

In Vitro Synthesis and Processing of Tomato Fruit Polygalacturonase¹

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ABSTRACT

The *in vitro* processing of tomato fruit polygalacturonase (PG) (poly[1,4- α -D-galacturonide]glucanohydrolase, EC 3.2.1.15) was studied. Complete chemical deglycosylation of a mixture of mature, purified PG 2A and PG 2B isozymes (45 and 46 kilodaltons; respectively) with trifluoromethane sulfonic acid yielded a single polypeptide of 42 kilodaltons. Similarly, N-terminal amino acid sequencing of the PG 2A/2B isozyme mixture yielded a single 21 amino acid N-terminal sequence, suggesting that the two isozymes result from differential post-translational processing of a single polypeptide. Translation of PG mRNA *in vitro* results in the synthesis of a single polypeptide with an apparent molecular weight of 54 kilodaltons. Nucleotide sequence analysis of a full-length PG cDNA clone indicates that the large size difference between the PG *in vitro* translation product and the mature isozymes is due to the presence of a 71 amino acid (8.2 kilodaltons) domain at the N-terminus of *in vitro* translated PG, consisting of a hydrophobic signal sequence followed by a highly charged prosequence. To determine the precise cleavage site of the signal sequence, PG mRNA was translated *in vitro* in the presence of canine pancreas microsomal membranes. This resulted in the production of two glycosylated PG processing intermediates with apparent molecular weights of 58 and 61 kilodaltons. The PG processing intermediates were shown to be sequestered within the lumen of the microsomal membranes by protease protection and centrifugational analysis. Deglycosylation of the PG processing intermediates with endoglycosidase H yielded a single polypeptide with an apparent molecular weight of 54 kilodaltons. The production of two distinct, glycosylated processing intermediates from the single *in vitro* translated PG polypeptide suggests a mechanism by which the differential glycosylation observed for the mature PG 2A and PG 2B isozymes may occur. Edman degradation of ³H-labeled 58 and 61 kilodalton PG processing intermediates indicates that the site of signal sequence cleavage is after amino acid 24 (serine). These results suggest that the proteolytic processing of PG occurs in at least two steps, the first being the co-translational removal of the 24 amino acid signal sequence and the second being the presumed post-translational removal of the remaining highly charged 47 amino acid prosequence.

softening process. The large body of evidence in support of this has recently been reviewed (2). PG activity increases dramatically during ripening (5) due to *de novo* synthesis of the enzyme (5, 9). Recent work with PG cDNA clones has shown that during the ripening process the steady state level of PG mRNA increases over 2000-fold (3, 9).

The PG activity extracted from ripe fruit is due to the presence of three closely related isozymes, PG 1, PG 2A, and PG 2B (6). All three isozymes are glycoproteins and antibodies to PG 2A have been shown to cross-react with both PG 2B and PG 1 (1). PG 2A and PG 2B also have identical isoelectric points, and similar mol wt (45 and 46 kD, respectively). The PG 1 isozyme is an approximately 100 kD holoenzyme which, when electrophoresed under denaturing conditions, yields a polypeptide identical in size to PG 2A (45 kD). Digestion of PG 1 and PG 2A with trypsin and chymotrypsin yields nearly identical peptide patterns (20, 23). Though the exact relationship of the three isozymes is at present unclear, the similarities of their biochemical and physical characteristics suggest that they may arise by differential post translational modifications of a single polypeptide chain.

Although three PG isozymes are observed *in vivo*, immunoprecipitation of ripe tomato fruit *in vitro* translation products with PG antiserum yields a single 54 kD polypeptide (4, 9, 19). Because PG is a secreted enzyme, the large size difference between the PG *in vitro* translation product and mature isozymes (7–9 kD) can be accounted for, in part, by the presence of an N-terminal signal sequence presumably required for the initial co-translational transport of the protein into the lumen of the endoplasmic reticulum. In addition to confirming the presence of a signal sequence, nucleotide sequence analysis of full-length PG cDNA clones has identified, immediately following the signal sequence, a highly charged prosequence of approximately 50 amino acids (5 kD) that is not found in the mature PG proteins (2, 11, 20). The function of this prosequence is at present unknown as is the exact cleavage site of the signal sequence. As an initial step in understanding the mechanisms involved in PG secretion and posttranslational processing we have characterized the *in vitro* N-terminal cleavage and glycosylation of PG by canine pancreas microsomal membranes. We also present data supporting the view that PG 2A and PG 2B are derived from post-translational modification of a single polypeptide.

MATERIALS AND METHODS

Source of Materials. L-[4,5-³H]Leucine at 158 Ci/mmol, L-[4,5-³H]isoleucine at 106 Ci/mmol, L-[³⁵S]methionine at 1260 Ci/mmol and canine pancreatic microsomal membranes were purchased from Amersham Corp. Nuclease-treated rabbit reticulocyte lys

The ripening of tomatoes is a complex, developmentally regulated process involving a large number of coordinated biochemical and physiological changes in the tissues involved (7, 8). The softening of tomato fruits during ripening has been studied extensively and a single cell wall enzyme, polygalacturonase (poly[1,4- α -D-galacturonide]glucanohydrolase, EC 3.2.1.15) or PG,² has been implicated as the major enzyme mediating the

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² Abbreviations: PG, polygalacturonase (EC 3.2.1.15); PG 1 and PG 2 are high and low mol wt isozymes, respectively; PTH, phenylthiohydantoin; TFMS, trifluoromethane sulfonic acid.

ate and RNasin were purchased from Promega Biotec. Vectors, reagents, and enzymes for *in vitro* transcription were obtained from Stratagene Cloning Systems (La Jolla, CA).

