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The properties of trehalose hydrolysing enzymes from tissues extracts of *Ascaris suum* (Nematoda)

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Summary

The enzymes of trehalose hydrolysis from muscle, intestine and reproductive system of *Ascaris suum* differ in their properties. Enzymes from the muscles and intestines are acid trehalases with the highest activity at pH 4.3 and 5.0, respectively. The enzyme from the reproductive system is an alkaline trehalase with the optimum pH at 7.4. The optimum temperature of muscle trehalase is 45°C, for the other enzymes 55°C. The enzyme from the reproductive system did not loose its activity after 15 min preincubation without substrate at 65°C, intestinal and muscles trehalases at 55°C and 45°C, respectively. The influence of Zn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, EDTA and PCMB differentiates studied trehalases. Trehalases from the muscles and intestines were localised mainly in lysosome while that from the reproductive system in the mitochondrial fraction. The above properties suggest that trehalases of *Ascaris* are tissue-specific enzymes.

Key words: trehalase; trehalose; α-glucosidase; disaccharidases; *Ascaris suum*; nematode

Introduction

Trehalase (α,α-trehalose glucohydrolase; EC 3.2.1.28) hydrolyses the α-1→1-glucoside binding of trehalose. As a result of that reaction, two molecules of glucose are released. In the numerous of organisms, trehalase is the major, or even the sole, enzyme participating in the catabolism of trehalose (Behm, 1997). The situation is similar in case of the intestinal parasite of swine *Ascaris suum*, where the hydrolytic path plays the major role in decomposition of trehalose. In the tissues of *A. suum* phosphorylase of trehalose (EC 2.4.1.64), the enzyme degrading trehalose along phosphorolytic path, is also present but its activity is 10-times lower than that of trehalase (Dmitryjuk, Żółtowska, 2004).

A. suum is the only nematode in case of which the distribution of trehalase in the body has been studied. The initial studies by Feist *et al.* (1965) showed trehalase only in the intestine of the nematode. Later, its presence was also found in other tissues as the muscles and the reproductive system (Fukushima, 1967, Lapp & Mason, 1978). Our studies have also shown a high activity of that enzyme in the parasite's cuticle (Dmitryjuk & Żółtowska, 2004). Absence of trehalase in hemolymph of the nematode confirmed in all studies is explained by the fact that trehalose is a "circulating" sugar similar as in the case of insects. Absence of the enzyme allows the maintainance its high concentration in that body fluid (Behm, 1997).

Trehalose is a compound fulfilling a number of important functions in nematodes: it is an energy carrier material, it is a circulating sugar and it protects cellular structures of embryos and mature forms during environmental stress allowing closing of the life cycle. As a consequence of the above, a better knowledge of the properties of the major enzyme decomposing that disaccharide – trehalase seems important.

The paper aims at comparing the properties of the enzymes present in three different tissues of *A. suum*: muscles, intestines and reproductive system. Presenting the differences among those enzymes may, besides pure knowledge expansion, be used in the future in medical or veterinary practice. The metabolism of trehalose may by considered a potentially good targets for drugs against those parasites – it is important for the parasite while its role for the host is marginal (Boczoń, 1995).

Material and Methods

1. Preparation of enzymatic extracts from A. suum tissues

The material for the study consisted of mature, fertilized

female of *A. suum* Goeze, 1782. Extracts were prepared of muscles, intestines and reproductive systems isolated from the nematodes. The tissues were homogenised with 0.9 % NaCl at 1:4 w/v in a glass homogenizer. The homogenates were centrifuged at 1500 × *g* for 15 min at 4°C. Supernatants were dialysed for 18 hours against 0.9 % NaCl at 4°C. Dialyzates were centrifuged at 1500 × *g* for 10 min. In the supernatants the properties of trehalases from the three studied sources were examined.

2. Enzymatic activity

The activity of trehalase in the enzymatic extracts was identified by the method of Dahlqvist (1968). The reaction mixture contained: 0.1 ml of the tested extract, 0.1 ml 50 mM trehalose and 0.8 ml 70 mM veronal/sodium acetate buffer. The controls contained 0.1 ml distilled water instead of the substrate. The reaction was carried at 37°C for 1 hour. Glucose released by the enzyme from trehalose during the reaction was measured using glucose oxidase reagent (LTS-120 glucose test by Cormay, Lublin – Poland).

