Storage-temperature-dependent Time Separation of Softening and Chlorophyll Loss from the Autocatalytic Ethylene Pathway and Other Ripening Events of 'Anjou' Pears

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ABSTRACT. Mature 'Anjou' pears (*Pyrus communis* L.) continuously stored at 20 °C or –1 °C before transfer to 20 °C exhibited differences in the sequence of ripening events up to 100 days. Pears continuously held at 20 °C showed little change in ripening characteristics (chlorophyll, firmness, titratable acidity) for 14 to 28 days, then these characteristics decreased at a daily rate of 1.4% thereafter. A 40% increase in soluble polyuronides paralleled the firmness loss, while ACC did not exceed 0.5 nmol·g⁻¹ until the 84th day, and internal ethylene did not exceed 0.2 µL·L⁻¹ until after 90 days, whereas ACC oxidase activity (and total protein) peaked after 63 days. 'Anjou' pears stored at –1 °C showed no changes in chlorophyll, firmness, protein, or total polyuronides for at least 84 d. Despite essentially no change in firmness during –1 °C storage, there was a slow but steady increase (≈15%) in soluble polyuronides. ACC oxidase activity, expressed as ethylene production, rose to 71 nL·g⁻¹·h⁻¹ and the ACC content increased to almost 1.0 nmol·g⁻¹ by the 84th day. Internal ethylene slowly increased and levelled to 1 µL·L⁻¹ by the 56th day. Satisfaction of a chilling requirement thus appears to favor the development of ethylene synthesis capacity, which on transfer from cold storage to higher temperatures results in enough internal ethylene to rapidly drive the associated ripening mechanisms. Fruit for which the chilling requirement (≥70 days at –1 °C) was met softened in response to accelerated internal ethylene production on transfer to 20 °C for 7 days. However, pears that were not chilled or partially chilled did not sustain the increased ACC levels or ACC oxidase activity. Chemical name used: 1-aminocyclopropane-1-carboxylate (ACC).

'Anjou' pears normally require 50 to 60 d in –1 °C cold storage to ripen to acceptable texture when transferred to 20 °C (Chen and Mellenthin 1981, 1982; Chen et al., 1981, 1982) unless treated with exogenous ethylene (Wang and Hansen, 1970; Wang et al., 1972). During storage at –1 °C, before accelerated ethylene production was noted, the ACC concentration increased (Blankenship and Richardson, 1985; Morin et al., 1985; Toumadje 1984; Vaz 1984; Wang et al., 1985) along with increased activity of ACC oxidase (Blankenship and Richardson, 1985).

'Anjou' pears gain competency to ripen even when held continuously at 20 °C. Loss in firmness at 20 °C precedes and is time-separated from the climacteric rise in respiration (Porritt, 1964) or the increase in autocatalytic ethylene production (Gerasopoulos and Richardson, 1995). When shifted to 20 °C for <2 weeks, partially chilled 'Anjou' pears exhibited a slightly higher but still low and unsustained ethylene production, a loss in firmness, and a depletion of the ACC pool (Toumadje 1984; Vaz 1984). Softening is associated with solubilization of cell wall polyuronides (Wang et al., 1985) and reduction in cell wall neutral sugar content (mainly galactose, arabinose, or both) (Jermyn and Isherwood, 1956; Knee, 1973).

While 'Anjou' pears with high sugar and organic acid content are associated with good postharvest quality, these constituents are not related to storage duration, whereas high protein content at

harvest usually results in short storage life (Mellenthin and Wang, 1976). All these constituents fluctuate from season to season and from one location to another and do not appear to be closely associated with postharvest life (Chen et al., 1982). Titratable acidity generally decreases during fruit ripening and storage (Chen and Mellenthin, 1981; Li and Hansen, 1964). Chlorophyll usually degrades with fruit maturity and ripening, and, thus, carotenoids contribute more to the color (Fidler et al., 1973; Goodwin, 1976).

