CHAPTER III

RESULTS

3.1 Purification of peroxidase from cassava parenchyma

3.1.1 Ammonium sulfate precipitation

Peroxidase from cassava parenchyma was purified according to the methods outlined in section 2.4. The crude enzyme prepared from 4500 g of parenchyma was sequentially precipitated with ammonium sulfate at 0-40% and 40-80% saturation. The highest specific activity was found in the precipitate from 40-80% ammonium sulfate saturation (Fig. 7). This step gave 103% activity yield and 2.02 folds of purification (Table 2).

3.1.2 Concanavalin A column chromatography

Further purification of the enzyme was performed by affinity chromatography on Concanavalin A Sepharose 4B column. Most of the proteins (peak I) did not bind to the Concanavalin A column and contained only small amount of peroxidase activity (Fig. 8). The bound fractions containing peroxidase was eluted as single major peak (peak II) with 0.3 M α-D-methyl glucopyranoside. When the column was further eluted with 0.5 M α-D-methyl glucopyranoside, no more peroxidase activity was detected.

When the unbound peak was reloaded to the same Concanavalin A column which was re-equilibrated, the protein peak with the peroxidase activity remained unbound. Elution with 0.3 M α-D-methyl glucopyranoside yielded no bound fractions with peroxidase activity (Fig. 9), indicating that peak I from the first

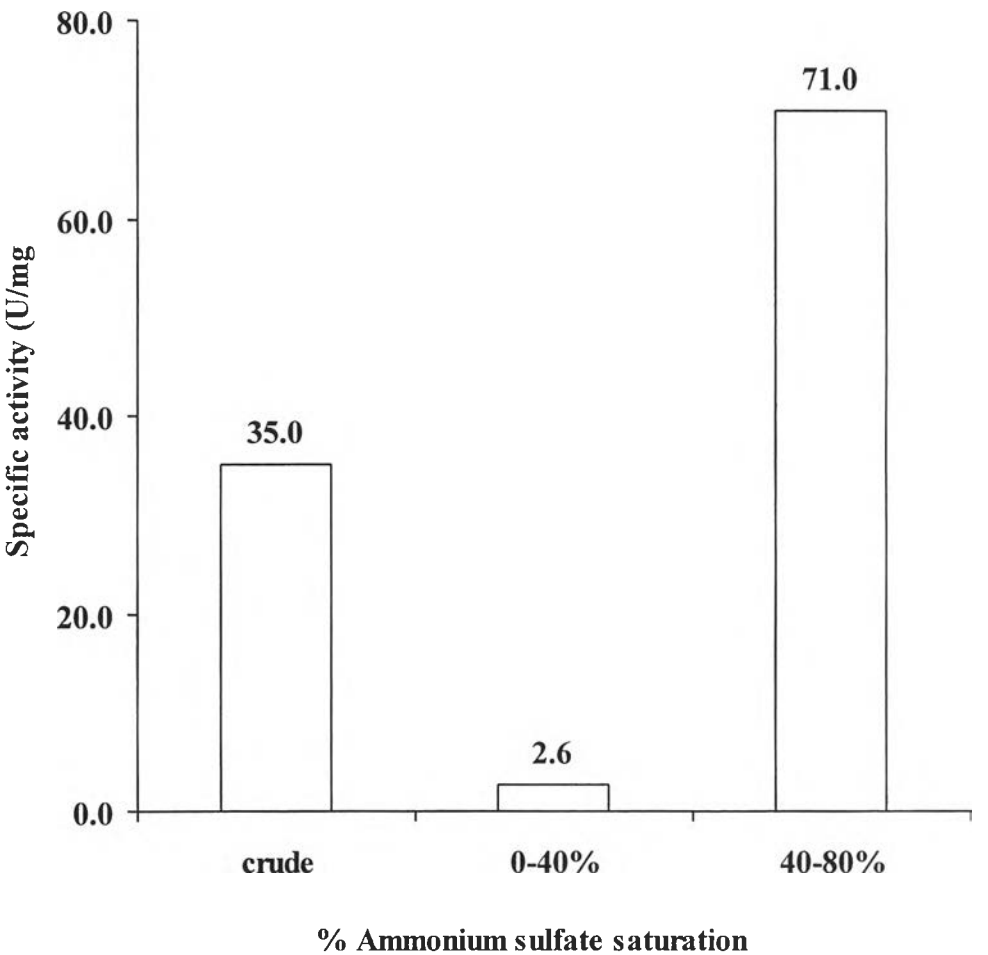


Fig. 7 The specific activity of peroxidase in the crude extract of cassava parenchyma and the fractions obtained by ammonium sulfate fractionation

U = Unit activity = Change of one absorbance unit at 465 nm per min

Table 2 Purification of peroxidase from cassava tubers

Sample	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)	% Yield	Purification fold
Crude enzyme	429	15028	35	100	1
40-80%$(NH_4)_2SO_4$	218	15472	71	103	2
Con-A Sepharose	3.9	1932	495.3	12.8	14
Sephadex G-200	1.9	1077.4	560.6	7.2	16

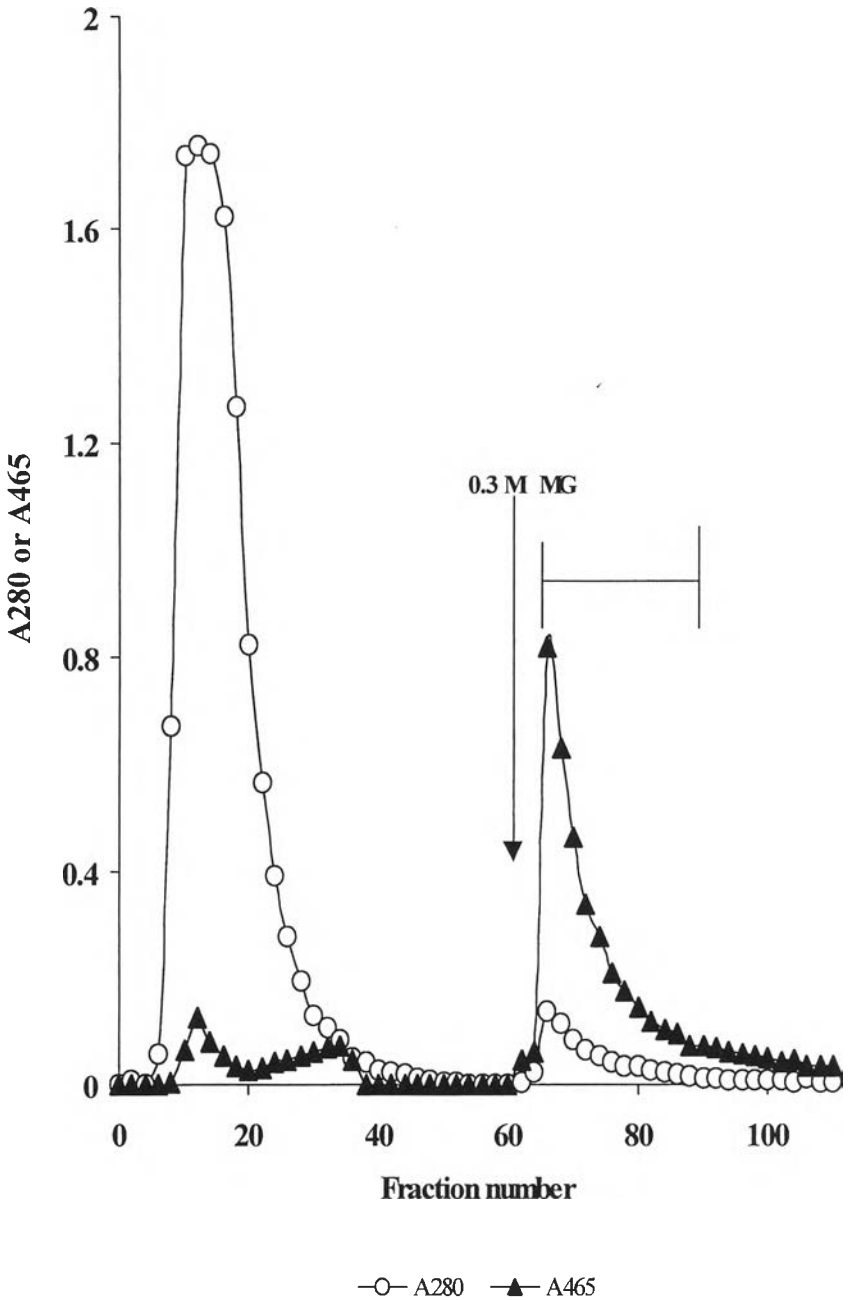


Fig. 8 Concanavalin A Sepharose chromatographic profile of 40-80% ammonium sulfate precipitated fraction of peroxidase from cassava root parenchyma.

protein A280, peroxidase activity A465

the peroxidase fractions being pooled

MG = methyl α-D-glucopyranoside

Fraction size = 3 ml

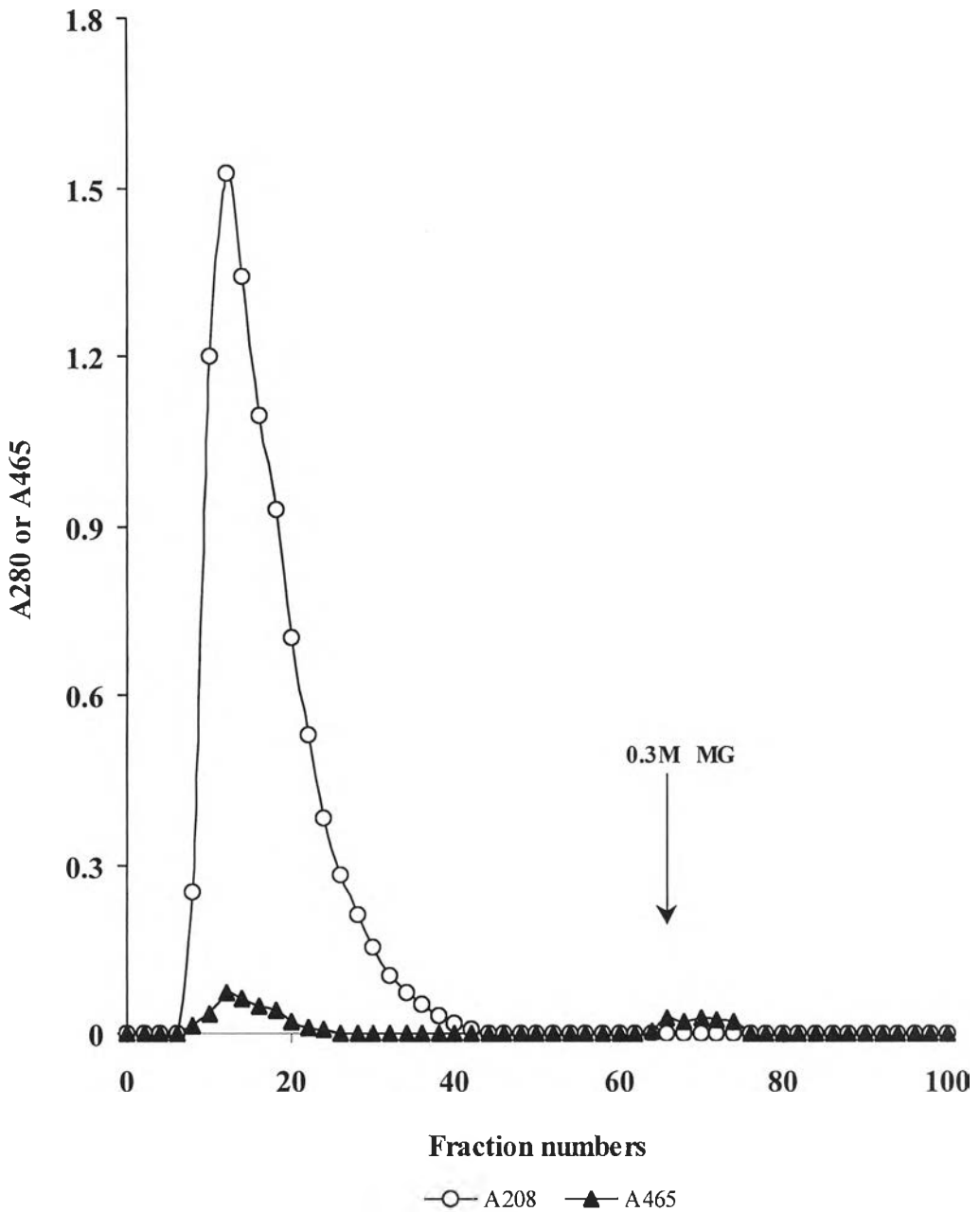


Fig. 9 Concanavalin A Sepharose chromatographic profile of reloaded unbound protein peak

protein A280 peroxidase activity A465

MG = methyl α-D-glucopyranoside

Fraction size = 3 ml

run was not a result of overloading proteins. Therefore, there were two fractions of the peroxidase activity, namely Con-A bound and Con-A unbound peaks (Fig. 9). Since the bound fraction had a higher specific activity, it was further purified.

3.1.3 Sephadex G-200 column Chromatography

The bound fraction from the Concanavalin A column was dialyzed and concentrated by using aqua-sorb before loaded onto a Sephadex G-200 column. The protein and activity profiles were shown in Fig. 10. The fractions with the peroxidase activity were pooled. The purification of cassava parenchyma peroxidase was summarized in the Table 2. The enzyme was purified 16 folds with 7.2% activity yield.

3.1.4 Non-denaturing and SDS-PAGE

The non-denaturing PAGE of the enzyme fractions from each step of the purification was performed as described in section 2.8.2. The activity stain of the peroxidase in each purification step revealed a series of peroxidase bands (Fig. 11). One major band (slow moving) was retained throughout the purificaton. The fast moving band was lost during the purification. Protein staining of the enzyme was barely detectable (Fig. 12).