The enzymatic unit [u] represents the quantity of enzyme releasing 1 nmol glucose from trehalose within 1 hour at 37°C. The enzymatic activity was converted to 1 mg of protein marked using the Bradford's method (1976) and was expressed as u/mg protein. The presented results represent the mean value from five independent experiments.

3. Characteristics of trehalases from muscles, intestines and reproductive system of A. suum

3.1. Influence of storage of the tissues upon the activity of the enzyme

Fresh muscles, intestines and reproductive systems were divided into 200 mg samples that were stored for 22 weeks at –10°C. Every week five samples of each material were unfrozen and used for preparation of enzymatic extracts. The results were expressed as percent of maximum activity (the value of which was taken for 100 %).

3.2. Intracellular distribution of trehalases

Fractions of subcellular structures were obtained by differential centrifugation (Van den Bossche & Borgers, 1973). Muscles, intestines and reproductive systems freshly isolated from the nematode were homogenised with a 0.25 M solution of sucrose. Sediments of individual fractions were obtained by centrifugation: nuclear at 800 × *g* for 10 min, mitochondrial at 10 000 × *g* for 10 min, lysosomal at 15 000 × *g* for 15 min. After centrifugation at 100 000 × *g* for 2 hours sediment of microsomal fraction and cytosol were obtained. Sediments of subcellular fractions were diluted in 0.9 % NaCl and dialysed for 1 hour against distilled water at 4°C.

3.3. The effect of pH on enzymes

The tests were carried in 70 mM veronal/sodium acetate buffer within the pH range from 3.2 to 9.2. The reaction mixture contained 0.1 ml of the extract, 0.1 ml 50 mM of trehalose solution and 0.8 ml of the buffer.

3.4. Identification of optimum temperature for enzyme activity

Mixtures of buffered enzymatic extracts at optimum pH: 4.3 pH for muscles, 5.0 pH for intestines or 7.4 pH for the reproductive system and 50 mM trehalose were incubated for 1 hour in water bath at from 25° to 95°C. Controls without trehalose were treated in the same way.

3.5. Thermal stability of the enzyme

Samples containing 0.1 ml of the tested extract and 0.8 ml of buffer at pH optimum for trehalase from a given material were pre-incubated for 15 min in water bath at from 25° to 95°C. After cooling the test samples were supplemented with 0.1 ml 50 mM trehalose solution while the controls with 0.1 ml distilled water and incubated for 1 hour at 37°C.

3.6. Influence of chemical compounds

The 0.1 M water solutions of the following compounds were applied: EDTA, PCMB (p-chloromercuribenzoate), magnesium, potassium, zinc and iron chlorides and zinc sulphate. The samples contained 0.1 ml of enzymatic extract, 0.1 ml 50 mM trehalose, 0.7 ml 70 mM veronal/sodium acetate buffer and 0.1 ml tested salt solution (10 mM). The controls contained 0.1 ml of distilled water instead of the effectors. The activity of control was assumed as 100 %. For each compound control tests without substrate were also prepared. In that way the influence of a given salt on the enzymes present in the glucose determining set.

Results

The mean activity of the enzyme in fresh muscles of the nematode was 801 ± 86.5 u/mg. After the first and second weeks of storage at –10°C the activity of trehalase was lowered by a half from that in the initial material (Fig. 1). During the following weeks from 3 to 6, the activity of trehalase increased. After six weeks of storage it was 2.5 times higher than in the fresh muscles. That level of treha-

