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Note

Analysis of abscisic acid in wheat leaves by a combination of high-performance liquid chromatography and radioimmunoassay

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Radioimmunoassay (RIA)[1] and high-performance liquid chromatography (HPLC)[2] with UV detection have been used in the quantitation of abscisic acid (AbA). RIA relies on antibody specificity and HPLC relies on sample purification. By using a combination of HPLC[3] and RIA, specificity and a high degree of purification can be combined to obtain reliable estimates of hormones. Weiler[4] used RIA to estimate hormones in crude aqueous extracts; however, in crude extracts other constituents may interfere with AbA–antibody interaction. Some sample purification is therefore desirable particularly when different tissues are analysed and compared. In the present investigation HPLC was used to purify and RIA to estimate AbA content in wheat leaf tissue. Accuracy of AbA estimations made by RIA was verified by simultaneous estimations of AbA by HPLC in the same tissue.

EXPERIMENTAL

Preparation of hapten conjugate

Weiler's[4] procedure was used: 66 mg of *cis*-(±)-AbA were dissolved in 1.5 ml of dimethylformamide–water (2:1). 125 mg of human serum albumin (HSA) were dissolved in 3 ml of water and the pH was adjusted to 8.5 with 0.01 *M* sodium hydroxide. The AbA solution was added to the HSA solution while the pH was maintained at 8.0 with 0.01 *M* sodium hydroxide. Four 26-mg portions of N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide were added to the mixture over a period of 90 min. The mixture was incubated in the dark at 4°C for 24 h. The conjugate was dialysed against distilled water in a 2-l beaker with a change of water each day for 4 days in the dark at 4°C. Aliquots of the conjugate were lyophilized and kept at −70°C. The procedure binds HSA to AbA via a carboxyl group[2,4].

Preparation of AbA antisera

The AbA–HSA conjugate was taken up in a 1:1 mixture of phosphate buffer, pH 7.4, and Freund's complete adjuvant. Aliquots (0.3 ml) equivalent to 1 mg of protein were injected intradermally at four locations into three male New Zealand

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rabbits (12 weeks old). Two further booster injections in the foot pads were made at monthly intervals. Two weeks after the final injection the titre of the serum was determined by collecting a small volume of blood through the ear vein from each of the animals. Samples of blood (30 ml) were collected from animals which gave a titre at 1:500 dilution which bound 40% of [^{3}H](±)-AbA. In the assay antiserum was further diluted by four times. Aliquots of the serum were frozen and stored at −70°C.

Radioimmunoassay

The assay was carried out in 75 × 12 mm I.D. disposable glass culture tubes. The incubation mixture contained the following: 300 μl of phosphate buffered saline solution (0.01 *M* sodium phosphate, 0.15 *M* sodium chloride, pH 7.4) containing bovine serum albumin at a concentration of 1 mg/ml, 50 μl of [^{3}H](±)-AbA, *ca.* 10,000 cpm, 50 μl of standard or sample and 100 μl of diluted antiserum. After mixing, the tubes were incubated at 4°C for at least 2 h. Following incubation, 1 ml of ammonium sulphate (10 volumes of saturated solution and 1 volume water) was added and precipitation was permitted for 1 h at room temperature. After centrifugation the supernatant was discarded and the tubes were kept inverted for 15 min. Any droplets remaining on the wall of the tubes were removed by suction. The precipitate was taken up in four aliquots of 100 μl of water into a scintillation vial, and 4.5 ml of Aquasol scintillator were added. The solution was allowed to stand overnight at room temperature and the radioactivity was then counted. Estimations were done in triplicate. Cross-reactivity of phaseic acid (PA) isolated from wheat leaf and *trans*-AbA separated from a *cis–trans* AbA mixture by HPLC were tested in the assay system.

HPLC apparatus and analysis

A Model 204 liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) which included a Model 440 UV absorbance monitor, two Model 6000 pumps and a Model 600 solvent flow programmer, was used.

Preliminary purification was similar to Durley *et al.*[5]. The first HPLC step was modified to reduce the AbA retention time. Chromatographic conditions are given in the figure captions. A portion of the AbA fraction collected after purification through the C_{18} column was analysed using a silica column with UV detection and chloroform–acetonitrile–acetic acid (94:6:1) as the solvent. The remainder was analysed by RIA after appropriate dilution.

Plant material

Plant tissue was obtained from eight wheat varieties (Wascana, Wakooma, Botno, Cando, Columbus, 3RB2, Sinton and Thatcher) grown in the field. Fifteen different tissue samples were used in the comparison of HPLC estimation with RIA.

Chemicals

cis-[^{3}H](±)-AbA, specific activity 33.2 Ci/mmol (Amersham); human serum albumin, dimethylformamide, N-ethyl-N'-(3 dimethylaminopropyl)-carbodi-imide hydrochloride (all Sigma); Freund's complete adjuvant (Difco); Aquasol (New England Nuclear).

