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CHANGES IN THE ACTIVITY OF ENZYMES OF PHENYLPROPANOID METABOLISM IN TOMATOES STORED AT LOW TEMPERATURES

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato fruit; chilling injury; phenylpropanoid metabolism; hydroxycinnamate CoA; quinate hydroxycinnamyltransferase (hydroxycinnamyltransferase).

Abstract—A large increase in the activity of an enzyme involved in chlorogenic acid metabolism, hydroxycinnamyltransferase occurs in tomatoes stored at low temperatures. In contrast, the activity of the enzyme remains constant or falls slightly during normal ripening at 20°. The rise in activity occurs at temperatures below 10° and fails to occur at 15° or 20°. This increase in activity during low temperature storage occurs with fruit at all stages of ripening from mature green to fully ripe. The hydroxycinnamyltransferase of chilled tomatoes falls rapidly on transfer to 20° with a lag of about 4–8 hr and within 48 hr returns to that of unchilled fruit. The effects of such warming treatments are reversible since when a chilling period is resumed following warming to 20°, the rise in hydroxycinnamyltransferase activity is also resumed. Of the 5 other enzymes of phenylpropanoid metabolism studied, only PAL shows a similar increase in activity during low temperature storage although the activity of the other enzymes was maintained at higher levels in fruit at 2° than at 20°. The possible relationship between the behaviour of hydroxycinnamyltransferase activity at various temperatures and the known susceptibility of tomatoes to chilling injury is discussed.

INTRODUCTION

Tomatoes, in common with many other fruit and vegetables, exhibit marked physiological disorder when stored at temperatures above their freezing point but below a certain critical threshold temperature. In the tomato the critical temperature is about 12° and the symptoms of injury include inhibition of the colour changes normally associated with ripening and increased suceptibility to decay organisms. These disorders have been termed collectively as chilling injury [1] even though the symptoms of the disorder vary from species to species. Temperature dependent phase changes in the cellular membranes have been postulated as the primary response of sensitive species to chilling temperatures and these are thought to lead to changes in membrane permeability and in the activity of membrane bound enzymes with the accumulation of toxic intermediates and hence cellular damage [1]. In chilling sensitive leaves, the importance of leaf dehydration rather than phase changes in the membranes as the primary temperature induced effect has been stressed [2, 3].

We have undertaken a study of some of the secondary changes induced under chilling stress to gain understanding of the internal compensation reactions which may account for differences in the chilling sensitivity between different varieties in some sensitive species [4]. The metabolism of phenolic compounds is often enhanced in plant tissues under types of stress such as mechanical damage [5] or infection by micro-organisms [6]. We have investigated the possible role of phenylpropanoid metabolism in tomatoes under chill stress conditions by studying changes in the activity of a range of enzymes of phenylpropanoid metabolism in tomatoes stored at chilling and non-chilling temperatures.

RESULTS

Figure 1 shows the changes in activity of a number of enzymes of phenylpropanoid metabolism in tomatoes picked at the 'breaker' stage and stored at 2°, 20° or at 2° followed by a subsequent transfer to 20°. Tomatoes stored at 20° ripened fully within 7 days but at 2° the colour changes associated with ripening are almost completely suppressed. After prolonged exposure to 2° and subsequent transfer to 20° the tomatoes show the characteristic symptoms of chilling injury with suppression of ripening and a marked increase in susceptibility to fungal infection. In normal ripening at 20°, the activities of phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (CAH), coniferyl alcohol: NADP oxidoreductase (aromatic ADH) and *o*-diphenol: O_2 oxidoreductase (phenolase) shows a marked fall. In contrast there is a rise of about 2 fold in the activity of *p*-coumarate CoA ligase during normal ripening at 20°, while under the same conditions the hydroxycinnamyl CoA: quinate hydroxycinnamyltransferase (hydroxycinnamyltransferase) [7] shows very little change. During storage at 2° the level of phenolase falls but more slowly than at 20°. Both CAH and *p*-coumarate CoA ligase activities fall during storage at 2°. The aromatic ADH activity does not change significantly under these conditions but both PAL and hydroxycinnamyltransferase

