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Cell Wall and Cytoplasmic Isozymes of Radish β-Fructosidase Have Different N-Linked Oligosaccharides

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ABSTRACT

When 36-hour-old dark grown radish seedlings are transferred to far-red light, there is a decrease in cytoplasmic β-fructosidase (βF) and an increase in cell wall βF compared to the dark controls. Cytoplasmic and cell wall-bound β-fructosidase are both glycoproteins and exhibit high antigenic similarities, but differ according to charge heterogeneity and carbohydrate microheterogeneity. Growth of radish seedlings in the presence of tunicamycin results in a partial inhibition of βF glycosylation but nonglycosylated βF still accumulates in the cell wall under far-red light. Thus, glycosylation is not necessary for intracellular transport, for correct targetting, or for wall association of an active βF. The nonglycosylated cytoplasmic and cell wall βF forms have the same relative molecular mass but glycosylated forms have different oligosaccharide side-chains, with respect to size and susceptibility to α-mannosidase and endoglycosidase D digestion. The oligosaccharides of both forms are partly removed by endoglycosidase H when βF is denatured. Isoelectric focusing analysis of βF shows that the cell wall-associated isozymes are more basic than the cytoplasmic isozymes, and that the charge heterogeneity also exists within a single plant. A time course of changes in βF zymograms shows a far red light stimulation of the appearance of the basic forms of the enzyme. However, the more basic cell wall specific βF forms are not present when *N*-glycosylation is prevented with tunicamycin. These results indicate that cytoplasmic and cell wall βF probably have common precursor polypeptides and basic cell wall forms arise via processing events which are tunicamycin sensitive.

A number of enzymes are present in both the cytoplasm and the cell wall of plant cells, and in some cases it has been shown that the cell wall isozymes differ from the cytoplasmic ones (20). We have studied the cytoplasmic and cell wall forms of βF[1] in radish seedlings and have shown that they are antigenically related but differ in their affinity for ConA and in their isoelectric points (12). Analysis of the isozymes of βF by isoelectric focusing shows that the cell wall contains several basic isozymes not present in the cytoplasm. When radish seedlings are grown in the dark, βF first accumulates in the cytoplasm, then slowly increases in the cell wall. A shift from darkness to FR light after 36 h of growth results in a rapid increase in cell wall βF at the expense of cytoplasmic βF. This accumulation occurs even when protein synthesis is inhibited by cycloheximide, indicating that growth under FR light is accompanied by an active secretion of cytoplasmic enzyme into the cell wall (13, 33) and the concomitant appearance of the basic forms of βF (9).

In this paper we present results on the oligosaccharide structure of βF and the role of processing in the generation of the various isozymes. The results support the conclusion that charge heterogeneity of cytoplasmic enzyme resides in the polypeptides, while the formation of basic cell wall forms occurs as a result of post-translational modifications that can be inhibited by tunicamycin. Experiments with tunicamycin-treated seedlings show that neither glycosylation nor glycan processing are prerequisites for intracellular transport or association of an active βF with the wall.

MATERIALS AND METHODS

Plant Material and Enzyme Preparations. Radish (*Raphanus sativus*, var *Longue rave saumonée*) seeds were germinated in complete darkness on filter paper moistened with distilled H_2O. After 36 h, seedlings were either maintained in the same conditions or transferred under FR illumination. Temperature was 25 ± 0.5°C for germination or seedling development. In some experiments seeds were germinated in tunicamycin (30 $\mu g \cdot ml^{-1}$). Cytoplasmic and cell wall-bound βF were extracted from radish seedling axes and assayed as previously described (12).

Digestion of βF with Glycosidases and Alkaline Phosphatase. Cell wall and cytoplasmic βF were incubated with different glycosidases; α-D-mannosidase from *Canavalia ensiformis* (Boehringer Mannheim), endo D from *Diplococcus pneumoniae* (Miles), endo H from *Streptomyces plicatus* (Miles), Neuraminidase from *Clostridium perfringens* (Sigma), or alkaline phosphatase from *Escherichia coli* (Sigma). βF incubation with these different enzymes was performed at 37°C for times varying between 8 and 18 h. Buffers used were either 50 mM citrate/phosphate buffer adjusted from pH 4.8 to pH 6.5 and supplemented according to glycosidase activity and stability requirements, or 50 mM Tris-HCl, pH 7.5 buffer for alkaline phosphatase. Incubations were performed under a toluene atmosphere in the presence of 1 mM PMSF. Controls were run with βF incubated in the same conditions but without glycosidase. Molecular mass markers were incubated simultaneously with glycosidases to verify from their mobility in SDS-PAGE that proteases were absent from glycosidase preparations. After digestion, βF activity was assayed and binding capacity to ConA-Sepharose (Pharmacia) columns determined.

SDS-PAGE, Protein Transfer, and Detection onto Nitrocellulose Sheet. SDS-PAGE was done according to Laemmli (18) using a 5% acrylamide stacking gel and a 12.5% acrylamide running gel. After SDS-PAGE, proteins were transferred electrophoretically at 6 V/cm for 18 h to a nitrocellulose sheet (Schleicher and Schuell, BA85) according to Towbin *et al.* (30).

