THE PURIFICATION AND CHARACTERIZATION OF β-GALACTOSIDASE FROM RAT MAMMARY GLAND

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ABSTRACT

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OF β-GALACTOSIDASE FROM RAT MAMMARY GLAND

By

Chao-Hen Kuo

A β-galactosidase has been purified 640 fold (hydrolase activity) from the 18th-day lactating female rat mammary gland by acid precipitation, $(NH_4)_2SO_4$ precipitation, gel filtration, and affinity chromatography. Alternatively, a 22-fold (hydrolase activity) or 27-fold (transferase activity purification was obtained by $(NH_4)_2SO_4$ precipitation, organic solvent precipitation and DEAE-cellulose column chromatography. This enzyme displayed hydrolysis activity toward p-nitrophenyl β-D-galactopyranoside (PNPG), o-nitrophenyl β-D-galactopyranoside (ONPG) and lactose. The transferase activity of the enzyme catalyzed the synthesis of 6-O-β-D-galactopyranosyl *myo*-inositol (6-β-galactinol) by the transfer of the galactose moiety from lactose, PNPG or ONPG (good galactosyl donors); or lactulose, 1-O-methyl β-galactoside (poor galactosyl donors) to *myo*-inositol.

Hydrolase and transferase reactions were carried out by one protein as judged by pH profile, thermal inactivation

rate, heavy-metal-ion inhibitory effect and the parallel increase of both specific activities during the purification steps. The enzyme had an acid pH optimum for either hydrolase or transferase activity. Mono- and di-valent cations had little or no stimulatory effect. The apparent K_m for PNPG was 1.85×10^{-4} M (hydrolase activity). For the synthesis of 6-β-galactinol (transferase activity) the apparent K_m for lactose was 37 mM; *myo*-inositol, 380 mM (DEAE-cellulose fraction) or 345 mM (affinity-chromatography fraction and the presence of α-lactalbumin).

Hg^{2+} (1 mM), Ag^{+} (1 mM) and p-chloromercuribenzoate (1 mM) powerfully inhibited the enzymatic activities indicating that SH groups are necessary for the rat mammary β-galactosidase activities. D-Galactose, D-galactono-β-lactone, 1-galactosylamine and phenyl β-D-thiogalactoside, but not D-glucose, were good inhibitors for the transferase activity.

The purified enzyme (affinity-chromatography fraction) was free from β-galactosaminidase, β-glucuronidase, β-glucosaminidase, β-glucosidase, α-mannosidase, α-galactosidase and α-glucosidase. The molecular weight of rat mammary β-galactosidase was determined as 200,000 (pH 5.0) or 110,000 (pH 7.0) by gel filtration on Sephadex G-200. The subunit molecular weight was estimated as 63,000 (major band) by the SDS polyacrylamide gel technique. A pH-dependent intermolecular converstion of rat mammary β-galactosidase was therefore postulated.

Finally, another *myo*-inositol containing disaccharide,

mannosyl inositol isolated from yeast, was identified as 6-O-α-D-mannosyl *myo*-inositol by gas-liquid chromatography, mass spectrometry and enzymatic hydrolysis.

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DEDICATION

This thesis is dedicated to my parents and to Pearl, my wife.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

A *myo*-inositol containing disaccharide, 6-O-β-D-galactopyranosyl *myo*-inositol (6-β-galactinol), was found in rat mammary gland, rat milk and human milk (1,2). In preliminary experiments, β-galactosidase of a crude homogenate of rat mammary gland was shown to carry out the biosynthesis of 6-β-galactinol, *in vitro*, by using lactose and *myo*-inositol as the galactosyl donor and acceptor, respectively.

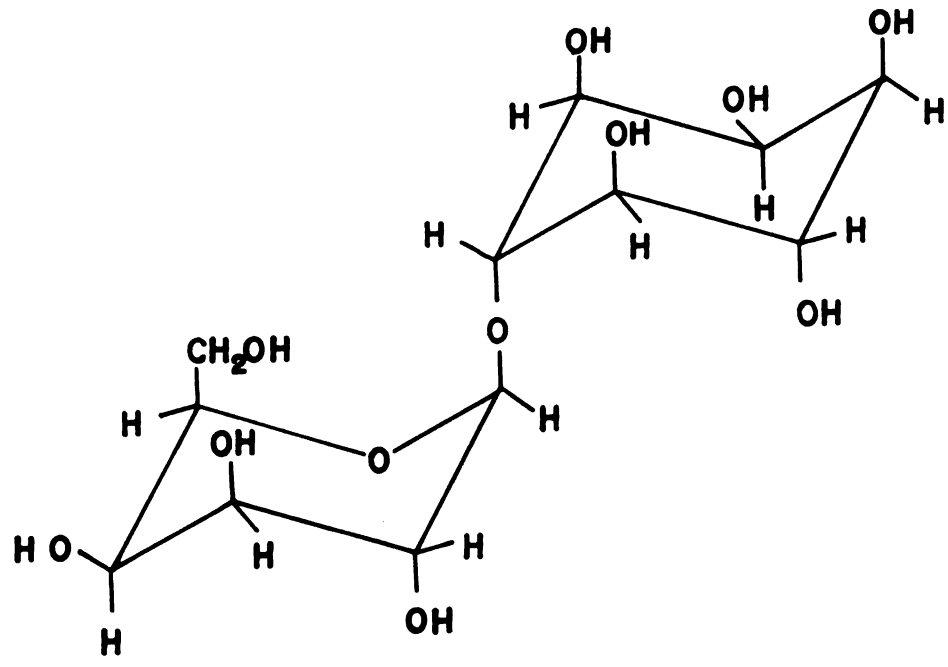
β-Galactosidase has been reported in microorganisms (27-46), plants (47-54) and animals (54-83). In animals several tissues have been investigated, especially small intestine (74-83). However, rat mammary-gland β-galactosidase was not previously purified and characterized. In this study I attempted to purify this enzyme and study its hydrolase activity and, particularly, transferase activity (i.e., the biosynthesis of 6-β-galactinol).

II. LITERATURE REVIEW

A. 6-O-β-D-Galactopyranosyl *myo*-Inositol (6-β-Galactinol)

6-β-Galactinol (Figure 1), a new disaccharide, was first found in rat milk, rat mammary gland during lactation, and the milk of lactating human females (1,2). The level of 6-β-galactinol in either rat mammary gland or rat milk increased concomitantly with *myo*-inositol levels during lactation (2). The content of 6-β-galactinol also dropped gradually as the level of *myo*-inositol in human milk decreased (3). This new compound was not detected in other tissues of lactating female rats nor in rat testes (2).

In addition to 6-β-galactinol, several naturally occurring glycosides of *myo*-inositol have been reported. Phosphatidyl *myo*-inositol mannoside was isolated and identified from *Mycobacterium tuberculosis* (4-7). Sphingolipids of *myo*-inositol phosphate were found in plant seeds (8) and yeast (9). Some indole-acetyl-*myo*-inositols were also isolated and identified from plant (10,11). *myo*-Inositol-containing trisaccharides including digalactosyl *myo*-inositol (12), dimannosyl *myo*-inositol (13), and diglucosyl *myo*-inositol (14) have been reported.



6-O-β-D-Galactopyranosyl myo-Inositol

(6-β-Galactinol)

Figure 1. The structure of 6-O-β-D-galactopyranosyl *myo*-inositol (6-β-Galactinol).

Besides the above *myo*-inositol involved compounds, there is a group of naturally occurring disaccharides of *myo*-inositol. 1L-1-O-α-D-Galactopyranosyl *myo*-inositol (1-α-galactinol) has been isolated and identified from sugar beet juice (15,16). α-D-Mannosyl *myo*-inositol was found in *Saccharomyces cerevisiae* (17). β-D-Glucosyl *myo*-inositol was detected in potato (18) and mung beans (14). Thus, 6-β-galactinol can be classified in this group.

B. The Possible Routes of 6-β-Galactinol Biosynthesis

After the discovery of 6-β-galactinol in the rat mammary gland and rat milk during lactation, the possible biosynthesis route of 6-β-galactinol (the degradation from larger molecules was also considered, if synthesis was not the case) was investigated. Established biosynthetic patterns of disaccharides have been considered, and these patterns will be reviewed.

1. Biosynthesis of Lactose

Lactose is detected exclusively in milk and mammary glands. The synthesis of lactose is catalyzed by lactose synthetase which consists of two protein components (19). One part is galactosyl transferase, which normally catalyzes the transfer of galactose from UDP galactose to an N-acetyl-glucosamine residue on glycoproteins. The second component is the milk protein α-lactalbumin (20), which regulates the glucose-binding properties of the galactosyl transferase so that lactose synthetase shifts to catalyze the synthesis

of lactose, (UDP-galactose + glucose ———> lactose + UDP), at the physiological concentration of glucose (21,22). In the absence of α-lactalbumin, lactose synthetase can still catalyze the synthesis of lactose, but the affinity of glucose to the enzyme is very low and has a Michaelis-Menten constant of 2,260 mM (23). If the proper amount of α-lactalbumin is added, the K_m for glucose drops to 2.1 mM.

2. Biosynthesis of 1-O-α-D-Galactopyranosyl *myo*-Inositol (1-α-Galactinol)

Frydman and Neufeld (24) had reported that an enzyme preparation from unripe pea seeds catalyzed the transfer of a galactosyl moiety from UDP-D-galactose to *myo*-inositol to form a disaccharide with the properties of 1-α-galactinol (UDP-D-galactose + *myo*-inositol ———> 1-α-galactinol + UDP). The same enzyme preparation could also catalyze galactosyl transfer to some isomers of *myo*-inositol: *scyllo*-, *dextro*- and *levo*-inositol to yield galactosides which are different from 1-α-galactinol. The optimal pH of the transferase was found to be 5.6 in 50 mM acetate or phosphate buffer, and the K_m for *myo*-inositol was 5 mM. The absence of Mn^{2+} and the presence of EDTA at a concentration of 2.5×10^{-4} M completely inhibited transferase reaction.

Tanner and his colleagues (25) later discovered the physiological role of 1-α-galactinol in higher plants. The galactosyl residue of 1-α-galactinol could be transfered to sucrose, raffinose, etc. to produce raffinose, stachyose,

etc. by 1-α-galactinol:galactosyl transferase (25).

3. Biosynthesis of 5-O-β-D-Galactopyranosyl *myo*-Inositol and 1(5)-O-β-D-Glucopyranosyl *myo*-Inositol

Enzyme extracts or growing cultures of *Sporobolomyces singularis*, which catalyze galactosyl or glucosyl transfer from lactose or cellobiose to *myo*-inositol has been reported by Gorin, Horitsu and Spenser (26). 5-O-β-D-Galactopyranosyl *myo*-inositol was the main product of transfer from lactose to *myo*-inositol and the major component was 1-O-β-D-glucopyranosyl *myo*-inositol when cellobiose was served as a glucosyl donor. 5-O-β-D-Glucopyranosyl *myo*-inositol (the minor product) was also synthesized.

4. Biosynthesis of Disaccharides by β-Galactosidases

Most of the studies on β-galactosidase are concerned with the hydrolase activity using synthetic or natural substrates. However, there are several reports on its transferase activity. The biosynthesis of disaccharides catalyzed by β-galactosidase will be reviewed in a separate section of "Transferase Activity".

At first, Wells and Iritani attempted to find enzymatic activity in rat mammary gland capable of synthesizing 6-β-galactinol following patterns similar to those of lactose and 1-α-galactinol biosynthesis. It was not possible to demonstrate analogous activity in rat mammary gland by using UDP-D-galactose and *myo*-inositol as galactosyl donor and acceptor, respectively.

Later, Wells and his co-workers found that 6-β-galactinol occurs only in mammary gland and milk (2). As lactose is also only found in milk and mammary glands, it was considered a most likely candidate for the galactosyl donor. A preliminary experiment showed that the extract of rat mammary gland during lactation could synthesize 6-β-galactinol in the presence of lactose and *myo*-inositol at low pH and rat mammary β-galactosidase was discovered to be the enzyme responsible for 6-β-galactinol biosynthesis. Since the enzyme from mammary tissue has not been previously isolated, we, therefore, undertook to purify it and study its properties.

C. β-Galactosidase (β-D-Galactoside Galactohydrolase, EC 3.2.1.23)

Enzymes that bring about the hydrolysis of the β-galactosidic linkage are termed β-galactosidases. They are among the most extensively studied glycosidases. The enzymatic activity has been found in microorganisms (27-46), plants (47-54) and animals (54-83).

1. Microbial β-Galactosidase

β-Galactosidase occurs in bacteria (27-40), yeasts (41-45), and protozoa (46). The enzyme was purified and crystallized from *Escherichia coli* strain ML309 (27) and strain ML308 (28). β-Galactosidase from *Escherichia coli* K12 has also been isolated and characterized by Lederberg (29), and Kuby and Lardy (30). Furthermore, a fractionation procedure

was developed by Graven *et al.* to isolate large quantities of β-galactosidase from *Escherichia coli* K12 in a high state of purity (31). Recently, β-galactosidase was purified from *Escherichia coli* B in Japan (32). Arraj *et al.* isolated ebg β-galactosidase (the product of the ebg gene) from *Escherichia coli* K12 strain LC110, which is a Lac^+ revertant of a mutant with a deletion of the lac β-galactosidase gene (33). This new ebg β-galactosidase activity was shown to be due to a discrete protein, immunologically unrelated to lac Z β-galactosidase. The enzyme shows a general preference for binding to monosaccharides rather than β-galactosides, suggesting the gene which regulates this new ebg β-galactosidase probably is modified from a gene that is involved with the metabolism of a monosaccharide. In addition to *Escherichia coli*, β-galactosidase has also been investigated in other bacteria, for example, *Aeromonas formicans* (34), *Diplococcus pneumoniae* (35), *Aerobacter cloacae* (36), *Streptococcus lactis* (37), *Bacillus megaterium* (38), *Staphylococcus aureus* (39), and *Propionibacterium shermanii* (40). To *Staphylococcus aureus* the first product of the metabolism of lactose is 6-phosphogalactosylglucoside (6-phospholactose), which is synthesized during the passage of lactose across the cell membrane by a phosphotransferase system. The 6-phospholactose molecule is then split by a special class of β-galactosidase, i.e., a 6-phosphogalactosidase.

β-Galactosidase has also been found in yeasts and fungi, for instance, *Saccharomyces lactis* (41), *Aspergillus foetidus*

(42), *Aspergillus niger* (43), *Sporobolomyces singularis* (44), *Asperillus oryzae* (45), and a protozoan, *Trichomonas foetus* (46).

2. Plant β-Galactosidase

In addition to microorganisms, β-galactosidases are found in plants. The enzyme was purified from jack bean (47,48). α-Galactosidases and β-galactosidases of *Cajamus indicus* have been separated by CM-cellulose chromatography (49). β-Galactosidase was resolved into three different forms which exhibited separate characteristic features, including Michaelis-Menten constant for synthetic substrate, o-nitrophenyl β-D-galactoside (ONPG), and the extent of hydrolysis of lactose and methyl-β-galactoside. β-Galactosidase activity was also detected in oats *(Avena sativa)* coleoptiles, using p-nitrophenyl β-D-galactoside (PNPG) as a substrate (50). Johnson and his co-workers observed that indoleacetic acid (IAA) treatment could enhance cell wall β-galactosidase activity *in vivo*. IAA has been known to promote cell elongation. The following hypothesis was, therefore, proposed to explain IAA effect on cell elongation: IAA first stimulates β-galactosidase and/or other glycosidase activities which then regulate cell growth. Evans (51) tested this hypothesis by examining the effect of inhibitors of β-galactosidase and β-glucosidase. Severe inhibition of measurable β-galactosidase or β-glucosidase activity was found to have no effect on IAA-promoted growth. In ripening apples

β-galactosidase activity has been identified in soluble and cell wall preparations from cortex tissue (25). The enzyme was able to degrade pectin galactan and PNPG. While apples softened with ripening, soluble polygalacturonide increased, and these changes were proceeded by an increase in soluble and cell wall β-galactosidase activity and a decrease of galactose content from the cell wall. These data indicate that β-galactosidase could play a role in apple softening. β-Galactosidase was also detected in the cell wall-degrading extracts of ripening tomato fruits (53) and almond emulsin (54).

3. Animal β-Galactosidase

β-Galactosidase has been well studied in many mammals. There are reports on enzymes of some invertebrates, especially in snails and insects.

a. Nonmammalian β-Galactosidase

Most β-galactosidases which have been investigated in invertebrates were obtained from the gut. The enzyme was reported in digestive juices of snail, *Helix pomatia* (54,55), in salivary glands, hepato-pancrease and crop wall and other tissues of *Helix aspersa* (56).

In some insects, such as locusts (*Locusta migratoria, Schistocera gregaria)* and cockroach (*Periplanata americana)*, enzyme activity was in the digestive juice (54). The gut β-galactosidase (lactase) of *Locusta migratoria* was isolated and characterized with respect to the substrate, lactose (57).

This lactase activity was found to be indistinguishable from a gut glucosidase, i.e., cellobiase as judged by: (a) the same pattern is obtained in isoelectrofocusing; (b) D-glucono-α-lactone is an inhibitor of bor enzymes and exhibits the same Ki value (58); (c) the two enzymes have similar temperature coefficients for heat denaturation (59). In addition to lactase, hetero β-galactosidase (p-nitrophyl β-D-galactosidase) was also observed in *Locusta migratoria* (59) and was separated into four components by isoelectrofocusing. Morgan (60) reviewed carbohydrases of the gut of locusts and grasshoppers and reported β-galactosidase activity in seven species. Hori (61) examined some phytophagous insects and confirmed the presence of lactase in the midgut but not in the salivary gland. Although lactase has been found in insect guts, its physiological role is unclear since lactose is not present in their natural food.

b. Mammalian β-Galactosidase

Mammalian β-galactosidase activity has been investigated in numerous tissues from different sources. Conchie *et al.* (62) surveyed the tissues of rats and mice and confirmed that β-galactosidase was widely distributed in many tissues including liver, spleen, kidney, preputial gland, testes, seminal vesicle, epididymis, ovary, uterus and vagina. Adult epididymis, kidney and spleen in both species are particularly rich sources. The enzyme of rat epididymis was partially purified and shown to have an acid pH optimum (63). Two β-galactosidases, acrosomal and lysosomal isoenzymes, were also

identified in rat testis (64). In addition to rats and mice, β-galactosidase has been reported in other mammalian species, e.g., ram testis (65), rabbit brain (66), human liver (67), pig kidney (68), bovine testis (69), monkey liver (70), guinea-pig epidermis (71), human skin fibroblasts (72) and human brain (73).

In mammals, the enzymes of the small intestine were studied extensively. Doell and Kretchmer (74) investigated the enzyme of rat and human small intentine during development and reported maximal lactase activity in jejunum and moderate amounts in duodenum and ileum. They also found that lactase activity in the intestine of the developing rat and rabbits increased rapidly and attained a maximum near the termination of gestation or shortly after birth and the activity then declined gradually. Due to different pH optima and substrate specificity, two β-galactosidases were distinguished in the small intestinal mucosa of rats and rabbits. Dahlquist and Asp (75) by using lactose and four hetero β-D-galactosides including phenyl β-D-galactosides (PG), ONPG, PNPG and 6-bromo-2-naphthyl β-D-galactosides (BNG) as substrates also confirmed at least two β-galactosidases were present in rat small intestine. One enzyme with a pH optimum of 3-4 mainly hydrolyzed hetero β-D-galactosides. The other enzyme with a pH optimum of 5-6 hydrolyzed lactose much more rapidly than hetero β-D-galactosides. These two β-galactosidases could be separated by gel filtration on Sephadex G-200. The enzyme with an acidic pH optimum (pH optimum at

3-4) was further fractionated into two components by DEAE-cellulose chromatography. Alpers (77) also isolated two β-galactosidases from rat small intestinal mucosa and found their location. He concluded that one was a lysosomal enzyme which had a pH optimum of 3.0 and hydrolyzed ONPG and BNG; another enzyme from the brush borders of intestinal mucosa with a pH optimum of 6.0 hydrolyzed lactose readily. In the same publication he confirmed the presence of a lysosomal enzyme and a lactase in human small intestine. Semenza *et al.* (78) reported two lactases partially separated from human intestine by gel filtration. Gray and his co-workers (79) isolated three β-galactosidases from human intestine by gel filtration and density gradient ultracentrifugation. One has a pH optimum of 6.0 and specifically hydrolyzes lactose. The second enzyme had a pH optimum of 4.5 and hydrolyzes both synthetic substrates and lactose at the same rate. The third one is a hetero β-galactosidase which has a pH optimum at 6.0 and hydrolyzes only the synthetic β-galactosides (BNG).

Multiple forms of β-galactosidases have also been found in the small intestine of other mammals. Swaminathan and Radhakrishnan (80) suggested that two different β-galactosidases in monkey intestine catalyzed the hydrolysis of lactose and ONPG separately based on evidence from the distribution pattern, heat inactivation, differential distribution on centrifugation and selective inhibition by Tris buffer. The same group (81) later partially purified two hetero β-galactosidases from monkey small intestine. One particulate enzyme

with pH optimum at 4.5 catalyzed the hydrolysis of lactose, whereas, a supernatant enzyme with pH optimum at 7.0 has no catalytic activity toward lactose. A hetero β-galactosidase was also reported in rabbit small intestine (82). This hetero enzyme is located in the cytosol fraction, has a neutral pH optimum (6.0-6.5), and has activity toward β-galactosides and β-glucosides but not toward lactose. Recently, Sato and Yamashina (83) reported three β-galactosidases in hog small intestine: lactase in the brush borders, hetero β-galactosidase in cytosol and acid β-galactosidase in lysosomes.

4. Enzymatic, Physical and Chemical Properties of β-Galactosidase

β-Galactosidase expresses hydrolase and transferase activities following the same reaction mechanism. The sequences of reactions during these two activities can be demonstrated as follows:

(1) G-O-R + E·H <———> G-O-R/E·H

(2) G-O-R/E.H <———> G·E + H-O-R

(3) G·E + H-O-R' <———> G-O-R' + E·H

where G-O-R represents synthetic or natural substrates; E·H, a β-galactosidase; G-O-R/E·H, a Michaelis complex; G·E, a galactosyl-enzyme complex; and H-O-R', an acceptor. If R' represents a hydrogen atom, then equations (1), (2) and (3) represent hydrolysis activity. If R' represents an alkyl, aryl or glycosyl residue then transferase activity takes place with G-O-R' being a transferase product.

Though all β-galactosidases from different sources are able to use synthetic and/or natural β-galactosides as substrates, the diversities of the properties of β-galactosidases occur among the species and different tissues of the same species.

a. Hydrolase Activity

(1) pH Optimum

A wide range of pH optima (2.2-8.0) can be found in β-galactosidases. All β-galactosidases are briefly separated into three groups. One group of enzymes has an 'acid' pH optimum of 2.2-4.5. A second and third group have a 'neutral' pH optimum (5.0-7.0) and a 'basic' pH optimum (7.0-8.0), respectively. Lactases which belong to 'neutral' enzymes, preferentially hydrolyze lactose and exclusively localize in the brush borders of small intestinal mucosa. These enzymes have been reported in rat (75,84,85), human (79,77), monkey (86) and locust gut (57). In addition to lactases, other 'neutral' enzymes are probably in cytosol, for instance monkey liver (70), human liver (87), beef liver (88), human intestine (79), monkey intestine (70,81), rabbit small intestine (82) and protozoan (46). In animals 'acid' β-galactosidases can be detected in almost any tissue: for example, snail (56), slug crop (54), rat epididymis (63), ram testes (65), rabbit brain (66), human liver (67,87,89), cat liver (90), rat intestine (75,77,84,85), human intestine (77,79), bovine testis (69), beef liver (88), and monkey small intestine (81). This group of enzymes probably localize in lysosomes.

Plant β-galactosidases have lower pH optimum, such as almond (54), jack bean (48), ripening apple (52), oat (50,51) and *Cajanus indicus* (49). On the contrary, 'basic' enzymes are only found in microorganisms (30,33,39,40,41,91). Some species of *Aspergillus* have low pH optimum (42,43,45).

(2) Metal Ion Effect

The requirement of metal ions for enzymatic activity is variable. In general, mono- and divalent cations express stimulatory effects or no effect, whereas, heavy metal ions invariably inhibit the enzymes.

The effects of metal ions on *E. coli* β-galactosidase have been well studied. Sodium ion stimulated enzymes from several *E. coli* strains (30,92,93) but had no effect on a mutant strain of *E. coli* K12 (33). Potasium ion also activated enzymes with ONPG, lactose or allolactose as the substrates (91,92,93). At low concentrations of Cs^+ (<10 mM), the *E. coli* enzyme was stimulated, however, there was no effect at high concentrations of Cs^+ (>10 mM) (92).

Divalent cations have also shown some effects on *E. coli* enzyme. Mg^{2+} was absolutely requried for enzymatic activity of β-galactosidase from *E. coli* ML308 (28). Ullmann and Monod (94) studied the renaturation of β-galactosidase from *E. coli* and discovered that the denatured enzyme could be renatured to an inactive form, which had a sedimentation coefficient of 16 S--the same as the native tetrameric enzyme, by dialysis in the absence of Mg^{2+}. The activity could be restored by adding Mg^{2+}. At high concentration of Mg^{2+} the

inhibition of enzymatic activity was observed (95). This inhibitory effect was due to competition for the site on the free enzyme which the activator Na^+ occupied. In the presence of EDTA (1 mM), 85% of ebg β-galactosidase activity was inhibited and this inhibition could be partially restored by the addition of Mg^{2+} (1 mM).

