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A Competitive Enzyme-Linked Immunosorbent Assay to Quantify Acetaldehyde-Protein Adducts That Accumulate in Dry Seeds during Aging[1]

Ming Zhang[2,3], Shigeyuki Nagata, Kae Miyazawa, Haruhisa Kikuchi, and Yohji Esashi*

Laboratory of Environmental Biology, Botanical Garden, Faculty of Science, North Campus, Tohoku University, Kawauchi, Aobaku, Sendai 980–77, Japan (M.Z., K.M., H.K., Y.E.); and Kitasato Medical Center, Kitamotoshi, Arai 364, Japan (S.N.)

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 $NaCNBH_3$, 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.

Acetaldehyde is an intermediate of alcoholic fermentation in plants. The toxic effects of acetaldehyde may arise from its nonenzymatic binding with proteins (Gaines et al., 1977; Stevens et al., 1981; Donohue et al., 1983) and DNA (Restow and Obe, 1978; Fraenkel-Conrat and Singer, 1988). Israel et al. (1986) found that the immunization of mice with acetaldehyde conjugated to human plasma proteins results in the production of a polyclonal antibody, which reacts with various APAs. The production of acetaldehyde in tissue culture is thought to cause cell damage (Perata et al., 1988; Perata and Alpi, 1991). Perata et al. (1992) tried to detect APA from suspension-cultured carrot cells using a direct ELISA method. However, APAs were detected only when both ethanol and a reducing agent ($NaCNBH_3$) were supplied in the culture medium, and only trace amounts of endogenous APA were detected without the presence of a reducing agent.

Among the many kinds of volatile compounds that have evolved from dry seeds, acetaldehyde and ethanol are common components in the various seed species (Zhang et al., 1993). Acetaldehyde is effective in causing the rapid deterioration of seeds when it is applied as a gas to seeds during storage periods (Zhang et al., 1994). We proposed a hypothesis that seed aging may be caused by a modification of seed proteins by endogenously produced acetaldehyde (Zhang et al., 1995). In this paper we report the detection of endogenous APA from five species of dry seeds using a sensitive, competitive ELISA technique and demonstrate the increased accumulation of APA in aged seeds.

MATERIALS AND METHODS

Seed Materials and Storage Methods

Seeds with high viability (90% or more) from lettuce (*Lactuca sativa* L. cv Aochirimen), pea (*Pisum sativum* L. cv Alaska), cocklebur (*Xanthium pennsylvanicum* Wallr.), soybean (*Glycine max* Merr. cv Miyagishirome), and carrot (*Daucus carota* L. cv Natsumaki 5 Sun) were harvested in 1993 and stored at −3.5°C until use. Seeds with intermediate viability (50-83%) were harvested in 1977 (pea), 1987 (cocklebur and lettuce), 1988 (carrot), or 1989 (soybean). The seeds were stored in sealed containers at about 50% RH and at room temperature, except pea seeds, which were stored at about 15°C. The seeds with low viability (12–37%) were seeds that had been harvested in 1993, which were stored in sealed containers at 75% RH at 23°C for 7 months before use.

Cocklebur seeds were stored with 1 mM gaseous acetaldehyde at 23°C and 53% RH for 4 months. For RH experiments, lettuce seeds were stored at 23°C and 12 to 75% RH for 30 d with or without 2 mM gaseous acetaldehyde. For

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[2] M.Z. was supported by a Japanese government scholarship.

[3] Present address: U.S. Department of Agriculture-Agricultural Research Service National Seed Storage Lab, 1111 South Mason Street, Fort Collins, CO 80521.

* Corresponding author; fax 81–22–263–9279.

Abbreviations: AOA, acetaldehyde-ovalbumin adduct; AOA10, AOA1, AOA0.1, AOA0.01, and AOA0, AOA that is synthesized at 10, 1, 0.1, 0.01, or 0 mM acetaldehyde, respectively, in the presence of 10 mM $NaCNBH_3$; APA, acetaldehyde-protein adduct.

