and significantly improved the purification procedure.

After ion-exchange and affinity chromatography, the ACE preparation contained minor contaminants with molecular mass of 60 kD; these were readily removed by subsequent gel filtration. The resulting preparation of bovine atrial ACE (0.6 mg) was electrophoretically homogenous (Table 1). Its molecular mass determined by electrophoresis under denaturing conditions was 180 kD (Fig. 1). This is consistent with the molecular masses of other somatic ACEs isolated from bovine lung (140-180 kD) [28, 29] and human heart (150 kD) [18]. However, this value is lower than the molecular mass of bovine atrial dipeptidyl-carboxyhydrolase (240 kD) [19].

In the presence of 10% glycerol, the activity of atrial ACE remained unchanged during storage at 4°C for at least 3 months. However, storage at −20°C for 1 month was accompanied by dramatic loss (90%) of the enzymatic activity.

Bovine atrial ACE exhibited maximal activity with Cbz-Phe-His-Leu as substrate at pH 7.2 (Fig. 2, curve *1*). This is consistent with the pH optimum for bovine lung ACE (pH 7.1-7.4) [29, 30]. Usually the pH optimum for Hip-His-Leu hydrolysis by ACE from various sources is more alkaline. For example, bovine lung and human heart ACEs exhibited the highest activity with this sub-

Table 1. Purification of bovine atrial ACE

Stage	Volume, ml	Protein content, mg	Total activity, U	Specific activity, U/mg protein	Purification degree	Yield, %
Extract (Triton X-100)	780	2570	1.0	0.00039	1	100
Ion-exchange chromatography (DEAE-Toyopearl 650M)	120	85	0.69	0.0081	21	69
Affinity chromatography (lisinopril-agarose)	5	0.68	0.24	0.35	897	24
Gel filtration (Sephadex G-100)	16	0.58	0.21	0.37	948	21

strate at pH 8.75 [29] and 7.8 [17], respectively. However, the pH optimum for Hip-His-Leu hydrolysis catalyzed by bovine atrial dipeptidyl-carboxyhydrolase was 7.3 [19]. Bovine atrial ACE exhibited maximal activity with this substrate at pH 8.3 (Fig. 2, curve *2*).

ACE is a zinc-dependent hydrolase and its activity depends on the presence of zinc cations in the reaction medium. The chelators EDTA and *ortho*-phenanthroline (1 mM) totally inhibited ACE activity. The optimum activity determined for bovine atrial ACE was observed at 1 μM concentration of zinc ions; this value coincides with that found for the lung enzyme [30].

Kinetic parameters (k_{cat} and K_m) for hydrolysis of the synthetic tripeptide substrates Cbz-Phe-His-Leu and Hip-His-Leu (C-terminal analogs of angiotensin I), FA-Phe-Phe-Arg (C-terminal analog of bradykinin), and FA-Phe-Gly-Gly catalyzed by atrial ACE were determined under nearly physiological conditions (pH 7.5, 150 mM NaCl). Table 2 shows these values together with recently obtained data for bovine lung ACE [31]. The kinetic parameters of FA-Phe-Gly-Gly hydrolysis catalyzed by the two enzymes were almost identical. The lung enzyme was 4 times more effective catalyst in hydrolysis of FA-Phe-Phe-Arg because the k_{cat} value was 2 times higher

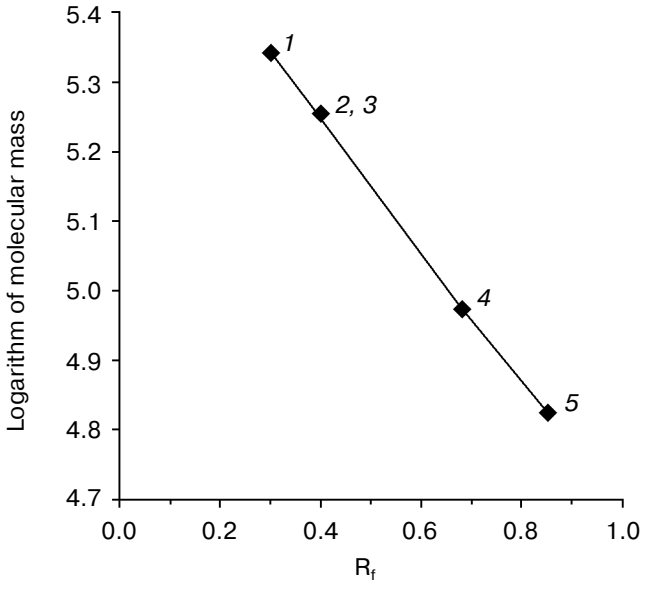

Fig. 1. Determination of molecular mass of the bovine atrial enzyme by electrophoresis under denaturing conditions: *1*) ferritin (monomer) (220 kD); *2*) bovine lung ACE (180 kD); *3*) bovine atrial ACE (180 kD); *4*) phosphorylase *b* (94 kD); *5*) albumin (67 kD).

