Purification and Kinetic Characterization of an Anionic Peroxidase from Melon (*Cucumis melo* L.) Cultivated under Different Salinity Conditions

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The partial characterization of an anionic peroxidase in melon fruit is described. Four melon peroxidase (MPX) isoenzymes were detected in crude extracts after isoelectric focusing. The major MPX isoenzyme (pI = 3.7) was partially purified by including hydrophobic and anion-exchange chromatography in the purification scheme. The sample obtained was used to characterize MPX. This peroxidase did not show activity on ascorbic acid but oxidized guaiacol at a high rate, showing an optimum pH of 5.5 when acting on this last reducing substrate. Melon fruits grown under highly saline conditions showed slightly increased levels of this anionic isoenzyme. Kinetic studies using 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid) (ABTS) as reducing substrate showed that increased salinity in the growth medium did not modify the kinetic parameters of melon peroxidase on both hydrogen peroxide and reducing substrate.

Keywords: *Melon; peroxidase; salinity; peroxidase isoenzyme; enzyme kinetics.*

INTRODUCTION

Melon is an important crop that is increasingly cultivated using low-quality saline waters in semiarid regions. Studies show that different cultivars vary from salt sensitive to moderately tolerant (Mangal et al., 1988; Medlinger and Pasternak, 1992a,b). However, sensitivity or tolerance can differ according to the culture medium, type of salinity, and plant growth stage (Nukaya et al., 1980). In any case, evaluation of the effect of salt is complicated by the fact that growth rates among cultivars are inherently different, and viability differs depending upon age and source of nutrient (Shannon et al., 1984). Furthermore, muskmelon quality parameters are affected to varying degrees by salinity (Shannon and Francois, 1978). Although there are a few studies that correlate salinity with melon fruit quality (Artes et al., 1993), no studies have been carried out on the effect of salinity on the biochemical parameters, which might explain the differences found in fruit quality. Peroxidases, enzymes whose primary function is to oxidize hydrogen donors at the expense of peroxides, are highly specific for H_2O_2. However, they accept a wide range of hydrogen donors (Dunford and Stillman, 1976).

Flesh firmness is considered a quality factor in melon fruit (Artes et al., 1993). Such firmness could be affected by salinity, which would cause changes in the structural components of the cell wall, such as a different proportion and composition of pectins and other noncellulosic polymers and an increase in protein (Adams and Ho, 1989; Adams, 1991). It has been proposed that peroxidase isoenzymes catalyze the cross-linking between the ferulic acid substituents of pectins (Fry, 1986). In addition, a clear correlation has been found between peroxidase activity and the synthesis of lignin and suberin polymers (Lagrimini et al., 1993).

Observation of any changes in peroxidase isoenzyme expression induced by stress could aid in the assignment of a functional role to a given peroxidase group. Our objective was to isolate MPX from melons grown under different saline conditions and study the characteristics of the enzyme. Methods for purification and identification of MPX in nondenaturing IEF gels are proposed. Moreover, the major anionic isoenzyme isolated from melon fruit has been kinetically characterized.

MATERIALS AND METHODS

Plant Material and Chemicals. The experiment was carried out in a greenhouse equipped with an automatic regulated computer system for drip irrigation. Muskmelon plants cv. Galia obtained from a commercial seedbed were transplanted into 1.2 m length perlite sacks. The base nutrient solutions used for irrigation had the following composition (in mmol L^{-1}): NO_3^-, 12; $H_2PO_4^-$, 1.5; SO_4^{2-}, 1; Ca^{2+}, 4; K^+, 8. The following micronutrients were added in concentrations of 1, 0.5, 0.5, 0.25, 0.02, and 0.01 mg L^{-1} for Fe, Mn, Zn, B, Cu, and Mo, respectively. The experiment consisted of a control (2.1 dS m^{-1} EC; M1) and a salt treatment (8 dS m^{-1}; M2). Each treatment was replicated four times and consisted of three sacks of perlite, each containing six plants and three 2 L h^{-1} emitters about 40 cm apart. The salinity treatment began two weeks after transplanting and involved adding the appropriate

amount of NaCl to the control solution. The pH and the conductivity of the nutrient solution were controlled during each irrigation period, while the amount of nutrient solution applied depended on the demand detected in the appropriate trays. During the middle of the harvest period, two uniform mature fruits per treatment were selected from each replication. The fruit were peeled, rinsed free of seeds, and frozen at −20 °C. Samples were freeze-dried and ground before determining peroxidase activity.

