

An Invertase Inactivator in Maize Endosperm and Factors Affecting Inactivation^{1,2}

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T. A. JAYNES³ AND O. E. NELSON⁴

Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana 47907

ABSTRACT

A protein present in the developing endosperm of maize (*Zea mays* L.) causes a loss of invertase activity under certain conditions of incubation. This protein, designated an inactivator, inactivates invertase I of maize even in the presence of other proteins. No inactivation of invertase II of maize or yeast invertase has been observed. The inactivator and invertase I are found only in the endosperm. The quantity of inactivator increases in the normal endosperm during development while invertase I activity decreases. However, the altered levels of invertase I activity in several endosperm mutant lines do not result from different quantities of inactivator. The inactivator can decrease invertase I activity during a preincubation period before addition of sucrose; inactivation is noncompetitive. Invertase I activity decreases curvilinearly with an increase in inactivator concentration. At high buffer concentrations or low inactivator concentrations in the reaction mixture, a latent period is observed when invertase I is not inactivated. Inactivation increases with an increase in temperature and a decrease in pH.

The life span of an enzyme may be much less than that of a whole cell, so enzymes in the cell undergo degradation as well as synthesis. The half-life of enzymes in the liver has been estimated at 3 days while the life span of liver cells may be on the order of months (6, 9). Some plant enzymes are known to be short-lived. For example, in potato tuber disks, phenylalanine ammonia-lyase appears and then disappears after exposing the tissue to light. A proteinaceous inactivator of phenylalanine ammonia-lyase that would cause the loss of activity following induction has been proposed (10). Despite the importance of enzyme degradation in higher organisms, little information is available about the factors responsible (1).

By enzyme assay alone it is difficult to distinguish an enzyme degrading system from one simply inhibiting an enzyme. Considerable study has been made on the inhibition of protein-

ases by specific proteins present in plants as well as animals. Other enzymes besides proteinases can be inhibited by proteins. A protein inhibitor of potato invertase was detected when an inhibitor-containing enzyme extract exhibited a nonlinear increase in activity with a linear increase in protein concentration. It was proposed that inhibition occurs by irreversible binding of the inhibitor to the enzyme; inhibition was noncompetitive. A metabolic role for the inhibitor was suggested when large amounts of the inhibitor and low invertase activity were found in tubers stored at 18 C and the reverse at 4 C (3-5).

Three forms of invertase are present in corn endosperm (2). The activity per endosperm of invertase I is greatest 12 days after pollination. Activity decreases 37-fold by the 28-day stage. This decrease is in contrast to the increase in soluble protein content and accumulation of starch after the 12-day stage. A protein has been found which is capable of inactivating invertase I *in vitro*. This protein accumulates in the normal endosperm during development while invertase I activity decreases. This inactivator decreases invertase I activity of corn endosperm only under specific conditions. This report discusses some of the factors affecting inactivation.

MATERIALS AND METHODS

Preparation of invertase I and the general method of assay are as given in the accompanying report (2).

Extraction of Inactivator The inactivator was extracted from 2.5 g of corn endosperms (*Zea mays* L.). The endosperms were ground in 2.5 ml of 0.05 M phosphate buffer (pH 8.0) with a mortar and pestle. After centrifuging this extract at 22,000g for 5 min, the supernatant fraction was retained, and the pellet was resuspended in 2 ml of buffer and centrifuged again. Both supernatant fractions were combined and heated at 95 C in a centrifuge tube for 5 min. Next the tube was cooled on ice for 5 min and then centrifuged at 22,000g for 5 min. The supernatant fraction, which contained the inactivator, was dialyzed 18 hr against water at 5 C. After dialysis the solution was brought to a volume of 10 ml. The inactivator was stable in this solution when stored at 5 C.

Quantitative Analysis of Inactivator. The amount of invertase inactivator during development was expressed as a percentage of the inactivator per normal endosperm at the 22-day stage. The linear portion of the standard inactivation curve was determined by varying the amount of inactivator of 22-day normal corn from 5 μ l to 12 μ l. To analyze for the inactivator, a volume of a preparation was determined which would inactivate invertase I at some point on the standard curve. For instance, 25 μ l of a 12-day inactivator preparation might result in the same percentage of invertase I activity remaining as 8 μ l of a preparation from 22-day endosperms. The corresponding amount of normal inactivator, 8 μ l, was divided by the amount of 12-day endosperm preparation used, 25 μ l, and the

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³ Present address: Department of Agronomy, University of Nebraska, Lincoln, Neb. 68503. Submitted to Purdue University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

⁴ Present address: Department of Genetics, University of Wisconsin, Madison, Wis. 53706.

