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Analysis of Free Amino Acid in Different Male 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 various morphs of adult male's morphs I-V of Callosobruchus analis by paper chromatography technique. For identification of amino acids in each band, their Rf value and Colours were compared with those of stranded map of known 20 amino acids. The colour intensity of bands was also recorded. The five morphs I-V of adult males comprise 9, 11, 8, 8 and 8 spots respectively. The amino acid pattern in the various morphs of adult males of Callosobruchus. analis has revealed difference in them. The difference observed during the present studies has been discussed in relation to their role in various life processes like metamorphosis, sex differentiation and polymorphism.***

Keywords: Chromatography, Rf value, *callosobruchus analis*, Morph

1. Introduction

Chromatography is a technique which helps in the separation of the chemical ingredients of a biological sample. The importance lies primarily in its use as an analytical tool. Paper chromatography did not become much popular until the work of Consden *et al.* (1944) who not only introduced, but also demonstrated its applications.

The molecular details of a biology process cannot be fully understood until the various bimolecular have been isolated and characterized. For this purpose chromatography techniques have provided an extremely good tool for separation, isolation and purification of all biomolecules.

This method was developed in by Russian botanist Michael Tswett (1906) who separated the plant pigments. Chromatography techniques, however, did not become a serious technique until the work of Martin and Synge (1994) who received the Nobel Prize for developing methodology of partition chromatography.

Geneticists, Botanists, Zoologists and a variety of other workers have employed paper chromatography for the separation of amino acids. Proteins, carbohydrates, vitamins and many other substances in the biological system. The advantage of the technique over other is in the quickness, small amount of material required the experiment and sensitivity of analysis.

The major development events such as growth, moulting etc are the result of the process of cell differentiation which in turn involves the synthesis of proteins .These are the most important molecules in the cell. Constituting about half or more of their dry weight. There are hundreds of proteins in each cell which are specific for a specific function. These are made up of one or more polypeptide chains, each consisting of many types of alpha-amino acids residues covalently linked by peptide bond. It is in this respect that the importance of the study of free amino acids becomes evident. This higher concentration of free amino acids is believed to play an important role in osmoregulation and make the insect more radio resistant than mammals (Beadle & Shaw, 1950). Other than buffering of the blood, the main function of the free amino acids is the synthesis of proteins.
The present studies on different morphs of Callosobruchus analis have been carried out due to the lack of information on this aspect in this beetle. This beetle along with *C. maculates* and *C. chinensis* are the important pests of stored grain .The chromatographic analysis of free amino acids in the life cycle of various insects constitute a promising field of research. It is believed that the present study of free amino acids in the various developmental stages like eggs, larvae, pupae and also on the various morphs of male species would add to the existing information in the area of research (Dhindsa, K.S. and Saini, D. 2014).

2. Material and Methods

Material

The adult males of *Callosobruchus analis* comprised the material for the present studies. *C. analis* is a common pest of 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 know in the literature that *C. analis* occurs in polymorphic forms based on the color pattern of elytra pygidium and the genetalia. In the cultures maintained in the Laboratory, five different morphs on the basis of their colour pattern, were identified, The characteristics of different male morphs is as follows:

Male Morphs

1. Pygidium - Black
2. Pygidium - Brown with lateral side black,
3. Pygidium - Brown with lateral and posterior side black,
4. Pygidium - With white seta and brownish back ground
5. Pygidium - Reddish brown

The present were designed to find out whether these polymorphic forms are because of the different in their amino acid patterns or not.

The adult individuals of *C. analis* were collected from the infested seed of *Phaseous aureus* (Moong). They were reared in the laboratory on the fresh seeds of *P. aureus* to analyze the present of free amino acids in them. The insects

were cultured in plastic jars at a controlled temperature of 30°C maintained in the B.O.D chamber.

For the study of qualitative and quantitative difference in free amino acids pattern in the different polymorphic forms of *C. analis*, the whole body of the insects was analysed chromatographically. To keep an accuracy of the spots, the extract was prepared from a fixed number of individuals.