It is clear that there also is an increase in soluble protein content during the climacteric of pome fruit (Hansen, 1966). Knee (1987) showed that mRNA for ACC synthase is formed in 'Conference' pears during –1 °C storage. Wilson et al. (1990) noted that accumulating mRNA specific for encoding polypeptides of 38, 44, and 45 kD in response to low-temperature storage of 'Conference' pears indicates altered gene expression.

The purpose of our study was to investigate the time course of the changes of ethylene biosynthesis and other biochemical constituents associated with nonchilled compared to chilling-induced ripening of 'Anjou' pears at 20 °C after they have been subjected to –1 °C storage for various time durations.

Materials and Methods

FRUIT MATERIAL. 'Anjou' pears from the Mid-Columbia Experiment Station, Hood River, Ore., were harvested based on 67-N flesh firmness (147 d from full bloom). After harvest, fruit free of cork spot or other blemishes were transferred to perforated, polyethylene film-lined, 20-kg cardboard cartons and placed into a –1 °C storage room. No fungicides or antiscald chemicals were

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applied. Fruit samples were taken at harvest, and, after 25, 40, 55, 70, and 85 d of storage at -1°C , they were transferred to 20°C in 20-L jars to ripen. Humidified air flow at $1000\text{ mL}\cdot\text{min}^{-1}$ was provided to each 20-L jar to ensure that CO_2 accumulation was maintained well below 0.5% in the jar atmosphere (to avoid ethylene synthesis inhibition) and fruit water loss was minimized.

Five individual fruit replicates were used for each sampling (three times weekly) for internal ethylene concentration and flesh firmness measurements. On three of these fruit, ACC, ACC oxidase activity, peel chlorophyll, titratable acidity, soluble or total polyuronides, and total protein were measured weekly.

INTERNAL ETHYLENE CONCENTRATION (IEC) AND FLESH FIRMNESS. A 1-mL internal atmosphere sample was drawn by syringe from water-immersed pears, and IEC was measured with a flame ionization gas chromatograph (model 311; Carle, Fullerton, Calif.) equipped with a 2.0-m, 80–100-mesh, activated alumina column. The same five fruit were used for flesh firmness measurements on two pared surfaces on opposite sides of each fruit at the equator by a fruit firmness tester equipped with a Hunter force gauge model (Western Industrial Supplies) and an 8-mm tip, in a Univ. of California–Davis apparatus.

ACC ANALYSIS. ACC was extracted and prepared according to Miller et al. (1988) with some modifications. Pear flesh tissue (5 g/pear) were homogenized in 15 mL 90% acetone with a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, N.Y.). The homogenate was filtered through Whatman no.1 filter paper into a scintillation vial, and the extract was stored at -20°C until it was analyzed. An aliquot equivalent to 1 g fresh mass tissue then was passed through a 2-mL, Dowex 50W $\times$ 8, 50–100 mesh, 1.7 meq $\cdot\text{mL}^{-1}$, wet resin (strongly acidic cation exchanger, Sigma-Aldrich Chemical Co., St. Louis, Mo.) H^+ form ion exchange column. The column was washed with 20 mL water, and ACC was eluted with 1 N NaOH and assayed according to Lizada and Yang (1979).

ACC OXIDASE ACTIVITY. ACC oxidase activity was determined by adding known amounts of ACC to tissue plugs, allowing ACC to convert to ethylene that was measured by gas chromatography according to Blankenship and Richardson (1985) with minor modifications. From three replications (individual pears), 12 plugs of flesh from each pear were cut with a cork borer (0.5-cm inside diameter). Six of these plugs ($\approx 3\text{ g}$) were weighed and vacuum-infiltrated (90 s, water aspirator) with 0.35 M mannitol (control). The excess liquid was drained, the plugs were blotted dry, and a partial vacuum was applied for 10 s to free trapped ethylene. The plugs then were placed in 25-mL Erlenmeyer flasks, sealed with serum caps, and incubated for 30 min at 20°C . Then, 1 mL atmospheric gas samples were withdrawn by syringe, and ethylene was measured as previously described. The other six plugs from the same fruit were treated identically, except they were vacuum-infiltrated with 0.05 mM ACC in 0.35 M mannitol. Control plug (0.35-M mannitol treated) ethylene was subtracted from that produced by ACC-treated plugs to calculate ACC oxidase activity and was expressed as C_2H_4 production in $\text{nL}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$.

