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ANALYSIS OF FREE AMINO ACIDS IN DEVELOPMENTAL STAGES AND DIFFERENT FEMALE MORPHS OF *CALLOSOBRUCHUS ANALIS*

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ABSTRACT :

The Present studies deals with the analysis of free amino acids in the development stages and also the different adult female morphs I-III of Callosobruchus analis by paper chromatography technique. For identification of amino acids in each band, their Rf value and Colors were compared with those of stranded map of known 20 amino acids. The color intensity of bands was also recorded. The three morphs I-III of adult females comprise 11, 9 and 10 spots respectively. The amino acid patterns in the various morphs of adult females of C. analis has revealed 14, 12 and15 amino acids in them. The difference in amino acids observed during the present studies has been discussed in relation to differential gene activation in various life processes like metamorphosis, differentiation and polymorphism.

KEYWORDS : *Chromatography, Rf value, Callosobruchus analis, Morph.*

1. INTRODUCTION:

The fine details of any biological process cannot be fully understood until various biomolecules have been isolated and characterized. For this task, chromatography technique has provided an excellent tool for separation, isolation and purification of biomolecules. Martin and Synge (1944) received the Noble prize for developing methodology of partition chromatography. Various Zoologists, Botanists, Geneticists and other scientists have employed paper chromatography for separation of various types of biomolecules.

The main development events in insects such as growing, moulting etc. are the results of the process of cell differentiation which in turn are regulated by protein which is the most important molecule in the cell. These proteins constitute about half or more of their dry weight. These proteins are made of amino acid residues, covalently linked by peptide bond. Also, the high concentration of free amino acids (FAA) is believed to play an important role in regulation and making the insect more radio resistant than mammals (Beadle and Shaw 1950). The present studies on different female morphs of *Callosobruchus analis* have been carried out under insufficiency of information on this aspect.

2. MATERIAL AND METHODS

2.1 Material

The adult females of *C. analis* comprised the material for the present studies. It is a common pest in stored pulses and usually occurs in association with *C. maculates* and *C. Chinensis*. The adults do not take any food, whereas larvae feed voraciously. It is known in the literature that *C. analis* occurs in polymorphic forms based on the color patterns of elytra pygidium and the genetalia. In the cultures

maintained in the Laboratory, three different morphs on the basis of their color patterns were identified. The characteristics of different female morphs are as follows:

1. Black elytra and black pygidium,
2. Brown elytra and black pygidium,
3. Brownish elytra and black pygidium.

The present were designed to find out whether these polymorphic forms are because of the difference in their amino acid patterns or not. The adult individuals of *C. analis* were collected from the infested seeds of *Phaseous aureus* (Moong). They were reared in the laboratory on the fresh seeds of *P. aureus* to analyze the presence of free amino acids in them. The insects were cultured in plastic jars under controlled temperature of 30°C, maintained in the B.O.D incubator. For the study of qualitative and quantitative differences in free amino acids pattern in the different polymorphic forms of *C. analis*, the whole body of the insects was analyzed chromatographically. To keep an accuracy of the spots, the extract was prepared from a fixed number of individuals.

2.2 Methods

The extract for the present studies were prepared as recommended by Mick (1956). A fixed number of insects were taken in a test tube having 1 ml of distilled water and crushed with the help of electrical homogenizer. The material was then centrifuged at 2000rpm for 10 minutes and the supernatant retained for further processing. To the above supernatant, equal volume of 95% ethanol was added to precipitate the protein and centrifuged again at 2000 rpm for 10 minutes. The supernatant so obtained was then mixed with three volumes of chloroform followed by subsequent centrifugation done at 1000rpm for 15-30 minutes. The upper layer of aqueous fraction was collected with the help of a dropper for the analysis of the free amino acids.

Separation

The various requirements for the separation of free amino acids are chromatographic chamber, solvent and location/detection reagent.

Chromatographic chamber

It consists of two Petri dishes, each having diameter of 10cm. In the lower Petri dish, the solvent was placed which is covered over by the second Petri dish. The solvent was kept in the chamber for 10-15 minutes before starting the experiments, so that the chamber gets saturated with solvent vapors.

A. Solvent

Solvent used for Chromatographic analysis consist of a mixture of butanol, acetic acid and distilled water in the ratio 60:25:15 respectively. It is considered to be the best solvent for the separation of amino acids from the sample.

