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entitled

STUDIES ON BETA - GALACTOSIDASE FROM BACILLUS CIRCULANS, MODIFIED BY GLUTARALDEHYDE

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	Absorbance(s)/Ampere
mA	Milliampere
A^{o}	Angstrom(s)
α	Alfa
β	Beta
BSA	Bovine Serum Albumin
^{o}C	Degree Centrigrade(s)
cm	Centimeter(s)
DTE	Dithioerythritol
EDTA	Ethylene diamine tetra-acetate
Fig.	Figure
g	Gram(s)
h	Hour(s)
[I]	Inhibitor concentration(s)
IAA	Iodoacetamide
k	Rate constant
K_m	Michaelis constant
K_i	Inhibition constant
2-ME	2-Mercaptoethanol

M	Molar concentration(s)
M.W.	Molecular weight
mg	Milligram(s)
ug	Microgram(s)
min	Minute(s)
ml	milliliter(s)
ul	Microliter(s)
mM.	Millimolar(s)
mol	Mole(s)
u mol	Micromole(s)
N	Normal concentration(s)
nm	Nanometer(s)
O.D.	Optical density
ONPG	o-Nitrophenyl- β-D-galactopyranoside
pH	-Log of hydrogen ion concentration
pK	-Log of apparent equilibrium constant K for ionization
PCMB	p-Chloromercuribenzoic acid
r.p.m.	Revolution per minute
-SH	Sulfhydryl group
Sec	Second(s)

[S]	Substrate concentration(s)
SDS	Sodium dodecyl sulfate
TEMED	Tetramethyl (ethylene diamine)
V	Reaction velocity
V_{max}	Maximal velocity
V_o	Void volume
V_e	Elution Volume
w/v	Weight/Volume
%	Percentage

abstract

β-Galactosidases (EC 3.2.1.23) from Bacillus circulans were purified and separated into two isozymes, β-galactosidase-1 (M.W. 2.4×10^5) and β-galactosidase-2 (M.W. 1.6×10^5). They showed similar isoelectric point of about 4.5 and the same optimum pH of 6.0, whereas they were considerably different in K_m values, substrate specificity, temperature stability, and particularly transgalactosidation activity. β-Galactosidase-1 produced only trisaccharide with a maximum yield of 6.6% of the total sugar, while β-galactosidase-2 produced many galacto-oligosaccharides with a maximum yield of 41.5%, during the hydrolysis of 4.56% lactose. Modification of β-galactosidases with glutaraldehyde changed the transgalactosidation activity. Immobilized and, or glutaraldehyde-treated β-galactosidase-1 and β-galactosidase-2 produced galacto-oligosaccharides with a maximum yield of 40% and 42% of the total sugar respectively, during the hydrolysis of 4.56% lactose.

introduction

The enzymes bringing about the hydrolysis of the galactosidic linkage are termed galactosidases. There are two types of them, called α-galactosidases and β-galactosidases depending on the configuration of the anomeric carbon atom of the substrate molecule. β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) is referred to frequently as lactase. It is a glycoprotein (Catherine et al. 1988). Most of the β-galactosidases are extracellular enzymes (Park et al. 1979).

β-Galactosidase is present in a wide variety of sources including plants, animals, and microorganisms (Shukla, 1975). A number of β-galactosidases have been purified and characterized, including those from Escherichia coli (Craven et al. 1965), Kluyveromyces lactis (Giec, 1982), and Aspergillus niger (Greenberg and Mahoney, 1981).

Physical properties of β-galactosidase have been studied. β-Galactosidase from the fungus Curvularia inaequalis has the isoelectric point 4.4, temperature optimum 60°C, and molecular weight 115,000-126,000 (Tikhomirova and Zagustina, 1976) and, from Aspergillus niger has isoelectric point 4.6, pH optima between 2.5 and 4.0, and molecular weight 73,000, 124,000 and 150,000 (Widmer and Leuba, 1979). Mahoney and Whitaker (1978) found that β-galactosidase from Kluyveromyces fragilis has 9 to 10 subunits and molecular weight 201,000; it has isoelectric point 5.1, partial specific volume 0.715, and molar extinction coefficient 1.58 cm^2/mg protein at 280 nm.

Higher temperature inactivates the enzymes. Thermostable β-galactosidases can tolerate higher temperature above 60°C (Widmer and Leuba, 1979 ; Takenishi et al. 1983). β-Galactosidases from some sources have temperature optima within 40° to 60°C (Zagustina and Tikhomirova, 1976 ; Catherine et al. 1988).

Activity of β-galactosidase is markedly influenced by pH. β-Galactosidases from some sources are stable in acidic pH range (Zagustina et al. 1975) and some in basic pH range (Borglum and Sternberg, 1972).

β-Galactosidase catalyzes not only the hydrolysis of the β-1,4-glycosidic bond in lactose to yield glucose and galactose but also the transgalactosidation reaction to produce galacto-oligosaccharides (Roberts and Pettinati, 1957 ; Burvall et al. 1979). The reversion reactions of β-galactosidase produce β-galactosylgalactoses and β-galactosyl glucoses (Huber and Hurlbert, 1986).

A study was implemented to quantitate the hydrolase and transglycosylase activities of β-galactosidase with lactose as the substrate and to investigate various factors which affect these activities (Huber et al. 1976). Production of oligosaccharides depends on substrate concentrations (Wierzbicki and Kosikowski, 1973c ; Greenberg and Mahoney, 1983). The yield of oligosaccharides (Roberts and Pettinati, 1957; Burvall et al. 1979) increases with lactose concentrations in the hydrolysis with β-galactosidases.

The structures of disaccharides (Toba and Adachi, 1978) and oligosaccharides (Jeon and Mantha, 1985; Kwak and Jeon, 1986) formed by transgalactosylation reaction of β-galactosidase have been determined. Characterization of the major constituents of tri-, tetra-, and pentasaccharides and their structure elucidation have been carried out by Toba et al (1985).

β-Galactosidase catalyzes not only lactose but lactosides (Akasaki et al. 1976) and β-galactosides (Huber et al. 1975; Widmer and Leuba, 1979). β-Galactosidase activity depends on the kind of substrates and sources of the enzyme. Activity for the substrate o-nitrophenyl-β-D-galactopyranoside is two times higher than that for lactose (Takenishi et al. 1983).

K_m of β-galactosidase varies with the kinds of substrates. K_m for o-nitrophenyl-β-D-galactopyranoside (ONPG) and lactose was studied (Wierzbicki and Kosikowski, 1973b). V_{max} of β-galactosidase depends on the kinds of substrates as well as sources of the enzyme (Park et al. 1979).

β-Galactosidase is activated by glycerol, -SH, and p-hydroxymercuribenzoate (Iwasaki et al. 1971). Metal ions, Na^+, K^+, Ba^+, Ni^+, Mg^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+} and Hg^{2+} also activate this enzyme (Smart and Richardson, 1987; Catherine et al. 1988).

Metal ions Na^{+}, Ag^{+}, Cu^{2+}, Hg^{2+} and Mg^{2+} and organic molecules, nitrophenyl thiogalactoside, galactonolactone, -SH, L-cysteine, monoiodo acetic acid, trisamino methane, EDTA, p-chloromercuribenzoate and mercaptide inhibit the activity of β-galactosidase (Iwasaki et al. 1971; Mahoney and Whitaker, 1978). p-Aminophenyl-β-D-thiogalactopyranoside competitively inhibits (Takenishi et al. 1983) the activity of β-galactosidase. Galactose causes product inhibition (Smart et al. 1985) of the enzyme.

The mechanism of action of β-galactosidase for the transfer of a D-galactose residue from galactoside to an accepter was studied (Wallenfels and Malhotra, 1961). Hydroxyl groups at positions 3,4, and 6 are important for binding to the galactose site of β-galactosidase (Huber and Gaunt, 1983). All the oligosaccharides produced during the enzymatic hydrolysis of lactose are formed by attachment of galactosyl residues in β-(1 → 6) linkages (Burvall et al. 1980).

Chemical modification of native enzyme is of great importance to obtain an enzyme with the desired operational parameters, such as optimum pH, temperature, stability and activity. Sugiura et al (1980) first modified the tryptophan residues of β-galactosidase with N-bromo-succinimide. Some investigators have reported the modificational effects of glutaraldehyde on β-galactosidase properties (Nakanishi et al. 1983).

Enzymes have been immobilized by a variety of methods (Silman and Katchalski, 1966; Gekas, 1985). By procedures such as adsorption (Okos et al. 1978), encapsulation (Ostergarred and Martiny, 1973), and covalent bonding (Woychik and Wondolwski, 1972; Morisi et al. 1973) it is now possible to stabilize enzymes, overcome their high initial cost by repeated reuse, and remove them from the final product.

Chemical modification of immobilized enzymes by crosslinking agents, in most cases, ensures greater efficiency of the system. The most common bifunctional reagent used for intra- and intermolecular crosslinking is glutaraldehyde (Olson and Stanley, 1979). β-Galactosidase from Bacillus circulans was immobilized by adsorption onto the support and crosslinked with glutaraldehyde (Nakanishi et al. 1983).

Recovery of activity (Hustad et al. 1973b) and the stability of the immobilized enzyme are the major considerations. Stability of the immobilized β-galactosidase is a rather difficult parameter to assess. pH, thermal (Ohmiya et al. 1975), storage (Hustad et al. 1973a), and operational stability (Nakanishi et al. 1983) have been studied.

Lactose hydrolysis has found an increasing interest these past few years. It allows milk consumption by lactase-deficient populations (Holsinger, 1978). β-Galactosidase is used as a therapeutic agent for lactose intolerant people (Sugiura et al. 1978). Exogenous β-galactosidase eliminate lactose malabsorption and gastrointestinal discomfort (Burvall et al. 1980) in them.

β-Galactosidase is used in the food industry to prevent crystallization of lactose in custards, milk concentrates (Giec et al. 1985), ice creams, food syrups (Wierzbicki and Kosikowski 1973a), etc. β-Galactosidase from Streptococcus thermophilus is being an useful enzyme for the hydrolysis of lactose in dairy products because of its thermostability (Ramana Rao and Dutta, 1981; Greenberg and Mahoney, 1982, 1983). Oligosaccharides, the trangalactosidation product, have recently become of interest, since these oligosaccharides might be useful as bifidus growth factor (Tanaka et al. 1983).

Immobilized β-galactosidase is used for the large scale production of low lactose dairy products (Ohmiya et al. 1975, 1977). One advantage of using such a preparation is that milk should not be contaminated with foreign protein which might cause allergic reactions to lactose intolerant people on oral administration of β-galactosidase. To avoid bacterial contamination of the dairy products and enzyme inactivation due to contaminants milk is treated with thermostable immobilized β-galactosidase at a temperature above 50°C (Kobayashi et al. 1978).

aim of the investigation

The application of /β-galactosidase to the hydrolysis of lactose to D-glucose and D-galactose in dairy products is of importance for sensory qualities and, particularly in patients with lactose intolerance. Recently the production of galactooligosaccharides using the transgalactosidation reaction has become of interest since such compounds might be useful as a bifidus growth factor.

The properties of partially purified /β-galactosidase from Bacillus circulans have been investigated. So far studied, the oligosaccharide-producing activity of /β-galactosidase from this species was higher than that of different sources.

In order to use /β-galactosidase efficiently for practical applications, various techniques of immobilization have been applied. Adsorption of /β-galactosidase from B.circulans to phenolformaldehyde resin has been studied. Less work has been done on Silica gel matrix. Immobilization of /β-galactosidase from B. circulans by covalent attachment is yet to be investigated. Covalent attachment of enzyme to the support provides the most secured method of immobilization. At present, glutaraldehyde is the only reagent employed commercially for the preparation of covalently immobilized enzymes.

Immobilization of biomolecules on inorganic carrier by metal-link method has become of importance. Activation of Silica, pumice stone, and other inorganic supports by transition metal have been studied. Hydrous metal oxide materials have proved to be suitable for immobilization of several biomolecules such as amino acids and peptides, enzymes, and etc.

Hybrid method of adsorption and covalent attachment of enzymes on supports has become of interest for the increasing efficiency of the preparation. Hence the aim of the present investigation is to study the properties of purified β-galactosidase from B. circulans covalently attached to the transition metal activated Silica gel, and to compare with that of the free form.

materials and methods

A. Materials

Crude β-galactosidase from Bacillus circulans was supplied by Daiwa Kasei K.K. (Osaka, Japan). Lactose, o-nitrophenyl-β-D-galactopyranoside (ONPG), 1,6-diaminohexane, and $FeCl_2$ were purchased from BDH Chemicals Ltd., U.K. A Glucostat reagent kit and a galactose ultraviolet test kit were obtained from Worthington Biochemical corporation and Boehringer Mannheim GmbH, respectively. Sephadex G-150, Sephadex G-200, QAE-Sephadex A-50, polybuffer exchanger PBE-94, and Octyl-Sepharose Cl-4B were products of Pharmacia Fine Chemicals, Sweden. Silica gel (pore size 500 Å) was purchased from E. Merck, F.R.G. Glutaraldehyde was obtained from Sigma Chemical Company, U.S.A. All other chemicals were of analytical grade, and purchased either from BDH Chemicals Ltd. or E. Merck.

B. Methods

1.0 Estimation of protein by the Folin Ciocalteau method (Lowry et al. 1951)

Reagents :

Alkaline copper reagent - A. 2% Na_2Co_3 in 0.1N NaOH, 2 g anhydrous Na_2Co_3 was dissolved in 100 ml 0.1N NaOH. B. 1% $CuSO_4$ solution in distilled water was mixed with an equal volume of 2% sodium potassium tartrate before use.

Working alkaline copper reagent - 50 ml of A. and 1.0 ml of B. were mixed immediately before use.

Folin Ciocalteau reagent (FCR) - 1.0 ml of FCR was diluted with 1.0 ml of distilled water.

Standard BSA solution -
10 mg of crystalline BSA was dissolved in distilled water and diluted to 100 ml.

Method : 0.1 ml portion from appropriately diluted sample and 0.1 to 1.0 ml from standard BSA solution were taken in a series of test tubes and made upto volume 1.0 ml with distilled water. 4.0 ml alkaline copper reagent was added to each tube and incubated at 40°C for 15 min. 0.5 ml of FCR was then added to the reaction mixture and was shaked vigorously. All the tubes were taken at room temperature for 30 min in order to develop color. Absorbance was taken at 660 nm against a blank.

2.0 Purification of β-Galactosidase

All operations were carried out at 0 to 4°C. Absorbance of each fraction eluted from various columns was measured at 280 nm using an UV-spectrophotometer, and the β-galactosidase activity was determined using ONPG as a substrate, as described later. After each step, the pooled active fractions were concentrated by ammonium sulfate precipitation (55 % to 75% saturation). Following each concentration, the concentrate was dialyzed exhaustively against the appropriate buffer.

The crude enzyme preparation (15g) was suspended in 100 ml of 0.1 M sodium phosphate buffer, pH 6.0 (assay buffer)and centrifuged at a speed of 8,000 r.p.m., 10 min.

2.1 Gel filtration using Sephadex G-150

Sephadex G-150 (40 g) was allowed to swell in 1 liter of distilled water for 24 h. Water was decanted and washed two times with the assay buffer (0.1M phosphate buffer, pH 6.0). Sephadex was then packed by gravity into the column (5.5 cm x 40 cm). After equilibrating for 24 h the column was ready for use.

The supernatant was applied onto the column and eluted with assay buffer. Fractions were collected in test tubes and stored at 4°C.

2.2 Chromatography on QAE-Sephadex A-50 anion exchanger

QAE-Sephadex A-50 (10g) was allowed to swell in 1 liter of distilled water for 24 h. Water was decanted and washed cyclically with the reagents as follows :

(a) 1.0 M NaOH (b) Water (c) 0.5M HCl (d) Water (e) 1.0 M NaOH (f) Water - (until excess alkali has been removed from the filtrate).

Washed adsorbent cake was equilibrated on suspending in sufficient starting buffer (0.01M Tris- acetate buffer, pH 8.0). A suitable slurry of the adsorbent (usually about 10 volumes of buffer per volume of washed adsorbent cake) was packed by gravity flow into the column (2.5 cm x 42 cm) initially filled with the starting buffer.

The active fractions obtained by the gel filtration were applied onto the column which was equilibrated with 0.01M Tris-acetate buffer, pH 8.0. The enzymes were eluted by a stepwise increase of NaCl concentration (0.1 M, 0.2M, and 0.3M) with constant 0.01M Tris-acetate buffer, pH 8.0.).

2.3 Isoelectric focusing by polybuffer exchanger PBE 94

A. Solution to prepare the density gradient for the column (1.5 cm x 25 cm) :

	Dense soln.	Less dense soln.
Carrier Ampholite (40%, w/v) ...	1.5 ml	0.3 ml
Protein solution (0.25mg/ml) + Distl. water	24 ml	31 ml
Sucrose	15 g	

B. Electrode solutions

Anode at the top of the column :

Concentrated sulfuric acid - 0.1 ml

Distl. water - 25 ml

Cathode in the central tube :

2M Sodium hydroxide - 4 ml

Distl. water - 56 ml

Sucrose - 44 g

Column was filled with the gradient solutions. Filling rate with gradient mixer was 5 ml/min.

The fraction rich in β-galactosidase, eluted by buffer containing 0.2M NaCl, was applied to the column, which was equilibrated with the starting buffer of 0.025 M piperazine - HCl, pH 5.5. Electrophoretic run was accomplished with a current supply of 20 mA for 24 h. The enzyme was eluted (flow rate, 4 ml/min) by feeding polybuffer 74-HCl (pH 4.0) which formed a pH gradient through the column.

2.4 Fraction with ammonium sulfate

Active fractions eluted after isolectric focusing were divided into eight equal sub-fractions (each fraction, 12 ml). Polybuffer in each fraction was removed from the enzymes for ammonium sulfate precipition as follows :

Each fraction was treated with solid ammonium sulfate so that it becomes 55% saturated with respect to ammonium sulfate. The pellet was collected by centrifugation at 15,000 r.p.m. for 30 min in Sorvall RC-5B refrigerated superspeed centrifuge. The supernatant and the pellet were collected separately. The enzyme activity and the protein content of both supernatant and the pellet were collected separately. The enzyme activity and the protein content of both supernatant and pellet (the latter being redisolved in 0.1 M phosphate buffer, pH 6.0) were determined. The pellet was stored at $4^{\circ}C$ and the supernatant was raised to 75% saturation with respect to ammonium sulfate. The pellet obtained after centrifugation was redissolved in 0.1M phosphate buffer, pH 6.0, and the enzyme activity and the protein content were determined.

2.5 Dialysis

The active fractions obtained by ammonium sulfate fractionation were dialyzed. Dialysis was carried out in a seamless cellulose tubing available in Visking Co., Chicago. Diameter and length of the tubing depend on the

amount of solution to be dialyzed. The dialysis tubing or bag was placed in a beaker of 1 liter capacity containing 0.1M phosphate buffer, pH, 6.0, the temperature of which was maintained at 0°to 4°C. The buffer was kept cold by placing the 1 liter beaker in another beaker of 5 liter capacity which was filled with ice. A magnetic stirrer was used to stir the buffer at a speed of 30 r.p.m. The dialyzate was placed in fresh buffer after each 4 to 5 h. During the night the dializing bag was placed in fresh buffer and kept in the refrigerator.

2.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Reagents :

A. Lower gel buffer, pH 8.8

Tris base	46.0g	0.375M (final concentration)
SDS	4.0g	0.4% "

were dissolved in 750 ml deionized water. pH was adjusted to 8.8 with HCl and volume made to 1 liter with deionized water. It was stored at 4°C.

B. Acrylamide - Bis-acrylamide solution

Acrylamide	30g	30% (final concentration)
Bis	0.82g	0.82% "

were dissolved in 100 ml deionized water, filtered through whatman No.1 and stored at 4°C in brown bottle.

C. Ammonium persulfate solution

Ammonium persulfate, 10g 10% (final concentration)

was dissolved in 100 ml deionized water.

Resolving gel - 7.5% Acrylamide gel :

A.	10.6 ml
B.	8.0 ml
C.	50 ul
TEMED	25 ul
Deionized water	13.4 ml

Overlay for lower gel - 0.1% SDS

SDS	100 mg
Water	100 ml

D. Upper gel buffer, pH 6.8

Tris base	7.5 g	0.125M (final concentration)
SDS	2.0 g	0.4% "

were dissolved to 300 ml of deionized water. pH was adjusted to 6.8 with HCl and volume made to 500 ml. It was stored at 4°C.

Stacking gel - 4.8% acrylamide gel :

D.	2.5 ml
B.	1.6 ml
C.	30.0 ul
TEMED	10.0 ul
Water	5.9 ml

E. Sample buffer, pH 6.8

Tris base	75 mg	0.0625M (final concentration)
Glycerol	1 g	10% (w/v) "
2-ME	500 mg	5% "
SDS	230 mg	2.3% "

were dissolved in 7.5 ml of water. pH was adjusted to 6.8 with HCl and made upto 10 ml. It was stored at 4°C.

Bromophenol blue solution :

Bromophenol blue	0.1g
Deionized water	100 ml

It was stored at room temperature.

F. SDS - running buffer, pH 8.3

Tris base	3.03g	0.025M (final concentration)
Glycine	144.0g	0.192 M "
SDS	10.0g	0.1% "

The volume was made upto 1 liter. It was stored at 4°C. The running buffer was diluted 10 times with deionized water before use.

Protein stain :

Coomassie brilliant blue R-250	0.25g
Methanol	125 ml
Glacial acetic acid	25 ml
Water	100 ml

Dye was first dissolved in methanol, then acid and water were added.

Destaining solution :

Methanol	50 ml
Glacial acetic acid	75 ml
Water	875 ml

Procedure :

Separating gel mixture was poured into the assembled plates and carefully overlayed with 0.1% SDS solution, and allowed to set for 30-60 min.

Stacking gel was poured on top of the separating gel after draining of the water. The comb was inserted and allowed to set for an hour. After removing the comb the wells were rinsed with distilled water.

About 50 ug of the sample mixed with sample buffer (50 ul) was boiled in water-bath and cooled. Then cooled samples were loaded into the wells with a micropipette. In one sample, bromophenol blue dye was added.

The gel electrophoresis cell and top chamber was filled with running buffer. Electrical supply was set to a constant current of 20 mA for stacking gel and 30 mA for separating gel.

After about 3 hours, when the bromophenol blue dye front had reached the bottom, the electrical supply was disconnected.

The gel was removed from between the glass plates and was immersed in the protein stain. The gel was left in the stain with gentle agitation until the dye had penetrated the gel.

The gel was then removed into the destaining solution and kept in a hot-pack. With a few changes of destaining solution, the gel background became colorless and protein bands were visible as blue bands.

2.7 Affinity chromatography by octyl-Sepharose-CL-4B

Appropriate amount of octyl-Sepharose-CL-4B gel was packed in the column (1.5 cm X 10 cm) and equilibrated with 0.2M $(NH_4)_2SO_4$ and 0.5 M NaCl in 0.05 M sodium phosphate buffer, pH 6.0 to remove a trace amount of contaminating protein.

Dialyzed protein from gel electrophoresis was applied onto the column and the fraction rich in β-galactosidase was eluted by the same assay buffer.

2.8 Preparative polyacrylamide gel electrophoresis

Preparation of reagents :

(i) Electrode or running buffer, 0.05M, pH 8.8

6.32g Tris base and 3.94g glycine were dissolved in water making 1 liter solution.

(ii) Resolving gel buffer, pH 9.4

36.3g Tris base and 40 ml 1N HCl were dissolved in water making 100 ml solution.

