CHAPTER IV

DISCUSSION

Cassava in one of the most economically important crops which is produced in large scale and most of it is exported from Thailand. One of the major problem of cassava trade is the rapid physiological deterioration of post-harvested cassava root. One of the observed changes associated with the deterioration is an increase activity of a peroxidase isozyme called isozyme form A in cassava tuber after cassava tuber was stored for one week. This work aimed at the characterization of this peroxidase isozyme in cassava parenchyma.

4.1 Purification of cassava peroxidase from cassava tubers

Peroxidases are widely distributed in plants. To characterize the peroxidase isozyme A in the post-harvested cassava root, it was necessary to purify it. The purification procedure used in our experiments were based on previous methods on purification of peroxidases from other sources as summarized below.

Patcharakorn Rattanapume et al. (54), purified peroxidase from *Hevea brasiliensis* leaves by using ammonium sulfate precipitation followed by DEAE Sephacel and Sephadex G-75 chromatography. The enzyme was puified to about 115 folds. Maria Rosaria Elia (17) also purified the enzyme from potato tubers using ammonium sulfate precipitation followed by DEAE Sephacel and hydroxyhapatite column chromatography. The purification was about 130 folds. Gazaryan et al. (55) purified a peroxidase from tobacco leaf with DEAE cellulose and gel filtration column chromatographies. They obtained 10 folds purification with specific activity of 4600 U/mg. Suriyaprom (56) obtained peroxidase from cassava leaves by

ammonium sulfate precipitation, followed by chromatographies on Con-A Sepharose and Sephadex G-200. The enzyme was purified about 74 folds.

In the present study, ammonium sulfate was used as the first step to purify the enzyme. However, other compounds, particularly plant phenolic compounds, may be trapped in the precipitation as well. Therefore, PVPP was included in the extraction buffer to remove the phenolic compounds. PMSF was also added to eliminate protease action. An affinity column of Con-A Sepharose was used in the next step in which the peroxidase were separated into 2 fractions: an unbound peak and bound peak (Fig. 8). The unbound peak contained rather low peroxidase specific activity for further characterization. Therefore, concentrated on the bound fraction. The binding of the cassava peroxidase to the Con-A Sepharose column suggested that the enzyme was probably a mannose-bearing glycoprotein (43). The unbound fraction was reloaded on the Con-A column and it still did not bind to the column, suggesting that it was not a result of overloading of the column. The unbound peroxidase was likely to be another isozyme with no mannose-containing carbohydrates in the enzyme. since the cassava leaf peroxidase can be purified to homogeneity by gel filtration (56) and our enzyme preparation contained very little protein, it was decided to short cut ion exchange column and employed gel filtration as the next and final step of purification. From the purification (Table 2), the ammonium sulfate step can remove half of the proteins while total peroxidase activity was retained. In the Con-A Sepharose column, the peroxidase activity dropped significantly to one eighth of that obtained from previous step. Part of the decrease in peroxidase activity may be that eluted in the unbound fraction and uncollected activity less than 0.05 U in bound fraction. However, a higher proportion of proteins were also eliminated. Sephadex G-200, further eliminated 50% of the protein, retaining about 60% of the enzyme

activity. Some of the bound fractions eluted from ConA Sepharose were other glycoproteins which were not peroxidases as shown in the PAS stain (Fig. 18, lane B). SDS gel electrophoresis showed that the 50% loss of protein in the gel filtration eliminated many contaminating proteins which were not the peroxidase (4,5, Fig. 7). Sephadex G-200 yielded a fraction which showed up as single band on SDS-PAGE. The purified protein did not show up well on protein staining of non-SDS PAGE but appeared on peroxidase activity stained as one major band and at least 3 minor bands which could be other isozymes. Since, the peroxidase enzyme assay or the activity stain were quite sensitive and readily detectable, they were mostly employed to follow the peroxidase. In some techniques such as ND-PAGE, PAS staining and IEF gel proteins in the purified fractions were barely detectable or not detectable at all (Figs 12, 16). Loading excess amount of proteins resulted in overloading of activity stain with little improvement on protein stain.

