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QUANTIFICATION OF LUPIN PEROXIDASE ISOENZYMES BY DENSITOMETRY*

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RESUMEN

Cuantificación de las isoenzimas de la peroxidasa de altramuz por densitometría

La formación del producto final de la oxidación de la benzidina («benzidine-brown») por las isoenzimas de la peroxidasa de altramuz separadas por isoelectroenfoque, se utilizó para cuantificar las actividades enzimáticas de cada una de las isoenzimas en un mismo gel. Se encontraron como condiciones de reacción más adecuadas, el uso de tiempos de reacción de 3 h, concentraciones de H_2O_2 del orden de 1 mM, y concentraciones de benzidina a saturación en tampones de pH 5.0. Bajo estas condiciones, la actividad de cada isoperoxidasa puede ser cuantificada como formación (y/o acumulación) de dicho producto en el estado estacionario de la reacción, siendo éste medido como área de cada pico registrado por barrido densitométrico a 460 nm. La validez de este método densitométrico para la cuantificación de las isoenzimas de la peroxidasa de altramuz, está apoyada por la existencia de una relación lineal entre la cantidad total de actividad peroxidasa depositada sobre el gel, y el área correspondiente a cada pico de isoperoxidasa separada por isoelectroenfoque.

Palabras clave: Altramuz, cuantificación, densitometría, isoperoxidasas.

SUMMARY

The formation of benzidine-brown end product by lupin peroxidase isoenzymes, separated by gel isoelectric focusing, is used in order to quantify isoenzyme activities on the same gel. Incubation times of 3 h, H_2O_2 concentration of 1 mM, benzidine as saturated solution, and pH 5.0, are the best assay conditions. Under these conditions, isoperoxidase activity can be quantified as formation (and/or accumulation) of the benzidine-brown end product in the steady-state of the reaction. The product can be measured as the peak area recorded by scanning densitometry at 460 nm. Linear relationship between the amount of total peroxidase activity loaded on the top of the gel, and the area of each isoperoxidase peak resolved by gel isoelectric focusing support the validity of this densitometric method for the quantification of lupin peroxidase isoenzymes.

Key words: Densitometry, isoperoxidases, lupin, quantification.

INTRODUCTION

Peroxidase (EC 1.11.1.7; donor:H_2O_2 oxidoreductase) is an enzyme which appears with a large number of isoforms in most plants. Although the indole-3-acetic acid catabolism has been considered for a long time the main physiological role of this enzyme, other roles related to plant cell wall differentiation have been recently recognised (MADER, 1985).

Plant peroxidase isoenzymes are differentially expressed during plant cell development

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(ROS BARCELÓ & SABATER, 1986), and they are frequently used as biochemical markers of some physiological stages of plant crops (KAHLEM, 1975). In these studies it is important to know quantitatively the relative activity of each peroxidase isoenzyme, and the contribution it makes to the total peroxidase activity in each developmental stage, therefore the quantification of isoperoxidase levels is frequently desirable. However, direct methods of isoperoxidase quantification are scant.

A simple method was devised by LIU (1973) for quantifying peroxidase isoenzymes. It was based on a chronometric assay, first proposed by GREGORY (1966), involving the coupled reaction of ascorbic acid with the products of the peroxidase-mediated oxidation of benzidine. In this reaction, when ascorbic acid is coupled to the benzidine-H_2O_2 peroxidase assay, it reduces the blue oxidized form (cation radical) of benzidine, and colour development is stopped until the ascorbic acid is completely oxidized. This method has some drawbacks. In the first place, ascorbic acid can be a substrate of peroxidase isoenzymes, and it cannot be assumed that all isoperoxidases oxidize ascorbic acid with equal efficiency (CASTILLO & GREPPIN, 1986). Secondly, this method reads initial rates of oxidation, and therefore it is neccesary to purify peroxidase activity in order to avoid the action of time-course peroxidase inhibitors, which are frequently found in plant cells and homogenates (CASTILLO *et al.*, 1981), and can migrate together with the isoperoxidases during their electrophoretic separation (LEGRAND *et al.*, 1978).

