Shichi and Hackett have isolated 3 b-type cytochromes from etiolated mung bean seedlings using conventional methods of ammonium sulfate fractionation and column chromatography (3, 5). Because of the turbidity of some of their early extracts, they were unable to measure the difference spectrum of their samples and therefore could not calculate the overall yield and purity of their cytochrome preparations. We have repeated some of their work using the scattered transmission accessory (Cary model 1462) for the Cary Model 14 recording spectrophotometer to measure the difference spectra of strongly scattering suspensions. From the heights of the \( \alpha \) bands we have calculated the b-type cytochrome content at each step during the purification procedure.

Materials and Methods

Cytochromes \( b-555 \) and \( b-561 \) were purified from 50 pounds of etiolated mung bean seedlings according to the method of Shichi and Hackett (3). Protein determinations were made using the Folin-phenol reagent as described by Lowry and co-workers (2).

The b-type cytochrome content of each fraction was calculated according to the formula (1):

\[
\text{Concentration} = \frac{\Delta A_{\lambda_{\text{max}}} - A_{\lambda_{\text{reference}}}}{\Delta E}
\]

For \( \Delta E \) we chose the molar extinction coefficient of the \( \alpha \) band of reduced cytochrome \( b-555 \) or \( 2.27 \times 10^4 \) liters per mole \( \times \) cm (4). To obtain the numerator in the above expression we subtracted the absorbancy at 575 m\( \mu \) from that at 555 m\( \mu \) or 561 m\( \mu \) in the hydrosulfite-reduced versus oxidized spectrum. The milligrams b-type cytochrome in each fraction was obtained by multiplying the concentration of cytochrome by the assumed molecular weight of 14,000 g per mole (4).

Subcellular particles were precipitated from the initial mung bean seedling extract at pH 5. The amount of b-type cytochrome remaining in solution represented the nonparticulate cytochrome and this amount was used as the standard for comparing relative cytochrome purity and yields during the purification procedure. We found that some of the b-type cytochrome was denatured when passed through buffer-washed DEAE-cellulose. This denaturation could be alleviated by washing the DEAE-cellulose with distilled water during its preparation for chromatographic purposes.

Results and Discussion

The results of the spectrophotometric determination of b-type cytochrome content, shown in tables I and II, indicate that 1 pound of etiolated mung bean seedlings will yield 1 to 2 mg of soluble b-type cytochrome in the 0 to 60% saturation ammonium sulfate fraction. Of the total amount of b-type cytochrome in the initial blended extract, 74% was precipitated by pH 5 treatment (table II). Of the 26% remaining in the supernatant fraction, only 12% was recovered after DEAE-cellulose chromatography and three-fourths of this material consisted of cytochrome \( b-555 \) (table I). In the overall purification procedure through the DEAE-cellulose chromatography step, cytochrome \( b-555 \) was purified 8.8-fold while the \( b-561 \) was only 1.3 times more pure than in the pH 5 supernatant.

Shichi and Hackett purified the cytochromes further by chromatography on hydroxylapatite and electrophoresis on starch (3). Although we have not repeated these purification steps, we have calculated the purity of the cytochrome samples after such treatment from their data. The results of such calculations (table III) show that Shichi and Hackett purified cytochrome \( b-555 \) 619-fold over that of the pH 5 supernatant fraction in tables I and II. Cytochrome \( b-561 \), however, was only 11 times purer than it was initially in our preparations. It is apparent, therefore, that the Shichi and Hackett procedure is primarily a method for isolating pure cytochrome \( b-555 \) from etiolated mung bean seedlings. Additional purification steps must be undertaken to obtain a preparation of cytochrome \( b-561 \) that approaches the purity of the \( b-555 \) sample.
Table I. Purification of b-Type Cytochromes from 50 Pounds of Etiolated Mung Bean Seedlings

