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STUDIES ON CASSAVA, *MANIHOT UTILISSIMA* POHL—I.

CYANOGENESIS: THE BIOSYNTHESIS OF LINAMARIN AND LOTAUSTRALIN IN ETIOLATED SEEDLINGS

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Abstract—No HCN could be detected in seeds of one cultivar of *Manihot utilissima* Pohl whereas seeds of two other cultivars contained 5·2–8·5 μg HCN/g fresh weight. However, 10–14-day-old seedlings of all three cultivars contained 156–260 μg HCN/g fresh weight. Thus a rapid biosynthesis of cyanogenic material occurred during germination. Chromatographic analysis showed that linamarin, 2(β-D-glucopyranosyloxy) isobutyronitrile, accounted for 93 per cent, while lotaustralin, 2(β-D-glucopyranosyloxy) 2-methylbutyronitrile, accounted for 7 per cent of the total HCN evolved by autolysing etiolated seedlings. L-Valine^{14}C (U) and L-isoleucine^{14}C (U) were incorporated by etiolated seedlings into the aglycone moieties of linamarin and lotaustralin respectively, indicating that the amino acids are effective precursors of these glucosides in *M. utilissima*. Seedlings of all three cultivars contained linamarase, the β-glucosidase which catalyses the hydrolysis of both glucosides. A crude preparation of the enzyme from leaves showed strong activity against linamarin and lotaustralin, mild activity against salicin, and weak activity against β-methyl glucoside and amygdalin.

INTRODUCTION

THE cassava plant, *Manihot utilissima* Pohl, is widely distributed in the tropics where it has achieved a considerable economic importance as a source of industrial and dietary carbohydrate. A biochemical feature of the plant is that it synthesizes and accumulates cyanogenic material in the edible tubers. Generally, "sweet" and "bitter" varieties of the plant are distinguished on the basis of a low and a high concentration of cyanogenic material in tubers.[1]

In a biochemical and physiological study of the plant, it was considered desirable to examine the question of cyanogenesis, in the light of recent studies of the phenomenon in many plant species of economic importance. Since Dunstan, Henry and Auld[2] isolated and identified linamarin, 2(β-D-glucopyranosyloxy) isobutyronitrile (1), in cassava tubers, it has generally been considered that this cyanogenic glucoside occurs alone in the plant. However, Butler[3] has recently shown that plants which have previously been shown to contain linamarin generally also contain its higher homologue lotaustralin, 2(β-D-glucopyranosyloxy) 2-methylbutyronitrile (2). Further, it has been shown that in *Linum usitatissimum*,[4] *Lotus arabicus* and *L. tenuis*,[5] the aglycone moieties of linamarin and lotaustralin are synthesized from valine and isoleucine respectively.

The present paper presents evidence for the occurrence of both linamarin and lotaustralin in seedlings of *M. utilissima*, as well as for the incorporation of uniformly labelled L-valine^{14}C and L-isoleucine^{14}C into the aglycone moieties of the respective glucosides. The preparation of crude linamarase from leaves of the plant is also described.

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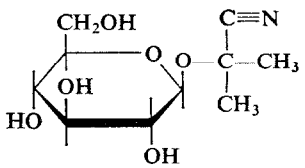
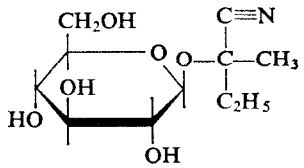


1. Linamarin



2. Lotaustralin

RESULTS

When seeds of a cultivar of *Manihot utilissima* obtained from Ghana were frozen in liquid nitrogen, ground, suspended in water or linamarase solution and aerated for 24 hr, no HCN could be detected. On the other hand seeds of a Brazilian cultivar (Santa Catarina) and seeds obtained commercially (OE) gave small quantities of HCN under similar treatment. However, 10–14-day-old seedlings of all three cultivars gave large amounts of HCN (Table 1). This shows that a rapid biosynthesis of cyanogenic material occurs during germination.

