Changes in Potato Tuber Invertase and Its Endogenous Inhibitor After Slicing, Including a Study of Assay Methods¹, ²

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Elmer E. Ewing, Maria Devlin,³ Deborah A. McNeill,⁴ Martha H. McAdoo,⁵ and Anne M. Hedges

Department of Vegetable Crops, Cornell University, Ithaca, New York 14853

ABSTRACT

The increase in the invertase activity of extracts from freshly cut potato (Solanum tuberosum L.) by “foaming,” caused by selective denaturation of an endogenous invertase inhibitor, did not occur in extracts made from thin disks 2 days after slicing. Rather, foaming such extracts decreased invertase activity. Apparently, the inhibitor disappeared after slicing, and the enzyme became more labile to foaming. Such disappearance of inhibitor could account for up to 15% of the dramatic increase in total invertase activity that had occurred within 2 days after slicing. The difference between extracts from 0-day and 2-day slices was mainly in the first of two peaks of invertase activity eluted from diethylaminoethyl-cellulose columns. This peak was increased by foaming 0-day extracts, but even when foamed was much smaller than in 2-day extracts. The apparent loss in inhibitor was not caused by a decreasing susceptibility of the enzyme to the inhibitor. Both the increase in total invertase activity and the apparent loss of inhibitor after slicing were partially blocked by actinomycin D and completely blocked by cycloheximide.

The presence of the inhibitor can lead to serious errors in the usual whole disk method of assay for invertase in slices. Ethyl acetate treatment reduces the solubility of the enzyme but does not inactivate the inhibitor.

Slices of storage tissue undergo manifold metabolic changes if kept moist and well aerated after cutting. Among these is a dramatic increase in invertase activity (1, 7, 21, 22). The increased activity may be the result of de novo synthesis of invertase inasmuch as it is inhibited by chemicals that interfere with RNA or protein synthesis (3, 7, 23) and the buoyant density of an invertase fraction from carrot disks was increased by incubation in deuterium oxide (24). On the other hand, Pressey has demonstrated the presence of an endogenous invertase inhibitor in potatoes (13, 14), red and sugar beets (15), and sweet potatoes (15). He pointed out (15) that there might be an inactivation of the inhibitor after slicing which could account for at least part of the increase in invertase activity.

We wanted to investigate Pressey’s suggestion but found it necessary first to consider the effects of various assay procedures on the apparent activity of the enzyme and inhibitor. The most common method of assaying invertase in slices has been to place the slices into the assay medium, usually after treatment with ethyl acetate (1). Vaughan and MacDonald (21) compared invertase assays of intact slices, of slices treated with ethyl acetate, and of soluble and insoluble fractions obtained by homogenization. The activity of the insoluble invertase was similar to that of untreated whole disks, whereas ethyl acetate-treated disks gave higher values, approximating the sum of the soluble and insoluble reaction rates. They concluded that ethyl acetate rendered the soluble enzyme within the cell accessible to the sucrose of the assay medium. The same authors stated (22) that freezing and thawing beet slices prior to assay gave results similar to ethyl acetate treatment.

A serious drawback to the whole disk method of assay is the difficulty of estimating how much the inhibitor is reducing the total invertase activity. To date, the only valid test for the presence of the endogenous inhibitor is “foaming” (9, 13, 17). The inhibitor, which is probably a protein (14), can be selectively inactivated through surface denaturation if conditions of foaming are carefully controlled (9). The foaming procedure did not seem adaptable to the whole disk method of assay; furthermore, we did not know what effect the ethyl acetate treatment would have on the inhibitor. The procedures used in previous inhibitor studies (13, 17), namely homogenization of the tissue and assay of the soluble invertase before and after foaming, seemed questionable for tuber slices because of evidence that the solubility of invertase in slices changes after cutting (21). Therefore, we began our study with an examination of the effects of ethyl acetate treatment and various other assay procedures on slices assayed at intervals after cutting.

In the course of examining the assay procedures, it appeared that there were qualitative as well as quantitative changes in the invertase activity with time after slicing. We report our preliminary investigation of the nature of these qualitative changes. As to the original question concerning inactivation of inhibitor as an explanation for increasing enzyme activity, both our experiments on assay techniques and our experiments on changes in the nature of the invertase show that although the endogenous inhibitor disappears after slicing, such disappearance can account for only a fraction of the increase in invertase activity.