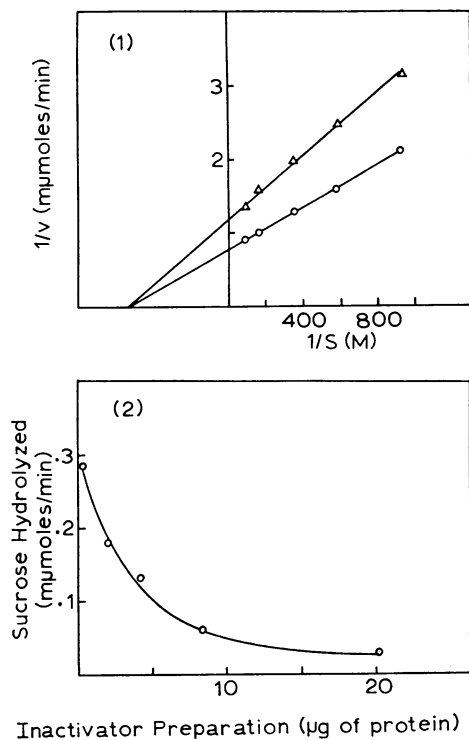


FIG. 1. Lineweaver-Burk plot of invertase I activity assayed with (Δ) or without (\circ) the inactivator. Invertase I was preincubated with inactivator and buffer for 30 min. Sucrose was added at zero time, T_0 . Reaction time was 15 min; reaction temperature was 37 C. Each point is a mean of four replications. Volume of reaction mixture was 0.175 ml containing: invertase I preparation from normal endosperms at 14-day stage, 6 μ g of protein; inactivator preparation from normal endosperms at 22-day stage, 1.3 μ g of protein; acetate buffer (pH 5.0), 5.7 mM.

FIG. 2. Effect of inactivator concentration on invertase I activity. Inactivator was preincubated with invertase I and buffer for 15 min at room temperature. Sucrose was added at T_0 . Reaction time was 30 min; reaction temperature was 37 C. Each point is a mean of two replications. Volume of the reaction mixture was 0.25 ml containing: invertase I prepared from $sh_2/sh_2/sh_2$ endosperms at 22-day stage, 7.6 μ g of protein; inactivator preparation from $su_2/su_2/su_2$ endosperms at 22-day stage; acetate buffer (pH 5.0), 20 mM; sucrose, 4 mM.

number of kernels in the inactivator preparation at the 12-day stage. This was then compared with inactivator activity in 22-day normal corn expressed on a similar per endosperm basis.

RESULTS

Nature of the Inactivator. The inactivator was originally detected after DEAE⁵-cellulose column fractionation of a dialyzed protein extract from endosperms at the 22-day stage. The inactivator eluted from the column with a peak activity in fraction number 14, whereas invertase I eluted in later fractions (the column fractionation is described in the accompanying report). In fractionation on Sephadex G-75, the inactivator eluted after the exclusion volume so its molecular weight is less than 75,000. A small amount of inactivator preparation (as measured by protein content) inhibited invertase to a great extent, although, as will be shown, this amount depended on the conditions of assay. The inactivation can be described as non-competitive (Fig. 1).

⁵ Abbreviations: DEAE: diethylaminoethyl; *shrunken*: *sh*; *sugary*: *su*.

Several lines of evidence suggest the inactivator is a protein. For one, the inactivator could be precipitated by ammonium sulfate. Also, although the inactivator in the protein extract was stable to boiling at pH 8.0, it could be denatured by heat in dilute solutions at pH 5.0. To test the protein nature of the inactivator, an experiment was designed in which 5 mg of pronase were added to 7.5 ml of a dialyzed protein extract, and the mixture was incubated 2½ hr. After this incubation the extract and a control without pronase were boiled, centrifuged, and the supernatant fractions were tested for inactivator. The pronase-treated extract had no inactivator remaining while the control had a normal level of inactivator.

