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PURIFICATION AND SOME PROPERTIES OF β-GALACTOSIDASE OF *BACILLUS SUBTILIS*

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SUMMARY

1. β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) was isolated from *Bacillus subtilis* and partially purified.

2. When the measurements were made at saturating or nearly saturating concentrations of substrate there was a linear relationship between the quantity of enzyme and the amount of product formed, and a linear formation of product with time. The enzymic activity was not influenced by ions.

3. The optimum pH for hydrolytic activity was 6.5.

4. The optimum temperature for hydrolysis was 50°. The enzyme was relatively heat-stable. The enzyme was not inactivated during 1 h at 50°. At 55° the inactivation was 15% after 30 min of exposure.

5. The affinity constant for lactose was $7.1 \cdot 10^{-1}$. The constants for the common substrates of the β-galactosidase of *B. subtilis* were different from those of the *Escherichia coli* enzyme.

INTRODUCTION

The hydrolytic enzyme β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) of *Escherichia coli* has been extensively investigated. Several authors have described its purification[1–3] and crystallization[4,5]. The genetic control of the regulation of the biosynthesis of the enzyme in this organism has been analysed by recombination[6–8] and transduction[9–11] experiments. LANDMAN[12] studied some properties and the induction of the enzyme in *Bacillus megatherium*. CLAUSEN AND NAKAMURA[13] investigated the enzyme of *Shigella sonnei*. So far, no attention has been paid to the β-galactosidase of *Bacillus subtilis*, although the organism can be genetically analysed by transformation[14,15] and transduction[16] experiments. In this paper a procedure for the purification of the β-galactosidase of *B. subtilis* is presented, and some of its properties are described.

Abbreviations: ONPG, *o*-nitrophenyl-β-D-galactoside; ONP, *o*-nitrophenol; IPTG, isopropyl-β-D-thiogalactoside.

MATERIALS AND METHODS

Organism and medium

The strain used was a constitutive mutant isolated from the Marburg strain by the method of COHEN-BAZIRE AND JOLIT[17]. Wild-type cells were grown alternately 8 h in synthetic medium containing 0.5% glucose and 16 h in synthetic medium containing 0.5% lactose. After one month a constitutive mutant was isolated which had a 25-fold β-galactosidase production in comparison with the wild type grown in a medium supplemented with lactose.

The cells were grown at 37° on reciprocal shakers in the following medium (g/l): $(NH_4)_2SO_4$, 2; KH_2PO_4, 6; K_2HPO_4, 14; $MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3 \cdot 6H_2O$, 0.01; $MnSO_4 \cdot 4H_2O$, 0.00025; peptone, 0.2; lactose · H_2O, 5. The pH of the medium was 7.0.

Enzyme assay

Enzymic activity was assayed with a modification of the method of REVEL, LURIA AND ROTMAN[9]. A mixture of 0.01 ml of enzyme (max. 600 units); 1 ml 0.1 M sodium–potassium phosphate buffer (pH 6.5) and 0.6 ml of distilled water were brought to 50°. Then 1 ml prewarmed 0.25 M sodium–potassium phosphate buffer (pH 6.5) containing 14 mg ONPG was added. After 5 min incubation at 50° the reaction was stopped by addition of 1.3 ml 1 M sodium carbonate. The absorbancy of the chromogen was measured at 420 mμ in a Zeiss spectrophotometer. One unit of enzyme is defined as the amount that releases 1 mμmole of ONP per min at 50° and pH 6.5. A solution of 1 mμmole of ONP per ml has an absorbancy of 0.005 under the above conditions (final pH 10.2; 10 mm light path).

Protein determination

Protein was determined spectrophotometrically by measuring the absorption at 280 and 260 mμ (ref. 18).

Reagents

ONPG was obtained from Sigma Chemical Company, IPTG from Mann Research Laboratories and Streptomycin sulphate from Mycofarm, Delft.

