D-ALDOHEXOPYRANOSIDE DEHYDROGENASE OF AGROBACTERIUM TUMEFACIENS*

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Growing cultures of Agrobacterium tumefaciens convert D-glucosides and D-galactosides to D-ribo- and D-xylo-hexopyranoside-3-ulose derivatives ("ketglycosides") respectively (Bernaerts et al., 1958, 1960a, 1960b, 1961, 1963a, 1963b; Feingold et al., 1961; and Fukui et al., 1963a, 1963b, 1963c). Sucrose-grown resting cells of the organism convert D-glucose to D-ribo-hexos-3-ulose (Fukui, 1965; Grebner, 1964). In addition, formation of ketsucrose from sucrose by resting cells has been reported (Fukui et al., 1963c). However, there has been no report of the behavior of resting cells of A. tumefaciens toward other sugars, nor of enzyme preparations which catalyze formation of 3-uloses. The latter two aspects are discussed in this paper.

Materials and Methods: Carbohydrates were commercial products except as noted below. Methyl α-D-ribo-hexopyranoside-3-ulose was a gift from Dr. O. Theander, Swedish Forest Products Institute, Stockholm. β-D-Fructofuranosyl-α-D-ribo-hexopyranoside-3-ulose (ketsucrose) was prepared as previously described (Fukui et al., 1963a).

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Ketsucrose was assayed by adding 0.1 ml of a soln containing up to 0.30 μmoles of ketsucrose to 3.4 ml of 0.1N NaOH and measuring the absorbance at 330 mμ 2.5 min after mixing. Under these conditions 0.30 μmole of ketsucrose give an absorbance of 0.50. Protein was determined by the method of Waddel (1956).

Paper chromatography was performed in butanone–acetone–acetic acid–H_2O (20:10:6:9). Sodium phosphate buffer (0.1M) pH 6.0, was used in all steps of enzyme purification.

Enzyme activity in extracts was assayed by following the decrease of absorbance at 600 mu of 0.04M 2,6–dichlorophenol indophenol (DIP) in the presence of 0.03M sucrose and 0.1M sodium phosphate buffer, pH 6.0, at 25 C. An enzyme unit is defined as the amount of enzyme required to reduce one μmoleof DIP per min. A molar absorptivity of 14.9×10^3 $cm^2 \times mole^{-1}$ was used for DIP.

A. tumefaciens, virulent strain, was grown with rotary agitation at 28 C in baffled flasks in a synthetic medium (Kraght et al., 1953) containing 2% sucrose until a Klett reading of 400–500 (Filter 42) was obtained. Cells were washed three times with cold H_2O and suspended in sufficient 0.1M sodium phosphate buffer, pH 6.0, so that a 1/10 dilution gave a Klett reading of 650 (Filter 42). (Resting Cell Suspension, RCS)

Results: When air was bubbled through RCS in 0.026M sucrose at 30 C, ketsucrose was produced at a linear rate (approx. 45 μmoles per hr per ml RCS), which dropped sharply after 2 hr. Ketsucrose was identified by its paper-chromatographic and electrophoretic mobility and by specific color reactions (Fukui et al., 1963a).

Each of the following carbohydrates was converted by RCS to a derivative which gave an absorption peak at 330 mμ in 0.1N NaOH, and which had the chromatographic mobility and color reactions of the corresponding ketglycoside: lactose, maltose, and trehalose (Fukui et

al., 1963b), melibiose and raffinose (Grebner, 1964). Unexpectedly, no detectable ketglycoside was formed from D-galactose, cellobiose, or α-methyl-D-glucopyranoside by RCS. This aspect was further investigated by preparing RCS from cells grown with each of the above three compounds as carbon source. D-galactose-grown cells showed no activity with D-galactose, D-glucose, or sucrose. Cells grown on α-methyl-D-glucopyranoside converted sucrose to ketsucrose but showed no activity with the growth substrate. Cellobiose-grown cells similarly converted sucrose to ketsucrose but failed to form ketcellobiose from the growth substrate.

For preparation of cell-free extracts, RCS was held at 0-4 C for 12 hrs and then was disrupted in a French pressure cell at 20,000 lb/in^2. Debris was removed by centrifugation in the cold at 105,000 x g for 1 hr. (Except where otherwise noted all subsequent operations were performed at 0-4 C.) Active extracts were obtained from cells grown on D-glucose, sucrose, α-methyl-D-glucopyranoside, and cellobiose. However, cells grown on D-galactose had no detectable activity. Nucleoproteins were precipitated from the centrifuged extract by addition of 0.05 vol of 0.5M $MnCl_2$. The supernatant fluid obtained by centrifugation was dialyzed overnight against buffer, and the precipitate which formed was removed by centrifugation. The resulting supernatant fluid was held at 55 C for 10 min, and then immediately cooled in ice. Denatured protein was removed by centrifugation and the supernatant fluid was fractionated by addition of solid $(NH_4)_2SO_4$. The fraction which precipitated between 45-55% saturation was collected and dissolved in a small volume of buffer.

