Conformational changes on ligand binding in wild-type and mutants from *Spodoptera frugiperda* midgut trehalase

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**Abstract**

Trehalase specifically hydrolyses trehalose into two glucose units and is most important in insects and fungi. Previous evidence suggested that *Spodoptera frugiperda* midgut trehalase (wild type, WT) has substantial conformational changes on binding different substances. Our goal is to understand this mobility. For this, two deletion mutants were produced, lacking regions supposed to be the cause of mobility ([102 residues from the N-terminus (NT) and this portion plus 31 residues from the C-terminus (NCT)]. Circular dichroism spectra before and after denaturation of the enzymes support the assertion that they are appropriately folded. The overall results show that the removal of 102 or 133 amino acids does not greatly change the interaction with the substrate and competitive inhibitors, but leads to a considerable decrease in kcat/Km values from WT 74300 M⁻¹ s⁻¹ to NT 647 M⁻¹ s⁻¹ and NCT 1044 M⁻¹ s⁻¹. Diethyl pyrocarbonate His modification only occurs in wild and truncated trehalases in the presence of some ligands. Looking for changes in folding WT, NT, and NCT were incubated with different compounds in the presence of Sypro Orange, that binds to hydrophobic regions increasing its fluorescence. The dye fluorescence is affected by 2 compounds when WT is present, and at least by 5 compounds when NT or NCT are present, suggesting that conformational changes caused by ligand binding occur only in the vicinity of the active site. These data provide physical evidence in favor of a change in folding around the active site caused by ligand binding, in agreement to prior chemical modification and other kinetic data and challenging the hypothesis that N- and C-terminal are the mobile regions.

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**1. Introduction**

Trehalase specifically hydrolyses trehalose to the constituent glucose units. As trehalase is the most important circulating sugar in insects, this enzyme is present in all insect tissues [2]. Trehalase inactivation affects insect performance and is a good target for insect control. In this connection, Silva et al. [15,16] and Gomez et al. [7] showed that several plant glucosides and their aglycones are able to inhibit trehalases from different tissues in a variety of insect orders. As most of those inhibitors are competitive, adaptation to them may be achieved by an increase in haemolymph trehalose concentration. Another adaptation to the presence of trehalase inhibitors is an increase in trehalase activity [16].

Trehalases are important not only for insects, but also for nematodes and fungi. Nevertheless, midgut insect trehalases are the ones best studied [17]. The only trehalase to have its 3D-structure resolved was the periplasmic trehalase (Ter37A) from *Escherichia coli* [6]. Based on the crystallographic data the authors hypothesized that two carboxylic residues (Asp 312 and Glu 496) are the catalytic ones and noticed that 3 Arg residues are in the trehalase active site. Site-directed mutagenesis in the midgut soluble trehalase from *S. frugiperda* (SfTre1, GenBank accession no. DQ447188) confirmed that Asp 322 and Glu 520 (homologous to the active residues of *E. coli* trehalase) are the catalytic residues and that 3 Arg residues (R169, R222 and R287) are essential to catalysis [14] and not to substrate binding as previously proposed [6]. The residue Arg222 has its pKa value affected by a His residue in a similar way as that of the proton donor [15]. This explains the previous misinterpretation of the proton donor as an Arg residue [15]. These findings also explain the earlier implication of His residues in assisting a carboxyl group acting as a proton donor [10,18,19].

Some results indicated that trehalases has substantial conformational changes on binding different substances. Thus, *S. frugiperda* trehalase is inactivated by diethyl pyrocarbonate (a modifier of His residues) only in the presence of a small competitive inhibitor like methyl-α-glucoside, which binding probably

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leads to a conformational change in the enzyme. Besides, trehalase is strongly inhibited by amygdalin (glucose β-1,6-glucose β-mandelonitrile), whereas gentiobiose (glucose β-1,6-glucose) is not inhibitory, even in concentrations as great as 10 fold the Ki value for amygdalin [15,7], indicating that the mandelonitrile moiety binds in an enzyme region that opens the active site to receive the gentiobiose portion of amygdalin. Furthermore, crystallographic data on E. coli trehalase showed that the inhibitor bound to its active site is completely surrounded by the protein, meaning that there must be a large conformational change to free it [6].

