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# Changes of Cyanide Content and Linamarase Activity in Wounded Cassava Roots[1]

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## ABSTRACT

**When cassava (*Manihot esculenta* Crantz) root was cut into blocks and incubated under laboratory conditions, the blocks showed more widespread and more even symptoms of physiological deterioration than those under natural conditions. Thus, the tissue block system has potential for biochemical studies of natural deterioration of cassava root. The changes in cyanide content and linamarase (linamarin β-D-glucoside glucohydrolase; EC 3.2.1.21) activity in various tissues during physiological deterioration were investigated. Total cyanide content increased in all parts of block tissue after 3-day incubation. The degree of increase in cyanide was most pronounced in white parenchymal tissue, 2 to 3 millimeters thick, next to the cortex (A-part tissue), where no physiological symptoms appeared. On the other hand, linamarase activity was decreased in all parts of block tissue after a 3-day incubation. A time course analysis of A-part tissue indicated a clear reciprocal relationship between changes in total cyanide and linamarase activity; total cyanide increased, while linamarase activity decreased. Free cyanide constituted a very small portion of the total cyanide and did not change markedly.**

Cassava (*Manihot esculenta* Crantz) root is perishable; roots begin to deteriorate soon after harvest (12–14). The deterioration consists of two types. The first is physiological deterioration characterized by internal root discoloration. The second is microbial deterioration caused largely by a wound pathogen (1). The mechanism and cause of the deterioration, however, have not yet been elucidated thoroughly.

Under natural conditions, the roots show rather local and spotted deterioration symptoms and that has hindered biochemical studies. In the present study, we cut the cassava root into blocks and incubated them under laboratory conditions. The blocks showed more widespread and more even physiological deterioration symptoms than those under natural conditions, indicating the potential for biochemical study of deterioration.

Cassava is a cyanogenic plant and contains linamarin (isobutyronitrile-β-D-glucoside) as a main component (2). In this paper, we investigated the changes in cyanide content and linamarase (linamarin β-D-glucoside glucohydrolase; EC 3.2.1.21) activity during deterioration of cassava root to get information on the metabolism and biological significance of cyanogenic glucoside in the deteriorating cassava root.

Previously, the tissue distributions of the cyanogenic glucoside, dhurrin, and enzymes involved in its metabolism in leaves of *Sorghum bicolor* were studied (9). Dhurrin was localized exclusively in the epidermis, whereas two enzymes responsible for its catabolism resided primarily in the mesophyll tissue. The bundle sheath strands contained neither dhurrin nor its degradative enzymes.

Therefore, we also examined in cassava roots the tissue distributions of cyanogenic glucoside and its degradative enzyme, linamarase, in comparison with those in sorghum leaves. The results suggest the different sequestering mechanisms of cyanogenic glucosides and their degradative enzymes in cassava roots and sorghum leaves.

## MATERIALS AND METHODS

**Plant Materials.** Cassava roots (*Manihot esculenta* Crantz cv Golden Yellow), 8 months old, 30 to 35 cm long and 5 to 6 cm in diameter at center, were harvested from the experimental farm of the Philippine Root Crop Research and Training Center (PRCRTC), Visayas State College of Agriculture (ViSCA), and were immediately used for experiments.

**Sampling and Incubation.** Transversely cut blocks (1 to 2 cm thick) with about equal weight were prepared from the adjacent parts of one root and incubated under laboratory conditions, with temperature ranging from 26 to 30°C and RH ranging from 78 to 92%. After incubation, the blocks were weighed and then cut transversely into thin slices to make it easier to distinguish the tissues by their color. The tissues in the slices were separated by hand with scissors into four parts (Fig. 1): the cortex and A-, B-, and C-part tissues. The cortex could be removed easily from parenchymal tissue by hand. The periderm on the cortex was discarded. A-part tissue was a white parenchymal tissue which was 2 to 3 mm thick next to the cortex. B-part tissue was a yellow parenchymal tissue, 7 to 8 mm thick, next to A-part tissue, where physiological deterioration symptoms appeared exclusively in an earlier stage of deterioration. C-part tissue, 16 to 18 mm thick, was the remaining core tissue. Each separated tissue fraction was weighed. The entire fractions were used for analysis in most experiments. In some experiments the separated tissues were cut into small pieces and mixed well, and the parts of the mixtures were used for analysis.

