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Tissue Distribution of β-Cyanoalanine Synthase in Leaves[1]

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ABSTRACT

β-Cyanoalanine synthase, which catalyzes the reaction between cysteine and HCN to form β-cyanoalanine and H_2S, was assayed in leaf tissues from cyanogenic (*Sorghum bicolor* × *Sorghum sudanense* [sorghum]) and noncyanogenic (*Pisum sativum* [pea], *Zea mays* [maize], and *Allium porrum* [leek]) plants. The activity in whole leaf extracts ranged from 33 nanomoles per gram fresh weight per minute in leeks, to 1940 nanomoles per gram fresh weight per minute in sorghum. The specific activities of β-cyanoalanine synthase in epidermal protoplasts from maize and sorghum and in epidermal tissues from peas were in each case greater than the corresponding values for mesophyll protoplasts or tissues, or for strands of bundle sheath cells.

The tissue distributions for this enzyme were determined for pea, leek, and sorghum: the mesophyll protoplasts and tissues in these three plants contained 65% to 78% of the enzyme, while epidermal protoplasts and tissues contained 10% to 35% of the total leaf activity. In sorghum, the bundle sheath strands contained 13% of the leaf activity. The presence of β-cyanoalanine synthase in all tissues and species studied suggests a fundamental role for this enzyme in plant metabolism.

β-Cyanoalanine synthase (EC 4.4.1.9), which catalyzes the reaction between cysteine and HCN to form β-cyanoalanine and H_2S (4), is widely distributed in higher plants (4, 11, 16). Although the enzyme has been purified and its kinetic properties examined (1, 12), its role(s) in plant metabolism have not yet been defined (see 8 for review). β-Cyanoalanine hydratase (EC 4.2.1.65) catalyzes the hydration of β-cyanoalanine to form asparagine (6). The sequential action of these two enzymes constitutes an effective mechanism for detoxifying HCN while conserving the nitrogen atom in the form of amide nitrogen. Such a role for these enzymes in cyanide detoxification is supported by studies (4, 5, 17) showing that gaseous, ^{14}C-labeled HCN is incorporated in high yields into asparagine when administered to either cyanogenic or noncyanogenic species. In addition, experiments demonstrating the detoxification of HCN produced *in vivo* during the metabolic turnover of cyanogenic glycosides in *Lotus* sp., *Nandina domestica*, and *Manihot utilissima* have been described (2, 3, 17). Asparagine synthesis in *Asparagus officinalis* has also been attributed to this two-step metabolic sequence (9), but the source of the HCN required as a substrate was not identified.

Recent studies by Peiser *et al.* (19) have provided evidence for the formation of $^{14}CN^-$ from [1-^{14}C]-1-aminocyclopropane-1-carboxylic acid ([1-^{14}C]ACC) during its conversion to ethylene *in vivo*. The radioactivity liberated during the metabolism of [1-^{14}C]ACC was converted in 62% yield to asparagine. HCN and β-cyano-L-alanine presumably were intermediates in the conversion. This important observation makes desirable more information regarding the localization of β-cyanoalanine synthase in plants. We report here the tissue localization of this enzyme in leaves from one species, sorghum, which contains a cyanogenic glycoside, and from three species, peas, maize, and leeks, which do not.

MATERIALS AND METHODS

Chemicals and Buffers. Cellulysin and Macerase were purchased from Calbiochem; all other biochemicals were from Sigma. The buffer used for preparation of cell-free extracts consisted of 50 mM Tris-HCl (pH 8.0) with 1% PVP-40, 10 mM 2-mercaptoethanol, and 0.01% Triton X-100.

Plant Materials. Seeds of *Zea mays* (var Golden Hybrid Blend), *Pisum sativum, Argentum* mutant (15), and a sorghum (*Sorghum bicolor* [L.] Moench)-sudan grass (*S. sudanense* [Piper] Stapf) hybrid (cv WAC Forage 99) were soaked in aerated water at room temperature. Sorghum and maize were planted in water-saturated vermiculite; peas were planted in soil. Seeds were germinated under a light/dark regime under conditions previ-

Table I. *Distribution of β-Cyanoalanine Synthase Activity in Leaf Tissues of Leek*

Results are the means of three separate experiments.

Tissue	Activity (nmol/min)		
	/mg protein	*/g fresh wt tissue*[a]	*/g fresh wt leaf*[b]
Outer epidermis	12	120	3.2
Inner epidermis	10	94	2.2
Mesophyll	12	24	19
Whole leaf	11	32	32

[a] Activity in 1 g of the tissue indicated.
[b] Activity in the tissue indicated which could be isolated from 1 g fresh weight of leaf.

