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Analytical Biochemistry 362 (2007) 151–153
ANALYTICAL BIOCHEMISTRY
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Notes & Tips

High-throughput colorimetric method for the parallel assay of glyoxylic acid and ammonium in a single extract

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Received 31 October 2006
Available online 22 December 2006

Glyoxylate and ammonia are signature metabolites of a major plant metabolic pathway, the C_2 oxidative carbon cycle, also known as photorespiration. This complex salvage pathway recycles most of the carbon that is lost from the Calvin cycle in the form of phosphoglycolate as a consequence of the oxygenation reaction of RuBP carboxylase–oxygenase (RuBisCO)[2] and constitutes a significant metabolic flux in photosynthesizing leaves of C_3 plants [1]. Mutations in enzymes of this pathway usually are lethal, underlining the importance of this salvage pathway [2–4]. Although all enzymes and some of the metabolite transporters involved in this highly compartmentalized pathway have been identified [5–9], information about the majority of the transport proteins and the processes regulating the pathway is still missing [1,4], possibly because mutations in the corresponding genes cause subtle metabolic phenotypes. Thus, a high-throughput method to quantify metabolites known to accumulate in photorespiratory mutants, glyoxylate and ammonium, is desirable. Here we present improved versions of two colorimetric methods for the quantification of ammonia and glyoxylate that make these procedures suitable for high-throughput and sensitive quantification of these signature metabolites in plant tissues.

A colorimetric method for measuring glyoxylate has been described previously [5,10,11]. In this method, a 1,5-diphenylformazancarboxylic acid derivative of glyoxylic acid is produced through mild oxidation of a glyoxylic acid hydrazone in the presence of excess phenylhydrazine. The formazan of glyoxylic acid shows maximal absorption at 520 nm (Fig. 1). The quantification of ammonium (based on the Berthelot reaction [12,13]) from the same plant extract as glyoxylate has been described previously in tobacco [5]. Ammonia reacts with hypochlorite and forms a monochloramine, which reacts with phenol to an indophenol derivative that has deep blue color and displays maximal absorbance at 620 nm (Fig. 1). Although both methods have been used previously to determine glyoxylate and ammonia contents in plant tissues [5,14], they are hampered by interfering compounds quenching the signal. In addition, many plant tissues accumulate anthocyanins (especially under stress conditions), which absorb light at similar wavelengths (Fig. 1) and therefore introduce noise to the assays. To address these problems and to render the assay suitable for high-throughput analysis, we have developed a new protocol optimized for use in a 96-well format that allows the colorimetric quantification of glyoxylate and ammonia from a single plant extract. Extract purification with chloroform and activated charcoal efficiently removed interfering compounds, thereby making the procedure appropriate for plant extracts.

Plant tissue is harvested and snap-frozen in liquid nitrogen and is ground to a fine powder under liquid nitrogen. Then 100 mg of the powder is extracted in 1 ml of 100 mM HCl, and subsequently 500 µl of chloroform is added. The samples are rotated for 15 min at 4 °C, and the phases are separated by centrifugation (12,000*g*, 10 min, 8 °C). The aqueous phase is transferred to a fresh tube containing 50 mg of acid-washed activated charcoal, thoroughly mixed, and centrifuged (20,000*g*, 5 min, 8 °C). For glyoxylate quantification, 450 µl of the supernatant is mixed with 50 µl of a freshly prepared 1% (v/v) solution of phenylhydrazine in 100 mM HCl, and the sample is incubated at 95 °C for 2 min and cooled on ice for 6 min. The sample is acidified by adding 250 µl of concentrated HCl and is divided into three 225-µl aliquots in cavities of a 96-well plate; two technical replicates are oxidized with 25 µl of a solution of 1.6% (w/v) $K_3Fe(CN)_6$ in water, and one aliquot serves as control

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[2] *Abbreviation used:* RuBisCO, RuBP carboxylase–oxygenase.

0003-2697/$ - see front matter
doi:10.1016/j.ab.2006.12.033

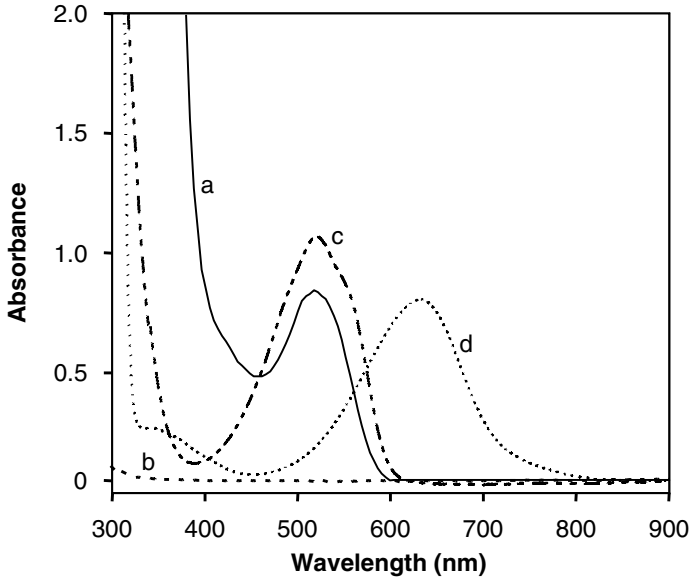

Fig. 1. Absorption spectra of a crude acidic extract of *Arabidopsis* leaves (a), of a partially purified acidic extract of *Arabidopsis leaves* (b), of the formazan of glyoxylic acidic (c), and of the indophenol derivative of ammonia (d) as described in the text.

with 25 µl of water added. Color formation is measured exactly 6 min after the addition of $K_3Fe(CN)_6$ solution because the reaction reaches a plateau after 4 min and the colored reaction product slowly decomposes with time afterward.

