Analysis of 3-(*N*-Oxalyl)-L-2,3-diaminopropanoic Acid and Its α-Isomer in Grass Pea (*Lathyrus sativus*) by Capillary Zone Electrophoresis

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A method was developed for the simultaneous quantitative determination of the neurotoxic nonprotein amino acid 3-(*N*-oxalyl)-L-2,3-diaminopropanoic acid (β-ODAP) and its nontoxic α-isomer 2-(*N*-oxalyl)-L-2,3-diaminopropanoic acid (α-ODAP) in seeds of grass pea (*Lathyrus sativus*) by capillary zone electrophoresis (CZE). Samples of 0.5 g of seed flour were extracted in 2 volumes of 10 mL of ethanol–water (6:4) by tumbling for 45 min each time. Hippuric acid was used as an internal standard. The separation was performed in a 50 μm i.d. uncoated capillary at 515 V/cm and 40 °C in 20 mM Na_2HPO_4 buffer at pH 7.8 with direct UV detection at 195 nm. A linear response was recorded in the concentration range 0.015–1.8 mM. This corresponds to a detection limit of 0.1 g kg^{-1}. Comparison between the CZE method and a standard colorimetric method for measuring total ODAP in 38 lines of grass pea gave a coefficient of correlation of 0.994.

Keywords: *3-(N-oxalyl)-L-2,3-diaminopropanoic acid; β-ODAP; BOAA; capillary electrophoresis; Lathyrus sativus*

INTRODUCTION

Grass pea (*Lathyrus sativus*) is an important grain legume in Bangladesh, India, and Ethiopia, where it is a major protein source for people in the lowest income groups (Spencer *et al.*, 1986; Tekle-Haimanot *et al.*, 1993). However, excessive ingestion of this pulse can lead to irreversible paralysis of the legs—a disease known as neurolathyrism (Roy *et al.*, 1989, and papers cited therein). The causative agent for lathyrism is currently believed to be 3-(*N*-oxalyl)-L-2,3-diaminopropanoic acid (β-ODAP) (Roy and Spencer, 1989; Roy *et al.*, 1989). This nonprotein amino acid is also known as β-(*N*-oxalylamino)-L-alanine (BOAA). The α-isomer of ODAP, 2-(*N*-oxalyl)-L-2,3-diaminopropanoic acid, has been shown to be nontoxic to chicks and mice (Wu *et al.*, 1976). Studies conducted by Chase *et al.* (1985) with rat spinal cord also led to the conclusion that the α-isomer is neither acutely nor chronicly toxic.

Lathyrus sativus, *Lathyrus cicera*, and *Lathyrus ochrus* show good adaptation to the low rainfall conditions of southwestern Australia, especially on the fine textured, neutral to alkaline soils (Siddique and Walton, 1992). The development of these species as grain crops for human consumption and as protein-rich feed for monogastric animals necessitates that the toxicity problem be overcome. This can be achieved through either plant breeding or post-harvest detoxification. In either case there is a need for a simple quantitative analytical method by which the β-ODAP content in seeds and other samples can be determined with accuracy and rapidity and at low cost. The method that is most commonly used for screening of plant breeding material at present is the colorimetric method of Rao (1978) and modifications thereof. This method has become widely used because large sample numbers can be handled at a low cost and only standard laboratory equipment is needed. The colorimetric method is approximately 2 orders of magnitude less sensitive than the reversed-phase HPLC method of Kisby *et al.* (1989), where a linear response was found in the concentration range 0.11–5.7 μM. The improved sensitivity was achieved by precolumn derivatization of the amino acid with the fluorophor 9-fluorenylmethyl chloroformate and fluorescence detection. Expensive consumables and specialized equipment are needed for the latter method. Both methods are relatively labor intensive and the two isomeric forms of ODAP are not separated. Separation of α- and β-ODAP was acheived by Khan *et al.* (1993) using reversed-phase HPLC, precolumn derivatization with phenyl isothiocyanate, 3 μm particles of octadecyl silica comprising the column, and a binary gradient mobile phase. This method also was seen as time-consuming, and consequently it was considered desirable to develop a method for determination of α- and β-ODAP using capillary zone electrophoresis (CZE) and direct UV detection. The method presented here is equally as sensitive as the above-mentioned colorimetric method and provides a simple, fast, inexpensive, and accurate alternative to existing methods for seed screening purposes.

