STUDIES ON THE UNFERMENTABLE SUGARS (VI~VII)

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(VI) The Oligosaccharides Synthesized from Maltose by *Schizosaccharomyces Pombe*

(VII) The Synthesis of Isomaltose from Glucose

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Introduction

In 1953, Gjertsen (1) reported that isomaltose was present in the beer paper chromatogram but not in the wort, and it is possible that it is not present in the wort, but is formed during fermentation. Another contribution towards the elucidation of this topic was made in 1954 by Blair and Pigman (2), who reported on the fermentation of maltose by *Fleishman's baker's yeast.* Namely pure maltose is fermented incompletely and the results obtained are consistent with the occurence of a polymerization reaction as one of a series of competing reactions involved in the fermentation of maltose.

Some workers furnished evidence that the higher saccharides were in fact formed directly from maltose ; the enzyme responsible was named amylomaltase by them and was shown to catalyse the reaction n maltose $\leftrightarrows$ n glucose + (glucose)n. The new enzyme, like phosphorylase, amylosucrase, and other enzymes responsible for the synthesis of oligosaccharides, was a transglucosidase.

In the previous communications (3), we investigated on the sugars contained in Saké (the widely used alcoholic beverage of the Japanese. The rice starch of the raw material of Saké is converted to fermentable sugars by mold enzyme hydrolysis and its subsequent fermentation by Saké yeast is carried out at the same time.) and found glucose and many oligosaccharides, namely sakébiose, kojibiose, isomaltose, panose and dextrantriose besides a few unidentified sugars. We also studied on the mechanism of the production of those oligosaccharides and found that they were synthesized by the action of fungal enzymes from

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maltose and glucose. From the facts, therefore, it is supposed that the oligosaccharides in Saké are mainly the sugars synthesized by enzymic action from maltose and glucose which are derived from rice starch.

Examining the residual sugars of fermentation of isomaltose, it was found (3) by means of paper chromatography that the spot of isomaltose disappeared gradually by many yeasts, but by *Schizo. Pombe*, its spot disappeared quickly and yet the spot of glucose appeared and then gradually disappeared. From the facts, it was considered that the isomaltase activity of *Schizo. Pombe* was strong.

In this communication, we wish to report the results obtained with *Schizosaccharomyces Pombe* No. 1 and No. 2, during the studies on the transglucosidation of many yeasts.

Washed and acetone preparation cells of *Schizosaccharomyces Pombe* No. 1 and No. 2 have been suspended in the buffered maltose solution (pH=4.7) and the reaction mixtures were incubated for 1～2 hours at 28～30°C. We have demonstrated qualitatively by paper chromatography that *Schizosaccharomyces Pombe* synthesized sakébiose, kojibiose, isomaltose, panose and dextrantriose from maltose. From glucose and sucrose, isomaltose was synthesized with the same method by *Schizosaccharomyces Pombe*. The digests of maltose was fractionated by Whistler and Durso's method (4) which involved passage of the solution through a charcoal column and subsequent elution of the column with aqueous solutions containing increasing concentrations of ethanol. We shall report in the next communication on the identification of those oligosaccharides obtained from each fractions.

Experimental

(1) Synthesis of Oligosaccharides from Maltose

The medium (30 ml of Koji extract, Bollg. 10, pH=5.0), placed in a 100 ml Erlenmyer flask, was inoculated with *Schizo. Pombe*. The inoculated medium was incubated for four days at 28～30°C. The cells were collected in a centrifuge and washed by sterilized water until the reducing power disappeared.

The cells (dry matter was approximately 0.07 g) were suspended in 5 ml of approximately 10 per cent maltose solution and to this was added 1 ml of McILVAINES buffer solution. The resulting mixture contained 7.63 g of maltose per 100 ml (pH=4.7), and was incubated for 1～72 hours at 28～30°C.

