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Comparative Study of Amino Acids in the Life Stages of Silkworm *(Bombyx mori L.)*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The silkworm *(Bombyx mori L.)* undergoes distinct life stages, including egg, larva (caterpillar), pupa (chrysalis), and adult moth(imago), each with specific physiological requirements. Amino acids are essential for the growth and development of the silkworm. In this study, we work in paper chromatography to compare the amino acid profiles in different life stages of the silkworm. Samples were collected from eggs, larvae, pupae, and adult moths. Amino acids were extracted and separated on chromatographic paper using a suitable solvent system. The developed chromatograms were analyzed to determine the relative concentrations of specific amino acids. Our results revealed variations in the amino acid composition across the life stages. The amino acids

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such as glycine, methionine, leucine, and aspartic acid were common in all life stages of silkworms. These differences may be attributed to the physiological changes associated with metamorphosis and reproduction in adult silkworms. Overall, this study provides valuable insights into the dynamic changes in amino acid profiles during the life stages of silkworms, highlighting the importance of amino acids in their growth, development, and silk production. And, this study demonstrates the utility of paper chromatography as a cost-effective and reliable method for analyzing amino acid composition in complex biological samples, providing valuable insights into the nutritional dynamics of the silkworm lifecycle.

Keywords: Silkworm; aminoacids; chromatography; life cycle.

1. INTRODUCTION

“Asia is the world’s top silk-producing region, contributing for 98% of worldwide output. Silkworm rearing is a profitable and successful commercial activity that needs little input and can improve the economic situation of rural people. During the last three years, the rise in silk production has not fulfilled the global demand for silk. This decrease in silk production is due to low-quality silk seed, unavailability of mulberry leaves, low prices, and poor quality of cocoons with fewer marketing facilities.

Silkworm *(Bombyx mori L.)* is a monophagous lepidopteron insect that is reared for silk production under a special controlled environment. Silkworm derives all essential nutrients from mulberry leaf for growth and development. The growth and development of silkworm larvae is greatly influenced by the mulberry leaves quality. The production of cocoons is also affected by different factors including environmental conditions, planting and pruning, and soil characteristics that affect mulberry leaf quality. The silkworm's dietary and nutritional requirements, including water, vitamins, carbohydrates, proteins, fats, and ascorbic acid, are met by digestion and assimilation of mulberry leaves. Moreover, mulberry leaf proteins are directly responsible for over 70% of the silk production by silkworms, subsequently affecting the overall cocoon production” [1].

“Breeding of the silkworm *(Bombyx mori L.)* has been carried out to grow healthy larvae and produce more silk for practical use. Many studies on amino acid metabolism in this insect have been concerned with the synthesis of silk protein. There is another series of studies on amino acid metabolism about pigment formation. Data are also available on the ingestion and utilization of amino acids for larval growth and silk production and on the accumulation of free amino acids in the hemolymph of silk gland atomized larvae. The amino acid nutrition of the silkworm is closely related to the synthesis of silk protein as well as to the growth of silk glands. These studies have in part dealt with the nutritional aspects of amino acids, but information on absolute amino acid requirements has been inconclusive. A method using paper chromatography for the analysis of the amino acid requirements of the silkworm has been adopted rather recently. Such a diet ensures larval growth and cocoon-spinning, although it is inferior to a diet containing mulberry leaves. Nutritional requirements for amino acids are deeply connected with their metabolic function. Recently, increased attention has focussed on gaining an understanding of amino acid nutrition of the silkworm on the biochemical or metabolic level with due regard to the nutritional role and metabolic function of amino acids in the silkworm attention has focussed on gaining an understanding of amino acid nutrition of the silkworm on the biochemical or metabolic level with due regard to the nutritional role and metabolic function of amino acids in the silkworm” [1].

1.1 Objective of Study

The main objective of this project is to study comparatively the amino acids present in every life stage of a silkworm. Amino acids are essential as they are the biological buffers and help in the formation of other compounds. Essential amino acids are biosynthesized only when precursors are provided in the diet under natural conditions, these precursors are not formed in the silkworm and are formed only to a small extent

1.2 Systematics of Mulberry Silkworm

Kingdom: Animalia
Phylum : Arthropoda

Subphylum : Mandibulata
Class: Insecta
Sub class: Pterygota
Division: Endopterygota
Order: Lepidoptera
Sub-order: Macrolepidoptera\Heterocera
Family : Bombicidae
Genus : *Bombyx*
Species: *mori*

Bombyx mori L. popularly called the Mulberry silkworm moth. The silk is a proteinaceous fiber produced by the larva of the silkworm. It is regarded as the 'Queen of Textiles' due to its lustrous nature. The rearing of silk moths and the production of raw silk is known as sericulture. The mulberry silkworm is a completely domesticated insect. The silk moth is dioecious. Fertilization is internal, preceded by copulation. The development includes a complicated complete metamorphosis, consisting of four stages (egg, larva, pupa, and adult).

