STUDIES ON THE UNFERMENTABLE DISACCHARIDES IN KOJI EXTRACT Part IV, ISOLATION AND IDENTIFICATION OF α,α- AND α,β-TREHALOSE

STUDIES ON THE UNFERMENTABLE DISACCHARIDES IN KOJI EXTRACT Part IV* ISOLATION AND IDENTIFICATION OF α,α- AND α,β-TREHALOSE

By

Kazuo MATSUDA

Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai, Japan

(Received October 30, 1955)

The author has previously isolated two unfermentable disaccharides, *i. e.* isomaltose and sakébiose, from Koji extract (1). The latter was proved to be identical with nigerose and *y*-sugar (2), the structure of which had been established by Barker *et al* as 3-*O*-α-D-glucopyranosyl-D-glucopyranose (3).

One more gluco-disaccharide, designated as kojibiose, was left unidentified. However, all attempts to isolate this sugar from isomaltose were hitherto unsuccessful by either carbon or Magnesol : Celite chromatography. Since the structure of this sugar was assumed to be 1,2-α linked disaccharide, I have tried to isolate it by treating with phenylhydrazine. By this treatment, 1,2-linked sugar gives phenylhydrazone, while the other disaccharides having *e.g.* 1,3-, 1,4-, or 1,6-linkage give water insoluble phenylosazone. If kojibiose is 1,2-linked disaccharede, it may be possible to isolate this sugar from the others by this treatment.

According to this idea, a mixture of kojibiose and isomaltose (fractionated by carbon column) was treated with phenylhydrazine hydrochloride and sodium acetate. The precipitated phenyloszone was filtered off and the filtrate was then treated with benzaldehyde to remove the excess of phenylhydrazine. The precipitated benzaldehyde phenylhydrazone was once more filtered off, and the filtrate was further purified by extraction with ether, treatment with ion exchanger and *etc.* The resulted solution and the original solution were compared by paper chromatography (PPC), and it was found that initially existed isomaltose completely disappeared by this treatment while kojibiose was distinctly detected by aniline hydrogen phtalate (AHP). So it can be suggested that kojibiose makes no phenylosazone and may be a 1,2-linked disaccharide.

* Studies on the Unfermentable Sugars (XVII)
Original Report : J. Agric. Chem. Soc. Japan, **30**, in press.

The resulted sugar solution was evaporated and the residue was acetylated as usual. The acetylated sugar was then fractionated by Magnesol: Celite chromatography. A crystalline sugar acetate was obtained. At first, I have considered this sugar acetate to be kojibiose octaacetate. But quite unexpectedly, the physical constants of this substance were in very close agreement with those of neo-trehalose (α,β-trehalose) octaacetate reported by Haworth *et al.* (4).

This sugar acetate was then deacetylated as usual. A crystalline free sugar was obtained, which showed no reducing power against Fehling's solution. On the paper chromatogram, this sugar showed a weak spot by ammoniacal silver nitrate, but none by AHP reagent. From the above results, it became clear that this sugar was not kojibiose but belonged to a disaccharide of nonreducing trehalose type. To identify this sugar, α,β-trehalose was synthesized by the method of Micheel and Hagel (5). And it was proved that the sugar acetate obtained by the author was identical with the synthetic specimen.

As to the preparation of α,β-trehalose, many studies have been hitherto reported, but the properties of those samples were quite different from each other and the identity of this sugar has been quite obscure.

In early days, Vogel and Debowska-Kurnicka obtained a sugar acetate, m.p. 68~70°C, $[\alpha]_D$ + 68.1 (chloroform), by condensing 2,3,4,6-tetra-*O*-acetyl-D-glucose in the presence of zinc chloride and phosphorous pentoxide (6). The product obtained was assumed to be α,β-trehalose octaacetate, since the $[\alpha]_D$ was in close agreement with that expected on the basis of Hudson's rules of isorotation (7).

Haworth and Hickinbottom (4) obtained a disaccharide heptaacetate by reacting 2,3,4,6-tetra-*O*-acetyl-β-D-glucose in benzene solution with Brigl's anhydride (1,2-anhydro-3,4,6-tri-*O*-acetyl-D-glucose). Octaacetate of this sugar was obtained by further acetaylation, m.p. 141°C, $[\alpha]_D$ + 82 (chloroform). They suggested to name this new nonreducing disaccharide neo-trehalose and assumed this to be α,β-trehalose.

