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α-3-Ketoglucosidase of *Agrobacterium tumefaciens*

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A 3-ketosucrose-degrading enzyme was purified 80-fold from the sonic extracts of *Agrobacterium tumefaciens* IAM 1525 grown on a sucrose-containing medium. The enzyme catalyzes hydrolysis of α-3-ketoglucosides such as 3-ketosucrose, 3-ketotrehalose, 3-ketomaltose, and 3-ketoglucose-1-phosphate but not of β-3-ketoglucosides, β-3-ketogalactosides, and other glycosides such as sucrose, trehalose, maltose, glucose-1-phosphate, cellobiose, lactose, or raffinose. From the strict substrate specificity of this enzyme, the name α-D-3-ketoglucoside 3-ketoglucohydrolase (trivial name, α-3-ketoglucosidase) was proposed. K_m values for 3-ketosucrose and 3-ketotrehalose were 3.9×10^{-3} M and 4.8×10^{-3} M, respectively. Optimum *p*H was 8.0 to 8.3. 3-Ketoglucose, a reaction product from α-3-ketoglucosides by the enzyme, behaved as a strong inhibitor. Physiological significance of this enzyme in the disaccharide metabolism of this bacterium was discussed.

A characteristic metabolism of sugar, including 3-ketosugar as a first intermediate, had been demonstrated in a strain of *Agrobacterium tumefaciens* (6-9), a plant tumor-inducing bacterium. In the previous paper (10), purification and properties of D-glucoside-3-dehydrogenase which catalyzes the oxidation of D-glucose or D-glucosides to the corresponding 3-keto compounds were reported, and physiological significance of the enzyme was discussed. When sucrose was used as carbon source, the bacterium accumulated a 3-ketosugar compound, α-D-*ribo*-hexopyranosyl-3-ulose-β-D-fructofuranoside (3-ketosucrose), in high yield in the growth medium, followed by the rapid degradation of the 3-ketosugar accumulated (7).

In this report, we deal with a 3-ketosucrose-degrading enzyme which catalyzes hydrolysis of α-3-ketoglucosides.

MATERIALS AND METHODS

Sugars. Crystalline preparations of the following 3-ketosugars were prepared by the methods described previously: 3-ketosucrose (α-D-*ribo*-hexopyranosyl-3-ulose-β-D-fructofuranoside; reference 8), 3-ketotrehalose [α-D-*ribo*-hexos-3-ulose-(1 → 1)-α-D-glucopyranoside; reference 6], 3-ketomaltose (α-D-*ribo*-hexos-3-ulose-(1 → 4)-D-glucopyranoside; reference 6), 3-ketoglucose-1-phosphate (α-D-*ribo*-hexopyrnosyl-3-ulose-1-phosphate; reference 3), 3-ketocellobiose [β-D-*ribo*-hexopyranosyl-3-ulose-(1 → 4)-D-glucopyranoside; reference 11], and 3-ketoglucose (D-*ribo*-hexos-3-ulose; reference 5). Other sugars were commercial products. Uniformly labeled ^{14}C-sucrose (5.3 mc/mmole) was purchased from Daiichi Pure Chemicals Co., Ltd., Tokyo. Radioactive 3-ketosucrose was prepared from the labeled sucrose (9).

Microorganism and cultivation. *A. tumefaciens* IAM 1525, a strain of plant tumor-inducing bacterium, was cultured in the medium of McIntire et al. (13), with the omission of zinc ion, in which sucrose, trehalose, maltose, lactose, or succinate was used as carbon source. Cultivation was at 27 C on a rotary shaker. After the cultivation for indicated periods, cells were collected by centrifugation, washed twice with cold 0.05 M phosphate buffer (*p*H 7.0), and suspended in the same buffer. The suspension was used as the resting cells.

Sonic extract. Cells were disrupted by sonic oscillation (10 kc) for 5 min, followed by centrifugation at 100,000 × *g* for 2 hr to remove the intact cells and subcellular particles. The supernatant fraction was used as a source of 3-ketosucrose-degrading enzyme.

