Polyamines in Soybeans

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ABSTRACT

Putrescine, spermidine, and spermine were three main polyamines isolated from soybeans and partially characterized. Occurrence of polyamines in soybeans was established by separating trichloroacetic acid extracts of soybeans by cationic exchange column chromatography, identification with thin layer chromatography, paper electrophoresis, mass spectral analysis, reactions with ninhydrin and Dragendorff reagents, and spectrophotometric characteristics. Soybeans contained a minimum of 29.0 micrograms of polyamines per gram of full-fat flour. The alcohol-soluble fraction of soybeans contained polyamines also. Resting seeds contained spermidine in higher concentration than either putrescine or spermine. Spermine appeared to be present in lowest concentration. Preliminary experiments suggested that some polyamines were possibly in bound forms.

Polyamines are widely distributed in plants, microorganisms, and animal tissues. Their distribution in microorganisms, conjugation with glutathione, nucleic acids, lipids, and viruses, function as a growth factor, and abilities to stabilize cells, protoplasts, and mitochondria have been studied and reviewed. Since then, increasing attention has been given to their effects on metabolism and biological functions in animal tissues. Investigations on plant tissues are relatively few. Putrescine \( \text{H}_2\text{N(CH}_2\text{)}_4\text{H} \), spermidine \( \text{H}_2\text{N(CH}_2\text{)}_3\text{NH(CH}_2\text{)}_2\text{H} \), spermine \( \text{H}_2\text{N(CH}_2\text{)}_4\text{NH(CH}_2\text{)}_2\text{NH(CH}_2\text{)}_4\text{H} \), and cadaverine \( \text{H}_2\text{N(CH}_2\text{)}_4\text{NH} \) have been found in cereal grains; putrescine, spermidine, and spermine in higher plants; and animal tissues. Their distribution in microorganisms, plants, and animal tissues is not known. Their occurrence in soybeans indicates that they are endorsed or recommended by the Department of Agriculture.

Soybean Flour and Alcohol Extraction of Soybeans. The beans (stored at 4 C) were ground into full-fat flour with an Alpine mill and kept at 4 C for further use. For the alcohol extractable fraction the full-fat soy flour was extracted with 80% (v/v) ethanol at a solvent-to-flour ratio of 5:1 at room temperature for 1 hr. The process was repeated once more with half the amount of ethanol. The combined alcohol extracts were evaporated under reduced pressure and lyophilized.

Extraction of Polyamines. One kilogram of soybean flour was stirred in 4 liters of 5% trichloroacetic acid for 1 hr at room temperature. After centrifugation to separate the extract, the residue was reextracted with 2 liters of 5% trichloroacetic acid. The trichloroacetic acid in the combined extracts was removed by shaking the solutions five times with several volumes of ether. After removing residual ether under reduced pressure, the solution was adjusted with NaOH to pH 13 to free the polyamines. The alkaline solution was then extracted five times with 100 ml of 1-butanol. The butanol solution (500 ml) was lyophilized and the resulting solids were dissolved in water (200 ml). The solution was neutralized with HCl and then centrifuged to remove insoluble materials. The clear supernatant contains the polyamines and is called crude polyamine extract.

Lyophilized alcohol extractables from 1 kg of soybean flour were dissolved in 200 ml of water, extracted with diethyl ether to remove oily materials, adjusted to pH 13 with sodium hydroxide, and extracted with 1-butanol. The butanol extracts were lyophilized, and further treatment was as for preparation of crude polyamine extract.

Column Chromatography. Crude polyamine extract was passed through a 2 × 20 cm column of Dowex-50 resin, and the column was first washed with 200 ml 50 mM sodium EDTA followed with 1 liter of distilled water. The water and EDTA were derivatives of putrescine. All three compounds are malodorous. Their presence in soybeans may be significant to the physiology of the seed but may adversely affect flavors of soybean proteins and products. For these reasons polyamines in soybean seeds were investigated. Isolation of putrescine, spermidine, and spermine as chloride salts are reported here.

MATERIALS AND METHODS

Kanrich variety of soybeans was used. Putrescine, spermidine, and their chloride or phosphate salts were purchased from Nutritional Biochemical Company, Cleveland, Ohio. Precoated thin layer plates, Silica Gel F-254, used for chromatography of polyamines were bought from Drinkerham Instruments Inc., Westbury, New York. Dowex-50 (X8, 200-400 mesh, H\(^+\)) for column separation of polyamines came from Bio-Rad Laboratories, Berkeley, California.

