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# Preparation of linamarase solution from cassava latex for use in the cassava cyanide kit

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**Abstract**

A simple method is described for the preparation of linamarase from cassava latex (sap). Latex, obtained from the end of the petiole (stalk) of cassava leaves, is mixed with water and the solution filtered to give a crude solution of enzyme. This solution may be stored indefinitely in the deep freeze. It may be used without any purification in kits for determination of cyanogens in cassava tubers and cassava products (flour, gari, etc). Immobilisation of linamarase in filter paper discs in the presence of a stabiliser (gelatin/PVP-10) reduces its activity to 25% and in the absence of stabiliser to only 4%. © 1999 Elsevier Science Ltd. All rights reserved.

## 1. Introduction

The exposure of some 600 million people in the tropics, for whom cassava is a staple food, to the potentially deleterious health effects due to the cyanide that it contains, is a serious public health problem. In order to measure the extent of the problem, we developed a simple picrate method that may be used by non-chemists in developing countries to monitor their own cassava roots and cassava products such as flour and gari (Bradbury, Egan & Bradbury, 1999; Egan, Yeoh & Bradbury, 1998). The picrate kit method was shown (Djazuli & Bradbury, 1999; Egan et al., 1998) to give comparable results to an intrinsically more accurate acid hydrolysis method (Bradbury, Bradbury & Egan, 1994; Bradbury, Egan, & Lynch, 1991), which like all the other 'accurate' methods is too difficult and/or too expensive to use and/or requires equipment that is not readily available in most developing countries. For routine measurements of total cyanogen content or of the amounts of the various cyanogens present (linamarin, acetone cyanohydrin and cyanide) the simple picrate method is quite adequate. This has been shown by results obtained in studies in Mozambique in 1996 (Cardoso, Ernesto, Cliff, Egan & Bradbury, 1998) and 1997 (Ernesto, Cardoso, Cliff & Bradbury, 1999) and in Indonesia (Djazuli & Bradbury 1999). These studies have shown mean total cyanogen levels of about 50 ppm in cassava flour (the WHO safe level is 10 ppm; FAO/WHO, 1991) with 5–10% of cases having levels of 100–200 ppm. These simple picrate kits prepared in our laboratory, are available free of charge to workers in developing countries, funded by the Australian Centre for International Agricultural Research (ACIAR).

The simple picrate kit method uses the enzyme linamarase (which is immobilised in a small filter paper disc) to catalyse the hydrolysis of linamarin to acetone cyanohydrin, which then hydrolyses in slightly alkaline conditions to acetone and HCN, that reacts with the picrate paper. The linamarase may be obtained commercially, but is normally too expensive for those working in developing countries. Other methods used for the preparation of linamarase (Cooke, Blake & Battershill, 1978; Eksittikul & Chulavanatol, 1988; Mpkong, Yan, Chism & Sayre, 1990; Wood, 1966; Yeoh, 1989) require equipment that may not be available in laboratories in developing countries. A simple method has been developed using cassava leaves that requires no special equipment, and uses a phenyl Sepharose short column to partially purify the linamarase (Yeoh, Bradbury & Egan, 1997).

Nambisan (1999), has recently developed a method based on the use of cassava latex. In this paper we report a simplification of her method for the preparation of

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linamarase solution, that can either be used directly as solution or immobilised in filter paper discs for use in the picrate kit method.

## 2. Materials and methods

Linamarin and guanidine hydrochloride were obtained from the Sigma Chemical Co. Leaves and latex of cassava plants were obtained from the Plant Culture Centre at the Australian National University. About 10–50 mature leaves and petioles were broken off from the main branches of the cassava plant. Latex on the end of each stalk (petiole) was collected in a small weighed bottle. The amount of latex varied from one variety to another and also varied with the time of the year. The weight of the latex was determined and an amount of distilled water was added on the basis of about 10 ml water for each 0.1 g of latex. In early work, 0.1 M phosphate buffer at pH 6.0 was used instead of distilled water. The latex plus water was stirred to dissolve linamarase, allowed to stand for 5 min and filtered through Whatman No 1 filter paper. In two cases (cvs TMS 91934 and TMS 30572), the leaves attached to the stalks from which the latex had been removed were cut into small pieces with scissors and ground in a pestle and mortar in 5 ml 0.1 M phosphate buffer at pH 6/g leaf together with 50 mg polyvinylpolypyrollidone/g leaf. After 30 min the slurry was filtered through a Whatman No 1 filter paper. The crude enzyme preparations from cassava latex and leaves were stored in the deep freeze at −20°C until assayed.

