Isolation and Identification of the Precursor of Ethane in *Phaseolus vulgaris* L.1, 2

**ABSTRACT**

Ethane production by homogenates of *Phaseolus vulgaris* L. cv. Harvoster was studied. The precursor of ethane was identified as linolenic acid. The liberation of ethane was optimum at pH 4.2 and was highest from homogenates of leaves and apical buds. When roots were homogenized in linolenic acid solution, ethane and ethylene production were stimulated. In corn root homogenates, ethylene biosynthesis was stimulated nearly 8-fold by linolenic acid. The enzyme responsible for ethane production from oat root homogenates was soluble and had a high molecular weight.

Although ethane is a natural product of plant metabolism (1, 5), it is usually produced in small quantities when compared to ethylene (6). When plant tissue is homogenized, ethylene production ceases and ethane is liberated (1, 5). When cytoplasmic particles from apple fruits were incubated with a thio-acid, ethane was liberated in large quantities (5). The lipid component of the cytoplasmic particles was essential to the production of ethane. Indirect evidence that linolenic acid was the precursor of ethane was provided when this fatty acid effectively substituted for the lipid fraction of the cytoplasmic particles (5). Other fatty acids also stimulated the production of ethane by the cytoplasmic particles and no attempt was reported to identify the precursor of ethane in plant tissue by isolation from the lipid extract. The experiments reported here were initiated to identify the precursor of ethane in *Phaseolus vulgaris* and to characterize further the ethane-producing system in plant tissue.

**MATERIALS AND METHODS**

Seedlings of *P. vulgaris* L. cv. Black Valentine or cv. Harvester were grown in vermiculite in a greenhouse for 6 to 8 days. Etiolated seedlings were grown in vermiculite in darkness for 7 to 9 days. Tissue was excised from various parts of the seedlings, weighed, homogenized in 1 ml of deionized H2O, and ethane liberation from the homogenate was determined by gas chromatography as reported (1). Seeds of *Zea mays* L. WF9X38-11, *Pisum sativum* L. cv. Alaska, *Avena sativa* L. cv. Noble, *Triticum vulgare* L. cv. Arthur, and *Helianthus annuus* L. cv. Mammoth were germinated on paper towels in covered casserole dishes. After 4 days in darkness at 28 C, five seedlings with roots of uniform size were selected. The entire roots were excised, weighed, homogenized in McIlvaine buffer (pH 4.2) with and without linolenic acid (1.5 mm), sealed in a glass syringe, and ethane and ethylene production determined after 2 hr in darkness at 28 C by gas chromatography.

Acetone powders of apical explants from 7-day-old *P. vulgaris* L. cv. Harvoster were grown in vermiculite in a greenhouse for 6 to 8 days. After moth were vester was grown in vermiculite in a greenhouse for 6 to 8 days. Acetone was removed from the extract and prepared by homogenizing in cold acetone (−20 C, 5 ml/g fresh wt) with a stainless steel Sorvall Omni-Mixer, filtering through Whatman No. 5 filter paper, and rinsing thoroughly with cold acetone. The acetone extract was stored in a sealed container at −20 C. Ethane production was tested by adding 1 ml of test solution to 10 to 15 mg of acetone powder in a 5-ml glass syringe and determining ethane liberation after 2 hr at 28 C by gas chromatography.

Acetone was removed from the extract in vacuo. 2 volumes of methanolic NaOH (10% w/v) were added to the residue, and the mixture refluxed for 20 min. The methanol was evaporated in vacuo. 1 volume of deionized H2O added, and the pH adjusted to 1 with concentrated HCl. Activated charcoal was added (2%, w/v), the mixture stirred for 15 min and filtered through Whatman No. 5 filter paper with suction. The charcoal was eluted with 4 volumes of acetone and the acetone was evaporated in vacuo. The residue was partitioned between ethyl ether, 0.1 n NaOH, and 5% HCl as described (8) to obtain an ether-soluble neutral, ether-soluble acidic, and ether-soluble basic fraction. The ether was evaporated, the residue dissolved in a small volume of petroleum ether and fractionated on a silicic acid column as described (3). The active fraction was further purified by TLC with Silica Gel H (250 μ E. Merck AG) developed with petroleum ether-diethyl ether-glacial acetic acid (90:10:1, v/v/v). The Rf of the bands was visualized by exposing test plates to either iodine vapors or heat after spraying with H2SO4. Corresponding bands were scraped from TLC plates and eluted with acetone. Bioassay for the ethane precursor at each step of the purification was accomplished by removing an aliquot from each fraction, evaporating the organic solvent in vacuo, dissolving in McIlvaine buffer (pH 4.2), adding 1 ml of this solution to 10 to 15 mg of acetone powder and monitoring ethane liberation by gas chromatography.

