Plant Physiol. (1987) 84, 1296–1300
0032-0889/87/84/1296/05/$01.00/0

The Linamarin β-Glucosidase in Costa Rican Wild Lima Beans (*Phaseolus lunatus* L.) Is Apoplastic[1]

Received for publication March 12, 1987 and in revised form May 11, 1987

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ABSTRACT

Analysis of mesophyll protoplasts and cell wall extracts of leaf discs of Costa Rican wild lima bean (*Phaseolus lunatus* L.) shows that the linamarase activity is confined to the apoplast. Its substrate linamarin, together with the related enzyme hydroxynitrile lyase, is found inside the cells. This compartmentation prevents cyanogenesis from occurring in intact tissue, and suggests that linamarin has to be protected during any translocation across the linamarase rich apoplast.

Upon hydrolysis by a β-glycosidase cyanogenic glycosides yield a sugar moiety and a hydroxynitrile which in turn dissociates spontaneously or enzymically to HCN and a carbonyl compound (aldehyde or ketone). This process, called cyanogenesis, occurs upon crushing the tissues of cyanogenic plants. Since the cyanogenic glycoside remains largely intact in the intact leaf, this compound must be compartmentalized from its catabolic enzymes.

The tissue and the cellular distribution of the enzymes involved in the metabolism of cyanogenic glycosides are known in several species (6, 10, 12, 18). The aim of this work was to localize the glucoside linamarin and its degrading enzymes linamarase and acetone cyanohydrin lyase (hydroxynitrile lyase) in lima bean. This was approached by comparing whole leaves with mesophyll protoplasts prepared from primary leaves. The results obtained raise questions about the mechanism whereby linamarin appears to be transported from the seed into the seedling during germination (5).

MATERIALS AND METHODS

Plant Material. We used the same collection of lima bean seeds as previously (5). In 1986 the germination rate of these seeds was 63.5% ($n = 895$). Seeds were germinated in vermiculite after surface sterilization in sodium hypochlorite (0.5–1%, w/v) for 10 min, rinsing three times in distilled water, pricking the seed coats with a razor blade, and soaking in aereated water overnight. The growth chamber settings were 11 h of mixed incandescent and fluorescent light of approximately 40000 lux at plant level and 30°C. In the dark the temperature was 18°C. The primary leaves were collected 18 to 30 d after onset of germination.

Mesophyll Protoplasts and Whole Leaf Extracts. The lower epidermis of primary leaves was abraded according to Beier and Bruening (2), and the midribs of the leaves were excised. Six leaves were floated on 15 ml of 250 mM sorbitol containing 2 mM $CaCl_2$, 1 mM PEG 4000, 5 mM Mes adjusted to pH 5.5 with NaOH (protoplast medium) supplemented with 2% (w/v) Cellulysin (Calbiochem) and 0.13% (w/v) Pectolyase (Sigma) in a plastic Petri dish (15 cm i.d.). After 2 h of incubation in a shaking waterbath (60–80 oscillations min^{-1}) at 30°C, the protoplasts were gently separated from the epidermis with a Pasteur pipette and the addition of some fresh protoplast medium. The resulting protoplast suspension was filtered through four layers of cheesecloth. The protoplasts were then pelleted at 50*g* for 5 min and resuspended in about 4 ml protoplast medium per three leaves. This suspension was loaded on discontinuous gradients of Percoll (Sigma) in protoplast medium (two layers: 5 and 25% [v/v]) and centrifuged at 200*g* for 2 min followed by 500*g* for 5 min. Protoplasts banding at the 5/25% Percoll interface were collected, diluted with one to two volumes of 50% Percoll in protoplast medium, overlayered with 25% Percoll in protoplast medium and, on top, with protoplast medium alone, and centrifuged as above. The protoplasts at the topmost (0/25% Percoll) interface were collected, diluted with protoplast medium, counted and pelleted at 500*g* for 5 min. The protoplast pellet was dissolved in cold Hepes-Triton X buffer (10 mM Hepes pH 7.5 [HCl] containing 0.1% [v/v] Triton X-100, 0.5 mM phenylmethyl sulfonyl fluoride) at a concentration of 1 ml per leaf and kept on ice until used for the assays. Whole leaf extracts were obtained by treating five primary leaves exactly the same way as for protoplasts except that cellulase and pectinase were not added to the protoplast medium. After incubation on the shaking water bath was terminated, the Petri dish was kept at room temperature throughout the time needed to purify the protoplasts. Then these leaves were blotted dry, homogenized in 10 ml of cold Hepes-Triton X buffer and placed on ice. All enzyme assays of the protoplast and whole leaf extracts were performed the same day.

