Enzyme Immunoassay (EIA) of Endogenous Cytokinins in *Citrus*

Gary A. Barthe and Ivan Stewart*

Two sensitive enzyme immunoassays (EIA) for the determination of zeatin riboside (ZR), isopentenyladenosine (IPA), and closely related cytokinins are described. ZR and IPA were conjugated to bovine serum albumin and used as immunogen for the production of two antisera. The assays utilized alkaline phosphatase–cytokinin tracers which were stable for at least 8 months. Only N^6 substituted purines were detected; common naturally occurring purine bases and nucleosides do not interfere. As little as 50 pg of cytokinin could be detected by this method. For the analysis of cytokinins in plant tissues, extracts were fractionated by high-performance liquid chromatography (HPLC) and quantified by EIA. ZR, IPA, and isopentenyladenine (IP) were detected in Valencia orange and Ruby Red grapefruit flower buds. The levels of all three compounds were found to increase during maturation of Valencia orange blossoms.

The study of endogenous cytokinins has, to a large extent, been limited by the lack of suitable analytical methods. Bioassays have primarily been used for quantification of these compounds (Letham, 1978). However, these assays are relatively unspecific and often difficult to reproduce (Reeve and Crozier, 1980). Analytical methods, such as high-performance liquid chromatography (HPLC) (Heindl et al., 1982), gas chromatography (GC) (Kemp et al., 1982; Stafford and Corse, 1982), and gas chromatography–mass spectroscopy (GC–MS) (Hashizume et al., 1979; MacLeod et al., 1976) have been developed for the separation and quantification of cytokinins. The major drawback with these methods is the need for numerous purification steps. As a result, these procedures are not only time consuming but also result in extensive losses of the compounds of interest.

The immunoassay is a sensitive and precise technique widely used in clinical medicine and endocrinology for the quantification of a wide range of compounds. The radioimmunoassay (RIA) procedure has recently been applied to the quantification of a number of plant constituents, including plant growth regulators (Weiler, 1983). The RIAs for cytokinin analysis are both sensitive and specific, allowing for the detection of picomolar amounts without interference from related purines (Kahn et al., 1977; Weiler, 1980; Weiler and Spanier, 1981; Vold and Leonard, 1981; MacDonald et al., 1981). Although specific for N^6 substituted nucleosides and bases, these assays cross-react with a number of naturally occurring cytokinins, making direct analysis feasible only in plants with a simple cytokinin complement. MacDonald et al. (1981) developed an HPLC–RIA procedure in which the cytokinins were fractionated on a reverse-phase column and subsequently quantified by RIA.

The objective of the present study was to develop a sensitive procedure for the analysis of naturally occurring cytokinins which would eliminate the major drawbacks of the RIA and GC–MS, namely the need for radioisotopes or for sophisticated equipment. This paper reports the development of an enzyme immunoassay (EIA) for the quantification of zeatin riboside (ZR), isopentenyladenosine (IPA), and closely related compounds. Prior to EIA analysis, the various cytokinins were fractionated with HPLC. When this approach is used, a single sample can be analyzed for several cytokinins simultaneously.

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MATERIALS AND METHODS

Materials. IPA, isopentenyladenine (IP), zeatin (Z), alkaline phosphatase (AP) type VII-S (catalogue no. P5521), and *p*-nitrophenyl phosphate (Sigma 104) were purchased from Sigma Chem. Co., St. Louis, MO. 2-(Methylthio)zeatin riboside (2-MeS-ZR) was a gift from T. Sugiyama and 2-(methylthio)isopentenyladenosine (2-MeS-IPA) was a gift from R. A. Anderson. ZR, dihydrozeatin (dHZ), other cytokinin standards, and bovine serum albumin (BSA) were purchased from Calbiochem-Behring, San Diego, CA. EIA microelisa plates (flat bottom, catalogue no. 76-381-04 and flat bottom Immulon 2, catalogue no. 011-010-3450) were purchased from Flow Labs, McLean, VA, and Dynatech, Alexandria, VA, respectively. Assay plates were incubated in a Flow Labs forced air incubator and the absorption at 405 nm was read with a Model 307 EIA reader (Biotek, Burlington, VT).

