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Communication

An Avidin-Biotin Solid Phase ELISA for Femtomole Isopentenyladenine and Isopentenyladenosine Measurements in HPLC Purified Plant Extracts

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ABSTRACT

A solid phase enzyme immunoassay was developed for isopentenyladenine (iP) and isopentenyladenosine (iPA) quantitation in HPLC purified plant extracts. It was performed on antigen-coated microtitration plates on which bound antibodies were indirectly labeled by the means of a biotinylated goat anti-rabbit antibody and an avidin-alkaline phosphatase conjugate. Less than 3 femtomoles of iP or iPA were easily detected and the measuring range extended from 3 femtomole to 1 picomole. The reproducibility has been tested and intra- and interassay variations did not exceed 5.0%. The specificity of iPA antibodies was good, as determined by cross-reactivity measurements with other adenylic compounds. The specificity of the measurements for iP and iPA was demonstrated by analysis of the immunoreactivity of fractions obtained by HPLC separation of a methanolic tobacco leaf extract.

Immunoenzymatic assays have been shown to be very powerful tools for plant growth substances analysis (12). Compared with other techniques, they offer the best compromise with regard to the possibility to perform many measurements within a short period (up to several hundreds per day) combined with a very high sensitivity and a very good precision due to the specificity of antibodies. We recently described a solid phase immunoenzymatic method, based upon an indirect antibody labeling system which involves the avidin-biotin interaction complex, for the determination of several plant hormone levels within the same HPLC purified plant extract (11). In this paper, we report on the extension of this immunological procedure to the analysis of iP[1] and iPA in plant samples. The efficiency of the assay is discussed in relation with its sensitivity, its reproducibility, and its specificity. An immunohistogram has been established from ELISA analysis of HPLC purified fractions of a tobacco leaf extract and is given as an example of the use of this methodology to test its iPA and iP content.

[1] Abbreviations used: iP, isopentenyladenine; iPA, isopentenyladenosine; ELISA, enzyme linked immunosorbent assay; Z, (trans-)zeatin; *t*- and *c*-ZR, *trans*- and *cis*-zeatin riboside; DHZ, dihydrozeatin; DHZR, dihydrozeatin riboside; Ado, adenosine; BAP, 6-benzylaminopurine; PBS, isotonic phosphate buffer (pH 7.4); OVA, ovalbumin.

MATERIALS AND METHODS

Chemicals. BSA and OVA were obtained from Sigma (USA). Cytokinins were from Sigma (Z, DHZ, DHZR, iP) or were synthesized (iPA, t-ZR, and c-ZR) as previously described (4). [^{3}H]iPA, specific activity 0.70 TBq/mmol was a gift from Dr. M. Laloue (Gif/Yvette, France). Avidin was supplied by Reactifs IBF (France), and alkaline phosphatase was from Boerhinger (France). All solvents were of analytical grade.

Preparation of Antiserum. The immunogenic iPA-BSA conjugate was prepared according to Erlanger and Beiser (5). iPA molecules (6.3) were bound with 1 BSA molecule according to the UV spectrophotometric measurement of Inouye *et al.* (8). A rabbit was immunized with 1.0 mg iPA-BSA conjugate diluted with complete Freund's adjuvant. Booster injections were done with 0.1 mg conjugate in incomplete Freund's adjuvant at 3 week intervals. No purification of the anti-iPa serum was required prior to perform the immunoenzymatic assay.

Plant Material. Tobacco (*Nicotiana tabacum*, cv Xanthi) seeds were sown on sterile Murashige and Skoog agar medium. Plants were grown at 25°C under 16 h light (44 ± 2 $\mu E/m^2 \cdot s$) per day. Thirteen days after sowing they exhibited two fully expanded leaves. Leaves were then excised, dropped into liquid N_2, and kept at −40°C until extraction for hormone analysis.

