

Lactose-Hydrolyzing Enzymes of *Lactobacillus* Species¹

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β -Galactosidase (β -gal, EC 3.2.1.23) and β -D-phosphogalactoside galactohydrolyase (β -Pgal) activities were observed in all of 13 *Lactobacillus* species studied except *L. casei* and *L. buchneri*. Only the latter enzyme was detected in nine strains of *L. casei*. The β -gal from *L. thermophilus* and the β -Pgal from *L. casei* were purified and characterized. In comparison with β -gal, the β -Pgal was slightly less active (V_{max} values were 28.9 and 50.0 μ moles per mg per min, respectively), but the substrate affinities were similar (K_m values were 1.69×10^{-3} M and 1.59×10^{-3} M, respectively). Although the two enzymes had similar amino acid compositions, the molecular weight of β -gal was 5.4×10^5 and that of β -Pgal was 1.3×10^5 . The β -gal from *L. thermophilus* and the β -Pgal from *L. casei* had optimal temperature and pH activity values of 55 C at pH 6.2 and 37 C at pH 5.0, respectively. The complete absence of β -gal from a homofermentative *Lactobacillus* species of industrial importance is further evidence of the heterogeneity of this genus.

Group N streptococci, with the exception of one strain (2, 17-22), utilize lactose by the phosphoenolpyruvate (PEP) phosphotransferase system. Kundig et al. (12) first described this enzyme system in *Escherichia coli*, and it also has been shown (4, 7-9, 11, 13, 14) to be involved in lactose utilization by *Staphylococcus aureus*. Recently, Romano et al. (26) examined the distribution of this pathway among bacteria for glucose metabolism, pointing out that it allows for conservation of energy among organisms carrying out anaerobic glycolysis.

A key enzyme in the PEP lactose phosphotransferase system is β -D-phosphogalactoside galactohydrolyase (β -Pgal), as first named by Laue and MacDonald (13, 14). This enzyme hydrolyzes lactose phosphate, the phosphorylation of which is mediated by PEP, to liberate glucose and galactose-6-phosphate. Because no studies of this enzyme have been reported for *Lactobacillus* species, the present investigation was undertaken. Results of a survey of lactobacilli for β -galactosidase (β -gal) and β -Pgal are presented, as well as the comparative properties of partially purified preparations of these enzymes from *L. thermophilus* and *L. casei*,

respectively.

MATERIALS AND METHODS

Organisms. The *Lactobacillus* cultures used were obtained from the American Type Culture Collection, from V. Bottazzi in the Dairy Research Department of the Catholic University in Milan, Italy, from H. C. de Klerk, Department of Microbiology, University of Pretoria, Pretoria, South Africa, and from the culture collection maintained in the Department of Microbiology, Oregon State University.

Media and growth conditions. Cultures were maintained by weekly transfers in sterile nonfat milk and incubated at their optimal temperatures for 12 to 14 hr; between transfers, they were held at 2 C. Cultures for enzyme studies were transferred (1.0%, v/v) from milk into lactic broth (5) containing lactose as the only energy source, and were incubated for 10 to 12 hr. The *L. thermophilus* Farr strain was incubated at 45 C (it grew well at 55 C but not at 60 C); the *L. casei* strains were incubated at 30 C.

Harvesting cells. Cells grown in the lactic broth were harvested by use of an RC-2 Sorvall refrigerated centrifuge (1 C) at $3,000 \times g$. The cells were washed three times with 0.1 M sodium phosphate buffer at pH 7.0.

Extraction and purification of β -gal from *L. thermophilus* Farr. Cells harvested from 7 liters of broth were broken by sonic treatment (Raytheon, 10-kc) for 30 min and were centrifuged for 20 min at $15,000 \times g$; the sediment was discarded. A 3-ml amount of 50% streptomycin sulfate (Sigma Chemical Co.) was then added to 50 ml of supernatant

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fluid which was stirred for 30 min at 5 C. After centrifugation at $15,000 \times g$ for 30 min, the enzyme extract was 65% saturated with reagent-grade (Mallinckrodt Chemical Works) ammonium sulfate. The precipitated protein was dissolved in 5 to 10 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.7) containing 5×10^{-4} M $MgSO_4$ and 0.002 M mercaptoethanol. The preparation then was dialyzed against three 2-liter changes of Tris buffer for successive 10-hr periods.