### 2.1. Linamarase assay

Aliquots (0, 2, 5, 10, 20 and 40 μL) of the crude cassava latex enzyme solution were transferred to small, flat bottomed plastic vials (25 mm diam, 50 mm high) and 0.5 ml of 0.1 M phosphate buffer at pH 6 was added to each vial. A small, square filter paper previously loaded with 40 μg HCN equivalent of linamarin (Bradbury et al., 1999) was added to one vial followed by a picrate paper (prepared previously by the method of Bradbury et al., 1999) and the vial was immediately closed with a screw cap. The vial was left at 30°C for exactly 15 min and then 200 mg of guanidine hydrochloride was added to the solution to inactivate the linamarase. The vial was immediately closed again and the guanidine hydrochloride dissolved by careful circular movement of the vial and kept at 30°C for about 3 h to allow complete reaction of HCN in the vial with the picrate paper. The picrate paper was then removed from the vial, separated from the plastic backing strip and the picrate paper was immersed with occasional shaking in 5 ml of distilled water for about 30 min. This process was repeated for the other five experiments. The absorbances of each of the solutions was measured at 510 nm against a yellow blank produced from the first vial, which contained no added enzyme. The amount of HCN evolved in μg from each experiment was obtained by multiplying the absorbances by 39.6 (Bradbury et al., 1999). The crude enzyme extract from cassava latex was tested for linamarin using the picrate method, with a negative result.

A graph was made of the amount of HCN (μg) liberated in 15 min against the volume of enzyme added (μL) which was linear up to 20 μl enzyme (Fig. 1), and usually showed slight downward curvature at larger amounts of enzyme used (40 μl and above). The activity of crude enzyme solutions prepared from cassava leaf was very much lower than that from cassava latex, hence amounts of solution (0, 40, 80, 160, 500 μl) were needed in order to obtain appreciable absorbances, see Fig. 2.

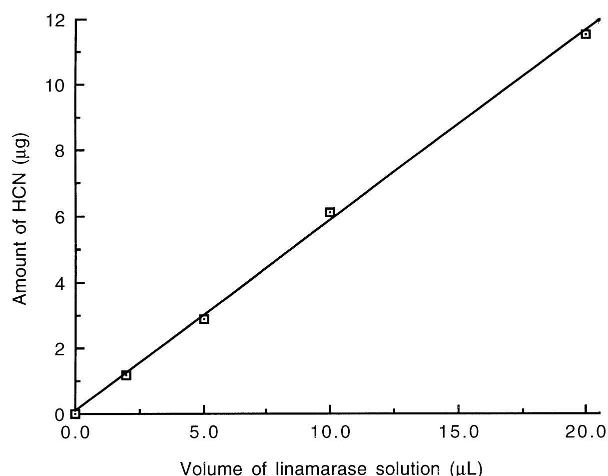


Fig. 1. Graph of amount of HCN (μg) produced from 40 μg linamarin in 15 min against the volume of linamarase solution (μl) produced from cassava latex of cv TMS 91934, ($r^2 = 0.999$).

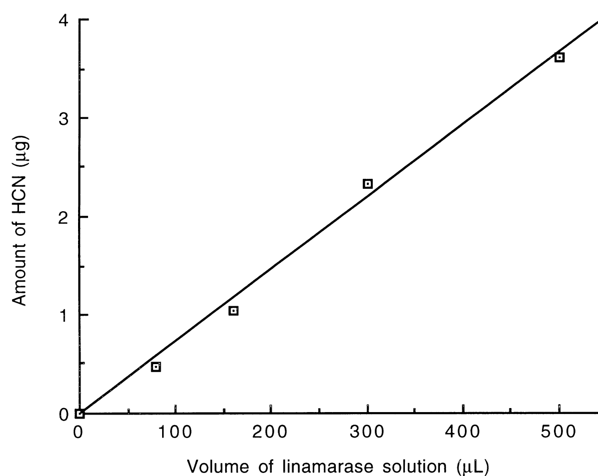


Fig. 2. Graph of amount of HCN (μg) produced from 40 μg linamarin in 15 min against the volume of linamarase solution (μl) produced from cassava leaves of cv TMS 91934, ($r^2 = 0.997$).