Preparation of Immunogen. ZR and IPA were coupled, via the *vic*-hydoxy groups of the ribose moiety, to free amino groups of BSA by the procedure of Weiler (1980); Weiler and Spanier (1981). From spectrophotometric analysis, a coupling ratio of 11 mol of ZR/mol of BSA and 6 mol of IPA/mol of BSA were determined for the ZR conjugate and IPA conjugate, respectively.

Immunization and Isolation of Immunoglobulins (IgG). Randomly bred 16-week-old rabbits were immunized with an emulsified mixture of either ZR or IPA immunogen by the procedure described by Jourdan et al. (1983). Serum was collected and stored at −18 °C. IgG was isolated by ammonium sulfate precipitation and DEAE-cellulose chromatography as outlined by Clark and Adams (1977), lyophilized, and stored at −18 °C. A stock solution of the IgG (100 μg/mL) was prepared in 50 mM $NaHCO_3$, pH 9.6, and stored at 4 °C with no loss in activity for at least 3 months.

Preparation of Enzyme Tracer. Coupling of ZR and IPA to alkaline phosphatase was done as follows: 3.3 mg (10 μmol) of IPA or 4.0 mg of ZR (11 μmol) was dissolved in 0.5 mL of methanol to which 0.01 M $NaIO_4$ (1.7 mL for IPA, 2.0 mL for ZR) was added dropwise over 7 min. After an additional 13 min, the formation of cytokinin dialdehyde was stopped with 0.2 mL of ethylene glycol. The solution was adjusted to pH 4 with 0.1 N HCl, applied to a C_{18} Sep-Pak cartridge (Waters), and washed with 4 mL of distilled water. The dialdehyde was eluted with 2 mL of methanol, concentrated, and diluted with 25% *N,N*-dimethylformamide (DMF) in H_2O (1 mL final volume). This solution was then added with constant stirring to AP, previously dialyzed against 1 L of PBS (phosphate buffered saline, 50 mM Na_2HPO_4, 10 mM NaCl, 1 mM $MgCl_2$,

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0.2% NaN_3, pH 7.5), to give a final molar ratio of 10 ZR or IPA:1 AP. The pH was maintained at 9.5 to 9.8 by dropwise addition of 5% K_2CO_3. The reaction was allowed to proceed at 4 °C for ca. 16 h, after which the conjugate was dialyzed against 1 L of 25% DMF and then against 3 × 3 L of PBS (pH 9.5) for 3 days at 4 °C. The tracers were diluted with an equal volume of glycerol and stored at −18 °C for up to 8 months with a minimal loss of activity.

Immunoassay Procedure. Standard solutions of Z, ZR, IP, and IPA were prepared by serial dilutions (with distilled water) from stock solutions (100 μg/mL in 25% ethanol stored at −18 °C). The standards were stored at 4 °C and remained stable for a minimum of 2 weeks. EIA plates were coated with a 4 μg/mL solution of either anti-ZR or anti-IPA IgG (200 μL/well) in 50 mM $NaHCO_3$, pH 9.6. Each well was filled with 200 μL of the appropriate IgG solution and the plate was sealed with parafilm and incubated for either 3 h at 24 °C or 16 h at 4 °C. The solution was then decanted off and the wells were washed two times with distilled water. To saturate the remaining protein adsorption sites, the coated wells were then incubated at 24 °C for 15 min with a 0.01% solution of BSA in Tris-HCl buffered saline (TBS) and washed as above. To each antibody-coated EIA well was added 100 μL of TBS (50 mM Tris-HCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.02% NaN_3, pH 7.5), 50 μL of cytokinin standard or sample, and 50 μL of appropriately diluted tracer (200-fold for AP–ZR tracer and 125-fold for AP–IPA tracer for the ZR or IPA assay, respectively). For the determination of the amount of tracer bound in the absence of standard or sample (Bo), 50 μL of water was used in place of standard. For the determination of the unspecific binding (Ub), an excess of antigen was added (200 ng/50 μL of ZR or 200 ng/50 μL of IPA). The plate was sealed, mixed gently, and incubated either at 25 °C for 90 min or at 4 °C for 16 h. The contents of the wells were then discarded and each well was rinsed three times with 10 mM NaCl and twice with 0.01% Tween 20 in 10 mM NaCl. For the detection of AP activity, 200 μL of *p*-nitrophenyl phosphate (1 mg/mL in 1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) was added to each well and the plates were incubated for 60 min at 38 °C in a forced air incubator. The substrate reaction was stopped by the addition of 50 μL of 2 N NaOH and the absorbance at 405 nm was read. Calculations for the EIA were performed as described by Weiler et al. (1981).

