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A membrane-bound trehalase from *Chironomus riparius* larvae: purification and sensitivity to inhibition

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A preparation of a membrane-bound trehalase from the larvae of the midge *Chironomus riparius* (Diptera: Chironomidae) was obtained by detergent solubilization, ion-exchange chromatography and concanavalin A affinity chromatography. Trehalase was purified 1080-fold to a specific activity of 75 U mg^{-1}. The initial rate of trehalase activity followed Henri–Michaelis–Menten kinetics with a K_m of 0.48 ± 0.04 mM. Catalytic efficiency was maximal at pH 6.5. The activity was highly inhibited by mono- and bicyclic iminosugar alkaloids such as (in order of potency) casuarine (IC_{50} = 0.25 ± 0.03 μM), deoxynojirimycin (IC_{50} = 2.83 ± 0.34 μM) and castanospermine (IC_{50} = 12.7 ± 1.4 μM). Increasing substrate concentration reduced the inhibition. However, in the presence of deoxynojirimycin, Lineweaver–Burk plots were curvilinear upward. Linear plots were obtained with porcine trehalase. Here, we propose that deoxynojirimycin inhibits the activity of trehalase from *C. riparius* according to a ligand exclusion model. Inhibition was further characterized by measuring enzyme activity in the presence of a series of casuarine and deoxynojirimycin derivatives. For comparison, inhibition studies were also performed with porcine trehalase. Results indicate substantial differences between midge trehalase and mammalian trehalase suggesting that, in principle, inhibitors against insect pests having trehalase as biochemical targets can be developed.

Keywords: Chironomus riparius / iminosugar alkaloids / inhibition / trehalase

Introduction

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is the main circulating sugar in the hemolymph of most insects (Elbein et al. 2003). Insect tissues possess the enzyme α,α-trehalase (EC 3.2.1.28), which promotes the irreversible hydrolysis of trehalose into two molecules of D-glucose (Thompson 2003). The enzyme has been mainly found in brain and thoracic ganglia (Strang and Clement 1980), asynchronous (Reed and Sacktor 1971) and synchronous flight muscle (Gussin and Wyatt 1965; Wegener et al. 2003), midgut cells (Azuma and Yamashita 1985; Silva et al. 2004), midgut surrounding visceral muscle (Mitsumasu et al. 2005), follicle cells (Shimada and Yamashita 1979), spermatophore (Yaginuma et al. 1996) and hemolymph (van Handel 1978). Trehalase has been proved to exist in two distinct forms, a membrane-bound form and a soluble form (Wyatt 1967). The membrane-bound form has been described in flight muscle, ovary cells, spermatophore and midgut. In flight muscle cells, the situation is further complicated by the fact that part of the membrane-bound enzyme is inactive and present in a latent form that can be activated by treatments that destroy membrane integrity (Wegener et al. 2003). The soluble trehalase has been purified from the midgut, where it is distributed in the cavity of the goblet cells (Mitsumasu et al. 2005), and from the hemolymph, even though the presence in the latter compartment has been questioned in some insects (Vaandrager et al. 1989).

For its role in glucose metabolism, trehalase has been regarded as an interesting target for the development of new agents against insect pests (Asano et al. 1990; Wegener et al. 2003). Inhibition studies revealed that several compounds are able to specifically block trehalase activity in a micromolar or submicromolar range. Among the most powerful compounds, there are validoxylamine A (Asano et al. 1990), trehazolin (Ando et al. 1995) and suidatrestin (Knuesel et al. 1998). Injection of these compounds into insect hemolymph is lethal (Wegener et al. 2003) since it produces unsuccessful metamorphosis (Asano et al. 1990). In addition, trehalase is inhibited by iminosugars that strongly inhibit other glycosidases (Asano 2003), such as the mono- and bicyclic alkaloids deoxynojirimycin and castanospermine (Asano et al. 1996), as well as by a variety of glycosides (Silva et al. 2004). Together with the selection of new potential insecticides, inhibition studies have been proved useful to elucidate structural features of the enzyme active site (Asano et al. 1996; Gibson et al. 2007; Cardona et al. 2009).

Most of what we know on insect trehalase derives from studies performed on terrestrial species such as Lepidoptera,

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Diptera, Blattaria and Orthoptera (Thompson 2003; Wegener et al. 2003; Mitsumasu et al. 2008). Little is known on trehalase from aquatic insects. Chironomidae, commonly known as midges, are widely distributed and often represent the most abundant invertebrate taxon in freshwater ecosystems. In view of their importance as potential sentinel organisms (Callaghan et al. 2001; Forcella et al. 2007), it is of concernment that our knowledge of their physiology is as wide as possible. *Chironomus riparius* represents a good model for biochemical studies, and we have recently demonstrated that its resistance to stress factors is highly dependent on trehalose catabolism. Larval exposure to sublethal concentrations of the two insecticides fenitrothion and carbofuran has effects on the catabolism of trehalose and on the trehalase activity (Forcella et al. 2007). This result has prompted us to assume that trehalase could be considered as a target for new and specific molecules having insecticide activity.

Results

Trehalase purification

About 38% of total trehalase activity in the midge larva homogenate is from membrane-bound trehalase. Activity was recovered upon solubilization of the membrane pellet obtained after centrifugation of the crude homogenate at 100,000 × *g* for 1 h. Extraction from membranes was obtained incubating the pellet with the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS). The purification of trehalase from *C. riparius* larvae is summarized in Table I. The solubilized crude extract was subjected to three chromatographic steps. The first one was included in order to remove greenish pigments present in the preparation. After Mono Q, the trehalase was concentrated in three 1-mL fractions. The final step was based on data from literature describing the membrane form of the trehalase as glycosylated. The molecular mass of the purified trehalase was estimated to be 67 kDa both by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (not shown) and by high-performance liquid chromatography IC_{50} 50% inhibitor concentration (HPLC) Superdex 200 HR 10/30 (Figure 1A). In order to verify if the preparation might be a mixture of isoforms, native gel electrophoresis with staining activity was performed. As shown in Figure 1B, a single band was detected, confirming the presence of a single trehalase isoform (the faint band above is represented by small quantities of unsolubilized material unable to enter the running gel). The apparent molecular mass was higher than that estimated by SDS-PAGE, and the discrepancy could be explained by the presence of glycosylated residues which reduce the mobility of the protein within the gel under non-denaturating conditions.

Kinetic studies

Trehalase showed high specificity for trehalose and displayed barely detectable activity or no activity at all against some disaccharides and trisaccharides such as lactose (0.1%), sucrose (0.08%), cellobiose (0.05%), maltose (0%) and raffinose (0.9%). Preliminary experiments showed that maximal activity was recorded at slightly acidic pH values. Therefore, kinetic experiments were performed at three different pH values (5.5, 6.5 and 7.5). In all instances, the initial rate of trehalase activity measured as a function of trehalose concentration showed saturation kinetics fitting with a simple Henri–Michaelis–Menten equation (Figure 2). Kinetics parameters are reported in Table II. Apparently, pH mainly affects the maximal velocity with no significant modification of the affinity for trehalose. Catalytic efficiency decreased 4.1-fold when pH increased from 6.5 to 7.5. To check whether this decrease was due to buffer effect, initial rate measurements at saturating concentration of trehalose were repeated in 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethansulfonic acid (HEPES)–2-amino-2-hydroxymethyl-1,3-propanediol (Tris), pH 6.5: the specific activity was slightly lower but not significantly different from that measured in acetate buffer (3.95 ± 0.15 vs 4.15 ± 0.09, respectively). These data are compatible with the slightly acidic pH found in the hemolymph of this insect species (unpublished observations).