Reagent grade H_2O_2 (30% v/v) was obtained from BDH Laboratory Supplies (Lutterworth, Leichestershire, UK), and its concentration was calculated by iodide titration in the presence of horseradish peroxidase isoenzyme C (Cotton and Dunford, 1973). ABTS, catechol, MBTH, tropolone, and guaiacol were purchased from Sigma, and ascorbic acid was obtained from Merck. Stock solutions of the reducing substrates were prepared in 0.15 mM phosphoric acid to prevent autoxidation. All other chemicals were of analytical grade and supplied by Merck.

Purification of Melon Peroxidase. Tissue homogenization, centrifugation, and dialysis were carried out between 4 and 8 °C. Chromatography purification steps were performed at room temperature. Ten grams of lyophilized melon was ground with 100 mL of extraction buffer comprising 0.1 M phosphate (pH 7.3) and 6% (w/v) TX-114 for 5 min with a blender operated at maximum sped. After 30 min at 4 °C the homogenate was centrifuged at $100000g$ for 15 min. The supernatant was collected and used as source of melon peroxidase (MPX). It was subjected to temperature-induced phase partition by increasing the TX-114 concentration by an additional 8% (w/v) at 4 °C and then warming to 35 °C for 15 min. The solution became turbid due to the formation, aggregation, and precipitation of large micelles of detergent that contained hydrophobic proteins and phenolic compounds. This solution was centrifuged at $8000g$ for 15 min at 25 °C. The detergent-rich phase was discarded, and the supernatant was subjected to an additional phase-partitioning step with 8% (w/v) TX-114. The protocol was repeated once more to remove the remaining phenols. The clear supernatant containing soluble MPX was acidified to pH 5.0. The acidified supernatant was kept for 1 h at 4 °C before being centrifuged at $100000g$ for 30 min. The pellet was discarded and the supernatant brought to 15% saturation with solid ammonium sulfate under continuous stirring. After 30 min the solution was centrifuged at $80000g$ for 30 min and the pellet discarded. Additional ammonium sulfate was added to the clear supernatant to give 60% saturation and stirred overnight. The solution was centrifuged at $100000g$ for 30 min and the pellet dissolved in a minimal volume of deionized water. The enzyme was applied in a Phenyl-Shepharose CL4B column (PS-CL4B) that had been equilibrated with 50 mM phosphate buffer (pH 7.0) and 1 M ammonium sulfate. The column was prewashed with 200 mL of equilibrating buffer, and then 1.5 L of a step gradient comprising 1.0 to 0 M ammonium sulfate was used to separate the different peroxidase isoenzymes. The major isoenzyme (pI = 3.7) was further purified by FPLC (ÄKTAexplorer 100, Pharmacia) using anion-exchange chromatography on a Resource Q column (Pharmacia) equilibrated with 25 mM Bis-Tris buffer (pH 6.5) and eluted with sodium chloride (0−0.5 M gradient).

Isoelectrofucosing. IEF was performed using the Pharmacia Fast Gel system on prepoured gels (pH 3−9) with the following standards: trypsinogen (pI 9.3), lentil lectin-basic (pI 8.65), middle (pI 8.45), and acidic (pI 8.15), myoglobin-basic (pI 7.35), human carbonic anhydrase B (pI 6.55), bovine carbonic anhydrase B (pI 5.85), β-lactoglobulin A (pI 5.20), soybean trypsin inhibitor (pI 4.55), and amyloglucosidase (pI 3.50). The pI's of the standards were assumed to be as designated by the supplier (Pharmacia). IEF gels containing MPX were placed in glass dishes containing 20 mL of rinsing buffer (50 mM acetate buffer, pH 4.5) and equilibrated at room temperature with gentle shaking for 30 min. After repeating the rinsing procedure two more times, the gels were transferred into 20 mL staining solution which contained the rinsing buffer supplemented with 1 mM hydrogen peroxide, 0.5 mM

MBTH, and 3 mM catechol. Tropolone, a polyphenoloxidase inhibitor (Kahn, 1985), was added in order to avoid the oxidation of catechol by polyphenol oxidase. After staining, the gels were stored in water.

