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Biochemical changes associated with the ripening of hot pepper fruit

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Hot pepper (*Capsicum annuum* L. cv. Chooraehong) fruit underwent a respiratory climacteric during ripening. However, the rate of ethylene production was low, reaching a maximum of approximately 0.7 μl kg^{-1} h^{-1} at the climacteric peak when the surface color was 30 to 40% red. Ripening was accompanied by a loss of galactose and arabinose residues from the cell wall. The content of uronic acid and cellulose in the wall changed only slightly during ripening. The average molecular weight of a cell wall hemicellulosic fraction shifted progressively toward a lower molecular weight during ripening. Total β-galactosidase (EC 3.2.1.23) activity increased 50-fold from the immature green to the red ripe stage. No polygalacturonase (EC 3.2.1.15) activity was detected at any stage of ripeness. Thus, the loss of galactose and arabinose residues from the cell wall, as well as the observed modification of hemicelluloses during ripening, seem to be unrelated to active polygalacturonase. Soluble polyuronide content remained relatively constant at approximately 60 μg (g fresh weight)$^{-1}$ as fruit ripended.

Additional key words – Capsicum annuum, cell wall, ethylene, β-galactosidase, polygalacturonase, respiration, soluble polyuronide.

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Introduction

As a result of many studies on ethylene production and respiration during the ripening of various fruits, it is now widely accepted that an increase in ethylene evolution slightly precedes or occurs simultaneously with an increase in respiration in climacteric fruits, while in non-climacteric fruits, no significant increase in respiration or ethylene production occurs (McGlasson 1985).

In studies on the ripening behavior of 'Chooraehong' hot pepper from the Republic of Korea, we observed that the fruit, which are dry and dehiscent at maturity, underwent a respiratory climacteric, but produced only relatively small amounts of ethylene throughout ripening. This paper reports our findings on ripening-related changes in ethylene and CO_2 production, cell wall composition, hemicellulose modification, soluble polyuronide content, polygalacturonase (EC 3.2.1.15) activity, and β-galactosidase (EC 3.2.1.23) activity of hot pepper fruit during ripening.

Abbreviation – ddH_2O, distilled-deionized water.

Materials and methods

Plant material

'Chooraehong' hot pepper plants were grown in a greenhouse under a temperature regime of 27/21°C day/night without supplemental lighting. Plants were staked

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in 20 l pots containing 15 g of Osmocote (14–14–14), Ball Mix II (a commercial soilless medium) and fertilized weekly. Flowers were pollinated by hand at anthesis and tagged. Fruit maturity was based on number of days after pollination, as well as surface coloration. Ripeness stages were as follows: Immature Green (IG), 20 days post-pollination; Mature Green (MG), 30 days post-pollination; Turning (T), 48 days post-pollination, 30–40% red surface color; Red Ripe (RR), 60 days post-pollination, 100% red surface color. Fruit were freshly harvested for each experiment or analysis.

Ethylene and carbon dioxide measurement

Initially, gas analysis was performed using a flow-through system. Fruit were hand-harvested at the mature green stage, rinsed with ddH_2O, weighed and individually placed into clear chambers (Polyvinyl chloride pipe; 2.8 cm i.d. by 12.5 cm). Respiration and ethylene production were measured by gas chromatography using an automatic, flow-through system interfaced to a minicomputer as previously described (Watada and Massie 1981). A Bendix Model 2300 chromatograph equipped with a 3.2 mm × 44 cm stainless steel column packed with Poropak Q 50/80 was used.

In subsequent experiments, a static, closed system was employed to determine ethylene evolution, since the relatively low amounts of ethylene produced were not detectable using the flow-through system. This procedure involved enclosing indivual fruit in containers with 3 ml of 5 *M* KOH to absorb evolved CO_2 and 5 ml of H_2O to maintain a high relative humidity. After being sealed for 18 h at 20°C, an aliquot of the enclosed atmosphere of each container was analyzed for ethylene content. The container was then opened, flushed with fresh air for 6 h, and resealed.

Cell wall extraction and analysis

Outer pericarp tissue was homogenized in 3 volumes of 80% ethanol using a Polytron homogenizer. After filtration through Miracloth, the residue was extracted sequentially with 20 m*M* N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid-NaOH (pH 6.9), chloroform-methanol (1:1, v/v), and acetone (Gross 1984), with the addition of an α-amylase (Sigma Chemical Co., Type III) treatment before the chloroform-methanol step to remove starch (Gross and Sams 1984). Cell wall material was dried over P_2O_5 in vacuo at 37°C to a constant dry weight prior to analysis.

