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Ketose Reductase Activity in Developing Maize Endosperm

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DOUGLAS C. DOEHLERT
Agricultural Research Service, United States Department of Agriculture, Northern Regional Research Center, 1815 North University Street, Peoria, Illinois 61604

ABSTRACT

Ketose reductase (NAD-dependent polyol dehydrogenase EC 1.1.1.14) activity, which catalyzes the NADH-dependent reduction of fructose to sorbitol (D-glucitol), was detected in developing maize (*Zea mays* L.) endosperm, purified 104-fold from this tissue, and partially characterized. Product analysis by high performance liquid chromatography confirmed that the enzyme-catalyzed reaction was freely reversible. In maize endosperm, 15 days after pollination, ketose reductase activity was of the same order of magnitude as sucrose synthase activity, which produces fructose during sucrose degradation. Other enzymes of hexose metabolism detected in maize endosperm were present in activities of only 1 to 3% of the sucrose synthase activity. $CaCl_2$, $MgCl_2$, and $MnCl_2$ stimulated ketose reductase activity 7-, 6-, and 2-fold, respectively, but had little effect on NAD-dependent polyol dehydrogenation (the reverse reaction). The pH optimums for ketose reductase and polyol dehydrogenase reactions were 6.0 and 9.0, respectively. K_m values were 136 millimolar fructose and 8.4 millimolar sorbitol. The molecular mass of ketose reductase was estimated to be 78 kilodaltons by gel filtration. It is postulated that ketose reductase may function to metabolize some of the fructose produced during sucrose degradation in maize endosperm, but the metabolic fate of sorbitol produced by this reaction is not known.

In the developing maize kernel, there are two known mechanisms for the breakdown of sucrose arriving from the phloem. Sucrose may be hydrolyzed by invertase (β-fructofuranoside fructohydrolase EC 3.2.1.26.) to form glucose and fructose. Alternatively, sucrose may be metabolized by sucrose synthase (EC 2.4.1.13.) in the presence of UDP to form fructose and UDP-Glc. In either case, fructose is a product. It would be expected that tissues that are actively catabolizing sucrose would have the capacity to metabolize fructose readily, but it is not clear how fructose is subsequently metabolized. It is generally accepted that fructose is phosphorylated by a hexokinase (19), but we have found, as have Tsai *et al.* (18), consistently low hexokinase activity relative to sucrose synthase activity in developing maize endosperm. Although it is possible that hexokinase activity is limiting the utilization of sucrose in these tissues, we have investigated the possibility that alternative pathways for the utilization of fructose may exist. Subsequent experiments have led to the discovery of a ketose reductase (NAD-dependent polyol dehydrogenase, EC 1.1.1.14.) in developing maize endosperm that will reduce fructose to sorbitol in the presence of NADH. This report describes the characterization of this enzyme partially purified from maize endosperm and discusses its possible significance in the utilization of fructose produced by sucrose degradation.

MATERIALS AND METHODS

Plant Material. Maize (*Zea mays* L. inbred W64A) was grown in the field in Peoria, IL, in the summer of 1985. Ears were hand pollinated, harvested and stored at −90°C until use.

Enzyme Extraction and Partial Purification. Enzymes were extracted from isolated maize endosperms. For measuring enzyme activities in crude extracts, endosperms were dissected from lyophilized kernels and homogenized in 50 mM Hepes (pH 7.0) containing 5 mM $MgCl_2$ and 1 mM DTT, at 0.05 g dry weight/ml buffer. Crude homogenates were centrifuged at 27,000*g* for 15 min and dialyzed in extraction buffer before assay of enzymes.

