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# ANGIOTENSIN CONVERTING ENZYME IN BRUSH-BORDER MEMBRANES OF AVIAN SMALL INTESTINE

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## SUMMARY

Angiotensin converting enzyme activity was identified in brush-border membranes purified from the small intestinal epithelium of the common grackle, *Quiscalus quiscula*. Angiotensin converting enzyme was enriched 20-fold in the membrane preparation, compared with intestinal epithelial cell scrapes, and was coenriched with the brush-border markers, alkaline phosphatase and aminopeptidase *N*. The kinetics of hydrolysis of *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG) gave a $V_{max}$ of $907 \pm 41$ units $g^{-1}$ and a $K_m$ of $55 \pm 6\,\mu mol\,l^{-1}$. The avian intestinal angiotensin converting enzyme was inhibited by the antihypertensive drug, Ramipril, with a median inhibitory concentration ($IC_{50}$) of $1\,nmol\,l^{-1}$. In the light of previous studies on angiotensin converting enzyme in mammalian epithelia, these results may implicate a physiological role for angiotensin converting enzyme in regulating electrolyte and fluid uptake in bird small intestines.

## INTRODUCTION

Angiotensin converting enzyme (ACE, kininase II, peptidyldipeptidase; E.C. 3.4.15.1) converts angiotensin I to angiotensin II, and hydrolyses a variety of bioactive peptides such as bradykinin and enkephalins. Historically ACE has been associated with pulmonary endothelial tissue and, more recently, with epithelial and neuroepithelial tissue in mammals (Defendini *et al.* 1983). Angiotensin converting enzyme's presence in the blood and lung has established it as an important physiological regulator of blood pressure *via* its control of vasoactive peptides (Yang, Erdos & Levin, 1971). Recently, however, ACE has been immunohistochemically identified as a major membrane-bound protein of the microvillus membrane of epithelial cells of mammalian small intestine and renal proximal tubule (Bruneval

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*et al.* 1986; Naim, Sterchi, Hauri & Lentze, 1985; Ward, Sheridan, Hammon & Erdos, 1980; Ward & Sheridan, 1983). It is notable that many of ACE's substrate peptides originate locally in the intestine (Tapper, 1983), and regulate the physiological events of electrolyte and fluid transport (Crocker & Munday, 1970; Davies, Munday & Parsons, 1970; Levens, 1986; Turnberg, 1983). Avian intestine is a critical site for absorption/resorption of ions and water (Crocker & Holmes, 1971), and thus ACE activity of avian enterocytes may play an important role in mediating the physiological status of the avian gastrointestinal tract. An alternative role for intestinal lumen ACE may involve oligopeptide digestion for the processing of nutrient nitrogen. In this study we have identified and partially characterized ACE activity in brush-border membranes purified from the small intestine of the grackle, *Quiscalus quiscula*. Furthermore, membrane ACE activity was probed using Ramipril, a potent and selective inhibitor of angiotensin converting enzyme (Becker, Scholkens, Metzger & Schultz, 1984; Bunning, 1984; Scholkens, Becker & Kaiser, 1984; Unger *et al.* 1986).

## MATERIALS AND METHODS

### *Purified brush-border membranes*

Small intestinal membrane vesicles were purified from intestinal mucosal scrapings obtained from adult common grackles *Quiscalus quiscula* of mean mass $90 \pm 6$ g ($N = 6$). Birds were killed using halothane. The small intestines were immediately excised, chilled in ice-cold 1·02% (w/v) saline, and then scraped of mucosa. One gram samples of mucosa from each bird were stored in cryotubes in liquid nitrogen, and used later to prepare brush-border membranes based on a $Ca^{2+}$ aggregation technique (Stevens, Ross & Wright, 1982; Stevens, Kaunitz & Wright, 1984). Briefly, each gram of mucosa was homogenized for 20 s, using a Polytron homogenizer (Brinkman, Westbury, NY) at setting no. 10, with 8 ml of buffer containing 350 mmol $l^{-1}$ mannitol and 1 mmol $l^{-1}$ Hepes/Tris, pH 7·5. $CaCl_2$ was added to the crude homogenate ($H_1$) to make a 10 mmol $l^{-1}$ solution, which was stirred for 20 min at 5 °C. This was centrifuged for 5 min at 1500 ***g***; the supernatant was collected and centrifuged again in the same manner. The second supernatant was centrifuged at 45 000 ***g*** for 30 min. The resulting pellet was homogenized (glass/Teflon) in buffer containing 350 mmol $l^{-1}$ mannitol and 10 mmol $l^{-1}$ Hepes/Tris, pH 7·5. This was then centrifuged at 45 000 ***g*** for 30 min. The final pellet containing brush-border membranes vesicles (BBMV) was homogenized in the 350 mmol $l^{-1}$ mannitol buffer to give a final protein concentration adjusted to 9·5 $\mu g\, \mu l^{-1}$. Protein concentration was determined using the Bio-Rad reagent (Bio-Rad, Richmond, CA). Samples of membranes were stored frozen in cryotubes in liquid nitrogen.