The monosaccharide constituents of non-cellulosic polysaccharides of cell walls were quantified by capillary gas chromatography-mass spectrometry. Polysaccharides were hydrolyzed with 2 *M* trifluoroacetic acid using the procedure of Jones and Albersheim (1972). Aldononitrile acetate derivatives of monosaccharides were prepared according to Lehrfeld (1981). Sugar derivatives were separated on a Hewlett-Packard 12.5 m WCOT (0.2 mm i.d.) OV-101 dimethylsilicone capillary column as previously described (Gross 1983) using a Hewlett-Packard 5880 gas chromatograph/5970A Mass Selective Detector (electron impact mass spectrometer). Quantification of individual sugars was performed using selected ion monitoring (m/z 145) at an ionization potential of 70 eV; *myo*-inositol was the internal standard.

Cellulose content of cell wall material was estimated according to the procedure of Updegraff (1969) as previously described (Gross 1984). Uronic acid content was determined by dissolving 10 mg of wall material in concentrated H_2SO_4 (Ahmed and Labavitch 1977) and assaying aliquots for uronic acids using carbazole (Dische 1947).

Soluble polyuronide determination

Soluble polyuronide was determined using a modification of the method described by Conway and Sams (1983). Ten g of outer pericarp tissue were homogenized in 20 ml of 0.1 *M* K_2CO_3 using a Polytron homogenizer. The homogenate was filtered through Miracloth and the residue discarded. The filtrate was mixed with 0.15 ml of 40% (w/v) $MgCl_2$ and centrifuged for 15 min at 27 000 *g*. The pellet was suspended in 10 ml of 100% ethanol and recentrifuged. After dissolving the resulting pellet in 20 ml of ddH_2O (80°C) samples were cooled to 20°C in an ice bath and recentrifuged. The supernatant was then assayed for uronic acids using carbazole (Dische 1947).

Enzyme extraction and assay

For extraction of polygalacturonase and β-galactosidase, 50 g of outer pericarp tissue were homogenized in 100 ml of ddH_2O at 4°C. The homogenate was stirred slowly for 30 min. Solid NaCl was added to a concentration of 1.0 *M*, and the pH adjusted to 6.0 with 0.5 *M* NaOH. After 1 h, the homogenate was filtered through Miracloth. Solid $(NH_4)_2SO_4$ was added to the filtrate and the protein, insoluble between 30 and 80% saturation, was collected by centrifugation at 27 000 *g* for 15 min. The pellet was dissolved in 0.15 *M* NaCl and dialyzed (Spectra/Por 1; 6–8000 MWCO) against 0.15 *M* NaCl for 18 to 25 h. All extraction procedures were carried out at 4°C.

Polygalacturonase (EC 3.2.1.15) activity was estimated by measuring the hydrolytic release of reducing groups from polygalacturonic acid under assay conditions described previously (Gross 1982) using 2-cyanoacetamide (Honda et al. 1982). β-Galactosidase (EC 3.2.1.23) activity was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-galactoside. Reaction mixtures (1 ml total volume) contained 0.1 ml of enzyme, 60 m*M* Na-acetate (pH 4.0), 15 m*M* NaCl, 0.06% bovine serum albumin (w/v), and 1.0 mg of substrate. Tubes were incubated at 30°C for 10 min and the

reaction stopped by adding 2 ml of 0.2 *M* Na_2CO_3. The absorbance at 400 nm was determined in order to measure liberated *p*-nitrophenol. Blanks (0-time) were run by adding Na_2CO_3 prior to the addition of substrate. Product formation was linear with time and proportional to the amount of enzyme in the reaction.

Hemicellulose extraction and chromatography

Hot pepper fruit cell walls were extracted with 50 m*M* 1,2-cyclohexanediaminetetracetic acid for 6 h and 50 m*M* Na_2CO_3 for 20 h at 25°C to remove pectic polysaccharides, and then with 4 *M* KOH containing 100 m*M* $NaBH_4$ to solubilize a hemicellulosic fraction as described in detail elsewhere (Gross 1984).

Gel filtration of the hemicellulosic fraction extracted from hot pepper cell walls was performed at 20°C using a column of Sephacryl S-500 (2.8 cm × 42 cm; 260 ml total bed volume) equilibrated in 50 m*M* 2-(N-morpholino)ethanesulfonic acid-NaOH (pH 6.5) containing 0.1 *M* NaCl and 5 m*M* EDTA. A 3 ml sample containing 5 mg of the hemicellulosic fraction was applied to the column; 4 ml fractions were collected at a flow rate of 0.4 ml min^{-1}. Since the predominant sugars in the hemicellulosic fraction are hexoses, fractions were analyzed for total hexose using anthrone (Spiro 1966). The column was calibrated with Blue Dextran 2000, dextrans of molecular weight 10^5 and 10^4, and glucose.

