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PHYTOHORMONE REGULATION OF ISOPEROXIDASES IN *CATHARANTHUS ROSEUS* SUSPENSION CULTURES

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Key Word Index—*Catharanthus roseus*; Apocynacea; cell suspension; phytohormones; peroxidase isoforms; purification.

Abstract—Peroxidase (POD) activity was investigated in *Catharanthus roseus* cell suspensions cultured under different hormonal conditions. Depletion of 2,4-dichlorophenoxyacetic acid (2,4-D) from the culture medium enhanced POD activity in cells and spent medium. Addition of phytohormones, in particular the auxin 2,4-D, reduced POD activity in medium and cellular compartments and enhanced ionically cell-wall bound POD. The differential modulation of POD is due to hormone effects on synthesis and/or accumulation of POD, rather than on the secretion process. Qualitative analysis showed that 2,4-D, but not cytokinins, regulated the synthesis of a basic isoform. The cytokinin treatment seemed to affect acidic rather than basic isoforms. The presence of basic POD is correlated with the capacity of cells to produce indole alkaloids. The major extracellular basic isoperoxidase was purified to homogeneity from culture medium of *Catharanthus roseus* cell suspensions. The isolated peroxidase is a haem protein with a M_r of 33,000 and a pI close to 9. The effect of pH on peroxidase activity was studied using guaiacol as substrate and the optimum pH determined at 25° was 6.0. This enzyme acted on guaiacol, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), *o*-dianisidine, *o*-phenylenediamine (*o*-PD) and pyrogallol, but had no effect on syringaldazine or coniferyl alcohol substrates.

INTRODUCTION

POD enzymes (EC 1.11.1.7) widely present in plants, fungi and yeast, catalyse the oxidation of various organic compound substrates in the presence of hydrogen peroxide. These enzymes which have been the subject of numerous studies, are distinguished according to their structural and catalytic properties. PODs are used in many industrial processes. In fact, they represent the main component in clinical diagnosis [1] and microanalytical immunoassay kits [2]. They are also implicated in the degradation of a wide variety of phenolic or aromatic compounds from waste [3] or from olive mill wastewaters [4]. In fact, plants constitute the most important source of POD isoenzymes, where they are present at a high level. Plant POD are associated with various biochemical and physiological processes such as growth [5], cell wall formation [6], fruit development and ripening [7], ethylene biosynthesis [8], pathogen resistance [9] and general stress response [10]. Biosynthesis, structure and physicochemical properties of several POD are well established. They are composed of several isoforms; each isoform having a specific role in metabolism. Acidic POD are thought to be involved in lignin biosynthesis processes [11, 12]. Until recently, it was proposed that the basic ones are implicated in IAA catabolism via a decarboxylation step, but actually a novel metabolic pathway has been established [13, 14]. However, little is known about the exact function and regulation of each isoenzyme [15]. POD play a role in indole alkaloid biosynthesis. In *Catharanthus roseus* leaves, basic POD targeted to vacuoles and cell-wall, is implicated in the coupling of vindoline and catharanthine into α-3′,4′-anhydrovinblastine [16–19]. In cell suspension cultures, POD activities catalyse the oxidation of ajmalicine to serpentine [20]. On the other hand, several studies have shown that hormones affect alkaloid production. Thus, transferring 2,4-D-dependent cells on auxin-free medium induced alkaloid production [21, 22]. Cytokinins stimulated alkaloid production, induced by removing 2,4-D [23, 24]. Analysis of cytokinins and/or auxin effects on gene expression showed a correlation between the synthesis of two cytokinin-enhanced polypeptides and alkaloid production capacities of *C. roseus* cells. However the function of these polypeptides remains undetermined [24, 25].