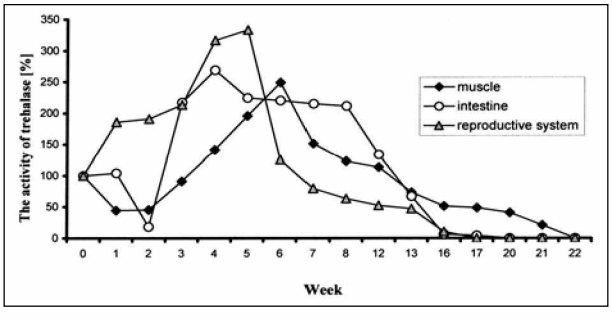

Fig.1. Influence of storage time on the activity of *A. suum* trehalase. The initial activity of enzyme (100 %) was 801 ± 86.5 u/mg for muscle, 728.5 ± 30.4 u/mg for intestine and 434.3 ± 31 u/mg for reproductive system

lase activity, higher than initial, continued until the 12 week. The enzyme lost its activity entirely after 22 weeks of storage (Fig. 1). Similar to the muscles, the activity of intestinal trehalase decreased after two weeks from the initial 728.5 ± 30.4 u/mg to 132.4 ± 15.5 u/mg, to increase during the following weeks to the maximum (Fig. 1). The intestinal enzyme lost its activity faster than the muscle one and after 16 weeks of storage it showed only 6 % of its initial activity. Trehalase from the reproductive system of the nematode behaved different than the muscle and intestinal trehalases. Its activity increased three times over the initial value of 434.3 ± 31 u/mg within 5 weeks of storage. Following that period, the enzyme rapidly lost its activity and after 16 weeks it was inactive (Fig. 1).

Intestinal and muscle trehalases appeared mainly in subcellular fractions – lysosomal and mitochondrial. The reproductive system trehalase was associated with the mitochondrial fraction and, to a lesser degree, microsomal one (Fig. 2). Very low activity of the enzyme in cytosol obtained from all studied tissues is interesting (Fig. 2).

Enzymes from whole extracts of muscles and intestines were active within the entire investigated range of pH from 3.2 to 9.2 (Fig. 3). The optimum pH for muscle trehalase is pH 4.3, while for the intestinal trehalase it is pH 5. Both trehalases show high levels of activity (exceeding 75 %) in the acid environment (pH 4.1 - 6.1) (Fig. 3). On that basis they can be classified to the group of acid trehalases. In the alkaline environment the muscle trehalase showed a slightly higher activity than the intestinal enzyme. Trehalase from the reproductive system of *A. suum* is an alkaline enzyme, susceptible to both low and high values of pH. The enzyme showed no activity within the range of pH from 3.2 to 6.1. It was active for pH range from 6.7 to 8.5 reaching the maximum activity in slightly alkaline environment at pH 7.4 (Fig. 3).