Attempts of our research group to crystallize the recombinant S. frugiperda trehalase failed. Since the failure could be a consequence of large mobile sequences on the enzyme, we hypothesized that this mobility would explain the changes associated with ligand binding. To test this hypothesis, the wild-type and two truncated trehalases were evaluated regarding their conformational changes on ligand binding. The results showed that changes in folding occurs in the vicinity of the active site both in the wild-type and in the truncated trehalases, thus challenging the initial hypothesis.

2. Materials and methods

2.1. Chemicals

Trehalose, buffer salts, diethyl pyrocarbonate (DEPC), inhibitors, and protein molecular weight markers were purchased from Sigma-Aldrich (USA). Other reagents were of the higher grade available and acquired from Sigma or Merck (Darmstadt, Germany).

2.2. Construction of expression vectors of cDNAs coding for wild type (WT), N-truncated (NT) and N- and C-truncated (NCT) type trehalases

The cDNAs coding for trehalase and truncated trehalases were amplified by polymerase chain-reaction (PCR) with the primers listed in Table 1, using the plasmid pGEMT containing the mature (without the signal peptide) coding sequence of trehalase (WT, which is SfFtre1, GenBank accession no. DQ447188) as a template. The PCR was performed in a thermocycler (AB Applied Biosystems) with Taq DNA polymerase (Invitrogen TM Life Technologies), according to manufacturer’s instructions, with 30 cycles of amplification (94 °C for 30 s; 50 °C for 45 s; 72 °C for 120 s), followed by 10 min at 72 °C. The purified PCR products were ligated into the pET SUMO vector (Invitrogen, Life technologies). The constructions were used to transform One Shot Mach 1T T1 phage chemically competent E. coli. The transfected cells were plated in LB agar containing kanamycin (50 μg/mL) and were grown overnight. Selected colonies were grown overnight in LB-kanamycin medium and used as a source of the different constructions. The sequences were confirmed by sequencing both strands, using an ABI automated sequencer (Applied Biosystems, Boston, USA) with primers listed in Table 2.

Afterwards, the constructions WT-pET SUMO, NT-pET SUMO and NCT-pET SUMO were used to transform BL2(DE3) E. coli competent cells (Invitrogen). The transfected cells were grown overnight at 37 °C in LB medium containing kanamycin 50 μg/mL. This culture was diluted 1:20 in 100 mL of LB medium supplemented with kanamycin 50 μg/mL and grown at 20 °C until the absorbance at 600 nm reached about 0.6. Isopropyl-β-D-thiogalactoside (IPTG) was then added to a final concentration of 1 mM, and the culture was incubated at 20 °C for 20 h. The cells were harvested by centrifugation at 4000 g for 20 min at 4 °C and stored at −80 °C. The pellet was suspended in 1 mL of lysis buffer [20 mM sodium phosphate buffer pH 7.0, 300 mM NaCl, 20 mM imidazol, 1 mM phenylmethylsulfonyl fluoride]. Disruption was performed by sonication for 3 cycles of 15 s using a Branson Sonifier Model 450 (Branson Ultrasonics, Danbury, USA) set at power level 3. The crude extract was centrifuged at 10,000 g for 30 min at 4 °C and the supernatant fraction was loaded onto a Ni-NTA agarose column (800 μL resin volume), previously equilibrated in lysis buffer and unbound proteins eluted with six column volumes of the same buffer. The bound recombinant proteins were eluted with 1 mL of lysis buffer supplemented with 0.3 M imidazol. His tag and SUMO were removed using SUMO protease according to the manufacturer’s instructions (Invitrogen, Life Technologies). The samples were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) [9].

