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Partial Purification of an Ethylene-binding Component from Plant Tissue[1]

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ABSTRACT

An ethylene binding component(s) has been partially purified from mung bean sprouts. Tissue was homogenized in 0.3 molar sucrose and 0.2 molar potassium phosphate buffer (pH 7.0). The homogenate was centrifuged, and resuspended fractions were assayed by incorporating them onto cellulose fibers (0.7 grams per milliliter). These were exposed to [^{14}C]ethylene (3.7×10^{-2} microliters per liter of 120 millicurie per millimole) in the presence or absence of 1000 microliters per liter unlabeled ethylene. The cellulose was transferred to separate containers and the [^{14}C]ethylene was absorbed in mercury perchlorate and counted. Distribution of ethylene binding to various fractions was: 0 to 3,000*g*, 3%; 3,000 to 12,000*g*, 4%; 12,000 to 100,000*g*, 69%; cellular debris, 24%; 100,000*g* supernatant, 0%. Adjustment of the pH to 4.0 precipitates the ethylene-binding component. Neutralization, addition of Triton X-100, and readjustment of the pH to 4.0 "solubilized" most of the binding component. Further purification was obtained by chromatography on CM-Sephadex in 10 millimolar potassium acetate buffer, (pH 5.0) containing 1% Triton X-100. Elution was with 200 millimolar potassium phosphate (pH 6.0) containing 1% Triton X-100. Upon treatment of the Triton "solubilized" component with cold acetone, over 90% of the binding capacity was lost. Extraction of the acetone-precipitated residue with 2% Triton X-100 restored some of the binding capacity which was found in the soluble fraction. The pH optimum for binding is 6.0. Passing the Triton X-100 extract of the acetone powder through Sepharose 6B provides considerable purification. The binding component moved ahead of most of the protein.

C_2H_4 has long been known to have an effect on plants, and many tests have been devised to measure its effect (1). Recently, a method of directly measuring C_2H_4 binding has appeared (11, 12). This method measured the amount of ^{14}C-labeled C_2H_4 present in tissue in the presence and absence of unlabeled C_2H_4. The difference provided a quantitative measure of the C_2H_4-binding sites. Another recent report of apparent compartmentation of C_2H_4 in plant tissue may, in fact, represent C_2H_4 binding (5). Evidence is not yet available to link the binding capacity to the physiological action of C_2H_4; however, since C_2H_4 must bind to some site in order to act, and even though binding concentrations are similar to physiological action concentrations, a further characterization of the binding component seems necessary.

A value of 3.5 nM was obtained for the concentration of C_2H_4-binding site in tobacco leaves (11). Based on an assumed mol wt of 100,000 and one binding site/molecule, this would amount to 350 μg binding site/kg of tissue. The binding site appears to be membrane derived (13). Because of the difficulties usually encountered with purifying membrane components and the low concentration, it was decided to develop a partial purification and concentration method. This should serve as a preparation from which various additional purification techniques can be tested. Inasmuch as large amounts of tissue are required, I used mung beans as a source of the binding component.

MATERIALS AND METHODS

Plant Material. Mung bean sprouts (*Phaseolus aureus*) were obtained by germinating mung beans in a plastic container at 22 C. Seeds were soaked overnight in tap H_2O, washed with 0.2% NaOCl for 5 min and placed in a plastic container with drainage holes covered with screen wire. After about 4 days, the screen wire was removed to give more aeration. The seeds were washed twice daily with tap H_2O. At about 7 days the sprouts were harvested.

Measurement of Binding. Material to be assayed was exposed to [^{14}C]C_2H_4 (3.7×10^{-2} μl/l of 120 mCi/mmol) in the presence or absence of 1000 μl/l unlabeled C_2H_4. Cellulose fiber (Sigma Chemical Co., St. Louis, Mo.) was added to cellular fractions (0.7 g/ml) to facilitate the diffusion of C_2H_4 to the binding sites. Samples to be treated were placed in desiccators and exposed to [^{14}C]C_2H_4. After 3 h, the samples were removed and placed in 250-cc containers with a 5-ml scintillation vial containing 0.3 ml mercury perchlorate (11). The transfer required approximately 30 s at room temperature. Approximately 75% of the unbound C_2H_4 ([^{14}C]C_2H_4 present in the presence of unlabeled C_2H_4) diffused from the sample in 10 min at 22 C. About 5% of the bound C_2H_4 diffused from the samples in 10 min at 22 C, so only a very small amount of bound C_2H_4 would be lost during the 30-s transfer. The samples were heated to 60 C for 4 to 12 h, the vials were removed from the containers, 4 ml scintillation fluid was added to each (4 g scintillator, 25% Triton X-100, and toluene to 1 liter), and the samples were counted. These times gave optimum counting rates. The difference in count rates between samples exposed to [^{14}C]-C_2H_4 in the presence and absence of 1000 μl/l unlabeled C_2H_4 was used as a measure of binding (11).