Endosperms for enzyme purification were dissected from frozen kernels and homogenized in the same extraction buffer described above at a ratio of 0.1 g fresh weight/ml buffer. Crude homogenates were centrifuged at 27,000*g* for 15 min and the pellet was discarded. The supernatant was brought to 30% (w/v) saturation with $(NH_4)_2SO_4$, centrifuged at 27,000*g* and the pellet was discarded. The supernatant was further brought to 60% saturation with ammonium sulfate and centrifuged as before. The supernatant was discarded, and the pellet was resuspended in extraction buffer. A portion of the resuspension was loaded on an Ultrogel[1] AcA 34 column (2 × 120 cm) equilibrated with 10 mM Hepes (pH 7.0), 5 mM $MgCl_2$, and 1 mM DTT. The column was eluted with the same buffer, and 5 ml fractions were collected. Fractions were assayed for ketose reductase activity, and those containing activity were pooled. Ultrogel fractions were then loaded on a Fast Protein Liquid Chromatography Mono Q column (Pharmacia) equilibrated with 10 mM Hepes (pH 7.0), 5 mM $MgCl_2$, and 1 mM DTT, and eluted with a linear NaCl gradient from 0 to 300 mM. Ketose reductase was assayed, and those fractions containing peak activity were pooled and used for enzyme characterization experiments. The resulting preparation had an increase in specific activity of about 104-fold from the crude extracts with an overall yield of about 24%. In all purification steps, ketose reductase activity co-chromatographed with NAD-dependent polyol dehydrogenase activity.

Molecular mass of ketose reductase was estimated on an Ultrogel AcA 34 column (1 × 60 cm) equilibrated with 10 mM Hepes (pH 7.0), containing 5 mM $MgCl_2$ and 1 mM DTT. Standards used to calibrate the column were ferritin (467 kD), catalase (232 kD), alcohol dehydrogenase (147 kD), BSA (64 kD), and carbonic anhydrase (29 kD). The elution position of standards from the column was determined by A_{280}, whereas the position of ketose reductase was determined by activity.

Enzyme Assay. Ketose reductase activity was measured spectrophotometrically by monitoring the continuous oxidation of NADH at 340 nm in the presence of fructose (11). The reaction mixture contained 50 mM Mes (pH 6.0), 5 mM $MgCl_2$, 400 mM fructose, 0.2 mM NADH, and an aliquot of the ketose reductase

[1] Names of products are included for the benefit of the reader and do not imply endorsement or preferential treatment by the United States Department of Agriculture.

preparation. Blanks contained no fructose. NAD-dependent polyol dehydrogenase was assayed spectrophotometrically by following the reduction of NAD at 340 nm in the presence of sorbitol (11). The reaction mixture contained 50 mM Bicine (pH 8.5), 5 mM $MgCl_2$, 80 mM sorbitol, 1 mM NAD, and an aliquot of ketose reductase. Blanks contained no sorbitol.

Hexokinase activity was assayed spectrophotometrically by coupling the production of glucose 6-P with glucose 6-P dehydrogenase (6). Reaction mixtures contained 50 mM Bicine (pH 8.5), 5 mM $MgCl_2$, 2 units/ml glucose 6-P dehydrogenase (Sigma G-4134), 1 mM NADP, 1 mM ATP, and 5 mM hexose. Blanks contained no hexose. Either glucose or fructose was used as hexose substrate. Fructokinase assays also contained 2 units/ml phosphoglucose-isomerase (Sigma P-5381). For UTP-dependent hexokinase, 1 mM UTP was substituted for ATP. The increase in A_{340} relative to the blank was used to derive the enzyme activity.

Sucrose synthase was assayed in the direction of sucrose degradation using a fixed time course determination of fructose produced from sucrose in the presence of UDP. Reaction mixtures contained 50 mM Mes (pH 6.0), 400 mM sucrose, and 8 mM UDP. Blanks contained no UDP. Fructose produced was determined by the procedure of Nelson (13). Reactions were terminated by adding the Nelson copper reagent. Reactions were linear with time over the time interval assayed (10 min).

For determination of the pH response of ketose reductase and sorbitol dehydrogenase, a buffer containing 200 mM citrate, 200 mM Hepes, and 200 mM glycine was prepared, and aliquots were adjusted to pH values from 5 to 10. These buffers were used in place of the Mes (pH 6.0) in the ketose reductase assay and the Bicine (pH 8.5) in the NAD-dependent polyol dehydrogenase assay. For all other assays, substrate, substrate concentration and cations were varied as indicated in the text.

