STUDIES ON MODE OF OCCURRENCE OF α-GLUCOSIDASE ACTIVITIES IN MOLD (*ASPERGILLUS ORYZAE*)

By

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CONTENTS

Introduction

Starch is the main reserve carbohydrate in plants and is quantitatively the

[Journ. Facul. Agr., Hokkaido Univ., Sapporo, Vol. 52, Pt. 3, 1962]

most important sources of energy in our life. As the first stage of metabolism of starch in organisms depends on the cleavage of α-1, 4-glucosidic linkages, it is not too much to say in the field of enzyme chemsitry amylase is one of the enzymes which have been most extensively investigated.

It is well known that amylase is generally classified into α-type and β-type, from the aspects of the extent of hydrolysis of starch or the rotation of the hydrolysis products. The occurrence of these two types of amylase varies from organism to organism, and in micro-organisms β-amylase is rarely found.

Among micro-organisms, mold has very strong activity in decomposing starch and is a most suitable material for an investigation on diastic enzymes system. Especially in Japan Koji has been closely related to our life and, as a consequence, amylase studies also have been greatly developed in scientific as welll as in economical fields in this country.

In molds too, as in other micro-organisms, the exsistence of β-amylase is not completely clear[32,33,83]. However, in recent years glucose-forming amylase was discovered and separated in crystalline state from various molds, and the properties of this enzyme have been almost clarified under the names of amyloglucosidase, gluc-amylase, γ-amylase, Taka-amylase B and saccharogenic amylase. (In present study the name amyloglucosidase was adopted, because this name represents most pertinently the difference of properties from β-amylase and the present study is a comparative study of α-glucosidase activities in mold).

Amyloglucosidase resembles β-amylase in certain aspects of its action and properties. Like β-amylase, amyloglucosidase appears to attack the non-reducing ends of the glucosidic chains of its substrate, but unlike β-amylase it produces glucose, not maltose, and in addition it also attacks maltose.

The striking fact that amyloglucosidase has an ability to hydrolyze maltose raises questions as to whether the maltose attacking activity in mold depends upon amyloglucosidase alone or a member of the class of α-glucosidase as oligosidase also exists. To solve this problem is a significant and an interesting work for resolution of the diastic enzyme systems in mold. This consideration directed the author to start the present study.

Of course the investigation on mold α-glucosidase, in terms of maltase, has been conducted by many workers but the results are conflicting, not only in aspects of substrate specificity[59,75,91] but also in aspects of discussions whether β-amylase and maltase in mold are due to a single enzyme or not[32,33,83].

From the literature hitherto published, it may be said that such divergent views were brought about by using crude materials as enzyme sources and by inadequate selection of substrates, among which maltose was principally used. Under such considerations the author adopted α-heteroglucoside as a guide for

following α-glucosidase activities. This selection of substate was a major factor of success in the elucidation of such enzyme systems.

In thus viewing the occurrence of α-glucosidase activities of mold in the present studies the existence of maltase besides amyloglucosidase was demonstrated. In addition, a new type of amyloglucosidase which is able to hydrolyze α-heteroglucosides was discovered.

Among the properties of each enzyme investigated the transglucosidation action of maltase and the multiple nature of amyloglucosidase were most important and interesting in this field of enzyme chemistry.

It is probable to classify the transglucosidation reactions into three types: (a) glucosyl transfer in which sugar-nucleotide participates, (b) glucosyl transfer in which phosphoryl ester of sugar participates and (c) glucosyl transfer in which phosphorylation of sugar is not involved.

The discovery and expansion of reaction (a) were mainly due to the results of Leloirs and his coworkers[40]. This type of glucosyl transfer in which uridinediphosphate sugar is the donor is a most important in aspect of utilization of free energy in saccharide synthesis. Sucrose[41], trehalose[13] and lactose[12] are formed by this reaction, and glycogen synthesis is also catalyzed by this type of transglycosylase[42,43,44].

$$\text{UDPG} + \text{Fructose} \rightleftarrows \text{UDP} + \text{Sucrose}$$
$$\text{UDPG} + \text{Primer} \rightleftarrows \text{UDP} + \alpha(1\rightarrow4)\text{-glucosyl-primer}$$

Reaction (b) is catalyzed by phosphorylase.

$$\text{Glucose-1-phsphate} + \text{primer} \rightleftharpoons \text{Glucosyl-}\alpha(1\rightarrow4)\text{-primer} + \text{P}$$

Maltosephosphorylase will also be involved in this class. Polysaccharides to be

TABLE 1. Polysaccharide Synthesis by Transglucosidation in which Phosphates do not participate

Enzyme	Substrate	Product	Literature
dextransucrase	maltose, isomaltose	dextran	(5)
dextransucrase	sucrose	dextran	(27) (61)
levansucrase	sucrose	levan	(28) (29)
D-enzyme	malto-triose	malto-pentaose	(77)
Q-enzyme	amylose	amylopectin	(6) (30)
1,4→1,6 transglucosidase	amylose	glycogen	(14) (49)
nigeran-producing enzyme	maltose	nigeran	(7) (8)
amylomaltase	maltose	α-1,4-polymer	(15) (95)

TABLE 2. Oligosaccharide Synthesis by Transglucosidation in which Phosphates do not participate

Origin	Substrate	Products	Literature
Asp. oryzae	maltose	α-1,6-oligosacch.	(67) (68)
Aspergilli	maltose	α-1,6-oligosacch.	(73)
Asp. niger	maltose	α-1,6-oligosacch.	(85)
yeast	maltose	α-1,6-oligosacch.	(81) (82)
yeast	sucrose	1-α-glu-fruct, turanose, isomaltulose	(2)
yeast	sucrose	unknown disacch, maltulose, turanose	(96)
Tetrahymena pyriformis	maltose	α-1,4 and α-1,6-oligosacch.	(4)
Cladophora rupenstris	celluobiose	α-1,4, 1,3, 1,1-oligosacch.	(16)
Sacch. fragilis	allolactose	trisacch.	(69)
liver	maltose or polysacch.	α-1,4-oligo-and polysacch.	(74) (79)
bovine blood	maltose or polysacch.	α-1,4-oligo-and polyshacch.	(53) (54)
rat liver	maltose	α-1,4-oligosacch.	(21)
sea weed	maltose	α-1,4-oligosacch.	(17)
Phaseolus radiatus	maltose	α-1,4-oligosacch.	(60)
sugar beet	sucrose	glucosyl fructotetraose	(3)

synthesized by these reactions are starch and glycogen but in vivo the reaction participating UDPG probably takes a major part of such polysaccharides synthesis. There is a report on sucrose formation from glucose-1-phosphate and fructose[86].

Reaction (c), which does not participate in sugar phosphate as well as sugar nucleotide, is a case of transglucosidation being catalyzed by mold maltase observed in present study. In Table 1 and Table 2, the polysaccharides-forming and oligosaccharides-forming enzymes are listed. The former exhibits no hydrolysis action while the reaction to be catalyzed by the latter is a reversible reaction.

As seen in Table 2, it is generally recognized that the enzymes of microorganism produce α-1, 6-linked oligosaccharides while the enzymes of animals and higher plants act on maltose to lengthen the chain of α-1, 4-linkage.

Besides the reactions described above, there are reports of a riboflavine-participating transglucosidation reaction. This reaction has been extensively

studied by KATAGIRI et al.[35),36)] and others[92)]. According to KATAGIRI it is assumed that the reaction may participate in polysaccharide synthesis and that riboflavin competes with phosphate and as a result it maintains a balance between oxidation of the sugar and glycolysis.

Amyloglucosidase has been extensively investigated since its description by PHILLIPS[63)] and it occurs widely in various molds. The mode of action of the enzyme has also been clarified, and according to FUKUMOTO[19)] amyloglucosidases are classified as Rhizopus delemar and Aspergillus niger types with respect to the extent of hydrolysis of starch or β-limitdextrin. (Table 3)

TABLE 3. Outline of Investigations on Amyloglucosidase (This table is mainly according to Fukumoto (19))

Investigator	Origin	Name	Hydrolysis extent of substrate, (%)				Literature
			Maltose	Iso-maltose	-limit dextrin	starch	
Phillips et al.	R. delemar	glucamylase	+	−	+	90	(63)
Fukumoto et al.			100	−	100	100	(18)
Ueda	Asp. oryzae	debranching factor	100		100	100	(88)
Weil et al.	Asp. niger	amyloglucosidase	100		100	100	(90)
Barker el al.		glucamylase	100	−	+	98	(6) (7)
Kitahara et al.	Asp. usamii	γ-amylase	±		+	70	(34)
Kerr	Asp. niger	amyloglucosidase	+			80	(39)
Okazaki	Asp. oryzae	Takaamylase B	100	−	50	80	(62)
Fukumoto et al.	Asp. niger	amyloglucosidase	100	−	40	78	(18)
Hazashida	Asp. kawachii	saccharogenic amylase	100		20	80	(25)

Recently UEDA[88)] fractionated two types of amyloglucosidase from a material of Aspergillus awamorii having different debranching activity on starch.

At any rate these investigators used essentially starch and its decomposition products as substrates. From the standpoint of α-glucosidase activity of mold, a re-examinatin of amyloglucosidases is necessary, along with maltase, and should result in some important findings involving α-heteroglucosidase activity due to one of amyloglucosidases and their multiple nature.

Recent developments of column chromatography, besides electrophoretic techniques, have brought about successful results on the separation, fractionation and further purification of proteins. By supplying such techniques the author could demonstrate the exsistence of modifications among amyloglucosidases. Such phenomena have already been observed and reported on ribonuclease, cytochrome C, Takamaltase, insulin and recently β-glucosidase.

The present study is, in a word, a reanalysis and re-examination of the diastic enzyme systems of mold, with special reference to α-glucosidase activities. Descriptions of the fractionation and purification of the constituents of this series of enzymes and on their detailed characteristics are presented. Furthermore the subject of the nomenclature of such enzymes is discussed.

The author wishes to express here his sincere gratitude to Prof. Dr. Y. NAKAMURA for his guidance and encouragement throughout the course of present study, and also to Prof. Dr. Y. OBATA, Prof. Dr. Y. SASAKI, Prof. Dr. Y. ISHIZUKA and Dr. T. SHIMOMURA for their valuable advice and encouragement. The author wishes also to express his thanks to Sankyo Co. Ltd. and Mr. M. SATO of Sapporo Shusei Kogyo Co. Ltd, for kindly supplying the Takadiastase and Koji used in this work, and also to Prof. Dr. K. ASO. of Tohoku University for his kind gift of pure oligosaccharides and to Mr. T. ITO for kindly supplying the pure potato β-amylase. Acknowledgement is also due to Mr. T. SEKIGUCHI, for his assistance in carrying out a part of this work.

Experimental Part

General Methods and Materials:

Takadiastase and Koji were kindly supplied by Sankyo Co. Ltd. and Mr. M. SATO of Sapporo Shusei Kogyo Co. Ltd., respectively.

Monosaccharides and most of disaccharides were from commercial sources. Pure preparations of isomaltose, panose and malto-oligosaccharides were kindly supplied by Prof. K. ASO. Malto- and isomalto-oligosaccharides were prepared by partial acid hydrolysis of potato amylose and dextran according to the method described by WHELAN et al.[93] and TURVEY et al.[87], respectively. Maltulose and isomaltulose were prepared by treating the corresponding aldo-disaccharides with lime water at room temperature for 24 hrs.[76] and separating the resulting ketosaccharides by carbon column chromatography. β-Limitdextrin was prepared by the usual method using potato starch and β-amylase of sweet potato, which was kindly supplied by Mr. T. ITO.

Alkyl-α-glucosides were synthesized from glucose and the corresponding alcohols in the presence of hydrogen chloride. Aryl-α-glucosides were synthesized by HELFERICH's principle which condense β-pentaacetylglucose with the corresponding aryl-alcohols in the presence of zinc chloride.

Calciumtriphosphate gel was prepared by the method described by KEILIN and HARTREE[37]. The carbon used in column chromatography was a "Shirasagi"-brand Takeda commercial product and cellulose powder of Toyoroshi Co. Ltd. Cellulose derivatives were prepared according to the method described by

PETERSON et al.[65]

Reducing power was measured by adopting Somogyi's copper reagents[38,78].

Butanol-pyridine-water (6 : 4 : 3) was used as the solvent for paperchromatography and aniline-phthalic acid or anisidine phosphoric acid was used as the color reagents.

PART I.

α-Glucosidase activity due to maltase.

In part I, the existence of maltase, besides amyloglucosidase, as one of maltose-hydrolyzing enzymes of mold was demonstrated by fractionation. And the method of fractionation and substrate specificity are described. Of the properties of crystalline maltase, priority was given to investigations of the transglucosidation reaction because of the significance of the reaction itself and because of its most striking distinctness from amyloglucosidase which exhibits only hydrolytic action.

Chapter 1.

Fractionation of Maltase and Amyloglucosidase in Molds and Crystallization of Maltase.

§ I-1-1. Preface :

Separation of maltase free from amylase activity to give the evidence for exsistence of α-glucosidase besides amyloglucosidase as polyase, is the first work in this series of study.

The author succeeded in the fractionation of maltase and amyloglucosidase activities in Koji and Takadiastase, and maltase was crystallized for the first time by further purification.

§ I-1-2. Crystallization of maltase from Koji mold (*Aspergillus oryzae*).

Step 1. Preparation of α-amylase-free enzyme solution from Koji, and fractionation of amyloglucosidase and maltase. (Fig. 1.)

Enzymes were extracted with 50% ethanol. The purpose of this procedure was the removal of α-amylase. That is, α-amylase was adsorbed by residual starch of Koji mold in a fifty percent alcoholic solution, while maltase and amyloglucosidase were not adsorbed[84].

When α-amylase was contaminated in the 50% ethanol-extract the treatment with residual starch of Koji (the extracted residue was washed thoroughly with 1% sodium chloride) was repeated.

Polished rice Koji, 3 kg.
| extracted with 5 L. of 50% ethanol, centrifuged,
50% ethanol extract
| added equal volume of ethanol, centrifuged,
Precipitate
| dissolved in 150 ml. of water, added kaolin and pH adjusted to 4.6~5.0 with N-CH_3COOH, centrifuged,
Supernatant
| neutralized to pH 6.6~6.8 with N/1 KOH,
| passed through a column of calcium triphosphate-gel (mixed with an equal amount of Celite), washed with water,
Calcium triphosphate Column
| washed with water thouroughly, poured through M/15 KH_2PO_4, collected each 20 ml. of eluate until maltose-decomposing activity disappeared,

M/15 KH_2PO_4 fraction (Amyloglucosidase) | M/10 $(NH_4)_2HPO_4$ fraction (Maltase)

Fig. 1. Fractionation of Mold Amyloglucosidase and Maltase.

As is shown in Table 4, these two enzymes, amyloglucosidase and maltase, are completely fractionated by acidic and basic phosphate, respectively.

The order of the elution must follow the above mentioned definition, since diammoniumphosphate elutes both amyloglucosidase and maltase. No concentration of diammonium phosphate solution higher than 0.1 M was required to reduce the elution range.

Elution was made by collecting 20 ml. portions of each eluate and in case further purification and crystallization were planned, parts having weak activities were discarded. Fig. 1. is an example of the procedure, and yields of maltase activities obtained in each stage of purification are shown in Table 4.

TABLE 4. Yields of Maltase Activity in each Stage of Purification

	Recovery against 50% ethanol extract (%)
1. 50% ethanol extract	100
2. 75% ethanol ppt.	95 ~ 97
3. M/10 $(NH_4)_2HPO_4$ eluate (only parts having strong activity collected)	50 ~ 60
4. 70% ethanol ppt.	48 ~ 55
5. dialyzed solution	50 ~ 55
6. 50% acetone ppt.	20 ~ 36
7. First crystallization	8 ~ 20

Diammoniumphosphate eluate

| Amberlite IR45 (pre-treated with sodium acetate) treatment, concentrated under diminished pressure, added alcohol, the 50~70% alcohol precipitate dissolved in 50 ml. of 0.5 M acetate (pH 5.0) and alcohol (70%) treatment was repeated,

Precipitate

| dissolved into a minimum quantity of 0.5 M acetate, added a 4-folds volume of saturated $(NH_4)_2SO_4$ sol., precipitate discarded,

Filtrate

| dialyzed against running water (3~4 days), treated with Amberlite IR-45, precipitated with alcohol (75%),

Precipitate

| dissolved in 25 ml. of 0.5 M acetate (pH 5.0), discarded insoluble substances by centrifugation, added an equal volume of acetone, centrifuged,

Supernatant

| further addition of acetone up to the concentration of 67%, stood in ice-box,

Crystalline Maltase.

Fig. 2. Crystallization of Mold Maltase from Diammoniumphosphate Fraction.

Step 2. Further purification and crystallization of maltase from diammoniumphosphate eluates. (Fig. 2)

The purpose of treatment with Amberlite IR-45 was the exchange of phosphate or sulfate for acetate. The large quantities of impurities were precipitated by salting-out with ammonium sulfate while maltase still remained in the solution. It is not clear whether this behavior of maltase to salting-out effectiveness is due to the experimental conditions or its specific property.

At the final stage of purification, acetone was used instead of ethanol, and by use of this solvent, crystallization occurred. Crystals of the enzyme appeared after standing for five to fifteen hours in the ice-box. For crystallization, this precipitate was dissolved in a minimum volume of water and acetone was added until the solution became turbid at 0°C. Further addition of acetone was made after turbidity disappeared and this procedure was repeated until the concentration of acetone increased about 60%. However complete removal of amorphous substances was difficult even after thrice recrystallization.

§ I-1-3. Crystallization of maltase from Takadiastase.

A. Method of measurement of maltase activity:

Phenyl-α-glucoside was used as the substrate for the measurement of the enzyme activity. Maltose was inadequate as a substrate, because of maltose-

hydrolyzing activity of amyloglucosidase. However, the rate of hydrolysis of phenyl-α-glucoside was quite low and subsequently it made the period of reaction one hour incubation. The accurate comparison of enzyme activity in each stage of purification had not been expected.

The enzyme activity was measured as follows: 0.5 ml. of enzyme solution, 4 ml. of 1% phenyl-α-glucoside and 0.5 ml. of 0.1 M acetate buffer (pH 4.8) were mixed and the solution was kept at 45°C for one hour. An unit of maltase activity was represented as the estimated mg. of glucose liberated in 2 ml. of the incubated mixture.

B. Crystallization of Takamaltase:

Fig. 3 is a brief description of the procedure for crystallization of Takamaltase and the yield in each stage of purification is shown in Table 5.

