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Biochemical and Spectroscopic Characterization of Morning Glory Peroxidase from an Invasive and Hallucinogenic Plant Weed *Ipomoea carnea*

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A novel heme peroxidase MGP from the latex of *Ipomoea carnea* subsp. *fistulosa* (morning glory) belonging to the Convolvulaceae family was purified to homogeneity using ammonium sulfate precipitation, anion exchange, hydrophobic interaction, and gel filtration chromatography. The enzyme is glycosylated and has a molecular mass of 42.06 kDa (MALDI-TOF) and an isoelectric point of pH 4.3. The enzyme has high yield, broad substrate specificity, and a high stability toward pH, temperature, chaotrophs, and organic solvents. The extinction coefficient ($\varepsilon_{280}^{1\%}$) of the enzyme was estimated as 20.56 and it consists of 13 tryptophan, 9 tyrosine, and 8 cysteine residues forming 4 disulfide bridges. There is significant effect of inhibitors targeting S−S bridges (mercaptoethanol, L-cysteine, glutathione), as well as of inhibitors targeting heme (sodium azide and hydroxylamine) on peroxidase activity, whereas inhibition was not observed with ethylmaleinimide due to the absence of reduced cysteine in the enzyme. Polyclonal antibodies against the enzyme have been raised in rabbit, and immunodiffusion suggests that the antigenic determinants of MGP are unique. The N-terminal sequence of MGP (D-E-A-C-I-F-S-A-V-K-E-V-V-D-A) exhibited considerable similarity to the sequence of other known plant peroxidases. Spectroscopic studies (absorbance, fluorescence, and circular dichroism) reveal that MGP has secondary structural features with α/β type with approximately 20% α-helicity.

KEYWORDS: Convolvulaceae; heme extraction; polyclonal antibodies; N-terminal sequence; fluorescence; secondary structure

INTRODUCTION

Peroxidases (donor: H_2O_2 oxidoreductase, EC 1.11.1.7), a ubiquitous class of plant proteins, are enzymes that oxidize a variety of hydrogen donors at the expense of hydrogen peroxide and alkyl peroxides as oxidants (*1*). In vivo they are one of the key enzymes controlling plant growth, differentiation, and development. Heme-containing peroxidases are widespread in nature and perform important physiological functions that include lignifcation, suberization, and stress/pathogen response (*2*). Peroxidases are involved in lignin biosynthesis (*3*), auxin metabolism (*4*), disease resistance (*5*), wound healing (*6*), and protection of plants from air pollutant stress (*7*). In addition, these enzymes are also widely employed in enzymatic immunoassays, clinical diagnosis, biosensors, and organic synthesis (*8*−*10*). Attributed to the catalytic activity of the enzyme, several novel applications have been suggested including treatment of waste-water containing phenolic compounds, synthesis of various aromatic chemicals, and removal of peroxide from foodstuffs and industrial wastes (*11*−*14*).

Non-animal heme peroxidases have been classified on sequence comparisons and enzyme localization into three classes: class I contains intracellular enzymes [e.g., yeast cytochrome *c* peroxidase (CcP), ascorbate peroxidase (APX), and bacterial gene-duplicated catalase-peroxidases], class II consists of secretory fungal enzymes (e.g., manganese peroxidase and lignin peroxidase), and class III contains the secretory plant peroxidases [e.g., horseradish peroxidase (HRP-C) (*15*)]. All members of the class III peroxidases share a heme prosthetic group and catalyze a multistep oxidative reaction involving hydrogen peroxide as the electron acceptor. Furthermore, a unique feature is that class III peroxidases contain four conserved disulfide bridges and two conserved calcium binding sites (*16*). These enzymes share a similar catalytic cycle in which H_2O_2 reacts with the resting ferric enzyme to form an intermediate compound **I** carrying two oxidizing equivalents. Compound **I** is subsequently reduced by two molecules of the reducing

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substrate. The first molecule of the reducing substrate generates a second intermediate, compound **II**, which is further reduced by the second molecule of the reducing substrate to regenerate the native ferric enzyme. Peroxidases are found in various plant tissues, but horseradish roots have been the traditional source for the commercial production of peroxidases. An ideal peroxidase for large-scale biocatalysis would be the one that is readily abundant, possesses wide substrate specificity, and has better stability over a wide range of pH, temperature, and sensitivity. Therefore, the searches for such novel peroxidases are of immense research interest.

Ipomoea carnea subsp. *fistulosa* of the Convolvulaceae family [morning glory (MG)] is a toxic plant (weed) found in abundance in India, Brazil, the United States, and other countries. *I. carnea*, and aggressive weed in wetlands, is toxic to cattle and difficult to eradicate (*17*). The water extract of the leaves causes drunkenness and smoked leaves are hallucinogenic. The plant has an allelopathic effect; boiled roots are used as a laxative, and it provokes menstruation. It is used by traditional healers for skin disease treatment, and the milky juice of the plant has been used for the treatment of leucoderma and other related skin diseases. The toxic principles of the plant had been identified as two nortropane alkaloids, calystegines B2 and C1, and an indolizidine alkaloid, swainsonine (SW) (*18*). The first two are powerful glycosidase inhibitors affecting α-glucosidase and β-galactosidases, leading to human genetic lysosomal storage defects, Gaucher's and Fabry's diseases, respectively (*19*), whereas the latter (SW) is a potent inhibitor of lysosomal mannosidase. Aqueous and 80% ethanol extracts of *I. carnea* spp. *fistulosa* exhibited HIV type 1 reverse transcriptase inhibitory activity, and therefore it may be useful in the treatment of AIDS (*20*). During the screening of different parts of the *I. carnea* subsp. *fistulosa* (morning glory) plant for peroxidase activity, the latex exhibited a considerable amount of peroxidase activity. In this paper, the identification, purification, and biochemical characterization of a heme peroxidase from the latex of morning glory is reported. Owing to its high purity, high yield, and broad stability range, the morning glory peroxidase may have immense industrial/biomedical applications.

EXPERIMENTAL PROCEDURES

Materials. Latex was collected by superficial incisions of young stems of *I. carnea* subsp. *fistulosa* (morning glory) found abundantly on the Banaras Hindu University campus, Varanasi, India. *o*-Dianisidine, guaiacol (2-methoxyphenol), *o*-phenylenediamine, sodium azide, L-cysteine, glutathione, *N*-ethylmaleimide, BSA, ribonuclease A, hen egg white lysozyme, hemoglobin, DTNB, $HgCl_2$, GuHCl, glycerol, urea, EDTA, EGTA, β-mercaptoethanol, *N*,*N*′-methylenebis(acrylamide), TEMED, Coomassie brilliant blue R-250, Freund's complete and incomplete adjuvants, and agarose were obtained from Sigma Chemical Co. H_2O_2 (30%, v/v) was from Loba Chemie Pvt Ltd. India; acrylamide, pyrogallol (1,2,3-trihydroxybenzene), aminoantipyrine, ammonium sulfate, and TFMS were purchased from Spectrochem Pvt Ltd. India. Methanol, ethanol, isopropanol, butanol, DMSO, and acetonitrile were of HPLC grade. Ampholine carrier ampholytes were from Bio-Rad Biolytes. All other chemicals were of the highest purity available commercially.