The dialyzed enzyme was placed in 150 ml of diethylaminoethyl (DEAE) A25 Sephadex (Pharmacia Chemical Co.) slurry and washed with 500-ml volumes of sodium chloride at 0, 1.0, 1.5, 2.5, and 4.0% salt concentrations. The most active fractions were combined, and the enzyme was precipitated by the addition of 40% ammonium sulfate (w/v). The protein was recovered by centrifugation ($15,000 \times g$ for 25 min) and was resuspended in 1.0 ml of Tris buffer. The solution was dialyzed as before and then chromatographed on a column (2.7 by 46 cm) of DEAE A25 Sephadex with linear gradient; 0.5 liter at 1.0% NaCl and 0.5 liter at 3.0% NaCl were used in the two mixing vessels. The active fractions (10.0 ml) were combined, brought to 65% ammonium sulfate saturation, and centrifuged. The resuspended protein (5 to 10 ml in Tris buffer) was dialyzed for 36 hr, layered on a 5 to 20% sucrose gradient, and centrifuged at $42,000 \times g$ for 25 hr. Fractions (1 ml) were collected and assayed for activity. Protein was measured by the method of Lowry et al. (16).

Extraction and purification of β -Pgal from *L. casei*. The procedures followed in purification of this enzyme were the same as for β -gal except that Sephadex G-200 chromatography was used in place of sucrose gradient centrifugation after the DEAE Sephadex treatment. Also, the G-200 column was buffered to pH 5.0, the optimal pH for the enzyme.

Enzyme assays. The assay used for β -gal was described earlier (2). β -Pgal assays were performed at pH 5.0 as indicated by McKay et al. (20). The *o*-nitrophenyl- β -D-galactopyranoside (ONPG) and *o*-nitrophenyl- β -D-galactopyranoside-6-phosphate (ONPG-6-P) were obtained from Sigma Chemical Co., St. Louis, Mo., and NK Laboratories, Jersey City, N.J., respectively. A unit of enzyme was defined as the number of micromoles of *o*-nitrophenol (ONP) liberated per milligram of enzyme protein per minute under the assay conditions used.

Enzyme properties. An aluminum temperature gradient block with a range from 25 to 65 C was used to determine the optimal temperature; ONP release was measured after 1 min of incubation. The influence of pH on enzyme activity was determined by adjusting 0.1 M NaH_2PO_4 to pH values from 4.0 to 8.0 by addition of 0.1 M Na_2HPO_4 and measuring enzyme activity at different pH values at 37 C.

To determine stability to heat, purified enzyme in the liquid state was held at different temperatures and sampled at intervals for activity determination.

The effect of ethylenediaminetetraacetic acid (EDTA) was determined by dialyzing enzyme preparations for 12 hr at 5 C against 1 liter of EDTA

ranging in concentration from 0.005 to 0.5 mg/ml.

An estimation of the molecular weight was made by use of the procedure of Andrews (1) as confirmed by Dasgupta et al. (3).

To determine amino acid composition, purified enzyme was hydrolyzed for 24 hr at 110 C in concentrated hydrochloric acid. Samples were analyzed on a Beckman Spinco model 170B amino acid analyzer.

