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A comparative study of silkworm (*Bombyx mori* L.) rearing under different sources of peptides isolated from Dudhiya and S1 mulberry leaves

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Abstract

Silkworm is a domestic monophagous insect, produces only natural animal fibre. Growth of silkworm larvae depends on the nutritional components of mulberry leaves. In present study one attempt was made to investigate the role of low molecular weight (0.5-3 kDa) mulberry peptides on silkworm rearing. For peptide extraction, two different types of mulberry leaves were chosen, one from S1 which was preferred by larvae for feeding, another is a germplasm named Dudhia, refused by larvae. Peptides isolated from young S1 leaves showed higher larval growth followed by peptides isolated from mature and senescence leaves. High ERR% along with enhanced weight of single cocoon and single shell was observed in silkworm fed with S1 peptide treated mulberry leaves as compared with the same by Dudhia peptides. Elevated antioxidant activities were exhibited by S1 peptides than Dudhia at all maturity stages (young, mature and senescence). Significant correlation was obtained between antioxidant activities of S1 peptides and economical attributes of silkworm rearing such as ERR %, weight of single cocoon, weight of single shell etc. From our observation it might be stated that the farmers would have been benefited if they could use mulberry leaves treated with antioxidant enriched peptides as a food for silkworm rearing.

Keywords: Oligopeptides, *Bombyx mori*, HPLC, antioxidant, Mulberry leaf.

Introduction

The *Bombyx mori* L. is an important sericigenous insect due to their golden fibre and it plays an important component of sericulture industry which contributes to the economic development of India and Bangladesh. The nutritional quality and quantity of mulberry leaves have a direct consequence on silkworm growth and development and subsequent cocoon production (Seidavi *et al.* 2005). Recently scientists are trying to improve silkworm rearing by feeding them different mulberry leaf supplementary products. The effects of different types of dietary protein on silkworm growth were determined by using semi-synthetic diets. Several reports stated that protein acts as an essential ingredient in silkworm diet (Horie and Watanabe 1983; El-Sayed and Nagda 1999). Smaller proteins less than 10 kDa have also been considered as peptides, therefore it can be predicted that these peptides might also have significant impact on the growth and development of silkworm. In present study, a scientific attempt was made to find out the effect of peptide(s) at low molecular weight ranges (0.5-3 kDa) isolated from mulberry leaves. Dudhia is a germplasm of mulberry and S1 is a cultivar used for peptides extraction at different maturation stages. As silkworm larvae have feeding preference on leaves of S1 cultivars than Dudhia germplasm (primitive), this study was undertaken by comparing the rearing efficiency and antioxidant activity of oligopeptides isolated from the two above mentioned sources of mulberry leaves.

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Material and methods

Plant culture

Leaves of S1 cultivars of mulberry and Dudhia were collected from Sericulture Farm of Malda, West Bengal, India. Leaves were selected at different maturity stages namely young, mature and senescence leaves at same season and same time. Young, mature and senescence leaves were selected on the basis of the biochemical attributes (chlorophyll and protein content) and the morphological parameters (length and breadth) of the leaves.

Isolation and purification of low molecular weight peptide(s)