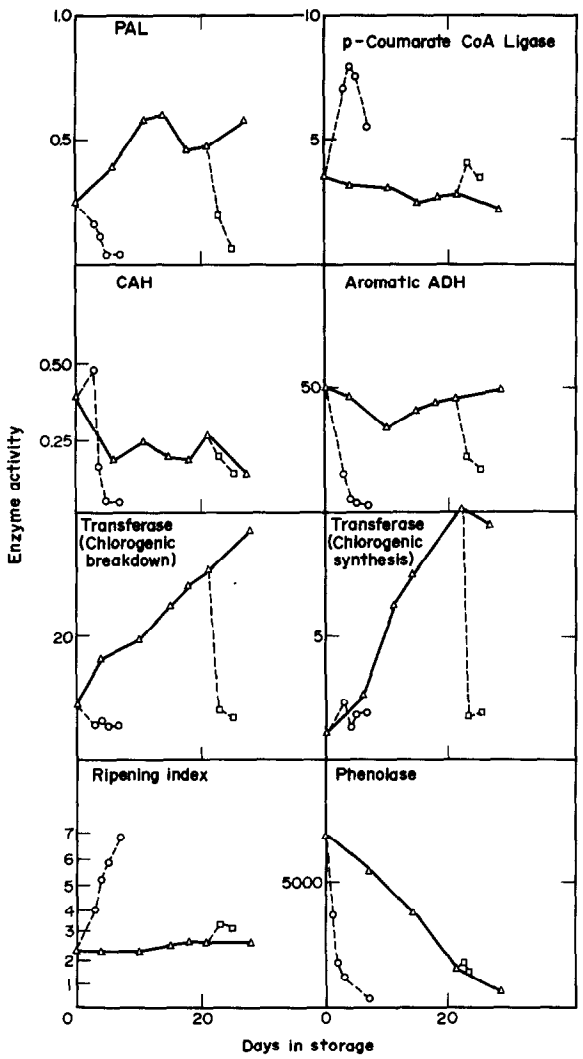

Fig. 1. Time course of changes in activity of six enzymes of phenylpropanoid metabolism during storage of 'breaker' tomatoes at 2° (△——△), 20° (○·····○) or 2° followed by 20° (□- - -□). The enzyme activity is expressed as nmoles/min/mg protein except in the case of aromatic ADH and phenolase when enzyme units/mg protein and enzyme units/10 g fresh weight are employed respectively. In these cases the enzyme unit is defined as the amount of enzyme giving a change of 0.001 absorbance/minute under the defined conditions. The changes in the ripening index of the tomatoes under the three storage conditions is also shown.

show considerable increases in activity. In 28 days storage at 2°, the activity of PAL rises just over 2 fold while a much greater increase in hydroxycinnamyltransferase (4–10 fold) activity occurs during the same period. The hydroxycinnamyltransferase shows the same dramatic increase in activity whether measured in the direction of chlorogenic acid breakdown or chlorogenic acid synthesis [see 7]. On transfer to 20° following storage for 21 days at 2° the levels of PAL and hydroxycinnamyl transferase fell dramatically and within 48 hr return to the levels found in unchilled tomatoes. The aromatic ADH also shows a marked fall in activity on transfer to 20° while *p*-coumarate CoA ligase shows a small increase and the activities of phenolase and CAH are largely unaffected by the transfer.