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2 Headquarters for the Northern Marketing and Nutrition Research Division, Agricultural Research Service, United States Department of Agriculture.
removed metals, salts, and materials not attached to the column. Amino acids and monoamines were eluted with an increasing gradient of HCl solution. The gradient elution was similar to the one used by Tabor et al. (13); 300 ml of 2.5 N HCl was added dropwise to 300 ml of water in a mixing vessel (500-ml Erlenmeyer flask). Immediately after the addition of the 2.5 N HCl solution, 300 ml of 6 N HCl was added, again dropwise, without changing the solution in the mixing vessel. The effluent was collected as 5-ml fractions with a Beckman fraction collector. Amine peaks were located with TLC. Fractions containing polyamines were pooled and lyophilized. Lyophilized solids were usually dissolved in a small volume of water and neutralized before diluting to 10 ml. This solution was used in assaying for polyamines with paper electrophoresis and by spectrophotometric measurements.

TLC. TLC (5) was used for assay of polyamines in the column effluents. After a 50-μl sample from 5-ml fractions was spotted on the plate was developed with solvent A (Methyl Cellose-propionic acid-water in a ratio of 70:15:15 v/v, saturated with NaCl). Occasionally, solvent B (Carbitol-propionic acid-water in a ratio of 70:15:15 v/v, saturated with NaCl) was used. When the solvent front reached about 13 cm (6-8 hr), the plate was dried, sprayed with 2% ninhydrin in butanol, and heated at 100 C for 15 min. The intensity of brown spots was monitored and traced with a Schoeffel spectrophotometer Model SD3000, wavelength setting at 550 nm. The tracings were cut out and weighed to obtain relative intensities due to the presence of polyamines.

Assay. The amounts of putrescine, spermidine, and spermine in the polyamine fractions pooled from column chromatography were estimated in two ways: reaction with 2,4-dinitrofluorobenzene (3) and nitrogen determination by micro-Kjeldahl method (9). The first was tedious for routine analysis but was used to standardize the second. For spectrophotometric determination of polyamines, 100 nmole of amino group in putrescine, spermidine, and spermine were reacted with 2,4-dinitrofluorobenzene for 20 min at room temperature; optical densities were measured with a Beckman spectrophotometer (3).

Electrophoresis. Paper electrophoresis of polyamines was carried out on a strip of Whatman No. 1 filter paper run at 300 v for 45 min in 50 mM citrate buffer, pH 4.5, in a Gelman paper electrophoretic chamber. The sample spots were detected the same way as TLC.

RESULTS

Chromatography of Polyamines. Cationic exchange resins and TLC have been used to separate polyamines (5, 13). However, as conditions change—differences in crosslinkage and mesh size of resin, type of effluent and thin layer plate—resolution of polyamines changes. Table I contains the results when authentic putrescine, spermidine, and spermine were chromatographed under given conditions. With 8% crosslinked Dowex-50 resins putrescine, spermidine, and spermine appeared in order with increasing HCl concentration from 3.2 to 4.9 N. When TLC was used to separate the same three polyamines with solvents A and B (5), R_f values in solvent B differed from those described by Hammond and Herbst (5). In solvent A, R_f values agreed with theirs except that spermine trailed badly. Since more time was required for the solvent front to travel 13 cm in solvent B than in A, solvent A was mainly used.

Figure 1 shows a chromatographic pattern of a crude polyamine extract from soybeans on a Dowex-50, X8, 200-400 mesh, H^+ form column and thin layer chromatography.

<table>
<thead>
<tr>
<th>Polyamines</th>
<th>Dowex-50 Resin (Column)</th>
<th>Silica Gel (Thin Layer)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCl conc for elution¹</td>
<td>Solvent A²</td>
</tr>
<tr>
<td>Putrescine³</td>
<td>3.2-3.7</td>
<td>0.60 (0.56)</td>
</tr>
<tr>
<td>Spermidine</td>
<td>3.7-4.9</td>
<td>0.43 (0.49)</td>
</tr>
<tr>
<td>Spermine</td>
<td>4.9</td>
<td>0.24 (0.41)</td>
</tr>
</tbody>
</table>

¹ A volume of 300 ml 2.5 N HCl was added dropwise to 300 ml of water in a mixing vessel. Immediately after the addition of the 2.5 N HCl solution, 300 ml of 6 N HCl was added, again dropwise, without changing the solution in the mixing vessel.