The SDS-PAGE of the enzyme fractions from each step of the purification was performed as described in section 2.8.1. The protein stain of the peroxidase in each purification step is shown in Fig. 13. Purified cassava peroxidase from parenchyma showed 1 protein band with the molecular weight in denaturing state of 54 kD.

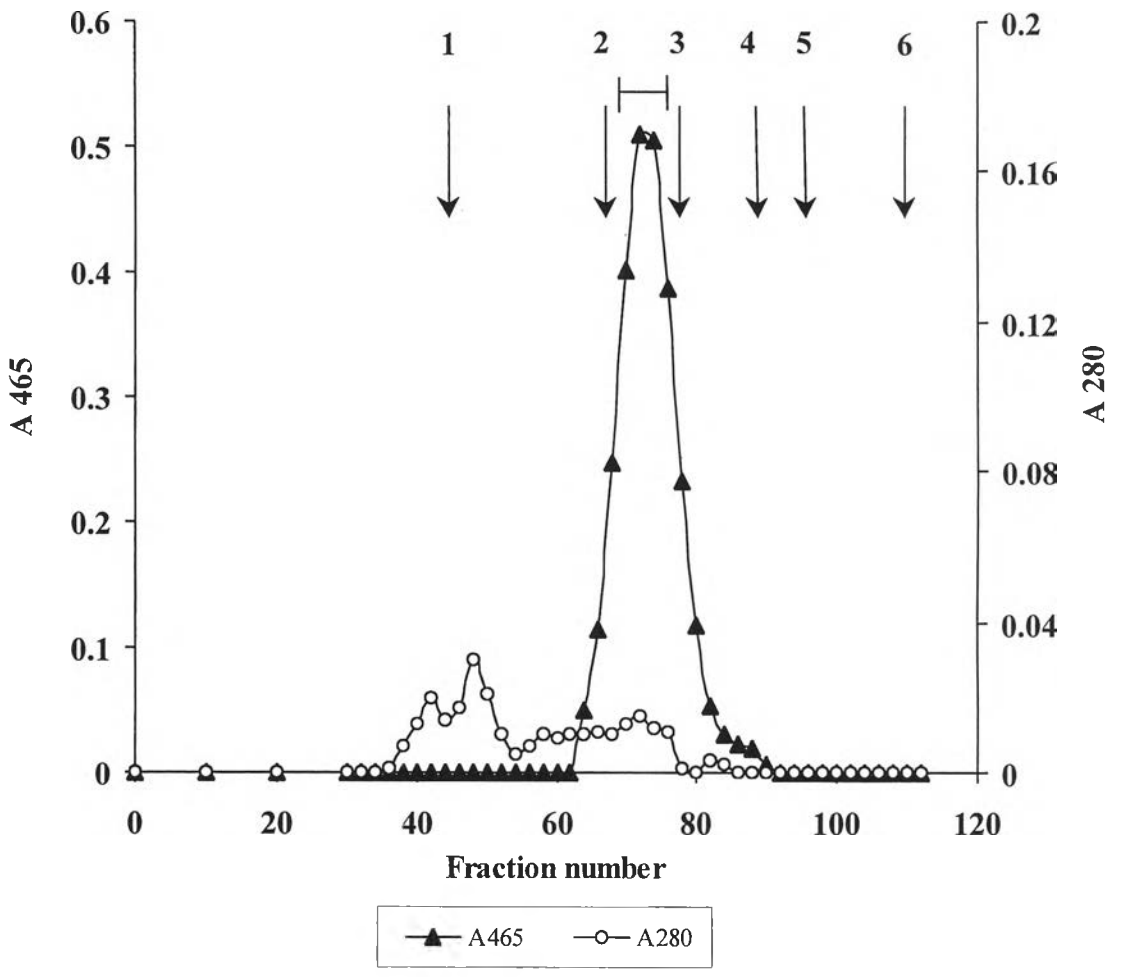


Fig. 10 Sephadex G-200 chromatographic profile of concentrated enzyme from Concanavalin A Sepharose column.

Fraction size = 2.4 ml

1 = Blue dextran

2 = Aldolase MW = 158 kDa

3 = Bovine serum albumin MW = 68 kDa

4 = Chymotrypsinogen MW = 27 kDa

5 = Ribonuclease MW = 13.7 kDa

6 = DNP-lysine

⊢⊣ = the peroxidase fractions being pooled

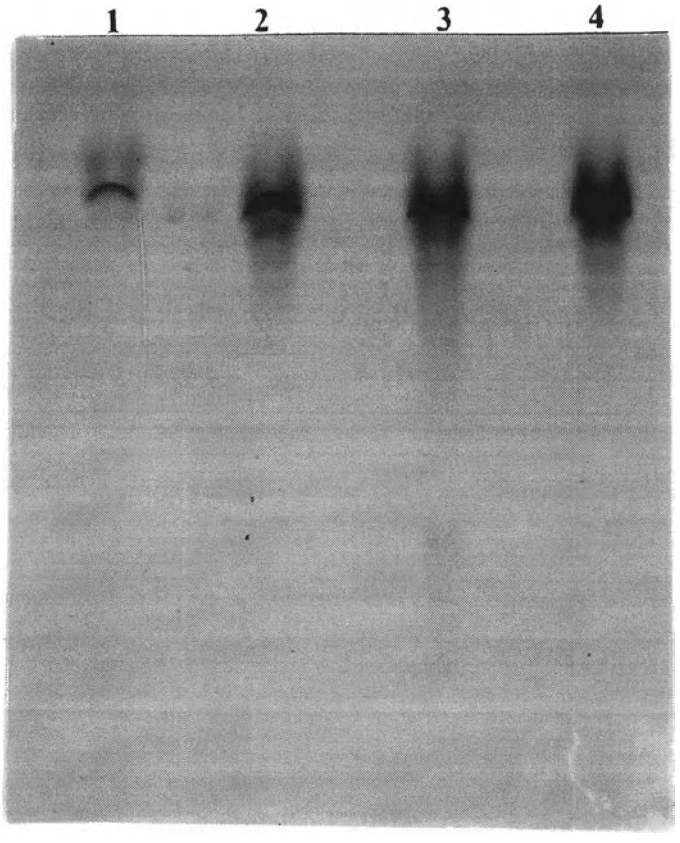


Fig. 11 Peroxidase activity stain of Non-denaturing PAGE of each purified proteins of cassava parenchyma

Lane 1 = Crude extract

Lane 2 = 40-80% ammonium sulfate precipitate

Lane 3 = Concanavalin A, bound peak

Lane 4 = Sephadex G-200

Amount loaded in lanes 2-4 = 0.2 U, lane 1 = 0.1 U

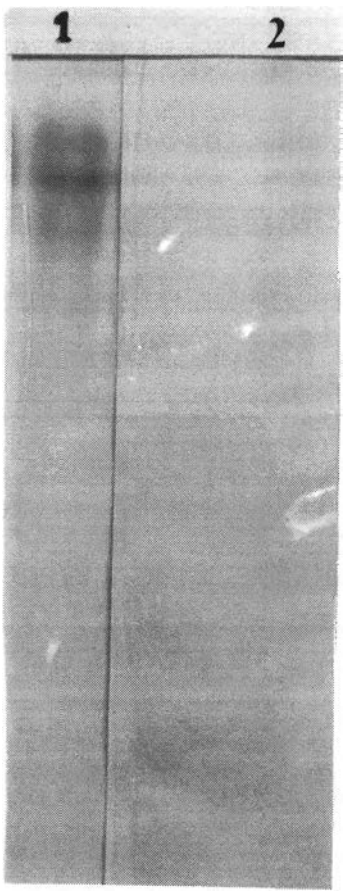


Fig. 12 Peroxidase activity and protein stain of Non-denaturing PAGE of purified protein from Sephadex G-200

Lane 1 = Peroxidase activity stain (0.2 U)

Lane 2 = Peroxidase protein stain (2 μg)

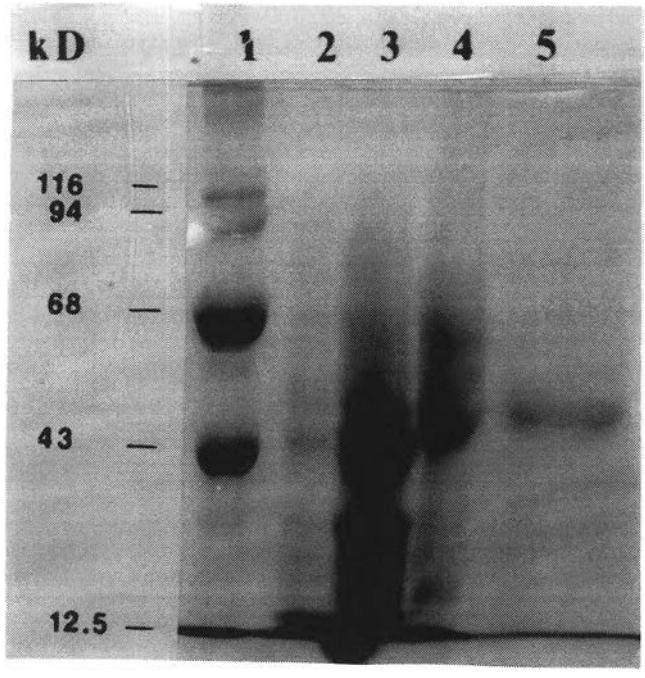


Fig. 13 Protein stain of SDS-PAGE (10%) of fractions from the purification of peroxidase from cassava parenchyma

Lane 1 = standard protein markers (10 μg)

Lane 2 = crude extract (10 μg)

Lane 3 = 40-80% ammonium sulphate percipitate (20 μg)

Lane 4 = Concanavalin A, bound peak (10 μg)

Lane 5 = Sephadex G-200 (10 μg)

3.2 Characterization of cassava parenchyma peroxidase

3.2.1 Determination of molecular weight

The native molecular weight of the purified peroxidase from cassava parenchyma was determined by chromatography on a Sephadex G-200 gel filtration column (Fig. 14). The column was calibrated with standard markers proteins as mentioned in section 2.5.3. From the calibration curve, the molecular weight of the cassava peroxidase was extimated at 105 kD. Its electrophoretic mobility on SDS-PAGE was compared with those of standard proteins under the same conditions (Fig. 13). A plot of Rf versus the logarithm of molecular weights of the standard proteins was linear (Fig. 15). Based on the Rf value, the purified cassava peroxidase had a subunit molecular weight of 54 kD. The data on the native and the subunit molecular weights indicated that the purified peroxidase from cassava parenchyma was a dimer of the 54 kD subunit.

3.2.2 Determination of isoelectric point (pI)

The purified cassava peroxidase was analyzed for its pI value by isoelectrofocusing on polyacrylamide gel as described in section 2.8.3. The peroxidase was detected by activity stain of IEF gel and the pattern was shown in Fig. 16, revealing 5 isoenzymes. Standard proteins were run on the same gel, but were stained for proteins with Coomassive blue and a standard curve was constructed as shown in Fig. 17. The major isozyme corresponded to pI 5.4 and pI's of other minor isozymes were 5.1, 5.2, 5.8 and 6 respectively.

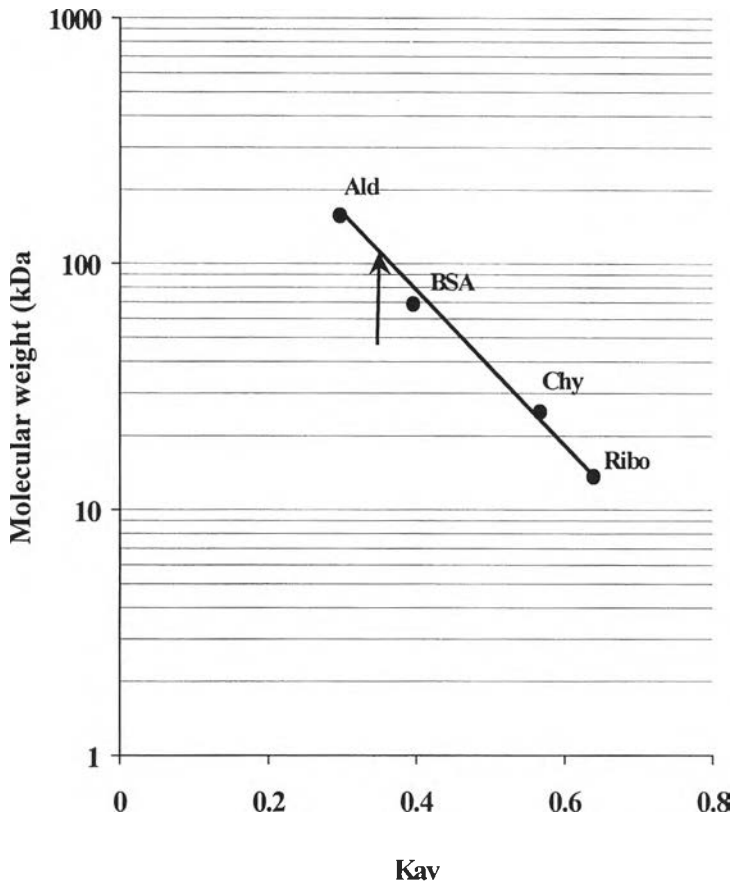


Fig. 14 Molecular weight calibration curve obtained from chromatography on Sephadex G-200 column

Ald = Aldolase MW = 158 kDa
BSA = Bovine serum albumin MW = 68 kDa
Chy = Chymotrypsinogen MW = 27 kDa
Ribo = Ribonuclease MW = 13.7 kDa
Arrow indicates the Kav of peroxidase from parenchyma