Mn^{2+} showed stimulatory effect on *E. coli* β-galactosidase (30,32,96). $MnCl_2$ (0.4 mM) activated β-galactosidase in the cell free extract and intact cells of *Propionibacterium shermanii* (40). It was also found to stimulate 'neutral' β-galactosidase of *Trichomonas foetus* (46), the enzymes from *Diplococcus pneumoniae* (35); inhibited 6-phospho-β-galactosidase of *Staphylococcus aureua* (39); and had no effect on *Aspergillus foetidus* (42) and *Aspergillus oryzae* (45). EDTA (20 mM) completely inhibited enzymatic activity of *Diplococcus pneumoniae* which was reactivated by the addition of Mn^{2+}, Ca^{2+} or Mg^{2+} (35). However, EDTA had no effect on the β-galactosidase of *Aspergillus foetidus* (42), *Aspergillus oryzae* (45), on the β-galactosidase II of *Trichomonas foetus* (46) and *Sporobolomyces singularis* (44). Monovalent (Na^+, K^+) and divalent (Mg^{2+}) ions have stimulatory effect on the lysosomal enzyme. However, KCl inhibited the soluble enzyme from rat liver (97). 'Acid' β-galactosidase of human liver has been reported to be stabilized by several monovalent and divalent cations (67). NaCl could activate the enzyme from human skin fibroblast (72).

Anions also affected the enzymatic activity. Cl^- has been reported to activate and stabilize the 'acid' enzyme and

inhibit 'neutral' enzyme of human liver at pH below 4.5 (89). Another publication confirmed that Cl^- could inactivate the first four isoenzymes from human liver and inhibit the fifth which had a pH optimum of 6.0 (87). CH_3OO^-, SO_4^{2-} and Cl^- all showed stimulatory effects on rat-liver enzyme (97).

Common heavy metal ions which have been used on β-galactosidases are Hg^{2+}, Ag^{2+} and Cu^{2+}. At a concentration of 1 mM, the three ions completely inhibited monkey-liver and intestine enzymes (70). The enzyme from rat epididymis was totally inhibited by Cu^{2+} (10 mM) and Hg^{2+} (0.4 μM); 50% activity was inhibited by Ag^+ (10 μM) (63). Ag^+ (5 μM), Hg^{2+} (0.5 μM) and Cu^{2+} (5 mM) supressed 50% of activity in the rat-liver homogenate (98). The enzymes from jack bean (48), *E. coli* (30,32), *Staphylococcus aureus* (39), *Diplococcus pneumoniae* (35) and *Aspergillus oryzae* (45) were all inhibited by heavy metal ions. The inhibition of heavy-metal ions indicates the requirement of sulfhydryl groups for the enzymatic activity of β-galactosidase.

(3) Inhibitors and Activators

A few compounds which can react with cystine residue to interfere with enzyme activity have been tested on the β-galactosidase activity. p-Chloromercuribenzoate (PCMB) was a good inhibitor for β-galactosidase prepared from *E. coli* ML309; however, N-phenylmaleimide (9.1×10^{-3} M) and N-ethylmaleimide (3×10^{-3} M) only partially inhibited the enzymes; at a concentration of 0.9 mM iodoacetamide (IA), no inhibition was observed (99). A β-galactosidase from

Propionibacterium shermanii was also inhibited by these agents (40). At a concentration of 0.5 mM, PCMB almost completely suppressed the activity; but when 10 mM N-ethylmaleimide was applied, only 70% of activity was inhibited. Iodoacetamide was a less efficient inhibitor. At a low concentration of iodoacetamide (1 mM) no inhibitory effect was found; only at higher concentrations, i.e., 20 mM, did an inhibitory effect occur. Of the β-galactosidases from rat small intestine, PCMB (0.1 mM) completely inhibited 'acid' enzyme but had no effect on 'neutral' activity (100). PCMB also selectively suppressed hetero β-galactosidase and acid β-galactosidase of hog small intestine and had no inhibitory effect on lactase (83). A hetero β-galactosidase of rabbit small intestine, which has activity against hetero β-D-galactosides and minimal activity toward lactase was markedly inhibited by PCMB at a concentration of 0.1 mM (82). Lactase and hetero β-galactosidase were separated from human small intestine by gel filtration and only the hetero β-galactosidase was selectively inhibited by PCMB (101). Hirsova *et al.* reported that monoiodoacetate exhibited noncompetitive inhibition on both neutral and acid β-galactosidases of the small intestine of suckling rats, while n-ethyl maleimide produced no inhibition (85). p-Hydroxymercuribenzoate was inhibitory on β-galactosidase of monkey small intestine, and this inhibition could be reversed by the addition of β-mercaptoethanol (81). 'Acid' β-galactosidases of human liver were also completely inhibited by PCMB at a concentration of 0.01 mM (67).

In addition to these sulfhydryl agents, some galactose and galactoside analogs have shown inhibitory effects. β-Galactono-γ-lactone, an analog of galactose is an inhibitor of β-galactosidases from *E. coli* (33), *Aspergillus foetidus* (42), bovine testes (69), rat small intestine, jack bean (48), and human liver (67). Levvy and his co-workers (103) found that D-galactono-γ-lactone could be converted to D-galactono-δ-lactone and δ-lactone was a much more powerful inhibitor than the γ-lactone. The nonhydrolyzable substrate analogs β-D-thio-galactosides, such as p-nitrophenyl β-D-thio-galactoside, isopropyl β-D-thio-galactoside and p-aminophenyl β-D-thiogalactoside are competitive inhibitors of β-galactosidases (42,48,69). The enzymes were not capable of hydrolyzing thio linkage but the association between enzymes and these analogs still took place. Galactal (1,2-deoxy-D-galactose) was also a reversible inhibitor (69,104). 1,2-Deoxygalactose could be converted to 2-deoxygalactose by β-galactosidase (105).

D-galactose is a common product from the hydrolysis of synthetic and natural β-galactosides catalyzed by β-galactosidases. This monosaccharide exhibited a very powerful inhibition of β-galactosidases (30,33,42,45,48,69,106), demonstrating a typical feedback inhibition by product. However, D-glucose, a co-product while lactose served as substrate, had either no effect (33,42,48) or even a stimulatory effect (69).

N-bromoacetyl-β-D-galactosylamine has been shown to

inhibit *E. coli* enzymes by the alkylation of a methionyl residue near the active site (107). This inactivation could be restored with β-mercaptoethanol. Replacement of this methionyl residue near the active site with norleucine protected the enzyme from inhibition by N-bromoacetyl-β-D-galactosylamine, indicating this methionine did not participate in catalysis. N-bromoacetyl-β-D-galactosylamine was also found to inhibit both 'acid' and 'neutral' β-galactosidases of human liver (61). But the inhibition is irreversible since the enzyme could not be reactivated with β-mercaptoethanol or dithiothreitol. In the presence of substrates the enzymes were protected against inhibition.

DNA, tRNA and other polynucleotides have been reported to inhibit 'acid' β-galactosidase of rat small intestine that could be protected by proteins (109). Moreno and Sols (110) reported that an acid resistant dialyzable factor from commercial albumin could increase the activity and stability of *E. coli* β-galactosidase. Recently an activator was isolated from human liver by Li and Li (111). The purified activator stimulated the hydrolysis of G_{M_1} by β-galactosidase. Chemical analysis identified the activator as a glycoprotein. The activator was heat-stable and the activity could be destroyed by pronase, indicating the protein part of the molecule was essential for its activity.

(4) Substrate Specificity

The strict requirements for the structure of the glycone part of the substrate molecules are observed in

β-galactosidase activity. Wallenfels and his co-workers have provided an excellent reivew of this area (112). β-Glycosidic linkage and D-pyranoside ring seems to be absolutely essential for the enzymatic activity. Replacement of the glycosidic oxygen with sulfur results in a remarkable interference in the enzymatic activity. However, the enzyme possesses a very high tolerance to variation of the aglycone part of a substrate molecule. These variations influenced the affinity of substrate to the enzymes. ONPG and PNPG usually have higher affinity in comparison with other β-galactosides, either hetero β-D-galactosides or lactose (30,35,42,45,58,59,67,69,70, 91,101,102). *E. coli* had a very high hydrolysis activity with ONPG, relatively less activity against PNPG, methylsalicylate-β-D-galactoside, PG, lactose and allolactose, as well as little activity against ethyl-β-D-galactoside and o-nitrophenyl thio-β-D-galactoside (99). Fungal enzymes (*Aspergillus foetidus*) could hydrolyze lactose and lactulose, but had no activity against melibiose and maltose (42). β-Galactosidase of yeast (*Sporobolomyces singularis*) showed hydrolytic activity toward lactose, ONPG and PNPG (44). The 'acid' enzyme from rat intestine hydrolyzed ONPG and BNG but was relatively inactive against lactose. On the contrary, 'neutral' enzyme (lactase) cleaved lactose more rapidly (77). Hetero β-galactosidase of monkey liver (70) hydrolyzed hetero β-D-galactosides (ONPG, PNPG, and PG) but not lactose. The similarity was also found in hog and human small intestine. Hetero β-galactosidase, one of three β-galactosidases from

hog intestine, had no activity on lactose; however, the other two enzymes, lactase and 'acid' β-galactosidase, could hydrolyze both lactose and hetero β-D-galactosides, and lactase preferentially hydrolyzed lactose (83). Two hetero β-galactosidases prepared from monkey small intestine (81) hydrolyzed hetero β-galactosides, but only one of these could use lactose as substrate. A hetero β-galactosidase which had activity toward hetero β-D-galactosides but minimal activity against lactose, was also found in rat small intestine (82). Therefore, different β-galactosidases of small intestine can be easily distinguished by the combination of substrate specificities and pH optima.

b. Molecular Weight

The molecular weight of native *E. Coli* K12 enzyme was accurately determined as 540,000 (31). This enzyme was a tetramer containing 4 identical subunits. The molecular weight of several microbial enzymes have been estimated as follows: 126,000 for *Aspergillus foetidus* by gel filtration (42), 105,000 and 106,000 for *Aspergillus oryzae* by gel filtration and sucrose density gradient centrifugation (45), and 140,000 for *Sporobolomyces singularis* (44). Lactase and aryl β-galactosidase of locust gut have been reported to possess molecular weights of 110,000 and 65,000, respectively (58). Several publications reported the molecular weight of mammalian β-galactosidases. By gel filtration on Sephadex G-200, molecular weight of 'acid' and 'neutral' β-galactosidases of small intestinal mucosa of infant rats were determined as

83,000-105,000 and 360,000-510,000, respectively (102). Three forms of β-galactosidases have been purified from human small intestine. 'Lactase' has a molecular weight of 280,000; 'hetero' galactosidase has a molecular weight of 80,000; and 'acid' enzyme has a molecular weight of 156,000 (79). Three forms of human liver β-galactosidases by gel filtration have the molecular weight of 23,000, 25,000 and 10,000 (89) G_{M1} ganglioside β-galactosidase A of human liver has an apparent molecular weight of 65,000 to 75,000 by gel filtration and a molecular weight of 72,000 by SDS gel (110). Therefore, there is no constant molecular weight for all β-galactosidases.

c. Multiple Forms

Polyacrylamide-gel electrophoresis of a β-galactosidase preparation from uninduced *E. coli* K12, only a single band of enzyme activity could be detected. However, when an enzyme preparation from *E. coli* induced with isopropyl-β-D-thiogalactoside (IPTG) was investigated, a multiple band of enzyme activity was observed on gel (113,114). The pattern of multiple bands was stable to dilution, freezing and thawing, dialysis and prolonged storage at -20°C. The most rapidly-moving band was purified and studied by sedimentation equilibrium. The molecular weight was 540,000. Only this band could be seen in the extract of uninduced *E. coli*. However, a mutant of *E. coli* 3310 did not form the multiple bands even when it was induced with IPTG (113).

These multiple bands were further studied by Marchesi *et al*. They reported some of them were 4 N (most rapidly-

moving band), 6 N, 8 N, 10 N, 12 N and 16 N (115). While heavy isoenzymes (> 4 N) were denatured by urea and renatured, only the tetramer was formed. If a purified mixture of heavy isoenzymes was stored at 4°C for long periods, the tetramer was also formed. Kaneshiro *et al.* (116) reported that β-galactosidase from *E. coli* ML308 and K12 3300 was dissociated into an inactive monomer in the presence of Ag^+, whereas, an active dimer was reformed by the addition of excess of dithiothreitol (116). In the comparative study of isoenzyme formation of bacterial β-galactosidase, Erickson and Steers (117) discovered the multiple forms of β-galactosidases from *Salmonella typhimurium, Serratia marcescens* and *Proteus mirabilis* but not from *Aeromonas formicans.*

Furth and Robinson (98) investigated several tissues of the rat and reported multiple forms of β-galactosidases occurring in rat liver, spleen, intestine, kidney and urine. Isoenzymes of small intestine were reported in several species. These have been reviewed in the previous sections. In addition to the small intestine, two isoenzymes of β-galactosidases from rat testes have been found. Isoenzyme I was localized in the lysosomes of precursor germinal cells and isoenzyme II was found in sperm acrosomes (64). Isoenzyme II was undetectable through the spermatocyte stage of development but increased in specific activity during the formation of spermatids (118). Following hypophysectomy of rat at the age of 26 days, or in adulthood, the specific activity of lysosomal isoenzyme I increased; however, the acrosomal isoenzyme II was

undetectable. When LH and FSH or testoterone was administered to hypophysectomized animals, the normal patterns of isoenzymes were restored (118). The multiple forms of β-galactosidase of human liver have been reported by several groups (67,87,89,110). Two β-galactosidases were reported in rabbit brain (66).

Multiple forms of mouse-liver enzyme were also demonstrated by polyacrylamide gel electrophoresis (119). However, while denatured with SDS, only one band can be observed in the SDS gel electrophoresis, having a subunit molecular weight of 63,000.

d. Transferase Activity

More often β-galactosidase was considered as a hydrolase to cleave disaccharides, oligosaccharides, glycoproteins, glycolipids and mucopolysaccharides. However, quite a few studies have been performed to show its transferase activity.

Aronson (120) reported that four oligosaccharides were formed during the hydrolysis of lactose by *Saccharomyces fragilis* lactase. In the same report he also confirmed the synthesis of oligosaccharides by *E. coli* enzyme, using lactose as a substrate. Pazur (121) indicated the formation of four oligosaccharides, i.e., allolactose, galactose-(1 ——> 6)-galactose, galactose-(1 ——> 6)-galactose-(1 ——> 4)-glucose and galactose-(1 ——> 6)-galactose-(1 ——> 6) glucose, when 'lactase B' was used as an enzyme source to utilize lactose. To understand the mechanism of enzymatic synthesis of galactosyl oligosaccharides, Pazur (122) carried out tracer experiments.

Based on the results, he suggested a two-step mechanism: a galactosyl-enzyme complex was formed first and galactosyl residue was then transferred to acceptors to form new oligosaccharides. Glucose, galactose, lactose and allolactose were all found to function as acceptors. A few years later, Pazur and his co-workers tried to determine the acceptor specificity on transferase activity of the enzyme from *S. fragilis*. The galactosyl residue was found to be transferred from lactose to planteose, sucrose and glucosamine (123). Pridham and Wallenfels (124) were also able to demonstrate transgalactosidase and transfucosidase activities in the synthesis of m-hydroxyphenyl β-D-galactoside (or m-hydroxphenyl β-D-fucoside) from lactose (or o-nitrophenyl β-D-fucoside) and resorcinol by *E. coli* β-galactosidase.

In the meantime, Gorin *et al.* (125) discovered the synthesis of β-D-galactosyl disaccharides in growing culture or the enzyme extract of yeast *(Sporobolomyces singularis)* by using lactose as a galactosyl donor and various acceptors. From the investigation of product structures they concluded that the galactosyl residue from lactose was transferred to a secondary hydroxyl oxygen rather than a primary hydroxyl oxygen in the acceptors. To understand whether the pyranoid-ring oxygen of glycosyl acceptors had effect on the transferase reaction, *myo*-inositol was tested as an acceptor and 5-O-β-D-galactosyl *myo*-inositol was produced either in the growing culture or enzyme extract (26). A few years later, Blakely and Mackenzie (44) purified a β-hexosidase from *Sporobolomyces*

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singularis and found the formation of oligosaccharides from lactose by this purified β-hexosidase. The maximal activity was found at pH 6.5, thus confirming the findings of Gorin *et al.* (125).

When Shifrin and Hunn (126) studied the effect of alcohols on the enzymatic activity and subunit association of *E. coli* β-galactosidase, they confirmed the transferase activity by the identification of methyl β-galactoside as one of the reaction products in the presence of ONPG, methanol and enzyme. In the presence of glycerol as an acceptor, Lehmann and Schröter (105) found that D-galactal can be transferred to glycerol to form glycerol 2-deoxy-β-D-galactoside by *E. coli* enzyme.

Allolactose has been reported as one of the transferase products by β-galactosidase on lactose (121,122,127,128). It was found as an important natural inducer of lac operon in *E. coli, in vivo* (127,129,130,131). Huber *et al.* (91) studies some enzymatic properties of *E. coli* enzyme, using allolactose as a substrate. At low concentration of allolactose ($\leq$ 50 mM) only galactose and glucose are produced; while at high concentration of allolactose (> 50 mM) transgalactolytic oligosaccharides were also synthesized. Recently, the same group (132) extensively studied the hydrolase and transferase activities of *E. coli* enzyme with lactose serving as a substrate. At low concentration of lactose (50 mM) the rate of glucose production was the same as the rate of galactose formation. With lactose concentrations higher than 50 mM, the rate of galactose production versus the rate of glucose production

decreased rapidly and the formation of trisaccharides and tetrasaccharides occurred. The acidity of reaction mixture and Mg^{2+} ion had effects on enzymatic activities. At higher pH (> 7.8), the transferase/hydrolase activity ratio increased markedly; it decreased at lower pH (< 6.0). At pH value between 6.0 and 7.8, the transferase/hydrolase ratio was relatively constant. The absence of Mg^{2+} decreased the transferase/hydrolase activity ratio. The anomeric configuration of lactose also altered the transferase/hydrolase activity ratio. α-Lactose decreased the ratio, while β-lactose increased the ratio.

Takano and Miwa (133) reported that the enzyme preparation from apricot and elder tree catalyzed the transgalactosidation by the transfer of galactose from PNPG to alcohols (methanol and ethanol) to form alkyl β-galactosides. With lactose serving as the substrate, protozan β-galactosidase could catalyze the transferase reaction (20).

Zilliken and his co-workers (134) found transferace activity of an enzyme preparation from *Lactobacillus bifidus* var *Penn* by the transfer of galactose from lactose to N-acetyl-D-glucosamine to form two disaccharides, 4-O-β-D-galactosyl N-acetyl-D-glucosamine and 6-O-β-D-galactosyl N-acetyl-D-glucosamine (135,136). When crude lactase of yeast was used as an enzyme source, 6-O-β-D-galactosyl N-acetyl-D-glucosamine was the main product. 3-O-β-D-Galactosyl N-acetyl-D-glucosamine was synthesized by the extract from bull testes, with a trace of the 4-isomer. Alessandrini *et al.* (137) furthermore found

an enzyme extract from 5th-day lactating rat mammary glands being able to catalyze the synthesis of three isomers: 3-O-β-D-galactosyl N-acetyl-D-glucosamine (90%), 4-O-β-D-galactosyl N-acetyl-D-glucosamine (9-10%) and 6-O-β-D-galactosyl N-acetyl D-glucosamine (trace) by the transfer of galactosyl residue from lactose to N-acetyl-D-glucosamine. Although the enzymes catalyzing the synthesis of these isomers were not isolated, these transferase reactions are likely catalyzed by β-galactosidase.

Wallenfels and Fisher (138) have purified a β-galactosidase from the intestinal mucosa of calf to 2,100 fold and found this purified enzyme capable of synthesizing allolactose and 3-O-β-D-galactosyl glucose by transgalactosylation reaction in the presence of lactose and glucose. An 'acid' galactosidase purified from bovine testis by Distler and Jourdian (69) also had the transferase activity. The purified enzyme could catalyze the transfer of galactose from PNPG to glucose, N-acetylglucosamine and N-acetylgalactosamine (good acceptors), as well as mannose, L-arabinose and L-fucose (poor acceptors). In addition to free sugar, this enzyme also catalyzed the transfer of galactose from PNPG to nucleotide monosaccharides to yield nucleotide disaccharides (139).

III. MATERIALS AND METHODS

A. Reagents

The following materials were obtained from the indicated sources: p-nitrophenyl β-D-galactoside, o-nitrophenyl β-D-galactoside, p-nitrophenyl α-D-galactoside, p-nitrophenyl β-D-glucoside, p-nitrophenyl α-D-mannoside, p-nitrophenyl α-D-glucoside, p-nitrophenyl N-acetyl-β-D-glucosaminide, p-nitrophenyl β-D-glucuronide, p-nitrophenyl β-D-galactosaminide, phenyl β-D-thiogalactoside, *myo*-inositol, D-raffinose, D-mannose, 3-O-methyl D-glucose, D-fucose, α,α-trehalose, UDP galactose, 1-O-methyl α-D-galactoside, 1-O-methyl β-D-galactoside, D-galactose, lactulose (Grade II, 90%), bovine serum albumin, L-histidine, phosphorylase a (rabbit muscle), β-glucuronidase (bovine liver, type B-3), hemicellulase (Grade II, Rhizopus mold), Trizma Base and 2-amino-2-methyl-1-propanol from Sigma Chemical Co. (St. Louis, MO); lactose and D-glucose from Mallinckrodt Chemical Works (St. Louis, MO); 1-α-galactinol and p-chloromercuribenzoate (PCMB) from Calbiochem (Los Angeles, CA); D-galactono-1,4-lactone and 1-methyl α-D-mannoside from General Biochemicals (Chagrin Falls, OH); D-xylose L-arabinose from Pfanstiehl Chemical Co. (Waukegan, IL); N-acetyl D-galactosamine from Nutritional Biochemicals Co. (Cleveland, OH);

Sephadex G-200 (40-120 μ), aldolase, ovalbumin, chymotrypsinogen A, ribonuclease A and blue dextran 2000 from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ); DEAE-cellulose DE52 (Whatman) from H. Reeve Angel, Inc. (Clifton, NJ); charcoal (Darco G-60) and ethylenediaminetetraacetic acid (EDTA) from Matheson, Coleman and Bell (Norwood, OH); celite from Johns-Manville Products Company (Lompoc, CA); glutamate dehydrogenase, α-mannosidase (Jack Bean), glycerate-3-phosphodehydrogenase and β-galactosidase (*E. coli*) from Boehringa Mannheim; alcohol dehydrogenase from Worthington Biochemical Co. (Freehold, NJ); acrylamide (99%) from Ames Co. (Elkhart, IN); sodium dodecyl sulfate from Pierce Chemical Co. (Rockford, IL); N,N'-methylene bisacrylamide (Bis) and N,N,N',N'-tetramethyl-ethylenediamine (TEMED) from Canal Industrial Co. (Rockville, MD); ammonium persulfate and $K_3Fe(CN)_6$ from Fisher Scientific Co. (Fair Lawn, NJ); bromophenol blue from Allied Chemical Co. (Morristown, NJ); 1-fluro-2,4-dinitrobenzene (FDNB) from Eastman Organic Chemicals (Rochester, NY); ion exchange resin MB-3 from Rohm and Haas (Philadelphia, PA); S & S Collodian Bag (No. 100) from Schleicher and Schuell, Inc. (Keene, NH); Diaflow ultrafiltration membrane UM2 from Amico Co. (Lexington, MA); agarose-p-amino-phenyl-β-D-thiogalactoside from P-L Biochemicals, Inc. (Milwaukee, WI); 3% XE-60 on Gas-Chrom Q (100-120 mesh) and 30% OV-1 on chromosorb W (100-120 mesh) from Applied Science Lab., Inc. (State College, PA); 3% OV-1 on Supelcoport (80-100 mesh) and 5% SP 2401 on Gas-Chrom Z (80-100 mesh) from Supelco, Inc. (Bellefonte, PA).

1-α-Galactosylamine was a gift from Dr. Bernard Axelord. 6-O-α-D-Glucosyl *myo*-inositol was kindly provided by Dr. Robert S. Bandurski who had obtained it from Dr. H. E. Carter.

B. Animals

Frozen rat mammary glands were obtained from 18th-day lactating female Sprague Dawley rats (Spartan Research, Haslett, MI) and stored at -80°C until use.

C. Methods

1. Assays of Hydrolase Activity

The hydrolase activity of β-galactosidase was determined by two alternative methods using p-nitrophenyl-β-D-galactopyranoside as a substrate.

a. Method 1.

The reaction mixture, consisting of 50 mM sodium phosphate-citrate buffer, pH 5.0, 3 mM p-nitrophenyl-β-D-galactopyranoside and enzyme in a final volume of 0.5 ml, was incubated at 37°C for 1 hr. The reaction was terminated by adding 1.0 ml of 2.7% trichloroacetic acid and the precipitate was discarded by centrifugation. An aliquot (1.0 ml) of the supernatant was then added with 1.0 ml of sodium borate-sodium hydroxide solution (0.108 M $Na_2B_4O_7 \cdot H_2O$-0.133 N NaOH) to a final pH of 9.3. The p-nitrophenol ion produced was determined at 410 nm by spectrophotometer $\varepsilon_{410\ nm} = 15.5\ cm^2/\mu mole$.

b. Method 2.

A final volume of 500 µl reaction mixture, generally containing 50 mM sodium citrate buffer, pH 3.4, 5 mM p-nitrophenyl-β-D-galactopyranoside, and enzyme was incubated at 37°C for 10-15 minutes. The reaction was stopped by the addition of 1.0 ml of 1.0 M glycine-NaOH buffer, pH 10.0. The chromogenic product, p-nitrophenol ion, was measured spectrophotometrically at 400 nm. The molar extinction coefficient determined with the standard p-nitrophenol solution was 1.82×10^4. A unit of enzyme was defined as that amount which released 1 nmole of p-nitrophenol from p-nitrophenyl-β-D-galactopyranoside in one minute under the assay condition described above.

2. Assays of Transferase Activity (Lactose: *myo*-Inositol Galactosyltransferase Activity)

For the determination of transferase activity of rat mammary enzyme, in general, two kinds of reaction mixtures were used.

a. Method 3.

The reaction mixture, containing 50 mM sodium acetate buffer, pH 3.6, lactose (175-200 mM), *myo*-inositol (250-375 mM) and enzyme in a final volume of 200 µl, was incubated at 37°C for 4.0 hr and a 50 µl aliquot of the reaction mixture was terminated by the addition of 0.3 ml of 0.3 N $Ba(OH)_2$ and 0.3 ml of 5% $ZnSO_4 \cdot 7H_2O$. A known amount of α,α'-trehalose was added as an internal standard prior to centrifugation at 1,600 x g for 5 minutes. An aliquot of the supernatant was

deionized with MB-3 resin, was dried either by a rotary evaporator or by a nitrogen evaporator, trimethylsilylated with TMS reagent (140), and gas chromatographed at 195°C on a 3 mm x 1.8 m (or 2 mm x 1.8 m) glass column packed with 3% XE-60 on Gas-Chrom Q (100-120 mesh).

b. Method 4.