The enzyme had a sharp pH optimum at 6.0. Sucrose hydrolase, present in the crude extract, was absent from the purified material. A summary of the purification procedure is presented in Table 1. A number of carbohydrates were tested in the enzyme assay. Relative reaction rates are listed in Table 2.

TABLE 1

Enzyme Purification

Fraction	Specific Activity (Units / mg protein)	Total Activity (Units)
Crude extract	5.2	475
$MnCl_2$ dialysis	16.9	364
Heat	31.4	352
$(NH_4)_2SO_4$	79.3	148

TABLE 2

Relative Rates of DIP-reduction with D-glucose, D-galactose, and Glycosides

Substrate	Relative Rate
Cellobiose	100
Methyl- α -D-glucopyranoside	89
Lactose	81
Maltose	81
D-glucose	76
D-galactose	58
Sucrose	65
Melibiose	55
α -D-glucopyranosyl phosphate	43
α -D-galactopyranosyl phosphate	43
Trehalose	23
Raffinose	19

Aldopentoses, D-fructose, L-rhamnose, L-fucose, D-fucose, D-galacturonic acid, D-glucitol, m-inositol, D-mannose and L-galactose did not reduce DIP in this test.

No chromatographically-demonstrable ketsucrose was produced by purified enzyme without artificial hydrogen acceptor. In the presence of the hydrogen acceptors DIP or phenazine methosulfate (PMS) ketsucrose was produced by the enzyme from sucrose. DIP, however, further reacts with ketsucrose, resulting in poor recovery of ketsucrose from reaction mixtures. Use of catalytic quantities of the autoxidizable PMS under aerobic conditions permitted extensive enzymic conversion of sucrose to ketsucrose.

Reaction mixtures, each containing 100 μmoles of one of the substrates listed in Table 2, 0.1 μmoles of PMS, 0.5 mg of enzyme, and 100 μmoles of buffer in a total volume of 0.1 ml were incubated in open tubes in an H_2O-saturated atmosphere at 25 C for 4 hrs. The mixtures were then subjected to radial paper chromatography. Each of the glycosides (except cellobiose and the two sugar phosphates) yielded a product identical in chromatographic mobility and spray reactions with the corresponding, known ketglycoside. Cellobiose yielded a product which on the basis of relative chromatographic mobility and spray reactions presumably was ketcellobiose; however, since ketcellobiose has not yet been described it was not available for reference.

The product of the reaction of α-D-glucopyranosyl phosphate was isolated by descending chromatography on Whatman 3 MM paper. It contained bound phosphate (Bandurski et al., 1951), and reduced alkaline triphenyltetrazolium (Wallenfels, 1950). Hydrolysis with seminal acid phosphatase released inorganic phosphate and a compound with the chromatographic mobility of ketglucose. The unhydrolyzed compound was reduced with $NaBH_4$ to yield a non-reducing organic phosphate which had the paper-electrophoretic mobility at pH 5.8 of α-D-glucopyranosyl phosphate. Hydrolysis of this substance (1N HCl, 100 C, 10 min) released inorganic phosphate and two carbohydrate components with the paper-electrophoretic mobilities in 0.1N NaOH of glucose and allose. These data tentatively identify the compound as α-D-ribo-hexopyranosyl-3-ulose phosphate.

With D-glucose and D-galactose no 3-ulose could be demonstrated, although other chromatographically-detectable products were obtained. These probably resulted from further reaction of the 3-uloses with PMS. When purified enzyme (0.4 mg protein) was incubated with 0.001 μmoles of uniformly C^{14}-labelled (80 μc/μmole) D-glucose in the absence of PMS the reaction mixture yielded a radioactive substance with the chromatographic mobility of ketglucose.

The enzyme may function as a stoichiometric electron acceptor in this case.

This is the first report of an enzyme which converts hexoses and hexosides to their 3-uloses. The enzyme preparation does not operate without an artificial hydrogen acceptor, probably because the integrity of the electron-transport system was destroyed during disruption of the cells.

On the basis of tests with a limited number of substrates, the enzyme obtained from sucrose-grown cells seems specific for D-glucose, D-galactose, and their glycosides. Since D-gluco-and-D-galactopyranosides are substrates, D-glucose and D-galactose react as the pyranoside forms. We therefore propose to call the enzyme D-aldohexopyranoside dehydrogenase.

A. tumefaciens grown on D-glucose, sucrose, and other glucosides possesses dehydrogenase activity. However, D-galactose-grown cells or cell-free extracts prepared from them have no demonstrable activity. These observations indicate that D-aldohexopyranoside dehydrogenase is an inducible enzyme.

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