Tetramethylbenzidine Polyvinylpyrrolidone Platinum Reaction for Detection of Tracer Horseradish Peroxidase

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Received for publication November 6, 1997

3,3',5,5'-tetramethylbenzidine polyvinylpyrrolidone platinum (TMB-PVP-PT) reaction was moderately sensitive to horseradish peroxidase (HRP), and gave an improved image of the tracer localization. When HRP-injected mouse liver and submandibular gland specimens were treated with this reagent, a blue reaction product in fine granular shape was formed only at the sites where the tracer was localized, while no endogenous peroxidase activity was detected at all. Some attempt to enhance the sensitivity of this reagent to peroxidase was also made. This reaction was suitable for making permanent microscopic preparations.

Key words: TMB-PVP-PT reaction, Tracer HRP, Mouse liver, Submandibular gland, TMB-PVP-PT-H2O2 reaction

I. Introduction

Tetramethylbenzidine (TMB) has been used as a hydrogen donor chromogen in combination with hydrogen peroxide (H2O2) as a hydrogen acceptor for detection of tracer horseradish peroxidase (HRP) [9-15]. Indeed this reaction is highly sensitive to detect a small quantity of the tracer, but it has some drawbacks in that its colored reaction product fades away rather rapidly and in that its product takes the form of needle crystals to make the decision of tracer localization difficult. Some attempts have been made to overcome the first point by stabilizing its reaction product [1, 8, 15], but little effort has been paid so far to improve the second point. This paper is concerned with this point, and deals with a new procedure for accurate detection of the tracer localization by using TMB as a hydrogen donor chromogen, polyvinylpyrrolidone (PVP) as both mild hydrogen acceptor and crystal formation inhibitor, and potassium platinous chloride (K2PtCl4) as color stabilizer. This TMB-PVP-PT reaction gave a blue colored stable reaction product of minute granular shape only at the sites of tracer HRP localization, no endogenous peroxidase activity being detected at all. These improved images of tracer detection were shown in HRP-injected mouse liver and submandibular gland specimens.

Some attempts were made to increase the sensitivity of the TMB-PVP-PT reaction by adding small amounts of hydrogen peroxide to the reagent (TMB-PVP-PT-H2O2 reaction), and also to apply these reactions to electron microscopy.

II. Materials and Methods

Biological specimens

Adult mice of SPF MCH (ICR) strain were used as the material. They were injected with 20 ml volume of physiological saline solution, then with 5 ml volume of HRP (Sigma Chem. Co., St. Louis) dissolved in mouse serum at 8 mg/ml from the left ventricle. It took 2 min for this HRP injection. Immediately after the injection, the liver and the submandibular gland were excised, and were fixed by immersing in a cold 4% glutaraldehyde dissolved in 0.1 M phosphate buffer of pH 7.4 for 1 hr. After being washed thoroughly with the buffer, they were cut into sections at 15 micra with a freezing microtome.

Reagents

Stock solutions for preparing the TMB-PVP-PT reagent were as follows: TMB (Dôtite, Wako Pure Chem. Co., Osaka) was dissolved at 20 mM in absolute ethanol and was diluted to a half strength with distilled water. K2PtCl4 (Nakarai Chem. Ltd., Osaka) was dissolved at 10 mM in distilled water, and PVP (K90, extra pure reagent, Nakarai) at 10% (w/v) in 0.1 M Sörensen's dibasic sodium citrate-hydrochloric acid buffer of pH 4.0. The reagent was prepared just before use by mixing 1.25 ml of 0.1 M Sörensen's buffer (pH 4.0), 2.5 ml of PVP (K90), 0.25 ml of TMB and 1.0 ml of K2PtCl4 solu-
tion (TMB-PVP-PT reagent). Besides this standard reagent, modified reagents of different pH values were prepared by substituting McIlvain’s buffer for Sorensen’s one. Those of different PVP concentrations, those of different TMB concentrations, those of different PT to TMB ratios and those containing PVP of different polymerization degrees, namely, K30 (extra pure reagent), K25 (guaranteed reagent) or N-vinyl-2-pyrrolidone monomer (extra pure reagent, Nakarai), were prepared for specified purposes.

Procedures

The specimens were treated with the standard TMB-PVP-PT reagent at 20°C for 20 min under continuous stirring. After the reaction was stopped by transferring the specimens to 50% ethanol, the stained specimens were dehydrated with a graded series of ethanol, infiltrated with xylol and mounted in Diatex (Becker A.B. Sweden). Besides this standard staining procedure, the reaction was carried out at a different temperature, for a different duration to trace the course of the color development.