(2) Qualitative Analyses of the Synthesized Oligosaccharides

The products, which were synthesized by suspension of *Schizo. Pombe* from matose, were examined from time to time by paper chromatography. The method used in our laboratory for the identification of sugars by paper chromatography was reported previously (3). For the developing solvent, a mixture of pyridine, buthanol, and water (2 : 3 : 1.5 respectively) as described

by Allene Jeanes et al (5) and four times multiple technique was used. For the spray agent, aniline hydrogenphtalate was used. After preliminary drying, the papers were heated for 10 to 15 minutes at 125°C in a special oven to develop the weak reducing oligosaccharides. The obtained chromatograms are shown in Fig. 1.

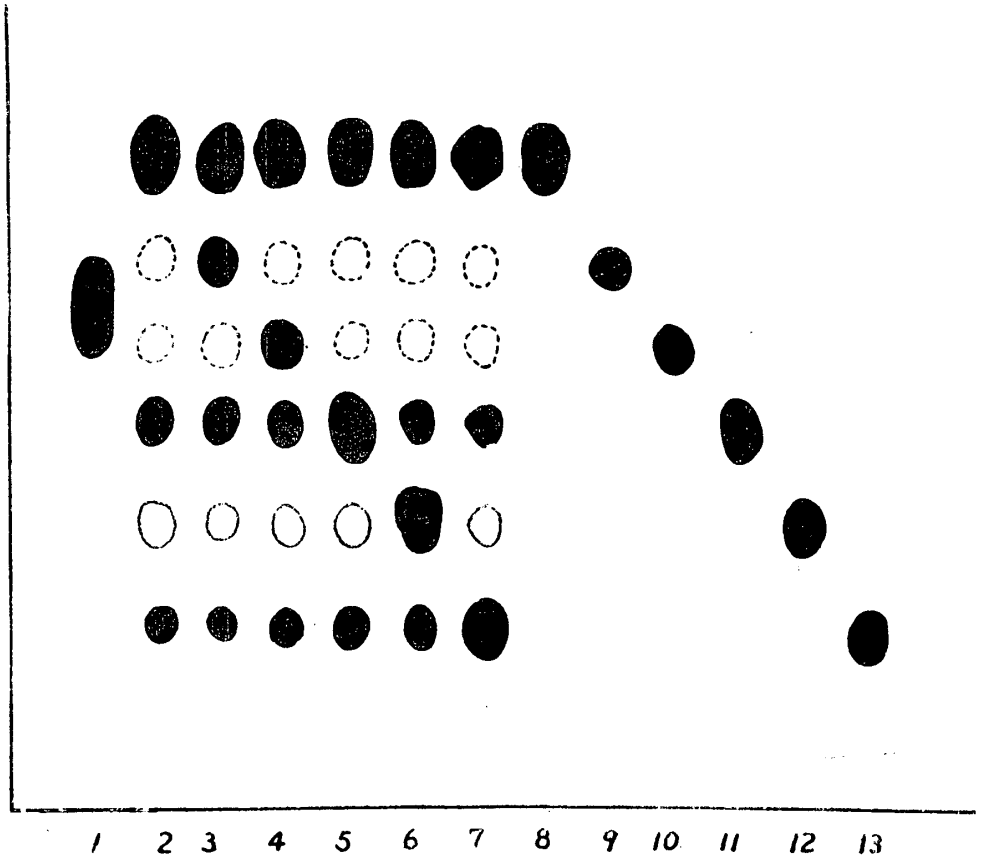

Fig. 1. Multiple Paper Chromatograms of the Digestion of Maltose.

