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Monosaccharide composition and properties of a deglycosylated turnip peroxidase isozyme

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Abstract

A neutral peroxidase isozyme (TP) purified from turnip (*Brassica napus* L. var. purple top white globe) was partially deglycosylated, using chemical and enzymatic treatment. A 32% carbohydrate removal was achieved by exposing TP to a mixture of PNGase F, *O*-glycosidase, NANase, GALase III and HEXase I, while *m*-periodate treatment removed about 88% of TP carbohydrate moiety. The glycoprotein fraction of the TP contained a relatively high mannose and fucose content (37 and 31%, w/w, respectively), 16% (w/w) galactose, and 15% (w/w) GlcNAc. Thus, the carbohydrate moiety was classified as a hybrid type. Partially deglycosylated TP had reduced activity (by 50–85%), was more susceptible to proteolysis, and showed a slight decrease in thermostability compared to the native enzyme. Circular dichroism studies strongly suggested that although the carbohydrate moiety of TP did not influence the conformation of the polypeptide backbone, its presence considerably enhanced protein conformational stability toward heat. Removal of oligosaccharide chains from TP caused a decrease in K_m and V_{max} for hydrogen peroxide. Native and chemically deglycosylated TP were similarly immunodetected by rabbit polyclonal antibodies raised against TP. The results suggest that the carbohydrate moiety of TP is important for peroxidase activity and stability.

Keywords: *Brassica napus* L. var. purple top white globe; Cruciferae; Turnip peroxidase; Enzymatic deglycosylation; Chemical deglycosylation

1. Introduction

Plant peroxidases (peroxidase: E.C. 1.11.1.7) are heme-containing oxidoreductases, having up to 20% (w/w) carbohydrates. The carbohydrate moiety of the glycoproteins is usually heterogenous with a number of individual units, as well as various degrees of branching and distribution along the polypeptide chain. These sugars usually form oligosaccharide chains N-linked to asparagine residues of the protein, such as found in peroxidase from horseradish (Welinder, 1985), and peanut (Hu and van Huystee, 1989a). Carbohydrates may modulate physicochemical properties of the glycoproteins, mediate their biological activity (Rademacher et al., 1988), and affect their storage stability (Tigier et al., 1991; Sánches-Romero et al., 1994; Nie et al., 1999).

The removal of the carbohydrate moiety normally affects peroxidase kinetics (Tigier et al., 1991), antigenicity and resistance to protease attack (Hu and van Huystee, 1989a), and thermal stability (Sánchez-Romero et al., 1994). However, neither activity nor thermal stability were affected when Nie et al. (1999) used *N*-glycopeptidases on a fungal peroxidase, indicating that most were *O*-linked glycans. Studies of plant peroxidase deglycosylation has shown a wide variety of results in peanut (Hu and van Huystee, 1989a), seeds of peach fruit (Tigier et al., 1991), avocado leaves (Sánchez-Romero et al., 1994) and horseradish (HRP; Tams and Welinder, 1995).

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This study aimed to investigate the possible role of the carbohydrate moiety of a neutral peroxidase isozyme purified from turnip roots (Duarte-Vázquez et al., 2001), using both chemical and enzymatic treatments. The extent of changes in deglycosylated peroxidase thermal stability, immunological, structural and kinetic properties was also investigated.

2. Results and discussion

2.1. Enzymatic deglycosylation

The carbohydrate content of TP was 9.1%, measured according to the method of Dubois et al. (1956). After enzymatic treatment (TP1), no changes in native TP relative mobility and residual activity were observed (Table 1). PNGase F cleaves the bond between the Asn residue of the protein and the *N*-acetylglucosamine (GlcNAc) residue that links the carbohydrate to the protein. NANase II is a broad specificity sialidase used to release sialic acid normally located at the end of the oligosaccharide chain. *O*-Glycosidase is highly specific for cleaving Ser/Thr linked Gal(β1,3)GalNAc(α1); other sugars render the oligosaccharide chain resistant to cleavage. Plant peroxidases have been shown to contain a large fucose content (Sun et al., 1997; Kim and Kim, 1995; McManus et al., 1988), which if α-1,3 linked to GlcNAc is resistant to PNGase F cleavage (Dwek, 1996). In this context Kurosaka et al. (1991), successfully used PNGase A to cleave the glycan moiety from pepsin digested HRP peptides. This enzyme was also used to cleave the glycan chain from Asn-144 of a tryptic digested peanut peroxidase peptide (Shaw et al., 2000).