PG Purification, Antibodies, and N-Terminal Sequence Analysis. The purification of PG from red ripe tomato fruit (1) and the production and characterization of PG antibodies has been described elsewhere (9). PG protein purified by this method contained a mixture of both PG 2A and PG 2B isozymes. The relative amount of each isozyme present in the sample was quantified as follows. Two μg of the purified PG isozyme mixture was separated by SDS-PAGE and stained with Coomassie brilliant blue G-250. After destaining, the relative amounts of each isozyme were determined by scanning the gel at 550 nm with a laser densitometer. Two hundred μg of the purified PG isozyme mixture was subjected to N-terminal amino acid analysis by sequential Edman degradation using a Beckman 890C liquid phase sequencer. PTH-amino acids were identified and quantified by GLC.

Isolation and Sequence Analysis of a Full-Length PG cDNA Clone. A previously constructed ripe tomato fruit cDNA library (9) was rescreened in search of full-length PG cDNA clones. First, a large number of PG cDNAs were selected by screening at high density with the 550 base pair ^{32}P -labeled insert of pPG16 (a previously characterized PG cDNA clone [9]). Positive colonies were plated at low density and screened with an end labeled 35-base synthetic oligodeoxyribonucleotide (5'-TCITAIGTIT-TICCITCICCTTIGCICCAAACT-3') corresponding to amino acid residues 9 through 20 of the N terminus of PG 2A and PG 2B. The oligonucleotide contained inosine (I) at redundant positions. Numerous cDNA clones were identified and two (pPG1.8 and pPG1.9) were further characterized by restriction mapping and nucleotide sequence analysis. DNA sequencing was carried out by the dideoxy chain termination method (18).

In Vitro Transcription of PG mRNA. Pure PG mRNA for use in translations was produced by *in vitro* transcription. The 1.75 kb insert of the full-length PG cDNA clone pPG1.9 was subcloned into a transcription vector (Bluescribe vector, Stratagene, LaJolla, CA) in such a way that sense PG mRNA would be transcribed by T7 RNA polymerase. The resulting plasmid (pBS1.9) was linearized by digestion with Hinc II and used as a template for transcription reactions. The transcription reactions contained 40 mM Tris (pH 8.0), 8 mM MgCl_2 , 2 mM spermidine, 50 mM NaCl, 400 μM of each ribonucleotide, 30 mM dithiothreitol, 25 units RNasin, 1 μg of linearized pBS1.9 template, and 10 units of T7 RNA polymerase in a total volume of 25 μl . The reaction was incubated at 37°C for 30 min at which time 5 units of RNase-free deoxyribonuclease I was added and the reaction incubated for a further 15 min at 37°C. The reaction was then phenol extracted and precipitated with 0.4 volumes of 5.0 M ammonium acetate (pH 5.5) and 2.5 volumes of ethanol at -20°C. The transcribed PG mRNA was collected by centrifugation for 30 min at 0°C in a microfuge, dried under vacuum, resuspended in diethylpyrocarbonate treated water, quantified by UV absorption, and used for *in vitro* translations. Such transcription reactions yielded 6 to 10 μg of PG mRNA per μg of template.

In Vitro Translations and Co-Translational Processing. *In vitro* transcribed PG mRNA was translated in a nuclease-treated rabbit reticulocyte lysate cell-free translation system (Promega Biotec) following the instructions supplied by the manufacturer except that the final concentration of K^+ and Mg^{2+} in all reactions was adjusted to 90 and 0.7 mM, respectively. The final concentration of PG mRNA in the reaction mixture was 15 to 25 ng/ μl . Co-translational processing of PG was performed as follows. A 40 μl reaction mixture contained either [^{35}S]methionine (50 μCi), [^3H]leucine (50 μCi), or [^3H]isoleucine (17 μCi), RNasin (5 units), canine pancreatic microsomal membranes (5 μl), PG mRNA (1

μg), 1 μl of a 1 mM amino acid mixture (minus the respective radiolabeled amino acid), and nuclease-treated rabbit reticulocyte lysate (17.5 μl). The components were added in the order listed and incubated at 30°C for 60 min. When necessary, co-translationally processed, membrane-associated PG *in vitro* translation products were separated from soluble nonprocessed PG translation products by first centrifuging for 30 min at 0°C in an Eppendorf microfuge, removing the supernatant, resuspending the pellet in PBS, and recentrifuging for 30 min at 0°C. The resulting membrane pellet was either analyzed directly by SDS-PAGE or resuspended in the appropriate buffer for immunoprecipitations or endoglycosidase H digestion. Immunoprecipitations, gel electrophoresis, and fluorography were performed as described (9).

Proteinase K Treatment. Total translation products from co-translational processing experiments (10 μl) were placed into three microfuge tubes. One tube was untreated as a control, 5 μg of proteinase K was added to the second tube and 5 μg of proteinase K and 1 μl of 10% (v/v) Triton X-100 was added to the third tube. The reactions were incubated at 0°C for 1 h after which 1 μl of 100 mM phenylmethylsulfonyl fluoride was added to each tube and the incubation continued for 5 min. Forty μl of electrophoresis sample buffer (2% SDS, 5% 2-mercaptoethanol, 62 mM Tris, 10% glycerol [pH 6.8]) was then added, the samples boiled for 3 min, and analyzed by SDS-PAGE and fluorography.

