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Alpha-glucosidases from Non-hematophagous Organisms Crystallize Heme in Vitro

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Abstract: Hemozoin (Hz), a heme crystal first known as a malaria pigment, reduces heme toxicity to hematophagous organisms such as protozoans, worms, and insects. The mechanism of Hz synthesis remains poorly understood, but studies on the blood-sucking insect *Rhodnius prolixus* indicate the involvement of an α-glucosidase enzyme. The objective of this study was to test the hypothesis that α-glucosidases from other organisms also have the ability to form Hz *in vitro*. This hypothesis was tested using protein extracts from non-hematophagous insects and non-insect organisms. Our results indicate that protein extracts from several sources have the potential to crystallize heme *in vitro*. This activity is likely associated with α-glucosidases. Thus, these enzymes seem to have the secondary capacity of detoxifying heme even in nonhematophagous organisms. This latent function may represent a crucial pre-adaptive evolutionary step in the adoption of hematophagy in hemoparasites.

Keywords: Hemozoin, glycoside-hydrolases, heme biocrystallization.

1. Introduction

The glycosidases or glucoside hydrolases, comprise a group of enzymes that participate in the degradation of polysaccharides, in the biosynthesis and modification of cellular α-glycoproteins, and in the catabolism of peptidoglycan and other glycoconjugates (Bhatia et al., 2001, Withers, 2010). These enzymes, which are present in mammals, plants, insects, and microorganisms, play important roles in cell signaling, during viral invasion, in energy storage and use, and in glycoprotein biosynthesis, among other functions (Zechel & Withers, 2002). Among the glycosidases, α-glucosidases (EC 3.2.1.20) catalyze the hydrolysis of the □-1,4 glycoside linkage present in some oligo and polysaccharides, releasing □-D-glucose monomers as their primary function (Zechel & Withers, 2002).

Various groups of insects express α-glucosidases in the digestive tract, where its activity is primarily associated with carbohydrate degradation (Terra & Ferreira, 1994; Carneiro et al., 2004; Kubota et al., 2004; Damasceno-Sá et al., 2007; Zibaee et al., 2009; Santos et al., 2008; Fonseca et al., 2010; Ghadamyari, 2010; Vale et al., 2012; Watanabe et al., 2013; Fialho et al., 2013; Mehrabadi, 2011; Souza-Neto et al., 2007). In aphids, α-glucosidases control the osmolarity of sucrose diets (Ashford et al., 2000; Salvucci, 2000). The fact that these enzymes also act as receptors (Silva-Filha et al., 1999) suggests a high degree of functional plasticity. In *Rhodnius prolixus*, which is a blood sucking hemipteran, an α-glucosidase present in perimicrovillar membranes may play a role in heme detoxification, participating in hemozoin (Hz) biosynthesis (Mury et al, 2009). Heme crystallization into Hz provides a defense against the oxidative effects of heme generated during the digestion of vertebrate hemoglobin in the intestinal lumen of this hematophagous insect. In other triatomine insects, α-glucosidases also act in Hz biocrystallization (Oliveira et al., 2007) a pre-adaptive evolutionary step that enabled these animals to overcome the challenges of hematophagy (Mury et al, 2009).

The parasites *Plasmodium* (Slater et al., 1991), *Schistossoma* (Oliveira et al., 2000) and *Hemoproteus* (Chen et al., 2001) also produce Hz. In *Plasmodium*, the heme detoxification protein (HDP) participates in oxidative protection (Jani et al., 2008), but in *Schistosom*a, lipids take part in Hz formation

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(Corrêa-Soares, et al., 2007; Hoang et al., 2010). In *Plasmodium*, Hz nucleation happens at the digestive vacuole inner membrane, with crystallization occurring in the aqueous rather than the lipid phase (Kapishnikova et al., 2012).

In this work, we tested the hypothesis that α-glucosidases from organisms other than bloodsucking insects also form Hz. Thus, we evaluated both activities *in vitro* using protein extracts from a variety of biological sources. The association between Hz-forming and hydrolytic activities was tested by using inhibitors.