A Gilford model 2000 multiple-channel recording spectrophotometer was used to follow the kinetics of hydrolysis of ONPG and ONPG-6-P. A Hakke temperature control unit was attached to the jacket of the cuvette chamber to maintain the temperature at 37 C. A wavelength of 420 nm was used. When lactose

TABLE 1. Specific activity of β -galactosidase (β -gal) and β -D-phosphogalactoside galactohydrolase (β -Pgal) in crude cell-free extracts of *Lactobacillus* species

Organism	Origin ^a	Specific activity ^b	
		β -gal	β -Pgal
<i>L. acidophilus</i> Farr ^c	OSU	4,999	136
<i>L. arabinosus</i> OSU	OSU	1,640	185
<i>L. brevis</i> XI	NCDO 473	300	50
<i>L. buchneri</i> G1	OSU	0	0
<i>L. buchneri</i> BC1	NCDO 110	0	0
<i>L. bulgaricus</i> 12278	ATCC	2,363	158
<i>L. casei</i> 9595	ATCC	0	412
<i>L. casei</i> 316	UP	0	200
<i>L. casei</i> 356	UP	0	153
<i>L. casei</i> C17	UP	0	419
<i>L. casei</i> C5	NCDO 151	0	348
<i>L. casei</i> C2	UP	0	575
<i>L. casei</i> OSU	OSU	0	432
<i>L. casei</i> 300	UP	0	420
<i>L. cellobiosus</i> G1	NCDO 927	1,360	225
<i>L. helveticus</i> 8019	ATCC	3,140	150
<i>L. helveticus</i> OSU	OSU	744	114
<i>L. helveticus</i> M699	CU	473	117
<i>L. helveticus</i> MAU95	CU	2,230	104
<i>L. helveticus</i> i 166	CU	770	123
<i>L. lactis</i> 39A	OSU	3,948	102
<i>L. lactis</i> L5b	CU	2,481	150
<i>L. lactis</i> Z18	CU	1,592	115
<i>L. lactis</i> L86	CU	6,800	134
<i>L. leichmannii</i> 7830	ATCC	4,539	235
<i>L. plantarum</i> OSU	OSU	1,330	260
<i>L. salivarius</i> OSU	OSU	387	40
<i>L. thermophilus</i> Farr	OSU	7,014	135

^a OSU, Oregon State University, Department of Microbiology culture collection; NCDO, National Collection of Dairy Organisms, Reading, England; ATCC, American Type Culture Collection; UP, University of Pretoria, South Africa, courtesy of H. C. de Klerk, Department of Microbiology; CU, Catholic University, Milan, Italy, courtesy of V. Bottazzi, Dairy Research Department.

^b Expressed as micromoles of *o*-nitrophenol liberated from *o*-nitrophenyl- β -D-galactopyranoside or *o*-nitrophenyl- β -D-galactopyranoside-6-phosphate per milligram of enzyme protein per minute under the assay conditions used. These preliminary screening data were obtained at pH 7.0.

^c The report of Miller et al. (23) indicated that this organism may not be *L. acidophilus*.

was used as the substrate, the rate of glucose liberated was measured by use of Glucostat (Worthington Biochemical Corp.). The Michaelis constants were determined from plots of the data made according to Lineweaver and Burk (15).

RESULTS

Enzyme distribution studies. Results of a survey of *Lactobacillus* species for β -gal and β -Pgal appear in Table 1. There were negligible phosphatase activities in the cell-free extracts with *p*-nitrophenylphosphate as substrate under the conditions used for the β -gal and β -Pgal assays. Thus, the β -Pgal activities recorded in Table 1 appear independent of phosphatase which might liberate ONPG for subsequent hydrolysis by β -gal. From Table 1, it is clear that most strains contain β -Pgal, but not all strains, notably *L. casei* and *L. buchneri* (a nonfermenter of lactose), have β -gal activity.

Enzyme purification. Data from a typical enzyme purification experiment for β -Pgal from *L. casei* ATCC 9595 appear in Table 2; the same data for purification of β -gal from *L. thermophilus* Farr appear in Table 3. The greatest purification (50-fold) was realized with

the β -gal of the *L. thermophilus* strain (Table 3).

Kinetic data. Figure 1 shows a reciprocal plot of the enzyme velocity data at different substrate concentrations; typical first-order kinetics were observed, and the V_{max} and K_m values for both enzymes are recorded in Table 4. The two enzymes had similar K_m values or affinity for substrate. Neither ONPG nor lactose was a substrate for β -Pgal from either *L. thermophilus* or *L. casei*.