Mulberry leaves from Dudhia germplasm and S1 cultivar amounting 1 kg each at three different maturation stages were surface sterilized. For peptide isolation the leaves were separately crushed with liquid nitrogen by a grinder and extracted with a measured amount chilled distilled water by blender at 4^0 C. The extract was cold centrifuged at 10,000 rpm for 30 minutes using protease inhibitor PMSF. The supernatant was subjected to ether wash at acidic pH to remove endogenous hormonal impurities, fats, lipids and oil as impurities. It was then passed through separate cation exchanger resin (Dowex-50; 900 meq. in glass column 60 × 2.9 cm) to get anionic hormone free solution, like indole 3-acetic acid (IAA), abscisic acid (ABA) and gibberellic acid (GA_3). The sample obtained after cation resin was dissolved in water with little acidic pH and again passed through anion resin (700 meq-Sigma Chemical Co. USA filled in glass column 60 cm × 2.9 cm, 1.6 meq / ml) for trapping amphoteric molecules like proteins, peptides and amino acids. Then concentrated aqueous acidic column eluents were washed 4 times with equal volume of peroxide free ether to remove traces of IAA, ABA, and GA_3. After discarding anionic hormones, the extracts were filtered through Millipore Ultrafiltration device with 10 kDa (YM 10), 3 kDa (YM3) and 0.5 kDa (YC 05) cut off ultrafiltration membranes (Amicon made) under 1.5 kg/cm^2 N_2 gas pressure. The samples were repetitively filtered and lyophilized. The obtained peptide extract was dissolved in 50 mL distilled water and stored in freeze at -20° C for further analysis.

HPLC analysis

The semi purified concentrated peptide(s) from different maturity status of leaves were passed through C_{18} HPLC Waters™ 486 reverse phase column with 10% methanol as running solvent fitted with 515 HPLC pump, runtime 60 minutes, absorbance at 250 nm, column length 3.9 × 150 mm, injection volume 20µl, flow rate 0.5-1.0 ml / minutes with pump pressure of 4000 psi. The peptide(s) appeared at different retention time were repeatedly loaded and purified, concentrated *in vacuo* and stored in deep freeze under -20^0 C. Each peak was isolated with their retention time and re-injected into the column to check its repetitive occurrence.

Sequencing of bioactive peptide separated through HPLC was performed through Shimadzu PPSQ-31A automated protein sequencer with 15 cycles operation, reactor temperature 60^0C, column temperature 37^0 C with mobile phase by 10% methanol. HPLC characterization of PTHs made use of a steel-walled C_{18} analytical column. After each cycle of Edman degradation, the PTH-derivatives were identified through Shimadzu UV-Vis SPD-20A Detector with detecting wavelength at 289 nm. System integrator calibrated the maximum probable sequence of amino acids.

Feeding trail

Present experiment was conducted in the laboratory under optimum temperature (27^0-29^0 C) and humidity (70 ± 5%). For the diseases free laying (DFL) of silkworm rearing, 5th instar F1 hybrid (Nistari × bivoltine) of silkworm larvae were selected and reared according to Jha *et al.* (2014). As a control, fifty larvae were reared by feeding with S1635 mulberry leaves (mulberry stem shocked under distilled water for 4 hrs). Side by side similar experiment was conducted with peptides treated S1 mulberry leaves separately. Peptide(s) isolated from young (P_y), mature (P_m), and senescence (P_s) leaves in both range 0.5-3 kDa and 3-10 kDa was diluted 20 times by distilled water. Leaves were soaked in peptide(s) for 30 minutes before feeding them to the larvae and air-dried and given to silkworm. Six separate groups, with 10 larvae were kept and fed by different peptide(s) treated leaves in separate plastic tray. Each experimental set was highly maintained contamination free. Each four hours duration larvae were fed by elicited leaves (4 times every day) until cocoons formation would be started. The larval weight was recorded by weighing them each day and growth rate pattern of the larvae was calculated. After cocoon formation, the cocoon weight of each set was recorded. When moths were released out from cocoon, cocoon shell weight was also measured. Growth rate, effective rearing rate (ERR), shell ratio, weight of single cocoon and single shell

was calculated by formulae used in Jha *et al.* (2015).

$$\text{Shell ratio (\%)} = \frac{\text{Single shell weight (gm)}}{\text{Single cocoon weight (gm)}} \times 100$$

$$\text{ERR \%} = \frac{\text{Total no. of cocoons harvested}}{\text{Total no. of larvae brushed}} \times 100$$

$$\text{Weight of single cocoon} = \frac{\text{Weight of 5 male cocoons + Weight of 5 female cocoons (gm)}}{\text{No. of cocoons taken (10)}} \times 100$$

$$\text{Single shell weight} = \frac{\text{Total shell weight of 5 male cocoons + 5 female cocoon shells (gm)}}{\text{Total no of cocoons taken (10)}} \times 100$$