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Total volume</th>
<th>Total protein</th>
<th>Total b-type cytochrome*</th>
<th>Specific amount of b-type cytochrome</th>
<th>Increase in specific amt**</th>
<th>Yield***</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract*</td>
<td>17,500</td>
<td>255,000</td>
<td>nil</td>
<td>14.9</td>
<td>[\text{ng cytochrome/mg protein}]</td>
<td>(m g)</td>
</tr>
<tr>
<td>2. pH 5 Supernatant</td>
<td>14,500</td>
<td>116,000</td>
<td>98</td>
<td>8.42</td>
<td>(\times 10^4)</td>
<td>%</td>
</tr>
<tr>
<td>3. 0-60% Ammonium sulfate supernatant</td>
<td>17,500</td>
<td>76,500</td>
<td>86</td>
<td>11.2</td>
<td>(\times 10^4)</td>
<td>%</td>
</tr>
</tbody>
</table>

* See text.
** Based on the specific amount of b-type cytochrome in the pH 5 supernatant fraction.
*** Based on the mg cytochrome in the pH 5 supernatant.

Table II. Early Steps in the Purification of b-Type Cytochromes

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total volume</th>
<th>Total protein</th>
<th>Total b-type cytochrome</th>
<th>Specific amount of b-type cytochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract*</td>
<td>17,500</td>
<td>255,000</td>
<td>381</td>
<td>14.9</td>
</tr>
<tr>
<td>2. pH 5 Supernatant</td>
<td>14,500</td>
<td>116,000</td>
<td>98</td>
<td>8.42</td>
</tr>
<tr>
<td>3. 0-60% Ammonium sulfate supernatant</td>
<td>17,500</td>
<td>76,500</td>
<td>86</td>
<td>11.2</td>
</tr>
</tbody>
</table>

* The crude extract was obtained from 50 pounds of etiolated mung bean seedlings.

Table III. Purity of b-Type Cytochromes after Electrophoresis on Starch

<table>
<thead>
<tr>
<th>Cytochrome</th>
<th>Absorbancy of reduced (\alpha) band*</th>
<th>Protein conc</th>
<th>Molar conc of cytochrome**</th>
<th>Conc of cytochrome***</th>
<th>Specific amount of cytochrome</th>
<th>Increase in specific amount(^t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(b-555)</td>
<td>0.063</td>
<td>0.07</td>
<td>(27.7 \times 10^4)</td>
<td>(3.86 \times 10^2)</td>
<td>5570</td>
<td>61,900</td>
</tr>
<tr>
<td>(b-561)</td>
<td>0.055</td>
<td>3.4</td>
<td>(24.2 \times 10^4)</td>
<td>(3.36 \times 10^2)</td>
<td>98.8</td>
<td>1100</td>
</tr>
</tbody>
</table>

* Calculated from the data given in figure I and figure 4, reference (3).
** \(\text{OD} = \frac{\epsilon_m}{\epsilon_{\alpha}}\) Calculated for both \(b-555\) and \(b-561\) on the basis of \(\epsilon_{\alpha} = 2.27 \times 10^4\) for the reduced \(\alpha\) band of \(b-555\).
*** Assuming a MW of 14,000 for both \(b-561\) and \(b-555\) (4).
\(^t\) Calculated on the basis of specific amount = \(9 \times 10^4\) mg cytochrome/mg protein for the pH 5 supernatant (see tables I and II).

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Summary

Spectrophotometric determination of the yields and purity of b-type cytochromes obtained from etiolated mung bean seedlings by the method of Shichi and Hackett indicated that cytochrome b-555 was obtained in greater quantities and was more pure than cytochrome b-561. The cytochrome content of turbid fractions was determined from hydrosulfite-reduced versus oxidized spectra measured with the scattered transmission accessory to the Cary 14 recording spectrophotometer.

Acknowledgment

The authors wish to thank Mr. Frank Barley for his conscientious assistance during the course of this work.

Literature Cited