The brush-border marker enzymes, alkaline phosphatase and aminopeptidase N were assayed by previously published methods (Stevens, Kempner & Wright, 1986; Hanna, Mircheff & Wright, 1979).

*Assay for angiotensin converting enzyme*

Measurement of angiotensin converting enzyme activity was based on the hydrolysis of *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG). FAPGG is a well-established definitive substrate for angiotensin converting enzyme (Bunning, Holmquist & Riordan, 1983; Holmquist, Bunning & Riordan, 1979).

Brush-border membranes were thawed in the cryotubes at 22°C, and a 95-$\mu$g sample of membrane protein was preincubated for 30 min in a plastic cuvette containing 50 mmol $l^{-1}$ Hepes/Tris, pH 7·5, 300 mmol $l^{-1}$ NaCl, 10 $\mu$mol $l^{-1}$ $ZnCl_2$ and 0·2 % (w/v) polyoxyethylene-9-lauryl ether detergent. The reaction was started at time zero by adding the same buffer also containing (final reaction concentration, pH 7·5): FAPGG (0·01–1·5 mmol $l^{-1}$), Ramipril-diacid (0–40 $\mu$mol $l^{-1}$) and NaOH (19 $\mu$mol $l^{-1}$). The Ramipril-diacid was added from a stock dissolved in 0·1 mol $l^{-1}$ NaOH; control reactions contained the same amount of NaOH lacking Ramipril-diacid.

Enzyme activity at 22°C was based on the initial linear rate of change in absorbance ($\Delta$A) recorded using a digital spectrophotometer (model DU-7HS, Beckman Instruments, Irvine, CA) with a sampling rate of 10 Hz. Data were corrected for the slight spontaneous self-hydrolysis of FAPGG in the blank (buffer with no protein). Hydrolysis was monitored at 328 nm using a cuvette with either a 1·0 cm light path (for [FAPGG] $\leqslant$ 100 $\mu$mol $l^{-1}$) or a 0·1-cm light path (for [FAPGG] $\geqslant$ 100 $\mu$mol $l^{-1}$). The FAPGG hydrolysis rate (specific activity = units g protein$^{-1}$ = $\mu$mol min$^{-1}$ g protein$^{-1}$) in the 1-ml sample was calculated using the equation:

$$\text{units g}^{-1} = \Delta A/(\Delta\epsilon \text{ min g}),$$

where we determined that $\Delta\epsilon = 2764$ mol $l^{-1}$ cm$^{-1}$ in our reaction mixture. $\Delta\epsilon$ represented the measured difference between the molar extinction coefficients for FAPGG substrate and furylacryloylphenylalanine product at 328 nm (Holmquist *et al.* 1979).

*Hydrolysis kinetics*

The kinetics of FAPGG hydrolysis was measured using a concentration range from 10 $\mu$mol $l^{-1}$ to 1·5 mmol $l^{-1}$. The data were fitted to the Michaelis–Menten equation (Segel, 1975):

$$V = \frac{V_{max}[\text{FAPGG}]}{K_m + [\text{FAPGG}]},$$

and the kinetic parameters were solved simultaneously by a Gauss–Newton nonlinear regression computer program (Stevens & Wright, 1987).

Ramipril (Hoe-498) manufactured by Hoechst AG, Frankfurt, FRG, was kindly supplied by M. I. Phillips of the Department of Physiology, University of Florida. Ramipril was always used in the diacid form. All other reagents were obtained from Sigma Chemical Co. (St Louis, MO).

