Enzymes as marker molecules

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Immunohistological staining procedures with enzymes as markers make use of enzyme substrate reactions which convert colorless chromogens into colored and insoluble end products. Enzyme labels offer the advantage of instant visualization and great sensitivity. Furthermore, enzyme markers may be utilized for both light and electron microscopic studies. Generally, either *direct* or *indirect* labeling techniques can be employed. Regardless of whether a direct or an indirect immunohistological method will be used, the choice of of the label is often a crucial decision. The most employed enzymes, their advantages, disadvantages as well as the various chromogens and substrates are listed in Table 1.

Table 1: Choice of enzyme labels in immunohistology (further details see *Enzyme cytochemical substrate solutions*)

Enzyme	Detection substrate [a]	Advantage	Disadvantage
Peroxidase (*horseradish*)	DAB (3,3'-diamino-benzidine) *plus* H_2O_2 AEC (3-amino-9-ethyl-carbazole) *plus* H_2O_2 CN (4-chloro-1-naphthol) plus H_2O_2 p-Phenylenediamine dihydrochloride (Hanker-Yates reagent)	Easy conjugation with antibodies giving 1:1 conjugates Good enzyme substrates available Dual or multilabeling of antigens with differently colored substrates	High endogenous peroxidase activities in blood cells and tissues
Alkaline phosphatase (*calf intestine*)	BCIP (Bromo-Chloro-Indolyl-Phosphate *plus* Nitro Blue Tetrazolium Naphthol AS-MX *plus* Fast Red TR Naphthol AS-MX *plus* Fast Blue BB Naphthol AS-BI *plus* New Fuchsin	Good enzyme substrates available giving different colors Sensitive coupled enzyme substrates available	Large conjugation products with antibodies Endogenous enzyme activities in many cells and tissues
Glucose oxidase (*Aspergillus niger*)	NBT (Nitro Blue Tetrazolium) *plus* Phenazine methosulfate INT (Iodophenyl-Nitro-phenyl Tetrazolium) *plus* Phenazine metho-sulfate TNBT (Tetra Nitro Blue Tetrazolium) *plus* Phenazine methosulfate	Good enzyme substrates available No endogenous enzyme activity in mammalian tissues Dual-labeling of anti-gens with differently colored substrates Enhanced sensitivity by use of enzyme ampli-fication with coimmo-bilized HRP as secon-dary system enzyme	Large conjugation products with antibodies Sensitivity lower than with peroxidase or alkaline phospha-tase (disadvantage can be compensated by two-step enzyme amplification or by DAB-nickel method

| Beta-galactosidase (*E. coli*) | BCIG (Bromo-Chloro-Indolyl-Galactoside) *plus* ferri-/ferrocyanide | Good substrate available No interference with mammalian enzyme activity when used at its optimal pH of 7.0 to 7.5 | High molecular weight will give large conjugates with antibodies Endogenous enzyme activity is easier to inactivate than other enzymes |

[a]   The most widely employed substrates are mentioned

Enzymes for immunohistological purposes must be available in pure form. Furthermore, enzymes must be highly specific for a given substrate and possess high catalytic efficiency. Enzymatic activity may depend on several variables such as substrate concentration, pH, salt concentration of the buffer milieu and temperature. When enzymes have to be attached covalently (by chemical linkage) or noncovalently (bridging techniques) to other molecules such as antibodies, their catalytic activity must be conserved. Also, under the experimental conditions of cytochemical staining, enzymes must be stable. Upon reaction with its substrate, an enzyme will form a transient *enzyme-substrate complex* at its prosthetic site (active site) which will finally result in characteristic products. The latter must be insoluble in the subsequent steps and colored in order to be observed in the microscope.

Peroxidases

Peroxidases are found in multiple isozyme forms that catalyse the oxidation of numerous substrates. Due to its high sensitivity and the availability of a variety of colorimetric, fluorescent and chemiluminescent substrates make horseradish peroxidase (HRP; donor:H_2O_2 oxidoreductase I.U.B. 1.11.1.7) the most widely used enzyme marker in immunoserological and histochemical assays. The enzyme is readily purified from the root of the horseradish plant (Amoracia rusticana) and can be purchased from many commercial sources. HRP is a hemoprotein that belongs to the ferroprotoporphyrin goup of peroxidases. HRP is a single chain polypeptide containing 18% carbohydrate. Its molecular weight is approx. 40 kDa; at least seven isozymes of HRP exist. All contain photohemin IX as prosthetic group. HRP interacts with hydrogen peroxide and the resultant complex (HRP-H_2O_2) oxidizes a variety of chromogen electron donors to yield detectable products; it can also utilize chemiluminescent (e.g. luminol) and fluorogenic substrates (e.g. tyramine). A wide variety of hydrogen donors have been utilized in peroxidase assays. An improved assay has been adopted using 4-aminoantipyrine as hydrogen donor. One unit results in the decomposition of one micromole of hydrogen peroxide per minute at 25°C and pH 7.0. The enzyme is stable in the pH range of 5.0 to 9.0, the pH optimum is pH 7.0.

