α-GALACTOSIDASE ACTIVITY IN CELL-FREE EXTRACTS OF ESCHERICHIA COLI*

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In vivo, the α-galactosidase (E.C. 2.3.1.22) activity of melibiose-induced cells of Escherichia coli can be easily measured by the hydrolysis of o-nitrophenyl-α-D-galactopyranoside (α-ONPG) (1,2,3). However, all previous attempts to demonstrate this activity in cell-free extracts have been unsuccessful. In this communication, we describe the conditions for extraction of α-galactosidase together with the mapping of a gene controlling its biosynthesis.

Chemicals: The substrate, α-ONPG, was prepared according to Porter et al. (1). Ethyl-1-thio-α-D-galactopyranoside was a gift of Dr. D. S. Hogness. Melibiose (6-O-α-D-galactopyranosyl-D-glucose) and glutathione (reduced) monosodium were purchased from Mann Research Labs., Inc., dithiothreitol (DTT) and dihydrostreptomycin from CalBiochem. Calcium phosphate gel, pH 6.8 (33 mg dry weight per ml) was prepared according to Keilin and Hartree (4).

Bacterial growth and enzyme extraction: A mutant (W 4680) of E. coli K12, unable to synthesize β-D-galactosidase (E.C. 3.2.1.23) due to a deletion in the Lac region (5) was employed for the preparation of

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extracts. Cells were grown in a medium containing: KH_2PO_4, 50 m$\underline{M}$; K_2HPO_4, 15 m$\underline{M}$; $MgSO_4$, 0.41 m$\underline{M}$; $(NH_4)_2 SO_4$, 7.6 m$\underline{M}$; melibiose, 8.3 m$\underline{M}$; 0.03% Difco Casamino Acids. The cultures (one liter) were incubated at 37° on a rotatory shaker until a density of 3×10^9 cells/ml was reached. Cells were harvested by centrifugation at 12,000 x g for 10 minutes. The centrifugation and all subsequent operations were conducted between 0° and 4°. After one washing with distilled water, the bacteria were resuspended in tris-Cl buffer, 50 m$\underline{M}$, pH 7.5 (measured at 37°) to a density of about 2×10^{11} cells/ml. The cells were lysed by one passage through a French press (6) at 10,000 p.s.i. The resulting extract was freed of cell debris by sedimenting at 27,000 x g for 15 minutes.

Enzyme assay: The assay mixture, in a total volume of one ml, contained tris-Cl buffer, 50 m$\underline{M}$, pH 7.5 (37°); freshly dissolved manganese dichloride, 5 m$\underline{M}$; DTT, 5 m$\underline{M}$; α-ONPG, 2 m$\underline{M}$. Samples of 0.1 ml containing the enzyme were added at zero time. After incubating at 37° until the yellow color of o-nitrophenol became clearly visible, the reaction was stopped by the addition of 1.5 ml of 0.2 $\underline{M}$ sodium carbonate containing 10 m$\underline{M}$ EDTA. The latter chelates manganese ions which otherwise interfere with the assay by forming a colored reaction product with DTT under alkaline conditions. The hydrolysis proceeded linearly for at least 15 minutes at rates of about 25 mμmoles/min. The amount of o-nitrophenol liberated in the reaction was determined by the optical density at 420 mμ ($\epsilon = 4.7 \times 10^3$). One unit of α-galactosidase is the amount of enzyme which releases one mμmole of o-nitrophenol/min. under our assay conditions.

Results: Our preliminary experiments with E. coli were in agreement with previous observations (1,2,3) that no conventional method gave extracts with α-galactosidase activity. Subsequently, we found that extracts prepared in the French press (7) exhibited significant α-galactosidase activity when bacterial suspensions with relatively high

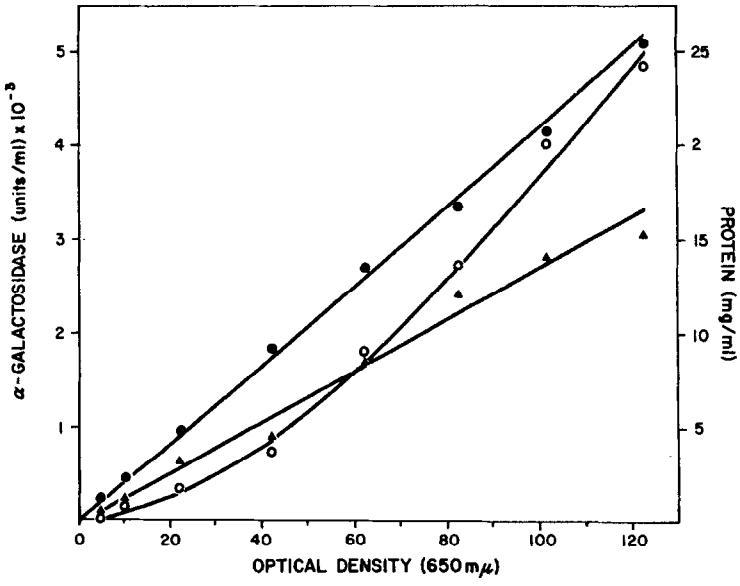

FIG. 1: RELATIONSHIP BETWEEN ACTIVITY OF α-GALACTOSIDASE IN CELL-FREE EXTRACTS AND DENSITY OF THE EXTRACTED BACTERIAL SUSPENSION.

