Properties of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in third-stage larvae of the nematode *Anisakis simplex* – preliminary studies

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Accepted for publication 13 October 2014

Summary. The activities of trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) were determined in third-stage larvae (L3) of *Anisakis simplex*. The optimum pH of TPS and TPP was 7.0 and optimum temperatures were 55 and 40°C, respectively. The thermostability of TPP was found to be higher than that of TPS. The activity of TPS at 20-50°C was approximately 20% of the maximum activity; at 65°C the enzyme was inactivated. The activity of TPP at 25-30°C was approximately 40% of the maximum activity; at 60°C the enzyme was inactivated. Effects of chemical compounds on the enzymes were determined. Supernatants used for enzyme preparations were obtained from homogenised worms spun for 15 min at 4°C and 1500 *g*. TPS activity increased up to 25-fold under the influence of trehalose. In the case of fructose and sorbitol an inverse relationship was shown between concentration and enzyme activity. Proline was demonstrated to be another TPS inhibitor. TPS was activated by 20 mM $MgCl_2$, NaCl and KCl. On the other hand, it was inhibited by $CuCl_2$, $CaCl_2$ and $CoCl_2$. TPP was activated by 10 mM $MgCl_2$, $CaCl_2$, $CoCl_2$, $ZnCl_2$ and NaCl and 20 mM $FeCl_3$, ZnCl, KCl and ethylenediaminetetra-acetic acid.

Key words: enzyme activity, pH, thermostability, third larval stage.

Anisakis simplex is a parasitic nematode that occurs mainly in fish (particularly cod, herring, sardines, sole and mackerel), as well as in dolphins and sea lions. In a human infected with *A. simplex*, larvae of the parasite stay alive in the body for a relatively short period, but the human host sometimes becomes highly sensitised. Hypersensitivity is particularly common in areas where there is a custom of eating raw, undercooked or improperly cooked fish (Danek & Rogala, 2005). The form of the parasite that invades humans is the third-stage larvae (L3), whose natural host is fish that live in salt or brackish waters. The larvae of this stage are extremely viable and resistant (Adams, 1999; Audicana *et al.*, 2002; Molina-Garcia, 2002). Although this nematode is a serious epidemiological problem, there are still much data lacking on its metabolism, with metabolism of carbohydrates being particularly understudied. This is important for endoparasites due to the oxygen-poor habitat in which they live (Lee & Atkinson, 1976). Trehalose is their main energy source (Behm, 1997). Studies on changes in this sugar add to knowledge of the metabolism of the nematode. Łopieńska-Biernat *et al.* (2007) conducted research on the changes of carbohydrate in L3.

Understanding the metabolism of trehalose can provide an opportunity to enrich knowledge on the physiology and biochemistry of *Anisakis* and to develop treatments for diseases caused by these parasitic nematodes. Trehalose (α-D-glucopyranosyl-1-1-α-D-glucopyranoside) is a non-reducing disaccharide, composed of two glucose molecules with an unusual α-1,1-glycosidic bond. The bond combines the two molecules, eliminating the reducing properties. The disaccharide therefore has a high hydrophilicity and chemical stability. Hydrogen bonds formed between the hydroxyl

group of the sugar and phosphate of head groups of membrane phospholipids result in a very flexible glycoside bond (Wolska-Mitaszko, 2001), where several essential physiological functions are discharged, such as energy reserve, glucose uptake, and protection against stress (Solomon *et al.*, 2000; Benoita *et al.*, 2009). Trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) are the two key enzymes involved in trehalose synthesis. TPS catalyses the transfer of glucose from uridine diphosphate glucose (UDP)-glucose to glucose-6-phosphate to give trehalose-6-phosphate and UDP, while TPP removes phosphate to produce free trehalose (Behm, 1997; Elbein *et al.*, 2003). In nematodes Dmitryjuk *et al.* (2012, 2013) characterised the properties of TPS and determined the activity of TPP in *Ascaris suum* and Kushwaha *et al.* (2011) determined the activity of TPP in *Brugia malayi*. Amongst nematodes, TPP was first identified in *C. elegans* where loss of its function caused lethal structural and metabolic defects in the nematode intestine (Kormish & McGhree, 2005), demonstrating its noteworthy significance in nematode species.

The aim of this study was to determine the optimum pH, optimum temperature, thermal stability and the impact of chemicals on the enzyme activity of TPS and TPP in L3 of *A. simplex*. Our results will help to better understand the physiology of the parasite and its response to various factors, which may be helpful in the development of appropriate drugs to combat anisakiasis.

