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Changes in Pectic Substances and Cell Wall Degrading Enzymes During Tomato Fruit Ripening

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Abstract: Changes in pectic substances and the activity of the cell wall degrading enzymes, such as pectinesterase (PE), polygalacturonase (PG) and cellulase, were investigated during the ripening of ‘Strain-B’ and ‘UC-82’ tomato fruits. Respiration rate progressively increased in a climacteric pattern with peak of respiration at the table-ripe stage. Total and insoluble pectins progressively decreased with fruit ripening, while the soluble pectins increased from the mature-green stage up to the canning-ripe stage. Fruits of ‘UC-82’ had higher total and insoluble pectins and more firm texture at harvest and during all stages of ripening than fruits of ‘Strain-B’. A positive correlation was obtained between total and insoluble pectins and fruit flesh firmness. The protein content and PE activity in both cultivars followed the climacteric pattern of respiration and had increased up to the table-ripe stage and then decreased. PG and cellulase activity progressively increased in a similar manner in both cultivars with a high correlation between increase in enzyme activity and stages of ripening.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetable crops in the Sudan. It is grown successfully in every state in the country. Its annual production was 243 thousand tons in 2001, representing 25% of total vegetable production (FAO 2001).

Fruit softening is characterized by changes in flesh firmness and has long been associated with ripening (Dostal 1970). These changes in flesh firmness determine shelf-life and quality of the commodity. Softening reflects chemical and physical changes in cell wall. It is associated with
cell wall disassembly and modifications in the pectin fraction, which are some of the most apparent changes that take place in the cell wall during ripening (Marin-Rodriguez 2002). Softening is generally accompanied by solubilization of pectin, involving the action of the enzymes pectinesterase (PE), polygalacturonase (PG) and pectate lyases (PL) (White 2002). This notion was supported by reports of changes in cell wall pectic material in ripening tomato (Besford and Hobson 1972), pear (Ahmed and Labavitch 1980a) and mango (Roe and Bruemmer 1981). PE and PG were reported as the major hydrolases involved, with cellulase as a secondary contributor (Crookes and Grierson 1983).

This study was conducted to investigate changes in pectic substances and the activity of the cell wall degrading enzymes PE, PG and cellulase during tomato fruit ripening.

MATERIALS AND METHODS

Experimental material
Two of the most important tomato cultivars in the Sudan, ‘Strain-B’ and ‘UC-82’, were selected for this study. Mature-green tomatoes were harvested from the Demonstration Farm of the Faculty of Agriculture in Shambat (15°40’N, 32°22’E). The fruits were washed, air-dried and stored in carton boxes at 20±1°C and 85%-90% RH. Twenty fruits, in four replications, were selected from each stage of ripeness based on the external fruit colour: mature-green, breaker, light-pink, dark-pink, table-ripe and canning-ripe (U.S.D.A. 1976).

Respiration rate, flesh firmness and total protein
Respiration rate was determined in the samples in each stage of fruit ripeness, using the total absorption method of Charlimers (1956), and expressed in mg CO₂/kg-hr. Flesh firmness was measured by the Magness and Taylor Firmness Tester (D. Ballauf Meg. Co) equipped with an 8-mm diameter plunger tip. Two readings were taken from opposite sides of each fruit after the peel was removed. Flesh firmness was expressed in kilogrammes per square centimeter. Total protein in fruit extract was determined by the protein-dye binding procedure of Bradford (1976) and expressed in grammes per 100 g fresh weight.
Pectin assay
Alcohol insoluble residues were prepared by the technique described by McCready and McComb (1952). Hundred grammes of tomato tissues were added to 430 ml of boiling 95% ethanol and heated for 20 minutes. After cooling, the sample was homogenized and filtered. The residue was extracted with 250 ml of 80% ethanol by refluxing for 20 minutes, filtered, washed with acetone and dried under vacuum.

