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Fluorescent Methods for the Histochemical Demonstration of Esterhydrolases

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A short review is made on the attempts to develop suitable fluorescent procedures for the histochemical detection of the enzymes, hydrolyzing organic or inorganic acids esters, i.e. the ester hydrolases. The main requirements, which must fulfill a fluorogenic substrate for such an enzyme, are summarized. The major directions for the design and development of new fluorescent methods for the above enzymes are browsed. Personal observations on the applicability of some of the possible methods are also included. A conclusion is drawn out, that the principles for developing new methods for these enzymes are already established and it remains to look for appropriate fluorochromes to synthesize new substrates and test them.

Key words: esterhydrolases, fluorogenic substrates, fluorescent methods, enzyme histochemistry.

Fluorescent methods for the visualization of enzyme activity have many advantages in comparison to chromogenic methods. They are two orders of magnitude more sensitive, small quantities of the final reaction product are needed to localize the enzyme, the fluorescent emission of the probe can be observed at a zero background noise [8, 26]. So, an important direction in the development of enzyme histochemistry is design of new fluorogenic substrates and their use for the visualization of enzymes.

The first fluorescent histochemical procedure proposed by B u r s t o n e [2] was intended for alkaline phosphatase. The method allowed precise enzyme localization, but due to the expensive substrate, it was not accepted for routine use.

After that, many fluorogenic substrates for non-specific esterases (nE), non-specific acid (aP) and alkaline (alkP) phosphatases were synthesized and tested. Some of them met applications in biochemistry, flow cytometry, for the visualization of isoenzyme bands at isoelectric focusing, etc. However, in enzyme histochemistry the requirements for the substrates are difficult to fulfill. Those requirements formulated originally by S t o w a r d [24] and updated for fluorescent methods by R a a p [20] are as follows: 1. The final reaction product should localize the sites of the enzyme activity on a cell level. 2. The fluorochrome as a final reaction product should have: high quantum yield, high photostability, an excitation maximum at $\lambda > 450$ nm (because if the excitation is possible only in the low ultraviolet range, the highly energetic irradiation may cause its decomposition), a large Stokes shift (allowing to escape the interference of the tissue

autofluorescence, which is principally characterized by low Stokes shifts [1]). 3. The samples should be reasonably permanent to allow observations and studies.

The substrates for nE, aP, alkP and arylsulfatases are based on fluorochromes, which possess free hydroxyl group in their structures. This group is to be esterified by organic acids (acetic, chloroacetic, butyric) to obtain substrates for nE or by inorganic acids (ortophosphoric, sulfuric) to obtain substrates for nP and arylsulfatases respectively. The widely used fluorochromes, such as fluorescein, methylumbeliferon, unsubstituted naphthols and the recently synthesized furanone [3, 16] and acridine [15] derivatives, have a considerable water solubility and cannot be used in enzyme histochemistry. One possible solution of the problem with the solubility of those fluorochromes is the gel incubation media. Substrates based on methylumbeliferone were used for the visualization of the enzymes in cells, fixed to glass slides and embedded in a half-dry agar gel [7]. Even though, a notable delocalization of the reaction product was still observed. Fluorescein suffers of two more disadvantages – a very low photostability and electron spectra, which closely overlap with those of the tissue fluorescence [9]. Fluorinated methylumbeliferones are less soluble and have better fluorescence properties than the non-fluorinated compound. Nevertheless, they can't be used in enzyme histochemistry [6]. More useful for histochemistry are the substrates, based on naphthols AS – BI, - MX, - TR etc. However, in alkaline media the hydroxyl group exists in a salty form, which causes a substantial water solubility of the compound. At acid pH values the hydroxyl group is protonated and the final reaction product is practically insoluble in water. So, the above compounds can be used as the bases of substrates only for acid hydrolases. Yet, the fluorescence intensity decreases in acid solutions, thus depriving the fluorescent method of its greatest advantage – the sensitivity.

Summing the above, it becomes clear, that the fluorescent methods, which do not use capture reactions, cannot be applied in the enzyme histochemistry, because of the high solubility, highly crystalline appearance and low photostability of the fluorochromes, employed thus far. According to the capture reaction concept, the fluorophore, liberated upon the enzyme hydrolysis should couple quickly with a capture agent to give a practically water-insoluble fluorescent end product, which precipitates at the sites of the enzyme activity and marks them. A capture agent should fulfil the following requirements: 1. It should couple quickly to the primary reaction product upon the incubation conditions. 2. It should not react with the tissues by producing fluorescent compounds or, if such compounds result, their fluorescence should be in such spectral area, where it could not interfere with the specific emission. 3. Non-fluorescent capture agents should be preferred [20].

