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A liquid chromatography–tandem mass spectrometry assay for the detection and quantification of trehalose in biological samples

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ABSTRACT

Trehalose is an important disaccharide that is used as a cellular protectant by many different organisms, helping these organisms better survive extreme conditions, such as dehydration, oxidative stress, and freezing temperatures. Methods to detect and accurately measure trehalose from different organisms will help us gain a better understanding of the mechanisms behind trehalose's ability to act as a cellular protectant. A liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay using selected reaction monitoring mode for the detection and quantification of trehalose using maltose as an internal standard has been developed. This assay uses a commercially available LC column for trehalose separation and a standard triple quadrupole mass spectrometer, thus allowing many scientists to take advantage of this simple assay. The calibration curve from 3 to 100 μM trehalose was fit best by a single polynomial. This LC–MS/MS assay directly detects and accurately quantifies trehalose, with an instrument limit of detection (LOD) that is 2–1000 times more sensitive than the most commonly-used assays for trehalose detection and quantification. Furthermore, this assay was used to detect and quantify endogenous trehalose produced by *Escherichia coli* (*E. coli*) cells, which were found to have an intracellular concentration of 8.5 ± 0.9 mM trehalose. This method thus shows promise for the reliable detection and quantification of trehalose from different biological sources.

1. Introduction

Trehalose, or α-D-glucose(1 → 1)α-D-glucose (**1**, Fig. 1a), is a disaccharide biosynthesized by many different organisms, including fungi, plants, insects, bacteria, and eukaryotic microorganisms [1–5]. Both intra- and extracellular trehalose allows these organisms to better survive environmental stresses such as reactive oxygen species, freezing temperatures, and almost complete drying [1–7]. It has been hypothesized that trehalose provides protection by replacing the water inside cells, thus stabilizing proteins and lipid membranes via hydrogen-bonding, and by forming a stable glass (a liquid of high viscosity) at room temperature, thus reducing the rates of damaging biochemical reactions [4,8]. These protective properties of trehalose, coupled with its low cellular toxicity, have generated interest in using this carbohydrate as a general cellular protectant [1,3–9].

To function as a cellular protectant, trehalose must be present inside cells [1–7]. Certain desiccation-tolerant plants, such as *Selaginella lepidophylla*, accumulate intracellular trehalose at levels up to 12% of their dry weight during periods of drought [10]. *Escherichia coli* (*E. coli*) cells increase their production of intracellular trehalose when exposed to cold temperatures, thus allowing these bacteria to better survive cold shock [11]. Mammalian cells, unfortunately, do not naturally biosynthesize trehalose. When mammalian cells are loaded with exogenous trehalose, however, they have greatly improved survival rates after freezing or drying compared to mammalian cells that contain no intracellular trehalose [7,12]. Exogenously-administered trehalose has also been shown to provide neuroprotective effects in animal models of Huntington disease, Parkinson disease, and amytrophic lateral sclerosis (ALS) [13–16]. Although there are several hypotheses that describe how trehalose exerts its protective effects, the exact mechanisms by which trehalose protects cells are not well characterized. To better understand the role of trehalose in cellular protection, it is crucial to be able to detect and accurately quantify trehalose from organisms exposed to various environmental conditions.

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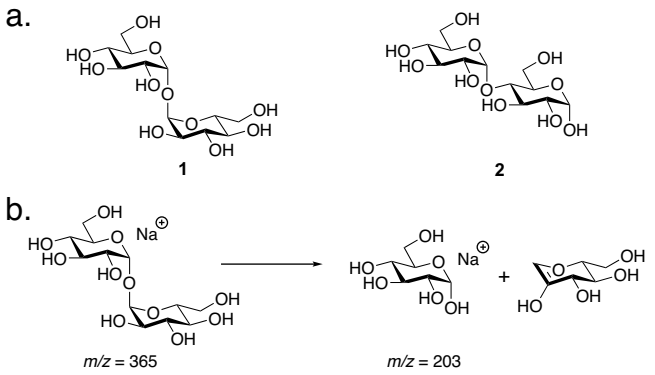

Fig. 1. (a) Structures of trehalose (**1**) and maltose (**2**). (b) Parent trehalose ion (m/z = 365) and daughter glucose ion (m/z = 203) detected by our LC–MS/MS method.

A number of methods exist for the detection and quantification of trehalose from biological sources. One of these assays uses anthrone, a reagent that reacts with trehalose to give a colorimetric change that can be quantified spectrophotometrically [17]. However, anthrone reacts with sugars other than trehalose, which may lead to an overestimation of trehalose concentration [17–20]. Another method for detecting and quantifying trehalose uses the enzyme trehalase, which cleaves trehalose into two glucose monomers [20,21]. An enzymatic assay is then used to determine the concentration of glucose, from which the original concentration of trehalose can be calculated after subtracting out the amount of endogenous glucose found in the sample. Unfortunately, this assay may also overestimate trehalose concentrations since one commonly-used trehalase enzyme from porcine kidney also hydrolyzes the disaccharide maltose as well as trehalose [20]. Moreover, both the anthrone reagent and the trehalase enzymatic assay do not measure trehalose levels directly. High performance liquid chromatography (HPLC), coupled with detection by a pulsed electrochemical detector or a refractive index detector, has also been used to detect and quantify trehalose [19,22–25]. One problem with these HPLC methods is that there is no way to conclusively identify trehalose based on the refractive index or electrochemical signals. Furthermore, trehalose can coelute with other biological compounds, thus causing an overestimation of the trehalose concentration [20,26]. Gas chromatography-mass spectrometry (GC–MS) provides high sensitivity and allows for the identification of trehalose in biological samples; however, it requires the derivatization of trehalose with trimethylsilyl ethers, thus adding an extra, time-consuming step to this method and possibly introducing additional error [26,27].