Results and discussion

Respiration and ethylene production

In previous studies, Lee et al. (1975) and Lee and Kim (1973) observed that hot pepper fruit underwent a respiratory climacteric, but produced no detectable ethylene during ripening. Since this was unusual for a climacteric type fruit, we undertook a study to characterize the ripening process in these hot peppers. Twenty-four fruits were harvested at the mature green stage and placed in individual containers at 20°C. Humidified air was metered through the containers at a rate such that the CO_2 level was maintained at 0.4% (v/v). Carbon dioxide and ethylene production by each fruit were monitored every 4 h for 10 days. The rates of CO_2 and ethylene production of a representative fruit are shown in Fig. 1. Although the fruit underwent a respiratory climacteric no ethylene was detected; a similar pattern was observed with all 24 hot pepper fruits. The respiratory climacteric peak occurred when fruit were approximately 50% red (data not shown).

In the event that fruit were producing low levels of ethylene which were undetectable using the flow-through system, we studied the production of ethylene using a static system that allowed ethylene to accumulate for 18 h prior to analysis. Five fruits showing incipient coloration (10% red color) were harvested and placed in individual containers (75 ml volume) with 3 ml of 5 *M* KOH and 5 ml of ddH_2O as described in Materials and methods. Ethylene evolution by individual fruit was determined each day for one week. Using this approach, a relatively low rate of ethylene production was evident; the rate from each fruit increased and

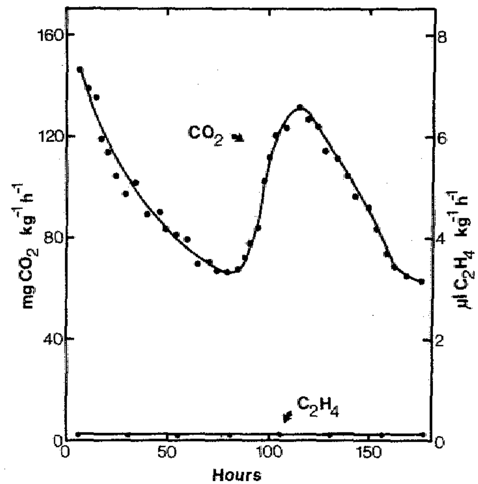

Fig. 1. Ethylene and CO_2 evolution by one of 24 individual 'Chooraehong' hot pepper fruits after harvest at the mature green stage of ripeness (30 days post-pollination). Gases were analyzed at 20°C with an automated sampling system using gas chromatography as described in Materials and methods.

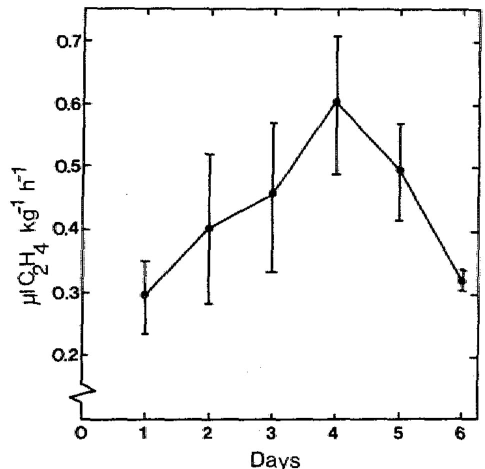

Fig. 2. Ethylene evolution of hot pepper fruit which were harvested when 10% red and held at 20°C for 1 week. Ethylene was analyzed using a static system which allowed gas concentration to increase for 18 h prior to analyzing the atmosphere using gas chromatography. Data are the mean ± SD of 5 fruits after shifting the peak production rate of all fruit to day 4 on the x-axis.

reached a peak within 5 days. Figure 2 depicts the average rate of ethylene evolution of the 5 fruits after shifting the peak rate of each fruit to day 4 on the x-axis, so that day 4 represents the maximum rate for all fruit. These results show that 'Chooraehong' hot peppers do exhibit a climacteric pattern of ethylene production, but the rate was only 0.7 µl kg^{-1} h^{-1} at the climacteric peak.

Because of the low rate of ethylene production, the relatively small increases during ripening were not apparent using the flow-through system. The amount of ethylene produced by hot pepper fruit was low when compared to other climacteric-type fruit, where maximum rates of climacteric ethylene production (Biale 1960) range from 10 µl kg^{-1} h^{-1} (tomato) to 380 µl kg^{-1} h^{-1} (passion fruit).

