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A New Stain for Copper–Protein Complexes: Its Use with Crustacean Hemocyanins

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A cyanide–tetrazolium medium is suggested as a rapid histochemical stain for copper. Red bands clearly mark the site of copper–protein complexes on polyacrylamide gels. The stain is darker, more specific, and more permanent than other methods currently in use and is particularly useful in the electrophoretic characterization of crustacean serum.

In the course of a study of serum proteins in diseased blue crabs, *Callinectes sapidus* (1), we observed faint pink bands on electrophoresed acrylamide gels that had been incubated in tetrazolium media for oxidoreductases. Because the bands appeared only at sites ascribed to hemocyanin by stains for copper and for peroxidatic activity, we speculated that a tetrazolium medium might provide a more satisfactory stain for copper–protein complexes. We had found stains currently in use to be either unstable or not sufficiently sensitive to detect the small amounts of hemocyanin copper in the serum of diseased or otherwise stressed crabs.

Of the available methods, Pearse (2) has said that rubeanic acid (dithiooxamide) is probably the most important in mammalian histochemistry. Cobalt and nickel also form rubeanates, but these salts are soluble in the presence of ethanol and acetate ions, leaving only copper rubeanate as a visible precipitate. Horn and Kerr (3) and Whittaker (4) have used versions of this stain for work with invertebrate hemocyanins. Manwell and Baker (5) preferred the dianisidine (dimethoxy benzidine) stain (6) to the rubeanic in their study of marine arthropods, on the grounds of greater color development. Indeed, Pearse pointed out that for demonstrating copper in invertebrate tissues, the tolidine (dimethyl benzidine) reaction shows a higher degree of sensitivity than any of the other methods, including rubeanic acid. In our hands, however, both tolidine and dianisidine stains produced pale and unstable bands, whereas copper rubeanate bands (3), although not very intense, were stable.

At the time the pink bands were observed, the tetrazolium stains in use contained nitro blue tetrazolium, phenazine methosulfate, and cyanide in a buffer with substrate and nucleotide coenzyme specific for the enzymes under study. Subsequent testing of the first three compounds revealed that copper could be readily detected within 0.5 hr at all pH's tested (4.7–10.0), in acetate, phosphate, and Tris buffers (0.1–0.5 M) and even in distilled water. Shifting the pH toward the alkaline side speeded color development, both of copper bands and of background. Phosphate buffers produced too dark a formazan background; Tris buffers at high concentrations slowed the reaction somewhat, but dilute Tris provided us with a needed measure of control. For uniformity, we selected the buffer rather than distilled water or 1 mM KOH, which were also effective. Optimal proportions of the reactants are reported below.

METHODOLOGY

Electrophoresis

Serum from healthy blue crabs and solutions of three copper–protein preparations of varying purity were subjected to electrophoresis on polyacrylamide-gel columns. Sample concentrations are noted below. Electrophoresis was performed at 4°C on 7% acrylamide, pH 9.1, with photopolymerized stacker and sample gels of 3% acrylamide, pH 5.2. Electrode buffers were Tris–glycine, pH 8.3. Both gel formularies and electrophoretic procedure are based on the work of Davis (7) and have been fully described by Gould and Medler (8). After electrophoresis, the gels were removed from the glass columns and placed in test tubes for staining.

Copper–Protein Samples

All samples were prepared in stacker gel solutions (SG) immediately prior to use.

Blue crab serum samples were made 0.04 ml in 1.0 ml of SG, and 0.30 ml of the resulting sample gel was used per column. The hemocyanin (HCy) content of the serum was taken as 4% and the copper content of crustacean HCy as 0.2% (9,10), resulting in a calculated 0.96 μg of copper applied per column; subsequent analysis of the sample gel solutions by atomic absorption corroborated the calculations within 2% (15.5 μg for 16.0 μg).

Purified ceruloplasmin (Type IV, human serum; 160,000 molecular weight and 7 Cu/mole) (11,12); partially purified uricase (hog liver; 12,000 molecular weight and 1 Cu/mole) (13); and partially purified diamine oxidase (hog kidney; 185,000 molecular weight and 2 Cu/mole)

(13) were also electrophoresed and stained for copper. The enzyme preparations were obtained from Sigma Chemical Company, St. Louis, MO.[1] Analysis by atomic absorption showed that copper was placed on the columns in the following amounts: ceruloplasmin, 1.04 and 0.104 μg; uricase, 2.70 and 1.35 μg; and diamine oxidase, 1.35 and 0.9 μg.

Stain

Reagents. KCN, 0.06 M, pH 8; *p*-nitro blue tetrazolium chloride (NBT), 5 mg/ml in H_2O; phenazine methosulfate (PMS), 3 mg/ml in H_2O; polyvinylpyrrolidone, Type NP-K30 (PVP); and 0.05 M tris(hydroxymethyl)aminomethane–HCl buffer, pH 8 (Tris). NBT and PMS were from Nutritional Biochemicals Corp., Cleveland, OH, and PVP was obtained from General Aniline and Film Corp., New York, NY.[1]

Solution. In a stoppered 50-ml Erlenmeyer flask, 18 ml of KCN and 10 ml of Tris buffer are mixed with about 0.5 g of PVP. Within an hour of use, NBT is solubilized (50 mg in 10 ml of H_2O) and added to the flask. The PMS can be made up ahead of time and stored in a refrigerator for as long as a week, but it must not be added (0.6 ml) to the cyanide–tetrazolium solution until just before use. Proportions should be kept close to (buffer) 5:(KCN) 9:(NBT) 5:(PMS) 0.3.