2. Materials and Methods

2.1 *Biological samples*

The midguts of the adult and larvae of *Callosobruchus maculatus* and *Tribolium castaneum* were kindly donated by Dr. Elenir Amâncio and Dr. Gustavo Rezende, respectively (LQFPP, CBB, UENF). The midgut *Rhodnius prolixus* females was used as a positive control (Silva et al., 2007). Midguts were washed in deionized water to remove luminal content and homogenized. The same procedure was applied to *Dysdercus peruvianus* and *Quesada gigas.*

The circulating adult form of *Schistosoma mansoni*, obtained through the courtesy of Dr. Marcus Fernandes de Oliveira (IBqM, UFRJ), was washed in deionized water and then centrifuged at 8000 x*g* for 15 min at 4 ºC, after which the pellet was used in the protein extraction procedure described below. *Arabidopsis thaliana* leaves, kindly provided by Dr. Gonçalo Apolinário de Souza Filho (LBT, CBB, UENF), were macerated in liquid nitrogen and solubilized in distilled water. Log phase cells of *Saccharomyces cerevisiae*, courtesy of Dr. Lev Alexandrovitch Okorokov (LFBM, CBB, UENF), *Bacillus thuringiensis*, kindly provided by Dr. Marilia Amorim Berbert de Molina (LBT, CBB, UENF), *Toxoplasma gondii*, and Vero cells (ATCC® CCL-81™), both courtesy of Dr. Renato DaMatta (LBCT, CBB, UENF) were harvested, washed in deionized water, and centrifuged at 8000 x*g* for 15 min at 4 ºC.

2.2 Protein Extraction

Each sample was homogenized in distilled water using a Potter-Elvehjem glass homogenizer, followed by five cycles of freezing and unfreezing in liquid nitrogen. Between each cycle, samples were centrifuged at 15,000 x*g* for 30 min at 4 ºC. Membrane-bound proteins were obtained by treatment of the pellet with 0.1% Nonidet P-40 (NP-40) for 12 h at 4ºC in 20 mM sodium phosphate buffer, pH 7.4, 5 mM imidazole, 1 mM PMSF and 1 mM benzamidine (Silva et al., 2007). Samples were incubated overnight in the same buffer at 4 ºC with agitation. After centrifugation at 15,000 x*g* for 30 min at 4 ºC, protein concentration was determined by bicinchoninic acid (BCA) assay (Smith et al., 1985)

2.3. Hemozoin formation assay

A sample corresponding to 10 µg/mL of protein (protein extract) was incubated for 24 h at 28 ºC in 0.5 M sodium acetate, pH 4.8, in the presence of 100 mM heme as previously described (Sullivan et al., 1996). After incubation, the reaction mixture was centrifuged at 15,000 x*g* for 15 min at 25 ºC. The pellet was washed three times with 1 mL of 0.1 M NaHCO3, 2.5% SDS, pH 9.1, and twice with deionized water. The final pellet was solubilized in 0.1 M NaOH, and the amount of heme was determined at 400 nm in a Shimadzu/UV-1240 spectrophotometer.

Alternatively, the specific α-glucosidase inhibitors erythritol (100 mM) and diethylpyrocarbonate (DEPC) (10 mM) or the specific hemozoin inhibitor chloroquine (20 mM) were incubated with the sample and the amount of Hz formed was evaluated.

2.4 α-Glucosidase activity assay

Enzyme activity was determined using □-nitrophenyl α-D-glucopyranoside (10 mM) (Sigma Aldrich Co., USA) in 100 mM citrate phosphate buffer, pH 5.5, as substrate and by following the formation of p-nitrophenolate, according to the method of (Terra et al., 1979). All assays were performed at 37 ºC. Incubations were carried out for four different periods of time (15, 30, 45 and 60 min). Reactions were stopped with 200 mL of 0.5 M Na2CO3 and initial rates of hydrolysis were calculated. The absorbance of released □-nitrophenolate was read at 405 nm. Alternatively, erythritol (100 mM), DEPC (10 mM) or chloroquine (20mM), were incubated with the samples and α-glucosidase activity was measured by the formation of ρ-nitrophenolate.

2.5 Fourier transformed infrared (FTIR) spectroscopy

To assess whether the heme pellet obtained as described above had the spectrum characteristics of Hz, samples were subjected to infrared spectroscopy (Slater et al., 1991) using Nicolet Magna 550 spectrometer.

2.6 Statistical analysis

Significant differences between groups were determined with the non-paired Student's *t* test using the GraphPad Prism software. For all tests, a difference of $P < 0.05$ was considered to be significant.

