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Development of a Highly Selective Fluorogenic Histochemical Substrate for Dipeptidyl Peptidase II

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

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

Dipeptidyl peptidases II (DPP II) and IV (DPP IV) are prolyl-specific enzymes, hydrolyzing similar substrates with proline or alanine as penultimate moiety. Thus, it is difficult to differentiate the two enzymes locations in tissue sections of mammalian organs. In the present study, two fluorogenic histochemical substrates for DPP II — Lys-Ala-4-hydrazido-N-hexyl-1,8-naphthalimide and Nle-Nle-4-hydrazido-N-hexyl-1,8-naphthalimide are used for the visualization of the enzyme activity in tissue sections of Balb/c mice kidney and small intestine. The first substrate gives a visible co-reaction with dipeptidyl peptidase IV. Oppositely, the second substrate is shown to visualize only DPP II activity and appears to be highly specific for this enzyme. A conclusion is drawn that the Nle-Nle-based substrate is valuable for the selective localization of DPP II activity in tissue sections and could be used for the distinctive study of this enzyme.

Key words: dipeptidyl peptidase II, dipeptidyl peptidase IV, fluorogenic substrates, enzyme histochemistry.

Introduction

Dipeptidyl peptidase II (DPP II; E.C. 3.4.14.2) is a lysosomal serine-type amino peptidase belonging to the family of prolyl-specific dipeptidases along with dipeptidyl peptidases IV and VIII. The enzyme catalyzes the hydrolysis of dipeptides from the free amino-terminal of low-molecular oligopeptides and synthetic peptide substrates at pH optimum 5.5 [2]. DPP II activity is inhibited by cations like puromycine and Tris [1]. The physiological role of the enzyme is not yet elucidated. It is supposed to be involved in cell differentiation, in the degradation of collagen, myofibrillar proteins and short neuropeptides like substance P and casomorphin (for review see [7]). Aberrant expression of DPP II is reported in squamous cell lung carcinoma [4], atherosclerosis [6] and celiac disease [8]. DPP II activity has been studied using different synthetic peptide substrates. High hydrolysis rate is found towards the substrates, possessing Lys-Pro- or Ala-Pro- amino acid sequences. However, these substrates are also hydrolyzed by dipeptidyl peptidase IV (DPP IV, E. C. 3.4.14.5). Lys-Ala-substrates are more specific for DPP II and are cleaved to a lesser extent by DPP IV. Recently we proposed a novel fluorogenic histochemical substrate for DPP II — Lys-Ala-4-hydrazido-N-hexyl-1,8-naphthalimide (Lys-Ala-HHNI), which was used successfully for the localization of the enzyme in rat small intestine, colon, kidney and epididymis

[9]. However, a substantial co-reaction of DPP IV was observed in kidney and jejunum. The detailed study of L e i t i n g et al. [5] on the enzyme specificity has shown that DPP II can hydrolyze Nle-Nle-rhodamine, whereas DPP IV does not cleave this substrate.

In the present paper we present a novel fluorogenic substrate for DPP II - Nle-Nle-4-hydrazido-N-hexyl-1,8-naphthalimide (Nle-Nle-HHNI) and compare its specificity for the visualization of the enzyme in tissue sections of mouse jejunum and kidney with this one of the previously proposed substrate — Lys-Ala-HHNI.

Material and Methods

The DPP II substrate Lys-Ala-HHNI was synthesized as described previously [9].

Synthesis of DPP II substrate Nle-Nle-HHNI. 4-Hidrazino-N-hexyl-1,8-naphthalimide (HHNI) was synthesized as described earlier [9]. The substrate Nle-Nle-HHNI was obtained by the N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-N-methylmethanaminium tetrafluoroborate N-oxide — method (TBTU, Fluka) after [3]. Briefly, Boc-Nle (Bachem) was coupled to the hydrazine group of HHNI using TBTU as coupling reagent, diisopropyl ethylamine (DIPEA, Sigma-Aldrich) as proton acceptor and hydroxybenzotriazole (HOBt, Fluka) in dimethyl formamide (DMF) for 2 hours at room temperature. The Boc-protection was cleaved with 4 N HCl/ dioxane for an hour at room temperature and the obtained HCl-salt of Nle-HHNI was isolated by diethyl ether. Boc-Nle was coupled to Nle-HHNI using the same procedure and the Boc-protected substrate was treated with HCl/dioxane as above. Thus, the substrate Nle-Nle-HHNI was obtained as HCl-salt.

Tissue treatment. Mature Balb/c mice of both sexes were decapitated under ether anesthesia. Pieces of kidney and jejunum were fixed in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0 for 18 hrs. at +4°C. Then, they were washed in Holt's solution (15% sucrose, 1% gum arabic in distilled water) for 24 hours at +4°C 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 1% celloidin for a minute at room temperature.

