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Cyanide liberation from linamarin

III. Separation of the linamarases of manioc rind by DEAE-cellulose chromatography

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Abstract : Our previous studies showed that manioc rind contained two linamarin hydrolysing enzymes, linamarase A and B. Studies using DEAE—cellulose chromatography of rind extracts of eight cultivars of *Manihot esculenta* Crantz showed the presence of 4 to 6 cyanide liberating activity peaks, the major activity peaks being due to linamarase A and B in all but one case. Studies also showed that linamarase A could be a sub-unit of linamarase B. Some properties of linamarase C and D are also described.

1. Introduction

Multiple cyanogenic glucosidases have been reported in a few cyanogenetic plant species. Hughes[1] reported that the callus tissue of white clover stems contained two β glucosidases capable of hydrolysing linamarin, one specific and the other non-specific which differed widely in their Michaelis constants. Stevens and Strobel[5] isolated two linamarases from a psychrophilic basidiomycete. The β glucosidases, which were separated on a DEAE-cellulose column, showed a marked variation in their Michaelis constants, pH optima, temperature stability and energy of activation.

Our previous studies[4] showed that two cyanide liberating enzymes could be separated by DEAE-cellulose chromatography from rind extracts of *Manihot esculenta* Crantz (manioc). The initial aim of this study was to determine if these two enzymes were present in all common cultivars of manioc. The paper describes the linamarase activity profile of DEAE-cellulose column eluates of manioc rind extracts of 8 cultivars of manioc and compares some of the more important properties of linamarase A, B, C and D.

2. Experimental

2.1 Plant material

Manioc cultivars were obtained from the Central Agricultural Research Institute, Gannoruwa, by courtesy of Dr. (Mrs). C. Breckenridge.

2.2 Crude preparations of linamarase

These were prepared by acetone precipitation as described by Wood.[6]

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2.3 DEAE—cellulose chromatography

This was performed, by the procedure described previously[4], directly on the dialysed acetone precipitated fraction.

2.4 Assays

Absolute enzyme activity of fractions off the DEAE-cellulose column was measured as described previously,[4] cyanide being determined (in the presence of glucose) by the method of Wood.[2,3,6]

2.5 Properties of the enzymes

Temperature stability, temperature optimum and energy of activation were determined on linamarase C and D as described previously for linamarase A and B.[4] The effect of concentration on enzymic activity was tested by incubating linamarin (20 mM). 0.08M citrate buffer (pH 5.6) and linamarase in varying concentrations. Reaction time varied so that the same amount of cyanide would be liberated in all cases if an enzyme concentration effect was absent. Reaction volume was 0.43 ml and the reaction was carried out at room temperature (29°C).

3. Results

3.1 Studies on cultivars

Crude preparations of the rind of 10 cultivars of manioc were obtained by homogenisation in acetate buffer followed by acetone precipitation.[6] The cultivars used were MU-71, Selection 3, MU-18, MU-46, MU-51, JAVA, MU-22, MU-64, MU-10 and MU-44. The concentrated protein precipitate was dialysed against 0.01M acetate buffer, pH 5.5 and activity measured. Linamarase activity of cultivars MU-18 and MU-10 was very low and therefore further studies were done only with the other 8 cultivars. DEAE-cellulose chromatogaphy showed that there were 4 consistent linamarase activity peaks with varying degrees of activity at elution volumes of 104, 116, 128 and 166 ml respectively, which have been called linamarase D, A, B and C respectively. In addition, other minor peaks were observed. The four main activity peaks were present in all cultivars except the cultivar JAVA, where only the activity peaks of linamarase D and A were present (figure 1). The activity peaks most prevalent were those of linamarase A and B. It was found during the course of these studies that reproducibility observed with the DEAE-cellulose column used was very high.

3.2 Sub-unit structures of linamarase B

DEAE-cellulose chromatography of extracts of linamarase A and B in the presence of 4M urea showed that, whereas linamarase A maintained its position on the DEAE-cellulose elution pattern in the presence of urea, linamarase B split up into two peaks one each corresponding to linamarase A and B (figure 2). This showed that linamarase A was a modified form of linamarase B, probably an active sub-unit.

