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POST-HARVEST BIOCHEMICAL CHANGES ASSOCIATED WITH THE SOFTENING PHENOMENON IN *CAPSICUM ANNUUM* FRUITS

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Key Word Index—*Capsicum annuum*; Solanaceae; fruits; bell pepper; biochemical changes; cell-wall hydrolases; alcohol-insoluble carbohydrates; neutral sugars.

Abstract—Changes in the activities of various cell-wall hydrolases were followed during ripening of bell pepper (*Capsicum annum*). The activity of polygalacturonase (PG) increased, while that of pectin methyl esterase (PME) decreased. The texture of the fruit declined concomitantly with the increase in PG activity. The behaviour of the glycanases, namely cellulase, xylanase, mannanase, glucanase and galactanase, and glycosidases, like α-D-mannosidase and β-D-galactosidase, were monitored. The influence of low storage temperature (8°) on the enzyme activities was studied in which PG, PME, cellulase, mannosidase and galactosidase were suppressed to different levels. Sugar composition of the alcohol-insoluble carbohydrate residue was also analyzed during ripening. Galactose (10%) was the major sugar followed by arabinose (4.2%). The amount of the neutral sugar which was initially 25% decreased to 15% during ripening, resulting in a nearly 40% loss; the changes mainly involved reduction in galactosyl and arabinosyl residues.

INTRODUCTION

Bell pepper (*Capsicum annuum*) is one of the commercially important crops which is classified under vegetable fruits. The texture, in particular the crispness of the pepper is an important quality attribute to consumers. The major post-harvest problem with this crop is excessive softening that may cause shrinkage, drying and pathological disorders which severly reduce the quality and acceptability of the product.

The softening that occurs in any fruit is primarily due to a change in cell-wall carbohydrate metabolism, resulting in a net decrease in certain structural components [1,2]. The changes in cell-wall composition result from the action of hydrolytic enzymes produced by the fruit. Prominent among the enzymes implicated are polygalacturonase (PG) and pectin methyl esterase (PME), because striking changes in wall pectin content are observed in ripening fruits and activities of these two enzymes often increase as ripening continues [3]. In addition, a variety of glycanases and glycosidases have been assigned roles in fruit cell-wall metabolism [3]. Although bell pepper softens extensively during ripening [4], relatively little work has been done to characterize post-harvest changes in its cell-walls. This paper describes measurements of the activity of a variety of carbohydrate-degrading enzymes in extracts of ripening bell pepper, a few of which are correlated with texture loss. Also, the influence of low storage temperature on these enzymes is reported, since temperature is the most effective environmental factor preventing ripening phenomenon [5].

Apart from solubilization of cell-walls, a net loss of non-cellulosic neutral sugar residues also occurs during the ripening of pear, apple, strawberry and tomato [6]. However, unlike changes in cell-wall polyuronides, the neutral sugar composition of fruits has been studied only in a limited number of species. Our paper also gives an account of the net loss of neutral sugars in EtOH-insoluble carbohydrates during ripening of bell pepper, which is associated with the enzyme activities.

RESULTS AND DISCUSSION

Pectolytic activities of pepper, measured at four stages of ripening are depicted in Fig. 1A. PME activity was present at Stage I (1st day of harvest) and was maximal at Stage II (7th day of harvest). It declined with further ripening and was negligible at Stages III (14th day of harvest) and IV (21st day of harvest). PG activity, which developed during ripening was maximal at Stage III (Fig. 1A). These results suggest that the action of PME is to prepare the pectic substances for PG to act upon. The degree of pectin methylation is