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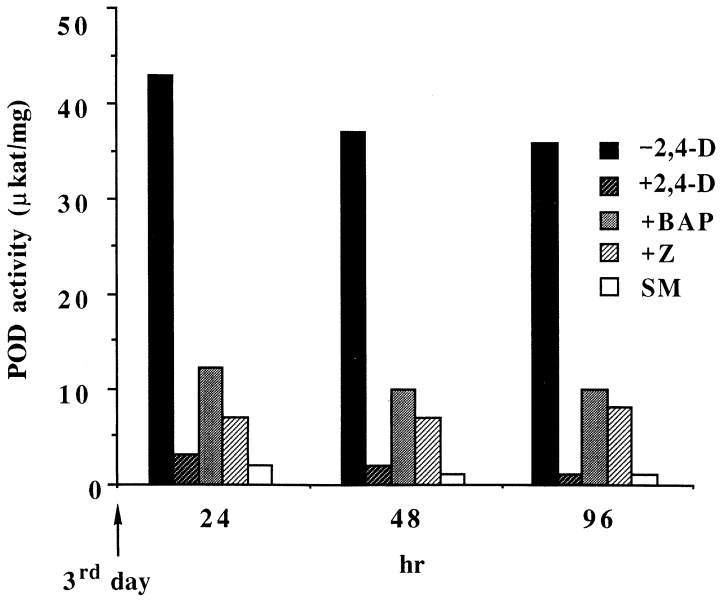

Fig. 1. Effect of various phytohormones on extracellular POD activities of *Catharanthus roseus* cells harvested after 24, 48 or 96 h treatments. Assays were performed in triplicate, SE did not exceed 10%. (+) with or (−) without hormone, (SM) subculture medium.

In this paper, we studied the variations in POD activities in *Catharanthus roseus* cell suspensions versus phytohormone treatments supporting or not alkaloid production. Our aim is to know whether specific isoperoxidase activity is correlated with alkaloid synthesis. Both activity and electrophoretic patterns have been analysed in the three compartments: cell, cell-wall and medium. Emphasis was placed on basic POD isoforms which accumulated in the culture medium after 2,4-D depletion (i.e. induction of alkaloid synthesis). We report the purification and partial characterization of the major basic POD, isolated from 2,4-D-free medium.

RESULTS AND DISCUSSION

Role of auxin and cytokinins in the regulation of POD activities secreted from Catharanthus roseus *cell suspensions*

Catharanthus roseus cells were maintained in 2,4-D containing B5 medium. For all experiments, cells were subcultured in 2,4-D-free medium for one passage and subjected on the 3rd day to one of the following treatments: cells untreated with hormone, addition of 2,4-D, addition of 6-benzylaminopurine (BAP) or zeatin (Z). POD activity was tested on protein extracts from culture media harvested at different times after treatment: 24, 48 or 96 h (Fig. 1). In the absence of any hormone, high specific POD activity was found in the extracellular medium. The secreted activity was maintained at a high level, even though it decreased slightly during the late stage of growth. In hormone-treated cultures, POD activities were strongly reduced. Addition of BAP or zeatin decreased POD activities about three times versus untreated culture, whereas the 2,4-D treatment led to a marked reduction in secreted POD activity. Thus, 2,4-D was more effective than cytokinins in decreasing activity. The lowest level of POD activity was reached at the end of the culture. It was identical to that of subculture medium. From these results, it may be concluded that phytohormone effects on secreted POD levels were unmodified according to the stage of culture. The level of POD activity tested 24 and 48 h after phytohormone treatments and those obtained after 96 h were similar. As the amounts of secreted proteins were more important during the late log stage of growth (day 7), cells were harvested on day 7 of growth which allowed high protein recovery and made POD analysis easier.

In Table 1, are reported data of protein amounts, total and specific POD activities in the three compartments (medium, cell-wall and cell) of *Catharanthus roseus* cell suspensions cultured under different conditions. Regardless of hormone treatments, total POD activities and protein amounts were lower in culture medium and cell-wall than in cells. The highest specific POD activities were observed in extracellular protein extracts. 2,4-D treatment reduced both extracellular and cellular POD activities whereas protein amounts were increased in the three compartments. The decrease induced by 2,4-D was stronger in the case of extracellular activities. On the contrary, 2,4-D treatment led to a great increase in the POD activity ionically bound in the cell-wall. From these experiments, we can speculate that 2,4-D affects synthesis and/or secretion of POD activities, the drastic decrease in POD activities of culture medium is

Table 1. Variation of protein content, total and specific POD activities in medium, cell-wall and cellular compartments of treated or untreated *Catharanthus roseus* cultures. Protein amounts and total POD activities values were expressed in mg and μkat respectively per liter of cell suspension cultures

	Protein (mg)			Total activity (μkat)			Specific activity (μkat mg^{-1})		
	−2,4-D	+2,4-D	+BAP	−2,4-D	+2,4-D	+BAP	−2,4-D	+2,4-D	+BAP
cell	527	664	668	2674	1050	2400	5	2	4
cell-wall	38	106	49	38	581	64	1	5	1
medium	11	18	12	407	23	132	36	1	11
Total	576	788	729	3119	1650	2600	—	—	—

accompanied by an accumulation of POD activities in cell-wall.

