BASIC ENZYMOLOGY

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■ Immunoenzymatic staining methods utilize enzyme-substrate reactions to convert colorless chromogens into colored end products. Of the enzymes used in these applications, only horseradish peroxidase and calf intestine alkaline phosphatase will be considered in some detail. Because of its low sensitivity, glucose oxidase *(Aspergillus niger)* is only rarely used today.

This chapter will also discuss the various chromogens and substrates that can be used in conjunction with peroxidase and phosphatase, along with suggested procedures for the preparation of some substrate solutions.

ENZYMES

■ Enzymes are proteinaceous catalysts peculiar to living matter. Hundreds have been obtained in purified and crystalline form. Their catalytic efficiency is extremely high–one mole of a pure enzyme may catalyze the transformation of as many as 10,000 to 1,000,000 moles of substrate per minute. While some enzymes are highly specific for only one substrate, others can attack many related substrates. A very broad classification of enzymes would include hydrolytic enzymes (esterases, proteases), phosphorylases, oxidoreductive enzymes (dehydrogenases, oxidases, peroxidases), transferring enzymes, decarboxylases and others.

Enzymatic activity is dependent upon several variables, such as enzyme and substrate concentrations, pH, salt concentration of the buffer milieu, temperature and light. Many enzymes also possess non-proteinaceous chemical portions termed prosthetic groups. Typical prosthetic groups are the iron-protoporphyrin of peroxidase, and biotin of $C0_2$ transferases. In addition, many enzymes require the presence of metal ions such as Mg^{++}, Mn^{++} and Zn^{++}, which function as electrophilic (electron-attracting) agents.

The general formula, which describes the reactions of an enzyme with its substrate, may be written as follows:

1. Enzyme (E) + Substrate (S) = ES complex
2. ES $\rightarrow$ E + Products (P)

Thus, before formation of the product, a transient enzyme-substrate complex is formed at the “active site” (prosthetic group) of the enzyme.

Substances that interfere with the specific binding of the substrate to the prosthetic group are specific inhibitors and differ significantly from agents which cause nonspecific denaturation of enzyme (or any protein). Two basic types of inhibition are recognized, competitive inhibition and non-competitive inhibition. Competitive inhibition is the result of a reversible formation of an enzyme-inhibitor complex (EI):

E + Inhibitor (I) + S = EI + S

The formation of the complex EI can be reversed by a change in the concentration of either the substrate or the inhibitor, unless the affinity of I for E is greater than of S for E. The action of carbon monoxide or azides on the heavy metals of respiratory enzymes is a typical example of competitive inhibition.

In noncompetitive inhibition, the inhibition depends solely on the concentration of the inhibitor and generally, is not reversible. Noncompetitive inhibition may or may not involve the prosthetic group of the enzyme and manifests itself by slowing down or halting the velocity of the enzyme’s reaction upon the substrate.

E + I + S $\rightarrow$ E I S

Selecting the enzyme most suitable for a particular immunohistochemical application depends on a number of criteria:

- The enzyme should be available in highly purified form and be relatively inexpensive.
- Conjugation (covalent binding to antibody or avidin, for example) or noncovalent binding should not abolish enzyme activity, although it may diminish it.
- The bound enzyme should be stable in solution.
- Endogenous enzyme activity should interfere only minimally with specific antigen-related staining.
- The products of the enzymic reactions should be readily detectable and stable.

Horseradish peroxidase and calf intestine alkaline phosphatase meet most of these criteria and the following will list their properties in more detail.

■ **HORSERADISH PEROXIDASE (HRP):** This enzyme (molecular weight 40 kD) is isolated from the root of the horseradish plant. HRP has an iron-containing heme group (hematin) as its active site and in solution is colored brown. The hematin of HRP first forms a complex with hydrogen peroxide and then causes it to decompose resulting in water and atomic oxygen. HRP oxidizes several substances, two of which are polyphenols and nitrates. Like many other enzymes, HRP and some HRP-like activities can be inhibited by excess substrate. The complex formed between

HRP and excess hydrogen peroxide is catalytically inactive and in the absence of an electron donor (e.g. chromogenic substance), is reversibly inhibited. It is the excess hydrogen peroxide and the absence of an electron donor that brings about quenching of endogenous HRP activities. Cyanide and azide are two other strong (reversible) inhibitors of HRP.

HRP can be attached to other proteins either covalently or noncovalently. The covalent binding of HRP to other proteins can be performed using either one-step or two-step procedures involving glutaraldehyde. The chemical 4,4'-difluoro-3,3'-dinitrophenyl sulfone (FNPS) is less commonly used for this purpose. In all cases, the epsilonamino groups of lysine and N-terminal amino groups of both proteins are involved in this reaction. The two-step conjugation procedure is preferred because, relative to the antibody molecule, the HRP molecule has few reactive groups. As a consequence, adding glutaraldehyde to a solution containing an admixture of HRP and antibody will result in more antibody molecules being conjugated to each other than to the enzyme. In the two-step procedure, HRP reacts with the bifunctional reagents first. In the second stage, only activated HRP is admixed with the antibody resulting in much more efficient labelling and no polymerization.

