Protein Patterns of Silkworm *Bombyx mori* L. Fed on Leaves of Wild and Cultivated Mulberry Varieties

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Abstract: Sodium dodecyl sulphate polyacrylamide gradient gel electrophoresis (SDS-PAGE) was used to determine the protein profiles of two mulberry variety leaves: the cultivated *Morus alba* var. Morittiana and the wild one *Morus laevigata* and their effects on haemolymph and silkgland protein patterns in final larval instar of silkworm *Bombyx mori* L. Results showed some differences in the number and abundance of protein bands in the two studied mulberry variety leaves as they were 5 bands in *Morus alba* var. Morittiana ranged between (47.27- 344.47 kDa), while, were 10 bands in *Morus laevigata* leaves ranged between (17.79- 350.11 kDa). However there were close similarity in haemolymph and silkgland protein patterns of larvae fed on the both studied mulberry varieties, which registrad 12 protein band in haemolymph and 15 protein bands in silkgland of larvae fed on the two mulberry varities.

Keywords: *Bombyx mori*, mulberry varieties, haemolymph protein patterns, silkgland protein patterns.

INTRODUCTION

Proteins are the building blocks of organisms therefore, proteins are important in all biological systems and play a wide variety of structural and functional roles. Proteins are also compounds of fundamental importance for all functions in the cell. The protein budget of the cell can be considered as an important analyte in evaluating the physiological standards of the cell. The high protein concentration is an indication of a greater metabolic activity of the tissue. So improvement of the food value is probably determined by the protein quantity and quality (Li *et al*., 2012; Rajitha and Savithri, 2013; Madhu Babu *et al*., 2014).

Mulberry has an economic importance in silk industry due to its foliage, which constitutes the chief food for the silkworm, *Bombyx mori* L. Because of its richness with protein the nutritive value of the mulberry leaves have a considerable influence on the growth of the silkworm. In addition, other components of the leaves are helping the silkworm to meet its dietary requirements. Nearly 70 percent of the silk produced by silkworm is directly derived from proteins of mulberry leaves (Machii and Katagiri, 1991).

Most methods used to evaluate the leaf quality or variations between different varieties are biochemical methods, phytochemical analysis or nutritive contents of different mulberry genotypes (Jyothi *et al*., 2014; Manjula and Kumari, 2015). On the other hand the performance of the *Bombyx mori* is based on economic, biological characters and productivity. These assessments have been conducted to assess the effect of different mulberry cultivars on silkworm rearing (Babu *et al*., 2014; Prieto-Abreu *et al*., 2016). However other studies on the biochemical components and bioassay of different varieties of mulberry leaves fed by silkworm in relation to silk production were done by Zannoon *et al*. (2008).

Recent studies concerned with the protein quantification by SDS-PAGE gel electrophoresis were carried out to demonstrate the variability in protein profiles between different mulberry cultivars only (Madhu Babu *et al*., 2014; Jyothi *et al*., 2016), on the other hand, the comparative proteomic maps approach was applied to assess many factors that affect the silkworm haemolymph and posterior silkgland protein patterns (Li *et al*., 2012; Zhang *et al*., 2014; Dong *et al*., 2016). Meanwhile the effect of cultivated mulberry varieties on haemolymph protein patterns was investigated by El-Akkad *et al*. (2008). On other hand no study was carried out to find the relation and effects of the protein patterns of different mulberry varieties on silkworm larvae protein patterns.

The present study was carried out to compare the protein profile of leaves of cultivated and wild mulberry varieties, and their effect on silkworm haemolymph and silkgland proteins patterns. Results can be used to assess as index in silkworm feeding program.

MATERIALS AND METHODS

Experimental silkworm rearing

The experiment was conducted at the Plant Protection Department, Faculty of Agriculture, Suez Canal University Ismailia, Egypt. Experimental silkworm eggs of four-way hybrid (KK × Hesa 1) × (Vesletz 2 × Gergana 2), was imported from Sericulture and Agriculture Experiment Station, Vratza, Bulgaria.