4.2 Characterization of purified cassava tuber peroxidase

4.2.1 Structural properties

The molecular weight of the purified enzyme determined from the calibration curve of standard proteins on Sephadex G-200 was estimated at 105 Kd (Fig. 14) However, its molecular weight determined on SDS-PAGE was found to be 54 kD (Fig. 5). The result suggested the root peroxidase existed as dimer. This result was similar to the reported finding of cassava leaf peroxidase with native molecular weight of 112 kD and subunit molecular weight of 56 kD (56), but different from other plant peroxidases. Horseradish peroxidase was reported to have a native molecular weight of 42 kD (57), para rubber (*Hevea brasiliensis*) bark peroxidase was 50 kD (27) and *Araucaria araucana* seeds peroxidase existed in as

two cationic forms with molecular weights of 83 kD and 145 kD (58). From the accumulated data on molecular weight, most plant peroxidases have the molecular weight in the range of 40-56 kD.

Isoelectricfocusing gel of the cassava root peroxidase revealed 5 bands with pI's 5.1, 5.2, 5.4, 5.8 and 6.0 (Fig. 16), with major forms at pI 5.4. They were in the same pI range as the peroxidase from cassava leaf (pI's 6.25 and 6.4 (56)) and those of Barley leaf (pI's 6.3 and 6.8 (27)). There were several other plant peroxidases reported with very acidic pI's : *Heavea* burk (pI 3.5, (27)), *Arabidopsis thaliana* (pI 3.5, (72)), Poplar xylem (pI 3.1-3.8, (61)). Horseradish peroxidase were reported to have basidic pI around 10-12 (62) and carrot peroxidase also had pI greater than 9.3 (63).

It has been known that reactions catalysed by peroxidases take place at the heme prosthetic group. Removal of heme causes total loss of peroxidase activity (64). The purified cassava root peroxidase showed absorption at 398 nm which was within the absorption range of heme. Other plant peroxidases were reported to show absorption around 402-405 nm (60).

Table 5 summarized the comparative structural properties of cassava tuber peroxidase and other plant peroxidases.

There were reports on the glycoprotein nature of several peroxidase (5,6). Deglycosylation of peanut peroxidase showed the change in electrophoretic mobility of peanut peroxidase (69). Preliminary report on cassava tuber peroxidase showed that deteriorating tubers showed changes in peroxidase patterns which differed in electrophoretic mobility on ND-PAGE, it was interesting to see if glycosylation played any role in the observed changes. The binding of the cassava tuber peroxidase to the Con-A Sepharose column indicated its glycoprotein nature.

Table 5 Peroxidases in various plant tissues

Source	No. of Isozyme (major)	Molecular Weight	pI
Horseradish (19)	3	40 kD**	10-12
Cassava parenchyma (This work)	3	105 kD* 54 kD**	5-6
Cassava leaf (56)	>2	112 kD* 56 kD**	6.3, 6.4
Japanese root radish (71)	ND	28 kD*	ND
Potato tuber (17)	ND	30 kD**	ND
Pea leaves (21)	ND	49 kD**	ND
Hevea bark (27)	ND	50 kD*	3.5
Araucaria seeds (58)	2	80 kD*, 145 kD*	ND
Carrot root (63)	ND	ND	>9.3
Poplar xylem (61)	6	46-54 kD***	3.1-3.8

Reference sources as indicated in ( ).

*determined by Sephadex G-200

**determined by SDS-polyacrylamide gel electrophoresis

***determined by Mono-Q Sepharose

ND = no data

Determination of carbohydrate content of the purified tuber enzyme showed that the enzyme contained high amount of carbohydrate up to 300% w/w (Figs 18 and 19). Therefore, it was interesting to study the significance of the high content of carbohydrate by comparative characterization of the native and deglycosylated forms.

4.2.2 Effect of pH and temperature

The purified peroxidase was used to study the effect of pH and temperature on its activity. Experiments were performed to test both the pH and temperture which the enzyme had maximum activity and the pH and temperature at which the enzyme remained stable for a long period.

The experiment on optimum pH (Fig. 28) showed that the cassava tuber peroxidase was active at slightly acidic pH (pH 5). This value was similar to the peroxidase of those reported in the leaves of other plants: *Hevea brasiliensis* (pH 5.4) (65), petunia leaf peroxidase (pH 5) (60), tea leaf peroxidase (4.5-5.0) (36). Horseradish peroxidase also had acidic optimum pH 4.3 (62) and *Hevea* bark enzyme has a broad pH optimum in the range 5-7 (27). The cassava tuber peroxidase was stable in a broad pH range from 5-11 (Fig. 29). At least 60% of the activity remained when incubated in pH 5-11 for 24 hr while 100% activity retained at pH 7. In the temperature range of 4-80°C, the purified peroxidase from tubers was most active at 60°C (Fig. 30), similar to peroxidase from cassava leaves which had an optimum temperature of 60°C (56). The peroxidase from *Hevea* leaves had an optimum temperature in the range of 60-70°C (54). In *Gracilaria* species, the optimum temperature of its peroxidase was absolute at 60°C (68). The activity of the cassava peroxidase from tubers was rather stable at high temperature (Fig. 31). The enzyme retained about 80% of its activity during incubation at temperature up to