Extraction and Purification of Samples. Aliquots of the sample (100 mg fresh weight) were homogenized in 5 ml 80:20 (vol:vol) methanol:distilled water containing 40 mg/L butylhydroxytoluol (BHT) as an antioxidant. [^{3}H]iPA (about 1 kBq) was added in the extract which was stirred overnight at 4°C in darkness. The extract was passed through a Millipore prefilter connected with a Sep-Pak C18 cartridge (Waters, USA). The prefilter and the Sep-Pak cartridge were rinsed with 10 ml of 80% methanol. The bulk eluates were reduced to about 50 μl by rotary evaporation, taken up with 1 ml acidified water (0.6 M acetic acid), and injected into a reverse phase HPLC column (ODS C18, Chrompack, The Netherlands). The elution was conducted with a Beckman 114 M HPLC system following an acidified water:methanol gradient (11). Tributylamine (10 mM) added in solvents resulted in an improved iP and iPA separation. Fractions (400 μl, 1 min elution time) were collected in 1.5 ml Treff microcentrifuge test tubes (Treff AG, Switzerland) and evaporated to dryness in a Speed-Vac concentrator (Savant, USA). All fractions were then taken up in 1 ml PBS and aliquots were submitted either to scintillation counting for recovery measurement of radioactivity or to immunoassay.

Preparation of Reagents. iPA-OVA conjugate was prepared according to the same procedure as for iPA-BSA. However, OVA was previously treated with glutaraldehyde to bind lysine residues to the free amino groups of OVA. iPA was consecutively linked to the so-realized spacer-arm. This procedure was developed to improve the sensitivity of the immunotest, as demonstrated for ZR immunoassay (11). Avidin-alkaline phosphatase conjugate preparation was performed with glutaraldehyde, according to Avrameas and Ternynck (1) and was described in Maldiney *et al.* (11).

ELISA Procedure. The method employed has already been described (9, 11). In brief, polystyrene microtitration plates (Nunc, Denmark) were coated with iPA-OVA conjugate (5 μg/ml in 0.05 M carbonate/bicarbonate buffer, pH 9.6). A limited amount of specific antibody and iP or iPA standard or sample were then added. The plates were incubated for 1 h at 4°C, in darkness. During this period, a competition occurred for antibody between iPA bound to the plate and free hormone in solution. After four washings with PBS containing 0.1% Triton X-100, anti-iPA antibody bound on the plate was quantitated by the means of the avidin-biotin interaction system: the plate was incubated for 1 h with an excess of biotinylated goat anti-rabbit antibody prepared as described by Guesdon *et al.* (7), washed, and subsequently incubated for 1 h with avidin-alkaline phosphatase conjugate. After four washings to eliminate the excess of conjugate, paranitrophenyl phosphate (1 mg/ml in 1.0 M diethanolamine buffer [pH 9.8], supplemented with 0.01 M $MgCl_2$) was added and the phosphatase activity bound to the plate was measured spectrophotometrically at 405 nm with a MR 600 (Dynatech, USA) spectrophotometer. The results were analyzed with an Apple (USA) Lisa microcomputer. Calculations were made by reference to a calibration curve established, on each microtitration plate, by a curvilinear regression of magnitude 4 obtained from the average of four standard curves. The iPA levels measured were deduced from the amount of added iPA as radioactive standard.

RESULTS

Figure 1 illustrates typical standard curves obtained for iPA and iP enzyme immunoassays. Calibration curves for other cytokinins were also represented on the same figure in order to facilitate comparison of cross-reactivities. Each curve was calculated as a linear regression from four replications. Less than 3 fmol of iP or iPA were easily detected and the measuring range extended from 3 fmol to 1 pmol per assay. The medium point of the standard curves, which corresponds to 50% maximum enzyme activity (*i.e.* maximum iPA antibody level) bound was obtained with 60 fmol iPA and 100 fmol iP, respectively. Within the measuring range, the curves' were almost linear and the precision was good, as shown by standard error of the means which were maintained within 0.2 and 5.0%. The standard curves obtained with other cytokinins clearly show that N-6-substituted adenine compounds cross-react with iPA. Relative molar cross-reactivity of these compounds was expressed as a percentage of iPA level required to produce the same effect as the level of purines which resulted in 50% maximum anti-iPA antibodies bound (Table I). With natural cytokinins, these values were very low (maximum 3.0% with c-ZR), thus providing good argument for a good specificity of anti-iPA antibodies.