A typical and detailed procedure is as follows: (In this experiment 300 g of the material was used and also, this is a part of the experiments which were conducted for entire fractionation of diastic enzymes system, as will be described later.)

300 g of Takadiastase was dissolved into 2,000 ml. of water and centrifuged, and insoluble substances were removed by washing. To the combined extracts (3,300 ml.) 10% calcium acetate was added up to one per cent in final concentration and the precipitate formed were removed by centrifugation. To the supernatant (3,500 ml.) solid ammonium sulfate was added in the proportion of 600 g to 1,000 ml. and centrifuged for 30 min. at 3,000 r.p.m. after standing overnight in centrifuge-tubes. The supernatant was still strongly turbid but a clear solution was rapidly obtained by filtration. The precipitates were collected and dissolved in water, and the resulting solution (1,500 ml.) was dialyzed against running water for 4 days. The cellophane bags were renewed every day. To the dialyzed solution (2,600 ml.) about 50 ml. of 4% rivanol solution was added untill α-amylaserivanol complex began to appear. The dark-yellow precipitates were removed by centrifugation. Further addition of rivanol to the supernatant was continued until the precipitation of yellow complex with α-amylase ceased. After centrifugation (yellow precipitates were used for crystallization of α-amylase according to the method described by AKABORI et al.[1]), excess rivanol was removed by adding 150 g of kaolin and stirring the mixture for 30 min. To the slightly yellow solution (2,750 ml.) obtained by filteration, ammonium sulfate was added (at the same concentration as in first salting-out) and held for 24 hrs. The precipitate were collected on a filter paper and dialyzed against running water for 3 days. Cellophane bags were also renewed every day in this stage. The dialysate (330 ml.) was then

Takadiastase (200 g.)
| Extracted with water and treated with 10% Ca-acetate (final conc. 1%), centrifuged (3,000 r.p.m., 10 min.)
Supernatant
| Added $(NH_4)_2SO_4$ (60 g./100 ml.), centrifuged (3,000 r.p.m. 30 min.)
Precipitate
| Dialyzed and treated with rivanol solution, centrifuged (2,000 r.p.m., 5 min.),
Supernatant
| Added $(NH_4)_2SO_4$ (60 g./100 ml.), filtered,
Precipitate
| Dialyzed and diluted with equal volume of M/15 citrate buffer (pH = 4.0)
Maltase and Amyloglucosidase fraction
| Chromatographed on Dowex 50-2 (Fig. 6)
Maltase fraction (No. 22~No. 25)
| Dialyzed, precipitated with alcohol (75%), centrifuged, (3,000 r.p.m.,) 10 min.)
Precipitate
| Dissolved in water, added alcohol dropwisely to 60% concentration
Crystalline Maltase
Recrystallize from alcohol at 50% concentration.

Fig. 3. Crystallization of Takamaltase.

TABLE 5. Purification of Takamaltase

	Volume (ml)	U.N./0.5 ml	U.N./mg N	Yield (%)
Original extract	2,000	1.2	—	100
1st. $(NH_4)_2SO_4$ ppt.	1,500	1.0	0.01	60~70
After rivanol treatment	1,700	0.9	0.04	55~60
2nd. $(NH_4)_2SO_4$ ppt.	150	9.8	—	50~60
Dowex 50 treatment (No. 22~25)	80	7.8	34	30~35
Alcohol ppt. (75%)	15	22.0	35	20~30
1st. crystallization	10	16.7	40	10~25

concentrated under diminished pressure to about 75 ml. and the concentrate was diluted with an equal volume of McILVAINE buffer (pH 3.8). The solution thus obtained contained maltase and an amyloglucosidase. They were fractionated by chromatography on a column of Dowex 50-2 in the following manner. The enzyme solution (150 ml., pH 3.8) was passed through the Dowex 50-2 (3 × 20 cm, treated previously with same McILVAINE buffer) and the development

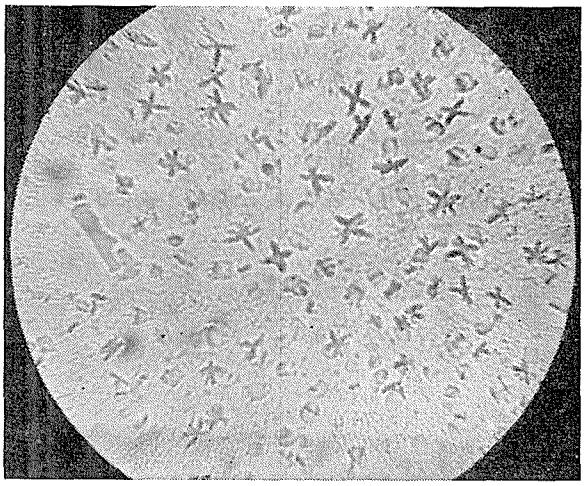


Fig. 4. Crystalline Maltase from Koji.

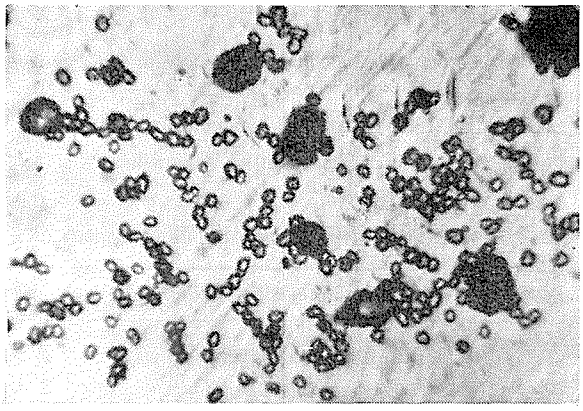


Fig. 5. Crystalline Maltase from Takadiastase.

of the enzymes adsorbed were also made by the same buffer (pH 3.8) until amylase activity had disappeared from the eluate. After finishing the elution of amyloglucosidase the pH of the buffer was raised to 5.0 and eluates were collected at every 20 ml. fraction. Only portions having strong activity of maltase (No. 2～No. 4 in this experiment) were collected and alcohol was added to the combined eluates to 75% concentration at an ice-cold temperature. The precipitates formed were centrifuged and dialyzed against running water for 3～4 days. The precipitation with alcohol was repeated again. The precipitates were then dissolved in 10 ml. of water and alcohol was added drop by drop to the cooled solution. At the 50% concentration the alcoholic solution was

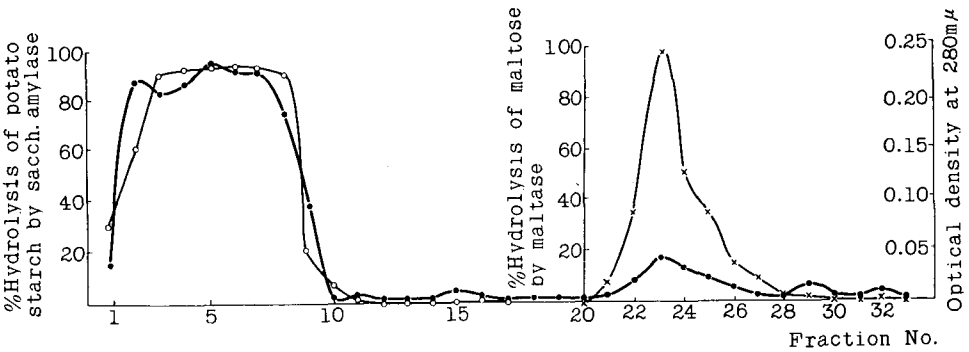


Fig. 6. Chromatography of maltase-saccharogenic amylase mixture on a column of Dowex 50-2.
●——● Optical density at 280 mμ with 0.1 ml. of eluate.
o——o Sacch. amylase activity with 0.5 ml. of pH 4.0 eluate.
×——× Maltase activity with 0.2 ml. of pH 4.8 eluate.

kept at 0°C. Crystalline maltase thus obtained was recrystallized from 50% alcohol. (Fig. 6)

The properties of maltase and amyloglucosidase will be described in Part I and Part II, respectively;

§ I-1-4. Summary.

1). The exsistence of maltase, as one of the maltosecleaving enzymes besides amyloglucosidase in mold, was demonstrated by their fractionation.

2). Maltase was crystallized for the first time from Koji and Takadiastase by using adsorption techniques or column chromatography.

Chapter 2.

Substrate Specificity and General Properties of Crystalline Mold Maltase.

§ I-2-1. Preface.

In regard to the maltose-hydrolyzing activity in mold, it was undoubted, as was seen in Chapter 1, that the two enzymes, maltase and amyloglucosidase, are responsible. From the view of α-glucosidase activity amyloglucosidase has been considered to be quite different from maltase because amyloglucosidase has no activity of transglucosidation and it is inactive to α-heteroglucosides. However the author found in the present study a new type of amyloglucosidase from Takadiastase which is able to hydrolyze the α-heteroglucosides. Thus because of the occurrence of such a combined system of α-glucosidase activity

in mold a lack of agreement on the substrate specificity of mold α-glucosidase had been reported by several workers.

MYRBACK[59] and PRINGSHEIM[64] reported that mold maltase hydrolyzes maltose but not α-heteroglucosides and according to Weidenhagen[91] maltose as well as phenyl- or methyl-α-glucoside was hydrolyzed by his enzyme.

Chapter 2 deals with the specificity of crystalline mold maltase on various α-heteroglucosides and oligosaccharides, pH optima, temperature dependence and inhibitors.

§ I-2-2. Experimental Results obtained by using Crystalline Takamaltase.

A. Action of the Enzyme on Alkyl- and Aryl-α-glucosides:

Methyl-, ethyl-, phenyl-, p-nitrophenyl- and p-cresyl-α-glucosides were used as substrates. Twenty ml. of 0.01 M substrate soln., 1m l. of 0.1 M acetate buffer (pH 5.0) and 0.5 ml. of 0.1% enzyme sol. were mixed and made up to 25 ml. with water. The resulting solution was kept at 40°C and 5 ml. aliquots of the reaction mixture were pipetted out for analysis. The analytical procedure was described previously. The rate of the hydrolysis was represented as the percentage of decomposition of the substrates.

As shown in Fig. 7, the hetero-α-glucosides tested were cleaved by this

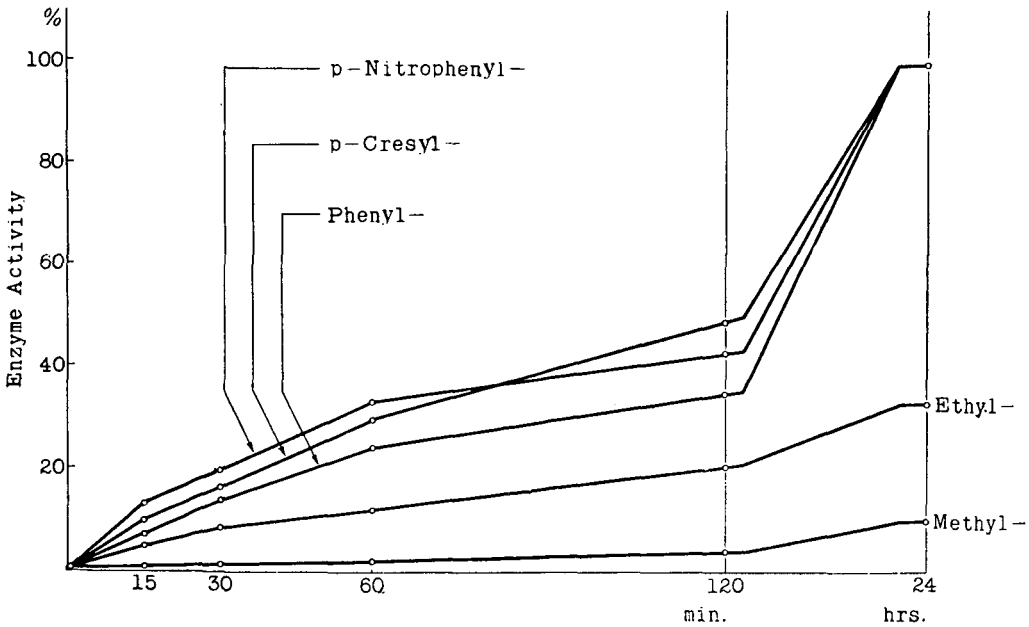


Fig. 7. Action of Crystalline Takamaltase on Alkyl- and Aryl- -glucosides.

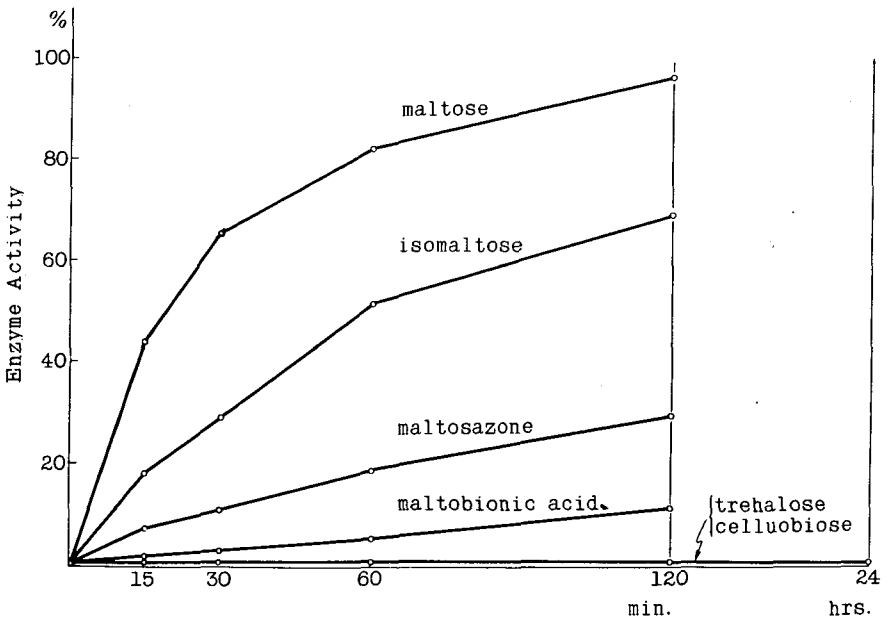


Fig. 8. Action of Crystalline Takamaltase on Gluco-disaccharides and Maltose-derivatives.

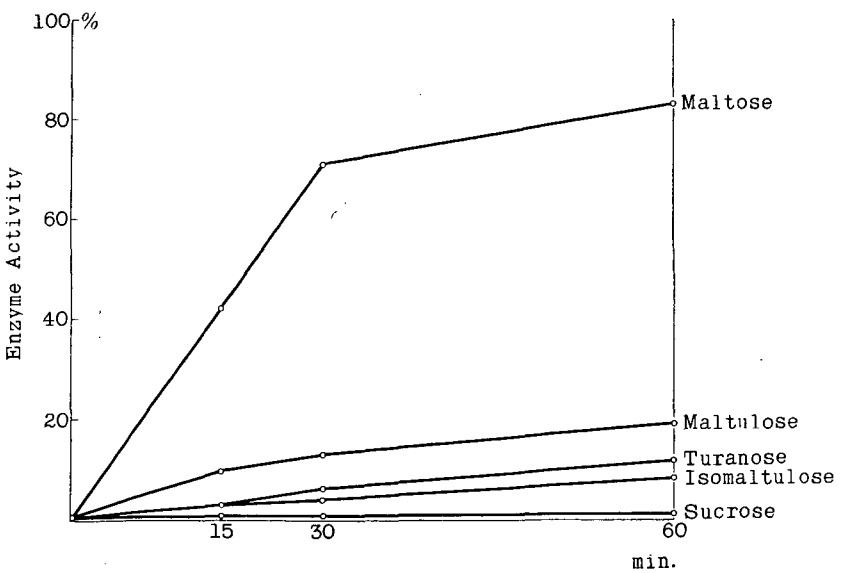


Fig. 9. Action of Crystalline Takamaltase on Glucosylfructose.

enzyme and the rate of hydrolysis of alkyl-α-glucosides was much lower than that of aryl-α-glucosides.

B. Action of the Enzyme on Gluco-disaccharides, Glucosyl-fructoses, and Malto- and Isomalto-oligosaccharides:

The conditions of the enzyme reaction and the analytical procedure were the same as described in the section of α-heteroglucosides.

As shown in Fig. 8, isomaltose was also hydrolyzed by this enzyme but the rate of hydrolysis was lower than that of maltose. Trehalose was not hydrolyzed while maltosazone and maltobionic acid were attacked at a slow rate.

Among the glucosides with fructose the nonreducing sugar sucrose was not hydrolyzed and turanose, maltulose and islmaltulose were the substrates for this enzyme. (Fig. 9)

To observe the lmiting length of substrate to be attacked by this enzyme as an oligosidase, tests were made by using malto- and isomalto-oligosaccharides. The data shown in Fig. 10 and Fig. 11 indicated that in both series of oligosaccharides the sugars above seven to eight units of hexose would not be hydrolyzed and that the rate of hydrolysis decreased with the increase of polymerization degree.

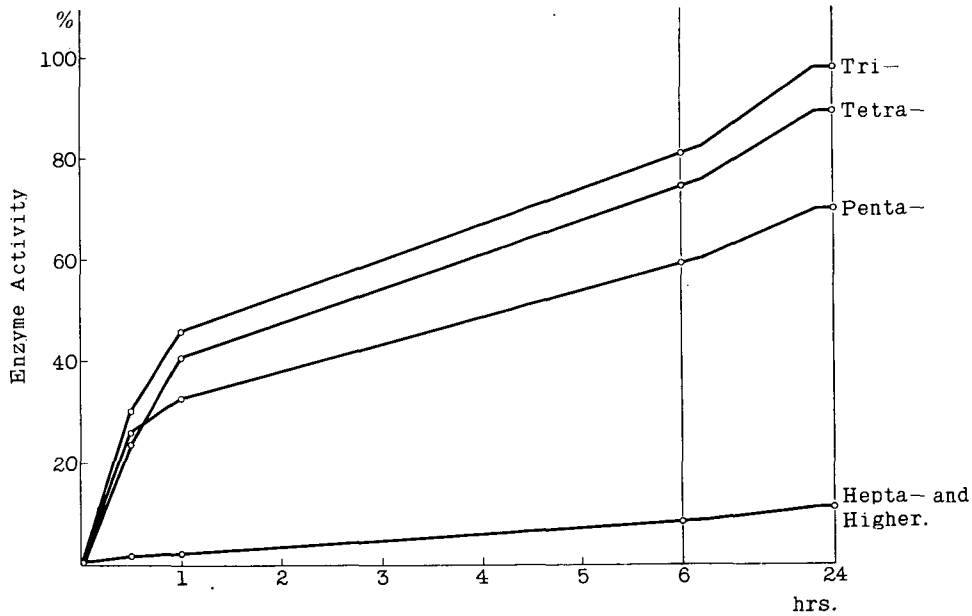


Fig. 10. Action of Crystalline Takamaltase on Malto-oligosaccharides.

