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Tetrazolium Salts Method for the Histochemical Demonstration of Aminopeptidase A

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A new substrate for aminopeptidase A (APA; EC 3.4.11.7) — L-Glutamic acid-1-(1-hydroxy-4-naphthylamide) (Glu-HNA) is synthesized and applied for the histochemical localization of the enzyme by a tetrazolium salt procedure with Nitro Blue Tetrazolium (NBT) or Tetra Nitro Blue Tetrazolium (TNBT) as visualization agents. Using the newly developed tetrazolium method, the enzyme is successfully demonstrated in tissue sections from rat organs. The histochemical technique proposed here escapes the disadvantages of the diazonium salts method and permits the precise localization of the enzyme in all the organs studied.

Key words: Aminopeptidase A, Tetrazolium salts method, Enzyme histochemistry

Introduction

Aminopeptidase A (Angiotensinase A; APA, EC 3.4.11.7) is a membrane-associated enzyme, hydrolyzing α-glutamyl or α-aspartyl residues from the N-terminal of different peptides, e.g. angiotensins. The enzyme activity level depends on the presence of calcium ions and is optimal at pH 7.6. Formerly, APA has been identified by G l e n n e r and F o l k [5]. Its activity distribution in the kidney of different mammalian species has been thoroughly studied by Kugler [7, 8, 9, 10, 11] and in the other mammalian organs — by L o j d a and G o s s r a u [12]. The APA activity is usually demonstrated using α-Glu-2-naphthylamide or α-Glu-4-methoxy-2-naphthylamide as substrates according to the azo-dye method with different diazonium salts (Fast Blue B, freshly hexazotized pararosaniline or new fuchsin) as visualization agents [12, 11, 9]. Though the tetrazolium salts are generally considered to be better auxiliary reagents than the diazonium salts [13], no tetrazolium method for the demonstration of APA has been proposed thus far.

Previously, we introduced a new tetrazolium principle for the histochemistry of aminopeptidases, based on newly designed substrates, bearing 1-hydroxy-4-naphthylamine (HNA) as a chromogenic leaving group. The method was successfully

applied for the histochemical visualization of dipeptidyl peptidase IV and gamma-glutamyl transpeptidase [2, 3]. In the present study we describe the synthesis of L-glutamic acid-1-(1-hydroxy-4-naphthylamide) (Glu-HNA) and its application as a new substrate for the histochemical demonstration of APA according to the tetrazolium salts principle.

Material and Methods

Synthesis of the substrate

The synthesis of 1-hydroxy-4-naphthylamine (HNA) was performed by the reduction of 4-sulfophenyl-azo-4-hydroxynaphthalene (the azo dye Orange I) with sodium hydrosulfite after [4]. The protected amino acid Z-Glu-OtBu (purchased from Bachem, Heidelberg, Germany) and HNA were coupled by the dicyclohexyl carbodiimide method [1] using N-ethylmorpholine as a hydrogen chloride acceptor in an inert atmosphere (argon). The tert-butyl-ester protection of the amino acid was cleaved using 6 N hydrogen chloride in dioxan and, after that, the Z-protection was cleaved by a catalytic hydrogenation in ethanol/ HCl with Palladium on activated carbon as a catalyst. Thus, the APA substrate — L-Glu-1-(1-hydroxy-4-naphthylamide) (Glu-HNA) was obtained as a hydrochloric salt.

Tissue treatment and incubation media

Mature Wistar rats of both sexes were killed by decapitation under ether anaesthesia and pieces of different organs (kidney, liver, heart and jejunum) were frozen immediately in liquid nitrogen. Ten micrometers thin sections were prepared on a cryotome 2800N, Reichert Jung, Nussloch, Germany at -20°C. The sections were mounted on glass slides. Freeze-dried sections from the liver were also prepared and mounted on albuminized glass slides. Then, all the sections were fixed in acetone for 5 min at -20°C, air-dried and incubated. The incubation solution consisted of 1 mM substrate (Glu-HNA), 2 mg/ml tetrazolium salt — NBT (Fluka, Switzerland) or TNBT (Serva, Heidelberg, Germany) and 10 mM calcium dichloride, all dissolved in 0.1 M cacodylate buffer, pH 7.6. The substrate and the tetrazolium salt were predissolved in minimal amounts of dimethylformamide. The substrate medium was filtrated before use. The incubation was performed for 20 to 45 minutes at 37°C. After that, the sections were fixed in neutral formaldehyde and embedded in glycerol-jelly.

