Phytochemistry, Vol. 33, No. 6, pp. 1327–1331, 1993
Printed in Great Britain.

0031-9422/93 $6.00+0.00
© 1993 Pergamon Press Ltd

A β-D-FUCOSIDASE FROM *ASCLEPIAS CURASSAVICA* LATEX

ROGER GIORDANI* and LAURENCE LAFON

Centre de Biochimie et de Biologie Moléculaire, CNRS, 31 Chemin Joseph Aiguier, 13009 Marseille, France

(*Received in revised form* 14 *December* 1992)

Key Word Index—*Asclepias curassavica*; latex; non-articulated laticifers; β-D-fucosidase.

Abstract—A β-D-fucosidase was isolated from *Asclepias curassavica* latex by anion exchange and gel filtration chromatography. The enzyme was purified 136-fold. The enzyme is a monomer and its M_r is close to 50000. It shows optimal activity at pH 5.5 and at 50°. The enzyme hydrolyses *p*-nitrophenyl-β-D-fucopyranoside with apparent K_m and V_{max} values of 6.58 mM and 304 mM min^{-1} mg^{-1} of protein, respectively, at the optimum pH. *p*-Nitrophenyl-β-D-fucopyranoside is the best substrate. D(+)-fucose acts as competitive inhibitor with a K_i value of 7.36 mM at pH 5.5. The hypothesis that β-D-fucosidase represents an enzymatic marker of laticifer differentiation and of the cell wall degradation process is discussed.

INTRODUCTION

A milky fluid or latex is exuded from secretory cells called laticifers. These cells have been classified into two types: articulated and non-articulated [1]. Articulated laticifers are formed from a series of initials in which the terminal walls may undergo partial or complete perforation. Non-articulated laticifers originate from single cells which develop into a tube-like structure by continued growth. The latex exuded from non-articulated laticifers of *Asclepias curassavica* is a vacuolar sap in which polyterpenic granules accumulate [2]. The vacuolar and lysosomal nature of this latex has been established [3]. Unlike *Hevea brasiliensis* latex, which has been the subject of many studies, most of the biochemical studies of latices exuded from non-articulated laticifers have been directed only to the detection of enzymatic activities. Thus it has been shown that they contain alkaline phosphatase, esterase [4], RNase, peroxidase [3] and several glycosidases [4] among which is a β-D-fucosidase [5]. Only the acid phosphatase [4, 6], associated with the autophagic process [7], and proteases [8–15] have been isolated and characterized. We have now purified to homogeneity and determined the properties of a β-D-fucosidase of *A. curassavica* latex. As an extension of the investigation of the glycosidases of latices and especially in order to compare it with the strict β-D-fucosidase isolated from *Lactuca sativa* latex exuded from articulated laticifers [5]. The release of fucose from cells walls incubated with this enzyme suggested to us that a strict β-D-fucosidase may be implicated in the degradation of plasmalemmic vesicles released from breaking cell wall areas during the breakdown process characteristic of the differentiation of articulated laticifers [7, 16]. In a tissue devoid of secretory cells, the pith, latency of a strict β-D-fucosidase has been demonstrated [17] and supports the hypothesis about the tissue specificity of the strict β-D-fucosidase of latex from articulated laticifers. In order to demonstrate this hypothesis it is necessary to examine the possible latency of a strict β-D-fucosidase in latex from non-articulated laticifers such as those of *A. curassavica*.

*Author to whom correspondence should be addressed.

Abbreviations: pNP-β-D-Fuc, *p*-nitrophenyl-β-D-fucopyranoside; pNP-β-D-Glu, *p*-nitrophenyl-β-D-glucopyranoside; pNP-α-D-Gal, *p*-nitrophenyl-α-D-galactopyranoside; pNP-β-D-Gal, *p*-nitrophenyl-β-D-galactopyranoside; pNP-β-D-GlcNAc, *p*-nitrophenyl-β-D-glucosaminide.

RESULTS AND DISCUSSION

Purification of the enzyme

The latex supernatant was deposited at the top of a DEAE-Trisacryl M column as indicated in the Experimental. A small amount (3%) of the β-D-fucosidase activity was not retained and was recovered in the rinsing volume. The rest (32% of the initial activity) was recovered in a single peak eluted from the column at about 0.08 M NaCl. The β-D-fucosidase activity peak was symmetrical with the corresponding $A_{280\,nm}$ peak (Fig. 1). No further activity was detected in other fractions or by further rinsing with higher molarities of sodium chloride. The active fractions were pooled, concentrated by ultrafiltration and dialysed for 24 hr against distilled water. The resulting solution (3 ml) was then subjected to gel exclusion chromatography on a Sephadex G 100 column. Twenty-four per cent of the initial activity was recovered with a purification factor of 136. The purification procedure is summarized in Table 1. The homogeneity of the preparation was confirmed by SDS-PAGE (Fig. 2).