C. pH Optima:

A mixture of 5 ml. of 0.01 M maltose solution, 4 ml. of McIlvaine buffer and 1 ml. of enzyme soln. was incubated at 40°C. After 30 min. the percentage of hydrolysis was measured by using 5 ml. aliquots of the incubated mixture.

The pH optima is in the range of pH 4.2~4.6. (Fig. 12)

D. Temperature Dependence:

Five ml. of 0.01 M maltose soln., 1 ml. of McIlvaine buffer (pH 4.0) and 3 ml. of water were mixed and kept for 10 min. at various test temperature. Then 1 ml. of enzyme soln. was added and after 20 min. the sample was assayed. The optimal temperature for the enzyme action was approximately 55°C. (Fig. 13)

E. Effects of Metal Ions and SH-Reagents on Crystalline Takamaltase:

The procedure used for determining the effectiveness of metal ions at a concentration of 10^{-3} M on the enzyme was as follows; 5 ml. of 0.01 M maltose soln., 1 ml. of enzyme soln., 2 ml. of McIlvaine buffer (pH 4.0) and 1 ml. of 0.01 M metal ions, e. g. $HgCl_2$, $CuSO_4 \cdot 5H_2O$, $BaCl_2 \cdot 2H_2O$, Pb-acetate$\cdot 2H_2O$, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $NiSO_4 \cdot 7H_2O$ and Co-acetate. After 30 min. incubation at 40°C, a 5 ml. aliquot was pipetted and the enzyme activity

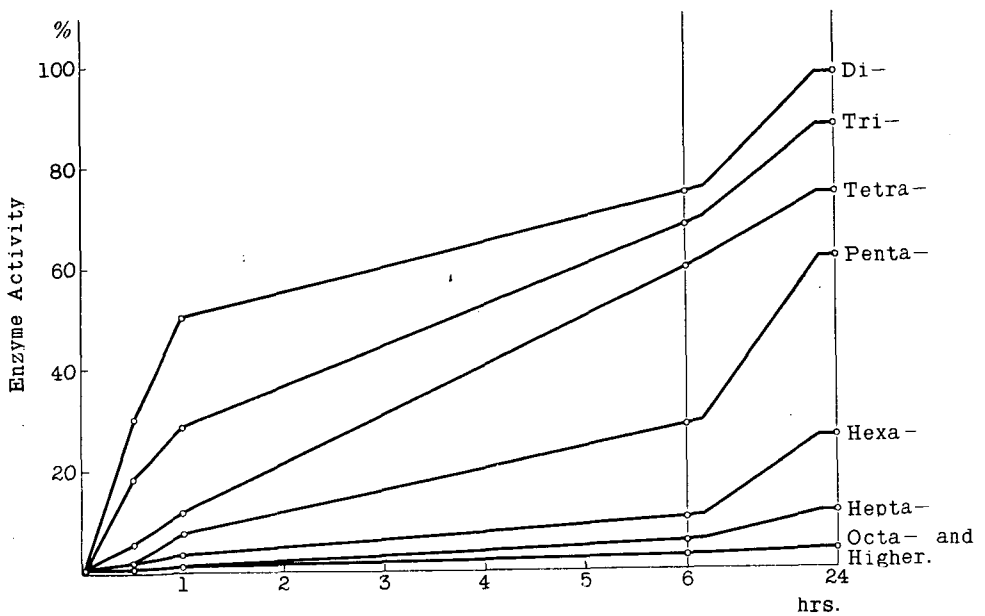


Fig. 11. Action of Crystalline Takamaltase on Isomalto-oligosaccharides.

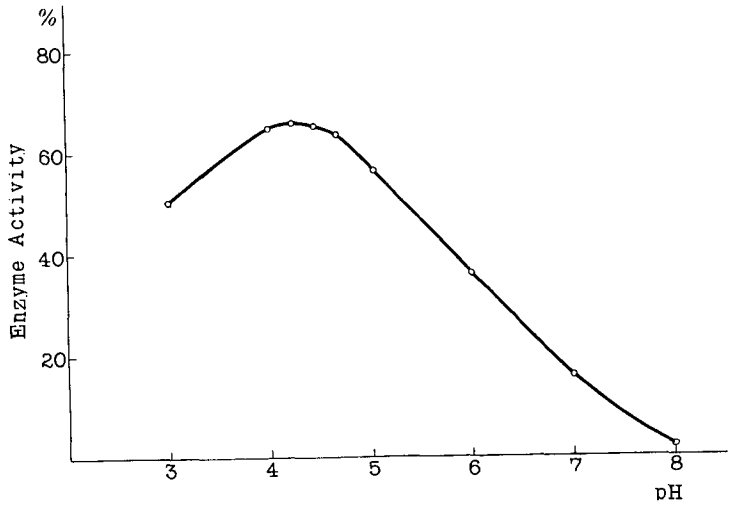


Fig. 12. pH-Activity Curve in Takamaltase Action.

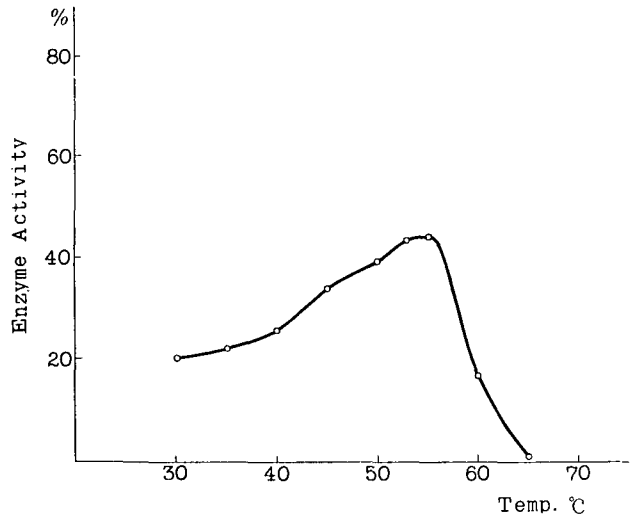


Fig. 13. Temperature-Activity Curve in Takamaltase Action.

was assayed. In this period of incubation Hg^{++} and Cu^{++} inhibited the enzyme at a extent of 100% and 80%, respectively. All the other metal ions tested exhibited no effect on the enzyme activity.

Sulfhydryl reagents, e. g. p-chloromercury-benzoate and iodoacetate were also used for observing the inhibitory action on the enzyme. At 10^{-4} and

2×10^{-4} M concentrations of the reagents they had no effects on the activity and glutathione exhibited no accelerating action. The procedure used for determining the effects was the same as that for metal ions except for the concentrations of the sulfhydryl reagents.

§ I-2-3. Experimental Results obtained by using Crystalline Maltase of Koji- Mold, and Comparison with purified Amyloglucosidase:

Crystalline maltase of Koji hydrolyzes maltose and phenyl-α-glucoside.

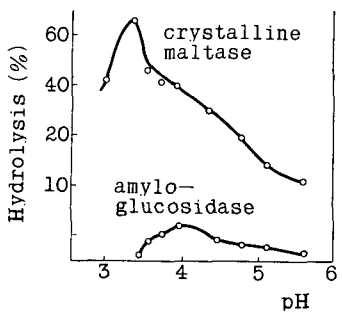


Fig. 14. Activity-pH Curves of Maltase and Amyloglucosidase of Koji.

0.5% enzyme 0.5 ml., 2.3% maltose 2 ml., acetate (0.5 M) 5 ml: Total 12.5 ml: 37°C, 30 min.

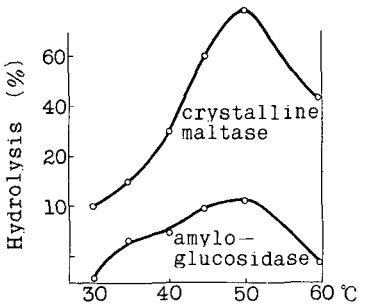


Fig. 15. Activity-Temperature Curves of Maltase and Amyloglucosidase of Koji.

pH 3.4 for maltase, 4.1 for amyloglucosidase. Contents of the incubation flasks are the same as Fig. 14; 20 min.

TABLE 6. Substrates of Mold Maltase and Amyloglucosidase

crystalline maltase			
Time of hydrolysis hours,	maltose %	phenyl-α-glucoside %	methyl-α-glucoside %
0.5	62	±	0
1		4	0
18		63	3

amyloglucosidase			
Time of hydrolysis hours,	maltose %	phenyl-α-glucoside %	methyl-α-uglcoside %
0.5	21	0	0
1		0	0
18		0	0

0.5% enzyme soln.: 1.5 ml. substrates: 0.05 M-3 ml.: accetate: 8 ml.; Total 17.5 ml. pH: 3.4 for maltase, 4.1 for amyloglucosidase; temp: 40°C

Hydrolysis of phenyl-α-glucoside is markedly slower than that of maltose. Optimum pH is 3.4 (Fig. 14) and optimum temperature 50°C. (Fig. 15)

A comparison of both activities of maltose-hydrolysis in mold, as shown in Table 6 and Fig. 14, indicates that there are considerable differences in substrate specificity and pH optimum. Phenyl- and methyl-α-glucoside are not hydrolyzed by amyloglucosidase and the opt. pH of this enzyme is 4.1. However, the opt. temperature is the same for both enzymes (Fig. 15).

§ I-2-4. Discussion.

As stated in the introduction of this Chapter, the α-glucosidase activity in mold is dependent upon a combined system, including maltase and various types of amyloglucosidase. And, a remarkable variation in activity of each enzyme in such a diastic enzyme system is generally observed among the materials to be assayed. Such facts had led to a lack of agreement in previous studies on the substrate specificity of mold α-glucosidase. LEIBOWITZ[45] classified maltases from various sources into glucosido-type (yeast, animal) and gluco-type (mold). Recently MATSUSHIMA[55] has reported on the substrate specificity of purified Takamaltase freed from amylases and the results were practically identical with the findings obtained in present study.

The present experiments using crystalline Takamaltase showed that the enzyme acts widely upon α-oligosaccharides and α-glucosides. These results as well as the difference of rate of hydrolysis of these substrates lead to a conclusion that Takamaltase has affinities not only to the glucon moiety but also to the aglucon moiety of the substrate.

Among α-glucosides, the rate of hydrolysis of alkyl-α-glucosides was extremely low as compared with that of saccharides or aryl-α-glucosides. The size of the aglucon moiety of alkyl-α-glucosides seemed to be related to the enzyme action and this was also observed with aryl-α-glucosides. Thus the hydrolysis rate of substrate increases with the size of the aglucon moiety.

In saccharides the enzyme hydrolyzed isomaltose in addition to maltose. This was also the case with both series of the oligosaccharides, thus indicating the cleavage of both α-1,4 and α-1,6 linkages.

The enzyme ceased its action on malto- and isomalto-oligosaccharides at seven to eight units of hexose in the molecule. This shows one of the most distinct differences between maltase as oligosidase and amyloglucosidase as polyase. As will be described in Chapter 4, mold maltase acts on maltose to synthesize many oligosaccharides, either dextran series or panose series of saccharide. The highest polymers of such saccharides formed were dextranheptaose and 4-α-dextranheptaosyl-D-glucose. It has not been clarified whether

or not such hepta and octasaccharides were the limiting factors of polymerization. But now, considering the transglucosidation reaction as being reversible, it can be concluded that the highest polymer to be synthesized is hepta- to octasaccharide.

In maltose derivatives maltosazone and maltobionic acid were substrates for this enzyme. According to Matsushima[56], methyl-α-maltoside was hydrolyzed by purified Takamaltase, forming glucose and methyl-α-glucoside. Those maltose derivatives which were masked in their reducing group seemd to be attacked by this enzyme but the rate of hydrolysis decreased remarkably. Among glucosylfructoses the cleavage of α-glucosidic linkage was observed in turanose, maltulose and isomaltulose. The cleavage of α-glucosidic linkage in sucrose has been observed only in yeast α-glucosidase action.

α-Heteroglucosides, and reducing α-disaccharides and their derivatives generally were attacked by mold maltase. These compounds have a semiacetal linkage on the glucon side and an etheric linkage on the aglucon side of the oxygen bridge. On the other hand, in non-reducing disaccharides, which were quite inert to the enzyme, both sides of the oxygen bridge are semiacetal linkage. Such a difference of mode of existence of linkages would give an explanation of substrate specificity of mold maltase. That is, it is neccessary for the enzyme action that both the existence of α-cofiguration and the existence of a semiacetal and an etheric linkage be present in both sides of oxygen bridge.

The optimal pH range of crystalline Takamaltase was 4.2～4.6. This was quite different from 3.8 for crystalline maltase isolated from Koji mold. This is probably due to the difference of the strain of Aspergillus oryzae. But the substrate specificity was identical in both. Takamaltase lost its activity on the alkaline side and heat stability was considerably higher. These findings were also practically identical with maltase from Koji mold.

By exhibiting no inhibition by sulfhydryl reagents, Takamaltase does not appear to be a sulfhydryl enzyme.

§ I–2–5. Summary.

The substrate specificity of crystalline mold maltase was investigated.

The enzyme acts widely upon α-heteroglucosides or saccharides. Aryl-α-glucosides were hydrolyzed much faster than alkyl-α-glucosides. The enzyme still acts on the maltose derivatives masked in the reducing group. But in glucosylfructoses turanose, maltulose and isomaltulose were attacked at a slow rate while the enzyme was quite inert to sucrose. Malto- and isomalto-oligosaccharides were also hydrolyzed and the enzyme ceased its action at seven to

eight units of hexose in both series of oligosaccharides.

The optimum pH range of Tkamaltase was 4.2～4.6 and its optimum temperature 50°～55°C. Cu^{++} and Hg^{++} strongly inhibited the enzyme activity but the other metal ions tested had no effect. The enzyme was thought to be not a sulfhydryl enzyme because of the effects of SH-reagents on its activity.

The optimum pH of maltase of Koji-mold was 3.8 and different from 4.2～4.6 range for Takamaltase. Substrate specificity and temperature dependence were practically same as Takamaltase.

Chapter 3

Transglucosidation Action of Crystalline Mold Maltase. (I) Transfer of Glucose from α-Heteroglucoside to Alcohols and Sugars.

§ I-3-1. Preface :

In Chapter 2 the substrate specificity of mold maltase was almost clarified by using the crystalline enzyme. But in certain aspects such as the comparative rates of hydrolysis of saccharides and α-glucosides or the limitation of polymerization of saccharides to be attacked, the results obtained were essentially identical with those of the crystalline enzyme described as "transglucosidase" of Aspergillus niger[85].

On the other hand, in many glucosidases it has been found that the enzyme acts on the substrate in the presence of some acceptors to transfer the glucosyl residue of the substrate to these acceptors. Accordingly hydrolysis and transglucosidation may be interpreted in terms of acceptor difference.

From such point of view investigations of transglucosidation action of mold maltase were indicated. The experimental results showed that the enzyme acts on α-heteroglucoside to transfer the glucosyl residue to several alcohols and sugars.

These findings (this chapter) and the results of experiments on transglucosidation on hologlucoside, maltose (chapter 4) or on glucosyl transfer to acceptor fructose (chapter 5) showed that the crystalline maltase of mold may be considered as a transglucosidase.

Chapter 3 deals with the transfer of glucose from α-hetero glucosides to acceptors.

§ I-3-2. Glucosyl Transfer from p-Nitrophenyl-α-D-Glucoside (PNPG) to Fatty- and Aryl-alcohols :

For a quantitative test, PNPG was used as the glucosyl donor and methanol,

ethanol, isopropanol, n-butanol, ethyleneglycol, phenol and p-cresol as acceptors.

Conditions of the reaction were as follows; PNPG, M/300; acceptor, M/150; acetate buffer, (pH 5.0) M/100; enzyme soln. (0.5%), 0.5 ml. in 10 ml. of reaction mixture. The mixture was incubated at 40°C for two hours. After incubation liberated glucose and PNP were determined. p-Nitrophenol liberated from the donor, PNPG, was determined electrophotometrically at 400 mμ and the liberated glucose by reduction.

The degree of transfer of glucose is expressed as follows;

$$\text{Degree of glucosyl transfer } (\%) = \frac{(\text{p-nitrophenol, liberated}) - (\text{glucose, liberated})}{\text{p-nitrophenol, liberated}} 100$$

TABLE 7. Transfer of glucose from PNPG to fatty- and arylalcohols by the action of crystalline mold maltase

Acceptor	Glucose, liberated %	PNP liberated %	Degree of transfer %
none	8.88	8.95	0.8
methanol	3.60	9.58	62.4
ethanol	5.85	20.19	71.0
isopropanol	4.80	19.05	74.7
n-butanol	4.05	10.65	61.9
ethyleneglycol	2.25	10.95	79.5
phenol	2.50	9.78	74.4
p-cresol	2.85	9.41	69.7

The condition of reaction is described in the text.

The results (Table 7) indicated that the action of glucosyl transfer occurred. No selection among the acceptors was found in these experiments and the degree of transfer was almost 60~70%. In case of the absence of the acceptor the amount of PNP liberated was higher than that of glucose liberated and, this also indicated that transglucosidation action had occurred. In this case some parts of the glucose liberated act as an acceptor of glucosyl residue of PNPG to produce the disaccharide, isomaltose. This reaction will be described in later section.

§ I-3-3. Isolation of α-Ethylglucoside formed by Enzymic Transfer of Glucose:

An experiment on the isolation of the transglucosidation product was

performed to demonstrate direct evidence for enzyme action. In this experiment phenyl-α-glucoside was used as the donor and ethanol as the acceptor. Two g. of phenyl-α-glucoside was dissolved in 100 ml. of water and 5 ml. of ethanol were added. Five ml. of the enzyme solution was added to the mixture and the pH was adjusted to 4.0 with 1N-acetic acid. The reacion mixture was incubated at 40°C for thirty hours and during this period phenyl-α-glucoside was completely split. The solution was subsequently heated in a boiling water bath for ten min. and filtered. The filtrate was shaken with ether repeatedly to remove phenol and then glucose was removed from the subsequent solution with bakers' yeast. After centrifugation the supernatant was heated in a boiling water bath and filtered. The filtrate was concentrated to dryness under diminished pressure and extracted with 20 ml. of hot ethanol. Ethanol was evaporated to dryness on a water bath and the residue was treated with 5 ml. of boiling water and the extract was cooled. The ethyl-α-glucoside obtained was recrystallized from water. Yield; ca. 50 mg, m.p. 111~114°C. Published value. 113~114°C.