EXPERIMENTAL PROCEDURES

Chemicals. All buffer and standard solutions used for capillary electrophoresis were made from double glass distilled water. For all other purposes distilled water was used. All chemicals were of analytical grade. 3-(*N*-Oxalyl)-L-2,3-diaminopropanoic acid (β-ODAP), hippuric acid, DL-2,3-diaminopropionic acid (DAP), L-homoarginine, and *o*-phthaldialdehyde (OPA) were obtained from Sigma (St. Louis, MO). L-Asparagine (L-Asn) and other protein amino acids were from BDH Chemicals Ltd. (Poole, U.K.).

Plant Material. ICARDA accessions of *L. sativus* (29 lines), *L. cicera* (2 lines), and *L. ochrus* (2 lines) were provided

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by K. H. M. Siddique, Department of Agriculture, South Perth, WA., Australia. Four lines of *L. sativus* were received from C. G. Campbell, Agriculture Canada Research Station, Morden, MB, Canada. The seeds were ground to a flour using a Tecator Cyclotec 1093 sample mill with a 1 mm screen.

Sample Extraction. The flour (0.5 g) was extracted in 2 volumes of 10 mL of ethanol–water (6:4) by tumbling in capped plastic vials (14 mm i.d. × 10 cm) for 45 min each time. Hippuric acid (12.84 mM), which was predissolved in dimethyl sulfoxide (DMSO, 3% v/v of final volume) was used as internal standard, and 200 μL of solution was added to the flour prior to the first extraction. The tubes were vortexed before tumbling. The extracts were centrifuged at 1400*g* for 10 min. The pooled extracts were filtered prior to CZE analysis using Gelman Nylon Acrodisc syringe filters (0.45 μm).

Capillary Zone Electrophoresis. The capillary electrophoresis (CE) instrument (Hewlett-Packard 3DCE) was equipped with a diode array detector and was operated via the HP 3DCE ChemStation software. The capillary (HP G1600-60211) of uncoated fused silica had the dimensions 48.5 cm × 50 μm and an effective length of 40 cm. On-column UV detection was performed at 195 nm.

The analyses were performed at a constant voltage of 25 kV at 40 °C in an electrolyte of 20 mM Na_2HPO_4 buffer at pH 7.8. The capillary was conditioned prior to each run by flushing it with 0.1 M NaOH for 2 min and with the electrolyte for 3 min. The electrolyte was replenished every third run. Filtered seed extracts were injected for 4–12 s at 25 mbar depending on the concentration of the extract.

Spectrophotometry. Seed extracts were analyzed for α- and β-ODAP according to the spectrophotometric method of Rao (1978) as modified by C. G. Campbell (1993, Agriculture Canada, personal communication). For each sample four solutions (A_1 to A_4) were prepared as described in the following. Hydrolysates were prepared by boiling 2 mL of seed extract in 4 mL of 3 M KOH for 30 min. Centrifuged hydrolysates (0.25 mL) were pipetted into 0.75 mL of water, and for the A_3 solution 2 mL of OPA reagent (100 mg of OPA, 200 μL of mercaptoethanol, 1 mL of absolute ethanol, 99 mL of 0.05 M $Na_2B_4O_7$, pH 9.9) was added, whereas 2 mL of 0.05 M $Na_2B_4O_7$, pH 9.9, was added for the A_4 solution. Likewise, another pair of tubes was prepared with unhydrolyzed extract to correct for any background absorbance (A_1 with OPA reagent, A_2 without OPA reagent). The solutions were kept at 40 °C for 2 h, and the absorbance was read at 425 nm. The formula $(A_3 - A_4) - (A_1 - A_2)/3$ was used for the calculation of the absorbance change that was ascribed to ODAP. A standard curve based on dilutions of a DAP stock solution (2.89 mM in ethanol–water, 6:4) was used for the quantification.

