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ON THE ISOLATION AND IDENTIFICATION OF CANAVANINE AND ETHANOLAMINE CONTAINED IN THE YOUNG LEAVES OF BLACK LOCUST, *ROBINIA PSEUDOACACIA*, LETHAL FOR THE LADY BEETLE, *HARMONIA AXYRIDIS*

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ハリエンジュの若葉に含まれ，ナミテントウムシに致死的なカナバニンとエタノールアミンの分離と同定について

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The oriental pea aphid, *Aphis craccivora*, living on black locust, *Robinia pseudoacacia*, is toxic to the lady beetle, *Harmonia axyridis*. The lethal compounds were isolated from the young leaves of black locust. They were soluble in water and were identified as canavanine and ethanolamine by fractionation of free amino acids, bioassay of each fraction, and amino acid analysis. The contents of canavanine and ethanolamine in the young leaves of black locust and in the oriental pea aphid living on them were determined.

ハリエンジュに寄生するマメアブラムシはナミテントウムシに有毒である。ハリエンジュの若葉から，水溶性致死化合物の分離を試み，遊離アミノ酸の分画，各画分の生物検定，アミノ酸分析によってカナバニンとエタノールアミンと同定した。ハリエンジュ若葉とそれに寄生するマメアブラムシ中のカナバニンとエタノールアミンの含量を定量した。

Introduction

In Japan, the lady beetle, *Harmonia axyridis* PALLAS, is one of the most important useful insects, because this species of beetle distributes widely and preys upon many species of aphids which are injurious to crops, flowers, vegetables, fruits, and forest trees.

In spite of such general evaluation as a useful insect, several papers[1-5] reported that the larvae of *H. axyridis* died within several few days when they fed the oriental pea aphid, *Aphis craccivora* KOCH, living on the young leaves of black locust, *Robinia pseudoacacia* L., while the aphid living on *Vigna sinensis* or *Vicia hirsuta* was not toxic to the beetle.

The present authors assumed that the young leaves of black locust contained some compounds which made the oriental pea aphid toxic. The present paper is dealt with the isolation of lethal compounds from the young leaves of black locust, detection of toxicity by means of bioassay, identification of the toxic compounds, and determination of them in the young leaves of black locust and oriental pea aphid.

Material, Bioassay and Fractionation Methods

Materials. I. Young leaves of black locust.

The young shoots of black locust which were planted as street trees in Takamatsu city were cut off during mid May and late June and taken to the laboratory, then stored in a freezing chamber kept at −20°C. After that time,

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they were taken out from the chamber and young but still not-fully developed leaves on them were detached. Detached young leaves were freed from the parasitic aphids and dusts, freeze-dried, and pulverized to pass 100–200 mesh sieve and they were stored under the condition of −20°C until extracting experiment.

II. Aphid.

The shoots of black locust with aphids collected at Takamatsu city were taken to the laboratory from early July to early August. These shoots were stored in a room kept at 10°C for a while. Then the aphids parasitized on the shoots were swept off from the shoots with hair-pencil in that cool room. Other species of aphids than the oriental pea aphid and dusts were removed carefully. The gathered material aphids were freeze-dried and pulverized to pass 100–200 mesh sieve and they were stored as powder at −20°C.

III. Control food for rearing the beetle.

Larval drone honeybees powder (LDHP)[6] was used as control food for rearing the beetle and for bioassay with the larvae of the beetle. LDHP was donated by Laboratory of Applied Entomology, Faculty of Agriculture, Tamagawa University, at Machida, Tokyo. The powder was stored at −20°C until use.

Bioassay methods. I. Bioassay procedure.

Various fractions fractionated from the young leaves of black locust as described in Fractionation method were mixed thoroughly with the control food such as LDHP or steam-heated, freeze-dried, and powdered apple grain aphid, *Rhopalosiphum prunifoliae*, living upon oat, *Avena sativa*, which is the best food for the beetle. The fractions were added in the ratio mainly at 1 to 5, and sometimes at 1 to 10 or 1 to 50 to prepare the test food. When a fraction was obtained in very small amount, it was dissolved in water and added to the control food.