(1) Multiple chromatogram of the buffered maltose solution
(2) Multiple chromatogram of the digestion of maltose
(3) Mixed chromatogram with sakébiose
(4) 〃 with kojibiose
(5) 〃 with isomaltose
(6) 〃 with panose
(7) 〃 with dextrantriose
(8) Multiple chromatogram of glucose
(9) 〃 of sakébiose
(10) 〃 of kojibiose
(11) 〃 of isomaltose
(12) 〃 of panose
(13) 〃 of dextrantriose

● intense
○ middle
◌ weak

From the Rf values, the colors and the mixed paper chromatograms with the authentic sugars, the spots of paper chromatograms were identified. No spot appeared on the chromatograms of the digestion without maltose, and only the maltose spot appeared on that of the buffered maltose solution. As soon as the cells were suspended in the buffered maltose solution, the spot of glucose and the weakly colored spot of isomaltose appeared on the chromatograms of the digestion. After about 30 minutes, the spot of maltose became very weak, and disappeaed after 60 to 100 minutes. On the contrary, the spots of oligosaccharides appeared increasing their color from time to time after 30 minutes.

In digestion of maltose by ordinary yeast — distiller's, brewer's and baker's yeasts — we could not recognized the spot of glucose on the chromatograms and yet the maltose spot remained a very long time. In the case of *Schizo. Pombe*, the maltose spot disappeared promptly on the contrary and yet the glucose spot remained for a long time without fermentation. The oligosaccharides synthesized from maltose were decomposed remarkably after 10 to 24 hours, and completely decomposed with fermentation after 48 hours. After two hours, six spots appeared on the four times multiple chromatograms of the digested solution. They are designated as A, B, C, D, E and F according to their Rf values. Comparative tests (Rf value, color, overlap with two sugars and etc) with the pure sugars by the technique of the mixed chromatography showed that A, B, C, D, E and F was glucose, sakébiose, kojibiose, isomaltose, panose and dextrantriose respectively. We obtained the same chromatograms from both *Schizo. Pombe* No. 1 and No. 2.

When aniline hydrogenphthalate was used as the spray reagent, there were remarkable differences in their color according to the constructions, of each oligosaccharide though they are similarly glucose polymers and some of them changed the color gradually. For example, the color of the spots on the chromatograms of a homologous series of 1, 4–α-linked glucosidic polymers obtained from starch sirup partially saccharified by malt, which contained maltose, maltotriose, maltotetraose, maltopentaose and etc., changed into the same green (105°C, 8 minutes heating) or greenish brown (115°C, 10 minutes heating) with each other after 24 hours. On the other hand, isomaltose and dextrantriose (isomaltotriose) which consisted of 1, 6–α-glucosidic linkage gave dark brown from the begining in the spots thus being different with glucose and maltose. The spot of panose, which has both 1, 4-α and 1, 6-α-linkages gave the same greenish brown as 1,4-α-linked polymer. We supposed that this fact was due to the presence of the reducing radical in glucose of 1,4-α-linkage. Kojibiose has been under identity and characterization, but the previous experiments show that it may be probably the 1,2-α-linked diglucosaccharide. This sugar gave pink (105°C, 3 minutes heating) or reddish brown (115°C, 10

minutes heating) in the spot which thus did not agree with those of maltose and isomaltose. Sakébiose which is supposed to be 1,3-α-glucosidic linked disaccharide gave brown color and thus resembled glucose in the spot.

As shown in Table 1 quantitative analyses of glucose and isomaltose in the digestion have been made by modifications of the method described by Flood et al (6) after two hours incubation. Namely the respective spots of sugars on the four times multiple paper chromatogram were cut off by guide strips and then extracted with water. The extracted sugars were analysed using the method of Stark and Somogyi (7).

Table1 1. Analysis of Sugars Synthesized from Maltose

Culture hours	*Schizo. Pombe* (1)		*Schizo. Pombe* (2)	
	Glucose g/100 ml	Isomaltose g/100 ml	Glucose g/100 ml	Isomaltose g/100 ml
1	4.49	0.89	5.28	0.43
5	4.32	0.59	2.94	0.12

Initial maltose concentration was 7.63 per cent. of sugars were fermented in the case of suspension.

(3) Synthesis by Acetone Preparation of *Schizo. Pombe*

50 g of suldge of *Schizo. Pombe* was washed by sterilized water until the reducing power disappeared. 250 ml of acetone was added to the yeasts and it was stirred for ten minutes. After acetone was filtered off by a glass-filter, 200 ml of acetone was added and stirred for 3 minutes. Then the acetone washed cells were treated twice with 200 ml of ether and dried in a vacuum desiccater for 24 hours.