3. Results

3.1 Hz-forming activity in epithelium protein extracts from Dysdercus peruvianus and Quesada gigas

Our hypothesis that the α-glucosidase from non-hematophagous insects forms Hz *in vitro* was tested using the luminal content of two non-hematophagous Hemiptera in Hz formation assays (Figure 1). Luminal content samples derived from *D.peruvianus*, a cotton seed bug (Figure 1A), and *Q.gigas*, the sap-sucking cicada (Figure 1B), had pellets but not supernatants with biocrystallization activity. The FTIR spectra from both *D.peruvianus* and *Q.gigas* samples displayed peaks characteristic of Hz at 1207 and 1660 cm-1, as previously described for *R.prolixus* (Oliveira et al., 1999) and for synthetic β-hematin (Chen et al., 2001). Thus, the perimicrovillar membranes from two non-hematophagous Hemiptera had a non-soluble fraction with Hz-forming activity.

3.2. α-Glucosidase hydrolytic and Hz-forming activities in protein extracts from non-hemipteran insects

The hydrolytic (Figure 2A) and Hz-forming (Figure 2B) activities of α-glucosidase were measured in the soluble and non-soluble fractions of the midgut epithelia from two non-hemipteran insects, *C.maculatus* (larvae and adult) and *T.castaneum* (larvae and adult). *R. prolixus* material was used as a positive control. The non-soluble fractions of *C. maculatus* and *R. prolixus* displayed higher α-glucosidase activity than their soluble fractions. *T.castaneum* samples had low activity, with no significant difference between the soluble and non-soluble protein fractions. Hz-forming activity was substantially higher in non-soluble compared to soluble fractions (Figure 2B) and was higher in *R.prolixus* and *T.castaneum* than in *C.maculatus* larval midgut extracts.

3.3. α-Glucosidase hydrolytic and Hz-forming activities in protein extracts from non-insects

Soluble and non-soluble protein fractions were also obtained from a group of non-insect organisms, including the yeast *S.cerevisiae*, the bacterium *B.thuringiensis*, the protozoan *T.gondii*, the platyhelminth *S.mansoni*, the plant *A.thaliana*, and cultured Vero cells. Both fractions were used in the assays of α-glucosidase hydrolytic and Hz-forming activities (Figure 3A and 3B). Regarding hydrolytic activity, the only significant difference was the high level observed in the non-soluble protein fraction of *S.mansoni* in comparison to all other samples (Figure 3A). Hz-forming activity was significantly higher in the non-soluble fraction from all non-insect organisms (Figure 3B).

3.4. α-Glucosidase hydrolytic and Hz-forming activities in protein extracts from insects in the presence and absence of inhibitors

We assessed the effects of chloroquine, an Hz synthesis inhibitor, and of DEPC and erythritol, both α-glucosidase inhibitors, on α-glucosidase hydrolytic and Hz-forming activities in various insects and non-insects (Table 1). The activity of α-glucosidase in *C.maculatus* and *R.prolixus* but not *T.castaneum* extracts was sensitive to erythritol. The same activity in extracts of *C.maculatus* larvae, *T.castaneum* larvae, and *R.prolixus* was significantly reduced in the presence of DEPC. Chloroquine reduced the hydrolytic activity of *C.maculatus* adult and *R.prolixus* extracts. The hydrolytic activity in *S.cerevisae*, *S.mansoni*, and Vero cell extracts was sensitive to erythritol and chloroquine and DEPC did not inhibit the hydrolytic activity of non-insect protein extracts except for that of *S.mansoni* extract. Hz-forming activity was significantly reduced in the presence of erythritol, DEPC and chloroquine in all extracts from insect and non-insect organisms.

4. Discussion

In Triatomines insects, □-glucosidase participates in Hz biocrystallization (Oliveira et al., 1999; Oliveira et al., 2007). This process occurs in the intestinal lumen, and probably represents a first line of defense against the oxidative effects of heme generated by digestion of vertebrate hemoglobin. Here, we show that midgut protein extracts from two non-hematophagous hemipteran insects, *D.peruvianus* and *Q.gigas*, also have Hz-forming activity (Figure 1). Our results suggest that Hz formation could be a secondary activity of other α-glucosidases, and not a specific characteristic of these enzymes in hematophagous Hemiptera. To test this hypothesis further, we assessed the presence of this activity in other organisms. We chose two coleopteran insects, *C.maculatus* and *T.castaneum.* Larvae of both insects had higher α-glucosidase hydrolytic activity than adults (Figure 2A). A residual Hz forming activity was found in these extracts if compared with the high activity of *T.castaneum* and *R.prolixus* extracts (Figure 2B).

We broadened the screening for Hz synthesis associated to α-glucosidase to non-insect protein extracts. Among non-insects, high activity was found in the non-soluble protein extract from *S.mansoni*. All other extracts had low hydrolytic activity. However, Hz-forming activity was easily detectable in all extracts except in that from *T.gondii*, which had low activity only in the nonsoluble fraction of the extract.

