J. Natn. Sci. Coun. Sri Lanka 1976 **4**(1) : 29—47

Cyanide liberation from Linamarin

II. Purification and some properties of the cyanide liberating enzymes of manioc rind *

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(*Paper accepted* : 31 *March* 1976)

Abstract : The cyanide liberating activity in crude extracts of the rind of *Manihot esculenta* Crantz were subjected to purification. The methods used were acetone and ammonium sulphate precipitation and Sephadex-gel and DEAE-cellulose chromatography. A considerable purification was achieved and two enzymes (called linamarase A and B in this paper) capable of liberating cyanide from the major cyanogenic glucoside of manioc rind (linamarin) were separated. Although the Km values of the two enzymes were similar, their temperature optima, activation energies and electrophoretic mobilities showed distinct differences. The pH activity curves and temperature stability characteristics showed only very minor differences.

1. Introduction

A wide variety of plants contain cyanogenic glucosides as well as the corresponding hydrolysing enzymes that release cyanide from them.

There are two main classes of β glucosidases which act on cyanogenic glucosides,[6] (1) the emulsin type and (2) the linamarase type. The former acts on cyanogenic di-glucosides (amygdalin) and aromatic cyanogenic glucosides while the latter acts specifically on aromatic and aliphatic cyanogenic glucosides but not on the di-glucosides.

The linamarase type has been extracted from linseed[4] and clover[7] in addition to manioc[12] (*Manihot esculenta* Crantz). While the linamarase of clover leaf has stability problems and cannot be purified in the same way as linseed linamarase,[6] the callus tissue of white clover stems contain two β glucosidases with linamarase activity which differ in their Michaelis constants.[7]

The main cyanogenic glucoside of manioc is linamarin[12] [2(β—D—glucopyranosyl oxy) iso butyronitrile]. Manioc also contains lotaustralin[2,9] [2(β—D—glucopyranosyl oxy) 2 methyl iso butyronitrile] in quantities of less than 10%. Linamarase acts on linamarin to produce 2-hydroxy isobutyronitrile which is then acted upon by hydroxynitrile lyase[6] (also present in manioc) to produce acetone and HCN.

* J. Natn. Sci. Coun. Sri Lanka **2** (1) : 57—66 is now considered as part I of this series.

‡ This paper forms a part of the Ph.D. Thesis, (University of Sri Lanka, Colombo Campus) of Nirmala Pieris.

Studies on the linamarase from the rind of manioc by Wood[12] (with a purified enzyme preparation of about 5—10 fold purification) showed that the enzyme had a pH optimum of 5.5 and that it was inhibited by δ- gluconolactone. In this study, the linamarase activity of manioc rind extracts have been subjected to more extensive purification and two enzymes capable of hydrolysing linamarin were separated. Neither of these two enzymes show a pH activity curve similar to that reported by Wood.[12] Although the two enzymes have similar Michaelis constants, they have different activation energies and electrophoretic mobilities.

2. Experimental

2.1 Purification of linamarase

2.1.1. *Acetone precipitation*

Crude extracts of linamarase were prepared by the method of Wood[12] using manioc rind. The method involves homogenisation of the tissue in acetate buffer (0.1M) followed by precipitation with 70% acetone. The precipitate was extracted with acetate buffer (0.2M) and the resulting aqueous solution was used for further purification.

2.1.2 *Salt precipitation*

$(NH_4)_2SO_4$ precipitation was used for fractionation. The 40%—65% saturated fractions contained most of the cyanide liberating activity.

2.1.3. *Sephadex-gel chromatography*

Sephadex (G-100) equilibrated in 0.1M acetate buffer, was packed into a column to give a bed of height 43 cm and diameter 2 cm. A 30 ml sample of enzyme extract (~1000 μmoles CN^-/min) was introduced into the column and eluted with 0.1M acetate buffer (pH 5.5) containing 0.2M NaCl, 3 ml fractions were collected at an elution rate of 18 ml/h.

2.1.4. *DEAE cellulose chromatography*

Fifteen ml of the dialysed enzyme extract (~600 μmoles CN^-/min) in 0.01M acetate buffer (pH 5.5) was introduced into a DEAE-cellulose column of bed volume 34 ml. The column was eluted using the following eluents at a rate of 25 ml/h.

(1) 0.01M acetate buffer (pH 5.5) (50 ml)
(2) 0.01M acetate buffer containing 0.1M NaCl (15 ml)
(3) 0.01M acetate buffer and 0.15M NaCl (15 ml)
(4) 0.01M acetate buffer and 0.2M NaCl (15 ml)
(5) 0.01M acetate buffer and 0.25M NaCl (15 ml)
(6) 0.01M acetate buffer and 0.3M NaCl (30 ml)
(7) 0.01M acetate buffer and 0.4M NaCl (30 ml)

The first 75 ml (which contained no enzyme activity) was discarded, following which 50 fractions of 2 ml were collected.

