J. Natn. Sci. Coun. Sri Lanka 1974 **2** (1) : 57--65.

Cyanide Liberation from Linamarin

E. R. JANSZ, E. E. JEYARAJ, NIRMALA PIERIS* AND D. J. ABEYRATNE

Ceylon Institute of Scientific and Industrial Research (CISIR), P. O. Box 787, Colombo 7, Sri Lanka.

(*Paper accepted : 29 April* 1974)

Abstract : Several plant materials were tested for cyanide liberating capacity. Two of these materials were able to liberate cyanide from purified linamarin. However, the mechanism of liberation appears to be dissimilar to that of the linamarase of manioc. Although ginger cannot liberate significant amounts of cyanide from purified linamarin, some samples have been found to release it from boiled manioc. In this case too this effect does not appear to be due to a "manioc-type" linamarase. Coliforms cannot liberate HCN from linamarin. A reputed antidote for manioc poisoning, guava leaf extract, contains a potent linamarase inhibitor. Further details on acid and enzymic hydrolysis of linamarin are also reported.

1. Introduction

Linamarin is one of a group of compounds known as the cyanogenic glucosides (Figure 1). These compounds occur widely in plants, notably in manioc, *Manihot esculenta* Crantz. Linamarin (R_1, $R_2 = CH_3$) is the major cyanogenic glucoside of manioc, comprising 93 % of the total cyanogenic glucosides,[4] the rest being made up by lotaustralin ($R_1 = CH_3$, $R_2 = C_2H_5$).

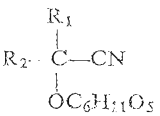

FIGURE 1.

Linamarin on hydrolysis produces hydrogen cyanide ; this reaction is of interest in connection with : (1) methods of assay of total cyanide and (2) the fate of linamarin after ingestion.

Assay of linamarin is usually performed after acid hydrolysis or enzymic hydrolysis (linamarase). Some studies on the assay of linamarin have been previously communicated by this group.[2,7,8] This paper presents further results using model systems.

Processed manioc contains considerable quantities of cyanogenic glucosides and it has been shown that in some instances a lethal dose of cyanide can be produced if all the linamarin ingested is hydrolysed to HCN. [7] It has been suggested that uncooked vegetables,[6] gut flora[5] or even intestinal enzymes[1] can bring about this reaction. This

*The studies on boiled manioc form a part of a M.Sc. dissertation (University of Sri Lanka, Colombo Campus) of Nirmala Pieris.

paper includes a preliminary survey of the cyanide liberating capacity of some plant materials which are generally consumed in a raw or semi-cooked state, where the method of preparation may not be expected to destroy all enzyme activity.

2. Experimental

2.1. Preparation of material

Linamarin and linamarase were prepared by the method of Wood.[11] Boiled manioc was prepared as described previously.[7] Vegetable extracts (from 10 to 15 g fresh weight) were prepared either by homogenisation in a Waring blendor or by grinding in a mortar with aqueous 0.1M citrate buffer at approximately tissue pH.

2.2 Acid hydrolysis of linamarin

Linamarin (30 to 40 mg) was steam distilled with acid of the appropriate normality keeping the volume of the distilled fluid unchanged. Total time for the operation was 90 min within which 200 ml distillate was collected in 25 ml of 2.0% NaOH. Residual linamarin was determined by adjusting the pH to 5, adding 300 units of linamarase and 10 ml of citrate buffer (0.2M, pH 5.0) followed by incubating for 18 h at room temperature. The HCN formed was distilled and collected as described above.

2.3. Assays

Linamarin, linamarase and HCN were assayed as described previously.[7,10,11]

3. Results

3.1. Studies with model systems

The basic method of Wood,[11] modified as reported previously,[7] for studying the linamarase-linamarin enzyme system by direct determination of HCN in the reaction mixture was tried out. The standard error was within 6%. Typical time-course curves for the reaction at two substrate concentrations which are (a) limiting and (b) sufficient to saturate the enzyme are shown in Figure 2.

The method has been applied in this study to test the effect of vegetable proteins on linamarase activity and the effect of some bacteria on linamarin. It has not been more widely used because, unless the material under consideration is freed of sugars and other small molecules which interfere with the picrate test, blanks are unacceptably high. For this reason hydrogen cyanide liberation from crude extracts was always assayed after distillation into Na_2CO_3.[7]

3.2. Acid hydrolysis

Results of these studies have been reported earlier.[2] More detailed studies have since revealed that the yield of HCN by hydrolysis of linamarin by linamarase was quantitative (100% recovery) and that the 83% yield reported in that paper[2] for the enzyme reaction was due to an error in the standard curve caused by effect of NaOH

on the picrate-cyanide colour reaction. Due to this the values for acid hydrolysis increase proportionately ; the best yield by acid hydrolysis being brought about by 7N H_2SO_4 which gives a yield of 68 %. It should, however, be noted that the use of lower acid concentrations results in considerable quantities of residual glucoside. Better overall yields might be obtained by increasing the reaction time when using the lower acid concentrations.