Table 1. Effect of storage of 'breaker' tomatoes for 20 days at various temperatures on the activity of enzymes of phenylpropanoid metabolism

	Storage Temperature (20 days storage period)				
	Initial	0°	2°	5°	10°
p-coumarate CoA ligase (nmol/min/mg protein)	0.63	0.71	0.74	0.21	0.40
caffeate CoA ligase (nmol/min/mg protein)	0.51	0.49	0.48	0.15	0.28
Aromatic ADH (EU/mg protein)	26.7	23.2	29.2	10.9	4.7
Hydroxycinnamyltransferase (nmol/min/mg protein)	6.2	15.1	18.7	16.5	20.5
PAL (nmol/min/mg protein)	0.15	0.43	—	0.08	0.07

Table 1 shows the activity of five enzymes in 'breaker' tomatoes stored at 0°, 2°, 5° and 10° for 20 days. The *p*-coumarate CoA ligase, caffeate CoA ligase and aromatic ADH show little change over the initial value during storage at 0° and 2° but fall at 5° and 10°. PAL shows a marked increase in activity at 0° but falls in relation to the initial value at 5° and 10°C. Hydroxycinnamyltransferase shows a large increase in activity (between 2.4–3.3 fold) at all of these temperatures which are below the

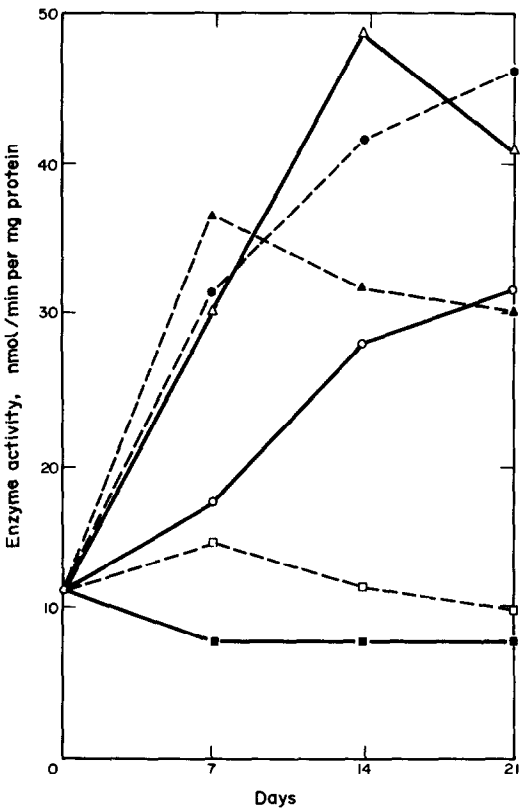

Fig. 2. Time course of changes in hydroxycinnamyltransferase activity in 'breaker' tomatoes stored for up to 21 days at 0° (○——○), 2° (●- - -●), 5° (△——△), 10° (▲- - -▲), 15° (□- - -□) and 20° (■——■).

threshold for chilling injury. In view of the dramatic changes in hydroxycinnamyltransferase activity under these low temperature conditions its behaviour was investigated further.

Figure 2 shows the time courses of changes in hydroxycinnamyltransferase activity in 'breaker' tomatoes stored at 0°, 2°, 5°, 10°, 15° and 20° for up to 21 days. At 15° and 20° the activity of hydroxycinnamyltransferase remains constant or falls slightly. At 5° and 10° the enzyme activity rises to a peak and then declines slightly while at 0° or 2° the activity of the enzyme increases steadily throughout the 21 day storage period. After 21 days storage the activity of hydroxycinnamyltransferase compared with the initial value had risen by 2.9, 4.2, 3.7 and 2.7 fold at 0°, 2°, 5° and 10° respectively while at 15° and 20° it had fallen by 7 and 27% respectively. The pattern of changes in hydroxycinnamyltransferase is unrelated to the changes in ripening under these storage temperatures. At 15° and 20° ripening proceeds normally, at 5° and 10° it is retarded so that after 21 days the 'orange' and pink stages respectively are reached while at 0° and 2° the colour changes associated with ripening are almost completely suppressed.

Table 2 shows the rise in hydroxycinnamyltransferase, 5.8 fold in 21 days, in 'breaker' tomatoes stored at 2° and the effect of interrupting the chill period by either a single 24 hour period at 20° after 7 days or two 24 hr periods at 20° after 7 and 14 days. During the warm up periods the activity of hydroxycinnamyltransferase falls but on return to 2° the increase in activity resumes at about the same rate as before the transfer to 20°. After 21 days the activity of hydroxycinnamyltransferase in tomatoes which had received a single period at 20° was 66% of the control maintained at 2° throughout while those that had had two periods at 20° had only 54% of the control level of activity.