A buffered maltose solution containing maltose hydrate (30 g), McILVAINES buffer solution (40 ml) and distilled water (160 ml) was prepared. The resulting mixture contained 11.78 per cent of maltose (13.10 per cent as glucose) and had pH=4.7. To the solution was added 7.0 g of acetone preparation of *Schizo. Pombe* and was incubated at 40°C for one and half hours. After removal of the cells in the centrifuge, the supernatant liquid was boiled to inactivate the enzyme. The synthesized oligosaccharides were examined by paper chromatography as described previously. (Four times multiple development technique) The obtained paper chromatograms were the same as shown in Fig. 1. The synthesized oligosaccharides were revealed to be glucose, sakébiose, kojibiose, isomaltose, panose and dextrantriose. The spot of maltose disappeared perfectly. The quantitative analyses of these oligosaccharides were made as shown in Table 2.

It is a very interesting result that *Schizo. Pombe* synthesized the same oligosaccharides in nearly almost the same proportions as each other from maltose

and glucose as *Asp. niger, Asp. oryzae* and etc. which were described previously (3). Especially both *Schizo. Pombe* and fungi synthesized sakébiose and kojibiose which had rare glucosidic linkage. It is reported often that *Schizo. Pombe* can ferment dextrin, but there is no report about hydrolase of *Schizo. Pombe*. From the above results, we shoud like to investigate hydrolase and transglucosidase of *Schizo. Pombe* in comparison with fungi.

Table 2. Analysis of Oligosaccharides Synthesized from Maltose

Oligosaccharides	g/100ml
Glucose	10.06
Sakébiose	0.28
Maltose	0.00
Kojibiose	0.43
Isomaltose	1.59
Panose	0.33
Dextrantriose	0.37
Total	13.06

6 g of maltose hydrate, 55 ml of water and 1.5 g of acetone preparation of *Schizo. Pombe* were shaken together and were divided into 5 ml portions, then placed in test tubes. To these were added 1 ml of McILVAINES buffer solution and each pH of them were adjusted to 3.3, 4.1, 4.6, 5.0, 6.1, 7.0, and 8.1. These digestions were incubated for 1 hour at 40°C. The synthesized oligosaccharides were examined qualitatively by paper chromatography. The quantitative analyses of glucose, maltose and isomaltose in the digestions were made. The results are shown in Table 3.

Table 3. Analysis of Sugars Synthesized from Maltose

pH		3.3	4.1	4.6	5.0	6.1	7.0	8.1
Glucose	g/100 ml	4.98	5.88	6.02	5.38	4.17	2.75	2.43
Maltose	〃	0	0	0	0	0.09	—	1.44
Isomaltose	〃	0.48	0.50	0.56	0.60	0.78	0.85	1.09

From a perusal of the chromatogram, it was shown that the synthesized oligosaccharides are glucose, sakébiose, kojibiose, isomaltose, panose and dextrantriose in each pH portions. At pH of 6 and 8 there appeared the spots of residual maltose. At pH 8.1 in Table 3, the quantity of isomaltose is at the maximum and glucose is at the minimum, and the quantity of residual maltose is at the maximum. From the above result in (2), it was shown that *Schizo. Pombe* synthesized many oligosaccharides from maltose but soon after fermented them perfectly. Namely the culture of *Schizo. Pombe* contain both synthesizing and hydrolyzing enzymes. Somogyi et al (8) reported in their method for analyzing mixtures of sugars that glucose and maltose were removed by fermentation with baker's yeast at a pH of 4.8, while only glucose was removed by the same organism at a pH of 8.4.

From the above results, we should not decide that the optimum pH of transglucosidase is approximatly 8.1, even if isomaltose is at the maximum.

On the contrary, the synthesized oligosaccharides would not be almost decomposed judging from the minimum yield of glucose.

