หอสมุดกลาง สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

MATERIALS AND METHODS

2.1 Plant materials

Cassava tubers were purchased from Pak Klong Ta Lad market, Bangkok, Thailand.

2.2 Chemicals

Name of Chemicals	Company
Ammonium sulfate $(NH_4)_2SO_4$	Sigma
Polyvinylpolypyrrolidone (PVPP)	Sigma
Acrylamide	Sigma
Concanavalin A sepharose 4B	Sigma
N,N-methyl-bis-acrylamide	Sigma
Mercaptoethanol	Sigma
IEF marker kit	Sigma
Standard marker protein for SDS-PAGE	Sigma
Coomassie brilliant blue R-250	Sigma
3,3-diaminobenzidine tetrahydrochloride (DAB)	Sigma
Bovine serum albumin (BSA)	Sigma
Tris[hydroxymethyl]aminomethane	Sigma
Glycine	Sigma
Sodium dodecyl sulfate (SDS)	Sigma
Guaiacol	Sigma
Pyrogallol	Sigma
o-Dianisidine	Sigma
Coniferyl alcohol	Sigma
Syringaldazine	Sigma
Quercetin	Sigma
Sodium azide	Sigma
α-D-methylglucopyranoside	Sigma
Bromophenol blue	Sigma

Name of Chemicals	Company
Pharmalyte pH 3-10	Sigma
DNP-lysine	Sigma
Sephadex G-200	Pharmacia
Hydrogen peroxide 35% (H_2O_2)	Merck
Dimethylsulfoxide (DMSO)	Merck
Methanol	Merck
Sodium carbonate	Merck
Folin-ciocalteau's phenol reagent	Merck
Glacial acetic acid	Merck
potassium monohydrogen phosphate	Merck
potassium dihydrogen phosphate	Merck
Ethanol	Merck
Hydrochloric acid (HCl)	Merck
Ascorbic acid	National Biochemical Corp
N,N,N′,N′-tetramethyl ethylene diamine (TEMED)	Fluka
Thiourea	Fluka
Copper sulfate	May and Backer
Ammonium persulfate	May and Baker
Potassium cyanide	Carlo-Erba
Glycerol	Carlo-Erba
Sulfuric acid	Carlo-Erba
Trifluoromethane sulfonic acid (TFMS)	Sigma
Pyridin	Merck

2.3 Equipments

Equipments/Model	Company
Centrifuge/2K 15	Sigma
Centrifuge/RC 5C plus	Sorvall
Electrophoresis Unit/Might Small II	Hoefer pharmacia Biotech, USA
Fracton collector/LKB 7000 Ultrorac	Pharmacia LKB, Sweden
Peristatic pump/P-1	Pharmacia Biotech, Sweden
Eletrophoresis power supply/2197	LKB, Bromma
Gyrotary water bath shaker /B76D	New Brunswick Scientific, USA
Spectrophotometer/uv-160	Shimazu
Spectrophotometer/uv-250/ PC	Shimazu

2.4 Preparation of crude cassava parenchyma peroxidase

Cassava root (4,500 gm) stored for 7 days was peeled and the cortex was removed. The parenchyma was chopped and homogenized in a blender in ice cold 0.1 M potassium phosphate buffer pH 6.8, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2% w/w of PVPP at the tissue to buffer ratio of 1:1 (w/v). The homogenate was filtered through filter cloth and centrifuged at 10,000 x g for 30 min at 4°C in Sorvall RC-5C plus refrigerated centrifuge. The supernatant was collected as crude extract for further experiments.

2.5 Purification of cassava parenchyma peroxidase

2.5.1 Ammonium sulfate precipitation

The crude extract was fractionated by slowly adding solid fine ammonium sulfate to 40% saturation with continuous stirring at 4°C. Precipitation was allowed to form for 4 hours at 4°C. Afterward, the precipitate was collected by centrifugaton at 10,000 × g for 30 min at 4°C. The supernatant was raised to 80%

saturation by further adding an appropriate amount of ammonium sulfate. Precipitate from each step was redissolved in a small volume of 20 mM potassium phosphate buffer pH 6.8 and dialyzed overnight against a large volume of the same buffer. Any precipitate formed during dialysis was removed by centrifugation at 10,000 × g for 20 min at 4°C.

2.5.2 Concanavalin A-Sepharose 4B column chromatography

Concanavalin A-Sepharose 4B was washed 4 times in distilled water to remove the ethanol used to preserve the gel. The settled gel was then suspended in 2 volumes of 50 mM potassium phosphate buffer, pH 6.8. The gel suspension was packed into a glass column (1.8 x 8 cm) using a peristaltic pump at a flow rate of 15 ml/hr. The column was pre-equilibrated with 10 column volumes of 50 mM potassium phosphate buffer, pH 6.8 containing 0.2 M NaCl at a flow rate of 15 ml/hr.