PNGase A was difficult to obtain and we decided to proceed with an enhancement kit treatment. The partially deglycosylated TP (TP2) migrated slightly faster than untreated TP, and both gave a positive carbohydrate stain (Table 1). A molecular weight 1 kDa lower than native TP was observed, accounting for 32% oligosaccharide removal and a drastically reduced TP2 activity (Table 1). This indicated that intact oligosaccharide chains are required for full peroxidase activity, in contrast to previous reports in which partial carbohydrate removal produced an unstable avocado peroxidase (Sánchez-Romero et al., 1994). We conclude that TP is therefore a glycoprotein whose enzymatic activity decreases after carbohydrate removal.

Table 1
Effect of glycosidase treatment on activity (% in parenthesis relative to the native enzyme), and molecular weight of turnip peroxidase

	Activity (kat/kg)	Molecular weight (kDa)	Carbohydrate removal (%)[a]
Native TP	37.8 (100%)	33.9	–
TP1[b]	36.5 (96%)	33.9	0
TP2[c]	18.8 (50%)	32.7	32

[a] Estimated by difference in M_r relative to native TP, using SDS-PAGE.
[b] TP1 = TP after initial enzymatic deglycosylation.
[c] TP2 = TP after treatment with the deglycosylation enhancement kit.

2.2. Monosaccharide composition of TP

The relative abundance of monosaccharides released from TP were GlcNAc 1.0, galactose 1.1, fucose 2.0 and mannose 2.4. The fraction of fucose was relatively high for plant peroxidases (Sun et al., 1997; Kim and Kim, 1995; Tams and Welinder, 1995). Plant glycoproteins usually have fucose residues linked to the proximal GlcNAc residues, while this is not a common feature in animal glycoproteins. Due to this linkage a treatment with PNGase A was probably needed. Thus, the use of the enhancement kit allowed partial carbohydrate removal from TP. Since TP glycoprotein had on a w/w basis 37% mannose, 31% fucose, 16% galactose, and 15% GlcNAc, it can be classified as a hybrid type (Freeze, 2000). At least 30% mannose has also been found for Korean radish (*Raphanus sativus*) peroxidase (Kim and Kim, 1995) and HRP (Sun et al., 1997). Other monosaccharides commonly found in plant peroxidases are xylose and arabinose; however, our standards did not contain either of these compounds and therefore were not identified in TP glycoprotein. We have obtained a cDNA fragment of TP coding for 265 amino acids, containing eight potential *N*-glycosylation sites (Asn-X-Ser/Thr, where X could be any amino acid apart from Pro; Dwek, 1996).

2.3. Periodate oxidation and effect of deglycosylation on TP kinetics

Periodate oxidation of the *vic*-hydroxyl group of native TP carbohydrate moiety achieved about 88% carbohydrate removal and a maximum activity loss after 20 h treatment (Table 2). This was confirmed by SDS-PAGE, using the carbohydrate stain where only a faint band was observed (results not shown). Extensive TP deglycosylation might have been achieved by using trifluoromethanesulfonic acid (TFMS), leaving only the *N*-linked GlcNAc at the expense of losing most TP activity (Edge et al., 1981). However, according to Tams and Welinder (1995), HRP besides losing all of its activity after TFMS treatment, showed a high number of negative charges indicative of protein conformational changes. Other reports using the periodate method have shown high activity retention, while achieving a 40–51% deglycosylation (Wasserman and Hultin, 1981; Marcone and Yada, 1997). Thus, periodate was used to retain most of the enzyme activity to test the properties of deglycosylated versus native TP.