MATERIALS AND METHODS

The research material consisted of L3 of *A. simplex* from fresh Baltic herring (*Clupea harengus*). The fishes were acquired in the marketplace at Olsztyn. L3 were washed several times in 0.65% NaCl solution, then dried on filter paper. Samples (100 mg) of larvae homogenised in hand by a glass homogeniser, then followed by an electric homogenizer in 1:1 (w/v) ratio in TBS buffer. The homogenate was centrifuged at 1500 *g* for 15 min at 4°C.

The activity of TPS wad determined using a method described by Giaever *et al.* (1988) and activity of TPP by Kaasen *et al.* (1992). The end-product of the reaction (trehalose) was assayed by high-performance liquid chromatography using a method described by Dmitryjuk *et al*. (2009). The activity of TPS and TPP was expressed in unit (U) per mg protein measured with the Bradford (1976) method. One unit (U) defines the quantity of trehalose (μ mol) synthesis during 1 min at 37°C. The effect of pH on the enzymes was determined in 0.1 M acetic acid-ammonia buffer within the range of pH 3.0-9.0.

Study of the optimum temperature of the enzymes was carried out in the temperature range 15-70°C in 0.1 M acetic acid-ammonia buffer at pH 7.0. For each subsequent sample, the temperature was increased by 5°C. To study the thermal stability of the enzyme, test samples containing buffered enzymatic protein were preincubated for 15 min at temperatures from 15-70°C. Next, the samples were cooled or warmed to 37°C and an enzymatic reaction was started by introduction of substrates: UDPG and G6P for TPS and T6P for TPP. Glucose, fructose, sorbitol, trehalose and proline were used as effectors for TPS activity. Sugars and amino acids were present in concentrations of 100, 200, 300 and 400 mmol. As effectors $MgCl_2$, $CaCl_2$, $CuCl_2$, $CoCl_2$, KCl_2, $NaCl_2$ were used with divalent ions present in concentrations of 2, 5, 10, 15, 20 and 25 mmol and monovalent ions at concentrations of 10, 20, 50, 100, 200, 400 mmol l^{-1}. For assaying the influence of chemical compounds for activity of TPP 2, 5, 10 and 20 mmol l^{-1} solutions of the compounds $MgCl_2$, $CaCl_2$, $CoCl_2$, $ZnCl_2$, $FeCl_3$, NaCl, KCl and ethylenediaminetetra-acetic acid (EDTA) were used. The activity of control sample, which contained water instead of the effector, was assumed at 100%.

Statistical analyses were conducted using the Statistica (Statsoft, V 9.0) software package. The data were analysed using the ANOVA, Post Hoc test; the results were considered statistically significant at $P < 0.005$.

RESULTS

Effect of pH on the enzymes. The optimum pH for both enzymes was 7.0. Under acidic conditions at pH 3.0-4.5, TPS had no activity, and TPP had about 50% of the maximum activity. TPS and TPP activities were reduced to only 20% and 40%, respectively, at pH 7.5. At pH 8.5 an increase in activity of TPS to about 60% was observed; however, activity of TPP decreased to 20% of the maximum activity (Fig. 1).

Optimum temperature of enzyme activity. The activity of TPP was high (> 75%) at a relatively low temperature of 30°C. TPP showed optimal activity at 40°C. The activity of this enzyme decreased rapidly at a temperature higher than 50°C. By contrast, TPS became more active when the temperature rose above 45°C, its peak of activity being recorded at 55°C (Fig. 2).

Table 1. Effect of chemical compounds on the activity of trehalose-6-phosphate synthase (TPS) from third-stage larvae of *A. simplex*.

Chemical compounds	**Activity of enzyme [%]**			
[mmol l^{-1}]	100	200	300	400
control	100.00			
Glucose	54.5±16.44	34±5.02[a]	114.06±83.7	25.9±26.2
Fructose	234.5±212.9[a]	214.7±191[a]	36.7±10.42	–
Sorbitol	302.5±71.91[a]	95.8±90.2	9.11±1.48[a]	18.07±16.9[a]
Trehalose*	1578±463[a]	2376±276[a]	2494±111[a]	–
Proline	21.15±11.6[a]	65.6±11.6[a]	–	–

The activities of the control sample without effectors (21.547 ± 2.23 U mg^{-1} of protein) were taken to be 100%; mean ± SD; n = 6; a – indicate significant difference means of the control and chemical groups. * – difference of control (inactivated enzyme preparation) and activated enzyme.