DPP II and DPP IV localization. For the visualization of DPP II activity, the sections were incubated in solutions, consisting of 0.5 mM substrate (Lys-Ala-HHNI or Nle-Nle-HHNI) and 1 mg/ml piperonal in 0.1 M phosphate buffer, pH 5.5. For the visualization of DPP IV activity, the sections were incubated in solutions, containing 0.5 mM substrate (Lys-Ala-HHNI or Nle-Nle-HHNI) and 1 mg/ml piperonal in 0.1 M phosphate buffer, pH 7.6. For both enzymes the incubation lasted 60 min with Lys-Ala-HHNI and 24 h with Nle-Nle-HHNI at 37°C.

Histochemical controls. Control sections were pre-incubated in 50 mM Tris/HCl, pH 5.5 (for the inhibition of DPP II) or in 50 mM $Pb(NO_3)_2$ in water (for the inhibition of DPP IV) for 30 minutes at 37°C. After that, they were transferred in the full substrate media supplied with 50 mM of the corresponding inhibitor and incubated as described above.

All the samples were post-fixed in 4 % formalin and embedded in glycerol-jelly.

The sections were studied under fluorescent microscope OPTON IM 35 with filter G546 FT580 LP 590. The photos were made on Konica Minolta (Japan) 200 colorful films.

Results

The substrate Lys-Ala-HHNI, previously synthesized by us was quickly hydrolyzed and considerable amounts of final reaction product are deposited in the section of mouse

kidney and jejunum. However, most of the fluorescent product was restricted to the apical part of the cells, i.e. in the brush border regions of enterocytes and kidney epithelial cells of the convoluted tubules. Fluorescent precipitates were also observed in the glomeruli, which is typical for DPP IV, but not for DPP II (Fig.1a, b, e, f). The enzyme reaction of similar pattern was obtained with this substrate in phosphate buffer, pH 7.6 — the optimal pH of DPP IV. Lead ions (Pb^{2+}), which are known to inhibit DPP IV but not DPP II, suppressed the reaction to a great extent, leaving only single fluorescent granules within the cells. Oppositely, pre-incubation of the sections in Tris buffer did not restrain the enzyme reaction. Obviously, Lys-Ala-substrate was simultaneously cleaved by the two enzymes and the DPP IV reaction dominated despite the low pH of the substrate medium. Still, DPP IV is known to be active over a broad pH range.

The substrate Nle-Nle-HHNI was slowly hydrolyzed — 24 hours incubation was necessary to obtain fluorescent staining in the sections. This is probably due to the fact, that norleucine is an atypical amino acid. On the other hand, the final fluorescent product was densely distributed within the epithelial cells of convoluted tubules in kidney and small intestinal enterocytes. The glomeruli were negative (Fig. 1c, d, g, h). This pattern is characteristic for DPP II and most likely correlates with the lysosomal localization of the enzyme. The reaction was fully inhibited by Tris — the specific DPP II inhibitor and was not suppressed by Pb^{2+}. Using this substrate in phosphate buffer, pH 7.6 no fluorescent product was deposited in the sections. This data showed that the reaction was entirely due to the substrate hydrolysis by DPP II.

Discussion

Prolyl-specific peptidases like DPP II and IV appear to be an important protease family since a number of biological processes like signaling by peptide hormones are regulated by proline-specific N-terminal processing [7]. However, the high specificity of the two peptidases towards substrates, possessing proline as a second amino acid as Lys-Pro or Ala-Pro makes it difficult to differentiate their activities in tissue sections. Lys-Ala- substrates are commonly used for the localization of DPP II activity, but it is important to note that alanine as a penultimate moiety is the second most preferred residue for DPP IV. For example, DPP IV endogenous substrates GLP-1 (glucagon-like peptide-1) and GIP (glucose-dependant insulinotropic polypeptide) both possess Ala in P1 position [10]. In view of the fact that many suggestions are made on possible functions of the enzymes depending on their localization (for review see [7]), it is important to develop specific substrates for DPP II. Recent finding that DPP II is able to hydrolyze Nle-Nle- dipeptides from the amino-terminal of synthetic substrates (rhodamines) [5], whereas DPP IV does not cleave such substrates, opens new possibilities in this area.

The substrate for DPP II Lys-Ala-HHNI, recently synthesized by us [9] could be used for the visualization of the enzyme activity in the presence of Pb^{2+} ions, since they inhibit DPP IV. This is especially important for kidney and jejunum, where DPP IV co-reaction was clearly visible. Our newly developed substrate Nle-Nle-HHNI avoided the use of inhibitor, because it was not hydrolyzed by DPP IV and was specific for DPP II. This makes it very convenient for the study of changes in the level and distribution of the enzyme activity in normal biological or disease-related processes.

