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INCREASED HEXOKINASE LEVELS IN ENDOSPERM OF MUTANT MAIZE

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Abstract—Hexokinase activity was measured in endosperms of shrunken-2 (sh_2) and starchy maize. Initial increases in hexokinase were observed for developing endosperms of both genotypes, and the enzyme declined in both as the seeds matured. A higher level of hexokinase was observed in developing sh_2 than in starchy endosperm. This difference persisted throughout maturation and occurred also in germinating seeds. Soluble hexokinase activity per endosperm continued to increase in sh_2 for about 8 days (22–30 days after pollination) after the enzyme in starchy endosperm had attained maximum activity and begun to decline. Hexokinase was predominantly soluble in both genotypes so the differences observed are not due to altered distribution of enzyme between particulate and soluble fractions.

INTRODUCTION

The shrunken-2 (sh_2) mutant of maize is of particular interest for studies of carbohydrate metabolism and gene action at the enzyme level. The weight of mature sh_2 seeds is about 75% of normal and the endosperms are collapsed because starch content is reduced to only 25–30% of normal.[1–3] The diminished starch accumulation is apparently due to a greatly decreased level of ADP-glucose pyrophosphorylase[3,4] which forms ADP-glucose, susbtrate of starch synthetase. The enhanced sucrose content of sh_2 could result from this metabolic block; enzymes that form sucrose via UDP-glucose are not reduced in sh_2 endosperm.[3] The complex nature of this mutation is indicated by the reports that although the activity of one enzyme is decreased in developing sh_2 endosperm, the activity of two others—phosphorylase and invertase—is higher than in starchy maize.[5,6]

Our current research deals with hexokinase, an enzyme of great importance to carbohydrate metabolism in maize endosperm. Translocated sucrose enters endosperm cells as glucose and fructose,[7] and further metabolism of these hexoses is dependent upon phosphorylation by hexokinase. Hence, the hexokinase reaction may be considered the first step in the sequence leading to endosperm reserve substances—starch, protein, and lipid,[8–10] and to reactions necessary for growth. It follows that hexokinase activity should be highest

[1] R. G. Creech, *Genetics* **52,** 1175 (1965).
[2] J. R. Laughnan, *Genetics* **38,** 485 (1953).
[3] C. Y. Tsai and O. E. Nelson, *Science* **151,** 341 (1966).
[4] D. B. Dickinson and J. Preiss, *Plant Physiol.* **44,** 1058 (1969).
[5] P. H. Jennings and C. L. McCombs, *Phytochem.* **8,** 1357 (1969).
[6] T. A. Jaynes and O. E. Nelson, *Plant Physiol.* **47,** 623 (1971).
[7] J. C. Shannon, *Plant Physiol.* **43,** 1215 (1968).
[8] R. Bressani and R. Conde, *Cereal Chem.* **38,** 76 (1961).
[9] A. Dalby, in *Proceedings of the High Lysine Corn Conference* (edited by E. T. Mertz and O. E. Nelson), p. 74, Corn Industries Research Foundation, Washington, D.C. (1966).
[10] J. W. Evans, *Cereal Chem.* **18,** 468 (1941).

during periods of growth and accumulation of storage materials. Indeed, reports from two laboratories[11,12] showed that endosperm hexokinase increased rapidly from 10 to 22 days after pollination, a period of rapid kernel enlargement and starch accumulation.

The earlier study of hexokinase included a survey of enzyme levels in endosperms of seeds germinated for 5 days.[11] Hexokinase activity observed in clarified extracts of sh_2 endosperms was several times greater than in similar extracts from starchy maize. Whether this difference arose during seed maturation or during germination was not established, nor was it known whether these genotypes differed in content of particulate or latent hexokinase. The present study was undertaken to determine at which time during development the difference in hexokinase level appears and to investigate the nature of this difference.