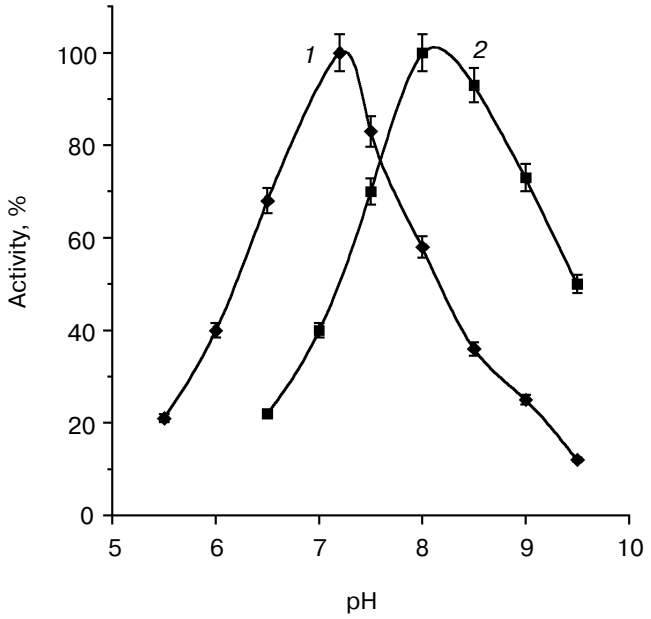

Fig. 2. The pH dependence of bovine atrial ACE. The reaction medium contained 20 mM Mes, 20 mM Hepes, 20 mM borate, 0.15 M NaCl, 1 μM $ZnCl_2$, and one of the substrates: 50 μM Cbz-Phe-His-Leu (*1*) or 50 μM Hip-His-Leu (*2*).

Table 2. Kinetic parameters of hydrolysis of synthetic substrates catalyzed by bovine lung and artial ACE at 25°C in reaction medium containing 50 mM Hepes, pH 7.5, 150 mM NaCl, and 1 μM $ZnCl_2$

Substrate	Atrial ACE			Lung ACE [31]		
	k_{cat}, sec^{-1}	K_m, mM	$k_{cat}/K_m \times 10^4$, $M^{-1}\cdot sec^{-1}$	k_{cat}, sec^{-1}	K_m, mM	$k_{cat}/K_m \times 10^4$, $M^{-1}\cdot sec^{-1}$
FA-Phe-Gly-Gly	315 ± 16	0.98 ± 0.04	32	280 ± 11	0.70 ± 0.03	40
FA-Phe-Phe-Arg	30 ± 1	0.100 ± 0.04	30	60 ± 3	0.050 ± 0.002	120
Cbz-Phe-His-Leu	100 ± 4	0.140 ± 0.05	71	58 ± 2	0.25 ± 0.01	23
Hip-His-Leu	6.5 ± 0.3	1.80 ± 0.07	0.36	12.0 ± 0.5	0.90 ± 0.04	1.3

and K_m 2 times lower than in atrial ACE. However, the latter hydrolyzed Cbz-Phe-His-Leu more effectively than lung ACE, which in turn was more effective a catalyst of Hip-His-Leu hydrolysis. In both cases different catalytic efficacy was due to differences in k_{cat} and K_m values. These data suggest the existence of some differences in substrate specificity of atrial and lung ACEs. It is possible that these differences can be explained by different glycosylation degree of ACE secreted in various organs. This seems likely because a recent study demonstrated [28] that change of this parameter in lung ACE influenced its substrate specificity.

Thiorphan and phosphoramidon were rather weak inhibitors of bovine atrial ACE (IC_{50} values were 0.1 and 1 μM, respectively), whereas the highly specific ACE inhibitors lisinopril and captopril effectively inhibited this enzyme. The K_i values were 0.6 ± 0.05 and 0.3 ± 0.02 nM, respectively. This is consistent with K_i values of 0.18 ± 0.02 and 0.6 ± 0.05 nM for inhibition of bovine lung ACE by lisinopril and captopril, respectively [21].

Activation of catalytic activity in the presence of anions (especially of chloride) is a characteristic feature of ACE. Figure 3 shows effects of chloride, nitrate, and sulfate on the activity of bovine atrial and lung ACE. It should be noted that even in the absence of added anions, the catalytic activities of the atrial and lung enzymes were different. If enzyme activity in the presence of 0.15 M NaCl is referred to as the maximally possible (100%); the "residual" activities of the lung and atrial enzymes determined in the absence of anions were 5 and 25% of the maximal activities.