2.1.5. *Electrophoretic mobility*

This was done using an Elphor power pack and tank. The purified fractions (~200μg protein) A and B were streaked on Whatman No. 1 chromatography (9.5 × 4 cm) paper and electrophoresis was conducted at 200V (60 mA) for 1½ h. in 0.025 M acetate buffer (pH 5.9). The protein bands were detected using Amido Black 10B.[13] The corresponding areas on unstained paper were tested for linamarase activity by incubation with linamarin, testing for cyanide with the picrate-sodium carbonate reagent.[12]

2.2 Purification of linamarin

The glucoside was prepared by the method of Wood.[12] The product obtained was further purified by paper chromatography using solvents capable of separating linamarin and lotaustralin. Approximately 0.1 μmoles of the glucoside was streaked on Whatman No. 1 paper and chromatographed using propanol : water (70 : 30). Two bands were observed on spraying with $AgNO_3$ reagent.[14] These bands were eluted with butanol : acetic acid : water (60 : 15 : 25) and restreaked on Whatman No. 1 chromatography paper. Chromatography of this material with ethyl methyl ketone : acetone : water (30 : 10 : 6) as solvent resulted in two $AgNO_3$ positive spots of R_f values 0.71 and 0.51. The latter spot did not give a cyanide positive reaction with linamarase. The cyanide positive spot was eluted with acetone and chromatographed once again in the above solvent system to test for purity. This resulted in one spot with $AgNO_3$ reagent but with a R_f value corresponding to that of lotaustralin as reported by Butler and Conn.[4] This gave a positive cyanide reaction after incubation with linamarase.

In order to confirm the identity of the glucoside, linamarin (20 mg) was hydrolysed with linamarase (1 unit) for ½ h. and heated at 100°C while aspirating with N_2. The volatile ketone was trapped as a 2 : 4 dinitrophenyl-hydrazone and chromatographed (n heptane saturated with methanol) with the 2 : 4 dinitrophenylhydrazones of acetone and propanone. These chromatographic studies showed that the only ketone formed by the hydrolysis of the glucoside was acetone and therefore that the glucoside was linamarin and not lotaustralin.

The purity of the glucoside was confirmed by quantitative estimation of the amount of HCN and glucose liberated from a known weight of linamarin. Linamarin (3.48 μmoles) incubated with 1 unit linamarase for 3 h. liberated 3.44 ± 0.10 μmoles CN^-. Linamarin (1.74 μmoles) incubated for a similar period with linamarase liberated 1.70 ± 0.04 μmoles glucose. Glucose was estimated by the method modified from Nelson[11] using the appropriate standard curve at 730 nm. The M.P.

of linamarin used was 138.6°C (uncorr.). Values of the M.P. of linamarin reported as 138°C (uncorr.) for the product isolated from manioc[12] and 140—142° (corr.) for the synthetic product.[12]

2.3. Enzyme activity

Enzyme activity is expressed as μmoles CN^- released/min. 1 unit of activity being the μmoles CN^- released in 1 min. Enzyme activity was measured in two ways : (1) relative activity by "syringe method" (as described previously)[10] (2) true activity• In the "syringe" method, concentration of the glucoside was 4 mM and was therefore only an estimation of relative activity since the rate of reaction was about 70% Vm. Rates were calculated by following a time course of the reaction and using the linear part of the curve. Calculation using this method is termed "relative activity" in this paper and has been used in order to get a time course of the reaction without the use of large quantities of substrate. The true activity of the enzyme was measured using substrate concentration of 25 mM in a reaction volume of 0.3—0.4 ml (all other conditions remaining the same). The reaction was stopped after about 10—15 μg (0.5 μmole) of cyanide was liberated and activity was calculated in these instances with only one incubation time. The latter method was used mainly for the assay of the fractions from columns.

2.4. Protein estimation

Protein was estimated using the Folin Ciocalteu Reagent.[8] A standard curve (casein) was used for the estimation.

2.5. Properties of enzymes

2.5.1. *Michaelis constants*

The kinetic characteristics of the enzymes were studied using the "syringe method"[10] at linamarin concentrations ranging from 0.4 mM to 4.0 mM. Linamarase activity used was about 0.4 units. Reaction volumes of 5 ml and 10 ml and aliquots of 1 ml and 2 ml were used for 1—4 mM and 0.4 to 1 mM concentrations respectively. The experiments were done at 29°C and pH 5.6.

2.5.2. *pH activity curve.*

The reaction mixtures contained linamarin (25 mM) citrate buffer (0.1M) and linamarase (approx. 0.4 units), in a reaction volume of 0.32 ml. The temperature of the reaction was 29°C.

2.5.3. *Temperature activity curve*

The reaction mixture contained linamarin (25 mM) citrate buffer (pH 5.6, 0.08 M) and linamarase (0.1 units CN^-/min.). The reaction volume was 0.37 ml and reaction time was 5 min.

2.5.4. *Temperature stability*

Linamarase A and B were incubated separately for 10 min. in a thermostated water-bath. The cooled enzymes were then incubated for 10 min. with linamarin (25 mM) and citrate buffer (pH 5.6, 0.1M) in a reaction volume of 0.43 ml.

3. Results

3.1. Purification of linamarase

Homogenisation in a Waring blendor resulted in the extraction of about 50% of the protein in manioc rind. On subjecting the extract of the rind to the acetone purification step of Wood,[10] a 6.5 fold purification was achieved while retaining 74% of the activity (Table 1A). The resulting extract can be stored at 0—4°C for several months without significant loss in activity. As the extract used for the results quoted in Table 1A was low, it was decided to continue further purification with another extract of much higher activity. The aging process was repeated with the extract and it was found that during the storage a protein precipitate was formed (mainly in first three weeks) which was removed by centrifugation resulting in an increase in specific activity. The supernatant was then subjected to Sephadex-gel (G—100) chromatography which showed the presence of shoulders on the activity peak (Figure 1) and suggested the presence of more than one enzyme with cyanide liberating properties. Due to this, care was taken to retain as much activity as possible, during the purification steps, in order to prevent the possible selective loss of one of these enzymes.