To determine whether synthesis and/or secretion was affected by 2,4-D treatment, qualitative analysis was performed using isoelectric focusing (IEF) electrophoresis (Fig. 2). In extracellular samples, POD activities were distributed in two main groups; (i) one acidic group, referred to as (A), that displayed at least three isoforms A_1, A_2 and A_3. In the absence of any hormone, only one A_2 isoform, highly active, was observed. This isoform was also present at a lower level in hormone-treated cultures. A_1 isoform was detected when 2,4-D was added to cultures, whereas A_3 isoform was found only in BAP-treated culture; (ii) The basic group, referred to as (B), was highly active in the control culture and to a lesser extent in BAP-treated ones. Addition of 2,4-D led to a drastic decrease of this activity. It should be noted that B group displayed few isoforms with pI greater than 8.5 (results not shown). In cell-wall proteins isolated from 2,4-D-treated cultures, one isoform with a pI similar to A_2 was clearly observed; however, activity of the basic group was weak. Cell-wall POD activities were strongly reduced in untreated and cytokinin-treated cells. IEF profiles obtained from cellular proteins showed some qualitative differences in acidic POD group (A). A_1 and A_3 isoforms were not detected in phytohormone-treated cells, whereas A_2 isoform was poorly active in untreated cells.

The present study deals with POD activities distribution in *C. roseus* cell suspension cultures subjected to different hormonal treatments. The increase in extracellular activity of untreated cultures was not due to cell lysis because activities and POD isoform patterns are different in culture medium and cells. This was confirmed by cell viability analysis and by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of proteins which showed a small number of polypeptides (data not shown). Our results showed that the decrease in the level of cellular and secreted POD activities was related to 2,4-D treatment of *C. roseus* cultures. This inhibition was mainly on the

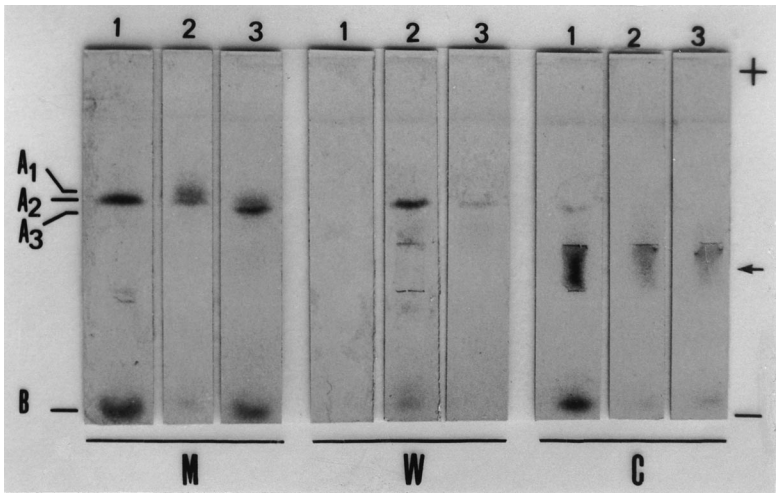

Fig. 2. IEF gel profiles of POD isoforms found in medium (M), cell-wall (W) and cell (C) extracts after 96 h treatment. Lane 1: −2,4-D, Lane 2: +2.,4-D, Lane 3: +BAP. Acidic POD referred (A_1, A_2 and A_3) and Basic POD referred (B). For the 3 hormonal treatments, the protein amounts of medium, cell-wall and cell loaded onto the gel were (50 μg, 70 μg and 100 μg) respectively. POD activity was revealed with benzidine/guaiacol. The arrow marked the origin of the run.