Incubation of Leaf Discs with Linamarin. Discs (8 mm o.d., about 18 mg fresh weight per disc) of primary leaves were rinsed in distilled water and blotted dry. Each leaf disc was submersed in a small dish (12 mm i.d.) containing 1 ml of 1 mM linamarin in 50 mM Mes (pH 5.5). All dishes were placed in an dessicator for infiltration. Vacuum was applied for 3 min followed by 3 min atmospheric pressure to allow good infiltration of the leaf discs with buffer. Timing of the linamarase reaction was started at the end of the infiltration procedure; incubation was at room temperature. During min 1 of the incubation period an aliquot of 750 μl of the infiltration buffer of each sample was removed and discarded; this was done to decrease the volume of the incubation buffer into which any leakage of linamarin from the discs might occur and therefore increase the sensitivity of detecting the leakage. Incubated samples were fixed at timed intervals by adding 400 μl of chilled 0.2 M NaOH and quickly homogenizing. They were then centrifuged for 3 min in a Beckman microfuge after which aliquots of the supernatants were assayed for HCN. As a control, an equal number of leaf discs was treated

[1] This work was supported by a Swiss National Foundation Fellowship to M. F. and in part by NSF Grant DMB-8517190.

in exactly the same way but without the addition of linamarin in order to monitor any cyanide produced from linamarin leaking out of the cells during the infiltration procedure. Two additional series of leaf discs (one without and one with 1 mM linamarin) were homogenized in 250 μl of 50 mM Mes buffer (pH 5.5) per disc directly after the infiltration period and incubated similarly to the intact leaf disc samples. This permitted determination of the maximum rates of HCN release in the samples containing only endogenous linamarin, and samples with added linamarin, 1 mM, respectively.

Extraction of Cell Walls. Cell walls of leaf discs (8 mm o.d.) were extracted by the method of Terry and Bonner (21). The leaf discs were washed in distilled water and then submerged in 2 ml of 10 mM Mes buffer (pH 5.5), containing 100 mM $MgCl_2$ (infiltration buffer). The infiltration itself was performed as described above. The leaf discs were blotted dry and placed in centrifuge tubes as shown in Figure 1. The samples were spun at 1000*g* for 10 min. This infiltration and centrifugation procedure was repeated with each leaf disc up to eight times. The combined cell wall extracts of each leaf disc were made up to 100 μl with infiltration buffer. Each leaf disc was then separately homogenized in 2 ml of infiltration buffer. The extracts and homogenates were assayed for linamarase, glucose 6-P isomerase, and NAD-malate dehydrogenase.

Enzyme Assays. The activities of α-mannosidase and β-*N*-acetylglucosaminidase were assayed using the corresponding *p*-nitrophenyl substrates. Assay mixtures contained 50 μl enzyme sample and 100 μl buffer (0.1 M citrate-phosphate (pH 4.0), for α-mannosidase or 0.5 M Mes (pH 5.5) for β-*N*-acetylglucosaminidase) containing 1 mg ml^{-1} BSA and 2 mg ml^{-1} of the corresponding substrate. The assays were incubated up to 120 min at 34°C and stopped at 30 min intervals by adding 1 ml of 0.2 M Na_2CO_3 to each tube. The nitrophenol released was measured by its absorption at 400 nm. Two different blanks were prepared for each enzyme sample: one blank lacked the enzyme and the other lacked the substrate. The NAD-malate dehydrogenase and glucose 6-P isomerase activities were assayed according to Kluge and Osmond (11) and to Bergmeyer (3), respectively.

