Institute of Experimental Morphology and Anthropology

Acta morphologica et anthropologica, 8
Sofia • 2003

A Novel Anthraquinonyl Hydrazine — Based Substrate for the Histochemical Detection of Gamma-Glutamyl Transpeptidase

R. Krieg, M. Dimitrova*, A. Dikov**

Institute of Anatomy II, Friedrich Schiller University Jena, Jena
**Institute of Experimental Morphology and Anthropology, Bulgarian Academy of Sciences, Sofia*

A new substrate for gamma-glutamyl transpeptidase (GTP; EC 2.3.2.2) — L-Glu-4-(1-anthraquinonyl hydrazide) is synthesized and applied for the histochemical detection of the enzyme in tissue sections from rat and human organs. The enzyme hydrolysis liberates the water insoluble deeply red 1-anthraquinonyl hydrazine, which marks the enzyme locations. The newly developed method for GTP does not use any auxiliary reagents in the incubation medium and permits the incubation to be performed at the optimal pH of the enzyme. A second reaction step — incubation in an acid medium with 4-dimethylamino benzaldehyde is performed to improve the quality of the preparations. The new method is used with success for the visualization of GTP activity in the organs studied.

Key words: Gamma-glutamyl transpeptidase, anthraquinonyl hydrazine, enzyme histochemistry.

Introduction

Gamma glutamyl transpeptidase (GTP; EC 2.3.2.2) catalyses the transfer of gamma-glutamyl moieties from different peptides, including glutathione, to other peptides, amino acids or water. It is a membrane-associated transferase with an alkaline pH optimum of about 8.2. GTP is widely distributed in mammalian organs and tissues (for review see [5]) and is found to increase its activity in some tumor cells [8, 9, 11]. Histochemically, GTP is usually demonstrated using gamma-L-Glu-2-naphthylamide or gamma-L-Glu-4-methoxy-2-naphthylamide as substrates with different diazonium salts as visualization agents according to the azo-dye method [4, 10]. Previously, we introduced a convenient tetrazolium method for the histochemical visualization of GTP [3]. However, no histochemical method without any auxiliary reagents in the incubation solution is still available for GTP.

In the present study we describe the synthesis of a novel substrate for GTP - L-Glu-4-(1-anthraquinonyl hydrazide) (Glu-AH) and its use for the histochemical detection of the enzyme in a procedure not using auxiliary reagents in the incubation medium. We give also a possibility of improvement of the histochemical picture by a second incubation step and present the localization of GTP in several rat and human organs, obtained by the newly developed method.

Material and Methods

Synthesis of the substrate

The colorful compound 1-anthraquinonyl hydrazine was synthesized by boiling 1-chloroanthraquinone (Merck, Darmstadt, Germany) and hydrazine hydrate in pyridine after M o e h l a u [7]. The 1-anthraquinonyl hydrazine was coupled with Z-L-Glu-OBzl (Bachem, Heidelberg, Germany) by the dicyclohexylcarbodiimide method [1]. The two protective groups of the amino acid (Z-group and the benzyl ester group) were cleaved simultaneously by a catalytic hydrogenation in ethanol/HCl with Pd on activated carbon as a catalyst. Thus the substrate - L-Glu-4-(1-anthraquinonyl hydrazide) (Glu-AH) was obtained as a HCl-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. Pieces of human kidney cortex were taken at autopsy from a person, killed in an accident, 12 hours after the death and were frozen in liquid nitrogen too. 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 1 mM substrate (Glu-AH) (pre-dissolved in a minimal amount of dimethyl formamide), 5 mM glycyl-glycine and 0.1 M Tris/HCl buffer, pH 8.2. The incubation was carried out at 37°C for 30 to 90 minutes (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 4-dimethylamino benzaldehyde. The second incubation was performed at room temperature for 4 to 6 hours. Then, the sections were post-fixed in neutral formaline and embedded in glycerol-jelly.

Two types of controls were prepared — thermally inactivated control sections were heated at 80°C for 10 min in water and incubated in the complete substrate solution, whereas the other controls were incubated only in buffered 4-dimethylamino benzaldehyde at pH 4.5.