Triton X-100 (1 ml of 20%) was added to all samples which had the lipid removed by acetone precipitation prior to incorporating them onto cellulose. Except where indicated, the samples were 0.1 M with respect to phosphate buffer at pH 6.0. Cellulose fibers, Triton X-100 and K-phosphate mixtures alone do not bind C_2H_4.

C_2H_4 Treatment. [^{14}C]C_2H_4 was obtained from Amersham/Searle Corp., Arlington Heights, Ill., and converted to the mercury-perchlorate complex (11). The desired amount of the [^{14}C]-C_2H_4-mercury-perchlorate complex was placed in a 25-ml Erlenmeyer flask containing a magnetic stirring bar. After closing the desiccator, 1 ml saturated LiCl was injected to release the C_2H_4 from the complex. A magnetic stirrer was used to facilitate diffusion of the C_2H_4 from the solution.

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Identification of the Binding Product. To ensure that the mercury perchlorate was not selectively concentrating some impurity from the radioactive C_2H_4, the product of the binding was examined. Using a Triton X-100 extract, trapping the product in mercury acetate and subjecting it to paper chromatography (3) revealed a single radioactive spot which was coincident with authentic C_2H_4 in two solvent systems. The product was also trapped in mercury perchlorate at 60 C. After 4 h, the trapped product was released by adding LiCl and retrapped in mercury acetate. Again, only a single peak was observed when subjected to paper chromatography. About 2% of the trapped ^{14}C was not released by LiCl. It is not known if this was an impurity or a breakdown product generated from the radioactive C_2H_4 by heating to 60 C for 4 h.

Protein Determination. Protein was determined by a microbiuret method (6, 7) because Triton X-100 interferes with many of the other methods.

CM-Sephadex Chromatography. CM-Sephadex (40 to 120 μm obtained from Sigma Chemical Co., St. Louis, Mo.) was hydrated in 10 mM K-phosphate (pH 5.0), containing 1% Triton X-100. After 24 h, the CM-Sephadex was placed in a column 2.5 × 25.0 cm.

RESULTS

Extraction of Sprouts. Sprouts were blended with 0.3 M sucrose, buffered with 0.2 M K-phosphate (pH 7.0), then strained through nylon cloth, and subjected to centrifugation to localize the C_2H_4 binding component. Distribution of C_2H_4 binding to various fractions was: 0 to 3,000*g*, 3%; 3,000 to 12,000*g*, 4%; 12,000 to 100,000*g*, 69%; cellular debris, 24%; 100,000*g* supernatant, 0%. The 12,000 to 100,000*g* fraction has not yet been subjected to further purification techniques to identify it with a particular cellular structure.

Precipitation of the Binding Component. The amount of tissue required for assays is large and 137 dpm/g of tissue was obtained under the assay conditions (Table I). I found that the binding component was stable to pH precipitation. Tissue was blended in a Waring Blendor at pH 7.0. After straining through nylon and adjusting the pH to 4.0, the binding component was recovered in the precipitate by centrifugation at 10,000*g* for 10 min. Although this step did not give a large purification, it reduced the volume considerably and made it possible to handle large quantities of tissue. This fraction (Fraction B, Table I) may be stored at −15 C.