Table 1. Purification of the β-D-fucosidase

Step	Protein (mg)	Total activity (U ml^{-1})	Specific activity (U mg^{-1})	Purification factor	Overall recovery (%)
Crude latex serum	42	882	21	1	100
DEAE-Trisacryl eluate	8	282	35	1.7	32
Sephadex G 150 eluate	0.075	214	2853	136	24

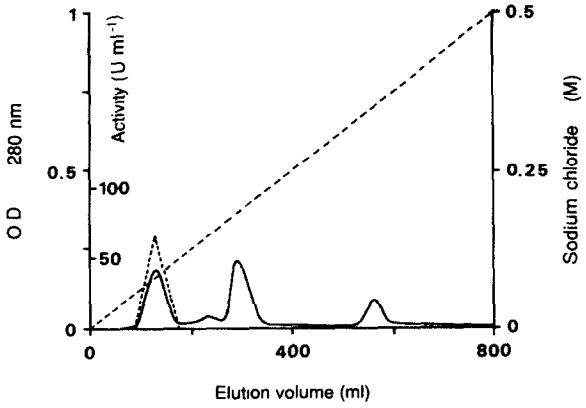

Fig. 1. Elution profile of latex β-D-fucosidase from a DEAE-Trisacryl M gel anion exchange column. The components were monitored by A at 280 nm (—) and fractions were assayed for β-D-fucosidase activity (- - - - -).

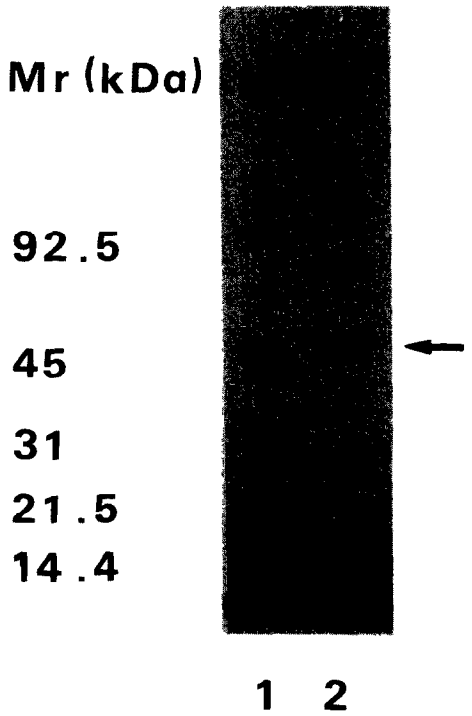

Fig. 2. SDS-PAGE of purified latex β-D-fucosidase on 10–15% PhastGel gradient. Lane 1, marker proteins: phosphorylase b (92 500), ovalbumin (45 000), carbonic anhydrase (31 000), trypsin inhibitor (21 500) and lysozyme (14 400). Lane 2, β-D-fucosidase (22 nM); the arrow shows the position of the enzyme (51 000). Proteins were revealed by Coomassie blue G250 staining.

M_r

Molecular sieving through a Sephadex G 100 column gave a M_r of about 49 000. In SDS-PAGE the purified enzyme showed a single faint protein band corresponding to a M_r of 51 000 indicating that the enzyme is made up of only one polypeptide chain (Fig. 2). This M_r is somewhat smaller than that of *Aspergillus phoenicis* (57 000) [18] but much larger than that of *L. sativa* latex (37 000) [16].

pH optimum and kinetic data

The enzymatic activity was measured from pH 4 to 7 using two different 0.1 M buffers (succinate from pH 4 to 6 and phosphate from pH 6 to 7). The pH optimum for β-D-fucosidase activity was found to be 5.5. At this pH and at 30° the K_m for *p*NP-β-D-fucopyranoside was 6.58 mM and the V_{max} 304 μmol min^{-1} mg^{-1} protein. These parameters were determined by linear regression fitting to a double-reciprocal plot using a computer program.

