ISOLATION AND PURIFICATION OF CYTOKININ BINDING PROTEIN FROM TOBACCO LEAVES BY AFFINITY COLUMN CHROMATOGRAPHY

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Cytokinin binding protein from tobacco leaves was isolated and purified to a single protein by means of affinity chromatography on benzyladenine-linked Sepharose column combined with polyacrylamide gel electrophoresis. In vitro binding of this protein to [^{14}C] benzyladenine was inhibited remarkably by cold benzyladenine and kinetin and slightly by adenine, but not adenosine. The molecular weight of the protein was determined to be about 4,000 daltons by gel filtration and SDS polyacrylamide gel electrophoresis.

Cytokinins enhance cell division (1) and retard senescence of various plant tissues (2-3). These actions of cytokinin seem to be closely related with RNA and protein synthesis (3-5), although the precise mechanisms of action are still unknown. Recently, Matthyssee and Abrams (6) indicated that cytokinin enhanced RNA synthesis in the presence of chromosomal protein factors. The presence of receptors for other phytohormones, auxin and gibberellins (7-8), as well as for mammalian hormones (9-10), has also been reported. To understand the mechanisms of action of phytohormones, the isolation and purification of their receptor proteins should be indispensable. Unfortunately, attempts at isolation and purification of phytohormone receptor proteins (6-8) clearly fall behind those of mammalian hormone receptors (9-10).

Affinity chromatography (11) offers a simple but potent means for isolation of specific molecules. In this paper, we describe a successful application of this technique for the isolation of the cytokinin binding protein in tobacco leaves.

Abbreviations: BA, benzyladenine; SDS, sodium dodecylsulphate; V_o; void volume.

MATERIALS AND METHODS

Plant Materials: Tobacco plants (Nicotiana tabacum var. Bright Yellow) were grown in a green house for 3 to 4 months. Fully expanded leaves (15-20 cm in width and 20-30 cm in length) were used.

Preparation of Crude Extracts: About 20 g (fresh weight) of tobacco leaves with main veins removed were homogenized in a Homo blendor (Sakuma, Tokyo, Japan) at maximum speed for 2 min with 100 ml of 0.1 M phosphate buffer (pH 7.2) containing Polyclar AT (0.1 g/g leaf). The homogenate was squeezed through Tetron cloth (Tore Co., Tokyo, Japan) and centrifuged at 15,000 g for 20 min (12). The supernatant was frozen, thawed and then centrifuged at 15,000 g for 20 min. The resultant supernatant was used as crude extracts. All operations were carried out at below 4° C.

Preparation of BA-Sepharose Column: Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) activated with cyanogen bromide was washed first with 10^{-4} M HCl, then thoroughly with water and stirred in 10^{-4} M BA solution overnight at room temperature in order to link BA covalently to Sepharose 4B. The BA-Sepharose obtained was packed into a column (6 x 1.4 cm), washed with 50 mM ethanolamine and then extensively with water.

Affinity Column Chromatography: About 20 ml of crude extracts of tobacco leaves was layered onto the BA-Sepharose column. The column was extensively washed with distilled water to remove non-adsorbable proteins. Adsorbable protein fraction was released from the column by the addition of about 10 ml of 0.1 N KOH. After neutralization with 1 N HCl, the eluate was dialyzed against distilled water for 10 hr at 4° C. The dialyzed sample was immediately used or lyophilized and kept in a deep freezer until use.

Assay of Binding: Protein fraction and [^{14}C] BA (Radiochemical Centre, Amersham, England, 12.8 mCi/mmole) were incubated for 2 hr 0° C. The incubation mixture was applied onto Sephadex G-25 (Pharmacia Fine Chemicals) column (10 x 1.2 cm) and eluted with water (13). After background subtraction, counts under a protein peak at around void volume (Fraction No. 16-18 in Fig. 2) were taken as bound BA to proteins. Amount of bound BA was a linear function of that of added binding protein (see Fig. 3(a)).

Ultrogel Filtration: Lyophilized binding protein or marker proteins were dissolved in a 0.2 M NaCl solution, pH 6 and applied onto a Ultrogel AcA 34 (LKB, Bromma, Sweden) column (50 x 2 cm) which had previously been equilibrated with 0.2 M NaCl solution. Elution with 0.2 M NaCl solution was carried out at room temperature.

