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BIOCHEMICAL ANALYSIS AND PROTEIN PROFILE THROUGH SDS-PAGE IN LEAVES OF FOUR (RC-1, RC-2, G-2 AND G-4) MULBERRY (*Morus alba L.*) CULTIVARS

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ABSTRACT

Quantitative, qualitative analysis of proteins, total reducing sugars and amylase activity from the leaves of RC-1, RC-2, G-2 and G-4 mulberry cultivars were conducted. Significant differences were observed among different leaves of four varieties. SDS-PAGE of proteins from tender, medium and coarse leaves of various cultivars revealed that the tender leaves had several proteins than other three mulberry cultivar leaves. Leaf protein profiles of four mulberry cultivars have revealed 14, 55 102kDa proteins were common in all the leaf samples with quantitative variations. SDS-PAGE gel electrophoresis clearly, demonstrated the variability in biochemical traits in four various mulberry cultivars.

INTRODUCTION

India is the second largest producer of silk after China. Hence the scope for increasing the silk production in our country is immense. Mulberry is widely distributed in Asia, Europe, North and South America and Africa, and it is cultivated extensively in East, Central, and South Asia for silk production. In India the major states concentrating on silk production are Karnataka, Andhra Pradesh, Tamil Nadu, West Bengal and Jammu & Kashmir. Sericulture activities are agro-based and the industrial sector. The agro-based part involves mulberry cultivation and silkworm rearing. Various factors have shown to affect the success of silkworm cocoon production including environment, mulberry leaf quality, rearing technique, silkworm race, silkworm egg quality and others (Mukul Deka, 2011). Mulberry (genus Morus), a perennial tree or shrub, is an economically important plant used yielding foliage and is the sole food for the domesticated silkworm, *Bombyx mori*. Improvement in food value which has been a breeding objective in mulberry is mainly determined by the protein quantity and quality.

Nutrition plays an important role in improving the growth and development of Bombyx mori L. The growth and development of the silkworm larvae and subsequently cocoon production are greatly influenced by nutritional quality of mulberry leaves (Anonymous, 1975., Krishnaswami, 1978). The quality of mulberry leaf depends on many factors, such as: species, variety, type of the field, leaf age, time of harvesting, fertilizers, mode of food administration, etc. Bose and Bindroo (2001) reported that different quality traits such as leaf moisture content, protein content, carbohydrate content, nitrogen content, amino acid content and chlorophyll content are responsible for leaf quality. It is stated that silk production is dependent on the larval nutrition and nutritive value of mulberry leaves, which plays a very effective role in producing good cocoons (Indira Bhojne, 2014). Proteins are compounds of fundamental importance for all functions in the cell (Das and Sikdar, 1970). Proteins are an important in all biological systems playing a wide variety of structural and functional roles. All of the enzymes the catalysts in biochemical transformations are protein in the nature. Proteins form the frame work of cells and can also be broken-down for the release of energy. The protein budget of the cell can be considered as an important analyte in evaluating the physiological standards of the cell (Young,U.R., 1970). The protein content of leaves decreases and the carbohydrate content increases with the maturity of leaves, fiber, fat and ash constituents also increased.

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Multiple forms of several different starch degrading enzymes have been found in almost all of the organs studied in any detail. These include endo- and exoamylases (a- and b-amylases, respectively), glucosidases, debranching enzymes, starch phosphorylase, and disproportionating enzyme (Trethewey and Smith, 2000). The nature of the enzyme amylase is responsible for the primary attack on the starch granule and converts the starch into glucose (Alison *et al*., 2003).

In silk formation two major proteins are involved: serecin and fibroin. The molecular weight of sericin ranges in between 70-200kDa (Dash 2007; Kundu, 2008). Fibroin protein in muga silk (A. assama), consists of two units having molecular weights of about 220kDa and 20kDa respectively (Ahmad, 2004; Kasoju, 2009), whereas the sericin protein is about 66kDa. *M.alba* possess serecin protein, whose molecular weight is same as that of A. assama but fibroin consists of two units, having molecular weights of 97kDa and 45kDa (Rajkhowa, 2000; Mandal, 2008). The present study is an attempt to investigate the total protein content, total reducing sugars, amylase activity and molecular weights of proteins in leaves of various mulberry cultivars and their importance in silk formation.

