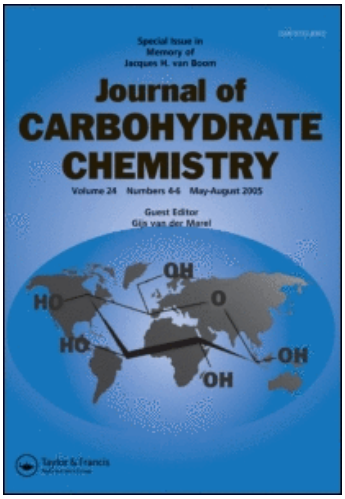

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Deoxymannojirimycin Inhibits *De novo* Synthesis of Complex *N*-Glycans and Delays Tomato Fruit Ripening[1]

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DEOXYMANNOJIRIMYCIN INHIBITS *DE NOVO* SYNTHESIS OF COMPLEX *N*-GLYCANS AND DELAYS TOMATO FRUIT RIPENING[1]

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ABSTRACT

Unconjugated *N*-glycans (UNGs) of the high mannosyl-type extracted from tomato fruit delay ripening of fruit pericarp discs by 2 days when applied exogenously. Since the amount of high mannosyl-type UNGs decreases while the amount of complex-type UNGs increases during early ripening, we postulated that high mannosyl UNGs are converted to complex-type by Golgi enzymes to relieve inhibition of ripening-inhibition by the high mannosyl-type UNGs. In this paper, we investigated the effect of deoxymannojirimycin, an inhibitor of mannosidase I involved in complex *N*-glycan synthesis. Deoxymannojirimycin, applied to pericarp discs, both inhibited synthesis of complex-type *N*-glycans and delayed ripening, supporting our hypothesis on modulation of tomato ripening by UNGs.

INTRODUCTION

The unconjugated *N*-glycans $Man_5GlcNAc$ and $Man_3(Xyl)GlcNAc(Fuc)GlcNAc$ delay tomato fruit ripening at 10 ng (g fresh wt)$^{-1}$ when tested on intact fruit or on tomato pericarp discs.[2,3] $Man_5GlcNAc$ affects ripening only when applied on discs prepared from fruits at the mature green 2 (MG2; mature, but no visible signs of ripening) stage of ripeness, while discs prepared from slightly advanced fruits at the MG4 stage (just prior to initiation of ethylene production) were unaffected by the treatment.[4] High mannosyl-type UNGs extracted from tomato pericarp had an effect similar to $Man_5GlcNAc$, whereas the complex-type UNGs

extracted from tomato fruit did not delay ripening as did $Man_3(Xyl)GlcNAc(Fuc)GlcNAc$. While following the occurrence of these two types of UNGs during ripening using MG2 tomato pericarp discs, we observed that the amounts of [^{14}C]glucosamine-labeled high mannosyl and complex-type UNGs changed inversely during the first three days. These changes did not occur in MG4 discs, a stage when discs are unaffected by $Man_5GlcNAc$.[4] We postulated that high mannosyl-type *N*-glycans, which inhibit ripening, are converted by Golgi enzymes into complex-type *N*-glycans, thereby relieving inhibition and initiating fruit ripening.[4] In the present study, we tested this hypothesis by using deoxymannojirimycin (*d*MM), an inhibitor of mannosidase I. This is the initial enzyme acting in the Golgi complex, and its inhibition blocks processing of glycans.[5] Changes in the skin color of unripe tomato pericarp discs from green to red was used to monitor progression of tomato fruit ripening.[2-4]

RESULTS AND DISCUSSION

MG2 pericarp discs treated with 50 µL of 10 mM *d*MM started showing color change (chlorophyll degradation and lycopene synthesis) three days after treatment, whereas changes in coloration of H_2O-treated discs (control) was observed after one day (Figure 1). From day 3 on, the rate of red coloration of the *d*MM treated discs was similar to the rate of coloration of the controls with a one day delay (Figure 1). The occurrence and separation of [^{14}C]-labeled high mannosyl- (SR; strongly retained) and complex-type (WR; weakly retained) UNGs was observed upon Con A chromatography as previously described.[6] After *d*MM treatment, no complex type UNGs were observed until day 3. A summary of Con A profiles of discs treated with *d*MM or H_2O is presented in Table 1. The processing of high mannosyl *N*-glycans recovered after 3 days, which correlated with the recovery of ripening as reflected by color change of the skin (Figure 1). In previous work, we hypothesized that high mannosyl-type *N*-glycans must be converted to complex-type by Golgi enzymes in order to allow ripening to proceed, since discs treated with high mannosyl-type UNGs from tomato pericarp showed a 2-day delay in ripening, probably due to the inability of Golgi enzymes to process this exccess amount of exogenously applied high mannosyl UNGs.[4] Since we did not observe any effect of complex-type UNGs, we postulated that high mannosyl-type *N*-glycans were converted to complex-type, relieving the inhibition of fruit ripening.