The ammonium sulfate fraction at 80% saturation was dialysed against an excess volume of the 50 mM potassium phosphate buffer, pH 6.8 containing 0.2 M NaCl overnight at 4°C. The dialyzed sample was centrifuged at 10,000 ×g for 20 min at 4°C. The supernatant fraction (15 ml, about 30 mg protein) was loaded onto the column. The column was washed with the starting buffer for 10 column volumes, using flow rate of 15 ml/hr. The elution of bound proteins can be achieved by using a stepwise gradient of α-D-methyl glucopyranoside (at 0.3 M and 0.5 M) in the starting buffer over 10 column volumes, at the same flow rate. Fraction of 3 ml each were collected. The elution profile was monitored for protein by measuring the absorbance at 280 nm and the peroxidase activity was determined as described in section 2.7. The fractions containing the bound peroxidase activity were pooled for further purifications.

2.5.3 Sephadex G-200 column

Sephadex G-200 was swollen in distilled water for 5 hours at 90°C. The settled gel was then suspended in 2 volumes of 20 mM potassium phosphate buffer pH 6.8. The gel suspension was degassed and packed into a glass column (52 × 2.5 cm) using a peristaltic pump at flow rate of 15 ml/hr. The column was calibrated with a molecular weight marker kit containing aldolase (Mr = 158,000), bovine serum albumin (Mr = 68,000), chymotrypsinogen (Mr = 27,000), and ribonuclease (Mr = 13,700).

The partially purified bound enzyme from the Concanavalin A Sepharose 4B column (section 2.5.2) was dialyzed against the starting buffer. An aliquot (1 ml, about 2 mg) was loaded onto the column and eluted at a flow rate of 12 ml/hr. Fractions of 2 ml each were collected. The elution profile was monitored for protein by measuring the absorbance at 280 nm and peroxidase actvity was determined as described in section 2.7. The fractions containing peroxidase were pooled and used for further characterization.

2.6 Protein determination

Protein concentration was determined by the method of Lowry et al (45) and bovine serum albumin (20-100 μg) was used as standard protein.

The assay mixture contained 100 μl of diluted protein solution and 3 ml of freshly prepared alkaline copper solution (100 ml of 2% Na_2CO_3 in 0.1 M NaOH was mixed with 1 ml of 1% potassium sodium tartrate and 1 ml of 0.5% copper sulfate). The mixture was left at room temperature for 10 min and then 0.3 ml of 1 M Folin reagent was added and mixed in thoroughly. The mixture was left for another 30 min

and then the absorbance at 650 nm was measured using a Shimadzu uv-160 spectrophotometer.

When amount protein was low, protein concentration was determined by the method of Bardford (44) using bovine serum albumin as standard. One hundred microtitres of sample was mixed with 1 ml of Coomassie blue reagent and left for 5 minutes before measured absorbance at 595 nm. One litre of Coomassie blue reagent was the mixture of 100 mg Coomassie blue G-250, 50 ml of ethanol, 100 ml of 85% phosphoric acid and 850 ml distilled water.

2.7 Assay of peroxidase activity using DAB

The peroxidase activity was measured by the modified method of Herzog and Fahimin (46) by following the initial rate in the conversion of freshly prepared 3,3′-diaminobenzidine (DAB) to the product which has absorbance at 465 nm using a Shimadzu uv visible spectrophotometer. The assay mixture (3 ml) contained 0.25 mM DAB in 0.1 M potassium phosphate buffer pH 6.8, enzyme extract and the reaction was started by the addition of 1 mM H_2O_2 (blank containing DAB and enzyme extract the absence of H_2O_2). The enzyme activity was expressed as A_{465}/min. One unit was defined as a change of one absorbance unit at 465 nm per min.

2.8 Gel electrophoresis

2.8.1 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

The peroxidase preparation from each step of purification was analyzed by denaturing gel electrophoresis on a slab gel system according to the modified method described by Laemmli (47). The slab gel system consisted of a stacking gel (10 × 2 × 0.001 cm) of 3% acrylamide and separating gel with 10% (w/v)

acrylamide (10 × 8.8 × 0.001 cm). The preparations of both separating gel and stacking gel were described in Appendix A.

