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EXTRACELLULAR MALTASE OF BACILLUS SUBTILIS.

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Extracellular maltase of *Bacillus subtilis*

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TABLE OF CONTENTS

INTRODUCTION

The existence of maltases was first demonstrated in 1880. Since then, there has been a number of studies on maltases (Gottschalk, 1950). Mammalian tissues, yeasts, and molds have received the most attention.

Although several studies have been conducted on bacterial maltases, only two extracellular bacterial maltases have been discovered; both were clostridial enzymes (Hockenhull and Herbert, 1945; Whelan and Nasr, 1951). Only one bacterial extracellular maltase, also clostridial, has been partially characterized (French and Knapp, 1950).

There should be many more bacteria not yet discovered which can manufacture extracellular maltases. Therefore, the prime purpose of this work was to isolate bacteria which were able to produce extracellular maltase(s) and to recover, purify, and characterize the maltase-like enzyme(s). To achieve this goal, a simple and easy method for screening the desired bacterial strains was needed. Two screening methods were developed.

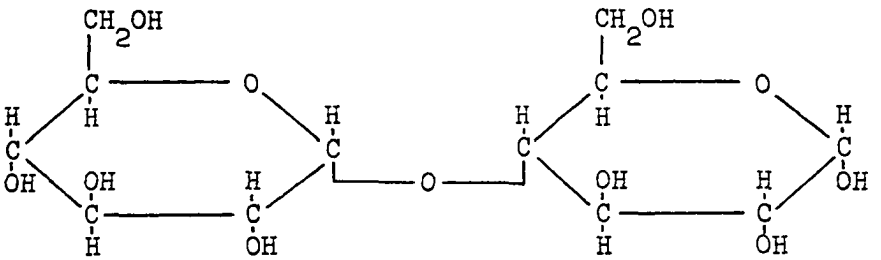
LITERATURE SURVEY

According to Gottschalk's review (1950), the enzymatic hydrolysis of maltose into glucose was first demonstrated in 1880. Porcine pancreas extracts and dried, shredded intestinal walls were used as the enzyme sources. Several years later, maltose-splitting enzymes were also found in Aspergillus niger, Aspergillus oryzae, and brewer's yeast. Thereafter, many investigators have worked on the purification and characterization of the maltose-splitting enzymes (maltases) from microorganisms, plants, and mammalian tissues.

Maltases are alpha-D-glucosidases, a family of enzymes catalyzing the hydrolysis and/or transfer of the alpha-D-glucosyl residue of alpha-D-glucosidically linked derivatives. Maltases not only catalyze the hydrolysis of maltose but some are also able to transfer the alpha-D-glucosyl residue of maltose and alpha-D-glucosides to suitable acceptors (Nisizawa and Hashimoto, 1970).

Substrate Specificity

The biological substrate for the enzyme maltase (E.C. 3.2.1.20) is maltose (4-alpha-D-glucopyranosyl-D-glucose), and is one end product of amylase action on amylose, amylopectin, and glycogen. Besides maltose, a number of maltose derivatives and some alkyl and aryl alpha-D-glucopyranosides are acted upon by maltases. The common feature of all these compounds is a nonsubstituted alpha-D-glucopyranosyl residue (Fig. 1). Besides the glucosidic oxygen, the hydroxyl groups at carbon



Glucon Moiety Aglucon Moiety

Fig. 1. Maltose (4-alpha-D-glucopyranosyl-D-glucose)

atoms 2, 3, 4, and 6 of the glucon moiety are involved in the enzyme-substrate complex (Gottschalk, 1950; Kelemen and Whelan, 1966). The hydroxyl group at carbon 3 of the glucon moiety is required for enzyme activity (Halvorson and Ellias, 1958). Contact between the enzyme protein and aglucon will vary with the chemical structure of the latter. Table 1 shows the substrate specificities of maltases of various origins. Differences between maltases of various origins with regard to their respective specificities toward the aglucon moiety of maltose are small. Most maltases can tolerate certain structural changes in the aglucon moiety of maltose and can act on alpha-D-glucosides (heterosides).

Mode of Action

A "true" maltase is an enzyme specific for maltose and without action on alpha-glucosides in general (e.g., sucrose, nigerose, etc.). The theoretical reaction products are glucose. Up to the present, no "true" maltase has been discovered.

Table 1. Substrate specificities of maltases of various origins.

Origin	Substrate	Reference
Yeasts		
Saccharomyces cerevisiae	maltose, alpha-phenyl glucoside	Spiegelman, Sussman, and Taylor, 1950
Saccharomyces cerevisiae	maltose, maltotriose	Cook and Phillips, 1957
Saccharomyces cerevisiae	maltose, sucrose, turanose, maltotriose, maltotriitol, 2-O-alpha-D-glucopyranosyl-D-erythrose	Phillips, 1959
Saccharomyces cerevisiae	maltose, sucrose	Khan and Eaton, 1967
Saccharomyces logos	maltose, phenyl-alpha-glucoside, turanose, nigerose, kojibiose, methyl-alpha-maltoside, isomaltose, phenyl-alpha-glucoside, maltotriose, maltotetraose, maltopentaose	Chiba, Saeki, and Shimomura, 1973a & 1973b
Candida tropicalis var. *japonicus*	sucrose, maltose, alpha-methyl glucoside	Sawai, 1956
Molds		
Taka-diastase (*Aspergillus oryzae*)	maltose, phenyl-alpha-glucoside, alpha-phenyl maltoside	Matsushima, 1960
Mucor rouxii	maltose (the only substrate test)	Flores-Carreon and Ruiz-Herrera, 1972
Mucor javanicus	maltose, alpha-methyl-maltoside, soluble starch	Yamasaki, Miyake, and Suzuki, 1973b

Table 1. (continued)

Origin	Substrate	Reference
Bacteria		
Clostridium acetobutylicum	maltose, isomaltose, sucrose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose	French and Knapp, 1950
Klebsiella aerogenes	maltose, isomaltose, methyl-beta-maltoside, trehalose, methyl-alpha-glucoside	Barker *et al.*, 1966
Bacillus cereus	maltose, nigerose, phenyl-alpha-maltoside	Yamasaki and Suzuki, 1974
Animal		
Equine serum	maltose, methyl-beta-maltoside, phenyl-alpha-glucoside, turanose, isomaltose, glycogen, starch	Lieberman and Eto, 1957
Human liver, heart, and muscle	maltose, glycogen	Hers, 1963
Cattle liver	glycogen, maltose, isomaltose, dextrin	Bruni, Auricchio, and Covelli, 1969
Rat liver	maltose, isomaltose, glycogen	Jeffrey, Brown, and Brown, 1970
Rabbit muscle	maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, glycogen, p-nitro-phenyl-alpha-maltoside, methyl-beta-maltoside, panose	Palmer, 1971a; Carter and Smith, 1973

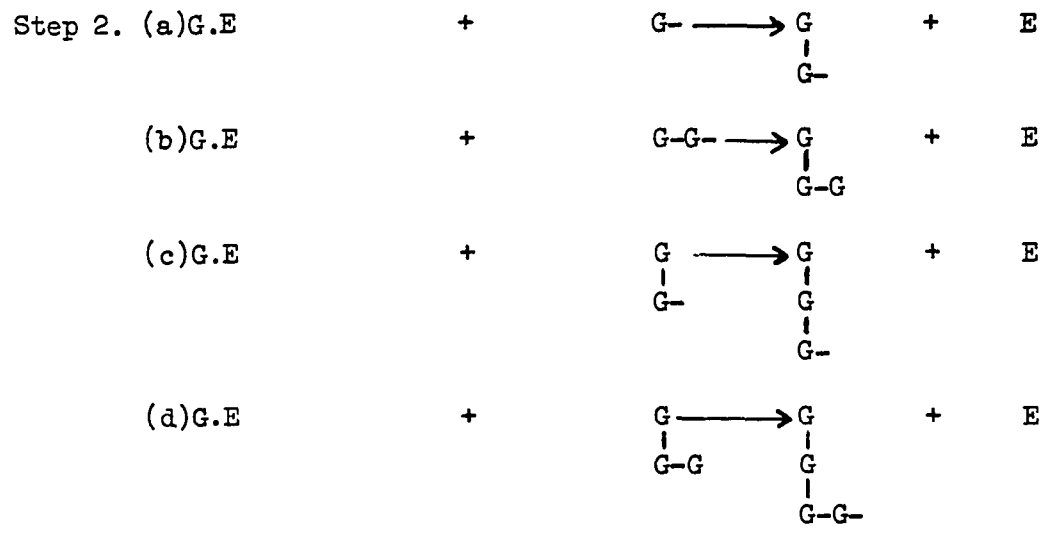
Table 1. (continued)

Origin	Substrate	Reference
Plant		
Barley malt	maltose, isomaltose, panose, isomaltotriose, maltotriose, starch dextrin	Jørgensen and Jørgensen, 1963; Jørgensen, 1963
Buckwheat	soluble starch, amylopectin, glycogen, beta-limit dextrin, maltose, panose, isomaltose, methyl-alpha-glucoside, methyl-alpha-maltoside, maltotriose, maltotetraose, maltopentaose	Takahashi and Shimomura, 1972
Rice	maltose, starch	Takahashi and Shimomura, 1973

The mode of action of the maltases found so far can be classified into two groups. The maltases of the first group are purely hydrolytic and produce lower saccharides. Examples of this group of enzymes are the maltases from Clostridium acetobutylicum (French and Knapp, 1950), Escherichia coli (Pontieri, 1955), Mucor rouxii (Flores-Carreon and Ruiz-Herrera, 1972; Reyes and Ruiz-Herrera, 1972), Saccharomyces cerevisiae (Spiegelman et al., 1950), Saccharomyces logos (Chiba et al., 1973b), and Candida tropicalis var. japonica (Sawai, 1956). The second group of maltases, besides catalyzing the hydrolysis of substrates, can transfer the alpha-glucosyl residue to suitable acceptors and form higher saccharides with or without the involvement of a vitamin. Examples of this group of enzymes are maltases from E. coli (Doudoroff et al., 1949; Katagiri, Yamada, and Imai, 1957, 1958; Pontieri, 1955; Wiesmeyer and Cohn, 1960b). Micrococcus sp. (Kawai, Yamada, and Ogata, 1971), Aspergillus oryzae (Pazur and French, 1952), Taka-diastase (Matsushima, 1960), Mucor javanicus (Yamasaki et al., 1973a, b), rat liver (Jeffrey et al., 1970; Stetten, 1959), rabbit muscle (Palmer, 1971b), barley malt (Jørgensen, 1964), and buckwheat (Takahashi and Shimomura, 1972).

After studies on the transglucosidase of Aspergillus oryzae, Pazur and French (1952) proposed a mechanism for the synthesis of oligosaccharides from maltose without the involvement of riboflavin or pyridoxine. The reaction can be conveniently represented as occurring in two steps:

Step 1. $G\text{-}G\text{-} + E \longrightarrow G.E + G\text{-}$



(Horizontal lines represent 1,4-glucosidic bonds, vertical lines 1,6-glucosidic bonds, G-, glucose units with a free aldehyde groups, and E, the enzyme). Wiesmeyer and Cohn (1960b) indicated that E. coli maltase releases the reducing end of the maltose molecule as free glucose, whereas the nonreducing end is contributed toward the formation of the polymer.

As to the formation of oligosaccharides with the involvement of riboflavin, Katagiri et al. (1957, 1958) proposed a mechanism based on studies with E. coli enzyme. The mechanism is illustrated by the following equations, where B_2 represents riboflavin, E, enzyme, and $(glucose)_n$, oligosaccharide:

$$B_2 + E \longrightarrow B_2E$$

$$B_2E + \text{maltose} \longrightarrow (B_2E)\text{glucose} + \text{glucose}$$

$$(B_2E)\text{glucose}_n + \text{maltose} \longrightarrow (B_2E)\text{glucose}_{n+1} + \text{glucose}$$

$$(B_2E)\text{glucose}_{n+1} \longrightarrow (B_2)\text{glucose}_{n+1} + E$$

Thus, riboflavin (B_2) plays a significant role as a glycosyl carrier in the biochemical process of oligosaccharide synthesis by E. coli maltase.

Another vitamin involved in the synthesis of oligosaccharides is pyridoxine (B_6). Kawai et al. (1971) showed that the alpha-glucosidase from Micrococcus sp. No. 431 could catalyze the transfer of the glucose residue of maltose, sucrose, or phenyl-alpha-D-glucoside to pyridoxine to form pyridoxine glucoside.

Location of Maltase

Most of the microbial, plant, and animal maltases are located in the cytoplasm, either in a soluble form or bound to subcellular particles (Doolin and Panos, 1969; Flores-Carreon, Reyes, and Ruiz-Herrera, 1969; Halvorson and Ellias, 1958; Khan and Eaton, 1967; Marshall and Taylor, 1971; Palmer, 1971a, b; Robertson and Halvorson, 1957; Sugimoto, Amemura, and Harada, 1974; Sutton and Lampen, 1962; Vojtkova-Lepsikova, 1969; Yamasaki et al., 1973a). Burger and Pavlasova (1964) reported that the maltase in the cytoplasm of a wild type of E. coli was in a soluble form. In mutant strains, however, the maltase was associated with insoluble structures and was inactive toward maltose. This inactive maltase could be liberated in active form from the insoluble material by the action of ribonuclease or toluene.

Aspergillus niger secretes its alpha-glucosidase into the medium and the enzyme can be separated from the mycelium by filtration (Reese, Maguire, and Parrish, 1968). In Mucor rouxii, one of the alpha-

glucosidases is inducible and is bound to the cell wall of the fungus in a manner possibly similar to the way in which yeast invertase is bound to the yeast cell wall (Flores-Carreon et al., 1970). This cell-wall bound alpha-glucosidase cannot be released by treatment with trypsin but can be released by a cell-wall lytic enzyme under a hypertonic condition which preserves the integrity of the protoplast, suggesting that this inducible alpha-glucosidase is associated with the cell wall and is thus protected from proteolysis.

So far, bacterial extracellular maltases have been found only in Clostridium spp. French and Knapp (1950) recovered a maltase from cell-free filtrates of liquid cultures of Clostridium acetobutylicum. The maltase was separated from amylase activity by adsorbing the amylase onto starch. Maltase activity was also detected in cell-free filtrates when a strain of Clostridium butyricum was cultured in a medium containing maltose as the inducer. In the absence of inducer, only amylase activity could be detected (Whelan and Nasr, 1951).

The only other extracellular maltases described are from the cellular slime mold, Dictyostelium discoideum (Every and Ashworth, 1973) and the stomach fluid of the oyster, Ostrea edulis L (Mathers, 1973).

MATERIALS AND METHODS

Isolation of Extracellular Maltase Producers

Soil samples from which extracellular maltase producers were isolated were obtained from the Iowa State University Soil Testing Laboratory. Two methods were used in the isolation of extracellular maltase producers. The first method was to overlay petri dishes containing isolated colonies with Lactobacillus sanfrancisco cell suspensions in agar. Lactobacilli that can utilize maltose but not glucose as the sole source of carbon (Kline and Sugihara, 1971; Ng, 1972) were used in the overlays. If a colony secreted maltase into the medium, the maltose surrounding the colony would be hydrolyzed to glucose. Since the lactobacilli cannot grow on glucose, there will be clear zones around maltase-producing colonies.

The medium for the isolation of maltase producers was composed of 1.5% agar, 0.5% peptonized milk, 0.2% yeast extract, and 0.5% maltose. Appropriate dilutions of soil samples were surface-plated on the maltose-milk agar and the plates were incubated at 30 C. After colonies had developed, the petri plates were overlaid with about 7 ml of tomato juice agar or glycerol-phosphate buffer agar, pH 6.8, containing 0.5% maltose and 0.1 ml of Lactobacillus sp. culture. After incubation overnight at 30 C, the plates were observed for zones where no growth appeared in the overlay.

Two strains of Lactobacillus sanfrancisco were used. One was isolated from a San Francisco sour-dough bread starter culture

(Microlife Products) and designated strain SORDO-L. The other, MM-2, was isolated from spoiled corn (McMahon, 1972). Both lactobacilli were maintained on microassay agar stabs containing 1% maltose. Inocula for the agar overlays were prepared by transferring cells from stab cultures to tubes of microinoculum broth containing 0.5% maltose; the broth was incubated overnight (12 to 18 hours) at 30 C before use.

The Lactobacillus overlay method seemed to be successful. Seven colonies apparently secreted extracellular maltase into the medium (produced zones of no growth of the indicator lactobacilli) and were isolated for further tests. A year later, however, we attempted to repeat the Lactobacillus screening method for isolation of extracellular maltase producers. In five different trials no maltase-producing bacteria were observed among soil isolates, and the method failed to detect a previously isolated and proven maltase producer. Clear zones were observed around several colonies and these colonies were purified. All of those isolates produced antibiotics inhibitory to the indicator lactobacilli; none produced an extracellular maltase. The reasons for failure to reproduce the maltase bioassay are unknown. Possibly the indicator lactobacilli lost their specific affinity for maltose; however, transfers from freshly opened lyophiles also were unsuccessful. The Lactobacillus bioassay is still under investigation by Dr. P. A. Hartman.