Deglycosylation of Proteins. Deglycosylation of the purified PG 2A and 2B isozyme mixture was carried out enzymically and chemically. Prior to enzymic deglycosylation with endoglycosidase H, the protein sample was denatured by heating to 100°C in the presence of 1% (w/v) SDS. Following this pretreatment the sample was diluted five-fold with 150 mM sodium acetate (pH 5.7), 10 munits of endoglycosidase H was added, and the mixture incubated at 37°C. After 6 h, an equal volume of 2 times electrophoresis sample buffer was added (125 mM Tris (pH 6.8), 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol), the mixture heated in boiling water for 3 min, and an aliquot analyzed by SDS-PAGE. Chemical deglycosylation of the PG isozyme mixture was carried out by anhydrous treatment with TFMS as described (10). Membrane-associated *in vitro* processed PG translation products were enzymically deglycosylated with endoglycosidase H as follows. After *in vitro* translation and processing were completed the membranes from a 120 μl reaction were collected by centrifugation as described above. The resulting membrane pellet was resuspended in 20 μl of 150 mM sodium acetate (pH 6.0) containing 0.5% (v/v) Triton X-100. The solubilized membranes were split into two equal samples and pretreated with SDS at 100°C as described above. Ten munits of endoglycosidase H were added to one sample and both samples were incubated for 2 h at 37°C. At the end of incubation an equal volume of 2 times electrophoresis sample buffer was added to each sample followed by boiling and analysis by SDS-PAGE and fluorography.

N-Terminal Analysis of PG Processing Intermediates. *In vitro* translation reactions with canine pancreas microsomes were scaled up (20- to 30-fold) and performed as described earlier except that either [^3H]leucine or [^3H]isoleucine was included in place of [^{35}S]methionine. The ^3H -labeled, membrane-associated PG processing intermediates were collected by centrifugation as described above. The membrane pellets were resuspended in PBS, immunoprecipitated with PG antiserum, and released into 150 μl of electrophoresis sample buffer as described (9). The immunoprecipitated proteins were diluted to 3.0 ml with H_2O and dialyzed overnight against 4 L of 1 mM Na_2HPO_4 (pH 7.6) with three buffer changes. The protein samples (74,000 cpm [^3H]isoleucine and 48,000 cpm [^3H]leucine) were then lyophilized and subjected to N-terminal sequencing. The radioactivity re-

moved by each sequencing cycle was determined by liquid scintillation counting.

RESULTS

Deglycosylation and N-Terminal Sequence Analysis of PG 2A and PG 2B. Our purification scheme represents an extension of the basic procedure described by Ali and Brady (1) with minor modifications (9). PG protein purified by this method contained a mixture of both PG 2A and PG 2B isozyme as judged by SDS-PAGE (Fig. 1, lane 1). The levels of each isozymes present in this mixture were quantified by scanning laser densitometry of an electrophoretically separated, Coomassie-stained sample (see O.D. trace to the left of Fig. 1, lane 1). By this method the mixture was found to contain 80% PG 2A (45 kD isozyme) and 20% PG 2B (46 kD isozyme).

To determine if the size difference between the PG 2A and PG 2B isozymes resulted from differential glycosylation of a single polypeptide we performed enzymic and chemical deglycosylation experiments to determine the size of the isozymes' respective polypeptide chains. Enzymic deglycosylation of the PG 2A/2B isozyme mixture with endoglycosidase H did not alter the electrophoretic mobilities of the proteins (Fig. 1, lane 2). Therefore, we chose to chemically deglycosylate the isozyme mixture by anhydrous treatment with TFMS. Chemical deglycosylation of the PG 2A/2B isozyme mixture yielded a single polypeptide with increased electrophoretic mobility and an apparent mol wt of 42 kD (Fig. 1, lane 3). This size is in good agreement with that of 41,833 D deduced from nucleotide sequence analysis of a full length PG cDNA clone (20). This result suggests that PG 2A and PG 2B have a polypeptide chain of identical size and that the different apparent mol wt of the isozymes result from differential glycosylation.

Further similarity between the PG 2A and PG 2B isozymes is indicated by N-terminal sequence analysis of the two proteins. Sequential Edman degradation of a 5 nmol sample of the PG 2A/2B isozyme mixture yielded the single 21 residue N-terminal sequence shown in Figure 2 (boxed sequence). The yield of PTH amino acid derivatives from initial sequencing cycles was 4.9 nmol (98% yield) indicating that both isozymes present in the mixture were being sequenced. Had either of the isozymes not been sequenced the expected yield would have been much less than 98%. Furthermore, if the isozymes possessed different N-terminal sequences at least some sequencing cycles would have

yielded two different PTH amino acid derivatives in an approximate 80:20 ratio. Because only a single PTH derivative was observed for all 21 cycles and because the yield from sequencing cycles indicated that the entire sample (*i.e.* both isozymes) was being sequenced we concluded that PG 2A and PG 2B have an identical N-terminal amino acid sequence.

Isolation and Characterization of a Full-Length PG cDNA Clone. A full-length PG cDNA (pPG1.9) containing a 1.75 kb insert was isolated from a ripe tomato fruit cDNA library by screening with a previously characterized PG cDNA clone (pPG16), (9) and a synthetic oligodeoxyribonucleotide corresponding to part of the N-terminal amino acid sequence of PG 2A and PG 2B. This synthetic oligonucleotide hybridized specifically to a 5' *HindIII/XbaI* fragment of both pPG1.8 and pPG1.9 (Fig. 2). Nucleotide sequence analysis of this *HindIII/XbaI* fragment revealed the presence of a 21 amino acid sequence which corresponded exactly to the 21 amino acid N-terminal sequence determined from PG 2A and PG 2B (boxed amino acids, Fig. 2). Nucleotide sequencing also identified a start codon 70 bases from the 5' end of the cDNA that was in frame with the sequence corresponding to the N terminus of PG 2A and PG 2B. Comparison of the deduced amino acid sequence of pPG1.9 with the N-terminal amino acid sequence of PG 2A and PG 2B indicated the presence of a 71 amino acid N-terminal domain containing a typically hydrophobic signal sequence immediately followed by a highly charged prosequence of about 50 amino acids, neither of which are present in the mature PG 2 isozymes (Fig. 2). A preliminary report of this sequence (2) was incorrect in several details but has been corrected here.