The method here described has been tested for hormonal analysis of a HPLC purified methanolic tobacco leaf extract in which [^{3}H]iPA was added prior to purification as internal standard (Fig. 2). Immunoreactivity of each fraction (1 min elution time) was submitted to ELISA with iPA as a reference. Radioactivity distribution within the ODS column eluted fractions of the extract was measured and 80% of radioactive iPA added prior to purification was recovered within the immunoreactive peak corresponding to iPA. Controls were done on the same column with Z, ZR, iP, and iPA, checked with a UV detector at 254 nm, and the respective corresponding elution zones have been indicated by small horizontal bars on Figure 2. In tobacco leaves, 5.4 pmol/g fresh weight of iPA were measured. This value was deducted from the amount of radioactive iPA recovered.

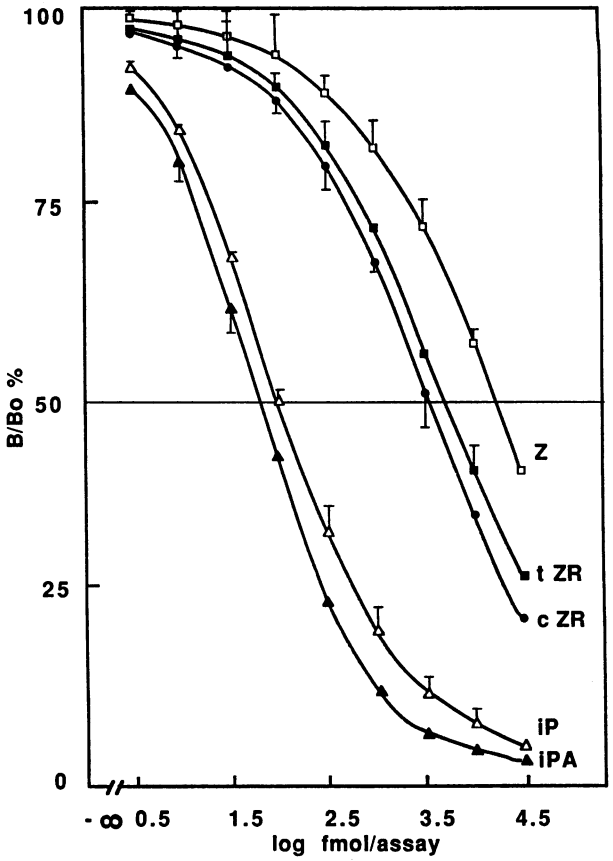

FIG. 1. Typical standard curves obtained with several cytokinins according to the avidin-biotin based ELISA procedure. B, Enzymic activity bound in the presence of hormone; Bo, enzymic activity bound without hormone in solution. The curves resulted from competition with iPA bound to the microtitration plate and free iPA, iP, c-ZR, t-ZR, or Z in solution for a limited amount of anti-iPA antibodies. Each curve resulted from a curvilinear regression of magnitude 4 calculated from four experimental standard curves. Bars, SE expressed as a percentage of maximum enzymatic activity bound. When lower than 0.5%, bars are included in symbols.

Table I. *Relative Molar Cross-Reactivity of Purines*

The concentration of purines at 50% relative enzyme activity bound are expressed as a percentage of iPA level required to produce the same effect.

Compound	fmol	Cross-Reaction
		%
iPA	60	100.0
iP	100	60.0
t-ZR	6000	1.0
Z	20000	0.3
c-ZR	2000	3.0
DHZR	8000	0.8
DHZ	6000	1.0
Ado	>30000	0.0
kinetin	2000	3.0
BAP	200	30.0

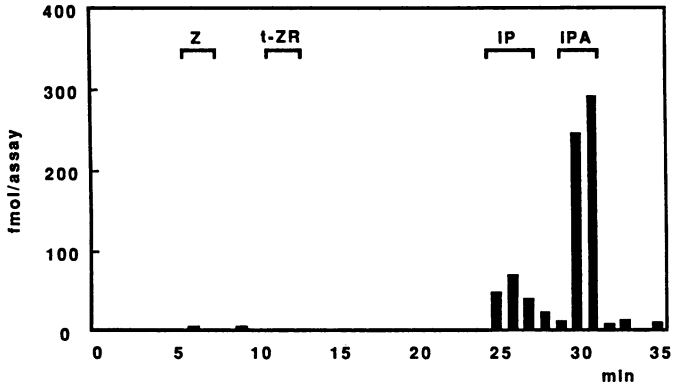

FIG. 2. Immunohistogram obtained from immunoreactivity measurements in HPLC separated fractions from a tobacco leaf extract. Typical elution zones for Z, t-ZR, iP, and iPA standards were determined with UV absorbance at 254 nm and represented with horizontal bars. Each fraction corresponded to 0.4 ml (1 min elution time).