A reaction mixture of 250 µl containing lactose (200 mM), *myo*-inositol (500 mM), BSA (1 mg/ml) and 50 mM sodium citrate buffer, pH 4.0, was incubated at 37°C for 1-5 hr. It was terminated by adding 0.2 ml of 0.3 N $Ba(OH)_2$ and 0.2 ml of 5% $ZnSO_4 \cdot 7H_2O$) to a 50 µl aliquot of the sample. The rest of the procedures were the same as Method 3. A unit of enzyme was defined as that amount which synthesized 1 nmole of 6-O-β-D-galactopyranosyl *myo*-inositol by the transfer of D-galactose from lactose to *myo*-inositol in one minute under the assay conditions described above.

3. Assay of Protein

The protein was determined by the method of Lowry *et al.* (141) and the method of Murphy and Kies (142), using bovine serum albumin as the standard.

4. Purification Procedures of Rat Mammary β-Galactosidase

Two purification procedures were used.

a. Primary Purification Procedure

Rat mammary gland (45.6 g) was thawed and homogenized (Tekman Tissuemizer) in 90 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose at 0-4°C. The subsequent purification

steps were performed following the procedure of Meisler (67) through the DEAE-cellulose step. A 1.75 l gradient from 0 to 0.3 M NaCl was applied with the eluant buffer (5 mM sodium phosphate, 50 mM Tris Buffer, pH 7.3) on a DEAE-cellulose column (2.5 cm x 39.5 cm) at a flow rate of 0.83 ml/min. The hydrolase activity in each fraction was assayed and the enzyme-rich fractions (8th-17th tubes) were then pooled and the protein was precipitated by the addition of ammonium sulfate at a concentration of 374 g/l. The precipitated protein was stored in a small volume (0.78 ml) of 5 mM sodium phosphate-Tris buffer, pH 6.0, at 4°C until use.

b. Secondary Purification Procedure

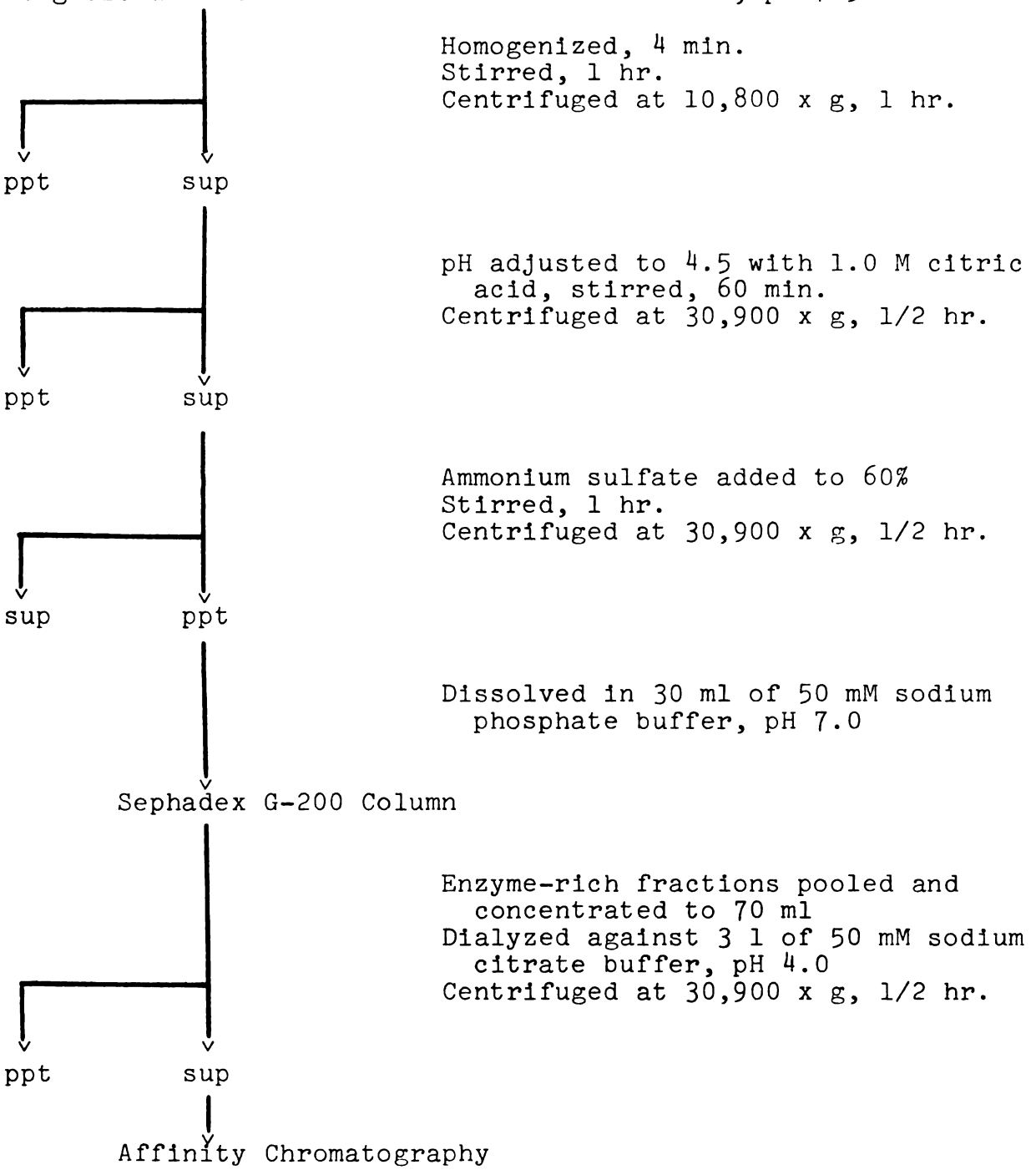
All preparations were carried out at 0-4°C (see Flow Chart 1).

Step 1: Homogenization. Forty grams of rat mammary gland (stored at -80°C) from 18th-day lactating female Sprague Dawley rats were thawed and homogenized in 4 volumes of 20 mM Tris-HCl buffer, pH 7.5, with a Tekmar Tissuemizer for four minutes and gently stirred for one hr. Precipitate was discarded after centrifugation at 10,800 x g, 1 hr in a Sorval RC-2B centrifuge.

Step 2: Acid Precipitation. The supernatant fraction was adjusted to pH 4.5 by gradually adding 1.0 M citric acid and then stirred for one hr. The precipitate was discarded after centrifugation at 30,900 x g for 30 minutes.

Step 3: Ammonium Sulfate Precipitation. The supernatant from Step 2 was brought to 60% saturation with

Flow Chart 1. Purification Procedure of β-Galactosidase from Rat Mammary Glands



$(NH_4)_2SO_4$ and then stirred for one hour. After centrifugation at 30,900 x g for 1 hr, the precipitate was saved and dissolved in an approximate 30 ml of 50 mM sodium phosphate buffer, pH 7.0.

Step 4: Sephadex G-200 Column. A column (5 cm x 88 cm) of Sephadex G-200 was equilibrated with 50 mM sodium phosphate buffer, pH 7.0, overnight. The enzyme preparation from Step 3 was then loaded onto the column and eluted with the same buffer. A flow rate of 18 ml/hr was applied and fractions were collected every 8.6 ml. The activity-rich fractions were pooled for further purification.

Step 5: Affinity Chromatography. A column (1 cm x 15 cm) of agarose-p-aminophenyl-β-thiogalactoside was washed sequentially with 50 mM Tris-HCl buffer, pH 7.5; distilled H_2O and 50 mM sodium citrate buffer, pH 4.0, before use. The enzyme preparation from the Sephadex G-200 step was concentrated to a volume of approximately 70 ml by using Diaflo ultrafiltration membrane UM2 under the pressure of 40 psi and dialyzed against 3 l of 50 mM sodium citrate buffer, pH 4.0, overnight. After centrifugation at 30,900 x g for 30 min, the supernatant was pumped at a flow rate of 18 ml/hr onto the affinity column and followed sequentially with 100 ml of 50 mM sodium citrate buffer, pH 4.0, and 50 ml of 200 mM lactose in the same buffer. The fractions (2 ml) were collected and the hydrolase activity of each fraction was assayed. The activity-rich fractions were pooled, concentrated to a small volume by using S & S collodion bags, and 10% sucrose was

added to protect the enzyme during storage at 4°C.

5. Gel Filtration

The molecular weight of β-galactosidase from rat mammary gland was determined by gel filtration on the Sephadex G-200 columns with two different pH elution buffers.

a. Sodium Phosphate Buffer (pH 7.0)

A column of Sephadex G-200 (2.5 cm x 90 cm) was equilibrated overnight with 50 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. A constant flow rate of 12 ml/hr was applied throughout the experiment and the fraction of 2.25 ml (i.e., 50 drops/tube) were collected. Marker proteins, including 4 mg β-glucuronidase, 7 mg aldolase, 6 mg ovalbumin, 4 mg chymotrypsinogen A and 6 mg ribonuclease A, were prepared in 3 ml of 50 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 6.6% sucrose. To measure column volume, 4.0 mg of blue dextran (Kd = 0) and 1.5 mg of $K_3Fe(CN)_6$ (Kd = 1) were also included in the sample. The elution pattern of β-glucuronidase was determined by the enzyme assay. 500 µl of incubation mixture contained 5 mM p-nitrophenyl β-D-glucuroside, 50 mM sodium acetate buffer, pH 4.8, and 50 µl effluent. The reaction mixtures were incubated at 37°C for 1 hr. The reactions were terminated and the p-nitrophenol ions were measured at 400 nm the same as Method 2. Aldolase (143), Chymotrypsinogen A (143) and ribonuclease A (144) were also determined by enzyme assays. $K_3Fe(CN)_6$, blue dextran and ovalbumin were determined by absorbancy at 420 nm, 630 nm and

280 nm, respectively. The same condition was used when the sample contained only blue dextran, $K_3Fe(CN)_6$ and partially purified β-galactosidase, which was prepared from 4 g mammary gland after the $(NH_4)_2SO_4$ step following the secondary purification procedure β-galactosidase activity in the fractions was determined by hydrolase assay. The distribution coefficient (Kd) for each protein was determined and plotted against molecular weight according to the method of Porath (145).

b. Sodium Citrate Buffer (pH 5.0)

A column of Sephadex G-200 (2.5 cm x 93 cm) was equilibrated overnight with 50 mM sodium citrate buffer, pH 5.0, containing 0.1 M NaCl. A constant flow rate of 12 ml/hr was applied and 3.05 ml/fraction was collected. The same marker proteins except aldolase, which was dissociated under this condition, and β-galactosidase preparation as the above, were used.

6. Disc Gel Electrophoresis

Disc gel electrophoresis was performed at pH 4.3 on 7% gels which were prepared as follows:

Stock solutions: (1) Concentrated AcBis solution: 28 g Acrylamide; 0.735 g Bis; H_2O to 100 ml; (2) Buffer, pH 4.3: 48 ml of 1 N KOH; 17.2 ml glacial acetic acid; 1.15 ml TEMED; H_2O to 100 ml; and (3) Ammonium persulfate solution: 2.8 g was dissolved to 100 ml H_2O.

The gels (0.5 cm x 10.5 cm) were prepared by the combination of 2 parts of solution (1), 4 parts of solution (2) and

2 parts of solution (3).

Electrophoresis solution: (1) Stock solution (pH 4.5): 31.2 g β-alanine; 8 ml glacial acetic acid; H_2O to 1 l; (2) Dilution of the stock solution (1:10) was prepared for use.

Electrophoresis procedure: 20 µg of purified enzyme (affinity-column fraction) was applied to each gel. The electrophoresis was performed at constant current (4 mA/gel) and constant temperature (4°C) for 8.5 hr. One gel was frozen at -20°C for 20 min after the electrophoresis and cut into 1.35 mm slices and assayed for hydrolase activity in a 2.0 ml reaction mixture containing 0.1 M sodium citrate buffer, pH 3.6, and 2.5 mM PNPG and one slice of gel. The incubation mixture was incubated at 37°C for 11.5 hr and terminated by the addition of 1.0 ml of 0.1 M glycine-NaOH buffer, pH 10, and measured at 400 nm spectrophotometrically. The other gel was stained with coomassie blue (146) to determine the location of protein on the gel and stored in 7.5% acetic acid.

7. SDS Gel Electrophoresis

The SDS gel electrophoresis of purified enzyme and the maker proteins was carried out in the parallel gels according to the procedure of Fairbanks *et al.* (147). The amount of protein was used as follows: 26 µg purified enzyme (affinity-column fraction), 10 µg β-galactosidase (*E. coli*), 25 µg phosphorylase a (muscle), 10 µg BSA, 10 µg glutamate dehydrogenase (liver), 20 µg alcohol dehydrogenase (liver), 10 µg aldolase, 10 µg ovalbumin, 10 µg glyceraldehyde-3-phosphodehydrogenase

(muscle) and 10 µg chymotrypsinogen A. The electrophoresis was performed at a constant current of 8 mA/gel for 2.5 hr, using bromophenol blue as a tracking dye.

8. Isolation of 6-O-β-D-Mannosyl *myo*-Inositol

The cells of *Saccharomyces cerevisiae* (Anheuser-Busch, Inc., St. Louis, MO) were grown aerobically in 10 l of sterilized medium (148) at 30°C for 40 hr. The cells (260 g wet weight) were harvested by centrifugation at 15,000 rpm for 20 min. The mannosyl *myo*-inositol was isolated essentially according to the method of Tanner (149). The washed cells were heated in 300 ml of H_2O at 90°C for 20 min and then centrifuged at 15,000 rpm for 10 min. The supernatant fraction was mixed with 0.9 g of charcoal for 30 min and filtered through a layer of celite. The charcoal was washed with 80 ml of 10% ethanol. About 380 ml of filtrate (including 80 ml of 10% ethanol) was dried under vacuum, resuspended in H_2O, then pumped into a column (4.5 cm x 50 cm) of charcoal-celite (1:1 w/w). After the column was washed with 8.2 l of H_2O, mannosyl *myo*-inositol was eluted with 2% ethanol. The mannosyl *myo*-inositol-rich fractions (39th-44th fractions) were concentrated to a small volume and streaked onto the sheets (25 cm x 45 cm) of Whatman 3MM paper and chromatographed by an ascending technique using four passes of a solvent system consisting of n-butanol-pyridine-water-acetic acid (6:4:3:0.3 by volume). Sugars were detected by the silver nitrate procedure (150). The band moving just ahead of authentic l-α-galactinol was

eluted from the paper with water and saved for further analysis.

9. Identification of 6-O-α-D-Mannosyl *myo*-Inositol

The purified disaccharide was fully trimethylsilated (140) and analyzed by gas chromatography on a 3 mm x 1.8 m glass column packed with 5% SP 2401 on Gas Chrom Z (80-100 mesh) at 240°C. The purified sugar was also hydrolyzed in 2 N H_2SO_4 at 100°C for 1.5 hr. After neutralization with 0.3 N $Ba(OH)_2$ and deionization with MB-3 Resin, the sample was dried under vacuum at 40-50°C and trimethylsilylated (140) with an internal standard of 1-methyl α-D-mannosyl pyranoside. Permethylation, hydrolysis and reduction of the hydrolysis products were carried out as previously published (151-153). Combined gas-liquid chromatography-mass spectrometry of the permethylated sugar and its hydrolysis-reduction procudts were recorded at an electron energy of 70 ev with an LKB mass spectrometer. The relative abundance of fragments was displayed as bar graphs by means of an on-line data acquisition and processing program (154). The source temperature was 290°, accelerating voltage 3.5 KV, and theionizing current 60 μA. The sample was introduced via the inlet of a gas-chromatograph by using a glass coil (3 mm x 1.8 m) packed with 3% OV-1 on Supelcoport (80-100 mesh). The column temperature was 240°C.

Enzymatic hydrolysis of purified disaccharide was carried out by jack bean α-mannosidase or hemicellulase. The reaction mixture, consisting of 100 mM sodium acetate buffer, pH 4.6, 10 mM $ZnSO_4$, and 50 units (μmoles p-nitrophenol produced/min)

of α-mannosidase (or 4 units hemicellulase), was incubated at 37°C for 6 hr (or 24 hr). The reaction was terminated by boiling for 3 min. After the addition og galactinol as an internal standard, the sample was deionized with MB-3, dried and gas chromatographed at 240°C on a 3 mm x 1.8 m glass column packed with 5% SP 2401 on Gas-Chrom Z (80-100 mesh).

IV. RESULTS

A. Purification of Rat Mammary β-Galactosidase

The purification of β-galactosidase from the 18th-day lactating rat mammary gland through the primary purification procedure is summarized in Table 1. The hydrolase activity was purified 22-fold with recovery of 3% of the original activity. The transferase activity was increased 27-fold with recovery of 4% of the original activity. The ratio of the specific activity of hydrolase reaction versus the specific activity of transferase reaction in each purification step approached a constant of 2. Prior to the DEAE-cellulose step, hydrolase activity was purified only 2.5-fold and transferase activity 2.8-fold. The DEAE-cellulose chromatography of rat mammary enzyme was shown in Figure 2. Only a single peak of β-galactosidase activity was observed. This activity peak was eluted with the first peak of protein at a concentration of near 0.05 M NaCl.

The purification of rat mammary β-galactosidase following the secondary purification procedure is summarized in Table 2. β-Galactosidase (hydrolase activity) was purified 640-fold with recovery of 9% of original activity. Prior to step 5, hydrolase activity increased only 4.3-fold. However, about 150-fold

Table 1. Primary Purification of β-Galactose from Rat Mammary Glands[a]

Fraction	Total Volume ml	Protein Conc. mg/ml	Total Protein mg	Total Units p-NP[b] nmole/min	Total Units 6-β-G[c] nmole/min	Sp. Activity p-NP nmole/min/mg	Sp. Activity 6-β-G nmole/min/mg	Sp. A. p-NP / Sp. A. 6-β-G
Crude Homogenate	107	36.2	3,870	7,550	3,330	1.95	0.86	2.27
1st $(NH_4)_2SO_4$ ppt.	38.3	50.8	1,840	4,520	2,370	2.46	1.29	1.91
Solvent ppt.	37.3	5.4	201	740	410	3.68	2.06	1.79
2nd $(NH_4)_2SO_4$ ppt.	2.8	51.7	145	720	350	4.93	2.43	2.03
DEAE-cellulose	0.9	5.7	5	210	210	42.60	23.20	1.84

[a] The enzyme was purified following primary purification procedure described in the Methods section.

[b] Hydrolase assay was carried out by method 1 described in the Methods section. p-NP represents p-nitrophenol which is the product of the hydrolysis of PNPG.

[c] Transferase assay was carried out by method 3 described in the Methods section. The incubation reaction contained *myo*-inositol (250 mM) and lactose (175 mM). 6-β-G represents 6-β-galactinol.

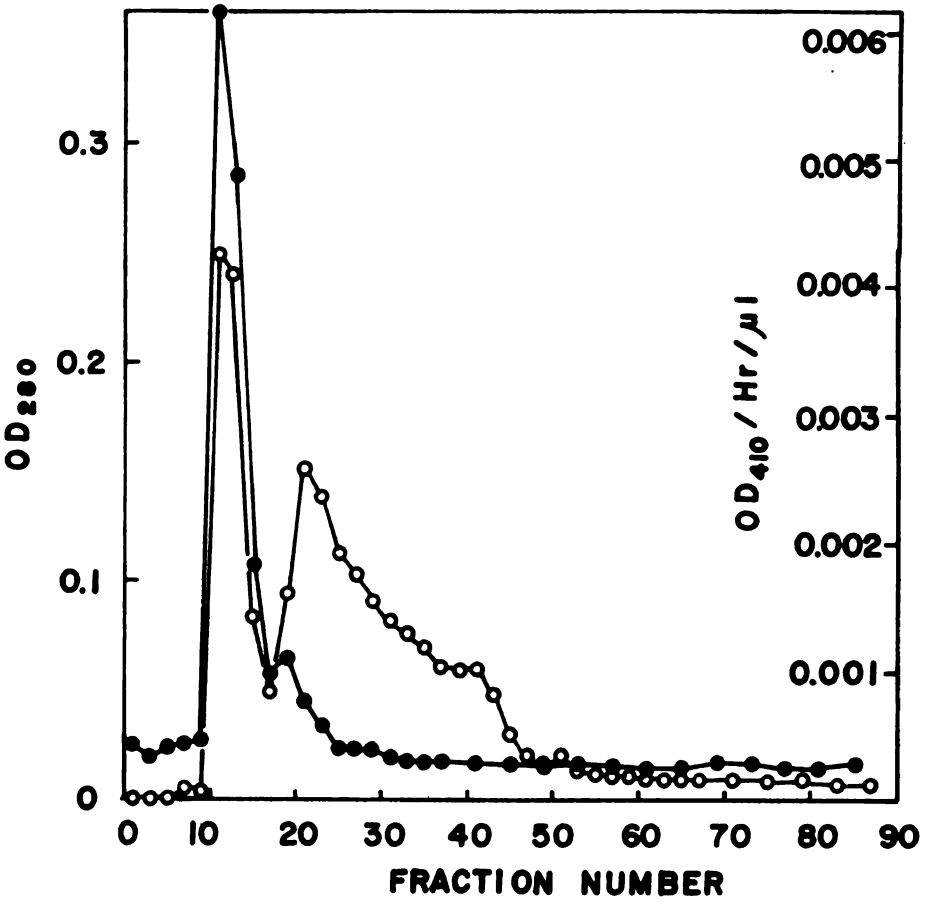


Figure 2. DEAE-cellulose chromatography of rat mammary-gland β-galactosidase. The partially purified enzyme (2nd $[NH_4]_2SO_4$ fraction, table 1) dialyzed against 5 mM phosphoric acid-Tris buffer, pH 6.0, was applied to a column (2.5 x 39.5 cm) of DEAE-cellulose. The proteins were eluted with a sodium chloride gradient (1.75 L) from 0 to 0.3 M in 5 mM phosphoric acid-50 mM Tris buffer, pH 7.3. Hydrolase assays were carried out as described in method 1. o——o, protein (OD_{280}); ●——●, hydrolase activity (OD_{410}/hr/µl).

Table 2. Secondary Purification of β-Galactosidase from Rat Mammary Glands[b]

Fraction	Volume (ml)	Total Protein (mg)	Total Units[b] (nmole/min)	Sp. Activity (units/mg)	Recovery %
Crude Homogenate	169	2,324	6,498	2.8	100
Extraction, pH 4.5	158	1,035	4,427	4.3	68
Ammonium Sulfate, 60%	34	679	4,051	6.0	62
Sephadex G-200	279	262	3,104	11.9	48
Affinity Chromatography	0.52	0.34	613	1,802.9	9

[a]The enzyme was purified following secondary purification procedure described in the Methods section.

[b]Hydrolase assay was carried out by method 2. The product of hydrolysis (p-NP) was measured at OD_{400} by spectrophotometer.

purification was obtained through the affinity-chromatography step.

Some proteins which had either higher or lower molecular weight than β-galactosidase were eliminated through Sephadex G-200 chromatography, while only the fractions within the bar were used for the further purification steps (Figure 3). In the affinity chromatography of the rat mammary enzyme, most proteins were eluted with sodium citrate buffer at pH 4.0. However, β-galactosidase was retained on the column until 200 mM lactose with the same buffer was applied to the column (Figure 4). After the affinity column, the purified enzyme was investigated and compared with the crude enzyme at original homogenate by using several p-nitrophenyl glycosides as the substrates (Table 3). The glycosidases examined were companion lysosomal enzymes, and in the homogenate preparation, N-acetyl β-glucosaminidase and β-glucuronidase had much higher activities than that remaining after the affinity column step. The remaining enzymes determined, including β-glucosidase, β-galactosaminidase, α-mannosidase, α-glucosidase, α-galactosidase, were also virtually eliminated after affinity-chromatography.

B. Stability

Rat mammary β-galactosidase was not stable in very acidic condition, either as the purified enzyme or the crude extract (Figure 5). When the pH was below 2.5, the samples

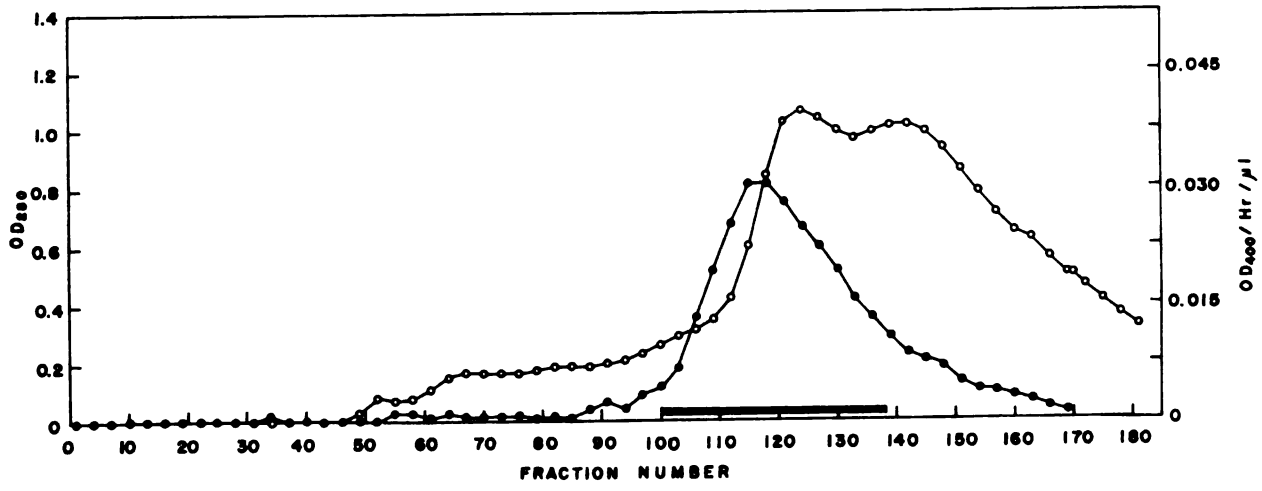


Figure 3. Sephadex G-200 filtration of rat mammary-gland β-galactosidase. An enzyme preparation from $(NH_4)_2SO_4$ step was applied to a Sephadex column (5 x 88 cm). The proteins were eluted with 50 mM sodium phosphate buffer, pH 7.0, at a flow rate of 18 ml/hr. Hydrolase assays were carried out by method 2. o——o, protein (OD_{280}); ●——●, activity (OD_{400}/hr/µl).