(iii) Stacking gel buffer, pH 7.2

2.85g Tris base and 12.8 ml 1M H_3PO_4 were dissolved in water making 100 ml solution.

These buffers (i, ii, iii) were stored at room temperature.

(iv) Stock acrylamide - bis acrylamide solution

20g Acrylamide and 1.0g Bis were dissolved in water making 100 ml solution and filtered with whatman No.1 filter paper and stored in a dark bottle.

(v) x 2 Stock sample buffer, pH 7.2

(0.05 M Tris - HCl, 0.001% Bromophenol blue, and 10% glycerol and 2-mercaptoethanol)

0.73g Tris base, 2 mg Bromophenol blue and 20 ml glycerol were dissolved in water making 90 ml solution, the pH of which was adjusted with HCl to 7.2. 2-Mercaptoethanol was added freshly before each run in the ratio of 10% (v/v).

(vi) Components of separating gel

5% Acrylamide, 0.25% Bis, pH 9.4

Stock solutions (each made upto 100 ml) :

A.	IN HCl,	40 ml
	Tris base	36.3g
	TEMED	0.23 ml, resulting pH 9.4

B. Acrylamide, 20 g
Bis, 1.0 g

C. Riboflavin, 4.0 mg

Working solution :

1 Part A., 2 Parts B., 1 Part C. and 4 Parts distl. water, making 140 ml solution. Photopolymerization was done by exposing to fluorescent light.

(vii) Components of spacer gel

2.5% Acrylamide, 0.625% Bis, pH 7.2

Stock solutions (each made up to 100 ml) :

A. 1M H_3PO_4, 12.8 ml
Tris base, 2.85 g
TEMED, 0.10 ml, resulting pH 7.2

B. Acrylamide, 10 g
Bis, 2.5 g

C. Riboflavin, 4.0 mg

D. Sucrose, 40 g

Working solution :

1 part A., 2 parts B., 1 part C., and 4 parts D., making 20 ml solution. Photopolymerization was done by exposure to fluorescent light.

(viii) Staining solution

Amido Black dye (napthol blue black), 1 g

7.5% acetic acid, 100 ml

(ix) Destaining solution

7.5% acetic acid solution.

Sample preparation and application :

Protein samples were mixed with 10 ul of bromophenol blue solution (sample buffer solution). Approximately 10-50 ug of protein was applied to each gel.

Procedure :

70 ml separating gel was placed in each slab (0.5 cm x 15 cm) and allowed to polymerize with a few ul of water-butanol solution at the top. After removing water-butanol, 10 ml spacer gel was added on the resolving gel of each slab and allowed to polymerize with a 'comb' set at the upper gel. After completion of polymerization, comb was removed carefully ensuring the slots unbroken.

25, 50, 75, 100 ul of different standard proteins were placed in different slots of the slab. Similarly, for sample proteins, the fractions which had a relatively high activity towards ONPG (fractions 1 and 2) or lactose (fractions 7 and 8) were placed in the slots of the slab.

The electrode or running buffer was poured onto the top of the gel chamber. Electrophoresis was run for 8 h at 4°C with a constant current of 30 mA.

Enzymatically active zones were stained with 6-bromo-2-napthyl-β-D-galactopyranoside coupled with Diazo blue B. A major band was cut out with a fine knife, crushed and suspended in a minimal amount of the cold assay buffer, and finally dialyzed against the same buffer.

3.0 Assay of β-D-galactosidase activity

The activity of β-galactosidase was determined either using ONPG (final concentration, 0.245%) or lactose (final concentration, 4.56%) as a substrate dissolved in the assay buffer (0.1 M sodium phosphate buffer, pH 6.0) by a method described by Nakanishi et al (1983). One unit of β-galactosidase activity was defined as the amount of enzyme producing 1 umol of 2-nitrophenol or D-glucose per min at 40°C and pH 6.0.

Enzyme activity towards ONPG was measured as follows :

The reaction was started by the addition 50 ul enzyme solution, appropriately diluted with the assay buffer, to 4.95 ml of 0.247% ONPG solution. After 10 min incubation, 1 ml of the sample solution was removed and added to 1 ml 10% Na_2CO_3 solution to stop the reaction. The concentration of 2-nitrophenol produced was determined by measuring the absorbance at 420 nm.

Enzyme activity towards lactose was measured as follows :

When measuring the activity towards lactose substrate, 0.4 ml of the enzyme solution, appropriately diluted with the assay buffer, was combined with 9.6 ml of a 4.75% (w/v) lactose solution and incubated at 40°C. After 10 min incubation or at various time intervals, 0.5 ml of the reaction mixture was removed and poured into 1 ml 0.375 N NaOH solution to stop the reaction. After storage at 0°C for 2h, this mixture was adjusted to pH 6.0 with acetic acid and phosphate buffer, and divided into three portions for the determination of D-glucose, D-glactose and oligosaccharides content. D-Glucose and D-galactose were determined with a Glucostat reagent and a Galactose UV test kit, respectively.

Values for the Michaelis-Menten constant K_m, maximum specific activity k_2, and inhibitor constant K_i were determined from reciprocal plots of substrate concentrations versus initial reaction rates (Lineweaver and Burk, 1934). Phosphate buffer was used to measure the pH dependence of the activity. Optimum temperature for the reaction was determined by measuring the activity at 10 min incubation, changing temperatures between 20° to 70°C.

3.1 Stability experiment

The effects of temperature on the stability of the enzymes were studied by measuring the remaining activity at 40°C

after incubating the enzyme solutions for one hour at different temperatures (20^{o}-60^{o}C).

To determine the effects of metal ions and group specific reagents on the β-galactosidase activity, the enzyme solutions were incubated in the assay buffer containing an appropriate amount of metal ions or reagents for 10 min at 20^{o}C and the enzyme activity was determined towards ONPG as a substrate at 40^{o}C, pH 6.0.

4.0 Determination of molecular weight

Molecular weights of purified β-galactosidases were determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE) as described by Hames (1982) and by gel filtration on a Sephadex G-200 column.

4.1 Gel filtration using Sephadex G-200

Sephadex G-200 (7.5 g) was allowed to swell in 1 liter of distilled water for 24 hours. Then water was decanted and washed a couple of times with the assay buffer (0.1 M sodium phosphate buffer, pH 6.0). The Sephadex was then packed by gravity into the column (1.5 cm x 40 cm). To support the column a teflon net was used at the bottom. After equilibrating with assay buffer at 4^{o}C for 24 h the column was ready for use.

The void volume (V_o) of the column was determined by passage of Blue Dextran-2000 (M.W. 2×10^{6}). The column

was standardized by using bovine serum albumin (BSA), aldolase, catalase, β-galactosidase (from E.coli) and thyroglobulin. For the determination of elution volumes (V_e) for all the marker proteins, 1.0 ml containing 1.0 mg of each marker was separately charged on the column. Only 1.0 ml solution was used to have a narrow starting zone for better separation, and fractions of 2.0 ml eluate were collected. To each fraction, 1.0 ml of 0.1 M phosphate buffer, pH 6.0, was added and mixed well. For marker proteins and sample, absorbances were measured in a Carl Zeiss UV spectrophotometer model PM QII, M_4 QIII.

5.0 Analysis of Saccharides

In the measurement of the course of the hydrolysis of lactose (final concentration, 4.56%), the amounts of glucose, galactose, lactose, and oligosaccharides produced were measured. At different times small portions of the reaction mixture were removed into a test tube and then heated in a boiling water bath for 2 min to stop the reaction.

The amount of glucose was determined with a glucostat reagent (Glucose oxidase - peroxidase method) :

The reagent was dissolved in 180 mM Tris phosphate buffer, pH 7.8. To 0.02 ml of a suitable dilution of sample (or eluate) (<4 mg glucose/ml) 2.0 ml of reagent solution

were added and incubated at 25°C for 60 min. The absorbance at 510 nm was determined in a spectrophotometer.

The amount of galactose was determined with a galactose UV test kit (UV Method) :

0.20 ml of sample (or eluant) diluted to contain about 0.1 mg galactose was transferred to a cuvette. 3.0 ml of 0.2M phosphate buffer, pH 7.5, and 0.10 ml of NAD solution (13 mM) were added. The absorbance (A_1) was measured at 340 nm. When a stable reading was obtained, 0.02 ml galactose dehydrogenase (20 U/ml) was added and the absorbance (A_2) was measured at 340 nm after 40 min. The difference ($A_2-A_1 = \Delta A$) of the absorbance is the actual absorbance of interest. For standardization a 0.20 ml galactose solution ($<$ 0.5 mg/ml) was then added and the absorbance was measured.

Lactose and other oligosaccharides were separated by paper chromatography on whatman 3 MM paper and they were identified by comparing the R_f values with those for standard substances. A measured amount of the reaction mixtures (in duplicate) was applied on the paper and the saccharides were separated using four 24 hours developments in solvent system butanol : pyridine : water (6:4:3). From each duplicate sample, one portion was cut and stained by silver dip reagent (Trevelyan et al. 1950), the

corresponding sugars were eluted in water, and the saccharide content was determined by the phenol - sulfuric acid method (Dubois et al. 1951). The Orange - yellow color produced by adding sulfuric acid (5 ml) to the sugar solution (2 ml) containing phenol was permanent; its optical density measured at 490 nm when reffered to a standard curve gave the concentration of the sugar.

5.1 Preparation of standard oligosaccharides

Oligosaccharides were separated from a lactose hydrolyzate. The lactose hydrolyzate was prepared as follows. One liter of a 4.56% lactose solution dissolved in deionised water was combined with β-D-galactosidase from B. circulans immobilized onto activated Silica gel and incubated at 40°C. When the D-glucose concentration in the reaction mixture reached 10 mg/ml, the activated Silica gel was removed with a glass filter. The filtrate was concentrated to 100 ml using a rotary evaporator at 40°C and the concentrate applied to an activated charcoal column (5.5 cm x 40 cm). The activated charcoal was preactivated with 15% acetic acid in boiling water, then packed in a column and finally washed with a large volume of distilled water until the pH of the eluate was neutral. The oligosaccharides were eluted using aquous 5, 15 and 20% ethanol solutions. The oligosaccharides corresponding to trisaccharide and tetrasaccharide appeared mostly in the fractions eluting at 10% and 15% ethanol solutions, respectively. These fractions were further purified by

paper chromatography and their chemical compositions were determined by the enzymatic hydrolysis. Oligosaccharides (0.5 - 0.8 mg/ml) separated by paper chromatography were incubated at 40°C with β-galactosidase from B. circulans (3 units/ml, determined by lactose as a substrate) partially purified by ultrafiltration for 48 h. Then their glucose and galactose compositions were determined. To prevent microbial contamination during the enzyme reaction 0.15% (w/v) methyl p-hydroxy benzoate was added to the reaction mixture. Complete hydrolysis of the oligosaccharides was confirmed by paper chromatography as described earlier. The major components for di-, tetra-, and pentasaccharides obtained were used as standard substances. The oligosaccharides were found to be composed of one molecule of glucose and one to four molecules of galactose.

6.0 Preparation of transition metal activated inorganic support

Support derivatization procedures by Cabral et al (1981b):

A. Oxychloride derivative : Silica gel (Pore size 500 Å) was steeped in 15% (w/v) $FeCl_2$ (2.5 ml/g matrix) for 30 h in an oven at 45°C to dryness. This derivative is unstable and must be immediately reacted with suitable agents for enzyme coupling.

B. Alkylamine derviative: The oxychloride derivative was aminated at 45°C for 30 min with 1% (w/v) 1, 6-diaminohexane solution (5 ml/g matrix)in carbon tertachloride. The alkylamine derivative was washed with methanol (3 x 5 ml/g matrix) and distilled water (3 x 5 ml/g matrix), in order to remove the excess amine reagent and solvent.

C. Carbonyl derivative: The alkylamine derivative was reacted with a 5% (v/v) glutaraldehyde solution (5 ml/g matrix) in 0.05 M pyrophosphate buffer pH 8.6, for 1 h at 25°C. The carbonyl derivative obtained by a schiff's base formation was then washed with distilled water (3 x 5 ml/g matrix).

7.0 Preparation of immobilized enzymes

β-D-Galactosidases from B. circulans was immobilized onto activated Silica gel and using a method described by Nakanishi et al (1983). An optimum yield of immobilization was obtained under the following experimental conditions. An appropriate amount of activated Silica gel which had been equilibrated overnight in the assay buffer with occasional decantation, was in contact with enzyme solutions of various concentrations for 24 h at 35°C under shaking. Then, the enzyme solution was decanted, and the activated Silica gel was put in contact with 3% glutaraldehyde

(final concentration) diluted with the assay buffer for 2 h at 4°C under gentle shaking. The resulting immobilized enzyme was washed with a large volume of the assay buffer.

7.1 Assay of immobilized β-D-galactosidase activity

A measured amount of the wet immobilized enzyme derivative was incubated with 4.56% lactose solution at 40°C under vigorous mixing with a magnetic stirrer. After 10 min incubation, or at various time intervals, a soluble portion of the reaction mixture was carefully removed from the test tube without contamination by immobilized enzyme derivative, and the amount of D-glucose produced was assayed with the Glucostat reagent (Nakanishi et al. 1983).

One unit of immobilized β-galactosidase activity was defined as the amount of wet immobilized enzyme producing 1 u mol of D-glucose per minute at 40°C and pH 6.0.

7.2 Elution of saccharides from the activated Silica gel column

Twenty percent lactose solution dissolved in the assay buffer (pH 6.0) was fed continuously for 24 h at 40°C to various columns containing either 1.5g of activated Silica gel, immobilized β-galactosidase-2 onto activated Silica gel (15 or 240 units/g of wet gel) or inactivated immobilized β-galactosidase-2 onto activated Silica gel (before inactivation with 20 mM mercuric chloride, 240 units/g of

wet gel). With immobilized columns the initial percentage of conversion of lactose at the outlet of the column was fixed at around 50% to 60%. After 24 h feeding the lactose solution was stopped. Then the assay buffer was fed with the constant flow rate at 40°C and the effluent from the columns was taken at certain times by a fraction collector. In one experiment with a column containing 1.5 g of immobilized β-galactosidase onto activated Silica gel (240 units/g of wet gel), the enzyme was inactivated after 24 h of continuous reaction by feeding 20% lactose containing mercuric chloride (100 mM) for 3 h at 40°C before the assay buffer was fed. The saccharide content of each fraction was measured by phenol-sulfuric acid method (Dubois et al. 1951). β-Galactosidase-1 followed this method.

The amount of saccharides remaining in the support was also measured by this method after the immobilized enzyme was washed on a glass filter using 500 ml of the assay buffer.

7.3 Modification of β-galactosidases with glutaraldehyde

β-Galactosidase in the assay buffer (0.1 M sodium phosphate buffer, pH 6.0) was treated with glutaraldehyde (final concentration, 0.025% to 3%) and incubated at 4°C for 2 h. The final enzyme concentration was 0.98 mg/ml. The enzyme solution treated with 0.25% or 3% glutaraldehyde

was dialyzed against the assay buffer for 48 h at 4°C. One milliliter of the enzyme solution treated with 0.025% glutaraldehyde solution was mixed with 0.025 ml of 3% ethylamine, and incubated at 4°C for 1 h to block the free aldehyde groups. Then this enzyme mixture was dialyzed as above. One portion of the dialyzed enzyme solution was further treated with glutaraldehyde (final concentration, 0.075%) for 2 h and them with ethylamine for 1 h to prepare the highly modified enzyme without intermolecular crosslinking. This enzyme preparation was dialyzed exhaustively as above.

The protein concentration of the native and modified enzymes was measured by the method of Lowry et al (1951). Polyacrylamide gel electrophoresis of the enzyme preparations was done as described previously.

8.0 Measurement of K_m value

The Michaelis - Menten constant K_m, of the enzyme treated with glutaraldehyde was evaluated from reciprocal plots of substrate concentrations versus initial reaction rates (Lineweaver and Burk, 1934). The experiment was done at 40°C with lactose as the substrate in the concentrations from 20 to 160 mM.

results

1.0 Purification of β-galactosidases

From a crude enzyme preparation of 15 g, 954 mg of protein was obtained with specific activities of 18.0 and 33.6 units per mg of protein when lactose and ONPG were used as substrates, respectively. The results of gel and ion-exchange chromatography are summarized in Table I. The β-galactosidase activity was eluted as a single peak by gel chromatography on Sephadex G-150. By this step, 533 mg of protein was recovered with specific activities of 24.0 and 37.0 units per mg protein with lactose and ONPG as substrates, respectively.

Ammonium sulfate precipitation and dialysis recovered 446 mg of protein with specific activities of 56.1 and 39.5 units per mg.of protein with lactose and ONPG as substrates, respectively.

In the case of ion-exchange chromatography on QAE-Sephadex A-50, most of the β-galactosidase activity was eluted as a single peak at 0.2 M NaCl. Total protein recovery was 322 mg. The specific activities towards lactose and ONPG were 69.8 and 55.3 units per mg of protein, respectively.

The β-galactosidase activity was eluted as a single peak by isoelectric focusing with polybuffer exchanger PBE 94 as shown in Fig.1.

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The β-galactosidase activity was eluted as a single peak by isoelectric focusing with polybuffer exchanger PBE 94 as shown in Fig. 1.

Table I. Summary of the partial purification of β-galactosidase from Bacillus circulans

Purification Steps	Total protein (mg)	Specific[a] activity (LU)	Yield (%)	Specific[b] activity (OU)	Yield (%)	Ratio of activity (OU/LU)
Crude	954	18.0	100	33.6	100	1.9
Sephodex G-150 Chromatography	533	24.0	74.5	37.0	61.5	1.5
Ammonium sulfate precipitation (55% saturation & dialysis)	446	56.1	145.7	39.5	55.0	0.7
QAE-Sephadex A-50 ion-exchange Chromatography	322	69.8	130.9	55.3	55.6	0.8

a. umol glucose/min/mg protein.

b. umol O-nitrophenol/min/mg protein.

OU - ONPG used as substrate.

LU - Lactose used as substrate.

Figure 1. Isoelectric Focusing of β-Galactosidase from B. circulans.

β-Galactosidase activity was measured during isoelectric focusing by polybuffer exchanger PBE 94 with the starting buffer of 0.025M piperazine-HCl, pH 5.5. The column was eluted by polybuffer 74-HCl, pH 4.0, forming a pH gradient.

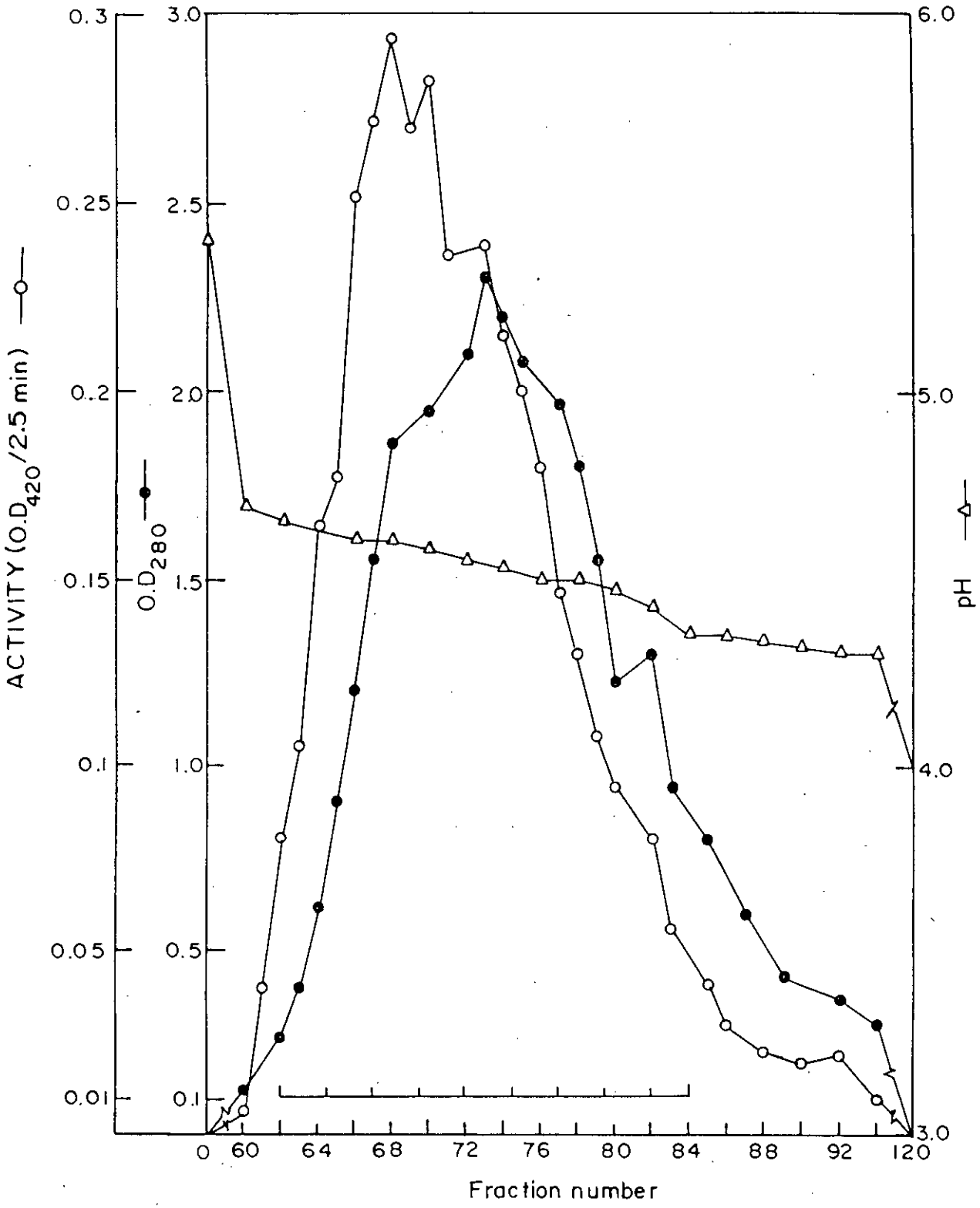

ACTIVITY (O.D420/2.5 min)
O.D280
pH
Fraction number
0.3
0.25
0.2
0.15
0.1
0.05
0.01
3.0
2.5
2.0
1.5
1.0
0.5
0.1
6.0
5.0
4.0
3.0
0
60
64
68
72
76
80
84
88
92
120

Fig. 1.

Polyacrylamide gel electrophoresis of eight sub-fractions eluted by polybuffer exchanger PBE 94 clearly showed two major bands as shown in Fig.2, though the color intensity between them was different for each fraction. In addition to the variation in proportion of the two major protein bands, specific activities towards lactose and ONPG for each fraction varied as shown in Fig.3.

Hydrophobic chromatography on Octyl-Sepharose CL-4B of each fraction gave a single elution peak.

Fractions 1 and 2, enriched with protein of low mobility, and fractions 7 and 8, with major proteins of high mobility, were separately subjected to preparative polyacrylamide gel electrophoresis. Each fraction showed two separate bands in the gel. The enzyme in each major band from each gel was extracted as described in 'Materials and Methods' and was further purified by hydrophobic chromatography on Octyl-Sepharose CL-4B.

A β-galactosidase separated from fractions 1 and 2 with low mobility was denoted β-galactosidase-1, and that from fractions 7 and 8, β-galactosidase-2.