Table 3 shows the time course of disappearance of hydroxycinnamyltransferase activity when 'breaker' tomatoes stored for 21 days at 2° are transferred to 20°. In separate experiments it was shown that it took about 4 hours for the centre of the tomatoes transferred from 2° to 20° to reach temperature equilibrium. The activity of hydroxycinnamyltransferase falls rapidly after a lag period of about 8 hr and by 48 hr had almost returned to the level of the unchilled tomatoes.

Table 2. Effect on the level of hydroxycinnamyltransferase activity of storing 'breaker' tomatoes under different regimes of storage at 2 and 20°

Periods at various temperatures (day numbers) 2°	20°	Total storage period (days)	Enzyme activity (nmol/min/mg protein)
0	0	0	5.4
0–7	0	7	21.2
0–14	0	14	27.1
0–21	0	21	30.9
0–7	8	8	13.5
0–7, 9–14	8	14	17.0
0–7, 9–21	8	21	20.3
0–7, 9–14	8, 15	15	10.1
0–7, 9–14, 16–21	8, 15	21	16.7

Table 3. The time course of disappearance of hydroxycinnamyltransferase activity in 21 day chilled tomatoes on transfer to 20°

Days at 2°	Hours at 2°	Enzyme activity (nmol/min/10 g)
0	—	151
7	—	341
14	—	416
21	—	462
21	4	432
21	8	457
21	12	369
21	24	331
21	48	174

Figure 3 shows the effect on hydroxycinnamyltransferase activity of storing at 2° tomatoes harvested at the mature green, 'breaker', orange, pink and fully ripe stages. At all stages, there is an increase in hydroxycinnamyltransferase during storage at 2°. In relative terms, the increases at the various stages after 21 days storage vary from 6.1 fold for mature green fruit to 4.1 fold for fully ripe fruit. In absolute terms the increases are greatest at the 'breaker' and orange stages and up to 50% less at the mature green, pink, fully ripe stages. In view of the differences in physiological state from mature green to fully ripe fruit it is significant that there is an essentially similar increase in hydroxycinnamyltransferase activity during low temperature storage in fruit at all stages of ripening.

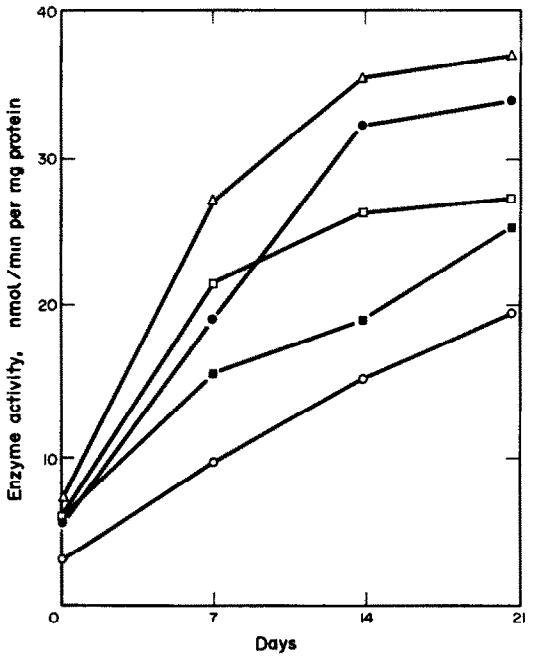

Fig. 3. Time course of changes in hydroxycinnamyltransferase activity during storage at 2° of tomatoes harvested at the mature-green (○——○), 'breaker' (●——●) 'orange' (△——△), pink (□——□), full red (■——■) stages of ripeness.

