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Increase of Glycolytic Enzymes in Peanuts during Peanut Maturation and Curing: Evidence of Anaerobic Metabolism

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Previously, a substantial increase in the activity of alcohol dehydrogenase (ADH) during peanut maturation and curing was found. It was hypothesized that the increase of ADH is primarily due to the increased activities of glycolytic enzymes preceding ADH in the alcohol fermentation pathway. To verify this hypothesis, color assays were developed for detection of the following glycolytic enzymes: (1) aldolase; (2) glyceraldehyde-3-phosphate dehydrogenase; (3) pyruvate decarboxylase; and (4) ADH. Results showed that the activities of these enzymes increased significantly during peanut maturation and curing. The increased enzyme activities suggest that peanut maturation and curing are processes associated with anaerobic conditions. Enzyme activities were significantly higher in cured peanuts than in noncured peanuts, indicating that anaerobic conditions were more severe in the former. The potential contribution of water stress to the severity of anaerobic conditions is discussed.

Keywords: *Peanut maturation and curing; glycolytic enzymes; aldolase; ADH; PDC; GAPDH; color assays; anaerobic and stress conditions*

INTRODUCTION

In many organisms (including man), lactic acid is a prominent end product of glycolysis or anaerobic metabolism. An unfavorable consequence of lactate accumulation in plants is cytoplasmic acidosis, a decrease in cellular pH that may finally lead to cell death (Roberts et al., 1984a). As an alternative, plants shift from lactate to ethanol production in the alcohol fermentation pathway (Figure 1) (Bucher et al., 1994; Umeda and Uchimiya, 1994). Plants can sustain ethanol fermentation because, unlike lactate, ethanol production does not result in cytoplasmic acidosis (Roberts et al., 1984b), and instead of accumulating to toxic levels in the tissues, ethanol diffuses into the surrounding medium (Good and Muench, 1993). In addition, high ethanol production and low lactate synthesis have been shown to correlate with anoxia tolerance (Hole et al., 1992; Muench et al., 1993).

Under anaerobic or stress conditions, many of the glycolytic enzymes in the alcohol fermentation pathway have been shown to be induced in plants (Russell et al., 1990; Russell and Sachs, 1992; Umeda and Uchimiya, 1994; Millar and Dennis, 1996; Sachs et al., 1996). Among the enzymes induced are alcohol dehydrogenase (ADH), pyruvate decarboxylase (PDC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and aldolase (ALD). ADH, an enzyme in the final step of the fermentation pathway, catalyzes the conversion of acetaldehyde to ethanol (Figure 1). Because ADH is preceded by the other glycolytic enzymes in the pathway, it is likely that if there is an increase of these preceding enzymes, ADH may increase in level as well. Indeed, several studies have shown a correlation between increases of ADH and PDC in plants under anaerobic condition (Chen and Chase, 1993; Bucher et al., 1994; Shelp et al., 1995).

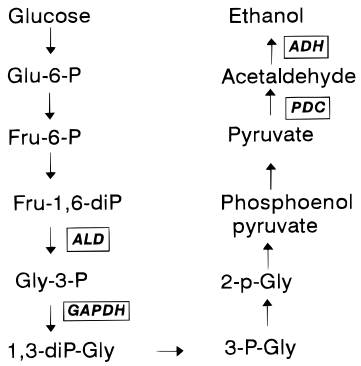

Figure 1. Enzymes in the alcohol fermentation pathway. Enzymes indicated are ALD, GAPDH, PDC, and ADH.

Recently we have shown that ADH increases substantially in activity during peanut maturation and curing (Chung et al., 1996). The objective of this study is to determine if activities of other glycolytic enzymes (preceding ADH in the fermentation pathway) increase as well. Development of color assays for the enzymes is also described.