Substrate specificity

The specificity of the pure β-D-fucosidase was tested with other *p*-nitrophenyl-(pNP-)glycosides known to be used by other fucosidases [16, 19, 20]. Our results indicate that in addition to *p*NP-β-D-Fuc, *p*NP-β-D-Glu, *p*NP-α-D-Gal, *p*NP-β-D-Gal and *p*NP-β-D-GlcNAc were hydrolysed by the enzyme (Table 2) recalling similar observations with β-D-fucosidases of animal and microbial origin [21–26]. The purified β-D-fucosidase has a β-D-fucosidase/β-D-glucosidase/*N*-acetyl-β-D-glucosaminidase/β-D-galactosidase/α-D-galactosidase activity ratio of 100 : 16 : 16 : 15 : 11 and no activity toward other *p*NP-glycosides tested. At the substrate concentrations used the enzyme appears to be strictly specific for the anomeric β carbon (Table 2). The β-D-fucosidase from *A. curassavica* latex is, therefore, not a strict β-D-fucosidase such as the one described in *L. sativa* latex exuded from articulated laticifers [16] and the highly specific β-D-fucosidase from *A. phoenicis* [18].

Inhibition by various glycosides

Whereas β-fucosidase activity is generally displayed by β-galactosidase [27] on account of the close structural

Table 2. Relative rates of hydrolysis of various pNP-glycosides substrates

Substrate	Relative rate of hydrolysis (%)
NP-β-D-Fucopyranoside	100
NP-α-L-Fucopyranoside	0
NP-α-D-Glucopyranoside	0
NP-β-D-Glucopyranoside	16
NP-Acetyl-β-D-glucopyranoside	16
NP-α-D-Galactopyranoside	11
NP-β-D-Galactopyranoside	15
NP-α-D-Mannopyranoside	0
NP-β-D-Mannopyranoside	0
NP-α-L-Arabinofuranoside	0
NP-β-D-Xylopyranoside	0

Glycosidic activities were determined under standard test conditions with 2.5 mM substrates. The relative hydrolysis rates are given as percentages of rate of hydrolysis obtained with pNP-β-D-fucopyranoside.

similarity of fucosides with galactosides, it is unlikely that a β-D-fucosidase possesses α-D-galactosidase activity as a secondary activity. Considering the purity of the isolated enzymic preparation as shown by SDS-PAGE (Fig. 2), the presence of a contaminating α-D-galactosidase cannot be excluded. Considering the several reactions catalysed (hydrolysis of *p*NP-β-D-Fuc, *p*NP-β-D-Glu, *p*NP-α-D-Gal, *p*NP-β-D-Gal and *p*NP-β-D-GlcNAc), it was interesting to know whether these can be considered as reactions occuring at the same catalytic site, or whether the enzyme has multiple catalytic sites. To resolve this problem a detailed kinetic study was carried out at 30° using the corresponding glycosides [D-(+)-fucose, D-(+)-galactose, D-(+)-glucose and D-(+)-glucosamine] at a final concentration of 100 mM and *p*NP-β-D-Fuc as substrate at several concentrations (5, 10, 15 and 20 mM).

D-(+)-fucose was found to be a competitive inhibitor for the enzyme, the inhibition constant K_i was 7.36 mM at pH 5.5. The β-D-fucosidase activity was inhibited by D-(+)-glucose (non-competitive inhibition; $K_i = 9.30$ mM at pH 5.5), by D-(+)-galactose (non-competitive inhibition; $K_i = 8.85$ mM at pH 5.5) or by D-(+)-glucosamine (non-competitive inhibition; $K_i = 11.10$ mM at pH 5.5). Therefore, fucose is the most efficient as an inhibitor of all glycosides tested.

In conclusion, it can be said that on the basis of these inhibition studies the β-D-fucosidase from *A. curassavica* latex possesses at least two separate catalytic sites on its polypeptide chain: one for β-D-fucosidase activity and one for α-D-galactosidase, β-D-galactosidase, β-D-glucosidase and *N*-acetyl-β-D-glucosaminidase activities. In order to establish whether or not the α-D-galactosidase, β-D-galactosidase, β-D-glucosidase and *N*-acetyl-β-D-glucosaminidase activities occur on the same catalytic site or on several catalytic sites, it will be necessary to carry out further kinetic studies using pNP-α-D-Gal, pNP-β-D-Gal, pNP-β-D-Glu and pNP-β-D-Glc-NAc as substrates.

Effect of ions and temperature

The metal complexing reagent EDTA at a final concentration of 25 mM inhibits the β-D-fucosidase (only 17% of the initial activity was recovered) suggesting that the enzyme is a metalloprotein and that metallic ions are probably implicated in the catalytic process. In fact, under the standard assay conditions, the chloride salts of various cations have an inhibitory or stimulatory effect on enzymatic activity. Thus β-D-fucosidase activity was stimulated by several cations such as Mg^{2+}, Mn^{2+}, Sr^{2+}, Ca^{2+}, Na^+, Li^+, K^+ (608–31% stimulation) and inhibited by Hg^{2+} and Fe^{3+} (100% inhibition), Ba^{2+}, Zn^{2+}, Cu^{2+} and La^{3+} (89–52% inhibition); Ni^{2+} on the other hand had no effect on enzyme activity (Table 3). These results are in contrast to those obtained with strict β-D-fucosidases previously purified from *L. sativa* latex [16] for which EDTA has neither an inhibitory nor a stimulatory effect. Our results are similar to those reported for several β-D-glycosidases for which metal ions play an essential role in enzymatic hydrolysis.