Table 2. Effect of divalent ions on the activity of trehalose-6-phosphate synthase (TPS) from third-stage larvae of *A. simplex*.

Chemical compounds	**Activity of enzymes [%]**					
[mmol l^{-1}]	2	5	10	15	20	25
control	100					
$MgCl_2$	81.9±2.4	80.22±14.7	92.67±7.5	61.63±1.7[a]	135.05±0.9[a]	129.02±3.2[a]
$CaCl_2$	32.53±6.5[a]	28.6±0.7[a]	81.12±2.6	59.9±5.7	85.08±7	90.27±10.1
$CoCl_2$	50.79±21.1[a]	13.64±0.3[a]	33.2±3.9	36.69±0.4	49.46±16.9	43.96±0.9
$CuCl_2$	60.86±36.7[a]	16.99±0.4[a]	20.83±11.8[a]	39.6±5.2	82.6±3.8	54.65±13.9

The activities of the control sample without effectors (21.547 ± 2.23 U mg^{-1} of protein TPS) were taken to be 100%; mean ± SD; n = 6; a – indicate significant difference means of the control and chemical groups.

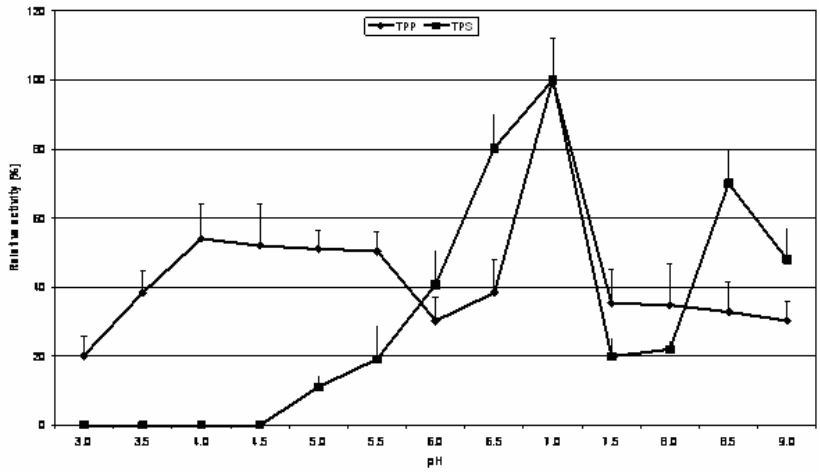

Fig. 1. Effect of pH on the activity of trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) from third-stage larvae of *Anisakis simplex*. The activities of samples in pH 7.0 (0.41 ± 0.02 U mg^{-1} of protein for TPS) and (0.44 ± 0.02 U mg^{-1} of protein for TPP) were taken to be 100%. n = 9 (the number of independent enzyme preparations), error bars = standard deviation (SD).

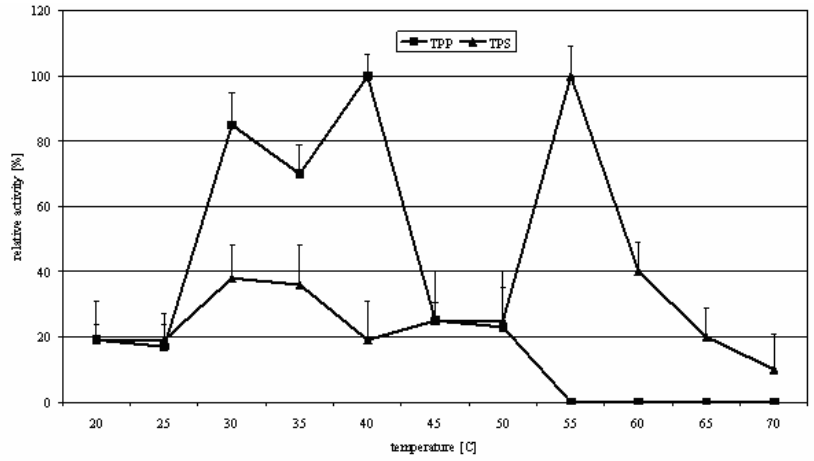

Fig. 2. Effect of temperature on the activity of trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) from third-stage larvae of *Anisakis simplex*. The activities of samples at 55°C (23.45 ± 2.12 U mg^{-1} of protein) and 40°C (0.375 ± 0.02 U mg^{-1} of protein) were taken to be 100%. n = 9 (the number of independent enzyme preparations), error bars = standard deviation (SD).

