Invertase Inhibitors From Red Beet, Sugar Beet, and Sweet Potato Roots

2940

By

Russell Pressey

Red River Valley Potato Processing Laboratory[1], East Grand Forks, Minnesota 56721

Invertase Inhibitors From Red Beet, Sugar Beet, and Sweet Potato Roots

Russell Pressey

Red River Valley Potato Processing Laboratory[1], East Grand Forks, Minnesota 56721

Received June 24, 1968.

Abstract. **Invertase inhibitors have been isolated and partially purified from red beets, sugar beets, and sweet potatoes. These inhibitors are thermolabile proteins with molecular weights of 18,000 to 23,000. They do not inhibit yeast and *Neurospora* invertases, but they are reactive with potato tuber invertase and other plant invertases with pH optima near 4.5. There are differences in reactivity of the inhibitors with some of the plant invertases, however. For most invertases, red beet and sugar beet inhibitors are most effective at pH 4.5 while sweet potato inhibitor is most effective at pH 5.**

The first protein inhibitor of invertase was isolated from potato tubers (3, 5). This inhibitor is effective for not only potato tuber invertase but also many other plant invertases with pH optima near 4.5 (5). The wide reactivity of the potato inhibitor indicates similarities in invertases from diverse plant sources. By analogy, if invertase inhibitors exist in other plants, it is probable that some of them are also similar and their reactivities are not limited to their natural invertases. It should then be possible to detect invertase inhibitors by assaying with any one of the invertases reactive with potato inhibitor. Using potato tuber invertase as the assay enzyme, this approach has led to the discovery of invertase inhibitors in red beet, sugar beet, and sweet potato roots.

This paper describes the isolation and partial purification of the 3 new invertase inhibitors. Their properties are compared with those of potato invertase inhibitor.

Materials and Methods

The red beets and sweet potatoes were obtained at local supermarkets during January, 1968. The sugar beets were provided by the American Crystal Sugar Company, East Grand Forks, Minnesota and had been stored in their root cellar for 5 months. Extracts of all 3 tissues were prepared the same way. The roots were washed, peeled, and passed through a juicerator (Acme Manufacturing Company, Lemoyne, Pennsylvania). A ml of 0.8 M sodium sulfite, pH 6, was added to each 100 ml of juice to prevent enzymatic darkening. After cooling to 0°, the extracts were clarified by centrifugation at 8500*g* for 20 minutes and dialyzed against 0.2 M NaCl. All of the purification steps were carried out at 4°, unless indicated otherwise.

Protein was determined by the biuret method and absorption at 280 mμ (2). The inhibitors were assayed according to the procedure described earlier (5) using partially purified potato tuber invertase. A unit of inhibitor is defined as that amount which inhibits one unit of potato invertase 50 % at pH 4.5.

Extracts of leaves were prepared as described earlier (5).

Purification of Red Beet Invertase Inhibitor. Acetone (160 ml) was added to 320 ml of dialyzed red beet juice while cooling to −10°. The precipitate was removed by centrifugation and 160 ml of acetone was added to the supernatant while cooling to −14°. The precipitate was collected and dissolved in 0.2 M NaCl (acetone fraction). The acetone fraction was then applied in 10 ml aliquots to a 2.5 × 95 cm column of Sephadex G-100, previously washed with 0.2 M NaCl. Elution was made with 0.2 M NaCl and 12 ml fractions were collected. Only a single fraction with the highest specific activity was saved from each of the 3 runs (Sephadex G-100 fraction). A summary of the purification is presented in table I.

Purification of Sugar Beet Invertase Inhibitor. The dialyzed sugar beet juice was adjusted to pH 1.5 with 4 N HCl. The insoluble material was removed by centrifugation and the supernatant was adjusted to pH 5.5 with 4 N NaOH (pH 1.5 fraction). The protein in the solution was concentrated before beginning the acetone fractionation. Solid ammonium sulfate was added to 80 % of saturation, the precipitate was collected, dissolved in 150 ml of 0.2 M NaCl, and the solution was dialyzed against 0.2 M NaCl. Acetone (75 ml) was then added while cooling to −10°. After centrifuging to remove the precipitate, 85 ml of acetone was added to the

[1] A laboratory cooperatively operated by the Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture; Minnesota Agricultural Experiment Station; North Dakota Agricultural Experiment Station; and the Red River Valley Potato Growers' Association.

