Purification and Some Properties of Two Types of
β-Fructofuranosidase from Tomato Fruit

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At least two distinct sucrose-splitting enzymes such as β-fructofuranosidase (β-FFase) E and L have been demonstrated in the tomato cell walls. Both β-FFase were purified by means of ammonium sulfate fractionation, chromatography on DEAE-Sephadex A-50 and the gel filtration on Sephadex G-200 column. The highly purified β-FFase E (early fraction in the DEAE-Sephadex chromatography) and L (late fraction) thus obtained, contained carbohydrate at about 5.3 and 9.1%, respectively. Both enzymes had the same optimal pHs of 4.5 and 5.1 for sucrose and raffinose, respectively, and were completely inactivated by the incubation at 55°C for 5 min. Activation energies were 7,370 and 17,300 cal/mole for β-FFase E and L, respectively. For both enzymes, Km values for raffinose were higher than those for sucrose. The activities of both enzymes were strongly inhibited with p-chloromercuribenzoate and silver nitrate (1×10⁻⁴M). β-FFase E but L was activated by potassium ferrocyanide (1×10⁻⁴M).

Marked increase in the activity of β-D-fructofuranoside fructohydrolase (β-FFase) (E. C. 3.2.1.26) located in cell walls, occurred during ripening process of tomato fruit." The increase of the activity would be derived from de novo synthesis. The occurrence of multiple molecular forms of β-FFase and the change of these forms during the ripening process of tomato fruit have been suggested. The change in molecular form may lead to a proposal that β-FFase play vital roles in the control of sucrose metabolism in tomato fruit. The knowledge concerning the nature of highly purified β-FFase in higher plant is still scanty.

This paper communicates the purification of two types of tomato β-FFase and the comparison of the nature of these enzymes.

MATERIALS AND METHOD

Plant material. Mature green tomato fruits were ripened under the condition as described previously. The increase of the activity would be derived from de novo synthesis. The occurrence of multiple molecular forms of β-FFase and the change of these forms during the ripening process of tomato fruit have been suggested. The change in molecular form may lead to a proposal that β-FFase play vital roles in the control of sucrose metabolism in tomato fruit. The knowledge concerning the nature of highly purified β-FFase in higher plant is still scanty.

This paper communicates the purification of two types of tomato β-FFase and the comparison of the nature of these enzymes.
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detected with phloroglucinol-hydrochloric acid reagent (Putman et al. [8]).

Disk electrophoresis. Disk electrophoresis was performed by the methods according to Ornstein [9] and Davis [10].

Sedimentation determination. Sedimentation analysis of the purified enzymes was kindly performed at Institute of Applied Microbiology, University of Tokyo, using Hitachi Analytical Ultracentrifuge.

Dialysis. Unless otherwise stated, samples in cellulose tubing (Visking Comp.) were dialyzed against 10 mM potassium phosphate buffer (pH 7.4) with continuous stirring overnight at 4°C. The insoluble material was discarded by centrifugation.

RESULTS

Preparation of crude extract

The crude extract was prepared as described previously [11].

First ammonium sulfate precipitation

Solid ammonium sulfate was slowly added to the crude extract to 90% saturation. By centrifugation for 10 min at 6000 g, the precipitate was collected, and was dissolved in a small amount of distilled water, followed by the dialysis.

Second ammonium sulfate precipitation

The insoluble material in the dialyzate was discarded by centrifugation. To the supernatant was added solid ammonium sulfate to give 30% saturation. After removal of the precipitate formed by centrifugation, solid ammonium sulfate was added to the supernatant to 90% saturation, and the colloidal solution was recentrifuged. The precipitate collected was dissolved and dialyzed.

DEAE-Sephadex A-50 column chromatography

The dialyzed enzyme solution thus obtained was applied to a DEAE-Sephadex A-50 column, previously equilibrated with 10 mM potassium phosphate buffer (pH 7.4). A linear gradient elution was performed by a two chambers system with 10 mM potassium phosphate buffer (pH 7.4) in the first chamber and 750 mM sodium chloride in 10 mM potassium phosphate buffer (pH 7.4) in the second one. Elution profiles in protein and β-FFase activity are shown in Fig. 1. Two peaks of enzyme activity were detected, and the former and the latter fraction in the eluate were termed as fraction E and L, respectively.

![Fig. 1. Typical Elution Pattern of the Precipitate at (NH₄)₂SO₄ 30–90% in the DEAE-Sephadex A-50 Chromatography.](image)

Basic protein, 2540 mg in 78 ml was applied to a column (2.7 x 35 cm) and eluted with a linear gradient of sodium chloride concentration from 0 to 750 mM in 10 mM potassium phosphate buffer, pH 7.4. The experimental conditions are described in the text.

