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Metabolic profiles of pathogen-challenged mulberry silkworm, *Bombyx mori* L. as a tool for disease diagnosis

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ABSTRACT

Metabolic profiles were detected in healthy and flacherie (inoculated with *Bacillus* sp.) and grasserie (inoculated with polyhedral bodies)- diseased *Bombyx mori* larvae. Free amino acids were separated and quantitatively determined by using hydrolysis method. Further, nitrogen (N) percentage was estimated with micro-Kieldahl method. Whereas, phosphor-molybdate method was applied for phosphorus (P) analysis. Results revealed that distinct differences in the amounts of eighteen individual amino acids in healthy and diseased silkworm 5th larval instar were found. The highest amino acids concentration was recorded in healthy larvae (224.05 mg/g tissue), while the lowest concentration was found in Gr-diseased larvae (177.77 mg/g tissue). Fl-diseased larvae inoculated with *Bacillus* sp. showed elevation in certain amino acids comparing with Gr-diseased larvae inoculated with of Nuclear Polyhedrosis Virus (NPV) occluded bodies. N and P percentages in Fl and Gr diseases recorded (N = 2.57%, P = 0.29%), (N = 3.86%, P = 0.32%) respectively, compared with healthy larvae (N = 1.63%, P = 0.23%). The present study reveals that a decrease in amino acids in fifth-larval stage implies an increase in N and P percentages in both bacterial and viral infected larvae. It is concluded that the determination of biochemical responses in silkworm against infections may be considered as bio-analytical markers, which are useful for early disease diagnosis and developing disease resistant breeds.

Keywords: *Bombyx mori*, Flacherrie disease, Grasserie disease, amino acids, nitrogen, phosphorus.

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INTRODUCTION

Bombyx mori L. has an economic importance because of the commercial value of its silk (Meeramaideen et al., 2017). Silkworm diseases are considered the direct cause for the sericulture damage (Ponnuvel et al., 2003). Bacterial and viral infections cause severe diseases in *B. mori* larvae and create a serious loss to silk industry (Rao et al., 2011). Sericulture development depends on understanding the metabolic, molecular, genetic and immunological modulation especially upon infection (Babu et al., 2009). The study of metabolic profile of an organism at a given time is known as metabolomics (Rouse, 2005). Recently, metabolomic has been utilized in medical sector to detect biomarkers and diagnose diseases (Yoshida et al., 2012). Taha and Kamel (2012) used gas chromatography/mass spectroscopy to analyze the metabolic products resulted from bacterial infection to discover novel biomarkers for early diagnosis of silkworm bacterial disease.

The most wide-spread diseases to *B. mori* in Egypt are Flacherrie (Fl) disease, which was caused by Infectious Flacherrie Virus (IFV) followed by bacterial infection, and Grasserie (Gr) disease, which was caused by Nuclear Polyhedrosis Virus (NPV) (Taha, 2002). During early stages of infection, it is difficult to detect disease because the clear symptoms can be recognized only at late stages (Shamim et al., 1996). Once the symptoms are observed in the rearing culture, the only practical method is to discard large number of larvae to prevent occurrence and

spread of diseases (Acharya et al., 2002).

The progress of bacterial and viral pathogens can be tracked by observing the alterations in different biochemical parameters inside the insect (Yao et al., 2006). However, before stepping into disease control, it is important to understand host-pathogen interactions and the development of antimicrobial proteins in insects (Deb, 2015). Pathogen can affect the basic metabolic system-related genes pathways, leading to wide-system changes in gene expressions (Huang et al., 2009). Thus, several biochemical alternations have been reported as appropriate biomarkers, such as changes in total proteins (Etebari et al., 2007) including protein, lipoprotein and glycoprotein patterns (Taha, 2007), enzymes (Mahesha et al., 2009) and glucose level (Mahesha and Thejaswini, 2013).