RESULTS AND DISCUSSION

Separation Conditions. Separation parameters were varied to obtain acceptable resolution of analytes at the shortest run time possible. The pH of the electrolyte was critical for the quality of the electropherograms. Tailing of the β-ODAP peak was observed at pH 8.0 and above, whereas fronting became pronounced at pH 7.7 and below. However, in a narrow range around pH 7.8 an acceptable peak shape was obtained. Fast separations could be obtained with buffer concentrations ranging from 20 to 25 mM. The low buffering capacity implied, however, that frequent replenishment of the buffer was necessary, and the buffer was exchanged every third run on a routine basis. This did not pose a problem as a feature of the Hewlett-Packard CE is that buffer can be automatically replenished from a reservoir during the caustic conditioning phase of each sample run. This practice also ensures migration times are reproducible between runs. Splitting of peaks was occasionally observed with a phosphate concentration of 20 mM when the amount of analytes injected was too high. This could be overcome by decreasing the injection volume or by increasing the buffer concentration or by a combination of both. The choice of the capillary temperature (40 °C) was a compromise between shorter migration times with increasing temperature on the one hand and distortion of peaks becoming significant at temperatures above 40 °C on the other. A typical electropherogram of a sample obtained under the separation conditions chosen is shown in Figure 1. The numbers of theoretical plates for the peaks corresponding to hippuric acid and α- and β-ODAP were 334 000, 271 000, and 159 000, respectively.

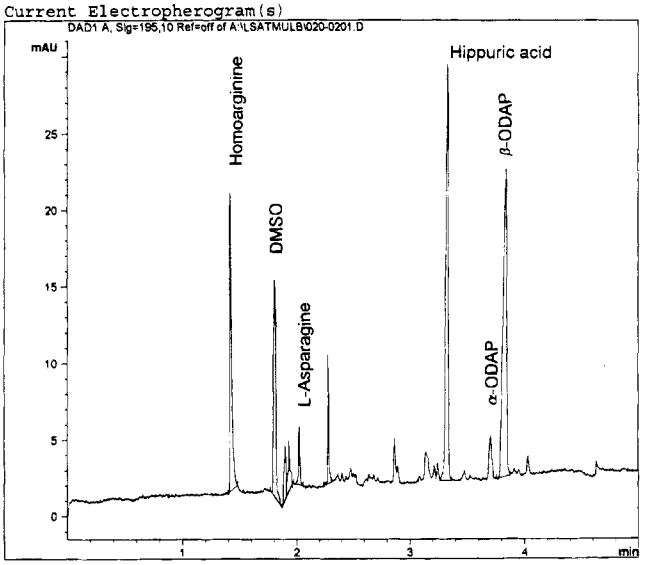

Figure 1. Electropherogram of a crude ethanol extract of *L. sativus* obtained by capillary zone electrophoresis (40 cm × 50 μm i.d., 20 mM phosphate buffer, pH 7.8, 25 kV, 40 °C, 195 nm).

Peak Identification. According to literature information interconversion between α- and β-ODAP takes place in solution (Abegaz *et al.*, 1993). CZE analyses of fresh standard solutions of β-ODAP showed one major peak with a migration time (Mt_{rel}) of 1.18 relative to that of the internal standard (hippuric acid $Mt_{rel} = 1$) and a minor peak moving slightly more quickly ($Mt_{rel} = 1.13$). The area of the latter accounted for 2.6% of the combined area of the two (Figure 2a). When the solution was kept at 55 °C for 3 days, the ratio of the minor to the major peak changed to 34:66 (Figure 2b). As the equilibrium concentration between α- and β-ODAP has been reported to be in the ratio of 30:70 at room temperature and 40:60 at 55–60 °C (Abegaz *et al.*, 1993; De Bruyn *et al.*, 1993), the minor peak was assigned to α-ODAP and the major peak to β-ODAP.

