Isolation of Crystalline Phosphoglucose Isomerase from Brewers' Yeast*

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In a program to elucidate the mechanism of the catalytic isomerization between glucose 6-phosphate and fructose 6-phosphate, isolation of the enzyme from several sources was pursued as the basis for quantitative studies of its protein nature. The comparative investigation of several heteroenzymes appears to be a valuable tool in identifying the structure of an enzyme at and around the active site since it will provide clues as to catalytically important features of the protein molecule.

Yeast phosphoglucomutase has been previously isolated in this laboratory in crystalline form from rabbit skeletal muscle (2), and from bovine skeletal muscle. Earlier attempts at isolation of phosphoglucomutase from brewers' yeast (3) had met with only partial success when a highly purified but still amorphous enzyme preparation was obtained. This communication now reports the isolation in crystalline form of phosphoglucomutase from brewers' yeast (Saccharomyces carlsbergensis). It will be of interest also to include in these comparative studies the crystalline enzyme from bakers' yeast; thus far however, the isolation procedure for this enzyme has only appeared in the form of a short note (4).

MATERIALS

Dried brewers' yeast for enzyme work was purchased from Anheuser-Busch, Inc., St. Louis, Missouri. It was stored at -18° and used within 1 to 12 months after delivery. In the fall of 1963, changes were made at the Anheuser-Busch plant in the processing of the yeast, the details of which could not be ascertained. These changes resulted in markedly different behavior of the yeast autolysate during purification and it became necessary to introduce additional steps in order to remove gummy material as well as stubborn contaminations of yellow protein in later stages of the procedure. Since then the procedure as described here has been successfully carried through for more than a dozen preparations.

* This is the third paper in the series dealing with studies on phosphohexose isomerases. The work was supported in part by Public Health Service Research Grant AM 07203 from the National Institute of Arthritis and Metabolic Diseases, and by Cancer Research Funds of the University of California. 1 p-Glucose 6-phosphate ketol-isomerase, EC 5.3.1.9 (1). 2 E. A. Noltmann, unpublished work, 1963.

METHODS

Enzyme activity was measured spectrophotometrically by recording the change in absorbance at 340 mμ produced by NADPH in a coupled enzyme system with fructose 6-phosphate as substrate and glucose 6-phosphate dehydrogenase as the indicator enzyme. Measurements were made in a Gilford model 2000 multiple sample absorbance recorder attached to a Beckman model DUR monochromator equipped with thermostatic controls for the cell compartment; the temperature was maintained at 30° with a constant temperature circulating water bath. Details of the assay and of required calculations are given in a previous paper (2). Occasionally, the activity was also measured in the forward reaction from glucose 6-phosphate to fructose 6-phosphate by a continuously recording pH-stat assay, coupling phos-
phosphofructokinase as the indicator enzyme with the phosphogluco-
ose isomerase reaction (8).

Protein was determined in crude solutions by the colorimetric
biuret procedure of Gornall, Bardawill, and David (9) with use
of a factor of 32 mg of protein per absorbance unit at 540 \text{nm}
for a 10.0-ml reaction volume and a light path of 10 mm (2, 10).
Except for the initial autolysate it was found necessary first to
precipitate the protein with 10% trichloroacetic acid before the
biuret procedure could be carried out. Moreover, centrifuga-
tion of the trichloroacetic acid-treated samples in a clinical desk
centrifuge proved insufficient to precipitate all the protein and
high speed centrifugation at 20,000 $\times g$ for 30 minutes was
routinely used to obtain complete precipitation. The colloidal
suspension of part of the protein is probably caused by the high
content of polysaccharides and nucleic acids in the yeast com-
pared with the particular ionic composition of the various frac-
tionation steps.

After elution from DEAE-cellulose the protein concentration
was estimated spectrophotometrically from its absorbance at
280 \text{nm} in 0.01 m phosphate of pH 7.0. An absorbance coefficient
of 1.11 for 1 mg of protein per ml and for a light path of 1 cm
was obtained by calibration against the biuret method. This
value should be considered preliminary, however, and a final
figure will be given after a more detailed study of the spectral
properties of the enzyme.