Table I. Purification of the membrane-bound trehalase from *C. riparius* larvae

Procedure	Total proteins (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	1404	194	0.138	100	1
DEAE	312	162	0.52	83.5	3.8
Mono Q	5.96	84	14.1	51.7	102
Concanavalin A	0.193	28.7	149.7	34.2	1080

Inhibition studies with glycosidase inhibitors

In order to characterize the sensitivity of this enzyme, inhibition by a panel of compounds was considered, and dose–response curves were established for each of them. Considering that we expected a competitive effect, experiments were performed at two substrate concentrations, one close to the K_m value (0.5 mM) and the second one at a 10-fold higher concentration. Inhibitor concentrations varied in the experiments according to their degree of inhibition as assayed in a preliminary screening. Figure 3 shows dose–response curves for deoxynojirimycin, castanospermine and casuarine at fixed trehalose concentration (0.5 mM). Initial rates as a function of inhibitor concentration were fitted to the following equation:

$$\frac{v_i}{v} = \frac{1}{1 + \left(\frac{[I]}{IC_{50}}\right)^n} \quad (1)$$

where v_i and v are the initial rate in the presence and in the absence of inhibitor, respectively; $[I]$ is the inhibitor concentration; IC_{50} is the inhibitor concentration producing half-maximal inhibition; and n is the Hill coefficient.

Among the compounds tested, casuarine has been proved to be the most potent inhibitor with a calculated IC_{50} = 0.248 ± 0.003 μM, whereas the dose–response curves for deoxynojirimycin and castanospermine fitted to Eq. (1) returned IC_{50} values of 2.83 ± 0.34 and 12.4 ± 1.14 μM, respectively. In the presence of 5 mM trehalose, all dose–response curves shifted to higher IC_{50} values as expected for competitive inhibition: the calculated IC_{50} values were 2.93 ± 0.84, 39.6 ± 2.84 and 132 ± 15 μM for casuarine, deoxynojirimycin, and castanospermine, respectively.

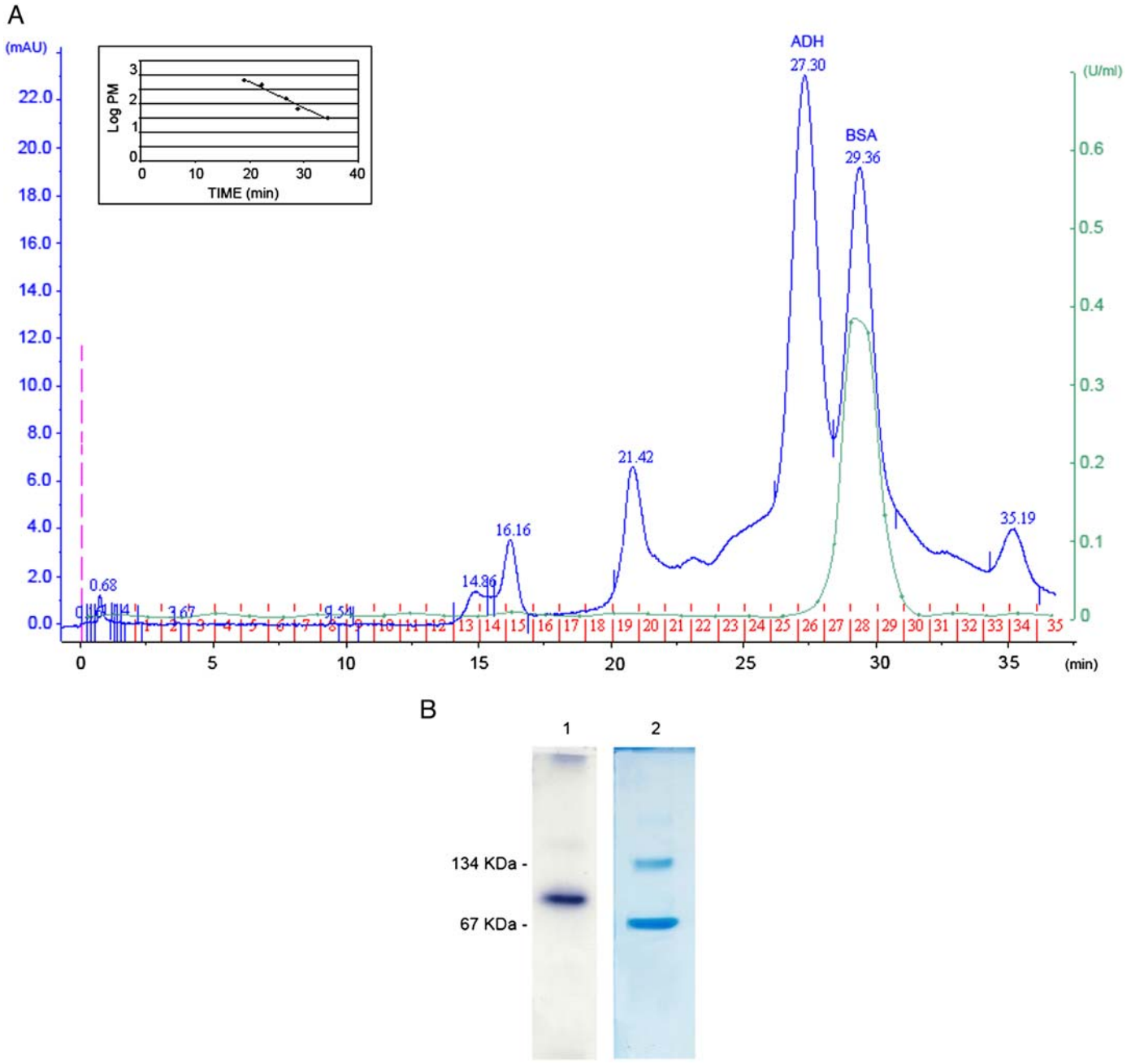


Fig. 1. Molecular mass of *C. riparius* trehalase. (**A**) Gel filtration co-elution profile of Mono Q active fractions from *C. riparius* homogenate and two standard proteins, bovine serum albumin (BSA) and alcohol dehydrogenase (ADH), with a known molecular weight of 67 and 150 kDa, respectively. The trehalase activity of each fraction was determined (–●–). In the box, the calibration curve of Superdex 200 HR 10/30 with thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa) and deoxyribonuclease (31 kDa) as standard is reported. (**B**) Non-denaturating PAGE of pooled fractions eluted in correspondence with BSA (see A) stained for trehalase activity.

