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β-GALACTOSIDASE FROM SUGAR CANE

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Key Word Index—*Saccharum officinarum*; Gramineae; sugar cane; β-galactosidase; cell wall.

Abstract—β-Galactosidase activity occurs in all of the organs of the sugar cane plant, and is also of general occurrence among different cultivars and species. Most of the activity was associated with the cell wall, and only *ca* 12–16% was an intracellular form. Both activities posess similar optimum pH and K_m, both are activated by Mn^{2+} and ethanol, and inhibited by Hg^{2+}, and both attack the same substrates.

INTRODUCTION

Although the glycosidases from sugar cane are of physiological and practical importance, few workers have studied these enzymes. Hitherto, the only well characterized glycosidases from sugar cane are the invertases [1–3], trehalase [4–6] and β-amylases [7, 8]. However, other glycosidases also occur in the plant, such as polygalacturonase, α-glucosidases, α- and β-galactosidases [9]. The present paper reports for the first time a study of the β-galactosidase from sugar cane.

RESULTS

Soluble β-galactosidase

A soluble β-galactosidase occurs in leaf blades from sugar cane. The enzyme gave only one peak on gel filtration (Sephadex G-100 and G-150) and one active band after polyacrylamide gel electrophoresis [10, 11]. The enzyme did not precipitate after 1 hr of centrifugation at 105 000 *g*.

Effect of pH

The enzyme has an optimum pH 4.25 and is active between pH 3 and 6. The optimum pH resembles that of some plant β-galactosidases [12–16] and differs from the optimum found for bacterial β-galactosidases which tends to be at pH 7 [17, 18]. Organisms such as *Neurospora crassa* have two isoenzymes with optimum pHs of 4 and 7 [19]. Certain moulds tend to have an acid β-galactosidase like higher plants [20, 21].

Effect of substrate concentration

The soluble β-galactosidase showed simple Michaelis Menten kinetics which corresponds with a K_m of $(2.7 \pm 0.51) \times 10^{-3}$ M (average of 7 determinations). In this the enzyme is similar to the β-galactosidase from *Lupinus luteus* [22], but it is less efficient than the enzymes from *Petunia hybrida* [14], and *Canavalia ensiformis* [23].

Effect of temperature

Arrhenius plots were straight-line graphs with an activation energy of 18 000 cal/mol. The β-galactosidase from *E. coli* gives a biphasic curve, with an inflection point at 30°, and the activation energy is lower at higher temperatures [17]. Furthermore, the enzyme from sugar cane was relatively stable at 47° with a loss of *ca* 42% of activity after 60 min at pH 4. Heating for 50 min at 50° produced total inactivation. The maximal stability of the enzyme at 37° occurs at pHs between 4.5 and 6.7.

Specificity of enzyme

The enzyme attacked lactose, phenyl-β-D-galactoside, *o*-nitrophenyl-β-D-galactoside and various galactans (Table 1).

Tissue distribution

The enzyme was found in all tested tissues from sugar cane (Table 2). However, the enzyme appears to predominate in the stalk apex. These determinations were performed as described in the Experimental. Similar determinations with apex tissues of several varieties and species of sugar cane showed that β-galactosidase was in all of the assayed plants (*S. officinarum*, *S. spontaneum*, cvs Tuc. 68–19, NA 63–90 and CP 70-321).

Effect of chemicals

Among sugar cane metabolites tested as effectors, 90 mM glucose produced 15% inhibition, fructose had no effect at 90 mM and sucrose produced 10% inhibition at 30 mM and had an activating effect (6%) at a concentration of 350 mM. Cysteine inhibited by 7.5% at a concentration of 8 mM and activated 10% with increasing concentrations (16–24 mM). The activating effect of cysteine may be due to the -SH group, when 75 mM 2-mercaptoethanol was tested, 13% activation was found. Substances which have activity on *E. coli* β-galactosidase, such as 75 mM NaCl and KCl and 125 mM Be^{2+} have no activity on the sugar cane enzyme. Tris, an inhibitor of many enzymes [24–26] was without effect on the β-galactosidase at concentrations of up to 125 mM. Ions as 30 mM Mg^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+} produced 20, 20, 10 and 6% activation, respectively. Although each effector has a small effect on the enzyme, a mixture of 30 mM Mg^{2+}, 300 mM sucrose and 75 mM 2-mercaptoethanol

