Pergamon

PII: S0031-9422(97)50466-4

Phytochemistry, Vol. 46, No. 8, pp. 1331–1334, 1997

Printed in Great Britain
0031-9422/97 $17.00+0.00

TREHALOSE, AN EXTREME TEMPERATURE PROTECTOR OF PHOSPHOENOLPYRUVATE CARBOXYLASE FROM THE C_4-PLANT *CYNODON DACTYLON*

GEORGE SALAHAS, BASILIOS PESLIS, CHRISTOS D. GEORGIOU*† and NIKOS A. GAVALAS‡

Department of Biology, Laboratory of Plant Physiology, University of Patra, Patra-26100, Greece; * Department of Biology, Section of Genetics, Cell and Developmental Biology, University of Patra, Patra-26100, Greece

(*Received in revised form* 28 *April* 1997)

Key Word Index—*Cynodon dactylon*; Gramineae; phosphoenolpyruvate carboxylase; cold and heat inactivation; enzyme stabilization; cosolutes.

Abstract—High concentration (1.25 M) of trehalose fully protects the activity of phosphoenolpyruvate carboxylase (PEPC, E C 4.1.1.31) from the C_4-plant *Cynodon dactylon* against cold inactivation (at 0°) and heat inactivation (at 37°). The concentration of glycerol needed for the same degree of protection was much higher (2.7 M). Phosphates at 100 mM only partially protect the enzyme against cold inactivation and at 60 mM fully protect it against heat inactivation. At temperatures higher than 45° the enzyme was better protected by trehalose than glycerol or phosphates. © 1997 Elsevier Science Ltd

INTRODUCTION

Certain compounds, known as natural cosolutes, have been widely used as stabilizers during extraction and preservation of animal and plant oligomeric proteins, protecting them against a variety of adverse conditions *in vitro* [1–3]. Among them there is the nonreducing disaccharide trehalose, widely distributed among living organisms, which has been used as stabilizer for nonplant proteins and organelles. It is found at particularly high concentrations (20% dry weight) in unhydrobiotic organisms [4]. It is also found in desiccation tolerant organisms [5] and in resurrection plants [6, 7], suggesting that this molecule may be involved in the desiccation tolerance of these organisms. In the last decade, the stabilizing effect of trehalose as cosolvent has been used in cryopreservation of mouse embryos [8], yeast cells [9] and as stabilizing agent on membrane phospholipids [10]. It has also been used as a stabilizer of the activity of oligomeric enzymes such as methanol dehydrogenase from *Hypomicrobium* X [11] and rabbit muscle phosphofructokinase [12]. So far, trehalose has not been used as a stabilizer of enzymes from plant sources. This work attempts for the first time to use this natural cosolute as stabilizer of the plant oligomeric enzyme phospoenolpyruvate carboxylase (PEPC) which is known to be especially sensitive under low and high temperatures [13, 14].

RESULTS AND DISCUSSION

In preliminary experiments, time courses of cold or heat inactivation where performed in the absence of stabilizers and at various protein concentrations in order to determine the concentration of proteins in which a considerable loss of activity was observed. For cold inactivation experiments, samples of dilute PEPC (14 μg ml^{-1} protein) were incubated for 45 min at 0° in the presence of increased concentrations of trehalose (0.2 to 1.25 M), and the % residual activity was measured (Table 1). The concentration of tre-

Table 1. Effect of trehalose, glycerol and phosphates on PEPC cold inactivation. PEPC was incubated at 0°C and pH 7.2 for 45 min in the presence of trehalose, glycerol and phosphates. Enzyme concentration was (14 μg ml^{-1} protein)

Added substance	% residual activity
None	34±3
Trehalose (0.2 M)	41±2
Trehalose (0.5 M)	67±2
Trehalose (1.0 M)	81±4
Trehalose (1.25 M)	91±5
Glycerol (2.7 M)	91±2
Phosphate (0.1 M)	62±3
Phosphate (0.1 M)+Trehalose (1.25 M)	90±4
Phosphate (0.1 M)+Glycerol (2.7 M)	93±3

† Author to whom correspondence should be addressed.
‡ Deceased, September 1995.

halose that resulted in a near full protection (91%) of PEPC activity against cold inactivation was 1.25 M. Other additives were tested in place of trehalose as indicated in Table 1. Glycerol gave the same degree of protection as trehalose at a much higher concentration (2.7 M), while high concentration of phosphates (100 mM) only partially protected (62%) the enzymic activity. Phosphates in mixture with trehalose or glycerol did not improve the protective effect that these cosolvents had when used alone.