Amino acids, the building units of proteins, are essential organic constituents of all living organisms (Rajitha and Savithri, 2014). The amino acids and their by-products are responsible for different functions inside the cell such as management of cell growth and biosynthesis of different components like hormones, nucleotides, porphyrins and some vitamins (Rodwell, 1993). Free amino acids were found to regulate numerous functions inside insects, such as protein synthesis (Nation, 2002), haemolymph osmo-regulation (Evans, 2009) and cocoon formation (Kunz et al., 2016). The physiological state of the cell can be understood by means of amino acid quality as the best diagnostic tool (Adibi, 1980; Kimura et al., 2009).

On the other hand, nitrogen and phosphorus play important roles in the insect biochemical functions, their metabolism are the basic pathways that maintain the biological balance of an organism (Li-Xia et al., 2003). Amino acid synthesis requires a source of endogenous nitrogen, which is most likely supplied by transamination from existing amino acids (Nation, 2002). While, phosphorus is principal component of adenosine triphosphate (ATP) and important for acid-base balance in the nucleic acids (Soetan et al., 2010). Accordingly, the metabolomic profile of an organism could provide a helpful vision about the disturbance factors and the healthy state of an organism (Fiehn, 2002; Simpson et al., 2011).

The present investigation was undertaken to study alterations in amino acid profiles and nitrogen and phosphorus percentages in silkworms upon bacterial and viral infections. It may provide some insights into the mechanisms involved in the metabolic alternations. As well as, it may suggest biomarkers as an alternative tool instead of the costly molecular techniques for early disease diagnosis and further restoration of sericulture output.

MATERIALS AND METHODS

Disease-free eggs ofm univoltine *Bombyx mori* local susceptible hybrid were obtained from the Sericulture Research Department, Plant Protection Research Institute (PPRI), Agricultural Research Centre (ARC). The newly emerged larvae were reared according to the rearing technique of Krishnaswami (1978). Naturally diseased mulberry silkworm with flacherrie (bacterial disease) and Grasserie (viral disease) were collected from the rearing culture.

Isolation of bacteria

Bacterial isolation and identification was carried out in Agricultural Microbiology Department, ARC, Egypt. Bacterial pathogens were collected from black thorax Septicemia-diseased larvae, the diseased larvae were crushed by using mortar and pestle. The homogenate was then filtered with silica filter. The filtrate was centrifuged at 5000 g for 10 min. The supernatant was discarded, and the pellet was used for bacterial culture after re-suspending in distilled water (Aneja, 2003).

Preparation of Luria agar (LA) medium and bacterial culture

Bacterial sample was streaked in LA under aseptic conditions in a laminar air flow chamber with the help of streaking loop then incubated at 37°C overnight. After 24 h the bacterial growth was noticed, and further it was sub cultured. A sample of bacteria was taken with the help of a loop and centrifuged for 15 min at 5000 g. By discarding the supernatant, pellet deposited at the bottom of the tube was dissolved in distilled water. The presence of bacteria was confirmed by staining with basic dye crystal violet (Suparna et al., 2011).

Isolation of virus

The polyhedral isolation was carried out at the Central lab, Virology Department, Cairo University, Egypt. The *Bombyx mori* nuclear polyhedrosis virus (BmNPV) was collected from the haemolymph of naturally infected larvae by cutting the pro-legs. The purification was carried-out according to (Harrap et al., 1977) as follow; the haemolymph containing polyhedra was filtered through cheese cloth. Larval debris were removed by pelleting in a centrifuge at 10000 g for 1 min. viral particles and polyhedra in the supernatants were purified by centrifugation at 9000 g for 3 h at 4°C in 25 to 60% (w/w) liner sucrose gradient. The white pellets containing polyhedra were collected washed twice in distilled water and precipitated at 20000xg for 30 min. The pellet was finally re-suspended in 1 ml of distilled water.

