Ethanolic Fermentation Enzymes, their Products and Transcription of Alcohol Dehydrogenase from Bell Pepper Fruit Held under Various Low Oxygen Atmospheres

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Summary

The concentrations of ethanol and acetaldehyde in bell pepper fruit were very low during storage at 1%, 3%, 5%, and 10% O₂, and air at 20 °C. At 0% O₂, ethanol and acetaldehyde to a lesser extent, rapidly accumulated in its tissue. The activity of alcohol dehydrogenase (ADH) was about 10 times that of pyruvate decarboxylase (PDC) during storage. The ADH activity greatly increased in the fruit exposed to 1% and 3% O₂, but in those exposed to 0%, 5%, and 10% O₂ it was slightly higher than that of the control. The PDC activity greatly increased in the fruit exposed to 1%, 3%, and 5% O₂, while those of fruits exposed to 0% and 10% O₂ were slightly higher than that of the control. ADH transcripts in the fruit exposed to 5% and 10% O₂ or air were not detectable, whereas those in the fruit exposed to 1% O₂ were significantly higher than those in 0% and 3% O₂ on day 1 at 20 °C. The discrepancy of the enzyme activities and their product levels are discussed.

Key Words: acetaldehyde, alcohol dehydrogenase, alcohol dehydrogenase transcript, ethanol, pyruvate decarboxylase.

Introduction

Exposing fresh fruits and vegetables to low O₂ and/or high CO₂ can be beneficial or harmful, depending on concentrations of these gases, temperature, and exposure duration. The benefits of controlled atmosphere (CA) or modified atmosphere (MA) storage include extending storage life and maintaining the quality of commodity. During storage, the physiological effects of low O₂ are as follows: decrease in respiration rate and ethylene evolution, delay in the onset of the climacteric rise and the retardation of ripening (Kader, 1986). However, exposing products to stress O₂ levels for a period longer than tolerable, can lead to abnormal ripening, browning of tissues, and accumulation of ethanol and acetaldehyde (Ke and Kader, 1992).

Most commodities require a minimum of 1% to 3% O₂ in MA storage to avoid a shift to anaerobic metabolism; O₂ levels as low as 0.2% in the plant cell may result in anaerobic respiration. During fermentation, acetaldehyde which is produced through pyruvate decarboxylation by pyruvate decarboxylase (PDC) is converted to ethanol by alcohol dehydrogenase (ADH) using NADH. Hence, ethanol is usually the major end-product of the pathway in low O₂-stressed commodities.

Hypoxia can also induce and/or inhibit protein synthesis, e.g. it may induce synthesis of anoxic proteins in fruits and vegetables and enable tissues to survive periods of anoxia by allowing continued production of high-energy compounds through glycolysis. However, hypoxic conditions can inhibit the synthesis of mRNA and proteins associated with wound responses in potato slices (Butler et al., 1990).

In this study, we investigated changes in concentrations of acetaldehyde and ethanol, activities of PDC and ADH, and the induction of ADH transcripts in bell pepper fruits under different O₂ atmosphere storage. The mode of action of low O₂ on fermentation is discussed.

Materials and Methods

1. Plant materials and treatments

Bell pepper fruits (*Capsicum annuum* cv. Kyomidori) were obtained from a local wholesale distributor in Sakai, Osaka transported to our laboratory, and kept overnight at 20 °C. Twenty-five fruits selected for uniformity of size, shape, and freedom from defects were placed in 7-liter glass jars that were ventilated with humidified air or a desired gas mixture at 250 ml·min⁻¹ flow rate for up to 7 days at 20 °C. The gas mixtures were: 0%, 1%, 3%, 5%, and 10% O₂ (balance N₂). Oxygen levels were routinely checked by using a gas chromatography (Yanaco Model G80, Tokyo), equipped with a thermal conductivity detector and a stainless steel column (3mm × 1.0m) containing

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molecular sieve 5A. The flow rate of argon gas, the carrier gas, was flow rate of $40 \text{ ml} \cdot \text{min}^{-1}$. The oven was set at 60°C and the injector was kept at 125°C . Each treatment was triplicated and the each measurement repeated at least three times.

Tissues from the equatorial area, including the skin, were frozen in liquid nitrogen at various intervals of storage and stored at -80°C until analyzed.

2. Ethanol and acetaldehyde determination

Ethanol and acetaldehyde levels were determined according to the method of Sugiura and Tomana (1983).

3. Enzyme extraction and assay

Enzymes were extracted according to Ke et al. (1995), with the following modifications. Frozen tissue (5 g) was homogenized with a chilled mortar and pestle in 20 ml of 100 mM 2-(*N*-morpholino)ethane-sulfonic acid (MES) buffer (pH 6.5) containing 2 mM dithiothreitol and 1% (w/v) polyvinylpyrrolidone (PVP). The homogenate was filtered through two layers of Miracloth and the filtrate centrifuged at $13,000 \times g$ for 25 min. PDC and ADH activities were assayed as described by Ke et al. (1995). Protein was assayed according to Bradford (1976), using bovine serum albumin as the standard.