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Experimental samples were combined with sample buffer containing 60 mM Tris–HCl buffer pH 6.8, 2.5% (w/v) SDS, 0.36 mM β-mercaptoethanol, 0.5 mM EDTA, 10% (v/v) glycerol, and 0.005% (w/v) bromophenol blue. The samples were heated for 5 min at 95 °C in a water bath, before being loaded onto a 12% (w/v) polyacrylamide gel slab containing 0.1% SDS [9] and the electrophoresis were run at constant voltage of 200 V. Protein staining was done with a solution of 0.1% (w/v) Coomassie Blue R in 10% acetic acid and 40% methanol for 30 min and stained with several washes in a solution containing 40% methanol and 10% acetic acid or with water. Molecular-mass markers used were: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), and phosphorylase b (97.4 kDa).

2.4. Protein determination and standard trehalase assay

Protein was determined according to Bradford [3], using ovalbumin as a standard. Trehalase activity was determined by measuring the release of glucose according to Dahlqvist [5]. The enzyme was assayed in 25 mM citrate–sodium phosphate buffer pH 6.0, with 7 mM trehalose as a substrate. Enzyme assays were performed at 30 °C under conditions such that activity was proportional to protein concentration and to time. Controls without enzyme or without substrate were included. Km and Vmax values were determined fitting the data in Michaelis–Menten equation, using the software OriginPro 8 (OriginLab Corporation, Northampton, MA). One unit of enzyme (U) is defined as the amount that hydrolyzes 1 μmol of substrate/min.

Table 2

| Primer                | Sequence          |
|----------------------|-------------------|
| Sumo forward         | 5’-AGATCTCTTACAGGCGTCC-3’ |
| T7 reverse           | 5’-TACGTTTCACCGGCTAAT-3’ |
| Internal forward     | 5’-GATTACAAAAAGCAGTATG-3’ |
| Internal reverse     | 5’-ATCCTAGGTTCATCCGTTAACC-3’ |
2.5. Inhibition analysis

For Ki value determinations, purified trehalase was incubated with at least 5 different concentrations of inhibitor in 8 different concentrations of the substrate. The concentrations used were: trehalose: 0.2, 0.3, 0.4, 0.6, 0.8, 1.2, 1.6, 2 mM; amygdalin: 0.1, 0.2, 0.3, 0.5, 0.785 mM; Man: 0, 8, 14, 26, 32, 38, 44 mM; 1-deoxyxojirimicin: 0, 0.48, 0.8, 2.8, 4.8 μM. Ki values were determined...
2.7. Tryptophan intrinsic and Sypro Orange dye extrinsic stopped by a 2-fold dilution of reaction mixtures with 12 mM His. TEMED buffer pH 6. The chemical inactivation reactions were the buffer used in modifications.

Attempts to detect conformational changes of wild and truncated trehalases were done by analysis of tryptophan intrinsic fluorescence measurements.

Attempts to detect conformational changes of wild and truncated trehalases were done by analysis of tryptophan intrinsic fluorescence spectra at different conditions (excitation, 295 nm; emission determined in the range 300–400 nm; scan speed 60 nm/min, with slits of 5.0 nm) in a spectrofluorimeter F-7000 (Hitachi, Tokyo, Japan). The average of 3 spectra was recorded.

Trehalase was submitted to the action of DEPC, a reagent that modify His residues [12]. Purified trehalase was dialyzed against the buffer used in modification reaction. Trehalase was incubated at 30 °C for several periods of time with 6 mM DEPC in 100 mM TEMED buffer pH 6. The chemical inactivation reactions were stopped by a 2-fold dilution of reaction mixtures with 12 mM His.

Table 3
Kinetic parameters of wild type and mutant trehalases.

|        | kcat (s⁻¹) | Km (mM) | kcat/Km (M⁻¹s⁻¹) |
|--------|------------|---------|-------------------|
| WT     | 82         | 1.1 ± 0.1 | 74,545           |
| NT     | 0.55       | 0.85 ± 0.07 | 647             |
| NCT    | 0.71       | 0.68 ± 0.05 | 1044            |

from replots of slopes and/or intercepts of Lineweaver–Burk plots against inhibitor concentration [13].

