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By

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(IV) Action of β–Glucosidase on the Unfermentable Disaccharides
(V) Isolation of Isomaltose and Sakébiose from Koji Extract

(IV)* Action of β-Glucosidase on the Unfermentable Disaccharides

In the previous report (1) we have pointed out the presence of isomaltose, panose and two other unidentified sugars (B_1 and B_2) in Saké and Koji (mold rice) extract.

The Rf value of B_1 was a little higher than that of maltose, and B_2 just agreed with the spot of cellobiose even though on the triple chromatogram, and therefore, we have considered B_2 as cellobiose. But afterwards, the identity of B_2 with cellobiose became questionable from the following facts.

1) The spot of B_2 on the chromatogram sprayed with aniline hydrogen phthalate showed a clear pink colour somewhat like that of pentose, while cellobiose showed a brown colour.
2) No β-glucosidic linked saccharide has been found in the enzymic hydrolyzate of starch, whereas cellobiose is a β-linked disaccharide.

The present study has been done to clarify the question whether B_2 is identical with cellobiose or not. For this purpose, the action of β-glucosidase (emulsin) upon these sugars has been examined. And it beacme clear that these sugars were not hydrolyzed by β-glucosidase, that is to say, these were both α-glucosidic sugars. So we should like to correct the mistake and designate these sugars herein as "sakébiose" (B_1) and "kojibiose" (B_2) respectively.

Recently, Barker *et al* (2) claimed to have isolated 3-*O*-α-D-glucopyranosyl-D-glucopyranose from the partial hydrolyzate of mycodextran (nigeran). According to their report, this sugar has an Rf value almost equal to that of laminaribiose (3-*O*-β-D-glucopyranosyl-D-glucopyranose).

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Then we compared these sugars on the paper chromatogram with laminaribiose which was kindly furnished by Drs. S. A. Barker and E. J. Bourne. Sakébiose showed very close Rf value to laminaribiose.

From these results, we surmise that sakébiose may be identical with 3–*O*–α-D-glucopyranosyl-D-glucopryanose though a more exact identification must be done from other aspects.

Experimental

(1) Preparation of Sugar Samples

The Koji extract was fermented with yeast. The fermented solution was treated with basic lead acetate to remove protein and other extraneous materials and deionized by ion exchange columns. The resulted solution was concentrated *in vacuo* to a sirup. The sirup was diluted to about 10% solution and spotted on the filter paper and developed three times with butanol : pyridine : water (3 : 2 : 1.5). The corresponding zones of B_1 and B_2 were sectioned and eluted with water and the eluates were concentrated *in vacuo* to a sirup. Thus obtained sugar samples were about 5 mg from one sheet of paper.

(2) Detection of Component Sugars of Disaccharides by Acid Hydrolysis

5 ml of 0.5 N sulfuric acid was added to the above sugar samples and heated in a boiling water bath for an hour. The hydrolyzate was neutralized by barium carbonate and concentrated *in vacuo*.

The sugars in the hydrolyzate was detected by paper chromatography. Only glucose was detected in the hydrolyzates of both sugars. No spot was revealed by either resorcinol (for the dectetion of ketoses) or ninhydrin (for the detection of amino sugar) reagent.

(3) Prepraration of Emulsin

Emulsin was prepared according to the method of Tauber (3).

100 g of almond seed was steeped in warm water (50°C) for 20 minutes to remove the peel, and crushed to a paste. The paste was extracted with ether for 30 hours to remove the oil, and dried in an oven at 30°C. The yield of oil free powder was about 45 g.

This powder was extracted with 350 ml of 33% ethanol for 6 minutes, then a same amount of 95% ethanol was added to the extract. The precipitated crude enzyme was centrifuged and dried in a vacuum desiccator. Yield 0.7 g. The enzyme solution was prepared as follows : 10 mg of above crude enzyme was dissolved in 5 ml of acetate buffer (pH. 4.4) and centrifuged after being kept standing several hours.

