Alcohol Dehydrogenase Expression and Alcohol Production during Pear Ripening

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ABSTRACT. Regulation of alcohol dehydrogenase (ADH), activity of pyruvate decarboxylase (PDC) and accumulation of acetaldehyde and ethanol in 'Packham's Triumph' pears (*Pyrus communis*, L.) subsequent to different storage regimes were investigated. Pears were stored for two months at -1°C either in air (Air) or under hypoxia at 3 kPa O_2 (Hyp) and subsequently warmed and allowed to ripen in air at 20°C . One set of fruit stored in air at -1°C was subjected to 3 days of hypoxia at -1°C (Air+Hyp) before ripening in air. Acetaldehyde, ethanol and methanol levels increased in all fruit in a similar fashion during ripening and did not reflect differences in storage treatments. During ripening, ADH activities in posthypoxic samples were generally twice that of air samples. PDC activities increased for ≈ 6 days during ripening then declined slightly but did not differ significantly among treatments. Upon transfer to 20°C in air, slightly higher levels of *Adh* mRNA were observed in samples treated with hypoxia than in air controls. Over the following 2 days at 20°C , the *Adh* transcription was markedly induced in Air and Air+Hyp samples. Although all *Adh* mRNAs returned to control levels within 4 days, ADH activities remained higher in hypoxia-treated fruit than in controls for up to 18 days. These results suggest that, in ripening pears, ADH does not limit ethanol production, and that the expression of this enzyme comprises post-transcriptional regulations. GenBank accession numbers of the *Adh* cDNAs are AFO 31899 and AFO 31900.

Pears, like many other fruit, produce acetaldehyde and ethanol during the ripening phase (Janes and Frenkel, 1978; Nanos et al., 1992). The ethanol pathway branches from pyruvate, through decarboxylation of the pyruvate by PDC to form acetaldehyde which is then reduced to ethanol by ADH.

In the pome fruit industry controlled atmospheres (CA) and/or low temperature regimes are commonly used to increase shelf life and maintain fruit quality. But they have also been reported to be detrimental to aroma (Yahia, 1994). ADH, which is induced by CA in pears (Nanos et al., 1992), may play various roles leading to aroma changes by i) producing alcohols, which are precursors of esters (themselves important aroma compounds, Yahia, 1994) or ii) maintaining the fruit tissues in a reduced state, while many oxidation processes are necessary to aroma production (Yahia, 1994, and references therein). Some work has been undertaken to examine the effects of controlled atmospheres on the physiology of stored pear fruit. 'Bartlett' pears stored under a hypoxic regime (0.25 kPa O_2 at 20°C) (Ke et al., 1994; Nanos et al., 1992) have increased levels of ethanol together with increased ADH activity, the latter being retained during subsequent ripening in air.

To extend these studies and to investigate the apparent retention of ADH activity in hypoxic-treated fruit, we have examined 'Packham's Triumph' pear fruit stored under two different regimes of combined low temperature and hypoxia (one with long term and one with short term hypoxic induction). Levels of ethanol were determined and were compared with the activity of ADH in the fruit. As PDC activity is a limiting factor in ethanol formation

in hypoxic tobacco leaves (Bucher et al., 1994), we also monitored PDC activity as well as acetaldehyde levels.

To examine further whether the activity of the ADH enzyme was a direct function of gene activity, cDNAs to two ADH mRNAs were prepared from pear fruit mRNA and were used as hybridization probes to measure mRNA abundance in the ripening fruit subsequent to the various storage treatments. The mRNA abundance was compared with ADH activities in the fruit.

Materials and Methods

PLANT MATERIAL. 'Packham's Triumph' pears were obtained from a local grower on the day of harvest and were stored for 2 months under either air (Air) or 3 kPa O_2 (Hyp) or air followed by 3 d at 3 kPa O_2 (Air + Hyp), all at -1°C , before the ripening trials. In the hypoxic treatments, the oxygen level was set at 3 kPa (CA storage of Packham's pears in Australia commonly uses O_2 levels of between 2 and 3 kPa). The CO_2 level was kept under 0.2 kPa over the whole storage period, in all treatments, as the object of the experiments was to study the effects of hypoxia, not those due to high CO_2 levels. Ripening was induced by transferring the fruit to 20°C in air (day 0 for all three treatments). At various times during the ripening phase, peeled and cored fruit were diced into liquid nitrogen. Samples were kept frozen in freezer bags at -75°C until analysis.

