Glyceride Structure and Biosynthesis of Natural Fats

Part III. Biosynthetic Process of Tryglycerides in Maturing Soybean Seed

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Changes in the contents and compositions of lipid classes and water-soluble components were examined in maturing soybean seed. At the first stage of seed development, triglyceride formation was very slow, and considerably large quantity of free fatty acids, phosphatidic acid, monoglyceride, and diglyceride were accumulated. At the second stage triglycerides were produced rapidly and the other components decreased. Glycerophosphate also appeared in the maturing seed. These results suggest that Kennedy's pathway to triglycerides and other side pathway occur in seed. Positional distribution of fatty acids in mono-, di-, and triglycerides was also determined, and synthetic process of glyceride structure was discussed.

INTRODUCTION

Oil-bearing plants have been shown to form fatty acids of a definite composition and a specific pattern of glyceride structure in their seed oils on the basis of the species of the source plants. Thus, the changes in component lipids of seed that occur when a plant proceeds toward maturity are closely associated with biosynthetic mechanism of individual fatty acid and with biosynthetic process of specific glyceride structure. Several workers1–4) studied the physical and chemical changes of oils in maturing seed of some plants. Recently, Sims and coworkers5,6) measured changes in composition of lipid classes in maturing seeds of flax and safflower plants. Miwa and coworkers7) also observed the mode of epoxyoleic acid biosynthesis by quantitative measurements of epoxyoleic acid and co-occurring fatty acids in maturing Vernonia anthelmintica (L.) Willd. seed.

In the present study, changes in lipid classes in maturing soybean seed were investigated in order to observe the biosynthetic process of triglycerides. Gross changes in the contents of glycerides, phospholipids, and water-soluble components were followed, and changes in the composition of these lipids were also measured. From these data and additional experiments, biosynthetic process of glyceride structure was discussed.

MATERIAL AND METHOD

Seed preparation.

Soybean seeds (Tokati Nagaba) were collected eight times, beginning 12 days after flowering and ending after 77 days when seeds were considered to be mature, and sample number was given as shown in the Table I.

Extraction of Lipids and Water-Soluble Components.

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The weight per thousand seeds was determined for each sample. Seeds were homogenized in a mixture of CHCl₃-MeOH (2:1, v/v) using homogenizer, and filtered. The residue was further re-extracted with hot water by the same technique. The water extract was slowly added into the above organic solvent extract in separating funnel. After standing for a few hours, two solvent layers separated. The upper aqueous phase was concentrated under vacuum, and the resulting dry matter was used for examination of water-soluble components. The chloroform phase was filtered through a Tōyō No. 2 filter paper, and on the vacuum distillation gave crude lipids.

**Fractionation of Crude Lipids.**

Free fatty acids were isolated from crude oil by general method on the basis of neutralization and extraction. The residual lipid was fractionated in silica gel column according to a modified technique of the procedure described by Quinlin, in which benzene, 10% ether in benzene, diethyl ether, and methanol were run through silica gel column as the eluting solvents, and triglycerides, diglycerides, monoglycerides, and phospholipids were eluted, respectively.

**Analyses of Fatty Acid and Glyceride Composition.**

Fatty acid composition was analyzed by "bromazon zone" method of paper chromatography. The separation of triglycerides was performed by paper chromatographic method as follows. Triglycerides were placed on paper impregnated with silicon oil (or liquid paraffin), and then developed with a mixture of aceton and acetic acid as moving solvent, using a multiple development technique, for 2 hours and successively 14 hours at 20°C. The spots were visualized by dipping the chromatogram in aqueous 0.5% solution of KMnO₄ for about one minute. Individual triglyceride was well separated on the basis of PC value of the triglyceride. Quantitative measurement of spots was also carried out by densitometer, and a possible composition of triglycerides was calculated from these values. Partial glycerides were analyzed by paper chromatography and thin-layer chromatography.

**Chromatography of Phospholipids and Other Components.**

Marinetti-type paper chromatography was employed for separation of phospholipids. Aqueous 0.5% solution of KMnO₄, Hanes-Isherwood reagent, ninhydrin, and periodate-schiff reagent were used as color reagents. Thin-layer chromatography was also effectively applied for analysis of phospholipids in crude oil.

Sugars and water-soluble phosphate was followed by paper chromatography according to the method reported by Glegg and Hübscher, respectively.

**Other Analytical Methods.**

Positional distribution of fatty acids in di- and triglycerides was determined by the technique of lipase hydrolysis as described in the previous paper. Proportion of monoglyceride isomers was examined by the periodate oxidation method.

**RESULTS**

The weight per fresh thousand seeds at different stages of maturation, the amount of crude oils and phospholipids are shown in Fig. 1. Rapid oil production occurred on 20th and 22nd day after flowering and between 27th to 43rd day. A large portion of total oil in fully mature seed was produced at the late stage. Phospholipid content showed specific change which might be associated with oil formation.