2.6. Chemical modification studies

Trehalase was submitted to the action of DEPC, a reagent that modify His residues [12]. Purified trehalase was dialyzed against the buffer used in modification reaction. Trehalase was incubated at 30 °C for several periods of time with 6 mM DEPC in 100 mM TEMED buffer pH 6. The chemical inactivation reactions were stopped by a 2-fold dilution of reaction mixtures with 12 mM His.

2.7. Tryptophan intrinsic and Sypro Orange dye extrinsic fluorescence measurements

Attempts to detect conformational changes of wild and truncated trehalases were done by analysis of tryptophan intrinsic fluorescence spectra at different conditions (excitation, 295 nm; emission determined in the range 300–400 nm; scan speed 60 nm/min, with slits of 5.0 nm) in a spectrofluorimeter F-7000 (Hitachi, Tokyo, Japan). The average of 3 spectra was recorded.

The Sypro Orange dye fluorescence increases in intensity when the dye binds to hydrophobic areas of proteins [8]. Thus, it may be used to detect conformational changes of wild and truncated trehalases caused by addition of different compounds to the reaction media. For this, the enzymes were incubated in the presence of a 5000-fold dilution of Sypro Orange dye in 25 mM citrate–sodium phosphate buffer pH 6.0, with or without one of the following compounds: trehalose (7 mM), glucose (1 mM), gentiobiose (20 mM), and several trehalase inhibitors like methyl-α-mannoside (62 mM), methyl-α-glucoside (178 mM), amygdalin (2.1 mM), prunasin (9.2 mM), mandelonitrile (11.5 mM) and mandelonitrile plus gentiobiose (11.5 plus 20 mM, respectively). The reactions were carried out in a 96-well Optical Reaction Plate (Applied Biosystems) and the Sypro Orange dye fluorescence emission measured in 7300 Real Time PCR System (Applied Biosystems, USA) at 30 °C with data collected through to filter B, used in standard applications for SYBR. The fluorescence was recorded after 5 min of incubation. Three determinations were accomplished and blanks were used for correcting the determinations. Blanks contained all compounds except the enzyme.

2.8. Homology modeling

Homology modeling was performed using the server SWISS–Model [1] and the results were analyzed with PYMOL (De Lano Scientific LLC).

2.9. Circular dichroism

CD spectra were recorded using a Jasco J-815 spectropolarimeter (Jasco Instruments, Tokyo, Japan) equipped with Peltier thermostatting cuvette holder, over the range of 190–260 nm (far-UV). CD spectra of native (4.2 μg/mL), NT (3.7 μg/mL) and NCT (3.7 μg/mL) were measured in 5 mM Tris–HCl buffer pH 7, in a quartz cuvette of 1 cm of path-length, at 25 °C and represents the average of eight scans. Data were also collected using the same parameters for cuvettes containing the relevant buffer to allow subtraction of the buffer contribution. The results were expressed as the mean residue ellipticity, [θ], defined as [θ] = θ obs x M/(10. C.ln), where θ obs is the CD in millidegrees, M is the molecular mass (Da), C is the protein concentration (mg/mL), l is the path length of the cuvette (cm), and n is the number of amino acid residues.

2.10. Temperature-induced unfolding

Thermal denaturation measurements for native (4.2 μg/mL), NT (3.7 μg/mL) and NCT (3.7 μg/mL) were monitored by far-UV CD spectroscopy, using a Peltier apparatus. The measurements were carried out in the range 25–90 °C, with a scanning rate of 1 °C/min and at 5 °C intervals. After denaturation, new measurements were obtained at 25 °C.

