RADIO-IMMUNOASSAYS FOR PLANT GROWTH SUBSTANCES IN AVOCADO

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OPSOMMING

Die beginsels van radio-immuno-bepalingsmetodes (RIB) en die ontwikkeling van tegnieke is kortliks beskryf. Konyne is gei'mmuniseer met bepaa/de p/antgroeireguleerders (PGR's) toegevoeg met bees serum albumien, om antisera te ontwikkel wat vir RIB geskik is. RIB's is vir die sitokiniene isopenteniel adenosien en isopentenieladenien, asook virabsisiensuurgevestig. Diesitokinien RIB's wassensitief tussenkonsentrasies van 100 pg en 100 ng, enabsisiensuurtussen 10 pg en 10 ng. Die RIB's is op ruwe avokado-ekstrakte gebruik om PGR status te bepaal en was vry van besmettingsfaktore.

SUMMARY

The principles of RIA and the development of the techniques are briefly described. Antiserum suitable for RIA was raised in rabbits inoculated with a PGS-BSA conjugate. RIA's have been established for the two cytokinins isopentenyl adenosine and isopentenyl adenine, as well as abscisic acid. The measuring range for the cytokinin RIA's was from 100 pg to 100 ng while the abscisic acid RAI was sensitive from 10 pg to 10 ng. The assays were found to be free from contaminants when used to assay crude avocado extracts for PGS.

INTRODUCTION

Under normal conditions plants grow and develop in an orderly, organised manner. This control, essential in growth and development, is governed by the genetic constitution of the plant and is thus endogenous. One of the important growth controlling systems in plants is provided by plant growth substances (PGS) or plant hormones. PGS may be produced in specific plant parts or organs, and are translocated in low concentrations to other plant parts where they have profound effects in regulating growth (Hill 1980). Exogenous applications of PGS frequently elicit several different effects upon the plant, depending upon other circumstances such as nutrition. Therefore, analysis of PGS is desirable with a view to possible PGS manipulation and hence manipulation of plant

growth.

Quantitation of PGS is usually dependent on some form of physico-chemical method and/or bioassay. All well-established techniques require highly purified plant extracts, usually achieved by solvent extraction and at least one chromatographic step. Established physico-chemical methods are generally specific and reproducible but of low sensitivity. Bioassays on the other hand have increased sensitivity but reduced specificity and reproducibility. Problems encountered with PGS analyses were extensively reviewed by Brenner (1981) and Hogan (1981).

Radio-immunoassays (RIA) were originally developed for human and animal hormone quantitation and later applied to drugs (Chard, 1978). RIA also appears to be ideally suited to PGS determination because of its simplicity (once techniques have been developed), sensitivity, and in most cases, specificity (Landon & Moffat, 1976). These advantages are desirable for the routine analysis of relatively crude plant extracts.

A radio-immunoassay requires three major reagents: antibody (or binder) of appropriate specificity; radiolabelled tracer PGS; and standard PGS preparations (Fig 1). The antibody and excess tracer are mixed in known predetermined concentrations with a number of different concentrations of standard unlabeled PGS. Due to competition for the limited binding sites on the antibody, the quantity of tracer (radiolabelled) PGS binding to the antibody is inversely proportional to the quantity of unlabeled PGS present (Fig 2). A standard Curve (Fig 4) can thus be established. Thereafter unknown plant extracts are substituted in placed on the standard. The reduction in tracer binding to the antibody is directly related to the amount of unknown PGS present. By reading from a standard curve, a direct answer can be obtained.

Probably the single most important aspect of RIA ¡s the production of good antibodies. Molecules with a relatively small molecular mass (<900) are unable to induce the production of antibodies in the host animal (in this research, rabbits) unless covalently attached to the larger molecules prior to immunization (Turk & Parker, 1977). The format for attachment of the PGS to a large protein varies with the functional group or groups available or selected on the PGS. The higher the molar ratio of PGS to protein, the greater the possibility of raising good antibodies (antisera). Antibodies have been raised to most PGS (Weiler, 1982) although the quality of antisera produced varies considerably among researchers. A typical antibody production schedule is presented in Fig 3.

The general principles and problems of RIA are extensively discussed in two recent reviews (Landon & Moffat, 1976; van Vunakis, 1980).

This paper is a progress report on the development of RIA's for a range of PGS, using techniques tailored specifically to our needs. This novel technique is being used to investigate certain avocado problems where PGS are believed to have an important controlling role.
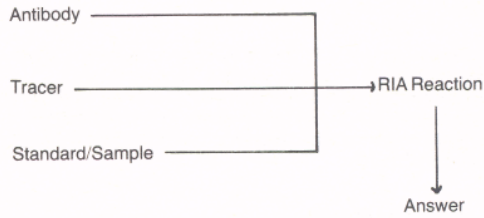
BASIC PRINCIPLE OF RIA

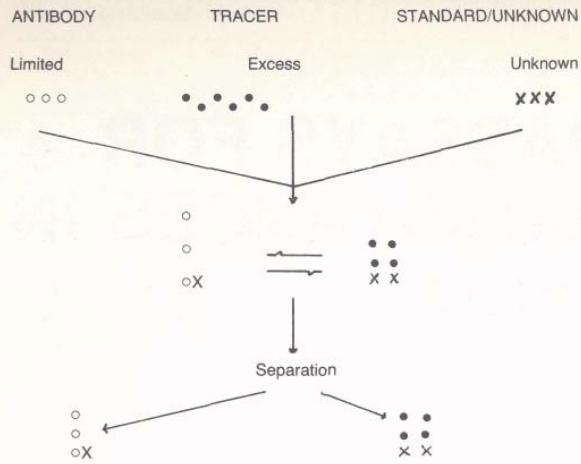FIG 1. Principle components of Radio-immunoassay.