Using the following Cheng–Prusoff relationship, $IC_{50} = K_i\left(1 + \frac{[S]}{K_m}\right)$, the inhibition constant was calculated, and these results are reported in Table III together with data obtained using porcine trehalase, a mammalian counterpart. Fitting to Eq. (1) returned n values higher than 1 for all three inhibitors, suggesting that more than one molecule of inhibitor could bind to the enzyme (in inhibition study, the n coefficient is not an index of cooperativity). To further explore this aspect, we performed kinetics of trehalase activity as a function of trehalose in the presence of increasing concentrations of deoxynojirimycin. Results are illustrated in Figure 4. The family of curves of the double reciprocal plot converged to the same V_{max} value, suggesting a competitive mechanism of inhibition. However, in the presence of deoxynojirimycin, the plot was curvilinear upward when *C. riparius* trehalase was used. The plot was linear for porcine trehalase, and for simplicity, only the results obtained in the presence of the highest concentration of deoxynojirimycin (5 μM) are illustrated (Figure 4, inset). The non-linearity of the Lineweaver–Burk plot could be explained by three possible scenarios: (i) the enzyme is a dimer, (ii) the

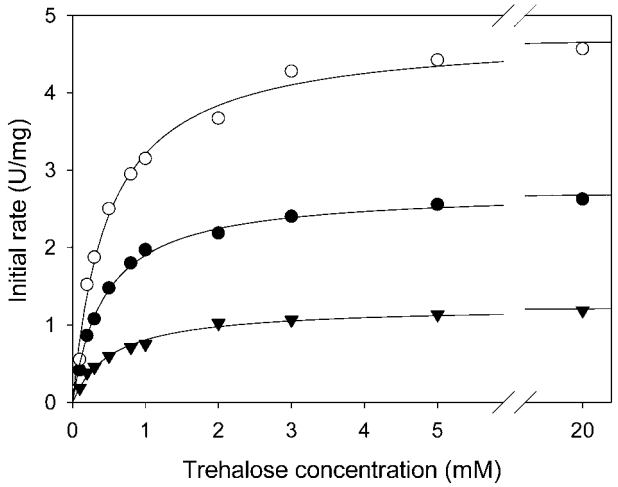


Fig. 2. Kinetics of *C. riparius* trehalase. Activity was measured as a function of medium pH: initial rates were at pH 7.5 (▼), 6.5 (○) and 5.5 (●).

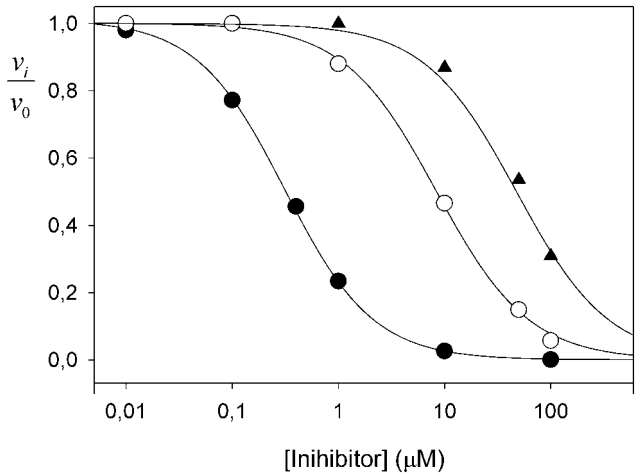


Fig. 3. Dose–response curves of glycosidase inhibitors on trehalase activity from *C. riparius* larvae. The enzyme was assayed in the presence of the indicated inhibitor concentrations and in the presence of 0.5 mM trehalose at pH 6.5. Symbols refer to casuarine (●), deoxynojirimycin (○) and castanospermine (▼). The assay was started by the addition of trehalose after 10 min of pre-incubation. Data are plotted according to Eq. (1).

inhibitor is a slow-binding inhibitor or (iii) a more complex non-linear inhibition pattern is present. From SDS-PAGE and gel filtration, we have no evidences that trehalase is a dimer. Therefore, to discriminate between the other two alternatives, the progress curve of product formation in the presence of an inhibitor was followed for a prolonged period of time in assay mixtures in which the reaction was started either by the addition of enzyme or substrate. According to literature, in the presence of a slow-binding inhibitor, in the first case the relatively rapid initial rate decreases to a slower steady-state rate, while in the second case there is a slow release of inhibitor, and ultimately a steady state is reached (Sculley et al. 1996). The reaction was followed over 30 min, and none of such slow-binding behaviors were observed: the time course of the reaction was in all instances linear. Deoxynojirimycin was chosen because its slow-binding inhibition is well documented toward sucrase-isomaltase, where the aforementioned effects are evident within 5–10 min of incubation (Hanozet et al. 1981). We conclude that the explanation of the observed results should be sought among complex non-linear inhibition patterns: this aspect will be discussed below.

Inhibition studies with deoxynojirimycin derivatives

The difference in the affinity for trehalose and in the inhibition pattern between midge trehalase and porcine trehalase suggests the presence of a different structure for the substrate/inhibitor binding site. If this were to be confirmed, it may be helpful in developing specific inhibitors for the insect enzyme. A series of inhibiting compounds were synthesized, introducing a substituent of the iminoglucitol ring of deoxynojirimycin. Their structure is reported in Figure 5. Eight compounds were assayed on trehalase activity, and their effect is reported in Table IV. The most active molecule was compound **(4)**, and its inhibition was partially displaced by the enzyme substrate. Compound **(1)** was the second most active, but its effect seemed to be non-competitive as well as that of inhibitors **(3)** and **(2)**. The remaining four compounds did not inhibit trehalase activity. For comparison, the same assay was carried out using porcine kidney trehalase, and no inhibition was observed for all but compound (**4**). Moreover, the degree of inhibition of compound **(4)** on porcine trehalase was significantly lower than that on midge trehalase. Inhibition kinetics was performed on midge trehalase for compound **(4)** (Figure 6). Fitting the data to the Henri–Michaelis–Menten equation returned the following kinetic parameters: K_m (mM) = 1.67 ± 0.12 and 0.34 ± 0.05, V_{max} (U/mL) = 0.25 ± 0.01 and 0.24 ± 0.02, in the presence and in the absence of an inhibitor, respectively. Competitive inhibition was confirmed by double reciprocal plot (Figure 6, inset). Using the Cheng–Prusoff relationship for competitive inhibition, the value K_i = 25 μM was calculated.

Table II. Kinetic parameters of trehalase activity from *C. riparius* larvae. Fixed amounts of trehalase were incubated in the presence of increasing concentrations of trehalose as illustrated in Figure 1 and described in Materials and methods. Data are means ± SE of three independent experiments

pH of the medium	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m
5.5	0.44 ± 0.02	2.73 ± 0.04	6.2
6.5	0.48 ± 0.04	4.57 ± 0.09	9.5
7.5	0.52 ± 0.04	1.22 ± 0.02	2.3

Inhibition studies with casuarine derivatives

To further explore the potentiality of the pyrrolizidine ring of casuarine, a number of derivatives were assayed as inhibitors of trehalase activity from *C. riparius*. The structure of molecules included in this study is illustrated in Figure 7, and their effect is reported in Table V. None of the compounds tested was proven to be more effective than casuarine itself in inhibiting trehalase activity; thus, the hydroxyl group in position 7 of casuarine seems to play a role in enhancing inhibitory activity. In particular, substitution of the hydroxyl group with a hydroxy methyl group dramatically increased the K_i value (273-fold in *C. riparius*), whereas removing the hydroxyl group (7-deoxycasuarine) had a relatively slight effect with

Table III. Inhibition of trehalase activity. Fixed amounts of trehalase from *C. riparius* larvae and from porcine kidney were incubated in the presence of fixed trehalose concentrations at K_m value and increasing concentrations of the indicated inhibitors. Parameters were calculated as described in the text. Data are means ± SE of three independent experiments

	C. riparius			Porcine kidney		
Inhibitor	IC_{50} (μM)	*n*	K_i (μM)	IC_{50} (μM)	*n*	K_i (μM)
Casuarine	0.25 ± 0.03	1.59	0.12 ± 0.01	12[a]	–	–
Deoxynojirimycin	2.83 ± 0.34	1.25	1.39 ± 0.18	5.96 ± 0.62 4.3[b]	1.08	2.98 ± 0.32
Castanospermine	9.93 ± 1.80	1.19	4.86 ± 0.91	2.5[b]	–	–

[a]Data from Kato et al. 2003.
[b]Data from Asano et al. 2003.

a 4.9-fold increase of the K_i value. Also, this latter compound had a K_i value 19.8-fold lower for trehalase from *C. riparius* larvae than that for trehalase from porcine kidney. Reducing the size of the heterocyclic ring from pyrrolizidine to pyrrolidine (as in 6-deoxy-DMDP and DAB-1) drastically decreased the inhibitory activity. In this case, the value of the inhibition constants for *C. riparius* larvae and porcine kidney was similar.