Other peaks in the electropherograms of *L. sativus* seed extracts were identified by spiking and by comparing the peak spectra generated by the diode array of the CE instrument. The fastest migrating peak was assigned to homoarginine (Figure 1). This compound was not fully separated from arginine, which had a slightly higher migration time. The peak corresponding to DMSO, which was used for solubilization of the internal standard, was observed at the beginning of the solvent front and can be used as a neutral marker. The peak corresponding to L-asparagine appeared immediately after the solvent front, and this amino acid was one of the minor constituents of the extract (Figure 1). The dominating compounds in the aqueous ethanol extracts of *L. sativus* seeds were β-ODAP and homoarginine, which is in agreement with literature information (Lambein *et al.*, 1992).

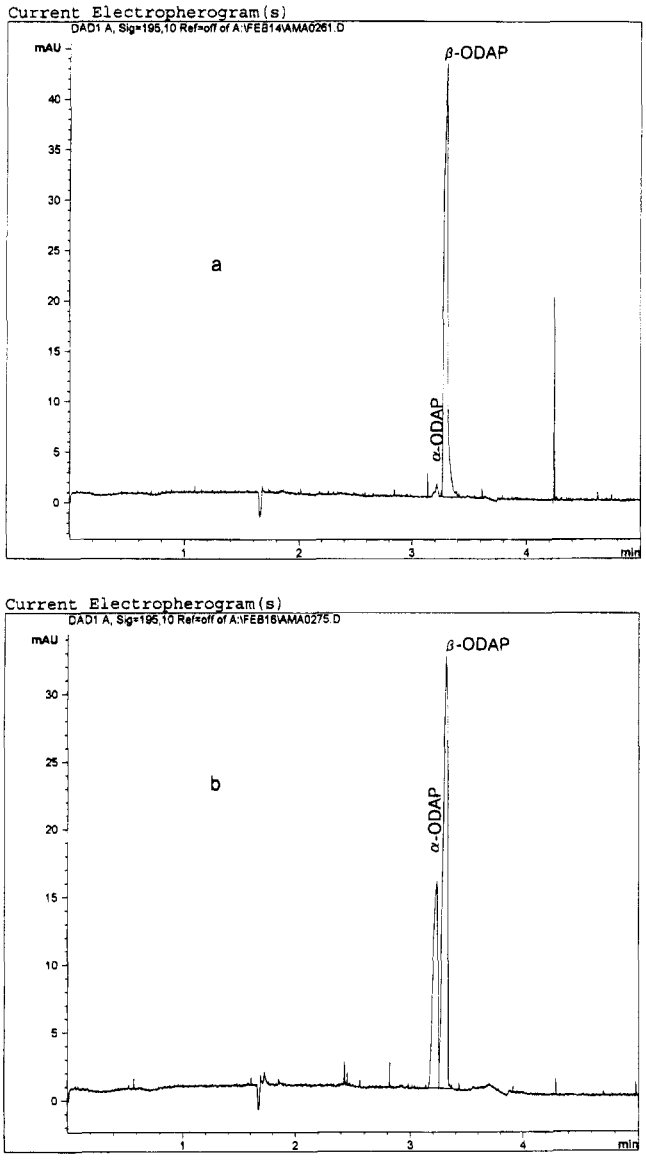

Figure 2. CZE electropherograms of β-ODAP standard solutions that were (a) freshly prepared or (b) heated at 55 °C for 3 days.

Extraction Procedure. The extraction efficiency of α- and β-ODAP was determined by three repeated extractions of the same seed sample. In the first extract 86% (n = 3) of total ODAP was recovered. This yield was confirmed in another series of measurements and the coefficient of variation (CV) was 4% (n = 10). The extraction yield of the first and second extracts in combination was 98% (n = 3).

Internal Standard. Compounds with suitable charge to mass ratios were tested in the search for an internal standard. Hippuric acid was the compound that best met the criteria of an internal standard. It is not a natural component in legume seeds, it is stable, it appears in the electropherogram as a symmetrical peak, and it has a migration time different from those of major constituents in the extract. However, it does coelute with a minor peak, which in the genotypes analyzed constituted up to about 2% of the hippuric acid peak area under the described separation conditions. Hippuric acid was added to the seed flour prior to extraction, and it was quantitatively extracted with 2 volumes of 10 mL of 60% ethanol–water. The relative standard deviation (rsd) for the combined content of α- and β-ODAP in a line of *L. sativus* with an ODAP level of 5