Extraction and Purification of Samples. Fully developed unopened blossoms of Valencia orange and Ruby Red grapefruit were used for the analysis of immunoreactivity, while for the analysis of cytokinin content during blossom development, blossoms were separated into five size groups from immature 4-mm diameter blossoms to open flowers. One-gram samples were weighed, frozen in liquid N_2, pulverized, ground in a Sorval blender with 50 mL of 80% ethanol, and extracted for 24 h at −18 °C. The extract was filtered through infusorial earth, and the tissue was reextracted in 50 mL of 80% ethanol. The two filtrates were combined, concentrated in vacuo at 50 °C to 10 mL, acidified to pH 2 with 1 N HCl, and filtered. The pH was adjused to 8.5 with 1 N NH_4OH and the extracts were partitioned three times with an equal volume of water saturated butanol. The butanol was removed by azeotropic distillation with water in vacuo at 50 °C. The resulting aqueous extract was diluted with an equal volume of 10% methanol (pH 3.2 with H_3PO_4 and stored at −18 °C).

HPLC Separation. The separation of cytokinins was performed on a Waters dual pump HPLC system with a Model 660 solvent programmer and LDC SpectroMonitor set at 267 nm. The stainless steel column (1 cm × 25 cm) was slurry packed (Micrometrics Model 705 packer) in isopropyl alcohol with Nucleosil 10 C_{18} packing for reverse-phase chromatography. The solvents used were 10% methanol adjusted to pH 3.2 with H_3PO_4 (solvent A) and 100% methanol (solvent B). The 1-mL sample was injected onto the column and eluted with a step gradient as follows: flow rate 3 mL/min; initial conditions 20% solvent B for 30 min, followed by 35% solvent B for 45 min and a linear gradient to 100% solvent B in 15 min to purge the column of remaining material. Under these conditions the retention times of the four cytokinins of interest were Z 12 min, ZR 25 min, IP 48 min, and IPA 63 min. Fractions were collected at 2-min intervals, neutralized with 0.1 N NH_4OH, frozen, and concentrated to 1 mL by lyophilization. Each fraction was assayed either in duplicate or triplicate with the EIA. All cytokinin values were expressed as ng of cytokinin/fresh weight of tissue.

Table I. Assay Parameters for the ZR and IPA EIAs

	ZR antisera	IPA antisera
tracer immunoreactivity[a]	19%	26%
IgG coating concentration, μg/mL	4	4
optimum pH range for EIA	6.0–9.0	6.0–9.0
unspecific binding (Ub)[b]	6%	12%
detection limit (ng, pmol)	0.05, 0.14 ZR 0.10, 0.46 Z	0.10, 0.30 IPA 0.20, 0.98 IP
standard curve measuring range, ng	0.05–20 ZR 0.1–50 Z	0.1–20 IPA 0.1–20 IP

[a] Trace immunoreactivity equals the percentage of the cytokinin–AP conjugate which could be bound in the presence of an excess of antibody. [b] Unspecific binding equals the amount of activity bound in the presence of excess antigen, expressed as a percent of the total bound activity (Bo).

