Purification of the Internal Invertase of Yeast*

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SUMMARY

Most of the studies on yeast invertase have been concerned only with the external enzyme (a mannan protein which is localized in the cell wall), but recently it has been reported that the invertase inside the cytoplasmic membrane is a smaller form. This paper presents a comparative study on the distribution of invertase isozymes in two stains of yeast and, after the selection of the best conditions and source, the purification of the internal or small enzyme. The major purification is obtained by chromatography on diethylaminoethyl Sephadex, although ammonium sulfate precipitation and gel filtration in Sephadex G-200 are also used to separate the large and small enzymes. The purified internal invertase is homogeneous on the basis of chromatographic criteria and its behavior in the analytical ultracentrifuge.

A considerable amount of information has accumulated on the characteristics, localization, and secretion of yeast invertase (β-fructofuranoside fructohydrolase, EC 3.2.1.26). It has been established (2-5) that in derepressed cells most of the invertase is external (i.e. cell-bound but outside the cytoplasmic membrane); only a small proportion of the enzyme is located inside the membrane and is not accessible to the substrate. In fully repressed cells all the invertase is intracellular (4). Recently invertase isozymes have been found in Neurospora (6-9) and in the snail enzyme (10, 11, and dithiothreitol were purchased from Calbiochem; 2-mercaptoethanol, from Eastman Organic Chemicals; and the snail enzyme (16) has compiled these data on yeast strain FH4C. A characteristic of the external invertase relevant to this study is its glycoprotein nature. The enzyme contains 50% mannose and 30% glucosamine. Eylar (16) has compiled a considerable amount of evidence that most mammalian extra cellular proteins contain carbohydrate. Soodak (17) believes that, in order for protein secretion to occur, covalently bound carbohydrate must be present in the protein in question. In our laboratory we are studying the secretion of invertase in a proto-plast system (11, 18). In this context it is desirable to know the properties of the internal invertase.

This communication is concerned with (a) the distribution of invertase isozymes in yeast strain 303-67 and the repression-resistant mutant FH4C when grown in high or low levels of glucose and (b) the purification of the internal invertase from strain FH4C. The properties of the purified external and internal enzymes are compared in a companion paper (19).

EXPERIMENTAL PROCEDURE

**Materials**—Glucose oxidase (pure) was obtained from Nutritional Biochemicals, peroxidase (POD-II) and lactate dehydrogenase, from Boehringer; and o-dianisidine, from Sigma. Yeast extract (Arthrobacter Z) was purchased from Yeast Products, Inc., Paterson, New Jersey. Sephadex G-200, diethylaminoethyl Sephadex A-50, sulfoethyl Sephadex C-50, and blue dextran were obtained from Pharmacia. Protamine sulfate, Aquacide 11, and dithiothreitol were purchased from Calbiochem; 2-mercaptoethanol, from Eastman Organic Chemicals; and the snail enzyme (16) has compiled these data for yeast strain FH4C. A characteristic of the external invertase relevant to this study is its glycoprotein nature. The enzyme contains 50% mannose and 30% glucosamine. Eylar (16) has compiled a considerable amount of evidence that most mammalian extra cellular proteins contain carbohydrate. Soodak (17) believes that, in order for protein secretion to occur, covalently bound carbohydrate must be present in the protein in question. In our laboratory we are studying the secretion of invertase in a proto-plast system (11, 18). In this context it is desirable to know the properties of the internal invertase.

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**Invertase Assay**—Before the assay, the enzyme was diluted in 0.02 M Tris-HCl, pH 7.5, and incubated at 30° for 10 min to ensure maximum activity (19). Hydrolysis of sucrose and assay of the glucose formed were carried out in two steps at 30°. In the first step, 50 μl of the diluted enzyme were added to 100 μl of 0.1 M acetate buffer, pH 5.0, and 50 μl of 0.5 M sucrose. The reaction was stopped after 10 min, unless otherwise indicated, by addition of 300 μl of 0.2 M dibasic potassium phosphate, and the tubes were immediately placed in a boiling water bath for 3 min. The glucose formed was assayed by a method similar to that of Keston (20) by addition of 2 ml of 0.1 M phosphate buffer, pH 7.0, containing 200 μg of glucose oxidase, 10 μg of peroxidase, and 600 μg of o-dianisidine. After 30 min of incubation, the reaction was stopped by addition of 2.5 ml of 6 N HCl. The
color produced was read at 540 nm. For fractions containing both glucose and invertase, an extra sample was run, in which the invertase was first inactivated at 100°C for 5 min. This sample provided a measure of glucose present, which was subtracted from the assay. One unit of invertase is defined as the amount of enzyme which hydrolyzes 1 μ mole of sucrose in 1 min at 30° in 0.05 M sodium acetate buffer, pH 5.0, containing 0.125 M sucrose.