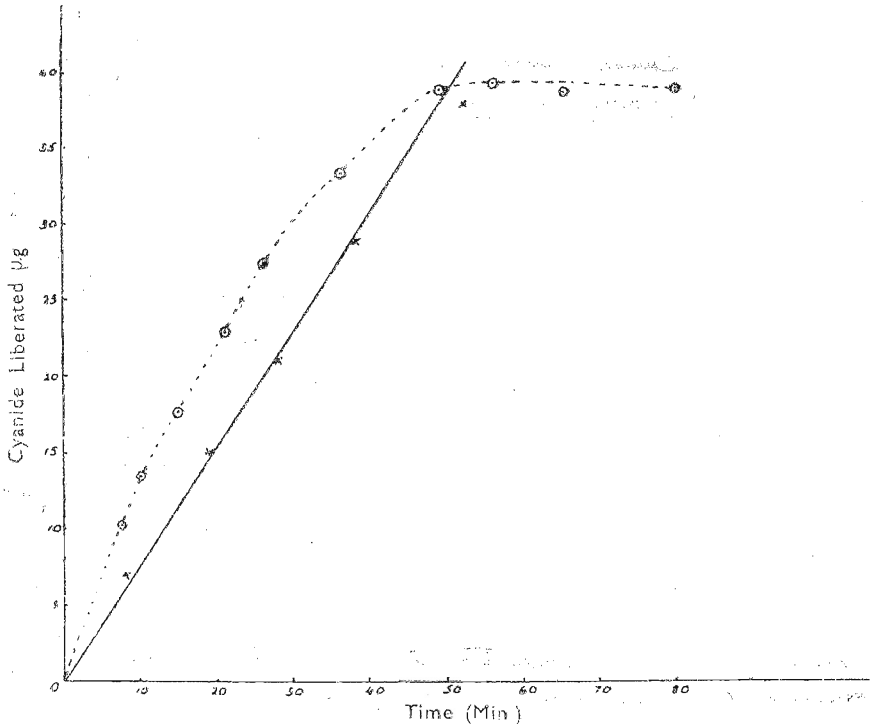

FIGURE 2. Liberation of cyanide from linamarin.

The reaction mixtures contained:

(*a*) 0——0 linamarin, 3.5 mg ; linamarase, 15 units ;

(*b*) x——x linamarin, 5.0 mg ; linamarase, 4 units ;

in a reaction volume of 5 ml, 0.08 M with respect to citrate buffer (pH 5.0).

Aliquots used for assay were 0.5 and 1 ml respectively.

3.3. Effect of vegetable juices on linamarin

Seventeen vegetables, chosen because they are commonly eaten raw, or semi-cooked, or because they are in some way associated with manioc consumption, were tested for cyanide liberating capacity. In a preliminary set of results (Table 1), it was found that only in 4 cases was cyanide liberated from linamarin. The results also show that in

many of the 17 cases it was possible to account for all the linamarin added (within 10%) either as cyanide or residual linamarin. Deviations were observed with ginger rhizome, carrot and karapincha (positive deviations) and guava leaf, red chillies and betel (negative deviations). Ginger, betel and red chillies gave high blanks and hence the deviations were not surprising. In all the other 14 cases, blanks were in the order of 2 to 10% total optical density reading.

TABLE 1. Cyanide liberation from linamarin by plant material.

Tissue	Name of species	Cyanide liberated (%)	Glucoside remaining (%)
Turmeric rhizome	*Curcuma Longa* L.	0	92
Murunga leaf	*Moringa pterygosperma* Gaertn.	0	105
Katurumurunga leaf	*Sesbania grandiflora* Pers.	64	37
Ginger rhizome	*Zingiber officinale* Rosc.	0	143
Gotukola leaf	*Centella asiatica* L.	0	93
Gotukola leaf	*Hydrocotyle asiatica* Urb.	5	85
Mukunuwenna leaf and stem	*Alternanthera sessilis* R. Br.	100	0
Red onion bulb	*Allium Cepa* L.	0	93
Chillie capsule (green, fresh)	*Capsicum annuum* L.	0	110
Chillie capsule (red, dry)	*Capsicum annuum* L.	0	51
Kankun leaf and stem	*Ipomoea aquatica* Forsc.	95	06
Betel leaf	*Piper Betle* L.	0	69
Karapincha leaf	*Murraya Koenigii* Sprang.	0	122
Carrot tuber	*Daucus Carota* L.	0	164
Coconut endosperm	*Cocos nucifera* L.	83	12
Guava leaf	*Psidium Guajava* L.	0	14
Japan-batu leaf	*Phyllanthus sp.*	0	108