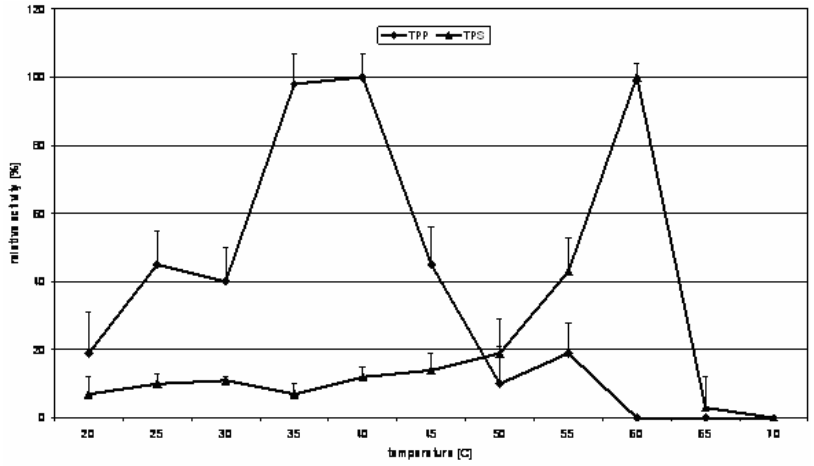

Fig. 3. Thermostability of trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) from third-stage larvae of *Anisakis simplex*. The activities of samples at 60°C (15.34 ± 1.23 mg^{-1} of protein) and 40°C (0.17 ± 0.05 mg^{-1} of protein) were taken to be 100%. n = 9 (the number of independent enzyme preparations), error bars = standard deviation (SD).

Thermal stability of enzyme activity. The study was conducted in a temperature range of 20-70°C. TPS proved to be very thermolabile. TPS activity did not change over the 15 min pre-incubation period (without substrate) at temperatures of up to 50°C, with enzyme activity being in the range of 10-20% of the maximum activity. At 55°C TPS activity increased to about 40% of the maximum activity. At higher pre-incubation temperatures rising from 50 to 60°C there was an increase in the rate of enzyme activity. However, a pre-incubation temperature above 65°C resulted in the inactivation of the enzyme (Fig. 3). The standard deviations within a given temperature are small.

The thermal stability curve of TPP was different. An increase in pre-incubation temperature from 25 to 45°C was accompanied by increased activity of this enzyme. Temperature higher than 50°C resulted in inactivation of TPP.

Influence of carbohydrates and proline on activity of trehalose-6-phosphate synthase. Trehalose was the most potent activator of this enzyme at all tested concentrations. At a concentration of 300 mmol l^{-1} increase in the activity of TPS was up to 25-fold compared with the activity of the reference sample that did not contain the chemical compound. Also, fructose at concentrations of 100 and 200 mmol l^{-1} increased enzyme activity by more than 2-fold (Table 1). In the case of fructose and sorbitol, activity was found to be related to concentration. TPS was inhibited by

sorbitol at concentrations of 300 and 400 mmol l^{-1}, having 9 and 18% of the activity of the reference sample, respectively. This chemical compound activated TPS by about 3-fold at concentration of 100 mmol l^{-1} (Table 1). Enzyme activity was inhibited to a lesser extent by proline at concentrations of 100 and 200 mmol l^{-1} having approximately 20 and 65% of its maximum activity. TPS was inhibited by glucose, with activity decreasing by about 70% at concentration of 200 mmol l^{-1} (Table 1).

Influence of chemical compounds. TPS. Of the divalent ions analysed, only Mg^{2+} ions were found to be activators of TPS. TPS activity increased by approximately 30% under the influence of Mg^{2+} ions at concentrations of 20 and 25 mmol l^{-1} (Table 2). It was observed that an inhibitory effect on TPS was exerted by heavy-metal ions, *i.e.*, Cu^{2+} and Co^{2+}. Cu^{2+} and Co^{2+} ions inhibited this enzyme activity by 83% even at a concentration of 5 mmol l^{-1} of salt. Also, Ca^{2+} ions at concentrations of 2 and 5 mmol l^{-1} caused inhibition of TPS activity by approximately 70% (Table 2).

TPP. The activity of TPP increased by 60% at the Mg^{2+}concentration of only 10 mmol l^{-1} (Table 3). This enzyme activity was strongly stimulated by Co^{2+}, Ca^{2+} and Zn^{2+}ions at 10 mmol l^{-1}. The presence of trivalent ions Fe in the reaction mixture elevated TPP activity by about 40% (Table 3).