Egg: After fertilization, each female moth lays about 300 to 400 eggs. These eggs are placed in clusters on the leaves of mulberry tree. The female covers the eggs by a gelatinous secretion which glues them to the surface of the leaves. The eggs are small, oval, and usually slightly yellowish. The egg contains a good amount of yolk and is covered by a smooth hard chitinous shell. After laying the eggs the female moth does not take any food and dies within 4- 5 days. In the univoltine (a single brood per year) they may take months because overwintering takes place in this stage but the multivoltine broods come out after 10-12 days. From the egg hatches out a larva called the caterpillar.

Larva: The larva of the silkworm moth is called the caterpillar larva. The newly hatched larva is about 4-6 mm in length. It has a rough, wrinkled, hairless and yellowish white or greyish worm-like body. The full-grown larva is about 6.00 to 8.00 cm in length. The body of larva is distinguishable by a prominent head, a distinctly segmented thorax and an elongated abdomen. The head bears a mandibular mouth and three pairs of ocelli.

The larva is a voracious eater and strongly gregarious. In the beginning, chopped young mulberry leaves are given as food but with the advancement of age entire and matured leaves are provided as food. The larva moves in a characteristic looping manner. The larval life lasts for 2-3 weeks. During this period the larva moulds four times. The stage of the larva between the two successive moulting is called instar. Thus, normally five instars occur.

Pupa: The thread becomes wrapped around the body of the caterpillar larva forming a complete covering or pupal case called the cocoon. The cocoon formation takes about 3-4 days. The cocoon is a white or yellow, thick oval capsule which is slightly narrow in the middle. It is formed of a single long continuous thread. The pupa lies dormant but undergoes very important active changes which are referred to as metamorphosis. The larval organs such as abdominal pro-legs, anal horn and mouth parts are lost. The adult organs such as antennae, wings and copulatory apparatus develop.

Adult: The adult moth emerges out through an opening at the end of the cocoon in about 2 to 3 weeks of time. Immediately before emergence, the pupa secretes an alkaline fluid, that softens one end of the cocoon and after breaking its silk strands, a feeble crumpled adult squeezes its way out. Soon after emergence, the adult silk moths' mate, lay eggs and die.

Study area: Department of sericulture, Melagaram, Nannagaram, Tenkasi , 627 811.

Chromatography: Chromatography was first proposed in Russia by M.S.Tswett in 1903. The feature common to them all is that two mutually immiscible phases are brought into different movements of the sample components in these two phases is responsible for their ultimate separation from each other. The identification of a given compound is made on the contact with each other, one of these phases is stationary, while the other is mobile. The efficient of the given solute is known as the retardation factor and is constant for a given basis of thedistance moved by the solvent front.

2. MATERIALS

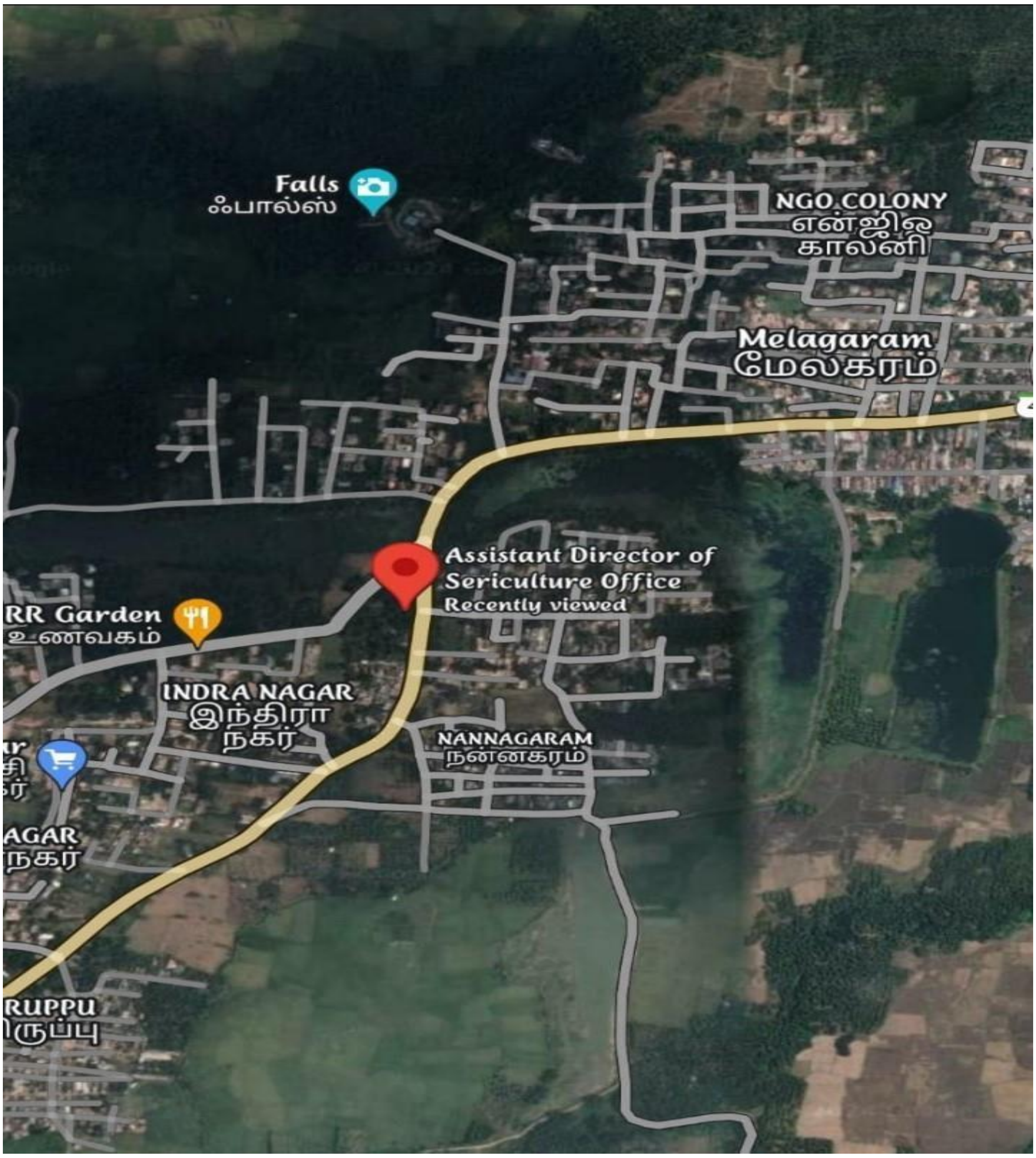
Whatman No:1 paper [2 cm x 10 cm]