The *in vitro* cold inactivation of PEPC from C_4 plants is well documented from previous workers [13, 15, 16]. Cold temperature, by weakening the hydrophobic intramolecular interactions and/or changing the pK values of ionizable groups, decreases the enzymic integrity, leading to inactivation (via dissociation) of the protein molecule [13, 16]. This structural instability is independent of, and additive to that caused by dilution-mediated deoligomerization [13]. There is evidence consistent with the idea that PEPC exists at a simple equilibrium between an active tetrameric form and an inactive (or less active) dimeric form [17, 18]. Dilution (through mass action) favours the conversion to the dimeric form and, thus causes inactivation. There have been many reports with various enzymes of inactivation due to dilution and/or cold-mediated deoligomerization [3, 19, 20].

In addition, the *in vitro* heat inactivation has been also studied in several oligomeric enzymes with a wide range of thermal sensitivities [21, 22]. Heat inactivation of PEPC from *Escherichia coli* and of the leaves of the C_4 plant *Eleusine indica* has been also studied [14, 23]. In subsequent experiments, portions of the enzyme (14 μg m^{-1} protein) were heated at 37°C for various intervals of time up to 30 min and the residual activity was recorded [Fig. 1(a)]. For testing the effect of trehalose on PEPC heat inactivation, we chose 20 min as the incubation control interval, at which the residual activity of the enzyme was 17%. The effect of different concentrations of trehalose and glycerol [Fig. 1(b)] as well as those of phosphates [Fig. 1(c)] were tested on the residual activity of the enzyme that was incubated under the previous control conditions. The inclusion of 1.2 M trehalose, 2.7 glycerol and 60 mM phosphates during incubation showed a 93 to 98% conservation of activity during heating. Having established the optimal concentrations of these stabilizing agents under these conditions, we tested them at different temperatures (Fig. 2). The unprotected enzyme completely lost its activity at 45°, while in the presence of the tested additives it preserved 80 to 98% of its activity up to 45°. At higher temperatures (47 and 50°) trehalose proved to be a better protectant of PEPC than glycerol and phosphates. Both cold and heat inactivation of oligomeric enzymes, as well as their destabilization due to dilution, has been diminished or prevented by the inclusion of high concentrations of natural cosolutes (glycerol, proline, sorbitol, PEGs and sugars) [17, 20, 21] and of phosphates [24] into the enzymic preparations.

The data presented in this work clearly show that trehalose at high concentrations (above 1.2 M) acts as a very effective natural cosolute by protecting diluted

