Enzyme-Amplified Microtiter Plate Assay for Ethanol: Its Application to the Detection of Apparent Ethanol in Peanuts

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A colorimetric microtiter plate assay amplified by aldehyde dehydrogenase, in the presence of alcohol dehydrogenase, nicotinamide adenine dinucleotide, diaphorase, and *p*-iodonitrotetrazolium violet was developed for detection of protein-bound apparent ethanol (i.e., ethanol and other primary alcohols) in peanuts of differing maturity and curing (stackpole) stages. Data showed that at each curing stage mature peanuts had a lower level of protein-bound ethanol than immature peanuts and that at each maturity stage the level of protein-bound ethanol decreased during curing. This change in the level of protein-bound ethanol suggests that peanut maturity and curing have an effect on the binding of ethanol to peanut proteins. The implication of this is that the extent of flavor binding might contribute to the consequent difference in flavors between mature/cured and immature/cured peanuts.

Keywords: *Ethanol; alcohol dehydrogenase; aldehyde dehydrogenase; enzyme amplification; microtiter plate assay; peanuts*

INTRODUCTION

Ethanol has been known to be the major constituent of volatiles in peanuts (Pattee et al., 1970) and is probably among the metabolites such as proteins (Chung et al., 1994) and carbohydrates (Vercellotti et al., 1994) that change in levels during peanut maturation and curing. As part of our investigation into the effect of curing on flavor volatiles, we determined ethanol (used as a representative of volatiles) from peanuts of differing maturity and curing stages. The current method for determination of ethanol is by use of gas chromatography (GC) (Chen and Johns, 1994) or is based on the measurement at 340 nm of nicotinamide adenine dinucleotide (reduced) (NADH) produced from the reduction of nicotinamide adenine dinucleotide (NAD^+) by ethanol in a reaction catalyzed by alcohol dehydrogenase (ADH) (Dominguez et al., 1993; Johansson et al., 1993). While the GC method is sensitive, it is costly in terms of equipment, sample preparation, and time. The method of detection using ADH and NAD^+ is more popular due to its simplicity and cost effectiveness. Recent developments in immunoassays have converted this method into a colorimetric assay, which, instead of measuring ethanol, is used as an amplifier to detect bioactive proteins (Weatherbee and Meyer, 1994) and hormones (Johannsson et al., 1986; Stanley et al., 1988). In the colorimetric method, additional reagents such as diaphorase (a flavin-bound enzyme) and *p*-iodonitrotetrazolium violet (INT-violet) (a substrate for diaphorase and a precursor of colored product) are needed besides ethanol, ADH, and NAD^+.

To date, there has been no report on the application of the above colorimetric method to the detection of ethanol in food products. To make it more cost effective and sensitive, we modified and increased the sensitivity of the colorimetric method by incorporating an aldehyde dehydrogenase (ALDH) (as an amplifier) into the assay system and converted the system into a microtiter plate assay. Using this ALDH-amplified microtiter plate assay, we determined apparent ethanol (i.e., ethanol and other primary alcohols) that binds to peanut proteins.

MATERIALS AND METHODS

Materials. Aldehyde dehydrogenase (100 units), alcohol dehydrogenase (30 000 units), diaphorase (20 units), nicotinamide adenine dinucleotide, *p*-iodonitrotetrazolium violet, ethylenediaminetetraacetic acid (EDTA), and ethanol were purchased from Sigma Chemical Co. (St. Louis, MO). Microtiter plates of Immulon I and plate reader M700 were purchased from Dynatech Laboratories, Inc. (Chantilly, VA). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL). Peanuts (*Arachis hypogaea* L. var. Florunner) were planted at the USDA-ARS National Peanut Research Laboratory (Dawson, GA), dug 160 days after planting, and subjected to stackpole curing for 0, 10, 20, and 40 days, respectively. After curing, the peanuts were sand blasted, shelled, sorted for maturity, and stored at −80 °C. Peanut maturity (e.g., "yellow" for immature; "orange" for medium immature; "brown" for medium mature; and "black" for mature) was determined according to the hull-scrape method (Williams and Drexler, 1981).