Translation of *In Vitro* Transcribed Polygalacturonase mRNA. PG mRNA transcribed *in vitro* by T7 RNA polymerase was used to program a rabbit reticulocyte lysate cell-free translation system and resulted in a severalfold stimulation over background incorporation (data not shown). Analysis by SDS-PAGE and fluorography of the radioactive translation products synthesized in the absence or presence of PG mRNA are shown in Figure 3 (lanes 1 and 2, respectively). The main product resulting from translation of PG mRNA is a 54 kD polypeptide (Fig. 3, lane 2) identical in size to the PG *in vitro* translation product previously characterized from ripe tomato fruit poly(A)⁺ RNA translation products by immunoprecipitation with PG antiserum (9). The identity of the 54 kD polypeptide produced from *in vitro* transcribed PG mRNA was further verified by its immunoprecipitation with PG antiserum (Fig. 3, lane 3). The characteristics of the 54 kD polypeptide produced from *in vitro* transcribed PG mRNA are consistent with previously published reports identifying the PG *in vitro* translation product (4, 9, 19). Other smaller polypeptides produced during translation (Fig. 3, lane 2) of PG mRNA are also recognized by PG antiserum (Fig. 3, lane 3). These smaller translation products most likely represent premature termination products of either PG mRNA transcription or translation.

Characterization of the Co-Translational Processing of PG by Canine Pancreas Microsomal Membranes. Addition of canine pancreas microsomal membranes to the PG cell-free translation system at the beginning of translation resulted in the appearance of two new polypeptides (Fig. 4, lanes 3–5) which had altered electrophoretic mobilities relative to the 54 kD unprocessed PG *in vitro* translation product (Fig. 4, lane 2). The prominent processed PG polypeptide had an apparent mol wt of 61 kD while the minor processing product migrated at 58 kD. The appearance of the 58 and 61 kD polypeptides was directly related to the amount of canine pancreas membranes present in the translation reaction (Fig. 4, lanes 3 through 5) and both the 58 and 61 kD polypeptides could be immunoprecipitated with PG antiserum (results not shown).

To determine whether the modification of PG translation prod-

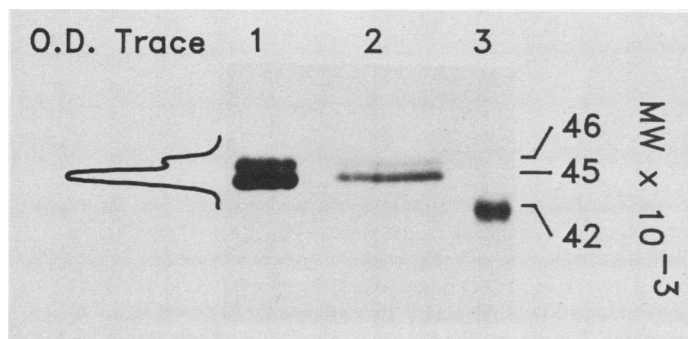


FIG. 1. Quantification and deglycosylation of purified PG 2A and PG 2B isozymes. Two μg of the purified PG 2A and PG 2B isozyme mixture (lane 1) was quantified by laser densitometry as described in the text. A densitometric scan is shown to the left of lane 1. The isozyme mixture was deglycosylated enzymically with endoglycosidase H (lane 2) or chemically with TFMS (lane 3). Lane 1, 2 μg untreated PG isozyme mixture; lane 2, 2 μg of PG isozyme mixture, pretreated with 0.2% SDS and boiling followed by digestion with endoglycosidase H; lane 3, TFMS treated PG isozyme mixture (approximately 1 μg).

FIG. 2. Nucleotide and deduced amino acid sequence of the 5' end of the full length PG cDNA clone, pPG 1.9. A physical map of pPG16, pPG1.8, and pPG1.9 is shown. A start codon is found at base 70. The nucleotide sequence shown extends 30 bases past the *Xba*I site. The boxed region indicates the N-terminal sequence of PG 2A and PG 2B determined by sequential Edman degradation of the PG isozyme mixture shown in Figure 1 (lane 1). Comparison of the nucleotide sequence with the N-terminal amino acid sequence of mature PG 2A and PG 2B isozymes indicated the presence of a deduced amino acid sequence (boxed area) identical to the N-terminal sequence of the mature PG isozymes. Immediately preceding the boxed region is an additional 71 amino acid domain not found in the mature proteins.