In this report, we have adapted the densitometric method of LEE (1973), previously employed for quantifying tobacco isoperoxidases, to lupin isoenzymes separated by electrofocusing. Assay conditions and reaction times have been checked so that isoenzyme quantification can be achieved on the same electrofocusing gel that is used to resolve and identify lupin isoperoxidases.

MATERIAL AND METHODS

PLANT MATERIAL

Eleven and fifteen-days old lupin (*Lupinus albus* L., cv. multolupa) hypocotyls were grown as described previously (ROS BARCELÓ *et al.*, 1987).

PEROXIDASE FRACTIONS

Lupin hypocotyls were homogenized and fractionated as reported ROS BARCELÓ *et al.* (1987). Soluble (from eleven-days old hypocotyls), and cell wall (from fifteen-days old hypocotyls) peroxidase fractions were dialysed against 50 mM Tris-HCl pH 7.2, for 24 h at 4° C, and stored at −20° C.

SEPARATION OF ISOPEROXIDASES BY ISOELECTROFOCUSING

Isoelectrofocusing was achieved as described by WRIGLEY (1971), in 4.0-9.0 pH gradients, using Ampholines® pH 3.5-10 (LKB 1809) as a mixture of carrier ampholytes. Cylindrical gels (60 mm × 5 mm) were prepared in media containing 7.5% (w/w) acrylamide, and photopolymerized in presence of riboflavine for 30 min at 3700 lux and 25° C. Prior to formation of the pH gradient, 0.3 nkat of peroxidase activity (assayed using guaiacol as substrate; ROS BARCELÓ *et al.*, 1987), were loaded on the top of the gel, and isoelectrofocusing was allowed to run for 2 h at 1 mAmp gel and 4° C.

ISOPEROXIDASE STAIN AND QUANTIFICATION

At the end of the isoelectrofocusing, the pH gradient was determined by cutting the gel into 0.5 cm segments, and by equilibrating the gel sections in deionized water for 30 min prior to pH measurement. The other gels of the same run were rinsed in water, and at once incubated in presence of 1 mg ml^{-1} benzidine and, unless otherwise noted, 1 mM H_2O_2 in 0.1 M Tris-acetate buffer of suitable pH. Reaction times were within 6 h. Benzidine reaction was stopped by rinsing the gels in water, and in order to allow the formation of the «benzidine-brown» precipitate and ensure both stability and reproducibility of the isoenzyme stain patterns, the gels were kept overnight at 25° C. Then, they were soaked in isopropanol: 0.1 M acetate buffer pH 5.0 (3:7, v/v), and kept at 4° C in darkness, eventually remaining in water.

The benzidine-stained gels were finally scanned at 460 nm, using a Joyce-Loebl MKII scanner densitometer, equipped with a linear transport. The area (cm^2) under each isoperoxidase peak was measured with a planimeter.

NATURE OF THE ISOPEROXIDASE ASSAY

Benzidine (p-diaminodiphenyl) is a well-know hydrogen donor in the peroxidase catalysed reduction of H_2O_2. It possesses several advantages compared to other substrates suitable for this enzyme, such as the stability of the end products of the oxidation, and the fact that several peroxidase isoenzymes oxidize this compound with similar k_M values (GIBSON & LIU, 1978; MADER *et al.*, 1977). Moreover, the end products are insoluble in aqueous buffers (SIEGEL *et al.*, 1978), and it can be supposed that this compound is a good hydrogen donor for the detection and quantification of isoperoxidases separated by electrophoretical techniques (KLISURSKA & DENCHEVA, 1980).

