

# MULTIPLE FORMS OF THE PROTEINACEOUS INHIBITOR OF ETHYLENE SYNTHESIS IN MUNGBEAN SEEDS

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Ethylene is probably the smallest organic substance that is produced by a number of plant tissues and affects many physiological as well as biochemical processes in plant tissues (1-3). The list of ethylene regulated phenomenon includes; breaking dormancy of potato tuber, regulation of swelling and elongation, hypertrophy, induction of adventitious roots, epinasty, apical hook closure, inhibition of leaf expansion, control of flower induction, exudation, senescence, abscission and fruit ripening (1,2).

Since ethylene has a wide spectrum of physiological action and is regarded as plant hormone, studies on the regulatory mechanism of ethylene biosynthesis are of great significance to elucidate the controlling mechanism of plant processes.

It is well known that auxin stimulates production of ethylene in plant tissues (4-12). Treatment with auxin greatly increases the rate of ethylene production in hypocotyl segments of etiolated mungbean by inducing synthesis of proteinins that are necessary for ethylene production (13). In a previous paper (14), we showed that auxin-induced ethylene synthesis by mungbean hypocotyl segments was inhibited by a protein in the same tissue, and we have isolated and purified the proteinaceous inhibitor in a pure form. Furthermore, it has been shown that the proteinaceous inhibitor inhibits endogenous ethylene production as well as auxin-induced ethylene synthesis (15).

Although it has not yet been clarified the physiological role of the proteinaceous inhibitor in the tissue, it may be an attractive working hypothesis that the proteinaceous inhibitor participates in the inactivation of the ethylene producing system *in vivo*.

The present paper reports the occurrence of multiple forms of the proteinaceous inhibitor of ethylene synthesis in mungbean seeds.

## MATERIALS AND METHODS

### *Plant material*

Mungbean (*Phaseolus aureus* Roxb.) seeds were purchased from a local bean-sprout producer. Method for the preparation of etiolated mungbean seedlings was described in a previous paper (13).

### *Assay of inhibitory activity*

Ten 6-mm long, subapical hypocotyl segments excised from 3-day-old mungbean seedlings were incubated with 0.5 ml auxin solution (0.5

mM IAA\* in 50 mM phosphate buffer, pH 6.8, with 50  $\mu$ g/ml chloramphenicol and 0.2 mM chlorogenic acid added) in a 25-ml glass vial sealed with a silicon stopper. The inhibitor preparation was included in the incubation medium. Chlorogenic acid was added to the incubation medium to inhibit oxidative degradation of IAA by peroxidase in the inhibitor preparation. The inhibitory activity was not impaired by chlorogenic acid added (14). After 6 hr incubation at 30°C, the ethylene content in the gas phase was measured with a gas chromatograph (063; Hitachi Ltd., Tokyo).

#### *Extraction of the inhibitor and Sephadex G-200 gel filtration*

Mungbean seeds were ground to a fine powder with a motor-driven pulverizer. Four g of finely powdered seeds were homogenized with 40 ml of 0.1 M phosphate buffer, pH 6.8, containing 5 mM  $\beta$ -mercaptoethanol with a glass homogenizer. The resultant homogenate was centrifuged at 10,000 $\times$ g for 10 min, and 20 ml of the supernatant obtained was applied to Sephadex G-25 column (bed volume 200 ml), equilibrated with 50 mM phosphate buffer (pH 6.8). The column was eluted with the above mentioned buffer, and total protein was quantitatively collected in a 50-ml eluate. For activity measurement of the inhibitor, 0.25 ml of the eluate was added to the incubation medium.  $(\text{NH}_4)_2\text{SO}_4$  was added to the eluate to give 80 % saturation, and the precipitate formed was collected by centrifugation, dissolved in distilled water, and dialyzed against 50 mM phosphate buffer, pH 6.8, for 18 hr with several changes of buffer. Any precipitate formed during dialysis was removed by centrifugation. The resultant supernatant corresponding to about 1.8 g of finely powdered seeds was applied on a Sephadex G-200 column (2.6 $\times$ 75 cm) previously equilibrated with the phosphate buffer, and elution was conducted with an upward flow with a flow rate of 15 ml/hr. Three-ml portion of the eluate was fractionated, and 0.1 ml of these eluate was used for activity measurement of the inhibitor. All procedures were carried out at 4°C.

#### *Protein determination*

The amount of protein was measured by the method of Lowry *et al.* (16), with bovine serum albumin as standard.