TABLE 1A—Purification of Linamarase—Part I

Step	Total Protein (mg)	Volume (ml)	Total Activity (μmoles CN^-/min)	Specific Activity (μmoles CN^-/mg protein/min)	Fold Purification	Yield %
(1) Crude extract	9.81	250	373	0.38	1	100
(2) Acetone ppt	46.3	40	318	0.69	1.8	84.5
(3) Centrifugate	11.0	40	269	2.45	6.5	74

Concentration by salt precipitation also resulted in an increase in specific activity. A detailed $(NH_4)_2 SO_4$ precipitation profile is shown in Figure 2. After dialysis the selected fractions (40%—65%) were separated on DEAE-cellulose. This resulted in the separation of two enzymes with cyanide liberating activity (Figure 3) showing specific activities of 58 and 61 μmoles CN^-/mg protein/min. respectively. The enzymes which are eluted at 123 ml and 139 ml have been called linamarase A and B respectively, in the paper.

If we are permitted to assume that the enzyme preparation used in Table 1B would have had a final specific activity of 6.2 units/mg protein, then at least a 60 fold purification of the enzyme was achieved (Table 1B). Other studies have shown that Sephadex G—200 cannot separate these two enzymes (Figure 4). While the capacity for DEAE-cellulose to do this separation is confirmed in another set of experiments

(Figure 5). The different ratios of linamarase A and B in this experiment can probably be accounted for by the fact that DEAE-cellulose chromatography was done after a much more extensive purification (where the recovery of activity was only in the order of 6%) and the extract used had probably undergone selective loss of linamarase B.

TABLE 1B—Purification of Linamarase—Part 2

	Step		Total Protein (mg)	Volume (ml)	Total Activity (μmoles CN^-/min)		Specific Activity (μmoles CN^-/mg protein/min)		Fold Purification		Yield %	
(3)	Centrifugate		152	30	946		6.2		1		100	
(4)	Aging (1 month) centrifugation		78.8	30	923		11.6		1.88		98	
(5)	Sephadex-gel chromatography (G-100)		50.0	30.25	765		15.4		2.50		83	
(6)	$(NH_4)_2SO_4$ Precipitation		28.5	14	567		20.0		3.25		61	
(7)	DEAE Cellulose chromatography	A	2.5	8	146	259	58.1	59.7	9.44	9.73	15	27
		B	1.8	8	113		61.2		10.02		12	

3.2 Preliminary studies on the enzyme

Progress curves of linamarase A and B measured at substrate concentration of 25 mM are shown in Figure 6.

The effect of concentrates on enzyme activity showed that increased concentration of protein resulted in a more than proportionate increase in activity (Figure 7). Repeated experiments showed that this increase though small was real.

3.3. Michaelis constants of the enzymes

Lineweaver-Burk plots of the enzymes (Figure 8) showed that their Km values were very similar, 2.2 and 2.2—2.5 respectively. The 'apparent' Vm values (not significant as enzymes were not fully purified) were 106 and 135 μ moles CN^-/mg protein/min. respectively.

3.4 Effect of pH on enzymic activity

Both enzymes had very similar pH-activity curves, with broad optima from pH 5.6 to 6.6. Enzyme B appeared to have a peak at about pH 6.2 but this peak was small and not significant as the error in estimation of the method of assay was in the order of 5% (Figure 9).

3.5. Effect of temperature on enzyme activity

Linamarase A and B have different activation energies, 5.7 Cal/mole and 3.3 Cal/mole respectively (Figure 10). Although the rates of reaction of linamarase A and B declined at 45°C and 65°C respectively, temperature stability studies in the absence of linamarin showed that the enzymes were 50% deactivated at 60° and 62°C respectively (Figure 11).