Total carbohydrate was measured by the phenol-sulfuric method of Dubois et al. (21). Total protein was estimated by the procedure of Lowry et al. (22) or by absorption at 280 nm, with purified internal invertase as standard. The amount of enzyme in the standard was determined from the amino acid analysis data (19).

Analytical Gel Filtration—The quantitative analysis of external and internal invertases and determination of the relative proportions of the two forms was performed according to Gascón and Ottolenghi (14) in an analytical column of Sephadex G-200 (2 × 47 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5. The same column was used for the estimation of the molecular weight of the internal enzyme by the method of Andrews (23).

Ultracentrifugal Analysis—A Beckman Spinco model E ultracentrifuge equipped with a constant temperature device and schlieren optics was used. Plates were read with the aid of a Nikon shadowgraph. A partial specific volume of 0.72 was used. Plates were run at 50,000 rpm in the ultracentrifuge at a speed of 30,000 rpm. The ultracentrifugal data were analyzed according to the method of Hearst and co-workers (24).

Yeast Strains and Culture Conditions—Two yeast strains were used, Saccharomyces strain 303-67 and mutant FH4C derived from it. The parent Saccharomyces strain 303-67, in which the distribution of invertase isozymes has been studied previously (14), is diploid and homozygous for the Su allele (K of Wingé and Roberts (25)). It forms invertase in appreciable amounts only after the glucose has disappeared from the medium (4), and does not hydrolyze maltose (25). The mutant strain FH4C was obtained by Symington and Lampen (26) by ultraviolet irradiation of strain 303-67. It produces high levels of external invertase even when growing in the presence of glucose (26).

For growth of the FH4C strain on a large scale, the following medium was used: glucose, 4%; ammonium sulfate, 0.2%; and yeast extract, 1%. The pH was adjusted to 5.5. The yeast were normally grown in 500-liter lots. The fermenter was inoculated aseptically with 5 liters of logarithmically growing yeast. Growth was continued for 16 hours at 28°C, by which time all the glucose had disappeared from the medium. The yeast were harvested in a Sharples centrifuge. Approximately 14 kg of cell paste were obtained per run.

Formation of Protoplasts—To 5 ml of yeast suspension containing approximately 8 × 10^6 cells per ml, the following additions were made: 0.05 M Tris-HCl buffer, pH 7.5, 1 ml; 1.2 ml KCl, containing 0.02 M magnesium sulfate, 6 ml, small enzyme, 0.5 ml, and 1 ml 2-mercaptoethanol, 0.2 ml. The mixture was incubated with shaking at 35°C, and after 3 hours more than 90% of the cells had been converted into protoplasts.

RESULTS

Distribution of InvertaseIsozymes

A study was undertaken of the distribution of the invertase isozymes in two strains of yeast. We were especially interested in determining whether mutant FH4C would be a better source of internal invertase than the parent 303-67 strain. Some of the results are presented in Table I. Besides the known sensitivity of the level of external invertase of strain 303-67 to the growth conditions (4) and the comparatively small effect of the glucose concentration on the production of external enzyme by the FH4C strain (26), it is worthwhile to note that there is approximately the same proportion of internal invertase in the FH4C cells as in the low glucose cells of strain 303-67. The low glucose FH4C cells have approximately twice as much external invertase as the high glucose ones. This is probably related to the finding of Lampen et al. (1) that protoplasts of FH4C secrete twice as much invertase in the presence of 0.005 M glucose than at 0.05 M or greater concentrations of sugar.

