Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 107 (2008) 576–579
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical Methods

Initial evaluation of a field-friendly incubation procedure for the colorimetric assay of cassava *linamarase*

Gerard M. O'Brien [a,*], Angel SH Ip [b]

[a] *Northern Ireland Centre for Food and Health (NICHE), University of Ulster, Cromore Road, Coleraine BT52 1SA, Northern Ireland, United Kingdom*
[b] *Department of Obstetrics and Gynaecology, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, The New Territories, Hong Kong*

Received 12 January 2007; received in revised form 29 June 2007; accepted 15 August 2007

Abstract

The Cooke colorimetric assay of cassava *linamarase* activity is temperature- and time-inflexible, making "real time" monitoring of *linamarase* activity in remote cassava-processing sites practically impossible. A modified incubation procedure is described, in which the 30 °C *linamarase* incubation step is terminated through acidification, yielding a stable cyanohydrin solution. Using partially purified *linamarase* as a "standard extract", the solution – held for up to 21 days at ambient/refrigeration temperatures before colorimetry – showed reductions of up to 21% compared with the standard Cooke assay. In a separate trial, a strong linear relationship ($r^2 > 0.95$) was observed between recorded *linamarase* activity values and incubation temperature in the 25–40 °C range, indicating that incubation may take place in remote processing sites without a water bath, and resulting data may reliably be adjusted in keeping with the standard 30 °C incubation assay. The novel procedure thus appears to offer a satisfactory "field-friendly" means of assaying *linamarase* activity.

Keywords: Cassava; *Linamarase*; Cyanogens; Cyanide; Cyanogenic potential; Assay; Colorimetric; Quantitative; Field-friendly; Remote site

1. Introduction

The tropical root crop cassava (*Manihot esculenta* Crantz) originated in the Americas, and is particularly suitable for subsistence farmers throughout the tropics, being drought and pest resistant, and able to grow in low-fertility soils (Cock, 1985).

Cassava root parenchyma contains variable amounts of cyanogenic glucoside (mainly linamarin), lending it a cyanogenic potential (CNP), depending on the cultivar, ranging from below 10 mg kg^{-1} to over 500 mg kg^{-1}HCN on a fresh weight basis (O'Brien, Wheatley, Iglesias, & Poulter, 1994). While low-CNP cultivars do not pose a significant toxicity risk, high-CNP cultivars require extensive processing, during which the glucoside is hydrolysed, *via* an intermediate cyanohydrin compound, to HCN, mainly under the hydrolytic action of the endogenous enzyme *linamarase* (O'Brien, Mbome, Taylor, & Poulter, 1992).

As more than 20-fold variability in root parenchymal *linamarase* activity has been reported among roots from different cultivars, with no apparent significant correlation between CNP and *linamarase* activity (Bradbury & Egan, 1994; O'Brien et al., 1994), both CNP and *linamarase* activity are important.

The assay of cassava *linamarase* activity, first developed by Cooke in the late 1970s, involves the incubation (pH 6/30 min/30 °C) of parenchymal extract with a known concentration of linamarin solution, followed by pH-induced release of HCN, which is measured colorimetrically (Cooke, 1979; Essers, Bosweld, Van der Grift, & Voragen, 1993). The assay requires that cassava samples be brought to the laboratory; hence in-field monitoring of *linamarase* activity is not possible. Therefore, *linamarase* activity in many cassava processing situations cannot be measured using this reliable method.

* Corresponding author. Tel.: +44 28 7032 4156; fax: +44 28 7032 4965.
E-mail address: gm.obrien@ulster.ac.uk (G.M. O'Brien).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.08.039

The modified incubation methodology presented here is aimed at enabling *linamarase* activity to be sampled at any location, and then quantified using the Cooke colorimetric assay.

2. Materials and methods

2.1. Chemical reagents

Partially-purified freeze-dried cassava *linamarase* (EC 3.2.1.21) preparation was a gift from CIAT (Centro Internacional de Agricultura Tropical – Cali, Colombia). Chloramine T (GPR grade) was obtained from BDH Ltd (Poole, UK); isonicotinic acid (purum grade) and 1,3-dimethylbarbituric acid (puriss.grade) were obtained from Fluka Chemie AG (Buchs, Switzerland); linamarin was obtained from Toronto Research Chemicals (North York, Canada); potassium cyanide (ACS grade) was obtained from Riedel-De Haën (Seeize, Germany). All other reagents used were of analytical grade.