50°C for 4 hr but can be kept longer than 24 hr at 25°C with 85% activity remaining. By comparison, cassava leaf peroxidase was stable up to 65°C with 70% activity for 24 hr (56). *Hevea* bark peroxidase was stable up to 60°C with 100% activity while 75% of the activity still remained at 70°C when incubated for 24 hr (27). Fox-tail millet A3p peroxidase was also stable up to 65°C at pH 7.4 (66) and lignin peroxidase from Phanerochaete chrysosporium was relatively stable for 6 hr at 60°C (67). It seemed that the cassava peroxidase from tubers should operate well in the extreme conditions (high pH and high temperature) in which most other enzymes may be inactive. Both the heat and pH stabilities of the enzyme should widen the range of the peroxidase applications. These characteristics were also observed in other plant peroxidases as mentioned before, indicating that they may be the special properties of most plant peroxidases.

4.2.3 Kinetic study of cassava tuber peroxidase

Several compounds were tested as substrate of the purified peroxidase. The enzyme had Km for H_2O_2 of 0.28 mM, higher than exhibited by cassava leaf peroxidase but lower than root of cassava plantlet (0.33 mM, (71)). The Km for H_2O_2 reported in other plants were: 0.26 mM for *Hevea brasillensis* bark, 1.1 mM for *Hevea* leaf, 0.03 mM for potato ascorbate peroxidase. Its K_m for DAB (0.03 mM) was lower than cassava leaf (0.09 mM) and *Hevea* bark (1.67 mM) but higher than root of cassava plantlet (0.07 μM).

The anionic peroxidase associated with the suberization response in potato tuber showed the following order of substrate preference: feruloyl (o-methoxyphenol) > caffeoyl > p-coumaryl ≈ syringyl. These compounds accumulated in tubers during wound healing (70). This enzyme preferred guaiacol over ascorbate

as substrate. The peroxidase from Poplar xylem existed as isoenzymes PXP_1, PXP_2, PXP_3, PXP_4, PXP_5. They can oxidize ABTS and DAB where as only PXP_3, PXP_4, PXP_5 can oxidize the lignin monomer analog, syringaldazine. PXP_{3-4} and PXP_5 were suggested to be involved in lignin polymerization (61). In *Hevea brasiliensis*, the peroxidase is more specific for o-dianisidine than ABTS and pyrogallol and the K_m values for *o*-dianisidine was 0.12 mM (54).

Therefore, the cassava tuber peroxidase was a classical peroxidase because it preferentially oxidized organic phenolic compounds and can also used guaiacol a substrate. It contained heme group and carbohydrate. (Partial deglycosylated peroxidase from peach seeds was decrease in activity and stability of peroxidase therefore the glycan portion is important for activity and stability of peroxidase isozyme).

4.3 Characterization of deglycosylated peroxidase

4.3.1 Deglycosylation of purified cassava tuber peroxidase.

There were several reports on glycoprotein nature of some peroxidases and deglycosylation led to many changes. Since our finding showed that the cassava tuber peroxidase contained high amount of carbohydrate, it was interesting to study the changes in its deglycosylated form. Attempts were made to deglycosylate the enzyme using carbohydrate hydrolysing enzymes such as endoglycosidase N-glyconase, endo M and endo F without success. Tams and Welinder (50) reported failure in deglycosylating HRP isozyme C by endoglycosidase but successful when they used chemical such as TFMS. We tried deglycosylaion with TFMS and found that 3% and 6% TFMS can remove 50% and 90% of the carbohydrate (Fig. 22).

However, using 6% TFMS caused nearly complete loss of activity whereas 3% TFMS decreased the activity to 50%.