**ISOLATION PROCEDURE**

General conditions for purification were the same as those
employed in the isolation of the enzyme from rabbit muscle (2):
all operations, except when otherwise stated, were performed in
a cold room at 2° or in an ice bath. Required amounts of am-
monium sulfate to obtain a certain degree of saturation, am-
monium sulfate concentrations in dissolved ammonium sulfate-
precipitated pellets, and volumes of solvent additions including
those of saturated ammonium sulfate solutions were calculated
as described previously (6).

The isolation proceeds according to the sequence given in
Fig. 1 with the individual fractionation steps performed as
follows.

**Fraction I**—Dried brewers' yeast, 600 g, is ground to a fine
powder in a porcelain mortar and suspended with vigorous
stirring in 3 liters of 0.2 m sodium bicarbonate, prewarmed to
40°. The suspension is stirred for 30 minutes at room tem-
perature and is then kept in an incubator for 5 hours at 36° after
which period the cell debris is removed by centrifugation for 20
minutes at 12,000 $\times g$ (e.g. Servall model GSA rotor). The
volume of the brownish-yellow, slightly turbid solution (Fraction
I) is approximately three-fourths of that of the bicarbonate
solution used.

**Fraction II**—An equal volume of ice-cold distilled water is
added to Fraction I followed by a quantity of 1 m zinc acetate
calculated to give a final concentration of 0.1 m with respect to
zinc acetate. During this addition the pH drops from 7.1 to
5.6. Approximately 650 ml of ice-cold 1 N NaOH are used to
adjust the pH to 8.5. Stirring is continued for 30 minutes at room
temperature and is then kept in an incubator for 5 hours at 36° after
which period the cell debris is removed by centrifugation for 20
minutes at 12,000 $\times g$ (e.g. Servall model GSA rotor). The
volume of the brownish-yellow, slightly turbid solution (Fraction
I) is approximately three-fourths of that of the bicarbonate
solution used.

**Fraction III**—The pH of Fraction II is adjusted to 7.5 with
30 to 40 ml of ice-cold 2 m acetic acid, and bentonite is added
with vigorous stirring, at a ratio of 15 g of bentonite per g of
protein. Stirring is continued for 30 minutes whereafter the
suspension is centrifuged for 30 minutes at 12,000 $\times g$. The pH of
the clear yellow supernatant liquid (Fraction II) is approximately
8.4. The volume at this stage is approximately 5 liters.

**Fraction IV**—The pH of Fraction III is adjusted to 7.5 with
30 to 40 ml of ice-cold 2 m acetic acid, and bentonite is added
with vigorous stirring, at a ratio of 15 g of bentonite per g of
protein. Stirring is continued for 30 minutes whereafter the
suspension is centrifuged for 30 minutes at 12,000 $\times g$. The
supernatant fluid (Fraction III) is clear and has only a greenish-
yellow tint.

**Fraction V**—As a consequence of the bentonite treatment
the protein concentration in Fraction III will be only 0.5 mg
per ml or less. Precipitation of the enzyme is achieved by the

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**Fig. 1. Flow sheet for the purification of phosphogluco-
ose isomerase from brewers' yeast. Vertical arrows indicate discarded
precipitates, horizontal arrows discarded supernatant liquids.
For details refer to the text.**
addition of an equal volume of acetone (chilled to $-10^\circ$) in a $-10^\circ$ bath with extreme caution not to exceed $0^\circ$ in the sample during any time of the addition period. The solution is kept for at least 30 minutes at $-10^\circ$ before the precipitate is collected by centrifugation (15 minutes at 12,000 × g) at a “running” temperature setting of $-10^\circ$. The pellets are taken up in 0.05 M magnesium acetate ($\frac{1}{2}$ the volume of Fraction III) and the mixture is stirred for approximately 2 hours until a homogeneous suspension is obtained. It is very important to keep the temperature at or slightly below $0^\circ$ at all times because the high acetone content of the suspended precipitate will otherwise cause denaturation of the enzyme. The suspension is then centrifuged for 20 minutes at 12,000 × g, at a running temperature setting of $-5^\circ$; the precipitate is discarded, and the pale yellow supernatant fluid is retained as Fraction IV.

