CHARACTERIZATION OF ALPHA-GALACTOSIDASE FROM *CORYNEBACTERIUM MURISEPTICUM* AND MECHANISM OF ITS INDUCTION

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In *C. murisepticum*, alpha-galactosidase (EC 3.2.1.22) was induced during growth in the presence of melibiose or raffinose, and to a much lesser extent in the presence of galactose, as a single carbon source. Evidence was obtained to show that the enzymes induced by any of the sugars were identical with one another. The principal inducer of *C. murisepticum* alpha-galactosidase is melibiose. Raffinose elicits induction only indirectly by prior extracellular hydrolysis to melibiose. The enzyme was purified to homogeneity from melibiose-grown cells by column chromatographic fractionation of cell-free extract on Sephadex G-200, followed by that on QAE-Sephadex A-25, and finally by ultracentrifugation in a 5–20% sucrose density gradient. The purified *C. murisepticum* alpha-galactosidase is a homotetramer of 320 kDa molecular mass, with a K_m for melibiose, 2 mM; for *p*-nitrophenyl-alpha-D-galactopyranoside (PNPG), 0.17 mM; and pH optimum of 7.5. Under optimal conditions of induction, 2% of total protein synthesized by *C. murisepticum* cells was alpha-galactosidase (as determined by selective immunoprecipitation of radio-labelled enzyme protein).

The enzyme alpha-galactosidase (EC 3.2.1.22) hydrolyzes terminal nonreducing alpha-D-galactose residues in alpha-galactosides and galactose containing oligosaccharides, e.g., melibiose, raffinose, stachyose, and verbascose, which are widely distributed in nature (3).

Biochemical and genetic aspects of alpha-galactosidase induction, which may shed light on certain intriguing modes of melibiose and raffinose utilization, have so far been studied in only a few microorganisms. Most of our knowledge on

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melibiose and raffinose assimilation has been obtained from studies on *E. coli* (1, 2, 4, 14).

This paper describes some aspects of the mode of induction of alpha-galactosidase and its characterization in *C. murisepticum*.

MATERIALS AND METHODS

Chemicals. Raffinose, melibiose, galactose, casamino acids, *p*-nitrophenyl-alpha-D-galactopyranoside (PNPG), O-dianisidine, glucose oxidase, peroxidase, bovine serum albumin (BSA), nonidet P-40 (NP-40), tris(hydroxymethyl)-amino methane (TRIS), ethylene diamine tetraacetic acid (EDTA), 2,5-diphenyloxazole (PPO), 1,4-bis[2-(5-phenyloxazolyl)] benzene: 2,2-*p*-phenylenebis(5-phenyl oxazole) (POPOP), Freund's complete adjuvant, Coomassie brilliant blue, acrylamide, *N,N'*-methylene bis-acrylamide, *N,N,N',N'*-tetramethyl ethylene diamine (TEMED), sodium azide, and molecular mass standards for proteins were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Tryptone, yeast extract, and glucose were obtained from Difco Laboratories (Detroit, Michigan, U.S.A.). Sephadex G-200, QAE-Sephadex A-25, Sephacryl S-400 were purchased from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade.

³⁵S-methionine (sp. act. >800 Ci/mmol) from Amersham (England) was used for radiotracer experiments.

Bacterial strains. *Corynebacterium murisepticum* ATCC 21374 and *Streptococcus faecalis* ATCC 8043 were obtained from American Type Culture Collection (U.S.A.).

Klebsiella pneumoniae M5a1 was obtained from Dr. Christina Kennedy, Sussex, U.K.

Escherichia coli D 1073 and D 1148 were obtained from Dr. R. Schmitt, West Germany.

Streptococcus cremoris NCIM 2179 and *Leuconostoc mesenteroides* NCIM 2073 were obtained from National Chemical Laboratory (Pune, India).

Staphylococcus aureus Cowon I 8550 was obtained from Radiation Medicine Centre (Bombay, India).