4. RNA extraction and cDNA cloning

Total RNA was extracted by phenol-LiCl method according to Liu et al. (1998).

Two degenerate oligonucleotide primers were used for reverse transcription-polymerase chain reaction (RT-PCR) of ADH. These primers were designed from the conserved amino acid sequences of the tomato ADH2 (Longhurst et al., 1994), the potato ADH1 (Matton and Brisson, unpublished), the rice ADH2 (Xie and Wu, 1990), the barley ADH1 (Trick et al., 1988) and *Arabidopsis* ADH (Chang and Meyerowitz, 1986): forward, 5'-AA(A/G)GCIGCIGTIGCITGGGA-3' and reverse, 5'-ACICC(C/T)TCICCIACIC(C/G)(A/T)TC-3' (where "I" denotes inosinic acid).

For RT-PCR analysis, the first-strand cDNA was synthesized using 10 mM Tris-HCl, 25 mM MgCl_2 , 10 mM deoxynucleotide triphosphate, 1,500 units of RNase inhibitor, 10 units of avian myeloblastosis virus reverse transcriptase (Takara), 100 mM oligo(dt) primer, and 4 μg of total RNA at 42°C for 30 min, 99°C for 5 min and 5°C for 5 min. PCRs were performed with 25 μl volume, containing PCR buffer (Takara), 2.5 mM each deoxynucleotide triphosphate, 100 mM of each primer pairs, 1 μl first-strand cDNA, and 5 units of EX Taq polymerase (Takara). PCR amplification was 30 cycles of 95°C for 0.5 min, 53°C for 1 min, and 72°C for 1 min. After PCR, the RT-PCR products were separated by electrophoresis in a 1.5% agarose gel, and TA-cloned in pT7 Blue T-Vector (Novagen), and then introduced into *Escherichia coli* JM109. After screening, target cDNAs

were sequenced using a DNA sequencer (Applied Biosystems).

5. Southern blot analysis of transcript

The first-strand cDNA synthesized by RT from RNA were used as a template for RT-PCR with two specific primers as described below. Two specific primers were designed from the conserved regions of the tomato *Adh2* gene sequence (Longhurst et al., 1994), the potato *Adh1* gene sequence (Matton and Brisson, unpublished), the rice *Adh2* gene sequence (Xie and Wu, 1990), the barley *Adh1* gene sequence (Trick et al., 1988) and *Arabidopsis* *Adh* gene sequence (Chang and Meyerowitz, 1986): forward, 5'-GCAAAGCTGCTGTGGCATG-3' and reverse, 5'-TCTCCTTCTCCAACACTCTC-3'.

ADH RT-PCR product was sequenced to verify its identity by using the direct DNA sequence method with dye-terminator chemistry and automated sequencing system. The desired bands were obtained by DNA sequencing reaction; the nucleotide sequence of the ADH RT-PCR product was completely identical to that of cDNA fragment TA-cloned in this study. That only this ADH RT-PCR product was cloned using a pair of specific primers confirms the code is a part of the ADH gene. It was chosen as the probe of the ADH gene expression analysis.

To determine the expression patterns for the ADH gene in bell pepper fruits, we carried out the RT-PCR Southern analysis as described below. PCR amplifications were subjected to 18 cycles of 94°C for 0.5 min, 50°C for 0.5 min, and 72°C for 1 min. After PCR, the RT-PCR products were separated by electrophoresis in a 1.5% agarose gel and blotted on nylon membranes (Hybond-N from Amersham), according to the method of Shimizu et al. (1997). As a probe, RT-PCR products were labeled by a PCR DIG probe synthesis kit (Pharmacia). The membrane was hybridized in $5 \times \text{SSC}$ ($1 \times \text{SSC}$ consists of 150 mM NaCl, and 0.015 M sodium citrate), 1% (w/v) blocking reagent (Boehringer Mannheim), 0.1% (w/v) *N*-lauroylsarcosine, and 0.02% (w/v) sodium dodecyl sulfate (SDS) at 68°C . Following hybridization, the membrane was washed in $2 \times \text{SSC}$ and 0.1% (w/v) SDS for 5 min at room temperature, then in $0.1 \times \text{SSC}$ and 0.1% (w/v) SDS at 68°C for 15 min. The washed membranes were developed with an immunostaining system, according to the manufacturer's instructions.

As a control for the RT-PCR analysis, the levels of actin transcript were measured. Two primers of actin were designed from the primers which Marshall et al. (1996) reported: forward, 5'-ACTGGAATGGTCAAGGC-3' and reverse, 5'-GTGTGACTCACACACCATC-3'.