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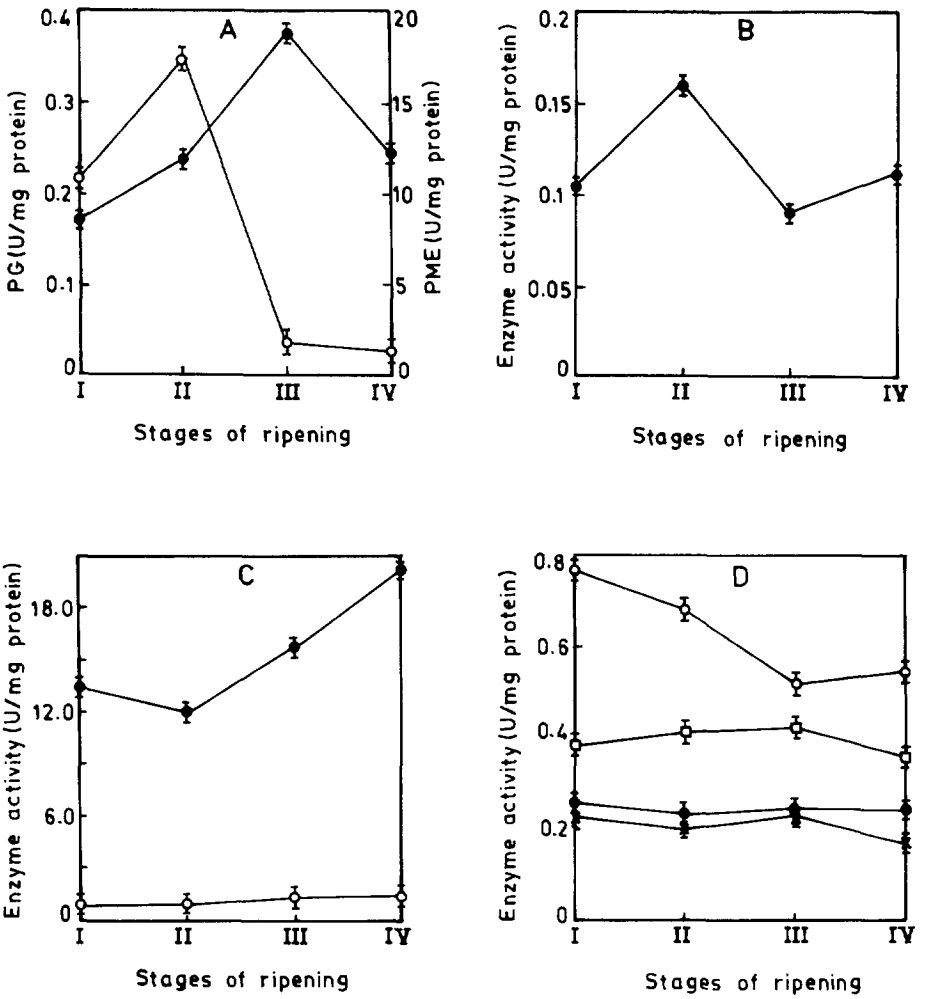

Fig. 1. Activities of cell-wall hydrolases of bell pepper at different stages of ripening (day of harvest). I – 1st day (colour: dark green); II – 7th day (light green); III – 14th day (turning); IV – 21st day (red); (A) ●—● polygalacturonase; ○—○ pectin methyl esterase, (B) ●—● cellulase, (C) ●—● α-D-mannosidase; ○—○ β-D-galactosidase, (D) ○—○ xylanase; ●—● mannanase; ×—× glucanase; □—□ galactanase. Vertical bars – s.d.

important for the action of PG [7]. PME was suppressed by *ca* 21% at low storage temperatures as compared with the activity in freshly harvested fruits (Table 2). The pectolytic enzyme activities in ripening pepper paralleled those of tomato [8], both belonging to the same family, Solanaceae. An increase in wall-associated polygalacturonase activity and soluble polyuronide accompany the ripening of a variety of fruits [2,9–11].

The texture index of pepper fruit declined with advancing ripening. There was an overall texture loss of 20±1% in fruits stored for 20 days at room temp (RT), whereas the loss was only 2±1% in the case of fruits stored for the same period at 8° (Table 1). This indicates that the textural softening is prevented at low storage temperature which may be due to various reasons like reduction in respiration and transpiration rates, inhibition of cell-wall hydrolytic enzymes, decreased ethylene production, etc. [12].