Table I. *Purification of Red Beet Invertase Inhibitor*

Fraction	Vol	Protein	Sp. act.	Yield
	ml	*mg*	*units/mg protein*	%
Crude extract	320	550	30	100
Acetone	30	176	72	76
Sephadex G-100	36	4.7	810	23

supernatant while cooling to −14°. The precipitate was collected and taken up in 0.2 M NaCl (acetone fraction). This solution was applied in 10 ml aliquots to a 2.5 × 95 cm column of Sephadex G-100 as described for red beet inhibitor. The best single fraction was saved from each of the 5 runs (Sephadex G-100 fraction). A summary of the purification is presented in table II.

Table II. *Purification of Sugar Beet Invertase Inhibitor*

Fraction	Vol	Protein	Sp. act.	Yield
	ml	*mg*	*units/mg protein*	%
Crude extract	1000	1100	10.5	100
pH 1.5	1020	765	23	153
Acetone	50	121	98	102
Sephadex G-100	60	4.8	1040	44

Purification of Sweet Potato Invertase Inhibitor. The pH of the dialyzed sweet potato juice was adjusted to 1.5 with 4 N HCl. A small amount of precipitate formed and was removed by centrifugation. The supernatant (pH 1.5 fraction) was adjusted to pH 5.5 with 4 N NaOH and fractionation was performed with ammonium sulfate. The precipitate obtained between 15 and 35 % of saturation was collected and dissolved in 0.2 M acetate, pH 6 (ammonium sulfate fraction). After dialysis of this solution against 0.2 M NaCl, it was treated with 2 g solids of alumina Cγ gel. The gel was collected by centrifugation, the inhibitor was eluted with 30 ml of 0.2 M phosphate buffer, pH 6, containing 0.5 M NaCl, and the eluate was dialyzed against 0.2 M NaCl (alumina Cγ gel fraction). Acetone was added to the dialyzed solution to a concentration of 47 % while cooling to −12°. After the precipitate was removed, the acetone concentration was increased to 56 % at −15°. The precipitate was collected and taken up in 150 ml of 0.2 M NaCl (acetone fraction). A summary of the purification is presented in table III. Because the crude extract was turbid, it was not possible to determine its protein content by the biuret method. The yields of inhibitor, therefore, are based on the inhibitor level in the pH 1.5 fraction.

Table III. *Purification of Sweet Potato Invertase Inhibitor*

Fraction	Vol	Protein	Sp. act.	Yield
	ml	*mg*	*units/mg protein*	%
Crude extract	710	...	...	...
pH 1.5	720	8400	18	100
Ammonium sulfate	355	6600	22	96
Alumina Cγ gel	340	3700	38	93
Acetone	150	1800	60	71

Results

Purification of the Inhibitors. High specific activities of red beet and sugar beet inhibitors were obtained with relative ease. Filtration through Sephadex G-100 is an effective step in the purifications of both inhibitors and typical elution patterns are shown in figure 1. Further purifications of these inhibitors were not attempted.

The amount of inhibitor in crude extracts of sugar beets was increased about 50 % by the acid treatment. This effect was obtained between pH 1.0 and 2.2 at 4° during the 20 minutes of centrifugation following acidification. Longer periods at low pH and warmer temperatures did not produce higher levels of inhibitor. A possible explanation for the increased level of inhibitor is that dissociation of an inactive complex consisting of the inhibitor and the

