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Metabolism of Hydrogen Cyanide by Higher Plants[1]

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ABSTRACT

A survey has been made of the occurrence and distribution of three enzymes which metabolize cyanide in a variety of higher plants including both cyanogenic and non-cyanogenic species. The enzymes investigated were β-cyanoalanine synthase, rhodanese and formamide hydrolyase. β-Cyanoalanine synthase was found to be present in every higher plant tested whereas rhodanese was found to occur far less commonly in plants. Formamide hydrolyase activity was not detected in any of the higher plants tested.

In addition, quantitative analyses have been made of the potential hydrogen cyanide content of each plant investigated. A general trend was apparent between the hydrogen cyanide potential and cyanide metabolizing activity, in that the higher the hydrogen cyanide potential, in general, the higher the cyanide metabolizing activity.

Many plants have the ability to produce HCN; more than 2,000 species have been demonstrated to be cyanogenic. The mechanism for the production of HCN, in most species, is the degradation of cyanogenic glycosides (7). The present study investigates the occurrence and distribution of cyanide metabolizing enzymes in a variety of higher plants, including both cyanogenic and non-cyanogenic species. This information in turn is compared with the HCN potential of each plant studied. The HCN potential is a reflection of the concentration of cyanogenic glycosides in the plant which, upon degradation, leads to the release of HCN.

The enzymes selected for investigation were β-cyanoalanine synthase (EC 4.4.1.9), rhodanese (EC 2.8.1.1), and FHL[3] (EC 4.2.1.66). The reactions they catalyze are shown in equations 1–3, respectively:

$$\underset{\text{cysteine}}{HSCH_2CHNH_2CO_2H} + HCN \rightarrow \underset{\beta\text{-cyanoalanine}}{NCCH_2CHNH_2CO_2H} + H_2S \quad (1)$$

$$S_2O_3^{2-} + CN^- \rightarrow SO_3^{2-} + SCN^- \quad (2)$$

$$HCN + H_2O \rightarrow HCONH_2 \quad (3)$$

The enzyme β-cyanoalanine synthase has been shown to be present in several plant species (1, 9) and in some bacteria (4, 8). Rhodanese, however, has been extensively investigated from animal sources and some bacteria (23) but only little studied in higher plants (5, 22). FHL, an enzyme found to be present in several species of fungi (11, 12), has been reported from only two plant sources, Japanese apricot (*Prunus Mume*, Sieb. et Zucc.) and loquat (*Eryobotrya japonica*, Lindl) (19).

MATERIALS AND METHODS

GROWTH OF PLANTS

When seedlings were used, the seeds were soaked and aerated in water for approximately 16 h and then placed on damp Vermiculite, except in the case of *Sorghum bicolor* (Linn.) Moench where seedlings were grown on cheesecloth. All seedlings were grown in the dark at room temperature. The age of the seedlings used for enzyme assays is indicated in Tables I and II. For *Phaseolus lunatus*, seed of Costa Rican wild lima beans were germinated and apical leaves of light grown plants were used. Samples of *Nandina domestica* and mature fruits of loquat (*Eryobotrya japonica*, Lindl) were obtained from plants growing in the Davis campus. Leaves of *Brassica oleraceae* var. capitata were obtained from local markets. *Gloeocercospora sorghi*, a generous gift of Dr. W. E. Fry, was cultured on V-8 juice (17).

PREPARATION OF CELL-FREE EXTRACTS

In most cases plant samples were ground to a fine powder in liquid N_2 and then placed in the extracting buffer. For seedlings of *Lupinus augustifolia*, *Glycine max*, *Phaseolus aureus*, mature leaves of *Brassica oleraceae* var. capitata and for the mesocarp of mature loquat fruits, extracts were prepared by homogenization in a Waring Blendor for 45 s. The crude homogenate, from both methods, was squeezed through cheesecloth and the filtrate centrifuged at 12,000*g* for 10 min. The resulting supernatant liquid (hereafter termed the enzyme extract) was used for enzyme assays. All steps in the preparation of the enzyme extract were carried out below 4 C.

ENZYME ASSAYS

All substrate solutions were prepared immediately prior to the enzyme assay. Enzyme activities were determined in every instance by reference to the appropriate boiled control. Controls containing no substrate(s) and controls containing no enzyme extract were also performed.