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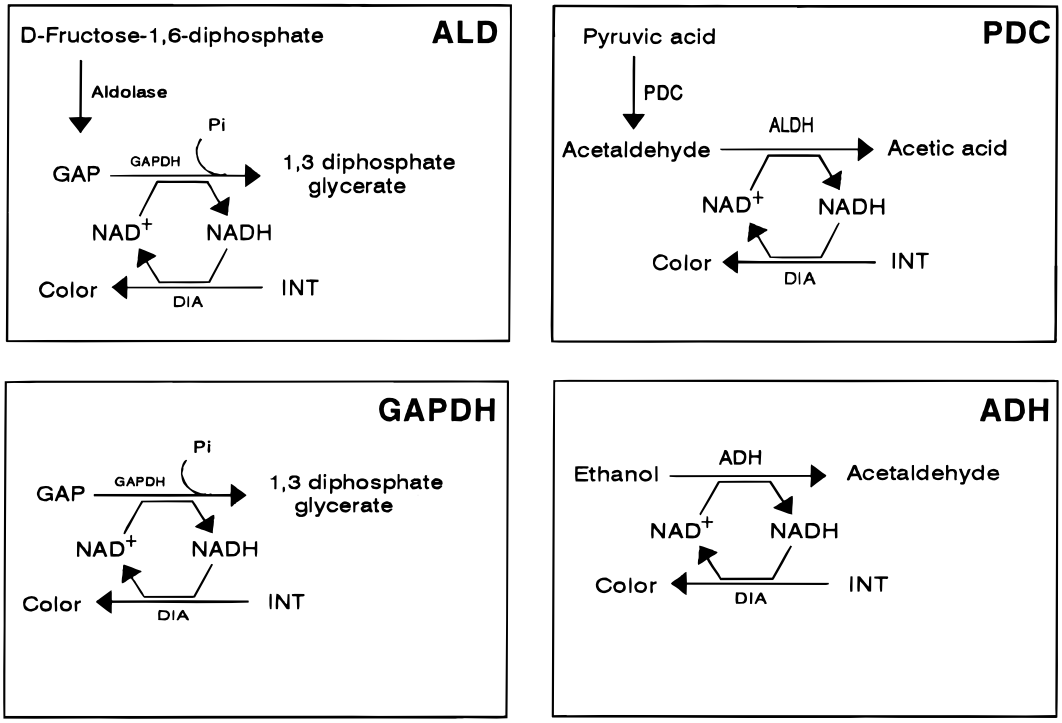

Figure 2. Principle of color assays for ALD, GAPDH, PDC, and ADH.

MATERIALS AND METHODS

Apparatus. CERES 900C microtiter plate reader was from Bio-Tek Instruments, Inc. (Winooski, VT). UV−visible 1601 spectrophotometer was from Shimadzu Scientific Instruments, Inc. (Columbia, MD).

Reagents. Microtiter plates of Immulon I were purchased from Dynatech Laboratories, Inc. (Chantilly, VA). Ethanol, nicotinamide adenine dinucleotide, aldehyde dehydrogenase (100 units), *p*-iodonitrotetrazolium violet (INT-violet), diaphorase (20 units), glyceraldehyde-3-phosphate, GAPDH, D-fructose-1,6-diphosphate, and pyruvic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemical Co. (Rockford, IL). Peanuts (*Arachis hypogaea* L. var. Florunner) were planted at the USDA-ARS National Peanut Research Laboratory (Dawson, GA), dug 120 days after planting, and subjected to windrow drying, from which samples were taken 0, 1, 2, 3, and 4 days later and day 4 samples were further dried to 10% moisture content with heated air. Day 0 samples refer to samples before curing. Day 4 samples refer to samples after curing. At each sample date, peanuts were subjected to gentle abrasion to remove the exocarp, sorted by pod color, and hand shelled, and seeds were stored at −80 °C. Peanut maturity (defined as yellow, orange, brown, and black) was based on the visual hull-scrape color method (Williams and Drexler, 1981).