Results

1. Changes in ethanol and acetaldehyde production

The concentration of ethanol in bell pepper fruit was

very low and did not change during storage at 1%, 3%, 5% and 10% O₂, and air. However, ethanol concentrations of the fruit kept in 0% O₂ significantly increased 77-fold and 203-fold on days 3 and 7, respectively, compared with the other treatments (Fig. 1).

The acetaldehyde concentration in bell pepper fruit was much lower than that of ethanol (Fig. 2). Acetaldehyde concentrations in the fruit stored at 1%, 3%, 5%, and 10% O₂, remained very low throughout the storage period, but in those fruit exposed to 0% O₂, the concentration of acetaldehyde increased 7-fold by day 1; they gradually increased afterward.

2. Changes in ADH and PDC activities

The ADH activity in bell pepper fruit was about 10 times higher than that of PDC during storage (Fig. 3 and 4). The ADH activity in the fruit exposed to 1% O₂ increased 13 times by day 3 and 24 times by day 7 compared with those kept in air (Fig. 3). ADH activity in fruit exposed to 3% O₂, increased 7 and 15 times by days 3 and 7, respectively. However, ADH activity in fruits exposed to 0%, 5%, and 10% O₂ was slightly higher than that of the control.

PDC activities in fruit exposed to 1% O₂ increased 2 and 3 times greater than those in air by days 3 and 7, respectively (Fig. 4). PDC activities in fruit exposed to 3% and 5% O₂, increased 1.5 and 2.4 times greater than those in air by days 3 and 7, respectively, whereas its activities in fruits exposed to 0% and 10% O₂ were just slightly higher than that of the control.

Fig. 1. Effects of various oxygen levels on ethanol production in bell pepper fruit held at 20 °C. Fruit were previously kept in 0% (◇), 1% (△), 3% (○), 5% (□), 10% (▽) O₂ and Air (×). Mean ± standard error (SE) from three experiments with at least three assays for each determination.

3. Levels of ADH transcript

Using degenerate primers designed from conserved amino acid sequences of five plant ADH cDNAs, three cDNA fragments approximately 200 bp in size were obtained. Nucleotide sequences of each fragment were

Fig. 2. Effects of various oxygen levels on acetaldehyde production in bell pepper fruit held at 20 °C. Fruit were previously kept in 0% (◇), 1% (△), 3% (○), 5% (□), 10% (▽) O₂ and Air (×). Mean ± SE from three experiments with at least three assays for each determination.

Fig. 3. Effects of various oxygen levels on ADH activity in bell pepper fruit held at 20 °C. Fruit were previously kept in 0% (◇), 1% (△), 3% (○), 5% (□), 10% (▽) O₂ and Air (×). Mean ± SE from three experiments with at least three assays for each determination.

cytoplasmic acidification, induction of glycolytic activity, and accumulation of toxic glycolytic end products (Ricard et al., 1994). Fermentative metabolism results in the accumulation of acetaldehyde and ethanol by the actions of the enzymes PDC and ADH under low oxygen concentration (Ricard et al., 1994). Exposure to O₂ levels below the tolerance limits caused acetaldehyde and ethanol to accumulate in apples (Ke et al., 1991), pears (Ke et al., 1990), stone fruits (Ke and Kader, 1992), oranges (Ke and Kader, 1990), carrots (Leshuk and Saltveit, 1991), and sweet potatoes (Chang et al., 1982).

We demonstrated that the bell pepper fruit kept in 0% O₂ for up to 7 days at 20 °C accumulated acetaldehyde and ethanol but those kept in 1%, 3%, 5%, and 10% did not (Fig. 1 and 2). Recently, Luo and Mikitzel (1996) reported that no increase in ethanol level occurred in bell peppers stored in 1%, 3%, and 5% O₂ atmospheres for 4 weeks at 20 °C. This indicates that no anaerobic respiration and no low O₂ injury occurred during storage under these conditions. Our results also indicated that ADH activity increased markedly whereas PDC activity slightly increased in fruit exposed to 1% and 3% O₂, while the enzyme activities did not significantly increase in fruit exposed to 0% O₂ (Fig. 3 and 4). Our results demonstrate that the changes in the activities of these two fermentation enzymes did not necessarily correlate with the levels of corresponding end-products. Discrepancies between the ethanol accumulation and apparent activity of either PDC or ADH were reported in sweet potato (Chang et al., 1982) and pear fruits (Ke et al., 1994). Roberts et al. (1989) reported that ethanol production rate in hypoxic maize root tips was correlated with ADH activity when the enzyme level was very low, but at high levels of ADH activity, ethanol production was independent from its activity. Xia and Saglio (1992) suggested that the activity of ADH was not the rate limiting factor for the accumulation of ethanol in some plant tissues. Jonson (1989) also found that hypoxia, rather than anoxia, induced a high level of ADH activity in maize root tips.