1.1 Homogeneity of the enzyme

Polyacrylamide gel electrophoresis of the two enzyme preparations (β-galactosidase-1 and β-galactosidase-2)

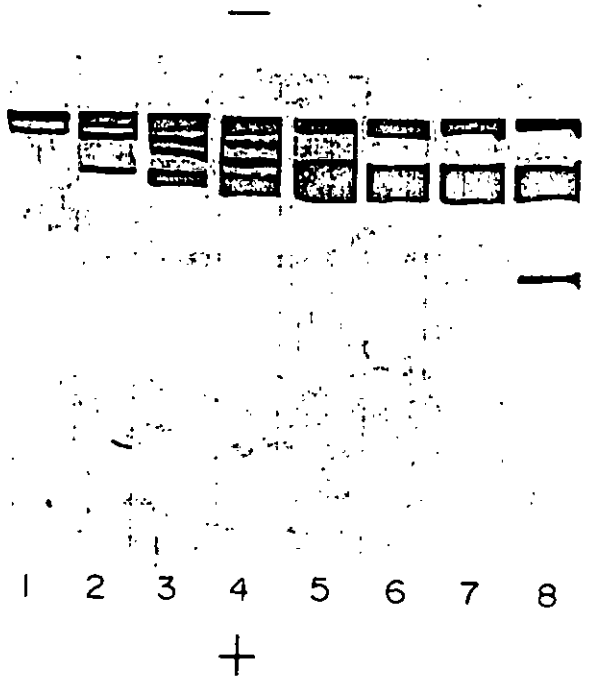


Fig. 2. Polyacrylamide Gel Electraphoresis of Eight Sub-fractions of β-Galactosidase Eluted by Polybuffer Exchanger PBE 94.

Electrophoresis was performed at room temperature using 7.5% acrylamide gel. Final concentrations of buffers were as follows: stacking gel, 0.125 M Tris-HCl, pH 6.8; resolving gel, 0.375 M Tris-HCl, pH 8.8; reservoir buffer, 0.025 M Tris, 0.192 M glycine, pH 8.3. Protein was stained by Coomassie blue, as described in methods for SDS-PAGE.

Figure 3. β-Galactosidase activities of eight sub-fractions eluted by polybuffer exchanger PBE 94.

Enzyme activities were measured under standard assay conditions.

—●— , activity towards lactose ;

—○— , activity towards ONPG.

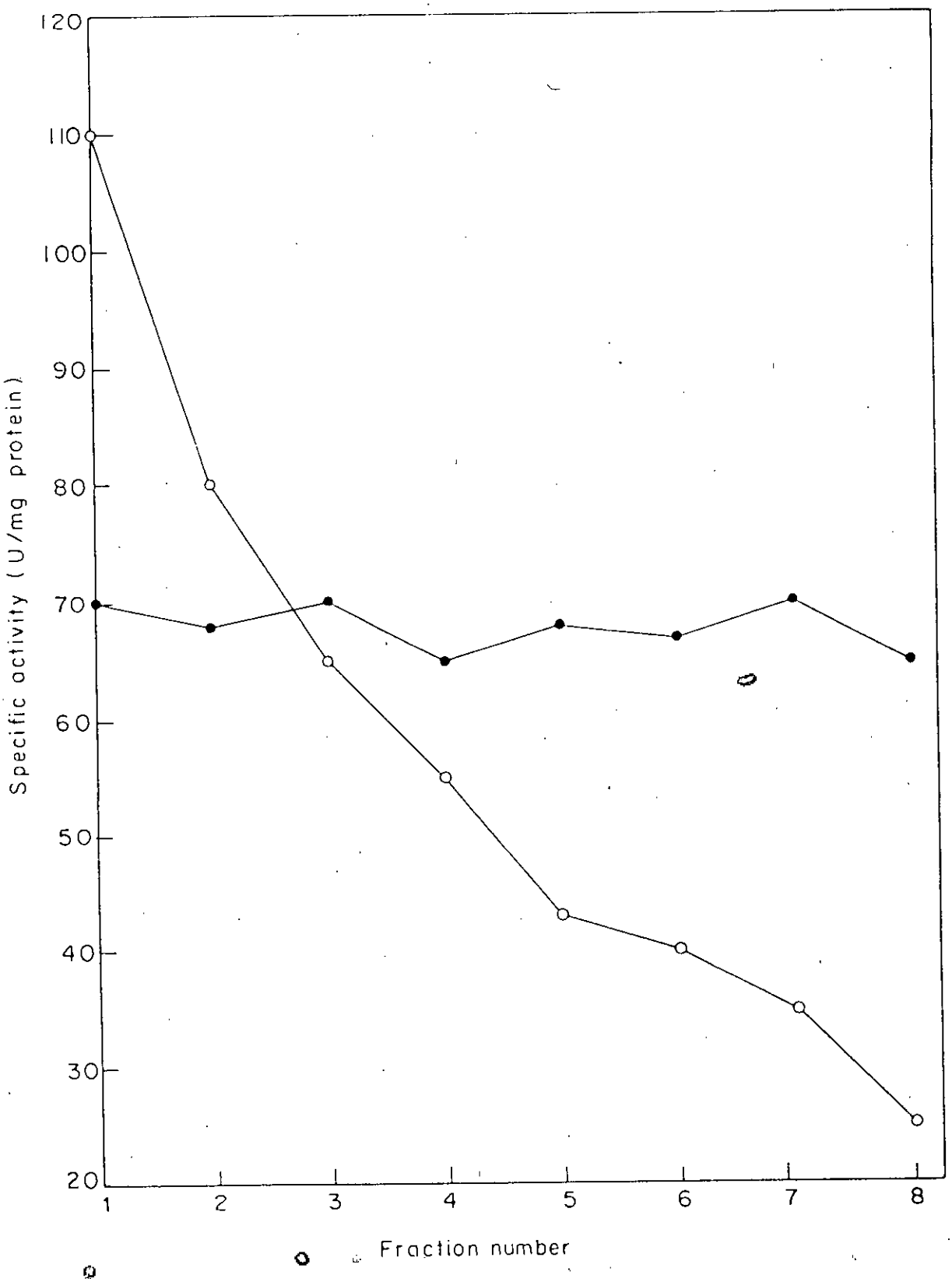

120
110
100
90
80
70
60
50
40
30
20
Specific activity (U/mg protein)
1
2
3
4
5
6
7
8
Fraction number

Fig. 3.

gave one predominant protein band each, which also coincided with the positions of crude enzyme preparation as shown in Fig.4.

1.2 Determination of molecular weight of the enzyme

The molecular weights of β-galactosidase-1 and β-galactosidase-2 were determined to be approximately 2.4×10^5 and 1.6×10^5, respectively, by gel filtration on a Sephadex G-200 column and SDS-gel electrophoresis. Marker proteins were bovine serum albumin (M.W. 68,000), aldolase (M.W. 158,000), catalase (M.W. 232,000), β-galactosidase (M.W. 540,000) (E.coli), and thyroglobulin (M.W. 669,000). The relationship, V_e/V_o versus Log Molecular weight gave a straight line, except for very small and very large molecular as shown in Fig.5.

1.3 Effect of pH on the enzyme activity

Figure 6 shows the pH dependency of the activities towards lactose and ONPG for β-galactosidase-1 and β-galactosidase-2 at 40°C. Experiments were performed with 0.1 M phosphate buffer at pH range 4.0 to 10.0. The enzymatic activities in all preparations were determined and expressed as percentage of relative activity. The observed major activity peak was at pH 6.0. Above and below this pH, activity declined gradually.

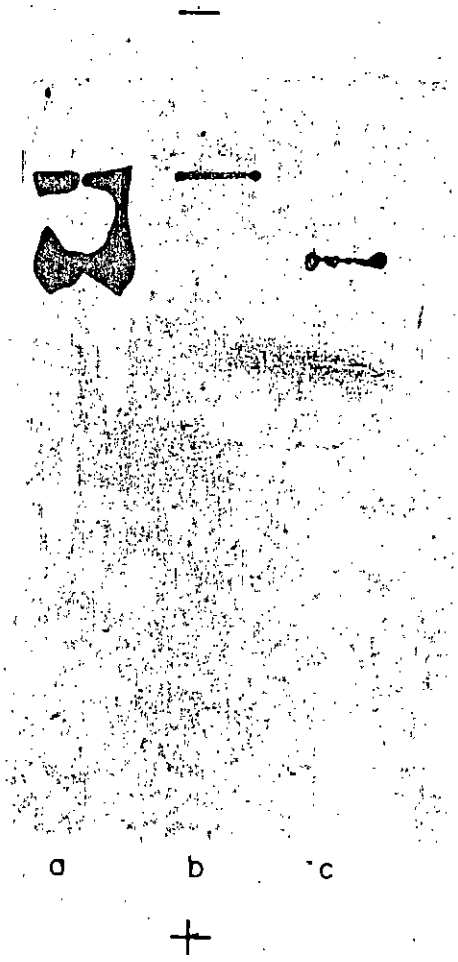


Fig.4. Polyacrylamide Gel Electrophoresis for the Crude and Purified β-Galactosidases from B. circulans. a) crude enzyme; b) β-galactosidase-1; c) β-galactosidase-2. The conditions for electrophoresis are the same as in Fig. 2.

Figure 5. Ve/Vo versus Log Molecular weight plot.

A Sephadex G-200 column (1.5 cm x 40 cm) was calibrated with known protein standards ; A) aldolase (158,000) ; B) bovine serum albumin (68,000) ; C) catalase (232,000) ; G) β-galactosidase from E.coli (540,000), and T) thyroglobulin (669,000). For the determination of void volume (Vo) Blue dextran-2000 was used. E1 and E2 are denoted β-galactosidase-1 and β-galactosidase-2, respectively.

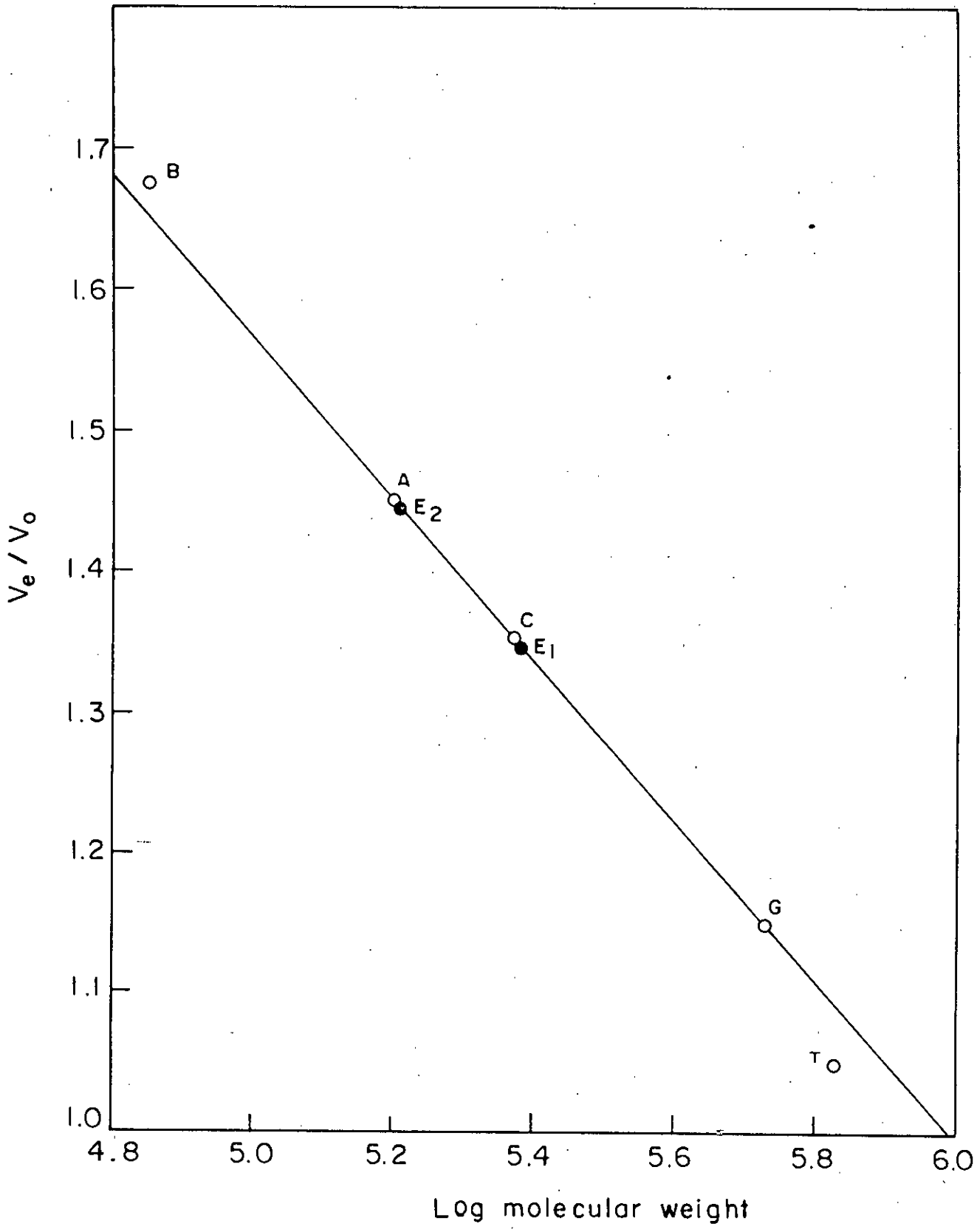

1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
V_e / V_o
B
A
E_2
C
E_1
G
T
4.8
5.0
5.2
5.4
5.6
5.8
6.0
Log molecular weight

Fig. 5.

Figure 6. pH dependence of the enzyme activity.

Activity was measured at 40°C and different pHs by using phosphate buffer, with (a) lactose as a substrate (final concentration ; 4.56%) and (b) ONPG as a substrate (final concentration ; 0.245%). The solid line (——●——) and the broken line (- - -o- - -) represent the pH profiles of β-galactosidase-1 and β-galactosidase-2, respectively.

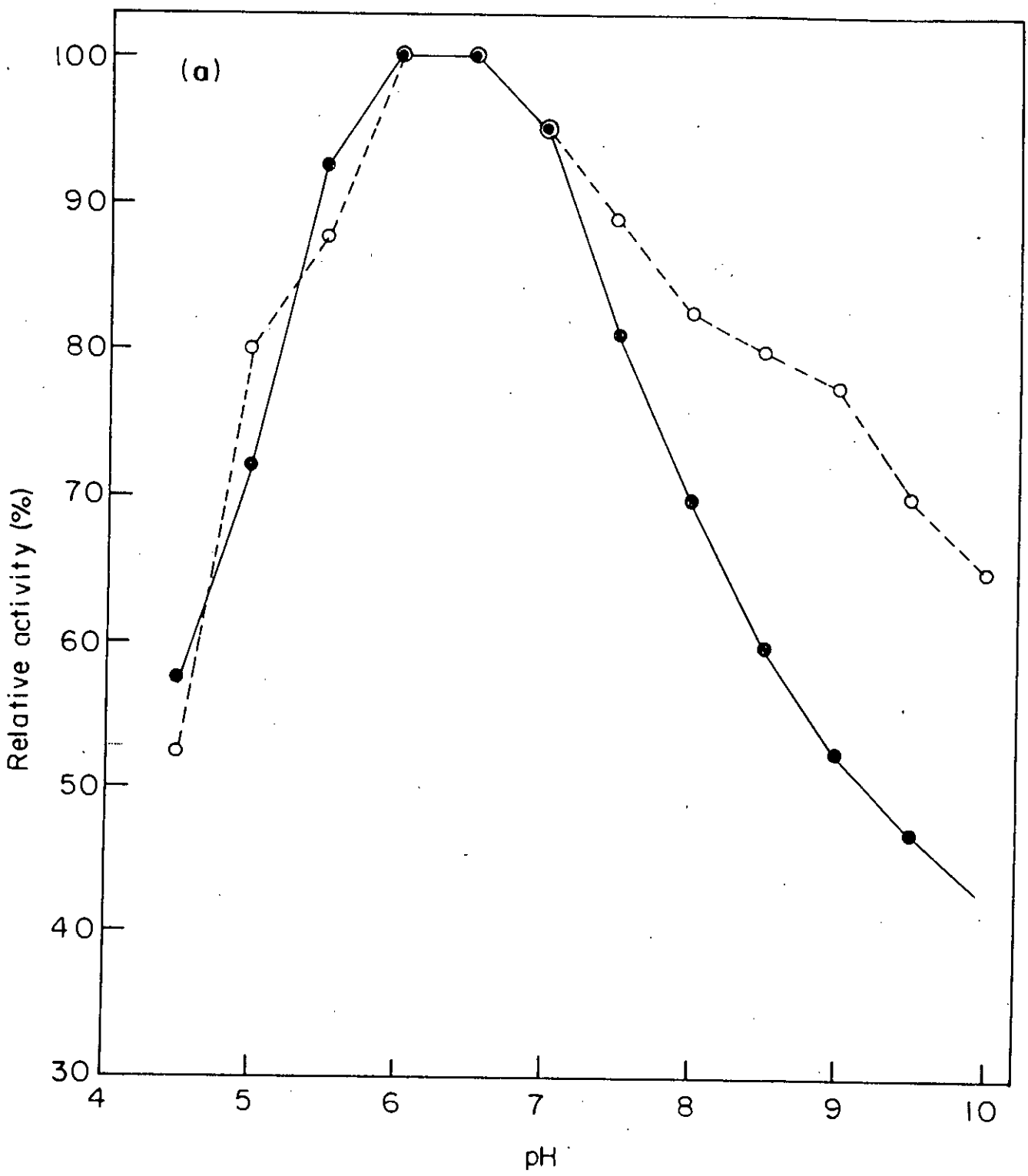


Fig. 6.

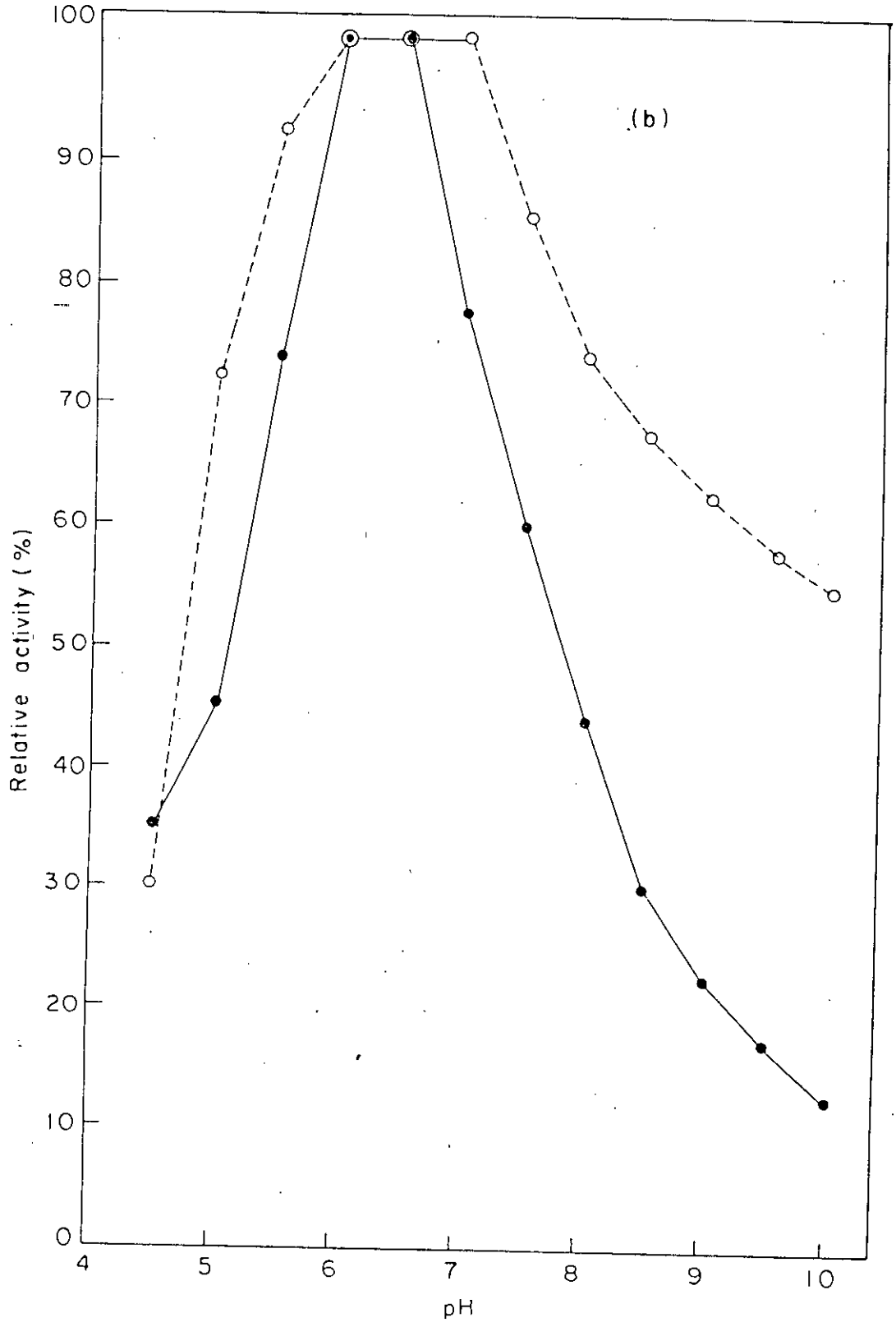


Fig. 6.

Optimum pH for both the enzyme forms was 6.0 for ONPG and lactose.

1.4 Effect of temperature on the enzyme activity

The activities were determined at the temperature range from 20° to 80°C. Enzyme preparations were incubated separately at each temperature for 10 minutes following which the activity was determined at pH 6.0 by usual procedure. The temperatures at which the maximal activity attained is shown in Fig.7. The optimum reaction temperatures for β-galactosidase-1 with lactose and ONPG as substrates were 60° and 45°C, respectively, whereas for β-galactosidase-2 it was 60°C. Above 60°C both enzymes lost their activity completely.

1.5 Thermal stability of the enzyme

Figure 8 shows a plot of the remaining activity versus temperature for β-galactosidase-1 and β-galactosidase-2. Enzymes were incubated at different temperatures for one hour at pH 6.0. Activity was expressed as percentage of relative activity. The β-galactosidase-1 was stable up to 40°C, while β-galactosidase-2 was stable to 50°C.

1.6 Effect of metal ions and reagents on the enzyme activity

The effects of metal ions and some group specific reagents on the activity of the two enzymes were similar

Figure 7. Optimal temperature of the enzyme activity.

The enzyme solutions were incubated with substrates for 10 minutes at the temperatures indicated, and the enzyme activities were measured at pH 6.0 with (a) lactose and (b) ONPG as substrate. The solid line (——●——) and the broken line (- - -o- - -) represent the temperature profiles of β-galactosidase-1 and β-galactosidase-2, respectively.

