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Analytical Methods Section

Quantitative analysis of linamarin in cassava using a cassava β-glucosidase electrode

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A simple protocol was developed for the quantitative analysis of linamarin in cassava roots using a cassava β-glucosidase (linamarase) electrode. A steady state value could be obtained within 15–20 min and the limit of detection was 0·1 mM linamarin. The linamarin content of 18 cassava varieties determined by this procedure ranged from 24 to 395 mg per 100 g fr. wt. root. These values were comparable to those obtained using a spectrophotometric method. The enzyme electrode method is reliable and simple to perform. Furthermore, it does not require the use of hazardous chemicals like pyridine which is needed by the spectrophotometric procedure.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is widely cultivated in the tropics, primarily for the storage roots, though the young leaves are also eaten (Lancaster *et al.*, 1982). Both the leaves and edible roots of these plants contain linamarin, a cyanogenic glycoside (Wood, 1966; Nartey, 1968). When the plant tissue is damaged, the linamarin is readily hydrolysed by the endogenous linamarase (β-glucosidase), resulting in the release of cyanide, which is the primary cause of toxicity in the plant. As cassava roots represent an important food and energy source in the developing countries, there is great interest among cassava growers and breeders to screen their germplasm collection for varieties with low linamarin (cyanide) content. Varieties with high linamarin content are also sought after as they tend to show greater resistance to pests and diseases.

Various methods of linamarin determination in cassava incorporate the use of linamarase or sulphuric acid to hydrolyse the linamarin, followed by spectrophotometric determination of the cyanide released (Cooke, 1978; Nambisan & Sundaresan, 1984; Bradbury *et al.*, 1991). These methods require the additions of many reagents, including the hazardous chemical pyridine, and are time-consuming. Recently, the construction of a cassava β-glucosidase electrode for linamarin determination was reported (Yeoh, 1992). The procedure seemed rapid and simple. Such an electrode would be useful for laboratories and industries dealing with cassava. Thus, it is worth assessing its potential as an analytical tool for the quantitative determination of linamarin (cyanide) in cassava roots.

In this paper, we describe a protocol for the use of the cassava β-glucosidase electrode for the quantitative analysis of linamarin in cassava. We report the linamarin content for 18 cassava varieties using this procedure, and compare the data against those obtained with a spectrophotometric method (Nambisan & Sundaresan, 1984).

MATERIALS AND METHODS

Plant materials

Cassava varieties used in this study come from the cassava germplasm collection of the Philippines Root Crop Research and Training Centre, Visayas State College of Agriculture, Philippines. Plants were grown in a spacing of 0·5 × 0·5 m² and roots of 10-month-old plants were used. Because of the longitudinal and

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radial linamarin gradients in the roots, and variation in linamarin content among roots from the same plant (Cooke, 1978; Bradbury *et al.*, 1991), we have consistently sampled only the mid-region of the roots (two roots per plant) from at least two plants for all our analyses.

Preparation of cassava extract

All steps were carried out at room temperature which ranged from 30 to 35°C. The mid-region of freshly harvested cassava root (parenchyma tissue, 10 g) was homogenised in 0·1 M *o*-phosphoric acid for 3 min. The ratio of root (g) to extraction medium (ml) was 1:5 w/v. For cassava varieties known to have high linamarin content, 1:10 w/v root to extraction medium ratio could also be used. The homogenate was filtered through Whatman No. 2 filter paper and 10 ml of the filtrate was then adjusted to pH 9·5 with NaOH. To maintain minimum dilution to the cassava extract (less than 10%), we used 3–4 M NaOH, followed by 1 M NaOH for pH adjustment. For enzymatic/spectrophotometric determination of linamarin, the cassava extract was adjusted to pH 6.

Preparation of enzyme electrode

The preparation of cassava leaf linamarase, its immobilisation in photocrosslinkable prepolymer resins (ENT-4000) and the set-up of the enzyme electrode were carried out as previously describe (Yeoh, 1989, 1992). Although the enzyme membrane could be reused, for this work a new enzyme membrane was used for each determination. Since the enzyme membranes were stored at 4°C, they were allowed to equilibrate to room temperature before use. Used enzyme membranes were soaked in 100 mM Na citrate buffer, pH 6, for washing and reuse (Yeoh, 1992). The sensing element of the cyanide selective electrode was kept shiny by polishing it daily after use.