Optimization of ALDH-Amplified Assay for Ethanol. Various concentrations of NAD^+ (1.0–2.5 μM) and ADH (0.05–0.4 units/μL) and various ratios of ALDH:diaphorase (2.25–9) were respectively tested to determine the optimal concentrations for detection of ethanol. Here, the optimal concentration was defined as the highest concentration achieved before the curve reached a plateau. The resulting optimization gave the following protocol for assay of ethanol. To the well of a microtiter plate was added a mixture containing the buffer (36 μL), diluted ethanol standard (20 μL), ADH (3 μL; 0.2 units/μL), diaphorase (3 μL; 30 units/mL), and 1.98 mM INT-violet (28 μL; dissolved in buffer, heated at 65 °C, and cooled before use). This was followed by addition of 2.5 μM NAD^+ (50 μL; in 0.02 M sodium phosphate buffer containing 0.1 M KCl, pH 8) and ALDH (10 μL; 0.2 units/μL). The plate was then shaken for 30 s, and the absorbance was read at 15 min against a blank (that contained all of the reagents except ethanol) at 570 nm using a plate reader. The total volume for the assay was 150 μL.

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Preparation of Protein and TCA-Treated (Protein-Free) Extracts from Peanuts. Prior to extraction, peanuts of differing maturity and curing stages were deskinned and defatted by grinding in sequence with cold acetone and hexane. After air-drying, the resultant defatted peanut meals were stored at −80 °C or used for preparation of protein extract and trichloroacetic acid (TCA)-treated extract. The protein extract was prepared by stirring overnight at 4 °C 0.1 g of defatted peanut meals in 1.2 mL of 20 mM sodium phosphate buffer containing 0.1 M KCl, 2 mM EDTA, and 0.04% sodium azide, followed by centrifuging at 10 000 rpm for 15 min. The resultant supernatant (i.e., protein extract) was used for assay of total ethanol content. *Note*: Globular proteins, which have to be extracted with a high salt content, were not investigated because they make the sample cloudy and milky and consequently interfered with the color assay. The TCA-treated (protein-free) extract was prepared by further treatment of the protein extract (100 μL) with cold TCA (10 μL of a 50% solution) at a final concentration of 5%, followed by centrifuging at 10 000 rpm for 15 min and neutralizing the supernatant with 10 μL of 5 M Tris. The resultant TCA-treated extract was used for determination of free ethanol content.

Determination of Protein-Bound Ethanol in Peanuts. Protein-bound ethanol (expressed as nanomoles per milligram of protein) was determined as the difference between the levels of total and free ethanol, divided by the amount of proteins in the peanut extract. Levels of total and free ethanol were determined, respectively, from the protein and TCA-treated (protein-free) extracts using the optimized ALDH-amplifed assay as described above. Briefly, the protein or TCA-treated extract (20 μL) was added to the enzyme solution (in a well of a microtiter plate) consisting of 0.02 M sodium phosphate buffer (36 μL, pH 8; contained 0.1 M potassium chloride), ADH (3 μL; 0.2 unit/μL), diaphorase (3 μL; 30 units/mL), and 1.98 mM INT-violet (28 μL), followed by the addition of 2.5 μM NAD^+ (50 μL) and ALDH (10 μL; 0.2 units/μL). The reaction (i.e., color development) was allowed to proceed for 15 min before the absorbance was read at 570 nm using a plate reader against a blank consisting of the extract and reagents except ADH and ALDH. Protein concentration was determined using the BCA protein assay kit from Pierce.

A standard curve was made by adding 20 μL of a diluted ethanol standard (0.004−0.012%) and 20 μL of a heat-treated peanut extract to the assay system (i.e., ADH, diaphorase, INT-violet, ALDH, and NAD^+). The heat-treated extract was needed to provide the same matrix effect as the untreated peanut sample in the assay. The heat-treated extract was prepared by incubating the protein extract (in an Eppendorf tube with the lid open) in a hot water bath (95 °C) for 20 min to allow evaporation of residual ethanol and precipitation of proteins, followed by centrifugation at 10 000 rpm for 15 min. For conversion to molar concentration, the density (0.785) and molecular weight (46.07) of ethanol were used.

For recovery study, samples were spiked with or without ethanol standards, and then analyzed using the ALDH-amplified assay system. The percent recovery was calculated as $(E_1 - E_2) \times 100/E_3$, where E_1 was the concentration of ethanol in the spiked peanut sample, E_2 the concentration of ethanol in the nonspiked peanut sample, and E_3 the known spike concentration.