Plant extracts (from 15 g fresh weight) were incubated with linamarin (25 mg) for 24 h. Cyanide liberated was determined after distillation. The glucoside remaining was determined after incubation with linamarase (150 units) for 24 h. In each case appropriate controls (with linamarin omitted) were used to obtain blank values which were subtracted before calculations were made.

After some of the experiments microbial spoilage was evident. The experiments were therefore repeated with sterilized apparatus. The negative results obtained above were essentially the same; but no release of cyanide was obtained with coconut endosperm and katurumurunga leaf (Table 2). The cyanide liberating activity of kankun and mukunuwenna was destroyed by boiling for 10 min and deactivated to a considerable extent in semi-cooked preparations such as "mallung".

TABLE 2. Cyanide liberation from linamarin.

Material	Cyanide liberated (mg)			
	Boiled (10 min)	Semi-cooked*	uncooked	blank†
Mukunuwenna leaf	0.11	0.51	4.07	0.23
Kankun leaf	0.00	0.04	3.98	0.00
Katurumurunga leaf	0.00	0.13	0.07	0.16
Coconut endosperm	0.00	0.00	0.20	0.03

Plant material extracts (from 5g fresh weight) incubated with linamarin (40 mg) for 24 h.

* Cooking for about 3 min as in the preparation of "mallung".

† Control value for plant material incubated and distilled in the absence of linamarin.

3.4. Effect of coliform bacteria

As it appeared possible that in at least some cases the release of cyanide from linamarin might be due to microbial action, coliform bacteria were tested for their ability to hydrolyse linamarin. Tests showed that none of the 7 coliform types tested were able to release a significant amount of cyanide from linamarin (Table 3).

TABLE 3. Cyanide liberation from linamarin by coliform bacteria.

Culture	Coliform type (I M V_i C)	Cyanide released (µg/ml) 0 h	4 h	24 h	Glucoside remaining (µg/ml as cyanide) 0 h	4 h	24 h
A	+ + — —	0	0.0	1.5	49.5	50.0	54.0
D	+ + + —	0	0.0	0.5	47.0	49.0	50.0
F	— + — +	0	0.0	0.5	50.0	52.0	47.5
J	+ + + +	0	0.0	1.0	51.5	48.0	51.5
L	— + + +	0	0.0	0.0	51.0	47.5	47.5
M	— — — +	0	0.5	2.0	50.0	50.0	50.0
O	— — + +	0	0.0	1.0	49.0	46.0	47.5

Concentration of linamarin added : 48.5 µg/ml as cyanide. Standard deviation for glucoside estimation: ± 3 µg/ml as cyanide. Values obtained for cyanide release are not significant.

3.5. Further studies with ginger

Repeat experiments with several samples of ginger showed that incubation with linamarin released, if at all, only small amounts of cyanide. Similar results were obtained with purified ginger, proteins and with different protein fractions isolated from ginger. In fact, ginger proteins inhibit the linamarase-linamarin reaction slightly. No activity was obtained with ginger essences.

However, incubation with boiled manioc led to the release of cyanide in the case of some ginger samples ; this active principle was heat labile. Another ginger sample gave no release. The conflicting results were rather puzzling, especially as in all cases fresh ginger samples were used and therefore there was little chance of the loss of an active principle. It is, however, likely that the divergent results obtained were due to differences in varieties of ginger used. In addition, a sample which released cyanide from boiled manioc did not release cyanide from linamarin (Table 4).

3.6. Further studies with mukunuwenna

Although mukunuwenna showed very high cyanide releasing activity from linamarin, cyanide release from boiled manioc was very much less and this too only after 24 h (Table 5). Similar results were obtained with kankun. Katurumurunga, however, did not release cyanide from boiled manioc. Results similar to katurumurunga were also obtained in the case of coconut. This reduction in activity was somewhat puzzling and further investigation revealed that the release of cyanide from linamarin by mukunuwenna was non-linear, a distinct lag being observed (Table 6). This activity was not inhibited in the presence of the microbial inhibitor p. hydroxy ethyl benzoate (1000 p.p.m.).