B. Location/ Detection reagent

For the detection of different amino acids on the chromatogram, ninhydrin solution in acetone (0.3%) was sprayed using a fine nozzle atomizer.

The Circular chromatography technique is also called Rutter method or horizontal chromatography technique.

i. Application of the sample
ii. Solvent run /Developing

(i) Application of the Sample:

In the centre of the circular filter paper (whatmann No.1), a circle of diameter one cm was drawn with the help of a sharp lead pencil and a Compass. Three crosses were marked on this circle at

equal distances. This paper was withheld using a clean forceps followed by transferring to the Petri dish before application of sample with the aid of a very thin capillary tube. The spotting was done 15 times in order to obtain concentrated spots. However, allowing the previous spot to dry before applying the next one. Excessive spreading of the spot was avoided, keeping the spot confined to a small area.

(ii) Solvent Run Developing:

After the application of the sample 15 times for various developmental stages and 10 times for standard amino acids sample, the spots were allowed to dry in air and then in centre a hole was made in the circle using a forceps. A paper wick cut out from another filter paper of the same quality in the form of a triangle was then inserted in the hole of the filter paper. Afterwards, the filter paper was placed in chromatographic chamber for the solvent development/run, ensuring the wick was dipping in the solvent. The solvent was allowed to run till it reached the edges of the petri dish. The filter paper and the wick was removed with the help of forceps after removing upper petri dish and the solvent front was marked using lead pencil. The paper was then dried in air.

3. DETECTION

0.3% ninhydrin solution in acetone was used as a detector. By holding the filter paper with the help of a forceps at a distance, the ninhydrin solution was uniformly sprayed using an atomizer with a fine nozzle. To obtain the colored bands of the amino acids on the filter paper it was first air dried and then warmed in the oven at 100ºC for 10-15 minutes. The colored bands so obtained were studied for the estimation of various FAAs in the experimental sample.

3.1 Preservation of the amino and chromatograms:

As the colored spots fade away within an hour, it is necessary to keep some of the chromatograms from spraying with ninhydrin for getting good resolutions in photographs as and when required. For this the chromatograms could be stored at room temperature in clean cardboards or wooden boxes for few days.

3.2 Photography:

The chromatograms were sprayed with ninhydrin solution, dried in the open and placed in the oven at 100°C for 10-15 minutes. Afterwards, they were taken out and boundaries of different spots were marked and photographed.

3.3 Identification of the Colored arcs/bands /spots:

For the identification of colored bands, standard map of known 20 amino acids were first prepared under the same experimental conditions as that used for the insect's under investigation. The Rf value (reference front) of the spots was calculated as follows

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}} \times 100$$

The Rf value as mentioned above is usually infractions but is convenient to prefer it in %age or number (Rf value x100).Thus, the Rf value has been calculated in number throughout the present course of investigations.

3.4 Preparation of Chromatograms of standard amino acids:

Each standard amino acid was weighed 1.5 mg individually. The solution was prepared by dissolving it in one ml of 10% isopropanol. The solution of each amino acid was spotted 10 times on circular filter paper (whatmann No.1). After the solvent run, it was then sprayed with 0.3% ninhydrin solution (300mg/100ml acetone) and dried first in open and then in oven at 100ºC for 10-15 minutes.

The color of the spot was noted and its Rf value was calculated. In the same way, the Rf value, patterns and intensity of colors of the 20 known standard amino acids were noted. To identify the amino acids from the various bands of the sample under analysis, the Rf value and color of all the bands were noted. Thus, by comparing the Rf value and color of each spot with that of standard amino acid, the amino acids in each band were identified and recorded.

4. OBSERVATIONS

To identity amino acids in various bands of the sample, the Rf value of all the spots were recorded and calculated using chromatogram. For the purpose of identification, both the Rf value and the color of spots were taken into consideration.

Thus, by comparing Rf value and color of each spot with that of standard amino acids, the amino acids in each spot were identified and recorded.

4.1 Standard map of 20 known free amino acids:

Some variations in the Rf value of the known free amino acids were observed, when compared to those obtained by earlier research workers. The variations may be due to the influence of various factors. The influence of various factors may be due to run of the solvent front, volume of solvent used, temperature, the nature of the solvent mixture, the grade of paper used etc. Therefore, to minimize experimental variations, the standard solutions of the amino acids were run individually under the same laboratory conditions as the material during the present studies. The name, color, Rf value and base triplet code of 20 standard amino acids are given in Table1.

