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UNCONJUGATED $MAN_5GLCNAC$ OCCURS IN VEGETATIVE TISSUES OF TOMATO

HERMONA YUNOVITZ, J. NORMAN LIVSEY and KENNETH C. GROSS*

USDA, Agricultural Research Service, Horticultural Crops Quality Laboratory, Building 002, 10300 Baltimore Avenue, Beltsville, MD 20705-2350, U.S.A

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; glycoprotein; high-mannosyl type *N*-glycans; high-pH anion-exchange chromatography; oligosaccharide; oligosaccharin; pulsed amperometric detection; senescence.

Abstract—Unconjugated Man $\alpha 1 \rightarrow 6$(Man $\alpha 1 \rightarrow 3$)Man $\alpha 1 \rightarrow 6$(Man $\alpha 1 \rightarrow 3$)Man $\beta 1 \rightarrow 4$GlcNAc ($Man_5GlcNAc$) delayed ripening when added exogenously at 10 ng g^{-1} fr. wt to both whole tomato fruit and excised pericarp tissue discs. In addition, $Man_5GlcNAc$ was one of 10 unconjugated *N*-glycans purified from tomato pericarp tissue. When applied exogenously at a concentration of 10 nM, $Man_5GlcNAc$ prevented the delay of ripening induced by tunicamycin, an inhibitor of its *de novo* synthesis. Since it was possible that this unconjugated *N*-glycan might be specifically related to ripening, we checked to see if it also occurred in fully expanded leaf and stem tissues using high-pH anion-exchange chromatography with pulsed amperometric detection. This method allowed us to detect 1 ng of oligosaccharide and to quantify accurately amounts in the range 60–600 ng per injection, significantly more sensitive than the amine-bonded HPLC method previously used to separate and quantify unconjugated *N*-glycans in tomato fruit pericarp tissue. Four high-mannosyl type unconjugated N-glycans were detected in tomato vegetative tissues, including $Man_5GlcNAc$. These results support a potential role of $Man_5GlcNAc$ and/or other unconjugated *N*-glycans in plant developmental processes, in addition to its apparent role as a modulator of senescence of fruit. However, an initial experiment showed no effect of $Man_5GlcNAc$ on senescence of tomato leaf discs, as measured by chlorophyll loss.

INTRODUCTION

Man $\alpha 1 \rightarrow 6$(Man $\alpha 1 \rightarrow 3$)Man $\alpha 1 \rightarrow 6$(Man $\alpha 1 \rightarrow 3$)Man $\beta 1 \rightarrow$ GlcNAc($Man_5GlcNAc$) was one of several high-mannosyl type unconjugated *N*-glycans found in the urine of patients with mannosidosis, a genetically inherited disease [1]. This *N*-glycan also stimulated and inhibited ripening when infiltrated into intact tomato fruit at 1 and 10 ng g^{-1}, respectively [2]. A delay in ripening was also observed when excised pericarp discs were infiltrated with this oligosaccharide at a concentration of 10 ng g^{-1} fr. wt [3].

While examining the *in situ* occurrence of unconjugated *N*-glycans (UNGs) in tomato fruit, Priem *et al.* [4] isolated 10 UNGs, including the high-mannosyl type *N*-glycans $Man_{5-8}GlcNAc$; 10 ng g^{-1} fr. wt of the high-mannosyl type mixture delayed ripening as much as $Man_5GlcNAc$ alone, whereas complex-type UNGs extracted from tomato pericarp showed no activity [5]. Use of Con A-Sepharose chromatography [4] to separate UNG types, revealed that the amount of ^{14}C-labelled high-mannosyl and complex-type UNGs changed inversely during ripening [5], suggesting that active metabolism of these oligosaccharides occurs during senescence of tomato fruit tissue. Also, tunicamycin, an inhibitor of *N*-glycosylation, which also delays fruit ripening when added exogenously to pericarp discs [6], lost it inhibitory effect on ripening two days after application, when both *de novo N*-glycan synthesis and ripening resumed [5]. Also, application of 10 nM $Man_5GlcNAc$, in conjunction with tunicamycin, prevented the delay of tomato pericarp disc ripening by tunicamycin [5]. These results supported a potential role for $Man_5GlcNAc$ in fruit ripening. However, $Man_5GlcNAc$ and Man $\alpha 1 \rightarrow 6$(Man $\alpha 1 \rightarrow 3$)(Xyl $\beta 1 \rightarrow 2$)(Man $\beta 1 \rightarrow 4$)-GlcNAc$\beta 1 \rightarrow 4$(Fuc $\alpha 1 \rightarrow 3$GlcNAc) (Man_3(Xyl)GlcNAc(Fuc)GlcNAc) had previously been isolated from the extracellular medium of *Silene alba* cell cultures [7] and the xylomannoside, Man_3(Xyl)GlcNAc(Fuc)GlcNAc, had been shown to stimulate elongation of flax hypocotyls at relatively low concentrations, while having anti-auxin activity at higher concentration [8]. In addition, UNGs were found

*Author to whom correspondence should be addressed.

