A competitive enzyme-linked immunosorbent assay (ELISA) was developed to quantify endogenous acetaldehyde-protein adducts (APAs) produced in plant seeds at low acetaldehyde concentrations without exogenous reducing agents. The key point of this technique is the use of a gelatin-acetaldehyde adduct, which is synthesized under 1 mM acetaldehyde and 10 mM NaCNBH₃ to pre-coat plate wells to obtain the proper binding parameters for the quantification of APA in seed proteins. Compared with the traditional, direct ELISA method, the competitive one has higher sensitivity and less background. Using competitive ELISA, we determined the accumulation of endogenous APAs in seeds in relation to the loss of seed viability. Lettuce seeds were exposed to 2 mM gaseous acetaldehyde during storage for 30 or 45 d; the relative humidity and temperature of storage were studied independently. Viability decreased only in acetaldehyde-treated seeds, as either the temperature or the relative humidity increased. A loss in viability was accompanied by an increase in the accumulation of APA. The APA content also increased as viability decreased in five species of seeds, which were aged naturally without exposure to acetaldehyde. It is suggested that the modification of functional seed proteins with endogenously evolved acetaldehyde may be an important cause of seed aging.
temperature experiments, lettuce seeds were first equilibrated at 53% RH and 23°C for 2 weeks and then stored at 15 to 35°C for 45 d with or without 2 mm gaseous acetaldehyde. The control of RH and the application of acetaldehyde have been described previously (Zhang et al., 1994).

**Synthesis of APA**

The acetaldehyde-BSA adduct was synthesized by combining 1 mm acetaldehyde and 10 mm NaCNBH$_3$ with 2 mg mL$^{-1}$ BSA in 50 mm carbonate buffer (pH 9.5) for the immunization of rabbits. The solution was sterilized by passing it through a 0.2-µm filter and incubating it at 23°C for 3 d. Other APAs were synthesized in 2 mg mL$^{-1}$ protein. Acetaldehyde-gelatin adducts were synthesized by combining gelatin with 0.5 to 16 mM acetaldehyde in the protein. Acetaldehyde-gelatin adducts were synthesized and incubated at 23°C in darkness. Germinated seeds were counted and discarded each day. Tests were continued for 10 d or more.

**Extraction of Seed Proteins and Seed Viability Test**

Two grams of seeds (lettuce or carrot) or 0.2 to 0.5 g of axes (soybean, pea, or cocklebur) were homogenized in 15 mL of PBS and centrifuged at 15,000 g for 10 min. The supernatant was precipitated using 80% saturated ammonium sulfate and dialyzed against PBS buffer. No reducing agent was used during the extraction or immunooassay of the seed proteins. The proteins were quantified by the Bradford method (Bradford, 1976). The protein solution was diluted to 2.5 to 10 mg mL$^{-1}$ in PBS and mixed with the antibody for assay of APA with the competitive ELISA. The protein concentration of the antigen-antibody solution was adjusted to 20 mg mL$^{-1}$ with ovalbumin. A 50-µL antibody-antigen solution was incubated in the pre-coated plate wells for 1 h at 25°C after the wells were blocked with 0.5% gelatin and washed with PBS. After the plate wells were washed with PBS-Tween, 80 µL of the second antibody (same as in direct ELISA) was added to each well and incubated for 1 h at 25°C. The plate wells were washed with PBS-Tween and rinsed with distilled water, and 80 µL of the alkaline phosphatase substrate was added to each well and reacted at 25°C for 30 min. The reaction was stopped by the addition of 80 µL of 2.5 N KOH. The absorbance in the presence of competitive antigen (standard [AOA] or sample [seed proteins]), and $A_B$ is the background absorbance determined in the presence of excess antigen (AOA10). The protein concentration of the antigen-antibody solution was adjusted to 20 mg mL$^{-1}$ with ovalbumin as described above. The standard lines were obtained by the logit-transformation of $B/B_c$% as follows:

$$\logit\left(\frac{B}{B_c}\right) = \ln\left(\frac{B/B_c}{1 - B/B_c}\right).$$

**RESULTS**

**Direct ELISA for the Assay of APA**

The direct ELISA was tested for the assay of APA in a model system in which ovalbumin was used as a carrier protein for the synthesis of AOA at acetaldehyde concent-
tractions from 0 to 1 mM (Israel et al., 1986; Perata et al., 1992). It was possible by the direct method to distinguish AOA1 from other AOAAs produced at lower acetaldehyde concentrations (Fig. 1). On the other hand, it was difficult to differentiate AOA0.01 from the control (AOA0) by direct ELISA. Based on our previous paper (Zhang et al., 1994), the concentrations of endogenous acetaldehyde in various seeds are about 1 to 100 μM. Therefore, we thought it was inadequate to use direct ELISA for comparing the amounts of endogenous APA between vigorous and aged seeds.

