BIOLOGIA PLANTARUM 53 (1): 129-132, 2009

BRIEF COMMUNICATION

Determination of oxalate in plant tissues with oxalate oxidase prepared from wheat

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Abstract

A method for determination of oxalate with oxalate oxidase (OxO, EC 1.2.3.4) prepared from wheat bran, is based on specific oxidation of oxalate to produce H_2O_2. The H_2O_2 formed was colorimetrically determined using horseradish peroxidase-catalyzed oxidation of 4-aminoantipyrine and N,N-dimethylaniline by H_2O_2. The new method was tested on rice, buckwheat, soybean and oxalis leaves, showing it is precise, sensitive, inexpensive, highly reproducible and simple to perform. Good agreement could be obtained between this method and the HPLC.

Additional key words: ascorbic acid; enzymatic analysis; interference

Oxalic acid is a dicarboxylic acid which commonly exist in plants, animals and microorganisms. Oxalate has been suggested to be involved in seed germination, calcium storage and regulation, ion balance, detoxification, structural strength, and insect repulsion in plants (Horner and Wagner 1995, Lane 1994). Overall, establishing a simple and effective method of oxalate determination is of practical importance.

So far, the methods for determination of oxalate in plant include titration (Baker 1952), capillary electrophoresis (Trevaskis and Trenerry 1996), gas chromatography (Ohkawa 1985), high performance liquid chromatography (HPLC) (Yu *et al*. 2000), and enzymatic analysis (Trevaskis and Trenerry 1996). Most of the procedures are time-consuming and/or require the apparatus not easily available. Enzymic colorimetric determination of oxalate is simple, fast and no special apparatus must be needed, but this method was mainly used in measurement of oxalate in urine, because colour inhibition may be caused by reducing substances, such as ascorbate. Moverover, OxO prepared from mosses, barley seedlings, beet stem and banana fruit peel suffers from interference with Cl^- and NO_3^-. The problem has been overcome by using a Cl^- and NO_3^- insensitive OxO purified from grain, sorghum leaves (Pundir and Satyapal 1998). However, a bulk quantity of the enzyme is required for a large number of clinical samples, which makes it expensive.

This paper reports an enzymatic analysis, in which the OxO was very readily prepared from wheat bran and believed to be highly purified. Such OxO, as naturally immobilized on the cellulose and/or semi-cellulose, is much more stable than ever reported to temperature, pH and storing duration, and is unaffected by 150 mM NaCl and 6 mM $NaNO_3$.

Oxalic acid standard sample (*Sigma*, USA),4-amino-antipyrine (*Fluka*, Switzerland), horseradish peroxidase (POD; *BioLife Science & Technology Co.*, Shanghai, China), OxO (prepared from wheat bran by ourselves and had applied for patent), other chemicals and biochemicals were of the highest grade available unless stated.

The pregerminated seeds of rice (*Oryza sativa* L.) were grown in Kimura B complete nutrient solution (pH 4.8), soybean (*Glycine max* L.) and buckwheat (*Fagopyrum esculentum* Moench) were grown in Hogland nutrient solution about two weeks in greenhouse (average day/night temperature of 30/25 °C night, relative humidity 60 - 80 %, photosynthetically active radiation 600 to 1000 μmol m^{-2} s^{-1} and 14-h photoperiod). The leaves of oxalis were harvested from the wild plants (*Oxalis corniculata* L.).

Fresh samples (0.2 g) were homogenized with 1.6 cm^3 HCl (0.5 M), then heated the homogenate in graduate tubes with boiling water for 20 min and shook the solution several times during heating. After cooling,

Received 16 February 2007, *accepted* 25 August 2007.
Abbreviations: HPLC - high performance liquid chromatography; OxO - oxalate oxidase; POD - peroxidase.
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distilled water was added to the tube until reaching 10 cm^3, then stayed overnight. The next day, about 1 cm^3 homogenates were clarified by centrifugation (15 300 *g*, 10 min) at 4 °C, then was added 0.016 cm^3 NaOH (2 M) accurately to 0.5 cm^3 supernatant, the mixture can be used to determine oxalate content.

First, 0.02 U OxO (about 20 mg dry powder) was added to each tube, followed by adding 0.11 cm^3 distilled water, 0.8 cm^3 colour reagent and 0.04 cm^3 POD (150 U cm^{-3}). 0.05 cm^3 oxalate extracts were added at last to initialize the reaction, and 0.05 cm^3 distilled water was added as the control. After incubation at room temperature for 90 min, the absorbance at 555 nm (A_{555}) was read and content of oxalate was determined from standard curve. The colour reagent consisted of 10 mg 4-aminoantipyrine, 0.025 cm^3 N, N-dimethylaniline per 100 cm^3 125 mM succinate-NaOH buffer which containing 75 % alcohol (v/v), pH 4.0.