ACETALDEHYDE AND ETHANOL DETERMINATION. Analyses were performed according to Tajima and LaRue (1982), with the following modifications. Frozen tissue was ground in a cold Waring blender containing CaCl_2 at $0.33 \text{ g}\cdot\text{g}^{-1}$ frozen tissue to inhibit enzyme activities during thawing (Buttery et al., 1987). One gram of frozen powder was weighed into a 2-mL Eppendorf tube and held at -75°C for temperature equilibration. The sample was then centrifuged at 1°C (15 min, $12,000 g_n$) and 5 μL of thawing juice was removed and injected immediately into a gas chromatograph (Shimadzu CR14A, Shimadzu, Kyoto, Japan). One gram of fresh tissue yielded $0.875 \pm 0.025 \text{ mL}$ of juice, determined on the basis

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of mass difference between fresh and freeze-dried tissue ($n=10$). Where appropriate, values are given $\pm$ standard deviation. Acetaldehyde (retention time (RT) = 8.5 min) and ethanol (RT = 15 min) were separated on a Porapak PS column (length 2 m, mesh 100/120, O.D. 3.2 mm). The injector temperature was 115 °C and the oven was subjected to a temperature program (65 °C for 12 min, 65 °C to 85 °C in 0.5 min, 85 °C for 5.5 min, then a cleaning step at 200 °C for 30 min). The carrier gas was N₂ (100 kPa) and detection was performed by flame ionization (210 °C). Acid washing of the glass injection liner was required after about every 20 analyses.

Acetaldehyde and ethanol detection by gas chromatography (GC) was compared with enzymatic assays using Boehringer kits (Boehringer, Mannheim, Germany), as the GC method was used for the first time with fruit juice. For acetaldehyde, the linear regression between responses obtained by Boehringer kits versus GC, ranging from 0 to 10 $\mu\text{mol}\cdot\text{mL}^{-1}$, had a slope of 0.44 and a correlation coefficient of 0.94 ($n = 14$). The difference (2.27 times more with GC) may be due to an overestimation by the chromatographic method or an underestimation by the enzymatic one (as acetaldehyde is highly volatile and the enzyme assays are run at 20 °C, even with cuvettes stoppered with Parafilm, as described in Boehringer's protocol). For ethanol, the linear regression ranging from 0 to 20 $\mu\text{mol}\cdot\text{mL}^{-1}$ had a slope of 1.03 and a correlation coefficient of 0.99 ($n = 14$).

ENZYME ASSAYS. All extractions and desalting steps were carried out below 4 °C. Extractions were performed according to Nanos et al. (1992), with the following modifications. Frozen tissue (5 g) was ground in a Waring blender and homogenized in 10 mL extraction buffer for 20 s using a Polytron homogenizer. The homogenate was centrifuged at 20,000 g_n for 20 min. Before assaying, the supernatant (2.5 mL) was desalted by passing through a 10 mL Sephadex G-50 column. ADH and PDC were assayed as described by Nanos et al. (1992). Blanks for ADH assays without the extract or without the substrate gave <5% activity. In the ADH assays, NADH oxidation was 99% inhibited by 60 $\mu\text{mol}\cdot\text{L}^{-1}$ methyl pyrazole, which inhibits ADH activity (Perata and Alpi, 1991). Protein content was determined by the Bradford assay (Bradford, 1976), using bovine serum albumin as the standard. Enzyme activities were calculated both on a fresh mass basis and as specific activities but have been reported in the figures on a per fresh mass basis to relate directly with metabolite levels. Specific activities followed the same pattern (data not shown). For determination of the optimum pH for enzyme activity, samples of day 2, 4, and 7 were mixed in equivalent amounts for each treatment.

RNA EXTRACTION. Total RNA was extracted from the pear fruit tissue by the proteinase K method described by Speirs and Longhurst (1993). We found it optimal to extract 4 g of freeze-dried tissue in 20 mL extraction buffer. At the end of the protocol, the pellets of total RNA were further purified according to Manning (1991) as follows. After the first ethanol precipitation and centrifugation, RNAs were resuspended to an approximate concentration of 2.5 $\mu\text{g}\cdot\mu\text{L}^{-1}$ in 100 $\text{mmol}\cdot\text{L}^{-1}$ NaCl, then 2-butoxyethanol was added to 25% of the final volume. The sample was cleared by centrifugation and additional 2-butoxyethanol was added to the supernatant to a final concentration of 50% of the final volume. The RNA yield from freeze-dried tissue was $95 \pm 51 \mu\text{g}\cdot\text{g}^{-1}$ ($n = 24$).