4.3.2 Comparative properties of native and partial deglycosylated cassava tuber peroxidase

Comparisons of the structural and kinetic properties of the deglycosylated enzyme with the native enzyme showed that mobility of the deglycosylated on ND-PAGE did not show distinctive changes using the partial deglycosylated form, but the isozyme patterns on IEF gel showed observable change. Deglycosylated enzyme had lost activity of pI's 5.1, 5.2, 5.8, 6.0 and new band appeared at pI 5.3. The different isozymes may contain different amount of carbohydrate contents. Deglycosylation may change the pI of the pI 5.1, 5.2, 5.4, 5.8, 6.0 which either move to the same positions as existing bands or became the newly detected band at pI 5.3. Unfortunately, the preparation of highly deglycosylated enzyme contained too little activity was not suitable for comparative study. If the preparation of the highly deglycosylated form can be improved to a level detectable by activity staining or protein staining, it may provide a clear explanation for the changes in pI pattern and may also yield a distinct change in electrophoretic mobility on ND-PAGE.

It was speculated that there may be some correlation of peroxidase B which predominated in fresh cassava tuber but disappeared in 7 days post-harvested tuber while form A gradually increased. ND-PAGE of the native enzyme compared with partial deglycosylated and highly deglycosylated from (Fig. 24) showed that removing carbohydrate part did not change its electrophoretic mobility significantly. Therefore, this peroxidase form A was unlikely to be the glycosylated form of form B. However, it may be an aggregated form of peroxidase B or a newly synthesized

enzyme. Peroxidase activity in cassava tuber gradually increased from day 1 to day 7 post-harvested, after which the root deteriorated to the condition that it cannot be used to study further. Monitoring some characteristics of the peroxidases more frequently during day 1 to day 7 may provide some useful information on the role of the glycosylated part on the activity of the enzyme. Comparative study of form A and form B peroxidases would also provide knowledge into the observed changes in the activity of form A and B during storage.

The deglycosylated peroxidase showed a decrease in temperature stability especially at 40°C or higher. pH stability profile showed a shift to the more acitic side for deglycosylated form. Carbohydrate portion in cassava tuber peroxidase may play some role in the folding or packaging of the enzyme. Removing some of the carbohydrate may result in higher susceptibility to pH and temperature changes. Partial deglycosylated avocado peroxidase and peach peroxidase were also reported to show a similar decrease in thermostability (41,42). The optimum pH and temperature profiles of the deglycosylated enzyme did not change significantly from that observed in native form suggesting the carbohydrate did not significantly affect the catalytic reaction of the enzyme.

The partial deglycosylated cassava tuber peroxidase seemed to show a significant decrease in Km for H_2O_2 in comparison to the native form, is similar to the report on deglycosylated peach peroxidase (41). From the series of substrates tested (Table 6), the deglycosylated enzyme appeared to gain affinity for all the substrates except syringaldazine to a different degree. The most significant changes in Km's were observed for H_2O_2, guaicol and pyrogallol. Therefore, it seemed that carbohydrate part may have some role in enhancing the binding of substrates to the enzyme. However, the decrease in V_{max} with all substrates seemed

Table 6 The K_m of plant peroxidase for various substrates

Substrates	K_m (mM)					
	Cassava parenchyma (This work)	Cassava leaves (56)	Cassava plantlet (71)	*Hevea* bark (270	*Hevea* leaf (54)	Potato tuber (17)
H_2O_2	0.28	0.09	0.33	0.26	1.1	0.03
DAB	0.03	0.09	$7x10^{-5}$	ND	ND	ND
Guaiacol	19.12	5.52	ND	ND	ND	ND
o-Dianisidine	0.07	0.04	ND	ND	ND	ND
Pyrogallol	2.63	0.89	ND	ND	ND	ND
Syringaldazine	0.05	0.08	ND	ND	ND	ND
Quercetin	-	0.05	ND	ND	ND	ND
Ascorbate	-	-	ND	ND	ND	ND

Reference source as indicated in ( )

- = not utilized

ND = no data

to point to a decrease rate of product formation catalyzed by the deglycosylated enzyme. The removal of carbohydrate moiety may enhance the binding of substrate to the active site (decrease K_m). It has been reported that heme group was involved in the reaction of most peroxidases and the cassava tuber peroxidase was shown to contain heme group which decreased upon deglycosylation.

The decrease in V_{max}, therefore, may occur from the lost of heme groups. The substrate binding site may involve the heme group. Lost of heme groups observed in the deglycosylated enzyme may be caused by either the effect of the TFMS or the removal of carbohydrate. From this study, it could only be concluded that peroxidase form A in cassava tuber parenchyma was highly glycosylated and its activity increased with post-harvested storage time. Deglycosylation of the enzyme seemed to increase affinity for substrates but decrease in rate of catalysis. It has to be further investigated that the observed changes in deglycosylated enzyme was totally the effect of the carbohydrate part removed or there was also the effect of treatment with TFMS.