To determine standard curve known amount of oxalate was added to the reactive system to initialize the reaction, the concentration of oxalic acid in these samples reached 2, 4, 6, 8, 10 μg cm^{-3}, respectively. When internal standard curve was made, known amount of solution was added to homogenate during trituration, the oxalic acid ultimate concentration in these oxalate extracts reached 0, 40, 80, 120, 160 μg cm^{-3}, respectively, then the oxalate content was determined according to the above procedure and calculating the regression equation of A_{555} *vs*. oxalate content.

For each treatment, data were statistically analyzed using *MS Excel for Windows*, significant difference analysis used Student's *t*-test.

The method addressed here is based on oxidation of oxalate catalysed by OxO to H_2O_2 and CO_2. Then the H_2O_2 is coupled with oxidation and conjugation of 4-aminoantipyrine and N,N-dimethylaniline with catalysis by POD to form chromogen which could be measured colorimetrically by the difference in A_{555} (Zhang *et al*. 1996). A_{555} was read every half hour and it showed no distinct difference between 0.5 and 3 h when 2 μg cm^{-3} oxalate was included in the reaction mixture (Fig. 1). The absorbance levelled off after 1.5 h if there was 10 μg cm^{-3} oxalate in the reaction mixture. It took a longer time to level off if oxalate concentration was increased to 20 μg cm^{-3}, but samples could be properly diluted to make the content lower than 10 μg cm^{-3}. So in practice for measuring samples, 1.5 h reaction time is enough to let the reaction complete.

To test the accuracy of this enzymatic analysis, it was compared with the well-established HPLC method (Yu *et al*. 2000) (Table 1) in terms of measuring oxalate content in leaves of rice with different treatment. The results of oxalic acid by enzymatic analysis (y) were in good agreement with HPLC (x), the regression equation: $y = 1.1216x - 0.1248$, $r^2 = 0.945$, and no significant difference between these two analysis was observed (in each treatment $P > 0.05$).

Firstly, 2, 4, 6, 8, 10 μg oxalate was directly added to the reaction mixture, respectively, after incubation the reaction solution at room temperature for 90 min, A_{555} was read and the regression equation of A_{555} *vs*. oxalate content was calculated (Fig. 2*A*). The results showed the absorbance (y) was linearly correlated with oxalate content (x), the regression equation being $y = 0.0567x + 0.014$, $r^2 = 0.9982$. In addition, the absorbance (y) was also well linear with oxalate content (x) for the internal curves (Fig. 2*B*), the regression equation being $y = 0.0577x - 0.0026$, $r^2 = 0.9998$, its slope was very close to that of the external curve, meaning the oxalate recovery was nearly 100 %.

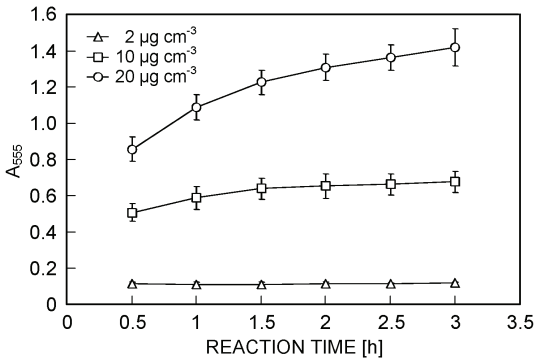

Fig. 1 Dependence of absorbance on reaction time during the enzymatic assay.

An absorbance change of 0.152 corresponded to 16 μM oxalate concentration in this reaction system. A similar commercially available kit was exploited by *Sigma*, 100 μM oxalate was needed to reach the same absorbance (0.15). By enhancing the detecting sensitivity of the spectrophotometer, Ladwig *et al*. (2005) was able to detect as low as 1 μM oxalate. It suggested that the method we described here had a better sensitivity.

The oxalate content was measured in rice, buckwheat, soybean and oxalis leaves by this protocol 20 times, then their standard deviation and coefficient of variation were analyzed. Oxalate contents were: 3.22 ± 0.179 (rice), 4.39 ± 0.163 (buckwheat), 2.11 ± 0.111 (soybean) and 34.56 ± 1.179 mg g^{-1}(f.m.) (*Oxalis*). The coefficients of variation were all less than 6 %. It suggested that this procedure had a good precision and reproducibility.

Known amount of oxalate was added to the tissue samples during oxalate extraction and the oxalate concentration in these samples was determined by this method to obtain percentage of oxalate recovery (Table 2). The mean recovery of the added oxalate was 99.8 %, showing the enzymatic analysis is dependable.