Sample were treated with a solubilizing buffer with a final concentration 1% (w/v) SDS, 10% glycerol, 5% (v/v) β-mercaptoethanol and small amount of bromophenol blue in 63 mM Tris-HCl, pH 6.8, and boiled for 1-2 min. Electrophoresis was performed with 0.1% SDS in 25 mM Tris-glycine pH 8.3 as electrode buffer at constant voltage of 100 v until the dye marker reached the bottom of the gel. The gel was removed and stained for protein with 0.2% Brilliant blue R-250 as described in Appendix A.

Molecular weight marker kit containing (β-galactoglobulin Mr = 116,000, phosphorylase b Mr = 94,000, bovine serum albumin Mr = 68,000, ovalbumin Mr = 43,000, cytochrome C Mr = 12,500) was parallelly run for molecular weight determination of the sample protein.

2.8.2 Non-denaturing gel electrophoresis

Non-denaturing gel electrophoresis was used to study the electrophoretic pattern of peroxidase isozymes. The system used was a modification of that reported by Cameo and Blaquier (48). The gel 5% separating was prepared from a stock solution of 30% (w/v) of acrylamide and 0.8% (w/v) of N, N-bis-methyleneacrylamide, whereas the stacking gel contained 3% acrylamide. The gel was polymerized chemically by the addition of TEMED and ammonium persulfate. The preparation of both separating and stacking gel were described in Appendix A.

Protein sample was mixed with a sample buffer containing 62 mM Tris-HCl pH 6.8, 10% glycerol and small amount of bromophenol blue in the ratio 5:1. The running buffer was 25 mM Tris-glycine pH 8.3. The gel was run at a

constant voltage of 100 V until the dye marker reached the bottom of the gel. The gel was removed and stained for peroxide activity as described in section 2.8.4

2.8.3 Isoelectric focusing on polyacrylamide gel (IEF)

The pI of the purified protein was determined by isoelectric focusing on a BIO-RAD mini gel IEF according to the method in the BIORAD Handbook (49).

First; one glass plate was attached with gel supported film on the side that contact of the gel.

Second; the acrylamide gel was prepared from a stock solution of 24.25% (w/v) of acrylamide and 0.75% (w/v) of N,N-bis-methyleneacrylamide. The acrylamide slab gels (10 ml) contained 5% acrylamide, 5% (w/v) glycerol, 5% ampholyte, pH 3-10. The gel was polymerized chemically by the addition TEMED and ammonum persulfate.

Third, the gel solution was layered on the casting tray and covered with a support film attached to a glass plate. The gel was allowed to set about 2 hr and after the gelling was complete, the casting tray was removed.

Fourth; 2 μl sample was loaded onto the gel attached to the support film and focusing was performed at 100 V for 15 min, 200 V for 15 min and 450 V for 60 min, successively. The standard pI marker used were glucose oxidase (pI = 4.2), trypsin inhibitor (pI = 4.6), β-lactoglobulin ApI = 5.1), carbonic anhydrase I (pI 5.4, pI 5.9), carbonic anhydrase I (pI = 6.6).

After electrophoresis, the protein bands in the focused gel were fixed in a solution of 30% methanol, 5% trichloroacetic acid and 3.5% sulfosalicyclic acid for 15 min and then transferred into a solution of 27% ethanol, 10% acetic acid and 0.04% Coomassie brilliant blue R-250 for 2-4 hr at room temperature for protein

stained. The other part of gel stained for enzyme activity band as described in section 2.8.4.

2.8.4 Staining for peroxidase activity

After electrophoresis, the gel was submerged in phosphate buffer pH 6.8 for 5 minutes prior to immersing in reaction mixture containing 0.5 mM DAB (freshly prepared), 1 mM H_2O_2 in 0.1 M phosphate buffer pH 6.8 until activity band appeared (approximately 10-15 minutes).

2.9 Deglycosylation of cassava peroxidase

Purified cassava parenchyma peroxidase was deglycosylated by a modification of the method reported by Tams and Welinder (50). Cassava parenchyma peroxidase (100 μl; 10 μg) was incubated with 3% or 6% of trifluoro-methanesulfonic acids (TFMS) at 0°C; 30 min. After that the sample was neutralized with 60% pyridine (100 μl) and the TFMS was removed by dialysis against 20 mM potassium phosphate buffer pH 6.8 overnight with frequent changes of buffer. After dialysis, the deglycosylated peroxidase was assayed for protein content as described in section 2.6 and assayed for peroxidase activity as described in section 2.7.