Inhibition of TMB-PVP-PT reaction

The inhibition of the reaction was tested using the specimens which had been heated at 90°C for 10 min. Modified TMB-PVP-PT reagents supplemented with KCN at 1 × 10⁻³ M, or with NaN₃ at 1 × 10⁻² M were prepared. Other modified reagents deprived of either PVP or K₂PtCl₄ from the standard reagent were also prepared. The pretreatment of the specimens with 0.1 mg/ml catalase (Sigma) dissolved in Sorensen’s buffer of pH 4.0 for 30 min, and the subsequent immersion in a modified TMB-PVP-PT reagent supplemented with catalase at the same concentration were carried out.

Model study for sensitivity to peroxidase of some reactions

A comparative model study for sensitivity was carried out using peroxidase conjugated affinity pure goat anti rabbit IgG (Jackson Immuno Res. Lab., Avondale) as the material. The IgG powder was dissolved in 2 ml of distilled water, and was taken as the solution of 1× strength. The solution was diluted with a 1 mg/ml bovine serum albumin (Amour Lab.) solution from 1 × 10⁻² to 1 × 10⁻⁵ strengths. Fifteen μl volume of each diluted solution was applied on a piece of acetylcellulose film (Schleicher und Schull, Dassel) and dried under an infrared lamp. These specimens were treated with 1 ml of each of the three kinds of peroxidase reagents at 20°C for 20 min. The reagents tested were as follows: 1) the TMB-H₂O₂-sodium nitroprusside reagent [11], 2) the TMB-PVP-PT reagent and 3) the TMB-PVP-PT-H₂O₂ reagent. The third reagent was prepared by supplementing the second one with H₂O₂ at the final concentration of 5 × 10⁻⁴ M. The stained specimens were washed briefly with water, dried and made lucent by immersion in liquid paraffin. They were measured for the optical density of their reaction products, and for the circular area occupied by their products. The amounts of the products were obtained by multiplying the optical density by the area in mm². The more dilute the specimen producing the colored product was, the higher the sensitivity of the reagent concerned was.

Estimation of reaction intensity

The reaction intensity was determined with a multipurpose spectrophotometer equipped with a microscope of 250 × magnification (MPS-5000, Shimadzu Co., Kyoto) through a pinhole of 5 μm diameter at the absorption peak wave lengths, namely, at 610 nm for the TMB-H₂O₂-sodium nitroprusside reaction and at 580 nm for the TMB-PVP-PT and the TMB-PVP-PT-H₂O₂ reactions.

Fading test

The stability of the colored product formed by the TMB-PVP-PT reaction was tested by measuring the optical density at 580 nm at proper time intervals at the specified points of those liver sections which were subjected to three different environments respectively. The first preparation was kept at 4°C in the dark, the second one at 37°C in the dark and the third one at 37°C under continuous illumination of a fluorescent lamp at 200 erg · cm⁻² · per sec.

III. Results

Parameters for optimal TMB-PVP-PT reaction

The TMB-PVP-PT reaction produced a blue-colored stable product in the presence of tracer HRP. The product showed an absorption peak at 580 nm (Fig. 1). The reaction revealed an optimal pH at pH 4.0 (Fig. 2, ①), an optimal TMB concentration at 5 × 10⁻⁴ M (Fig. 2, ②), an optimal color intensity at 5% of PVP concentration (Fig. 3, ③), a bright blue color at PT to TMB ratio of 4 (Fig. 3, ④), an optimal color intensity at 5% of PVP concentration (Fig. 3, ④), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑤), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑥), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑦), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑧), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑨), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑩), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑪), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑫), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑬), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑭), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑮), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑯), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑰), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑱), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑲), an optimal color intensity at 5% of PVP concentration (Fig. 3, ⑳), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㉑), a bright blue color at PT to TMB ratio of 4 (Fig. 3, ㉒), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㉓), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㉔), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㉕), a bright blue color at PT to TMB ratio of 4 (Fig. 3, ㉖), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㉗), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㉘), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㉙), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㉚), a bright blue color at PT to TMB ratio of 4 (Fig. 3, ㉛), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㉜), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㉝), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㉞), a bright blue color at PT to TMB ratio of 4 (Fig. 3, ㉟), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㊱), a bright blue color at PT to TMB ratio of 4 (Fig. 3, ㊲), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㊳), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㊴), a bright blue color at PT to TMB ratio of 4 (Fig. 3, ㊵), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㊶), a bright blue color at PT to TMB ratio of 4 (Fig. 3, ㊷), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㊸), a bright blue color at PT to TMB ratio of 4 (Fig. 3, ㊹), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㊺), a bright blue color at PT to TMB ratio of 4 (Fig. 3, ㊻), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㊼), a bright blue color at PT to TMB ratio of 4 (Fig. 3, ㊽), an optimal color intensity at 5% of PVP concentration (Fig. 3, ㊾), a bright blue color at PT to TMB ratio of 4 (Fig. 3, ㊿).

