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Evidence for N- and C-terminal processing of a plant defense-related enzyme: Primary structure of tobacco prepro-β-1,3-glucanase

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ABSTRACT **Tobacco glucan endo-1,3-β-glucosidase (β-1,3-glucanase; 1,3-β-D-glucan glucanohydrolase; EC 3.2.1.39) exhibits complex hormonal and developmental regulation and is induced when plants are infected with pathogens. We determined the primary structure of this enzyme from the nucleotide sequence of five partial cDNA clones and the amino acid sequence of five peptides covering a total of 70 residues. β-1,3-Glucanase is produced as a 359-residue preproenzyme with an N-terminal hydrophobic signal peptide of 21 residues and a C-terminal extension of 22 residues containing a putative N-glycosylation site. The results of pulse–chase experiments with tunicamycin provide evidence that the first step in processing is loss of the signal peptide and addition of an oligosaccharide side chain. The glycosylated intermediate is further processed with the loss of the oligosaccharide side chain and C-terminal extension to give the mature enzyme. Heterogeneity in the sequences of cDNA clones and of mature protein and in Southern blot analysis of restriction endonuclease fragments indicates that tobacco β-1,3-glucanase is encoded by a small gene family. Two or three members of this family appear to have their evolutionary origin in each of the progenitors of tobacco, *Nicotiana sylvestris* and *Nicotiana tomentosiformis*.**

Glucan endo-1,3-β-glucosidases (β-1,3-glucanase; 1,3-β-D-glucan glucanohydrolase; EC 3.2.1.39) have been implicated in the defense reaction of plants against pathogens (1). They are induced in response to the stress hormone ethylene (2, 3), to infection (4–10), and to elicitors (9, 11) and have antifungal activity *in vitro* (12). We have described a basic β-1,3-glucanase of ≈33 kDa, which is a major component (5–10%) of the soluble protein of cultured tobacco tissues and the lower leaves and roots of tobacco plants (13, 14). This β-1,3-glucanase exhibits complex hormonal and developmental regulation. It is induced in tobacco leaves treated with ethylene (3) or infected with tobacco mosaic virus (8, 10) and is down-regulated at the mRNA level in cultured tobacco cells by combinations of the plant hormones auxin and cytokinin (3, 14–16).

We have isolated and sequenced five partial cDNA clones that together cover the entire coding region for tobacco β-1,3-glucanase. Our results show that the primary translation product is a preproenzyme with an N-terminal hydrophobic signal peptide and a C-terminal extension. An N-glycosylated proenzyme is formed as an intermediate that is processed to the mature form with the loss of the oligosaccharide and the C-terminal peptide.

MATERIALS AND METHODS

Plant Material and Culture Methods. *Nicotiana tabacum* L. cv. Havana 425, *Nicotiana sylvestris*, and *Nicotiana tomentosiformis* were grown from seed in a greenhouse. The cloned line of Havana 425 pith tissue, 275N (17), was cultured under conditions that induce β-1,3-glucanase accumulation (15). Suspensions of leaf protoplasts were prepared from axenic Havana 425 shoot cultures and cultured in liquid K3 medium as described by Paszkowski *et al.* (18).

Construction and Screening of a cDNA Library. A cDNA library was prepared from poly(A)$^+$ RNA isolated from 275N tissue (15) as described (19) and inserted into the *Pst* I site of plasmid pBR322 by the homopolymeric poly(dC·dG) tailing method (20). Recombinant colonies were screened by colony hybridization with the ^{32}P-labeled *Pst* I insert of the tobacco β-1,3-glucanase cDNA clone pGL43 (15) as a hybridization probe.

Deoxynucleotide Sequence Analysis. Restriction fragments were cloned into the M13 vectors mp18 and mp19 (21) and sequenced in both orientations by the dideoxynucleotide chain-termination method (22). Nucleotide sequences were analyzed with the computer program of Queen and Korn (23).

Southern Blot Analysis. DNA (5 μg) prepared from leaves by the method of Murray and Thompson (24) was digested to completion with the restriction endonucleases *Eco*RI, *Hin*dIII, or *Xba* I. The resultant fragments were separated by electrophoresis on a 0.8% agarose gel, transferred to a Zeta-Probe nylon membrane (Bio-Rad), and hybridized as recommended by the manufacturer. The final wash was at 50°C in 0.2 × SSC (1× SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7)/0.1% $NaDodSO_4$. The *Pst* I insert of the tobacco β-1,3-glucanase cDNA clone pGL43 (15) was labeled by using a random-primer labeling kit (Boehringer Mannheim).