The linamarase assay contained 100 μl 0.1 M linamarin, 500 μl enzyme sample, and 400 μl 0.5 M Mes buffer (pH 5.5), containing 2 mg ml^{-1} BSA. Incubation was at 25°C; at timed intervals, aliquots of 20 to 50 μl were withdrawn from the assay mixture and immediately ejected into 180 to 150 μl of 0.2 M NaOH. Cyanide was then determined as described below. Controls were performed similarly but with the addition of water instead of linamarin.

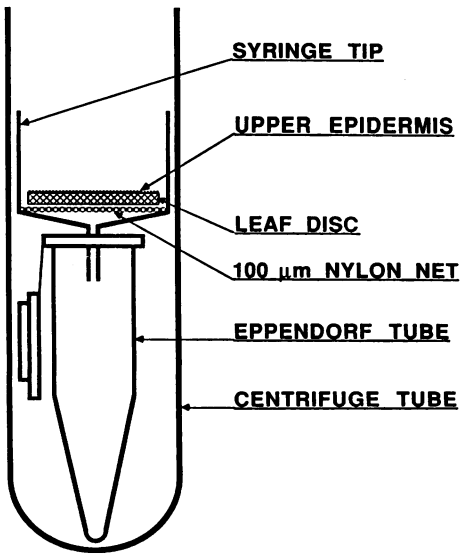

FIG. 1. Centrifugation of leaf discs. This simple arrangement allowed the extraction of buffer soluble cell wall constituents of vacuum infiltrated leaf discs by centrifugation at 1000*g* for 10 min.

The hydroxynitrile lyase activity was determined by modifying slightly the method of Selmar (18). Prior to use, the substrate (acetone cyanohydrin, Aldrich Chemical Co., Inc.) was stored overnight in a dessicator containing solid sodium hydroxide to remove any residual HCN. A freshly prepared solution of 10% (v/v) cyanohydrin in 0.1 M citric acid was used in the assay which contained 200 μl enzyme sample and 790 μl 0.1 M phosphate-citrate buffer (pH 5.5). The reaction (at room temperature) was started with the addition of 10 μl of the 10% substrate solution. At timed intervals, typically, 0, 5, 10, 15, and 20 min, a 20 μl aliquot of the assay was withdrawn and ejected into a mixture of 50 μl 1 M acetic acid, and 1.25 ml of the succinimide/*N*-chlorosuccinimide reagent used in the cyanide assay. HCN was then determined as described below. The rate of spontaneous decomposition of the substrate, determined in a control assay with water instead of enzyme sample, was used to calculate the enzymic release of HCN.

Estimations. The cyanide potential (HCN-p) of a sample was determined in a sealed vial containing 500 μl sample, 0.5 IU linamarase (BDH Chemicals Ltd.), 300 μl 0.1 M phosphate buffer (pH 6.8), and, in a center well, 300 μl 0.2 M NaOH to trap the HCN released over night at 34°C. Aliquots of the center well were made up to 200 μl with 0.1 M NaOH and the cyanide therein determined.

Cyanide was determined by the Lambert method (13) with the following modifications: Fifty μl of 1 M acetic acid and 1 ml of the succinimide/*N*-chlorosuccinimide reagent were added sequentially to a sample containing 0 to 25 nmol of cyanide in 200 μl of 0.1 to 0.2 M NaOH and mixed; then 200 μl of the barbituric acid/pyridine reagent was added, mixed, and the absorbtion at 580 nm was read after about 10 min. Calibration curves were linear up to 30 nmol HCN per assay.

Chl was estimated by the method of Arnon (1).