(V) Transglucosidation by Filtrate

It was shown that the cells of *Schizo. Pombe* had a transglucosidase. Next we investigated the presence of transglucosidase in the filtrates. *Schizo. Pombe* was incubated in Koji extract for seven days at 28~30°C. After removal of the cells in the centrifuge, the supernatant liquid was filtered with filter-aid (Celite) several times. The perfectly cleared filtrate was dialysed with cellophane for 24 hours in water stream. Examining by Fehling solution and paper chromatography that there was no sugar, the filtrate was used. A digest, containing 10 ml of filtrate, 2 ml of McILVAINES buffer solution (pH =4.7), 1 g of maltose hydrate and 0.5 ml of toluene was incubated for 24 hours at 40°C. It was shown by paper chromatography that glucose, isomaltose, panose and dextrantriose were synthesized from maltose. We could not recognize the spots of sakébiose and kojibiose, because they were overlaped by the large spot of residual maltose. It seemed that transglucosidase activity of the filtrate was weaker than that of the cells.

(VI) The Oligosaccharide Synthesized from Glucose and Sucrose

It is interesting to make an investigation of a scheme for the mechanism of the transglucosidation whether oligosaccharides are synthesized from glucose.

A digest, containing 11.32 per cent of glucose instead of maltose, buffered solution and acetone preparation of *Schizo. Pombe* was incubated for 48 hours at 40°C with the same method as described in (2). It was shown by paper chromatography that isomaltose was synthesized from glucose. Isomaltose was sythesized also from sucrose (9.40 per cent) as glucose.

Summary

We studied on the synthesis of oligosaccharides by *Schizosaccharomyces Pombe* No. 1 and No. 2 from maltose, glucose and sucrose, and obtained the following results.

(1) The washed cells of *Schizo. Pombe* were suspended in the buffered maltose solution (pH=4.7) and the reaction mixture was incubated for one to two hours at 28~30°C. We have demonstrated by paper chromatography that *Schizo. Pombe* synthesized sakébiose, kojibiose, isomaltose, panose and dextrantriose from maltose.

(2) The acetone preparation of *Schizo. Pombe* also synthesized the same oligosaccharides from maltose. A quantitative analyses of these sugars have been made. Initial maltose concentration was 11.78 per cent. Yield ; sakébiose 0.28 g, kojibiose 0.43 g, isomaltose 1.59 g, panose 0.33 g and dextrantriose 0.37 (in 100 ml).

(3) From glucose and sucrose, isomaltose was synthesized by *Schizo. Pombe* with the same method.

Acknowledgment

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(VII) The Synthesis of Isomaltose from Glucose by *Asp. niger*.*

In previous communications (1), we reported that isomaltose was synthesized from glucose and matose by fungal enzyme, but not from arabinose, xylose, galactose, fructose, mannit, sucrose and inulin. Pfannmuller (2) reported that isomaltose and a smaller quantity of an oligosaccharide containing more than two glucose units per molecule were synthesized from glucose by a purified precipitated enzyme preparation derived from *Asp. oryzae*. Pan *et al* (3) reported on the synthesizing action of *Asp. niger* enzymes on maltose and showed no formation of oligosaccharides from glucose. Tsuchiya *et al* (4) have demonstrated by chromatographic techniques that *Asp. niger* enzymes synthesize oligosaccharides from maltose but direct synthesis from glucose was not demonstrated. Pazur *et al* (5) reported that the enzyme prepared from *Asp. oryzae* did not synthesize oligosaccharides from glucose-l-phosphate and glucose or from glucose alone.

We have investigated on the sugars synthesized from glucose by the action of fungal enzyme. The method was the same as in the previous communication (1) described on the sugars synthesized from maltose. Buffered 7 per cent glucose solution were mixed with the culture filtrate of *Asp. niger sp.* as enzyme solution and incubated for 72 hours at 55°C. The synthesized oligosaccharides

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in the digestion were revealed to be isomaltose and a smaller quantity of sakébiose and kojibiose by paper chromatography. Then the digestion of glucose was fractionated by Whistler and Durso's method (6) which involved the passage of the solution through a charcoal column and subsequent elution of the column with aqueous sloutions containing increasing concentrations of ethanol. Each of the eluates, with the exception of the first, was concentrated to a small volume, filtered and dried up to a white solid. From the eluates of 6 per cent ethanol, isomaltose was obtained as a white powder which was a very hygroscopic solid, and its octaacetate and phenylosazone were obtained.