Acetone was removed from the active TLC eluate and the residue methylated with freshly distilled diazomethane as described (7). The methylated samples were dissolved in chloroform and the fatty acid components identified by GLC using glass columns packed with 15% HI-EFF-IBP on 60/80 mesh Gas-chrom P (Applied Science Laboratories). The percentage of individual fatty acids was determined by normalizing the peaks of the chromatograms with a compensating polar planimeter. After evaluation of detector response to increments of reference standards, the recorder response for esters of C12 through C18 and unsaturated C18 fatty acids was normalized without correction for detector response. Chromatographically pure fatty acids were used as standards (Nu Chek Prep., Inc., Elysian, Minn.).
RESULTS AND DISCUSSION

When bean seedlings were dissected and homogenized, ethane production was highest from homogenates of apical bud explants and leaf sections of both green and etiolated seedlings (Table I). Much lower ethane production from the more mature tissue of the seedlings than from young rapidly growing leaves and buds suggests that on a fresh weight basis, meristematic tissue has the greatest ability to liberate ethane. The liberation of ethane from the homogenates was facilitated by acidic pH (Fig. 1). McIlvain buffer (pH 4.2) was used in subsequent experiments.

Since ethane production was highest from apical bud and leaf tissues, acetone powders were made from apical explants. When the acetone extract was dissolved in buffer and a portion added to the acetone power, ethane was liberated. Heating the acetone powder reduced its ability to mediate the reaction, whereas heating the acetone extract led to increased ethane production. There was no ethane liberated from the acetone extract in the absence of acetone powder. Bromine treatment caused the acetone extract to lose its ability to liberate ethane in the presence of acetone powder. Saponification of the acetone extract had no effect on its ability to stimulate ethane production. The active fraction from the saponified extract was absorbed to charcoal and eluted with acetone. When this eluate was partitioned to yield ether-soluble fractions (8), the acidic fraction stimulated ethane production by acetone powders while the basic fraction inhibited, and the neutral fraction had no effect on ethane production. When the ether-soluble acidic fraction was eluted from a silicic acid column, 82% of the original activity was found in the free fatty acid fraction (3). When the active fraction from

Table 1. Ethane production by various parts of P. vulgaris seedlings after tissue homogenization. Tissues were homogenized in 1 ml of buffer and ethane production was assayed for 2 hr at 28°C by gas chromatography. Control was unhomogenized tissue.

<table>
<thead>
<tr>
<th>Type of Tissue and Treatment</th>
<th>Ethane Production (ml of ethane/mg protein)</th>
<th>Buffer</th>
<th>Etilated</th>
<th>Homogenized</th>
<th>Etiolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>green</td>
<td>Etiolated</td>
<td>11.7</td>
<td>5.6</td>
<td>60.4</td>
<td>12.9</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td>0.2</td>
<td>0.6</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>etiolated</td>
<td>Homogenized</td>
<td>2.7</td>
<td>17.7</td>
<td>53.6</td>
<td>181.0</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td>0.4</td>
<td>0.2</td>
<td>0.5</td>
<td>0.3</td>
</tr>
</tbody>
</table>

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Table II. GLC determination of fatty acid composition of the active band at Rf 0.41 on TLC plates and ethane production by oat roots. The acetone extract was dissolved in buffer and a portion added to the active fraction (Table II). Unlike the system reported by others (2, 5), this system was capable of producing ethane only when linolenic acid was present. These results support the suggestion of others (9) and lend us to conclude that linolenic acid was the precursor of ethane.

Root tissues were homogenized in buffer or linolenic acid solution to determine their ability to produce ethane (Table III). The legumes liberated high levels of ethane, the cereals were intermediate, and corn was low. The comparatively low production of ethane by sunflower roots may be due to phenolic interference with the ethane-liberating enzymes. Not only did the addition of linolenic acid to the homogenization medium stimulate ethane production, it also caused a marked increase in the amount of ethylene liberated by the homogenates. This was especially apparent with corn root homogenates which liberated nearly 8-fold more ethylene in the presence of linolenic acid.

When oat roots were fractionated (4), the enzyme responsible for ethane production from linolenic acid remained in the supernatant fraction after an 80,000 × g × 0.5 hr centrifugation (Table IV). The enzyme was retained by an Amicon ultrafiltration membrane with a 100,000 mol wt exclusion with no loss of activity and a 300,000 mol wt exclusion membrane with a substantial loss of activity. The enzyme responsible for ethane production by oat root homogenates is a soluble, high mol wt protein.

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LITERATURE CITED


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FIG. 1. Determination of optimum pH for ethane production by tissue homogenates of P. vulgaris L. cv. Harvester. Five apical bud explants were excised, homogenized in 1 ml of buffer, sealed in a glass syringe for 2 hr at 28°C, and ethane liberation determined by gas chromatography.