

# Solubilization and Partial Characterization of Homogalacturonan-Methyltransferase from Microsomal Membranes of Suspension-Cultured Tobacco Cells<sup>1</sup>

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The transfer of a methyl group from *S*-adenosyl-L-methionine onto the carboxyl group of  $\alpha$ -1,4-linked-galactosyluronic acid residues in the pectic polysaccharide homogalacturonan (HGA) is catalyzed by an enzyme commonly referred to as pectin methyltransferase. A pectin methyltransferase from microsomal membranes of tobacco (*Nicotiana tabacum*) was previously characterized (F. Goubet, L.N. Council, D. Mohnen [1998] *Plant Physiol* 116: 337–347) and named HGA methyltransferase (HGA-MT). We report the solubilization of HGA-MT from tobacco membranes. Approximately 22% of the HGA-MT activity in total membranes was solubilized by 0.65% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid containing 1 mM dithioerythritol. The addition of phosphatidylcholine and the methyl acceptors HGA or pectin (30% degree of esterification) to solubilized enzyme increased HGA-MT activity to 35% of total membrane-bound HGA-MT activity. Solubilized HGA-MT has a pH optimum of 7.8, an apparent  $K_m$  for *S*-adenosyl-L-methionine of 18  $\mu$ M, and an apparent  $V_{max}$  of 0.121 pkat  $mg^{-1}$  of protein. The apparent  $K_m$  for HGA and for pectin is 0.1 to 0.2  $mg\ mL^{-1}$ . Methylated product was solubilized with boiling water and ammonium oxalate, two conditions used to solubilize pectin from the cell wall. The release of 75% to 90% of the radioactivity from the product pellet by mild base treatment showed that the methyl group was incorporated as a methyl ester rather than a methyl ether. The fragmentation of at least 55% to 70% of the radiolabeled product by endopolygalacturonase, and the loss of radioactivity from the product by treatment with pectin methylesterase, demonstrated that the bulk of the methylated product produced by the solubilized enzyme was pectin.

Pectins are a family of complex polysaccharides present in all primary walls. To date, three pectic polysaccharides have been identified in all plants investigated: homogalacturonan (HGA), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) (O'Neill et al., 1990). Each of these pectic polysaccharides can be *O*-methylated (O'Neill et al., 1990; An et al., 1994) and *O*-acetylated (Ishii, 1997). For example, the  $\alpha$ -1,4-linked  $\beta$ -galactosyluronic acid residues of HGA (O'Neill et al., 1990) and RG-II (Pellerin et al.,

1996) are partly methylesterified, whereas RG-I contains 4-*O*-methyl-GlcUA (An et al., 1994) and RG-II contains 2-*O*-methyl-Fuc and 2-*O*-methyl-Xyl (O'Neill et al., 1990, 1996).

Enzymes that methylate pectin, typically referred to as pectin methyltransferases (PMTs), have been studied in mung bean (Kauss et al., 1967; Crombie and Reid, 1998), flax (Vannier et al., 1992), tomato (Crombie and Reid, 1998), and tobacco (*Nicotiana tabacum*) (Goubet et al., 1998). These PMTs use *S*-adenosyl-L-methionine (SAM) as a methyl donor in vitro. The main difference reported between these PMTs is the effect of the addition of exogenous methyl acceptors, pectin or HGA, on in vitro membrane-bound PMT activity. The membrane-bound PMT from mung bean is activated by HGA (Kauss et al., 1967), whereas the membrane-bound PMT from flax is activated by HGA following freezing and thawing of the membranes (Bruyant-Vannier et al., 1996). The membrane-bound PMT from tobacco is not activated by HGA or pectin, irrespective of whether the membranes have been frozen or not (Goubet et al., 1998).

The PMT from flax has been solubilized with 0.5% (w/v) Triton X-100, and the solubilized enzyme is activated by HGA (Bruyant-Vannier et al., 1996). The amount of activation by HGA of solubilized flax PMT and previously frozen membrane-bound flax PMT are comparable (Bruyant-Vannier et al., 1996; Bourlard et al., 1997). Bruyant-Vannier et al. (1996) reported that flax PMT had a pH optimum of 7.1, an apparent  $K_m$  for SAM of 0.5  $\mu$ M, and an apparent  $K_m$  for HGA of 0.5 to 0.7  $mg\ mL^{-1}$ . However, Bourlard et al. (1997) more recently reported that the solubilized- and HGA-activated PMT from flax has a pH optimum of 5.5, an apparent  $K_m$  for SAM of 20  $\mu$ M, and a  $K_m$  for HGA was 0.25  $mg\ mL^{-1}$ . The reasons for the differences in the pH optimum and kinetic constants for PMT(s) from flax have not been explained.

In vitro studies of HGA biosynthesis suggest that the galactosyluronic acid residues in HGA are methylesterified after polymerization of at least some of the GalA residues in the HGA chain (Kauss and Swanson, 1969; Doong et al., 1995; Goubet et al., 1998). The glycosyltransferase that synthesizes HGA, HGA 4- $\alpha$ -galacturonosyltransferase (HGA-GalAT), has been studied in mung bean (Villemez et al., 1966), sycamore (Bolwell et al., 1985), and tobacco (Doong et al., 1995). The tobacco enzyme has been characterized in microsomal membranes (Doong et al., 1995), and a galac-

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turonosyltransferase has been solubilized from these membranes using the detergent CHAPS (Doong and Mohnen, 1998). HGA with degrees of polymerization (DP) greater than 9 are required as exogenous acceptors to obtain appreciable solubilized galacturonosyltransferase activity. Membrane-bound HGA-GalAT generates a product that is approximately 50% esterified (Doong et al., 1995); at least 40% of the esters have been shown to be methyl esters (Doong et al., 1995). The notion that HGA is methylesterified following its polymerization is supported by the finding that the *in vitro* methylation of HGA in microsomes by SAM is increased by the inclusion of UDP-GalA (Goubet et al., 1998). In contrast, the methyl donor SAM does not increase the rate of HGA formation by HGA-GalAT (Kauss and Swanson, 1969; Doong et al., 1995).