² Solvent A: ethylene glycol monomethyl ether (Methyl Cellose)-propionic acid-water (70:15:15). Solvent B: diethylene glycol monomethyl ether (Carbitol)-propionic acid-water (70:15:15). Both solvent systems were saturated with NaCl. R_f values in parenthesis are from Hammond and Herbst (5). Spermine trailed considerably.

³ Polyamines and their chloride salts gave similar results.

Abbreviation: TLC: thin layer chromatography.
also indicates that the separated polyamines are free from contaminants; however, after putrescine, spermidine, and spermine were pooled into three fractions (I, II, III in Fig. 1) and then analyzed with paper electrophoresis, results were somewhat different (Fig. 4).

The putrescine fraction contains at least two components migrating the same distance as spermidine and spermine. It is difficult to believe that so much spermidine and spermine occur as contaminants in the putrescine fraction. At present cadaverine or higher homologs of diamines are being investigated as possible contaminants. Cadaverine has been reported in soybeans (1), and diamines will appear with putrescine on a Dowex-50 column (13). The spermidine fraction contains trace amounts of spermine and vice versa in the spermine fraction. Neither fraction II nor III contains putrescine.

Supporting evidence for polyamines in soybeans was obtained from mass spectral data. Results of analyzing authentic putrescine and spermidine are given in Table II. As seen in Table II, putrescine yields ion peaks of m/e 88, 71, 59, 43, and 30. The parent ion (88) is not stable since its relative intensity is 1% of the base peak (m/e 30). Spermidine produces ion peaks of m/e 145, 101, 87, 44, and 30. Again, the parent ion of m/e 145 is less intense than the other peaks. When fractions I and II were analyzed, mass spectral evidence indicated that fraction I contained cadaverine and possibly higher homologs. The occurrence of cadaverine was deduced from the presence of m/e 85 (30%) and 73 (10%) in addition to the expected

Fig. 2. TLC of crude polyamine extract from soybeans compared with standards: A: putrescine; B: spermidine; and C: spermine. Solvent system A: Methyl Cellosolve-propionic acid-water (70: 15:15 v/v) saturated with NaCl.

Fig. 3. TLC of separated polyamines from fractions I, II, and III in Figure 1 with solvent A. A, B, and C are standards as in Figure 2.
I. Putrescine, Spermidine, & Spermine

Fig. 4. Paper electrophoresis of polyamines separated into fractions I, II, and III as in Figure 1. Citrate buffer pH 4.5 0.05 M at 300 v for 45 min with a Gelman paper electrophoresis apparatus.

peaks for putrescine in fraction I. Putrescine does not lose 3 mass units to form the m/e 85 ion; this ion probably forms by loss of mass 17 (NH₃) from cadaverine (102–17). Also, beta bond-cleavage of cadaverine will produce m/e 73. These data and those from paper electrophoresis suggest that cadaverine is present with putrescine in fraction I. Spectra from fraction II and that of authentic spermidine trichloride are essentially identical.

Other evidence to support that the materials isolated from soybean seeds and seedlings are polyamines include: (a) reaction with fluorodinitrobenzene and measurement of the spectra of the derivatives (3)—putrescine peaked at 350 nm while spermidine and spermine did at 360 nm; (b) positive reaction with Dragendorff reagent (2); and (c) crystallization as chloride salts; however, low yields have prevented quantitative elemental analysis.

Estimation of Polyamines in Soybeans. Polyamine contents of cereals are, for example, 394 µg and less than 20 µg per gram of fresh wheat germ and endosperm, respectively (10). Soybeans contain 28.7 µg of polyamines per gram of flour estimated by nitrogen analysis in the pooled fractions of Figure 1 (Table III). Because polyamines dissolve in alcohol, ethyl alcohol extraction of soybeans was also tried. Because only putrescine was found in the alcohol extract, the acidic effects resulting from trichloroacetic acid extraction were simulated. The alcohol-extractables were adjusted to pH 2.0 before adding NaOH, but difficulties resulted with the Dowex-50 resin columns. Large amounts of white precipitate appeared when the samples were placed on the columns, and the columns became plugged. Preliminary information indicates the presence of acidic polysaccharides, lipids, and oily materials. The higher amount of putrescine (Table III) in the alcohol-extractables may suggest the possibility of more polyamines in soybeans. Polyamines found in the trichloroacetic acid extract may represent only a fraction of the total. However, the lack of spermidine and spermine in the alcohol-extractables may indicate that the alcohol extraction method is selective and less satisfactory. Therefore, an improved method for isolation may be needed.