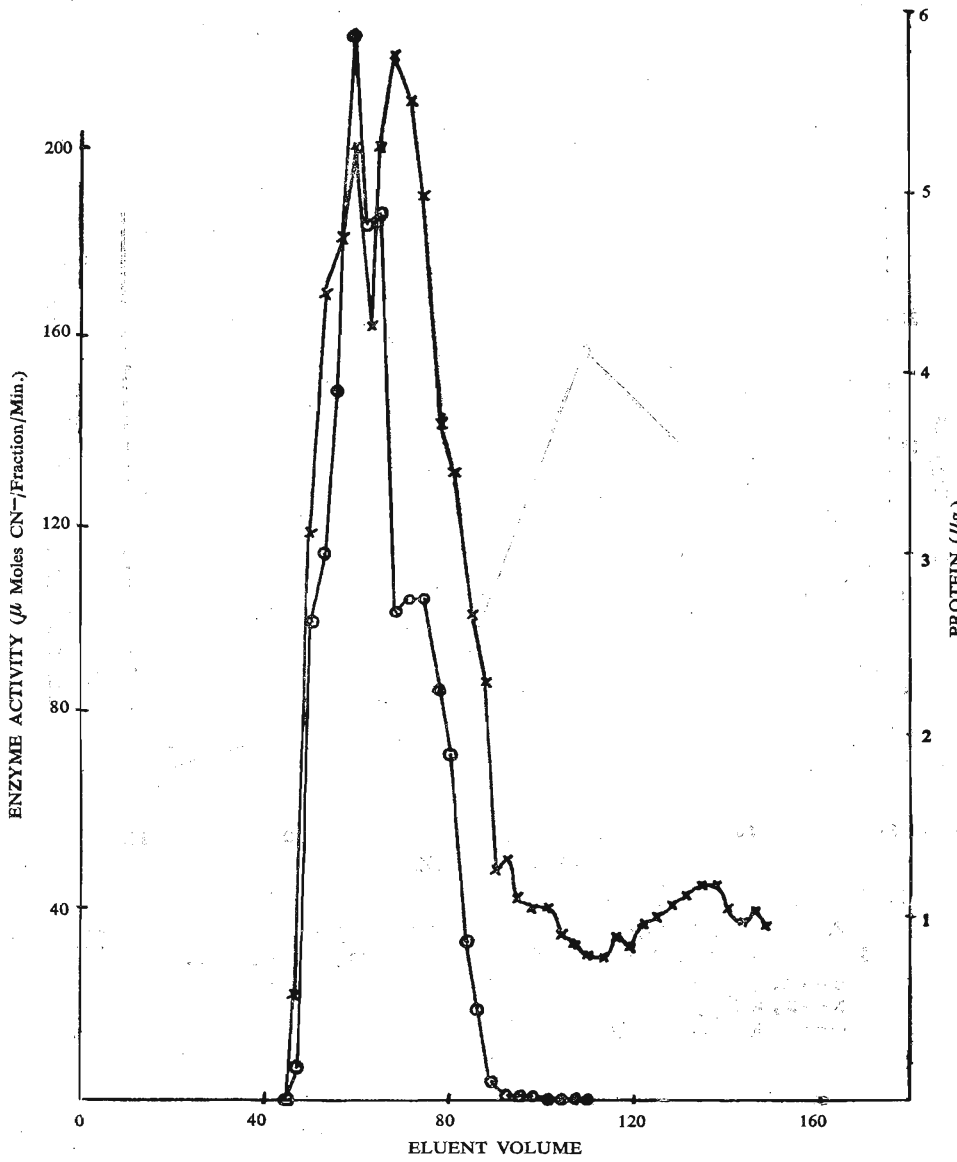

Figure 1. Purification by Sephadex-gel (G-100) Chromatography

o — o, activity

x — x, protein

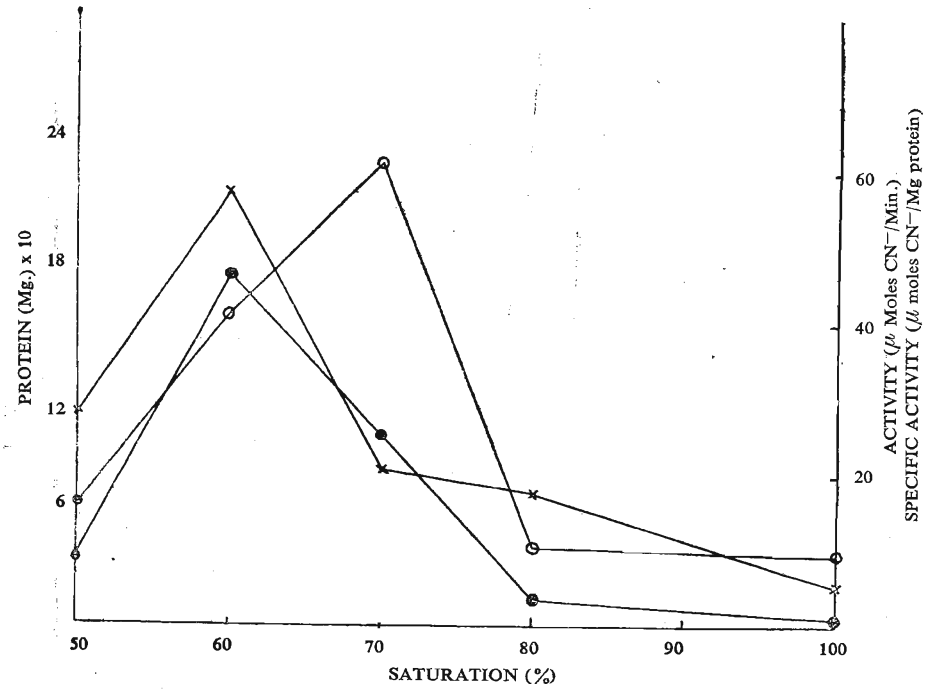

Figure 2. Salt precipitation profile

Ammonium sulphate was used as the precipitant. The figure above shows % protein precipitated at varying degrees of saturation of the protein extract.

o — o, protein
x — x, activity
● — ●, specific activity.