ISOLATION OF cDNAs ENCODING TWO PEAR ADH ENZYMES. PolyA⁺ RNA was isolated from a mixture of total fruit RNAs representing 33% of each of the three treatments, extracted from day 0 samples. One microgram of the polyA⁺ RNA was reverse transcribed from a specific oligo-d(T) primer as described by Frohman et al. (1990) and the single stranded cDNA was used as the template for PCR amplification. ADH encoding sequences were selectively ampli-

fied by PCR using a redundant 5' primer and a 3'-specific primer (Frohman et al., 1990). The redundant 5' primer (GAY GTH KAC TTC TGG GAD GCY AAG GG, where Y = C/T, H = A/C/T, K = T/G, and D = A/T/G) was constructed on the basis of a conserved region of 13 *Adh* genes (sequences obtained from GenBank, NCBI, Bethesda, Md.). Two cDNAs, PcADH3 and PcADH4 with sizes of ≈ 1.16 and 1.14 kb were isolated. The numbers associated with the names are trivial and do not correspond to particular alleles. The pear cDNA sequences are recorded in the GenBank database with accession numbers AFO 31899 and AFO 31900. Sequence comparisons were conducted by GAP analysis (a general method allowing for gaps, Needleman and Wunsch, 1970) using default parameters.

The cDNAs, PcADH3 and PcADH4 have open reading frames encoding partial ADH sequences which are lacking the N-terminal 50 amino acids. The encoded peptides have 90% similarity to each other, 89% and 98% similarity, respectively, to ADH from apple (*Malus $\times$ domestica* Borkh. GenBank accession no. Z48234) and 89% and 95% similarity, respectively, to ADH from grape (*Vitis vinifera* L. accession no. U36586).

NORTHERN HYBRIDIZATION. Aliquots (4.5 μg) of the RNAs were fractionated on denaturing gels by the method of Fournery et al. (1988) without the addition of ethidium bromide. A duplicate set of RNAs were run separately on the same gel and were stained with ethidium bromide, to allow quantitative comparison of the RNA loadings. The unstained RNAs were transferred to charged nylon membrane (Zeta Probe, BioRad, Hercules, Calif.) for hybridization. The membranes were hybridized with an equal mixture of both pear *Adh* cDNAs, ³²P-labelled. The filters were prehybridized and hybridized in 0.25 $\text{mol}\cdot\text{L}^{-1}$ Na₃PO₄, 240 $\text{mmol}\cdot\text{L}^{-1}$ of sodium dodecyl sulfate (SDS), 1 $\text{mmol}\cdot\text{L}^{-1}$ ethylenediaminetetraacetic acid (EDTA) at 65 °C, and washed 2 $\times$ 15 min with 40 $\text{mmol}\cdot\text{L}^{-1}$ Na₃PO₄, 1 $\text{mmol}\cdot\text{L}^{-1}$ EDTA, 170 $\text{mmol}\cdot\text{L}^{-1}$ SDS, and 2 $\times$ 15 min with 40 $\text{mmol}\cdot\text{L}^{-1}$ NaPO₄, 1 $\text{mmol}\cdot\text{L}^{-1}$ EDTA, 35 $\text{mmol}\cdot\text{L}^{-1}$ SDS, all at 65 °C. Washed filters were blotted dry and were quantified by phosphoimaging (Storm 860 Phosphoimager; Molecular Dynamics, Sunnyvale, Calif.).

Ethidium bromide staining was quantified by scanning with a Relisys scanner (model VM 3511; AIM Inc., Milpitas, Calif.) image acquisition using the software Picture Publisher (Micrographx Inc., Richardson, Tex.) and band intensity measurement using the software SigmaScan (SPSS Inc., Chicago).

The intensities of the hybridization bands were corrected according to the RNA loading given by ethidium bromide fluorescence of the ribosomal RNA bands.

STATISTICAL ANALYSES. All ANOVAs (factor 1: CA treatment, factor 2: time during ripening, and interaction 1 $\times$ 2) were performed with Genstat 5 (3.2) (Lawes Agricultural Trust, IACR-Rothamstead, U.K.). LSDs were calculated at $P = 0.05$, $\text{LSD} = t \times \text{SED}$, with $\text{SED} = \sqrt{2 \times \text{RMS}/n}$ ($t = 1.96$ as residual degrees of freedom always >30; SED = standard error of the difference of means; RMS = residual mean square). Unless noted otherwise, only results significant at $P \leq 0.05$ are discussed.