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in relatively large quantities in unfertilized eggs of a freshwater fish [9, 10]. Therefore, we undertook a study to determine if the existence of UNGs in tomato plants was restricted to reproductive tissue by examining if unconjugated $Man_5GlcNAc$ was also present in vegetative tissues, such as stems and leaves.

In previous work, Priem *et al.* [4] purified UNGs obtained from Con A fractionation by HPLC on an amine-bonded silica column equilibrated with 65% acetonitrile. Oligosaccharides were detected by monitoring UV absorbance at 200 nm during a 1-hr isocratic run. This detection was insensitive to concentrations in the nanogram range (detection threshold was *ca* 0.5 μg $Man_5GlcNAc$ per injection) and was, therefore, less than optimal for detecting trace levels of UNGs. Separation of asparagine-linked oligosaccharides in mammalian tissues by high-pH anion-exchange chromatography with pulsed amperometeric detection became the method of choice in recent years due to its sensitivity, which enables detection of picomole levels of oligosaccharides [11]. However, this technique had not been used, to our knowledge, to study oligosaccharides in plants. Modification of the technique used to detect asparagine-linked oligosaccharides by Townsend *et al.* [12] allowed us to detect and quantitate UNGs in vegetative tissues of tomato. This method was clearly superior to the previous method used.

RESULTS AND DISCUSSION

For identification of the UNGs present in tomato tissues, an oligosaccharidic extract of tomato fruit pericarp tissue, which had been metabolically labelled with ^{14}C-glucosamine, provided radiolabelled standards to determine the retention time of high-mannosyl type *N*-glycans during Con A-Sepharose chromatography (Fig. 1). Desalted stem and leaf extracts were then subjected to Con A-Sepharose chromatography according to Priem *et al.* [4]. Fractions eluting with 300 mM α-methylglucoside (fractions 18 and 19), corresponding to the strongly retained (SR) fraction, were collected, desalted using HW-40S and subjected to Dionex CarboPac™ PA-100 column chromatography. Commercially available standards (Accurate Chemical & Scientific Corp., U.S.A.) of $Man_2GlcNAc$, $Man_5GlcNAc$ and $Man_9GlcNAc$ were used to determine the retention times of the oligosaccharides. The lower detection limit for $Man_5GlcNAc$ was *ca* 1 ng, but the detector response was not closely correlated with oligosaccharide amount at levels up to 20 ng. However, a standard curve for amounts of $Man_5GlcNAc$ ranging from 60 to 600 ng showed a linear detector response which was proportional to the amount of oligosaccharide.

Fractionation of the SR UNG fractions from leaf and stem tissue gave four distinct peaks eluting between 7.4 and 12.6 min after injection (Figs. 2a and b), resulting in a profile similar to that obtained from chromatographing a tomato fruit pericarp SR fraction on an amine-bonded silica HPLC column [4]. The extracts

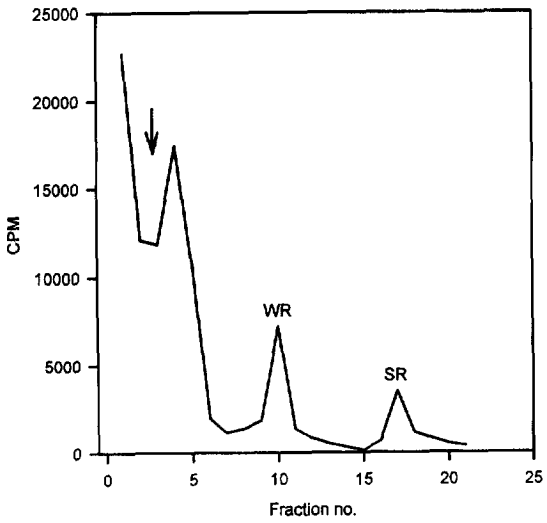

Fig. 1. Con A-Sepharose chromatography of oligosaccharides obtained from tomato pericarp tissue radiolabelled with ^{14}C-glucosamine. The arrow indicates void volume (V_0) and the beginning of successive step elutions with 0.01 and 0.3 M α-methylglucoside, resulting in elution of weakly (WR) and strongly retained (SR) fractions, respectively. The 5 mM NaOAc (pH 5.2) buffer contained 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.02% NaN_3.

of both leaves and stems revealed four peaks corresponding to $Man_5GlcNAc$, $Man_6GlcNAc$, $Man_7GlcNAc$ and $Man_8GlcNAc$. Peak 1 had the same retention time as authentic Man_5(GlcNAc (Fig. 2).