Since the results of bioassay with LDHP and dried aphid powder as control food were almost the same throughout the experiment, and as the preparation of dried aphid powder in large amount was difficult, most of the bioassay was carried out with LDHP as control food.

Each of newly hatched larvae of the beetle was put in each polyethylene container and starved for 24 hours. Test food moistened with water was put on a piece of filter paper and was fed to each of larvae for every 24 hours, and 4 individual larvae composed one test plot.

A proper bioassay procedure was used to simulate the transfer of lethal compounds from the young leaves to the beetles through aphids. The cut green leaves of oat were put in large test tubes and were cultured on 5 ppm kinetin solution which contained 0.1 m*M* of studied compounds. Then, the apple grain aphids which were let to parasitize for 7–10 days on the leaves cultured with the said solution were fed to the beetle to examine the transfer of compounds and their toxicity. This new procedure established in the present work was named by us as TCA (Toxication of Cultured Aphid) method.

II. Elucidation of bioassay results.

The efficiency of each fraction was classified from the results of bioassay on death rates and growth inhibition of the larvae of beetle. The classification is shown in Table 1.

Table 1. Classification of bioassay results

Relative evaluation	Death rates	Growth inhibition
−	0%	None
±	0–30%	Without obvious growth inhibition
+	20–60%	With growth inhibition to some extent
++	40–80%	With obvious growth inhibition
+++	70–100%	With marked growth inhibition

Fractionation method. Amino acid fraction.

The present authors assumed that some compounds in the phloem sap of black locust made the oriental pea aphid toxic to the beetle. Therefore, in proper way, lethal compounds should be isolated chemically from the phloem sap. But it is difficult to obtain phloem sap from black locust in large amount[7]. As the lethal compounds from the freeze-dried young leaves were soluble in water[8], the authors used the young leaves as the starting material. Since it was known from a preliminary experiment[9] that the lethal compounds belonged to amino acids or related compounds, the compounds of amino acids group were obtained and fractionated as follows[10].

Freeze-dried and powdered young leaves of black locust were mixed with Polyclar-AT powder[11] to remove polyphenols and then with 5% trichloroacetic acid solution. After steeping overnight, the suspension was centrifuged at 12,000 rpm for 15 minutes, and the supernatant was poured onto a column with cation exchanger, Amberlite IR-120 B (H^+ form). After washing the column with water, free amino acid fraction including purines, pyrimidines, guanidino compounds, and non-volatile amines were eluted from the column with 2 *N* ammonia.

After evaporating free amino acid fraction under vacuum to remove ammonia, this fraction was further fractionated into acidic, neutral, and basic amino acids fractions by eluting from the columns with Dowex 21 K (acetate form) with 2 *N* acetic acid, from Amberlite IR-120 B (ammonia form) with water and then with 2 *N* ammonia, respectively. Basic amino acid fraction was fractionated again into imidazolic and aliphatic basic amino acids fractions by eluting the column with Amberlite IRC-50 (ammonia form) with water and 2 *N* ammonia, respectively.

Each fraction was freed from acetic acid or ammonia by evaporation under vacuum and stored at -20°C.

Imidazolic amino acid fraction was further fractionated by eluting from the columns with CM Sephadex A-25 with 0.1 *M* sodium acetate and then with DEAE Sephadex A-25 with increasing concentration of sodium acetate. These fractions were desalted with ion exchange resins before bioassay.

Aliphatic amino acid fraction was fractionated again by eluting from the column with Dowex 50 W×8 (H^+ form) with increasing concentration of ammonia. Ammonia was removed from each fraction by vacuum evaporation.

Each amino acid fraction was fractionated as above also from the oriental pea aphid collected from black locust. In this case, acetone-extracted aphid was mixed with same amount of Polyclar-AT powder, because aphid bodies contained large amounts of polyphenols and pigments.

Amino acids in each fraction were identified and determined by amino acid analyzer, KLA-5, Hitachi, with authentic amino acids as standard.

