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ISOLATION AND CHARACTERIZATION OF β-GALACTOSIDASE FROM *SACCHAROMYCES LACTIS*

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SUMMARY

1. β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) was purified 78.6-fold from wild type *Saccharomyces lactis*, strain M-12 by $(NH_4)_2SO_4$ fractionation, column chromatography on Sephadex G-100 and DEAE–Sephadex A-50.

2. The purified enzyme showed a pH optimum of 7.2 and a Mg^{2+} optimum of 2 μmoles per ml.

3. The Michaelis constant was determined and found to be 1.18 mM using *o*-nitrophenyl-β-D-galactopyranoside as the substrate.

INTRODUCTION

Previous purification procedures regarding β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) in microorganisms have been reported from a number of strains of *Escherichia coli*. One of the earliest was carried out by MONOD, TORRIANI AND GRIBETZ[1] in 1948 and by COHN AND MONOD[2] in 1951. KUBY AND LARDY isolated the enzyme from *E. coli* strain K 12 and obtained a 99.5-fold purification. WALLENFELS *et al.*[4] obtained a 13.6-fold purification from *E. coli* ML 309 and were able to crystallize their product. HU, WOLFE AND REITHEL[5] also isolated and crystallized the enzyme from *E. coli*. CRAVEN, STEERS AND ANFINSEN[6] produced a material from *E. coli* K 12 that was electrophoretically pure and showed no contamination on ultracentrifugal analysis.

One of the most recent attempts to purify β-galactosidase was reported by MCFETERS, SANDINE AND ELLIKER[7]. These investigators used *Streptococcus lactis* as a source for the enzyme. Some investigations have also been attempted on yeast with the strain *Saccharomyces fragilis*[8,9].

The present work describes a procedure for the purification of β-galactosidase from yeast *S. lactis* and a study of some of its properties.

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EXPERIMENTAL MATERIALS AND METHODS

Stockculture of wild type *S. lactis* strain M-12 was obtained through the courtesy of Dr. NORMAN EATON, Brooklyn College, Brooklyn, N.Y. The substrate *o*-nitrophenyl-β-D-galactopyranoside A grade was obtained from Calbiochem, Los Angeles, Calif. $(NH_4)_2SO_4$, a product of Mann Research Laboratories, New York, N.Y., was enzyme grade while all other reagents used were of analytical grade. The liquid medium for the growth of the yeast cells consisted of 2% lactose, 2% peptone, and 1% yeast extract.

The yeast was grown by inoculating stockculture into test tubes containing 5 ml of sterilized media. This was permitted to develop at room temperature for 24 h. At this time 1 ml of this inoculum was pipetted into Fernbach flasks containing 1 l of sterilized media. The product was harvested after 48 h incubation at a constant temperature of 30° in a rotary shaker and washed twice with distilled water.

Enzyme activity was determined by following the continuous release of *o*-nitrophenol from *o*-nitrophenyl-β-D-galactopyranoside. The assay mixture contained enzyme, 0.02 mmole of phosphate buffer (pH 7.2), 2 μmoles of $MgCl_2$, 0.02 mmole of 2-mercaptoethanol, 0.6 μmoles of *o*-nitrophenyl-β-D-galactopyranoside in a total volume of 1 ml. The absorption of *o*-nitrophenol was determined at 400 mμ using a Beckman DU spectrophotometer equipped with a Gilford absorbance indicator model 1220 and automatic cuvette positioner model 210.

A unit of enzyme activity was defined as a change of absorbance of 0.001 per min of reaction time in a 1-cm light path at room temperature. Protein was determined by a modified biuret method of ZAMENHOFF[10] at 310 mμ. Sephadex G-100 and DEAE–Sephadex A-50 were obtained from Pharmacia Fine Chemicals and prepared with 0.05 M phosphate buffer (KH_2PO_4–Na_2HPO_4) according to the manufacturer's instructions.

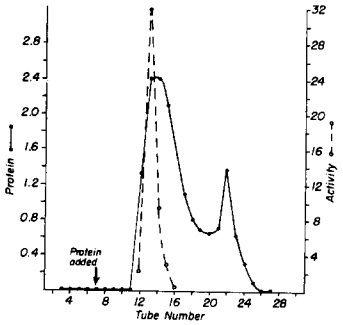
Polyacrylamide-gel electrophoresis was performed with the 7.5% 'standard gel' at 5° and 3 mA per tube according to the directions supplied by Canal Industries Corp. (Bethesda, Maryland). Following the electrophoresis procedure the gels were stained with Amido Schwarz.