Peroxidase Purification. All steps for the purification of the enzyme were carried out at 4 °C. All solutions were prepared using ultrapure water produced by a Milli-Q Plus system (Millipore).

Step 1: Gum Removal. Latex was collected by superficial incisions made on young stems of *I. carnea* plants and collected into 0.05 M sodium acetate buffer, pH 5.5, and stored at −20 °C for 24 h. The latex was thawed at room temperature and centrifuged at 12000*g* for 20 min using a Sorvall RC-5C Plus to remove any insoluble material and gum. The clear supernatant thus obtained was referred to as crude latex and used further.

Step 2: Ammonium Sulfate Precipitation. Crude latex from the above step was subjected to 80% (w/v) ammonium sulfate precipitation at 4 °C with continuous stirring. After 24 h, the resultant precipitate was collected by centrifugation (10000*g* for 20 min at 4 °C) with a Sorvall RC-5C Plus, dissolved in 0.05 M Tris-HCl buffer, pH 8.0, and dialyzed against the same buffer. The peroxidase activity and protein concentration of the dialyzed protein were measured to estimate the specific activity as well as the total protein obtained in this step.

Step 3: Anion Exchange Chromatography. The dialysate from the previous step was subjected to anion exchange chromatography on a DEAE-Sepharose Fast Flow column (5.0 × 5.0 cm) pre-equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. The bound proteins from the column were eluted with a linear gradient of NaCl from 0.0 to 0.5 M in the same buffer. Fractions of 3 mL volume were collected at a flow rate of 3 mL/min. All of the fractions were assayed for protein content by measurement of absorbance at 280 nm, activity using hydrogen peroxide as first substrate along with aminoantipyrine/phenol as a second substrate, and the extent of homogeneity by SDS-PAGE. The elution profile of the bound proteins was resolved into two peaks as the first peak (fractions 25−75), which is active as well as higher in protein content but heterogeneous on SDS-PAGE analysis, followed by second peak (fractions 80−125) with less protein content, and peroxidase activity was observed in the ascending shoulder. SDS-PAGE analysis of the second peak showed the presence of more impurities. Therefore, fractions 25−75 of the earlier peak were pooled together and subjected to further purification on hydrophobic interaction chromatography.

Step 4: Hydrophobic Interaction Chromatography. The fractions of the first peak (25−75) with peroxidase activity were pooled and dialyzed against 0.05 M MES buffer, pH 6.5, and 4.0 M ammonium sulfate solution was added to a final concentration of 1.5 M ammonium sulfate. After centrifugation, the supernatant was applied to an Ether-Toyopearl 650S (TOSOH) (2.0 cm × 7.0 cm) column pre-equilibrated with 1.5 M ammonium sulfate in the same buffer. The bound proteins were eluted by a linear reverse ammonium sulfate concentration gradient from 1.5 to 0 M in the same buffer at a flow rate of 2 mL/min. A serine protease named carnein (*21*) and two other proteins of the chitinase family were obtained to homogeneity in the eluted fractions. Peroxidase activity was not found in the eluted fractions, whereas the unbound fractions showed high protein concentration and peroxidase activity. The unbound fractions were pooled and subjected to size exclusion chromatography.

Step 5: Gel Filtration on a Sephacryl S-200 Column. The unbound protein fractions obtained after the HIC step were pooled and dialyzed against 0.05 M MES buffer, pH 6.5, and concentrated using a Viva Spin concentrator (MWCO 10000 Da) The concentrated sample was subjected to gel filtration on a Sephacryl S-200 (Pharmacia) column (1.2 × 120 cm) pre-equilibrated with 0.5 M NaCl in 0.05 M MES buffer, pH 6.5, and eluted isocratically with the same at a flow rate of 0.25 mL/min. Fractions of 1 mL were collected and assayed for protein contents and peroxidase activity. The fractions with peroxidase activity were pooled, concentrated, desalted using the Viva Spin, and further analyzed on SDS-PAGE for homogeneity (**Figure 2a**, lane 5). The pooled protein solution was scanned on a UV−Vis spectrophotometer in the wavelength range of 240−700 nm, and the RZ ratio (A_{403nm}/A_{280nm}) was found to be 3.2. The protein obtained at this step was pure and homogeneous and thus stored at 4 °C for further biochemical and biophysical characterization as well as N-terminal amino acid sequencing. The enzyme was named morning glory peroxidase (MGP).

Analytical Methods. *Protein Concentration.* Protein concentration was determined spectrophotometrically (absorbance at 280 nm) as well as by Bradford assay (*22*) using bovine serum albumin as standard.

Electrophoresis. Homogeneity of the proteins in different steps was judged by SDS-PAGE under reducing conditions (*23*), and the gel was stained with Coomassie brilliant blue R-250. For activity staining native PAGE was performed and the gel was incubated at 37 °C with 0.17 mM H_2O_2 as substrate I and one of the hydrogen donors guaiacol, aminoantipyrine/phenol, *o*-dianisidine, pyrogallol, or *o*-phenylenediamine as substrate II.

Isoelectric Focusing. The isoelectric point (p*I*) of the purified MGP was determined by isoelectric focusing on polyacrylamide disk gels (0.6 × 10.0 cm) as described (*24*).

Electrophoretic runs were carried out with ampholine carrier ampholytes in the pH range of 4.0−6.0 at 300 V for 2 h using 0.1 M NaOH as catholyte and 0.1 M orthophosphoric acid as anolyte. Enzyme in IEF-PAGE gel was visualized by Coomassie staining G-250 and activity staining. For activity staining, the IEF tube gel was incubated at 37 °C in 50 mM phosphate buffer, pH 7.0, containing 0.17 mM H_2O_2 and 0.12 mM aminoantipyrine with 0.17 M phenol. The pink color band appears with the passage of time in approximately 10−15 min (gel not shown).

Mass Spectrometry. The molecular weight of the purified peroxidase was determined by mass spectrometry (MALDI-TOF MS). Samples were dissolved at a concentration of 10 pmol/μL in 1:1 (v/v) 1% aqueous formic acid and methanol. Positive ionization was used for the sample analyses with an electrospray voltage of 1.0 kV. A sampling cone voltage of 40 V and an MCP detector of 2700 V was adjusted. Nitrogen gas was employed as the API gas, and data were acquired over the appropriate *m*/*z* range at a scan speed of 3.0 s in continuum mode. An external calibration was made using horse heart myoglobin (MW 16951.5 Da), and data were processed using Mass Lynx of the software program supplied with the mass spectrometer.

Assay for Peroxidase Activity and Substrate Specificity. The peroxidase activity of MGP has been determined spectrophotometrically by using hydrogen peroxide in combination with a second substrate. The substrates used in the present study were guaiacol, aminoantipyrine/phenol, *o*-dianisidine, pyrogallol, and *o*-phenylenediamine.

