Institute of Experimental Morphology and Anthropology

Acta morphologica et anthropologica, 7
Sofia • 2002

Fluorescent Histochemical Demonstration of Alanine Aminopeptidase Using a Newly Synthesized Substrate

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New fluorogenic substrate for alanineaminopeptidase (APN)- L-Ala-2-anthraquinonylamide (Ala-AA) is synthesized and used for the histochemical localization of the enzyme by a direct fluorescent method. The enzyme hydrolyses the substrate to release the water-insoluble fluorochrome 2-aminoanthraquinone, which precipitates on the enzyme sites, marking them by a strong orange-red fluorescence, when excited by a green light (510-580 nm). A precise enzyme localization in tissue sections from rat organs is achieved using the new fluorescent method. The selected fluorochrome is promising for future applications in the other peptidases histochemistry as well.

Key words: Alanine aminopeptidase, 2-aminoanthraquinone, fluorogenic substrate, enzyme histochemistry

Introduction

Alanine aminopeptidase (aminopeptidase N, APN; EC 3.4.11.2) is a membrane-associated enzyme, hydrolyzing a number of amino acids from the NH_2-terminal of di- or oligopeptides at neutral pH values (optimal pH=7.2). The enzyme is widely distributed in mammalian and human organs (4). It has been identified as CD13 in monocytes membranes (9) and plays an important role as a member of enkephalin-degrading enzymes family (6,7). The histochemical localization of APN is performed using different artificial substrates- L-Ala-amides with chromogenic or fluorogenic tags (4). One of the fluorescent methods, tested by now, is introduced by Dolbeare and Smith (2). It is based on L-Ala-4-methoxy-2-naphthylamide (Ala-MNA) as a substrate and 5-nitrosalicylaldehyde (NSA) as a coupling agent. The method is useful for the demonstration of the enzyme in cultured cells [3], but is of little or no importance in tissue sections [8]. No other fluorescent histochemical method is available now.

In the present study we describe the synthesis of a new fluorogenic substrate for APN-L-Ala-2-anthraquinonylamide (Ala-AA) and its application for the enzyme localization in tissue sections of rat organs.

Materials and Methods

*Synthesis of the substrate.*The APN substrate -L-Ala-2-anthraquinonyl amide (Ala-AA) was synthesized as described by B e y e r m a n et al. [1]. Fmoc-Ala-chloride was obtained by boiling Fmoc-Ala-OH (N-fluorenyl-methyloxycarbonylalanine -Bachem, FRG) and thionylchloride in dichloromethane. After that, the Fmoc-Ala-chloride and the purified by a vacuum sublimation 2-aminoanthraquinone (AA- Fluka, Switzerland) were coupled by boiling them in benzene. The Fmoc-protection of the amino acid was cleaved using piperidine in dimethylformamide. Thus, Ala-AA was obtained as a free base.

Tissue treatment and incubation media. Wistar rats were decapitated and pieces of the kidney and small intestine were frozen in liquid nitrogen. Tissue sections (12μm) were cut on a cryotome Reichert Jung, Nussloch, FRG. The sections were freeze-dried, mounted on albuminized glass slides and embedded in 0.5% celloidin. The sections were incubated in a substrate medium, consisting of 0.5 mM substrate (Ala-AA) (dissolved in a minimal amount dimethylformamide) in 0.1 M phosphate buffer, pH 7.0. The incubation was carried out at 37 °C for 2-3 hours. Then, the sections were washed in buffer and embedded in Karion F (Merck, Darmstadt, Germany).

Two types of controls were used — sections, heated to 80 °C in distilled water for 10 min and incubated in the full substrate medium and sections, incubated in the lack of substrate. The fluorescence was viewed on OPTON IM 35 fluorescence microscope by G 546 filter for excitation and LP590 filter for emission.The sections were photographed using FUJIFILM NEOPAN Professional 400 black and white film.

Results

In order to synthesize the substrate of APN - Ala-AA, we had to use the acid chlorides method for activation of the amino acid carboxy group, because AA is a very weak base (pKa = 1.3) and the milder methods of peptide synthesis appeared to be unsuccessful. The substrate itself did not fluoresce,which shows that the fluorescence properties of AA fully depend on the presence of the free NH_2-group.

The thermally inactivated control sections did not show any non-specific precipitations. In the sections, incubated only in buffer, the tissue autofluorescence was very low upon excitation in the green area of the visible spectrum (510-580 nm).

The new substrate — Ala-AA was readily hydrolysed by the enzyme and the practically insoluble fluorochrome AA precipitated at the enzyme sites, marking them by an intensive orange-red fluorescence. Freeze-drying-celloidin-mounting proved to be necessary for the correct localization of APN. The final reaction product - AA formed amorphous deposits on the sections. In the kidney, the enzyme activity was restricted to the brush borders of the proximal convoluted tubules, whereas the glomeruli were negative (Fig.1). In the small intestine, a high enzyme activity was visualized in the brush border of the enterocytes and, also,in the stromal macrophages (Fig. 2). Exactly the same localization of APN in rat kidney and small intestine has been described previously [4].

Fig. 1. Kidney. APN is localized in the brush-borders of the proximal convoluted tubules (× 500)

Fig. 2. Small intestine. High APN activity in the brush-border of the enterocytes and macrophages of the lamina propria (× 500)

Discussion

Fluorescent methods for the visualization of the enzyme activity are two orders of magnitude more sensitive than the chromogenic methods [8]. Unfortunately, the fluorescent methods for peptidases, proposed thus far, are unsatisfactory, especially when applied to tissue sections. The methoxynaphthylamine-nitrosalicylaldehyde (MNA-NSA) method of D o l b e a r e and S m i t h [2] is based on the production of a fluorescent Shiff base, which forms coarse crystals on the sections [3]. Furthermore, the primary reaction product (MNA) is water-soluble and the accuracy of the localiza-

tion depends on the rate of the second reaction step — the Shiff base formation, which is quick enough only in an acid medium. So, the method is limited to the determination only of acid peptidases [8]. Another drawback is the use of NSA as a coupling agent. It reacts with tissue components, thus causing a considerable background noise to appear, which interferes with the enzyme visualization. A direct fluorescent method for peptidases with substrates, based on naphthylamine AS has been proposed [5], but not accepted in routine practice, because it also suffers a lot of disadvantages — the substrates are water-insoluble; they are very slowly hydrolysed by the enzymes and the reaction product -naphthylamine AS is rough crystalline [4].

The novel fluorogenic substrate for APN, synthesized by us — Ala-AA is used to visualize the enzyme activity in a one-step fluorescent method. The new histochemical method escapes most of the disadvantages of the methods in use. A very important advantage of the method is, that the substrate itself does not fluoresce. Thus, the unavoidable absorption of the substrate by tissues cannot cause the appearance of a fluorescent background. Ala-AA is easily hydrolyzed by APN to release the fluorochrome AA, which precipitates on the enzyme locations in an amorphous form, marking them by a highly intensive and stable orange-red fluorescence. Tissue autofluorescence is very low at such excitation wavelength. So, the fluorescent background is hardly visible on the sections and does not interfere with the enzyme determinations. Another advantage of the new method is that no coupling reagent is needed. Thus, all the drawbacks connected with the use of auxiliary reagents are now escaped.

The fluorochrome, employed in the present study proved to be very successful for the enzyme histochemistry. That is why the syntheses of other peptidasis substrates, based on it are now in progress.

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