1972

Solubility of (β-Glucosidase in Homogenates of Sweetclover Leaves and Bean Hypocotyls

T. A. Jaynes
Agricultural Research Service, United States Department of Agriculture

Francis A. Haskins
University of Nebraska-Lincoln, fhaskins@neb.rr.com

H. J. Gorz
University of Nebraska-Lincoln

A. Kleinhofs
Agricultural Research Service, United States Department of Agriculture

Plant Physiol. (1972) 49, 277–279

Short Communication

Solubility of β-Glucosidase in Homogenates of Sweetclover Leaves and Bean Hypocotyls[1]

Received for publication June 28, 1971

T. A. JAYNES, F. A. HASKINS, H. J. GORZ, AND A. KLEINHOFS[2]
Department of Agronomy, University of Nebraska, and Plant Science Research Division, Agricultural Research Service, United States Department of Agriculture, Lincoln, Nebraska 68503

In a recent paper Eberhart and Beck (4) suggested that the aryl-β-glucosidase of *Neurospora* conidia is a mural enzyme. Similarly, Nevins (11) reported that β-glucosidase is strongly associated with cell walls during extraction of bean hypocotyls at pH 4.6 and suggested that this enzyme is wall-bound *in situ*, and Keegstra and Albersheim (9) noted that β-glucosidase could be effectively washed from the surface of sycamore cells grown in liquid culture. On the basis of histochemical evidence, Ashford (1) suggested that β-glucosidase in corn roots is associated with a reticulate network of strands and particles, and Ashford and McCully (2) concluded that this network is located peripherally in the cells.

Sweetclover homogenates contain a β-glucosidase which hydrolyzes the β-glucoside of *cis-o*-hydroxycinnamic acid (*cis-o*-HCA)[3] (10). Under acidic conditions the aglycone lactonizes spontaneously, forming coumarin. The sweetclover enzyme also can hydrolyze the commonly used aryl-β-glucosidase substrate, PNP-G (12).

This report deals with the distribution of β-glucosidase activity in fractionated homogenates of sweetclover leaves and bean hypocotyls prepared under various conditions. The data presented indicate that binding to cell walls is not the only factor involved in determining these distribution patterns.

MATERIALS AND METHODS

Sweetclover β-glucosidase was extracted from young, fully expanded leaves harvested from greenhouse-grown plants of biennial white-flowered sweetclover (*Melilotus alba* Desr.) of the *CuCuBB* genotype. The derivation of this genotype has been described elsewhere (5). Leaves (0.5 g) were ground in a mortar with 5 ml of 50 mM borate buffer, pH 8.5, or with 5 ml of 50 mM acetate buffer, pH 5.0. Homogenates were centrifuged at 17,000*g* for 10 min to yield supernatant and pellet fractions. Supernatant fractions from pH 8.5 homogenates were used in preincubation experiments. These fractions were deionized prior to use by washing through a column of Sephadex G-25[4] (Pharmacia) with water.

β-Glucosidase was extracted from the hypocotyls of beans (*Phaseolus vulgaris* L. var. Red Kidney) which had been allowed to germinate for 5 days at room temperature between moist paper towels. The bean seeds were obtained from a local market. Extraction and centrifugation procedures were similar to those used for sweetclover preparations.

The cellular debris used in some preincubation experiments was prepared from sweetclover leaves which had been frozen in liquid nitrogen, pulverized in a mortar, lyophilized, and stored in a freezer. A 1-g portion of this material was suspended in 20 ml of 50 mM borate buffer, pH 8.5, and the suspension was centrifuged at 17,000*g* for 10 min. The pellet was subjected to three additional washings with buffer, one with water, and a final washing with acetone. The washed pellet was air-dried.