Total pectin was determined according to the method of McCready and McComb (1952). A 0.1g of alcohol-insoluble residue was moistened with 95% ethanol and then suspended in 50 ml of 0.5% versene. The pH was adjusted to 11.5, and the sample was incubated at 25°C for 30 minutes. The mixture was then acidified to pH 5.0 and 10 mg of fungal pectinase was added, incubated at 25°C for one hour and then filtrated. The filtrate was analyzed for anhydrogalacturonic acid by the carbazole method of Rouse and Atkins (1955). Water-soluble pectin was prepared by taking 0.1 g of the alcohol-insoluble residue and extracted with 50 ml of distilled water at 25°C for two hours. The suspension was centrifuged, and the insoluble material was washed with a small amount of distilled water. The anhydrogalacturonic acid content of the combined supernatants was measured by the carbazole method of Rouse and Atkins (1955). Insoluble pectin was calculated by subtracting the water-soluble pectin from the total pectin percentage.

Enzyme assay
The enzymes were extracted according to the procedure described by Ahmed and Labavitch (1980 b). Tomato fruits at the designated stage of ripeness were peeled and 100 g of flesh were homogenized in two volumes of 100 mM sodium acetate buffer pH 6.0 containing 0.2% sodium dithionite (Na2S2O4) and 1% polyvinyl pyrrolidone (PVP, MW 44 000) for one minute using Sanyo Solid State blender. The homogenate was centrifuged at 10 000 rpm for 20 minutes. The supernatant was stored at –12°C (buffer extract).

The residue was suspended in two volumes of 1M sodium acetate buffer pH 6.0 containing 6% NaCl. The pH of the suspension was adjusted to 8.2 with 2 N NaOH. The sample was kept over night at 4°C with continuous stirring and then centrifuged at 10 000 rpm for 20 minutes.
The supernatant was filtered twice using Whatman No.1 filter paper. The filtrate (salt extract) was dialyzed against distilled water for 48 hours with four changes and concentrated against polyethylenglycol. All operations were carried out in an ice bath. This dialyzed sample constituted the enzyme extract.

Pectinesterase (PE) activity was determined according to the technique described by Nagel and Patterson (1967). The substrate used was a 1% (w/v) solution of pectin (citrus; 150 grade; H.P.Bulmer Ltd. Herford, England). The pH of the pectin solution was adjusted to 7.0 with 0.02 N NaOH. The reaction mixture contained 25 ml of crude enzyme, 5 ml of 0.2 M sodium oxalate and 25 ml of substrate. The reaction mixture was incubated at 30°C and continuously stirred by bubbling CO₂-free air through it. During the course of the reaction, the pH of the reaction mixture was maintained at 7.0 with 0.02 N NaOH. The amount of 0.02N NaOH added was recorded every 15 minutes. The enzyme activity was expressed in milliequivalents of ester hydrolysed per minute per kg fresh weight.

Polygalacturonase (PG) activity was determined by measuring the reducing groups released from polygalacturonic acid (Orange; Sigma Chem.Co.). Reducing groups released were measured according to the technique described by Somogyi (1952). The reaction mixture, containing 0.25 ml of crude enzyme, 0.25 ml of 100 mM sodium acetate buffer pH 4.5 and 0.5 ml of 0.1% polygalacturonic acid solution, was incubated at 37°C for 30, 60, 90, 120 and 150 minutes. At the end of each incubation period, the amount of reducing groups released was determined. A calibration curve was obtained using D-galacturonic acid (Sigma Chem. Co.) as a standard. PG activity was determined as µ moles of galacturonosyl reducing groups liberated per minute per kg fresh weight. The activity was then expressed in units. One unit of PG activity will liberate one µ mole of galacturonosyl reducing groups per minute per kg fresh weight.

Cellulase activity was determined by measuring the reducing groups released from carboxymethyl cellulose. The concentration of the reducing groups was determined, with D-glucose as a standard, as in the PG assay. The reaction mixture contained 0.25 ml of crude enzyme, 5 ml of 0.1 %
carboxymethyl cellulose and 0.25 ml of 100 mM sodium acetate buffer pH 5.0. Incubation was carried out at 37°C for 2, 4, 6 and 12 hours. Cellulase activity was determined as µ moles of glucosyl reducing groups catalyzed per hour per kg fresh weight. The activity was then expressed in units. One unit of cellulase activity will liberate one µ mole glucosyl reducing groups per hour per kg fresh weight.

