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Partial purification and characterization of enzymes degrading trehalose in mycobacteria

Wstępne oczyszczanie i charakterystyka enzymów degradujących trehalozę w prątkach

Trehalose (α–D–glucopyranosyl–α–glucopyranoside) is the major hemolymph sugar of insects (1), storage compound and stress protectant in yeast and other fungi (13), stress protectant and carbon and enegy source in some bacteria (11). Biosynthesis of trehalose is catalyzed by trehalose–6––phosphate synthase/phosphatase enzyme system (4), while its degradation is mainly mediated by a widespread hydrolic enzyme – trehalase (EC 3.2.1.28), which is present in some organism in two forms: regulated (by cAMP) and non–regulated one (2).

In mycobacteria, trehalose occurs both as a free sugar in cytosol and as a constituent of cell wall glycolipids, like for instance trehalose dimycolate (cord factor) and other acylated and/or sulphated trehaloses (9, 10). Here, too, its biosynthesis is catalyzed by a similiar enzyme system as described above (3, 7). Trehalase has also been detected in mycobacteria, yet the enzyme is suggested to be membrane–bound (8).

In our study on overall metabolism of trehalose and its derivatives in mycobacteria, we have found two enzyme activites degrading this disaccharide. In this report preliminary results of their characterization are presented.

MATERIAL AND METHODS

Trehalase preparations. The enzymes were prepared from *Mycobacterium* sp. 279, grown for 4–5 days at 37°C on a liquid glucose–glutamate–citrate–salts medium (pH 7.0) in Roux flasks (15). The bacteria (60 g) were suspended in 300 ml 10 mM phosphate buffer, pH 7.5 containing 1 mM 2–mercaptoethanol, sonicated 2 x 5 min (MSE, 24 kHz/100 W) and centrifugated for 30 min at 17,000 x g. The cell–free extract was incubated with DN–ase (1 mg/100 ml, Sigma) at 4°C overnight, then treated with ammonium sulfate to obtain 0.35 – 0.40 saturation fraction (AS–Fraction). The AS–fraction was dialyzed overnight against 25 mM phosphate buffer, pH 7.5 containing 1 mM

2–mercaptoethanol and applied on a DEAE–cellulose column (2.5 x 23), which was equilibrated with the same buffer. The column was washed with the buffer, then the enzymes were eluted with a linear gradient (400 µl) of NaCl 0 – 0.5 M in the buffer. The trehalase activity peak fraction were pooled, concetrated by ultrafiltration (Amicon) and dialyzed aganist the extraction buffer.

Assays. Trehalase I was assayed in a standard system (40 µl) containing 3 mM MgCl2, 50 mM trehalose, 50 mM phosphate buffer, pH 7.5 and enzyme preparations. Trehalase II was assayed in a standard system (40 µl) containing 50 mM trehalose, 25 mM HEPES, pH 7.5 and enzyme. The incubation at 37°C lasted 10 to 30 min. Then, samples were kept in the boiling water bath for 5 min and centrifuged. A reducing power of the liberated glucose was determined with a reducing method.

Protein concentration was determined by the method of Lowry et al. (5). Paper chromatography was performed using Whatman no 3 paper, butanol: pyridine: water (4/3/1.5, v/v) as solvent and alkaline silver nitrate reagent for localization of sugars.

RESULTS AND DISCUSSION

Trehalose is present in mycobacteria both as a free sugar and as a constituent of cell–wall acylated and sulfated trehaloses (9, 10). The previous report (8) suggested the presence in mycobacteria a membrane–bound trehalase which could participate in the breakdown of this disacharide. In our study on metabolism of trehalose in mycobacteria, we have found only soluble trehalase in this microorganisms. To further characterize the enzyme, purified preparations were needed.

Glucose–grown cells of *Mycobacterium* sp. 279 were disrupted by sonication and resulted cell––free extract was fractionated at first with ammonium sulfate, then by means of a DEAE–cellulose column. Figure 1 shows the latter fractionation, using a linear gradient of NaCl concentration (0 – – 0.5 M). One can see two clearly separated peaks of trehalase activity which we named trehalase I and II. These peaks were separately pooled, concentrated and purified further on a Chelating Sepharose column (not shown). At this stage, trehalase I proved to be very labile and could be preserved only in the presence of phosphate ions.