Effect of Other Proteins on Inactivation. Extraneous proteins added to the assay mixture did not affect inactivation. Several concentrations (1 μ g–50 μ g) of bovine albumin were added to reaction mixtures containing inactivator and invertase I. Inactivation was the same at all concentrations of bovine albumin. The possible effect of the inactivator on enzymes other than invertase I was of interest. The inactivator had no effect

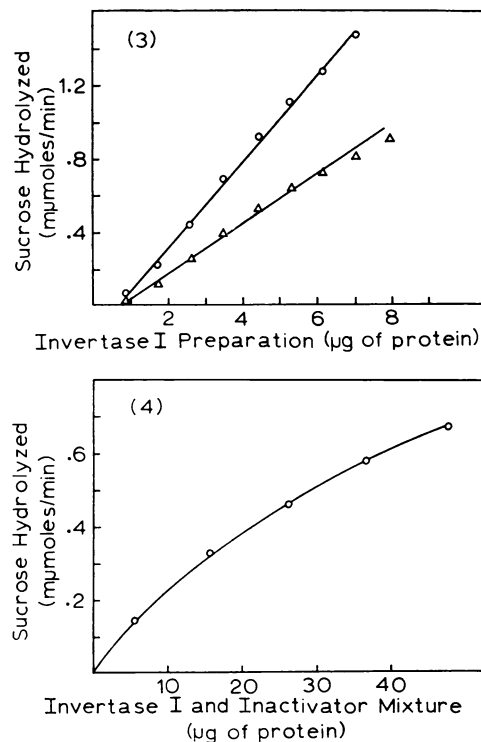


FIG. 3. Invertase I activity as a function of the quantity of invertase I added to a reaction mixture with (Δ) or without (\circ) the inactivator. Reaction time was 15 min; reaction temperature was 37 C. Each point is a mean of two replications. Volume of the reaction mixture was 0.175 ml containing: invertase I preparation from normal endosperms at 16-day stage; inactivator preparation from $su_2/su_2/su_2$ endosperms at 22-day stage, 1.87 μ g of protein; acetate buffer (pH 5.0), 28.6 mM; sucrose, 5.7 mM.

FIG. 4. Invertase I activity as a function of the concentration of a protein mixture containing invertase I and inactivator. The mixture was made by adding an inactivator preparation containing 58 μ g of protein to an invertase I preparation containing 216 μ g of protein. Invertase I was prepared from $sh_2/sh_2/sh_2$ endosperms at the 22-day stage and fractionated on DEAE-cellulose. Inactivator was from $su_2/su_2/su_2$ endosperms at 22-day stage, heat treated as usual but then dialyzed against 0.01 M tris-maleate buffer (pH 7.0) and fractionated on DEAE-cellulose. Inactivator containing fraction was dialyzed against water. For assay of activity sucrose was added at T_0 . Reaction time was 30 min; reaction temperature was 37 C. Each point is a mean of three replications. Total volume of the reaction mixture was 0.33 ml containing: acetate buffer (pH 5.0), 30.3 mM; sucrose, 3 mM.

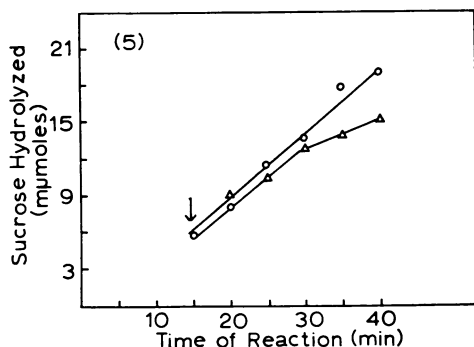


FIG. 5. Latent period in the inactivation of invertase I after addition of inactivator during the reaction period. Inactivator (Δ) or an equal volume of water (\circ) was added to the reaction mixtures 15 min after T_0 . Reactions were stopped at 5-min intervals up to 40 min. Each point is a mean of two replications. Reaction temperature was 37 C. Volume of reaction mixture was 0.3 ml containing: invertase I preparation from *sh₂/sh₂/sh₂* endosperms at 22-day stage, 11.7 μ g of protein; inactivator preparation from *su₂/su₂/su₂* endosperms at 22-day stage, 1.75 μ g of protein; acetate buffer (pH 5.0), 16.7 mM; sucrose, 3.3 mM.

on an α -amylase in corn endosperm or on the other soluble invertase in corn, invertase II. Neither did it inactivate yeast invertase.