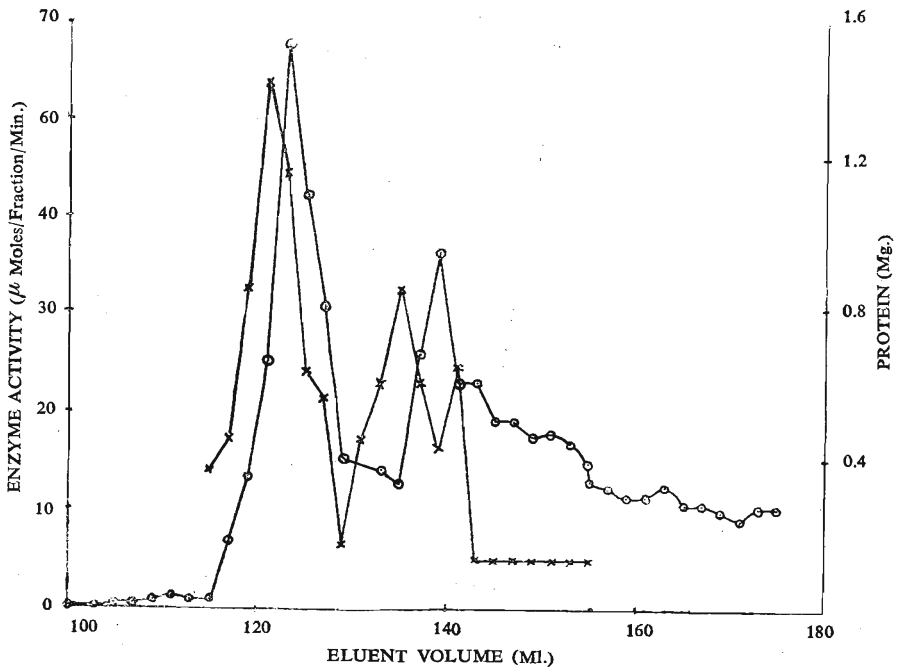

Figure 3. Separation of linamarase A and B by DEAE-cellulose chromatography
The eluent volume of linamarase A and B were 123 ml and 139 ml respectively.

o — o, activity
x — x, protein

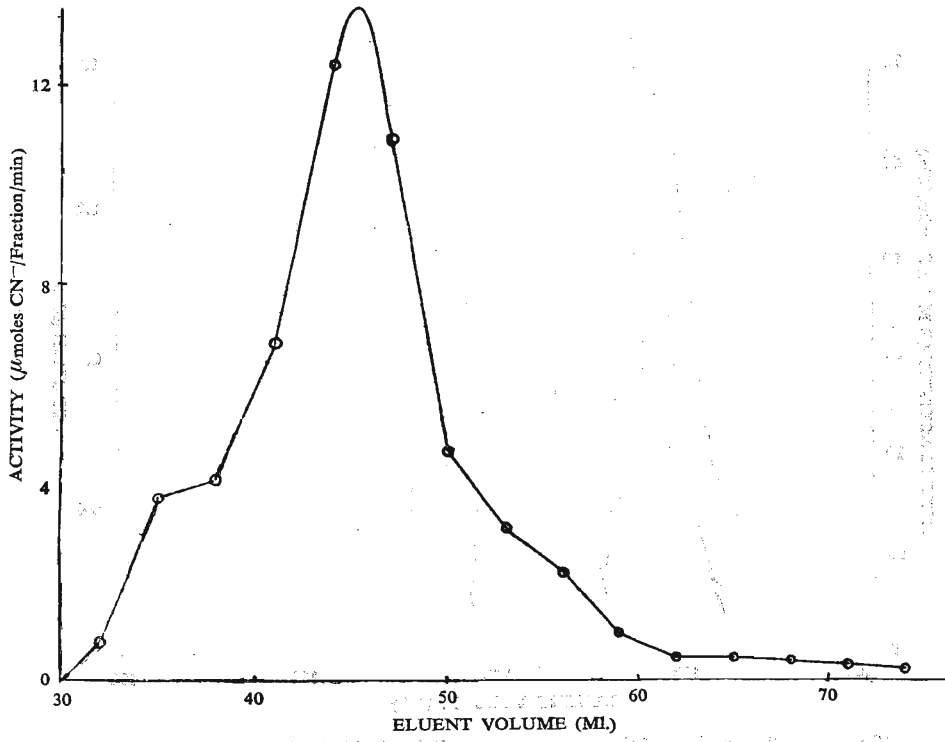

Figure 4. Activity peak obtained using Sephadex-gel (G-200) chromatography.

Column height, 45 cm ; column diameter, 2 cm ; sample size, 6 ml ; eluent rate, 9 ml/hr. Other details as in 2.1.3.