TABLE 1. CONCENTRATION OF CYANOGENIC GLUCOSIDES IN TISSUES OF THREE CULTIVARS OF *M. utilissima*

Cultivar	Tissue analysed	Concentration of cyanogenic glucosides (μg HCN/g fresh weight tissue)
Ghana	Seeds	0·0
Ghana	Seedlings	260·0
Ghana	Leaves	468·0
Ghana	Roots	126 0
Brazil (Santa Catarina)	Seeds	8·5
Brazil (Santa Catarina)	Seedlings	156·0
Brazil (Santa Catarina)	Leaves	239·0
Brazil (Santa Catarina)	Roots	79·0
Brazil (Santa Catarina)	Tubers	282·0
Commercial (OE)	Seeds	5·2
Commercial (OE)	Seedlings	252 0

1–3 g tissue was frozen in liquid nitrogen, ground and suspended in water or linamarase solution. Suspensions were aerated for 24 hr and released HCN collected in 0·1 N NaOH and estimated by titration with 1 mM $AgNO_3$ or by the colorimetric method of Aldridge.[12] Figures represent mean values from six estimations for each tissue.

Paper chromatographic analysis of aqueous extracts from seedlings showed that both linamarin and lotaustralin were present. With 2-propanol: water (7:1 v/v), both glucosides were located at R_f 0·80, glucose and valine at R_f 0·50, and isoleucine at R_f 0·57. When the glucosides were eluted and rechromatographed with propanol: water (7:3 v/v), linamarin (R_f 0·65) was well separated from lotaustralin (R_f 0·74); methyl-ethyl ketone: acetone: water (30:10:0·6 v/v),[4] also separated the glucosides (R_f 0·59 and 0·72 respectively). Occasionally, a modification of the last solvent in the ratio 30:11:6 v/v was employed to further purify the glucosides which were located at R_f 0·34 and R_f 0·48 respectively. Quantitative estimations of the resolved glucosides showed that linamarin accounted for 93 per cent while lotaustralin accounted for only 7 per cent of the total cyanogenic material present in the extracts.

To confirm the occurrence of lotaustralin, and to determine whether valine and isoleucine

are effective precursors of linamarin and lotaustralin respectively in *M. utilissima*, uniformly labelled L-valine^{14}C and L-isoleucine^{14}C were fed to 10–14-day-old etiolated seedlings for 48 hr under constant illumination. Seedlings were subsequently extracted, and extracts evaporated to dryness *in vacuo* at 35°. Residues were dissolved in 5-ml aliquots of 10 per cent 2-propanol and 500 μl aliquots separated chromatographically. In all cases, standards of authentic cyanogenic glucosides were run on the same paper. The glucosides were located by their R_f values or by radioautography.

Regions on the chromatograms corresponding to the positions of the glucosides were eluted. Eluates were dried in planchets and counted for radioactivity. Planchets were then treated with 500 μl aliquots of linamarase, incubated overnight, dried and counted again and it was found that most of the activity in linamarin and lotaustralin had been lost. It will be noticed in Table 2 and that there was some residual radioactivity after the removal of the radioactive volatile aglycone moieties. This was probably due to radioactive impurities with R_f's similar to those of the glucosides, or due to labelled glucose, since both valine and isoleucine are known to be glucogenic.

TABLE 2. TOTAL RADIOACTIVITY OF ELUATES FROM THE POSITIONS OF LINAMARIN AND LOTAUSTRALIN ON CHROMATOGRAMS OF EXTRACTS FROM 10–14-DAY-OLD SEEDLINGS FED L-VALINE^{14}C (U) OR L-ISOLEUCINE FOR 48 hr, BEFORE AND AFTER TREATMENT WITH LINAMARASE

Compound fed	Glucoside	Radioactivity (cpm × 10^{-2})		
		Before treatment with linamarase	After treatment with linamarase	In aglycone
L-Valine	Linamarin	266·0	32·0	234·0
	Lotaustralin	6·0	4·0	2·0
L-Isoleucine	Linamarin	19·0	19·0	0·0
	Lotaustralin	125·0	24·0	101·0

Aqueous extracts of seedlings and leaves of all three cultivars of *M. utilissima* were found to possess strong hydrolytic activity against linamarin and lotaustralin. The enzyme was obtained in crude form by acetone precipitation of aqueous extracts prepared from the acetone powder of leaves. The bulk of the enzyme was precipitated with cold acetone

TABLE 3. HYDROLYSIS OF β-GLUCOSIDES BY CRUDE LINAMARASE FROM LEAVES OF *M. utilissima*

β-Glucoside	Per cent hydrolysis
Linamarin	81·5
Lotaustralin	79·1
Salicin	11·4
β-Methyl glucoside	1·4
Amygdalin	0·2

Reaction mixtures contained 5 mg substrate and 400μl enzyme solution (234 μg protein) in a total volume of 2 ml 0·067 M phosphate buffer, pH 6. Mixtures were incubated under toluene at 30° and samples analysed for liberated glucose at 10 hr by the method of Nelson.[14] Protein was estimated by the method of Lowry *et al.*[15]

(−40°) at 75 per cent by volume. A solution of this precipitate dissolved in phosphate buffer, pH 6, was used for spraying chromatograms prior to location of the glucosides. In activity tests against different β-glucosides, it was found that the crude enzyme rapidly hydrolysed linamarin and lotaustralin. It showed mild activity against salicin but only weakly hydrolysed β-methyl glucoside and amygdalin (Table 3).