Invertase I could still be inactivated when included with all other proteins in an extract from 12-day normal corn. For example, when 0.5 ml of an inactivator preparation was added to a dialyzed homogenate, incubated at room temperature for 15 min, and then cooled rapidly and fractionated on a DEAE-cellulose column, the amount of invertase I activity recovered was reduced to 57% of the control. Invertase I activity could be reduced further with a longer period of preincubation or by the addition of more inactivator.

Effect of Concentration on Inactivation. Inactivation of invertase I was dependent on the concentration of the inactivator in the reaction mixture. When increasing amounts of the inactivator were added to a constant amount of invertase I, invertase I activity decreased curvilinearly (Fig. 2). The extent of inactivation with high concentrations of the inactivator was not constant but depended on the method of assay. In the experiment of Figure 2, invertase I was added to the inactivator and buffer before the assay was initiated with the addition of sucrose. However, when invertase I was added at zero time, T_0 , as was done in the experiment of Figure 11, the plot was different. Under conditions of that experiment there was no inactivation below a threshold level of the inactivator. The threshold is related to the latent period in inactivation to be described later.

Invertase I activity increased linearly with an increase in concentration of invertase I (though not always from the origin). When a constant amount of inactivator was included in an assay with increasing amounts of invertase I, then activity was reduced but remained linear with the increase in invertase I concentration (Fig. 3). Even in experiments at very low concentrations of invertase I, the inactivator did not cause a complete loss of invertase I activity. However, if different amounts of invertase I were reacted with a relatively low and constant concentration of inactivator, then activity was reduced slightly and was parallel to the control.

Inactivation was examined by increasing simultaneously the concentrations of invertase I and inactivator. This was accomplished by adding an amount of inactivator to invertase I and measuring invertase I activity with increasing amounts of the mixture. The line relating activity to concentration was curvilinear (Fig. 4). Inactivation was greater at higher concentrations of the mixture.

Latent Period of Inactivation. Under some conditions (high buffer or low inactivator concentrations) of assay, a latent period was evident before invertase I was inactivated. Even if the inactivator was added during the course of the reaction of invertase I with sucrose (Fig. 5), invertase I activity did not decrease immediately but only after an additional 15 min. Under these conditions, if invertase I was added at T_0 to a mixture of inactivator, sucrose, and buffer, no inactivation would be apparent at the end of a 15-min reaction period.

The effect of inactivator concentration on the latent period is illustrated by the experiment of Figure 6, a time-course study using three concentrations of inactivator. The latent period increased with a decrease in inactivator concentration. In parallel experiments the latent period increased with an increase from 25 mM to 50 mM in the buffer concentration of the reaction mixture. The concentration of acetate buffer had no effect on invertase I alone. Also, preincubation of invertase I alone in buffer for different periods of time had no effect on the response of invertase I to inactivator added after this preincubation. A decrease in inactivation was noticed, likewise, using citrate buffer in the assay rather than acetate buffer. A low

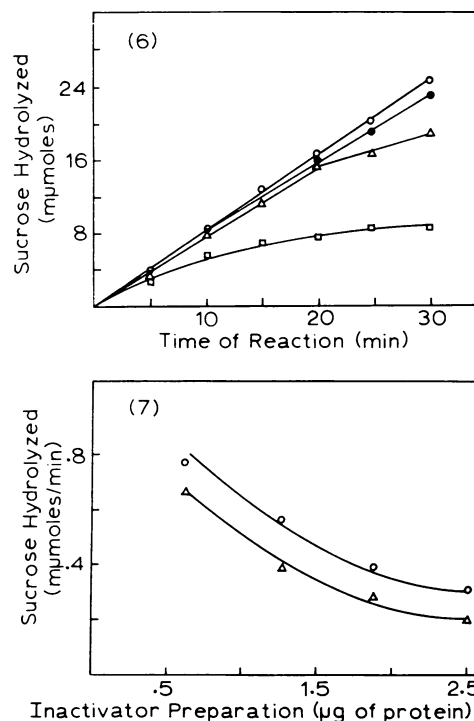


FIG. 6. Effect of inactivator concentration in a time-course analysis of invertase I activity. Invertase I was added at T_0 . The reactions were stopped at 5-min intervals until 30 min. Reaction temperature was 37 C. Volume of reaction mixture was 0.155 ml containing: invertase I preparation from normal endosperms at 12-day stage, 2.65 μ g of protein; inactivator preparation from normal endosperms at 22-day stage, no inactivator (\circ), 5.40 μ g (\square), 2.70 μ g (Δ), or 1.35 μ g (\bullet) of protein; acetate buffer (pH 5.0), 12.9 mM; sucrose, 6.4 mM.

