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Fluorescent Histochemical Localization of Dipeptidyl Peptidase IV Activity

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A novel fluorogenic substrate for dipeptidyl peptidase IV (DPP IV) – Ala-Pro-4-hydrazido-N-(4′-*iso*-butoxy)phenyl-1,8-naphthalimide (Ala-Pro-BuOPHNI) and its application for the localization of the enzyme in tissue sections of rat organs are presented. The substrate permits a precise detection of DPP IV activity *in situ* and might find various applications in the studies of the diseases for which DPP IV is a marker enzyme.

Key words: Dipeptidyl peptidase IV, 1,8-Naphthalimide derivatives, Fluorescent histochemical methods, Enzyme histochemistry.

Introduction

Dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) is a membrane-bound serine protease hydrolyzing Xaa-Pro dipeptides from the NH_2-terminal of oligopeptides and synthetic substrates at pH optimum 7.8. The enzyme has multiple functions. It participates in the digestion of different proteins, modulates the activities of biologically active peptides and acts as a co-stimulatory protein, adhesion molecule or receptor molecule [4]. DPP IV is accepted as a diagnostic marker for thyroid carcinoma and regarded as a supporting marker for the assessment of the severity of various diseases [1].

In the present study we present a novel fluorogenic substrate for DPP IV – Ala-Pro-4-hydrazido-N-(4′-*iso*butoxy)phenyl-1,8-naphthalimide (Ala-Pro-BuOPHNI) and its application for the localization of the enzyme in tissue sections of rat organs. The substrate permits a precise detection of DPP IV activity *in situ*.

Materials and Methods

DPP IV substrate and inhibitor. The substrate Ala-Pro-4-hydrazido-N-(4′-*iso*butoxy) phenyl-1,8-naphthalimide (Ala-Pro-BuOPHNI) was synthesized from 4-hydrazino-N-(4′-*iso*-butoxy)phenyl-1,8-naphthalimide (BuOPHNI) and Boc-Ala-Pro-OH (Bachem)

after TBTU-method in dimethylformamide with diisopropylethylamine as HCl-acceptor, isolated customarily and Boc-protection cleaved by HCl /dioxane [3]. DPP IV inhibitor N-(H-Phe-Pro-)-O-(4-nitrobenzoyl)hydroxylamine hydrochloride (Phe-Pro-NHONb) was synthesized according to Demuth [2].

Animals, tissue treatment and incubation media. Mature Wistar rats of both sexes were decapitated, peaces of different organs were extracted and frozen in liquid nitrogen. Sections (10 μm) were cut on cryocut Reichert-Jung (FRG) at –25 °C, air-dried and covered by celloidin (1% celloidin in ethanol:chloroform:aceton 3:3:4) for a minute at room temperature. The sections were incubated in 0.1 M phosphate buffer (pH 7.8) containing 0.5 mM substrate (Ala-Pro-BuOPHNI) and 2.5 mM 2,3,4-tri-methoxy benzaldehyde (TMBA) for 30-90 min at 37 °C, then fixed in 4 % neutral formalin, stained with hematoxyline and embedded in glycerol/jelly.

Inhibitor controls. Sections, incubated in 0.5 mM inhibitor Phe-Pro-NHONb in phosphate buffer, pH 7.8 for 45 min at room temperature were transferred to a full substrate medium, supplied with 0.5 mM inhibitor for an hour at 37°C and then treated as above.

All the preparations were studied under light and fluorescent microscopes and photographed on Konica 200 ASA colourful films.

Results and Discussion

DPP IV substrate, developed by us (Ala-Pro-BuOPHNI) was used according to the principle in Scheme 1. The enzyme hydrolysis releases the fluorochrome BuOPHNI, which reacts immediately with the aromatic aldehyde TMBA to form an insoluble fluorescing hydrazone. The last compound precipitates on the sites of the enzyme activity and marks them by a bright red fluorescence. The substrate proposed here represents a modification of the already reported by us fluorogenic DPP IV substrate Gly-Pro-4-hydrazido-N-hexyl-1,8-naphthalimide (Gly-Pro-HHNI) [3]. Introduction of the second aromatic ring in the substrate molecule (see Scheme 1) provides a substantial decrease of the solubility of the primary reaction product (BuOPHNI is more insoluble than HHNI), which allows an increased precision of the enzyme localization. On the other hand, the elongated conjugated system provides a very bright fluorescence and the possibility to use different aromatic aldehydes as visualization agents. The histochemical method, presented here allowed us to achieve precise detection of the enzyme activity in all its known locations in tissue sections of rat organs at the lack of background noise in a short incubation time (Fig. 2). The specificity of the reaction was proved by the use of the irreversible DPP IV inhibitor Phe-Pro-NHONb. This inhibitor abolished almost fully the enzyme activity. Residual activity was observed only in the brush-borders of the small intestinal entherocytes, signified by isolated fluorescent granules (Fig. 2 – A1, B1).

