A REVISED METHOD OF GIBBERELLIN ASSAY BY OAT ENDOSPERM TISSUES

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Introduction

Gibberellins have been known to exert a potent promoting effect on plant growth. In particular, the growth of dwarf mutants of some plants as pea[2], French bean[1], maize[16] and rice[13] was greatly enhanced. Accordingly, the use of these mutants as a tool in detection and estimation of plant gibberellin has been advocated. In the face of the evidence stated above, these bioassays are not always well suited for investigation of natural gibberellins due to poor availability of these seeds.

On the other hand, gibberellins also enable to enhance the release of reducing sugar from cereal endosperm tissues[3),14),19)]. According to the fact that the amount of reducing sugar released from the tissues were proportional to logarithm of gibberellin concentration applied, these endosperm tissues provided a higher sensitive bioassay system for gibberellin compared to the dwarf mutant bioassays[10),12),15)].

Conventionally, oat seeds have long been obtained quite easily in common laboratories as useful materials for auxin[9] and cytokinin[18] assays. The following experiment is worthwhile to make certain the possibility, therefore, that the endosperm of oat seed may also provide a valuable material for gibberellin bioassay.

Materials and Methods

Bioassay for gibberellin activity

Seeds of oat (*Avena sativa* L. cv. Victory) stored for 3 years were hulled by hand and selected for uniformity. The hulled seeds were cut in half transversely and the halves with embryo were discarded. Unless otherwise specified, the following procedures were as described below. The havles with endosperm, henceforth referred to as endosperms, were sterilized with 0.4% sodium hypochlorite solution for 5 min, rinsed 5 times with sterile

water and then blotted briefly on sterilized filter paper. Four fragments of the endosperms were transferred to a 30-ml Erlenmeyer flask with 2 ml test solution containing 50 mM phosphate buffer, pH 6.8, and sample to be tested, and then incubated for 48 hr at 25°C in the dark. Prior to incubation, the flasks were sealed with double layered aluminum foils and autoclaved for 3 min. After incubation period, the solution was passed through a DEAE-cellulose filter paper to remove amino acids and insoluble impurities such as starch grains. Aliquot of the filtrate (usually 0.2 ml) were taken for measurement of reducing sugar by the method of NELSON[11].

Extraction of gibberellin from plant materials

Ninety fragments of first internodes obtained from etiolated patato shoots were collected and homogenized with sufficient amount of ethanol to produce a final 70% ethanolic extract and kept overnight at 4°C. After filtration, the filtrate was evaporated to remove ethanol, adjusted to pH 2.5 with 1 N HCl and then extracted three equal volumes of ethyl acetate. The combined ethyl acetate extracts were evaporated to reduce its volume and shaken with three half volumes of 1 M phosphate buffer (pH 6.2). The combined buffer extracts were adjusted to pH 2.5 with HCl and re-extracted with ethyl acetate. The extract was dried over sodium sulphate anhydride and evaporated. The resulting residue was subjected to paper chromatography developed with isopropanol : ammonia : water (10 : 1 : 1 v/v). After drying, the chromatograms were cut into 10 equal Rf strips and each of them was placed directly into test solution.

Results and Discussion

Sterilization of endosperm

To reduce the incidence of microbial contamination, some workers[5),12)] sterilized barley endosperms with calcium or sodium hypochlorite solution over 3 hr. If such a severe sterilization is liable to injure aleurone layers where α-amylase is synthesized in response to gibberellin, lowering of sensitivity to gibberellin should occurre in the treated endosperm tissues. Therefore, in following experiment, brief durations of sterilization period were tested to circumvent this problem. The oat endosperms were divided into three groups and each of them was sterilized with 0.4% sodium hypochlorite solution for 1, 5 and 10 min respectively, succeeded by incubation in the test solutions. Amounts of reducing sugar liberated from these endosperms were shown in Fig. 1. No sign of contamination was observed in any flask during incubation periods. The result indicates that 1-minute sterilization

is sufficient to prevent contamination and 10-minute sterilization decreased in the amount of reducing sugar. Consequently, sterilization with sodium hypochlorite solution for 5 min was employed in all subsequent experiments.