Sugars and glycosides were determined by gas-liquid chromatography as described by Frehner *et al.* (7) with the following modifications. The vials containing mixtures of the hydroxylamine reagent and samples were sonicated for 7 min prior to incubation at 75°C for 7 min; no further heating occurred for the final derivatization step. The gas chromatograph was a Packard model 417 (Becker). Helium at a flow rate of 60 ml min^{-1} and a temperature program from 150 to 320°C at a rate of 15°C min^{-1} with a final hold of 3 min were used.

The osmotic pressure of leaves was determined with a Wescor model 5100 C vapor pressure osmometer. Leaves were squeezed out in a 1 ml disposable syringe, and the resulting sap was used for osmometry.

RESULTS

The only detailed study on the subcellular organization of cyanogenesis has been done on *Sorghum* by Kojima *et al.* (12). Experiments with lima beans in this laboratory suggested that, in contrast to *Sorghum*, the β-glucosidase in this species may be associated with the cell wall (E Wurtele, unpublished results). We therefore decided to investigate the subcellular localization of cyanogenesis in wild lima bean by comparing protoplasts and whole leaf extracts. To obtain protoplasts from primary leaves of lima bean, the protocol of Martinoia *et al.* (14) was followed except that Cellulysin was substituted for the Onozuka cellulase. It was impossible to strip off the lower epidermis of lima bean leaves, but the abrasion method of Beier and Bruening (2) proved to be successful. The optimum concentration (300 mM) of the sorbitol osmoticum was slightly higher than the osmolarity of the press sap of leaves (around 280 mOsmol kg^{-1}). Microscopic

examination of the protoplasts released during incubation revealed that all the intact protoplasts contained chloroplasts and thus that their origin was the leaf mesophyll. It has not been possible to obtain epidermal protoplasts to allow a comparison of two different tissues of leaves of lima bean as was possible with *Sorghum* (12, 23).

This procedure resulted in yields of Chl as great as 58% of that in the leaves (Table I); this figure is taken as the protoplast yield. Two enzymes of primary metabolism, NAD-malate dehydrogenase and glucose 6-P isomerase, nearly paralleled this high yield of Chl with 40 and 52%, respectively (Table I). On the other hand, the yield of the vacuolar enzymes α-mannosidase and β-*N*-acetylglucosaminidase (4) were low (7–19%, Table I), probably due to an uneven distribution of the enzymes among different cell types present in the leaf. The carbohydrates sucrose and glucose had recoveries of 21 to 42% and 17 to 40%, respectively. These values, intermediate between the two groups of enzyme described above, demonstrate the integrity of the protoplast membranes in retaining small molecules.

The amount of hydroxynitrile lyase that catalyzes the rapid release of HCN in cyanogenesis, recovered in the mesophyll protoplasts was only 6 to 7% of that of the leaf (Table I). Since this is similar to that of the α-mannosidase and of β-*N*-acetylglucosaminidase (7–19% and 8–16%, respectively, Table I), the hydroxynitrile lyase is also considered to be an intracellular, possibly vacuolar, enzyme. The recovery of linamarin, measured as HCN-p or as linamarin, was also low (5–19%, Table I) and similar to those of the vacuolar enzymes. These findings suggest the main tissue localization of linamarin to be elsewhere, possibly in the epidermis as in the case of dhurrin in *Sorghum* leaves (12).

Most surprising was the finding that linamarin β-glucosidase was absent in mesophyll protoplasts of lima beans (0%, Table I). This indicates an extracellular localization and contrasts with the situation in *Sorghum*. The presence of linamarase in the apoplast is supported by the ability of leaf discs to hydrolyze externally supplied linamarin (Fig. 2a). Controls show that the infiltration method used did not affect the integrity of the cell membranes; without the addition of linamarin, no HCN was released by intact leaf discs (Fig. 2a). On the other hand, a homogenate of leaf discs released HCN very efficiently, both with and without added linamarin (Fig. 2b). The relatively lower rate of production of HCN from leaf discs infiltrated with linamarin (Fig. 2a) as compared to the rate of homogenized leaves (Fig. 2b) is probably due to the incomplete and uneven infiltration of the cell wall space. This obviously affects the amount of enzyme reached by the substrate linamarin. In addition the infiltrated linamarin will be diluted by an unknown factor in the extracellular fluid. Both effects lead to a lower HCN production (Fig. 2a, linamarin added) than expected from the control experiments (Fig. 2b).