Product Analysis. Products of the fructose reductase and NAD-dependent sorbitol dehydrogenase reactions were determined by HPLC. The reaction mixture for the fructose reductase reaction contained 50 mM Mes (pH 6.0), 5 mM $MgCl_2$, 10 mM fructose, 10 mM NADH, and an aliquot of enzyme (0.03 mg protein). The reaction mixture for the NAD-dependent sorbitol dehydrogenase reaction contained 50 mM Bicine (pH 8.5), 5 mM $MgCl_2$, 10 mM sorbitol, 10 mM NAD, and an aliquot of enzyme (0.03 mg protein). Reactions were initiated by adding enzyme. Aliquots (0.4 ml) were taken at 0, 0.5, 1.0, and 4.0 h and reactions were terminated by boiling. An internal standard (mannitol) was added to each fraction, which was then passed through a 0.5 ml Whatman DE-52 mini-column equilibrated and eluted with distilled, deionized H_2O. One ml eluent was collected for each sample and filtered through 0.45 μ membrane (Schleicher & Schull). Aliquots (40 μl) were subjected to HPLC equipped with a Spectra-Physics SP8780XR autosampler. The separation of reaction products was achieved by a Sugar-Pak I column (Waters) at 90°C, with 0.1 mM $Ca \cdot Na_2 \cdot EDTA/H_2O$ as the mobile phase at 0.5 ml/min and a model 410 (Waters) differential refractometer.

Concentrations of sorbitol and fructose in equilibrium mixtures were quantified by HPLC to determine the equilibrium constant (K_{eq}) by means of the equation:

$$K_{eq} = \frac{[\text{sorbitol}][\text{NAD}]}{[\text{fructose}][\text{NADH}]}.$$

Sorbitol and NAD concentrations were assumed to be equal as were fructose and NADH concentrations, because the reactions were initiated with equal molar concentrations.

RESULTS

Activities of Hexose-Metabolizing Enzymes. Sucrose synthase activity is high in maize endosperm and may comprise as much as 2.8% of the soluble protein in this tissue (16). In this study, the highest sucrose synthase activity was found in 15 DPP[2] endosperms (Table I). Hexokinase activity was low relative to sucrose synthase activity. ATP-dependent fructokinase activity was only 1.2% of the sucrose synthase activity at 15 DPP. UTP-dependent fructokinase activity was detected at levels higher than ATP-dependent fructokinase (at 10–20 DPP) but at 15 DPP it was only 3% of the sucrose synthase activity. ATP-dependent glucokinase activity was generally higher than fructokinase activity, but it was about equal to the UTP-dependent fructokinase activity at 10 and 15 DPP. There was no detectable UTP-dependent glucokinase activity in any samples tested.

Ketose reductase activity was high relative to the hexokinases and was in the same order of magnitude as sucrose synthase activity from 10 to 25 DPP (Table I). After 25 DPP there was a sharp drop in the ketose reductase activity.

Properties of Ketose Reductase. Divalent cations had strong activating effects on ketose reductase activity (Fig. 1). Calcium and magnesium ions had the strongest activating effects, stimulating activity from 6- to 7-fold. Manganese ion stimulated activity only 2-fold. K_m values for $CaCl_2$, $MgCl_2$, and $MnCl_2$ were calculated to be 0.78, 1.18, and 0.40 mM, respectively. Divalent cations had little effect on the NAD-dependent polyol dehydrogenase activity (data not shown).

Ketose reductase and NAD-dependent polyol dehydrogenase activities had distinctly different pH optima (Fig. 2). Ketose reductase activity had optimal activity at pH 6.0, whereas NAD-dependent polyol dehydrogenase activity had optimal activity at pH 9.0. The specific activity of ketose reductase at its optimal pH was also much higher than the NAD-dependent polyol dehydrogenase activity at its pH optimum.

HPLC analysis of the products of the ketose reductase reaction with fructose as substrate confirmed that sorbitol is the product of fructose reduction (Fig. 3A). Fructose was also shown to be the product of the NAD-dependent polyol dehydrogenase reaction when sorbitol was the substrate (Fig. 3B).

