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New Synthetic Fluorescent Substrate for Histochemical Localization of the Enzyme Dipeptidyl Peptidase II on the Base of 1,8-Naphthalimides

R. Todorova, I. Ivanov, M. Dimitrova*

Institute of Experimental Morphology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia
** Faculty of Biology, St. Kl. Ohridsky University of Sofia, Sofia*

A novel fluorescent substrate L-Lys-L-Ala-6-hydrazido-N-hexyl-1,8-naphthalimide for the histochemical visualization of activity distribution of Dipeptidyl peptidase II is proposed. By now, a fluorescent determination of the DPP II activity is realized only in cell homogenates and cultivated cells, whereas fluorescent method for the enzyme localization in tissue sections is missing. The results of our work show that the new method permits precise localization and visualization of dipeptidyl peptidase II in tissue sections from several Wistar rat organs.

Key words: Dipeptidyl peptidase II, 1,8-naphthalimide derivatives, fluorescent histochemical methods, synthetic fluorescent substrates.

Introduction

Dipeptidyl peptidase II (DPP II; EC 3.4.14.2) belongs to serine peptidases family. Biochemical investigations show the lysosomal localization of the enzyme. Preferably, DPP II catalyzes the liberation of dipeptides, with lysine, phenylalanine or leucine at the N-terminal, and alanine or proline at penultimate position [2] from tripeptides, oligopeptides and from synthetic substrates at pH 5.5. The enzyme activity is inhibited by protonated Tris [3].

Here we propose a novel histochemical fluorescent substrate L-Lys-L-Ala-6-hydrazido-N-hexyl-1,8-naphthalimide (Lys-Ala-HHNI) for Dipeptidyl aminopeptidase II and use the substrate, based on 1,8-naphthalimide compound, to investigate the distribution of the enzyme activity in rat kidney, small intestine, colon and epididymis.

Materials and Methods

*Synthesis of N-hexyl-6-hydrazino-1,8-naphthalimide(HHNI).*The fluorochrome HHNI was synthesized as follows: Acenaphthene (Merck, Germany) was brominated by N-

bromo succinimide in dimethylformamide. The obtained 6-bromo acenaphthene was oxidized to 6-bromonaphthalanhydride by a standard procedure with sodium dichromate in acetic acid. The last compound was coupled with hexylamine by boiling it in absolute ethanol for 12 hours to give 6-bromo-N-hexyl-1,8-naphthalimide. The fluorochrome — HHNI was obtained from the brom-containing compound and hydrazine monohydrate in dimethylsulphoxide at 60°C using potassium fluoride as auxiliary reagent and tetrabutyl ammonium sulfate as a catalyst by a novel procedure, which will be published elsewhere. *Synthesis of the substrate.* The DPP II substrate — L-Lys-L-Ala-6-hydrazido-2-hexyl-1,8-naphthalimide (Lys-Ala-HHNI) was synthesized in two steps: First HHNI was coupled with Boc-Ala-OH (Bachem, Switzerland) by the well known N,N,N',N'-Tetramethyl-O-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU) procedure. The Boc-protection was cleaved in 4N HCl/dioxane. Then the obtained L-Ala-HHNI.HCl was coupled with Boc-Lyz(Boc)-OH (Bachem, Switzerland) again by the TBTU-method. The Boc-protection was cleaved as above and the substrate was obtained as dihydrochloride salt.

Tissue treatment and incubation. Mature male Wistar rats were decapitated under ether anesthesia. Pieces of kidney, small intestine, colon and epidydimis were removed and fixed in 2% formaldehyde in 0.1 M cacodylate buffer, pH 7.0 for 18 hrs. at +4°C. After that, the pieces were washed in Holt solution for 24 hrs. and frozen in liquid nitrogen. Ten μM sections were cut on cryotome Reichert Jung (Nussloch, Germany) at —25°C and mounted on gelatinized glass slides. The sections were covered with 0,5 % celloidin for one minute at room temperature and incubated in solutions, consisting of 0,5 mM substrate (Lys-Ala-HHNI) and 1 mg/ml aromatic aldehyde (piperonal), dissolved in 0.1 M phosphate buffer, pH 5.5. The incubation lasted 30-60 min at 37°C. The samples were post-fixed in 4 % neutral formaline and embedded in glycerol-jelly. Control sections were incubated in buffered aldehyde (in the absence of substrate) or in the full substrate medium supplied with 50 mM Tris.

The sections were studied under fluorescent microscope OPTON IM 35 with filter combination G 546 FT 580 LP 590. The photos were made on Konica Minolta (Japan) VX 200 colorful films.

Results and Discussion

DPP II is thought to play important role in the breakdown of some oligopeptides such as substance P and casomorphin [1]. The enzyme has certain requirements to substrate peptide sequence. It hydrolyzes Lys-Pro and Ala-Pro peptide derivatives, but shows higher specificity to Lys-Ala substrates. Besides, Dipeptidyl peptidase IV co-reaction is avoided by using Lys-Ala substrates. Therefore, we synthesized Lys-Ala derivative of 6-hydrazino-N-hexylamine-1,8-naphthalimide as fluorescent substrate for DPP II.

