Role of carbohydrate moieties in peanut (*Arachis hypogaea*) peroxidases

Chunfang HU and Robert B. VAN HUUSTEE*

Department of Plant Sciences, University of Western Ontario, London, Ontario, Canada N6A 5B7

The activities of a cationic (C.PRX) and an anionic peroxidase isolated from peanut (*Arachis hypogaea*)-cell suspension culture were drastically reduced when they were deglycosylated with glycopeptidase F or oxidized by 10 mM-periodate. In contrast with the controls, the deglycosylated or the oxidized peroxidases were much more susceptible to proteolytic degradation. In radiolabelling experiments with [³⁵S]methionine, the non-glycosylated C.PRX was synthesized in the tunicamycin-treated cultures and secreted into the medium. Examination of the C.PRX polypeptides by SDS/polyacrylamide-gel electrophoresis followed by fluorography showed that the non-glycosylated form had an M_r of approx. 31 000, which is about 78 % of that of the glycosylated form. Our results suggest that carbohydrates may not be essential for peroxidase secretion, but that stabilization of the peroxidase molecules and acquisition by these isoenzymes of a catalytically active conformation is linked directly or indirectly to glycosylation.

INTRODUCTION

Plant peroxidases (EC 1.11.1.7) are glycoproteins that contain *N*-linked oligosaccharide chains, as shown for horseradish (*Armoracia lapathifolia*) peroxidase C (HRP C), turnip (*Brassica napus*) peroxidase (Welinder, 1985) and peanut (*Arachis hypogaea*) peroxidase (Ravi *et al.*, 1986). Many peroxidases occur in the cell wall or in the extracellular space (van Huystee, 1987). They are probably synthesized on rough endoplasmic reticulum, processed in the Golgi complex and then secreted in the same way as many other glycoproteins (van Huystee & Chibbar, 1987). Incorporation of [³H]mannose into the C.PRX was reduced by tunicamycin, an efficient inhibitor of *N*-glycosylation (Ravi *et al.*, 1986). In HRP C, eight oligosaccharide chains are exposed on the surface of the molecule (Welinder, 1985). The carbohydrate moieties of peanut peroxidase are readily recognized by antibodies raised against the native isoenzymes (Hu & van Huystee, 1988). However, relatively little is known about the biological significance of the oligosaccharide chains for the isoperoxidases. We therefore investigated the possible roles of these side chains in C.PRX and in the anionic peroxidase (A.PRX).

EXPERIMENTAL

Materials

Concanavalin A (Con A), PNGase F (EC 3.2.2.18), trypsin and tunicamycin were purchased from Boehringer Mannheim Biochemica, HRP from Sigma Chemical Co., [³⁵S]methionine (42.59 TBq·mol⁻¹) from New England Nuclear Corp., and IgGSorb from the Enzyme Center (Malden, MA, U.S.A.).

Cell culture and peroxidase purification

Peanut cells were maintained in suspension culture as described by Hu *et al.* (1987a). The C.PRX and the

A.PRX were isolated from the conditioned medium by ion-exchange chromatography (Chibbar & van Huystee, 1984) and polyclonal antibodies were raised to each (Cairns *et al.*, 1980), and monoclonal antibodies (McAb) to the C.PRX (Hu *et al.*, 1987b).

In experiments where the effects of tunicamycin were reversed, peanut cells were cultured with or without tunicamycin (1 µg/ml) added to the medium at day 0 of subculturing. After 6 days of culture, the culture medium was withdrawn. The cells were washed twice with normal medium not containing tunicamycin and allowed to grow in this medium for another 6 days. Peroxidase activity was monitored both before and after withdrawal of tunicamycin from the medium.