Suspensions of melibiose-induced W 4680 previously adjusted to different optical densities (650 mμ) were extracted and centrifuged as indicated in the text. The α-galactosidase activity of the intact cell suspension (● - ●), the α-galactosidase activity (O - O), and the protein content (▲ - ▲) (6) of the cell-free extract are plotted against the optical density of the cell suspension. A unit of optical density corresponds to 1.4×10^9 cells per ml.

cell density were employed. As shown in Fig. 1, the enzymatic activity of cell-free extracts increased proportionally to the cell density in the original suspension if the suspension had more than 10^{10} cells/ml. About 95% of the activity present in the intact cells was recovered in extracts prepared with suspensions containing more than 2×10^{11} cells/ml.

In contrast to the α-galactosidase activity of the intact cell, the activity of the cell-free extracts decayed rapidly. At 0° it had a half life of three days. Additions, such as albumin, gelatin, cell extracts (both from E. coli and Aerobacter aerogenes) and sucrose did not stabilize the enzyme. Manganous ions were found necessary for maximal enzymatic activity (Table 1).

TABLE 1: MANGANESE REQUIREMENT FOR α-GALACTOSIDASE ACTIVITY

Additions	Activity (units/ml)
none	18.3
Mn^{++} (8 mM)	141
EDTA (2 mM)	0
EDTA (2 mM) plus Mn^{++} (8 mM)	133

Tubes containing equal amounts of enzyme and DTT (5 mM) in tris-Cl (50 mM), pH 7.5, were supplemented (figures indicate final concentrations) as shown. After 5 minutes equilibration at 37°, the assay was initiated by the addition of α-ONPG.

The presence of both manganous ions and reducing agents, namely glutathione and DTT (8), served to diminish losses which occurred during incubation at 37° (Table 2).

TABLE 2: STABILIZATION OF α-GALACTOSIDASE BY MANGANESE IONS AND DTT

Minutes at 37°	Activity (units/ml) No addition	DTT (5 mM)	Mn^{++} (5 mM)	DTT plus Mn^{++} (5 mM each)
0	65	62	65	63
15	40	41	32	62
30	32	35	27	57
60	22	26	20	50

Each of four tubes containing equal amounts of cell-free extract were made up to 1.5 ml with tris-Cl (50 mM), pH 7.5, and supplements as indicated. After placing the tubes at 37°, 0.25 ml samples were withdrawn at intervals from each tube and added to 0.75 ml of assay mixture containing the necessary amounts of buffer, DTT, Mn^{++} and α-ONPG to obtain the concentrations required in our standard assays of α-galactosidase.

Another difference between the α-galactosidase in cell-free extracts and that of intact cells was that the latter lost activity when assayed in dilute solutions (Table 3).

TABLE 3: LOSS OF ENZYMATIC ACTIVITY UPON DILUTION

	Intact Cells			Extracts		
	Units/ml			Units/ml		
Dilution	Expected	Observed	Loss (%)	Expected	Observed	Loss (%)
1 : 1	102	102	0	135	135	0
1 : 2	51	52	- 2	67.5	45	24
1 : 4	25.5	26	- 2	34	14	59
1 : 10	10.2	10	2	13.5	3	78
1 : 20	5.1	5	2	7	0.4	94

The samples containing α-galactosidase were diluted into the assay mixturn described above to obtain the indicated ratio. The expected values were calculated from that obtained in the 1 : 1 dilution.

Heavy losses in enzymatic activity occurred when purification by conventional methods was attempted. Fractionation by column chromatography with Sephadex (G25 and G75) and with DEAE-Sephadex resulted in complete loss of activity. Ammonium sulfate fractionation caused a 70% loss without gain in specific activity. Removal of nucleic acids by ultracentrifugation and precipitation with streptomycin followed by calcium phosphate gel adsorption did not cause significant loss of activity (Table 4). Surprisingly, these extracts could be lyophilized and stored at 4^{o} for periods up to two months without noticeable loss of activity.

The α-galactosidase of the cell-free extracts had maximal activity at pH 7.5. The kinetics of hydrolysis of α-ONPG by the extracts followed a Michaelis-Menten curve with a K_m of 3×10^{-4} M.

The α-galactosidase of E. coli differs from that of A. aerogenes in several respects. The latter (2) can be extracted by conventional methods, it is stable in cell-free extracts, and has been purified about 200 fold (D. S. Hogness, personal communication). In crude extracts, the enzyme from A. aerogenes does not require manganous ions for maximal

TABLE 4: ENZYME PREPARATION

Preparation	Units/ml	Units/O.D.$_{280}$	Total Activity
Crude extract	2780	30	13,900
Supernatant of ultra-centrifugation (114,000 x g; 90 min.)	2880	34	13,500
Supernatant after dihydro-streptomycin (23 mg/ml)	2420	44	11,900
Supernatant after calcium phosphate gel (3 mg/ml)	2140	86	10,900

activity. Ethyl-1-thio-α-D-galactopyranoside, which is an inducer of α-galactosidase in A. aerogenes (2), does not induce E. coli.

Three mutants unable to utilize melibiose (mel$^-$) were isolated after treating Hfr$_1$ cells (W 1895) with N-methyl-N'-nitro-N-nitroso-guanidine and selecting by the penicillin method (9, 10). No detectable α-galactosidase activity or TMG permease II (11) was found in these mutants after gratuitous induction with melibiose in the presence of 0.1% lactate. Genetic analysis of the mutants by recombination frequency with different markers and by determination of chromosome transfer in interrupted mating experiments indicated that the mel locus is located between xylose and arabinose on the E. coli chromosome close to the methionine A marker. Detailed genetic data will be presented elsewhere. The concomitant loss of α-galactosidase and TMG permease II in the mel$^-$ mutants together with the fact that the two functions are induced simultaneously by several α-galactosides suggests that they are part of an operon.

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