The second method used to screen for maltase production was enzymatic. PGO enzymes (Sigma Chemical Co., St. Louis, Mo.) were used. PGO reagent is an enzyme mixture of peroxidase and glucose oxidase and

contains o-dianisidine as a chromogen. In the presence of glucose, peroxide is formed; the peroxide reacts with o-dianisidine to produce a color. When maltase-producing colonies were overlaid with PGO reagent dissolved in melted and cooled 0.5% agar-0.5 M Tris buffer, pH 7.5, red zones developed around the colonies within 30 minutes, as shown in Fig. 2. The PGO procedure was superior to many alternative procedures that were attempted, such as pouring liquid PGO-buffer on plates or spraying plates with PGO-buffer.

The PGO-buffer-agar overlay method was used to double-check maltase-producing colonies obtained from the Lactobacillus method. The best maltase producer of 7 isolates, strain P-11, was characterized and used for further studies.

Fermentation Tests for Extracellular Maltase Production

Tests for the effects of nitrogen and carbon sources, and their concentrations, on extracellular maltase production by strain P-11 were conducted in shake culture. After sterilization (121 C, 15 minutes), 250-ml Erlenmeyer flasks, each containing 100 ml of medium, were inoculated with 1 ml of seed culture and shaken (200 rpm) for 24 hours at 37 C. The seed culture was prepared by inoculating 1 loopful of cells into a medium containing 0.5% peptonized milk and 0.2% yeast extract, and shaking for 6 hours at 37 C. After the best nitrogen source was determined, the peptonized milk was replaced by phytone. Other modifications of procedure are described in the appropriate RESULTS sections.

The relationships between extracellular maltase activity, cell growth, and incubation period were determined by using 2.8-ℓ Fernbach flasks, each containing 1.0 ℓ of medium. After being inoculated with 10 ml of seed culture, flasks were shaken (180 rpm) at 37 C. Samples were taken periodically and assays for maltase activity were conducted as described in RESULTS.

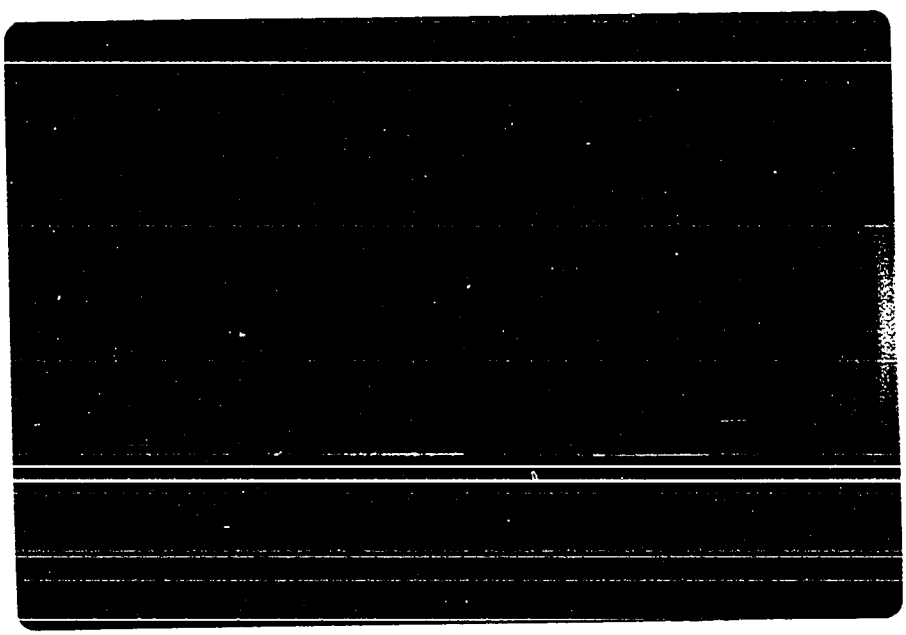


Fig. 2. Color reaction pattern of extracellular maltase producers by the PGO reagent method. Colonies were overlaid with PGO reagent in 0.5% agar-0.5 M Tris buffer, pH 7.5. Red zones develop around extracellular maltases-producing colonies within 30 minutes.

Enzyme Assays

To determine extracellular maltase activity, the reaction mixture contained 1 ml of cell-free filtrate and 9 ml of 3.3 mM maltose solution in M/15 phosphate buffer, pH 6.5. After incubation at 40 C for 30 minutes, the reaction mixture was stopped by heating in a boiling water bath for 5 minutes. The amount of glucose produced in the reaction mixture was determined with PGO reagent (Sigma Technical Bulletin No. 510, 1969). One unit of maltase activity is defined as the amount of enzyme which produces 1 µmole of glucose from maltose under the conditions used.

The amylase activity was determined by a blue-value method, which measures the disappearance of iodine-starch blue color due to amylase action (Robyt and Whelan, 1968).

The semi-quantitative analysis of proteinase activity was determined by a disc-plate method. Clear zones due to proteinase action were formed around paper discs on milk agar plates (Lewis et al., 1957).

The qualitative assay of transglucosylase activity was conducted by a paper chromatographic method. Substrate digests were spotted on Whatman No. 3 filter paper and developed ascendingly three times with n-butanol-pyridine-water (6: 4: 3 by volume). The chromatograms were developed by the silver nitrate dip method, according to Robyt and French (1963).

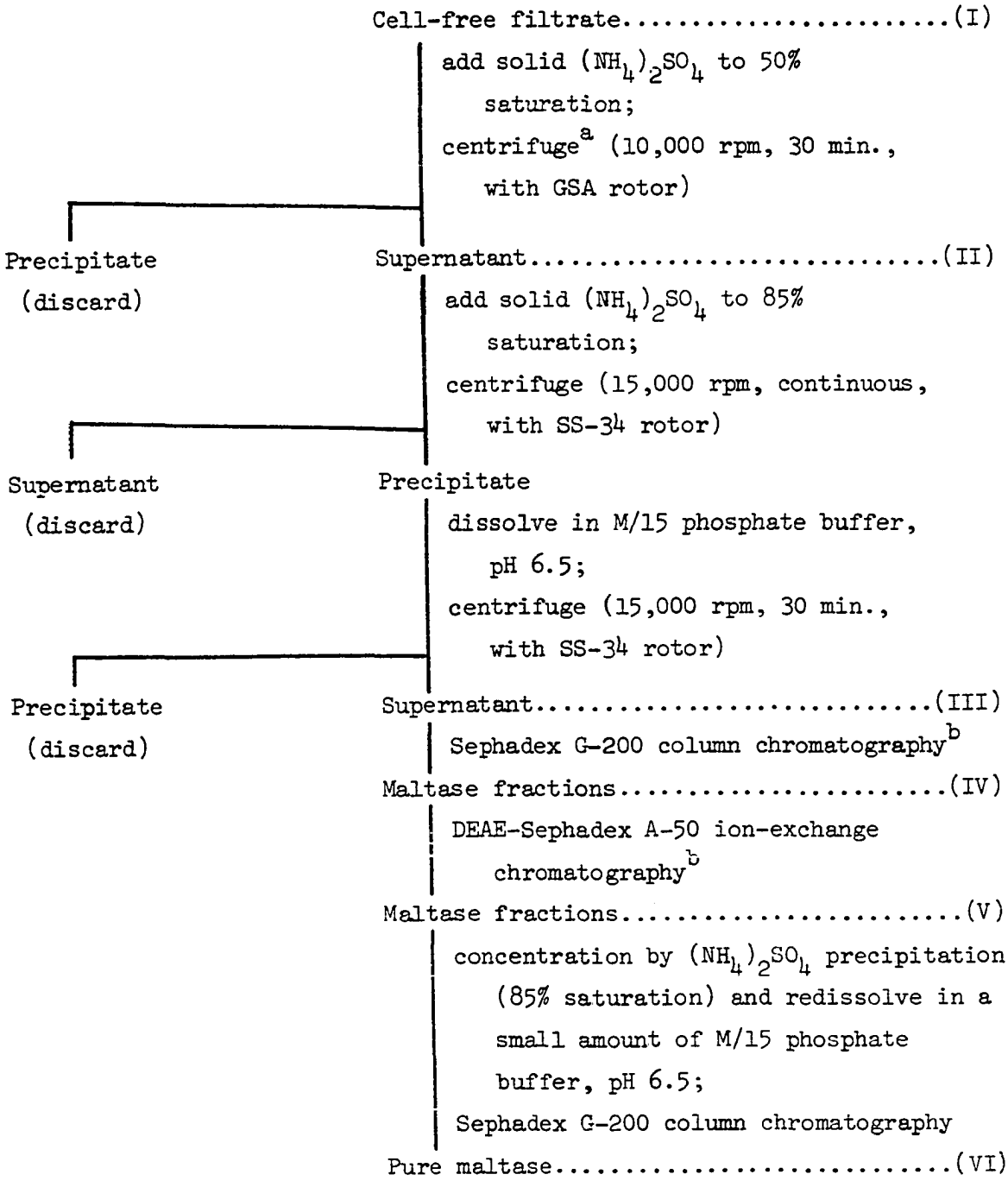
Protein Determination

The protein concentration was determined by the Folin-phenol method

(Lowry et al., 1951). Bovine serum albumin was used as the standard. The protein concentration of each fraction during column chromatography was estimated by observing the absorbance at 280 nm.

Purification of the Extracellular Maltase

Based on a number of preliminary experiments, a protocol was developed for maltase purification. The purification of the extracellular maltase was conducted according to the schema shown in Fig. 3.



[a]Sorval RC-2B superspeed centrifuge, 10 C.
[b]Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Fig. 3. Procedure developed for purification of the extracellular maltase of B. subtilis, strain P-11.

RESULTS

Identification of Maltase-producing Isolate, P-11

Seven colonies capable of producing extracellular maltase were obtained from the Lactobacillus screening method. After being double checked with the enzymatic PGO reagent method, the best maltase producer, P-11, was selected for further studies. The taxonomical characteristics of strain P-11 are shown in Table 2. The properties of strain P-11 were identical to those of Bacillus subtilis described in Bergey's Manual (8th edition).

Table 2. Characterization of maltase producer, P-11.

Morphology:	Gram positive
	Rod-shaped, 0.6-0.7 x 3-4 μ
	Motile, with lateral flagella
	Endospore formation in AK #2 medium (BBL) after 48 hours, subterminal and sporangia not swollen
Cultural and Physiological Characteristics:	Gelatine stab, stratiform liquefaction
	Starch hydrolysis +
	Catalase +
	Nitrite formation +
	Indole -
	Growth in 7% NaCl-Trypticase soy broth
	Milk agar streak plate: wide zone of hydrolysis of casein
	Thioglycollate broth: surface growth with pellicle
	Optimum temperature, 37 C

Development of Media for Extracellular Maltase Production by B. subtilis P-11

The effects of carbon and nitrogen sources on extracellular maltase production were conducted in shake culture (200 rpm) at 37 C. As shown in Tables 3 and 4, phytone and methyl-α-D-glucoside were the best nitrogen and carbon sources, respectively, for maltase production. Other nitrogen sources such as trypticase-soy and tryptone, supported adequate maltase production (Table 3). Other carbon sources, such as maltose, trehalose, and xylose, also gave good results, although trehalose, xylose, and methyl-α-D-glucoside are not the substrates of the maltase.

Different concentrations of methyl-α-D-glucoside, phytone, and yeast extract were examined for their effects on extracellular maltase production by B. subtilis P-11 (Table 5). The best combination tested was 2.0:0.5:0.2 (methyl-α-D-glucoside:phytone:yeast extract). Another combination, 1.0:0.5:0.2, also resulted in very substantial production of extracellular maltase.

Fig. 4 shows the relationships between extracellular maltase activity, cell growth, and incubation period in the medium that was developed previously (Table 5). Maltase activity appeared in the cell-free filtrate during the initial (early logarithmic) phase of growth (Fig. 4). Maltase increased approximately parallel to growth, reached maximum levels at the early stationary phase, and then decreased gradually. The release of maltase into the culture medium did not seem

Table 3. Effect of nitrogen source on extracellular maltase production by *B. subtilis* P-11.

Nitrogen source[a]	Concentration (%)	Extracellular maltase activity (units/ml)[b]
Phytone	0.5	6.4
Trypticase-soy	0.5	5.1
Tryptone	0.5	4.9
Peptone	0.5	4.5
Peptonized milk	0.5	4.3
Skim milk	0.5	4.3
Casamino acids (vitamin free)	0.5	3.7
Proteose-peptone	0.5	3.5
Trypticase	0.5	3.3
NH_4NO_3[c]	0.2	3.3
NZ-case	0.5	3.1
Urea[c]	0.1	2.6
Soytone	0.5	2.0
$(NH_4)_2SO_4$[c]	0.3	1.7

[a] 1.0% maltose and 0.2% yeast extract were used as sources of carbon and growth factors, respectively, in all media.

[b] Average of samples from duplicate flasks, taken after incubation for 24 hours.

[c] Levels of inorganic nitrogen sources were selected so that the nitrogen content was approximately the same as that in the organic nitrogen sources.

The inorganic nitrogen sources were sterilized separately in an autoclave and added to the media.

Table 4. Effect of carbon source on extracellular maltase production by B. subtilis P-11.

Carbon source[a] (1.0%)	Final pH[b]	Extracellular maltase activity (units/ml)[c]
Methyl-α-D-glucoside	6.9	6.1
Maltose	6.7	5.3
Trehalose	6.8	4.4
Xylose	6.7	4.4
Raffinose	6.8	3.6
Soluble starch	6.7	2.8
Glycogen (oyster)	6.8	2.8
Galactose	7.2	2.4
Lactose	7.2	2.5
Cellobiose	6.8	2.3
Sucrose	6.8	2.2
Glucose	6.5	2.1
Fructose	6.5	2.1
Mannose	6.8	1.9
Inulin	6.7	1.7
Amylopectin	6.7	1.7
Dimalt	6.3	1.1
Dextrin	6.9	0.6
Ribose	6.6	0.6
None	8.3	0

[a]All media also contained 0.5% phytone and 0.2% yeast extract.

[b]The initial pH was 6.8.

[c]Average of samples from duplicate flasks after incubation for 24 hours.

Table 5. The effects of various concentrations of methyl-α-D-glucoside, phytone, and yeast extract on extracellular maltase production by B. subtilis P-11.

Methyl-α-D-glucoside (%)	Phytone (%)	Yeast extract (%)	Final pH[a]	Extracellular maltase activity (units/ml)[b]
0	0.25	0.05	8.3	0
0	0.25	0.1	8.3	0
0	0.25	0.2	8.5	0
0	0.5	0.05	8.4	0
0	0.5	0.1	8.5	0
0	0.5	0.2	8.5	0
0	1.0	0.05	8.4	0
0	1.0	0.1	8.6	0
0	1.0	0.2	8.5	0
1.0	0.25	0.05	7.4	1.39
1.0	0.25	0.1	7.5	1.39
1.0	0.25	0.2	7.0	5.00
1.0	0.5	0.05	7.4	1.39
1.0	0.5	0.1	7.0	5.28
1.0	0.5	0.2	6.9	6.11
1.0	1.0	0.05	7.4	2.50
1.0	1.0	0.1	7.3	4.44
1.0	1.0	0.2	7.2	1.95
2.0	0.25	0.05	7.2	1.39
2.0	0.25	0.1	7.4	1.95
2.0	0.25	0.2	6.8	3.61
2.0	0.5	0.05	7.1	1.95
2.0	0.5	0.1	7.4	2.50
2.0	0.5	0.2	6.7	6.94
2.0	1.0	0.05	7.2	2.50
2.0	1.0	0.1	7.0	1.39
2.0	1.0	0.2	7.1	1.39

[a]The initial pH was 6.8.

[b]The incubation period was 24 hours.

Fig. 4. Relationship between extracellular maltase activity, cell growth, and incubation period.

The experiment was conducted in 2.8-liter Fernbach flasks, each containing 1 liter of medium composed of 2% methyl-alpha-D-glucoside, 0.5% phytone, and 0.2% yeast extract. The shaking speed and temperature were 180 rpm and 37 C, respectively.

Key: (•——•) maltase, (o——o) viable counts, (▫——▫) absorbance, and (x——x) pH.

ABSORBANCE (605 nm)
0
2
4
6
8
VIABLE COUNT (log10 cells/ml)
7
8
9
INCUBATION PERIOD (hours)
0
10
20
30
0
2.5
5.0
7.5
10.0
EXTRACELLULAR MALTASE ACTIVITY
(units/ml)
5.5
6.0
6.5
pH

to require the action of autolytic enzymes because maximum enzyme levels were attained before appreciable cell lysis occurred.

Maltase preparations used in all of the subsequent experiments were produced by shaking culture in Fernbach flasks. A medium comprised of 2% methyl-α-D-glucoside, 0.5% phytone, and 0.2% yeast extract was used, and the fermentation beer was collected after shaking the culture (180 rpm) for 12 hours at 37 C. After the removal of cells by centrifugation, the crude maltase preparation was made by ammonium sulfate precipitation.

Purification of the Extracellular Maltase of B. subtilis P-11

The crude maltase preparation obtained from the above procedure contained at least three kinds of enzyme activity: maltase, amylase, and proteinase. When the enzyme preparation was incubated with maltose and soluble starch, separately, at various pH values, the two curves shown in Fig. 5 were obtained. Proteinase activity also could be detected in the preparation by the paper disc method described by Lewis et al. (1957). Therefore, subsequent purification procedures were designed to eliminate amylase and proteinase activities and other proteins from the maltase preparation.