Determination of sugars. 3-Ketoglycosides were determined by measuring the optical density at 340 nm after alkaline treatment (in 0.1 N NaOH; reference 4). With this method, 3-ketosucrose, 3-ketotrehalose, 3-ketomaltose, and 3-ketoglucose-1-phosphate gave molar extinction coefficients of 6.50×10^3, 6.20×10^3, 5.51×10^3, and 3.85×10^3 M^{-1} cm^{-1}, respectively, whereas 3-ketoglucose gave no absorption. 3-Ketoglucose was determined from the reduction rate of 2,6-dichloroindophenol in the presence of phosphate buffer (*p*H 7.0) at 20 C (4). The assay system for 3-ketoglucose was as follows: 1.0 M phosphate buffer (*p*H 7.0), 0.3 ml; 3×10^{-4} M 2,6-dichloroindophenol, 0.5 ml; sample (3-ketoglucose, 0.25 to 2.0 μmoles), 0.1 ml; and distilled water, 2.1 ml. The reduction rate of 2,6-dichloroindophenol was proporional to the amount of 3-ketoglucose. In this method, 0.5 μmole of the sugar reduced the optical density (at 600 nm) at a rate of 0.14 per 2 min (4), whereas 3-ketosucrose did not show any color reduction. Reducing sugars other than 3-ketosugars were estimated by Somogyi-Nelson method (14), and a Glucostat was employed for the determination of glucose (16).

Paper chromatography. For separation of ketosugars, the following solvent system was used: acetone-acetic acid-water (4.0:1.2:1.0, v/v; references 2 and 10). Ketosugars were detected with the urea phosphate reagent (17).

Enzyme assays. (i) For assay of 3-ketosucrose-degrading enzyme, 1 ml of reaction mixture contained 10 μmoles of 3-ketoglycoside, 100 μmoles of phosphate buffer (*p*H 7.0), and enzyme solution. Reaction was at 30 C for 10 min. After the reaction, a sample of the reaction mixture containing 0.1 to 0.4 μmole of 3-ketoglycoside was diluted to 1.0 ml with distilled water, followed by the addition of 2.0 ml of 0.15 N NaOH. The amount of the substrate remaining was determined from the absorbancy at 340 nm. In some experiments, formation of 3-ketoglucose was determined from the reduction rate of 2,6-dichloroindophenol. When 3-ketodisaccharide containing a D-glucose moiety was used as substrate, free glucose formed was also determined by use of a Glucostat.

(ii) For the assay of glycosidase, 1 ml of reaction mixture contained 10 μmoles of dissacharide, 100 μmoles of phosphate buffer (*p*H 7.0), and an appropriate amount of enzyme solution. The reaction was at 30 C for 5 min and was stopped by heating for 5 min in boiling water. Then free glucose and increased reducing power were determined by use of a Glucostat and Somogyi-Nelson methods, respectively.

(iii) For assay of phosphatase, 1 ml of reaction mixture contained 10 μmoles of glucose-1-phosphate, 30 μmoles of Tris-chloride buffer (*p*H 8.2), and an appropriate amount of enzyme solution. The reaction was at 27 C for 10 min. Orthophosphate formed was determined by the method of Allen (1).

(iv) The activity of D-glucoside-3-dehydrogenase was estimated by colorimetry with 2,6-dichloroindophenol as hydrogen acceptor, by the method used previously (10). A 1-ml amount of assay mixture contained 10 μmoles of sucrose, 0.35 μmoles of 2,6-dichloroindophenol, 100 μmoles of phosphate buffer (*p*H 7.0), and an appropriate amount of enzyme solution. The reaction was at 20 C. The reduction rate of 2,6-dichloroindophenol was measured spectrophotometrically at 600 nm.

(v) The uptake of ^{14}C-sucrose and of ^{14}C-3-ketosucrose from a medium by the resting cells was determined by the vacuum filtration method described in the previous paper (9). A 1-ml amount of reaction mixture contained 200 μg (as dry cells) of the resting cell suspension, 100 μmoles of phosphate buffer (*p*H 7.0), and 1.0 μmole of radioactive substrate. The reaction was carried out at 27 C without shaking.