§ I-3-4. Evidence for Absence of Synthetic Reaction from Glucose and Alcohols by the Enzyme Action:

Although the results described above present evidence for the glucosyl transfer, there still remains the question of whether or not the synthetic reaction of glucoside, and not the glucosyl transfer, with the alcohol used as the acceptor and glucose liberated by the enzyme action had taken place.

An attempt to resolve this question was made using a solution of glucose

TABLE 8. Evidence for the Absence of Shynthesis of Glucoside from free Glucose and Alcohol by Enzyme Action

	Time of incubation	Glucose determined
pH 3	3 hrs.	10.2 mg
	15	10.1
	24	10.1
pH 5	3	10.2
	15	10.5
	24	10.3
pH 7	3	10.1
	15	9.9
	24	10.3

and methanol. One ml. of 5% glucose solution, 0.25 ml. of enzyme soln. and 0.5 ml. of M/15 citrate buffer were mixed and made up to 4 ml. with water. To this solution 1 ml. of methanol was added and incubated at 37°C. No decrease of glucose was observed after twenty-four hrs. incubation in the range of pH 3~7, indicating that the synthesis of glucoside had not taken place.

§ I-3-5. Transfer of Glucose from PNPG to Glucose, Fructose and Mannose:

In the hydrolysis of PNPG in the absence of acceptor alcohols, as described in the preceding section, the amount of PNP liberated was higher than that of glucose liberated. This observation is to be interpreted as a result of transglucosidation action and subsequent formation of oligosaccharide.

In order to demonstrate the oligosaccharide formation, experiments were conducted by using glucose, fructose and mannose as acceptors and the product was checked by paperchromatography.

Thirty mg. of PNPG was dissolved in 5 ml. of water and then were added 0.5 ml. of 0.5% enzyme soln, 0.5 ml. of M/15 citrate buffer (pH 4.0) and 1 ml. of 5% glucose soln.. The reaction mixture was incubated at 30°C and its reducing power was estimated at intervals. The decrease in reducing power (calculated as glucose) after fifteen hrs. was 3% and 14% after 24 hrs. Glucose and isomaltose were detected by paperchromatography after 24 hrs. incubation.

The same experiments were conducted by using fructose and mannose as

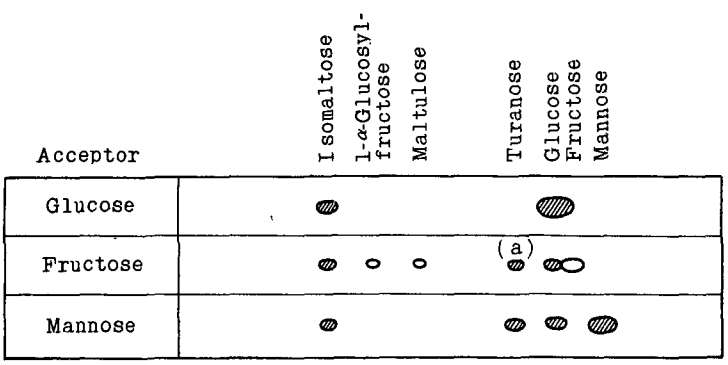


Fig. 16. Paperchromatogram of Sugars produced from PNPG and Aceptor Sugars by Crystalline Mold Maltase.

Solvent: Butanol-pyridine-water (6.4 : 3) Multiple development by ascending.

○: detected with aniline-phthalic acid.

▨: detected with anisidine-phsphoric acid.

(a) was detected with both the above reagents.

the glucosyl acceptors. Paperchromatography showed the formation of certain oligosaccharides by the detection of three unknown spots as well as spots of the monosaccharides and isomaltose, when fructose was used as acceptor. However, in the case of mannose there was only one spot which was assumed to be a transglucosidation product other than isomaltose. (Fig. 16)

These three unknown sugars which were formed in the presence of fructose were identified as maltulose, turanose and 1-α-glucosylfructose by their isolation as described in Chapter 5.

The detection of isomaltose by paperchromatography in this experiment leads us to the conclusion that the glucosyl residue of substrate is transferred to the 6-position of the acceptor glucose. This has been further confirmed by isolation and identification of many oligosaccharides possesing α-1,6-glucosidic linkages produced by the action of this enzyme on maltose, which will be described in the following chapter.

§ I–3–6. Summary.

1. Glucosyl transfer from p-nitrophenyl-α-D-glucoside to several alkyl- and arylalcohols was observed with crystalline mold maltase. α-Ethylglucoside was isolated as a transglucosidation product, indicating the evidence of such action.

2. No reaction of α-glucoside synthesis from free glucose and alcohols could be observed with mold maltase.

3. By using glucose, fructose and mannose as acceptors, the glucosyl residue of p-nitrophenyl-α-D-glucoside was transferred to these acceptor sugars. Paperchromatography showed the formation of isomaltose from glucose, and maltulose, turanose and 1-α-glucosylfructose from fructose, and one unidentified oligosaccharide from mannose, respectively.

Chapter 4.

Transglucosidation Action of Crystalline Mold Maltase. II. Action of the Enzyme on Maltose and Isolation of Transglucosidation Products by Carbon Column Chromatography.

§ I–4–1. Preface :

In the preceding Chapter the author described the glucosyl transfer from α-heteroglucosides to alcohols and sugars by crystalline mold maltase. The enzyme was recognized as a transglucosidase.

Transglucosidases of mold have already been described by PAZUR and FRENCH[67,68], PAN, NICHOLSON and KOLACHOV[73], and TUJISAKA and FUKUMOTO[85].

These workers have investigated the action of transglucosidase on maltose principally and observed the formation of oligosaccharides from the disaccharide.

The author accordingly examined the transglucosidation action of maltase on holo-glucoside, maltose, as a continuance of the previous experiment on heteroside. The results indicated that maltase of Koji or Takamaltase is identical with the transglucosidase described by the above investigators.

However, in present experiment, penta-, hexa- and heptasaccharides and presumably octasaccharide were found and isolated. These have hitherto not been observed in the maltose-transglucosidase reaction.

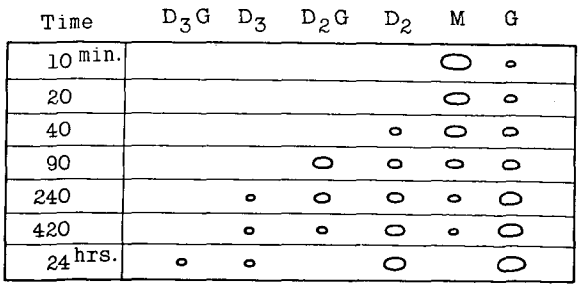
This Chapter deals with the qualitative and quantitative observation of maltase-maltose reaction, and the separation of products by carbon column chromatography. Furthermore a discussion on the mechanism of transglucosidation is also presented.

Abbreviations :

G : glucose, M : maltose, D_2 : isomaltose, D_3, D_4, D_5, D_6 and D_7 : dextrantri-, tetra-, penta-, hexa-, and heptaose, D_2G : panose, D_3G, D_4G, D_5G, D_6G and D_7G : 4-α-[-dextrantri-, tetra-, penta-, hexa- and heptaosyl-]-D-glucose.

§ I–4–2. Paperchromatographical Observation of Transglucosidation Action of Crystalline Maltase on Maltose.

Preliminary experiments were performed by application of paperchromatographical analysis. The chromatogram showed that the enzyme acted on maltose to form several oligosaccharides as well as the hydrolysis product, glucose, and isomaltose formed remained throughout the incubation period, while panose almost disappeared after twenty-four hrs. The detection of oligosaccharide higher than D_4 was unsuccessful in this experiment, although these sugars were isolated by column chromatography as described in later section. (Fig. 17).



Maltose, 0.1 M; 0.5% enzyme solution 1 ml; acetate (pH 4.0) 3 ml. 10^{-2} M in 7 ml. of the reaction mixture. 45°C.

Fig. 17. Paperchromatogram of the products by maltose-maltase reaction in various periods of incubation.

Quantitative analysis of the products was also made by paperchromatography and subsequent extraction of each sugar. Saccharides higher than panose were estimated as a whole, because of a lack of distinctness in the detection of spots. (Table 9)

TABLE 9. Quantitative analysis of the products observed in paperchromatogram (Fig. 14)

Time of reaction	Glucose	Maltose (g)	Isomaltose (mg)	Panose	Dextran-triose and others (mg)
0 min.		0.26			
10	0.01	0.25			
40	0.02	0.27			
90	0.05	0.20	0.01	0.02	0.02
240	0.10	0.09	0.04	0.03	0.01
420	0.16		0.05	0.03	0.01
24 hrs	0.19		0.04	0.00	0.03

The sugars were extracted with 5 ml. of boiling water from the corresponding spot of the chromatogram; spotted with 0.01 ml. soln.; Conditions of the reaction were the same as those shown in Fig. 14.

§ I-4-3. Transglucosidation Action of the Enzymes at various pH values and Temperatures and in the presence of Metal Ions:

In the course of the experiments on pH and temperarture dependence of the enzyme and on effects of metal ions or SH-reagents, described in Chapter 2, the transglucosidase activity of the enzyme was examined along with its hydrolysis activity.

Paperchromatographical observation on oligosaccharides formation showed that the two activities always paralleled each other.

§ I-4-4. Oligosaccharides Formation by Transglucosidation Action in the Presence of Glucose Oxidase:

Five ml. of 2% maltose, 2 ml. of 0.2 M acetate buffer (pH 5.0) and 2.5 ml. of 2% glucose oxidase solution were mixed and 0.1 g of calcium carbonate was added to the mixture. The mixture was made up to 12.5 ml. with water and kept for 30 min. at 37°C and aerated. Then 0.5 ml. of 0.1% crystalline Takamaltase solution was added and allowed to stand at the same temperature under aeration. After 2 hrs. incubation the reaction mixture was heated in a boiling water bath for 5 min. and filtered. The filtrate was concentrated to

a syrup and the sugars were extracted with 70% hot alcohol. After removal of alcohol by destillation the sugars were assayed by paperchromatography.

Paperchromatograms showed that in both cases in which glucose oxidase was present or absent isomaltose, panose and probably higher oligosaccharides were formed while the glucose was completely split off in the presence of glucose oxidase. A quantitative determination of each sugar was not conducted.

§ I-4-5. Separation of Transglucosidation Products in Maltose- Maltase Reaction by Carbon Column Chromatography :

The experiment was first performed using 5 g. of maltose for the purpose of the separation of the reaction products and quantitative estimation. In the next experiment the author attempted to prepare the products on a large scale and thus several saccharides which did not appear in the first experiment were found.

A. *Experiment 1.*

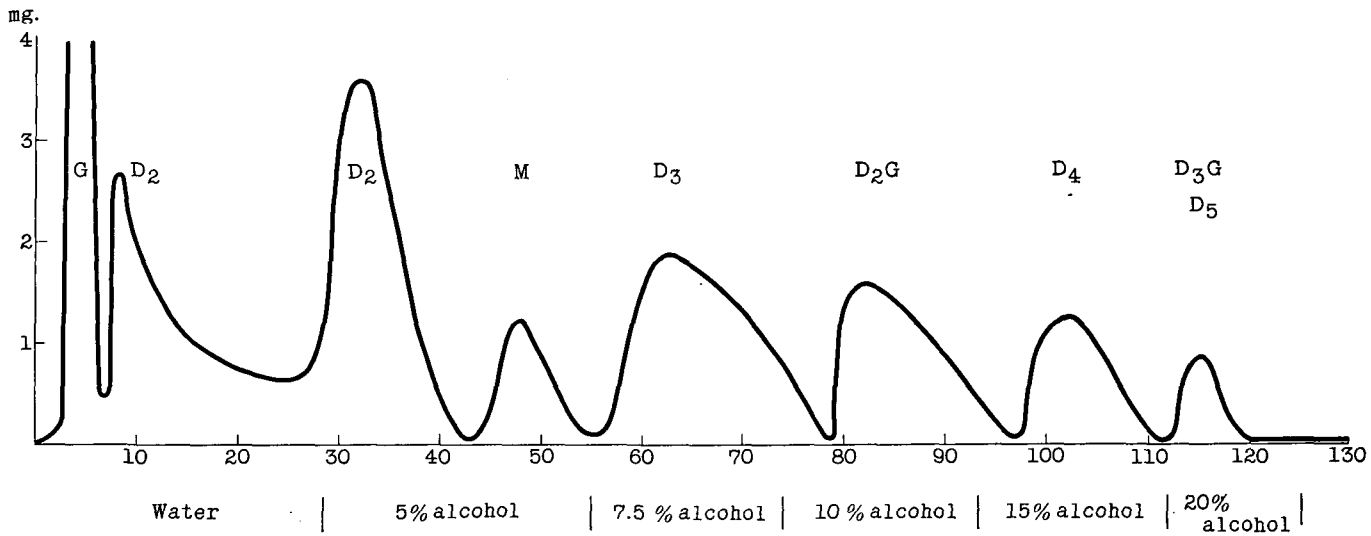
Five g. of maltose in 100 ml. of 0.01 M acetate buffer (pH 4.0) was treated with 10 mg. of crystalline maltase isolated from "Koji". The reaction mixture was allowed to stand at 35°C for twenty hrs. At the end of this period about 18% of maltose remained in the reaction mixture. (Table 10) The reaction

TABLE 10. Determination of amounts of the products obtained by the maltase-maltose reaction

Glucose	1.79 g	35.8%
Isomaltose	1.09	21.8
Maltose, remaining	0.91	18.2
Dextrantriose	0.46	9.2
Panose	0.46	9.2
Dextrantetraose	0.42	8.4
4-α-[dextrantriosyl]-D-glucose and dextranpentaose	0.12	2.4

Calculated as glucose after hydrolysis with 4 N-HCl, 6 hrs.

products were adsorbed and chromatographed on a carbon-celite column (34 × 180 mm). (Fig. 18) Elution was first made with water to remove glucose, and isomaltose appeared in the successive water-effluents soon after the end of elution of glucose. Subsequently, the peak of the isomaltose fraction was obtained in two parts, in both water and 5% alcohol effluents. In the last fraction, eluted with 20% alcohol solution, D_3G and D_5 were detected by paper-chromatography. These two sugars were separated by paperchromatography



Each 10 ml. of effluents were collected, except for each 50 ml. of No. 1 to No. 15.
Sugars were determined reductometrically and calculated as glucose.

Fig. 18. Chromatogram of Transglucosidation Products on Carbon Celite Column. (Experiment 1)

and prepared by subsequent extraction, as will be described later.

The amounts of each saccharide formed were determined as glucose after the acid hydrilysis of a portion of each fraction collected. (Table 10)

Isomaltose: The combined eluates (No. 7～41) were concentrated under diminished pressure and the syrup obtained was dissolved in hot methanol. The filtered methanol solution was evaporated to dryness in vacuo. As the residue was still syruppy, it was treated with ethanol. Isomaltose was obtained as a white powder. Yield, 0.7 g. $[\alpha]_D = +117.7°$C. Published value, 120°[50], 119°[68]. Its mobility in paperchromatography was identical with pure isomaltose.

Dextrantriose: The combined eluates (No. 58～77) were treated in the same manner as in the isomaltose fraction. After partial acid hydrolysis, G, D_2 and the remaining D_3 were detected by paperchromatography. $[\alpha]_D = +132°$, Published value, +134°[73]. Yield, 310 mg.

Panose: Panose was crystallized from methanol after undergoing the same treatment as fraction D_2. The Rf value was in accordance with pure panose. $[\alpha]_D = +152°$, published value, +154°[73], +150°[68]. Yield, 300 mg.

Dextrantetraose: Small amounts of panose were contaminated in this fraction (No. 97～110). The contaminant was removed by re-chromatography on a carbon column. The isolation technique of D_4 was the same as described above. After partial acid hydrolysis, G, D_2, D_3 and the remainig tetraose were identified by paperchromatography. $[\alpha]_D = +149°$. Yield, 250 mg.

4-α-[Dextrantriosyl]-D-glucose : This fraction (No. 114～118) was concentrated under diminished pressure. The syrup obtained was placed in a streak on filterpapers (5 × 35 cm) and the resolution of the saccharides was accomplished by the multiple ascending technique. Then both sides of the resulting chromatogram were cut, sprayed and used as a mark to determine the corresponding bands of the two sac-

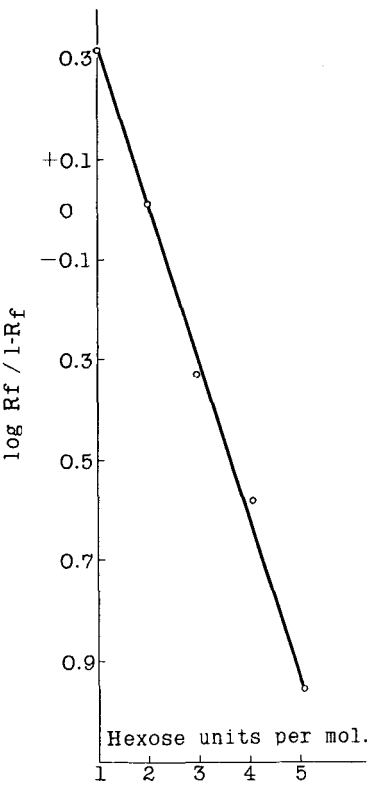


Fig. 19. Linear Relation of Mobilities of the Dextran Series of Transglucosidation Products on Paper.

charides. The saccharides were extracted with boiling water from the paper. The combined extracts from eleven chromatograms were concentarted under diminished pressure and the saccharides were precipitated with ethanol and dried. Yield; D_3G, 40 mg. and D_5, 50 mg. The acid-hydrolysis products were G, M, D_2, D_2G, D_3 and the remainig tetraose from the former, and G, D_2, D_3, D_4 and the remainig pentaose from tha latter.

In this experiment D_5 was the highest polymer of glucose possessing only 1,6-α-glucosidic linkages, and mobilities of the dextran series of oligosaccharides on paper showed a linear relation, which had been experimentally found by French et al. (Fig. 19)

Maltase Action on Transglucosidation Products: By treating the transglucosidation products with maltase it was observed with paperchromatography that these saccharides function as cosubstrates in the maltase-maltose reaction. For instance, D_3 was formed from D_2, and D_3 and D_3G from D_2G. Whereas with D_5 or D_3G no any substances except hydrolysis products were found. These results as well as the acid-hydrolysis products and specific rotation values are summarized in Table 11.