(3) Action of Emulsin on the Sugars

0.1 ml of above prepared enzyme solution was added to the samples prepared as (1) together with a few drops of toluene, and incubated at 30°C for 72 hours. The paper chromatogram of the samples before and after the reaction was

compared. As a control, 10 mg of cellobiose was treated simultaneously in the same condition. Though after 72 hours, these sugars were both immune to β-glucosidase, while cellobiose was completely hydrolzyed to glucose.

Summary

1) Action of β-glucosidase on the unfermentable disaccharides in Koji extract (B_1 and B_2) has been examined and it became clear that these sugars were both immune to β-glucosidase.

2) We suggested to designate these sugars "sakébiose" and "kojibiose" respectively and surmised the former to be 3-*O*-α-D-glucopyranosyl-D-glucopyranose by comparing these sugars with laminaribiose on the paper chromatogram.

Acknowledgement

We should like to acknowledge our indebtedness to Drs. S. A. Barker and E. J. Bourne for the sample of laminaribiose.

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2) Barker, S. A., Bourne, E. J. and Stacey, M. (1953) ; Chem,. and Ind., 756.
3) Tauber, H. (1932) : J. Biol. Chem., **99**, 257.

(V) *Isolation of Isomaltose and Sakébiose from Koji Extract

We have reported in the previous communication, that two unidentified sugars in Koji extract were both α-glucosidic linked disaccharides and designated them sakébiose and kojibiose respectively. And we have surmised sakébiose to be 3-*O*-α-D-glucopyranosyl-D-glucopyranose.

Attempts have been made on the isolation of these sugars, and we have been able to isolate sakébiose and isomaltose from the Koji extract as their crystalline octaacetates by employing carbon column chromatography of free sugars together with Magnesol : Celite column chromatography of sugar acetates.

Fukimbara *et al* (1) reported on the isolation of these sugars from Saké by the application of paper chromatography. They isolated isomaltose and panose as crystalline free sugars and two other sugars corresponding to our sakébiose and kojibiose as amorphous powder. But the physical constants of the sugar "*x*" corresponding to sakébiose were not described.

Barker *et al* (2) have isolated a new disaccharide from the partial hydrolyzate of mycodextran (nigeran) extracted from the mycelium of *Asp. niger*. They concluded that this sugar was 3-*O*-α-D-glucopyranosyl-D-glucopyranose from

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the identity of its osazone with that of turanose (3-*O*-α-D-glucopyranosyl-D-fructose), and suggested to name this sugar "nigerose".

The properties of our sakébiose very much resemble to those of the disaccharide of Barker (nigerose) as shown in Table 1, except the melting point of the acetate.

Table 1. Properties of Disaccharides considered to be 3-*O*-α-D-glucopyranosyl-D-glucopyranose

Name	m.P. (°C)	$[\alpha]_D$ (H_2O)	m.P. (°C) of acetate	$[\alpha]_D$ of acetate ($CHCl_3$)	m.P. (°C) of osazone
Amylolyose					160~162
Disaccharide synthesized by Gakhokidze	162 102 (dihydrate)	$[\alpha]_D^{18} = +84.4$	149	$[\alpha]_D^{18} = +41.3$	202
Nigerose		+136	111~113		204~206
Y			155~157	$[\alpha]_D^{25} = +78$	
Sakébiose		$[\alpha]_D^{12} = +135$	150	$[\alpha]_D^{23} = +80$	205

Recently, Thompson *et al* (3) have also obtained a new disaccharide octaacetate from the reversion products of D-glucose and designated it *y*-acetate. The properties of this sugar acetate are also very close to our sakébiose octaacetate.

As to 1,3-α-glucosidic disaccharide, Nakamura (4) reported in 1941 that he had obtained this sugar from the enzymic hydrolyzate of starch as its phenylosazone and designated it "amylolyose".

In 1946, Gakhokidze (5) claimed to have synthesized 3-*O*-α-D-glucopyranosyl-D-glucopyranose by condensing α-D-glucopyranose 2:3:4:6 tetraacetate with 4:6-*O*-benzylidene-1 : 2-*O*-isopropylidene-D-glucopyranose, and then removing the protecting groups.