Control sections were prepared in the same way, but incubated in the lack of substrate (only in buffer, containing the tetrazolium salt).

Results

Control sections did not show any non-specific diformazan precipitations.

The APA substrate — Glu-HNA was hydrolyzed quickly by the enzyme, so that an incubation time of 20—45 minutes was enough to reveal the enzyme locations in all the organs studied. The 1-hydroxy-4-naphthylamine, released upon the enzyme hydrolysis, reduced immediately the tetrazolium salt to give deeply coloured diformazan deposits exactly on the APA locations. The enzyme visualization was equally good with both tetrazolium salts. In the case of NBT, the

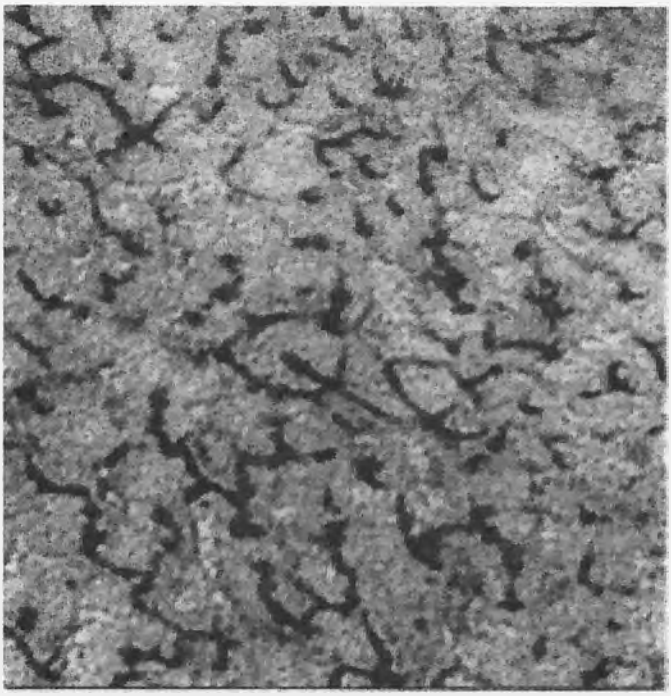

Fig. 1. APA in the rat liver. Glu-HNA and NBT. Freeze-dried, acetone fixed section. Diformazan deposits in the bile capillaries (× 500)

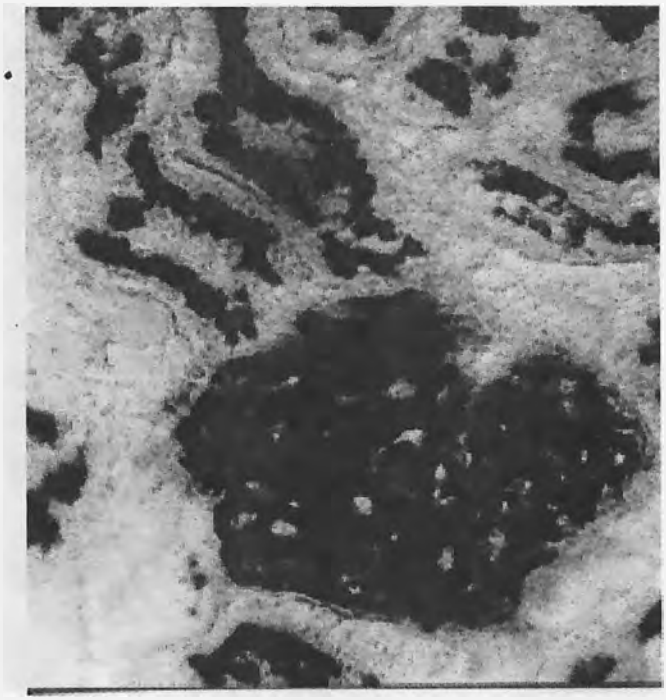

Fig. 2. APA in the kidney. Glu-HNA and NBT. Fresh-frozen acetone fixed sections. Reaction for APA in the brush borders of the convoluted tubules, glomerular endothelium and podocytes (× 500)