TABLE 11. Products obtained from by partial acid hydrolysis of maltase action on transglucosidation products, and their specific rotation values

	Products		$[\alpha]_D$
	Acid hydrolysis	Maltase action	
Isomatose	G, (D_2)	G, (D_2), D_3	+117.7°
Dextrantriose	G, D_2, (D_3)	G, D_2, (D_3), D_4	+132°
Panose	G, D_2, M, (D_2G)	C, D_2, D_3, (D_2G), D_3G	+152°
Dextrantetraose	G, D_2, D_3, (D_4)	G, D_2, D_3, (D_4), D_5	+149°
4-α-[dextrantriosyl]-D-glucose	G, M, D_2, D_2G, D_3, (D_3G)	G, D_2, M, D_3, (D_3G)	+191°
Dextranpentaose	G, D_2, D_3, D_4, (D_5)	G, D_2, D_3, D_4, (D_5)	+158°

Acid hydrolysis: 0.1 N-H_2SO_4, 3 hrs, 100°C

Maltase action: 40 mg. of substrate, 0.5 ml. of 0.5% enz. soln.

Brackets represent the remaining saccharides.

B. *Experiment 2.*

The purpose of this work was preparation of the transglucosidation products on a large scale. As the reaction of maltase has a tendency to proceed to hydrolysis when the ratio of the enzyme to the substrate is high the ratio was markedly decreased.

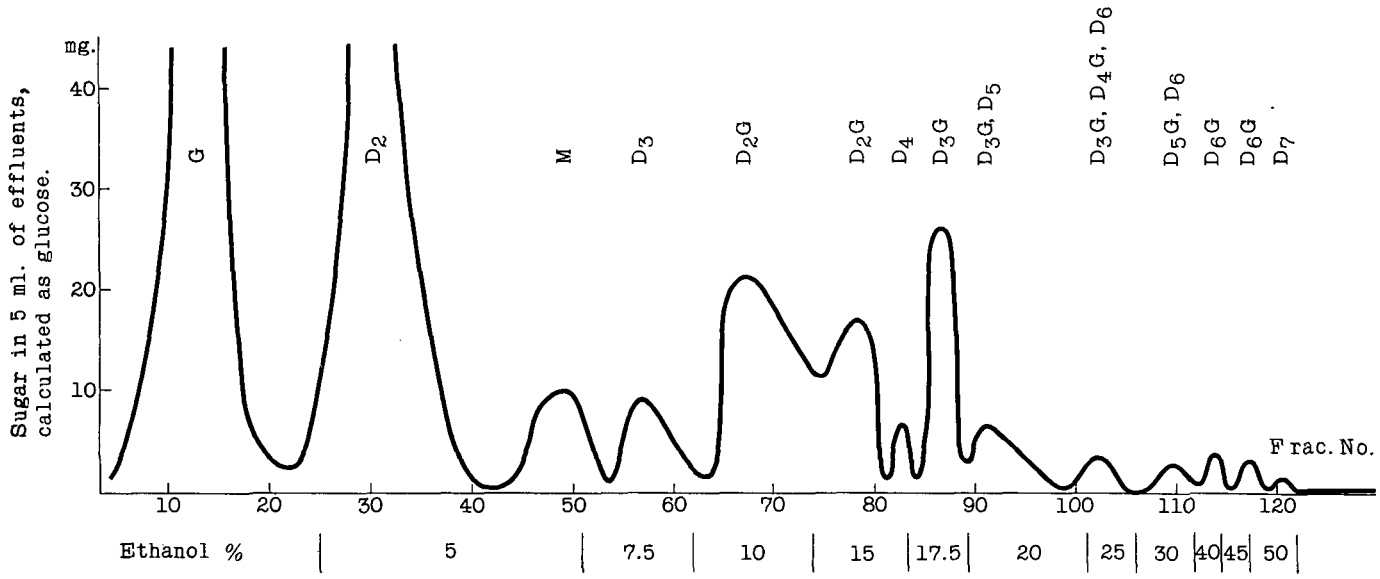


Fig. 20. Experiment 2. Chromatography of Transglucosidation Products on Carbon Celite Column.

Fifteen mg of crystalline Takamaltase and 115 g. of maltose were dissolved in 1.5 l. of water and buffered with 15 ml. of 0.5 M acetate (pH 5.0). The reaction mixture was allowed to stand for thirty hours at 30°C.

The reaction products were adsorbed and chromatographed on a carboncelite column (10 × 50 cm) in the same manner as described in Experiment 1. The effluents were collected in each 250 ml. portion and a elution curve was made by measuring the reducing power (calculated as glucose). (Fig. 20)

The bulk of D_3G was separated from D_5 at a 17.5% concentration of ethanol (cf. Experiment 1).

The procedures of preparation of D_2, D_3, D_4, D_5, D_2G and D_3G were the same as described in Experiment 1. Yield: D_2, 24 g; D_3, 8 g; D_2G, 21 g; D_4, 2.6 g; D_3G, 11.5 g; and D_5, 2 g. The approximate estimation of these sugars formed, calculated from reducing power, was as follows: G, 30.1%; M, 2.5%; D_2, 22.9%; D_3, 7.4%; D_2G, 17.4%; D_4, 2.3%; D_3G, 9.5%, and D_5, 1.8%.

25% Ethanol Fraction (No. 97～100): Three sugars were detected in this fraction by paperchromatography. Although the resolution and isolation were accomplished by the paperchromatographic technique described previously, the multiple descending technique was adopted.

One of saccharides obtained was D_3G contaminated. Yield; 65 mg. The other two saccharides were D_4G and D_6, and their tentative structure were suggested from the production of G, M, D_2, D_2G, D_3, D_3G and D_4 from D_4G, and D_2, D_3, D_4 and D_5 from D_6 by acid hydrolysis. Yield; D_4G, 220 mg.; D_6, 100 mg. $[\alpha]_D$: D_4G +212°; D_6, +178°.

30%～35% Ethanol Fraction (No. 103～107): This fraction consisted of a mixture of two saccharides, D_5G and D_6. In the paperchromatographical method for isolation of these sugars, the solvent was allowed to flow down for 48 hrs. The structure of D_5G was presumed from the production of G, M, D_2, D_2G, D_3, D_3G, D_4, D_4G and D_5 by hydrolysis with acid. D_6 was identified from its mobility on paper and its hydrolysis product from a comparison with D_6 isolated from the 25% ethanol fraction. Yield; D_5G, 80 mg.; D_6, 60 mg.

40% Ethanol Fraction (No. 109～110) and 45% Ethanol Fraction (No. 112～113): The paperchromatogram indicated that only one saccharide is contained in both fractions and that mobilities were the same. The saccharide was a heptasaccharide, D_6G, which produced C, M, D_2, D_3, D_4, D_5, D_6, D_2G, D_3G, D_4G and D_5G by acid hydrolysis. Yield; 370 mg (combined.). $[\alpha]_D$ = +226°.

50% Ethanol Fraction (No. 114～120): In this stage of elution, it was

difficult to observe the appearance of the peak by measurement of reducing power. After No. 119 practically no saccharides were found. Accordingly, the collection of 50% alcoholic eluates was made by combining Nos. 114～120 as a whole. The saccharide was prepared by the concentration of combined eluates and precipitation with alcohol. Yield; 300 mg. G, M, D_2, D_3, D_4, D_5, D_6, D_2G, D_3G, D_4G, D_6G were found by partial acid hydrolysis. Mobility on paper was less than D_6G. From these results, the saccharide obtained from this fraction was presumed to be an octasaccharide. However, as described below, the presence of D_7 in this saccharide is most probable.

In paperchromatography, the transglucosidation products moved on paper in the following order; G, M, D_2, D_2G, D_3, D_3G, D_4, D_4G, D_5, D_5G, D_6, D_6G, and D_7G. From this observation as well as the order of elution from the column, the probable appearance of D_7 was expected. However the mobility on paper was extremely low and was quite similar in such saccharides of higher molecular weight in the solvent system used. Subsequently by means of this method it was practically difficult to separate D_7 from D_7G.

§ I–4–6. Discussion

In a previous chapter on a study of the glucosyl transfer action of crystalline mold maltase from α-heteroglucoside to acceptors, it was reported that the enzyme is a transglucosidase. In the present study, this has been further confirmed by isolation and identification of many oligosaccharides produced by the action of this enzyme on α-hologlucoside, maltose.

The saccharides produced from maltose were di-, tri-, tetra-, penta-, hexa-, hepta- and octasaccharides: D_2, D_3, D_2G, D_4, D_3G, D_5, D_4G, D_6, D_5G, D_6G and D_7G. Among these eleven sugars, the saccharides possessing higher molecular weights such as dextranhexaose and 4-α-[dextran tetra-, penta-, hexa- and heptaosyl-]-D-glucose have been found and described for the first time.

Although a conclusive determination of the structure by preparing and analyzing the derivatives has not been accomplished, the experimental results present some evidence for the structure of these products formed from maltose. The mobilities of the saccharides on paper were in accord with those of pure substances and their specific rotation values were also practically identical with published values.

Most of the saccharides of higher molecular weight, which were found in this study, were analyzed by partial hydrolysis with acid and by subsequent paperchromatographical identification of the hydrolysis products. Naturally, in the partial acid-hydrolysis of such saccharides, some of the hydrolysis products obtained were the same as those characterized previously and tentatively in the

same manner (Experiment 2). That is, most of the saccharides used as standards for the mobilities on paper were those that had been first isolated in the present study and yet had no conclusive structure. However the chromatographical technique was useful enough for characterizing such saccharides as transglucosidation products with mold maltase. From the results previously described by many investigators[68,70,85], it is natural to expect that a series of two types of sugar would appear during the reaction, corresponding to the difference of glucosyl acceptors-hydrolysis products, glucose, or the substrate, maltose itself.

An interesting phenomenon was found experimentally in paper- and column chromatography, and this observation also served as another basis for characterizing the saccharides. A series of sugars such as $D_2 \sim D_6$ which only contain 1,6-linkages and another type such as $D_2G \sim D_6G$ which possesses a 1,4-linkage in the reducing terminal besides 1,6-linkage were eluted from the carbon-celite column and moved on paper alternately in the regular manner, as described below :

Order of elution : G, D_2, M, D_3, D_2G, D_4, D_3G, D_5, D_4G, D_6, D_5G
Order of mobility : G, M, D_2, D_2G, D_3, D_3G, D_4, D_4G, D_5, D_5G, D_6

In the two experiments on separation of the transglucosidation products, the amounts of each product differed greatly. This may be explained from difference in reaction conditions, especially, the ratio of enzyme to the substrate maltose. Since the reaction proceeds mildly when the ratio is low, the opportunities for liberated glucose to act as a glucosyl acceptor would be less than those of substrate maltose itself and, the products may also function as cosubstrates. Accordingly in such case, the panose series of saccharides would accumulate in preference to the dextran series.

Octasaccharides were found to be the highest polymer among the transglucosidation products. But there remained the possibility of contamination of dextranheptaose in this fraction as it was expected from the order of elution from the carbon column. At any rate, the yields of these saccharides of higher molecular weight were extremely low, as compared with the yields of di-, tri-, tetra- and pentasaccharides. Whether such degree of polymerization as octasaccharide is a limiting factor of polymerization or not, is a problem left for further investigation.

However, in view of the observation that the limitation of oligosaccharides as the substrate of this enzyme was hepta$\sim$octasaccharides, and furthermore, that transglucosidation is a reversible reaction, it might be concluded that the octasaccharides which were produced by transglucosidation in the present

experiments is the highest polymer to be synthesized in such reaction as transglucosidation by mold.

There are several reports on the mechanism[98,70,79] of oligosaccharide formation by enzymic catalysis in terms of transglucosidation. The following schematical representation is generally recognized in such a type of reaction as in mold maltase.

Step 1. $E+G-G^* \rightleftarrows E.G-G^*$

$E.G-G^* \longrightarrow EG+G^*$ (1)

Step 2. $G.E+G^* \rightleftarrows \begin{matrix} G \\ | \\ G^* \end{matrix} +E$ (2)

$G.E+G-G^* \rightleftarrows \begin{matrix} G \\ | \\ G-G^* \end{matrix} +E$ (3)

Step 3. $GE+\begin{matrix} G \\ | \\ G^* \end{matrix} \longrightarrow \begin{matrix} G \\ | \\ G \\ | \\ G^* \end{matrix} +E$ (4)

$\begin{matrix} G \\ | \\ G-G^* \end{matrix} \rightleftarrows \begin{matrix} G \\ | \\ G \\ | \\ G-G^* \end{matrix} +E$ (5)

E; Enzyme, G; Glucosyl residue, G*; Reducing terminal glucose, G-G*; α-1,4-Linkage, $\begin{matrix} G \\ | \\ G^* \end{matrix}$; α-1,6-Linkage.

Step 1. in the above equations shows that the glucosyl donor is glucosyl enzyme complex and that the acceptor is free glucose formed by hydrolysis of substrate.

In the present experiments no evidence was obtained which might be considered as contrary to the mechanism mentioned above. However an additional mechanism in such a type of reaction, especially in initial stage, might be proposed.

Notwithstanding that the reaction was proceeded in the presence of glucose oxidase, even under conditions in which free glucose does not exist throughout the reaction process, the transglucosidation products were formed.

From the results it could most probably be said that at least in the initial stage of reaction transglucosidation is not dependent on the equations (1) and (2) and that the acceptor is maltose, not glucose. Accordingly as in equation (3) panose will be first produced, but as the α-1,4-linkage is hydrolyzed much faster than the α-1,6-linkage isomaltose will also appear. (equation (6)).

Another possible mechanism is based on the paperchromatographical observation that isomaltose appears ahead of panose. If equation (7) represents this reaction[79], it necessitates proposing a reaction which is quite different from transglucosidation hitherto reported. Thus, it may be possible to consider that two moles of maltose may condense, forming α-1,6-linkage, on the surface of enzyme molecule and simultaneously may be hydrolyzed at two positions of α-1,4-linkage at the same time, resulting in the formation of isomaltose and two moles of glucose.

$$\mathrm{G.E+G{-}G^{*} \longrightarrow E+ \begin{matrix} \mathrm{G} \\ | \\ \mathrm{G{-}G^{*}} \\ \downarrow \\ \mathrm{G} \\ | \\ \mathrm{G^{*}} \end{matrix} + G^{*}} \qquad (6)$$

$$\mathrm{E+2\,(G{-}G^{*}) \longrightarrow E\begin{pmatrix} G{-}G^{*} \\ G{-}G^{*} \end{pmatrix} \longrightarrow \begin{matrix} \mathrm{G} \\ | \\ \mathrm{G^{*}} \end{matrix} + 2G^{*}} \qquad (7)$$

§ I-4-7. Summary

Isomaltose, dextrantri-, tetra-, penta- and hexasaccharides, panose, and 4-α-[dextrantri-, tetra-, penta-, hexa- and heptaosyl]-D-glucose were isolated as transglucosidation products formed in the reaction of crystalline mold maltase with maltose.

Chapter 5.

Transglucosidation Action of Crystalline Mold Maltase (III). Enzymic Synthesis of 1-α-Glucosyl-fructose, Maltulose and *Turanose*, and Determination of the Structure of 1-α-Glucosyl-fructose by Chemical Synthesis.

§ I-5-1. Preface:

As was described in Chapters 3 and 4, the transfer of the glucosyl residue of the substrate to the primary alcohol group, 6-position, of acceptor glucose or maltose, by the mold maltase results in the formation of oligosaccharides.

As the next step in the investigation of synthesis of gluco-oligosaccharides, fructose was now used as glucosyl acceptor for the purpose of a comparative study on acceptor specifity in transglucosidation action.

AVIGAD[2] has reported recently on the synthesis of glucosyl fructose from sucrose by an α-glucosidase of yeast and isolated maltulose, isomaltulose and 1-α-glucosylfructose. But according to YASUMURA[96] the glucosylfructoses formed

in such a reaction system were turanose, maltulose and an unknown disaccharide (this is probably 1-α-glucosylfructose).

In the present study on the crystalline Takamaltase-isomaltose reaction in the presence of fructose, the author isolated 1-α-glucosylfructose, maltulose and turanose. The same results had also been obtained by ISHIZAWA[31].

However, among glucosylfructoses, 1-α-glucosylfructose had the only probable structure and in the present investigation it was determined by chemical synthesis from α-acetobromoglucose and β-diacetonefructose in nitromethane in the presence of mercury-cyanide.

§ I–5–2. Separation of a Mixture of Disaccharides by Carbon Column Chromatography from the Reaction Mixture of Isomaltose, Fructose and crystalline Takamaltase.

Isomaltose, instead of maltose, was used as glucosyl donor for the reason that the glucooligosaccharides to be expected to form simultaneously from isomaltose would be only the dextran series. As a consequence, it reduces difficulties in the separation techniques of each sugar from reaction mixture, including gluco- and glucosylfructooligosaccharides.

Twenty g. of isomaltose, 20 g. of fructose, 80 ml. of 0.1–m acetate buffer (pH 4.5) and 2 mg. of enzyme were mixed and made up to 800 ml. (5% at final concentration of sugars) with water. The mixture was allowed to stand for 60 hrs. at 30°C. Throughout the course of the reaction the reducing power was increased but little and glucosylfructoses were detected by paperchromatography, indicating the development of transglucosidation action.

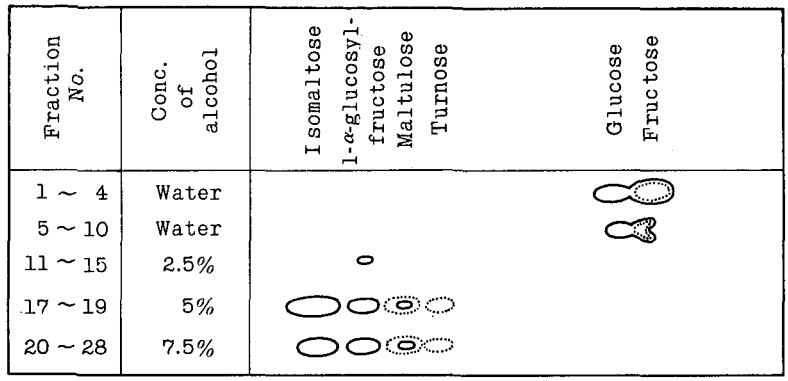
Paperchromatographical observation indicated that glucosylfructoses, in an incubated mixture, which were assumed to be disaccharides had similar Rf values to each other and existed between monosaccharides and isomaltose in the solvent system used. From the experience this observation further suggested that satisfactory separation of each sugar by carbon column chromatography would not be expected.

The author first tried to separate them as a mixture of disaccharides from other higher saccharides, expecting to facilitate resolution of each sugar by the application of cellulose column chromatography to the disaccharides mixture to be obtained.