Table 2. Purification steps of the extracellular basic peroxidase of suspension-cultured *Catharanthus roseus* cells

Purification step	Total protein (mg)	Total activity (μkat)	Specific activity (μkat mg^{-1})	Purification fold	Recovery (%)
Culture medium	17	607	36	1	100
Enzyme extract	11	414	38	1	68
Chromatofocusing	4.5	254	56	1.6	42
CM-sepharose	1.5	125	84	2.3	21
TSK phenyl-5PW	0.026	34	1330	37	6

basic isoforms. Particularly, 2,4-D inhibited the synthesis but not the secretion of some basic isoforms. On the contrary, 2,4-D treatment was found to increase specifically the synthesis and the accumulation of acidic POD isoforms in the cell-wall compartment. Cytokinin treatment lowered total and specific POD activities in cells and culture medium. The cytokinin-dependent decrease was less marked when compared with the 2,4-D effect. IEF analysis showed that cytokinins affected only slightly the basic group POD, whereas 2,4-D led to a drastic decrease of these isoforms. So, the partial decrease of POD activity in presence of cytokinins would concern acidic POD. This result is of particular interest since: (i) basic group corresponds to several POD isoforms, (ii) basic PODs were found in cells cultured on untreated or cytokinin-treated media which support alkaloid synthesis. Consequently we can assume that removing 2,4-D induced the synthesis of the basic POD, implicated in alkaloid synthesis, and these isoforms remained unmodified after addition of cytokinins. The high levels of basic POD found in hormone-free media may also represent, at least in part, a stress response of *Catharanthus roseus* cells to the suppression of 2,4-D. Further studies are necessary to elucidate the phytohormone effects on synthesis and/or secretion of the main basic POD isoform and its relationship with alkaloid synthesis. For this reason we decide to purify and partially characterize this isoform.

Purification of extracellular basic peroxidase

All purification steps are summarized in Table 2. The basic POD isoforms were mainly secreted with high activity in 2,4-D-free medium from *Catharanthus roseus* cells. In fact, the enzyme extract subjected to purification, was prepared as described in Experimental. The suspension-cultured *Catharanthus roseus* cells released into culture medium a large amount of polysaccharide (20 g l^{-1}, unpublished results). This was precipitated with $CaCl_2$ and removed from culture medium by centrifugation. The supernatant containing proteins was filtered on a 0.45 μm nitrocellulose filter and concentrated by precipitation with $(NH_4)_2SO_4$ saturated to 85%. The amount of the extracted proteins was very low (12 mg^{-1} of culture medium). This may be explained by the fact that the polysaccharide bound a significant amount of the protein. The enzyme extract was loaded onto a chromatofocusing column. Two protein peaks (I and II) were separated according to their pI. Peak I with pI value higher than 8.3 did not bind to the column and was present in the washing fractions. 60% of the total POD activity was found in this peak, which was used for further purification of basic peroxidase. Peak II showed a low level of POD activity and corresponded to acidic POD. The eluate containing basic POD, was applied to a CM-sepharose column. The major POD activity was eluted at 150 mM of NaCl (Fig. 3). Fractions with high specific activity were loaded onto a Progel-TSK^{TM} phenyl-5PW column using an HPLC system. Only the last peak eluted by buffer D without $(NH_4)_2SO_4$, exhibited POD activity. The active eluate was dialysed against 20 mM NH_4OAc buffer (pH 5) and concentrated by lyophilization. The purity of the isolated POD was further controlled by gel electrophoresis under denaturing conditions, followed by silver staining.

Physico-chemical properties

Only a single protein band could be detected in SDS-PAGE. The M_r of the denatured POD was 33,000 (Fig. 4). The pI of this enzyme, as determined by IEF, was 8.8. The optimum pH was determined by incubating the purified POD with guaiacol in 100 mM NaOAc buffer for pH 3 to 5, in 100 mM Na-Pi buffer for pH 6 to 7 and in 100 mM Tris-HCl buffer for pH 8 to 9. The enzyme exhibited maximum activity at pH 6.0. It lost 50% of its activity at pH 4.5 or pH 8.5. This enzyme was able to oxidize guaiacol, *o*-PD, ABTS, *o*-dianisidine and pyrogallol, but had no effect on syringaldazine and coniferyl alcohol substrates, suggesting their non-involvement in lignin biosynthesis.

Five soluble POD as previously reported [16], were isolated from *Catharanthus roseus* cells according to their pI (7.8; 8.0; 8.3; 9.0; 10.5). The most basic isoform showed a M_r of 15,000, whereas the M_r of the other enzymes was 37,000. This M_r was slightly greater than our purified POD, but the pI was almost the same.