Table 1: Name, color, Rf value and base triplet code of 20 standard amino acids.

Sr. No.	Name of amino acids	Color	Rf value	Base triplet code**
1	L-Histidine *	Blue violet	12	CAU,CAG
2	L-Arginine	Purple	13	AGA,AGG
3	L-Ornithine	Blue violet	16	___
4	L-Cysteine	Violet pink	18	___
5	L-Lysine	Purple	26	AAA,AAG
6	DL-Serine	Purple brown	27	AGU, AGC
7	Glycine	Purple	31	GCC, GCA
8	DL- Aspartic acid	Violet Pink	33	GAU, GAC
9	DL-Theonine	Dark Purple	36	ACU, ACC ,ACA, ACG
10	L-Glutamic acid	Purple	40	GAA, GAC
11	L- Tyrosine	Purple Light	42	UAA ,UAC
12	DL-Tryptophan	Brown	46	UGG
13	DL-Alanine	Purple Brown	47	GCU , GCC ,GCA
14	L- Proline	Yellow	51	CCU , CCC, CCA ,CCG
15	DL- Methionine	Purple	53	AUG
16	DL-Butyric acid	Purple Pink	57	___
17	DL- Valine	Purple Brown	65	GUU, GUC,GUA,GUG
18	DL-Phenylalanine	Purple Dark	72	___
19	L-Leucine	Purple	77	UUA , UUG
20	DL- Isoleucine	Purple	84	___

*These amino acid are as monohydrochlorides

** Nirenberg and Methaei (1961)

4.2 Free amino acids in eggs of *C. analis*:

Circular chromatograms of the eggs of *C. analis* has revealed 7 spots with varying colors i.e. Violet pink, Purple & Purple brown and Rf value ranging from 14 to 68. The 12 amino acids which could be identified in these spots are given in Table II (Fig.1) with their different color intensities.

Table II. Color, Color intensity, Rf value and name of free amino acids present in eggs of *C. analis*:

Spot No.	Color	Color Intensity	Rf value	Name of free amino acids present
1	Purple	++	14	l- Histidine, L- Arginine, L- Ornithine
2	Violet	++	18	L- Cysteine
3	Purple	++	25	L- Lysine, DL- Serine
4	Violet Pink	+	33	Glycine, DL- Aspartic Acids
5	Purple Brown	++	40	L-Glutamic acid, L- Tyrosine
6	Purple	+	53	DL- Methionine
7	Purple	+	68	DL- Valine

4.3 Free amino acids in fourth instar larvae of *C. analis*:

Circular chromatograms of the fourth instar of *C.analis* has revealed 9 spots with varying colors i.e. Brown, Purple and Violet and Rf values ranging from 18 to 84. The 14 amino acids which could be identified in these spots are given in Table III (Fig.2) along with their different color intensities.

Table III: Color, color intensity, Rf value and name of free amino acids present in fourth instar larvae of *C. analis.*

Spot No.	**Color**	**Color Intensity**	**Rf value**	**Name of free amino acids present**
1	Violet	++++	18	L-Cysteine, L- Ornithine
2	Purple	++	24	L-Lysine
3	Purple	++	31	Glycine, DL –Aspartic acid
4	Purple	++	38	DL- Threonine, L- Glutamic acid
5	Brown	+++	46	DL –Tryptophan, DL-Alanine
6	Purple	+++	56	DL- Butyric acid
7	Purple	++	62	DL-Valine
8	Purple	+	75	L-Leucine, DL-Phenylalanine
9	Purple	+	84	DL- Isoleucine

4.4 Free amino acids in the pupae of *C. analis*:

Circular chromatograms of the pupae of *C. analis* has revealed 11 spots with varying colors of Yellow, Brown, Violet and Purple and Rf values ranging from 15 to 85. The 15 amino acids which could be identified in these spots are given in Table IV (Fig.3) with their different color intensities. An unidentified yellow colored band with Rf value 65 was also observed.