Periodate oxidation

Periodate treatment was conducted as detailed by Biroc & Etzler (1978). The purified peroxidases (1 mg/ml) were incubated in the dark at 4 °C with 10 mM-sodium metaperiodate in 50 mM-sodium acetate buffer, pH 4.5. The reaction was stopped at specific time intervals by adding ethylene glycol (EG) to a final concentration of 16 %. The mixture was dialysed against 50 mM-phosphate buffer, pH 7.0, at 4 °C before analysis for peroxidase activity or for binding ability to Con A. The same procedure was used for the controls, except that periodate was omitted or added after the addition of EG, or EG was added alone. In the case of peroxidase binding to Con A, the procedures described by Hawkes (1982) were adapted with minor modifications. Briefly, the peroxidases were resolved by SDS/PAGE and blotted to nitrocellulose membrane (Hu *et al.*, 1987b). The blots were incubated with blocking buffer [2 % (w/v) BSA in 20 mM-Tris/HCl (pH 7.4)/200 mM-NaCl (TBS)] for 30 min before probing with Con A (50 µg/ml) in the same buffer supplemented with 1 mM-CaCl₂ and 1 mM-

Abbreviations used: HRP C, horseradish peroxidase C; C.PRX, cationic peroxidase; A.PRX, anionic peroxidase; PNGase F, glycopeptidase F; TFMS, trifluoromethanesulphonic acid; McAb, monoclonal antibody; Rz, A_{405}/A_{280} ratio; EG, ethylene glycol; Con A, concanavalin A; PAGE, polyacrylamide-gel electrophoresis; TBS, Tris-buffered saline (composition and pH given in the text); BSA, bovine serum albumin.

* To whom correspondence and reprint requests should be addressed.

MnCl₂ for another 30 min. The blots were washed with TBS thrice, 15 min each and then incubated with HRP C (50 µg/ml) for 30 min. After washing with TBS, the HRP C ligated through Con A was revealed by incubating with *o*-dianisidine and H₂O₂ in TBS (Towbin *et al.*, 1979). The same procedure was used for the control, except that 0.1 M-mannose was included in 2% BSA/TBS for blocking.

Deglycosylation of peroxidases

The native C.PRX or A.PRX (1 mg/ml) was incubated with PNGase F at a concentration of 12 units/mg of protein in 0.1 M-sodium phosphate buffer, pH 8.0, at 37 °C for increasing periods by the method of Tarentino *et al.* (1985).

Radiolabelling and immunoprecipitation

Peanut cells at the exponential stage of growth were preincubated with or without tunicamycin (1 µg/ml) for 1 h, and then labelled with [³⁵S]methionine in the presence or absence of the same concentration of tunicamycin (Ravi *et al.*, 1986). At various time intervals, the cells were recovered, homogenized and extracted as described previously (van Huystee & Lobarzewski, 1982). The radioactivity incorporated into total proteins was measured after precipitation with cold 10% (w/v) TCA (Mans & Novelli, 1961). The C.PRX was precipitated from the medium or the cellular extracts with specific rabbit antiserum (Ivarie & Jones, 1979). After preclearing with rabbit pre-immune serum and protein A (IgGSorb), aliquots of 1 ml of medium or 0.5 ml of cellular extract were incubated at 4 °C with 100 µl of rabbit anti-C.PRX serum. After 1 h, 300 µl of IgGSorb was added to each sample and the mixture was kept at 4 °C for another 30 min before centrifugation. The pellets were washed thrice with TBSE [10 mM-Tris/HCl (pH 7.6)/0.15 M-NaCl/1 mM-EDTA] supplemented with 0.5% Nonidet P40. The washed pellets were dissolved in scintillation liquid for radioactivity measurement or resuspended in SDS sample buffer [62.5 mM-Tris/HCl (pH 6.8)/2% (w/v) SDS/5% (v/v) 2-mercaptoethanol/10% (v/v) glycerol]. In the latter case, 40 µl of sample buffer was added to each pellet, boiled and centrifuged for 3 min. The supernatant obtained was used for SDS/PAGE and fluorography.

PAGE, immunoblotting and fluorography

SDS/PAGE on 12% polyacrylamide gels, transferring and Western blotting of the separated proteins were done as described by Hu *et al.* (1987b). Fluorographs were prepared by the method of Bonner & Laskey (1974).

Other assays

Protein contents were measured as described by Lowry *et al.* (1951), with BSA as standard. Peroxidase activity assay was performed using guaiacol and H₂O₂ as substrates (van Huystee & Lobarzewski, 1982).

Trypsin digestion

The peroxidases pretreated with or without PNGase F or sodium periodate were hydrolysed with trypsin at 1% (w/w) final concentration in 0.1 M-sodium phosphate buffer, pH 8.0 for 30 min or 1 h (Allen, 1981). Tryptic digests were then subjected to SDS/PAGE and Western blotting.