Extraction with Triton X-100. The pH 4.0 precipitated material was neutralized to pH 7.0 with K_2HPO_4 and Triton X-100 was added (100 ml 20% Triton X-100/1,000 g of initial tissue). The material was suspended in a blender and subjected to 20,000*g* centrifugation for 30 min. The supernatant fluid was removed and the pellet was blended with 30 ml 20% Triton X-100/1,000 g initial tissue. Centrifugation was repeated and a third extraction with 30 ml Triton X-100 was made. The desired amount of H_2O was also added to the precipitated material before centrifugation. The combined extracts were adjusted to pH 4.0 and subjected to 10,000*g* for 10 min. This procedure "solubilized" most of the binding component (Table I) with considerable purification. This fraction (fraction C, Table I) may be stored at −15 C.

CM Sephadex Chromatography. The Triton X-100 extract was made 50% with respect to $(NH_4)_2SO_4$ and centrifuged at 10,000*g* for 10 min. The floating lipid-like fraction was collected and dialyzed against distilled H_2O. The dialyzed extract, made to 10 mM in K-acetate, (pH 5.0) containing 1% Triton X-100 was passed through the column containing CM-Sephadex at a rate of approximately 50 ml/h. About 80% of the binding component remained on the column. The binding component was eluted with approximately 300 ml 200 mM K-phosphate (pH 6.0). Subsequently, the binding component was removed from solution by making it 50% saturated with respect to $(NH_4)_2SO_4$. After centrifugation, the floating material was collected and dialyzed for 2 days against distilled H_2O. This preparation (fraction D, Table I) is stable and contains sufficient binding capacity on a protein basis to be suitable for electrophoresis (14).

Acetone Powder. The Triton X-100 extract, after centrifugation at pH 4.0, was poured into 5 volumes of cold acetone (−15 C) and the precipitate was collected by filtering in a Büchner funnel. After washing with cold 1-butanol and rewashing with acetone, the powder was dried at room temperature. Extraction with phosphate buffer at pH 6.0 and assaying the total extract revealed that over 90% of the binding capacity had been lost, although a small, but definite, amount was retained. If the acetone powder were extracted with 2% Triton X-100, approximately 30% of the binding capacity was restored. Evaporation of the acetone and adding this fraction to the assay mixture does not seem to be superior to Triton X-100 alone. When 20% Triton X-100 was used in the extraction, the supernatant fluid separated into two phases. The upper phase appeared oily and contained the binding component. The lower phase was watery and had little or none of the binding component.

Sepharose 6B Chromatography. When the 2% Triton X-100 extract of the acetone powder was dialyzed and subjected to chromatography on Sepharose 6B, the binding component eluted ahead of most of the protein and this resulted in a considerable amount of purification (Fig. 1). Fractions containing the binding component were collected and the binding component was concentrated by $(NH_4)_2SO_4$ precipitation. In the presence of 45% saturated $(NH_4)_2SO_4$, the binding component came out of solution, floated, and was greasy in appearance. It readily redissolved in 0.1 M K-phosphate (pH 6.0). This preparation contained sufficient binding capacity on a protein basis to be suitable for electrophoresis.

Use of Chaotropic Ions. Chaotropic ions have been used to extract proteins from membranes (4), and their use may be of value in extracting the C_2H_4-binding component. An attempt was made to extract the component with 2 M sodium perchlorate and 2 M sodium trichloroacetate. Although some binding component

Table I. *Purification of C_2H_4-binding Component from Mung Bean Sprouts*

	Fraction	Binding Original Tissue	Protein	Binding
		dpm/1,000 g	*g*	*dpm/g protein*
	Sprouts	137,600		
A	Crude	102,700	15	6,846
B	pH 4.0 precipitate	79,060	11	7,186
C	Triton X-100 extract of fraction B	91,500	0.500	183,000
D	CM-Sephadex	54,431	0.050	1,088,620

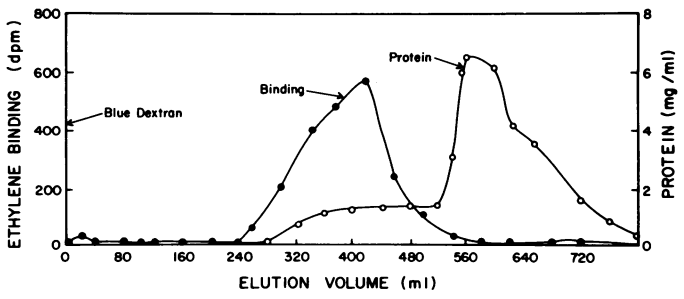

FIG. 1. Elution of C_2H_4-binding component from Sepharose 6B column. Column (5 × 55 cm) contained 1 liter Sepharose 6B. Elution was with 2% Triton X-100.