Table IV: Color, color intensity, Rf value and name of free amino acids present in pupae of C. analis:

Spot No.	Colour	Colour Intensity	Rf value	Name of free amino acids present
1	Violet	++++	15	L- Ornithine
2	Violet	++	21	L-Cysteine
3	Purple	++	27	L-Lysine, DL- Serine
4	Violet	+	34	DL-Aspartic acids
5	Purple	++	40	L-Glutamic acid, L-Tyrosine
6	Brown	+++	45	DL-Tryptophan, DL-Alanine
7	Purple	++	50	DL-Methionine, L-Proline
8	Purple	++++	57	DL-Butyric acid,
9	Yellow	+	65	Unidentified
10	Purple	+	74	DL-Phenylalanine, L-Leucine
11	Purple	+	85	DL-Isoleucine

4.5 Free amino acids in the adult Female morph I (Black elytra and Black Pygidium) of *C. analis:*

Circular chromatograms pattern in adult and female morph I of analis has revealed 11 spot with varying colours of Purple Brown, Purple and Violet and Rf value ranging from 19 to 86. The 14 amino acids which could be identified in these spots are given in Table V (Fig.4) with their different colour intensities.

Table V: Colour, colour intensity Rf value and name of free amino acids ,present in Female morph I (Black elytra and Black Pygidium) of *C. analis*.

Spot No.	Colour	Colour Intensity	Rf value	Name of free amino acids present
1	Violet	+	19	L-Cystine
2	Purple	+++	21	L-Lysine
3	Purple	++	25	DL- Serine
4	Purple	++	40	L-Glutamic acid, L-Tyrosine
5	Purple Brown	+	45	DL-Trytophan, DL-Alanine
6	Purple	++	51	DL-Methionine, L-Proline
7	Purple	+++	58	DL- Butyric acid
8	Purple	++	65	DL-Valine
9	Purple	+	74	DL-Phenylalanine
10	Purple	+	80	L-Leucine
11	Purple	+	86	DL-Isoleucine

4.6 Free amino acids in the adult female morph II (Black elytra and Black Pygidium) of *C. analis.*

Circular chromatogram pattern in the adult female morph II of *C. analis* has revealed 9 spots with varying colors of Brown, Purple Brown, Purple yellow and Violet blue with Rf values ranging from 15 to 73. The 12 amino acids which could be identified in these spots are given Table VI (Fig.5) with their different color intensities.

Table VI: Color, color intensity, Rf value and name of free amino acids present in female morph II (Black elytra and Black Pygidium) of *C. analis.*

Spot No.	Color	Color Intensity	Rf value	Name of free amino acids present
1	Violet Blue	+++	15	L-Arginine , L-Ornithine
2	Purple	++	23	L-Lysine
3	Purple Brown	++	27	DL-Serine
4	Purple	++	36	DL-Threonine
5	Purple Brown	+	42	DL-Tyrosine, L-Glutamic acid
6	Brown	++	47	DL-Tryptophan DL-Alanine
7	Yellow	+	52	L-Proline
8	Purple	+	64	DL-Valine
9	Purple	+	73	DL-Phenylalanine

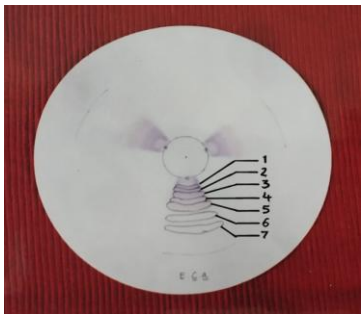

Fig. 1Spots of free amino acids in eggs of *C. analis*.

Fig. 2 Spots of free amino acids in larvae of *C. analis*.

Fig. 3 Spots of free amino acids in pupae of *C. analis*.

Fig.4 Spots of free amino acids in morph I of *C. analis*.

Fig.5 Spots of free amino acids in morph II of *C. analis*.

Fig. 6 Spots of free amino acids in morph III of *C. analis*.

4.7 Free amino acids in the adult female morph III (Black elytra and Black Pygidium) of *C. analis.*

Circular chromatogram pattern in the adult female morph III of *C. analis* has revealed 10 spots with varying colors of Brown, Purple Brown, Purple yellow and Violet blue with Rf values ranging from 14 to 74. The 15 amino acids which could be identified in these spots are given Table VII (Fig.6) with their different color intensities.