Table II. *Distribution of β-Cyanoalanine Synthase Activity in Leaf Tissues of Pea*

Results are the means of two separate experiments.

Tissue	Activity (nmol/min)		
	/mg protein	*/g fresh wt tissue*[a]	*/g fresh wt leaf*[a]
Upper epidermis	55	610	18
Lower epidermis	23	150	11
Mesophyll	5.4	120	54
Whole leaf	3.8	66	66

[a] See footnotes in Table I.

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Table III. *Distribution of β-Cyanoalanine Synthase Activity in Leaf Tissues of Sorghum*

Results are the means of six separate experiments.

Tissue	Activity (nmol/min)			
	/10⁶ cells	*/mg protein*	*/mg Chl*	*/g fresh wt leaf*[a]
Epidermal protoplasts	35	183		129
Mesophyll protoplasts	32	102	756	1050
Bundle-sheath strands		48	473	186
Whole leaf		75	1560	1940

[a] Calculated from the relative Chl contents of mesophyll and bundle-sheath tissues, as determined by quantitative recovery of total leaf Chl from these tissues and from the ratio of mesophyll to epidermal cells in sorghum leaf blades (13).

Table IV. *Distribution of β-Cyanoalanine Synthase Activity in Leaf Tissues of Maize*

Results are the means of two separate experiments.

Tissue	Activity (nmol/min)			
	/10⁶ cells	*/mg protein*	*/mg Chl*	*/g fresh wt leaf*
Epidermal protoplasts	32	300		
Mesophyll protoplasts	21	37	260	
Bundle-sheath strands		65	790	
Whole leaf		44	570	530

ously described (22).

Sorghum seedlings were harvested at 5.5 d after planting and were 3 to 7 cm tall with two expanded leaves. Maize seedlings were 7 d old at harvest. Fully expanded pea leaves were removed from 20- to 30-d-old plants. Leeks (*Allium porrum*) were obtained from a local market.

Epidermal and Mesophyll Tissues of Peas and Leeks. Both adaxial and abaxial epidermal layers were peeled from the mesophyll layer of leaves of peas and leeks. Epidermal and mesophyll tissues were weighed and immediately frozen in liquid nitrogen, homogenized in Tris buffer, and desalted by rapid centrifugation through Sephadex G-25 which was equilibrated with the identical buffer.

Epidermal and Mesophyll Protoplasts and Bundle Sheath Strands from Sorghum and Maize. Abraded sorghum seedling leaf blades (13) and transversely cut segments of the second leaves of maize seedlings (10) were digested enzymically to yield a mixture of mesophyll and epidermal protoplasts (13). Epidermal and mesophyll protoplasts were purified by a Ficoll density gradient (Wurtele and Nikolau, unpublished). Epidermal protoplasts were collected from the 0%/7% interface. Cross-contamination between epidermal and mesophyll protoplasts was monitored by microscopic examination using a hemocytometer (13). Bundle sheath strands were isolated by a sedimentation/flotation procedure (13). Protoplasts were lysed with 50 mM Tris buffer. Whole leaves and bundle sheath strands were frozen in liquid nitrogen and homogenized in the Tris buffer. All samples were immediately centrifuged through Sephadex G-25 columns, equilibrated with Tris buffer, to remove small molecules.

β-Cyanoalanine Synthase Assays. Cell-free extracts were assayed immediately after passage through Sephadex G-25, or were frozen in liquid nitrogen in small aliquots which were thawed as needed. Enzyme activity was not altered by rapid freezing in liquid nitrogen. β-Cyanoalanine synthase activity was determined colorimetrically by measuring the reduction of methylene blue by the H_2S released from cysteine (4). Identical assays lacking enzyme, lacking substrate, and containing boiled enzyme were used as controls. Assays for the different plant species and tissues were linear with respect to time and substrate concentration. Triton X-100 (0.01%) in the assay did not affect β-cyanoalanine synthase activity.

Relative Chl levels in mesophyll and bundle-sheath tissues in sorghum leaves were determined by comparing the Chl content of whole leaves and of mesophyll protoplasts and bundle-sheath strands following the quantitative digestion of sorghum leaves (Wurtele and Nikolau, unpublished).