Pathogen inoculations

Around 1500 healthy newly emerged 5^{th} larval stage were divided into three groups named; control, bacterial and viral each with 500 larvae/group. Each group with five replicates, each replicate containing 100 larvae. The second group, silkworms were inoculated with the bacteria by smearing the bacterial solution (7×10^7 cells/ml) on a known weight (~20 g) of mulberry leaf surface and dried in shade then fed to larvae. The same procedure was done for viral infection with polyhedral occlusion bodies (OBs) (2×10^5 OBs/ml) (the third group) as recommended by Khurad et al. (2004). The control group (first group) was fed with distilled water smeared mulberry leaves. For the diseased groups, infected leaves were provided during the first feed on first day and thereafter the larvae were fed with normal leaves (Mahmoud et al., 2012). Both control larvae and treated one that ate all the treated leaves, were shifted on fresh mulberry leaves for subsequent rearing. On the seventh day from inoculation, the bacterial and viral diseased larvae (~30

larvae from each group) with obvious symptoms were collected randomly and dried for further analytical tests.

Determination of free amino acids

Free amino acids of *B. mori* larvae were separated and determined quantitatively according to the Hydrolysis method (Pellet and Young, 1980) using Eppendorf LC 3000 amino acid analyzer at Central Lab of Desert Research Centre.

Nitrogen (N) and phosphorus (P) analysis

The samples were wet digested in H_2SO_4-H_2O_2 mixture and total N was determined by micro-Kjeldahl method (Kalra, 1997). P was determined colorimetrically with phosphor-molybdate method according to ASTM (2002) using UV/Visible Spectrophotometer, Unicam UV 300, Thermo- Spectronic, USA.

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA), IBM SPSS V.20. Means were compared using Tukey post-hoc test ($\alpha \leq 0.05$).

RESULTS AND DISCUSSION

Changes in amino acid composition fractions

Using hydrolysat chromatogram separation method for whole tissues of healthy, Fl- and Gr- diseased 5th instar larvae of *B. mori* revealed the presence of 17 amino acids which were separated according to their retention time at wavelength 570 nm, as illustrated in Figure 1, 2 and 3. In addition, proline amino acid was separated at 440 nm. Chromatogram pattern showed no obvious qualitative changes in amino acid profiles of healthy, Fl- and Gr-diseased larvae. Seventeen types of amino acids besides proline were present in all tested samples. However, Aboul-Ela et al. (1991) noticed 15 and 14 amino acid in healthy and bacterial treated larvae of *Plodia interpunctella*, respectively.

In the present study, the total amounts of free amino acids were decreased in both Fl- and Gr-diseased larvae (199.96 and 177.77 mg/gm tissue, respectively) compared to healthy larvae (224.05 mg/gm) as illustrated in Table 1.

These findings agree with Chadwick (1977) who recorded a reduction in free amino acids in *B. mori* larvae after Nuclear Polyhedrosis Virus infections. Cheung and Grula (1982) and Govindan et al. (1998) attributed that reduction to the cessation of larval feeding and difficulty to process the food material. As well as, the usage of specific proteins for virus multiplication which induce distortion in the protein metabolism in the host cells via the virus proteins (Gururaja et al., 1999). On the other hand, Kumar et al. (2011) concluded that the decrease in proteins in inoculated larvae may be because of strong degeneration of structural proteins. Nevertheless, they found no elevation in amino acid content which suggested that the degraded proteins might be used for the pathogen development.

The pathogenic conditions alter the metabolism of the infected larvae which may lead to impairments of midgut function and lowering in food consumption, therefore, the protein content was decreased as suggested by Savithri et al. (2006). It is assumed that, infected larvae counteract the insufficiency of energy because of pathogen infection stress with accelerated degradation of proteins to amino acids and entering them to TCA cycle as a keto-acid (Etebari et al., 2005). Lakshmi and Purushotham (2011) suggested that decrease in the free amino acid content in fat body may indicate the possibility of active feeding of amino acid in TCA cycle and glycolytic pathway to meet the emergent energy needs as well as their utilization in the production of some new proteins synthesized to cope with stress.

In the present study, the amino acid pattern in a whole-body tissue of healthy *B. mori* larvae revealed that neutral amino acids glycine, alanine and serine are the most abundant amino acids along with the basic amino acids histidine, lysine and arginine, and these results agree with that reported by Parenti et al. (1985). Besides, the acidic amino acids aspartic and glutamic acid were abundant in the healthy larval tissues.