Changes of fatty acid composition in crude oil is given in Fig. 2. The values represent percentage of individual acid in whole fatty acids. At early stage, the content of total saturated acids increase and then successively total unsaturated acids increase rapidly. A similar change in fatty acid composition was

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12) O. Hirayama, J. Biochemistry, in press.
16) O. Hirayama, This Journal, 28, 395, 201 (1964).
also observed at late stage. Although all saturated acids gave similar curves each other, unsaturated acids showed considerably different formation curves in which maximum increase of oleic and linolenic acid occurred on 20th and 22nd day and between 43rd to 77th day respectively and linoleic acid increased continuously after 16 days.

Composition of free acids and partial glycerides were summarized in Table II. Large content of the free fatty acids at the earliest stage decreased rapidly as seed matured, whereas monoglyceride percentage increased till 20th day (sample No. 3) and then decreased abruptly. Diglycerides content showed an increase between 16th and 20th day and between 22nd and 27th day. It was interesting that free acids and partial glycerides were accumulated at early stages which corresponded to the period of slow production of oil.

Fig. 3 shows the optical density curves of the paper chromatograms of triglycerides. The spots were distributed among PC values of 36, 38, 40, 42, 46, and 48. Main triglyceride in the spots of these values was determined by analyzing fatty acid composition of each spot, and shown in Fig. 3. At the earliest stage, triglycerides were divided into two groups of highly unsaturated (PC value
and lowly unsaturated (PC value 44~48) glycerides.

The former group may be protoplasmic triglycerides, because the triglyceride composition was similar to that of leaf triglycerides. Fig. 3 further suggests that triglyceride composition in maturing seeds showed great change at early stage, but little change after 22 days of flowering.

**TABLE II. COMPOSITION OF GLYCERIDE FRACTION**

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>FFA</th>
<th>MG</th>
<th>DG</th>
<th>TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37.2</td>
<td>36.6</td>
<td>16.3</td>
<td>9.9</td>
</tr>
<tr>
<td>2</td>
<td>36.5</td>
<td>48.8</td>
<td>10.7</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>14.1</td>
<td>65.5</td>
<td>14.1</td>
<td>6.3</td>
</tr>
<tr>
<td>4</td>
<td>3.2</td>
<td>2.9</td>
<td>4.1</td>
<td>93.0</td>
</tr>
<tr>
<td>5</td>
<td>—</td>
<td>2.7</td>
<td>32.2</td>
<td>65.1</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>2.5</td>
<td>15.3</td>
<td>82.2</td>
</tr>
<tr>
<td>7</td>
<td>—</td>
<td>0.6</td>
<td>9.3</td>
<td>90.1</td>
</tr>
<tr>
<td>8</td>
<td>—</td>
<td>0.1</td>
<td>—</td>
<td>99.9</td>
</tr>
</tbody>
</table>

* FFA, free fatty acid; MG, monoglyceride; DG, diglyceride; TG, triglyceride.

**TABLE I. ACID VALUE AND IODINE VALUE OF CRUDE OIL**

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Days after flowering</th>
<th>A. V.</th>
<th>I. V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>62.2</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>34.6</td>
<td>—</td>
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<tr>
<td>4</td>
<td>22</td>
<td>8.0</td>
<td>117.2</td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>3.3</td>
<td>125.2</td>
</tr>
<tr>
<td>6</td>
<td>33</td>
<td>2.9</td>
<td>110.1</td>
</tr>
<tr>
<td>7</td>
<td>43</td>
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</tr>
<tr>
<td>8</td>
<td>77</td>
<td>1.1</td>
<td>137.5</td>
</tr>
</tbody>
</table>

* unpublished results.

**FIG. 2. Change of Fatty Acid Composition in Oil Soybean Seeds.**

**FIG. 3. Optical Density Curve of Paper Chromatography of Triglycerides.**

The technique of paper chromatography is described in text. PC value correspond to total carbon numbers minus total unsaturation numbers of three fatty acids in triglyceride molecule. S, saturated; O, oleic; L, linoleic; Ln, linolenic. No., sample No.
Table III shows the positional distribution of fatty acids in glycerides of samples 6 and 8 obtained by pancreatic lipase hydrolysis. Both triglycerides from samples 6 and 8 gave a similar pattern of distribution in which saturated acids were preferentially esterified at 1 and 3 positions and unsaturated acids at 2 position. In sample 6, fatty acids esterified at 2 position of diglyceride showed similar composition with those of triglyceride, and the fatty acid composition of monoglyceride approximately corresponded to that at 1 and 3 positions of triglyceride. The examination of crude oil by the periodate oxidation technique also suggested that original monoglycerides might be 1-isomer.

Paper chromatograms of phospholipids were shown in Fig. 4, which suggests that phospholipid components was considerably different among the stages of seed maturation. Spot of $R_F$ 0.71 was positive for periodate-schiff reagent and KMnO$_4$ solution, and slightly positive for Hanes-Isherwood reagent. This spot lipids also gave $\alpha$-glycerophosphate by mild saponification. These results indicate that the spot contained phosphatidic acid and some lipid which was positive for periodate-reagent. Other spots were also identified to be phosphatidyl ethanolamine ($R_F$ 0.58), lecithin (0.47), and inositol phospholipid (0.34) from stain behaviour, $R_F$ values of authentic phospholipids, and their deacylation products. Similar results were also obtained by thin-layer chromatography.

