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ENZYMIC ACTIVITIES OF THE BRUSH BORDER MEMBRANE OF RAT INTESTINE HYDROLYZING β-NAPHTHYLAMIDES OF AMINO ACIDS, LEUCINAMIDE AND DIPEPTIDES

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SUMMARY

Brush border membranes isolated from rat intestine hydrolyzed β-naphthylamides of many amino acids, L-leucinamide and L-phenylalanyl–L-alanine with high specific activities.

These enzymic activities, appearing to be intrinsic to that subcellular fraction, were characterized as regards pH optimum, ion dependence and puromycin inhibition.

Only the enzyme hydrolyzing γ-L-glutamyl-β-naphthylamide was not activated by metal ions, or inhibited by puromycin; after papain solubilization, it was separated by gel filtration on Sephadex G-200 from the enzyme(s) responsible for the hydrolysis of the other studied substrates.

INTRODUCTION

Intestinal mucosa, as well as other tissues, contains several enzymes (arylamidases) which are able to hydrolyze β-naphthylamides of leucine and other amino acids[1–5]. L-Leucyl-β-naphthylamidase activity of the small intestine is predominantly but not exclusively localized in the brush border of the enterocyte[6–9]. In contrast, only a small percentage of the enzymic activities of intestinal mucosa which hydrolyze dipeptides is present in the brush border region[10–11].

The present note describes some properties of the enzymic activities of the brush border membrane of rat intestine hydrolyzing amino acyl-β-naphthylamides, L-leucinamide and dipeptides. Solubilization of these enzymic activities by papain digestion and separation of γ-glutamyl transpeptidase activity from the other arylamidase and peptidase activities of the membrane are also reported.

EXPERIMENTAL

Enzyme preparation

Total homogenates of intestinal mucosa, crude and purified brush borders, and microvillus membranes were prepared according to Forstner *et al.*[9]. Membrane

preparations were found to be homogeneous by electron microscopic examination. All enzymic activities were determined on brush border membranes unless otherwise indicated. The enzymic activities of intestinal lumen were measured according to Rhodes *et al.*[10].

Enzyme assays

The incubation mixture for the assay of arylamidase activities contained in a total volume of 200 μl: (1) substrate, at the level of 0.2 μmole of α-L-glutamyl- and γ-L-glutamyl-β-naphthylamide, 0.025 μmole of L-seryl-L-tyrosyl-β-naphthylamide, and 0.04 μmole of the β-naphthylamides of the other amino acids. (2) 4 μmoles of phosphate–acetate–borate buffer[12] at the optimal pH, when known, and at pH 8.0 for all other substrates. (3) Enzyme solution, and other components when indicated. Incubation was carried out at 37 °C for 30 and 60 min and the amount of β-naphthylamine liberated was determined by the procedure of Goldbarg and Rutenburg[13].

Dipeptidase activities and the activity hydrolyzing L-leucinamide were determined according to Auricchio *et al.*[14]. The assay mixture for the activity hydrolyzing L-phenylalanyl-L-alanine contained 2 μmoles of dipeptide instead of 0.4 μmole, in a total volume of 100 μl.

Incubation mixtures were at pH 8.0 for the hydrolysis of L-leucylglycine and at the optimal pH for the hydrolysis of the other substrates.

A unit of enzymic activity hydrolyzes 1 μmole of substrate per minute.

All enzymic activities were proportional to the incubation time and to the enzyme concentration when the assay mixtures contained 1 to 6 munits of peptidase and 0.2 to 1 munit of arylamidase activity.

The pH optima for the hydrolysis of the substrates were found to be: 6.9 for L-phenylalanyl-β-naphthylamide, and α-L-glutamyl-β-naphthylamide, 7.4 for L-leucyl-β-naphthylamide, 8.1 for L-lysyl-β-naphthylamide, 8.6 for γ-L-glutamyl-β-naphthylamide, 6.9 for L-phenylalanyl-L-alanine, and 7.9 for L-leucinamide.