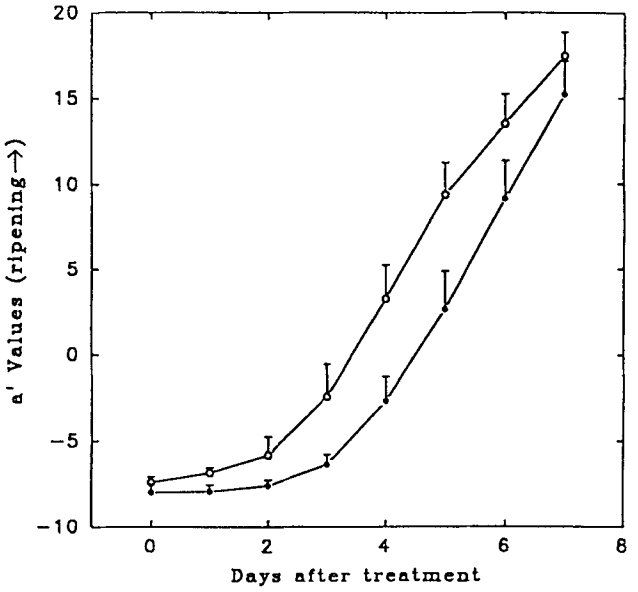

Figure 1. Effect of 285 µg/gfw deoxymannojirimycin (●) on ripening of mature green pericarp discs. Controls were treated with H_2O (○). Data are the mean of 6 discs ± SE.

Table 1. Radiolabeling of weakly retained (WR) and strongly retained (SR) Con A-Sepharose fractions for 5 days after [^{14}C]glucosamine application to discs from MG2 fruit pretreated with 285 µg/gfw deoxymannojirimycin versus control. Data are expressed as % of total ^{14}C-labeled *N*-glycan in WR or SR fractions relative to total ^{14}C detected in the desalted fraction.

Time(d)	%WR		%SR	
	Control	*d*MM	Control	*d*MM
1	0.5	ND[a]	2.4	2.9
2	0.6	ND	1.2	0.9
3	8.3	0.9	3.0	0.6
4	2.8	2.4	9.7	1.1
5	NA[b]	5.0	NA	2.5

a. ND = none detected; b. NA = not done

We observed that *d*MM treatment delayed the accumulation of [^{14}C]glucosamine in complex-type *N*-glycans, indicating that inhibition of mannosidase 1 affected processing of high mannosyl-type *N*-glycans to complex-type (Figure 1, Table 1). This observation supports our hypothesis concerning a role of UNGs in initiation of fruit ripening. Ripening, which is the beginning of senescence, represents the final stage of a tomato plant's life in which the fruit become fragrant, tasty, and soft. This results in eventual seed dispersal through consumption and seed elimination by animals or by colonization by various microorganisms, ensuring a new generation of progeny. Therefore, it is important for fruit not to ripen before reaching full size with viable seed. Our results suggest a role for UNGs in modulating the ripening process.

EXPERIMENTAL

Plant and chemical materials. Tomato plants (*Lycopersicon esculentum* Mill., cv. 'Rutgers') were grown in a greenhouse without supplemental lighting. Flowers were pollinated using a mechanical vibrator and tagged at anthesis. Ripeness stages were determined as previously described.[7] Con A-Sepharose (Type III-A), *d*MM, and α-MeGlc were from Sigma (St. Louis, MO). [U-^{14}C]-D-Glucosamine hydrochloride (286 mCi/mmol) was from Amersham (Arlington Heights, IL). The UNG fractions WR and SR were prepared from tomato fruit as previously described.[6]

Pericarp disc preparation and treatment. After surface-sterilization of fruit with 0.525% Na-hypochlorite, pericarp discs were prepared using the procedure described in detail elsewhere.[4] However, for detection of radiolabeled UNGs, the skin was first removed from fruit prior to disc preparation. Treatment groups consisted of 6 discs (14-mm in diameter), each from one of six fruits. Treatment solutions were sterilized using 0.45 μm filters and were applied to discs 24 h after excision to allow metabolism to equilibrate prior to treatment. The areas between culture plate wells were partially filled with sterile H_2O to maintain a relatively humid environment. A 10 mM stock solution of *d*MM was prepared, and 50 μL (or 50 μL H_2O) were applied to the cut surface of discs to give a final concn of 265 μg (g fresh wt)$^{-1}$. For metabolic labeling, 1 μCi of [^{14}C]glucosamine was dissolved in a 50 μL of either 10 mM *d*MM or dd H_2O. The increase in red coloration of discs, which is closely

correlated with ripening of intact fruit,[2,8] was quantified by determining the a' value every 24 to 48 h using a Hunter XL-800 colorimeter. Experiments were performed in an ethylene-free room using a Swingcat 50 ethylene scrubber (Tubamet AG, Switzerland). After metabolic [^{14}C]-labeling for 1, 2, 3, 4 or 5 days, discs were frozen in liquid N_2.

Oligosaccharide extraction. [^{14}C]-Labeled UNGs were prepared as previously described.[4,6] Briefly, mature green pericarp discs (1.4 g), which had been incubated with [^{14}C]glucosamine, were lyophilized, placed into 15 mL of boiling 80% EtOH, capped, and stirred for 15 min. Samples were then homogenized, filtered, and dried with a rotary evaporator *in vacuo*. Five mL of 0.01% (v/v) acetic acid containing 0.04% (w/v) NaN_3 were added and the samples desalted using a Waters TSK-HW-40S glass HPLC column. A peak which eluted between 43 and 54 min included desalted oligosaccharides and was therefore collected for Con A-Sepharose chromatography as previously described.[6] Elution of UNG fractions with 10 mM α-MeGlc gave a weakly retained (WR) fraction that includes the complex-type UNGs, whereas 300 mM α-MeGlc eluted a strongly retained fraction (SR) that contained high mannosyl-type UNGs.[6] The fractions were lyophilized, dissolved in 0.5 mL of H_2O, and 10 mL of scintillation cocktail were added for [^{14}C] counting.

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