RESULTS

Preliminary experiments with maturing dent kernels established the need for separating endosperm from other tissues in order to obtain a valid estimate of endosperm hexokinase activity. For instance, the pericarp contained 5-fold more hexokinase than did the endosperm at 10 days after pollination, approximately the same amount of hexokinase at 14–42 days, and one-half as much hexokinase at 50 days. In contrast to these results, 90% of maize seed ADP-glucose pyrophosphorylase is found in the endosperm.[4]

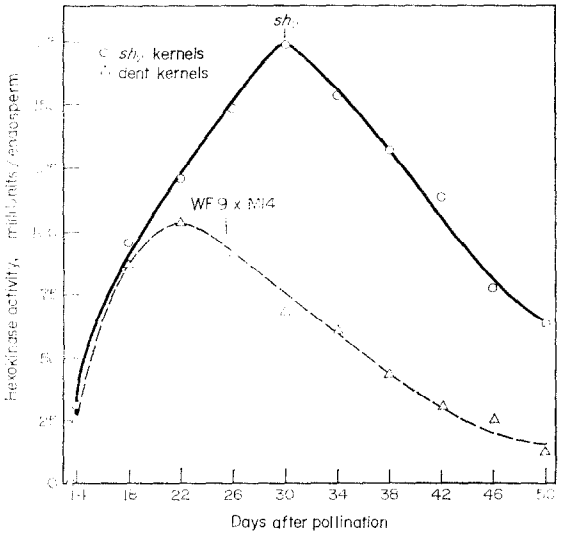

FIG. 1. PATTERN OF ENDOSPERM HEXOKINASE ACTIVITY IN sh_2 AND DENT KERNELS DURING DEVELOPMENT. POINTS ARE MEANS OF TWO REPLICATIONS.

Figure 1 shows that the difference in hexokinase level per endosperm between shrunken-2 and dent appears during maturation. Shrunken-2 resembled dent at 14 and 18 days, and enzyme activity increased about 3-fold during that time. There was very little subsequent increase in the dent enzyme, but the shrunken-2 enzyme continued to increase at a nearly linear rate until 30 days. Activity declined continuously in both genotypes after maximum activity was reached. The enzyme decreased at a greater absolute rate in shrunken-2, but

[11] E. L. Cox and D. B. Dickinson, *Phytochem.* **10**, 1771 (1971).
[12] C. Y. Tsai, F. Salamini and O. E. Nelson, *Plant Physiol.* **46**, 299 (1970).

the decreases were quite similar when expressed as percentages of the maximum activity for each genotype. The shrunken-2 enzyme had decreased to one-half of its maximum activity by about 15 days after the maximum was observed; the dent enzyme declined to one-half of its maximum by about 14 days after the maximum. Thus, the striking difference in hexokinase levels occurs because the increase in enzyme is maintained for about 1 week longer in the endosperm of the mutant.

During early germination of shrunken-2 (Table 1, days 1–4) there is an apparent increase in hexokinase which is partly accounted for by the increased extraction of soluble protein. This pattern is somewhat different from the constant decrease in hexokinase observed for germinating dent seeds.[11] Even at the earliest stage of germination, the shrunken-2 enzyme is higher than was observed for dent, and it seems likely that this difference has persisted through the drying and rehydration of the seed.

TABLE 1. HEXOKINASE ACTIVITY, DRY WT, AND PROTEIN CONTENT OF ENDOSPERM FROM GERMINATING SHRUNKEN-2 KERNELS

Days germinated	Dry wt (mg/endosperm)	Protein (mg/endosperm)	Hexokinase (mU/endosperm)	(mU/mg protein)
1	71·9	0·26	21	81
2	56·5	0·43	25	58
3	56·9	0·42	26	62
4	40·6	0·40	41	102
5	31·3	0·29	19	65

The data for soluble hexokinase (Fig. 1) are a valid estimate of total endosperm hexokinase as indicated by the results in Table 2. For both genotypes the proportion of total activity in the initial supernatant was 86–87% at 14 days and essentially 100% by 34–38 days. The particulate enzyme was partially solubilized by Tween 80, but there was no enhancement of enzyme activity from the Tween treatment. Thus, the difference between genotypes is not due to altered ratios of soluble to particulate hexokinase or to a large amount of latent hexokinase in the particulate fraction of dent endosperm. The maize

TABLE 2. DISTRIBUTION OF HEXOKINASE IN THE EXTRACT OF ENDOSPERM FROM DEVELOPING MAIZE KERNELS

Kernel age	Endosperm phenotype: Shrunken % Activity in Supernatant	Shrunken % Activity in Pellet*	Dent % Activity in Supernatant	Dent % Activity in Pellet*
14	86·1	13·9	86·8	13·2
18	90·8	9·2 (60·3)	95·6	4·4 (0·0)
26	92·1	7·9 (46·0)	95·5	4·5 (6·1)
34	—	—	100·0	0·0
38	97·1	2·9	—	—
42	100·0	0·0	—	—

* Each value in parenthesis is the percentage of pellet activity that was solubilized by extraction with 5% Tween 80. Details of procedure are given in Experimental.

endosperm enzyme is predominantly soluble and differs in this respect from hexokinase in several other plant tissues. Hexokinase in lentil roots,[13] pea hypocotyls,[14] wheat germ, and potato tubers[15] is present predominantly in the particulate fraction.