Discussion

Insect trehalase has been purified from several species belonging to main insect orders, and new trehalases continue to be added to this list (Jin and Zheng 2009). The enzyme is of increasing interest in insect physiology since the soluble isoform of the enzyme is under hormonal regulation (Tatun et al. 2008; Gu et al. 2009). For its central role in glucose catabolism, trehalase has been considered (Asano et al. 1990) and continues to be regarded as a possible target for insect pest control (Jin and Zheng 2009). The larvae of the midge *C. riparius* play a fundamental role in the freshwater food web and are included as sentinel organisms for their ability to cope with polluted and anoxic environments (Rosenberg and Resh 1993). *C. riparius* larvae, as other insects, contain both soluble and membrane-bound isoforms of trehalase, known as trehalase-1 and trehalase-2, respectively. It is believed that the membrane isoform represents a latent or inactive form that can be activated in vitro by detergents (Wegener et al. 2003). In *Bombyx mori*, the predominant trehalase switches from trehalase-2 to trehalase-1 at the onset of spinning, marking the beginning of the prepupal period (Yamashita et al. 1974). Previous studies performed in our laboratory suggested that anoxia might trigger activation processes on trehalase-2 in *C. riparius* larvae (Forcella et al. 2007).

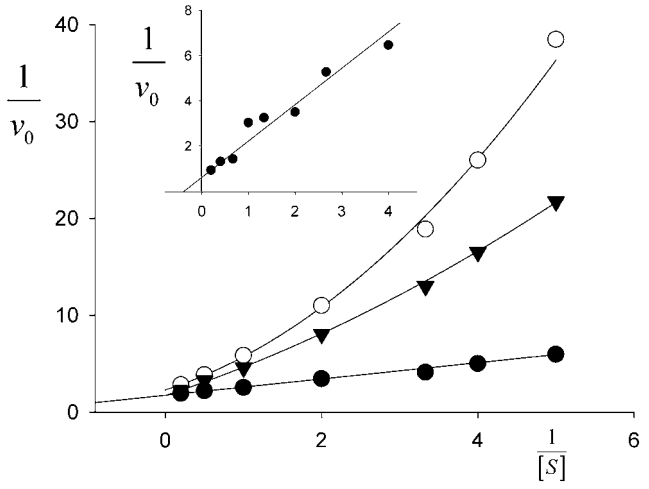


Fig. 4. Lineweaver–Burk plot in the presence of deoxynojirimycin. Main panel: trehalase kinetics was measured as a function of deoxynojirimycin: 0 μM (●), 2.5 μM (▼) and 5 μM (○). Inset: kinetics of porcine kidney trehalase in the presence of 5 μM deoxynojirimycin.

Trehalase-2 purified from midge larvae is a glycoprotein as demonstrated by its retention on concanavalin A affinity column. Its apparent molecular mass is 67 kDa as determined with SDS-PAGE and gel filtration. Also, the enzyme preparation is apparently homogeneous and made of a single isoform as determined by native gel electrophoresis. This latter aspect is particularly important for the interpretation of the kinetic results. First of all, as expected, the larval enzyme is highly specific for trehalose with an affinity about 5-fold higher when compared to the mammalian trehalase. Secondly, the enzyme is competitively inhibited by casuarine, deoxynojirimycin and castanospermine. However, we observed that, in the presence of glycosidase inhibitors, in particular deoxynojirimycin, the double reciprocal plot of the initial rate as a function of trehalose concentration was curvilinear upward. This phenomenon has neither been described for other trehalases from insect tissues nor from other organisms. In fact, it is not evident in inhibitory studies performed with porcine kidney trehalase. In principle, this pattern suggests the presence of a multisite system. For example, it could be compatible with a situation in which the enzyme is a dimer and the inhibitor *I* binds at the interface between the two subunities, inducing a conformational change that excludes the substrate *S* from both catalytic sites. If the binding of *S* to one catalytic site prevents *I* from binding, then the *v* versus [*S*] is sigmoidal, and the Lineweaver–Burk plot is non-linear (Segel 1975, p.385). This scenario is ruled out because there is no evidence that trehalase from *C. riparius* is a dimer or oligomer in its active form: after gel filtration, the enzyme eluted as a single peak in correspondence of the expected molecular weight as determined with standard proteins.

Among complex inhibitory patterns which can be compatible with our experimental data, the "hit-and-run" substrate-inhibitor displacement system proposed by Reiner (1969) and further developed by Fisher et al. (1970) as a ligand exclusion theory seems to give a plausible explanation of our observations. The model assumes the presence of subsites for the binding of sub-