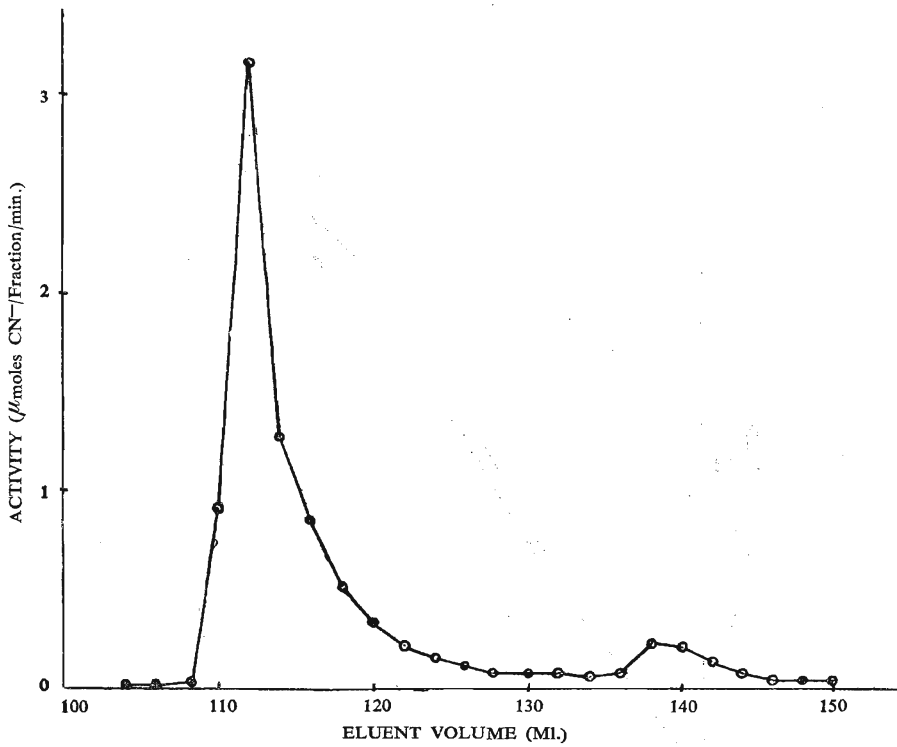

Figure 5. Separation of linamarase A and B by DEAE-cellulose chromatography

Volume of column, 35 cm ; sample size, 3.7 ml ; eluent rate, 25 ml/hr. Eluents used 0.01 M acetate buffer containing NaCl in the following set of concentrations (i) no NaCl (50 ml). (ii) 0.1 M (30 ml). (iii) 0.2 M (30 ml). (iv) 0.3 M (30 ml) (v) 0.5 M (30 ml).

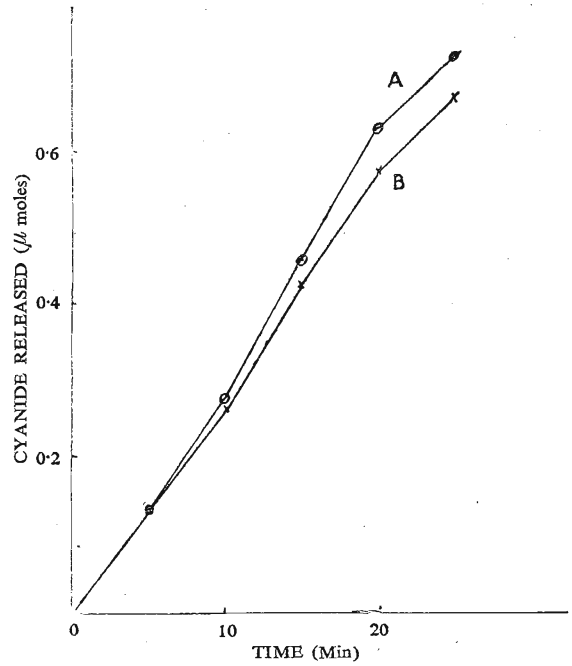

Figure 6. Time course of initial reaction by linamarase A and B.

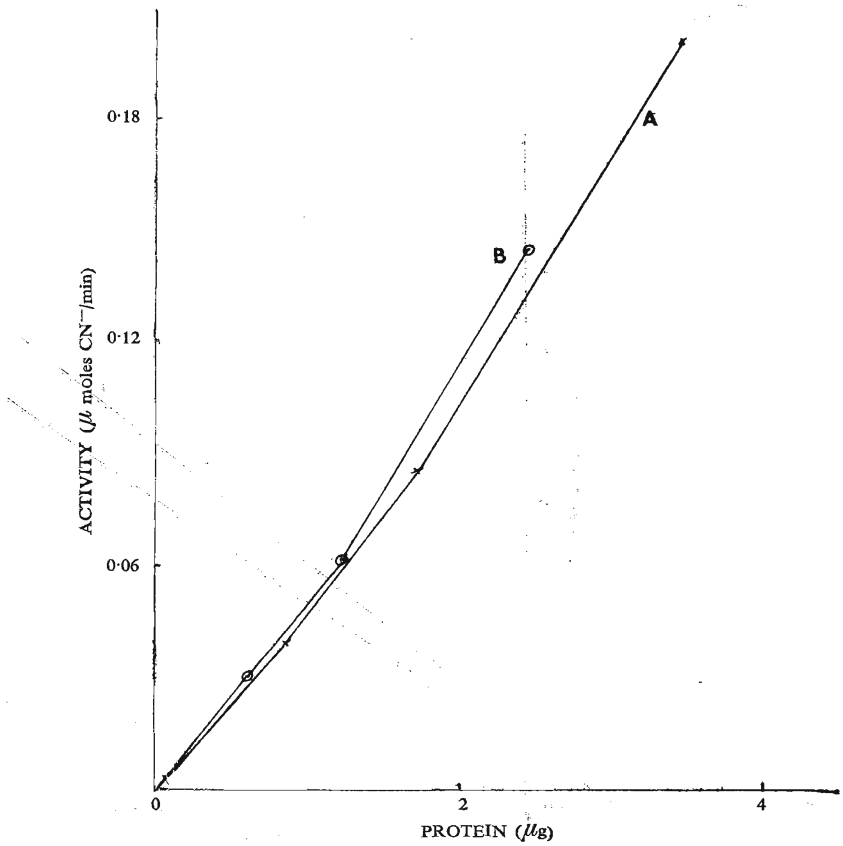

Figure 7. Effect of concentration on activity of linamarase A and B.