Methods

The extract for the present studies was prepared as recommended by Mick (1956). A fixed number of insects were taken in test tube having 1 ml of distilled water and crushed with the help of electrical homogenizer. The materiel was then centrifuged at 2000rpm for 10 minutes and the supernatant retained for further processing .To the above supernatant equal volume of 95% enathol 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 and 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 Chromatographic chamber, Solvent and Location/Detection reagent.

Chromatographic chamber It consists of two petridishes of equal size having 10cm diameter. In the lower Petridis, the solvent was placed which is covered over by the second Petridis. The solvent was kept in the chamber for 10-15 minutes before starting the experiments so that the chamber gets saturated with solvent vapours.

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 the different amino acids on the chromatogram, ninhydrin solution in acetone (0.3%) was sprayed using a fine nozzle atomizer as recommended by Toennies and Kolb (1951).

The Circular chromatography technique is also called Rutter method or horizontal chromatography technique .It was carried out in the following steps as recommended By Swarup *et al*. (1981).

A. Application of the sample
B. Solvent run /Developing

A. 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 a sharp lead pencil and a Compass. Three crosses were marked on this circle at equal distance .This paper was then with held the help of a clean forcep and palced on the petridish before the sample was applied with the help of a very thin capillary tube. The spotting was done 15 times so that a concentrated spot was obtained, allowing the previous spot to dry before applying the next one. Much spreading of the spot was avoided keeping the spot confined to a small area.

B. Solvent Run Developing

After the application of the sample 15 times for various developmental stages 10 times for standard amino acids sample, the spots were allowed to dry in air and then in a centre hole was made in the above circle with the help of a forcep. A paper wick was cut out from another filter paper of the same quality in the form of a triangle and inserted in the hole of the filter paper. The filter paper was placed in chromatogphic chamber for the solvent development/run, taking care that the wick was dipping in the solvent. The solvent was allowed to run till it reached the the edges of the petridish. After removing upper petridish the filter paper and the wick was removed with the help of forcep and the solvent front was markedd with the help of the lead pencil. The paper was then dried in air.

3. Detection

0.3% ninhydrin solution in acetone was used as a detector. For this 300 mg of ninhydrin was dissolved in 100 ml for acetone. Then holding the filter paper with the help of a forceps at a distance, the ninhydrin solution was uniformly sprayed using an automizer with affine nozzle. To obtain the coloured bands of the amino acids on the filter paper it was first dried in the open air and then warmed in the oven at paper for 10-15 minutes. The colored bands so obtained were studied for the estimation of various FAAs in the experimental sample.

Preservation of the amino and chromatograms:

As the colored spots fade away with in a hours it is necessary to keep some of the chromatograms without spraying them with ninhydrin for getting the photographs as and when required. For this the chromatograms can be storted at room temperature in clean cardboard or wooden boxes for a few days.

Photography:

The chromatograms were sprayed with ninhydrin solution. Dried in the open and then placed in the oven at 100C for 10-15 minutes. They were taken out and boundaries of different spots marked and photographed.

Identification of the Coloured arcs/bands /spots:

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

$$R_f = \frac{Distance\ \ travelled\ \ by\ spot}{distance\ \ travelled\ \ by\ solvent} \times 100$$

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

Preparation of Chromatograms of standard amino acids:

Each standard amino acid was weight 1.5 mg separately. The solution was prepared by dissolving it in one ml of 10% isopropanol .The solution of each amino acid was spotted 10 time circular filter paper whatmann No.-1. After the solvent run and .it was then sprayed with 0.3% ninhydrin solution (300mg/100ml acetone) and dried first in open and then in oven at 100C for 10-15 minutes. The colour of the spot was noted and its Rf value calculated. In the same way, the Rf value, pattern and intensity of colours' of the 20 known standard amino acids were noted. To identify the amino acid from the various bands of the sample under analysis, the Rf value and colour of all the bands were noted. Thus by comparing the Rf value and colour of each spot with that of standard amino acid, the amino acids. The amino acids in each band identified and recorded.