Experimental

(1) Synthesis of Oligosaccharides from Glucose

A surface culture of *Asp. niger sp.* (isolated strain from soil in our laboratory ; it had a maximum yield of isomaltose in the previous communication (1) was prepared in a medium containing the following ingredients per liter ; corn 20 g, ammonium sulfate 2 g, ferrous sulfate 0.01 g, calcium carbonate 5 g, potassium dihydrogen phosphate 1 g, and magnesium sulfate 0.5 g. The medium was divided into 100 ml portions, placed in 300 ml Erlenmyer flasks, sterilized and inoculated with spores of the mold (*Asp. niger sp.*). The inoculated medium was incubated for five days at 28~30°C. One liter of filtrate of the fungal culture was added to one liter of a buffered glucose solution. The resulting mixture contained approximately 70 mg of glucose per 1 ml and 10 ml of McILVAINES buffer solution (pH=4.5) per 100 ml. A small amount of toluene was added and the reaction mixture was incubated for 72 hours at 55°c.

(2) Qualitative Analyses of the Synthesized Oligosaccharides

The products, which were synthesized by culture filtrates of *Asp. niger sp.* from glucose, were examined by paper chromatography. The method used in our laboratory for the identification of sugars by paper chromatography was reported previously (1). For the developing solvent, a mixture of pyridine, buthanol and water (2 : 3 : 1.5 respectively) and four times multiple technique was used. For the spray agent, aniline hydrogenphtalate was used. The obtained chromatograms are shown in Fig. 1.

From the Rf values, colors and the mixed paper chromatograms with the authentic sugars, the spots of paper chromatograms were identified. No spot appeared on the chromatograms of the culture filtrate, and only the glucose spot appeared on that of the buffered glucose solution. Four spots appeared on the four times multiple chromatograms of the digestion. Comparative tests with the pure sugars by the techniques of the mixed chromatography showed that they were glucose, sakébiose, kojibiose and isomaltose respectively. The colors of the spots of sakébiose and kojibiose were very weak.