[1] Abbreviations: βF, β-fructofuranosyl fructohydrolase (EC 3.2.1.26), also called invertase; endo H, endo-β-*N*-acetylglucosaminidase H; endo D, endo-β-*N*-acetylglucosaminidase D; ConA, concanavalin A; PMSF, phenylmethylsulfonylfluoride; Ip, isoelectric point; M_r, relative molecular mass; FR, far red light; TBS, Tris-buffered saline; TTBS, Tween 20—containing TBS; IEF, isoelectric focusing.

Proteins transferred onto the nitrocellulose sheet were stained 2 min in 0.2% amidoblack, 10% acetic acid, 45% methanol, and destained in 2% acetic acid and 90% methanol. Glycoprotein detection on nitrocellulose filter was performed with ConA and peroxidase. After saturation of residual binding capacity (quenching) with 500 mM NaCl, 80 mM Tris-HCl, pH 7.6 (TBS) containing 0.1% Tween 20 (TTBS), the nitrocellulose sheet containing transferred glycoproteins was first incubated with 25 $\mu g \cdot ml^{-1}$ of ConA in TTBS, then with 50 $\mu g \cdot ml^{-1}$ of horseradish peroxidase in TTBS. The bound peroxidase was visualized in the presence of 0.01% H_2O_2 and 0.3 $mg \cdot ml^{-1}$ 3-3′-diaminobenzidine in 50 mM sodium acetate buffer (pH 5.0). The specificity of staining for glycoproteins was demonstrated by incubation with ConA in the presence of 0.3 M α-methyl-D-mannoside.

Enzyme-linked immunoblotting detection of denatured βF after SDS-PAGE and transfer onto nitrocellulose sheet was performed essentially as described (23). After quenching with TTBS, the nitrocellulose sheet was incubated successively with anti-βF serum, and partially purified enzyme, both diluted in TTBS. The native and active enzyme was thus immunochemically bound to denatured inactive nitrocellulose-bound βF. β-Fructosidase activity was located on the blot with 'sandwich type' incubation. An indicator gel was made of 1% low gelling temperature agarose A37 (Pharmindustrie), buffered with 85 mM citrate/phosphate (pH 6.5) and mixed at 45°C with 0.1 M sucrose, 0.02 $mg \cdot ml^{-1}$ glucose oxidase (grade 1, Boehringer Mannheim), 0.12 mg/ml^{-1} horseradish peroxidase (Sigma), and 0.3 mg/ml^{-1} 3-3′-diaminobenzidine (grade 2, Sigma). The gel was poured on a Gel Bond (Marine Colloids) sheet and then laid on the nitrocellulose sheet for βF detection.

Isoelectric Focusing of βF. IEF in polyacrylamide gels and staining procedure for βF used in this system have been described (9, 11). IEF in thin layer agarose gels was performed in 0.4 mm thick gels made of 1% agarose (Isogel, Marine Colloids) containing 12% sorbitol and either 0.33 ml LKB Ampholines pH 3.5 to 10, 0.06 ml ampholines pH 5 to 8 and 0.06 ml ampholines pH 7 to 9 ('a' gels), or 0.47 ml of Pharmacia Pharmalyte pH 3 to 10 ('b' gels). The gels were cast on a sheet of Gel Bond in a mould consisting of two glass plates (12.5 × 12.5 cm) separated by a 0.4 mm plastic spacer. The gel attached to Gel Bond was removed from the gel former and stored for 18 h at 5°C before use. Electrode strips made of glass fiber support GF/B (Whatman) were soaked in either 0.5 M acetic acid and 1 M NaOH (carrier ampholytes from LKB) or 50 mM H_2SO_4 and 1 M NaOH (carrier ampholytes from Pharmacia), then blotted to remove excess fluid and laid on the anodic and cathodic sides of the gel, respectively. The samples (5 μl) were applied 1.8 cm from the cathode (except for direct focusing of tissue extracts; see below), using a silicone rubber strip (Desaga, Heidelberg) slightly greased with silicone oil DC200 (Serva). IEF was performed at 10°C for 75 min (a gels) or 50 min (b gels) on a flat bed apparatus at constant power (6 W) with 1500 V and 15 mamp as maxima. Sample applicators were removed after 30 min. At the end of the run, the pH gradient was measured with an Ingold surface electrode. The gel portions which had been in contact with the electrode strips were cut off and discarded. The gel attached to gel bond was then overlaid with a 1.5 mm thick indicator gel prepared as described above. This sandwich-type incubation was performed as described (8); its sensitivity allowed optimal βF detection in the range 6 to 13.10^{-3} enzyme units. After about 15 min incubation at 37°C, the enzyme reaction was stopped by soaking the gel for 5 min in 10% TCA; the gel was washed with distilled H_2O, pressed, and dried.

Direct Focusing of Tissue Extracts. To perform βF focusing without preliminary enzyme extraction, we introduced some variations to the above described procedure. One radish seedling hypocotyl was pressed between two glass fiber strips (0.5 × 0.2 cm) in the presence of one or two drops of distilled H_2O. These glass fiber applicators were layered separately on the agarose gel, 1 cm from the anode to prevent pH gradient shift observed from cathodic application of salt-containing samples. Focusing was first performed 10 min with limited voltage (300 V), then continued for an additional 75 min in the conditions described above (a gels). Sample applicators were discarded after 55 min focusing. At the end of the run, βF detection was performed as above.