TP inhibition due to excess H_2O_2 was observed in the deglycosylated form at much lower concentrations than

Table 2
Effect of periodate oxidation on peroxidase activity

Conditions	Remaining activity (mkat)
TP in buffer[a]	53.3±0.32[c]
TP in buffer + EG[b] + $NaIO_4$	52.2±0.53
Periodate treatment (TP3; 4 °C)	
1 h	46.2±0.68
4 h	38.2±0.30
20 h	7.95±0.23

[a] 50 mM acetate buffer, pH 4.5.
[b] EG = ethylene glycol.
[c] Standard deviation.

for the native enzyme (Fig. 1). This may have occurred via one electron transfer to compound I, generating compound II which reacted with excess H_2O_2 to produce compound III (oxyperoxidase, $Fe^{II}O_2$), which has a slow rate of conversion back to the peroxidatic reaction (Nicell and Wright, 1997). Thus, carbohydrate removal could facilitate exposure of the heme goup, followed by TP inhibition. The H_2O_2 K_m value for partially deglycosylated TP was 25 μM, lower than that obtained for the native TP (55 μM). Saturation curves for TP using ABTS as substrate showed a similar trend, with a K_m decrease from 1.1 to 0.9 mM for native and deglycosylated TP, respectively. Enzymatic or m-periodate deglycosylations produced similar effects on TP kinetics. Subtle conformational changes in the binding site resulting from glycosidic chain removal may explain these results. Sánchez-Romero et al. (1994) found similar results for avocado peroxidase. Comparing peroxidases with known sequences from different sources, there is a highly conserved asparagine glycosylation site in the vicinity of two conserved amino acids: arginine and tyrosine (Henriksen et al., 1998; Schuller et al., 1996). These two amino acids are essential for peroxidase activity since they are involved in substrate binding (Buffard et al., 1990). If they were present in TP, this site could have been affected by carbohydrate removal. After removal of this carbohydrate moiety, substrates probably had better accessibility to their binding sites, which may explain their increased affinity for deglycosylated TP. Lige et al. (2001) evaluated the contribution of individual *N*-linked glycans from a cationic peanut peroxidase to its function. They concluded that all three oligosaccharide chains were required for a stable conformation of the protein but only two were necessary for full expression of the catalytic activity. These findings support the hypothesis that each glycosylation site may play a distinct function in the overall behavior of enzyme glycoproteins.

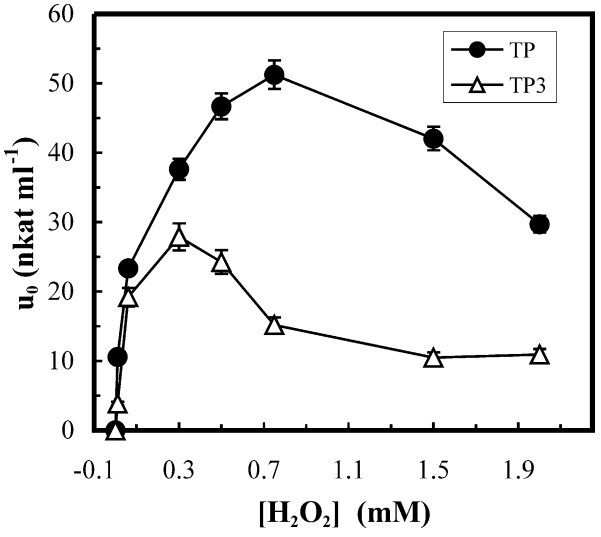

Fig. 1. Activity of native (TP) and chemically deglycosylated (TP3) turnip peroxidase at different hydrogen peroxide concentrations.

2.4. Stability of deglycosylated and native TP to trypsin

A 10% decrease in native TP activity was observed after 3 h incubation with trypsin, while the chemically deglycosylated TP (TP3) only retained 36% of its original activity (Table 3). Therefore, the carbohydrate moiety appears to exert a protective effect against proteolysis of native TP, as had been noted for other glycoproteins (Varki, 1993). The carbohydrate moiety may block the access of proteases to the potential proteolytic cleavage sites or may stabilize the conformation of the domain where the cleavage sites are exposed (Schwarz and Datema, 1982). In addition, carbohydrate removal may produce protein conformational changes exposing amino acid sequences recognized by proteases and eventually destroyed (Barriocanal et al., 1986).