Determination of antioxidant activity of isolated peptide(s)

ABTS$^+$ scavenging activity

The spectrophotometric analysis of ABTS$^+$ radical cation(s) scavenging activity was determined according to Re *et al.* (1999) method with some modifications. The ABTS$^+$ was obtained by reacting 7 mM ABTS$^+$ radical cation(s) in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for 12-16 hrs. Before usage, the ABTS$^+$ solution was diluted to get an absorbance of 0.750 ± 0.025 at 734 nm with sodium phosphate buffer (0.1 M, pH 7.4). Then, 2 mL of ABTS$^+$ solution was added to 1 mL of the aqueous extract. After 30 min, absorbance value was recorded at 734 nm, relative to a blank absorbance. The percentage inhibition of the samples was calculated as:

Inhibition % $= (1 - A/A_0) \times 100$

Where A_0 is the absorbance at 734 nm of the control, *A* is the absorbance at 734 nm of the sample mixture.

DPPH Scavenging activity

Antioxidant activity of LMW peptide was examined by using capacity of free radical scavenging effect of stable DPPH free radical. The radical scavenging activity of the aqueous extracts was measured by DPPH method (Blois 1958). In this assay ascorbic acid was used as a standard compounds. The absorbance was measured at 517 nm. A reaction mixture without test sample was taken as control. The free radical scavenging activity of tested sample were expressed as percentage of inhibition and were calculated according to these equation:

% inhibition of DPPH activity$=[(A_0-A_1)/A_0]\times 100\%$

Where A_0 is the absorbance values of the blank sample i.e. control reaction and A_1 is the absorbance value of the tested sample. A curve of inhibition percent or percent scavenging rate against sample concentrations was determined from where IC_{50} (concentration of the sample required to inhibit 50 % of free radicals) of tested sample were calculated.

Reducing power

The assay was performed according to the method of Oyaizu (1986) with some modifications. To determine reducing power activity of peptide, 1% potassium ferricyanide solution was used. Fluorescent green colour was appeared and absorbance of the final solution was recorded at 700 nm.

Nitric oxide Scavenging assay

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction (Marcocci 1994). For this reaction 320 µL extract, 360 µL (5 mM) sodium nitroprusside-PBS solutions, 216 µL Greiss reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% napthyl ethylene diamine dihydrochloride) was mixed and incubated at 25°C for one hour. Finally 2 mL water was added and absorbance was taken at 546 nm.

Superoxide anion radical scavenging activity

The superoxide radical scavenging activity was measured by the method of Nishikimi *et al.* (1972) with slight modifications. The reaction mixture contained 1 mL of NBT solution, 1 mL of NADH solution and 1 mL of methanolic extract of different concentrations. After 5 min incubation, 100 µL of PMS was added to the reaction mixture. The reactant was illuminated at 25°C for

30 min and the absorbance was measured at 560 nm against methanol as control.

Result and Discussion

Comparative analysis of effects of peptides on silkworm rearing system

The larval growth and development depends on the essential nutrients in exact ratio (Kanafi *et al.* 2007). It was earlier reported that mulberry leaves supplemented with different nutrients

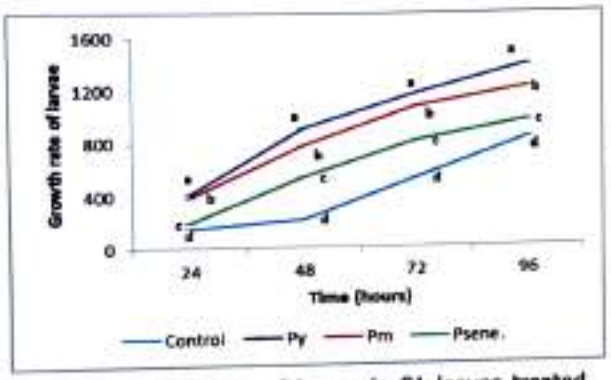


Figure 1: Growth rate of larvae in S1 leaves treated with 0.5-3 kDa peptide isolated at different maturity stages of leaves of S1 and control (only S1 leaves). Results are represented as mean ± SEM, n = 3. Values with different letters (a, b, c & d) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