For labeling purposes, only highly purified products should be used, for example HRP with RZ 3 or higher (RZ = Reinheitszahl). RZ is the absorbance ratio A_{403}/A_{275}, a measure of hemin content of the peroxidase. Peroxidase is easily attached to other proteins either covalently (e.g. glutaraldehyde, periodate oxidation) or noncovalently. As with other enzymes, covalent coupling of HRP with antibodies is preferentially done by two-step procedures. Usually, the epsilon-amino groups of lysine and N-terminal amino groups of both proteins (HRP and antibody) are involved in this reaction. Other peroxidases such as from soybean may work as well.

There exist several electron donors which become colored products upon oxidation. The original histochemical staining techniques is based on the oxidation of 3-amino-9-ethyl-

carbazole (AEC) and 3,3'-diaminobenzidine (DAB). The latter is especially useful for light and electron microscopic studies because it gives an amorphous, brownish colored reaction product which is highly insoluble in ethanol and other organic solvents. Oxidation of DAB causes an osmiophilic polymerization product which readily reacts with osmium tetroxide, and, thus, increasing its staining intensity and electron density. Also, several other methods were described for the intensification of oxidized DAB at the light microscopic level.

With respect to DAB as cytochemical tool, the following comment will give a useful information on the versatility of this chromogen in histology: apart from its use as enzyme substrate in cellular immunoperoxidase staining, DAB can be readily photooxidized by fluorescent probes, thus, enabling correlation of fluorescence, light and electron microscopy. Although fluorescent molecules are not in themselves visible in the electron microscope, they can be used to drive the oxidation of DAB to produce an electron dense reaction product (MARANTO AR, 1982). This technique, which is known as *fluorescence photooxidation,* works by exploiting the reactive oxygen which is generated when fluorescent molecules are excited by high-intensity photon illumination.

Alkaline phosphatases (AP)

Alkaline phosphatases, orthophosphoric-monoester phosphohydrolase I.U.B. 3.1.3.1 (alkaline optimum) are widely distributed enzymes in a large number of species and tissues. In mammals, two forms are known which are distributed (a) in a variety of tissues or (b) only in the intestine. The two forms are affected differently by inhibitors and heat inactivators. Usually, endogenous AP activity can be inhibited under milder conditions than those required for peroxidases. While levamisole (1 mM in the substrate buffer) will inhibit tissue alkaline phosphatases, intestinal AP may be inhibited by treatment of tissue sections with acetic acid or with periodic acid followed by potassium borohydride.

AP conjugates are preferred over HRP conjugates in immunohistological assays where high endogenous peroxidase activities are difficult to be inhibited. From the known phosphatases, calf intestinal alkaline phosphatase is the label of choice because endogenous activity of non-intestinal AP can be easily inhibited by 1 mM levamisole. The enzyme is isolated from the intestinal mucosa and can be purchased from several commercial sources, e.g. 1,000 to 6,000 units/mg protein. One unit is the amount of enzyme which catalyzes the hydrolysis of one micromole of p-nitrophenyl phosphate per minute at 37°C and pH 9.8. The molecular weight of alkaline phosphatase is about 140 kDa. The enzyme is stable in the pH range of 6.5 to 11.0 and at 4°C for at least two years.

Alkaline phosphatase (AP) is readily conjugated with antibodies. For labeling purposes, AP with high specific activity should be used. While AP conjugates are often employed in serological immunoassays, the unlabeled anti-alkaline phosphatase (APAAP) procedure is preferred in immunocytochemistry even if the molecular weights of those complexes are quite high (approx. 560 kDa). The APAAP technique is particularly recommended for use on blood and bone marrow smears. Endogenous alkaline phosphatase activity can be inhibited by the addition of 1 mM levamisole to the substrate solution. In tissue preparation from the intestine, however, AP is not adequately inhibited by levamisole. In that case, the endogenous enzyme activity may be inhibited by treatment of the tissue sections prior to staining with acetic acid (or with periodic acid), followed by potassium borohydride.

Alkaline phosphatase is a generic name for phosphomonoesterases that hydrolyze orthophosphate at an alkaline pH. For the histochemical detection of these enzymes,

simultaneous coupling azo dyes methods were first developed in 1944 and which have been modified repeatedly since then. In principle, the phosphate-substrate is hydrolyzed and then coupled to the diazonium salt such as Fast Blue BB (see Table 1). In the meantime, a large variety of chromogenic substrates are commercially available for the detection of AP in histochemical staining, and one can chose between different colors. Some of them are even stable in organic solvents.