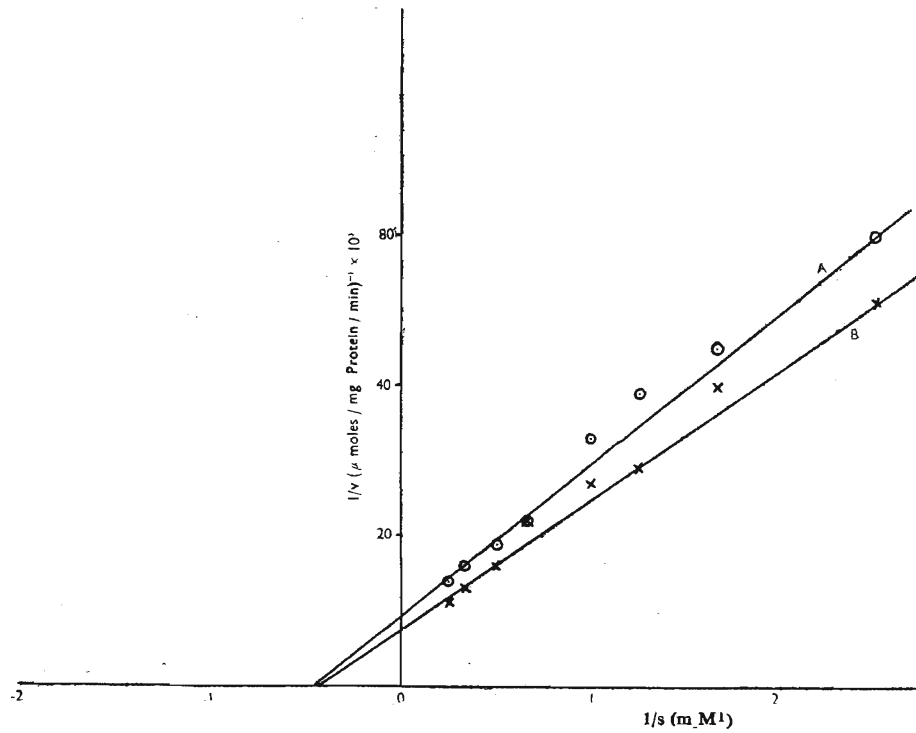

Figure 8. Lineweaver-Burk plots of linamarase A and B.

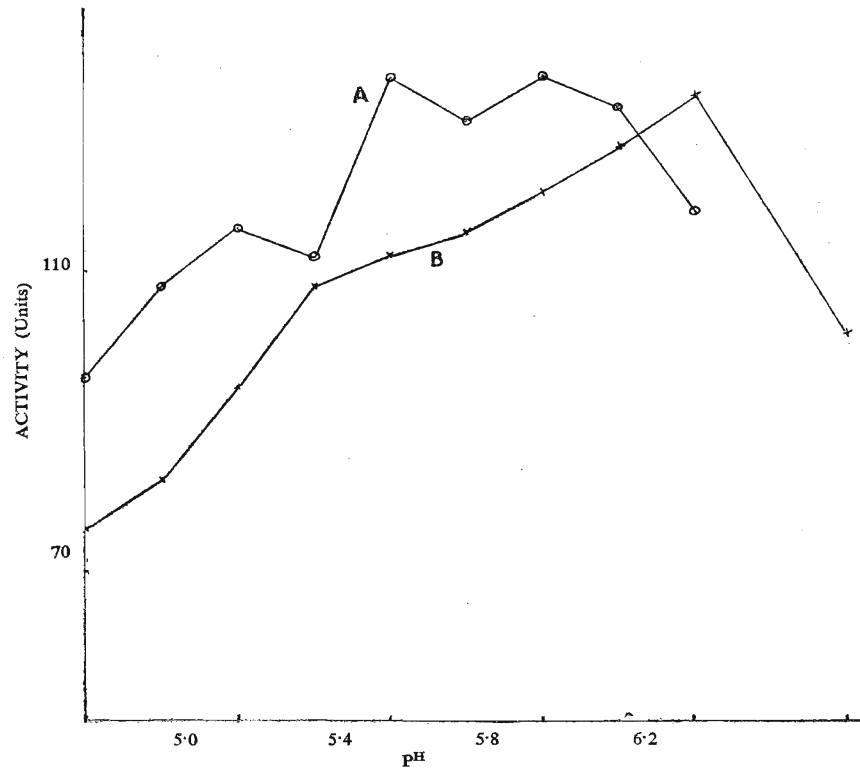

Figure 9. Effect of pH on activity of linamarase A and B.