EXPERIMENTS AND RESULTS

The cells were centrifuged at the end of the logarithmic phase, washed once with saline and a second time with 0.01 M phosphate buffer (pH 7.0). The bacteria were then packed at 27 000 × g in a Servall RC-2 centrifuge. The wet cells (100 g) were resuspended in 200 ml 0.01 M phosphate buffer (pH 7.0) and ruptured in batches of 24 ml with 16 g ballotini beads No. 15 (English Glass Company, Leicester) in the Cell homogenizer of MERKENSCHLAGER[19] at 2500 rev./min during 3 min. The cell debris was spun down at 27 000 × g during 30 min. The brownish red crude extract could be stored for at least 3 weeks at −20° without loss of activity.

Purification

Step 1 Nucleic acid precipitation. To the extract (120 ml) 7 ml of a 50% streptomycin sulphate solution in 0.01 M phosphate buffer (pH 7.0) were slowly added with stirring at 4°. The mixture was allowed to stand in ice for 20 min and centrifuged in the cold for 30 min at 27 000 × *g*. The clear yellow supernatant was retained for protein precipitation, and the nucleic acid containing residue was discarded[4].

Step 2. First ammonium sulphate fractionation. The solution was brought to 45% saturation with $(NH_4)_2SO_4$ by slow addition with stirring of a saturated salt solution (pH 7.2), kept in the cold for 25 min, and centrifuged at 4° for 30 min at 27 000 × *g*. The supernatant was brought to 65% saturation with the $(NH_4)_2SO_4$ solution. After 25 min the mixture was centrifuged and the precipitate was dissolved in 32 ml 0.01 M sodium–potassium phosphate buffer (pH 7.0).

The fractionations were performed in rapid succession because inactivation of the enzyme in the $(NH_4)_2SO_4$ solutions was observed.

Step 3. Acetone precipitation. Prechilled acetone (16 ml) was added slowly with stirring to 32 ml of the enzyme solution of Step 2. The mixture was allowed to stand for 15 min in a −10° bath and centrifuged at −10° at 27 000 × *g* for 25 min. To the supernatant 8 ml of acetone were added. After 15 min at −10° the suspension was centrifuged as before. The supernatant was discarded and the precipitate dissolved in 10 ml of phosphate buffer (pH 7.0). This fraction was dialysed with stirring against 500 ml of the same buffer at 4° for 23 h.

TABLE I

PURIFICATION OF β-GALACTOSIDASE

Step	*Total volume (ml)*	*Total units* × 10^{-3}	*Total protein (mg)*	*Units/mg protein* × 10^{-3}	*Yield (%)*	*Overall purification*
Crude extract	120	4262	360	12	100	—
Nucleic acid precipitate	127	4262	360	12	100	—
First $(NH_4)_2SO_4$ fractionation	33	4208	121	35	98	2.9
Acetone precipitate	10	2240	18	119	52	10.1
Second $(NH_4)_2SO_4$ fractionation	4	1434	6	239	33	20.3

Step 4. Second ammonium sulphate fractionation. The dialysed solution was brought to 45% saturation by addition of saturated $(NH_4)_2SO_4$ (pH 7.2), kept in an ice bath for 25 min and centrifuged at 4° for 25 min at 27 000 × *g*. The supernatant was brought to 55% saturation, allowed to stand for 25 min and centrifuged. The precipitate was dissolved in 4 ml 0.01 M phosphate buffer (pH 7.0), and dialysed for 25 h against three batches of 750 ml of the same buffer. Table I summarizes the recovery and purification for each step. A 20-fold overall purification was achieved with about 33% recovery of enzyme activity. The purified extract could be stored below −20° for at least 3 months without loss of activity. Further attempts at purification by means of absolute methanol were unsuccessful, because this alcohol rapidly inactivated the enzyme.

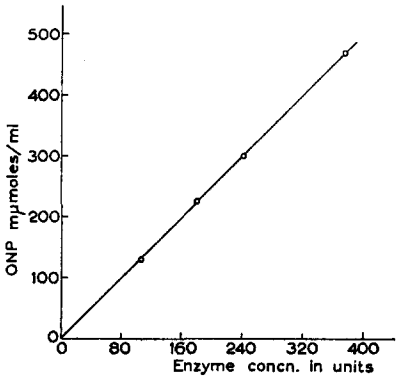

Fig. 1. Relationship between enzyme concentration and rate of hydrolysis. Various concentrations of enzyme were tested under optimal conditions in the standard assay as described under MATERIALS AND METHODS.

