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Glycyl-L-Prolyl-1-Anthraquinonyl hydrazide — a New Substrate for the Histochemical Detection of Dipeptidyl Peptidase IV

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Glycyl-L-Prolyl-1-anthraquinonylhydrazide (Gly-Pro-AH) was synthesized and used as a new substrate for the histochemical detection of dipeptidyl peptidase IV (DPP IV). The enzyme hydrolysis liberates the insoluble dark red 1-anthraquinonyl hydrazine, which marks the enzyme sites. After a post-incubation with some aromatic aldehyde in an acid medium, 1-anthraquinonyl hydrazine is converted to the respective hydrazone. The most convenient aromatic aldehyde is selected to give a deeply colored and amorphous hydrazone, thus improving the histochemical picture considerably. The new method is used to demonstrate the enzyme in tissue sections from different rat organs. It is found to allow an accurate DPP IV localization in all its locations.

Keywords: Dipeptidyl peptidase IV, 1-anthraquinonyl hydrazine, hydrazone formation, enzyme histochemistry.

Introduction

Dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) — a serine type aminoexopeptidase [3] is found in the cell surface membranes in many tissues [8, 12]. It cleaves aminoacyl-Pro-dipeptides from the amino-terminal of oligopeptides at pH optimum 7.8. DPP IV is identical to T-cell activation marker CD 26 [19]. Presently, this enzyme is a subject of a vast interest, due to the variety of biological functions it has in the organism, such as digestion of natural polypeptides [17] and collagen [1], activation or inactivation of peptide hormones [16], activation of T- and B-lymphocytes [18, 20], etc.

Histochemically, DPP IV is usually determined using Gly-L-Pro-4-methoxy-2-naphthylamide as a substrate with either diazonium salts according to the azo-coupling method [9, 13] or 5-nitrosalicylaldehyde as a fluorogenic reagent [10, 11]. However, the above methods suffer a lot of drawbacks [12]. Recently, we developed a convenient chromogenic method for the histochemical detection of DPP IV, based on the substrate Gly-L-Pro-1-hydroxy-4-naphthylamide and tetrazolium salts as visualization reagents [4].

In the present paper, we propose a new chromogenic substrate for DPP IV — Gly-L-Pro-1-anthraquinonyl hydrazide (Gly-Pro-AH), which we use to localize the enzyme in tissue sections of different rat organs. The new technique is developed to escape most of the disadvantages of the methods in use and to provide a tool for the precise determination of the enzyme.

Material and Methods

Synthesis of the substrate

1-Anthraquinonyl hydrazine was synthesized from 1-chloroanthraquinone (Merck, Darmstadt, Germany) and hydrazine in pyridine as described by Moehlau [15]. Boc-Gly-L-Pro-OH (Bachem, Heidelberg, Germany) was coupled to 1-anthraquinonyl hydrazine by the dicyclohexylcarbodiimide method after [2]. The Boc-protective group of Gly was cleaved with hydrogen chloride in dioxane to obtain the substrate Gly-L-Pro-1-anthraquinonyl hydrazine (Gly-Pro-AH) as a hydrochloric salt.

Tissue treatment and incubation media

Mature Wistar rats of both sexes were killed by decapitation and pieces of different organs were immediately frozen in liquid nitrogen. Tissue sections, 10 μm thin, were cut on cryotome 2800M Reichert-Jung, Nussloch, Germany. They were mounted on glass slides and fixed in acetone for 5 min at —20°C or, otherwise, they were freeze-dried, mounted on albuminized glass slides and covered by 1 % celloidin for a minute at room temperature. All the sections were incubated in a substrate medium, containing 0.5 mM substrate (Gly-Pro-AH), pre-dissolved in a minimal amount of dimethyl formamide, and 0.1 M Tris/HCl buffer or phosphate buffer, pH 7.8. The incubation was carried out at 37°C for 20 to 60 min (longer incubation was necessary for the celloidin-embedded sections). After that, the sections were transferred into a second incubation medium, consisting of acetate buffer, pH 4.5, and 1 mg/ml aldehyde — 4-dimethylaminobenzaldehyde, 4-methoxybenzaldehyde, 4-nitrobenzaldehyde or 4-diethylaminobenzaldehyde. The second incubation was performed at room temperature for 4 to 6 h (or, better, overnight). Then, the sections were post-fixed in neutral formaline and embedded in glycerol-jelly.