But the constants of amylolyose and Gakhokidze's sugar are fairly different from those of sakébiose, nigerose and *y*.

The structure of sakébiose is not yet established, but further study of identification is now in progress.

(1) Preparation of Unfermentable Sugars from Koji Extract

Koji (mold rice). prepared from 7Kg of rice, was mixed with 20*l* of water and digested at 55°C for 10 hours. The hydrolyzate was filtered, diluted to the sugar concentration of about 15%, sterilized at 100°C for 30 minutes and fermented with Saké-yeast (Kyôkai No. 7) at 25°C for 4 days. The fermented solution was neutralized to pH. 5.8 with sodium carbonate and concentrated under diminished pressure to about 1.5 *l*. 3.5 *l* of ethanol was added to the concentrated solution and allowed to stand overnight. The precipitate was

removed by decantation and the supernatant was again concentrated *in vacuo* to a thick sirup. Yield 380 g. The above sirup was dissolved in 1 *l* of water and treated with basic lead acetate. The excess of lead ion was precipitated with hydrogen sulfide, and the filtrate was passed through the ion exchange columns (Amberlite IR-120 and IR-A-410) to remove the ionic materials. The resulted light yellow solution was concentrated *in vacuo* to a sirup. Yield 280 g.

(2) Fractionation of Sugars by Charcoal Column

An amount of the solution containing about 35 g of the above unfermented sugar was diluted to 500 ml and placed on a chromatographic column (400×80 mm diam.) containtng 360 g of charcoal (Darco G-60) and the same amount of Celite (No. 545), and washed with water with suction. The effluent was caught in 1000 ml quantities and concentrated to about 10 ml. The sugars in eluate were detected by paper chromatography. The elution was continued until the sugars in effluent became only a trace. Then the solvent for elution was changed to 5% ethanol, and the elution was continued as before. The solvent was then changed to 10% ethanol and finally 25% ethanol. The results are shown in Table 2.

Table 2. Fractionation of Sugars by Charcoal Column

Solvent used for elution	Fraction No.	Probable Sugar Component by Paper Chromatography	Yield
water	1~2	No sugar	
〃	3	No sugar ; Very viscous substance probably glycerine	
〃	4	Xylose, Arabinose, Glucose, Galactose,	3.2g
〃	5	Xylose, Arabinose, Glucose, Galactose, Isomaltose	2.6g
〃	6~27	Isomaltose	12.2g
〃	28~51	Isomaltose, Kojibiose	3.8g
5% Ethanol	52~55	Isomaltose, Kojibiose	2.6g
〃	56	Kcjibiose, Maltose	0.8g
〃	57~61	Maltose, Sakébiose	3.6g
〃	62~74	Sakébiose	1.7g
〃	75~79	Dextrantriose ?	0.8g
10% Ethanol	77~79	Dextrantriose ?	
〃	80	Sakébiose, Dextrantriose ?	1.7g
〃	81~82	Sakébiose, Panose	
〃	83~85	Panose	
〃	86~88	Panose, A trace of other trisaccharides	
25% Ethanol	89~92	Panose and other tri- or more higher saccharides	1.3g

(3) Identification of Isomatose

Fraction No. 6~27 was combined and concentrated under reduced pressure to a sirup. The sirup was dissolved in hot methanol and the insoluble matter

was filtered off. The filtrate was again concentrated and dried in a vacuum desiccator. Isomaltose was obtained as white amorphous powder. Yield 12.2 g.

1 g of above crude isomaltose was acetylated with 0.6 g of fused sodium acetate and 5 ml of acetic anhydride at 110°C for two hours. The reaction mixture was cooled and poured into 150 ml of ice and water. After the hydrolysis of residual acetic anhydride, the sugar acetate was extracted with chloroform. The extract was washed with aqueous solution of sodium carbonate and water, dried over anhydrous sodium sulftae and evaporated to a sirup. Yield 1.9 g. This sirup was crystallized from ethanol and was recrystallized from the same solvent m.p. 145~146°C, unchanged on admixture with the authentic specimen, $[\alpha]_D^{23} = +97$ (c, 2.8 ; chloroform).