β-Cyanoalanine Synthase. The β-cyanoalanine synthase assay was based on the reduction of methylene blue by H_2S (1). Tris-HCl (50 mM) (pH 8.5) was used as the extracting buffer; 2.5 ml of the buffer was used per 1 g of fresh weight of plant material. For samples of *N. domestica* the extracting buffer was 0.2 M Tris-HCl (pH 8.5) containing Amberlite XAD-4 resin (14) (2:1 w/w resin: plant material), and the supernatant liquid, after centrifugation, was passed through a Sephadex G-25 column (Pharmacia column PD-10). For samples of loquat mesocarp the extracting buffer was 0.2 M Tris-HCl (pH 8.9). NaCN and L-cysteine were dissolved in 100 mM Tris-HCl (pH 8.5) to a final concentration of 50 and 10 mM, respectively, and the pH adjusted to 8.5 immediately prior to

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[3] Abbreviation: FHL: formamide hydrolyase.

Table I. *β-Cyanoalanine Synthase Activity in Cyanogenic Plants*

Duplicate samples were performed for each experiment. In most cases the enzyme activity was established more than once for each plant, but the results given are for one experiment only. For the determination of HCN potential, 4 replicates were done for each plant whether positive or negative.

Plant	Tissue and Growth Conditions	β-Cyanoalanine Synthase Activity	β-Cyanoalanine Synthase Activity	HCN Potential
		nmol H_2S/g fresh wt tissue·min	*nmol H_2S/mg protein·min*	*μmol HCN/g fresh wt tissue*
Nandina domestica	Buds	1050 ± 42	113 ± 24	205 ± 37
	Berries	51 ± 2	10.0 ± 0.9	0.28 ± 0.04
Phaseolus lunatus	Leaves	869 ± 50	131 ± 11	27.5 ± 5.2
Sorghum bicolor moench	3-day seedlings:			
	shoots	600 ± 20	115 ± 5	38.3 ± 1.7
	roots	123 ± 1	63.7 ± 1.8	4.62 ± 0.23
Linum usitatissimum	6-day seedlings:			
	shoots	91.5 ± 0	19.8 ± 0.3	17.4 ± 1.5
	roots	26.5 ± 0	11.3 ± 0.1	15.6 ± 0.2
Lotus tenuis	9-day seedlings:			
	whole seedlings	88.0 ± 0.3	31.9 ± 2.4	4.3 ± 0.7
Eschscholzia californica	5-day seedlings:			
	whole seedlings	80.0 ± 0.6	26.6 ± 1.4	2.87 ± 0.01
Trifolium repens	5-day seedlings:			
	whole seedlings	31.9 ± 0.3	13.8 ± 0.4	0.13 ± 0.01
Vicia sativa	10-day seedlings:			
	whole seedlings	28.1 ± 0.3	5.0 ± 0.6	0.05 ± 0.02
Eryobotrya japonica	Mature mesocarp	3.3 ± 0.1	1.7 ± 0.2	0.17 ± 0.02

Table II. *β-Cyanoalanine Synthase Activity in Non-Cyanogenic Plants*

Duplicate samples were performed for each experiment. In most cases the enzyme activity was established more than once for each plant, but the results given are for one experiment only.

Plant	Tissue and Growth Conditions	β-Cyanoalanine Synthase Activity	β-Cyanoalanine Synthase Activity	HCN Potential
		nmol H_2S/g fresh wt tissue·min	*nmol H_2S/mg protein·min*	*μmol HCN/g fresh wt tissue*
Lupinus augustifolia	10-day seedlings:			
	whole seedlings	24.0 ± 0.6	5.0 ± 1.0	ND[a]
Phaseolus aureus	5-day seedlings:			
	whole seedlings	14.1 ± 0.3	2.7 ± 0.1	ND
Medicago sativa	5-day seedlings:			
	whole seedlings	7.8 ± 0.3	2.2 ± 0.1	ND
Glycine max	4-day seedlings:			
	whole seedlings	3.92 ± 0.04	0.101 ± 0.006	ND
Brassica oleraceae var. capitata	4-day seedlings:			
	whole seedlings	75.6 ± 3.8	14.5 ± 1.1	ND
	mature leaves	130.6 ± 3.1	29.7 ± 2.5	ND
Brassica juncea var. foliosa	4-day seedlings:			
	whole seedlings	25.0 ± 1.3	8.5 ± 1.0	ND