Some properties of the enzyme

Enzyme concentration and time course. Under optimal conditions, there was a linear relationship between the rate of hydrolysis and the concentration of the enzyme (Fig. 1).

When ONPG was hydrolysed by the enzyme during various incubation times at 50° and optimal pH, the amount of product formed was linear with time during the first 15 min (Fig. 2).

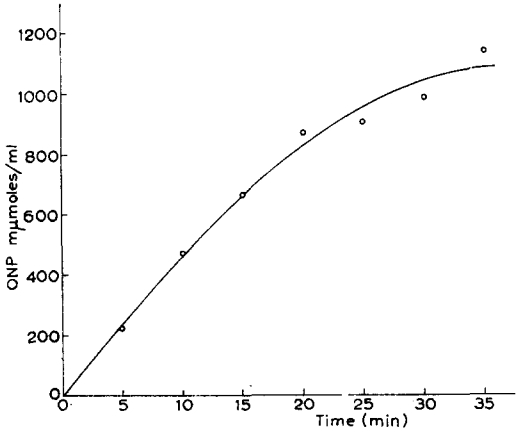

Fig. 2. Rate of hydrolysis of ONPG during incubation under optimal conditions.

Activity at various pH values. The activity of the enzyme was measured in sodium–potassium phosphate buffers of various pH values. The maximal activity of the enzyme was at pH 6.5 (Fig. 3).

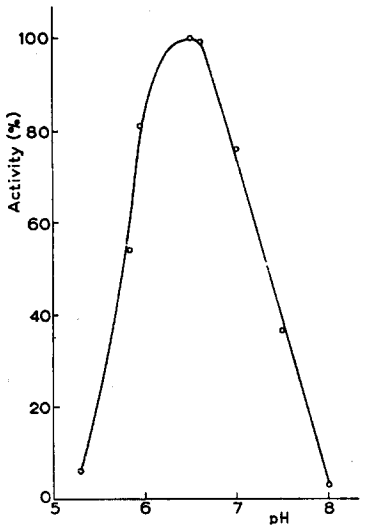

Fig. 3. Effect of pH on enzyme activity. The phosphate buffer (pH 6.5) of the standard reaction mixture was substituted by phosphate buffers of various pH values.

Ion effects. Variation of the concentration of the sodium–potassium phosphate buffer had no influence on enzyme activity. Whether sodium phosphate or potassium phosphate buffer were used, the activity was unaffected. No influence of the following cations and anions could be detected in the standard reaction mixture: NH_4^+, Ba^{2+}, Ca^{2+}, Li^+, Fe^{3+}, Mn^{2+}, Mg^{2+}; Cl^-, SO_4^{2-}, CO_3^{2-}, NO_3^-.

Temperature optimum and thermal inactivation. No β-galactosidase activity was detected in preliminary experiments in cells grown with lactose as carbon and energy source. In early experiments the enzyme activity was routinely measured at 28°. In later experiments it was found that the activity of the enzyme at this

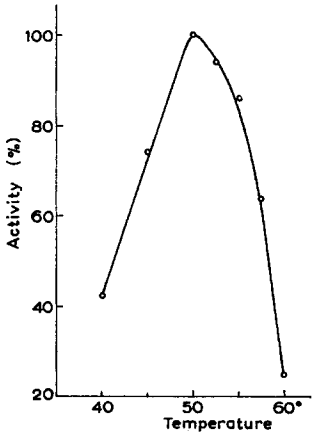

Fig. 4. Effect of temperature on enzyme activity. Standard reaction mixtures were incubated at various temperatures during 5 min.

temperature was very low. The optimal temperature of the enzyme was determined under otherwise optimal conditions (pH 6.5, enzyme concentration, etc.) and found to be 50° (Fig. 4).

An exposure to 50° caused no loss of activity of the enzyme even after 90 min. At 55° the inactivation was 15% after 30 min of exposure, whereas at 60° the inactivation was 50% after 2 min.