Potentiometric measurements

An aliquot (100 μl) of the cassava extract (pH 9·5) was pipetted onto the surface of the enzyme membrane placed over the sensing element of the cyanide selective electrode. The reference electrode was then brought into contact with the sample. A Corning pH/ion meter 150 was set to record mV readings at 5 min intervals. As the enzyme electrode response took 15–20 min to reach steady state, we used the 20 min mV reading for all calculations.

Linamarin standards (0·1 mM to 20 mM) prepared in the 0·1 M *o*-phosphoric acid adjusted to pH 9·5 with NaOH were used to calibrate the enzyme electrode. Potentiometric measurements were carried out as described for the cassava extract. We would like to emphasise that since the activity of cyanide ion depends on the ionic strength and nature of the medium, it is important that both the linamarin standards and the cassava extract be prepared with the same medium.

Spectrophotometric determination of linamarin

The cassava extract (pH 6) was incubated with 0·1 unit of linamarase for 15 min at room temperature, followed by detection of the cyanide with chloramine T and barbituric acid–pyridine reagent, according to the procedure of Nambisan and Sundaresan (1984). In using this procedure, it would be more relevant to use the calibration curve based on linamarin rather than on cyanide. The linamarase used was prepared from cassava leaf (Yeoh, 1989).

RESULTS AND DISCUSSION

Cassava varieties were selected from the cassava germplasm collection of the Philippines Root Crop Research and Training Centre based on available information on the qualitative cyanide level in these varieties as obtained by the rapid screening method of Bradbury and Egan (1992). Selection was aimed at providing us with samples representing the range of cyanide level found in the germplasm collection. Table 1 shows the linamarin content for 18 cassava varieties analysed by the enzyme electrode procedure and by a spectrophotometric method (Nambisan & Sundaresan, 1984). We have also included in Table 1 the qualitative level of linamarin content (indicated as low, medium or high in terms of cyanide released) for the varieties as determined by the rapid screening method of Bradbury and Egan (1992).

The linamarin content for 18 cassava varieties as determined by the enzyme electrode method ranged from 24 to 395 mg per 100 g fr. wt. root. This is equivalent to 2·5 to 41·5 mg cyanide per 100 g fr. wt. root. The values obtained were comparable to those obtained by the spectrophotometric procedure. The latter ranged from 27 mg to 350 mg per 100 g fr. wt. root. It was also observed for several varieties, such as PRC 443, PRC 480, PRC 319, PRC 398, PRC 368 and PRC 60a, that the values for linamarin content determined by the spectrophotometric method were more varied compared to values obtained by the enzyme electrode procedure (Table 1). In this respect, the enzyme electrode method appeared more reliable than the spectrophotometric method. Overall, however, there is good correlation between both methods of analysis. The analyses also revealed that variations in linamarin content among the roots from the same plant and from plants of the same variety were a common occurrence, as previously reported by other workers (Bradbury *et al.*, 1991).

The results also showed good correlation between the qualitative estimate of cyanide level and the actual linamarin content as determined by the enzyme electrode method. Low or medium cyanide varieties gave linamarin contents less than 52 mg per 100 g fr. wt.

Table 1. Linamarin contents of cassava roots[a]

Cassava variety		Linamarin/cyanide assay methods			
Accession number	Local name	Rapid assay	Enzyme electrode		Spectrophotometric
		Cyanide release	Linamarin (cyanide equiv.)		Linamarin
			(mg per 100 g fr. wt. root (mean ± SD))		
PRC 377	K31	low	24·3 ± 7·0	(2·5 ± 0·7)	26·7 ± 7·6
PRC 443	C-CS-341	medium	24·9 ± 4·7	(2·6 ± 0·5)	29·5 ± 19·0
PRC 476	C-CS-1984	low	25·7 ± 3·4	(2·7 ± 0·4)	34·3 ± 6·7
PRC 365	K 12	low	34·3 ± 14·9	(3·6 ± 1·6)	44·7 ± 2·8
PRC 480	C-CS-2583	low	35·7 ± 17·4	(3·7 ± 1·8)	36·2 ± 24·7
PRC 319	—	medium	41·4 ± 10·9	(4·3 ± 1·1)	40·9 ± 25·7
PRC 379	K 34	low	50·0 ± 21·2	(5·2 ± 2·2)	56·2 ± 27·6 (n=3)
PRC 446	C-CS-354	low	51·2 ± 28·5	(5·4 ± 3·0)	56·2 ± 26·6
PRC 398	C-CS-37	medium	51·5 ± 29·1	(5·4 ± 3·0)	42·8 ± 37·1
PRC 522	Bilu-bilu	high	64·0 ± 6·5	(6·7 ± 0·7)	80·9 ± 9·5
PRC 380	K 35	low	68·8 ± 25·5	(7·2 ± 2·7)	87·6 ± 39·9
PRC 518	Amarilto 1	high	72·4 ± 40·3	(7·6 ± 4·2)	115·2 ± 54·2
PRC 462	C-CS-461	low	80·0 ± 43·7	(8·4 ± 4·6)	120·9 ± 26·6
PRC 368	K15	high	93·3 ± 50·4	(9·8 ± 5·3)	118·0 ± 87·6
PRC 450	C-CS-379	high	123·6 ± 89·1	(13·0 ± 9·4)	129·5 ± 1·9
PRC 60a	Java Brown 2	high	216·3 ± 131·4	(22·7 ± 13·8)	195·2 ± 138·9
PRC 329	—	high	349·3 ± 139·6	(36·7 ± 14·6)	323·8 (n=2)
PRC 2	Rayong 3	high	395·4 ± 154·9	(41·5 ± 16·3)	350·0 (n=2)