Enzyme properties. The influence of pH and temperature on activity of the enzymes is shown in Fig. 2 and 3, respectively. Enzyme from *L. thermophilus*, with the higher optimal growth temperature, revealed an expected higher temperature requirement for maximal enzyme activity. Figure 4 shows the effects of storage and heating on the stability of the enzymes; the β -Pgal from *L. casei* was less stable to heat than the β -gal from *L. thermophilus*. EDTA had no effect on the stability of β -Pgal from *L. casei* but caused an immediate decrease in activity by 50% when added (50 μ g/ml) to β -gal of *L. thermophilus*. Storage did not increase the 50% inactivation.

TABLE 2. Typical data resulting from purification of β -D-phosphogalactoside galactohydrolase from *Lactobacillus casei* 9595

Fraction	Vol (ml)	Protein (mg)	Units ^a	Yield (%)	Specific activity (units/mg)	Purification (-fold)
Crude extract	40	264	234,000	100	890	1.0
Streptomycin sulfate supernatant	40	264	234,000	100	890	1.0
Ammonium sulfate precipitate	2.5	90	146,000	69.3	1,620	1.8
DEAE Sephadex eluate	40	11.8	72,000	30.8	6,130	6.9
Sephadex G-200 eluate	200	0.4	11,200	4.7	28,000	31.5

^a Defined as micromoles of *o*-nitrophenol liberated from *o*-nitrophenyl- β -D-galactopyranoside or *o*-nitrophenyl- β -D-galactopyranoside-6-phosphate per milligram of enzyme protein per minute under the assay conditions used.

TABLE 3. Typical data resulting from purification of β -galactosidase from *Lactobacillus thermophilus* Farr

Fraction	Vol (ml)	Protein (mg)	Units ^a	Yield (%)	Specific activity (units/mg)	Purification (-fold)
Crude extract	40	1,100	2,530,000	100	2,300	1.0
Streptomycin sulfate supernatant	40	1,100	2,530,000	100	2,300	1.0
Ammonium sulfate precipitate	4	250	1,450,000	59	5,750	2.5
First DEAE Sephadex eluate	8	54	970,000	40	18,000	8.0
Second DEAE Sephadex eluate	3	20	600,000	28	30,000	13.0
Sucrose density gradient	20	1.9	22,000	11	120,000	50.0

^a Defined as micromoles of *o*-nitrophenol liberated from *o*-nitrophenyl- β -D-galactopyranoside or *o*-nitrophenyl- β -D-galactopyranoside-6-phosphate per milligram of enzyme protein per minute under the assay conditions used.

The amino acid compositions of the enzymes from *L. thermophilus* and from *L. casei* appear in Table 5. They contain similar amino acids with similar quantitative relative orders for each amino acid; cystine was absent. Figure 5 shows the molecular-weight data; β -gal from *L. thermophilus* had a molecular weight of 5.4×10^5 , and β -Pgal from *L. casei* revealed a value of 1.3×10^5 .

DISCUSSION

Organisms of the *Lactobacillus* genus are widely distributed in nature, and both homofermentative and heterofermentative species can be isolated from plants (25). Those important in milk fermentations are infrequently isolated from green plants (25) and presumably occur in the intestinal tract, where lactose may be available; however, no recent definitive study on species present in the human intestine has been made. The species used in this

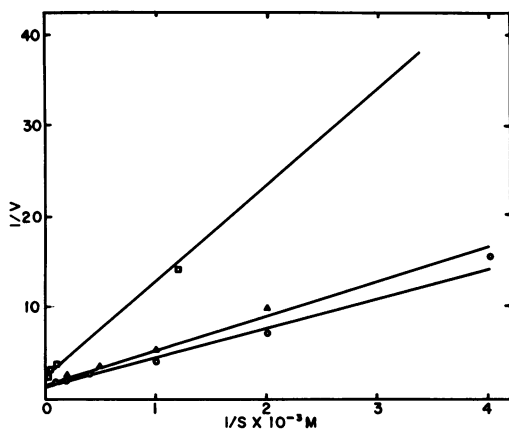


FIG. 1. Lineweaver-Burk plot of the effect of substrate concentration on velocity of enzyme reaction. The substrate for β -D-phosphogalactoside galactohydrolase from *L. casei* was *o*-nitrophenyl- β -D-galactopyranoside-6-phosphate (O); that for β -galactosidase from *L. thermophilus* was lactose (\square) or *o*-nitrophenyl- β -D-galactopyranoside (Δ).