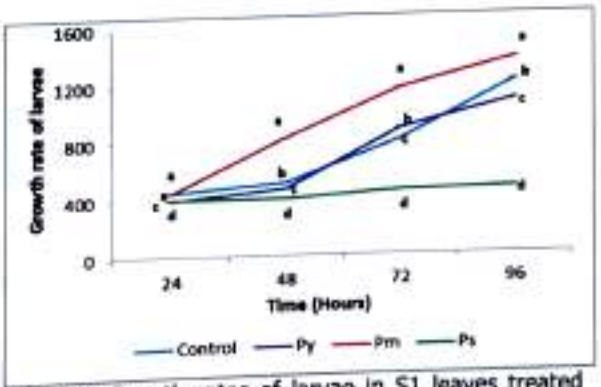


Figure 2: Growth rates of larvae in S1 leaves treated with 0.5-3 kDa peptide isolated at different maturity stages of leaves of Dudhia and control (only S1 leaves). Results are represented as mean ± SEM, n = 3. Values with different letters (a, b, c & d) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

improved silkworm larval growth (Sarker *et al.* 1993). Also previous findings supported that peptides at low molecular weight range had an effect on silkworm larval growth and silk production (Jha *et al.* 2014, 2015). Paralytic peptides of silkworm larvae had *in vitro* effects on both in hematopoietic regulation and silkworm larval haemocyte immune reaction (Nakahara *et al.* 2003). Literature survey also reveals that oligopeptides from different plants had a role on larval growth and their metabolic activity. Our study has unveiled that low molecular weight S1 mulberry peptides influence the larval growth and other economic attributes of silkworm rearing system. Dudhia peptide showed comparatively reduced effects on larval growth than S1. On the other hand, effects of Dudhia peptides significantly improved over control. These observations clearly indicate that short protein or peptides can influence the silkworm larval growth and silk production and also it was source dependent. Highest larval growth rate was recorded at 96 hrs after 4th moulting under all peptides treatment. Similar occurrence was also reported earlier by Jha *et al.* (2014). Larval growth rate was gradually increased from 24 hrs to 96 hrs under all peptides treatment as well as in control (Figure 1). In case of S1 peptides treatment, larval growth and economic attributes of Py (peptides isolated from young leaves) was followed by Pm and Ps (peptides isolated from mature and senescence leaves respectively), and all of their bioactivities improved over control. Conversely when Dudhia peptides were considered, only Pm showed effective growth rate over control but Py and Ps had no positive effects (Figure 2). Weight of single cocoon and single shell was high under Py treatment of S1 peptides followed by Pm and Ps (Table 1). In case of Dudhia peptide treatment, highest economic attributes like cocoon weight and single cocoon shell weight was recorded under Pm treatment followed by Py and Ps. Mulberry leaves supplemented with soybean flour had effects on larval weight. Sridhar and Radha (1986) found significant larval growth and enhanced economic attributes after feeding mulberry leaves treated with amino acids. Amala Rani (2011) reported that mulberry leaves supplemented with protein had significant effect on larval growth and different economic parameters of silkworm rearing system. Our experimental data stated that low molecular weight peptides influenced the

