Properties of trehalose-6-phosphate phosphatase from *Ascaris suum* muscles – preliminary studies

Małgorzata Dmitryjuk, Elżbieta Łopieńska-Biernat and Bogumił Sawczuk

[1] **Department of Biochemistry, Faculty of Biology and Biotechnology, University of Warmia and Mazury, Oczapowskiego 1A, Olsztyn 10-957, Poland; e-mail: m.dmit@uwm.edu.pl**

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Summary. The activity and preliminary properties of trehalose-6-phosphate phosphatase (TPP) were detected in muscles of *Ascaris suum* (Nematoda). The optimum temperature of the enzyme activity was established at 35°C in the optimal pH 7.0. Without the protection of the substrate, the enzyme rapidly loses activity at higher than room temperatures and was inactive at 55°C. TPS was activated by 10 mmol $FeCl_3$, NaCl, $MgCl_2$, $CaCl_2$ and $ZnCl_2$. In addition, it was inhibited partly by KCl and completely by EDTA.

Key words: nematodes, trehalose metabolism, trehalose-6-phosphate.

In many nematodes including *Ascaris suum*, α-trehalose, a disaccharide of glucose, is present in much higher concentrations than free glucose (Fairbairn, 1958; Dmitryjuk *et al*., 2009). Trehalose fulfils several important functions in these invertebrates: it play crucial roles in protecting cellular structures against most environmental stresses such as desiccation and freezing, oxidative and osmotic stress; it provides energy, is the major circulating blood sugar and it is also important in the process of hatching (Perry, 1989; Behm, 1997; Elbein *et al*., 2003).

In most eukaryotes (including nematodes) the synthesis of trehalose is catalysed by two enzymes: trehalose-6-phosphate synthase (TPS, EC2.4.1.15), which catalyses the transfer of glucose from uridine diphosphate (UDP)-glucose to glucose-6-phosphate to produce trehalose-6-phosphate (T6P), and trehalose-6-phosphate phosphatase (TPP, EC3.1.3.12), which can transform T6P to free trehalose and P_i (Behm, 1997). The activity of TPS and TPP was observed in muscles, individual parts of the reproductive system and hemolymph of *A. suum*, the parasite of pigs' intestine. It was noticed, that the highest activity of both enzymes was in muscles. Synthesis of trehalose was not observed in the cuticle and intestine of nematodes (Dmitryjuk *et al*., 2009). There is a lack of information about the trehalose-6-phosphate phosphatase in nematodes. By contrast, genetic aspects of trehalose metabolism have been studied in detail. The *tps* genes in *Caenorhabditis elegans* and filarial nematodes have been examined (Pellerone *et al*., 2003) and the occurrence of *tps* genes was also shown in *Aphelenchus avenae* (Goyal *et al*., 2005). Kormish & McGhee (2005) were the first to demonstrate the existence of genes encoding T6P phosphatase (TPP) in nematodes. Besides encoding TPP in *C. elegans* by *gob-1* gene, it was observed that concurrent inhibition of *tps1* and *tps2* genes abolish the lethal phenotype of *gob-1*. Development of lethal phenotype, gob-1, allowed authors to demonstrate that trehalose-6-phosphate accumulation leads to death of nematodes. These research data indicate that trehalose-6-phosphate phosphatase could play a key role in the degradation of T6P (probably toxic for nematodes) and contribute to the survival of nematodes. Confirmation of this could be the fact that trehalose-6-phosphate was used to eradicate onchocerciasis (Oke & Watt, 1998).

Therefore, the pathway of trehalose-6-phosphate utilisation occurring in nematodes may play an important role for therapeutic aims. This pathway can be a target for anti-nematode drugs, especially as T6P phosphatase has not been found in mammals (Pellerone *et al*., 2003). Determination of T6P phosphatase properties in tissue muscles of *Ascaris* may be a useful start to such studies.

MATERIALS AND METHODS

Enzyme extraction and activity assays. The material for the study consisted of adult female pig roundworms *A. suum* Goeze, 1782. The extracts

were prepared from muscles by homogenisation with 0.9% NaCl (1:4 w/v) in a YellowLine DI 18 basic homogeniser (Ikawerke, Germany). The crude extract was obtained by centrifugation at 1500 *g* for 15 min at 4°C. The activity of trehalose-6-phosphate phosphatase (TPP) was determined in the supernatant using the method of Kaasen *et al*. (1992). The end product of the reaction, trehalose, was determined by using HPLC according to procedure described by Dmitryjuk *et al*. (2009). The activity of TPPe was expressed in units per mg of proteins marked by Bradford's method (Bradford, 1976). The enzymatic unit (U) corresponds to the volume of enzyme releasing 1 nmol of trehalose from trehalose-6-phosphate (Sigma, Germany) during 1 min at 37°C.