Larvae were reared in plastic containers placed in a chamber where the temperature was at 25 ± 1°C and the relative humidity was 75 ± 5%, fed on fresh mulberry leaves from hatching to the fifth instar four times per day. Leaves were collected from healthy plants at Faculty of Agriculture Farm. Two groups of mulberry variety were used, namely the cultivated mulberry variety *Morus alba* L. var. Morittiana and the wild one, *Morus laevigata* which was grafted on *Morus alba* L. var. Morittiana branches each group divided to 3 replicates.

Electrophoretic analysis of proteins (SDS-PAGE)

Mulberry leaves preparation and proteins analysis

Healthy leaves were collected from medium portions of mulberry plants as pooled samples with three replicates of each variety. 1 gm of leaf samples were weighed and homogenized with 5 ml of extraction

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buffer. The samples were centrifuged at 10,000 rpm for 20 minutes. The supernatants of were collected and used for the electrophoresis protein profile estimation.

Samples were mixed with loading the buffer and heated at 95°C for 5-10 minutes. The sandwich was clipped to the electrophoresis apparatus filled with Tris-glycine-SDS Buffer in the lower chamber. 100 µg of each sample was loaded into the bottom of the walls using micropipette fitted with long tip. The apparatus was connected to the power supply started electrophoresis process at 50 V for the first 30 min and then increased the voltage to 100 V. The gel was transferred in Coommassie blue staining solution and incubated overnight, then transferred to a distaining solution (200 ml methanol, 50 ml acetic acid and 750 ml distilled water) and left for several hours until the protein bands appeared (Jyothi *et al*., 2016).

Haemolymph and silkgland proteins preparation and analysis

According to Bovilla *et al*. (2016), the maximum protein concentration was observed on the 7th day of the fifth larval instar. Therefore, haemolymph of ten 5th instar larvae (7-day old) from each replicate were collected by cutting caudal horn and stored at -80°C for further use. Subsequently, posterior silkglands were collected from the same larvae, homogenized in 0.01 M Tris-HCl (pH 8.8) buffer. The homogenates were filtered and the filtrates were centrifuged for 10 min. The supernatant was collected and washed in 0.75% ice-cold physiological salt solution, blotted on a filter paper, allowed to freeze in liquid nitrogen immediately, and stored at -80°C for further use.

One-dimensional sodium dodecyl sulfate-polyacrylamide electrophoresis (1D SDS-PAGE) were carried out according to (Zhou *et al*., 2008; Li *et al*., 2009) for haemolymph protein analysis, while the procedure established by Vasudha *et al*. (2006) was used for silkgland proteins electrophoresis analysis.

The electrophoresed 1D gels were visualized by Coomassie Brilliant Blue R250 (CBB-R250, Sigma, St. Louis, MO, USA). Densitometric analysis Gels were analyzed comparing the banding patterns abundance (raw volume percentage) and their molecular mass against protein molecular markers run parallel along with samples using gel image analysis software-SynGene Gene Tools - File version: 4.03.05.0 installed and photographed in gel documentation unit in the Institute of Biotechnology for Post Graduate and Research at Suez Canal University.

RESULTS

Electrophoretic patterns of mulberry leaves proteins

The qualitative analysis of separated protein bands from the *M. alba* var. Morittiana and *M. laevigata* leaves was done by SDS-PAGE. Electrophoresis analysis separated only 5 bands in *M. alba* var. Morittiana leaves while in *M. laevigata* leaves, 10 protein bands were separated with molecular weight ranged from 17.79 kDa to 345 kDa (Table 1 and Figure 1). Protein bands with molecular weight of 345.11 kDa, 100.00 kDa, 87.40 kDa, 74.16 kDa, 67.74 kDa, 57.36 kDa, 47.71 kDa, 40.80 kDa, 31.41 kDa and 17.79 kDa were shown in analysis of *M. laevigata* variety leaves, while they were 344.47 kDa, 77.33 kDa, 67.02 kDa, 57.18 kDa and 47.27 kDa in analysis of *M. alba* var. Morittiana leaves. The dominant proteins bands in the leaves of the two mulberry varieties were 350, 67, 57 and 47 kDa, while the more abundant protein was 47 kDa. Protein bands molecular weight with 100, 87, 74, 40, 31, 17 kDa were detected in *M. laevigata* leaves but were absent in *M. alba* var. Morittiana leaves profile.