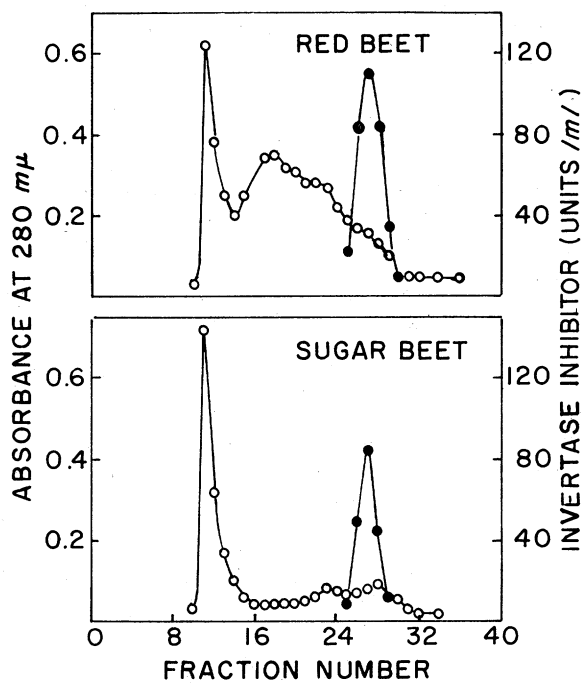

FIG. 1. The elution patterns from a Sephadex G-100 column of the acetone fractions of red beet and sugar beet inhibitors. Protein was measured by absorption at 280 mμ and the inhibitors were measured by the standard assay. Experimental conditions are described in the text. ○——○, absorbance at 280 mμ; ●——●, invertase inhibitor (units/ml). Other peaks of inhibitor activity were not detected.

enzyme invertase occurs during the acid treatment. Some of the enzyme is either inactivated or removed in the precipitate that forms, resulting in a greater amount of excess inhibitor.

Only a 3-fold increase in the specific activity of sweet potato inhibitor was obtained. Chromatography of the acetone fraction on Sephadex G-100 yielded essentially a single protein peak which coincided with the inhibitor activity.

Stability. The partially purified inhibitors are stable at 4° for at least 2 months and at 37° for at least 2 hours. At higher temperatures, sweet potato inhibitor is the most stable and it possesses maximum heat stability between pH 4.5 and 6.5. At pH 6, 50 % of the inhibitor is inactivated by heating to 75° for 5 minutes. Red beet and sugar beet inhibitors possess maximum heat stabilities at about pH 4 and 50 % of their activities are lost by heating to 60° for 5 minutes.

Inactivation by Trypsin. About 6 units of each inhibitor were incubated for 8 hours with 1 mg of trypsin (Sigma Chemical Company, St. Louis) at pH 7 and 25°. Aliquots were withdrawn, treated with an excess of trypsin inhibitor, and assayed for residual invertase inhibitor. All of the inhibitors were slowly but completely inactivated by trypsin.

Molecular Weights. The molecular weights of the inhibitors were estimated by comparing their elution volumes from a 2.5 × 95 cm column of Sephadex G-100 with those of 3 proteins of known molecular weights, according to the method of Whitaker (8). The column was washed and eluted with 0.2 M NaCl. The reference proteins, cytochrome *c*, ovalbumin, and horse serum albumin, were assayed by absorption at 280 mμ and the inhibitors were assayed by the standard method using potato invertase. The elution volumes were: cytochrome *c*, 351 ml; ovalbumin, 246 ml; horse serum albumin, 207 ml; red beet inhibitor, 324 ml; sugar beet inhibitor, 323 ml; and sweet potato inhibitor, 303 ml. From these data, the molecular weights are: red beet inhibitor, 17,800; sugar beet inhibitor, 18,100; and sweet potato inhibitor, 22,900 (using molecular weights of 13,000 for cytochrome *c*, 45,000 for ovalbumin, and 70,000 for horse serum albumin).

Reactivity With Potato Invertase. Red beet inhibitor is similar to potato invertase inhibitor in its action on potato tuber invertase. At pH 4.5, potato invertase is completely inhibited by both of these inhibitors at about 5 units inhibitor/unit enzyme (fig 2). Sugar beet and sweet potato inhibitors readily inhibit about 90 % of the potato invertase but the remaining activity is inhibited only by excessive levels of these inhibitors.

The effects of the inhibitors on the pH optimum for potato invertase are shown in figure 3. Low concentrations of inhibitors broaden the pH optimum. At higher concentrations of potato, red beet, and sugar beet inhibitors, inhibition is greatest at about pH 4.5, resulting in 2 peaks of residual invertase activity. The sweet potato inhibitor differs somewhat from the other inhibitors in its effect on potato invertase. It is most effective at pH 5 and the residual invertase activity is much lower on the alkaline than acid side of pH 5.