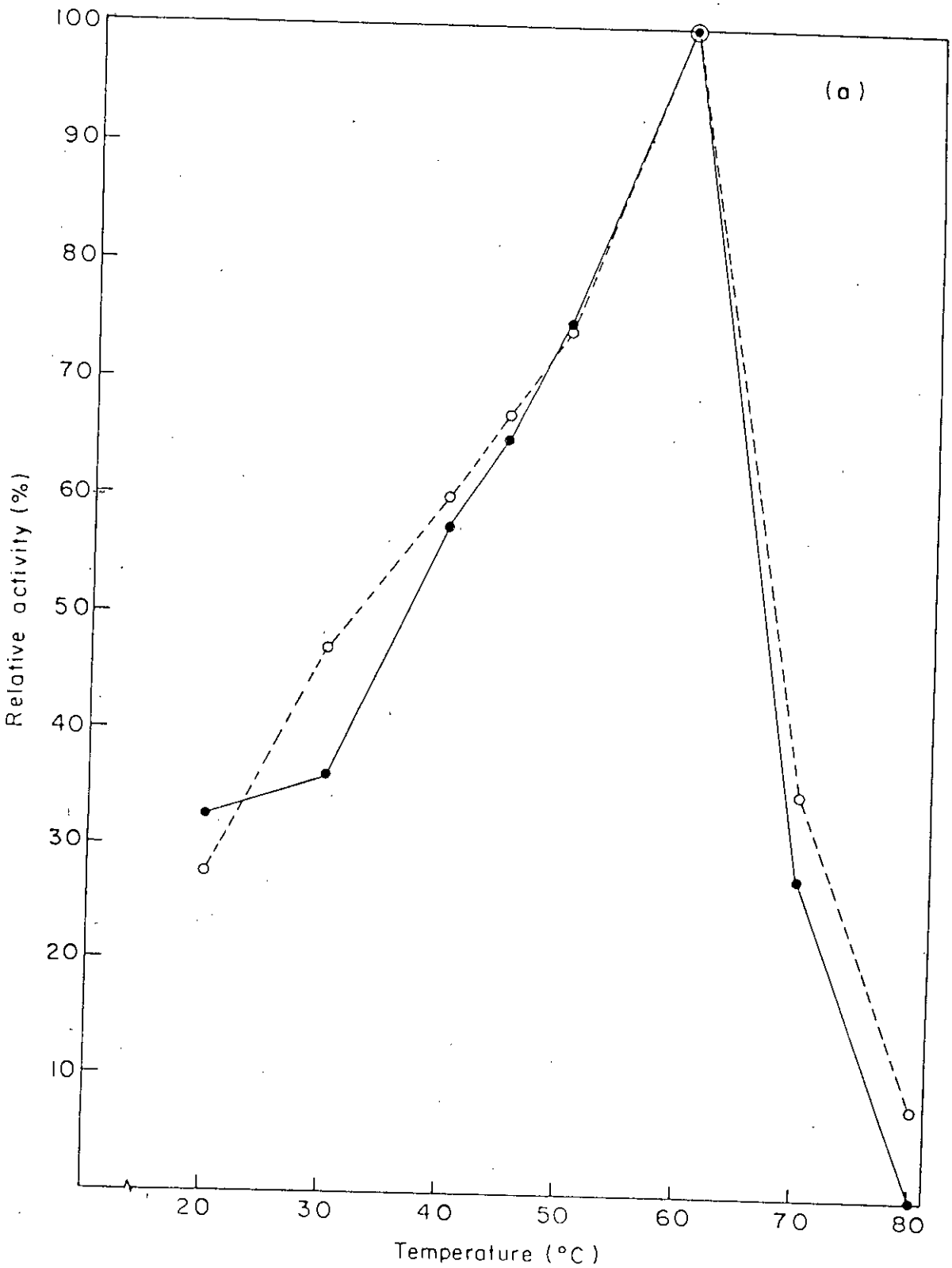

(a)
Relative activity (%)
100
90
80
70
60
50
40
30
20
10
20
30
40
50
60
70
80
Temperature (°C)

Fig. 7.

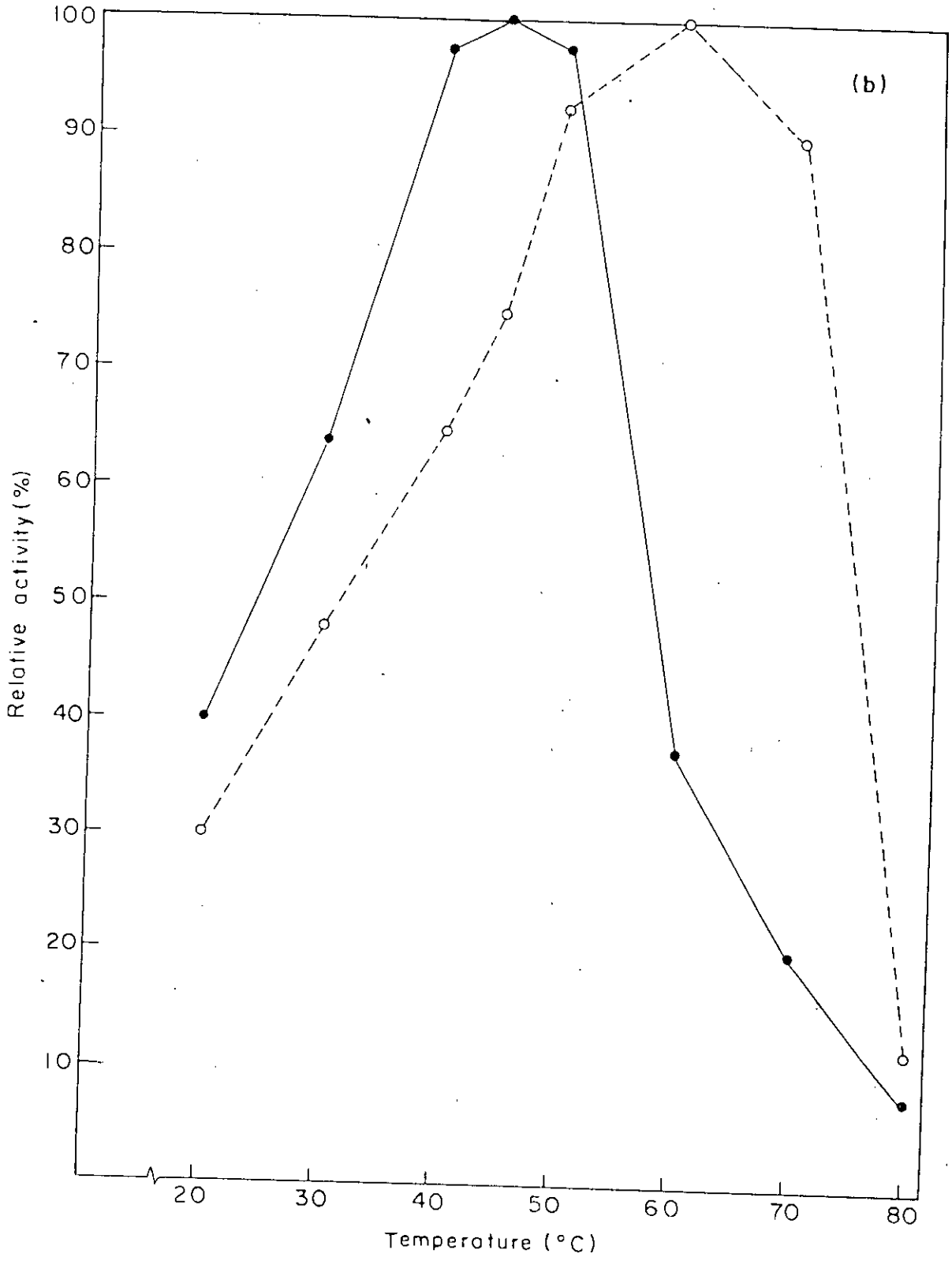

(b)
Relative activity (%)
100
90
80
70
60
50
40
30
20
10
20
30
40
50
60
70
80
Temperature (°C)

Fig. 7.

Figure 8. Effects of temperature on the stability of β-galactosidase-1 and β-galactosidase-2. The enzyme solutions were incubated in the assay buffer (pH 6.0) at various temperatures for 1h. The remaining activities were then measured under the assay conditions using (a) lactose and (b) ONPG as substrates. The solid line (——●——) and the broken line (— — -o- — —) represent the temperature profiles of β-galactosidase-1 and β-galactosidase-2, respectively.

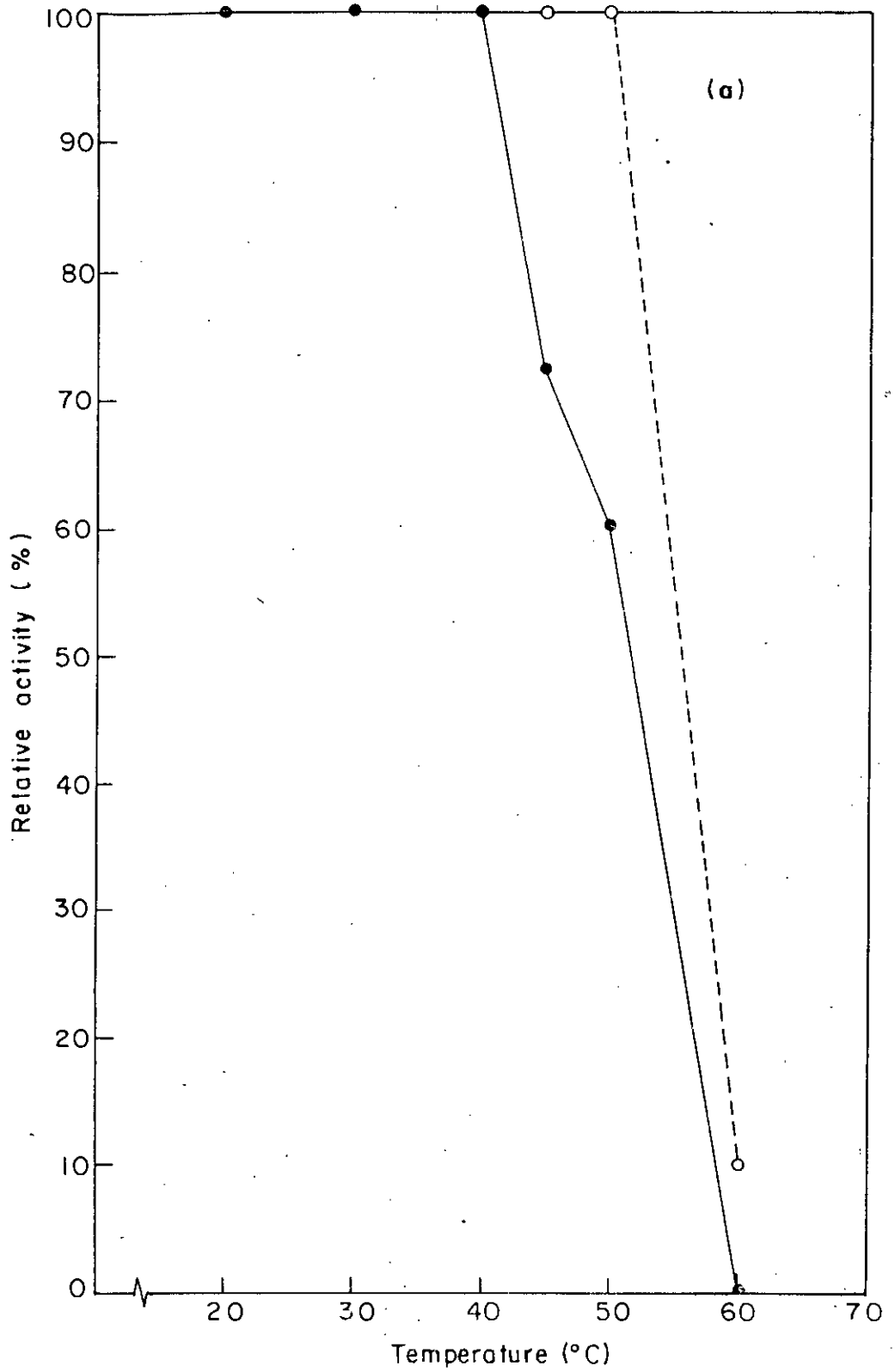

(a)
Relative activity (%)
100
90
80
70
60
50
40
30
20
10
0
20
30
40
50
60
70
Temperature (°C)

Fig. 8.

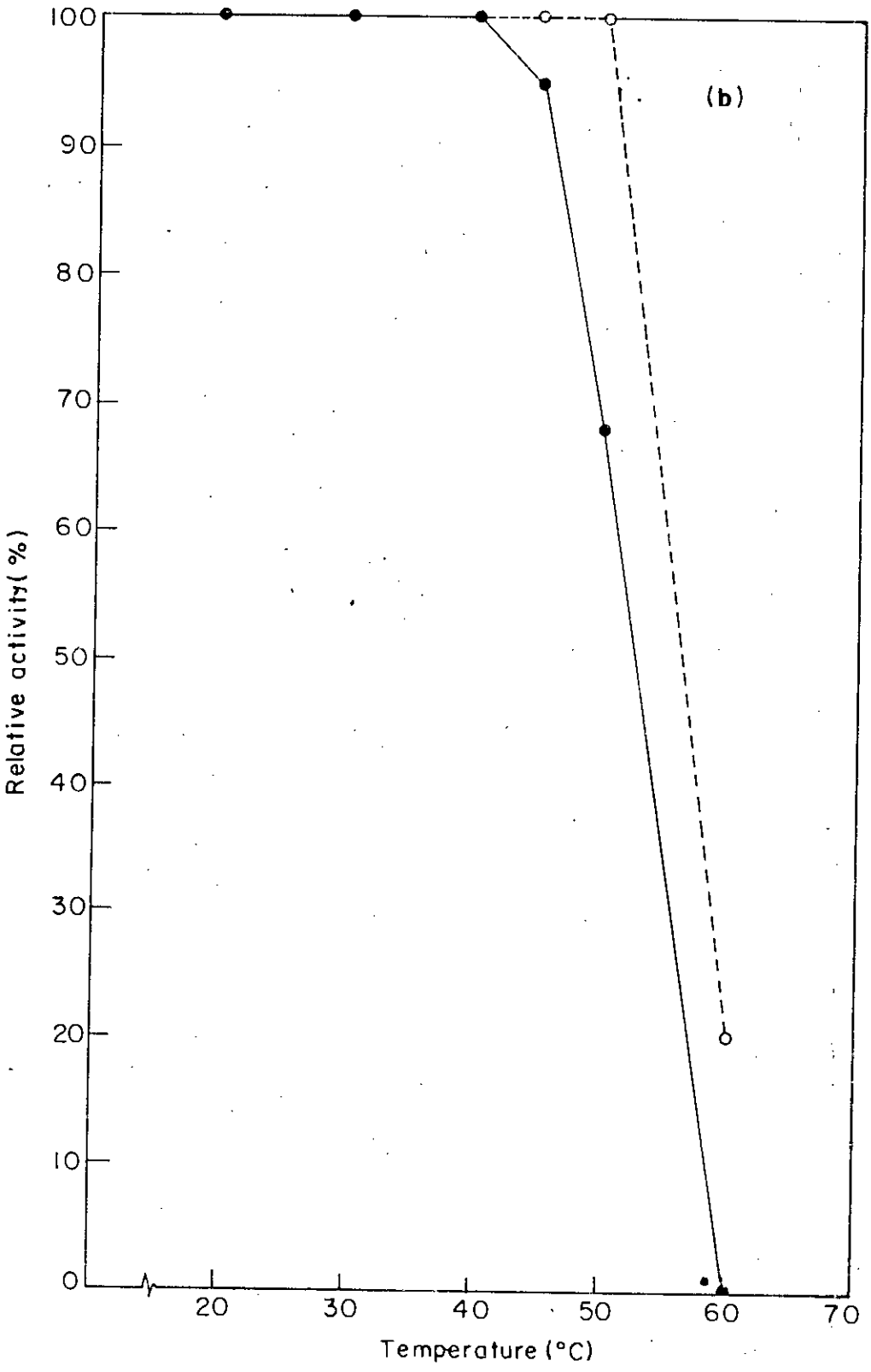

(b)
Relative activity(%)
100
90
80
70
60
50
40
30
20
10
0
20
30
40
50
60
70
Temperature (°C)

Fig. 8.

except that of $AgNO_3$, as shown in Table II. $AgNO_3$ inhibited β-galactosidase-1 activity completely and β-galactosidase-2 partially. Both enzymes were completely inhibited by $HgCl_2$. p-Chloromercuribenzoic acid did not show any inhibition for these two enzymes. Both enzymes were inhibited to some extent by EDTA.

1.7 Kinetics of the enzyme

Table III shows the kinetic parameters of β-galactosidase-1 and β-galactosidase-2. The K_m values were determined from the double-reciprocal plot (Lineweaver-Burk plot) of activity versus substrate concentration (Fig. 9,10). K_m values obtained for β-galactosidase-1 with ONPG (Fig. 9a) and lactose (Fig. 9b) were lower than the K_m values for β-galactosidase-2 with ONPG (Fig. 10a) and lactose (Fig. 10b), respectively. β-Galactosidase-1 showed a much higher activity towards ONPG than lactose, while β-galactosidase-2 had the opposite tendency. β-Galactosidase-1 was found to be competitively inhibited by galactose (K_i, 150 mM) with lactose as a substrate, whereas β-galactosidase-2 was inhibited by neither galactose nor glucose (Fig. 9').

Table II. Effects of metal ions and group specific reagents on activity of β-galactosidases from B. circulans.

Metal ions and reagents	Relative activity[a] (%)	
	β-galactosidase-1	β-galactosidase-2
Control	100	100
$AgNO_3$ (1 mM)	0	68
$HgCl_2$ (1 mM)	0	0
$MgSO_4$ (1 mM)	100	100
NaCl (10 mM)	122	125
KCl (10 mM)	110	134
PCMB (1 mM)	100	105
DTE (1 mM)	105	100
IAA (1 mM)	87	76
2-ME (1 mM)	100	116
EDTA (1 mM)	63	75

a. β-Galactosidases, 0.15 ml (6.25×10^{-5} mM, β-galactosidase-1; 12.5×10^{-5} mM, β-galactosidase-2) were preincubated at 20°C with metal ion or reagent for 10 min. Then 50 ul of the enzyme solutions were incubated with 2.5 ml ONPG (final concentration, 0.245%) for 10 min at 40°C. The enzyme activity towards ONPG in the assay buffer without metal ions or reagents were taken as control. Concentrations of metal ions or reagents during preincubation are shown in parentheses.

Table III. Kinetic properties and substrate specificities of β-galactosidases from B. circulans[a].

Enzyme	Substrate	Km (mM)	V_{max} Units/mg protein	V_{max}/Km	$OU_{V_{max}}$ / $LU_{V_{max}}$
β-galactosidase-1	Lactose	15.50	50.00	3.23	2.57
	ONPG	5.00	128.60	25.72	
β-galactosidase-2	Lactose	50.00	79.50	1.59	0.71
	ONPG	10.00	56.50	5.65	
β-galactosidase-1	Lactose	150[b]			
β-galactosidase-2	Lactose	∝			

a. The reactions were performed at 40°C, pH 6.0, and 0.1M phosphate buffer, in the concentration range 10 to 160 mM with lactose and 1 to 16 mM with ONPG as substrates.

b. K_i value determined with galactose as competitive inhibitor; ∝ means no inhibition.

OU - ONPG used as substrate.

LU - Lactose used as substrate.

Figure 9. Double reciprocal plot of substrate concentration versus activity. Km and k_2 values for β-galactosidase-1 were determined at 40°C, pH 6.0, in concentration range (a) 1 to 16 mM with ONPG, and (b) 10 to 160 mM with lactose as substrates.

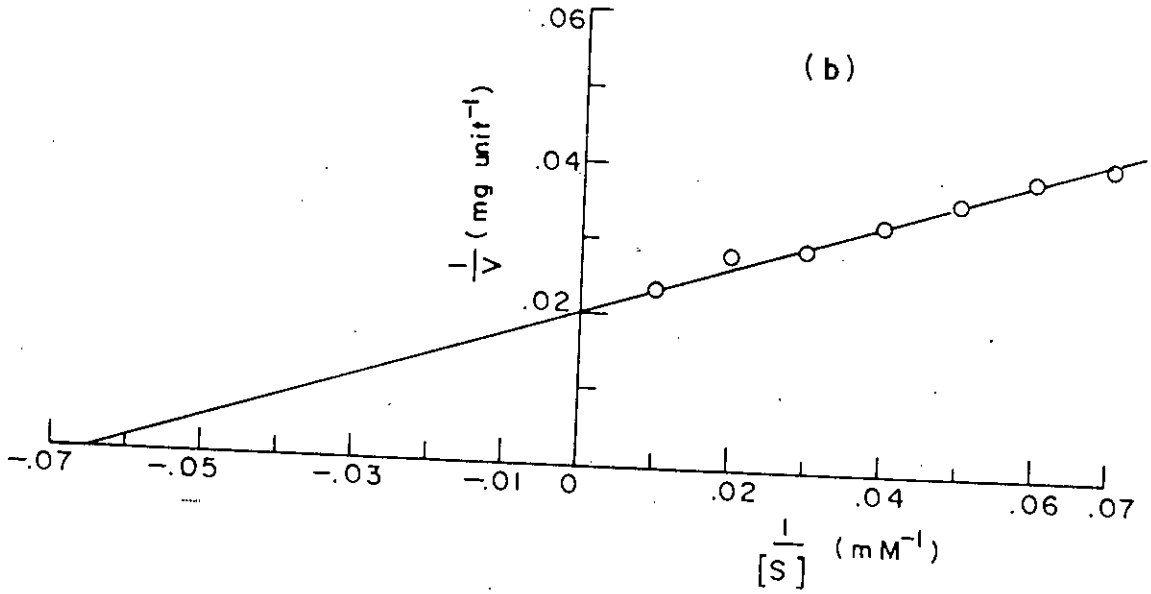

(b)
.06
.04
.02
$-\frac{1}{v}$ (mg unit^{-1})
-.07
-.05
-.03
-.01
0
.02
.04
.06
.07
$\frac{1}{[S]}$ (mM^{-1})

Fig. 9.

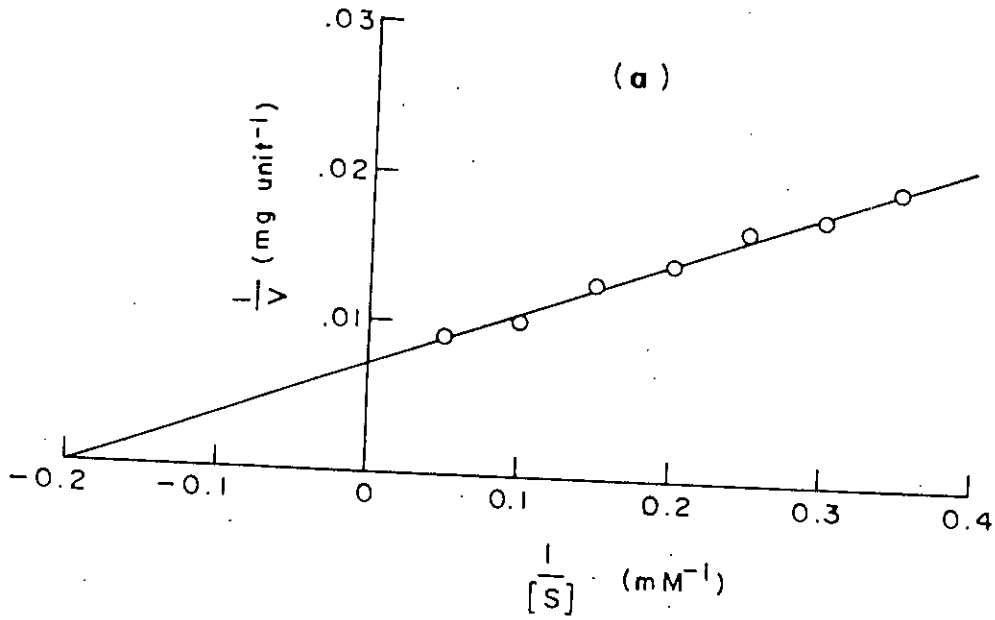

(a)
.03
.02
.01
$-\frac{1}{v}$ (mg unit^{-1})
-0.2
-0.1
0
0.1
0.2
0.3
0.4
$\frac{1}{[S]}$ (mM^{-1})

Fig. 9.

Figure 9'. Double reciprocal plot of substrate concentration versus activity. Ki value for /β-galactosidase-1 was determined at 40°C, pH 6.0, in concentration range 10 to 160 mM with lactose as substrate. The concentration of galactose as an inhibitor was 75 mM.