2.5. Effect of deglycosylation on secondary structure

Native TP had a circular dichroism spectrum showing two valleys with minima at 208 and 222 nm, typical of an α-helix with a small fraction of β-sheet (Manavalan and Johnson, 1983, Fig. 2). Chemical (*m*-periodate) or enzymatic deglycosylation did not produce significant

Table 3
Susceptibility of native (TP) and enzymatically deglycosylated (TP2) peroxidase to trypsin attack (% in parenthesis relative to TP)[a]

Sample	Remaining activity (mkat)
Control (TP)	
0 h	56.3 (100%)
2 h	53.0 (94%)
3 h	50.7 (90%)
Deglycosylated (TP2)	
0 h	28.0 (100%)
2 h	19.7 (70%)
3 h	10.1 (36%)

[a] Mean of three replicates with standard deviation within 5% of the mean.

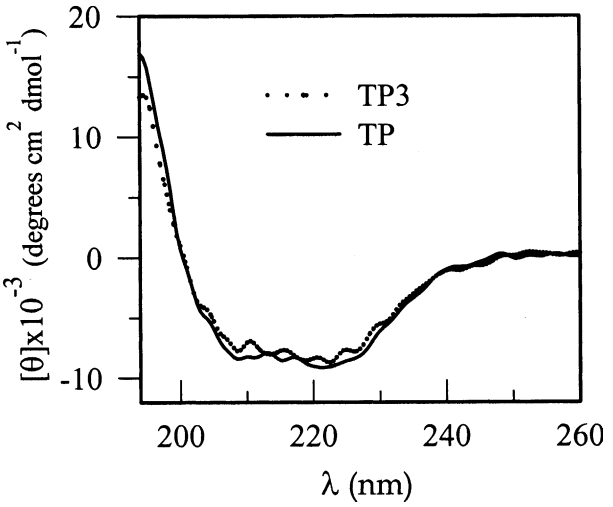

Fig. 2. Far-UV circular dichroism spectroscopy of native (TP) and chemically deglycosylated (TP3) peroxidase.

changes in the secondary structure of native TP, since their CD spectra were similar (Fig. 2). The mean content of α-helix, β-sheet and random structures for both deglycosylated and native TP were 31, 11 and 58%.

Enzymatic deglycosylation of yeast invertase, bovine serum fetuin and glucoamylase from *Aspergillus niger* did not show any significant changes in secondary structure (Wang et al., 1996). Similar results were observed after *N*-glycosidase F action on cowpea globulin, where 50% carbohydrate removal was achieved (Aluko et al., 1997). In contrast with these results, dramatic changes in amaranth globulin secondary structure were attributed to the removal of the carbohydrate moiety of this protein by Marcone and Yada (1997).

2.6. Effect of deglycosylation on thermal stability

Peroxidase thermal stability based on the rate of activity loss showed that enzymatically deglycosylated TP (TP2) was inactivated at a higher rate than the native TP, when heated at 65 °C (Fig. 3). This was attributed to the crucial role played by the carbohydrate portion on the thermal stability of the protein, as indicated by Nie et al. (1999) and Tigier et al. (1991).

Based on protein structural properties using CD spectroscopy significant differences in thermal stability of native and chemically deglycosylated TP were observed (Fig. 4). After heating up to 70 °C unfolding was completely irreversible for all TP samples. The difference in the T_m value of TP3 (57±0.25 °C) and native TP (59±0.25 °C) was small. However, a clear difference in onset temperature (where the loss in ellipticity signal is 5% of the original; T_O) was observed (Fig. 4). T_O for native TP was about 40 °C, while chemical carbohydrate removal decreased this value to 35 °C.