Media and culture conditions. For studying induction of alpha-galactosidase, *C. murisepticum* cells were grown in M9 minimal medium (Na₂HPO₄ 6 g, KH₂PO₄ 3 g, NH₄Cl 1 g, CaCl₂·2H₂O 0.02 g, MgSO₄·7H₂O 0.2 g, distilled water 1 l, pH 7.0) supplemented with one of the three sugars—melibiose, raffinose, or galactose (final concentration of 0.4%)—as the sole carbon source. The cultures were incubated with aeration at 30°C on an orbital shaker set at 200 rpm. The cell growth during the period of induction was followed by measuring the absorbance at 600 nm.

For immunological studies, *C. murisepticum*, *K. pneumoniae*, *E. coli*, *S. cremoris*, *S. faecalis*, and *L. mesenteroides* were grown in Luria broth (Tryptone 10 g, yeast extract 5 g, NaCl 5 g, distilled water 1 l, pH 7.0) supplemented with 0.4% melibiose at 30°C for 16 h with aeration in an environmental orbital shaker set at

200 rpm.

For studying the rate of total protein synthesis and immunoprecipitation of radioactively labelled alpha-galactosidase, *C. murisepticum* cells were grown in M9 minimal medium supplemented with raffinose or melibiose (0.4% final conc.) and ^{35}S -methionine (100 $\mu\text{Ci}/\text{ml}$, final conc.).

Enzyme assay. Induced enzyme levels were assayed with PNPG as the substrate by toluenizing the cells (0.5 ml).

Alpha-galactosidase activity in cell extracts was assayed by measuring hydrolysis of 1 mM PNPG as the substrate in 50 mM sodium phosphate buffer pH 7.5 (0.5 ml) at 37°C. The reaction was terminated by addition of 1 M Na_2CO_3 (0.5 ml), and the amount of *p*-nitrophenol released was determined from the absorbance at 420 nm. One unit of alpha-galactosidase is the amount of enzyme required to liberate one nmol of *p*-nitrophenol in one min at 37°C.

Melibiose (0.2 mM–4 mM) hydrolysis by alpha-galactosidase was measured indirectly by determining the amount of glucose released in the reaction using the coupling enzyme system of glucose-oxidase and peroxidase (8).

Protein determination. Protein concentrations were determined by the method of Lowry et al. using bovine serum albumin as standard (6).

Thin-layer chromatography (T.L.C.). T.L.C. was performed for detection of sugars. After 3 h growth in the presence of raffinose, cells were centrifuged at $10,000 \times g$ for 10 min and the supernatant was lyophilized, dissolved in 50% ethanol and chromatographed on 0.5 mm thin-layer silica gel plate using chloroform–acetic acid–water (60:70:10 by vol) as the solvent system. The plate was sprayed with 1% *p*-aminophenol and 4% phosphoric acid in distilled alcohol to locate the position of sugar bands after baking at 100°C for 30 min (16).

Purification of alpha-galactosidase. All the procedures were carried out at 4°C unless otherwise stated. Cells of *C. murisepticum* (15.57 g wet weight) were harvested after 24 h growth at 30°C in M9 minimal medium supplemented with melibiose and casamino acids, at $3,000 \times g$ for 10 min. The cells were washed, suspended in 50 ml of 50 mM phosphate buffer pH 7.5, and disrupted by ultrasonication. Nucleic acids were precipitated with streptomycin sulfate (0.7%, final conc.). The sonicate was centrifuged at $27,000 \times g$ for 60 min. The crude enzyme was fractionally precipitated by ammonium sulfate at 35–60% saturation and the precipitate was collected by centrifugation at $12,000 \times g$ for 15 min. The pellet containing the enzyme was dissolved in 7 ml of the 50 mM phosphate buffer pH 7.5 and loaded onto a Sephadex G-200 column (2.5 cm $\times$ 81.5 cm) equilibrated with 50 mM phosphate buffer pH 7.5 and eluted with the same buffer. Fractions of 5 ml were collected and analyzed at 280 nm for protein. The enzymatically active fractions were pooled and loaded onto a QAE-Sephadex A-25 column (1.5 cm $\times$ 45 cm). The bound protein was eluted by a linear gradient of 0–0.5 M NaCl, and fractions of 5 ml were collected and analyzed for absorption at 280 nm and for enzyme activity. Enzymatically active fractions were pooled, dialyzed against 50 mM phosphate buffer pH 7.5, and loaded onto a second QAE-Sephadex A-25