In another experiment, cell walls of leaf discs were extracted *in vivo* by infiltrating the tissue with buffer and recovering the extracellular fluid by centrifugation as suggested by Terry and Bonner (21). Up to 32% of the linamarin β-glucosidase activity present in the leaf discs was extracted in eight infiltration and centrifugation sequences (Fig. 3). However, only negligible amounts of the intracellular marker enzymes glucose 6-P isomerase (<0.5%) and NAD-malate dehydrogenase (<0.3%) were extracted by this technique.

DISCUSSION

Protoplasts in plant physiology have become a popular tool to investigate the compartmentation of metabolites and metabolic pathways within cells and tissues (22). We attempted to localize the main cyanogenic glucoside linamarin and its catabolic enzymes in leaves of the Costa Rican wild lima bean (*Phaseolus lunatus* L.). The method used to obtain protoplasts involved common cell wall degrading enzymes (Cellulysin and Pectolyase Y-23). Preliminary tests showed that these preparations contained enzymes capable of hydrolyzing linamarin, thereby resembling linamarase, one of the enzymes we wanted to localize. But the two gradients used after obtaining the protoplasts purified them very efficiently since no linamarase activity was detected in the resulting protoplast fraction. Their content of small molecules and various enzymes proved the protoplasts to be intact and viable (Table I). Microscopic examination showed that all protoplasts were intact and contained chloroplasts, demonstrating their origin from the mesophyll tissue of the primary leaves.

Since there was no linamarase activity associated with the protoplasts it was important to compare the mesophyll protoplasts with extract of whole leaves (Table I) even though such extracts contained the components of other tissues with distinct physiological functions and metabolism. After correction for the protoplast yield (27–58% of Chl), such comparison shows that the mesophyll protoplasts contain only 19 to 36% of the linamarin (Table I), as compared to glucose 6-P isomerase (68–89%) or to Chl (100%). It is unlikely that this low value is due to the storage of linamarin in the extracellular space since the linamarase located there would inevitably hydrolyze any accessible linamarin (see below). An extremely fast metabolism of linamarin during the protoplast isolation procedure which would lower the linamarin content is not very likely as the leaves which the protoplasts are compared with (Table I) have been stressed in a similar way: incubation buffer, osmoticum, temperature, and duration of these possible stress factors were the same for both the protoplasts and the homogenized leaves, the only difference being the cell wall digesting enzymes. Such results suggest that, as in the case of *Sorghum* (12), the linamarin concentration in the epidermal cells of the lima bean leaf may be much higher than in the mesophyll cells.

It is not obvious that the same conclusion can be drawn for

Table I. *Comparison of Mesophyll Protoplasts and Whole Leaf Extract of Primary Leaves of Lima Bean*

	Chl	α-Manno-sidase	β-*N*-Acetyl-glucos-aminidase	NADH-Malate Dehydro-genase	Glucose 6-P Isomerase	Linamarase (β-Glu-cosidase)	Hydroxy-nitrile Lyase	HCN-p	Lina-marin	Sucrose	Glu-cose	Proto-plasts
	μg	*milliunits*						*nmol*				*No.*
Extract[a]	113	43.8	50.2	344	148	25.4	648	1171	961	88.6	597	ND[e]
Protoplast fraction[a]	59.5	6.05	8.23	139	60.0	0.08	44.4	181	183	34.5	241	$8.79 \cdot 10^5$
Recovery in protoplast fraction[b]	27–58%	7–19%	8–16%	23–40%	18–52%	0%	6–7%	5–15%	5–19%[c]	21–42%	17–40%	$4.54 \cdot 10^5$–$8.79 \cdot 10^5$
In protoplasts[b,d]	100%	26–33%	28–31%	54–83%	68–89%	0–1%	11–25%	14–29%	19–36%[c]	72–76%	54–77%	ND

[a] Absolute figures of only one experiment as example; amounts per leaf. [b] Total variation of three experiments. [c] Two experiments only. [d] Corrected for Chl content. [e] Not determined.