Competitive ELISA for the Quantification of APA

We developed a new, competitive ELISA system for the quantification of APA. Gelatin was used as a carrier protein for the antigen to pre-coat the immunoplate wells, because the acetaldehyde-gelatin adducts gave the lowest background values compared with other proteins (data not shown).

The optimal concentration of acetaldehyde to prepare acetaldehyde-gelatin adducts in the presence of 10 mM NaCNBH₃ was determined to achieve the highest sensitivity for the competitive ELISA. First, the suitable concentrations of the antibody were determined by a direct ELISA method (titer test) when plates were coated with various acetaldehyde-gelatin adducts (Fig. 2). If 1.2 to 1.4 of A₄₉₅ was selected as the maximal absorbance (A₄₉₅), the suitable concentration of antibody should be 1/20, 1/60, 1/120, 1/200, 1/300, or 1/400 when the plate was coated with acetaldehyde-gelatin adducts synthesized at 0.5, 1, 2, 4, 8, or 16 mM acetaldehyde, respectively. Thereafter, 2 mg mL⁻¹ AOA10 and AOA0.1 were used as competitive antigens and mixed with the antibody at a suitable concentration, determined in Figure 2, to test the binding ratio when the plate was pre-coated with various acetaldehyde-gelatin adducts (Fig. 3). AOA10 gave almost 0 of B/B₀% under all pre-coating conditions. However, AOA0.1 failed to prevent the antibody from binding with acetaldehyde-gelatin adduct synthesized at more than 4 mM acetaldehyde. Efficient competition was obtained only when the plate well was pre-coated with acetaldehyde-gelatin adduct prepared at 0.5 or 1 mM acetaldehyde. Therefore, the acetaldehyde-gelatin adduct prepared at 1 mM acetaldehyde was adopted for pre-coating immunoplate wells in the following experiments.

AOA1, AOA0.1, AOA0.01, and AOA0 were used as the standards to draw the standard lines for the quantitative analysis of endogenous APA. As shown in Figure 4, logit (B/B₀%) had a linear relationship with the log concentration of the competitive antigens (AOAs), especially when B/B₀% was below 70%. AOA1 was used as a standard for the quantification of APA in seed proteins in the following experiments. According to this method, even AOA prepared with the acetaldehyde concentration as low as 0.01 mM could be detected. However, ovalbumin reacted only

Figure 1. Direct ELISA of acetaldehyde-ovalbumin adducts prepared at 1, 0.1, 0.01, and 0 mM acetaldehyde in the presence of NaCNBH₃. Error bars indicate ±SE (n = 6).

Figure 2. Titer test of an antiserum against APA. The immunoplate was coated with the acetaldehyde-gelatin adduct prepared at 0.5 to 16 mM acetaldehyde in the presence of NaCNBH₃. Data are means of six determinations.

Figure 3. Competitive ELISA for determination of an acetaldehyde concentration that is suitable for the synthesis of the acetaldehyde-gelatin adduct by which the immunoplate well was pre-coated. AOA10 (●) and AOA0.1 (○) were used as competitive antigens. Error bars indicate ±SE (n = 6).
with 10 mM NaCNBH₃ in the absence of acetaldehyde also caused a low, competitive reaction (Fig. 4).

The competitive method was compared with the direct method for detecting APA in proteins extracted from cocklebur seeds (Table I). With the direct ELISA, little difference in absorbance was detected between acetaldehyde-treated and nontreated seeds, although both caused higher absorbances. Background was shown in the direct ELISA when the anti-APA antibody was blocked by AOA10. On the other hand, the competitive method succeeded in showing the significant difference of ELISA absorbance between acetaldehyde-treated and control seeds. APA amounts were also calculated from the standard line of AOA1, as shown in Figure 4. These results clearly show that the competitive ELISA is an adequate method to detect APA from seed proteins.