Characterisation of the enzyme. The effect of pH on the enzyme. The studies were performed by employing 0.1 mol acetic acid – ammonia buffer in the pH range 3.0-9.4.

The optimum temperature of enzyme activity. Incubation of samples of TPS was carried out in the temperature range 20-65°C in 0.1 mol acetic acid – ammonia buffer in pH 7.0. The temperature was increased by 5°C for each subsequent sample.

The thermal stability of TPP. Test samples containing buffered enzymatic protein were preincubated for 15 min at temperature from 20° to 65°C. Next, the samples were cooled or warmed to 37°C and an enzymatic reaction was started by the introduction of substrate T6P.

Influence of chemical compounds. For assaying the effects of selected chemical compounds, 10 mmol solutions of the following compounds, $CaCl_2$, NaCl, $ZnCl_2$, $FeCl_3$, $MgCl_2$, KCl and EDTA, were used. First, samples contain buffered enzymatic protein as well as suitable agents were preincubated for 15 min at temperature 37°C. Next, an enzymatic reaction was started by introduction of the substrate T6P. The activity of the control sample, which contained deionized water in place of the effector, was defined as 100%.

RESULTS

Trehalose-6-phosphate phosphatase from *Ascaris* muscle was an inactive enzyme in strong acid pH (3.0-3.8; Fig. 1). At pH 5.8, the activity of this enzyme was half that of the maximum. The optimum activity was at pH 7.0. In pH above the optimum the activity of TPP was clearly reduced and at pH 8.6 it was less than 40% of the maximum. In pH 9.4 the enzyme was inactive (Fig. 1).

The enzyme was active at temperatures between 20-55°C. The optimum temperature for activity of this TPP was 35°C (Fig. 2). A rapid decrease of enzyme activity was noticed at 40°C, with only 46% of maximum activity. In higher temperatures the activities of the enzyme decreased and at 60°C the enzyme was inactive (Fig. 2).

TPP from muscles of *Ascaris* is not a thermostable enzyme. The enzyme was active in a range of 20-50°C during incubation without substrates. The maximum activity (100%) was at 20°C. With increasing temperature, the activity of the enzyme gradually declined and only 8.14% of the maximum activity was achieved at 50°C. This enzyme was inactive at higher temperatures (Fig. 2).

The 10 mmol solution of $FeCl_3$ was the strongest activator of TPP followed by NaCl, $MgCl_2$, $CaCl_2$ and $ZnCl_2$. The presence of $FeCl_3$ in the reaction mixture elevated TPP activity almost 6-fold (Table 1). Activity of TPP was also strongly increased by 10 mmol NaCl (about 3.5-fold). The well-known activator of phosphatases, $MgCl_2$, as well as calcium and zinc chlorides activated this TPP almost 2-fold (Table 1). EDTA and KCl were inhibitors of the TPP. The presence of 10 mmol EDTA in the reaction mixture resulted in 100% inhibition of TPP. A significant decrease in enzyme activity (below 50% of activity in the control) was observed in the presence of KCl (Table 1).

Table 1. Influence of different effectors and inhibitors on the activity of trehalose-6-phosphate phosphatase from *Ascaris suum* muscles.

The agent[a]	Activity of TPP U mg^{-1}	Relative activity of enzyme[b] (%)
I. Activators		
$FeCl_3$	3446.75 ± 1248.7	610.01
NaCl	2066.14 ± 121.31	365.67
$MgCl_2$	1648.69 ± 379.36	291.79
$CaCl_2$	1516.32 ± 173.78	268.36
$ZnCl_2$	1230.93 ± 366,9	217.85
II. Inhibitors		
EDTA	0.0	0.0
KCl	261.76 ± 8.3	46.32

[a]The agent concentration was 10 mmol.

[b]The activities of control sample without effectors (565,03 ± 121,3 U mg^{-1} of protein) were taken as 100%; Mean ± SD; n = 6.