Chromatographic chamber

Solvent-n-butanol, acetic acid and water [4:1:1]

0.1% Ninhydrin in acetone

Amino acids standards (Glutamic acid, methionine, lysine, alanine, tryptophan, glycine, threonine, histidine, tyrosine, isoleucine, leucine, phenylalanine, valine, aspartic acid, proline, arginine, asparagine,, cysteine ,serine, histidine, glutamine).



Map 1. Map showing study location

Sample preparation: They were pounded in a mortar and pestle into a pulp and then extracted with the solvent. The best solvent for extracting free amino acids from biological samples is 80% ethanol in water. The pooled extracts were centrifuged at 3000 rpm for 10 minutes, and the clear supernatant was concentrated. This concentrate can be used as a sample to determine the free Amino acids.

Procedure:

1. Whatman No:1 filter paper was taken and a pencil line was drawn parallel to the short edge at a distance of 2.5 cm.

2. Three points were marked along the line leaving equal distance between the points and a margin was left on either side.

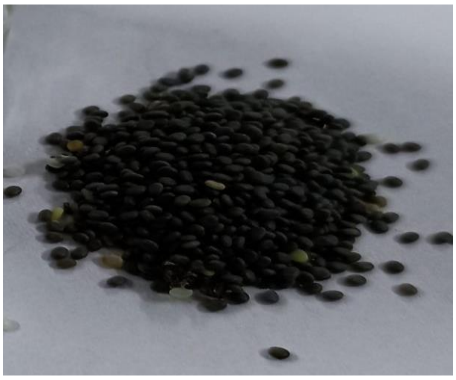
3. At each point a drop of a particular amino acids mixture was spotted with a capillary tube and the central point of the biological sample was spotted.

4. The n-butanol-acetic acid water mixture [4:1:1] was prepared and shaken well. A small amount of it was taken in a beaker and left in the chromatographic c saturation.

5. After half an hour the spotted filter paper with the edge parallel and nearer to the spotted amino acids was placed in the chamber supported by a glass rod and hung in such a way that it did not touch the sides of the chamber.

6.80 ml of the running solvent was then poured into the chamber. Close the chamber airtight and allow to run for 4 hours.

7. The filter paper was removed, and the solvent front was marked off with a pencil and allowed to dry at room temperature by hanging from clips withthe starting end downwards.



Picture 1. Egg

Picture 2. Larva

8. When it was completely dry it was developed with 0.IN Ninhydrin in acetone. The development was done by spraying the ninhydrin solution with a pipette.

9. The paper was dried at room temperature and then at 100'e for one or two minutes, the spots were marked off as circles, and the Centre of each circle was noted. The distance between the center of the spot and the starting line gives the distance traveled By the amino acids.

10. The distance between the starting line and the solvent front gives the distance traveled by the solvent. Therefore

Rf = Distance traveled by the solute Distance traveled by the solvent

By comparing the Rf values of the amino acids in the given fluid with those of the standards the amino acids are identified.