Results

The weight of the young leaves of black locust decreased to about 20% of the fresh weight after freeze-drying showing that moisture content of young leaves was about 80%. The weight of the aphid decreased to about 30% of the fresh weight after freeze-drying showing that moisture content of the aphid was about 70%.

Pulverizing freeze-dried young leaves of black locust was found to be superior to homogenizing the fresh leaves in juicer or grinding them in mortar; lethal compounds were extracted from the freeze-dried and pulverized material with good yield.

Fractionation and lethal activity of amino acid fractions.

Yields of each amino acid fraction and its relative evaluation of lethal activity for the beetle are shown in Figure 1. Results of bioassay of each amino acid fraction are shown in Table 2.

Since the basic amino acid fraction had the strongest lethal activity among 3 amino acid fractions, imidazolic and aliphatic basic amino acid fractions were further fractionated and lethal activity of each fraction was determined as shown in Figures 2 and 3, and in Tables 3 and 4.

Amino acids analysis given in Figure 3 and Table 4 showed that the C-3 and C-4 fractions contained relatively large amount of canavanine, and C-2 fraction contained ethanolamine as main amine. Since canavanine and

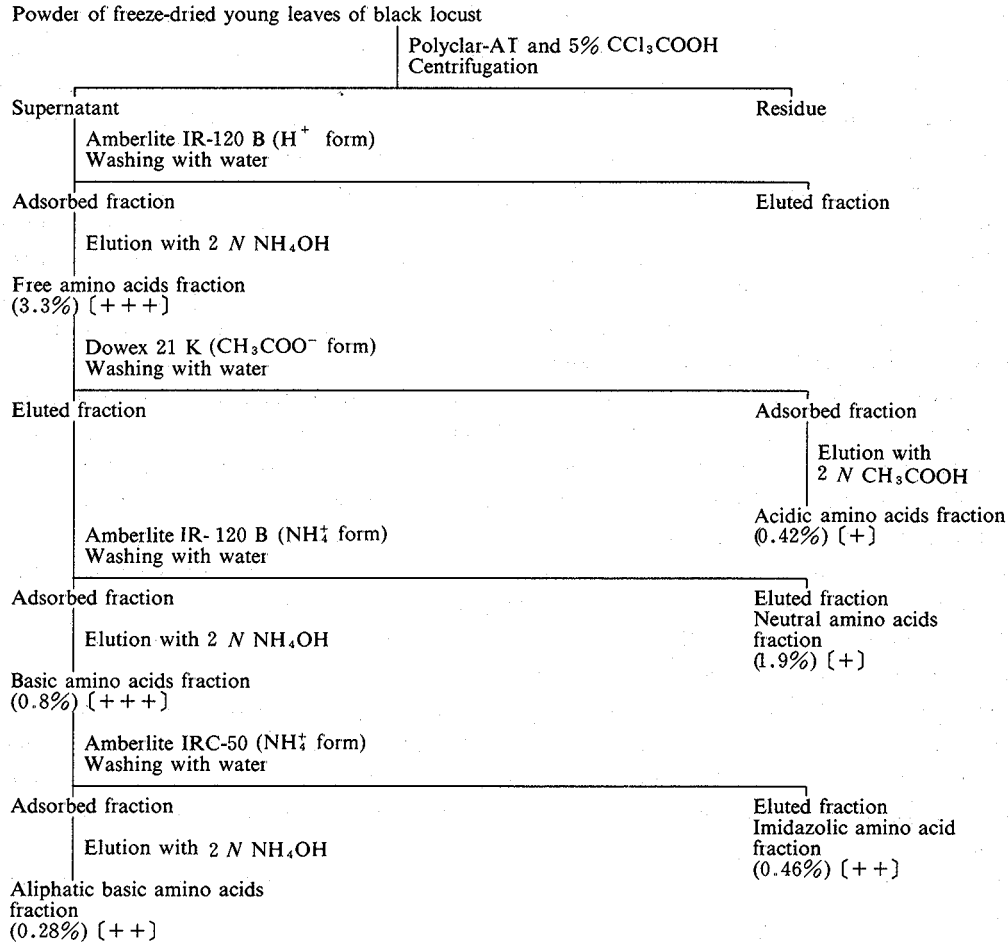

Figure 1. Preparation of free amino acids fractions from young leaves of black locust
( )=Yield %
[ ]=Lethal activity shown by relative evaluation

