BIOLOGIA PLANTARUM 39 (3): 473-478, 1997.

BRIEF COMMUNICATION

The double staining of plant peroxidase and other proteins in the same polyacrylamide gel

V. REPKA and I. FISCHEROVÁ

Laboratory of Molecular Biology and Virology, CRIVE, Matúškova 25, SK-833 11 Bratislava, Slovakia

Abstract

We present a simple and rapid technique for the double staining of plant peroxidase and other proteins in the same polyacrylamide gel using the principle of iodide oxidation followed by Coomassie Blue counterstain. The colored bands of peroxidase isozymes and proteins are easily distinguishable. An additional benefit of the method is the use of the low cost chemicals, as well as it eliminates the need for a potentially hazardous reagents frequently used in the detection of peroxidase isozymes.

Additional key words: Coomassie Blue, *Cucumis*, electrophoresis, starch/KI reagent.

Peroxidases have been associated with a plethora of physiological processes in plants: leaf and flower abscision, apical dominance, dormancy, aging and senescence, fruit development and ripening, germination and early development, as well as cold tolerance (Gaspar *et al.* 1982, Wellinder *et al.* 1993). Moreover, peroxidase has frequently been employed as a marker for monitoring plant resistance (Hammerschmidt *et al.* 1982, Dalisay and Kuc 1995). Specific isozymes have been implicated in auxin catabolism or thought to participate in lignification (Gaspar *et al.* 1985).

Peroxidase decomposes hydrogen peroxide by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Campa 1991, Gaspar *et al.* 1991). While

Received 12 September 1996, *accepted* 15 January 1997.

Abbreviations: AEC - 3-amino-9-ethylcarbazole; DAB - 3,3′-diaminobenzidine; EDTA - ethylenediamintetraacetic acid; ICF - intercellular fluid; PAGE - polyacrylamide gel electrophoresis; PVP - polyvinylpyrrolidone; SDS - sodiumdodecylsulphate; TEMED - N,N,N′,N′ - tetramethylethylenediamine, TNV - tobacco necrosis virus.

Phone: (+421-7) 375929, fax: (+421-7) 375436.

the basic chemical reactions of peroxidase are well established, the questions of how many isozymes function and how their action is controlled remain largely unanswered. Polyacrylamide gel electrophoresis accompanied with in gel activity staining is widely used to address such questions. Several methods have been developed for enzymatic staining of peroxidase isozymes in gel electrophoresis (Shaw and Prasad 1970). In this work, we describe an optimized protocol for the rapid and simple double staining of both peroxidase and nonperoxidase protein in the same gel. This method could be very useful in the peroxidase gene expression studies, especially in the field of plant molecular biology, physiology as well as in plant pathology.

Cucumber (*Cucumis sativus* L. cv. Laura) seeds were surface sterilized with 0.8 % *Domestos* (*Henkel*, Bratislava, Slovakia), washed with sterile distilled water and germinated under aseptic conditions in Petri dishes containing filter paper discs soaked with half-strength MS medium (Murashige and Skoog 1962) lacking phytohormones. The seeds were placed into a humid cultivation chamber and allowed to germinate at 25 °C under various irradiances (65 - 260 W m^{-2}) for 6, 8 and 11 d. Fully expanded cucumber cotyledons (7-d-old) were abrased and inoculated with a suspension of TNV as described elsewhere (Repka and Slováková 1994). The cotyledons bearing necrotic lesions were harvested 7 d after inoculation and ICF was prepared following the method described in Repka *et al.* (1993a).

Plant material was homogenized with a mortar and pestle in appropriate volumes of ice cold (4 °C) TRISEPAC buffer (50 mM Tris-HCl, pH 8.0, 500 mM saccharose, 1 mM Na_2EDTA, 0.2 % insoluble PVP, 6 mM ascorbic acid and 0.1% cysteine). The homogenate was immediately centrifuged at 10 000 *g* for 20 min (4 °C). The supernatant was concentrated using *Microcon-3* microconcentrators (*Amicon GmbH.*, Witten, Germany) and kept at -20 °C until further use.

Discontinuous, nondenaturing PAGE gels (10 %) were prepared according to Laemmli (1970) with the exception that gels did not contain SDS and β-mercapto-ethanol. Slab gels (14 × 16 × 0.15 cm) were prepared and run using the *SE-600* apparatus (*Hoefer Scientific Instruments Inc.*, San Francisco, USA). Samples containing 5 - 100 μg of total proteins were applied per lane and run at 4 - 6 °C.