The analysis of variance and Fisher’s protected LSD test with a significance level of $P \leq 0.05$ were performed on the data (Gomez and Gomez 1984).

RESULTS AND DISCUSSION

Changes in respiration rate
The respiration curves of the two tomato cultivars exhibited a typical climacteric pattern. Respiration rate progressively increased from the mature-green stage in a climacteric pattern with a climacteric peak at the table-ripe stage and then deceased at the canning-ripe stage (Fig.1). A similar pattern was reported in mango (Abu-Goukh and Abu-Sarra 1993) and guava (Bashir and Abu-Goukh 2003). Respiration rate was slightly higher in ‘Strain-B’ than ‘UC-82’.

Changes in fruit flesh firmness
Fruit flesh firmness of the two tomato cultivars showed a progressive decline during ripening (Fig.2). Most of this decline occurred between the light-pink and canning-ripe stages. A similar drop in flesh firmness was reported in guava (Bashir and Abu-Goukh 2003), banana (Abu-Goukh et al. 1995) and mango (Abu-Goukh and Abu-Sarra 1993). ‘Strain-B’ had less flesh firmness at harvesting time and softened most rapidly than ‘UC-82’.
Fig. 1. Changes in respiration rate during ripening of 'Strain-B (ÜÜ) and 'UC-82' (----) tomato fruits. Vertical bars represent LSD.
Fig. 2. Changes in flesh firmness during ripening of 'Strain-B' (●●) and 'UC-82' (----) tomato fruits. Vertical bars represent LSD.
Changes in pectic substances
Total and insoluble pectins progressively decreased with fruit ripening, while the soluble pectins increased from the mature-green stage to the canning-ripe stage (Fig. 3). The conversion of insoluble pectins to soluble forms could be attributed to the sharp decrease in total and insoluble pectins and to the increase in soluble pectins (Pressy et al. 1971). ‘UC-82’ had a higher total and insoluble pectins at harvest and during all stages of ripening than ‘Strain-B’. This could explain the more firm texture of ‘UC-82’ over ‘Strain-B’ at all ripening stages (Fig. 2). A positive correlation coefficient ($r^2 = 0.988$) was obtained between total and insoluble pectins and fruit flesh firmness. This is in agreement with previous reports in pear (Ahmed and Labavitch 1980a) and mango (Nour 1978). Hobson (1968) attributed the progressive loss of firmness with ripening to the gradual solublization of protopectin in the cell wall to form pectin and other products. This was confirmed by Pressy et al. (1971).

Changes in total protein
Total protein content in both tomato cultivars followed the climacteric pattern of respiration. It increased up to the table-ripe stage and then decreased (Fig. 4). Similar changes in total protein during fruit ripening were reported in mango (Abu-Goukh and Abu-Sarra 1993) and guava (Bashir and Abu-Goukh 2003). The increase in protein content during the climacteric phase coincided with increased activity of PE (Fig. 5), PG (Fig. 6) and cellulase (Fig. 7). The decline in protein content at the over-ripe stage was explained as due to breakdown of protein during senescence, which again supported the view that proteins in the ripening fruits are mainly enzymes required for the ripening process (Frenkel et al. 1968). ‘Strain-B’ had higher protein content than ‘UC-82’ (Fig. 4). This was also reflected in higher enzyme activity of PE, PG and cellulase (Figs. 5, 6 and 7).
Fig. 3. Changes in total pectins ( ■ ), insoluble pectins ( ▲ ) and soluble pectins ( ○ ) during ripening of 'Strain-B' ( --- ) and 'UC-82' ( ---- ) tomato fruits. Vertical bars represent LSD.
Fig. 4. Changes in total protein content during ripening of 'Strain-B' (---) and 'UC-82' (----) tomato fruits. Vertical bars represent LSD.
Fig. 5. Changes in pectinestrase (PE) activity during ripening of 'Strain-B' (-----) and 'UC-82' (- - - -) tomato fruits. One unit of PE activity will cleave one millequivalent of ester linkages per kg fresh weight. Vertical bars represent LSD.
Fig. 6. Changes in polygalacturonase (PG) activity during ripening of 'Strain-B' (-----) and 'UC-82' (----) tomato fruits. One unit of PG activity will liberate one µmole galacturonsyl reducing groups per min. per kg. fresh weight. Vertical bars represent LSD.
Fig. 7. Changes in cellulase activity during ripening of 'Strain-B' ( --- ) and 'UC-82' ( ---- ) tomato fruits. One unit of cellulase activity will liberate one μ mole glucosyl reducing groups per hr. per kg. fresh weight. Vertical bars represent LSD.
Changes in enzyme activity
Enzyme activity was measured both in the buffer extract and the salt extract. The buffer extract contained large amounts of reducing sugars, which resulted in high background readings in the reducing group assay. Although this problem can be lessened by dialysis of the extract, the time required was excessive. Activities in the buffer extract were extremely low compared with those of the corresponding salt extract. This is probably due to the fact that most cell wall enzymes are bound to the cell wall. To release them, either salt concentration and high pH or both are necessary. Only the data of enzyme activities in the salt extract are presented.