Conditions under which assays for maltase activity should be run and information on enzyme stability were determined prior to the development of further purification procedures. Figs. 5 and 6 show the pH ranges of the crude preparation for optimum activity (Fig. 5) and relative stability (Fig. 6), respectively. The optimum pH for maltase activity in the crude preparation was 6.5 (Fig. 5).

Fig. 5. Effect of pH on maltase and amylase activities in the crude preparation obtained by ammonium sulfate precipitation.

Relative maltase activities, (•——•), were expressed on the basis of the activity at pH 6.5 as 100%. Amylase activities, (o——o), were expressed in blue value units, which are defined as $\frac{(D - D')}{D}$, where D and D' are the absorption (660 nm) of the iodine complex of the digest at zero time and at t minutes of hydrolysis.

RELATIVE MALTASE ACTIVITY(%)
100
50
0
0
3
5
7
9
pH
MALTASE
AMYLASE
PHOSPHATE
ACETATE
GLYCINE
.4
.3
.2
.1
0
AMYLASE ACTIVITY (blue value)

To determine the pH stability, the enzyme preparation was held at 40 C for 30 minutes in buffer of various pH values. Then the pH was neutralized to 6.5 and the residual activity was determined. The maltase activity was stable at pH 5.0 to 7.0 (Fig. 6); less than 10% of the activity was lost within this pH range. Temperature stability of the maltase was determined by incubating the enzyme preparation at various temperatures; the residual activity was determined at various time intervals. The data in Fig. 7 show that the maltase activity was stable at 25 C and fairly stable at 40 C. Only 20% of the activity was lost when the crude preparation was incubated at 45 C for 90 minutes; even greater losses occurred at higher temperatures.

The maltase activity was stable to freezing and frozen storage. Even repeated freezing and thawing did not result in loss of activity. The maltase activity was unstable, however, when stored at temperatures above freezing. In the refrigerator (6 C), for example, about 10% of the maltase activity was lost per day during storage for 3 or 4 days; after this time, the preparation seemed to stabilize and enzyme activity losses were slight. The reason for substantial losses of activity initially followed by relative stability during refrigerated storage is not known.

After the aforementioned conditions of handling the enzyme were determined, attempts at enzyme purification could proceed. Preliminary experiments led to the development of a five-step purification procedure (Fig. 3).

Fig. 6. pH stability of maltase activity in the crude preparation.

The crude preparation was held at 40 C for 30 minutes in buffers of various pH values; samples were adjusted to pH 6.5 and the residual activities were measured.

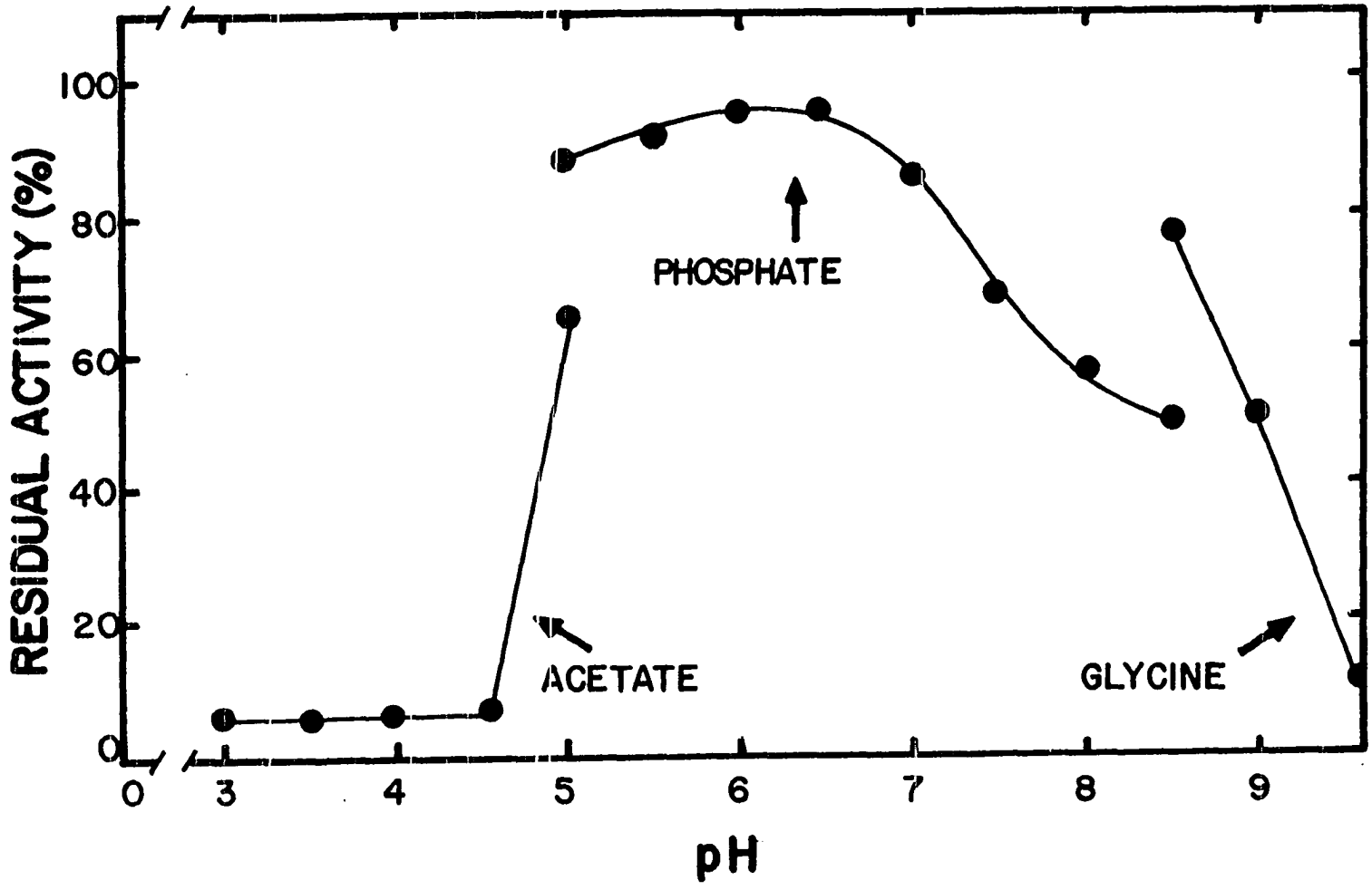
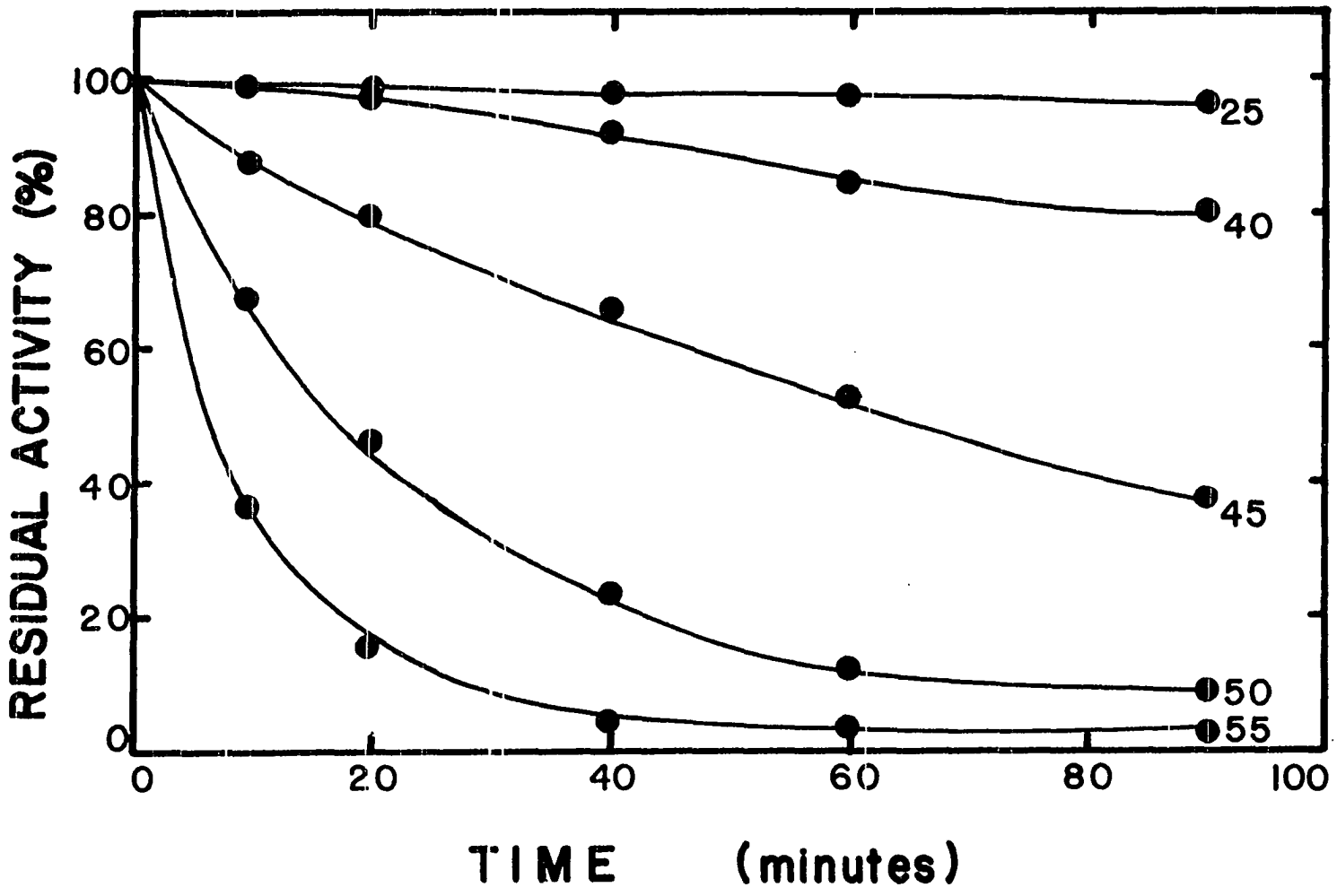

RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
0
3
4
5
6
7
8
9
pH
PHOSPHATE
ACETATE
GLYCINE

Fig. 7. Temperature stability of maltase activity in the crude preparation.

The crude preparation was diluted in phosphate buffer, pH 6.5, and held at temperatures of 25, 40, 45, 50, and 55 C. Residual activities were measured at various time intervals.


RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
0
20
40
60
80
100
TIME (minutes)
25
40
45
50
55

Step 1 was concerned with preparation of cell-free supernatants. After inoculation with seed culture, 2.8- Fernbach flasks, each containing 1 liter of medium composed of 2.0% methyl-α-D-glucoside, 0.5% phytone, and 0.2% yeast extract were shaker-cultured (180 rpm) at 37 C for 12 hours. The cells were removed from the fermentation beer by centrifugation and the cell-free supernatant was adjusted to pH 6.5 and stored in a cold room (0 to 2 C), ready for the next step.

Step 2 involved ammonium sulfate fractionation of the cell-free enzyme preparation. Substantial losses in activity were obtained when the enzyme was concentrated by acetone or ethanol precipitation, and considerable activity also was lost when enzyme concentration was affected by Diaflo ultrafiltration. Ammonium sulfate precipitation was the best method to obtain maltase concentrates. Table 6 shows a precipitation profile of extracellular maltase by ammonium sulfate. Most maltase activity was precipitated at between 60 and 85% saturation with ammonium sulfate.

For the production of larger quantities of maltase, solid ammonium sulfate was added to 11.5 liters of cell-free supernatant to attain 50% saturation; 7.5% of the protein and only 1% of the maltase activity were precipitated. After standing in the cold room at 0 to 2 C for about 4 hours, the precipitate was removed by centrifugation and discarded. More ammonium sulfate was added to the supernatant solution to bring it to 85% saturation. After standing overnight in the cold, the precipitate was collected in an SS-34 rotor by continuous

Table 6. Fractional precipitation of extracellular maltase of *B. subtilis* P-11 by ammonium sulfate[a].

% Saturation of ammonium sulfate	% Maltase activity precipitated	% Protein precipitated
20	0	0.5
30	0	0.5
40	0	6.7
50	1.3	7.5
60	3.6	24.9
70	40.0	30.9
80	70.0	40.8
85	81.0	62.8

[a]Solid ammonium sulfate was gradually added to 100 ml of cell-free supernatant containing 4 units/ml of maltase activity and 1.6 mg/ml protein. After the indicated % saturation had been attained, the preparation was allowed to stand for 4 hours at 0 to 2 C and then the precipitates were collected by centrifugation. Each precipitate was redissolved in M/15 phosphate buffer, pH 6.5, for the enzyme activity and protein determinations.

centrifugation at 15,000 rpm. The precipitate was dissolved in 131 ml of M/15 phosphate buffer, pH 6.5.

Step 3 involved passage through a Sephadex G-200 column. Sephadex G-200 was hydrated, packed in a 2.5 x 90 cm column, and equilibrated with 25 mM phosphate buffer, pH 6.5. For each chromatographic run, 10 ml of the concentrated crude enzyme solution obtained from Step 2 were applied to the column. Elution was carried out with the same buffer used for equilibration. The head pressure was maintained

constant; the surface of the buffer reservoir was 12 cm higher than the outlet of the column. Fractions of 5 ml each were collected. Fig. 8 shows the elution profile. Maltase activity was not retained by the column and appeared in the early fractions, associated with the void volume. Proteinase activity could be detected in fractions 35 to 70; therefore, the maltase was still contaminated with proteinase and probably other proteins. Amylase activity was well separated from the maltase, appearing in fractions 78 to 100.

DEAE-Sephadex A-50 column chromatography was used for Step 4 of the maltase purification process. DEAE-Sephadex A-50 was hydrated, packed in a 2.5 x 35 cm column, and equilibrated with 25 mM phosphate buffer, pH 6.5. The maltase-containing fractions (fractions 30 to 50), collected from Step 3, were applied to the column to be adsorbed by DEAE-Sephadex. The proteinase activity was washed out and appeared in the eluate early, before the NaCl gradient was applied. As shown in Fig. 9, the maltase activity was eluted at NaCl concentrations of 0.10 to 0.15 M. The maltase was now devoid of detectable amylase and proteinase activities. Probably, the second peak shown in Fig. 9 is inactive maltase.

Step 5 consisted of a second Sephadex G-200 column chromatograph. Fractions 45 to 65 of Step 4 were collected, concentrated by enzyme grade ammonium sulfate precipitation (85% saturation), and passed through a Sephadex G-200 column. There was a single maltase-containing peak, as shown in Fig. 10. The peak (Fig. 10) was shifted from the

Fig. 8. Elution pattern of the initial Sephadex G-200 chromatograph of the extracellular maltase of Bacillus subtilis P-11.

Column: 2.5 x 90 cm; flow rate: 10 to 15 ml/hour; void volume: 170 ml; fraction volume: 5 ml.

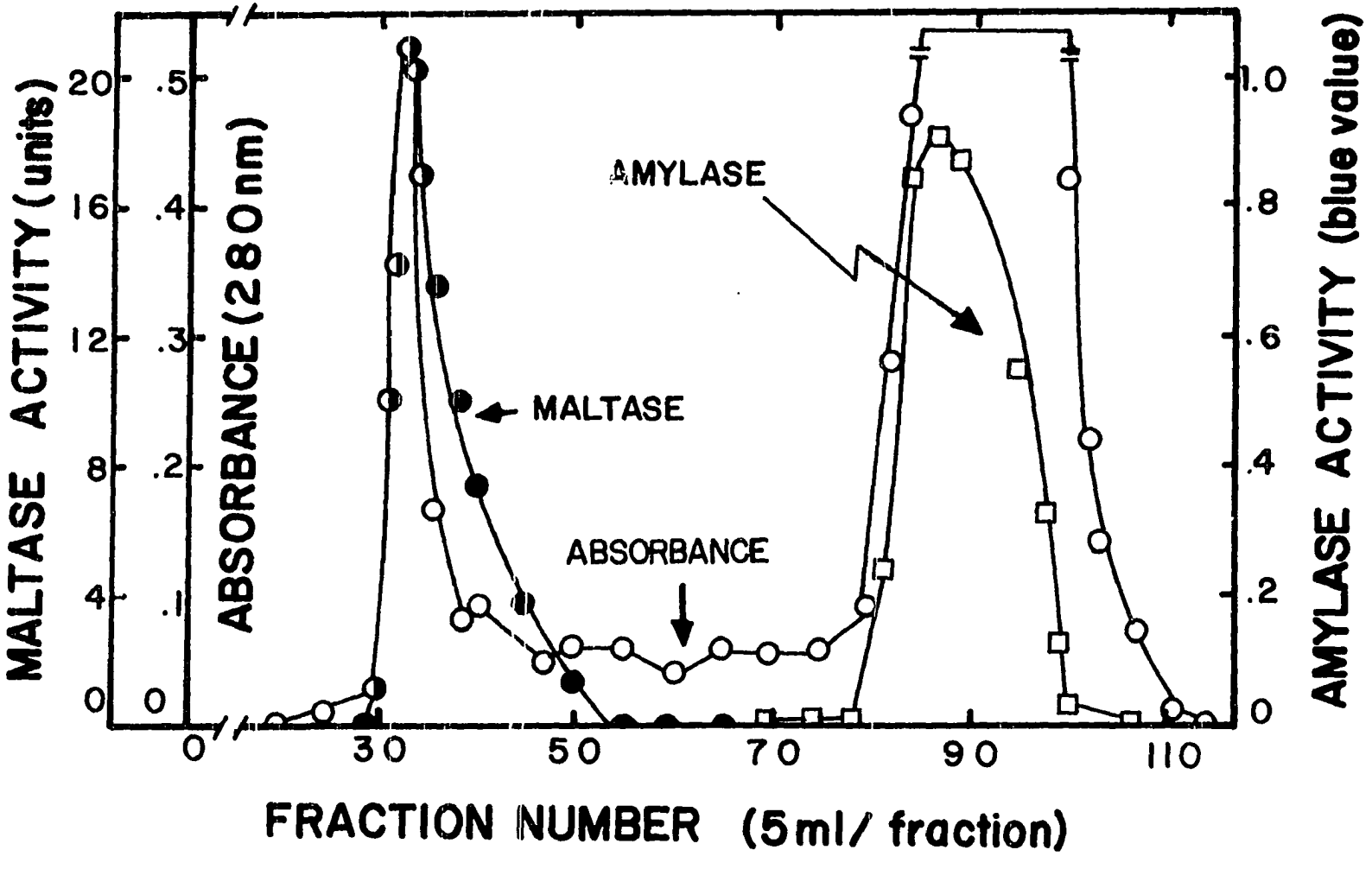

MALTASE ACTIVITY (units)
ABSORBANCE (280nm)
AMYLASE ACTIVITY (blue value)
AMYLASE
MALTASE
ABSORBANCE
FRACTION NUMBER (5ml/ fraction)
0
4
8
12
16
20
.1
.2
.3
.4
.5
.2
.4
.6
.8
1.0
30
50
70
90
110

Fig. 9. Elution pattern from a DEAE-Sephadex A-50 ion-exchange column of the extracellular maltase of Bacillus subtilis P-11.