Chromatographic Procedures. Gel filtration was performed on a Sephadex G-100 (Pharmacia) column (47 × 0.9 cm) equilibrated with 85 mM citrate phosphate buffer (pH 6.5) containing 0.5 M NaCl. The column was run at room temperature; the flow rate (12.5 ml h^{-1}) was controlled by peristaltic pump; 0.7 ml fractions were collected. Column calibration was controlled in each chromatographic step by addition of molecular weight markers to the βF sample (0.15 ml final volume).

RESULTS

Charge Heterogeneity of Native βF. The analysis of radish βF charge properties with IEF in polyacrylamide gels shows extensive polymorphism and large differences in isozyme patterns of βF obtained from cytoplasm and the cell wall (Fig. 1). However, since extracts from hundreds of seedlings were required for enzyme analysis in this focusing system, it was not clear whether the polymorphism was due to the presence of different alleles in the population or was present in each individual. Greater sensitivity of visualization of the isozymes after IEF in an agarose gel allowed us to demonstrate that the charge heterogeneity was also present in a single seedling (Fig. 2). Thus, polymorphism of radish βF is not due to manipulations during extraction or to genetic variability of the plant material.

Time-Course of Appearance of Basic Forms. Radish seedlings were grown for 36 h in the dark, and either left in the dark or transferred to FR. Tissue homogenates were fractionated into cytoplasmic and cell wall fractions and the cell wall associated enzyme extracted with salt. Samples of each enzyme preparation were then fractionated by IEF, and the βF visualized (Fig. 3). The position of hemoglobin, which was used as a reference, is

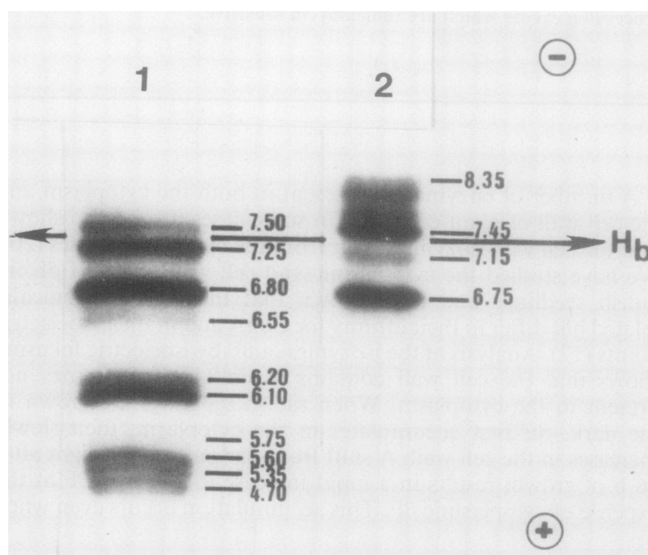

FIG. 1. Polyacrylamide gel IEF of cytoplasmic (1) and cell wall (2) βF from 72 h old seedlings grown under FR. Samples tested here contained 1.2 enzyme units. Rabbit hemoglobin (H_b), was used as internal standard and pI values presented are the mean of at least five experiments.

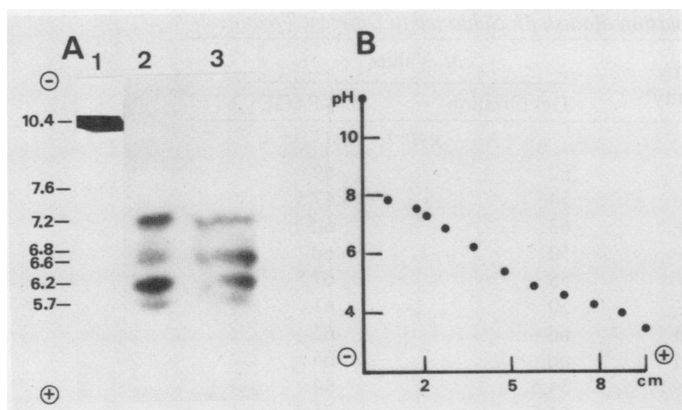

FIG. 2. IEF of cytoplasmic βF from single seedling. A, Lanes 1 and 3 are direct tissue focusing from a single 72 h old radish seedling hypocotyl. Lane 1 is stained for peroxidase activity, lane 3 for βF. Lane 2 is a focusing pattern obtained for cytoplasmic βF extracted from several 72 h old seedling hypocotyls according to a standard procedure. B, pH gradient observed at the end of focusing *versus* distance from cathode.

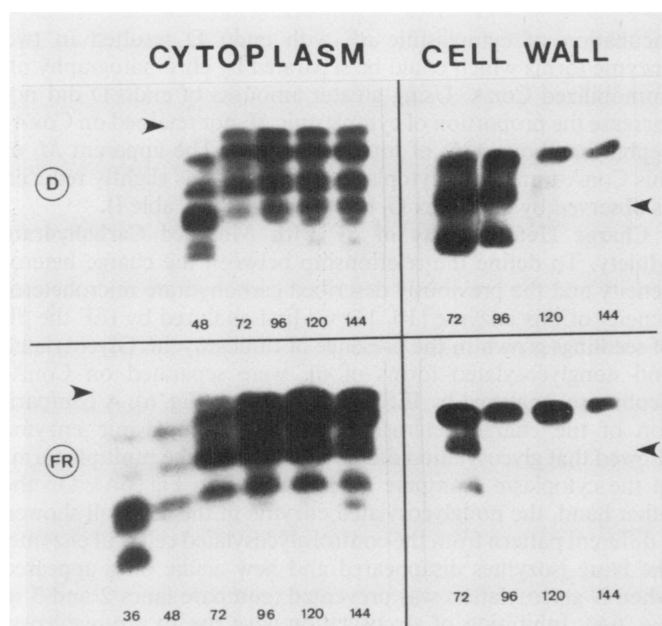

FIG. 3. Focusing patterns in thin-layer agarose gels for cytoplasmic βF and cell wall bound βF obtained from radish axes along the time course of seedling development from 36 to 144 h either under FR or in darkness (D). Here, carrier ampholytes 'a' were used; cathode is at the top of each plate. The position of rabbit hemoglobin is indicated with an arrow on each plate.