Control sections were prepared in the same manner, but heated to 80°C for 10 min in water before the incubation (thermally inactivated controls). Other controls, incubated only in buffered aromatic aldehyde at pH 4.5 were also prepared.

Results

Thermally inactivated controls as well as the controls, incubated only in aromatic aldehyde solutions, were free of any non-specific staining.

The newly synthesized DPP IV substrate — Gly-Pro-AH was hydrolyzed readily by the enzyme to release 1-anthraquinonyl hydrazine. The last compound, being practically insoluble in aqueous media, precipitated on the sites of the enzyme activity, marking them precisely by a deeply red color. In the acetone fixed sections, the hydrazine deposits were grainy to microcrystalline. Much better results for all the organs studied were obtained in the freeze-dried celloidin-mounted sections,

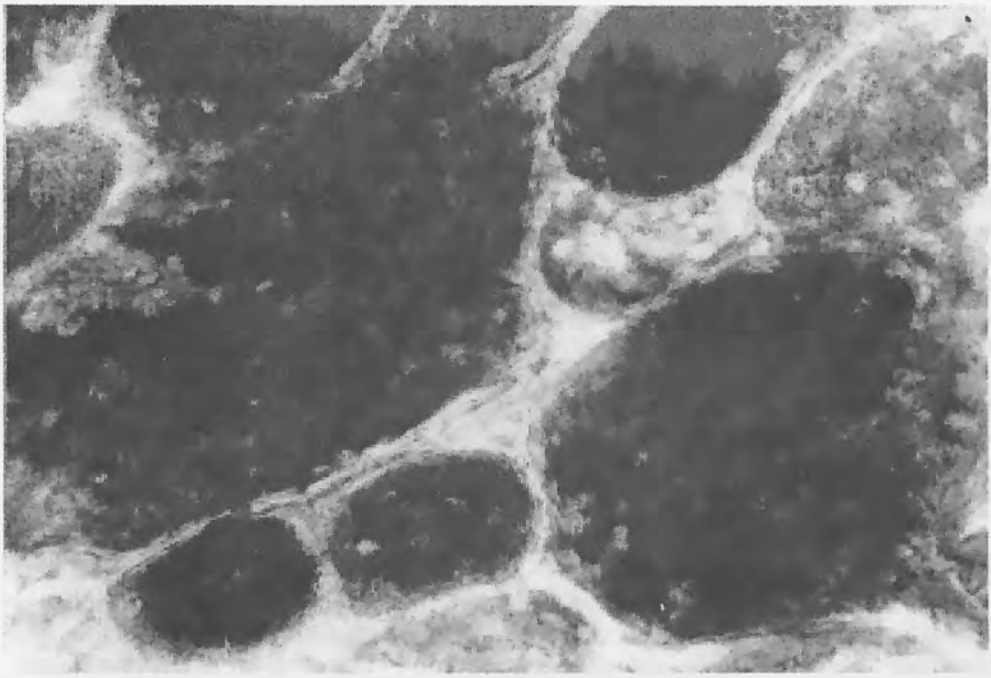

Fig.1. DPP IV in the kidney. Gly-L-Pro-AH and 4-dimethylaminobenzaldehyde. Reaction product in the glomeruli and in proximal renal brush border (× 500)