Analysis of Proteins. β-1,3-Glucanase was purified from 275N tissue through the CM-Sephadex G-50 chromatography step for chitinase (16). Fractions containing β-1,3-glucanase were pooled, concentrated by ultrafiltration (immersible CX-10 ultrafilter; Millipore), adjusted to 100 mM NaCl/10 mM Tris·HCl, pH 8.4, and passed over a column containing regenerated chitin (25) to remove traces of chitinase. The final product was >95% pure as judged by $NaDodSO_4$/PAGE with Coomassie blue R-250 staining. To prepare tryptic peptides, β-1,3-glucanase (320 μg in 1 ml) was adjusted to 8 M urea and dialyzed against 2 M urea/0.1 M NH_4HCO_3. Trypsin (7 μg; Worthington) was added and the mixture was incubated for 6.5 hr at 37°C. Additional trypsin (7 μg) was added and the reaction was stopped after 24 hr by adjusting the pH value to 3 with HCl. The procedures for CNBr cleavage, fractionation of peptides by HPLC, amino acid analysis, and amino acid sequencing were as described (26).

Pulse–Chase Experiments. Leaf protoplasts (5 × 10^4 cells) were incubated for 45 min at 26°C in the dark with shaking (50 rpm) in wells of a Falcon 24-well tissue culture plate (Becton Dickinson) containing 500 μl of K3 medium without phytohormones and ≈20 μCi of L-[^{35}S]methionine (specific activ-

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ity, ≈1275 Ci/nmol; 1 Ci = 37 GBq; Amersham). Where indicated, the pulse labeling was followed by incubation for 11.75 hr with methionine (final concentration, 1 mM) added to the wells. A replicate set of wells was supplemented with tunicamycin (20 μg/ml) (Calbiochem) added 20 min before the L-[^{35}S]methionine. Protein extracts were prepared by adding 100 μl of a solution of 6 mM dithiothreitol, 6% (vol/vol) Triton X-100, and 200 mM Tris·HCl (pH 8.0) to each well, incubating for 10 min at 26°C, and freezing the samples in liquid N_2. The extracts were divided into two aliquots. The protein in one aliquot was precipitated with acetone and analyzed by NaDodSO_4/PAGE (17). The second aliquot was incubated with rabbit anti-tobacco β-1,3-glucanase IgG (27) bound to protein A-Sepharose CL-4B (Pharmacia), and the immunoadsorbed polypeptides were analyzed by NaDodSO_4/PAGE (15). Urs Voegeli (Botanical Institute, Basel) kindly provided [^{35}S]methionine-labeled *in vitro* translation products obtained with a rabbit reticulocyte system. Total RNA from 275N tissues induced to produce β-1,3-glucanase was the source of mRNA (15). Gels were calibrated with low molecular weight standards (Bio-Rad).

RESULTS

Deoxynucleotide Sequence of β-1,3-Glucanase cDNAs. A cDNA library prepared from 275N tobacco tissue induced to produce β-1,3-glucanase was screened with the insert of pGL43, a partial cDNA clone for tobacco β-1,3-glucanase (15). Although β-1,3-glucanase mRNA is an abundant component of the poly(A)$^+$ RNA used to prepare the cDNA libraries (15), only a small number of partial cDNA clones could be identified in several libraries. Five of the seven positive recombinant clones with the longest cDNA inserts were sequenced (Fig. 1). Unexpectedly, three of the clones did not include 3′ regions of the mRNA. The composite sequence has a single uninterrupted open reading frame of 1083 nucleotides beginning at position 1 (Fig. 2). The consensus sequence for the start of translation in plants is AACAAUGGC, in which the purine at position −3 is important for efficient translation in a reticulocyte lysate system but is not important in a wheat germ system (28). The first available initiation codon in the frame is at position 7, which gives the sequence UCAAAUGCG. Translation starting with this codon generates a polypeptide 359 residues long with a M_r 39,173 corresponding in size to the primary translation product of tobacco β-1,3-glucanase (15, 29). The sequence of four tryptic peptides and a CNBr peptide of the pure enzyme covering a total of 70 amino acid residues was the same as the deduced sequences providing unambiguous evidence that the cloned cDNAs were derived from the mRNA for β-1,3-glucanase (Fig. 2). Additional amino acids were found at two positions of the polypeptide, 284 and 287, that were not represented in the cDNA clones isolated.

