

UDC 579.842.24:577.15:547.458.34
ISSN 1330-9862

preliminary communication

(FTB-1257)

Kinetic Properties of α -Galactosidase and the Localization of Total Proteins in *Erwinia chrysanthemi*

Leonard Vuyani Mabinya^{1*}, John Morgan Brand¹,
Ndjoko Yei Osée Muyima¹ and Ganka Lubenova Pironcheva²

¹Department of Biochemistry and Microbiology, University of Fort Hare,
Alice 5700, Eastern Cape, South Africa

²Institute of Molecular Biology, BU-1113 Sofia, Bulgaria

Received: August 5, 2003
Accepted: February 16, 2004

Summary

Erwinia chrysanthemi is an enterobacterium that causes soft-rot in plants in general, resulting in enormous economic losses annually. For the pathogen to survive in the host plant, it has to use the readily assimilable compounds from the host fluids and degrade the host tissue. To accomplish this, *E. chrysanthemi* produces several extracellular and intracellular enzymes. Among the intracellular enzymes there is a special digestive class, the galactosidases, which can be either periplasmic or cytoplasmic. α -Galactosidase is known to degrade melibiose and raffinose into glucose and galactose, and into galactose and sucrose respectively. The aim of the present study was to investigate the kinetic properties of α -galactosidase in *E. chrysanthemi*, and the localization of total proteins, after culturing it in the presence of raffinose and melibiose. The α -galactosidase that degrades melibiose seems to be the same enzyme that is also responsible for the breakdown of raffinose in *E. chrysanthemi*. It is localized mainly in the cytoplasm with a fraction of between 2.4 and 5.4 % localized in the periplasm. The majority of *E. chrysanthemi* proteins have cytoplasmic localization.

Key words: *Erwinia chrysanthemi*, α -galactosidase, periplasm, melibiose, raffinose

Introduction

Bacterial cells utilize a variety of mechanisms in the transport of carbohydrates and their subsequent metabolic degradation. Although bacteria lack discrete cellular compartments such as organelles, they do possess subcellular organization at the level of protein localization. Nutrients that cross the envelopes of Gram-negative bacteria have to traverse a complex structure, consisting of a pair of membranes (periplasmic and cytoplasmic) interspersed by the periplasm and cytoplasm, respectively. The two membranes have points of contact with one another, suggesting a more complex structure

(1–3). The surface of the cell wall has proteins (porins) incorporated into it that function as hydrophilic pores of various diameters through which water and water-soluble solutes can migrate (4).

Among the proteins found in the periplasm is a special class of digestive enzymes that cleave cytoplasmic membrane-impermeable molecules into molecules whose translocation across the cytoplasmic membrane is catalyzed by the presence of specific enzymes called permeases. These transport proteins are localized primarily in the cytoplasmic membrane and they exhibit specific-

* Corresponding author; Phone: ++27 40 652 24 44; Fax: ++27 40 602 23 64; E-mail: Lmabinya@ufh.ac.za

ties for different nutrients such as sugars, vitamins, amino acids, nucleotides and many other compounds (5,6).

Erwinia soft-rot species of plant pathogens are enterobacteria that cause diseases, typified by the maceration of host tissues on a wide range of plants (7,8). Soft-rot *erwinias* produce several extracellular enzymes including pectinases, cellulases and proteases that can attack different components of the plant cell wall. The secretion of pectinases is responsible for the maceration of the plant tissue by soft-rot *erwinias*. Some characteristics of the disease are still not well understood. For example, the mode of action involved in the systemic spreading of *Erwinia* within the plant is not known (8,9).

Melibiose and raffinose are known to constitute a carbohydrate source in plants (10,11), and may provide nutritional resources for *E. chrysanthemi* during infection (12). The survival of *E. chrysanthemi* in the host plant depends, among other things, on its ability to degrade and assimilate the host tissue. This requires the presence of specific degradative enzymes and the existence of various transport systems. In this study, the localization of the proteins within the bacterial cell as well as the kinetic properties of α -galactosidase produced by this pathogen are investigated.

