Pergamon

0031-9422(94)00878-7

Phytochemistry, Vol. 39, No. 2, pp. 323–326, 1995

0031-9422/95 $9.50 + 0.00

THE BIOSYNTHESIS OF CYANOGENIC GLUCOSIDES IN ROOTS OF CASSAVA

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(*Received in revised form* 21 *September* 1994)

Key Word Index—*Manihot esculenta*; Euphorbiaceae; cassava root; cyanogenic glucosides; biosynthesis.

Abstract—Linamarin is the main cyanogenic glucoside of cassava. *De novo* synthesis of linamarin in cassava roots was demonstrated *in vivo* by feeding [^{14}C]valine to excited segments of phelloderm. *In vitro*, a microsomal enzyme system isolated from cassava roots was shown to catalyse the conversion of valine to acetone cyanohydrin, the aglucone of linamarin. An antibody raised against cytochrome $P450_{TYR}$, the enzyme which catalyses the initial step in the biosynthesis of the cyanogenic glucoside dhurrin in sorghum, cross-reacts with a major polypeptide of similar molecular mass in cassava microsomes. Cyanogenic glucosides are known to accumulate in cassava roots, but hitherto *de novo* synthesis has only been demonstrated in the leaves, suggesting translocation of cyanogenic glucosides from leaves to roots. Our results show that at least part of the cyanogenic glucosides are synthesized in the roots. The data demonstrate that acyanogenic cassava roots cannot be obtained solely by blocking the transport of cyanogenic glucosides to the roots from other parts of the cassava plant.

INTRODUCTION

Cyanogenic glucosides are amino acid-derived plant constituents present in more than 2500 plant species. Cassava contains two major cyanogenic glucosides linamarin (2-β-D-glucopyranosyloxy-2-methylpropiononitrile) and lotaustralin [(2*R*)-2-β-D-glucopyranosyloxy-2-methylbutyronitrile] derived from valine and isoleucine, respectively. The ratio of linamarin and lotaustralin in leaves and roots is about 93:7. Cassava is an important crop in tropical and subtropical regions where its large tuberous and starchy roots form the major staple food for over 300 million people [1].

In cassava, cyanogenic glucosides are known to accumulate in all parts of the plant, but *de novo* synthesis of cyanogenic glucosides has only been demonstrated *in vivo* in the primary leaves of young seedlings [1, 2]. In *Hevea brasiliensis*, the translocation of cyanogenic glucosides are suggested to proceed by the 'linustatin pathway' [3, 4]. According to this pathway linamarin and lotaustralin are initially glucosylated to produce the diglucosides linustatin and neolinustatin which then serve as transient forms resistant to the hydrolysis by β-glucosidases present in apoplastic spaces [3, 4]. Based on isolation of trace amounts of linustatin and neolinustatin from cassava seedlings, Lykkesfeldt and Møller have suggested that the linustatin pathway is also involved in the translocation of cyanogenic glucosides from the leaves to the roots in cassava plant [2].

In vitro biosynthesis of cyanogenic glucosides in cassava has been demonstrated by Koch *et al.* using a microsomal enzyme system isolated from etiolated cassava seedlings [5]. This microsomal enzyme system catalyses the conversion of valine and isoleucine to the corresponding cyanohydrins which dissociate into the corresponding aldehydes or ketones and HCN, the end products obtained *in vitro*. *In vivo*, the cyanohydrins are glucosylated into linamarin and lotaustralin by a soluble UDP-glucose:glucosyltransferase [1]. The involvement of cytochrome P450 in biosynthesis of cyanogenic glucosides has been demonstrated in the microsomal enzyme systems isolated from etiolated seedlings of sorghum and cassava [5, 6]. In the present paper, we demonstrate *de novo* synthesis of linamarin in cassava roots both *in vivo* and *in vitro*. Our results show that at least part of the cyanogenic glucosides are synthesized in the roots.

RESULTS AND DISCUSSION

Cassava roots consist of three distinct regions: the phelloderm which is the 1–4 mm thick readily removable peel, the cortex which consists of parenchyma cells and constitutes the region of carbohydrate storage, and the inner core which is the central vascular cylinder [1]. In

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the present study, the HCN potential of cassava roots of different varieties and at different developmental stages was found to vary between 20 and 50 μg HCN g^{-1} fr. wt. In old roots (12 months), the highest concentration of cyanogenic glucosides is found primarily in the phelloderm, and with a gradient of decreasing concentration of cyanogenic glucosides from the phelloderm towards the inner core. Similar results have been obtained by Bokanga [7]. In young roots (4 months), the cyanogenic glucosides are more evenly distributed across the tubers (data not shown).

For examination of *in vivo* biosynthesis of linamarin in cassava roots, [^{14}C]valine was administered to excised pieces of phelloderm from young and old roots of sweet and bitter varieties. After incubation, cyanogenic glucosides were extracted from the tissues and analysed by TLC. All extracts contained radioactively labelled linamarin as evidenced by co-migration with authentic linamarin as exemplified by the data from old roots of the sweet variety (Fig. 1). Under the experimental conditions used, where non-saturating concentrations of substrate were used, similar incorporation rates were obtained independent of age and variety. The identification of linamarin was confirmed by release of HCN from the corresponding position on the TLC plates upon spraying with a solution of β-glucosidase and monitoring using Feigl/Anger paper (data not shown). The *in vivo* data demonstrate *de novo* biosynthesis of linamarin in the cassava root.