- - - , O.D. at 280 μμ; ○ -- ○, β-FFase activity; - - - , NaCl concentration.
(I) Purification of fraction E

Third ammonium sulfate precipitation. The most active fractions of the fraction E were collected and were added with ammonium sulfate to 50% saturation. The precipitate was collected by centrifugation, dissolved in a small amount of water and dialyzed against 25 mM potassium phosphate buffer (pH 6.5).

First Sephadex G-200 gel filtration. The concentrated enzyme solution obtained was applied onto a Sephadex G-200 column equilibrated with 25 mM potassium phosphate buffer (pH 6.5) and eluted with the same buffer. The most active fractions were pooled.

Fourth ammonium sulfate precipitation. Solid ammonium sulfate was added slowly to the pooled solution to 50% saturation. The precipitate formed was collected by centrifugation, dissolved in a small amount of water.

Second Sephadex G-200 gel filtration. The concentrated enzyme solution was rechromatographed by the same manner as described in the first Sephadex G-200 gel filtration. The results obtained are illustrated in Fig. 2. The most active fractions were pooled and concentrated with ammonium sulfate.

(II) Purification of fraction L from tomato fruit

Third ammonium sulfate precipitation. The pooled solution of the fraction L was fractionated by the addition of solid ammonium sulfate, and the precipitate between 50 and 90% saturation was collected by centrifugation and was dialyzed against 25 mM potassium phosphate buffer (pH 6.5).

First Sephadex G-200 gel filtration. The concentrated enzyme solution obtained was applied onto a Sephadex G-200 column equilibrated with 25 mM potassium phosphate buffer.

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**Fig. 2. Second Chromatography of Tomato β-FFase E on Sephadex G-200.**

An elution pattern of the precipitate (0–50% saturation of (NH₄)₂SO₄) in a Sephadex G-200 column (2.7 x 63.5 cm) chromatography. The elution procedure are described in the text.

- ●, O.D. at 280 mµ; ○—○, β-FFase activity.

**Fig. 3. Second Chromatography of Tomato β-FFase L on Sephadex G-200.**

An elution pattern of an (NH₄)₂SO₄ precipitate (50–90%) from a Sephadex G-200 column (2.7 x 63.5 cm). The elution procedure are described in the text.

- ●, O.D. at 280 mµ; ○—○, β-FFase activity.
TABLE I. PURIFICATION OF TOMATO \( \beta \)-FFase FRACTION E AND L

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Activity (units)</th>
<th>Protein (mg)</th>
<th>Carbohydrate (mg)</th>
<th>Specific activity (mg protein per mg carbohydrate)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>8150</td>
<td>19,500</td>
<td>3940</td>
<td>1025</td>
<td>4.95</td>
<td>19</td>
</tr>
<tr>
<td>1st ((NH_4)_2SO_4) 0~90% precipitate</td>
<td>425</td>
<td>22,900</td>
<td>3040</td>
<td>525</td>
<td>7.54</td>
<td>43.6</td>
</tr>
<tr>
<td>2nd ((NH_4)_2SO_4) 30~90% precipitate</td>
<td>78</td>
<td>22,400</td>
<td>2540</td>
<td>330</td>
<td>8.81</td>
<td>67.9</td>
</tr>
</tbody>
</table>

**Fourth ammonium sulfate precipitation.** The most active fractions were combined.

**Second Sephadex G-200 gel filtration.** The combined solution was added with solid ammonium sulfate, and the precipitate between 50 and 90\% saturation was collected by centrifugation, and dialyzed.

**Summary of purification experiments.** Table I shows the summary of the purification experiments from 10 kg of mature tomato pericarp.

**Properties of purified fraction E and L.**

**Sedimentation analysis.** Fractions E as well as L moved as a single symmetrical peak at 60,000 rpm with sedimentation coefficients of 8.25 and 8.96 S, respectively (Fig. 4).

**Disk electrophoresis.** The purified fractions E and L were subjected to disk electrophoresis in polyacrylamide gel at pH 8.3 in 300 mM Tris-HCl buffer with a current of 2 mA per tube (0.5 x 7 cm). Each of fractions E and L showed a single band when stained with amide black. They show the same mobility in disk electrophoresis as shown in Fig. 5.