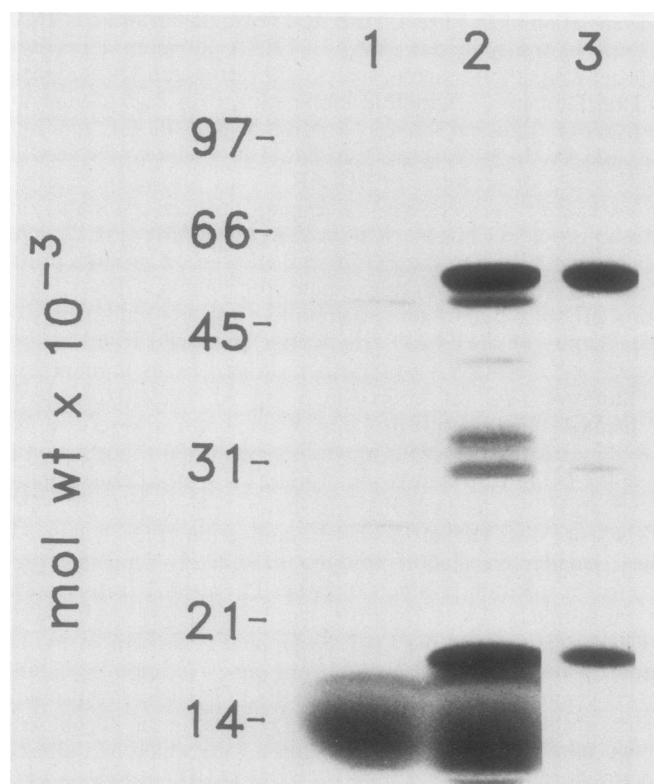
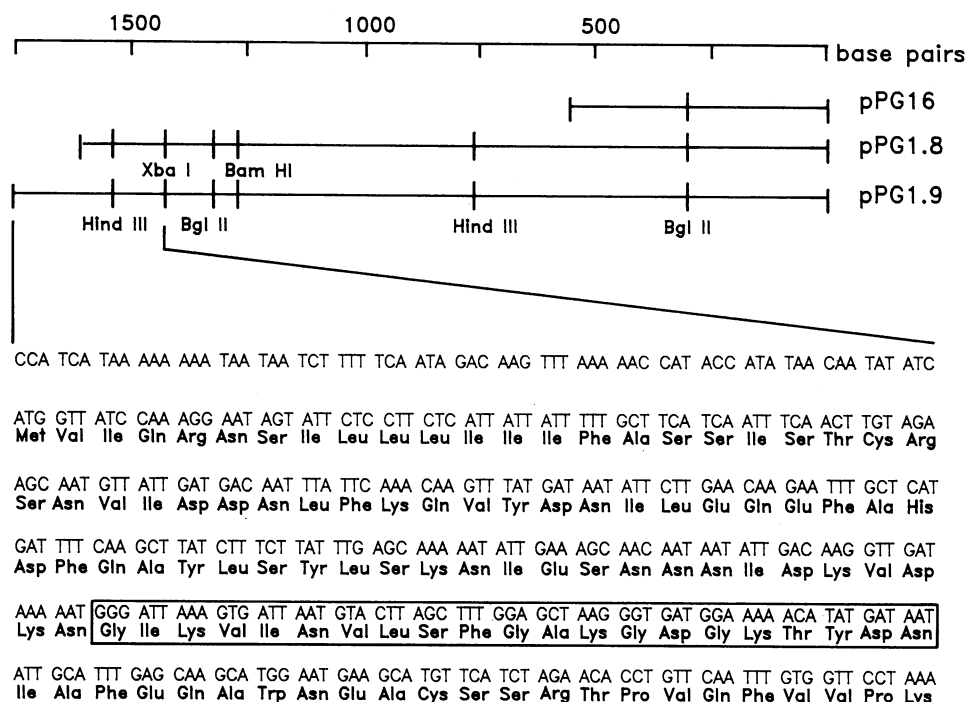


FIG. 3. Translation of *in vitro* transcribed PG mRNA in a rabbit reticulocyte lysate cell-free system. PG mRNA was translated in a nuclease-treated rabbit reticulocyte lysate as described in "Materials and Methods." Lane 1, Translation products in the absence of added mRNA; lane 2, translation products in the presence of *in vitro* transcribed PG mRNA; lane 3, immunoprecipitation of lane 2 with 2.5 μ l of polygalacturonase antiserum. Proteins were separated by SDS-PAGE and detected by fluorography.

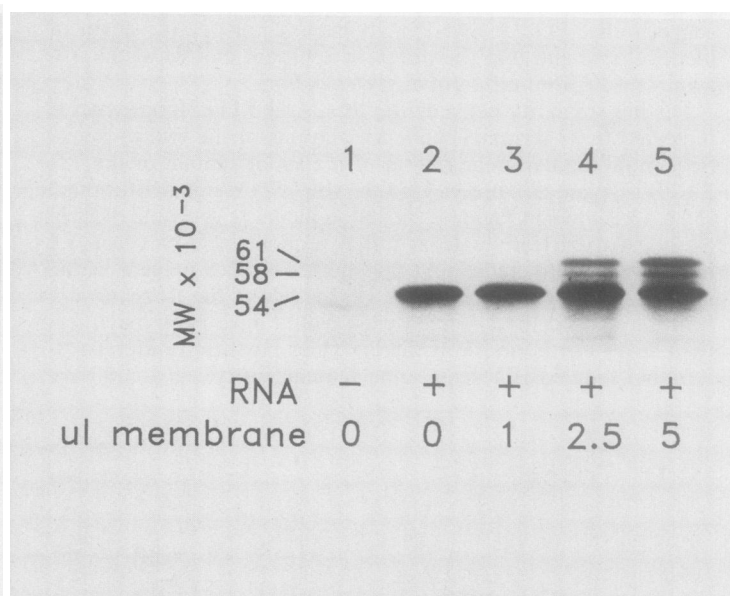


FIG. 4. Effect of translating polygalacturonase mRNA in the presence of increasing amounts of canine pancreas microsomal membranes. The indicated amounts of canine pancreas microsomal membranes were added at the start of *in vitro* translations. Equal volumes of each reaction were separated by SDS-PAGE (10–16% gradient gels) and analyzed by fluorography. Lane 1, No RNA; lane 2, translation with *in vitro* transcribed PG mRNA; lanes 3 through 5, translation with *in vitro* transcribed PG mRNA and 1.0, 2.5, and 5.0 μ l (respectively) of canine pancreas microsomal membranes.

