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PURIFICATION OF CASSAVA LINAMARASE

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Abstract—Linamarase was purified from parenchymal tissue of cassava by extraction with acetate buffer, fractional precipitation with ammonium sulphate, followed by column chromatography on DEAE-cellulose and Sepharose-6-B gel filtration. The specific activity is increased 350 fold with 35% recovery. The K_ms for linamarin and *p*-nitrophenyl β-D-glucoside are 1.45×10^{-3} M and 0.46×10^{-3} M, respectively. The pH optimum in 50 mM NaPi is pH 6 and the specific activity is 26.5 nkat/mg. The enzyme can be prepared from cassava peel using the same procedure and has similar properties.

INTRODUCTION

Cassava roots are an important staple food in the tropics [1] but contain varying and often large amounts of the cyanogenic glucosides linamarin, 2(β-D-glucopyranosyloxy) isobutyronitrile, and to a lesser degree lotaustralin, 2 (β-D-glucopyranosyloxy) 2-methylbutyronitrile [2]. Quantitative hydrolysis (or autolysis) of these cyanogenic glucosides is difficult and existing methods of assay are unreliable and lack sensitivity [3]. This paper reports the purification and characterisation of cassava linamarase (linamarin β-D-glucoside glucohydrolase; EC 3.2.1.21) with the aim of developing an enzymic essay for the cyanide content of cassava.

Wood [4] reported the preparation of linamarase from cassava peel using acetone precipitation; the sp. act. was only slightly greater than that of the crude extract. The enzyme has been purified from linseed (*Linum usitatissimum L*) meal by alcohol precipitation [5], stepwise DEAE-cellulose chromatography and Sephadex G200 followed by analytical scale column electrophoresis on Sephadex G25 [6]. The column electrophoresis gave a 105 fold purification; little purification resulted from the DEAE-cellulose and G200 stages. Linamarase from white clover (*Trifolium repens L.*) has been prepared by stepwise DEAE-cellulose chromatography, but no purification data was presented [7]. The β-glucosidases from the cyanophoric plants almond [8] and sorghum [9] have been purified, but these are specific for aryl cyanogenic glycosides [6].

RESULTS AND DISCUSSION

Purification of linamarase

The enzyme was extracted from cassava parenchymal tissue (peeled root) and subsequently from cortex tissue (cassava peel) by homogenisation in acetate buffer. The inclusion of 5mM dithiothreitol, EDTA, polyvinylpyrrolidone (PVP) (1%) or diethyldithiocarbamate (DIECA) did not alter the activity of these crude extracts. These compounds can reduce enzyme inhibition by phenolic compounds [10] and inclusion of DIECA and cysteine improved the linamarase yield from white clover leaves [11].

Fractionation of the crude extract from cassava parenchymal tissue with 60% $(NH_4)_2SO_4$ gave a 14 fold increase in sp. act. (Table 1). $(NH_4)_2SO_4$ was reported to be unsuitable for fractionation of β-glucosidase from alfalfa (*Medicago sativa L.*) [12], and alcohol or acetone precipitation was used for the initial purification of linamarase from linseed [5], white clover [7] and cassava peel [4]. Chromatography of the $(NH_4)_2SO_4$ fraction was monitored by assaying with both linamarin and *p*-nitrophenyl β-D glucoside since it has been suggested [6] that linamarase is specific for linamarin/lotaustralin and that hydrolysis of other glucosides is due to different β-glucosidases. Fig. 1 shows that the two activities follow one another closely. The breakdown of linamarin to HCN has been assumed [2] to depend on two enzymes: linamarase which produces hydroxyisobutyronitrile (acetone cyanohydrin) and hydroxynitrile lyase which catalyses the dissociation of the cyanohydrin. The latter enzyme has been purified from sorghum [13] and acts on *p*-hydroxymandelonitrile. Acetone cyanohydrin is less stable and dissociates readily at the alkaline pH used to stop the linamarase activity (Experimental) and this assay does not require such a secondary enzyme activity.