SDS Polyacrylamide Gel Electrophoresis: Slightly modified method of Laemmli (14) was used. Lyophilized protein samples or marker proteins were dissolved in a mixture of 0.0625 M Tris-HCl pH 7, 2 % SDS, 5 % glycerol and 2 % mercaptoethanol and boiled for 3 min. After cooling, the dissolved protein samples were applied onto 7.5 % gel column (5 x 0.6 cm) without spacer gel, which were previously polymerized in the presence of 0.025 % TEMED, 0.1 % SDS, 0.28 % Bis and 0.15 % ammonium persulfate. Electrophoresis was carried out in a mixture of 0.025 M Tris, 0.192 M glycine and 0.1 % SDS, pH 8.3, for 1 hr at 3 mA/gel column. After the run, the gels were stained with 0.04 % Coomassie Brilliant Blue in 10 % acetic acid and 25 % methanol and destained with 10 % acetic acid plus 25 % methanol. For molecular weight determination, 15 % polyacrylamide gel columns were used.

Reagents: Proteins as molecular weight markers, i. e. ovalbumin, myoglobin, cytochrome C and bacitracin were obtained from Schwarz-Mann (New York, U.S.A.). Pronase and Polyclar AT were purchased from Kaken Co. (Tokyo, Japan) and Gokyo Sangyo Co. (Nagoya, Japan), respectively.

RESULTS

Isolation of Binding Protein - Fig. 1 shows a typical result of BA-Sepharose column chromatography of leaf proteins. The adsorbable fraction, which is

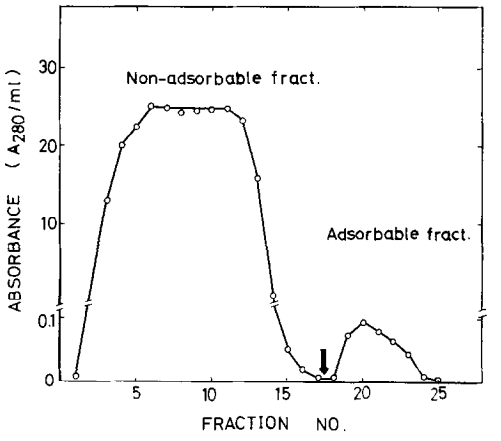
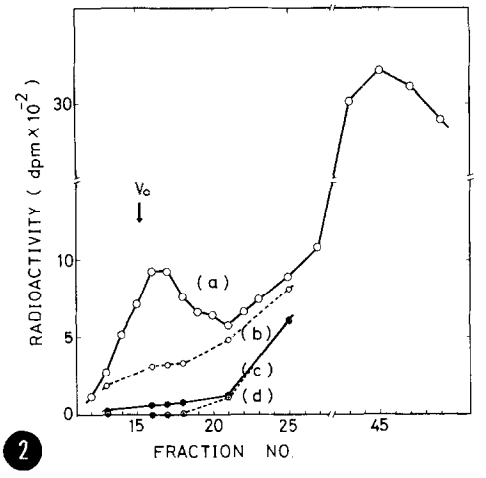


Fig. 1. Isolation of cytokinin binding protein by BA-Sepharose 4B affinity column chromatography. Crude extracts from tobacco leaves were layered onto the column. After non-adsorbable fraction was throughly washed out with distilled water, adsorbable fraction was eluted with 0.1 N KOH. The arrow indicates the start of KOH addition. The flow rate was regulated at 20 ml/hr. 3 ml eluate was collected in each tube.

believed to contain the cytokinin binding protein, was not released from the column by acetic acid or NaCl, but was eluted by 0.1 N KOH solution. [^{14}C] BA binding activity of each protein fraction was assayed by Sephadex G-25 gel filtration as shown in Fig. 2. Bound [^{14}C] BA was eluted at void volume while elution of unbound [^{14}C] BA was extremely retarded (curve (a)). The non-adsorbable fraction hardly bound to [^{14}C] BA (curve (d)). On the other hand, binding activity of the adsorbable fraction was about 40 times as high as that of the crude extracts (compare curve (a) with curve (c)). Specific binding activities of the adsorbable fraction and the crude extracts were 15,000 and 380 dpm [^{14}C] BA/mg protein, respectively. When the adsorbable fraction was incubated with pronase (80 μg/ml) for 60 min at 30° C, its binding activity was remarkably decreased (curve (b)). This together with the UV absorption curve obtained for the adsorbable fraction (data not shown) clearly indicates that a protein component was responsible for the binding.