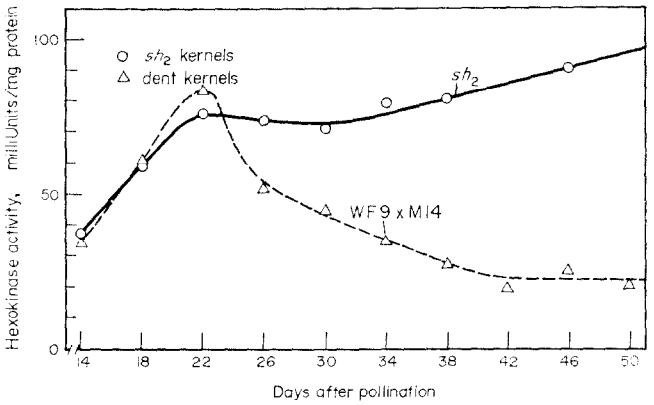

FIG. 2. PATTERN OF HEXOKINASE SPECIFIC ACTIVITY IN sh_2 AND DENT KERNELS DURING DEVELOPMENT. POINTS ARE MEANS OF TWO REPLICATIONS.

The specific activity of hexokinase was calculated (Fig. 2). Changes in total enzyme may also be compared to changes in soluble protein (Fig. 3) and fresh and dry wts (Fig. 4). From 14 to 22 days the genotypes have similar specific activities, but a difference is observed thereafter (Fig. 2). Specific activity of the dent enzyme declines after 22 days while that of shrunken-2 does not decline. The significance of this result is not clear because the soluble protein content exhibits a complex pattern during development (Fig. 3). Soluble proteins

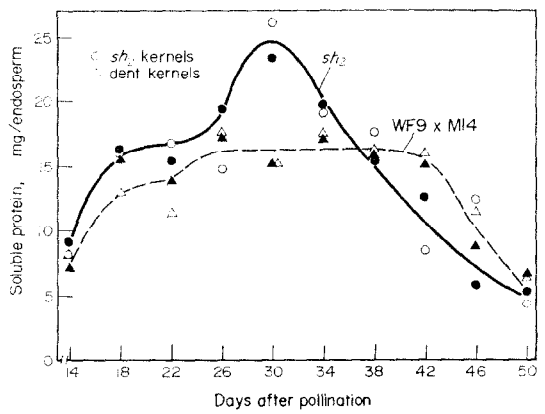

FIG. 3. PATTERN OF ENDOSPERM SOLUBLE PROTEIN CONTENTS DURING DEVELOPMENT OF sh_2 AND DENT KERNELS. OPEN SYMBOLS ARE REPLICATION ONE; FILLED SYMBOLS ARE REPLICATION TWO.

are a small proportion of the total endosperm protein. The observed changes in soluble proteins may result from differences in extraction of the relatively insoluble reserve proteins rather than altered levels of soluble cytoplasmic proteins.

[13] J. C. Meunier, A. M. Moustacas and J. Ricard, *Compt. Rend.* **267D**, 1445 (1968).
[14] A. A. Abdul-Baki and P. M. Ray, *Plant Physiol.* **47**, 537 (1971).
[15] P. Saltman, *J. Biol. Chem.* **200**, 145 (1953).

The two genotypes also differ in the pattern of fresh and dry wts (Fig. 4). The increased hexokinase in sh_2 endosperms is only partly accounted for by the greater fresh wt of sh_2. At 26 days post-pollination and thereafter the sh_2 enzyme exceeds the dent enzyme when the data is expressed as enzyme units per g fresh wt. Also, the differences in fresh and dry wt accumulation are not well correlated with the different patterns of enzyme activity. The difference between genotypes in enzyme content per endosperm appears several days later than the difference in fresh wt. The sh_2 enzyme increases considerably between 22 and 30 days, a period when the rate of dry matter accumulation is decreasing. Conversely, activity of the dent enzyme drops continuously during the same period although dry matter accumulates at a constant rate. The dry matter data are similar to published values.[1,5,10,16] The increased fresh wt of sh_2 at 3 weeks after pollination was noted earlier,[3,4] but there seem to be no reports on change in fresh wt of sh_2 during development.