MATERIALS AND METHODS

Mulberry Cultivation

The experiment was conducted in the Botanical garden of Botany Department, Sri Krishnadevaraya University. Mulberry (*Morus alba L*) varieties-RC-1, RC-2, G-2 and G-4 cuttings were collected from eight-months-old healthy plants, Regional Sericultural Research Station (Central Silk Board-RSRS), Rapthadu, Anantapuramu, A.P, India. The cuttings made were of 5-6 inches long with a minimum of three to four active buds. The cuttings were brought to the laboratory and immediately planted in red soil containing FYM in 3:1 proportions. Each variety was divided into four sets and arrange in Randomized complete Experimental Block Design (REBD). The plants were kept under natural photoperiod of about 12-13hrs with a temperature of 28 ± 4^{o}C.

Leaf sample preparation

Four popularly cultivated indigenous mulberry (RC-1, RC-2, G-2 and G-4) varieties were chosen. The healthy leaves were collected from tender, medium and coarse portions of mulberry plants. 1gr of leaf samples were weighed and homogenized with 5ml of extraction buffer. The samples were centrifuged at 10,000rpm for 20 minutes. The supernatants of the samples were collected and used for the estimation of total proteins by Lowry's method and protein profile through the electrophoresis.

Electrophoretic analysis of proteins (SDS-PAGE)

Dried glass plates were assembled and sealing was done using molten agarose. It was checked for leakage by filling water between the plates and later discarded. The gel mix was prepared and poured between the plates till the level reached 3/4th of the capacity. Immediately it was overlaid with water to an additional height of 0.5cm. Gel was allowed to polymerize for nearly 30mins. After polymerization the excess water was drained completely. Stacking gel mix was prepared and poured onto to polymerized resolving gel and a clean dry comb was carefully inserted without trapping air bubbles. Gel was left for additional 30mins for complete polymerization.

Samples were mixed with sample loading buffer as per the table and heated at 95°C for 5-10 minutes. Spun down the samples in microfuge for 5 minutes. Clipped the sandwich to the electrophoresis apparatus filled with Tris-glycine-SDS Buffer in the lower chamber. Care was taken not to introduce any air bubbles between the bottom of the gel and the buffer. The comb was carefully removed from the gel and filled the top of the apparatus with Tris-glycine-SDS Buffer. 100 µg of each of the samples were loaded into the bottom of the wells using microlitre syringe or micropipette fitted with long tip. Carefully recorded the contents of each well. The apparatus was connected to the power supply started electrophoresis at 50 V for the first 30 min and then increased the voltage to 100V. 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 shaking for several hours until the protein bands appeared. The gels were observed under gel documentation system and photographed.

Determination of total reducing sugars

Estimation of reducing sugar was done by the method of Miller (1972). 500 mg of fresh leaves were homogenized with 10.0ml of 80% ethanol. The sample was centrifuged at 2000rpm for 20 minutes and the supernatant was collected separately. Alcoholic extract was used for estimation of reducing sugars. To 3.0ml of alcoholic extract, 3.0ml of 0.1% DNSA reagent was added. The mixture was heated for 5 minutes in a boiling water bath. After color had developed, 1.0ml of 40% Rochelle salt was added when the contents of the tubes were still warm. The tubes were cooled under running tap water. Absorbance was recorded using spectrophotometer at 575nm against blank.

Estimation of amylase activity

Amylase activity was measured in the leaves of various mulberry cultivars according to the method of Sridhar and Ou (1972). The fresh leaves were extracted into 10ml of cold 1M acetic acid sodium acetate buffer (pH 6.0) by macerating in a mortar with pestle. The homogenate was filtered through a muslin cloth and the filtrate was transferred into centrifuge tubes and centrifuged at 5000rpm in a refrigerated high speed centrifuge for 20min at 0^{o}C. The pellet was discarded and the supernatant was taken. 5.0ml of enzyme extract was taken into a conical flask, 10ml of 1M acetic acid sodium acetate buffer (pH 6.0) and 5ml of 1% starch were added to it. The contents were mixed and incubated at 37^{0}C for 24hrs. Amylase activity was measured in µg of reducing sugars released per mg of protein.

RESEULTS AND DISCUSSION

In this study to determine the proteins, reducing sugars quantity and amylase activities in leaves of various genotypes of mulberry (RC-1, RC-2, G-2 and G-4) cultivars were taken for the test and their nutritional values were estimated and recorded in the table I. The graphical comparison was described in the charts (fig.1-3) for easy understanding.