We recently identified a PMT in microsomal membranes of suspension-cultured tobacco cells (Goubet et al., 1998). The PMT methylates HGA and has been named HGA methyltransferase (HGA-MT) to distinguish it from methyltransferases that methylate the pectic polysaccharides RG-I and RG-II. We now describe the solubilization of tobacco HGA-MT and the partial characterization of the solubilized enzyme and the products its synthesizes.

## MATERIALS AND METHODS

### Chemicals

The chloride salt of *S*-adenosyl-L-Met, GalA, tri-GalA, the ammonium salt of UDP-GalA, polygalacturonic acid (referred to as HGA in this paper), DL-phosphatidylcholine, DL-phosphatidylinositol, and DL-phosphatidylethanolamine were purchased from Sigma. Pectins of 31% to 93% degrees of esterification (DE) were purchased from Sigma or obtained as a gift from the Hercules Corporation (Wilmington, DE). Dextran standards were purchased from Pharmacia. [<sup>14</sup>C]Methyl-*S*-adenosyl-L-Met (specific activity 55 mCi/mM) was purchased from Amersham. CHAPS was purchased from Pierce. Triton X-100, Triton X-114, and Genapol X-080 were purchased from Boehringer Mannheim. Oligogalacturonides of DP 7 to 23 were prepared as described (Doong et al., 1995). Partially purified decagalacturonides and pentagalacturonides were prepared by fractionation of oligogalacturonides (DP 7–23) using Q-Sepharose anion-exchange resin as described previously (Lo et al., 1994; Doong and Mohnen, 1998).

### Plant Material

Suspension-cultured tobacco (*Nicotiana tabacum* L. cv Samsun) cells, originally isolated from pith callus tissue (Eberhard et al., 1989), were grown on Murashige and Skoog medium supplemented with 4.5  $\mu$ M 2,4-D and 90 mM Suc and subcultured every 13 to 14 d (Doong et al., 1995).

### Preparation of Membranes from Tobacco Cell Suspension Cultures

Membranes were prepared by a modification of the method of Villemez et al. (1966). Tobacco cells (75 g) were

collected by filtration 3 or 4 d after transfer to fresh medium. The cells were homogenized with a polytron at maximum speed for 2 min at 4°C in 100 mL of grinding buffer (50 mM Tris-HCl, pH 7.3, 0.4 M Suc, 1% (w/v) BSA, and 1 mM EDTA). The homogenate was strained through a nylon cloth (50- $\mu$ m pore size) and the filtrate centrifuged at 3,500g for 15 min. The supernatant was centrifuged for 1 h at 100,000g to produce a membrane pellet. The pellet was resuspended with a Potter-Elvehjem glass homogenizer (Fisher Scientific) in 5 mL of storage buffer (0.4 M Suc and 50 mM Tris-HCl, pH 6.8) and kept at -80°C prior to use. The protein content of the membranes was determined using a Bradford (1976) assay with BSA as a standard.

### Solubilization of Membrane Proteins

Membranes (1 mL, 4 mg mL<sup>-1</sup> of protein) were mixed with 1 mL of 1.3% (w/v) CHAPS and 1 mM dithioerythritol (DTE), vortexed, and the suspension incubated for 2 min at 4°C. The suspension was then centrifuged for 45 min at 180,000g. The supernatant was used as solubilized enzyme. The pellet was resuspended in 1 mL of storage buffer (0.4 M Suc and 50 mM Tris-HCl, pH 6.8) and used as non-solubilized enzyme. The protein content of the soluble fraction and the pellet were determined using the method of Bradford (1976) and DC protein assays (Bio-Rad) with BSA as a standard. The relative protein content of the soluble versus membrane proteins were the same using these two assays. The Bradford assay gave absolute protein values 33% lower than the DC assay.

### HGA-MT Assay

The HGA-MT assay was a modification of that described by Kauss and Hassid (1967). An aliquot (25  $\mu$ L, approximately 25  $\mu$ g of protein) of the soluble fraction or resuspended membrane pellet was added to 25  $\mu$ L of concentrated reaction buffer (50 mM Tris-MES, pH 8.5, 25  $\mu$ g DL-phosphatidylcholine, 0.4 M Suc, 50  $\mu$ g HGA or pectin of 30% DE, 8  $\mu$ M [<sup>14</sup>C]methyl-SAM [0.01  $\mu$ Ci], and 12  $\mu$ M SAM) and incubated at 30°C for 4 h. The reaction was stopped and the methylated products precipitated by the addition of 50  $\mu$ L of 20% (w/v) TCA and 5  $\mu$ L of a 10% (w/v) BSA solution. The resulting suspensions were centrifuged 5 min at 4,000g. Unincorporated SAM was removed by washing the pellets twice with 200  $\mu$ L of 2% (w/v) TCA. The washed pellets were resuspended in 300  $\mu$ L of water, and the radioactivity incorporated into product was measured by liquid scintillation counting using scintillation cocktail (Scintiverse BD, Fisher Scientific).

### Uronic Acid Assay

Uronic acids were determined by a *meta*-hydroxybiphenyl assay (Blumenkrantz and Asboe-Hansen, 1973).