While interesting, these results raise new questions. Hz activity could be related to other components of the protein extract such as the lipids present in the non-soluble fraction. (Stiebler et al., 2011). reviewed the factors involved in Hz synthesis in diverse biological systems. In *R.prolixus* midgut, both lipid and protein factors from PMM cooperate to promote Hz synthesis (Stiebler et al., 2010). In *S.mansoni*, Hz is also produced physiologically, and lipids seem to be the main agents of Hz formation (Corrêa-Soares et al., 2007). In *P.falciparum*, a heme detoxification

protein (HDP) as well as lipids were implicated in Hz synthesis (Jani et al., 2008; Fitch et al., 1999) Faced with these possibilities, we tested the effects of inhibitors of α-glucosidase hydrolytic activity on Hz synthesis. We also tested the effects of a known inhibitor of Hz synthesis on the hydrolytic activity of α-glucosidase.

DEPC inhibits several enzymes by modifying histidine residues (Coan & DiCarlo, 1990) or by linking the ε-amino group in lysine residues to the carboxyl groups of glutamic and aspartic acid residues (Wolf et al., 1970).

DEPC treatment inhibits the activity of *C.elegans* α-glucosidase by 40% (Torre-Bouscoulet et al., 2005). Erythritol is a competitive inhibitor of α-glucosidases (Ridleyet al., 1992). Both inhibitors were used here to assess the presence of α-glucosidase activity in association with Hz synthesis in the extracts (Table 1). The results show that not all extracts showed □-glucosidase activities sensitive to the inhibitors. Quinoline drugs, such as chloroquine, act against *Plasmodium* through Hz synthesis inhibition (Sullivan et al., 1996; Oliveira et al, 1999; Ridley et al., 1997). These drugs inhibit Hz synthesis in vitro and under physiological conditions (Slater & Cerami, 1992). The action of these antimalarial drugs in *S.mansoni* (Corrêa-Soares et al., 2009), through an identical mechanism, opens a new front of chemotherapy development.

Here, chloroquine inhibited the Hz-forming activity of all protein extracts (Table 1). On the other hand, chloroquine also reduced hydrolytic activity in *C.maculates, R.prolixus, S.cerevisae, S.mansoni* and Vero cells. Unexpectedly, the α-glucosidase inhibitors were also able to decrease Hz forming activity in almost all protein extracts except Vero Cell extract. These results suggest a potential physical association of both activities within the enzyme structure. Moreover, our findings indicate that chloroquine and the other inhibitors can be used to assess both α-glucosidase hydrolytic and Hz forming activities.

Our group has shown that α-glucosidase present in the PMM of *R.prolixus* may be related to the process of heme detoxification and Hz synthesis (Mury et al., 1999). Heme molecules interact with PMM, binding to them in regular intervals, which would facilitate the formation of crystals. Interaction with PMM is mediated by α-glucosidase located in these membranes (Ferreira et al., 1988; Smith et al., 2004). During the formation of heme crystal, the nucleation process is a limiting step. α-Glucosidases would promote the nucleation process and Hz formation at the interface between PMM and the intestinal lumen (Mury et al., 1999; Stiebler et al., 2011). Once Hz crystals are formed in the PMM in the early stages of the digestive process, further heme crystallization becomes physiologically very effective. This increased effectiveness certainly explains why 97% of the heme in the midgut of *R.prolixus* is found in the form of Hz (Stiebler et al., 2010).

We presented an initial hypothesis that □-glucosidases from organisms other than bloodsucking insects would have, as a secondary activity, the capacity to crystallize heme. Indeed, we showed here that protein extracts derived from non-hematophagous insects such as *C.maculatus* and *T.castaneum* as well as those from non-insects, including *S.mansoni*, *A.thaliana*, *T.gondii*, and *S.cerevisae*, exhibited heme biocrystallization activity. Furthermore, this activity was likely associated with □-glucosidases. This catalytic potential provides further evidence of the plasticity of □-glucosidases, and may represent a crucial pre-adaptive evolutionary step in the adoption of hematophagy in hemoparasites.

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Legends

Table 1: *In vitro* assessment of α-glucosidase and Hz forming activities in the non-soluble fraction of protein extracts from insects and non-insects, in the presence or absence of inhibitors erythritol, DEPC and chloroquine.