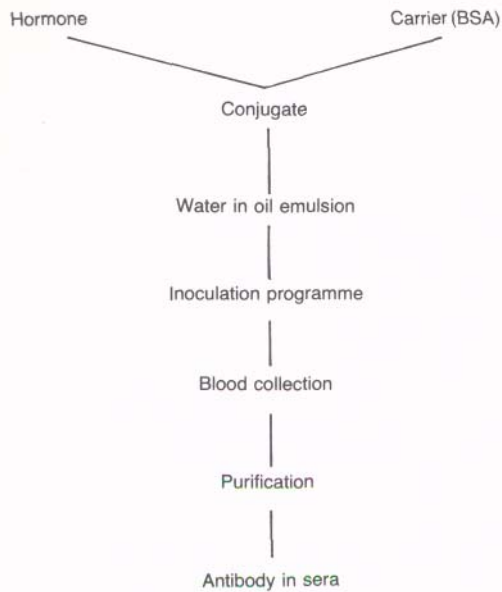FIG 2. Basic principle of Radio-immunoassay.

TYPICAL ANTIBODY PRODUCTION

FIG 3. Schematic antibody production flow chart.

(i) IPA slope = 2,2
r = 0,998
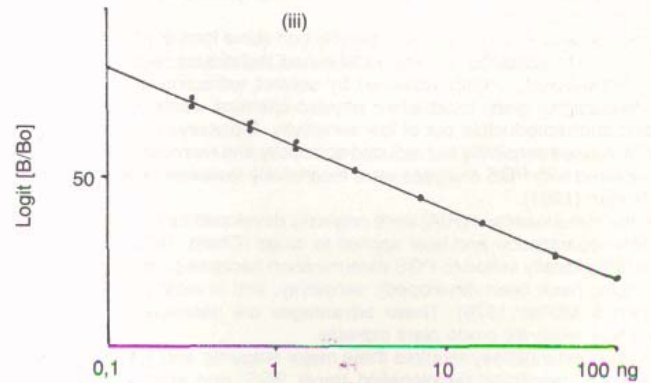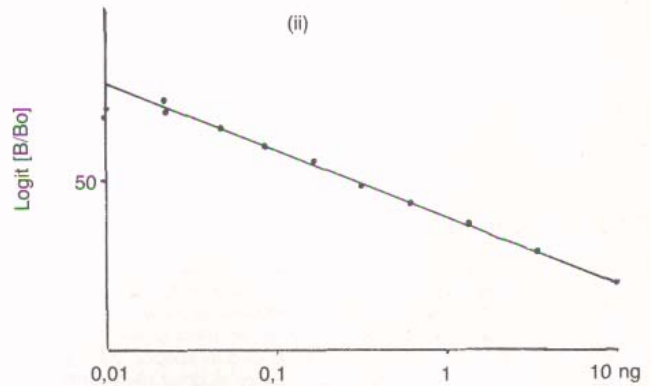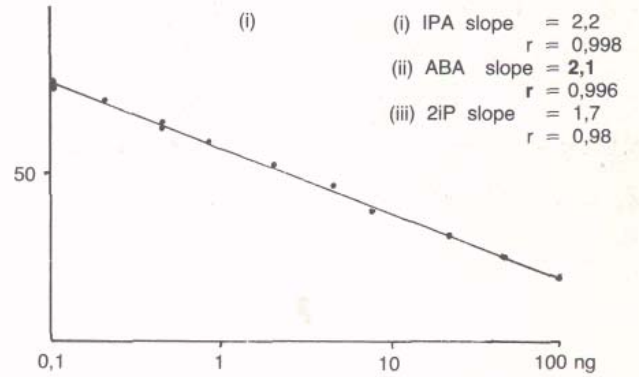(ii) ABA slope = 2,1
r = 0,996
(iii) 2iP slope = 1,7
r = 0,98

FIG 4. Standard curves (log-logit format) for (i) Iso-pentenyl adenosine, (ii) Abscisic acid and (iii) Iso-pentenyl adenine. Logit [B/Bo] = ln

$$\frac{B/Bo}{100 - B/Bo}$$

MATERIALS AND METHODS

Coupling PGS to a Protein Carrier:

For cytokinins only the riboside moiety is used for immunogen synthesis at present. Attachment is to the hydroxyl groups on the ribose unit via the sodium periodate reaction (Erlanger & Beiser, 1964). Ratios for isopentenyl adenosine to BSA were 16,4:1 and for zeatin riboside to BSA 15,8:1.