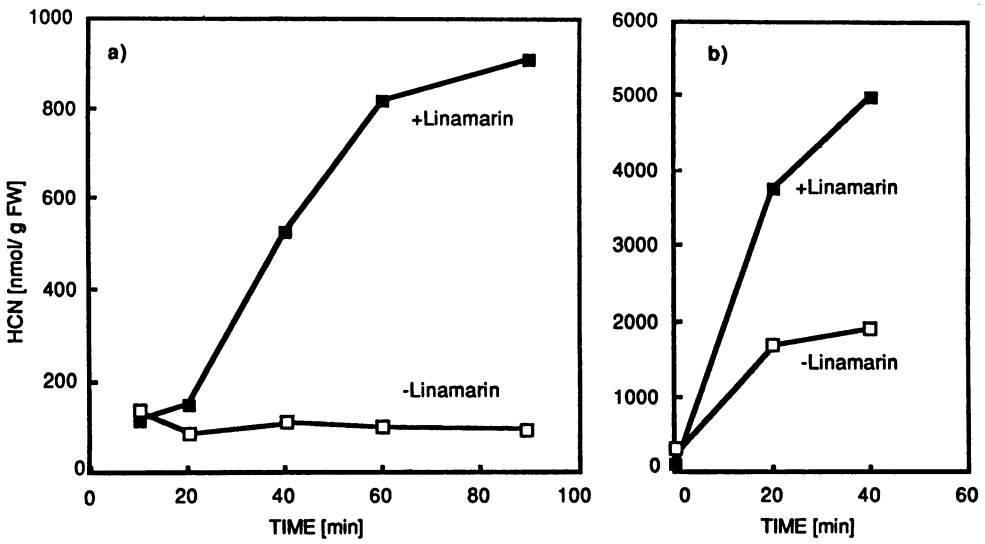

FIG. 2. Evolution of HCN in leaf discs. Discs (about 18 mg each) of primary leaves were infiltrated with buffer containing 1 mM linamarin (■) and with buffer alone (□). a) Intact leaf discs; b) homogenized leaf discs.

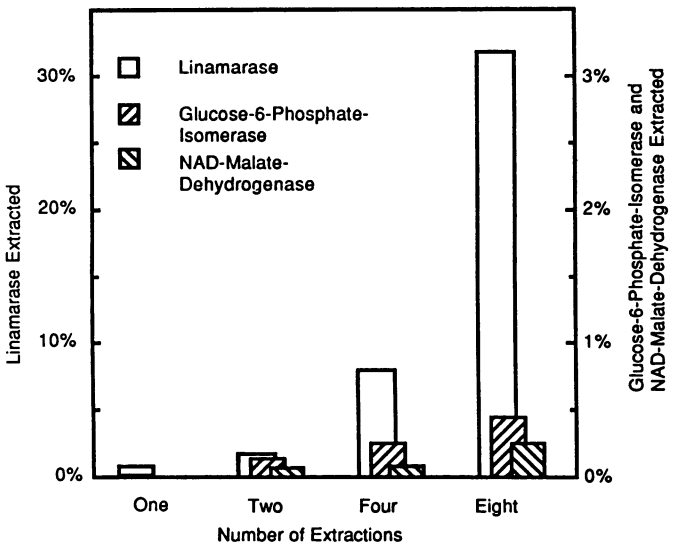

FIG. 3. Activity of linamarase (left scale), glucose 6-P isomerase and NAD-malate dehydrogenase (right scale) recovered in cell wall extracts. Discs (about 18 mg each) of primary leaves of lima bean were repeatedly vacuum infiltrated with buffer and extracted by centrifugation at 1000*g* for 10 min. Each set of infiltration and centrifugation represents a single leaf disc. Enzyme activities are in relative units: whole leaf disc (sum of total extracted and total residual activity) = 100%.