Figure 1: Hemozoin synthesis in the protein extract from the epithelia of *D.peruvianus* (A) and *Q.gigas* (B). The intestinal epithelium samples were collected and subjected to protein extraction. The hemozoin synthesis assay was performed using 12 µg of protein supernatant (S) or pellet (P). Insets show the insects used and the FTIR spectra of the crystals produced in vitro.

Figure 2: α-Glucosidase activity (A) and hemozoin formation (B) in soluble (S) and nonsoluble (NS) fractions of protein extracts from different insects. C – Control; Cm.L – *C.maculatus* Larva; Cm.A. - *C.maculatus* Adult; Rpro – *R.prolixus* Adult. Tc L. – *T.castaneum* Larva; Tc A – *T.castaneum* Adult. Hemozoin formation assays were carried out for 24 h at 28 oC. Hemozoin formation activity is expressed as nmol heme aggregated in 24 h for 12 µg of protein extract. α-Glucosidase activity is expressed as nmol ρ-nitrofenolate released in 1 min. Results shown are means ±SEM (n = 4) of three experiments run in triplicate. *($p < 0..05$); ***($p < 0.001$).

Figure 3: α-Glucosidase activity (A) and hemozoin formation (B) in soluble (S) and nonsoluble (NS) fractions of protein extracts from different non-insect organisms. C – Control; Yeast – *S.cerevisiae*.. Bt – *B.thuringiensis*; Schist. – *S.mansoni*. Vero – Vero Cells; Ara – *A.thaliana*. Toxo – *T.ghondii*. Hemozoin formation assays were carried out for 24 h at 28ºC. Hemozoin formation activity is expressed as nmol heme aggregated in 24 h for 12 µg of protein extract. α-Glucosidase activity is expressed as nmol ρ-nitrofenolate released in 1 min. Results shown are means ±SEM (n = 4) of three experiments run in triplicate. ***($p < 0.001$).

Table 1

Sample	Insect extracts - α-Glucosidase activity (mU/mg)						
	Control	Erythritol	ρ - value	+	ρ - value	+Chloroquine	ρ - value
C. maculatus	1047.03	112.5	0.0232*	600.18	0.036 *	900.18	0.4228
C. maculatus adult	89.40	23.52	0.0128*	154.58	0.2691	115.58	0.031*
R. prolixus adult	287.04	26.465	0.0007***	104.96	0.0017**	89.69	0.0031**
T. castaneum	101.17	105.40	0.7456	50.77	0.001**	90.81	0.1461
T. castaneum	41.99	82.76	0.0503	30.15	0.4587	70.23	0.0544
Sample	Non-insect extracts - α-Glucosidase activity (mU/mg)						
	Control	Erythritol	ρ - value	DEPC	ρ - value	Chloroquine	ρ - value
S. cerevisae	93.35	28.94	0.0148*	103.76	0.1565	47.73	0.0142*
B. thuringiensis	62.64	12.88	0.0549	47.9	0.3083	46.23	0.3194
S. mansoni	380.86	37.93	0.0005***	199.99	0.0025**	198.99	0.0042**
Vero Cells	72.93	17.17	0.0048**	78.8	0.1907	42.34	0.0171*
T. gondii	43.05	40.93	0.623	39.25	0.1476	26.84	0.1099
A.thaliana	126.17	27.93	0.1257	77.05	0.3283	61.76	0.2375
Sample	Insect extracts - Hz forming activity						
	Control	Erythritol	ρ - value	DEPC	ρ - value	Chloroquine	ρ - value
C. maculatus	0.21	0.13	0.0002***	0.09	0.0001***	0.03	0.0001***
C. maculatus adult	6.52	0.23	0.0001***	1.70	0.0003***	0.06	0.0001***
R. prolixus adult	9.98	0.17	0.0001***	1.16	0.0001***	0.02	0.0001***
T. castaneum	0.32	1.18	0.0001***	0.32	0.0001***	0.03	0.0001***
T. castaneum	1.22	1.32	0.0013**	1.22	0.0024**	0.03	0.0001***
Control	0.03	0.03		0.03		0.03	
Sample	Non-insect extracts - Hz forming activity						
	Control	Erythritol	ρ - value	DEPC	ρ - value	Chloroquine	ρ - value
S. cerevisae	1.14	0.27	0.0001***	2.03	0.0001***	0.04	0.0001***
B. thuringiensis	9.98	0.17	0.0001***	1.16	0.0003***	0.02	0.0001***
S. mansoni	5.97	1.51	0.0001***	3.20	0.0001***	0.05	0.0001***
Vero Cells	7.27	4.14	0.0029**	8.20	0.1578	0.05	0.0001***
T. gondii	2.81	1.47	0.0001***	2.81	0.0001***	0.04	0.0001***
A.thaliana	3.36	0.40	0.0002***	2.88	0.0001***	0.05	
Control	0.03	0.03		0.03		0.03	

