PREPARATION OF GIBBERELLIN AFFINITY ADSORBENTS*

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Received 16 September 1975

1. Introduction

The transport and mechanism of action of animal hormones are mediated mostly by receptor proteins [1,2]. These proteins are present in target tissues in very low amounts. Much success in the investigation of these proteins and the enzymes of hormone metabolism has been achieved by introduction of affinity chromatography [3–7]. Contrary to animal hormones for plant hormones far less is known on receptor proteins and enzymes of hormone metabolism. First attempts to introduce affinity chromatography have been made by Venis [8,9] coupling the synthetic auxin 2,4-dichlorophenoxyacetic acid via a lysine spacer molecule to agarose.

The present publication shows different methods of linking gibberellic acid (GA_3) to agarose in order to get suitable affinity adsorbents for the plant hormone group of gibberellins.

2. Material and methods

Agarose (Sepharose 4-B) was purchased from Pharmacia, GA_3 from Phylaxia Budapest.

[U-^{3}H] GA_3 (16 mCi/mmol) was prepared by a catalytic Wilzbachexchange reaction [10] and purified by different column chromatographic methods.

GA_3-anhydride was synthesized according to [11].

[U-^{3}H] GA_3 aminoalkylamides were prepared by reaction of [U-^{3}H] GA_3 anhydride with the corresponding diamines [12]. All other chemicals used were of reagent grade.

Radioactivity was counted in a Packard liquid scintillation spectrometer Tricarb 3365. Radioactivity of solid agarose derivatives was measured after disaggregation of the dried materials in a Micro-Mat BF 5010 (Friesecke and Hoepfner/Berthold). The activation of agarose with cyanogen bromide and the synthesis of ω-aminoalkyl-agarose and carboxyalkyl-agarose derivatives were realized by methods described by Cuatrecasas [13]. The *N*-hydroxysuccinimide ester of carboxyethyl-agarose was synthesized according to Cuatrecasas and Parikh [14].

2.1. *Preparation of carrier-fixed gibberellins*

(a) 1.5 ml aminoalkyl-agarose gel suspended in 4 ml 0.1 M $NaHCO_3$ (pH 8.5) were treated for 30 h at 4°C with 36.5 μmol [U-^{3}H] GA_3 anhydride (0.92 mCi/mmol) in 1.0 ml 1.4-dioxane. The reaction product was washed extensively with 0.1 M $NaHCO_3$ and water.

(b) 12 ml agarose gel activated with cyanogen bromide (200–270 mg BrCN/ml gel) were suspended in 15 ml 0.1 M $NaHCO_3$ and treated for 20 h at 4°C with 344 μmol [U-^{3}H] GA_3 4-amino-*n*-butylamide (32.6 μCi/mmol). After washing out the excess of unreacted amide the gel was incubated with 10 ml of 1 M ethanolamine in 0.1 M $NaHCO_3$ to remove remained active sites at the agarose. For the further purification 0.1 M $NaHCO_3$ and water were used.

(c) 1 ml of *N*-hydroxysuccinimide ester of carboxyethyl-agarose in 3 ml 0.1 M acetate buffer pH 6.3 was treated for 15 h at 4°C with 48 μmol [U-^{3}H] GA_3 4-

* Dedicated to Professor Dr Dr h. c. mult. K. Mothes on the 75th anniversary of his birthday.
Gibberelline, XL. Part XXXIX: Lischewski, M. and Adam, G. (1975), Tetrahedron Letters, in press.

amino-*n*-butylamide (32.6 μCi/mmol). The gel was washed extensively with 0.1 M $NaHCO_3$ and water. In all cases the purification procedure was continued until the washings were free of radioactivity.

3. Results and discussion

By applications of affinity chromatography the importance of introducing spacer arms with optimal chain length between carrier-matrix and ligand has been shown [15,16]. In this way steric hindrance of the protein–ligand interaction due to the matrix may be minimized. Therefore, also the gibberellin molecules were separated from the agarose matrix by spacer arms of different chain length (aliphatic diamines or ω-amino acids). There are generally two possibilities for coupling the ligand to the matrix via a spacer arm. The spacer arm may be coupled to the matrix and then the ligand is attached to the free functional group of the spacer arm or, alternatively, the ligand may be coupled to the spacer arm prior to the immobilization at the matrix. Both ways have been chosen for synthesis of gibberellin affinity adsorbents.

As is shown in fig.1 agarose was activated by cyanogen bromide and aliphatic diamines (with 4 to 6 carbon atoms) were attached by one of the amino groups to the activated carrier. By an aminolytic reaction the aminoalkyl derivatives of agarose split [U-^{3}H] GA_3-anhydride forming a stable amide bond. The resulting affinity adsorbents contain 3.5 to 9.5 μmol of covalently bound GA_3/ml agarose gel, calculated from the radioactivity.