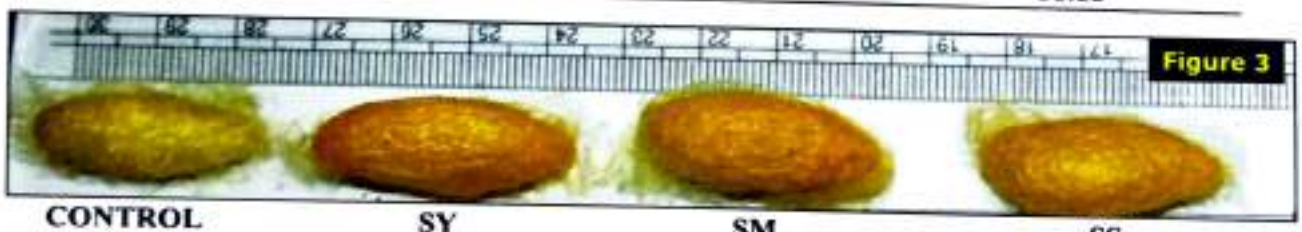
economical parameters of the silkworm rearing system. Figure 3 and 4 shows the comparative accounts of cocoons obtained after S1 and Dudhia peptide treatment.

Comparative studies of antioxidant activity of isolated peptide(s)

The results of $ABTS^{\cdot+}$ and DPPH free radical-scavenging activity of purified peptides are shown in Figure 5 and 6 respectively. Both free radical-scavenging activity of peptide samples isolated from Dudhia and S1 mulberry leaves, increased in a concentration-dependent manner. High scavenging activity was recorded in SY (peptides isolated from S1 young leaves). Low IC_{50} values mean high antioxidant activities. In case of DPPH and $ABTS^{\cdot+}$ scavenging assay, S1 showed higher scavenging activity than Dudhia at each maturity stages. Peptides isolated from young leaves exhibited higher antioxidant activity than Pm and Ps in both sources of mulberry leaves. Nitric oxide is responsible for numerous physiological processes like vasodilation, immune response, neural signal transmission etc (Wink *et al.* 1991). Our experiments revealed that Pm had higher nitric oxide scavenging activity rather than Py and Ps in both Dudhia and S1 peptides (Figure 7). Superoxide is considered as an initial free radical, formed from mitochondrial electron transport systems and creates other cell-damaging free radicals, such as hydrogen

Table 1: Effect of S1 and Dudhia peptides on various economic attributes of silkworm rearing system (where SY, SM & SS means peptides isolated from S1 young, mature and senescence leaves respectively and DY, DM & DS means peptides isolated from Dudhia young, mature and senescence leaves respectively).

Treatment	Weight of single cocoon	Weight of single Shell	Shell ratio (%)	ERR (%)
Control (S1 leaves)	0.66 ± 0.027	0.098 ± 0.019	14.85	100.00
SY	0.78 ± 0 .015	0.178 ± 0.02	22.82	100.00
SM	0.72 ± 0.018	0.115 ± 0.01	15.97	100
SS	0.74 ± 0.016	0.12 ± 0.005	15.75	90
DY	0.688 ± 0.018	0.16 ± 0.011	23.84	41.67
DM	0.71 ± 0.009	0.114 ± 0.007	16.06	83.33
DS	0.57 ± 0.006	0.113 ± 0.01	19.82	58.33