Column: 2.5 x 35 cm; flow rate: 15 to 20 ml/hour; fraction volume: 5 ml.

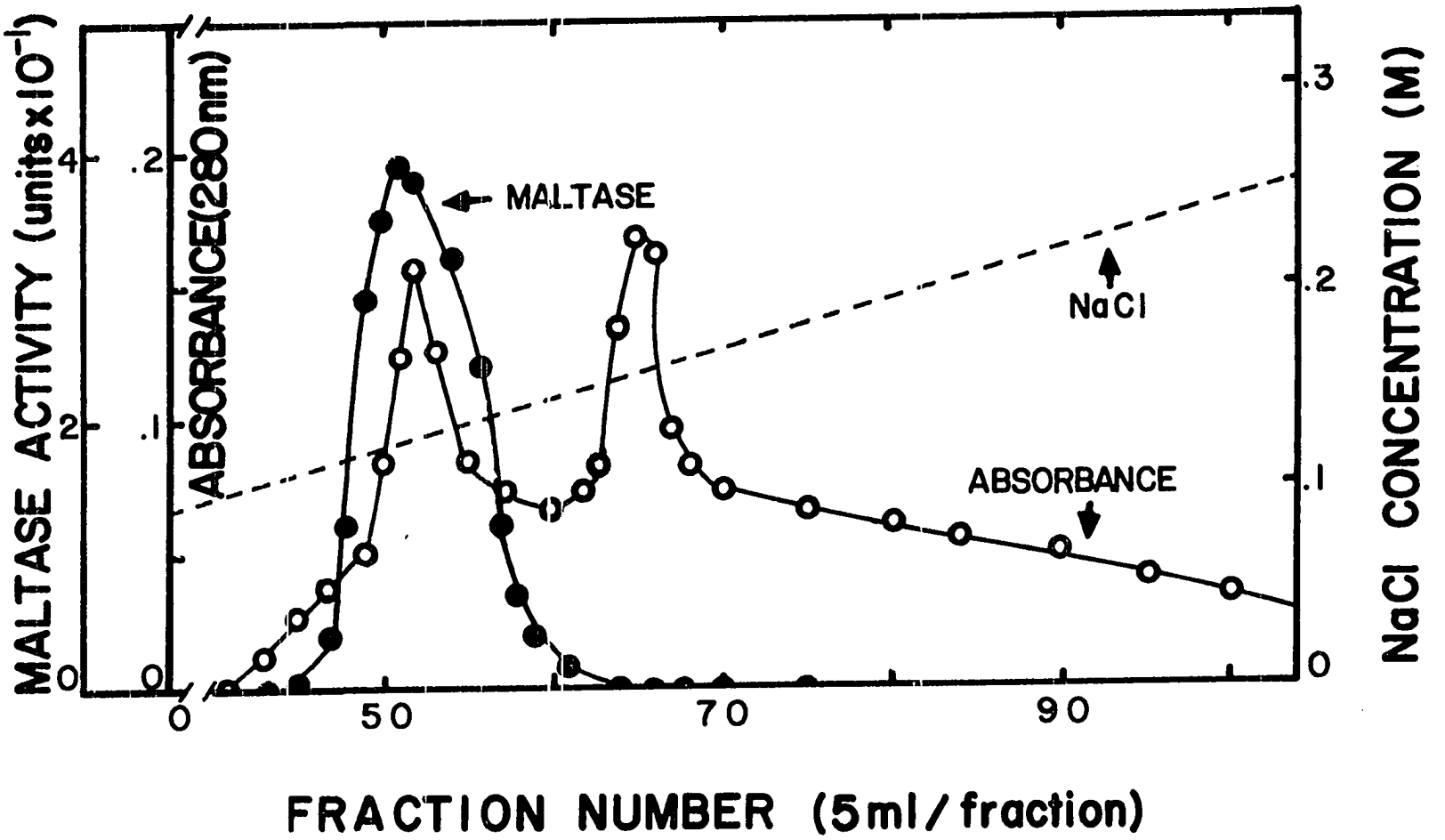
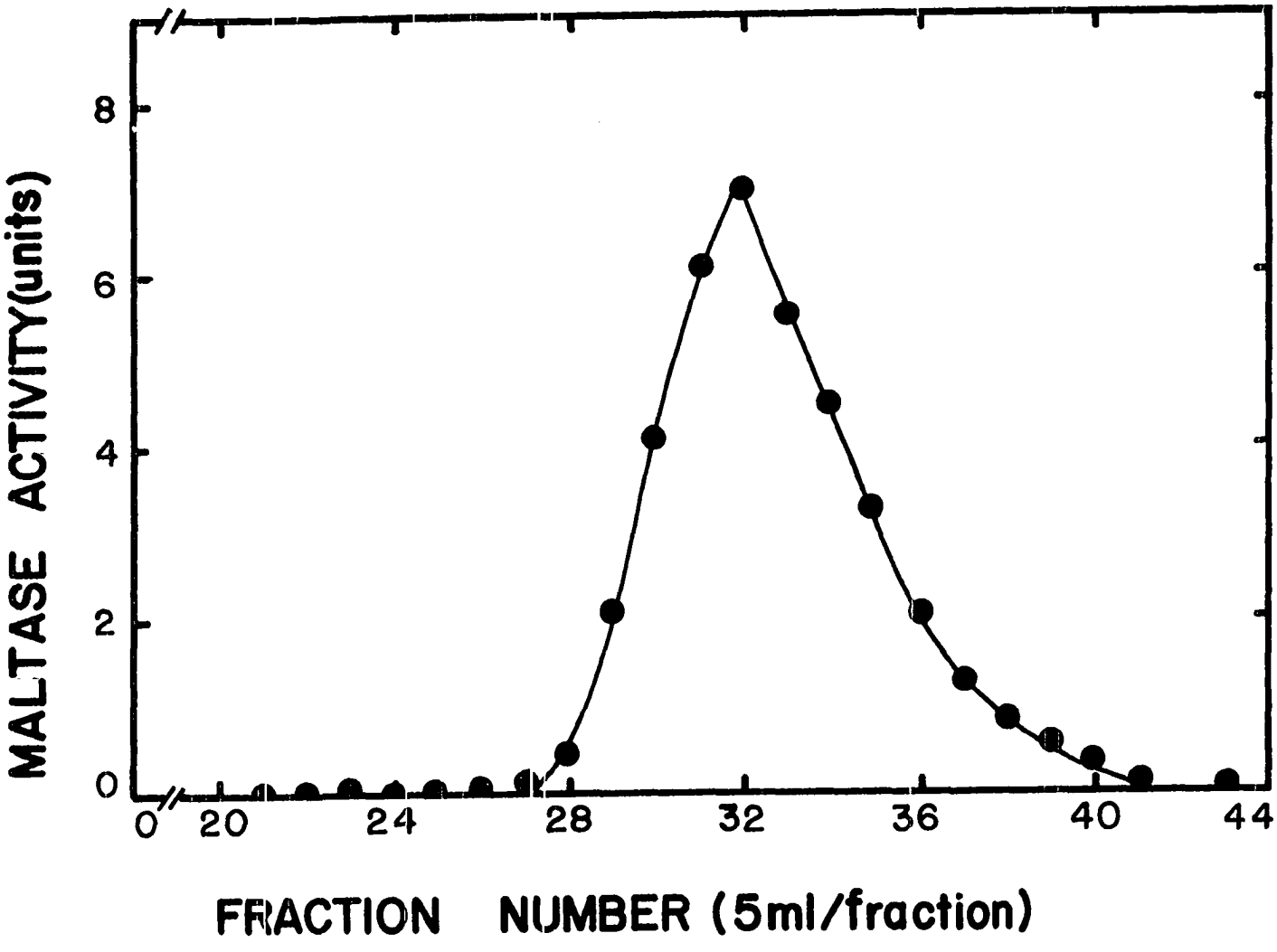

MALTASE ACTIVITY (units x 10⁻¹)
ABSORBANCE(280nm)
NaCl CONCENTRATION (M)
MALTASE
NaCl
ABSORBANCE
0
2
4
.1
.2
.3
50
70
90
FRACTION NUMBER (5ml/fraction)

Fig. 10. Elution pattern of the second Sephadex G-200 column chromatograph of the extracellular maltase of Bacillus subtilis P-11.

Column: 2.5 x 40 cm; flow rate: 10 to 15 ml/hour; void volume: 75 ml; fraction volume: 5 ml.


MALTASE ACTIVITY(units)
0
2
4
6
8
0
20
24
28
32
36
40
44
FRACTION NUMBER (5ml/fraction)

void volume to near the position originally occupied by amylase in the first Sephadex G-200 fractionation (Fig. 8). I will enlarge upon this observation in the DISCUSSION.

Table 7 shows the specific activities and other parameters at each stage of purification. As indicated in the table, the final preparation was 942 times purer than the original fermentation beer. The yield, however, was less than 20%.

Characterization of the Extracellular Maltase of B. subtilis P-11

As shown in Fig. 11, the pH optimum for maltase activity was 6.0; this was one half unit lower than that of the crude preparation (Fig. 5). As shown both in Figs. 5 and 6, substantial activity was retained at pH values between 5.0 and 7.0.

The temperature optimum for the extracellular maltase, as shown in Fig. 12, was about 45 C when the incubation time was 30 minutes. At temperatures much above 45 C, the enzyme activity decreased sharply.

The pH stability of the extracellular maltase was determined by a procedure identical to that used for the crude preparation, except that the incubation temperature was changed to 45 C. The extracellular maltase of B. subtilis P-11, as shown in Fig. 13, was stable at pH 6.0 to 6.3; this was a narrower pH-stability range than was observed for the crude preparation (Fig. 6). Only 10% of the maltase activity was lost after incubation for 30 minutes at 45 C and pH 6.0 (Fig. 13). Residual enzyme activities decreased sharply as the pH

Table 7. Parameters observed during the purification of the extracellular maltase of *B. subtilis* P-11.

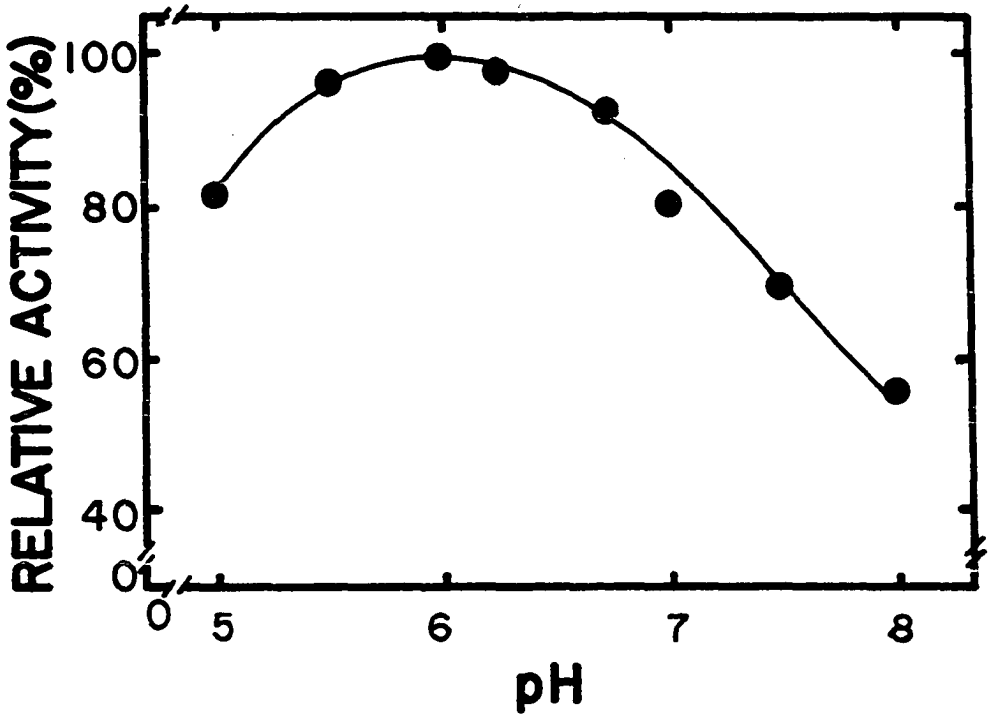
Fraction number in Fig. 3	Volume (ml)	Maltase activity (units/ml)	Total units ($x10^3$)	Protein (mg/ml)	Specific activity (units/mg)	Yield (%)	Purification
I	11,500	3.8	43.2	2.1	1.7	100	1
II	13,000	3.3	42.6	1.8	1.9	98.6	1.1
III	131	233.3	30.6	9.0	25.2	70.6	15.2
IV	710	25.1	17.8	0.3	76.1	41.2	44.5
V	110	76.4	8.4	0.1	694.5	19.5	406.1
VI	103	80.6	8.3	0.05	1,611.2	19.2	942.2

Fig. 11. pH-activity profile of the purified extracellular maltase of Bacillus subtilis P-11.

The enzyme activity at pH 6.0 was taken as 100%.

Fig. 12. Temperature-activity profile of the purified extracellular maltase of Bacillus subtilis P-11.

The enzyme activity at 45 C was taken as 100%.