### RESULTS AND DISCUSSION

The extract of mungbean seeds was freed from low molecular contaminants by Sephadex G-25 treatment. As shown in Table 1, this extract had a significantly high inhibitory activity of auxin-induced ethylene synthesis. It was also found that the inhibitory activity was not changed by a heat treatment over boiling water bath for 5 min as

\*IAA: Indoleacetic acid

TABLE 1.  
Effect of crude proteins on auxin-induced ethylene synthesis by mungbean hypocotyl segments.

Additions	C <sub>2</sub> H <sub>4</sub> produced nl/10 seg/6 hr	% Inhibition
None	203	
Crude proteins	31	85
Crude proteins, boiled	31	85

in the case of the inhibitory activity found in a crude extract of mungbean hypocotyls (14).

When crude proteins from mungbean seeds were subjected to gel filtration with Sephadex G-200, the inhibitory activity was distributed over four fractions. The elution profile and the distribution of the inhibitory activity are shown in Fig. 1. A minor peak of the inhibitory

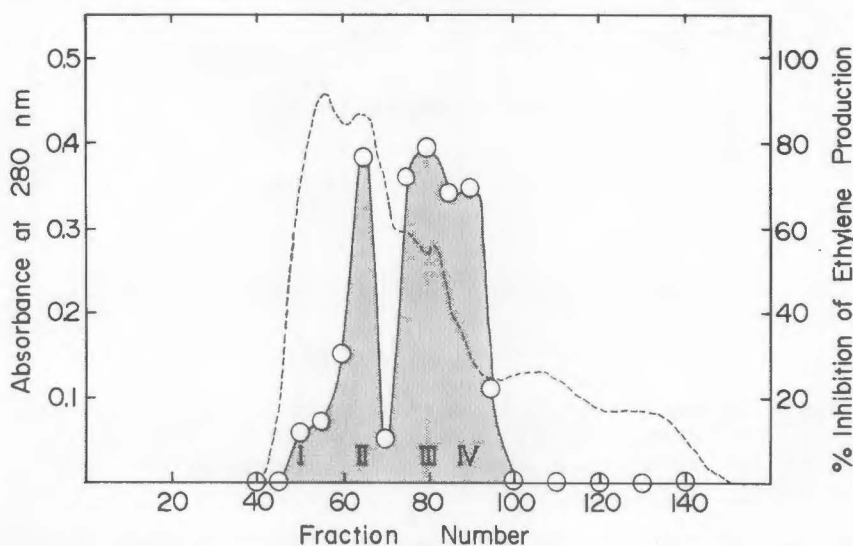


Fig. 1. Elution profile of crude proteins from mungbean seeds over a Sephadex G-200 column. Dotted line, absorbance at 280 nm; open circle, inhibitory activity of auxin-induced ethylene synthesis by mungbean hypocotyl segments.

activity at fraction number of 50 was named as inhibitor I, and other three peaks of the inhibitory activity at fraction numbers of 65, 80 and 90 were named as inhibitors II, III, and IV, respectively. Although the activity of inhibitor I was very low, the activities of inhibitors II, III and IV were considerably high. The sum of the activities of inhibitors III and IV formed more than 60 % of total inhibitory activity in the extract.

A very high-molecular form of the inhibitor, inhibitor I, eluted corresponding to the void volume of Sephadex G-200 column. Therefore

its molecular weight could not be estimated. However, apparent molecular weights of inhibitors II, III and IV were determined by gel filtration with Sephadex G-200. From the data of gel filtration and the calibration curve made with standard proteins, molecular weights of about 316,000, 112,000 and 56,000 were estimated for inhibitors II, III and IV, respectively (Fig. 2). The molecular weight of inhibitor II corresponded