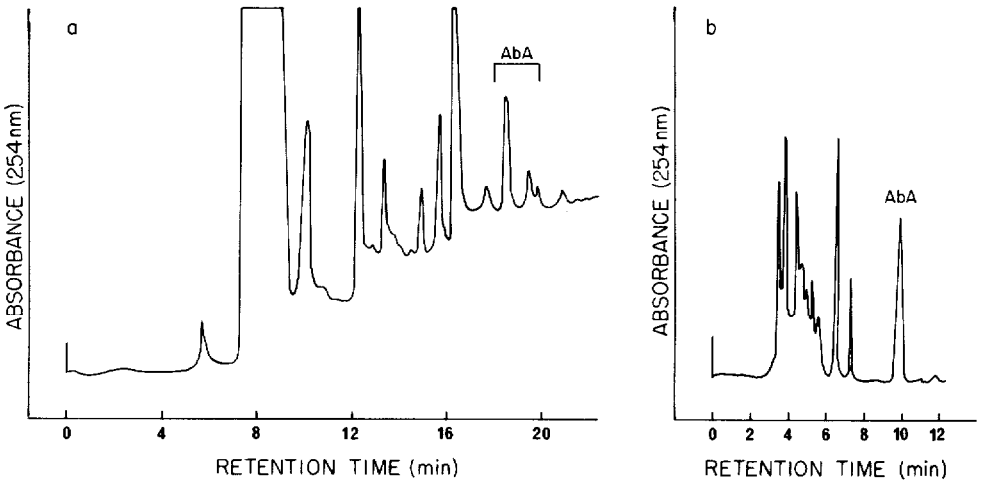

Fig. 1. (a) Purification of wheat leaf tissue samples on a reversed-phase HPLC column. Column, 25 × 1 cm I.D., Beckman Ultrasphere ODS; mobile phase, convex gradient (5) over 25 min starting with water–methanol–acetic acid (60:40:0.5) and ending with composition (30:70:0.5); flow-rate, 1.6 ml/min. Fraction collected for AbA is indicated in the figure. At the end of the collection solvent composition was changed to (20:80:0.5) to remove late eluting impurities. (b) HPLC of AbA. Column, 25 × 0.46 cm I.D., Beckman Ultrasphere Si; mobile phase, chloroform–acetonitrile–acetic acid (94:5:1); flow-rate, 1 ml/min; detection by UV absorption.

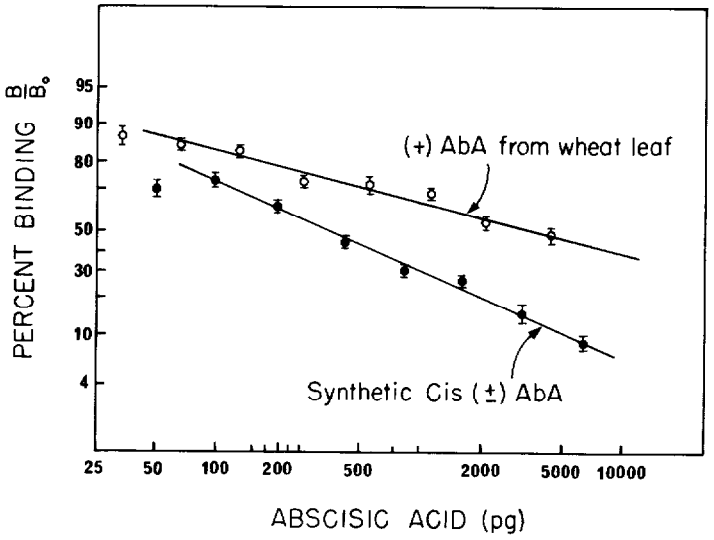

Fig. 2. Standard curves for (±)-AbA and (+)-AbA isolated from wheat leaf drawn on logit-log paper. B = Binding (radioactivity, cpm in the precipitate) in the presence of a given amount of unlabelled AbA. B_0 = binding in the absence of unlabelled AbA.