The gradient ($g_r$) of the line was obtained from the graphs shown in Figs. 1 and 2 and the number of enzyme units (U) per μl of enzyme solution was calculated by the equation

$$\text{Activity of enzyme solution (U/μl solution)} = g_r/27 \times t \quad (1)$$

where $t$ = time in minutes over which the linamarase was active (15 min) and one enzyme unit (U) is defined as the amount of linamarase required to hydrolyse one micromole of linamarin (liberating one micromole of HCN) in 1 min. The number of enzyme units (U) per g latex (or leaves) was calculated by the equation

$$\text{Activity of latex (U/g latex)} = \frac{g_r \times \text{vol water used (μl)}}{27 \times t \times w} \quad (2)$$

where $w$ = weight of latex (or leaves) in g.

### 2.2. Immobilisation of linamarase in small filter paper discs

Whatman 3 MM, 21 mm diameter filter paper discs were loaded with 0, 5, 10, 20, 40, and 80 μl of linamarase solution. The same linamarase solution was mixed with an equal volume of a solution containing 2% (w/v) gelatin and 10% (w/v) polyvinylpyrollidone-10. This second solution was also loaded on to Whatman 3 MM filter paper discs using the same volumes as above. The papers were allowed to dry overnight and then were used (as described above using enzyme solution) to determine the enzyme activity. Both sets of papers were also used (with 40 μg HCN equivalents of linamarin added) to determine their ability to liberate fully the HCN from linamarin in 16 h at 30°C.

## 3. Results and discussion

The results of the enzyme assays on linamarase solutions from latex and from leaves are given in Table 1. For cv TMS 91934 it is seen that extraction of latex using pH 6 phosphate buffer gave the same result within experimental error as using distilled water, hence buffer solution was discontinued. Another comparison showed that latex from tender young leaves of M Col 1468 gave about the same linamarase activity as from mature leaves. The results of two experiments on latex and leaves from the same leaves and stalks, show that the activity of linamarase solution prepared from 1 g latex is on average about 1600 times greater than the activity of linamarase solution prepared from 1 g of leaves.

Table 1
Linamarase activities of crude enzyme solution from cassava latex and leaves

| Cassava cultivar | Activity of latex solution (U/ml) | Activity of latex solution (U/g latex) | Activity of latex from one leaf (U/leaf) | Activity of leaf solution (U/g leaf) |
|---|---|---|---|---|
| TMS 63397 | 5.1 | 510 | 0.18 | – |
| M Aus 7 | 3.9 | 390 | 0.27 | – |
| SM 1-150 | 3.7 | 370 | 0.29 | – |
| TMS 60506 | 2.5 | 250 | 0.86 | – |
| M Col 1468[a] | 1.9 | 190 | 0.33 | – |
| M Col 1468[bc] | 1.6 | 160 | 0.22 | – |
| TMS 4(2)1425 | 1.6 | 160 | 1.1 | – |
| TMS 91934 | 1.2 | 120 | 1.8 | – |
| TMS 91934[c] | 1.5 | 150 | 1.9 | – |
| TMS 71693 | 1.1 | 110 | 0.46 | – |
| TMS 50395 | 1.1 | 110 | 0.85 | – |
| TMS 30572 | 0.98 | 98 | 1.2 | – |
| TMS 91934[b] | – | – | – | 0.085 |
| TMS 91934[d] | 1.4 | 140 | 1.7 | 0.081 |
| TMS 30572[d] | 1.3 | 130 | 0.74 | 0.091 |

[a] Mature leaves were used.
[b] Tender young leaves were used.
[c] Extraction with pH 6 phosphate buffer instead of distilled water.
[d] Latex and leaves from the same stalks and leaves were used.

Nambisan (1999) estimates the linamarase activity from latex to be about 300 times that of linamarase from leaf and rind (root cortex). The linamarase activities of the crude enzyme solutions from latex (col 2 of Table 1) are of the same order of magnitude as those previously obtained from cassava leaves after a partial purification procedure (Table 1 of Yeoh et al., 1997). Furthermore, the range of enzyme activities from leaves of different cultivars in previous work was nearly 1000 fold, which meant that the method was not useful with leaves from certain cultivars such as M Col 1468. However, in the method described here, there is only a five fold range of linamarase activity, which means that all cultivars may be used.