Fig. 1. Effect of periodate oxidation on the binding of peanut peroxidases to Con A

The A.PRX (a) and the C.PRX (b) were incubated in the presence (+) or absence (–) of 10 mM-NaIO₄ for 1 or 18 h, followed by dialysis against 50 mM-sodium phosphate, pH 7.0. The peroxidases were probed with Con A after SDS/PAGE and transferring to nitrocellulose. (c) Contained control A.PRX (A) and C.PRX (B), and was probed with Con A after it had been incubated with 0.1 M-mannose in 2% BSA/TBS. Lane S contained protein standards. Note only the glycoprotein ovalbumin was stained in lane S.

RESULTS

Carbohydrate requirement for the expression of enzyme activity in peroxidases

The purified C.PRX and the A.PRX were first treated with periodate, which oxidizes *vic*-hydroxy groups of carbohydrates (Dyer, 1956). The activities of both isoenzymes were found to be greatly reduced upon incubation with 10 mM-sodium metaperiodate (Table 1). A rapid decrease in the enzyme activity occurred in the first 10 min, followed by a gradual reduction. The A.PRX appeared to be more sensitive to periodate oxidation, since its activity was lost more rapidly and more completely than that of C.PRX. In the meantime, a slight reduction of peroxidase activity was also observed in the controls with EG added before periodate. However, this reduction could be accounted for by incubating the peroxidase with EG alone (Table 1). The ability of the peroxidase isoenzymes to bind Con A were eliminated by treatment with periodate (Fig. 1). These results thus suggest that intact carbohydrate chains are probably required for peroxidase activity.

Confirmation of the need of glycosylation for expression of peroxidase activity was found in experiments where the enzymes were treated with PNGase F, which acts on the *N*-glycosidic linkage and generates a carbohydrate-free peptide and intact oligosaccharide chains (Tarentino *et al.*, 1985). Purified C.PRX or A.PRX peroxidase was hydrolysed by PNGase F under native conditions and peroxidase activity was assayed at various time intervals. Under these conditions, the *M_r* of the peroxidases were about 1000 lower than those of the controls (Fig. 2). However, peroxidase activities of the isoenzymes were both reduced. The peroxidase activity

Table 1. Effect of periodate oxidation on peroxidase activity

The C.PRX ($R_z = 2.5$) or the A.PRX ($R_z = 2.0$) in 50 mM-sodium acetate buffer, pH 4.5, at 1 mg/ml, was mixed with an equal volume of 20 mM- NaIO_4 (sodium metaperiodate) and incubated at 4 °C in the dark. The reaction was stopped at the times indicated by the addition of 16% EG, followed by dialysis against 50 mM-phosphate, pH 7.0. For the controls, the same procedure was performed, except that the peroxidases were incubated with sodium acetate buffer alone, with EG in the buffer, or with NaIO_4 added after the addition of EG. The values are means $\pm$ S.D. ($n = 3$).

Conditions	Peroxidase activity ($A_{470} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$)	
	C.PRX	A.PRX
Controls		
1. Buffer alone	593.3 $\pm$ 10.2	602.1 $\pm$ 41.1
2. Buffer + EG + 10 mM- NaIO_4		
0 min	591.9 $\pm$ 9.3	604.2 $\pm$ 51.2
10 min	521.6 $\pm$ 21.3	467.7 $\pm$ 42.8
1 h	492.6 $\pm$ 43.4	534.4 $\pm$ 36.8
2 h	466.9 $\pm$ 18.6	393.4 $\pm$ 26.2
4 h	486.5 $\pm$ 14.6	400.6 $\pm$ 20.4
20 h	415.4 $\pm$ 42.3	430.1 $\pm$ 19.4
3. Buffer + EG		
10 min	554.5 $\pm$ 35.9	553.1 $\pm$ 20.4
4 h	527.2 $\pm$ 58.7	416.3 $\pm$ 31.2
20 h	563.0 $\pm$ 17.8	453.4 $\pm$ 25.7
Treatment (10 mM- NaIO_4)		
0 min	521.3 $\pm$ 52.5	522.6 $\pm$ 5.8
10 min	320.1 $\pm$ 28.2	162.0 $\pm$ 5.7
1 h	281.5 $\pm$ 22.1	112.1 $\pm$ 0.7
2 h	238.7 $\pm$ 9.6	80.5 $\pm$ 7.8
4 h	200.9 $\pm$ 12.8	66.0 $\pm$ 5.2
20 h	163.0 $\pm$ 13.1	11.9 $\pm$ 3.2

of the A.PRX was reduced gradually and became nearly undetectable after overnight incubation with PNGase F at a concentration of 12 units/mg of protein (Table 2). Conversely, the C.PRX did not lose its activity as severely under the same conditions.

