STUDIES ON FRACTION I PROTEIN

Kuan-Jen Yang

INTRODUCTION

The protein constituents of spinach leaf were first investigated by Wildman and Bonner (1947). Two separate classes were distinguished by the different electrophoretic mobilities; these were designated as "Fraction I" and "Fraction II". In tobacco, spinach and other green leaves, Fraction I protein makes up a major portion of the total leaf protein. Several studies have shown that this protein has some distinctive properties in addition to its abundance. It has a large molecular weight of approximately 500,000 and exhibits ribulose-1, 5-diphosphate carboxylase activity (Dorner et al., 1957; Lyttleton and T'oso, 1958; Park and Pon, 1961; Trown, 1965; Kuehn et al. 1969).

The isolation and characterization of Fraction I protein from spinach (Trown, 1965; Paulsen and Lane, 1966), rice (Mendiola and Akazawa, 1964) and chemosynthetic microorganisms (Kuehn and McFadden. 1969) have been reported. To my knowledge, the purification of the protein from tobacco leaf chloroplast to a homogeneous state has never been carried out. Here the author wishes to report the isolation from tobacco leaves of a purified Fraction I protein which is essentially homogeneous by ultracentrifugal analysis and disc electrophoresis. A preliminary account of this work has appeared in Pacific Slope Biochemical Conference, Eugene, Oregon (1967).

Some investigators (Spencer and Wildman, 1963; Francki, Boardman and Wildman, 1965) have described a system, consisting of chloroplast isolated from tobacco leaves which, when incubated aerobically in Tris buffer in the presence of Mg, ATP and ATP generator, were capable of incorporating appreciable quantities of 14C-amino acid into protein. 70 S ribosomes which located in the mobile phase of chloroplast are mainly responsible for this incorporating activity. This system is inhibited by RNase, puromycin, and chloramphenicol. The characteristics of the incorporating activity are similar to those of the conventional ribosome preparation obtained from such organisms as Escherichia coli (Nirenberg and Matthaei, 1961), wheat (Bamji and Jagendorf, 1966) and pea (Racette 1961).

Preliminary evidence is presented in this communication to indicate that tobacco chloroplasts are capable of incorporating 14C-amino acids into a typical chloroplast protein, namely, Protein I protein.

MATERIALS AND METHODS

Protein Purification

The methods of isolation and fractionation of Fraction I protein were based on the report of Mendiola and Akazawa (1964). Crude tobacco leaf chloroplast extract precipitated with (NH₄)₂SO₄ at 50% saturation was first applied to Sephadex G-100 column, and then to Sephadex G-200 gel filtration. The eluting solution was 0.1 M Tris buffer (pH 7.5) containing 1 mM mercaptoethanol. Protein fractions were collected and reprecipitated at 50% saturation of (NH₄)₂SO₄. After being desalted by passage through a column of Sephadex G-25, the protein was next applied to a column of DEAE-cellulose and eluted with 0.01 M Tris buffer (pH 7.5) containing 0.1 mM
mercaptoethanol and NaCl in a linear gradient (0-1 M)

**Ultracentrifugal Analyses**

Analyses were carried out in a Spinco Model E analytical ultracentrifuge at 42,020 rpm and temperatures from 2 to 10°C, using a standard 12-mm cell.

**Amino Acid Analyses**

Fraction I protein was purified to homogeneity as outlined above. Prior to amino acid analysis protein solutions were dialyzed 24 hours against four changes of 500 ml distilled water. After freeze-drying, 2 mg of protein were transferred to hydrolysis vials containing an equal volume of HCl, degassed in vacuo, and then sealed under N₂. Hydrolysis was conducted at 110-113°C. All hydrolysates were analyzed with a Beckman-Spinco automatic amino acid analyzer.

**Electrophoresis**

The purity of Fraction I protein prepared from tobacco leaf chloroplast was examined by polyacrylamide gel disc electrophoresis. Polyacrylamide gel column were prepared with running-gel, stacking-gel, and sample-gel sections according to the general procedure of Davis (1964). All running-gel stacks were prepared at pH 8.0 with runs at pH 8.3. A current of less than 5 mA/gel was applied at room temperature until a tracking dye (bromophenol blue) was approximately 0.2 cm from the bottom of the gel. Gels were stained in amido black (1 g in 200 ml of 7% acetic acid) for two hrs and then destained electrophoretically at current of 3 mA/gel.