The Michaelis constant for the hydrolysis of L-phenylalanyl-L-alanine was 9.5 mM.

Sucrase activity was measured by the method of Auricchio *et al.*[15].

Protein concentration

This was estimated by the method of Lowry *et al.*[16], with bovine albumin as a standard.

Reagents

Substrates and reagents used for the assays of peptidase and arylamidase activities were described in a previous report[14]. Crystalline papain was obtained from Mann Biochemicals, New York, N.Y., U.S.A., and Sephadex G-200 from Pharmacia, Uppsala, Sweden.

RESULTS AND DISCUSSION

Puromycin inhibition

1 mM puromycin in the incubation mixture reduced by more than 80 % the arylamidase activities. Only γ-glutamyl transpeptidase activity was unaffected.

Thiol and metal dependence

0.3 mM EDTA in the incubation mixture reduced by 90 % or more the arylamidase and peptidase activities. Only the hydrolysis of γ-L-glutamyl-β-naphthylamide was unaffected by the presence of EDTA, 0.3 to 2.5 mM. Mg^{2+}, Co^{2+}, Zn^{2+} and Mn^{2+} as chlorides in concentrations in the incubation mixture ranging from 1 to 0.01 mM, as well as 2-mercaptoethanol in concentrations ranging from 2.5 to 50 mM, did not enhance arylamidase and peptidase activities of the membranes.

In order to demonstrate an activating effect of metal ions on amino acyl-β-naphthylamides, L-leucinamide and dipeptides hydrolysis, it was necessary to incubate the membrane preparation at 37 °C for 1 h in 10 mM EDTA and 80 mM phosphate–acetate–borate buffer, pH 7.2. A dialysis against 10 mM EDTA pH 7.4 was carried out at 4 °C for 24 h, followed by a second dialysis against water for a similar period of time. After this treatment more than 50 % of the enzymic activities disappeared, with the sole exception of γ-glutamyl transpeptidase activity, which was unaffected.

Co^{2+}, Ca^{2+}, Mn^{2+} or Zn^{2+} in a concentration of 1 mM fully restored the enzymic activities. No or very small activating effect was demonstrable for Mg^{2+}.

The results on ion activation are consistent with those of Rhodes *et al.*[10] on the enzymic activity of brush border membrane of hamster intestine hydrolyzing L-leucyl-β-naphthylamide and L-leucylglycine and with those of Emmelot and Visser[17] on the aminopeptidase(s) of rat liver plasma membranes.

TABLE I

HYDROLYSIS OF β-NAPHTHYLAMIDES OF DIFFERENT AMINO ACIDS BY BRUSH BORDER MEMBRANE

The enzymic activities are given as percentages of the activity hydrolyzing L-alanyl-β-naphthylamide.

Substrate	*Relative activity* *	
	Mean	*Range*
At pH 8.0		
L-Ala-β-naphthylamide	100.0	(100–100)
L-Leu-β-naphthylamide	48.3	(44–56)
L-Phe-β-naphthylamide	18.3	(17–20)
L-Lys-β-naphthylamide	8.6	(8–10)
α-L-Glu-β-naphtylamide	12.0	(6–16)
γ-L-Glu-β-naphthylamide	9.6	(8–12)
L-His-β-naphthylamide	4.0	(3–5)
L-Ile-β-naphthylamide	2.6	(2–3)
L-Tyr-β-naphthylamide	25.6	(20–30)
L-Val-β-naphthylamide	1.3	(0.8–2)
L-Ala–L-Ala-β-naphthylamide	31.3	(20–38)
L-Cystine-bis-β-naphtylamide	0.012	(0–0.02)
L-Ser–L-Tyr-β-naphthylamide	12.6	(9–17)
At optimal pH		
L-Leu-β-naphthylamide	58.3	(46–69)
L-Phe-β-naphthylamide	80.0	(72–88)
α-L-Glu-β-naphthylamide	32.3	(23–38)
γ-L-Glu-β-naphthylamide	8.0	(6–9)

* The specific activities of the three different preparations of brush border membrane for the hydrolysis of L-alanyl-β-naphthylamide were 2.03, 2.36 and 2.26, respectively.