## RESULTS

### *Activity of angiotensin converting enzyme*

The activity of angiotensin converting enzyme of grackle small intestinal brush-border membranes is illustrated in Fig. 1. The time-dependent decrease in absorbance indicates that first-order hydrolysis of FAPGG substrate occurred during the measurement period. In this example $50\,\mu mol\,l^{-1}$ FAPGG was hydrolysed at $280\,\mu mol\,min^{-1}\,g^{-1}$. Fig. 1 also demonstrates that ACE activity was arrested when Ramipril-diacid was added to the buffer at time zero. Subsequent experiments were based on measurements made in this manner.

### *Co-purification of ACE with brush-border marker enzymes*

The grackle intestinal ACE activity was 20 times higher in the brush-border membrane preparation than in the corresponding mucosal/enterocyte scrapes, as demonstrated in Table 1. Also shown in Table 1 are the activities and enrichments for the brush-border membrane marker enzymes, alkaline phosphatase and amino-peptidase *N*. The coenrichment of ACE and marker enzymes in the final BBMV pellet indicated that ACE was bound to the brush-border membrane.

### *Hydrolysis kinetics*

The enzyme hydrolysis kinetic parameters were estimated by a Gauss–Newton nonlinear regression, giving a maximal velocity of the reaction, $V_{max}$, of $907 \pm 41\,units\,g^{-1}$, and an apparent Michaelis–Menten constant, $K_m$, of

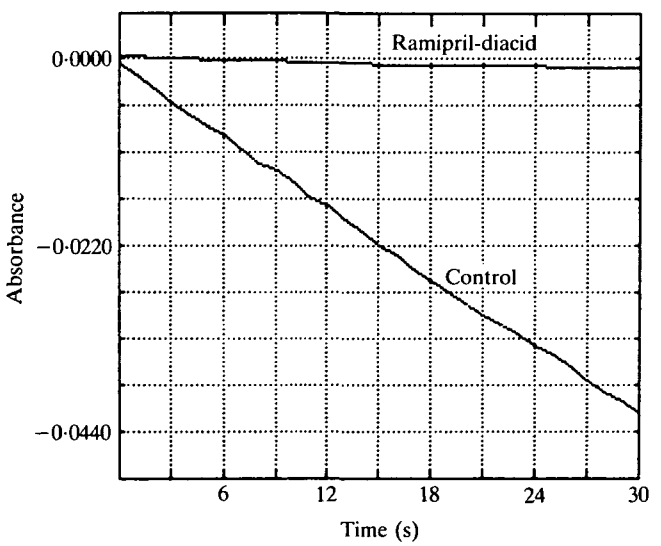


Fig. 1. Hydrolysis of $50\,\mu mol\,l^{-1}$ FAPGG substrate by angiotensin converting enzyme in grackle small intestinal purified brush-border membranes. Reaction conditions are outlined in the text; the Ramipril-diacid concentration was $40\,\mu mol\,l^{-1}$. In this example, the control rate (no Ramipril) was $280\,\mu mol\,min^{-1}\,g^{-1}$ measured at 22 °C. The rate in the presence of Ramipril was zero, after correcting for the self-hydrolysis of FAPGG.

Table 1. *Co-enrichment of avian brush-border membrane vesicle (BBMV) enzymes compared with mucosal crude homogenates ($H_1$)*

| | Enzyme activity ($\mu$mol min$^{-1}$ g$^{-1}$) | | |
|---|---|---|---|
| | Angiotensin converting enzyme* | Alkaline phosphatase† | Aminopeptidase N‡ |
| BBMV | 336 ± 46 (7) | 1100 ± 30 (3) | 122 ± 4 (3) |
| $H_1$ | 17 ± 1 (7) | 40 ± 1 (3) | 8 ± 1 (3) |
| Enrichment | 20-fold | 28-fold | 15-fold |

Values are mean ± S.E. (number of experiments) measured at 22°C.
Substrate concentrations: *FAPGG (50 $\mu$mol l$^{-1}$); †*p*-nitrophenylphosphate (5 mmol l$^{-1}$); ‡alanine-*p*-nitroanilide (1 mmol l$^{-1}$).