column (1.5 cm $\times$ 11 cm) for step-elution with 0.5 M NaCl. Fractions of 1 ml were collected, assayed for enzyme activity and loaded on the top of a 12 ml-linear 5–20% continuous sucrose density gradient, in 50 mM phosphate buffer pH 7.5 containing 0.5 M NaCl, for the final purification step. Gradients were centrifuged at $155,000 \times g$ for 16 h at 4°C using SW40 rotor. Following the centrifugation, the gradients were fractionated from the bottom into 24 equal fractions (23 drops/fraction). An aliquot of each fraction was assayed for enzyme activity. Purity of protein was ascertained by SDS-polyacrylamide gel electrophoresis. Fractions containing pure enzyme protein were pooled, dialyzed against 50% glycerol in 50 mM phosphate buffer pH 7.5, and stored at -20°C .

SDS-polyacrylamide gel electrophoresis. This was performed as described by Weber et al. (17).

Gel filtration. Molecular mass of purified alpha-galactosidase was determined by gel filtration using Sephacryl S-400 column (1.5 cm $\times$ 104.5 cm).

Amino acid composition. Phenyl-thiocarbamyl (PTC) derivatization of amino acids and their separation was carried out according to the procedure described by Stone and Williams (15).

Preparation of immunized serum against C. murisepticum alpha-galactosidase. Antiserum for alpha-galactosidase was obtained in a rabbit by subcutaneous injection of purified enzyme protein (300 μg) emulsified with Freund's complete adjuvant. Two booster doses of 100 μg each were given at two-week intervals. Ten days after the second booster dose, the animal was bled and antiserum obtained was stored at -20°C .

Ouchterlony immunodiffusion plate technique. This was performed as described by Ouchterlony (12).

Neutralization of enzyme activity with antibodies. Cell extracts of alpha-galactosidase preparations from *C. murisepticum*, *K. pneumoniae*, *E. coli*, *S. cremoris*, *S. faecalis*, and *L. mesenteroides* were treated separately, either with normal serum from a nonimmunized rabbit or the antiserum against purified alpha-galactosidase from *C. murisepticum*, for 4 h on ice. Antigen-antibody complexes were removed by centrifugation at $10,000 \times g$ for 5 min, and the supernatant was assayed for enzyme activity.

Preparation of immunoglobulin fraction (IgG) from immunized serum. Immunoglobulin fraction (IgG) was prepared from the immunized serum by DEAE-cellulose column chromatography (7).

Immunoprecipitation of radioactively labelled alpha-galactosidase. Formaldehyde-fixed protein A-bearing *S. aureus* cells were prepared, and immunoprecipitation of the labelled enzyme was carried out essentially according to the method described by Kessler (5).

RESULTS AND DISCUSSION

The modes of induction of alpha-galactosidases exhibit significant variation

Fig. 1. Time-course of alpha-galactosidase induction.

Corynebacterium murisepticum cells were grown in M9 minimal medium with melibiose (Δ), raffinose ($\circ$) or galactose ($\bullet$) as the sole carbon source, at 30°C with aeration. Induced enzyme levels were assayed with PNPG as the substrate in toluenized cell suspension (0.5 ml). Amount of *p*-nitrophenol released and cell density were measured at 420 nm and 600 nm, respectively.

among microorganisms. Some are inducible either by melibiose, raffinose, or galactose or with all three, with or without catabolite repression. *Corynebacterium murisepticum* could grow in M9 minimal medium with melibiose, raffinose, or galactose as the sole carbon source. As can be seen in Fig. 1, the rate of alpha-galactosidase induction, as studied by toluenizing the cells, was linear for more than 2 h with any one of the three sugars, although maximum induction was achieved with melibiose as the carbon source.

Alpha-galactosidases induced in raffinose- and melibiose-grown *C. murisepticum* cells were partially purified and characterized for their biochemical parameters. Compelling evidence was obtained to suggest that in *C. murisepticum* alpha-galactosidase induced in melibiose-grown cells is the same as that induced in raffinose-grown cells, for instance the K_m for the substrates, PNPG (0.17 mM) and melibiose (2 mM), pH optimum 7.5, and electrophoretic mobility detected by activity staining.