Table 3. Effect of divalent and trivalent ions on the activity of trehalose-6-phosphate phosphatase (TPP) from third-stage larvae of *A. simplex*.

Chemical compounds	**Activity of enzymes [%]**			
[mmol l^{-1}]	2	5	10	20
control	100			
$MgCl_2$	59.2±4.5	81.7±12.2	158.6±10.8[a]	80.9±8.9
$CaCl_2$	6.9±5.2	72.2±12.9	139±23.9	70.7±12.8
$ZnCl_2$	140±3.8	76.4±12.4	150±12.8[a]	164±23.8[a]
$CoCl_2$	63±12.5	84.5±12.4	139±12[a]	64.3±12.9
$FeCl_2$	39.9±12.9	28±4.7[a]	79.6±23.9	137.7±23[a]

The activities of the control sample without effectors (0.257 ± 0.02 U mg^{-1} of protein TPP) were taken to be 100%; mean ± SD; n = 6; a – indicate significant difference means of the control and chemical groups.

Table 4. Effects of monovalent ions and ethylenediaminetetra-acetic acid (EDTA) on the activity of trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) from third-stage larvae of *A. simplex*.

Chemical compounds	**Activity of enzymes [%]**							
[mmol l^{-1}]	**2**	**5**	**10**	**20**	**50**	**100**	**200**	**400**
TPS								
KCl	–	–	101.75±22	74.61±5.5	91.59±5.9	85.43±2.17	100.75±5.3	145.77±1.8[a]
NaCl	–	–	45.07±5.4	292.12±0.08[a]	466.65±50[a]	254.93±11.3[a]	33.76±8.2	27.07±1.9
TPP								
KCl	131±12	80±12	50±34	124±12[a]	–	–	–	–
NaCl	174±23	126±23	204±54[a]	83±12	–	–	–	–
EDTA	42±12.89	90±23.08	60±23.9	110±34.9[a]	–	–	–	–

The activities of the control sample without effectors (21.547 ± 2.23 U mg^{-1} of protein TPS; 0.257 ± 0.02 U mg^{-1} of protein TPP) were taken to be 100%; mean ± SD; n = 6; a – indicate significant difference means of the control and chemical groups.

Among the monovalent ions, Na^+ ions were activators of TPS at concentrations of 20, 50 and 100 mmol l^{-1}. Under the influence of Na^+, enzyme activity was significantly increased, with 2- to 4-fold higher activity as compared with the reference sample (Table 4). K^+ ions did not affect the activity of this enzyme. Only K^+ ions at the concentration of 400 mmol l^{-1} increased enzyme activity by approximately 45% compared with the activity of the reference sample (Table 4). Na^+ ions increased TPP activity by 2-fold at the concentration of 10 mmol l^{-1}; however, K^+ ions activated this enzyme by 20% at the concentration of 20 mmol l^{-1}. The activity of TPP was stimulated only by 10% with a 20 mmol l^{-1} concentration of EDTA (Table 4).

DISCUSSION

Carbohydrate metabolism plays a vital role in sustaining the processes of life in many nematodes, including *A. simplex*. The synthesis of trehalose during the development of parasites protects them from external factors so that they can survive in harsh conditions. The increase in the incidence of anisakiasis, due to the popular consumption of raw fish, has attracted the interest of researchers seeking new anti-parasitic drugs. Scientific evidence indicates that blocking the proteins involved in the enzymic synthesis of trehalose contributes to the death of nematodes, which can be used in developing measures to combat parasitic diseases (Honda *et al.*, 2010). The first need is to know the properties of these proteins.