**Tryptic Digestion and Peptide Mapping**

The oxidation of Fraction I protein was carried out in a way described by Sanger (1949). 50 mg protein was dissolved in 9 ml formic acid and treated with one ml of 30% hydrogen peroxide for 15 minutes at room temperature. The mixture was diluted with 10 ml of water, concentrated in vacuo to a volume of 1 ml and the oxidized protein was precipitated with acetone. The precipitated material was washed free from formic acid by repeated centrifugation of acetone suspension and then dried in the air. 50 mg of the oxidized protein was dissolved in 5 ml of water at pH 10 and then adjusted to 8.3. The solution which contained a fine suspension was incubated at 38°C in pH-stat. 0.2 ml of trypsin solution (5 mg/ml in 0.001 M HCl) (Worthington Biochemical Corporation) was added. The mixture was maintained at pH 8.3 by a Automatic Titrator Model TTTI (Radiometer, Copenhagen, Denmark). Hydrolysis was completed within 20 minutes as judged by the appearance of plateau on Radiometer SBR 2 Recorder (Radiometer, Copenhagen, Denmark). The solution was then adjusted to pH 6.5 and the insoluble material was washed twice with 0.5 ml of water. The washed solutions were combined with the supernatant solution of peptide and lyophilized. The peptides in the soluble fraction of the tryptic digestion were separated by a two-dimensional process of paper electrophoresis and paper chromatography. Paper electrophoresis on water-cooled matel plate insulated by 0.1 inch sheet of polyethylene was carried out by modifying the method of Katz et al (1959). About 1.5 mg of the lyophilized tryptic digest was dissolved in 100 ul of pyridine-acetic acid-water (10:0.4:90) electrophoresis buffer at pH 6.5 and this was transferred with a capillary pipette to the starting area on a 40 X 55 cm sheet of Whatman 3 MM filter paper. The paper was wetted with the electrophoresis buffer and blotted. 20 cm wicks of two layer of filter paper were overlapped, 2.5 cm at each end of the paper and immersed in the electrode vessels. One layer of buffer-wetted dialysis tubing was inserted between each which served to reduce
the flow of buffer from the vessels into the paper. The paper was covered with a 2-cm thick plexiglass. Electrophoresis was carried out at 1.00 volts for two hours. At the end of this period, the paper was removed, dried, and after turning 90° from the direction used in electrophoresis, subjected to chromatography for 20 hours in n-butanol-acetic acid-water (4:1:5). After drying, the chromatogram was usually sprayed with ninhydrin and the spots observed were outlined with pencil.

**Preparation of Tobacco Leaf Chloroplasts**

Method for the isolation of chloroplasts from young tobacco leaves (Nicotiana tabaccum var. Turkish Samsun) was similar to that described by Spencer and Wildman (1964). Fresh weight 5g samples of deribbed leaf fragment were chopped into a fine mince with 6 ml of Honda medium, containing 2.5% Ficoll, 5% dextran, 0.25 M sucrose, 0.025 M Tris at pH 7.8, 1.0 mM magnesium acetate, and 4.0 mM mercaptoethanol. The crude extract was filtered through 4 layers of fine cheesecloth and the resultant cell-free extract was centrifuged at 1,000 g for 10 minutes. The supernatant was discarded and the pellets were resuspended in 0.4 ml Tris-Mg:Sh medium which consists of 0.01 M Tris at pH 7.8, 15 mM magnesium acetate and 3.0 mM mercaptoethanol.

**Reaction Mixture for Protein Synthesis**

The standard reaction mixture for these experiments contained the followings, in a total volume of 3.0 ml: 1.5 ml of chloroplasts, 100 u moles Tris-buffer, pH 7.5; 40 ug pyruvate kinase, 30 u moles ATP, 0.15 u moles each of UTP, GTP and CTP; 18.75 u moles phosphoenolpyruvate, 20 moles magnesium acetate, 50 u moles KCl, 100 ul of 14C-amino acids mixture. The reaction for protein synthesis was initiated by the addition of chloroplasts. After incubating at 37°C for 20 minutes, 0.5 ml of 0.1 u moles each of non-radioactive amino acids was added and further incubated for 50 minutes.