Ten micrograms of enzyme was added to 2 mL of buffer containing 0.17 mM of H_2O_2 (substrate I) with various concentrations of hydrogen donor (substrate II) dissolved in the corresponding buffer. Guaiacol in 50 mM acetate buffer, pH 5.5; aminoantipyrine with 0.17 M phenol in 50 mM phosphate buffer, pH 7.0; *o*-dianisidine HCl in 50 mM tris HCl, pH 8.0; pyrogallol in 50 mM phosphate buffer, pH 6.0; and *o*-phenylenediamine in 50 mM acetate buffer, pH 5.5, were used as second substrates. The rate of change of absorbance at 470, 510, 460, 420, and 445 nm was monitored, respectively (*25*).

When the affinities of MGP for different substrates were studied, the activity was measured under optimal conditions by varying each of the second substrates as described above. H_2O_2 was freshly prepared, and the concentration was determined by absorbance at 240 nm using $\varepsilon = 43.6\ M^{-1}\ cm^{-1}$ (*26*). The apparent Michaelis−Menten constant (K_m) was deduced from a Lineweaver−Burk plot (*27*). The value of the catalytic constant (K_{cat}) was also estimated by dividing the V_{max} value by the K_m value. One unit of enzyme activity is defined as the amount of peroxidase required for a change of 0.1 unit absorbance per minute under the assay conditions. The specific activity is expressed as the units of activity per milligram of protein.

Effect of Inhibitors on Peroxidase Activity. The effect of different compounds on the peroxidase activity has been studied using sodium azide, β-mercaptoethanol, L-cysteine, glutathione, hydroxylamine, *N*-ethylmaleimide, and sodium sulfide. In each case, 10 μg of MGP enzyme was incubated in the presence of increasing concentration of the inhibitors for 60 min at 37 °C and assayed with guaiacol as well as aminoantipyrine/phenol substrates. A control assay was done with enzyme solution without inhibitors, and the resulting activity was considered to be 100%.

Tyrosine and Tryptophan Content. The tyrosine and tryptophan contents of the MGP were measured in alkaline condition spectrophotometrically using the method of Goodwin and Morton (*28*). The absorbance spectra of the enzyme in 0.1 M NaOH was recorded between 220 and 320 nm using a Beckman DU 640 B spectrophotometer, and the absorbance values at 280 and 294.4 nm were deduced from the spectra. The formula given by Goodwin and Morton was used to estimate the tryptophan and tyrosine contents. For calculations, the formula $w = (A_{280nm} - x\varepsilon_y)/(\varepsilon_w - \varepsilon_y)$ was used, where w is the estimated tryptophan content in moles per liter, A_{280nm} is the absorbance at 280 nm from the protein spectra, and ε_w and ε_y are molar extinction coefficients of tryptophan and tyrosine in 0.1 M NaOH at 280 nm ($\varepsilon_w = 5225$ and $\varepsilon_y = 1576$), respectively. The total tyrosine and tryptophan content in the protein, x, was calculated using $\varepsilon_{294.4} = 2375$. The number of a particular amino acid residue per molecule of the protein was calculated from the ratio of the molar concentrations of the amino acid residues to that of the total protein.

To validate these measurements, tyrosine and tryptophan contents of some standard proteins such as papain, ribonuclease A, BSA, and lysozyme were determined in a similar manner.

Free and Total Sulfhydryl Contents. The exposed and total cysteine residues of MGP were estimated by Ellman's (*29*) method using DTNB reaction, where the release of thionitrobenzoate (TNB) due to reduction of the thiol with DTNB was determined by increase in the absorbance at 412 nm. The molar extinction coefficient of TNB anion at 412 nm is 14150 $M^{-1}\ cm^{-1}$ (*30*). For exposed sulfhydryl group estimation, the purified enzyme was activated with 0.01 M β-mercaptoethanol for 15 min, in 0.05 M Tris-HCl buffer, pH 8.0, and dialyzed against 0.1 M acetic acid at 4 °C for 24 h with frequent changes. For the estimation of total sulfhydryl content, the enzyme was reduced with 0.01 M β-mercaptoethanol in the presence of 6 M GuHCl for 15 min at 37 °C and dialyzed against 0.1 M acetic acid. DTNB reaction was carried under alkaline conditions to estimate free and total sulfhydryl contents of the protein. The number of disulfide bonds, in the protein, was deduced by comparison of the number of free and total cysteine residues. To validate the current measurements, exposed and total sulfhydryl contents of papain, ribonuclease, BSA, and lysozyme were determined in a similar way.

Extinction Coefficient. The extinction coefficient of MGP was determined according to a spectrophotometric method (*31*). Several solutions of the enzyme were prepared in serial dilutions, and the concentration of the enzyme in each sample was determined by using the Bradford method. The absorbance of each sample at 280 nm was measured, and the extinction coefficient of the enzyme was calculated using Beer−Lambert's law. In the spectrophotometric method, the extinction coefficient was determined using a formula given by Aitken and Learmoth (*31*).

Heme Content and Heme Extraction. The heme presence was inferred from absorbance spectra in the wavelength range of 220−720 nm, and the heme content was estimated by atomic absorption spectroscopy. One milligram of lyophilized protein was taken in 10 mL of nitric acid and perchloric acid in the ratio of 6:1 (v/v) in a conical flask free of any metals and digested slowly on a hot plate until white fumes appeared. Five milliliters of 1 N nitric acid was added, and the resulting solution was used for atomic absorption spectroscopy. A similar procedure was also followed for preparation of the blank without protein. The sample was analyzed on a Shimadzu atomic absorbance spectrum, and the heme content was deduced from the standard Fe plot.

The cleavage of the heme−protein linkage was achieved by acid methyl ethyl ketone as described by Teale (*32*) with some minor modifications. Heme extraction was carried out at 4 °C. The pH of the MGP solution was adjusted to 2.2 with 0.1 M HCl and mixed with an equal volume of 2-butanone. The reaction mixture was vortexed and centrifuged to separate the heme (organic phase, orange color) and apoprotein (aqueous phase). The aqueous phase was dialyzed against 50 mM Tris-HCl, pH 7.8, to remove any residual butanone and centrifuged again to remove any precipitate.

Carbohydrate Content and Deglycosylation. The carbohydrate content of MGP was determined using a phenol−sulfuric acid method (*33*). Different amounts of protein and galactose (standard) were taken in an ELISA plate (96 wells), and the absorbance was taken at 492 nm in an ELISA plate reader. The carbohydrate content of MGP was extrapolated from the standard plot generated from galactose measurement. The deglycosylation of MGP was done by a chemical method using TFMS (*34*), which breaks the bond between monosaccharides and also *o*-glycosidic bonds between carbohydrate and proteins (but not the N-glycosidic bond between aspargine and GlcNAc). The lyophilized MGP protein was incubated with 0.39 mL of anisole and 0.59 mL of TFMS at 4 °C for 4 h under nitrogen followed by neutralization with 1.57 mL of 60% aqueous ice-cold pyridine. The product obtained was dialyzed overnight against water at 4 °C and then extracted with anhydrous ether. The aqueous phase was lyophilized