**Fraction V**—The protein concentration of Fraction IV should be 5 to 8 mg per ml. The pH (approximately 8) is adjusted down to 6.0 with ice-cold 2 M acetic acid (approximately $\frac{1}{2}$ the volume of Fraction IV is required). In a $-10^\circ$ bath, cold 95% ethanol is slowly added with efficient stirring to bring the ethanol concentration to 10% (v/v with respect to 95% ethanol) and with care to avoid raising the temperature in the sample above $0^\circ$. Thirty minutes after the last addition of ethanol, the solution is centrifuged (20 minutes at 12,000 × g) with the running temperature set at $-10^\circ$. The precipitate is discarded and the ethanol concentration in the supernatant liquid is slowly increased to 23% (v/v of 95% ethanol). The solution is again kept for 30 more minutes in the $-10^\circ$ bath before centrifuging for 20 minutes (12,000 × g, $-10^\circ$). The precipitate is suspended with ease in 0.05 M magnesium acetate ($\frac{1}{2}$ the volume of Fraction IV) and, after stirring for 30 minutes, insoluble material is removed by centrifugation for 15 minutes at 12,000 × g. The supernatant fluid is dialyzed (Visking thin wall seamless cellulose tubing, 18/36-inch inflated diameter) overnight at $2^\circ$ against three 6-liter portions of distilled water. The light yellow dialysate is Fraction V.

**Fraction VI**—The total protein in Fraction V is determined and an adsorption cake is prepared from the calculated amount of DEAE-cellulose (20 g of the dry exchanger per g of protein) as described under “Methods.” Fraction V is allowed to permeate into the pre-equilibrated cellulose pad which is then washed with 0.005 M sodium phosphate, pH 5.6 (50 ml per g of dry DEAE-cellulose) with the result that most of the yellow protein contaminations are removed. The enzyme is eluted with 0.1 M phosphate of pH 6.0 applied in five portions of the following volumes (in terms of milliliters per g of dry DEAE-cellulose): 1, 5, 5, 2.5, and 2.5. Gentle suction is employed and after passage of each buffer portion the cake is freed of as much liquid as possible. Usually, the first and last fractions contain only very small amounts of enzyme and can be discarded. The remaining eluates are combined to give the almost colorless Fraction VI.

**Fraction VII**—Concentration of the enzyme in Fraction VI is achieved by dialysis against saturated ammonium sulfate for at least 12 hours. Salt penetration into the dialysis bag containing the enzyme solution and water movement out of the bag against the concentration gradient act concurrently to effect a very mild precipitation of the enzyme protein. The precipitate is collected by centrifugation for 2 hours at 30,000 × g and is discarded.

Occasionally, when the protein concentration of the DEAE-eluates was too low, difficulty was experienced in collecting all the enzyme activity at 30,000 × g. In these cases centrifugation was...
solved in a small volume of cold 0.01 M sodium phosphate buffer, pH 7.0, to give a final protein concentration of 15 to 20 mg per ml. The slightly yellow solution is designated as Fraction VII.

Crystallization—The ammonium sulfate concentration in Fraction VII is estimated (cf. (6)) and neutralized saturated ammonium sulfate solution is slowly added to bring the saturation in the sample to 0.60. Some turbidity will occur which is immediately removed by centrifugation (15 minutes at 25,000 × g). The ammonium sulfate saturation is then increased in 0.005 saturation increments and at a rate of not more than 0.01 per day until 0.63 saturation is reached. Between 0.58 and 0.60 saturation the enzyme precipitates initially in the form of amorphous or at best semicrystalline granules. Crystallization is normally completed after approximately 2 weeks. The change from the granular to the crystalline form is accompanied by a very characteristic change in the macroscopic and microscopic appearance of the enzyme suspension. In the beginning the granular precipitate settles within 4 to 6 hours and, on swirling, displays only a weak and dull metallic appearance which, however, is distinctly different from the milky white and microscopic appearance of the enzyme suspension. In the transformation into very fine needles. Crystallization is normally completed after approximately 2 weeks.