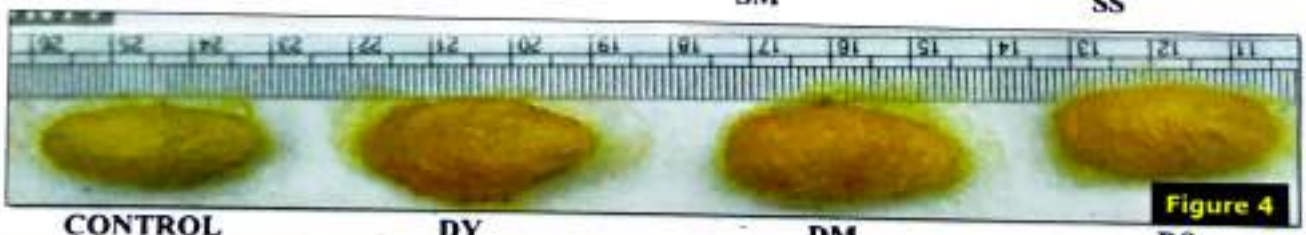


Figure 3: Cocoon obtained after S1 peptide treatment and from control (after untreated mulberry leaves). Here, SY, SM and SS stands for peptides isolated from young, mature and senescence leaves of S1 mulberry leaves respectively.
Figure 4: Cocoon obtained after Dudhia peptide treatment and from control (after untreated mulberry leaves). Here, DY, DM and DS stands for peptides isolated from young, mature and senescence leaves of Dudhia leaves respectively.

peroxide, singlet oxygen or hydroxyl radical (Bloknina *et al.* 2003). Peptides can protect the cells against toxic effect of some superoxide free radicals (Comfort *et al.* 2011).The results shown in Figure 8 clearly indicates that peptides isolated from S1 young and mature leaves have high potential superoxide scavenging activity than the Dudhia peptides.

The reducing capacity of a biological compound plays a significant indicator of its potential antioxidant activity in reducing power determining assay (Kallithraka *et al.* 2001). As shown in Figure 9, Py showed high antioxidant activity than Pm and Ps in case of both. All peptides isolated from S1 leaves exhibited higher antioxidant activity than their respective counterparts of Dudhia peptides.

IC_{50} values of S1 peptides like DPPH, $ABTS^{\cdot+}$, nitric oxide, superoxide was negatively correlated with different economical parameters of silkworm rearing system such as ERR%, WSC (weight of single cocoon), WSS (weight of single shell) etc. As the IC_{50} values of different free-radical scavenging components were negatively associated with antioxidant property, economic attributes were directly related to antioxidant activity of isolated peptides, which means that antioxidant rich peptide from mulberry leaves might elicit the growth of silkworm and facilitate metamorphosis from larval stage to pupa as well as cocoon production.

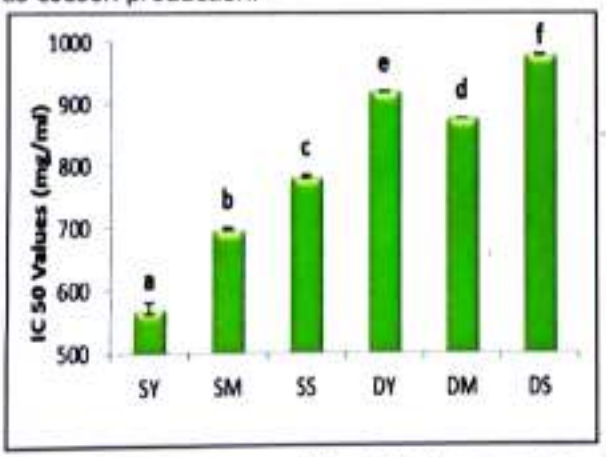


Figure 5: Comparative analysis of $ABTS^{\cdot+}$ scavenging activity of peptide(s) isolated from S1 and Dudhia leaves at three maturity stages. Results are represented as mean ± SEM, n = 3. Values with different letters (a-f) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

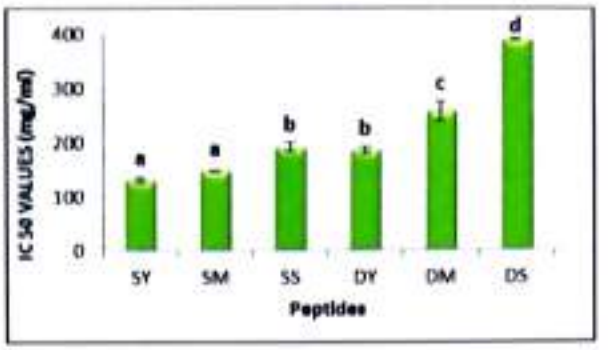


Figure 6: Comparative analysis of DPPH scavenging activity of peptide(s) isolated from S1 and Dudhia leaves at three maturity stages. Results are represented as mean ± SEM, n = 3. Values with different letters (a-d) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