(4) Isolation of Sakébiose

Fraction No. 62~74 was combined and treated as before. 1.7 g. of white amorphous sugar was obtained, $[\alpha]_D^{12} = +135$ (c, 1.2 ; water).

Acetate :

0.5 g of the above sugar was acetylated with 0.3 g of sodium acetate and 3 ml. of acetic anhydride by the same procedure as described above. Yield 0.9 g of sirup. This sirup was crystallized from ethanol and was recrystallized from the same solvent, m.p. 150°C, $[\alpha]_D^{23} = +80$ (c, 3.1 ; chloroform).

Anal. Cal. for $C_{28}H_{38}O_{19}$: C, 49.55 ; H, 5.64. Found : C, 49.67 ; H, 5.87.

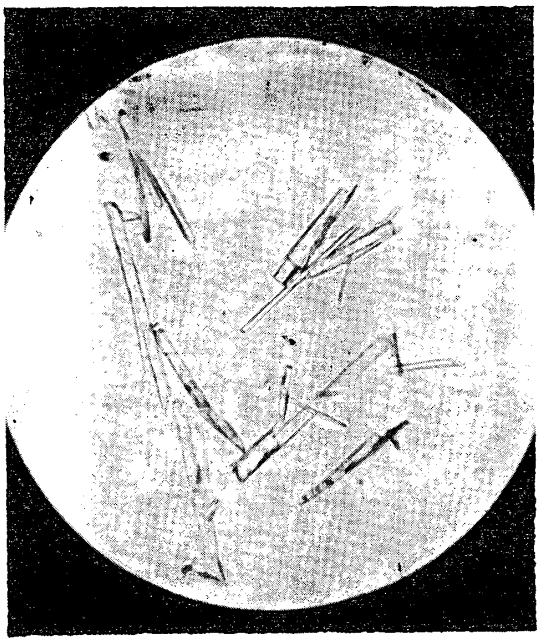

Fig. 1. Sakébiose octaacetate

Osazone :

0.1 g of above sugar, 0.2 g of phenylhydrazine hydrochloride and 0.3 g of sodium acetate were heated with 2 ml of water on a boiling water bath for 1.5

hours. After cooling, yellow precipitate was filtered and washed with water, which was crystallized from ethanol and was recrystallized twice from the same solvent, m.p. 205°C.

Anal. Cal. for $C_{24}H_{32}O_9N_4$: N, 10.76. Found : N, 10.25.

(5) Chromatographic Resolution of Sakébiose and Maltose Octaacetates

Fraction No. 57~61 was combined and treated as before, 3.6 g of amorphous sugar was obtained which consisted of sakébiose and maltose. 0.5 g of this mixed sugar was acetylated as above, 0.9 g of mixed acetates were obtained.

These mixed acetates were dissolved in 10 ml of benzene and placed on a 210×35 mm (diam.) column of Magnesol : Celite (5 : 1 by weight) and developed with 2500 ml of benzene-t-butyl alcohol (100 : 1 by volume). The column was extruded and located by the streak indicator (1% $KMnO_4$ solution in 2.5 N NaOH). A clear zone appeared 30–95 mm from the top, which was sectioned, eluted with acetone and evaporated to a sirup. Yield 0.5 g. This sirup was crystallized from ethanol and was recrystallized from the same solvent, m.p. 150°C, agreed with that of sakébiose octaacetate. The effluent from the column was evaporated to a sirup. Yield 0.2 g. This sirup was crystallized from ethanol, and was recrystallized from the same solvent, m.p. 158~159°C, unchanged on admixture with known β-maltose octaacetate.

Summary

1) The sugars in the fermented solution of Koji extract were fractionated by carbon column chromatography. The results are shown in Table 2.

2) Sakébiose (probably 3-*O*-α-D-glucopyranosyl-D-glucopyranose), isomaltose and maltose were isolated as crystalline octaacetates by employing above method and Magnesol : Celite column chromatography.

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