[a] ND: not detected. For the determination of HCN potential, four replicates were done for each plant whether positive or negative.

use. The enzyme extract and substrate solutions were equilibrated separately at 30 C for 5 min and then 0.5 ml of the buffered NaCN solution was added to the enzyme extract (1.0 ml) followed immediately by 0.5 ml of the buffered L-cysteine solution. After 10 min, in most cases, or 30 min with extracts having only a low activity, the reaction was stopped by the addition of acidic *N,N*-dimethyl-*p*-phenylenediamine sulfate and ferric chloride and the color developed as previously described (1). The samples were then centrifuged at 1,050*g* for 5 min to remove precipitated proteins and the *A* at 650 nm recorded using a Beckman DU spectrophotometer. Sodium sulfide was used as the standard reference. When necessary, enzyme extracts were diluted with 50 mM Tris-HCl (pH 8.5) prior to assay.

Rhodanese. The rhodanese assay was based on the formation of a colored complex between thiocyanate and ferric nitrate according to the method of Sorbo (21). Tris-HCl (50 mM) (pH 8.5) was used as the extracting buffer; 2.0 ml of the buffer was used per 1 g fresh weight plant material. KCN and $Na_2S_2O_3$ were dissolved in 100 mM Tris-HCl (pH 8.5) to a final concentration of 0.125 M each and the pH adjusted to 8.5 immediately prior to use.

To 1.0 ml of the buffered KCN solution was added 1.0 ml of the buffered $Na_2S_2O_3$ solution at 30 C; the solutions were mixed and 0.5 ml of enzyme extract was added. Samples were incubated at 30 C for 0, 30, 60, and 90 min (except for extracts of *S. bicolor* which were incubated for 0, 5, 10, and 15 min), after which time 0.5 ml formaldehyde (37% w/v) was added followed by 2.5 ml ferric nitrate reagent (6). The samples were centrifuged at 1,050*g* for 5 min and the *A* at 460 nm recorded. Sodium thiocyanate was used as the standard reference.

KCN will react spontaneously with $Na_2S_2O_3$ to form thiocyanate (SCN^-); the rate of this spontaneous reaction increases with increasing temperature and pH. Formaldehyde inhibits this spontaneous reaction. It is, therefore, necessary to choose carefully the correct controls when performing the rhodanese assay particularly at elevated temperatures and pH.

FHL. The FHL assay was based on the formation of an hydroxamate between formamide and hydroxylamine (12). Tris-HCl (50 mM) (pH 8.0) was used as the extracting buffer; 2.0 ml of the buffer was used per 1 g fresh weight plant material. NaCN (100 mM) in 50 mM Tris-HCl (pH 8.0) was adjusted to pH 8.0 immediately prior to use. To 1.0 ml of enzyme extract was added 1.0 ml of the buffered NaCN solution. Samples were incubated for 2 h at 25 C after which time 1.0 ml was used for formamide analysis by the hydroxamate method (12, 20) and 1.0 ml for ammonia analysis (see below) in order to detect any conversion of HCN to NH_3 as reported by Shirai (19).

For the ammonia analysis, 1.0 ml of the reaction mixture was placed in the outer chamber of a 25-ml Erlenmeyer flask fitted with a center well. H_2SO_4 (0.5 ml of 1 N) was added to the center well. To the outer chamber was added 1.0 ml of a saturated solution of K_2CO_3 and the flask sealed and shaken at 30 C for 2 h. The H_2SO_4 was removed and the center well rinsed with 0.5 ml 0.1 N H_2SO_4. To the combined H_2SO_4 fractions (1.0 ml) was added 5 ml of Nesslers' reagent (Sigma ammonia color reagent, diluted times 10 with deionized, distilled H_2O). After 15 min the *A* at 490 or 420 nm was recorded. Ammonium chloride (also subjected to microdiffusion) was used as the standard reference.