TABLE 4. Cyanide liberation by ginger.

Materials and treatment	Cyanide release (μg)
A. Manioc (1) + Ginger (1) 4 h incubation	821
B. Manioc (1) + Ginger (1) 24 h incubation	2190
C. B followed by linamarase action (Total residual glucoside)	645
D. Manioc (1) : Total cyanide	2674
E. Manioc (1) + boiled ginger (1) 24 h incubation	N.D.*
F. Ginger (1) + linamarin 24 h incubation	13
G. Manioc (2) + Ginger (1) 24 h incubation	975
H. Manioc (2) + Ginger (2) 24 h incubation	N.D.*
I. Manioc (2) : Total cyanide	2129

Ginger (10 g fresh weight) was incubated with boiled manioc (10 g dry weight) or linamarin (20 mg).

Free cyanide in the manioc samples was in the order of 100 μg. Ginger blanks were in the order of 1000 μg; values given above have been corrected for ginger blanks.

* N.D. not detected ; optical density was less than that of blank.

TABLE 5. Cyanide liberation from boiled manioc by leaf extracts.

Plant tissues	Cyanide released (or equivalent O.D) (μg)		
	Control* 4 h incubation	4 h incubation	24 h incubation
Mukunuwenna leaf	26	157	306
Kankun Leaf	28	151	312
Katurumurunga leaf	108	252	167**

Fresh leaf extract (from 10 g fresh weight) was incubated with boiled manioc (10 g dry weight). Free cyanide in boiled manioc was 113 μg. Total potential cyanide was approximately 2500 μg (Most of this can be recovered by manioc linamarase).

* Control : Plant material incubated and distilled in absence of manioc.

** The low value may be due to some cyanide binding by cyanide utilizing reactions, but this is not important as the cyanide liberated is in any case very low.

TABLE 6. Cyanide release from linamarin by mukunuwenna extract.

Experiment	Time of incubation (h)	Cyanide liberated (μg)
Experiment A	2	0
	4	75
	6	150
	16	1116
	24	1344
Experiment B	22	1150
	22 + Microbial inhibitor *	1233

Linamarin (approximately 15 mg) was incubated with mukunuwenna extract (from 5 g fresh weight). Total volume of reaction mixture was 100 ml (0.02 M with respect to citrate buffer).

* Inhibitor, p. hydroxy ethyl benzoate (1000 p.p.m.)

3.7. Further studies with guava leaf

Results in Table 1 show that the recovery of linamarin from an incubation mixture with guava leaf was only 14%. This could have been due to : (1) binding of HCN formed and (2) inhibition of linamarase used to estimate the residual glucosides. Addition of more linamarase and an increase of incubation time resulted in the release of more cyanide. This showed that the effect was most likely due to an inhibition of linamarase ; the enzyme was inhibited to at least 90% by an extract from 15 g of guava leaf.

This was further confirmed by time-course studies on the effect of guava extract on the linamarin-linamarase reaction (Table 7). These studies showed that guava leaf contained a potent inhibitor of linamarase. This factor was both heat stable and dialysable. A similar inhibition was observed when boiled manioc was used as a source of linamarin (Table 8).

TABLE 7. Inhibition of linamarase action on linamarin by guava extract.

Conditions	Cyanide liberated (μg)					
	Incubation time 0.5 h	1 h	2 h	4 h	6 h	18 h
(i) Guava extract omitted	1240	1790				
(ii) Guava extract		32	60	180	240	540
(iii) Guava extract dialysate		672				

Guava leaf extract (from 10 g) was incubated with linamarin (18 mg) and linamarase (140 units). Total volume of reaction mixture was 100 ml, 0.04 M with respect to citrate buffer (pH 5.0).

TABLE 8. Inhibition of cyanide liberation from boiled manioc.

Materials	Cyanide liberated (μg)
Boiled manioc	84
Boiled manioc, linamarase	1596
Boiled manioc, linamarase, guava extract	0

Boiled manioc (10 g dry weight) was incubated for 4 h in the above manner. Weight of guava leaf used was 10 g (fresh weight). Linamarase activity: 140 units.

4. Discussion

Studies comparing the relative efficiency of acid and enzymic hydrolysis of linamarin have established that the latter method is superior. However, the method of acid hydrolysis at low acid concentrations for an increased period of time warrants further investigation. On comparison of the results of one such technique,[9] that of Rajaguru, with the method used in this study, it was found that enzymic hydrolysis gives values 5 to 10% higher. However, as $AgNO_3$ (which was used in the acid hydrolysis studies) overestimates cyanide by 8 to 14%[12,11] it appears that the relative efficiency of this method of acid hydrolysis is in the region of 80 to 85%.