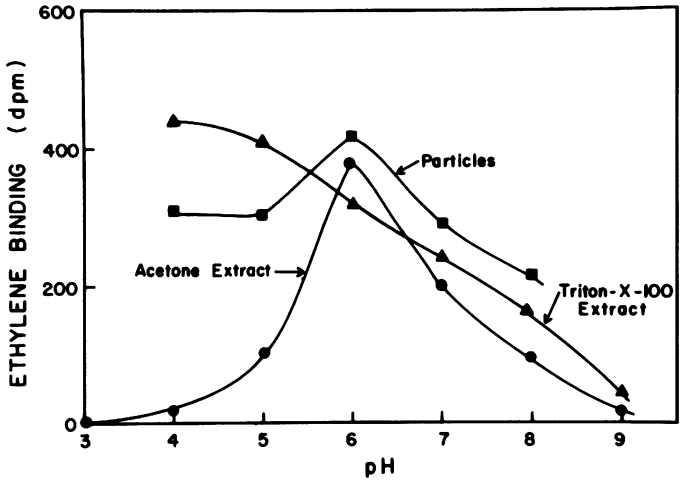

FIG. 2. Effect of pH on various C_2H_4-binding preparations. The "particle" preparation was obtained by precipitation at pH 4.0. The Triton X-100 extract was a Triton extract of the particles. The acetone extract was a Triton X-100 extract of an acetone powder. See text for details. Buffers were: pH 3 to 6, citrate; pH 6 to 7, K-phosphate; and pH 7 to 9, Tris-sulfate.

was extracted with these reagents, they were not nearly as effective as Triton X-100.

Effect of pH on Binding. The effect of pH on the binding of C_2H_4 with different preparations was determined. With isolated particles, a maximum binding value was found at pH 6.0 which decreased as the pH was raised or lowered (Fig. 2). With the initial Triton X-100 extract (fraction C, Table I), binding continued to increase as the pH was lowered but declined as it increased toward pH 9.0. With an acetone powder extract, a maximum was observed at pH 6.0, and a marked decline was observed as the pH was raised or lowered. Probably the binding component is associated with more lipid material in the particle preparation and in the Triton X-100 extract and, thus, are not as strongly affected by hydrogen ions as in the acetone powder extract from which most of the lipids had been removed.

DISCUSSION

A partial purification procedure for the C_2H_4-binding component is presented. This procedure consists of precipitation by adjusting the pH to 4.0, centrifugation, adjusting the pH to 7.0, and adding Triton X-100. After readjusting the pH to 4.0 and centrifugation, the binding component is "solubilized." After concentrating with $(NH_4)_2SO_4$ and dialysis, further purification is achieved by chromatography on CM-Sephadex. The eluted and concentrated preparation contains sufficient binding capacity on a protein basis to be a useful starting source for other purification procedures such as electrophoresis. The usual methods of cell fractionation and subsequent purification of plant material have been reviewed recently (9). There is a need to identify binding with specific organelles and this usually requires density gradient centrifugation. The amount of material that can be obtained by this procedure is very limited and the method presented here is more practical for preparing the large amounts of material needed to obtain a sufficient amount of C_2H_4-binding component for further purification work. In addition to the above method, an alternate procedure using an acetone powder and Sepharose 6B is presented.

The C_2H_4-binding component appears to be a lipophilic protein based on the following evidence. It is located in the 100,000*g* fraction and adjusting the pH to 4.0 causes it to be precipitated from solution. It is solubilized with a nonionic detergent and it is separated from solution by 50% saturated $(NH_4)_2SO_4$. It is precipitated by 50% acetone and is insoluble in butanol. It is a high mol wt component, as is evidenced by gel filtration chromatography. It moves as a broad band upon electrophoresis and appears as a broad band between pH 5.5 and 6.5 by isoelectric focusing (14). Binding capacity is destroyed by heating at 100 C for 2 min, and some of the binding capability is destroyed by trypsin digestion (14). These results are typical of those obtained with many membrane proteins (2, 8, 10).

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