Materials and Methods

Erwinia chrysanthemi strain and culture media

Erwinia chrysanthemi strain (EC-C) used was isolated from corn (7). Luria Bertani and Minimal M63 media were prepared according to Miller (13). All media were adjusted to the desired pH before sterilizing at 101 kPa (121 °C) for 20 min. The sugar solutions were made in bidistilled water and filter-sterilized through a 0.22- μ m membrane filter (Millipore Corp., USA). *o*-Nitrophenyl- α -D-galactopyranoside (20 mM) was made in bidistilled water. Buffers: (i) 0.1 M CH₃COONa at pH=3.7, 4.8 and 5.6, (ii) 0.1 M NaH₂PO₄ at pH=6.4, 7.0, 7.4 and 8.0, (iii) 0.1 M Tris-HCl, pH=9. The solution of MgSO₄·7H₂O was added to all buffers to a final concentration of 0.1 %.

α -Galactosidase assay

The assay method for α -galactosidase activity was a modified version of the method of Gray (12). An overnight *E. chrysanthemi* culture grown in Luria Bertani (30 °C, 200 rpm) was used to inoculate minimal M63 medium supplemented with either 0.05 % glycerol and 0.4 % melibiose, or 0.05 % glycerol and 0.4 % raffinose. The cultures were incubated at 30 °C with shaking (200 rpm) until an absorbance at $\lambda=600$ nm reached 0.8. Cultures supplemented with glycerol only (0.4 %) were used as controls. The assay mixture contained 0.1 mL of cells, 0.5 mL of Tris buffer, 0.3 mL of bidistilled water and 25 μ L of toluene. After vortexing and equilibrating at 30 °C for 5 min, the reaction was started by the addition of 0.1 mL of *o*-nitrophenyl- α -D-galactopyranoside (20 mM). The reaction was stopped after 15 min by the addition of 1 M Na₂CO₃ solution. After determination of the absorbance at 420 and 550 nm, respectively, a correction for turbidity was made by subtracting 1.711 \times $A_{550\text{nm}}$ from the $A_{420\text{nm}}$ reading in a Milton Roy Spectronic 1201 spectro-

photometer (Milton Roy, New York, USA). Enzyme activity was calculated according to Miller (13), as follows:

$$\begin{aligned} \text{Activity units (A.u.)} &= \\ &= (1000 \times \text{Nett } A_{420\text{nm}}) / (t \times v \times A_{600\text{nm}}) \end{aligned}$$

where:

Nett $A_{420\text{nm}}$ = average $A_{420\text{nm}}$ – average $A_{550\text{nm}} \times 1.711$,

t = time of the reaction in min,

v = volume (mL) of culture used in the assay.

Kinetic properties of α -galactosidase

The kinetic properties of α -galactosidase were determined using *o*-nitrophenyl- α -D-galactopyranoside (α -ONPG) as substrate. These properties included the pH optimum, the pH stability, the temperature optimum, the temperature stability, the Michaelis constant (K_m) and maximum reaction rate (v_{max}) of this enzyme. A linear enzyme assay curve was achieved in the following way: phosphate buffer, pH=7.4 (2.5 mL), was mixed with bidistilled water (2.2 mL) and α -ONPG (0.25 mL) in a 25-mL Erlenmeyer flask, the mixture was equilibrated at 30 °C for 5 min prior to the addition of 0.25 mL of cell suspension. Samples (0.5 mL) were taken at 5-min intervals over a period of 45 min and immediately mixed with 1 M Na₂CO₃ (1 mL) to stop the reaction. Absorbance was determined at 420 nm in a Milton Roy Spectronic 1201 spectrophotometer (Milton Roy, New York, USA). Control was prepared by replacing the substrate with bidistilled water. All subsequent kinetic determinations were done on the basis of the enzyme assay curve.