ucts by canine pancreas membranes resulted in the sequestering of the 58 and 61 kD polypeptides within the microsomal membrane vesicles, membrane translation mixtures were subjected to proteolytic digestion or centrifugation analysis at the conclusion of translation. When the translation products from co-trans-

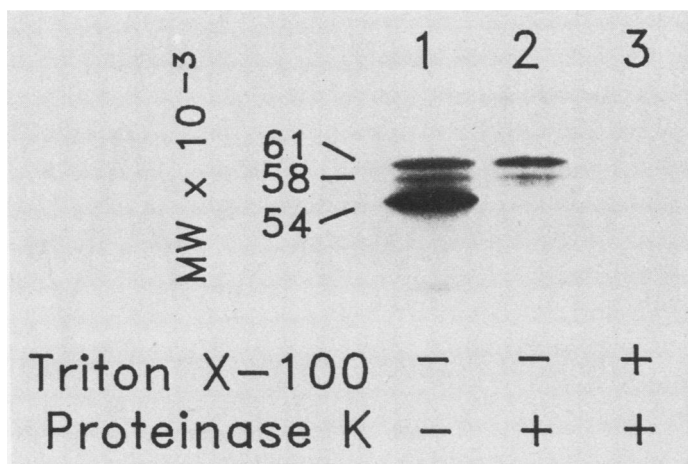


FIG. 5. Effect of proteinase K on PG *in vitro* processing intermediates. *In vitro* transcribed PG mRNA was translated in the presence of canine pancreas microsomes. After translation was completed, equal amounts of the reaction mixture were subjected to the following treatments. Lane 1, Untreated translation products; lane 2, treated with 5 µg of proteinase K; lane 3, treated with 5 µg of proteinase K in the presence of 1% (v/v) Triton X-100. Proteins were analyzed by SDS-PAGE and fluorography.

lational processing experiments (Fig. 5, lane 1) were subjected to digestion with proteinase K (Fig. 5, lane 2) the 58 and 61 kD polypeptides remained intact, indicating that the polypeptides were indeed sequestered within the microsomal vesicles. This contrasts with the complete digestion of the 54 kD (unprocessed) PG translation product in the reaction mixture (Fig. 5, lane 2). Protease digestion in the presence of 1% (v/v) Triton X-100 (Fig. 5, lane 3) resulted in digestion of the 58 and 61 kD polypeptides as well as the 54 kD polypeptide. The collection of membranes from processing reactions by centrifugation yielded similar results. When co-translational processing reactions (Fig. 6, lane 3) were centrifuged to pellet the microsomal membranes, the 58 and 61 kD polypeptides were always found associated with the membrane pellet (Fig. 6, lane 4). In subsequent experiments this method was employed to collect membrane-associated proteins.

Deglycosylation of *In Vitro* Processed PG. In addition to the primary activities of translocation and signal sequence cleavage, canine pancreas microsomal membranes also contain other enzymic functions, most notably glycosylation activity (25). Although core glycosylation of polypeptides is not required for translation, translocation and signal sequence cleavage (17), the addition of high mannose core oligosaccharides to specific asparagine residues is a common occurrence during *in vitro* processing of proteins (15, 16). Nucleotide sequence analysis of a full length PG cDNA has identified the presence of four possible *N*-glycosylation sites (11, 20). Thus, the large size of the PG processing intermediates (58 and 61 kD) relative to the nonprocessed form (54 kD) might result from the co-translational addition of high mannose oligosaccharides to the protein. To test whether the increased apparent mol wt of the processing intermediates is due to glycosylation, the 58 and 61 kD proteins (Fig. 7, lane 4) were subjected to enzymic deglycosylation with endoglycosidase H (Fig. 7, lane 5). Treatment with endoglycosidase H decreased the apparent mol wt of the 58 and 61 kD proteins by 4 and 7 kD, respectively, resulting in a single polypeptide that migrated at 54 kD, the same mol wt as unprocessed PG. Because processing by canine pancreas microsomes should result in cleavage of the signal sequence we had expected that endoglycosidase H digestion would reduce the apparent size of the PG processing

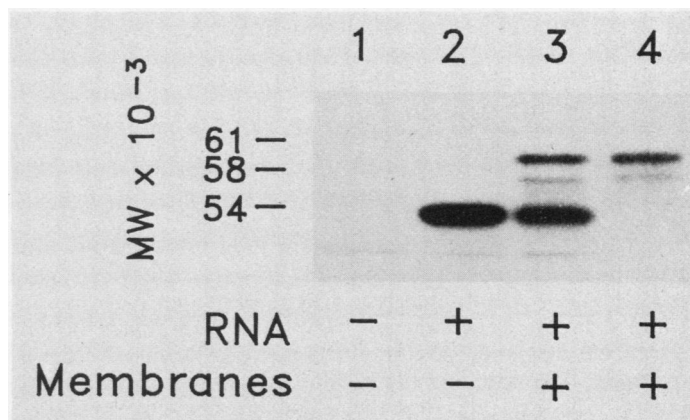


FIG. 6. Isolation of membrane-associated PG processing intermediates by centrifugation. After co-translational processing was completed, microsomal membranes were collected by centrifugation. Lane 1, Translation products with no RNA; lane 2, translation products with PG mRNA; lane 3, translation products with PG mRNA in the presence of canine pancreas microsomal membranes; lane 4, same as lane 3 except membranes were collected by centrifugation as described in "Materials and Methods."