Picture 3. Pupa

Picture 4. Adult

3. RESULTS

Culturing of mulberry silkworm *Bombyx mori L.:* The adult silkworms were collected from Nannagaram, courtallam, and Tamil Nādu. The lifecycle of the mulberry silkworm is observed. The adults were allowed to mate and lay eggs. A female lays more than 350 eggs. The eggs were laid in clusters and were covered with gelatinous secretion of the female moth. After 10 days egg is hatched into a larva which consumes a huge amount of mulberry leaves. The larval growth is marked by four molting and five instar stages. The full-grown caterpillar develops a pair of silk glands. These glands secrete silk which consists of an inner tough protein, fibroin, enclosed by a water-soluble gelatinous protein, sericin. The next stage is pupa which is the inactive resting stage of silkworm. The pupal period lasts for 8-14 days after which the adult moth emerges slitting through the pupal skin and piercing the fibrous cocoon shell with the aid of the alkaline salivary secretion that softens the tough cocoon shell. The adult of *bombyx mori L.* is about 2.5 cm in length and pale creamy white. In males eight abdominal segments were visible; while in females seven. The female has comparatively smaller antennae. Just after emergence, male moths copulate with females for about 2-3 hours and die after that. The female starts laying eggs just after copulation (Pictures 1-5).

Comparative study of amino acids: The presence of amino acids in different life stages of silkworm *Bombyx mori L.* was analyzed using paper chromatography and compared the number of free amino acids [2]. Paper chromatography is an analytical method used to separate colored chemicals or substances.

Picture 5. Life Stages

Table 1. The amino acids present in the silkworm egg

S.NO	Amino Acids	Egg Rf value
1	Glycine	0.208
2	Isoleucine	-
3	Methionine	0.521
4	Proline	-
5	Alanine	0.228
6	Leucine	0.9
7	Glutamic acid	0.202
8	Threonine	0.260
9	Aspartic acid	-
10	Lysine	0.086
11	Arginine	0.146
12	Tyrosine	0.066