RELATIVE ACTIVITY(%)
100
80
60
40
0
0
5
6
7
8
pH

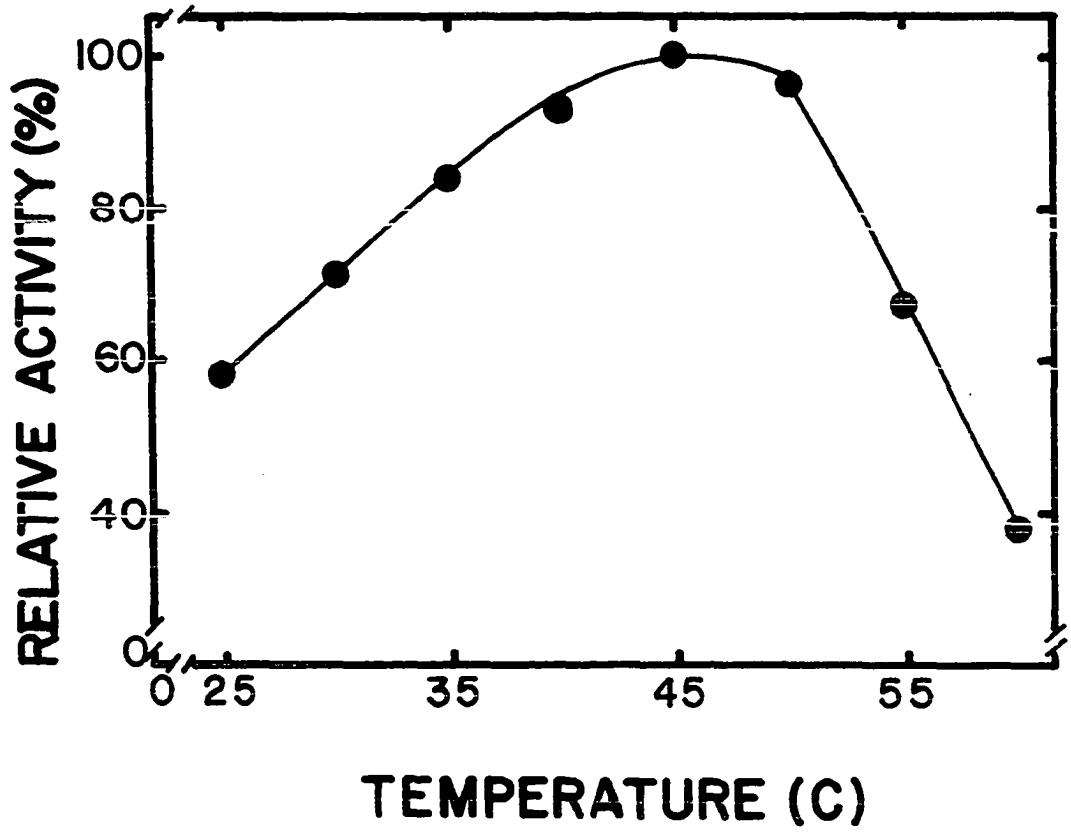
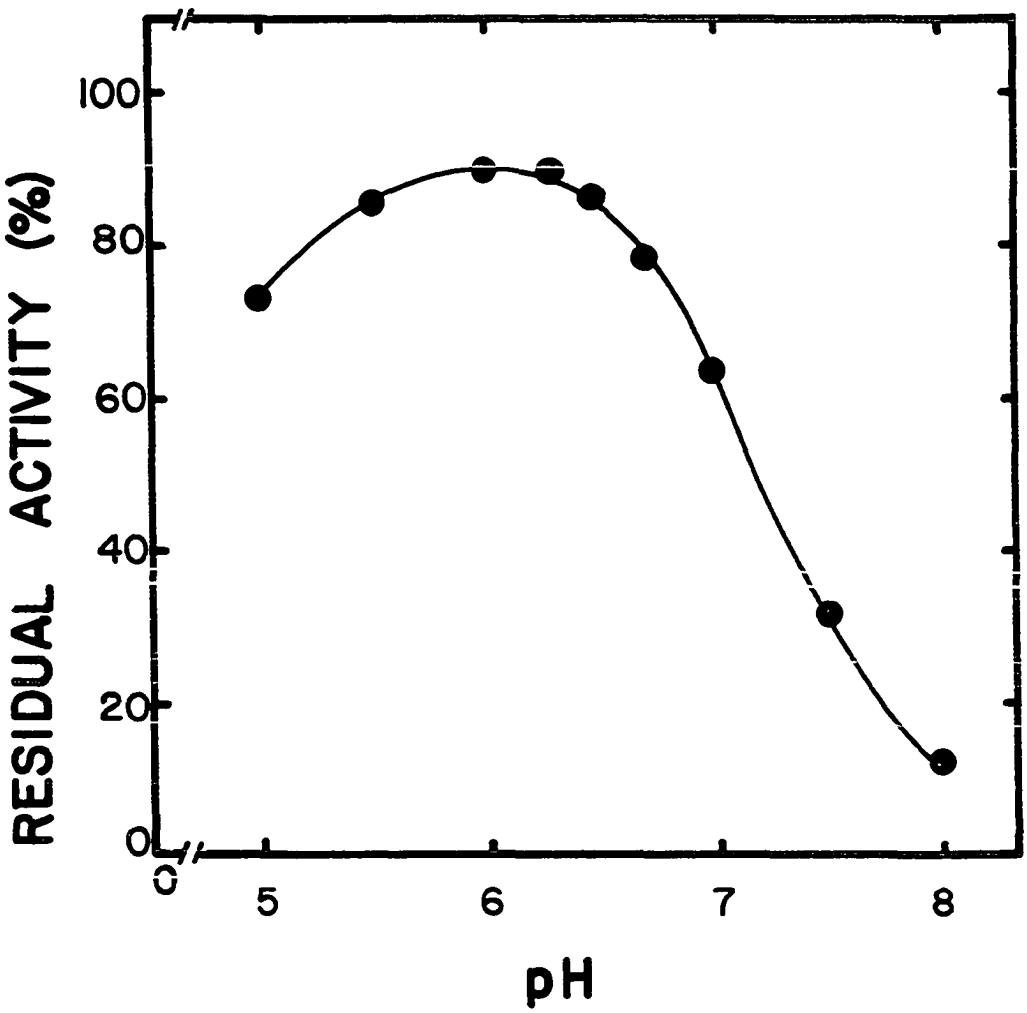

RELATIVE ACTIVITY (%)
100
80
60
40
0
0
25
35
45
55
TEMPERATURE (C)

Fig. 13. pH stability of the extracellular maltase of Bacillus subtilis P-11.

The maltase was held at 45 C for 30 min. in buffers of various pH values and then was neutralized to pH 6.0 for measurement of residual activity.


RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
0
5
6
7
8
pH

values increased from 6.3 to 8.0; therefore, the enzyme was unstable at alkaline pH values.

As shown in Fig. 14, the extracellular maltase was quite stable to high temperatures; it was more stable than the crude preparation (Fig. 7). About 20% of the activity of the purified maltase was lost when the enzyme was held at 45 C for 2 hours (Fig. 14). At 50 C, almost half of the activity was lost after 2 hours of incubation, while more than 90% of the activity in the crude preparation was lost under the same conditions (Fig. 7).

To determine whether the phosphate buffer that I had used was the most appropriate, a comparison was made between six kinds of buffers. The buffers examined were citric acid-sodium citrate, McIlvaine's, NaH_2PO_4-Na_2HPO_4, Maleic-NaOH, HCl-sodium arsenate, and KH_2PO_4-NaOH (control). A constant quantity of purified maltase was diluted in each of the buffers and residual activity was measured. There were only slight differences in enzyme activity between the buffers used to dilute the maltase. Neither the type nor concentration of buffer, up to 0.5 M, affected the enzyme activity.

The molecular weight of the extracellular maltase of B. subtilis P-11 was estimated by Sephadex G-200 gel-filtration, according to Andrew (1965). Aldolase (M.W. 158,000), ovalbumin (M.W. 45,000), and chymotrypsinogen A (M.W. 25,000) were used as the standard molecular weight markers. The results are shown in Fig. 15. The molecular weight of the enzyme was estimated to be 33,000.

Fig. 14. Temperature stability of the extracellular maltase of Bacillus subtilis P-11.

The maltase was held at various temperatures in phosphate buffer, pH 6.0. Residual activities were measured at various time intervals.

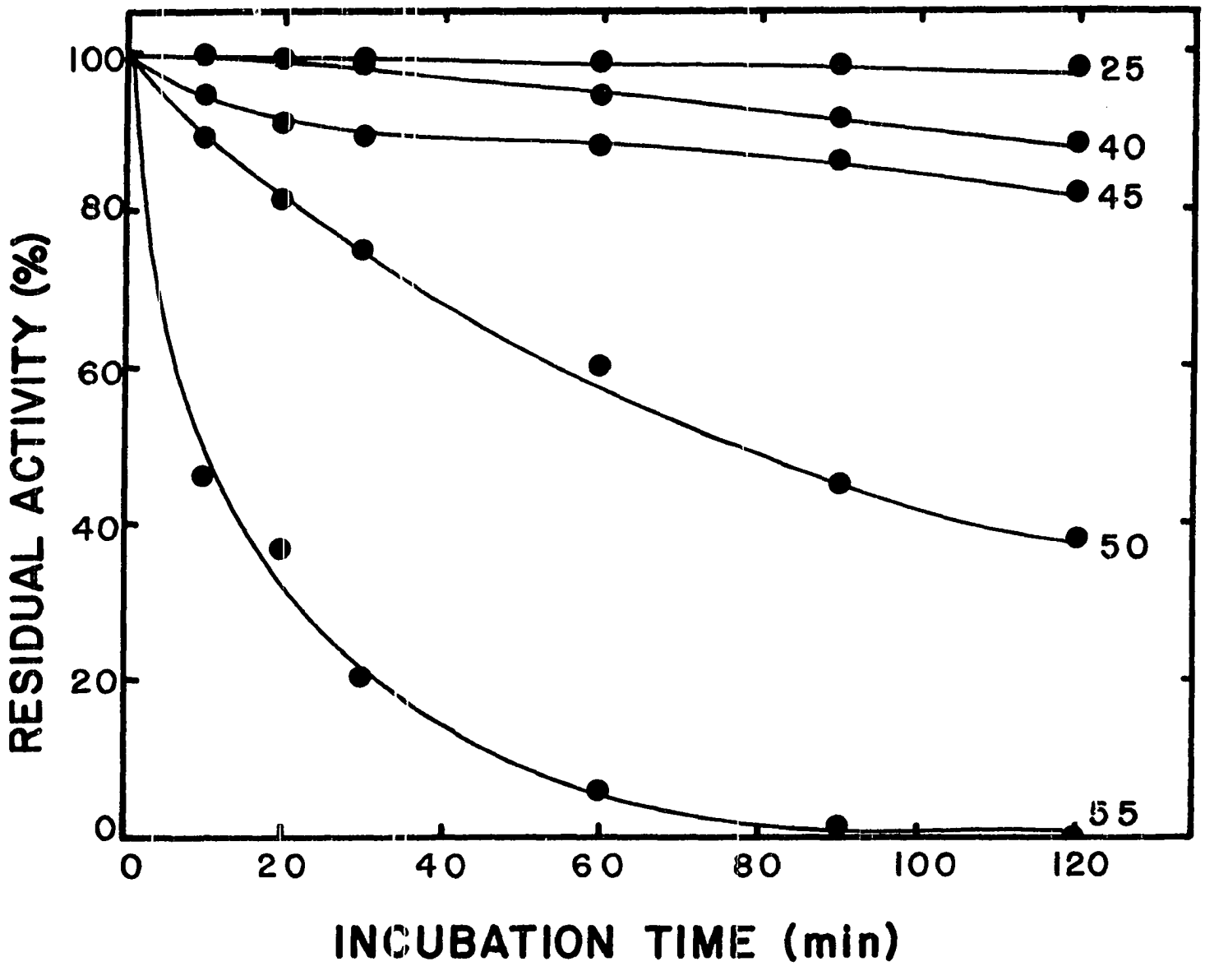
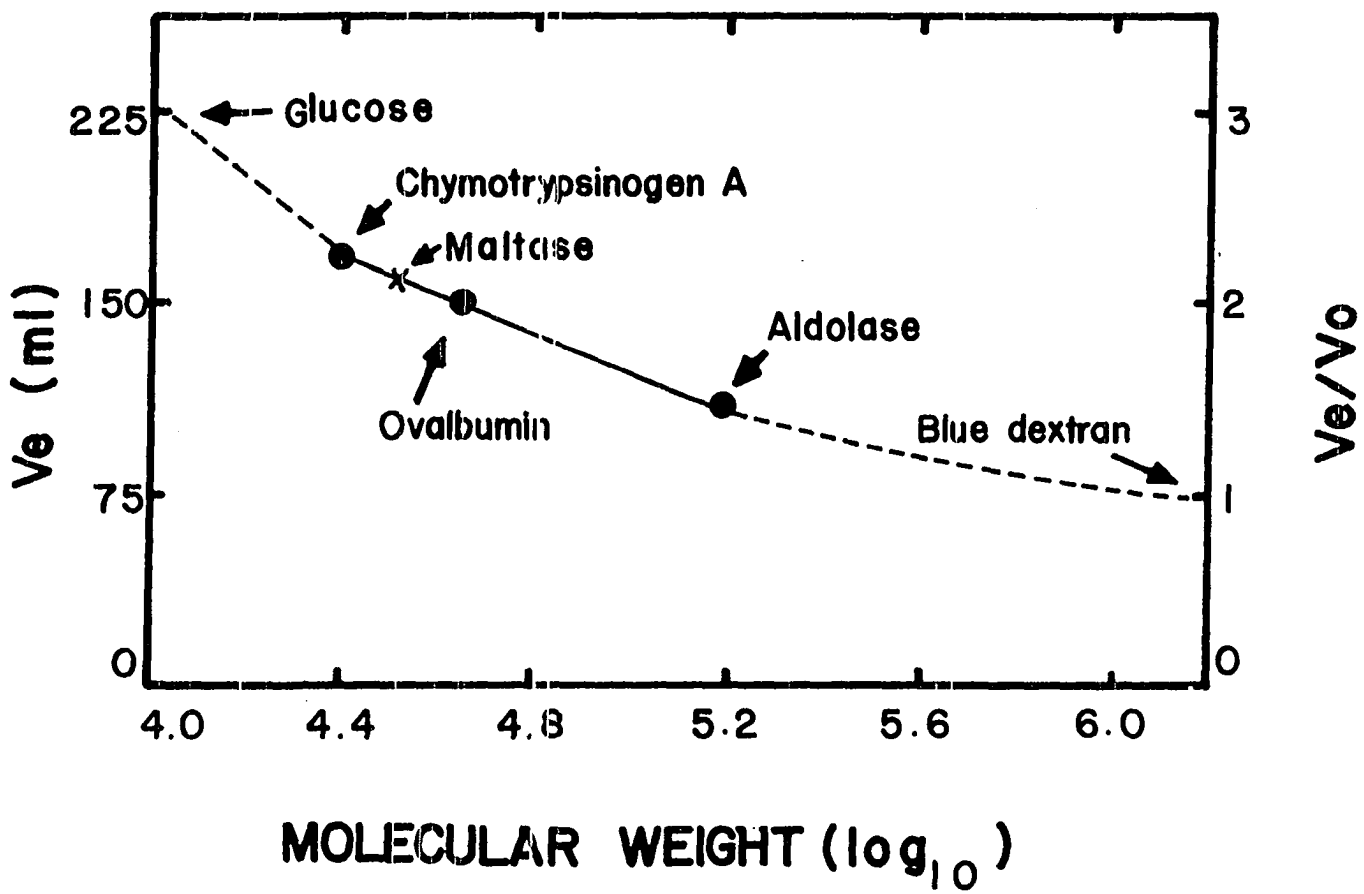

RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
INCUBATION TIME (min)
25
40
45
50
55

Fig. 15. Molecular weight estimation of the extracellular maltase of Bacillus subtilis P-11 by using the Sephadex G-200 gel-filtration method of Andrew, 1965.

Conditions: column: 2.5 x 40 cm; flow rate: 10 to 15 ml/hour; fraction volume: 5 ml.

Molecular weights of the standard proteins: aldolase 158,000; ovalbumin 45,000; chymotrypsinogen A 25,000.

V_e: elution volume (ml), V_o: void volume (measured with blue dextran 2000) 75 ml.


Glucose
Chymotrypsinogen A
Maltase
Aldolase
Ovalbumin
Blue dextran
225
150
75
0
Ve (ml)
3
2
1
0
Ve/Vo
4.0
4.4
4.8
5.2
5.6
6.0
MOLECULAR WEIGHT (log_{10})

The isoelectric point of the extracellular maltase of B. subtilis P-11 was measured by a gel-isoelectric focusing technique. Focusing gel was prepared according to Ortec Application Note AN 32A (Techniques for high resolution electrophoresis). A 5% focusing gel was used. A sample of maltase (1 ml, containing 250 μg of protein) was mixed with the focusing gel (making a total volume of 4 ml) rather than in the cap gel before polymerization. Ampholines (LKB Produktor AB, Broma, Sweden) with a pH range 3.5 to 10.0 were used. The isoelectric point of the enzyme was about pH 6.0.

The absorption spectrum of the extracellular maltase of B. subtilis P-11 was taken by using a Beckman Model DB spectrophotometer with a recorder (Fig. 16). The enzyme exhibited maximum absorption at 275 nm, and the ratio of absorption at 280/260 was 1.39. The extinction coefficient was $A^{1\%}_{1\ cm} = 46.0$.

Table 8 shows the substrate specificity of the maltase. Maltose was the best substrate for the enzyme. The rate of hydrolysis decreased as the degree of polymerization of the substrate increased. Maltotriose was attacked at about one-fifth the rate of maltose, and maltotetraose at one-fourth the rate of maltotriose. No maltopentaose was available for use. The enzyme could not hydrolyze polysaccharides bearing α-1,4-glucosyl bonds, such as amylose, amylopectin, and glycogen. Although there was a trace of activity towards soluble starch, this was probably because the starch was contaminated with smaller oligosaccharides.

Fig. 16. Absorption spectrum of the extracellular maltase of Bacillus subtilis P-11.

A Beckman Model DB spectrophotometer with recorder was used. The enzyme concentration was 250 µg/ml in 0.3 M NaCl-0.05 M phosphate buffer, pH 5.93. The maximum absorption wavelength was 275 nm and the extinction coefficient $A^{1\%}_{1cm}$ = 46.0 at 280 nm. The ratio of absorption at 280/260 was 1.39.