Table VII: Color, color intensity, Rf value and name of free amino acids present in female morph III (Black elytra and Black Pygidium) of *C. analis.*

Spot No.	Color	Color Intensity	Rf value	Name of free amino acids present
1	Purple	+++	14	L-Histidine, L-Arginine
2	Violet	++	19	L-Ornithine, L- Cystine
3	Purple	++	23	L-Lysine
4	Purple Brown	++	28	DL-Serine
5	Purple	++	34	DL-Aspartic acid, DL-Threonine
6	Purple	++	41	L-Glutamic acid, L-Tyrosine
7	Purple	+++	50	DL-Alanine
8	Yellow	++	53	L-Proline
9	Purple	+	66	DL-Valine
10	Purple	+	74	DL-Phenylalanine, L-Leucine

Table VIII: Amino acids present in eggs, larvae, pupae and various morphs (I-III) of *C. analis* among with their intensities.

S. No.	Name of amino acids	Eggs	Larvae	Pupae	Females morphs		
					I	II	II
1	L-Histidine	P(++)	A	A	A	A	P(+++)
2	L-Arginine	P(++)	A	A	A	P(+++)	P(+++)
3	L-Ornithine	P(++)	P(+++)	P(++++)	A	P(+++)	P(++)
4	L-Cysetine	P(++)	P(++++)	P(++)	P(+)	A	P(++)
5	L-Lysine	P(++)	P(++)	P(++)	P(+++)	P(++)	P(++)
6	DL-Serine	P(++)	A	P(++)	P(++)	P(++)	P(+++)
7	Glycine	P(+)	P(++)	A	A	A	A
8	DL-Aspartic acid	P(+)	P(++)	P(++)	A	A	P(++)
9	DL-Threonine	A	P(++)	P(++)	A	P(++)	P(++)
10	L-Glutamic acid	P(++)	P(++)	P(++)	P(++)	P(+)	P(++)
11	L-Tyrosine	P(++)	A	P(++)	P(++)	P(+)	P(++)
12	DL-Trytophan	A	P(+++)	P(+++)	P(+)	P(++)	A
13	DL-Alanine	A	P(+++)	P(+++)	P(+)	P(++)	P(+++)
14	L-Proline	A	A	P(++)	P(++)	P(+)	P(++)
15	DL-Methionine	P(+)	A	P(++)	P(++)	A	A
16	DL-Butyric acid	A	P(+++)	P(++++)	P(+++)	A	A
17	DL-Valine	P(+)	P(++)	A	P(++)	P(+)	P(+)
18	DL-Phenylalanine	A	P(+)	P(+)	P(+)	P(+)	P(+)
19	L-Leucine	A	P(+)	P(+)	P(+)	A	P(+)
20	DL-Isoleucine	A	P(+)	P(+)	P(+)	A	A

5. DISCUSSION:

The present work was designed to determine the free amino acid composition in the various developmental stages i.e. egg, larvae, and pupae in the various polymorphs of adult female of *C. analis*. It is with a view to find out the role of free amino acids in development, if any, in this insect.

In the present investigation, eggs, larvae and pupae have revealed 7, 9 and 11 spots respectively. These developmental stages carried 12, 14 and 16 amino acids. L-Ornithine, L-Cystine, L-Lysine, DL-Aspartic acid and L-Glutamic acid have been identified in all the developmental stages. Although, these amino acids are present in all the developmental stages but their intensities differ with

respect to each other. Dang and Pant (1964) observed variation in free amino acids in developmental stages of different insects. The three morphs I-III of adult females have revealed 11, 9 and 10 spots respectively. These morphs I-III carried 14, 12, and 15 amino acids respectively in them (Table VIII to XII and XV). L-Lysine, DL-Serine, L-Glutamic acid, L-Tyrosine, DL-Alanine, L-Proline, DL-Valine and DL-Phenylalanine have been observed in all the adult female morphs. Although, these amino acids are present in all the morphs but their intensities differ with respect to each other. For instance, L-Lysine and DL-Valine is maximum in morph no. I, DL-Serine and DL-Alanine in morph no. III whereas, DL-Proline and L-Glutamic acid in morph no.I and III. Handa *et al.* (1996) reported qualitative and quantitative differences in free amino acids in the adults of *Zygogramma bicolorata* collected from different localities.

6. CONCLUSION:

The findings revealed that not only the different developmental stages but different morphs of the same species also vary in their free amino acids content and concentration. These differences may be due to various life supporting processes such as metamorphosis, differentiation, polymorphism and differential gene activities.

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