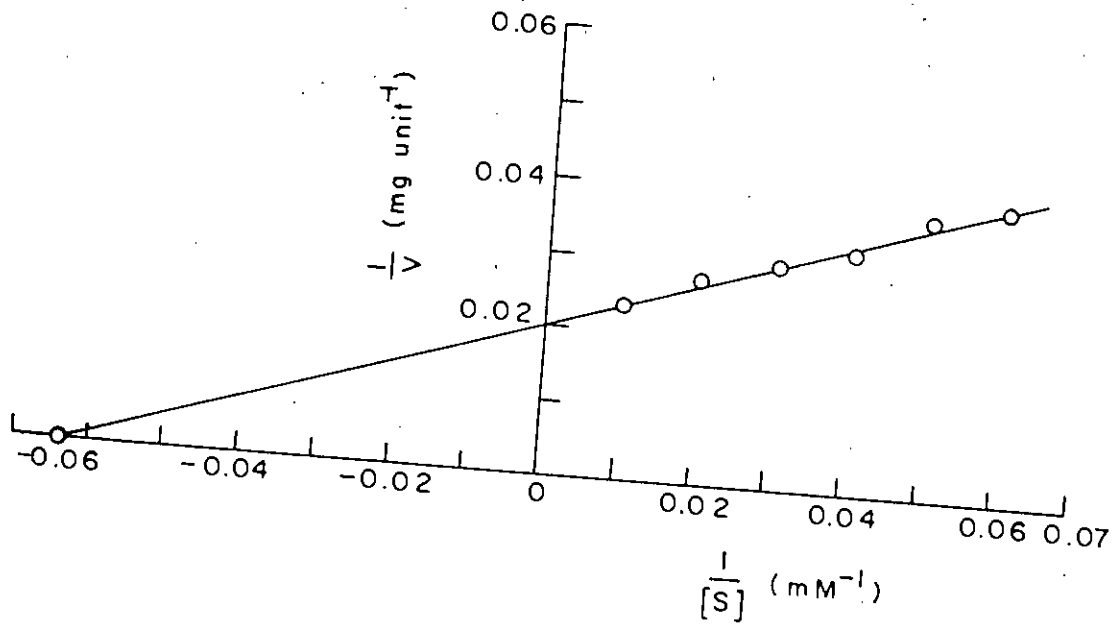

0.06
0.04
0.02
-0.06
-0.04
-0.02
0
0.02
0.04
0.06
0.07
$-\frac{1}{v}$ (mg unit^{-1})
$\frac{1}{[S]}$ (mM^{-1})

Fig. 9

Figure 10. Double reciprocal plot of substrate concentration versus activity. Km and K_2 values for β-galactosidase-2 were determined at 40°c, pH 6.0, in concentration range (a) 1 to 16 mM with ONPG, and (b) 10 to 160 mM with lactose as substrates.

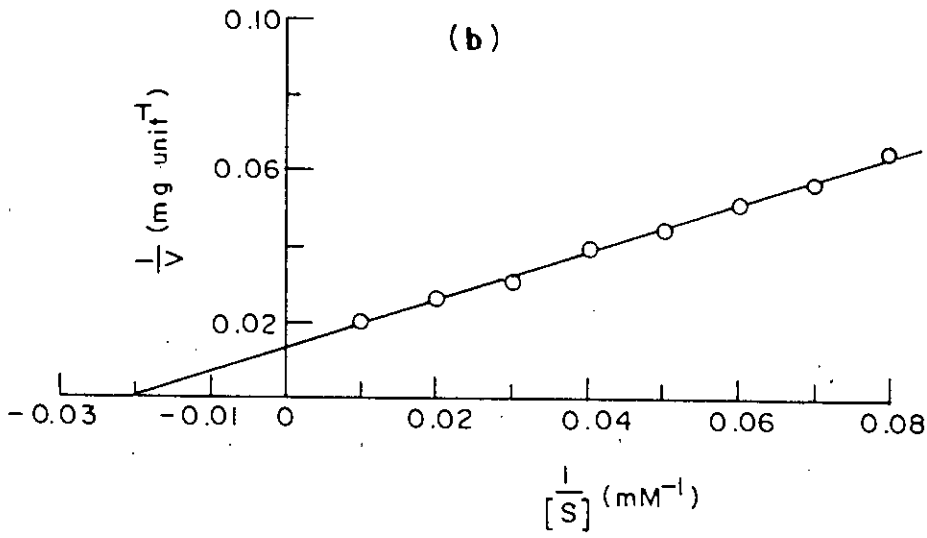

(b)
0.10
0.06
0.02
$\frac{1}{V}$ (mg unit^{-1})
-0.03
-0.01
0
0.02
0.04
0.06
0.08
$\frac{1}{[S]}$ (mM^{-1})

Fig. 10.

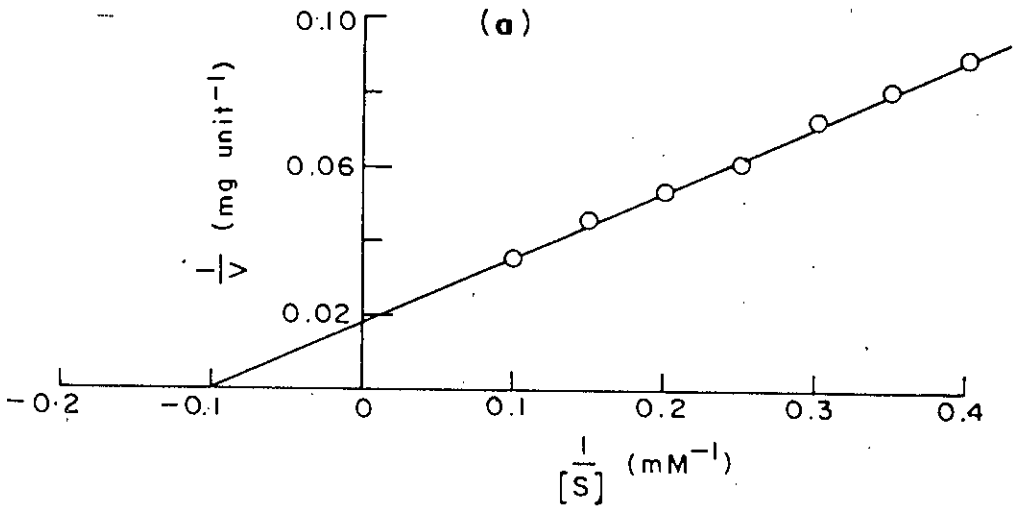

(a)
0.10
0.06
0.02
$\frac{1}{V}$ (mg unit^{-1})
-0·2
-0.1
0
0.1
0.2
0.3
0.4
$\frac{1}{[S]}$ (mM^{-1})

Fig. 10.

1.8 Course of hydrolysis of lactose by the enzyme

Figure 11 shows the course of the hydrolysis of lactose in the assay buffer (pH 6.0) at 40°C with the β-galactosidase-1. During the course of the reaction only a small amount of oligosaccharide was detected. The maximum amount of oligosaccharides was 6.6% of the total sugar at 24% conversion of 4.56%lactose at 15 min, and latter it disappeared completely. The oligosaccharide produced was a trisaccharide with one molecule of glucose and two molecules of galactose. On the other hand, β-galactosidase-2 produced many more oligosaccharides, as shown in Fig. 12 a, b. As the reaction proceeded, a considerable amount of trisaccharide, tetrasaccharide, and pentasaccharides was identified. The maximum amount of oligosaccharides was 41.5% of the total sugar at 66.3% conversion of 4.56% lactose at 70 min. Two kinds of disaccharide other than lactose with a slightly different mobility on paper chromatography were also observed. Oligosaccharides were detected by comparing the R_f values with those for standard substances as described in 'Materials and Methods'. These oligosaccharides were found to be composed of one molecule of glucose and one to four molecules of galactose. The amount of trisaccharide produced reached a maximum value of 26.3% of the initial lactose concentration after 45 min from the start of

Figure 11. Course of hydrolysis of lactose with β-galactosidase-1.

Hydrolysis of lactose (final concentration; 4.56%) with purified β-galactosidase-1 (final concentration; 12.5×10^{-5} mM) was done at 40°C and pH 6.0.

——●—— , glucose; ——○—— , galactose;
——×——, lactose; ——△——, trisaccharide.

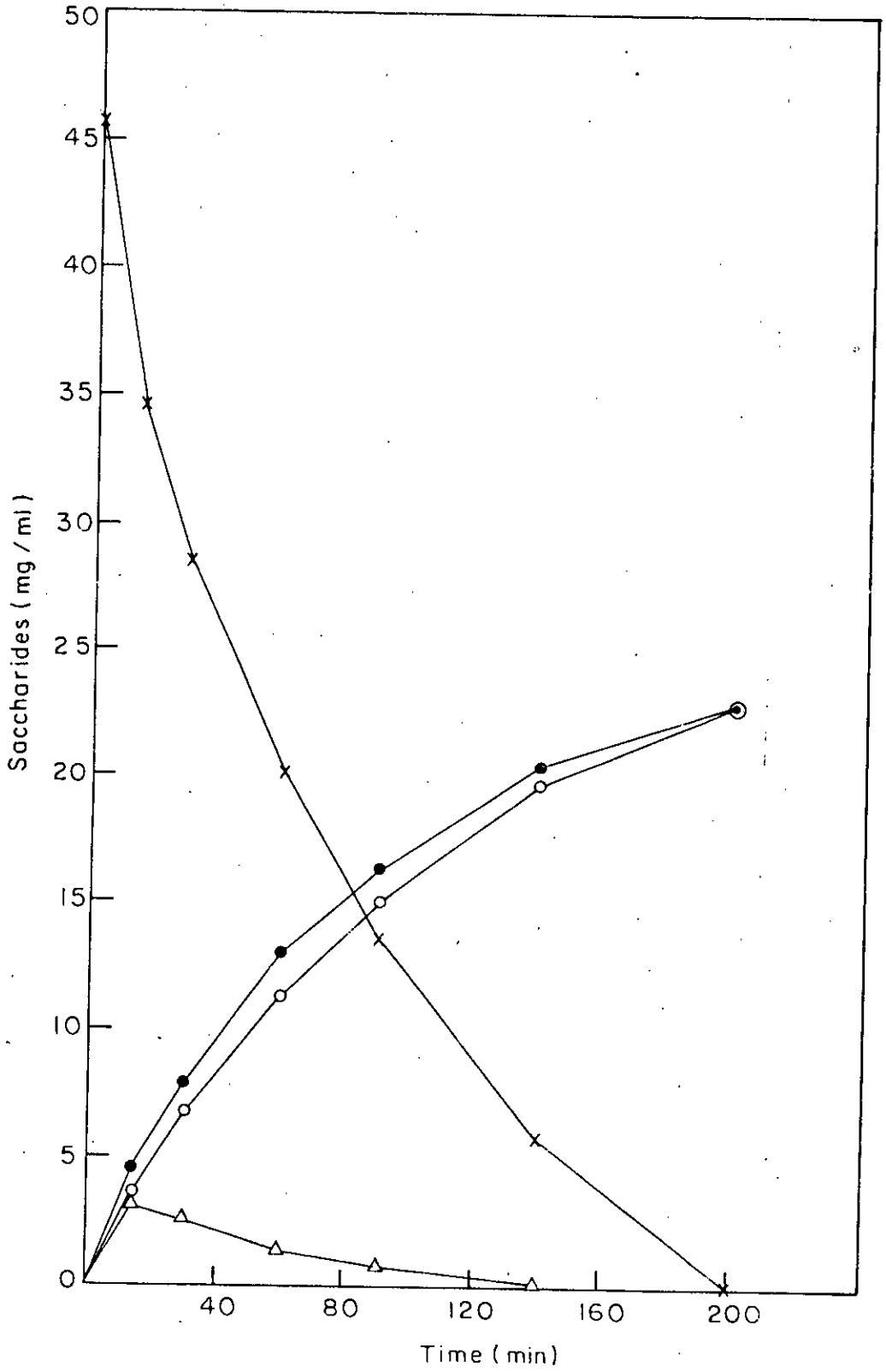

50
45
40
35
30
25
20
15
10
5
0
Saccharides (mg / ml)
40
80
120
160
200
Time (min)

Fig. II.

Figure 12. Course of hydrolysis of lactose with β-galactosidase-2.

Hydrolysis of lactose (final concentration ; 4.56%) with purified β-galactosidase-2 (final concentration; 25 x 10^{-5} mM) was done at 40°C and pH 6.0. (a) Course of reaction up to 200 min. (b) Course of reaction after 200 min.

——●——, glucose ; ——○——, galactose ; ——×——, lactose ; - - -●- - -, disaccharide-1 ; - -○- -, disaccharide-2 ; ——△——, trisaccharide ; ——□——, tetrasaccharide ; ——■——, pentasaccharide.

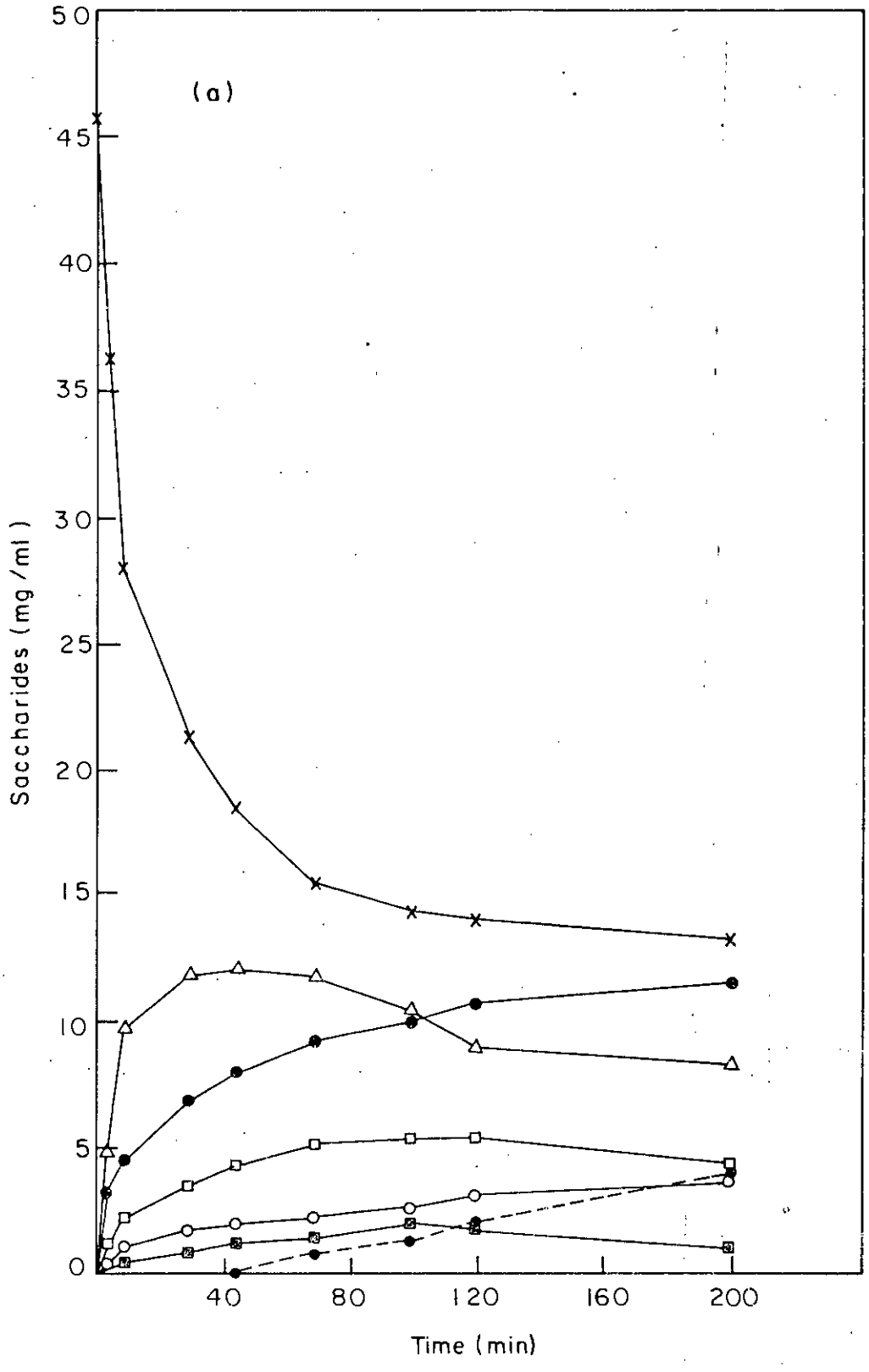

(a)
Saccharides (mg/ml)
Time (min)
0
5
10
15
20
25
30
35
40
45
50
40
80
120
160
200

Fig. 12.

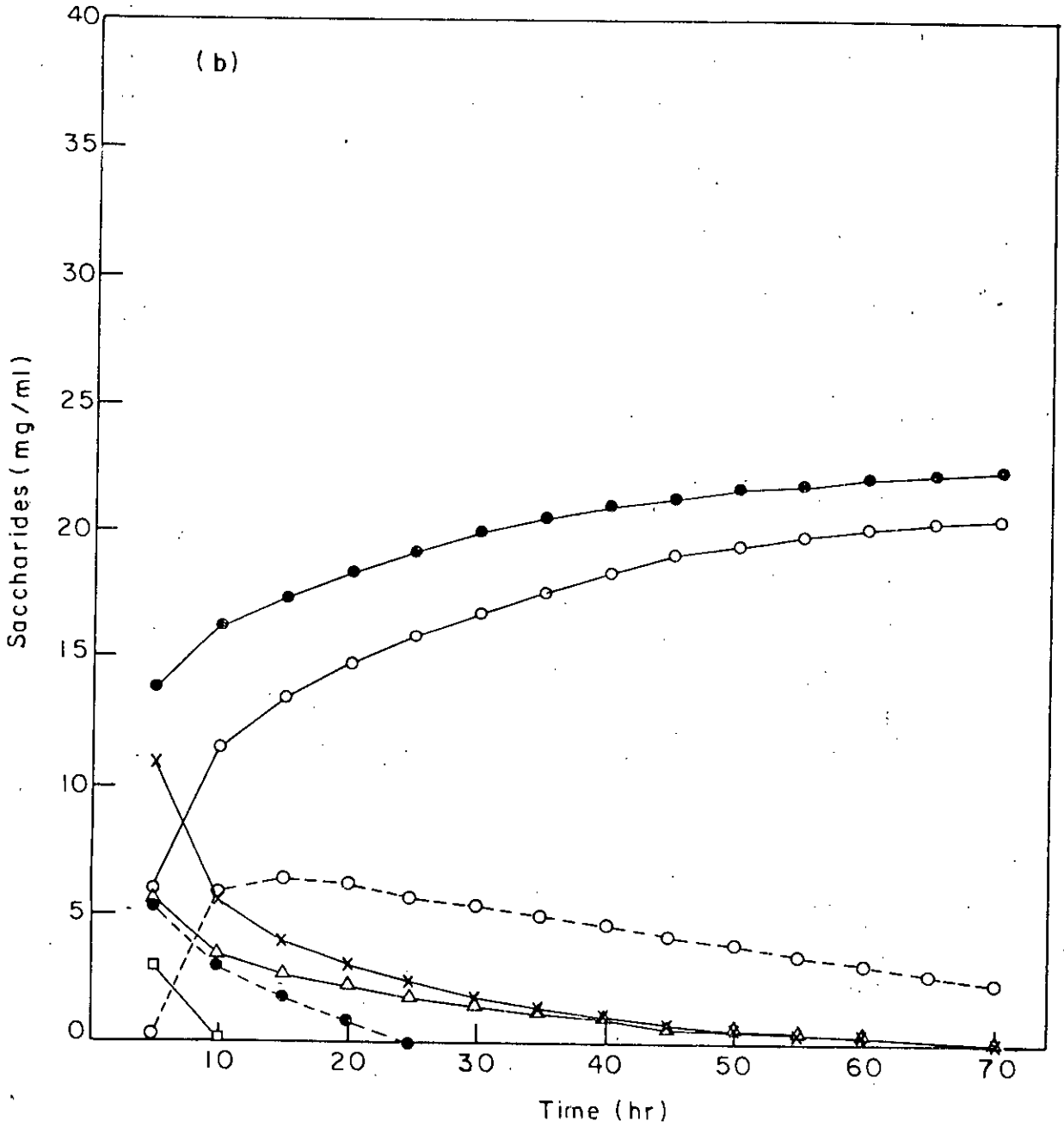

(b)
Saccharides (mg/ml)
Time (hr)
0
5
10
15
20
25
30
35
40
10
20
30
40
50
60
70

Fig. 12.

reaction, then gradually decreased, and finally disappeared after 45 h. When trisaccharide started to decrease, a disaccharide (disaccharide-1) appeared which was different from lactose, presumably in the type of linkage. The mobility of disaccharide-1 was slightly higher than lactose in paper chromatography. Tetrasaccharides and pentasaccharides were at maxima after 100 min. with 11.7% and 4.1% of the initial amount of lactose, respectively, and disappeared after 10 h. At 5 hours of reaction, other kind of disaccharide (disaccharide - 2) was formed with a slightly lower mobility than lactose in paper chromatography. This did not disappear even after 72 h.

2.0 Immobilization of β-galactosidase-1

β-Galactosidase-1 was immobilized onto metal activated Silica gel (controlled pore). The immobilized enzyme produced many oligosaccharides with much higher yield than did the free enzyme. Figure 13a shows a plot of the percentage yield of saccharides versus percentage conversion of lactose for the native β-galactosidase-1. The immobilized enzyme produced di-, tri-, tetra-, and pentasaccharides with a maximum yield of 40% at around 60% conversion of lactose(Fig.13b). The yield of oligosaccharides and their R_f values on paper chromatography were similar as those of free β-galactosidase-2 from B. circulans during hydrolysis of 4.56% lactose at 40°C and pH 6.0.

Figure 13. Relationship between conversion of lactose and yield of saccharides. The 4.56% lactose was hydrolyzed at 40°C and pH 6.0 with β-galactosidase-1 (a) as a free enzyme (b) immobilized onto activated Silica gel (12.5 units/g of wet gel).

——●—— , glucose ; ——○——, galactose ; - - -●- - -, disaccharide-1 ; - - -○- - - , disaccharide-2 ; ——△—— , trisaccharide ; ——□——, tetrasaccharide; ——▣—— , pentasaccharide ; ——▲—— , total oligosaccharide.

The yield of saccharides is defined as the amount of saccharides produced as a percentage of the amount of lactose used as the substrate.