The whole is thoroughly mixed and poured over the gels; the tubes are stoppered, inverted several times, and placed in the dark at room temperature for 30–40 min. After band development, the gels are thoroughly rinsed in tap water.

Although the pink bands are not dark initially, they will deepen in color with time. It is advisable, therefore, to change the water in which the gels are stored, two or three times at 15-min intervals, to leach most of the as-yet-unreduced tetrazolium from the gels; otherwise the formazan background (reduced tetrazolium) will be too dark. Contrast between bands and background depends largely upon development time and subsequent washings. Final rinse should be with distilled water to minimize formation of air bubbles.

DISCUSSION

Normally pink to deep rose, the copper bands are occasionally edged with yellow. The NBT–PMS combination alone can produce faint yellow bands on an electrophoresed gel after an overnight incubation, without the help of cyanide. The strongly nucleophilic cyanide ion, however, accelerates the displacement of copper from its protein complex and promotes the rapid precipitation of the copper bands.

[1] Use of trade names is merely to facilitate identification. It does not constitute an endorsement by the National Marine Fisheries Service, N.O.A.A.

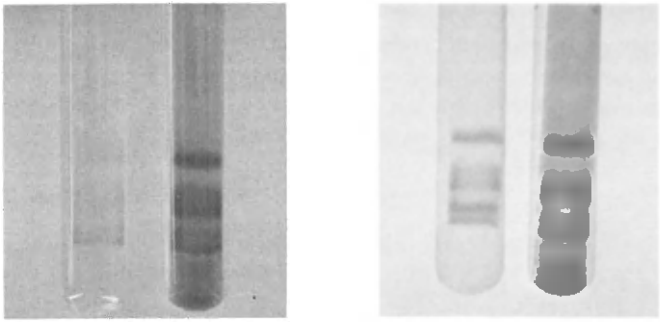

FIG. 1. Copper stains on acrylamide-gel columns, indicating sites of hemocyanin from the serum of blue crab, *Callinectes sapidus*. (A) is a picture taken in reflected light and (B), in transmitted light. In each case, the left-hand column shows copper rubeanate bands deposited by the rubeanic acid stain (3), and the right-hand column shows copper bands deposited by the tetrazolium–cyanide stain described in this report.

Of the copper proteins tested, hemocyanin gave the strongest color deposition for the amount of copper in the sample. Slightly less than a microgram of HCy-Cu produced the three major bands typical of blue crab serum (Fig. 1): The strong β monomer and the two fast α dimers (1). The same pherogram pattern, still easily discernible, can be obtained with less than 0.5 μg of HCy-Cu.

One microgram of ceruloplasmin-Cu produced a single very strong band, approximately twice the width of the strong β-HCy of the blue crab, and 0.1 μg of ceruloplasmin-Cu produced a faint but detectable thin pink band. The other two copper–protein preparations tested, uricase and diamine oxidase, contained hemoglobin and other impurities that obscured the copper bands, both with the cyanide–tetrazolium and with the rubeanic acid stain.

Deep purple bands may also be seen on gels containing the blue crab HCy and stained for copper with this medium, indicating sites of a tetrazolium reductase (14) that does not require pyridine nucleotide coenzyme for activation.

A bleached area immediately surrounding the copper bands is also usually seen and is particularly noticeable when the copper concentration is low, as in the 0.1-μg ceruloplasmin-Cu and in the α-HCy's of nanogram amounts of Cu; indeed, sometimes bleached bands appear with no apparent copper in association. If the incubation is allowed to proceed in the light, the bleached areas become more pronounced against the dark formazan background. This effect should not be attributed to superoxide dismutase (15); it is more probably due to the formation of either H_2O_2 or perhydroxy ions, both of which have been

suggested as intermediates in the transfer of electrons by copper ions in hemocyanin (16) and laccase (17).

Although the work reported here was with acrylamide gels exclusively, the tetrazolium–cyanide medium should be an effective copper stain with other electrophoretic media as well; tetrazolium stains are a tool widely used by workers with starch-gel and agar-gel electrophoresis. Because of the strong evidence for the involvement of imidazole groups in the binding of copper in hemocyanin (18), however, and the weakening of these bonds by photooxidation of histidine residues (19), it may be that photopolymerization of the acrylamide sample gel increases the stain's efficiency by rendering the copper ions more readily removable from the hemocyanin molecule.

The cyanide–tetrazolium stain has proved particularly useful in examining the hemolymph of several crabs other than *Callinectes*. Work is presently under way to compare the number and migration rate of hemocyanins under uniform assay conditions and to discover their gross meric structure.

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