Stability of the isoperoxidases

Periodate-oxidized peroxidase demonstrated an increased susceptibility to attack by trypsin (Fig. 3). Whereas the oxidized peroxidases were hardly detectable on the immunoblots after 30 min incubation with trypsin,

the controls were clearly visible. Similarly, the partially deglycosylated peroxidases were much more susceptible to trypsin (Table 3). About 12% of the original peroxidase activity was detectable in each isoenzyme after 1 h and no activity remained by the end of the 2 h incubation with 1% trypsin at 37 °C. Conversely, the native peanut peroxidases retained about 80% activity after 2 h incubation. Again, the partially deglycosylated peroxidases were not retained on the gel after trypsin treatment when analysed by SDS/PAGE (results not shown). Therefore the carbohydrate appears to exert a

Table 2. Peroxidase activity after deglycosylation with PNGase F

Purified C.PRX ($R_z = 3.2$) or A.PRX ($R_z = 2.6$) was incubated with glycopeptidase F at a concentration of 12 units/mg of protein for the time indicated and then assayed for peroxidase activity. The values are means $\pm$ S.D. ($n = 3$).

	Period of incubation (h)	Peroxidase activity ($A_{470} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$)		
		Control	PNGase F-treated	As % of control
C.PRX	2	553 $\pm$ 43.0	271 $\pm$ 27.2	49.0
	4	602 $\pm$ 9.5	219 $\pm$ 19.1	36.4
	8	608 $\pm$ 4.0	229 $\pm$ 8.8	37.7
	20	610 $\pm$ 34.6	227 $\pm$ 38.4	37.2
A.PRX	2	595 $\pm$ 35.7	489 $\pm$ 1.4	82.2
	4	681 $\pm$ 11.1	354 $\pm$ 49.5	52.0
	8	657 $\pm$ 36.0	252 $\pm$ 11.0	38.4
	20	601 $\pm$ 52.0	4 $\pm$ 0.6	0.7

Fig. 2. SDS/PAGE of PNGase F-treated peroxidases

The C.PRX and the A.PRX were incubated with (+) or without (-) PNGase F for 18 h before they were resolved by SDS/PAGE and stained with Coomassie Blue.

Fig. 3. Trypsin treatment of peroxidases after periodate oxidation

The cationic (a) or the anionic (b) peroxidase oxidized with NaIO_4 for 0 (-) or 20 h (+) were digested for 0 or 0.5 h with trypsin, followed by SDS/PAGE and Western blotting with their respective antisera.

protective effect against proteolysis of peanut peroxidase isoenzymes, and this in turn increases the stability of the enzyme molecules.

Formation and distribution of the C.PRX in the presence of tunicamycin

The C.PRX is the major peroxidase isoenzyme released into the culture medium (Maldonado & van Huystee, 1980). A study of the effects of tunicamycin on the

Table 3. Trypsin treatment of deglycosylated peroxidases

The activity ($A_{470} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) of the control was 870 for the C.PRX ($R_z = 3.6$) and 1500 for the A.PRX ($R_z = 2.9$) at zero time; that for the treatment (incubation with PNGase F for 16 h) was 260 for the C.PRX and 455 for the A.PRX at zero time.

	Peroxidase activity (% of initial)	
	C.PRX	A.PRX
Control		
0 h	100	100
1 h	88	98
2 h	79	81
PNGase F		
0 h	100	100
1 h	12	13
2 h	0	0

biosynthesis and distribution of C.PRX may yield information on the role of carbohydrates in secretion of the peroxidase molecule. We reported that there was a remarkable decrease of peroxidase activity in the culture medium supplemented with tunicamycin ($5 \mu\text{g/ml}$) and a reduction of incorporation of radioactive precursors into the released C.PRX (Ravi *et al.*, 1986). It remained unclear, however, whether the decrease in C.PRX in the medium was due to a reduced secretion or to inhibition of biosynthesis of the protein moiety.