Fruit softening during ripening is frequently attributed to the enzymatic degradation of cell wall materials (Ahmed and Labavitch 1980b) The current theory is that PE removes the methyl groups of the galacturonic acid polymer (Lee and MacMillan 1970), which then enables PG to depolymerise the de-esterified polygalacturonide chain and reduce its molecular weight (Benkova and Markovic 1976). Cellulase cleaves the β-1, 4 glucosidic bonds of cellulose (Babbitt et al. 1973). Recently, Marin-Rodriguez et al. (2002) reviewed the role of pectate lyases in fruit softing. Pectate lyases (PL) catalyse the Ca$^{2+}$-dependent cleavage of de-esterified pectin, which is a major component in the primary cell walls of many higher plants. PL activity has been obtained directly from banana pulp with a substantial increase in activity during ripening (Marin-Rodriguez 2001). Fruits of tomato, strawberry and grape all express PL, where they play a significant role in fruit softening (White 2002). The exact sequence of events and the contribution of each of these enzymes to softening in fruit is still not clear.
Extractable PE activity in both tomato cultivars increased steadily up to the dark-pink stage, and then decreased (Fig.5). This agrees with the reports for mango (Roe and Bruemmer 1981) and guava (Abu-Goukh and Bashir 2003). However Abu-Sarra and Abu-Goukh (1992) found inconsistent patterns of PE changes during ripening of some mango cultivars. In contrast, PE activity increases during ripening of tomato (Buescher and Tigchelaar 1975) and banana (Hultin and Levine 1965).

Pectic enzymes are apparently important in the softening of fruit tissues during ripening (Ahmed and Labavitch 1980b). Due to the pronounced change in cell wall components and in the degree of esterification of pectin, which are generally known to occur during fruit ripening, the softening process was thought to be a consequence of de-esterification of pectin catalyse by PE, followed by pectin depolymerization catalysed by PG (Roe and Bruemmer 1981; Ahmed and Labavitch 1980b). Consequently, the slow rate of fruit softening observed by Abu-Sarra and Abu-Goukh (1992) in ‘Abu-Samaka’ mango fruit, in spite of its high PG activity, suggested a key role for PE in controlling the rate of fruit softening during fruit ripening.

The softening of tomato has been shown to be closely associated with the increase in PE. Buescher and Tigchelaar (1975) reported that PE activity increased during ripening of normal tomato fruit, while that of rin fruit (non-ripening mutant) remained constant. The failure of the non-ripening tomato mutant (rin) was evaluated in terms of PE activity (Buescher and Tigchelaar 1975). PE activity has been reported to increase during ripening of tomato (Grierson and Kader 1986) and banana (Hultin and Levine 1965). Matto and Modi (1969) related the softening of chilling injured mango fruits to the increased activity of PE.