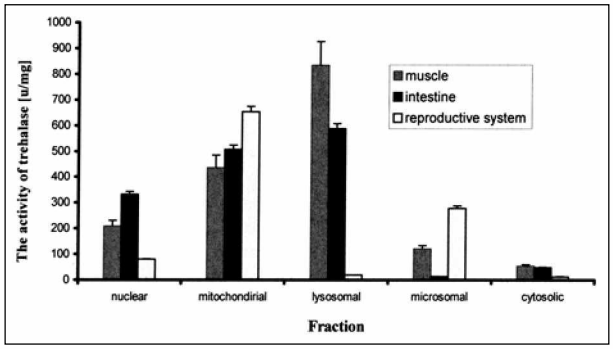

Fig. 2. Subcellular distribution of trehalase from *A. suum*

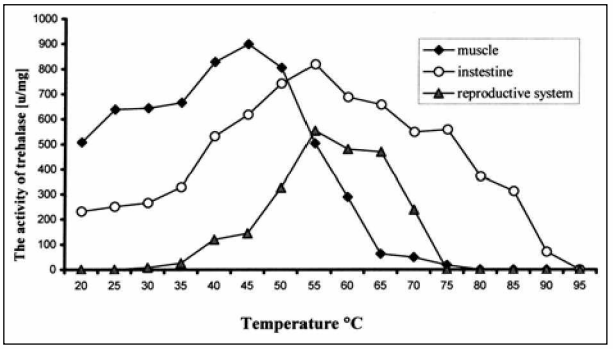

Fig. 4. Dependence of the activity of trehalase from *A. suum* on temperature

The character of the curve of dependence of trehalase activity on temperature was different for each of the studied trehalases (Fig. 4). Muscle trehalase of *A. suum* was active within the temperature range from 20° to 65°C (Fig. 4). The optimum temperature for activity of that enzyme was 45°C where its activity reached the maximum of 899.7 ± 97.2 u/mg. In higher temperatures the enzyme lost its activity rapidly. Incubated at 55°C it had 56 %, and at 65°C only 7 % of its maximum activity. Intestinal trehalase had the optimum temperature 10°C higher than the muscle one. At 55°C its activity was 819.1 ± 102.7 u/mg. At 85°C the intestinal enzyme still showed 38 % of its maximum activity and lost it entirely only at 95°C (Fig. 4). Trehalase from the reproductive system, on the other hand, was practically inactive up to 35°C (Fig. 4). In higher temperatures

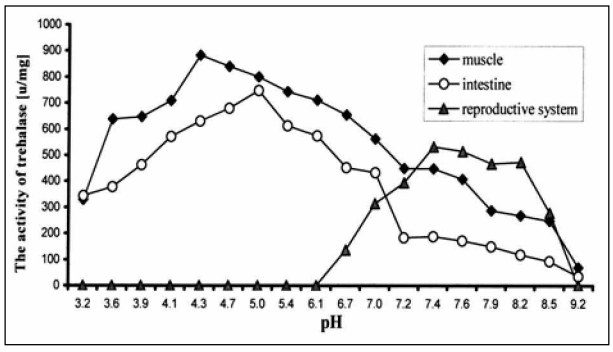

Fig. 3. Influence of pH on the activity of trehalase from *A. suum*

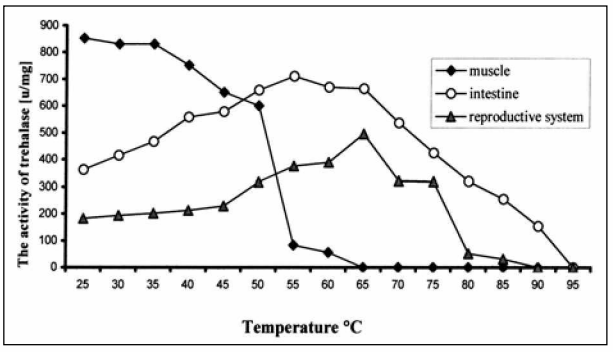

Fig. 5. Thermal stability of the activity of trehalase from *A. suum*

Table 1. The influence of chemical compounds on the activity of trehalase from *Ascaris suum* tissues

The influence on all studied trehalases	Chemical compounds	The activity of enzyme [%]* from		
		muscle	intestine	reproductive system
Inhibition	$FeCl_3$	64.70 ± 9.16	89.56 ± 8.6	32.73 ± 1.4
	EDTA	19.40 ± 9.2	45.01 ± 19.1	79.58 ± 3.45
Activation	$ZnSO_4$	110.85 ± 11.5	113.10 ± 3.8	197.09 ± 8.5
Differentiated	PCMB	158.80 ± 19.1	91.28 ± 4.1	71.75 ± 17.1
	$MgCl_2$	147.05 ± 15.9	123.17 ± 17.8	80.01 ± 3.4
	$CaCl_2$	123.50 ± 3.3	118.81 ± 12.3	33.19 ±1.44
	$ZnCl_2$	109.3 0 ± 14.2	98.2 ± 4.9	270.66 ± 11.7
	KCl	197.05 ± 19.7	110.87 ± 9.8	73.18 ± 13.2

* The activities of control samples was taken as 100 %. Their activities were 825.3 ± 41.8 u/mg, 719.6 ± 45.2 u/mg, and 470.66 ± 24.8, respectively for trehalase from muscle, intestine and reproductive system

its activity increased gradually. The maximum activity of that enzyme was reached at 55°C (554.2 ± 50.8 u/mg). Further increase of incubation temperature by 20°C caused total inactivation of that enzyme (Fig. 4).