RESULTS AND DISCUSSION

Characteristics of the Cytokinin EIAs. The competitive enzyme-linked immunosorbent assays developed in this study utilized antisera produced against cytokinin–BSA conjugates and cytokinin–AP tracers. In this method, free cytokinin and cytokinin–AP tracer compete for a fixed number of antibody sites on the walls of the polystyrene wells. Following the removal of unbound reagents, enzyme activity is determined with the use of *p*-nitrophenyl substrate with the amount of color produced being inversely proportional to the quantity of free cytokinin in the sample.

For analysis, the amount of tracer used was chosen to give a final absorbance of 1.0 at 405 nm. Under these conditions, the Ub gave an absorbance of 0.06 and 0.10 for the ZR and IPA assays, respectively. Typical standard curves for Z, ZR, IP, and IPA are given in Figure 1. The effective range and the sensitivity of the assays are comparable to the published RIA procedure (Weiler, 1980; Weiler and Spanier, 1981; MacDonald et al., 1981) allowing for the determination of picomolar concentrations of cytokinins. Some of the basic characteristics of the two assays are given in Table I.

Assay Specificity. The antibodies produced in the procedure were polyclonal and hence were not specific for the cytokinins against which the antisera were developed. This phenomenon, however, has the advantage of permitting analysis for more than one cytokinin in a single sample. The degree to which various cytokinins are bound to the antibodies and displaced by tracer varies. This variation or cross-reactivity (CR) of a compound is determined by the following formula: $CR = (x/y)100$ where

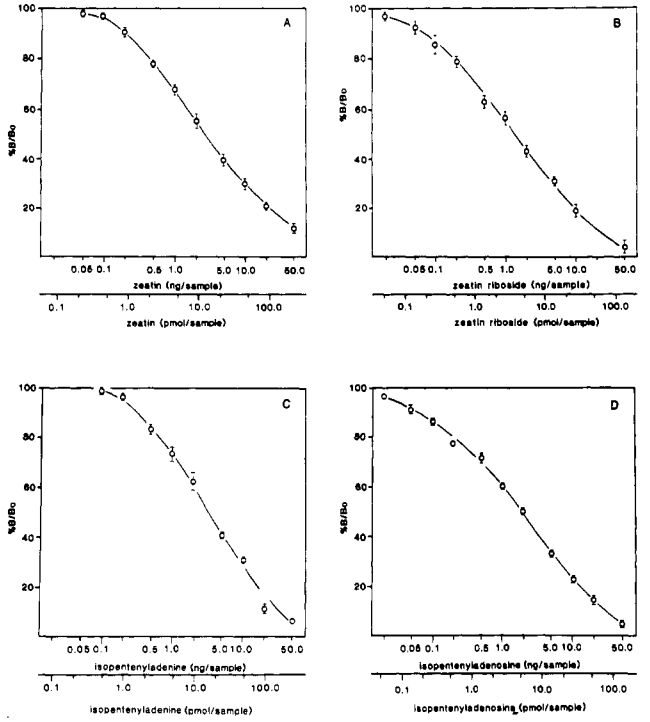

Figure 1. Typical standard curves obtained for the ZR + IPA enzyme immunoassays. Anti-ZR sera and ZR–AP tracer were used for A and B, and anti-IPA sera and IPA–AP tracer for C and D. The standards used were zeatin (A), zeatin riboside (B), isopentenyladenine (C), and isopentenyladenosine (D). The bars represent ± standard deviations of triplicates, B equals the binding of tracer to antibody in the presence of standard as measured by the absorbance at 405 nm, and Bo equals the binding in the absence of standard.

y = the molar amount of compound needed to inhibit tracer bindings by 50% and x = the molar amount of ZR or IPA needed for the same inhibition (Chard, 1978). The primary determinant for immunoreactivity for both antisera was the N^6 side chain. Thus, the N^6 substituted purines tested, with the exception of purines containing a short side chain (methylamino or hydroxyethylamino), were all found to cross-react to varying degrees, while purine bases and their nucleosides showed virtually no CR (Table II). This specificity for N^6 substitution was also found in antisera developed by others (Weiler, 1980; Weiler and Spanier, 1981; MacDonald et al., 1981).