Results from CD studies strongly suggest that although the carbohydrate moiety of TP did not influence the

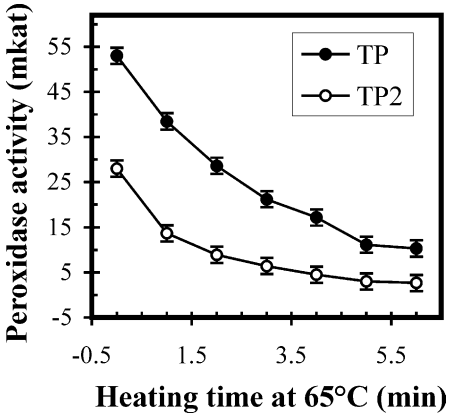

Fig. 3. Thermal stability of native (TP) and enzymatically deglycosylated (TP2) peroxidase.

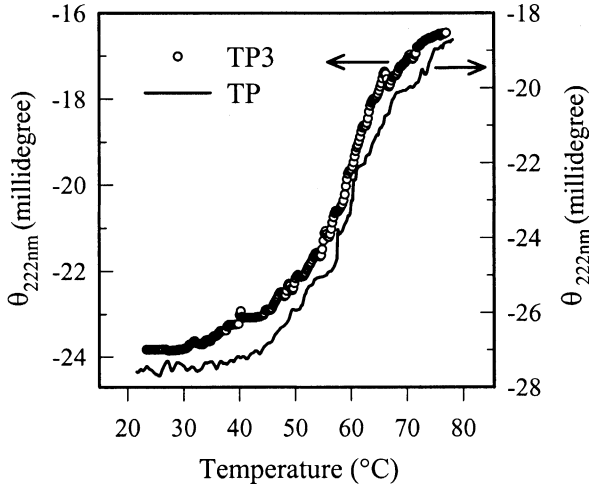

Fig. 4. Thermal stability of native (TP) and chemically deglycosylated (TP3) turnip peroxidase. CD was used to follow the changes in ellipticity at 222 nm, using a heating rate of 1 °C/min.

conformation of the polypeptide backbone, its presence considerably enhanced protein conformational stability toward heat. This was probably due to hydrogen bonding with the polypeptide backbone (Wang et al., 1996). Studies on glucose oxidase crystal structure, where *N*-linked mannose residues formed strong hydrogen bonds with the carbonyl oxygen of glutamic acid, are further evidence of this suggestion (Hecht et al., 1993).

2.7. Effect of deglycosylation on immunological properties

Very small amounts of TP (4 μg) could be used to raise the antibodies (10 mg of IgG per μg of injected TP), because they were directly injected into the lymphatic system. Other immunization protocols have used 20–100 μg of antigen (Vaitukaitis et al., 1971; Hu and van Huystee, 1989b). Despite the small oligosaccharide

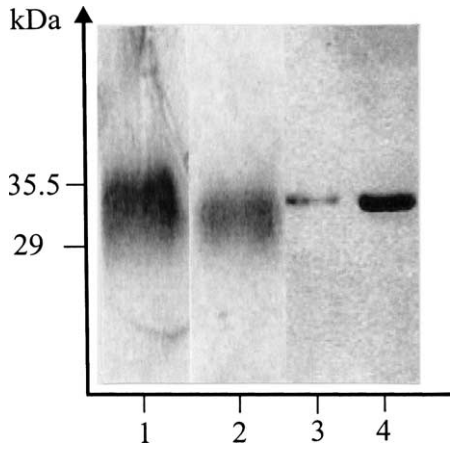

Fig. 5. Chemiluminescence immunodetection of native (TP) and partially deglycosylated (TP3) turnip peroxidase, using an antibody raised against TP. Lane 1: TP (5 μg); lane 2: TP3 (5 μg). Both samples were electrophoresed and transferred to a PVDF membrane. Lane 3: SDS-PAGE of TP (0.4 μg), silver stained; lane 4: SDS-PAGE of TP3 (3 μg), silver stained.