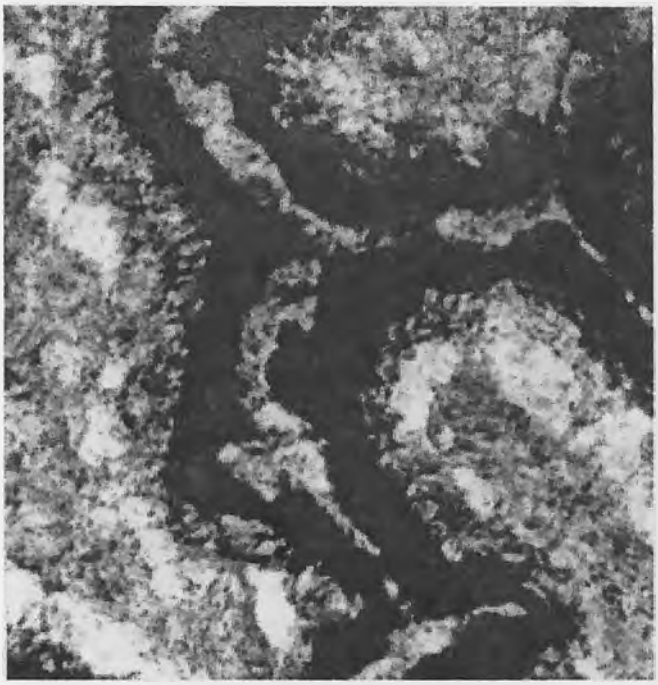

Fig. 3. APA in the jejunum. Glu-HNA and NBT. Fresh-frozen acetone-fixed section. High APA activity in the brush borders of the entherocytes (× 500)

Fig. 4. APA in the heart muscle. Glu-HNA and NBT. Fresh-frozen acetone-fixed section. Diformazan deposits in the heart capillaries (× 500)

diformazan precipitate was bluish-violet in colour. When TNBT was used, the diformazan was reddish-brown.

Good results were obtained in non-freeze-dried sections from all the organs except the liver, for which the freeze-drying appeared to be obligatory. In the liver, the final reaction product (the NBT or TNBT diformazan) was deposited in the bile capillaries (Fig. 1). In the kidney, APA activity was visualized in the brush borders of the convoluted renal tubules as well as in the glomerular endothelial cells and podocytes (Fig.2). In the jejunum, the brush borders of the enterocytes were highly APA-positive (Fig.3). In the myocardium, the diformazan precipitates were visible on the capillary endothelium (Fig.4).

Discussion

Aminopeptidase A is widely distributed in mammalian organs and tissues, where it plays an important role in peptide metabolism (for review see [12]). For example, it is present in the brush borders of the small intestinal enterocytes and proximal renal tubules, where an active absorption take s place. Furthermore, the enzyme is found in the capillary endothelium of every organ studied and is considered a marker enzyme for capillary bed endothelium [6, 12]. The only method for the histochemical determination of APA still remains the azo-coupling procedure, though it suffers a lot of drawbacks, caused by the use of diazonium salts as visualization agents. The diazonium salts are enzyme inhibitors. They decompose especially at pH above 7, not permitting the incubation at the optimal pH of the membrane-bound alkaline peptidases. In contrast, the tetrazolium salts are known to inhibit the enzymes to a much lesser extent [13]. The ditetrazolium salts, such as NBT and TNBT are very convenient for use in alkaline media. However, no tetrazolium method for APA has been proposed thus far. Here we propose the first tetrazolium method for APA, based on the new substrate L-Glu-1-(1-hydroxy-4-naphthylamide). The substrate is readily hydrolyzed by the enzyme to release a highly active reducing agent — 1-hydroxy-4-naphthylamine, which reduces quickly and quantitatively NBT or TNBT to the respective diformazans. The last compounds, being highly water- and buffers insoluble, precipitate on the enzyme locations, marking them accurately by a deep colour (bluish-violet or reddish-brown). A very important advantage of the method is that it permits incubation at the optimum pH of the enzyme (7.6). Thus, the new method escapes the disadvantages of diazonium salts methods and allows the precise localization of the enzyme.

With the present study, we expand our work on the new tetrazolium principle for the histochemical visualization of aminopeptidases, introduced recently by us [2, 3].

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