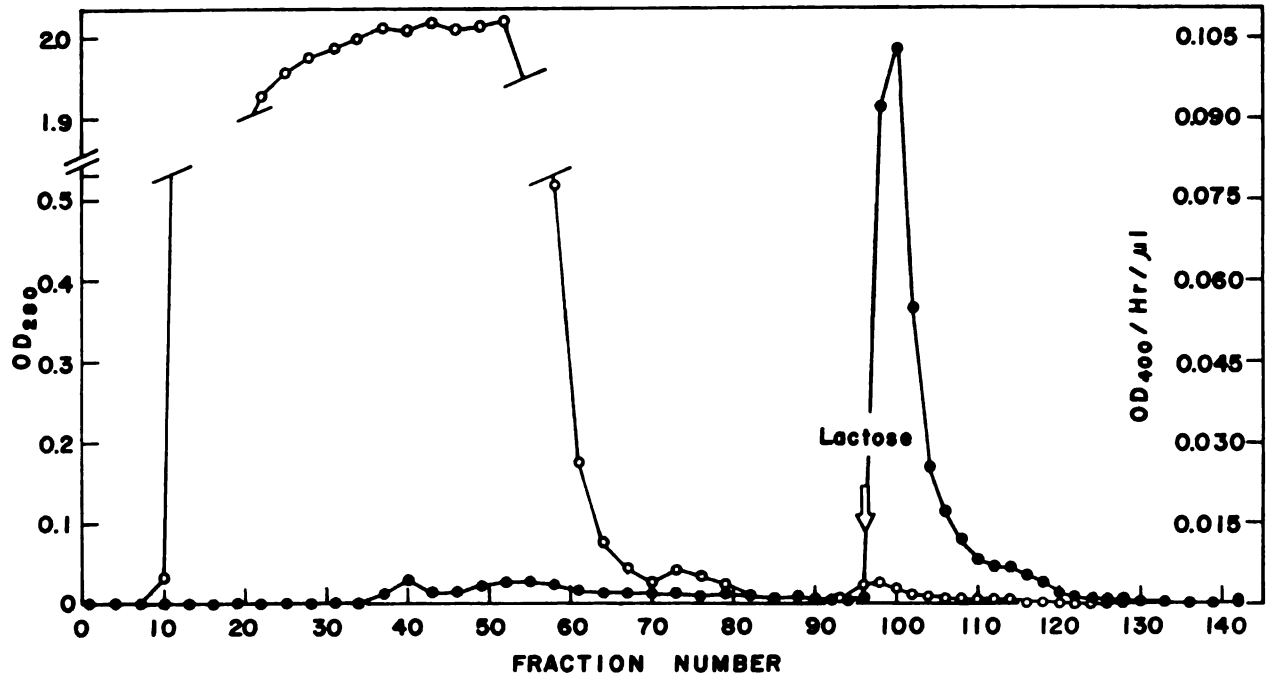


Figure 4. Affinity chromatography of β-galactosidase. The sample from Sephadex-column fraction (table 2) was concentrated, dialyzed against 50 mM sodium citrated buffer, pH 4.0, overnight and applied to a column (1 x 15 cm) of agarose-p-amino-phenyl-β-D-thiogalactoside. The proteins were eluted with 100 ml 50 mM sodium citrated buffer, pH 4.0, and then 50 ml of 200 mM lactose in the same buffer was used to elute β-galactosidase at a flow rate of 18 ml/hr; 2 ml fractions were collected. Hydrolase activity was determined as described in method 2. o——o, protein (OD_{280}); ●——●, activity (OD_{400}/hr/µl).

Table 3. Substrate Specificity of Purified Enzyme

Substrates (p-Nitrophenyl)	Conc. (mM)	Relative Activity	
		Homogenate[a] (%)	Purified Enz.[a] (%)
β-D-galactoside[b]	5.0	100	100
N-acetyl-β-D-glucosaminide	3.6	248	0.8
β-D-glucuronide	5.0	275	0
β-D-glucoside	5.0	29	0.5
N-acetyl-β-D-galactosaminide	2.4	20	0
α-D-mannoside[c]	3.6	26	0.3
α-D-glucoside	5.0	6	0.1
α-D-galactoside	5.0	38	0.3

[a]Homogenate and purified enzyme were taken from crude-homogenate fraction and affinity-chromatography fraction as described in table 2.

[b]Assays of β-galactosidase, N-acetyl-β-glucosaminidase, β-glucuronidase, β-glucosidase, α-galactosidase and N-acetyl-β-galactosaminidase were carried out in the following buffers: sodium citrate buffer, pH 3.4; sodium citrate buffer, pH 4.4; sodium acetate buffer, pH 4.8; sodium citrate buffer, pH 5.0; sodium citrate buffer, pH 5.2; and sodium citrate buffer, pH 4.6, respectively.

[c]α-Mannosidase activity was assayed in sodium citrate buffer, pH 4.6, containing 0.1 mM $ZnCl_2$. α-Glucosidase was carried out in sodium citrate buffer, pH 4.0, with 0.2 M KCl.

[b,c]All the buffers had a concentration of 50 mM. The reaction mixture was incubated at 37°C for 15 min (purified enzyme) or 17 min (homogenate). p-Nitrophenol produced was measured at OD_{400} by spectrophotometer as described in method 2.

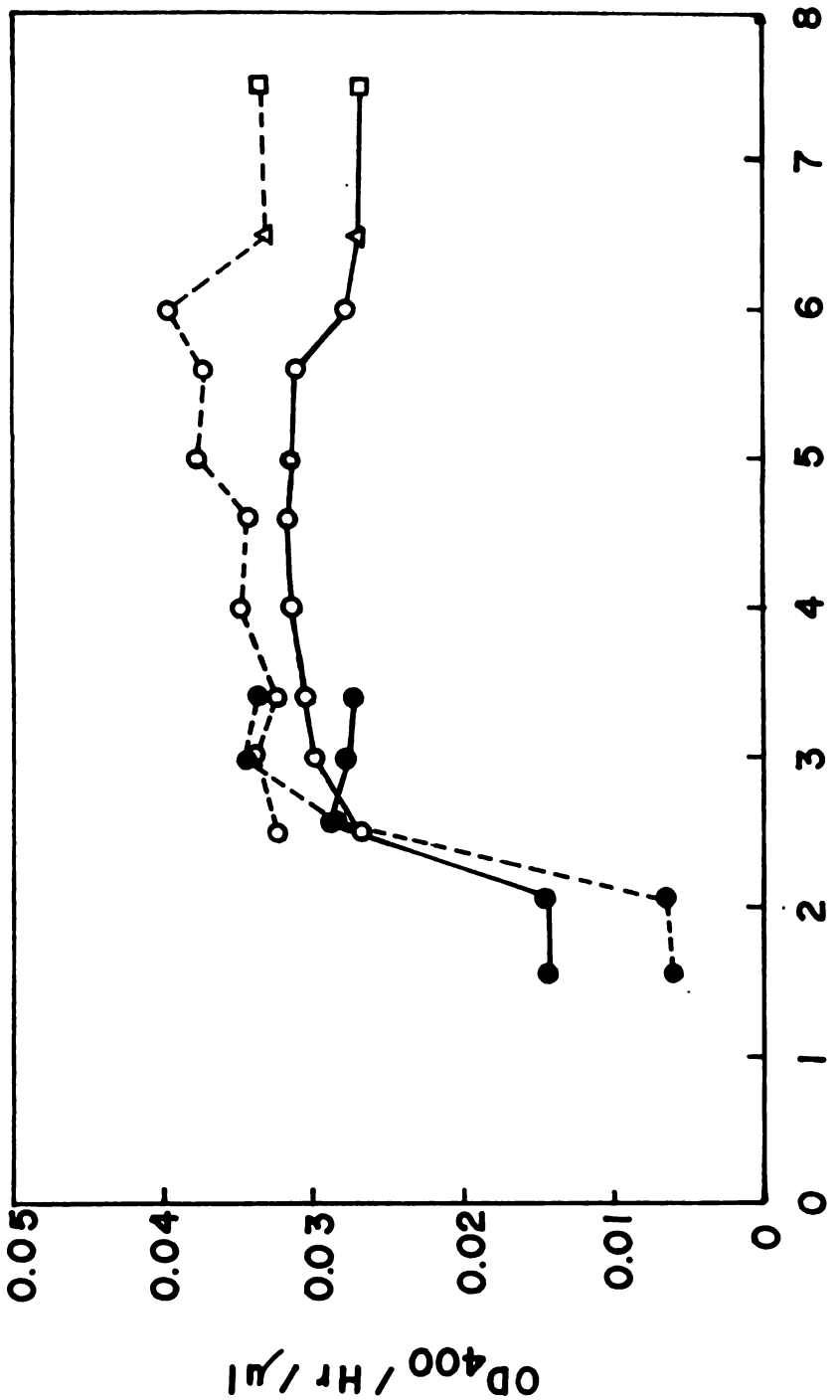


Figure 5. Effect of pH on β-galactosidase stability. Purified enzyme (——, affinity-column fraction) and homogenate (---) were stored in different pH buffer at 4°C, 18 hrs. The samples were brought back to pH 3.0. The residual hydrolase activity was determined in 50 mM Glycine-HCl buffer, pH 3.0, with 5 mM PNPG as substrate. (●), 50 mM Glycine-HCl buffer; (o), 50 mM sodium citrate buffer; (Δ), 50 mM sodium phosphate buffer; (□), 50 mM Tris-HCl buffer.

lost about two thirds of its activity after 18 hr storage at 4°C. However, when the pH was above 2.5, the stability of the enzyme was similar up to 7.5. However, if 10% sucrose was added, the enzyme could be kept in 50 mM sodium citrate buffer, pH 4.0, at 4°C for several months and only small amounts of activity were lost.

C. pH Optima

Four different buffer systems were tested to determine the pH profile of hydrolase activity with either p-nitropehnyl β-D-galactoside (Figure 6) or o-nitrophenyl β-D-galactoside (Figure 7) as the substrate. The rat enzyme displayed high hydrolase activity from pH 2.5 to 4.5. When p-nitrophenyl β-D-galactoside was used as a substrate, the enzyme had a pH optimum of 3.4 in sodium citrate buffer, and a pH optimum of 2.7-3.0 in glycine-HCl buffer. When o-nitrophenyl β-D-galactoside served as substrate, the pH optimum was about the same as that for PNPG in glycine-HCl buffer. In sodium citrate buffer, the mammary β-galactosidase had a pH optimal plateau from 2.5 to 4.5 and highest hydrolase activity at 3.0 by using ONPG as the substrate.

The pH profile of transferase activity of rat mammary β-galactosidase was also investigated. Purified enzyme (DEAE-cellulose fraction) exhibited a pH optimum of around 4.0 when sodium citrate buffer was used (Figure 8). While at a pH below 3.4 or above 4.6, the activity dropped rapidly. However,

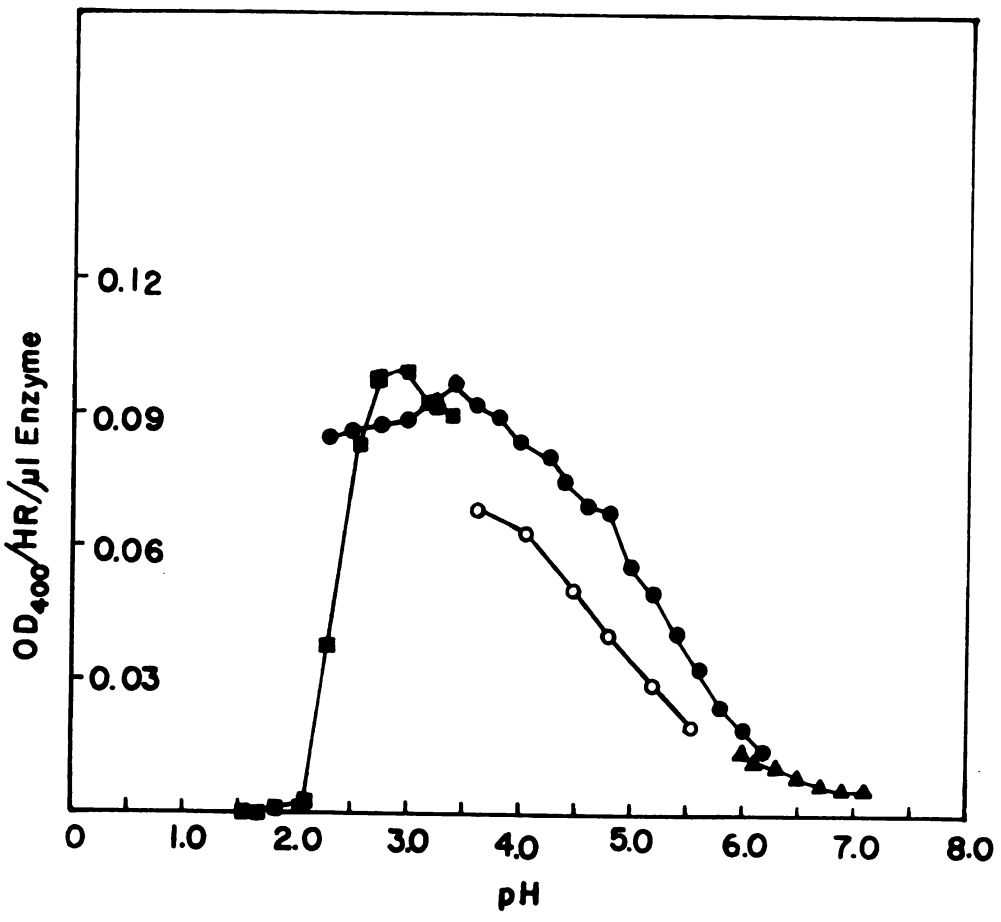


Figure 6. Effect of pH on hydrolase activity of β-galactosidase with PNPG as a substrate. The reaction mixtures consisting of 50 mM buffer, 5 mM PNPG and 20 µl crude enzyme were incubated at 37°C for 35 or 70 min. Hydrolase activity was then determined as described in method 2. (■——■), glycine-HCl buffer; (o——o), sodium citrate buffer; (●——●), sodium acetate buffer; (▲——▲), sodium phosphate buffer.

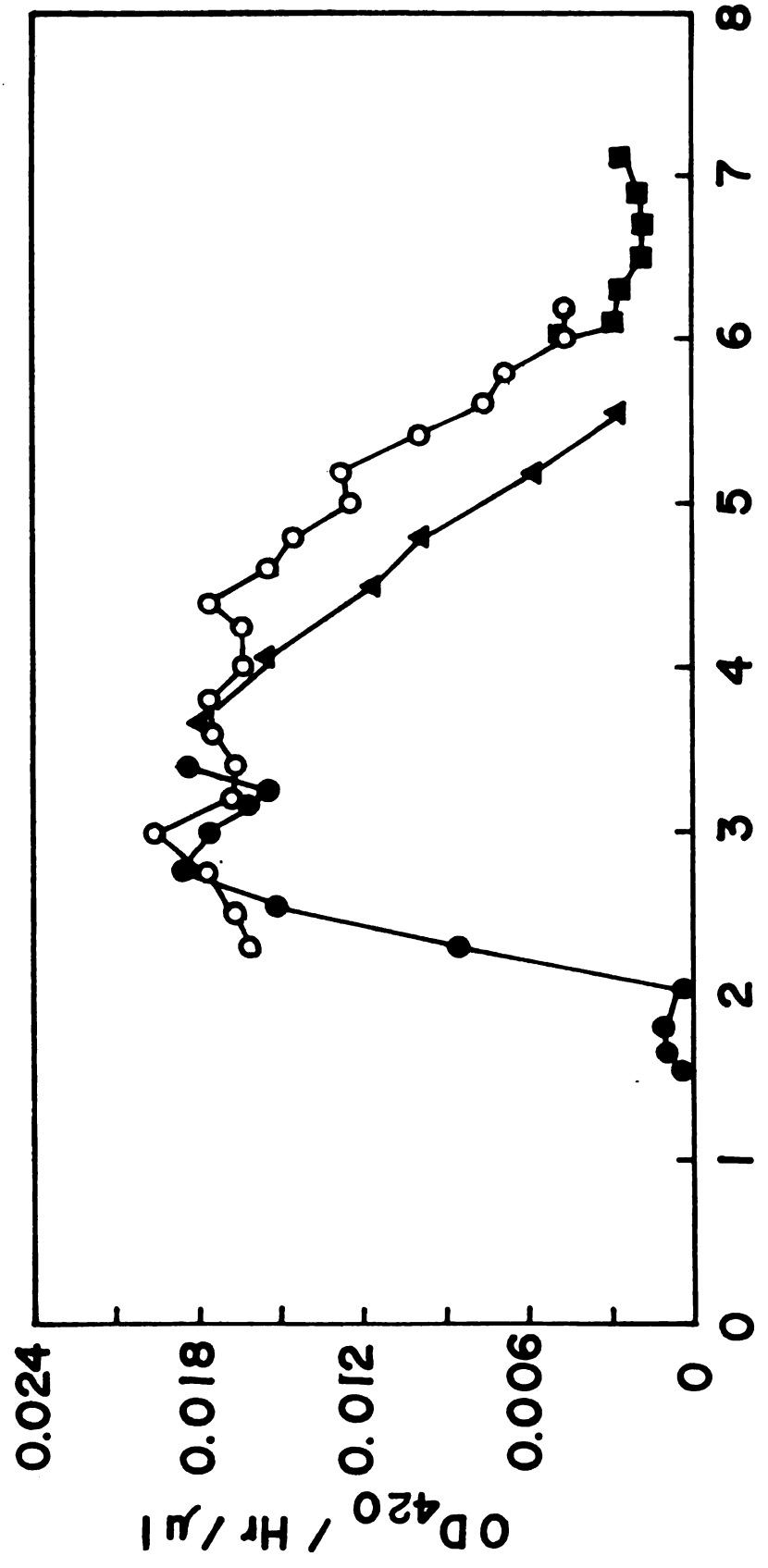


Figure 7. Effect of pH on hydrolase activity of β-galactosidase with ONPG as a substrate. Reaction mixtures consisting of 50 mM buffer, 5 mM ONPG and 10 µl crude enzyme were incubated at 37°C for 30 or 60 min. Hydrolase activity was then determined as described in method 2. (●——●), glycine-HCl buffer; (o——o), sodium citrate buffer; (Δ——Δ), sodium acetate buffer; (■——■), sodium phosphate buffer.

with crude extract as the source of β-galactosidase, transferase activity was still high even at a pH down to 3.0, but at a high pH, β-galactosidase loss transferase activity dramatically (Figure 9). The pH profile of transferase activity of purified enzyme (affinity-chromatography fraction) was also determined in the presence of 1 mg/ml of BSA in the same four buffer systems previously studied (Figure 10). The enzyme activity was almost totally lost below pH 2.0. The highest activity was observed from pH 2.5 to 3.0 in glycine-HCl buffer, and there was a plateau of transferase activity from 2.0 to 4.0 in sodium citrate buffer. The enzyme activity decreased while pH increased above 4.0.

D. Effect of Incubation at 37°C on the Activities of Rat Mammary β-Galactosidase

The hydrolase activity of purified enzyme (affinity-chromatography fraction) was linear with the time course of incubation at 37°C at least up to 15 min and then gradually lost (Figure 11). On the other hand, in the presence of 1 mg/ml BSA, 200 mM lactose and 500 mM *myo*-inositol, the transferase activity of purified enzyme (affinity-chromatography fraction) was linear at 37°C for 5 hr (Figure 12).

E. Molecular Weight

The molecular weight of β-galactosidase was determined by gel filtration on Sephadex G-200 column with standard

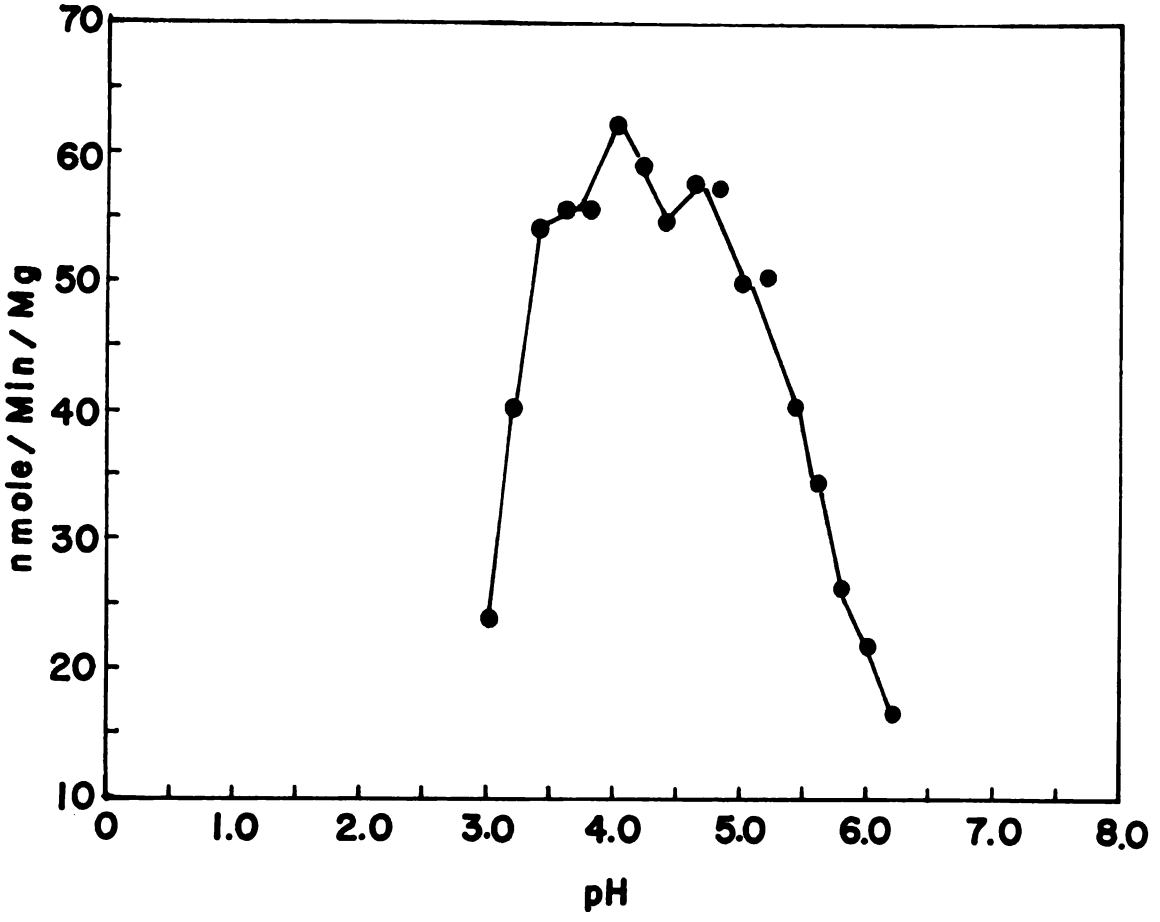


Figure 8. Effect of pH on transferase activity of purified β-galactosidase. Reaction mixtures containing 50 mM sodium citrate buffer, 200 mM lactose, 675 mM *myo*-inositol and 5 µl purified enzyme (DEAE-cllulose-column fraction) were incubated at 37°C for 6 hr. 6-β-Galactinol was determined by gas chromatography with α,α-trehalose as an internal standard. (●——●), transferase activity (nmole 6-β-galactinol/min/mg).

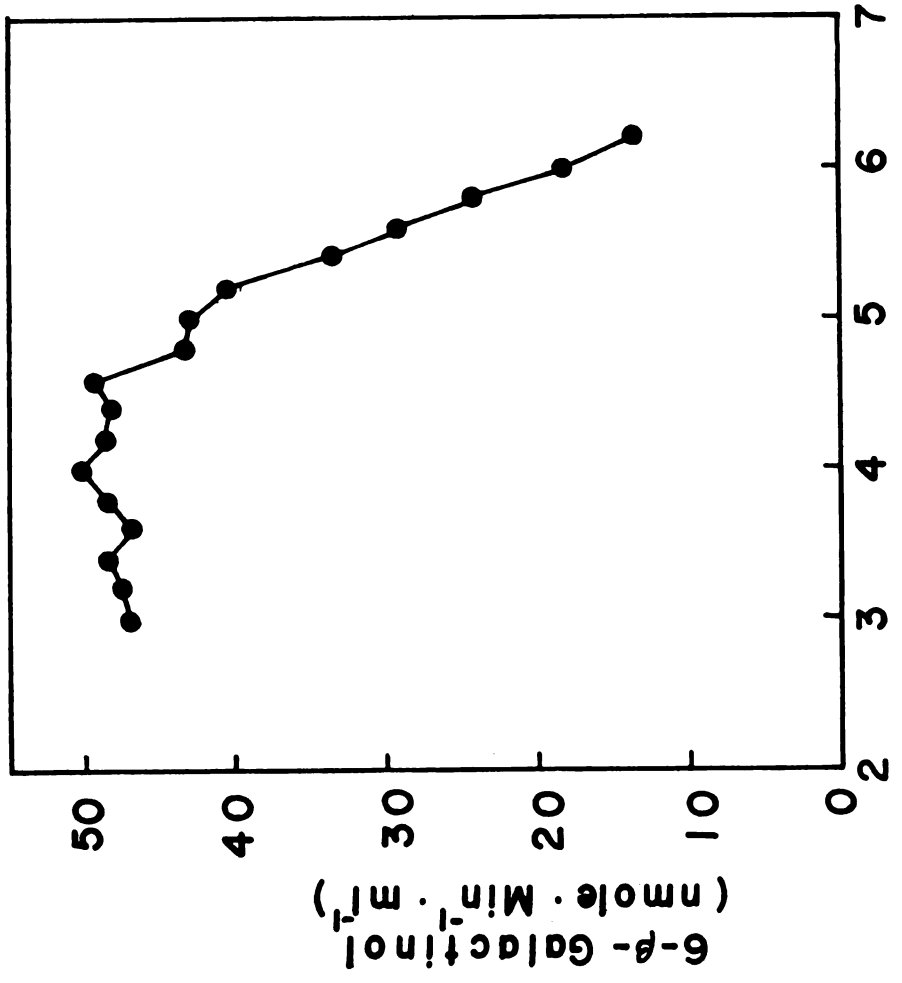


Figure 9. Effect of pH on transferase activity of crude β-galactosidase. Reaction mixtures contained 50 mM sodium citrate buffer, 150 mM lactose, 650 mM *myo*-inositol and crude enzyme. Samples were incubated at 37°C for 6 hr. 6-β-Galactinol was determined by gas chromatography with α,α-trehalose as an internal standard.