[a] Number of samples analysed, n, ranged from 4 to 8 roots except where indicated otherwise.

root, this being equivalent to 5·4 mg CN^- per 100 g fr. wt. root. Two varieties, PRC 380 and PRC 462, regarded as low cyanide varieties, however, were found to contain linamarin levels (69 mg and 80 mg per 100 g fr. wt. root, respectively) which were within the range of values for the high cyanide varieties, the range being 64 mg to 395 mg linamarin per 100 g fr. wt. roots. As both quantitative methods of linamarin analysis gave similar levels of linamarin for these two varieties, it was unlikely that the quantitative value would be due to an error. Thus is might be important that qualitative information should at some stage be supported by quantitative data.

The protocol developed for the enzyme electrode assay of linamarin involved extraction of cassava root in *o*-phosphoric acid, filtering the extract, adjusting the pH of the extract to 9·5 and taking the potentiometric readings of cassava extract. Each analysis took about 20 min and we could easily carry out 16 to 18 analyses per day, which was roughly the same number as we could handle using the spectrophotometric method.

The main advantage of the enzyme electrode method is the simplicity of the procedure. Compared to the various spectrophotometric methods of linamarin (cyanide) determination (Cooke, 1978; Nambisan & Sundaresan, 1984; Bradbury *et al.*, 1991), the enzyme electrode analysis of linamarin involves less preparation of reagents and avoids the use of pyridine. With the spectrophotometric method, it is necessary to prepare several dilutions of the cassava extract so that the final absorbance reading falls within the linear portion of the standard curve. For example, with 16 samples we need to prepare at least 48 samples, assuming three dilutions for each sample, for the determination. Thus, in our experience we found the spectrophotometric procedure tedious and error prone.

The analyses are reliable and reproducible. However, there are two points worth noting. First, both the cassava extract and linamarin standards must be prepared in the same medium. We found that 0·1 M borate–NaOH, pH 9·5, as employed by Yeoh (1992) to prepare the linamarin standards, gave a lower electrode response compared to the linamarin standards prepared in 0·1 M *o*-phosphoric acid, adjusted to pH 9·5. Second, the enzyme membrane usually stored at 4°C must be allowed to equilibrate to room temperature prior to use. On the day of use, enzyme membranes could be left at room temperature (30–35°C) for the whole day without any loss in enzyme activity. This is because the immobilised enzyme is very stable (Yeoh, 1991).

As previously reported, the enzyme electrode is easy to construct (Yeoh, 1992). With photocrosslinkable prepolymer resins (ENT-4000), a sheet of 6 × 6 cm^2 enzyme membrane could be prepared within 15 min without need of sophisticated instrumentation. Thirty-eight membrane disks of 9 mm diameter could be cut out from a membrane of this size. Enzyme membranes could be reused many times and have been stored in Na citrate buffer, pH 6, at 4°C for as long as 2 years with negligible loss in enzyme activity. In using the enzyme electrode procedure, the only investment is the cyanide selective electrode and its reference electrode, or a combination cyanide electrode since most laboratories would possess a pH meter with absolute mV measurement capability. The cost of the electrode is certainly cheaper than purchasing a reasonably good spectrophotometer. With fewer reagents used, there will be savings in costs of consumables.

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