3. Results

3.1. Production of trehalase mutants

Two truncated trehalases were produced to evaluate the role of putatively mobile N- and C-terminal regions of trehalase in its conformational changes on ligand binding. One lacks 102 N-terminal residues (NT), whereas the other lacks, in addition to those residues, 31 C-terminal residues (NCT) (Fig. 1). Homology modeling was performed here only to show the relative positions of the lacking regions. A detailed homology modeling of SfTre1 was published elsewhere [14].

In order to produce truncated trehalases, constructs containing the cDNA coding for wild type trehalase (WT), NT and NCT enzymes were cloned in the expression vector pET SUMO fused with His residues. BL21(DE3) cells were transformed and the production of the enzymes was induced by IPTG. The proteins were purified using a Ni-NTA agarose column and Sumo was removed according to manufacturer's instructions. The WT, NT and NCT enzymes were expressed with different yields. From one L of culture medium we obtained 0.83 mg of WT, 0.29 mg of NT and 0.08 mg of NCT. The molecular weight of the purified proteins after
SDS–PAGE (Fig. 2) are in agreement with the theoretical values calculated taking into account the amino acid sequences (WT, 64.7 kDa; NT, 52.6 kDa; NCT, 49.6 kDa). SfTre1 mass (67 kDa, [15]) is slightly larger than WT (64.7 kDa) probably due to in vivo glycosylation. WT, NT and NCT have, respectively, 32.86%, 33.09% and 33.58% of identity with the E. coli enzyme used as the template for structure modeling, indicating that the internal portion of the S. frugiperda trehalase is slightly more similar to E. coli trehalase than its N- and C-terminals.

### 3.2. Kinetic properties of wild and truncated enzymes

The truncated mutants have kcat/Km values two orders of magnitude lower than the value for the wild type enzyme, due to the small kcat value, since the Km values are similar for the three enzymes (Table 3). It is interesting to note that the enzyme lacking the N-plus C-terminal portions (NCT) has higher kcat and lower Km values than the enzyme that lacks only the N-terminal portion.

In order to probe the geometry of the active site of the 3 enzymes, the type of inhibition and Ki values for some inhibitors were determined for several compounds. The results for amygdalin inhibition of the wild type trehalase are shown in Fig. 3. The same procedures were used for the other compounds and enzymes. All the compounds used are competitive inhibitors of the enzyme purified from S. frugiperda midguts [15] and the kind of inhibition is not changed in the recombinant WT or truncated enzymes. The calculated Ki values are shown in Table 4.

The second best inhibitor for S. frugiperda midgut soluble trehalase is amygdalin [15] and the Ki values for the 3 recombinant enzymes are the same, suggesting that amygdalin is bound to the same residues in the enzyme and with the same intensity. This indicates that the geometry of the active site is not changed by the N-terminal or N- plus C-terminal deletions. Similar results were found using methyl α-mannoside (MαGlu) or 1-deoxynojirimycin as inhibitors.

### 3.3. Modification with DEPC

No DEPC inactivation of wild-type trehalase is observed (Fig. 4). Nevertheless, when a concentration equivalent to twice the Ki value of MαGlu is present in the assay, trehalase is inactivated up to a residual activity of approximately 50% of the initial activity (Fig. 4A). This result is similar to that obtained with the trehalase
3.4. Extrinsic binding. Occurs only at His residues, as Tyr residue modification by the lack of tetranitromethane modification are widely used to monitor conformational changes of proteins.

3.5. Circular dichroism of WT, NT, and NCT. Wherein the truncated enzymes are affected by 5 (NT) or 6 (NCT) NT and CNT. Thus, WT is affected only by glucose and gentiobiose, whereas amygdalin, prunasin and mannitol plus gentiobiose change the emission gentiobiose, WT and NCT; whereas amygdalin, prunasin and man- nitol plus gentiobiose change the emission.