### Chemical Extraction of Radiolabeled Product

The pellets obtained after TCA precipitation were treated with boiling water, 0.5% (w/v) boiling ammonium oxalate,

or 0.1 N NaOH at 20°C to 25. The water and oxalate treatments were performed three times for 1 h. The NaOH treatment was performed for 4 h, followed by neutralization with 0.1 N HCl. The suspensions were centrifuged, and the amount of radioactivity in the supernatant and pellet determined.

### EPGase and Pectin Methyltransferase (PME)

#### Digestion of Product

Methylated product was demethylated by treatment for 4 to 12 h at 25°C with 1 to 4 units ( $\mu$ moles of methanol released per minute) of a purified cloned *Aspergillus aculeatus* PME expressed in *Aspergillus oryzae* (Christgau et al., 1996). Methylated product was fragmented by treatment for 4 to 12 h at 25°C with 1 to 4 units (micromoles of reducing ends produced per minute) of a purified EPGase from *Aspergillus niger*. Both enzymes were purified from culture filtrates (gifts of Carl Bergmann, Complex Carbohydrate Research Center, Athens, GA).

#### HPLC of $^{14}\text{C}$ -Labeled Products

Radiolabeled methanol released from the  $^{14}\text{C}$ -labeled product by treatment with PME or NaOH was collected by distillation and separated using a Rezex ROA-organic acid column (Phenomenex, Torrance, CA) eluted with water (flow rate 0.6 mL min<sup>-1</sup>) on a chromatography system (DX 500, Dionex). Non-radiolabeled methanol was detected by electrochemical detection with post-column addition of 400 mM NaOH (0.2 mL min<sup>-1</sup>). Fractions were collected manually and radioactivity was determined by liquid scintillation counting using scintillation cocktail (Fisher Scientific).

#### Size-Exclusion Chromatography of $^{14}\text{C}$ -Labeled Products

Radioactive products obtained after TCA precipitation in the HGA-MT assay were solubilized with 0.3 M potassium acetate, pH 5.2, containing 6 mM imidazole. Half of the solubilized product was treated for 8 h with 2 units of a purified EPGase (748 units mL<sup>-1</sup>) from *Fusarium moniliforme* (gift of Carl Bergmann, Complex Carbohydrate Research Center, Athens, GA). Intact and digested product, non-radiolabeled pectin, and HGA standards were filtered through 0.2- $\mu$ m nylon membranes (Rainin Instrument, Woburn, MA) and then fractionated using a Superose 12HR 10/30 size-exclusion column (Pharmacia) in 0.3 M potassium acetate, pH 5.2 (flow rate 0.48 mL min<sup>-1</sup>). Fractions (0.48 mL) were collected and radioactivity measured by liquid scintillation counting using scintillation cocktail (Fisher Scientific). The uronic acid content of non-radiolabeled standards separated under the same conditions was determined colorimetrically (Blumenkrantz and Asboe-Hansen, 1973).

## RESULTS

### Solubilization of HGA-MT Activity and Optimization of the Solubilization Procedure

#### Solubilization of HGA-MT Using CHAPS

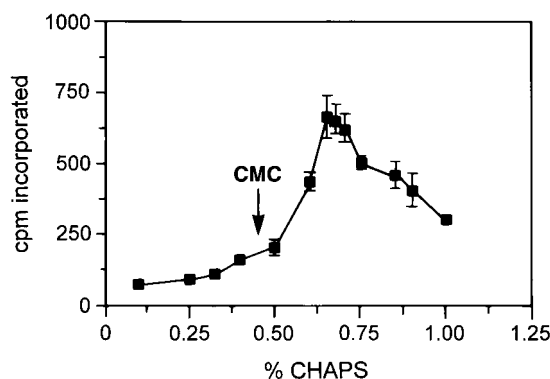
Four detergents previously shown to be useful for the solubilization of membrane-bound enzymes (Jones et al., 1987; Waldron et al., 1989), Triton X-100, Triton X-114, CHAPS, and Genapol X-080, were separately added to tobacco membranes at a final concentration of 0.5% (2 mg mL<sup>-1</sup> of protein). CHAPS, Triton X-100, and Triton X-114 solubilized an average of 6% of the total HGA-MT activity. No solubilized HGA-MT activity was recovered using Genapol X-080. The solubilization of HGA-MT by CHAPS was optimized since it caused the least loss of total HGA-MT activity in the detergent-dispersed enzyme and because the high critical micelle concentration (CMC) of CHAPS would facilitate the effective removal of the detergent by dialysis during protein purification.

The maximal solubilization of HGA-MT was obtained with 0.6% to 0.7% (w/v) CHAPS (Fig. 1), corresponding to 1.2 to 1.5 times CMC. The solubilized HGA-MT activity was inhibited in a concentration dependent manner at greater than 1.2 to 1.5 times the CMC of CHAPS. Thus, 0.65% (final concentration, w/v) CHAPS was used to solubilize HGA-MT.

The highest recovery of HGA-MT activity was obtained with 2 min of CHAPS treatment. When the solubilization time was increased the amount of solubilized HGA-MT activity decreased (Fig. 2). The addition of detergent followed by the immediate centrifugation of the membranes resulted in a low recovery of HGA-MT. The HGA-MT activity after a 2-min solubilization time was stable for at least 5 d at 3°C, whereas the HGA-MT after a 30-min solubilization was unstable, with only 50% of the HGA-MT activity recovered after 2 d at 3°C.