Fig. 1 shows the behavior of large and small invertase on gel filtration in Sephadex G-200. The examples chosen are extreme, but intermediate ratios of external and internal enzymes can be obtained. The enzyme released into the medium by actively growing yeasts, as well as the invertase released during the formation of protoplasts, is of the large type. Also in the large form is the enzyme secreted by protoplasts (1). Predominantly small enzyme is released by lysis from protoplasts of Saccharomyces strain 303-67 high glucose cells. This confirms the idea that the small invertase is internal with respect to the cytoplasmic membrane. Incomplete removal of the cell wall probably accounts for the presence of the large form found in protoplast lysates (14). This is supported by observation that the invertase which is not solubilized during the lysis, but is subsequently released by sonic treatment of the residue, is in the large form.

Internal invertase, determined as the difference between total activity and the activity of osmotically stabilized protoplasts, represents only a minimal value. The recovery of small enzyme in the supernatant of lysed protoplasts was equal to or greater than this (Table I); therefore, it is concluded that most of the internal invertase is in the small form. The possibility cannot be ruled out, however, that some of the large invertase revealed by lysis of the protoplasts was internal.

We have also studied the quantitative distribution of invertase isozymes in Saccharomyces cerevisiae strain LK2G12, which has been used in our laboratory for extensive studies on invertase and its secretion (5, 15, 29). The results were similar to those described for strain 303-67 and we have not included these data in Table I.

Purification of Internal Invertase

The high production of external invertase which characterizes strain FH4C is accompanied by a corresponding increase in the level of internal enzyme. Mutant FH4C is the richest available source of internal invertase and was used for preparation of the purified enzyme.

Table II summarizes the purification from this strain. Evaluation of the first steps in purification is quite complicated, since the ratio of external to internal invertase in the crude extract is about 25:1. Estimation of internal invertase in a sample involves double precipitation with ammonium sulfate at 75% saturation and subsequent gel filtration of the precipitate in an
The yeasts were inoculated into flasks containing 100 ml of Wickerham's medium (28) and incubated on a rotary shaker at 28° for 16 hours. The medium for high glucose cells contained 8% glucose and had more than 3% left in the culture supernatant at the time of harvesting. For low glucose cells, 1% glucose was used, and this disappeared before 14 hours of cultivation. The cells were harvested and washed twice with distilled water by centrifugation at 5,000 × g for 10 min. The protoplasts, obtained as indicated in "Experimental Procedure," were centrifuged at 10,000 × g for 10 min and then resuspended and washed twice in 0.05 M phosphate buffer, pH 6.0, containing 0.6 M KCl and 0.01 M magnesium sulfate. Protoplasts were lyzed by reformation to 0.05 M Tris-HCl buffer, pH 7.5, and the supernatant was obtained by centrifugation at 30,000 × g for 20 min. For estimation of invertase activity in intact protoplasts, all solutions were supplemented with 0.6 M KCl. Total invertase is the value obtained with protoplasts that had been frozen and thawed twice; that with intact protoplasts, external invertase; the difference gave the value for internal invertase. The relative proportions of large and small invertase were estimated as indicated in Fig. 1. All values for activity are expressed as invertase units per 6 × 109 cells or protoplasts, or their equivalent in the case of the supernatants.

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The procedure described below first separates the internal invertase from the external enzyme by repeated precipitation with ammonium sulfate. The internal enzyme is precipitated at 70% saturation, whereas most of the external form remains in the supernatant. The ammonium sulfate precipitate is dissolved, treated with protamine sulfate, batch-adsorbed on DEAE-Sephadex A-50, and passed through a column of the same material. The active fractions are dialyzed and applied to a column of sulfoethyl Sephadex C-50. When examined by column chromatography and analytical ultracentrifugation, the active peak is free of detectable contamination. The purification obtained is in the order of 2500-fold in terms of protein. The purification obtained in terms of protein was small, but the bulk of the carbohydrate remained in the supernatant and, what is more significant, 70 to 80% of the external invertase remained in the supernatant. The precipitate (approximately 3 kg, wet weight) was dissolved in 12 liters of cold, distilled water, and 436 g of ammonium sulfate were added per liter (70% saturation). After 20 hours the solution was centrifuged at 10,000 × g for 15 min, and the precipitate was redissolved and again precipitated at 70% ammonium sulfate saturation. The precipitate was dissolved in 4 liters of distilled water and dialyzed against distilled water until the conductivity of the solution was similar to that of 1 M NaCl. At this point in the purification, the ratio between external and internal invertase was about 1:1, and for further steps of purification it was not necessary to assay each fraction for internal enzyme by filtration through Sephadex G-200.