On an Ouchterlony immunodiffusion plate, alpha-galactosidases from melibiose- and raffinose-grown cells gave a single precipitin line with antibodies to alpha-galactosidase purified from melibiose-grown *C. murisepticum* cells (see below) (Fig. 2). The precipitin lines for alpha-galactosidases from the two cell extracts merged completely with each other, without any spur confirming a complete immunological identity between the two—in an analogy to their resemblance in enzyme kinetic properties.

Fig. 2. Examination of immunological cross-reactivity between alpha-galactosidases from melibiose-grown and raffinose-grown *C. murisepticum* cells.

Ten microliters of antiserum against purified alpha-galactosidase from melibiose-grown *C. murisepticum* cells were placed in the center well of 1% agarose gel. Ten microliter extracts of cells grown on melibiose (A1, A2) and raffinose (B1, B2) were placed in the outer wells and incubated for 16 h at 30°C. The gel was stained with Coomassie brilliant blue.

The mechanisms by which raffinose- and melibiose-grown cells synthesize one and the same enzyme were explored. Thin-layer chromatography of cell-free supernatant of raffinose-grown cells revealed the presence of melibiose and fructose (Fig. 3). This would suggest that raffinose is not hydrolyzed extracellularly by alpha-galactosidase (for which the products should have been sucrose and galactose) but by an invertase acting at beta-1,2 linkage (results not shown). This would imply that in *C. murisepticum* the induction of alpha-galactosidase by raffinose could be indirect via melibiose generated by extracellular hydrolysis of raffinose. Galactose, a constituent of melibiose, induces the same alpha-galactosidase as that induced by melibiose or raffinose (results not shown), though the efficiency of induction was low (see Fig. 1). Taken together these results suggest that in *C. murisepticum*, alpha-galactosidase is specified by only one operon located in a bacterial chromosome. Since in *C. murisepticum*, no plasmid was detectable (as ascertained by SDS-lysis procedure followed by agarose gel electrophoresis), the existence of a plasmid-coded substrate-utilizing ability could be ruled out.

Using ^{35}S -methionine as the radioactive precursor and antibodies to *C. murisepticum* alpha-galactosidase, the efficiency of induction of alpha-galactosidase protein by melibiose and by raffinose was determined. In analogy with Fig. 1, melibiose proves to be an efficient inducer of the enzyme (Fig. 4). Under optimal conditions, 2% of the total protein synthesized was alpha-galactosidase, which is indeed a highly efficient rate of induction.

Alpha-galactosidase from melibiose-grown *C. murisepticum* cells was purified from the cytoplasmic fraction using ammonium sulfate precipitation, followed by successive column chromatography on Sephadex G-200 and QAE Sephadex A-25. The final step of 5–20% sucrose density gradient ultracentrifugation yielded electrophoretically homogeneous enzyme protein (Fig. 5).

The results of the enzyme purification are summarized in Table 1.

Analysis of the molecular mass of the enzyme protein by gel filtration and

Fig. 3. Thin-layer chromatography of sugars in culture supernatant of raffinose-grown *C. murisepticum* cells.

Ten microliters of cell-free supernatant of raffinose-grown culture (B) were applied on silica gel plate. Chromatographic separation and detection of sugars was performed as described in Materials and Methods. (A) Spots for standard sugars (from bottom: raffinose, melibiose, sucrose, galactose, glucose, and fructose).

Fig. 4. Rate of incorporation of ^{35}S -methionine into total protein and alpha-galactosidase in *C. murisepticum* cells growing on melibiose or raffinose.

C. murisepticum cells were incubated in M9 minimal medium containing melibiose (Δ) or raffinose (○) (0.4% final conc.) as the sole carbon source along with ^{35}S -methionine (100 $\mu\text{Ci}/\text{ml}$, final conc.) at 30°C with aeration. At various times aliquots were removed, and radioactivity incorporated into total proteins and alpha-galactosidase was monitored as described in Materials and Methods. A: Total protein synthesis. B: Immunoprecipitation of alpha-galactosidase from raffinose-grown (○) and melibiose-grown (Δ) cells.

Fig. 5. SDS-polyacrylamide gel electrophoresis of purified alpha-galactosidase from *Corynebacterium murisepticum*.

Twenty micrograms of denatured alpha-galactosidase sample protein was loaded on 7.5% polyacrylamide gel containing 1% SDS and electrophoresed at a constant current of 8 mA per tube for 5 h at 30°C.