4. Observations

To identity amino acids in various or bands of the sample, the Rf value of all the spots was calculated in search chromatogram. For the purpose of identification both the Rf value and the colour of spot were taken into consideration .Thus by comparing Rf value and colour of each spot with that of standard amino- acids, the amino acids in each spot were identified and recorded.

Standard map of 20 known free amino acids:

There has been observed some variation in the Rf value of the known free amino acids 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 great of paper used etc. (Saifer and Oreskes 1953 and Swarup *et al.* 1981) Therefore to minimize experimental variations ,the standard solution of the amino acids were run individually under the same laboratory conditions as the material during the present studies. Name, colour,Rf value and base triplet code of 20 standard amino acids are given in Table1 (Figs. 1-4).

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

Sr. No.	*Name of amino acids*	*Colour*	*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-Cystine	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)

Free amino acids in the adult male morph I (Pygidium-Black) of *C. analis*.

Circular chromatogram pattern in the adult female morph I of *C. analis* has revealed 9 spots with varying colours of Brown, Purple Brown, and Purple Violet pink and yellow and Rf value ranging from 17 to 84. An unidentified band with Rf value 69 and yellow in colour is also observed .The 12 amino acids which could be identified in these spots are given Table 2 (Fig.1) with their different colour intensities.

Table 2: Colour ,colour intensity, Rf value and name of free amino acids present in male morph I (Pygidium- Black) of *C. analis*

Spot No.	*Colour*	*Colour Intensity*	*Rf value*	*Name of free amino acids present*
1	Violet Pink	++	17	L-Ornithine, L-Cystine
2	Purple	+++	22	L-Lysine
3	Purple Brown	++	28	DL-Serine
4	Brown	+	44	L-Tyrosine
5	Purple	+	53	DL-Methionine, L-Proline
6	Purple	+++	62	DL- Valine
7	yellow	++	69	Unidentified
8	Purple	+	74	L-Leucine
9	Purple	+	84	DL-Isoleucine

Free amino acids in the adult male morph II (Pygidium-Brown with lateral black) of *C. analis.*

Circular chromatogram pattern in the adult female morph II of *C. analis* has revealed 11 spots with varying colours of Brown, Purple Brown, and Purple Violet pink and yellow and Rf value ranging from 14 to 87.The 16 amino acids which cloud be identified in these spots are given Table 3 (Fig. 2) with their different colour intensities. An unidentified spot with Rf value 59 and yellow in colour is also observed.

Table 3: Colour ,colour intensity, Rf value and name of free amino acids present in male morph II (Pygidium- Brown with lateral black) of *C. analis*

Spot No.	*Colour*	*Colour Intensity*	*Rf value*	*Name of free amino acids present*
1	Purple	+++	14	L-Histidine,L- Arginine
2	Violet	++	20	L-Cystine
3	Purple Brown	++	27	DL-Serine,L-Lysine
4	Purple	+	33	DL-Aspartic acid, Glycine
5	Purple	++	38	DL-Threonine, L-Glutamic acid
6	Light Brown	++	46	DL-Tryptophan, DL-Alanine
7	Purple	++++	55	DL-Methionine,DL-Butyric acid
8	Yellow	++	59	Unidentified
9	Purple	+	66	DL-Valine

10	Purple	+	77	L-Leucine
11	Purple	+	87	DL-Isoleucine

Free amino acids in the adult male morph III (Pygidium-Brown with posterior and lateral black) of *C. analis*

Circular chromatogram pattern in the adult female morph III of C. analis has revealed 8 spots with varying colours of Brown, Purple Brown, and Purple Violet pink and yellow and Rf value ranging from 13 to 78.The 14 amino acids which could be identified in these spots are given Table 4 (Fig.3) with their different colour intensities (Dhindsa, K.S. and Saini, D. 2014).