PG activity progressively increased during ripening in a similar manner in both tomato cultivars (Fig. 6). Strong correlation was obtained between PG activity and the stage of ripening. Similar results were reported during ripening of tomato (Grierson and Kader 1986; Hobson 1964), mango (Abu-Sarra and Abu-Goukh 1992; Roe and Burmmer 1981), pear (Ahmed and Labavitch 1980b), peach (Pressy et al. 1971) and guava (Abu-Goukh and Bashir 2003).
Although ‘Stain-B’ had lower values of total and insoluble pectins than ‘UC-82’, it had higher contents of soluble pectins (Fig. 3). This could be due to the higher activity of PG in ‘Strain-B’ than in ‘UC-82’ (Fig.6). Grierson and Kader (1986) reported that the increased activity of PG during tomato fruit ripening was closely associated with loss in galacturonic residues from the cell wall. They suggested PG as the most important softening enzyme in tomato fruits. Tigchelaar and McGlasson (1977) stated that the inhibition of ripening in rin and nor, sigle-gene non-ripening mutants of tomato, is proposed to occur directly through the effects of these genes on fruit PG biosynthesis or activation. The physiological disorder “blotchy ripening” in tomato was also evaluated in terms of PG activity. The red areas of blotchy fruit contain less PG than even-red fruits, and the activity is even lower in non-red areas (Hobson 1964). Ahmed and Labavitch (1980a) reported that treatment of cell walls from unripe pear fruits with highly purified PG changed the sugar composition to resemble that of cell wall from ripe fruits. Pressy et al. (1971) stated that, in peaches, fruit firmness begins to decrease before PG is detected; however, the fruit softened rapidly after the appearance of the enzyme. Miller et al. (1987) related the softening of mechanically stressed cucumbers to the increase in PG, PE and xylanase activities.

Cellulase activity increased progressively during ripening of the two tomato cultivars from the mature-green stage to the canning-ripe stage in a similar pattern (Fig.7). This is in agreement with the previous reports in tomato (Hobson 1968), date (Hasegawa and Smolensky 1971), avocado (Pesis et al. 1978) mango (Abu-Sarra and Abu-Goukh 1992) and guava (Abu-Goukh and Bashir 2003).

The role of cellulase in fruit softening is not fully understood. Babbitt et al. (1973) found that tomato fruits treated with gibberellic acid showed very low PG activity, while cellulase activity increased steadily. Due to the loss of firmness in the treated fruits, they suggested that softening is initiated by the action of cellulolytic enzymes and that pectolytic enzymes were involved in subsequent changes of texture. Pesis et al. (1978) reported that the cellulase activity in detached avocados is directly correlated with fruit softening. In contrast, Hobson (1968) found that cellulase activity is not correlated with loss of firmness during ripening of tomatoes. The enzyme activity was reported to increase during ripening of
normal tomato fruit and similar change was observed in *rin* and *nor* non-ripening mutants (Buescher and Tigchelaar 1975). No cellulase activity was detected in papaya fruits (Selvaraj *et al.* 1982) and pears (Ahmed and Labavitch 1980b). However, during maturation and ripening, the cellulase activity increased markedly in ‘Deglet Noor’ date fruits (Hasegawa and Smolensky 1971). The amount of fibre contents, which consist of cellulose, hemicellulose and lignin, decreased during maturation and ripening of dates (Mustafa *et al.* 1986). The cellulose in the ripening dates is converted into glucose (Barrevelled 1993).

Cellulase activity steadily increased with the decrease in tissue firmness with high correlation in mango (Abu-Sarra and Abu-Goukh 1992) and guava (Abu-Goukh and Bashir 2003). In this study, cellulase activity increased during the ripening of both tomato cultivars with a similar pattern (Fig.7). Therefore, in spite of activity and the loss of resistance to shearing force, no significant role in tissue softening can be ascribed to cellulase at the present time.

**REFERENCES**


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بتاريخ 5/1 بـ. دراسة للعلاقة بين تغيرات الثمرة أثناء النضج throma – يظهر نتائجها معاؤها وصلوًا وتمائمها كالأحماض السكرية أثناء النضج. وتشمل هذه الدراسة الدراسة بـ 82. (أ) 82. (B) 82. (C) 82. (D) 82. (E) 82. (F) 82. (G) 82. (H) 82. (I) 82. (J) 82. (K) 82. (L) 82. (M) 82. (N) 82. (O) 82. (P) 82. (Q) 82. (R) 82. (S) 82. (T) 82. (U) 82. (V) 82. (W) 82. (X) 82. (Y) 82. (Z)