Analytical Biochemistry 498 (2016) 47–52

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A reliable method for spectrophotometric determination of glycine betaine in cell suspension and other systems

Ma Guadalupe Valadez-Bustos [a], Gerardo Armando Aguado-Santacruz [b], Axel Tiessen-Favier [c], Alejandrina Robledo-Paz [a], Abel Muñoz-Orozco [a], Quintin Rascón-Cruz [d], Amalio Santacruz-Varela [a, *]

[a] Campus Montecillo, Colegio de Postgraduados, C.P. 56230, Montecillo, Estado de México, Mexico
[b] Unidad de Biotecnología, Campo Experimental Bajío-Inifap, C.P. 38010, Celaya, Guanajuato, Mexico
[c] Laboratorio de Metabolómica y Fisiología Molecular, Cinvestav Unidad Irapuato, C.P. 36821, Irapuato, Guanajuato, Mexico
[d] Facultad de Ciencias Químicas, Universidad Autónoma de Chihuahua, C.P. 31170, Chihuahua, Chihuahua, Mexico

ARTICLE INFO

Article history:
Received 12 May 2015
Received in revised form
27 November 2015
Accepted 18 December 2015
Available online 14 January 2016

Keywords:
Glycine betaine
Cell cultures
Stress
Bouteloua
Quaternary ammonium compounds

ABSTRACT

Glycine betaine is a quaternary ammonium compound that accumulates in a large variety of species in response to different types of stress. Glycine betaine counteracts adverse effects caused by abiotic factors, preventing the denaturation and inactivation of proteins. Thus, its determination is important, particularly for scientists focused on relating structural, biochemical, physiological, and/or molecular responses to plant water status. In the current work, we optimized the periodide technique for the determination of glycine betaine levels. This modification permitted large numbers of samples taken from a chlorophyllic cell line of the grass *Bouteloua gracilis* to be analyzed. Growth kinetics were assessed using the chlorophyllic suspension to determine glycine betaine levels in control (no stress) cells and cells osmotically stressed with 14 or 21% polyethylene glycol 8000. After glycine extraction, different wavelengths and reading times were evaluated in a spectrophotometer to determine the optimal quantification conditions for this osmolyte. Optimal results were obtained when readings were taken at a wavelength of 290 nm at 48 h after dissolving glycine betaine crystals in dichloroethane. We expect this modification to provide a simple, rapid, reliable, and cheap method for glycine betaine determination in plant samples and cell suspension cultures.

On a global scale, water shortage is the main ecological problem for food production in rain-fed agriculture. Consequently, a significant portion of genetic improvement efforts are aimed at obtaining plants with higher tolerance to water stress. Tolerance to water stress in plants is controlled by several genes that act in an additive manner [1,2].

A substantial increase in the cellular concentrations of osmotically active compounds, termed compatible solutes, has been observed in a vast number of organisms in response to salinity or drought stress [3–5]. Inorganic solutes such as K^+, Na^+, and Cl^- can also increase during osmotic stress, but Na^+ and Cl^- interfere with cellular activities and need to be compartmentalized to the vacuole [6]. For a solute to be compatible, a considerable increase in its concentration must not interfere with the normal metabolic functions of the cell. Some of the best-known osmolytes contain quaternary ammonium (glycine betaine), amino acids (proline, glycine, and taurine), polyols (glycerol, inositol, and sorbitol) and their derivatives (methyl-inositol), or sugars (mannitol, sorbitol, sucrose, and trehalose), among other compounds. How these compatible solutes protect cells against damage from osmotic stress is still a matter of debate [7,8]. An early hypothesis was that compatible solutes may help cells to conserve remnant water by biophysically functioning as water-attracting or water-conserving molecules, thereby maintaining cell turgor. A widespread hypothesis points to the interactions between elements of the ternary complex water–osmolytes–proteins as the underlying phenomenon [9], where osmolytes modulate biochemical reactions leading to the preferential exclusion mechanism, where the stabilizing solutes do not bind to proteins; on the contrary, they are excluded from a protein's hydration layer through conformational changes to fold up more

Abbreviations: GB, glycine betaine; PEG 8000, polyethylene glycol 8000; RWC, relative water content; SWC, soil water content; OD, optical density.

* Corresponding author.
E-mail address: asvarela@colpos.mx (A. Santacruz-Varela).

http://dx.doi.org/10.1016/j.ab.2015.12.015
0003-2697/

compactly in order to reduce exposure to thermodynamically unfavorable interactions with the stabilizing solute [10]. A third hypothesis is that compatible solutes function as scavengers of reactive oxygen species [11].

Glycine betaine (GB) is an amphiphilic compound with a hydrophobic positive end and a hydrophilic negative end, and it is electrically neutral over a wide range of pH values [12]. This osmolyte is synthesized in several families of plants such as Chenopodiaceae, Amaranthaceae, Avicenniaceae, Convolvulaceae, Plumbaginaceae, Solanaceae, Leguminosae, Asteraceae, Malvaceae, Poaceae, Portulacaceae, and Caryophyllaceae. However, few families are able to accumulate quantities of this osmolyte sufficient to achieve an osmotic effect [13]. In higher plants, the enzymes that synthesize GB are found in the chloroplast stroma [14,15].

GB is the most effective compatible solute for the improvement of salinity and drought tolerance in higher plants [16]. By interacting with both hydrophilic and hydrophobic domains of macromolecules, this osmolyte is involved in reducing lipid peroxidation [17], neutralizing high NaCl concentrations [10], maintaining thylakoid membrane integrity [18], and stabilizing the structure of proteins from the damaging effects of abiotic factors such as drought, salinity, and freezing [12].