RESULTS

Existence of 3-ketodisaccharide-degrading enzymes. When the bacterium was grown on a medium containing disaccharide as carbon source, the formation of a 3-ketodisaccharide-degrading enzyme was induced, but no enzyme activity was detected in cells grown on a succinate medium. The degrading activities on 3-ketosucrose, 3-ketotrehalose, and 3-ketomaltose were observed with the sonic extract prepared from the cells grown on a sucrose, trehalose, or maltose medium (Table 1). In a lactose medium, the activity toward both 3-ketosucrose and 3-ketomaltose was induced, but the activity toward 3-ketotrehalose was not induced. 3-Ketolactose-degrading activity was not detected in the sonic extract of disaccharide-grown cells, whereas, with the resting cells obtained from a lactose medium, the activity was demonstrated (footnote *d*, Table 1). Therefore, 3-ketolactose-degrading enzyme was considered to be very unstable with the sonic treatment. Glycosidase activities in the sonic extract are also listed in Table 1.

Purification of 3-ketosucrose-degrading enzyme. All procedures for the purification of 3-ketosucrose-degrading enzyme were carried out at 4 C.

First ammonium sulfate fractionation. A 20-g amount of solid ammonium sulfate was added to the soluble part of a sonic extract (95 ml) prepared from wet cells (35 g) which were harvested in the stationary growth phase (36-hr culture) after growth on sucrose medium. After standing for 14 hr, the precipitate that formed was removed by centrifugation at 15,000 × *g* for 10 min. A 20-g amount of solid ammonium sulfate

TABLE 1. *Activities of 3-ketodissaccharide-degrading enzymes[a] and glycosidases[b] in the sonic extract of the cells grown in various carbon sources*

Substrate	Carbon source in growth medium (10^{-2} M)[c]				
	Succinate	Sucrose	Trehalose	Maltose	Lactose
3-Ketosucrose	0	5.9	6.6	7.8	7.0
3-Ketotrehalose	0	7.2	10.2	11.7	0
3-Ketomaltose	0	5.4	9.3	12.5	3.3
3-Ketolactose	0	0	0	0	0[d]
Sucrose	3.6	10.6	12.3	12.1	2.1
Maltose	2.5	9.6	5.7	6.5	4.0
Trehalose	1.2	5.8	6.4	6.8	1.4
Cellobiose	1.1	0.8	3.3	3.7	6.2
Lactose	0	0.2	1.8	2.2	0
Raffinose[e]	0	0	0	0	0

[a] One unit of 3-ketoglycoside-degrading enzyme is defined as the amount that degrades 1 μmole of substrate per min.

[b] One unit of glycosidase is defined as the amount that degrades 1 μmole of glycoside per min. Amount of glycoside degraded was calculated from glucose liberated.

[c] Results expressed as units per gram of dry cells.

[d] Resting cells showed a 3-ketoglactose-degrading activity of 13.2 units/g of dry cells. For determination of the activity, the resting cells were used in place of enzyme solution (*see* assay method for 3-ketoglycosidase activity).

[e] Raffinose-degrading activity was measured by the method of Somogyi-Nelson.

was added to the supernatant fraction. The precipitate that formed was dissolved in 5×10^{-2} M phosphate buffer (*p*H 7.0) to make a total volume of 40 ml.

Protamine treatment. To the enzyme solution obtained above was added 0.7 ml of 5% protamine sulfate solution, followed by dialysis against 600 ml of 1.0×10^{-2} M phosphate buffer, *p*H 7.0. After dialysis for 18 hr, the precipitate that formed was removed by centrifugation. The supernatant fluid was diluted to approximately 200 ml with 5×10^{-2} M phosphate buffer (*p*H 7.0) to give an absorbancy of 6.0 at 280 nm.

Second ammonium sulfate fractionation. To the diluted supernatant fluid was added 56 g of solid ammonium sulfate. After standing for 2 hr, the precipitate that formed was removed by centrifugation, and then 14 g of solid ammonium sulfate was added. The precipitate that formed within 2 hr was collected by centrifugation, dissolved in 15 ml of 5×10^{-2} M phosphate buffer (*p*H 7.0), and dialyzed against 2 liters of 5×10^{-3} M phosphate buffer (*p*H 7.0) for 1 day. Most α-glycosidase activity was removed with the first precipitate in this fractionation.