The activating effect of Co^{2+} recalls the properties of the particle bound renal aminopeptidase of the rabbit[18,19]. Furthermore, this enzyme, probably localized in the brush border of the cells of the proximal tubule[20], hydrolyzes L-alanyl-β-

TABLE II

SPECIFIC ACTIVITY FOR THE ENZYMIC HYDROLYSIS OF AMINO ACYL-β-NAPHTHYLAMIDES, L-LEUCINAMIDE AND L-PHENYLALANYL-L-ALANINE IN SUBCELLULAR FRACTIONS OF RAT INTESTINE

Expt No.	*Substrate*	*Units of enzymic activity per mg of protein* *			
		Homogenate	*Crude brush border*	*Purified brush border*	*Membrane*
1	Sucrose	0.086	0.66	1.30	2.34
	L-Leu-β-naphthylamide	0.049	0.42	0.81	1.37
	L-Lys-β-naphthylamide	0.021	0.047	0.14	0.10
	L-Phe-β-naphthylamide	0.052	0.55	1.21	1.81
2	Sucrose	0.077	0.420	2.00	2.56
	γ-L-Glu-β-naphthylamide	0.0035	0.0181	0.088	0.120
3	Sucrose	0.099	0.783	1.26	1.97
	α-L-Glu-β-naphthylamide	0.090	0.223	0.297	0.468
4	Sucrose	0.075	0.704	1.23	2.00
	L-Leucinamide	0.060	0.350	0.563	0.834
5	Sucrose	0.029	0.493	0.740	1.31
	L-Phe–L-Ala	0.237	2.233	3.500	6.714

* The recovery of sucrase of the total homogenate ranged in 5 different preparations between 43 and 77% (mean = 57.4%) for the crude brush border, 21 and 38% (mean = 29.0%) for the purified brush border, and 16 and 25% (mean = 20.2%) for the membrane. The recovery of the activity of the total homogenate hydrolyzing γ-L-glutamyl-β-naphthylamide was in two different experiments 41 and 42% for the crude brush border, 27 and 19% for the purified brush border, and 18 and 13% for the membrane.

TABLE III

SOLUBILIZATION OF ENZYMIC ACTIVITIES OF BRUSH BORDER MEMBRANE

Substrate	*Enzymic activity (% of original)* *	
	Total amount after papain digestion	*Soluble amount*
L-Leucyl-β-naphthylamide	88	67
L-Phenylalanyl-β-naphthylamide	110	99
L-Lysyl-β-naphthylamide	73	67
α-L-Glutamyl-β-naphthylamide	60	45
γ-L-Glutamyl-β-naphthylamide	98	87
L-Leucinamide	87	80
L-Leucylglycine	80	60
L-Phenylalanyl-L-alanine	64	53
Sucrose	105	103

* Mean values of four different experiments. The activities of the papain solubilized enzymes were assayed in the presence of 1 mM $CoCl_2$ with the exception of the assay mixture for the enzymic activity hydrolyzing γ-L-glutamyl-β-naphthylamide, which contained no metal ions.

naphthylamide more rapidly than L-leucyl-β-naphthylamide[18,19], a result similar to our observations with intestinal microvillus membranes (see Table I).

Subcellular localization

All specific activities increased from the crude brush border to the microvillus membrane, as did sucrase activity, which was also determined as a marker of the latter fraction (see Table II). Furthermore, in the lumen the specific activity for the hydrolysis of all substrates was lower than 1 % of that of the brush border membrane.