TOTAL AND WATER-SOLUBLE POLYURONIDES. Peeled and cored pear flesh tissue (10 g of each of three individual fruit) was homogenized in 90 mL acetone. The homogenate was placed in -20°C for 2 d for precipitation of the insoluble material. After centrifugation (2000 g_n for 20 min), the pellet was washed twice with 50 mL acetone, dried under vacuum, and stored in glass vials until further analysis. Before analysis, the insoluble solids (AIS) of the acetone pellet was dried overnight at 50°C . AIS (40 mg), then dissolved in 100 μL concentrated H_2SO_4 , stored overnight under N_2 at 4°C , and then diluted to 1 N H_2SO_4 . Then, 25 μL was further diluted with 175 μL 1 N H_2SO_4 and used for galacturonic acid determination with meta-hydroxydiphenyl reagent according to Blumenkranz and Asboe-Hansen (1973). Galacturonic acid in 1 N H_2SO_4 was used as a standard. The total polyuronic content was calculated as $\text{mg}\cdot\text{g}^{-1}$ of galacturonic acid equivalents on AIS basis. Another 40 mg of the AIS was suspended in 10 mL of water and heated for 30 min at 80°C . After cooling, water insoluble material was precipitated by centrifugation. The hot-water-soluble extract (80 μL) was analyzed for polyuronic content according to Blumenkranz and Asboe-Hansen (1973). Galacturonic acid in water was used as a standard and the water-soluble wall fraction was expressed as $\text{mg}\cdot\text{g}^{-1}$ of galacturonic acid equivalents on AIS basis.

PEEL CHLOROPHYLL DETERMINATION. The epidermis of three fruit replicates was peeled and 10 discs (1.1 cm in diameter) per fruit were cut with a cork borer. The peel tissue was immediately homogenized in 5 mL 95% acetone (final concentration 80% acetone) and insoluble material was pelleted by centrifugation. The absorption at 665 nm of the extract was determined spectrophotometrically (MacKinley, 1941).

TITRATABLE ACIDITY. An aliquot equivalent to 1 g of fresh pear tissue in the extract prepared for ACC analysis (three fruit replicates) was titrated to pH 7.2 using 0.001 N NaOH. The results are expressed as microequivalents per gram of organic acids on a fresh mass basis.

TOTAL PROTEIN DETERMINATION. AIS (40 mg) was suspended in 5 mL 1 N NaOH filtered, and 100 μL of this solution was neutralized with 100 μL 1 N HCl and the protein assayed with the Coomassie Blue reagent according to Bradford (1976) using bovine serum albumin as the standard. The results are expressed as milligram per gram of protein on a fresh mass basis.

Fig. 1. Changes in (A) flesh firmness and (B) soluble polyuronides of 'Anjou' pears during -1°C storage (closed symbols) or during ripening at 20°C following -1°C storage (open symbols) for 0, 25, 40, 55, 70, or 85 d.

Fig. 2. Changes in peel chlorophyll of 'Anjou' pears during -1 °C storage (closed symbols) or during ripening at 20 °C following -1 °C storage (open symbols) for 0, 25, 40, 55, 70, or 85 d.

experiment. Soluble polyuronides of pears held continuously at 20 °C slowly increased to 84 mg·g⁻¹ in 98 d. Pears stored for 25 d at -1 °C and then ripened at 20 °C showed almost the same pattern as fruit held continuously at 20 °C (Fig. 1B). However, after 70 d at 20 °C, soluble polyuronides rose to 88 mg·g⁻¹, and this increase coincided with autocatalytic ethylene onset. Soluble polyuronides increased to 90, 88, 95, and 100 mg·g⁻¹ on the 49th, 35th, 14th, and 14th day of ripening at 20 °C for pears held at -1 °C for 40, 55, 70, and 85 d, respectively (Fig. 1B).