The present study showed that, high significant differences were observed in total proteins, total reducing sugar contents and amylase activity of the tender, medium and coarse leaves of four (RC-1, RC-2, G-2 and G-4) mulberry cultivars were studied. Tender leaves of G-4 mulberry cultivar revealed highest total proteins (23.05mg/g) followed by RC-2, G-2 cultivars. Whereas, lowest protein content (20.1mg/g) was observed in coarse leaves of RC-1 mulberry cultivar. The maximum amount (8.34mg/g) of total reducing sugars were observed in coarse leaves of G-4 mulberry cultivar followed by RC-2, G-2 cultivars and a significant decrease (6.12mg/g) has been observed in tender leaves of RC1 mulberry cultivar. Higher level (3.08) of amylase activity was observed in coarse leaves of G-4 mulberry cultivar followed by medium, coarse leaves of G-4 cultivar, RC-2, G-2 cultivars and the lower levels (2.02) were observed in coarse leaves of RC-1 mulberry cultivar.

Table.1 Shows total proteins, reducing sugars content and amylase activity in leaves of various mulberry cultivars.

Mulberry cultivars	Total proteins (mg/g)			Total Reducing sugars (mg/g)			Amylase activity (µg of reducing sugars formed mg^{-1} protein min^{-1})		
	Tender	Medium	Coarse	Tender	Medium	Coarse	Tender	Medium	Coarse
RC-1	23.02 ±3.94	19.55 ±2.23	20.1 ±3.22	6.12 ±0.41	6.33 ±0.64	7.38 ±0.65	2.02 ±3.94	2.05 ±2.23	2.11 ±3.22
RC-2	24.25 ±2.26	24.01 ±3.46	22.95 ±2.35	6.23 ±0.52	6.56 ±0.53	7.02 ±0.86	2.75 ±2.26	2.71 ±3.46	2.75 ±2.35
G-2	25.15 ±3.52	21.05 ±1.32	20.05 ±2.31	6.36 ±0.63	6.78 ±0.48	7.56 ±0.72	2.45 ±3.52	2.45 ±1.32	2.46 ±2.31
G-4	28.05 ±3.43	25.2 ±4.36	23.05 ±3.45	7.04 ±0.14	7.35 ±0.21	8.34 ±0.66	3.05 ±3.43	3.12 ±4.36	3.08 ±3.45

Mulberry leaves are rich source of proteins, carbohydrates, total chlorophyll and total carotenoids, ascorbic acid and various mineral elements. Deficiency of certain nutrients or an imbalance of nutrients in leaves cause changes in the composition or metabolic activity of silkworm larval body (Ito, 1972). The carbohydrates, proteins and lipids play an important role in the biochemical process underlying growth and development of insects (Ito and Horie, 1959, Wyatt, 1961 and 1967). Carbohydrates particularly the sugar content in mulberry leaves in closely related to the health of the silkworm. Mulberry leaves with high sugar content field's good results of rearing (Kichisaburo minamizawa, 1970).

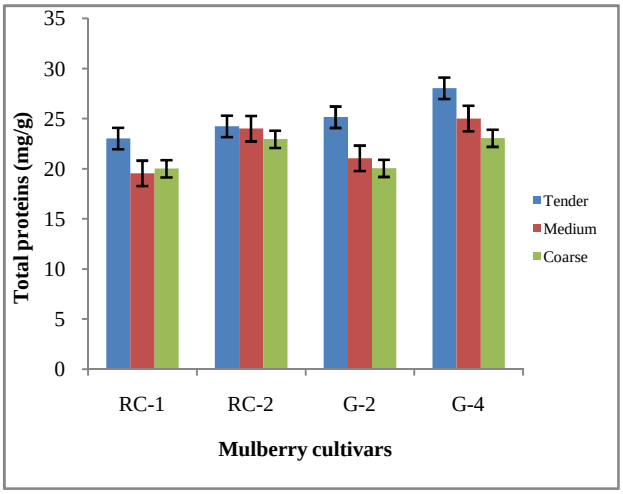


Fig.1 Comparison of total proteins in leaves of various mulberry cultivars

Thomsen *et al*., (1991) and Freeze (1996) found that proteins are the primary component of living things. The presence of higher protein level in these plants pointed towards their possible increased food value. Machii, & Katagiri, (1991) opined that increased protein content beyond the optimal level in mulberry leaves leads to a marginal improvement in cocoon productivity. Protein and amino acids are of particular importance for the silkworm larvae because of their active utilization for the synthesis of silk proteins. Carbohydrates are estimated based on the amount of sugar and starch content available in leaves (Bose and Bindroo, 2001). If the leaves have high carbohydrate content, silkworms gain more energy and in turn may enhance the synthesis of silk protein.