Activity of trehalase from extracts of muscles of the nematode was not changed by 15 min preincubation without substrate up to 35°C (Fig. 5). Preincubation at 50°C resulted in loss of ca. 30 % of that enzyme's activity. Higher temperature resulted in a drastic decrease of the enzyme's activity. The behaviour of trehalase from intestinal extracts was different. An increase in preincubation temperature from 25°C to 55°C was accompanied even by an increase of its activity (Fig. 5). Only above 65°C the enzyme's activity decreased. It should be underlined that intestinal trehalase is the enzyme with a clearly higher thermal stability than the enzymes from the two other sources. Following preincubation at 80°C it still maintained 43 % of activity while the total inactivation was observed only at 95°C (Fig. 5). On the other hand, preincubation of the extract from the reproductive system at up to 45°C did not change its activity. At these temperatures it showed ca. 40 % of maximum activity found at 65°C (Fig. 5). Preincubation at 50° – 65°C resulted in an increase in its activity. Higher temperatures caused a partial loss of activity of that trehalase (Fig. 5).

Both similarities and major differences were observed in the reactions of studied trehalases to presence of investigated chemical compounds in the environment of the reaction (Table 1). In case of trehalases from all the sources, 10 mM EDTA and $FeCl_3$ acted as inhibitors while $ZnSO_4$ was an activator. $MgCl_2$, $CaCl_2$ and KCl activated muscle and intestinal trehalases while those compounds inhibited trehalase from the reproductive system. On the other hand, that last trehalase was strongly stimulated by both zinc salts (Table 1). The reaction of trehalases to PCMB differed. It acted a strong inhibitor in case of trehalase from the reproductive system, had no influence upon the intestinal enzyme and activated muscle trehalase 1.5-times (Table 1).

Discussion

In the available literature no information was found on specificity of trehalase from tissues of nematodes, except for some data from papers by Lapp and Mason (1978) concerning neutral muscle trehalase. On the basis of own results it can be stated that enzymes present in individual tissues of *A. suum* differ in properties. Similar tissue specificity is characteristic for human trehalases. In humans expression of trehalase genes was observed in three organs – kidneys, small intestine and liver. It was proven that human trehalases are coded by single genes that are expressed in individual organs. As a consequence, a different isoform of the enzyme is produced in each of the organs (Ishihara *et al.*, 1997). It cannot be excluded that a similar situation exists also in *Ascaris,* but it was not confirmed experimentally.

Trehalases from *A. suum* differ in their subcellular distribution (Fig. 2). The muscle and intestinal enzymes of the nematode are mainly lysosomal. Their relatively high activity was also found in the mitochondrial fraction, which may result from its contamination with lysosomes. The trehalase from the reproductive system was mainly located in mitochondria while microsome was the second fraction showing its activity. On the other hand, activity of muscle and intestinal trehalases in this fraction was minimal. The above results suggest that *Ascaris* trehalases are present in subcellular structures, as the activity of the enzyme in cytosol from all the studied organs of *A. suum* was negligible. Similarly, trehalase of *C. elegans* is linked to cellular membranes (Behm, 1997). Also trehalases of mammals are enzymes linked to membranes (Takesue *et al.*, 1986, Ruf *et al.*, 1990, Ishihara *et al.*, 1997, Murray *et al.*, 2000, Oku & Nakamura, 2000).

Individuality of properties of tissue enzymes is confirmed by our results on stability of the enzyme during storage of the material. The enzyme of the reproductive system stored for a week at –10°C increased its activity 4-times. Muscle trehalase lost a half of its activity during that time while in-

testinal trehalase did not change. On the other hand, trehalase from the reproductive system was subject to the earliest inactivation. The enzyme from the muscles of the parasite was most durable. This study has proven that storage of material in frozen state exceeding three weeks has a positive influence upon activity of trehalase. The muscle enzyme reached the highest activity after 6 weeks of storage, the one from the reproductive system after 5 and the intestinal enzyme after 4 weeks. Feist *et al.* (1965) also observed an increase in activity of trehalase from intestines of *A. suum* after freezing and unfreezing the tissue. Similarly, Fukushima (1967) found that freezing caused an almost 2-times increase in activity of trehalase from the intestine of the nematode while it influenced the increase of muscle enzyme activity to a lesser degree. Increase of trehalase activity observed during storage of the material is surely a result of decomposition of cellular structures. Presence of a factor lowering the activity of trehalases in fresh tissues cannot be also excluded. Their presence is also, indirectly, indicated by the results of thermal stability of trehalases. This is particularly well visible in case of the enzymes from the reproductive system and intestines. Enzyme from both sources reached its maximum stability at relative high temperatures of 65° and 55°C, respectively. That indicates the possibility of thermal denaturation of such hypothetical inhibitor. Confirmation of their presence and identification of their role *in vivo* in the tissues of *A. suum* require further focused research.