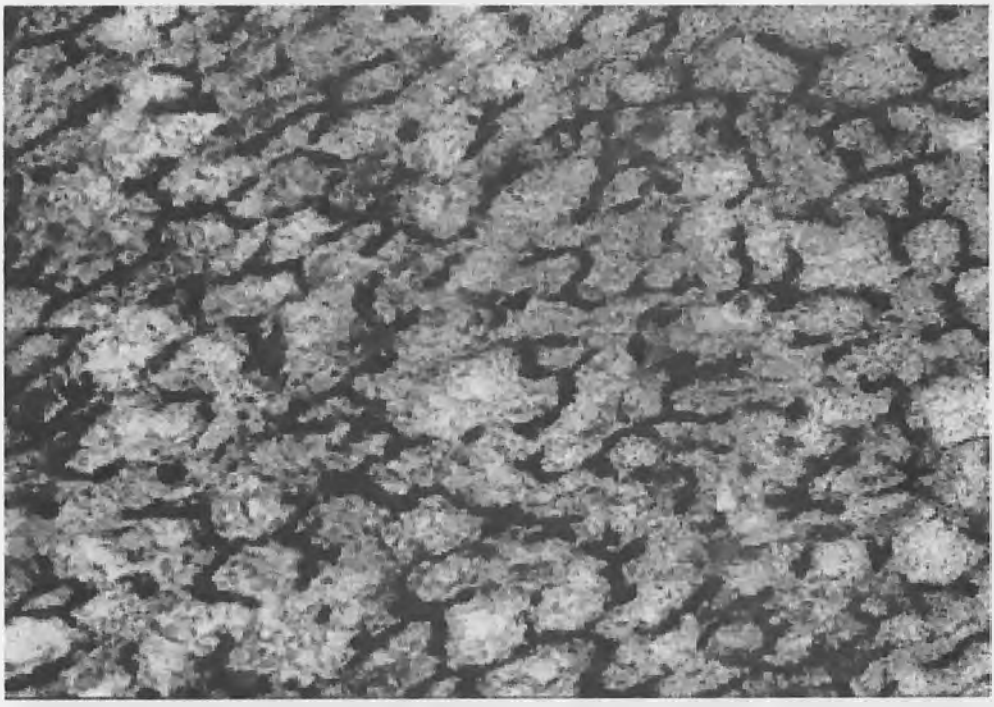

Fig.2. DPP IV in the liver. Gly-L-Pro-AH and 4- dimethylaminobenzaldehyde. High enzyme activity in the bile capillaries and sinus endothelial cells (× 500)

where the hydrazine deposits were amorphous. Better results were obtained using Tris/HCl buffer. As it was described above, the sections were transferred into acetate buffer, supplied with an aromatic aldehyde for the post-coupling procedure. During the second incubation, 1-anthraquinonyl hydrazine was converted into hydrazone, which color was different, depending on the aromatic aldehyde employed: dark blue with 4-dimethylaminobenzaldehyde, dark violet with 4-methoxybenzaldehyde, red-orange with 4-nitrobenzaldehyde and blue to black with 4-diethylaminobenzaldehyde. The last two aldehydes were rejected for further use, since with them granular to crystalline hydrazones were formed. In contrast, 4-methoxybenzaldehyde and 4-dimethylaminobenzaldehyde were found appropriate, because they gave finely amorphous and very dark in color hydrazones with 1-anthaquinonyl hydrazine. The histochemical contrast was much improved by this conversion. In the kidney (Fig.1), a high DPP IV activity was visualized in the proximal renal brush border and in renal corpuscles. In the liver (Fig. 2), the final product was visible in bile capillaries as well as in sinus endothelial cells. In the epididymis (Fig. 3), it was observed in stereocilia and apical parts of the duct epithelial cells. In the seminal vesicles (Fig. 4), DPP IV was highly active in the epithelial cells.

Discussion

Recently, it is widely recognized, that the mostly used method for the histochemical detection of DPP IV — the azo-coupling method — suffers a lot of disadvantages. Diazonium salts are powerful enzyme inhibitors. They decompose in the incubation media, especially at pH > 7.0, thus causing precipitation artifacts and not permitting the incubation at the optimal pH of the enzymes [12]. In a previous study [4] we introduced a tetrazolium method for the histochemical detection of DPP IV. Tetrazolium salts are known to inhibit the enzymes to a much lesser extent than the diazonium salts [14]. No histochemical method for DPP IV without auxiliary reagent in the incubation solution is still available.