Results and Discussion

ETHANOL AND ACETALDEHYDE CONTENTS OF RIPENING FRUIT AFTER STORAGE TREATMENTS. The ethanol content of the ripening fruit in all the treatments started to rise markedly between days 4 and 7 (Fig. 1a). There was no effect of the CA treatment, but time and the interaction of CA treatment $\times$ time had significant influence on ethanol levels. The effect of time is obvious, and the significance of the interaction might be seen in the reversed ethanol

Fig. 1. Variations in the ethanol, acetaldehyde and methanol contents during pear ripening, as functions of the time elapsed since the cold storage under air or hypoxia ($n = 6$, bars show LSDs for $P = 0.05$).

levels at days 10 and 18, in Air and Hyp samples. At day 10, the higher level in Hyp may be due to higher ADH activity as observed previously (Nanos et al., 1992). At day 18, the lower ethanol level in Hyp samples may reflect a slower ripening of the fruit, with the onset of fruit softening occurring around day 4 for Air and Air+Hyp samples and day 5 in Hyp samples (data not shown). Acetaldehyde content of the pear tissues decreased in the first 7 d after transfer to 20 °C and then increased as the fruit matured (Fig. 1b), only time had a significant influence. Janes and Frenkel (1978) have described a similar late increase in acetaldehyde in 'Bosc' pears ripening after hypobaric storage at 0 °C but did not note the early drop in acetaldehyde as seen in Fig. 1b. The initial level of acetaldehyde (at day 0, Fig. 1b) may be due to rewarming and associated oxidation processes.

While measuring acetaldehyde and ethanol, we observed an additional volatile peak ($RT = 6.2$ min) which was subsequently identified by GC-MS as methanol. The abundance of methanol in the fruit was observed to increase over the ripening period in all three treatments (Fig. 1c). While time was the only factor significantly influencing methanol levels, CA treatment had an F probability = 0.08 in the analysis of variance. The lower methanol content of Hyp fruit relative to Air and Air+Hyp, over the last days

of the ripening period, may reflect a delayed or slowed ripening, as stated previously. Methanol has been observed in other pear cultivars (Phan, 1968) and its abundance in 'Bartlett' pears, measured in the fruit after canning, has been related to the softness of the fruit before canning (Luh et al., 1955). It is generally believed that the increased methanol abundance in ripening fruit is a function of pectin deesterification associated with fruit softening (Luh et al., 1955).

ADH AND PDC ACTIVITIES. ADH activity in Air fruit, on a fresh mass basis, was $\approx 0.5 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ upon removal of the fruit from cold storage and then declined slowly throughout the course of the experiment (Fig. 2a). The ADH activities in Hyp and Air+Hyp samples were more than twice the Air control activities from day 4 to day 12. The elevated levels of activity in these two treatments were sustained throughout the course of the measurements and appeared to be independent of the duration of the hypoxic induction. The Hyp samples showed higher activities from day 0 onwards, whereas Air+Hyp started with activities in the range of Air samples and reached the levels of Hyp samples after 2 to 4 d.

Nanos and Kader (1993) found that the pH of pear juice decreased as the fruit underwent normal ripening whereas in fruit subjected to a prior hypoxic treatment the juice pH remained at higher levels. While these differences may reflect changes in vacuolar pH as opposed to cytosolic pH, we considered the possibility that different treatment regimes may lead to the accumulation of various ADH isozymes with differing pH optima. In our studies, the optimum pH for all the ADH extracts, measured *in vitro*, was 6.0 (data not shown) and there was no detectable difference among treatments.

All three treatments, Air, Hyp and Air+Hyp, generated similar ethanol levels (Fig. 1a), but different levels of ADH activity, as measured *in vitro* (Fig. 2a). If the *in vivo* activity of the ADH is similar to the average activity measured *in vitro* ($0.5 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$; Fig. 2a), the fruit would have the potential to produce ethanol at a rate of $>700 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{d}^{-1}$, 2 to 3 orders of magnitude greater than was

Fig. 2. Variations in alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) activities during pear ripening, as functions of the time elapsed since the cold storage under air or hypoxia ($n = 6$, bars show LSDs for $P = 0.05$).

Fig. 3. Levels of hybridization of a mix of two pear *Adh* cDNA probes with total pear RNA, as a function of the time elapsed since the cold storage under air or hypoxia ($n = 2$, bar shows LSD for $P = 0.05$).

observed. Taking into account that the *in vivo* enzyme activity will be subject to some degree of metabolic regulation, the potential ADH activities in fruit from all three treatments would still appear to be greatly in excess of requirements. Either the *in vivo* ADH activity differs markedly from the activity measured *in vitro* or the ADH enzyme is not rate limiting in the ethanol production pathway or a combination of both. Ethanol accumulation in the tissue will also be modulated to some extent by volatilization and/or transformation into esters.