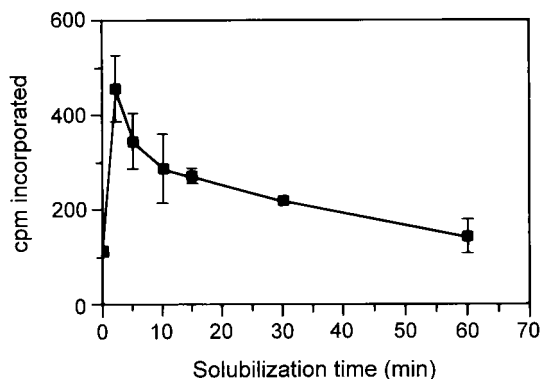
#### Optimization of the HGA-MT Solubilization Protocol

The inclusion of the reducing agent DTE during enzyme solubilization increased the amount of total solubilized



**Figure 1.** Effect of CHAPS concentration on the solubilization of HGA-MT. The data are the average counts per minute ( $\pm$ SE) of product recovered from duplicate samples from at least four independent experiments except for the 0.1% and 1% (w/v) CHAPS points, which are from duplicate samples from one experiment.





**Figure 2.** Effect of solubilization time on the solubilization of HGA-MT. The data are the average counts per minute ( $\pm$ SE) incorporated into product in triplicate samples from one experiment for the 0-, 30-, and 60-min points and from three independent experiments for the other points.

HGA-MT activity recovered to  $159\% \pm 25\%$ , (mean  $\pm$  SD, 0.5 mM DTE) and  $161\% \pm 24\%$  (5 mM DTE) of that recovered upon solubilization in the absence of DTE. Low concentrations of the Cys and Ser protease inhibitor PMSF (0.1 mM) did not affect the solubilization of HGA-MT activity. In contrast, inclusion of 1 mM PMSF and 5 mM EDTA in the solubilization buffer reduced the amount of total HGA-MT activity recovered to  $83\% \pm 12\%$  and  $87\% \pm 3\%$ , respectively. Low concentrations of  $\text{MnCl}_2$  (0.05–0.5 mM) during solubilization did not affect the recovery of solubilized HGA-MT, but the recovered enzyme activity was lowered to 50% at 5 mM and 30% at 10 mM  $\text{MnCl}_2$ .  $\text{MgCl}_2$  (12 mM) reduced the amount of total solubilized HGA-MT activity by 30%. The amount of total HGA-MT activity recovered was not increased by the addition of 0.1 mg  $\text{mL}^{-1}$  of HGA or 2.5 to 10  $\mu\text{M}$  UDP-GalA in the solubilization buffer (data not shown).

Under our experimental conditions, the most effective procedure for the solubilization of HGA-MT from tobacco membranes was to treat the membranes (4 mg  $\text{mL}^{-1}$  of protein) with 1 mL of 1.3% (w/v) CHAPS and 1 mM DTE for 2 min at 4°C, followed by centrifugation for 45 min at 180,000g.

### Optimization of the HGA-MT Assay for Solubilized HGA-MT

#### *Precipitation of the Methylated Product Generated by Solubilized HGA-MT*

A protein carrier (e.g. BSA) and a short precipitation period increased the yield of product generated by solubilized HGA-MT (Table I). Attempts to precipitate the product produced by solubilized HGA-MT using ethanol (Vannier et al., 1992) and methanol/chloroform (Doong et al., 1995) were not effective. Methanol/chloroform precipitation followed by ethanol washes gave variable counts per minute with high background counts per minute, and the precipitation of the product by ethanol followed by ethanol-NaCl washes gave a low yield of product (results not shown). The method used to assay solubilized

HGA-MT was the precipitation of product with 20% (w/v) TCA containing 500  $\mu\text{g}$  of BSA and the removal of unincorporated SAM by washing the pellet twice with 2% (w/v) TCA.

#### *Effect of Potential Stimulatory Agents on Solubilized HGA-MT Activity*

To test the effect of the reducing agent DTE on HGA-MT activity, HGA-MT activity in reactions with and without 1 mM DTE was assayed using HGA-MT solubilized in the absence of DTE. The inclusion of DTE in the reaction did not inhibit or stimulate solubilized HGA-MT activity. Thus, the stimulatory effect achieved by adding DTE during the solubilization of HGA-MT was likely due to a stabilization of the solubilized enzyme and not to an activation of the enzyme.

$\text{MgCl}_2$  (1 mM) inhibited the solubilized HGA-MT by 60%, while Mn activated the solubilized HGA-MT with maximum activation (1.7-fold) at 10 to 20 mM  $\text{MnCl}_2$ .  $\text{MnCl}_2$  inhibits HGA-MT at concentrations above 50 mM. Solubilized HGA-MT was not affected by GTP or ATP at 0.1 to 1 mM, and was inhibited by 50% at 10 mM.

The ability of exogenous lipids to stimulate solubilized HGA-MT activity was determined. Phosphatidylcholine and phosphatidylethanolamine (0.25–0.5 mg  $\text{mL}^{-1}$ ) activated HGA-MT activity by 20% to 45%, and 10% to 15%, respectively. Phosphatidylinositol (0.5 mg  $\text{mL}^{-1}$ ) inhibited solubilized HGA-MT by 50%. At concentrations greater than 0.5 mg  $\text{mL}^{-1}$  all the lipids inhibited HGA-MT activity.

#### *Effect of Potential Substrates on Solubilized HGA-MT Activity*

The effect of the exogenous acceptors HGA and pectin on total solubilized HGA-MT activity was determined. HGA (DE = 0) and pectin with 30% DE both increased total solubilized HGA-MT activity (Table II). The ability of pectins with higher DE to increase total solubilized HGA-MT activity could not be determined, since TCA does not effectively precipitate pectins with a high DE (10% of pectin

**Table I.** Optimization of the precipitation procedure for recovery of the products generated by HGA-MT

Solubilization was performed with 0.65% (w/v) CHAPS for 2 min at 4°C. After centrifugation, the solubilized HGA-MT in the supernatant was incubated for 4 h in reaction buffer containing 10  $\mu\text{M}$  SAM (20,000 cpm [ $^{14}\text{C}$ ]SAM), 50  $\mu\text{g}$  HGA, or pectin (30% DE), and 25  $\mu\text{g}$  of phosphatidylcholine, and the methylated products were precipitated by 20% (w/v) TCA with or without BSA for 5 min or 12 h. The solutions were centrifuged and the pellets were washed twice with 200  $\mu\text{L}$  of 2% (w/v) TCA. The data are the averages  $\pm$  SD from triplicate samples from at least two independent experiments. n.d., Not determined.