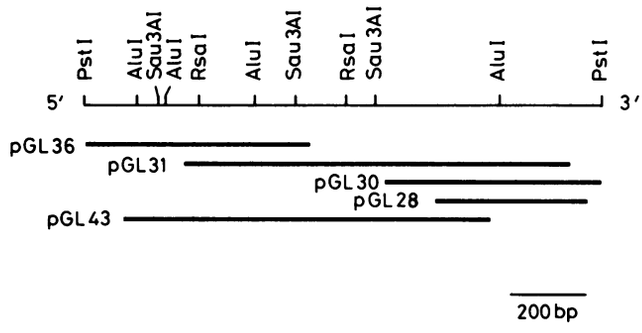

FIG. 1. Partial restriction map showing the lengths and positions of the cDNA clones of tobacco β-1,3-glucanase that were sequenced. bp, Base pair(s).

The overlapping sequences of the inserts of clones pGL28, pGL30, and pGL31 were identical suggesting that they represent the same mRNA. Comparison of the inserts of pGL31 and pGL36, which overlap for a length of 328 nucleotides, showed two silent substitutions at positions 555 and 564 and two substitutions at positions 547 and 548 resulting in a single amino acid substitution of threonine for valine. The sequence of the pGL43 insert differed from the sequences of the pGL31 and pGL36 inserts at 13 positions; 12 differences occurred in the third wobble position of the codon and were silent and 1 difference at position 1006 resulted in the conservative change from leucine to isoleucine that was also detected by amino acid sequencing of the mature enzyme.

The composite cDNA sequence had two putative polyadenylylation signals for plants, AAGAAA at position 1285 and ATTAAT at position 1321 (30). The overlapping sequences of the cDNA inserts from pGL28 and pGL30 were identical up to position 1310, where the poly(A) tail of pGL28 started 18 nucleotides downstream from the first polyadenylylation signal. The poly(A) tail of pGL30 started 24 nucleotides downstream from the second polyadenylylation signal. This suggests that there is an alternative polyadenylylation site for the β-1,3-glucanase transcripts as reported for other plant genes (31).

Southern Blot Analysis. Southern blot analysis was performed with DNA isolated from tobacco (*N. tabacum*) and its progenitor species, *N. sylvestris* and *N. tomentosiformis* (32, 33). The restriction endonuclease fragments that hybridized to the insert of tobacco β-1,3-glucanase cDNA clone pGL43 are shown in Fig. 3. Each of the restriction endonucleases generated four hybridizing fragments with tobacco DNA. The *Eco*RI and *Xba* I fragments corresponded in size and intensity of hybridization to two fragments generated with DNA from each of the progenitor species. *Hin*dIII generated fragments with *N. sylvestris* DNA that did not correspond in size to the fragments generated with tobacco DNA. Analyses with combinations of restriction nucleases and other hybridization probes suggest that differences in intensity of hybridization reflect differences in homology rather than copy number.

Primary Structure of Prepro-β-1,3-Glucanase. Using the rules for protein processing of Von Heijne (34), a highly probable cleavage site was identified between Ala-21 and Gln-22 of the amino acid sequence deduced from cDNA clones. Processing at this site is consistent with our observation that the N-terminal end of mature β-1,3-glucanase is blocked. N-terminal glutamines are often blocked by spontaneous or enzymatic conversion to a pyroglutamyl residue (35). We purified an N-terminally blocked peptide obtained by CNBr cleavage of the mature enzyme with the composition $Glu_{0.6}Ser_{1.0}Ile_{1.0}Gly_{2.0}Val_{1.1}Tyr_{1.0}Hse$, where Hse is homoserine, corresponding to the N-terminal sequence Gln-Ser-Ile-Gly-Val-Cys-Tyr-Gly-Met. These results indicate that the primary translation product for β-1,3-glucanase has a hydrophobic signal peptide 21 residues long and that the N-terminal end of the mature enzyme is Gln-22.