Immediately after the separation was completed, gels were stained for peroxidase activity using DAB, AEC or guaiacol as described elsewhere (Repka and Slováková 1994, Repka and Jung 1995). For the starch-KI procedure gels were soaked for 30 min at room temperature in an aqueous solution of 100 mM potassium iodide containing 4 % of potato starch. The gels were transferred to an acetate buffer (50 mM, pH 5.2), containing 0.3 % hydrogen peroxide for 5 min. The gels were rinsed for 45 s with distilled water and transferred to a starter solution (40 % methanol and 7 % acetic acid) for a period until the reddish-brown bands started to appear. Isozyme patterns were further developed until optimal in a tray containing distilled water. The gels were photographed immediately and then put into a Coomassie Blue solution (0.1 % Coomassie Blue R-250, 40 % methanol and 7 % acetic acid) for 10 min. Several gels were stained only with the Coomassie Blue solution. Gels were destained with a solution containing 25 % methanol and 10 % acetic acid for 10 - 15 min and photographed again.

The parameters were adjusted for assaying the peroxidases and are fully comparable to those used in appropriate colorimetric assays (Table 1). There was the only exception. It was shown to be absolutely necessary to start the peroxidating activity by an intensive acidifying of the reaction conditions. The need for acidic conditions is due to the low buffering capacity of the starch/KI reagent.

Table 1. Step-by-step optimized protocol to stain anionic peroxidases using the starch/KI reagent

Step	Incubation solution	Time [min]
I	4 % potato starch + 100 mM KI	30
II	sodium acetate buffer (50 mM, pH 5.2) + 0.3 % H_2O_2	5
III	rinse with distilled H_2O	0.45
IV	40 % methanol, 7 % acetic acid	1 - 5[a]
V	distilled H_2O	1 - 10[b]

[a] - incubation of the gel in a starter solution (IV) is optional and depends mainly on the amount of protein (peroxidase) applied per lane.
[b] - development periode of the peroxidase isozymes pattern in distilled water depends either on the amount of peroxidase applied to the gel or on the sensitivity of detection needed.

To test the validity and sensitivity of the starch/KI method, several leaf extracts containing soluble peroxidase (*e.g.* ICF from cucumber cotyledons reacting hypersensitively to infection with tobacco necrosis virus) were applied to PAGE gels (Fig. 1*A*). The same pattern was obtained if DAB, AEC or guiacol were used as a substrate (data not shown). Serial dilution of the ICF extract (ranging from 10 to

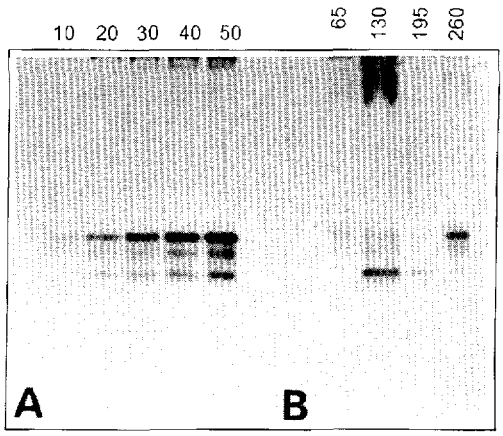

Fig. 1. Electrophoretogram of cucumber anionic peroxidase patterns visualized with starch/KI-specific peroxidase stain: *A*) - serial dilution (10 - 50 μg) of an ICF extract prepared from cucumber cotyledons reacting hypersensitively to inoculation with tobacco necrosis virus; *B*) - patterns of peroxidase accumulation in cucumber seedlings germinated for 11-d under various irradiance (65 - 260 W m^{-2}). To each lane of the gel 50 μg of total protein were loaded.

50 μg of total proteins) also demonstrated that the method of peroxidase staining using the starch/KI reagent was sensitive enough to obtain a reproducible patterns.