RESULTS AND DISCUSSION

Principle of ALDH-Amplified Microtiter Plate Assay. The mechanism for production of colored products in the ALDH-amplified or nonamplified assay is shown in Figure 1. In the nonamplified assay (Figure 1a), ethanol is oxidized in an ADH-catalyzed reaction to acetaldehyde by NAD^+, which in turn is reduced to NADH. The NADH thus formed takes part in another reaction catalyzed by diaphorase, in which INT-violet is reduced to a colored (red) product and NAD^+ is regenerated in the redox cycle. The color thus formed is read at 570 nm in a microtiter plate using a plate reader. In the ALDH-amplified assay, a higher sensitivity is achieved because two reactions (i.e., ADH in Figure 1a and ALDH in Figure 1b; both producing colored products) are involved instead of one in the nonamplified assay (i.e., Figure 1a). In other words, the ALDH-amplified assay is a combination of two reactions catalyzed by ADH and ALDH, respectively. In the ALDH-catalyzed reaction (Figure 1b), colored products are formed because NADH is produced through oxidation of acetaldehyde (a product derived from ethanol oxidation in Figure 1a) by NAD^+ and subsequently activates the diaphorase-catalyzed reaction (i.e., involving INT-violet) to produce colored products.

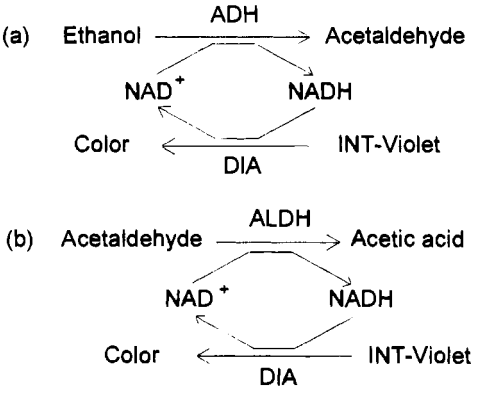

Figure 1. Principle of ALDH-amplified microtiter plate assay for ethanol. Amplified assay includes steps a and b. Nonamplified assay contains step a only. ADH, ALDH, DIA, and INT-violet stand for alcohol dehydrogenase, aldehyde dehydrogenase, diaphorase, and *p*-iodonitrotetrazolium violet, respectively.

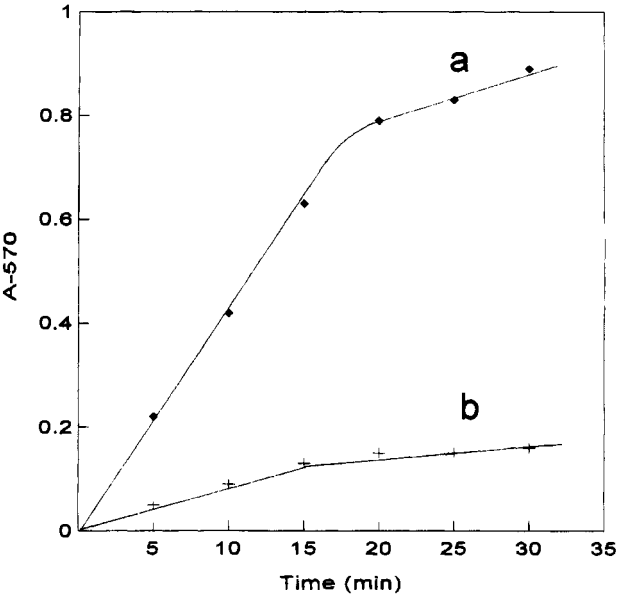

Figure 2. Comparison of ALDH-amplified and nonamplified microtiter plate assays for ethanol (0.012%). (a) Rate of color development from ALDH-amplified assay. (b) Rate of color development from nonamplified (no ALDH added) assay.

Comparison of ALDH-Amplified and Nonamplified Assays. To show the difference in the sensitivity between the ALDH-amplified and nonamplified assays, ethanol at a concentration of 0.012% (v/v) was tested using each of the assays. A profile of the rates of color development derived from the assays is shown in Figure 2. As shown, the ALDH-amplified assay had a steeper curve or a higher rate of color development than the nonamplified assay. Comparison of the slopes of the curves indicated that a 4-fold increase in the rate of color development was seen using the ALDH-amplified assay.

Optimal Assay Conditions. Factors affecting the rate of color development in the detection of ethanol

Table 1. Protein-Bound Ethanol[a] (Nanomoles per Milligram of Protein) in Peanuts of Differing Maturity and Curing Stages

	stage of stackpole curing			
maturity[b]	day 0	day 10	day 20	day 40
Ye	60.08	65.60	47.46	43.42
Or	52.35	54.88	38.74	35.20
Br	34.88	37.67	25.74	22.46
Bl	31.22	35.16	19.06	16.28

[a] Mean of duplicate determinations. [b] Yellow (Ye) for immature; orange (Or) and brown (Br) for medium; black (Bl) for mature.

include NAD^+, ADH, ALDH, and diaphorase. To determine the optimal conditions for the ALDH-amplified assay, various concentrations of the individual factor were tested. Figure 3 shows the effect of concentrations of NAD^+ and ADH and the ALDH:diaphorase ratio on the rate of color development, respectively. In each case, the rate of color development was shown to increase with the concentration. The optimal concentration (defined as the highest concentration before a plateau was reached) for each individual factor was established as follows: NAD^+, 2.5 μM; ADH, 0.2 unit/μL; and ratio of ALDH:diaphorase, 9:1. Under these assay conditions, the curve or rate of color development was linear within the first 15 min. A standard curve based on the measurement of ethanol at various concentrations (0.004–0.016%) was made (Figure 4). The detection limit for the assay was 0.004% ethanol. Using the standard curve, the mean percent recovery of ethanol determined from spiked and nonspiked peanut samples was found to be 97% ($n = 2$).