The loss in texture was found to be consistent with the increase in pepper PG activity. Softening in pepper does not correlate with PME activity since PME declined as softening increased. The texture loss in tomato during ripening has also been correlated with increase in PG activity [13]. The amount of soluble polyuronide extracted from pepper pericarp tissue increased from 56±3 μg (Stage I) to 71±4 μg (Stage IV) per g fresh weight during ripening. The rise in PG

Table 1. Texture analysis of ripening peppers

	Shear force (kg g^{-1})		Texture loss (%)	
Days of storage	RT	8°	RT	8°
0	41.25	41.25	0.00	0.00
5	39.98	41.11	3.08	0.34
10	38.25	40.87	7.27	0.92
15	35.59	40.52	13.72	1.77
20	32.67	40.15	20.80	2.67

RT, Room temperature.
The data given are means of three values.

Table 2. Effect of low temperature (8°) on activities of pectolytic and other carbohydrate-hydrolyzing enzymes

Enzymes	Activity in units mg^{-1} protein	
	Freshly harvested fruits	Fruits stored at 8° for 20 days
Polygalacturonase	0.171	0.129
Pectin methyl esterase	10.8	8.5
Cellulase	0.105	0.092
α-D-mannosidase	13.5	11.7
β-D-galactosidase	0.84	0.57
Mannanase	0.254	0.249
Xylanase	0.763	0.752
Glucanase	0.232	0.234
Galactanase	0.382	0.375

Results are means of three independent experiments.

activity coincides with the conversion of cell-wall pectic polysaccharides to H_2O-soluble forms. Also, there was nearly a 25% suppression in the level of PG in fruits stored at 8° for 20 days when compared with the levels in freshly harvested fruits (Table 2). In addition, there was a net loss of nearly 56% in the yield of the pectinic acid fraction of EtOH-insoluble carbohydrates from the fresh fruits as compared to the fruits stored at RT for 20 days (Table 3). All these results suggest a key role for PG in the wall changes which accompany pepper-ripening.

Cellulase activity increases with the ripening of many fruits [14, 11]. Cellulolytic enzymes could degrade both cellulose and the β-1,4-glucan backbone of xyloglucan, a hemicellulosic polysaccharide prominent in walls of dicotyledons [15]. Cellulase activity was maximum at Stage II and gradually decreased thereafter (Fig. 1B). There was no difference in the yield of the cellulosic fraction of cell walls (Table 3) between the fresh and the softened fruits. Cellulase was suppressed by *ca* 12% in activity at low temperature (Table 2). Activities of xylanase were maximum at Stage I and gradually reduced as the ripening progressed (Fig. 1D). Mannanase, glucanase and galactanase activities were present constantly in significant amounts in all the stages, with a slight increase in Stage III (Fig. 1D) of ripening. The glycanases did not show significant suppression of activity at 8° (Table 2). Arabinanase and rhamnanase activities were not followed due to the non-availability of the respective substrates.

There was a steady increase (*ca* 50%) in α-D-mannosidase during ripening, whereas, β-D-galactosidase increased by nearly 70% from Stage I to Stage III and remained constant thereafter (Fig. 1C). Changes in various glycosidase activities have been described for many fruits [11, 16, 17]. The most complete records are those of Pharr *et al.* [17] and Wallner and Walker [18] who described eight and seven glycosidases, respectively in tomato. The cellular role of this array of glycosidases is not clear. Yet, α-D-mannosidase was found to be present in abundance and also was the most active among the glycosidases checked in bell pepper and, hence, was purified, followed by enzymological and immunological characterization (unpublished data). Under low storage temperature, mannosidase and galactosidase were suppressed by *ca* 13% and 32%, respectively as compared to freshly harvested fruits (Table 2). As with isolated hemicelluloses, galactose, glucose, xylose and mannose were the predominant neutral sugars with smaller amounts of rhamnose and arabinose. The decrease in their levels during ripening