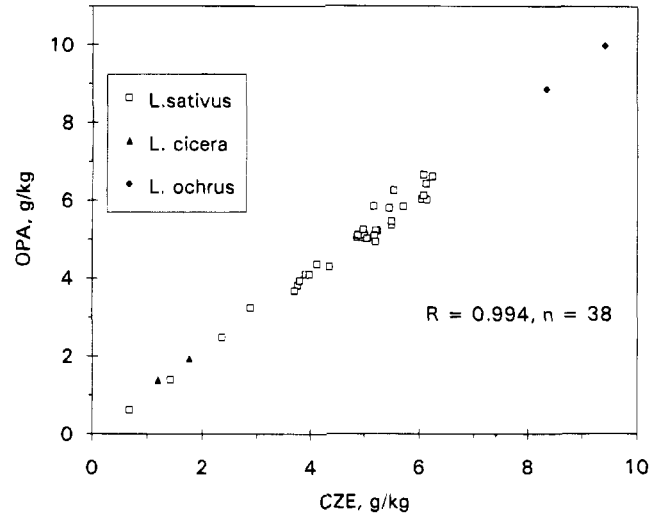

Figure 3. Comparison between the CZE and OPA method for measuring total ODAP (grams per kilogram) in seeds of selected lines of *L. sativus*, *L. cicera*, and *L. ochrus*.

g kg^{-1} was 3.2% (n = 10 extracts). The rsd for repeated ODAP analyses of the same extract was 1.3% (n = 10 injections).

Linearity. A linear relationship between the concentration of β-ODAP and the corresponding peak area was found in the concentration range 2.7–320 mg L^{-1} (0.015–1.8 mM). An ODAP concentration of 2.7 mg L^{-1} approached the limit of detection, and when a solvent to mass ratio of 40 mL/g of seed was applied for the extraction, this level corresponds to 0.1 g kg^{-1} β-ODAP in the plant material. A linear increase in the peak area of β-ODAP was observed when increasing volumes of a standard solution of β-ODAP was injected by application of pressure in the range 100–500 mbar.s. Accordingly, improvements of the detection limit can be obtained by increasing the injection volume or by using extended light path capillaries.

Stability. The stability of ODAP in aqueous ethanol was demonstrated by leaving eight extracts at room temperatures exceeding 25 °C for 3 days. The total concentration of α- and β-ODAP was unchanged, but the percentage of α-ODAP increased by 5% on average. In fresh seed extracts α-ODAP typically constituted 6–11% of the total ODAP concentration.

Method Verification. The validity of the results obtained by the CZE method was verified by an established method. Thus, for selected samples the same extracts were analyzed for α- plus β-ODAP using the colorimetric method of Rao (1978, with Campbell's modifications, OPA method) as well as the CZE method (Figure 3). The total concentration of ODAP in the material analyzed ranged from 1 to 10 g kg^{-1} and the coefficient of correlation (r) between methods was 0.994 (n = 38).

Conclusion. Baseline separation of α- and β-ODAP was obtained by the CZE method developed. This is important because the β-ODAP is considered toxic, whereas α-ODAP is so far known to be inocuous. The between-batch precision (rsd 3.2%) and within-batch precision (rsd 1.3%) of the CZE method have been shown. The CZE method has also been validated against an established colorimetric procedure. Crude aqueous ethanol extracts were injected directly into the CZE system, making sample preparation and overall analysis a simple and fast procedure. The autosampler facility makes it possible to perform analyses un-

attended, and with total run times of 9 min per analysis, large sample numbers can be handled. Inexpensive aqueous buffers and capillaries were used for the procedure, keeping the costs of operation low. The detection limit of 0.1 g kg^{-1}, although sufficient for our breeding program, can be improved 5 times by using extended light path capillaries and increasing the injection volume. Even greater sensitivity should be possible using fluorescent labeling (Wu and Dovichi, 1992; Albin *et al.*, 1991). In summary, CZE provides a means for reliable, simple, fast, and cheap analyses for α- and β-ODAP in plant breeding material.

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