Micro Methods for Determination of 3-Ketosucrose and 3-Ketoglucose

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Methods for differential determination of 3-ketosucrose and 3-ketoglucose were established. For determination of 3-ketosucrose, alkaline treatment with 0.1 N NaOH was found to be most effective. In this method, 3-ketosucrose gave a characteristic absorption spectrum with a molar extinction coefficient of $6.5 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 340 m μ , while 3-ketoglucose did not show a significant absorption spectrum within a range from 300 to 400 m μ .

By mixing with 0.2 M phosphate buffer, pH 7.0, 3-ketoglucose gave a characteristic absorption spectrum with a molar extinction coefficient of $3.8 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 310 m μ , while 3-ketosucrose showed little absorbance.

From the reduction rate of 2,6-dichloroindophenol with 3-ketoglucose, the ketosugar was determined. 3-Ketosucrose was not able to reduce the reagent at all.

The methods established here were not affected by fructose.

Recently, 3-ketoglycosides have been proved to be the first intermediates in the metabolic pathway of carbohydrate by *Agrobacterium tumefaciens*, a plant tumor inducing bacterium.^{1~5)} As one of their general properties, it was found that every 3-ketoglycoside so far isolated showed strong reducing power and could reduce readily cupric ion to cuprous form by the Somogyi-Nelson reagent at room temperature. On the basis of this finding, a new determination method for 3-ketosugar was reported in our previous paper.⁶⁾ On the other hand, Feingold *et al.*⁷⁾ indicated the

possibility of 3-ketosucrose determination by alkaline treatment with 0.1 N NaOH. In the study of 3-ketosucrose-hydrolyzing enzyme,⁸⁾ a novel enzyme named as α -D-3-ketoglucosidase, differential determination methods for the substrate, 3-ketosucrose, and the product, 3-ketoglucose, were required to establish the stoichiometry of the reaction.

In this paper, we describe new methods for 3-ketosucrose and 3-ketoglucose determinations.

METHODS AND RESULTS

Determination of 3-ketosucrose

For determination of 3-ketosucrose, the alkaline treatment was employed. By the treatment, 3-ketosucrose showed a characteristic absorption spectrum having a maximum at 340 m μ .⁹⁾

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Standard. Crystalline 3-ketosucrose ($C_{12}H_{20}O_{11} \cdot 3H_2O$, α -D-ribo-hexopyranosyl-3-ulose- β -D-fructofuranoside) was prepared as described in our previous paper.¹¹ Freshly prepared $1.0 \times 10^{-3} M$ 3-ketosucrose solution was used as a standard solution.

Reagent. 0.15 N NaOH solution.

Procedure. To 1.0 ml of a sample containing 0.05 to 0.4 μ mole of 3-ketosucrose was added 2.0 ml of 0.15 N NaOH solution. After standing for 3 min at room temperature, optical density at 340 $m\mu$ was measured. The maximum intensity was obtained after 3 min treatment. Under these conditions, the sugar gave a molar extinction coefficient of $6.5 \times 10^3 M^{-1}cm^{-1}$. Other 3-ketoglucosides so far isolated such as 3-ketotrehalose, 3-ketomaltose, 3-ketocellobiose and 3-ketoglucose 1-phosphate were also determined by this method, and their molar extinction coefficients were 6.2, 5.5, 3.55 and $3.85 \times 10^3 M^{-1}cm^{-1}$ at 340 $m\mu$, respectively. The hydrolyzate of 3-ketosucrose with yeast invertase, *i.e.*, a mixture of equimolar amounts of 3-ketoglucose and fructose, showed little absorbance at a range from 325 to 400 $m\mu$ as seen in Fig. 1. Fructose did

FIG. 1. Absorption Spectra of 3-Ketosucrose and 3-Ketoglucose Measured in 0.1 N NaOH.

Curve 1, absorption spectrum of 3-ketosucrose (0.17 μ moles); curve 2, absorption spectrum of 3-ketoglucose (0.26 μ moles).