**Substrate specificity.** Non-reducing saccharides were tested at pH 4.7 under the standard assay conditions except that the final concentration of test sugar was 50 mM. The same concentration was used for reducing saccharides which were tested at the same pH and were assayed by paper chromatography. The results are summarized in Table II. A substituted \( \beta \)-fructofuranoside (melezitose), 4-O-\( \alpha \)-glucopyranosyl-D-glucose (maltose)
FIG. 4. Sedimentation Pattern of β-FFase E (upper) and L (lower).

The pictures were taken at a phase plate of 60° at 11, 26, 41 and 56 min after the maximum speed was reached. The direction of the sedimentation is toward the left. The respective concentrations of β-FFases E and L were 4.75 mg and 6.5 mg per ml in 100 mM potassium phosphate buffer, pH 6.0. Both preparations appear to be monodisperse.

FIG. 5. Disk Electrophoresis of Purified Tomato β-FFase Fraction E and L.

Disk electrophoresis patterns of purified tomato β-FFase fraction E and L in polyacrylamide gel (7.5%) at pH 8.0 in 3 mA. Protein was stained with amide black.

and 4-O-β-D-galactopyranosyl-D-glucose (lactose) were not attacked by fractions E nor L. The substrate specificity is thus consistent with that for a β-fructofuranosidase (β-FFase), as shown in other higher plants.4,5)

pH Dependency. The activities of purified β-FFases E and L were assayed at various pH values (Fig. 6 and Fig. 7). β-FFases E and L have the same optimal pHs of 4.5 and 5.1 for sucrose (Fig. 6) and for raffinose (Fig. 7), respectively.

Heat sensitivity. Heat stability was ascertained in terms of a percentage enzymic activity (at standard assay conditions) remaining after a 5-min preincubation at a given
TABLE II. SUBSTRATE SPECIFICITY OF TOMATO \( \beta \)-FFASE E AND L

Activities are expressed relative to the rate of sucrose hydrolysis.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>( \beta )-FFase E</th>
<th>( \beta )-FFase L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Raffinose</td>
<td>54.5</td>
<td>41.5</td>
</tr>
<tr>
<td>Melezitose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maltose</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lactose</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**FIG. 6.** pH Dependency toward Sucrose Hydrolysis.

The reaction proceeded on 73 mM sucrose. Citrate phosphate buffer (250 mM) was used under a pH range of 2.6 to 6.8. Potassium phosphate buffer was used above the pH of 7.2.

**FIG. 7.** pH Dependency toward Raffinose.

The reaction proceeded on 50 mM raffinose. Acetate buffer (250 mM) was used under a pH range of 3.6 to 5.1. Potassium phosphate buffer (250 mM) was used at pH range above 5.5.

**FIG. 8.** Heat Sensitivity.

The determination of sucrose-hydrolyzing activity was carried out at 0, 5, 10 and 15 min at various temperatures. In Fig. 9 the logarithm of the initial velocity is plotted against the reciprocal absolute temperature. The values for the activation energies of \( \beta \)-FFases E and L calculated from Fig. 9 are 7.37 and 17.3 kcal/mole, respective-
ly. The higher activation energy was obtained with $\beta$-FFase L than E.

**Effect of pH on Km values.** Lineweaver Burk plot of both $\beta$-FFases E and L for sucrose and raffinose as substrate were illustrated in a pH range of 3.6 to 5.1. $K_m$ values for both enzymes at various pH values were summarized in Table III. Both enzymes showed higher affinity for raffinose.

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_m$ of $\beta$-FFase E (mM)</th>
<th>$K_m$ of $\beta$-FFase L (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sucrose</td>
<td>Raffinose</td>
</tr>
<tr>
<td>3.6</td>
<td>5.89</td>
<td>125</td>
</tr>
<tr>
<td>4.5</td>
<td>3.45</td>
<td>29.4</td>
</tr>
<tr>
<td>5.1</td>
<td>2.17</td>
<td>20.4</td>
</tr>
</tbody>
</table>

The presence of two distinct sucrose-splitting enzymes was confirmed by the lower activation energy with $\beta$-FFase L than E.

**Effect of some reagents.** The enzyme preparations were incubated with $p$-chloromercuribenzoate ($p$CMB) for 5 min at 30°C in a solution containing 250 mM acetate buffer, pH 4.5. In the $p$CMB-sucrose incubation the enzyme sample was added to a buffered sucrose solution containing $p$CMB.