To study the interference of ascorbic acid, 20, 40, 60 μg ascorbic acid was added to 0.13 g rice leaves during oxalate extraction, the results showed that ascorbic acid added had no significant effect on the determinations of oxalate in the leaves of rice, although ascorbic acid inhibit the colour reaction catalyzed by peroxidase if directly included in reaction mixture. It suggested that ascorbic acid may be degraded or inactivated during sample processing.

Table 1. Determination of oxalate [mg g^{-1}(f.m.)] in rice leaves by reversed phase high performance liquid chromatography and the enzymatic analysis. Means ± SE, n = 3.

	1	2	3	4	5	6	7
HPLC method	1.71±0.115	3.74±0.433	4.42±0.548	8.11±0.606	2.74±0.162	8.85±0.092	5.12±0.427
Enzymatic analysis	1.93±0.137	3.46±0.346	5.11±0.051	9.62±0.005	3.11±0.645	8.77±0.183	5.70±0.041

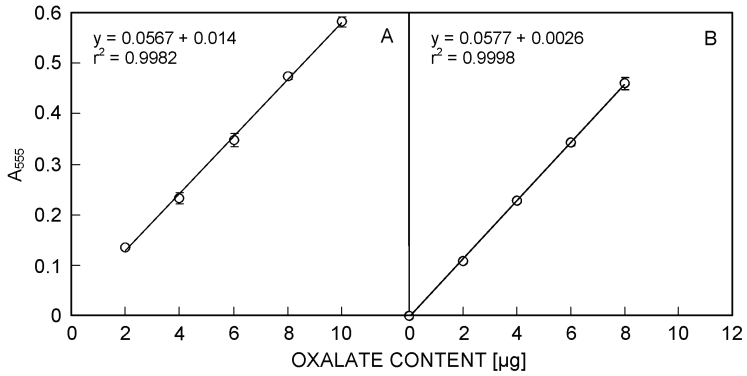

Fig. 2. The external (*A*) and internal (*B*) standard curve of oxalate determined by the enzymatic method.

A wide variety of methods have been proposed for the measurement of oxalic acid, each with its advantages and disadvantages. A major problem was encountered in the enzymatic assay is the interference (Buttery *et al*. 1983). There have been many studies done in the past in order to solve the interference problem (Crider and Curran 1984, Berckmans and Boer 1988, Potezny *et al*. 1983). Interference by urinary ascorbate in both enzymatic and nonenzymatic methods during measuring oxalate is a major impediment to the development of a simple assay for urinary oxalate (Inamdar *et al*. 1991). In the coupled method involving OxO and peroxidase, part of the H_2O_2 generated in the reaction mixture is consumed to oxidize ascorbate to dehydroascorbate, so that not all of the H_2O_2 is available for the peroxidase reaction, leading to underestimation of oxalate. Also, when alkaline conditions are used for oxalate analysis, ascorbate can be converted to oxalate nonenzymatically at pH > 4.0, the higher the pH the more rapid the conversion, leading to overestimation of oxalate (Mazzachi *et al*. 1984, Crawford *et al*. 1985, Wilson and Liedtke 1991). The sample of pH in our experiment was lower than 2 all the time. Moreover, we heated the homogenate with boiling water for 20 min and then stayed overnight during sample processing. So 60 µg ascorbic acid added had no significant effect on the determinations of oxalate, suggesting that ascorbic acid may be degraded or inactivated during sample processing.

Table 2. The recovery of oxalate from the samples. Means ± SE, n = 3.

Oxalate added [µg cm^{-3}]	Found [µg cm^{-3}]	Recovery [%]
40	38.45±0.539	96.1±1.35
80	80.78±1.335	101.0±1.67
120	120.75±3.716	100.6±3.10
160	162.26±7.747	101.4±4.84

Here we used OxO isolated from wheat bran. This enzyme is advantageous over the other source of OxO because of its ready availability, its greater resistance to inhibition by high salt concentrations, and its stability. The procedure could be completed within 2 d, but the system allows for routine handling of many samples. Analytical recovery of oxalate for the whole assay procedure is 99.8 % (Table 2), it suggested that the procedure described here markedly decreases the influence of interfering substances, even ascorbic acid, in the colour development. We have not encountered any interference with the sample in our oxalic acid assay. Moreover, the levels of oxalic acid in rice leaves of different treatment were determined by enzymatic assay were in good agreement with those determined by HPLC, so the method we described here is dependable.

In conclusion, we describe reliable spectrophotometric oxalate assay that is less time-consuming and technically demanding than the assay used previously. Extracting oxalate from plant tissues by 0.5 M HCl and keeping the pH of the sample lower than 2 allows for eliminating ascorbate interference, using OxO which was readily and cheaply prepared from wheat bran makes it inexpensive, and easy to perform in every laboratory for various biomaterials, foods and other materials.

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