Binding Conditions - In order to see optimum conditions for the binding, pH and protein content of the assay mixture and the incubation time and temperature



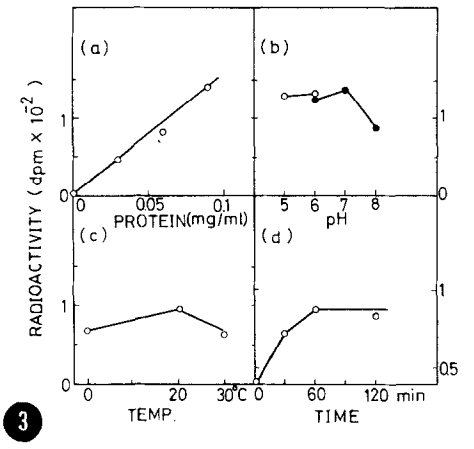


Fig. 2. Binding of proteins to [^{14}C] BA. Protein fraction (each 250 μg protein) were mixed with [^{14}C] BA (1/60 μCi) and kept at 0° C for 2 hr. The mixture was applied onto a Sephadex G-25 gel column. The flow rate was regulated at 1.5 ml/min. 0.5 ml eluate was collected in each tube. (a) Adsorbable fraction, (b) Adsorbable fraction previously treated with pronase, (c) Crude extracts, (d) Non-adsorbable fraction. Adsorbable and non-adsorbable fractions were separated as in Fig. 1. Pronase (80 μg/ml) treatment was carried out at 30° C for 60 min. Results of four separate experiments were combined to construct this figure.

Fig. 3. Effects of protein concentration, pH, temperature and incubation time on binding of adsorbable fraction protein to [^{14}C] BA. (a) Effect of concentration of adsorbable fraction protein, (b) Effect of pH, pH 5-6: 0.1 M citrate buffer, pH 6-8: 0.1 M phosphate buffer, (c) Effect of incubation temperature, (d) Time course of binding reaction. Standard incubation procedure: protein (about 50 μg) was mixed with [^{14}C] BA (1/60 μCi) in 3 ml distilled water, pH 6 and incubated for 2 hr at 0° C.

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were changed as shown in Fig. 3. The amount of bound [^{14}C] BA increased linearly with that of the adsorbable fraction protein (Fig. 3(a)). Binding activity was almost constant at pH 5 to 7 (Fig. 3(b)) and at temperature 0 to 30° C (Fig. 3(c)). Saturation of binding was reached by a 60 min incubation as shown in Fig. 3(d). At pH 5 to 7, changes in ionic strength from 0 to 0.1 M had no effect on the binding. Subsequently, therefore, the standard binding procedure used as follows: test protein (about 50 μg) was mixed with [^{14}C] BA (1/60 μCi) in 3 ml distilled water, pH 6, and incubated for 2 hr at 0° C.

Binding Specificity - As shown in Table 1, binding of the adsorbable fraction

Table 1. Binding specificity of adsorbable fraction protein.

Binding Mixture	Bound [^{14}C] BA (dpm)	% of Control
Adsorbable fraction only (Control)	3,220	100
Adsorbable fraction + BA (10^{-5}M)	1,260	39
Adsorbable fraction + kinetin ($5x10^{-5}$M)	1,080	33
Adsorbable fraction + adenine ($5x10^{-5}$M)	2,470	77
Adsorbable fraction + adenosine (10^{-4}M)	3,670	114

Adsorbable fraction to BA-Sepharose column was mixed with [^{14}C] BA in the presence or absence of adenine derivatives. After 2 hr incubation at 0° C, the mixture was subjected to Sephadex G-25 gel filtration.

protein to [^{14}C] BA was inhibited by both cold BA and kinetin by more than 60 %. Adenosine, however, rather slightly promoted the binding, and adenine suppressed it only by 20 %.

Molecular Weight of Binding Protein - In order to determine the molecular weight of the binding protein, the adsorbable fraction was subjected to Ultrogel column filtration. Fig. 4(a) clearly indicates that a protein component of the adsorbable fraction combined to [^{14}C] BA, no other protein components being detectable in the elution profile. Molecular weight of the binding protein was estimated at about 4,000 daltons from a calibration curve constructed with standard marker proteins of known molecular weights(Fig. 4(b)).