A capture procedure, which is considered promising for the histochemical demonstration of hydrolases is the tertazolium reaction, based on the cyan-substituted tertazolium salts (TS), synthesized by S t e l l m a c h [23]. The substrate represents a compound, which upon the enzyme hydrolysis gives a product of a high reduction potential. This primary product reduces the tetrazolium salt, supplied in the incubation medium to an insoluble highly fluorescent formazan. The Stellmach's TS are reduced to formazans, fluorescing brilliant red. Unfortunately, those formazans are coarse crystalline and their crystals cannot be diminished by neither of the known histochemical techniques. In our laboratory, the synthesis of new tetrazolium salts with long saturated chains (hexyloxy-, pentyloxy- or isopentyloxy-groups) was attempted [4]. The assumption was that the long mast chain might make formazans less crystalline. It was shown that such a chemical treatment leads to the formation of microcrystalline, but not amorphous formazans. A conclusion was drawn out, that other groups, reducing the crystals to a greater extent must be used. In brief, the fluorescent formazan conception is still to be explored in the future.

A fluorescent procedure for the visualization of alkaline phosphatase (alkP) with the use of naphtol – AS-MX phosphate as a substrate and the diazonium salt Fast Red TR, was proposed [25]. The authors assert that the azo dye fluoresces in brilliant red and allows a specific localization of alkP in fixed mouse embryos. Other possibilities of using naphthol-AS-based substrates in combination with different diazonium salts to produce highly fluorescent product were recently discussed by H a l b h u b e r et al. [10]. Diasonium salt Fast Red Violet LB was used in combination with 5-bromo-4-chloro-indolylphosphate as a substrate for alkP. Without diazonium salt, this substrate would produce non-fluorescent indigo [22]. The azo-group is known as one of the powerful quenchers of the fluorescence [8, 26]. That is the main reason why the fluorescent emission of azo-compounds is usually doubted. Some authors assume that the fluorescence in such cases is due to certain impurities, always present in the commercial diazonium salts [5]. Even if this is not the case, the diazonium salts are readily absorbed by the tissue components and can react with them, as well. This would lead to the appearance of a strong fluorescent background [13]. Thus, the modification of azo dye technique for the fluorescent observation is still discordant.

Another not much exploited possibility for the fluorescent visualization of ester hydrolases, represents the classical Gomori method. Using the idea, originally introduced by R a a p and van D u j i n [21], M u r r a y and E w e n [17] use calcein phosphate as substrate for alkP according to a Gomori-type reaction. Calcium ions cause the precipitation of calcein as a highly water insoluble, strongly fluorescent complex. However, the method failed to demonstrate the places with low enzyme activity.

2-Aryl-substituted derivatives of 4(3H)-quinazolinone are well known as fluorescent compositions of inks and enamels. These dyes exhibit stable fluorescence in the solid state. Furthermore, they possess large Stokes shifts and remarkable photostability. H a u g l a n d et al. [11] synthesized 2-(2´-hydroxyphenyl)-4(3H)-quinazolinone (HPQ) and some of its derivatives. The esters with organic (acetic) or inorganic (ortophosphoric, sulfuric) acids of this fluorochrome were regarded as potential substrates for nE, nP and arylsulphatases [18], but they were not tested for the histochemical demonstration of the enzymes concerned. We synthesized HPQ using isotoic anhydride and salicylamide after H a u g l a n d et al. [11]. Then, we synthesized also 2-(2´-phosphoryloxyphenyl)-4(3H)-quinazolinone after N a l e w a y et al. [18] and tested it as a substrate for the histochemical detection of aP in sections from rat organs. Our study showed that those compounds are not applicable, due to the rough-crystalline precipitates, which were formed early during the incubation (not published personal observations). The insertion of certain substituents (e.g. chlorine) results in a higher insolubility of the compound, but increases its crystalline appearance. 2-(5´-Chloro-2´-phosphoryloxyphenyl)-6-chloro-4(3H)-quinazolinone is a commercial product, used for the fluorescent visualization of antibodies, marked with alkP [12, 14, 19]. In immunochemistry, though, the amounts of the end product are too small, which prevents the influence of crystallization. However, the same compound is absolutely inapplicable in enzyme histochemistry. This fact is very unfortunate, since with the use of the above substrate, most of the disadvantages of the substrates in use might be avoided. For example, its solubility is good, whereas the fluorochrome, released by the enzyme activity, is practically insoluble. This property is to be attributed to the presence of an intermolecular hydrogen bond in the fluoro-chrome structure. The quinazolinone is very stable upon excitation – about 500 times more stable than fluorescein. Finally, the fluorescent spectrum of the compound excludes any autofluorescent interference by virtue of its large Stokes shift (λ_{excit} = 360 nm, λ_{emiss} = 530 nm) [14]. In the patent [11] other quinazolinones are also described, with substitutents such as methoxy- and nitro-groups, which might decrease the crystallinity of the compound. Some of them may decline the quantum yield of the fluorochrome, but not sub-

stantially (personal observations). These derivatives might open new possibilities in the application of the quinazolinone-based substrates. Thus, the use of substituted quinazolinones in enzyme histochemistry is still to be tested.

The overview of the available methods for the histochemical study of ester hydrolases reveals the lack of convenient methods for the detection of these enzymes, some of which are very important in the clinical and scientific investigations. Nonetheless, the main bases of the research in this field are already formulated and it remains to test different fluorochromes and different histochemical techniques to find a successful histochemical method.

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