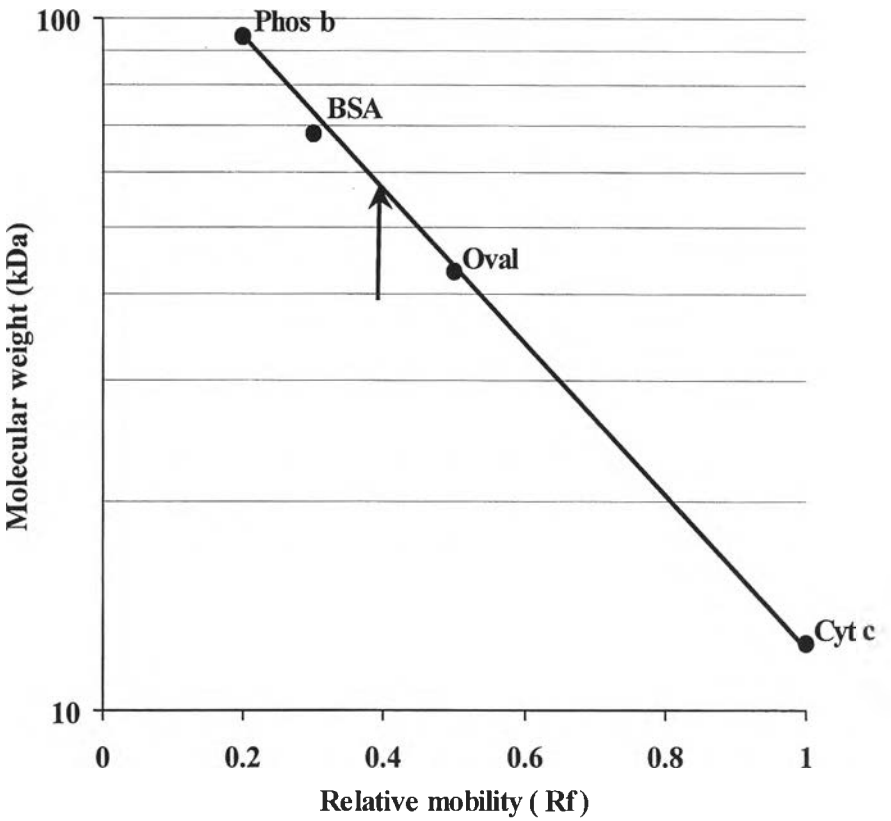


Fig. 15 Molecular weight calibration curve obtained from SDS-PAGE

Phos b = Phosphorylase b MW = 94 kDa

BSA = Bovine serum albumin MW = 68 kDa

Oval = Ovalbumin MW = 43 kDa

Cyt C = Cytochrome C MW = 12.5 kDa

Arrow indicates the position of peroxidase from parenchyma

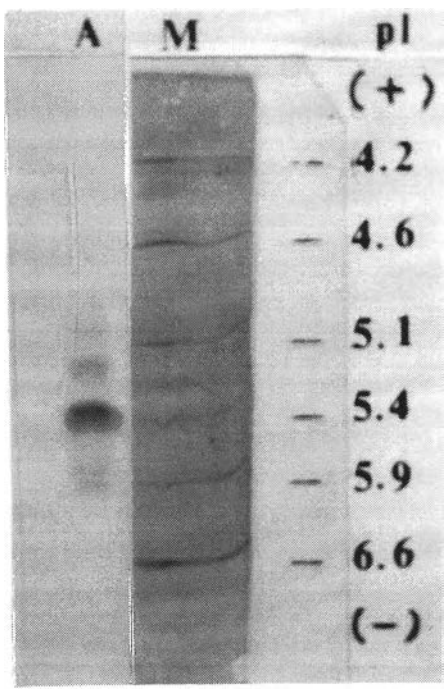


Fig. 16 Isoelectrofocusing gel electrophoresis of purified peroxidase isozyme A from cassava tubers.

Lane A = purified peroxidase

Lane M = standard pI markers

Glucose oxidase pI = 4.2

Trypsin inhibitor pI = 4.6

β-Lactoglobulin A pI = 5.1

Carbonic anhydrase II pI = 5.4

Carbonic anhydrase II pI = 5.9

Carbonic anhydrase I pI = 6.6

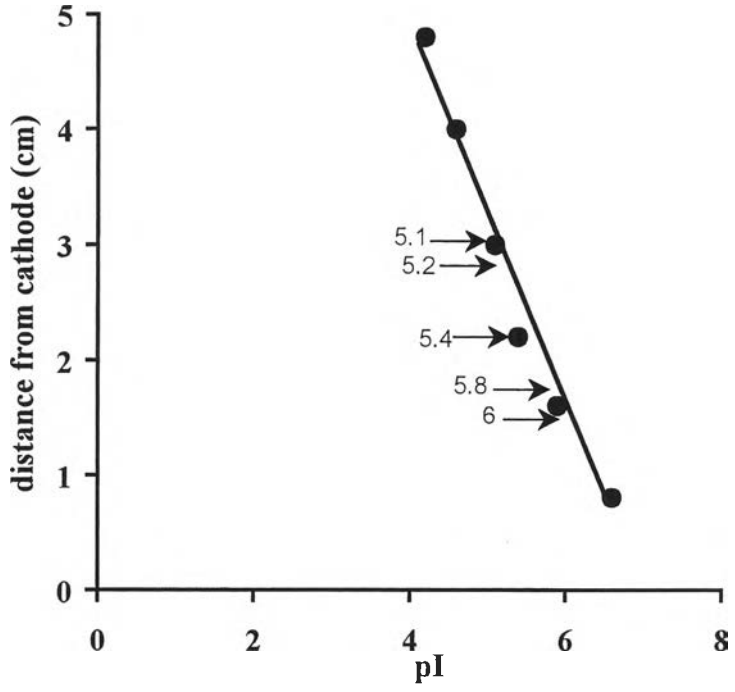


Fig. 17 Calibration curve of standard pI markers from isoelectrofocusing gel electrophoresis

Arrows indicate the positions of bands of peroxidase from parenchyma

3.2.3 Determination of carbohydrate content

To determine whether the cassava peroxidase was a glycoprotein, carbohydrate content of the enzyme was monitered both qualitatively and quantitatively by PAS staining of the peroxidase on polyacrylamide gel and neutral sugar determination by phenol-sulfuric method respectively.

3.2.3.1 PAS stain of the enzyme

The proteins from each purification step was electrophoresed on SDS-PAGE with standard proteins and stained for glycoproteins using PAS stain as described in section 2.18. Standard proteins Phosphorylase b, bovine serum albumin, ovalbumin and cytochrome C were used as negative control. The fraction bound Con-A was used as positive control. The purified peroxidase was positive for PAS stain while non of the standard proteins showed up on PAS stain (Fig. 18).

3.2.3.2 Determination of neutral sugar content by phenol-sulfuric method

The neutral sugar content of the purified cassava peroxidase was determined by the phenol-sulphuric acid method as described in section 2.15. Mannose at different concentrations were used to construct a standard curve (Appendix D). Peroxidase from cassava was found to contain high amount of carbohydrate. Determination of carbohydrate content of horseradish peroxidase was also included in the experiment as positive control (Fig. 19), the result was shown in Table 3. Parallel determination of carbohydrate content of HRP gave the value of 10.9% w/w which agreed with the value reported by Silva (73).

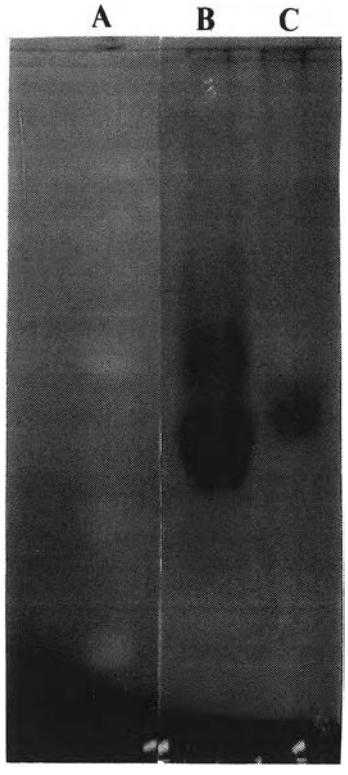


Fig. 18 PAS staining of purified peroxidase from cassava parenchyma (SDS; 10% gel)

Lane A = standard markers; Negative control

Lane B = Con-A, bound peak; Positive control (20 μg)

Lane C = Purified peroxidase from G-200 (5μg)

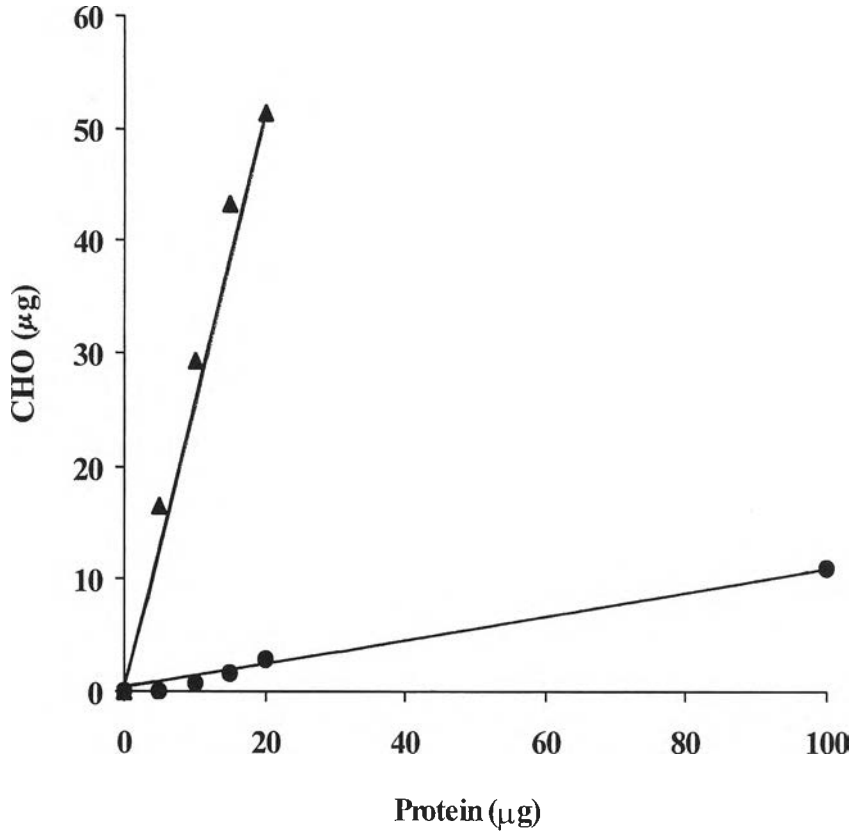


Fig. 19. Total carbohydrate (CHO) of cassava peroxidase and horseradish peroxidase (HRP) by phenol-sulfuric acid method

-●- horseradish peroxidase

-▲- cassava parenchyma peroxidase

Table 3 Carbohydrate content of cassava peroxidase

Protein	% of carbohydrate (w/w)
Horse radish peroxidase	10.9 (73)
Cassava peroxidase	292.3
3% TFMS-treated peroxidase	146.2
6% TFMS-treated peroxidase	29.4

w/w = weight of carbohydrate/weight of protein

3.2.4 UV-visible absorption spectrum

Light absorption of the purified cassava peroxidase and horse radish peroxidase were performed in the range 200 to 800 nm and their spectra were shown in Fig. 20. Horse radish peroxidase exhibited a distinct absorbance peak at 402 nm. Purified cassava peroxidase spectra exhibited a distinct absorbance peak at 398 nm.

3.3 Partial deglycosylation of cassava peroxidase

Purified cassava peroxidase were deglycosylated with TFMS as described in section 2.9. To determine the suitable temperature for performing deglycosylation, a preliminary experiment was performed on deglycosylation of peroxidase with 3% TFMS at 0°C and 37°C (Fig. 21). It was found that deglycosylation at 37°C caused greater activity loss therefore, further experiment on deglycosylation were performed at 0°C. Varied amounts of the enzyme were treated with 3% and 6% (v/v) TFMS at 0°C and the carbohydrate contents determined. Phenol sulfuric assay showed that the carbohydrate content of cassava peroxidase decreased to 50% and 90% when treated with 3% and 6% TFMS respectively (Fig. 22), and % of carbohydrate was calculate in Table 3. The peroxidase activity of the 6% TFMS treated enzyme was almost completely lost while the activity of 3% TFMS treated fraction decrease to 50% (data not shown).