Sharp *et al* (8) have recently questioned the identity of this substance and have reported on the preparation of neo-trehalose octaacetate, m.p. 120°C, $[\alpha]_D$ + 67 (chloroform), both by reaction of β-acetofluoro glucose with silver carbonate, anhydron and iodine in chloroform and by treating 2,3,4,6-tetra-*O*-acetyl-α-D-glucose with phosphorous pentoxide in chloroform.

More recently, Micheel and Hagel (5) reported a very simple preparation of this sugar. Namely, they obtained α,β-trehalose octaacetate together with β,β-trehalose octaacetate by reacting acetobromo glucose with about 0.4 M of water in the presence of mercuric cyanide. The constants of the obtained sugar acetate, m.p. 140°C, $[\alpha]_D$ + 80 (chloroform), are in close agreement with those reported by Haworth *et al.*

Subsequently, Bredereck *et al* (9) reported to have obtained α,β- and β,β-trehalose octaacetates by treating 2,3,4,6-tetra-*O*-acetyl-β-D-glucose with zinc chloride. The constants of α,β-trehalose octaacetate obtained by them were as follows : m.p. 140°C, $[\alpha]_D$+64.4 (chloroform).

Last year, Lemieux and Bauer (10) have reinvestigated the reaction of Haworth *et al* by using anomeric 2,3,4,6-tetra-*O*-acetyl-D-glucose. They obtained α,β-trehalose octaacetate, m.p. 141~142°C, $[\alpha]_D$+81.8(chloroform) together with α,α-trehalose octaacetate which had not been hitherto obtained by the synthetic prepration. These values are also in good agreement with those obtained by Haworth *et al.*

As the presence of α,β-trehalose is proved, the presence of α,α-trehalose is naturally expected. Therefore, I attempted to isolate α,α-trehalose and was able to obtain this sugar in the same way from the earlier fraction in which only isomaltose had been detected by PPC.

Thus two types of nonreducing trehalose were isolated from Koji extract and identified. α,α-Trehalose was isolated from yeast in fairly good yield (11), but α,β-trehalose has been hitherto prepared only synthetically and no one has reported either on the isolation of this sugar from natural substance or on its biochemical preparation. It is not certain, whether these sugars were extracted from mycelial felts of yeast or produced by the socalled trans-glucosidation of *Asp. oryzae.* In any case, it is very interestng that α,β-trehalose is obtained by the non synthetic method and the presence of this sugar in other natural substances may be expected.

Experimental

〔1〕 *Isolation of α,β-Trehalose.*

6.4 g of sugar mixture, in which isomaltose and kojibiose were detected by PPC, was heated on a water bath for four hours with 13 g of phenylhpdrazine hydrochloride 19 g of sodium acetate and 130 ml of water. After cooling, the yellow precipitate of phenylosazone was filtered off. The filtrate was treated with 10 ml of benzaldehyde and precipitated benzaldehyde phenylhydrazone was filtered off. The filtrate was then extracted with ether to remove the excess of benzaldehyde and passed through an ion exchanger Amberlite IR 120 and IR A-410 to remove the ionic substances. The resulted solution was finally decolorized with carbon and concentrated *in vacuo* to a sirup. The sirup was dissolved in hot methanol and the insoluble material was filtered off. The filtered solution was again concentrated *in vacuo.* 0.8 g of amorphous powder was obtained. On the paper chromatogram of this sample, kojibiose and glucose was detected by AHP. The latter is presumed to be produced by the hydrolysis during the course of the above treatment. This powder was then acetylated as usual and 1.3 g of acetate was obtained.

The acetylated sugar was dissolved in 10 ml of benzene and poured into a 190×35 mm (diam.) column of Magnesol : Celite (5 : 1) and developed with 2500 ml of benzene : *t*-butyl alcohol (100 : 1). Two zones were located on the extruded column by alkaline permanganate (1 percent solution in 4 N NaOH). One is 45~95 mm and the other 110~140 mm from the top. Each zone was sectioned and eluted with acetone and evaporated. From the upper zone, 0.3 g of sirupy acetate was obtained which was crystallized from ethanol and was recrystallized from the same solvent, m. p. 140~141°C, $[\alpha]_D^{17}$+ 82 (*c*, 3.0; chloroform).