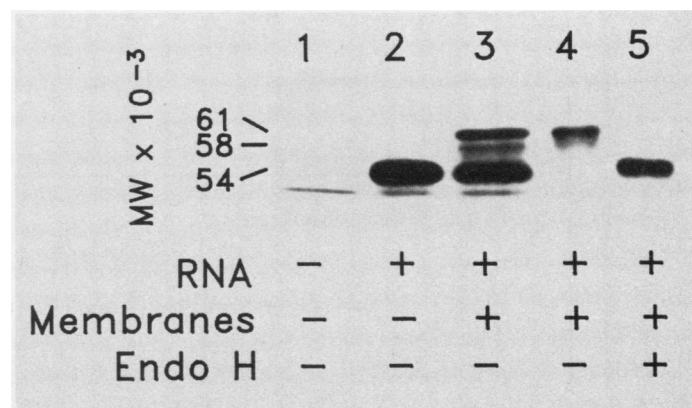


FIG. 7. Endoglycosidase H treatment of membrane-associated PG processing intermediates. Co-translational processing experiments were carried out as described in "Materials and Methods." Lane 1, Translation products with no RNA; lane 2, translation products with PG mRNA; lane 3, translation products with PG mRNA and canine pancreas microsomes; lane 4, same as lane 3 except membranes were collected by centrifugation; lane 5, digestion of lane 4 with 10 munits of endoglycosidase H for 2 h at 37°C.

intermediates to a level somewhat less than 54 kD. However, because endoglycosidase H cleaves high mannose oligosaccharide side chains between the two proximal GlcNAc residues (22), leaving a single GlcNAc residue still attached to the protein, after deglycosylation the 58 and 61 kD proteins might still contain as many as four GlcNAc residues. The presence of such residues would likely alter the electrophoretic mobility of the protein and might account for the larger than expected size of the deglycosylated processing intermediates. Other possible explanations include the lack of a proper oligosaccharide core structure for digestion by endoglycosidase H or lack of oligosaccharide accessibility to endoglycosidase H digestion. The latter is unlikely as pretreatment of the PG processing intermediates by heating

at 100°C in the presence of SDS and/or extended incubation of the PG processing intermediates with endoglycosidase H (up to 20 h) did not alter the electrophoretic mobility of the deglycosylated proteins (results not shown). A final possible explanation for the identical electrophoretic migration of the unprocessed PG protein and the processed, deglycosylated 58 and 61 kD proteins is that canine pancreas microsomes did not efficiently cleave the signal sequence of PG.

N-Terminal Sequence Analysis of *In Vitro* Processed PG Protein. To determine whether the PG *in vitro* processing intermediates did indeed undergo signal sequence cleavage during translocation into the lumen of microsomal membranes we subjected the PG processing intermediates to N-terminal radiosequencing. PG processing intermediates were labeled *in vitro* with either [³H]leucine or [³H]isoleucine and the radioactivity released at each sequencing cycle was determined (Fig. 8). With [³H]leucine labeling, discrete radioactive peaks were released at cycles 7 and

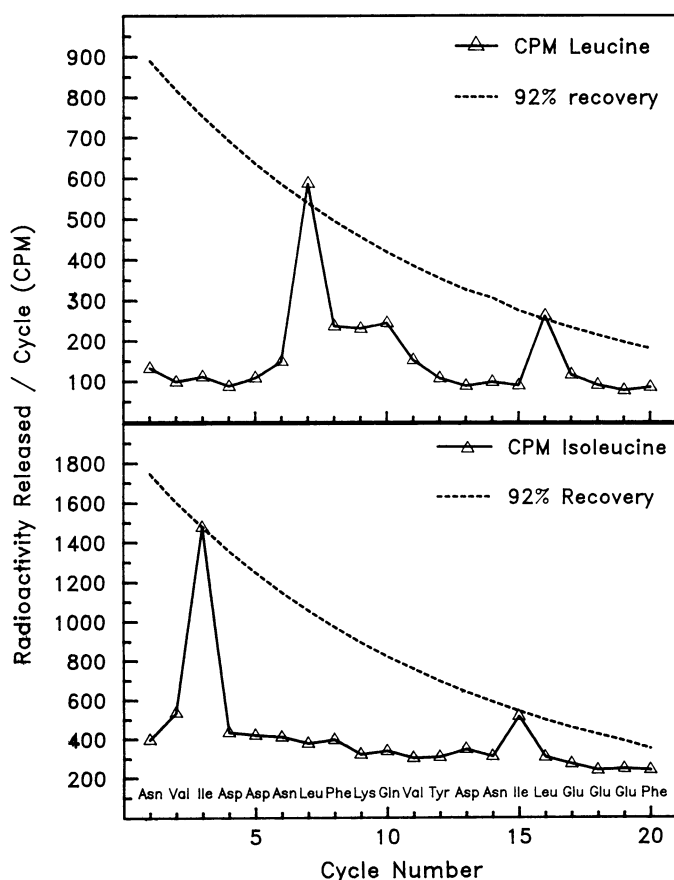


FIG. 8. Analysis of the N-terminal amino acid sequence of PG processing intermediates. *In vitro* transcribed PG mRNA was translated in a rabbit reticulocyte lysate cell-free system containing canine pancreatic microsomes and either [³H]leucine or [³H]isoleucine. Following translation membranes were collected by centrifugation and the PG processing intermediates purified by immunoprecipitation and subjected to automated sequential Edman degradation. Upper panel, N-terminal sequence analysis of [³H]leucine labeled PG processing intermediates (48,000 cpm of [³H]leucine-labeled polypeptides were introduced into the spinning cup). Lower panel, N-terminal sequence analysis of [³H]isoleucine-labeled PG processing intermediates (74,000 cpm of [³H]isoleucine-labeled polypeptides were introduced into the spinning cup). The deduced amino acid sequence of PG (residues 25 through 44) is shown at the bottom of the lower panel. The dotted line represents a theoretical curve based on a 92% repetitive yield for each cycle of the Edman degradation.