study included the homofermenters used in starter cultures in the manufacture of fermented dairy products. Among these are *L. acidophilus*, *L. bulgaricus*, *L. helveticus*, and *L. lactis*. Other homofermentative species included those such as *L. casei* and *L. plantarum*, which are important in bringing about further changes in products such as Cheddar cheese during curing by virtue of their lower optimal growth temperatures.

Although it was not surprising to find β -gal and β -Pgal missing in *L. buchneri*, a nonfermenter of lactose, the complete absence of β -gal from eight different *L. casei* strains was surprising, particularly because all other lactose-fermenting strains revealed both β -gal and β -Pgal activities. Whether or not this is of evolutionary significance remains to be seen. It also was noteworthy that the range of specific activities for β -gal (300 to 7,014) was considerably greater than for β -Pgal (40 to 575).

One of the first studies on a lactose-hydrolyzing enzyme from a *Lactobacillus* organism was made by Monod and Cohen (24), who found that the β -gal of *L. bulgaricus* was activated by monovalent cations as well as divalent metal ions such as Mg^{2+} , Mn^{2+} , and Fe^{2+} . Recently, Iwasaki et al. (10) purified 34-fold the β -gal from *L. bifidus* (*Bifidobacterium bifidum*); the enzyme had an optimal pH of 7.0 and maximal activity at 50 C. It also was activated by Mn^{2+} and Fe^{2+} .

To our knowledge, no reports on β -Pgal in lactobacilli have been made. Molecular weights of 540,000 and 130,000 have been reported (17) for β -gal in *Streptococcus lactis*; these were the approximate molecular weights determined in the present study for β -gal and β -Pgal, respectively. Also, McFeters et al. (17) observed a molecular weight of 500,000 for the monomer form of β -gal from *S. lactis* 7962. McFeters (Ph.D. thesis, Oregon State University, Corvallis, 1967) also noted a quantitative similarity in amino acid content between *Escherichia coli* and *S. lactis* 7962 β -gal, and

TABLE 4. Kinetic data obtained for β -galactosidase (β -gal) from *Lactobacillus thermophilus* Farr and β -D-phosphogalactoside galactohydrolase (β -Pgal) from *L. casei* 9595

Substrate	β -Pgal		β -gal	
	V_{max}^a	K_m	V_{max}^a	K_m
<i>o</i> -Nitrophenyl- β -D-galactopyranoside	0	—	50.0	1.69×10^{-3}
<i>o</i> -Nitrophenyl- β -D-galactopyranoside-6-phosphate	28.9	1.59×10^{-3}	0	—
Lactose	0	—	42.0	0.6×10^{-2}

^a Micromoles per milligram per minute.