There was only a slight decrease (3%) in peel chlorophyll during 100 d of -1 °C storage (Fig. 2). Pears held at 20 °C, without cold storage, had only slight chlorophyll loss the first 14 d, followed by a progressive degradation that resulted in nearly complete loss by the 98th day. Fruit stored for 25, 40, 55, 70, and 85 d at -1 °C and then ripened at 20 °C lost nearly all chlorophyll by the 70th, 49th, 35th, 21st and 21st day, respectively (Fig. 2).

'Anjou' pear titratable acidity at harvest was 33 µeq·g⁻¹ (Fig. 3). The titratable acidity of pears stored at -1 °C slowly decreased to 25 µeq·g⁻¹ in 98 d. However, for fruit directly held at 20 °C, titratable acidity decreased even more to 20 µeq·g⁻¹ between the 45 and 90 d of storage. Titratable acidity of fruit ripening at 20 °C after cold storage decreased to ≈20 to 22 µeq·g⁻¹ at faster rates the longer they were held in cold storage (Fig. 3).

After the initial decrease to a minimum ethylene level, the IEC began to rise reaching 0.1 µL·L⁻¹ by day 21 and, thereafter, continued to rise slowly to 1.85 µL·L⁻¹ over the duration of 98 d (Fig. 4A).

The pears held continuously at 20 °C eventually entered the autocatalytic phase of ethylene production rising to 30 µL·L⁻¹ on the 98th day of ripening. Fruit held at -1 °C for 25 d showed a considerable delay (63 d) before 0.03 µL·L⁻¹ was exceeded at 20 °C (Fig. 4A). Within the next few days, the IEC rose about the same time as control fruit (i.e., by the 90th day; Fig. 4A) and rapidly rose to 30 µL·L⁻¹. Pears held 40 or 55 d at -1 °C also had delayed climacteric ethylene production, up to 34 and 23 d, respectively (Fig. 4A). Once the pears held at -1 °C for at least 70 d had warmed to 20 °C, sustainable IEC >1 µL·L⁻¹ and a peak of to 40 µL·L⁻¹ was attained in ≈14 d (Fig. 4A).

ACC at harvest was ≈0.1 nmol·g⁻¹ and remained at low concentration through 35 d of -1 °C storage. Then, it increased progressively to 0.9 nmol·g⁻¹ (70 d) of -1 °C storage, leveled for 14 d more, and began to decrease after the 91st day as climacteric ethylene (sustainable) increased (Fig. 4B). Pears held continuously at 20 °C had ACC levels that remained <0.1 nmol·g⁻¹ through 91 d and then increased to 0.42 nmol·g⁻¹ during the next 7 d. 'Anjou' pears stored at -1 °C for 25, 40, 55, 70, or 85 d, then transferred to 20 °C initially had 0.19, 0.28, 0.20, 0.35, or 0.21 nmol·g⁻¹ ACC and peaked on the 63rd, 42nd, 28th, 21st, or 14th day of ripening at 20 °C, respectively (Fig. 4B). 3

ACC oxidase activity at harvest was 1.4 nL·g⁻¹·h⁻¹. During -1

STATISTICAL ANALYSIS. Least significant differences (LSDs) for comparisons between treatments at a particular time during ripening were calculated with the Number Cruncher Statistical Software (J. Hintze, Kaysville, Utah) statistical computer software. LSDs for comparing firmness, soluble pectin, peel chlorophyll, titratable acidity, IEC, ACC, ACC oxidase, and total protein were obtained by applying factorial analysis.