Electrophoretic patterns of haemolymph proteins

The electrophoretic pattern of the 5th instar haemolymph proteins (Table 1 and Figure 1) showed 12 and 15 polymorphic bands for larvae fed on *M. laevigata* and *M. alba* var. Morittiana leaves, respectively, with molecular weights range from 161.59 to 21.15 kDa.

Qualitative analysis of proteins exhibited obvious variations in the number and position of bands between the larvae reared on each of the two investigated mulberry varieties.

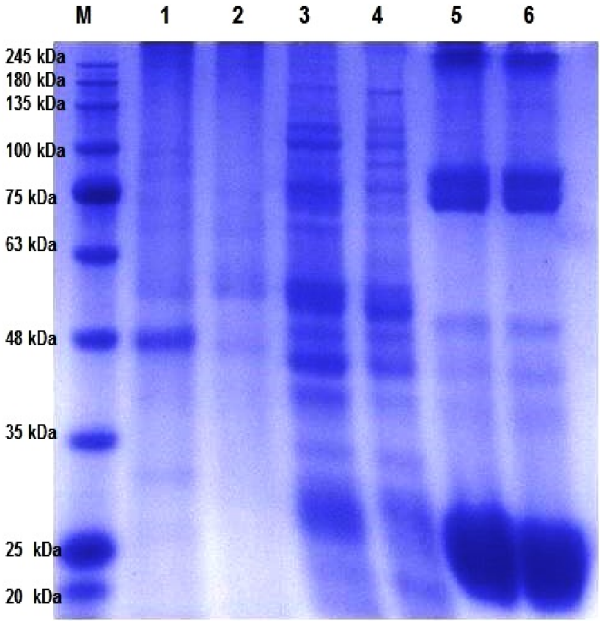

Figure (1): Protein pattern profiles of mulberry leaves, larvae haemolymph and silkgland of larvae fed on the two mulberry varieties.

M - Protein marker;
Lane 1 - *M. laevigata* mulberry leaves
Lane 2- *M. alba* var. Morittiana leaves
Lane 3- larvae haemolymph fed on *M. laevigata*
Lane 4- larvae haemolymph fed on *M. alba* var. Morittiana
Lane 5- Posterior silk gland in larvae fed on *M. laevigata*
Lane 6- Posterior silk gland in larvae fed on *M. alba* var. Morittiana.

Table (1): Proteins molecular weights and raw volume percentage of mulberry leaves and larvae haemolymph and silkgland fed on the two mulberry varieties

Protein marker		**Lane1**		**Lane2**		**Lane3**		**Lane4**		**Alne5**		**Lane6**	
Molecular weight	**Abundance %**	**Molecular weight**	**Abundance %**	**Molecular weight**	**Abundance %**	**Molecular weight**	**Abundance %**	**Molecular weight**	**Abundance %**	**Molecular weight**	**Abundance %**	**Molecular weight**	**Abundance %**
245.00	1.331	350.11	13.923	344.47	11.809	155.88	0.256	161.59	2.391	270.05	32.203	278.96	35.106
180.00	2.762	100.00	3.299	77.33	1.384	115.40	2.925	114.62	2.015	134.08	0.226	135.00	0.006
135.00	4.310	87.40	1.037	67.02	6.540	103.47	5.547	103.47	4.138	98.78	0.038	98.78	0.024
100.00	5.636	74.16	2.198	57.18	5.217	77.33	10.023	91.23	1.748	80.72	32.265	84.25	12.787
75.00	19.754	68.74	0.219	47.27	21.049	67.97	3.252	77.33	4.682	74.37	10.734	73.75	9.635
63.00	7.447	57.36	5.174			55.42	11.375	72.92	0.183	50.30	9.815	50.62	6.279
48.00	11.135	47.71	63.658			52.23	9.019	67.78	1.402	42.59	7.457	42.59	7.457
35.00	19.481	40.80	0.041			49.06	2.775	55.42	6.090	38.14	4.027	37.90	3.334
25.00	23.414	31.41	8.999			44.59	10.848	52.55	22.145	25.15	32.235	25.15	27.504
20.00	4.730	17.79	1.453			39.93	5.162	48.60	2.470				
						33.69	4.365	43.78	6.362				
						29.45	34.452	39.21	1.600				
								32.63	3.736				
								29.45	32.867				
								21.15	3.857				