BSA	Precipitation Time	cpm in the Pellet
0	5 min	200 $\pm$ 33
	12 h	n.d.
500 $\mu\text{g}$	5 min	450 $\pm$ 21
	12 h	311 $\pm$ 18

**Table II.** Effect of pectins with different degrees of methylesterification and oligomerization on total solubilized HGA-MT activity and a comparison of their precipitation by TCA

Pectin (% DE)	HGA-MT Activity <sup>a</sup>	Exogenous Acceptor <sup>b</sup> Precipitated
	%	
0%	159 ± 12	95 ± 5
30%	155 ± 11	92 ± 3
55%	109 ± 9	13 ± 1
68%	107 ± 10	2 ± 1
73%	104 ± 14	9 ± 1
93%	88 ± 11	38 ± 12
OGA <sup>c</sup>	82 ± 14	20 ± 2

<sup>a</sup> The percentage activity is the total HGA-MT activity in the presence of exogenous pectin divided by the total HGA-MT activity in the absence of exogenous pectin × 100. The cpm incorporated into product per reaction in the absence of exogenous pectin was 300 ± 40. The results are the averages ± SE of triplicate samples from at least three independent experiments. <sup>b</sup> The percentage of pectin (50 μg) precipitated represents the percentage of uronic acid-positive material precipitated based on standard curves generated using the same pectin. The data are the average percentages ± SE of pectin precipitated from triplicate samples from at least two independent experiments. <sup>c</sup> OGA, Oligogalacturonides of DP 7 to 23.

[55%–73% DE] precipitated; 30% of pectin [93% DE] precipitated) or a low  $M_r$  (20% of OGAs [DP 7–23] precipitated).

Attempts to increase HGA-MT activity further by using pectin in combination with MnCl<sub>2</sub> resulted in a 70% to 100% decrease in total HGA-MT compared with the activity detected in absence of these compounds. In fact, Mn made the pectin gel at concentrations of 0.1 to 50 mM MnCl<sub>2</sub> and 0.1% to 1% (w/v) HGA or pectin (30% DE). UDP-GalA, an activator of membrane-bound HGA-MT (Goubet et al., 1998), did not stimulate solubilized HGA-MT activity either in the presence or absence of MnCl<sub>2</sub>.

In summary, approximately half of the protein and 30% to 40% of the total HGA-MT activity was solubilized using the HGA-MT solubilization and assay procedures summarized in Table III.

### Characterization of Solubilized HGA-MT

The temperature optimum, pH optimum, and enzyme kinetics were established for solubilized HGA-MT in the

presence of exogenous HGA and independently for solubilized HGA-MT in the presence of exogenous pectin (30% DE). The characteristics of solubilized HGA-MT acting on the two different exogenous acceptors were similar. The combined results from reactions containing either HGA or pectin (30% DE) as an exogenous acceptor are presented.

The temperature optimum for solubilized HGA-MT was between 25°C and 30°C. Solubilized HGA-MT was completely inactivated by treatment for 1 h at 60°C or for 5 min at 100°C. Solubilized HGA-MT was stable for at least 1 month at –80°C, and for up to 5 d when stored at 3°C, but was reduced by 50% when stored at 6°C for 1 d. Similar levels of stability for frozen xyloglucan fucosyltransferase (Hanna et al., 1991) and glucuronosyltransferase (Waldron et al., 1989) have been reported.

The effect of reaction pH ranging from 5.5 to 8.6 on solubilized HGA-MT activity was determined (Fig. 3). A major peak of activity was obtained at pH 7.8 to 8.0, and minor peaks occurred at pH 6.8 to 7.3 and pH 8.0 to 8.3.

The rate of incorporation of <sup>14</sup>C into product was linear during the first 4 h (Fig. 4). The apparent  $K_m$  of solubilized tobacco HGA-MT for SAM was 18 ± 3 μM (Fig. 5A). The apparent  $K_m$  of solubilized HGA-MT for HGA or pectin (30% DE) was 0.1 to 0.2 mg mL<sup>-1</sup> (Fig. 5B), which represents approximately 21 μM HGA assuming an average DP of 40 for HGA. The apparent  $V_{max}$  of solubilized HGA-MT was 0.121 ± 0.02 pkat mg<sup>-1</sup> of protein (Fig. 5).

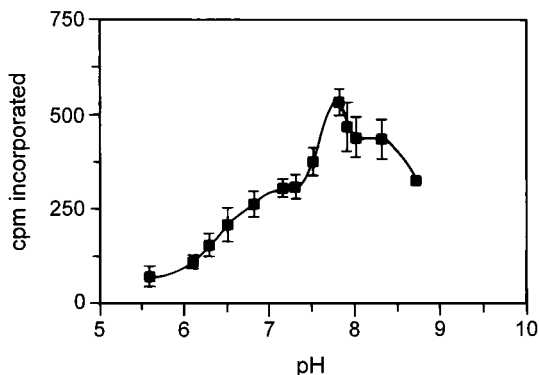
### Characterization of the Total Product Produced by Solubilized HGA-MT