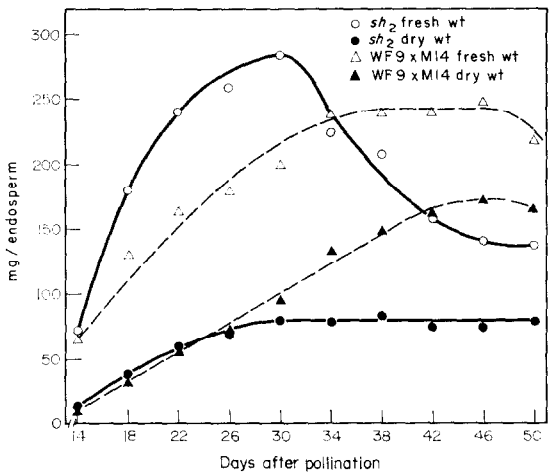

FIG. 4. PATTERN OF ENDOSPERM FRESH AND DRY WTS DURING DEVELOPMENT. POINTS ARE MEANS OF TWO REPLICATE DETERMINATIONS.

Enzyme assays were done immediately after extraction in our experiments reported above. Some of the extracts were stored in ice and assayed later. The sh_2 enzyme was relatively stable since 93% of initial activity was retained after 24 hr and 72% after 7 days (averages of 13 extracts from all stages of development). The isolated dent enzyme seemed less stable, particularly at later stages of development. Dent extracts from 40- and 50-day tissues retained about 82% of initial activity after 24 hr and only 37% after 7 days. It was not established whether the altered enzyme stability was a property of the enzyme itself or was due to altered levels of protective or destructive components of the crude extract.

DISCUSSION

The pattern of hexokinase activity observed here is similar to the pattern for invertase[6] in that invertase content per endosperm tends to increase or remain high in sh_2 during a period (12–20 days) when the invertase in normal maize is continuously decreasing. Unlike hexokinase, the invertase eventually decreases quite rapidly in sh_2, and the difference

[16] J. INGLE, D. BEITZ and R. H. HAGEMAN, *Plant Physiol.* **40**, 835 (1965).

between sh_2 and normal disappears by 28 days after pollination. The difference in phosphorylase between sh_2 and normal appears earlier in development[5] than the difference observed for hexokinase. The study of phosphorylase ended at 27 days after pollination. Hence, there is no information whether sh_2 phosphorylase remains higher than normal during the entire course of maturation and subsequent germination as is observed for hexokinase.

Since the sh_2 hexokinase seems more stable *in vitro* than the dent enzyme, the increased hexokinase level in sh_2 endosperm may be due to greater *in vivo* enzyme stability. However, this suggestion seems to be contradicted by the similar *in vivo* rates of enzyme decay after maximum activity is attained. The pattern of hexokinase activity in developing endosperm may result from an initial period of enzyme synthesis which terminates at the time of maximum enzyme activity. Enzyme synthesis in sh_2 might continue about 1 week longer than in dent to produce the observed difference in enzyme levels. Such a prolonging of enzyme synthesis could be related to elevated sugars and other metabolites in sh_2 and thus be an indirect result of the decreased ADP-glucose pyrophosphorylase activity. Whether the level of hexokinase in dent endosperm could be increased by external application of sugars or sugar phosphates is not known. The level of hexokinase in castor bean cotyledons is greatly enhanced by externally applied sucrose, glucose, and fructose.[17] The enhancement is thought to be a stimulation of enzyme synthesis although the mechanism is not known.

Carbohydrate metabolism in sh_2 endosperm may be altered by the increased hexokinase. One consequence could be a more rapid formation of hexose phosphates which would give rise to UDP-glucose and then sucrose. In this case the increased sucrose in sh_2 would result from both the blockage in ADP-glucose formation and an enhanced capacity to form sucrose precursors. A positive feedback mechanism may be operating if enhanced sucrose content (due initially to reduced ADP-glucose) causes increased hexokinase levels which in turn cause increased sucrose formation.