Table 1. Purification of alpha-galactosidase from *Corynebacterium murisepticum*.

Step	Total enzyme (units)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Cell lysate (supernatant after 27,000 × g centrifugation)	336,000	631	532	100.0	1.00
Supernatant after 35% sat. Ammonium sulfate	326,000	467	699	97.1	1.30
35–60% saturation Ammonium sulfate precipitate	263,000	ND	—	78.0	—
Sephadex G-200 column chromatography	202,000	49.5	4,090	60.0	9.68
QAE Sephadex A-25 column chromatography	108,000	3.10	34,800	32.0	65.4
5–20% sucrose density gradient ultracentrifugation	71,900	1.25	57,500	21.4	108

SDS-polyacrylamide gel electrophoresis showed *C. murisepticum* alpha-galactosidase to be a homotetrameric protein with a subunit molecular mass of 83 kDa.

It would be of interest to compare structure-functions of alpha-galactosidase of *C. murisepticum* and modes of its induction with those of *E. coli* and other microorganisms. In *E. coli*, melibiose utilization by alpha-galactosidase is linked to

the presence of *mel* operon, located at 93 min on the *E. coli* genetic map. This operon consists of at least two structural genes *melA* and *melB*, which code for the alpha-galactosidase and melibiose transport carrier protein, respectively (4), and its expression is controlled by cAMP-CRP regulatory circuit (10).

In *E. coli*, the inducible functions of *mel* operon cannot support utilization of raffinose. However a class of transmissible plasmids has been found which confers to *E. coli* the ability to grow on raffinose (11). This is achieved by acquisition of three new functions, with regard to alpha-galactosidase, raffinose permease, and invertase, necessary for metabolism of raffinose. These are all under a common control designated as *raf* operon (1). The *raf*-alpha-galactosidase of *E. coli* differs from alpha-galactosidase encoded by chromosomal *mel* operon (mel-alpha-galactosidase) in substrate affinities, cofactor requirements, stability, and amino acid composition. The molecular mass of mel-alpha-galactosidase is 200 kDa with a subunit molecular mass of 50 kDa (4), whereas that of *raf*-alpha-galactosidase is 320 kDa with a subunit molecular mass of 82 kDa (14).

Although alpha-galactosidase of *C. murisepticum* is not directly induced by raffinose, it nevertheless resembled the *E. coli* *raf*-alpha-galactosidase in terms of molecular mass, subunit composition, and enzyme kinetic parameters, such as K_m for melibiose and PNPG and pH optimum (Table 2). Similarly, like *raf*-alpha-galactosidase of *E. coli*, it did not show any cofactor requirement for enzyme assay or stability. *Bacillus stearothermophilus* isoenzyme II is also reported to be similar to *E. coli* *raf*-alpha-galactosidase in pH optimum, molecular mass, and subunit structure (13).

Like *E. coli* *raf*-alpha-galactosidase—but unlike *E. coli* mel-alpha-galactosidase—*C. murisepticum* alpha-galactosidase could be assayed after toluenization of bacterial cells, indicating that cellular localization of the enzyme (and/or, perhaps,

Table 2. Biochemical properties of *C. murisepticum* alpha-galactosidase and two *E. coli* alpha-galactosidases.

Criteria	<i>C. murisepticum</i> alpha-galactosidase	<i>E. coli</i>	
		Alpha-galactosidase specified by plasmid-located <i>raf</i> operon (14)	Alpha-galactosidase specified by chromosomally located <i>mel</i> operon (14)
Molecular mass of the active enzyme	320 kDa	320 kDa	200 kDa
Molecular mass of the subunit	83 kDa	82 kDa	50 kDa
Cofactor requirement	None	None	NAD
Ion requirement	None	None	Mn ²⁺
pH optimum	7.5	7.2	7.5
K_m for PNPG	0.17 mM	0.14 mM	3.0 mM
K_m for melibiose	2 mM	3.2 mM	10 mM
Toluene sensitivity	No	No	Yes

Table 3. Amino acid composition of purified alpha-galactosidases from *C. murisepticum* and *E. coli*.