The products synthesized by solubilized enzyme in the absence of exogenous acceptor (i.e. only endogenous acceptor present) or in the presence of exogenous HGA and pectin (30% DE) were partially solubilized by boiling water and boiling ammonium oxalate (Table IV). These same two treatments partially solubilized the products generated by membrane-bound HGA-MT (Goubet et al., 1998) and are commonly used to solubilize pectin from isolated cell walls (O'Neill et al., 1990; Goubet et al., 1993). Water solubilized a similar percentage of the product (77%–81%) produced using endogenous acceptor or exogenous HGA, while only 55% of the product produced using pectin (30% DE) was solubilized (Table IV). Ammonium oxalate solubilized a greater percent of the product synthesized using exogenous HGA (72%) compared with product made using endogenous acceptor (40%) or pectin of 30% DE (53%). Be-

**Table III.** Recovery of total HGA-MT activity and fractionation of protein during the solubilization of HGA-MT

The percentage of solubilized and membrane-bound (pellet) HGA-MT activities in the presence of HGA were compared with the membrane-bound HGA-MT activity. The data are the averages ± SE of protein and HGA-MT activity values from triplicate samples from at least six independent experiments.

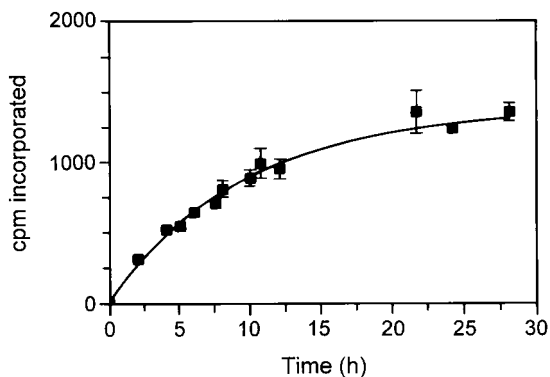
Source	Protein mg/fraction	Total HGA-MT Activity pmol s <sup>-1</sup>	HGA-MT Activity %
Membranes	4.3 ± 0.3	0.235 ± 0.015	100
Solubilized enzyme	2 ± 0.1	0.0835 ± 0.004	35 ± 3
Pellet	2.2 ± 0.1	0.0196 ± 0.007	8 ± 2



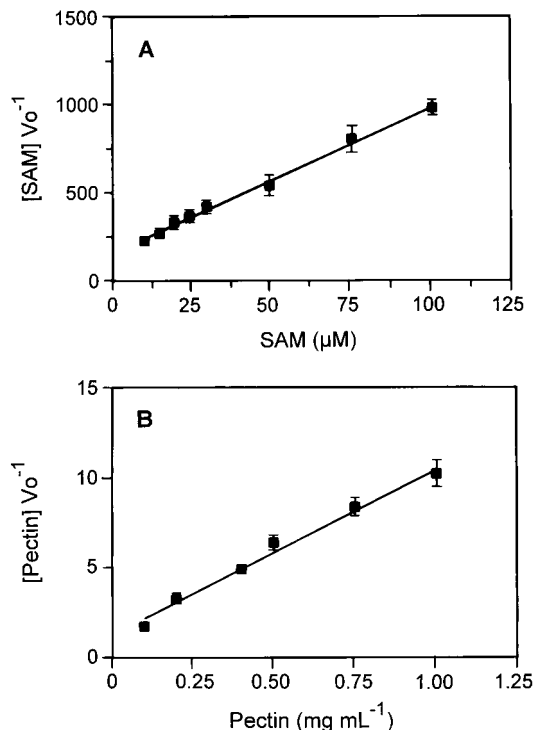
**Figure 3.** Effect of pH on solubilized HGA-MT activity. The data are the average counts per minute ( $\pm$ SE) incorporated into product in duplicate samples from five independent experiments.

tween 75% and 90% of the radioactivity in the total product produced using HGA, pectin (30% DE), or endogenous acceptor (Table IV) was released by treatment with 0.1 N NaOH. The product was identified as methanol by HPLC (data not shown). Thus, the bulk (>75%) of the methyl group was incorporated as a methyl ester rather than as a methyl ether.

Additional evidence that the radiolabeled product was indeed methylesterified HGA was obtained by treating  $^{14}\text{C}$ -methylated product with EPGase and PME. HGA and pectin (30% DE) are precipitated by TCA (Table II) while OGAs and highly methylated pectin are not effectively precipitated. Thus, radiolabeled and partially methylated HGA oligomers released from HGA by EPGase or methanol released from the product by PME would not be precipitated by TCA. The material generated by the solubilized enzyme was resuspended in water or treated with EPGase or PME and then precipitated by with TCA. Approximately 37% of the product produced using endogenous acceptor or exogenous HGA acceptor was not precipitated by TCA, indicating that it had been fragmented by EPGase (Table V). These results suggest that at least 38% of the product produced in the presence of exogenous HGA was methylated pectin. Further evidence that  $^{14}\text{C}$ -methyl



**Figure 4.** Time course of the incorporation of  $^{14}\text{C}$ -methyl into precipitable product. The data are the average counts per minute ( $\pm$ SE) incorporated into product in duplicate samples from three independent experiments.



**Figure 5.** Hanes-Woolf plot of the production of methylated product by solubilized HGA-MT. A, Hanes-Woolf plot for SAM. Reactions contained with  $1 \text{ mg mL}^{-1}$  of pectin (HGA or pectin [30% DE]). B, Hanes-Woolf plot for pectin (HGA and pectin [30% DE]). Reactions contained  $100 \mu\text{M}$  SAM. The data represent the average initial velocities ( $\pm$ SE) in duplicate samples from at least two independent experiments. Initial velocity ( $V_0$ ) is in picomoles of methyl incorporated per second per milligram of protein.

