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α-GALACTOSIDASE OF *POTERIOOCHROMONAS MALHAMENSIS*

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Key Word Index—*Poterioochromonas malhamensis*; alga; α-galactosidase; isofloridoside; osmotic regulation; stability.

Abstract—In cell-free extracts of *Poterioochromonas malhamensis*, α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) was stable at pH 8. The pH optimum of the enzyme was 7. The enzyme was purified 10-fold through chromatographic steps involving DEAE-cellulose, hydroxylapatite and Sephadex G-200. The apparent MW was 360 000 by sucrose density-gradient centrifugation. All activity was lost on subjecting the enzyme to polyacrylamide-gel electrophoresis. The substrate specificity of the enzyme was examined and some kinetic values determined. The enzyme displayed an unusual activity curve with respect to isofloridoside.

INTRODUCTION

The golden-brown alga, *Poterioochromonas malhamensis* Peterfi (syn: *Ochromonas malhamensis* Pringsheim) is a unicellular, wall-less flagellate which displays a special mechanism of osmotic regulation [1–3]. Osmotic solutes cause the cell to shrink within 1–3 min, though during the following 1–2 hr the cell volume recovers [4]. This recovery is mainly mediated by an increase in the internal concentration of isofloridoside (IF; *O*-α-D-galactopyranosyl-(1 → 1)-glycerol) [4–6]. On the other hand, reducing the osmotic pressure outside the cell (produced by dilution with water) causes a rapid fall in the internal IF level [1, 2]. During the latter process the carbon of both glycerol and galactose moieties is incorporated into a reserve β-(1 → 3)-glucan in the cell [4–6].

Pulse–chase experiments showed that a fast turnover of IF occurs even at constant size of IF-pool [7]. An α-galactosidase which is implicated in the breakdown of IF into galactose and glycerol has been detected in crude extracts of the alga, and it has been shown that the activity of the enzyme in the cell increases under conditions of elevated external osmotic pressure. This was considered to be due to *de novo* synthesis of the enzyme [8]. In the present communication we describe the isolation of the α-galactosidase and some of its properties.

RESULTS AND DISCUSSION

Isolation of α-galactosidase and its stabilization

The enzyme extract, prepared from the algal cells using a French press had a lighter green colour and higher specific activity (1.25-fold) compared to that obtained using the sonicator; the total activity in the former enzyme preparation was also higher by 1.7-fold, therefore this technique was used. The intact cell suspension displayed no enzyme activity showing the absence of extracellular α-galactosidase and that the 4-methyl-umbelliferyl substrate was unable to diffuse through the cell membrane. The enzyme extract from cells of 5-day-old cultures showed the highest specific activity (2.3 m-units/mg protein); thus the cultures of this stage of growth were used for further experiments.

The stability of the enzyme in crude preparation at pH 7.2 is shown in Fig. 1a; at 0° it lost 55% of its activity in 1 hr and this loss was 85% at 25°. There was no protection of the loss by bovine serum albumin (BSA) and phenylmethyl sulphonyl fluoride; the latter is known to retard proteolytic activity. The SH-protecting reagents, dithiothreitol (DTT) and mercaptoethanol (ME), stabilized the enzyme to some extent (Fig. 1b) but the loss of activity was total on overnight storage at 4°. α-Galactosidase of *Escherichia coli* is also unstable and several investigators failed to demonstrate its activity in cell-free extracts [9–12]. It was later shown that the enzyme requires NAD^+ and Mn^{2+} for activity [13–15]. In the case of *P. malhamensis*, these co-factors showed no stabilizing effect. On the other hand, α-galactosidases from higher plants are generally stable in a pH range of 4–6 and do not require any co-factors [16].

The effect of glycerol, sorbitol, sucrose, $CaCl_2$ and NaCl in stabilizing the enzyme was also examined; no significant protection was shown. In all cases there was complete inactivation on overnight storage. The stabilization was finally achieved by increasing the pH of the enzyme extract to 8 (Fig. 1c). The pH of the extraction buffer was therefore altered to 8 in future experiments (cf. Experimental).