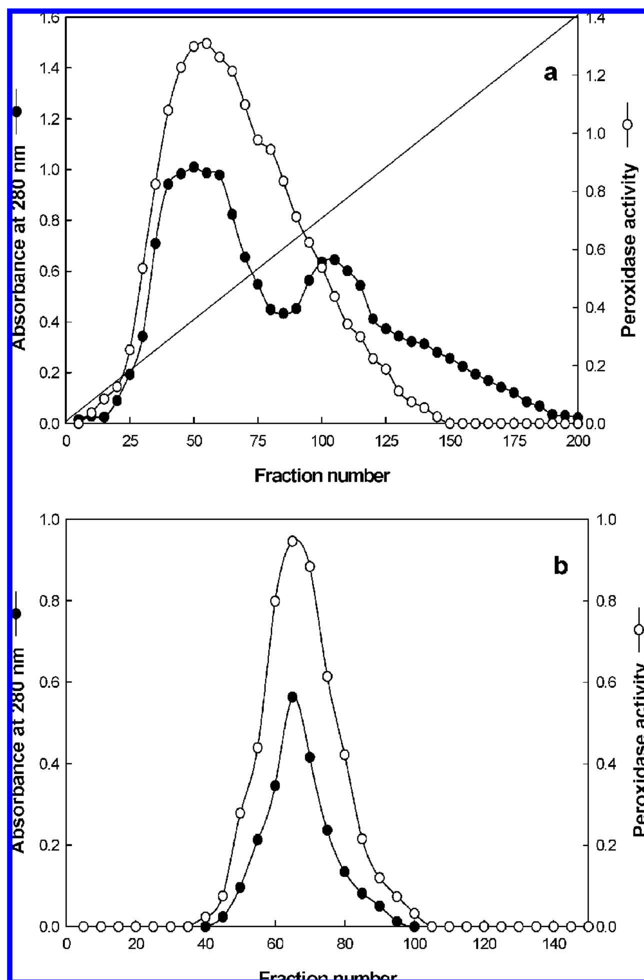

Figure 1. (**a**) Elution profile of MGP by anion exchange chromatography on a DEAE column. The bound proteins were eluted with a linear salt gradient of 0.0−0.5 M NaCl in 50 mM Tris-HCl, pH 8.0. Fractions of 3 mL were collected at a flow rate of 3 mL/min. All fractions were assayed for activity (○) and for protein content (●) (**b**) Elution profile of MGP purification on gel filtration chromatography using Sephacryl S-200 equilibrated with 50 mM MES, pH 6.5, containing 0.5 M NaCl and eluted with the same after injection of the unbound protein pool from the previous HIC step. All fractions were assayed for activity (○) and for protein content (●).

and dissolved in SDS-PAGE sample buffer for electrophoresis. The electrophoresis gel was stained with Schiff reagent specific to glycoprotein (*35*).

pH and Temperature Stability. The stability of the peroxidase as a function of varying pH was monitored. Ten micrograms of MGP was incubated with 50 mM buffer of desired pH in the range of pH 0.5−12.0 for 24 h at 25 °C and assayed as described above. The buffers used were KCl−HCl (pH 0.5−1.5), glycine−HCl (pH 2.0−3.5), sodium acetate (pH 4.0−5.5), sodium phosphate (pH 6.0−7.5), Tris−HCl (pH 8.0−10.0), and glycine−NaOH (pH 10.5−13.0). The concentration of the buffer was 50 mM in all cases.

Similarly, the stability of the enzyme at different temperatures was also measured. The reaction mixture consisting of 10 μg of MGP was incubated in 50 mM acetate buffer, pH 5.5, at different temperatures in the range of 20−99 °C for 60 min, and the activity was assayed.

Effect of Chaotrops, Organic Solvents, and Metal Ions on Stability. The ability of MGP to retain its activity following exposure to extreme pH, strong denaturants, temperature, detergents, and organic solvents was studied by exposing the enzyme to the respective conditions. Ten micrograms of enzyme was incubated with various concentrations of denaturants, detergents, organic solvents, and metal salts for 24 h at 25 °C and assayed for peroxidase activity.

Antigenic Properties. Antibodies against the purified MGP enzyme were raised in a male albino rabbit (1 kg body weight) as described (*24*), and the presence of antibodies was confirmed by immunoassays. The pure enzyme (500 μg) in 0.05 M acetate buffer, pH 5.5, was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously at multiple sites. After 1 week, enzyme (500 μg) was emulsified with an equal volume of Freund's incomplete adjuvant and administered as a booster dose. Furthermore, two similar doses were administered at intervals of 7 and 15 days. At 7 days after the last dose, the rabbit was bled through the marginal ear vein. Blood was allowed to clot initially for 1 h at room temperature and later for 12 h at 4 °C; supernatant was collected by centrifugation. Preimmune serum was obtained from the rabbit prior to the first injection of antigen, and all of the sera were stored at −20 °C. The presence of antibodies was confirmed by immunodiffusion studies. Ouchterlony's double immunodiffusion was performed as described by Ouchterlony and Nilsson (*36*); 1% agarose in phosphate-buffered saline was solidified in Petri dishes, and appropriate holes were punched into it. MGP and the other three proteins purified from the latex of the morning glory were loaded into the peripheral well, and anti-MPG serum was loaded in the central well to check the specificity of the raised antibody.

Amino-Terminal Sequence Analysis. Ten picomoles of the purified MGP enzyme (dialyzed against distilled water) was applied on an Applied Biosystems Procise Sequencer, and the N-terminal sequence was determined by Edman's automated degradation. The N-terminal sequence of MGP was compared with other known plant peroxidases using NCBI-BLAST and CLUSTAL W.

Spectroscopic Studies: Absorbance, Fluorescence, and Circular Dichroism. Absorbance measurements were carried out on a Beckman DU 640B spectrophotometer. The absorbance spectra of the protein (native as well as denatured) were recorded in the wavelength region from 220 to 320 nm using appropriate blanks for baseline corrections. The protein concentration used was 0.5 mg/mL. For denaturation studies the proteins were incubated for 24 h in the presence of 6 M GuHCl dissolved in 50 mM phosphate buffer, pH 7.0.

Fluorescence measurements were carried out on a Perkin-Elmer LS 50B spectrofluorometer. The protein concentration used was 0.01 mg/mL for fluorescence measurements. Tryptophan was selectively excited at 292 nm in native as well as denatured conditions. The emission was recorded from 300 to 400 nm with 10 and 5 nm slit widths for excitation and emission, respectively.

The circular dichroism (CD) spectra of MGP under native and denatured conditions were recorded on a JASCO 500A spectropolarimeter, precalibrated with 0.1% *d*-10-camphorsulfonic acid solution. The conformational changes in the secondary structures of the protein were monitored in the far-UV region between 200 an 260 nm, with a

Table 1. Purification of Morning Glory Peroxidase (MGP) from *Ipomoea carnea*

step	total amount (mg)	total activity[a] (units)	specific activity (units/mg)	purification factor (fold)	yield (%)	RZ ratio (A_{403nm}/A_{280nm})
crude extract	96.6	5040	52.17	1.00	100.0	0.23
ammonium sulfate precipitation	81.3	4730	58.18	1.12	93.9	0.64
AEC on DEAE	48.0	2918	60.80	1.17	57.9	1.18
HIC on ETP	26.5	1859	70.15	1.35	36.9	1.80
gel filtration on Sephacryl S-200	9.4	1415	150.53	2.89	28.1	3.20

[a] The activity measurement was done using 0.17 mM H_2O_2 and 0.12 mM aminoantipyrine with 0.17 M phenol in 50 mM phosphate buffer, pH 7.0, as described under Experimental Procedures.