FIG. 2. Determination Curve for 3-Ketosucrose in Alkaline Treatment.

not show any absorbance at this range in 0.1 N NaOH. When the sample contained both 3-ketosucrose and 3-ketoglucose, the amount of 3-ketosucrose was determined by subtracting the absorption value at 400 $m\mu$ from that at 340 $m\mu$, because 3-ketoglucose gave equal values at these two wavelengths. The determination curve is shown in Fig. 2.

Determination of 3-ketoglucose

Method A. 3-Ketoglucose solution in distilled water did not show any absorbance at a range from 260 to 400 $m\mu$, but in the presence of a high concentration of phosphate buffer at a neutral pH, the ketosugar have a characteristic absorption spectrum having a maximum at 310 $m\mu$ (Fig. 3).

Standard. 3-Ketoglucose ($C_6H_{10}O_6 \cdot 1/2H_2O$, D-ribo-hexos-3-ulose) was isolated in a crystalline form in the previous paper.¹⁰ The compound was hygroscopic in atmosphere and was unstable in an aqueous solution, so that the hydrolyzate of 3-ketosucrose with yeast invertase was used as a standard solution of

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FIG. 3. Absorption Spectra of 3-Ketoglucose and 3-Ketosucrose Measured in 0.2 M Phosphate Buffer, pH 7.0.

Curve 1, 3-ketoglucose (0.26 μ moles); curve 2, 3-ketosucrose (0.17 μ moles); curve 3, 3-ketoglucose (0.26 μ moles) in 0.1 M phosphate buffer, pH 6.0.

3-ketoglucose in this paper. To 1.0 ml of 10×10^{-3} M 3-ketosucrose solution in distilled water was added 10 μ g of yeast invertase (Grade VI, Sigma Chem. Co.). Complete hydrolysis of 3-ketosucrose took place during the treatment with the invertase for 5 min at room temperature without addition of a buffer. The hydrolyzate thus obtained was used as a standard solution of 3-ketoglucose. The ketosugar is so unstable that the standard solution should be freshly prepared.

Reagent. 1.0 M phosphate buffer, pH 7.0.

Procedure. To 1.6 ml of a sample containing 0.05 to 0.4 μ mole of 3-ketoglucose was added 0.4 ml of the phosphate buffer and mixed. The mixture was allowed to stand for 3 min at room temperature, and then optical density at 310 m μ was measured against a reagent blank. The intensity reached maximum after 3 min standing (Fig. 4). Fructose did not show any absorbance at 310 m μ in this method. Under these assay conditions, 3-ketoglucose gave a molar extinction coefficient of 3.8×10^3 M $^{-1}$ cm $^{-1}$, whereas 3-ketodi-

FIG. 4. Time Course of Intensity Change in Absorbance at 310 m μ in Method A (3-ketoglucose, 0.21 μ moles).

FIG. 5. Determination Curve for 3-Ketoglucose in Method A.

saccharides such as 3-ketosucrose, 3-ketotrehalose, 3-ketomaltose and 3-ketolactose showed no absorbance at 310 m μ . The optical density depended on both concentration and pH of the phosphate buffer used. The maximum value of intensity was obtained in 0.1 M or higher concentration (final concentration) of phosphate buffer at pH 7.0. The determina-

tion curve is shown in Fig. 5. Completely same curve was obtained with crystalline 3-ketoglucose as standard.

Method B. When 3-ketoglucose was incubated with 2,6-dichloroindophenol in a phosphate buffer, pH 7.0, at room temperature, obvious reduction of the color was observed, while 3-ketosucrose showed little reducing power under these conditions. By applying this finding, the following two procedures were developed for the determination of 3-ketoglucose.

Standard. Same as in Method A.

Reagents. (i), 3×10^{-4} M 2,6-dichloroindophenol solution.

(ii), 1.0 M phosphate buffer, pH 7.0.

Procedure 1. To a mixture of 0.5 ml of the 2,6-dichloroindophenol solution, 0.3 ml of the phosphate buffer and 1.2 ml of distilled water

FIG. 6. Reduction of 2,6-Dichloroindophenol with 3-Ketoglucose in Method B (Procedure 1).