### 3.1. Preparation and stability of linamarase/buffer filter paper discs for cyanide kits

Currently two kits are available. Kit A for determination of total cyanogens in cassava roots and kit B for determination of all forms of cyanogens (linamarin, acetone cyanohydrin and HCN/CN-) in cassava products. Both of these kits contain 21 mm diameter Whatman 3MM filter paper discs loaded with pH 8 phosphate buffer and linamarase (Egan et al., 1998). There is a loss of linamarase activity due to immobilisation in the Whatman 3MM filter paper matrix which may be mitigated by mixing the enzyme with a gelatin/polyvinylpyrrolidone-10 solution, prior to applying it to

the filter paper (Egan et al., 1998; Yeoh, Lim & Woo, 1996). In order to check the extent of this loss, Whatman 3MM filter paper discs were loaded with various known amounts of enzyme solutions, some of which had been premixed with an equal volume of gelatin/PVP-10 solution (see experimental). The linamarase activity of the enzyme solution was then compared with that of the linamarase immobilised in the filter paper discs. The results are shown in Fig. 3. The activity of the linamarase solution given by the gradient in graph 1 in Fig. 3, is reduced when the linamarase is mixed with stabiliser and immobilised in filter paper (graph 2) and even further reduced when the stabiliser is absent (graph 3). If the enzyme activity of the solution is set at 100, then the relative activities obtained from graphs 2 and 3 are 25 and 4, respectively. Thus, the enzyme activity of the linamarase immobilised in filter paper is 25% in the presence and only 4% in the absence of stabiliser.

The stability of linamarase immobilised with stabiliser in Whatman 3MM filter paper discs and stored at room temperature (about 20°C) over a period of 7 months was checked for six different samples using 40 μg HCN equivalents of linamarin (see experimental). There was no loss of linamarase activity of filter paper discs after 7 months storage at room temperature, which confirms the stability of linamarase immobilised in filter paper.

### 3.2. *Methodology for preparation of linamarase from cassava latex and use in cyanide kits*

About 10–50 mature leaves and stalks were collected from the cassava plant. A drop of sap (latex) was collected from the end of each stalk in a small weighed bottle. The weight of the latex was determined and an amount of water was added equal to 10 ml water per 0.1 g latex. The solution was stirred and after 5 min was filtered through Whatman No 1 filter paper. The crude enzyme solution was stable indefinitely in the deep freeze at −20°C, but only for one week at 2–4°C and it lost 12% of its activity after one week at 20–30°C in the dark.

In laboratory measurements using the kits for determination of cyanogens in cassava roots and cassava products it is recommended that the linamarase solution be used directly. The volume of enzyme solution needed was determined by adding 5, 10, 20, and 40 μl aliquots of enzyme solution to 0.5 ml water buffered at pH 8 with a square paper containing 40 μg HCN equivalents of linamarin. A picrate paper was added, the bottle closed and after 16 h at room temperature the picrate paper was removed and added to 5 ml of water. After 30 min the absorbance (A) of the solution was measured at 510 nm against a yellow blank solution produced from an unreacted picrate paper. The amount of HCN in μg was equal to 39.6 A (Bradbury et al., 1999). If, for example, the results with 5, 10, 20,and 40 μl enzyme were 31, 40, 42 and 41 μg HCN, then clearly 10 μl linamarase would be sufficient. If the linamarase were to be used in field experiments that required the use of linamarase immobilised in Whatman 3MM filter paper discs, then it would be necessary to stabilise the enzyme using gelatin/PVP-10 (see above). The detailed methodology for this procedure is given in a kit (Kit C) which describes the preparation of linamarase from cassava latex and is available free to workers in developing countries.

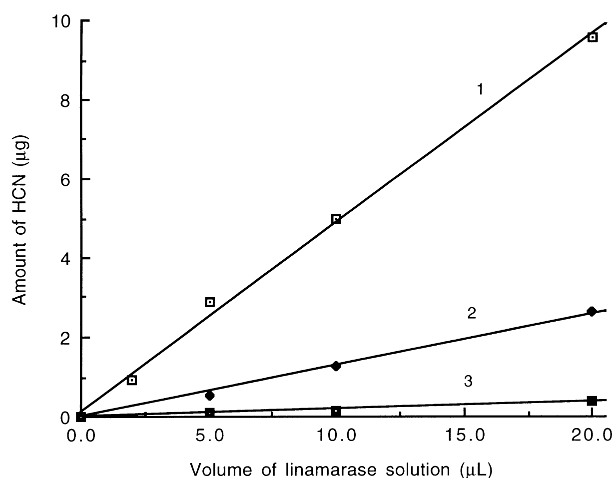


Fig. 3. Graphs of amount of HCN (μg) produced from 40 μg HCN equivalents of linamarin in 15 min against (1) the volume of linamarase solution (latex prepared from TMS 30572), (2) the volume of linamarase solution (plus an equal volume of gelatin/PVP-10) immobilised in filter paper and (3) the volume of linamarase solution immobilised in filter paper.

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