Gibberellic Acid—Binding Proteins from Pea Stems

R. Konjević, D. Grubišić, R. Marković, and J. Petrović
Institute of Botany, Faculty of Science and Institute for Biological Research, Takovska 43, 11000 Beograd, Yugoslavia

Summary. The formation of complexes of gibberellic acid (GA₃) and proteins under in vitro conditions was studied. It was shown that labelled GA₃ binds to soluble cytoplasmic proteins, although a considerable amount of radioactivity remains in the pellet containing nuclei and cell debris. GA₃-protein complexes are excluded from Sephadex G-10 column with the void volume. They sediment in linear sucrose density gradients as three distinct peaks, having higher S values than bovine serum albumin, used as a marker. Soluble GA₃-protein complexes can be separated into four zones of radioactivity upon ion exchange chromatography on DEAE-Sephadex A-50 column, each of them eluting with a different KCl concentration. Agarose gel electrophoresis of GA₃-protein complexes reveals two zones of radioactivity at the anodic part of the electrophoretogram. After extraction of the complex with ethanol, more than 90% of radioactivity is found in the ethanolic phase, which indicates that the binding is not covalent. GA₄⁺ and GA₁₃ decrease the binding of GA₃ to cytoplasmic proteins for 30%, suggesting that some common binding sites exist at the same binding proteins.

Introduction

Investigations of the primary step in the action of steroid hormones in animal systems have revealed the existence of specific binding proteins in target tissues (Jensen et al., 1971). Some reports indicate that plant hormones, auxins (Matthysse and Phillips, 1969) and cytokinins (Matthysse and Abrams, 1970), enhance RNA synthesis directed by isolated pea nuclei or chromatin in the presence of protein mediators. Attachments of indolyl-3-acetic acid (IAA) and benzyladenine (BAP) to s-RNA have also been reported (Bendaña et al., 1965; Fox, 1966). The close chemical resemblance of IAA to an amino acid, and the purine nature of BAP, makes it difficult to estimate whether the binding to macromolecules is either simply due to that similarity or represents a prerequisite for their hormone action.

Gibberellins are a distinct class of compounds, having no similarity to basic metabolites and, therefore, evidence demonstrating the existence of gibberelin—protein complexes could be more meaningful for elucidation of the mechanism of hormone action. Recent findings of Stoddart et al. (1974) show the existence of two protein fractions in total homogenate of dwarf pea seedlings with an affinity for GA₁₃.

Although it is known that GA₃ is not the native hormone of pea seedlings (Railton and Reid, 1974; Frydman et al., 1974) this substance has been used in this study, since it greatly affects pea stem growth. In this paper we present the evidence on the binding of GA₃ to cytoplasmic proteins and data concerning some characteristics of these complexes.

Materials and Methods

Material

In all experiments pea seeds (Pisum sativum L. cv. Alaska) were surface sterilized with 5% calcium hypochlorite, imbibed in water for five hours and then sown in moist vermiculite. Seedlings were grown in darkness at 26°C for seven days and then harvested. Methylene [8-¹⁴C] gibberellic acid, specific activity 6.1 mCi/mM, was purchased from Radiochemical, Amersham, England. Sephadex G-10 and DEAE-Sephadex A-50 were obtained from Pharmacia, Uppsala, Sweden and prepared as recommended by the supplier. Samples of GA₄⁺ and GA₁₃ were kindly supplied by Dr. Broadbent, I.C.I., U.K.

Preparation of Pea Cytosol

Stems were homogenized in TSS solution (0.25 M sucrose, 0.025 M KCl, 0.010 M MgCl₂, 0.050 M TRIS; HCl, pH 7.55) supplemented by 0.001 M β-mercaptoethanol. The crude homogenate was filtered

Abbreviations: BAP = benzyladenine; GA = gibberellic acid; IAA = indolyl-3-acetic acid; TSS = tris-sucrose-salts
through miracloth, then incubated with \(^{14}C\)GA\(_3\) (0.1 \(\mu\)Ci per ml of homogenate) for 30 min at room temperature, centrifuged at 10,000 \(\times\) g. The pellet and supernatant radioactivity was measured in a Beckman CPM-100 scintillation counter in Bray's (1960) scintillation liquid. Protein content was determined according to Lowry et al. (1951) using bovine serum albumin as a standard.