Table 2. Bioassay of each amino acid fraction obtained from young leaves of black locust

Test fraction added to the control food	Numbers of larvae tested	Numbers of test plot	Death rates	Remarks
None	7	2	0	
Free amino acids	8	2	100	
Acidic amino acids	8	2	50	
Neutral amino acids	8	2	50	
Basic amino acids	8	2	87.5	
Aliphatic basic amino acids	4	1	75	Growth inhibition
Imidazolic amino acid	4	1	75	Growth inhibition

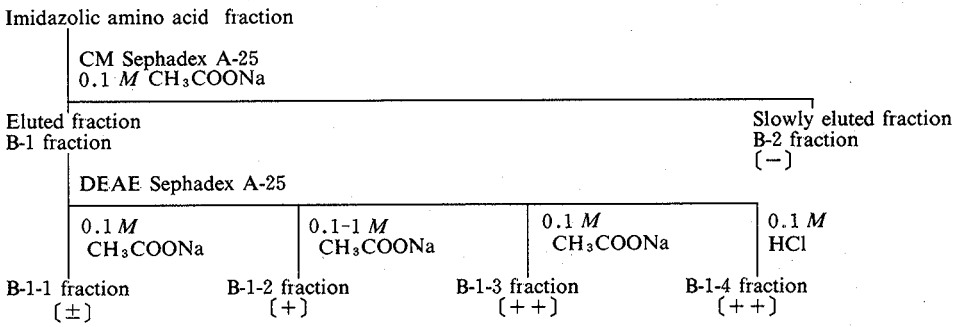

Figure 2. Fractionation of imidazolic amino acid fraction
( )=Lethal activity shown by relative evaluation

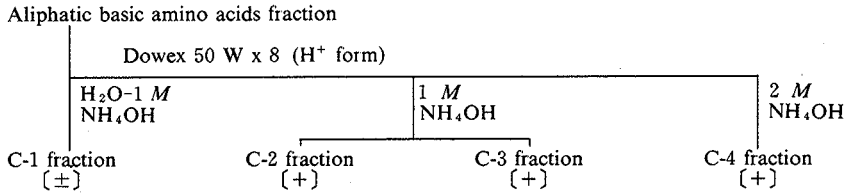

Figure 3. Fractionation of aliphatic basic amino acids fraction
( )=Lethal activity shown by relative evaluation

Table 3. Bioassay of each fraction from imidazolic amino acid fraction

Test fraction added to the control food	Numbers of larvae tested	Numbers of test plot	Death rates	Remarks
None	10	3	0	
Basic amino acids fraction	4	1	100	
B–2 fraction	4	1	0	
B–1–1 fraction	4	1	25	
B–1–2 fraction	4	1	50	
B–1–3 fraction	10	1	80	Growth inhibition
B–1–4 fraction	10	1	80	Growth inhibition

Table 4. Bioassay of each fraction from aliphatic basic amino acids fraction

Test fraction added to the control food	Numbers of larvae tested	Numbers of test plot	Death rates	Remarks
None	4	1	0	
Basic amino acids fraction	4	1	75	Growth inhibition
C–1 fraction	4	1	25	
C–2 fraction	4	1	50	Growth inhibition
C–3 fraction	4	1	50	Growth inhibition
C–4 fraction	4	1	50	Growth inhibition

Table 5. Contents of main free amino acids from young leaves of black locust, oriental pea aphid living upon black locust, and LDHP