indicated by an arrow in each gel. Only qualitative comparisons can be made between lanes because they were not necessarily loaded with the same amount of enzyme. The results show that there was considerable heterogeneity in the cytoplasmic enzyme whether plants were kept in the dark (D) or transferred to FR. In addition, growth in the dark or in FR is accompanied by a gradual increase in the basic forms of the enzyme in the cytoplasm, especially between 48 and 72 h in the dark, and between 36 and 48 h in FR. The cell wall-associated βF consists of more basic isozymes, and at the end of the developmental period the most basic form predominates. Thus, growth is associated with a gradual increase in basic isozyme forms, both in the cytoplasm and the cell wall.

Relative Molecular Mass of βF. The M_r of both cytoplasmic and cell wall βF was estimated either by gel filtration on Sephadex G-100 or by SDS-PAGE followed by immunodetection of the enzyme. Both methods showed that the cell wall and the cytoplasmic enzymes, which are heterogeneous by IEF, have but a single M_r. However, with both methods the cell wall βF had a greater M_r than the cytoplasmic βF (Table I). The two methods did not give the same value which is not surprising, considering the enzymes are glycoproteins (11). An example of the results obtained with SDS-PAGE is shown in Figure 4, and the small difference in M_r between the cytoplasmic enzymes (Fig. 4B, lane 1) and the cell wall enzyme (Fig. 4B, lane 3) is clearly visible. When both forms of the enzyme were denatured with SDS at 20°C for 8 h, we observed the presence of a partly unfolded βF with smaller M_r (Fig. 4A, arrowhead on lanes 2 and 5). Under these conditions of mild denaturation the mol wt standards had the same mobility as when they were treated at 90°C for 3 min (data not shown). The M_r of these partly unfolded active βF forms is 54,000 for cytoplasmic βF and 56,000 for cell wall βF. These M_r values are close to the ones estimated from gel filtration for undenatured enzyme (Table I), and this result probably reflects a high conformational stability of the enzyme.

The M_r of cytoplasmic βF remained the same when tissue extracts were prepared in a medium containing 0.1 M EDTA which inactivates radish α-mannosidase (Fig. 4B, lane 2). This indicates that mannose residues were not removed from the βF by the endogenous α-mannosidase during the standard extraction procedure.

Relative-Molecular-Mass of βF with Modified Carbohydrate Moiety. The contribution of the carbohydrate unit to the molecular mass of βF was investigated in cytoplasmic and cell wall βF by growing the seedlings in the presence of tunicamycin. In animals and plants, tunicamycin inhibits formation of lipid-linked oligosaccharides and prevents *N*-glycosylation (1, 14, 21, 27). The level of tunicamycin was adjusted to be consistent with radish seed germination and development. In these conditions, only partial inhibition of βF glycosylation is obtained; glycosylated and nonglycosylated forms of the enzyme extracted from tunicamycin treated seedlings were separated by chromatography on ConA-Sepharose columns which binds glycosylated βF but did not retain the nonglycosylated form of this enzyme. Glycosylated and nonglycosylated βF were found in the cytoplasm as well as in the wall, indicating that glycosylation is not necessary for transport to the cell wall or for association with the wall polymers. The nonglycosylated cytoplasmic and cell wall βF exhibited the same apparent M_r of 50,000 as determined by gel filtration, and glycosylated forms obtained from tunicamycin treated seedlings displayed approximately the same M_r as control glycosylated enzymes (Table I).

To gain further information about the structure of the glycans, we treated βF with endo- or exoglycosidases, and examined the effect of this treatment on the M_r of βF, and on its ability to bind to ConA-Sepharose. Native cytoplasmic and cell wall βF were resistant to digestion with endo H as judged from their unchanged affinity for ConA, and their unchanged M_r (Fig. 5A, lanes 9 and 10). In contrast, M_r of cytoplasmic and cell wall βF was significantly reduced when subjected to SDS-heat denaturation prior to treatment with endo H (Table I). Thus, as described previously for several glycoproteins (2, 5), the oligosaccharide moiety in the native molecule of βF is not accessible to endo H.