For the study of the pH optimum, the determination of enzyme activity was done in triplicates at each pH value. The assay procedure was similar to the one used in establishing the enzyme assay curve, but scaled down in volume and carried out in Eppendorf microcentrifuge tubes. The reaction mixtures were incubated at 30 °C for 30 min before determining the absorbance at 420 nm. For determining pH stability, the crude enzyme was incubated with individual buffers in equal volumes and left at room temperature for 3 h before its activity was assayed in phosphate buffer (pH=7.4).

Temperature optimum was obtained by determining enzyme activity at different temperatures ranging from 0 (ice-water slurry) to 100 °C (boiling water bath). The assay procedure was as described above for pH optimum, except that phosphate buffer at pH=7.4 was used. For determining temperature stability, 0.5 mL of cell suspension was dispensed into Eppendorf microcentrifuge tubes and pre-incubated at various temperatures for 1 h before assaying for enzyme activity in phosphate buffer (pH=7.4) at 30 °C.

The K_m and v_{max} were measured at 30 °C in phosphate buffer (pH=7.4) over a substrate concentration range of 0.24 to 0.96 mM. The assay procedure was similar to that reported for determining α -galactosidase activity (12).

Protein determination and enzyme localization studies

The localization of periplasmic proteins (14,15) was investigated after culturing the cells in M63 medium supplemented with 0.001 M MgSO₄ and either with me-

libiose or raffinose (0.4 %), to an absorbance value of 0.6 at $\lambda=600$ nm. The cells were conditioned for osmotic shock by adding 1 M NaCl and 1 M Tris-HCl buffer (pH=7.3) to a final concentration of 0.03 M each. They were harvested by centrifugation (4500 rpm) for 10 min at room temperature and the pellet resuspended in 7.5–10 volumes of 0.03 M Tris-HCl buffer, pH=7.3. After mixing by inversion, an equal volume of 0.03 M Tris-HCl, 40 % sucrose, and 2 mM Na-EDTA were added with stirring and the cells were collected by centrifugation. The cell pellet was resuspended in 20 mL of bidistilled water and 1 M $MgCl_2$ was added to a final concentration of 1 mM. The cells, after salt stress, were removed by centrifugation (4500 rpm) and the supernatant represented the periplasmic fraction.

The pellet was washed twice in 0.03 M Tris-HCl buffer and resuspended in 1 mL of bidistilled water prior to adding a drop of toluene and incubating at 37 °C for 1 h. After harvesting the cells by centrifugation (4500 rpm), the supernatant was collected for the assay of cytoplasmic proteins.

To isolate the membrane bound proteins, the pellet was washed twice in bidistilled water, resuspended in 1 mL of bidistilled water and the cells disrupted by sonication on ice at 10 s for 3 min with intervals of 20 s. After centrifugation (4500 rpm) in an Eppendorf microcentrifuge for 20 min at room temperature, the supernatant was collected and assayed for membrane bound enzymes. Total protein was determined by using the Bio-Rad protein microassay procedure with bovine serum albumin (Sigma, St. Louis, Missouri, USA) as a standard protein.

Results and Discussion

The kinetic properties of *Erwinia chrysanthemi* α -galactosidase were determined using α -ONPG as a substrate. These properties include pH optimum and stability, temperature optimum and stability, Michaelis constant and maximum reaction rate. The K_m and v_{max} were determined using the Lineweaver-Burk double reciprocal plot. In order to obtain an accurate measurement of the initial reaction rate, standard enzyme assay curves were constructed within time intervals. The localization of proteins was investigated using both the osmotic shock method described by Willis *et al.* (15) and the chloroform shock method described by Ames *et al.* (14).

A pH optimum of 7.4 was obtained for α -galactosidase. Its pH stability ranged between pH=6.4 and 8.0. The enzyme exhibited a temperature optimum of 50 °C and was stable at this temperature for 1 h. Similar kinetic properties of α -galactosidase produced in the presence of melibiose and raffinose respectively were obtained (Table 1).