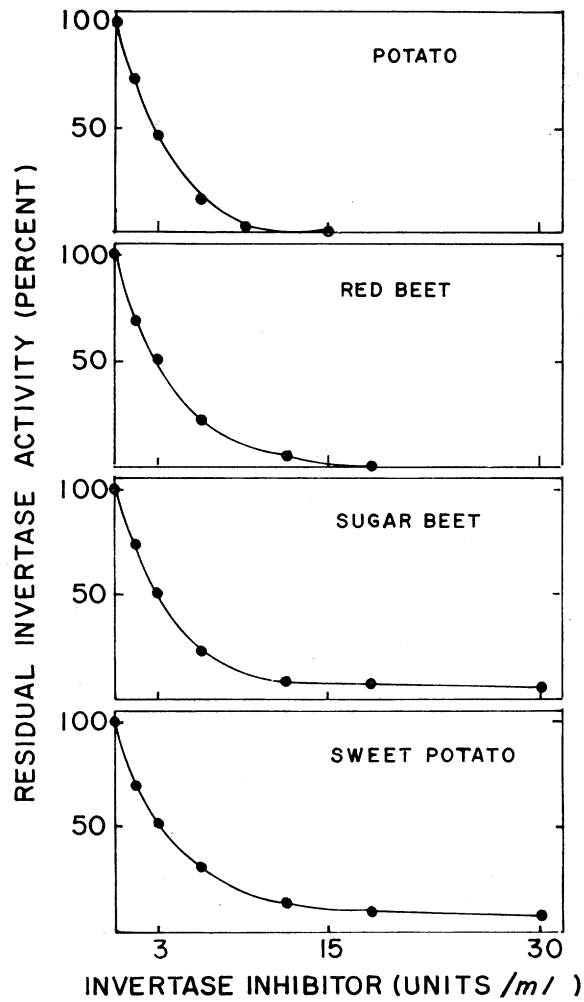

FIG. 2. The effects of increasing concentrations of potato, red beet, sugar beet, and sweet potato inhibitors on potato tuber invertase. The reaction mixtures contained 3 units invertase/ml in 0.04 M acetate buffer, pH 4.5, and were incubated for 1 hour at 37°.

Reactivity With Potato Leaf Invertase. All of the inhibitors inhibit about 50 % of the total invertase activity in extracts of potato leaves (fig 4). The inhibitors are equally effective on this invertase and low levels are required to attain maximum inhibition. It is clear that extracts of potato leaves contain at least 2 invertases, 1 of which is reactive with all 4 invertase inhibitors.

Reactivity With Red Beet Leaf Invertase. Red beet and sugar beet inhibitors are equally effective for red beet leaf invertase and they completely inhibit this enzyme. Potato and sweet potato inhibitors are less effective and do not inhibit about 20 % of the total activity. Red beet leaf invertase

possesses a pH optimum at 4.7. Potato, red beet, and sugar beet inhibitors inhibit this invertase most effectively at about pH 4.2. As in the case of potato invertase, double peaks of residual activity are obtained at moderate levels of inhibitors. The peaks of residual activity are larger above than below pH 4.2 for red beet and sugar beet inhibitors. In contrast, sweet potato inhibitor is most effective for this invertase at pH 5 and the residual activity above pH 5 is low.

Reactivity With Sweet Potato Leaf Invertase. Sweet potato inhibitor is the only 1 of the 4 inhibitors that is reactive with the invertase in extracts of sweet potato leaves, but it inhibits only about