By newly developed substrate and histochemical method based on it, we managed to determine DPP II activity in rat kidney, small intestine (duodenum, jejunum, ileum), colon and epididymis. Non-specific staining was not observed in control sections, lacking the substrate. Single fluorescent granules were scare in the sections, inhibited by Tris.

For kidney, short incubation period was needed. The enzyme was localized in epithelial cells of the convoluted tubules. There was no reaction in the glomeruli (Fig. 1). We found that, the DPP II activity was higher in the convoluted tubules near to the medulla than in this, which are far off it. DPP II showed high activity levels in small intestine too. We did not observe enzyme activity differences between duodenum, jejunum and ileum. DPP II was localized in the entherocites (Fig. 2). At prolonged incubation, there was a weak Dipeptidyl peptidase IV co-reaction in ileum

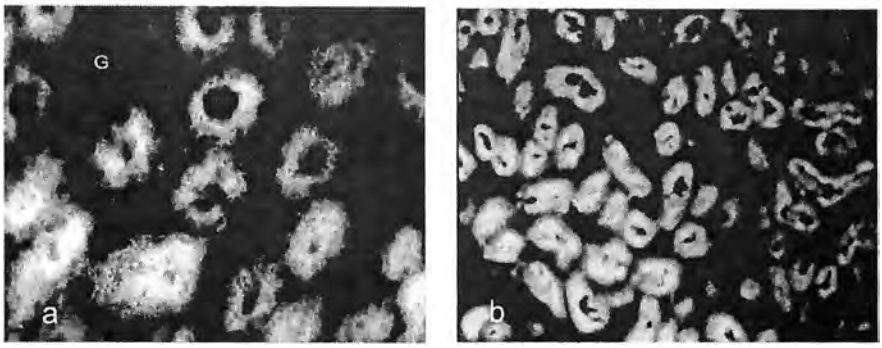

Fig. 1. Kidney. DPP II activity in epithelial cells of convoluted channels
a — No DPP II activity in glomeruli (G), × 500; b — Higher activity in the convoluted channels near to the medulla, × 200

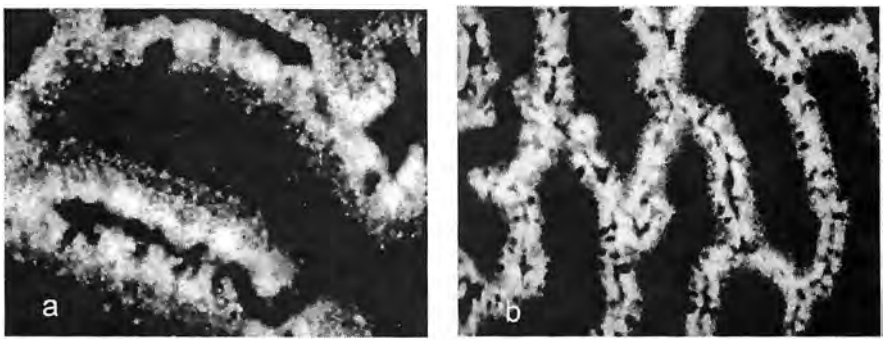

Fig. 2. Small intestine (jejunum). Strong reaction for DPP II in the entherocytes.
a — × 500; b — × 200

Fig. 3. Epididymis. DPP II in the principal and basal cells of the channels. × 500

(not shown here). Dipeptidyl peptidase II reaction in colon was missing. The DPP II activity was comparatively lower in epididimys and the incubation period had to be longer (60 min). Granular histochemical pattern which, probably corresponds to lysosomal localization of the enzyme was obtained. DPP II activity was observed exactly in the principal and basal cells of the channels (Fig. 3).

The results of our work demonstrate that 6-hydrazino-N-alkyl-1,8-naphthalimides are proper for substrate synthesis, and histochemical method based on them is appropriate for localization of the lysosomal peptidase DPP II. The newly proposed fluorescent histochemical substrate for DPP II can be used for further investigations of the enzyme activity distribution.

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References

1. Hisazumi, A., L. Yao-Hua, Y. Yamamoto, Y. Haneda, M. Nishi, R. Kikkawa. Immunohistochemical Localization of Dipeptidyl Peptidase II from the Rat Kidney and Its Identity with Quiescent Cell Proline Dipeptidase. — J. Biochem., **129**, 2001, 279-288.
2. Lojda, Z., R. Gossrau, T. H. Schiebler. A laboratory manual. — In: Enzyme Histochemistiy. New York, Springer, 1979, 169-170.
3. Maes, M., A. Lambeir, K. Gilany, K. Senten, P. Van der Veken, B. Leiting, K. Augustyns, S. Scharpé, I. De Meester. Kinetic investigation of human dipeptidyl peptidase II (DPPII)-mediated hydrolysis of dipeptide derivatives and its identification as quiescent cell proline dipeptidase (QPP)/dipeptidyl peptidase 7 (DPP7). — Biochem. J., **386**, 2005, 315-324.