The phosphorolysis was the major or only route of glucan degradation in chloroplasts because of difficulty in detecting chloroplastic amylase activities against a background of high extra-chloroplastic activity (Stitt *et al*., 1978).

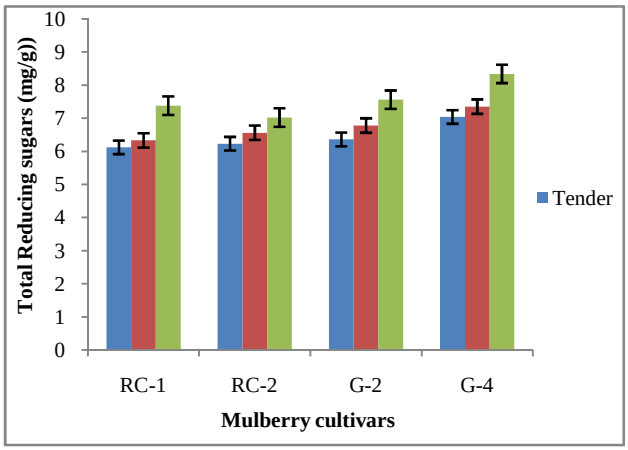


Fig.2 Comparison of total reducing sugars in leaves of various mulberry cultivars

It is now clear that many chloroplasts and other starch containing plastids possess both a- and b-amylases in addition to phosphorylase (da Silva *et al*., 1997; Lao *et al*., 1999), and are able to degrade starch into glucose hydrolytically as well as phosphorolytically (Schleucher *et al*., 1998).

Protein profile of mulberry leaves

The qualitative analysis of proteins in the tender, medium and coarse leaves of RC-1, RC-2, G-2 and G-4 cultivars were done by SDS-PAGE. Gel electrophoresis analysis separated 10-12 bands in different stages of four mulberry cultivars. About 12 clearly detectable mulberry protein bands over a wide range of molecular weight 14kDa to 102kDa were recognized. The number of protein bands gradually decreased from tender leaves to coarse leaves. Especially, the bands migrated in the

high (35-66kDa) and low molecular weight zones (below 27kDa).

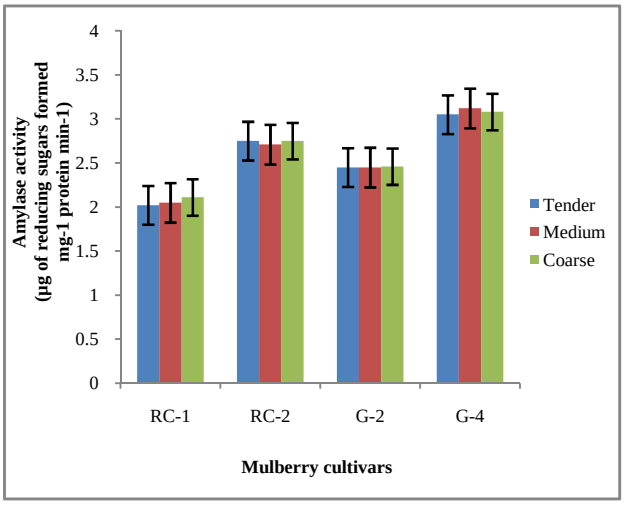


Fig.3 Comparison of amylase activity in leaves of various mulberry cultivars

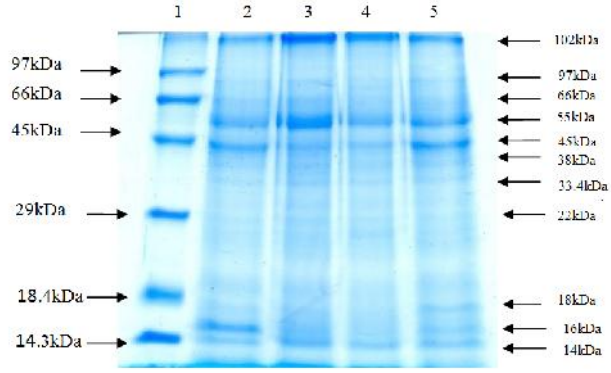


Fig.4 SDS-PAGE analysis of proteins from tender leaves of Four mulberry cultivars.
Lane 1: Protein Marker; lane 2: RC-1; lane 3: RC-2;
Lane 4: G-2; lane 5: G-4 cultivars.

