THE USE OF MELDOLABLUE IN ISOZYME STAINS AFTER ELECTROPHORESIS

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1. Introduction

Electron transfer dye staining methods which employ phenazine methosulphate (PMS) as an intermediary catalyst are commonly used for the detection of isozymes after electrophoresis [1]. Most of these staining mixtures contain the tetrazolium salt methyl thiazolyl blue (MTT), which is rapidly reduced by electron donors in the presence of PMS to form a dark blue–purple insoluble formazan. A wide variety of methods has been built up around a standard MTT–PMS mixture for the detection of enzyme reactions which lead to the generation of the reduced forms of the coenzymes NAD and NADP. The same mixture is also used to detect certain oxidases such as xanthine oxidase. However, MTT–PMS mixtures are light sensitive and incubation must be carried out in the dark. Also the stains tend to deteriorate if prolonged incubation is necessary for the detection of weak enzyme activity.

Recently, a new reagent, 7-dimethylamino-1,2-benzophenoxazinium chloride (Meldolablue, Fast New Blue 3R) has been marketed by Boehringer Mannheim GmbH as a substitute for PMS in the determination of reduced pyridine coenzymes. This new compound has been tested in spectrophotometric assay procedures for various dehydrogenases and appears to be relatively much less sensitive to light and more stable than PMS [2]. This paper describes the use of Meldolablue (MLB) instead of PMS in staining mixtures for the detection of isozymes after starch gel electrophoresis or isoelectric focusing in polyacrylamide gels and evaluates the new reagent in direct comparison against PMS. Tests involving 30 different enzymes showed that MLB is generally superior to PMS in its stability and sensitivity as an intermediary catalyst in tetrazolium stains for isozymes.

2. Materials and methods

Tests were carried out on enzymes present in human red cell lysates and tissue homogenates using the methods of enzyme electrophoresis described [1,3]. The intensity and clarity of the isozyme patterns obtained with the standard MTT–PMS stains were compared with those obtained when MLB was substituted for PMS. The working pH of the staining mixtures used for the various enzymes (listed in table 1) covered a wide range, but the effects of pH on the stability and sensitivity of MLB and PMS were also deliberately compared using a simple agar spot plates method with NADH as the electron donor. Agar plates (2 mm thick) were made up with Difco Agar Noble at final conc. 1% (w/v) dissolved in 0.5 M citrate/phosphate buffer [4] ranging from pH 3.0 to 8.0 at intervals of one pH unit, which contained MTT (100 μg/ml) and PMS (100 μg/ml) or MLB (2 μg/ml). A series of circular wells (2 mm diam.) was cut in each plate and the reaction was initiated by the introduction of 50 μl aliquots of standard NADH solution (ranging from 1 μM to 1 mM) to the wells in each plate at timed intervals. The size of the formazan rings around each well was measured after a standard period of incubation in the dark at 37°C and the intensity of background staining on each plate was assessed visually.

3. Results

Preliminary experiments were carried out using

human alcohol dehydrogenase isozymes as the test enzyme. PMS was used at a standard 100 μg/ml and MLB was substituted at various concentrations (100 ng/ml to 100 μg/ml) in the staining mixture. The MTT concentration was kept fixed at 100 μg/ml as were the other ingredients of the stain (viz. ethyl alcohol or amyl alcohol 0.4 ml and NAD 40 mg in 30 ml 0.02 M Tris/HCl buffer (pH 8.6) and 20 ml 2% aqueous agar). Optimum results were obtained with MLB at 2 μg/ml and this was the amount used in testing the series of enzymes listed in table 1.

MLB was found to be suitable for the detection of NADH and NADPH, generated in the primary reaction of the direct stains listed in table 1, or in the secondary reactions catalysed by exogenous linking enzymes in the indirect stains. However, the overall speed of the reaction leading to the appearance of the isozymes was much slower with MLB than with PMS. The period of incubation necessary for adequate staining using MLB was about double the usual time required with PMS.

The end results obtained with MLB in most of the NAD- and NADP-linking staining methods were frequently superior to those with PMS. These enzyme stains are indicated by ++ in table 1. Some were more or less equivalent, indicated by +, but a few NAD-linked enzymes (lactate, glycerol-3-phosphate, sorbitol, and malate dehydrogenases) showed less

Table 1
Enzyme staining methods tested using Meldolablue (MLB) as a substitute for phenazine methosulphate (PMS)