The volume of the enzyme solution was brought to 12 liters, convenient. Microscopic estimation is difficult because of the extensive clumping that occurs with this yeast.

### Table I

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>High glucose cells</th>
<th>Low glucose cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>303-67</td>
<td>FHHC</td>
</tr>
<tr>
<td></td>
<td>Invertase</td>
<td>Small invertase</td>
</tr>
<tr>
<td>Cells</td>
<td>units</td>
<td>%</td>
</tr>
<tr>
<td>Culture supernatant</td>
<td>1.5</td>
<td>94</td>
</tr>
<tr>
<td>Enzyme released during protoplast formation</td>
<td>0.0</td>
<td>260</td>
</tr>
<tr>
<td>Protoplasts</td>
<td>0</td>
<td>730</td>
</tr>
<tr>
<td>External enzyme</td>
<td>0.6</td>
<td>56</td>
</tr>
<tr>
<td>Internal enzyme</td>
<td>2.0</td>
<td>14</td>
</tr>
<tr>
<td>Supernatant of lysed protoplasts</td>
<td>2.5</td>
<td>95</td>
</tr>
</tbody>
</table>

* After protoplast formation, the recovery of invertase was always greater than 100% because of the synthesis and secretion of enzyme which occurred (5).

* The values given for external enzyme are maximal. Part of the activity probably originated from protoplasts lysed during handling of the sample and assay of the enzyme. The values for internal invertase (total minus external) are therefore minimal.

* Part of the invertase was not solubilized, and remained attached to the protoplast residues. After ultrasonic disruption of the pellet, the solubilized enzyme behaved as large invertase.

Ammonium Sulfate Precipitation and Protamine Sulfate Treatment—To the crude extract, 516 g of solid ammonium sulfate per liter were added (80% saturation), and the solution was stored overnight at 4°. The precipitate, which contained the small invertase, was collected in a Sharples centrifuge. The purification obtained in terms of protein was small, but the bulk of the carbohydrate remained in the supernatant and, what is more significant, 70 to 80% of the external invertase remained in the supernatant. The precipitate (approximately 3 kg, wet weight) was dissolved in 12 liters of cold, distilled water, and 436 g of ammonium sulfate were added per liter (70% saturation). After 20 hours the solution was centrifuged at 10,000 × g for 15 min, and the precipitate was redissolved and again precipitated at 70% ammonium sulfate saturation. The precipitate was dissolved in 4 liters of distilled water and dialyzed against distilled water until the conductivity of the solution was similar to that of 1 M NaCl. At this point in the purification, the ratio between external and internal invertase was about 1:1, and for further steps of purification it was not necessary to assay each fraction for internal enzyme by filtration through Sephadex G-200.

The volume of the enzyme solution was brought to 12 liters, convenient. Microscopic estimation is difficult because of the extensive clumping that occurs with this yeast.

* During purification, the concentration of crude enzyme solutions was achieved by precipitation with ammonium sulfate to 70% saturation. Pure or highly purified enzyme was concentrated by dialysis against sodium salts of carboxymethyl cellulose (Aquanide 11) or by rendering the enzyme insoluble at pH 5.0 by dialysis against distilled water, or both.
and the pH was adjusted to 6.5. Three liters of a 2% solution of protamine sulfate in water were added with vigorous stirring, and the solution was kept at 4° overnight. The inactive precipitate was collected at 10,000 x g for 15 min. The protamine sulfate treatment removed not only inactive protein from the extract, but also particulate material and non-protein contaminants.