Table 4: Colour ,colour intensity, Rf value and name of free amino acids present in male morph III (Pygidium- Brown with posterior and lateral black) of *C. analis*

Spot No.	*Colour*	*Colour Intensity*	*Rf value*	*Name of free amino acids present*
1	Purple	+++	13	L-Histidine,L- Arginine
2	Violet	++	18	L-Ornithine,L-Cystine
3	Purple Brown	++	27	DL-Serine,L-Lysine
4	Purple	++	35	DL-Aspartic acid, DL-Threonine
5	Purple Brown	++	47	DL- Tryptophan,DL-Alanine
6	Purple Yellow	+	52	L-Proline, DL Methionine
7	Purple	+	66	DL-Valine
8	Purple	+	78	L-Leucine

Free amino acids in the adult male morph IV (Pygidium-Brown with posterior and lateral black) of C. analis.

Circular chromatogram pattern in the adult female morph III of *C. analis* has revealed 8 spots with varying colours of Light Brown, Purple, and Purple Violet pink and Rf value ranging from 17 to 84.The XI amino acids which could be identified in these spots are given Table 5 (Fig. 4) with their different colour intensities.

Table 5: Colour ,colour intensity, Rf value and name of free amino acids present in male morph IV (Pygidium- Browishn with black background with white seta of C. analis

Spot No.	*Colour*	*Colour Intensity*	*Rf value*	*Name of free amino acids present*
1	Violet Pink	+++	17	L-Cystine,L- Ornithine
2	Purple	++	24	L-Lysine, DL-Serine
3	Purple	+	35	DL-Threonine, DL- Aspartic Acid
4	Purple	+	39	L-Glutamic acid
5	Light Brown	+	46	DL-Trytophan,DL-Alanine
6	Purple	++++	54	DL-Methionine
7	Purple	+	68	DL-Valine
8	Purple	+	84	DL-Isoleucine

Free amino acids in the adult male morph V (Pygidium-Brown) of C. analis.

Circular chromatogram pattern in the adult female morph V of *C. analis* has revealed 8 spots with varying colours of Purple , Purple Violet, and Violet pink and Rf value ranging from 18 to 84.The 12 amino acids which could be identified in these spots are given Table 6 (Fig.5) with their different colour intensities(Dhindsa, K.S. and Saini, D. 2014).

Table 6: Colour ,colour intensity, Rf value and name of free amino acids present in female morph V (Pygidium- Brown) of *C. analis*.

Spot No.	*Colour*	*Colour Intensity*	*Rf value*	*Name of free amino acids present*
1	Violet Pink	+	18	L-Lystine, L-Ornithine
2	Purple	++	24	L-Lysine , L-Serine
3	Purple Violet	++	33	DL-Aspartic acid , Glycine
4	Purple	++	43	L-Tyrosine
5	Purple	++++	54	DL-Methionine , L-Proline
6	Purple	++	60	DL-Butyric acid
7	Purple	+	73	DL-Phenylalanine
8	Purple	+	84	DL-Isoleusine

5. Discussion

Paper chromatography has been emphasized by various workers as an important tool in the establishment of development, taxonomical, genetical and many other kinds of relationships among various organisms. The present work was designed to determine the free amino acid composition in the various developments stages i.e egg, larvae, pupae and in the various polymorphs of adult males and 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.