**Table IV.**  $^{14}\text{C}$ -Methylated product solubilized using different chemical treatments

Results are the percentages of radioactivity solubilized from the product pellet compared with the radioactivity present in the pellet before treatment. Results from reactions with endogenous substrate and HGA as exogenous substrate are the averages  $\pm$  SD of the percentage of cpm of product solubilized from at least two samples from at least two independent experiments. Results for reactions with pectin of 30% DE as exogenous substrate are the average percentage cpm of product solubilized from duplicate samples from one experiment.

Treatment	% Radioactivity Released from the Product Pellet		
	Endogenous acceptor	HGA as exogenous acceptor	Pectin of 30% DE as exogenous acceptor
		%	
Water	81 $\pm$ 8	77 $\pm$ 10	55
Oxalate	40 $\pm$ 2	72 $\pm$ 16	53
NaOH	90 $\pm$ 10	75 $\pm$ 15	87

**Table V.** Fragmentation of intact methylated product by EPGase and PME

The cpm  $\pm$  SE recovered upon precipitation of intact or enzyme-treated product is shown. Results are the average  $\pm$  SD cpm recovered from at least duplicate samples from at least two independent experiments.

Product	Precipitated Endogenous Acceptor	Precipitated Exogenous HGA Acceptor
	<i>cpm</i>	
Total intact	363 $\pm$ 7	475 $\pm$ 20
EPGase-treated	233 $\pm$ 13	295 $\pm$ 15
PME-treated	243 $\pm$ 9	298 $\pm$ 12

was incorporated into HGA was obtained by the treatment of the  $^{14}\text{C}$ -methylated product with PME. Approximately 33% to 37% of the radioactivity was not precipitated following PME treatment (Table V). Since the PME used here catalyzes the hydrolysis of 75% to 85% of the methyl groups in highly esterified pectin (Christgau et al., 1996), these results confirm that at least 37% of the product produced in the presence of exogenous HGA is methylated pectin.

#### Characterization of the Water- and Oxalate-Solubilized Product Produced by Solubilized HGA-MT

The ability of EPGase and PME to fragment  $^{14}\text{C}$ -labeled product solubilized from the total product pellet by boiling water or boiling oxalate (see Table IV) was also tested (Table VI). The bulk (>70%) of the EPGase-treated water- and oxalate-solubilized product produced using endogenous acceptor or exogenous HGA as an acceptor was not precipitated by TCA. The susceptibility to EPGase suggests that 55% to 70% of the total product produced by solubilized HGA-MT in the presence of endogenous acceptor or exogenous HGA was pectin. Sensitivity of the water- and oxalate-solubilized product to PME treatment confirms that the bulk (>64%) of the solubilized product was pectin and that at least 51% of the total product was sensitive to PME. The fact that a greater percentage of the water- and oxalate-solubilized product compared with total product was sensitive to hydrolysis by EPGase and PME likely reflects a greater accessibility of the enzymes to products that have been extracted from total product prior to enzyme treatment. Interestingly, a smaller percentage of the product methylated in the presence of exogenous pectin

(30% DE) was not precipitated following treatment of water- and oxalate-solubilized product with EPGase (53%) and PME (43%). These results suggest that either the exogenous pectin (30% DE) itself is not as amenable as HGA or the endogenous acceptor to complete hydrolysis by these enzymes, or that the in vitro-methylated pectin is not as accessible.

The intact and EPGase-treated radiolabeled products produced by the solubilized enzyme in the presence of exogenous pectin (30% DE) acceptor were separated by size-exclusion chromatography over a Superose HR 10/30 column. The retention times of the radiolabeled product were compared with the retention times of commercially available pectin (30% DE) and HGA (data not shown). We were unable to recover intact radiolabeled methylated product from the size-exclusion column despite repeated attempts using different chromatographic conditions. At least half of the intact radiolabeled product bound to the spin filter used to remove particulates prior to injection onto the column. The remaining radioactive product apparently remained bound to the column. However, EPGase treatment of the intact product gave peaks that co-eluted with the intact pectin acceptor, others that co-eluted in the range of oligogalacturonides of DP 2 to >15, and some radioactivity that eluted with retention times similar to Glc and methanol. For example, in one reaction with pectin (30% DE) as the exogenous acceptor, 63% of the EPGase-fragmented product recovered from the column eluted as radioactive peaks with retention times between those of the exogenous pectin (30% DE) acceptor and digalacturonic acid. The appearance of radioactive peaks following EPGase treatment of intact  $^{14}\text{C}$ -labeled product was reproducible, although the retention times of the radioactive peaks and the amount of radioactivity recovered varied between different experiments. Nevertheless, these results confirm that solubilized HGA-MT methylates pectin and suggest that the degree and/or pattern of HGA methoxylation may vary in product produced in different in vitro enzymatic reactions.

## DISCUSSION

HGA-MT transfers a methyl group from SAM onto the carboxyl group of GalA in the HGA of pectin (Goubet et al., 1998). Solubilization of the membrane-bound HGA-MT from tobacco with the detergent CHAPS resulted in a

**Table VI.** Fragmentation of oxalate- and water-solubilized methylated product by EPGase and PME

The cpm  $\pm$  SD recovered upon precipitation of intact or enzyme-treated product is shown. Results are the average  $\pm$  SD cpm from at least two samples from at least two independent experiments. Results for reactions with pectin (30% DE) as exogenous acceptor are the average cpm from duplicate samples from one experiment.

Product	Precipitated Endogenous Acceptor	Precipitated Exogenous HGA Acceptor	Precipitated Exogenous Pectin (DE 30%) Acceptor
	<i>cpm</i>		
Nontreated	300 $\pm$ 40	286 $\pm$ 17	450
EPGase-treated	42 $\pm$ 20	84 $\pm$ 9	211
PME-treated	105 $\pm$ 15	96 $\pm$ 12	256



reduction of total HGA-MT activity. Detergents also inhibit membrane-bound PMT in flax (Bruyant-Vannier et al., 1996). The activity of solubilized tobacco HGA-MT is increased by the addition of HGA or pectin (30% DE), which is consistent with the identification of the solubilized enzyme as HGA-MT. The described solubilization and assay procedures allow for the recovery of approximately 35% of the tobacco membrane-bound HGA-MT activity as soluble enzyme.