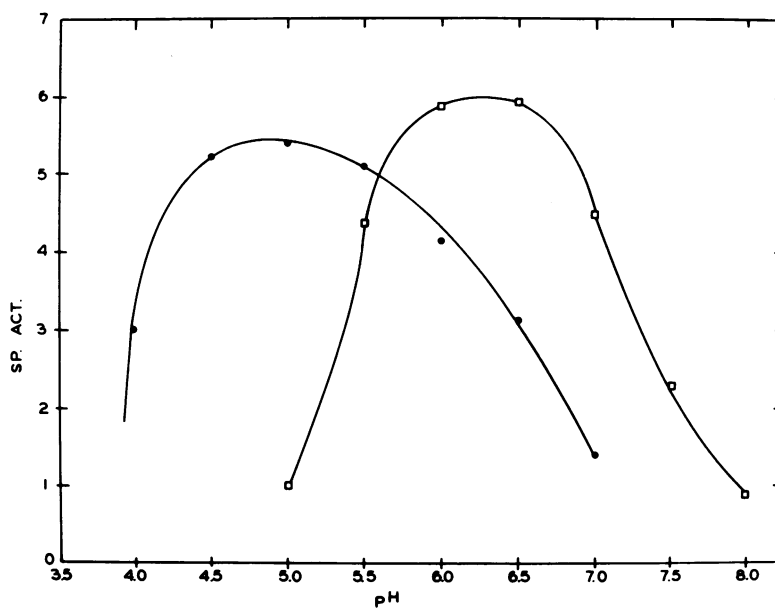


FIG. 2. Effect of pH on the specific activity of β -D-phosphogalactoside galactohydrolase from *L. casei* (●) and β -galactosidase from *L. thermophilus* (□).

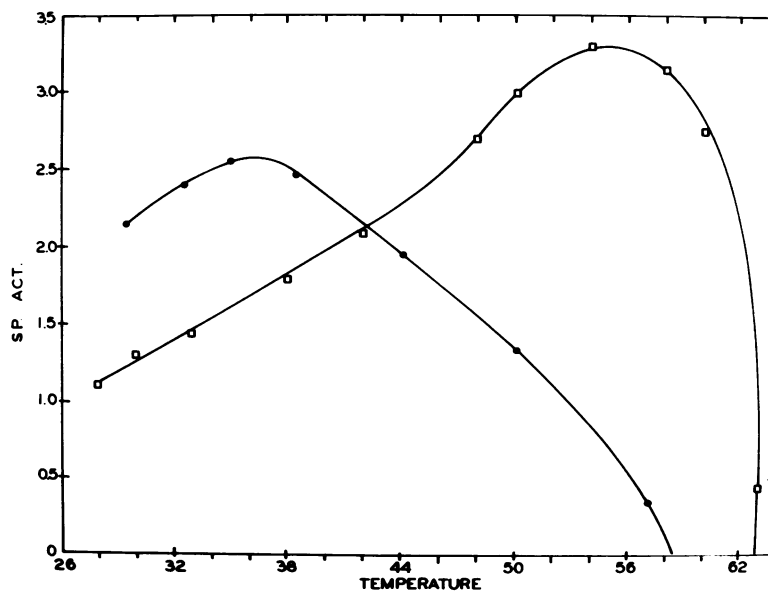


FIG. 3. Effect of assay temperature on the specific activity of β -D-phosphogalactoside galactohydrolase from *L. casei* (●) and β -galactosidase from *L. thermophilus* (□).

the same trend was noted in this study (Table 5) for β -gal and β -Pgal.

Enzyme purification data (Tables 2 and 3) indicated that β -Pgal was less easily purified to as high a specific activity as β -gal. Storage and kinetic data also suggested that the former

enzyme was less stable (Fig. 4) and less active, with a lower V_{max} value but the same affinity for substrate. Also, ONPG was preferred over lactose as a substrate for β -gal, (Fig. 1), but purification of this enzyme with ONPG as the substrate may have influenced these results. In