The K_m values obtained in the presence of melibiose (0.32 mM) and raffinose (0.35 mM) are shown in Table 1. This suggests that the same enzyme may be responsible for the hydrolysis of both melibiose and raffinose in *E. chrysanthemi*. α -Galactosidase is a cytoplasmic enzyme. Raffinose has less effect on the activation of α -galactosidase as compared to melibiose, as seen from Table 2. This finding is consistent with the observations made by

Table 1. Determination of kinetic properties of α -galactosidase produced by *E. chrysanthemi* grown on either melibiose or raffinose using α -ONPG

Substrate	$t/^\circ C$		pH		K_m mM	v_{max} A. u.
	optimum	stability	optimum	stability		
melibiose	50	0–50	7.4	6.4–8.0	0.32	0.39
raffinose	50	0–50	7.4	6.4–8.0	0.35	0.41

Table 2. Localization of α -galactosidase produced by *E. chrysanthemi*

Substrate	A.u. (α -Galactosidase)			
	periplasm	cytoplasm	membrane-bound	extracellular
melibiose	0.67	33.10	n.d.	n.d.
raffinose	0.34	5.89	n.d.	n.d.

n.d. – enzyme activity was not detected

Rios *et al.* (16) that the affinity of an enzyme decreases as the length of the substrate molecule increases. In *E. coli*, α -galactosidase is a cytoplasmic enzyme inducible by melibiose and raffinose and is subject to catabolite repression by other simple sugars (17). However, Wong (18) has reported an α -galactosidase in *Azotobacter vinelandii* that was located outside of the cell membrane. Our results (Table 2) clearly show that 0.67 and 33.10 activity units of α -galactosidase are found in the periplasm and cytoplasm respectively, when melibiose is used as a substrate.

As seen from Fig. 1, about 55 % of *E. chrysanthemi* proteins have cytoplasmic localization. In addition, a small fraction of proteins is detected in the extracellular medium, whilst substantial amounts (~400 $\mu g/mL$) are membrane-bound. The periplasmic fraction constitutes about 11 % of total proteins (Fig. 1). The periplasmic proteins of Gram-negative bacteria usually comprise 10 to 15 % of the total cell proteins and are defined as those

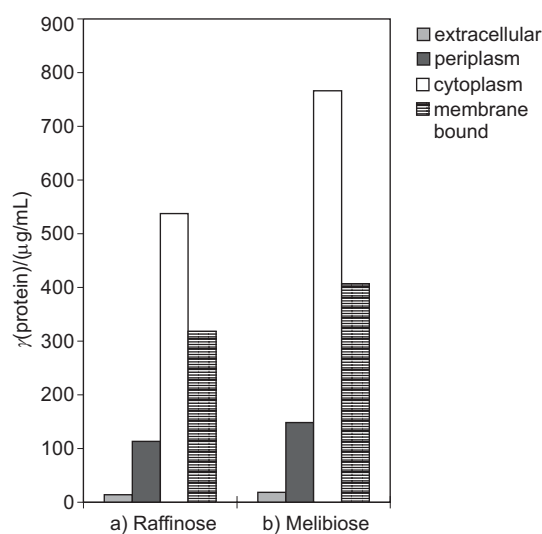


Fig. 1. Distribution of total proteins in *E. chrysanthemi*
a) *E. chrysanthemi* was cultured in M63 medium supplemented with raffinose
b) *E. chrysanthemi* was cultured in M63 medium supplemented with melibiose

proteins that are released into the medium by mild osmotic shock (14,15). The separation of these proteins from the bulk of the cell protein is a very important step, characteristic for mutations that affect periplasmic protein genes (19).

Our data are consistent with reports that both α - and β -galactosidase are known mostly as cytoplasmic proteins in Gram-negative bacteria (14). In addition, the present study also shows an apparent presence of a small fraction of 2 and 5.4 % of α -galactosidase in the periplasm when melibiose and raffinose are used as substrates, respectively (Table 2). For the first time we show that in *Erwinia chrysanthemi*, between 2 and 5.4 % of α -galactosidase is localized in the periplasm of the cell, while the major quantity of it is in the cytoplasm.