The primary translation product of β-1,3-glucanase is ≈4 kDa larger than that of the mature enzyme (15, 29). The presence of a signal peptide could only account for a difference of ≈2 kDa, suggesting the protein undergoes additional processing. Cleavage of the enzyme with CNBr yielded a peptide that lacked homoserine indicating that it was the C-terminal end of the mature protein. Its amino acid composition and sequence were determined, and the results showed it to correspond to residues 308–337 of the deduced sequence (Fig. 2). Since the stop codon occurs 22 amino acid residues downstream of Gly-337, we conclude that a C-terminal extension is removed during processing.

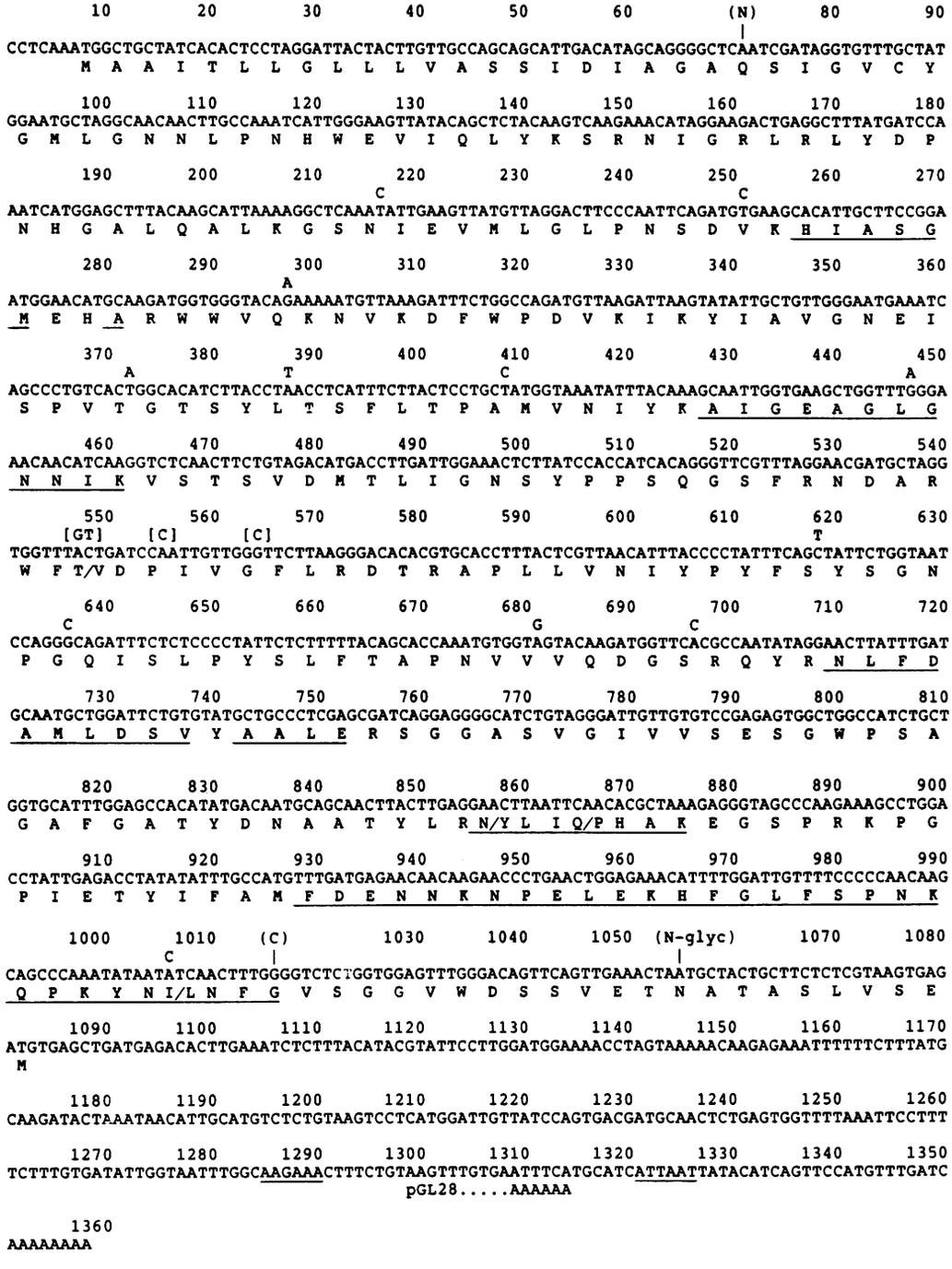

FIG. 2. Nucleotide sequence and deduced amino acid sequence of a composite cDNA for tobacco β-1,3-glucanase mRNA obtained by combining pGL30, pGL31, and pGL36. Nucleotides unique to pGL36 (letters in brackets above the sequence) and pGL43 (letters above the nucleotide sequence) are indicated. Putative polyadenylylation sequences are underlined. (N) and (C), N- and C-terminal amino acids of mature β-1,3-glucanase; (N-glyc), putative N-glycosylation site. The positions of amino acids confirmed by peptide sequencing are underlined. Heterogeneity in amino acid sequence is depicted by a slash.