DEPC - diethylpyrocarbonate. One mU of α-Glucosidase activity is defined as one nMol of *ρ*-nitrofenolate released per min. Results shown a means ±SEM (n = 4) of two experiments run in triplicate. *($p < 0.05$); **($p < 0.01$). ***($p < 0.001$).
Hz forming assays were carried out for 24 h at 28ºC. Hz formation activity is expressed as nmol heme in 24 h for 12 µg protein extract. Resul shown are means ±SEM (n = 4) of two experiments run in triplicate. *($p < 0.05$); **($p < 0.01$). ***($p < 0.001$).

Figure 1

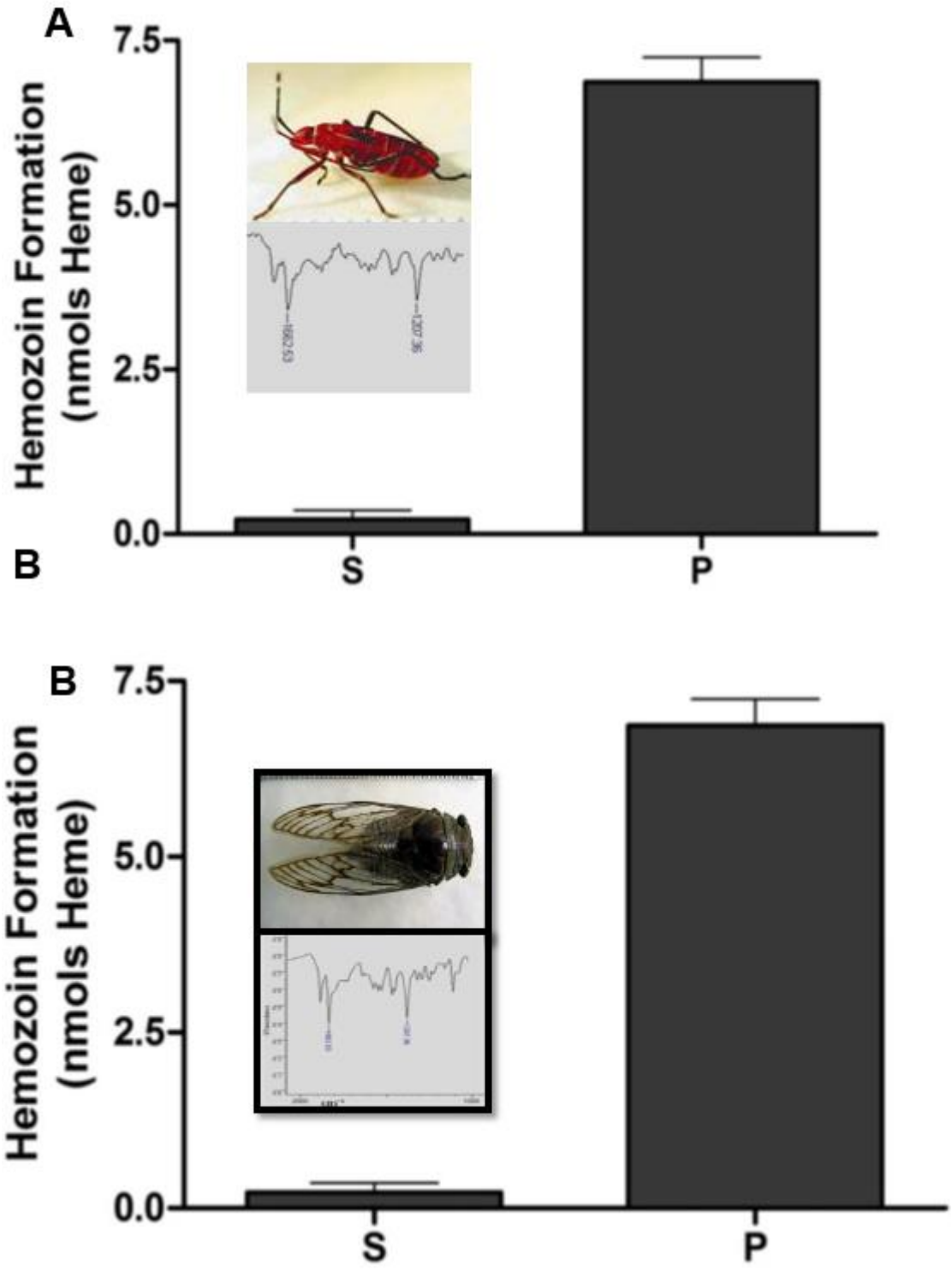

A
Hemozoin Formation
(nmols Heme)
7.5
5.0
2.5
0.0
S
P
B
Hemozoin Formation
(nmols Heme)
7.5
5.0
2.5
0.0
S
P