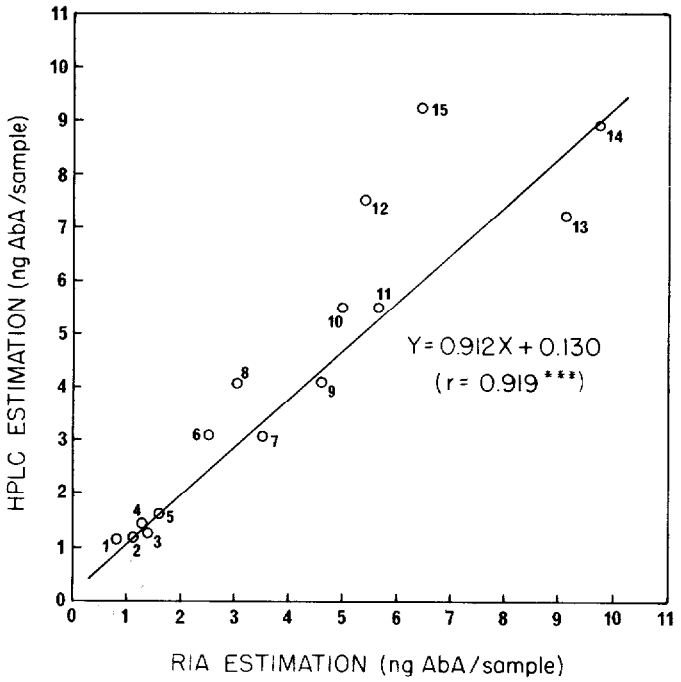

Fig. 3. Correlation between AbA levels per sample estimated by RIA and by HPLC for fifteen different samples taken from eight different wheat varieties. Sample numbers: 1 = Wascana$_1$; 2 = Wascana$_2$; 3 = Wakooma; 4 = Botno$_1$; 5 = Botno$_2$; 6 = Cando$_1$; 7 = Columbus$_1$; 8 = Columbus$_2$; 9 = 3RB$_1$; 10 = 3RB$_2$; 11 = Columbus$_3$; 12 = Cando$_2$; 13 = Sinton; 14 = 3RB$_3$; 15 = Thatcher. r = correlation coefficient.

RESULTS

As shown by the UV absorption at 254 nm, most of the impurities were excluded from the AbA fraction following C_{18} reversed-phase column chromatography (Fig. 1a). Subsequent analysis of this fraction on a normal-phase HPLC silica column gave a distinct peak which had a retention time identical to AbA (Fig. 1b). The height of this peak was used to estimate the AbA content. Identity of the peak as AbA was determined by gas chromatography–mass spectrometry. The peak was methylated with diazomethane and run on a DB-5 capillary column connected to a quadropole mass spectrometer. The fragmentation pattern obtained for methylated AbA under a 70-eV electron impact was similar to that of AbA, methyl ester[6].

Two standard curves were obtained for (±)-AbA purchased from Sigma and for (+)-AbA isolated and quantified by HPLC from wheat leaf (Fig. 2). The standard curve obtained for (±)-AbA was steeper than that obtained for (+)-AbA. The ranges of estimation for the two curves were 50 pg to 6 ng for (±)-AbA and 75 pg to 5 ng for (+)-AbA.

The AbA content of the fraction collected off the C_{18} reversed-phase column was estimated separately by chromatography on a silica column with UV detection

and by RIA using the (+)-AbA standard curve (Fig. 3). AbA estimations by the two methods were highly significantly correlated ($r = 0.91^{***}$). Correlation was further improved ($r = 0.96^{***}$) if samples containing less than 6 ng AbA were analyzed. The cross-reactivity of PA and *trans*-AbA in the radioimmunoassay were 0.2% and 0.5%, respectively.

DISCUSSION

The present study combines the purification capability of HPLC with the high sensitivity of RIA for AbA analysis. Excellent HPLC purification greatly reduces the errors of estimation due to cross-reactivity of antibody due to non-specificity. Cross-reactivity of antibody towards PA was 0.2%. Since PA elutes well before (±)-AbA in C_{18} reversed-phase chromatography[3] any interference by it in the AbA estimation is not expected. Cross-reactivity towards *trans*-AbA was slightly higher than PA, however in plants *trans*-AbA is not expected to occur at significant levels[7]. Weiler[1] and Walton *et al.*[2] tested a number of closely related compounds for cross-reactivity towards an antibody raised in rabbits against AbA. Some of these compounds, such as AbA glucose esters, had cross-reactivity; however, due to polarity differences they are expected to elute much earlier than AbA in C_{18} chromatography. To test whether there was interference by other compounds RIA estimation was compared with HPLC. There was a highly significant correlation ($r = 0.91^{***}$) between HPLC and RIA estimations.

Antibody raised against HSA and (±)-AbA conjugate was more sensitive to (±)-AbA than to (+)-AbA isolated from wheat leaves. This is shown by the steeper standard curve for (±)-AbA than for (+)-AbA. Weiler[1] and Walton *et al.*[2] also noted that antibodies formed against HSA and (±)-AbA conjugate react more towards (−)-AbA than towards (+)-AbA. To overcome this problem and to obtain accurate estimations Walton *et al.*[2] suggested the use of (+)-AbA to obtain standard curves which can then be used to estimate naturally occurring (+)-AbA. However, according to Weiler[1] this will result in loss of assay sensitivity. In the present study we used (+)-AbA isomer isolated from wheat leaf to obtain standard curves. As noted by Weiler[1], the sensitivity of the assay was lowered, however the accuracy of the AbA estimation was not affected. This was shown by the highly significant correlation ($r = 0.91^{***}$) between RIA and HPLC estimations.

RIA is sensitive enough to detect AbA at picogram levels. This reduces the amount of tissue required to be processed for hormone analysis. Small amounts of tissue can be purified easily and rapidly by HPLC, thus making the combination of HPLC and RIA an effective analytical method for AbA analysis.

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