Journal of Chemical Ecology, Rapid Communication, Published September 24, 2004

QUANTIFICATION OF INVERTASE ACTIVITY IN ANTS UNDER FIELD CONDITIONS

MARTIN HEIL,* RITA BÜCHLER and WILHELM BOLAND

Dept. of Bioorganic Chemistry, Max-Planck-Institute for Chemical Ecology, Hans-Knöll-Str. 8, D-07745 Jena, Germany

(Received June 29, 2004; revised September 17, 2004; accepted September 18, 2004)

Abstract—Invertases (EC 3.2.1.26) are hydrolases that cleave sucrose into the monosacccharides, glucose and fructose. They play a central role in carbohydrate metabolism of plants and animals. Methods presented so far to quantify invertase activity in ants or other animals have been hampered by the variability in both substrates and products of the enzymatic reaction in animals whose carbohydrate metabolism is highly active. Our method is based on a spectrophotometric quantification of the kinetics of glucose release. We first obtained an equilibrium state summarizing reactions of any carbohydrates and enzymes that are present in the extract. Sucrose was then added to quantify invertase activity as newly released glucose. Invertase activities differed significantly among species of ants. Variances were lowest among individuals from the same colony and highest among different species. When preparations were made from ants of the same species, invertase activity was linearly related to the number of ants used for extraction. Our method does not require ants to be kept on specific substrates prior to the experiment, or expensive or large equipment. It, thus, appears suitable for dealing with a broad range of physiological, ecological, and evolutionary questions.

Key Words—β-Fructosidase, carbohydrate metabolism, saccharase, sucrose, sugar.

*To whom correspondence should be addressed. E-mail: Heil_Martin@web.de

© 2004 Plenum Publishing Corporation

INTRODUCTION

Invertases (β-fructofuranosidase, EC 3.2.1.26, also termed ß-fructosidase, saccharase, or sucrase) are glycoside hydrolases (EC 3.2.1.-) that catalyze the cleavage of sucrose (α-D-Glucopyranosyl-β-D-fructofuranoside) into the two monosaccharides, glucose and fructose (Henrissat and Bairoch, 1993; Sturm and Tang, 1999; Naumoff, 2001). Carbohydrates ingested by heterotrophic organisms undergo several metabolic steps, in the first of which polymeric carbohydrates are cleaved into their monomers, which then can pass through membranes. Invertase, thus, appears to be a particularly important enzyme for both plants and animals.

Feeding on animals as well as plant material, ants can metabolize many different food sources. Early investigations have indicated that differences among species in the activity of digestive enzymes may reflect the feeding habits of the ants (Ayre, 1967; Ricks and Vinson, 1972). Many ants feed on carbohydrate-rich liquid food sources, such as honeydew of homopterans, plant sap, and extrafloral nectar (i.e., nectar secreted on vegetative organs that is not functionally involved in pollination, see Heil and McKey, 2003). As sucrose is common in all these food sources, invertase activity is required to make use of such food sources.

Given this general importance, surprisingly few studies have tried to quantify invertase activity in ants (Ayre, 1963, 1967; Ricks and Vinson, 1972) or other animals (Martínez del Rio, 1990; Zhang et al., 1993). This might be due to the particular methodical problems arising from the quantification of invertase in animals whose carbohydrate metabolism is highly active. Invertase usually is quantified via the release of glucose from sucrose. Both substrate and product of the enzymatic reaction can be present at varying concentrations in the extract used to quantify enzyme activity. Earlier investigations (Ayre, 1963, 1967; Ricks and Vinson, 1972; Zhang et al., 1993) have been based on endpoint determinations of glucose after adding sucrose to extracts of insects (or parts of insects) and likely are hampered by the presence of glucose and/or sucrose in the reaction preparation prior to the enzymatic reaction. These problems might explain why even recent studies of digestive enzymes in ants have chosen indirect methods, such as feeding selected di- or oligosaccharides to living ants and quantifying monosaccharides as putative reaction products in the ants themselves (Boevé and Wäckers, 2003). The drawback of this method is that selected carbohydrates must be fed to cultivated ants, which have no access to other food sources. Otherwise, direct uptake of the monosaccharides, which are quantified as putative reaction products, would alter the outcome of the experiment. Moreover, carbohydrate metabolism does not stop at the level of monosaccharides, which are further metabolized in living organisms. Quantifying intermediate products can at best reveal semi-quantitative results concerning the velocity of the first step, since their quantities are dependent on both the reactions that lead to their production and by later reactions for which they serve as substrates.