The optimum temperature for β-D-fucosidase activity assayed at temperatures in the range 30–70° was found to be 55° at pH 5.5. Stability tests were carried out by incubating the enzyme for 20 min at various temperatures before performing the standard test at 30°. No activity loss could be observed up to 50°; at 55° only 10% of initial activity was recovered. At higher temperatures, the enzyme was totally inactivated.

In conclusion, the *A. curassavica* latex contains a β-D-fucosidase which, in contrast to that of *L. sativa* latex [16] exuded from articulated laticifers, does not exhibit a strict specificity towards pNP-β-D-fucopyranoside as substrate. This absence of a strict β-D-fucosidase from *A. curassavica* latex, where any cell wall degradation process

Table 3. Effects of cations on the β-D-fucosidase activity

Substance added (25 mM)	Relative β-D-fucosidase activity (%)
None	100
Fe^{3+}	0
La^{3+}	11
Ba^{2+}	52
Ca^{2+}	135
Cu^{2+}	12
Hg^{2+}	0
Mg^{2+}	608
Mn^{2+}	154
Ni^{2+}	103
Sr^{2+}	140
Zn^{2+}	48
K^+	131
Li^+	136
Na^+	136

The results are expressed as percentages of the activity recorded in the presence of buffer alone. The cations were used in the form of chloride salts.

occurs, and from articulated laticifer-free tissue, the pith [17], supports the hypothesis about the specificity of the strict β-D-fucosidase of latex from articulated laticifers and, therefore, the hypothesis that this enzyme represents an enzymatic marker for articulated laticifer differentiation and for cell wall degradation, as judged from the *in vitro* parietolytic action of this β-D-fucosidase [16].

These results present the first direct evidence for the involvement of a strict β-D-fucosidase in articulated laticifer differentiation, especially in the cell wall perforation process.

EXPERIMENTAL

Materials. Asclepias curassavica latex was collected from plants (growing at Marseille) by making repeated cuts along the stems using a razor blade. The latex was collected in Eppendorf tubes and stored at −18° until required. The milky product was clarified by centrifugation (14 000 *g*, 10 min) and the clear latex serum separated to give the start juice.

Protein determination. Protein was estimated by the method of Bradford [28] using BSA as standard.

Enzymatic activity. The standard incubation medium (0.6 ml, 30°) contained 0.1 M succinate buffer (pH 6.0), 0.02% NaN_3 and 2.5 mM pNP-β-D-Fuc. After incubation, the reaction was stopped by adding 0.25 ml 0.2 M Na_2CO_3. The yellow colour developed from the liberation of *p*-nitrophenol was estimated by measurement of $A_{400\,nm}$ and compared with a control sample containing only substrate. One unit (U) of enzyme was defined as the amount which liberated 1 μmol of *p*-nitrophenol min^{-1}. Other glycosidic activities (α-D- and β-D-glucosidase, α-D- and β-D-galactosidase, α-D- and β-D-mannosidase, α-L-arabinosidase, β-D-xylosidase and *N*-acetyl-β-D-glucosaminidase) were tested utilizing the appropriate *p*-nitro phenyl glycosides as substrates under the same experimental conditions as those used with *p*-nitrophenyl-β-D-fucopyranoside.

Enzyme purification. A DEAE-Trisacryl M anion exchange chromatography column (2.1 × 16 cm) was equilibrated with 0.01 M Pi buffer (pH 6.8) containing NaN_3 (0.02%). After deposition of the latex sample (clear latex serum obtained after centrifugation), the column was rinsed with 200 ml of the same buffer. Elution was carried out with a linear 0–0.5 M gradient (800 ml) of NaCl in phosphate buffer. The frs (10 ml) collected were assayed for β-D-fucosidase activity. The active fractions were pooled and concd by ultrafiltration through a PM 10 membrane in an Amicon Diaflo apparatus at a pressure of 3 kg cm^{-2}. The concd fr. (4 ml) was loaded onto a Sephadex G 100 column (2.5 × 110 cm) equilibrated with 0.1 M succinate buffer (pH 6.0) containing 0.1 M NaCl. Three ml frs were collected and assayed for β-D-fucosidase activity. The column was calibrated for M_r determinations using α-chymotrypsin (24 700), pepsin (34 700), ovalbumin (45 000) and bovine serum albumin (68 000) as standards.