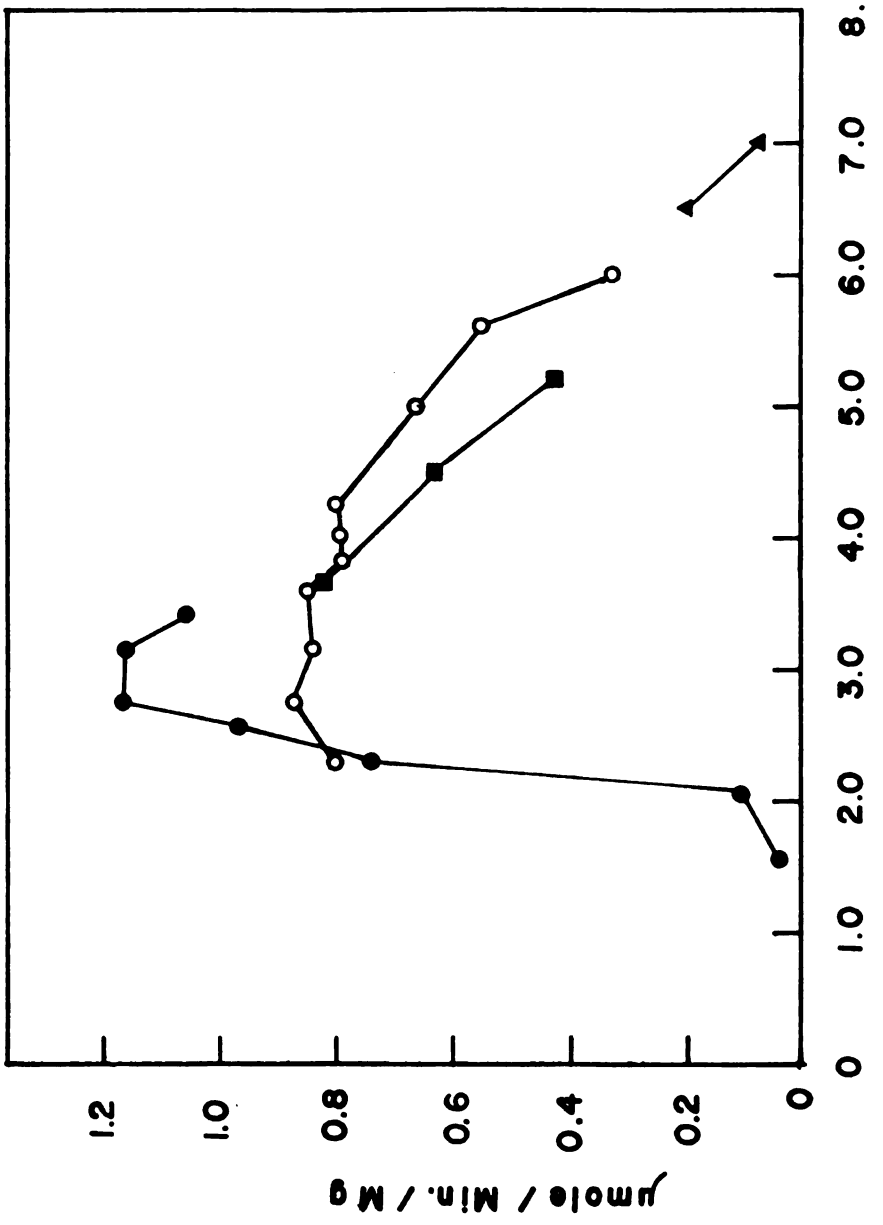


Figure 10. Effect of pH on transferase activity of purified β-galactosidase. The reaction mixtures consisting of 50 mM buffer, 200 mM lactose, 500 mM *myo*-inositol and 2 μl pufified enzyme (affinity-chromatography column) were incubated at 37°C, 4.5 hr. The amount of 6-β-galactinol synthesized was determined by gas-liquid chromatography as described in method 4. (●——●), glycine-HCl buffer; (o——o), sodium citrate buffer; (■——■), sodium acetate buffer; (▲——▲) sodium phosphate buffer.

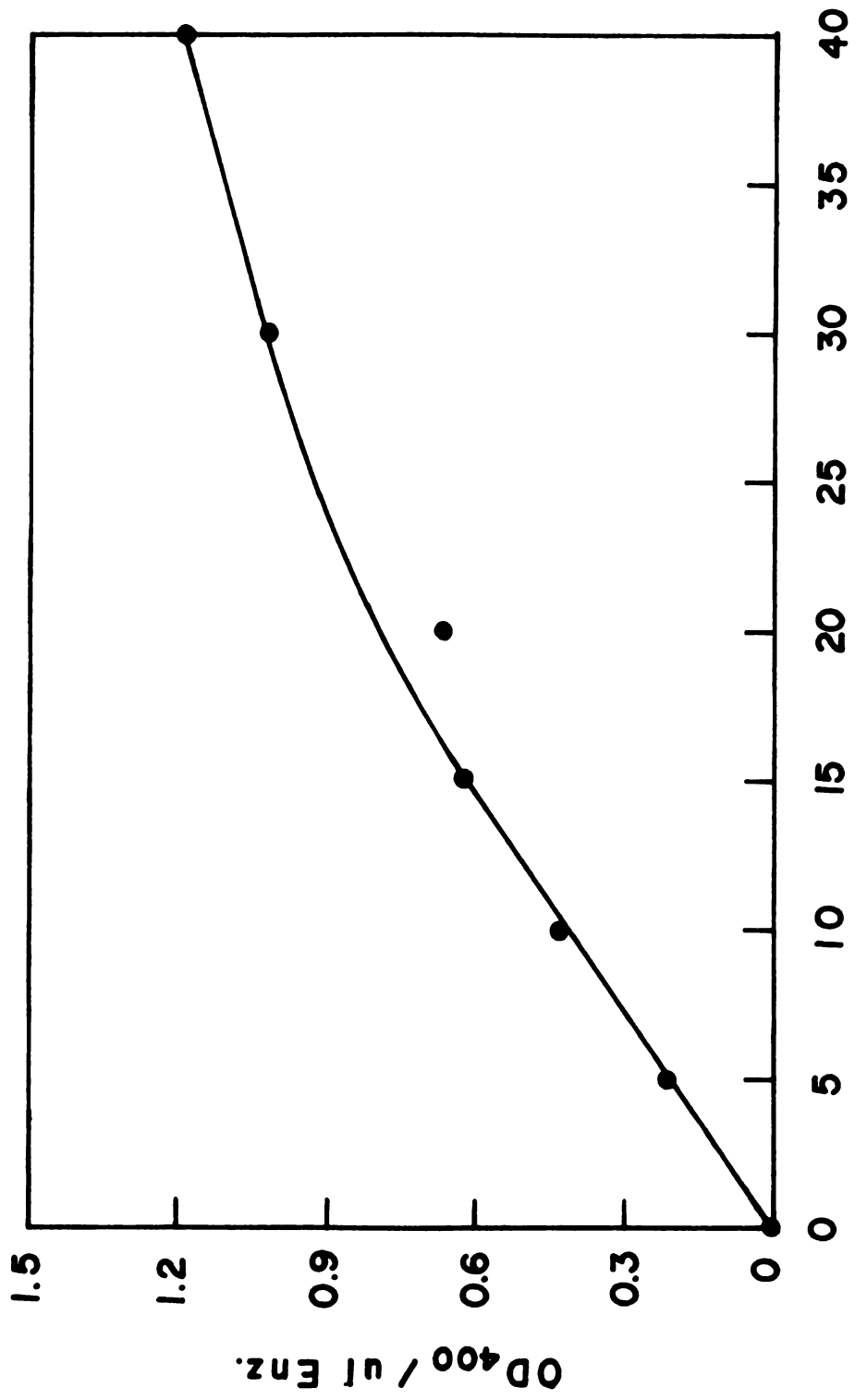


Figure 11. Effect of incubation at 37°C on the hydrolase activity of β-galactosidase. The reaction mixtures (500 µl) contained 50 mM sodium citrate buffer, pH 3.4, 5 mM PNPG and 1 µl purified enzyme (affinity-chromatography fraction). The assays were carried out the same as described in method 2.

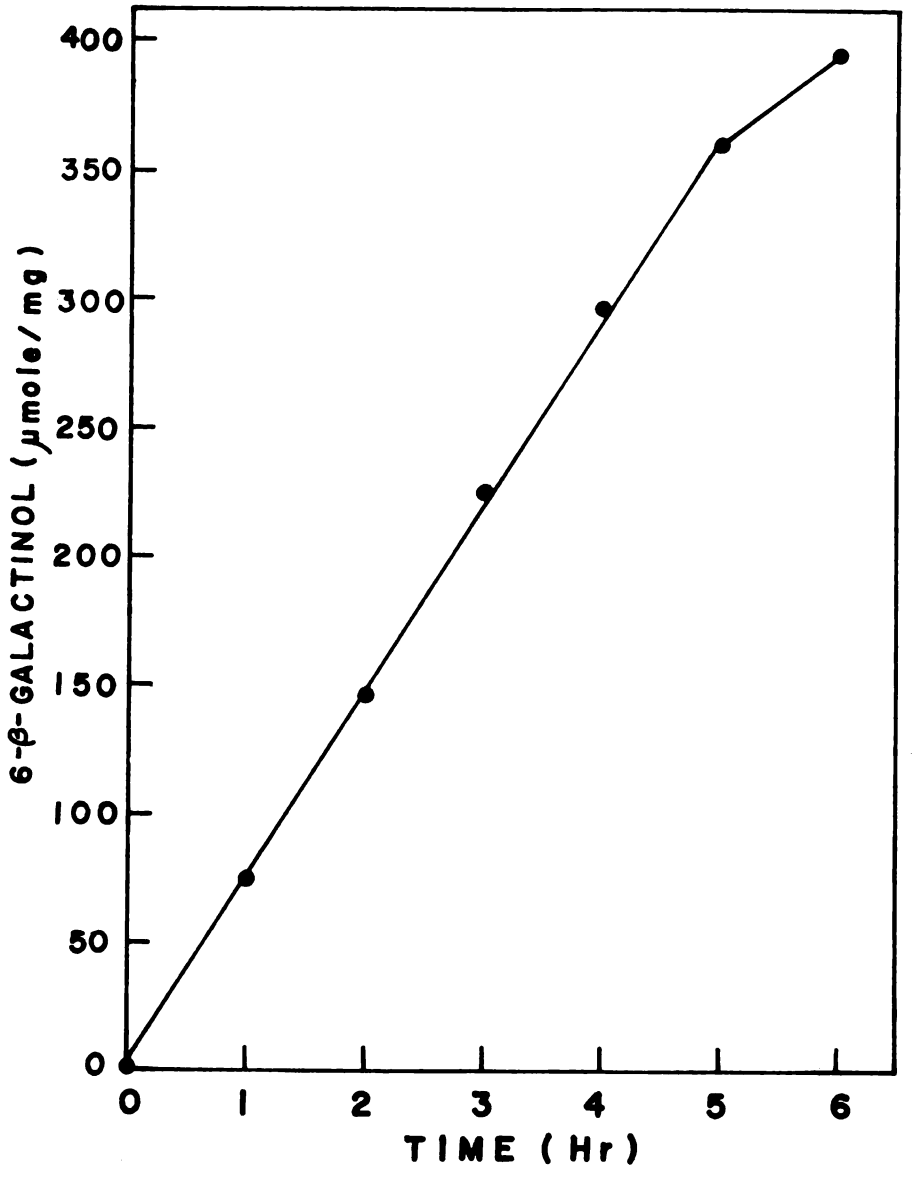


Figure 12. Effect of incubation at 37°C on the transferase activity of β-galactosidase. The reaction mixtures (250 μl) included 50 mM sodium citrate buffer, pH 4.0, 200 mM lactose, 500 mM *myo*-inositol, 1 mg/ml BSA and 2 μl purified enzyme (affinity-chromatography fraction). The assays were carried out the same as described in method 4.

proteins of known molecular weights. Chromatography was carried out in the presence of 0.1 N NaCl with different pH buffers. The elution pattern of β-galactosidase and standard proteins from Sephadex G-200 at neutral pH was investigated. The relationship between the elution volume of each protein and its molecular weight was plotted according to the method of Porath (145). From this plot a molecular weight of 110,000 could be estimated for rat mammary β-galactosidase at pH 7.0 (Figure 13). However, the molecular weight determined by gel filtration on Sephadex G-200 at pH 5.0 was different from that at pH 7.0 and had a value of 200,000 (Figure 14).

F. Subunit Molecular Weight

The subunit molecular weight of rat mammary β-galactosidase was determined by SDS-gel electrophoresis. Several proteins of known subunit molecular weight were treated as standards. The relationship between the log of molecular weight of subunits and the relative mobility of each protein was plotted in Figure 15. In addition to the major band, two minor bands were observed on the gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 16). The molecular weight of 63,000 of the major band was estimated according to the plot of Figure 15. Two minor bands had molecular weights of 52,000 and 43,000, respectively.

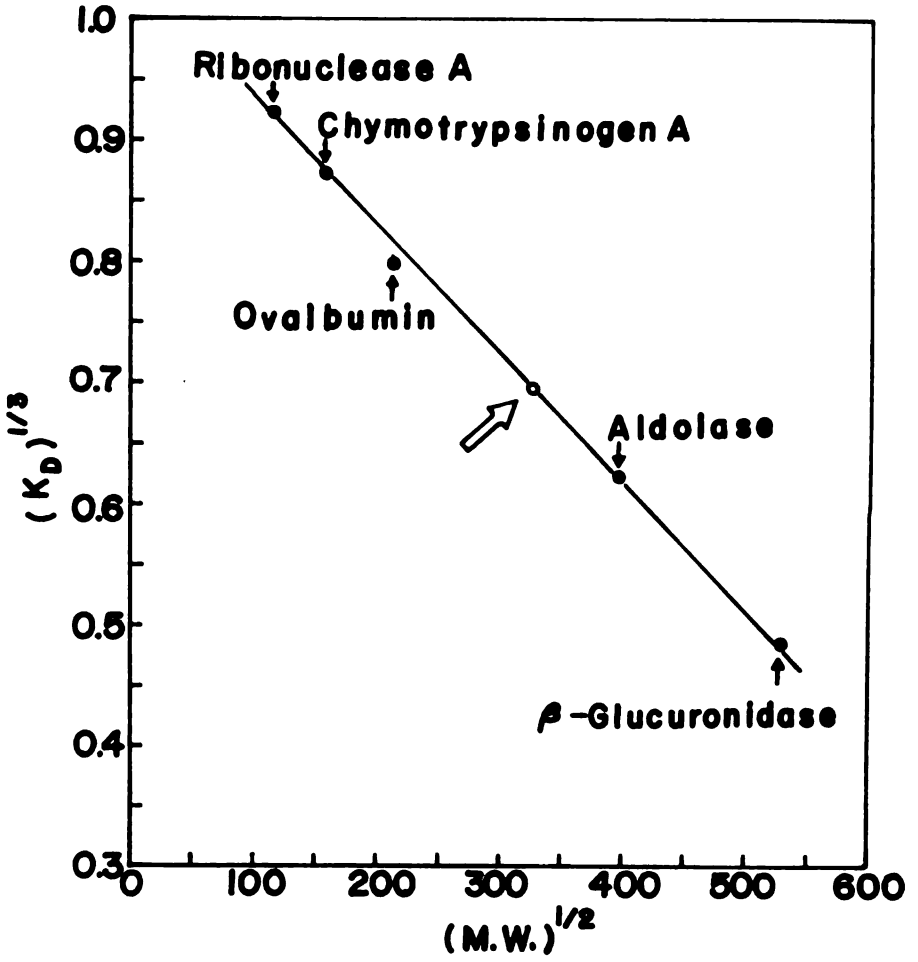


Figure 13. Gel filtration of β-galactosidase on Sephadex G-200 at pH 7.0. 3 ml of sample containing β-galactosidase (ammonium-sulfate fraction, table 2) was chromatographed on a column (2.5 x 90 cm) of Sephadex G-200 as described in the Methods section. The following proteins were used as markers: (a) β-glucuronidase (M.W.-280,000); (b) aldolase (M.W.-158,000); (c) ovalbumin (M.W.-45,000); (d) chymotrypsinogen (M.W.-25,000); and (e) ribonuclease A (M.W.-13,700). The column was eluated with 50 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl at a flow rate of 12 ml/hr. (●) standard proteins; (o) rat mammary β-galactosidase.

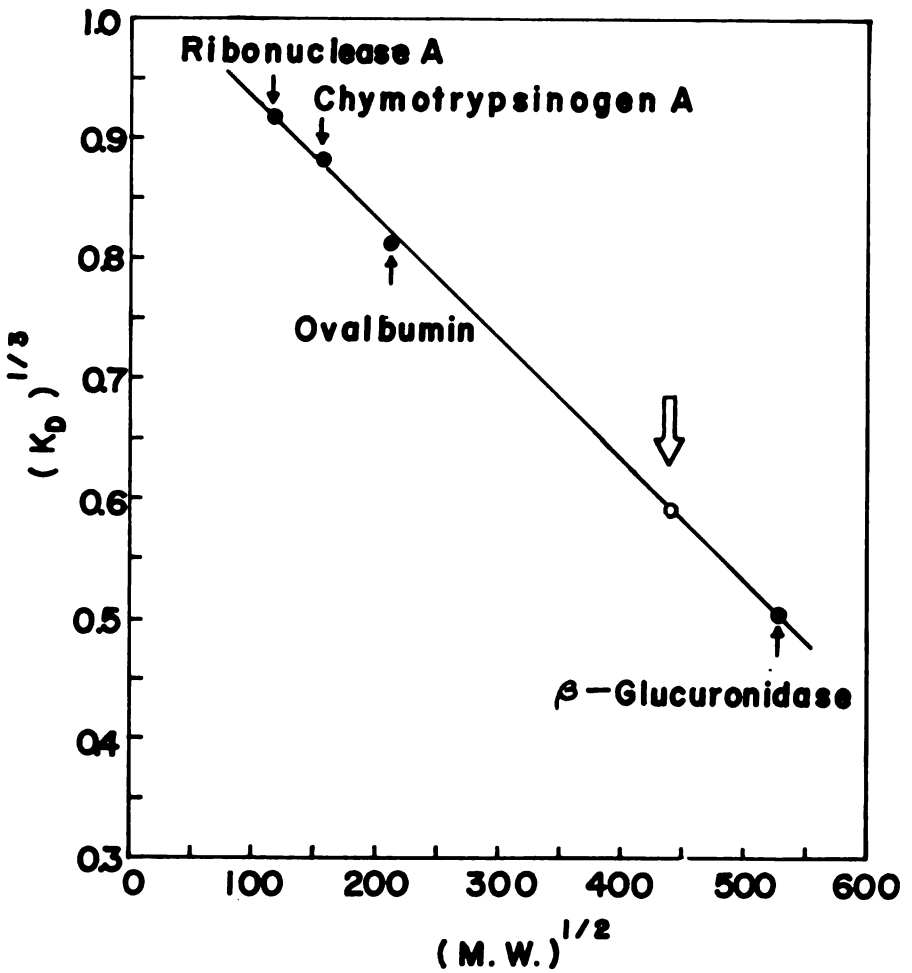


Figure 14. Gel filtration of β-galactosidase on Sephadex G-200 at pH 5.0. 3 ml of sample containing β-galactosidase (ammonium-sulfate fraction, table 2) was chromatographed on a column (2.5 x 92 cm) of Sephadex G-200 with 50 mM sodium citrate buffer, pH 5.0, containing 0.1 M NaCl at a flow rate of 12 ml/hr. 3.05 ml fractions were collected. The following proteins were used as markers: (a) β-glucuronidase (M.W.-280,000); (b) ovalbumin (M.W.-45,000); (c) chymotrypsinogen A (M.W.-25,000); (d) ribonuclease A (M.W.-13,700). (●) standard proteins; (o) rat mammary β-galactosidase.

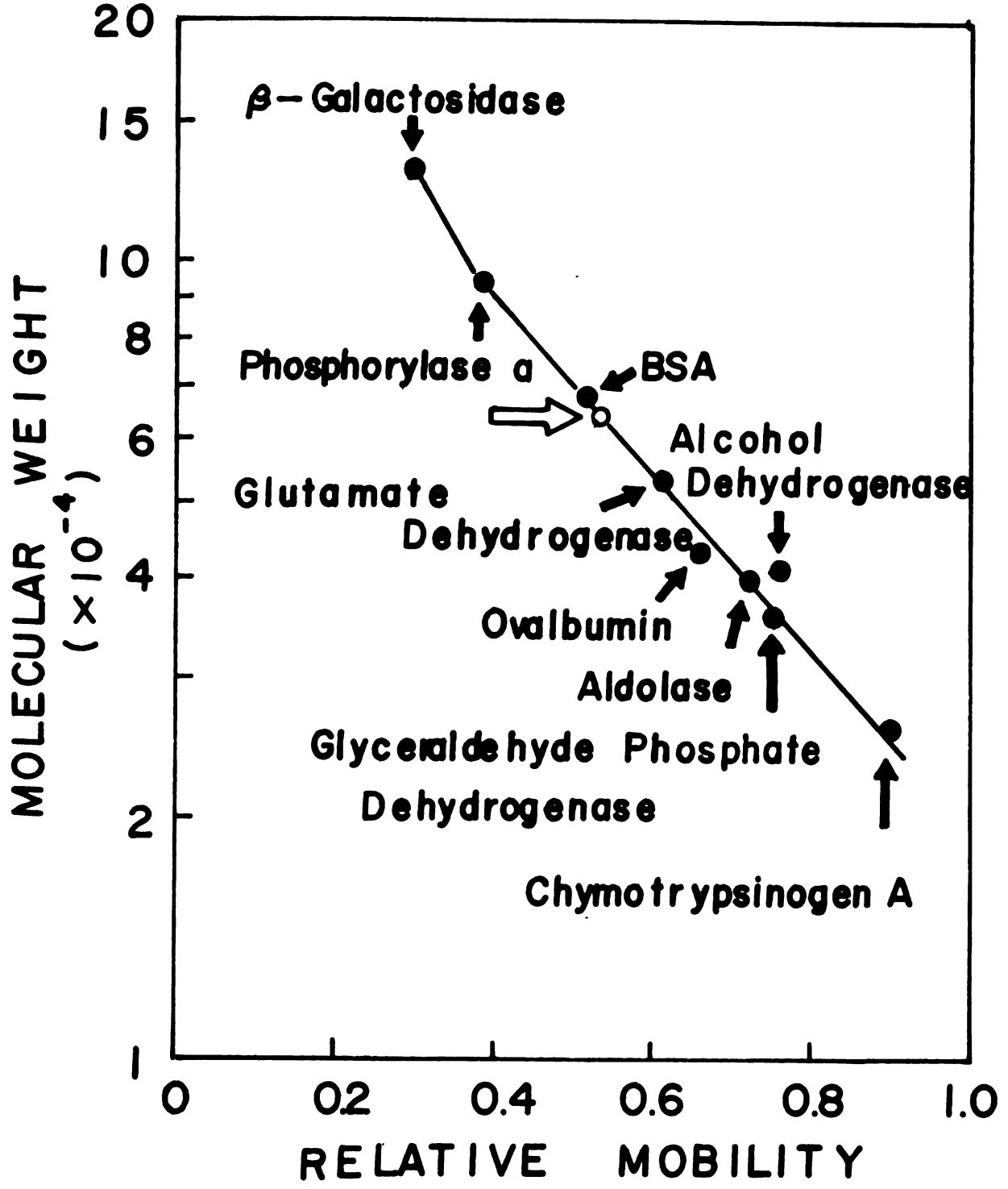


Figure 15. Disc gel electrophoresis of purified β-galactosidase in the presence of sodium dodecyl sulfate at pH 7.4. Electrophoresis was carried out as described in the Methods section. The mobility of each protein relative to bromophenol blue was plotted against log of molecular weight. (●), standard proteins; (○), the major band of β-galactosidase (affinity-chromatography fraction).

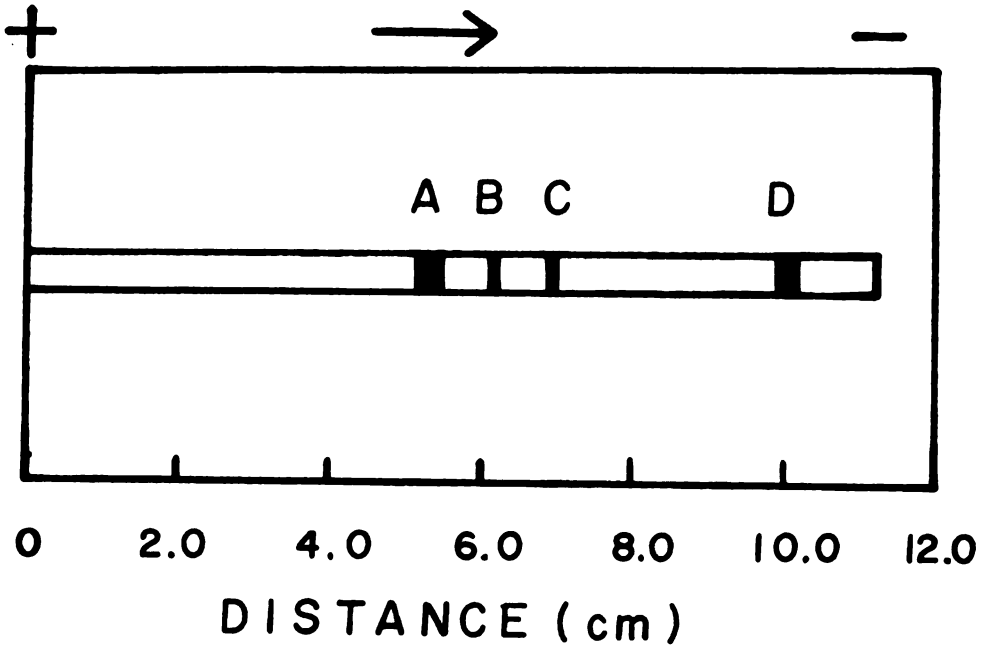


Figure 16. Disc gel electrophoresis of purified β-galactosidase (affinity-chromatography fraction) in the presence of sodium dodecyl sulfate at pH 7.4. Electrophoresis was carried out as described in the Methods section. A = major band; B and C = two minor bands; D = bromophenol blue.