EXPERIMENTAL

Plant material. Maize plants (*Zea mays* L.) of gentoype sh_2sh_2 (var. Illini Chief Super Sweet) or Sh_2Sh_2 (dent inbreds WF9 and M14) were grown in a field nursery. Ears were covered with bags and hand pollinated to insure that all kernels were of known genotype. The endosperm genotypes were $sh_2sh_2sh_2$ (sh_2sh_2 selfed) and $Sh_2Sh_2Sh_2$ (WF9 × M14). Samples were collected at 4-day intervals commencing on the 14th day after pollination and continuing until the 50th day. Each sample consisted of 60 kernels and was obtained by taking 20 kernels from an ear on each of three different plants. The kernels were taken at random. Replicate samples were harvested from different plantings made in May and June 1970. The harvested kernels were kept on ice in the field, then in the laboratory were rinsed with cold distilled water, blotted dry, frozen in liquid N_2, and stored at −20° until used. The time from harvest until freezing did not exceed 15 min. Preliminary experiments established that freezing and thawing did not alter the amount of hexokinase extracted from the endosperm tissue. Enzyme level did not change during frozen storage; similar activities were observed whether samples were extracted soon after being frozen or one year afterward. For germination studies, mature seeds from hand pollinated sh_2 ears were surface sterilized for 5 min with 2% NaOCl, exhaustively rinsed, and germinated with 10^{-4} M $CaCl_2$ in darkness at 27 ± 1° as described by Ingle *et al.*[18]

Preparation of the crude extract. The endosperm tissue was dissected from 30 kernels, weighed, and ground to a fine powder with mortar and pestle under liquid N_2. A sample of the frozen powder was taken for the dry wt determination, and the remainder was extracted with 2 vols. (2 ml/g powder) of 50 mM phosphate buffer, pH 7·5, containing 5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA and 1 mM dithiothreitol.[11] After 30 min incubation with stirring, the mixture was squeezed through 8 layers of cheesecloth and the filtrate centrifuged for 20 min at 25000 *g*. The supernatant was used undiluted in the hexokinase assays. The temperature of the tissue and extract was kept below 4° at all times. In several experiments the centrifuged pellet was re-extracted to determine the efficiency of the extraction procedure. First the pellet was

[17] E. Marré, M. P. Cornaggia, F. Alberghina and R. Bianchetti, *Biochem. J.* **97,** 20p (1965).
[18] J. Ingle, L. Beevers and R. H. Hageman, *Plant Physiol.* **39,** 740 (1964).

dispersed in a volume of extraction medium equal to the volume of supernatant which had been removed from the pellet. After 10 min the mixture was centrifuged (5 min, 400 *g*) and a sample of cloudy supernatant withdrawn for hexokinase assay. The tube was recentrifuged (20 min, 25000 *g*), and the supernatant was decanted and assayed for hexokinase. The pellet was resuspended in one supernatant-volume of extraction medium that contained 5% (v/v) Tween 80. The mixture was incubated in ice for 20 min, then centrifuged for 20 min at 25000 *g*. The supernatant was assayed and the pellet discarded. For germination studies, the scutellum and embryo were removed from the germinated kernels and the remainder (endosperm and seed coat) ground under liquid N_2. The frozen powder was handled as described above. 20–25 kernels, giving 1–1·5 g of fresh material, were used for each experiment.

Hexokinase assays. Enzyme activity was assayed spectrophotometrically by coupling the hexokinase reaction to glucose 6-phosphate dehydrogenase and measuring the NADP reduced at 340 nm. The reagents and techniques used were described previously.[11] Enzyme activity is expressed in International Units. 1 Unit = 1 μmol glucose-6-P formed/min at 30°; 1 milliUnit (mU) = 1 nmol glucose-6-P formed/min.

Dry matter and protein content. Dry matter content of the endosperm tissue was calculated from the wt of the powder sample after drying to a constant wt at 60° in a circulating oven (48 hr). Soluble protein in the initial supernatant fraction was determined by the method of Lowry *et al.*[19] on the material precipitated by 10% TCA; the standard was crystalline bovine serum albumin.

Acknowledgements—This investigation was supported by U.S. National Science Foundation Grant No. GB 8764 and the Illinois Agr. Exp. Sta.

[19] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193,** 265 (1951).