Both *A. simplex* enzymes examined (TPS and TPP) had optimum activity at pH 7. TPS was active at pH 5-9; however, TPP was active in the pH range of 3-9. The curves of TPS and TPP activity under different pH conditions are clearly marked with two maxima at pH 7.0 and 8.5 for TPS and pH 7.0 and 4.0 for TPP (Fig. 1), which may indicate the presence of isoforms of these enzymes. TPS from the muscle of the parasitic nematode *A. suum* shows the highest activity at acidic pH, with the optimum pH being 4.2. The enzyme exhibits at pH 7 is only about 29% of the maximum activity, and the enzyme in an alkaline environment is inactive (Dmitryjuk *et al.*, 2013). TPS from *Aphelenchus avenae* and *Saccharomyces cerevisiae* is present in a complex with TPP, with optimum TPS activity at pH 8 and 8.5, respectively (Loomis *et al.*, 2005; Chaudhuri *et al.*, 2009). Also, according to the research of Pan *et al.* (2004) carried out in *Mycobacterium smegmatis*, the optimum activity of the enzyme is at pH 7. Trehalose synthase from *Pseudomonas putida* was examined and the enzyme was found to have its highest activity at pH 7.4 (Ma *et al.*, 2006). In the thermostable bacterium *Thermus thermophilus* RQ-1 synthase is an enzyme that is most active in a slightly acidic environment, having optimum activity at pH 6 (Silva *et al.*, 2005). Valenzuela-Soto *et al.* (2004) investigated the properties of TPS and determined the environment for the optimum activity of the enzyme. The enzyme derived from *Selaginella lepidophylla* showed the highest activity at pH 7 and was stable within a pH range of 5-10. Similar to TPP of *A. simplex*, optimum pH was found to be 7.0 for *A. suum* (Dmitryjuk *et al.*, 2012) and for *B. malayi* phosphatases (Kushwaha *et al.*, 2011) and *T. thermophilus* (Silva *et al.*, 2005). These data indicate that most of these enzymes require a neutral environment for proper activity.

The optimum temperature for TPS is 55°C, while that for TPP is 40°C. TPP activity depends on the temperature; as for optimum pH there seem to be two forms of the enzyme, which have optimum temperatures of 30 and 40°C. Similarly, TPP from muscle of *A. suum* and *B. malayi* and *Escherichia coli* has an optimum temperature of 35°C (Seo *et al.*, 2000; Kushwaha *et al.*, 2011; Dmitryjuk *et al.*, 2012). In a study conducted by Dmitryjuk *et al.* (2013) TPS was inactive at 55°C; the temperature for optimum activity was 35°C. Also TPS from *E. coli* exhibits its highest activity at 35°C (Lee *et al.*, 2005). Silva *et al.* (2005) showed that in *T. thermophilus* RQ-1 TPS-1 at 30°C is not activated, and the temperature for optimum activity is 98°C. At 102°C the enzyme has 80% of its maximum activity.

There are also differences in the thermal stability of enzymes in different species. TPS from *A. simplex* is the most stable at the temperature of 60°C. Dmitryjuk *et al.* (2013) showed that for enzymes of *A. suum* the highest thermal stability occurs at a temperature of 35°C. The difference in stability of TPS from *A. simplex* and *A. suum* may be indicative of the occurrence of enzyme in a complex with another protein that alters the properties of *A. simplex* TPS. Also, Ma *et al.* (2006) have demonstrated thermal stability of trehalose synthase of *P. putida*. Silva *et al.* (2005) studied the thermostable bacteria with a half-life of the enzyme at 100°C for 31 min. TPP from *A. simplex* represents a quite thermostable enzyme. Without the protection of substrate, the enzyme lost activity at just 50°C and had about 10% of its maximum activity. Similarly, TPP from *B. malayi* and *A. suum* retained significant activity at 50°C for 15 min. Other phosphatases were more thermostable. The phosphatases from *Phormia regina* showed 70-90%

of activity at 60°C. Also, TPP from *E. coli* retained 90% of its activity at 50°C (Seo *et al.*, 2000). Mycobacterial and *T. thermophilus* RQ-1 enzymes also tolerated high temperatures (Klutts *et al.*, 2003; Silva *et al.*, 2005).