Ke et al. (1995) indicated that fermentative metabolism can be regulated in avocado fruit: 1) molecular control of PDC and ADH, and 2) metabolic control of these enzymes in plant tissue under low-O₂ stresses. They suggested that a molecular induction of the expression of these fermentation enzymes is not the major mechanism for regulating fermentative metabolism and that the metabolic control of these enzymes by changes in pH and concentrations of substrates, cofactors, and/or inhibitors may also be important for their regulation. Moreover, Bucher and Kuhlemeier (1993) gave several explanations for the discrepancy between the activities of these two fermentation enzymes and the production of corresponding end-products. Firstly, pyruvate may preferentially enter the tricarboxylic acid cycle via pyruvate dehydrogenase and,

thus, may not be available for fermentation at a low O₂ level; secondly, PDC activity might be inhibited by an unfavorable cytoplasmic pH and, thus, acetaldehyde, the substrate for ADH, would be not produced; and thirdly, an ADH inactivator might be present.

On the other hand, increases in the activities of these enzymes by low O₂ atmospheres have been attributed to increased transcription and translation, that result in new mRNA synthesis and *de novo* synthesis of the corresponding enzyme proteins (Good and Crosby, 1989). In our study, both ADH activity and ADH transcript were elevated at low O₂, with the exception of ADH activity of 0% O₂. At 1% O₂ the induction of ADH in both the enzyme activity and gene expression level was near maximal (Fig. 3 and 6). Imahori et al. (1998) reported that at 1% O₂, the respiration rate of bell peppers was minimal and that hypoxia-induced injury was not observed so that storage life was prolonged. For bell peppers, 1% O₂ is close to the aerobic-anaerobic transition point, but, for those kept in 0% O₂, an increase in ADH activity was small compared to the increase in ADH transcripts (Fig. 3 and 6). This difference between ADH activity and ADH transcript level may be explained by the existence of the ADH gene family in bell pepper fruit. Furthermore, anaerobiosis can result in an initial decline in protein synthesis (Ricard et al., 1994). This may reflect polysome dissociation; even the anaerobic mRNAs may not be translated in direct proportion to their relative amounts. We found that ADH transcript level is not correlated with ADH activity in fruit kept in 0% O₂. The disparity between relative levels of mRNA and concurrent accumulation of translation product is recognized as a feature of posttranscriptional regulation of gene expression (Gallie, 1993). Posttranscriptional regulation of the anaerobic response involves a selective translation of mRNAs to encode anaerobic proteins and/or repression of translation of mRNAs to encode normal aerobic proteins (Bailey-Serres and Freeling, 1990). The ADH transcripts in the fruit kept in 0% O₂ may not be translated, and the protein expression may be blocked without the turnover of translation product.

In conclusion, our results indicate that in bell pepper fruit the PDC and ADH activities and ADH transcript increased at low levels of O₂, especially at 1% O₂, which might be the key point in the induction of both ADH activity and ADH transcript. The increase in ADH transcript and ADH activity did not correlate with acetaldehyde and ethanol accumulation in the fruits kept in 0% O₂ at 20 °C. Further experimentation is needed to seek how posttranscriptional regulation under low oxygen stress controls ethanolic fermentation.

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種々の低酸素濃度下におけるピーマン果実のアルコール発酵関連酵素活性とその生成物
およびアルコール脱水素酵素遺伝子の転写

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摘 要

1%, 3%, 5%, 10%O₂ 雰囲気および空気下で 20℃貯蔵したピーマン果実中のエタノールおよびアセトアルデヒド濃度は非常に低かったが, 0%O₂ 雰囲気ではこれらの成分が果実組織中に急速に増加した. エタノール濃度はアセトアルデヒド濃度より高かった. 貯蔵中のピーマン果実のアルコール脱水素酵素 (ADH) 活性は, ピルビン酸脱炭酸酵素 (PDC) 活性の約 10 倍であった. ADH の活性は, 1% および 3%O₂ 雰囲気で著しく増加したが, 0%, 5% および 10%O₂ にさらされ

た果実における活性の増加はわずかであった. PDC 活性は, 1%, 3% および 5%O₂ 貯蔵で著しく増加したが, 0% および 10%O₂ 貯蔵果実ではわずかな増加にとどまった. 20℃貯蔵 1 日で 1%O₂ にさらされた果実の ADH 転写量は, 0% および 3%O₂ にさらされた果実に比べて著しく多かった. 一方, 5%, 10%O₂ および空気下にさらされた果実では, ADH の転写は認められなかった. 酵素活性とその生成物量との矛盾についても議論した.