Preparation of Enzyme Extracts. Enzyme extracts were prepared by a modification of the method of Chung et al. (1996). Prior to extraction, seed coats were removed from peanuts of different maturity and curing stages, and peanuts (20 g) were defatted by grinding with a Wiley mill with acetone and then hexane (both in dry ice). After air-drying, the resultant defatted peanut meals were stored at −20 °C or used for preparation of crude enzyme extracts. Extracts were each prepared by suspending 0.1 g of defatted peanut meal in 0.7 mL of 0.02 M sodium phosphate buffer, pH 7, and stirring for 30 min at 4 °C. Crude cell extracts were centrifuged at 8500*g* for 10 min. The resultant supernatants were again centrifuged. The final fat-free supernatants were used for enzyme assays.

Color Assays of Enzymes. Assays were performed with a slight modification of the method of Chung et al. (1996). All assays were carried out in wells of a microtiter plate containing the followings: buffer (64 μL) (0.02 M phosphate, pH 7, or 0.1 M bicarbonate, pH 9.6); substrate (50 μL); 4 mM INT-violet (15 μL); 14.3 μM NAD^+ (10 μL); diaphorase (1 μL); and peanut extract (10 μL). For assay of PDC and ALD, additional chemicals (1 μL) such as aldehyde dehydrogenase (ALDH) and GAPDH were added, respectively, to the assay solution (in this case, buffer volume was 63 μL instead of 64 μL). To determine the optimal pH or final substrate concentration for each of the enzyme systems, various pH values or substrate concentrations were tested. In the standard assay, substrates (50 μL each) for ADH, GAPDH, PDC, and ALD were, respectively, ethanol (3 μL, plus 47 μL of 0.1 M bicarbonate buffer, pH 9.6), 7.5 mM glyceraldehyde-3-phosphate in 0.1 M bicarbonate buffer, pH 9.6, 60 mM pyruvic acid in 0.02 M phosphate buffer, pH 7, and 2 mM D-fructose-1,6-diphosphate in 0.1 M bicarbonate buffer, pH 9.6. The total volume for each assay was 150 μL. Enzyme reactions were allowed to proceed for 10−15 min, and absorbances (color ranging from pink to red) were read at 570 nm against a blank (which contained everything except the substrate) using a Bio-Tek CERES 900C microtiter plate reader. Enzyme activity was defined as A_{570} per minute per milligram of protein, and the mean ($n = 2$) was obtained from two separate determinations. Protein concentration was determined using the BCA protein assay kit from Pierce, and absorbance was read at 562 nm using the UV-1601 spectrophotometer from Shimadzu.

RESULTS AND DISCUSSIONS

In this study, we determined the activities of ADH and three other glycolytic enzymes in the fermentation pathway, namely, GAPDH, PDC, and ALD. The conventional method for assay of these enzymes is based on the measurement of NADH at 340 nm or of hydrazone at 240 nm (for ALD) (Worthington, 1988). To visualize the enzyme reactions, we developed color assays similar to those described by Chung et al. (1995, 1996), in which enzyme activities were determined on the basis of colored products produced from the reduction of a tetrazolium salt (i.e., INT-violet) by NADH in the presence of diaphorase. Using this method, a linear region representing the enzyme activity was obtained within 10−15 min (Chung et al., 1995).

Assay Principle. The principle of the assay for each of the enzymes is shown in Figure 2. In the assay of