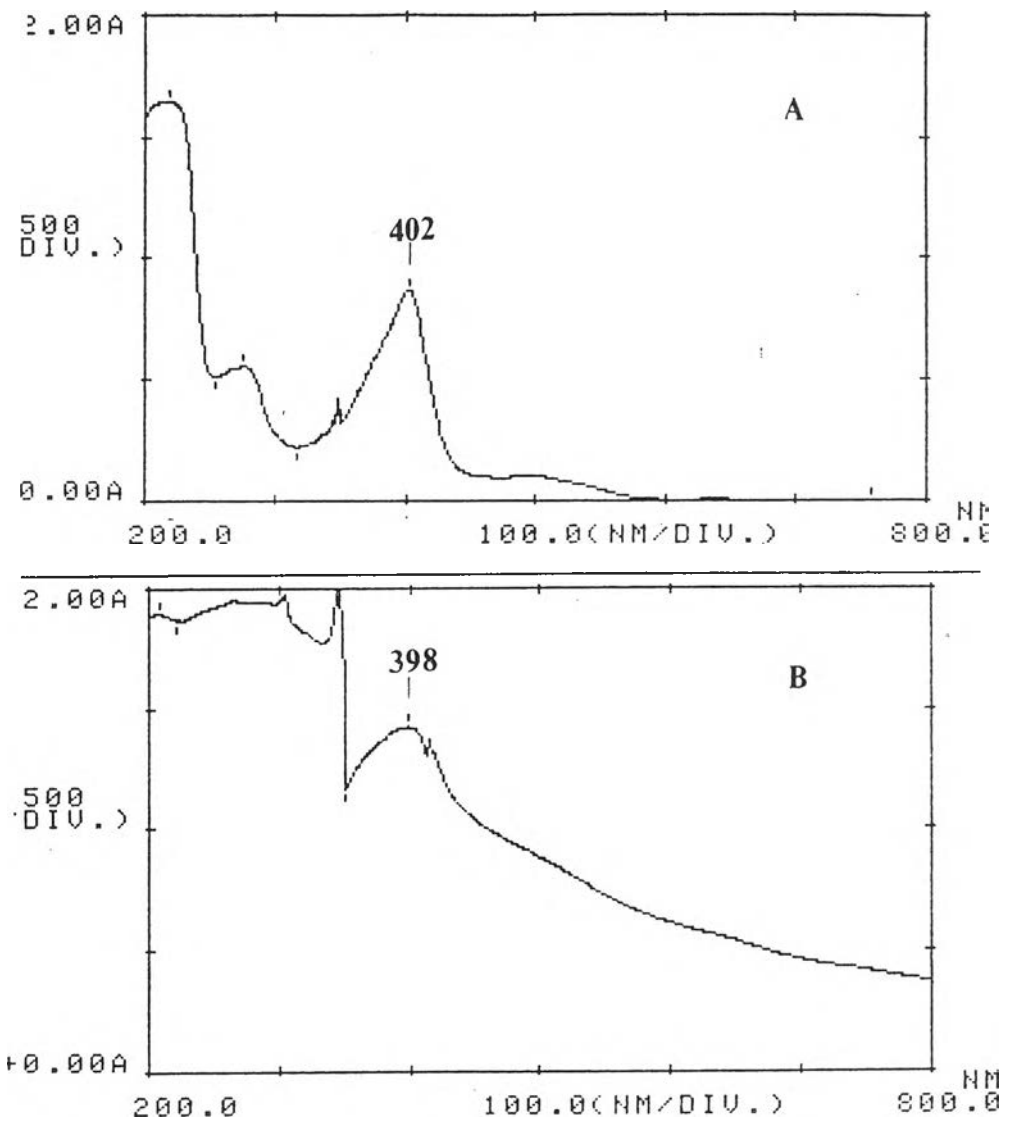


Fig. 20 UV-visible absorption spectrum of cassava peroxidase

(A) spectrum of horseradish peroxidase (800 μg)

(B) spectrum of purified cassava peroxidase (800 μg)

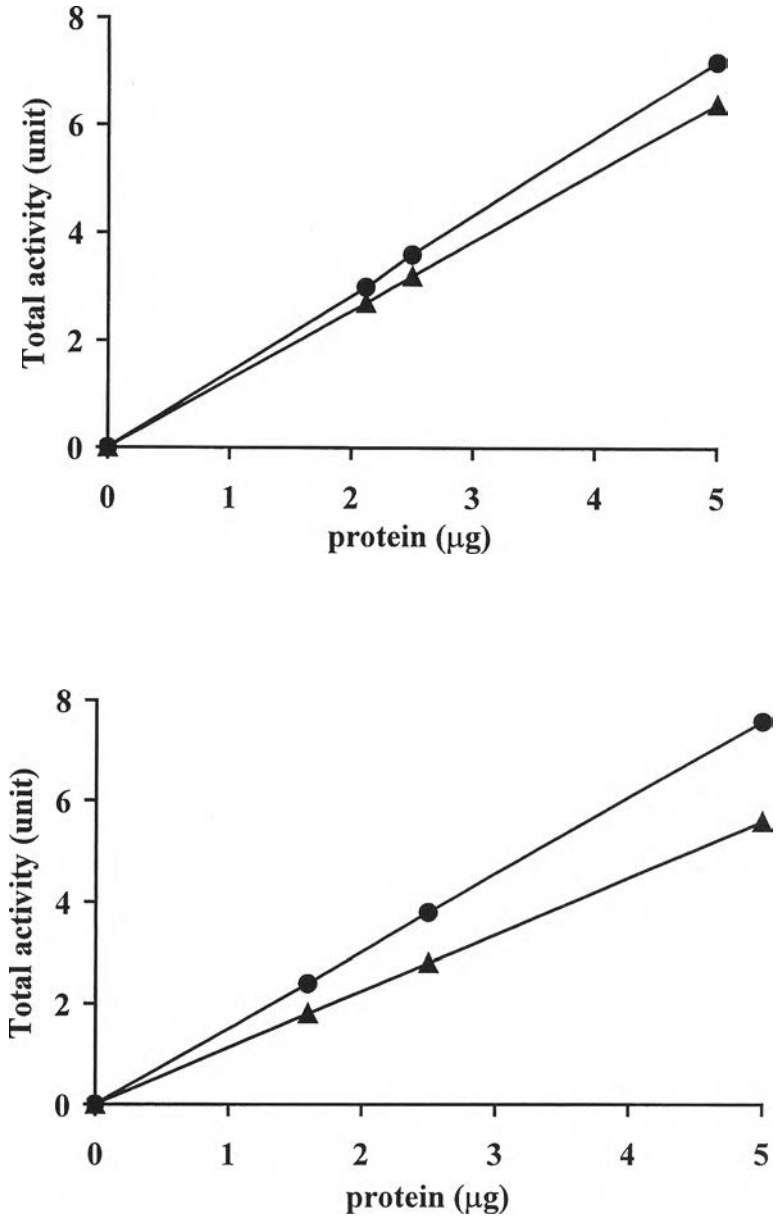


Fig. 21 Total activity of peroxidase from cassava parenchyma after partial deglycosylation by TFMS

(A) incubated at 0°C, 60 min

(B) incubated at 37°C, 60 min

● Native peroxidase

▲ 3% TFMS-treated peroxidase

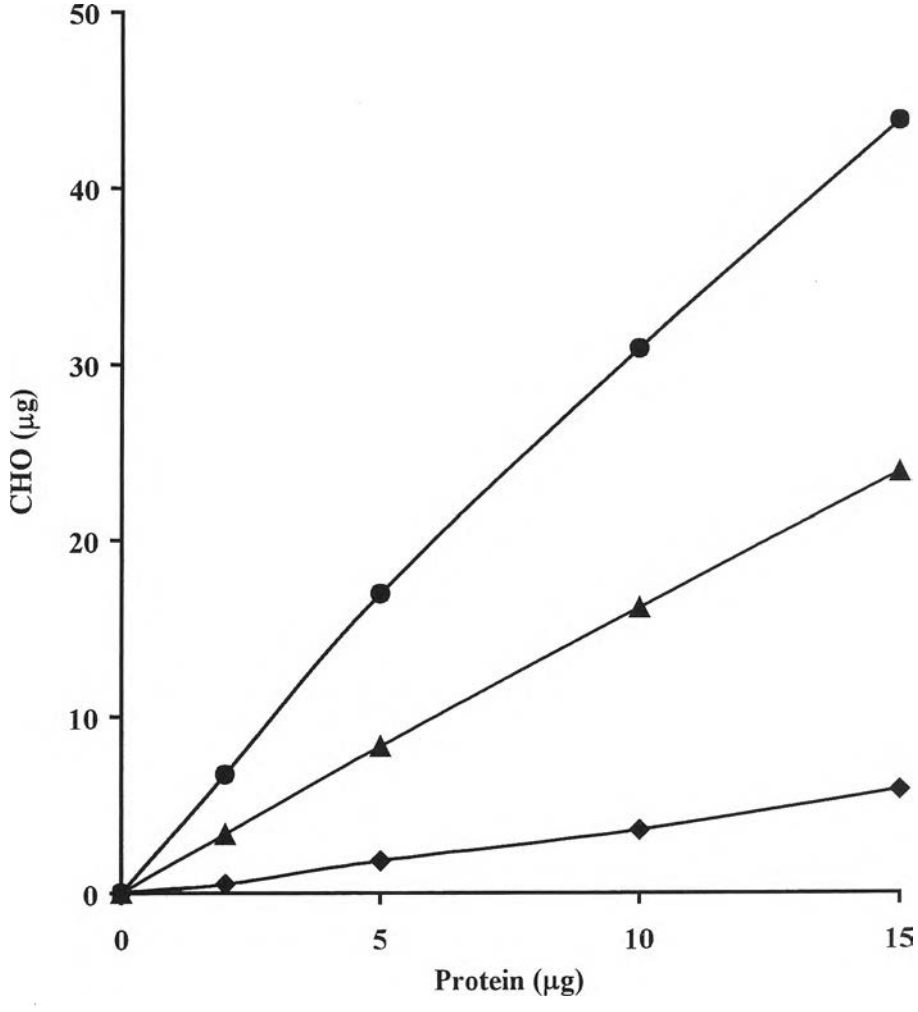


Fig. 22 Total carbohydrate (CHO) of peroxidase after partial deglycosylation with TFMS

● Native peroxidase at 0°C

▲ 3% TFMS-treated peroxidase at 0°C

◆ 6% TFMS-treated peroxidase at 0°C

3.4 Comparative characterization of native and deglycosylated cassava peroxidase

The enzyme deglycosylated with 3% TFMS was used for further studies in comparison with native enzyme. Although 6% TFMS removed more carbohydrate, the enzyme activity was greatly lost to a level which was not suitable for biochemical studies.

3.4.1 UV-VIS absorption spectrum

The uv-visible absorption spectrum of deglycosylated enzyme also changed. The distinct absorption at 398 nm decreased upon deglycosylation (Fig.23).

3.4.2 Mobility on ND-PAGE and SDS-PAGE

The native and deglycosylated enzyme were run on ND-PAGE and SDS PAGE, the peroxidase was detected by activity staining and the pattern was shown in Fig. 24. Mobility of deglycosylated enzyme on 5% ND-PAGE and 7-12% SDS-PAGE seemed to be slightly different from the native enzyme, but it was difficult to confirm the difference since the band intensity were also different. Sample used in SDS gel in the experiment was not heated because it was prepared for activity stained

3.4.3 Isoelectric point (pI)

Native and deglycosylated cassava peroxidase were analyzed for their pI values by isoelectrofocusing on polyacrylamide gel as described insection 2.8.3. The peroxidase was detected by activity stain of IEF gel and the pattern was shown in Fig. 25. Standard proteins were run on the same gel, but were stained for proteins with Coomassive blue and a standard curve was constructed as shown in Fig. 26. The major isozyme with pI 5.4 of deglycosylated peroxidase was not different from native isozyme, the new band isozyme corresponded to pI 5.3 was found in deglycosylated peroxidase but pI's of other minor isozyme were the same in native isozyme.

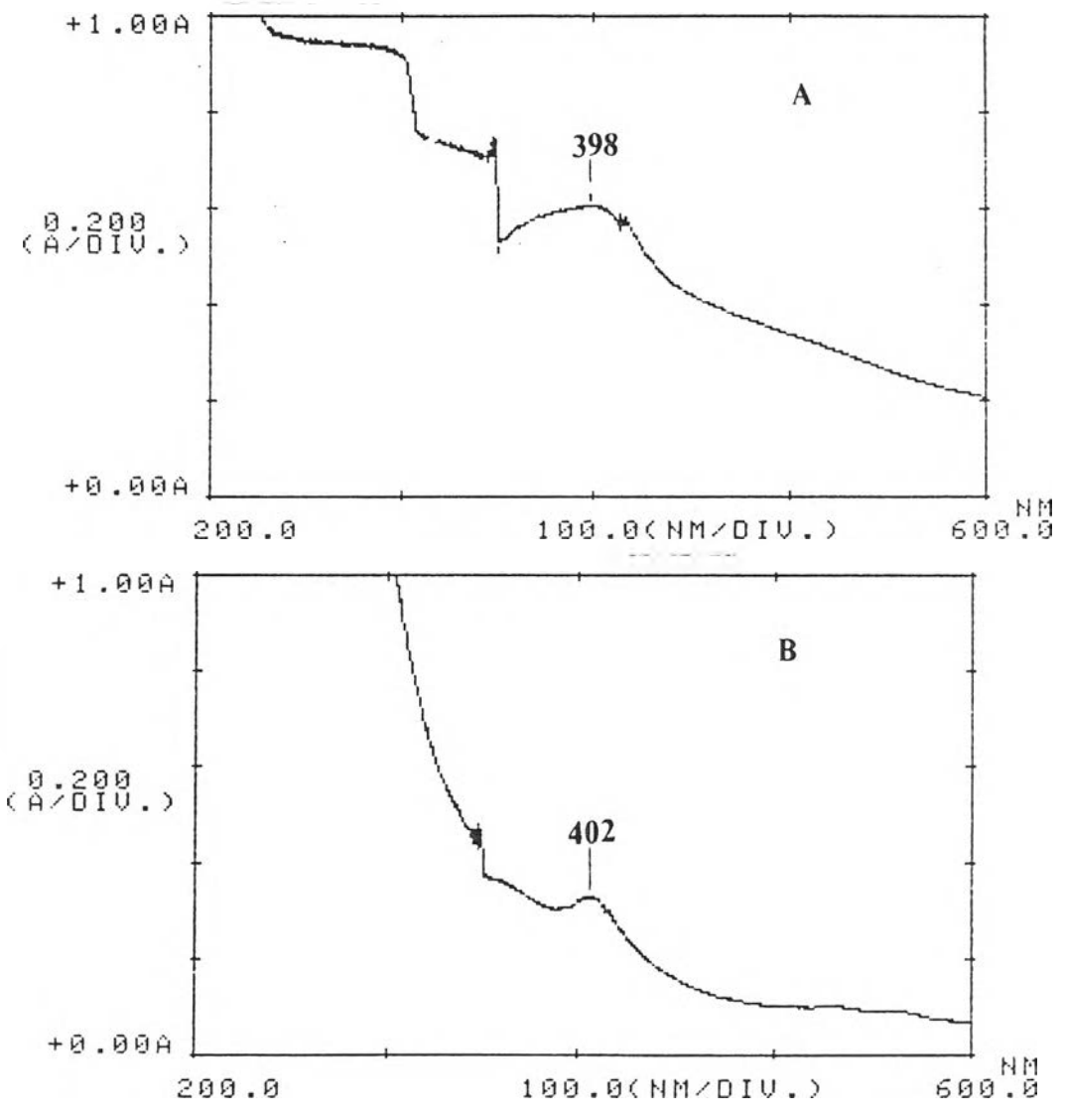


Fig. 23 UV-visible absorption spectrum of purified cassava parenchyma peroxidase

(A) Native peroxidase (200 μg)