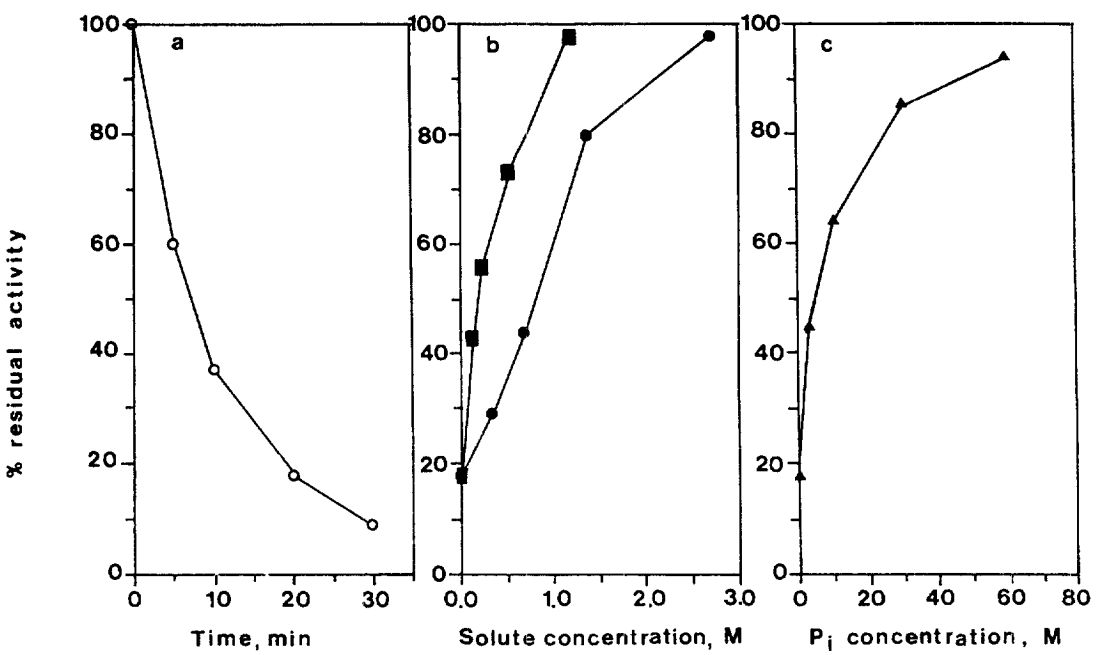

Fig 1. Heat inactivation of PEPC at 37° and pH 7.2 in the presence of trehalose, glycerol and phosphates. (a) Enzyme heated at different times in the absence of cosolutes. (b) Enzyme heated for 20 min in the presence of different concentrations of trehalose (■) and glycerol (●). (c) Enzyme heated for 20 min in the presence of different concentrations of phosphate. The concentration of enzyme was 14 μg ml^{-1} protein.

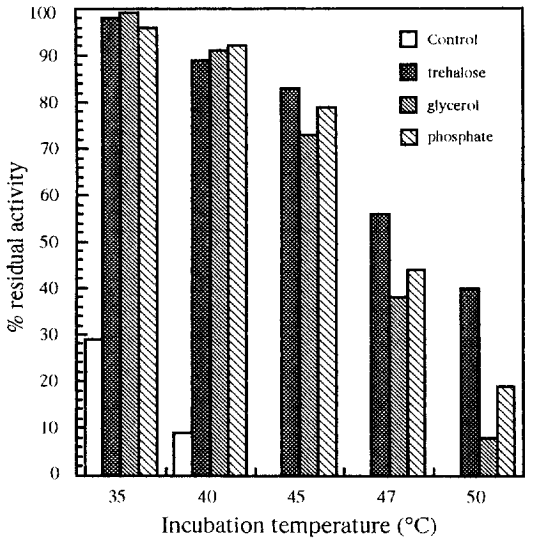

Fig 2. A histogram showing heat inactivation of PEPC. The enzyme (14 μg ml^{-1} protein) was incubated for 20 min at different temperatures at pH 7.2 in the presence and absence of 1.25 M trehalose, 2.7 M glycerol and 0.1 M phosphate.

PEPC preparations from *Cynodon dactylon* leaves from cold and heat inactivation. It confers better protection than glycerol or phosphates during cold (Table 1) and heat treatment (Figs 1 and 2). The molecular basis of trehalose action on the quaternary structure of diluted PEPC could be interpreted by the exclusion volume theory [25]. According to this theory, trehalose stabilizes the enzyme through preferential exclusion from the protein domain, resulting in a mutual repulsion between the enzyme and the sugar molecule. The enzyme molecules are confined into a small fraction of the total volume and the weak intrinsic forces maintaining their structural integrity are strengthened, leading to increased apparent protein concentration. The use of natural cosolutes as stabilizers, acting by increasing the apparent enzymic protein, makes the *in vitro* studies more relevant to the *in vivo* situation in a way that mimics experimentally the aqueous microenvironment of the cell.

EXPERIMENTAL

Cynodon dactylon (L.) Pers. plants, grown in pots in a greenhouse under natural illumination, were used as experimental plants. For the partial purification of PEPC, 6 g of fully expanded leaves, taken during daylight, were ground in a prechilled mortar with purified sea sand, a small amount (0.6 g) of insoluble PVP (M_r 10 000) and 30 ml of extraction medium (100 mM Tris–HCl, pH 7.2, 1 mM EDTA, 10 mM $MgCl_2$, 3% PVP and 20% glycerol). The extract was centrifuged at 18 000 *g* for 10 min. PEPC from the supernatant was pptd between 7 and 30% PEG-6000. After centrifugation the pellet was dissolved in 2.5 ml of 40 mM K-Pi buffer, pH 7.2 (plus 10 mM $MgCl_2$ and 20% glycerol) and applied on a DEAE-cellulose column (1.8 × 10 cm) equilibrated with the same medium. Proteins were elluted with a linear 40–400 mM K-Pi buffer, pH 7.2 and 40 frs (4 ml each) were collected. The most active frs were pooled together and the total proteins were pptd with PEG-6000 (final concn of 30%) by centrifugation at 18 000 *g* for 10 min. The ppt was resuspended in 100 mM HEPES–KOH buffer, pH 7.2 (plus 10 mM $MgCl_2$ and 5 mM DTT) and desalted through a Sephadex G 25-300 column equilibrated with the same buffer. The collected PEPC that was used in our assays had sp. act. of 16 μmol min^{-1} mg^{-1} protein at pH 8.0. All the above steps were carried out at room temp. PEPC incubation conditions at low or high temp. are indicted in Figs and Table.