Results

Thermally inactivated controls did not show any colorful reaction product. The other controls also had no precipitation artifacts.

The substrate — Glu-AH was quickly hydrolyzed by the enzyme to release the deeply colored reaction product — 1-anthaquinonyl hydrazine, which precipitated on the sites of the enzyme activity and marked them in a red color. However, those preparations showed a tendency to distort upon storage, because the colorful precipitation in them used to become crystalline in time. So, we intended a second incubation with 4-dimethyldminobenzaldehyde in an acid medium (acetate buffer, pH 4.5), which was aimed to convert the 1-anthaquinonyl hydrazine into the respective hydrazone (4-dimethylaminobenzylidene-1-anthraquinonyl hydrazone) — a dark blue highly amorphous compound. Thus, the preparations became more contrasted and were stable on storage (permanent preparations). Good results were obtained

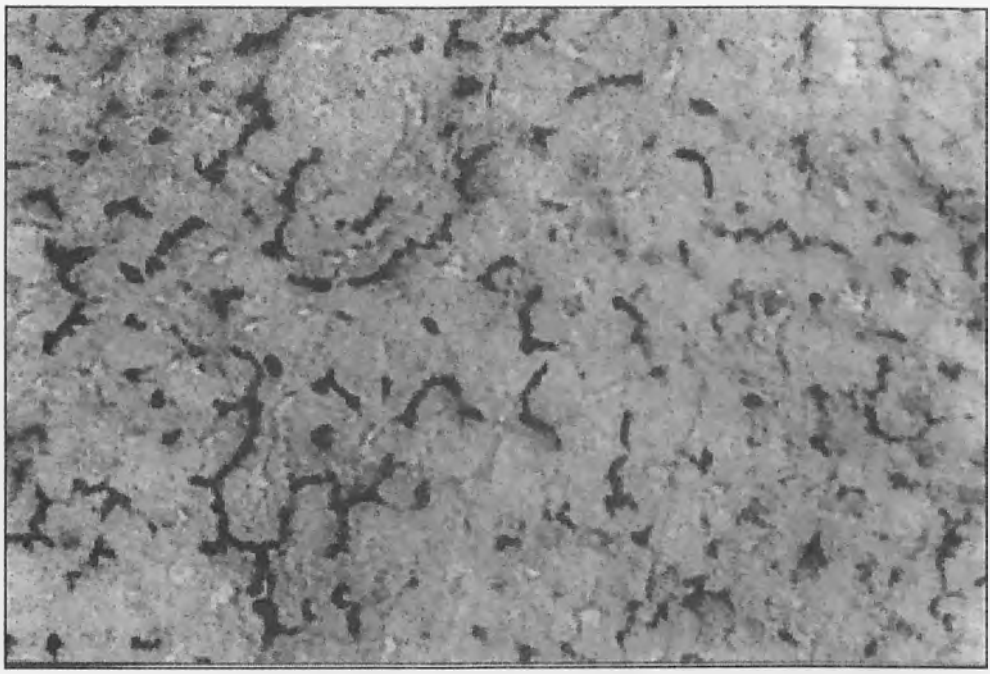

Fig. 1. GTP in the rat liver. Freeze-dried celloidin-mounted section. A small quantity of the final reaction product is visible in the bile capillaries (× 500)