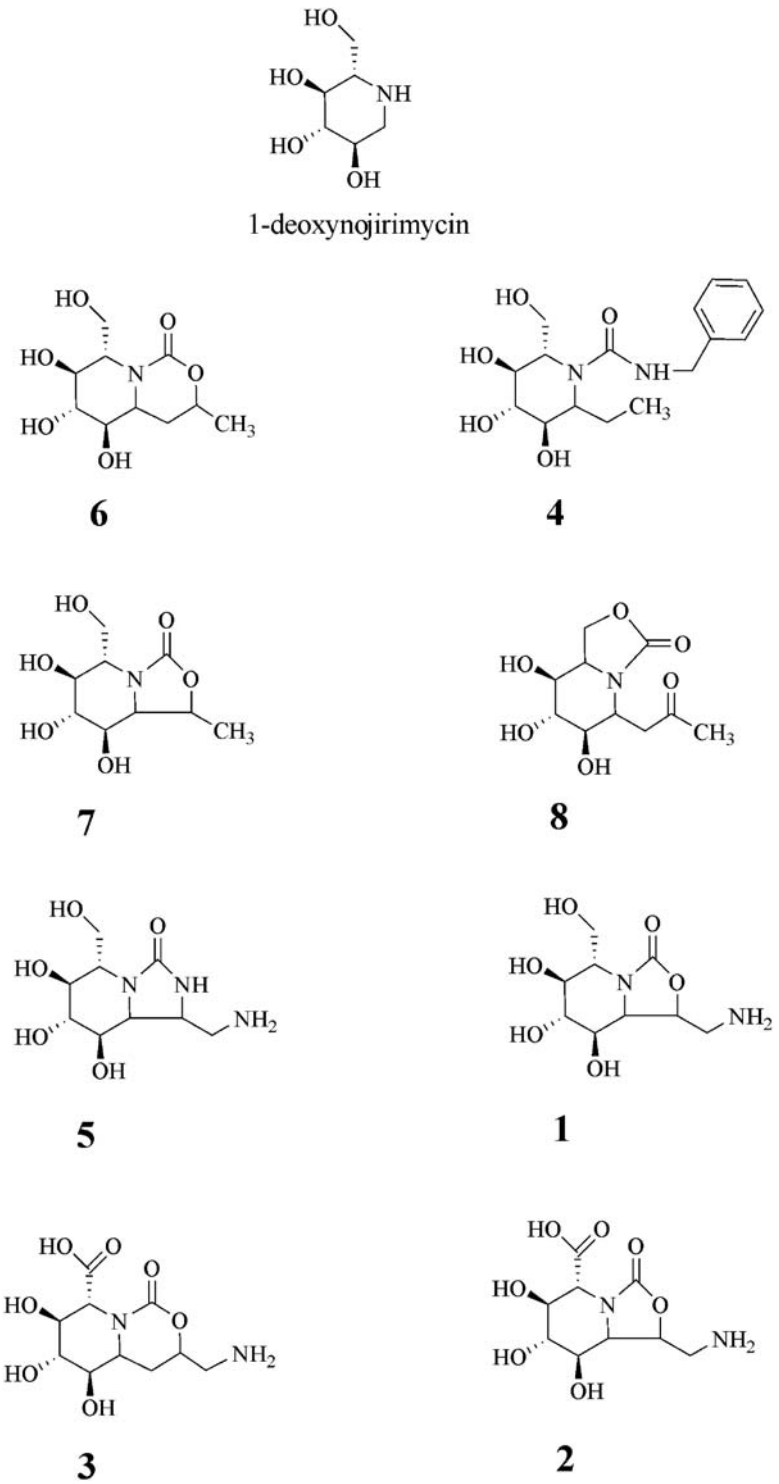
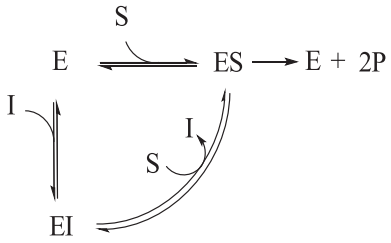


Fig. 5. Structure of deoxynojirimycin derivatives used as inhibitors of trehalase from *C. riparius* larvae and from porcine kidney.

Table IV. Inhibition by deoxynojirimycin derivatives of membrane-bound trehalase from *C. riparius* larvae and trehalase from porcine kidney. Initial rate of trehalase was measured in the presence of 100 μM inhibitor (#) at two fixed substrate concentrations, namely at the corresponding K_m value and in the presence of an excess of trehalose. Data represent relative activities (v_i,v) and are expressed as a percentage of a control without inhibitors

	Trehalose concentration			
	C. riparius		Porcine kidney	
Inhibitor (#)	0.5 mM	5 mM	2.5 mM	20 mM
(1)	50	51	103	103
(2)	68	67	97	100
(3)	59	63	106	102
(4)	46	63	75	91
(5)	101	102	94	98
(6)	91	83	102	103
(7)	100	95	98	100
(8)	95	96	103	104

strate and inhibitor, and there are two routes to the complex enzyme substrate according to the following equilibria (Segel 1975, p.836):



It is now well accepted that trehalase catalytic site contains two subsites (Asano et al. 1996; Silva et al. 2004; Gibson et al. 2007; Cardona et al. 2009), a catalytic site and a recognition site. It was demonstrated that deoxynojirimycin can bind to each subsite of the porcine trehalase (Asano et al. 1996). Our results can be interpreted with more than one deoxynojirimycin

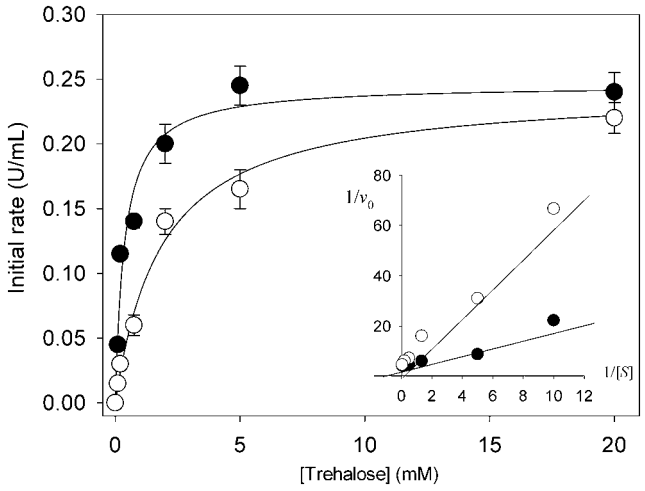


Fig. 6. Inhibition kinetics by compound **4** on *C. riparius* trehalase. Closed symbols represent a control in the absence of inhibitor, open symbols initial rates in the presence of 100 μM inhibitor. Inset: Lineweaver–Burk plot of the data.

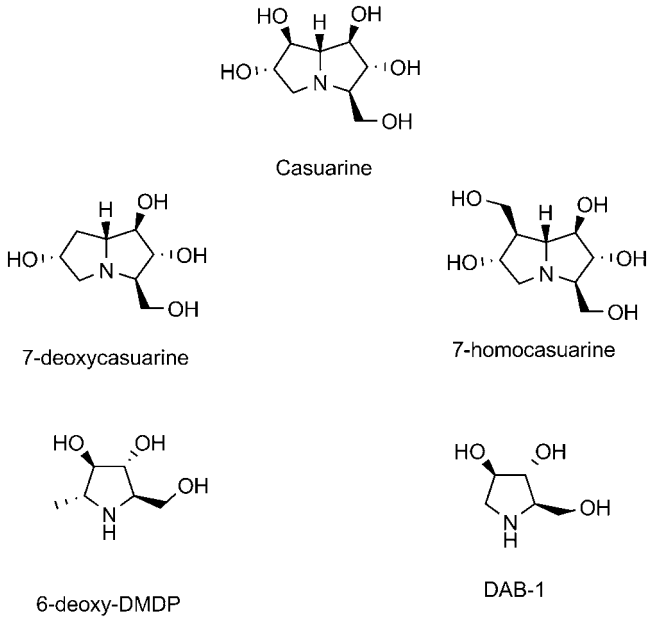


Fig. 7. Structure of casuarine and analogs used as inhibitors of trehalase from *C. riparius*.

binding to *C. riparius* trehalase and with binding of the inhibitor that modifies the kinetics as a function of trehalose concentration. A cartoon illustrating the interaction of deoxynojirimycin with trehalase from midge larvae is shown in Figure 8.

There is significant dissimilarity in the kinetic behavior between trehalase from *C. riparius* and trehalase from porcine kidney. This is clearly evidenced by several aspects: first of all, there is a significant difference in substrate affinity; second, the degree of inhibition caused by iminosugar alkaloids casuarine, castanospermine, deoxynojirimycin and their derivatives is markedly different between the two enzymes; third, the effect of deoxynojirimycin is different since it modifies the v versus [S] relationship from hyperbolic to sigmoidal in *C. riparius* trehalase. Taken together, these data support the idea that, although trehalase is an evolutionary old and well-conserved protein, the midge trehalase bears molecular features that could be exploited to develop compounds having insecticidal activity against dipterans. Comparative studies with trehalases from species belonging to other insect orders are needed to explore whether these features are shared also by major agricultural insect pests.