Two peaks of linamarase activity were eluted from DEAE-cellulose (Fig. 1), peak 1 being *ca* 24% the activity of peak 2. Linamarase from two other batches of parenchymal tissue eluted at about the same ionic strength (±15 mM NaCl) but peak 1 was 10 and 40% of peak 2 in these two cases. White clover linamarase was resolved into two types by its affinity for DEAE-cellulose in 60 mM Pi pH 6 [7]; one form is primarily associated with the seed (bound by the cellulose) and one form associated with young leaves (not bound by the cellulose). Stepwise elution of linseed linamarase from DEAE-cellulose gave only one peak [6], but such an elution scheme would not have resolved the peaks

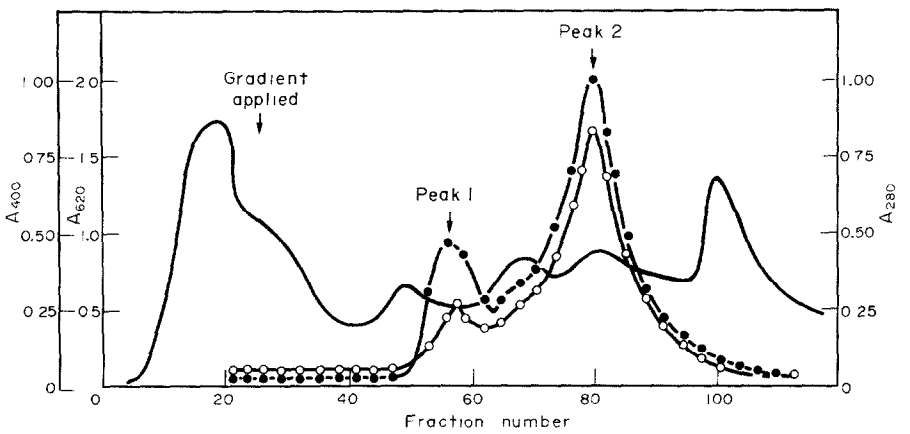

Fig. 1. Elution of linamarase from DEAE-cellulose by a linear NaCl gradient. A column (15 × 4.5 cm) of DEAE-cellulose was equilibrated in 10 mM Pi adjusted to pH 5.5 with 5N NaOH (buffer 1). 120 ml of the resuspended 60% $(NH_4)_2SO_4$ fraction was pumped (110 ml/hr) into the column which was then washed with 1 column vol. of buffer 1. A linear gradient of 1 l. of buffer 1 and 1 l. of buffer 1 containing 0.5 M NaCl was used to elute the proteins (started at fraction 22). The fraction size was 12.8 ml. Key to symbols: ——— A_{280} O——O A_{400} (glucosidase activity). ●——● A_{260} (linamarase activity).

shown in Fig. 1. A number of alfalfa seed β-glucosidase fractions were resolved by DEAE-cellulose chromatography [12].

The major peak (peak 2) from the DEAE-cellulose was eluted in the excluded volume from both Sephadex G150 and G200. Gel filtration on Sepharose 6-B gave a single peak with an apparent MW of *ca* 600000; this was not altered by the inclusion of 0.1 M NaCl in the 0.1 M acetate pH 5.5 buffer. This large apparent MW may be caused by an enzyme–carbohydrate complex, as has been suggested to explain the occurrence of different β-glucosidases in the same tissue [14, 12]. The recovery of activity from the column was almost 100%, but the enzyme was now more sensitive to $(NH_4)_2$ SO_4 precipitation (accounting for the loss recorded in the final stage). The final recovery was 35% with a 350 fold purification.