DETERMINATION OF THE HCN POTENTIAL

Plant material (approximately 0.5 g) was ground to a fine powder in liquid N_2 and placed in the outer chamber of a 50-ml Erlenmeyer flask fitted with a center well. NaOH (1.0 ml of 1 N) was placed in the center well and into the outer chamber was added 2–3 ml of an enzyme mixture capable of hydrolyzing the known cyanogenic glycosides and having the following composition: 50 mg commercial emulsin (Sigma), 5.0 ml linamarase obtained from flax seedlings (2), and 100 ml 100 mM Na-phosphate (pH 6.8). The flasks were sealed and shaken overnight at 37 C. The NaOH was removed and diluted to 2.0 ml with 0.1 N NaOH.

The HCN concentration was determined colorimetrically by a modified method of Lambert (13). After acidification of a 1.0 ml aliquot of the cyanide sample with 0.5 ml acetic acid (1.0 N), 5.0 ml of succinimide-*N*-chlorosuccinimide reagent was added. Finally, 1.0 ml of the barbituric acid-pyridine reagent was added and the *A* at 580 nm was recorded after 10 min.

PROTEIN DETERMINATION

Protein concentration was determined by the method of Lowry *et al.* (15). Enzyme extracts were passed through a G-25 column (Pharmacia PD-10) and the protein fraction collected before determination. BSA was used as a protein standard.

RESULTS

β-CYANOALANINE SYNTHASE

Values for β-cyanoalanine synthase activity in extracts of cyanogenic plants are shown in Table I. It can be seen that the enzyme was present in every higher plant tested. Moreover, there is a general trend between the enzyme activity and the HCN potential. The higher the HCN potential, in general, the higher the β-cyanoalanine synthase activity. Table II shows the results obtained for non-cyanogenic plants. The activity of β-cyanoalanine synthase in non-cyanogenic plants was found, in general, to be lower than that observed in cyanogenic plants. The exception was the seedlings and mature leaves of *B. oleraceae* var. capitata.

Because of results obtained for *B. oleraceae* var. capitata, alternative explanations for the production of H_2S involving enzymes other than β-cyanoalanine synthase in *Brassica* seedlings were considered. Several members of the Cruciferae have been shown to contain the enzyme cystine lyase (16); therefore, a pathway for the formation of H_2S involving this enzyme was considered possible (Fig. 1). In this scheme, cysteine may undergo oxidation to cystine which may then be further converted to thiocysteine, pyruvate and ammonia by the enzyme cystine lyase. The reactive product, thiocysteine, may then be converted back to cystine in the presence of cysteine with the simultaneous release of H_2S. Another conceivable mechanism for forming H_2S could be the enzyme cystathionine-γ-lyase (EC 4.4.1.1) acting on the substrate cysteine. This reaction would involve the direct conversion of cysteine to pyruvate, ammonia, and H_2S (equation 4).

$$HSCH_2CHNH_2CO_2H \rightarrow CH_3COCO_2H + NH_3 + H_2S \quad (4)$$

Since both reactions involve the formation of pyruvate, the production of this compound from cysteine was investigated. The total keto acid method of Friedemann and Haugen (10) was used to determine pyruvate production. Pyruvate production from cysteine was observed in extracts of seedlings of both *B. oleraceae* var. capitata and *B. juncea* var. foliosa. However, the production of pyruvate in both cases was inhibited completely when cyanide (at a final concentration of 12.5 mM), was present in the reaction mixture. We concluded that the values for H_2S production given for the *Brassica* species in Table II were the result of β-cyanoalanine synthase activity.

RHODANESE

Each species listed in Tables I and II was examined for rhodanese but activity was detected in only the four species given in Table III. In 3-day-old shoots of etiolated *S. bicolor* (Linn.) Moench, the enzyme activity was found to be very high. The HCN potential for *Sorghum* shoots was also high. Rhodanese activity was detected in only one other cyanogenic plant, namely *E. californica* but it was also observed in two species of non-cyanogenic plants, *B. oleraceae* var. capitata and *B. juncea* var. foliosa.