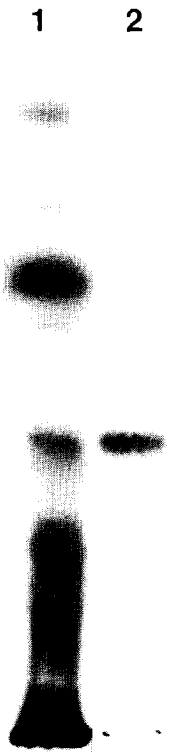

Fig. 1. TLC chromatogram demonstrating *in vivo* biosynthesis of linamarin in the phelloderm of cassava roots. Lane 1, autoradiogram of TLC plate with methanol extracts of phelloderm from old cassava roots of the sweet variety. Lane 2, linamarin standard.

In vitro biosynthesis of cyanogenic glucosides has been demonstrated in various cyanogenic plants using microsomal enzyme systems isolated from etiolated seedlings of the corresponding plants [8]. In the present study, a biosynthetically active microsomal enzyme system was isolated from cassava roots of both sweet and bitter varieties. Using valine as substrate, the microsomal enzyme system showed NADPH-dependent HCN formation (Table 1). This is the first demonstration of *in vitro* biosynthetic activity from cassava roots. The microsomal enzyme system isolated from the young sweet variety showed the highest biosynthetic activity. Lower levels of biosynthetic activity were obtained in older roots. This might reflect the increased difficulties in extracting biosynthetically active microsomes from increasingly woody roots. Based on the *in vitro* biosynthetic activity, the potential for biosynthesis of cyanogenic glucosides in young sweet roots can be calculated as 70 ng HCN g^{-1} fr. wt hr^{-1}. In the presence of a saturating amount of valine and no turn-over of the cyanogenic glucosides once synthesized, a synthesis period of *ca* two weeks will be sufficient to reach the endogenous level in four-month-old roots.

Cytochrome P450 enzymes are involved in the biosynthesis of cyanogenic glucosides as well as in many other metabolic pathways [8, 9]. Based on CO difference spectra, the total content of cytochrome P450 in the microsomal preparation from young sweet roots is *ca* 0.1 nmol mg^{-1} protein. A similar cytochrome P450 content has been reported in microsomal preparations from etiolated sorghum seedlings [6]. Dialysis of the cassava microsomal preparation for removal of endogenous HCN prior to measurement of HCN production resulted in loss of more than half of the amount of cytochrome P450. This indicates that the *in vitro* rate of biosynthesis obtained above represents a minimum rate. Polyclonal antibodies against cytochrome $P450_{TYR}$, the cytochrome P450 enzyme catalysing the multistep conversion of tyrosine to *p*-hydroxyphenylacetaldoxime in the biosynthesis of the cyanogenic glucoside dhurrin in sorghum [10], recognize a major polypeptide with an apparent M_r of 55 000 among the microsomal proteins of the cassava

Table 1. *In vitro* biosynthesis of linamarin by the microsomal enzyme system isolated from cassava roots. The biosynthetic activity was measured colorimetrically as HCN formation using valine as substrate

Cassava variety	ng HCN mg^{-1} microsomal protein hr^{-1}
young, sweet	70
young, bitter	32
old, sweet	24
old, bitter	34

root. A stronger cross reaction is obtained with microsomal proteins from the sweet variety compared to the bitter variety (Fig. 2). This correlates with the higher biosynthetic activity observed with the sweet variety compared to the bitter variety and suggests that the antibodies are recognizing a cyanogenic-specific protein.

In etiolated seedlings of cassava, cyanogenic glucosides are synthesized in the cotyledons and transported to root and hypocotyl [5]. In the mature cassava plant, a transport of cyanogenic glucosides from the green leaves to the roots has been postulated by de Bruijn based on ringing experiments which showed accumulation of cyanogenic glucosides in the stem cortex above the incision [11]. Our results show that the cassava root itself is actively synthesizing linamarin. The pool of free valine may vary greatly during the life cycle of the cassava plant and from one tissue to another. Consequently, the rates observed in the *in vitro* experiments cannot easily be converted into *in vivo* rates. Nevertheless, the high *in vitro* rates reported in this paper suggest that the biosynthesis of cyanogenic glucosides in the cassava root plays an important role especially in young roots. At later stages of development a large proportion of cyanogenic glucosides may be translocated from the green leaves to the roots. The higher biosynthetic activity measured in the young sweet variety is surprising, since sweet varieties are normally associated with a low HCN potential [7, 12]. Our results suggest that the HCN potential in the root does not necessarily correlate with the biosynthetic activity in the root and that transport of cyanogenic glucosides from the leaves to the roots may vary in different varieties and be dependent on the developmental stage. Liberation of HCN from cyanogenic glucosides constitutes a serious nutritional health hazard, if the cassava plant material is not properly processed before consumption [1]. Future attempts to reduce accumulation of cyanogenic glucosides in cassava roots by either blocking *de novo* synthesis in the roots and/or by preventing transport from leaves to roots require further investigations into the quantitative importance of synthesis in the root compared to the leaves.