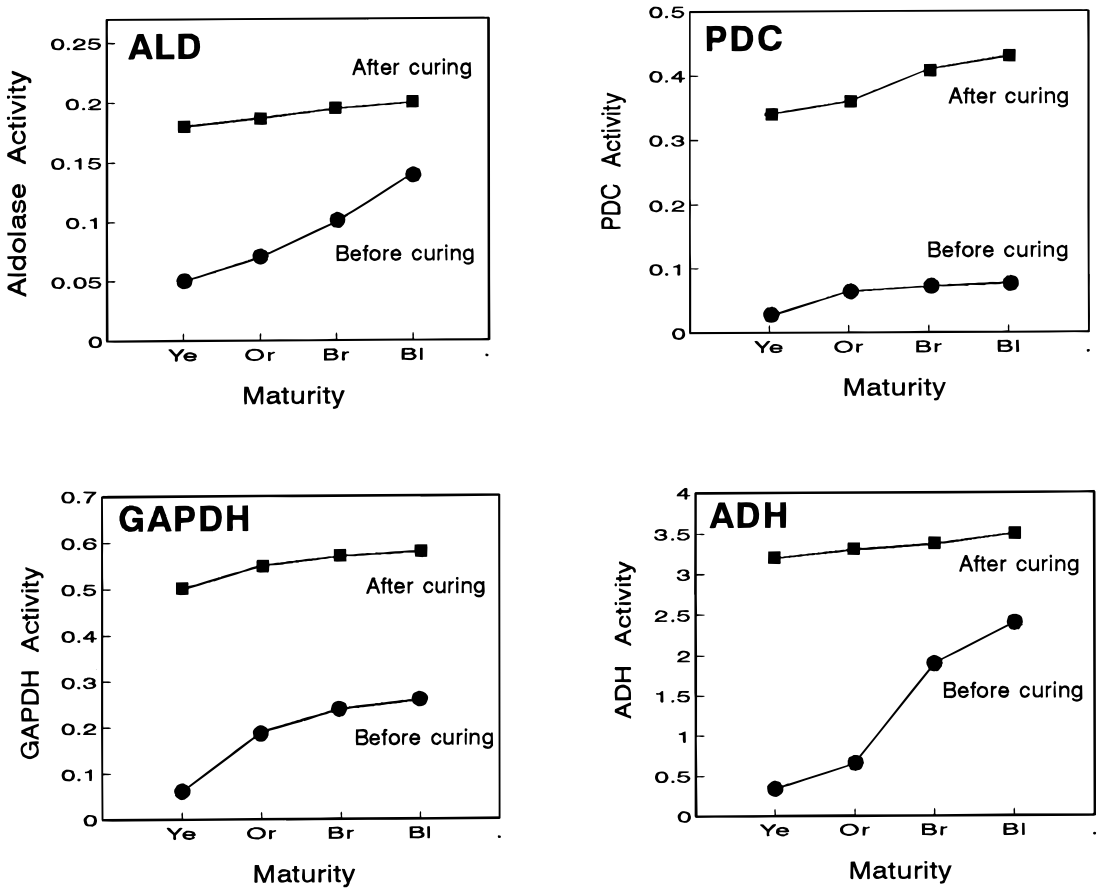

Figure 3. Activities [A_{570} min^{-1} (mg of protein)$^{-1}$] of ALD, GAPDH, PDC, and ADH at different peanut maturity stages. Samples were taken from 0 day (before curing) and 4 days after curing. Ye, yellow; Or, orange; Br, brown; and Bl, black.

GAPDH (or ADH), glyceraldehyde-3-phosphate (GAP) (or ethanol) is converted to 1,3-diphosphate glycerate (or acetaldehyde) by the enzyme and NAD^+. As a result, NADH is formed and in turn oxidizes, in the presence of diaphorase, INT-violet to a colored product (from pink to red), which is read at 570 nm in a microtiter plate using a plate reader. In the assay of PDC (or ALD), pyruvic acid (or D-fructose-1,6-diphosphate) is converted by the enzyme to acetaldehyde (or GAP), which is further oxidized, in the presence of acetaldehyde dehydrogenase (ADLH) (or GAPDH), to acetic acid (or GAP) by NAD^+. The NADH thus formed reduces INT-violet to a colored product in the presence of diaphorase. Absorbance was read as described above.