MATERIALS AND METHODS

The potato (Solanum tuberosum L.) cultivar ‘Katahdin’ was used in all experiments except the one summarized in Table II, where ‘Sebago’ was substituted. Using aseptic procedures, a core 2 cm in diameter was cut out along the axis of a tuber, from apical to basal end. Disks 0.75 mm thick were sliced from the core, omitting the ends. All slices in a given replication of an experiment were cut successively from the same tuber core, and treatments within each replication were randomized. The slices

¹ Supported by Hatch funds granted to Cornell University.
² Paper 678 of the Department of Vegetable Crops, Cornell University.
³ Present address: Department of Immunology, Rush-Presbyterian-St. Luke’s Medical Center, Chicago, Ill. 60612.
⁴ Present address: 1613 E. Genesee St., Syracuse, N.Y. 13201.
⁵ Present address: 62 Le Brun Court, Galveston, Tex. 77550.
were placed on moist filter paper supported by glass beads in Petri dishes (4) containing distilled H2O. The incubation temperature was 23 C. Checks for bacterial contamination were made by placing slices in Bacto nutrient broth.

Invertase assays of homogenates were carried out according to Pressey (13), with slight modification (9). Slices were homogenized by grinding in a mortar and pestle with a small volume of 0.8 m sodium sulfite (pH 6 and ionic strength approximately 1); the homogenate was buffered with 0.1 m sodium acetate (pH 4.75) and centrifuged. The precipitate was washed once with the acetate buffer, and the washings were added to the supernatant and dialyzed against 0.2 m NaCl to remove reducing sugars. Aliquots of the dialyzed solution were taken for assay. The precipitate was washed two more times and was treated 15 min with ethyl acetate, washed twice with ice water, and transferred to the sucrose assay medium. Foaming of extracts to inactivate the inhibitor was accomplished by bubbling N2 through the extracts (approximately 4 bubbles/sec) at room temperature (9). All other steps prior to the assay were carried out at about 3 C. Ethyl acetate assays were according to Bacon et al. (1). Disks were immersed 15 min in ethyl acetate that was cooled in ice, rinsed three times with ice water, and then placed in assay medium.

DEAE-cellulose chromatography of centrifuged homogenates was slightly modified from Sasaki et al. (19). There was no ammonium sulfate precipitation prior to chromatography, the column bed was 90 cm x 1.5 cm, and the NaCl elution step at the end of the pH gradient was omitted. There was good recovery of invertase activity applied to the column and no indication that part of the enzyme adhered, as reported by Sasaki et al. (19). Km values (10) for fractions eluted from the columns are means of four replications.

The assay medium—after addition of either extracts, cellular debris from homogenization, or whole slices—was 0.25 m with respect to sucrose and 0.1 m with respect to acetate buffer (pH 4.75). All assays were performed at 37 C for 1 hr, and reactions were stopped with the copper reagent used for the reducing sugars determination (20). Boiled blanks served as controls in all assays and gave values very close to those obtained from reagent blanks. Leaching of reducing sugars from ethyl acetate-treated tissue during assay (24) was not a factor. Invertase activities are expressed as µmol of reducing sugar formed/hr · g of original fresh wt of disks. Weights of disks are based on weighings made on similar disks at time of slicing.

RESULTS

First we examined three methods of treating disks prior to whole disk assay. One day after cutting, potato disks were either dipped in ethyl acetate, frozen by immersing the container in dry ice and acetone, or frozen and then lyophilized. Next the disks were rinsed in ice water and were assayed for invertase. The ethyl acetate, freezing, and lyophilization treatments resulted in activities of 23.1, 25.1, and 23.0 µmol reducing sugar/hr · g fresh wt, respectively. These values were not significantly different (LSD0.05 = 4.4).

The three methods also gave comparable values when used on freshly sliced disks or on disks taken 2 days after slicing (approximately 4 and 40 µmol reducing sugar/hr · g, respectively, by all three methods). However, examination of the ice water used to rinse the disks prior to placing them in the assay medium indicated that both invertase and inhibitor had been leached from the disks (Table I). The amount leached was greatest in the dialyzed rinse water from lyophilized slices and least in that from the ethyl acetate treatment, especially in the disks taken 2 days after slicing. Another difference apparent in Table I is that the invertase activity in the rinse water from fresh slices increased upon foaming, suggesting that the inhibitor was present; whereas foaming did not increase the activity in rinse water from slices which had been cut for 2 days, suggesting the absence of inhibitor.