The nature of liberation of cyanide from linamarin by plant tissues has proved to be somewhat complex. Heat labile factors in kankun and mukunuwenna can bring about this liberation, possibly by a mechanism quite distinct from the linamarase reaction. The latter is suggested by : (1) the lag in cyanide liberation and (2) liberation of very little cyanide from boiled manioc. The second observation suggests considerable inhibition of the cyanide liberating process by manioc substrate ; such levels of inhibition are not shown by manioc linamarase in the presence of manioc substrate.

The effect of ginger is yet more complex. Once again these studies show that the cyanide liberating factor is not a linamarase. It appears that ginger provides a heat labile factor that helps in cyanide liberation. The studies of Kodagoda *et al.*[3] suggest that ginger could be providing an activator to the deactivated manioc linamarase ; the results of this study are consistent with this suggestion. Alternatively, it is possible that the factor produced by ginger is an enzyme whose action is dependent on a minimum level of some unknown cofactor that could be present in either or both manioc or ginger. This alternative would explain the lack of liberation of cyanide from purified linamarin and the observation that some samples of ginger did not liberate cyanide from boiled manioc.

The inhibition of linamarase activity by guava leaf extracts is of significance because these extracts are used as an antidote for manioc poisoning in rural Sri Lanka. Although several extracts used in this study inhibit linamarase activity, none of them

is as effective as guava leaf. This potent inhibitor is a dialysable, heat stable molecule; it is interesting to note that gluconolactone is also an effective inhibitor of manioc linamarase.[11]

5. Conclusion

Extracts of some plant tissues can hydrolyse linamarin but the factors responsible are not the same as linamarase. It is difficult to extrapolate these results to obtain information on the effect of these materials on ingested linamarin, as the cyanide liberating process is very likely to involve an interplay of activators and inhibitors of enzymes superimposed on the complex physiological changes that occur in the digestive system. Further work is needed to determine the exact mechanism of the cyanide liberating processes. This study shows that plant tissue extracts can liberate cyanide from cyanogenic glucosides and, therefore, should serve as a deterrent to the consumption of manioc with raw or semi-cooked plant material.

Acknowledgements

The authors thank Miss N. T. Amerasinghe and Miss R. Kandage for technical assistance, and Mrs. I. Bandara and Miss D. Obadage for secretarial assistance.

References

1. CERIGHELLI, R. (1955) *In Cultures tropicales* I. Plantes vivrieres (Nouvelle Encycl. Agr., Paris, 1955) p. 367.
   Ref. quoted in JONES, W. O. (1959) *Manioc in Africa*. Stanford, California : Stanford Univ. Press.
2. JANSZ, E. R. & NETHSINGHA, C. (1973) *J. Natn. Sci. Coun. Sri Lanka* **1** (2): 83-96.
3. KODAGODA, N., MARKUS, V. & AMBALAVANAR, S. (1973) *Proc. Ceylon Ass. Advmt Sci.* **29** (1): 38.
4. NARTEY, F. (1968) *Phytochem.* **7** : 1307-1312.
5. NESTEL, B. & MACINTYRE, R., *eds.* (1973) *In Chronic cassava toxicity: proceedings of an interdisciplinary workshop, London, England,* 29-30 *January* 1973, pp. 159-162. Ottawa : International Development Research Centre. (IDRC-010e).
6. NICHOLLS, L. (1951) *In Tropical nutrition and dietetics*, 3rd edn. p. 381. London : Bailliere, Tindall & Cox.
7. PIERIS, N., JANSZ, E. R. & KANDAGE, R. (1974) *J. Natn. Sci. Coun. Sri Lanka* **2** (1) : 67 — 76.
8. PIERIS, N., PREMADASA, G. G. & JANSZ, E. R. (1973) *J. Natn. Sci. Coun. Sri Lanka.* **1** (2): 207-210.
9. RAJAGURU, A. S. B. (1973) *Private communication.*
10. WOOD, T. (1965) *J. Sci. Fd Agric.* **16** : 300-305.
11. WOOD, T. (1966) *J. Sci. Fd Agric.* **17** : 85-90.
12. ZITNAK, A. (1973) *In Chronic cassava toxicity : proceedings of an interdisciplinary workshop, London, England,* 29-30 *January* 1973, pp. 89-95. Ottawa : International Development Research Centre. (IDRC-010e).