2.10 Determination of pH stability

The pH stability was determined by incubating 2 μg of the enzyme in universal buffer (Gritton & Robinson Type) pH between 3-11 at 4°C for 24 hr then readjusted to pH 6.8 with 2.9 ml of 0.1 M sodium phosphate buffer pH 6.8. The peroxidase activity was assayed as described in section 2.7. The result was expressed

as the percentage of the enzyme activity remained with the maximum activity as 100%.

2.11 Determination of Optimal pH

The enzyme was determined for its optimm pH by performing the peroxidase assay (section 2.7) replacing the phosphate buffer with universal buffer at various pH ranging from 3-11.

2.12 Temperature stability

Enzyme stability was investigated over the temperature range of 25°C - 60°C. The purified peroxidase was incubated at various temperatures for 24 hrs. The enzyme solution was removed and cooled immediately to 25°C before measuring the remaining activity by the method described in section 2.7. The result was expressed as the percentage of the enzyme activity remained with the activity at 0 time as 100%.

2.13 Optimal temperature

To study the effect of temperature on enzyme activity, the purified enzyme was assayed at various temperatures (4°C, 20°C, 30°C, 50°C, 60°C, 65°C, 70°C) using DAB as substrate as described in section 2.7. The reaction mixture (in the absence of H_2O_2 and peroxidase) was incubated at the desired temperature. The reaction was started by the addition of 1 mM H_2O_2 and peroxidase. The result was expressed as the percentage of the enzyme activity, with the activity at the highest temperature obtained as 100%.

2.14 Spectral analysis

The uv-visible spectra of native purified peroxidase and horseradish peroxidase in range 200-800 nm were recorded using a Shimazu, uv-160 at 25°C. A cuvette (1 cm light path) containing 800 μg enzyme solution in 20 mM potassium phosphate buffer pH 6.8 was used.

The absorption spectrum of coniferyl alcohol was also recorded using a Shimazu, uv-250/PC at 25°C. Its spectrum obtained after the addition of 1 mM H_2O_2 and purified enzyme was similarly recorded.

2.15 Carbohydrate determination

Neutral sugar content of the peroxidase was determined by phenol-sulfuric acid method of Dubois, et al (51). Simple sugar or oligosaccharides with free reducing groups formed furfural derivatives in conc. H_2SO_4 which form complex with phenol giving varing color yields. The purified peroxidase solution (5, 10, 15, 20 μg/0.5 ml) (0.5 ml) was mixed with 0.3 ml of 5% phenol reagent. Concentrated sulfuric acid (2 ml) was then added rapidly and mixed immediately. After standing at room temperature for 30 min, the absorbance was read at 484 nm. A water blank and standards of 3-20 μg mannose were similarly incubated in the assay. Neutral sugar contents of peroxidase was estimated using a standard curve of the absorbance against the mannose (μg).

2.16 Glycoprotein staining

After SDS-PAGE electrophoresis, the gel was stained for glycoprotein by PAS staining (53). The gel was immersed in 12.5% trichloroacetic acid (25-50 ml/gel) for 30 min after which it was rinse lightly with distilled water. The gel was then immersed in 1% periodic acid for 50 min, and washed in 200 ml distilled water/gel with stirring or shaking for 10 minutes. The washing was repeated with 6 changes of water or the gel can be washed overnight with a few changes of water. The gel was then incubated in the dark in fuchin-sulfite stain for 50 min and washed 3 times with freshly prepared 0.5% metabisulfite (25-50 ml/gel) at 10 minute interval. Excess stain was removed by washing in distilled water with frequent changes and the gel was stored gel in 3-7.5% acetic acid.

2.17 Determination of kinetic constants

The native and deglycosylated peroxidase from parenchyma were used in the kinetic study to compare their K_m is for selected substrates. The substrates were used:

Substrate	Buffer/Solvent	Product absorbance (nm)
H_2O_2	0.25 mM DAB n 0.1 M phosphate buffer	465
DAB	0.1 M phosphate buffer	465
Guaiacol	0.1 M phoshate buffer	470
o-dianisidine	2% ethanol	460
Pyrogallol	0.1 M phosphate buffer	430
Coniferyl alcohol	5% ethanol	260
Syringaldazine	7% DMSO	530

H_2O_2 was fixed at 1 mM when Km's of other substrates were monitored by the method described in section 2.7 and concentrations of DAB or other substrates being studied were varied.

To determine K_m of H_2O_2, DAB concentration was fixed at 0.25 mM while H_2O_2 concentration was varied. The K_m's for all substrates were estimated by Lineweaver-Burk plots using Enzfitter computer program.

2.18 Effects of urea

The purified enzyme was incubated with various concentrations of urea at 25°C for 10 min. Then, the enzyme was assayed for the remaining activity in reaction mixture as described in section 2.7.