Curve 1, 3-ketosucrose (1.0 μ mole) in 0.1 M phosphate buffer, pH 7.0; curve 2, 3-ketoglucose (1.0 μ mole) in 0.1 M phosphate buffer, pH 7.0; curve 3, 3-ketoglucose (1.0 μ mole) in 0.1 M phosphate buffer, pH 5.6. Molar extinction coefficients of 2,6-dichloroindophenol were 19.1×10^3 M $^{-1}$ cm $^{-1}$ at pH 7.0 and 9.5×10^3 M $^{-1}$ cm $^{-1}$ at pH 5.6 (9).

FIG. 7. Determination Curve for 3-Ketoglucose in Method B (procedure 1).

was added 1.0 ml of a sample solution containing 0.5 to 2.0 μ moles of 3-ketoglucose, and mixed. Color reduction at 600 m μ was followed with a spectrophotometer for 5 min at 20°. As seen with in Figs. 6 and 7, after a lag period of approximately one minute, color reduction proceeded linearly and the rate was directly proportional to the amount of 3-ketoglucose. When 0.1 M phosphate buffer (final concentration) of pH 5.6 was used in place of the phosphate buffer of pH 7.0, only a little reduction of color was observed. The determination curve in this procedure is shown in Fig. 7.

Procedure 2. To 1.6 ml of a sample containing 0.4 to 2.0 μ moles of 3-ketoglucose was added 0.2 ml of the phosphate buffer, pH 7.0, and mixed. After standing for 3 min at room temperature, 0.2 ml of the 2,6-dichloroindophenol solution was added to the mixture, then the change in color intensity of the mixture at 600 m μ was followed with a spectrophotometer for 5 min at room temperature (Fig. 8). The decrease in color intensity during the initial two minutes of the reaction was proportional to the amount of 3-keto-

FIG. 8. Reduction of 2,6-Dichloroindophenol with 3-Ketoglucose in Method B (procedure 2).

Curve 1, without 3-ketoglucose; curves 2, 3, 4, and 5 are 3-ketoglucose 0.33, 0.67, 1.33 and 2.00 μ moles in 0.1 M phosphate buffer, pH 7.0, respectively; curve 6, 3-ketoglucose, 1.33 μ moles, in 0.1 M phosphate buffer, pH 6.0.

FIG. 9. Determination Curve for 3-Ketoglucose in Method B (procedure 2).

glucose. The determination curve is shown in Fig. 9.

DISCUSSION

3-Ketosucrose could be differentially deter-

mined by alkaline treatment in the presence of 3-ketoglucose. In the treatment, 3-ketoglucose also gave some absorbance at 340 $m\mu$, but the absorption disappeared within one minute after addition of the alkali solution. This might be due to the instability of 3-ketoglucose in an alkaline solution. When the treatment was performed with 0.01 N NaOH (final concentration), 3-ketosucrose showed the same absorption spectrum as that in 0.1 N NaOH, but the rate of decrease in the absorbance at 340 $m\mu$ given by 3-ketoglucose was very low.

Okazaki *et al.*¹¹⁾ reported that thymidinediphosphate 4-keto-6-deoxyglucose and 4-keto-6-deoxyglucose 1-phosphate in 0.1 N NaOH gave characteristic absorption spectra having maxima at 318 and 334 $m\mu$, respectively. So, it should be stated that differential determination methods for 3-ketoglycoside and 4-ketoglycoside are not yet established.

Under the assay conditions in the alkaline treatment, L-ascorbic acid which has a chemical structure similar to that of 3-ketoglucose showed a little absorbance at 340 $m\mu$, while in Method B the acid strongly interfered the determination of 3-ketoglucose because of the high reducing power of the acid toward 2,6-dichloroindophenol. When 10 or more $m\mu$ -moles of ascorbic acid was included in a sample, Method B could not be employed. In Method A, determination of 3-ketoglucose was not interfered even in the presence of 1 μ mole of ascorbic acid.

In the procedure 1 of Method B, there was a lag period before the appearance of a linear decrease in color reduction, while there was no lag in the procedure 2 of Method B. This observation may indicate that 3-ketoglucose is converted to a form having an ability to reduce 2,6-dichloroindophenol by the treatment with 0.02 M phosphate buffer, pH 7.0.

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