Oligosaccharides present in the four peaks were hydrolysed with 2 N trifluoroacetic acid at 121° for 1 hr and injected into a CarboPac™ PA-1 column for glycosyl composition analysis. Only mannose and *N*-acetylglucosamine were detected (Fig. 3) with relative Man:GlcNAc ratios as determined by area percentage to be 5:1 for peak 1 ($Man_5GlcNAc$) and 8:1 for peak 4 ($Man_8GlcNAc$). The amount of $Man_5GlcNAc$ was estimated from a standard curve prepared using peak areas resulting from chromatography of authentic $Man_5GlcNAc$ on the CarboPac™ PA-100 column. The amount was 65 ng g^{-1} fr. wt for leaf and 21 ng g^{-1} fr. wt for stem tissue.

An experiment was performed to determine if exogenously applied $Man_5GlcNAc$ could alter the rate of tomato leaf senescence by floating 10 mm diameter discs, adaxial side up, on 10 ng ml^{-1} $Man_5GlcNAc$ or double-distilled H_2O. No differences in green colour among treatments was apparent (data not shown).

However, high-mannosyl type UNGs clearly do seem to play an important role in tomato fruit-ripening. Inhibition of their *de novo* synthesis by tunicamycin delayed ripening [5, 6]. Interestingly, this delay was prevented if 10 nM $Man_5GlcNAc$ was applied together with tunicamycin [5], suggesting that newly synthesized $Man_5GlcNAc$ was essential for initiation of ripening. Also, high mannosyl type UNGs were converted into a complex type during the first days of ripening [5]. If this conversion, occurring in the Golgi [13], was

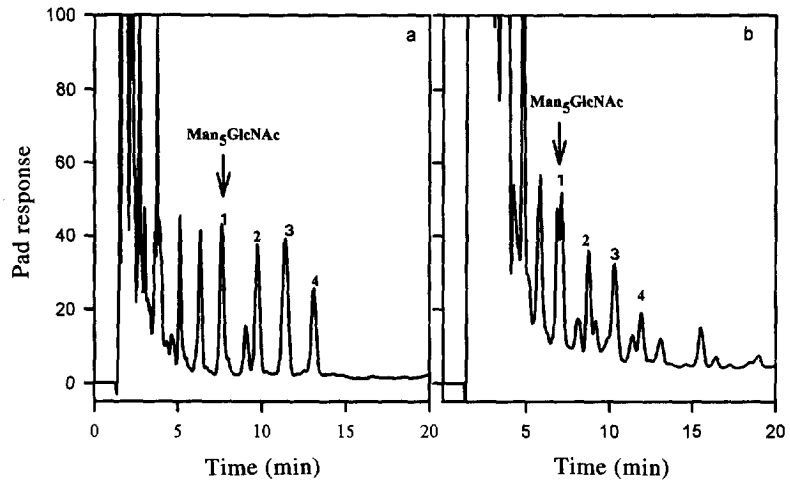

Fig. 2. High-performance anion-exchange chromatography of oligosaccharides in the SR fraction obtained from Con A-Sepharose chromatography of (a) leaf and (b) stem tissue using a Dionex CarboPac™ PA-100 column. See Experimental for detailed description of chromatography conditions.

stopped by deoxymannojirimycin, an inhibitor of mannosidase I, which is involved in the high-mannosyl to complex type *N*-glycan processing [14], then ripening was delayed until processing of high-mannosyl *N*-glycans resumed [15].

Also, a potential ripening-related interaction between $Man_5GlcNAc$ and indole-3-acetic acid (IAA) was shown [3], whereby exogenously added 10 ng g^{-1} fr. wt $Man_5GlcNAc$ prevented stimulation of ripening induced by 100 μM IAA. In concert, these findings suggest that high-mannosyl type *N*-glycans, especially $Man_5GlcNAc$, have two different roles in the ripening process: (1) to prevent premature ripening before fruit are physiologically mature [3, 15], and (2) newly synthesized $Man_5GlcNAc$ could act as a trigger for ripening to commence when fruits are mature and seeds viable [5].

The presence of $Man_5GlcNAc$ in tomatoes at 65 ng g^{-1} fr. wt and 21 ng g^{-1} fr. wt in leaf and stem tissue, respectively, versus 100 ng g^{-1} fr. wt in fruit [4], suggest that $Man_5GlcNAc$ could have some other function in plant growth and development. However, our preliminary experiment indicates leaf senescence (data not shown) does not seem to be one of them.