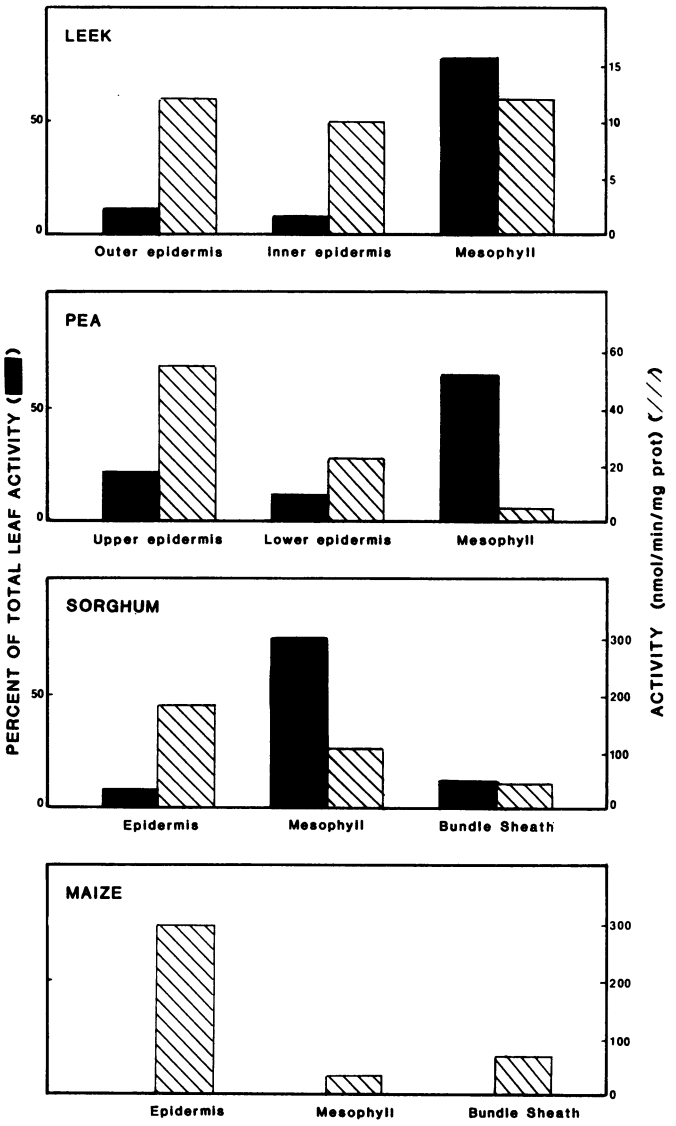

FIG. 1. Distribution of β-cyanoalanine synthase activity in leaves. β-Cyanoalanine synthase activity was compared in epidermal and mesophyll tissues from leeks and peas, and in epidermal and mesophyll protoplasts and bundle-sheath strands from maize and sorghum. Distribution of β-cyanoalanine synthase is presented as per cent of total leaf activity. For comparison, specific activites are also shown.

RESULTS AND DISCUSSION

β-Cyanoalanine synthase activities in leaf extracts ranged from 33 nmol·min^{-1}·g^{-1} fresh weight for leeks to 1940 nmol·min^{-1}·g^{-1} fresh weight for sorghum (Tables I–IV). The value for

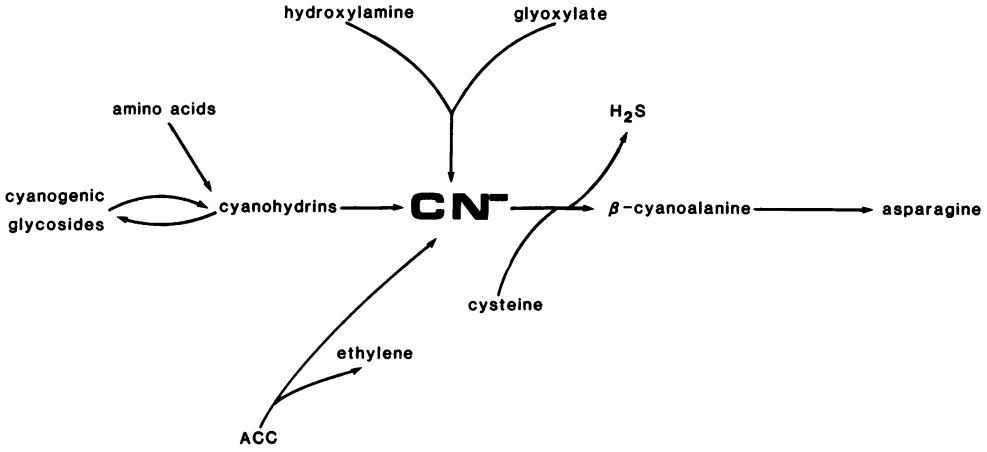

FIG. 2. Pathways of cyanide metabolism demonstrated in higher plants.

sorghum is similar to that reported in previous experiments with etiolated sorghum shoots (13).