**Preparation of Linamarase for Cyanide Assay.** Cassava root cortex of Hawaiian 5, bitter type, grown at PRCRTC, was extracted by the method of Cooke (3, 5). The extract was brought to 70% saturation with ammonium sulfate. The resulting precipitate

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was dissolved in 20 mM acetate buffer, pH 5.5, applied to a Sephadex G-25 column pre-equilibrated with 10 mM phosphate buffer, pH 6.0, and eluted by the same buffer. The fraction eluted at void volume was collected and assayed for linamarase activity at 30°C in a final volume of 6.0 ml by the method of Ikediobi *et al.* (8) using *p*-nitrophenyl-β-D-glucoside as the substrate. One unit of linamarase activity was defined as that amount of enzyme which produced a change of *A* of 0.001 at 400 μm/min. One hundred μl of linamarase preparation containing 230 units was included in all total cyanide assay mixtures. This amount of the enzyme hydrolyzed all cyanogenic glucosides in the samples to release HCN in 30 min at 30°C.

**Preparation of Cassava Extracts for Cyanide Analysis.** The cassava extracts for cyanide analysis were prepared using a modified method of Cooke (3, 5). The separated tissue was diced and homogenized in 0.1 M orthophosphoric acid in the ratio of 3 ml to 1 g tissue using a mortar and pestle chilled by ice. The homogenate was centrifuged at 2200*g* for 15 min. The mortar was rinsed and the precipitate was washed by centrifugation using 0.1 M orthophosphoric acid in the ratio of 1 ml to 1 g tissue. The supernatants of the two centrifugations were combined and the volume was measured. An aliquot of the mixture was used for cyanide analysis.

**Determination of the Total and Free Cyanide Content of the Extracts.** HCN was liberated from cyanogenic glucosides by the modified enzyme method of Cooke (3, 5) and distilled into NaOH solution according to the method of Reay and Conn (11). An aliquot (20 to 80 μl) of extract was incubated in a sealed vial containing 500 μl of 0.2 M phosphate buffer, pH 7.0, and 100 μl of linamarase preparation (230 units of enzyme activity) at room temperature. After 6 h of incubation, the vial was injected with 3 drops of 5 N $H_2SO_4$ and incubated for another 15 h at room temperature. The liberated HCN was distilled into 0.2 ml of 1 N NaOH solution in a small glass cup suspended in the vial. The NaOH solution and the washings were combined and 1 ml of the resulting solution was used for cyanide determination by the modified Lambert method (10).

In analyzing for free cyanide, linamarase preparation was replaced by 100 μl of 10 mM phosphate buffer, pH 6.0; otherwise, the procedures for determination were exactly the same as those for total cyanide analysis.

A standard curve was made for each experiment by treating standard KCN solution (2 mM in 0.1 M NaOH) in the same way as samples. A control was run without sample extract.

**Extraction of Linamarase and Assay of Its Activity.** The separated tissue was diced and homogenized in 0.1 M acetate buffer, pH 5.5, in the ratio of 3 ml to 1 g tissue using a mortar and pestle chilled by ice. The homogenate was centrifuged at 2200*g* for 15 min. The mortar was rinsed and the precipitate was washed by centrifugation using 0.1 M acetate buffer, pH 5.5, in the ratio of 1 ml to 1 g tissue. The supernatant and the washings were combined and the volume was measured. Two ml of extract was applied to a column of Sephadex G-25 (1.5 × 13 cm) pre-equilibrated with 10 mM phosphate buffer, pH 6.8, and eluted by the same buffer to remove lower mol wt compounds. The void volume fraction of the column was collected, adjusted to 4 ml by 0.1 M acetate buffer, pH 6.8, and used as the enzyme preparation.