The presence of $Man_5GlcNAc$ in white campion cell cultures [7] in concert with the interactions between $Man_5GlcNAc$ and IAA and $Man_5GlcNAc$ and the lectin Con A in tomato fruit [3], and between unconjugated $Man_3(Xyl)GlcNAc(Fuc)GlcNAc$ and IAA in *S. alba* cell cultures [8], suggest a potential involvement on UNGs in other plant processes.

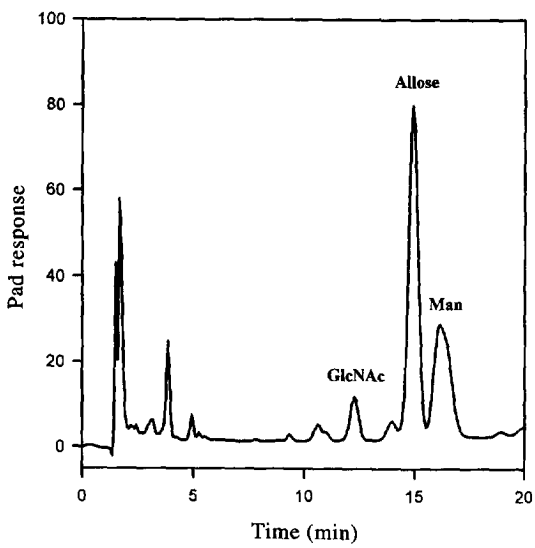

Fig. 3. High-performance anion-exchange chromatography of monosaccharide constituents (glycosyl composition) of oligosaccharides in peaks 1 and 4 after Dionex CarboPac™ PA-100 chromatography. Monosaccharides were separated on a Dionex CarboPac™ PA-1 column. See Experimental for detailed description of chromatography conditions.

EXPERIMENTAL

UNG extraction. Tomato plants (*Lycopersicon esculentum* Mill., cv. Rutgers) were grown in a greenhouse without supplemental lighting. Fully expanded leaves (236 g fr. wt) and stems (135 g fr. wt) were excised and briefly rinsed sequentially with 80% EtOH and dist. H_2O. Tissues were then frozen with liquid N_2, lyophylized, boiled in 80% EtoH for 15 min and homogenized with a Polytron apparatus. The EtOH extract was taken to dryness using a rotary evaporator and the residue dissolved in 20 ml 50% MeOH; 40 ml $CHCl_3$ were then added. The soln was shaken and centrifuged at 1000 *g* for 10 min. The upper phase

containing oligosaccharides was collected and 20 ml 50% MeOH was added to the lower phase. The mixt. was again shaken and centrifuged for 10 min. The upper phase was collected and combined with the first. This soln was taken to dryness using a rotary evaporator. HOAc (0.01%, 10 ml) was added to dissolve the oligosaccharides and the soln run through a 10-ml insoluble PVP (Sigma) column to remove phenols. The first 10 ml were collected, frozen and lyophylized before redissolving in 0.01% HOAc and desalting on HW-40S (TosoHaas) as previously described [4, 5]. The oligosaccharidic fr. was collected from the HW-40S column and fractionated using Con A-Sepharose according to ref. [4]. The fr. eluted with 300 mM α-methylglucoside was lyophilized and desalted on HW-40S. The sample was then lyophilized and resuspended in dist. H_2O prior to high performance anion-exchange chromatography.

High-performance anion-exchange chromatography. Desalted SR frs were purified using a Dionex CarboPac™PA-100 column (Dionex Corp.; 4 × 250 mm) as previously described [12], but with some minor changes. The system comprised a Dionex GPM pump and an ED40 electrochemical detector (pulsed amperometric detector) which was controlled using PeakNet software. Eluent 1 was H_2O, eluent 2 (250 mM NaOH) was not used, eluent 3 was 250 mM NaOAc and eluent 4 was 1 M NaOH. Samples were injected using a Rheodyne injector equipped with a 25-μl sample loop. The flow rate was 1 ml min^{-1} at ambient temp. The system was equilibrated with eluent 1–eluent 3–eluent 4 (8:1:1). This proportion was maintained for 5 min, at which time the proportion of eluent 3 was increased linearly to 20% during 15 min, held for 3 min and the column then re-equilibrated.

Glycosyl composition analysis. Desalted oligosaccharide frs obtained from the PA-100 column were lyophilized and hydrolysed with 2 N TFA at 121° for 1 hr in sealed glass tubes. Samples were then taken to dryness under a stream on N_2 and dissolved in dist. H_2O. Hydrolysed samples were chromatographed using Dionex CarboPac™ PA-1 according to ref. [16].

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