Both enzymes were activated by chloride, and in both cases maximal activity was observed at 0.15 M NaCl. However, subsequent increase of NaCl concentration inhibited only the atrial ACE (Fig. 3a, curve *1*), whereas the activity of the lung enzyme remained unchanged (Fig. 3b, curve *1*). The inhibitory effect of high concentrations of chloride was earlier found for the bovine ACE N-domain [31] and human heart ACE [18]; in the latter case maximal enzyme activity was observed at 0.3-0.5 M salt concentration.

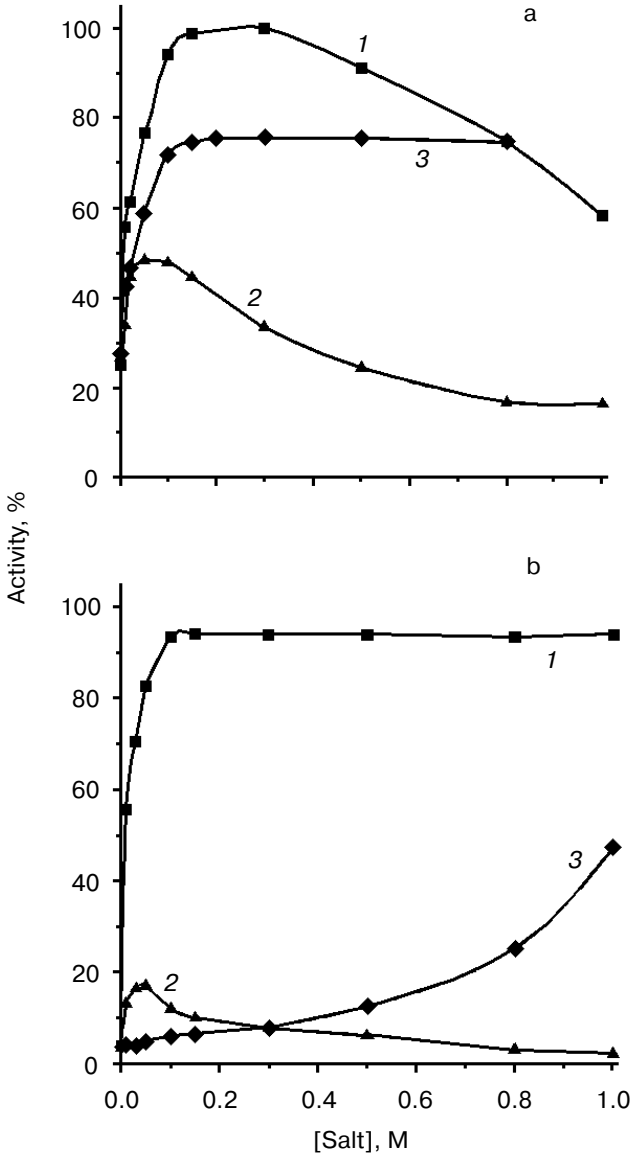

Fig. 3. Dependence of bovine atrial (a) and lung (b) ACE activity on salt concentration: *1*) NaCl; *2*) KNO_3; *3*) Na_2SO_4. The reaction medium contained 50 mM Hepes, pH 7.5, 1 μM $Zn(CH_3COO)_2$, 50 μM Cbz-Phe-His-Leu, and 0.1 nM ACE.

Nitrate exerted weaker effects on the activity of both enzymes (Fig. 3, curves *2*) than chloride. The maximal activation, observed at 0.1 M salt concentration, was only 20 and 50% (of maximally possible activity) for the lung and atrial enzymes, respectively. Increase of nitrate anion concentration was accompanied by inhibition of both enzymes. It should be noted that nitrate did not activate human heart ACE [16].

Activation of lung and atrial ACEs by sulfate was characterized by completely different concentration–response curves (Fig. 3, curves *3*). Lung ACE was insensitive to low sulfate concentration, and the activation profile was an abnormal curve with a pronounced lag; at 1 M salt concentration its activity was 50% of maximally possible [31]. Activation of atrial ACE by sulfate (70% of maximally possible) reached a plateau at 0.15 M and remained unchanged up to 1.0 M salt concentration. Activation of human heart ACE by sulfate was twofold higher than the activation by chloride [17]. In all cases the activity of bovine atrial dipeptidyl-carboxyhydrolase did not depend on the concentration and nature of the anion and was determined only by ionic strength of the reaction medium [19].

Thus, in the present study we have purified bovine atrial ACE to electrophoretic homogeneity. Some of its basic properties (substrate specificity, anion sensitivity) differ from those reported for ACE isolated from bovine lung and human heart. These differences obviously reflect tissue and species specificity of this enzyme.

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