Amino acid	Leaves	Aphid	LDHP
Methionine	0.80%	1.04%	0.24
Isoleucine	0.03	0.12	0.56
Tyrosine	0.99	0.91	1.91
Phenylalanine	0.63	2.79	0.42
Ethanolamine	0.05	0.05	none
Histidine	2.17	2.02	0.38
Lysine	0.70	3.84	1.48
Tryptophan	0.37	0.46	0.82
Canavanine	1.06	0.39	none

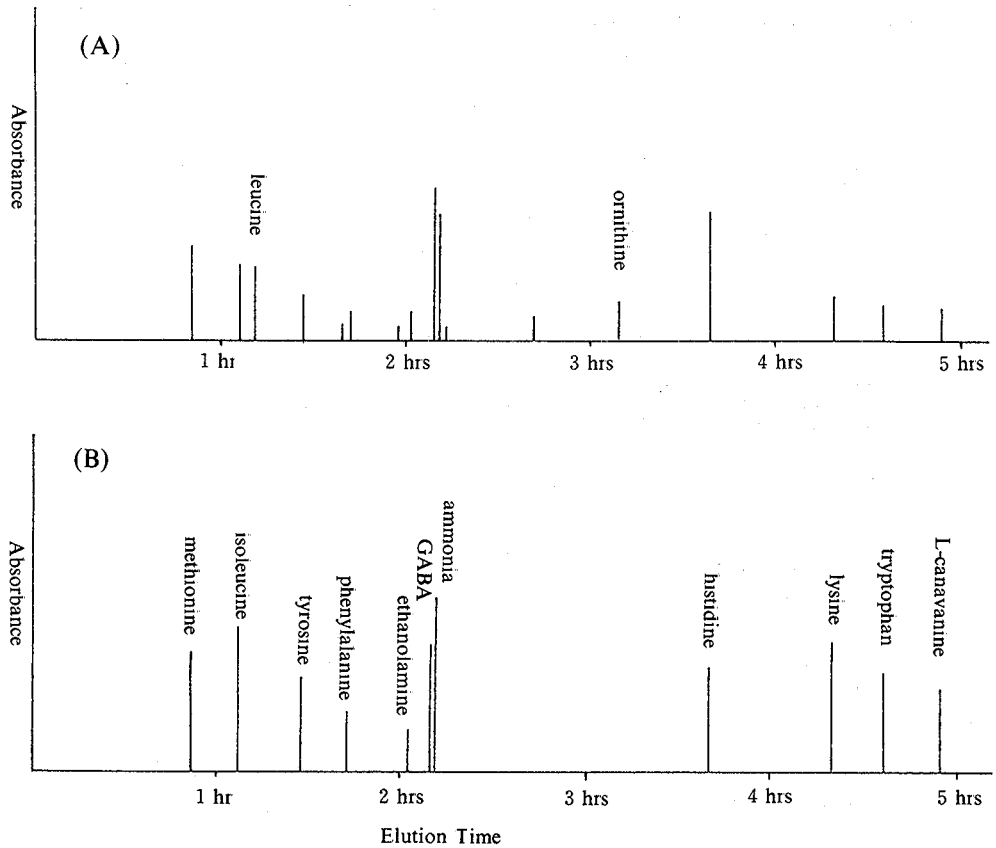

Figure 4. Amino acids analysis of free amino acids fraction from young leaves of black locust (A) and standard free amino acids (B)

ethanolamine were supposed to constitute the lethal compounds, the contents of canavanine, ethanolamine, and other main amino acids in the free amino acids fractions obtained from the young leaves of black locust, the oriental pea aphid living upon black locust, and LDHP were determined with amino acid analyzer as shown in Table 5. The elution pattern of amino acids obtained from the young leaves of black locust was compared with that of standard amino acids as shown in Figure 4. The bioassay results of identified main amino acids by the use of commercially

Table 6. Bioassay of main amino acids contained in the young leaves of black locust

Compound added to the control food	Numbers of larvae tested	Numbers of test plot	Death rates	Remarks
None	12	3	0	
Canavanine	8	2	75	Growth inhibition
Ethanolamine	8	2	62.5	Growth inhibition
Arginine	8	2	0	
Histidine	8	2	0	
Isoleucine	8	2	0	
Methionine	8	2	0	

Table 7. TCA bioassay of main free amino acids from young leaves of black locust

Compound added to culture medium	Numbers of larvae tested	Numbers of test plot	Death rates	Remarks
None	6	2	0	
Canavanine	6	2	33	Growth inhibition
Ethanolamine	6	2	33	Growth inhibition
Arginine	3	1	0	
Histidine	3	1	0	
Lysine	3	1	0	
γ-Amino-*n*-butyric acid	4	1	0	

Concentration of each compound was 0.1 m*M*.