**Sucrose Density Gradient Centrifugation**

A 25 ml gradient of sucrose(5-20% W/V) was prepared in 30 ml Spinco centrifugation tubes. The sucrose solution was prepared in 0.025 M Tris buffer, pH 7.8, containing 0.015 M magnesium acetate. Before the centrifugation, one ml of the chloroplast supernatant was placed on the top of the gradient. Centrifugation in the Spinco (Rotor SW 2.1) was maintained at 24,000 rpm for two hours at 4°C. The gradient was fractionated by piercing the base of the tube and collecting 10-drop fractions (about 1.0 ml). The protein was determined in each fraction from the extinction at 280 mu.

**Sephadex Gel Filtration**

The dextran gel Sephadex G-100 and G-200 were used and prepared on columns, similar to the procedure described by Mendiola and Akazawa (1964). Sephadex G-100 was suspended in 0.05 M Tris buffer at pH 7.5, containing 0.1 M NaCl and poured into a Sephadex Laboratory Column K 25 (2.5×45cm). After the gel had settled the soluble radioactive protein was carefully layered on the top of the gel bed underneath the buffer by means of a thin polyethylene tubing connected to a syringe. When all of the material had passed below the top layer of the gel bed, the column was connected to a buffer reservoir. The eluate was automatically collected in 5-ml fraction by a Beckman Model 132 Fraction Collector.

**RESULTS**

**Analysis of Chloroplast Protein in the Ultracentrifuge**
When protein isolated from chloroplasts was examined in the analytical ultracentrifuge, three peaks were seen to move away from the starting boundary after the rotor reach its operating speed of 42,040 rpm for 20 minutes (Fig. 1). The left two peaks are the protein boundaries usually observed in extracts of green leaves, have sedimentation coefficient of 18 S and 4 S, and have previously been designated as Fraction I and Fraction II proteins respectively by Eggman et al. (1953). The right small peak was found to have sedimentation coefficient of 70 S, corresponding to those seen by Lyttleton (1962) in extracts of spinach chloroplasts.

**Homogeneity Studies of Fraction I Protein**

The Fraction I protein was isolated from tobacco chloroplasts and purified in accordance with methods described by Mendola and Akazawa (1964). The analytical ultracentrifuge exhibited a single symmetrical peak during sedimentation (Fig. 2), indicating a high degree of homogeneity of the molecular weight of the components of this preparation. Further evidence of homogeneity was obtained through the analysis of disc electrophoresis. Fig. 3 shows that only one visible boundary appears.

**Amino Acid Composition**

The amino acid composition of the purified Fraction I protein expressed as um per cent are summarized in Table I. These analyses were made on samples that had been hydrolyzed for 24 hrs. No attempt has been made to correct the data for the destructive hydrolysis of serine, methionine and threonine or for the relatively slow liberation of leucine, isoleucine and valine from protein. In spite of these limitations, it is clear that there is no significant differences between two preparations. While the amino acid composition of tobacco Fraction I protein differs slightly from those of rice and spinach with respect to a few amino acid, the overall analyses are quite similar.

**Peptide Mapping**

Peptide map of the tryptic digestion of Fraction I protein reveals between 30 and 32 ninyhydrin-positive spots with no significant ninyhydrin-positive material at the origin. The result of this experiment is shown in Fig. 4. It will be noted that there is a large number of peptides which move relatively slowly by electrophoresis. Because of their rapid chromatographic mobility, it can be assumed these are hydrophobic in nature, consisting largely of such amino acid as glycine, alanine, proline, valine and isoleucine.

**Amino Acid Incorporation**

The results summarized in Table II indicate that the amino acid incorporating activity of chloroplast is dependent on the presence of ATP and ATP generator. The removal of ATP, phosphoenolpyruvate, and pyruvate kinase from the system causes the loss of 80% incorporating activity.

**Radioactive Protein Analyses**

In order to separate soluble radioactive protein from ribosomal protein, the chloroplasts were centrifuged down from the reaction mixture at 1,000 g for 10 minutes. The radioactive protein which present in the supernatant was layered on a sucrose density gradient column and centrifuged at 24,000 rpm for two hours at 4°C. The determination of radioactivity of soluble protein revealed that a appreciable quantity of protein has been released from ribosome, usually this represented 20% of total radioactive protein made by this system. The soluble radioactive protein which has been separated from ribosome by sucrose density gradient was filtrated through Sephadex
Fig. 1. Ultracentrifuge analysis of chloroplast proteins. Pattern obtained at a bar angle of 60° after 25 minutes at 42,040 rpm.