DISCUSSION

The threshold temperature for chilling injury in the tomato is about 12°. Symptoms of injury appear at temperatures below this critical temperature and there is evidence that a number of cellular functions of the tomato associated with membrane systems such as the permeability of chloroplasts [8], the rate of photosynthetic electron transport [9] and the activity of mitochondrial membrane bound enzymes [10] show marked changes in properties at about 12° which may indicate changes in metabolic patterns in the range of temperatures above and below this critical temperature. Temperatures below the threshold during storage lead to changes that ultimately result in the breakdown of the resistance of the tissue to invading microorganisms. Exposure to chilling for periods of 2–3 weeks normally leads to irreversible changes. However, the changes brought about by shorter chilling periods are normally reversible when the fruit is raised to a non-chilling temperature [11]. Moline [11] has shown that ultrastructural changes occur in chilled tomatoes which include interference with the conversion of chloroplasts to chromoplasts and the swelling and degeneration of both mitochondria and plastids. These ultrastructural changes were reversed when tomatoes stored at 2° for up to 15 days were transferred to 18° but beyond 15 days the changes became irreversible. It is also necessary to maintain the cold period for up to 15 days continuously to obtain the effect since to interject a period at a non-chilling temperature reversed the changes associated with chilling injury [1]. The effect of temperature of storage on chilling injury in tomatoes has been studied extensively by Tomkins [12] and the direct effects of chilling on ripening as opposed to sensitivity to fungal infection were studied using tomatoes stored in the presence of fungicide impregnated wraps [13].

In the present study of enzymes of phenylpropanoid metabolism the behaviour of hydroxycinnamyltransferase activity during the chilling periods is interesting since it seems to correlate with chilling injury. Hydroxycinnamyltransferase activity increases in storage at all the temperatures below the threshold for chilling injury (12°) tested i.e. 0°, 2°, 5° and 10° while no increases occur at temperatures above the threshold i.e. 15° and 20°. This increase in enzyme activity occurs independently of ripening changes since the rise in activity of the enzyme during storage at 2° occurs in fruit at all stages of ripening. Raising the temperature above the threshold of tomatoes previously stored at 2° leads to rapid fall in hydroxycinnamyltransferase activity after a lag of 4 8 hr. When tomatoes stored at 2° are warmed at 20° for short periods the hydroxycinnamyltransferase activity falls but continues its increase when returned to 2° at a rate that approximates to that of tomatoes left at 2° throughout. It is interesting of itself that an increase in enzyme activity is induced at low temperatures and prevented at higher temperatures. The rise in hydroxycinnamyltransferase activity suggests that relatively greater capacity to synthesise or breakdown quinate esters is developed during chilling storage. As has been pointed out previously, the role of hydroxycinnamyltransferase either in the synthesis or breakdown of quinate esters or both is undecided [7]. However in other studies increased metabolism of chlorogenic acid has been shown to occur in peppers [14], sweet potatoes [15] and tobacco [16] under chilling conditions.

PAL as well as hydroxycinnamyltransferase shows a rise in activity in low temperature storage. A similar rise in PAL occurs during the storage of peppers under chilling conditions [17]. None of the other enzymes of phenylpropanoid metabolism studied in the tomato showed a similar rise in activity at low temperatures. However all the enzymes except the *p*-coumarate CoA ligase maintain higher levels of activity in fruit stored at 2° compared with 20°. The behaviour of some of these enzymes (i.e. phenolase) not showing a rise in activity in the cold can be interpreted as a slowing down of changes normally associated with ripening during low temperature storage. Further experiments to elucidate the changing patterns of phenylpropanoid metabolism in tomatoes at different temperatures are under way.

The fact that the rise in hydroxycinnamyltransferase activity during storage at 2° occurs at all the stages of tomato ripening studied including fully ripe fruit may shed light on the role of temperature in controlling the level of hydroxycinnamyltransferase activity. The capacity for protein synthesis of fruits past their climacteric peak is generally very low [18] and thus it would seem unlikely that protein synthesis is involved in the rise in hydroxycinnamyltransferase activity. Some form of activation of a pre-existing protein may be involved and this aspect will be investigated in later work.