Linamarase activity was assayed using linamarin as the substrate. The enzyme was incubated at 30°C with 0.75 μmol of linamarin and 5 μmol of phosphate buffer, pH 6.8, in a total volume of 650 μl. After 10-min incubation, the reaction was terminated by addition of 350 μl of 0.3 N NaOH and the liberated cyanide was directly assayed by the modified Lambert method (10). Two controls were run; one was without linamarin and the other was without enzyme extract.

**Base of Comparison of Cyanide Content and Linamarase Activity.** The cassava root blocks were dehydrated to some extent and the tissues in the block lost weight during incubation under laboratory conditions. The fresh weights of various tissues in the block were calculated from the decrease in total weight of whole block by assuming that all tissues in the block were dehydrated to the same degree. The fresh tissue weights thus calculated were used as the basis of comparison of cyanide content and linamarase activity.

## RESULTS

Our preliminary experiments indicated that large variations in cyanide content and linamarase activity occur among roots, even from one plant, as reported by other workers (3, 6). Furthermore, there are both radial and longitudinal gradients in cyanide content in cassava root (3). The radial gradient is very steep, while the longitudinal gradient is shallower. Therefore, we investigated the changes in cyanide content and linamarase activity during incubation using the blocks prepared from the adjacent parts of one root. The experiments were repeated several times using different roots and different parts, that is, proximal, middle, or distal parts of the root. The tendency of all experimental results was constant, although the absolute values fluctuated. In this paper, we present representative results.

**Deterioration of Cassava Root Block Incubated under Laboratory Conditions.** When cassava root blocks (1 to 2 cm thick) were incubated under laboratory conditions, the blocks deteriorated rapidly. After a 3-d incubation, physiological deterioration symptoms appeared exclusively in B-part tissue (see "Materials and Methods") as in the early stage of physiological deterioration under natural conditions (Fig. 2). The symptoms of the incubated blocks were similar to those under natural conditions and were distributed more widely and evenly than the latter. Thus, the incubated blocks seemed to be a good system for biochemical study on the physiological deterioration, although it is difficult to

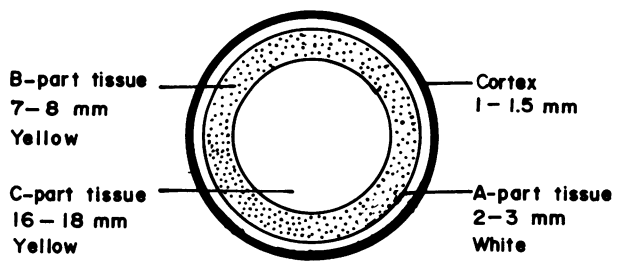


FIG. 1. A scheme of the various tissues in cassava (cv Golden Yellow) root block, 5 to 6 cm in diameter, used in determinations of cyanide content and linamarase activity.

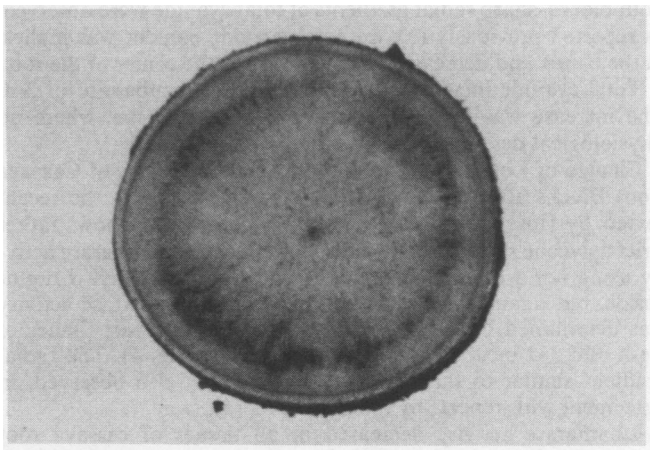

FIG. 2. Physiological deterioration of cassava root block incubated for 3 d under laboratory conditions. The dark parts show brownish discoloration.