FIG. 7. Increase in inactivation of invertase I between two periods of preincubation at different inactivator concentrations. Invertase I was preincubated at 37 C for 30 min (\circ) or 60 min (Δ) with four concentrations of inactivator. Sucrose was added at T_0 . Reaction time was 15 min; reaction temperature was 37 C. Each point is a mean of two replications. Volume of reaction mixture was 0.15 ml containing: invertase I preparation from normal endosperms at 14-day stage, 4.7 μ g of protein; inactivator preparation from normal endosperms at 22-day stage; acetate buffer (pH 5.0), 40 mM (preincubation with one-sixth this amount; the rest was added at T_0); sucrose, 5.3 mM.

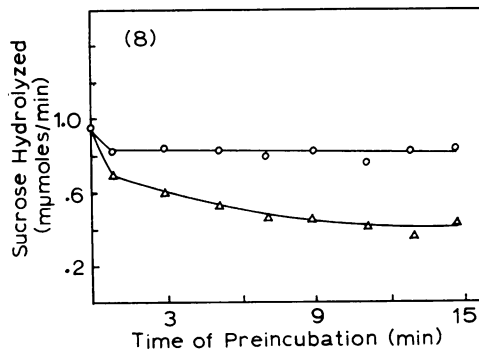


FIG. 8. Effect of preincubation temperature on inactivation of invertase I. Invertase I was preincubated at 0°C (○) or 37°C (△) with inactivator and buffer for several periods of time. Sucrose was added at T_0 . Reaction time was 15 min; reaction temperature was 37°C. Each point is a mean of two replications. Volume of reaction mixture was 0.16 ml containing: invertase I preparation from normal endosperms at 16-day stage, 4.3 µg of protein; inactivator preparation from $su_2/su_2/su_2$ endosperms at 22-day stage, 3.7 µg of protein; acetate buffer (pH 5.0), 28 mM; sucrose, 5.6 mM.

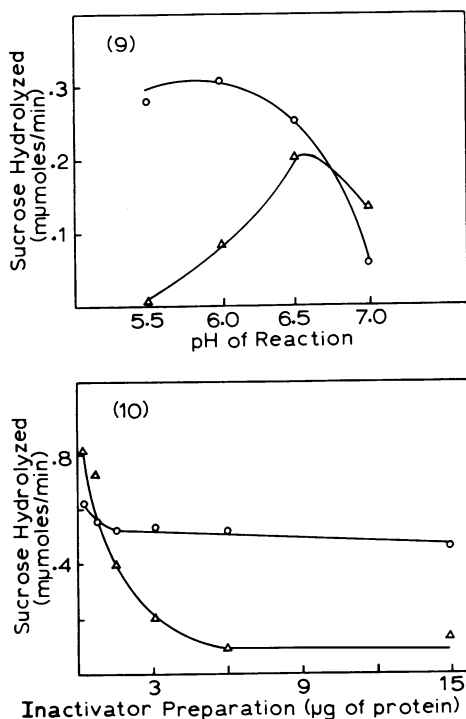


FIG. 9. Activity of invertase I assayed with (△) or without (○) the inactivator at different pH values. Reaction time was 30 min; reaction temperature was 37°C. Each point is a mean of two replications. Volume of reaction mixture was 0.19 ml containing: invertase I preparation from $sh_2/sh_2/sh_2$ endosperms at 22-day stage, 7.6 µg of protein; inactivator preparation from normal endosperms at 22-day stage, 6.0 µg of protein; MES buffer, 10.5 mM; sucrose, 5.3 mM.

FIG. 10. Effect of pH during reaction on the extent to which invertase I is inactivated. Invertase I was reacted with buffer, sucrose and several inactivator concentrations at a pH of 5.5 (△) or 6.5 (○). Reaction time was 30 min; reaction temperature was 37°C. Each point is a mean of two replications. Volume of reaction mixture was 0.22 ml containing: invertase I preparation from $sh_2/sh_2/sh_2$ endosperms at 22-day stage, 7.5 µg of protein; inactivator preparation from normal endosperms at 22-day stage; MES buffer, 9.1 mM; sucrose, 4.5 mM.

acetate buffer concentration and an increase in ionic strength by addition of NaCl or KCl decreased inactivation.