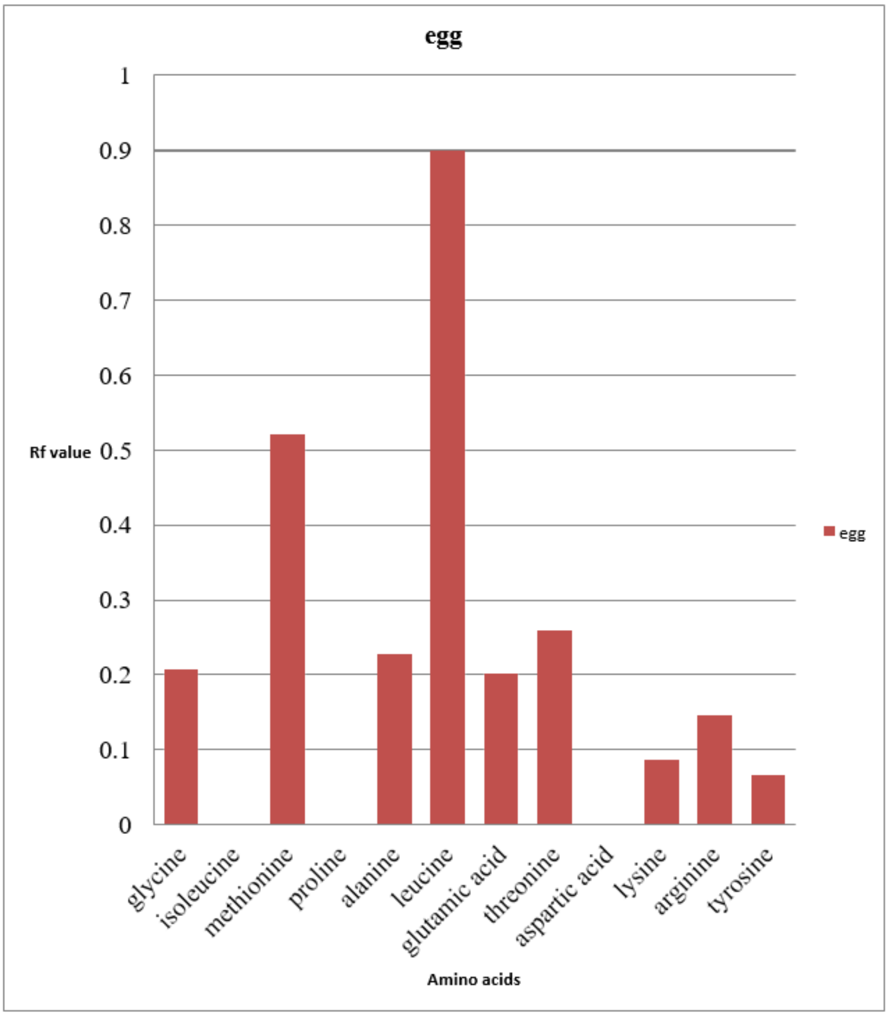


Fig. 1. Study of amino acids in Egg

Table 2. The amino acids present in the silkworm larva

S.NO	Amino Acids	Larva Rf value
1	Glycine	0.353
2	Isoleucine	0.971
3	Methionine	0.803
4	Leucine	0.956
5	Threonine	0.382
6	Aspartic acid	0.353
7	Lysine	0.075
8	Arginine	0.138
9	Valine	0.878
10	Phenylalanine	0.144
11	Tryptophan	0.837
12	Histidine	0.125

Among these free amino acids, glycine, alanine, and methionine were found in all life stages of silkworms. (Tables 1-4 and Figs. 1-4).

The calculated Rf values of samples were compared with those of the Rf values of the standard amino acids of silkworms. (Table 5 and Pictures 6-9).

Table 3. The Amino acids present in silkworm pupa

s.no	Amino acids	Pupa Rf value
1	Glycine	0.282
2	Methionine	0.716
3	Alanine	0.259
4	Leucine	0.819
5	Aspartic acid	0.197
6	Tyrosine	0.486
7	Valine	0.716
8	Phenylalanine	0.868
9	Butyric acid	0.578
10	Cysteine hydrochloride	0.833
11	Hydroxyproline	-
12	Tryptophan	0.615

Table 4. Amino acids present in the adult silkworm

S.NO	Amino Acids	Adult Rf value
1	Glycine	0.318
2	Isoleucine	0.970
3	Methionine	0.628
4	Alanine	0.692
5	Leucine	0.984
6	Aspartic acid	0.352
7	Arginine	0.267
8	Valine	0.791
9	Phenylalanine	0.940
10	Cysteine hydrochloride	0.921
11	Tryptophan	0.628
12	Serine	0.104

Table 5. Comparison of amino acids in *bombyxmori L.*

S.No	Amino acids	Egg	larva	pupa	Adult
1	Glycine	**+**	**+**	**+**	**+**
2	Isoleucine	-	+	-	+
3	Methionine	+	+	+	+
4	Proline	-	-	-	-
5	Alanine	+	-	+	+
6	Leucine	+	+	+	+
7	Glutamic acid	+	-	-	-
8	Threonine	+	+	-	-
9	Aspartic acid	-	+	+	+
10	Lysine	+	+	-	-
11	Arginine	+	+	-	+
12	Tyrosine	+	-	+	-
13	Valine	-	+	+	+
14	Phenylalanine	-	+	+	+
15	Butyric acid	-	-	+	-
16	Cysteine hydrochloride	-	-	+	+
17	Hydroxyproline	-	-	-	-
18	Tryptophan	-	+	+	+
19	Serine	-	-	-	+
20	Histidine	-	+	-	-