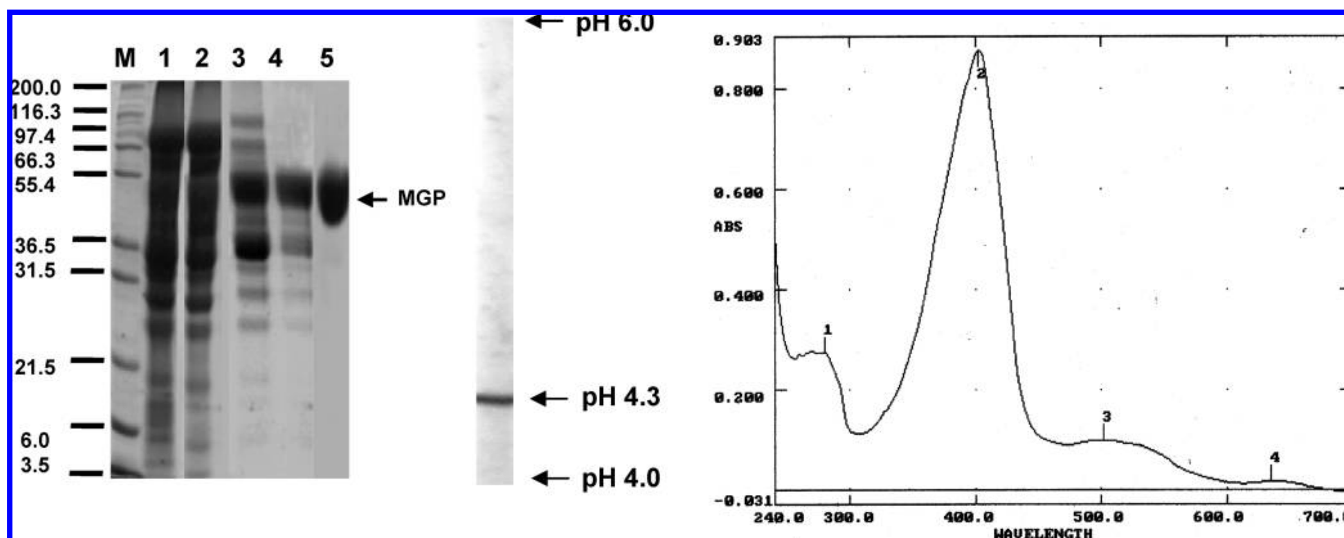

Figure 2. (**a**) SDS-PAGE of different purification steps. Ten microliters of Mark 12 molecular weight marker (lane M) and 20 μg of protein was loaded from each step (lanes 1−5) respectively. Lane 5 shows a homogeneous band of pure MGP. (**b**) Isoelectric focusing of MGP. Twenty micrograms of protein sample was loaded, and electrophoresis was performed using 5% polyacrylamide gels with ampholine carrier ampholytes (pH 4−6) for 2 h at a constant current of 2 mA/rod. A single band corresponding to pH 4.3 was observed. (**c**) Absorbance spectrum of pure MGP in UV−visible region showing peaks 1, 2, 3, and 4 at 280, 401, 498, and 637 nm, respectively. The RZ (A_{403nm}/A_{280nm}) value for pure MGP was determined as 3.2 from the spectrum.

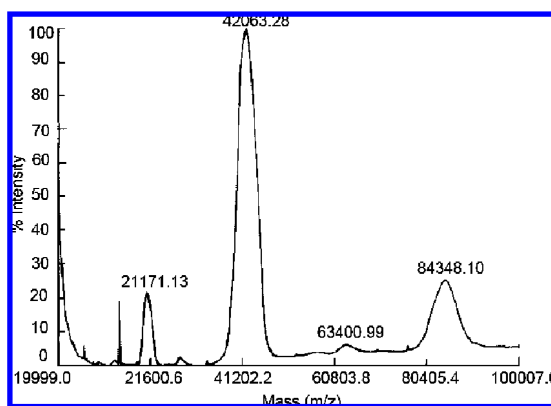

Figure 3. Mass spectrometry of MGP peroxidase by MALDI-TOF MS. Ten picomoles of MGP dissolved in 1:1 v/v aqueous formic acid and methanol was subjected to MALDI-TOF analysis. A calibration was done using horse heart myoglobin (MW 16951.5 Da).

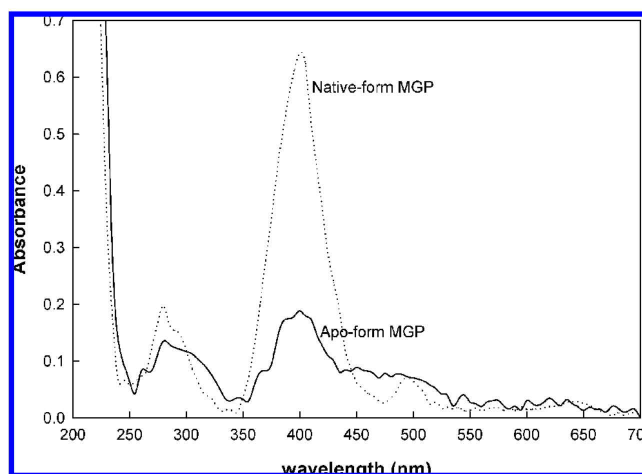

Figure 4. Absorption spectra of native enzyme and apoenzyme. The absorbance spectra of native MGP (dotted line) and apoenzyme (solid line) were obtained after heme extraction. In apoenzyme the Soret peak decreased significantly.

protein concentration of 0.1 mg/mL in a 1 mm path length cuvette. The results were expressed as mean residue ellipticity$[\theta]_{MRW,}$ using the equation

$$[\theta]\mathrm{MRW} = \theta_{obs}\mathrm{MRW}/10cl$$

where θ_{obs}, c, and l represent the observed ellipticity in degrees, the protein concentration in mg/mL, and the path length in cm, respectively. The mean weight of amino acid residues (MRW) was taken as 110 for the calculations.

The α-helicity of a protein can be determined using the simple calculation

$$\%\ \alpha\text{-helicity} = (\theta_{222} - \theta_{min}/\theta_{max} - \theta_{min}) \times 100$$

where θ_{222}, θ_{min}, and θ_{max} represent, respectively, the molar ellipticity at 222 nm, the minimum value (θ_{min} = 2340) of molar ellipticity at 222 nm calculated for the unordered fraction of five proteins, and the maximum value of molar ellipticity at 222 nm calculated for the helical fractions of the five proteins (θ_{max}= 30300).

RESULTS AND DISCUSSION

A novel heme peroxidase MGP has been purified from *I. carnea* latex and characterized biochemically as well as spectrophotometrically. *I. carnea*, an invasive and noxious weed, may become a new source of peroxidase for industrial and biotechnological applications. The purification procedure involved four steps: ammonium sulfate precipitation, anion exchange chromatography on a DEAE column, hydrophobic interaction chromatography on an Ether-Toypearl 650S column, and a polishing step of gel filtration chromatography on Sephacryl S-200.