Purification

The crude extract, prepared by using 10 mM Tris–HCl buffer, pH 8 that contained 5 mM ME from the cells of 5-day-old culture, was subjected to various purification

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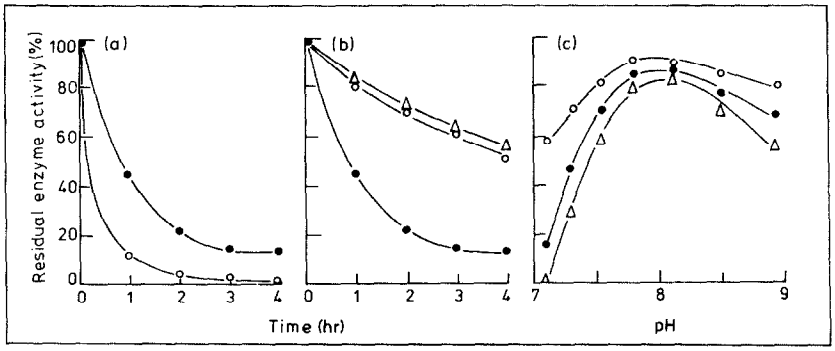

Fig. 1. Stability of α-galactosidase from *P. malhamensis*. (a) Effect of temperature: After holding the crude enzyme extract at 0° (●) and 25° (○), respectively, for various time intervals in 50 mM Tris–HCl buffer, pH 7.2, the activity was assayed as described in the Experimental. (b) Effect of SH-protecting reagents: Crude enzyme extract was held at 0° in the presence of 5 mM DTT (△) and 5 mM ME (○) in the buffer as above and the enzyme activity compared with that of control (●). (c) Effect of pH: Crude enzyme extracts were held at 0° at various pH values of 50 mM Tris–HCl buffer containing 5 mM ME, for 4 hr (○), 24 hr (●) and 72 hr (△), and then assayed for activity.

steps. Preliminary experiments showed that fractionation methods, using $(NH_4)_2SO_4$, acetone (at −10°) and lowering of pH, were ineffective due to rapid inactivation, though these purification steps have been successfully used in purifying higher plant α-galactosidases [16–19].

Ion-exchange filtration using DEAE-cellulose column (Whatman DE-52; 1 cm × 12 cm) equilibrated with the enzyme extraction buffer, pH 8, resulted in a 2-fold purification. The enzyme was not bound to the column but most of the pigments were retained. A CM-cellulose ion-exchanger could not be used because of the instability of the enzyme in acidic pH range. Further column chromatography of this enzyme preparation using hydroxylapatite brought about an additional 3.5-fold purification. The enzyme was eluted with 0.2 M NaCl. Finally, Sephadex G-200 gel filtration (using the extraction buffer for elution) gave active fractions that were in the exclusion volume of the elution profile; the enrichment of the enzyme was 1.5-fold in this step. Results of purification are summarized in Table 1. Active fractions from each step of purification were pooled and concentrated by ultrafiltration using XM-300 membrane (Amicon) which has a cut-off limit of MW 300 000.

General properties

The pH–activity curve of the purified enzyme shows a well-defined optimum at pH 7 (half maximal activity at pH 6.7 and 7.7), whereas higher plant α-galactosidases generally display pH optima in the acidic region [16]. When the pH of the enzyme preparation was brought to 5, a proteinous precipitate appeared which possessed the α-galactosidase activity. The precipitate could be redissolved in 10 mM Tris–HCl buffer, pH 8; however, the recovery of the enzyme was only 5%. As regards the stability, the purified enzyme behaved in an identical way as the crude preparation (see Fig. 1) hence the low recovery when precipitated at pH 5.

The enzyme was inhibited by *p*-chloromercuribenzoate (PCMB) and $HgCl_2$, and the nature of the inhibitory effect was competitive (PCMB, $K_i = 3\,\mu M$; $HgCl_2$, $K_i = 25\,\mu M$). This suggests involvement of SH groups in the enzyme activity. It is also worth noting that the SH-

Table 1. Purification of α-galactosidase from *P. malhamensis*

Step	Volume (ml)	Enzyme activity (m-units/ml)	Protein concn (mg/ml)	Specific activity (m-units/mg)	Purification* (-fold)	Yield* (%)
(1) Cell-free extract and concentration	39	9.6	4.2	2.3		
(2) DE-52 chromatography and concentration	10	33.6	7.3	4.6	2.0	90
(3) Hydroxylapatite chromatography and concentration	5	44.5	2.8	15.9	6.9	59
(4) Sephadex G-200 gel-filtration and concentration	5	38.0	1.6	23.7	10.3	51

The crude extract was prepared from 200 ml suspension made from a 5-day-old cell culture. Experimental details are given in the Experimental.