The Accumulation of APA and the Loss of Viability in Lettuce Seeds Stored with Gaseous Acetaldehyde

To demonstrate the possible involvement of acetaldehyde in seed aging, the relationship between the loss of viability and the accumulation of APA in dry lettuce seeds exposed to 2 mM gaseous acetaldehyde was examined in relation to RH (Fig. 5) and temperature (Fig. 6). Without exposure to acetaldehyde, seed viabilities in all cases were 95% or more, and APA content in the PBS-soluble proteins remained at low levels of 15 to 20 mg AOA1 g⁻¹ protein. When the seeds were subjected to acetaldehyde, however, the loss of viability and the accumulation of APA proceeded concomitantly with increasing RH, in spite of only a 30-d storage (Fig. 5). Even at 12% RH, the APA content of seeds stored with acetaldehyde was about 2-fold higher than the control, although their viability was relatively unchanged. In the presence of acetaldehyde the APA content increased further and seed viability declined as RH was elevated above 33%. At 75% RH, the APA content reached 125 mg AOA1 g⁻¹ protein, whereas the seed viability decreased to 7%. The production of APA also increased with increasing temperature during storage for 45 d (Fig. 6). At −15°C, the APA content in acetaldehyde-treated seeds was the same as that of the control, but it increased sharply when the storage temperature was elevated above 5°C. Seed viability did not change at −15°C or −5°C even if exposed to acetaldehyde, but it was rapidly lost above 5°C. At 35°C, the APA content increased to 287 mg AOA1 g⁻¹ protein, where all seeds completely lost viability.

The Relationship between the Accumulation of Endogenous APA and the Loss of Seed Viability

The relationship between the loss of seed viability and the accumulation of endogenous APA in PBS-soluble proteins is shown in Figure 7. Generally, the typical long-lived pea seeds contained less APA than did soybean and carrot seeds. However, the typical short-lived lettuce seeds contained more APA than did most other seeds tested. The accumulation of APA during storage was shown to be concomitant with the loss of seed viability for the accretion of APA in different seeds.

Table I. Comparison of the sensitivity of the APA assay between the direct and the competitive ELISA methods using cocklebur seeds stored with (+) or without (−) gaseous acetaldehyde

<table>
<thead>
<tr>
<th>Acetaldehyde</th>
<th>A₄₅₀ (Direct ELISA)</th>
<th>Competitive ELISA</th>
<th>AOA amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>Background</td>
<td>B/B₀,%</td>
<td>mg AOA1 g⁻¹ protein</td>
</tr>
<tr>
<td>−</td>
<td>0.732 ± 0.01</td>
<td>0.448 ± 0.01</td>
<td>63.9 ± 1.4</td>
</tr>
<tr>
<td>+</td>
<td>0.757 ± 0.02</td>
<td>0.441 ± 0.01</td>
<td>42.8 ± 1.5</td>
</tr>
</tbody>
</table>

Figure 4. Linear relationship between logit (B/B₀,%.) and concentrations of AOA as competitive antigens in the competitive ELISA. AOA1 (O), AOA0.1 ( ), AOA0.01 ( ), and AOA0 ( ) were used as competitive antigens. Data are means of six determinations.

Figure 5. Changes in viability and APA in dry lettuce seeds stored at 23°C under different RHs for 30 d with or without 2 mM gaseous acetaldehyde. The amount of APA was assayed by competitive ELISA and calculated on the basis of the standard line of AOA1 in Figure 4. Error bars indicate ±SE (n = 6) for APA; n = 3 for viability.

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seeds, which had relatively short longevities. In all tested seeds the APA content always increased with the loss of seed viability. In lettuce the seeds with 99% viability contained 16 mg AOA1 g⁻¹ protein. The APA content increased to 45 or 67 mg AOA1 g⁻¹ protein as the viability decreased to 83 or 30%, respectively (Fig. 7). In the same seeds exposed to 2 mM acetaldehyde (Figs. 5 and 6), the viabilities decreased to about 80 or 30%, whereas the APA content increased to 50 to 60 or 100 to 110 mg AOA1 g⁻¹ protein, respectively. Thus, it is suggested that the accumulation of APA may be an important cause of seed aging.

DISCUSSION

Quantification of APA by the Competitive ELISA

Acetaldehyde is an endogenous component evolved by plant seeds (Zhang et al., 1993), which suggests that APA may be present in both vigorous and aged seeds. In Table I the direct ELISA could not detect differences between acetaldehyde-treated and nontreated seeds. The main reason is that the direct ELISA showed fewer differences among different antigens than the competitive method, especially when the antigens were produced under low concentrations of acetaldehyde. In a model system, for example, the difference between AOA0.1 and AOA0.01 was 2.2-fold in the direct system (Fig. 1) but became 5.2-fold in the competitive system (Fig. 4). Moreover, high background color development in the direct ELISA also limited the sensitivity of the APA assay from the seed proteins (Table I).