PAGE. The purity of the β-D-fucosidase was checked by SDS-PAGE. *Ca* 22 ng of protein per lane was applied. The sample was denatured by heating at 100° in 30 mM Tris–HCl (pH 6.8) buffer and 4% SDS for 5 min prior to electrophoresis. A PhastSystem (Pharmacia) was used with a 10–15% PhastGel gradient. Lysozyme (14 400), trypsin inhibitor (21 500), carbonic anhydrase (31 000), ovalbumin (45 000) and phosphorylase *b* (92 500) were used as markers for M_r determinations. The protein bands were detected by Coomassie blue G 250 staining [29].

Acknowledgement—We are grateful to Dr M. L. Cardenas (Laboratoire de Chimie Bactérienne, CNRS, Marseille) for critical reading of the manuscript.

REFERENCES

1. Esau, K. (1965) in *Plant Anatomy*, p. 318. J. Wiley, New York.
2. Giordani, R. (1978) *Biol. Cell.* **33**, 253.
3. Giordani, R., Blasco, F. and Bertrand, J. C. (1982) *C. R. Acad. Sc., Paris, III* **295**, 641.
4. Lynn, K. R. and Clevette-Radford, N. A. (1987) *Phytochemistry* **26**, 939.
5. Giordani, R., Noat, G. and Marty, F. (1986) in *Plant Vacuoles: Their Importance in Solute Compartmentation in Cells and their Applications in Plant Biotechnology*. NATO ASI series, 134, (Marin, B., ed.), p. 383. Plenum Press, New York.
6. Giordani, R., Nari, J., Noat, G. and Sauve, P. (1986) *Plant Sci.* **43**, 207.
7. Giordani, R. (1980) *Biol. Cell.* **38**, 231.
8. Sugiura, M. and Sasaki, M. (1974) *Biochim. Biophys. Acta* **350**, 38.
9. Abraham, K. I. and Joshi, P. N. (1979) *Biochim. Biophys. Acta* **568**, 111.
10. Abraham, K. I. and Joshi, P. N. (1979) *Biochim. Biophys. Acta* **568**, 120.
11. Brockbank, W. J. and Lynn, K. R. (1979) *Biochim. Biophys. Acta* **578**, 13.
12. Lynn, K. R., Brockbank, W. J. and Clevette, N. A. (1980) *Biochim. Biophys. Acta* **612**, 119.
13. Lynn, K. R. and Clevette-Radford, N. A. (1983) *Biochim. Biophys. Acta* **746**, 154.
14. Lynn, K. R. and Clevette-Radford, N. A. (1984) *Phytochemistry* **23**, 682.
15. Lynn, K. R. and Clevette-Radford, N. A. (1985) *Phytochemistry* **24**, 925.
16. Giordani, R. and Noat, G. (1988) *Eur. J. Biochem.* **175**, 619.
17. Giordani, R. (1992) *C. R. Acad. Sc., Paris, III* **315**, 189.
18. Yu-Cheng, Z., Ya-Jun, G. and Shu-Zheng, Z. (1988) in *Enzyme Dynamics and Regulation* (Chock, P. B., Huang, C. Y., Tsou, C. L. and Wang, J. H., eds), p. 385. Springer.
19. Iijima, Y. and Egami, F. (1971) *J. Biochem.* (Tokyo) **70**, 75.
20. Carlsen, R. B. and Pierce, J. G. (1972) *J. Biol. Chem.* **247**, 23.
21. Lewy, G. A. (1960) *Nature* **187**, 1027.

22. Wiederschain, G. Y. and Prokopenkov, A. A. (1971) *Arch. Biochem. Biophys.* **158**, 539.
23. Rodringuez, J. A., Cabezas, J. A. and Calvo, P. (1982) *Int. J. Biochem.* **14**, 539.
24. Calvo, P., Santamaria, M. G., Melgar, M. J. and Cabezas, J. A. (1983) *Int. J. Biochem.* **14**, 685.
25. Colas, B. (1980) *Biochim. Biophys. Acta* **613**, 448.
26. Flowers, H. M. (1981) *Adv. Carb. Chem. Biochem.* **39**, 279.
27. Dey, P. M. and Del Campillo, E. (1984) *Adv. Enzymol.* **56**, 141.
28. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.
29. Heukeshowen, J. and Dernick, R. (1988) *Electrophoresis* **9**, 60.