

Enzymatic responses of a possible microbial symbiont in the digestive tract of *Hadenoeus subterraneus* to changes in diet.

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Cave crickets, *Hadenoeus subterraneus*, are well adapted to the cave ecosystem. A flora of microorganisms has been shown to live in the crop and hindgut of its digestive system which are believed to supply the cave cricket with essential digestive enzymes in order for it to survive. Assay of six different enzymes, amylase, xylanase, cellulase, chitinase, protease, and lipase, were conducted on three different groups of crickets. One group was assayed using crickets feed on their natural diet, another was feed on a high polysaccharide fruit diet, and the last was feed on a high protein diet. Enzyme activity levels shifted between diet types, indicative of microbial production of enzymes capable of digesting different substrates. A symbiotic relationship between *H. subterraneus* and the microorganisms inhabiting its digestive tract is discussed.

INTRODUCTION:

The relationship between insects and microorganisms has drawn a considerable amount of attention in the past decade. The symbiotic relationship between insects and microbes in the digestive system has been shown to have considerable biological significance (Martin and Martin, 1979, Bracke, *et al.*, 1979, Brlocher, 1982). Microbes supply vitamins, synthesize amino acids, fix nitrogen, degrade uric acid, and perform a variety of biochemical processes, such as assistance in digestion in these organisms (Martin, 1987). In the

digestive process, the microbes can perform a three fold function: they supply a concentrated source of digestible macronutrients such as proteins, lipids, and carbohydrates; they provide micronutrients such as vitamins and unsaturated acids, and they transform normally indigestible organic compounds into digestible metabolites (Martin, 1987).

Cavernicolous crickets in Kentucky, such as the cave cricket, *Hadenoeus subterraneus*, feed opportunistically upon plant detritus, decaying fruit, animal remains, decomposing wood, and dung (Levy, 1976). With this detritus they also ingest bacteria, and other microorganisms. If ingested microbes colonize in the digestive tract, they may aid in the digestive process by secreting extracellular enzymes. These microbially-produced enzymes may augment or extend the digestive and metabolic capabilities of the organism (Martin and Kukor, 1986). The ability of other Orthopeterans to survive under poor dietary conditions has been suggested to be dependent on microbial symbiosis (Trager, 1970, Meglitsch, 1975).

H. subterraneus is indigenous to the caves located in and around Mammoth Cave National Park, Kentucky. The Mammoth Cave system lacks large populations of bats, therefore troglobitic species must depend upon the input of fixed sources of carbon by floods and/or organisms which leave caves to forage (Poulson and Kane, 1981). *H. subterraneus* produces up the bulk of the biomass in the form of guano, eggs and carcasses in the cave system (Lavoie *et al.* 1991). *H. subterraneus* is restricted to a very narrow temperature range close to cave ambient in which it may leave to cave to forage for food (Studier and Lavoie, 1990). Based on their rate of weight loss, adult female crickets are required to feed every 11.5 days, and males are required to forage every 9.5 days (Studier, *et al.* 1986). Crickets eat frequently to the point of physical distortion by consuming up to 101% for females and 74% for males of their total body weight

during a single feeding (Studier *et al.*, 1986). These metabolic adaptations allow *H. subterraneus* to feed opportunistically, taking full advantage of immediate and sometimes infrequent access to food sources.

It has been demonstrated that at least six different microbial species inhabit the crop and hindgut of *H. subterraneus* (Phillipots, 1989). Microbes that have been found in the digestive tract of *H. subterraneus* grow best at an optimum temperature of 23°C (Phillipots, 1989), which is significantly higher than cave ambient of 13.5°C. It is believed that the cave cricket is restricted to a narrow temperature range in order to keep the growth of their crop microbes under control (Lavoie *et al.*, 1991). Observations by Studier and Lavoie have indicated that crops on some dissected crickets were visibly extended with gas, occasionally to the point of rupture (Lavoie, *et al.* 1991). When some of these crops were punctured, a distinctive odor of ethanol was released. Such changes could have been the result of exposure to increased temperatures which caused unregulated growth of microbes in the crop.

In this study, we measured the concentrations of enzyme activity in the digestive tract in response to differing diets of known composition, and correlated our findings to show enzyme activity which could result from microbial interaction. Microbially produced enzymes should show rapid shifts in activity to produce the necessary enzymes capable of digesting relative substrates in each diet.