Diethylaminoethyl (DEAE)-cellulose column chromatography. The dialyzed preparation was applied to a DEAE-cellulose column (1.7 by 28 cm) which had been equilibrated with 1.0×10^{-2} M phosphate buffer (*p*H 7.0) before use. Stepwise elution was carried out with KCl solutions prepared in 1.0×10^{-2} M phosphate buffer (*p*H 7.0) at an elution rate of 2 ml/min. The elution profile is presented in Fig. 1. The activity was recovered as a single peak in the eluate with 0.1 M KCl. Activity was in the tubes numbered 24 to 26 (each tube, 20 ml). These fractions were combined and immediately concentrated to 4.0 ml by treatment with Carbowax 6,000 (12). The activity of the DEAE eluate was very unstable, so that further purification was unsuccessful. The entire procedure is summarized in Table 2.

Identification of reaction products. To characterize the chemical reaction catalyzed by the 3-

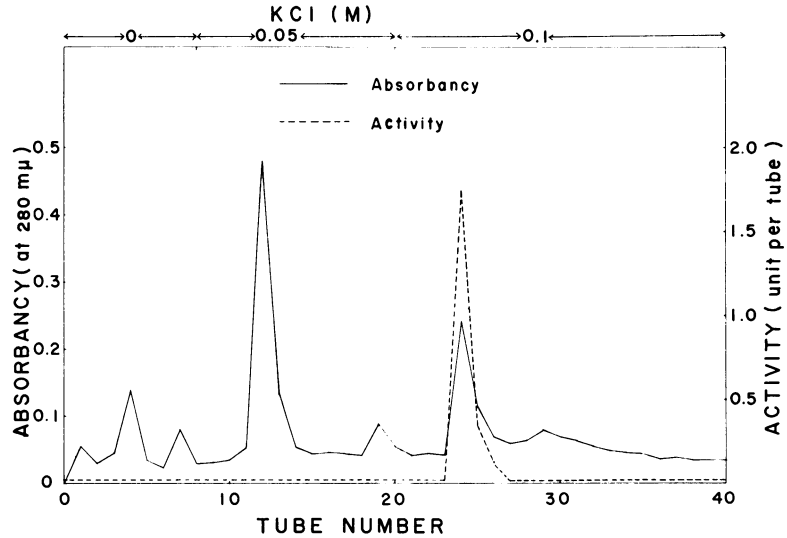

FIG. 1. *Elution profile of 3-ketosucrose-degrading enzyme on DEAE-cellulose column chromatography. Before use, the column had been equilibrated with 10^{-2} M phosphate buffer, pH 7.0.*

TABLE 2. *Purification of 3-ketosucrose-degrading enzyme*

Determination	Total vol	Total activity	Total	Specific activity	Yield
	ml	*unit*	A_{280}[a]	10^{-3} *unit*/A_{280}	%
Sonic extract	95	12.5	5,850	2.14	
1st Ammonium sulfate fractionation	40	12.0	2,700	4.44	96
Protamine treatment	200	11.4	1,223	9.33	91
2nd Ammonium sulfate fractionation	15	11.0	159	69.1	88
DEAE-cellulose column chromatography	1.6	1.9	11.4	167	15

[a] Absorbancy per cm at 280 nm.

ketosucrose-degrading enzyme obtained above, the reaction products were determined. To avoid spontaneous degradation of 3-ketoglucose, the enzyme reaction was performed in a phosphate buffer at *p*H 5.6 instead of *p*H 7.0. Formation of 3-ketoglucose and fructose from 3-ketosucrose was observed by paper chromatography. Other sugars were not detected. The amount of 3-ketoglucose formed was almost equimolar to the amount of 3-ketosucrose degraded (Table 3). As we have no suitable method for differential determination of fructose in the presence of both 3-ketosucrose and 3-ketoglucose, a molar ratio of 3-ketoglucose to fructose could not be obtained. When 3-ketotrehalose was used as substrate, it was observed that 1.57 μmoles of glucose was liberated with the formation of 3-ketoglucose with concomitant consumption of 1.61 μmoles of substrate. Accordingly, it may be concluded that the 3-ketosucrose-degrading enzyme is a hydrolase.