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 presence of 10 mM $NaCNBH_3$ in 50 mM carbonate buffer (pH 9.5) at 4°C for 1 week to pre-coat the immunoplate wells. AOAs were synthesized with 0, 0.01, 0.1, 1, or 10 mM acetaldehyde under the same conditions as the synthesis of the acetaldehyde-gelatin adducts. AOA0, AOA0.01, AOA0.1, and AOA1 were used as the standards. AOA10 was used as an excess antigen for blocking antibodies. All of the solutions were dialyzed against PBS (including 137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH 7.4) after the synthesis of these adducts and were concentrated by ultrafiltration.

Direct ELISA

Anti-APA antibody was produced by subcutaneous injections of acetaldehyde-BSA adducts into rabbits (Niemela and Israel, 1992).

ELISA was carried out at 25°C. Immunoplate wells were coated with 100 μL of prepared APA (AOAs or acetaldehyde-gelatin adducts) or seed proteins (100 μg mL^{-1}) for 2 h. The plate wells were then blocked by 0.5% gelatin in PBS for 2 h. Anti-APA antibody was diluted to a suitable concentration in blocking solution (0.5% gelatin-PBS) and incubated in the plate wells for 1 h after being washed with PBS. Thereafter, the plate wells were washed again with PBS-Tween (0.05% Tween 20 in PBS) and 100 μL of second antibody (goat anti-rabbit immunoglobulin bound to alkaline phosphatase), which was diluted in blocking solution, added to each well, and incubated for 1 h. Then, plate wells were washed with PBS-Tween and rinsed with distilled water, and 100 μL of alkaline phosphatase substrate (*p*-nitrophenyl phosphate, disodium) was added to each well and reacted for 10 to 30 min. The reaction was stopped by the addition of 100 μL of 2.5 N KOH. The developed color was assayed at 405 nm.

Competitive ELISA

Immunoplate wells were pre-coated at 25°C for 2 h with acetaldehyde-gelatin adducts (100 μg mL^{-1}), which were prepared at 1 mM acetaldehyde and 10 mM $NaCNBH_3$. Anti-APA antibody was diluted to a suitable concentration in PBS and mixed with the competitive antigen (standard [AOA] or sample [seed proteins]) at 4°C for 16 h. The protein concentration in 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 binding parameter ratio (B/B_0%) was calculated according to the method of Weiler et al. (1981):

$$B/B_0\% = \frac{A_S - A_B}{A_M - A_B} \times 100.$$

where A_M is the maximal A_{405} in 20 mg mL^{-1} ovalbumin-PBS solution without competitive antigen, A_S is 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_0% as follows:

$$\text{Logit}(B/B_0\%) = \ln\left(\frac{B/B_0\%}{100 - B/B_0\%}\right).$$

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 immunoassay 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. The seed protein concentration that produced 20 to 70% B/B_0% in the competitive ELISA was used for calculating the endogenous APA content according to the standard line of AOA1, because a stable, linear relationship occurs in this range of B/B_0%.

Seed viability was expressed as radicle emergence percentage. The germination test was performed in triplicate at 23°C in darkness. Germinated seeds were counted and discarded each day. Tests were continued for 10 d or more.

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 AOAs at acetaldehyde concen-

trations 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 AOAs 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 $NaCNBH_3$ 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 A_{405} was selected as the maximal absorbance (A_M), 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/B_0% 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/B_0%) had a linear relationship with the log concentration of the competitive antigens (AOAs), especially when B/B_0% 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

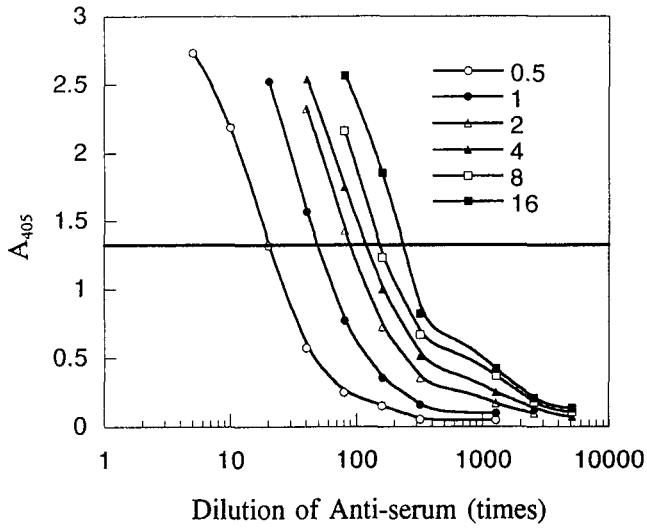

Figure 2. Titer test of an antiserum against APA. The immunoplate was coated with the acetaldehyde-gelatin adduct prepared at 0.5 to 16 mM acetaldehyde in the presence of $NaCNBH_3$. Data are means of six determinations.