Figure 2

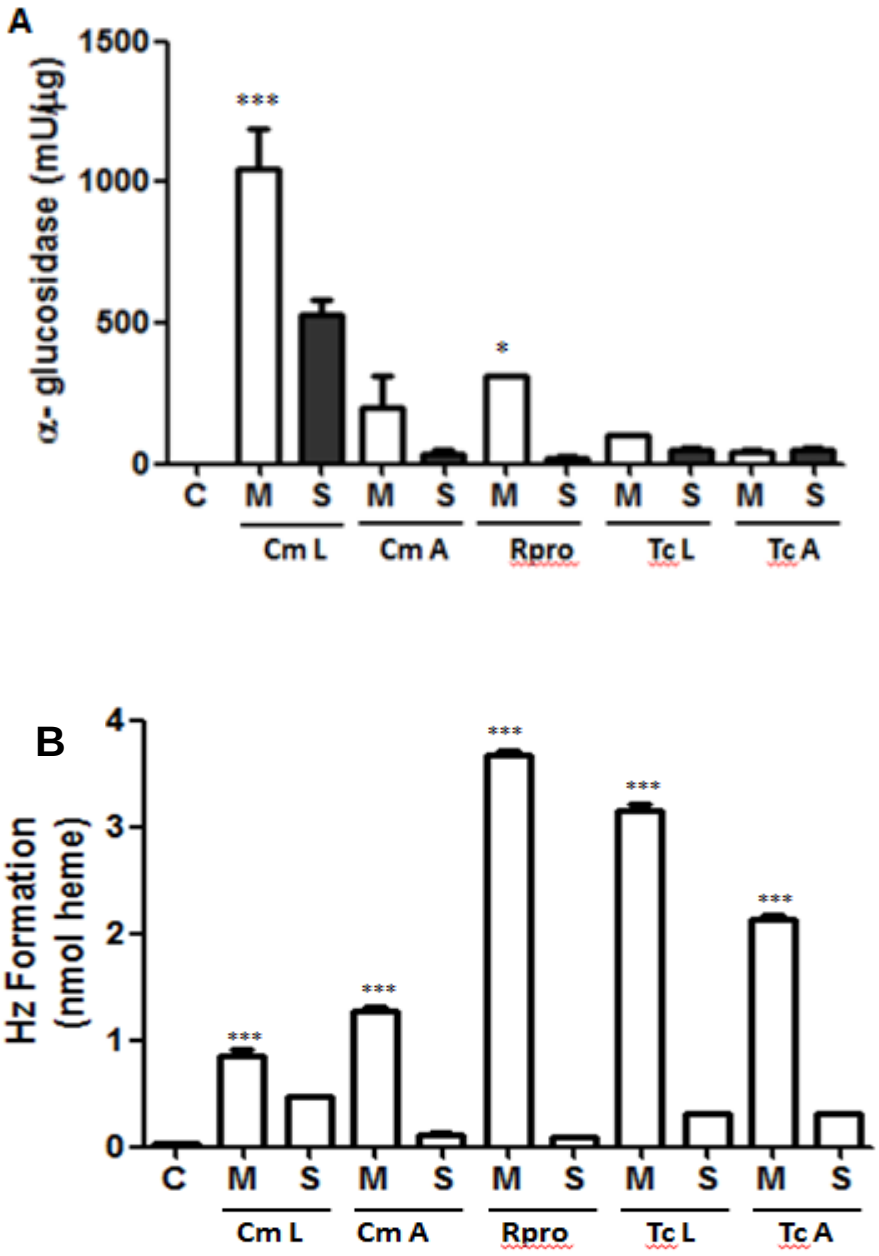
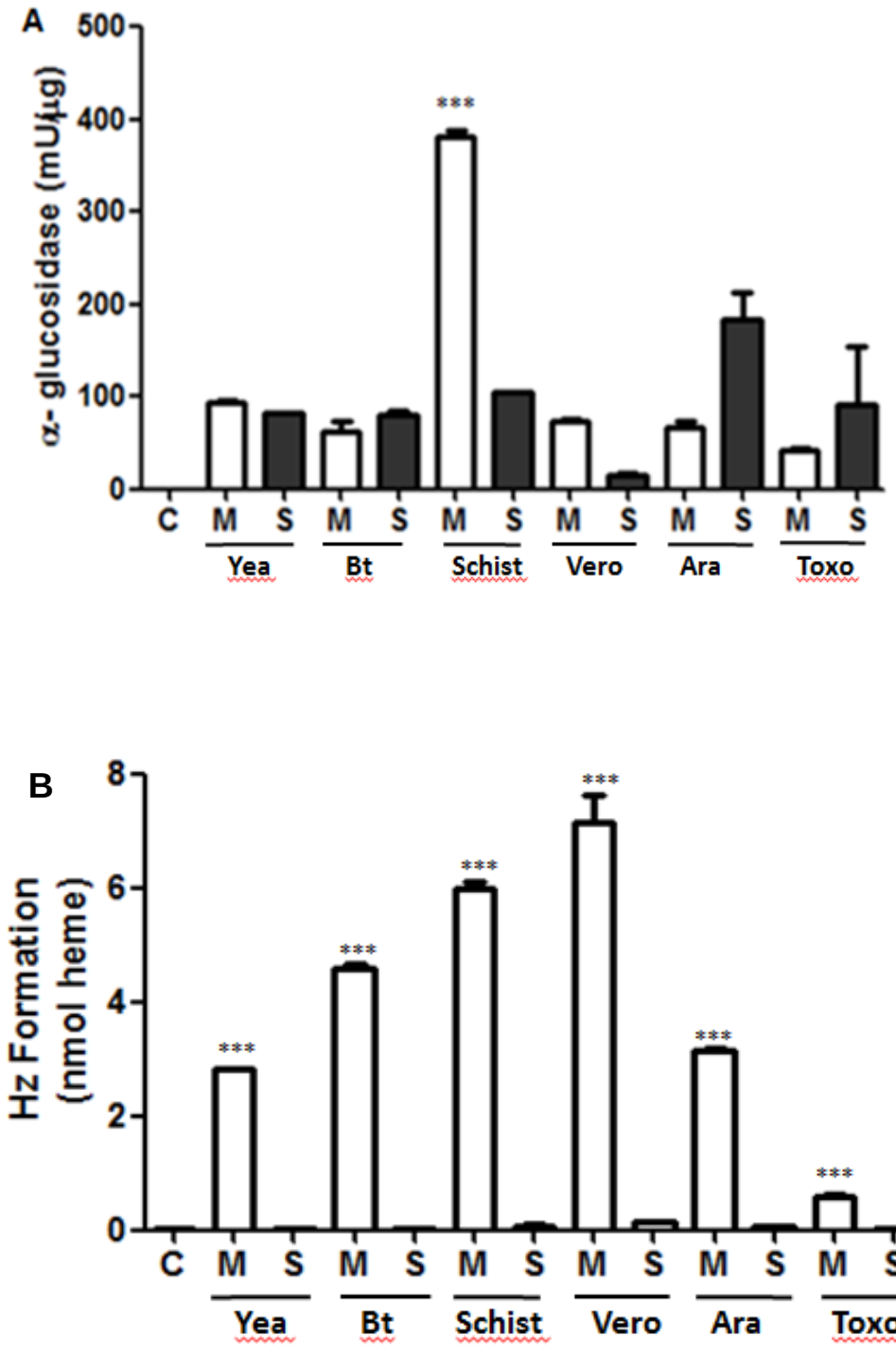

A
α- glucosidase (mU/µg)
1500
1000
500
0
***
*
C
M
S
M
S
M
S
M
S
M
S
Cm L
Cm A
Rpro
Tc L
Tc A
B
Hz Formation (nmol heme)
4
3
2
1
0
***
***
***
***
C
M
S
M
S
M
S
M
S
M
S
Cm L
Cm A
Rpro
Tc L
Tc A

Figure 3


A
α- glucosidase (mU/µg)
500
400
300
200
100
0
***
C M S M S M S M S M S M S
Yea Bt Schist Vero Ara Toxo
B
Hz Formation (nmol heme)
8
6
4
2
0
***
***
***
***
***
C M S M S M S M S M S M S
Yea Bt Schist Vero Ara Toxo