Amino acid	mol % in each subunit	
	<i>C. murisepticum</i> alpha-galactosidase	<i>E. coli</i> raf-alpha-galactosidase (14)
Aspartic acid	2.3	9.04
Glutamic acid	9.87	12.41
Serine	18.50	4.22
Glycine	19.72	3.90
Histidine	2.65	4.99
Arginine	1.46	12.17
Threonine	5.10	3.64
Alanine	9.44	4.79
Proline	7.89	4.69
Tyrosine	1.50	4.89
Valine	1.06	4.69
Methionine	3.47	3.29
Isoleucine	3.7	4.77
Leucine	2.89	10.00
Phenylalanine	1.34	5.57
Lysine	9.09	2.92
Cysteine	N.D.	N.D.
Tryptophan	N.D.	5.45

Table 4. Immunoneutralization of activities of alpha-galactosidases from various microorganisms by antiserum to *C. murisepticum* alpha-galactosidase.

Lysate	Alpha-galactosidase activity (units)	
	Control	Supernatant after immunoprecipitation
<i>C. murisepticum</i>	19.0	2.50
<i>K. pneumoniae</i>	8.88	2.44
<i>E. coli</i> D1073	6.50	6.50
<i>E. coli</i> D1148	4.80	4.80
<i>S. faecalis</i>	9.83	9.83
<i>S. cremoris</i>	9.83	9.83
<i>L. mesenteroides</i>	4.00	4.00

Twenty microliters of enzyme preparations from *C. murisepticum*, *K. pneumoniae*, *E. coli*, *S. faecalis*, *S. cremoris*, and *L. mesenteroides* were incubated on ice with 20 μ l of antiserum to *C. murisepticum* alpha-galactosidase. After 4 h, the samples were centrifuged at $10,000 \times g$ for 5 min., and enzyme activity in the supernatant was assayed.

its resistance to the inhibitory effect of toluene) is similar to that of *E. coli* raf-alpha-galactosidase.

Notwithstanding some striking similarities between raf-alpha-galactosidase and *C. murisepticum* alpha-galactosidase, these two enzymes do not exhibit any immunological relationship. The amino acid composition of the two enzymes also

Fig. 6. Immunological cross-reactivity of alpha-galactosidase from melibiose-grown *C. murisepticum* cells with the enzyme from *K. pneumoniae* cells.

Ten microliters of antiserum against purified alpha-galactosidase from melibiose-grown *C. murisepticum* cells were placed in the center well of 1% agarose gel. Ten microliters of 10 times concentrated extract of *K. pneumoniae* cells (A) and 10 μ l extract of *C. murisepticum* cells (B1, B2) were placed in outer wells. The plate was incubated for 16 h at 30°C and the gel was then stained with Coomassie brilliant blue.

differs widely (Table 3). The *C. murisepticum* enzyme revealed a relatively high percentage of glycine and serine residues. *E. coli* raf-alpha-galactosidase has a high percentage of glutamic acid and leucine (14), whereas *E. coli* mel-alpha-galactosidase contains high amounts of aspartic acid, glutamic acid, and alanine (9).

Immunological identities of alpha-galactosidases from *E. coli* D1148 (serotype A₁), *E. coli* D1073 (serotype A₂), *K. pneumoniae* (which exhibited no immunological relation to *E. coli* enzyme), *Streptococcus*, and *Leuconostoc*, with the *C. murisepticum* alpha-galactosidase, revealed that antibodies to *C. murisepticum* alpha-galactosidase failed to neutralize alpha-galactosidase activity from *E. coli*, *Streptococcus*, and *Leuconostoc*. However, the antibodies neutralized alpha-galactosidase activity from *K. pneumoniae* (Table 4), and on an Ouchterlony immunodiffusion plate *C. murisepticum* alpha-galactosidase exhibited partial immunological identity with *K. pneumoniae* alpha-galactosidase (Fig. 6).

Finally, it may be worth pointing out that the alpha-galactosidase in *C. murisepticum* is not coordinately controlled with invertase, which is found to be constitutive (results not shown). This is in contrast with *E. coli* harboring plasmid-borne raf operon, in which case both alpha-galactosidase and invertase are coordinately controlled. *E. coli*, devoid of the plasmid-borne operon, lacks either inducible or constitutive invertase.

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