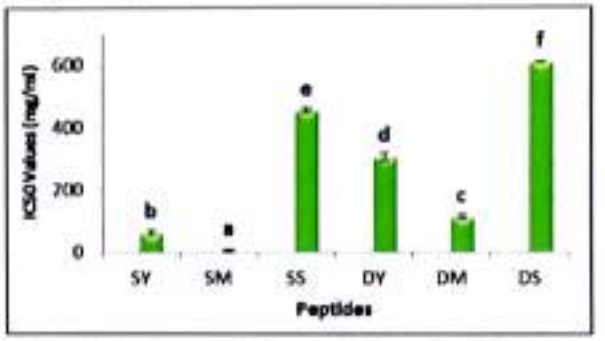


Figure 7: Comparative analysis of nitric oxide scavenging activity of peptide(s) isolated from S1 and Dudhia leaves at three maturity stages. Results are represented as mean ± SEM, n = 3. Values with different letters (a-f) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

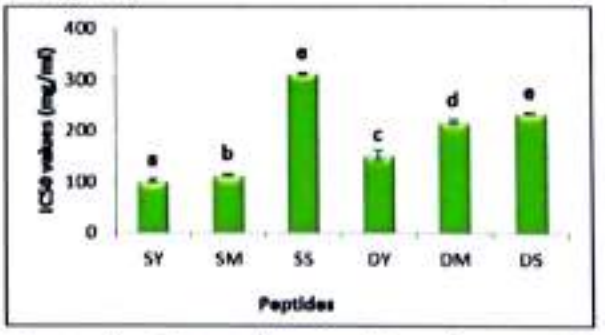


Figure 8: Comparative analysis of superoxide scavenging activity of peptide(s) isolated from S1 and Dudhia leaves at three maturity stages. Results are represented as mean ± SEM, n = 3. Values with different letters (a-e) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

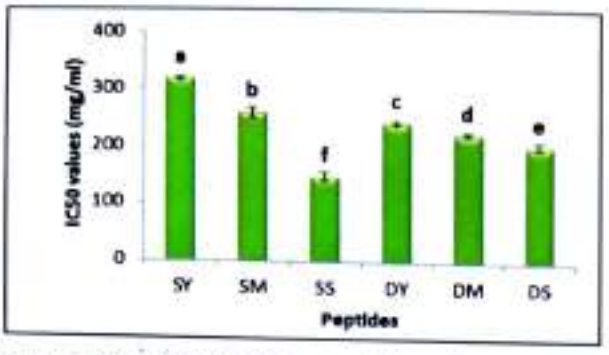


Figure 9: Comparative analysis of superoxide scavenging activity of peptide(s) isolated from S1 and Dudhia leaves at three maturity stages. Results are represented as mean ± SEM, n = 3. Values with different letters (a-e) are significantly ($P < 0.05$) different from each other by Duncan's multiple range test (DMRT).

HPLC and peptide(s) sequencing

Based on the retention time, isolated heterogeneous oligopeptides from different maturity stages of leaves exhibited different pattern in High Performance Liquid Chromatographic (HPLC) profile. Figure 10 (a and b) showed the HPLC chromatogram of Py from S1 and Dudhia respectively. As Pm and Ps of S1 showed better response in silkworm feeding and the same of Dudhia counterparts does not having remarkable effects on silkworm rearing, the comparative profile of these two peptides isolated