Preincubation mixtures consisted of 1.0 ml of deionized enzyme preparation; 0.5 ml of water, 81 mM acetate buffer, pH 5.0, or 81 mM borate buffer, pH 8.5; and, in some instances, 5 mg of cellular debris. Mixtures were allowed to stand for 5 min at room temperature and were then centrifuged. Supernatant fractions from this centrifugation were assayed for β-glucosidase activity. Pellets were resuspended in 1.5 ml of 50 mM borate buffer, pH 8.5, and recentrifuged. Assay of β-glucosidase activity in the supernatant fractions from this centrifugation provided a measure of the activity which was associated with the pellet at the end of the preincubation treatment.

Reaction mixtures for β-glucosidase assay contained the following in a volume of 0.5 ml: 30 mM acetate buffer, pH 5.0; 0.4 mM PNP-G (Sigma Chemical Company); and 0.01 to 0.10 ml of enzyme preparation. Mixtures were incubated at 30 C, usually for 15 min. The reaction was stopped by addition of 2 ml of 0.05 N NaOH, and absorbance at 400 nm was measured. Activity was calculated as μmoles of PNP-G hydrolyzed per min per ml of enzyme preparation (or, in some cases, per ml of preincubation mixture).

RESULTS AND DISCUSSION

The distribution of β-glucosidase activity following centrifugation of homogenates was strongly influenced by pH (Table

[1] Contribution from the Plant Science Research Division, Agricultural Research Service, United States Department of Agriculture, and the Nebraska Agricultural Experiment Station, Lincoln, Neb. Published with the approval of the Director as Paper No. 3173, Journal Series, Nebraska Agricultural Experiment Station. The work reported was conducted under Nebraska Agricultural Experiment Station Project No. 12–50, and was supported in part by National Science Foundation Grant GB-8280.

[2] Present address: Department of Agronomy, Washington State University, Pullman, Wash. 99163.

[3] Abbreviations: *cis-o*-HCA: *cis-o*-hydroxycinnamic acid; PNP-G: *p*-nitrophenyl-β-D-glucopyranoside.

[4] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

Table I. *Distribution of β-Glucosidase Activity*

β-Glucosidase was assayed following centrifugation of sweetclover leaf and bean hypocotyl homogenates prepared at pH 5.0 and 8.5.

Source	Extraction pH	Fraction Analyzed	β-Glucosidase		Protein
			Activity	Percentage of total	
			μmoles/min·ml		*mg/ml*
Sweetclover leaves	5.0	Supernatant	9.6	36	1.7
		Pellet	16.8	64	0.5
	8.5	Supernatant	29.2	99	2.6
		Pellet	0.3	1	0.4
Bean hypocotyls	5.0	Supernatant	0.5	16	0.9
		Pellet	2.7	84	1.8
	8.5	Supernatant	3.6	86	2.4
		Pellet	0.6	14	0.2

Table II. *Dissociation of Sweetclover β-Glucosidase from Pellets Obtained by Centrifuging pH 5.0 Homogenates*

Five 0.5-g portions of sweetclover leaves were ground in pH 5.0 buffer, and homogenates were centrifuged. Pellets were resuspended in water or the solutions listed (5 ml), and the suspensions were centrifuged. Supernatant fractions were assayed for β-glucosidase activity.

Resuspension Medium	β-Glucosidase Activity
	μmoles/min·ml
Water	0
50 mM Buffer, pH 5.0	0
50 mM NaCl	1.0
1.0 M NaCl	19.2
50 mM Buffer, pH 8.5	15.2

I). At pH 8.5, most of the activity was found in the supernatant fraction, but at pH 5.0, the pellet accounted for most of the activity. This pattern of distribution was observed for both sweetclover and bean homogenates. Total activities of the homogenates prepared at pH 8.5 were somewhat higher than totals for pH 5.0 homogenates, suggesting some loss of activity at pH 5.0.