To determine the effect of tunicamycin on total protein and the C.PRX synthesis in peanut cells, cultures were preincubated for 1 h with or without the inhibitor in the normal medium and then labelled with [^{35}S]methionine

Fig. 4. Effect of tunicamycin on the incorporation of [^{35}S]methionine into trichloroacetic acid-insoluble proteins and immunoprecipitated C.PRX

After 1 h preincubation with (----) or without (—) tunicamycin ($1 \mu\text{g/ml}$), 30 ml portions of peanut-cell culture were incubated with 2.22 MBq of [^{35}S]methionine each. Cells were harvested at the times indicated and the cellular extracts were prepared. The radioactivity in trichloroacetic acid-precipitated cellular (a) or medium proteins (c) and in the C.PRX immunoprecipitated from cellular extracts (b) or media (d) was measured.

Fig. 5. Fluorograph of SDS/polyacrylamide gels and electrophoresis of immunoprecipitated C.PRX from tunicamycin (1 µg/ml)-treated (+) and control (-) peanut-cell cultures

The cells were incubated with [³⁵S]methionine for 3.5 h. The C.PRX immunoprecipitated from 2 ml of 64 medium (a) or 0.5 ml cellular extract (b) was reduced and analysed by SDS/PAGE and fluorography.

for another 3.5 h. A rapid increase in radioactivity incorporated into the trichloroacetic acid-insoluble cellular proteins was observed in the first 1 h of radiolabelling for both the control and the treated sample (Fig. 4a). In agreement with this, the specific radioactivities in the C.PRX immunoprecipitated from the cellular extracts reached their maxima at 1 h and then levelled off (Fig. 4b).

The secretion of newly synthesized proteins into the medium was inhibited by tunicamycin treatment (Fig. 4c). The C.PRX was released quickly into the control medium after radiolabelling (Fig. 4d). In the tunicamycin-treated cultures, however, secretion of the non-glycosylated form of this peroxidase proceeded slowly over the labelling period and reached a level of about one-third that of control at 3.5 h (Fig. 4d). Rapid degradation of medium proteins is not probable, since no proteinase has been detected in the medium (van Huystee & Turcon, 1973).

Another consequence of tunicamycin treatment is the increased electrophoretic mobility of the polypeptide shown by immunoprecipitation with the specific antiserum to C.PRX (Fig. 5). This band had an M_r of about 31 000, which is 9000 lower than that of control. Such a comparatively lower M_r would be expected for a protein in which glycosylation is prevented (Hu & van Huystee, 1988; Ravi *et al.*, 1986). The C.PRX treated with trifluoromethanesulphonic acid (TFMS) gave a peptide with an M_r about 31 900 on SDS/PAGE (Hu & van Huystee, 1988). The M_r difference between the C.PRX isolated from tunicamycin-treated cultures and the TFMS-deglycosylated form is probably due to the *N*-acetylglucosamine residues left on the linkages.

The finding that the non-glycosylated C.PRX

Fig. 6. Western blots of medium and cellular proteins before (a) and after (b) removal of tunicamycin from the cultures

Crude cellular (1) or medium proteins (2) were separated on SDS/polyacrylamide gels, transferred to nitrocellulose membranes and probed with McAb 1B. -, Control cultures; +, tunicamycin-treated cultures. The amounts of the cellular (25 µg) or the medium proteins (5 µg) loaded in each lane were varied in accordance to the relative amount of the C.PRX present.

synthesized in the presence of tunicamycin continues to be transported and released, albeit at a lower rate into the medium, was confirmed by Western blotting. In this experiment, peanut cells were washed before subculturing in medium supplemented with or without tunicamycin (1 µg/ml). At the exponential cell-growth phase and peroxidase production (van Huystee & Lobarzewski, 1982), the cells were harvested, homogenized and extracted. Only a broad band with an approx. M_r of 40 000 (at the top) was detected from the control medium on the immunoblot probed by McAb-1B, which is specific for the protein part of C.PRX (Hu *et al.*, 1987b; Hu & van Huystee, 1988). Multiple polypeptide bands of approx. M_r 40 000, 38 800, 36 000, 33 500 and 31 200 reacting to

Table 4. Effect of tunicamycin on distribution of proteins and peroxidase activity

Peanut cells were cultured in the presence or absence of tunicamycin (1 µg/ml). The cells were harvested and extracted on day 6. The medium and the cellular extracts were used for protein and peroxidase activity assay. Values are means $\pm$ S.D. ($n = 3$).