Results

'Anjou' pear firmness at harvest was 70 N and decreased only by 6% during -1 °C storage for ≈100 d (Fig. 1A). Fruit held at 20 °C continuously retained their firmness of >65 N for 20 d, followed by a gradual decrease to 15 N on the 98th d. Fruit kept at -1 °C storage for 25, 40, 55, 70, and 85 d before transfer to 20 °C to ripen showed a dramatic firmness loss to ≈10 N on the 70th, 49th, 30th, 9th, and 9th day at 20 °C, respectively (Fig. 1A).

Total polyuronides were constant for all pear fruit in the range of 107 to 118 mg·g⁻¹ throughout the experiment (data not shown). Soluble polyuronides of 'Anjou' pears at harvest were 63 mg·g⁻¹ (Fig. 1B). During -1 °C storage, soluble polyuronides began to increase after 42 d and levelled at 72 mg·g⁻¹ at the end of the

°C storage, the ACC oxidase activity progressively increased to a maximum of $71 \text{ nL} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ on the 98th day (Fig. 4C). The ACC oxidase activity of fruit held at 20 °C without any prior cold storage steadily increased to $4.5 \text{ nL} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ and remained level between 35 and 63 d. On day 63 through 70 at 20 °C, ACC oxidase activity peaked at $13.4 \text{ nL} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$, then declined to $2.8 \text{ nL} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ on the 98th day. ACC oxidase activity for fruit stored at -1 °C for 25, 40, 55, 70, or 85 d on transfer to 20 °C was 17, 24, 52, 50, or $50 \text{ nL} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ with maximal activity on the 49th, 35th, 28th, 7th, or 7th day, respectively (Fig. 4C).

Total proteins during -1 °C storage decreased slightly from $0.35 \text{ mg} \cdot \text{g}^{-1}$ at harvest to $0.30 \text{ mg} \cdot \text{g}^{-1}$ the 14th day followed by a slight increase to $0.36 \text{ mg} \cdot \text{g}^{-1}$ by day 98 (Fig. 5). Fruit held directly at 20 °C also showed an initial decrease to $0.28 \text{ mg} \cdot \text{g}^{-1}$ the 21st day, followed by increases after 40 to 90 d (Fig. 5).

Discussion

'Anjou' pears kept at -1 °C for ≈ 100 d softened only slightly ($<7\%$, Fig. 1A) and showed minimal chlorophyll loss ($\approx 7\%$, Fig. 2). However, IEC build up to $1.5 \mu\text{L} \cdot \text{L}^{-1}$ (Fig. 4A), ACC up to $0.9 \text{ nmol} \cdot \text{g}^{-1}$ (Fig. 4B), and ACC oxidase activity up to $71 \text{ nL} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ (Fig. 4C). The soluble polyuronide fraction increased by $\approx 15\%$ (Fig. 1B), and titratable acidity decreased by 20% (Fig. 3) accompanied by a small net decrease (7%) in protein (Fig. 5). Changes in softening and ethylene synthesis characteristics during -1 °C storage of 'd' Anjou' pears have been reported previously (Blankenship and Richardson, 1985; Toumadje, 1984; Vaz, 1984; Wang et al., 1985) in addition to changes in the mRNA population (Wilson et al., 1990), including ACC oxidase and ACC synthase mRNA transcripts (Lelievre et al., 1996).

Fruit held at 20 °C after harvest with no cold treatment ripened in a characteristically different way—by showing a much earlier flesh firmness (Fig. 1) and chlorophyll (Fig. 2) losses 40 to 50 d ahead of the climacteric rise in ethylene (Fig. 4A). Climacteric ethylene production occurred on the 91st day of 20 °C storage, well after the fruit become edibly ripe and soft to thumb pressure (≈ 20 N). Chlorophyll loss accompanied the loss in firmness, and titratable acidity (Fig. 3) became half that at harvest. Total protein increased by $\approx 20\%$ (Fig. 5) before climacteric ethylene initiated

(Fig. 4A), along with the rise in ACC (Fig. 4B) in ≈ 90 d at 20 °C. ACC oxidase activity (Fig. 4C), however, increased from the beginning. While loss in firmness during storage at 20 °C has been reported by Porritt (1964), the ability of pears to eventually ripen and produce climacteric ethylene at 20 °C with no chilling has been overlooked. Winter pear ripening ability postharvest without chilling (at 20 °C) has been examined thereafter for only 20 (Toumadje, 1984; Wang et al., 1985) or 30 d (Gerasopoulos and Richardson, 1996).