The PDC activity increased in all treatments up to about day 8 then decreased slowly (Fig. 2b). The significance of this was confirmed by analysis of variance, in which time had a significant effect, but not CA treatment. As with ADH, the *in vitro* activity of the PDC enzyme is greatly in excess of the *in vivo* activity that would be required to produce the amounts of acetaldehyde and ethanol observed to accumulate in the fruit tissues (Fig. 1). Moreover, the production of acetaldehyde in fruit may not be due entirely to PDC activity as it has been shown to occur by direct oxidation of ascorbic acid and related compounds (Miyake and Shibamoto, 1995). Therefore PDC, like ADH, is unlikely to be rate limiting.

In recent experiments by Bucher et al. (1994), the introduction of a heterologous (*Zymomonas mobilis* Lindner) gene for PDC into tobacco (*Nicotiana tabacum* L.) resulted in a 10- to 35-fold enhanced production of acetaldehyde and an 8- to 20-fold enhancement of ethanol production relative to basal levels in tissues under hypoxia, while no enhancement was noted in the absence of hypoxic stimulation. This would indicate that the fermentation pathway is strongly dependent on the glycolytic flux. Such may also be the case in ripening pears. Nanos et al. (1994) have shown, with ripening 'Bartlett' pears, an increase in pyruvate kinase activity which correlates with an increase in ethanol content but in the absence of an increase in ADH activity (Nanos et al., 1992). In the case of ripening pears, the glycolytic flux is increased at the

climacteric peak of respiration (Brady, 1987).

***Adh* mRNA AND ADH ENZYME STABILITY.** In order to examine whether the sustained activity of ADH in the fruit treated with hypoxia was regulated at the gene level, we undertook to examine *Adh* mRNA levels in the ripening fruit from the various treatments. Total RNAs from fruit from the three storage regimes (sampled during the first 4 d of ripening) were hybridized with a probe containing both of the pear *Adh* cDNAs (Fig. 3). We used a mixed probe in order to maximize hybridization. Both factors (time and CA treatment) and their interaction showed significant effects. Both Hyp and Air+Hyp treated fruit had slightly higher levels of *Adh* mRNA upon removal from storage than did Air treated fruit, but the levels in Air and Air+Hyp fruit rapidly increased within 24 h while the levels in Hyp fruit increased only slowly. The enhanced levels in Air fruit, evident at day 1, appear to have been induced by the removal of the fruit from low temperature storage but were not observed in the Hyp fruit. The slow increase in the Hyp fruit may reflect a delayed recovery of the fruit metabolism from low temperature hypoxic conditions. In all three treatments, the enhanced levels of *Adh* mRNA declined within 2 to 4 d after transfer of the fruit to air at 20 °C.

The rapid increase in ADH activity in Air+Hyp samples, relative to the activity in the Air control fruit (Fig. 2a), appears to be correlated with the rapid increase in *Adh* mRNA in these fruit (Fig 3). In the Hyp fruit, subjected to hypoxia throughout storage, a slightly elevated level of *Adh* mRNA was already evident upon rewarming and was reflected in a stable high level of ADH activity. In contrast the Air samples, in which the transcription of *Adh* mRNAs was induced (day 1, Fig. 3) did not show a proportional increase in ADH activity. This may be due to post-transcriptional regulation of synthesis or activation of the enzyme(s), as shown in other plants (Fennoy and Bailey-Serres, 1995, and refs therein). The sustained high ADH activities observed in the hypoxia-treated fruit do not appear to be a function of sustained transcription, but instead may reflect regulated translation of the mRNAs or high enzyme stability.

Nanos et al. (1992) observed a difference in ADH isozymes in air and hypoxia-treated fruit suggesting that different genes may be involved in their synthesis. In the experiments described here, we have not tested whether the *Adh* mRNA in Air fruit is transcribed from the same gene(s) as the mRNA found in fruit treated with hypoxia. However, in tomato, there is no evidence for the involvement of more than one gene in the synthesis of ADH in normally ripening or hypoxia treated fruit (Tanksley, 1979; Tanksley and Jones, 1981; Longhurst et al., 1990).

Conclusions

Our results suggest that neither ADH nor PDC are limiting factors for the accumulation of ethanol during pear ripening. Moreover the induction of ADH is complex and is probably regulated at some post-transcriptional level (e.g., induction of *Adh* mRNAs in Air, without concomitant increase in ADH activity). Whether or not this is going to influence the development of flavour in the ripening fruit is as yet unclear, although ADH is involved in the balance of some flavour-associated compounds (Yahia, 1994). We are currently conducting studies on a wide range of aroma components after such treatments.

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