* Expressed in comparison to step (1).

Table 2. Rates of hydrolysis of various substrates by α-galactosidase from *P. malhamensis*

Substrate	Substrate concn in assay (mM)	Enzyme in assay (mg/ml)	Rate of hydrolysis (nmol/min/mg)
Isofloridoside	10	1.4	3.1
4-Methylumbelliferyl α-D-galactoside	0.9	0.014	20.5
p-Nitrophenyl α-D-galactoside	0.9	0.14	24.0
Isofloridoside phosphate	10	2	0
Floridoside	10	2	0
Raffinose	10	2	0

Methods of enzyme assay were the same as described in the Experimental.

protecting reagents, DTT and ME, confer some stability (Fig. 1b). At 3.5 mM, galactose and EDTA inhibited the enzyme by 33 and 65%, respectively. Inorganic ions K^+, Na^+, Ca^{2+} and Mg^{2+} had no apparent effect on the enzyme activity up to 0.1 M. The metabolite, glycerol-3-P, inhibited the enzyme by 45% at 0.1 M. This concentration is rather high and the effect may not be significant in the overall regulation of the enzyme. Glycerol, mannitol and sucrose had no effect at this concentration.

Unlike α-galactosidase-I of *Vicia faba*, the purified enzyme (at pH 8) lost no activity on freezing and thawing. It was also stable to freeze-drying (cf. ref. [20]). The enzyme solution could be stored in 30% glycerol or 10% sucrose at −20° for 3 months without any significant loss of activity.

When the enzyme was subjected to polyacrylamide gel electrophoresis using 10 mM Tris–HCl buffer, pH 8.7 (see ref. [21]), no enzyme activity could be detected corresponding to any of the six protein bands that were obtained. Electrophoresis using 1 mM Tris–HCl buffer and 1 mM phosphate buffer of pH 8 also did not produce an active band. It is possible that during electrophoresis some factor(s) is removed which is otherwise essential for the activity of the enzyme.

The enzyme was eluted in the exclusion volume when subjected to gel-filtration using Sephadex G-200 (exclusion limit of MW 8×10^5) and Sepharose 6B (exclusion limit of MW 4×10^6). This would imply that the MW of the enzyme is higher than 4×10^6, however, it holds true only if the protein is globular in nature. The MW was also determined by the sucrose density-gradient centrifugation method [22] and the apparent value was found to be 360 000. The marker proteins were urease (480 000), catalase (240 000), alcohol dehydrogenase (141 000) and BSA (67 000); the values in parentheses are the respective MWs. Thus the results from the above gel chromatography studies only tentatively indicate that the α-galactosidase is either not a globular protein or undergoes aggregation during gel filtration.

Substrate specificity

The rates of hydrolysis of some substrates by the purified enzyme are shown in Table 2. Synthetic substrates were hydrolysed by the enzyme from *P. malhamensis* at faster rates than the natural substrate IF, as also observed with other α-galactosidases [23, 24]. It is of much interest that, whereas the enzyme hydrolyses IF, no detectable hydrolysis occurs of the structurally related isofloridoside phosphate (IFP) and floridoside, even on longer incubation using higher enzyme concentrations. The enzyme also showed no measurable hydrolysis of galactinol, guar galactomannan, melibiose, planteose, sesamose and stachyose which are generally hydrolysed by higher plant α-galactosidases [3, 25].

The K_m values for *p*-nitrophenyl α-D-galactoside and 4-methylumbelliferyl α-D-galactoside were 0.71 and 0.45 mM, respectively. Unlike some higher plant α-galactosidases [16], this enzyme showed no inhibition at higher substrate concentrations. However, when IF was used as the substrate, the curve of the rate of hydrolysis vs substrate concentration was S-shaped, but with a relatively high activity at the lowest IF concentrations used. This may be of particular significance in the *in vivo* catabolism of IF, especially when the level of this metabolite builds up in response to high osmotic pressure outside the cell [8].