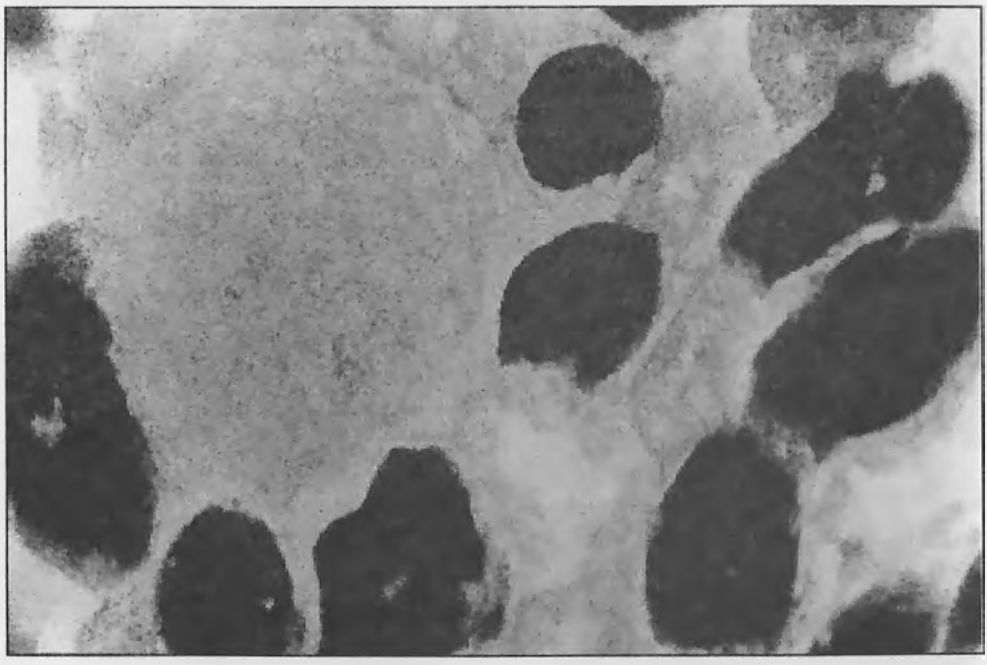

Fig. 2. GTP in the rat kidney. Fresh-frozen acetone-fixed section. A very strong reaction for GTP in the brush borders of the convoluted tubules. No reaction product in the glomeruli (× 500)

Fig. 3. GTP in the human kidney. Fresh-frozen acetone-fixed section. Large quantities of hydrazone in the brush borders and the basal membranes of the renal convoluted tubules (× 500)

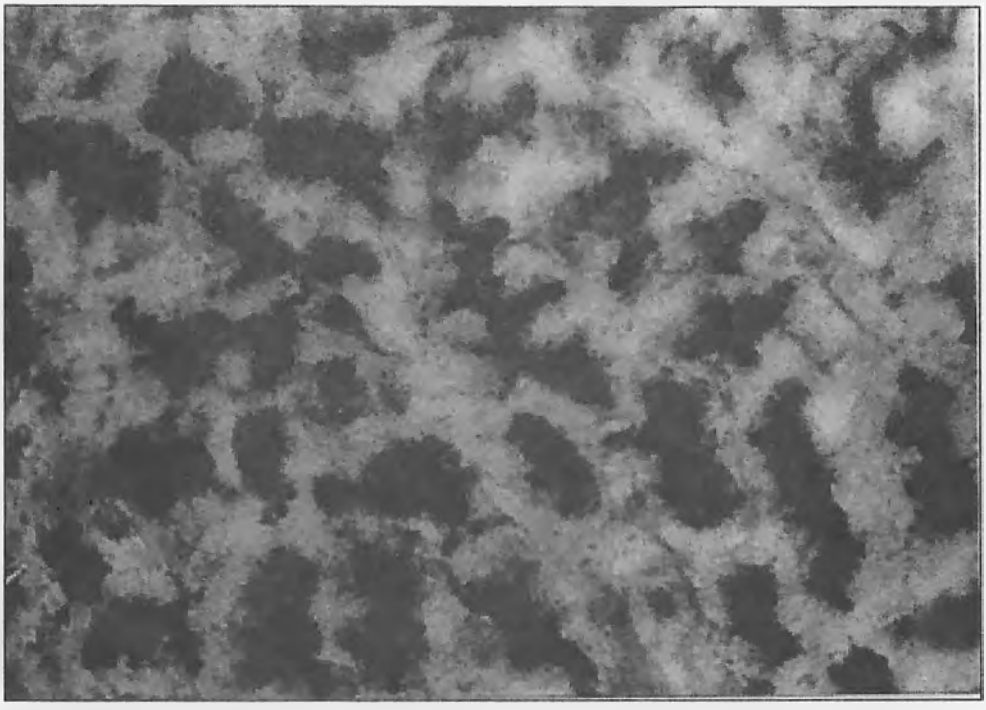

Fig. 4. GTP in the rat pancreas. Freeze-dried celloidin-mounted section. The main reaction for GTP is visible in the apical poles of the acinar cells (× 500)