With a crude sonic extract of sucrose-grown cells as source of 3-ketosucrose-degrading enzyme, the formation of an almost equimolar amount of 3-ketoglucose from 3-ketosucrose consumed was also observed. It was therefore concluded that the 3-ketosucrose-degrading enzyme in the sonic extract was a 3-ketosucrose-hydrolyzing enzyme.

Substrate specificity. With a number of carbohydrates, the specificity of 3-ketosucrose-degrading enzyme was examined, and the relative reaction rates are listed in Table 4. Of the 3-ketoglycosides, α-3-ketoglucosides such as 3-ketosucrose, 3-ketotrehalose, 3-ketomaltose, and 3-ketoglucose-1-phosphate served as substrates for the enzyme, whereas β-3-ketoglucoside (3-ketocellobiose) and β-3-ketogalactoside (3-ketolactose) did not. Among the 3-ketoglucosides used, 3-ketosucrose showed the highest reactivity. Invertase (yeast β-fructofuranosidase) hydrolyzed 3-ketosucrose but was inactive with any other α-3-ketoglucosides. None of the α-glucosides tested was hydrolyzed by the enzyme. Thus, 3-ketosucrose-degrading enzyme could be distinguished from α-glucosidase, β-fructofuranosidase, and phosphatase.

3-Ketosugars as inhibitors. 3-Ketoglucose, one of the reaction products derived from α-ketoglucosides, proved to be a strong inhibitor of the 3-ketosucrose-degrading enzyme, whereas glucose and fructose were not inhibitory (Table 5). When two kinds of 3-ketoglucoside were present together in a reaction mixture, one worked as a competitive inhibitor of the hydrolysis of another, and vice versa. For example, hydrolysis of 3-ketotrehalose was inhibited by both 3-ketosucrose and 3-ketoglucose-1-phosphate in a competitive fashion (Fig. 2).

TABLE 3. *Formation of 3-ketoglucose from 3-ketosucrose by 3-ketosucrose-degrading enzyme*[a]

Incubation time	3-Ketosucrose	3-Ketoglucose
min	*μmoles*	*μmoles*
0	5.67	0
60	2.97	2.53
Difference	−2.70	2.53

[a] Reaction mixture contained 5.7 μmoles of 3-ketosucrose, 50 μmoler of phosphate buffer (*p*H 5.6) and 0.13 unit of enzyme in a final volume of 0.5 ml. 3-Ketosucrose and 3-ketoglucose were determined by the methods described in the text.

TABLE 4. *Substrate specificity of 3-ketosucrose-degrading enzyme*

Substrate (10^{-2} M)	Relative activity
3-Ketosucrose	100
3-Ketotrehalose	46
3-Ketomaltose	10
3-Ketocellobiose	0
3-Ketoglucose-1-phosphate	60
3-Ketolactose	0
Sucrose	0
Trehalose	0
Maltose	0
Cellobiose	0
Lactose	0
Raffinose[a]	0

[a] Method of Somogyi-Nelson was used to determine reducing power.

TABLE 5. *3-Ketoglucose as inhibitor of 3-ketosucrose-degrading enzyme*[a]

Addition (mM)	Substrate[b] (mM)	Inhibition
		%
None	3KS (2.4)	
Glucose (11)	3KS (2.4)	6
Fructose (11)	3KS (2.4)	7
3-Ketoglucose (11)	3KS (2.4)	75
Sucrose (5)	3KS (2.4)	2
Maltose (5)	3KS (2.4)	9
3-Ketoglucose (9.7)	3KS (2.8)	75
3-Ketoglucose (9.7)	3KG-1-P (9.5)	80
3-Ketoglucose (9.7)	3KT (4.5)	93
3-Ketoglucose (9.7)	3KM (1.8)	33

[a] A 0.5-ml amount of the reaction mixture contained 50 μmoles of phosphate buffer (*p*H 7.0), 0.04 units of the enzyme, and saccharides. The enzyme activity was estimated from the rate of consumption of substrate.