NaCl added during the extraction of inactivator and then removed with dialysis did not decrease recovery of the inactivator. Also, salt at a concentration which prevented inactivation during a reaction period did not reverse inactivation when added after invertase I was inactivated.

A somewhat confusing observation is the effect of inactivator concentration on the rate of inactivation. At higher levels of inactivation, the increase in inactivation between two periods of preincubation was independent of inactivator concentration. This is shown in the experiment of Figure 7 where invertase I was preincubated with inactivator for 30 and 60 min. The two curves are parallel. The same result was noted in experiments with higher inactivator concentrations but shorter periods of preincubation.

Effect of Temperature on Inactivation. The effect of temperature on inactivation was examined in one experiment by preincubating invertase I with inactivator for various periods of time at 0°C or 37°C (Fig. 8). Little invertase I was inacti-

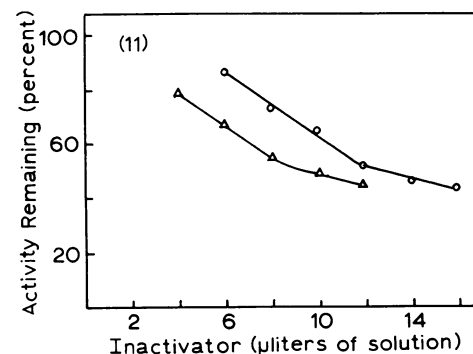


FIG. 11. Standard inactivation curve for the quantitative analysis of inactivator. The inactivator for the standard curve was from normal endosperms at 22-day stage (○). Also shown is a similar curve with the inactivator from $su_2/su_2/su_2$ endosperms at the 14-day stage (△). Reaction time was 15 min; reaction temperature was 37°C. Each point is a mean of four replications. Volume of reaction mixture was 0.147 ml containing: invertase I preparation from brittle-1 endosperms at the 10-day stage, 1.1 µg of protein; acetate buffer (pH 5.0), 13.6 mM; sucrose, 6.8 mM.

Table I. Amount of Inactivator in the Normal Endosperm and Three Endosperm Mutant Lines during Development

The amount is expressed as a percentage of inactivator per normal endosperm at the 22-day stage. Calculation of the amount is given in the text.

| Stage of Development | Inactivator/Endosperm | | | |
|----------------------|-----------------------------|------------------|------------------|------------------|
| | + / + / + | $sh_1/sh_1/sh_1$ | $sh_2/sh_2/sh_2$ | $su_2/su_2/su_2$ |
| days | % of normal at 22-day stage | | | |
| 12 | 8 | 11 | 13 | — |
| 14 | 30 | 24 | 27 | 51 |
| 16 | — | 25 | 68 | 73 |
| 18 | 62 | 63 | 91 | — |
| 20 | 68 | — | — | — |
| 22 | 100 | 103 | 125 | 74 |
| 24 | 50 | — | 108 | 96 |
| 26 | 47 | 78 | 125 | 74 |
| 28 | 67 | 74 | 80 | 61 |

vated during preincubation at 0 C. The effect of temperature was examined in another experiment using several temperatures of reaction. The difference in activity between a control mixture without inactivator and a mixture with it determined the effect of temperature just on inactivation. When inactivation is expressed as a percentage of the activity in the reaction mixture without inactivator, inactivation increased with an increase in temperature from 30 to 40 C. An increase from 50 to 55 C evidently denatured invertase I with or without the inactivator and the percentage of inactivation decreased.

Effect of pH on Inactivation. Invertase I was inactivated to a greater extent at a lower pH. Activity of invertase I assayed alone or with the inactivator in a typical experiment at four pH values is plotted in Figure 9. The pH optimum of invertase I in the presence of inactivator was shifted to a pH near 6.5 in the experiment.

The pH effect was further investigated by reacting invertase I at two pH values 5.5 and 6.5, with several inactivator concentrations (Fig. 10). Invertase I activity was less at pH 6.5 than pH 5.5 as shown by activity at the zero level of inactivator. The inactivator was more active at the lower pH, and the extent of inactivation, as shown by a high inactivator concentration, was greater at the lower pH.

Analysis of Inactivator during Endosperm Development. To analyze quantitatively for the inactivator during development, it was necessary to test if inactivation was additive. Inactivator preparations from two stages of endosperm development, containing different levels of inactivator, were combined. The amount of inactivation was intermediate between that in the preparations analyzed separately.