To overcome these problems, we developed a method for quantifying invertase in extracts of ants, which is based on repeated and specific quantification of glucose by a spectrophotometrical method. This allows kinetics to be measured, instead of relying on an endpoint determination. Consequently, an equilibrium is obtained prior to the enzymatic reaction, which reflects reactions of any carbohydrates and enzymes that may be present in the extract. Sucrose is added after the equilibrium has been reached to then quantify invertase activity as newly released glucose.

METHODS AND MATERIALS

Insects and Preparation. The experiment was carried out with ants freshly caught in their natural habitats in the vicinity of Puerto Escondido, Oaxaca, Mexico. Because these ants have different feedings strategies, they were likely to exhibit quantitative differences in invertase activity. Various glands that are located in the head or in the gaster may contribute differently to an ant's overall invertase activity (Ayre, 1967). Ants were, therefore, dissected in 'Insect Ringer' solution (10.4 g NaCl, 0.32 g KCl, 0.48 g $CaCl_2$, and 0.32 g $NaHCO_3$ in 1 l water) in order to remove the largest parts of the content of head and gaster. Care was taken to include maxillary and postpharyngeal glands and the midgut, while poison glands and acid glands were discarded or, at least, emptied. Thoraxes and all chitin components were discarded. The parts putatively containing invertase were weighed and transferred to a 50 mM sodiumphosphate buffer (pH 6.0). Preliminary experiments with different species of *Atta*, *Camponotus*, and *Crematogaster* revealed that this pH was close to the optimum for all ant species investigated. However, different pH conditions should be examined for other ant species in order to conduct the assays at an optimal pH. The contents of 1–10 individuals (equivalent to 8–28 mg fresh material) were combined and put into 300 μl buffer to which 50 μl solution of proteinase inhibitor (1 Complete Mini Tablet, Roche Diagnostics, Mannheim, Germany in 1.5 ml water) had been added. The material then was homogenized with sand and cleared by micromembrane filtration ('Rotilabo Spritzenfilter', 13 mm, 0.2 μm Nylon, Carl Roth, Karlsruhe, Germany). After clearing, the extracts were stored for ca 1 hr at 4°C.

Quantification of Invertase. The quantification of enzyme activity is based on quantifying glucose released from sucrose with the Glucose (HK) Assay Kit (Sigma-Aldrich, Steinheim, Germany. The kit combines hexokinase and glucose-6-phosphate dehydrogenase). Ten microliters of extract and 100 μl HK reaction solution (prepared according to manufacturer's protocol) were introduced into cuvettes (70 μl micro disposable cuvettes, Plastibrand®, Brand, Wertheim, Germany) and placed immediately into a photometer (Genesys 20 Spectrophotometer, ThermoSpectronic, Cambridge, UK). A mixture of 10 μl buffer and 100 μl HK reaction solution served as a blank. Absorption was quantified

at 340 nm every five min until a steady state was reached, usually by 30–45 min. In cases of very high initial absorptions (>0.3) and/or high increases in absorption, samples could be further diluted with HK reaction solution, since high initial values resulted from high amounts of glucose (absolute values) and/or sucrose and invertase (high increases) and, thus, were generally correlated with high invertase activities. After the steady state had been reached, 20 μl sucrose solution (250 mg sucrose in 1 ml water) were added, and absorption was further quantified at regular intervals for 1.5–2 hr. Invertase activity was calculated from the slope of the linear increase in absorption after adding sucrose. For each ant species, 5–7 quantifications were conducted with different ants (usually representing different colonies). For each sample (i.e., ant colony), two extractions were conducted with different numbers of ants, and two replicates per extract were used for analysis; means were calculated from these four measurements.

Standard Curves. Standardization was conducted by quantifying defined amounts of glucose (D-Glucose, Sigma-Aldrich; three replicates per glucose concentration) under the same experimental conditions, and by subjecting purified invertase to the same experimental procedure. Of a 0.01 units ml^{-1} solution of invertase in TRIS-HCl buffer, pH 4.5 (invertase from baker's yeast, ca. 300 units mg^{-1} solid, Sigma-Aldrich, pH according to supplier's suggestion), different volumes (0, 2.5, 5, 10, and 15 μl) were diluted with TRIS-HCL buffer to a volume of 20 μl and mixed with 200 μl HK reaction solution. Sucrose (20 μl of a 0.25 g ml^{-1} solution) was added after 15 min, and absorption was quantified every 10 min for at least the next 30 min (three replicates per concentration of invertase). Ant invertase activity, thus, can be expressed as ng glucose released per min and per mg ant fresh material, or as units (with purified invertase as standard).

