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A modified enzymatic method for measuring insect sugars and the effect of storing samples in ethanol on subsequent trehalose measurements

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ABSTRACT

A modified method for enzymatically measuring concentrations of glucose, fructose and trehalose in parasitoid wasps is described and evaluated. The method has high specificity, gives results comparable to high performance liquid chromatography (HPLC), and shows good correlations with HPLC. The enzymatic method is quicker and less expensive than HPLC, and is safer, faster and more sensitive than another commonly used insect sugar determination method, the anthrone test. The method has potential to measure additional sugars, and details for measuring sucrose are provided as Supplementary material. We also investigate if endogenous enzymes can retain activity in insects preserved in ethanol, thus potentially skewing insect sugar measurements. The results show that the enzyme trehalase, which converts the insect haemolymph sugar trehalose to glucose, can remain active in insect specimens that are stored whole in ethanol. Thus, sugar measurements from insects preserved in this way have potential for bias towards low trehalose and high glucose. In contrast, the activity of endogenous trehalase in insects that are either frozen at −20 °C, or crushed in ethanol is either much reduced or eliminated. If, prior to sugar analysis, insects are to be stored in ethanol at temperatures above −20 °C, then we recommend they be crushed.

1. Introduction

Parasitoid wasps are enormously valuable throughout the world as biological control agents of pest species (Greathead, 1986). Adults of many parasitoids benefit from access to sugar sources such as floral nectar, extrafloral nectar and hemipteran honeydew (Jervis et al., 1993; Russell, 2015; Tena et al., 2013). Some can also feed from host insects to obtain diverse nutrients including sugars (Giron et al., 2002; Jervis and Kidd, 1986). Ingested sugars are used for energy production, and stored as glycogen in the fat body, or as trehalose in the haemolymph (Rivero and Casas, 1999). Benefits to parasitoids of sugar-feeding can include longer lifespans (Heimpel et al., 1997; Olson and Andow, 1998), increased egg production (Heimpel et al., 1997; Olson and Andow, 1998), greater flight capability (Wanner et al., 2006), and increased time spent searching for and parasitising hosts (Araj et al., 2011). Many studies have attempted to enhance parasitoid suppression of pests by manipulating their habitats to increase parasitoids' access to sugar (Heimpel and Jervis, 2005; Tena et al., 2015; Winkler et al., 2009).

Sucrose is a disaccharide comprising the monosaccharides glucose and fructose. The presence of sucrose or fructose in a parasitoid is often used to deduce if it has fed because these sugars are not produced by parasitoids, but are present in parasitoid food sources such as nectar, honeydew, honey and fruit juices (Lee et al., 2004; Nicolson and Thornburg, 2007; Tena et al., 2016; Van Handel, 1984, 1970). Trehalose is a disaccharide comprising two molecules of glucose, and plays major roles in insect energy storage and other functions (Thompson, 2003). It is frequently the principal sugar found in insect haemolymph, which can also contain large proportions of glucose and/ or fructose (Thompson, 2003; Wyatt and Kalf, 1957).

Methods for measuring sucrose, glucose and fructose in parasitoids and other insects are often based on anthrone tests originally developed for mosquitoes (Van Handel, 1985, 1970, 1968, 1967); these tests can also measure trehalose (Giron et al., 2002; Van Handel, 1985) and the polysaccharide glycogen (Foray et al., 2012; Van Handel, 1985). The cold anthrone test (Van Handel, 1967) has frequently been used to measure fructose in parasitoids, and the hot anthrone test (Van Handel, 1985) to measure total sugars (Lee et al., 2004; Olson et al., 2000; Wyckhuys et al., 2008). Anthrone tests have the disadvantages that they may be insufficiently sensitive for minute arthropods (Wyckhuys et al.,

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2008), can take 4–5 h at 20 °C, and involve using and disposing of sulfuric acid.

High performance liquid chromatography (HPLC) has also been used to measure sugars in parasitoids and nectars because it offers increased sensitivity and the capability to quantify a wider range of sugars (Steppuhn and Wackers, 2004; Tena et al., 2016; Vattala et al., 2006; Wyckhuys et al., 2008). HPLC has the disadvantages that it requires sophisticated laboratory equipment, and is expensive and comparatively slow.

When storing insect samples prior to measuring sugars, Van Handel (1985) and Tennessen et al. (2014) cautioned that it is essential to store specimens at −20 °C or lower immediately after capture to prevent degradation of trehalose and glycogen to glucose by endogenous enzymes, which would skew the results. Moreover, the enzymes will likely still be active after samples are removed from the freezer (Van Handel, 1985). Many recent insect sugar studies have stored specimens in ethanol rather than a freezer (Hogervorst et al., 2007; Rusch et al., 2013; Steppuhn and Wackers, 2004; Tena et al., 2015, 2013; Vattala, 2005), although there is no information about the possibility that endogenous enzymes could remain active in ethanol. It is notable that at least two of these studies, which both used HPLC to measure sugar concentrations, recorded unexpectedly low (Vattala, 2005) or zero (Steppuhn and Wackers, 2004) trehalose concentrations in several parasitoid species.