Fig. 5 shows SDS polyacrylamide gel electrophoretic profiles of the crude extracts and the non-adsorbable and adsorbable fractions. The profile of the non-adsorbable fraction is essentially the same as that of the crude extracts. Note that the adsorbable fraction gave only one detectable protein band, in agreement with the result of the Ultrogel column experiment. Comparison of the electrophoretic mobility of this component with those of the standard marker proteins indicated that its molecular weight again about 4,000 daltons. Consistency of the molecular weight determined by SDS polyacrylamide gel electrophoresis with that determined by gel filtration without detergent suggests that no subunit structure is involved in the isolated binding protein.

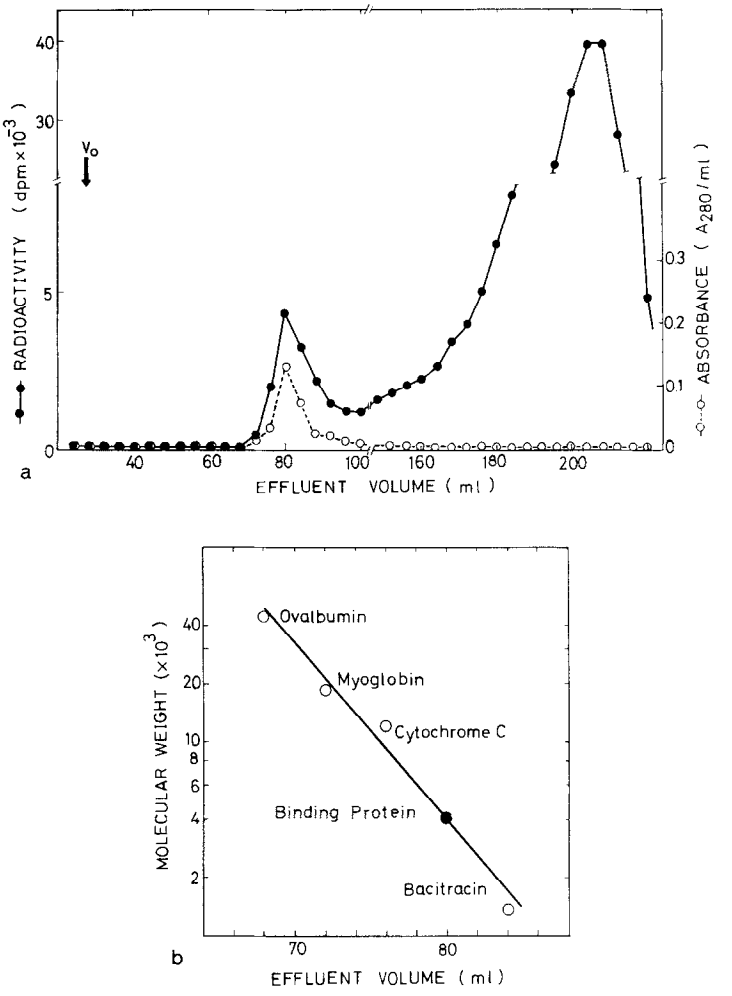

Fig. 4. Determination of molecular weight of cytokinin binding protein. (a) Gel filtration of binding protein. The adsorbable fraction separated by affinity column chromatography was incubated with $[^{14}C]$ BA for 2 hr 0° C. The reaction mixture was applied onto Ultrogel column. The flow rate was regulated at 15 ml/hr. 4 ml eluate was collected in each tube. (b) Calibration curve. Marker proteins were applied onto the Ultrogel column as above. Positions of the elution peaks were plotted against molecular weights.

DISSCUSSION

A protein has been isolated and purified from tobacco leaves which can preferentially bind to $[^{14}C]$ BA in vitro. The binding was rather easily broken by conformation destruction of the protein due to strong acid, alkali or ethanol, although relevant data were not presented.