G. Gel Electrophoresis of "Native" Enzyme

The result of the electrophoresis of the purified enzyme in 7% polyacrylamide gel, pH 4.3, is shown in Figure 17. Two parallel gels were run simultaneously. One gel with purified enzyme was used to determine protein location. Another one was sliced immediately after the electrophoresis and the hydrolase activity were assayed with p-nitrophenyl-β-D-galactoside as a substrate. The length of a broad-spread protein zone and the length of activity zone were the same, as well as the activity peak approximately corresponding to the darker place of protein zone.

H. Metal Ion Effect

The effects of several monovalent and divalent cations on hydrolase activity were investigated. The ions of sodium, lithium, calcium, barium and manganese displayed some activation of hydrolase activity of mammary β-galactosidase (Table 4). In the presence of K^+, NH_4^+ and Mg^{2+}, the hydrolase activity of β-galactosidase was about the same as that of the control. Also in the presence of EDTA, no significant difference from the control was observed.

Both hydrolase and transferase activities of rat mammary β-galactosidase were remarkably inhibited in the presence of heavy metal ions of Ag^+ and Hg^{2+} (Table 5). However, Cu^{2+} at a concentration of 1 mM did not significantly inhibit these two activities.

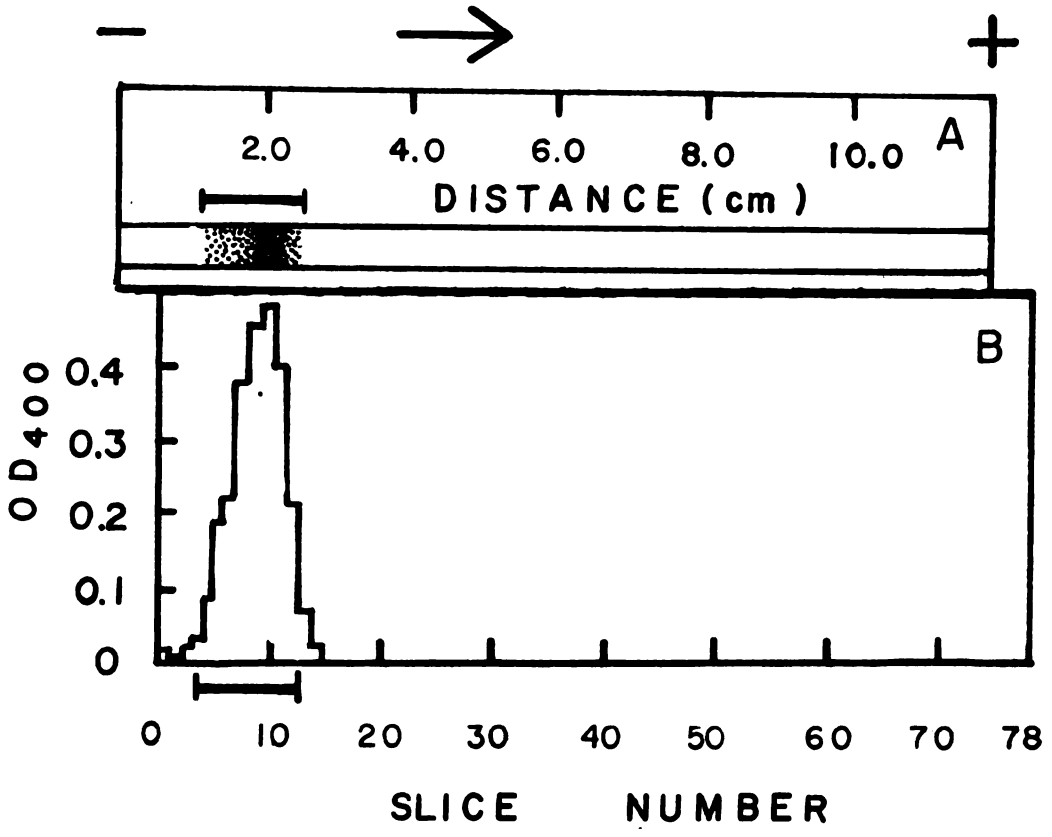


Figure 17. Polyacrylamide gel electrophoresis of purified β-galactosidase (affinity-chromatography fraction). The electrophoresis was carried out as described in the Methods section. A - protein was stained with Coomassie blue; B - enzymatic activity was determined as described in the Methods section by using PNPG as a substrate.

Table 4. Effect of Metal Ions on Hydrolase Activity[a]

Metal Ions	Concentration (mM)	Relative Activity (%)
Control	--	100
KCl	50	97
Nh_4Cl	50	106
NaCl	50	114
LiCl	50	126
$MgCl_2$	1	104
$CaCl_2$	1	115
$BaCl_2$	1	119
$MnCl_2$	1	129
EDTA	1	106

[a]Assays were carried out in 50 mM glycine-HCl buffer, pH 3.0, with 5 mM of p-nitrophenyl β-D-galactoside. The reactions were performed as described in method 2.

Table 5. Effect of Heavy Metal Ions on β-Galactosidase Activities

Metal Ions	Conc. (mM)	Relative Activity	
		Hyd. Act.[a] (%)	Trans. Act.[b] (%)
Control	---	100	100
$Cu(NO_3)_2$	1	98	93
$AgNO_3$	1	0	0
$HgCl_2$	1	3	0

[a]Assays of hydrolase activity were carried out in 50 mM sodium citrate buffer, pH 3.0, using 5 mM p-nitrophenyl β-D-galactoside as a substrate. The rest of reaction condition was the same as described in method 2.

[b]Assays of transferase activity were carried out in 50 mM sodium citrate buffer, pH 4.0, as described in method 4.

I. Substrate Specificity of Transferase Activity

Rat mammary β-galactosidase hydrolyzed lactose, p-nitrophenyl β-D-galactoside and o-nitrophenyl β-D-galactoside.

In the presence of lactose, *myo*-inositol and rat mammary β-galactosidase, 6-β-galactinol was synthesized, and the specificity of the galactosyl donors on transferase activity was investigated. In comparison with lactose, p-nitrophenyl β-D-galactoside was a much better galactosyl donor at 18.6 mM (Table 6). o-Nitrophenyl β-D-galactoside at 18.6 mM was about the same as lactose at 200 mM. However, at 100 mM lactulose and 1-o-methyl-β-D-galactoside were very poor donors producing 9 and 4% of the lactose activity, respectively. UDP gal, 1-o-methyl-α-D-galactoside, p-nitrophenyl-α-D-galactoside and galactose were not galactosyl donors.

J. Effect of Inhibitors on Transferase Activity

In the presence of p-chloromecuribenzoate (1 mM) transferase activity was completely inhibited (Table 7). D-galactono-1,4-lactone, D-galactose, phenyl-β-D-thiogalactoside and 1-galactosylamine partially suppressed the transferase activity.

Some compounds, such as Tris-Base, histidine, 2-amino-2-methyl-1-propanol and FDNB did not show significant inhibition of the transferase activity (Table 7).

Table 6. Substrate Specificity of Transferase Activity of β-Galactosidase[a]

Substrate	Conc. (mM)	Relative Activity (%)
Lactose	200	100
p-Nitrophenyl β-D-galactoside	18.6	150
o-Nitrophenyl β-D-galactoside	18.6	99
Lactulose	100	9
1-O-Methyl β-D-galactoside	100	4
UDPgal	74	0
1-O-Methyl α-D-galactoside	200	0
p-Nitrophenyl α-D-galactoside	18.6	0
D-Galactose	200	0

[a]Assays were carried out in 50 mM sodium citrate buffer, pH 4.0, using 500 mM *myo*-inositol as a galactosyl acceptor. The reaction condition was the same as described in method 4.

Table 7. Effect of Inhibitors on Transferase Activity of β-Galactosidase[a]

Additament	Conc. (mM)	Relative Activity (%)
None	--	100
p-Chloromercuribenzoate	1	0
D-Galactono-1,4-lactone	50	11
D-Galactose	50	19
Phenyl β-D-thio-galactoside	43	21
1-Galactosylamine	50	22
FDNB	< 5[b]	98
Histidine	5	93
2-Amino-2-methyl-1-propanol	5	100
Tris-Base	5	99

[a]Assays were carried out in 50 mM sodium citrate buffer, pH 4.0, with 500 mM *myo*-inositol and 200 mM lactose, as described in method 4.

[b]25 µl of saturated FDNB solution (25°C) was added in a final volume of 250 µl reaction mixture.

K. Effect of Monosaccharides on Transferase Activity

Several reports (70,87,108,155,156) have been published to show that β-galactosidase could hydrolyze, in addition to β-D-galactosides, some other p-nitrophenyl glycosides which contained the configuration of glycosidyl residue the same as that of galactose at C-1, C-2, and C-3. Therefore, the inhibitory effects of some monosaccharides which had this characteristic were investigated. Three other monosaccharides which did not have this common portion of the structure were also examined in order to compare the specificity of configuration (Table 8). L-arabinose, 3-O-methyl-D-glucose, D-fucose and D-glucose showed some inhibitory effect on transferase activity. But there was no effect when either D-xylose, D-mannose and N-acetyl-D-galactosamine was added.

L. Thermal Inactivation of Rat Mammary β-Galactosidase

Purified enzyme (affinity-chromatography fraction) was heated at 60°C for various periods and the residual hydrolase and transferase activities were then determined. The relationship between the log of percentage of original activity and the period of inactivation at 60°C is shown in Figure 18. The denaturation rates of transferase activity and hydrolase activity of rat mammary β-galactosidase were the same, indicating the activities were that of a single enzyme. The half life of either activity at 60°C was approximately 20 minutes.

Table 8. Effect of Monosaccharides on Transferase Activity of β-Galactosidase[a]

Monosaccharides	Conc. (mM)	Relative Activity (%)
Control	--	100
D-Fucose	50	92
D-Glucose	50	90
D-Xylose	50	104
L-Arabinose	50	77
D-Mannose	50	103
N-Acetyl-D-galactosamine	50	99
3-O-Methyl-D-glucose	50	82

[a]Assays were carried out in 50 mM sodium citrate buffer, pH 4.0, with 500 mM *myo*-inositol and 200 mM lactose as described in method 4.

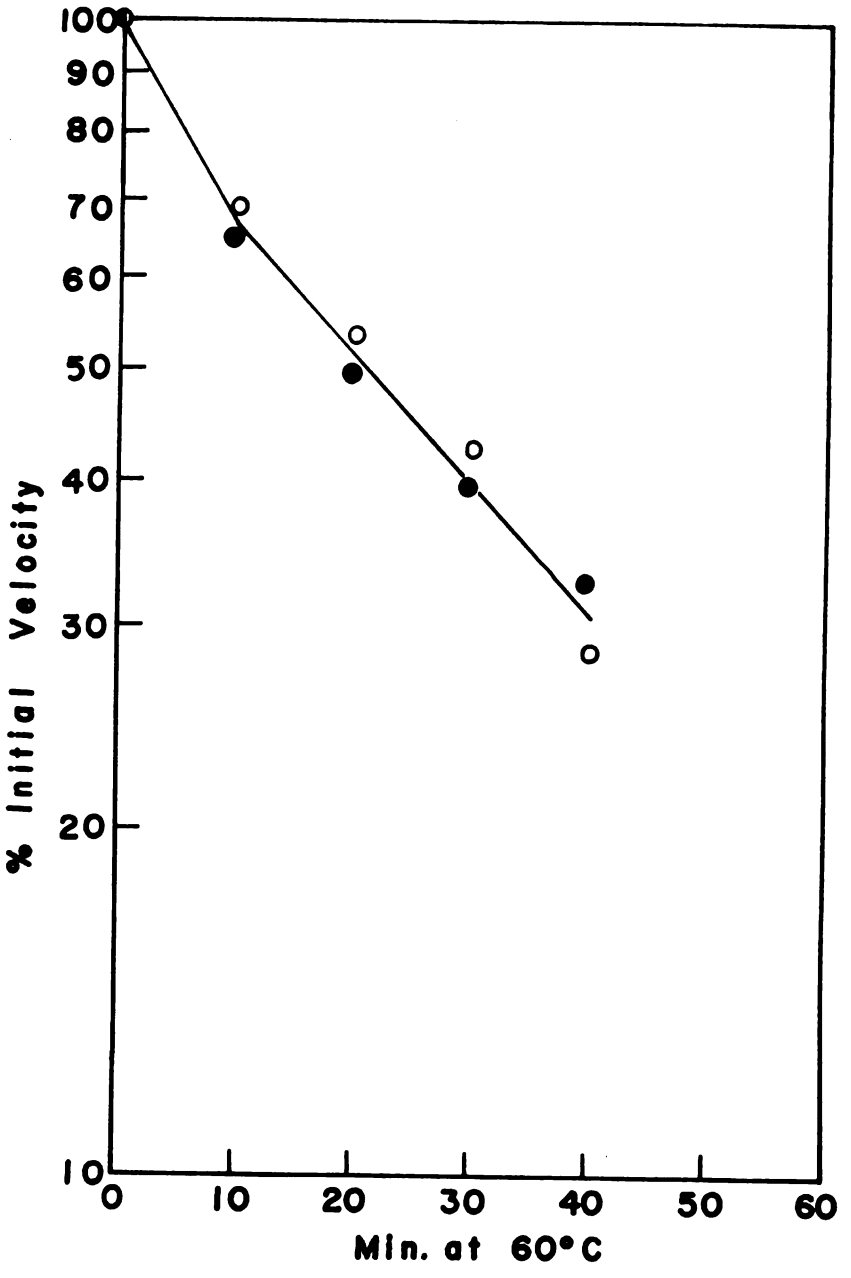


Figure 18. Heat inactivation of hydrolase and transferase activities of purified enzyme. β-Galactosidase (affinity-chromatography fraction) was preheated at 60°C in the presence of 1.09 mg/ml BSA. The assays of the residual transferase activity were carried out as described in method 4. The assays of hydrolase activity were carried out as described in method 2 in the presence of 0.45 mg/ml BSA. (o——o), transferase activity; (●——●), hydrolase activity.

M. Kinetic Properties of Rat Mammary β-Galactosidase

The apparent Km for p-nitrophenyl β-D-galactoside was determined in the presence of 50 mM sodium citrate buffer, pH 3.4, and purified enzyme (affinity-chromatography fraction) by a Lineweaver-Burk plot (Figure 19). The value was 1.85 x 10^{-4} M. By the same kind of plot, the apparent Km values for lactose and *myo*-inositol in the presence of 50 mM sodium acetate buffer, pH 3.6, and purified enzyme (DEAE-cellulose fraction) were determined as 37 mM and 380 mM, respectively (Figures 20 and 21). The apparent Michaelis-Menten constant for *myo*-inositol was also determined by the Lineweaver-Burk plot in the presence of 50 mM glycine-HCl buffer, pH 3.0, lactose (80 mM), α-lactalbumin (0.5 mg/ml) and purified enzyme (affinity-chromatography fraction). The Km value was 345 mM (Figure 22).

N. The Characteristics of 6-β-Galactinol

Gas-liquid chromatography of the fully trimethylsilylated of 6-β-galactinol isolated from rat mammary gland is shown in Figure 23. The GC analysis of 6-β-galactinol synthesized *in vitro* is also exhibited in Figure 24, and it has the same retention time as the natural compound. The paper chromatography of natural and synthesized 6-β-galactinol was shown in Figure 25. The band a corresponding to natural 6-β-galactinol contained 6-β-galactinol and another galactosyl *myo*-inositol which was also synthesized *in vitro* by

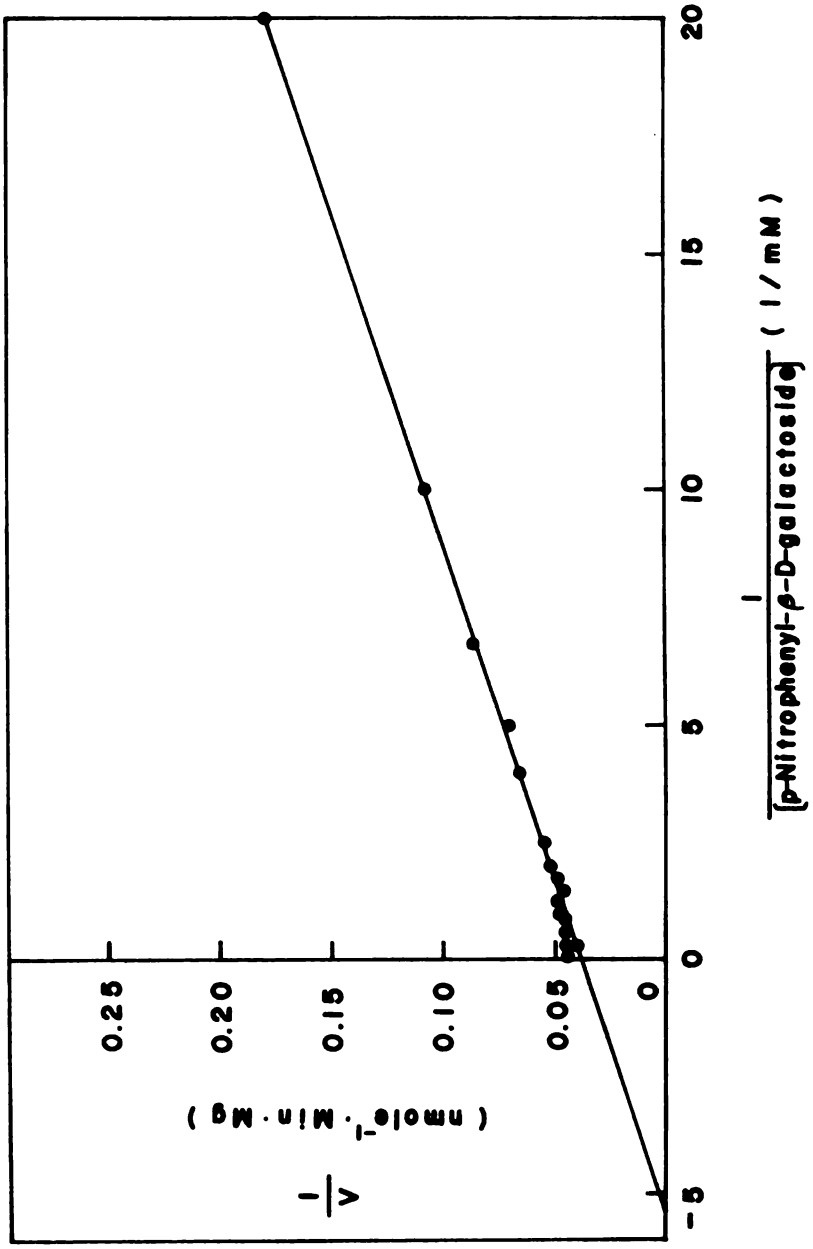


Figure 19. Lineweaver-Burk plot for p-nitrophenyl β-D-galactoside. The assays of hydrolase activity were carried out as described in method 2. The reaction mixtures consisting of 50 mM sodium citrate buffer, pH 3.4, PNPG (0.05-10 mM) and purified enzyme (affinity-chromatography fractions) were incubated at 37°C for 13 min.

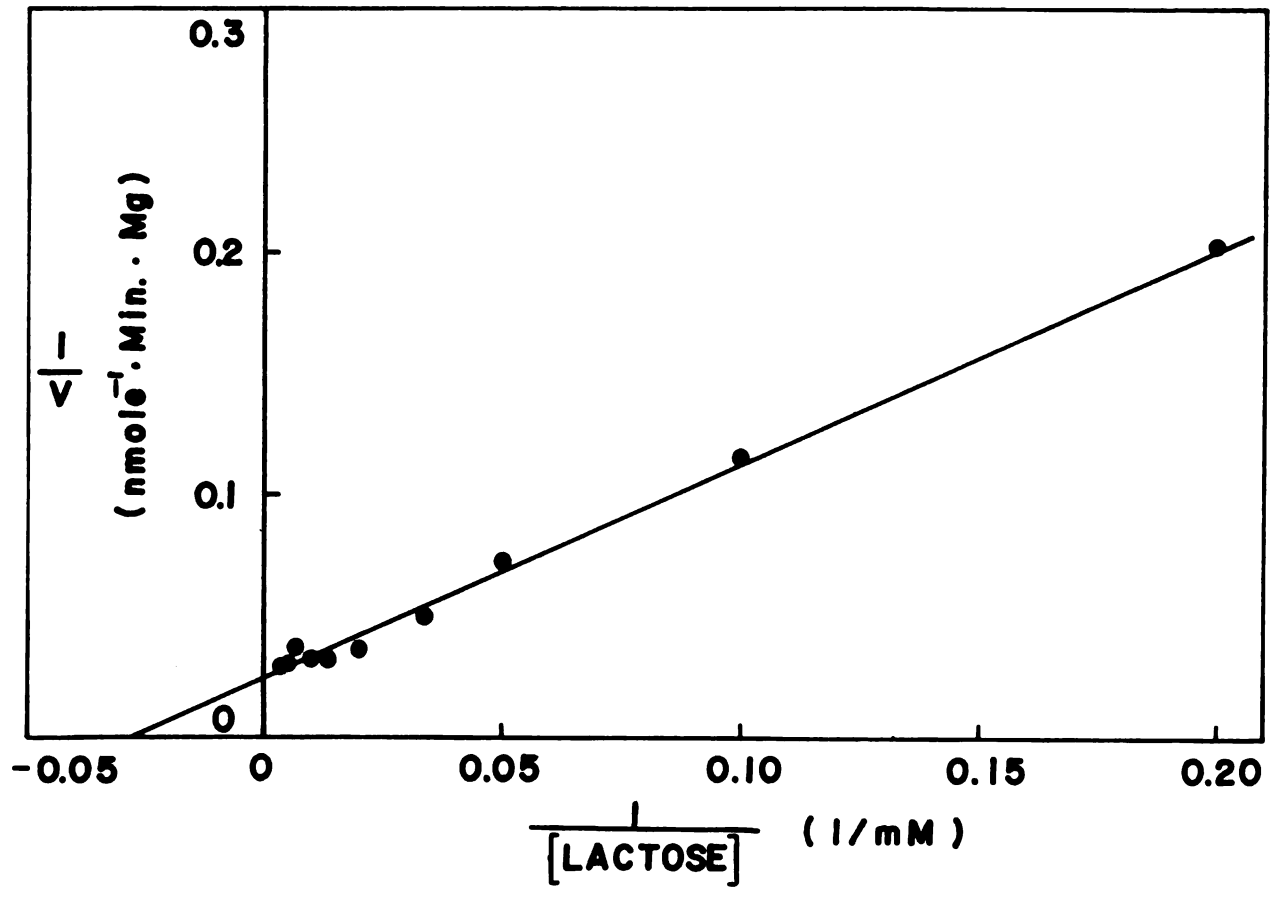


Figure 20. Lineweaver-Burk plot for lactose. The assays of transferase activity were carried out as described in method 3. A final volume of 200 µl reaction mixture consisting of sodium acetate buffer (50 mM, pH 3.6), *myo*-inositol (375 mM), lactose (5-250 mM) and purified enzyme (DEAE-cellulose fraction) was incubated at 37°C for 3 hr. The amount of 6-β-galactinol produced was determined by gas-liquid chromatography by using α,α-trehalose as an internal standard.

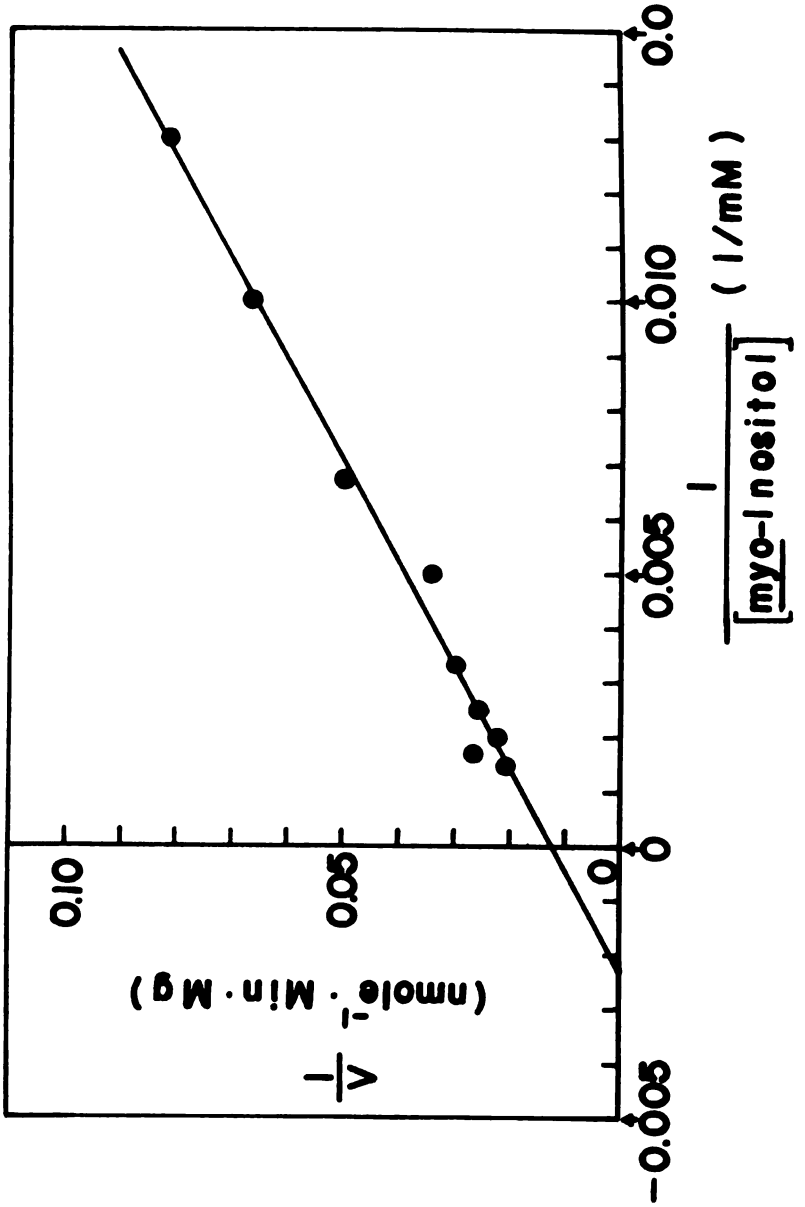


Figure 21. Lineweaver-Burk plot for *myo*-inositol. The assays of transferase activity were carried out as described in method 3. A final volume of 200 µl reaction mixture consisting of sodium acetate buffer (50 mM, pH 3.6), lactose (200 mM), *myo*-inositol (75-675 mM) and enzyme (DEAE-cellulose fraction) was incubated at 37°C for 3 hr. The amount of 6-ß-galactinol produced was determined by GC with α,α-trehalose as an internal standard.