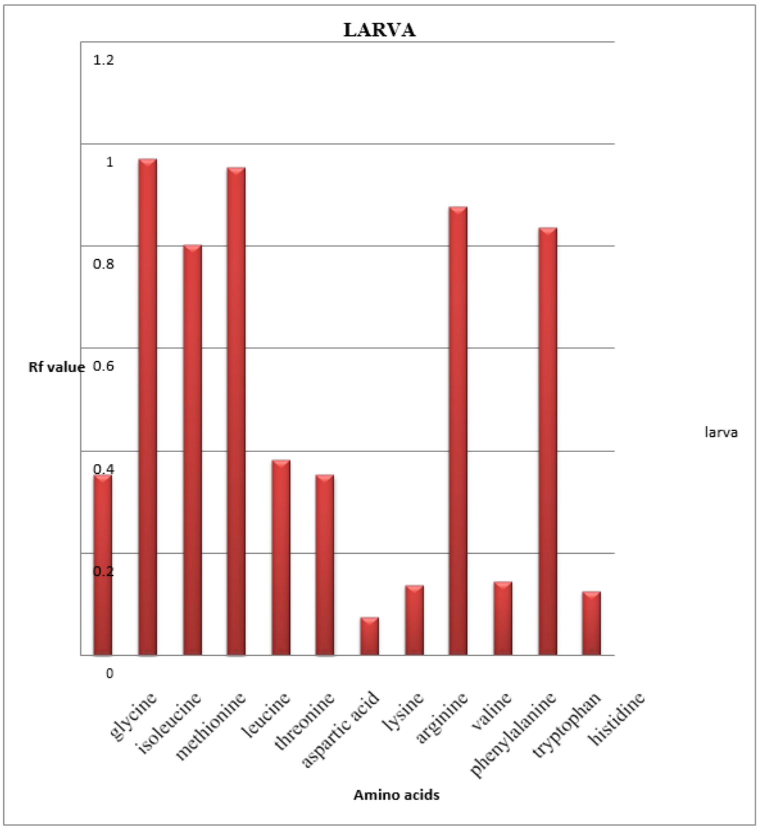


Fig. 2. Study of amino acids in silkworm larva

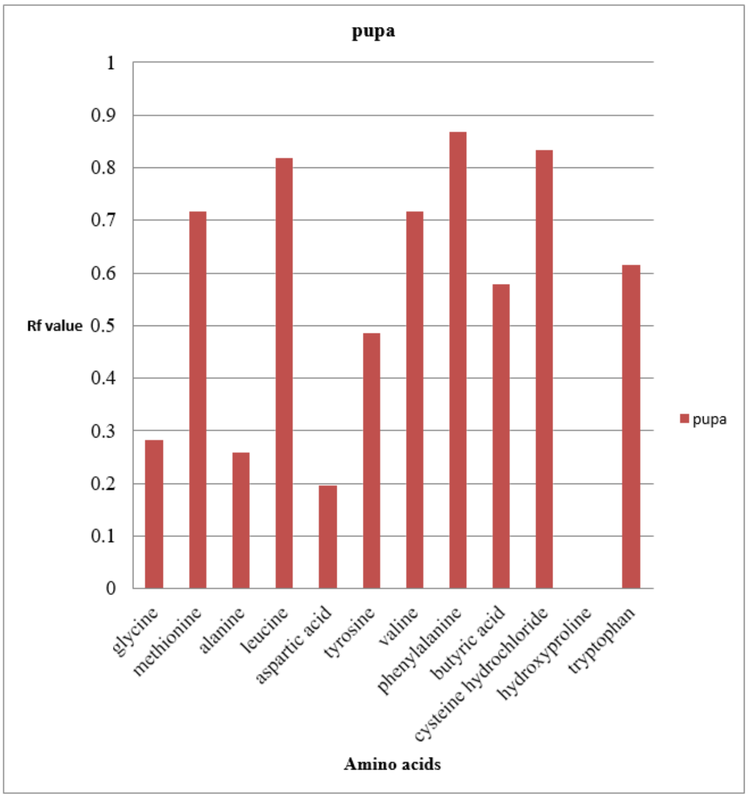