2.2. Preparation of solutions

Phosphate buffers (pH 3.0 and 6.0) were prepared using 0.1 M H_3PO_4, adjusted with 5 M NaOH. Barbiturate/isonicotinate (BI) reagent was prepared as described by Essers et al. (1993): 1,3-dimethylbarbituric acid (7.0 g) and isonicotinic acid (5.7 g) were dissolved in 0.46 M NaOH solution (200 ml), and pH adjusted to 7.5. Chloramine T reagent was prepared daily: 2 g chloramine T in 100 ml distilled water. Batches of *linamarase* preparation (8–9 EU/ml activity) were made up in phosphate buffer (pH 6.0). Before each trial, *linamarase* preparation was diluted (1/1000 by volume) in phosphate buffer (pH 6.0).

2.3. Calibration standards and tests

The colorimetric procedure used in this paper was calibrated using KCN standards, originating from KCN stock solution (0.25 g/l in 0.2 M NaOH). A series of test tubes was produced, each holding 0.25 μg–2.5 μg KCN (0.1–1.0 μg HCN equiv.) in 4 ml of phosphate buffer (pH 6.0). The mixtures underwent colorimetric assay and the equivalent absorbance of 1 μg HCN was calculated.

2.4. Measurement of linamarase enzyme activity, using the Cooke standard incubation procedure

Note: partially-purified cassava *linamarase* preparation was used throughout as a proxy for parenchymal *linamarase* extracts.

A 0.1-ml aliquot of dilute (1/1000) *linamarase* preparation was added to 0.5 ml of linamarin solution (5 mM in pH 6.0 phosphate buffer) in a test tube. The mixture underwent incubation at 30 °C for 30 min. To end the incubation (and convert cyanohydrin to CN^-), a 0.6 ml aliquot of 0.2 M NaOH was added. After 5 min, phosphate buffer (pH 6.0, 2.8 ml) was added, bringing the total volume to 4.0 ml.

Chloramine T solution (0.1 ml) was added, and the test tube was placed in an ice–water bath for 5 min. BI reagent (0.6 ml) was then added, and the tube was left for another 10 min for colour development to take place. 9 (Essers et al., 1993).

The activity of the enzyme preparation was calculated from its absorbance (A_{605}). A specimen calculation is detailed below:

Adjusted A_{605} value for enzyme = 0.750.
The equivalent value for 1 μg HCN = 0.876.
Hence, the equivalent amount of HCN present is: (0.750/0.876) = 0.856 μg.
Hence there were 0.032 μmol HCN present (MW of HCN is 27.02).

Thus, 0.032 μmol linamarin was hydrolysed by 0.1 ml of enzyme preparation (diluted by 1/1000) during an incubation period of 30 min.

Hence, the activity of 1 ml of the enzyme preparation, in its original undiluted form (measured in enzyme units [EU] – *i.e.*, μmol/minute/ml) would be:

$$\frac{(10 \times 1000 \times 0.032)}{30} = 10.67 \text{ EU}$$

2.5. Testing of modified procedures for in-field linamarase assay

The standard Cooke protocol was varied in two ways: incubation temperature (Experiment A); and post-incubation chemistry/subsequent storage (Experiment B), and the respective steps are detailed in Table 1.

2.6. Statistical analysis of data

All assays were performed either in quadruplicate (Experiment A – all assays; Experiment B – 'control' and Day 0 assay) or in duplicate (Experiment B – all storage assays). For each assay, both the arithmetic mean value (x) and the standard error ($\sigma_{n-1}/\sqrt{n}$) were calculated (Glantz, 1997). The assay results obtained were compared graphically, and linear regression models (Glantz, 1997) produced, to represent the apparent effects of incubation temperature on reported activity. Microsoft Excel was used for all analyses.

3. Results and discussion

3.1. Experiment A – Assay with ambient temperature incubation

The range of incubation temperatures tested (25–40 °C) represents much of the variation in daytime temperatures within the tropics, where cassava cultivation and processing takes place.