Treatment	Fresh cell weight (g)	Protein content (mg/g fresh wt.)	Specific PRX activity ($A_{470} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)
Control	6.2 $\pm$ 2.1		
Medium		3.5 $\pm$ 0.16	39.5 $\pm$ 2.8
Cell extract		5.5 $\pm$ 0.70	120.8 $\pm$ 13.8
Tunicamycin	3.6 $\pm$ 0.7		
Medium		2.9 $\pm$ 0.26	7.8 $\pm$ 2.4
Cell extract		5.4 $\pm$ 1.19	119.7 $\pm$ 7.1

Fig. 7. Recovery of peroxidase activity in the culture medium after tunicamycin treatment

Peanut cells were incubated with (---) or without (—) tunicamycin (1 µg/ml) for 6 days and then washed with, and continued to be cultured in, fresh normal medium.

this McAb showed up in the medium of tunicamycin-treated culture (Fig. 6a). In contrast with a drastic decrease of peroxidase activities in the medium of tunicamycin-treated cultures, the specific peroxidase activity of the treated cellular extracts remained virtually the same as that of control (Table 4). Since an inhibition of peroxidase activity in the medium as well as of cell growth in terms of fresh weight was noted after several days culture in the presence of tunicamycin (Table 4), a peroxidase-activity-recovery experiment was performed to examine the reversibility of tunicamycin effect. The results of such an experiment are shown in Fig. 7. Upon removal of tunicamycin, the accumulation of peroxidase activity in the medium resumed slowly and reached the same level as that of control at day 12 of culture. Western blots of medium proteins at this stage demonstrated no difference between control and treated samples (Fig. 6b).

DISCUSSION

The present study evaluates the role of carbohydrates in the enzyme activity, stability and release of peanut peroxidases. Our major findings are: (i) that deglycosylation or periodate oxidation of carbohydrates of the C.PRX and the A.PRX greatly reduces peroxidase activities and increases their susceptibilities to proteolysis and (ii) that the drastic inhibition of glycosylation of the C.PRX does not prevent its synthesis or secretion. Thus carbohydrates of these isoperoxidases seem to be essential for the expression of peroxidase activity and for the stability of their protein moieties.

It has become evident that protein glycosylation may contribute to, and be an intrinsic part of, the physiological activity of glycoproteins (Rademacher *et al.*, 1988). The evidence provided by periodate oxidation and enzymic deglycosylation of the C.PRX and the A.PRX under non-denaturing conditions indicates that carbohydrates are necessary for peroxidase activity (Tables 1 and 2). The elimination of binding of the periodate-oxidized peroxidases (Fig. 1) to Con A and preservation of their reactivities towards the antibodies (Fig. 3) indicate that the effect of the mild periodate treatment was directed to

carbohydrate rather than to protein. Native peanut peroxidase molecules seemed to be quite resistant to attack by PNGase F, since their M_r was approx. 1000 less than that of the controls after incubation with PNGase F overnight under the conditions described. The peroxidase activities were reduced, however, to about 37 and 0.7% of controls for the C.PRX and the A.PRX respectively (Table 2). These results suggest that not all of the oligosaccharide chains are required for the expression of peroxidase activities.

When examining the Western blot of the cellular extracts in Fig. 6(a), one may see that two polypeptides of M_r 40000 and 36000 were detected by McAB-1B from the cellular extracts of both control and tunicamycin-treated cultures with the higher band denser, and the lower one lighter, in the control. Since the non-glycosylated form of the C.PRX was found to have an M_r of 31000 (Fig. 5), the 40000 and 36000 bands are fully and partially glycosylated forms respectively. They may have assumed a catalytically active conformation, and thus the cellular extracts of both control and treatment showed the same level of peroxidase activity (Table 4). However, the reason why only these two forms of polypeptide of the C.PRX are detected in the cellular extracts is unclear. In contrast with enzyme activation, glycosylation in the C.PRX is not a prerequisite for its secretion (Figs. 4d, 5 and 6). Similar observations have been reported for other glycoproteins (Chrispeels *et al.*, 1982; Reinwald, 1985; Amri *et al.*, 1986).