Many proteases are now regarded as possible markers for the onset and progression of various malignant, immune, neurodegenerative and other diseases. Thus, development of fluorogenic substrates for the *in situ* analysis of proteolytic enzymes is considered an important tool for the study of those enzymes in normal and pathologically altered tissues. In this respect, our novel fluorogenic DPP IV substrate can find a variety of applications in diagnosis, response to therapy and prognosis of the diseases for which DPP IV is a marker enzyme.

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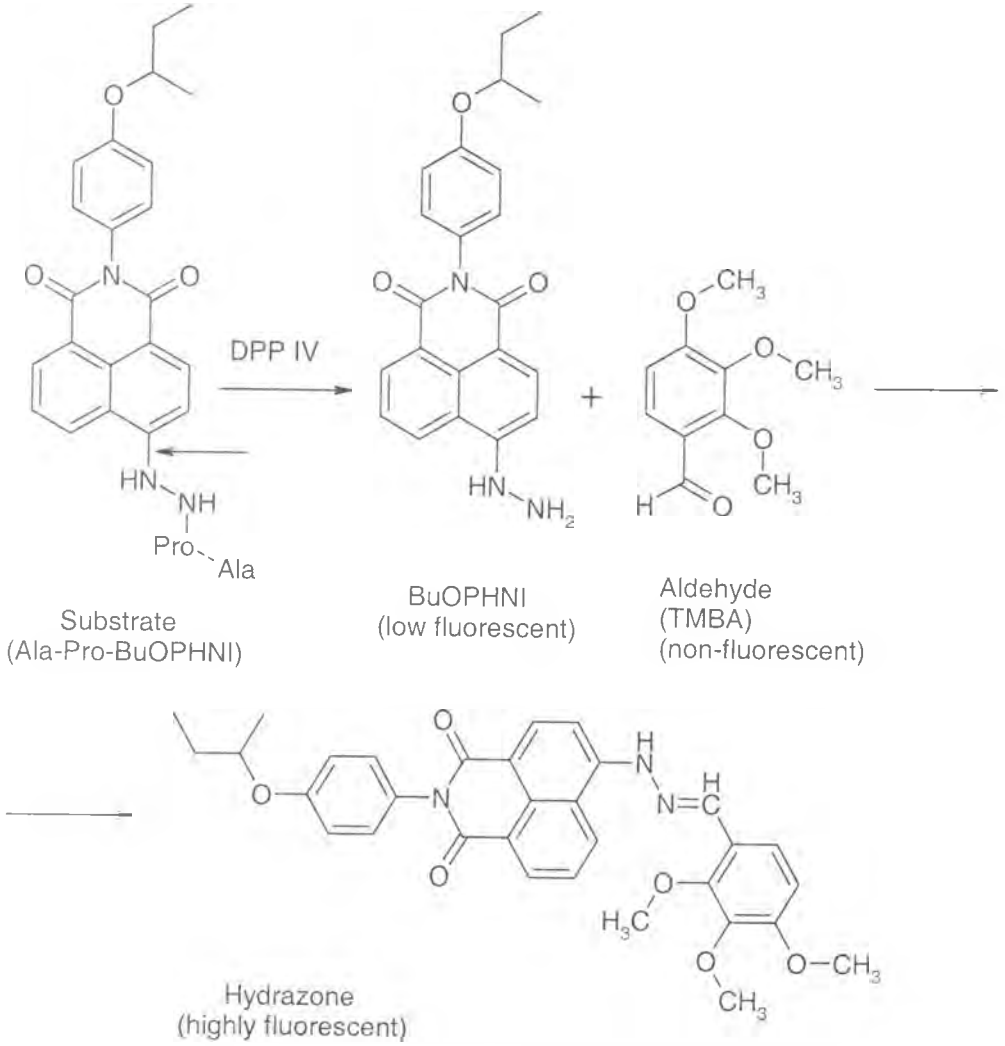