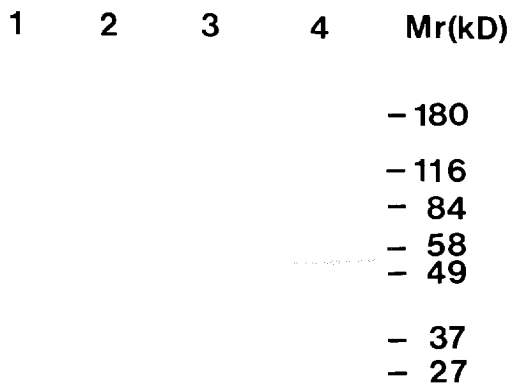

Fig. 2. Western blot of the microsomal enzyme preparations from cassava roots. SDS-solubilized microsomal proteins (20 μg) were separated on an 8–25% SDS-polyacrylamide gel, transferred to nitrocellulose filter and probed with anti-$P450_{TYR}$. Lane 1, old root of bitter variety; lane 2, old root of sweet variety; lane 3, young root of bitter variety; lane 4, young root of sweet variety.

EXPERIMENTAL

Materials. Roots of cassava (*Manihot esculenta* Crantz), harvested 4 and 12 months after planting, were obtained from The International Institute of Tropical Agriculture, Ibadan, Nigeria. The samples included the sweet variety TMS 4(2)1425 (4- and 12-months-old), the bitter variety TMS 91934 (4-months-old) and the bitter variety TMS 50395 (12-months-old). The freshly harvested roots were stored at 4° and analysed within two weeks.

In vivo *biosynthesis of linamarin in cassava roots.* Small cylinders with a diameter of 0.5 cm were cut from cassava roots and the outer 2–3 mm layer representing the phelloderm was excised. The segments were immediately placed in septum-covered vials containing 2 μCi of [U-^{14}C]valine (Amersham, 266 mCi mmol^{-1}) in 170 μl 50 mM tricine, pH 7.9. After incubation overnight at 30°, cyanogenic glucosides were extracted from the excised root tissue by boiling twice in 5 ml of 90% MeOH for 15 min as described earlier [2]. Aliquots of extracts and authentic linamarin were applied to TLC plates (Merck, Kieselgel 60 F_{254}) and separated by a solvent system of EtOH–Me_2CO–CH_2Cl_2–MeOH–H_2O (20:15:6:5:4). Radioactive bands on TLC plates were visualized using autoradiography. The authentic linamarin was visualized by spraying with 5% H_2SO_4 in MeOH followed by heating. Feigl/Anger paper was used for specific detection of HCN upon hydrolysis of linamarin after spraying β-glucosidase on the TLC plates [14].

In vitro *biosynthesis of linamarin in cassava roots using a microsomal enzyme system.* Cassava roots (100–200 g fr. wt) were carefully peeled and shredded into a prechilled mortar containing 2 vol. of isolation buffer (100 mM tricine, pH 7.9, 250 mM sucrose, 50 mM NaCl, 2 mM DTT, 1 mM PMSF), 0.1 vol. of PVPP, and 0.5 vol. of seasand. The preparation of microsomes was carried out as described by Koch *et al.* [5]. The microsomal preparation was resuspended in 5–10 ml of dialysis buffer (50 mM Tricine, pH 7.9, 2 mM DTT) and dialysed overnight under a nitrogen atmosphere.

Biosynthetic activity of the microsomal preparation was determined as production of HCN using valine as substrate. A typical reaction mixture contained 100 μl microsomal enzyme (5–9 mg protein ml^{-1}), 40 mM valine, 1.2 mM NADPH, in a final vol. of 200 μl of 50 mM Tricine, pH 7.9. The reaction mixtures were incubated for 30 min at 30° in a test tube closed with a silicone septum and the reaction was stopped by the injection of 40 μl of

6 M NaOH. The amount of HCN produced was determined colorimetrically directly in the reaction mixture as described previously [15].

Other analytical procedures. Analytical SDS–PAGE was performed using 8–25% linear gradient gels prepared as described [16]. Western blotting of microsomal proteins was carried out using the polyclonal rabbit antibodies against cytochrome $P450_{TYR}$ involved in the biosynthesis of dhurrin in sorghum [17]. The concentration of total cytochrome P450 enzymes was measured by difference spectroscopy using a molar extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ [18]. Protein concentrations were determined by the method of ref. [19].

Acknowledgements—Inga Olsen and Hanne Linde Nielsen are thanked for technical assistance. We are grateful to Prof. Eric Conn and Dr Dirk Selmar for the authentic samples of cyanogenic glucosides. This work was supported by the Commission of the European Communities Program Science and Technology for Development, by DANIDA and by the Danish Center for Plant Biotechnology.

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