DISCUSSION

The available literature does not have much information about the T6P phosphatase in Eucaryota. Much more information is available on this enzyme in prokaryotes. The T6P-phosphatase gene (otsB) was detected, mapped and located on

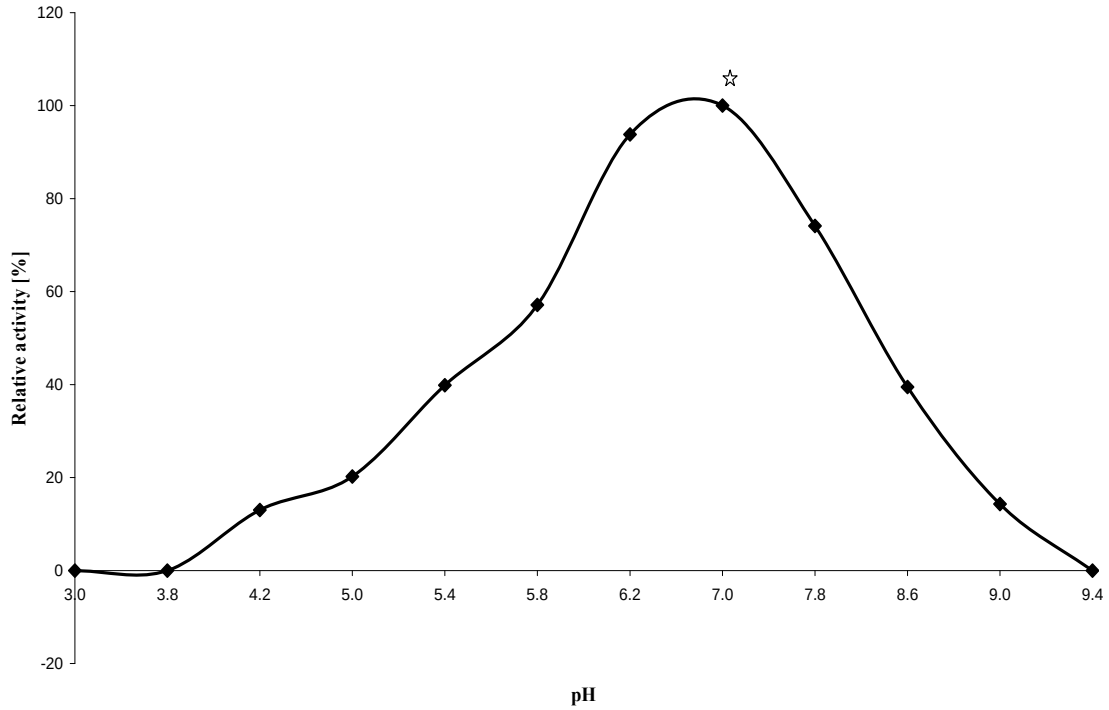

Fig. 1. Dependence of activity of trehalose-6-phosphate phosphatase from *Ascaris suum* muscles on pH. The activities of samples in pH 7.0 (672.24 ± 114.18 U mg^{-1} of protein) were taken as 100% (marked ☆).