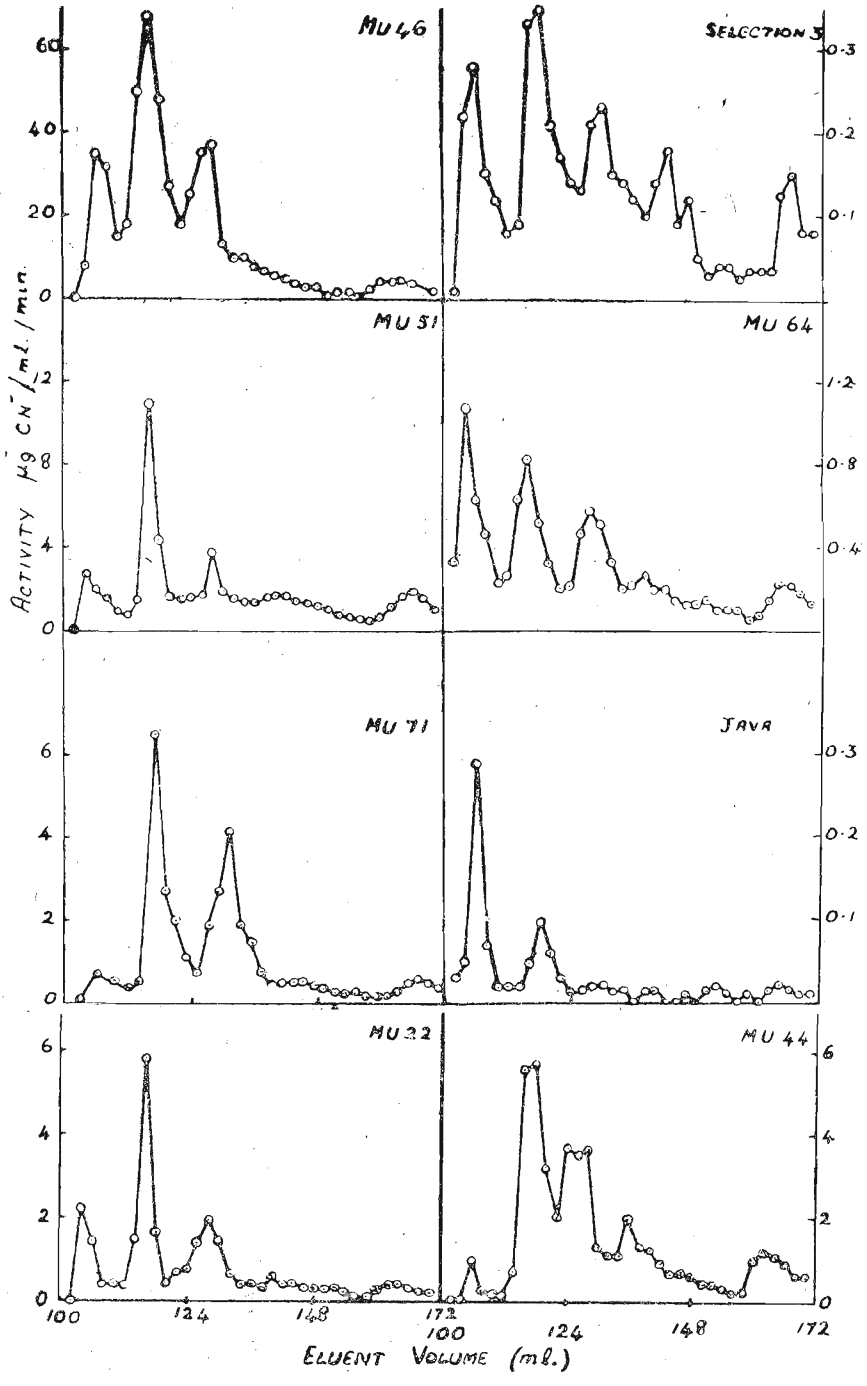

FIGURE 1—*DEAE-cellulose chromatography of rind extracts of various cultivars.*

Bed Volume, 34 ml; Rate of Elution 25 ml/h. Eluents used were as follows: 0.01M Acetate buffer pH 5.5 containing (*a*) no NaCl added (30ml) (*b*) 0.1M NaCl (15ml) (*c*) 0.15M NaCl (15ml) (*d*) 0.2M NaCl (15ml) (*e*) 0.25M NaCl (15ml) (*f*) 0.3M NaCl (30ml) (*g*) 0.4M NaCl (30ml). Symbols at top right hand corner of each inset refer to the name of cultivar.