The amount of inactivator at different stages of development was expressed as a percentage of the inactivator per normal endosperm at the 22-day stage of development. Under conditions in which the analysis was made, inactivation of invertase I was linear with the amount of inactivator to approximately 45 percent of initial invertase I activity remaining. The amount of inactivator was calculated by locating the percentage invertase I activity remaining when assayed with an unknown amount of inactivator on a plot made with the inactivator from 22-day normal endosperms (Fig. 11). One standard invertase I preparation was used for this analysis because invertase I preparations even from the same sample of kernels did not always respond identically to the inactivator. In the analysis of the inactivator the threshold amount necessary for inactivation varied with the amount of inactivator in the preparation. This is demonstrated in another plot made with a 14-day $su_2/su_2/su_2$ preparation which was considered to have a higher concentration of inactivator. The slopes of the two inactivation curves were similar. The threshold amount for $su_2/su_2/su_2$ was less than that for the preparation from normal corn.

Table I summarizes quantitative analyses of the inactivator in normal corn and the endosperm mutant lines, sh_1/sh_1 , sh_2/sh_2 and su_2/su_2 . The endosperm 12 days after pollination had only about 10% of the inactivator present at the 22-day stage. The greatest amount was found in the 22- to 24-day stages. The decreased inactivator after this period paralleled the decrease in soluble protein. While the amount of inactivator was increasing during endosperm development of normal corn, invertase I activity was decreasing.

DISCUSSION

Many factors can affect the level of enzyme activity in the cell: rate of enzyme synthesis, availability of substrate, presence of inhibiting metabolites and proteins, the microenvironment

of the cell, as well as the degradation of the enzyme molecule. The means by which enzymes are specifically degraded are unknown. Some of the factors observed here which affect invertase I inactivation may be critical in detecting such mechanisms. These experiments have not determined how invertase I in corn endosperm is inactivated.

The inactivator is probably a protein. It is degraded by pronase, has a high molecular weight, is denatured in very dilute solutions at pH 5.0 by heat, and is precipitated by ammonium sulfate. This protein is not competing with sucrose for a binding site on the invertase I molecule because the K_m of invertase I does not change when assayed with the inactivator. Also, invertase I is inactivated during a preincubation period in the absence of sucrose. The potato invertase inhibitor functions noncompetitively (3).

The inactivator could function by enzymatically reacting with invertase I. As discussed by Holzer (1) an inactivator could hydrolyze certain peptide bonds or the bond to other chemical groups, *i. e.*, carbohydrates, attached to the molecule and necessary for maintaining its configuration. If the inactivator were acting catalytically, it would be difficult to explain why the rate of inactivation does not increase with an increase in inactivator concentration (Fig. 7). However, other proteins in either the inactivator or invertase I preparation may be influencing the increase in inactivation with time.

Other mechanisms of inactivation can be proposed. Some of the kinetics of invertase I inactivation are similar to those described during subunit association for alkaline phosphatase of *Escherichia coli* (7, 8). Unassociated phosphatase monomers are not enzymatically active. Association, observed through an increase in enzyme activity, increases with time, is temperature dependent and influenced by ionic strength, type of buffer, and presence of metal ions. The rapid association of monomers even in protein extracts points to a high degree of recognition between monomers. Similarly, invertase I from corn endosperms is readily inactivated in the crude homogenate.

Because inactivation decreases with an increase in the salt concentration, formation of ionic bonds may be important in inactivation. An increase in the salt concentration or inactivator concentration of the reaction mixture causes a decrease in the latent period. Some of the kinetics of inactivation, including the latent period, are not explained.

An inverse relationship is apparent between invertase I activity in normal corn during development and the level of inactivator. However, several observations suggest invertase I activity *in vivo* does not depend alone on the level of inactivator in the endosperm. Normal and $sh_2/sh_2/sh_2$ endosperms at the 22-day stage have similar amounts of inactivator, but there is a large difference in their invertase I activities. Also, when two endosperm samples of different invertase I activities and similar inactivator levels are coextracted, total activity decreases to that of the lower sample (2). This suggests other factors in the endosperm could be active in regulating the level of invertase I in the coextract. Possibly the balance between enzyme synthesis and degradation depends on the metabolic state of the cell which in the endosperm mutants is not normal.

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