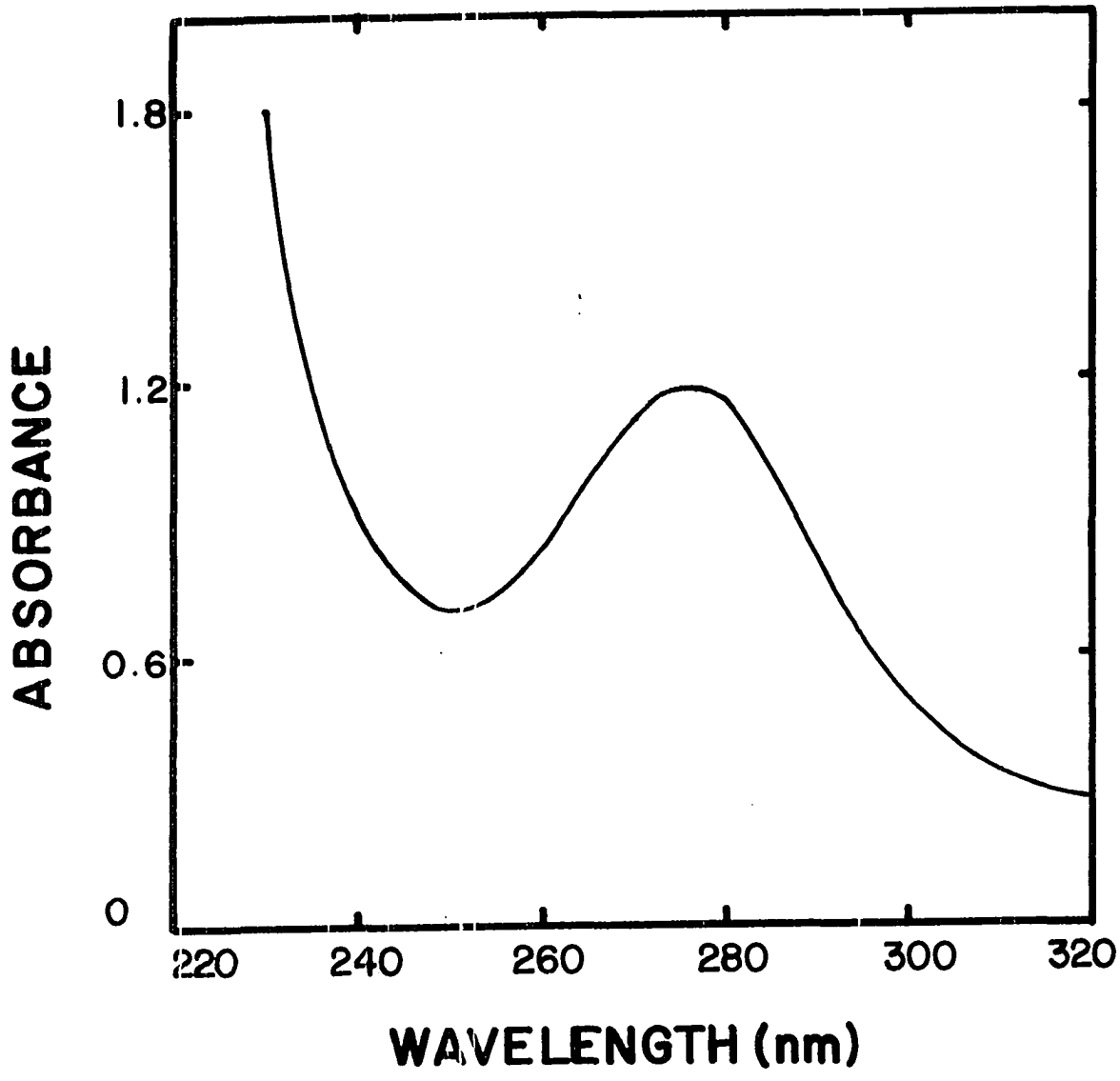

ABSORBANCE
1.8
1.2
0.6
0
220
240
260
280
300
320
WAVELENGTH (nm)

Table 8. Substrate specificity of the extracellular maltase from *B. subtilis* P-11.

Substrate[a]	Relative rate of hydrolysis (%)
Maltose	100
Maltotriose	16
Isomaltose	15
Maltotetraose	4
Soluble starch[b]	4
Trehalose	0
Cellobiose	0
Turanose	0
Sucrose	0
Melibiose	0
Salicin	0
Melezitose	0
Lactose	0
Methyl-α-D-glucoside	0
Raffinose	0
Methyl-α-D-galactoside	0
Methyl-α-D-mannoside	0
Phenyl-α-D-glucoside	0
Maltitol	0
p-nitro-phenyl-glucoside	0
Amylose[b]	0
Amylopectin[b]	0
Glycogen (rabbit liver)[b]	0

[a]Concentration: 3 mM; see footnote b.

[b]Concentration: 1 mg/ml. Amylose was prepared according to Robyt and French (1967).

If the glucose unit in the aglucon moiety of the maltose molecule was replaced with a methyl, phenyl, or p-nitro-phenyl group, or glucitol as in maltitol, the resulting saccharides no longer served as substrates for the extracellular maltase of B. subtilis P-11 (Table 8).

The enzyme possessed α-1,6-glucosidase activity because isomaltose was hydrolyzed (Table 8). The enzyme did not hydrolyze trehalose (αα-1,1-glucosidic linkage), cellobiose (β-1,4-glucosidic linkage), salicin (β-glucoside), turanose (α-1,3-glucosylfructoside), sucrose (α-1,2-glucosylfructoside), melibiose (6 [-D-galactoside] -D-glucose), melizitose (glucopyranosyl-glucopyranosyl-fructofuranose), methyl-D-mannose, methyl-D-galactoside, lactose (4-o-β-D-galactopyranosyl-D-glucopyranose), or raffinose (galactopyranosyl-glucopyranosyl-fructofuranose).

To examine the effects of various cations on maltase activity, two levels of each of 14 different compounds were added to maltase preparations in citrate buffer. Residual activity was measured (Table 9). The addition of $MnSO_4$ resulted in increased enzyme activity to the extent of about 50% at a concentration of 25 mM. Other compounds such as $CaCl_2$, $MgSO_4$, $ZnSO_4$, $LiSO_4$, $CoCl_2$, NaCl, and Pb-acetate resulted in slight (probably not significant) increases in enzyme activity. $CuSO_4$ increased the enzyme activity somewhat when at a low concentration, 1 mM, but this stimulation disappeared when the $CuSO_4$ concentration was increased to 10 mM. $HgCl_2$, $AgNO_3$, and $FeSO_4$ were

Table 9. The effects of various cations on the extracellular maltase of *B. subtilis* P-11[a].

Metallic ions	Concentration (mM)	Relative residual activity (%)
$MnSO_4$	5	134
	25	151
$ZnSO_4$	1	113
	10	113
$CaCl_2$ $2H_2O$	5	110
	25	113
$MgSO_4$	5	110
	25	110
NaCl	5	103
	25	106
$CoCl_2$	1	109
	10	115
Pb-acetate	1	103
	10	106
$LiSO_4$ H_2O	1	111
	10	104
$CuSO_4$ $5H_2O$	1	118
	10	98
Cd-acetate	1	94
	10	92
$Ba(OH)_2$	1	92
	10	90
$FeSO_4$	1	76
	10	35
$AgNO_3$	1	12
	10	0
$HgCl_2$	1	9
	10	0
None		100

[a]M/15 citrate buffer was used in this experiment.

strong inhibitors of the maltase; the enzyme was probably inactivated. $Ba(OH)_2$ and Cd-acetate were weak inhibitors.

The effects of certain organic compounds on maltase activity also were measured (Table 10). The compounds were preincubated with enzyme for 10 minutes at room temperature before the residual activities were determined. Table 10 shows that the reduced form of glutathione, mercaptoethanol, and dithiothreitol were very strong inhibitors of the maltase. The amino acid, cysteine, could inhibit the enzyme activity noncompetitively, as is shown later in Fig. 18a. Tris and glucose showed inhibition, and they were examined in more detail: the inhibition was probably competitive, as is shown in Fig. 18a. From the Lineweaver-Burk plot, the inhibitor constant, K_i, can be determined according to the equation,

$$K_i = \frac{i}{\frac{K_p}{K_m} - 1}$$

where i is the concentration of the inhibitor, K_m is the Michaelis constant without inhibitor, and K_p is the Michaelis constant in the presence of the inhibitor. The K_i values for glucose and Tris were 4.38 and 75.08 mM, respectively (Dixon and Webb, 1964). Mannose and galactose, which are glucose epimers with respect to carbon atoms 2 and 4, respectively, did not show any inhibition. Xylose, structurally similar to glucose except for carbon atom 5, did not inhibit the enzyme either. The disaccharides, turanose and sucrose, and alkyl- or aryl-D-glucoside, did not affect the enzyme activity,

Table 10. The effects of various organic compounds on the extracellular maltase of B. subtilis P-11.

Effector[a]	Concentration (mM)	Relative residual activity (%)
Mannose	1	100
	5	100
Galactose	1	100
	5	100
Ribose	1	100
	5	100
Xylose	1	100
	5	100
Sucrose	0.5	100
	1	100
Fructose	0.5	100
	1	100
Turanose	0.5	100
	1	100
Maltitol	0.5	100
	1	100
Phenyl-α-D-glucoside	0.5	100
	1	100
Inositol	0.5	103
	1	107
Sorbitol	0.5	102
	1	103
Erythritol	0.5	100
	1	100
Glucosamine-HCl	0.5	107
	1	111

[a]The effector was preincubated with enzyme for 10 minutes at room temperature, before enzyme assays were performed.

Table 10. (continued)

Effector	Concentration (mM)	Relative residual activity (%)
EDTA	5	103
	25	100
Histidine	5	103
	25	103
Methionine	5	107
	25	103
Δ-gluconolactone	0.5	100
	1	100
	10	86
4-chloromercuri-benzoic acid	0.5	100
	1	95
Diphenylamine	0.5	92
	1	89
Cysteine	1	99
	10	32
Tris	5	94
	25	76
Glucose	0.5	60
	1	52
Dithionthreitol	0.5	60
	1	0
Mercaptoethanol	0.25	17
	1	0
Glutathione(reduced form)	0.25	4
	1	0
Rose bengal	0.5 (mg/ml)	0
	1 (mg/ml)	0
None		100

although turanose is an inhibitor of the α-glucosidases from Bacillus cereus (Yamasaki and Suzuki, 1974) and other sources. The chelating agent, EDTA, had no effect on the maltase activity; this indicated that either the enzyme does not require a metal ion for its activity or the metal is tightly bound. Polyols, such as maltitol, sorbitol, inositol, and erythritol also showed no effect. A variety of other compounds also had no effect on the maltase activity.

The purified B. subtilis P-11 maltase possessed transglucosylase activity. To demonstrate this, 9 ml of substrate (50 mg) and 1 ml of enzyme solution were incubated at 45 C. At various time intervals, 25 µl samples of the hydrolyzates were applied to Whatman No. 3 filter paper. The chromatograms were developed three times by the ascending method (Yamasaki and Suzuki, 1974) with n-butanol:pyridine:water (6:4:3 by volume). After drying, the chromatograms were developed by the silver nitrate dip method (Robyt and French, 1963). Hydrolyzates of maltose, isomaltose, and maltotetraose are shown in Fig. 17a, b, and c, respectively. Maltose was hydrolyzed to produce glucose rapidly (Fig. 17a). In addition, transglucosylation products (trisaccharide, tetrasaccharide, etc.) were produced within one hour of incubation and their concentrations increased as the incubation time increased. For isomaltose (Fig. 17b), the products (glucose, maltose, tetrasaccharide, and pentasaccharide) increased as the incubation time was prolonged. When the maltase was incubated with maltotetraose (Fig. 17c), glucose production increased as the incubation time was extended. Very faint spots corresponding to

Fig. 17. Chromatographic analyses of action on (a) maltose, (b) isomaltose and (c) maltotetraose by the extracellular maltase of Bacillus subtilis P-11.

Each reaction mixture (10 ml) contained 9 ml (50 mg) of substrate and 1 ml of enzyme solution. After incubation at 45 C for various time intervals, 25 µl samples of the hydrolyzate were applied to Whatman No. 3 filter paper. The paper was developed three times by the ascending method with n-butanol: pyridine:water (6:4:3 by volume). After drying, the chromatogram was developed by the silver nitrate dip method.

Symbols: S: standard (mixture of oligosaccharides); IG_2: isomaltose; G_x: oligosaccharides with x glucose units; and G_x^y: hydrolyzate of G_x after y hours digestion.

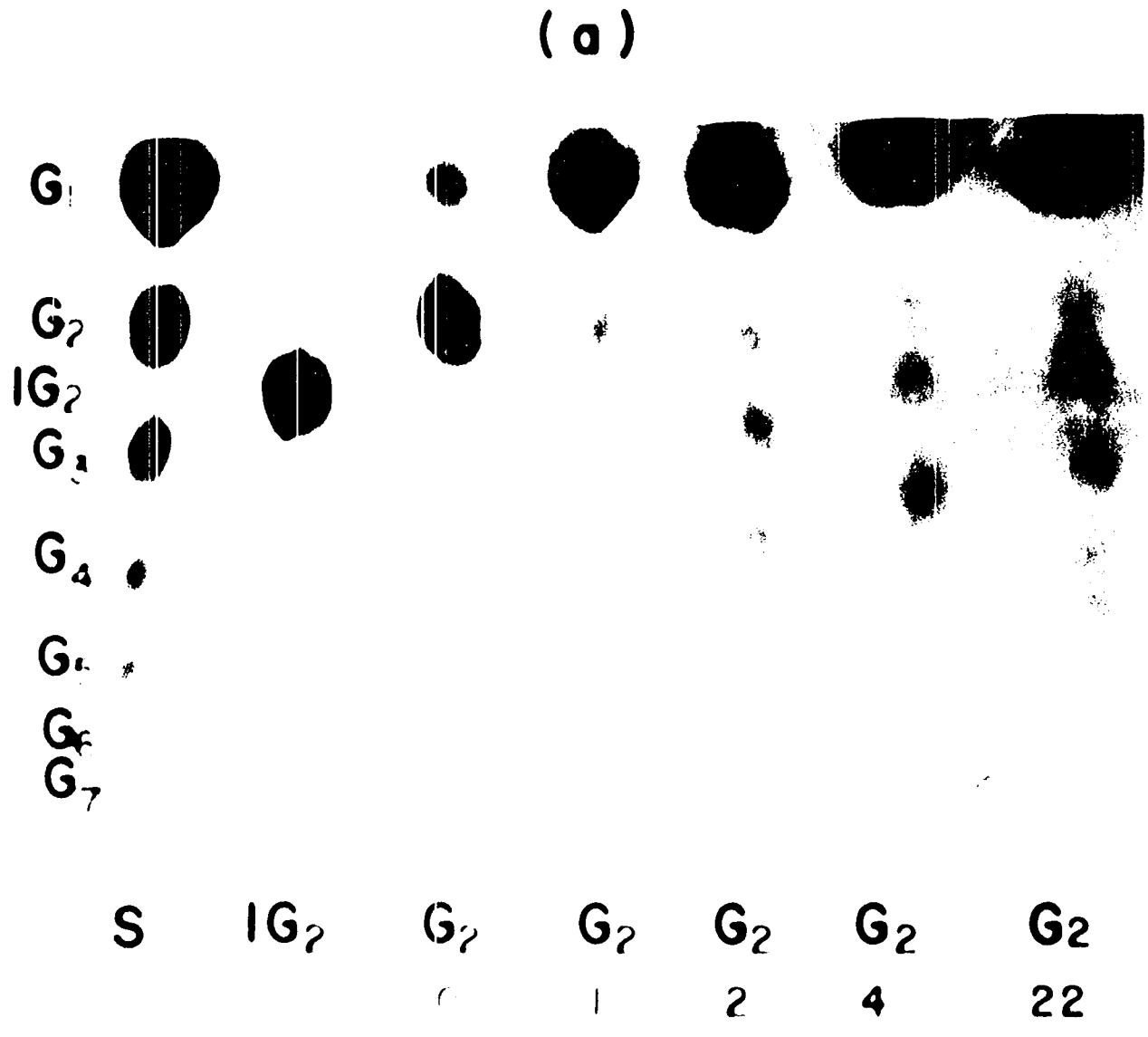

(a)
G1
G2
IG2
G3
G4
G5
G6
G7
S
IG2
G2
G2
G2
G2
G2
0
1
2
4
22

Fig. 17 (Continued).