Pulse–Chase Experiments. The processing of β-1,3-glucanase deduced from structural studies was confirmed in pulse–chase experiments. The major polypeptide labeled following pulse labeling of tobacco protoplasts with [^{35}S]-methionine for 45 min was immunoadsorbed by anti-β-1,3-glucanase antibody (Fig. 4). It had a molecular mass slightly larger than the *in vitro* translation product included on the same NaDodSO$_4$/polyacrylamide gel as a standard for the preproenzyme. After the chase, the majority of the immunoreactive material co-migrated with the mature form of β-1,3-glucanase, indicating that most of the precursor form of β-1,3-glucanase was processed to the mature form during the ≈12-hr chase.

The deduced sequence of the precursor has a single putative site for N-glycosylation, Asn-Xaa-Ser/Thr (36), starting at Asn-350 in the C-terminal extension. This suggested that processing of β-1,3-glucanase involves glycosylation. To test this hypothesis, replicate protoplast suspensions used in the pulse–chase experiments were incubated with tunicamycin, which inhibits the synthesis of lipid-linked oligosaccharides and is known to block N-glycosylation of plant polypeptides (37). Tunicamycin treatment inhibited [^{35}S]methionine incorporation into polypeptides by ≈50%. The immunoreactive polypeptide obtained after pulse labeling in the presence of tunicamycin had a molecular mass ≈2 kDa smaller than the polypeptide obtained with untreated

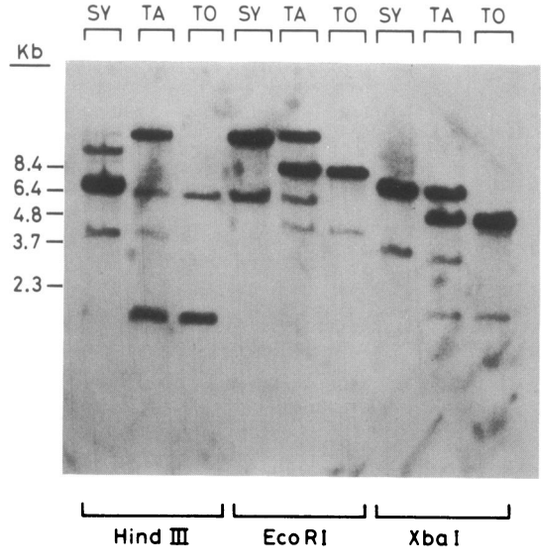

FIG. 3. Southern blot analysis of DNA prepared from leaves of tobacco (lanes TA), *N. sylvestris* (lanes SY), and *N. tomentosiformis* (lanes TO) digested with the restriction endonuclease indicated and hybridized with the insert of tobacco β-1,3-glucanase cDNA plasmid pGL43. Scale at left shows the size of marker DNAs in kilobases (kb).

protoplasts, as expected were tunicamycin to block the addition of an oligosaccharide side chain. On the other hand, the immunoreactive polypeptide obtained after the chase treatment in the presence of tunicamycin had the same molecular mass as mature β-1,3-glucanase. Plant *N*-glycans (37) and the N-terminal signal sequence of β-1,3-glucanase have the same molecular mass, ≈2 kDa. Therefore, the results indicate that the precursor loses the signal peptide and gains an oligosaccharide side chain. Later, the resultant proenzyme is deglycosylated and the C-terminal extension is lost to give the mature enzyme. Processing to the mature form, however, does not require glycosylation.