Both ketose reductase and NAD-dependent polyol dehydrogenase behaved according to Michaelis-Menten kinetics with respect to their substrate saturation responses (Figs. 4, 5). Ketose reductase had a K_m (fructose) of 136 mM and a V_{max} of 21.2 μmol NAD/min·mg protein, whereas NAD-dependent polyol dehydrogenase had a K_m (sorbitol) of 8.45 mM and a V_{max} of 5.87 μmol NADH/min·mg protein.

The molecular mass of ketose reductase was estimated to be 78 kD by gel filtration (Fig. 6).

Product analysis of an equilibrium mixture of the fructose reductase reaction and the NAD-dependent sorbitol dehydrogenase reaction allowed the calculation of equilibrium constants. At pH 6.0 K_{eq} was calculated to be 1.17, and at pH 8.5 K_{eq} was calculated to be 1.97.

Substrate Specificity. The substrate specificity of ketose reductase was tested by substituting various ketoses for fructose in the ketose reductase assay (Table II). All ketoses tested gave activity, with *D*-ribulose giving higher activity than fructose at 100 mM. No aldose tested (D-glucose, D-xylose, D-ribose) served as substrate for this enzyme. NAD-dependent polyol dehydrogenase was also substrate nonspecific, and would oxidize sorbitol (D-glucitol), adinitol, xylitol, L-arabitol, and L-threitol, although the highest activity at 50 mM was found with sorbitol. No activity was found using mannitol or *myo*-inositol as substrates.

DISCUSSION

These results demonstrate in developing maize endosperm the presence of a nonspecific ketose reductase, which may function to metabolize a portion of the fructose produced from sucrose

[2] Abbreviation: DPP, days post-pollination.

Table I. *Activities of Hexose Metabolizing-Enzymes in Maize (W64A) Endosperm*
Values are the average of 5 extractions.

DPP	ATP-Dependent Fructokinase	UTP-Dependent Fructokinase	ATP-Dependent Glucokinase	Ketose Reductase	Sucrose Synthase
d			*μmol (min·g dry wt)*$^{-1}$		
10	0.450	0.684	0.660	1.49	6.84
15	0.730	1.759	1.612	43.31	59.36
20	0.411	0.906	1.127	26.05	48.06
25	0.380	0.354	0.527	13.97	21.83
30	0.209	0.108	0.220	3.17	22.58
35	0.198	0.091	0.244	2.94	10.46

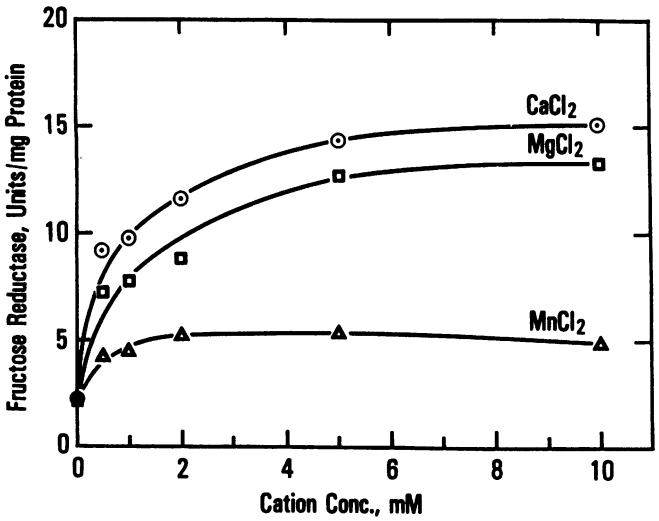

FIG. 1. The effect of divalent cation concentration on ketose reductase activity. Assays contained 400 mM fructose, 0.2 mM NAD, and were at 30 C, pH 6.0. Values are the average of four determinations.