(B) 3% TFMS-treated peroxidase (200 μg)

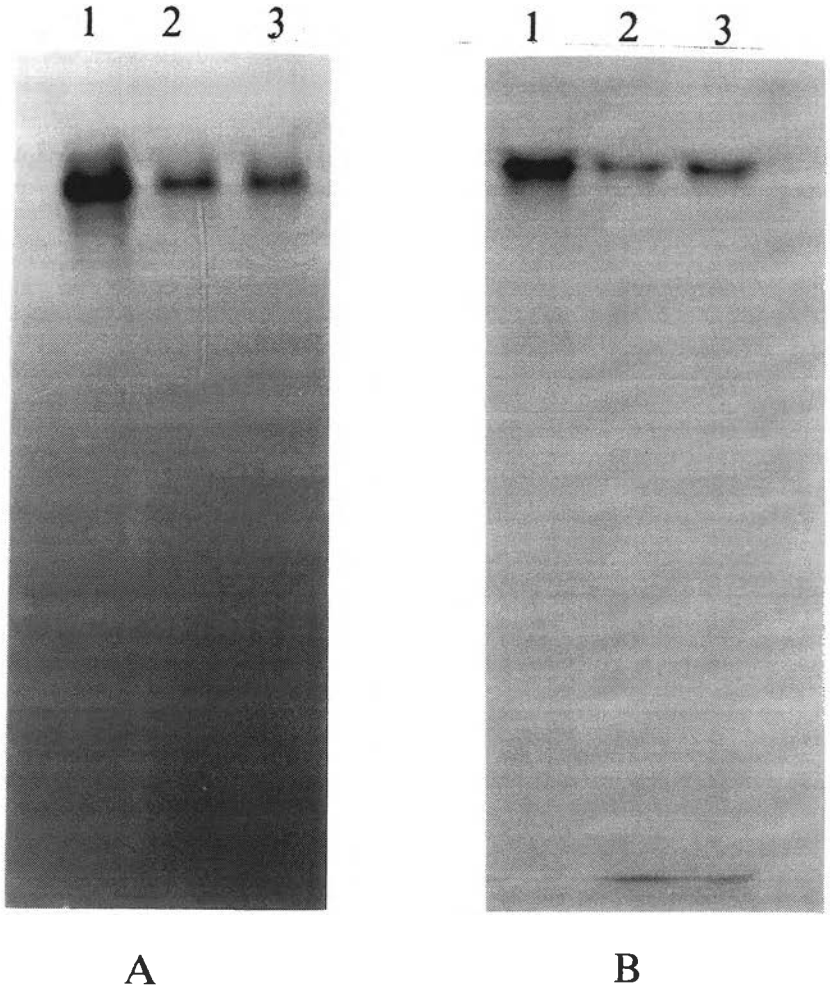


Fig. 24 Peroxidase activity stain of mobility on ND-PAGE and SDS-PAGE

1 = Native peroxidase

2 = 3% TFMS-treated peroxidase

3 = 6% TFMS-treated peroxidase

A = ND-PAGE (5%)

B = SDS-PAGE (7-12% gradient; without heating)

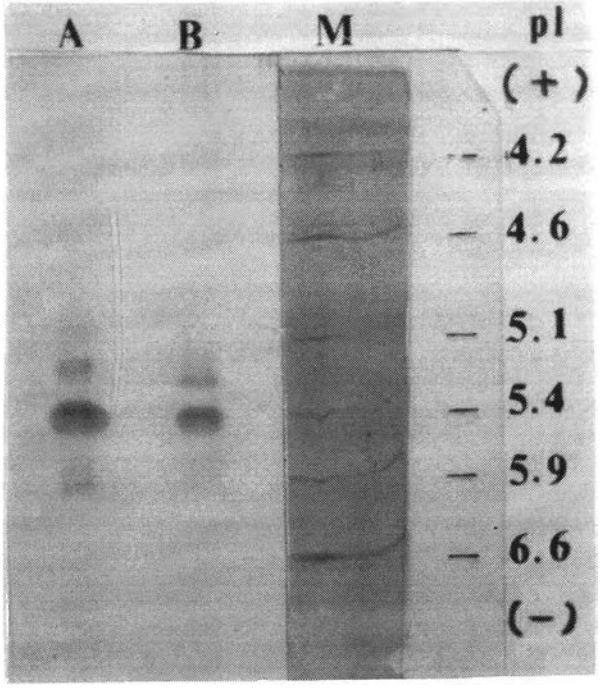


Fig. 25 Isoelectrofocusing gel electrophoresis of native and deglycosylated peroxidase from cassava peroxidase from cassava parenchyma

Lane A = Native peroxidase (0.1 μg)

Lane B = 3% TFMS-treated peroxidase (0.1 μg)

Lane M = standard pI markers

Glucose oxidase	pI = 4.2
Trypsin inhibitor	pI = 4.6
β-Lactoglobulin A	pI = 5.1
Carbonic anhydrase II	pI = 5.4
Carbonic anhydrase II	pI = 5.9
Carbonic anhydrase I	pI = 6.6

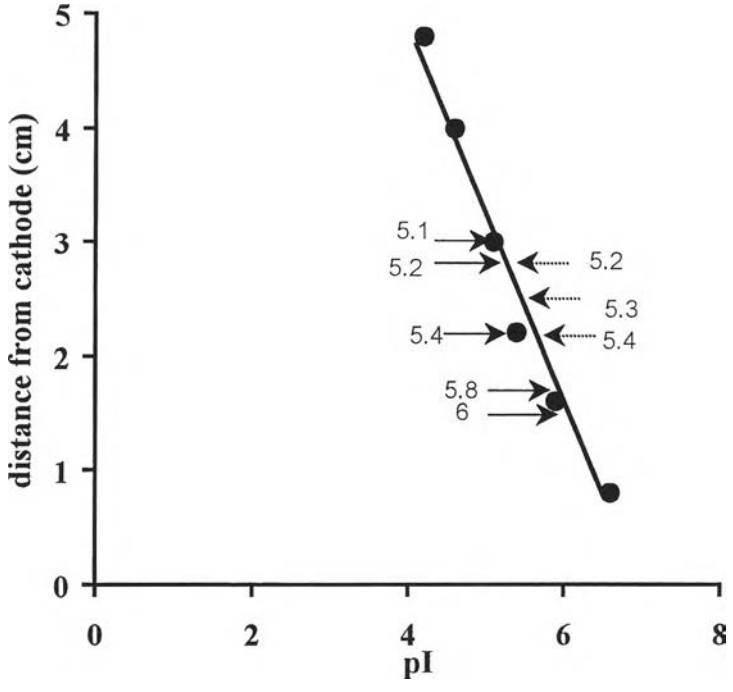


Fig. 26 Calibration curve of standard pI marker from isoelectric focusing gel electrophoresis

→ indicates the position of native peroxidase

⇢ indicates the position of 3% TFMS-treated peroxidase

3.4.4 Effect of urea

Native and deglycosylated cassava peroxidase 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. The results are shown in Fig. 27.

At 6 M, urea can inhibit activities of native and deglycosylated peroxidasse, the remaining activities were about 30% and 20% respectively.

3.4.5 Optimum pH

Native and deglycosylated cassava peroxidases were assayed at various pHs as described in section 2.11. The results were shown in Fig. 28. Both enzyme preparations showed similar patterns of pH optimum with highest activity at pH 5 and this was defined as 100% activity. In the acidic pH range of 3.0-5.0, the activity increased rapidly. When the pH was increased above 5, the activity dropped sharply reaching nearly zero at pH 8 and beyond.

3.4.6 pH stability

The effect of pH on the activity of the cassava peroxidase was determined by incubating aliquots of the purified peroxidase in universal buffer at various pH values (3 to 11) for 24 hr. The reaction mixtures were then adjusted to pH 6.8 for enzyme assay as described in section 2.10. It was found that the native peroxidase was quite stable in pH range of 3-11, with highest stability at pH 7. The partial deglycosylated peroxidase was more stable at pH 5 (Fig. 29).

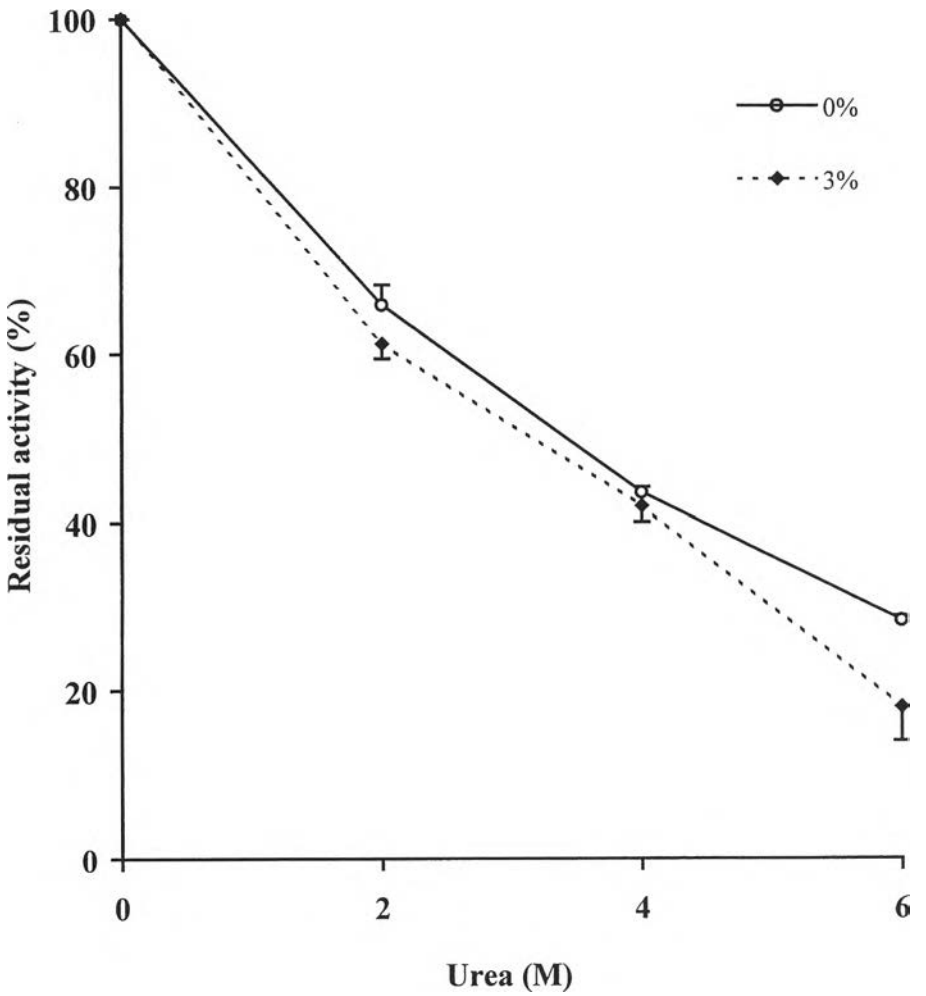


Fig. 27 Effect of urea on native and partial deglycosylated cassava peroxidase

Native and 3% TFMS-treated cassava peroxidase were incubated with various concentrations of urea at 25°C for 10 min. Then, the enzyme was assayed for the remaining activity.

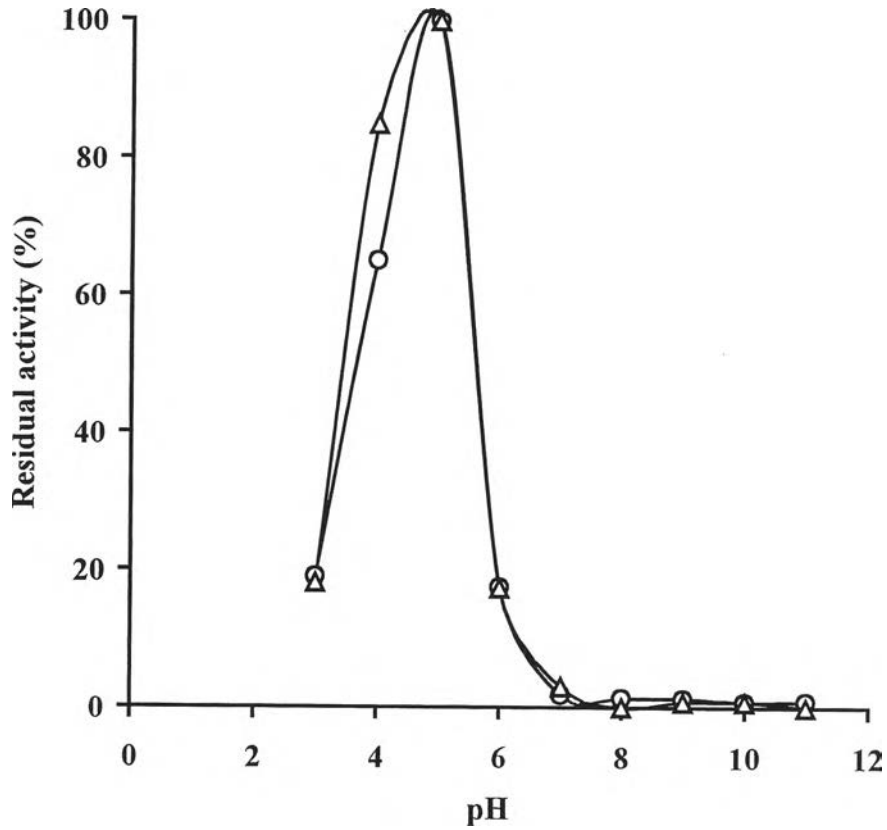


Fig. 28 pH optimum of native and partial deglycosylated cassava parenchyma peroxidase