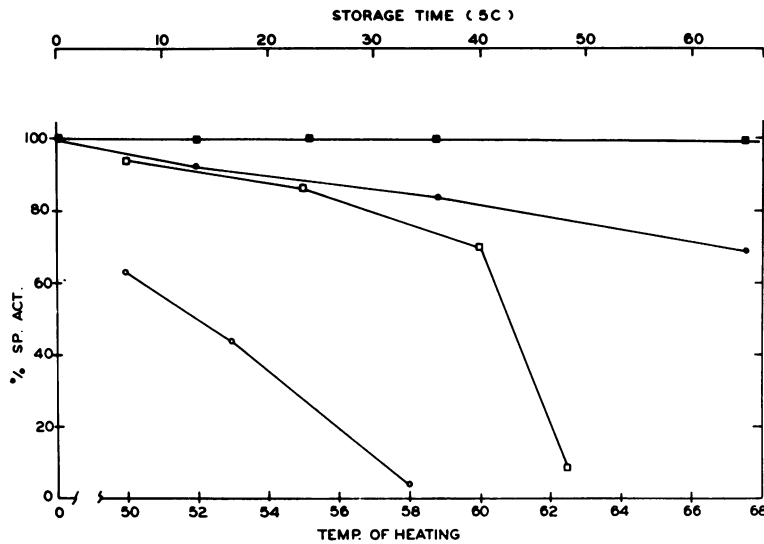


FIG. 4. Effect of heating (open symbols) and storage at 5 C (closed symbols) on specific activity of β -D-phosphogalactoside galactohydrolase from *L. casei* (O, ●) and on β -galactosidase from *L. thermophilus* (□, ■). Heated cultures were held at each temperature for 5 min prior to assay.

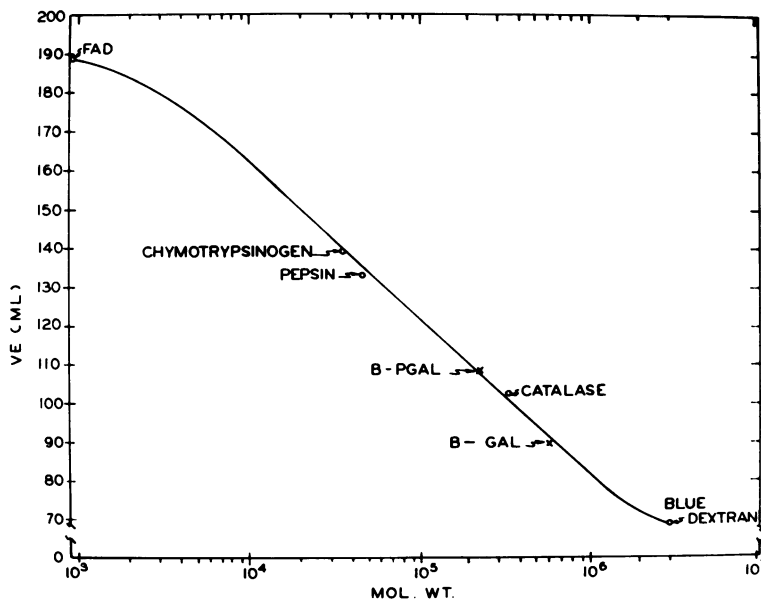


FIG. 5. Molecular-weight curve determined by measuring the elution volumes (V_e) of known proteins from a Sephadex G-200 column (2.5 by 40 cm) in comparison with β -D-phosphogalactoside galactohydrolase from *L. casei* and β -galactosidase from *L. thermophilus*.

this regard, it is of interest that Gray and Santiago (6) have reported the occurrence of distinct enzymes in human intestinal mucosa with different affinities for ONPG and lactose.

Further studies on β -Pgal will include comparative properties of the enzyme from dif-

ferent *Lactobacillus* species as well as an immunological study of the antigenic relatedness between β -gal and β -Pgal. The possibility that the low pH optimum of β -Pgal is important in releasing glucose from lactose in the presence of relatively large amounts of lactic

TABLE 5. Amino acid composition^a of β -galactosidase (β -gal) from *Lactobacillus thermophilus* and β -D-phosphogalactoside galactohydrolase (β -Pgal) from *L. casei*

Amino acid ^b	<i>L. thermophilus</i> β -gal	<i>L. casei</i> β -Pgal
Glutamic acid	1.255	1.137
Aspartic acid	1.180	1.175
Alanine	0.890	1.175
Leucine	0.940	0.762
Glycine	0.900	0.762
Lysine	0.735	0.675
Valine	0.808	0.537
Threonine	0.525	0.525
Serine	0.640	0.437
Isoleucine	0.515	0.475
Proline	0.480	0.450
Arginine	0.440	0.350
Phenylalanine	0.410	0.350
Tyrosine	0.420	0.350
Histidine	0.195	0.175
Methionine	0.155	0.159
Half cystine	0	0

^a Micromoles per milligram of protein.

^b Tryptophan destroyed by acid hydrolyses.

acid (>0.75%) also will be studied.

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