From our results, it may be argued that 2,4-D

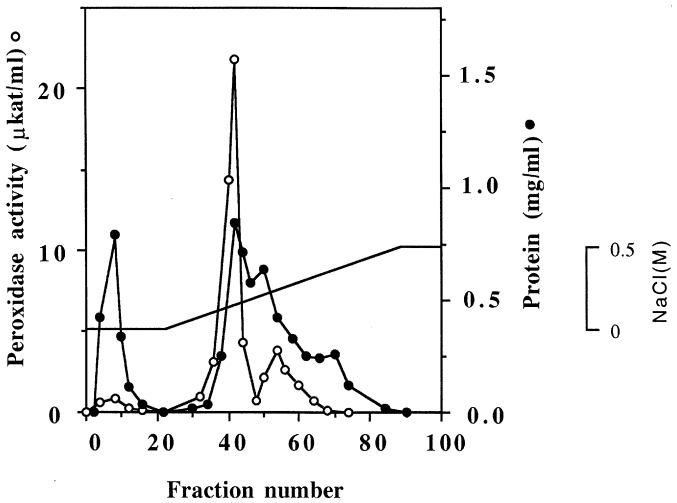

Fig. 3. Separation of basic isoperoxidases on CM-sepharose column. The proteins were loaded onto the column (5 × 95 mm) previously equilibrated with 25 mM NaOAc buffer (pH 5). The elution of bound proteins was carried out at 5 ml h^{-1} with a linear gradient of NaCl (0–500 mM) in the same buffer. The eluates (1 ml per fraction) containing POD activity were pooled and submitted to HPLC step.

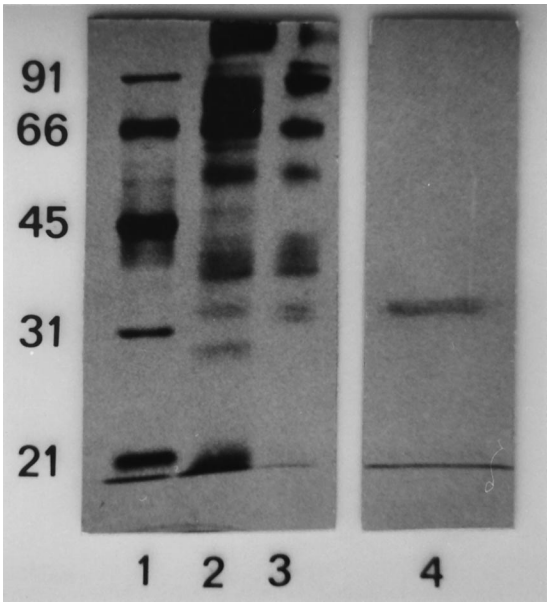

Fig. 4. SDS-PAGE profiles during the purification process of basic POD released in medium from *Catharanthus roseus* cell suspensions. Lane 1: M_r markers, Lane 2: protein extract after $(NH_4)_2SO_4$ precipitation (4 μg), Lane 3: Pooled fractions from CM-sepharose (1.5 μg), Lane 4: Purified basic POD from HPLC step (0.2 μg). The position of M_r expressed in k are shown on the left hand side. The proteins were silver stained.

inhibited the synthesis of the basic POD and increased the accumulation of an acidic isoform. The physiological significance of these results is at present unknown, but further experiments including the characterization of gene structure, its expression and modulation by phytohormones may help to elucidate the function of this basic POD as was reported for tobacco anionic POD [26].

EXPERIMENTAL

Buffers

Buffer A: 50 mM Tris-HCl (pH 7), buffer B: 25 mM Tris-HOAc (pH 8.3), buffer C: 25 mM NaOAc (pH 5), buffer D: 100 mM Na-Pi (pH 7).

Cell suspension cultures

Catharanthus roseus cells (strain C_{20}) were cultured in the B5 basal medium [27] containing 58 mM sucrose and 4.5 μM 2,4-D. They were maintained on a 7 day growth cycle at 24° in the dark [22]. Suspension of cells (50 ml) were incubated on a rotary shaker at 100 rpm. For experimental purposes 7-day-old cells were subcultured in a 2,4-D-free B5 medium containing 58 mM sucrose and were exposed on the 3rd day to one of the following treatments: no addition of hormones (control cells), addition of 4.5 μM 2,4-D, addition of 5 μM BAP or addition of 5 μM zeatin. Cells harvested at different time: 24, 48 and 96 h, were sepd from the medium by filtration through 50 μm nylon filter, washed with H_2O, frozen into liquid N_2, lyophilized and stored at −20° prior to use.