Activity of purified enzyme was determined at various pH values, 3-11 by using DAB as substrate. The percentage of relative activity was calculated by using maximum activity of enzyme as 100%

-⊖- Native peroxidase -△- 3% TFMS-treated peroxidase

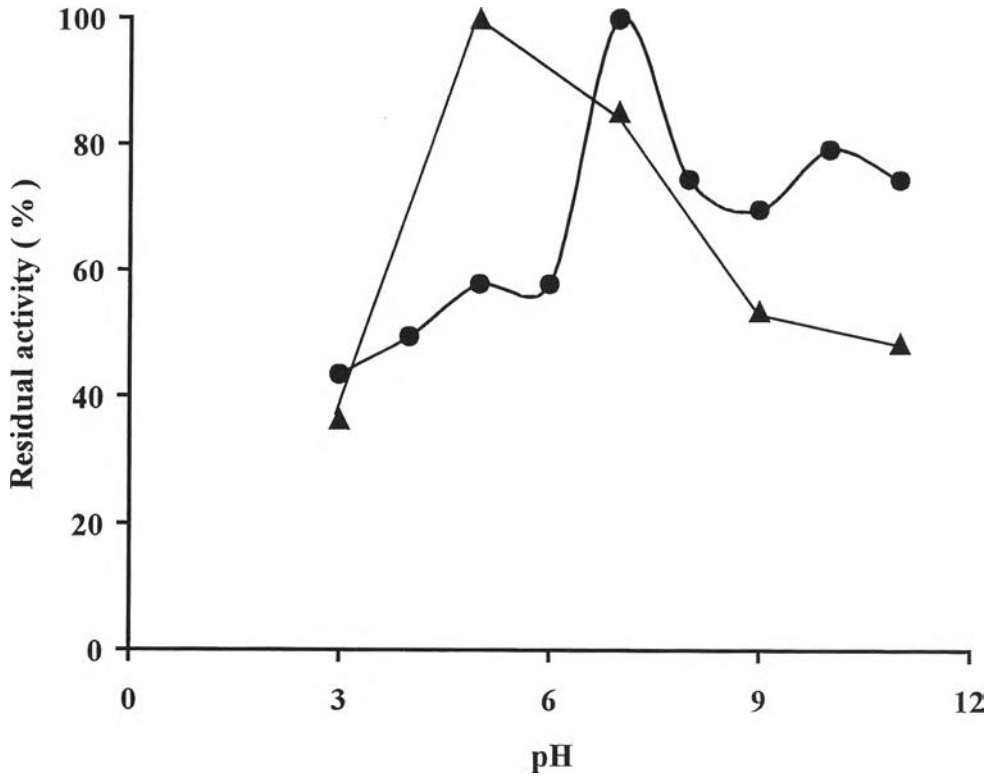


Fig. 29 pH stability of native and partial deglycosylated cassava parenchyma peroxidase

Aliquots of the enzyme were preincubated at 4°C for 24 hr at various pH values, 3-11 before being assayed for enzyme activity

● Native peroxidase ▲ 3% TFMS-treated peroxidase

3.4.7 Optimum Temperature

The activities of native and deglycosylated cassava peroxidase were assayed at various temperatures as described in section 2.13. The results were shown in Fig. 30. The activity of the native cassava peroxidase was highest at 65°C and partial deglycosylated enzyme was at 60°C. Increasing the assay temperature from 60°C to 70°C resulted in 20% decrease in both native and deglycosylated cassava peroxidases.

3.4.8 Temperature Stability

The stability of the cassava peroxidases at different temperatures were studied as descried in section 2.12. Native enzyme was fairly stable when kept at 25°C for 24 hr with about 15% loss of activity (Fig. 31). The enzyme showed a slight loss of its activity to 95% when incubated at 50°C for 4 hr but completely loss its activity at 24 hr. Incubation of the enzyme at 60°C resulted in about 95% activity loss at 4 hr and complete loss at 8 hr.

The partial deglycosylated peroxidase was also quite stable at 25°C with 30% activity loss at 24 hr. The activities at 40°C of both enzyme preparations decreased to 45% at 24 hr. The partial deglycosylated enzyme lost its activity much faster than native enzyme at high temperatures. At 50°C, the remaining activities were about 40% at 4 hr and 0% at 16 hr while at 60°C the enzyme activity was nearly zero at 4 hr (Fig. 31).

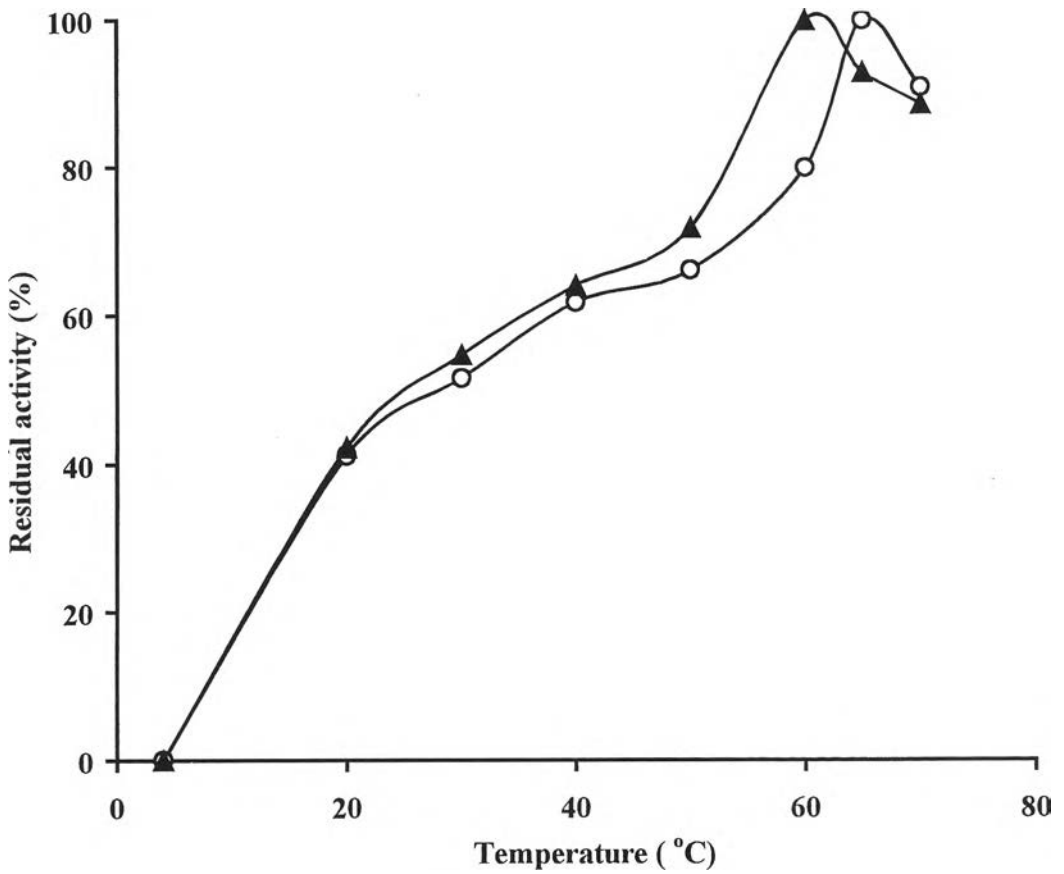


Fig. 30 Optimum temperature of native and partial deglycosylated cassava peroxidase

Activity of purified enzyme was determined at various temperatures by using DAB as substrate in phosphate buffer pH 6.8. The percentage of relative activity was calculated by using maximum activity of enzyme as 100%

Native peroxidase 3% TFMS-treated peroxidase

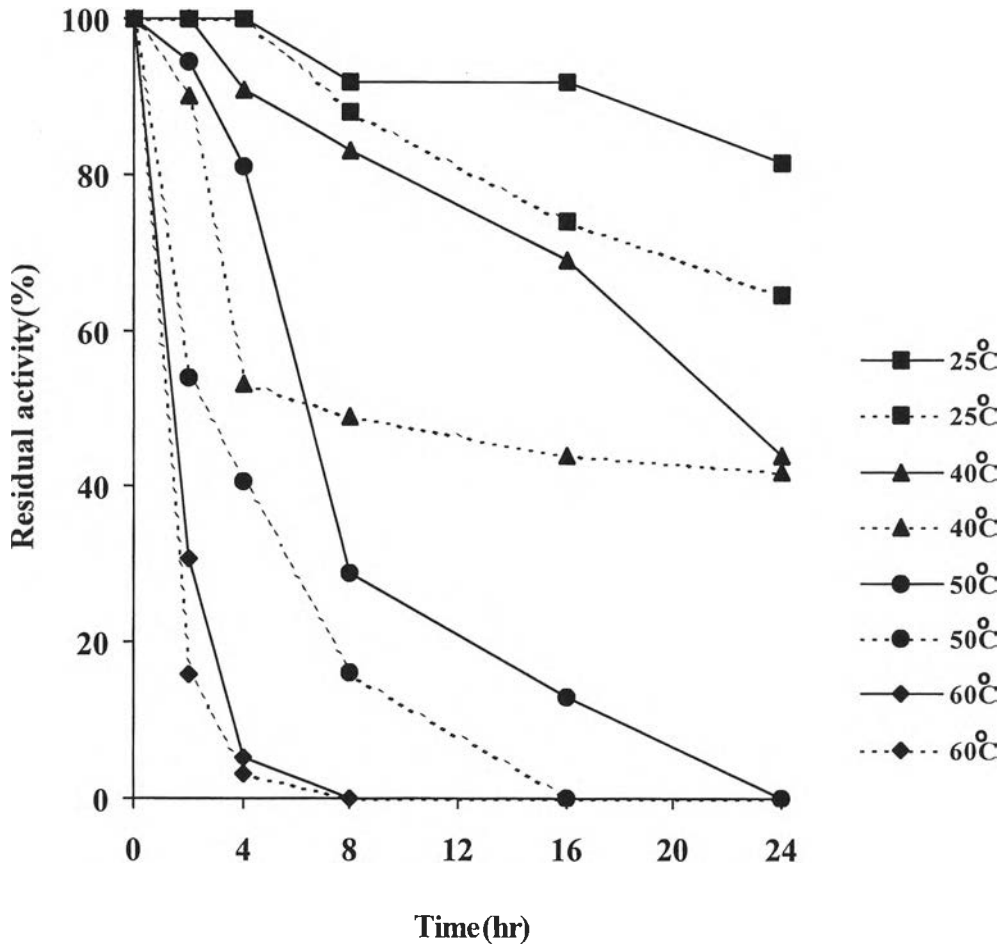


Fig. 31 Temperature stability of native and partial deglycosylated cassava peroxidase

The purified enzyme was incubated in various temperatures for 0-24 hr, then the enzyme activity was assayed.

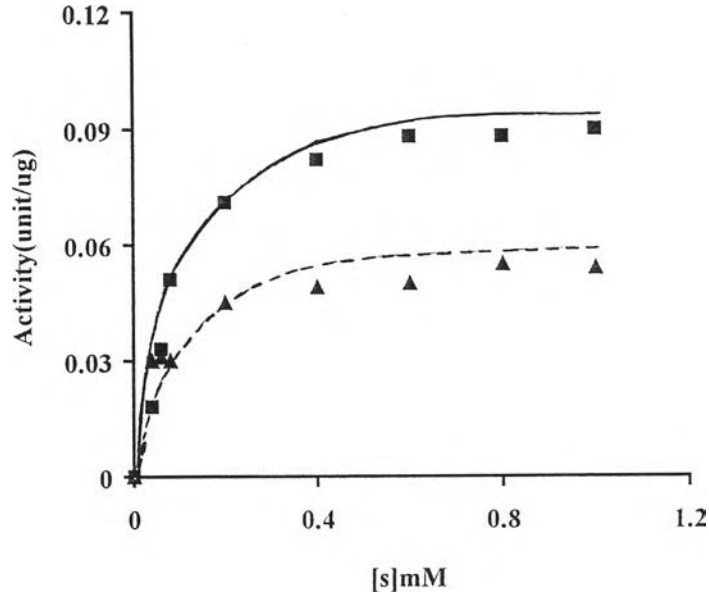
——Native peroxidase ····· 3% TFMS-treated peroxidase

3.4.9 Comparisons of kinetic constants

Varied concentrations of several known substrates for peroxidases namely H_2O_2, DAB, guaiacol, *o*-dianisidine, pyrogallol and syringaldazine were incubated with both native and deglycosylated purified cassava peroxidase as described in section 2.19. The data were plotted and kinetic constants determined using Enzfitter software. The results were shown in Figs. 32- 37. The calculated K_m and V_{max} for all substrates studied were summarized in Table 4.

The enzyme can also use coniferyl alcohol as its substrate but the saturation points and K_m cannot be determined (Fig. 38) due to interference of product measurement at substrate concentration higher than 7 μM as shown in the absorption spectrum (Fig. 39).