content in the chemically deglycosylated TP, this sample and native TP reacted equally with the antibodies (Fig. 5). This indicated the possible presence of binding sites within the peptide chain, involving not only the carbohydrate moiety in TP antigenicity. Similar results were found by Schmitz et al. (1997), and Tigier et al. (1991), for soybean coat and peach seed peroxidases, respectively. In contrast to these results, antibodies raised against peroxidase from peanut cell culture did not bind to the deglycosylated enzyme (Hu and van Huystee, 1989b). The importance of carbohydrates on the antigenicity of peanut peroxidase was demonstrated by Hu and van Huystee (1989a). In addition, three oligosaccharides from a cationic peroxidase were highly antigenic when coupled to bovine serum albumin (Wan and van Huystee, 1994). To clarify the immunological importance of the amino acid sequence, monoclonal antibodies could be used to obtain differences between native and deglycosylated peroxidases.

3. Experimental

3.1. Peroxidase purification and activity assay

Peroxidase neutral isozyme (TP) was extracted from turnip (*Brassica napus* L. var. purple top white globe) roots. It was purified using cation-exchange and hydrophobic-interaction chromatographies (Duarte-Vázquez et al., 2001). Protein concentration was determined using bicinchoninic acid (Smith et al., 1985), with horseradish peroxidase (HRP; Sigma Type VII) as protein standard. TP activity was evaluated at 25 °C, using the diammonium salt of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) as hydrogen donor, by the change in absorbance at 414 nm ($\epsilon_{414}=36$ $mM^{-1}cm^{-1}$; Childs and Bardsley, 1975). The final reaction mixture (1.5 ml) contained 1 mM ABTS, 5 mM H_2O_2, 1 μM enzyme and 10 mM potassium phosphate buffer, pH 6.0.

3.2. Polyacrylamide gel electrophoresis (SDS-PAGE)

It was conducted as described by Laemmli (1970), using a Mighty Small vertical chamber (SE 250, Hoeffer). The protein bands were silver stained (Ausubel et al., 1995), and the molecular weight was evaluated from an image analysis software 1D (Kodak digital science, Rochester, USA). The glycosylated moiety was stained using a glycoprotein detection kit (Sigma). This test is based on the oxidation of the oligosaccharides by periodic acid followed by staining of the aldehyde groups with Schiff reagent (Gerard, 1990).

3.3. Antibody production

Purified isozyme (4 μg) was used to raise rabbit (New Zealand white, 80 days old) polyclonal antibodies, by injecting the protein diluted in a 1:1 ratio with complete Freund's adjuvant into the popliteal lymph node (Jacob et al., 1986). Three subcutaneous booster immunizations with an equal amount of TP emulsified with incomplete Freund's adjuvant were applied every 12 days, and a final bleed was conducted after 50 days. The antibodies were precipitated with 33% saturated ammonium sulfate, re-suspended in phosphate buffer saline, pH 7.3 and dialyzed against the same buffer. This antibody fraction was used for immunological assays.

3.4. Immunoblotting

Protein was transferred onto a polyvinylidene difluoride (PVDF; Bio-Rad) membrane by using a semi-dry transfer unit (trans-blot SD, Bio-Rad). The immunoblot was conducted as depicted by Ausubel et al. (1995). The membrane was incubated with the rabbit antibodies for 3 h under constant shaking (rocking platform shaker, Lab-Line) at room temp, then washed with TBS containing 0.5% Tween 20 (TBST), and incubated for 50 min with an HRP-conjugated goat anti-rabbit secondary antibody (Zymed, CT, USA). Detection was accomplished by chemiluminescence using luminol and 4-iodophenol (BM chemiluminescence kit, Roche, Germany). After 20 min, a sheet of X-OMAT film (Kodak, Rochester, USA) was placed onto the blot, and exposed for 3 s.