α-Mannosidase digestion completely abolished the binding of cytoplasmic and cell wall βF to ConA-Sepharose, and the degraded forms had a smaller M_r (Fig. 5B). The M_r of cell wall βF decreased from 69,000 to 64,000, while that of cytoplasmic βF decreased only from 67,500 to 65,500 (Table I). This sensitivity of both forms of the enzyme to α-mannosidase degradation with simultaneous loss of affinity for ConA and the decrease in M_r, are consistent with a high mannose type or a one-side-free biantennary type structure for the βF oligosaccharide. For the

Table I. *M_r Values Determined for Cell Wall and Cytoplasmic Radish βF Subjected to Different Treatments*

Enzyme Form	Treatment	ConA Affinity[a]	M_r Values	
			Gel filtration	SDS-PAGE
			$\times 10^{-3}$	
Cell wall	None	+	62	69
Cytoplasmic	None	+	54	67.5
Cell wall	TM	+	63	nd
		−	50	nd
Cytoplasmic	TM	+	55	67.5
		−	50	61
Cell wall	endo H	nd	nd	65
Cytoplasmic	endo H	nd	nd	65
Cell wall	α-Mannosidase	−	54.5	64
Cytoplasmic	α-Mannosidase	−	54.5	65.5
Cell wall	endo D	+	Nondegraded	
Cytoplasmic	endo D	+	54	67.5
		−	52.5	nd

[a] + and − means retained and not retained by ConA; nd means not determined; enzyme was SDS and heat denatured prior to endo H digestion; the M_r of the cell wall and cytoplasmic βF remained the same when 2-mercaptoethanol was omitted from the denaturation medium prior to SDS-PAGE.

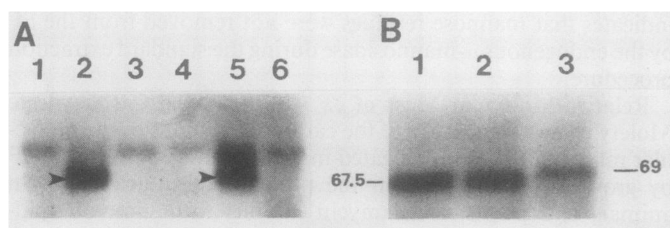

FIG. 4. M_r estimation of cytoplasmic and cell wall bound βF with SDS-PAGE and enzyme-linked immunoblotting detection on a nitrocellulose sheet. A, cytoplasmic βF (lanes 1–3) and cell wall βF (lanes 4–6) were denatured with SDS and 2-mercaptoethanol 3 min at 90°C (lanes 1 and 4), or 8 h at 20°C (lanes 2 and 5), or treated 3 min at 90°C with SDS but without 2-mercaptoethanol (lanes 3 and 6). The arrowheads indicate the positions of partly unfolded βF. B, Cytoplasmic βF (lane 1, 675 kD) and cell wall βF (lane 3, 69 kD) and were extracted according to standard procedure and denatured 3 min at 90°C with SDS and 2-mercaptoethanol. Cytoplasmic βF in lane 2 (67.5 kD) was treated as for lane 1 but endogenous radish α-mannosidase was inactivated during βF extraction.

controls, molecular mass markers were also incubated with α-mannosidase or endo H and then submitted to SDS-PAGE. Ovalbumin forms of reduced M_r appeared after incubation of mol wt standards with these glycosidases, as shown from protein staining on the blot (compare lanes 1 and 2 on panel A and B in Fig. 5). These forms with reduced M_r had lost affinity for ConA (Fig. 5; compare lanes 3 and 4 on panel A and B). Note on Figure 5B that part of the ovalbumin, the M_r of which was unchanged after α-mannosidase digestion (lane 2), could be stained with ConA-peroxidase (lane 4). However, the other non-glycosylated mol wt markers had the same electrophoretic mobility after digestion with these glycosidases. Thus, the shifts observed in the same conditions for denatured cell wall or cytoplasmic βF mol wt after endo H digestion (Table I) and for the native forms of βF after α-mannosidase digestion (Fig. 5B, lanes Sβ and Iβ) were not due to proteolysis but resulted from specific degradation of the carbohydrate moiety. On the other hand, unchanged M_r of native cytoplasmic and cell wall βF after incubation with endo H cannot be explained by the inactivity of the glycosidase because of ovalbumin digestion in the same conditions (Fig. 5A, lane 4).

When treated with endo D, cell wall βF was resistant to digestion but cytoplasmic βF was partly degraded. Typically, incubation of cytoplasmic βF with endo D resulted in two enzyme forms which could be separated by chromatography on immobilized ConA. Using greater amounts of endo D did not increase the proportion of cytoplasmic βF not retained on ConA-Sepharose above 43% of total βF activity. The apparent M_r of this ConA-unreactive cytoplasmic βF form was slightly reduced as observed by Sephadex G-100 gel filtration (Table I).

Charge Heterogeneity of βF with Modified Carbohydrate Moiety. To define the relationship between the charge heterogeneity and the previously described carbohydrate microheterogeneity of this enzyme (10, 11) we first analyzed by IEF the βF of seedlings grown in the presence of tunicamycin. Glycosylated and nonglycosylated forms of βF were separated on ConA-Sepharose, analyzed by IEF, and visualized (Fig. 6). A comparison of the charge heterogeneity of the cytoplasmic enzyme showed that glycosylation did not account for the multiple forms in the cytoplasm (compare lanes 4 and 5 in Fig. 6A). On the other hand, the nonglycosylated enzyme in the cell wall showed a different pattern from the control glycosylated cell wall enzyme: the basic isozymes disappeared and new acidic ones appeared when *N*-glycosylation was prevented (compare lanes 2 and 3 in Fig. 6A). Inhibition of glycosylation gave rise to a nonglycosylated cell wall βF zymogram closely related to the one observed for cytoplasmic βF (compare lanes 2 and 5 in Fig. 6A). As described before, both glycosylated and nonglycosylated βF are found in tunicamycin treated seedlings, and an examination of the glycosylated cell wall βF extracted from tunicamycin-treated seedlings (lanes 2 and 5, Fig. 6B) shows that the isozymes pattern is also modified as compared to control cell wall βF (lanes 3 and 6, Fig. 6B). The isozymes of glycosylated or nonglycosylated cell wall βF forms obtained from seedlings treated with tunicamycin appear similarly modified as compared to control cell wall βF, except for the presence of an isozyme with pI 8.6 (lanes 2 and 5 in Fig. 6B). Further attempts at characterization of processing events that may occur on the enzyme N-linked oligosaccharide chain structure and induce peculiar cell wall βF charge properties were unsuccessfull. Different glycosidases such as α-mannosidase, endo H, endo D, neuraminidase or alkaline phosphatase used for *in vitro* degradation of the cell wall βF did not induce any change in the enzyme charge heterogeneity. Experiments using immobilized lectins (peanut agglutinin, phytohemagglutinin, castor bean agglutinin, and wheat germ agglutinin) have also failed to show any other structural differences between