DISCUSSION

This work marks the first isolation of putrescine, spermidine, and spermine from soybean seeds. Polyamines are cationic bases and represent a different type of compound from other soybean constituents—phytates, saponins, isoflavones, and others (11). The presence of polyamines in soybeans, though at a low level of 28.7 µg per gram of flour, may play an important role in the physiology of resting and germinating seeds. Preliminary experiment on germinated soybean seedlings indicates a rapid increase of putrescine within 5 days. Concurrently, protein is synthesized rapidly during that period. Polyamines have been reported related to protein and nucleic acid synthesis (14). Resting seeds contain a lower level of putrescine than that of spermidine. Putrescine is a precursor of spermidine and spermine (13). Results obtained here and by others (1) indicate that cadaverine is also present. Perhaps cadaverine is also a source of primary amine.

Owing to their basic properties, polyamines may affect the

Table II. Mass Spectral Study of Spermidine and Putrescine

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ion Peaks</th>
<th>Relative</th>
<th>Positive Ions</th>
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<tbody>
<tr>
<td></td>
<td>m/e</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Spermidine</td>
<td>145</td>
<td>7</td>
<td>H₂N(CH₃)₂NH(CH₃)₂NH₂ (parent ion)</td>
</tr>
<tr>
<td></td>
<td>101</td>
<td>20</td>
<td>H₂N(CH₃)₂NHCH₂-</td>
</tr>
<tr>
<td></td>
<td>87</td>
<td>35</td>
<td>H₂N(CH₃)₂NH</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>100</td>
<td>CH₃NH—CH₃</td>
</tr>
<tr>
<td>Spermine</td>
<td>30</td>
<td>46</td>
<td>CH₂—NH₂</td>
</tr>
<tr>
<td>Putrescine</td>
<td>88</td>
<td>1</td>
<td>H₂N(CH₃)₂NH₃ (parent ion)</td>
</tr>
<tr>
<td></td>
<td>71</td>
<td>19</td>
<td>NH₂(CH₃)₂CH—CH₂</td>
</tr>
<tr>
<td></td>
<td>59</td>
<td>26</td>
<td>NH₂(CH₃)₂CH₄</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>52</td>
<td>CH₃ or CH₃N</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>100</td>
<td>CH₂—NH₂</td>
</tr>
</tbody>
</table>

Table III. Estimation of Polyamines in Soybeans

Kanrich full-fat soybean flour was used for analysis.

<table>
<thead>
<tr>
<th>Polyamines</th>
<th>Extraction Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5% Trichloroacetic acid</td>
</tr>
<tr>
<td>Putrescine</td>
<td>6.6</td>
</tr>
<tr>
<td>Spermidine</td>
<td>16.4</td>
</tr>
<tr>
<td>Spermine</td>
<td>5.7</td>
</tr>
<tr>
<td>Total</td>
<td>28.7</td>
</tr>
</tbody>
</table>

¹ Alcohol-extractables were isolated (see text), and polyamines were extracted with butanol after the pH was brought to 13 from neutrality.
solubilities of soybean constituents. They are associated with lipids, nucleic acids (6), probably phytate through the phosphate group, and also possibly with sugars by Amadori reaction (7). Another possibility is that they react with the $\gamma$-pyrone ring of isoflavones. Isoflavone is quite water insoluble, and possibly its $\gamma$-pyrone can react with ammonia or its derivative as polyamine to form $\gamma$-pyridone. A good example is the conversion of isomaltol to a corresponding pyridone (4). Polyamines may act as crosslinking agents to maintain such constituents as lipids, isoflavone, and phytates in solution. All these ideas are speculative, but they suggest the importance of polyamines in soybeans.

Free polyamines are malodorous although their chloride or phosphate salts are not. Despite the minute quantities of polyamines in soybeans, they may produce off-flavors in soybean flour and limit its use as food. Though their level in soybeans is somewhat in doubt, their existence is certain. Alcohol extraction seems to be an effective way to remove polyamines from soybeans. More experiments on polyamines in alcohol-extractables are underway.

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LITERATURE CITED