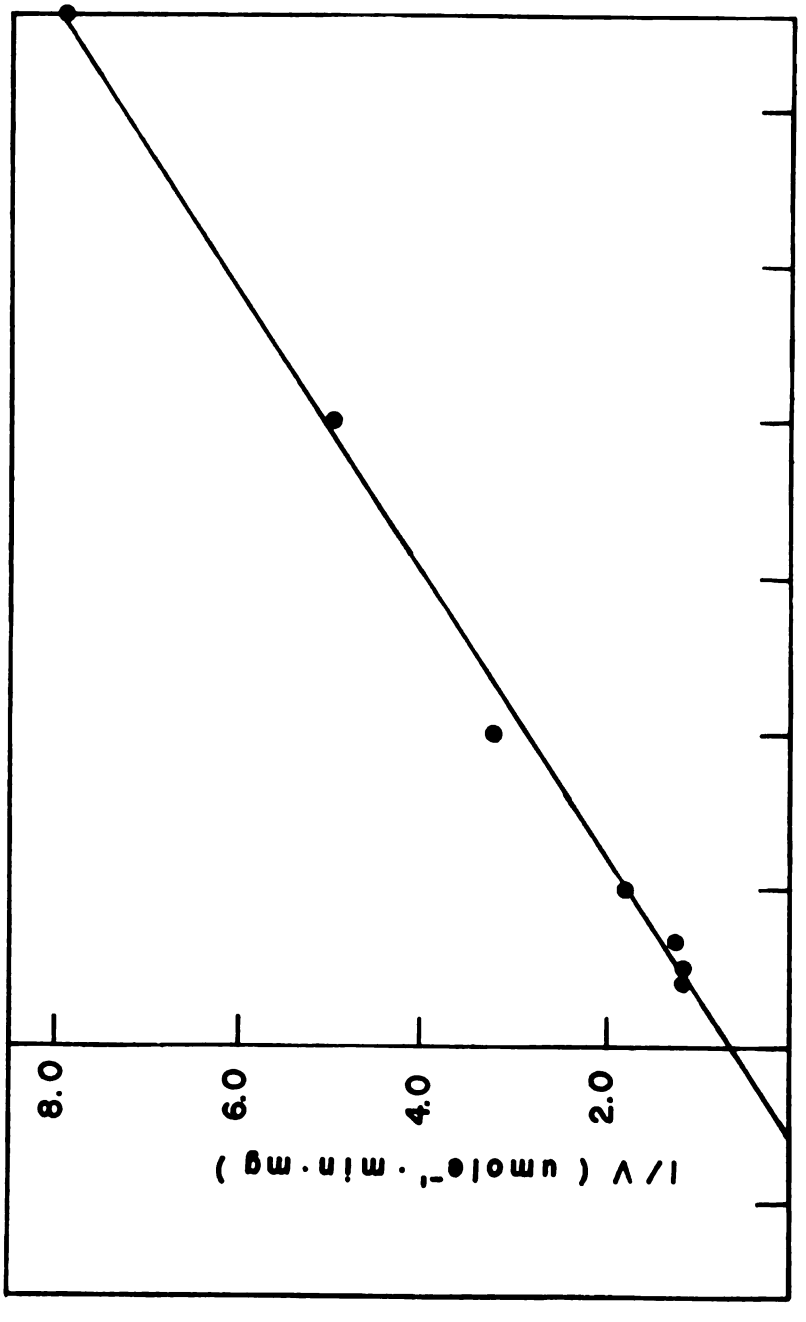


Figure 22. Lineweaver-Burk plot for *myo*-inositol. The reaction mixtures consisting of glycine-HCl buffer, pH 3.0 (50 mM), lactose (80 mM), *myo*-inositol (30-500 mM), α-lactalbumin (0.5 mg/ml, cow), purified enzyme (2 μl, affinity-chromatography fraction) were incubated at 37°C for 3.25 hr. The amount of 6-β-galactinol produced was measured the same as described in method 4.

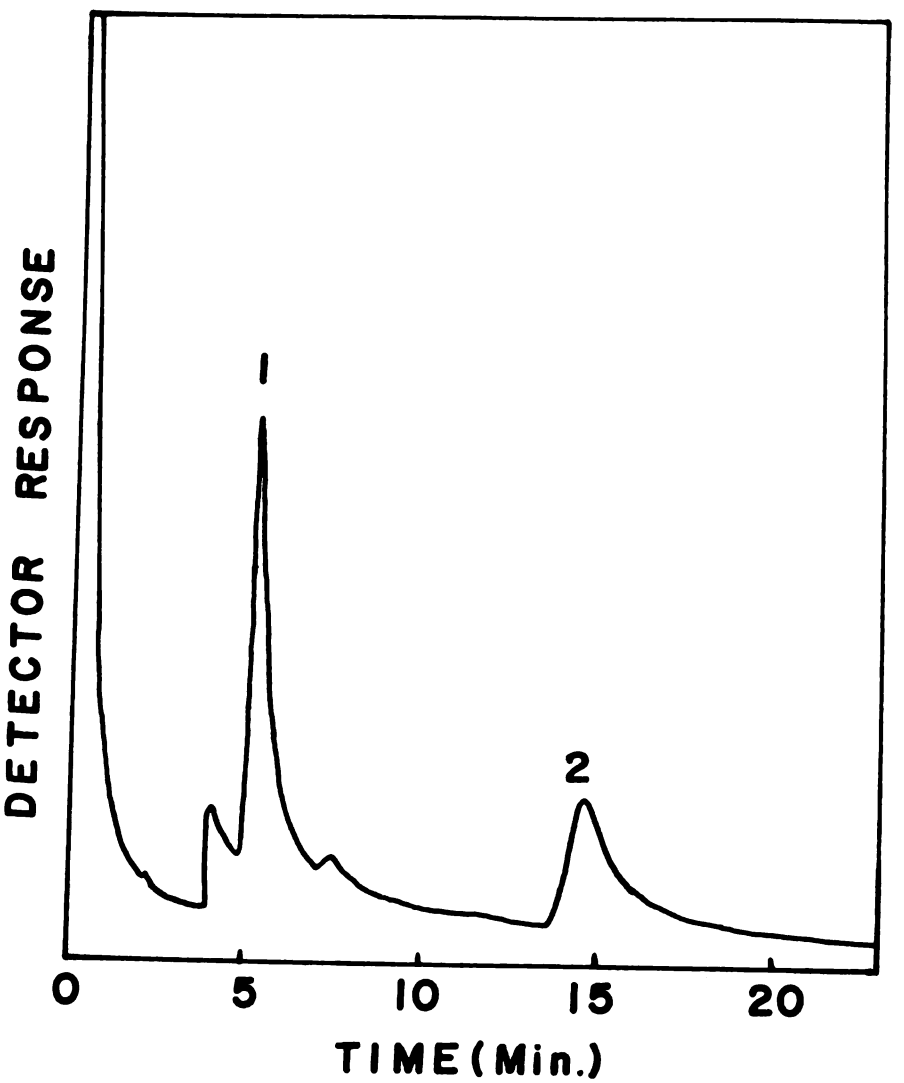


Figure 23. Gas-liquid chromatography of the fully trimethylsilylated 6-β-galactinol isolated from rat mammary gland during the 18th-day lactation. The experiment was carried out on a 2 mm x 1.8 m glass column packed with 3% XE-60 on Gas-Chrom Q, 100-120 mesh. The column temperature was 195°C. Peak 1 = α,α-trehalose (internal standard); Peak 2 = 6-β-galactinol.

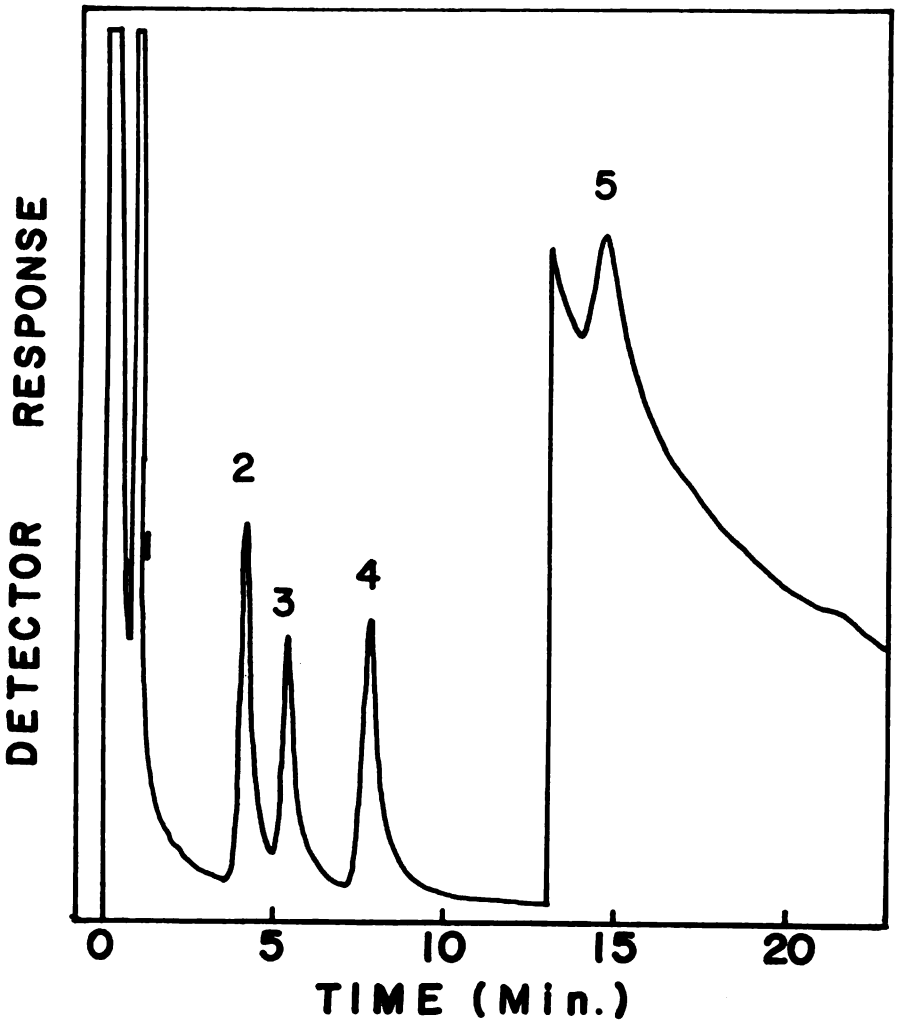


Figure 24. Gas-liquid chromatography of the fully trimethylsilylated 6-β-galactinol, which was synthesized from lactose and *myo*-inositol by purified β-galactosidase (affinity-chromatography fraction). The experiment was carried out on a 3 mm x 1.8 m glass column packed with 3% XE-60 on Gas-Chrom Q (100-120 mesh) at 195°C. Peak 1 = *myo*-inositol; Peak 2 and 4 = α- and β-lactose; Peak 3 = α,α-trehalose (ISTD); Peak 5 = 6-β-galactinol.

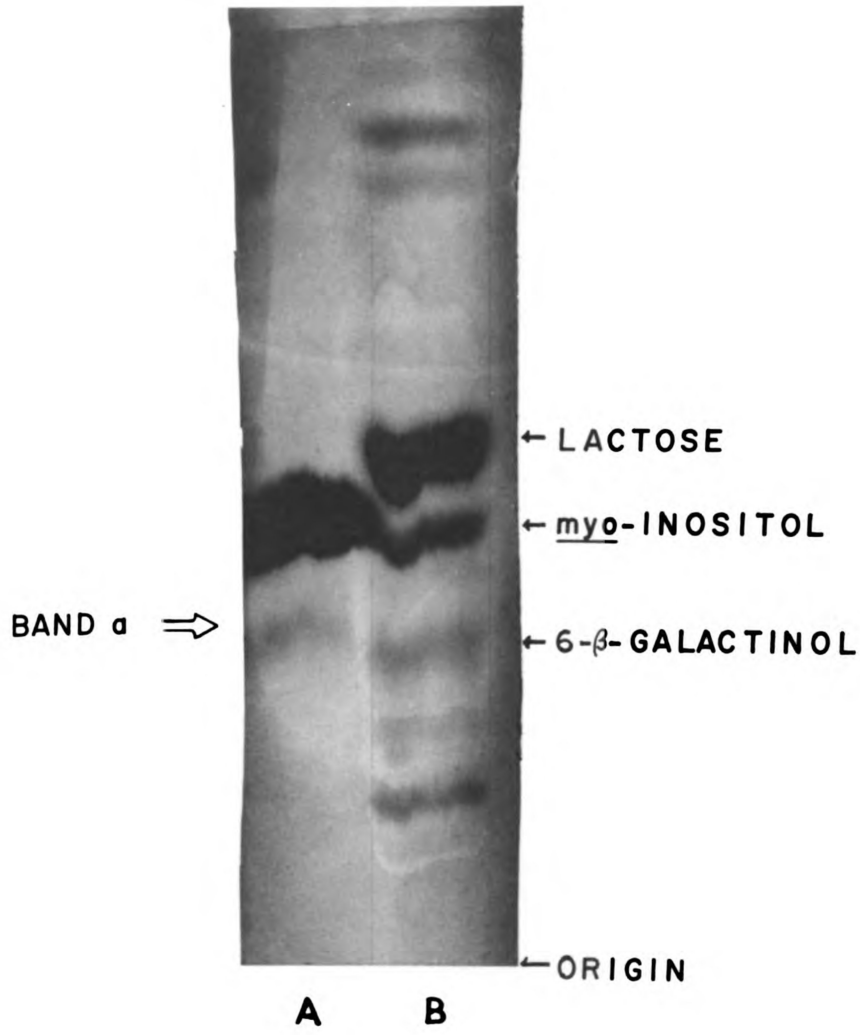


Figure 25. Paper chromatography of natural sugar isolated from rat mammary gland during the 18th-day lactation (B) and 6-β-galactinol synthesized by rat mammary β-galactosidase *in vitro* (A). Chromatography was developed on 3MM Whatman paper (25 x 45 cm) by an ascending technique using six passes of a solvent system (butanol/pyridine/water/acetic acid = 6/4/3/0.3, by volume). Sugars were detected by the silver nitrate procedure (150). The arrow (⇨) pointed to the band containing 6-β-galactinol.

β-galactosidase in the presence of lactose and *myo*-inositol and had a shorter retention time than 6-β-galactinol (Figure 26).

O. Identification of 6-O-α-D-Mannopyranosyl *myo*-Inositol

While searching for the possible route for the biosynthesis of 6-β-galactinol, we investigated a disaccharide, α-D-mannosyl *myo*-inositol which had been found and isolated from yeast. But the position of the glycosidic linkage on *myo*-inositol from the 1 position of mannose was then unknown. Therefore, we isolated this disaccharide from yeast through charcoal-celite column and paper chromatography (Figure 27). The gas chromatographic analysis of the fully trimethylsilylated α-D-mannosyl *myo*-inositol isolated from yeast was shown in Figure 28. When the disaccharide was acid hydrolyzed, mannose and *myo*-inositol were identified by gas chromatography of the trimethylsilylated derivatives as the only products and were present in a 1:1 molar ratio.

The permethyl and trimethylsilyl derivatives of mannosyl isolated, and two standards (1-α-galactinol and glucosylinositol) were investigated by gas chromatography (Table 9). The derivatives of mannosylinositol moved faster than the derivatives of standards either on 3% OV-1 or on 5% Sp 2401 and had the retention times of 7.6 minutes and 6.0 minutes.

In order to determine to which carbon atom of *myo*-inositol the mannosyl residue was linked, the permethyl derivatives

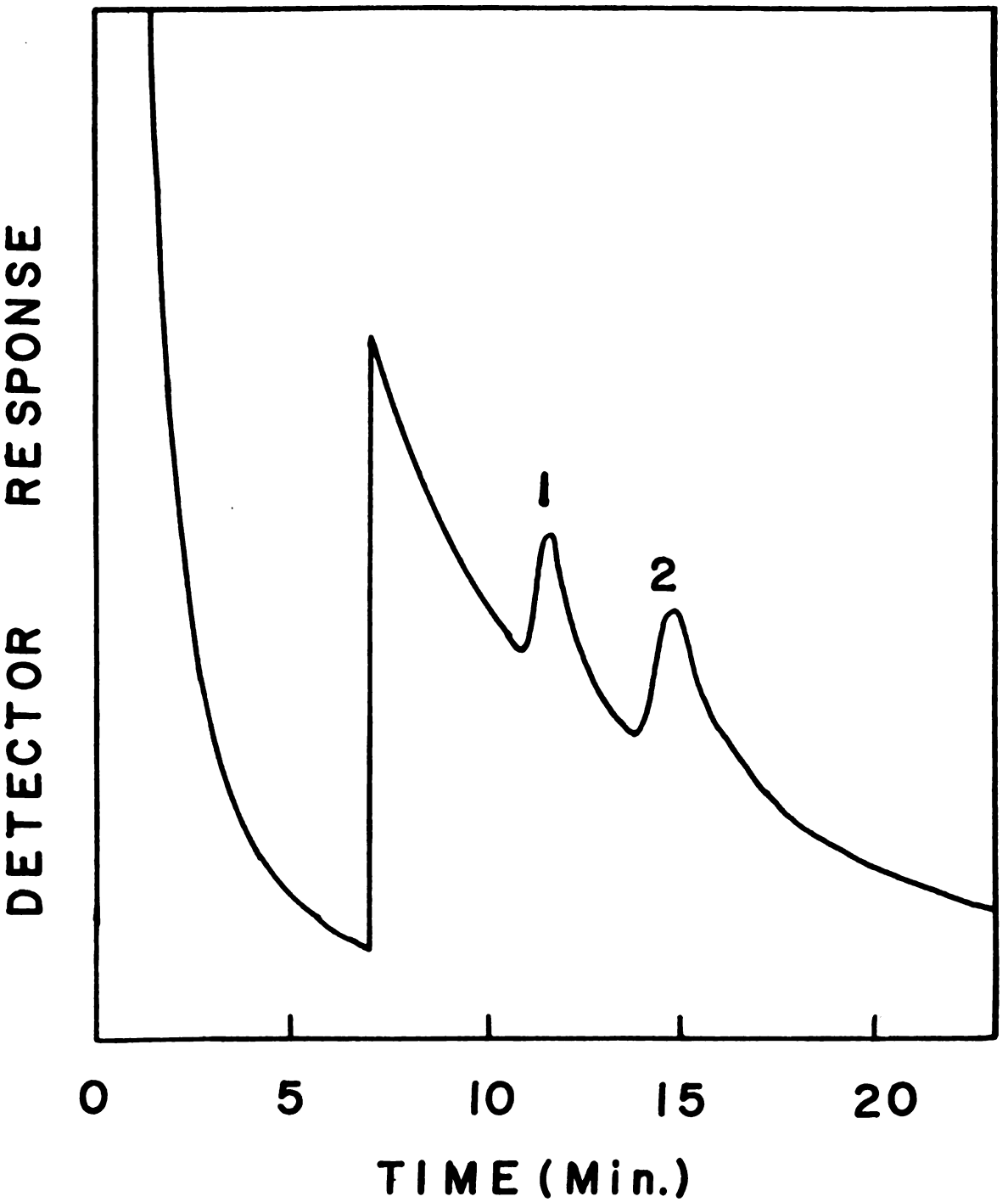


Figure 26. Gas-liquid chromatography of the fully trimethylsilylated the disaccharides which was eluted from band A (Fig. 26). The experiment was carried out on a 2 mm x 1.8 m glass column packed with 3% XE-60 on Gas Chrom Q (100-120 mesh). The column temperature was 195°C. Peak 1 = galactosyl *myo*-inositol (the isomer of 6-β-galactinol); Peak 2 = 6-β-galactinol.

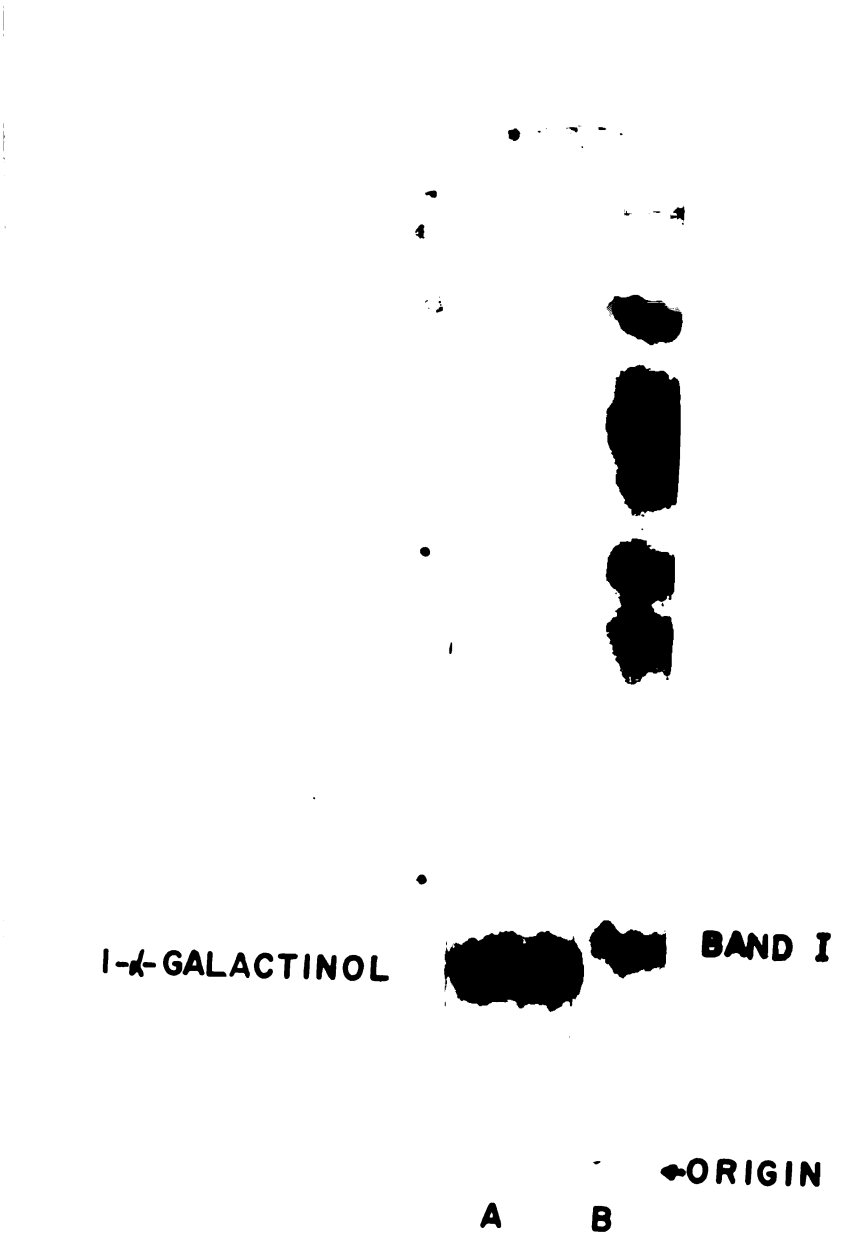


Figure 27. Paper chromatography of 6-O-α-D-mannosyl *myo*-inositol isolated from yeast as described in the Methods (B) and l-α-galactinol (standard) (A). Chromatography was developed on Whatman paper (25 x 45 cm) by an ascending technique using four passes of a solvent system (n-butanol:pyridine: water:acetic acid, 6:4:3:0.3, by volume). Sugars were detected by the silver nitrate procedure (150). Band I = mannosyl *myo*-inositol.