Paper chromatograms of water-soluble components gave the spots which showed the same $R_F$ value with glucose, sucrose, $\alpha$-phosphoglycerophosphate, and phytin, with several other unknown spots.

**DISCUSSION**

Fig. 2 showed a reversed relation between changes of oleic and linoleic acid contents in samples after 27 days. The results may suggest a conversion of oleic acid to linoleic acid in maturing seed as demonstrated by Yuan and Bloch\(^{18}\) in vitro. A characteristic relation was also observed between formation curves of linoleic and linolenic acids. Although a possible mode of unsaturated acid formation from saturated acid is considered, it was impossible to observe them in detail.

**TABLE III. POSITIONAL DISTRIBUTION OF FATTY ACIDS IN ORIGINAL AND TRIGLYCERIDES IN SAMPLES NO. 6 AND 8 OBTAINED PANCREATIC LIPASE HYDROLYSIS**

<table>
<thead>
<tr>
<th>No. of fatty acid</th>
<th>1,3-position of TG</th>
<th>1,2-position of DG</th>
<th>2,3-position of DG</th>
<th>1,2-position of TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample No. 6</td>
<td>1.6</td>
<td>5.3</td>
<td>1.0</td>
<td>5.1</td>
</tr>
<tr>
<td>16</td>
<td>21.8</td>
<td>7.8</td>
<td>27.1</td>
<td>9.3</td>
</tr>
<tr>
<td>18</td>
<td>8.5</td>
<td>3.0</td>
<td>8.4</td>
<td>2.0</td>
</tr>
<tr>
<td>18:1</td>
<td>26.6</td>
<td>28.3</td>
<td>12.9</td>
<td>34.1</td>
</tr>
<tr>
<td>18:2</td>
<td>30.0</td>
<td>47.1</td>
<td>33.5</td>
<td>42.1</td>
</tr>
<tr>
<td>18:3</td>
<td>9.5</td>
<td>12.2</td>
<td>12.8</td>
<td>11.3</td>
</tr>
<tr>
<td>total S</td>
<td>33.9</td>
<td>12.4</td>
<td>40.8</td>
<td>12.3</td>
</tr>
<tr>
<td>total U</td>
<td>66.1</td>
<td>87.6</td>
<td>59.2</td>
<td>87.7</td>
</tr>
</tbody>
</table>

S, saturated fatty acid; U, unsaturated fatty acid; MG, monoglyceride; DG, diglyceride; TG, triglyceride.

Original monoglycerides are presumed to be 1-isomer by the periodate oxidation technique.

**FIG. 4. Chromatograms of Phospholipids.**

Samples were run on silicic acid-impregnated paper using diisobutyrylketone-acetic acid-water (40:20:3) as the solvent system. The spots were visualized by dipping chromatograms in aqueous 0.5% solution of KMnO$_4$.

Kennedy and coworkers\textsuperscript{19} elucidated following pathway to triglycerides in animal tissue: 
\[ \alpha\text{-glycerophosphate} \rightarrow \text{phosphatidic acid} \rightarrow 1, 2\text{-diglyceride} \rightarrow \text{triglyceride}. \]
All these intermediates appeared in the present maturing seeds. The accumulations of the intermediates such as phosphatidic acids and diglycerides at the stage of slow formation of oil suggest that seed oil might be synthesized according to Kennedy’s pathway. However, the accumulation of monoglycerides is not explained from the pathway, because it does not contain them as the intermediate. On the other hand, specific changes of monoglyceride content as shown in Table II seem to suggest that the monoglycerides are not secondary products formed by lipase during the preparation of sample but original products as intermediate in oil synthesis. The discussion leads us to a speculation that Kennedy’s pathway and other side pathway which is associated with monoglyceride occurred in seed oil production. Recently, Clark and Hubscher\textsuperscript{20} suggested that monoglyceride was directly converted to di- and triglyceride by mitochondrial preparation of intestinal tissue. Pieringer and Hokin\textsuperscript{21} also demonstrated that monoglyceride was converted to liso-phosphatidic acid by microsomal preparation from guinea pig or calf brain. Either of these reactions might be associated with the sides pathway mentioned in seed oil formation.

Data obtained by the lipase hydrolysis (in Table III) indicate that specific distribution of fatty acids in triglyceride molecules has already occurred at early stages of seed maturation, and the pattern of distribution correspond to that of fully matured seed oil. These results lend support to the assumption that the formation of glyceride structure in the oil is attributed to specificities of the enzymes in normal synthetic pathway of the triglycerides. The distributions of fatty acids in partial glycerides also revealed that there are similarities in compositions of fatty acids esterified in the same positions of glycerols among mono-, di-, and triglycerides in immature seeds. The finding, in conjunction with specific changes of mono- and diglyceride contents in immature seeds, seems to provide us a possible synthetic process of triglycerides having specific positional distribution of fatty acids.