For ammonia quantification, the supernatant obtained after charcoal treatment is diluted 1:1 (v/v) in 100 mM HCl. Then 20 µl of this solution is mixed with 100 µl of a 1% (w/v) phenol–0.005% (w/v) sodium nitroprusside solution in water, and 100 µl of a 1% (v/v) sodium hypochlorite–0.5% (w/v) sodium hydroxide solution in water is added. The samples are incubated at 37 °C for 30 min, and light absorption at 620 nm is measured.

To develop the glyoxylate assay, we have recapitulated the original procedure of Dekker and Maitra [10] with varying concentrations of sodium glyoxylate in water. This protocol produces linear standard curves from 0.25 to 200 µM sodium glyoxylate.

We have tested the suitability of the Dekker and Maitra method [10] for plant extracts by quantifying glyoxylate in spiked plant extracts. The resulting internal standard curve is compared with the external standard curve prepared without the addition of plant extract. We observe a marked difference in slope between both curves, indicating the presence of one or more compounds in the plant extract that quench the signal (Fig. 2A). Plant tissues also frequently

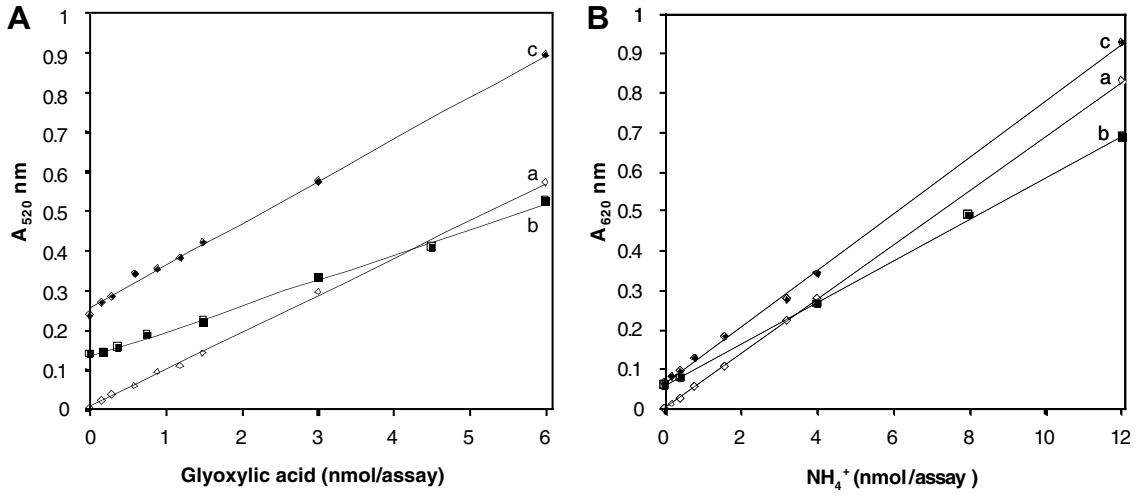

Fig. 2. Comparison of standard curves for glyoxylate (A) and ammonia (B) determination. The external standard curve was prepared in 100 mM HCl (a). The internal standard curves were prepared in crude acidic extract of *Arabidopsis* leaves and were either measured (b) or partially purified and measured (c). The glyoxylate and ammonia contents were determined as described in the text. For all curves, $R^2 > 0.99$.

contain varying amounts of anthocyanins and other pigments that might obscure the specific signal because they absorb light at a wavelength similar to that of the formazan (Fig. 1). When plant tissue is extracted with 100 mM HCl and chloroform (derived from Ref. [15]) and purified with activated charcoal, the coabsorbing compounds are removed (Fig. 1, trace b), as are the unknown interfering compounds without absorbing glyoxylic acid, given that the slope of the internal standard curve fits perfectly to the slope of the external standard curve (Fig. 2A). The shift between both curves represents the glyoxylic acid content in the plant tissue.

To develop the ammonium assay for plant tissue, we have adapted the Berthelot reaction as described in Weatherburn [12] to the 96-well format. The resulting standard curve is linear in the range of 0.01 to 3 mM ammonium. We next extracted finely ground plant tissue with 100 mM HCl and spiked the extract with varying concentrations of ammonium. Although this internal standard curve is linear in the range of 0.01 to 1 mM ammonium, its slope is markedly different from that of the external standard curve, indicating the presence of one or more interfering compounds (Fig. 2B). Husted and coworkers [16] reported that amino acids interfere with the Berthelot reaction when present in high concentration as compared with ammonium. We found that activated charcoal efficiently absorbed most of the amino acids from an aqueous solution (data not shown) This partial purification by charcoal makes the Berthelot reaction suitable for plant extracts. After partial extract purification with chloroform and activated charcoal, the slopes of the internal and external standard curves match, indicating that the level of amino acids and potential other interfering compounds is too low to interfere with the reaction (Fig. 2B). The protocol presented in Ref. [16] requires elaborate instrumentation, whereas the novel extraction and purification protocol presented here allows reliable and efficient quantification of ammonium with a spectrophotometer. However, if this new protocol is to be adapted for different tissues or plant species, we recommend optimizing the relative amounts of extract to activated charcoal to minimize interference as described above.

In summary, we have developed a novel purification protocol for acidic plant extracts in which interfering compounds and coabsorbing compounds were quantitatively removed by chloroform and charcoal extraction. This new method allows the simultaneous determination of two signature metabolites of photorespiration, glyoxylate and ammonium, from the same plant extract. The method has also been adapted to the high-throughput 96-well format and potentially can be robotized, thereby making it suitable for mutant screens.

Acknowledgment

This work was supported by National Science Foundation awards IOB-0548610, MCB-0618335, and MCB-0519740.

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