The chromatograms for purified MGP are shown in **Figure 1**. As chromatography steps increase, the purity, specific MGP activity, yield, and purification fold also increase (**Table 1**). Because the achieved purity and yield are very high and the plant is found abundantly, *I. carnea* may become a potent source of peroxidase. The achievement of peroxidase purification in different steps is visualized in SDS-PAGE gel (**Figure 2a**). The purified MGP migrated on the gel as a single band with an apparent molecular mass of 40−45 kDa. A molecular mass of 42.063 kDa was obtained by MALDI-TOF (**Figure 3**), and it

Table 2. Effect of Various Metal Salts on the Activity of Morning Glory Peroxidase[a]

metal	relative activity (%) 1 mM	10 mM
none	100.0	100.0
NaCl	96.7	94.8
KCl	93.6	92.6
CsCl	91.6	86.3
$MgCl_2$	94.2	83.5
$CaCl_2$	83.8	61.8
$HgCl_2$	64.5	0.0
$NiCl_2$	96.2	90.6
$CuSO_4$	94.6	80.8
$CoCl_2$	90.8	51.9
$MnCl_2$	97.2	93.2
$ZnCl_2$	96.4	95.0
$K_2Cr_2O_7$	102.3	108.8
$PbCl_2$	98.4	91.8
EDTA	96.7	87.3

[a] The activity measurement was done as described in **Table 1**.

Table 3. Effect of Chaotrophs and Organic Solvents on MGP Activity

chaotroph or organic solvent	concentration[a]	residual activity[b] (%)
GuHCl	3.0 M	84.2
GuSCN	1.5 M	82.4
urea	8.0 M	100.0
SDS	2 mM	81.7
methanol	70%	85.0
ethanol	65%	82.0
isopropanol	45%	80.0
butanol	30%	81.0
acetonitrile	60%	88.0
DMSO	30%	76.0
dioxane	50%	82.0

[a] The minimum concentration of chaotroph or organic solvent, where the residual activity is >80%. [b] The activity measurement was done as described in **Table 1**.

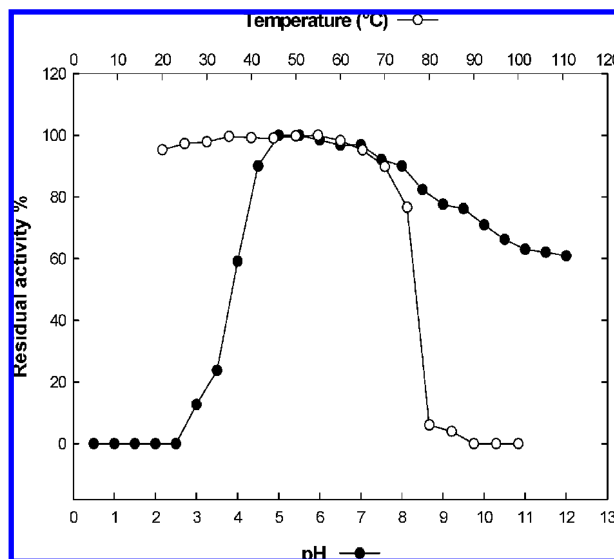

Figure 5. Stability studies against pH and temperature. After exposure for 24 h in different buffers, MGP retains >80% activity in the pH range of 4.0−9.0 (●). MGP retains its activity up to 75 °C, and activity decreases drastically at higher temperature (○).

was used for biochemical calculations. During the purification of peroxidase a serine protease carnein (*21*) (molecular mass = 80.24 kDa, N-terminal TTHSPEFLGLAESSGLXPNS) and two chitinases (I, 33.94 kDa; and II, 30.06 kDa; with GEIAIY-WGQNGGEGS and GEITIYXGQNGFEGS, respectively) were copurified.

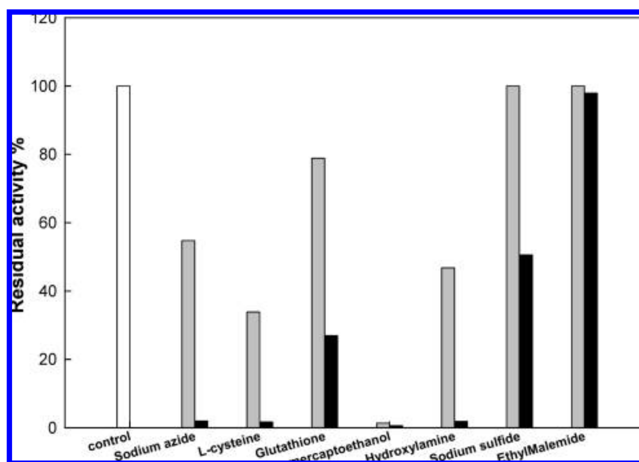

Figure 6. Effect of different compounds on peroxidase activity. Twenty micrograms of MGP was incubated with the compound for 1 h, and activity was monitored. Activity assay was monitored at 1 mM (gray bars) and 10 mM concentrations (black bars) of the compound. MGP without the addition of any compound was assayed and treated as a control (white bar).

Table 4. Relative Activity of Morning Glory Peroxidase with Different Substrates

substrate (AH)	pH [buffer] (50 mM)	[H_2O_2] (mM)	λ (nm)	K_m (mM)	V_{max} (units)	relative K_{cat} = V_{max}/K_m (units/mM)
aminoantipyrine	7.0	0.17	510	0.12	0.78	6.50
guaiacol	5.5	0.17	470	0.29	0.77	2.69
o-dianisidine	8.0	0.17	460	0.50	0.43	0.84
pyrogallol	6.0	0.17	420	1.63	0.83	0.51
o-phenylenediamine	5.5	0.17	445	2.02	0.69	0.34

In the isoelectric focusing pH gradient of 4−6, MGP migrated as a single sharp band with an apparent isoelectric point of 4.3, classifying it as an anionic peroxidase (**Figure 2b**). The most studied plant peroxidase, HRP-C, is a cationic peroxidase; however, a number of anionic peroxidases (p*I* < 7.0) were purified recently from soy, tobacco, tea, potato, sweet potato, palm tree, and broccoli (*37*).

The RZ value, the absorbance ratio A_{403nm}/A_{280nm} and an essential purity criterion for peroxidases, increased with each step of purification for MGP, and its value for pure enzyme was 3.2 (**Table 1**; **Figure 2c**). This value is in good

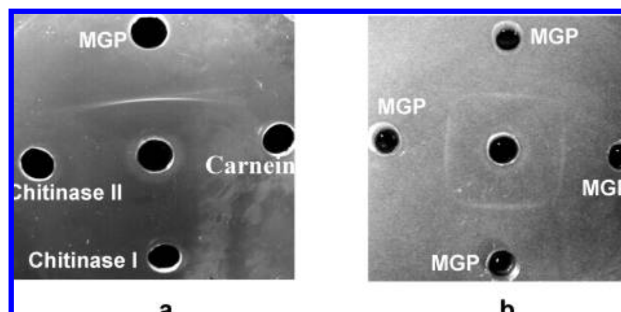

Figure 7. Ouchterlony's double immunodiffusion was carried out in 1% agarose dissolved in phosphate-buffered saline. Antiserum (100 μL) was added in the central well, and (**a**) 40 μg of four purified proteins (MGP, carnein, chitinase I, and chitinase II) was added in peripheral wells. (**b**) Forty micrograms of purified protein MGP was added in all of the peripheral wells. The precipitin band was observed after 24 h of incubation.