Preparation of cell-wall proteins and cellular soluble proteins

Cell-wall proteins and cellular soluble proteins were obtained using the protocol of Refs. [28, 29].

Preparation of extracellular protein extract

All steps were performed at 4°. To the culture medium, 132 mM of solid $CaCl_2$ was added with constant stirring and the resulting ppt was removed by centrifugation at 8000 *g* for 20 min. The supernatant was again filtered through 0.45 μm nitrocellulose filter, and desalted by dialysis against 50 mM Tris-HCl buffer (pH 7). The enzyme extract was concd by precipitation with solid $(NH_4)_2SO_4$ satd to 85%, and left overnight at 4°. The pellet was collected by centrifugation at 10,000 *g* for 20 min, resuspended in a small vol. of 50 mM Tris-HCl buffer (pH 7) and stored at −20°.

Purification of an extracellular basic peroxidase

Step 1: The protein extract from the last step isolated from culture medium of *Catharanthus roseus* cells cultured without hormones, was dialysed overnight against the start buffer 25 mM Tris-HOAc (pH 8.3) and applied to a chromatofocusing column (6 × 200 mm). The column was packed with a gel polybuffer exchanger PBE 94 and equilibrated with the start buffer. The column was washed with a start buffer until the A at 280 nm of eluate was less than 0.02. Fractions with peroxidase activity eluted during washing, were pooled and stored at −20°, whereas the bound proteins were eluted by the eluent buffer according to their isoelectric points.

Step 2: Following chromatofocusing column, the active frs were pooled, dialysed overnight at 4° against 25 mM NaOAc buffer (pH 5) and loaded onto a CM-Sepharose CL-6B column (5 × 95 mm) previously equilibrated in the same buffer. After sample application, the column was washed with 25 mM NaOAc buffer (pH 5) and the elution was carried out at 5 ml h^{-1} with a linear gradient of NaCl (0–500 mM) in the same buffer. All collected frs were assayed for peroxidase activity and those containing the highest activity (corresponding to peak eluted at 150 mM of NaCl) were conserved.

Step 3: These frs were then dialysed against buffer D containing 1.5 M $(NH_4)_2SO_4$. The dialysate was applied to a Progel-TSK™ phenyl-5PW column equilibrated in buffer E [buffer D containing 1.5 M $(NH_4)_2SO_4$]. Bound proteins were eluted with a linear gradient of $(NH_4)_2SO_4$ (from 1.5 to 0 M) in 60 min at a flow rate of 1 ml min^{-1}. All frs corresponding to the sepd peaks were collected and tested for POD activity. Only the peak eluted last exhibited a high peroxidase activity.

Peroxidase activity assay

POD activity was measured at 25° using guaiacol as hydrogen donor. The reaction mixt. contained 9 mM guaiacol, 19 mM H_2O_2, 50 mM Na-Pi buffer (pH 7) and 50 μl of enzyme soln in a total vol. of 1 ml. The reaction was initiated by the addition of H_2O_2 and the reaction velocity was monitored by measuring the increase in *A* at 470 nm. The peroxidase activity was expressed in units of μkatal (one μmol of guaiacol was oxidized per s in the presence of H_2O_2) under the assay conditions. A molecular extinction of ε_{470} nm = 26.6 mM^{-1} cm^{-1} was used for calculation [30]. All assays were carried out in triplicate.

Protein determination

The protein content was performed by the method of Ref. [31] using a Bio-Rad protein assay with bovine serum albumin as standard.

Electrophoresis

Na dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 10% polyacrylamide gel [32]. Bio-Rad low M_r standards were used. Proteins were revealed by silver staining [33]. Native IEF was performed in a Bio-Rad Mini IEF Cell (Model 111) using 5% polyacrylamide gel with a 1 mm thick (according to manufacturer's instructions). A commercially available Biolyte 3/10 (ampholytes 40%) were used. Aliquots of 20 μl protein extract were applied on the gel and were subjected to electrophoresis. After focusing, the gel was soaked for 30 min in 50 mM Na-Pi buffer (pH 7) with 150 mM NaCl to remove ampholytes and equalize pH throughout the gel. The peroxidase isoenzymes were visualized by incubating the gel for 5 min in 50 mM Na-Pi buffer (pH 7) containing 9 mM guaiacol, 1 mM benzidine followed by addition of 19 mM H_2O_2 [34].

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