Table 1. Specificity of β-galactosidase activities

Substrate	Concentration	Incubation time (hr)	Activities*	
			Soluble	Cell wall
o-Nitrophenyl-Galactoside	17.5 mM	1	9.25	2.17
Phenyl galactoside	17.5 mM	1	2.56	—
Lactose	12.5 mM	1	1.68	—
Reaction Wood gal.	0.25%	24	1.14	0.60
Larch gal.	0.25%	24	2.28	1.03
L.albus gal.	0.50%	24	1.38	0.86
Carrageenan	0.25%	24	1.00	0.73
Agaropectin	0.125%	24	1.71	0.86

* Results are expressed as μmol produced/ml of enzyme.

Table 2. Distribution of total β-galactosidase activity in different organs

Organ	Enzyme units/g fr. tissue
Leaf-blades	0.214 ± 0.050
Leaf-sheaths	0.132 ± 0.015
Stalk apex	0.361 ± 0.097
Root (elongation zone)	0.270 ± 0.005
Root (hair zone)	0.133 ± 0.048
Mature stalk	0.036 ± 0.004

Each value is the mean of 5 determinations performed with tissue cuttings.

produced *ca* 30% activation. A similar effect was observed for mixtures of cysteine and sucrose. A concentration of 5 μM $HgCl_2$ produced an inhibition of 93.5%, and 0.84 M ethanol activated by 32%. The activity of the enzyme was not affected by 5% ethyl acetate.

Effect of enzyme concentration

Reaction rates were proportional to the enzyme concentration from 0 to 50 μl of extract. According to these results there are no endogenous reversible inhibitors, as known for potato invertase [27].

Occurrence of cell wall bound β-galactosidase

Soluble β-galactosidase represents *ca* 12% of the total activity found in tissue cuttings. Tissues treated with ethyl acetate gave 16.1% more β-galactosidase activity than untreated tissues. As ethyl acetate increases the permeability of the membranes [28] the difference is attributable to a cryptic enzyme. Studies of cellular localization of the enzyme showed that β-galactosidase occurs in the supernatant on centrifuging at 40 000 *g* (26.1×10^{-4} enzyme units per g fresh tissue) and is absent from the cell membrane. Furthermore, cell wall preparations also showed β-galactosidase activity. The cell wall preparations were sonicated and successively washed with the homogenization buffer (M NaCl pH 7.5; 0.2 M Na-borate buffer pH 8.5 and 0.2 M NaPi-citric acid buffer pH 8.5, M NaCl, 50 mM EDTA, 1 mM 2-mercaptoethanol) without solubilization of the enzyme. All of these solutions have been used successfully in previous experimental work as a way of solubilizing cell wall enzymes [29–33]. According to these results the enzyme is firmly bound to the cell wall.

Properties of cell wall β-galactosidase

The optimum pH of the cell wall β-galactosidase was 4.4. The K_m was $(2.76 \pm 0.51) \times 10^{-3}$ M (mean of 7 determinations). The enzyme was activated 25% by 30 mM Mn^{2+} and 32% by 0.84 M ethanol, and is inhibited 64.5% by 5 μM $HgCl_2$; further, there are no significant differences between the K_m of the soluble and of the cell wall enzyme, ($P \geqslant 5\%$). According to these results both activities appear to correspond to the same enzyme. The differences of the inhibitory values are probably due to differences in the environment of each enzyme. The cell wall β-galactosidase attacked galactans containing β(1 → 4) bonds such as reaction wood, larch, and *Lupinus albus* galactan. Heterogalactans such as carrageenan from *Gigartina skottsbergi* [34] and agaropectin from *Corallopsis* species, which contain D-galactose, sulphated galactose, L-galactose and 3,6-anhydro-galactose with different types of bonds such as (1 → 4), (1 → 3) and (1 → 2), were also attacked by the enzyme.