Lane 1 - *M. laevigata* mulberry leaves Lane 2- *M. alba* var. Morittiana leaves Lane 3- larvae haemolymph fed on *M. laevigata* Lane 4- larvae haemolymph fed on *M. alba* var. Morittiana Lane 5- Posterior silk gland in larvae fed on *M. laevigata* Lane 6- Posterior silk gland in larvae fed on *M.alba* var. Morittiana.

Protein profile of larvae fed *on M. laevigata* leaves was characterized by the presence of bands with a molecular weight of 155.88 kDa, 115.40 kDa, 103.47 kDa, 77.33 kDa, 67.97 kDa, 55.42 kDa, 52.23 kDa, 49.06 kDa, 44.59 kDa, 39.93 kDa, 33.69 kDa and 29.45 kDa. While, protein bands observed in haemolymph of larvae fed on *M. alba* var. Morittiana leaves were 161.59 kDa, 114.62 kDa, 103.47 kDa, 91.23 kDa, 77.33 kDa, 72.92 kDa, 67.78 kDa, 55.42 kDa, 52.55 kDa, 48.60 kDa, 43.78 kDa, 39.21 kDa, 32.63 kDa, 29.45 kDa and 21.15 kDa.

Most protein molecular weights were dominant in the two varieties except 91.23 and 72.92 kDa which appeared in *M. alba* var. Morittiana leaves only. The protein band 30 kDa was the most abundant band in larvae fed on the leaves of the two mulberry varieties.

Electrophoretic patterns of silk gland proteins

9 bands with molecular weight ranged between 350.11 kDa to 25.15 kDa were detected in electrophoretic profiles of the silk gland proteins (Table 1 and Figure 1). Silkgland Protein molecular weights were 270.05 kDa, 134.08 kDa, 98.78 kDa, 80.72 kDa, 74.37 kDa, 50.30 kDa, 42.59 kDa, 38.14 kDa and 25.15 kDa in larvae fed on *M. laevigata* leaves. Similar bands were observed in silkgland of larvae fed on *M. alba* var. Morittiana leaves which were 278.96 kDa, 135.00 kDa, 98.78 kDa, 84.25 kDa, 73.75 kDa, 50.62 kDa, 37.90 kDa and 25.15 kDa. In all separated protein bands, the most abundant protein molecular weight was 25 kDa in larvae fed on the leaves of the two tested mulberry varieties.

DISCUSSION

Obtained results clearly indicated differences in protein band numbers in grafted wild variety *M. laevigata* leaves, which were 10 protein bands while were five proteins bands in cultivated variety *M. alba* var. Morittiana leaves. Protein bands with molecular weights of 345 kDa, 68.74 kDa, 57.36 kDa and 47 kDa were common in the two mulberry varieties, but with different abundance. The abundant proteins in leaves were 47 KDa which had a higher representation in *M. laevigata* in comparison to that in *M. alba* var. Morittiana leaves. Results are in agreement with that of Madhu Babu *et al.* (2014), who found that protein molecular weight of 48kDa and 55 KDa were rather common in all the five examined mulberry varieties and the 44kDa protein was the major component of the leaf proteins.