DISCUSSION

The fact that seeds from some cultivars of *Manihot utilissima* contain cyanogenic material, whereas seeds from other cultivars contain none may be associated with the distinction between the "sweet" and "bitter" varieties of the plant and is analogous with the occurrence of amygdalin in seeds of the "bitter" but not the "sweet" variety of *Prunus amygdalus*. Analysis of seeds for HCN may therefore be of taxonomic value in differentiating between the "sweet" and "bitter" varieties, since differentiation based on tuber analysis for HCN or on morphological structure of plants has proved inconclusive.[1]

Germinating seeds synthesize relatively large quantities of cyanogenic glucosides, and are therefore well suited for *in vivo* studies on the biosynthesis of these glucosides. The rapid biosynthesis of cyanogenic glucosides has also been shown to occur in seedlings of other plants.[4–6] Although linamarin has long been known to occur in *M. utilissima*, lotaustralin has hitherto not been reported as a constituent of this species; recently, Butler[3] reported the occurrence of both compounds in the roots of *M. carthaginiensis* Muell. Arg. where the former accounts for 96 per cent and the latter 4 per cent of the total cyanogenic glucosides. Their occurrence in *M. utilissima* in similar concentrations (93 per cent and 7 per cent) is in agreement with this observation.

The effective utilization of uniformly labelled L-valine^{14}C and L-isoleucine^{14}C as precursors of the aglycone moieties of linamarin and lotaustralin respectively, indicates that the general pathway of biosynthesis of the glucosides is similar in *M. utilissima*, *Linum usitatissimum*,[4] *L. arabicus* and *L. tenuis*.[5]

The ability of some plants to utilize HCN for the biosynthesis of asparagine,[5] and the rhythmic fluctuations in cyanogenic glucoside concentration noted in some of these plants[7] suggest that cyanogenic glucosides are active rather than inert constituents of these plants. During germination high concentrations of valine and isoleucine are made available by the action of proteinases and peptidases on reserve proteins. Under non-photosynthetic conditions in the dark, it is highly probable that these amino acids are catabolized through energy-yielding processes which lead to the formation and accumulation of high concentrations of the glucosides. The occurrence of highly active linamarase in seedlings provides the condition under which the glucosides may subsequently be hydrolysed and the hydrolytic products made available for respiratory break-down or incorporation into amino acids for protein synthesis.

Unlike certain strains of white clover which contain the glucosides but not the enzymes which hydrolyse them,[8] seedlings of all the three cultivars of *M. utilissima* investigated were found to contain the glucosides as well as linamarase with a wide substrate specificity. Crude and partially purified preparations of the enzyme from white clover[9] and linseed[10] have also been shown to exhibit wide substrate specificities.

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EXPERIMENTAL

Materials

Santa Catarina seeds and stem-cuttings were kindly supplied by the Instituto Agronomico, Campinas, Brazil, and those of the Ghana cultivar were obtained from the Crops Research Institute, Ghana Academy of Sciences. Seeds designated OE were purchased from Ohlsens Enke, Copenhagen. Plants were grown in the greenhouse of the Botanical Gardens of the University of Copenhagen at 22–25° and 60–70 per cent relative humidity.

Authentic cyanogenic glucosides used as standards were kindly donated by Professor E. E. Conn, Dr. G. W. Butler and Dr. R. C. Clapp. Solutions of L-valine^{14}C (U) and L-isoleucine^{14}C (U) were purchased from the Radiochemical Centre, Amersham.

Seed Germination

Seeds were filed slightly at the region of the micropyle and sterilized in 10 per cent H_2O_2 for 30 min. The H_2O_2 was replaced with tap water and seeds aerated overnight. After thorough rinsing with redistilled water, seeds were placed on a plastic net covered with three layers of muslin. The net was placed in a light-proof glass tank containing dilute mineral solution.[11] The tank was then placed in the dark room at 29° with continuous aeration of the mineral solution. After 10–14 days seeds had grown uniformly in thickness and height (10 cm).