RESULTS

Purification of the enzyme

The yeast was grown in 6 Fernbach flasks each containing 1 l of medium. After harvesting, the yield was crushed in a stainless steel press[11].

The ruptured cells were taken up in 0.05 M phosphate buffer (pH 7) which was made 0.01 M with respect to $MgCl_2$. The resulting suspension was centrifuged in a Servall centrifuge at 12 000 × *g* for 10 min. The precipitate was discarded and the supernatant which contained 5.9 g of protein was subsequently centrifuged in a Spinco Model L ultracentrifuge at 100 000 × *g* for 1 h. The supernatant was fractionated with enzyme grade $(NH_4)_2SO_4$ at 55% saturation. The mixture was centrifuged at 12 000 × *g* for 10 min and the residue dissolved in a minimum volume of 0.05 M phosphate buffer (pH 7) made 0.01 M with respect to $MgCl_2$ by the addition of solid $MgCl_2$ to buffer. The solution was dialyzed for 4 h in a cold room (4°) against 0.05 M phosphate buffer (pH 7) which contained 1 mM 2-mercaptoethanol. A precipitate of



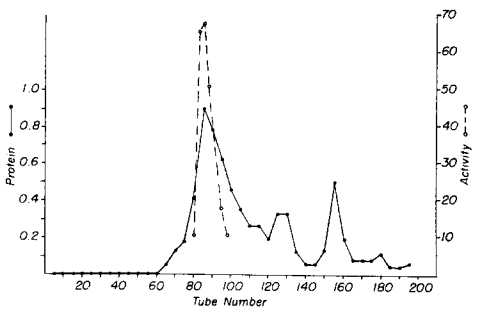
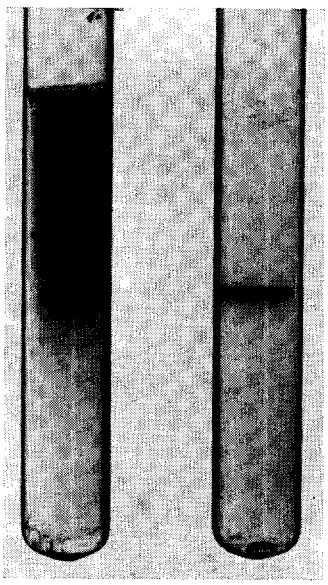


Fig. 1. A typical elution pattern on Sephadex G-100. Fractions of 10 ml were collected. ●—●, absorbance at 280 mμ; ○—○, activity of enzyme (Δ absorbance per min).

Fig. 2. Chromatography on DEAE–Sephadex A-50. Approx. 15 mg of protein applied in 25 ml of 0.05 M phosphate buffer. Fractions of 5 ml were collected with a linear gradient of 0 to 0.5 M NaCl in buffer. ●—●, absorbance at 280 mμ; ○—○, activity of enzyme (Δ absorbance per min).

magnesium ammonium phosphate was found developing outside of the dialysis bags. The dialyzed solution was further purified by adding it to a chromatography column (5 cm × 100 cm) containing Sephadex G-100 (Fig. 1). The column was eluted with 0.05 M phosphate buffer (pH 7) which contained 1 mM 2-mercaptoethanol. Fractions of 10 ml-volumes were collected. Tubes containing the most active samples were pooled and the enzyme concentrated by the addition of solid $(NH_4)_2SO_4$ brought to



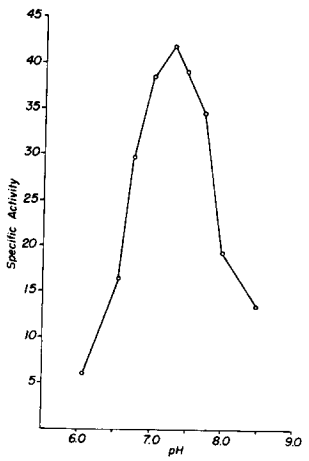


Fig. 3. Polyacrylamide-gel disc electrophoresis patterns of crude extract (left) and purified β-galactosidase (right).

Fig. 4. The effect of pH on enzyme activity. Ordinate is the Δ absorbance per min per mg protein using *o*-nitrophenyl-β-D-galactopyranoside as the substrate. Measurements were made in 0.1 M phosphate buffers.