Properties of the enzyme

The K_ms of the purified enzyme for linamarin and *p*-nitrophenyl-β-D-glucoside (PNP-glucoside) are 1.45 mM and 0.46 mM, respectively at pH 6. The sp. act. is the same for both substrates. No data is available for the linseed, white clover or cassava peel preparations described by earlier workers. The β-glucosidases from *Sorghum vulgare* [9] have K_ms for PNP-glucoside in the range 0.13–0.31 mM the corresponding figures for β-glucosidases from barley [15] are 0.2–0.5 mM whereas a purified β-glucosidase from almond [16] had a K_m of 2.5 mM. The pH optima for these 3 enzymes are reported as 4.8, 4–4.5 and 5.6 respectively. The pH optimum of the purified cassava enzyme in McIlvaine buffer [17] is pH 6 for linamarin and 6.4 for PNP-glucoside. The pH optimum is broad: the activities at pH 5 and 7 corresponding to 79 and 86% of the activity at optimum pH (with linamarin). The optimum of white clover linamarase is pH 5 [18]. The activity of the purified cassava enzyme in NaPi pH 6 is almost independent of buffer molarity in the range 5–500 mM with both substrates. The crude enzyme has a very similar pH and ionic strength dependence.

The activity of the purified enzyme is unaffected by inclusion of 10 mM glucose, KCN, EDTA, iodoacetate, dithiothreitol or cellobiose in the assay medium of either substrate. The lack of inhibition by cellobiose is in agreement with earlier studies [6, 19] indicating separate roles for β-disaccharase and β-glucosidase activities. The enzyme is inhibited competitively by glucono-(1–5)-lactone with a K_i of 0.15 mM (linamarin substrate) like other plant glycosidases [9, 15]. The PNP-galactosidase (EC 3.2.1.23) activity of the crude enzyme is *ca* 50% that of the PNP-glucosidase activity. This figure has been reduced to 5% in peak 2 (Fig. 1) and was not detectable after the Sepharose 6-B gel filtration. In contrast both galactosidase and glucosidase activities appear to be exhibited by multifunctional enzymes in almond [16] and white clover [7, 11], whereas β-glucosidase fractions with and without β-galactosidase activities have been isolated from alfalfa [12] and barley [15].

Linamarase from cassava peel

Preliminary studies on the extraction of linamarase from cassava peel confirmed that a greater concentration is present in peel than parenchyma [20] but the total activity in the latter exceeds the former because of its greater mass. The enzyme is precipitated by 60% $(NH_4)_2SO_4$, eluted from DEAE-cellulose as a single peak in about the same position (±20 mM NaCl) as peak 2 in Fig. 1. The peel enzyme resembles the parenchymal enzyme in the pH and ionic strength dependence and K_m for linamarin. The sp. act. of the crude extract (0.42 nkats/mg) and initial $(NH_4)_2SO_4$ concentrate (30 nkats/mg) are much higher than the corresponding figures for the parenchymal enzyme (Table 1). DEAE-cellulose chromatography gives a *ca* 2 fold increase in sp. act. The final purification of the peel enzyme is uncertain because of large losses encountered on $(NH_4)_2SO_4$ concentration of the purified enzyme. The greater sp. act. of the peel fractions is the subject of further investigations.

The purified parenchymal enzyme and the $(NH_4)_2SO_4$ concentrated peel enzyme are both sufficiently active

Table 1. Purification and yield of linamarase from cassava parenchymal tissue

	Volume (ml)	Protein (mg)	Units of activity (nkats)	Specific activity (nkats/mg)	Purification	Yield (%)
1. Crude extract (500 g tissue in 1600 ml acetate pH 5.5)	1500	4350	329	0.076	—	100
2. 60% $(NH_4)_2SO_4$ concentrate	120	276	294	1.07	14.2	90
3. Eluate from DEAE-cellulose Peak 1	20	—	53	—	—	16
after $(NH_4)_2SO_4$ concentration. Peak 2	28.6	91.6	220	2.4	31.7	66.8
4. Eluate of peak 2 from Sepharose-6-B after $(NH_4)_2SO_4$ concentration	14	4.37	116	26.5	351	35.2

to hydrolyse quantitatively dilute linamarin solutions and they are being evaluated for use in the determination of cyanide in cassava and cassava products. The $(NH_4)_2SO_4$ concentrated peel enzyme can be frozen and freeze-dried with negligible loss in activity; its sp. act. is *ca* 50 times greater than the peel preparation reported by Wood [4].