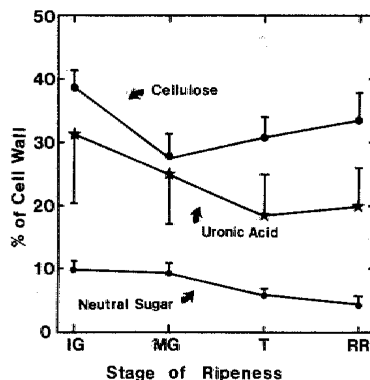

Fig. 3. Non-cellulosic neutral sugar, uronic acid and cellulose content of cell wall material extracted from hot pepper fruit during ripening. Stages of ripeness are described in Materials and methods. Data represent the mean ± SD of 3 separate analyses. IG, immature green; MG, mature green; T, turning; RR, red ripe.

Cell wall composition

An increase in wall-associated polygalacturonase activity and soluble polyuronide accompany the ripening of a variety of fruits (Huber 1983, Labavitch 1981, Pilnik and Voragen 1970, Pressey 1977). A concomitant loss of wall-associated galacturonosyl residues occurs in 'Bartlett' pears (Ahmed and Labavitch 1980) and tomatoes (Gross 1984). In addition to cell wall uronide solubilization, a net loss of neutral sugar residues occurs from most fruits during ripening, involving primarily galactosyl and arabinosyl residues (Ahmed and Labavitch 1980, Gross and Sams 1984, Knee 1973, Knee et al. 1977).

Changes in cell wall carbohydrate composition of hot peppers during ripening are shown in Fig. 3. Compared to a number of other fruit types (Gross and Sams 1984), 'Chooraehong' hot pepper fruit cell walls contained a relatively low amount (10%) of non-cellulosic neutral sugar. The total amount decreased by more than 50% during ripening. After an initial decrease from the immature green to the mature green stage, the amount of uronic acid in cell walls changed only little during ripening. The relative cellulose content also decreased during the period prior to maturity, but increased slightly during ripening.

The decrease in non-cellulosic neutral sugar residues primarily involved galactose- and arabinose-containing polymers (Tab. 1). The galactose and arabinose content of walls decreased by 76 and 38%, respectively, during ripening. A loss of glucose residues also occurred. However, this may have been due to a small amount of starch in immature green wall preparations, despite α-amylase treatment. These wall changes are qualitatively and quantitatively similar to changes observed during the ripening of Cayenne hot peppers (Gross and Sams 1984).

Soluble polyuronide and hemicellulose modification

Unlike many fruits, the amount of soluble polyuronide extracted from hot pepper pericarp tissue did not change significantly during ripening. Fruits at the mature green, turning, and red ripe stages of ripeness contained 57 ± 15, 69 ± 37, and 57 ± 34 µg (g fresh weight)$^{-1}$, respectively.

In addition to tomato cell wall compositional changes, Huber (1983) observed a marked change in the molecular weight distribution of cell wall hemicelluloses. During ripening, tomato fruit showed progres-

Tab. 1. The non-cellulosic neutral sugar composition of 'Chooraehong' hot pepper cell walls during ripening. Data represent the mean ± SD of 3 separate analyses. Stages (see Fig. 3 legend for abbrev.) of ripeness are described in Materials and methods. Rha, rhamnose; Ara, arabinose; Xyl, xylose; Man, Mannose; Glc, glucose; Gal, galactose.

Stage	mg (100 mg cell wall)$^{-1}$					
	Rha	Ara	Xyl	Man	Glc	Gal
IG	0.7±0.1	1.5±0.6	1.2±0.3	0.5±0.1	1.2±0.1	4.7±0.5
MG	0.6±0.1	1.3±0.4	1.4±0.3	0.4±0.1	0.9±0.2	4.6±1.3
T	0.6±0.1	1.0±0.3	1.5±0.3	0.4±0.1	0.9±0.2	1.6±0.5
RR	0.5±0.1	0.8±0.2	1.3±0.3	0.4±0.1	0.6±0.2	1.1±0.4