(b)

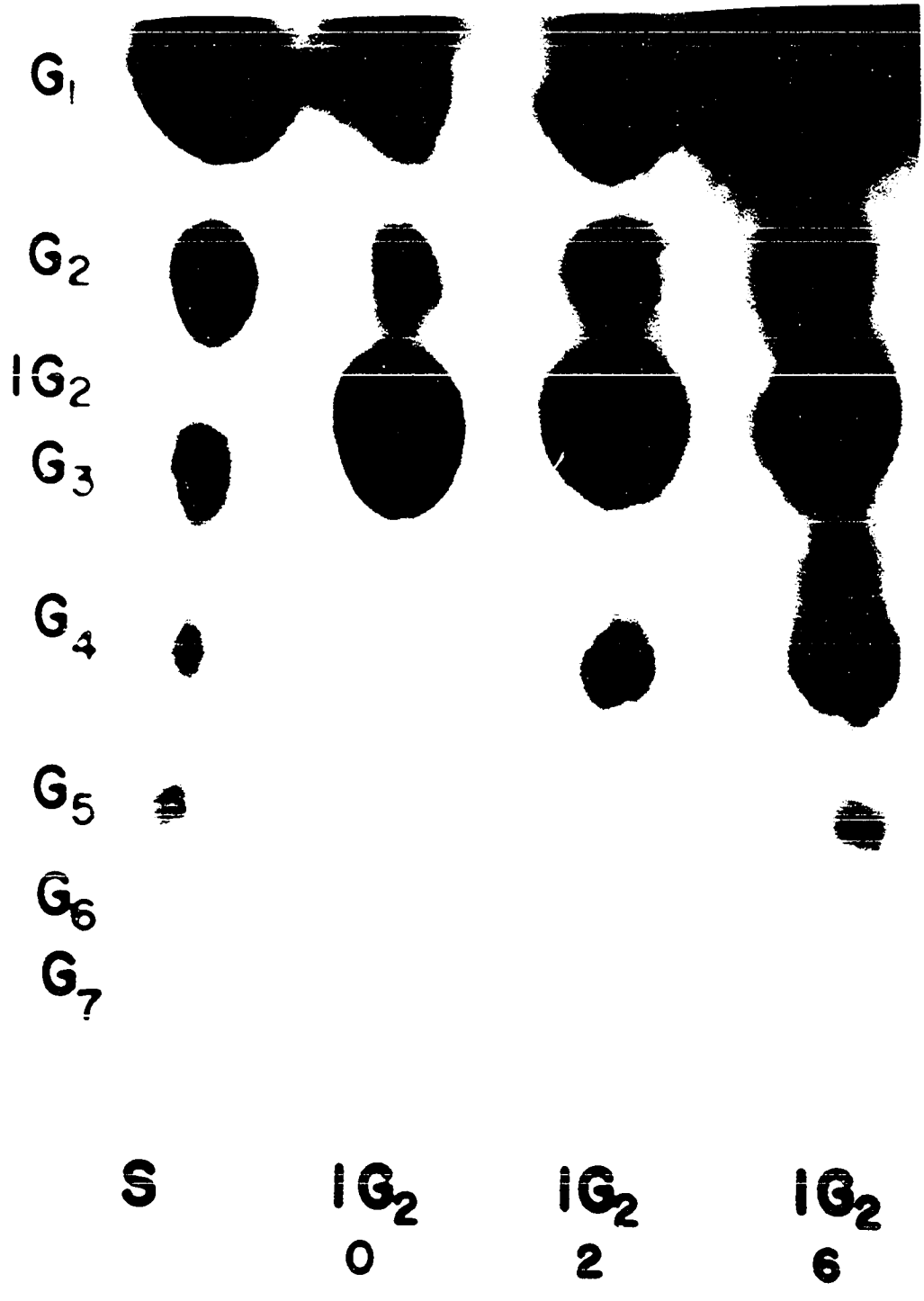


Fig. 17 (Continued).

(c)
S
G4 —
G4 2
G4 4
G4 22

maltose and maltotriose also could be seen on the chromatogram but did not show up in the photograph. Maltotriose also was examined as a substrate for the maltase (data not shown); maltose was trans-glucosylated to form maltotetraose, maltopentaose, and higher oligosaccharides.

Fig. 18 shows Lineweaver-Burk plots of the extracellular maltase on: (a) maltose, (b) maltotriose, and (c) isomaltose. The K_m values were 5 mM for maltose, 1 mM for maltotriose, and 10 mM for isomaltose.

Fig. 19 shows the effects of pH on K_m, V_{max}, and v_o of the maltase when maltose was used as the substrate. Within the pH range of 5.5 to 6.5, the K_m and V_{max} values, respectively, were almost the same. Outside this range, the K_m values increased while the V_{max} values decreased.

The rates of hydrolysis of maltose, maltotriose, maltotetraose, and isomaltose by the extracellular maltase were determined. Substrates in 3 mM concentration were incubated with the maltase at 40 C and the percent hydrolysis of the substrates was measured at various time intervals as shown in Fig. 20. After 24 hours of incubation, half of the initial quantity of maltose was hydrolyzed, while only 22%, 13%, and 10.5% of the initial quantities of maltotriose, maltotetraose, and isomaltose, respectively, were hydrolyzed.

Fig. 18. Lineweaver-Burk plots of the extracellular maltase on (a) maltose, (b) maltotriose, and (c) isomaltose.

Incubation was for 30 minutes at 45 C in M/15 phosphate buffer, pH 6.0. The K_m values were 5 mM for maltose (G_2), 1 mM for maltotriose (G_3), and 10 mM for isomaltose (IG_2). Fig. 18a also includes data showing competitive inhibition by glucose (G_1, 0.5 mM) and Tris buffer (Tris, 50 mM), and noncompetitive inhibition by cysteine (Cys., 3 mM).

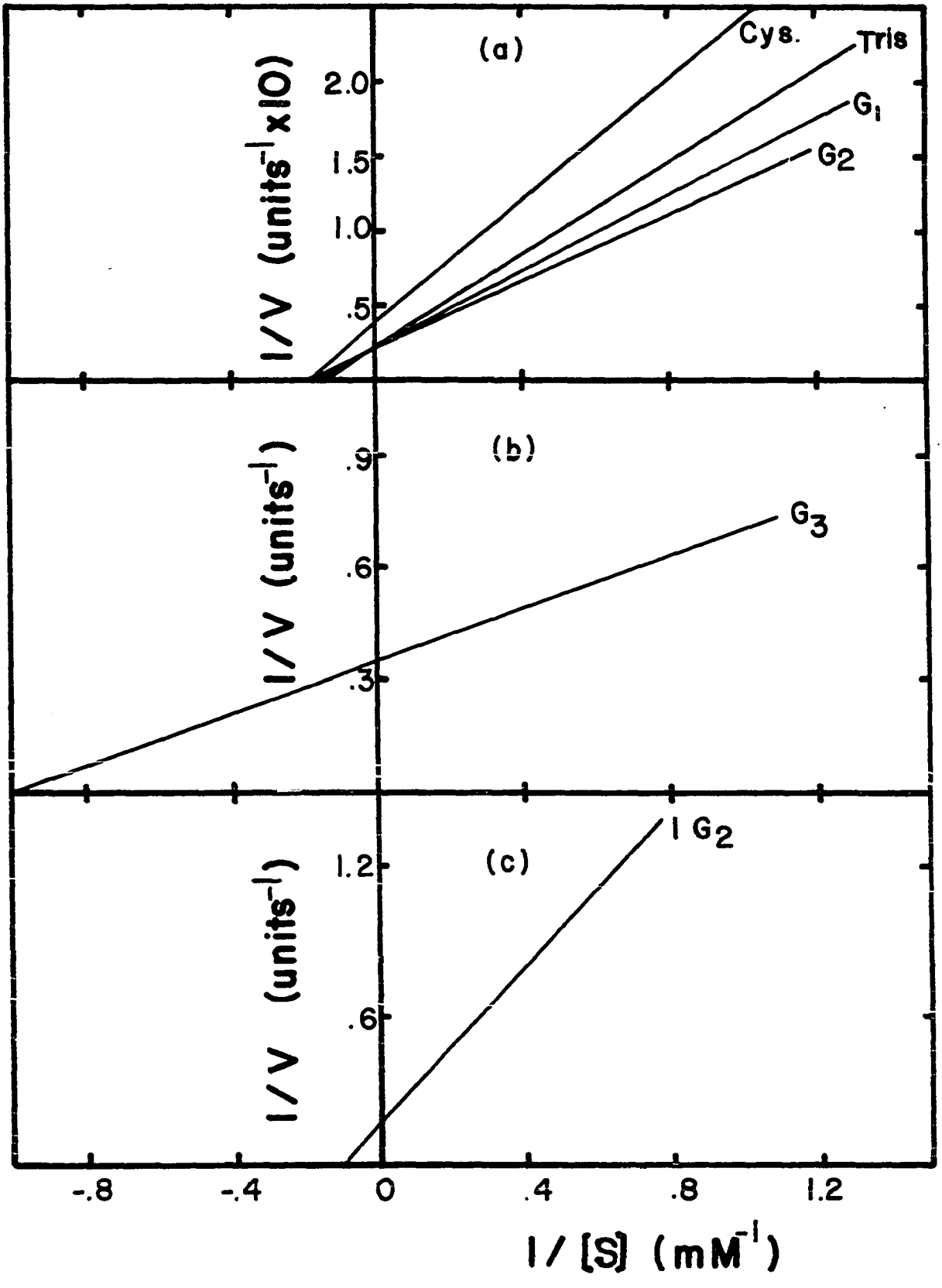

(a)
Cys.
Tris
G1
G2
2.0
1.5
1.0
.5
1/V (units⁻¹ x10)
(b)
G3
.9
.6
.3
1/V (units⁻¹)
(c)
1 G2
1.2
.6
1/V (units⁻¹)
-.8
-.4
0
.4
.8
1.2
1/[S] (mM⁻¹)

Fig. 19. Effects of pH on K_m, V_{max}, and v_o for the extracellular maltase on maltose.

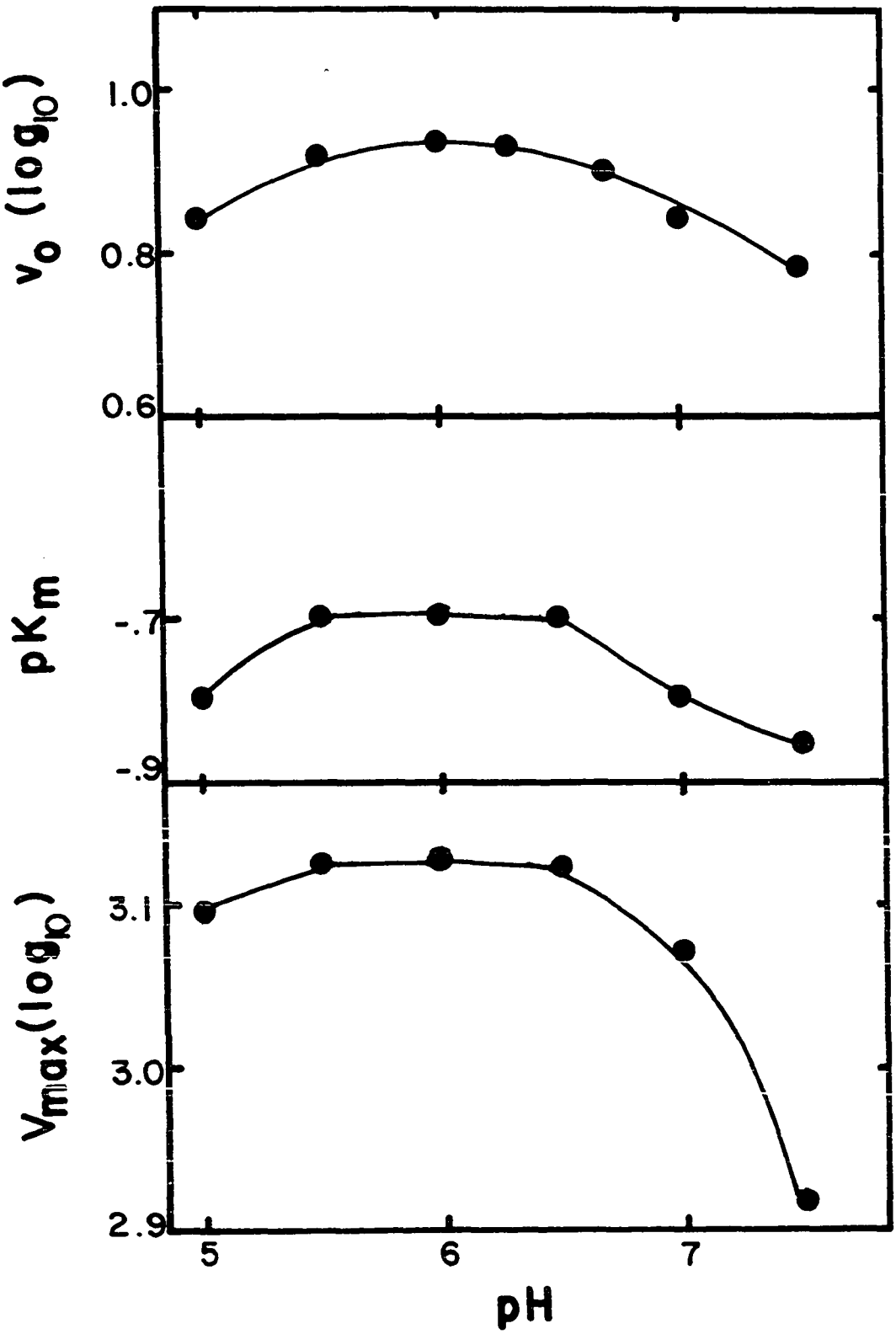
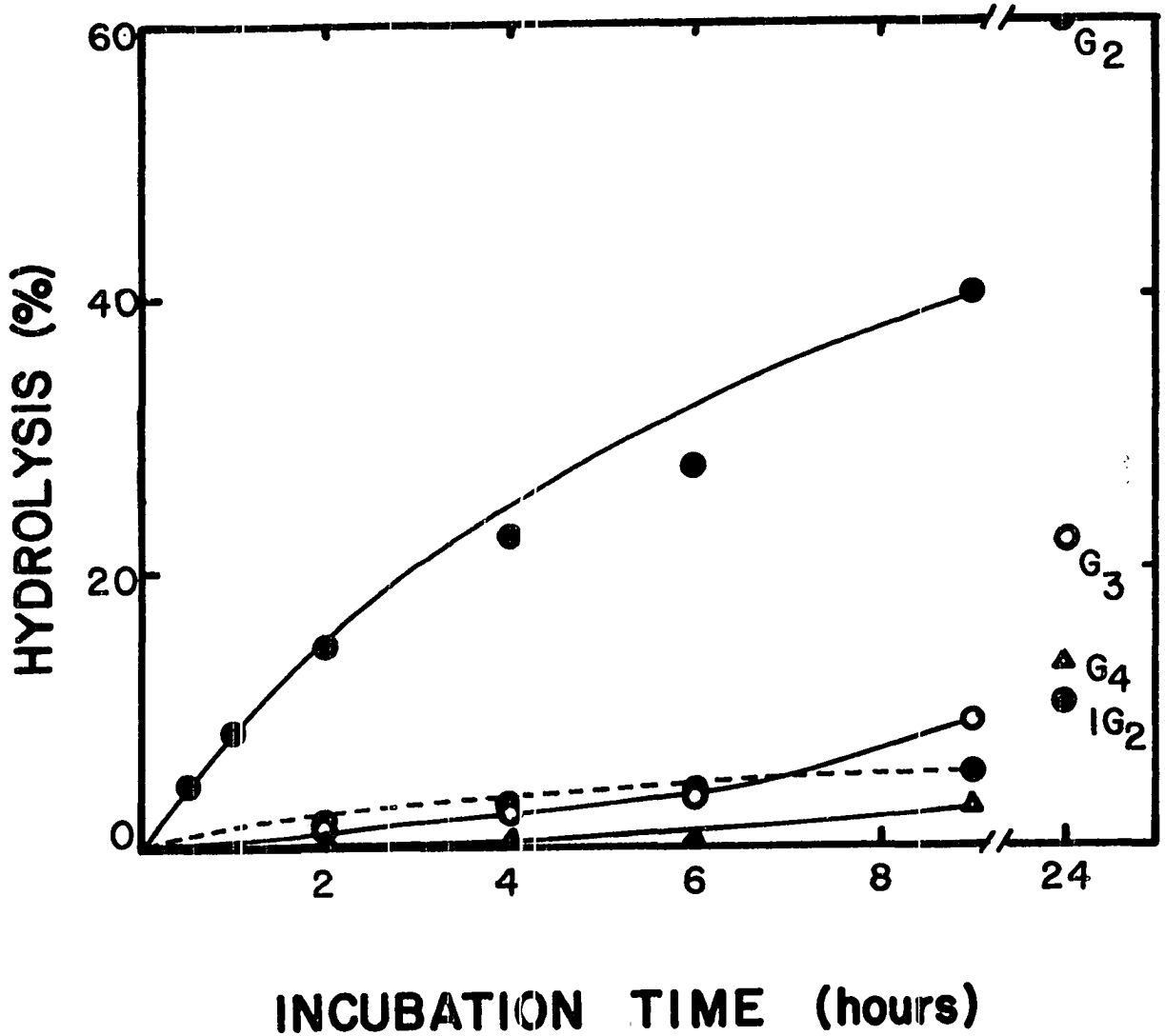

v_o (log_{10})
1.0
0.8
0.6
pKm
-.7
-.9
V_{max}(log_{10})
3.1
3.0
2.9
5
6
7
pH

Fig. 20. Comparison of the rates of hydrolysis of maltose, maltotriose, maltotetraose, and isomaltose by the extracellular maltase of Bacillus subtilis P-11.

Reaction mixtures containing 1 ml of enzyme and 9 ml of 3.3 mM substrate were incubated at 45 C. Toluene was used as a preservative, and the tubes were covered with paraffin film to retard evaporation. Portions of the reaction mixtures were withdrawn at various time intervals and the quantities of glucose formed were measured. (•——•) maltose, (o——o) maltotriose, (▲——▲) maltotetraose, and (•----•) isomaltose.


60
40
20
0
HYDROLYSIS (%)
2
4
6
8
24
INCUBATION TIME (hours)
G2
G3
G4
IG2

DISCUSSION

As mentioned earlier, only two extracellular bacterial maltases have been discovered. Both were clostridial enzymes (Hockenhull and Herbert, 1945; Whelan and Nasr, 1951). A simple and easy screening method would greatly facilitate the isolation of more bacteria that can manufacture extracellular maltases. The two methods that were developed, the Lactobacillus method and PGO enzymatic method, may meet these requirements, although the Lactobacillus method was not reproducible. The Lactobacillus method needs more study; non-reproducibility was probably due to the adaptation to grow on glucose by the Lactobacillus sp. used. The PGO method, however, was specific for extracellular maltase producers. It could differentiate the clear zones caused by antibiotic action from those caused by extracellular maltases; this was not true of the Lactobacillus method. The PGO method also can be used in the isolation of extracellular invertase producers, or to detect any hydrolytic enzyme that releases glucose as an end product, based on the same principle. For optimum results, the PGO reagent should be dissolved in 0.5% agar solution when used in the overlay. Otherwise, unlike the iodine-overlay used in the detection of amylase producers on starch agar plates, the PGO reagent stays at the surface of the agar and the colorful oxidized form of o-dianisidine diffuses all over the surface of the plate instead of moving into the agar. Soft agar (0.5%) holds the PGO reagents in place and prevents diffusion to other areas. Another

advantage of the PGO method was savings in time. It takes only 10 to 30 minutes to detect extracellular maltase-producing colonies.