The eluent volumes of linamarase A, B, C and D were 116, 128, 165 and 104 ml respectively.

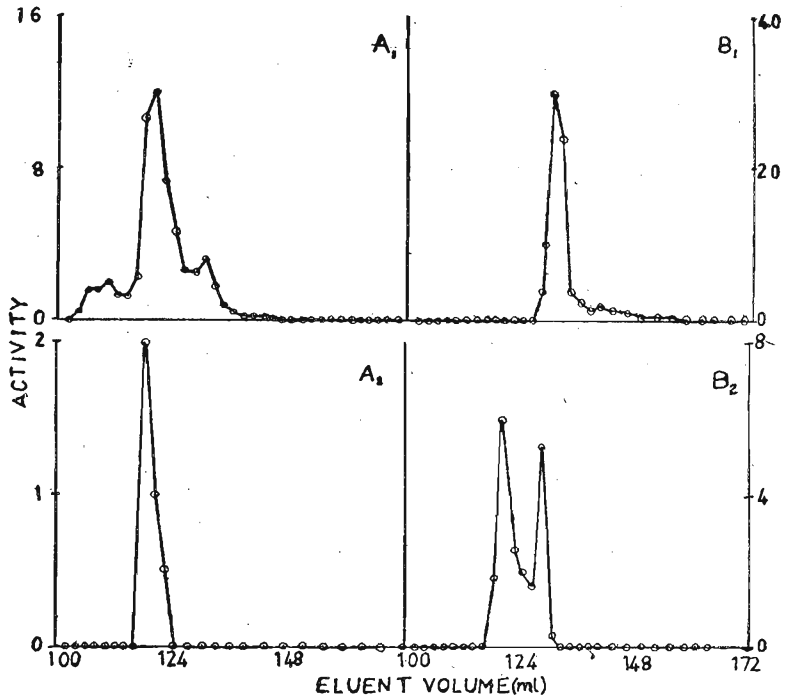

FIGURE 2—*Effect of 4M urea on linamarase A and B.*

A_1—Peak of MU 46 linamarase A, (see figure 1) rechromatographed on a DEAE-cellulose column.

A_2—Peak of A_1 rechromatographed on DEAE-cellulose after exposure to 4M urea.

B_1—Purified linamarase B rechromatographed on DEAE-cellulose.

B_2—Peak of B, rechromatographed on DEAE-cellulose after exposure to 4M urea.

Other details as in figure 1.

3.3 Some properties of linamarase C and D

The properties of linamarase A and B have been described in a previous communition.[4] Similar studies on linamarase C and D showed that like linamarase A and B, linamarase D had a disproportionate increase in activity with increased enzyme concentration. The opposite was found to be true for linamarase C which showed negative deviations (table 1). A time course of cyanide liberation of these two enzymes is shown in figure 3.

TABLE 1—Effect of enzyme concentration on activity of linamarase C and D.

Linamarase	Volume of extract (ml)	Cyanide released (umoles/min)	Activity (umoles/ml/min)
C	0.1	0.05	0.50
	0.2	0.098	0.49
	0.4	0.130	0.33
D	0.1	0.044	0.44
	0.2	0.098	0.49
	0.4	0.210	0.53

Concentration of protein in extract for enzyme D was 0.065 mg/ml. The corresponding value for enzyme C for this table of results is not available as the sample was lost in a laboratory accident.

For experimental details see section 2.