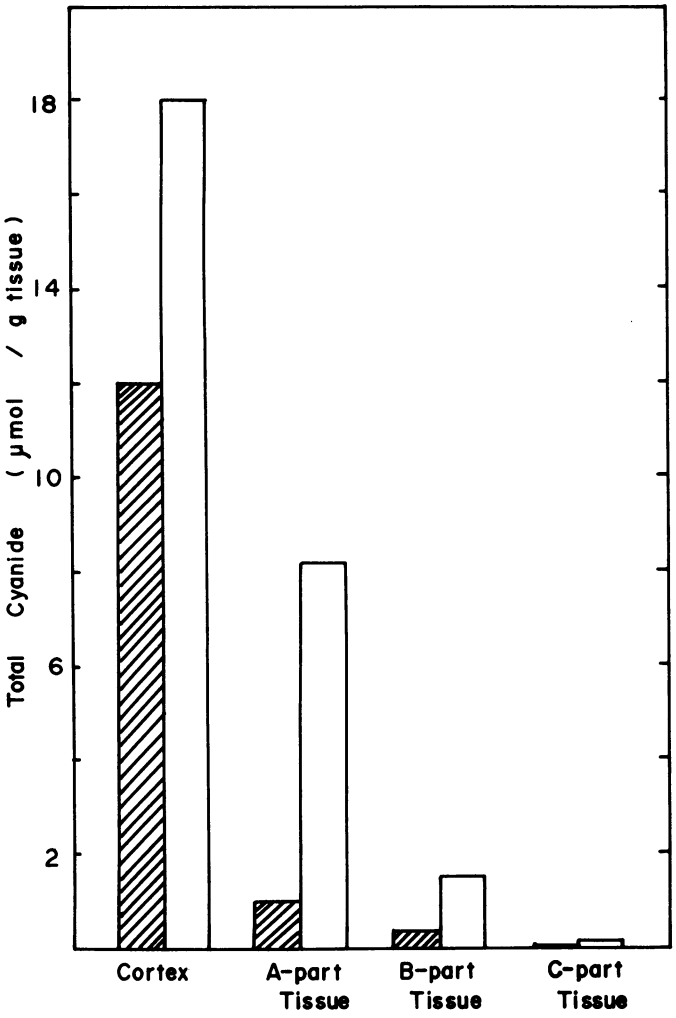


FIG. 3. Change of total cyanide content in various tissues of cassava root blocks incubated for 3 d under laboratory conditions. The shadowed and open bars indicate the total cyanide content of fresh and 3-d incubated tissues, respectively.

exclude the effect of wounding.

**Change in Total Cyanide Content of Various Tissues of Cassava Root Block after 3-d Incubation.** The total cyanide contents in the cortex and A-, B-, and C-part tissues of fresh and 3-d incubated cassava root blocks were determined and compared (Fig. 3). In both blocks, sharp radial gradients of total cyanide were observed, as reported previously (3); the total cyanide content was highest in the cortex and decreased sharply towards the center of the root.

Total cyanide increased in all tissues after incubation for 3 d. The increase was most pronounced in A-part tissue, where no physiological deterioration symptoms were observed.

**Change of Linamarase Activity in Various Tissues of Cassava Root Blocks after 3-d Incubation.** As emphasized in the recent review by Hosel and Conn (7), plant β-glucosidase shows rather strict aglycone specificity. Therefore, we assayed linamarase activity using not artificial substrates, such as *p*-nitrophenyl-β-D-glucoside, but linamarin, the natural substrate. Linamarase activity was determined in the cortex and A-, B-, and C-part tissues of fresh and 3-d incubated cassava root blocks (Fig. 4). The radial gradient similar to that for total cyanide was also observed, in agreement with reports by other workers (4).