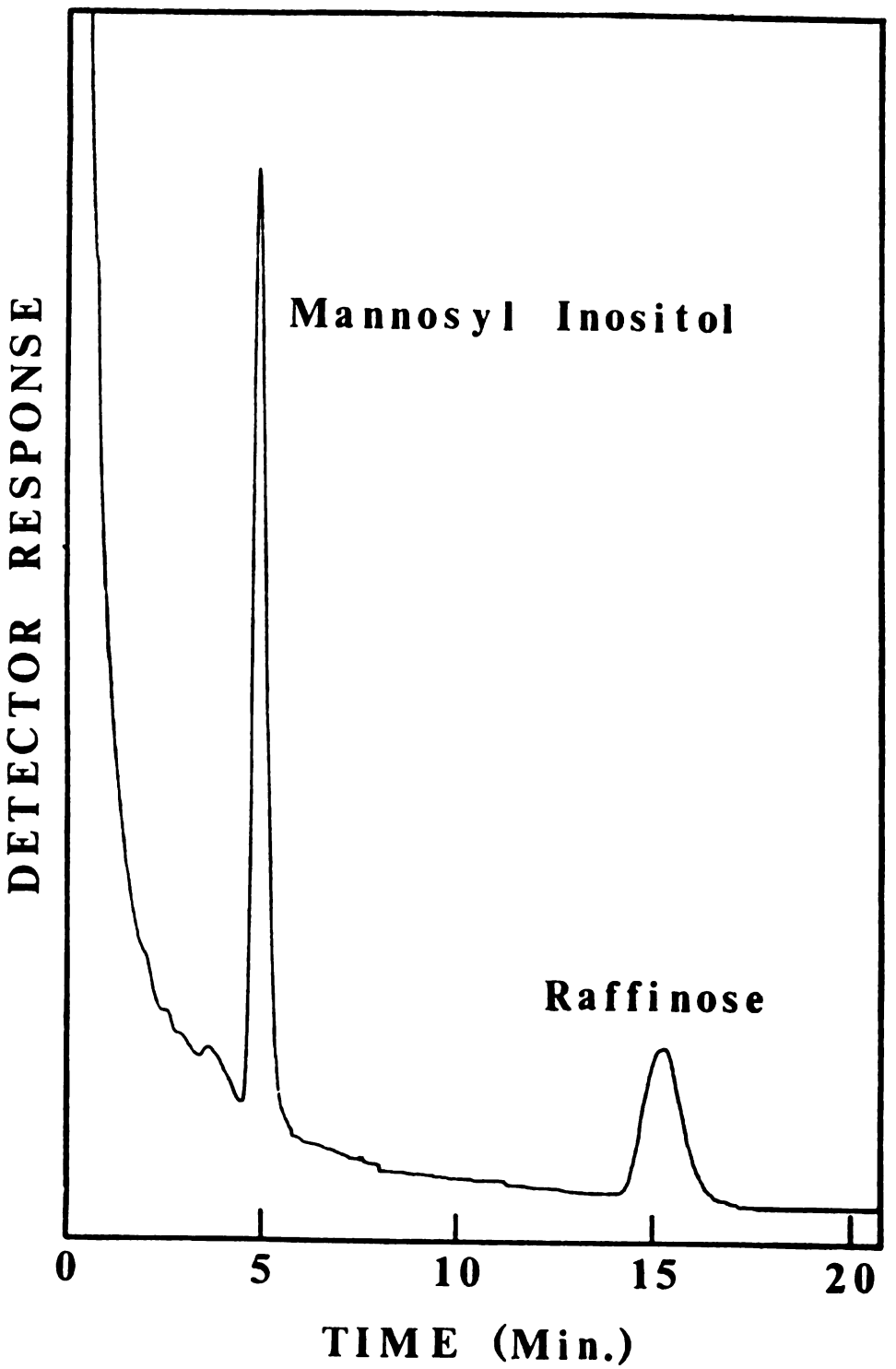


Figure 28. Gas-liquid chromatography of the fully trimethylsilylated 6-O-α-D-mannopyranosyl *myo*-inositol isolated from yeast. The experiment was carried out on a 3 mm x 1.8 m glass column packed with 5% SP 2401 on Gas-Chrom Z (80-100 mesh). The column temperature was 242°C. The raffinose derivatives were an added internal standard.

Table 9. Gas Chromatography of Derivatives of *myo*-Inositol Disaccharides

Derivatives	Retention Time (min)		
	1-α-Galactinol	Gluco-sylinositol	Manno-sylinositol
Permethyl[a]	11.8	7.7	7.6
Trimethylsilyl[b]	6.8	---	6.0

[a]Column was 3% OV-1, 3 mm x 1.8 m on Chromosorb W, 100-120 mesh, and the temperature was 220°C.

[b]Column was 5% SP 2401, 3 mm x 1.8 m on Gas-Chrom Z, 80-100 mesh, and the temperature was 240°C.

of mannosyl *myo*-inositol was prepared and then hydrolyzed in 2 N H_2SO_4 1.5 hr. Meanwhile two standards, 1-α-galactinol (glycolidic linkage occurring between C-1 of *myo*-inositol and C-1 of galactose), and glucosylinositol (glycolidic linkage between C-6 of *myo*-inositol and C-1 of galactose) were also prepared following the same procedure. The gas-liquid chromatography of these methylated hydrolysis products of *myo*-inositol-containing disaccharides was presented in Table 10. The mehtylated derivatives of mannosyl *myo*-inositol had the same retention time as that of *myo*-inositol from the permethyl derivatives of glucosyl-inositol, but not the same as that from the permethyl derivative of 1-α-galactinol. Therefore, the glycolidic linkage in mannosylinositol was confirmed at C-6 of *myo*-inositol.

Additional evidence that mannosyl *myo*-inositol is composed of mannose in glycosidic linkage with *myo*-inositol was obtained from the mass spectrometry of the permethylated derivative. The mass spectrum of the permethylated derivative was shown in Figure 29. The ions in the low mass region appeared at m/e 45, 75, 88, 101, 145 and in the high mass region at m/e 201, 233, 293. This mass spectrum has been shown to be the characteristic of 1-α-galactinol and 6-β-galactinol but not lactose (galactosyl glucose) (2).

To determine the configuration of glycosidic linkage in mannosyl inositol, enzymatic hydrolysis was carried out by using jack bean α-mannosidase (this part of the work was done by Dr. Wells and Rita Ray). The hydrolysis of the mannosyl

Table 10. Gas Chromatography of Hydrolysis Products of Permethylated *myo*-Inositol Disaccharides[a]

Compound Methylated	Hydrolysis Products	Retention Time (min)
1-α-Galactinol	2,3,4,6-tetra-O-methyl galactinol	6.8
	1,2,3,5,6-penta-O-methyl *myo*-inositol	4.8
Glucosylinositol	2,3,4,6-tetra-O-methyl glucitol	6.6
	1,2,3,4,5-penta-O-methyl *myo*-inositol	6.2
Mannosylinositol	2,3,4,6-tetra-O-methyl mannitol	6.7
	1,2,3,4,5-penta-O-methyl *myo*-inositol	6.2

[a]Column was 3% OV-1, 3 mm x 1.8 m on Chromosorb W, 100-120 mesh, and the temperature was 148°C.

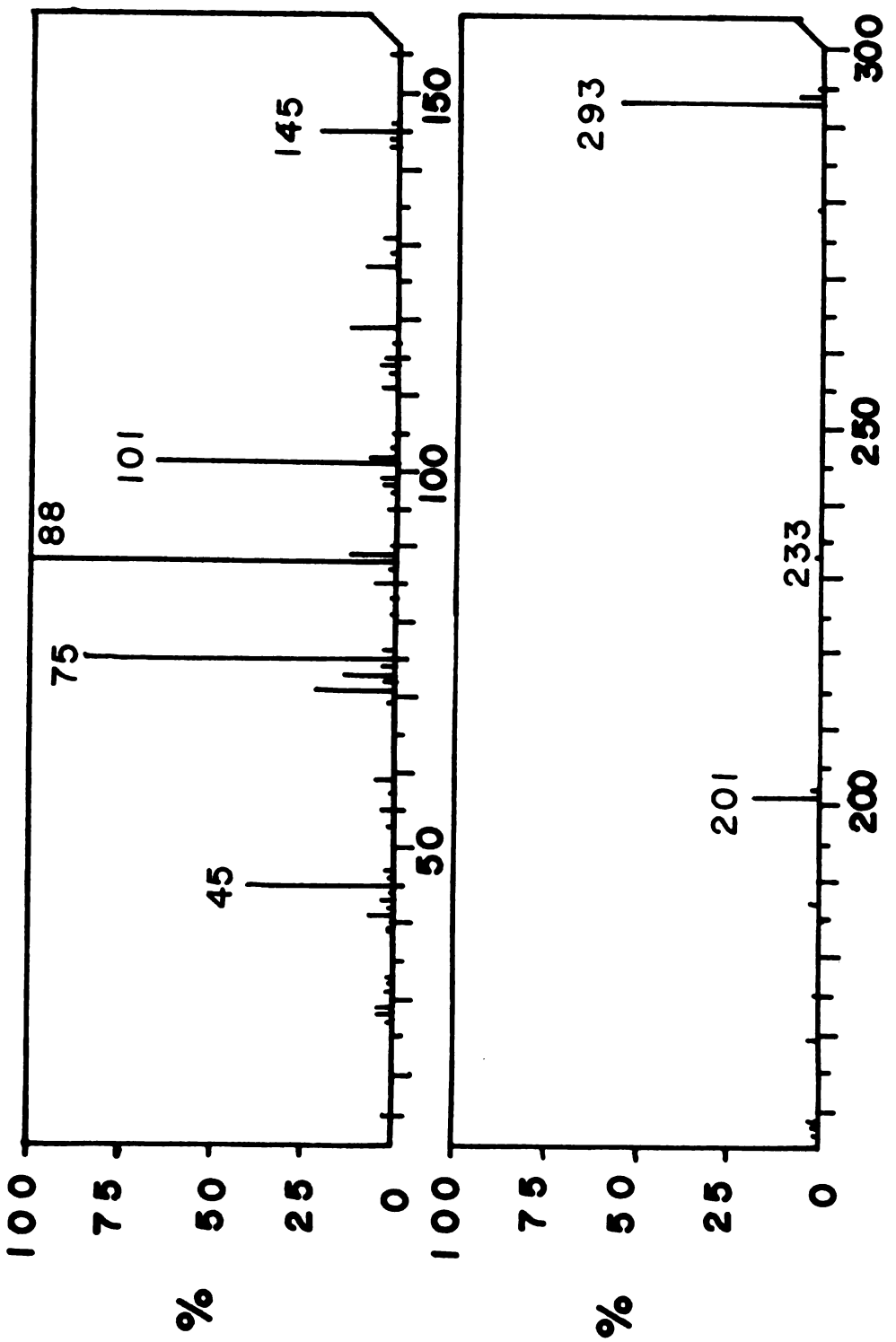


Figure 29. Mass spectrum of permethylated mannosyl *myo*-inositol. The spectrum was obtained as described tine the Methods section.

myo-inositol by 50 units of α-mannosidase was 0.7% in 6 hours. On the other hand, no hydrolysis of the mannoside was detected after 24 hr incubation with crude β-mannosidase which contained no activity toward p-nitrophenyl α-D-mannoside. Therefore, mannosyl inositol isolated from yeast was concluded to be of the α-configuration, which is in agreement with Tanner (149).

According to the results from the above experiments, the yeast mannoside could be identified as 6-o-α-D-mannopyranosyl *myo*-inositol.

V. DISCUSSION

The primary purification procedure as described in the "Methods" section was modified from the procedure which Meisler (67) used to purify β-galactosidase of human liver 225-fold. Nevertheless, only 22-fold (hydrolase activity) or 27-fold (transferase activity) purification was obtained from rat mammary β-galactosidase following virtually the same experimental procedure. During the purification of human liver β-galactosidase, a 9-fold increase of specific activity was achieved along with 18% loss of total activity in the solvent (ethanol:acetone:water, 15:4:1) precipitation step. But the same step resulted in the 84% loss of total hydrolase activity or 83% loss of total transferase activity and gained only a 1.5-fold of specific hydrolase activity or a 1.6-fold of specific transferase activity during the purification of rat mammary β-galactosidase. Therefore, the solvent precipitate step was not judged suitable for the purification of rat mammary enzyme.

In the DEAE-cellulose step, a gradient (1 liter) from 0.05 M to 0.2 M NaCl which had been reported to purify the β-galactosidase of human liver by Meisler (67) was first employed to purify rat mammary β-galactosidase. However,

the rat mammary β-galactosidase was directly eluted from the column. Later, we applied a much broader gradient to the DEAE-cellulose column (1.75 l, and from 0 to 0.3 M sodium chloride) and the enzyme still appeared in the early fractions of the effluent. In contrast to rat mammary β-galactosidase, the human liver β-galactosidase was eluted between 640-770 ml of a total volume of one liter of gradient solution (67).

As rat mammary β-galactosidase could not be well purified following the procedure of Meisler through DEAE-cellulose step, the secondary purification procedure (Table 2) was employed. The purification of rat mammary β-galactosidase through this procedure resulted in a 640-fold increase in specific activity and a 9% recovery of original total activity. Affinity-column chromatography was applied in the secondary purification procedure. This technique has been used to purify several microbial β-galactosidase (38,43,157), jack bean (47) and bovine testicular (69) β-galactosidases.

In general, the purification was based on a specific reversible binding between the enzyme or the protein of interest and a ligand (substrate or analogue inhibitor) which was chemically coupled to an inert support. When a mixture of protein was applied, only the enzyme or the protein of interest was bound to the column while the remaining proteins passed through with the eluate. In the present study rat mammary β-galactosidase was found to bind to agarose (inert support) on which p-aminophenyl β-D-thiogalactoside (analogue inhibitor) was covalently attached directly, i.e., without an arm between

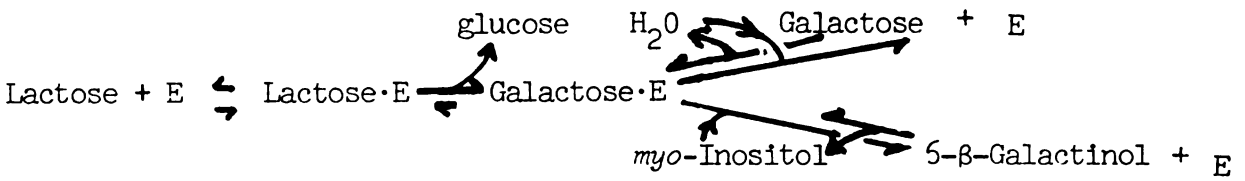
the ligand and the inert support.

In contrast to rat mammary β-galactosidase, *E. coli* β-galactosidase was not retarded on the column packed with agarose-p-aminophenyl β-D-thiogalactoside (157). As a long arm (succinyl 3,3'-diaminodipropylamine) was interposed between agarose and p-aminophenyl β-D-galactoside (158), *E. coli* β-galactosidase was selectively bound in the column when the remaining proteins passed through.

Steers *et al.* (157), therefore, considered that a distance of arm (about 21 Å) from the support matrix (agarose) was necessary for the occurrence of the binding between *E. coli* β-galactosidase and analogue inhibitor (p-aminophenyl β-D-galactoside). However, the long arm was not required for the binding of rat mammary β-galactosidase to affinity column. The similarity was also observed by Distler and Jourdian (69) when they attempted to purify bovine testicular β-galactosidase on an affinity column containing p-aminophenyl β-D-thiogalactoside. Though the regular purification of bovine testicular β-galactosidase was achieved on a column containing succinyl aminoalkyl Bio-Gel A-15 M covalently bound to p-aminophenyl β-D-thiogalactoside, 83% of the enzyme was still bound to the column packed with Bio-Gel A-15 M-phenyl-β-D-thiogalactosidase (69). After β-galactosidase was selectively bound to the affinity column, the enzyme was eluted usually with a pH shift (47,69,157). In theory, the enzyme should also be released from the column by the application of the substrates competing with the ligands. *E. coli* β-galactosidase could not be readily

eluted with the buffer containing lactose, ONPG or isopropyl β-D-galactoside (157). In contrast to *E. coli* β-galactosidase, rat mammary β-galactosidase could be eluted efficiently with buffer containing lactose (one of the substrates of rat mammary β-galactosidase).

Rat mammary β-galactosidase has been shown to catalyze the hydrolysis of β-galactosides, such as lactose, ONPG and PNPG; and the synthesis of 6-β-galactinol by the transfer of galactose from β-galactosides to *myo*-inositol. In fact, these two reactions can be expressed in the same reaction mechanism as follows, using lactose as a substrate.



Lactose can be either hydrolyzed to glucose and galactose or the galactose residue of lactose can be transferred to *myo*-inositol to form 6-β-galactinol by rat mammary β-galactosidase. These two activities have been proven to be carried out by the same protein based on the following evidences.

(a) Thermal inactivation of rat mammary β-galactosidase at 60°C resulted in the same decay rate of hydrolase and transferase activities.

(b) During the purification of rat mammary β-galactosidase following the primary purification procedure, the specific hydrolase activity increased in parallel with the specific transferase activity with a constant ratio of 2.

(c) The two activities have similar pH profiles.

(d) Heavy metal ions (Hg^{2+}, Cu^{2+} and Ag^{+}) displayed the same extent of inhibition on both activities.

The transferase activity of rat mammary β-galactosidase is not unexpected. The transferase activity has been reported for several microbial (120-126), calf intestinal (138) and bovine testicular (69) β-galactosidases.

In the presence of lactose and *myo*-inositol, rat mammary β-galactosidase can catalyze the transfer of galactosyl residue of lactose to *myo*-inositol to yield 6-β-galactinol, *in vitro*. Since UDP-Gal has been found not to be the galactosyl donor, this route becomes the most likely pathway for the synthesis of 6-β-galactinol, *in vivo*. *myo*-Inositol and β-galactosidase occur in other tissues besides mammary gland, but lactose is exclusively found in mammary gland; therefore, lactose seems to be the main factor that accounts for the localization of the synthesis of 6-β-galactinol in mammary glands and milk (2). However, the level of 6-β-galactinol varies with the concentration of *myo*-inositol in mammary gland and milk (2,3). Although β-galactosidase was found in mammary glands, the catalytic characteristics of rat mammary β-galactosidase on the transferase reaction may be different from other sources of β-galactosidase. Studies of the transferase reaction of β-galactosidase, Zilliken *et al*. (134-137), showed that the structure of the transfer products varied with the source of enzyme. For example, using N-acetyl glucosamine as an acceptor and lactose as a donor, the

β-galactosidase of *E. coli* yield 6-O-β-D-galactosyl-N-acetyl glucosamine (136), the β-D-galactosidase of *Lactobacillus bifidus* (intact cells) yielded 4-O-β-D-galactosyl-N-acetyl glucosamine (main) and 6-O-β-D-galactosyl-N-acetyl glucosamine (trace) (134-136), and the β-galactosidase of mammalian tissues yielded 3-O-β-D-galactosyl-N-acetyl-D-glucosamine (90%), 4-O-β-D-galactosyl-N-acetyl-D-glucosamine (9-10%), and 6-O-β-D-galactosyl-N-acetyl-glucosamine (trace) (137). In addition, when using *myo*-inositol as an acceptor, β-galactosidase of *Sporobolomyces singularis* synthesized 5-O-β-D-galactosyl *myo*-inositol (125). However, rat mammary β-galactosidase catalyzed the synthesis of 6-O-β-D-galactosyl *myo*-inositol and another isomer. The synthesis rate of the latter isomer was much slower than that of 6-β-galactinol. The apparent Michaelis-Menten constant for lactose on the synthesis of 6-β-galactinol is 37 mM and the concentration of lactose in rat milk is 70-76 mM (159,160). On the other hand, the apparent Km for *myo*-inositol is much higher and has a value of 345 mM (affinity-chromatography fraction) or 380 mM (DEAE-cellulose fraction). The concentration of *myo*-inositol in rat mammary gland and rat milk are 0.5-1.5 μmole/g (2, 161) and 0.5-5.5 mM (2,161,162), respectively. In comparison with the level of *myo*-inositol found in rat mammary gland and rat milk, the Km value for *myo*-inositol in 6-β-galactinol synthesis of the rat mammary β-galactosidase is high. However, a significant amount of 6-β-galactinol is still synthesized in the rat mammary gland during lactation. This

may be due to one of the following reasons or the combination of them.

(a) The synthesis of 6-β-galactinol is a long-term reaction under low concentration of *myo*-inositol and this product is stable in the mammary-gland cells and after secretion into the more neutral pH of raw milk.

(b) The distribution of *myo*-inositol in the rat mammary gland is not uniform. Some compartments of the cell may have a high concentration of *myo*-inositol.

(c) Some factors which exist in the rat mammary gland can regulate β-galactosidase to synthesize 6-β-galactinol more rapidly even in the low concentration of *myo*-inositol. α-Lactalbumin has been shown to regulate galactosyltransferase to synthesize lactose at the low concentration of glucose (23). However, incubation with α-lactalbumin (0.5 mg/ml) was tested and the result was negative (data not shown). Some cations including Ca^{2+} (6.2 g/100 ml), Na^{+} (100 mM), K^{+} (100 mM), Mg^{2+} (100 mM) and Mn^{2+} (100 mM) also did not show any effect on the synthesis of 6-β-galactinol (data not shown). Recently, some activators for the hydrolase activity of β-galactosidase have been found in commercial BSA (110) and human liver (111).

In addition to lactose, PNPG and ONPG have been shown to be good galactosyl donors in the synthesis of 6-β-galactinol. Surprisingly, lactulose and 1-O-methyl β-D-galactoside are very poor donors. The difference between these donors was apparently due to the variation of the aglycone portion of the β-galactosidase. As *E. coli*

β-galactosidase (112), β-configuration of galactosyl residue of the donors is necessary for the transferase activity of rat mammary β-galactosidase. Neither UPD Gal, 1-O-methyl α-D-galactoside nor p-nitrophenyl α-D-galactoside can serve as a galactosyl donor. In the presence of galactose and *myo*-inositol only, 6-β-galactinol could not be synthesized by β-galactosidase within a 4.5 hr incubation. Wallenfels *et al.* (163) have reported that after a few days incubation with *E. coli* β-galactosidase, (1 ——> 6), (1 ——> 4) and (1 ——> 3) β-D-galactosyl-D-glucose could be formed from galactose and glucose. Galactose did not serve as a galactosyl donor in the 4.5 hr incubation period reported herein but it is a very powerful inhibitor during the synthesis of 6-β-galactinol with lactose as a galactosyl donor. "Galactose" inhibition has been observed on the hydrolase activity of several β-galactosidase (30,33,42,45,58,69,106). In contrast to galactose, glucose is a very poor inhibitor (33,42,48). It is possibly explained based on the model of Wallenfels *et al.* (163). When β-galactosides bind with enzyme to form a Michaelis complex, only the galactosyl residue of β-galactosides occupies the active site tightly but the aglycone portion (such as glucose if lactose serves as the substrate) stays out of the active site during the transferase reaction. When free galactose molecules are added, they will compete with lactose by occupying the active site. On the contrary, glucose won't go into the active site to interfere the enzyme-substrate interaction.

Rat mammary β-galactosidase purified through the affinity-chromatography step was separated from several lysosomal enzymes including β-glucosaminidase, β-glucuronidase, β-glucosidase, β-galactosaminidase, α-mannosidase, α-glucosidase and α-galactosidase. A broad-spread band of β-galactosidase was observed on the polyacrylamide gel. After the denaturation of the enzyme with sodium dodecyl sulfate, one major band was observed on the SDS gel along with two minor bands. The existance of these two minor bands may be the result of contamination by other proteins or partial proteolysis of the native β-galactosidase by proteases during the purification procedure. The subunit molecular weight of the major band was estimated as 63,000. The molecular weight of rat mammary β-galactosidase was determined by gel filtration. The value of the molecular weight varies with the different pH of the eluant. A molecular weight of 110,000 was obtained at pH 7.0 or 200,000 at pH 5.0. Based on these data and the pH profile from either hydrolase or transferase activities, a pH-dependent molecular interconversion of rat mammary β-galactosidase can be proposed as follows.

	Tetramer	⟷	Dimer	⟷	Monomer
Molecular Weight	200,000		110,000		63,000
pH	3.0	⟷	7.0	⟷	?

While pH changes from neutral toward acid, the tetramer forms are gradually formed from dimers. Presumably, the tetramer is the more active form, and the enzymatic activities will

increase along with the decrease of pH. When pH reaches the 3.0-4.0 region, β-galactosidase shows its highest activities, and the tetramers are the major species in this range of pH. In the reverse direction, the enzymatic increases and under these circumstances the less active dimers predominate. If the pH is shifted to a more basic range, the dimers will probably dissociate into monomers. This kind of pH-dependent interconversion has also been observed in mouse liver β-galactosidase (119) and urinary β-galactosidase (164). Sometimes, determinations of the enzyme molecular weight have been carried out by gel filtration eluted with a buffer which has a pH different from the optimal pH of the enzyme catalyzed reaction. Under this condition the estimated molecular weights may not be the native form of the enzymes.

Rat mammary β-galactosidase has been shown to catalyze the hydrolysis of PNPG, ONPG and lactose. The pH optima of hydrolase activity are similar when using either PNPG or ONPG as a substrate. The apparent Michaelis-Menten constant for PNPG hydrolysis is 1.85×10^{-4} M. This value is close to that reported for *E. coli* β-galactosidase (0.93×10^{-4} M), (30), and human liver β-galactosidase (2×10^{-4} M) (67). In contrast to *E. coli* β-galactosidase (28) and rat liver β-galactosidase (98), Mg^{2+} ion did not affect the hydrolase activity of rat mammary β-galactosidase, in agreement with several other mammalian β-galactosidases (69,70). A few cations including Na^{+}, Li^{+}, Ca^{2+}, Ba^{2+} and Mn^{2+} exhibited some stimulatory effect. Of these ions Mn^{2+} had the greatest

effect. This cation has been shown to activate several microbial β-galactosidases including *E. coli* and *Propionibacterium shermanii* enzymes (30,32,35,40,46,96).

As in the case of other β-galactosidases (30,32,35,39, 45,48,63,70,98), rat mammary β-galactosidase was almost totally inhibited by Ag^{+} and Hg^{2+} ions indicating the involvement of the sulfhydryl group in the enzymatic activities. However, Cu^{2+} ion (1 mM) showed little inhibitory effect on either hydrolase or transferase activity. It has been reported that the inhibitory effect of Cu^{2+} ion on β-galactosidase required much higher concentration than Hg^{2+} and Ag^{+} (39,63,98). The concentration we used is probably not great enough to inhibit rat mammary β-galactosidase.

In addition to heavy metal ions, p-chloromercuribenzoate also suppressed the transferase activity of rat mammary β-galactosidase, the same as observed for several other β-galactosidases including *E. coli* (40,67,82,83,99,101), but not the "neutral" β-galactosidase of small intestinal mucosa (83,100,101).

In contrast to the "neutral" or "hetero" β-galactosidases of small intestine (85,101), rat mammary enzyme was not inhibited by tris-base. This property was the same as that of the "acid" enzyme of small intestinal mucosa.

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