Sensitivity to tracer peroxidase

A model study was carried out to check the sensitivity of allied peroxidase reagents containing TMB as a hydrogen donor chromogen. The result obtained was given in Fig. 4. Judging from the least peroxidase quantity detectable by these reagents, they were sensitive to peroxidase in the following order: the TMB-H₂O₂-nitroprusside reagent → the TMB-PVP-PT-H₂O₂ reagent → the TMB-PVP-PT reagent. The third reagent could be sensitized ten-fold by addition of H₂O₂ at 5 × 10⁻⁴ M.

Detection of tracer in mouse liver

When the specimens which had been injected with
Fig. 1. Absorption spectrum of TMB-PVP-PT reaction product for tracer HRP. Axis of ordinates denotes absorbance, and that of abscissas wave length in nm.

Figs. 2, 3. Reaction parameters for TMB-PVP-PT reaction.

Fig. 2. O, pH; □, temperature in °C; △, color development in min; ◊, final TMB concentration in M on logarithmic scale.

Fig. 3. ◊, final PVP concentration in %; △, PT : TMB ratio; ○, final ethanol concentration in %; ①, color fading at 4°C in the dark; ②, fading at 37°C in the dark; ③, fading at 37°C under continuous illumination. Axis of ordinates denotes relative activity in %.

Fig. 4. Model study for sensitivity of some TMB-containing reagents. ×, TMB-H₂O₂-nitroprusside reagent; ○, TMB-PVP-PT-H₂O₂ reagent; □, TMB-PVP-PT reagent. Axis of ordinates denotes relative amount of reaction product, and that of abscissas dilution factor on logarithmic scale.
tracer HRP were treated with the TMB-PVP-PT reagent, the tracer HRP turned blue. The branches of the hepatic artery, those of the portal vein and the central veins were stained blue along their wall. These two kinds of branches were stained denser than the central veins. The sinusoids were also stained blue along their wall, and frequently, they showed a decreasing gradient in color intensity from the periphery to the center of the liver lobule (Fig. 5). A close examination under high magnification revealed that the blood vessel wall and the sinusoid wall were stained evenly blue (Fig. 6, arrowheads). Some of the parenchyma cells took up the tracer. Those cells which in-

Figs. 5-8. Permanent preparations of HRP-injected specimens subjected to TMB-PVP-PT reaction.
Fig. 5. Distribution of tracer HRP in mouse liver, injected with HRP. C, central vein; P, portal area. 260 x.
Fig. 6. Close view of tracer incorporation in liver parenchyma cells. Arrows show vesicles or spherical bodies produced by endocytosis, and arrowheads sinusoid walls stained evenly. N, hepatocyte nucleus. 1050 x.
Fig. 7. Distribution of tracer HRP in mouse submandibular gland, injected with HRP. Arrowheads denote blood vessels loaded with tracer, and arrow shows blood vessel occupied by tracer except erythrocytes. 420 x.
Fig. 8. Close view of tracer HRP in capillary lumen of submandibular gland. Arrows show tracer HRP in fine granular shape. E, erythrocyte. Note negative pseudoperoxidase reaction in erythrocytes. 1600 x.
corporated the tracer revealed numbers of blue spherical bodies and vesicles in their peripheral region of cytoplasm lining the sinusoids (Fig. 6, arrows). This distribution of the tracer HRP demonstrated the transportation of the tracer in the liver. The tracer entered from the branches of the hepatic artery and of the portal vein, and spread via the sinusoids. It was incorporated in some of the parenchyma cells by endocytosis [17], and it was drained to the central veins. In contrast, neither endogenous peroxidase activity in Kupffer cells [7] and in leukocytes [2, 3] nor pseudoperoxidase one in erythrocytes were detected at all.

Detection of tracer HRP in mouse submandibular gland

When the tracer-injected specimens were subjected to the TMB-PVP-PT reaction, the blood vessels were stained homogeneously blue owing to the presence of HRP (Fig. 7, arrowheads) except the erythrocytes (Fig. 7, arrow). Examinations at higher magnification revealed that narrow capillaries were occupied by the deposits of fine blue granules of the reaction product (Fig. 8, arrows). In contrast, both serous acinar cells and leukocytes remained colorless [2, 3].

Inhibition of TMB-PVP-PT reaction

No blue reaction product was formed at all in the heat-injured specimens treated with the standard TMB-PVP-PT reagent. Both KCN at $1 \times 10^{-2}$ M and NaN$_3$ at $1 \times 10^{-2}$ M inhibited this reaction completely. A modified reagent deprived of PVP did not give any colored reaction product in the presence of HRP. The omission of K$_2$PtCl$_4$ from the standard reagent resulted in the formation of a transient unstable blue product, which faded away rapidly. The standard TMB-PVP-PT reagent containing PVP K90, and the modified reagents containing either PVP K30 or PVP K25 at 5% gave a blue stable reaction product of the same color intensity at the sites of tracer localization. In contrast, N-vinyl-2-pyrrolidone monomer did not give any positive reaction at all. Here, it must be emphasized that PVP K90 was the only PVP which made the reagent viscous. The pretreatment of the specimens with catalase followed by the subsequent immersion in a modified TMB-PVP-PT reagent supplemented with catalase failed to show any blue reaction product at all.