Benzidine is readily oxidized by peroxidases (JOSEPHY, 1986), and the oxidation proceeds via an one-

electron oxidized intermediate (cation radical), which is further oxidized to a quinone-diimine (fig. 1; JOSEPHY, 1986). The oxidation mechanism consistent with the spectroscopic evidence involves two sequential single-electron transfers yielding free radical and diimine metabolites (fig. 1; JOSEPHY, 1986), which slowly decays to the poorly characterized polymeric «benzidine-brown» end product(s). The formation of the last compound(s) is the basis of the isoperoxidase assay and quantification by densitometry described later.

NOMENCLATURE OF PEROXIDASE ISOENZYMES

Individual isoenzymes were named on the basis of their pI, A acidic (pI < 7.0), and B basic (pI > 7.0) isoperoxidases, with the number 1 asssigned to the one of lower pI. Phenol-induced lupin isoenzymes (conformers; ROS BARCELÓ *et al.*, 1987) were named as C species, and the subscript number was assigned as above.

RESULTS AND DISCUSSION

QUALITATIVE EFFECTS OF THE pH, ENZYME AND H_2O_2 CONCENTRATION

Peroxidase isoenzymes can differ significantly in a number of catalytic properties such as optimum pH and half-saturation H_2O_2 concentration (KLISURSKA & DENCHEVA, 1980).

In order to check these variables, peroxidase activity located in soluble fractions was subjected to isoelectrofocusing and bands of isoperoxidase activity were detected by placing the gels in pH 5.0 buffered solutions containing various concentrations of H_2O_2 (0.05 to 5 mM).