Table 3. Changes in non-cellulosic neutral sugar composition of various carbohydrate fractions obtained from the alcohol-insoluble residue of bell pepper

Alcohol-insoluble carbohydrate fraction	Stage	Yield (%)	Non-cellulosic neutral sugar (mg 100 mg^{-1} carbohydrate fraction)*						
			Total	Rha	Ara	Xyl	Man	Gal	Glu
C.W.S.	I	19.14	28.52	Tr.	9.04	Tr.	Tr.	19.31	Tr.
	II	2.32	13.25	Tr.	6.90	Tr.	Tr.	6.20	Tr.
H.W.S.	I	8.45	24.78	0.64	3.53	1.62	0.91	14.21	3.80
	II	10.21	12.38	0.89	1.89	1.54	0.83	4.56	2.64
H.E.S.	I	19.88	25.39	3.00	5.11	8.86	Tr.	4.09	4.27
	II	8.65	16.41	2.98	1.24	7.84	Tr.	1.31	2.85
Alkali-soluble									
Hem. A	I	2.27	21.35	Tr.	Tr.	8.88	Tr.	10.36	2.06
	II	3.33	13.10	Tr.	Tr.	8.47	Tr.	3.33	1.02
Hem. B	I	8.10	25.63	Tr.	1.14	Tr.	6.51	10.40	7.50
	II	8.39	14.64	Tr.	0.87	Tr.	6.07	3.44	4.24
Hem. C	I	2.76	26.78	6.70	6.43	3.75	4.48	2.16	3.20
	II	28.59	21.19	8.44	4.01	2.58	3.01	0.69	2.38

C.W.S., Cold water-soluble; H.W.S., Hot water-soluble; H.E.S., Hot EDTA-soluble (pectinic acid); Hem., Hemicellulose; Rha, Rhamnose; Ara, Arabinose; Xyl, Xylose; Man, Mannose; Gal, Galactose; Glu, Glucose (Fucose was not determined).

Stage I – 0 day of storage; Stage II – 20th day of storage.

Yield of alkali-insoluble cellulosic fraction: Stage I – 39.40%; Stage II – 38.51%.

*Data represent means of three analyses; triplicate injections were made of each sample.

Tr., present in trace amounts (less than 0.1 mg 100 mg^{-1} fraction).

(Table 3) may be due to the action of the glycanases with the combined effect of glycosidases.

The EtOH-soluble sugar (total) content increased from 120±5 mg (51 mg reducing sugar; 69 mg non-reducing sugar) to 440±8 mg (115 mg reducing sugar; 325 mg non-reducing sugar) per 10 g fresh weight during softening. The soluble sugar content of the fruits stored for 20 days at low temperature (114±5 mg per 10 g fresh weight) resembled that of fresh fruits which were analysed immediately after harvest.

The EtOH-insoluble carbohydrate fractions of bell pepper contained nearly 25% of non-cellulosic neutral sugar (Table 3). This decreased to nearly 15% during ripening, resulting in a 40% loss as compared to 39% and 56% loss of cell-wall neutral sugar in tomato and hot pepper, respectively [6]. Galactose was the major neutral monosaccharide present in unripe fruit accounting for 10% of the fractions, which is also true for tomato and hot pepper, where it constitutes 14% of the wall material [6]. The net loss of neutral sugar residues during ripening involved primarily galactose (*ca* 68% loss)- and arabinose (*ca* 41% loss)-containing polymers followed by glucosyl, mannosyl and xylosyl residues (Table 3).