HRP is also conjugated to (strept)avidin using the two-step glutaraldehyde procedure. This form is used in the LAB and LSAB procedures for example. Conjugation with biotin also involves two steps, as biotin must first be derivatized to the biotinyl-N-hydroxysuccinimide ester or to biotin hydrazide before it can be reacted with the epsilonamino groups of the enzyme.

Noncovalent binding of HRP to antibody (also known as unlabelled antibody binding) is described in great detail by Sternberger.[1] Instead of the use of bifunctional reagents, IgG-class antibodies to HRP are used to form a soluble semicyclic immune complex consisting of two antibody and three enzyme molecules. The molecular weight of the peroxidase-antiperoxidase or PAP complex is 400–430 kD.

■ **CALF INTESTINE ALKALINE PHOSPHATASE (AP):** Calf intestine alkaline phosphatase (molecular weight 100 kD) removes (by hydrolysis) and transfers phosphate groups from organic esters by breaking the P-0 bond; an intermediate enzyme-substrate bond is briefly formed. The chief metal activators for AP are Mg^{++}, Mn^{++} and Ca^{++}.

AP had not been used extensively in immunohistochemistry until publication of the unlabelled alkaline phosphatase-antialkaline phosphatase (APAAP) procedure.[2,3] The soluble immune complexes utilized in this procedure have molecular weights of approximately 560 kD. The major advantage of the APAAP procedure compared to the PAP technique is the lack of interference posed by endogenous peroxidase activity. Because of the potential distraction of endogenous peroxidase activity on PAP staining, the APAAP technique is recommended for use on blood and bone marrow smears. Endogenous alkaline phosphatase activity from bone, kidney, liver and some white cells can be inhibited by the addition of 1 mM levamisole to the substrate solution,[4] although 5 mM has been found to be more effective.[5] Intestinal alkaline phosphatases are not adequately inhibited by levamisole.

SUBSTRATES AND CHROMOGENS

■ **PEROXIDASE** As described above, HRP activity in the presence of an electron donor first results in the formation of an enzyme-substrate complex, and then in the oxidation of the electron donor. The electron donor provides the "driving" force in the continuing catalysis of H_20_2, while its absence effectively stops the reaction.

There are several electron donors that when oxidized, become colored products and are therefore called chromogens. This, and the property of becoming insoluble upon oxidation, make such electron donors useful in immunohistochemistry.

3,3'-DIAMINOBENZIDINE (DAB) produces a brown end product which is highly insoluble in alcohol and other organic solvents. Oxidation of DAB also causes polymerization, resulting in the ability to react with osmium tetroxide, and thus increasing its staining intensity and electron density. Of the several metals and methods used to intensify the optical density of polymerized DAB, gold chloride in combination with silver sulfide appears to be the most successful.[6]

3-AMINO-9-ETHYLCARBAZOLE (AEC), upon oxidation, forms a rose-red end product which is alcohol soluble. Therefore, specimens processed with AEC must not be immersed in alcohol or alcoholic solutions (e.g., Harris' hematoxylin). Instead, an aqueous counterstain and mounting medium should be used. AEC is unfortunately susceptible to further oxidation and, when exposed to excessive light, will fade in intensity. Storage in the dark is therefore recommended.

4-CHLORO-1-NAPHTHOL (CN) precipitates as a blue end product. Because CN is soluble in alcohol and other organic solvents, the specimen must not be dehydrated, exposed to alcoholic counterstains, or coverslipped with mounting media containing organic solvents. Unlike DAB, CN tends to diffuse from the site of precipitation.

p-PHENYLENEDIAMINE DIHYDROCHLORIDE/pyrocatechol (Hanker-Yates reagent) gives a blue-black reaction product which is insoluble in alcohol and other organic solvents. Like polymerized DAB, this reaction product can be osmicated. Varying results have been achieved with Hanker-Yates reagent in immunoperoxidase techniques.

ALKALINE PHOSPHATASE In the immunoalkaline phosphatase staining method, the enzyme hydrolyzes naphthol phosphate esters (substrate) to phenolic compounds and phosphates. The phenols couple to colorless diazonium salts (chromogen) to produce insoluble, colored azo dyes. Several different combinations of substrates and chromogens have been used successfully.

NAPHTHOL AS-MX PHOSPHATE can be used in its acid form or as the sodium salt. The chromogens Fast Red TR and Fast Blue BB produce a bright red or blue end product, respectively. Both are soluble in alcoholic and other organic solvents, so aqueous mounting media must be used. Fast Red TR is preferred when staining cell smears.