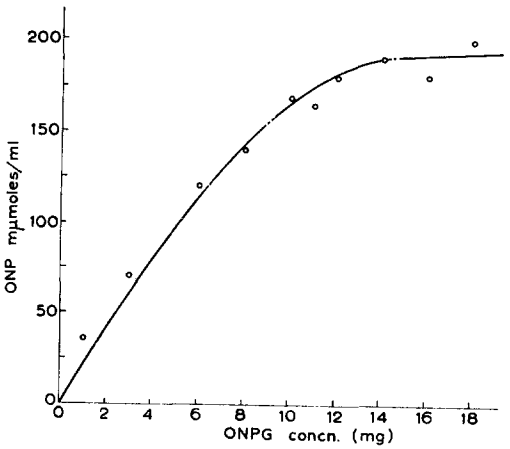

Fig. 5. Effect of ONPG concentration in the standard assay.

Affinity relations. The influence of ONPG concentration on the rate of hydrolysis is shown in Fig. 5. The amount of enzyme used in most of the experiments (160 units) was saturated with 14 mg ONPG in the reaction mixture.

The affinities of lactose, galactose and IPTG for the enzyme were measured in a series of tests, in which the inhibition of ONPG hydrolysis by a fixed concentration of inhibitor was estimated at various ONPG concentrations. The results, plotted by the LINEWEAVER–BURK method[20] (Fig. 6), gave the K values shown in Table II.

TABLE II

A COMPARISON OF SOME PROPERTIES OF THE β-GALACTOSIDASES OF *B. subtilis*, *E. coli* AND *B. megatherium*

	B. subtilis	*E. coli*	*B. megatherium*
pH optimum	6.5	7.0	—
Inactivation at 55° after 5 min	0%	100%	42%
K_m (ONPG)	$4.2 \cdot 10^{-2}$	$1.8 \cdot 10^{-4}$	$1.6 \cdot 10^{-4}$
K_I (lactose)	$7.1 \cdot 10^{-1}$	$1.1 \cdot 10^{-3}$	$1.8 \cdot 10^{-3}$
K_I (galactose)	$4.2 \cdot 10^{-2}$	$1.1 \cdot 10^{-3}$	$1.6 \cdot 10^{-2}$
K_I (IPTG)	$1.2 \cdot 10^{-1}$	$2.6 \cdot 10^{-4}$	$3.2 \cdot 10^{-4}$

K_m or K_I in moles/l.

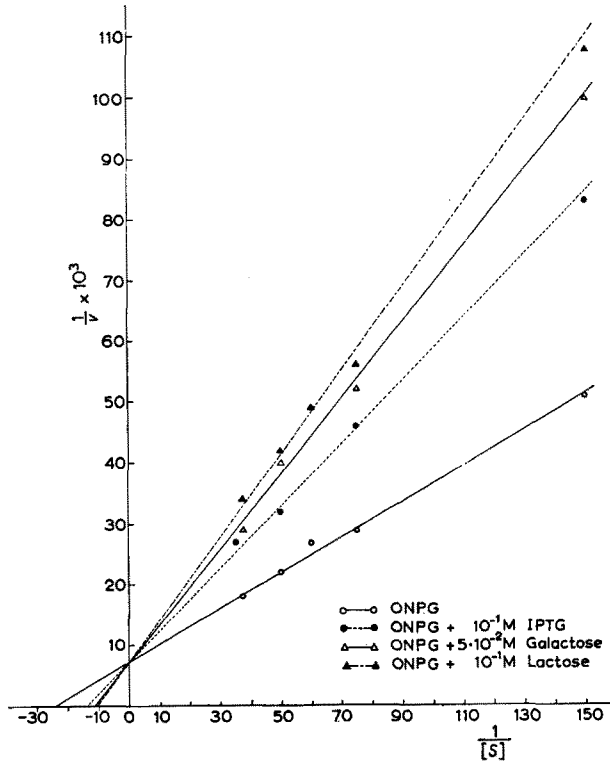

Fig. 6. LINEWAEVER–BURK plot for the determination of the Michaelis constant for ONPG and the inhibitor constants for lactose, galactose and IPTG.

DISCUSSION

There was a linear relationship between the quantity of enzyme and the amount of product formed, and a linear formation of product with time, when the measurements were made at saturating or nearly saturating concentrations of substrate, or when measurements were made at points where only a small fraction of the substrate had been converted. Similar results have been found with the β-galactosidase from *E. coli*[1].