A survey of many types of fruits revealed that 15 out of 17 experienced a net loss of non-cellulosic neutral sugar with ripening and 14 out of 15 experienced net loss of cell-wall galactosyl and/or arabinosyl residues [6]. The substantial loss of galactosyl and arabinosyl residues during ripening in bell pepper indicates changes in pectic polymers. However, lack of any change in the amount of rhamnosyl residues (Table 3), which form branch points in the backbone of rhamnogalacturonic acid polymers for attachment of galactan and arabinogalactan side-chains, indicates that there may be little change in the proportion of cell-wall rhamnogalacturonan polymers during ripening. The slight change in the amount of xylosyl, mannosyl and glucosyl residues indicates little change in hemicellulose composition (Table 3).

On the basis of available information, of the enzyme activities described above, PG can presently be assigned a definite role with the other enzymes playing an unspecified role in softening-associated cell-wall metabolism of bell pepper. This paper also demonstrates that many of the cell-wall compositional changes which occur during softening and senescence of bell pepper, including a decrease in galactosyl and arabinosyl residues are similar to those which occur in other rapidly-softening fruits, such as tomato, strawberry and blueberry.

EXPERIMENTAL

Material. Bell peppers (bull-nose wild variety) were freshly harvested from a local farm, brought to the laboratory and a batch of fruits was stored at room temp. for normal ripening and another batch at 8° as recommended in ref. [5].

Texture measurements. Texture measurements were conducted using a texture analyser (Instron 4301). Shear force was measured using a 500 kg load cell and a stroke speed of 200 mm min^{-1}. The firmness was expressed as kg shear force g^{-1} pepper fruit.

Activity of enzymes. Me_2CO dry powder was prepd from the fruits on the 1st, 7th, 14th and 21st day of harvest (fruit surface being dark green, light green, turning and red in colour, respectively) for following enzyme activities. Extraction of enzymes was performed for 12 hr at 4° with the respective buffers used for the assay (as given below) in the presence of 0.25 M NaCl. The resulting suspension was passed through four layers of cheesecloth and the filtrate centrifuged at 5,000 *g* for 30 min. The clarified filtrate served as crude enzyme extract.

PG. The reaction mixt. consisted of 0.2 ml enzyme in 0.15 M NaCl, 0.2 ml 0.2 M tris-acetate buffer (pH 4.5), 0.1 ml 0.01 M $CaCl_2$ and 0.5 ml 1% polygalacturonate (PGA, Sigma). A blank was prepd for each sample by boiling the reaction mixts before addition of substrate. After 1 hr at 37°, the reaction was stopped by heating at 100° for 3 min and 0.5 ml of each soln was analysed for reducing groups using the dinitrosalicylate method (DNS) [19]. One unit of activity is the amount which catalyses the formation of 1 μM of reducing group per hr [20]. *PME.* The rate of citrus pectin demethylation was measured at room temp by titration with 0.025 N NaOH; 50 ml of 1% (w/v) pectin (Sigma, from citrus fruits) in 0.1 N NaCl was used as substrate and adjusted to pH 7.0 before the addition of 1.0 ml of enzyme extract. One unit of PME activity is defined as the amount of enzyme capable of catalysing the consumption of 1 mM of base per hr under the assay conditions. *Cellulase.* Cellulase activity was determined by measuring the reducing groups released from carboxymethyl cellulose (CMC, Sigma) [21]. The reaction mixt. contained 0.25 ml of crude enzyme, 0.5 ml of 0.1% (w/v) CMC and 0.25 ml of 100 mM NaOAc (pH 5.0). Incubation was carried out at 37° for 2, 4, 6 and 12 hr. One unit is defined as the amount of the enzyme that catalysed the formation of 1 μM reducing group per hr. *α-D-mannosidase.* The enzyme reaction was performed at pH 5.6 in 0.1 M NaOAc buffer at 37° for 15 min in a total vol. of 3.0 ml. Enzyme activity towards 1.25 mM *p*-nitrophenyl α-D-mannopyranoside (Sigma) was determined by measuring the liberated *p*-nitrophenol at 420 nm after the addition of 0.5 M Na_2CO_3 to the reaction mixt. One unit of enzyme activity is defined as the amount of enzyme which hydrolyzed one μM of substrate per hr. *β-D-galactosidase.* The enzyme reaction was performed as described above at pH 6.6 in 0.1 M Pi buffer with *p*-nitrophenyl β-D-galactopyranoside (Sigma) as the substrate. *Other glycanases.* The standard reaction mixt. contained enzyme extract and 0.1% (w/v) substrate in 1.0 ml of 100 mM NaOAc (pH 5.0). Incubation was carried out at 37°. Substrates used were xylan, mannan, arabinogalactan and glucan (all obtained from Sigma) for xylanase, mannanase, galactanase and glucanase, respectively. One unit is defined as the amount of the