NEW FUCHSIN also gives a red end product. Unlike Fast Red TR and Fast Blue BB, the color produced by New Fuchsin is insoluble in alcohol and other organic solvents, allowing for the specimens to be dehydrated before coverslipping. The staining intensity obtained by use of New Fuchsin is greater than that obtained with Fast Red TR or Fast Blue BB.

OTHER SUBSTRATES AND CHROMOGENS Additional substrates include naphthol AS-BI phosphate, naphthol AS-TR phosphate and 5-bromo-4-chloro-3-indoxyl phosphate (BCIP). Other possible chromogens include Fast Red LB, Fast Garnet GBC, Nitro Blue Tetrazolium (NBT) and iodonitrotetrazolium Violet (INT).

Detailed descriptions and information for the preparation of the most commonly used substrate-chromogen mixtures for HRP[7] and AP[8] as well as their appropriate use and advantages or disadvantages are available.[9-12]

SUGGESTED PROCEDURES FOR SUBSTRATE-CHROMOGEN REAGENTS

PEROXIDASE

AEC SUBSTRATE SOLUTION (recommended for cell smears)

1. Dissolve 4 mg AEC in 1 mL N,N-dimethylformamide.
2. Add 14 mL 0.1 M acetate buffer, pH 5.2 and 0.15 mL 3% hydrogen peroxide.
3. Mix, and filter if precipitate forms.
4. Add solution to tissue and incubate for 5–15 minutes at room temperature.
5. Rinse with distilled water.
6. Counterstain and coverslip with aqueous-based medium.

DAB SUBSTRATE SOLUTION

1. Dissolve 6 mg DAB in 10 mL 0.05 M Tris buffer, pH 7.6.
2. Add 0.1 mL 3% hydrogen peroxide. Mix, and filter if precipitate forms.
3. Add solution to tissue and incubate for 3–10 minutes at room temperature.
4. Rinse with distilled water.
5. Counterstain and coverslip with either organic- or aqueous-based medium.

ALKALINE PHOSPHATASE

FAST RED SUBSTRATE SOLUTION (recommended for cell smears)

1. Dissolve 2 mg naphthol AS-MX phosphate, free acid (Sigma N 4875) in 0.2 mL N,N-dimethylformamide in a glass tube.
2. Add 9.8 mL 0.1 M Tris buffer, pH 8.2.
3. Add 0.01 mL of 1 M levamisole (Sigma L 9756) to block endogenous alkaline phosphatase. (Solution can be stored at 4°C for several weeks, or longer at -20°C.)
4. Immediately before staining, dissolve 10 mg Fast Red TR salt (Sigma F 1500) in above solution and filter onto slides.
5. Incubate for 10–20 minutes at room temperature.
6. Rinse with distilled water.
7. Counterstain and coverslip with aqueous-based medium.

NEW FUCHSIN SUBSTRATE SOLUTION (recommended for tissue sections)

1. Solution A: Mix 18 mL of 0.2 M 2-amino-2-methyl-1, 3-propanediol (Merck 801464) with 50 mL 0.05 M Tris buffer, pH 9.7 and 600 mg sodium chloride. Add 28 mg levamisole (Sigma L 9756).
2. Solution B: Dissolve 35 mg naphthol AS-BI phosphate (Sigma N 2250) in 0.42 mL N,N-dimethylformamide.
3. Solution C: Under fume hood, mix 0.14 mL 5% New Fuchsin (Sigma N 0638, 5 g in 100 mL 2 N HCl) with 0.35 mL of freshly prepared 4% sodium nitrite (Sigma S 2252, 40 mg in 1 mL distilled water). Stir for 60 sec.
4. Mix Solutions A and B, then add Solution C; adjust to pH 8.7 with HCl. Mix well and filter onto slides.
5. Incubate for 10–20 minutes at room temperature.
6. Rinse with distilled water.
7. Counterstain and coverslip with either organic- or aqueous-based medium.

NEW FUCHSIN SUBSTRATE SOLUTION (alternative procedure)

1. Solution A: In fume hood add 0.2 mL of 5% New Fuchsin (Merck 4041, in 2 N HCl) to 0.5 mL of fresh 4% sodium nitrite. Agitate for 30-60 sec. Add 100 mL of 0.05 M Tris buffer, pH 8.7, and 100 µL of 1 M levamisole to block endogenous alkaline phosphatase.
2. Solution B: Dissolve 50 mg naphthol AS-BI phosphate (Sigma N 2250) in 0.6 mL N,N- dimethylformamide.
3. Add Solution B to Solution A and mix well. Filter directly onto slides.
4. Incubate for 10–20 minutes at room temperature.
5. Rinse with distilled water.
6. Counterstain and coverslip with either organic- or aqueous-based medium.

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