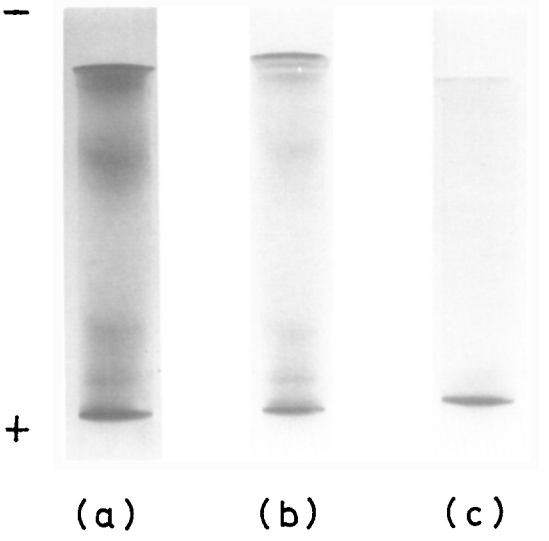

Fig. 5. Electrophoretic profiles of crude and non-adsorbable and adsorbable fractions from tobacco leaves. (a) Crude extracts (200 μg protein), (b) Non-adsorbable fraction to affinity column (150 μg protein), (c) Adsorbable fraction to affinity column (110 μg protein). 7.5 % polyacrylamide gel was used.

Inhibition by adenine derivatives of the binding of the protein to [^{14}C] BA (see Table 1) would probably be competitive in nature. It is, therefore, interesting to see that the degree of inhibition reflects the cytokinin activity of those adenine derivatives; adenine is slightly active but adenosine is not at all (1). This suggests that the binding protein plays an important role in regulation of cytokinin action.

Although the biochemical function of the cytokinin binding protein is not yet obvious, it seems possible that RNA polymerase activity is regulated by the binding protein because RNA synthesis has repeatedly been shown to be enhanced by cytokinin (3-5, 15). This possibility is also supported by the findings of chromosomal protein factors interacting with kinetin (6), and protein factors for auxin and gibberellin (7-8, 16-17). Recently, Fox and Erison (18) reported that BA binds to crude ribosomal proteins washable with 0.5 M KCl. Our preliminary study, however, suggests that our protein is of

different subcellular distribution from that of Fox and Erison's. Investigations of the relationship between the cytokinin binding protein and RNA polymerase is now in progress in our laboratory.

SDS polyacrylamide gel electrophoresis and gel filtration indicates that the cytokinin binding protein may consist of a single polypeptide chain of about 4,000 daltons. A possibility is not excluded that the binding protein was denatured during elution from the affinity column. However, the conformational modification, if any, must be negligible, since it quantitatively binds to [^{14}C] BA and this binding is preferentially inhibited by cytokinins. Furthermore, our recent experiment suggests that the binding protein of the same size, i. e. about 4,000 daltons, is present in the crude extracts as well.

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REFERENCES

1. Skoog, F., Mamizi, H. Q., Szweykowska, A. M., Leonard, N. J., Carraway, K. L., Fujii, T., Helgeson, J. and Loeppky, R. N. (1967) Phytochem., 6, 1169-1192.
2. Richmond, A. E. and Lang, A. (1957) Science, 125, 650-651.
3. Sugiura, M., Umemura, K. and Oota, Y. (1962) Physiol. Plantarum, 15, 457-463.
4. Osborne, D. J. (1962) Plant Physiol., 37, 595-602.
5. Roychoudhurry, R., Datta, A. and Sen, S. P. (1965) Biochim. Biophys. Acta, 107, 346-357.
6. Matthyssee, A. G. and Abrams, M. (1970) ibid., 199, 511-518.
7. Venis, M. A. (1971) Proc. Natl. Acad. Sci. USA, 68, 1824-1827.
8. Stoddart, J., Breidenbach, W., Nadeau, R. and Rappaport, L. (1974) ibid., 71, 3255-3259.
9. Jensen, E. V. and DeSombre, E. R. (1973) Science, 182, 126-134.
10. Cuatrecasas, P. (1974) Annu. Rev. Biochem., 43, 169-219.
11. Colowick, S. P. and Kaplan, N. O. ed., Method in Enzymology XXXIV, Affinity Techniques ed. by Jakoby, W. B. and Wilckek, M., Academic Press (1974).
12. Yoshida, K. (1974) Plant and Cell Physiol., 15, 441-456.
13. Fukui, S. and Miyairi, S. (1970) J. Bacteriol., 101, 685-691.
14. Laemmli, U. K. (1970) Nature, 227, 680-685.
15. Takegami, T. (1975) Plant and Cell Physiol., 16, 417-425.
16. Mondal, H., Mandal, R. K. and Biswas, B. B. (1972) Biochem. Biophys. Res. Commun., 49, 306-311.
17. Johri, M. M. and Varner, J. E. (1972) Proc. Natl. Acad. Sci. USA. 59, 269-276.
18. Fox, J. E. and Erison, J. E. (1975) Biochem. Biophys. Res. Commun., 64, 694-700.