Determination of Protein Concentration and Enzyme Assay. Protein concentrations were determined using the Bio-Rad protein assay procedure with bovine serum albumin as a standard. The peroxidase activity with ABTS as the reducing substrate was determined in a reaction mixture (1 mL) containing 50 mM sodium citrate buffer (pH 4.5), 1 mM ABTS, and 0.5 mM H_2O_2. The oxidation of ABTS was followed by observing the increase in absorbance at 414 nm ($\epsilon_{414nm} = 31.1$ mM^{-1} cm^{-1}). One unit of the enzyme is defined as the amount of enzyme that oxidized 1 μmol ABTS/min at 25 °C under the above assay conditions. The rate of guaiacol oxidation was measured using the some reaction mixture. Tetraguaiacol formation was followed at 470 nm ($\epsilon_{470nm} = 26.6$ mM^{-1} cm^{-1}).

Kinetic Data Analysis and Spectrophotometry. Steady-state kinetic constants were obtained by measuring the initial rates of ABTS oxidation at 25 °C at different H_2O_2 (0.05−0.5 mM) and ABTS (0.1−1.0 mM) concentrations in the above assay conditions. Data were fitted by linear regression to eq 1:

$$1/\nu_0 = (K_m^{ABTS}/V_{max})(1/[ABTS]) + (K_m^{H2O2}/V_{max})(1/[H_2O_2]) + (1/V_{max}) \quad (1)$$

Spectrophotometric assays were carried out with a Perkin-Elmer Lambda-2 spectrophotometer on-line interfaced with a compatible PC for further data analysis. Temperature was controlled at 25 °C using a Haake D1G circulating water bath equipped with a heater/cooler and controlled by a Cole-Parmer digital thermometer with a precision of ±0.1 °C.

Inactivation Kinetics. MPX-A2 was inactivated at 25 °C in 0.5 mL incubations of 50 mM sodium phosphate, pH 7.0, containing a fixed amount of the enzyme (0.5 unit). The reactions were started by the addition of H_2O_2 (over a range of concentrations). At specific time intervals, 10 μL aliquots of the incubation mixtures were transferred to cuvettes containing 1 mL of an assay mixture composed of 0.5 mM ABTS and 0.2 mM H_2O_2 in 50 mM sodium citrate buffer, pH 4.5. A minimum of four incubation assays for each peroxide concentration were made. The residual enzymatic activity (A_R) was taken as the remaining enzymatic activity (A_t) as a percentage of the initial activity (A_0).

RESULTS AND DISCUSSION

Melon Peroxidase Purification. Melons growing in different salinity conditions (M1 and M2; see Materials and Methods section) were used as peroxidase source. Peroxidase was extracted and partially purified by using two sequential phase partitionings with TX-114 followed by ammonium sulfate precipitation and hydrophobic chromatography. IEF of crude extracts obtained from M1 showed four peroxidase isoenzymes, two acidic pI's of 3.7 and 5.4 (MPX-A1 and MPX-A2, respectively), and two basic with pI's of 8.4 and 8.6 (MPX-B1 and MPX-B2, respectively) (data not shown). Melon peroxidase isoenzymes were detected in isoelectric focusing gels employing hydrogen peroxide, MBTH, and catechol in the presence of tropolone. Tropolone prevents the polyphenol oxidase-catalyzed oxidation of catechol (Kahn, 1985; Rodriguez-Lopez et al., 1994), doing the method specific for peroxidase activity. In the presence of hydrogen peroxide, MPX catalyzed the oxidation of both MBTH and catechol to their corresponding radical products (Ngo and Lenhoff, 1980). Both radical products react in a coupling reaction to give an orange and insoluble compound with a maximum at around 470 nm. The limited solubility of the MBTH/
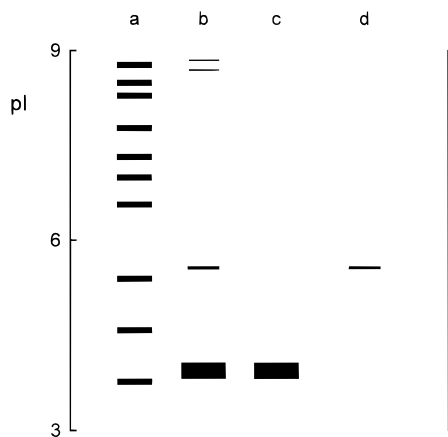
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Figure 1. Isoelectrofocusing of melon peroxidase. MPX was obtained from melon fruits grown in highly saline condition: lane a, market; lane b, crude extract; lane c, first eluted peak after elution in a phenyl-Sepharose CL4B column; lane d, second eluted peak after elution in a phenyl Sepharose CL4B column.