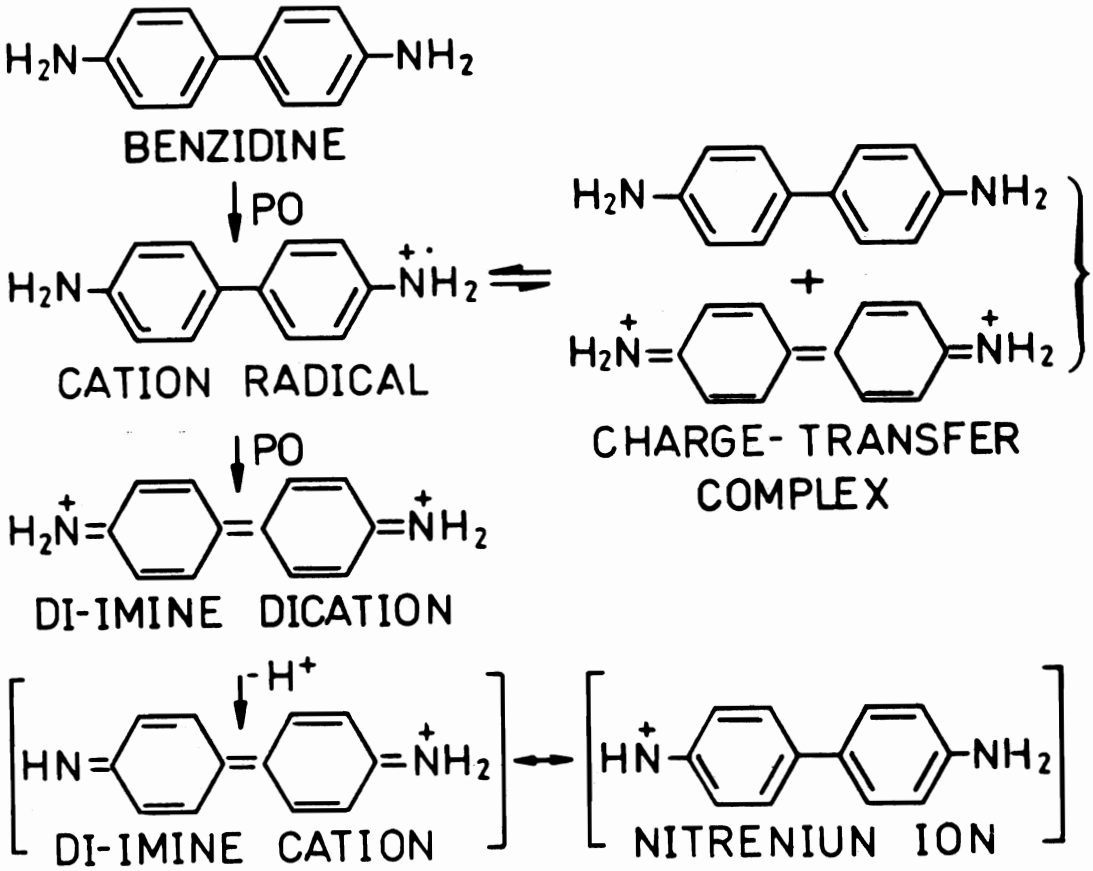

FIGURE 1. Mechanism of the oxidation of benzidine catalised by peroxidase (PO) and intermediate products. Taken from JOSEPHY (1986).

Mecanismo de la oxidación de la benzidina catalizada por la peroxidasa (PO) y productos intermedios de la reacción. Tomado de JOSEPHY (1986).

Densitometric recording of the gels (fig. 2) illustrates the effect of H_2O_2 concentration on the stain of each isoperoxidase. Based on their response to H_2O_2, a 1 mM concentration was regarded as the best (fig. 2). Lower substrate concentrations resulted in the appearance of only faint bands (fig. 2A and 2B), whereas a H_2O_2 concentration of 5 mM resulted in reduced intensity of all of them (data not shown).

Enzyme activity loaded on the gel was much less critical, and lowering or increasing the amount of peroxidase activity (from 0.1 to 0.6

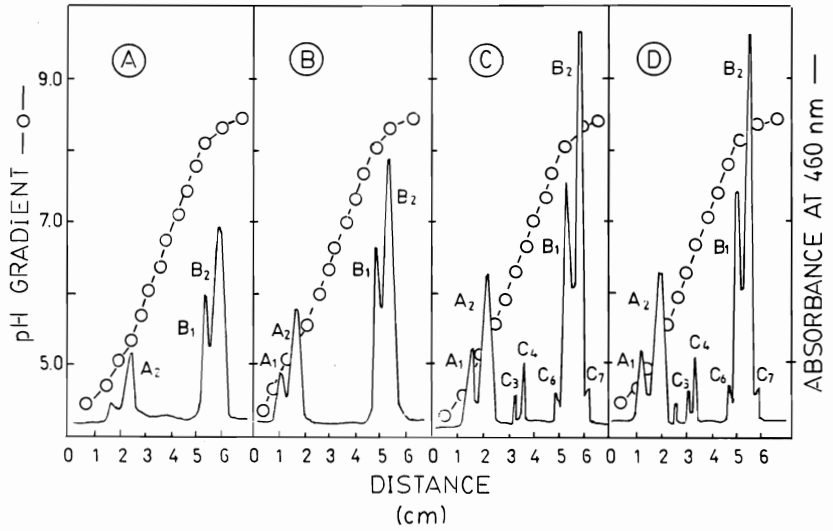

FIGURE 2. Densitometric recordings of the isoperoxidase patterns at different H_2O_2 concentrations. A, B, C and D, corresponding to 0.05 mM, 0.1 mM, 0.5 mM and 1.0 mM values, respectively. Stains were carried out at pH 5.0 for 3 h. 0.3 nkat of soluble peroxidase activity was loaded on the top of the gel.

Registros densitométricos de los patrones de isoperoxidasas revelados a diferentes concentraciones de H_2O_2. A, B, C y D, corresponden a las concentraciones de 0.05 mM, 0.1 mM, 0.5 mM, y 1.0 mM, respectivamente. La reacción de teñido fue llevada a cabo a pH 5.0 durante 3 h. En todos los casos 0.3 nkat de actividad peroxidasa soluble se depositaron sobre la superficie del gel.