DISCUSSION

Although β-galactosidases are well known enzymes in many higher plants, the enzyme from sugar cane was recently found in this laboratory, The enzyme must be of general occurrence among commercial varieties of sugar cane, because most of these cultivars have *S. officinarum* and *S. spontaneous* among their ancestors and these species contain β-galactosidase. The enzyme is present in all of the sugar cane tissues, but is mainly a leaf enzyme and an activity of growing tissues such as apex and root meristems. According to the K_m, optimum pH, Mn^{2+} and ethanol activations, and Hg^{2+} inhibition, the same protein appears to be present in the cell wall and in the cell. Determinations of enzyme localization using cuttings of tissues suggested that the activity is mainly extracellular, but as cell membranes did not show activity, the extracellular enzyme must be in the cell wall. Direct proof of this localization was obtained from cell wall

preparations. According to these facts it is possible that the cell wall enzyme originates in the cell, and afterwards is excreted and then bound to the cell wall. β-Galactosidases bound to cell wall are also known in some fruits, for example in apples [35] and tomato [36]. In these fruits, however, the enzyme is involved with other glycosidases in the softening process by alteration of the cell wall structure. The functions of the galactosidases from sugar cane are not so clear, but it is possible that the cell wall enzyme participates in the turnover of the cell wall. This supposition is further supported by the attack of various galactans with different structure by the cell wall enzyme. As there are no known soluble β-galactosides in sugar cane it is probable that the cryptic enzyme is a precursor of the cell wall enzyme. However, it is possible that the intracellular form acts on the galactolipids which were not studied.

EXPERIMENTAL

A cultivar of sugar cane (cv Tuc 68–19), cultivated in the field at the Estación Experimental Agrícola de Tucumán was used throughout this work unless otherwise stated.

Soluble enzyme preparation. Blades from leaf +1, +2 and +3 were used, where +1 is the youngest fully-expanded leaf. The leaves (200 g) were cut into small pieces and homogenized in 3 vol. of 10 mM NaPi buffer pH 7.2, 1 mM 2-mercaptoethanol, 5 μM $MnSO_4$ and 0.5% Na_2SO_3. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 12 100 *g* for 10 min. Solid $(NH_4)_2SO_4$ was added to the supernatant and a fraction which pptd between 30 and 60% satn was collected by centrifugation. The pellet was resuspended in 1.5 ml of 10 mM NaPi buffer pH 7.2, 1 mM 2-mercaptoethanol, and was dialyzed against the same buffer.

Cell wall preparation. Leaf blades without ribs (50 g) were homogenized in 300 ml buffer as stated. The homogenization of the fibrous material was repeated ×3, and the combined fractions were filtered through 2 layers of cheesecloth and centrifuged for 10 min at 270 *g*. The pellet was washed ×5 and resuspended in 30 ml of the homogenization buffer using a Potter. The suspension was sonicated, centrifuged and the pellet was washed ×5 and resuspended in 4 ml of the buffer.

Cell membranes were prepared from 100 g of leaf tissues following ref. [37].

Assay of cell β-galactosidase. Incubation mixture consisted of 30 μl 0.2 M NaOAc buffer pH 4.25, 70 μl 0.05 M *o*-nitrophenyl-β-D-galactoside, 25 μl enzyme prepn and H_2O in a final vol. of 200 μl. Reaction time was 30 min (unless otherwise stated) at 37°. The reactions were stopped by adding 2 ml M Na_2CO_3. The amount of *o*-nitrophenol released was measured at 420 nm.

Assay of cell wall β-galactosidase. Incubation miture consisted of 100 μl of cell wall suspension in 0.2 M NaOAc buffer pH 4.25, 100 μl of 0.05 M *o*-nitrophenyl-β-D-galactoside (pH 4.25). The incubations were performed for 30 min at 37°.

Assay of β-galactosidase in tissue cuttings. Tissue (100 mg) was cut into small strips 1–2 mm wide. Cuttings were washed for 5 min with H_2O. Washings were repeated ×3 and the tissues were suspended in 0.6 ml 0.05 M *o*-nitrophenyl-β-D-galactoside, 0.2 M NaOAc buffer pH 4.25, 5% EtOAc and incubated at 37°. Aliquots (100 μl) of the incubation mixture were taken every 15 min and the *o*-nitrophenol released was measured as stated in assay of soluble β-galactosidase. When an approximate measure of cell wall enzyme was required, EtOAc was omitted.