The linking of auxin to BSA is through either the carboxyl group (Fuchs, Mayak & Fuchs, (1972) whereby we obtained a 4,3:1 molar ratio, or through the active nitrogen (Pengelly & Meins, 1977) which yielded a conjugate with a molar ratio of 21,6:1. Abscisic acid (ABA) and gibberellic acid (GA_3) were linked through the carboxyl group using a modification of the method of Fuchs and Fuchs (1969). The molar ratio of gibberellic acid to BSA was 28:1, while that of abscisic acid has not yet been determined.

Antiserum Production

Smalt amounts of conjugate (1 mg per rabbit) were taken up in saline (1 ml per 1 mg conjugate) and emulsified in Freunds complete adjuvant (2 ml adjuvant per ml saline). The resultant stable emulsion was injected into ± 20 intradermal sites on the animal's back to spread the response. The rabbits also received two subcutaneous injections in the neck skin area. There were 5 rabbits in each group.

Blood was collected from the marginal ear vein 30 and 60 days after inoculation, and assayed for liter and cross-reactivity. Blood was purified by the method of Garvey, Cremer & Sussdorf (1977). Freshly drawn blood was incubated at 37 °C for two hours to promote clot formation, and stored at 4°C for 12 hours to permit clot contraction. Serum was decanted and cleared by centrifugation (1,000 ng for 10 min), removed and snap frozen (antisera were not pooled).

Tracer Production and Purification

Custom preparations of ^{3}H-IAA, ^{3}H-isopentenyl adenosine, ^{3}H-zeatin riboside and ^{3}H-gibberellic acid were supplied by the Radiochemical Centre (Amersham, UK). Only labile tritium was removed in the purchased product, therefore further purification was necessary. ^{3}H-cytokinin purification was achieved with Sephadex LH-20 (16 x 900 mm) in 40% ethanol. ^{3}H-IAA was purified by TLC (silica gel GF_{254} - solvent ethyl acetate: chloroform: methanol 50:10:1). ^{3}H-gibberellic acid has not yet been purified. ^{3}H-Abscisic acid was a catalogue item and did not require purification.

Extraction of Plant Material

Extraction was carried out by homegenising tissue and extracting for 24 hours in 80% ethanol or 90% ethanol (acidified with 1% acetic acid). The extract was cleared by centrifugaron (1 000 g for 10 min). Further purification, usually involving some form of TLC, was necessary, depending upon the type of PGS being analysed.

Radio-immunoassay

Assays were performed as follows: standards and samples were processed in

duplicate. The incubation mixture in each tube was: 100µl buffer (1% BSA in phosphate buffered saline pH 7), 100 µl dilute anti-serum (or water for MSB), 100 µl tracer solution and 100 µl standard or sample previously reduced to dryness in the tube. After mixing, the tubes were incubated for 10 to 15 min (depending on assay) at 37 °C. The reaction was allowed to stabilize in ice for 60 min after which 800 µl of dextran-coated charcoal was added to each tube. After centrifugation (1 500 g for 10 min) the supernatant (bound fraction) was decanted into 12,5 ml scintillant and counted for radioactivity.

RESULTS AND DISCUSSION

Radio-immunoassay for two cytokinins, isopentenyl adenosine (Cutting, Lish-man, Van der Hoven & Wolstenholme, 1983), and for isopentenyl adenine and abscisic acid have been developed. Standard curves have been plotted using the log-logit probability format especially developed for immunoassays. Typical standard curves for these three assays are presented (Fig 4).

The measuring range for ABA is 10 pg to 10 ng, while for the two cytokinins the range is 100 pg to 100 ng. The increased sensitivity of the ABA RIA is thought to be due to the higher specific activity of the radioactive tracer.

Various separation techniques have been applied to the assays. Precipitation techniques (Chard, 1978) using saturated ammonium sulphate or polyethylene glycol gave non-specific binding values in excess of 5%. However, this technique has proved suitable for other workers (Weiler, 1980). Double antibody precipitation proved too time-consuming although results were very reproducible. Dextran coated charcoal with relatively short incubation times proved the best, with non-specific binding between 1 and 3 per cent.

The assays have been applied to routine analysis of avocado extracts, particularly for ABA in fruit. As the ABA assay is non-specific for ABA, ABA methyl ester and ABA glucosyl ester, a composite consisting of bound and free fractions of ABA was obtained when assaying crude extracts. As the bound forms are physiologically inactive, separation of these forms is necessary to obtain meaningful results. This was achieved by TLC. By assaying the free fraction, free and total ABA can be determined.

Once assays for the other zeatin-type cytokinins, *as* well as auxin and gibberellic acid, are complete more thorough studies can be undertaken. Two topics being researched at present are etiolation physiology (as in avocado clonal rootstock propagation) and PGS changes in fruit development from flowering to harvest and during ripening. Practical applications from this type of research could be improved rooting of etiolated cuttings, and an understanding of the multifaceted fruit development and ripening sequence with the associated problems of stress, nutrition and fruit quality as applicable to the South African situation.

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