TPS activity increased under the influence of trehalose and fructose (Table 1). The enzyme derived from *S. lepidophylla* (Velenzuela-Soto *et al.*, 2004) and from *A. simplex* increased their activity under the influence of fructose. Dmitryjuk *et al.* (2013), by examining the substrate specificity of the purified synthase of trehalose-6-phosphate from *A. suum*, showed that the enzyme is not specific with respect to fructose. However, the effect of trehalose had the opposite reaction on the enzyme. According to Velenzuela-Soto *et al.* (2004), trehalose did not affect the activity of the enzyme. The present work showed that TPS from *A. simplex* under the influence of trehalose increases its activity 25-fold, and is the most potent activator of the enzyme of the compounds tested (Table 1). Different results were also obtained when studying the effect of sorbitol on TPS activity. This compound accumulates in the cells under the influence of thermal and osmotic stress. Physiological concentrations of sorbitol protect the protein from heat, and thus from aggregation and inactivation. The accumulation of sorbitol under the influence of thermal stress is analogous to the accumulation of trehalose in yeast (Salvucci, 2000). TPS activity in *A. simplex* is increased 3-fold by sorbitol at a concentration of 100 mmol l^{-1}, and then decreased with increasing concentrations (Table 1). The opposite effect of sorbitol on TPS activity of *S. lepidophylla* was demonstrated by Velenzuela-Soto *et al.* (2004). This compound is an activator of the enzyme. TPS activity increases with increasing concentration. At a concentration of 400 mmol l^{-1}, enzyme activity was 215% of relative activity. Proline accumulates in the cells of plants under osmotic stress, cooling, thermal stress, UV light and exposure to heavy metals (Özturk & Demir, 2002). According to Claussen (2005), proline as a substance protects soluble complex protein structures against denaturation, stabilises membrane through interaction with phospholipids, and can be a source of energy and nitrogen. Proline reduces the oxidation of membranes and protects cells from reactive oxygen species (Hoque *et al.*, 2007). High concentrations of proline can be harmful to plants (Ashraf & Foolad 2007). Research conducted by Velenzuela-Soto *et al.* (2004) confirms the inhibitory effect of proline on TPS activity from *S. lepidophylla*. Proline is also a TPS inhibitor in *A. simplex* (Table 1). In the present study it was found that glucose is one of the substrates that has an inhibitory effect on TPS enzyme activity (Table 1). It can be concluded that accumulation of the enzyme substrate reduces enzyme activity. However, Velenzuela-Soto *et al.* (2004) showed that glucose activates TPS. The enzyme activity in *S. lepidophylla* increases with increasing concentration. Dmitryjuk *et al.* (2013) investigated the substrate specificity of the purified enzyme from *A. suum* and demonstrated that TPS is not specific for glucose.

The divalent ion Mg^{2+} was found to be an activator of TPS. The strongest activity of TPS was shown at the concentrations of 20 and 25 mmol l^{-1}, with 130% relative activity (Table 2). Dmitryjuk *et al.* (2013) also showed that TPS activity from *A. suum* muscle under the influence of Mg^{2+} increased to approximately 160%. With another nematode, *Aphelenchus avenae*, Mg^{2+} ions were also an activator of the enzyme (Loomis *et al.*, 2005). TPS from *T. thermophilus* RQ-1 also is activated by Mg^{2+} ions (Silva *et al.*, 2005). In *E. coli* Mg^{2+} ions do not affect the activity of the enzyme, varying in the range of 95-107% (Lee *et al.*, 2005). Velenzuela-Soto *et al.* (2004) found that in *S. lepidophylla* the activity of TPS is increased 3-fold. On the basis of the present study and other research to date it is clear that Mg^{2+} ions play an important role in TPP catalysis, as they do in nematodes (*A. suum*, *B. malayi*) and bacteria (*M. smegmatis*, *T. thermophilus* RQ-1) (Pan *et al.*, 2004; Silva *et al.*, 2005; Kushwaha *et al.*, 2011; Dmitryjuk *et al.*, 2012). It has been shown that the heavy-metal ions significantly decrease the activity of the enzyme (Table 3). This is related to the toxic properties of these compounds. Moreover, in *P. putida* (Ma *et al.*, 2006), *E. coli* (Lee *et al.*, 2005) and *S. cerevisae* (Chaudhuri *et al.*, 2009) heavy-metal ions significantly decrease the activity of enzymes. Cu^{2+} ions at the concentration of 1 mmol l^{-1} will decrease enzyme activity by approximately 66% (Lee *et al.*, 2005). Otherwise, Co^{2+} ions at 10 mmol/l activated TPP by about 40%, a similar effect having been found at 2 mmol l^{-1} only in thermophilic bacteria (Silva *et al.*, 2005). Ca^{2+} ions affect TPS activity in many different ways. Concentrations of 2 and 5 mmol l^{-1} are inhibitors of the enzyme, whereas at higher concentrations activity is not altered (Table 2). Other results have been obtained by Dmitryjuk *et al.* (2013). These authors found that Ca^{2+} increases TPS activity of *A. suum* muscle by approximately 1.5-fold. The presence of Ca^{2+} ions is beneficial for enzyme activity in *A. avenae* (Loomis *et al.*, 2005). Also Velenzuela-Soto *et al.* (2004) showed that the concentration of 1 mmol l^{-1} of Ca^{2+} increased the

enzyme activity in *S. lepidophylla*, but at the concentration of 10 mmol l^{-1} the enzyme was inactive. Lee *et al.* (2005) showed that low concentrations of Ca^{2+} do not affect the activity of TPS in *E. coli*; however, the concentration of 20 mmol l^{-1} inhibits its activity.