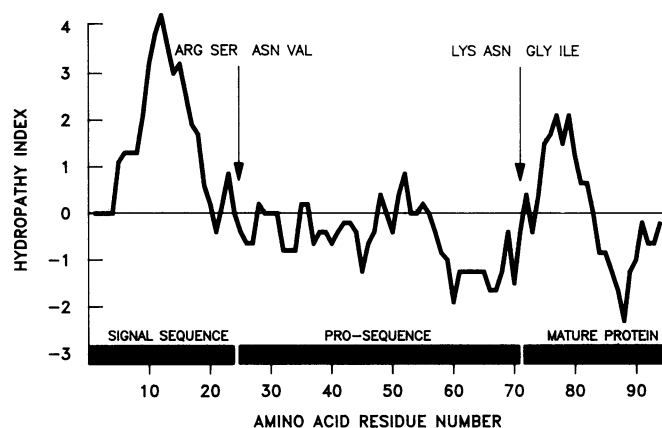


FIG. 9. Hydropathy plot of the first 93 amino acids of PG showing size, cleavage sites, and hydropathy of the signal sequence and propeptide.

16 (Fig. 8, upper panel). With [³H]isoleucine labeling, radioactivity was released at cycles 3 and 15 (Fig. 8, lower panel). Radiosequencing of [³H]leucine labeled, unprocessed PG yielded radioactivity at cycles 9, 10, and 11 (data not shown) as expected from the predicted sequence of the signal sequence (Fig. 2). Together, these results indicate that translocation of PG into the lumen of microsomal membranes was accompanied by proteolytic processing of the PG signal sequence. Furthermore, the relative positions of leucine (residues 7 and 16) and isoleucine (residues 3 and 15) in the processing intermediates indicate that the signal cleavage site is after amino acid 24 (serine). This determination allows a precise delineation of processing domains within the PG protein (Fig. 9). These domains consist of a typical hydrophobic signal sequence from residues 1 to 24 with the cleavage site between serine-24 and asparagine-25. This domain is followed by a 47 amino acid prosequence with a cleavage site between asparagine-71 and glycine-72. Glycine-72 is the N-terminal amino acid of the mature protein.

DISCUSSION

In this study we report the *in vitro* proteolytic processing and glycosylation of the tomato fruit cell wall enzyme polygalacturonase. Comparison of the N-terminal amino acid sequence of purified PG 2A and PG 2B with the deduced amino acid sequence of a full-length PG cDNA indicated the presence of a 71 amino acid N-terminal domain not present on the mature PG isozymes. This domain consists of a typically hydrophobic signal sequence and a highly charged prosequence of about 50 amino acids. To determine the extent of the co-translational proteolytic cleavage of PG and to determine the precise cleavage site of the signal sequence, we characterized the processing of PG by canine pancreas microsomal membranes. A recent report has demonstrated that a plant vacuolar protein (sporamin A) was identically processed by both canine pancreas microsomes and potato microsomes (12), indicating that canine pancreatic membranes are a suitable heterologous system for examining the co-translational processing of plant proteins.

Several groups have shown the PG *in vitro* translation product produced from tomato fruit poly(A)⁺ RNA to be a single polypeptide with an apparent mol wt of 54 kD (4, 9, 19). Translation of PG mRNA in the presence of canine pancreas microsomal membranes resulted in the production of two glycosylated, membrane-associated PG processing intermediates with apparent mol wt of 58 and 61 kD. The oligosaccharide side chains of these processing intermediates were shown, by virtue of their sensi-

tivity to digestion with endoglycosidase H, to be of the high-mannose type. However, the lack of endoglycosidase H sensitivity of the mature PG isozyme oligosaccharide side chains (Fig. 1, lane 2) indicates that the oligosaccharides are modified during the secretory process, presumably in the Golgi (13, 21).

The results from Edman degradation of [³H]leucine or [³H]isoleucine labeled PG processing intermediates (Fig. 8) indicated that the signal sequence of PG was indeed proteolytically removed during co-translational transport into the lumen of the microsomal vesicles. This cleavage occurred after serine-24 (Fig. 9), the site predicted by the statistical method of Von Heijne (24). Based on nucleotide sequence analysis of the full length PG cDNA pPG1.9 (Fig. 2), the removal of this 24 amino acid signal sequence should have reduced the apparent mol wt of the PG *in vitro* translation product by 2.66 kD. This decrease was not reflected in SDS-PAGE analysis possibly due to extensive co-translational glycosylation and incomplete deglycosylation of the processing intermediates by endoglycosidase H (Fig. 7, lane 5). Removal of the remaining 47 amino acid prosequence would further reduce the size of the polypeptide by an additional 5.56 kD.

The co-translational removal of the 24 amino acid hydrophobic signal sequence suggests that the remaining 47 amino acid prosequence is removed posttranslationally, most likely during subsequent processing and maturation steps in the endoplasmic reticulum and Golgi apparatus or after secretion to the cell wall. The function of the prosequence is at present unknown, but it may be involved in maintaining the protein in an inactive state prior to maturation or in directing the protein to the cell wall. In this regard, a recent report examining the targeting of carboxypeptidase Y/invertase fusion proteins to the yeast vacuole suggests that the targeting information for this protein resides in a discrete amino acid sequence near the N terminus of a prosequence associated with the protein (14).

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