The peak eluted before iPA corresponded to 1.7 pmol/g fresh weight as calculated from the iP calibration curve represented on Figure 1. No significant radioactivity nor immunoreactivity were detected in other fractions of this tobacco leaf extract.

DISCUSSION

Several immunoassays for iPA have already been described (2, 6, 10, 13). However, the methodology we used, based upon the avidin-biotin interaction complex and high specific activity alkaline phosphatase as label, has shown a very high sensitivity (3 fmol iPA or iP can be measured and lower amounts detected) associated with its adaptability to the analysis of several plant hormones with the same detector (11). Furthermore, we confirm here Weiler's observations about the highest sensitivity of enzyme immunoassays than radioimmunoassays for plant growth substances analysis (12, 13). Some ELISA we made with anti-iPA antibodies kindly supplied by Dr. Morris gave very similar results as with our own serum (data not shown), thus providing further good argument in favor of the pre-cited observations. Here we could point out the fact that an ELISA which is performed with antigen-coated microtitration plates appear very useful to compare the efficiency of several antisera from different sources. This could be of importance in testing antibodies secreted by hybridomas for the screening procedure when preparing monoclonal antibodies.

The sensitivity of the iPA and iP ELISAs was also associated with a good reproducibility, as demonstrated by the low intra-assay variations which never exceeded 5% and was generally comprised between 1 and 3% within the measuring range (*i.e.* 3 fmol–1 pmol). Inter-assay variations were tested from a series of experiments and were within the same range. Nevertheless, we always performed standardization on each microtitration plate in order to ascertain the best precision of measurements. One can notice also that the background level was always very low. This was due to the high dilution of antibodies, the absence within the serum of antibodies specific to the periodate linkage or to the carrier protein we used for the coating, and to the optimization of buffer composition adapted from Guesdon *et al.* (7) to prevent unspecific binding of avidin.

Considering cross-reactions, we have shown that some N-6-substituted naturally occurring cytokinins do cross-react with the iPA-ELISA, whereas adenosine was not recognized by anti-iPA antibodies (Fig. 1; Table I). Among cross-reactant compounds, the specificity of the reaction was determined by the base, as shown by comparison of standard curves obtained from free bases or ribosides. This confirms previous observations reported by Weiler (13), MacDonald *et al.* (10), and Barthe and Stewart (2). The ribose moiety of ribosides may act as a spacer-arm which could facilitate the base-antibody interaction (13). Standard curves illustrated on Figure 1 provide more information about cross-reactions than percentages calculated on molar basis at 50% specific antibody bound (Table I). It appears that relatively low levels (about 100 fmol) of cross-reactant hormones (zeatin and zeatin-related compounds) could be detected but relatively high amounts of these substances would be required to affect iP and iPA ELISAs. Therefore, a good specificity can be expected for iP and iPA quantitation by this method in diluted extracts. However, iP and iPA standard curves are very close to each other, thus making a high performance purification step a prerequisite prior to performing an accurate determination of the levels of these hormones in a plant extract.

The immunohistogram described in Figure 2 was made to test the presence of cross-reactant compounds (metabolites, conjugates of cytokinins, or unknown compounds) which could exist in a plant, be extracted by methanol with iP and iPA, and interfere with the ELISA. Only iP and iPA were significantly detected in the tobacco extract. However, slight differences could have been observed following another extraction procedure which could have prevented hydrolysis of eventually occurring ribotides (3). This method could be very useful to compare the efficiency of different extraction and purification procedures for cytokinins.

A radioactive internal standard is of great importance to check the efficiency of the purification procedure and to allow accurate quantitation of iPA. Recovery (80%) was obtained when freshly silanized glassware was used but lower recovery (60% or less) was obtained with untreated or badly silanized glassware. Care must be taken to add low amounts of very high specific activity radioactive standard to reduce relative interference in the ELISA and to deduce the measurements from the corresponding amount of recovered internal standard.

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