Assays for PEPC activity were run at 30° in 3 ml (final vol.) of 100 mM Tris–HCl, pH 8.0, 5 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.14 mM NADH, 4.5 units of malate dehydrogenase (pig heart, Sigma), phosphoenolpyruvate at 3.2 mM (substrate satn concn) and other additives as indicated in Figs and Table. The reaction was started with the addition of the enzyme [26] and the reaction rate was measured by the decrease in A at 340 nm (oxidation of NADH). Protein measurements were made with the Folin phenol reagent [27].

Acknowledgements—This work was partially supported by the Greek General Secretary of Research and Technology (project 91 ED 412).

REFERENCES

1. Pollard, A. and Wyn Jones, R. G., *Planta*, 1979, **144**, 291.
2. Rhodes, D. and Hanson, A. D., *Molecular Biology*, 1993, **44**, 357.
3. Bock, P. E. and Frieden, C., in *Trends in Biochemical Science*, 1978, **4**, 100.
4. Crowe, J. H. and Crowe, L. M., *Science*, 1984, **223**, 701.
5. Winkler, K., Kienle, I., Burgert, M., Wagner, J. C. and Holrer, H., *FEBS Letters*, 1992, **291**, 269.
6. Adams, R. P., Kendall, E. and Kartha, K. K., *Biochemical Systematics and Ecology*, 1990, **8**, 107.
7. Bianchi, G., Gamba, A., Limiroli, R., Pozzi, N., Elster, R., Salamini, F. and Bartels, D., *Physiologia Plantarum*, 1993, **87**, 223.
8. Seok, H. B., Lee, K. W. and Son, D. S., *Korean Journal of Animal Sciences*, 1992, **33**, 495.
9. Berny, J. F. and Hennebert, G. L., *Mycologia*, 1992, **83**, 805.
10. Rudolph, A. S., Crowe, J. H. and Crowe, L. M., *Archives of Biochemistry and Biophysics*, 1986, **245**, 134.
11. Argall, M. E. and Smith, G. D., *Biochemistry and Molecular Biology International*, 1993, **30**, 491.
12. Carpenter, J. F., *Archives of Biochemistry and Biophysics*, 1986, **250**, 505.

13. Angelopoulos, K. and Gavalas, N. A., *Journal of Plant Physiology*, 1988, **132**, 714.
14. Rathnam, C. K. M., *Planta*, 1978, **141**, 289.
15. Kleczkowski, L. A. and Edwards, G. E., *Zeitschurft für Naturfòrsching*, 1990, **45**, 42.
16. Krall, J. P. and Edwards, G. E., *Plant Cell Physiology*, 1993, **34**, 1.
17. Selinioti, E., Nikolopoulos, D. and Manetas, Y., *Australian Journal of Plant Physiology*, 1987, **14**, 203.
18. Wu, M.-X., Meyer, C. R., Willeford, K. O. and Wedding, R. T., *Archives of Biochemistry and Biophysics*, 1990, **281**, 324.
19. Kono, N. and Ueda, H., *Journal of Biological Chemistry*, 1973, **248**, 8603.
20. Salahas, G., Manetas, Y. and Gavalas, N. A., *Photosynthesis Research*, 1990, **26**, 9.
21. Gerlsma, S. Y. and Stuur, E. R., *International Journal of Peptide and Protein Research*, 1972, **26**, 65.
22. Paleg, L. G., Douglal, T. J., van Daal, A. and Keech, D. B., *Australian Journal of Plant Physiology*, 1981, **8**, 107.
23. Izui, K., Nishikido, T., Ishihara, K. and Katsuki, H., *Journal of Biochemistry*, 1970, **68**, 215.
24. Salahas, G. and Gavalas, N. A., *Photosynthetica*, 1997, **33**, 189.
25. Timasheff, S. N., in *Water and Life*, ed. C. B. Somero, C. B. Osmond and C. L. Bolis. Springer, Berlin, 1992 p. 70.
26. Angelopoulos, K., Stamatakis, K., Manetas, Y. and Gavalas, N. A., *Photosynthesis Research*, 1988, **18**, 317.
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., *Journal of Biological Chemistry*, 1951, **193**, 265.