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REFERENCES

1. Glasziou, K. T. and Gayler, K. R. (1972) *Bot. Rev.* **38**, 471.
2. Alexander, A. G. (1965) *J. Agric. Univ. P. R.* **49**, 287.
3. Sampietro, A. R., Prado, F. E. and Vattuone, M. A. (1979) *Plant Physiol. Suppl.* **53**, 11.
4. Glasziou, K. T. and Gayler, K. R. (1969) *Planta* **85**, 299.
5. Alexander, A. G. (1973) *Plant Cell Physiol.* **14**, 157.
6. Fleischmacher, O. L., Vattuone, M. A., Prado, F. E. and Sampietro, A. R. (1980) *Phytochemistry* **19**, 37.
7. Alexander, A. G. and Lebron, J. (1969) *Proc. Int. Soc. Sugar-Cane Technol.* **13**, 514.
8. Roughan, P. G., Waldron, J. C., Glasziou, K. T. (1972) *Proc. Int. Soc. Sugar Cane Technol.* **14**, 257.
9. Quiroga, E. N., Re de Maxud, T., Vattuone, M. A., Prado, F. E. and Sampietro, A. R. (1977) *Plant Sci. Letters* **8**, 135.
10. Ornstein, L. and Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 321.
11. Erickson, R. P. and Steers, E. (1970) *J. Bacteriol.* **102**, 79.
12. Agrawal, K. M. L. and Bahl, O. P. (1968) *J. Biol. Chem.* **243**, 103.
13. Rosenfield, C. L. and Matile, P. (1979) *Plant Cell Physiol.* **20**, 605.
14. Komp, M. and Hess, D. (1977) *Z. Pflanzenphysiol.* **81**, 248.
15. Hutson, D. H. and Manners, D. J. (1964) *Biochem. J.* **93**, 545.
16. Marbach, I., Mayer, A. M. and Maron, R. (1978) *Phytochemistry* **17**, 655.
17. Wallenfels, K. and Malhotra, O. P. (1961) *Adv. Carbohydr. Chem.* **16**, 239.
18. Anema, P. J. (1964) *Biochim. Biophys. Acta* **89**, 495.
19. Lester, G. and Byers, A. (1965) *Biochem. Biophys. Res. Commun.* **18**, 725.
20. Tanaka, Y., Kagamiishi, A., Kouchi, A. and Horiuchi, T. (1975) *J. Biochem.* **77**, 241.
21. Bahl, O. P. and Agrawal, K. M. L. (1969) *J. Biol. Chem.* **244**, 2970.
22. Matheson, N. K. and Saini, H. S. (1977) *Carbohydr. Res.* **57**, 103.
23. Li, S. C., Marzotta, M. Y., Chien, S. F. and Li, Y. T. (1975) *J. Biol. Chem.* **250**, 6786.
24. Hatch, M. D., Sacher, J. A. and Glasziou, K. T. (1963) *Plant Physiol.* **38**, 338.
25. Sampietro, A. R., Vattuone, M. A. and Prado, F. E. (1980) *Phytochemistry* (in press).
26. Good, N. E., Winger, G. D., Winter, W., Conolly, T. N., Izawa, S. and Singh, R. M. M. (1966) *Biochemistry* **5**, 467.
27. Pressey, R. (1966) *Arch. Biochem. Biophys.* **113**, 667.
28. Fekete, M. A. R. de and Vieweg, G. H. (1973) *Ber. Dtsch. Bot. Ges.* **86**, 227.
29. Kato, T. and Kubota, S. (1976) *Physiol. Plant.* **42**, 67.
30. Arnold, W. N. (1966) *Biochim. Biophys. Acta* **128**, 124.
31. Hisajima, S. H. and Arai, Y. (1978) *J. Jpn. Soc. Starch Sci.* **25**, 163.
32. Wray, J. L. and Brice, R. E. (1973) *Phytochemistry* **12**, 1917.
33. Parr, D. R. and Edelman, J. (1975) *Planta* **127**, 111.
34. Cerezo, A. S. (1967) *J. Chem. Soc.* 2491.
35. Bartley, I. M. (1977) *J. Exp. Botany* **28**, 943.
36. Wallner, S. J. and Walker, J. E. (1975) *Plant Physiol.* **55**, 94.
37. Sinensky, M. and Strobel, G. (1976) *Plant Sci. Letters* **6**, 209.