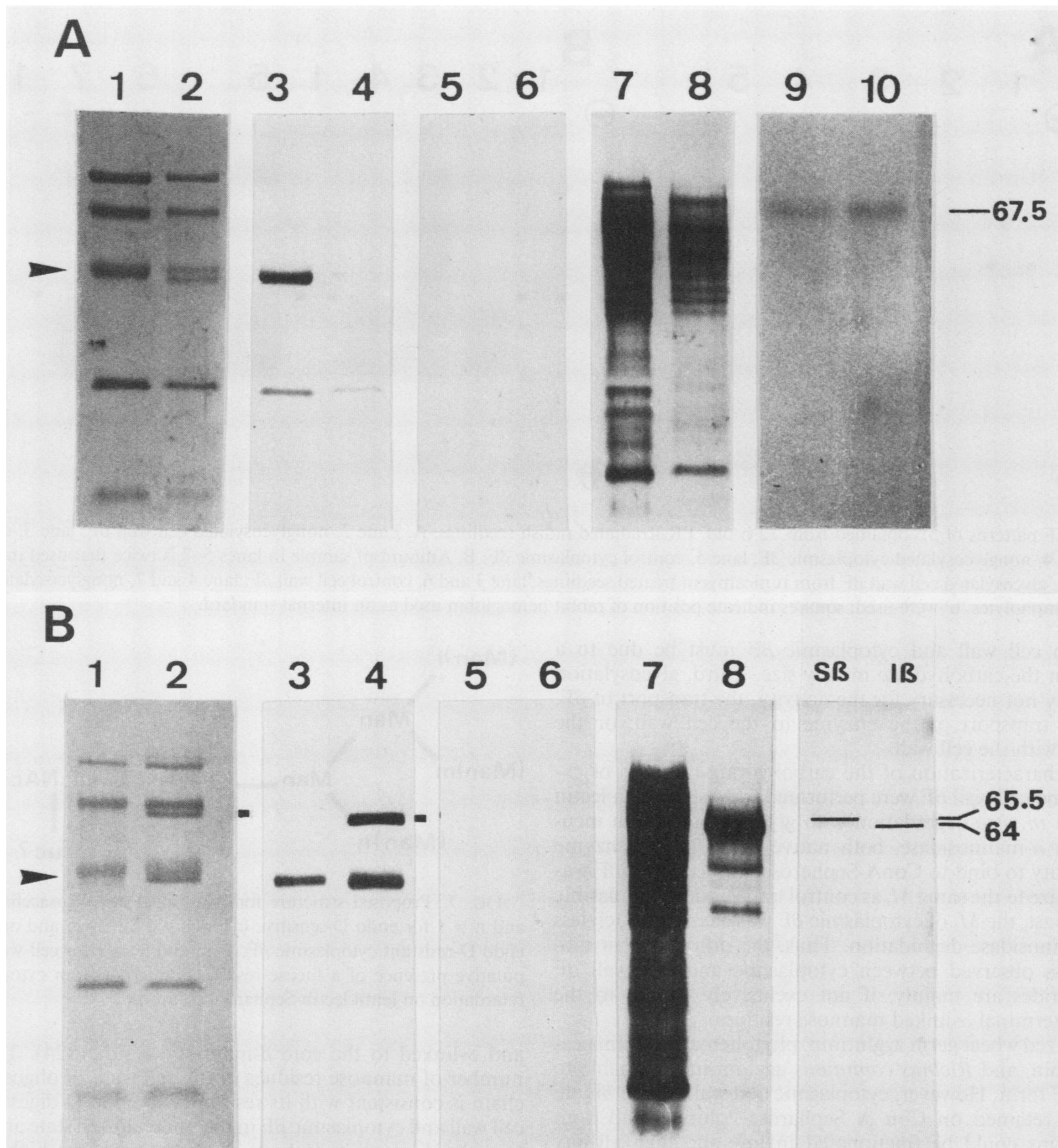

FIG. 5. Effect of glycosidase digestion on the M_r of βF. After incubation in the native enzyme form with endo H (A) or α-mannosidase (B), proteins were SDS-heat denatured, submitted to SDS-PAGE, transferred to paper, and detected on the nitrocellulose sheets. Samples were incubated in the presence (right lane in each panel) or in the absence (left lane) of glycosidase, except for lanes Sβ and Iβ (B) (see below). Mol wt standards of 94, 64, 43, 30, and 20.1 kD were stained with amido black (lanes 1, 2) or with ConA-peroxidase (lanes 3, 4). Lanes 6–10: polypeptides from cytoplasmic extract stained with ConA-peroxidase (lanes 7, 8) or for βF activity (lanes 9, 10). Lanes 5, 6 were stained for ConA-peroxidase but in the presence of 0.3 M α-methyl-D-mannoside. Lanes-Sβ and Iβ (B) are, respectively, cytoplasmic and cell wall βF incubated with α-mannosidase and stained for βF activity. The bands are too weak for photographic reproduction, but the original shows that cytoplasmic βF (Sβ) has an M_r of 65,000 and cell wall βF (Iβ) an M_r of 64,000 (see also Table I). Note the positions of ovalbumin (arrows) and of the heavy, ConA-reactive subunit of α-mannosidase (squares) and the presence of a ConA-reactive contaminant in the molecular standards particularly for lane 3 (A).

cytoplasmic and cell wall βF than the ones previously observed with ConA and lentil lectin (10, 11).

DISCUSSION

The results presented in this study show that cell wall and cytoplasmic βF have different carbohydrate moieties. We propose that these differences are generated during the secretion of the enzyme which occurs as a light-dependent process. Previous work from our laboratory (33) has shown that this secretory process is mediated by the intracellular endomembrane system (22, 26). Cell wall βF is slightly larger than cytoplasmic βF when both forms are normally glycosylated. However, the nonglycosylated forms synthesized in