Anal. Calc. for $C_{28}H_{38}O_{19}$: C, 49.55; H, 5.64; mol. wt., 678.
Found : C, 48.74; H, 5.50; mol. wt. (Rast) 657.

The m.p. of this substance is very close to that of β-isomaltose octaacetae, but on admixture with the latter the m. p. depressed to 119~122°C.

From the lower zone, 0.2 g of sirup was obtained. This substance is considered probably to be kojibiose acetate, but crystallization of this substance is not yet successful.

The effluent from the column was evaporated. The residue was crystallized from ethanol after several days. After being recrystallized from the same solvent the m. p. was 129°C, unchanged on admixture with known β-glucose pentaacetate.

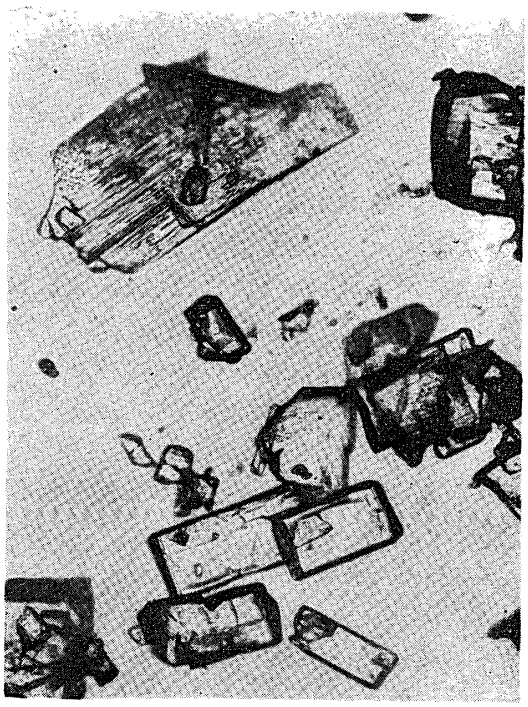

Fig. 1. α,β-Trehalose Octaacetate
(isolated from Koji extract)

〔2〕 *α,β-Trehalose*

50 mg of the above α,β-trehalose octaacetate was dissolved in 0.5 ml of 0.05 N sodium methoxide in methanol and kept at 0~5°C overnight. The solvent was evaporated slowly in a desiccator at room temperature. The crystallized free sugar was collected and recrystallized from aqueous ethanol in a refrigerator. 18 mg of crystalline free sugar was obtained. This free sugar sintered at 145~150°C and frothed at 153°C, but the exact m.p. could not be obtained. Haworth *et al* reported neo-trehalose to melt at 210~220°C with sintering at 145~150°C. Lemieux *et al* reported this sugar to melt at 195~210°C with sintering at 141~145°C. The crystal obtained sintered at 145°C, but all attempts to obtain the exact m.p. were unsuccessful. This sugar showed no reducing power against Fehling's solution, and on the paper chromatogram a spot was obtained which was very close to that of α,α-trehalose.

〔3〕 *Synthesis of α,β-Trehalose Octaacetate*

2.5 g of α-acetobromo glucose, 0.744 g of mercuric cyanide and 0.044 ml of water was dissolved in dry acetone and kept at 50°C for several minutes. After removal of the solvent *in vacuo*, the residue was heated on a water bath for three hours. The dark brown reaction mixture was extracted with three portions of 20 ml of benzene. The extract was washed with aqueous sodium bicarbonate and water, decolorized with carbon and dried over anhydrous sodium sulfate. After removal of the solvent 0.8 g of yellow sirup was obtained. This sirup was then dissolved in 10 ml of 0.05 N sodium methoxide in methanol and kept at 0~5°C overnight. The resulted solution was diluted with water and deionized by Amberlite IR-120 and IR A-410. From the resulted somewhat opaque solution a small amount of yellow preciptate, probably mercuric oxide, was deposited. The filtered solution was treated once with charcoal and evaporated *in vacuo* to a sirup which was dried azeotropically with methanol. 0.3 g of amorphous powder was obtained. This powder was acetylated as usual and 0.5 g of acetate was obtained. This sugar acetate was then fractionated chromatographically using 230×23 mm (diam.) column of Magnesol : Celite and 1000 ml of benzene : *t*-butyl alcohol (100 : 1). A continuous zone was located by alkaline permanganate on the upper part of the extruded column. The lower part of the zone was eluted with acetone and evaporated. 0.2 g of sirup was obtained which was crystallized from ethanol after being kept at room temperature for several days. After recrystallization from ethanol, m.p. was 139~140°C uuchanged on admixture with the specimen obtained from koji extract.