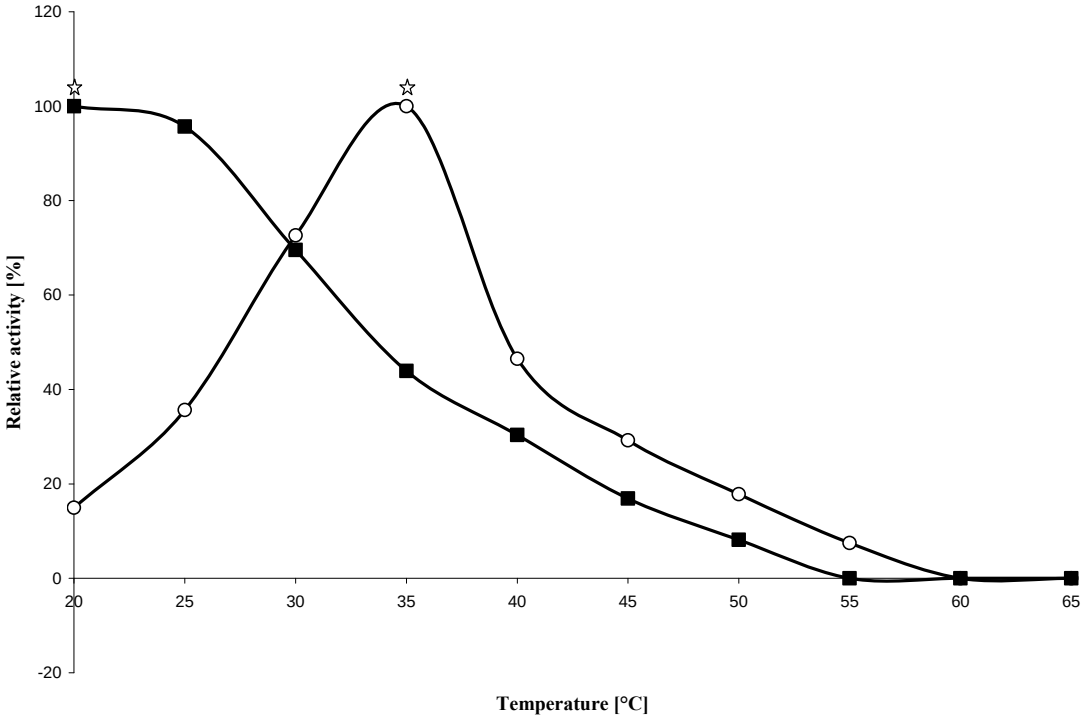

Fig. 2. The optimum temperature and thermostability of TPP from *Ascaris suum* muscles. Optimum temperature (—◦—) : The activities of samples in 35°C (731.98 ± 102.14 U mg^{-1} of protein) were taken as 100%. Thermostability (—▪—): The activities of samples in 20°C (700.11 ± 271.34 U mg^{-1} of protein) were taken as 100%.

☆

the *Escherichia coli* chromosome (Giaever *et al.*, 1988; Kaasen *et al.,* 1992). Synthase and phosphatase T6P in *E. coli* apparently do not exist as a complex because the two proteins could be expressed separately (Hengge-Aronis *et al.*, 1991; Seo *et al.*, 2000). A similar situation exists in *Mycobacterium smegmatis* (Klutts *et al*., 2003). The T6P phosphatase gene was also identified in the *Arabidopsis thaliana* genome by functional complementation of the yeast trehalose-P phosphatase (tps2) gene (Muller *et al*., 2001). Chary *et al.* (2008) demonstrated that class II genes (AtTPS5-AtTPS11) encode multifunctional enzymes having activity of synthase and phosphatase in *Arabidopsis*. In *Saccharomyces cerevisiae*, the synthase and the phosphatase purified as a complex. TPS and TPP purified with different chromatographic procedures, suggesting that they are part of a single bifunctional protein (Vandercammen *et al.*, 1989). The gene encoding the 100-kDa subunit of the trehalose-6-phosphate synthase/phosphatase complex in *S. cerevisiae* was TPS2 (De Virgilio *et al*., 1993).

In nematodes, the presence of two synthase genes has been demonstrated. In *C. elegans* two putative trehalose-6-phosphate synthase genes (*tps*1 and *tps*2) encode the enzymes that catalyse trehalose synthesis (Pellerone *et al*., 2003), as this has also been demonstrated in the anhydrobiotic nematode *A. avenae* (Goyal *et al*., 2005). Probably in *A. suum*, there are also two synthase T6P genes. We observed *tps*1 and *tps*2 mRNAs expression in muscles of *Ascaris* (unpublished data). Up to 2005, trehalose-6-phosphate phosphatase, the second enzyme in the trehalose synthetic pathway, had not been identified in nematodes. Kormish & McGhee (2005) discovered the lethal gut-obstructing *gob*-1 gene, which encodes the T6P phosphatase in *C. elegans*. The results of their work showed that nematode trehalose-6-phosphate phosphatases could provide especially promising control targets. The *gob*-1-gene is the only component of the trehalose synthesis pathway in nematodes that results in a strong lethal phenotype when its function is inhibited. The suppression of *gob*-1 lethality by ablation of TPS-1 and TPS-2 suggest that T6P phosphatase gene lethality results from a toxic build-up of the intermediate trehalose-6-phosphate, not from an absence of trehalose, as previously thought.

Results from the present study are the first reports on biochemical properties of this enzyme in nematodes.

The pH optimum of TPP from *A. suum* muscles was 7.0, as in the case with *Phormia regina*, the black blow fly (Friedman, 1960), and with *M. smegmatis* and *Thermus thermophilus* phosphatases (Matula *et al.*, 1971; Klutts *et al*., 2003; Silva *et al.*, 2005). The pH optima of OsTPP1 and OsTPP2 from rice were approximately 7.0 and 6.5, respectively (Shima *et al*., 2007). These data indicate that the phosphatase trehaloso-6-phosphate needs a neutral environment for proper operation. This is the opposite of many nonspecific acid and alkaline phosphatases that have maximum activity at around pH 5.0 or 9.0. The temperature optimum for the trehalose-6-phosphata phosphatase activity from *A. suum* muscles was 35°C and it was close to the temperature recorded for the host of *A. suum*, just as was the case T6P synthase from the same source (unpublished data). The enzyme from the flies had the highest activity at 46°C (Friedman, 1960). Bacterial enzymes have a very high optimum operating temperatures; for example, 60 and 70°C for *Rubrobacter xylanophilus* and *T. thermophilus*, respectively (Klutts *et al*., 2003; Nobre *et al*., 2008).