In this paper, a simple liquid chromatography–tandem mass spectrometry (LC–MS/MS)–based assay for the direct detection and accurate quantification of endogenous trehalose from biological samples using maltose (**2**, Fig. 1a) as an internal standard is described. LC–MS/MS is routinely used as a method for quantifying analytes from biological samples [28–30]. Moreover, LC–MS/MS analysis allows for the conclusive identification of the compound or compounds present in each chromatographic peak, thus ensuring that the signal used for quantification is produced by the target compound, rather than an unknown interfering compound. Although two LC–MS-based assays for the detection and/or quantification of trehalose have been reported in the literature, neither method detected nor quantified endogenous trehalose from a biological sample. Additionally, the reported methods rely either on a custom-produced, hand-packed silica column for trehalose separation or an expensive ion trap mass spectrometer [30,31]. This LC–MS/MS-based assay for the detection and quantification of trehalose uses a commercially available LC column for HPLC separation and a standard triple quadrupole mass spectrometer, thus allowing many scientists to take advantage of this simple assay. The method described here detects and accurately quantifies trehalose with high sensitivity and selectivity, with an instrument limit of detection (LOD) of 28 nM (560 fmol) trehalose and an instrument limit of quantification (LOQ) of 110 nM (2.3 pmol) trehalose. These values are 2–400 times lower than those reported for almost all trehalose detection and quantification assays and will allow researchers to use fewer cells when measuring trehalose concentrations in biological samples. Furthermore, this method is used to detect and quantify endogenous trehalose from *E. coli* cells, which has never been accomplished using an LC–MS/MS-based assay. The LC–MS/MS assay reported in this paper will be useful for the detection and quantification of trehalose from a variety of biological sources, thus aiding in the study of the role that trehalose plays in cellular protection.

2. Methods and materials

2.1. Materials

D-(+)Trehalose dihydrate (>99%), D-(+)-maltose monohydrate (99%), and LC–MS grade ammonium acetate (≥99%) were purchased from Fisher Scientific. Solvents (acetonitrile and water) were of LC–MS/Optima grade and obtained from Fisher Scientific. LB Broth (Miller), agar, ampicillin, (D)-(−)arabinose, and tetracycline were obtained from Fisher Scientific. RPMI-1640 medium was purchased from America Type Culture Collection (ATCC, Manassas, VA). Phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were obtained from Fisher Scientific.

2.2. Escherichia coli (E. coli) lysate preparation

E. coli cells (strain DH5α) were a generous gift from K. Fox (Union College). *E. coli* cells were plated on LB Broth (Miller)-agar plates and allowed to grow overnight at 37 °C. A single colony was picked from the plate, placed in 5 mL of sterile LB broth, and shaken (275 rpm) at 37 °C for 23 h. The cells were centrifuged (3000 rcf × 10 min), and the supernatant was removed. The cell pellet was resuspended in PBS (5 mL), centrifuged (3000 rcf × 10 min), and the supernatant was removed. This process was repeated one more time, after which the cell pellet was frozen at −80 °C until cell lysis.

The wild-type, trehalose knockout (Δ*otsA*) of the *E. coli* strain MC4100 was created by Kaasen et al. and was a generous gift from P. Woodruff (University of Southern Maine) [32]. The Δ*otsA* strain was plated on LB Broth (Miller)-agar plates containing 50 μM tetracycline and allowed to grow overnight at 37 °C. A single colony was picked from the plate, placed in 5 mL of sterile LB broth containing 50 μM tetracycline, and shaken (275 rpm) at 37 °C for 16 h. 1.5 mL of the Δ*otsA* strain starter culture were then used to inoculate 100 mL of sterile LB broth containing 50 μM tetracycline, and the culture was shaken (275 rpm) at 37 °C for 4 h. The cells were centrifuged (3000 rcf × 10 min), and the supernatant was removed. The cell pellet was resuspended in PBS (90 mL), centrifuged (3000 rcf × 10 min), and the supernatant was removed. The cell pellet was then resuspended in PBS (5 mL) and centrifuged (3000 rcf × 10 min) again. The supernatant was removed, and the cell pellet was frozen at −80 °C until cell lysis.

The arabinose-inducible OtsA/OtsB overproducer of the *E. coli* strain MC4100 was created by Frederick et al. and was a generous gift from P. Woodruff (University of Southern Maine) [33]. The arabinose-inducible OtsA/OtsB overproducer strain was plated on LB Broth (Miller)-agar plates containing 100 μM ampicillin and allowed to grow overnight at 37 °C. A single colony was picked