β-Cyanoalanine synthase activities were determined in extracts prepared from mesophyll (containing vascular strands) and epidermal tissues from leek (Table I) and pea leaves (Table II) and in mesophyll protoplasts, epidermal protoplasts, and bundle-sheath strands isolated from leaves of sorghum (Table III) and maize (Table IV). It should be noted that, for these two latter species, β-cyanoalanine synthase activity in guard cells and in extracellular material, other than that associated with bundle sheath strands, could not be measured except as part of the whole-leaf extract.

β-Cyanoalanine synthase activity was found in all leaf tissues examined (Table I–IV). Because of the differences in the protein and Chl content among the tissues of the four species, the data are presented on the three bases of protein, Chl, and fresh weight.

The experimentally observed distribution of β-cyanoalanine synthase in the different leaf tissues could be markedly different from the true distribution if there are tissue-specific factors which inhibit or activate the enzyme. To discount these possibilities, we prepared whole-leaf extracts following quick freezing of the leaf in liquid nitrogen. The activities (on the basis of either protein, Chl, or fresh weight) of β-cyanoalanine synthase of the whole-leaf extracts were intermediate between the activities of the separated tissues (Tables I–IV). In leaves of sorghum, pea, and leek, we could calculate the contribution each separated tissue makes to the total activity of the enzyme in the leaf. The sums of the activities in the different tissues are similar to those obtained for the activity in the whole-leaf extracts (Tables I–III). These two findings strongly suggest the lack of selective inhibition or activation of β-cyanoalanine synthase in any of the tissues examined.

The epidermal tissue of pea (Table II) and isolated epidermal protoplasts from sorghum (Table III) and maize (Table IV) contained higher activities of β-cyanoalanine synthase (on the basis of protein and fresh weight) than the other tissues of these leaves. This suggests a greater requirement for this enzyme in epidermal tissues of these leaves, possibly reflecting the relative capacity for cyanide production in this tissue.

Tissue distribution of β-cyanoalanine synthase activity were determined for leaves of leeks, peas, and sorghum (Fig. 1). The mesophyll tissue in these species contained the bulk of the β-cyanoalanine synthase activity in the leaf, reflecting in part the greater number of mesophyll cells per leaf. The specificity activity of the enzyme in different tissues of the four species is given for comparison.

In sorghum leaves, the occurrence of β-cyanoalanine synthase activity in all three major leaf tissues contrasts with the compartmentation of cyanogenic glycoside metabolism. Dhurrin and the majority of the UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase activity are located in the epidermal protoplasts (13, 22) while the β-glucosidase specific for dhurrin hydrolysis is in the mesophyll protoplasts (13, 21). Upon leaf injury, dhurrin is hydrolyzed and HCN is released. If a primary role of β-cyanoalanine synthase in sorghum is detoxification, this enzyme would be required by all cell types because of the gaseous nature of the large amounts of HCN which are released upon dhurrin hydrolysis.

The ubiquitous nature of β-cyanoalanine synthase, as demonstrated by *in vivo* (2, 3, 5) and *in vitro* studies (4, 9, 16, and this paper), implies that HCN is produced more widely in plants than has previously been recognized. Several sources of HCN in higher plants have previously been described (Fig. 2). More than 2,000 plant species from a wide variety of plant families and genera (7) are known which accumulate amino acid-derived cyanogenic glycosides. Other plant species, which do not accumulate cyanogenic glycosides but synthesize labile cyanohydrins from amino acids, are known (3, 18). The recent discoveries of the release of HCN during ethylene biosynthesis from 1-aminocyclopropane 1-carboxylic acid (19), and the conversion *in vitro* of glyoxylate and hydroxylamine to cyanide (20), provide two additional examples of pathways resulting in cyanide biosynthesis in plants.

In vivo involvement of cyanide in the regulation of plant metabolic processes has rarely been postulated (20), although the *in vitro* effects of cyanide are well known. For example, cyanide-resistant respiration is a pathway induced both by endogenous cyanide and exogenous ethylene (14). An examination of the relationship between cyanide formation, ethylene formation, β-cyanoalanine synthase activity and cyanide-resistant respiration might clarify some of the ambiguities (14) associated with the function of this alternative respiratory pathway.