Ascorbate, quercetin were also tested, but they can not be utilized by cassava peroxidase



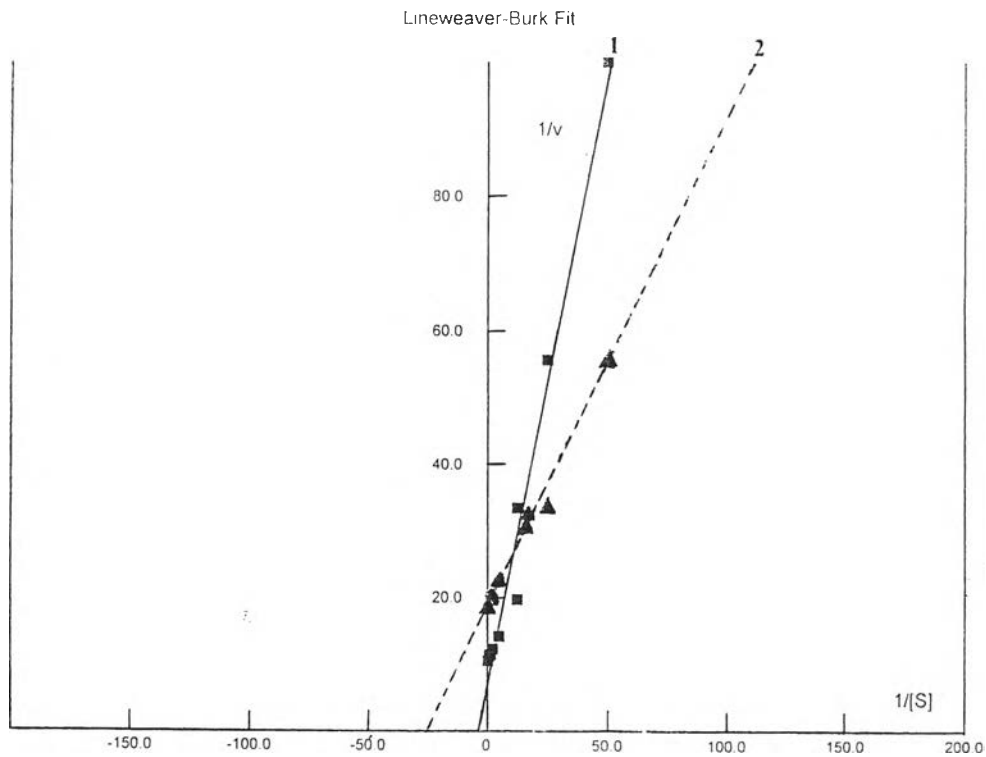
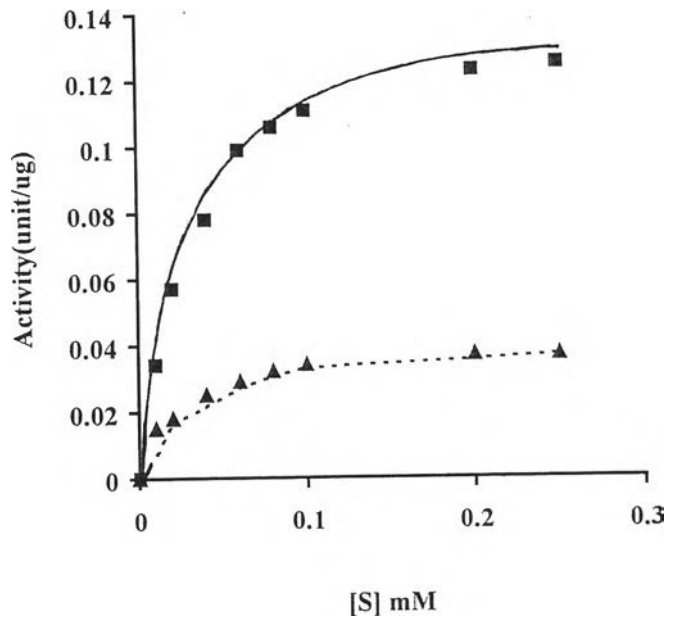


Fig. 32 H_2O_2 saturation curve of peroxidase (A) and its Lineweaver-Burk Plot (B)

1 The correlation coefficient = 0.98 — Native peroxidase

2 The correlation coefficient = 0.98 --- 3% TFMS-treated peroxidase



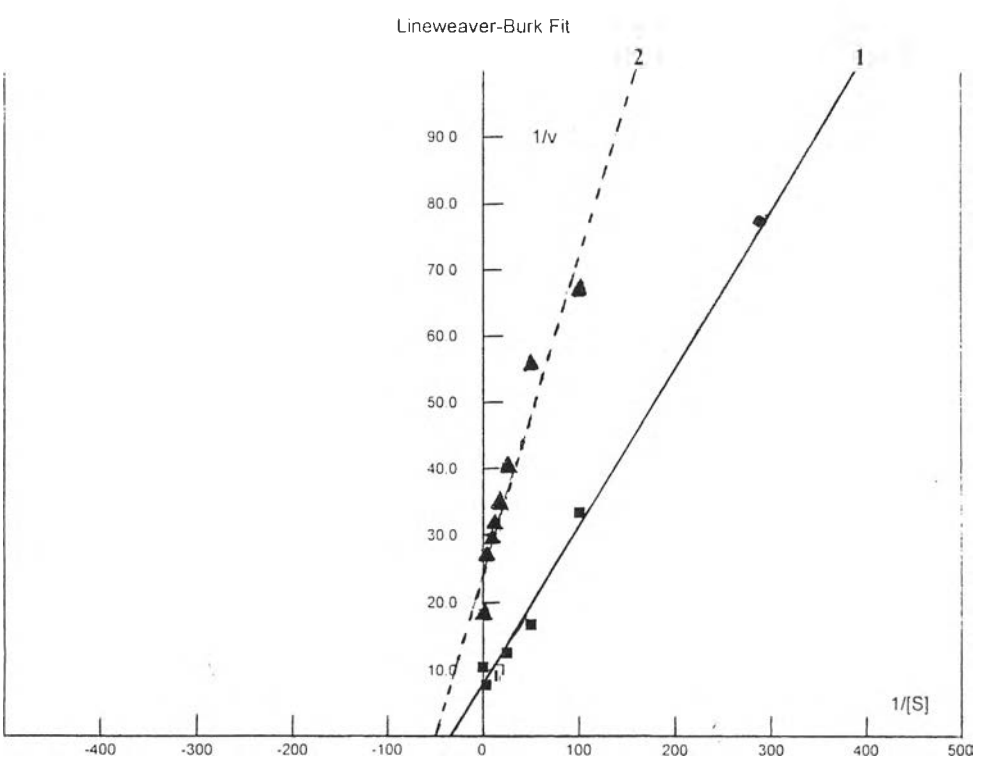
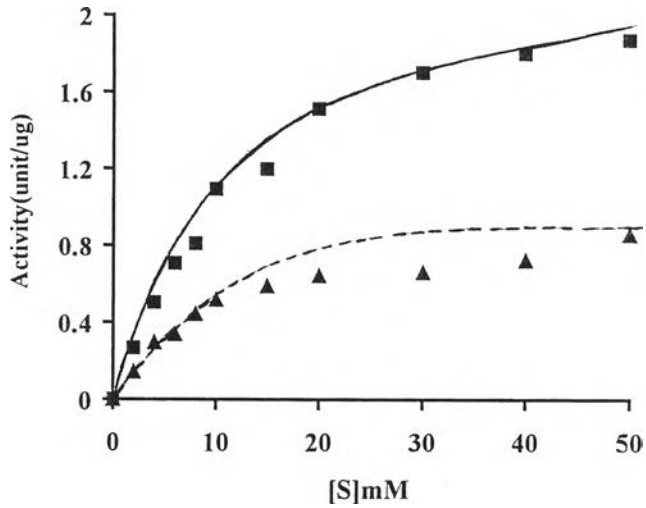


Fig. 33 DAB saturation curve of peroxidase (A) and its Lineweaver-Burk Plot (B)

1 The correlation coefficient = 0.98 ■ Native peroxidase

2 The correlation coefficient = 0.98 ▲ 3% TFMS-treated peroxidase



B

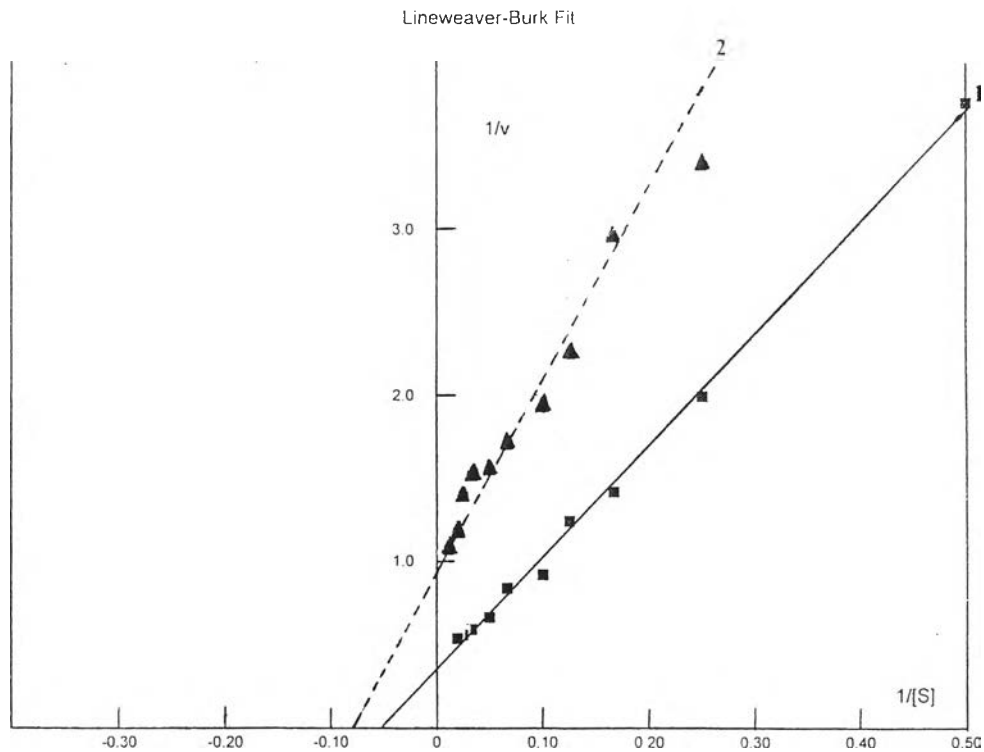
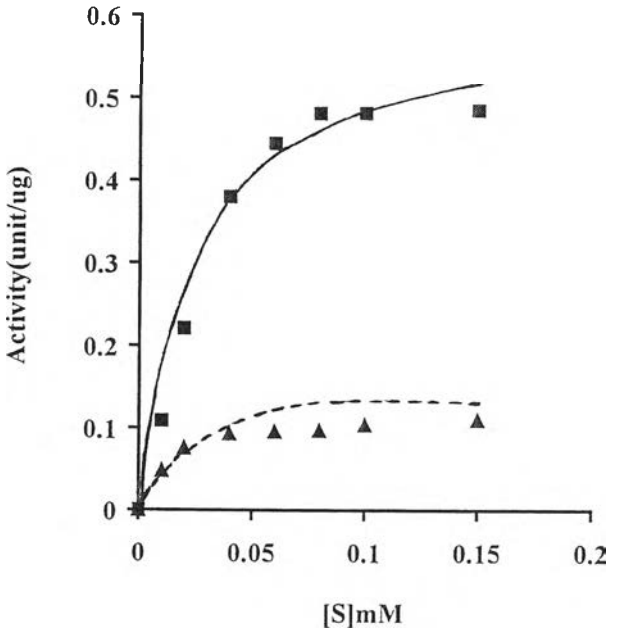


Fig. 34 Guaiacol saturation curve of peroxidase (A) and its Lineweaver-Burk Plot (B)

1 The correlation coefficient = 0.97 —■— Native peroxidase

2 The correlation coefficient = 0.97 ···▲··· 3% TFMS-treated peroxidase



A

B

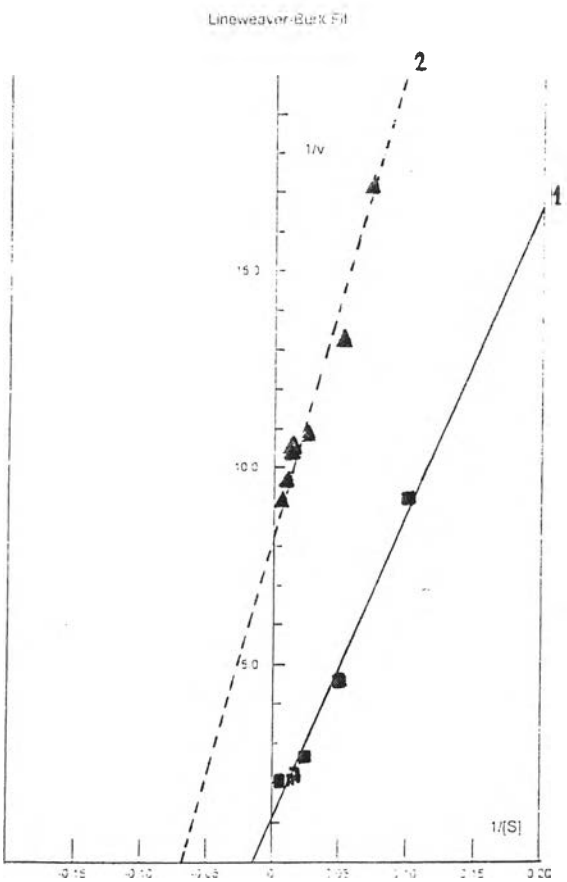
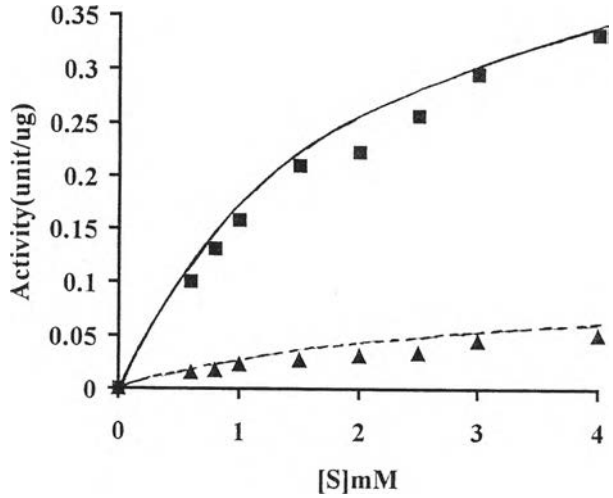