Based on the present research work, an attempt has been also made to compare the present data with that of known data either on this very group or on the other related animal groups. Difference in the free amino acids in various morphs (I-IV) of adult males of *C. analis*

In the present investigation, all the five morph I-V of adult males have revealed 9, 11, 8, 8 and 8 spots respectively. The five morph I-V carried 12, 16, 14, 12, and 12 amino acids respectively in them (Table VIII to XII and XV). L-Cystine, DL- Serine and DL- Methionone amino acids have been observed in allthe morphs. Although these amino acids are present inall the mprohs but their intensities differ with respect to each other, For Example L-Cystine is maximum in mproh no. IV to and L-Lysine in morph no. Iand DL-Methionine in morph nos II,IV and V. L-Histidine and L-Arginine could be seen in morph nos II and III only. L-Tyrosine and DL-Phenlalanione werealso seen in morph nos. I and V. Similarly , Glycine and DL—Butyric acid were present only in morphs nos .II and IV . l-Ornthine is present in all morphs except morph no. II ans DL-Isoleucine except no. II DL-Trytophan and DL- Valine is present in morph nos. I to IV.

These finding suggest that qualitative as well quantitative different of free amino acids exist among various morphs of adult males of *C. analis*. The difference in the amino acids result in varied biochemical activities in them resulting in the occurrence of different morphs both males and females of *C. analis* in nature (Dhindsa, K.S. and Saini, D. 2014).

6. Conclusion

The finding thus reveal that not only the different developmental stages, different sexes and different species vary in their FAA content but different body parts of the organisms also differ in the type, number and concentration

of FAA in them. This difference s may be attributed to the fact that the different metabolic functions are being carried out in different tissues and organs of the body of an individual. Hence to fulfill this requirement of the synthesis of harmones, enzymes and other structural and functional proteins, the composition of FAA also varies.

Table 7: Amino acids present in various morphs (I-V) of adult males of *C. analis* among with their intensities.

Sr. No.	*Name of amino acid*	*Male morphs (I-V)*				
		I	*II*	*III*	*IV*	*V*
1	L-Histidine	A	P(+++)	P(+++)	A	A
2	L-Arginine	A	P(+++)	P(+++)	A	A
3	L-Ornithine	P(++)	A	P(++)	P(+++)	P(+)
4	L-Cystine	P(++)	P(++)	P(++)	P(+++)	P(+)
5	L-Lysine	P(+++)	P(++)	P(++)	P(++)	P(++)
6	DL-Serine	P(++)	P(++)	P(+++)	P(++)	P(++)
7	Glycine	A	P(+)	A	A	P(++)
8	DL-Aspartic	A	P(++)	P(++)	P(+)	P(++)
9	DL-Threonine	A	P(++)	P(++)	P(+)	A
10	L-Glutamic acid	A	P(++)	A	P(+)	A
11	L-Tyrosine	P(+)	A	A	A	P(++)
12	DL-Trytophan	P(+)	P(++)	P(++)	P(+)	A
13	DL-Alanine	A	P(++)	P(++)	P(+)	A
14	L-Proline	P(+)	A	P(+)	A	P(++++)
15	DL-Methionine	P(+)	P(++++)	P(+)	P(++++)	P(++++)
16	DL-Butyric acid	A	P(++++)	A	A	P(++)
17	DL-Valine	P(+++)	P(+)	P(+)	P(+)	A
18	DL-Phenylalanine	P(+)	A	A	A	P(+)
19	l-Leucine	P(+)	P(+)	P(+)	A	A
20	DL-Isoleucine	P(+)	P(+)	A	P(+)	P(+)

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Figure 1: Rf value and name of free amino acids present in male morph I of *C. analis*

Figure 2: Rf value and name of free amino acids present in male morph II of *C. analis*

Figure 3: Rf value and name of free amino acids present in male morph III of *C. analis*

Figure 4: Rf value and name of free amino acids present in male morph IV of *C. analis*

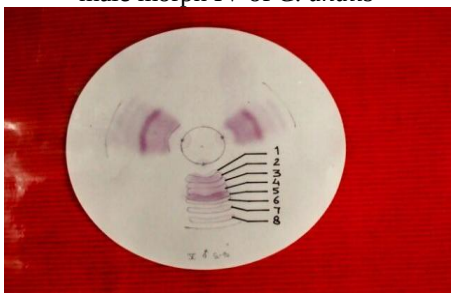

Figure 5: Rf value and name of free amino acids present in male morph V of *C. analis*