When characterizing the enzymes, it appeared that both of them were most active at pH 7.5 (Fig. 2). However, they distinctly differ with their demand for metal ions. Trehalase I was found totally dependent on Mg^{2+} ions, and other ions tested were without effect (Table 1). On the other hand, trehalase II was not activated by tested ions, and some of them (i.e. Cu^{2+} and Ni^{2+}) even inhibited the enzyme at the concentration used of 2.5 mM.

An interesting feature of trehalase I is its strict demand for phosphate ions. In the absence of these ions the activity of trehalase I is very low or absent (Fig. 3). The saturation of this enzyme with phosphate was obtained at a concentration of about 25 mM.

The phenomenon of phosphate dependence can suggest various explanations. One of them is, that a phosphorylase reaction is involved. However, the only case of phosphorylase occurrence is the one described by Marechal and Belocopitow (6) for *Euglena gracilis*, the organism which does not possess trehalase activity. Temporarily, we are not able to identify glucose–1–phosphate as one product of the postulated phosphorylase reaction (the other should be glucose). The only product we found was glucose. This dose not exclude a possibility of the phosphate ester breakdown, or its use in other reactions (i.e. polimerization). The other possibility is an activation of trehalase I by phosphate. Such an activation was described for trehalase in insect (1). Yet, the enzyme preparations from the insect *Galleria mallonella* neither contained phosphorylase, nor was activated by phosphates (1). In fungi

Table 1. Effect of metal ions on the activity of trehalase I and II from *Mycobacterium* sp. 279

Ion (2.5 mM)	Trehalase I	Trehalase II
	activity (%)	
Mg^{2+}	100	100
Ca^{2+}	0	102
Co^{2+}	0	86
Cu^{2+}	0	54
Ni^{2+}	0	36
Fe^{2+}	0	100
Fe^{3+}	0	80
Nil	0	78

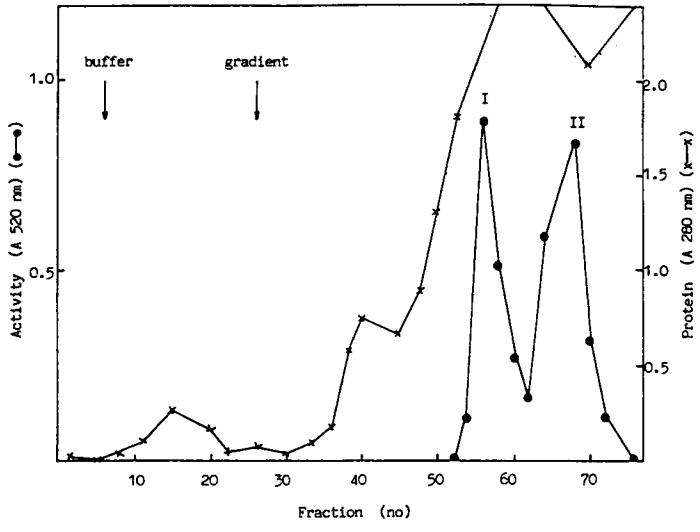

Fig. 1. Ion–exchange chromatography separation of trehalase I and II on DEAE–cellulose column. AS–fraction was applied on DEAE–cellulose column (2.5 x 23 cm) equilibrated with 25 mM phosphate buffer, pH 7.5, which contained 1 mM 2–mercaptoethanol. The enzymes were eluted with a linear NaCl gradient 0 – 0.5 M (400 ml) in the buffer

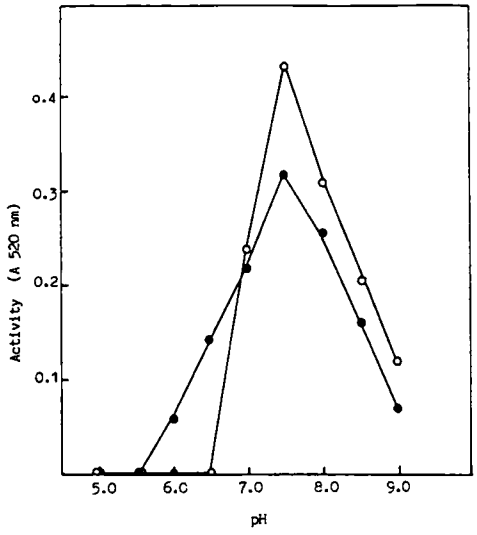

Fig. 2. Effect of pH on trehalase I (●–●) and II (o–o) activity. The buffers used were: acetate (pH 5.0), MES–NaOH (pH 5.5 – 6.5), HEPES–NaOH (pH 7.0 – 8.0) and glycine–NaOH (pH 8.5 – 9.0). In trehalase I 25 mM phosphate was present and pH was adjusted as shown above