References

1. T. R. Hirst, R. A. Welch, *Trends Biochem. Sci.* 13 (1988) 265–269.
2. J.-V. Höltje, *Microbiol. Mol. Biol. Rev.* 62 (1998) 181–203.
3. S. R. Lybarger, J. R. Maddock, *J. Bacteriol.* 183 (2001) 3261–3267.
4. M. H. Saier, Jr.: *Enzymes in Metabolic Pathways: A Comparative Study of Mechanisms, Structure, Evolution and Control*, Harper and Row, New York (1987).
5. M. H. Jr. Saier, *Bacteriol. Rev.* 41 (1977) 856–871.
6. P. W. Postma, J. W. Lengeler, *Microbiol. Rev.* 49 (1985) 232–269.
7. J. P. Mildenhall, *Fort Hare Pap.* 6 (1974) 29–33.
8. A. Kotoujansky, *Ann. Rev. Phytopath.* 25 (1987) 405–430.
9. A. K. Chatterjee, M. P. Starr, *Rev. Microbiol.* 34 (1980) 645–676.
10. C. M. Duffus, J. H. Duffus: *Carbohydrate Metabolism in Plants*, Longman, New York (1984).
11. P. M. Dey: α -Galactose-Containing Oligosaccharides. In: *Biochemistry of Storage Carbohydrates in Green Plants*, P. M. Dey and R. A. Dixon (Eds.), Academic Press, New York (1985) pp. 53–129.
12. J. S. S. Gray, W. A. Lindner, J. M. Brand, J. P. Mildenhall, *J. Bacteriol.* 168 (1986) 886–891.
13. J. H. Miller: *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1972) pp. 27–434.
14. G. F.-L. Ames, C. Prody, S. Kustu, *J. Bacteriol.* 160 (1984) 1181–1183.
15. R. C. Willis, R. G. Morris, C. Cirakoglu, G. D. Schellenberg, N. H. Gerber, C. E. Furlong, *Arch. Biochem. Biophys.* 161 (1974) 64–75.
16. S. Rios, A. M. Pedregosa, I. F. Monistrol, F. Laborda, *Fems Microbiol. Lett.* 112 (1993) 35–42.
17. J. J. Caguit, A. L. Wilson, A. O. Summers, *J. Bacteriol.* 181 (1999) 3462–3471.
18. T.-Y. Wong, *J. Bacteriol.* 122 (1990) 101–106.
19. B. D. Feilmeier, G. Iseminger, D. Schroeder, H. Webber, G. J. Philip, *J. Bacteriol.* 182 (2000) 4068–4076.

Kinetička svojstva α -galaktozidaze i lokalizacija ukupnih proteina u *Erwinia chrysanthemi*

Sažetak

Erwinia chrysanthemi je enterobakterija koja uzrokuje meko truljenje biljaka, što tijekom godine uzrokuje velike ekonomske gubitke. Da bi patogena bakterija mogla preživjeti u biljci, treba koristiti lako asimilirajuće spojeve iz sokova biljke te razgraditi njezino tkivo. Stoga *Erwinia chrysanthemi* proizvodi nekoliko ekstra- i intracelularnih enzima. Od intracelularnih enzima posebnu skupinu čine galaktozidaze, koje se nalaze u perioplazmi ili u citoplazmi. Poznato je da α -galaktozidaza razgrađuje melobiozu i rafinozu u glukozu i galaktozu, odnosno u galaktozu i saharozu. Svrha je ovoga rada bila ispitati kinetička svojstva α -galaktozidaze u *Erwinia chrysanthemi* te lokalizaciju ukupnih proteina nakon uzgoja u prisutnosti rafinoze ili melibioze. Čini se da je α -galaktozidaza koja razgrađuje melibiozu isti enzim kao i onaj koji razgrađuje rafinozu u *Erwinia chrysanthemi*. Enzim je smješten uglavnom u citoplazmi, a u perioplazmi je nađeno samo između 2,4 i 5,4 % α -galaktozidaze.