The incubated mixture was concentrated to one-thirds of its volume and the sugars were adsorbed on a column (5 × 50 cm) of carbon-celite mixture and eluted with alcoholic solution by the method described in Chapter 4. Elution was made by collecting each 200 ml. and the elution curve was followed with measurement of reducing power of each fraction. After collecting every peak

the sugars were assayed by paperchromatography.

Fructose and glucose appeared in water effluents, No. 1～10, and in No. 11～15 of 2.5% alcoholic effluents only a small amount of 1-α-glucosylfructose was detected, thus showing a slow elution. Most of disaccharides were eluted in the 5% and 7.5% alcoholic eluates. (No. 17～19 and No. 20～28) (Fig. 21).



Ascending, five times;
: detected with aniline phthalate.
: detected with anisidine phosphate.

Fig. 21. Paperchromatographical Assay of Clucosylfructoses in the Eluates obtained by Carbon Column Chromatography.

After finishing the elution of disaccharides, the concentration of alcohol was raised to 22.5% and the higher oligosaccharides remaining in column were eluted all together. This fraction contained two kinds of glucosylfructotrisaccharide and dextranoligosaccharides. Their separation and characterization will be described in later section. (See page).

§ I-5-3. Separation of Glucosylfructoses by Cellulose Column- and Paperchromatography:

Disaccharide fractions (No. 17～28) were combined and concentrated under diminished pressure to 80 ml. To remove isomaltose from the mixture, 10 g. of bariumcarbonate and 2.5 ml. of bromine were added to the concentrate and the mixture was allowed to stand for five hrs. with occasional shaking. (Fig. 21)

As isomaltose was completely oxidized in this period of reaction bromine was removed by aeration and the colourless solution obtained was filtered. Two hundred and seventy ml. of alcohol was added to the filtrate (90 ml.) after which it was stood overnight at room temp. After centrifugation the supernatant was concentrated to remove alcohol. The reducing power indicated the

existence of 2.2 g of glucosylfructoses, showing that about a 30% yield of total disaccharides existed before bromine treatment.

Five g of cellulose powder (200~300 mesh) was added to the concentrate, which was then dried over sulfuric acid in vacuum desiccator. This adsorbate was placed on a top of a cellulose column (2.5 × 25 cm) and the sugars were developed by a mixed solvent of butanol-ethanol-water (10:1:1). After the flow of about 3,000 ml. of the solvent the column was cut into 2 cm lengths and the sugars were extracted with 15% alcohol from each piece.

However, as was shown in Fig. 22, this technique for the resolution of sugars was not very effective. Therefore paperchromatography was used for complete resolution of the sugars in portions obtained as a mixture.

Thus No. 6 and No. 7~10 were collected respectively and paperchromatography was applied according to the method described previously.

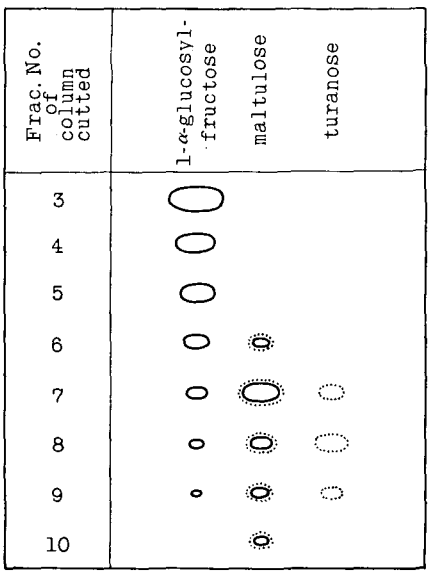


Fig. 22. Cellulose Column Chromatography of Glucosylfructoses.

The combined yields collected from each fragment were:

1-α-glucosylfructose 1.5 g; maltulose 200 mg; turanose 50 mg.

Identification of Maltulose and Turanose:

These two sugars were identified by their specific rotation.

maltulose $[\alpha]_D = +50.9°$ published value $+52.8°$
turanose $[\alpha]_D = +74.1°$ published value $+75°$

1-α-Glucosylfructose:

The sugar was thought to be 1-α-glucosylfructose for two reasons: (1) its mobility on paper was different from that of isomaltose and was somewhat lower (2) phenylhydrazone or α-methylphenylhydrazone was not formed by treating it with corresponding hydrazines.

However, specific rotation of this sugar was +34.5 and this value was considerably low as compared with the value +45.1 (2) or +49 (96) of the sugar which had hitherto been assumed to be 1-α-glucosylfructose and isolated as a transglucosidation product.

As the next step the author planned to synthesize chemically to determine the structure of this sugar.

§ I–5–4. Chemical Synthesis of 1-α-glucosylfructose from α-Acetobromoglucose and β-Diacetonefructose in Nitromethane in the presence of Mercury cyanide:

α-Acetobromoglucose was prepared according to the method described by SCHEURER et al.[80] and β-diacetonefructose according to PACSU et al.[72].

Four point five g of α-acetobromoglucose and 2.6 g of β-diacetonefructose were dissolved into 20 ml. of nitromethane and 1.2 g of powdered mercury cyanide was added to the mixture. The reaction mixture was shaken for seven hrs. at room temperature. The resulting dark brown solution was filtered and the residual insoluble matters were washed with a small quantity of nitromethane on the filterpaper. The filtrates were combined and the solvent was removed by destillation under diminished pressure. The syrup obtained (about 7 g) was dissolved in 20 ml. of hot benzene and kept at 0°C for 24 hrs. After filtration the benzene was distilled off under diminished pressure and the product was obtained in a syruppy state.

The syrup was dissolved in 50 ml. of methanol, and 1 ml. of 0.1 M sodium methylate solution was added. (At this time a small amount of insoluble yellow precipitate was formed and was removed by filtration). Deacetylation was performed by keeping the solution on a water bath for 15 min. just below its boiling temp. After neutralizing with acetic acid metanol was distilled off and then isopropylidene groups were removed by acetic acid according to the method described by PACSU et al.[72]. Thus 150 ml. of 5% acetic acid were added to the residue and heated on a boiling water bath for 4 hrs. The solution was neutralized with sodium hydroxide, concentrated, and the insoluble substances were filtered off. The aqueous solution thus obtained was chromatographed on a carbon-celite column (3.5 × 20 cm) by usual method.

The results of paperchromatographical assay are shown in Fig. 23. Glucose and fructose appeared in No. 3～6 (water) and unidentified, easily moved spots were detected in No. 6. 1-α-Glucosylfructose, which had the same mobility as the transglucosidation product, was eluted in a wide range of No. 8～16. However in No. 11～13 unknown and tailing spots were detected. Thus No. 8～10 and N0. 14～16 were combined and concentrated. 1-α-glucosylfructose was obtained as a snow-white powder by alcohol precipitation. Yield: about 60 mg. $[\alpha]_D = +32.5°$ (C=3)

Glucose and fructose were obtained by acid hydrolysis, and phenyl- or α-methylphenyl hydrozone was not formed.

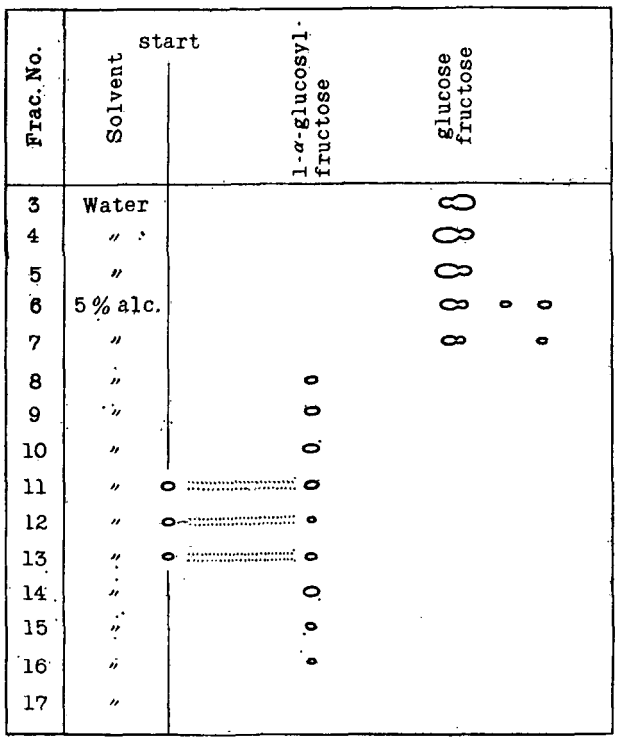
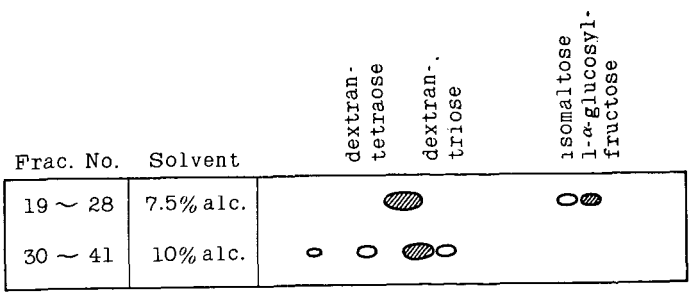


Fig. 23. Fractionation of Synthesized Sugar by Carbon Column Chromatography and Paperchromatographical Assay.



▨ : Glucosylfructo-oligosaccharide.

Fig. 24. Carbon- and Paperchromatograyhy of Transglucosidation Products other than Disaccharides.

§ I–5–5. Trisaccharides Other than Gluco-oligosaccharides:

Paperchromatographical assay of the sugars in 22.5% alcoholic eluate from carbon column chromatography showed the existence of dextran tri-, tetra- and pentaose, and twosorts of unkown saccharides which were assumed to be fructose-containing saccharides, judging from their behaviour toward coloring reagents and different mobilities on paper from dextran-oligosaccharides.

Resolution of these saccharides was conducted by using re-chromatography with a carbon column and subsequent application of paperchromatography.

A 22.5 per cent alcoholic fraction of the eluates obtained in the first passage of the carbon column was concentrated to remove alcohol. Eluation from the carbon-celite column (2 × 30 cm) was made by collecting each 20 ml. of eluate. (Fig. 24). A small amount of disaccharides was observed in the first 240 ml. of aqueous and then 80 ml. of 5% alcholic eluates.

The 7.5 per cent Alcoholic Fraction (200 ml.): An unknown trisaccharide and very small amount of isomaltose and 1-α-glucosylfructose existed in this fraction. The unknown sugar was separated by paperchromatography. Yield: 200 mg. Glucose, fructose, isomaltose and 1-α-glucosylfructose were determined by paperchromatography after acid hydrolysis (0.1–N H_2SO_4, 3 hrs, 100°C). This observation suggested that this sugar is 1-α-isomaltosyl fructose.

Ten % Alcoholic Fraction (220 ml.): In this fraction dextrantri- and tetraose and an unknown sugar which had a lower mobility than that of 1-α-isomaltosylfructose were detected by paperchromatography.

The products of partial acid hydrolysis (0.1 N H_2SO_4, 3 hrs, 100°C) of this unknown sugar were glucose, fructose, maltulose and isomaltose. Accordingly the tentative structure of the sugar was 4-α-isomaltosylfructose. Yield: 90 mg.

§ I–5–6. Discussion

1-α-glucosylfructose, maltulose and turanose were synthesized by the Taka-maltase-isomaltose reaction in the presence of fructose. 1-α-Glucosylfructose was obtained in the largest quantity of these three glucosylfructoses. This showed that fructose accepts the glucosyl residue of substrate at carbon- 1, 3 and 4 positions and the glucosyl transfer to 1-position, primary alcohol group, of fructose is far ahead of the other two secondary alcohol groups.

However, the glucosyl transfer to the primary alcohol group in 6-position of fructose was not observed. It is well known that in chemical reaction the primary alcohol group generally has preferential reactivity. In the transglucosidation action of mold maltase, such preferential reactivity was observed in the case of fructose as acceptor but it was rather surprising that selectivity of

primary alcohol groups, 1 and 6-position, was observed, especially considering the occurrence of glucosyl transfer to the 3 and 4-position of the secondary alcohol groups.

As was described in the previous Chapter the glucosyl residue of the substrate was transferred to acceptor glucose or maltose at only the primary alcohol group of 6-position (in maltose, 6-position of glucosyl moiety), forming isomaltose or panose.

YASUMURA[96] isolated 1-α-glucosylfructose, turanose and maltulose in the reaction of yeast α-glucosidase and sucrose, but according to AVIGAD[2] the products were 1-α-glucosylfructose, isomaltulose and maltulose, in which 1-α-glucosylfructose had no conclusive structure. Thus even in the reaction of the enzyme from same source the results obtained had not always been identical.

On explaining the acceptor specificity of transglucosidation of mold maltase, one could, at present, say only that it is phenomenal that glucose accepts the glucosyl residue of substrate only in 6-position, most distant from reducing group, while fructose accepts it at the nearest three positions around the reducing group.

Whether such behaviour generally occurs between aldose and ketose, regardless of configuration, or not, is an interesting and important problem left for further investigation.

It had been reported that hydrolysis of maltose by α-glucosidase is inhibited by some of monosaccharide, including glucose as a hydrolysis product[58]. In the present experiment the increase of reducing power was little throughout the course of incubation periods, while a lage amount of hydrolysis product, glucose, was formed. These findings demonstrated that such apparent inhibitory action of monosaccharides was not due to a competitive effect but rather to transglucosidation, involving synthesis of oligosaccharides.

Among glucosylfructoses, the structure of 1-α-glucosylfructose had not yet been determined and its probable structure was suggested by ; (1) inability to derive osazone ; (2) distinct Rf value on paperchromatography from other glucosylfructoses ; (3) dextro-rotation as compared with levorotation of 1-β-glu cosylfructose[76].

1-α-glucosylfructose prepared by transglucosidation as well as by chemical synthesis gave a remarkably lower $[\alpha]_D$ value than that previously reported. However, the specific rotation values were practically identical in both preparations in the present experiments.

Reports in the literature on the chemical synthesis of α-disaccharides were much less than those of β-disaccharides. 1-β-Glucosylfructose had already been synthesized by condensing acetobromoglucose and diacetonefructose[72] or dibenzal-

fractose[11] in the presence of silver oxide. But by using a mercury salt as a catalyzer it has been observed that α-disaccharide also formed with β-type[26,51,52]. The synthesis of 1-α-glucosylfructose was one of the examples of the availability of such a catalyzer in the synthesis of α-type of glucosylfructoses but the yield was very low. A good yield may be expected if BRIGL's anhydroglucose[10] is used as the starting material, by which maltose[46], sucrose[47] and trehalose[48] were successfully synthesized, but this method was not attempted.

Carbon column chromatography was applied to the final separation of the sugar synthesized chemically. As seen in Fig. (23), during the elution of the 1-α-glucosylfructose unknown and paperchromatographically immobile or tailing substances appeared.

These substances were not investigated and the author has no explanation of such abnormal behaviour on column chromatography at present.

§ I–5–7. Summary :

1). Using fructose as a glucosyl acceptor, 1-α-glucosylfructose, turanose, maltulose, 1-α-isomaltosylfructose and 4-α-isomaltosylfructose were synthesized by Taka-maltase and isomaltose as glucosyl donor.

2). Transfer of glucose to the 1-position of fructose was far ahead of that at the 3 and 4-positions.

3). The structure of 1-α-glucosylfructose, which had hitherto remained to be proved, was determined by chemical synthesis from α-acetobromoglucose and β-diacetonefructose in nitromethane in presence of mercury-cyanide.

PART II.

α-Glucosidase Activity due to Amyloglucosidase

In part I the properties of mold maltase were described. But in case of isolation of maltase from Takadiastase it was observed that considerable α-heteroglucoside-hydrolyzing activity remained, 30～60% of total activity among materials used, in the filtrate of salting-out. This suggested the existence of an another type of α-glucosidase in Takadiastase and experimental results showed that this activity of α-glucosidase was due to one of the amyloglucosidases. Amyloglucosidase having activity of α-heteroglucoside hydrolysis had not yet been reported.

Thus, the investigations on α-glucosidase of mold made it necessary to fractionate systematically the diastic enzyme system, including α-amylase, maltase and amyloglucosidases.

Among the results obtained, are two facts, which were newly found, and

are to be emphasized: the first, α-heteroglucoside-hydrolyzing activity was dependent upon one of amyloglucosidases, and the second, amyloglucosidase of mold exhibited multiple nature.

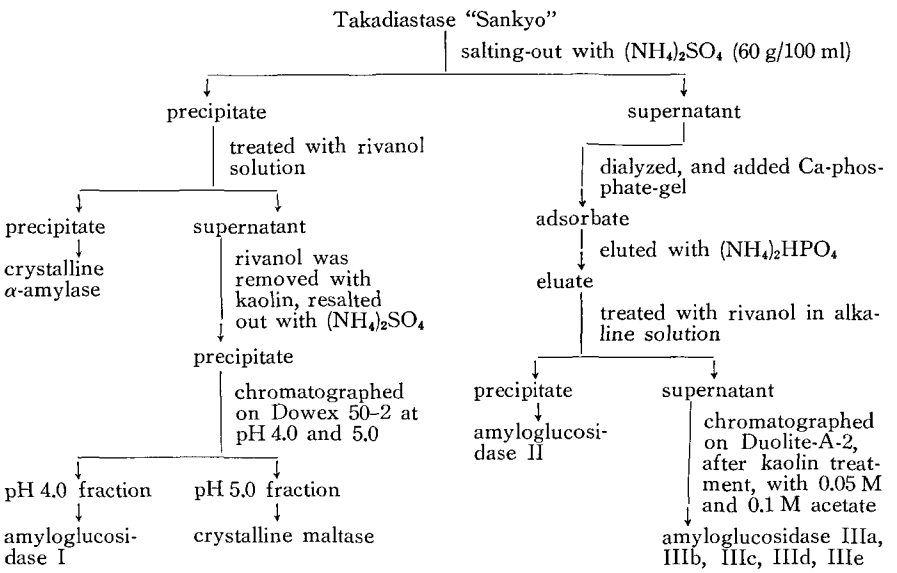
These results will be described in Chapters 6 and 7, respectively.

Chapter 1.

Fractionation and Purification of α-Amylase, Amyloglucosidases and Maltase from Takadiastase, and Multiple Nature of Amyloglucosidase.

§ II-1-1. Outline of Methods for Fractionation and Purification of the Enzymes:

The methods are summerized in following diagram. The names of amyloglucosidase I, II and III a, b, c, d, e were adopted tentatively in present study.