catechol adduct makes this method ideal for detecting MPX in nondenaturating polyacrylamide gels. MPX-A1 was found to be the major peroxidase band (ca. 88% of peroxidase activity as determined by optical densitometry at 470 nm) in extracts of melon cultivated under normal conditions. The same isoenzymatic pattern was observed for crude extracts obtained from M2 (Figure 1). Therefore, melons grown under highly saline conditions did not express any new peroxidase isoenzymes.

Anionic peroxidase induction has been related with different types of physiological stress in plants (Rothan and Nicolas, 1989; Repka and Fischerova, 1996). To investigate possible differences in the expression and properties of the anionic isoenzymes expressed under normal or saline conditions, we purified these peroxidase isoenzymes using hydrophobic chromatography. MPX-A1 and MPX-A2 showed different degrees of hydrophobocity and could be separated by PS-CL4B (Figures 2 and 3). The bulk of the peroxidases were eluted from the column in the free ammonium sulfate fractions. We used a step gradient in this purification step to avoid overdilution of the enzyme (Jen et al., 1980). Two peaks of peroxidase activity were observed; the first one eluted from the column was determined to have a pI of 3.7, corresponding to MPX-A1, whereas MPX-A2 (pI 5.4) eluted off immediately after (Figures 2 and 3). The level of both isoenzymes increased in melon cultivated in saline conditions, the amount of MPX-A1 and MPX-A2 being ca. (1.6 ± 0.2)- and (3.4 ± 0.3)-times higher, respectively, in melon fruit that had been subjected to salt stress. The above results correspond to the average of five separate purification experiments. To study the kinetic properties of the major isoenzyme expressed in melon fruit, MPX-A1 was further purified using anion-exchange chromatography.

Substrate Specificity and pH Optimum. One of the ways to distinguish different classes of peroxidase is to compare their relative enzymatic activities for guaiacol and ascorbic acid (Kvaratskhelia et al., 1997). MPX-A1 oxidized guaiacol at a high rate but showed no activity on ascorbic acid, demonstrating that this anionic isoenzyme in melon fruit seed is not an ascorbate peroxidase. When guaiacol was the substrate, the optimum activity of MPX-A1 was pH 5.5 (Figure 4). The pH profile was the same for the MPX-A1 purified from melons grown in highly saline conditions.
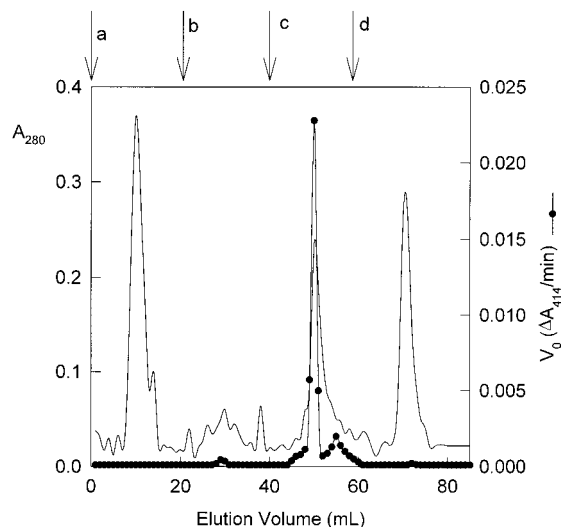