the presence of tunicamycin are either smaller or identical with respect to their M_r. First, these experiments show that the glycans are asparagine-linked oligosaccharides as found in many other plant glycoproteins (for review see Lehle and Tanner [19] and Sharon and Lis [28]). Second, since the nonglycosylated polypeptides synthesized in the presence of tunicamycin have the same M_r, the difference in

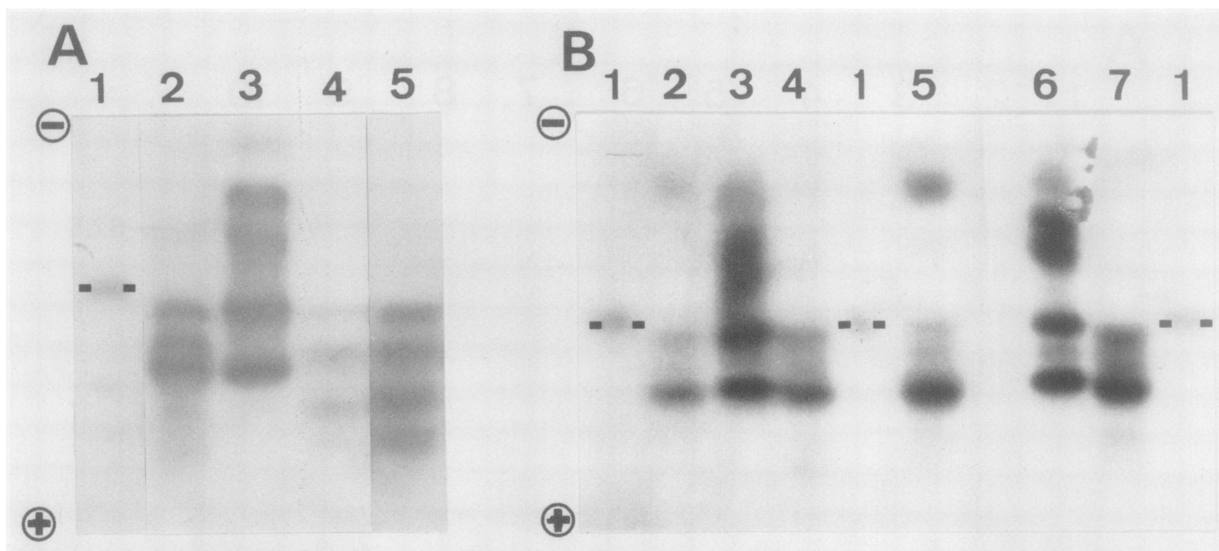

FIG. 6. IEF patterns of βF obtained from 72 h old, FR irradiated radish seedlings. A, Lane 2, nonglycosylated cell wall βF; lane 3, control cell wall βF; lane 4, nonglycosylated cytoplasmic βF; lane 5, control cytoplasmic βF. B, Amount of sample in lanes 5–7 is twice that used in lanes 2–4. Lane 2 and 5, glycosylated cell wall βF from tunicamycin treated seedlings; lane 3 and 6, control cell wall βF; lane 4 and 7, nonglycosylated cell wall βF. Carrier ampholytes 'b' were used; squares indicate position of rabbit hemoglobin used as an internal standard.

M_r between cell wall and cytoplasmic βF must be due to a difference in the carbohydrate moiety size. Third, glycosylation is apparently not necessary for the activity, the transport of βF, the correct transport of the enzyme to the cell wall, or the association with the cell wall.

Further characterization of the carbohydrate moieties of cytoplasmic and cell wall βF were performed from studies on lectin affinity and *in vitro* degradation with glycosidases. Upon incubation with α-mannosidase, both native forms of the enzyme lost the ability to bind to ConA-Sepharose, and cell wall βF was reduced in size to the same M_r as control undegraded cytoplasmic βF. In contrast, the M_r of cytoplasmic βF was changed much less after α-mannosidase degradation. Thus, the differences in molecular mass observed between cytoplasmic and cell wall βF oligosaccharides are mainly, if not exclusively, linked to the number of terminal α-linked mannose residues.