MATERIALS AND METHODS

This study was conducted during the last week of February 1992. Adult *Hadenoeus subterraneus* cave crickets (HFL>20) were collected from White Cave with the exception for those crickets assayed for lipase activity, which

came from the Frozen Niagara entrance located in Mammoth Cave National Park, Kentucky.

Assay of enzymes were made on hindgut and crops of freshly dissected crickets. Methods for assaying enzyme activity was modified from Martin *et al.*, 1981. Crickets were collected and placed into three separate groups. One group was used to assay enzymes from crickets fed on their natural diet ("natural"). Another group was fed on a high carbohydrate diet ("fruit") consisting of apples and bananas. The final group was fed on a high protein diet ("protein") consisting of previously dissected crickets (minus the crop and hindgut) from the first two groups. Ten females and ten males were used for each diet except for the natural which used fourteen males and ten females. Crickets were allowed to feed for approximately 48 hours. Prior to dissection, crickets were put on ice for 30 minutes to anesthetize them. Dissections were performed in Cleveland Avenue of Mammoth Cave. Hindgut and crops were assayed separately from males and females, with five individuals pooled for each assay, except for the natural diet which used seven individuals for the male assay. Samples were extracted in the cave, kept on ice and transported to the Maple Springs Research Center for assay.

Assays on extract were performed to determine activity of amylase, xylanase, hemicellulase, cellulase, protease, and lipase. Assays consisted of 0.5ml of crop or hindgut extract to 0.5ml of substrate-buffer solution. Each test assay was incubated in a dry bath at 23°C for six hours except for amylose and xylan which were incubated for three hours. Incubation was terminated for amylose and xylan by adding 1.0ml of DNSA (Brenfeld, 1955). Chitin, cellulose, and Azocoll were terminated by filtration through a millipore using a 5cc syringe. Extracts were then heated at 110°C in a dry bath for eight minutes, 1.5ml of distilled water was added and the solutions allowed to cool.

Each assay was measured for activity using a Spec20 set at 540nm (520nm for protease). Blanks of DNSA and substrate-buffer solution were boiled in a water bath for five minutes and brought to volume using distilled water. A calibration curve was made by using 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.7 and 1.0 mg of maltose in 1.0 ml of water; 1.0ml of DNSA was added to each solution and heated for eight minutes in a dry bath at 110°C, 1.5ml of water was added and solutions allowed to cool. Absorbance was measured at 540nm and a curve was constructed by determining $A_{540}(\text{test}) - A_{540}(\text{blank})$ (Figure 1). Enzyme activity was reported in mg maltose equivalents/100mg wet weight using the calibration curve.

RESULTS

Crop and hindgut extracts from each of the three diets (natural, fruit, and protein) exhibited activity toward all substrates tested (amylase, xylan, cellulose, chitin and protein). Data for enzyme activity measured in mg maltose equivalents per 100mg wet weight can be seen in Figure 1, 2, and 3, with average values of enzyme activity correlating with the diet. *A priori* assumption was that amylase, xylanase, and cellulase activity were expected to be greater in the fruit diet. Protease and chitinase activity were expected to be greater in the protein diet. Enzyme activity was analyzed via two-way ANOVA to consider the effect of diet type, location, and sex on activity. If interactions were found to be significant ($p < 0.05$), one-way ANOVAs were performed to determine the significance of each. All statistical analyses were performed using SYSTAT.

Analysis of the data for amylase activity showed no significant difference between amylase activity and diet type ($F \text{ stat} = 2.073$, $df = 2$, $p = 0.151$). A marginal interaction between diet type and location for amylase activity was

shown. However, as mentioned above, the one-way ANOVA for diet type was not significant, and the one-way ANOVA for location also showed no significant difference between activity and location (F stat=0.005, $df=1$, $p=0.947$). This a posteriori comparison disproves that initial assumption concerning amylase activity. Activity remained the same for each of the three diet types and was the same in the crop as well as in the hindgut. A significant difference may have been found with a larger sample size or with a shorter incubation period.

We found a significant diet-location interaction for xylanase activity (F stat=9.104, $df=2$, $p=0.002$). A one-way Anova showed no significant difference between location and xylanase activity (F stat + 0.938, $df=1$, $p=0.343$): meaning that xylanase activity was the same in the hindgut and the crop. However, one-way ANOVA did show a marginal significance between diet type and xylanase activity (F stat= 3.194, $df=2$, $p=0.062$). A significant difference between xylanase activity and diet may have been found as expected if the sample size had been larger or if a shorter incubation period was used.