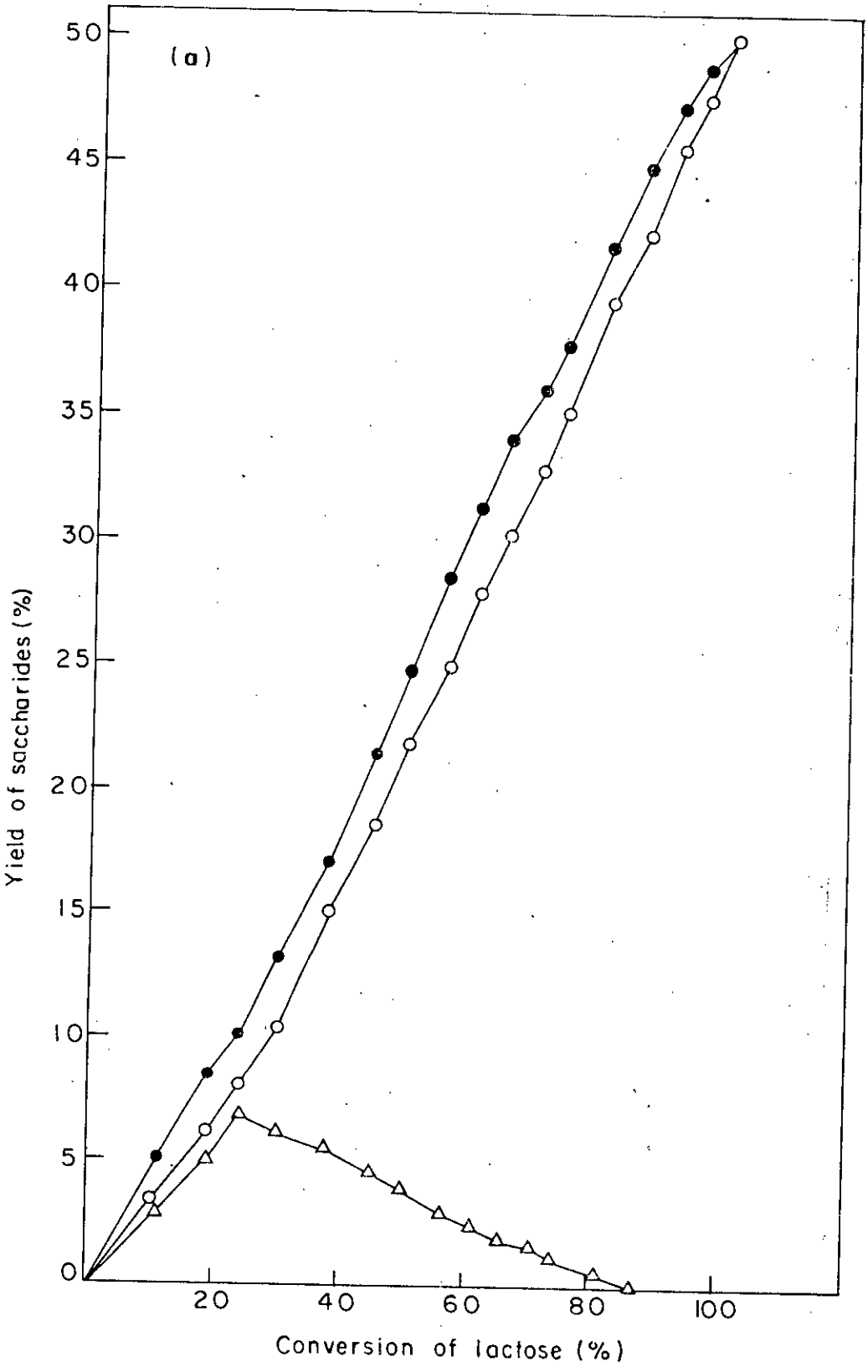

(a)
Yield of saccharides (%)
Conversion of lactose (%)
0
5
10
15
20
25
30
35
40
45
50
20
40
60
80
100

Fig. 13.

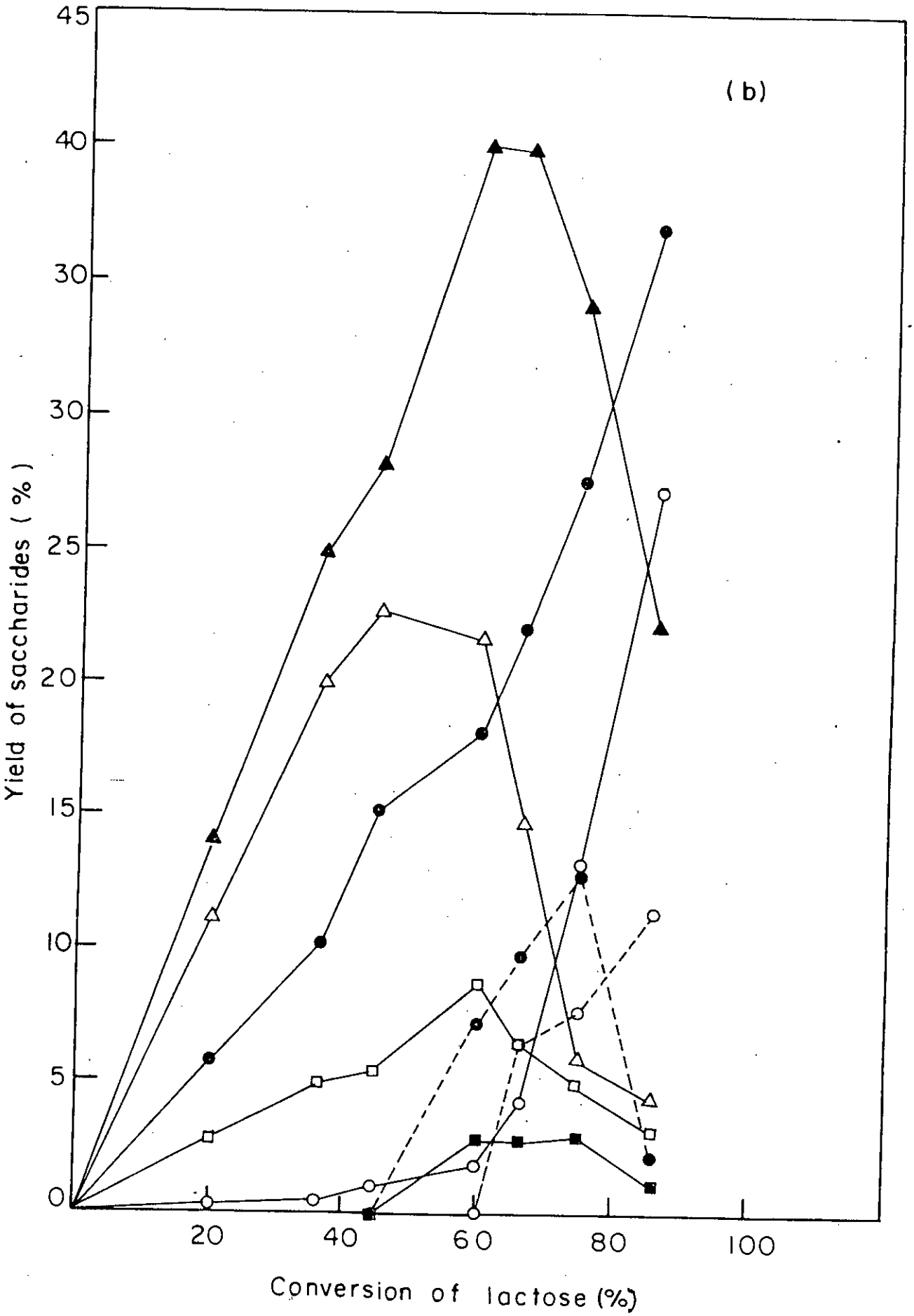

(b)
Yield of saccharides (%)
Conversion of lactose (%)
0
5
10
15
20
25
30
30
40
45
20
40
60
80
100

Fig. 13.

Disaccharide-1 and disaccharide-2 appeared after 44.2% and 60% conversion of lactose, respectively.

2.1 Modification of β-galactosidase-1

β-Galactosidase-1 was modified by glutaraldehyde of different concentrations. The enzyme was treated with 0.025%, 0.1%, 0.25% and 3% glutaraldehyde, respectively. In the case of 0.1% glutaraldehyde treatment, the enzyme was treated twice, first by 0.025% followed by 0.075% as described under 'Materials and Methods'. Figure 14a, b and c show the relationship between the percentage yield of saccharides and the percentage of lactose converted by the enzyme treated with 0.025, 0.1, and 3% glutaraldehyde, respectively. The enzyme treated with 0.25% glutaraldehyde produced the same kinds and amounts of oligosaccharides as that with 3%. The maximum yield of oligosaccharides reached to 40% at around 60% conversion of lactose. When the enzyme was treated with 0.025% and 0.1% glutaraldehyde, the maximum yields of oligosaccharides were 12.44% and 24.85%, respectively. No pentasaccharides appeared in the conversion of lactose by the enzyme treated with 0.025% and 0.1% except 3% glutaraldehyde.

Figure 14. Relationship between conversion of lactose and yield of saccharides. The 4.56% lactose was hydrolyzed at 40°C and pH 6.0 with β-galactosidase-1 (a) treated with 0.025% glutaraldehyde (b) treated with 0.1% glutaraldehyde (c) treated with 3% glutaraldehyde.

——●——, glucose ; ——○——, galactose ; - - -●- - -, disaccharide-1 ; - - -○- - - , disaccharide-2 ; ——△—— , trisaccharide ; ——□—— , tetrasaccharide ; ——▣—— , pentasaccharide ; ——▲—— , total oligosaccharide.

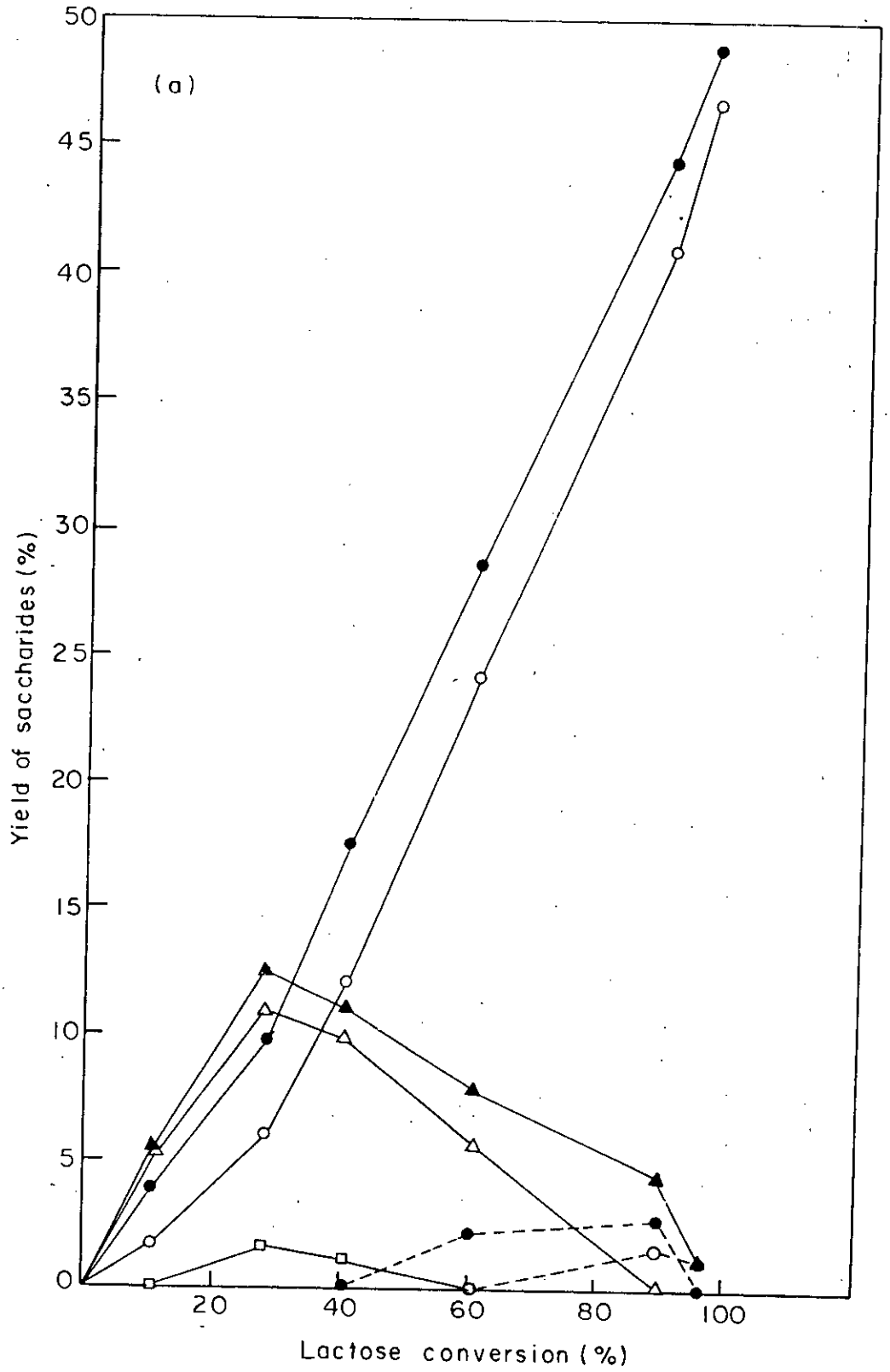

(a)
Yield of saccharides (%)
Lactose conversion (%)
0
5
10
15
20
25
30
35
40
45
50
20
40
60
80
100

Fig. 14.

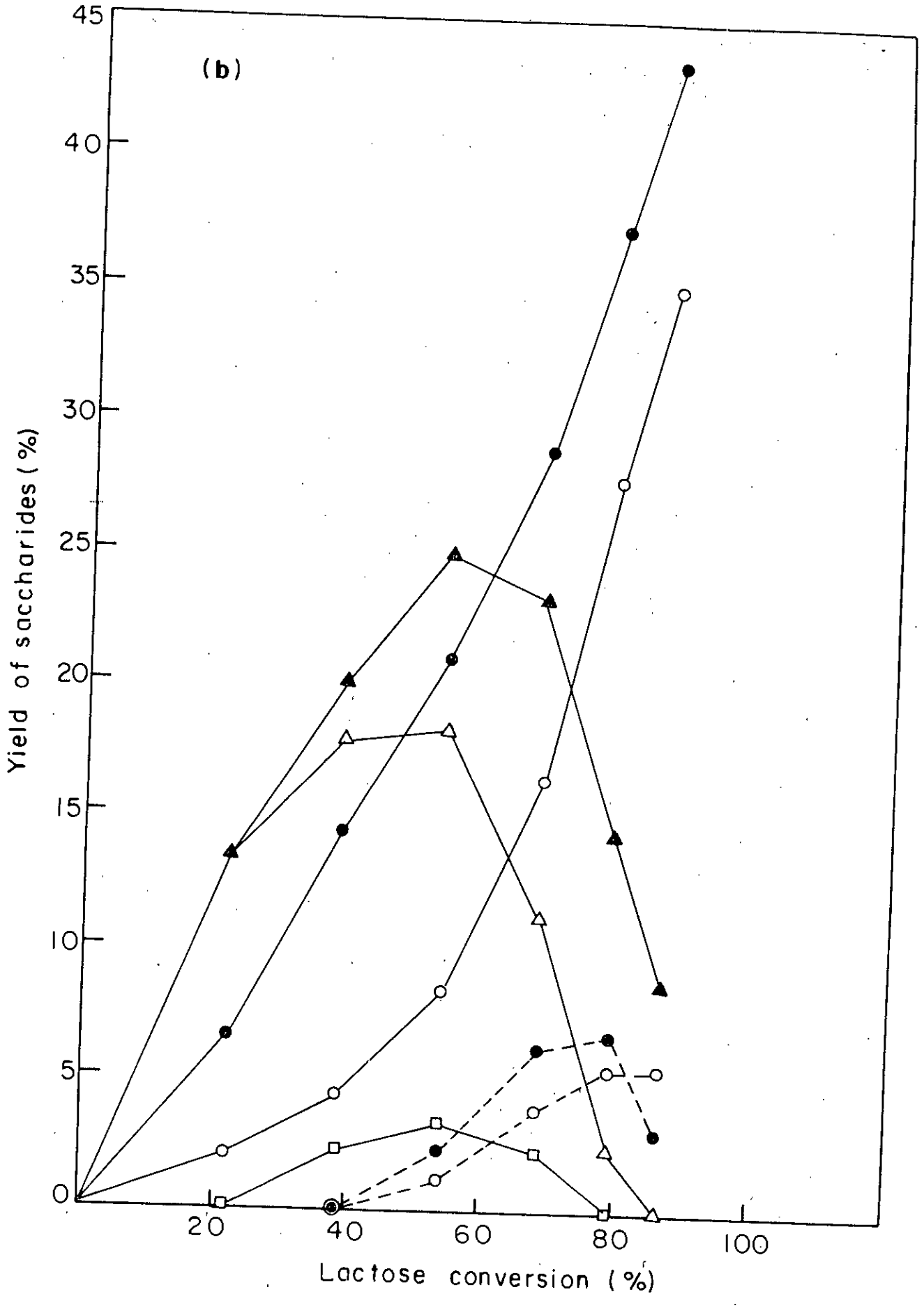

(b)
45
40
35
30
25
20
15
10
5
0
Yield of saccharides (%)
20
40
60
80
100
Lactose conversion (%)

Fig. 14.

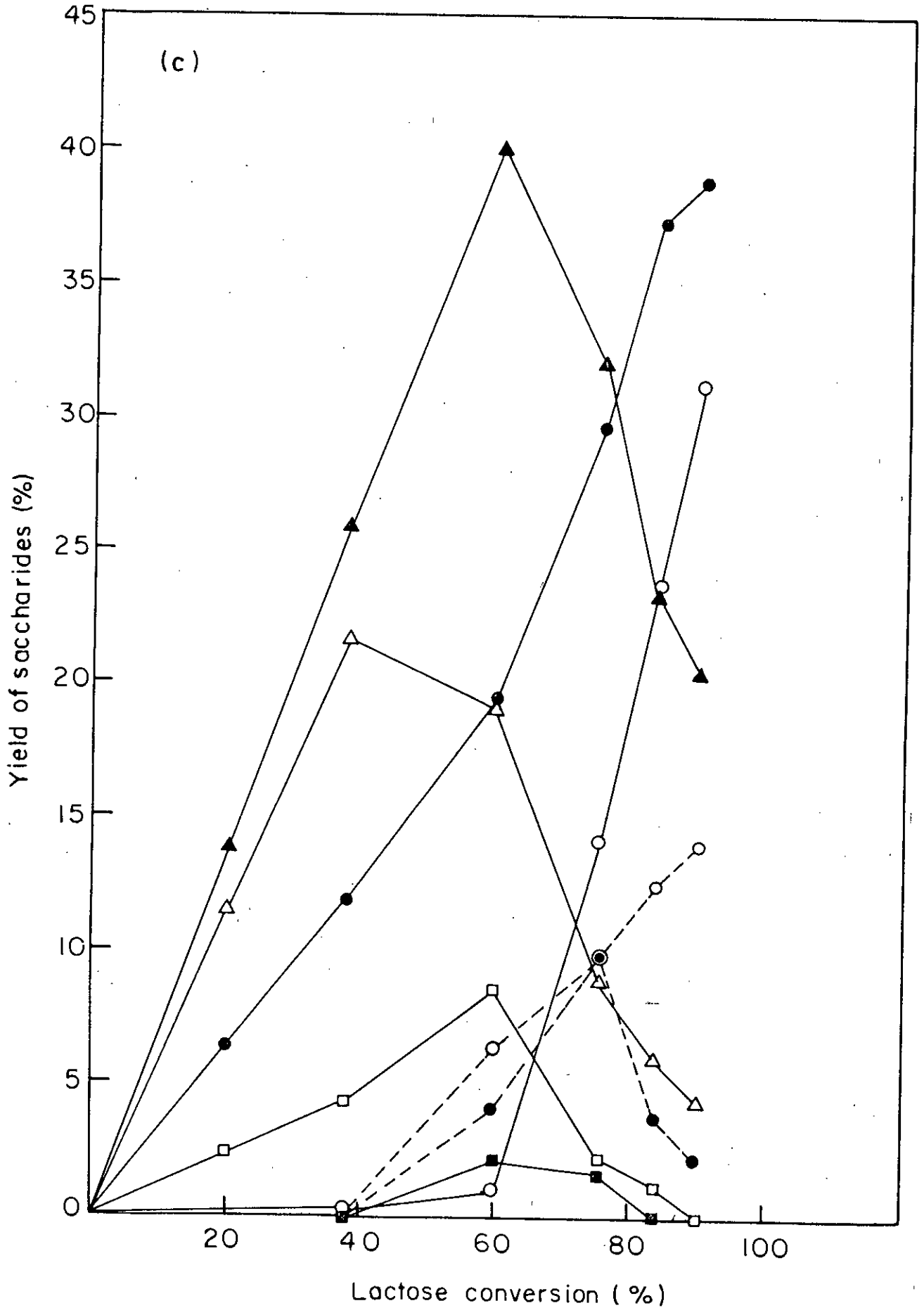

(c)
45
40
35
30
25
20
15
10
5
0
Yield of saccharides (%)
20
40
60
80
100
Lactose conversion (%)

Fig. 14.

3.0 Immobilization of β-galactosidase-2

Figure 15b is a plot of the percentage yield of saccharides produced during hydrolysis of 4.56% lactose with β-galactosidase-2 immobilized onto metal activated Silica gel (15 units/g of wet gel) against the percentage of lactose converted. The total percentage of oligosaccharides was about the same as with the free enzyme (Fig. 15a). The immobilized enzyme produced di-, tri-, tetra-, and pentasaccharides with a maximum yield of 42% at around 70.6% conversion of lactose. Their R_f values on paper chromatography were same as those of free enzyme. Disaccharide-1, disaccharide-2, and pentasaccharide appeared after 48.7%, 58.3%, and 33.6% conversion of lactose, respectively. All the saccharides disappeared at the completion of lactose conversion, except disaccharide-2.

3.1 Modification of β-galactosidase-2

The enzyme was treated with 0.025%, 0.1%, 0.25% and 3% glutaraldehyde, respectively. Figure 16a and 16b show the relationship between the percentage yield of saccharides and the percentage of lactose converted by the enzymes treated with 0.025% and 3% glutaraldehyde, respectively. The same kinds and amounts of oligosaccharides were

Figure 15. Relationship between conversion of lactose and yield of saccharides. The 4.56% lactose was hydrolyzed at 40°C and pH 6.0 with β-galactosidase-2 (a) as a free enzyme (b) immobilized onto activated Silica gel (15 units/g of wet gel).

——●——, glucose ; ——○—— , galactose ;
– – –●– – –, disaccharide-1 ; – – –○– – – , disaccharide-2;
——△——, trisaccharide ; ——□——, tetrasaccharide ;
——■—— , pentasaccharide ; ——▲—— , total oligosaccharide.