Linamarase activity decreased in all tissues of cassava root blocks after incubation for 3 d.

**Time Course Changes of Total and Free Cyanide Content and Linamarase Activity in A-part Tissue.** The changes in total and free cyanide content and linamarase activity during incubation were followed in A-part tissue (Fig. 5). The changes in total cyanide content and linamarase activity showed a reciprocal relationship; the total cyanide increased markedly in 1 d and continued to increase gradually, while the linamarase activity decreased in the opposite way. Free cyanide constituted a very small portion of total cyanide and did not change markedly during 3-d incubation. Consequently, it was inferred that the increase of total cyanide was caused mainly by the increase of cyanogenic glucosides. This inference was supported by the analysis using silica gel TLC. When the extracts from fresh and 3-d incubated tissues were chromatographed on silica gel thin layer plates using chloroform:methanol (5:1, v/v) and analyzed for total cyanide after elution with water, only one broad peak, which corresponded to linamarin and lotaustralin, was detected on both chromatograms (data not presented).

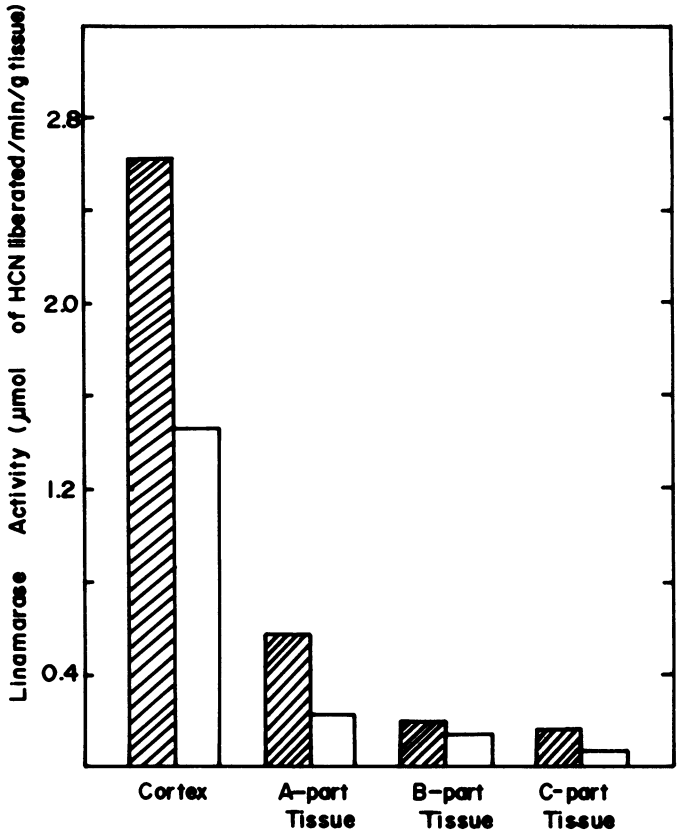


FIG. 4. Change of linamarase activity in various tissues of cassava root blocks incubated for 3 d under laboratory conditions. The shadowed and open bars indicate the linamarase activity of fresh and 3-d incubated tissues, respectively.

## DISCUSSION

In this paper, we analyzed the total cyanide content of cassava root by a two-step method. First, cyanogenic glucoside in the sample was hydrolyzed by linamarase from cassava root cortex. The HCN liberated was then distilled into NaOH solution and assayed by the modified Lambert method. By the method, the analytical results will be free from the interference of the contaminants in the sample.