Optimal Assay pH and Substrate Concentrations. Optimal pH and final substrate concentration for ADH have been shown to be 8 and 2% (ethanol), respectively, in an ALDH-amplified system (Chung et al., 1995). To simplify the assay for ADH, a nonamplified system was used (i.e., no ALDH, the amplifier, was added). In this case, the optimal pH for the ADH system was 9.6, and final substrate concentration 2%. In the cases of ALD, GAPDH, and PDC, optimal pH values for the respective enzyme systems were 9.6, 9.6, and 7 and optimal final substrate concentrations were 1.3, 2.5, and 20 mM.

Increase of Glycolytic Enzymes during Peanut Maturation. Figure 3 (lower curve) shows the activities of ALD, GAPDH, PDC, and ADH from peanuts (before curing) of different maturity stages. Maturity (ranging from immature to mature) is classified as *yellow* (immature), *orange*, *brown*, or *black* (mature), which are the peanut pod colors determined by the visual hull scrape/color method (Williams and Drexler, 1981). As shown, all of the enzymes increased in activities in the following maturity order: *black* > *brown* > *orange* > *yellow*. In other words, the activities increased during peanut maturation. Of the four enzymes, ADH was the most abundant and increased most substantially in activities during peanut maturation. Because of its association with the ripening process (Longhurst et al., 1990; Chen and Chase, 1993) and plant tolerance to environmental stresses (Dolferus et al., 1994; Umeda and Uchimiya, 1994; Millar and Dennis, 1996; Sachs et al., 1996), ADH may be related to the "maturation proteins" (Rosenberg and Rinne, 1988; Blackman et al., 1991), so-called because these proteins have been shown to accumulate during seed maturation and are important in the development of tolerance to water loss or stress (Rosenberg and Rinne, 1986; Mundy and Chua, 1988; Bewley and Marcus, 1990; Blackman et al., 1991).

The observed increase in activities of ALD, GAPDH, and PDC supports our hypothesis that the increase of ADH during peanut maturation and curing is related to the increased activities of preceding enzymes in the fermentation pathway. Whether ADH activity is affected by one or more than one of the glycolytic enzymes is unclear. Because ADH and PDC have been shown to be related (Bucher et al., 1994; Shelp et al., 1995), it is thought that PDC may be the rate-limiting enzyme regulating ADH activity. However, in this study, PDC and ADH do not seem to be related because the change in PDC activity is not as substantial as that in ADH activity during peanut maturation (Figure 3). Similar observations (i.e., no relationship) have also been made in studies of corn seeds and ripening tomatoes (Van Toai et al., 1985; Chen and Chase, 1993). This suggests that