Table V. Inhibition by casuarine derivatives of membrane-bound trehalase from *C. riparius* larvae and trehalase from porcine kidney. Initial rate of trehalase activity was measured in the presence of 0.5 mM trehalose (*C. riparius*) and 5 mM trehalose (porcine kidney) and increasing concentrations of the indicated inhibitors ranging from 0.1 to 200 μM. Data are means ± SE of three independent experiments

	C. riparius		Porcine kidney	
	IC_{50}(μM)	K_i (μM)	IC_{50}(μM)	K_i (μM)
7-Deoxycasuarine	1.2 ± 0.04	0.59 ± 0.02	21.5 ± 2.8	11.7 ± 1.4
7-Homocasuarine	65.5 ± 1.5	33.0 ± 0.8	214.0 ± 28.8	107.0 ± 14.4
6-Deoxy-DMDP	52.4 ± 2.2	25.6 ± 1.1	110.8 ± 16.4	55.4 ± 8.2
DAB-1	19.0 ± 0.9	9.3 ± 0.4	10.6 ± 2.4	5.3 ± 1.2

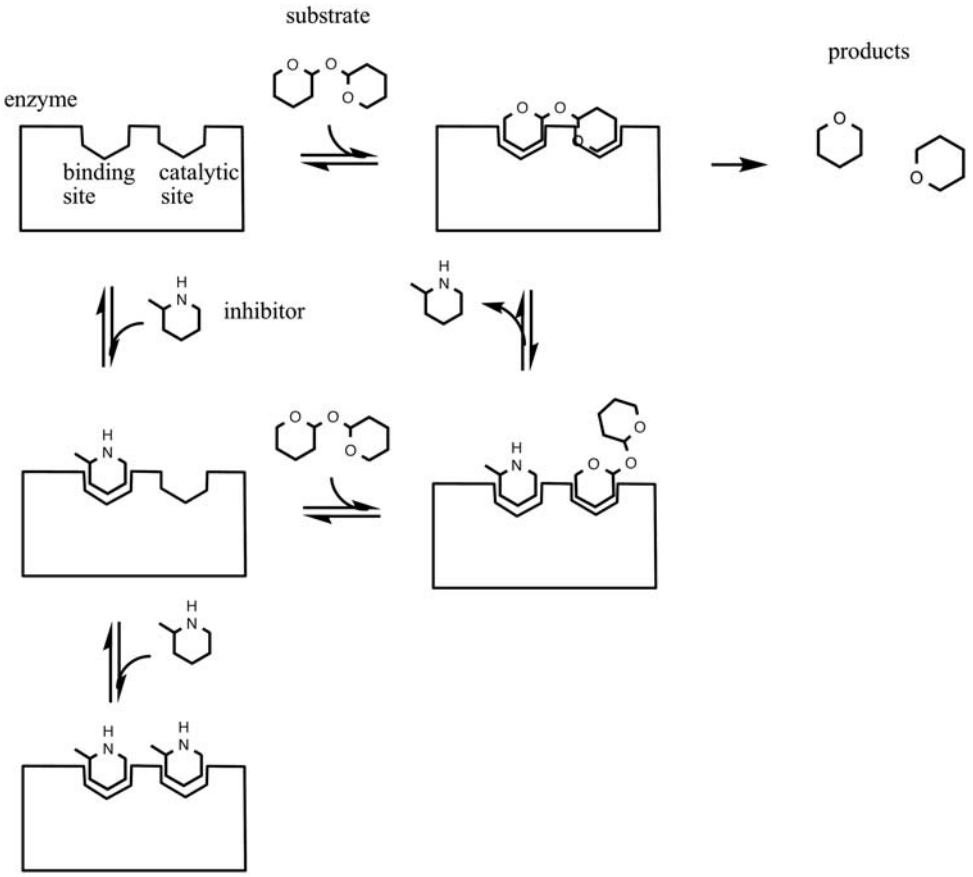


Fig. 8. Cartoon of the inhibition pattern by deoxynojirimycin on *C. riparius* trehalase. The schematic structure of trehalose (substrate), glucose (products), and deoxynojirimycin (inhibitor) are shown.

Materials and methods

Animals

C. riparius larvae were purchased alive from a wholesale bait and fish stocking farm (Eschematteo, Parma, Italy). Larvae were maintained at constant photoperiod (16 h light/8 h dark) and temperature (4°C) in large vessels containing 2 cm of dechlorinated tap water, continuously aerated, and reared with Tetramin or yeast powder. The water was changed as soon it became turbid. The larvae used for the experiments were in the fourth instar.

Chemicals

Tris, 4-morpholine-ethansulfonic acid (MES), HEPES, CHAPS, phenylmethylsulfonyl fluoride (PMSF), sodium azide, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) trehalase from porcine kidney, α-methyl-D-mannoside, deoxynojirimycin, castanospermine, salts, substrates and coenzymes were purchased from Sigma-Aldrich (Italy). The following compounds, (1*R*,2*R*,3*R*,6*S*,7*S*,7a*R*)-3-(hydroxymethyl) hexahydro-1*H*-pyrrolizine-1,2,6,7-tetrol (casuarine), (1*R*,2*R*,3*R*,6*R*,7a*R*)-3-(hydroxymethyl) hexahydro-1*H*-pyrrolizine-1,2,6-triol (7-deoxycasuarine), (1*R*,2*R*,3*R*,6*R*,7*R*,7a*R*)-3,7-bis (hydroxymethyl)hexahydro-1*H*-pyrrolizine-1,2,6-triol (7-homocasuarine), (2*R*,3*R*,4*R*,5*R*)-3,4-dihydroxy-2-(hydroxymethyl)-5-methylpyrrolidine (6-deoxy-DMDP) and (2*R*,3*R*,4*R*)-3,4-dihydroxy-2-(hydroxymethyl)pyrrolidine (DAB-1) were synthesized as described by Cardona et al. (2003), Merino et al. (2008) and Cardona et al. (2009). Deoxynojirimycin derivatives (5*R*,6*S*,7*S*,8*S*)-5,6,7-trihydroxy-8-(hydroxymethyl)-3-methylhexahydropyrido[1,2-c][1,3]oxazin-1(3H)-one (**6**), (3*R*,4*S*,5*S*,6*S*)-*N*-benzyl-2-ethyl-3,4,5-trihydroxy-6-(hydroxymethyl)piperidine-1-carboxamide (**4**), (5*S*,6*S*,7*R*,8*R*)-6,7,8-trihydroxy-5-(hydroxymethyl)-1-methyltetrahydro-1*H*-oxazolo [3,4-a]pyridin-3(5H)-one (**7**), (6*R*,7*S*,8*S*)-6,7,8-trihydroxy-5-(2-oxopropyl)tetrahydro-1*H*-oxazolo[3,4-a]pyridin-3(5*H*)-one (**8**), (5*S*,6*S*,7*S*,8*R*)-1-(aminomethyl)-6,7,8-trihydroxy-5-(hydroxymethyl) hexahydroimidazo [1,5-a]pyridin-3(5*H*)-one (**5**), (5*S*,6*S*,7*R*,*8R)*-1-(aminomethyl)-6,7,8-trihydroxy-5-(hydroxymethyl)tetrahydro-1*H*-oxazolo[3,4-a]pyridin-3(5*H*)-one (**1**), (5*R*,6*S*,7*S*,8*R*)-3-(aminomethyl)-5,6,7-trihydroxy-1-oxooctahydropyrido[1,2-c][1,3]oxazine-8-carboxylic acid (**3**) and (5*R*,6*S*,7*S*,8*R*)-1-(aminomethyl)-6,7,8-trihydroxy-3-oxohexahydro-1*H*-oxazolo[3,4-a]pyridine-5-carboxylic acid (**2**) were synthesized as previously described (Cipolla et al. 2007).