Figure 2. Hydrophobic chromatography of melon peroxidase using a phenyl-Sepharose CL4B column. Melon peroxidase was obtained from melon fruits grown in normal salinity conditions (M1). Elution was by stepwise decreasing gradients with 1 (a), 0.5 (b), and 0.0 M (c) ammonium sulfate in 50 mM phosphate buffer, pH 7.0, followed with deonized water (d). 2.5 mL fractions were collected.
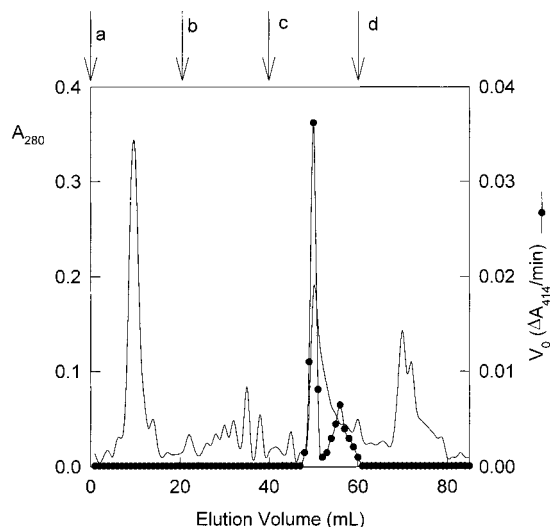

Figure 3. Hydrophobic chromatography of melon peroxidase using a phenyl-Sepharose CL4B column. Melon peroxidase was obtained from melon fruits grown in highly saline condition (M2). Elution was by stepwise decreasing gradients with 1 (a), 0.5 (b), and 0.0 M (c) ammonium sulfate in 50 mM phosphate buffer, pH 7.0, followed with deonized water (d). 2.5 mL fractions were collected.

Kinetic Studies. Our peroxidase assay used the noncarcinogenic substrate ABTS. Linear Lineweaver–Burk plots were obtained over a limited range of substrate concentrations (Figure 5). Primary and secondary plots were used to evaluate values of V_{max} and apparent K_m for H_2O_2 and ABTS (Figure 5). Such data, presented in Table 1, was useful for further characterization of MPX-A1 isoenzyme and in the comparison of this enzyme expressed under normal and highly saline conditions. Under steady-state conditions with ABTS as substrate, the MPX-A1 obtained from melon grown under both normal and highly saline conditions showed a high affinity for both ABTS and hydrogen peroxide and similar kinetic parameters (Table 1). Salinity in the growth medium did not modify the kinetic characteristics of this enzyme.
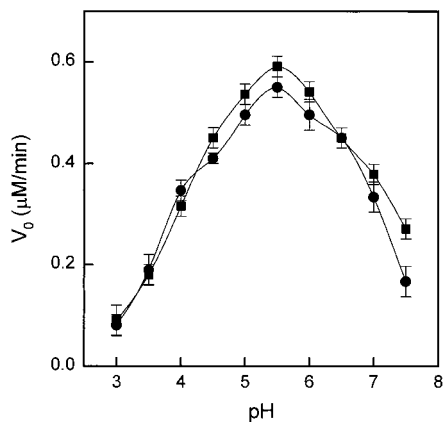
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Figure 4. Effect of pH on the enzymatic activity of MPX-A1 purified from melon grown under normal (●) and highly saline (■) conditions and with guaiacol as reducing substrate. Assay conditions are described in the Materials and Methods section. Each point represents the mean of five separate experiments.
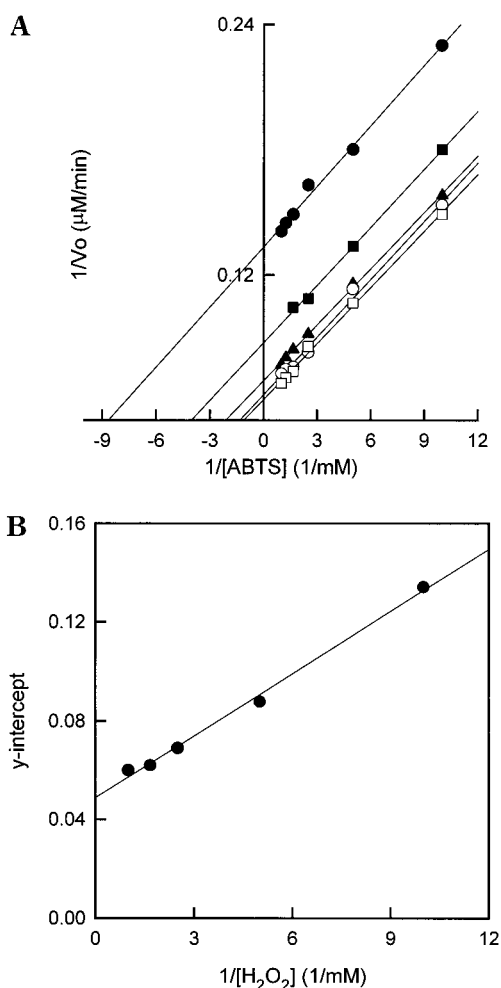