The ammonium sulfate concentration in solutions is estimated by graphs taken at the corresponding stages of crystallization (Figs. 2 and 3) support the above macroscopic description. For recrystallization the ammonium sulfate suspension is centrifuged at pH 8.5 by 0.03 M sodium phosphate buffer. For recrystallization the crystalline suspension is centrifuged for at least 1 hour at 30,000 × g, the mother liquor is removed by a transfer pipette, and the crystals are dissolved in approximately 1/2 the volume of the dissolved precipitate. It should be emphasized that the pH of the resulting solution is estimated, and neutralized saturated ammonium sulfate solution is added as before. For at least the first three crystallizations, it is advantageous to remove the initial turbidity which occurs at 0.58 saturation, by centrifugation for 5 minutes at 30,000 × g. Crystallization occurs in an analogous manner to that described for the first crystal but the upper limit of ammonium sulfate saturation may be dropped from 0.63 to 0.62 after the third crystallization.

The first three mother liquors usually display decreasing intensities of yellow, whereas the succeeding ones are colorless. Also, packed crystalline pellets become white in appearance after the second recrystallization. Five crystallizations are normally required until no further increase in specific activity of the enzyme is observed. Specific activities of 650 to 700 μmoles units per mg (determined at 30 °C with the NADP-glucose 6-phosphate dehydrogenase system) are routinely obtained in the initial purification step the response to the addition of divalent zinc is quite different as the muscle enzyme is precipitated at pH 5.4, whereas in the case of the yeast enzyme the zinc acetate concentration can be raised to 0.1 M with only little isomerase protein coming down with the precipitate. It should be emphasized that the pH of the resultant precipitate. It should be emphasized that the pH of the result-

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg protein)</th>
<th>Purification</th>
<th>Recovery</th>
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</thead>
<tbody>
<tr>
<td>I. Yeast autolysate</td>
<td>2.200</td>
<td>30.500</td>
<td>173,000</td>
<td>5.7</td>
<td>(100)</td>
<td></td>
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<tr>
<td>II. Zinc acetate supernatant</td>
<td>5.100</td>
<td>6.600</td>
<td>115,000</td>
<td>17.3</td>
<td>66</td>
<td></td>
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<tr>
<td>III. Bentonite supernatant</td>
<td>4.525</td>
<td>9.05</td>
<td>108,000</td>
<td>120</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>IV. Acetone precipitate, 50%</td>
<td>13.0</td>
<td>50.0</td>
<td>104,000</td>
<td>176</td>
<td>31</td>
<td></td>
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<tr>
<td>V. Ethanol precipitate, 25%</td>
<td>4.5</td>
<td>30.6</td>
<td>81,700</td>
<td>26/4</td>
<td>4/</td>
<td></td>
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<tr>
<td>VI. DEAE-eluate</td>
<td>95</td>
<td>82</td>
<td>68,000</td>
<td>331</td>
<td>58</td>
<td></td>
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<tr>
<td>VII. Ammonium sulfate precipitate</td>
<td>5.9</td>
<td>104</td>
<td>59,800</td>
<td>383</td>
<td>67</td>
<td></td>
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<tr>
<td>First crystals (GCBY-24)</td>
<td>5.5</td>
<td>71</td>
<td>35,000</td>
<td>501</td>
<td>88</td>
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<tr>
<td>First crystals (GCBY-19)</td>
<td>5.8</td>
<td>41</td>
<td>24,300</td>
<td>593</td>
<td>(97)</td>
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<td>Average</td>
<td>5.7</td>
<td>56</td>
<td>30,000</td>
<td>536</td>
<td>(94)</td>
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<tr>
<td>Second crystals, average</td>
<td>5.4</td>
<td>35</td>
<td>21,800</td>
<td>623</td>
<td>(108)</td>
<td></td>
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<tr>
<td>Third crystals, average</td>
<td>6.5</td>
<td>24</td>
<td>16,200</td>
<td>675</td>
<td>(118)</td>
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</table>

* Units are expressed as micromoles of glucose 6-phosphate formed per minute from fructose 6-phosphate at 30 °C, as measured with the NADP-glucose 6-phosphate dehydrogenase system.

† For recrystallization, individual preparations were frequently combined. The mean values of GCBY-19 and GCBY-24 are presented in this table to illustrate typical data for purification during recrystallization.