Haemolymph neutral amino acids (threonine, proline, aspartate and glutamate) are utilized directly by the silk glands for silk proteins biosynthesis (Parenti et al., 1985). Whereas, the basic amino acids are responsible for the osmotic pressure regulation of the haemolymph (Florkin and Jeuniaux, 1974). In healthy silkworms, the principal proteins found in silk were the fibroin protein, consists of the recurrent amino acids sequence (Gly-Ser-Gly-Ala-Gly-Ala) with high amounts of glycine and alanine 42.8 and 32.4 g/100 g protein, respectively. Sericin is the second importantly amino acid found in silk. The key amino acids in sericin are serine (30.1 g), aspartic acid (16.8 g), glutamic acid (10.1 g) and threonine (8.5 g) as evaluated by Kirimura (1999). Whereas, asparagine was used by silk glands to synthesize alanine via transamination (Seshachalam et al., 1992). Rajitha et al. (2013) suggested that the increase in free amino acid levels may be due to the continuous supply of free amino acids from haemolymph to the silk gland for the synthesis of silk proteins.

In the present investigation, a decrease was found in the amount of aspartic, threonine, serine, glycine, alanine, cysteine, valine, tyrosine, arginine, and NH_4 acids in all diseased larvae. However, the most affected amounts of amino acids were detected in Gr-diseased larvae. These results can explain the inability of the viral- and bacterial-infected larvae to produce silk. On the other hand, glutamine, proline, methionine, isoleucine, leucine, phenylalanine, histidine, and lysine increased in Fl-diseased larvae but decreased in Gr-diseased larvae.

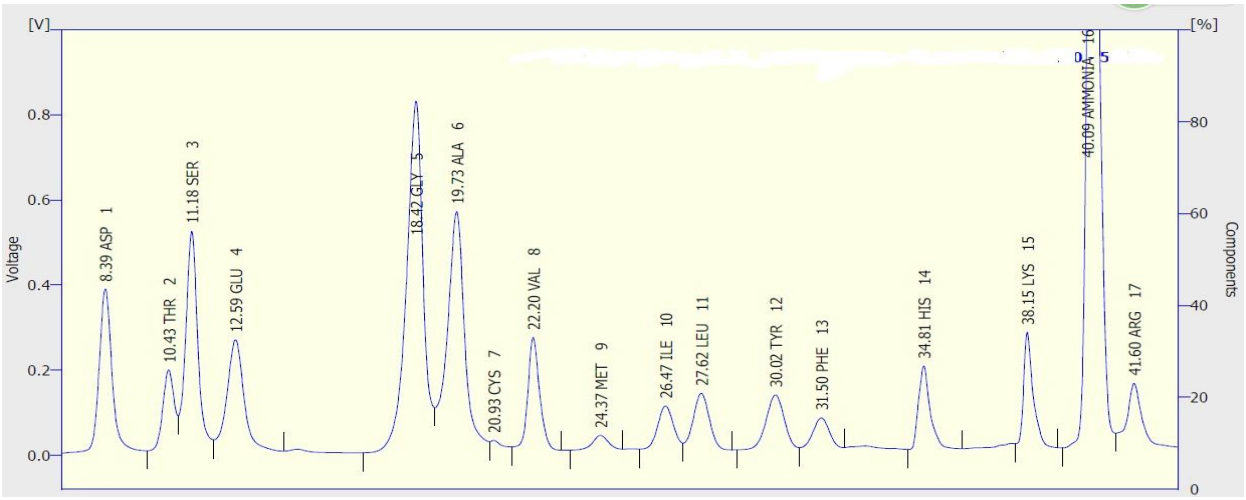

Figure 1. Amino acids chromatogram analysis of healthy *Bombyx mori* fifth larval stage.