Figure 5. (A) Variation of $1/V_0$ vs the inverse of the concentration of ABTS at different fixed values of the concentration of hydrogen peroxide. Conditions: $[\text{MPX-A1}]_0 = 0.5$ unit; $[\text{H}_2\text{O}_2] = 30, 50, 100\ 150$, and $200\ \mu\text{M}$. (B) Plot of y-axis values vs $1/[\text{H}_2\text{O}_2]$. Each point represents the mean of three separate experiments. Standard deviation bars were omitted for clarity.

Inactivation of MPX-A2 by Hydrogen Peroxide. Peroxidases suffer a suicide inactivation by H_2O_2 in the absence of reducing substrates (Arnao et al., 1990). MPX-A1 showed great resistance to H_2O_2, and Figure 6 shows the time-course of MPX-A1 inactivation at different H_2O_2 concentrations. There was a fast inacti-

Table 1. Apparent K_m Values for ABTS and Hydrogen Peroxide in Their Reaction with MPX-A1 Obtained from Melons Grown under Normal (M1) or Highly Saline (M2) Conditions[a]

enzyme	$K_{m\ (ABTS)}$ (mM)	$K_{m\ (hydrogen\ peroxide)}$ (mM)
MPX-A1 (M1)	0.25 ± 0.05	0.23 ± 0.06
MPX-A1 (M2)	0.20 ± 0.06	0.19 ± 0.05

[a] The values represent the kinetic parameters $\pm$ the standard deviation.

Figure 6. Time dependence of the inactivation of MPX-A1 on H_2O_2. MPX-A1 (0.5 units) was incubated with H_2O_2 at (●) 5.0 and (■) 50 mM. Each point is the average of five different inactivation experiments.

vation process (within the first minute of the reaction), while the remaining activity was dependent on the H_2O_2 concentration. At 50 mM H_2O_2 this remaining activity was ca. 65% of the initial activity. This behavior contrasts with the other plant peroxidases such as the cationic isoenzyme of horseradish peroxidase (HRPC), which showed a higher degree of inactivation at 50 mM H_2O_2 (Hiner et al., 1995). The resistance of peroxidases to H_2O_2 could be related with their physiological function. Thus, peroxidases expressed under stress conditions may be more resistant to H_2O_2. No differences in the susceptibility to H_2O_2 inactivation were found between MPX-A1 expressed in normal and highly saline conditions.

CONCLUSIONS

Peroxidase isoenzymes have been reported to be involved in many cell alterations. It has been proposed that peroxidases catalyze the cross-linking between tyrosine residues of the cell wall extensins and the ferulic acid substituents of pectins (Fry, 1986). Moreover, a clear correlation has been found between peroxidase activity and the synthesis of lignin and suberin polymers (Lagrimini et al., 1993). On the other hand, it has been reported that in cucumber plants peroxidase activity was enhanced as fruit matured, particularly in the external parts of the fruit (Repka and Ficherova, 1996). All these previous results address the question of the involvement of peroxidase in the cell wall changes that occur during cell adaptation to stress. The main contribution to cell wall peroxidase activity has been ascribed to anionic isoenzymes (Campa, 1991). In this report we observed a slight increase in the levels of anionic MPX isoenzymes induced by salinity. This increase in peroxidase content could be related with the increase in fruit firmness observed with increasing salinity (Petersen et al., 1998).

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ABBREVIATIONS USED

MPX, melon peroxidase; ABTS, 2,2′-azinobis(3-ethyl-benzothiazolinesulfonic acid); MBTH, 3-methyl-2-benzothiazoline hydrazone; tropolone, 2-hydroxy-2,4,5-cycloheptatrien-1-one; IEF, isoelectric focusing.

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