**DEAE-Sephadex Batch Adsorption and Chromatography**—The supernatant from the protamine sulfate treatment was adjusted to pH 7.5 with 0.5 M Tris, and distilled water was added to make the conductivity equivalent to 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl. The volume was adjusted to 38 liters with this buffer. DEAE-Sephadex (20 g, dry weight, previously equilibrated with the same buffer) was added with vigorous stirring, and the solution was kept at 4° overnight. The inactive precipitate was collected at 10,000 x g for 15 min. The protamine sulfate in water were added with vigorous stirring, and the pH was adjusted to 6.5. Three liters of a 2% solution of protamine sulfate in water were added with vigorous stirring, and the solution was kept at 4° overnight. The inactive precipitate was collected at 10,000 x g for 15 min. The protamine sulfate treatment removed not only inactive protein from the extract, but also particulate material and non-protein contaminants.

**Dialysis at pH 4.0 and Sulfoethyl Sephadex Chromatography**—The more active fractions from the DEAE-Sephadex column were pooled, concentrated, and dialyzed against 0.05 M acetate buffer, pH 4.0, containing 0.05 M NaCl. The purification in this step was approximately 20-fold, and most of the external invertase was also removed.

The pooled eluates from the batch adsorption were dialyzed against 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, and applied to a column of DEAE-Sephadex with the characteristics given in Fig. 2, which also illustrates the elution procedure. The batch adsorption and the column chromatography afforded a purification of 200-fold in terms of protein content.

**Dialysis against 0.05 M Tris-HCl Buffer, pH 7.5**—The external invertase was also removed.

The pooled eluates from the batch adsorption were dialyzed against 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, and applied to a column of DEAE-Sephadex with the characteristics given in Fig. 2, which also illustrates the elution procedure. The batch adsorption and the column chromatography afforded a purification of 200-fold in terms of protein content.

**Table II**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>External Invertase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>units</td>
</tr>
<tr>
<td>1. Yeast suspension</td>
<td>54,000</td>
<td>304,000</td>
<td>14,000,000</td>
</tr>
<tr>
<td>2. Crude extract</td>
<td>50,000</td>
<td>120,000</td>
<td>12,000,000</td>
</tr>
<tr>
<td>3. Ammonium sulfate (0.07)</td>
<td>6,000</td>
<td>54,700</td>
<td>185,000</td>
</tr>
<tr>
<td>4. Protamine supernatant</td>
<td>15,000</td>
<td>27,400</td>
<td>108,000</td>
</tr>
<tr>
<td>5. DEAE-Sephadex batch adsorption</td>
<td>2,000</td>
<td>1,200</td>
<td>2,000</td>
</tr>
<tr>
<td>6. DEAE-Sephadex column</td>
<td>200</td>
<td>44.4</td>
<td>0</td>
</tr>
<tr>
<td>7. Dialysis against pH 4 buffer (supernatant)</td>
<td>174</td>
<td>37.0</td>
<td>66,000</td>
</tr>
<tr>
<td>8. Dialysis against distilled water (precipitate)</td>
<td>5</td>
<td>8.4</td>
<td>24,000</td>
</tr>
<tr>
<td>9. Sulfoethyl Sephadex column</td>
<td>37</td>
<td>6.2</td>
<td>18,000</td>
</tr>
</tbody>
</table>

**Fig. 2. Fractionation of the internal invertase on DEAE-Sephadex A-50.** Fraction 5 of Table II was applied to a column (4 x 35 cm) previously equilibrated with 0.05 m Tris-HCl buffer, pH 7.5, containing 0.15 m NaCl. The column was washed with 300 ml of this buffer, and a linear gradient was established to 0.5 m NaCl in the same buffer. The total volume of the gradient was 4000 ml. The first peak of invertase, which appeared before the gradient was started, corresponds to the external enzyme. O--O, invertase activity; △--△, protein; △--△, carbohydrate; ---, molarity of the eluent.
taining 0.05 M NaCl. The solubilized enzyme was adsorbed on a sulfoethyl Sephadex C-50 column (1 × 10 cm) that had been equilibrated with a similar buffer. The column was eluted as shown in Fig. 3. The specific activity along the active peak was constant. The active fractions were pooled, dialyzed against 0.05 M Tris-HCl buffer, pH 7.5, and stored.