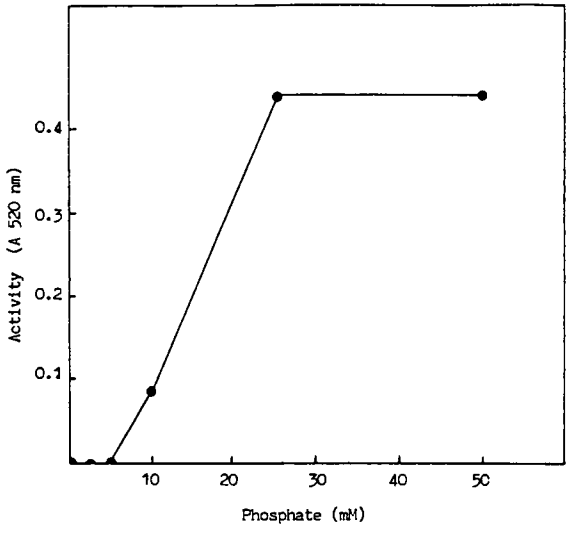

Fig. 3. Effect of phosphate concentration on trehalase I activity
The buffer was 100 mM HEPES–NaOH, pH 7.5

and yeast only trehalase is present (13). In the latter organisms two trehalases are present: acidic non–regulated trehalase of lysosome origin, and neutral cytoplasmic trehalase, an enzyme activated by cAMP (4). Thus, the mechanism of trehalase reaction in mycobacteria remains to be explained.

REFERENCES

1. Kalf G.F., Rieder S.V.: The purification and properties of trehalase. J. Biol. Chem., 230, 691, 1958.
2. Kopp M. et al.: Molecular analysis of the neutral trehalase gene from *Saccharomyces cerevisiae*. J. Biol. Chem., 268, 4766, 1993.
3. Lapp D. et al.: Properties of trehalose phosphate synthetase from *Mycobacterium smegmatis*. J. Biol. Chem., 246, 4567, 1971.
4. Londesborough J., Vuorio O.: Trehalose 6–phosphate synthetase (phosphatase complex from baker's yeast: purification of a proteolytically activated form. J. Gen. Microbiol., 137, 323, 1991.
5. Lowry O.H. et al.: Protein measurement with the Folin phenol reagent. J. Biol. Chem., 195, 265, 1951.
6. Marechal L.R., Belocopitow E.: Metabolism of trehalose in *Euglena gracilis*. J. Biol. Chem., 247, 3223, 1972.
7. Matula M. et al.: Partial purification and properties of a highly specific trehalose phosphate phosphatase from *Mycobacterium smegmatis*. J. Bacteriol., 107, 217, 1972.
8. Patterson B.W. et al.: Trehalase from *Streptomyces hygroscopicus* and *Mycobacterium smegmatis*. Meth. Enzymol., 28, 996, 1972.
9. Ratledge C.: [In:] Biology of Mycobacteria. Ratledge C. and Stanford J. (eds). London, 1982, Acad. Press, 185.
10. Sempre A.M. et al.: A triacyltrehalose containing 2–methyl–branched unsaturated fatty acyl groups isolated from *Mycobacterium fortuitum*. J. Gen. Microbiol., 139, 585, 1993.
11. Strom A.R., Kaasen I.: Trehalose metabolism in *Escherichia coli*: stress protection and stress regulation of gene expression. Mol. Microbiol., 8, 205, 1993.
12. Szumiło T.: A novel enzyme, tagatose kinase, from *Mycobacterium butyricum*. Biochim. Biophys. Acta, 660, 366, 1981.
13. Thevelein J.M.: Regulation of trehalase metabolism in fungi. Microbiol. Rev., 48, 42, 1984.

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STRESZCZENIE

W *Mycobacterium* sp. 279 znaleziono dwie aktywności trehalazy, enzymu katalizującego hydrolizę disacharydu trehalozy. Trehalaza I była aktywna jedynie w obecności jonów Mg^{2+} i HPO_4^{2-}. Natomiast aktywność trehalazy II była całkowicie niezależna od tych kofaktorów. Aktywności trehalazy I i II całkowicie rozdzielono na kolumnie jonowymiennej DEAE–celulozy. Optimum pH ich aktywności wynosiło ok. 7,5. W pracy dyskutowano mechanizm reakcji trehalazy I.