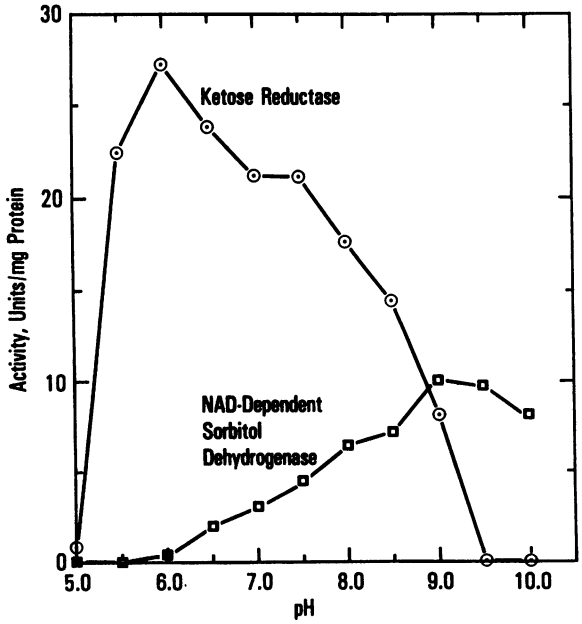

FIG. 2. The pH response of ketose reductase and NAD-dependent polyol dehydrogenase. Values are the average of four determinations.

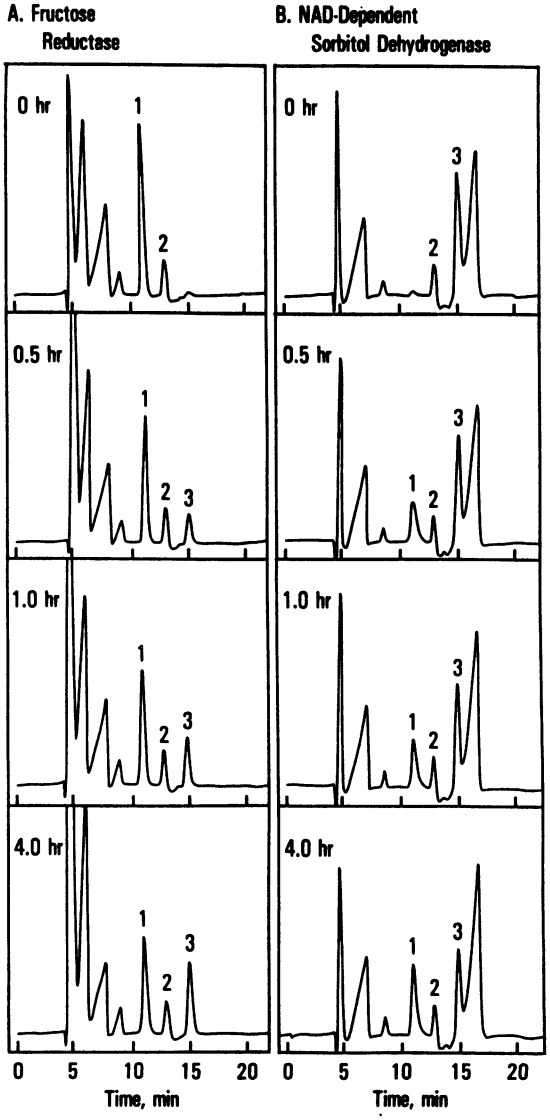

FIG. 3. HPLC analysis of the products of fructose reductase and NAD-dependent sorbitol dehydrogenase reactions. Reactions were terminated after 0, 0.5, 1.0, and 4.0 h as indicated. Peak 1 corresponds to the fructose standard, peak 3 corresponds to the sorbitol standard, peak 2 is an internal standard, mannitol. Additional peaks represent other reaction components such as buffers, salts and NAD (or NADH), that were not identified.

degradation. The product of this reaction would be sorbitol (Fig. 3). Sorbitol has been reported to be present in maize endosperm (3, 14) although the source and function of sorbitol was unknown. Whereas this report can offer a possible source of sorbitol,