In this paper, we describe a modification of existing enzymatic sugar measurement methods (e.g., Cairns, 1987, Campbell et al., 1999) for measuring glucose, fructose and trehalose in parasitoids. We focus on these three sugars because they usually comprise the majority of the sugars found in insect haemolymph, and glucose and fructose are particularly useful for distinguishing between fed and unfed parasitoids. The methods require only rudimentary laboratory equipment, are quicker and safer than anthrone tests, and are quicker and less expensive than HPLC. We also evaluate if trehalose can be converted to glucose by endogenous enzymes when parasitoids are stored in ethanol, and propose an improved method for storing parasitoids in ethanol prior to sugar analysis. The following sections: Present the reagents for the stains used to produce formazan from glucose, trehalose and fructose; assess the sensitivity and specificity of the reactions; compare sugar concentrations estimated by the enzymatic assay to those estimated by HPLC from the same parasitoid samples; evaluate if the enzymatic assay can distinguish between parasitoids provisioned with honey solution from those provisioned with water; and investigate if trehalose can be converted to glucose by endogenous enzymes when specimens are stored in ethanol.

2. Methods

The sugar measurement method exploits a short series of enzymatic reactions previously used to develop a colorimetric assay for evaluating the viability of small immobile arthropods (Phillips et al., 2013). In the viability assay, hexokinase phosphorylates glucose to glucose-6-phosphate in the presence of adenosine triphosphate (ATP) (Manchenko, 1994). Phillips et al. (2013) used the ATP that is present in living organisms to drive reactions that reduce the tetrazolium salt thiazolyl blue tetrazolium bromide (MTT) to a visible purple formazan (Fig. 1), which can either be quantified colorimetrically or assessed visually. The same reaction is used here for measuring sugars, except ATP is added to the reagents, but glucose is not, thus the amount of formazan produced is limited by sample glucose concentration (Fig. 1). Trehalose and fructose are measured in the same manner after enzymatically converting them to glucose. The reagents used to measure glucose, trehalose and fructose are given in Table 1, and a similar method for measuring sucrose is described in Supplement 1.

Our general approach, summarized in step 4 of Fig. 2, was to:

1. Measure glucose concentration in a sample aliquot (Fig. 1).
2. Enzymatically convert either trehalose or fructose to glucose in a separate sample aliquot.
3. Measure the concentration of glucose produced in the second reaction.
4. Calculate the concentration of either trehalose or fructose by subtracting the glucose measured in the first reaction from that measured in the second.

In this paper: 'Glucose stain' refers to the solution that produces visible formazan only from glucose; 'trehalose stain' refers to the solution that converts trehalose to glucose and produces visible formazan from the glucose; and 'fructose stain' refers to the solution that converts fructose to glucose and produces visible formazan from the glucose.

2.1. Reagents

The glucose stain (Table 1) uses any glucose present in the sample to reduce the tetrazolium salt MTT to produce a visible formazan (Fig. 1). The trehalose stain (Table 1) contains the same components as the glucose stain, plus the enzyme trehalase, which converts trehalose to glucose. Thus, the amount of formazan produced by the trehalose stain is proportional to the combined concentration of glucose and trehalose in the sample. The trehalose concentration is calculated by subtracting the formazan produced by the glucose stain from that produced by the trehalose stain, and the same principles are also applied to fructose (Fig. 2, step 4; Table 1).

2.2. Stain sensitivity

Twenty µl aliquots from separate dilution series (1.25, 2.5, 5, 10, 20, 40, 60, 80 and 100 µg/ml) of glucose, trehalose, and fructose were added to separate wells of a 96-well flat assay microplate (Nunc, Denmark), mixed with 20 µl of a freshly prepared stain (Table 1), incubated at room temperature for 20 min in the dark, then mixed with 40 µl of 10% SDS in 0.001 M HCl to stop the reaction (Fig. 1). Water and reagent blanks were included on each microplate as zero points for standard curves. Optical densities of solutions in each well were measured with a FLUOstar® Omega microplate reader (BMG Labtech, Germany) fitted with a 570 nm filter. Measurements were made within 30 min of stopping the reactions, and optical densities were recorded as the mean of two replicate wells.

2.3. Stain specificity

To obtain accurate assay results, it is essential that each stain (Table 1) produces glucose and formazan only from the sugar that it is intended to measure. Reactions between different sugars and stains were measured in a 5*4 factorial combination (sugars*stains), replicated three times. The sugars tested were 25 µl of 50 µg/ml solutions of fructose, glucose, sucrose and trehalose, plus water controls. The stains used were 25 µl of the fructose, glucose and trehalose stains, plus a water control, with each assay conducted in duplicate. All reactions were stopped 10 min after stains were added to wells using 10% SDS in 0.001 M HCl. Results from each combination of sugar and stain were measured in a microplate reader as previously described. Absorbance at 570 nm was read 5, 30, 60 and 120 min after stopping the reactions to verify that adding SDS and HCl immediately stopped the reaction, and the results were stable.

2.4. Comparison of results obtained from insect samples between enzymatic method and HPLC

This experiment used HPLC and enzymatic methods to measure glucose, trehalose and fructose concentrations in the same parasitoid samples. The parasitoids *Microctonus aethiopoides* Loan (Hymenoptera: Braconidae) and *M. hyperodae* Loan were obtained by vacuuming their