Glucose oxidase (GOD)

Glucose oxidase (GOD), β-D-glucose:oxygen 1-oxidoreductase I.U.B. 1.1.3.4, is isolated from *Aspergillus niger*. The enzyme catalyzes the oxidation of β-D-glucose, producing hydrogen peroxide and gluconic acid. GOD is a dimeric flavoenzyme consisting of two identical polypeptides which are disulphide-linked. Each subunit contains 1 mole of flavin-adenine dinucleotide (FAD). The enzyme is glycosylated (approx. 12% carbohydrate) and has a molecular weight of 160 kDa. Flavin undergoes reduction by accepting hydrogen from the oxidation of glucose and passing it on to a receptor such as oxygen; pH optimum is pH 5.5 with broad range of 4.0 to 7.0. One unit catalyzes the oxidation of 1 micromole of glucose to gluconic acid per minute at 25°C and pH 7.0 coupled with peroxidase and o-dianisidine. The enzyme can be purchased from several commercial sources, e.g. 260-350 U/mg protein, mainly as freeze dried powder; the enzyme is stable at 4°C for many years. It is important to choose GOD preparations with low catalase activity because catalase will destroy hydrogen peroxide in the reaction sequence.

In mammalian tissue, there is no GOD activity making this enzyme an excellent choice for tissue immunostaining with unambigous staining patterns. GOD is readily conjugated to proteins such as antibodies and can be utilized in labeled and unlabeled (bridge) antibody techniques as well as in avidin-biotin technology. There are several tetrazolium salts whose formazan reaction products are suitable for histochemical stainings. The chromogens Nitro Blue Tetrazolium (blue to black end product), Iodophenyl-Nitrophenyl Tetrazolium (red end product) and Tetra Nitro Blue Tetrazolium (brown end product) result in stable permanent preparations. Due to the differently colored end products, GOD staining is useful for both double or multi labeling experiments and cellular labelings together with HRP as marker.

Finally, the detection of tissue molecules with GOD as marker enzyme can be greatly enhanced by a modified technique which may be called *immobilized two-step enzyme system* where HRP and GOD are coimmobilized at the same cellular sites by immunological means or by bridge techniques. In this coupled enzyme system, GOD will function as peroxide donor in the peroxidative reactions of HRP with diaminobenzidine as chromogen. Then, the reaction product of the second enzyme (oxidized DAB) can be further intensified by addition of nickel sulfate and ammonium chloride to the substrate mixture.

Beta-galactosidase (ß-Gal)

Beta-galactosidase (β-Gal), β-D-galactoside galactohydrolase I.U.B. 3.2.1.23, is isolated from *Escherichia coli* with a molecular weight of about 540 kDa; β-Gal can hydrolyse a variety of galactopyranoside derivatives giving either water-soluble or water-insoluble products. The enzyme is tetrameric being composed of four identical subunits of 135 kDa, each with an active site which may be independently active; pH optimum in the range of 6.0 to 8.0. One unit causes the hydrolysis of one micromole of o-nitrophenyl-β-D-galactopyranoside per

minute at 25°C and pH 7.5. The enzyme is stable for several months when stored at 4-5°C (note: the enzyme is not stable when diluted, dilutions should be used immediately). β-Gal can be purchased as lyophilized powder or as suspension in 1.6 M ammonium sulfate; store at 2-8°C.

β-Gal has been successfully conjugated by a variety of cross-linkers to antibodies). This enzyme is mainly used in ELISA techniques but rarely in immunohistological stainings. Nevertheless, β-Gal is a sensitive and useful marker in applications where endogenous enzyme activities (f.e. peroxidase, alkaline phosphatase) are a persistent problem. The pH optimum of β-Gal from *Escherichia coli* is different from human β-Gal, and interferences can be avoided. Moreover, β-Gal is inactivated at temperatures which are reached during paraffin embedment, and appropriate staining of tissue sections from paraffin embedded specimens can be obtained.

The enzyme catalyzes the hydrolysis of ß-galactosides including lactose. The histochemical staining is based on 5-bromo-4-chloro-3-indolyl-ß-D-galatopyranoside (X-Gal) as chromogenic substrate; ß-Gal cleaves the glycosidic linkage in X-Gal, thus, producing a soluble and colorless indoxyl monomer. Subsequently, liberated indoxyl moieties form a dimer which is nonenzymatically oxidized. Dimerization and oxidation reactions require transfer of an electron which is facilitated by ferric and ferrous ions. The resultant halogenated indigo is a stable and insoluble dark blue compound.

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