Immobilized wheat germ agglutinin, phytohemagglutinin, peanut agglutinin, and *Ricinus communis* agglutinin are unable to bind any βF form. However, cytoplasmic and wall bound βF are completely retained on Con A Sepharose columns and both enzyme forms could be fractionated in low and high affinity molecular variants by differential elution (10). Furthermore, cell wall bound βF contains a greater amount of molecular variants with low affinity for ConA than cytoplasmic βF (11). From both forms, only cytoplasmic βF binds to lentil lectin with a specifically retarded elution on lentil lectin-Sepharose columns (10). According to the specificity of lentil lectin (17) the presence of a fucosyl residue on the core di-*N*-acetylchitobiosyl sequence may be the reason for the retardation of cytoplasmic βF on lentil lectin-Sepharose columns (16). Fucosylation has already been described for the phytohemagglutinin PHA (3, 4) and this processing event occurs in many other plant glycoproteins. From the sensitivity of βF to α-mannosidase degradation and from the oligosaccharide structures required for binding to the different lectins tested (6, 7, 25), we conclude that the results are consistent with a high mannose type structure, as shown in Figure 7. At least part of cytoplasmic βF was digested with endo D to an active but ConA-unretained form reduced in size down to 52.5 kD. Thus, the oligosaccharide structure of the cytoplasmic βF sensitive to this endoglycosidase contains a small number of mannose residues, and has one mannose which is unsubstituted

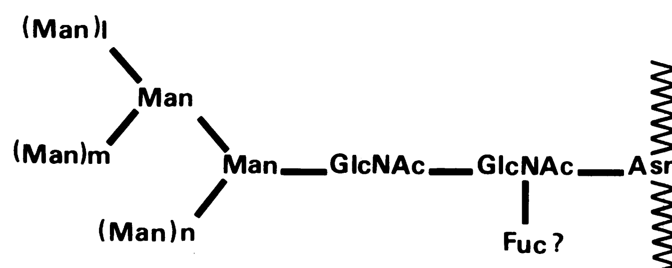

FIG. 7. Proposed structure for Asn-linked βF oligosaccharide. l, m and n = 1 for endo D-sensitive cytoplasmic βF; l, m and or n > 1 for endo D-resistant cytoplasmic βF; l, m and n ≫ 1 for cell wall βF. The putative presence of a fucose residue is inferred from cytoplasmic βF retardation on lentil lectin-Sepharose column.

and α-linked to the core β-mannosyl residue (24). The higher number of mannose residues in the cell wall βF oligosaccharide chain is consistent with its resistance to endo D digestion. Both cell wall and cytoplasmic βF in the undenatured state are resistant to endo H which hydrolyzes sugar chains of the high mannose type (29).

Both βF forms have increased electrophoretic mobility when endo H digestion is performed after denaturation by boiling with SDS. These results are consistent with descriptions in the literature of glycans resistant to *in vitro* glycosidase digestion because of sterically hindered accessibility (2, 5). Processing enzymes also have limited access to these glycans *in vivo*, and the glycans frequently remain as unmodified high-mannose type oligosaccharides (15, 31, 32). The presence of a second modified oligosaccharide chain, resistant to endo H even after protein denaturation, is indicated by the M_r differences observed between tunicamycin treated and endo H digested βF.

Preliminary studies performed with IEF in polyacrylamide gels revealed extensive polymorphism of βF, but the large amounts of enzyme required for detection with this technique was a limiting factor for further investigations. Using the increased sensitivity of agarose gel focusing (approximately × 100) we have shown that the heterogeneity of βF exists within a single plant and was not the result of the use of numerous plants when large-scale enzyme preparations are made. IEF shows that the isozymes

associated with the cell wall are more basic than those in cytoplasm and that there is a true light-dependent change in the pattern. A FR stimulation of the appearance of the basic forms of the enzyme is paralleled by the light-dependent appearance of βF in the cell wall (12). The polymorphism of nonglycosylated cytoplasmic βF obtained from tunicamycin treated seedlings was the same as that of glycosylated cytoplasmic βF. Thus, it can be concluded that cytoplasmic βF polymorphism is not due to glycosylation. Consequently, cytoplasmic βF forms shown previously to be antigenically related (9) most probably result from multigene families.

The results obtained with cell wall βF were quite different. Thus, nonglycosylated βF, produced when seedlings are grown in the presence of tunicamycin, is transported to the cell wall; the most basic enzyme forms disappeared and new acidic ones appeared when cell wall βF glycosylation was prevented. The zymogram of nonglycosylated cell wall βF closely resembled the one observed for cytoplasmic βF. Surprisingly analogous changes in charge properties were observed for part of the cell wall βF which is glycosylated under our conditions of tunicamycin treatment. Thus, certain charge properties of cell wall βF originate from posttranslational processing events which are inhibited in the presence of tunicamycin, but occur whether preliminary events of *N*-glycosylation take place or not.

In conclusion, the present results strongly indicate that neither glycosylation, nor basic charge properties are necessary for intracellular transport and cell wall association of an active βF. Cytoplasmic and cell wall βF were shown previously to be antigenically identical (11) and their differences in charge properties and mol wt are shown here to be the result of posttranslational events of processing occurring on common precursor polypeptides. That a precursor-product relationship really exists between the cytoplasmic and cell wall forms of this enzyme cannot be concluded from our results.

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