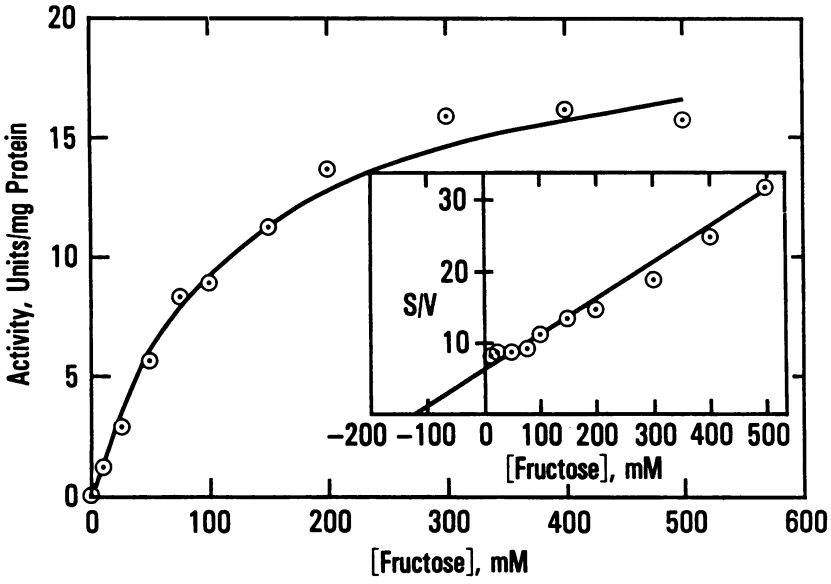

FIG. 4. Fructose saturation curve of ketose reductase at 30 C, pH 6.0. NADH concentration was 0.2 mM. Values are the average of four determinations. Inset: Hanes-Woolf replot of data. The K_m (fructose) was estimated to be 136 mM from this plot.

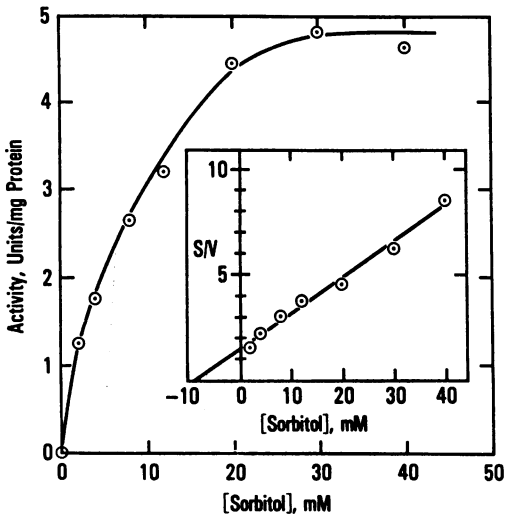

FIG. 5. Sorbitol saturation curve of NAD-dependent polyol dehydrogenase at 30 C, pH 6.0. NAD concentration was 1.0 mM. Values are the average of four determinations. Inset: Hanes-Woolf replot of data. The K_m (sorbitol) was estimated to be 8.45 mM from this plot.

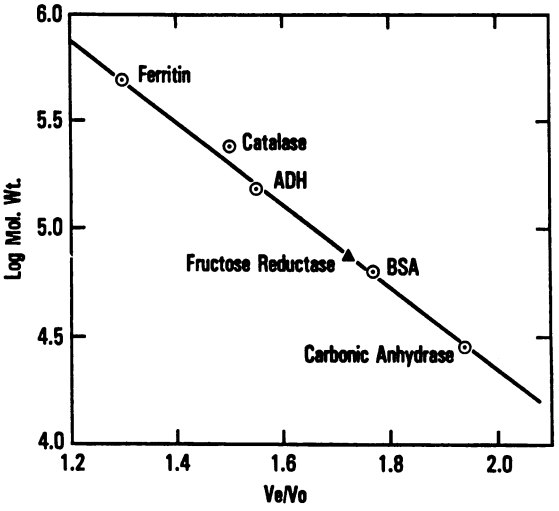

FIG. 6. Molecular mass estimation of ketose reductase on a calibrated Ultrogel AcA 34 column. See "Materials and Methods" for the elution conditions.

the fate of sorbitol produced by this reaction is still unknown. Fructose is produced in significant quantities in this tissue. The equilibrium constant of ketose reductase would appear to favor the conversion of fructose to sorbitol, although this conversion also depends on the NAD/NADH ratio. The fact that sorbitol does not accumulate to great extent in this tissue (14) suggests that it is further metabolized by other enzymes. Additional steps in sorbitol metabolism are presently under investigation.