**Molecular Sieving Chromatography**

In one series of experiments the supernatant obtained after centrifugation (\(S_{100}\)) was sieved through a Sephadex G-10 column, equilibrated with TSS solution. Fractions of 1 ml were collected and measured for protein content and radioactivity. Fractions containing both protein and radioactivity were pooled and dialysed overnight against low salt buffer supplemented by labelled GA\(_3\) (5 \(\mu\)Ci/ml) in order to prevent possible losses in specific activity of the hormone-protein complexes. Pooled fractions were then concentrated in a Sartorius membrane filter apparatus and used for further work.

**DEAE-Sephadex A-50 Chromatography**

DEAE-Sephadex A-50 was equilibrated with TSS solution. After application of the concentrated sample, the column was washed with TSS until the A\(_{280}\) of the eluate fell below 0.03 and then the elution was performed with increasing linear KCl gradient in TSS buffer. Fractions of 2 ml were collected and checked for protein content (Warburg and Christian, 1941), radioactivity and chloride concentration. Chloride concentration was determined by argentometric titration using \(K_2CrO_4\) as an indicator.

**Sucrose Density Gradient Centrifugation**

Aliquots of samples containing GA\(_3\)-protein complexes after molecular sieving were used for this analysis. The sucrose density gradient centrifugation was performed as follows: 0.3 ml of samples or bovine serum albumin were applied to a 4.6 ml of 5-20% linear sucrose gradient containing 0.025 M KCl, 0.001 M EDTA, 0.050 M TRIS; HCl, pH 7.55, and centrifuged for 20 h at 39,000 rpm, in the SW 39 rotor of a Spincos-Beckman model L3-50 ultracentrifuge. Fractions were collected after puncturing the bottoms of the centrifuge tubes directly into Bray's (1960) scintillation liquid and counted as above. The sedimentation profile of bovine serum albumin was determined by optical density reading at 280 nm.

**Agarose Gel Electrophoresis**

Gel preparation and electrophoresis were performed as described by Johansson (1972). After layering 0.8% agarose solution in 0.085 M veronal buffer, pH 8.6, onto a glass slide, a slit-forming device was placed along the gel plate to obtain 10 \(\mu\)l slits for application of the samples, ten \(\mu\)l of the concentrated samples containing GA\(_3\)-protein complexes were filled into the slits using a micropipette. Bovine serum albumin was also run on the same plate as a marker. The other gel plate was used for the electrophoretic run of the free hormone. Electrophoresis was carried out for 60 min at 160 mA, 220-240 V. The apparatus was cooled with tap water. After that the gel was divided into two parts, one of them stained for protein and bovine serum albumin localization, the other cut into 5-mm-long slices for radioactivity measurements. The gel slices were dissolved in 95% Protosol solution (N.E.N., U.S.A.) at 60°C for 2 h, and counted in toluene based scintillation liquid.

**Results**

Figure 1 shows the elution profile of \(S_{100}\) supernatant sieved through Sephadex G-10 column. As can be seen from the Figure, two zones of radioactivity were obtained. The first one corresponded to hormone bound to proteins, as judged by the protein content curve, and the second one was due to free GA\(_3\).

The results of DEAE-Sephadex A-50 chromatography are presented in Figure 2. This analysis resulted in the resolution of four zones of radioactivity: firstly in the fractions eluted between 0.05 and 0.12 M KCl, secondly between 0.14 and 0.20 M KCl, thirdly between 0.24 and 0.30 M KCl and fourthly between 0.31 and 0.34 M KCl. From OD and radioactivity profiles it can be seen that the protein classes forming

**Fig. 1.** The separation of GA\(_3\)-binding protein(s) from free hormone on Sephadex G-10 column. ○—○ protein content; ⋅—⋅ radioactivity

**GA\(_{4+7}\) and GA\(_{12}\) Competition Experiments**

\(S_{100}\) supernatant was pretreated with GA\(_{4+7}\) and GA\(_{12}\), in concentrations 100 times higher than the concentration of labelled GA\(_3\), for 5 min, and then incubated with \(^{14}C\)GA\(_3\) (0.5 \(\mu\)Ci per ml of cytosol) for 30 min at room temperature. The control sample was incubated with labelled gibberellin for the same period of time. The incubation was stopped by adding a saturating amount of (NH\(_4\))\(_2\)SO\(_4\) to the incubation mixture. Protein content and radioactivity were measured in the pellet obtained after centrifugation.

**Extraction with Ethanol**

The bound gibberellin was extracted by adding absolute ethanol into the cytosol preincubated with \(^{14}C\)GA\(_3\). The sample was then centrifuged and radioactivity was measured in the pellet as well as in the supernatant.