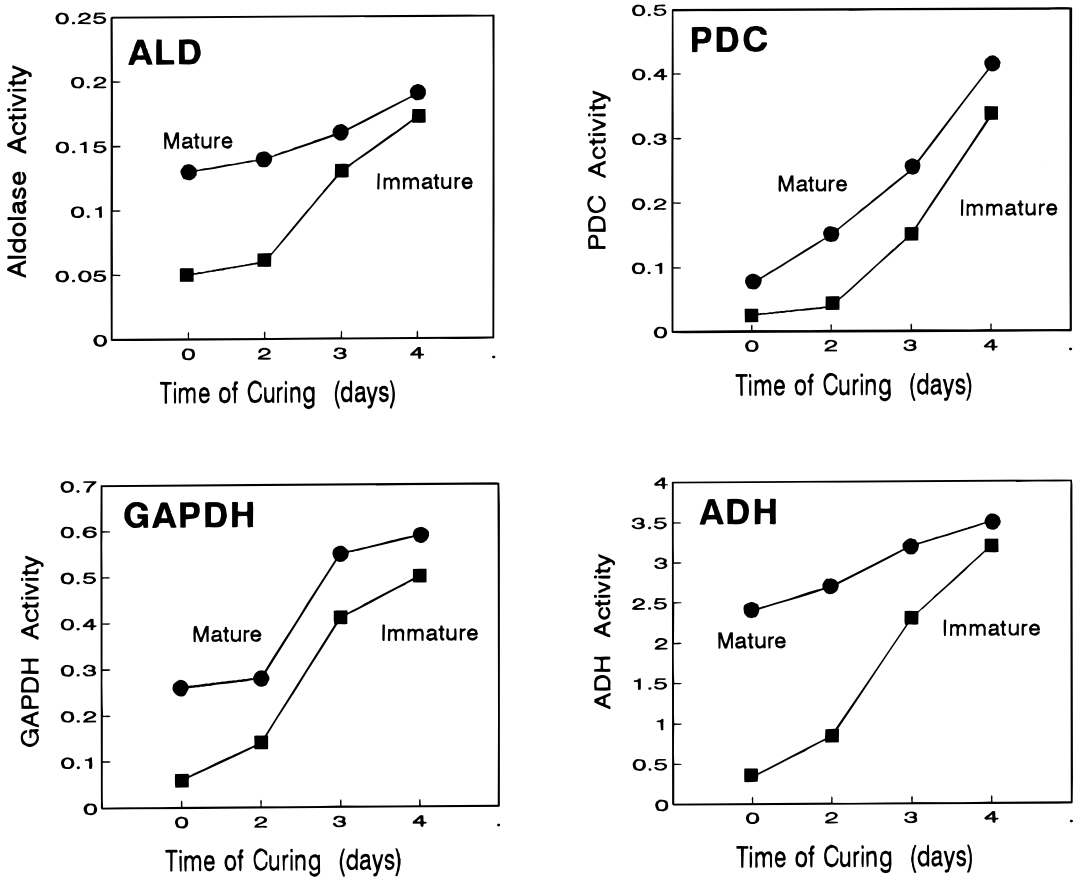

Figure 4. Activities [A_{570} min^{-1} (mg of protein)$^{-1}$] of ALD, GAPDH, PDC, and ADH at different times of curing (windrow drying). Mature and immature refer to seeds from black and yellow peanut pods.

ADH activity may not be influenced simply by one single enzyme such as PDC but, rather, by many enzymes in the glycolytic flux. Indeed, several studies (Schaaff et al., 1989; Cornish-Bowden, 1990; Pierce and Crawford, 1997) have shown that glycolytic flux may be modulated by many different glycolytic enzymes rather than by a single rate-limiting enzyme. The researchers claimed that it is very rare for any one enzyme in a metabolic system to merit the purely qualitative description of "rate-limiting".

The finding (i.e., increase of glycolytic enzymes) suggests that peanut maturation is a process associated with anaerobic conditions. Under these conditions, genes encoding ADH and other glycolytic enzymes [also called anaerobic proteins (ANPs)] have been shown to be rapidly turned on (Russell et al., 1990; Shelp et al., 1995; Millar and Dennis, 1996; Sachs et al., 1996). Evidence is accumulating that ANPs play a role in anaerobic survival. For instance, high rates of ethanol fermentation are known to be associated with high tolerance to anoxia (Millar and Dennis, 1996). Plants (mutants) that are null for ADH activity survive only a few hours of anoxia, compared to those that have ADH activity and survive up to 3 days of anaerobic treatment (Sachs et al., 1996). Moreover, the complexity of a plant's ANP pattern may correlate with its tolerance to anaerobic conditions. Russell et al. (1990) have shown that maize is more tolerant than soybeans and peas, presumably because maize has ≈20 ANPs, while soybeans and peas have 5−7 ANPs only.

Additionally, the anaerobic process is known to lead to a disturbance in the ionic balance of plant cells (Poovaiah and Reddy, 1993). For instance, cytosolic Ca^{2+} has been shown to increase in levels under anaerobic conditions (Subbaiah et al., 1994) and stabilize ADH (De Bolle et al., 1997). Also, a correlation between Ca^{2+} accumulation and induction of glycolytic enzymes has been established (Subbaiah et al., 1994). This suggests that Ca^{2+} may be a factor contributing to the increase of ADH and glycolytic enzymes during peanut maturation.