Table 8. Relative evaluation of lethal activity of main amino acids and amine for *Harmonia axyridis*

Compound	LDHP Bioassay	TCA Bioassay
None	−	−
Canavanine	++	+
Ethanolamine	++	+
Arginine	−	−
Histidine	−	−
Isoleucine	−	not tested
Methionine	−	not tested
γ-Amino-*n*-butyric acid	not tested	−

available authentic amino acids were shown in Table 6. Lethal activity of some amino acids and ethanolamine to the beetle larvae was determined by TCA method as shown in Table 7. Relative evaluation of lethal activity of main amino acids and amine were summarized from the data shown in Tables 6 and 7 as shown in Table 8.

Discussion

Bioassay.

The bioassay procedure utilizing LDHP or dried aphid powder as control food had advantages, that is, the procedure was simple and time-saving, so this procedure was assumed to be sufficient to screen the lethal compounds.

But there were several problems about the bioassay procedure. During bioassay, test food paste was spread on a filter paper slip, so that infusion of the lethal compounds from the test food to the filter paper was possible. Actually, a paper slip, which had test food paste on it for 10 days, colored faint purple if sprayed with ninhydrin solution.

Moreover, the homogeneous mixing of control food such as LDHP or dried aphid powder with lethal fraction or isolated lethal compounds was not guaranteed, especially if small amount of lethal compounds was used.

In addition, it was almost impossible to determine the amount of test food taken by the larvae of the beetle.

Although the TCA bioassay was not used extensively in this experiment, it enabled to prove that a supposed lethal compound in the liquid medium was absorbed by the oat leaf and then by the apple grain aphid, which was originally innoxious to the beetle, and made the aphid toxic to the beetle. The transfer of canavanine or ethanolamine from the young leaves of black locust to the beetle through aphids was clearly simulated by this procedure.

Canavanine.

Although the configuration of canavanine in the leaves of black locust was not determined, it was assumed to have L-configuration, because commercially available authentic L-canavanine showed almost the same lethal activity as the fractionated one. Canavanine is synthesized in the leaves of legumes[12] as an important nitrogen stock and is toxic to certain other forms of life[13]. Canavanine accounted for 31.8% of total nitrogen in phloem sap of black locust[14]. The contents of canavanine in total free amino acids from the young leaves of black locust and in oriental pea aphid living upon black locust were 1.0% and 0.4% as shown in Table 5, respectively.

Metamorphosis of silk worm, *Bombyx mori*, was inhibited by a food containing only 5 ppm canavanine[15]. Death rate of *Callosobruchus masculatus* increased markedly by adding 1% canavanine to artificial diet[16]. There were several reports on the toxicity of canavanine to tobacco horn worm, *Manduca sexta*[17,18]. Moreover, 36 m*M* canavanine in 1 kg artificial diet killed almost all *Tribolium castaneum*[19].

Ethanolamine.

Ethanolamine in distillates of plant material was assumed to be formed by degradation of larger molecules[20]. Since free amino acid fractions were obtained by rather mild extraction method, ethanolamine in this fraction was not assumed to be a decomposition artefact. Free ethanolamine was found in 41 species of higher plant[20], and its oral LD_{50} for rats is 2.1 g/kg[21].

An important problem on the fractionation of the lethal compounds was the decrease of death rate as the fractionation proceeded. This might suggest that death rate of the beetle was caused by the additive or synergic effect of several toxic compounds. This point of view needs further investigation.

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