Collection and Estimation of HCN

Seed testa were removed manually and intact kernels frozen in liquid N_2 and ground. The ground material was transferred to 50- or 100-ml Erlenmeyer flasks connected to wash-flasks which were in turn connected to micro-Kjeldahl flasks containing 10 ml 0·1n NaOH. Water or linamarase was added to each sample and the system aerated gently with purified air for 24 hr at 30°. HCN evolved during hydrolysis of glucosides in all tissues was collected in this manner and estimated by titration with 1 mM $AgNO_3$ or by the colorimetric method of Aldridge,[12] using a Zeiss Spectrophotometer Model PMQ 11.

Extraction of Cyanogenic Glucosides

Frozen seedlings or leaves were ground and covered with 10 volumes of boiling 80 per cent ethanol, boiled and then homogenized in a multi-mixer for 1 min. The slurry was filtered and washed with hot aqueous ethanol. The combined filtrates were evaporated to dryness and the residue re-extracted with hot methanol. The methanolic solution was evaporated to dryness, and the residue re-extracted with hot ethyl acetate from which a cream solid was obtained. Aliquots of a solution of the product in 10 per cent 2-propanol were streaked on Whatman No. 3 paper and developed by ascending chromatography with 2-propanol:water (7:1). Concentrated eluates from areas at R_f 0·80 were rechromatographed on Whatman No. 1 paper with methylethyl ketone:acetone:water (30:10:0·6) (descending). Eluates from areas at R_f 0·59 were dried and residues crystallized from hot ethyl acetate. The product, linamarin, melted at 139–140° and gave a positive test on incubation with linamarase in the presence of Na-picrate paper. Lotaustralin was not isolated. The glucosides were located by lightly spraying developed chromatograms with linamarase and incubating them in a perspex press at 30° with Na-picrate paper in adjacent position.[13] The orange–red spots which developed on the Na-picrate paper during the release of HCN marked the positions of the glucosides.

Proportions of glucosides were estimated by hydrolysing the resolved glucosides with linamarase and determining evolved HCN.

Feeding of Uniformly Labelled L-*Valine*^{14}C *and* L-*Isoleucine*^{14}C

Six intact and uniform etiolated seedlings weighing 3–4 g were placed in 5-ml beakers containing either 0·018 μM L-valine^{14}C (U) (2·5 μc) in 55 μl of 2% ethanol or 0·02 μM L-isoleucine^{14}C (U) (2·5 μc) in 45 μl of 2% ethanol, and illuminated continuously in a ventilated fume chamber at 29° for 48 hr and "washed in" with 2 ml redistilled water added to each beaker in aliquots of 200 μl during the period of feeding. Rinsed seedlings were frozen in liquid N_2, ground and extracted with 10 volumes of 80 per cent ethanol. Extracts were evaporated to dryness and residues dissolved in 5 ml 10 per cent 2-propanol. 500 μl aliquots were chromatographed with solvent 1. The glucosides were eluted and rechromatographed with solvent 3 or its modification. The positions of the glucosides were determined by reference to R_f values or radioautographs prepared by contact of chromatograms with Kodak X-ray film for 7 days. Seedlings of the Ghana cultivar were used for the labelling experiments, and radioactivity was measured with a Frieseke and Hoepfner windowless flow counter.

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Crude Linamarase from Leaves

In a typical experiment, 100 g fresh weight leaves were homogenized in cold acetone (−40°) and filtered. The residue was washed with cold acetone until the filtrate was clear of pigments. The product, a cream-coloured powder, was air-dried and suspended in 300 ml 0·067 M phosphate buffer, pH 7·3, containing 0·3 mM cysteine. The suspension was stirred for 1 hr, left standing overnight in the cold room and squeezed through three layers of muslin.

The filtrate, pH 6·75, was centrifuged and the supernatant treated slowly with cold acetone (−40°) to 75 per cent by volume. The precipitate which formed after standing for 1 hr was collected at the centrifuge, washed with cold 75 per cent acetone and dissolved in 500 ml phosphate buffer, pH 6. The solution was centrifuged to remove undissolved material and the supernatant used for spraying chromatograms and for the activity tests described. Linamarase activity was determined by incubation of aliquots of the crude enzyme with substrates under toluene at 30° and estimation of liberated glucose by the method of Nelson.[14] Protein was estimated by the method of Lowry *et al.*[15]

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