As shown in Table 1 quantitative analyses of glucose, sakébiose, kojibiose

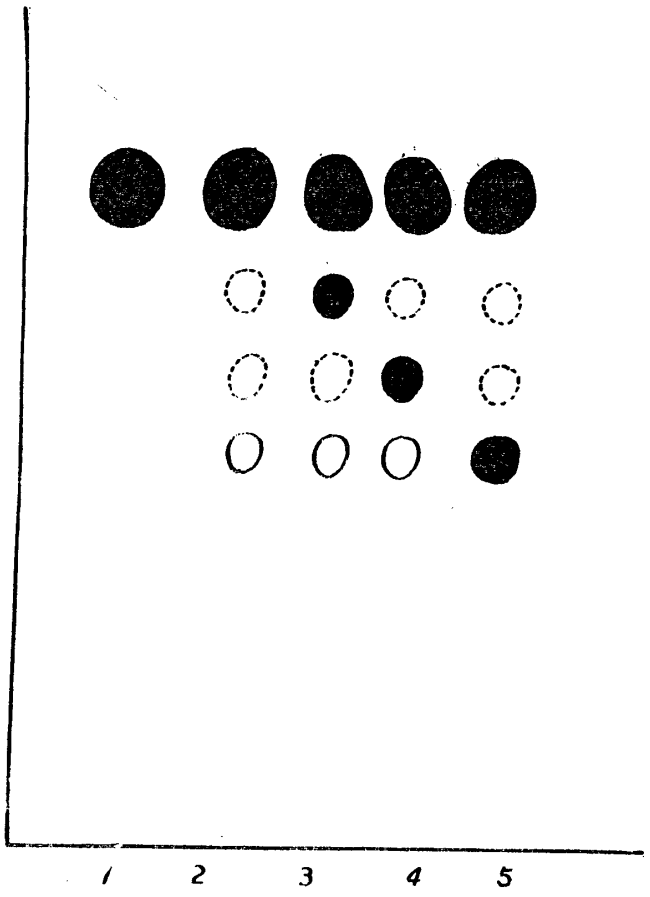

Fig. 1. Multiple Paper Chromatograms of the Digestion of Glucose.
(1) Multiple chromatogram of the buffered glucose solution
(2) Multiple chromatogram of the digestion of glucose
(3) Mixed chromatogram with sakébiose
(4) 〃 with kojibiose
(5) 〃 with isomaltose

● intense
○ weak
○ very weak

and isomaltose in the digestion have been made by the modifications of the method described by Flood et al (7). Namely the respective spots of sugars on the four times multiple paper chromatograms were cut off by guide strips and then extracted with water. The extracted sugars were analyzed using the method of Stark and Somogyi (8).

Table 1. Analysis of Sugars Synthesized from Glucose

Oligosaccharides	g/100 ml	%
Residual glucose	6.16	96.20
Sakébiose Kojibiose	0.05	0.79
Isomaltose	0.19	3.01
Total	6.40	100.00

(3) Isolation of Isomaltose

Furthermore we tried to isolate isomaltose. The digestion of glucose was fractionated on a charcoal column by Whistler and Durso's method (6). The

column was prepared in a glass cylinder 40×5 cm with fritted glass bottom. The adsorbent was made by mixing mechanically equal parts by weight of active carbon (Takeda) and Celite (Hyflo-Super cel). A slurry of the adsorbent in water was poured into the cylinder and was packed to form column 25 cm high. Sufficient suction was applied to draw the liquids through at a rate of 300 ml per hour. The effluent containing the desorbed material was caught at the bottom of the unit in a large suction flask in 1,000 ml quantities. 2,000 ml of the digestion was poured into these two cylinders. Each 1,000 ml of eluates of water with the exception of the first, was concentrated *in vacuo* to a volume of 5 ml. The 1 st~111 ed eluates were proved to contain only a large quantity of glucose. Glucose was removed by washing with 15 liter of water. After removal of the glucose, the isomaltose was desorbed by washing with 6 per cent ethanol. The 6 per cent ethanol fraction, in which isomaltose only was presented as sugar by its paper chromatography were concentrated *in vacuo* to sirup. To a colorless sirup methanol was added and the solution was then filtered. A filtrate was dried up *in vacuo* and a white powder was obtained. Only one spot of isomaltose was obtained on the paper chromatogram of this white powder. It was very hygroscopic.

After purification with absolute ethanol, all attempts to crystallize the substance failed. A part of it was treated with phenylhydrazine hydrochloride and phenylosazone of isomaltose was obtained. After recrystalization from hot water, the melting point was 206~208°C. Acetylation of isomaltose by newly fused sodium acetate and acetic anhydride gave an amorphous product. The melting point of the amorphous acetate purified with ethanol was 143~145°C and was in close agreement with the accepted values (M. P. = 143~144°C.) for isomaltose octaacetate (9, 10.).

Summary

Studying the synthesis of isomaltose by action of fungal enzymes on glucose, the following results were obtained.

Filtrates from fungal culture (*Asp. niger sp.*) were added as fungal enzymes to the buffered glucose solutions and the reaction mixtures were incubated for 72 hours at 55°C. The synthesized oligosaccharides were revealed to be isomaltose, sakébiose and kojibiose by paper chromatography. Then its quantitative analysis was made. Isomaltose was isolated from the digestion of glucose, using a modification of the method described by Whistler and Durso.

Isomaltose octaactate M. P. 143~145°C

Isomaltose phenylosazone M. P. 206~208°C

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