Coleman (1967), in his studies on the synthesis of extracellular enzyme formation by Bacillus subtilis, discovered that there was a low but definite production of exoenzymes from the moment the cells initiated growth until the end of the logarithmic growth phase. As the rate of increase in cell mass decreased, the rate of enzyme secretion increased to a high linear value which was maintained even into the stationary phase. He, then, proposed a mechanism whereby exoenzyme m-RNA formation and hence enzyme production is limited during growth by a limitation of nucleic acid precursors caused by the depletion of the precursor pool during rapid ribosomal RNA synthesis. When the growth rate decreases, so does ribosomal RNA synthesis; the nucleic acid precursor pool may then increase in size, thereby removing the limitation so that exoenzyme m-RNA and protein may be formed at maximum rates. The formation of the extracellular maltase of B. subtilis P-11, as shown in Fig. 4, did not follow Coleman's model. The maltase appeared in the medium at the early logarithmic phase and maltase levels increased approximately parallel to cell growth. Maltase activity reached the maximum levels at the late logarithmic phase and then decreased as the culture entered the stationary phase of growth. This growth-enzyme production relationship suggests that neither lytic enzyme action nor a delayed exoenzyme m-RNA mechanism was involved in the formation of the extracellular maltase. It seems that a mechanism associated with

active cell growth controls the formation of the enzyme.

The extracellular maltase of B. subtilis P-11 is an induced enzyme. Without the presence of an inducer, there was no enzyme formation at all, although the inducers were not highly specific, as was shown in Table 4. The hydroxyl group at carbon atom 3 of the glucon moiety is required for activity of some maltases (Halvorson and Ellias, 1958); it also could be required for an inducer to be active because ribose, with a configuration of carbon atom 3 that is different from that of glucose, induced the formation of only small amounts of enzyme. The microorganism did not necessarily obtain energy from the inducer because methyl-α-D-glucoside, which is not a substrate of the maltase and was not hydrolyzed, induced maltase production.

During purification of the extracellular maltase, attempts were made to use Diaflo ultrafiltration to concentrate and purify the enzyme. An XM-50 membrane, which permits molecules with molecular weights below about 50,000 daltons to pass through, was useful for this purpose. However, only 50% of the total maltase activity could be recovered. There was no detectable activity in the filtrate. Filtration under high pressure (90 atm, nitrogen gas) seemed to inactivate the enzyme. Because the enzyme inactivation might have been caused by foaming when the pressure was released, experiments were conducted by using very slow pressure release; these were unsuccessful in preventing loss of enzyme activity. Attempts also were made to use acetone or ethanol to precipitate the enzyme. Although the

precipitations were carried out at temperatures below 0 C, over half of the total maltase activity was lost. The extracellular maltase, however, was quite stable if ammonium sulfate was used to precipitate it, as was shown in Table 6.

Some anomalous properties of the maltase were observed during preliminary experiments with Sephadex G-200 and DEAE-Sephadex A-50 columns. The procedure outlined in Fig. 3 resulted in complete separation of maltase and amylase activities. In this procedure, the enzyme preparation was passed through a Sephadex G-200 column before it was passed through a DEAE-Sephadex column. Passage through the DEAE-Sephadex column altered the size, shape, or another property of the maltase so that the passage was retarded in the Sephadex G-200 column (compare Figs. 8 and 10). If the crude enzyme (ammonium sulfate precipitate of cell-free culture fluid) was passed through a DEAE-Sephadex column before passage through a Sephadex G-200 column, the maltase and amylase peaks overlapped and separation of the two enzymes was not possible. This indicates that the molecular weight of the maltase in the initial, cell-free culture fluid may be 200,000 daltons or greater, or that the maltase is associated with some nonmaltase material. This association or complex is disrupted during DEAE-Sephadex chromatography. The second peak shown in Fig. 9 could be this inert "fragment" or "carrier". Such a shift in position during column chromatography probably was not due to any change from tetramer to dimer or monomer. At least Robyt and Ackerman (1973)

discovered in their studies on multiple forms of B. subtilis α-amylase that the specific activity of the tetramer was higher than that of the trimer which was higher than that of the dimer with or without zinc ion.

The specific activity of the B. subtilis P-11 maltase was increased 9 times by the DEAE-Sephadex ion-exchange chromatography, as shown in Table 7. Therefore, by taking advantage of association of the maltase to a nonmaltase substance during an initial passage through a Sephadex G-200 column, dissociation of enzyme from the inert substance by ion-exchange chromatography, and a second passage through Sephadex G-200 (Fig. 10), the extracellular maltase could be freed of contaminating amylase and proteinase activities.

The purified enzyme was even more heat stable than the crude preparation, as shown in Figs. 7 and 14. This effect might be due to presence of the "carrier" on the crude enzyme or possibly because proteinase(s) were removed during the purification procedures.

When polyacrylamide gel electrophoresis was used to demonstrate the homogeniety of the purified enzyme, the enzyme would not pass into the gels. The maltase stayed at the top 0.5 cm, even when buffers of various pH values were used and direction of electrophoresis was changed. The same situation occurred when gel-isoelectric focusing was first used. If the enzyme was mixed with the focusing gel before polymerization, however, the maltase activity was concentrated into a broad band rather than a thin, sharp band. Furthermore, isofocusing times had to be extended to obtain sufficient concentration. After

the gels were stained with Coomassie blue, there was only one band, whose position was coincident to that of the maltase activity recovered by slicing the unstained gel and extraction with deionized water. Only one band of maltase activity was obtained after sucrose gradient (5% to 20%) ultracentrifugation (40,000 rpm/20 hours with SW-40 rotor), suggesting that the purified enzyme existed in only one monomeric or dimeric form.

Larner and Gillespie (1956) considered that the inhibition of α-glucosidases by Tris was due to the unionized amino group in Tris because tert-butylamine, the ultimate product of substitution of the hydroxylmethyl group of Tris by a methyl group, had no inhibitory action at pH 7.0. An alternative view, however, is that the inhibition is due to the hydroxyl groups. This would also explain the lack of inhibition by tert-butylamine. Therefore, Kelemen and Whelan (1966) tested analogues of Tris that lacked amino groups and found that pentaerythritol was inhibitory to α-glucosidases and that the inhibition was not pH dependent. They concluded that both explanations of the inhibitory power of Tris might be correct and the polyols exhibited one facet of the Tris inhibition. On the basis of the present study, the inhibition of maltase activity by Tris probably was due to the unionized amino group, rather than the hydroxyl groups, of Tris. As was shown in Table 10, the polyols, such as erythritol, sorbitol, and inositol, did not inhibit the enzyme activity.

A glycosidase has at least two types of functional groupings, a binding site and a catalytic site (Koshland, 1959). The binding site

determines the carbohydrate which will be hydrolyzed, the specificity being determined by the glucon in the substrate molecule. The catalytic site of the enzyme determines the nature of the bond which will be hydrolyzed, which in the case of a glycosidase is determined by the configuration of the bond binding the glycon to the aglycon. Therefore, a good competitive inhibitor of glycosidase activity should fit both the specific and catalytic sites, preventing the hydrolysis of any one α- or β-glycoside. Since glucose is a competitive inhibitor of maltase activity, with a K_i value 4.38 mM (Fig. 18a), but ribose, and the glucose epimers mannose and galactose, are not inhibitors of the maltase, carbon atoms 2, 3, and 4 of the glucose molecule must be necessary for the substrate-enzyme complex. The pentose, xylose, whose structure is similar to glucose except no primary alcohol, is not an inhibitor. Hence, carbon atom 6 is also required. When the aldehyde group of the glucon moiety was changed, either to an alcohol as in sorbitol or to a lactone group as in Δ-gluconolactone, the inhibitory property was largely lost. Therefore, the entire glucose molecule seems to be required to fit the specific site. Besides, an intact aglucon moiety is also required for this binding. That is the reason why phenyl-α-D-glucoside and maltitol were neither the substrates for, nor inhibitors of, the maltase. The specificity of the catalytic site must not be critically high because both α-1,4 and α-1,6 bonds could be cleaved by the maltase. However, the α-1,4-linkage is hydrolyzed more rapidly than the α-1,6-linkage. Bulky α-1,4-linked molecules such as amylose,

also seem not to fit the specific site.

The binding of the substrate to the enzyme molecule and the hydrolysis of the substrate are pH dependent (Fig. 19). From the plot of pK_m (-log K_m) vs pH, there were two ionic groups with pK_a values of 5.5 and 6.5, respectively, involved in or near the active center of the enzyme. These pK_a values correspond closely to the imidazole group of histidine. That histidine may reside at the catalytic site is substantiated by the finding that rose bengal, a reagent that modifies histidine residues, is a strong inhibitor of the maltase (Table 10), while 4-chloromercuribenzoic acid, specific for the cysteine residue, is a very weak inhibitor.

To compare the properties of the extracellular maltase of B. subtilis P-11 with those of maltases previously reported, a summary table was made (Table 11). Most of maltases reported are strongly inhibited by Cu^{++}, Pb^{++}, Zn^{++}, and Ni^{++}, but these cations did not appreciably affect the maltase of B. subtilis P-11. As shown in Table 11, cysteine, glutathione, and mercaptoethanol were stimulators for the α-glucosidase of Saccharomyces italicus, while these were inhibitors of the maltase of B. subtilis P-11. The amino acid residues involved in the active center were histidine for the maltases of B. subtilis P-11 and Mucor javanicus, and both cysteine and histidine for the maltase of B. cereus. Similarities and differences in several other properties, such as in pH optima, are summarized in Table 11.

After these comparisons were made, a question arose. Were the effects of reducing agents, such as glutathione, on the extracellular

Table 11. Comparison of the properties of the extracellular maltase of *B. subtilis* P-11 to those of maltases previously reported.

Source	Properties
B. subtilis P-11	Opt. pH, 6.0; opt. temp., 45 C (30 minutes); stable within pH 6.0 to 6.3 and up to 45 C; pI, pH 6.0 (by gel-isoelectric focusing); mol. wt., 33,000 (Sephadex G-200); Hg^{++}, Ag^{+}, Fe^{++}, glutathione, mercaptoethanol, dithiothreitol, rose bengal, strong inhibitors; glucose and Tris, competitive inhibitors; cysteine, non-competitive inhibitor; Mn^{++}, stimulator; K_m, 5.0 mM (maltose); histidine residue involved in active center.
B. cereus (Yamasaki and Suzuki, 1974)	Opt. pH, 6.8 to 7.3; opt. temp. 40 C (15 minutes); stable within pH 6.8 to 7.3 and up to 35 C; pI, pH 4.5; mol. wt., 12,000 (SDS-polyacrylamide electrophoresis); Cu^{++}, Zn^{++}, Hg^{++}, Ni^{++}, strong inhibitors; Tris and turanose, moderate inhibitors; K_m, 5.55 mM (maltose); histidine and cysteine involved in active center.
Escherichia coli (Wiesmeyer and Cohn, 1960a, b)	Opt. pH, 6.9; mol. wt., 124,000 (ultracentrifugation); Hg^{++}, Cu^{++}, Ni^{++}, Zn^{++}, potent inhibitors; methyl-α-D-glucoside, glucose, competitive inhibitors.
Mucor javanicus (Yamasaki *et al.*, 1973a, b)	Opt. pH, 4.6; opt. temp., 55 C (15 minutes); stable within pH 4.0 to 7.0 and up to 45 C; pI, pH 8.6; Cu^{++}, Hg^{++}, Pb^{++}, Zn^{++}, strong inhibitors; Tris and turanose, moderate inhibitors; K_m, 0.68 mM (maltose); histidine residue involved in active center.

Table 11. (continued)

Source	Properties
Saccharomyces italicus (Halvorson and Ellias, 1958)	Opt. pH, 6.6 to 6.8 (PNPG); stable within pH 6.0 to 7.8; mol. wt., 85,000 (ultracentrifugation); Cu^{++}, Hg^{++}, Ag^{+}, Pb^{++}, Zn^{++}, PCMB, strong inhibitors; cysteine, glutathione, mercaptoethanol, stimulators; K_m, 0.28 mM (PNPG).
Saccharomyces logos (Chiba et al., 1973a, b)	Opt. pH, 4.6 to 5.0; opt. temp., 40 C (30 minutes); stable within pH 3.6 to 6.6 and up to 40 C; mol. wt., 270,000 (Sephadex G-200); K_m, 7.7 mM (maltose).

maltase of B. subtilis P-11 (Table 10) due to the action of maltase itself or on the PGO reagent? An additional experiment was done. In this experiment, the concentrations of the effectors and procedures followed were the same as used before (Tables 9 and 10), except no maltase was added. After the addition of effectors to a known quantity of glucose (50 µg/0.5 ml), the mixtures were heated in a boiling water bath for 5 minutes. Then, the color reaction due to the action of PGO reagent on glucose was determined. Table 12 shows that the effectors did not alter the PGO reaction by more than ± 20% except for mercaptoethanol, glutathione, dithiothreitol, cysteine, and $FeSO_4$ (10 mM). Thus, the extent of inhibition or stimulation of maltase by these effectors cannot be determined by use of the PGO assay because they interfere with the assay itself. Furthermore, citrate buffer was not the best choice for experiments on the effects of various cations on maltase activity. It was pointed out to me after the experiments had been completed that chelation of cations by citrate might have affected the results obtained, especially at a low cation concentration. In future studies, another buffer (such as HCl-sodium arsenate described on page 48) should be used.

Table 12. Effect of cations and organic compounds on PGO reagents.

Effector	Concentration (mM)	Residual activity (%)
None		100
$MnSO_4$	5	106
	25	109
$CuSO_4$	1	82
	10	91
$AgNO_3$	1	82
	10	82
$HgCl_2$	1	82
	10	82
$FeSO_4$	1	88
	10	59
Rose bengal	0.5 (mg/ml)	80
	1 (mg/ml)	50
Glutathione	0.25	71
	1	36
Cysteine	1	64
	3	50
	10	14
Dithiothreitol	0.5	32
	1	0
Mercaptoethanol	0.25	36
	1	0

SUMMARY

Two methods were developed to detect extracellular maltase producers. The first method was to overlay isolated colonies with Lactobacillus sanfrancisco cell suspensions in soft (0.5%) agar containing maltose. The lactobacilli utilize maltose but not glucose as a source of carbon, and so zones of no growth are discernible around colonies that produce extracellular maltase. The second method was enzymatic. Petri plates are overlaid with PGO reagent (peroxidase-glucose oxidase-o-dianisidine), also in soft agar. Glucose produced by the hydrolysis of maltose surrounding colonies that excrete maltase, reacts with the PGO reagents to produce red zones around the colonies.

Seven extracellular maltase-producing bacteria were isolated. Strain P-11, which produced the most maltase, was identified as a strain of Bacillus subtilis. A medium containing 2% methyl-α-D-glucoside, 0.5% phytone, and 0.2% yeast extract provided the best production of extracellular maltase by strain P-11. The extracellular maltase, after the removal of cells, was precipitated by ammonium sulfate (85% saturation). The enzyme was purified by using the following procedures: Sephadex G-200 column chromatography, DEAE-Sephadex A-50 ion-exchange column chromatography, and a second Sephadex G-200 column chromatography. A highly purified maltase without amylase or proteinase activities was obtained.

Some properties of the extracellular maltase were determined: optimum pH, 6.0; optimum temperature, 45 C when the incubation time was 30 minutes; pH stability, stable within 6.0-6.3; heat stability, stable up to 45 C (20% of the acitivity was lost if the enzyme was held at 45 C for 2 hours); isoelectric point, pH 6.0 (by gel-isoelectric focusing); molecular weight, 33,000 daltons (by gel-filtration with Sephadex G-200); substrate specificity, the relative rates of hydrolysis of maltose, maltotriose, isomaltose, and maltotetraose were 100: 15: 14: 4, respectively and there was no activity toward alkyl- or aryl-α-D-glucosides, amylose, or higher polymers; heavy metals such as $HgCl_2$, $AgNO_3$, and $FeSO_4$, were strong inhibitors, $MnSO_4$ was a stimulator, glucose and Tris were competitive inhibitors with K_i values of 4.54 and 75.8 mM, respectively, cysteine was a noncompetitive inhibitor, transglucosylase activity was present; and the Michaelis constants were 5 mM for maltose, 1 mM for maltotriose, and 10 mM for isomaltose. A plot of pK_m ($-\log K_m$) vs pH revealed two deflection points, one each at 5.5 and 6.5. These data suggested that ionic groups with pK_a values of 5.5 and 6.5 were involved in or near the active center; they probably correspond to the imidazole group of a histidine residue. This assumption was supported by the strong inhibition of enzyme activity by rose bengal.

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