the hydroxynitrile lyase. The hydroxynitrile lyase assay is not very accurate for the low activity observed in protoplast extracts because there are relatively large values for the nonenzymic dissociation (in this case up to 85% of the apparent activity) which have to be subtracted from the apparent enzyme activity (18). Nontheless we suggest that the hydroxynitrile lyase is located within cells, most likely in the epidermis cells.

The presence of linamarase in the cell wall is not unequivocally proven by the experiments with the protoplasts (Table I). Two complementary experiments positively support the cell wall site of the linamarase: (a) the linamarase is freely accessible by linamarin introduced into the cell wall space by infiltration (Fig. 2a), and (b) linamarase activity can be extracted by simply 'flushing' the cell walls with a buffer (Fig. 3).

Although none of the experiments performed (Table I; Fig. 2, 3) permit one to conclude that the linamarase activity is exclusively confined to the apoplast, it seems unlikely that any significant amount of linamarase is intracellular: (a) we expect linamarin to be a component of the vacuole (15, 17). (b) intracellular β-glucosidase activity is usually found in the vacuole (4). As both the linamarin and the β-glucosidase (linamarase) are incompatible in the same compartment, we suggest that the linamarase in lima bean is associated exclusively with the cell walls. Kakes (10) found the linamarin β-glucosidase located in the cell walls of thin section of *Trifolium* leaves using immunofluorescence. Rissler and Millar (16) found the linamarin β-glucosidase, both intracellular and extracellular in leaves of *Lotus corniculatus*. Selmar (18) found the β-glucosidase in the apoplast of *Hevea* as he was able to wash this enzyme off the intact leaves.

Clegg *et al.* (5) have suggested that linamarin might be transported from the cotyledons to the growing seedling during germination of lima beans. Any such transport presumably would involve movement of the glycoside from inside a cell into the apoplast (containing linamarase activity) and then into another cell. One of the long distance transport systems in plants is the xylem stream which is part of the apoplast. The other possible transport system is the phloem which is symplastic. It is generally agreed that solutes have to cross the apoplast at the site of phloem loading and/or at the site of phloem unloading (8, 9, 20, 24). Neither of these two common transport mechanisms seem to be likely for linamarin, unless it is protected from the action of the apoplastic β-glucosidase. Recently Selmar (18) proposed a mechanism that allows linamarin to be transported and then degraded in *Hevea*. He found in *Hevea* the diglucoside of linamarin (linustatin) which is resistant to hydrolysis by the β-glucosidase in that species (19). He proposed that linamarin is glucosylated to linustatin prior to crossing the apoplast and being loaded into the phloem for transport to the target tissue (18). That tissue, in turn, contains a special glycosidase which converts the linustatin to gentiobiose and acetone cyanohydrin.

Our results are compatible with the transport mechanism of Selmar (18): we found high amounts of linamarase in cell walls and, in a preliminary experiment, a leaf homogenate of lima beans containing high linamarase activities failed to hydrolyze linustatin. Further experiments to elucidate the transport of linamarin in lima beans, including the identification of its transport form, are planned.

Acknowledgments—We wish to thank Dr. D. O. Janzen for providing the seed sample, Dr. W. J. Lucas for the use of the osmometer, and Dr. M. Scalet for technical assistance.