Fig. 3. Study of amino acids in pupa

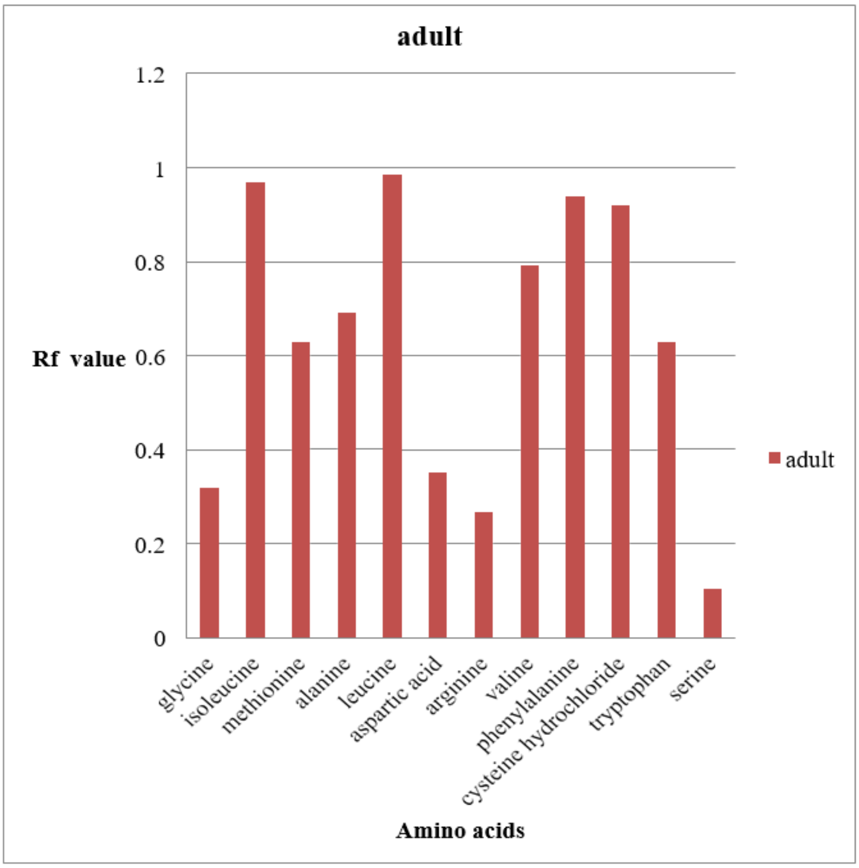
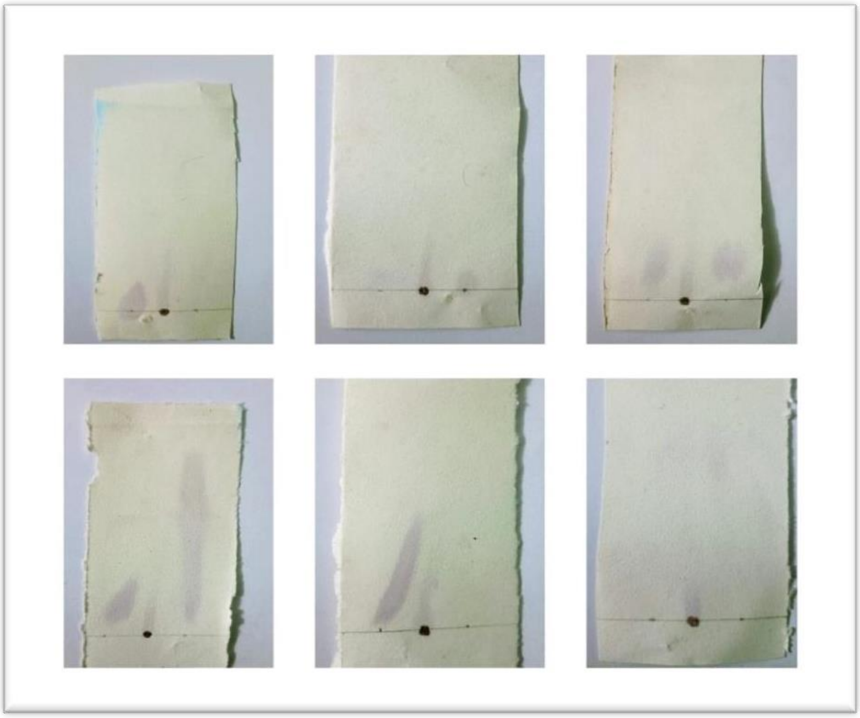
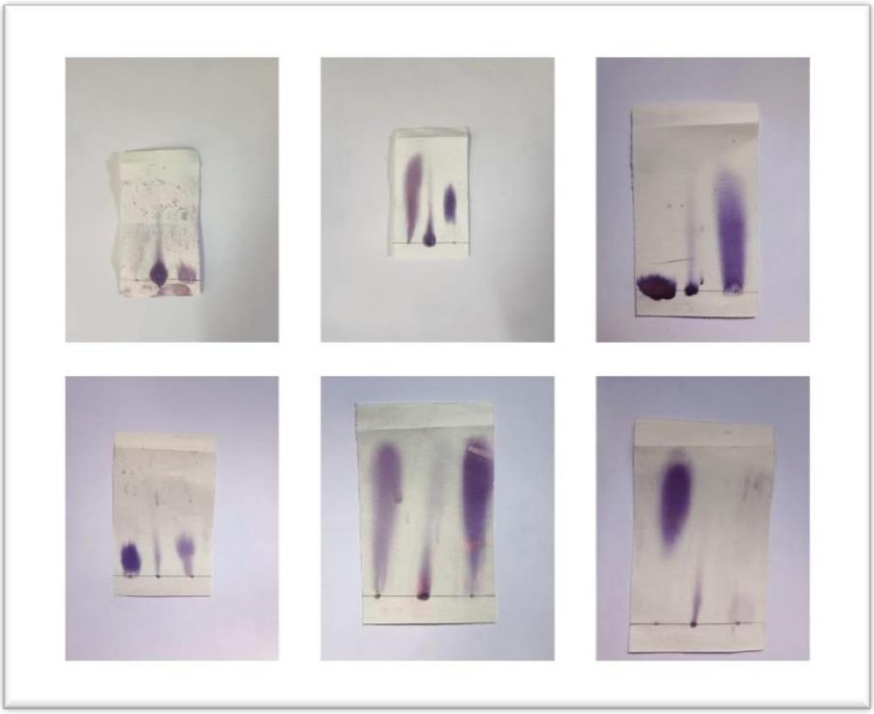