IFP is the precursor of IF, and the former's synthesis, under osmotic stress conditions, is regulated through proteolytic activation of IFP-synthase [26–28]. *In vivo* experiments showed that IF is converted into a β-(1 → 3)-glucan [5, 6]; this would therefore require α-galactosidase and galactokinase in the initial stages of conversion. Galactokinase has recently been detected in *P. malhamensis* and it has been shown that its activity increases when higher osmotic pressure is applied outside the cell [29].

In conclusion, it may be said that α-galactosidase plays a role in the osmoregulation of *P. malhamensis* by degrading IF and thus providing products for transformation into high MW substances.

EXPERIMENTAL

Maintenance and growth of P. malhamensis. The algal cells were maintained and grown in nutrient medium containing glucose (10 mg/ml) as described earlier [7]. The cells were harvested by centrifugation at 500 g for 10 min, resuspended in the 1:2.4-fold diluted nutrient medium (60–70 milliosmolal) so that the 1:50-fold dilution of this suspension in H_2O gives an A_{510nm} of 0.5 (10 mm light path, Pye-Unicam SP30). Prior to use, the cell suspension was aerated for 30 min under the conditions of growth.

Preparation of cell-free extract. A known vol. of the cell suspension was centrifuged at 500 *g* for 5 min and the pellet of cells was immediately resuspended in an equal vol. of ice-cold 10 mM Tris–HCl buffer, pH 7.2 containing 5 mM ME. Cells were disrupted in a Yeda press at a N_2 pressure of 100 kg/cm^2. For comparison, cells were also disrupted using an ultrasonic disintegrator (Sonic 300, Braun) at full power. The exposures were in 4 × 15 sec bursts, interspersed with cooling in ice. The homogenate was subsequently centifuged at 50 000 *g* for 10 min and the pale green supernatant was collected.

Analytical determinations. Protein was routinely estimated using Coomassie brilliant blue G-250 reagent [30] and calibrated against ovalbumin (Grade VII, Sigma). The activity of α-galactosidase was assayed by incubating 50 μl of the enzyme extract with a mixture of 0.5 ml, 50 mM Pi buffer, pH 7 (containing 5 mM ME) and 25 μl aq. soln of 4-methylumbelliferyl α-D-galactopyranoside (11.5 mM) at 25° for 5 min. The reaction was stopped by adding 1 ml 0.2 M Na_2CO_3 and the liberated aglycone was estimated fluorimetrically using an Eppendorf 1101-M spectrophotometer fitted with a fluorescence measuring device. Excitation filter, 313–366 nm and emission filter, 470–3000 nm were used. The substrate blanks were subtracted from the assay readings and, finally, quantitative values were calculated with the aid of a standard curve prepared under the assay conditions using 4-methylumbelliferone. In the assay, the release of the product was linear with time and was proportional to the amount of enzyme used. The hydrolysis of *p*-nitrophenyl α-D-galactoside was monitored under the above assay conditions by measuring the release of *p*-nitrophenol at 405 nm as described earlier [24]. One unit of α-galactosidase activity is expressed as the amount of enzyme catalysing the hydrolysis of 1 μmol of substrate/min. Sp. act. is expressed as m-units of enzyme per mg protein. Inhibition studies were carried out under the conditions of assay using 4-methylumbelliferyl α-D-galactoside as the substrate.

D-galactose, the hydrolysis product of the α-galactosidase action on the natural substrates as used in the specificity expts, was determined [31] using D-galactose dehydrogenase (Boehringer–Mannheim, W. Germany). The reaction mixture consisted of 750 μl 100 mM Tris buffer, pH 8.6; 25 μl 16.5 mM NAD^+ soln; 5 μl D-galactose dehydrogenase (25 U/ml) and 50 μl of the D-galactose-containing soln. This was incubated at 37° for 20 min and the A_{334nm} was measured. An appropriate control was run which had no galactose; the final quantitative value was determined by constructing a standard curve using galactose (0–100 nmol).