[b] Abbreviations: 3KS, 3-ketosucrose; 3KG-1-P, 3-ketoglucose-1-phosphate; 3KT, 3-ketotrehalose; and 3KM, 3-ketomaltose.

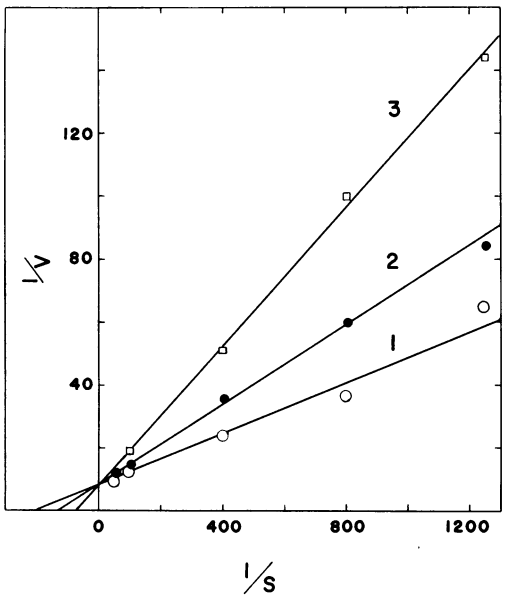

FIG. 2. *Competitive inhibition by 3-ketosucrose and 3-ketoglucose-1-phosphate on hydrolysis of 3-ketotrehalose by 3-ketosucrose-degrading enzyme. Reaction mixture contained 30 μmoles of phosphate buffer (pH 7.0), 0.3 unit of the enzyme, and various amounts of 3-ketotrehalose in a final volume of 0.3 ml. Hydrolysis of 3-ketotrehalose was estimated from an amount of glucose formed. Glucose was determined by Glucostat. Ordinate (1/v), reciprocal of formation velocity of glucose (v: micromoles of glucose formed per minute); abscissa (1/s), reciprocal of 3-ketotrehalose concentration (M); 1, no inhibitor; 2, 3-ketosucrose added (1.7×10^{-3} M); 3, 3-ketoglucose-1-phosphate added (1.1×10^{-2} M).*

Other properties. From Lineweaver-Burk plots at *p*H 7.0, K_m values of 3-ketosucrose-degrading enzyme for 3-ketosucrose and 3-ketotrehalose were calculated to be 3.9×10^{-3} M and 4.8×10^{-3} M, respectively. The *p*H dependency curve gave an optimum at *p*H 8.0 in phosphate buffer.

Culture age and 3-ketosucrose-degrading enzyme. Total activity of 3-ketosucrose-degrading enzyme increased as the culture grew during exponential growth phase and reached a maximum at an early stationary-growth phase, and then the activity began to decrease gradually (Fig. 3). The specific activity of 3-ketosucrose-degrading enzyme, however, remained constant at from 12 to 17 μmoles per g (dry matter) per min throughout the cultivation. On the other hand, D-glucoside-3-dehydrogenase activity was found at levels similar to that of the 3-ketosucrose-degrading enzyme only during exponential growth. The maximal activity of the dehydrogenase was observed in the extracts of cells harvested in the late exponential growth phase. Approximately one-third of the maximal activity was recovered in a 48-hr culture. However, the cells from the 48-hr culture showed a high activity of 3-ketosucrose uptake reaction (45.2 μmoles/g of dry cells per min) which corresponded to 10 times of the uptake rate given by the cells from a 24-hr culture (Table 6).