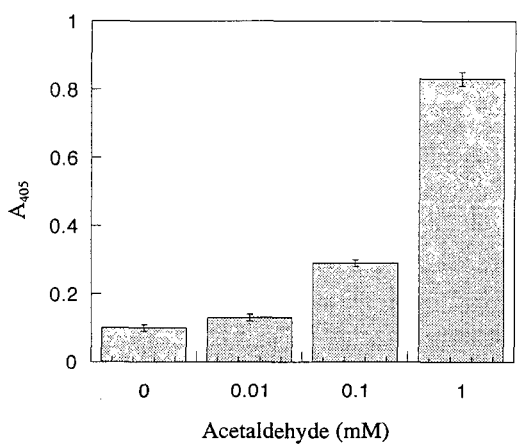

Figure 1. Direct ELISA of acetaldehyde-ovalbumin adducts prepared at 1, 0.1, 0.01, and 0 mM acetaldehyde in the presence of $NaCNBH_3$. Error bars indicate ±SE (n = 6).

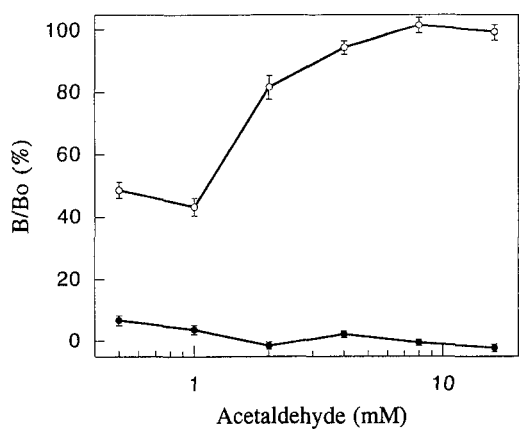

Figure 3. Competitive ELISA for determination of an acetaldehyde concentration that is suitable for the synthesis of the acetaldehyde-gelatin adduct by which the immunoplate well was pre-coated. AOA10 (●) and AOA0.1 (○) were used as competitive antigens. Error bars indicate ±SE (n = 6).

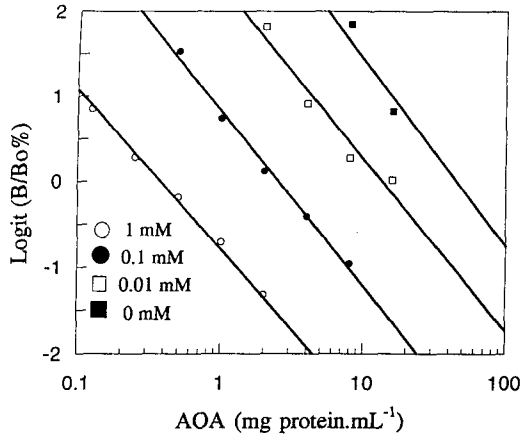

Figure 4. Linear relationship between logit (B/B_0%) and concentrations of AOA as competitive antigens in the competitive ELISA. AOA1 (○), AOA0.1 (●), AOA0.01 (□), and AOA0 (■) were used as competitive antigens. Data are means of six determinations.

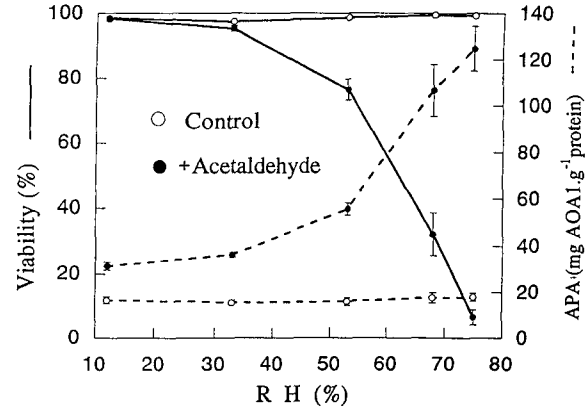

Figure 5. Changes in viability and APA in dry lettuce seeds stored at 23°C under different RHs for 30 d with or without 2 mM gaseous acetaldehyde. The amount of APA was assayed by competitive ELISA and calculated on the basis of the standard line of AOA1 in Figure 4. Error bars indicate ±SE (n = 6) for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

with 10 mM $NaCNBH_3$ 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° 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