Fig. 1. Principle of the visualization of DPP IV activity

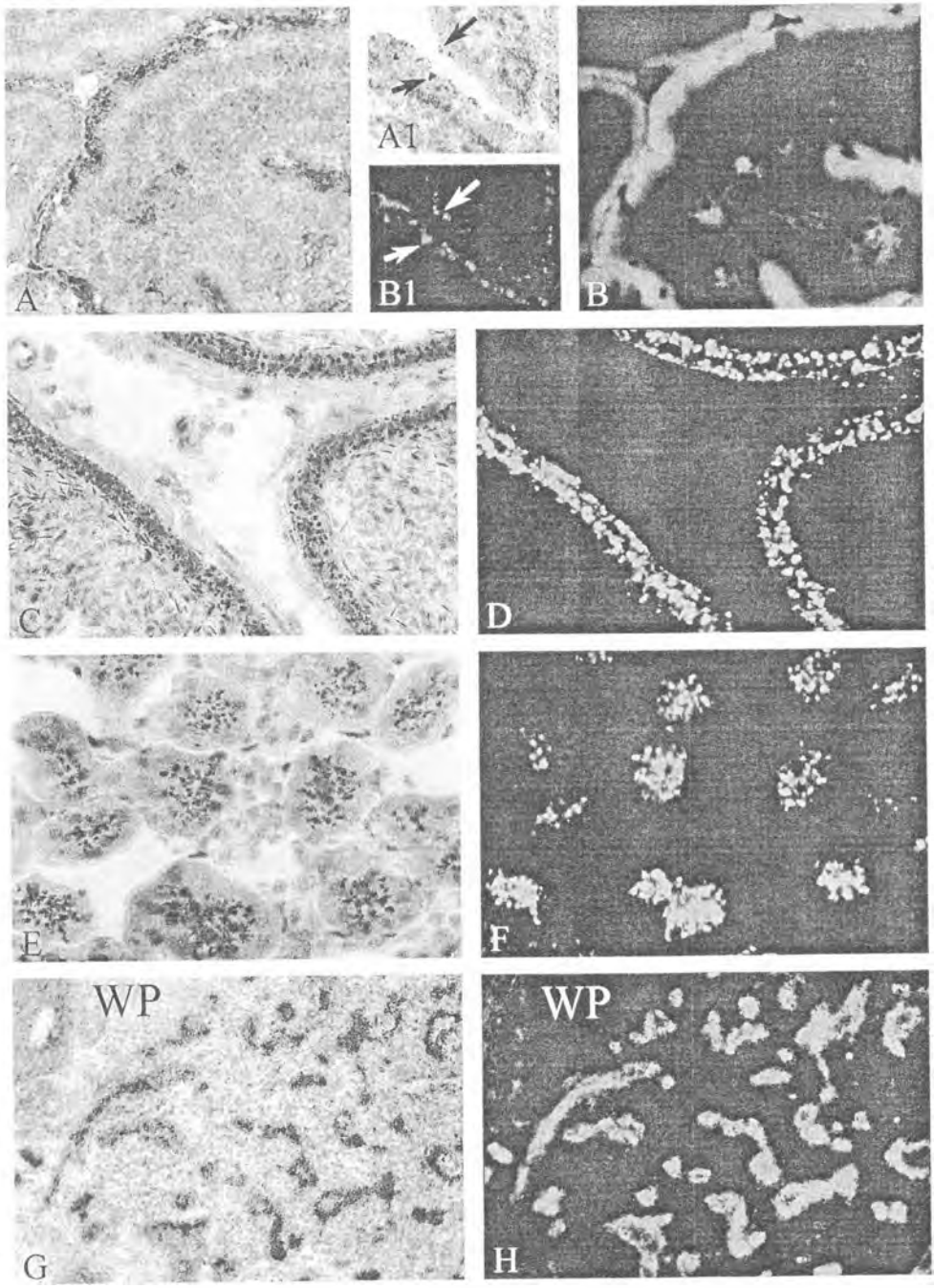

Fig. 2. DPP IV activity by the substrate Ala-Pro-BuOPHNI and TMBA in: A, B – rat intestine: reaction in the brush borders of the enterocytes, × 400; A1, B1 – inhibitor experiment in rat small intestine: a considerably decreased amount of the fluorescent reaction product in the brush border area of enterocytes (arrows), × 400; C, D – epididymis: epithelial cells of the channels are filled with the fluorescent deposits, × 400; E, F – kidney cortex: DPP IV reaction in the apical pole of the epithelial cells of convoluted tubules, × 400; G, H – rat spleen: DPP IV reaction in the red pulp veins and sinusoids; no reaction in white pulp (WP), × 160. A, A1, C, E, G – light microscopy; B, B1, D, F, H – fluorescent microscopy

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