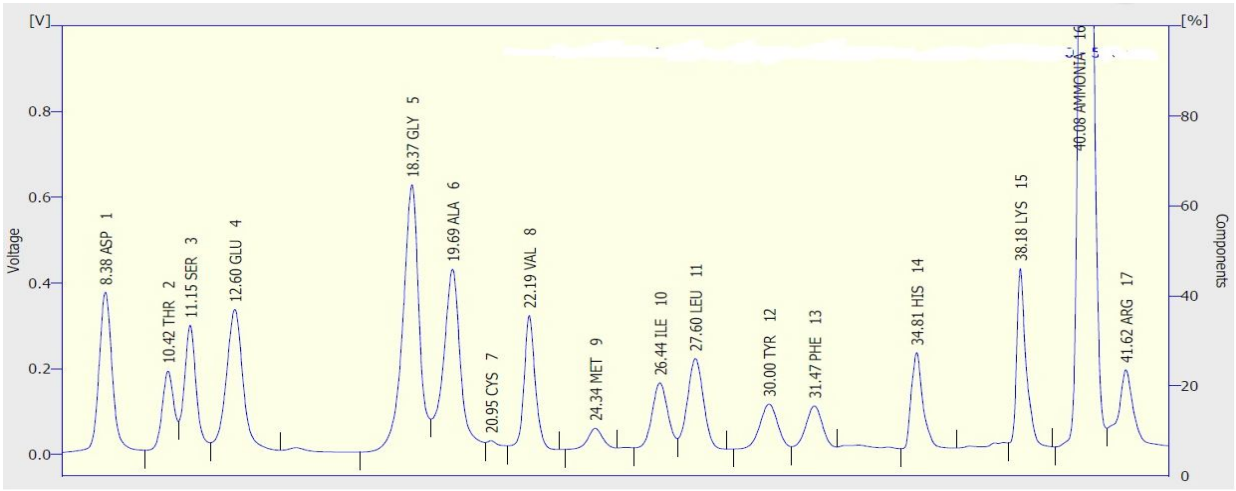

Figure 2. Amino acids chromatogram analysis of Fl-diseased *Bombyx mori* fifth larval stage.

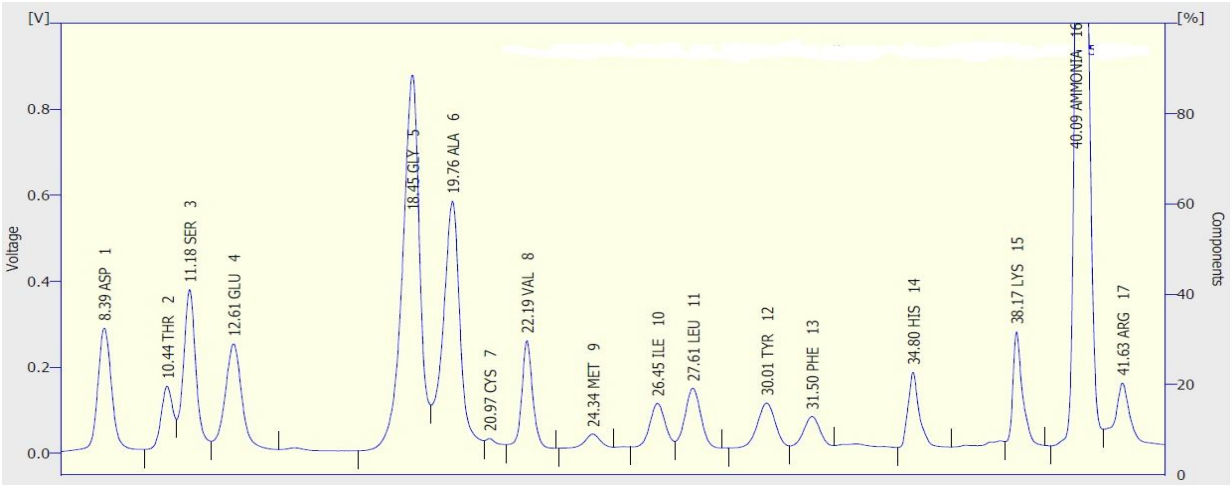

Figure 3. Amino acids chromatogram analysis of Gr-diseased *Bombyx mori* fifth larval stage.