Table 5. First 15 N-Terminal Sequence Amino Acid Residues of MGP and Comparison with Amino Acid Sequences of Other Known Plant Peroxidases

plant peroxidase	amino acid residues															identity %
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
>gi|MGP anionic peroxidase [*Ipomoea carnea*]	D	E	A	C	I	F	S	A	V	K	E	V	V	D	A	100
>gi|6002684|gb|AAF00094.1|AF1091231 anionic peroxidase Swpa3 [*Ipomoea batatas*]	D	E	A	C	I	F	S	A	V	K	E	V	V	D	A	100
>gi|6002682|gb|AAF00093.1|AF1091241 anionic peroxidase swpa2 [*Ipomoea batatas*]	D	E	A	C	V	F	S	A	V	K	E	V	V	D	A	93
>gi|37783269|gb|AAP42503.1| anionic peroxidase swpa4 [*Ipomoea batatas*]	D	E	D	C	I	F	S	A	V	K	E	V	V	D	A	93
>gi|33516947|sp|O04795|PERA|POBA anionic peroxidase precursor (SwPA1)	D	E	A	C	V	F	S	A	V	K	E	V	V	V	A	87
>gi|37783273|gb|AAP42505.1| anionic peroxidase swpa6 [*Ipomoea batatas*]	D	E	A	C	V	F	S	A	V	K	E	I	V	E	A	80
>gi|129807|sp|P15003|PER1SOLLC suberization-associated anionic peroxidase 1 precursor (TMP1)	P	E	A	C	V	F	S	A	V	R	A	V	V	D	S	67
>gi|129811|sp|P15004|PER2SOLLC suberization-associated anionic peroxidase 2 precursor (TMP2)	Q	E	S	C	V	F	S	A	V	K	G	V	V	D	S	67
>gi|110825730|sp|P12437|PERXSOLTU suberization-associated anionic peroxidase precursor (POPA)	P	E	A	C	V	F	S	A	V	R	G	V	V	D	S	67
>gi|58578270|emb|CAI48071.1| anionic peroxidase [*Capsicum chinense*]	Q	E	S	C	V	F	S	A	V	N	G	V	V	D	S	60
>gi|64976605|dbj|BAD98313.2| peroxidase [*Nicotiana tabacum*]	P	S	A	C	I	F	S	A	V	R	R	V	V	N	R	60
CLUSTAL W multiple-sequence alignment		.		*	:	*	*	*	*	.		:	*			

agreement with reported values of 2.5−3.5 for known plant peroxidases. Like other known plant peroxidases, the absorbance spectrum of MGP in the UV−visible region has maxima at 280, 401 (Soret band), 498, and 637 nm.

Atomic absorption spectroscopy suggests that the heme content in MGP is approximately 50−60 ppm/mg of protein. Heme extraction was performed according to Teale's method; in the absorption spectrum of apoenzyme the Soret peak decreased significantly compared to that of the native protein (**Figure 4**). The carbohydrate content of MGP was estimated to be 15−16% by the phenol−sulfuric method, suggesting it to be a glycoprotein like most of the plant peroxidases. The MGP was deglycosylated according to a chemical method and analyzed on SDS-PAGE stained using Schiff base with clear distinction between the glycosylated pink band and the deglycosylated band (data not shown). The tryptophan and tyrosine contents of the protein were 13 (measured value, 13.21) and 9 (measured value, 9.44), respectively. The total cysteine content of the MGP was found to be 8 (measured value, 8.16), forming four disulfide bridge, a characteristic of most plant peroxidases (*16*). The extinction coefficient measured by the spectrophotometric method was 20.56, which was used for all practical calculations. Because the protein concentration used in these calculations was estimated by using the Bradford method, there may be some disagreement in the actual and calculated values.

The effect of various metals, chaotrophs, organic solvents, pH, temperature, and inhibitors on MGP activity has been studied. $HgCl_2$ inhibits the total activity of the enzyme, whereas $CoCl_2$ and $CaCl_2$ result in significant reductions of peroxidase activity (**Table 2**). Generally, calcium ions appear to be important to maintain the activity as well as thermostability of most plant peroxidases. In the present case, some reduction in peroxidase activity of MGP enzyme is observed in the presence of 1 or10 mM $CaCl_2$, although the exact reason for such observation is not known but confirmed several times by our activity measurements. The following explanation may give some idea of the reduction in peroxidase activity. The enzyme may undergo conformational changes in the active site structure in the presence of calcium ions. The activity of a peroxidase enzyme depends on a catalytic triad of active site residues, heme propionates, and electrostatic repulsion of nearby residues. There may be extensive protonation of the enzyme in the presence of calcium cations, which may decrease the repulsion and, moreover, may allow an extra calcium cation to be bound and block access to the active site. Therefore, such binding of the calcium ion may block the active site residues so affecting the rate constant toward the hydrogen peroxide, resulting in activity reduction. However, similar results were reported with windmill palm tree peroxidase (WPTP) (*37*) and tobacco peroxidase (TOP) (*38*). The exact reason may be clear after structure determination of the peroxidase−calcium complex by X-ray crystallography. MGP retains activity when exposed to denaturants and organic solvents up to the concentration as mentioned in **Table 3**. MGP when exposed to different pH values for 24 h retains >80% activity in the pH range of 4.0−9.0. Under extreme acidic condition, MGP was very unstable. The optimum pH for the activity of MGP with the aminoantipyrine/phenol system was found between pH 5.0 and 7.0. Like other peroxidases, MGP showed a high thermal stability. MGP shows a reasonable activity up to 75 °C followed by an abrupt loss of activity at higher temperature (**Figure 5**). There is a significant effect of inhibitors targeting S−S bridges (mercaptoethanol, L-cysteine, and glutathione), as well as of inhibitors targeting heme (sodium azide and hydroxylamine), on peroxidase activity, whereas there was no inhibition by ethylmaleinimide as there are no reduced cysteines in the enzyme (**Figure 6**).

The relative affinity of MGP toward hydrogen donor peroxidase substrates was determined. The hydrogen peroxide serves as a suicide substrate for peroxidases; the hydrogen donor substrate may protect the peroxidase active site from the inactivating action of H_2O_2. Therefore, for the oxidation of each substrate, the concentration of H_2O_2 and substrate required for the maximum catalytic activity by MGP was determined. The maximum affinity was observed with aminoantipyrine/phenol (relative K_{cat} value = 6.5) followed by guaiacol (relative K_{cat} value = 2.7) (**Table 4**). Thus, among the studied substrates, aminoantipyrine/phenol was the superior substrate, whereas *o*-phenylenediamine was the worst one.