enzyme that catalysed the formation of 1 μM reducing group per hr [22].

Protein was estimated by Lowry's method [23] for the determination of the specific activity of enzymes.

Carbohydrate analysis. Analyses were carried out using the fruits immediately after harvest and after 20 days of storage at room temp.

Isolation of carbohydrate fractions. Fruits were washed with dist. H_2O, excised, sliced into small sections, placed into 80% EtOH and stored at 0° for up to 1 month prior to analysis. This procedure inactivates the enzymes and prevent compositional changes. Samples were homogenized with a Sorvall Omnimixer homogenizer, filtered through four layers of cheese cloth and the residues subjected to hexane–$CHCl_3$ (1:2) extraction; 10 ml g^{-1}; ×3 at 80° for 2 hr each. Free sugars from the defatted, depigmented material were extracted with 70% EtOH (10 ml g^{-1}, ×3) at room temp. The combined alcoholic extracts were purified by passage through Dowex 1 × 8 (H^+) and Dowex 50 W (OH^-) resins, concd and analysed chromatographically. Quantification of EtOH-sol. sugars was done by GC as TMSi ethers [24].

EtOH-insol. residues were extracted ×3 with dist. H_2O at 4° for 2 hr. After centrifugation, the cold H_2O-sol. fr. was recovered from the supernatant by precipitation with EtOH. The residue was then gelatinized by heating in H_2O at 90° for 2 hr. Centrifugation of the suspension gave a supernatant from which a hot H_2O-sol. gum was recovered by precipitation with EtOH. Three successive extractions with 0.5% EDTA at 70° for 2 hr, followed by precipitation, gave pectic substances. The resulting pectin-free residue was suspended in 10% carbonate-free NaOH and hemicelluloses (Hem) were extracted over a period of 5 hr in an N_2 atmosphere. Centrifugation and acidification (to pH 5.0) of the extract at ice-cold temp. with 50% HOAc precipitated Hem A, which was collected by centrifugation. Addition of EtOH (3 vols) to the clear centrifugate yielded Hem B. The supernatant was concd to give Hem C. The alkali-insol. residue left after alcoholic extraction represented the cellulosic fr. This fractionation procedure is a modification of the method described in ref. [25].

All rotary evapns were done at 40°. Total sugar, uronic acids and reducing sugars were estimated using the pHOH–H_2SO_4 [26], carbazole [27] and DNS [19] methods, respectively. Glucose, glucuronic acid and maltose served as the calibration standards for total sugar, uronic acid and reducing sugar determinations, respectively.

Non-cellulosic neutral sugar determination. All the frs obtained as described above, except for the cellulosic fr., were hydrolysed as follows. Each fr. (*ca* 5 mg) was suspended in H_2O (0.5 ml) for 2 hr, followed by dropwise addition of conc H_2SO_4 to 72% concn at 0°. After 30 min at 0°, it was dild to 8% acid and hydrolysis was continued by heating at 100° for 8–10 hr. Hydrolysates were neutralized (solid $BaCO_3$) to pH 7.0, deionized (Amberlite IR 120, H^+), concd and analysed by GC [28]. Quantitation of sugars was achieved by GC using the corresponding alditol acetates [29].

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