Table 1
Ways in which the *linamarase* assay protocol was varied in two separate experiments (Cooke standard procedure used as a 'control' in both experiments)

Standard Cooke *linamarase* assay ('control' in trials)	Experiment A: *Linamarase* assay with ambient temperature incubation	Experiment B: *Linamarase* assay with in-field incubation & storage of mixture
5 mM linamarin in phosphate buffer pH 6.0 (0.5 ml)	5 mM linamarin in phosphate buffer pH 6.0 (0.5 ml)	5 mM linamarin in phosphate buffer pH 6.0 (0.5 ml)
pH 6.0 root parenchyma extract* (0.1 ml)	pH 6.0 root parenchyma extract* (0.1 ml)	pH 6.0 root parenchyma extract* (0.1 ml)
30 min incubation at 30 °C	30 min incubation at 25/ 30/ 35/40 °C	30 min incubation at 30 °C
0.2 M NaOH (0.6 ml) (leave for 5 min)	0.2 M NaOH (0.6 ml) (leave for 5 min)	0.2 M H_3PO_4 (0.4 ml) (storage at 4 °C or ambient for up to 21 days)
		0.2 M NaOH (1.2 ml) (leave for 5 min)
Phosphate buffer pH 6.0 (2.8 ml)	Phosphate buffer pH 6.0 (2.8 ml)	Phosphate buffer pH 3.0 (1.8 ml)
Colorimetric procedure	Colorimetric procedure	Colorimetric procedure

* Note: For purposes of these experiments, partially-purified *linamarase* extracts from root cortex (7–12 EU/ml), diluted 1:1000 in pH 6.0 buffer, were used.

As seen in Fig. 1, the results of all three trials appear to indicate, within the 25–40°C range, an acceptably linear relationship ($r^2 \approx 0.95$ and above) between incubation temperature and reported *linamarase* activity. This indicates that the potential exists for in-field incubation at ambient temperature, without a thermostatically-controlled water bath, with subsequent arithmetical adjustment of the reported value to a 30 °C-equivalent "true value".

While the results from trials 1 and 2 produced virtually identical linear equations, the *linamarase* extract used in trial 3 clearly was of higher activity than in the other trials, and a somewhat different linear equation emerged. Further repetitions of the trials would hopefully provide important information regarding the extent to which the linear equation may be expected to vary.

3.2. Experiment B – Assay with in-field incubation/storage of mixture

To facilitate *linamarase* assay at a remote field site, there has to be some means of stabilising the mixture immediately after the incubation step. The methodology tested in Experiment B differed from the standard Cooke protocol, including an acidification step instead of alkali-addition, in order to (i) denature the *linamarase* enzyme, and (ii) stabilise the cyanohydrin intermediate produced *via* the enzyme's catalytic action.

As shown in Fig. 2, all reported activity values obtained with the modified in-field protocol were lower than those obtained with the laboratory-based Cooke protocol (note: the abnormally low value for Day 14/refrigerated storage apparently arose from pipetting error, and should be ignored). Indeed, even on Day 0, when the stabilised mixture did not undergo storage, and colorimetry took place straight after incubation, the in-field protocol yielded a reported activity that was around 6% lower (8.16 EU/ml) than that yielded by the standard Cooke protocol (8.70 EU/ml). This is not surprising: a small proportion of the cyanohydrin produced during the 30 minute incubation period at pH 6.0 would be expected to undergo spontaneous hydrolysis to HCN (Fomunyam, Adegbola, & Oke, 1985). With the Cooke protocol, the addition of 0.2 M NaOH to stop the incubation will, as well as hydrolysing the cyanohydrin present to HCN,conserve the small

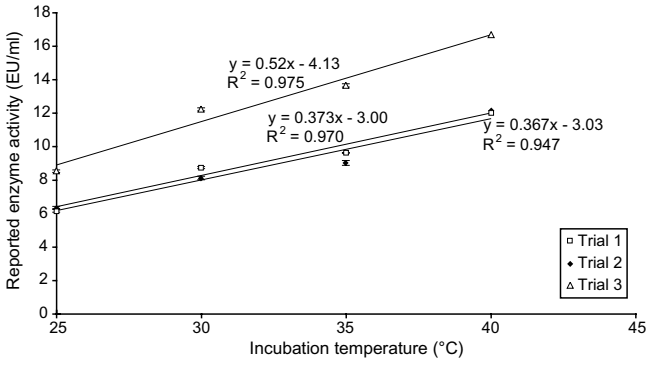

Fig. 1. *Linamarase* assay – effect of varied incubation temperature on reported activity (all assays quadruplicate; standard error around mean values shown).