There was a significant difference between cellulase activity and diet type (F stat= 22.768, $df=2$, $p<0.000$). There was marginal significance between activity and location (F stat = 3.836, $df=1$, $p=0.066$).

No significant difference was found between hemicellulase activity and diet (F stat= 1.659, $df=2$, $p=0.218$), nor between activity and location (F stat= 1.015, $df=1$, $p=0.321$).

A significant difference was found between protease activity and location (F stat = 9.248, $df =1$, $p=0.008$). Marginal significance was found between activity and diet type (F stat = 3.025, $df=2$, $p=0.077$). A significant difference may have been found between protease activity and diet as expected if the sample size had been larger, or if incubation time had been longer.

Following these initial statistical computations, Tukey HSD multiple comparisons were performed on data which proved to be significant according to the ANOVA. Tukey HAD performed by diet for cellulase activity showed significant differences between all possible pairwise comparisons of the three diet types (natural diet and fruit diet, $df=1$, $p<0.000$; natural diet and protein diet, $df=1$, $p=0.040$; fruit diet and protein diet, $df=1$, $p=0.002$). Cellulase activity was different between the three separate diets demonstrating greatest activity in the natural diet. Tukey HAD performed by location for protease activity showed a significant difference between activity in the hindgut and activity in the crop ($T \text{ stat}= 2.418$, $df=1$, $p=0.025$), with activity being greater in the hindgut. Tukey HAD was also performed on data shown to be only marginally significant; however, all probabilities resulted in acceptance of the null hypothesis and no significant difference was found.

Discussion

In this study a prior assumption was made that if microbes are producing enzymes in the digestive tract of the cave cricket, then enzyme activity should show rapid shifts between diets of known composition. We tested this hypothesis on three differing diet types; a natural diet, a high polysaccharide fruit diet, and a protein rich diet. If cellulase, amylase, and xylanase are under significant microbial regulation, then higher concentration of these enzymes should be seen in the fruit diet, since these are able to digest the structural polysaccharides of plant cell walls (Martin *et al.*, 1981). Lipase, protease, and chitinase should be greater in the protein diet if these are under microbial control.

Our results demonstrated that concentrations for xylanase showed a significant difference between diet type, with a higher concentration of xylanase

activity in the fruit diet in relation to the others. Cellulase activity was also statistically significant, however it was lower in the fruit diet than in the either the protein or natural diet. A more accurate indication of cellulase activity may have been acquired if incubation times were longer. Protease activity was also higher in the protein diet. These values may indicate that microbes are producing these enzymes in response to appropriate substrates. Only protease and cellulase activity showed significant difference between location and activity as well as diet type. It is unclear from our study if enzymes are produced from within the crop or the hindgut. These results may also suggest that enzymes are being produced by a flora of microbes present in both location.

Further studies are necessary to further demonstrate the effect of diet on enzyme activity. One possible experiment would be to capture crickets and allow them to use up previously digested foods before feeding on the experimental diets. Our method for assay does not account for the possibility of crickets not feeding on substrates provided and cannot ensure that crop and hindgut contents are from the experimental diets.

It has been suggested that microbes in the digestive system of *H. subterraneus* may aid the digestion of the variable diet of this species (Lavoie, *et al.* 1991). Initial evidence which has suggested the importance of microbial interaction in the digestive system of this species is based on its extreme sensitivity to temperature regimes above cave ambient. These crickets can only forage on nights which are close to the cave ambient conditions (Studier and Lavoie 1990). Lavoie and Studier have observed upon dissection that some crickets show crops visibly extended, to the point of rupture, with gas. When punctured a distinct smell of ethanol was released (Studier and Lavoie, personnel communication), it is believed that these crickets were exposed to temperature regimes higher than cave ambient and the gut microbes proliferated beyond control. Similar

observation were seen by our own study in which crickets dissected after unseasonable warm weather conditions with two days above 21°C, five crickets dissected out of twenty were extended to point of rupture with a gas having a distinctive odor of ethanol.

Our observation support the hypothesis that the cave cricket may be able to survive because of microbial symbionts which aid in digesting normally indigestible organic matter. Further investigations are needed using a similar experimental approach with more accurate incubation times to confirm these preliminary results. Experiments using microbial isolates from the crop and hindgut would be useful in determining if similar enzyme activity can be shown. Results from this study may have been more conclusive if crickets were selected for by crop and hindgut content, or by allowing crickets to exhaust the nutrient supply before feeding on experimental diets.

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