Next we compared the ethyl acetate whole slice assay with an assay of centrifuged homogenate prepared from slices. Again, we tested for invertase activity in the dialyzed rinse water obtained from the ethyl acetate-treated disks. To see whether all of the enzyme was extracted by homogenization, we centrifuged the homogenate, washed the cellular debris, treated the debris with ethyl acetate, rinsed with ice water, and transferred the debris to buffered sucrose medium for invertase assay. Results are presented in Table II. By either assay method, invertase activity increased greatly during a 2-day incubation period. As in the previous experiment, there was evidence of both invertase and inhibitor in the ice water rinse of the disks that were treated with ethyl acetate immediately after slicing; but again there was less enzyme and no detectable inhibitor in the rinses from the slices cut 48 hr before assay. Responses of the homogenate were similar to those of the ice water rinses: foaming greatly increased the invertase activity of supernatants from fresh slices but had no significant effect on supernatants from slices homogenized 2

| Table I. Invertase activity of leachates from disks treated by three methods, immediately or 2 days after slicing. |
|---|---|---|
| Treatment | Time foamed | Days after slicing |
| | min | wmoles per hr/g fr wt |
| Ethyl acetate | 0 | 0.28 | 0.23 |
| | 10 | 0.46 | 0.18 |
| | 20 | 0.53 | 0.11 |
| Pressing | 0 | 0.63 | 2.38 |
| | 10 | 1.28 | 2.26 |
| | 20 | 1.29 | 2.13 |
| Pressing + lyophilization | 0 | 0.33 | 3.34 |
| | 10 | 2.06 | 3.15 |
| | 20 | 2.00 | 3.30 |

| Table II. Invertase activity of ethyl acetate-treated whole disks, leachates from treated disks, centrifuged homogenate of untreated disks, and ethyl acetate-treated debris from homogenization. |
|---|---|---|
| Material assayed | Days after slicing | wmoles per hr/g fr wt |
| Whole disks, ethyl acetate-treated | 0.54 ± 0.06 | 43.81 ± 3.15 |
| Leachate from treated disks | 0.07 ± 7.12 | 0.05 ± 0.12 |
| Not foamed | 0.27 ± 0.10 | 0.10 ± 0.06 |
| Foamed 10 min | 0.52 ± 0.10 | 0.12 ± 0.06 |
| Foamed 20 min | 0.52 ± 0.10 | 0.12 ± 0.06 |
| Supernatant from untreated disks | 0.28 ± 0.02 | 38.53 ± 4.88 |
| Not foamed | 1.04 ± 0.59 | 38.37 ± 4.97 |
| Foamed 10 min | 2.03 ± 0.53 | 40.04 ± 5.15 |
| Foamed 20 min | 2.03 ± 0.53 | 40.04 ± 5.15 |
| Debris | 0.16 ± 0.03 | 4.05 ± 0.69 |

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purified invertase and inhibitor from tubers as previously described (9). We also prepared supernatant from disks 2 days after slicing. We took aliquots of the partially purified invertase and of the extract from 2-day slices such that each had approximately the same invertase activity, diluted the aliquots to a standard volume, and added inhibitor to half of the aliquots from each source. After preincubation (9) for 3 days at 2°C under toluene, we assayed for invertase activity. There were five replications. The inhibitor reduced the activity of the invertase from 2-day slices by 356 (from 697 to 341), and that from whole tubers by 345 (from 811 to 466) nmol reducing sugar/hr. The interaction was not significantly different at the 5% level; i.e., there was no evidence that the inhibitor was more effective in one set than in the other.

Another possible explanation is that the invertase produced during the 2 days after cutting is mainly bacterial and therefore differs in lability to foaming. Although aseptic technique was employed throughout, in some batches of tubers it was difficult to maintain complete freedom from bacteria; and in these cases up to 25% of freshly cut slices developed colonies when placed in nutrient broth. To examine the effect of contamination, we cut disks in half 2 days after slicing, placed one half of each disk in nutrient broth, treated the other half with ethyl acetate, and assayed for invertase. There was no significant difference at the 5% probability level between the activities of halves matching those which showed contamination and those matching halves which did not. Slices from other batches of tubers remained completely free from contamination, and these slices underwent the same increases in total invertase activity and in enzyme lability to foaming.