FHL

Formamide production from cyanide was not observed in any of the higher plants listed in Tables I and II. *Gloeocercospora*

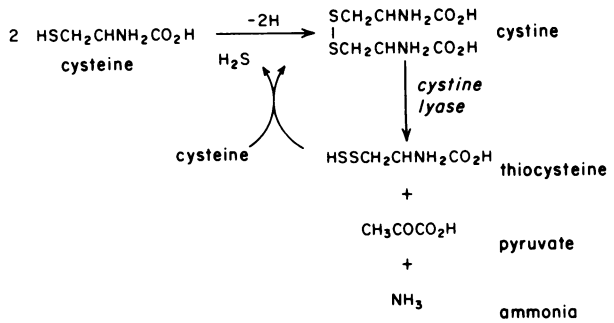

FIG. 1. A possible pathway for H_2S production involving the enzyme cystine lyase.

Table III. *Rhodanese Activity in Cyanogenic and Non-Cyanogenic Plants*

Plant	Tissue and Growth Conditions	Rhodanese Activity	Rhodanese Activity[a]	HCN Potential
		nmol thiocyanate/mg protein·min	*nmol thiocyanate/g fresh wt tissue·min*	*μmol HCN/g fresh wt tissue*
S. bicolor moench	3-day seedlings	252 ± 15	923	38.3 ± 1.7
E. californica	5-day seedlings: whole seedlings	2.49 ± 0.14	7.3	2.87 ± 0.01
B. oleraceae var. capitata	4-day seedlings: whole seedlings	7.73 ± 0.04	24.2	ND[b]
	mature leaves	1.73 ± 0.09	10.7	ND
B. juncea var. foliosa	4-day seedlings: whole seedlings	5.2 ± 0.3	9.7	ND

[a] The values given are the average of duplicate results.
[b] ND: not detected.

sorghi, a fungus pathogenic towards *Sorghum* sp. has previously been shown to have FHL activity (11). The mycelium and spores of this fungus were therefore also tested for the presence of FHL and found to be active when assayed under the same conditions as those used for higher plants.

If HCN is hydrolyzed to formamide in higher plants, it is possible that the formamide could be further hydrolyzed to formic acid and ammonia, with the result that formamide may not accumulate and be detected. Formamide and ammonia were therefore analyzed simultaneously. However, no ammonia production, above that of the boiled control, was detected for any of the plants listed in Tables I and II.

DISCUSSION

Every plant investigated in this study was capable of metabolizing HCN by one or more pathways and the pathway common to all plants tested was that involving β-cyanoalanine synthase. The results of the study also indicate that rhodanese occurs far less commonly in plants. Rhodanese has previously been reported in mature leaves of *B. oleraceae* var. capitata (22) and also, more recently, in *Sorghum* sp. (18). These findings were confirmed in the present study.

A general trend was apparent between cyanide metabolizing activity and HCN potential in higher plants; the higher the HCN potential, in general, the higher the cyanide metabolizing activity. Cyanide is a well known inhibitor of the terminal step in respiration. Since the degradation of cyanogenic glycosides leads to the release of HCN, it may therefore be advantageous for plants which contain cyanogenic glycosides to be capable of metabolizing cyanide, and for those plants which contain high levels of cyanogenic glycosides to have high levels of cyanide metabolizing activity.

The utilization of β-cyanoalanine synthase by plants for the metabolism of cyanide may also be advantageous since many plants (3) can further metabolize the product, β-cyanoalanine, to asparagine which can then be incorporated into the general metabolism of the plant.

In a recent study (19) Shirai has observed the conversion of HCN to NH_3 in extracts of Japanese apricot (*P. Mume*, Sieb. et Zucc.) and loquats (*E. japonica*, Lindl). Based on studies involving a partially purified protein fraction from loquat and work with [^{14}C]HCN, Shirai proposed that HCN is first converted to formamide by FHL and that the latter is then converted to formic acid and NH_3, possibly via formaldoxime as an intermediate. Another possibility is the direct hydrolysis of formamide to formate and NH_3 by the enzyme formamide amidohydrolase.

Using loquat mesocarp tissue of different stages of ripeness, however, we could not confirm the enzymic conversion of HCN to formamide or NH_3 as reported by Shirai (19). Since the enzymic conversion of HCN to either formamide or NH_3 was not observed in any of the other 14 species of plants examined in this survey, we could not establish the significance of FHL in the metabolism of HCN by higher plants.

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