4. Discussion

The evidence reviewed in the Introduction indicates that trehalase undergoes conformational changes on ligand binding. Since our past failures on crystallizing the recombinant S. frugiperda trehalase could be a consequence of large mobile regions in the enzyme, we looked for regions which might change in conformation. Homology modeling of S. frugiperda trehalase with the periplasmic trehalase from E. coli (PDB 2JF4, [6]) resulted in a model with overall quality in the range of x-ray determined structure [14]. The model shows that a long stretch of the N-terminal and a shorter stretch of the C-terminal correspond to external loops that might be mobile (Fig. 1B). Thus, two mutant trehalases were produced: one lacking 102 N-terminal residues (NT) and another lacking 31 C-terminal residues, in addition to those N-terminal residues (NCT). If these loops were responsible for the conformational changes on ligand binding, the truncated enzymes should be rigid.

Km and Ki values for different competitive inhibitors are similar or identical in the three enzymes indicating that the binding site in the active center is preserved in the truncated enzymes. Nevertheless, truncated trehalases kcat values are much lower than that of wild type trehalases. This means that the removal of stretches of trehalase far from the active site may affect the positioning of the catalytic (but not the binding) residues. This may result from misfolded truncated trehalases. Nevertheless, CD spectra before and after heat denaturation support the assertion that the truncated trehalases are appropriately folded. Another possibility for the low kcat values for the truncated trehalase is that the removal of N- or N- plus C-terminal sequences affects the contact pathways that connect far placed residues with active site residues. This may negatively impacting kcat values (see discussion in [11]).

His modification by DEPC occurs only after the binding of competitive inhibitors. This indicates that truncated trehalases undergo conformational changes on ligand binding, as previously discussed for wild type trehalase. Thus, the external loops of trehalase apparently are not involved in the conformational changes detected by chemical modification on ligand binding.

Looking for physical evidence of conformational changes on ligand binding, the intrinsic fluorescence of tryptophan of trehalases was studied at different conditions. The fluorescence spectra (excitation at 295 nm) of samples of wild and truncated trehalases, with or without ligands, showed no significant emission around 350 nm, where tryptophan is expected to fluoresce (results not shown). The experiments were repeated several times with different protein concentrations with the same results. No conclusion can be drawn about conformational changes from these experiments, mainly because the emission of Trp was very low. The amino acids Lys, Tyr, Gln, Asn, Glu, Asp, Cys and His are known as efficient quenchers of Trp fluorescence [4] and the relative position of Trp inside the protein may explain our results.
Experiments based on the extrinsic fluorescence of Sypro Orange dye were more informative. For this, WT, NT, and NCT were incubated with different compounds in the presence of the dye. The substrate trehalose did not cause any change in fluorescence. Glucose and gentiobiose increase the fluorescence of the dye when WT is present, whereas at least 5 compounds affected dye fluorescence in the presence of NT or NCT (Fig. 5). This means that WT was less affected by ligands than the truncated mutants or, in other words, the truncated enzymes are more mobile than the wild-type trehalase.

In the presence of NT, five of the added compounds change dye fluorescence, from which 4 decrease it. In the case of NCT, 6 compounds change fluorescence and 5 increase it. This indicates that most compounds led NCT to exposure hydrophobic residues, the contrary being true for NT.

The data suggest that conformational changes caused by ligand binding that affect kinetic parameters occur mainly in the vicinity of the active site. The wild type trehalase has extensive loops that may hinder the mobile region, which is more exposed to the dye in the truncated mutants. In accordance with this view, NCT, which lacks two segments, changes its emission in the presence of dye with more ligands than NT, which lacks only one segment. These data provide physical evidence in favor of a trehalase change in folding, anticipated by chemical modification and other kinetic data ([15], this paper) and suggest that the changes occur mainly in the neighborhood of the active site. This challenges the initial

Fig. 5. Fluorescence emission of Sypro Orange dye. The enzymes (WT, NT and NCT) were incubated with or without substrate, glucose and several inhibitors in the presence of the dye. αG, methyl α-glucoside; αM, methyl α-mannoside; A, amygdalin; G, glucose; Ge, gentiobiose; M, mandelonitrile; P, prunasin; T, trehalose.
suggestion, based on homology modeling, that the terminal loops are mobile.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.09.015.