EXPERIMENTAL

Tomatoes (var Eurocross BB) were grown in greenhouse at F.R.I. Harvested fruit was stored in rooms at the various temps in cabinets through which humidified air was passed to maintain a relative humidity of 95–98%. In the temp. transfer expts samples of fruit were transferred to similar cabinets housed in separate storage rooms at the required temp. During storage the ripeness of the samples was assessed by visual colour using a grading system of 1 for mature green tomatoes, 2 for 'breaker' stage, 3 for $\frac{1}{3}$ colour, 4 for $\frac{2}{3}$ colour, 5 for pink, 6 for full red and 7 for overripe. Samples of at least 20 fruit were used in the assessment of a ripening index, the sum of the ripeness score of each individual tomato divided by the total number in the sample. This is essentially similar to the grading system used by other workers [19]. Samples of 5 tomatoes were taken for each estimation of enzyme activity. Wall material (about 250–300 g) from these fruits was frozen in liquid N_2 and ground in liquid N_2 to a fine homogeneous powder in an Ultraturrax homogeniser. The powder was stored at liquid N_2 temp. until a suitable aliquot (10 g) was weighed out prior to enzyme extraction. In the earlier experiments in which CAH activity was measured unfrozen material was used since this enzyme which is present in the microsomal fraction is inactivated by freezing. All the other enzymes are unaffected by this treatment. In the first experiment using unfrozen material, 75 g of wall material was homogenized in a stainless steel roller mill in 300 ml of medium containing 0.1 M Tris–HCl, 0.5 M sucrose, 1 mM EDTA, 2 mM DTE, 2.4 g polyclar AT pH 8.0. The extract was filtered through miracloth and the filtrate centrifuged at 10000 *g* for 10 min in an MSE 18 centrifuge. The supernatant was decanted and made to 300 ml. 100 ml of the 10000 *g* supernatant was centrifuged at 165000 *g* for 1 hr in the T50 rotor of the Beckman Spinco L2 preparative ultracentrifuge. The 165000 *g* pellet was resuspended in 3 ml of 0.1 M phosphate, 0.25 M sucrose, 0.5 mM EDTA and 1 mM DTE pH 7.5. This microsomal suspension was used for assay of CAH activity by the method of Hill and Rhodes [20]. The remaining 200 ml of 10000 *g* supernatant was treated with $(NH_4)_2SO_4$ to a conc of 85% saturation and the pptd protein collected by centrifugation at 40000 *g* for 30 min in the MSE 18 centrifuge. The gelatinous pellet was taken up in 15 ml 0.1 M Tris, 0.1 mM DTE pH 7.45 and desalted by

chromatography on a column of Sephadex G25 as previously described [7]. This desalted fraction was used for the assay of PAL [21] Aromatic ADH [22], *p*-coumarate CoA ligase [23] and hydroxycinnamyl transferase [7] by published methods. In some of the later expts in which hydroxycinnamyltransferase was measured alone and in which aliquots of frozen powder were used, a simplified extraction procedure was employed. 10 g of frozen tomato powder was homogenised in 0.2 M phosphate, 1 mM EDTA, 2 mM DTE, 0.5 g polyclar AT pH 7.5 in an Ultra turrax homogeniser. The extract was filtered through miracloth and the filtrate clarified by centrifugation at 38000 *g* for 40 min. The supernatant was decanted and made to 50 ml, aliquots of this extract were used for estimation of the activity of hydroxycinnamyltransferase in chlorogenic acid breakdown. The enzyme was always assayed at at least two different levels of extract and in all cases good linearity between rate of caffeyl CoA formation and volume of extract was obtained. Good agreement was also obtained between replicate extractions of frozen tomato powder. In expts in which phenolase activity was measured, a variation of this simplified extraction procedure was employed in which the extraction medium consisted of 0.2 M KH_2PO_4, 1 mM EDTA pH 7.5 and homogenisation was carried out in the presence of 2 g Amberlite IR 938. Phenolase activity was measured by the method of Sisler and Evans [24].

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