Determination of Protein-Bound Ethanol in Peanuts. Like soy proteins (Arai et al., 1970; Malcolmson et al., 1987) and bovine serum albumin (BSA) (Beyeler and Solms, 1974), peanut proteins bind flavor volatiles. Some of these volatiles may hardly be removed by heat treatment or solvent extraction due to their strong interaction with the proteins. Their existence in proteins is thought to be responsible in part for the desirable or undesirable flavors. Treatment of soy proteins with proteolytic enzymes is known to be effective in removing undesirable flavors or lowering their affinity for the proteins (Fujimaki et al., 1968; Noguchi et al., 1970). This suggests that flavor quality can be affected by changes in the affinity or binding of volatiles to proteins. We hypothesized that similar changes may occur during curing or maturation of peanuts and could be responsible in part for the difference in flavor quality between peanuts of differing maturity or curing stages. To demonstrate changes in the affinity of volatiles for proteins of peanuts during curing and maturation, we used apparent ethanol (i.e., ethanol and other primary alcohols) as the index or representative of volatiles and determined its level in protein-bound form at each curing and maturity stage, using the ALDH-amplified microtiter plate assay.

Table 1 shows the levels of protein-bound ethanol (nanomoles per milligram of protein) in peanuts at differing maturity (i.e., yellow, orange, brown, and black) and curing stages (i.e., stackpole curing for 0, 10, 20, and 40 days). Proteins from windrow drying were not investigated due to the problem of aggregation (Chung et al., 1994), which led to sample cloudiness. In determining the effect of stackpole curing on ethanol, it was noted (Table 1) that levels of protein-bound ethanol at each maturity stage were approximately the same between 0 and 10 days of curing. This indicates that during the first 10 days of curing there was no significant change in the binding of ethanol to proteins.