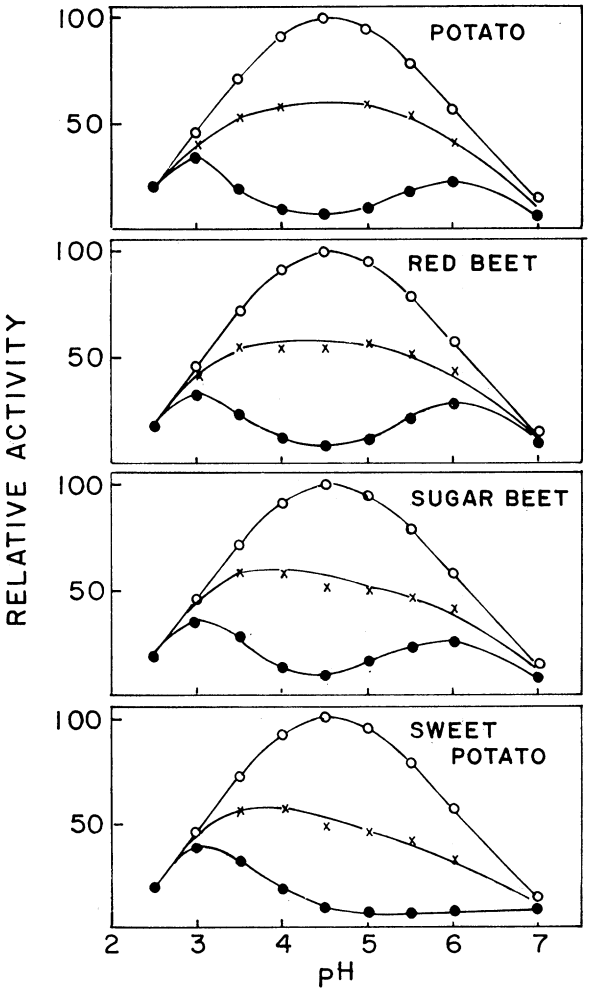

FIG. 3. Effects of potato, red beet, sugar beet, and sweet potato inhibitors on the pH optimum for potato tuber invertase. The reaction mixtures contained 3 units invertase/ml in 0.04 M citrate-phosphate buffers and were incubated 1 hour at 37°. ○——○, no inhibitor; X——X, 2.5 units of each inhibitor/ml; ●——●, 8 units potato inhibitor/ml, 10 units red beet inhibitor/ml, 12 units sugar beet inhibitor/ml, and 25 units sweet potato inhibitor/ml.

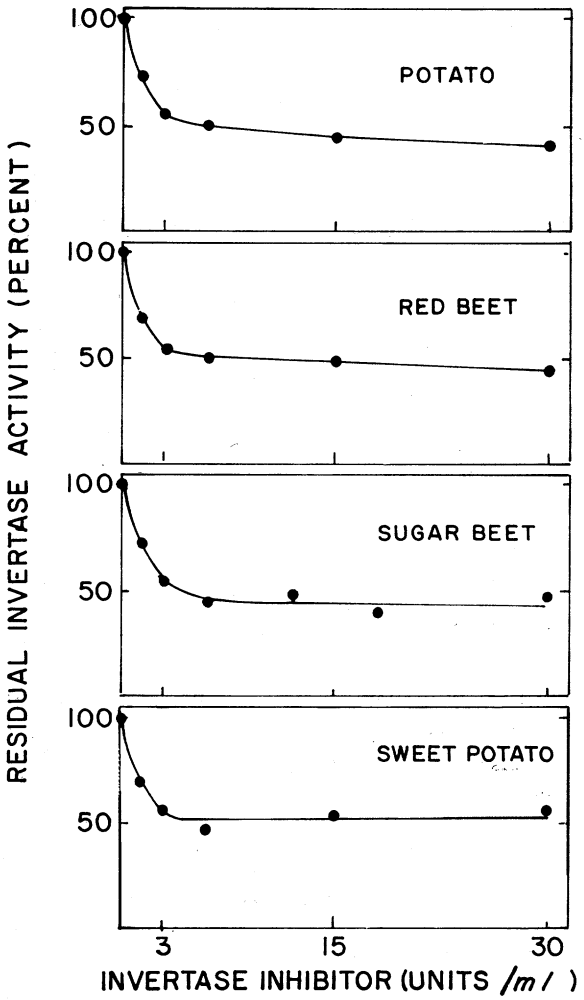

FIG. 4. The effects of increasing concentrations of potato, red beet, sugar beet, and sweet potato inhibitors on potato leaf invertase. The reaction mixtures contained 3 units invertase/ml in 0.04 M acetate buffer, pH 4.5, and were incubated 1 hour at 37°.

50 % of the total activity. About 10 units of inhibitor are required to inhibit 1 unit of invertase 50 %.