Floridoside was a gift from Professor W. Z. Hassid, University of California, Berkeley. IFP was prepared according to the method described earlier [32] and finally eluted from the electrophoretograms. IF was prepared by extracting the *P. malhamensis* cells which had previously been subjected to osmotic shock (with a final concn of 0.1 M NaCl) and stood in suspension for 5 hr (see ref. [4]) with boiling 80% EtOH. The EtOH extract was evapd to dryness and the residue dissolved in H_2O and washed with $CHCl_3$. The H_2O extract was concd, deionized with Dowex-1 and 50, and purified by HPLC; the IF fraction was finally freeze-dried.

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REFERENCES

1. Kauss, H. (1979) *Progress in Phytochemistry*, pp. 1–27, Pergamon Press, Oxford.
2. Kauss, H. (1979) *Ber. Dtsch. Bot. Ges.* **92**, 11.
3. Dey, P. M. (1980) *Adv. Carbohydr. Chem. Biochem.* **37**, 283.
4. Schobert, B., Untner, E. and Kauss, H. (1972) *Z. Pflanzenphysiol.* **67**, 385.
5. Kauss, H. (1967) *Z. Pflanzenphysiol.* **56**, 453.
6. Kauss, H. (1967) *Nature* **214**, 1129.
7. Kauss, H. (1973) *Plant Physiol.* **52**, 613.
8. Kreuzer, H. P. and Kauss, H. (1980) *Planta* **147**, 435.
9. Lester, G. and Bonner, D. M. (1957) *J. Bacteriol.* **73**, 544.
10. Porter, C. J., Holmes, R. and Crocker, B. F. (1953) *J. Gen. Physiol.* **37**, 271.
11. Hogness, D. S. and Battley, E. H. (1957) *Fed. Proc.* **16**, 197.
12. Pardee, A. B. (1957) *J. Bacteriol.* **73**, 376.
13. Burstein, C. and Kepes, A. (1966) *C. R. Acad. Sci.* **262**, 227.
14. Burstein, C. and Kepes, A. (1971) *Biochim. Biophys. Acta* **230**, 52.
15. Schmitt, R. and Rotman, B. (1966) *Biochem. Biophys. Res. Commun.* **22**, 473.
16. Dey, P. M. and Pridham, J. B. (1972) *Adv. Enzymol.* **36**, 91.
17. Malhotra, O. P. and Dey, P. M. (1967) *Biochem. J.* **103**, 508.
18. Dey, P. M. and Pridham, J. B. (1969) *Biochem. J.* **113**, 491.
19. Dey, P. M. and Wallenfels, K. (1974) *Eur. J. Biochem.* **50**, 107.
20. Williams, J., Villarroya, H. and Petek, F. (1978) *Biochem. J.* **175**, 1069.
21. Villarroya, H., Williams, J., Dey, P. M., Villarroya, S. and Petek, F. (1978) *Biochem. J.* **175**, 1079.
22. Martin, R. G. and Ames, B. N. (1960) *J. Biol. Chem.* **236**, 1372.
23. Malhotra, O. P. and Dey, P. M. (1967) *Biochem. J.* **103**, 739.
24. Dey, P. M. and Pridham, J. B. (1969) *Biochem. J.* **115**, 47.
25. Dey, P. M. (1978) *Adv. Carbohydr. Chem. Biochem.* **35**, 341.
26. Kauss, H. and Quader, H. (1976) *Plant Physiol.* **58**, 295.
27. Kauss, H., Thomson, K., Tetour, M. and Jeblick, W. (1978) *Plant Physiol.* **61**, 35.
28. Kauss, H., Thomson, K., Thomson, M. and Jeblick, W. (1979) *Plant Physiol.* **63**, 455.
29. Dey, P. M. (1980) *FEBS Letters* **112**, 60.
30. Sedmak, J. J. and Grossberg, S. E. (1977) *Analyt. Biochem.* **79**, 544.
31. Kurz, G. and Wallenfels, K. (1970) in *Methoden der Enzymatischen Analyse* (Bergineyer, H. U., ed.) pp. 1241–1244. Verlag Chemie, Weinheim.
32. Kauss, H. and Sochobert, B. (1971) *FEBS Letters* **19**, 131.