Results are in context with Jyothi *et al*. (2016) who detected 10-12 protein bands in leaves of four cultivars and explained the relationship between mulberry leaves and silkworm protein patterns. They added that mulberry leaves protein bands may belong to sericin and fibroin family, the main components of silkgland and silk fibers.

The present qualitative analysis of proteins in larvae haemolymph are not typical but similar in 11 protein bands molecular weights with variations between the larvae reared on the leaves of the two investigated mulberry varieties. These proteins were involved in a variety of cellular functions, including metabolism, development, nutrient transport and reserve. Protein band of 30 kDa, was the abundant protein in haemolymph of larvae fed on the leaves of the two varieties. This finding has been confirmed by Zhang *et al*. (2014) who reported that storage proteins 30 KDa constituted the most abundant groups of nutrient-storage proteins. Hyrsl and Simek (2005) have also reported that storage proteins, 30 kDa, were detected in protein patterns and were highly abundant at some developmental stages in haemolymph. These proteins are often called larval serum protein, larval specific serum proteins or larval haemolymph proteins. Comparative proteomics suggested that abundantly synthesized 30 K proteins in haemolymph of the fifth larval instar (the vigorous feeding stage) were taken up by and stored in fat body (storage tissue), to meet nutrient requirement of non-feeding stages, pupae, adults and eggs (Pakkianathan *et al*., 2012; Zhang *et al*., 2014).

The 9 bands with molecular masses of 278 to 25 kDa were detected in silkglands with similar patterns of larvae fed on leaves of the two mulberry varieties, while 25 kDa was the more abundant protein in the silkgland of larvae fed on the two mulberry varieties leaves.

Present findings were supported by finding of Bhat and Manjunatha (2014) and Jyothi *et al*. (2016). They reported that the chief compositions of silk fiber are fibroin and sericin where fibroin composed of two proteins; light chain (25 kDa) and heavy chains (325 kDa). These two chains are linked together by di-sulfide bonds, whereas large number proteins observed in the SDS-PAGE gel could be cellular proteins, which might support the glandular cells for fibroin synthesis (Bhat and Manjunatha, 2014). Hence, this 25 kDa protein band shall be fibroin L-chain as posterior silk gland involved in synthesis of only fibroin but not sericin.

The present study found that protein band numbers in the grafted *M. laevigata* variety leaves were more than that in *M. alba* var. Morittiana leaves. Hence, it is suggested that these bands may be related to enzymes or other components which make larvae gain the same weighs although it fed on leaves of mulberry variety that have a low proteins, nitrogen and free amino acids contents.

Although there are some differences in protein band numbers and abundance in the leaves of the two investigated mulberry varieties, the proteins pattern profile in haemolymph and silkgland of larvae fed on the leaves of both investigated mulberry varieties were closely similar which may mean that the grafted *M. laevigata* variety leaves can be used in silkworm *Bombyx mori* L. rearing system.

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شرائح البروتين لدودة الحرير التوتية التي تتغذى على أوراق صنفي التوت البري و المنزرع

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استخدم التفريد الكهربي لتحديد البروتين في أوراق صنفين من التوت: الصنف المنزرع (الرومي) والصنف البري (العماني) وتأثيرها على شرائح البروتين في سائل الدم وغدة الحرير لدودة الحرير التوتية خلال العمر اليرقي الخامس. أظهرت النتائج أن هناك اختلافات بين عدد شرائح البروتين وكميته في أوراق صنفي التوت قيد الدراسة حيث كانت ٥ شرائح في الصنف الرومي و١٠ شرائح في الصنف العماني بينما كان هناك تشابه بين شرائح البروتين في كل من سائل الجسم وغدة الحرير في حيث سجلت ١٢ شريحة بروتين في سائل الجسم بينما سجلت ١٥ شريحة بروتين في غدة الحرير لليرقات التي تغذت على أوراق كلا صنفي التوت قيد الدراسة.