Enzyme EC no.	Name	Cofactor			Type of Stain		Intensity with MLB
		NAD	NADP	Other	Direct	Indirect	
1.1.1.1	Alcohol dehydrogenase	+			+		++
1.1.1.8	Glycerol-3-phosphate dehydrogenase	+			+		(+)
1.1.1.14	Sorbitol dehydrogenase	+			+		(+)
1.1.1.27	Lactate dehydrogenase	+			+		(+)
1.1.1.37	Malate dehydrogenase	+			+		(+)
1.1.1.40	Malic enzyme		+		+		+
1.1.1.42	Isocitrate dehydrogenase		+		+		++
1.1.1.44	Phosphogluconate dehydrogenase		+		+		+
1.1.1.47	Glucose dehydrogenase	+			+		++
1.1.1.49	Glucose-6-phosphate dehydrogenase		+		+		++
1.1.3.1	Glycolate oxidase			+	+		(+)
1.2.1.12	Glyceraldehyde phosphate dehydrogenase	+			+		+
1.4.1.3	Glutamate dehydrogenase		+		+		+
1.4.3.1	D-Aspartate oxidase			+	+		–
1.4.3.3	D-Amino acid oxidase			+	+		–
2.4.2.1	Purine nucleoside phosphorylase			+		+	+
2.7.1.1	Hexokinase		+			+	+
2.7.3.2	Creatine kinase		+			+	+
2.7.4.3	Adenylate kinase		+			+	++
2.7.4.10	Nucleoside triphosphate-adenylate kinase		+			+	++
2.7.5.1	Phosphoglucomutase		+			+	++
3.4.11.*	Peptidases			+		+	–
3.5.4.3	Guanine deaminase			+		+	+
3.5.4.4	Adenosine deaminase			+		+	++
4.1.2.13	Aldolase	+				+	(+)
4.2.1.2	Fumarate hydratase	+				+	+
4.2.1.3	Aconitase		+			+	+
5.3.1.1	Triosephosphate isomerase	+				+	++
5.3.1.8	Mannose phosphate isomerase		+			+	+
5.3.1.9	Glucosephosphate isomerase		+			+	++

Stains which employ exogenous linking enzymes are referred to as indirect. The system of scoring the isozyme staining intensity with MLB is given in the text

clear staining using MLB as the electron transfer reagent and are indicated by (+) in table 1. The reasons for the variability among the pyridine nucleotide-linked enzyme stains are difficult to assess. The pH and the ionic composition of the staining mixtures and the gel buffer systems used for electrophoresis are extremely varied and may influence the efficiency of MLB.

The agar spot plate test showed that MLB was effective over a wide range of pH. Optimum sensitivty, with MLB at 2 μg/ml, was obtained between 6.0 and 7.0 when concentrations of NADH as low as 50 μM were detected by the generation of a definite formazan ring in contrast to PMS which gave scarcely detectable formazan precipitation at this concentration of NADH. The sensitivity of both reagents was less at pH 4.0–6.0 and very much lower at pH 2.4–4.0. MLB appeared to be superior to PMS in catalysing the reduction of MTT by NADH at pH 8.0, but this is partly attributable to the relatively very slow deterioration of the MLB/MTT mixtures compared to the PMS/MTT mixtures which darken rapidly in alkaline conditions.

The soluble and mitochondrial forms of superoxide dismutase which show up clearly as artefacts using standard MTT/PMS staining mixtures for most dehydrogenases, are not easily detected when MLB is substituted for PMS. This is a testament to the relatively high stability of the MLB mixture and the absence of background formazan precipitation on which the detection of the superoxide dismutase isozymes depends.

The stains such as those for the peptidases and the amino acid oxidases, which routinely employ peroxidase and *o*-dianisidine or aminoethyl carbazole in preference to PMS and MTT were all relatively inefficient when MLB was substituted for PMS in the staining mixture (indicated by (–) in table 1). However, the three enzymes (adenosine deaminase, purine nucleoside phosphorylase and guanine deaminase) which depend for their detection on a final reaction catalysed by xanthine oxidase were all satisfactory with MLB and indeed, the adenosine deaminase isozyme patterns were superior in total activity and contrast.

Most of our experiments were carried out using starch gel electrophoresis as the separation technique. However, the isozyme patterns of many of the enzymes listed in table 1 were also examined by flat bed isoelectric focusing; the results of staining with MLB were the same in the pH gradients as on the conventional gels.

4. Conclusions

Meldolablue is an excellent electron transmitter for the detection of the NAD(P)-dependent reactions of isozymes after electrophoresis. It is relatively much more stable to light than phenazine methosulphate and sensitive over a wider pH range without deterioration or excessive background staining. The tests on more than thirty different enzymes suggest that MLB is the reagent of first choice though in some cases, especially where the isozymes are particularly active, the conventional catalyst, phenazine methosulphate, may be preferred. Slight disadvantages associated with the use of MLB in isozyme staining are the failure to detect the superoxide dismutase isozymes and the rather slow reaction time compared with stains using PMS.

Recent work has shown that MTT and PMS are directly acting mutagens in two species of bacteria [5]. Preliminary studies by the same tests have shown that MLB is also mutagenic [6]. All three reagents should therefore be handled with care and appropriate precautions to minimise exposure in view of the strong qualitative correlation between mutagenicity and carcinogenicity.

References

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