The description of isolation of crystalline maltase in Chapter 1 of Part I is part of the series of fractionation studies and the method for crystallization of α-amylase was adopted essentially from the same method described by AKABORI et al. Accordingly this description is omitted in this chapter.

Determination of Enzyme Activity:

Enzyme activity was measured by determining the amount of glucose liberated in a 2 ml. aliquot of 10 ml. of incubated mixture, which contained 0.5 ml. of enzyme solution, 1 ml. of 0.01 M acetate (pH 4.8) and 100 mg. of

soluble starch, at 45°C for 15 min.

One unit of enzyme activity was represented by one mg. of glucose liberated.

§ II-1-2. Fractionation of α-Amylase, Amyloglucosidase I and Maltase Salting-out Precipitates of Takadiastase:

A. α-Amylase: α-Amylase was crystallized by the method described by AKABORI et al.

B. Maltase: See page. ( ).

C. Purification of Amyloglucosidase I:

The enzyme was purified from pH 3.8 eluates which were obtained from a Dowex 50-2 column (See page ( )).

Portions having strong activity of amylase (No. 2~6, 250 ml.) were collected and their pH was adjusted to 5.4 with N-NaOH. Solid ammonium sulfate was added to the solution (15 g/100 ml.) and the resulting precipitate was removed by centrifugation (12,000 r.p.m., 10 min.). To the supernatant ammonium sulfate was added up to a concentration of 55 g per 100 ml. and the precipitate was collected by centrifugation (12,000 r.p.m., 10 min.) and dialyzed against running water for 3 days. The dialysate (75 ml.) was diluted with an equal volume of water and shaken with 30 ml. of chloroform for 15 min. This procedure was repeated twenty five times, until the precipitate of denaturated protein almost disappeared. To the enzyme solution thus obtained ice-cold acetone was added and the precipitate formed was rapidly centrifuged off. Acetone was added to the supernatant up to a 70% concentration and the precipitate obtained by centrifuging was dissolved in 25 ml. of water. This enzyme solution was again shaken five times with 5 ml. chloroform, until the insoluble substances formed by chloroform almost completely disappeared. To

TABLE 12. Purification of amyloglucosidase I

	volume	unit/0.5 ml	unit/mg N	yield %
original extract	3,500	9.7	2.6	100
salting-out ppt. 1st	2,600	7.6	3.0	58.0
salting-out ppt. 2nd	330	18.0	17.0	17.7
pH 3.8 eluate	250	17.3	20.0	12.8
salting-out ppt. 3rd	150	16.5	25.0	9.9
chloroform treatment, 1st	110	13.9	38.0	4.6
70% acetone fraction	25	30.0	61.0	2.2
chloroform treatment, 2nd	37.5	14.4	67.0	1.6

the solution treated with chloroform (37.5 ml) 112.5 ml. of acetone was added after cooling and precipitate was dissolved in 5 ml. of water and precipitation at 50% concentration of acetone was repeated once again. The highly purified amyloglucosidase-I was obtained and preserved in dry state. (Table 12).

§ II-1-3. Fractionation of Amyloglucosidase II and Amyloglucosidase III from Salting-out Filtrates:

The filtrates which were obtained by the first precipitation with ammonium sulfate of Takadiastase was dialyzed against running water for 4~6 days. Although considerable ammonium sulfate remained in dialysate in this period of dialysis, adsorption of the enzyme on calciumphosphate-gel to be adopted in next stage was not affected. About 220 g of hard paste of fresh calciumphosphate was added to the dialysate (13,100 ml.) and the mixture was stirred for 20 min. The purpose of this procedure was to remove impurities. The quantity of the gel to be added was determined in advance by observing 5~10% loss of amylase activity by using a part of the dialysate.

The dark-coloured adsorbate was removed by centrifugation. Amylases were almost completely adsorbed by further addition of calciumphosphate gel (800 g). The adsorbate was washed with water and then the enzymes were extracted three times with 1,500 ml. of M/15 diammonium phosphate solution. The combined extract (4,500 ml.) was concentrated below 40°C to 450 ml., and 900 ml. of alcohol was added. The precipitates formed were separated rapidly from the supernatant by centrifugation and were then dialyzed for 3 days. However the removal of ammoniumphosphate by dialysis was rather difficult, and consequently the dialysate was treated with bariumacetate. The excess Ba^{++} was removed by sulfuric acid and concentrated below 40°C, and then alcohol precipitation and dialysis were repeated.

The dialysate was diluted with water to 400 ml. and, considering the remaining α-amylase, 8 ml. of 2% rivanol solution was added, and slight amount of precipitate formed was removed. The pH value of supernatant was adjusted to pH 10~11 with 3% sodium carbonate and 20 ml. of rivanol was added. A syrupy precipitate was obtained after standing for a while and amyloglucosidase II (precipitate) and III (supernatant) were fractionated by centrifugation.

§ II-1-4. Purification of Amyloglucosidase II:

The amyloglucosidase-rivanol complex which was formed in alkaline solutionwas dissolved in 100 ml. of 0.1 M acetate buffer (pH 4.8), and about 25 g of kaolin was added under stirring. The mixture was filtered on a Buchner funnel. To the yellow solution thus obtained (90 ml), an equal volume of ice-

TABLE 13. Purification of amyloglucosidase II

	volume	unit/0.5 ml	Unit/mg N	yield %
salting-out filtrate	13,000	5.3	—	100
Ca-phosphate pre-treatment	12,500	4.8	—	88
$(NH_4)_2HPO_4$ eluate	4,500	8.7	—	57
2nd dialysate	275	18.8	19	7.5
rivanol precipitate	90	23.4	46	3
50% alcohol fraction	30	23.6	45	1

cold alcohol was added and the precipitate collected by centrifugation was dissolved in 30 ml. of water and used as the enzyme solution (Table 13).

§ II–1–5. Evidence that Amyloglucosidase II is a different kind of protein from amyloglucosidase I. An electrophoretic study:

It was observed that α-heteroglucoside-hydrolyzing activity was concentrated only in the amyloglucosidase III fraction and that, as will be described in later section, amyloglucosidase I and II seemed to be a same kind of enzyme in activity principle, judging from their behavior towards several substrates. These two enzymes occur either in the salted-out fraction or in the non-salted-out fraction. As the salting-out procedure was not complete enough to isolate a protein it is most probable that amylase II was merely a part of amyloglucosidase I which remained in the salted-out filtrate. From this reason paperelectrophoresis was applied for the purpose of identification.

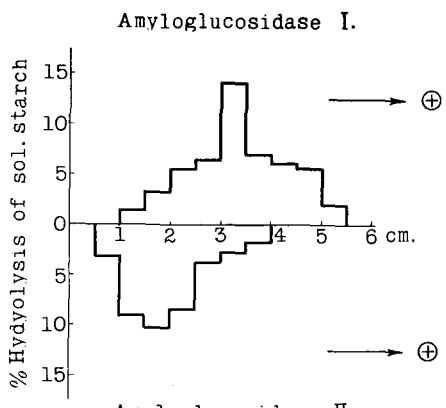


Fig. 25. Paperelectrophoresis of Amyloglucosidase III by Duolite.

Paperelectrophoresis was conducted as follows; enzyme solution 0.03 ml, width of paper 2 cm, 0.1 M veronal buffer pH 8.0, 300 V, 0.2 mA/cm.

After 5 hrs of electrophoresis the paper strip was airdried and cut in 0.5 cm sections. The enzyme was extracted from the pieces of paper with 3 ml. of 0.1 M acetate buffer (pH 4.8). To each extract 2 ml. of 2% soluble starch was added and the mixture was incubated at 30°C for 30 hrs. Glucose was determined reductometrically by using a 2 ml. aliquot of each mixture. A mobility figure as shown in Fig. 25 was obtained.

Both enzymes moved to the anode but the mobility of I was far greater than that of II. Accordingly, while I and II exhibited the same behavior on substrates, they were quite different in paper electrophoretical behavior.

§ II–1–6. Fractionation of Amylogucosidase III by Column Chromatography:

The supernatant which was obtained by removing the rivanol amylase complex still exhibited amylase activity and yet α-glucosidase activity (in terms of maltase) was concentrated only in this fraction.

The enzyme solution (40 ml.) which was adjusted to pH 6.0 with N–CH_3COOH and treated with caolin had 15.1 units of amylase activity. The yield of activity was 80% of the total activity in the original filtrate while 60% α-glucosidase activity remained in this fraction.

The author first presumed that these two activities were probably due to different enzymes, and some experiments were conducted on fractionation. The findings obtained from the preliminary experiments were:

(1) The enzymes were not adsorbed on Dowex 50–2 which was used for fractionation of amyloglucosidase I and maltase in the salted-out precipitate.

(2) The enzymes were also not adsorbed by corn-starch from aqueous solution, 33% alcohol or half-saturated ammonium aulfate solution.

(3) Fractional elution of the enzymes from calciumphosphate gel, by which the fractionation of amyloglucosidase and maltase of Koji-mold were successful, was not applicable. In the present experiment both activities appeared together in M/15 potassium diphosphate eluates.

(4) Amberlite IRC–50, IR–4B, IRA–400, diethylaminoethylcellulose and carboxymethylcellulose were all ineffective for fractionation.

From these results it was suggested that as regards α-glucosidase activity the enzyme in this fraction is of quite a different type from the maltase obtained from the salted-out precipitate.

However further investigations have led the author to a conclusion that both activities were due to a single enzyme: thus, it was found that when the enzymes were chromatographed by using Duolite A–2, amylase activity itself was fractionated in several fractions and yet α-glucosidase activity also appeared in each fraction having amylase activity.

A. *Experiment 1*:

Ten ml. of enzyme solution, adjusted to pH 4.8 by adding 0.05 M acetate buffer, was adsorbed on a column of Duolite A–2 (13 × 140 mm, buffered with 0.05 M acetate, pH 4.8) and chromatography was conducted by developing with

acetate buffer (pH 4.8) at 0.05 M and 0.1 M concentration and collecting each 2 ml. of eluate. Amylase and α-glucosidase activity as well as optical density at 280 mμ were measured in each fraction. The results (Fig. 26) show that four peaks were obtained and that each fraction had two activities.

B. *Experiment 2*:

This experiment was planned to prepare each enzyme on a large scale. It was found that at least five peaks having enzyme activities would appear in the amyloglucosidase III fraction.

In experiment 1, in spite of developing with pH 4.8 buffer, the pH value

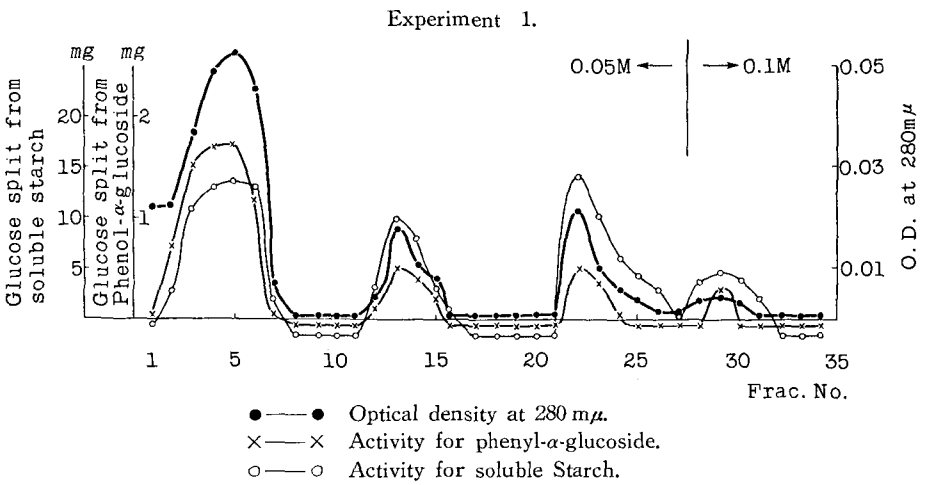
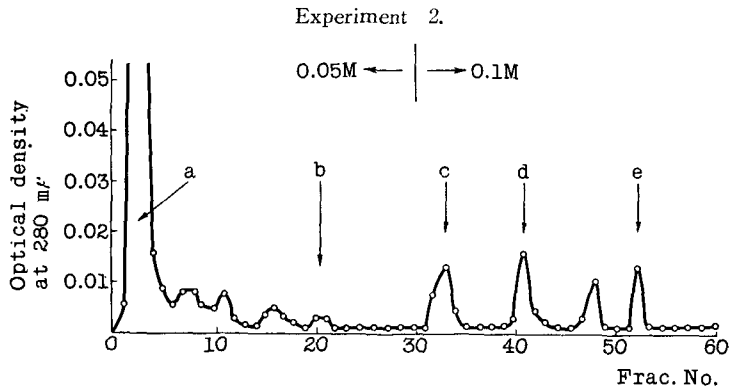


Fig. 26. Fractionation of Amyloglucosidase III by Duolite A_2.



a, b, c, d and e were peaks having enzyme activity.

Fig. 27. Fractionation of Amyloglucosidase III by Duolite A_2.

increased to 5.6～5.8 in the No. 1～5 fraction. This is probably due to the remaining inorganic substances being exchanged with the ion-exchanger used. Accordingly a preliminary purification was made in this experiment.

A volume of 250 ml. of the fraction was concentrated to 50 ml. at 30°C and 125 ml. of alcohol was added. The precipitate was rapidly collected by centrifugation and dialyzed for several days. The dialysate was diluted with an equal volume of 0.05 M acetate buffer (pH 4.8) and insoluble matters were filtered off. The 45 ml. of enzyme solution thus obtained was adsorbed and chromatographed on a Duolite A-2 column (3.5 × 23 cm) as described in Exp. 1. Eluates of each 50 ml were collected in No. 1～5 and 25 ml. after No. 6 (Fig. 27). The recovery was 89%, calculated from optical density at 280 mμ.

In Fig. 27, a, b, c, d and e were the peaks having enzyme activity but the b-fraction had only a slight activity. The units of amylase in each fraction were as follows: a: 5.3, c: 0.9, d: 1.2, e: 0.9; and the ratios of the

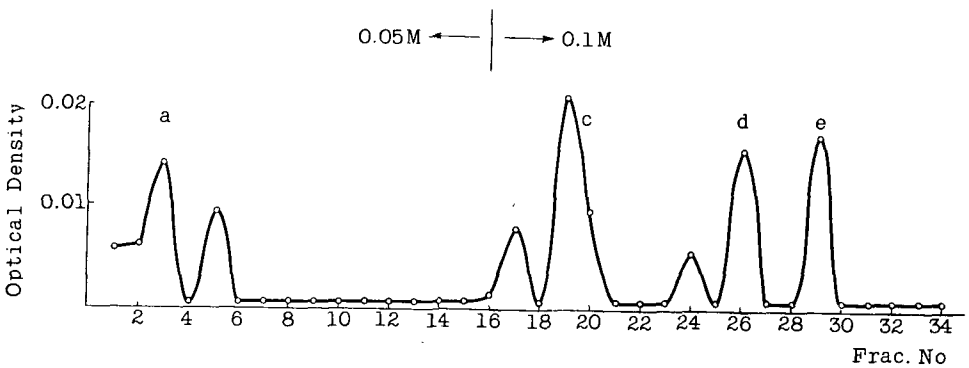
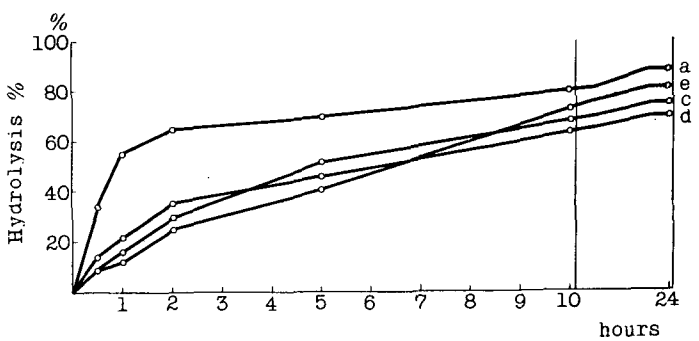


Fig. 28. Reproducibility of Chromatographic Behavior of Amyloglucosidase III.



0.5 ml. of each fraction was used.

Fig. 29. Action of Amyloglucosidase IIIa, IIIb, IIIc and IIId on Soluble Starch.

activity to a (=100) were 18.7, 22.4 and 18.7 in c, d and e, respectively.

When parts of a, c, d and e were combined and chromatographed in the same condition as above mentioned, peaks having enzyme activity were completely reproduced. (Fig. 28).

That the activity principle of these four fractions of enzyme is identical is suggested by following facts: (1) similar behavior toward soluble starch was observed, and the extent of starch hydrolysis was 70~80% in each case; (Fig. 29) (2) each enzyme had both amylase and α-glucosidase activity.

§ II-1-7. Discussion:

Amyloglucosidase I and II had almost the same behavior upon various substrates (the detailed results will be given in next Chapter) and these enzymes were supposed to be essentially the same in activity principle. However, as to the solubility, the former was easily salted-out by ammoniumsulfate while the latter was not salted-out even by saturation with the salting-out agent.

Furthermore, their individual electorphoretical behaviors were distinctly different each other. Amyloglucosidase I exhibited a greater mobility to the anode than did amyloglucosidase II.

From such results these enzymes cannot be regarded as originally the same but merely isolated separately by imperfect fractionating procedure. It is to be recognized that each enzyme has its specific property in the protein molecule.

On the other hand amyloglucosidase III was fractionated in several (at least four to five) fractions by treating with ion exchange resin and each fraction showing amylase activity also had α-glucosidase, especially α-heteroglucosidase, activity. Like I and II, the activity principle in amyloglucosidase IIIa~IIIe is supposed to be essentially the same and the difference of chromatographic mobility is to indicate their individuality as protein.

Undoubtedly amyloglucosidases would be classified into two types according to the marked difference of the extent of starch hydrolysis. This fact is confirmed by the present study on Takadiastase. But now the author would like to emphasize the existence of a modification of the protein in each of two types of amyloglucosidase.

The existence of a modification or multiple nature in enzyme protein has been reported on ribonuclease[23], cytochrome C[22], Takamaltase[55] and recently β-glucosidase[75].

It could be said that the new findings in the present study are partly due to the fractionation procedure adopted. The author planned the entire fractionation of mold diastic enzymes system without removing a particular enzyme by irreversible inactivation. The results were successful generally. This

demonstrates that in comparative biochemistry the systematic isolation of individual protein is necessary.