In Figure 3, 2 mg mL⁻¹ AOA10 resulted in very low B/B₅₀ values under all pre-coating conditions. AOA10, which included sufficient epitopes linked tightly with the antibody, prevented the antibody from binding to pre-coated acetaldehyde-gelatin adduct in the plate wells. On the other hand, B/B₅₀ of AOA0.1 was much greater than that of AOA10, because AOA0.1 had fewer epitopes than AOA10. It is interesting that AOA0.1 failed to prevent the antibody from binding with the acetaldehyde-gelatin adduct synthesized with acetaldehyde of 4 mM or more but brought about an efficient competition when the plate well was pre-coated with acetaldehyde-gelatin adduct prepared at 0.5 or 1 mM acetaldehyde (Fig. 3). It is conceivable that the antibody may bind much tighter with pre-coated acetaldehyde-gelatin adducts prepared at high concentrations of acetaldehyde than with AOA0.1. Thus, the antibody would have absolute superiority to link with the pre-coated acetaldehyde-gelatin adduct. In this case, competitive binding with the antibody between AOA0.1 in solution and acetaldehyde-gelatin adduct in the immunoplate well did not occur. On the other hand, the affinity of the antibody with AOA1 would be comparable to that with the acetaldehyde-gelatin adduct prepared at 0.5 or 1 mM acetaldehyde. Thus, the antibody could bind simultaneously with both the acetaldehyde-gelatin adduct on the plate well and AOA0.1 in the solution. As a result, the antigen in the solution can be quantified using these conditions on the basis of the relative binding parameter. The proper concentration of the antibody is 1/20 antiserum when the plate well is coated with the acetaldehyde-gelatin adduct prepared at 0.5 mM acetaldehyde (Fig. 2). The antibody concentration would be too high for ELISA analysis. Here, acetaldehyde-gelatin adduct prepared at 1 mM acetaldehyde was adopted to pre-coat immunoplate wells in our experimental system. We suggest that the proper pre-coating antigen and antibody concentration should be screened through the protocol described in Figures 2 to 4 when the antibodies against APA are newly produced.

Theoretically, the specificity of the competitive ELISA is better than that of the direct ELISA. Seed proteins assayed by the direct ELISA show a high background color (Table I). In the direct system the plate well was coated by seed proteins of numerous kinds. The polyclonal antibody against APA includes many kinds of antibodies that may bind with seed proteins coated on the plate well and cause background. Moreover, endogenous alkaline phosphatase in seed protein extracts may be coated on the plate well and...
also cause background color development. In the competitive system, however, the plate well was coated by an acetaldehyde-gelatin adduct (pre-coating) and then saturated by gelatin (blocking). Thereafter, the mixture solution of antiseraum and seed proteins was added to the plate well. Seed proteins absorbed on the plate wells, which may cause background of assay, should be greatly decreased. Gelatin itself has a very low background (data not shown), and thus, it becomes obvious that the competitive ELISA is an effective method for the assay of endogenous APA in dry plant seeds.

**Seed Aging in Relation to the Accumulation of APA**

Seed aging has generally been regarded as a result of oxidative processes in seeds, but seed aging still proceeds in O$_2$-deficient atmospheres (Priestley, 1986). Here we propose a possible mechanism of seed aging induced by the modification of seed proteins with endogenous acetaldehyde. The production of APAs increased with increasing RH and temperature (Figs. 5 and 6), which also accelerate the process of seed aging during storage.

Stable APAs are produced through the formation and stabilization of a Schiff’s base (Fig. 8). In water solution a Schiff’s base may be hydrolyzed if it cannot be stabilized by reducing agents. However, the Schiff’s base may be relatively stable in dry seeds because of the absence of free water. Therefore, the first product of APA in dry seeds may accumulate until reducing agents become available. The modification of seed proteins would cause proteins to denature, which may cause seed aging. In some in vitro experiments, it has been demonstrated that acetaldehyde attacks Lys residues (Tuma et al., 1987) and causes the loss of the catalytic activity of some enzymes (Mauch, et al., 1986).

In this experiment only the final, stable adducts (reduced Schiff’s base) would be caught, whereas Schiff’s bases may be hydrolyzed during the extraction of seed proteins. Although the Schiff’s base is produced through a dehydration process, the accumulation of stable APA was increased with increasing RH (Fig. 5). A possible reason may be that the stabilization of Schiff’s base easily occurs in seeds that are stored at high RHs, probably through the predominant supply of reducing agents and the increased movement of molecules in seeds stored at high RHs. It has been suggested that l-ascorbate (Tuma et al., 1984) and H$^+$, augmented by the oxidation of ethanol (Sorrell and Tuma, 1985), are the reducing agents for the formation of stable adducts in humans. The production system of endogenous reducing agents in dry seed is a future problem.

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**LITERATURE CITED**


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