The pH optimum of the *B. subtilis* enzyme was close to 6.5, whereas the optimum of the enzyme of *E. coli*[1,2,5] is about 7.0. The results of LANDMAN[12] with *B. megatherium* are not comparable with our results because he used the continuous method to measure ONPG hydrolysis. In this way it is only possible to measure the optimum pH of the coloured tautomer of ONP, and it is not possible to measure the optimum pH for enzyme activity.

Ions have a stimulating or inhibiting effect on the activity of the β-galactosidase of *E. coli*[1,2,21] but they had no influence on the activity of the enzyme of *B. subtilis*.

The stability of the three enzymes at high temperature is completely different. The *B. subtilis* enzyme was not inactivated at 50°. It was 15% inactivated during

an exposure of 30 min to 55°; and it was totally inactivated in a few minutes at 60°. The enzyme of *E. coli* is rapidly inactivated above 40° (ref. 5). At 50° about 94% of the enzyme is inactivated after 9 min of exposure, and at 55° the enzyme is totally inactivated in 1 min (ref. 2). On the contrary JANEČEK AND RICKENBERG[22] found no inactivation at 50° with a β-galactosidase extract from *E. coli* ML 30 after 12 min of exposure. A 5-min exposure of the β-galactosidase from *B. megatherium* in 0.5 M K_2HPO_4 resulted in 42% inactivation.

The method of LINEWEAVER AND BURK[20] was used to estimate the values of K_m or K_i for the various substrates of the three enzymes. The measurements were made at different temperatures: with *E. coli* at 30°, *B. megatherium* at 40° and *B. subtilis* at 50°. According to WALLENFELS and co-workers[21] there is not much influence of the temperature on the Michaelis constant for ONPG for the *E. coli* enzyme. Consequently it is possible to compare at least the values for K_m or K_i for the enzymes of *E. coli* and *B. subtilis*. These values show considerable differences (Table II).

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REFERENCES

1 J. LEDERBERG, *J. Bacteriol.*, 60 (1950) 381.
2 M. COHN AND J. MONOD, *Biochim. Biophys. Acta*, 7 (1950) 153.
3 S. A. KUBY AND H. A. LARDY, *J. Am. Chem. Soc.*, 75 (1953) 890.
4 A. S. L. HU, R. G. WOLFE AND F. J. REITHEL, *Arch. Biochem. Biophys.*, 81 (1959) 500.
5 K. WALLENFELS, M. L. ZARNITZ, G. LAULE, H. BENDER AND M. KESER, *Biochem. Z.*, 331 (1959) 459.
6 A. B. PARDEE, F. JACOB AND J. MONOD, *J. Mol. Biol.*, 1 (1959) 165.
7 M. RILEY, A. B. PARDEE, F. JACOB AND J. MONOD, *J. Mol. Biol.*, 2 (1960) 216.
8 F. JACOB AND J. MONOD, *J. Mol. Biol.*, 3 (1961) 318.
9 H. R. REVEL, S. E. LURIA AND B. ROTMAN, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 1956.
10 H. R. REVEL AND S. E. LURIA, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 1968.
11 H. R. REVEL, S. E. LURIA AND N. L. YOUNG, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 1974.
12 O. E. LANDMAN, *Biochim. Biophys. Acta*, 23 (1957) 558.
13 C. R. CLAUSEN AND M. NAKAMURA, *Nature*, 197 (1963) 570.
14 J. SPIZIZEN, *Federation Proc.*, 18 (1958) 957.
15 J. SPIZIZEN, *Proc. Natl. Acad. Sci. U.S.*, 44 (1959) 1072.
16 C. B. THORNE, *J. Bacteriol.*, 83 (1962) 106.
17 G. COHEN-BAZIRE AND M. JOLIT, *Ann. Inst. Pasteur*, 84 (1953) 937.
18 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
19 M. MERKENSCHLAGER, K. SCHLOSSMANN AND W. KURZ, *Biochem. Z.*, 329 (1957) 332.
20 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
21 K. WALLENFELS, J. LEHMAN AND O. P. MALHOTRA, *Biochem. Z.*, 333 (1960) 209.
22 J. JANEČEK AND H. V. RICKENBERG, *Biochim. Biophys. Acta*, 81 (1964) 108.