Sorbitol metabolism has been described extensively in apples and other Rosaceae where sorbitol is the primary product of photosynthesis and is transported in the phloem (1). Negm and Loescher (11) described a NAD-dependent sorbitol dehydrogenase from apple callus tissue which has subsequently been detected in apple leaf, fruit and cotyledons (9, 21). It was suggested that this enzyme functions in the direction of fructose production because its K_m (fructose) was very high (1.5 M). Other enzymes of sorbitol metabolism in apples include an NADP-dependent sorbitol dehydrogenase (23) that converts sorbitol to glucose, a NADP-dependent sorbitol 6-P dehydrogenase (12) that converts sorbitol 6-P to glucose 6-P, and a sorbitol oxidase (22) that converts sorbitol to glucose independent of either NAD or NADP. In maize, sorbitol is apparently not transported but rather synthesized in the kernel, because sorbitol is found only in the kernel (pedicel, embryo and endosperm) and not in the shank of the ear or in the cob (14). Maize endosperm ketose reductase may function to convert fructose to glucose with sorbitol as an intermediate, as occurs in mammals and fungi (1). This is apparently the first report of a ketose reductase in plants other than in the Rosaceae. Maize endosperm ketose reductase

Table II. *Substrate Specificity of Ketose Reductase and NAD-Dependent Polyol Dehydrogenase*

Conditions were as stated in "Materials and Methods."

Polyol	Activity	Ketose	Activity
50 mM	%	*100 mM*	%
Sorbitol	100	D-Fructose	100
Xylitol	91	D-Xylulose	57
Ribitol	73	D-Ribulose	167
L-Threitol	43	L-Sorbose	66
L-Arabitol	17		

is nonspecific as to the ketose it will reduce or to the polyol it will oxidize (Table II). In this respect it is similar to the NAD-dependent sorbitol dehydrogenase from apple callus (11) and to various polyol dehydrogenases from rat liver (2, 10), sheep liver (15), guinea pig seminal vesicle (20), *Candida utilis* (4), and *Bacillus subtilis* (7).

The activity of ketose reductase relative to hexokinase suggests that this enzyme may be important in hexose metabolism. Its pH optimum is 6.0, which is the same as sucrose synthase in the sucrose degradation direction (17). This is in contrast to maize endosperm hexokinase activity, which reportedly has optimal activity at pH 8.2 in crude extracts (5). Although the K_m (fructose) for ketose reductase is high (136 mM), the K_m (sucrose) for sucrose synthase is also high (40 mM) (18), and sucrose synthase appears to function in sucrose degradation. Perhaps the presence of these enzymes at such high activities has allowed them to function at physiological sugar concentrations at only one tenth of their potential V_{max}.

It is not clear whether the ATP-dependent fructokinase and the UTP-dependent fructokinase are actually different proteins, although their developmental activity profiles are quite different (Table I). Recently, Huber and Akazawa (8) described a UTP-dependent fructokinase activity from cultured sycamore cells that could not be resolved from ATP-dependent fructokinase. ATP-dependent fructokinase and ATP-dependent glucokinase activities have been separated in this laboratory (DC Doehlert, unpublished data) demonstrating that these are different proteins. The function of these enzymes is clearly dependent on their intracellular localization, which is not yet known. The variety of different hexose metabolizing enzymes might suggest specialization of function for each of these enzymes. The cell may use a different hexokinase or ketose reductase to partition carbon into different metabolic fates, such as starch synthesis, protein synthesis, respiration or lipid synthesis. The reported hexose K_m values for hexokinases from maize endosperm are 65 to 183 μM (6). Thus these enzymes would be operating at V_{max} at suboptimal hexose concentrations for ketose reductase. The significance of these observations is not yet clear and requires further investigation.

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