The properties of osmolytes are useful in agriculture, cell biology and biotechnology [10,17] because they are indicators of suboptimal environmental conditions. In particular, plant breeders interested in developing crops that are more tolerant of drought, salinity, and freezing require reliable, efficient, and affordable techniques for detecting osmolytes in a time-efficient, low-cost, and rapid manner. Experimental evidence shows that overexpression or incorporation of the biosynthesis of some osmolytes in the genome of some plants can result in increased tolerance to abiotic factors in plants [11].

Plant cell cultures are important systems for the study and isolation of genes related to water tolerance. Using these biological systems, several studies have been performed to analyze the physiological, molecular, and biochemical processes operating during saline stress [19], osmotic stress [20–24], and cold [25].

Cell cultures with high chlorophyll content, such as the chlorophyllic system studied in this work, offer additional advantages because certain enzymes of the plant's metabolism are located in chloroplasts [26,27]. Chloroplasts are important within the biotechnology of water stress [28,29] due to the confinement of certain compatible solutes (or enzymes involved in their biosynthesis) in these cellular compartments. For example, the osmoregulator glycine betaine is mainly located in chloroplasts [30], where it stabilizes the photosynthetic apparatus [31], and therefore the photosynthetic rate, during stressful conditions [32].

Determination of glycine betaine levels currently requires sophisticated and costly equipment such as a refraction index detector [33], a mass spectrometer [34,35], nuclear magnetic resonance spectroscopy [11], and pyrolitic instrumentation.

Grieve and Grattan [36], Stumpf [37], and Arakawa and coworkers [38] developed different methods for determining this compound using affordable equipment such as the spectrophotometer. Currently, the periodide method of Grieve and Grattan [36] is the method most widely used to precipitate quaternary ammonium compounds for glycine betaine determination. This technique, although successful in a large variety of species, is not efficient when applied to certain systems, such as the plant cell suspension culture analyzed here, because they contain large amounts of quaternary ammonium compounds and cause GB sedimentation, making this technique tedious and time-consuming.

The aim of this research was to optimize the periodide method for determining glycine betaine levels in recalcitrant samples such as chlorophyllic cell cultures of the grass *Bouteloua gracilis*.

Materials and methods

Chlorophyllic cell suspension growth kinetics

B. gracilis chlorophyllic cells were routinely cultivated in 125-ml flasks containing 25 ml of liquid MPC medium under optimal growth conditions [39]: continuous fluorescent light, 77 μmol s^{-1} m^{-2}, 33 ± 1 °C temperature, and shaking at 90 rpm. The MPC medium contained the basal salts and vitamins of MS medium [40], 2 mg L^{-1} 6-benzylaminopurine (BAP), 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 40 mg L^{-1} adenine, and 3% (w/v) sucrose. The pH of the medium was adjusted to 5.8 before sterilization (120 °C/15 min).

Growth kinetics were assessed by culturing the chlorophyllic cells under either normal or hyperosmotic conditions. Osmotic treatment was achieved by adding polyethylene glycol 8000 (PEG 8000) to the basal MPC medium at a concentration of 14 or 21% PEG. An initial 8-day stock cell culture was used to inoculate 0.4 g FW cells into 120-ml flasks containing 25 ml of the different liquid media. The cells from the initial stock culture were considered the day 0 material in the growth kinetics. All cell growth kinetics were assessed under the same environmental conditions described before for the routine culture of the chlorophyllic cells. After initial sampling at day 0, cells were further collected at days 3, 6, 9, and 12 after inoculation of the 120 flasks (10 repetitions per treatment). Complete cell growth kinetics, including MPC, 14% PEG, and 21% PEG treatments, were repeated at least four times. All harvested cell material was lyophilized for further glycine betaine determinations.

Plants of tomato and wheat grown under greenhouse

To further test this technique, glycine betaine analyses were performed in monocot and dicot species. Tomato (*Solanum lycopersicum*), CID hybrid, and wheat (*Triticum aestivum*) cv. Tlaxcala seeds were sown in 1-L containers with peat moss (Sunshine) and then transplanted into 1-L vessels with sandy loam soil. Plants were grown under greenhouse conditions (minimum/maximum air temperature of 26/55 °C and relative humidity between 8 and 90% at mid-day during the experimental period). Irrigation was applied every other day, and the drought treatment began 40 days after planting. Plants were subjected to two water regimes: (i) optimal regime (control), where plants were constantly irrigated to maintain 21% of soil moisture content (field capacity of this soil was 16.1%), and (ii) drought treatment, without irrigation until the substrate reached permanent wilting point (PWP for this soil was 9.5%).

After treatments, in addition to determination of GB in leaves and roots, relative water content (RWC) and soil water content (SWC) were evaluated. SWC was estimated by the gravimetric method as recommended by Ortiz-Villanueva and Ortiz-Solorio [41], whereas the estimation of RWC was based on the methods described by Salisbury and Ross [42].

GB determination in chlorophyllic cell suspension

The concentration of glycine betaine was determined for all treatments and sampling dates using a modification of the method described by Grieve and Grattan [36], which is described below. H_2SO_4 (1.5 ml of 2N) was added to 1 mg lyophilized cells, and the mixture was heated up to 60 °C in an Eppendorf ThermoMixer C for