Tissue homogenization and protein extraction

Midge larvae were suspended in 9 mL/g fresh weight of a hypotonic buffer (50 mM MES, pH 6.5) containing 0.1 mM PMSF and 0.01% sodium azide and homogenized at 4°C in a Warring mixer, set at 11,400 rpm, for two 2-min periods separated by a 2-min cooling interval. The suspension was then filtered through two layers of surgical gauze, and one small al-

iquot was withdrawn for enzyme assays. The crude extract was centrifuged at 100,000 × *g* for 1 h at 4°C (XL-90 Ultracentrifuge, Beckman), and the resulting supernatant was discharged. The sediment was resuspended in four volumes of homogenization buffer in a glass and teflon Potter-Helvehjem, two 6-strokes at 2000 rpm, separated by 1 min in ice and incubated with 30 mM CHAPS, stirring gently at room temperature for 30 min. The preparation was centrifuged at 100,000 × *g* for 1 h at 4°C, and the supernatant containing the solubilized membrane-bound trehalase was used for further processing.

Purification of membrane-bound trehalase

The supernatant containing membrane-bound trehalase activity was applied on a weak anion exchanger (DEAE Sephacel, Pharmacia Biotech). The column was equilibrated and washed with 20 mM Tris-HCl, pH 6.8, and trehalase was eluted by 1 M NaCl in the same buffer. Fractions containing trehalase activity were combined, dialyzed (cutoff 13 kDa) against 20 mM Tris-HCl, pH 6.8 and concentrated by ultrafiltration under N_2 at 200–300 KPa of pressure (Amicon unit, Millipore cellulose filter, 30 kDa). Fractions were then chromatographed on Mono Q anion exchange column in a fast protein liquid chromatography (FPLC) system (Pharmacia): the column was equilibrated and washed with 20 mM Tris-HCl, pH 6.8, and fractions were eluted, performing a linear gradient with increasing concentrations of sodium chloride (from 0 to 1 mol L^{-1}) at a flow rate of 1 mL min^{-1}. Fractions with trehalase activity were combined, dialyzed, concentrated on Centricon YM-30 (Millipore) and subjected to affinity chromatography using a concanavalin A column (Con A-Sepharose 4B, Amersham) according to Jahagirdar et al. (1990). The column was washed with 20 mM Tris-HCl, pH 6.8 containing 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and elution was achieved with 0.2 M α-methyl-D-mannoside in the same buffer. As before, trehalase fractions were combined, dialyzed against 20 mM Tris-HCl, pH 6.8 and concentrated on Centricon YM-30 (Millipore). Protein concentration was determined with the method of Bradford (1976) using bovine serum albumin as standard. The enzymatic activity was expressed in international units and referred to protein concentration.

Enzyme assay

Trehalase activity was measured through a coupled assay with glucose-6-phosphate dehydrogenase and hexokinase according to Wegener et al. (2003). All enzyme assays were performed in triplicates at 30°C using sample volumes varying from 5 to 20 µL in a 1-mL test and using a Cary3 UV–Vis Spectrophotometer. Enzyme activities were analyzed by Cary Win UV application software for Windows XP. The specific activity (U mg^{-1}) was expressed as µmol min^{-1} mg $protein^{-1}$. Values were expressed as mean ± SE of replicated.

Kinetic analyses

Kinetic experiments were performed using purified trehalase from *C. riparius* and commercial porcine trehalase measuring enzymatic activity at different trehalose concentrations from 0.1 to 20 mM. Kinetic experiments were carried out at different pH values in the following buffer systems: MES–Tris 20 mM pH 5.5, sodium acetate 20 mM, pH 6.5 and HEPES–Tris 20 mM, pH 7.5. The effect of inhibitors was evaluated as a function of trehalose concentrations from 0.1 to 5 mM in the presence of fixed inhibitor concentrations (see figure legends) and at fixed substrate concentration in the presence of increasing inhibitor concentrations as specified in the figure legends. Kinetic parameters were calculated using a multiparameter, iterative, non-linear regression program based on the Marquardt–Levenberg algorithm (Sigma Plot, Jandel, CA). Data are given ±SD of three independent experiments.

Determination of molecular mass

The molecular weight of membrane-bound trehalase was determined by SDS gel electrophoresis according to a standard protocol and by gel filtration on the Superdex 200 HR 10/30 (Amersham) column in 50 mM sodium phosphate buffer (pH 7) containing 150 mM NaCl into FPLC system, with the following proteins (from Sigma Aldrich) as standards: thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa) and deoxyribonuclease (31 kDa).

Native electrophoresis

Non-denaturating electrophoresis was done on gradient from 20% to 6% polyacrylamide gel at constant voltage of 60 V overnight at 4°C until equilibrium. No SDS nor β-mercaptoethanol was added, and the samples were not heated before being loaded onto the gel. After the run, trehalase activity was detected by incubating the gel at 37°C overnight in 120 mM sodium acetate, pH 6.5, 10 mM $MgCl_2$, 1 mM ATP, 0.6 mM $NADP^+$, 20 mM trehalose, 1.34 U/mL hexokinase, 0.86 U/mL glucose-6-phosphate dehydrogenase and 0.6 mM BCIP in 70% dimethylformamide and 0.5 mM NBT in dimethylformamide.

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Abbreviations

BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CHAPS, 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate; FPLC, fast protein liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethansulfonic acid; HPLC, high-performance liquid chromatography IC_{50} 50% inhibitor concentration; MES, 4-morpholine-ethansulfonic acid; NBT, nitro blue tetrazolium; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

References

Ando O, Nakajima M, Kifune M, Fang H, Tanazawa K. 1995. Trehazolin, a slow, tight-binding inhibitor of silkworm trehalase. *Biochim Biophys Acta*. 1224:295–302.