Reactivity With Other Invertases. None of the inhibitors inhibit invertases from yeast and *Neurospora.*

Discussion

The properties of invertase inhibitors from potatoes, red beets, sugar beets, and sweet potatoes show similarities but also some differences. They are proteins with low molecular weights; the molecular weight of sweet potato inhibitor is somewhat greater than that of the other inhibitors. Potato, red beet, and sugar beet inhibitors are most effective for reactive invertases near pH 4.5, while sweet potato inhibitor is most effective at pH 5. At moderate

levels of inhibitors, potato tuber and red beet leaf invertases exhibit double pH optima of residual activity. However, the peaks above pH 5 are small in the presence of sweet potato inhibitor. All of the inhibitors inhibit about half of the invertase activity in extracts of potato leaves. Sweet potato inhibitor is the most stable to inactivation by heat and it is the only inhibitor that inhibits sweet potato leaf invertase. This inhibitor therefore differs from the others in a number of ways.

Whereas the potato inhibitor regulates invertase activity in potato tubers (4), it is not certain whether the inhibitors isolated from red beets, sugar beets, and sweet potatoes are involved in similar roles in their respective tissues. Invertases from these sources are not available to determine their reactivities with the corresponding inhibitors. However, there is indirect evidence that these proteins function as invertase inhibitors. First, because the inhibitors are effective for invertases from at least several plant sources, it is likely that they are reactive with the invertases in their own tissues. Furthermore, invertase activities were not detected in the red beets, sugar beets, and sweet potatoes used for isolation of the inhibitors. Other workers have reported the absence of invertase activities in red beets (1, 7) and sugar beets (1). The absence of activity in extracts of each these tissues does not necessarily represent the absence of invertase, however. The enzyme may be present but its activity cannot be detected because of the presence of an excess of a reactive inhibitor.

It is known that invertase activity develops in slices of red beet during aging in water (1, 6, 7). The activity appears after a lag phase of 6 hours and reaches a maximum after 3 days. Vaughan and MacDonald (7) have found that the development of activity is strongly inhibited by fluorophenylalanine. This suggests that the appearance of invertase activity represents *de novo* invertase synthesis. However, now that an invertase inhibitor has been found in red beets, the results on invertase development in slices can be interpreted in terms of inhibitor destruction rather than enzyme synthesis. The appearance of invertase activity may represent, at least in part, inactivation of an excess of inhibitor. If this is the actual mechanism, the results with fluorophenylalanine (7) suggest that protein synthesis is required for inhibitor inactivation. Presumably, the inactivation involves a proteolytic enzyme specific for the inhibitor.

Acknowledgments

I am grateful to Eunice A. Paulson for technical assistance and to Dr. R. L. Metzenberg, University of Wisconsin, for a sample of *Neurospora* invertase.

Literature Cited

1. Bacon, J. S. D., I. R. MacDonald, and A. H. Knight. 1965. The development of invertase activity in slices of the root of *Beta vulgaris* L. washed under aseptic conditions. Biochem. J. 94: 175–82.
2. Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. In: Methods in Enzymology, Vol. III. S. P. Colowick and N. O. Kaplan, eds. Academic Press, New York. p 447–54.
3. Pressey, R. 1966. Separation and properties of potato invertase and invertase inhibitor. Arch. Biochem. Biophys. 113: 667–74.
4. Pressey, R. and R. Shaw. 1966. Effect of tempertaure on invertase, invertase inhibitor, and sugars in potato tubers. Plant Physiol. 41: 1657–61.
5. Pressey, R. 1967. Invertase inhibitor from potatoes: Purification, characterization, and reactivity with plant invertases. Plant Physiol. 42: 1780–86.
6. Vaughan, D. and I. R. MacDonald. 1967. Invertase development in storage tissue disks of *Beta vulgaris;* its nature, extent, and location. J. Exptl. Botany 18: 578–86.
7. Vaughan, D. and I. R. MacDonald. 1967. The effect of inhibitors on the increase in invertase activity and RNA content of beet disks during aging. J. Exptl. Botany 18: 587–93.
8. Whitaker, J. R. 1963. Determination of molecular weights of proteins by gel filtration on Sephadex. Anal. Chem. 35: 1950–53.