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*

Background of the direct ELISA was shown by blocking the antibody with AOA10. Seed proteins (10 mg mL^{-1}) were used for the competitive ELISA. APA amounts in the competitive ELISA were calculated on the basis of the standard line of AOA1 (Logit[B/B_0%] = −1.865 log[APA mg mL^{-1}] − 0.792) in Figure 4 (A_M = 1.403; A_B = 0.045). Data are means ± SE (n = 6).

Acetaldehyde	A_{405} (Direct ELISA)		Competitive ELISA		
	Total	Background	A_{405}	B/B_0 %	APA amount
1 mM					*mg AOA1 g^{-1} protein*
−	0.732 ± 0.01	0.448 ± 0.01	0.913 ± 0.02	63.9 ± 1.4	18.8 ± 1.4
+	0.757 ± 0.02	0.441 ± 0.01	0.626 ± 0.02	42.8 ± 1.5	54.6 ± 3.7

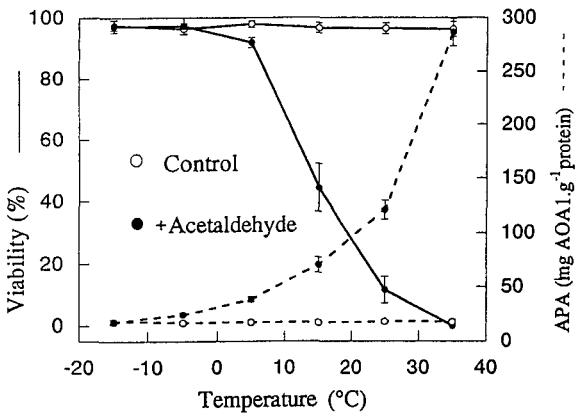

Figure 6. Changes in viability and APA content in dry seeds stored at 53% RH at different temperatures for 45 d with or without 2 mM gaseous acetaldehyde. APA was quantified as in Figure 5. Error bars indicate ±SE (n = 6) for APA; n = 3 for viability.

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/B_o% 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/B_o% 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 protocal 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

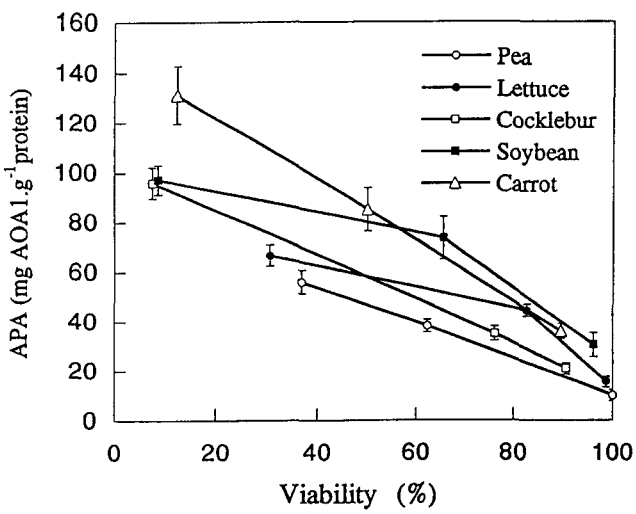

Figure 7. Loss of viability and accumulation of APA in the seeds of pea, lettuce, cocklebur, soybean, and carrot. APA was quantified as in Figure 5. Error bars indicate ±SE (n = 6).

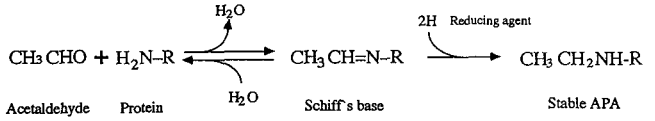

Figure 8. The process of APA production by reactions of the proteins with acetaldehyde and a reducing agent.

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 antiserum 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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