LITERATURE CITED

1. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
2. BEIER H, G BRUENING 1965 The use of an abrasive in the isolation of cowpea leaf protoplasts which support the multiplication of cowpea mosaic virus. Virology 64: 272–276
3. BERGMEYER HU1974 Methods of enzymatic analysis, 2nd English Ed. Verlag Chemie International, Deerfield Beach, FL, pp 501–502
4. BOLLER T 1982 Enzymatic equipment of plant vacuoles. Physiol Vég. 20: 247–257
5. CLEGG DO, EE CONN, DH JANZEN 1979 Developmental fate of the cyanogenic glucoside linamarin in Costa Rican wild lima bean seeds. Nature 278: 343–344
6. CONN EE 1980 Cyanogenic compounds. Annu Rev Plant Physiol 31: 433–451
7. FREHNER M, F KELLER, A WIEMKEN 1984 Localization of fructan metabolism in the vacuoles isolated from protoplasts of Jerusalem artichoke tubers (*Helianthus tuberosus* L.). Z Pflanzenphysiol 116: 197–204
8. GEIGER DR, BR FONDY 1980 Phloem loading and unloading: pathways and mechanisms. What's New Plant Physiol 11: 25–28
9. GIAQUINTA RT, W LIN, L SADLER, VR FRANCESCHI 1983 Pathway of phloem unloading of sucrose in corn roots. Plant Physiol 72: 362–367
10. KAKES P 1985 Linamarase and other β-glucosidases are present in the cell walls of *Trifolium repens* L. leaves. Planta 166: 156–160
11. KLUGE M, CB OSMOND 1972 Studies on phosphoenolpyruvate carboxylase and other enzymes of Crassulacean Acid Metabolism of *Bryophyllum tubiflorum* and *Sedum praealtum*. Z Pflanzenphysiol 66: 97–105
12. KOJIMA M, JE POULTON, SS THAYER, EE CONN 1979 Tissue distribution of dhurrin and of enzymes involved in its metabolism in leaves of *Sorghum bicolor*. Plant Physiol 63: 1022–1028
13. LAMBERT JL, J RAMASAMY, JV PAUKSTELIS 1975 Stable reagents for the colorimetric determination of cyanide by modified König reactions. Anal Chem 47: 916–918
14. MARTINOIA E, U HECK, A WIEMKEN 1981 Vacuoles as storage compartments for nitrate in barley leaves. Nature 289: 292–294
15. MATILE P 1984 Das toxishe Kompartiment der Pflanzenzelle. Naturwissenschaften 71: 18–24
16. RISSLER JF, RL MILLAR 1977 Biochemical evidence for the histochemical localization of linamarase activity in *Lotus corniculatus* infected with *Stemphylium loti*. Protoplasma 92: 57–70
17. SAUNDERS JA, EE CONN 1978 Presence of the cyanogenic glucoside dhurrin in isolated vacuoles from *Sorghum*. Plant Physiol 61: 154–157
18. SELMAR D 1986 Cyanogenese in *Hevea*. Zwei Wege zur Metabolisierung cyanogener Glycoside. PhD thesis. Technische Universität Carolo-Wilhelmina, Braunschweig FRG
19. SELMAR D, R LIEBEREI, B BIEHL, J VOIGT 1987 *Hevea* linamarase—a nonspecific β-glycosidase. Plant Physiol 83: 557–563
20. SERVAITES JC, LE SCHRADER, DM JUNG 1979 Energy-dependent loading of amino acids and sucrose into the phloem of soybean. Plant Physiol 64: 546–550
21. TERRY ME, BA BONNER 1980 An examination of centrifugation as a method of extracting an extracellular solution from peas, and its use for the study of indoleacetic acid-induced growth. Plant Physiol 66: 321–325
22. THAYER SS 1985 Protoplasts—for compartmentation studies. *In* HF Linskens, JF Jackson, eds, Modern Methods of Plant Analysis. Cell components, New Series, Vol 1. Springer-Verlag, Heidelberg, pp 54–65
23. THAYER SS, EE CONN 1981 Subcellular localization of dhurrin β-glucosidase and hydroxynitrile lyase in the mesophyll cells of *Sorghum* leaf blades. Plant Physiol 67: 617–622
24. WOLSWINKEL P, A AMMERLAAN, HFC PETERS 1984 Phloem unloading of amino acids at the site of attachment of *Cuscuta europaea*. Plant Physiol 75: 13–20