References

[1] K. Arnold, L. Bordoli, J. Kopp, T. Schwede, The SWISS–MODEL workspace: a web-based environment for protein structure homology modeling, Bioinformatics 22 (2006) 195–200.

[2] M. Bounias, A. Bahjou, L. Cordoux, R. Moreau, Molecular activation of a trehalase purified from the fat body of a coleopteran insect (Tenebrio molitor), by an endogenous insulin-like peptide, Biochem. Molec. Biol. Int. 31 (1993) 249–266.

[3] M.M. Bradford, Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.

[4] Y. Chen, M.D. Barkley, Toward understanding tryptophan fluorescence in proteins, Biochemistry 37 (1998) 9976–9982.

[5] A. Dahlqvist, Assay of intestinal disaccharides, Anal. Biochem. 22 (1968) 99–107.

[6] R.P. Gibson, T.M. Gloster, S. Roberts, R.A. Warren, I. Storch de Gracia, A. Garcia, J.L. Chiara, C.J. Davies, Molecular basis for trehalase inhibition revealed by the structure of trehalase in complex with potent inhibitors, Angew. Chem. – Int. Ed. 46 (2007) 4115–4119.

[7] A. Gomez, C. Cardoso, F.A. Genta, W.R. Terra, C. Ferreira, Active site characterization and molecular cloning of Tenebrio molitor midgut trehalase and comments on their insect homologs, Insect Biochem. Molec. Biol. 43 (2013) 733–741.

[8] A. Hawe, M. Sutter, W. Jiskoot, Extrinsic fluorescent dyes as tools for protein characterization, Pharm. Res. 25 (2008) 1487–1499.

[9] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[10] J.H. Lee, M. Tsuji, N. Nakamura, M. Okayama, H. Mori, A. Kimura, H. Matsui, S. Chiba, Puriﬁcation and identiﬁcation of the essential ionizable groups of honey bee Apis mellifera L. trehalase, Biosci. Biotechnol. Biochem. 65 (2001) 2657–2665.

[11] L.M.F. Mendonça, S.R. Marana, Single mutations outside the active site affect the substrate speciﬁcity in a β–glycosidase, Biochim. Biophys. Acta 1814 (2011) 1616–1623.
[12] E.W. Milles, Modification of hystidyl residues in proteins by diethylpyrrocarbonate, Meth. Enzymol. 47 (1977) 431–442.

[13] I.H. Segel, Enzyme Kinetics, Wiley, New York, 1975.

[14] M.C.P. Silva, W.R. Terra, C. Ferreira, The catalytic and other residues essential for the activity of the midgut trehalase from Sodoptera frugiperda, Insect Biochem. Mol. Biol. 40 (2010) 733–741.

[15] M.C.P. Silva, W.R. Terra, C. Ferreira, The role of carboxyl, guanidine and imidazole groups in catalysis by a midgut trehalase purified from an insect larvae, Insect Biochem. Mol. Biol. 34 (2004) 1089–1095.

[16] M.C.P. Silva, W.R. Terra, C. Ferreira, Absorption of toxic β-glucosides produced by plants and their effect on tissue trehalases from insects, Comp. Biochem. Physiol. 143 (2006) 367–373.

[17] W.R. Terra, C. Ferreira, Biochemistry and molecular biology of digestion, in: I. I. Gilbert (Ed.), Insect Molecular Biology and Biochemistry, Academic press–Elsevier, London, 2012, pp. 365–418.

[18] W.R. Terra, C. Ferreira, A.G. de Bianchi, Physical properties and Tris inhibition of an insect trehalase and a thermodynamic approach to the nature of its active site, Biochem. Biophys. Acta 524 (1978) 131–141.

[19] W.R. Terra, I.C.M. Terra, C. Ferreira, A.G. de Bianchi, Carbodiimide–reactive carboxyl groups at the active site of an insect midgut trehalase, Biochem. Biophys. Acta 57 (1979) 79–85.