The increased lability to foaming of invertase from 2-day slices suggested a change in the form of invertase. We therefore subjected centrifuged homogenates from 0-day and 2-day slices to chromatography on DEAE-cellulose columns (Fig. 1). Before chromatography, half of the 0-day homogenate was foamed by stirring 5 min in a VirTis blender set on medium speed. In most replications there were two peaks from all three kinds of sample. The first of these peaks eluted with the void volume. It was small or in some replications completely missing in 0-day homogenates that had not been foamed (Fig. 1a). The fractions collected from this elution zone showed much higher invertase activity after we bubbled N₂ through them, demonstrating that the enzyme had been contaminated with the inhibitor. Foaming the 0-day homogenate prior to chromatography greatly increased the size of the first peak but had no effect on the second peak (Fig. 1b). Chromatography of the unfoamed extract from the 2-day slices gave 95.2% ± 2.8% (mean of five determinations ± sp) of the invertase activity in the first peak (Fig. 1c), and foaming the eluate from the first peak decreased the level of invertase activity.

The Km for invertase activity in the first peak from the 2-day extract was not different from that for the first peak in the 0-day extract (foamed). Both values were 4 mm sucrose.

Matsushita and Uritani (12) showed that acid invertase activity is essentially absent in sweet potato roots, although it rapidly builds up after slicing. Invertase activity at pH 8 was present at slicing and did not increase (12). We found that potato disks assayed after ethyl acetate treatment showed optimum invertase activity at pH 4.75, with a secondary peak in activity between pH 6 and 7, whether assays were made immediately or 2 days after cutting (data not shown). The activities of fresh and 2-day slices assayed at pH 4.75 weased at 35 and 35 μmol/hr·g, respectively, compared to 0.38 and 6.42 μmol/hr·g assayed at pH 7. Tuber homogenates also have an optimum at 4.75, whether or not they have been foamed to destroy the inhibitor. The foaming response at a given pH is roughly proportional to the activity before foaming (Ewing and McNeill, unpublished data).

It is known that inhibitors of RNA synthesis or protein synthe-
sis will block the increase in invertase activity that follows cutting. Figure 2 shows the effects of actinomycin D concentration on invertase activity 2 days after slicing as assayed by the ethyl acetate-treated disk method. Based upon these results, we compared invertase activities of centrifuged homogenates from disks placed in water or in 20 μg/ml of actinomycin D for 0, 7, 24, or 48 hr after slicing. We also examined the response to foaming the supernatant from each treatment (Fig. 3). In the absence of actinomycin D, the expected results were obtained: the invertase activity of the supernatant increased with time after slicing, and the response to foaming decreased with time to the point where it became negative. The actinomycin D partially inhibited both trends.

Cycloheximide, which interferes with protein synthesis and may have other effects as well (8), produced more striking results than did actinomycin D. A preliminary concentration curve showed that 10 μM cycloheximide would stop the increase in invertase activity with time after cutting as assayed by the ethyl acetate-treated disk method. Figure 4 shows that this concentration of cycloheximide eliminated any changes with time, either in invertase activity of supernatants or in foaming response. Supernatants obtained from disks treated with cycloheximide for 48 hr had essentially the same invertase activity as supernatants from freshly sliced disks. Whereas foaming normally decreases the invertase activity of supernatants from 2-day slices, when such slices had been treated with cycloheximide the increase in invertase activity produced by foaming was equal to that produced in supernatants from fresh slices.
DISCUSSION

The well known increase in invertase activity after slicing could be accounted for by a very great increase in the activity of the enzyme that eluted just after the void volume from DEAE-cellulose columns. In contrast, the activity of later eluting invertase was similar whether extracts were made from fresh slices or from slices that were 2 days old. Foaming extracts from freshly cut slices either before or after column chromatography showed that the enzyme was present in the first peak, but that it was inactivated by the endogenous inhibitor. Our extracts from fresh slices gave different DEAE elution patterns than previously published profiles for tuber extracts (19). One explanation is that our tubers were dormant and had not been exposed to low temperatures, whereas Sasaki et al. (19) used sprouted tubers that were stored at 4°C for 3 weeks before extracting. Even so, it is surprising that we found that foaming increased activity in the first peak rather than in the second peak as reported by Sasaki et al. (19).