Micheel *et al* have obtained β,β-trehalose octaacetate simultaneously, but I failed to obtain this substance.

〔4〕 *Isolation of α,α-Trehalose*

3.7 g of sugar fraction, in which only isomaltose was detected by PPC,

was treated with 7.5 g of phenylhydrazine hydrochloride and 11 g of sodium acetate. The resulted solution was treated in the same manner as described in [1]. 0.5 g of sirupy sugar was obtained. On the paper chromatogram, a spot corresponding to α,α-trehalose was detected by the silver reagent. No spot was detected by AHP reagent.

This sirup was acetylated as usual and fractionated on the 200×35 mm (diam.) column of Magnesol : Celite with 2500 ml of benzene : *t*-butyl alcohol. Only one zone, 50~90 mm from the top, was located on the extruded column, which was sectioned and eluted with acetone. Removal of the solvent left 0.4 g of sirup which was crystallized from ethanol. The m.p. of the recrystallized crystal was 70~74°C, this value is quite different from the reported value (12). The m. p. of the α,α-trahalose octaacetate prepared from the authentic specimen (Tekeda) was also 70~75°C which did not raise though after several recrystallizations.

Bredereck (13) reported that the m.p. of α,α-trehalose octaacetate prepared by him was 70~75°C after twice recrystallization from ethanol, but the m.p. raised to 101~102°C (corr.) when the same sample was dried at 61°C *in vacuo* (12 mm).

The above obtained α,α-trehalose octaacetate was dried *in vacuo* over phosphorous pentoxide at 61°C for several hours, m.p. was 96~98°C, unchanged on admixture with the authentic specimen dried in the same way, $[\alpha]_D^{27}$ +160 (*c*, 1.2; chloroform).

Summary

α,α-and α,β-Trehalose were isolated from Koji extract as their crystalline octaacetates, and the latter was identified by mixed melting point with the synthetic specimen prepared by the method described by Micheel *et al.* Separation of these sugars from isomaltose, was performed by removal of the latter as its water insoluble phenylosazone.

Kojibiose also gives no phenylosazone and is presumed probably to be 1,2-α linked disaccharide, but neither the free sugar nor its octaacetate could be crystallized.

Acknowledgment

The author wishes to acknowledge his indebtedness to Prof. Aso for his kind guidance; to Mr. Shibasaki for his advice; to the Laboratory of Biological Chemistry of the Department of Chemistry of the Faculty of Science of our University for the measurment of $[\alpha]_D$, to the Department of Agricultural Chemistry of the Faculty of Agriculture of the Tokyo University for microanalysis.

This study was partly supported by a grant from the Scientific Research Expenditure of the Ministry of Education.

References

1) Matsuda K. and Aso K. (1954) : This Journal, **V**, No. 2, 125.
2) Matsuda, K., Hiroshima, G., Shibasaki, K. and Aso, K. (1954) : This Journal, **V**, No. 3, 243.
3) Barker, S.A., Bourne, E.J. and Stacey, M. (1953) : J. Chem. Soc., **1953**, 3084.
4) Haworth, W. N. and Hickinbottom, W. J. (1931) : J. Chem. Soc., **1931**, 2847.
5) Micheel, F. and Hagel, K.O. (1952) : Chem. Ber., **85**, 1087.
6) Vogel, H. and Debowska-Kurnicka, H. (1928) : Helv. Chim. Acta, **11**, 910.
7) Hudson, C.S. (1916) : J.Am. Chem. Soc., **38**, 1566.
8) Sharp, V.E. and Stacey, M. (1951) : J. Chem. Soc., **1951** 285.
9) Bredereck, H., Höschele, G., and Ruck, (1953) : Chem. Ber., **86**, 1277.
10) Lemieux, R.U. and Bauer, H.F. (1954) : Can. J. Chem., **32**, 340.
11) Sato, T. and Tsumura, S. (1953) : J. Agr. Chem. Soc. Japan, **27**, 412.
12) Hudson, C.S. and Johnson, J. (1915) : J. Am. Chem. Soc., **37**, 2748.
13) Bredereck, H. (1930) : Ber. **63**, 959.