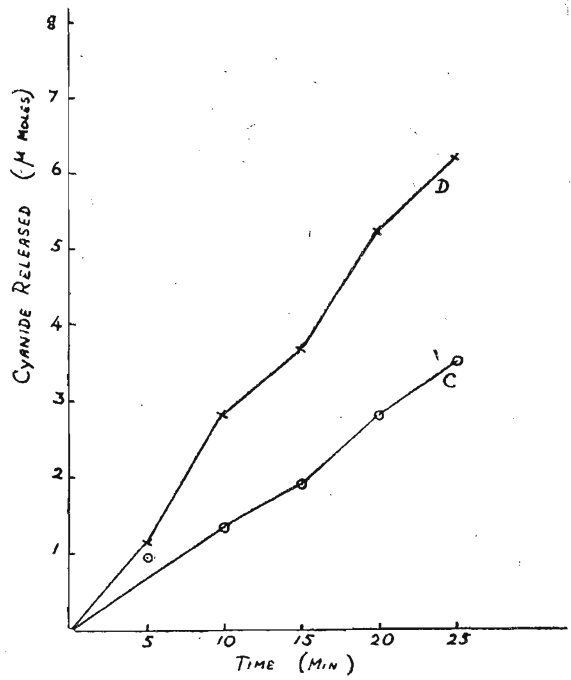

FIGURE 3—*Time course of initial reaction of linamarase C and D with linamarin*

The reaction mixture contained linamarin (25 mM) citrate buffer(0.08M, pH 5.6) and linamarase in a total volume of 0.37ml for each experimental point. The reaction which was carried out at room temperature was terminated by addition of Na_2CO_3—picric acid reaction mixture.[6]

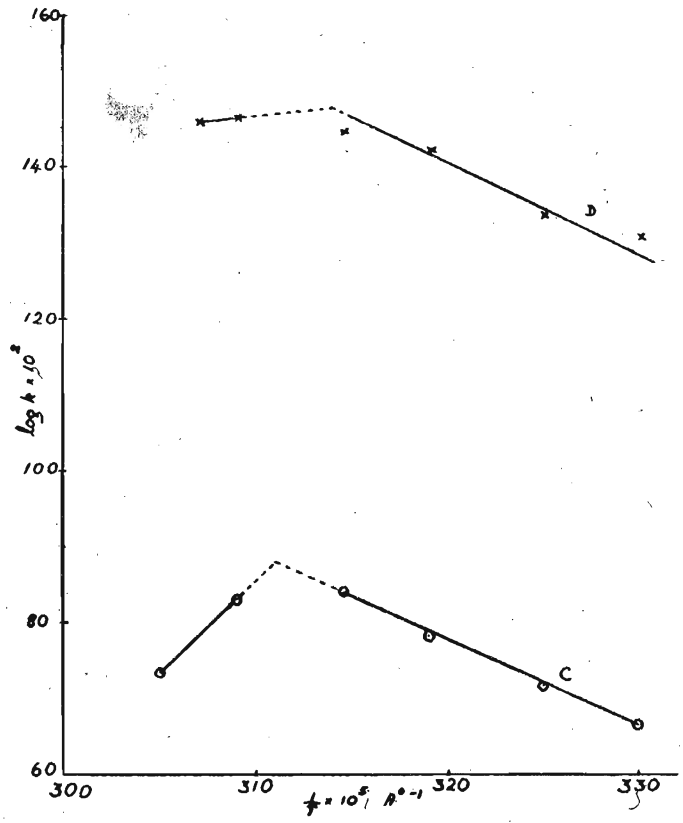

FIGURE 4—*Arrhenius plots of the activity of linamarase C and D at various temperatures.*

Experimental details as in figure 3. Reaction time was 10 min. and linamarase activity for C and D was 0.05 and 0.025 μmoles cyanide/min.

Linamarase C and D had activation energies of 5.2 Cal/mole and 5.4 Cal/mole respectively ; Arrhenius plots are shown in figure 4. Temperature optima of linamarase C and D were found to be 45°C and 55°C respectively, while temperature stability studies, in which the enzymes were incubated for 10 min. in a thermostated water bath prior to reaction with linamarin, showed that the two enzymes were 50% deactivated at 55°C and 66°C respectively, (figure 5).[4]

Table 2 gives a summary of some properties of linamarase C and D together with data obtained with linamarase A and B which were reported earlier.[4]