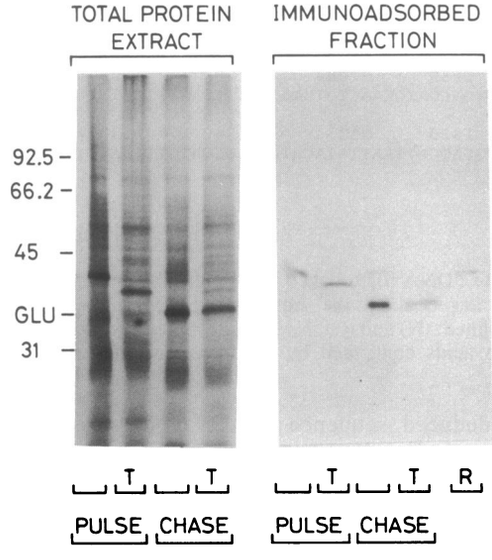

FIG. 4. Autoradiogram of polypeptides from tobacco protoplasts incubated for 45 min with [^{35}S]methionine (pulse) and then ≈12 hr with 1 mM methionine (chase). Immunoadsorption was performed with anti-tobacco β-1,3-glucanase. R, products obtained by *in vitro* translation of total RNA from cells induced to produce β-1,3-glucanase; T, protoplasts treated with tunicamycin. Scale at left shows the molecular mass of protein standards in kDa. GLU, position of purified β-1,3-glucanase.

DISCUSSION

Three classes of cDNA clones were isolated with similar coding sequences. In addition, there was heterogeneity in the amino acid sequence not represented in the mRNAs corresponding to the cDNA clones isolated. Thus, cultured tobacco cells induced to produce β-1,3-glucanase have at least four transcriptionally active β-1,3-glucanase genes. This finding and the small number of restriction fragments detected in Southern blot experiments indicate that tobacco β-1,3-glucanase is encoded by a small gene family. Modern tobacco arose by the hybridization of two ancestral species, *N. sylvestris* and *N. tomentosiformis* (32, 33). Comparison of the restriction fragments generated from DNA of tobacco and the ancestral species suggests that two or three members of the tobacco β-1,3-glucanase family have their evolutionary origin in each of these ancestors.

Computer searches of the European Molecular Biology Laboratory version 14 (38) and National Biomedical Research Foundation version 15 (39) data banks gave only one protein with significant sequence similarity to tobacco β-1,3-glucanase. After introducing three gaps into the sequence of the mature tobacco enzyme, the amino acid sequence of barley endosperm (1→3,1→4)-β-glucanase isozyme II (40) was identical at 47% of the positions. In addition, there are highly conserved regions (12 of 15 positions) at the N- and C-terminal ends of the mature proteins. The sequence of the first 22 N-terminal amino acids of a putative β-1,3-glucanase purified from *Nicotiana plumbaginifolia* (41) is identical to the N-terminal sequence of the tobacco enzyme except for the N-terminal amino acid, which is glutamic acid.

Pulse–chase experiments provide evidence that prepro-β-1,3-glucanase is processed sequentially. First, the N-terminal signal peptide is removed and an oligosaccharide side chain is added, as has been reported for the seed storage proteins phaseolin and Con A (42, 43). Later, the oligosaccharide and the C-terminal extension are removed to give the mature enzyme. Loss of C-terminal extensions has been reported for the plant proteins, prothaumatin (44), proCon A (43, 45, 46), and isolectin 3 of wheat germ agglutinin (47). No significant sequence similarities were found between these peptides and the C-terminal extension of the tobacco enzyme.

The distinctive feature of tobacco β-1,3-glucanase processing is that the proenzyme undergoes a loss of an oligosaccharide side chain and the C-terminal extension. Mature β-1,3-glucanase does not contain *N*-acetylglucosamine (13), which is a constituent of plant *N*-glycans (37). This observation and the fact that the only putative N-glycosylation site in the molecule is in the C-terminal extension suggest that processing to the mature form results from the loss of an N-glycopeptide. This mechanism has been reported for processing of isolectin 3 of wheat germ agglutinin (47); and, in the case of Con A, an N-glycoprotein is lost from an interior region of the proprotein followed by polypeptide ligation to give the mature protein (43, 45, 46, 48).

Processing is important in the intracellular transport and secretion of plant proteins (37). The intracellular location of tobacco β-1,3-glucanase is not known. Acidic isoforms of β-1,3-glucanase of apparent molecular mass of ≈40 kDa (49) are localized in the intercellular spaces of leaves infected with tobacco mosaic virus (8). The relationships of these isoforms and the possibility that they arise by differential processing are areas for further study.

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