Increase of Glycolytic Enzymes during Peanut Curing. Activities of enzymes (ALD, GAPDH, PDC, and ADH) from mature and immature peanuts cured at different stages (0, 1, 2, 3, and 4 days) were determined. As shown in Figure 4, all of the enzymes from both groups of peanuts increased in activity during curing. This again supports our hypothesis that there is a relationship between the activities of ADH and preceding glycolytic enzymes in the alcohol fermentation pathway. Unlike mature peanuts, immature peanuts exhibited a tremendous increase of glycolytic enzymes during curing. An approximate 10-fold increase in enzyme activities (i.e., ratio of day 0 to day 4 activities) (except for ALD) was seen in immature peanuts, as compared to an approximate 2-fold increase (except for PDC) in mature peanuts. However, at the final curing stage (day 4), activities between mature and immature peanuts were shown to be almost the same.

Results show that regardless of their maturity state, cured peanuts have significantly higher enzyme activities than noncured peanuts [Figure 3, before and after curing (the latter refers to 4 days after curing)]. This suggests that curing has an inducing effect on enzyme activities. A possible explanation for this inducing effect is given as follows. Four consecutive events are thought to occur. First, curing may induce water stress to peanuts (stress may result from water loss in peanuts;

water content drops from 40% to 10% during curing). Second, water stress in turn induces anaerobic condition (Sprent and Gallercher, 1976; Umeda and Uchimiya, 1994; Shelp et al., 1995; Sachs et al., 1996). Third, glycolytic enzymes or ANPs are genetically expressed due to anoxia (Millar and Dennis, 1996; Sachs et al., 1996). Fourth, as a result, there is an increase of enzyme activities. In summary, the order of events leading to the increase of enzymes presumably is as follows: curing → water stress → anaerobic condition → gene expression → increased enzyme activities. On this basis, curing is considered a process associated with not only anaerobic conditions but also stresses. Stresses probably enhance the severity of the anaerobic condition, thus giving rise to higher enzyme activities in cured peanuts compared to noncured peanuts.

Two hypotheses have been suggested to explain the functions of anaerobic or stress-induced proteins (Schobert, 1977; McCue and Hanson, 1990). In the first, accumulation of the proteins leads to "osmotic adjustment" through mass action, which results in increased water retention. The second hypothesis considers anaerobic or stress proteins as "compatible solutes". In this function, they could replace water as a solute in biochemical reactions or associate with lipids/lipoproteins to prevent membrane disintegration, dissociation of protein complexes, or the inactivation of enzymes. In brief, the functions of anaerobic or stress-induced proteins are to confer stress tolerance, maintain homeostasis, and protect cells from anaerobic or stress damage (Millar and Dennis, 1996; Sachs et al., 1996). Seeds with a higher tolerance to stresses are known to have a higher ADH activity and better quality than those with low tolerance (McDonald, 1980; Van Toai et al., 1985). In one study (Mangolin et al., 1994), ADH is considered as a marker of stress conditions.

Conclusions. In this study, we developed color assays for several glycolytic enzymes (ALD, GAPDH, PDC, and ADH) from the alcohol fermentation pathway. We found an increase in activities of these enzymes in peanuts during peanut maturation and curing. The increased activities of these enzymes (those preceding ADH in the fermentation pathway) probably account for the substantial increase of ADH activity. This also provides evidence that peanut maturation and curing are processes associated with anaerobic conditions. The anaerobic conditions appear to be more severe in cured peanuts than in noncured peanuts on the basis of the fact that higher enzyme activities occur in the former. Water stress is thought to contribute to the severity of anaerobic conditions.

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