Asano N. 2003. Glycosidase inhibitors: update and perspectives on practical use. *Glycobiology.* 13:93R–104R.
Asano N, Kato A, Matsui K. 1996. Two subsites on the active center of porcine kidney trehalase. *Eur J Biochem*. 240:692–698.
Asano N, Takeuchi M, Kameda Y, Matsui K, Kono Y. 1990. Trehalase inhibitors, validoxylamine A and related compounds as insecticides. *J Antibiot*. 43:722–726.
Azuma M, Yamashita O. 1985. Cellular localization and proposed function of midgut trehalase in the silkworm larva, *Bombyx mori Tiss. Cell*. 17:539–551.
Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein dye binding. *Anal Biochem*. 72:248–252.
Callaghan A, Hirthe G, Fisher T, Crane M. 2001. Effect of short-term exposure to chlorpyriphos on developmental parameters and biochemical biomarkers in *Chironomus riparius* Meigen. *Ecotoxicol Environm Safety.* 50:19–24.
Cardona F, Faggi E, Liguori F, Cacciarini M, Goti A. 2003. Total syntheses of hyacinthacine A_2 and 7-deoxycasuarine by cycloaddition to a carbohydrate derived nitrone. *Tetrahedron Lett*. 44:2315–2318.
Cardona F, Parmeggiani C, Faggi E, Bonaccini C, Gratteri P, Sim L, Gloster TM, Roberts S, Davies GJ, Rose DR, et al. 2009. Total syntheses of casuarine and its 6-*O*-alpha-glucoside: complementary inhibition towards glycoside hydrolases of the GH31 and GH37 families. *Chem Eur J*. 15:1627–1636.
Cipolla L, Reis Fernandes M, Gregori M, Airoldi C, Nicotra F. 2007. Synthesis and biological evaluation of a small library of nojirimycin derived bicyclic iminosugars. *Carbohydr Res*. 342:1813–1830.
Elbein AD, Pan YT, Pastuszak I, Carroll D. 2003. New insights on trehalose: a multifunctional molecule. *Glycobiology.* 13:17R–27R.
Fisher HF, Gates RE, Cross DG. 1970. A ligand exclusion theory of allosteric effects. *Nature*. 228:247–249.
Forcella M, Berra E, Giacchini R, Rossaro B, Parenti P. 2007. Increased alanine concentration is associated with exposure to fenitrothion but not carbamates in *Chironomus riparius* larvae. *Ecotoxicol Environm Safety.*. 66:326–334.
Gibson RP, Gloster TM, Roberts S, Warren RAJ, Storch de-Gracia I, García Á, Chiara JL, Davies GJ. 2007. Molecular basis for trehalase inhibition revealed by the structure of trehalase in complex with potent inhibitors. *Angew Chem Int Ed*. 46:4115–4119.
Gu J, Shao Y, Zhang C, Liu Z, Zhang Y. 2009. Characterization of putative soluble and membrane-bound trehalases in a hemipteran insect, *Nilaparvata lugens J Insect Physiol.*. 55:997–1002.
Gussin AES, Wyatt GR. 1965. Membrane-bound trehalase from *Cecropia* silkworm muscle. *Arch Biochem Biophys*. 112:626–634.
Hanozet G, Pircher HP, Vanni P, Oesch B, Semenza G. 1981. An example of enzyme hysteresis. *J Biol Chem*. 256:3703–3711.
Jahagindar AP, Downer RGH, Viswanatha T. 1990. Purification and characterization of trehalose-hydrolyzing enzymes from thoracic musculature of the American cockroach *Periplaneta americana*. *Insect Biochem*. 20:511–516.
Kato A, Kano E, Adachi I, Molyneux RJ, Watson AA, Nash RJ, Fleet GWJ, Wormald MW, Kizu H, Ikeda K, et al. 2003. Australine and related alkaloids: easy structural confirmation by ^{13}C NMR spectral data and biological activities. *Tetrahedron Asymmetr.*. 14:325–331.
Knuesel I, Murao S, Shin T, Amachi T, Kayser H. 1998. Comparative studies of suidatrestin, a specific inhibitor of trehalases. *Comp Biochem Physiol Part B*. 120:639–646.
Mitsumasu K, Azuma M, Niimi T, Yamashita O, Yaginuma T. 2008. Changes in the expression of soluble and integral-membrane trehalases in the midgut during metamorphosis in *Bombyx mori Zool Sci*. 25:693–698.
Jin L-Q, Zheng Y-G. 2009. Inhibitory effects of validamycin compounds on the termites trehalase. *Pest Biochem Physiol Sci*. 95:28–32.
Merino P, Delso I, Tejero T, Cardona F, Marradi M, Faggi E, Parmeggiani C, Goti A. 2008. Nucleophilic additions to cyclic nitrones en route to iminocyclitols—total syntheses of DMDP, 6-deoxy DMDP, DAB-1, CYB-3, nectrisine, and radicamine B. *Eur J Org Chem*. 17:2929–2947.
Mitsumasu K, Azuma M, Niimi T, Yamashita O, Yaginuma T. 2005. Membrane-penetrating trehalase from silkworm *Bombyx mori*. Molecular cloning and localization in larval midgut. *Insect Mol Biol*. 14:501–508.
Reed WD, Sacktor B. 1971. Localization of trehalase in flight muscle of the blowfly *Phormia regina*. *Arch Biochem Biophys*. 145:392–401.
Reiner JM. 1969. *Behavior of enzyme systems*.. N.Y: Van Nostrand Reinhold.
Rosenberg DM, Resh VH. 1993. *Freshwater biomonitoring and benthic macroinvertebrates*.. New York: Chapman and Hall. p. 488.
Sculley MJ, Morrison JF, Cleland WW. 1996. Slow-binding inhibition: the general case. *Biochim Biophys Acta*. 1298:78–86.
Segel H. 1975. *Enzyme kinetics*. NY: John Wiley & sons.
Shimada S, Yamashita O. 1979. Trehalose absorption related with trehalase in developing ovaries of the silkworm *Bombyx mori*. *J Comp Physiol*. 131:333–339.
Silva MCP, Terra WR, Ferreira C. 2004. The role of carboxyl, guanidine and imidazole groups in catalysis by a midgut trehalase purified from an insect larvae. *Insect Biochem Mol Biol*. 34:1089–1099.
Strang RHC, Clement EM. 1980. The relative importance of glucose and trehalose in the nutrition of the nervous system of the locust *Schistocerca americana gregaria*. *Insect Biochem.*. 10:155–161.
Tatun N, Singtripop T, Tungjitwitayakul J, Sakurai S. 2008. Regulation of soluble and membrane-bound trehalase activity and expression of the enzyme in the larval midgut of the bamboo borer *Omphisa fuscidentalis*. *Insect Biochem Mol Biol*. 38:788–795.
Thompson SN. 2003. Trehalose—the insect "blood" sugar. *Adv Insect Physiol*. 31:205–285.
Vaandrager SH, Haller TB, Van Marrewijk WJA, Beenakkers AMT. 1989. Fractionation and kinetic properties of trehalase from flight muscle and hemolymph of the locust *Locusta migratoria*. *Insect Biochem*. 19:89–94.
van Handel E. 1978. Trehalose turnover and the coexistance of trehalose and active trehalase in cockroach haemolymph. *J Insect Physiol*. 24:151–153.
Wegener G, Tschiedel V, Schlöder P, Ando O. 2003. The toxic and lethal effects of the trehalase inhibitor trehazolin in locusts are caused by hypoglycemia. *J Exp Biol*. 206:1233–1240.
Wyatt GR. 1967. The biochemistry of sugars and polysaccharides in insects. *Adv Insect Physiol*. 4:287–360.
Yaginuma T, Mizuno T, Mizuno C, Ikeda M, Wada T, Hattori K, Yamashita O, Happ GM. 1996. Trehalase in the spermatophore from the bean-shaped accessory gland of the male mealworm beetle, *Tenebrio molitor*: purification, kinetic properties and localization of the enzyme. *J Comp Physiol*. 20:1079–1085.
Yamashita O, Sumida M, Hasegawa K. 1974. Developmental changes in midgut trehalase activity and its localization in the silkworm, *Bombyx mori*. *J Insect Physiol*. 20:1079–1085.