The antisera showed only minimal specificity for the ribose moiety as seen by the immunoreactivity of ZR and Z in the ZR assay (CR of 100% and 80%, respectively) and IPA and IP in the IPA assay (CR of 100% and 49%, respectively). A lack of specificity for the ribose was expected since the ribose forms the bridge to the protein in both immunogen and tracer. As previously reported, the highest selectivity is normally obtained to the structural features away from this coupling region (Weiler, 1983).

A number of similar cytokinins, other than the free bases, were found to be immunoreactive. Thus, with the ZR antiserum, both 2-MeS-ZR and dHZ were immunoreactive. With the IPA antiserum, 6-(hexylamino)purine, 6-(benzylamino)purine and its riboside, and 2-MeS-IPA were immunoreactive. The broad antibody specificity of these assays allows these cytokinins, along with those for which the assay was developed, to be quantified. This is achieved by separating the cytokinins by HPLC and determining each individual compound with the EIA. The cytokinin value obtained by the particular EIA used (expressed as ZR or IPA equivalent) can then be corrected by the appropriate percent CR value to obtain the actual levels of the cytokinin present.

Table II. Cross-Reactivities of Cytokinins and Related Compounds

compd	cross-reactivity, %[a] ZR antisera[b]	IPA antisera[c]
t-zeatin riboside	100	3
t-zeatin	80	1
2-(methylthio)zeatin riboside	44	
dihydrozeatin	18	4
c-zeatin	0.6	<0.1
isopentyladenosine	5	100
isopentenyladenine	9	49
2-(methylthio)isopentenyladenosine	1	29
6-(benzylamino)purine	3	23
6-(benzylamino)purine riboside	4	100
6-(furfurylamino)purine	0.6	31
6-(hexylamino)purine	4	11
6-(hydroxyethylamino)purine	<0.05	<0.1
6-(methylamino)purine	0.05	0.05
6-(methylamino)purine riboside	<0.05	0.05
adenosine	<0.05	0.03
adenine	<0.05	
guanosine	<0.05	<0.03
guanine	<0.05	
cytidine	<0.05	<0.03
cytosine	<0.05	<0.03
xanthosine	<0.05	<0.03

[a] See text for explanation of cross-reactive. [b] Tracer = AP labeled ZR. [c] Tracer = AP labeled IPA.

Analysis of Plant Extracts. Fractionation of plant extracts prior to EIA analysis was achieved by the use of HPLC semipreparative columns (1 cm × 25 cm). Smaller analytical columns (4 mm × 25 cm) were found to have insufficient capacity to separate the amounts of extracts required for analysis. Several packing materials were tried but the best resolution was achieved with Nucleosil 10 C_{18} which was packed in our laboratory. Satisfactory HPLC fractionations of 1-g plant samples were obtained by using a preliminary butanol–water purification step.

HPLC fractionation of citrus blossom extracts showed major peaks of immunoreactivity cochromatographing with ZR, IP, and IPA. Both ZR and IPA have been identified from *Citrus* with GC–MS (Stewart and Barthe, 1984). For isolation of IPA, EIA was used as the means of detecting activity in crude extracts. By this method, sufficient amounts of the cytokinin were isolated from citrus extracts for cochromatography with known standards by using HPLC and GC and for GC–MS analysis. ZR had previously been identified in a similar manner by using soybean bioassay as a means of detection. Although IP has not been conclusively identified, the specificity of the EIA coupled with the presence of immunoreactive material coinciding with the HPLC retention time of IP presents strong evidence for its presence. Isolation and final identification by GC–MS are currently in progress.