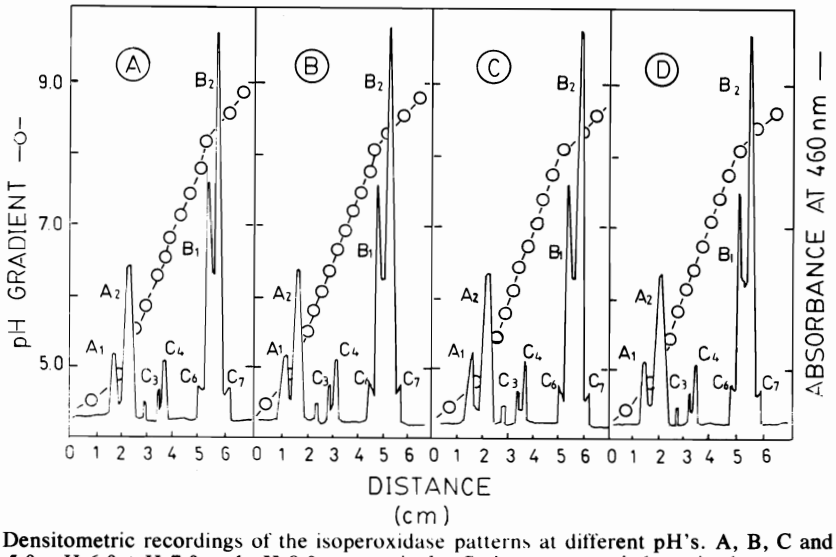

FIGURE 3. Densitometric recordings of the isoperoxidase patterns at different pH's. A, B, C and E, correspond to pH 5.0, pH 6.0, pH 7.0 and pH 8.0, respectively. Stains were carried out in the presence of 1 mM H_2O_2. The other conditions were the same as described in figure 2.

Registros densitométricos de los patrones de isoperoxidasas revelados a diferentes pH's. A, B, C y D, corresponden a tampones de pH 5.0, pH 6.0, pH 7.0 y pH 8.0, respectivamente. La reacción de teñido fue llevada a cabo en presencia de H_2O_2 1 mM. Otras condiciones de ensayo han sido descritas previamente en la figura 2.

nkat), only decreased on increased the intensity of staining of all bands, but had no effect on the total number of isoperoxidases (data not shown).

When the pH of the 0.1 M Tris-acetate buffer was varied in a range from 5 to 8, no qualitative changes were observed in the isoenzyme patterns (fig. 3). Only a weak background stain was observed with either neutral or basic buffer, although this stain increased with the time. For this reason the acidic buffer (pH 5.0) was chosen as the most suitable.

Buffer pH also influences the benzidine precipitation in the reaction times employed (3 h), being higher at either neutral or basic pH's. Benzidine solutions were henceforth used as saturated solutions, because reproducible results were obtained in that way.

ISOENZYME QUANTIFICATION

For the quantitative determination of isoperoxidases on electrophoreograms, the amount of enzyme-enzyme activity relationship should follow a linear correlation. The latter depends on the degree of the substrate oxidized, which ultimately depends on the reaction time. For this reason, both enzyme concentration and reaction times were studied in detail.

Figure 4 shows the time-course of isoperoxidase activity, measured as peak area for each isoenzyme. In this figure, it can be seen that steady-state rates are reached after 3 h for all isoperoxidases. Different lag times are observed for the benzidine-brown formation, and they vary from 20 min to 60 min for soluble isoperoxidases (A_{1-2} and B_{1-2}), being 90 min for the C_5 (cell wall-localized) isoperoxidase. These lag times can be due to either the presence of peroxidase inhibitors (CASTILLO *et al.*, 1981), or more probably to the two final non-enzymatic steps in the benzidine-brown formation.