DISCUSSION

It seems appropriate to comment briefly on the behavior of the phosphoglucose isomerases isolated both from brewers' yeast and from rabbit muscle (2) during their respective purification processes.

In the initial purification step the response to the addition of divalent zinc is quite different as the muscle enzyme is precipitated at pH 5.4 by 0.03 M zinc acetate, whereas in the case of the yeast enzyme the zinc acetate concentration can be raised to 0.1 M with only little isomerase protein coming down with the precipitate. It should be emphasized that the pH of the result-
highly purified muscle isomerase, tended to denature the yeast enzyme and appreciable losses in activity were observed after which could be used satisfactorily to precipitate crude as well as the situation with the muscle enzyme. Unfortunately, acetone, which causes the enzyme to precipitate in the form of its en-

zyme-magnesium-nucleate complex (cf. (6) and (11)).

Concentration of the highly diluted yeast enzyme prior to crystallization turned out to be somewhat difficult in analogy to the situation with the muscle enzyme. Unfortunately, acetone, which could be used satisfactorily to precipitate crude as well as highly purified muscle isomerase, tended to denature the yeast enzyme and appreciable losses in activity were observed after its use. Some trial experiments were performed with Aquacide (Calbiochem, Los Angeles, California), a high molecular weight hydrophilic, which in fact was found to be fairly efficient in absorbing water. Yet it appeared to release some polysaccharide-like material into solution and which seriously interfered with the succeeding crystallization so that it had to be abandoned as a means to concentrate the enzyme. Dialysis against saturated ammonium sulfate solution was finally resorted to as most suitable for precipitation of the yeast isomerase from the DEAE-

eluate.

The yeast enzyme resisted numerous attempts at crystalliza-
tion at a purity comparable to that at which crystallization of the muscle enzyme initiates. Thus, a DEAE-cellulose adsorp-
tion step was introduced to bring the specific activity prior to crystallization into the neighborhood of 400 units, i.e. twice the minimal purity needed for the first crystallization of the muscle enzyme. This step is also highly effective in removing contaminating nucleic acids as evidenced by the change of the absorbance ratio of 260 μμ:280 μμ which will rise, after the DEAE-cellulose treatment, from approximately 0.5 to 1.7. The application of DEAE-cellulose as a powerful tool to remove contaminating nucleic acids from enzyme preparations had previously been described and commented upon in purification procedures for both crystalline glucose 6-phosphate dehydrogenase (6) and nucleoside diphosphokinase (11).

In addition to the greater difficulties which are encountered in the crystallization of the yeast enzyme it should also be noted that the solubility in ammonium sulfate solutions differs for both enzymes as demonstrated by the different concentration ranges within which crystallization occurs. These are between 0.48 and 0.56 saturation for the muscle enzyme and between 0.58 and 0.63 saturation for the yeast isomerase.

Further evidence for structural differences between the two heteroenzymes is provided by a somewhat different shape of the crystals (under otherwise equal conditions for crystallization), by a greater stability of the yeast enzyme at slightly acid pH\textsuperscript{10} and by marked differences in their total amino acid composi-
tion.\textsuperscript{10} Amino acid analyses indicate a considerably lower content of basic amino acids for the yeast enzyme which should therefore have a lower isoelectric point than the muscle enzyme. This difference in over-all net charge may well be a contributing factor to the different solubility behavior of the two enzymes. Detailed comparative studies on the physical and chemical properties of both phosphoglucos isomerases are in progress and will be the subject for future communications.

SUMMARY

1. A procedure is described for the isolation of crystalline phosphoglucose isomerase from brewers' yeast. The following sequence of steps is utilized: autolysis in 0.2 M NaHCO₃, removal of inert protein by treatment with zine acetate and bentonite, fractionation with acetone and ethanol in the presence of Mg\textsuperscript{2+}, adsorption of the enzyme on DEAE-cellulose and elution with 0.1 M phosphate, pH 6.0, and crystallization and recrystallization from ammonium sulfate solutions.

2. Some comments are presented comparing this procedure with the isolation method for crystalline phosphoglucose isomerase from rabbit muscle (2).

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REFERENCES


\textsuperscript{10} Unpublished experiments.
Isolation of Crystalline Phosphoglucose Isomerase from Brewers' Yeast
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