Carbohydrates in peanut peroxidases exert a protective effect on the protein moieties (Fig. 3; Table 3). This is consistent with conformational studies of HRP C, in which the carbohydrate chains are found to occur on the surface of the molecule (Welinder, 1985). They probably act as a molecular shield, blocking the access of proteinases to the potential proteolytic cleavage sites or stabilize the conformation of the protein part in which the cleavage sites are exposed, as has been suggested by Schwarz & Datema (1982).

In summary, peanut peroxidases seem to be the first example of plant enzymes reported in which oligosaccharide units are likely to play a critical role for the acquisition of their catalytic activities and their stabilities.

We thank Ms. E. Eisen for her help in some of this work, and Mr. P. Sesto for providing peanut cell culture. This work was supported by a grant from Natural Sciences and Engineering Research Council of Canada.

REFERENCES

- Allen, G. (1981) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S. & Burdon, R. H., eds.), pp. 53–55, Elsevier/North-Holland, New York
- Amri, E.-Z., Vannier, C., Etienne, J. & Aihaud, G. (1986) *Biochim. Biophys. Acta* **875**, 334–343
- Biroc, S. L. & Etzler, M. E. (1978) *Biochem. Biophys. Acta* **544**, 85–92
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Cairns, E., van Huystee, R. B. & Cairns, W. L. (1980) *Physiol. Plant.* **49**, 78–92
- Chibbar, R. N. & van Huystee, R. B. (1984) *Plant Physiol.* **75**, 294–298

- Chrispeels, M. J., Higgins, T. J. V., Craig, S. & Spencer, D. (1982) *J. Cell Biol.* **93**, 5–14
- Dyer, J. R. (1956) *Methods Biochem. Anal.* **3**, 111–152
- Hawkes, R. (1982) *Anal. Biochem.* **143**–146
- Hu, C. & van Huystee, R. B. (1988) *Biochem. Biophys. Res. Commun.* **156**, 500–505
- Hu, C., Lee, D., Chibbar, R. N. & van Huystee, R. B. (1987a) *Physiol. Plant.* **70**, 99–102
- Hu, C., Carbonera, D. & van Huystee, R. B. (1987b) *Plant Physiol.* **85**, 299–303
- Ivarie, R. D., Jones, P. P. (1979) *Anal. Biochem.* **97**, 24–35
- Lowry, O. H., Rosebrough, N. J., Farr, L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mans, R. J. & Novelli, G. D. (1961) *Biochem. Biophys. Acta* **94**, 48–93
- Maldonado, B. A. & van Huystee, R. B. (1980) *Can. J. Bot.* **58**, 2280–2284
- Rademacher, T. W., Parekh, R. B. & Dwek, R. A. (1988) *Annu. Rev. Biochem.* **57**, 785–838
- Ravi, K., Hu, C., Reddi, P. S. & van Huystee, R. B. (1986) *J. Exp. Bot.* **37**, 1708–1715
- Reinwald, E. (1985) *Eur. J. Biochem.* **151**, 385–391
- Schwarz, R. T. & Datema, R. (1982) in *The Glycoconjugates*, vol. 3 (Horowitz, M. I., ed.), pp. 47–79, Academic Press, New York
- Tarentino, A. L., Gomez, C. M. & Plummer, T. H. (1985) *Biochemistry* **24**, 4665–4671
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- van Huystee, R. B. (1987) *Annu. Rev. Plant. Physiol.* **38**, 205–219
- van Huystee, R. B. & Chibbar, R. N. (1987) *Curr. Top. Biol. Med. Res.* **13**, 155–179
- van Huystee, R. B. & Lobarzewski, J. (1982) *Plant. Sci. Lett.* **27**, 59–67
- van Huystee, R. B. & Turcon, G. (1973) *Can. J. Bot.* **51**, 1165–1175
- Welinder, K. G. (1985) *Eur. J. Biochem.* **151**, 497–504

Received 2 March 1989/24 April 1989; accepted 2 May 1989