Fig. 2. Schlieren pattern of purified Fraction I protein. The picture was taken 30 min after reaching top speed (56,100 rpm).
Fig. 3. Polyacrylamide gel electrophoretogram and densitometer tracing of purified Fraction I protein.

Fig. 4. Peptide map prepared from tryptic peptides of Fraction I protein. Electrophoresis was conducted in the first dimension with the arrow pointing in the direction of the cathode.
Fig. 5. A tracing of typical tryptic peptide map of Fraction I Protein. Details of the mapping procedure are given in the text.

Fig. 6 Gel filtration on Sephadex G–100 of soluble radioactive protein.
### TABLE I

AMINO ACID COMPOSITION OF FRACTION I PROTEIN

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Preparation I</th>
<th>Preparation II</th>
<th>Previously Rice (Akazawa)</th>
<th>Reported Spinach (Barley)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>um%</td>
<td>um%</td>
<td>um%</td>
<td>um%</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.29</td>
<td>8.80</td>
<td>6.47</td>
<td>9.90</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.80</td>
<td>6.40</td>
<td>4.18</td>
<td>5.80</td>
</tr>
<tr>
<td>Serine</td>
<td>2.80</td>
<td>2.80</td>
<td>3.01</td>
<td>3.20</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>11.10</td>
<td>11.20</td>
<td>7.30</td>
<td>11.50</td>
</tr>
<tr>
<td>Proline</td>
<td>4.90</td>
<td>4.20</td>
<td>4.70</td>
<td>6.50</td>
</tr>
<tr>
<td>Glycine</td>
<td>11.60</td>
<td>10.70</td>
<td>6.86</td>
<td>11.00</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.60</td>
<td>7.70</td>
<td>6.29</td>
<td>10.10</td>
</tr>
<tr>
<td>Valine</td>
<td>6.80</td>
<td>6.80</td>
<td>4.34</td>
<td>9.90</td>
</tr>
<tr>
<td>Cystine</td>
<td>1.30</td>
<td>1.30</td>
<td>—</td>
<td>2.00</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.70</td>
<td>0.80</td>
<td>1.31</td>
<td>2.20</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.70</td>
<td>4.10</td>
<td>3.22</td>
<td>5.50</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.40</td>
<td>8.90</td>
<td>6.35</td>
<td>10.60</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.30</td>
<td>4.50</td>
<td>2.93</td>
<td>5.00</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.20</td>
<td>4.20</td>
<td>3.31</td>
<td>5.70</td>
</tr>
<tr>
<td>Lysine</td>
<td>5.40</td>
<td>5.50</td>
<td>9.46</td>
<td>6.80</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.00</td>
<td>2.10</td>
<td>5.73</td>
<td>3.30</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.20</td>
<td>5.30</td>
<td>18.35</td>
<td>5.80</td>
</tr>
</tbody>
</table>

### TABLE II

Effect of Various Components on Amino Acid Incorporation by the 1000-g Fraction of Tabacco Leaves

<table>
<thead>
<tr>
<th>Assay System</th>
<th>$^{14}C$-Amino Acid Incorporation (CMP/Assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete reaction mixture</td>
<td>1854</td>
</tr>
<tr>
<td>– GTP, UTP, and CTP</td>
<td>1465</td>
</tr>
<tr>
<td>– $^{14}C$ Amino Acid</td>
<td>1208</td>
</tr>
<tr>
<td>– PEP and pyruvate Kinase</td>
<td>1124</td>
</tr>
<tr>
<td>– ATP, PEP and Pyruvate Kinase</td>
<td>258</td>
</tr>
<tr>
<td>– All additions</td>
<td>125</td>
</tr>
</tbody>
</table>
Fig. 7  Gel filtration on Sephadex G-200 of radioactive Fraction I protein.