FUKUMOTO[19] classified amyloglucosidase in Aspergillus niger type and Rhizopus delemar type according to the extent of hydrolysis of starch. Furthermore Ueda pointed out two types of amyloglucosidase in a material of Asp. awamorii having different debranching activity to starch, and separated them from salted-out precipitate and filtrate respectively. Evidence for existence of such enzymes was also obtained in Takadiastase in the present experiment. However, although amyloglucosidase II was isolated from the filtrate by using the method described originally by OKAZAKI[62], (but the filtrate was not treated with mercury chloride to remove impurities and pre-treated with calciumphosphate) the behaviour of the enzymes upon starch or β-limit-dextrin was quite different. (Fig. 30). According to OKAZAKI the enzyme hydrolyzes β-limit dextrin to an extent of about 50% while amyloglucosidase II hydrolyzed it completely, and III, not precipitating with rivanol, was a similar kind of enzyme based on starch or β-limit dextrin hydrolysis (Fig. 29, 30).

As described in chapter 1 amyloglucosidase and maltase of koji-mold were fractionated by treating the calciumphosphate-gel adsorbate with acidic and then basic phosphate solution respectively. But it has often been observed that both activities appeared in the first eluate, e, g, acidic phosphate eluate. This was most probably due to the existence of amyloglucosidase III. The experimental results on Takadiastase will support above presumption, in view of the behavior of the enzyme on eluting agents (cf. preliminary experiments). Also, in Takadiastase, α-glucosidase activity varied remarkably among the materials used and, in addition, α-heteroglucoside hydrolyzing activity in the filtrate exhibited a remarkable variation, in a range of 30～60% of the total activity of original material.

From such results it is thought that a slight variation of cultural conditions of mold would bring about a remakable variation in the creation of each enzyme which comprises the diastic enzyme system.

§ II–1–8. Summary :

1) Methods of fractionation and purification of α-amylase, maltase and amyloglucosidases are described. Amyloglucosidases were fractionated in I, II and IIIa～IIIe.

2) It is suggested that amyloglucosidase I and II are essentially the same enzyme in activity principle. No activity on phenol-α-glucosides was observed in either enzyme. But the mobility in paperelectrophoresis was different and I moved faster to the anode than did II.

3) Amyloglucosidase III appears to be an another type of this group of enzymes. It was fractionated into several fractions possessing both amylase and α-heteroglucosidase activities.

4) It is thought that modifications of the enzyme molecule may exist among the amyloglucosidases in mold.

Chapter 2.

Properties of Amyloglucosidase III and a Comparative Study of Behavior of Amyloglucosidases towards Different Substrates.

§ II-2-1. Preface:

In the present study on the diastic enzymes system of mold, the author has been primarily concerned with α-glucosidases. This has brought about new findings on amyloglucosidase, especially on amyloglucosidase III which has an ability to hydrolyze α-heteroglucosidase.

Chapter 2 deals with comparative studies on the α-glucosides activity of amyloglucosidase III with maltase and on the amylase activity of other amyloglucosidases.

Experimental Results

§ II-2-2. Evidence that α-glucosidase and Amylase activity in Amyloglucosidase III fraction is due to a single enzyme:

As was described in Chapter 2, amyloglucosidase IIIa~IIIe were assumed

TABLE 14. Rate of Adsorption of Two Activities of Amyloglucosidase IIIa on Calcium Phosphate-gel

Gel, added ml.	Hydrolysis %		Ratio of Rate of Hydrolysis
	Sol. Starch	Maltose	
no addition	42.7	20.6	2.1
0.3	41.2	22.5	1.8
0.5	36.0	18.7	2.0
1.0	24.5	11.2	2.2

Calcium phosphate-gel suspension was added to 1.5 ml. of enzyme solution and made up 2.5 ml. with water. This mixture was stirred, centrifuged, and 1 ml. of supernatant was used as enzyme solution to be tested. Reaction condition: 2% substrate soln., 5 ml.; 0.1 M acetate buffer (pH 4.8) 1 ml.; water 3 ml.; enzyme solution, 1 ml.: 40°C. 30 min.

TABLE 15. Rate of Elution of Amyloglucosidase IIIa from Calcium Phosphate-gel Adsorbate by Phosphates

Eluting Agents	Hydrolysis %		Ratio of Rate of Hydrolysis
	Sol. Starch	Maltose	
M/100 KH_2PO_4	30.0	15.4	1.9
M/50 $(NH_4)_2HPO_4$	30.7	13.1	2.3
M/10 $(NH_4)_2HPO_4$	20.2	11.2	1.8

A 10 ml. volume of calciumphosphate-gel suspension was added to 5 ml. of enzyme solution. The mixture was stirred, centrifuged, washed with water and then the enzyme was extracted with 5 ml. of phosphates in order. The condition of reaction was the same as indicated in Table 14.

to be the same kinds of enzyme in activity principle. To confirm whether both activities depend on a single enzyme or not, the rate of adsorption to adsorbing agent and the rate of discharge from adsorbate were observed by using IIIa.

After the addition of different quantities of calcium phosphate-gel to the enzyme solution or after elution by different eluting agents from adsorbate, the remaining or discharged activities of each enzyme were estimated. The ratio of amylase to α-glucosidase activity was about 2 in both cases. (Table 14 and Table 15).

§ II-2-3. Behavior of Amyloglucosidase I, II and IIIa toward various Substrates :

I and II were used in 0.1% solution and IIIa was used in the combined eluates of No. 2~6 fractions which were obtained by chromatography. One ml. of enzyme solution, 1 ml. of 0.1 M acetate buffer (pH 4,8) and 10 ml. of 1% substrate solution except 2% phenyl-α-glucoside were mixed and kept at 40°C. An aliquot of the mixture was pipetted out periodically and the percentage of hydrolysis was calculated by measuring its reducing power. The sugars formed from panose were calculated as glucose.

A. Soluble starch : Any enzyme used ceased its action on soluble starch after 70~80% hydrolysis. By observing the initial velocity of the reaction and the period to reach the limitation of hydrolysis, I and II seemed to be similar in activity while IIIa exhibited a comparatively high velocity in the initial stage. (Fig. 30a)

B. β-Limitdextrin : β-Limitdextrin was completely hydrolyzed by I and II and was slowly attacked by IIIa. Even after 24 hrs. it was only by 70% hydrolyzed in the latter case. This fact indicated that III was clearly a dif-

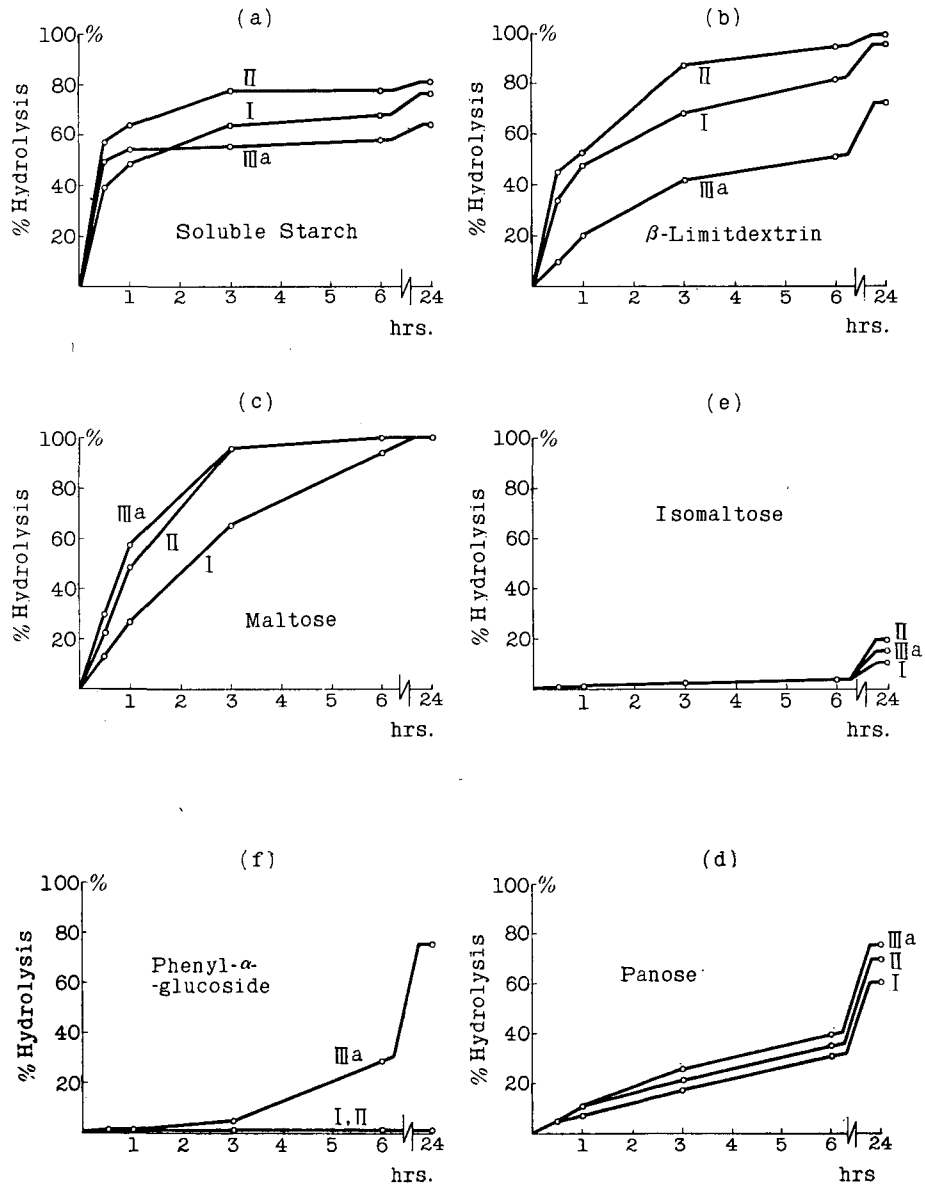


Fig. 30. Action of Amyloglucosidase I, II and IIIa on various Substrates.

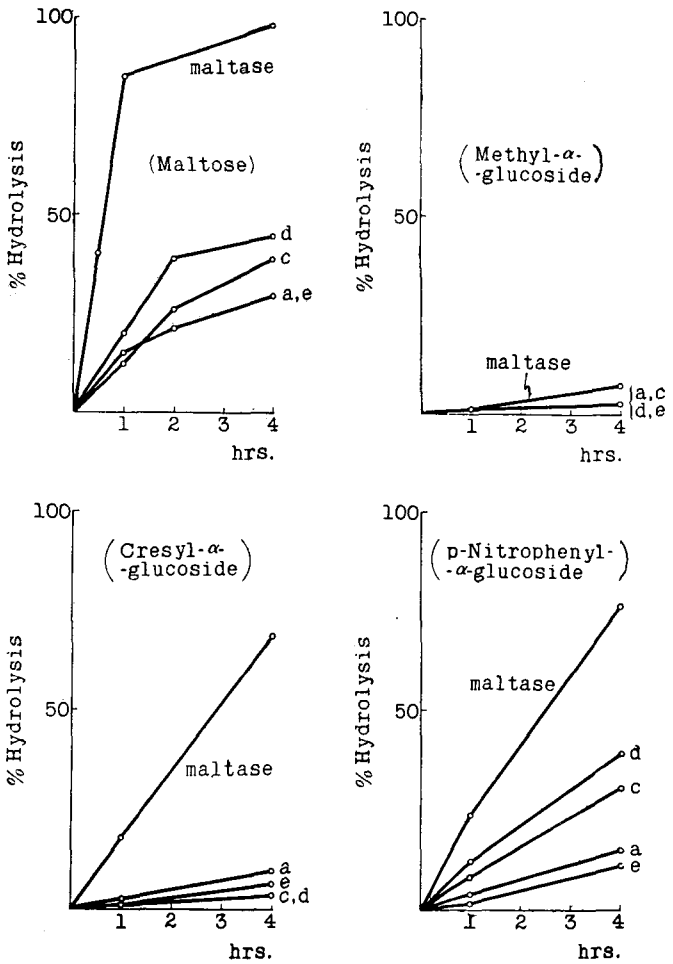


Fig. 31. Comparison of Amyloglucosidase IIIa, IIIc, IIId and IIIe with Maltase on their Activity toward α-Glucosides.

ferent type of amyloglucosidase from I and II. (Fig. 30b).

C. Oligosaccharides: Maltose was completely hydrolyzed (Fig. 30c) and panose was attacked at a considerably slower rate. (Fig. 30d). These enzymes also attacked isomaltose but rate of hydrolysis was extremely low (Fig. 30e).

D. Phenyl-α-glucoside: Only III acts on penyl-α-glucoside while I and II did not. Although the rate of hydrolysis by III was very low this is a most distinct and important difference among these enzymes from the stand-point of α-glucosidase activity. (Fig. 30f).

§ II–2–4. Comparative Study on Amyloglucosidase IIIa, c, d, e and Maltase on Their Activity toward α-Glucosides :

The conditions of reaction were the same as described in the preceding section. Fig. 31 shows that the activities of each of amyloglucosidases on the substrates generally resembled maltase but rate of hydrolysis was considerably lower.

§ II–2–5. Discussion :

In preceding chapter the multiple nature or existence of two types of amyloglucosidases was discussed. These findings were further confirmed by the present experiments.

As to α-heteroglucosidase activity it has been demonstrated that there exist at least two types of this enzyme in mold, one is maltase and the other is amylase-depending α-glucosidase. Existence of such an enzyme as amyloglucosidase III which has a wide range of substrate specificity, from α-1,4 linked polysaccharide to α-heteroglucosides has already been pointed out by LIEBERMAN et al. in maltase of equine serum. But the ratio of amylase activity to maltase activity of this enzyme was about 0.015 while that of amyloglucosidase III was about 2. LIEBERMAN et al. named his enzyme "maltase" but amyloglucosidase III is an amylase.

The enzymes from amyloglucosidase IIIa to IIIe had similar activites towards several α-glucosides, but the relative activity on each substrate was not completely identical. Although the author regards them, at present, as modified proteins which have the same activity principle, the fact that their relative activity is more or less different may be of greater importance, because it is most probable that such a modification of protein nature in an enzyme brings about a variation of mode of action, as was observed by column chromatography of α-glucosidase[75].

§ II–2–6. Summary :

1) A comparative study of amyloglucosidase I, II and IIIa was made. I and II exhibited essentially the same activities on various substrates used while IIIa was quite different, especially toward starch, β-limitdextrin, and α-heteroglucosides.

2) A comparative study of amyloglucosidase IIIa to IIIe and maltase was made. Some aspects on the behavior toward α-glucosides were quite similar.

General Discussion

Although the respective properties of each enzyme have already been discussed, certain other aspects should be pointed out.

Terminology: Substrate specificity and transglucosidation action of mold maltase indicated that this enzyme is the same kind of enzyme which bas been hitherto described as "transglucosidase" of mold. However, hydrolysis and transglucosidation may be interpreted in terms of acceptor difference, and furthermore such a concept is now generally recognized in the case of many glucosidases. Accordingly the author believes there is no necessity for adopting the name "transglucosidase".

As stated in the introduction the enzymes which belong to amyloglucosidase were reported under various names by different workers. The author advocates consolidating their names under a single name, amyloglucosidase, because (1) the enzymes act on starch to produce glucose, not maltose, and (2) some of them hydrolyze not only maltose but also α-heteroglucosides, which closely resembles maltase.

Multiple Nature of Enzyme: It was established in the present study that amyloglucosidases exhibit a dual nature in amylase activity as well as in α-glucosidase activity. These two types of amyloglucosidase exhibited a multiple nature with the protein molecule having probably the same mode of action.

On the other hand, the α-glucosidase activity of maltase and of one of the amyloglucosidase was similar in certain aspects. Thus it is possible to say that the α-glucosidase activity in mold is also of dual nature. MATSUSHIMA[55] recently pointed out the existence of modifications in Takamaltase by column chromatography, although they were not observed by the present author.

From such a point of view as described above, is it not possible to state that the diastic enzyme system of mold is constituted of a group of proteins among which a consecutive change of structure exists in each other?

Conclusion

Enzymes Participating in Maltose or α-glucosides Hydrolysis in mold

Enzyme	Substrate				Trans glucosi-dation
	Maltose	α-Hetero glucosides	Soluble Starch	β-limit Dextrin	
Amyloglucosidase I, II	+	−	80%	100%	−
Amyloglucosidase III	+	+	60%	70%	−
Maltase	+	+	−	−	+

1). For maltose or α-glucosides hydrolysis in mold, it was demonstrated that maltase and amyloglucosidases were responsible. Existence of such a combined system in α-glucosidase activities in mold was demonstrated by fractionating them from Koji-mold and Takadiastase.

2). The substrate specificity of mold maltase, of which there have been conflicting reports among investigators, was established by using crystalline enzyme. The rate of hydrolysis of α-heteroglucosides increased with the size of aglucon moiety. The enzyme cleaves α-1,6 as well as α-1,4-glucosidic linkage and ceases its action on malto- and isomalto-oligosaccharides at seven to eight units of glucose in the molecule. It is necessary for the enzyme to act on the α-configuration in the presence of a semiacetal- and etheric linkage in both sides of oxygen bridge.

3). Mold maltase is a transglucosidase. The enzyme acts on α-heteroglucosides in the presence of alcohols or sugars to transfer the glucosyl residue to the acceptors. When maltose is used as a substrate, many oligosaccharides linked with α-1,6-glucosidic chains are synthesized by the transglucosidation reaction. The transglucosidation products from maltose involve two types of sugars; one is the dextran series and the other is the panose series of saccharides. The highest polymer to be sythesized are hepta to octasaccharides.

When fructose is used as an acceptor, this ketose accepts, quite different for aldose, the glucosyl residue of the substrate at the three positions of the 1, 3 and 4 carbon atoms, forming 1-α-glucosylfructose, turanose and maltulose respectively.

4). A fractionation study on the diastic enzyme system in mold, with special reference to α-glucosidase activity, was performed. Amyloglucosidases exhibited a dual nature in mode of action on substrates and a multiple nature in protein character in each of the two types.

From the point of view of mode of action, amyloglucosidase I and II have higher debranching activity on starch or β-limitdextrin than amyloglucosidase IIIa～IIIe while the ability to hydrolyze α-heteroglucosides is observed only with amyloglucosidase IIIa～IIIe.

Amyloglucosidase I and II are supposed to be essentially the same enzyme in activity principle but, as to their solubility, the former is easily salted-out by $(NH_4)_2SO_4$ and the latter not even by saturation with the salting-out agent. Furthermore their electrophoretical behaviour is clearly distinct and different.

Amyloglucosidase III is a group of the enzyme proteins which have practically the same action on the substrate, and is fractionated in several fractions, IIIa～IIIe, by column chromatography with Duolite A_2.

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