RESULTS AND DISCUSSION

The use of purified invertase under the experimental conditions as described above resulted in a linear increase of absorption over time after addition of the enzyme's substrate, sucrose (Figure 1A). Increases in absorption were then linear for at least 1.5 hr (pers. observations), and the slopes of these curves were highly correlated with invertase activity. The resulting calibration curve is linear from 0 to ca 400 n units μl^{-1} and then tends to reach saturation (insert in Figure 1A).

Different ant species were used to quantify invertase activity in tissue of freshly caught animals. In contrast to the curves observed for purified invertase, ant extracts first showed an increase in absorption, which resulted from the presence of the substrate (sucrose) as well as the product (glucose) of the enzymatic reaction in the extract (Figure 1B). Depending on the species and the number of ants used, 15 to 45 min were required to reach the equilibrium, after which sucrose could be added. Addition of sucrose solution first resulted

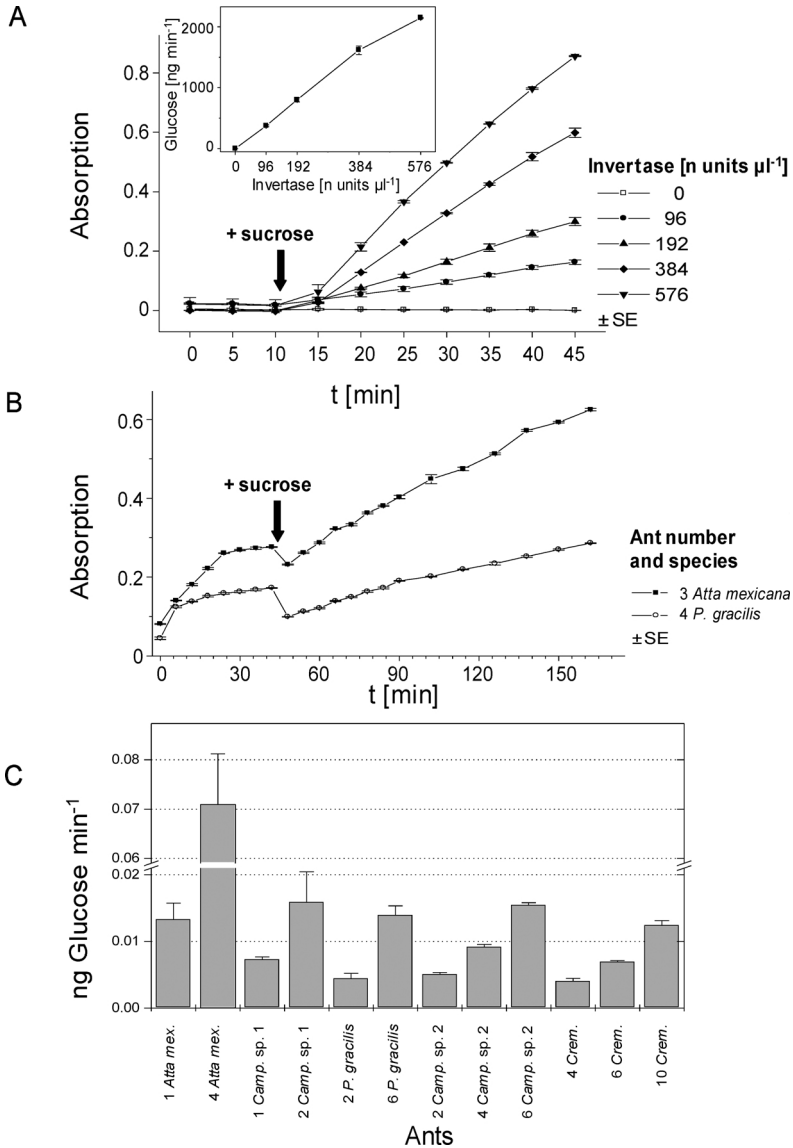

FIG. 1. Quantification of invertase and validation of the method. Time courses in absorption are given separately for purified invertase (A) and extractions of two ant species (B). Note the time at which sucrose has been added. Use of the slopes of the curves representing different amounts of purified invertase for quantification of activity revealed an almost linear calibration curve (insert in panel A). Use of different numbers of ants (C) revealed linear dose-activity relations. *Atta mex.*, *Atta mexicana; Camp.*, *Camponotus; Crem., Crematogaster*; *P. gracilis*, *Pseudomyrmex gracilis*.