Fig. 8  Elution profile of radioactive Fraction I protein on a 1×15cm DEAE-cellulose column.
Gel-100. Fig. 6 shows the elution pattern of soluble radioactive protein. The first peak represents the high molecular weight protein, namely, Fraction I protein, while the second corresponds to Fraction II protein which contains a considerable number of constituents. About 30 to 40% of radioactivity has been found to be associated with the first peak. In order to ascertain that the radioactivity revealing in the first peak is really associated with Fraction I protein, the author repeated this experiment and collected the protein and chromatographed on DEAE-cellulose column. The elution pattern shows that the curve obtained from radioactivity follows the protein content (Fig. 8). The degree of purity obtained with DEAE-cellulose was usually on the order of 90 to 95% as judged by ultracentrifuge analysis. This protein was eluted at 0.18 M sodium chloride while broken ribosome at 0.6 M. This shows that radioactivity associated with Fraction I protein is not due to the contamination by the broken \textsuperscript{14}C ribosomes.

**DISCUSSION**

The present studies present the first isolation of pure Fraction I protein from tobacco leaves. It is a major protein that comprises 30-50% of soluble protein in leaf cytoplasm. The protein has been associated with chloroplast and accounts for up to 60% of the readily solubilize protein of the chloroplast. In photosynthesis, it catalyzes the condensation of CO\textsubscript{2} with ribulose-1,5-diphosphate, forming two molecules of 3-phosphoglyceric acid.

The experiments described above clearly demonstrate that the preparation is homogeneous in the ultracentrifuge and upon polyacrylamide gel electrophoresis. Based on the data of amino acid analysis and peptide mapping, this protein may consist of about 20 to 24 subunits. Additional studies on the isolated chains and the number of active sites of the protein will certainly be required in order to clarify our knowledge of the structure and catalytic mechanism of the protein.

It is very clear that cell-free extracts from tobacco leaf chloroplast are capable of catalyzing the incorporation of the amino acid into protein. The results summarized in Table II indicate that the amino acid incorporating activity of chloroplast is dependent on exogenous adenosine triphosphate, and in addition requires Mg\textsuperscript{2+}, a mixture of amino acids, a mixture of guanosine, uridine, and cytidine triphosphate. Spencer and Wildman (1964) reported that this system is extremely sensitive to ribonuclease (0.02 ng/ml gives 50% inhibition), puromycin, and chloramphenicol, but is relatively insensitive to deoxyribonuclease and actinomycin D. Francki et al. (1965) confirmed that the material responsible for in vitro protein synthesis by chloroplast including s-RNA and activating enzymes are located in the mobile phase of the chloroplast.

Although a number of chloroplast amino acid incorporating system have been isolated from higher plant sources, relatively little was known of the nature of the protein formed by this system. Analysis of Sephadex gel filtration in Fig. 6 shows that about 30% to 40% of radioactive soluble protein is Fraction I protein. At the same time, some proteins with sedimentation coefficient between 18 S and 4 S have been actively synthesized. Since ribosomes have been separated from soluble proteins by sucrose density gradient, it is felt that the radioactivity associated with Fraction I protein is due to the presence of nascent protein, and does not represent protein bound to ribosomes or ribosomal subunit.
The determination of radioactivity of total incorporating activity revealed that only 20% radioactivity resided in soluble fraction and that more than 80% associated with particulate substances such as chloroplast membrane and ribosomes. Spencer (1965) presented evidence that large fraction of protein formed by chloroplast amino acid incorporating system is insoluble lamellar protein which consists primarily of internal chloroplast membrane. This may account for the low yield of Fraction I protein synthesized by tobacco chloroplast incorporating activity in these experiments.

ACKNOWLEDGMENT

The author expresses his sincere thanks to Dr. S. G. Wildman (Department of Botany and Plant Biochemistry, University of California, Los Angeles) for his valuable advice and discussions.

REFERENCES

1. Bamji, M. S. and A. T. Jagendorf
2. Dorner, R. W., A. Kahn and S. W. Wildman
   G. Wildman
   J. Biol. Chem. 234:2897-2905
7. Lyttleton, J. W.
9. Mendola, L. and T. Akazawa
    (1961).
11. Park, R. B., and N. G. Pon
12. Paulsen, J. M., and D. Lane
    Biochem. 5:2350-2357 (1966).
13. Racee, L. D.
14. Sanger, F.
15. Spencer, D., and S. W. Wildman
16. Trown, P. W.