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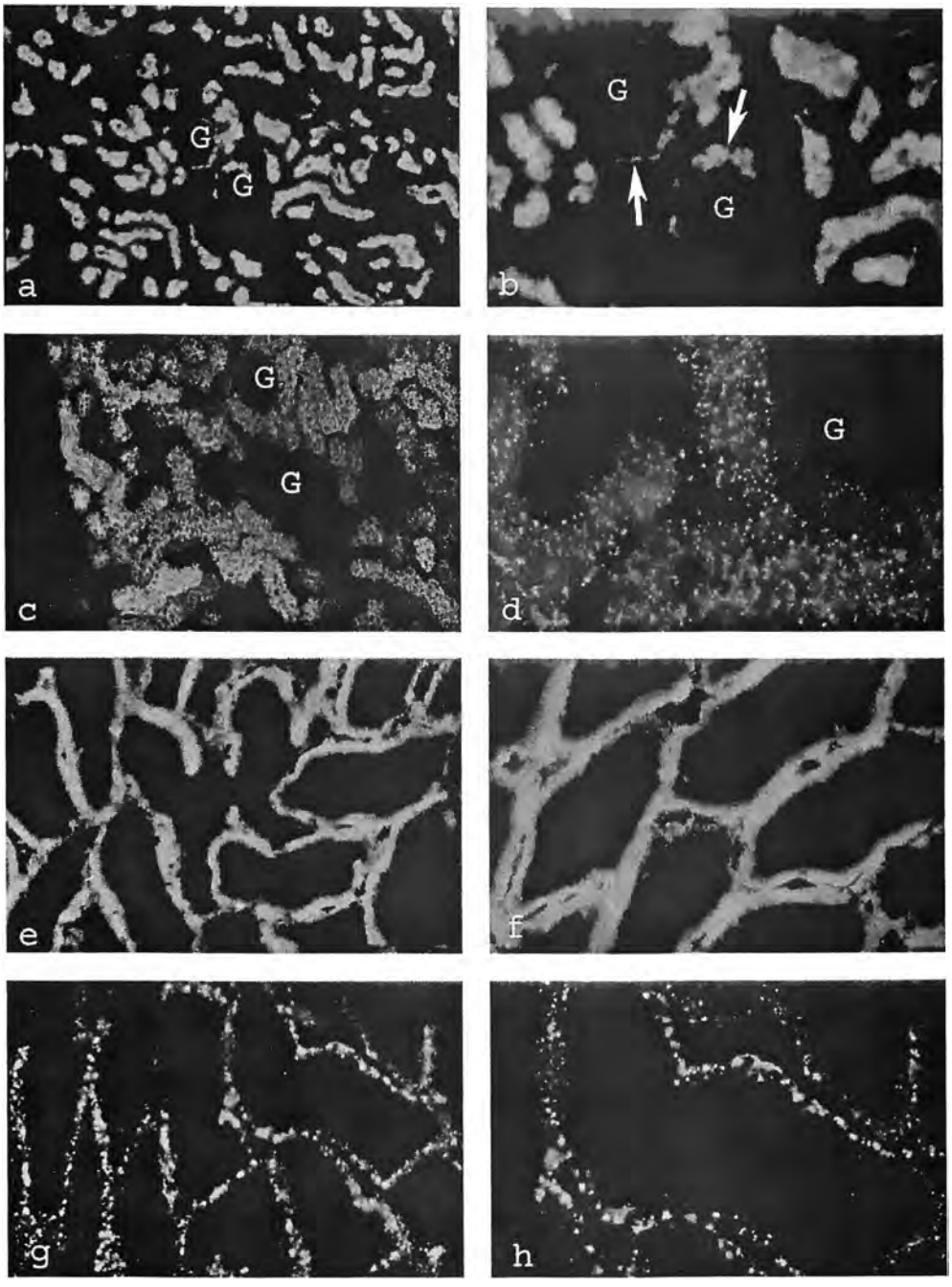

Fig. 1. Visualization of DPP II activity in Balb/c mouse kidney (a-d) and jejunum (e-h)
a, b — Lys-Ala-HHNI, amorphous fluorescent deposits in the apical part of epithelial cells of kidney convoluted tubules and in the glomeruli (arrows). G—glomeruli; c, d — Nle-Nle-HHNI, granular fluorescent product, densely distributed within the epithelial cells of convoluted tubules. No reaction in the glomeruli (G); e, f — Lys-Ala-HHNI, final reaction product, restricted to the brush border of small intestinal enterocytes; g, h — Nle-Nle-HHNI, fluorescent granules inside the enterocytes; Left ×200; Right ×500

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