Fig. 4. Study of Amino acids in adult silkworm

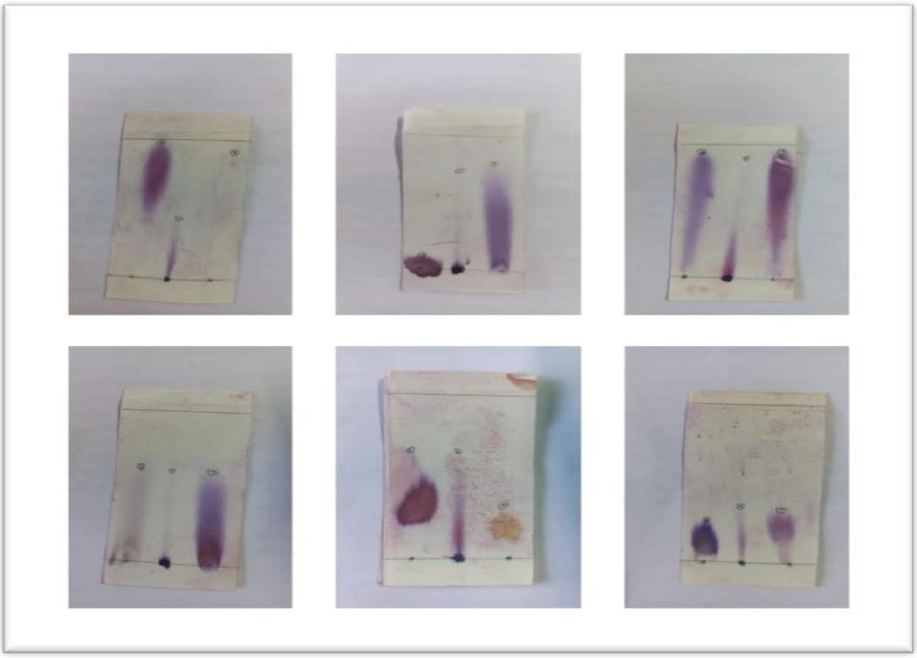


Picture 6. Egg

Picture 7. Larva



Picture 8. Pupa



Picture 9. Adult

4. DISCUSSION

This study comparatively studies the life stages of silkworms and the various Amino acids found in them (egg, larva, pupa, adult). The silkworms were reared, and the period of each life stage and the complete life cycle were observed.

Another study was on the various amino acids found in the different stages of the silkworm. The method was carried out by the method of paper chromatography [3]. For this, we collected the silkworm's egg, larva, pupa, and adult stages and randomly took 12 amino acids. Using paper chromatography found out the highest amino acid found in each life stage of the silkworm [4].

Through this, we have known the amino acid composition in the silkworm’s life stages. Here glycine, methionine, and leucine are dominant and abundant in the 4 stages of silkworm (egg, larva, pupa, adult). Aspartic acid, valine, phenylalanine, and tryptophan are abundant in 3 life stages of silkworms. (larva, pupa, adult) [5].

The present study has been undertaken to enhance the commercial and biological traits of silkworms by feed supplementation with amino acids (Alanine, Glycine, and Serine) fortified mulberry leaves. The increase in silk production in India will not only help to improve silk productivity to meet local silk demand but also increase silk exports which will directly impact India's economy [6].

Matsumoto (2008) demonstrated that silkworms reared at different temperatures (25 and 30) exhibited variations in the glycine and alanine content of their silk fibroin. Similarly, glycine and alanine show variation in the content [7].

In the body of silkworm, the concentration of glycine, alanine, glutamic acid, proline, arginine, and tyrosine are high, but the percentage is less in mulberry leaf proteins. Mulberry leaf contains 75% moisture and 20% leaf protein, while fibroin and sericin contain 100% protein. Thus, silkworm larvae convert mulberry leaf protein into its constituent amino acids and utilize them for its growth and synthesis of fibroin and sericin proteins [8].

The protein of silkworms is known as fibroin, the fibroin is covered with another gummy proteinaceous substance called sericin mainly the formation of a cocoon shell. The ideal food for silkworms is mulberry [9], the natural food mulberry leaf satisfies the dose requirements for essential amino acids (aspartic and glutamic acid) and proline best larval growth is obtained with an increase in the amino acids diet in the growth [10]. To make silkworms grow satisfactorily enough feed must be provided and feed should have all the necessary nutrients for the growth of silkworms. The important nutrients such as amino acids, lipids, carbohydrates, and requirements of vitamins and minerals are necessary for the growth and development of silkworms [11].

5. CONCLUSION

A few limitations are also associated with the practical adoption of silkworm feed supplements described in the present study. It is challenging to convince silkworm farmers, so that they may add amino acid supplements to the silkworm's feed [12]. Furthermore, it is important to use amino acid supplements at specific concentrations, as higher concentrations can lead to toxicity. Here it is concluded that amino acid fortification of silkworm (*Bombyx mori* L.) feed has great potential in the sericulture industry and if applied properly it can lead to the enhancement of overall productivity of this cottage-based industry [13]. The use of these amino acids as a food supplement in the diet of silkworms could be the possible reason for enhanced growth and better silk cocoon parameters. Alanine plays a vital role in the metabolism of organic acid, glucose, and tryptophan] [14]. It also boosts immunity and nourishes the brain, muscles, and central nervous system as well as plays a key role in the development of insect exoskeleton. Glycine and serine are involved in the regulation of cross-links between the fat body and silk gland. Furthermore, these amino acids can also regulate nutrient absorption and inhibitory neurotransmitter signaling [15].

COMPETING INTERESTS

The authors have declared that no competing interests exist.

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