The concentration of detergent is a critical condition for the solubilization of membrane-bound enzymes (Wasserman et al., 1989; Doong and Mohnen, 1998). The solubilization of HGA-MT was optimal at 0.65% (w/v) CHAPS, a concentration corresponding to 1.2 times the CMC. A similar effective CHAPS concentration was reported for the solubilization of glucan synthase, which was optimal at 0.6% (w/v) CHAPS (Wasserman et al., 1989). Bruyant-Vannier et al. (1996) reported the solubilization of flax PMT using 1% (w/v, 100 times the CMC) Triton X-100, however, no optimization of the concentration of Triton X-100 was presented.

Reducing agents, protease inhibitors, and chelators have been reported to increase the solubilization and/or the stability of membrane-bound enzymes (Wu et al., 1991; Von Jagow and Schägger, 1994). The inclusion of 0.5 to 5 mM DTE during solubilization increased the amount of solubilized HGA-MT activity recovered by 60%. In contrast, neither PMSF or EDTA increased the total solubilized HGA-MT activity. The activity of some soluble enzymes is increased by the presence of divalent cations during solubilization (Doong and Mohnen, 1998). MgCl<sub>2</sub> and MnCl<sub>2</sub> had no effect on membrane-bound HGA-MT (Goubet et al., 1998). On the contrary, solubilized HGA-MT activity was inhibited by 1 mM MgCl<sub>2</sub> and activated by 10 to 20 mM MnCl<sub>2</sub>. Some enzymes require nucleotides as cofactors (Fevre, 1983; Girard and Fevre, 1991). However, HGA-MT was not stimulated by GTP or ATP.

The methylation of HGA is believed to occur after the HGA chain is polymerized (Kauss and Swanson, 1969; Doong et al., 1995; Goubet et al., 1998). Thus, it was possible that the inclusion of the substrates for HGA synthesis, HGA and UDP-GalA, in the solubilization buffer would stabilize HGA-MT. However, the inclusion of UDP-GalA or HGA during solubilization had no effect on the amount of HGA-MT activity recovered.

Phospholipids are often required to stabilize solubilized membrane-bound enzymes. The stabilization can require one phospholipid or a mixture of phospholipids (Wasserman and McCarthy, 1986; Jones et al., 1987; Kasamo and Nouchi, 1987; Kasamo and Sakakibara, 1995). Solubilized tobacco HGA-MT activity was increased by the addition of phosphatidylcholine or phosphatidylethanolamine, whereas phosphatidylinositol inhibited HGA-MT activity. Positively charged phospholipids also enhance the recovery of solubilized glucan synthase activity from red beet (Wasserman and McCarthy, 1986) and soybean (Kauss and Jeblick, 1986).

We previously showed that TCA effectively precipitates the products generated by HGA-MT in tobacco cell membranes (Goubet et al., 1998), and that alternative procedures to precipitate pectin using ethanol (Vannier et al., 1992) and methanol/chloroform (Doong et al., 1995) were not effective. The latter two procedures were also not effective for the reproducible recovery of methylated product generated by solubilized HGA-MT. However, appreciable methylated product produced by solubilized HGA-MT was recovered by precipitation with TCA in the presence of BSA. No carrier was required to facilitate the precipitation of product synthesized by membrane-bound HGA-MT, and the length of the precipitation time was not critical. In contrast, the inclusion of BSA during TCA precipitation and a short precipitation time resulted in a greater, less variable recovery of product produced by solubilized HGA-MT. The loss of methyl groups by de-esterification in acid conditions (BeMiller, 1986) could account for the loss of radioactivity upon extended exposure of the product to acidic solutions. The methylated products produced by solubilized HGA-MT were in direct contact with the TCA, while the products produced by membrane-bound HGA-MT were likely surrounded by a membrane. Also, the protein content of the solubilized HGA-MT fraction (1 mg mL<sup>-1</sup> of protein) was lower than that in membranes (4 mg mL<sup>-1</sup> protein) and may lead to a less-effective coprecipitation of product by TCA.

Microsomal membranes contain endogenous polysaccharides (Goubet and Morvan, 1993). The lack of effect of exogenous pectin on membrane-bound HGA-MT may be due to the presence of endogenous polysaccharides in the membranes (Goubet et al., 1998) or to the inaccessibility of the exogenous pectin to the HGA-MT in the membranes. Upon solubilization of HGA-MT from the membranes, the

**Table VII.** Comparison of the characteristics of membrane-bound and solubilized HGA-MT from tobacco cells

Parameter	Membrane-Bound Enzyme <sup>a</sup>	Solubilized Enzyme
pH optimum	7.8–8.0	7.8–8.0
Temperature optimum	25°C–40°C	25°C–30°C
Apparent $K_m$ for SAM	38 $\mu\text{M}$	18 $\mu\text{M}$
Apparent $K_m$ for HGA	n.d. <sup>b</sup>	0.1–0.2 mg mL <sup>-1</sup>
Apparent $K_m$ for pectin (DE 30%)	n.d.	0.1–0.2 mg mL <sup>-1</sup>
Apparent $V_{max}$	0.81 pkat mg <sup>-1</sup> of protein	0.12 pkat mg <sup>-1</sup> of protein
Stimulation by UDP-GalA	Yes	No

<sup>a</sup> Data are from Goubet et al. (1998). <sup>b</sup>n.d., Not determined.





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