It was shown in the present study that Na^+ is beneficial for TPS activity at concentrations of 50 and 100 mmol l^{-1}, and TPP activity at 10 mmol l^{-1}. Similarly, a correlation was found for activity of TPP from *A. suum*; there Na^+ ions increased activity about 3-fold and TPS activity by nearly 1.5-fold (Dmitryjuk *et al.*, 2012, 2013). The activating effect of Na^+ on the synthesis of trehalose-6-phosphate was also shown by Loomis *et al.* (2005) in *A. avenae*. Enzyme activity under the influence of Na^+ ions was 2-fold. K^+ ions increased the activity of the enzyme at the concentration of 400 mmol l^{-1}, while lower concentrations did not alter the activity (Table 4). A similar relationship was observed by Velenzuela-Soto *et al.* (2004) and Dmitryjuk *et al.* (2013), who showed that K^+ ions are both an enzyme activator in *S. lepidophylla* as well as in the parasitic nematode *A. suum*. Also Loomis *et al.* (2005) have shown that K^+ ions improve TPS activity in *A. avenae* 2-fold. However, TPP activity, unlike TPS, increased only at the highest tested concentration of 20 mmol l^{-1}. For TPP from *A. suum* K^+ acts as an inhibitor, but at concentrations below 10 mmol (Dmitryjuk *et al.*, 2012). A similar surprise was the activation of TPP by trivalent ion at 20 mmol l^{-1} by 40%, while in *A. suum* a concentration of 10 mmol l^{-1} produced an activation of 6-fold (Dmitryjuk *et al.*, 2012).

The present study showed that the metal-chelating agent EDTA makes TPP a functionally stable enzyme at 20 mmol l^{-1} without change of activity. The results are different from those obtained in other nematodes. These differences may result from different concentrations of acid: the enzymes from *A. suum* and *B. malayi* were inhibited at 10 mmol l^{-1}, while in the present study activity of the enzyme from *A. simplex* at 20 mmol l^{-1} did not change. Perhaps at a higher concentration EDTA ceases to be an inhibitor of TPP, especially, as it is a non specific metal chelator. Confirmation of differences in response to this effector in *A. simplex* L3 is the activation of trehalase, the enzyme responsible for the hydrolysis of trehalose (Łopieńska-Biernat *et al.*, 2007).

In conclusion, the available literature is concerned with the prevalence of forms of the enzymes TPS and TPP in nematodes, as a single protein or as protein complexes catalysing the reactions of trehalose synthesis. Both enzymes have the potential to have isoenzymes. To further confirm this, the phenomenon is beginning to be studied at the molecular level. The present study has demonstrated significant differences in response to temperature and metal ions. It seems that the protein TPS is more sensitive than TPP.

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E. Łopieńska-Biernat, M. Czubak, E.A. Zaobidna and J. Rokicki. Особенности трегалоза-6-фосфат синтазы и трегалоза-6-фосфат фосфатазы у личинок 3-й стадии *Anisakis simplex* – предварительные наблюдения.

Резюме. У личинок 3-й стадии *Anisakis simplex* определяли активность ферментов трегалоза-6-фосфат синтазы (ТФС) и трегалоза-6-фосфат фосфатазы (ТФФ). Оптимум pH для ТФС и ТФФ составлял 7,0, а оптимум температур – 55°C и 40°C, соответственно. Было показано, что термостабильность ТФФ выше, чем у ТФС. Активность ТФС при температуре 20-50°C составляла около 20% максимальной активности, а при 65°C фермент инактивировался. Активность ТФФ при 25-30°C была около 40% максимальной активности, а инактивация наступала при 60°C. Определяли действие некоторых химических соединений на эти ферменты. Супернатанты для приготовления ферментов получали из гомогенизированных нематод после центрифугирования при 1500 *g* в течение 15 мин и температуре 4°C. Активность ТФС возрастала в 25 раз в присутствии трегалозы. Для фруктозы и сорбитола была показана обратная зависимость между концентрацией и активностью ферментов. Было показано, что пролин является еще одним ингибитором ТФС. ТФС активировалась при добавлении 20 mM $MgCl_2$, NaCl и KCl. С другой стороны, этот фермент ингибировался $CuCl_2$, $CaCl_2$ и $CoCl_2$. ТФФ активировался добавлением 10 mM $MgCl_2$, $CaCl_2$, $CoCl_2$, $ZnCl_2$ и NaCl, а также 20 mM $FeCl_3$, ZnCl, KCl и этилендиаминтетрауксусной кислоты.