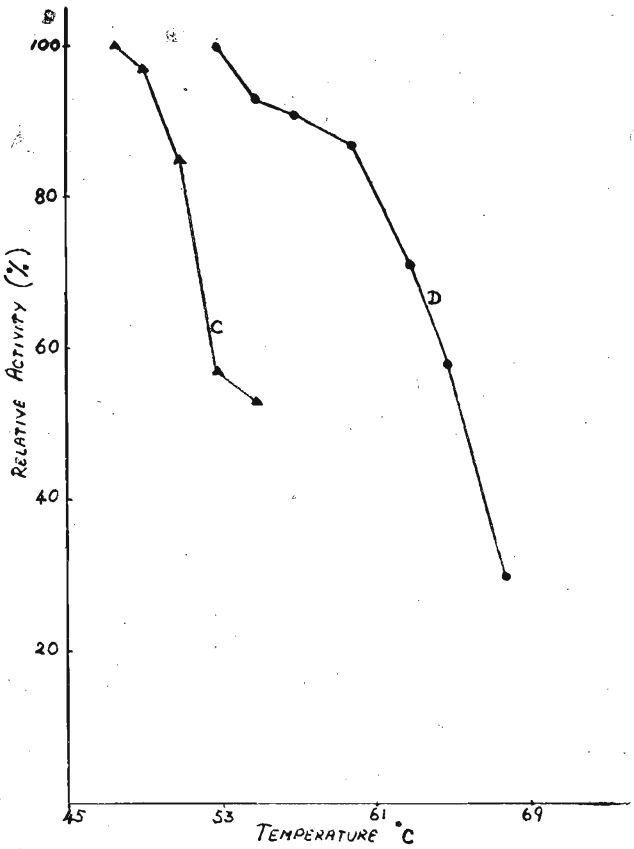

FIGURE 5—*Temperature stability of linamarase C and D.*

The enzymes were held for 10 min. in a thermostated water bath and then reacted at room temperature with linamarin as in figure 3. Reaction time was 20 min. and 10 min. for linamarase C and D respectively.

TABLE 2—Summary of properties of linamarase A, B, C and D.

Property	Linamarase			
	A*	B*	C	D
1. Position on DEAE-cellulose column	116	128	166	104
2. Migration to negative electrode on electrophoresis at pH 5.9 (mm)	23	08	—	—
3. Effect of enzyme concentration (deviation)	+ive	+ive	—ive	+ive
4. Michaelis Constant (K_m)	2.2	2.2—2.5	—	—
5. pH optimum	6—6.6	6.2	—	—
6. E Activation (K Cals/mole)	5.7	3.3	5.2	5.4
7. Temperature of 50% deactivation (°C)	60	62	55	66
8. Temperature optimum (to nearest 5°C)	45	60	45	55

*Published previously.[4]

—, No experimental point.

4. Discussion

Our previous communication[4] clearly demonstrated the existence of 2 linamarases in the extracts of manioc rind and the initial aim of these studies was to determine the distribution of these two enzymes in various cultivars of manioc.

Investigations revealed that multiple forms of linamarase activity were present in the rind of these cultivars. The enzymes were present in different proportions in the extracts of each cultivar. However, the absolute activity of each enzyme cannot be attributed much significance because of : (1) the possibility of selective loss of enzymes during the acetone purification stage and (2) the possibility that the ratios of the different enzymes could vary depending on factors such as environment and maturity of the tubers (on which no studies have been done). Nevertheless, these studies confirm the presence of several cyanide liberating enzymes which appear to vary in proportion from cultivar to cultivar.

A significant feature was that linamarase A and B represented the bulk of the activity in all cultivars. This fact together with the conversion of linamarase B to linamarase A in the presence of urea suggested that linamarase B is the major cyanoglucosidase of manioc rind while linamarase A is an artifact of acetone precipitation. However, this has to be confirmed since one line of evidence does not support this

conclusion. Namely, the formation of linamarase A from linamarase B does not result in an abnormal degree of loss of activity which leads to the conclusion that linamarase A (the sub-unit or modification) must be as active as the linamarase B (the enzyme *in vivo*). However, the temperature of deactivation of linamarase B is very comparable to temperature of loss of enzymic activity in the tissue (72°C) as reported by Joachim and Pandittasekere[2] giving some support to the theory that linamarase B is the form in which the enzyme is found in nature.

The presence of activity peaks C and D show that there are other proteins (in manioc rind) capable of hydrolysing linamarin. In one instance (MU 46) the activity of linamarase D was too large to be due to a non-specific β glucosidase and therefore the enzyme appears to be a true linamarase. Linamarase C is present only in small quantities in all cultivars and is probably a non-specific β glucosidase. In addition, DEAE column eluates have shown the presence of minor activity peaks.

From these studies it is concluded that multiple forms of linamarin hydrolysing enzymes are present in manioc rind. The significance of this situation is not understood but it will explain why cyanoglucosidase negative cultivars of manioc have not been demonstrated.

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