55 ± 6 $\mu$mol l$^{-1}$ FAPGG. The nonlinear regression analysis also indicated that only one enzyme species existed in our system. When the data were plotted in an Eadie–Hofstee plot (Fig. 2), the resulting single linear relationship indicated that one species of ACE enzyme was responsible for the hydrolysis of FAPGG (Segel, 1975). Linear regression of the Eadie–Hofstee data (Fig. 2) yielded the same values as the nonlinear analysis.

### *Ramipril inhibition*

Ramipril served as a highly potent and selective pharmacological probe of ACE activity (Becker *et al.* 1984; Bunning, 1984; Scholkens *et al.* 1984; Unger *et al.* 1986). Ramipril-diacid strongly inhibited grackle intestine brush-border ACE

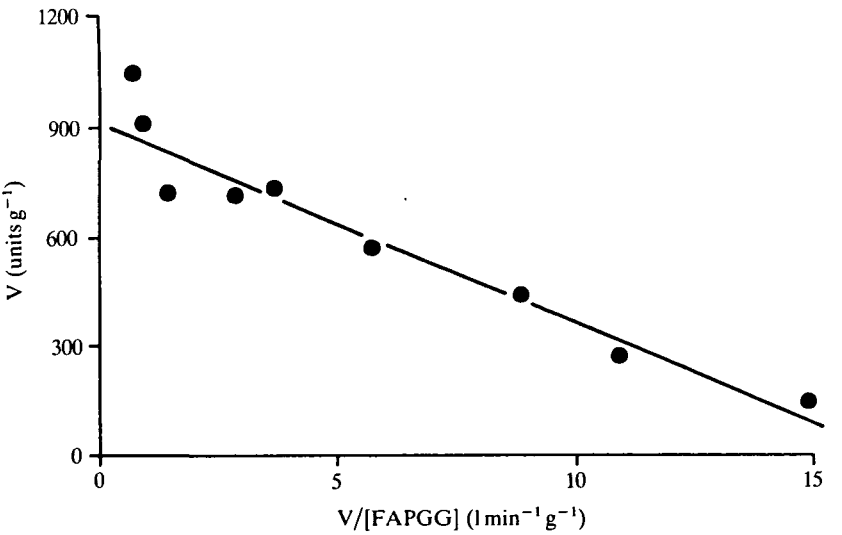


Fig. 2. Eadie–Hofstee plot of hydrolysis of FAPGG by grackle intestinal angiotensin converting enzyme at 22°C. $V_{max}$ is 907 ± 41 units g$^{-1}$ and $K_m$ is 55 ± 6 $\mu$mol l$^{-1}$ FAPGG, determined by computer nonlinear regression analysis based on nine points, where each point is the mean of three experiments.

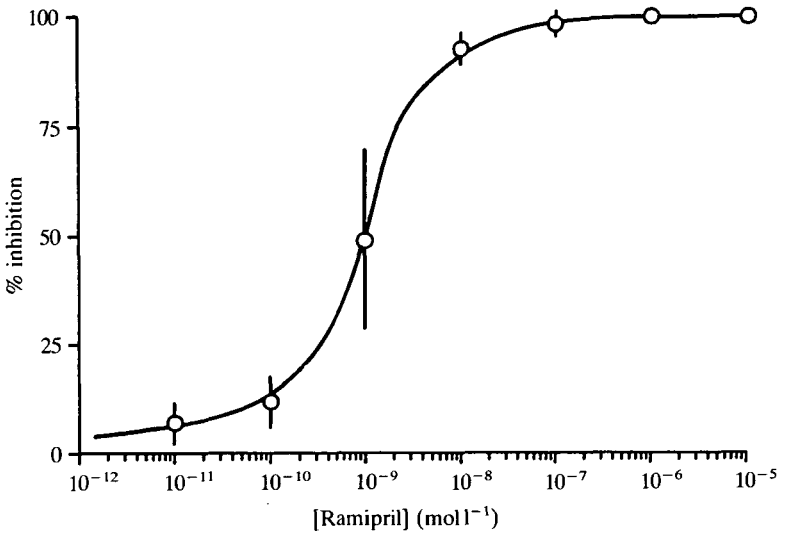


Fig. 3. Inhibition of activity of grackle brush-border membrane angiotensin converting enzyme by Ramipril-diacid at pH 7·5. The data represent the percentage inhibition of activity compared with control (no Ramipril) activity; points are mean ± S.E. ($N = 3$). FAPGG substrate concentration was 50 $\mu$mol $l^{-1}$. $IC_{50}$ was 1 nmol $l^{-1}$ Ramipril-diacid.