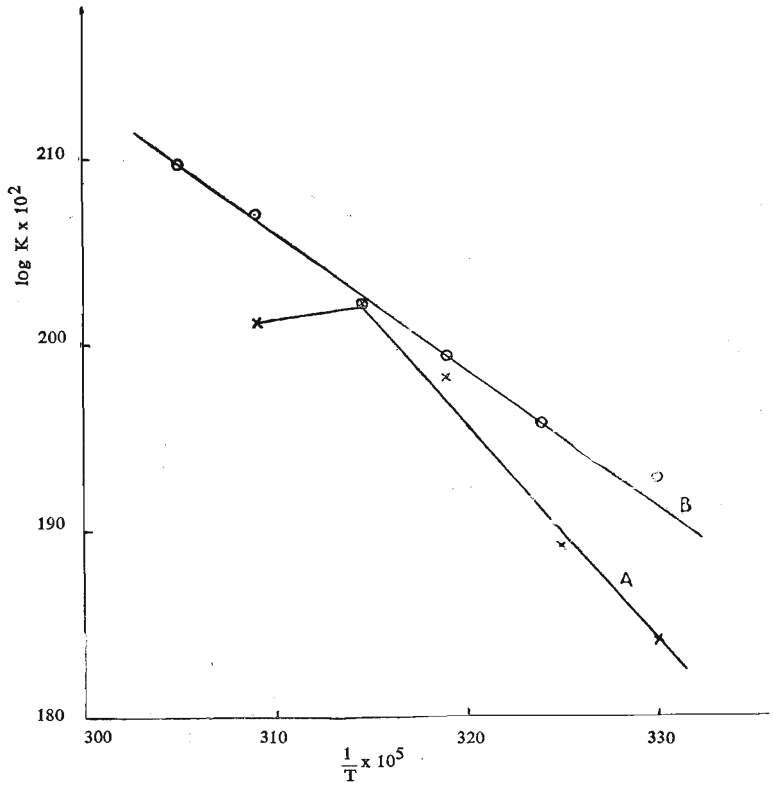

Figure 10. Arrhenius plots of rate of reaction at varying temperatures for linamarase A and B.

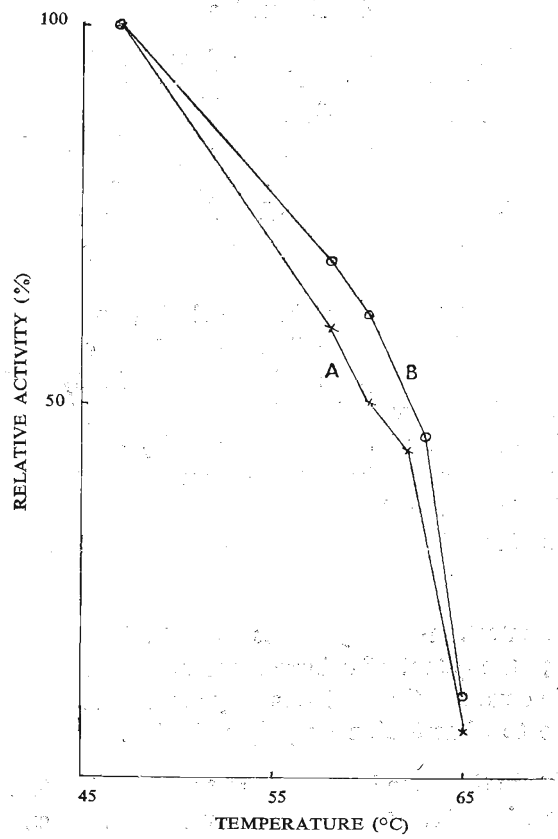

Figure 11. Effect of temperature on enzyme stability.
Activity of linamarase A and B after 10 min. incubation at various temperatures.

3.6. Electrophoretic mobility

Paper electrophoresis of linamarase A and B showed that they had a vastly different electrophoretic mobility. In each case only one protein band was detected. Linamarase A and B migrated 23 and 8 mm respectively after electrophoresis for $1\frac{1}{2}$ h.

4. Discussion

These studies confirm that the major cyanogenic glucoside of manioc is linamarin.[2, 9, 12] Some doubts were entertained on this point due to the incorrect R_f values reported in the literature.[5] However, the identification of acetone as a hydrolytic product of the glucoside confirmed that the glucoside was linamarin and not lotaustralin which would have given ethyl-methyl ketone on hydrolysis. The purity of the compound was illustrated by the liberation of theoretical amounts of glucose and HCN from a known quantity of linamarin as well as the melting point of the linamarin isolated.

The presence of at least two linamarin hydrolysing enzymes in manioc rind is illustrated by the separation on the DEAE-cellulose column. Electrophoretic studies show that these two enzymes migrate at distinctly different rates. The enzymes also show a major difference in their activation energies and minor variations in their pH activity curves and temperature activity curves. Although the rate of reaction of linamarase A and B begin to be depressed at 45° and 65°C respectively their rates of deactivation in the absence of substrate are very similar. This may be due to the relative stabilisation of linamarase B to temperature due to the presence of the substrate.

The enzyme concentration-activity curves of both linamarase A and B show positive deviations ; the extent of enhancement in the range of about 20% for a four fold increase in concentration and was very reproducible. It is possible that the enhancement is due to dimerisation or polymerisation.

The presence of more than one linamarase in the tissue is interesting especially as several kinetic properties of the two enzymes are similar. It suggests the presence of multiple genes for linamarase biosynthesis and might provide a reason for the absence of acyanogenic varieties in manioc, (whereas acyanogenic forms are present in several other cyanogenic plants).

Acknowledgements

The authors thank the Director, CISIR, for facilities provided, Mr. E. E. JeyaRaj, Head, Industrial Microbiology Section, CISIR, for encouragement, Dr. S. Senthe Shanmuganathan for advice for and carrying out electrophoresis experiments, Miss N. T. Amerasinghe for drawing the figures in this paper and Mrs. I. Bandara for secretarial assistance.

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