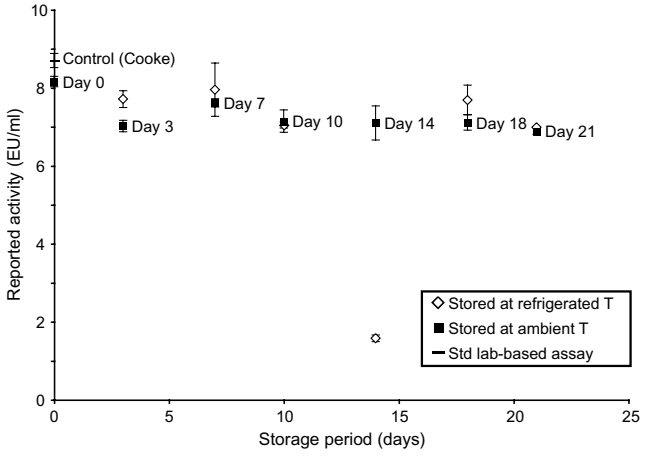

Fig. 2. Modified in-field *linamarase* assay: reported activity after storage of stabilised mixtures (Cooke and Day 0 assays quadruplicate, all others duplicate; standard error around mean values shown).

amount of HCN already present until colorimetry. In contrast, with the modified in-field protocol, the addition of 0.2 M H_3PO_4 to stop the incubation will, as well as stabilising the cyanohydrin present (for hydrolysis to HCN later, after storage), result in the loss of any HCN already present, through volatilisation.

After Day 0, reported activity values obtained with the modified in-field protocol dropped by up to 16% relative to the Day 0 value (up to 21% relative to the Cooke protocol value) over the 21-day storage period (Fig. 2), with no significant difference, apart from on Day 3, between ambient temperature storage and refrigeration temperature storage of the stabilised mixture.

This indicates that the modified incubation procedure has the potential to provide a reliable time-flexible means to assay for cassava *linamarase* activity in remote field sites located up to 21 days' travelling distance from a laboratory (provided that an adjustment factor and error margin are taken into account), and that refrigerated storage of the stabilised mixtures does not appear necessary.

In a spirit of caution, however, it is worth noting that the ambient storage temperatures that were involved in this experiment were in the temperate range of around 18–22 °C. Further repetitions of the trials at tropical ambient temperatures would provide information more precisely tailored to cassava-growing countries.

4. Conclusion

The feasibility of a reliable *linamarase* assay featuring incubation at tropical ambient temperatures, removing the need for a thermostatically-controlled water bath, appears likely in the 25–40 °C range, given the linearity observed in the relationship between incubation temperature and reported activity. Further studies should focus on building up more data, with a view to deriving a reliable predictive equation.

The feasibility of an assay protocol that would permit sampling and incubation in a field site far removed from the nearest laboratory, with completion of the assay some weeks later, also appears assured, with *linamarase* activity values losing no more than 21% of their expected value over a 21-day period, even without refrigerated storage. Again, more data should be gathered through further trials, with storage trials focusing more on tropical (rather than temperate) ambient temperature storage.

It is hoped that, after further exploration, both of the above-mentioned innovations may be combined in one "field-friendly" protocol for the assay of *linamarase* activity in remote field sites.

Acknowledgements

The authors wish to thank Dr. Archie McKinley (University of Ulster) for valuable statistical advice, and Dr. Hernán Ceballos and Mrs. Terésa Sánchez of CIAT (Cali, Colombia), for kindly preparing and supplying lyophilised cassava *linamarase*.

References

Bradbury, H. J., & Egan, S. V. (1994). Improved methods of analysis for cyanide in cassava and screening for low cyanide varieties in the Pacific. *Acta Horticulturae (ISHS), 380*, 237–242http://www.actahort.org/books/380/380_37.htm.

Cock, J. (1985). *Cassava: New potential for a neglected crop*. Boulder, USA: Westview Press.

Cooke, R. D. (1979). Enzymatic assay for determining the cyanide content of cassava and cassava products. Cali, Colombia: Centro Internacional de Agricultura Tropical 05EC-6.

Essers, A. J. A., Bosweld, M., Van der Grift, R. M., & Voragen, A. G. J. (1993). Studies on the quantification of specific cyanogens in cassava products and introduction of a new chromogen. *Journal of the Science of Food and Agriculture, 63*, 287–296.

Fomunyam, R. T., Adegbola, A. A., & Oke, O. L. (1985). The stability of cyanohydrins. *Food Chemistry, 17*, 221–225.

Glantz, S. A. (1997). *Primer of biostatistics* (4th ed.). New York, USA: McGraw-Hill.

O'Brien, G. M., Mbome, L., Taylor, A. J., & Poulter, N. H. (1992). Variations in cyanogen content of cassava during village processing in Cameroon. *Food Chemistry, 44*, 131–136.

O'Brien, G. M., Wheatley, C. C., Iglesias, C. I., & Poulter, N. H. (1994). Evaluation, modification and comparison of two rapid assays for cyanogens in cassava. *Journal of the Science of Food and Agriculture, 65*, 391–399.