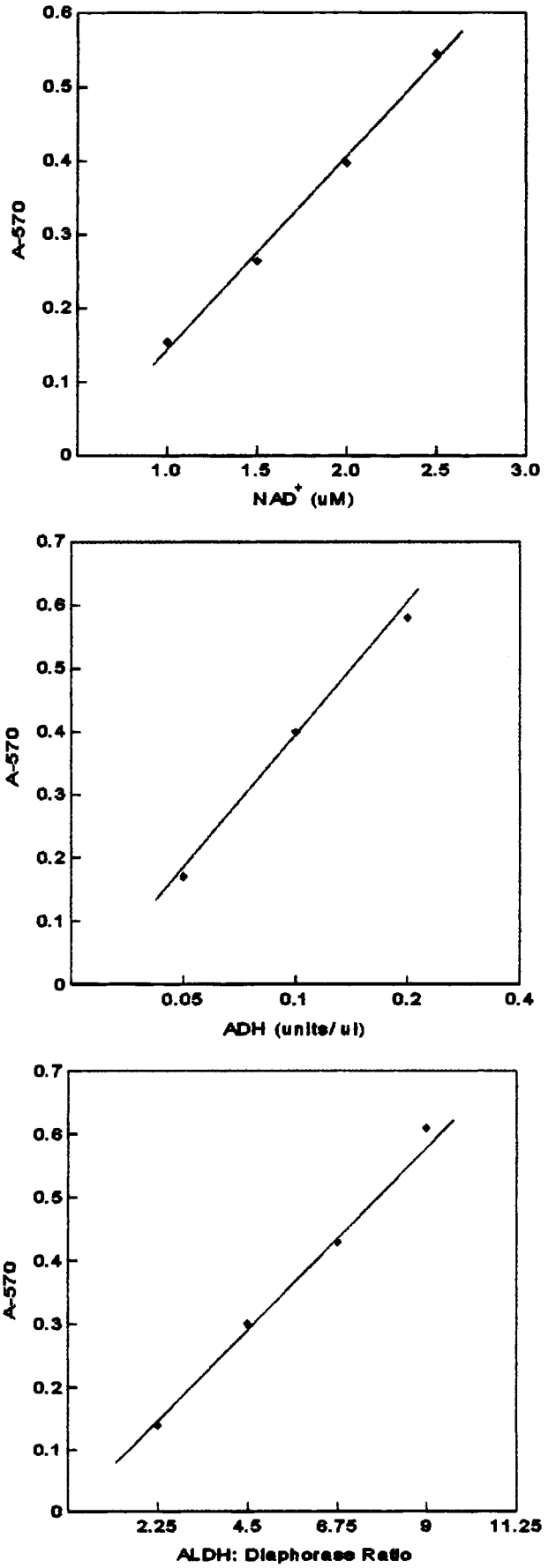

Figure 3. Effect of concentrations of NAD^+ and ADH and ALDH:diaphorase ratio on rate of color development in the ALDH-amplified microtiter plate assay.

However, as the curing process proceeded (i.e., 20–40 days), changes in levels of protein-bound ethanol were

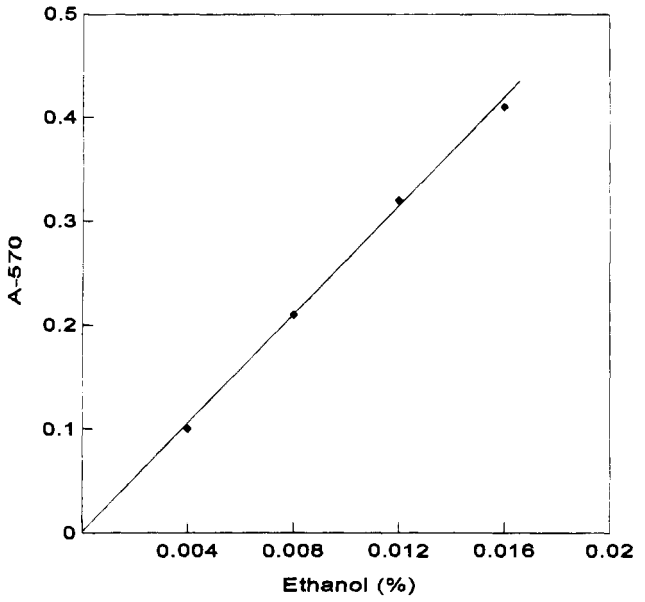

Figure 4. Standard curve of ethanol. Ethanol at the concentration indicated was determined using the optimized ALDH-amplified microtiter plate assay.

observed at each maturity stage and remained constant during 20- and 40-day curing. For instance, in the case of "yellow" (i.e., immature), the level of protein-bound ethanol at day 20 or 40 (curing) was approximately 45 nmol/mg of protein, as compared to 63 nmol/mg of protein at day 0 or 10. This decrease in the level of bound ethanol occurring at the final stages of curing (i.e., 20–40 days) and at each maturity stage is indicative of change in ethanol binding probably due to changes in the structure of proteins (Chung et al., 1994), which consequently lead to changes in their affinity for flavor volatiles (Damodaran and Kinsella, 1981).

The effect of peanut maturation on the binding of ethanol to proteins was also shown (Table 1). Levels of protein-bound ethanol were shown to decrease progressively with the maturity of peanuts. In other words, the more mature the peanuts, the lower the binding of ethanol to proteins. For instance, at day 0 (i.e., prior to curing), levels of protein-bound ethanol for yellow, orange, brown, and black were 60.08, 52.35, 34.88, and 31.22 nmol/mg of protein, respectively. In addition, changes in protein-bound ethanol were shown to occur also at each curing stage (i.e., 10-, 20-, or 40-day curing) (Table 1). This indicates that the binding of ethanol to peanut proteins can be affected by the maturation process.

In conclusion, a colorimetric ALDH-amplified micro assay was developed for determination of apparent ethanol (ethanol and other primary alcohols) in peanuts. The assay, conducted in a microtiter plate containing an ethanol standard, NAD^+, ADH, ALDH, diaphorase, and INT-violet, had a sensitivity 4-fold higher than a nonamplified assay. The detection limit for the assay was 0.004% ethanol, which in this study was used as a representative of flavor volatiles. Using the technique, changes in protein-bound ethanol during peanut curing or maturation were demonstrated. In this case, mature peanuts or peanuts cured for 20 or 40 days were found to have lower levels of protein-bound ethanol or volatiles. Whether this decrease in protein-bound volatiles is related to the enhancement of desirable flavor in mature or cured peanuts is not known. However, the fact that immature or uncured peanuts are low in flavor quality and the present finding that they are high in protein-bound volatiles suggest that there may be a relationship between peanut flavor quality and the extent of flavor binding to proteins.

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