We recently established the use of 1-anthraquinonyl hydrazine—based substrates in the histochemistry of peptidases. Using such substrates we managed to localize dipeptidyl peptidases I and II (DPP I, II) and tripeptidyl peptidase I (TPP I), the last enzyme had not been demonstrated histochemically before that [5, 6, 7]. Though the above substrates are hydrazides, not amides, they are readily hydrolyzed by peptidases. With the present work we expand the newly developed method to the demonstration of a membrane-associated peptidase. For the lysosomal enzymes, which have acidic pH optima, the second reaction step — the conversion of 1-anthraquinonyl hydrazine into hydrazone with some aromatic aldehyde, can be performed in the incubation medium (simultaneous coupling). The DPP IV optimal pH (7.8) does not permit a hydrazone production in a single step. Thus, a second incubation is needed. Anthraquinonyl hydrazine is highly water- and buffers- insoluble and almost amorphous in celloidin, but its color is not deep enough and it tends to form crystals upon a time. If it is converted to a hydrazone, the precipitations become very deeply colored, perfectly amorphous and do not change upon storage. We made a screening of several aromatic aldehydes to choose the most convenient one for the hydrazone formation step. The aldehydes of choice proved to be 4-methoxybenzaldehyde and 4-dimethylaminobenzaldehyde. The second incubation step proposes more advantages, since no auxiliary reagents are present in the incubation medium. Without the use of a second reagent one escapes all the disadvantages of the azo-coupling and tetrazolium methods (see above). Using the new method, we localized precisely DPP IV activity in all the organs studied.

Fig.3. DPP IV in the epididymis. Gly-L-Pro-AH and 4- dimethylaminobenzaldehyde. Hydrazone final reaction product in the stereocilia and the apical part of duct epithelial cells (× 500)

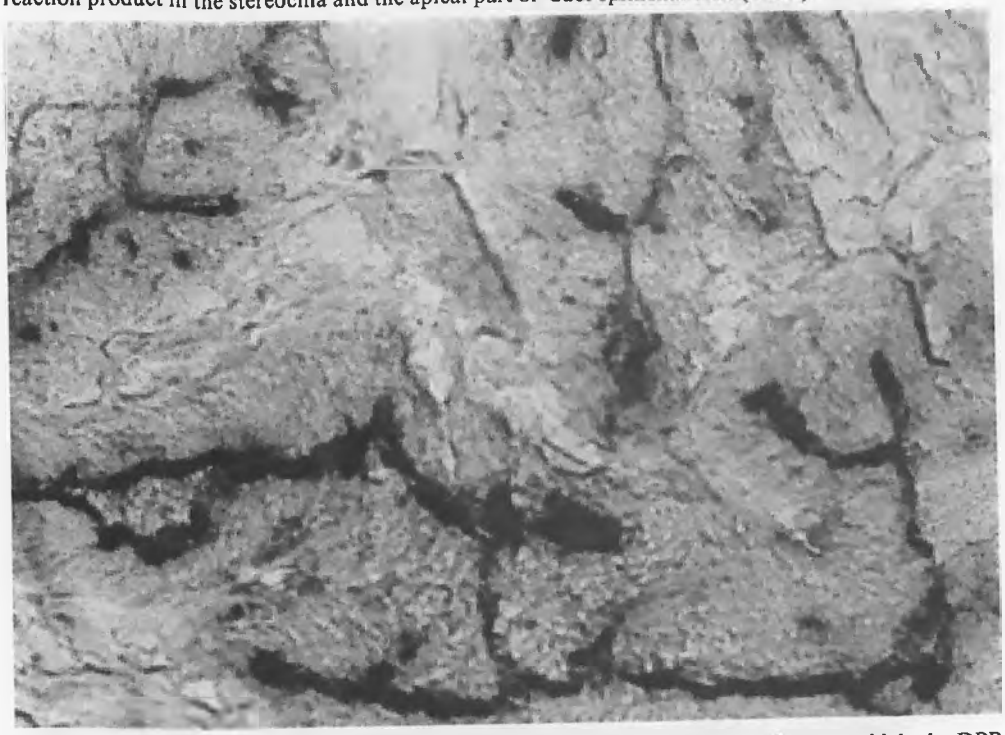

Fig.4. DPP IV activity in the seminal gland. Gly-L-Pro-AH and 4- dimethylamino-benzaldehyde. DPP IV activity in the seminal gland epithelial cells (× 500)

The novel histochemical method, proposed here, proved to be very appropriate for the visualization of the membrane-associated alkaline peptidase DPP IV. The synthesis of other aminopeptidases substrates, based on 1-anthraquinonyl hydrazine are in progress.

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