The cross-reactivity studies and the correlation of immunoreactivity with the HPLC retention times of cytokinin standards give an indication of the specificity of the assay. For the quantification of cytokinins in plant extracts, the validity of the HPLC–EIA procedure was demonstrated by showing parallelism of the dilution curves of HPLC fractions with the standard curve and by the recovery (96%) of ZR and IPA added to the extracts prior to EIA.

The EIA procedure is sufficiently sensitive to detect cytokinins in nanogram and picogram amounts. There are, however, problems in extracting these low amounts. Significant losses may occur from degradation and from adsorption on glassware during concentration of extracts.

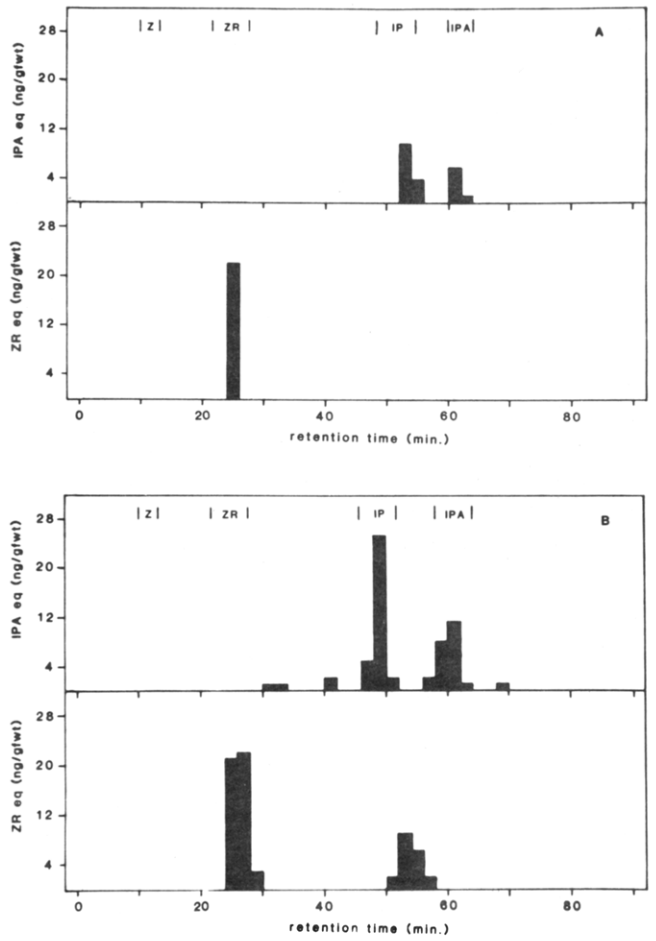

Figure 2. Distribution of immunoreactivity of Valencia orange blossom (A) and Ruby Red grapefruit blossom (B). Extracts were chromatographed on a Nucleosil C_{18} reverse-phase column (1 cm × 25 cm), and fractions were collected at 2-min intervals. See text for other chromatographic conditions. Each fraction was concentrated to 1 mL by lyophilization and analyzed with the cytokinin EIAs. ZR equiv equals zeatin riboside equivalents, IPA equiv equals isopentenyladenosine equivalents.

MacDonald et al. (1981) for example have reported a 90% loss of ZR standard when dried and redissolved in buffer. Therefore, in order to reduce analytical error, glassware was silylated, extracts were not taken to dryness, and a minimum of 1-g samples was used.

Cytokinins in Flower Buds of *Citrus*. The applicability of the HPLC–EIA procedure for the detection of cytokinins in citrus blossoms was demonstrated with the analysis of Valencia orange and Ruby Red grapefruit flower buds (fully developed, prior to opening). Extracts from both cultivars contain several immunoreactive compounds (Figure 2), including ZR (11 ng/g and 44 ng/g, respectively), IP (14 ng/g and 66 ng/g, respectively), and IPA (4 ng/g and 22 ng/g, respectively). In addition to these cytokinins, at least one unidentified immunoreactive compound was found in Ruby Red grapefruit extracts. Based on the previous CR studies, this compound is believed to be an N^6 substituted nucleoside.