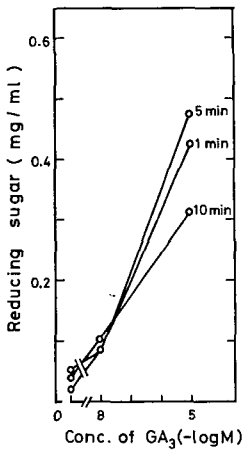

Fig. 1. Release of reducing sugar by oat endosperm incubated in GA_3 solutions after sterilizing with 0.4% sodium hypochlorite solution for 1, 5 and 10 min.

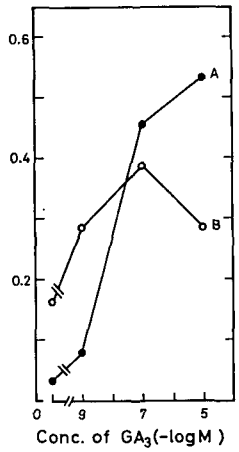

Fig. 2. Comparison of reducing sugar release by oat endosperm incubated in GA_3 solutions autoclaved (A) and added 100 μg/ml streptomycin sulphate (B).

Sterilization of test solution

To prevent the contamination by micro-organisms, sterilization of the test solution was also necessary. In considering the possibility that antibiotics may produce some detrimental effect on the *de novo* synthesis of α-amylase, an addition of antibiotic to the test solutions as stated by NICHOLLS and PALEG[12] and JONES and VARNER[8] seems to be not suited for these experiments. When the test solution was sterilized by autoclaving for 3 min in return the application of antibiotic, the amount of reducing sugar liberated into the solution were porportional to the logarithm of the GA_3 concentration (Fig. 2). Contrarily, the observed proportionality was disturbed by the addition of streptomycin sulphate at a concentration of 100 μg/ml. Thus it was assured that GA_3 is stable to autoclaving at this condition.

Depth of test solution

It has been reported that gibberellin-induced α-amylase synthesis in barley grain is dependent on aerobic condition[19], and decreasing the release of reducing sugar was observed with increasing depth of the test solution[5]. By using a 30-ml Erlenmeyer flask as a test vessel, an addition of 2 ml test

solution maintained depth of about 1 mm and some part of the added oat endosperm always projected above the surface of the solution. When the endosperms were completely submerged in the same volume of test solution (about depth of 5 mm) which was holded in a vial (24×50 mm), the amount of reducing sugar released was considerably decreased (Fig. 3). The result indicates that an aerobic condition is necessary to ensure a high sensitivity to gibberellin in oat endosperm assay.

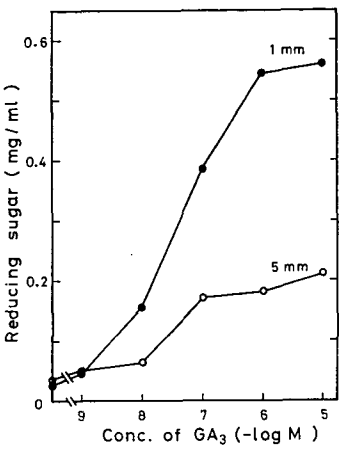

Fig. 3. Effect of depth of test solution on GA_3-induced reducing sugar release by oat endosperm. 1 mm : 2 ml test solution/30 ml Erlenmeyer flask. 5 mm : 2 ml test solution/vial (24×50 mm).