EXPERIMENTAL

Chemicals were purchased from the following companies: *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl β-D-galactoside, glucono-(1–5)-lactone and BSA from Koch-Light Ltd. Linamarin from Calbiochem. Bovine thyroglobulin, bovine heart lactate dehydrogenase from Sigma.

Enzyme preparation. Cassava roots were purchased from Kenya Horticultural Exporters (Nairobi) and air-freighted to London where they were peeled, sliced and deep frozen (−20°) on the day of receipt (within 7 days of harvest). The peeled storage parenchymal tissue was diced (1 cm cubes) and 100 g homogenized for 3 min at high speed in 200 ml of 0.1 M acetate pH 5.5 in a Waring Blendor, 600–800 g being treated in this manner for each prepn. The homogenate was centrifuged at 10000 *g* for 30 min and the supernatant brought to 60% satn of $(NH_4)_2SO_4$ and held at 4° for 16 hr. The ppt. obtained by centrifugation at 10000 *g* for 1 hr was dissolved in and dialysed (3 × 50 vol) against 10 mM Pi buffer 5.5. This soln was applied to a column of DEAE-cellulose as described in the legend to Fig 1. Fractions of the major peak (70–90) were pooled, pptd with $(NH_4)_2SO_4$ as above, and re-dissolved in 0.1 M acetate pH 5.5. Batches (0.9 ml) of this soln were applied to analytical columns of Sephadex G150, G200 and Sepharose 6B. Preparative fractionation on Sepharose 6B (58 × 2.7 cm) was carried out at a flow rate of 55 ml/hr and 6 ml fractions were collected. The linamarase peak fractions were pooled, and pptd with 60% $(NH_4)_2SO_4$ for 2 hr at 4°. The ppt. obtained by centrifugation at 10000 *g* for 1 hr was redissolved in 0.1 M acetate pH 5.5. The data shown in Table 1 are the mean of two separate purifications. *Ca* protein concns were measured by the *A* at 280 nm corrected for that at 260 nm [21]. Linamarase was prepared from cassava peel by homogenising 25 g of diced (0.2 × 0.5 cm) peel in 200 ml 0.1 M acetate pH 5.5 as described above.

Enzyme assays. Linamarase activity was assayed at 30° by adding 0.1 ml aliquots of the enzyme to tubes containing 2.5 μmol linamarin in 0.1 M NaPi pH 6 (final vol. 0.6 ml). The reaction was stopped after 30 min by addition of 0.6 ml 0.2 N NaOH. The HCN released was estimated by modification of Epstein's [22, 23] method as follows. 6.8 ml of 50 mM NaPi pH 6 was added to the tubes followed by 0.4 ml of 0.5% (w/v) chloramine T. The tubes were mixed and stood in iced water for 5 min. 1.6 ml of a soln containing 0.2 g bispyrazolone (3,3'-dimethyl-1,1'-diphenyl-(4,4'-bi-2-pyrazoline)-5,5'-dione) and 1 g of 3-methyl-1-phenyl-5-pyrazolone in Py (200 ml) was then added and mixed. The blue colour developed after storage for 90 min at room temp. was measured at 620 nm. The *A* at 620 nm did not change (<5%) between 60 and 130 min after addition of the Py reagent. Chloramine T incubation times from 3 to 15 min made no difference to the final A_{620}. KCN dried over conc H_2SO_4 was used to calibrate the *A* values, 5 μg KCN in the 10 ml final vol. gave *ca* 0.84 A_{620}. The rate of release of HCN was proportional to added enzyme in the range 0 to 1.2 A_{620}/30 min. PNP-glucosidase and PNP-galactosidase activities were determined using 10 mM substrates in 0.1 M NaPi pH 6. The reaction was stopped after 1 hr by addition of 0.2 M borate pH 9.8 and the *p*-nitrophenol measured at A_{400} [24].

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