β-Glucosidase activity which was associated with the pellet following centrifugation of sweetclover homogenates prepared at pH 5.0 was solubilized by suspending the pellet in NaCl solution or in pH 8.5 buffer (Table II). NaCl at a concentration of 1.0 M was much more effective than 50 mM NaCl in solubilizing the enzyme. Water and 50 mM buffer, pH 5.0, were ineffective. If sweetclover β-glucosidase is indeed wall-bound, responses such as these to changes in pH and NaCl concentration would not be surprising.

In further investigations of the apparent binding of β-glucosidase, cellular debris and deionized enzyme were prepared separately as described under "Materials and Methods." Portions of deionized enzyme preparation were preincubated, with and without added debris, at pH 5.0 or 8.5, after which the distribution of β-glucosidase activity was determined. Results (Table III) indicate clearly that at pH 5.0, the enzyme activity from both sweetclover and bean was associated primarily with the pellet, whereas at pH 8.5, activity was found predominantly in the supernatant fraction. Inasmuch as these distributions are similar to the distributions recorded in Table I, it appears that β-glucosidase may be reversibly shifted between pellet and supernatant fractions by varying the pH of the suspending medium. Furthermore, it appears that pH was the critical factor in determining β-glucosidase solubility; cellular debris had no effect on the distribution pattern (Table III). β-Glucosidase which had been precipitated by exposure to 30 mM buffer, pH 5.0, like the enzyme in homogenates prepared at pH 5.0, was solubilized by treatment with NaCl solutions or pH 8.5 buffer (compare Tables IV and II).

It could be argued that the Sephadex-treated enzyme preparation might still include a component capable of binding β-glucosidase at pH 5.0. In view of this possibility, sweetclover β-glucosidase was partially purified by chromatography. The chromatographic procedure employed a DEAE-cellulose column and an eluting buffer consisting of a NaCl gradient in 10 mM borate, pH 8.5 (8). β-Glucosidase activity eluted from the column as a single peak. The partially purified enzyme was subjected to preincubation at pH 5.0 with and without cellular debris. In the absence of cellular debris, activity was not precipitated at pH 5.0, even at levels of protein similar to those used in preincubations involving the Sephadex-treated enzyme. Possibly the DEAE-cellulose chromatography removed a protein which otherwise would have precipitated with β-glucosidase in the interisoelectric range (7). Preincubation of chro-

Table III. *Preincubation of Deionized β-Glucosidase from Sweetclover and Bean at pH 5.0 and 8.5 in the Presence and Absence of Cellular Debris*

Sample was passed over Sephadex G-25 in water prior to use.

Source	Additives		β-Glucosidase Activity	
	Buffer	Cellular debris	Supernatant fraction	Pellet fraction
		mg	*μmoles/min·ml*	
Sweetclover	0	0	11.3	0.8
	pH 5.0	0	2.5	6.1
	pH 5.0	5	2.5	6.0
	pH 8.5	0	11.8	0.5
	pH 8.5	5	11.6	0.8
Bean	0	0	0.8	0.2
	pH 5.0	0	0.1	0.7
	pH 5.0	5	0.1	0.7
	pH 8.5	0	0.9	0.1
	pH 8.5	5	0.8	0.1

Table IV. *Solubilization of β-Glucosidase from Pellets Made by the Addition of pH 5.0 Buffer to Deionized Enzyme Preparation*

Five 1.0-ml samples of deionized enzyme preparation were diluted to 1.5 ml with 90 mM acetate buffer, pH 5.0, and after an incubation period of 5 min, suspensions were centrifuged. Pellets were resuspended in water or the solutions listed (1.5 ml), and the suspensions were centrifuged. Supernatant fractions were assayed for β-glucosidase activity.

Resuspension Medium	β-Glucosidase Activity
	μmoles/min·ml
Water	0
50 mM Buffer, pH 5.0	0.5
50 mM NaCl	2.4
1.0 M NaCl	5.0
50 mM Buffer, pH 8.5	4.8

matographed enzyme with cellular debris at pH 5.0 resulted in partial precipitation of β-glucosidase activity.