IV. Discussion

TMB-PVP-PT reaction

The reaction was positive in HRP-injected specimens treated with the TMB-PVP-PT reagent to produce a blue, stable reaction product only at the sites where the tracer was localized. It was completely inhibited by the presence of KCN or NaN$_3$ [4]. Heat-injured specimens treated with the reagent also failed to give the positive reaction. From these facts, it seems clear that the positive result demonstrates the presence of tracer HRP in the specimens examined. Indeed this reaction was sensitive enough to detect tracer HRP, but was insensitive to prove endogenous peroxidase activity. Though various procedures have been proposed to discriminate the former from the latter activity [18-20], this discrimination is realized simply by using this reaction.

Use of platinochloride ions

To stabilize reaction product as durable blue compounds, varieties of stabilizers, such as sodium nitroprusside [11], potassium ferricyanide [1], diaminobenzidine and cobalt acetate [15] and ammonium molybdate [8] have been used during or after the reaction. In the TMB-PVP-PT reaction, platinochloride ions works as an effective color stabilizer to yield a blue stable product, as proved by the fact that the omission of K$_2$PtCl$_4$ from the reagent failed to produce any blue durable product. This TMB-PVP-PT reaction product was insoluble in usual solvents to allow the procedure for permanent microscopic preparations.

PVP as hydrogen acceptor

Though PVP has never been reported to work as hydrogen acceptor in the literature, PVP plays actually a role of hydrogen acceptor in the TMB-PVP-PT reaction. This conclusion was supported by the following facts; 1) A modified TMB-PVP-PT reagent deprived of PVP did not give any positive reaction product for HRP at all. 2) PVP of different polymerization degrees showed a similar ability of accepting hydrogen to produce a blue reaction product in the presence of TMB and tracer HRP. In contrast, vinylypyrrolidone monomer exhibited no accepting ability. 3) The presence of catalase prior to and during the incubation of HRP-injected specimens in a modified TMB-PVP-PT reagent failed to give a positive reaction for the tracer. 4) PVP is synthesized from its monomers by heat polymerization in the presence of H$_2$O$_2$ and ammonia as catalysts [21]. Based on these facts, it seems probable to conclude that a minute quantities of H$_2$O$_2$ may exist in the PVP polymers available from commercial sources as a remnant of the catalysts used, and this may be the principle constituent of PVP as a mild hydrogen acceptor.

PVP as preventive from crystal formation

PVP K90 made the reagent viscous, and this prevented the reaction product from growing into needle crystals [6]. The resulting reaction product obtained was in minute granular shape, and this feature permits a demonstration of an accurate localization of the tracer.

Sensitivity

A model study for sensitivity to peroxidase of some TMB-containing reagents proved that the TMB-PVP-PT reagent was moderate in sensitivity. The Mesulam's reagent [11], the most popular one in neurohistochemistry [11-14], was of high sensitivity. The TMB-PVP-PT reagent could be sensitized ten times to peroxidase by supplementing with H$_2$O$_2$ at the final concentration of $5 \times 10^{-4}$ M (TMB-PVP-PT-H$_2$O$_2$ reaction, Fig. 4).
Remarks on TMB-PVP-PT and TMB-PVP-PT-H₂O₂ reaction in comparison

Though the latter reaction was superior to the former one in sensitivity to peroxidase (Fig. 4), the latter is inferior to the former in the ability to produce a fine minute granular reaction product, and in the ability of discriminating tracer peroxidase from endogenous peroxidase activity. For example, the blue reaction product formed by the latter reaction in the capillaries of the HRP-injected submandibular gland was of rod shape, and was larger in size than the product formed by the former one. The endogenous peroxidase activity of Kupffer cells and of leukocytes in the HRP-injected liver, which was not detected by the former reaction, was demonstrated to exist by the latter one.

Suggestion to use TMB-PVP-PT reaction for electron microscopy

The TMB-PVP-PT reaction should be followed by postosmification with 1% OsO₄ at pH 7.4 for electron microscopic purpose, because the blue reaction product of this reaction dissolved slowly in epoxy resin [5, 10, 16].

V. Acknowledgments

The present author would like to express his sincere gratitude to Prof. K. I. Hirai, Department of Anatomy, Kanazawa Medical University, who put all his laboratory equipments at the author's disposal. His thanks are due to Dr. T. Ueda, the same Department, for his reliable collaboration during this investigation.

VI. References