The muscle enzyme is less thermostable and possesses a lower temperature of activity at 45°C than the other trehalases. It is also much more active than the other enzymes in the lower temperature range from 20° to 45°C. That range corresponds, on the one hand, with the temperature in the external environment (ca. 20°C), where early mobile larvae develop and on the other to the temperature in the body cavity of the host where the older developmental stages of parasite live. We suppose that may be a manifestation of ontogenic adaptation. The muscles develop and start functioning much earlier (already in L_1 larvae) than the alimentary or reproductive systems of the parasite, which become fully active only after infestation of the host's intestine, i.e. in the environment with a higher temperature. That may be reflected by the higher optimum temperate of activity in case of trehalases from those systems (Anya, 1976).

The differences between the studied enzymes are also reflected in the optimum pH. The optimum pH of researched trehalase from the intestine was lower than trehalase from the brush border of the intestine of *Ascaris* isolated by Gentner & Castro (1974) using tissue centrifuging at 15 000 × *g*, (5.5 – 6.0). Such cleaning of the intestinal enzyme probably influenced a shift of the optimum pH. A similar phenomenon was observed in case of acid trehalase from muscles of *A. suum*. The enzyme in the whole extract showed the highest activity at pH 4.3, while after isolation at pH 4.9 (Dmitryjuk & Żółtowska, 2003). This study confirms the suggestion by Lapp & Mason (1978) concerning presence of two types of trehalase in muscles of *A. suum* – the neutral which is soluble and acid bound to the membranes. In case of studies on whole extracts from muscles of the nematode, besides the maximum at pH 4.3, another lower peak of trehalase activity was observed at pH 6.1. The last result probably corresponds to the activity of the neutral enzyme (pH 6.0), described by Lapp & Mason (1978).

Trehalases from tissues of *A. suum* investigated during this study differ in their reaction with the examined chemical compounds (Table 1). Although EDTA, the chelator of bivalent cations is an inhibitor of trehalases from all tissues and its activity confirms the importance of those cations for the enzymes from *Ascaris*, individual trehalases are activated by different cations as proven by further studies. Zn^{2+} is necessary for the trehalase from the reproductive system. It is much weaker in activating the muscle and intestinal enzymes. The activity of muscle and intestinal trehalases increases extensively in presence of the magnesium cations and slightly in presence of calcium cations. On the other hand magnesium is an inhibitor for trehalase from the reproductive system. Fe^{3+} is inhibitor of trehalase from all three tissues but the degree of reduction depends on their origin. It inhibits 80 % of activity of the muscle enzyme, 55 % of the intestinal enzyme and only 20 % of the trehalase from the reproductive system. Influence of PCMB shows that groups –SH are only important for trehalases from the reproductive system. They do not play a major role in catalysis by intestinal trehalase while their modification stimulates the activity of trehalase from the muscles of the parasite. The presented examples of reaction of trehalases from individual tissues of the nematode upon the tested salts as well as the values of optimum pH of activity suggest that different groups important for catalytic functions are present in catalytic and regulatory sites of those enzymes.

Concluding it can be stated that trehalose in the tissues of *A. suum* is hydrolysed by specific trehalases differing in a number of properties: subcellular location, optimum pH and temperature, thermal stability and reaction to chemical effectors. What is more, comparative analysis of properties of trehalose hydrolysing enzymes from tissues of studied systems of *Ascaris* allows formulating a general conclusion that trehalases present in the muscles and intestine of the nematode, despite certain differences, are more similar to each other than to the enzyme from the reproductive system characteristics of which are definitely different.

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