Optimal pH of test solution

The solution of 10^{-5} M GA_3 was adjusted to different pH values with a McIlvain's citrate-phosphate buffer solution and tested. The result represented in Fig. 4 shows that the amount of reducing sugar released from the endosperms reached its maximum over a narrow pH range (pH 3.8-4.8). However, when various GA_3 concentrations were also tested at pH 4.8 and 6.8, the amounts of sugar released at pH 6.8 were more distinctly proportional

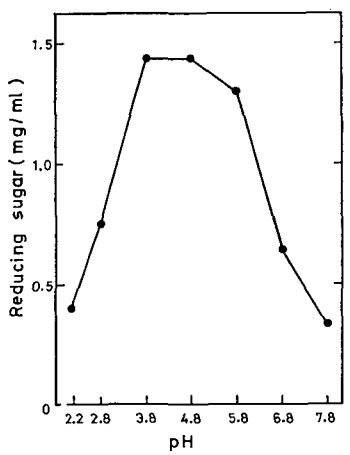

Fig. 4. Effect of pH of test solution on reducing sugar release by oat endosperm incubated in GA_3 solution of 10^{-5} M.

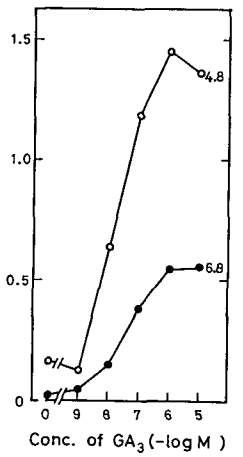

Fig. 5. Comparison of reducing sugar release by oat endosperm incubated in GA_3 solutions of pH 4.8 and pH 6.8.

to the logaithm of GA_3 concentration, especially at low concentration below 10^{-9} M, as compared to that at pH 4.8 (Fig. 5). In view of the well known fact that natural gibberellins were generally contained at low concentration in plant materials, pH 6.8 is considered to be well suitable for measurement of gibberellin in plant tissues.

Duration of incubation period

In order to determine a suitable incubation period, the time course change in the amount of reducing sugar released was examined at GA_3 concentrations of 10^{-8} and 10^{-5} M. As shown in Fig. 6, at both GA_3 concentrations, the amount of reducing sugar increased linearly with time from 24 hr of lag period onward. From the experiment, a 48-hr incubation is found to be appropriate for rapid determination of gibberellin activity.

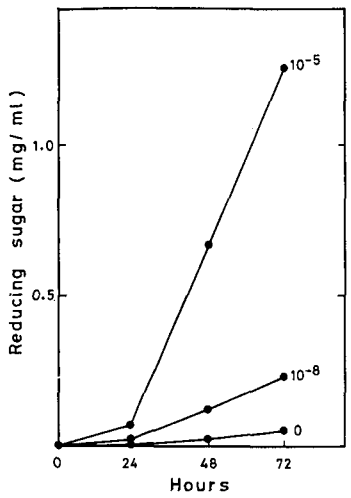

Fig. 6. Time course of reducing sugar release by oat endosperm incubated in GA_3 solution of 0, 10^{-8} and 10^{-5} M respectively.

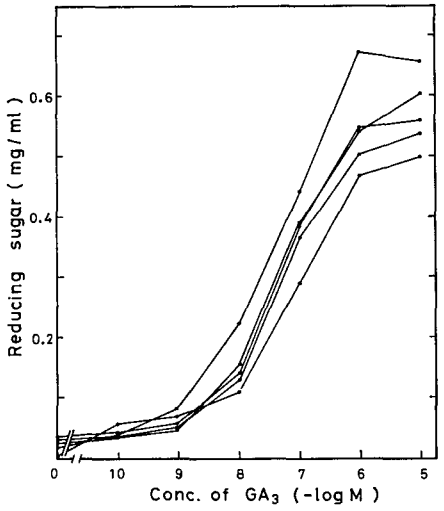

Fig. 7. Release of reducing sugar by oat endosperm incubated in solutions of various GA_3 concentrations in 5 replicated tests.