TABLE I

PURIFICATION OF *S. lactis* β-GALACTOSIDASE

Fraction	*Total volume (ml)*	*Protein (mg/ml)*	*Units (mg)*	*Total units* $\times 10^{-6}$	*% yield*	*Purification*
Original	67	88	1.82	10.72	—	—
Supernatant 100 000 × *g* centrifugation	46	77	4.22	14.95	139.0	2.3
Precipitate 55% satn. $(NH_4)_2SO_4$ fractionation	30	48	8.25	12.75	119.0	4.9
Sephadex G-100	123	2.7	43.7	14.51	135.0	24.0
DEAE–Sephadex	26	0.55	143.0	2.04	19.1	78.6

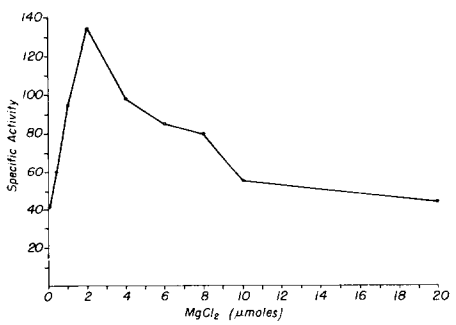
65% saturation. The precipitate was subsequently dissolved in 0.05 M phosphate buffer (pH 7) and dialyzed as in the previous procedure. The enzyme was further purified by column chromatography on a column (2.5 cm × 45 cm) charged with DEAE–Sephadex A-50 (Fig. 2). The column was eluted with 0.05 M phosphate buffer (pH 7) containing 1 mM 2-mercaptoethanol and a linear gradient of 0–0.5 M NaCl in buffer. The most active fractions were dialyzed against 0.05 M phosphate buffer (pH 7) containing 1 mM 2-mercaptoethanol. The final enzyme solution had a specific activity of 143 and showed a 78.6-fold purification. A summary of the purification procedure is given in Table I.

The purity of the enzyme was checked on polyacrylamide gel. The single band of β-galactosidase in comparison to the multi-banded gel of the crude extract is shown in Fig. 3.

Properties of the enzyme

pH optimum. The enzyme has a pH optimum at pH 7.2. Measurements were made in 0.1 M phosphate buffer in a range from pH 6 to 8.5 as shown in Fig. 4.

Optimum Mg^{2+} concentration. Mg^{2+} appeared to be necessary for optimal enzyme activity. Maximum activity was obtained after addition of 2 μmoles $MgCl_2$ as shown in Fig. 5.



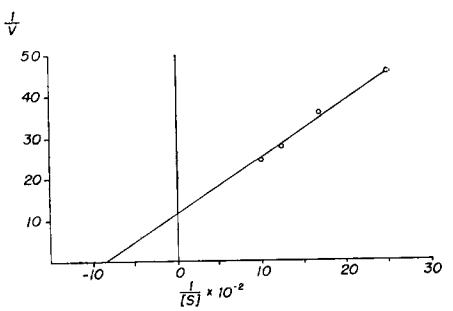


Fig. 5. The effect of varying concentrations of $MgCl_2$ on the activity of the enzyme using *o*-nitrophenyl-β-D-galactopyranoside as substrate.

Fig. 6. LINEWEAVER–BURK plot of the enzyme. *v* is expressed as absorbance increase per min [*S*] is expressed in M of *o*-nitrophenyl-β-D-galactopyranoside substrate.

Kinetic study. K_m was determined by the method of LINEWEAVER AND BURK using *o*-nitrophenyl-β-D-galactopyranoside as a substrate. The K_m for this enzyme was 1.18 mM. The results are illustrated in Fig. 6.

DISCUSSION

One of the major difficulties encountered in the purification is the instability of the enzyme as the isolation progresses. The β-galactosidase is stabilized in the presence of 3–5% $(NH_4)_2SO_4$. It was noticed that upon removal of the $(NH_4)_2SO_4$ by dialysis, the enzyme begins to lose its activity. This is not restored by the re-addition of $(NH_4)_2SO_4$.

The addition of the $(NH_4)_2SO_4$ apparently stabilizes and activates the enzyme as noted by the increase in total activity, Table I, Step 3 and 4. Similar stabilization and activation were also found in the purification of Streptococcus lactis β-galactosidase[7]. The increase in total activity in Step 2 may be attributed to the removal of an inhibitory substance.

Fig. 5 shows that addition of 2.0 μmoles of $MgCl_2$ to the assay mixture results in maximal stimulation of enzyme activity. REITHEL AND KIM[12] showed that purified β-galactosidase obtained from *E. coli* ML 308 had a Mg^{2+} and Na^+ requirement for maximum catalytic activity when *o*-nitrophenyl-β-D-galactopyranoside was used as a substrate.

CRAVEN, STEERS AND ANFINSEN[6] have shown the *E. coli* K12 enzyme to be stabilized with mercaptoethanol. This seems to be the case with our preparation and thus mercaptoethanol was used both in the isolation procedure and in the assay.

Further characterization of the *S. lactis* strain M-12 β-galactosidase is in progress.

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