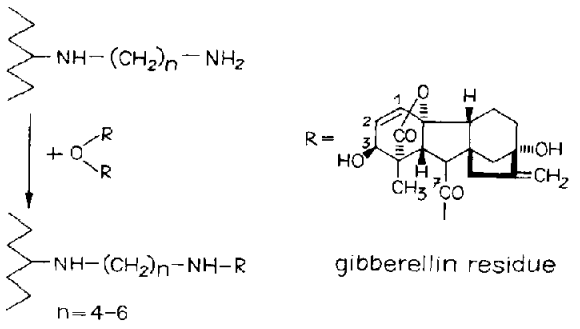

Fig.1. Reaction of aminoalkyl-agarose with GA_3-anhydride yielding carrier-fixed GA_3.

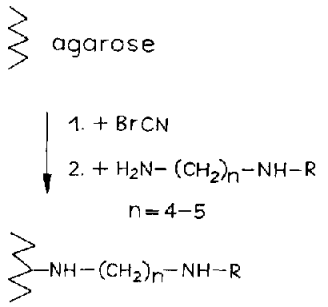

Fig.2. Reaction of GA_3-amino-n-alkylamides with cyanogen bromide-activated agarose. R see fig.1.

On the other hand [U-^{3}H] GA_3 aminoalkylamides were prepared [12] using aliphatic diamines with 4 to 5 methylene groups and [U-^{3}H] GA_3 anhydride as components of reaction. These GA_3-aminoalkylamides containing one primary amino group are suitable for direct coupling to cyanogen bromide-activated agarose (fig.2). By this method about 10 μmol GA_3/ml agarose gel were bound.

The method described above depends on an unprotonated primary amino group and, therefore, it works optimally only in strong alkaline solution (pH 9 to 11). Under these conditions the GA_3 molecule is rather instable and chemical changes of the attached gibberellin cannot be excluded.

A method working under mild conditions (4°C, pH 6.0 to 8.5) is the coupling of a ligand with its primary amino group to an activated ester derivative of agarose [14]. For this reason agarose was substituted by β-alanine and the resulting carboxy-ethyl-agarose esterified by *N*-hydroxysuccinimide. This activated ester was treated at pH 6.3 with [U-^{3}H] GA_3 4-amino-*n*-butylamide (fig.3). However, only 0.5 to 1 μmol GA_3/ml agarose gel were attached to the matrix indicating that in this case an unprotonated amino group is necessary, too.

Besides the demonstration of ligand binding by measurements of radioactivity the prepared affinity adsorbents were soluble in 85% sulfuric acid and showed in this state the typical fluorescence spectrum of GA_3 [17].

In all prepared gibberellin affinity adsorbents the 7-carboxylic group of the gibberellin molecule has been used for the attachment of the plant hormone to the agarose-carrier. From structure–activity rela-

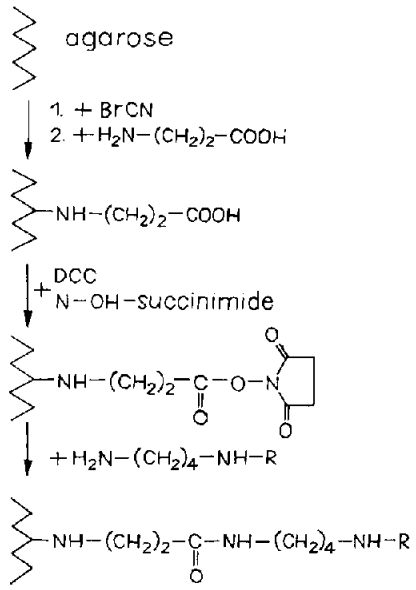

Fig.3. Scheme of reactions involved in the preparation of carrier fixed GA_3 by synthesis of *N*-hydroxysuccinimide ester of carboxyethyl-agarose and aminolysis with GA_3-4-amino-*n*-butylamide. R see fig.1; DCC = *N,N'*-dicyclohexylcarbodiimide.

tionships (cf. [18]) the unsubstituted 7-COOH group is known to be important for high biological activity. Therefore, the prepared adsorbents possibly may be of reduced efficiency in the isolation of receptor proteins. However, they may possess a high affinity to enzymes of gibberellin metabolism.

Attempts to synthesize gibberellin affinity adsorbents using the 3-hydroxyl group for linking the hormone to the carrier were unsuccessful because of instability of the formed ester bond. A useful but difficult way for the synthesis of gibberellin affinity adsorbents will be the coupling of the gibberellin molecule at positions 1 and 2, respectively, to the carrier. Substitution of these positions is nearly without influence on biological activity. Such adsorbents are in preparation.

First application of the described gibberellin affinity adsorbents in studies on enzymes of gibberellin metabolism in *Phaseolus coccineus* L. showed their ability to retard a protein fraction which could be eluted with 1 M sodium chloride solution. The significance of this fraction is now being investigated.

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