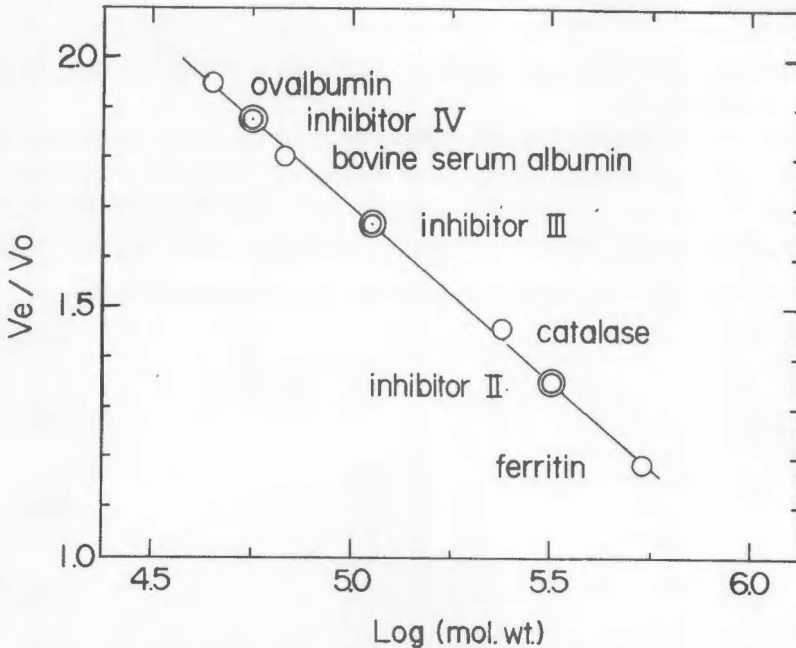


Fig. 2. Molecular weight estimation of the inhibitor by gel filtration with Sephadex G-200. Molecular weight of standard proteins: ovalbumin,  $4.5 \times 10^4$ ; bovine serum albumin,  $6.7 \times 10^4$ ; catalase,  $2.4 \times 10^5$ ; ferritin,  $5.4 \times 10^5$ .

to 2.8-fold that of the inhibitor purified from mungbean hypocotyls (14). The molecular weights of inhibitors III and IV were coincident with those of the inhibitor and subunit of it purified from mungbean hypocotyls (14).

As shown in Table 2, the activities of inhibitors II, III and IV were almost completely lost after 2 hr treatment of the preparations with trypsin. From these results, it seems likely that inhibitor III is the same proteinaceous inhibitor as purified from mungbean hypocotyls and inhibitor IV is a monomeric form of this inhibitor. Although inhibitor II is a proteinaceous inhibitor, it is uncertain whether inhibitor II is a bound form of inhibitor III or IV to other macromolecule or not.

In a previous paper (14), we reported that the inhibitory activity of ethylene synthesis was distributed over numerous fractions from a very

TABLE 2.  
Effect of trypsin on partially purified inhibitor preparations.

Additions	mg Pro/ml	C <sub>2</sub> H <sub>4</sub> produced nl/10 seg/6 hr	% Inhibition
None		196	
Inhibitor II	0.35	14	93
Inhibitor III	0.27	18	91
Inhibitor IV	0.19	21	89
Trypsin treated inhibitor II		186	5
Trypsin treated inhibitor III		201	0
Trypsin treated inhibitor IV		195	0
Boiled trypsin		197	0

One ml of the most active fraction of each inhibitor was mixed with 2 mg of trypsin (Miles Labs., Elkhart, Ill., U.S.A.) in 1 ml of 50 mM phosphate buffer, pH 6.8, and incubated at 37°C for 2 hr. After the incubation period, the mixture was heated over boiling water bath for 5 min and then cooled in an ice-water bath. The boiled reaction mixture was centrifuged, and 0.1 ml of the supernatant obtained was used for the assay of the inhibitory activity.

high-molecular fraction to one with a molecular weight corresponding to about 100,000 when a crude extract of mungbean hypocotyls was subjected to gel filtration with Sephadex G-200. However, we focussed our attention on the inhibitory activity of slow-moving fraction in gel filtration with Sephadex G-200, and purified the proteinaceous inhibitor with a molecular weight of 112,000 (14). In this paper, it was shown that the proteinaceous inhibitors with molecular weights of 316,000 and 56,000 were also contained in the extract of mungbean seeds. Therefore, there is a possibility that these inhibitory proteins are present in mungbean hypocotyls.

The inhibitory mechanism of ethylene synthesis by the proteinaceous inhibitors has not yet been clarified. However, in a previous paper (15), we showed that cells to produce ethylene in response to auxin were epidermal cells and reversible binding of the inhibitor to epidermal cells was enough to inhibit ethylene production. Since the proteinaceous inhibitor purified from mungbean hypocotyls is a macromolecule with a molecular weight of 112,000 and its inhibitory action is easily removed by washing the inhibited segments with a large volume of water (15), the inhibition is probably resulted from binding of the inhibitor to outer surface of cytoplasmic membrane. Although penetration of the inhibitor into epidermal cells cannot be ruled out, it seems unlikely that such a macromolecule easily goes in and out of plant cells. Therefore, I assume that the ethylene producing enzyme may be located in a membrane system and the binding of the inhibitor causes the change of membrane activity resulting in the inhibition of ethylene producing enzyme system.

## SUMMARY

The inhibitory activity of auxin-induced ethylene synthesis by mungbean hypocotyl segments was detected in the extract of mungbean seeds. When crude proteins from mungbean seeds were subjected to gel filtration with Sephadex G-200, four peaks of the inhibitory activity were observed. Although the nature of a very high-molecular form of the inhibitor was not clarified, other three forms were characterized to be the proteinaceous inhibitors with molecular weights of about 316,000, 112,000 and 56,000, respectively.

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