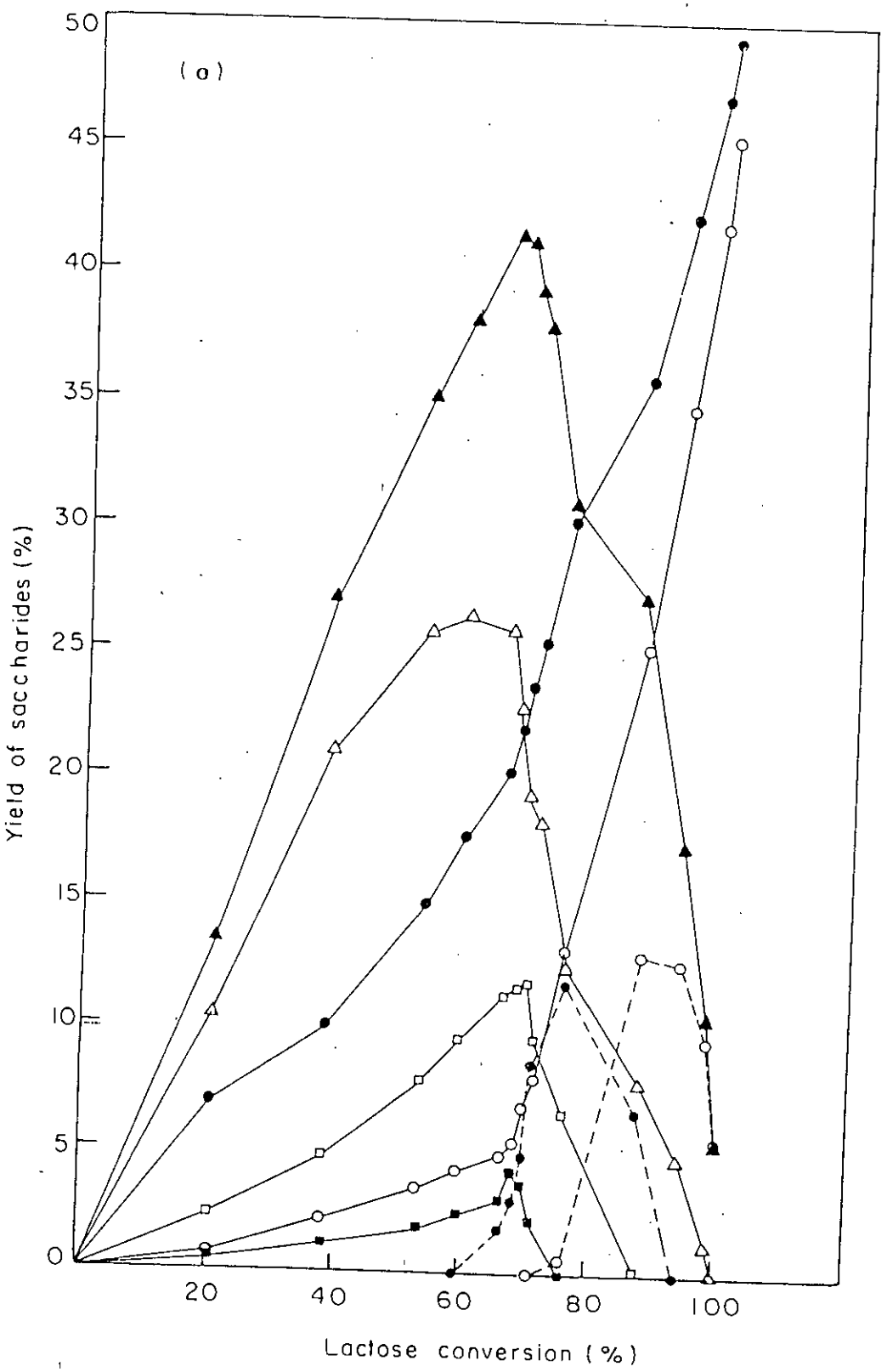

(a)
50
45
40
35
30
25
20
15
10
5
0
Yield of saccharides (%)
20
40
60
80
100
Lactose conversion (%)

Fig. 15.

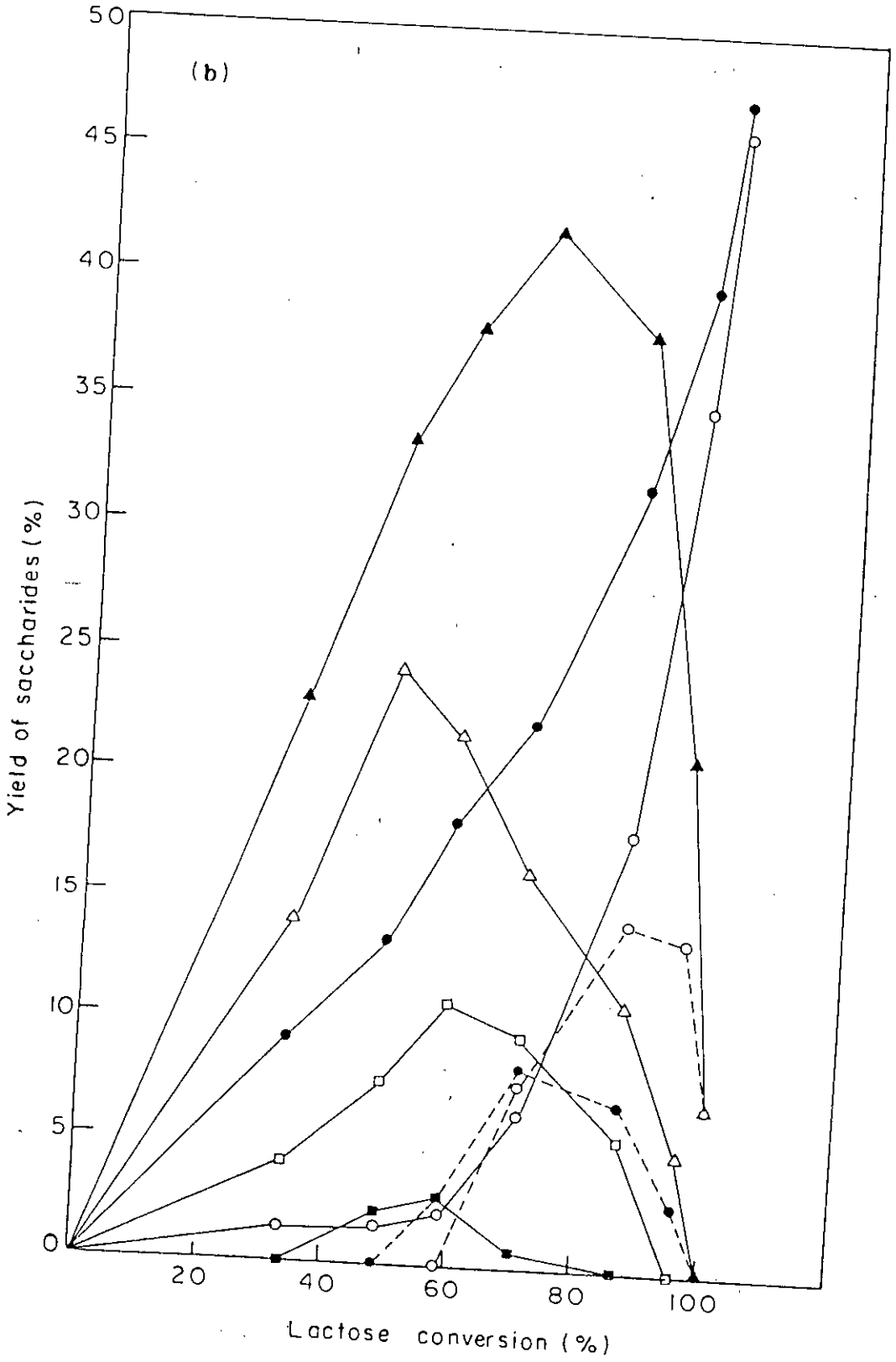

(b)
Yield of saccharides (%)
Lactose conversion (%)
0
5
10
15
20
25
30
35
40
45
50
20
40
60
80
100

Fig. 15.

Figure 16. Relationship between conversion of lactose and yield of saccharides. The 4.56% lactose was hydrolyzed at 40°C and pH 6.0 with β-galactosidase-2 (a) treated with 0.025% glutaraldehyde (b) treated with 3.0% glutaraldehyde.

—●—, glucose; —○—, galactose; ---●---, disaccharide-1; ---○--- , disaccharide-2; —△— , trisaccharide; —□—, tetrasaccharide; —▣— , pentasaccharide; —▲— , total oligosaccharide.

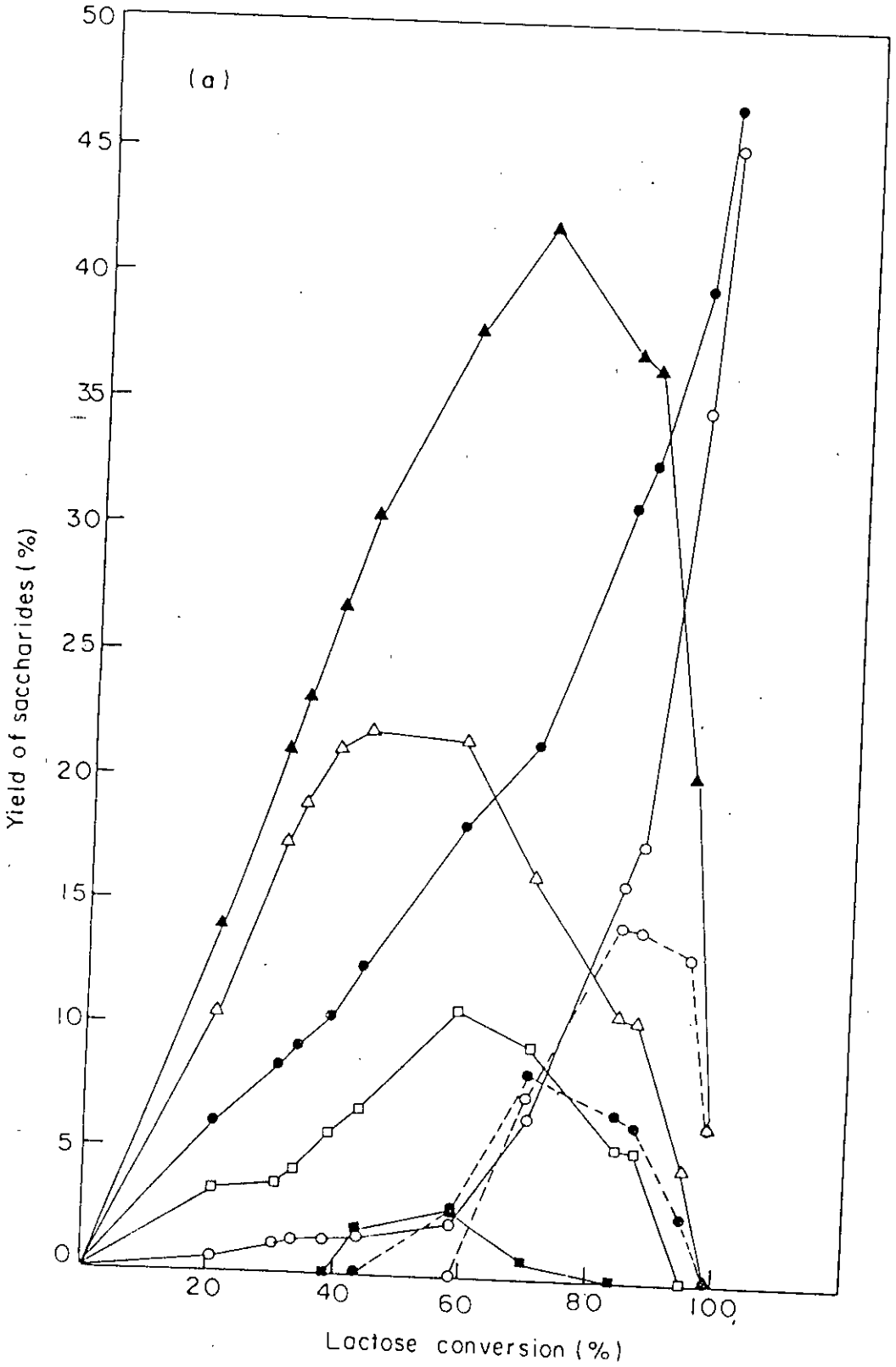

(a)
Yield of saccharides (%)
Lactose conversion (%)
0
5
10
15
20
25
30
35
40
45
50
20
40
60
80
100

Fig. 16.

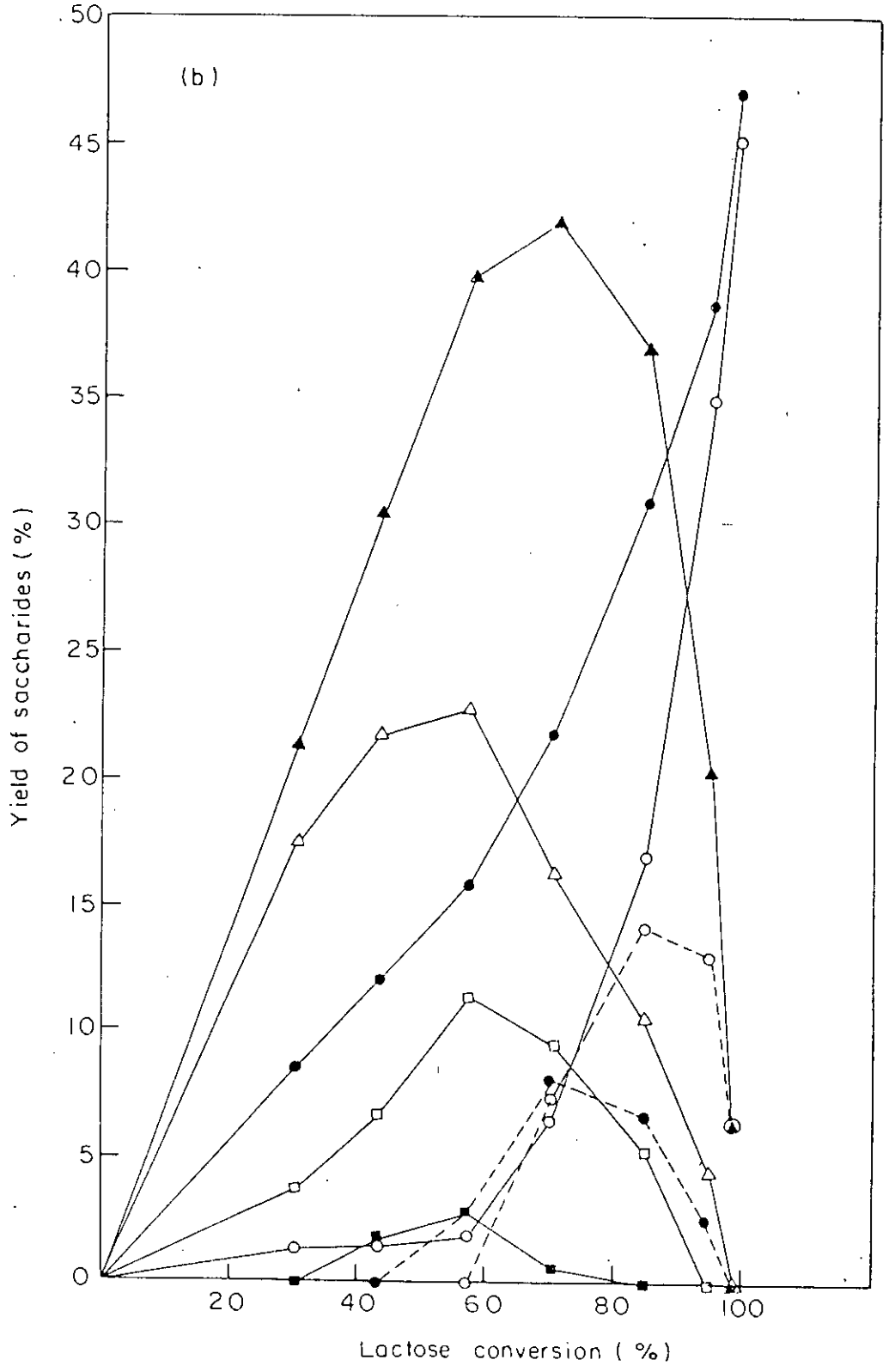

(b)
Yield of saccharides (%)
Lactose conversion (%)
0
5
10
15
20
25
30
35
40
45
50
20
40
60
80
100

Fig. 16.

Produced by these modifications. The maximum yield of oligosaccharides reached to 42% at around 70% conversion of lactose. Di-1, di-2, and pentasaccharides appeared after 43.8%, 57.5% and 38.7% conversion of lactose, respectively. Except disaccharide-2, all other saccharides disapper at the completion of conversion of lactose.

Figure 17. Relationship between conversion of lactose and total saccharides. The 4.56% lactose was hydrolyzed at 40°C and pH 6.0 with /3-galactosidase-1

——●——, free enzyme; ——○——, treated with 0.025% glutaraldehyde;---●---, treated with 0.1% glutaraldehyde;---○---, treated with 3% glutaraldehyde; ——△——, immobilized enzyme.

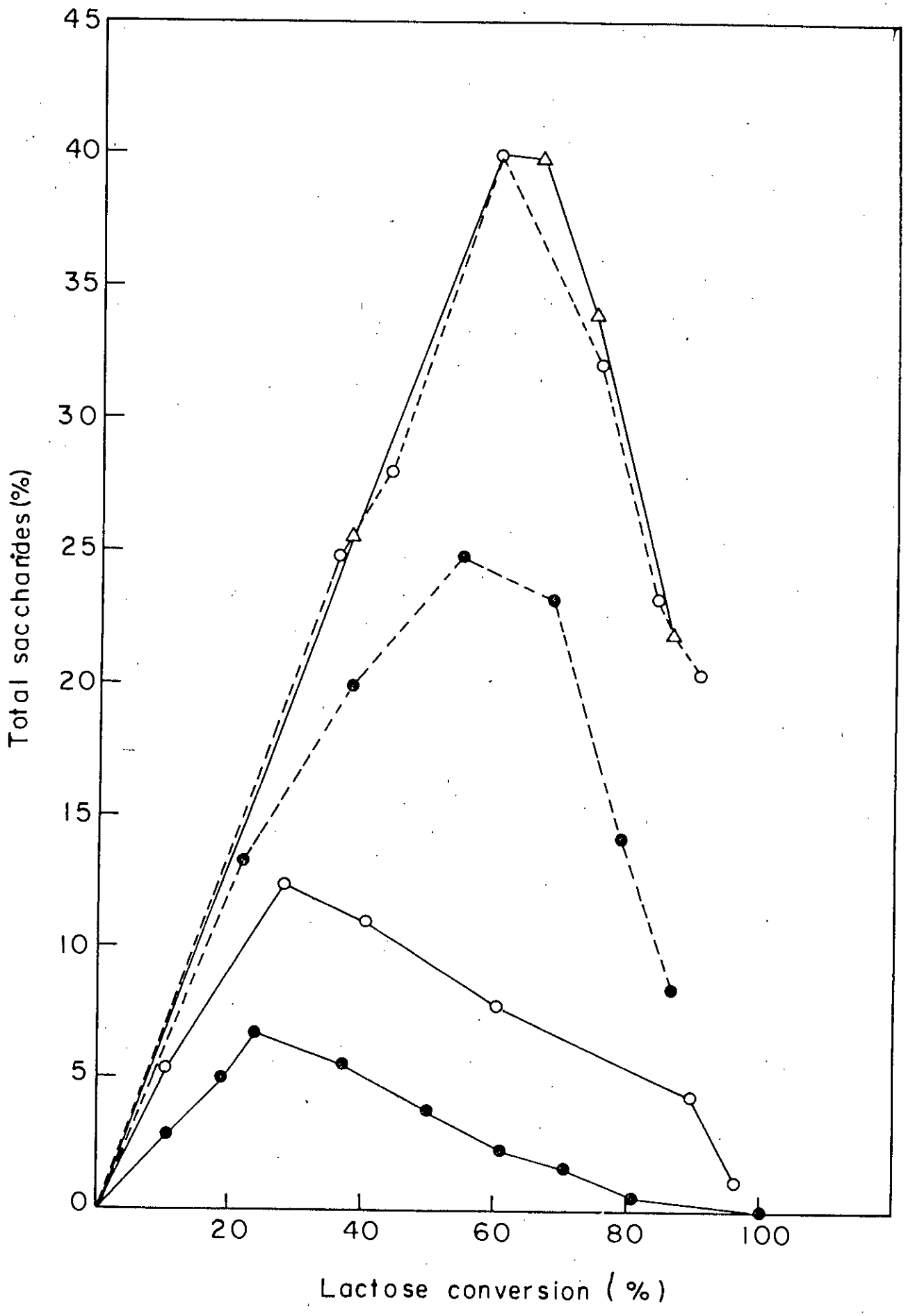

45
40
35
30
25
20
15
10
5
0
Total saccharides (%)
20
40
60
80
100
Lactose conversion (%)

Fig. 17.

Figure 18. Relationship between conversion of lactose and total saccharides. The 4.56% lactose was hydrolyzed at 40°C and pH 6.0 with β-galactosidase-2.

——●—— , free enzyme ; ——○—— , treated with 3% glutaraldehyde. For clarity enzyme treated with 0.025 and 0.1% glutaraldehyde, and immobilized enzyme showing similar pattern of hydrolysis have not been shown in the graph.

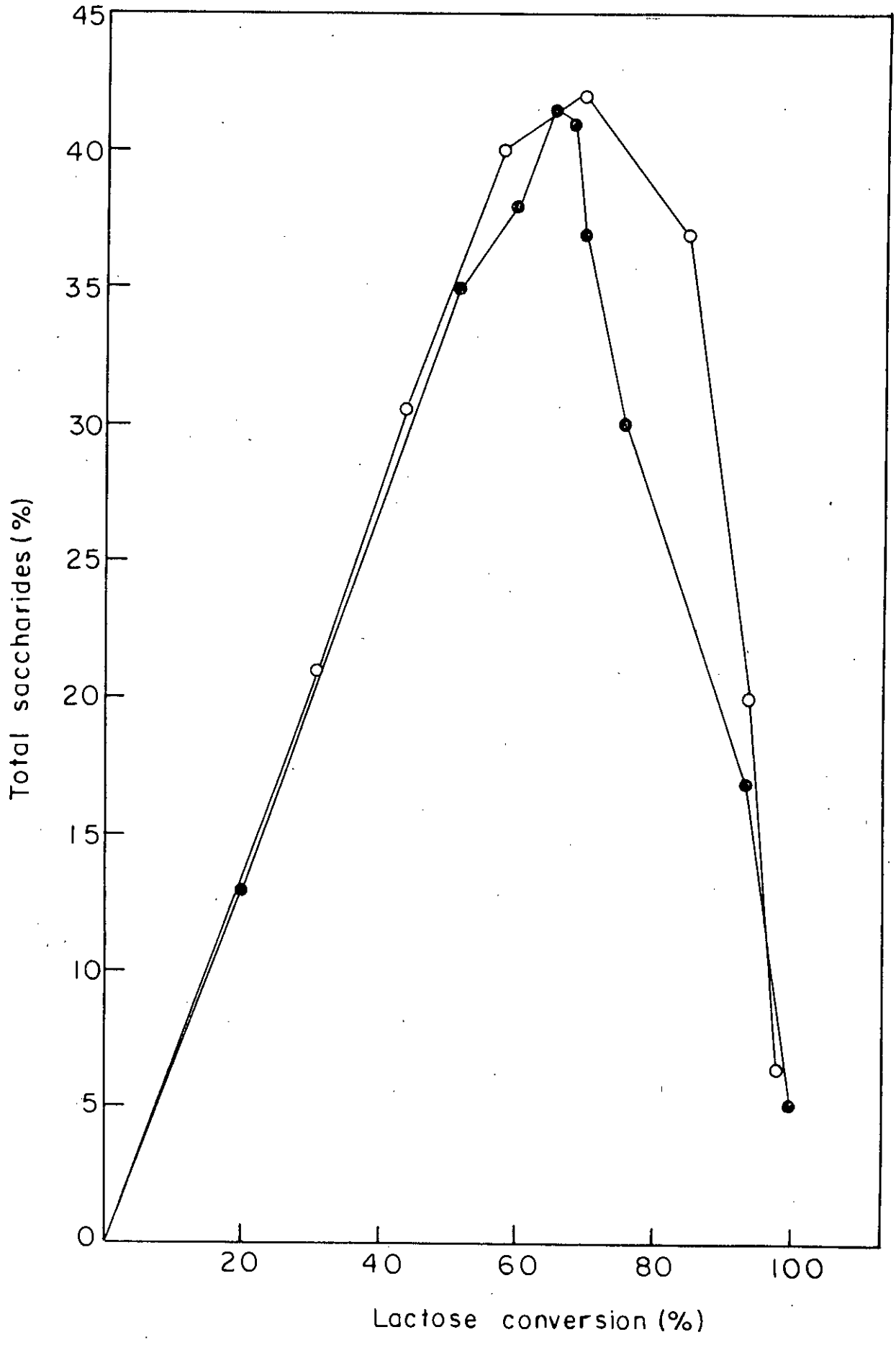

45
40
35
30
25
20
15
10
5
0
Total saccharides (%)
20
40
60
80
100
Lactose conversion (%)

Fig. 18.

discussions

The β-galactosidase activity was eluted as a single peak by gel chromatography on Sephadex G-150. By this step the specific activity was increased only slightly (Table I), since the crude enzyme preparation had been partially purified by ultrafiltration. After ammonium sulfate precipitation and dialysis, the total activity as well as the specific activity towards lactose was considerably increased in contrast with those towards ONPG. This increase in the activities towards lactose might be indicative of the removal of some endogenous inhibitors.