The ultraviolet absorption of the purified enzyme shows a maximum at 282 nm and a ratio of absorbances at 280 and 260 nm of 1.75, indicating the absence of nucleotides and nucleic acids.

Alternative Purification Procedures—The procedure for purification just outlined was developed for working with large batches of yeast. In preliminary studies, good purification of internal invertase was obtained on preparative columns of Sephadex G-200 (5 × 60 cm) with reversed flow. The bulk of the protein, carbohydrate, and external invertase was present in the fractions corresponding to the exclusion volume, whereas the internal invertase has an elution volume to exclusion volume ratio of approximately 1.7. This procedure proved impractical for large scale preparation, and after the protamine sulfate treatment the purification obtained by this method is relatively small. The gel filtration step was therefore omitted in routine purifications.

Rechromatography of the purified enzyme on DEAE-Sephadex at pH 7.5 and 8.6 gave no further purification.

Molecular Weight and Sedimentation Velocity Determination

Gel filtration of the purified internal invertase on Sephadex G-200 was carried out in order to estimate its molecular weight. The enzyme was dissolved in 0.05 M Tris-HCl buffer, pH 7.5, and added to an analytical column calibrated with lactate dehydrogenase and blue dextran, according to the method of Andrews (23). Fig. 1 shows the elution profile of the lactate dehydrogenase as well as that of a crude preparation containing predominantly internal invertase (A in Fig. 1). In the gel filtration profile of purified internal invertase, there was no material in the region of external or large invertase, and it gave a sharper profile than the one shown in Fig. 1 (See Fig. 5 in the accompanying paper (19)). The peak obtained with the purified enzyme and that of the lactate dehydrogenase overlap almost exactly. The elution volume of the internal enzyme suggested a molecular weight of 130,000 to 140,000.

Sedimentation velocity experiments revealed only one peak (Fig. 4) with $s_{20, w}$ of 8.8.

**DISCUSSION**

Invertase was first isolated by Berthelot in 1860 (30). Since then, the enzyme has been extensively studied and purified from a variety of sources and with different extraction methods (15). The existence of invertase inside protoplasts has been detected recently (4, 5, 29), and Sutton and Lampen (5) established that the internal enzyme had the same kinetic properties as the external (see also Gascón and Ottolenghi (14)). It is not surprising, however, that its presence has not been detected in the several preparations of invertase, in view of the small proportion of the internal enzyme in fully induced cells and the heterogeneous nature of invertase extracted by the usual autolytic procedures. In addition, owing to the different characteristics of both enzymes, it is highly probable that internal invertase will be lost in one of the preliminary steps of purification.

The distribution and characteristics of invertase isozymes of *Neurospora* are markedly different from our findings and those of Gascón and Ottolenghi (14), with invertase isozymes of yeast.
Metzenberg (i) has shown that in Neurospora the two isoenzymes can be interconverted, and has previously reported that the heavy form contains little if any carbohydrate (31). It is also known (8) that both isoenzymes can coexist outside the cytoplasmic membrane, although the heavy one predominates inside the proplastids. In contrast, in yeast the heavy and light invertases correspond to the internal and external enzymes, and the external enzyme contains carbohydrate (15) whereas the internal is devoid of it (19).

Hoshino, Kaya, and Sato (11) have reported three forms of invertase in baker's yeast, distinguishable by chromatography on DEAE-cellulose columns. Forms I and II were eluted from the column; the third form was retained and was not further studied because it only represented 2 to 6% of the total activity. Hoshino et al. used fully induced cells, and in view of the fact that Forms I and II contained mannan (10) and constituted more than 90% of the total invertase, they may represent the external invertase. Form III has two characteristics in common with our internal enzyme; that is, it represents a small fraction of the total, and it adsorbs very strongly to anionic exchangers; however, insufficient data have been published on the characteristics of this fraction to warrant further speculation on that possibility.

The purified internal invertase from strain FH4C is homogenous by column chromatography and ultracentrifugation, and its specific activity is in the same order of magnitude as that reported for external enzyme (15). In a companion paper (19) a comparative study of the properties of both enzymes is made with special emphasis on a possible precursor-product relationship of the two isozymes.

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