in the freshly frozen acetone fixed sections for all the organs studied except for the liver and pancreas, where the results were better in the freeze-dried celloidin-mounted sections. In the liver (Fig. 1) the small quantity of the final reaction product was observed in the bile canaliculi and only single sinusoidal cells were GTP-positive. These results are in agreement with the previous findings, that GTP has a low activity in the rat liver [5]. In the rat kidney (Fig. 2) a very strong reaction for GTP was seen in the epithelial cells of the renal convoluted tubules, whereas the glomeruli were perfectly negative. In the human kidney (Fig. 3) the same pattern of GTP was observed, however, the basal membranes of the tubules were well accentuated. In the rat pancreas (Fig. 4) a considerable quantity of hydrazone was visible in the apical parts of the acinar cells. In the epididymis (Fig. 5) the enzyme activity was located in the principal cells of the tubules, including stereocilia and the sperms within the channels were also GTP-positive.

Discussion

Gamma-glutamyl transpeptidase is an enzyme of a vast scientific interest, since it plays a significant role in degradation of natural polypeptides, the most important of which are glutathione and its derivatives. Other functions of the enzyme have also been suggested (for review see [6]). However, in order to understand the multiple functions of GTP it is important to know its precise localization in mammalian organs, the main tool for that purpose still remains the activity histochemistry. Till now, the mostly used histochemical method for the visualization of GTP is the azo-dye procedure, which suffers a lot of drawbacks (for review see [2]). The most im-

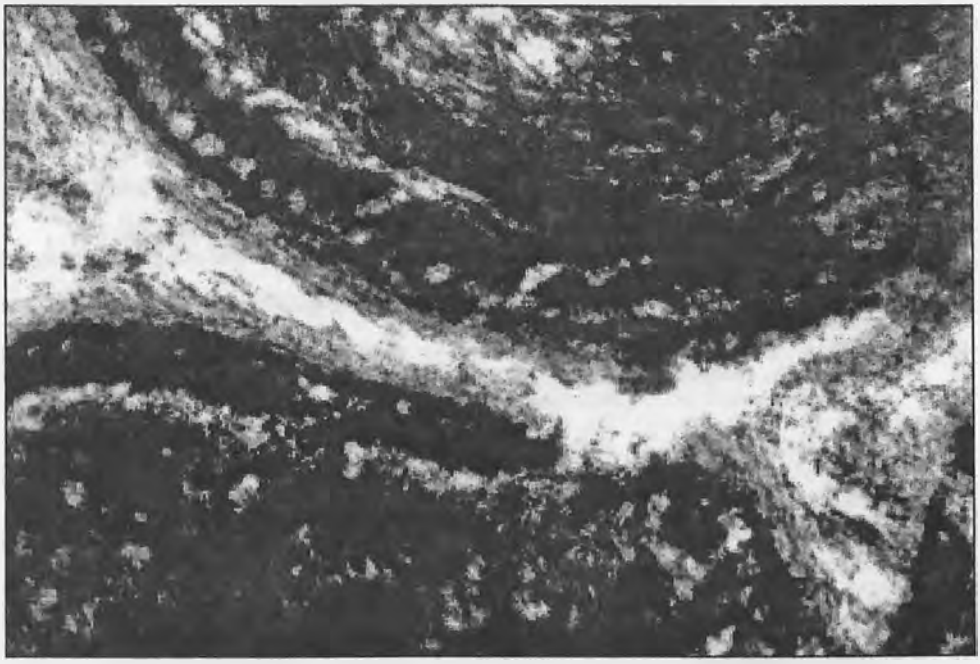

Fig. 5. GTP in the rat epididymis. Fresh-frozen acetone-fixed section. A very strong activity of GTP in the stereocilia and the whole principle cells of the channels, as well as in the spermatozoa within the channels (× 500)

portant disadvantage of this method is that the incubation can not be carried out at the optimal pH of the membrane-bound alkaline peptidases.