The evidence presented indicates that although cellular debris may be effective in precipitating partially purified β-glucosidase, specific binding to such debris (cell walls?) need not be invoked to explain the apparent insolubility of β-glucosidase in crude pH 5.0 homogenates of sweetclover leaves or bean hypocotyls. Although the conditions used by Nevins (11) were not identical to those used in the present work, it is suggested that the β-glucosidase solubility behavior he observed may have involved processes other than attachment to and release from cell walls.

The *bb* genotype of sweetclover has been reported to lack β-glucosidase activity (6, 12). These reports were based on assays employing water extracts of sweetclover leaves. Inasmuch as alkaline buffers are much more effective than water in extracting sweetclover β-glucosidase, a question arises as to whether *bb* plants might contain a β-glucosidase which is insoluble in water but soluble in alkaline buffers. Assay of leaf homogenates prepared in pH 8.5 buffer indicated relative β-glucosidase activities of 1 for the *bb* genotype, 238 for *BB*, and 114 for a homogenate of equal quantities of *bb* and *BB* leaves. Thus, the earlier conclusion regarding the effect of the *b* gene on β-glucosidase activity in sweetclover remains valid.

A role in cell wall extensibility has been proposed for glycosidases which hydrolyze cell wall polysaccharides (3, 9, 11). If sweetclover β-glucosidase has such a role, a pronounced effect of the *b* gene on plant height, for example, would be expected. The absence of significant height differences between *BB* and *bb* plants (5, 6) suggests that in sweetclover, this enzyme may not have a large influence on wall extensibility.

LITERATURE CITED

1. Ashford, A. E. 1970. Histochemical localization of β-glycosidases in roots of *Zea mays*. I. A simultaneous coupling azo-dye technique for the localization of β-glucosidase and β-galactosidase. Protoplasma 71: 281–293.
2. Ashford, A. E. and M. E. McCully. 1970. Histochemical localization of β-glycosidases in roots of *Zea mays*. II. Changes in localization and activity of β-glucosidase in the main root apex. Protoplasma 71: 389–402.
3. Cleland, R. 1971. Cell wall extension. Annu. Rev. Plant Physiol. 22: 197–222.
4. Eberhart, B. M. and R. S. Beck. 1970. Localization of the β-glucosidases in *Neurospora crassa*. J. Bacteriol. 101: 408–417.
5. Gorz, H. J. and F. A. Haskins. 1969. Absence of dominance of the *Cu* gene in influencing *o*-hydroxycinnamic acid content in *Melilotus alba*. Crop Sci. 9: 79–81.
6. Haskins, F. A. and H. J. Gorz. 1965. Absence of dominance of the *B* gene in influencing β-glucosidase activity in *Melilotus alba*. Genetics 51: 733–738.
7. Haurowitz, F. 1963. The Chemistry and Function of Proteins. Academic Press. New York.
8. Jaynes, T. A., F. A. Haskins, and H. J. Gorz. 1971. Oxidation of phenylpyruvate by sweetclover peroxidase. Phytochemistry. In press.
9. Keegstra, K. and P. Albersheim. 1970. The involvement of glycosidases in the cell wall metabolism of suspension-cultured *Acer pseudoplatanus* cells. Plant Physiol. 45: 675–678.
10. Kosuge, T. and E. E. Conn. 1961. The metabolism of aromatic compounds in higher plants. III. The β-glucosides of *o*-coumaric, coumarinic, and melilotic acids. J. Biol. Chem. 236: 1617–1621.
11. Nevins, D. J. 1970. Relation of glycosidases to bean hypocotyl growth. Plant Physiol. 46: 458–462.
12. Schaeffer, G. W., F. A. Haskins, and H. J. Gorz. 1960. Genetic control of coumarin biosynthesis and β-glucosidase activity in *Melilotus alba*. Biochem. Biophys. Res. Comm. 3: 268–271.