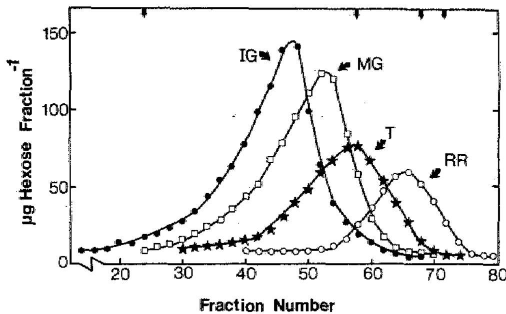

Fig. 4. Sephacryl S-500 gel-filtration profiles of a hemicellulosic fraction extracted from 'Chooraehong' hot pepper fruit at various stages of ripeness. Chromatographic conditions and stages of ripeness are described in Materials and methods. Arrows at the top of the figure represent the elution positions of (from left to right): Blue Dextran 2000, dextrans of average molecular weight of 10^5 and 10^4, and glucose. IG, immature green; MG, mature green; T, turning; RR, red ripe.

sively lower amounts of high molecular weight polysaccharides and higher amounts of low molecular weight polymers. These results suggest that substantial modification of cell wall integrity could occur without manifestation as net changes in cell wall composition. In view of the minimal change that occurred in cell wall polyuronide solubilization (Fig. 3), we studied hemicellulose modification of hot pepper cell walls using the general approach of Huber (1983).

In addition to the net neutral sugar compositional changes that occurred, a hemicellulosic fraction extractable from hot pepper fruit cell walls was modified during maturation and ripening resulting in a shift from higher (about 100 000) to lower (about 10 000) average molecular weight (Fig. 4). The change was similar to that observed in tomato (Huber 1983) and also in strawberry fruit (Huber 1984), which contain no detectable polygalacturonase activity and do not show the normal breakdown of cell wall pectins which occurs in most fruits during ripening and softening. The total amount of hexose positive material decreased during ripening, suggesting a change in hemicellulose hexose composition. This is currently under investigation.

Tab. 2. Polygalacturonase and β-galactosidase activities (mmol (g FW)$^{-1}$ h^{-1} in hot pepper fruit at various developmental stages. Data represent the means of 2 separate enzyme extractions and assays. Stages (see Fig. 3 legend for abbrev.) of ripeness are described in Materials and methods. ND, none detected.

Enzyme	Stage of ripeness			
	IG	MG	T	RR
	mmol (g FW)$^{-1}$ h^{-1}			
Polygalacturonase	ND	ND	ND	ND
β-Galactosidase	1.2	4.3	14.8	62.4

β-Galactosidase and polygalacturonase activities

Since the content of galacturonosyl residues did not decrease substantially during the ripening of 'Chooraehong' hot peppers, and since no increase in soluble polyuronide occurred, we attempted to estimate the activity of polygalacturonase during ripening. However, no polygalacturonase activity was detected throughout the ripening process (Tab. 2). The assay used to estimate polygalacturonase activity (Gross 1982) was recently used to measure previously undetectable polygalacturonase activity in bell peppers (Jen and Robinson 1984).

Pressey (1983) showed that total β-galactosidase activity in tomatoes, which remained relatively constant throughout ripening, was due to the presence of 3 isozymes whose individual levels of activity changed differentially during ripening. One isozyme (β-galactosidase II) increased in activity 3-fold during ripening. The increase in β-galactosidase II activity coupled with its ability to degrade, in vitro, a galactose-rich polysaccharide extracted from mature green tomato walls using polygalacturonase II and pectinesterase II (Pressey and Avants 1982) indicated that β-galactosidase II may be involved in fruit softening (Pressey 1983) by hydrolyzing β-1,4-galactan (Pressey and Himmelsbach 1984).

We studied the activity of β-galactosidase in hot peppers to determine the relationship between activity and the loss of galactosyl residues from the wall during ripening. Unlike total β-galactosidase activity in tomatoes, total activity increased 15-fold during the ripening of hot peppers (Tab. 2). Studies using Sephacryl S-200 gel-filtration and DEAE-cellulose chromatography showed that the total activity was due to the presence of at least 4 isozymes (S. D. Kim, K. S. Kim, and K. C. Gross, unpublished results). The characterization of these isozymes is currently under investigation.

In summary, the results of this study show that, unlike bell peppers which are non-climacteric (Saltveit 1977), hot peppers are a climacteric fruit but produce lower amounts of ethylene during ripening relative to most climacteric fruits. Possibly other types of fruit which are dry and dehiscent at maturity would follow a similar ripening pattern. They are also different from many climacteric fruit in that they produce no detectable polygalacturonase activity during ripening. Thus, the results suggest that the hemicellulose modification that occurs, as well as the loss of galactosyl and arabinosyl residues from the wall, are apparently not related to active polygalacturonase. In this respect, hot pepper fruit cell wall changes are similar to those of strawberry (Huber 1984).

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