Reproducibility of standard curve

To calculate gibberellin content in plant materials by means of a bioassay, it would be preferable that the standard curve to gibberellin concentration is reproducible. Five runs of the standard curves obtained with the similar procedure as described in the Methods are shown in Fig. 7. The result established that GA_3 can be detected precisely within the range from 10^{-9} M

(0.00035 μg/ml) up to 10^{-5} M (3.5 μg/ml). The reproducibility of this test is definitely better than that reported by COOMBE *et al.*[6] using barley endosperm.

Sensitivity to other plant growth regulators

The endosperms were incubated in mixed solutions of GA_3 (0, 10^{-8} and 10^{-5} M) and another plant growth regulator (0, 10^{-8} and 10^{-5} M). No significant effect on the release of reducing sugar was observed by the addition of either indol-3-acetic acid (IAA) or kinetin (Fig. 8). However, the release of the sugar was slightly inhibited by addition of abscisic acid (ABA). The present result was in agreement with the finding that the α-amylase production in aleurone layer of barley grain was inhibited by the addition of ABA[4),7),17)].

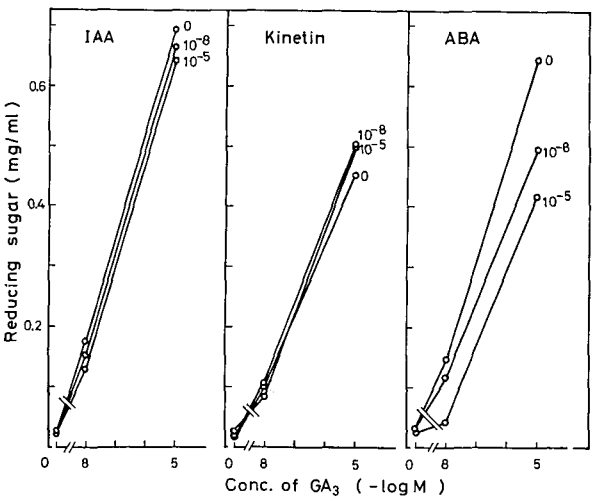

Fig. 8. Effect of another plant growth regulator on GA_3-induced reducing sugar release by oat endosperm.

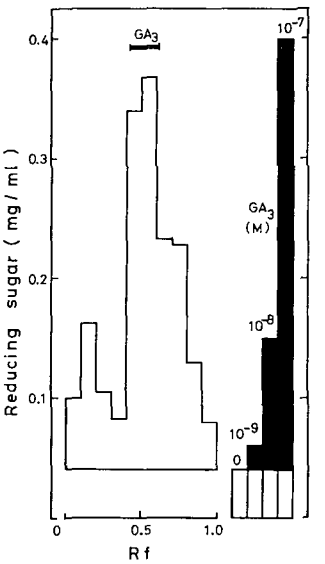

Fig. 9. Distribution of gibberellin activity of extract equivalent to 25 g in fresh weight of etiolated potato first internode. Gibberellin activity was detected by means of the oat endosperm assay. Paper chromatogram was developed with iso-propanol : ammonia : water (10 : 1 : 1 v/v).

Estimation of natural occurring gibberellins

With the intention of evaluating this assay system, extract of etiolated potato shoots was chromatographed on paper and measured their gibberellin activity. Two peaks of the activity could be detected at Rf 0-0.2 and Rf 0.4-0.6 (Fig. 9). The fast-moving peak (Rf 0.4-0.6) was found to co-chromatograph with GA_3. In terms of GA_3 equivalent, total amount of gibberellin per Kg of the fresh tissue was calculated to give about 6 μg.

Summary

A convenient bioassay method based on the gibberellin-induced release of reducing sugar from barley endosperm[12], was revised by use of oat endosperm in place of barley. The present method has advantages in convenient preparation of endosperms and a high sensitivity to gibberellic acid. Gibberellic acid can be detected within the range from below 10^{-9} M (0.00035 μg/ml) up to 10^{-5} M (3.5 μg/ml).

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