We suggest that the role of HCN and β-cyanoalanine synthase in plant metabolism may be more extensive than has previously been considered.

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LITERATURE CITED

1. AKOPYAN TN, AE BRAUNSTEIN, EV GORYACHENKOVA 1975 Beta-cyanoalanine synthase: Purification and characterization. Proc Natl Acad Sci USA 72: 1617–1621
2. ABROL YP, EE CONN 1966 Studies on cyanide metabolism in *Lotus arabicus* L. and *Lotus tenuis* L. Phytochemistry 5:237–242
3. ABROL YP, EE CONN, JR STOKER 1966 Studies on the identification, biosynthesis and metabolism of a cyanogenic glucoside in *Nandina domestica* Thunb. Phytochemistry 5: 1021–1027
4. BLUMENTHAL SG, HR HENDRICKSON, YP ABROL, EE CONN 1968 Cyanide

metabolism in higher plants III. The biosynthesis of cyanoalanine. J Biol Chem 243: 5302–5307
5. BLUMENTHAL-GOLDSCHMIDT S, GW BUTLER, EE CONN 1963 Incorporation of hydrocyanic acid labelled with carbon-14 into asparagine in seedlings. Nature 197: 718–719
6. CASTRIC PA, KJF FARNDEN, EE CONN 1972 Cyanide metabolism in higher plants. V. The formation of asparagine from β-cyanoalanine. Arch Biochem Biophys 152: 62–69
7. CONN EE 1979 Biosynthesis of cyanogenic glucosides. Naturwissenschaften 66: 28–34
8. CONN EE, GW BUTLER 1969 The biosynthesis of cyanogenic glycosides and other simple nitrogen compounds. *In* J B Harborne, T Swain, eds, Perspectives in Phytochemistry. Academic Press, New York, pp 47–74
9. COONEY DA, NH TARYARAM, SG SWENGROS, SC ALTER, M LEVINE 1980 The metabolism of L-asparagine in *Asparagus officinalis*. Int J Biochem 11: 69–83
10. DAY DA, CL JENKINS, MD HATCH 1981 Isolation and properties of functional mesophyll protoplasts and chloroplasts from *Zea mays*. Aust J Plant Physiol 8: 21–29
11. FLOSS HF, L HADWIGER, EE CONN 1965 Enzymatic formation of β-cyanoalanine from cyanide. Nature 208: 1207–1228
12. HENDRICKSON HR, EE CONN 1969 Cyanide metabolism in higher plants. IV. Purification and properties of the β-cyanoalanine synthase of blue lupine. J Biol Chem 244: 2632–2640
13. 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 65: 1022–1202
14. LATIES GG 1982 The cyanide resistant, alternative path in higher plant respiration. Annu Rev Plant Physiol 33: 519–555
15. MARX GA 1982 The argentum (Arg) mutant of *Pisum*: genetic control and breeding behavior. J Hered 73: 413–420
16. MILLER JM, EE CONN 1980 Metabolism of hydrogen cyanide of higher plants. Plant Physiol 65: 1199–1202
17. NARTEY F 1969 Studies on cassava, *Manihot utilissima* II. Biosynthesis of asparagine-^{14}C from ^{14}C-labelled hydrogen cyanide, and its relations with cyanogenesis. Physiol Plant 22: 1085–1096
18. OLECHNO JD, JE POULTON, EE CONN 1984 Nandinin, an acylated free cyanohydrin from *Nandina domestica*. Phytochemistry. In Press
19. PEISER G, TT WONG, N HOFFMAN, SF YANG 1983 Evidence for $^{14}CN^-$ formation from [1-^{14}C]ACC during *in vivo* conversion of ACC to ethylene. Plant Physiol 72: S-37
20. SOLOMONSON LP, AM SPEHAR 1981 Glyoxylate and cyanide formation. *In* B Vennesland, EE Conn, CJ Knowles, J Westley, F Wissing, eds, Cyanide in Biology. Academic Press, New York, pp 363–370
21. THAYER SS, EE CONN 1981 Subcellular localization of dhurrin β-glucosidase and hydroxynitrile lyase in the mesophyll cells of *Sorghum* leaf blades. Plant Physiol 43: 1919–1924
22. WURTELE ES, SS THAYER, EE CONN 1982 Subcellular localization of UDP-glucose:aldehyde cyanohydrin β-glucosyl transferase in epidermal plastids of *Sorghum* leaf blades. Plant Physiol 70: 1732–1737