In ion-exchange chromatography most of the β-galactosidase activity was eluted as a single peak at 0.2 M NaCl. The specific activities towards ONPG as well as lactose were increased somewhat by this stage (Table I). Although the degree of purification was significantly increased by these steps, multiple protein bands were still detected by polyacrylamide gel electrophoresis, including two major bands with β-galactosidase activity. Even by the method of isolectric focusing with polybuffer exchanger PBE 94, the two forms of β-galactosidase could not be separated. This is due to a similarity of the isoelectric points of the two proteins (Fig. 1).

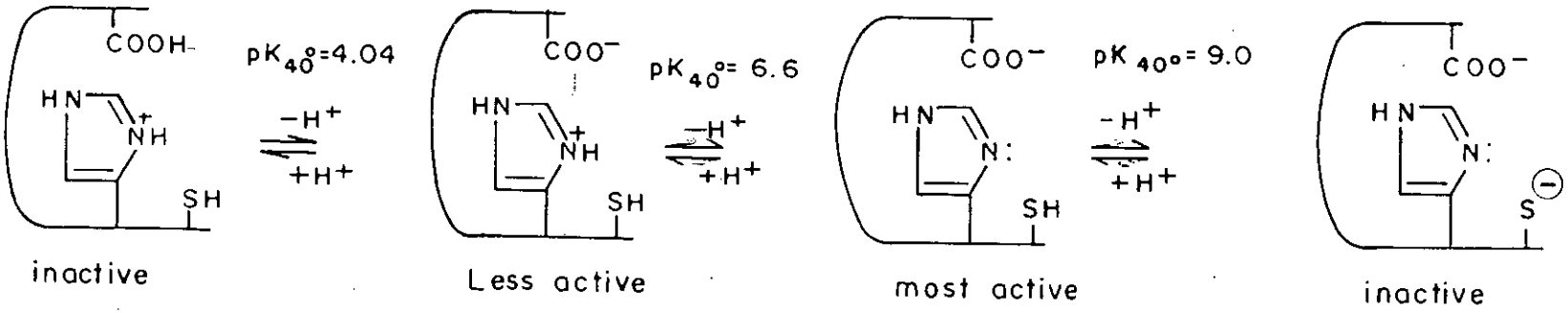
Polyacrylamide gel electrophoresis of eight sub-fraction eluted by polybuffer exchanger clearly indicated the existence of two major bands (Fig. 2). In addition to the variation in proportion of the two major protein bands, specific activities

towards lactose and ONPG for each fraction varied as shown in Fig. 3, which indicates the existence of two β-galactosidases with different substrate specificities. Widmer and Leuba (1979) reported three different forms of β-galactosidase from Aspergillus niger with similar isoelectric points. They succeeded in separating these three forms by hydrophobic chromatography, since the enzymes were quite different in carbohydrate content. In the present study, the two different β-galactosidases from Bacillus circulans were not separable by hydrophobic chromatography on Octyl-Sepharose CL-4B columns, presumably due to the small difference in carbohydrate content between them. The carbohydrate content for both enzymes was less than 1%.

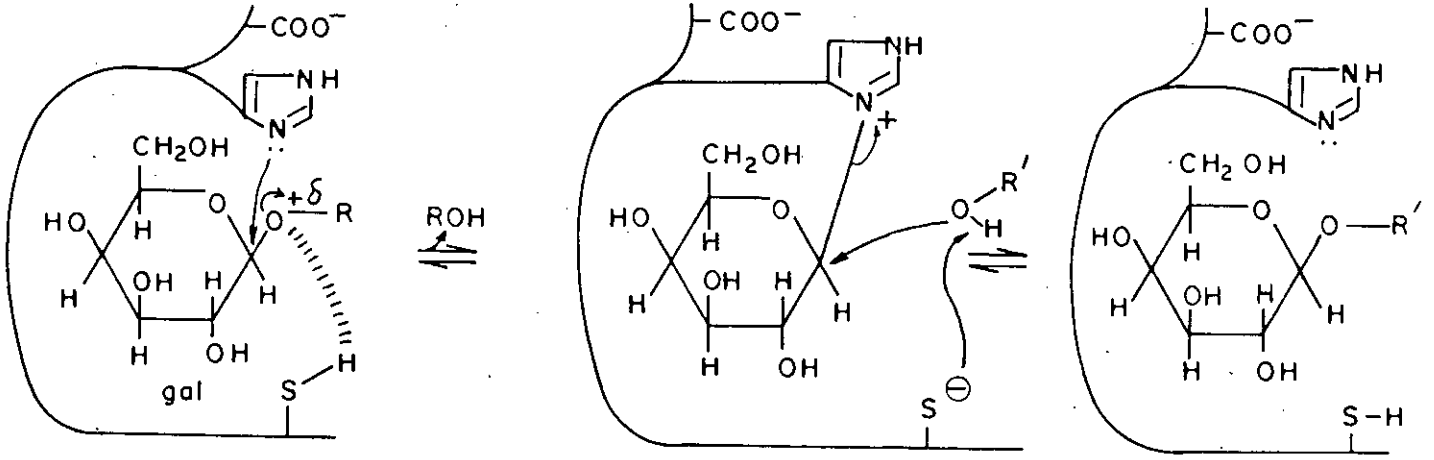
On preparative polyacrylamide gel electrophoresis fractions 1 and 2 showed a distinct major protein band of low mobility and, fractions 7 and 8 a distinct major protein band of high mobility which indicates the well separation of the two enzymes. After purification by hydrophobic chromatography with Octyl-Sepharose CL-4B to remove a small amount of contaminating protein these two enzymes were subjected to homogeneity test which was positive (Fig. 4). This indicates that the two enzymes have been fully purified. A β-galactosidase separated from fractions 1 and 2 with low mobility is denoted β-galactosidase-1, and that from the fractions 7 and 8 with high mobility is denoted β-galactosidase-2.

The molecular weights of β-galactosidase-1 and β-galactosidase-2 were determined to be approximately 2.4×10^5 and 1.6×10^5, respectively, by gel filtration on Sephadex G-200 column (Fig. 5). The SDS - gel electrophoresis method for estimation of molecular weight gave the same results, indicating that the enzymes are monomeric proteins.

The optimum pH for both the enzyme forms was 6.0 for ONPG and lactose (Fig. 6). Irrespective of substrates, β-galactosidase-2 was more stable in alkaline pH than β-galactosidase-1 at 40°C. These indicate that three dissociable groups (pK 4.04, 6.6 and 9.0, at 40°C) of these two enzyme molecules participate in the enzymic hydrolysis of ONPG and lactose. From a comparison of these pK values with those given by Edsall (1943) for various groups in proteins, the groups dissociating on the alkaline side of the pH optimum have been identified as an imidazolium group of a histidine residue and a sulfydryl group of a cysteine residue. The sulfhydryl group was supported by the inhibition of β-galactosidases with heavy metals (Table II). These groups are thus most probably an imidazolium group of a histidine residue and a sulfhydryl group of a cysteine residue. For the group dissociating on the acidic side, two possibilities arise, namely, a carboxyl group of a glutamic acid residue or a carboxyl group of an aspartic acid residue. The most possible group is a carboxyl group of a glutamic acid residue. The effect of pH on the enzymic hydrolysis of ONPG and lactose can thus be represented as shown in Scheme 1.



Scheme 1



Scheme 2

Proposed mechanism for β-galactosidase

The optimum reaction temperatures at pH 6.0 for β-galactosidase-1 with lactose and ONPG as substrates were 60° and 45°C, respectively, whereas for β-galactosidase-2 it was 60°C (Fig. 7). It shows that substrate might have some contribution in maintaining active conformation of β-galactosidase-1. On the other hand, β-galactosidase-1 was stable up to 40°C, while β-galactosidase-2 was stable to 50°C (Fig. 8). This indicates that β-galactosidase-2 is more heat-stable than β-galactosidase-1.

Table II shows that NaCl and KCl act as activators for both enzyme forms. These cations may depress K_m and increase V_{max} of these enzymes. Inhibition of the enzymes by heavy-metal ions such as Ag^+ and Hg^{2+} indicates that free sulfydryl groups are involved in the action of β-galactosidase-1 and β-galactosidase-2. Wallenfels and Fischer (1960) attributed this inhibition to a non-specific binding of metal ions, which results in changes in the tertiary structure of the enzyme.

p-Chloromercuribenzoate, a potent inhibitor for several microbial β-galactosidases (Wallenfels and Malhotra, 1961 ; Ramana Rao and Dutta, 1981) did not inhibit either of the β-galactosidases (Table II), suggesting that no free sulfhydryl group is present in the active site. A similar observation has been reported in the case of β-galactosidase preparations from A. niger (Widmer and Leuba, 1979). Of other sulfhydryl

reagents, dithioerythritol and 2-mercaptoethanol acted as activators, and iodoacetamide caused a little inhibition of the enzymes. From the above inhibition patterns it can be suggested that there are three types of sulfhydryl group present in β-galactosidase-1 and β-galactosidase-2 (Wallenfels and Malhotra, 1961).

Inhibition of the enzymes to some extent by the chelating agent, EDTA indicates that these two enzymes may contain a metal ion not in the active site. Probably, this intrinsic metal ion is important for maintaining the enzyme in an active conformation. β-Galactosidase of E.coli., ML 309 contains about 5 calcium atoms per molecule (M.W. 750,000). The effect of several chelating agents, which differed from each other in the stabilities of their calcium complexes, on the activity of the β-galactosidase of E. coli, ML 309, has been studied (Wallenfels and Malhotra, 1961).

Of the two enzymes β-galactosidase-1 was competitively inhibited by galactose (K_i, 150 mM) with lactose substrate (Table III). A competitive inhibition of the hydrolysis of ONPG with the enzyme of E. coli, K12, has been observed in the presence of D-galactose, lactose, and some other β-D-galactosides (Lederberg, 1950).

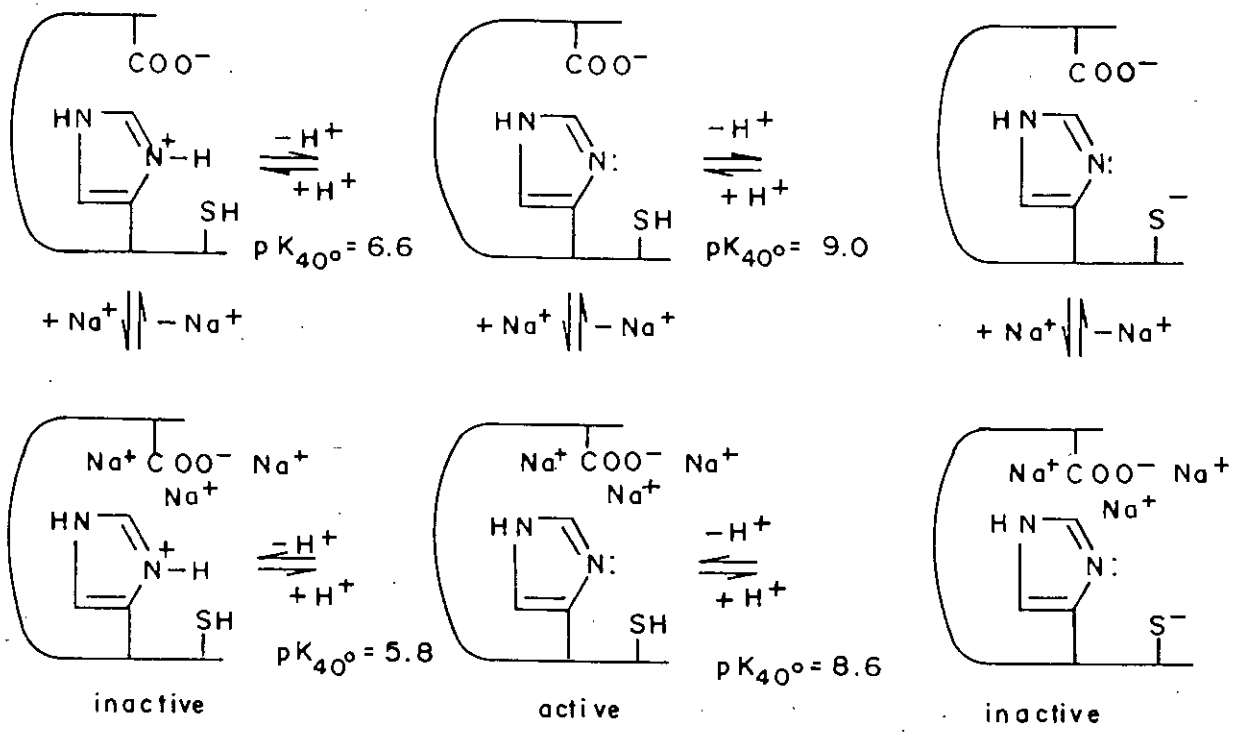
The biological substrate for β-galactosidases from all sources is probably lactose. This, however, is not always the best substrate. The relative hydrolyzabilities of lactose and ONPG

under the specified condition can be read off from Table III. V_{max}/K_m is a measure of the catalytic efficiency and specificity of an enzyme. ONPG was hydrolyzed at about 8 and 3.6 times the rate of lactose by β-galactosidase-1 and β-galactosidase-2, respectively. Table III also gives a comparison of the action of the enzymes, β-galactosidase-1 and β-galactosidase-2 from B. circulans. The ratio of V_{max} for ONPG to that for lactose was 2.57 for β-galactosidase-1 and 0.71 for β-galactosidase-2. It suggests that specificity is to be attributed to a specific configuration of the enzyme rather than to the relative stabilities of various glycosides.

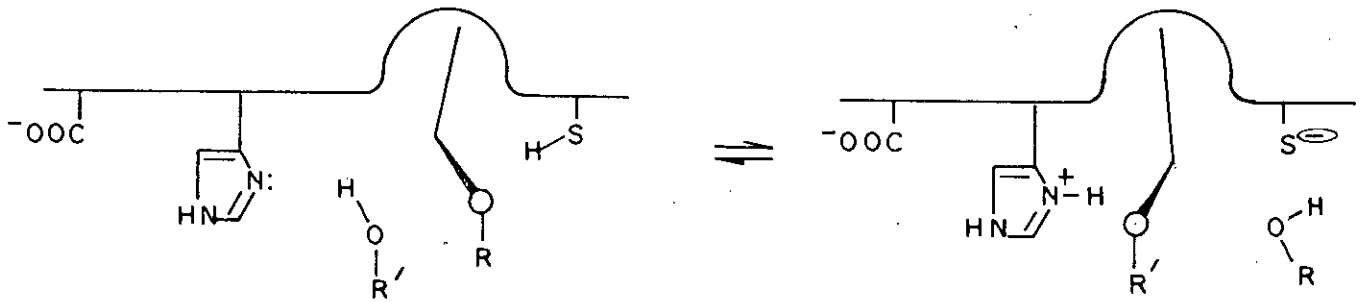
The aglycon determined not only the relative rates of hydrolysis but also the Michaelis constant (Table III). No correlation was, however, observed between K_m and V_{max}. Although the exact nature of K_m is not known in all cases, the data in Table III do suggest that the aglycon exerts a strong influence on the enzyme-substrate affinity. This part of the substrate molecule must, therefore, also be bound in some way to the active site, but probably less specifically than the glycon. From the results reported so far, it can be concluded regarding the glycon and aglycon specificities that β-galactosidases from B. circulans exhibit strict specificity requirements for the structure of the glycon as well as aglycon part of the substrate molecule.

The inhibition experiments and pH-activity studies have demonstrated the presence of three groups, a carboxyl, a sulfhydryl, and an imidazolium group, on the active site of the β-galactosidases of B. circulans. The following mechanism for the transfer of a D-galactose residue from galactoside to an acceptor can be proposed (Wallenfels and Malhotra, 1960) as shown in Scheme 2. The sulfhydryl group acts as a general acid to protonate the glycosidic oxygen atom and the imidazole group acts as a nucleophile in that it attacks the nucleophilic center at C(1) of the glycon. A covalent intermediate involving a C-N bond is proposed. In removal of the galactosyl group, the sulfhydryl anion (S^-) acts as a general base to abstract a proton from water which assists in the attack of OH^- at the C(1) position. There is no inversion of the anomeric carbon at either step in the reaction and the product retains the β-configuration around the anomeric carbon.

It might be possible that certain negatively - charged groups, for example, carboxyl groups, are in the neighborhood of imidazolium group. Such groups supress the dissociation of the imidazolium group and thus raise its pK value. The influence of alkali ions can then be readily understood, as these ions will surround the negatively - charged groups and thus shield the imidazolium group from their influence. The dissociation of the imidazolium group will be facilitated, and the pK value will shift toward the acidic side. This effect will evidently enhence



Scheme 3



Scheme 4

the enzyme activity, especially at lower pH. Although several negatively - charged groups must be involved in such an effect, it has been shown figuratively by only one carboxyl group near the imidazole ring in Scheme 3. The pK value of the sulfhydryl group is also displaced similarly, facilitating its dissociation. This, however, will result in inhibition of the enzyme activity at higher pH.

Huber et al (1976) reported that the sugar residue forming the glycon part of the substrate molecule may be transferred to water and, or to some other hydroxylic acceptors such as sugar or saccharides. On longer incubation, all of the products are hydrolyzed. β-Galactosidase-1 and β-galactosidase-2 with different transgalactosidation activity are shown in Fig. 11 and Fig. 12 a, b, respectively. β-Galactosidase-1 produced only trisaccharide while β-galactosidase-2 produced di-, tri-, tetra-, and pentasaccharides. These observations are readily explained if it is assumed that the aglycon and the acceptor occupy the same position on the enzyme molecule and that the aglycon is removed from the enzyme before the acceptor enters the common binding-site and reaction. The formation of products from the enzyme-substrate complex might be a one-step process rather than two-step process (as in Scheme 2) to explain the formation of different kinds of saccharides from an enzymic hydrolysis. The mechanism of the one-step process may be called a "Switch-over" mechanism (as shown in Scheme 4) which postulate

the existence of two similar sites (for aglycon and acceptor, respectively) near each other, because similar specificities are observed for aglycon and acceptor.

Maximum yields of oligosaccharides produced by β-galactosidase-1 and β-galactosidase-2 with lactose substrate were 6.6 and 41.5% of the total sugar, respectively (Fig. 11, 12, a, b). This indicates that transgalactosylase/hydrolase activity ratio of β-galactosidase-2 is much higher than that of β-galactosidase-1 (Huber et al. 1976). Formation of trisaccharide shows that these two enzymes probably utilized both lactose and allolactose as galactosyl acceptor (Huber et al. 1976). Allolactose could be formed in significant quantities by the transfer of galactose to the 6 position of free glucose (Huber et al. 1976 ; Greenberg and Mahoney, 1983). Maximum yield of trisaccharides was higher in β-galactosidase-2 (26.3%) than in β-galactosidase-1 (6.6%). On the other hand rate of production of trisaccharide was higher than that of tetra- and pentasaccharides, respectively in β-galactosidase-2. These show that the efficiencies of various sugars or saccharides as acceptor in transgalactosylation reaction are not the same for the two enzymes, indicating that acceptor specificity of an enzyme may vary with isozymes (Wallenfels and Beck, 1960).

After 60% conversion of lactose and at the declining phase of the formation of tri-, tetra-, and pentasaccharides, two kinds of disaccharides (disaccharide-1 and disaccharide-2) appeared

with the concomitant increased formation of glucose and galactose (Fig. 12 a,b). This indicates that the production of these two kinds of disaccharides is preferentially by the decomposition of oligosaccharides rather than by the reversion reactions of β-galactosidase (Huber and Hurlbert, 1986). The presence of disaccharide-2 at the complete conversion of lactose by β-galactosidase-2 indicates that disaccharide-2 is more stable than disaccharide-1 and the enzyme has lower affinity for this substrate to be hydrolyzed. Figure 12. a,b also shows that a much longer time was required to hydrolyze lactose completely into glucose and galactose by β-galactosidase-2 than by β-galactosidase-1 (Fig. 11), although in the case of β-galactosidase-2 twice as many units were used for hydrolysis. This fact indicates that the rate of decomposition of oligosaccharides is rather low and also that the hydrolysis of lactose might be inhibited by the presence of oligosaccharides.

β-Galactosidase-1 was immobilized onto metal activated Silica gel treated with glutaraldehyde in order to produce trisaccharide (galactosyl lactose) continuously. Nakanishi et al (1983) immobilized partially purified β-galactosidase from B. circulans onto fenolformaldehyde resin and obtained quite high activity for the hydrolysis of lactose. However, the immobilized enzyme produced many oligosaccharides with much higher yield than did the free enzyme. Data for the native enzyme (Fig. 13a) are from a previous study. The immobilized enzyme produced di-, tri-,

tetra-, and pentasaccharides with a maximum yield of 40% at around 60% conversion of lactose (Fig. 13b). The yield of oligosaccharides and their R_f values on paper chromatography were similar as those of free β-galactosidase-2 from B. circulans during hydrolysis of 4.56% lactose at 40°C and pH 6.0.

Since the yield of oligosaccharides was similar to the immobilized β-galactosidase-1 with different specific activities (12.5 to 136 units/g of wet gel), the change in the oligosaccharide-producing activity arising from the diffusional effect was considered small (Nakanishi et al. 1983). Therefore, it is thought that this change might be caused by the modification of the enzyme molecule by glutaraldehyde. The pattern of oligosaccharide produced by the enzyme treated with 3% glutaraldehyde (Fig. 14c) resembled that of the immobilized enzyme. The maximum yield of oligosaccharides reached to 40% at around 60% conversion of lactose. The enzyme treated with 0.25% glutaraldehyde produced the same kinds and amounts of oligosaccharides as the enzyme treated with 3% glutaraldehyde. The patterns of oligosaccharide produced by the enzyme treated with 0.025 and 0.1% glutaraldehyde (Fig. 14 a, b) were similar to the result obtained with the enzyme treated with 3% glutaraldehyde (Fig. 14c), although the yield of oligosaccharides was less. These findings might indicate that modification of some functional amino groups causes an increase in the oligosaccharide-producing activity of the β-galactosidase-1

(Fig. 17). Although the detailed mechanism of this increase in the yield of oligosaccharide with glutaraldehyde treated enzyme is not clear, some conformational change near the active site of the enzyme might be responsible.

Hydrolysis of 4.56% lactose with β-galactosidase-2 immobilized onto metal activated Silica gel (Specific activity, 15 units/g of wet gel) is shown in Fig. 15b. The total amount of oligosaccharides (42%) was about the same as with the free enzyme (41.5%). Data for the native enzyme (Fig. 15a) are from a previous study. Since only 15 units of enzyme per gram of activated Silica gel was immobilized, the intraparticle diffusion was probably less (Nakanishi et al. 1983). Slight increase of maximum yield of oligosaccharide with β-galactosidase-2 than that with free enzyme might be caused by the modification of amino groups of enzyme molecule by glutaraldehyde. The patterns of oligosaccharides produced by the enzyme treated with 0.025% (Fig. 16a) and 3% glutaraldehyde (Fig. 16b), respectively, resembled that of the immobilized enzyme. The maximum yield of oligosaccharides reached to 42% at around 70.6% conversion of lactose. Same kinds and amounts of oligosaccharides were produced when the enzyme was treated with 0.1% and 0.25% glutaraldehyde, respectively. These results indicate that modification of some amino groups of β-galactosidase-2 has no such influence on oligosaccharide- producing activity unlike the β-galactosidase-1 (Fig. 18). Probably, these amino groups have no functionality on the activity of the enzyme, β-galactosidase-2.

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