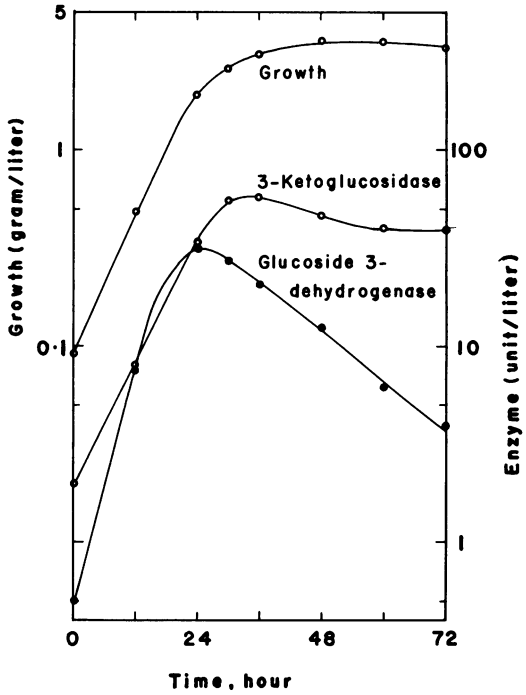

FIG. 3. *Effect of culture age on the levels of both 3-ketosucrose-forming enzyme and 3-ketosucrose-degrading enzyme. A sonic extract was used as enzyme preparation.*

TABLE 6. *Sucrose and 3-ketosucrose transport activities by resting cells*[a]

Entry of	Rate of transport reaction[b]	
	24-Hr cells	48-Hr cells
3-Ketosucrose	5.2	45.2
Sucrose	46.8	5.0

[a] Portions (0.1 ml) of the reaction mixture were filtered with a membrane filter (Millipore Corp., Bedford, Mass.) at 2 min of incubation. After washing the filter, radioactivity in cells collected on the filter was estimated. Values for 24-hr cultures have been published previously (9).

[b] Results expressed as micromoles per gram of dry cells per minute.

DISCUSSION

Evidence was presented for the existence of a novel enzyme catalyzing the hydrolysis of 3-ketosucrose in *A. tumefaciens* IAM 1525, a plant tumor-inducing strain. From studies on the substrate specificity and stoichiometry of the reaction, the enzyme should be named α-3-ketoglucoside-3-ketoglucohydrolase (trivial name, α-3-ketoglucosidase) according to the rule presented by the Enzyme Commission.

The data in Table 1 show that the bacterium grown in sucrose possesses α-glucosidase but lacks a β-fructofuranosidase. The α-glucosidase fraction which was obtained in the second ammonium sulfate fractionation had no activity toward 3-ketosucrose. α-3-Ketoglucosidase, therefore, seems to be the only enzyme which catalyzes the hydrolysis of 3-ketosucrose in this bacterium. Cells grown in maltose or trehalose medium also possess 3-ketoglucoside-degrading activity. Whether this enzyme is identical with that obtained from sucrose-grown cells is, however, unknown.

In a soluble fraction of the sonic extract of the bacterium, both D-glucoside-3-dehydrogenase and α-3-ketoglucosidase were found, and the respective reaction rates were estimated to be 16.8 and 16.5 μmoles/g of dry matter per min. Therefore, in the cell-free extract, a considerable part of the 3-ketosucrose once formed from sucrose should be hydrolyzed to fructose and 3-ketoglucose. However, in previous in vivo experiments (9), the quantitative conversion of sucrose to 3-ketosucrose was demonstrated with resting cells obtained from cultures in exponential growth. This observation indicates that α-3-ketoglucosidase does not work in the resting cells, although both enzymes are present in the extracts. At the present time, we do not have an explanation for the observation that 3-ketosucrose formed by D-glucoside 3-dehydrogenase is not hydrolyzed. Two possibilities may be considered. (i) The enzymes functional in the formation and degradation of 3-ketosucrose are separately located in the cells. It has been shown that the dehydrogenase can be extracted by osmotic shock (15) from the cells, whereas only a small amount of the glycosidase can be detected in the fluid (S. Fukui et al., *unpublished data*). (ii) The bacterium has a special transport system for the 3-ketosucrose exit, but has weak transport reaction for the 3-ketosucrose entry during the exponential growth phase. The results of the transport experiment (Table 6) indicate that the transport system for the entry of 3-ketosucrose may play a significant role in controlling the degradation of the extracellular 3-ketosucrose which was accumulated in the culture medium.

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