Table 1. Amino acid composition in healthy, Fl- and Gr-diseased *Bombyx mori* fifth larval stage.

Free amino acids (mg/g tissue)	Healthy larvae	Fl-diseased larvae	Percent difference (%)	Gr-diseased larvae	Percent difference (%)
Aspartic	21.72	19.15	-11.84	15.49	-28.69
Threonine	8.87	7.73	-12.80	6.29	-29.06
Serine	18.75	9.33	-50.26	12.38	-34.00
Glutamine	19.05	20.82	9.27	15.63	-17.96
Proline	5.50	6.28	14.09	4.28	-22.19
Glycine	31.52	20.67	-34.40	30.61	-2.87
Alanine	25.45	16.73	-34.26	22.62	-11.13
Cysteine	1.80	1.56	-13.47	1.61	-10.98
Valine	10.28	10.19	-0.88	7.95	-22.60
Methionine	2.16	2.59	19.54	1.74	-19.77
Isoleucine	6.14	8.05	30.94	5.27	-14.29
Leucine	8.21	11.51	40.26	7.47	-8.94
Tyrosine	12.66	9.00	-28.94	8.95	-29.33
Phenylalanine	5.84	7.14	22.21	5.00	-14.49
Histidine	10.00	10.12	1.18	7.61	-23.91
Lysine	9.71	13.26	36.61	8.40	-13.44
NH_4	12.17	12.08	-0.75	11.05	-9.21
Arginine	14.20	13.75	-3.12	5.42	-61.83
Total amount	224.05	199.96		177.77	

Percent difference indicate the percentage increase (+) or decrease (-) in diseased samples comparing with healthy samples.

Bacterial and viral propagation may be the reason for the amino acid disturbance in the diseased larvae. These findings agree with Huang et al. (2009) who recorded an increment in anti-microorganism's amino acids, including attacin, lebocin, enbocin, gloverin and moricin families, after infection with *Bacillus bombysepticus*. The elevation of the amino acid content indicates either low transaminase activity or high proteolytic activity of enzymes (Watanabe and Kobayashi, 1976). Rajasekhar et al. (1992) stated that the infected larvae may suffer from diarrhea and vomiting that leads to dehydration which resulted in the increment in amino acid levels.

Metabolic alteration in insects, in addition to different immune responses, is the tool of insects to fight infection (Shamitha and Purushotham, 2008). Amino acids and protein profiles of a tissue may be taken as a diagnostic tool to assess the physiological status of an insect (Kimura et al., 2009). As a result, more attention was directed to study the amino acid metabolism in silkworms under different stress conditions (Lakshmi and Purushotham, 2011; Rajitha and Savithri, 2014).

Changes in nitrogen (N) and phosphorus (P) contents

In the present study, total N and P percentages were measured in healthy, Fl- and Gr- diseased larvae. Bacterial infection resulted in an increment in both elements (N = 2.57, P = 0.29%), and the same trend was found in viral infection (N = 3.86, P = 0.32%), compared with healthy sample (N = 1.63, P = 0.23%), as represented in Table 2.

Insects contain approximately 10% nitrogen by weight, primarily in proteins and chitin (Behie and Bidochka, 2013). Large amount of nitrogen is used for the fibroin synthesis in the last larval instar of *B. mori,* approximately more than 65% of nitrogen to produce silk (Unni et al., 2000). Nakano and Monsi (1968) reported that 18% of the assimilated nitrogen in silk moth larvae goes to the production of eggs while 47% routed to the silk production. This may be the reason behind low nitrogen content in healthy larvae. Montgomery (1982) found that nitrogen requirements of *Lymantria dispar* become relatively lower compared with other dietary constituents during larval maturation. On the other hand, elevation in nitrogen percentage, upon bacterial or viral infection, might be due to the excessive formation of uric acid and ammonia. The toxicity of these compounds lead to death as suggested by Tojo (1971) and Hirayama et al. (1997). Prudhomme and Couble (2002) revealed that during the synthesis of alanine, glycine and serine in the silk glands, the sources of the nitrogen and carbon skeletons are glutamine, asparagine, glutamate and aspartate. This may be the reason behind the increment of nitrogen content in bacterial-infected larvae due to the increment of glutamine by 9.27% over healthy larvae. On the other hand, another explanation was proposed by Huang et al. (2009), who found that all nitrogen pathways regulated

Table 2. Nitrogen and phosphorus contents as percentages of body dry-mass in healthy, Fl and Gr-diseased *B. mori* fifth larval stage.