In the present work we propose a new method for GTP, which does not use any auxiliary reagent in the incubation medium. This point might be regarded as a very important advantage, because any additional compound in the solution may inhibit the enzyme at a certain extent (even tetrazolium salts, which are usually considered to be very weak enzyme inhibitors). Furthermore, the newly developed method permits the incubation at the optimal pH of GTP — 8.2. The novel GTP substrate — Glu-AH was readily hydrolyzed by the enzyme to release the deeply red water-insoluble 1-anthaquinonyl hydrazine, which marks the enzyme locations. We performed also a second incubation, in which 1-anthraquinonyl hydrazine is converted into a hydrazone with 4-dimethylaminobenzaldehyde, but this additional step is only for the sake of improvement of the preparations quality. The finally obtained preparations are of a very good contrast with the dark blue perfectly amorphous hydrazone. Moreover, they are permanent, i.e. they do not change upon storage.

With the newly developed method we managed to localize GTP in the same locations in the rat and human organs as it was described before. So, the new method, proposed here, is very promising for future application in other membrane-bound peptidases histochemistry as well.

Acknowledgement. This work was supported by Deutsche Forschungsgemeinschaft, Bonn — Germany.

References

1. Bodanszky, M., A. Bodanszky. The dicyclohexylcarbodiimide method. — In: The practice of peptide synthesis. Academie-Verlag Berlin, 1985, 143-144.
2. Dikov, A., R. Gossrau, H.-G. Frank. Menadiol diphosphate, a new substrate for non-specific alkaline phosphatase in histochemistry and immunohistochemistry. — Histochemistry, **94**, 1990, 217-223.
3. Dikov, A., M. Dimitrova, R. Krieg, K.-J. Halbhuber. New tetrazolium method for the histochemical demonstration of gamma-glutamyl transpeptidase. — Cell. Mol. Biol., **45**, 1999, 241-248.
4. Gossrau, R. Investigation of proteinases in the digestive tract using 4-methoxy-2-naphthylamine (MNA) substrates. — J.Histochem.Cytochem., **29**, 1981, 464-480.
5. Gossrau, R. Cytochemistry of membrane proteases. — Histochem. J., **17**, 1985, 737-771.
6. Meister, A., S. S. Tate. Glutathion and related gamma-glutamyl compounds: biosynthesis and utilization. — Ann. Rev. Biochem., **45**, 1976, 559-604.
7. Moehlau, R. Ueber eine neue Synthese der Anthrachinonyl-hydrazin. — Chem.Ber., **45**, 1912, 2244-2247.
8. Power, C. A., S. A. Griffiths, J. L. Simpson, Y. Laperche, G. Guellaen, M. M. Manson. Induction of gamma-glutamyl transpeptidase mRNA by aflatoxin B 1 and ethoxiquin in rat liver. — Carcinogenesis, **8**, 1987, 737-740.
9. Russell, J. J., E. F. Staffeldt, B. J. Wright, A. Prapuolenis, B. A. Carnes, C. Peraino. Effects of rat strain, diet composition and Phenobarbital on hepatic gamma-glutamyl transpeptidase histochemistry and on the induction of altered hepatocyte foci and hepatic tumors by diethylnitrosamine. — Canser Res., **47**, 1987, 1130-1134.
10. Ruttenburg, A. M., H. Kim, J. W. Fishbein, J. S. Hanker, A. L. Wassercrug, A. M. Seligman. Histochemical and ultrastructural demonstration of gamma-glutamyl transpeptidase activity. — J. Histochem. Cytochem., **17**, 1969, 517-526.
11. Sepulveda, A. R., G. M. Habib, A. Damjanov, S. Matacic, I. Damjanov, R. M. Lebovitz, M. W. Lieberman. Expression of gamma-glutamyl transpeptidase in midgestation mouse yolk sac and mouse visceral yolk sac carcinoma cells. — Exp. Cell Res., **219**, 1995, 494-498.