Foaming did not increase invertase activity of extracts made 2 days after slicing. On the contrary, the new invertase activity was highly labile to foaming. This suggested that it might represent a new form of the enzyme. Both extracts showed the same pH optima with respect to invertase activity; added inhibitor was equally effective in reducing the invertase activity of each; and the first peak eluted from DEAE-cellulose columns for the 2-day extract did not differ in Km from the equivalent peak in the 0-day (foamed) extract. Thus it is not clear whether a new form of invertase is produced after slicing, or whether there is some other explanation for the increased lability of the enzyme.

The disappearance of inhibitor, as monitored by response to foaming, with time after slicing seemed to be complete by the end of two days. This disappearance was blocked partially by actinomycin D at the concentration used and was blocked completely by cycloheximide. The fact that these chemicals reduced the disappearance of the inhibitor shows that it was not lost through leaching. It would seem that the decrease in endogenous inhibitor is yet another manifestation of the many metabolic changes set in motion by cutting the tuber and exposing the slices to a moist, aerated environment.

The portion of increased invertase activity attributable to inhibitor loss ranged from about 5% (calculated from the data in Table II) to about 15% (from Fig. 3). There is evidence (16, 17) that the amount of inhibitor present in potatoes varies with cultivar, storage temperature, and length of storage; and we found that with different batches of tubers the activity of ethyl acetate-treated, freshly sliced disks ranged from approximately 0.1 to 4 μmol reducing sugar/hr-g. Presumably much of this variation reflected variations in inhibitor content. Thus a considerable range is to be expected in the contribution of inhibitor decline to increased invertase activity. Because Pressley (15) found the inhibitor also in other storage tissues, it is reasonable to assume that the phenomenon may not be restricted to potatoes. The fact that the invertase activity for freshly cut, whole disks was much closer to the sum of the values for unfoamed supernatant plus debris than it was to the value for foamed supernatant plus debris (Tables II and III) indicates that the inhibitor and enzyme are present together in the whole slice and that the inhibitor is not an artifact of extraction.

The only known method for measuring "total" invertase activity when a tuber is present is to inactivate the inhibitor by boiling. Other methods used in the past are inappropriate because the inhibitor has a very high affinity for the enzyme (9). Foaming does not, as has been suggested (19), increase enzyme activity directly by unfolding the enzyme molecule rather than by inactivating the inhibitor, since foaming the partially purified inhibitor before it has been added to the enzyme renders the inhibitor ineffective (9).

We are unable to recommend any single assay method as best for invertase in potato slices. Ethyl acetate reduces the solubility of the enzyme and hence the leaching which occurs during rinsing. Because ethyl acetate does not inactivate the endogenous inhibitor, and because only a small portion of the inhibitor is leached out during the ice water rinse, apparent levels of invertase obtained by the ethyl acetate assay of freshly cut slices are less than the levels obtained by foaming homogenate and assaying. On the other hand, homogenization and extraction procedures, when taken alone, failed to account for the insoluble form of the enzyme. Here it may be noted that the pH and ionic concentration of the extraction medium may have a marked effect on the solubility of invertase (11, 18), but it is unlikely that any extraction procedure would render soluble all of the invertase in slices 2 days after cutting (5, 22). The ionic strength of our extraction medium was approximately 1, which was found to be optimal by Little and Edelman (11). Further increases in ionic strength and manipulation of pH (4.7, 6, and 7) of the extracting medium did not increase the invertase activity obtained from 2-day slices.

On balance, a reasonable compromise is to homogenize the tissue, centrifuge and wash the precipitate, and save both fractions. The supernatant plus washes, after dialysis to remove reducing sugars, may be divided into aliquots that are foamed for various intervals and then assayed to establish maximum activity. The precipitate may be treated with ethyl acetate, washed with ice water, and placed directly into the sucrose assay medium. Total invertase activity can then be determined as the sum of that obtained from the foamed extract and from the ethyl acetate-treated precipitate.

LITERATURE CITED