The presence of polyclonal antibodies in anti-rabbit serum was checked by immunodiffusion. The precipitin line started appearing after 10−12 h of incubation at room temperature and was distinctly visible after 24−30 h. A control experiment was

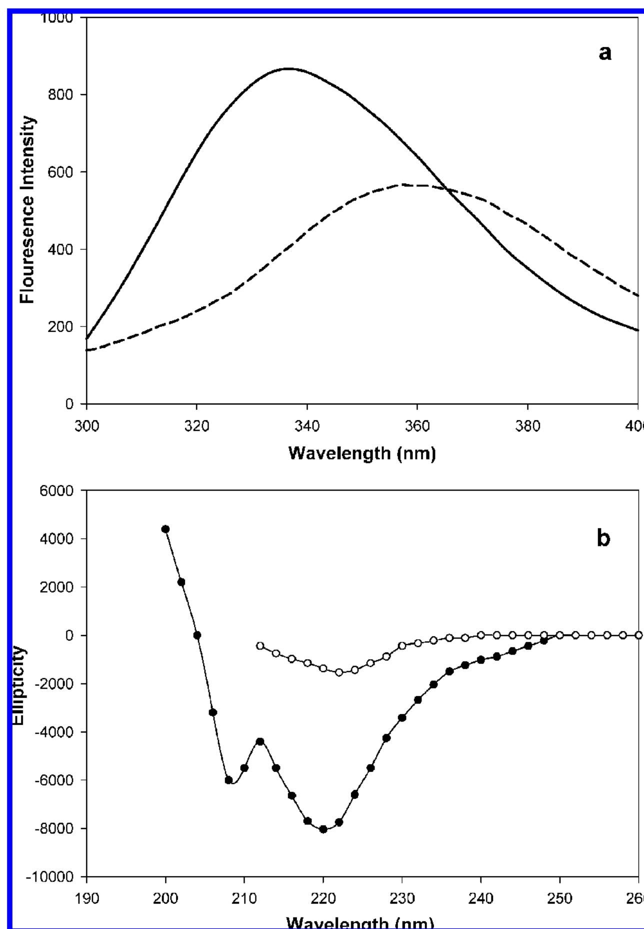

Figure 8. Spectroscopic studies of MGP peroxidase. For denaturation the protein was incubated under 6 M GuHCl in 50 mM, pH 7.0, buffers for 24 h at room temperature before the experiments were carried out. (**a**) Intrinsic fluorescence spectrum of MGP in native and denatured conditions. The protein concentration used was 0.01 mg/mL; excited at wavelength 292 nm and scanned in the 300−400 nm range for the native (solid line) as well as the denatured state (dashed line). (**b**) Circular cichroism spectrum of MGP in native and denatured conditions. The protein concentration used was 0.10 mg/mL; the spectrum has been taken in the near-UV region of 260−200 nm for the native (●) and denatured states (○).

performed with preimmune serum in the central well surrounded by MGP antigen in the peripheral wells, and no precipitin line was observed (data not shown). Antisera to MGP did not cross-react with another three proteins purified from the same plant source, suggesting that the antigenic determinants of MGP are unique (**Figure 7**).

The first 15 N-terminal amino residues (D-E-A-C-I-F-S-A-V-K-E-V-V-D-A) exhibited considerable similarity to those of other plant peroxidases when aligned using NCBI-BLAST and CLUSTAL W (**Table 5**). The highest similarity was observed with sweet potato peroxidases, which belong to the same Convolvulaceae family, whereas a significant similarity was also observed with tomato and potato peroxidases of the Solanaceae family.

The conformational changes of MGP under native and denatured (6 M GuHCl) conditions were studied by CD, fluorescence, and absorbance measurements. The absorbance spectra of MGP in native as well as denatured condition are similar, with no changes in the shape or intensity at 280 nm (data not shown). A fluorescence spectrum of proteins is a sensitive measure of the conformation of the protein under different conditions. The spectrum is resultant polarity in the environment of tryptophan and tyrosine residues and their specific interactions. The fluorescence emission suffers a red shift when the aromatic chromophores are exposed to solvent, and the quantum yield of the fluorescence intensity decreases. Intrinsic fluorescence spectra of MGP in native and denatured conditions are shown in **Figure 8a**. In the native state MGP exhibits a fluorescence emission maximum of 334 nm with a fluorescence intensity of 866 arbitrary units. The fluorescence intensity of the fluorescence decreases by 50% at 334 nm, whereas a wavelength shift of 24 nm in emission maximum from 334 to 358 nm is seen, as the protein is completely denatured in 6 M GuHCl, indicating a nonpolar environment around tryptophan residues. Such a red shift in the wavelength maximum indicates that more tryptophan residues of MGP are exposed to a polar environment, which is characteristic of protein unfolding.

In the far-UV region of 200−260 nm, the CD spectra of proteins are particularly sensitive to protein secondary structure. The secondary structural features of MGP in native and denatured conditions are shown in **Figure 8b**. MGP revealed two well-resolved negative peaks at 220 and 208 nm. The ellipticity at 220 nm is higher in magnitude in comparison to 208 nm. The mean residue ellipticity at 222 nm was around 8.0×10^3 deg cm^2 dmol^{-1}, and this value has been used for the estimation of α-helicity of the MGP peroxidase. The estimated α-helicity is approximately 20%. MGP in the native state retains all secondary features, suggesting the α/β class of proteins. However, the secondary structural features of MGP have been completely lost with the disappearance of all prominent peaks in the presence of 6 M GuHCl.

Broad substrate specificity and a high stability against pH, temperature, H_2O_2, and other organic solvents of the enzyme enables the development of H_2O_2-sensitive biosensors that can be employed under extreme conditions. As hydrogen peroxide determination is crucial in clinical diagnostics, the chemical and pharmaceutical industries, environmental control, and biological and medical sciences, the utility of peroxidases such as MGP may be immense. The conventional methods employed in titrimetry, UV−Vis spectroscopy, chemiluminescence, and electrochemistry, for H_2O_2 measurement are not as sensitive as biosensors developed using peroxidase-modified electrodes (*39*−*43*).

This is the first report of a peroxidase from the latex of the plant weed *I. carnea*. Further studies on the identification of physiological substrates, tissue localization, and biophysical and structural studies will give insight into the utility of the enzyme in biotechnology and the pharmaceutical industry.

SAFETY

The latex of *Ipomoea carnea* (morning glory) is toxic, so gloves were worn during collection. Acrylamide and *o*-dianisidine are potent neurotoxins and carcinogenic and were handled with safety gloves. The handling of phenol, H_2O_2, and TCA was done carefully because of their highly corrosive nature to skin. All other experiments were carried out with the utmost precaution and care.

ABBREVIATIONS USED

H_2O_2, hydrogen peroxide; RZ, Reinheitszahl; BSA, bovine serum albumin; BLAST, basic local alignment search tool; DEAE, diethylaminoethyl; DMSO, dimethyl sulfoxide; DTNB,

5,5μ-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(α-aminoethyl ether) tetraacetic acid; GuHCl, guanidine hydrochloride; GuSCN, guanidine isothiocynate; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MES, 2-(*N*-morpholino)ethanesulfonic acid; MWCO, molecular weight cutoff; NCBI, National Center for Biotechnology Information; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TEMED, tetramethylethylenediamine; TCA, trichloroacetic acid; TFMS, trifluoromethanesulfonic acid.

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