A more detailed investigation was made of the cytokinin content of Valencia orange flowers during development. Blossoms were separated into five development stages from immature buds to open flowers. ZR, IPA, and IP were detected in all stages (Figure 3), with the highest amounts occurring in fully developed flower buds and open blossoms (stages 4 and 5). Zeatin was not found in any of the tissues examined. Additional work is in progress to more fully characterize the changes in cytokinins occurring during flower and fruit development in *Citrus*.

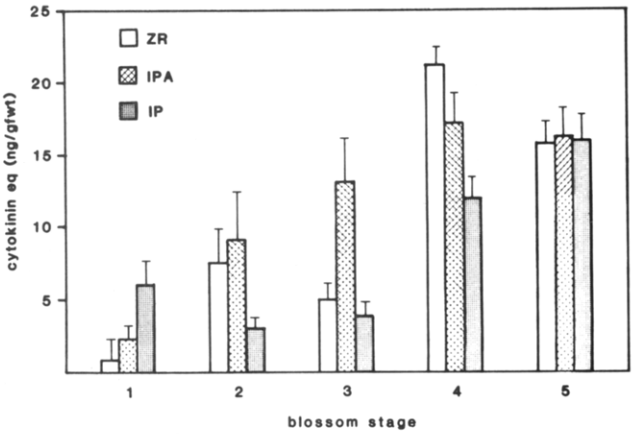

Figure 3. Changes in the cytokinin levels during blossom development. Developing blossoms of Valencia were separated into five stages, extracted, and analyzed. Blossom stages were (1) 4-mm diameter, (2) 5-mm diameter, (3) 7-mm diameter, (4) 8-diameter, elongated, prior to opening, and (5) open blossom. Values are the mean of two triplicate EIA analyses of each sample. Bars represent the standard deviation.

CONCLUSION

We have developed an EIA procedure which, when coupled with semipreparative HPLC, can be used to quantify the endogenous cytokinins in plant tissues. The sensitivity and specificity of the EIAs are comparable to that of the published cytokinin RIAs. The present procedure has the advantage of not requiring radioisotopes and can be performed with standard laboratory equipment. In addition to measuring ZR and IPA, for which antisera were developed against, the HPLC–EIA procedure can also be used to determine the levels of related compounds such as Z, 2-MeS-ZR, and IP. Furthermore, this procedure can also be a useful tool in the isolation and purification of N^6 substituted compounds which may have cytokinin activity.

Note: Following the submission of this paper, an EIA procedure for the analysis of Z and ZR was published by C. E. Hansen et al. in *Plant Physiol.* **1984**, *75*, 959.

Abbreviations Used: AP, alkaline phosphatase; Bo, amount of tracer bound in absence of standard; BSA, bovine serum albumin; CR, cross-reactivity; dHZ, dihydrozeatin; DMF, *N*,*N*-dimethylformamide; EIA, enzyme immunoassay; GC, gas chromatography; GC–MS, gas chromatography–mass spectroscopy; HPLC, high-performance liquid chromatography; IgG, immunoglobulin; IP, isopentenyladenine; IPA, isopentenyladenosine; 2-MeS-ZR, 2-(methylthio)zeatin riboside; 2-MeS-IPA, 2-(methylthio)isopentenyladenosine; PBS, phosphate buffered saline; RIA, radioimmunoassay; TBS, Tris-HCl buffered saline; Ub, unspecific binding; Z, zeatin; ZR, zeatin riboside.

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Registry No. ZR, 6025-53-2; IPA, 7724-76-7; IP, 2365-40-4; (*E*)-Z, 1637-39-4; 2-MeS-ZR, 53274-45-6; 2-MeS-IPA, 20859-00-1; dHZ, 23599-75-9; 6-(benzylamino)purine, 1214-39-7; 6-(benzylamino)purine riboside, 4294-16-0; 6-(furfurylamino)purine, 525-79-1; 6-(hexylamino)purine, 14333-96-1; alkaline phosphatase, 9001-78-9.