Isolated microvillus membrane of rat intestine has a high specific activity for the hydrolysis of L-leucinamide and L-phenylalanyl-L-alanine (see Table II). The enzymic hydrolysis of the dipeptide, assayed in optimal conditions, shows a specific

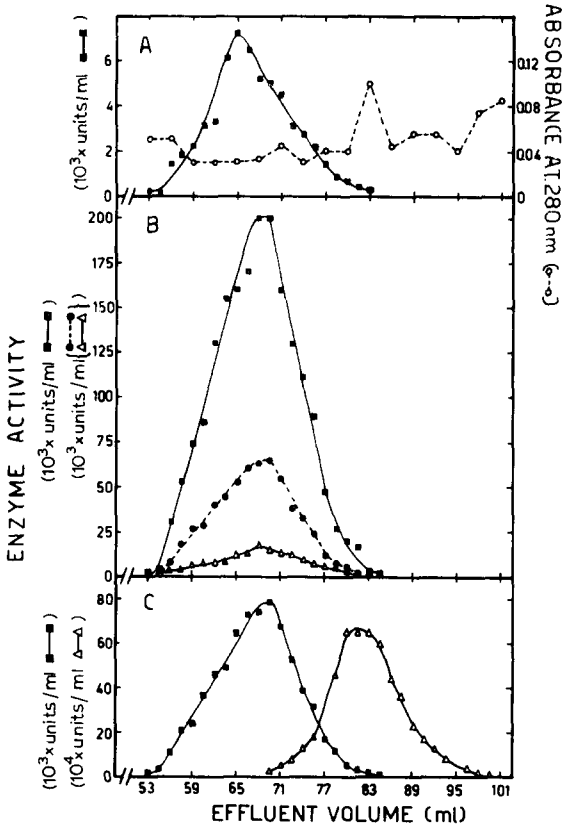

Fig. 1. Gel filtration pattern on Sephadex G-200 column (80 cm × 1.8 cm) of the enzymic activities of the brush border membrane hydrolyzing (A) L-lysyl-β-naphthylamide (■—■); (B) L-leucylglycine (△—△), L-phenylalanyl-L-alanine (■—■), L-leucinamide (●---●); (C) L-leucyl-β-naphtylamide (■—■), γ-L-glutamyl-β-naphthylamide (△—△). The enzymic activities hydrolyzing α-L-glutamyl- and L-phenylalanyl-β-naphthylamide were eluted together with L-leucyl-β-naphthylamidase activity and the ratio between them was found to be constant in the various fractions. 3.5 mg of membrane protein were incubated at 37 °C for 10 min with 0.75 mg of crystalline papain and 1 mg of cysteine in 2 ml of 0.1 M potassium phosphate buffer, pH 7.4. After centrifugation at 105000 × *g* for 90 min, approx. 1.5 ml of the supernatant were applied on the Sephadex G-200 column equilibrated with 10 mM potassium phosphate buffer (pH 7.4). The column was developed in the cold room with the same phosphate buffer at a flow rate of 9 ml/h. 1.5-ml fractions were collected.

activity 4 to 5 times higher than that of sucrase. This suggests that the intestinal hydrolysis of some dipeptides may be a physiological function of the microvillus membrane[10].

Papain solubilization and gel filtration

Papain solubilized arylamidase and peptidase activities of the brush border membrane (see Table III). Gel filtration on Sephadex G-200 of these solubilized enzymes separated a first activity peak containing all tested arylamidase and peptidase activities from a second one containing only γ-glutamyl transpeptidase activity (Fig. 1).

In conclusion, in the brush border membrane: (1) peptidase activities showing high specific activities may be involved in the terminal stages of the digestion of proteins. (2) The enzyme hydrolyzing γ-L-glutamyl-β-naphthylamide is different from that (or those) responsible for the hydrolysis of the α-amino acyl-β-naphthylamides, L-leucinamide, L-leucylglycine, and L-phenylalanyl-L-alanine. In fact, the former activity is not enhanced by metal ions, not inhibited by puromycin and is separable from the other studied arylamidase and peptidase activities by gel filtration on Sephadex G-200 of papain solubilized enzymes.

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