T6P phosphatase from pig worm muscles represents a non-thermostable enzyme. Without the protection of the substrate, the enzyme rapidly lost activity at higher temperatures and had only 8.14% of its maximum activity at 50°C. Similarly, T6P phosphatase from rice was non-thermostable in temperatures higher than 40°C (Shima *et al.,* 2007). Other phosphatases were more thermostable. The phosphatase from *P. regina* showed 75-90% loss of activity at 60°C. As expected, the enzyme from *T. thermophilus* it is very thermostable (to about 90°C; Silva *et a*l., 2005). Mycobacterial trehalose-6-phosphate phosphatase also tolerated high temperatures (Klutts *et a*l., 2003).

With the exception of K^+, metal cations clearly activated the phosphatase. Surprisingly, the best activator was 10 mmol $FeCl_3$, which raised the enzyme activity by almost 6-fold. By contrast, Fe^{3+} did not stimulate *R. xylanophilus* enzyme activity (Nobre *et al*., 2008). The chlorides of sodium, calcium and zinc, increased the activity of the enzyme from *Ascaris*. A well-known activator of phosphatases is magnesium ions. The phosphatase in the present study was also activated by Mg^{2+} (nearly 3-fold). We used a 10 mmol concentration of Mg^{2+} only because of the difficult accessibility and high cost of substrate trehalose-6-phosphate. Lower concentrations may have been better, as demonstrated Klutts *et al.* (2003) who showed that the optimum concentration of Mg^{2+} for the native TPP from *M. smegmatis* was about 1-2 mmol. On the basis of the present study, however, it is clear that the magnesium ions probably play an important role in T6P phosphatase catalysis, as they do in

bacteria *M. smematis, T. thermophilis, R. xylanophilis* (Matula *et al.,* 1971; Klutts *et al*., 2003; Silva *et al*., 2005; Nobre *et a*l., 2008) and yeast (Vandercammen *et al*., 1989). Two reagents, 10 mmol EDTA and KCl, were shown as inhibitors of TPP from pig worm muscles. Addition of KCl caused a decrease of T6P phosphatase activity by half; EDTA completely blocked the enzyme activity. TPP from yeast and *M. smegmatis* was also completely inhibited by EDTA (Matula *et al.*, 1971; Vandercammen *et al*., 1989). It is also worth noting that ethylenediaminetetraacetic acid was an inhibitor of other enzymes of trehalose metabolism in the muscles of *A. suum*: acid trehalase (responsible for the hydrolysis of trehalose; Dmitryjuk & Żółtowska, 2003) and T6P synthase (responsible for the synthesis of trehalose-6-phosphate; unpublished data). It could therefore be used as a blocker of trehalose metabolism in the muscles of parasite. Due to the fact that 10 mmol of EDTA partially inhibited the T6P synthase and completely inhibited TPP activity, using the concentration of this acid could lead to accumulation of T6P, which is toxic to nematodes and thus lead to the death of worms.

In conclusion, the results of this work have cognitive advantages as well as practical aspects. T6P phosphatase from *A. suum* is a non-thermostable enzyme acting in a neutral pH at a temperature that prevails inside the host. This may be one of the adaptations to the parasitic worm. On the other hand, the results of studies on EDTA could serve to develop highly specific targets for nematode control.

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Dmitryjuk, M., E. Łopieńska-Biernat, B. Sawczuk. Особенности трегалоза-6-фосфат фосафтазы из мышечных клеток *Ascaris suum* – предварительные данные.

Резюме. В мышечных клетках *Ascaris suum* выявлена активность трегалоза-6-фосфат фосфатазы. Исследованы свойства этого фермента. Температурный оптимумум для него - 35°C при оптимуме pH 7.0. В присутствии субстрата фермент быстро теряет активность при температурах выше комнатной и становится неактивным при 55°C. Трегалоза-6-фосфат синтаза активировалась в присутствии 10 mmol $FeCl_3$, NaCl, $MgCl_2$, $CaCl_2$ и $ZnCl_2$. Этот фермент частично ингибировался KCl и полностью ингибировался EDTA.