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Structure-Function Relationships in Food Proteins: Subunit Interactions in Heat-Induced Gelation of 7S, 11S, and Soy Isolate Proteins

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The nature of specific interactions occurring between soy protein subunits upon gelation following heating was studied by ultracentrifugation and two-dimensional gel electrophoresis. The results indicate that acidic subunit (AS) III in contrast to ASIV is an integral and essential component of glycinin and soy isolate gels, that the three subunits of β-conglycinin (7S) participate uniformly in gel matrix formation from conglycinin, and that there is a preferential interaction between the β subunit of 7S and the basic subunits of glycinin (11S) in soy isolate gels. Selective solubilization of gels indicated that hydrogen bonding and disulfide bonds were important in maintaining gel network structures.

The composition and oligomeric structure of soy proteins affect their functional behavior (Kinsella, 1979; Kinsella et al., 1985). The major globulins of soy protein are conglycinin (7S) and glycinin (11S). 7S is a trimeric glycoprotein (141 000–170 000 daltons) composed of six different combinations of three subunits, α (57 000), α′ (58 000), and β (42 000) associated via hydrophobic interactions (Thanh and Shibasaki, 1978). The 11S consists of two apposed hexagonal rings each containing three hydrophobically associated pairs of disulfide-linked acidic (35 000–37 000) and basic (20 000) subunits (Badley et al., 1975). Upon being heated, these subunits may dissociate and reassociate in different ways and form gels (German et al., 1982; Damodaran and Kinsella, 1981; Mori et al., 1982a,b).

The possible mechanisms involved in the heat-induced gelation and the association–dissociation behavior soy protein isolate and its constituent proteins of soybean have been studied by many workers (Mann and Brigs, 1950; Watanabe and Nakayama, 1962; Saio et al., 1968; Wolf and Tamura, 1969; Catsimpoolas et al., 1969, 1970; Aoki, 1970; Fukushima and van Buren, 1970; Hashizume et al., 1975; Hashizume and Watanabe, 1979; Yamagishi et al., 1980; Mori et al., 1982a; German et al., 1982; Damodaran and Kinsella, 1982; Nakamura et al., 1984a,b). However, most of these studies were carried out under conditions in which gel is not formed. Considerable research has recently been done on the mechanism of gelation and the role of the constituent subunits in the formation and properties of the gels formed from 11S (Mori et al., 1982a,b; Nakamura et al., 1984a,b; Utsumi et al., 1983). These studies led to the suggestion that the 11S globulin oligomers appear to undergo minor changes upon heating, retain quaternary structure, and reveal little obvious denaturation when associating to form a gel following heating at 100 °C (Mori et al., 1982a; Nakamura et al., 1984b). The contributions of the constituent subunits to the formation and properties of the 11S gel are not equal. Thus, acidic subunit (AS) III plays an important role in increasing the hardness of the gels (Mori et al., 1982b; Nakamura et al., 1984b). Similar results were obtained with respect to 11S globulin of broad bean (Utsumi et al., 1983). These results suggest that the contribution of the various soy subunits to the interactions involved in the formation of gel may be different. Recently, we showed that the subunits of 7S globulin interact electrostatically with the basic subunits (BS) of 11S globulin forming soluble complexes at low protein concentration (German et al., 1982; Damodaran and Kinsella, 1982; Utsumi et al., 1984). Similar interactions occur at high protein concentration and the main molecular forces involved in the formation of the gels from 7S, 11S, and soybean isolates are apparently different, suggesting that the protein–protein interactions in gels made from soybean isolate may be different from those made from the constituent proteins, 7S and 11S globulins (Utsumi and Kinsella, 1985).

In the preceding paper we showed that the soluble macromolecular complexes formed upon heating of soy isolates were composed mostly of basic subunits of 11S associated with β subunits of 7S, mostly via electrostatic interactions. Association of basic subunits via disulfide bonds also occurred (Utsumi et al., 1984).

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