TABLE 1. MEAN INVERTASE ACTIVITIES IN DIFFERENT ANT SPECIES

	Atta mex.	Camp. 1	P. gracilis	Camp. 2	Crem. spec.
Activity (mean)	46.2[a]	24.4[b]	28.6[b]	14.6[c]	17.3[c]
SE	5.4	3.9	6.3	0.9	2.3
N	6	4	5	6	5

Invertase activity is given in ng glucose released per mg ant tissue and per min; means marked with different letters are significantly different ($P < 0.05$ according to LSD *post hoc* analysis). *Atta mex.*, *Atta mexicana; Camp.*, *Camponotus; Crem., Crematogaster*; *P. gracilis*, *Pseudomyrmex gracilis*.

in a slightly reduced absorption due to dilution effects by the added volume of sucrose solution (Figure 1B). After 10–15 min, the resulting curves showed almost linear slopes, and invertase activity calculated from these slopes differed significantly among the species tested (Table 1). Variability among ant species was much higher than within a species, and in general the values obtained from ants belonging to the same colony (genetically similar and usually having fed on the same food sources) were more similar than those obtained from ants from different colonies. When varying numbers of ants were extracted, an almost linear ant number—invertase activity relationship was found (Figure 1C).

The method described here allows the easy quantification of invertase activity in extracts of the digestive tracts of ants, and it should be applicable to other arthropods. The method is easy, does not require expensive or elaborate equipment, and can be applied under field conditions. Because the method measures enzyme activity it is not suitable for identifying the presence of isoenzymes or for determining whether the enzyme is synthesized by the animal itself or, rather, is the product of endosymbiotic microorganisms. However, the method reliably measures the activity of a digestive enzyme and provides an analysis of the digestive capacity of the insect—or insect organ—used for extraction.

Acknowledgments—We thank Manfred Verhaag, Staatl. Museum für Naturkunde, Karlsruhe, for determining ant species, Emily Wheeler for critically reading an earlier version of this manuscript, and Flavio Roces (University of Würzburg) for provisioning of ants for preliminary studies. Financial support by the Deutsche Forschungsgesellschaft (DFG-grants He3169/2-2 and 3-1) and the Max-Planck-Gesellschaft is gratefully acknowledged.

REFERENCES

AYRE, G. L. 1963. Feeding behaviour and digestion in *Camponotus herculeanus* (L.) (Hymenoptera: Formicidae). *Entomol. Exp. Appl.* 6: 165–170.

AYRE, G. L. 1967. The relationships between food and digestive enzymes in five species of ants (Hymenoptera: Formicidae). *Canad. Entomol.* 99: 408–411.

BOEVÉ, J. L. and WÄCKERS, F. L. 2003. Gustatory perception and metabolic utilization of sugars by *Myrmica rubra* ant workers. *Oecologia* 136: 508–514.

HEIL, M. and MCKEY, D. 2003. Protective ant-plant interactions as model systems in ecological and evolutionary research. *Annu. Rev. Ecol. Evol. Syst.* 34: 425–453.

HENRISSAT, B. and BAIROCH, A. 1993. New families in the classification of glycosyl hydrolases based on amino-acid-sequence similarities. *Biochem. J.* 293: 781–788.

MARTÍNEZ DEL RIO, C. 1990. Dietary, phylogenetic, and ecological correlates of intestinal sucrase and maltase activity in birds. *Physiol. Zool.* 63: 987–1011.

NAUMOFF, D. G. 2001. β-Fructosidase superfamily: Homology with some α-L-arabinases and β-D-xylosidases. *Proteins: Struct. Funct. Genet.* 42: 66–76.

RICKS, B. L. and VINSON, S. B. 1972. Digestive enzymes of imported fire ant, *Solenopsis richteri* (Hymenoptera: Formicidae). *Entomol. Exp. Appl.* 15: 329–334.

STURM, A. and TANG, G. Q. 1999. The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. *Trends Plant Sci.* 4: 401–407.

ZHANG, J., SCRIVENER, A. M., SLAYTOR, M., and ROSE, H. A. 1993. Diet and carbohydrase activities in three cockroaches, *Calolampra elegans* Roth and Princis, *Geoscapheus dilatatus* Saussure and *Panesthia cribrata* Saussure. *Comp. Biochem. Physiol. A* 104: 155–161.