3.5. Deglycosylation

3.5.1. Enzymatic

To use the enzyme for further crystallization studies, a non-denaturing protocol was used, with the Bio-Rad

enzymatic deglycosylation kit. Native TP (100 μg) was incubated at pH 6.0, with a mixture of acetylneuraminyl hydrolase (NANase) II (1.00 U/mL) and endo-α-*N*-acetylgalactosaminidase (*O*-glycosidase) DS (0.100 U/ml) at 37 °C for 1 h. After adjusting the pH to 7.5, the TP was further incubated for 24 h with peptide: *N*-glycosidase F (PNGase F; 119 mU/mL). Further deglycosylation was conducted using the enhancement kit (Bio-Rad). The native TP was incubated with PNGase F as described above. Then TP was incubated at 37 °C for 2 h, pH 6.0, with a mixture of NANase II (417 mU/ml), exo-β-galactosidase (GALase) III (62.5 mU/ml), HEXase I (333 mU/ml; releases non-reducing terminal β-linked *N*-acetyl-glucosamine), and *O*-glycosidase DS (41.7 mU/ml). The samples run on a SDS-PAGE gel were tested for carbohydrates using the glycoprotein detection kit (Sigma), and silver stained to measure the protein mobility changes.

3.5.2. Periodate oxidation

The purified TP (0.40 mg/ml) was 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 time intervals by adding ethylene glycol (EG) to a final concentration of 16% (Yasuda et al., 1971). After dialysis at 4 °C against 50 mM phosphate buffer, pH 6, TP activity was determined. Controls were run without the addition of periodate. The carbohydrates removed were quantified by the colorimetric method of Dubois et al. (1956).

3.6. Monosaccharide composition of TP

We used a monosaccharide compositional analysis kit (Bio-Rad). Sialic acid was released using a final concentration of 0.1 N TFA, amine sugars with 4 N HCl, and neutral sugars with 2 N TFA. The fluorophore 2-aminoacridone (AMAC) was used to label the released sugars. A glycoprotein (bovine fetuin), an oligosaccharide (*N*-acetyllactosamine), sialic acid, monosaccharide standards, and the sugar samples were separated by electrophoresis in a borate buffer, according to manufacturer's instructions. The IPLAB Gel analysis and densitometry software (Scanalytics, VA, USA) was used to quantify the separated sugars.

3.7. Native and deglycosylated TP properties

3.7.1. Kinetic constants

The K_m values were obtained from double reciprocal plots, according to the Lineweaver–Burk method (Cornish-Bowden, 1995). H_2O_2 K_m values were obtained at concentrations between 0.011 and 5.0 mM, at saturating ABTS concentration (2.5 mM). ABTS concentrations ranging 0.05–3.2 mM were used at saturating H_2O_2 concentration (2.0 mM). Ionic strength, pH, and temp were kept constant.

3.7.2. Thermal stability

An aliquot (50 μl) of TP was added to 500 μL of 10 mM citrate buffer (pH 5.0) in small thin-walled test tubes (1.0 cm outside diameter×7.5 cm long), slightly shaken in a water bath and heated for a designated time at 65 °C. The samples were immediately cooled in ice-water, and the residual enzymatic activity was evaluated.

3.7.3. Proteolysis

TP solutions of 100 μg/ml in 50 mM phosphate buffer, pH 7.0 (800 μl), were mixed with 20 μl of 10 mg/ml trypsin solution (Sigma) and incubated at 37 °C. Aliquots were removed at specific time intervals and assayed for residual activity.

3.7.4. Secondary structure and heat stability

The effect of deglycosylation on protein conformation and conformational heat stability was investigated by using circular dichroism (CD). Far-UV CD spectra of TP solutions (0.1 mg/ml) were recorded using a JASCO J-715 spectrometer from 190 to 250 nm in a 0.1 cm pathlength quartz cell, fitted with a thermoelectric temp. control under constant nitrogen flush. The proportions of the secondary structure (α-helix, β-sheet and random) were calculated using the K2D program (Andrade et al., 1993). Thermal denaturation of TP was performed by heating the samples contained in 3 mL quartz cuvettes at a constant rate of 1 °C/min, while monitoring the ellipticity (θ) at 222 nm. Melting temp. (T_m) was defined as the midpoint of the transition from helical to random structure.

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