LEE (1973) quantified tobacco isoperoxidases separated by gel electrophoresis using a reaction time of 30 min. Under these conditions, the area of each isoperoxidase peak was poor (for the A_{1-2} and B_{1-2} isoperoxidases), or null (for the C_5 conformer), due principally to the absence of benzidine-brown formation, althoug «benzidine blue» products, corresponding to the formation of the benzidine cation radical

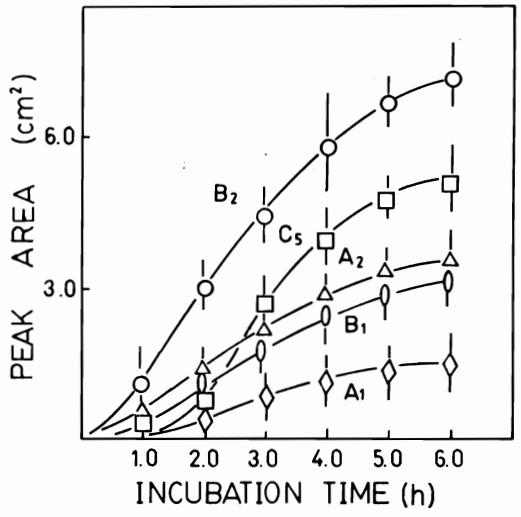

FIGURE 4. Time-course of the development of each isoperoxidase peak resoluted by isoelectrofocusing. Benzidine was used as saturated solution at pH 5.0, and H_2O_2 at 1 mM concentration. Other conditions were the same as described in figure 2. Bars show SE.

Curso temporal del desarrollo de cada pico de isoperoxidasa separada por isoelectroenfoque. Se utilizó la benzidina como disolución saturada a pH 5.0, y el H_2O_2 a la concentración de 1 mM. Otras condiciones de ensayo han sido descritas previamente en la figura 2. Las barras muestran el error estándard.

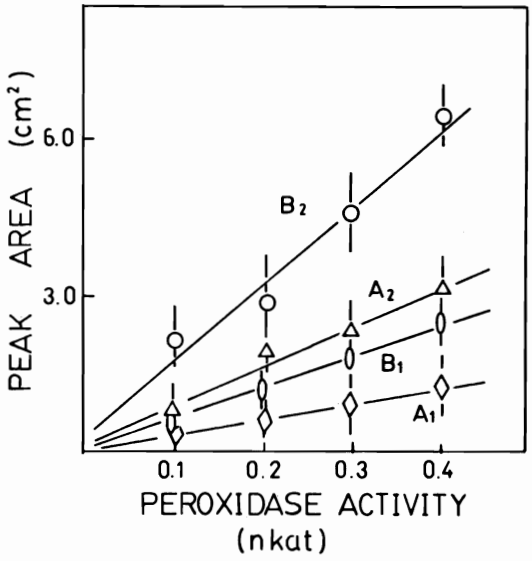

FIGURE 5. Relationship between total peroxidase activity loaded on the top of the gel, and the area of each isoperoxidase peak resolved by isoelectrofocusing. Assay conditions were the same as described in figure 4. Bars show SE.

Relación entre el nivel de la actividad peroxidasa total depositada sobre el gel, y el área de cada pico de isoperoxidasa separado por isoelectroenfoque. Las condiciones de ensayo fueron las mismas que las descritas en la figura 4. Las barras muestran el error estándard.

(see fig. 1), and at its stabilized charge-transfer complex (fig. 1), were ubiquitous in all the isoenzyme bands.

Finally, using incubation times of 3 h, a linear relationship was found between total peroxidase activity loaded on the top of the gel, and the area of each isoperoxidase peak in a wide range of enzyme concentrations (fig. 5; correlation coeffients for all isoperoxidases were within 0.99).

This technique would therefore be useful in studies which follow changes in peroxidase levels which are correlated with developmental processes (ROS BARCELÓ & SABATER, 1986). Thus, by measuring the percent contribution of each isoenzyme to the total peroxidase activity in each developmental stage, one can determine quantitatively the degree to which changes in peroxidase levels represent alterations in the expression of all or just a few of the peroxidase isoenzymes. For example, the application of this technique to quantify isoperoxidases during the lupin hypocotyl xylogenesis, shows that the increase in cell wall peroxidase activity during this developmental phase (ROS BARCELÓ & SABATER, 1986), is only due to the differential expression of the cell-wall A_1 isoperoxidase.

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