<table>
<thead>
<tr>
<th>Incubation with</th>
<th>Concentration of reagent (M)</th>
<th>Activity relative to control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100 100</td>
</tr>
<tr>
<td>$p$CMB</td>
<td>$1 \times 10^{-4}$</td>
<td>38 20</td>
</tr>
<tr>
<td>$p$CMB-Sucrose</td>
<td>$1 \times 10^{-4}$</td>
<td>65 45</td>
</tr>
<tr>
<td>Indaoacetamide</td>
<td>$1 \times 10^{-4}$</td>
<td>100 121</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>$1 \times 10^{-4}$</td>
<td>106 100</td>
</tr>
<tr>
<td>Sodium arsenite</td>
<td>$1 \times 10^{-4}$</td>
<td>116 100</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>$1 \times 10^{-4}$</td>
<td>7 2</td>
</tr>
<tr>
<td>Cupric sulfate</td>
<td>$1 \times 10^{-3}$</td>
<td>23 10</td>
</tr>
<tr>
<td>$1 \times 10^{-4}$</td>
<td></td>
<td>99 94</td>
</tr>
<tr>
<td>Potassium ferrocyanide</td>
<td>$1 \times 10^{-4}$</td>
<td>151 103</td>
</tr>
</tbody>
</table>

The presence of two distinct sucrose-splitting enzymes was confirmed by the lower activation energy with $\beta$-FFase L than E.
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Purification of enzymes such as β-FFases E and L was demonstrated in the tomato cell walls. Both enzymes were purified as preparations giving a single band in both electrophoretic and ultracentrifugal analysis. Different behaviors between the enzymes E and L were observed on the DEAE-Sephadex A-50 column chromatography and fractionation by ammonium sulfate, while no difference in mobility on disk electrophoresis at pH 8.3 was observed. In data not presented here, column calibration with proteins of known molecular size showed that the elution positions of both β-FFases E and L were close to that of bovine serum albumin, indicating a molecular weight near 75,000 for both enzymes. The sedimentation coefficients of β-FFases E and L were calculated with 8.25 and 8.95 S, respectively.

The tomato β-FFases E and L are glycoproteins containing 5.36 and 9.16% carbohydrate, respectively, indicated by the phenol-sulfuric acid reagent. Significant amounts of carbohydrate were detected in β-FFase of grape berries as well as yeast, Neurospora, and Bacillus subtilis.

The purification procedure outlined in this paper yielded highly purified β-FFase E and L with a specific activity of 42 and 80 units per mg protein, respectively. Although the specific activity of this β-FFase L is about 42 times that obtained from the previous purification procedure, the specific activity of our preparation is at best about one third of that reported with grape berries.

The substrate specificities of both tomato enzymes E and L indicate their β-FFase function. With sucrose and raffinose as the substrates, the pH dependencies of both enzyme activities were found to be similar. Each of β-FFases E and L has an optimum pH value at 4.5 and 5.1 for sucrose and raffinose, respectively. For sucrose, bean β-FFase had an optimum at pH 4.9, while the optimum for the potato enzyme was at pH 4.0 and beet at 4.5. A low and indeterminable pH optimum was observed in grape berries enzyme. The presence of two invertase was shown in sugar cane and in carrot root, and one is acid enzyme having respective optimal pHs at 5.0 and 4.5 and the other is neutral enzyme having respective optimal pH at 7.0 and 7.5.

Km value for yeast β-FFase was constant in a pH range from 4.0 to 8.0, while the difference in Km values at various pH values observed with the enzymes in potato, French-bean, grape, and tomato. The Km values of both tomato β-FFases E and L for sucrose and raffinose were also dependent on pH. For both enzymes, Km values for raffinose were higher than those for sucrose.

The similarity between tomato β-FFase E and L on the sensitivity with heat treatment was observed, whereas the temperature profile of these enzymes had two straight lines of different slopes as observed in the Arrhenius plot.

No marked differences between the effects on β-FFase E and L by the chemicals employed, except that potassium ferrocyanide could be positively established. Arnold reported only 41–50% inhibition of purified grape berries β-FFase by 25 mM silver ions, whereas only 0.01 mM of silver ions completely inhibited both bean and potato enzymes. The sensitivity of tomato β-FFases E and L to silver ions were found to be similar to that of bean and potato enzymes. The patterns of inhibition of tomato β-FFases E and L by pCM were also similar to that reported for the bean and potato counterpart. Up to 45–65% of the activity could be restored by addition of sucrose to reaction mixture. This effect has been occurred in Neurospora, bean and potato enzyme. Another thiol group inhibitor such as N-ethylmaleimide and iodoacetamide had no effect on both tomato enzymes. We lack enough data to assess whether the two enzymes play important and different roles in the control of sucrose metabolism in tomato fruit. It is possible that future investigation will show that tomato fruit synthesize some quite different β-FFase at different stages of development.
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