The total cyanide content was high in the cortex and decreased sharply towards the center of the root (Fig. 3). A somewhat similar tissue distribution of cyanide was also observed with young sorghum leaves; dhurrin (*p*-hydroxy-(*S*)-mandelonitrile-β-D-glucoside) was localized exclusively in epidermis and no significant amount of it was contained in inner tissues, namely, mesophyll

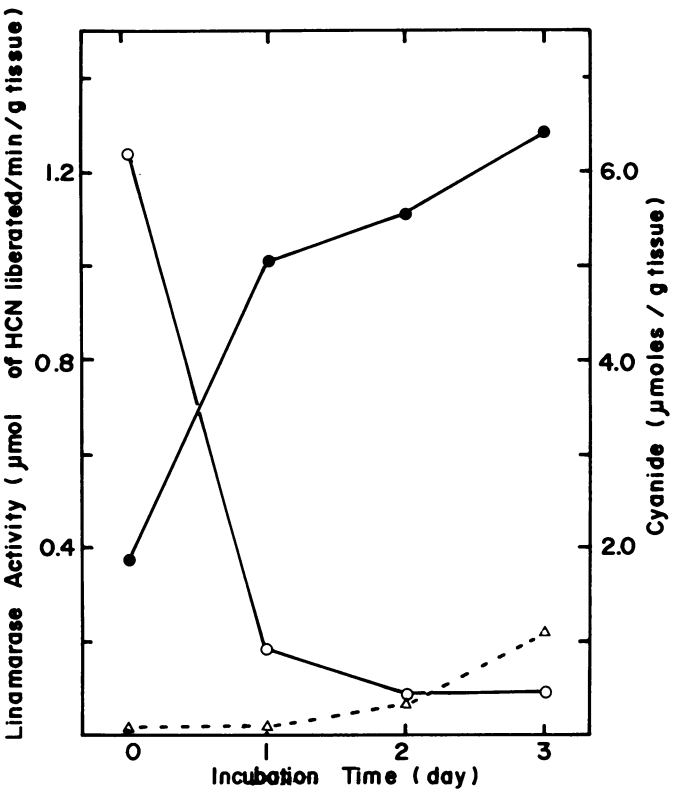


FIG. 5. Time course changes of the total and free cyanide content and of linamarase activity in A-part tissue of cassava root blocks incubated under laboratory conditions. (●——●), total cyanide content; (△- - -△), free cyanide content; (○——○), linamarase.

and bundle sheath strands (9). Thus, cyanogenic glucoside was localized in the outer tissues of both organs of two cyanogenic plants. Such tissue distribution of cyanide suggests that the cyanogenic glycosides are involved in defense action of the plants against pathogens and insects invading from outside. The increase of cyanide in deteriorating cassava root might be a defense action of the tissue against pathogens (Fig. 3). Our present finding that cyanide was increased in the deteriorated cassava root also is important from the point of food hygienics. Especially, it is noteworthy that the total cyanide content increased in inner tissues, A-, B-, and C-part tissues which are used as food (Fig. 3).

Linamarase showed a radial gradient in cassava root similar to that of total cyanide. The linamarase activity was highest in the cortex which contained the highest level of cyanide (Figs. 3 and 4). The tissue distribution patterns of linamarase and cyanide (mainly cyanogenic glucoside, linamarin) in cassava roots are in contrast to those in young sorghum leaves. In the leaves of sorghum, dhurrin was localized in the outmost tissue, the epidermis, whereas dhurrin β-glucosidase resided almost exclusively in the inner tissue, mesophyll, which contained no significant amount of dhurrin (9). Thus, it is inferred that different sequestering mechanisms of cyanogenic glucosides and their degradative enzymes exist in cassava roots and sorghum leaves.

Linamarase activity decreased in all tissues of cassava root blocks incubated under laboratory conditions (Fig. 4). The decrease in linamarase activity is unusual because other enzymes such as acid invertase, phenylalanine ammonia-lyase, and peroxidase increased in the deteriorated cassava root (12, 14).

The time course changes of total cyanide (mainly cyanogenic glucosides) and linamarase showed a clear reciprocal relationship (Fig. 5). The results suggest that linamarase plays a prime role in the regulation of cyanogenic glucoside level in cassava roots.

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