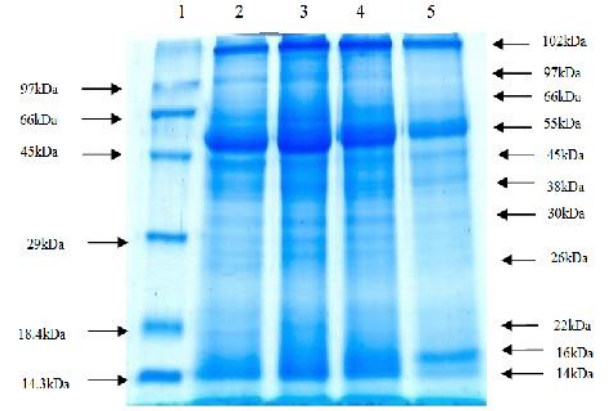
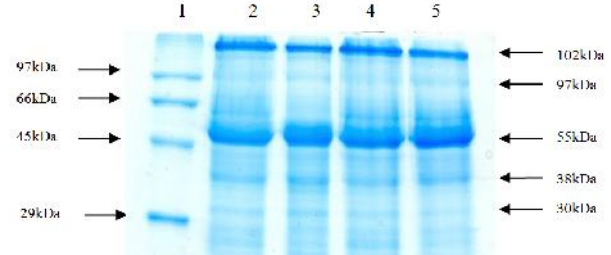


Fig.5 SDS-PAGE analysis of proteins from medium leaves of four mulberry cultivars. Lane 1: Protein Marker; lane 2: RC-1; lane 3: RC-2; lane 4: G-2; lane 5: G-4 cultivars.



Leaf protein profiles of four mulberry cultivars have revealed that 14, 55 and 102kDa proteins were common in all the leaf samples with quantitative variations. 16kDa protein was observed in tender leaves of RC-1, G-4 variety and medium leaves of G-4 mulberry cultivars. 18kDa protein was identified in tender leaves of G-4 variety and coarse leaves of RC-2 cultivar. 22kDa protein was observed in only coarse leaves of four mulberry cultivars. 26 and 28kDa proteins were identified in medium leaves of RC-2 and G-2 cultivars. 30kDa protein was presented in medium leaves of RC-2, G-2, G4 varieties and coarse leaves of four mulberry cultivars. 33.4, 66kDa proteins were observed in only coarse leaves of four mulberry varieties. 38kDa protein was identified in tender leaves of RC-2, G-4 varieties and medium, coarse leaves of four mulberry cultivars. 45kDa protein was observed in tender and medium leaves of all the four varieties. 66kDa protein was identified in tender leaves of all four varieties and medium leaves G-2, G-4 cultivars. 97kDa protein has been observed in tender leaves of RC-2, G-4 cultivars, medium leaves of RC-1, RC-2, G2 cultivars and coarse leaves of RC-2, G2 and G-4 mulberry cultivars.

In general silk is formed by serecin and fibroin proteins. The protein of molecular weight 66 KDa has been reported earlier and it was identified as serecin (Sujatha, 2015) and Fibroin is the major structural protein formed by two different polypeptide chains, i.e., heavy (H) and light (L) chains of molecular weights 350 kDa and 25 kDa respectively. These two chains are linked together by di-sulfide bonds (Vollrath, 2001). The proteins of similar molecular weights were noticed in leaves of mulberry cultivars may belong to serecin and fibroin family.

CONCLUSION

According to the proteomic, amylase activity and reducing sugars analysis, it is suggested that total protein content, reducing sugars were quantitatively declined in leaves of four mulberry cultivars. SDS-PAGE gel electrophoresis also clearly, demonstrated the variability in protein profiles in RC-1, RC-2, G-2 and G-4 cultivars at different maturity of leaves, which indicates that protein profiles of four mulberry cultivars were almost identical except for appearance/disappearance of two to five bands and significantly quantitative variations in accordance with the changes in amount of total protein, reducing sugars and amylase activity.

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