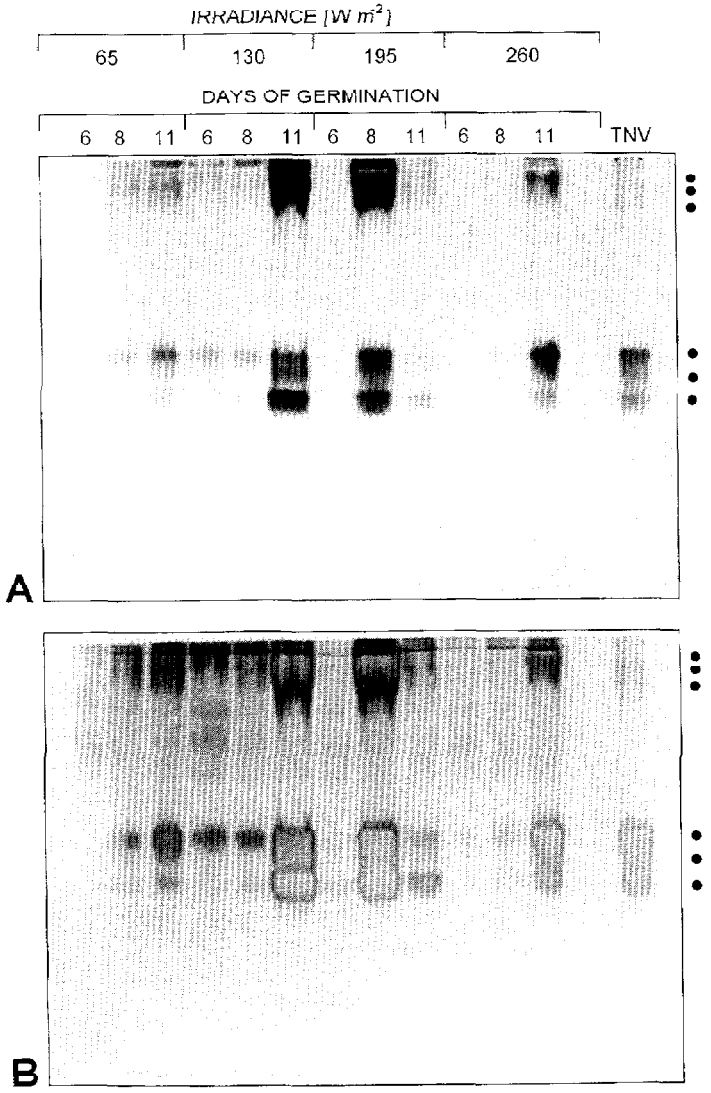

Fig. 2. Comparison of the starch/KI staining and double staining method. Two (10 %) discontinuous, nondenaturing polyacrylamide gels were loaded with protein extracts prepared from cucumber seedlings germinated for 6, 8 and 11 d at different irradiances (65 - 260 W m^{-2}). Each lane was loaded with 50 μg of total protein. Gel was stained with the starch/KI reagent (*A*) or it was double stained, first with the starch/KI reagent and then with Coomassie R-250 (*B*). Lane marked as TNV refers to extract prepared from tobacco necrosis virus infected cucumber cotyledons (25 μg of total protein). Peroxidase isozymes detected are demonstrated by the black dots.

The reproducibility of the method was further demonstrated on extracts prepared from cucumber seedlings germinated for 11 d under different irradiance (Fig.1*B*).

An example of the use of starch/KI method for the detection of isoperoxidases accumulated in cucumber seedlings which had been germinated at various irradiance for 6-, 8- and 11-d is documented in Fig. 2*A*. Fig. 2*B* represents a gel which was electrophoresed in parallel to the gel in Fig. 2*A* but stained with the double staining procedure. The isoperoxidase patterns were the same and nonperoxidase proteins appeared as pale blue bands. However, unspecific staining of protein bands with Coomassie Blue R-250 purchased from different vendors gave unreproducible patterns (data not shown).

Within our studies on peroxidase isozymes as a method for screening plant resistance and pathogenesis (Repka *et al.* 1993a,b, Repka 1996), we frequently needed a simple and sensitive procedure to visualize peroxidase isozymes present in plant extracts and at the same time detect and stain other proteins separated in the same polyacrylamide gel. Therefore we have adopted and modified a simple histochemical test for destruction of exogenous H_2O_2 assayed by a starch/I_2 procedure of Olson and Varner (1993). Although this test has a many potential applications *(e.g.* detection of polyamine oxidase or catalase), to our knowledge this method has not been used for the identification and characterization of peroxidases in native gels after electrophoretic separation.

The method presented in this work is based on the principle that peroxidase decomposes hydrogen peroxide and oxidizes iodide ions to iodine; the iodine is complexed by the starch to form a reddish-brown color. Although the results obtained by the using of the starch/KI reagent were shown to be comparable with those obtained with DAB or AEC, relative disadvantage is the instability of the peroxidase pattern due to the solubility of the end product. As with bands stained with guaiacol as hydrogen donor (Shimoni and Reuveni 1988), peroxidase bands stained with starch/KI fade rapidly. However, this disadvantage must be balanced against the use of nonhazardous and low cost chemicals needed to perform the staining.

A similar method was developed for staining and stabilizing peroxidase activity revealed with the aid of guaiacol (Shimoni and Reuveni 1988). It was demonstrated, however, that the Coomassie Blue R-250 purchased from different vendors gave unreproducible patterns. For example, while Coomassie Blue produced by *Sigma* or *Bio-Rad*, an original stain we have used in our procedure, appeared to be sufficient to perform the double staining method, the dye obtained from *Serva* or *BDH* gave undistinguishable patterns. Using the latter dyes both peroxidases as well as nonperoxidase proteins were visualized as an equally blue bands in the gel.

The double staining method described in this work enables us to visualize, in the same gel, peroxidases among other proteins present in the gel. The method also enables a direct comparison of the reddish-brown bands of peroxidase isozymes with other proteins, without the manipulation of protein concentration in tissue extracts.

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