	Healthy larvae (Mean ± SD)	Fl-diseased larvae (Mean ± SD)	Gr-diseased larvae (Mean ± SD)	*F*	*(df)*
Total nitrogen (%)	1.66 ± 0.31 [a]	2.60 ± 0.26 [b]	3.85 ± 0.46 [c]	37.7	(2,9)
Total phosphorus (%)	0.23 ± 0.07 [a]	0.29 ± 0.03 [a]	0.31 ± 0.03 [a]	2.52	(2,9)

A parameter (column) with the same letter are not significantly different at (α > 0.05).

genes were down regulated in *B. mori* larvae infected with *B. bombysepticus*, which resulted in reduction in nitrogen metabolism.

Phosphorus is an important element which contributes in the biological events inside silkworm. It occurs in a variety of biological molecules including DNA and RNA, ATP and other adenine nucleotides, phosphorylated metabolites, and phospholipids (Wiesenborn, 2013). Zaidi et al. (1985) reported that both testes and ovaries contain the greatest phosphorus' demand for maturation of the reproductive cells. Furthermore, the growing larvae have a high level of ribosomes in their cells, which consist of more than 50% rRNA, and that contains significant amounts of phosphorus in the sugar-phosphate backbone (Woods et al., 2002). Nakamura et al. (1982) estimated the phosphorus contents in the posterior silk glands of *B. mori* 5th instar correspond to one third of the phosphorus content in the entire silk gland. Moreover, Sakamoto and Horie (1979) reported that acid-soluble phosphate comprised a large portion of the total phosphate during 5th larval instar. Whereas, content of inorganic phosphate was approximately 10% of total phosphate as revealed by liquid chromatography. The present results showed that phosphorus content in healthy larvae was 0.23%, which agrees with the findings of Woods et al. (2004). They measured the phosphorus content in seven insect orders and found that P resembles 0.79% of the body dry-mass, but the same ratio is decreased in Lepidoptera.

The content of phosphorus compounds appears to be governed by the activities of phosphatases (acid and alkaline). The decrease in the activity of acid and alkaline phosphatases leads to increment in acid soluble phosphorus (Sridhara and Bhat, 1963). Additionally, Zaidi et al. (1985) reported that the elevation of phosphorus level might be due to the inhibition of certain enzymes because of necrosis or lysis of certain body structures after infection. Williams and Fraústo da Silva (1996) stated that extra free phosphate could drive phosphorylation reactions toward phosphate compounds, affect Ca and Mg regulation, alter energy compounds (ATP and ADP), or disturb intracellular regulation. Upon the above studies, high level of phosphate could disturb cell reactions and accumulate Phosphorus inside cells at high levels. Accordingly, high level of phosphates might lead to hyperosmotic haemolymph (Woods et al., 2002).

Upon the above results, the increase in the percentages of N and P in Fl- and Gr-diseased larvae, indicates that diseased silkworms assimilate proportionately less N and P than healthy ones. Another explanation for the increase of N is might due to the breakdown of amino acids which appears in the decrease of some amino acids content in the present study.

CONCLUSIONS

Changes in amino acid profiles, levels of nitrogen and phosphorus in diseased mulberry silkworms enhance knowledge in sericulture research. These findings are useful tools for early diagnosis of diseases and may lead to develop disease-resistant breeds. Furthermore, such changes in the infected larvae depict the possible cellular adjustment machinery of the host in response to the pathogen attack. It may have recommended for further studies to detect proteins and genes involved in conferring resistance to be able to modulate their expression in genetically-modified silkworm hybrids.

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