Fig. 35 *o*-Dianisidine saturation curve of peroxidase (A) and its Lineweaver-Burk Plot (B)

1 The correlation coefficient = 0.98 —■— Native peroxidase

2 The correlation coefficient = 0.98 ···▲··· 3% TFMS-treated peroxidase



A

B

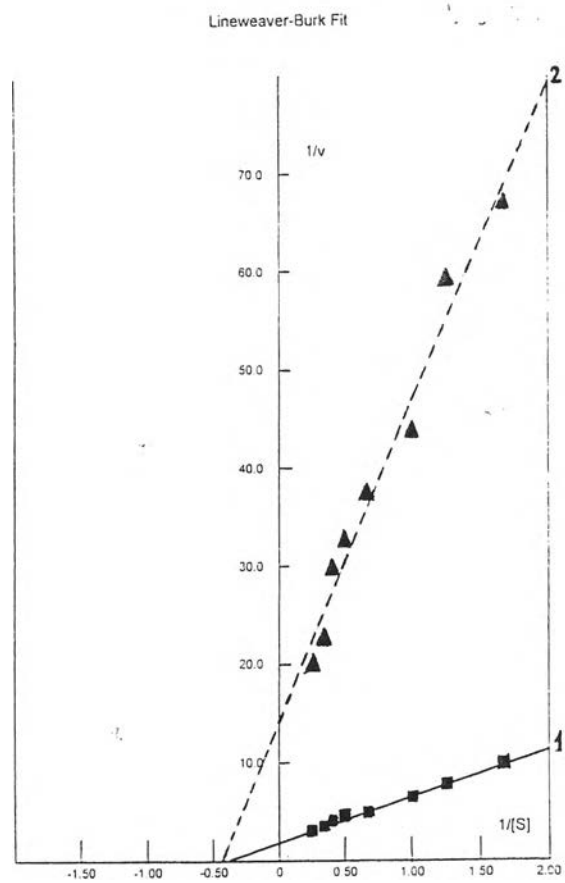
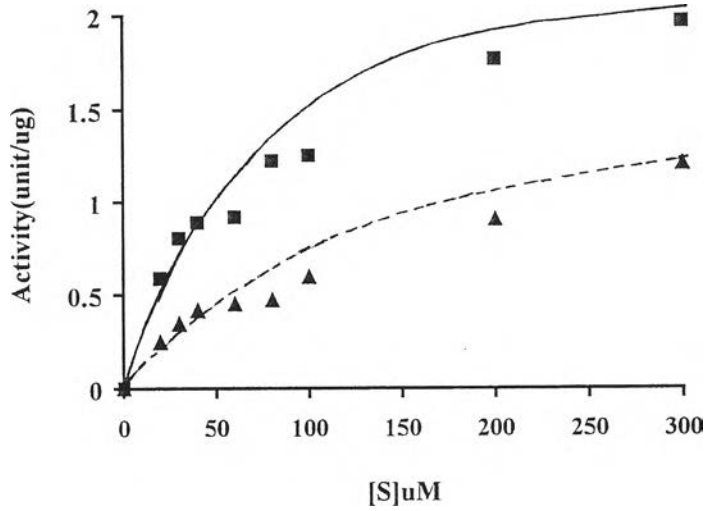


Fig. 36 Pyrogallol saturation curve of peroxidase (A) and its Lineweaver-Burk Plot (B)

1 The correlation coefficient = 1.00 ■ Native peroxidase

2 The correlation coefficient = 0.99 ▲ 3% TFMS-treated peroxidase



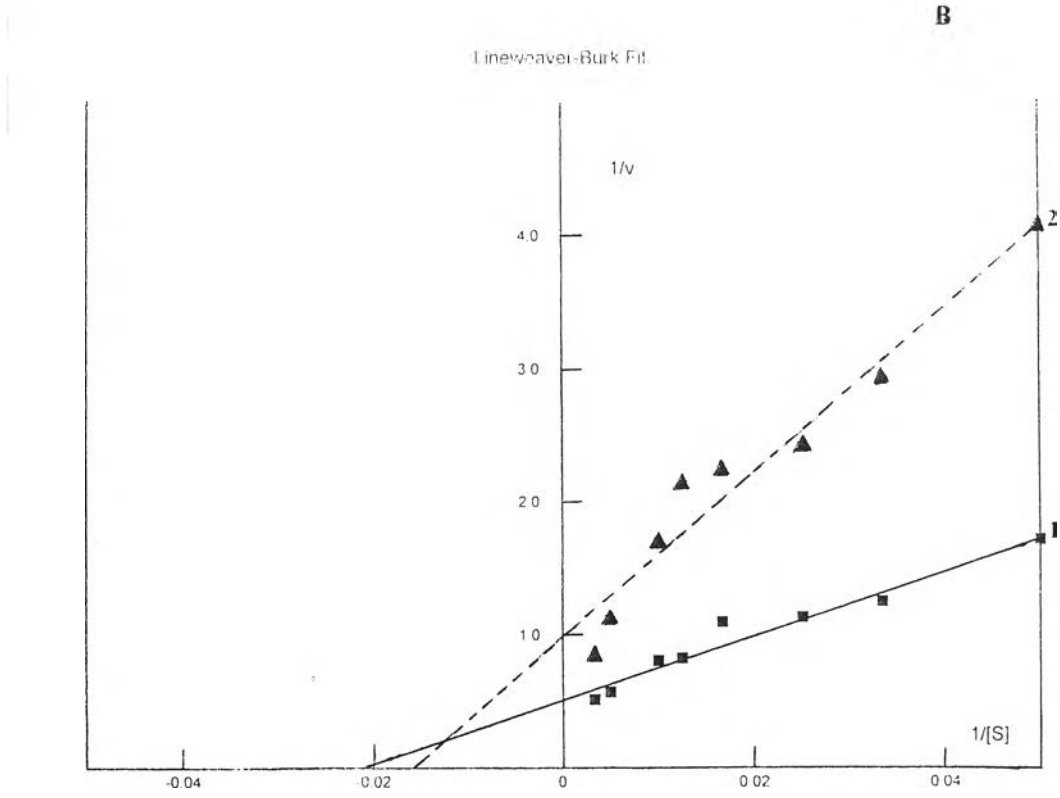


Fig. 37 Syringaldazine saturation curve of peroxidase (A) and its Lineweaver-Burk Plot (B)

1 The correlation coefficient = 0.98 — Native peroxidase

2 The correlation coefficient = 0.97 ···▲··· 3% TFMS-treated peroxidase

Table 4 K_m and V_{max} of cassava tuber peroxidase for various substrates

Substrate	V_{max} (U/μg)		K_m (mM)	
	Native (0%)	Deglycosylate (3%)	Native (0%)	Deglycosylate (3%)
H_2O_2	0.15	0.05	0.28	0.04
DAB	0.13	0.04	0.03	0.02
Guaiacol	2.56	1.08	19.12	12.64
o-Dianisidine	0.92	0.12	0.07	0.02
Pyrogallol	0.55	0.07	2.63	1.17
Syringaldazine	1.95	1.05	0.05	0.07

U = change of one absorbance unit at 465 nm per min

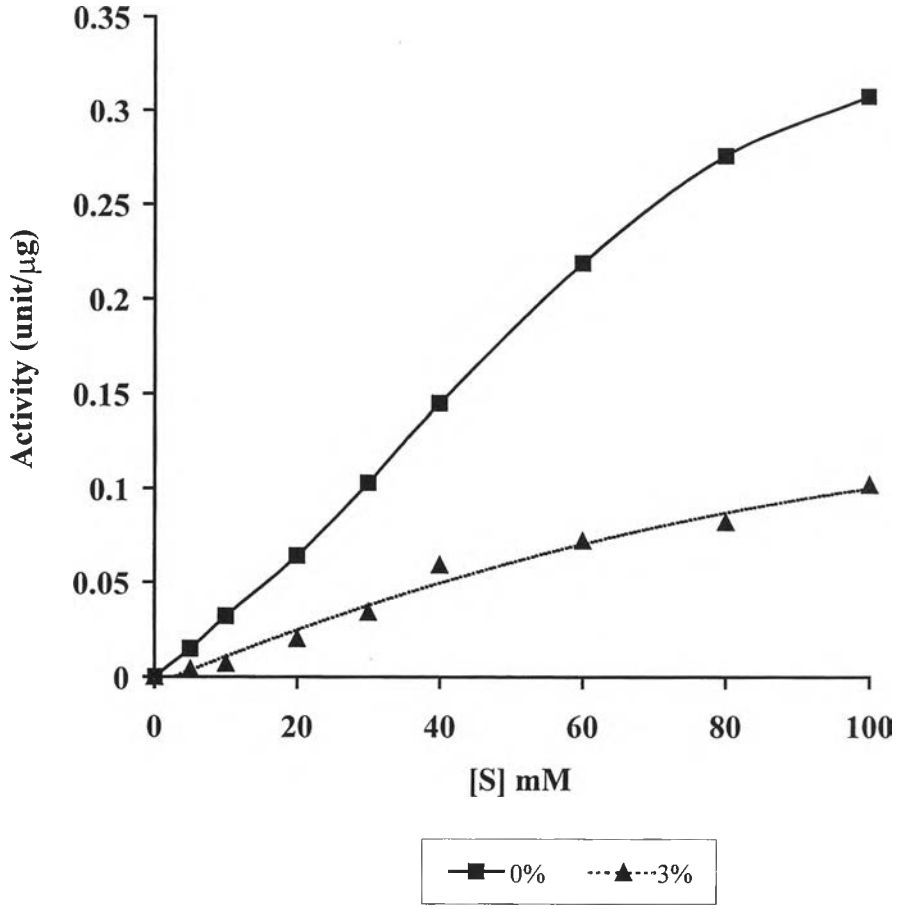


Fig. 38 Coniferyl alcohol saturation curve of cassava peroxidase

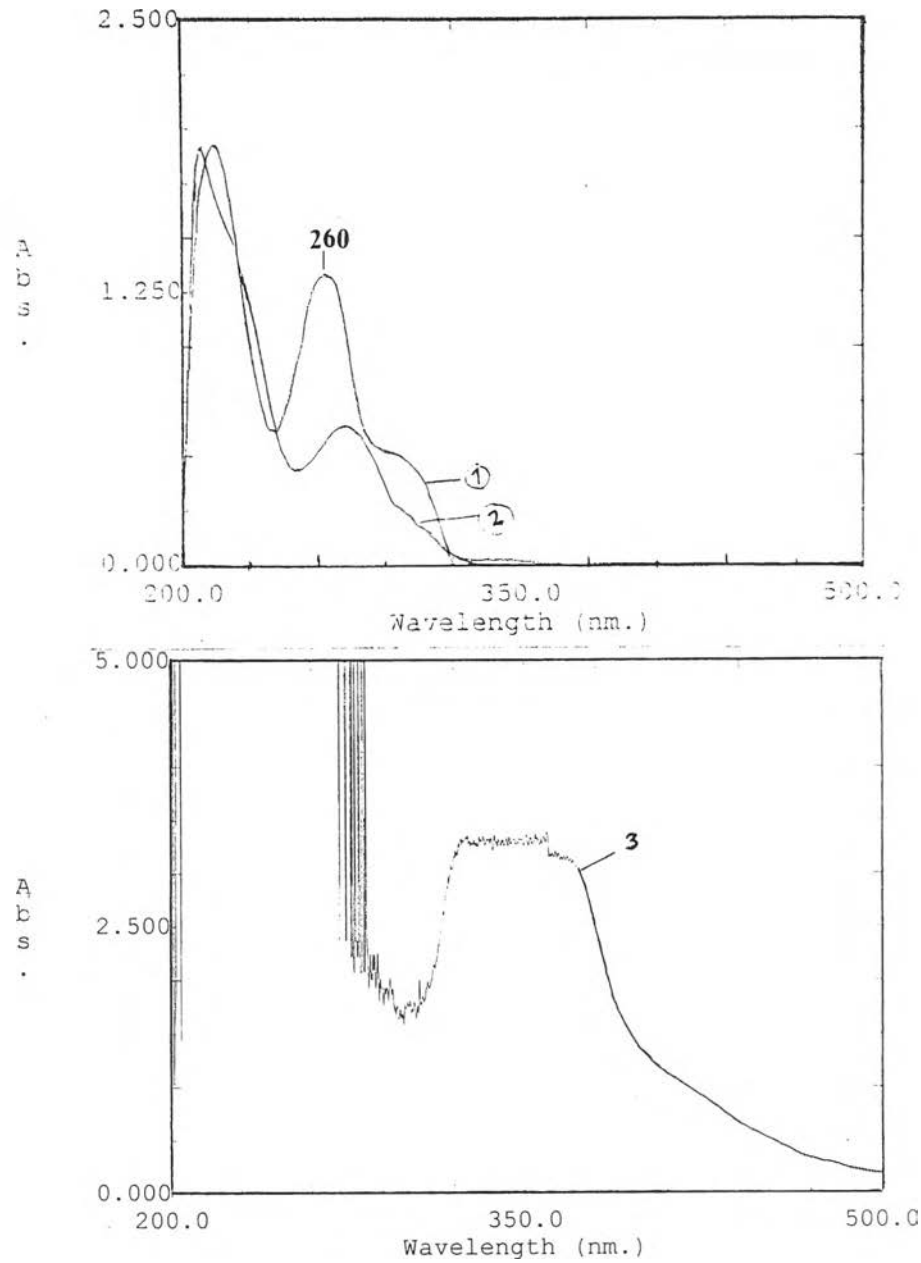


Fig. 39 The absorpton spectrum in coniferyl alcohol

1 = the absorption spectrum of coniferyl alcohol

2 = the absorption spectrum of reaction mixtured

(peroxidase 0.5 μg in 3 ml + 1 mM H_2O_2 + coniferyl alcohol

concentration of coniferyl alcohol = 0.007 mM

3 = the absorption spectrum of coniferyl alcohol at high concentration,

100 mM