Incompletely chilled 'Anjou' pear fruit after storage at -1 °C followed by transfer to 20 °C are not usually able to sustain the accumulated ACC (Blankenship and Richardson, 1985) or the high ACC oxidase activity. ACC concentration and ACC oxidase activity decreased to a certain level and then peaked before or during the climacteric rise in ethylene (Fig. 4 A-C). However, incompletely chilled pears showed a rise in ethylene when firmness was in the range of 25 to 15 N (Figs. 1A, 4A).

'Anjou' pears with increasingly satisfied chilling requirement when exposed to ripening temperatures (20 °C for our experiment) showed increasingly higher IEC (Fig. 4A), more ACC (Fig. 4B), and higher ACC oxidase activity (Fig. 4C). These pears also have shown increased sensitivity to propylene (Gerasopoulos and Richardson, 1997) and higher unsaturated : saturated fatty acid ratio (Gerasopoulos and Richardson, 1995). The chilling requirement was satisfied in cold storage when all those characteristics peaked. The fruit ripened in a characteristic way, with loss in firmness, green color, and organic acids and gained in protein and water-soluble polyuronides in response to rising climacteric ethylene. Treatment of 'Anjou' pears at harvest with $50 \mu\text{L} \cdot \text{L}^{-1}$ propylene (equivalent to ethylene at 0.4 to $0.5 \mu\text{L} \cdot \text{L}^{-1}$) resulted in slow softening from 67 to 10 N within 30 d without a rise in climacteric ethylene (Gerasopoulos and Richardson, 1996), suggesting that this low concentration of propylene stimulates the activity and/or production of wall softening enzymes, without triggering many other events normally associated with ripening. Other studies on 'Anjou' pears suggest that softening is sensitive to as little as ethylene at $0.08 \mu\text{L} \cdot \text{L}^{-1}$ (Wang et al., 1972). Thus, small or slightly increased amounts of ethylene above that that is always present in preclimacteric fruit tissue, depending on the temperature, can stimulate softening, which, in turn, leads to satisfaction of the chilling requirement and stimulation the climacteric (i.e., autocatalytic) rise in ethylene production.

ACC oxidase activity preceded the rise in ACC and the IEC in all cases. ACC oxidase activity of partially chilled (25 or 40 d), and for the nonchilled pears increased well before ACC or climacteric ethylene. Once chilling was satisfied (beyond 70 d of chilling), ACC oxidase activity started high then decreased. ACC increased in parallel with the initiation of ethylene for nearly all treatments. The fact that ACC accumulated in cold-stored pears implies either that it is compartmentally segregated from the ACC oxidase or, alternatively, that enzymes leading up to ACC were not as low-temperature inhibited as the enzymes converting ACC to ethylene. Our evidence (Fig. 4B) supports the latter alternative

Fig. 3. Changes in titratable acidity of 'Anjou' pears during -1 °C storage (closed symbols) or during ripening at 20 °C following -1 °C storage (open symbols) for 0, 25, 40, 55, 70, or 85 d.

Fig. 4. Changes in (A) internal ethylene, (B) ACC, and (C) ACC oxidase activity of 'Anjou' pears during -1°C storage (closed symbols) or during ripening at 20°C following -1°C storage (open symbols) for 0, 25, 40, 55, 70, or 85 d.

as ACC can disappear quite rapidly once chilling is satisfied and pears are placed at 20°C .

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Fig. 5. Changes in total protein of 'Anjou' pears during -1°C storage (closed symbols) or during ripening at 20°C following -1°C storage (open symbols) for 0, 25, 40, 55, 70, or 85 d.

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