activity in a log dose-dependent manner (Fig. 3), with a median inhibitory concentration ($IC_{50}$) of 1 nmol $l^{-1}$; the activity was completely inhibited by 40 $\mu$mol $l^{-1}$ Ramipril (Figs 1, 3).

## DISCUSSION

Angiotensin converting enzyme is a multifunctional enzyme which classically has been studied for its vasoactive properties. However, recent immunohistochemical studies have confirmed the prominence of ACE on epithelial brush-border membranes of mammalian kidney, choroid plexus and small intestine (Bruneval *et al.* 1986; Naim *et al.* 1985; Ward *et al.* 1980; Ward & Sheridan, 1983). The results of the present study using an avian intestinal preparation of enterocyte brush borders corroborate the immunohistochemical work conducted on mammalian tissues.

ACE predominately converts angiotensin I to angiotensin II, inactivates bradykinin and hydrolyses enkephalins (Yang *et al.* 1971). These peptides originate locally in these tissues, where they modulate salt and water transport (Bolton, Munday, Parsons & York, 1975; Crocker & Munday, 1970; Gaginella, 1984). Avian intestine is an important site for ion and water transport (Crocker & Holmes, 1971; Holmes & Phillips, 1985; Duke, 1986), and therefore epithelial membrane-bound ACE may play an important role in controlling the bioactive peptides which affect the physiological status of the gut. The present study indicates that the avian small intestinal epithelial brush-border membranes possess prominent ACE activity which is strongly inhibited by the ACE inhibitor, Ramipril.

Avian intestinal membrane-bound ACE displays efficient kinetic properties, compared with its mammalian lung counterpart. The lung has long been recognized as a source of ACE (Erdos & Skidgel, 1987). Utilizing FAPGG as a substrate, the avian intestinal $K_m$ value of 55 $\mu$mol $l^{-1}$ (Fig. 2) is an order of magnitude lower than the $K_m$ of 300 $\mu$mol $l^{-1}$ measured for rabbit lung ACE (Bunning *et al.* 1983; Holmquist *et al.* 1979; Bunning, 1984). This suggests that ACE in avian intestine may be better adapted than in mammalian lung to control bioactive peptides. We utilized FAPGG as the definitive synthetic substrate for ACE, because FAPGG demonstrates a greater selectivity and turnover efficiency than hippuryl-His-Leu, angiotensin I or bradykinin (Bunning *et al.* 1983).

The avian intestinal BBMV ACE was strongly inhibited by Ramipril (Fig. 1), a selective and potent ACE inhibitor originally developed as an orally administered antihypertensive drug (Becker *et al.* 1984; Bunning, 1984; Scholkens *et al.* 1984; Unger *et al.* 1986). Ramipril in the present study served as a definitive probe for ACE activity in the membranes. The $IC_{50}$ value of 1 nmol $l^{-1}$ (Fig. 3) is similar to the $IC_{50}$ of 3 nmol $l^{-1}$ found for Ramipril inhibition of plasma ACE (Unger *et al.* 1986), and similar to the competitive binding $K_i$ of 10 nmol $l^{-1}$ for rabbit lung ACE (Bunning, 1984).

In conclusion, we have shown that purified avian small intestinal brush-border membranes possessed considerable angiotensin converting enzyme activity, as demonstrated by hydrolysis of the ACE synthetic substrate FAPGG. The activity was strongly inhibited by nanomolar concentrations of Ramipril. The results suggest that bird intestinal ACE has some properties in common with its mammalian pulmonary vascular counterpart. Intestinal ACE may serve the avian intestine by controlling endogenous bioactive peptides which regulate electrolyte and water fluxes, or it may serve a digestive role in hydrolysing luminal nutrient oligopeptides which possess penultimate *N*-terminal prolyl residues.

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