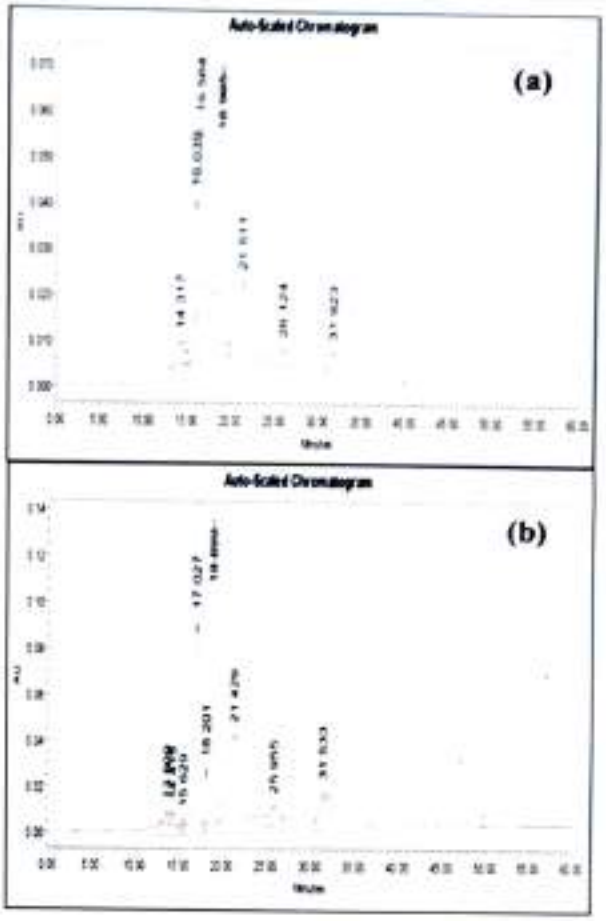
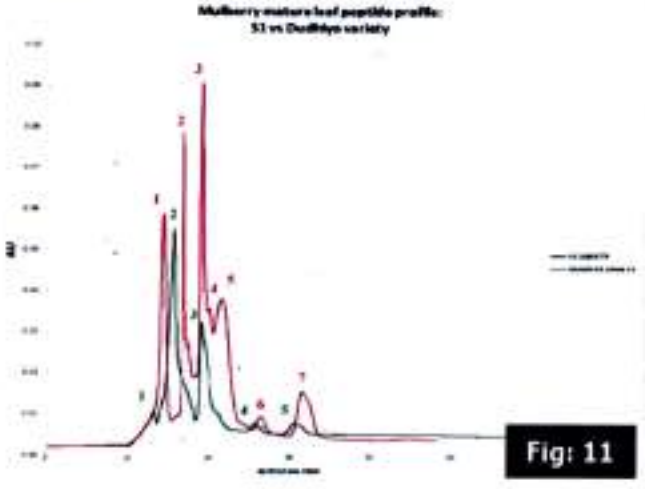


Figure 10: HPLC generated auto-scaled chromatogram of peptide(s) isolated from (a) S1 and (b) Dudhia young leaves.



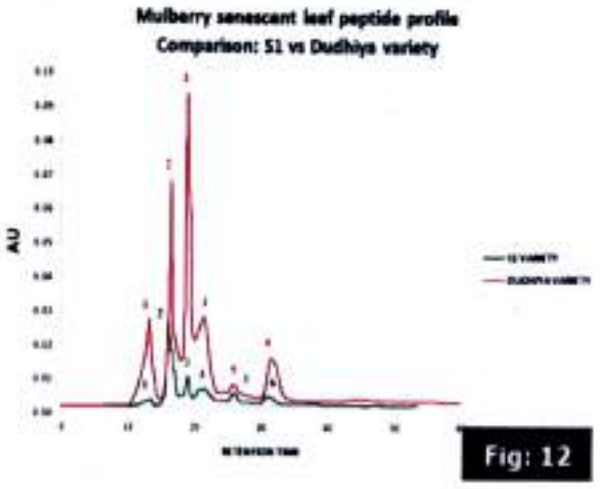


Figure 11: Comparative analysis of HPLC generated auto-scaled chromatogram of peptide(s) isolated from S1 and Dudhia mature leaves. Figure 12: Comparative analysis of HPLC generated auto-scaled chromatogram of peptide(s) isolated from S1 and Dudhia senescence leaves.

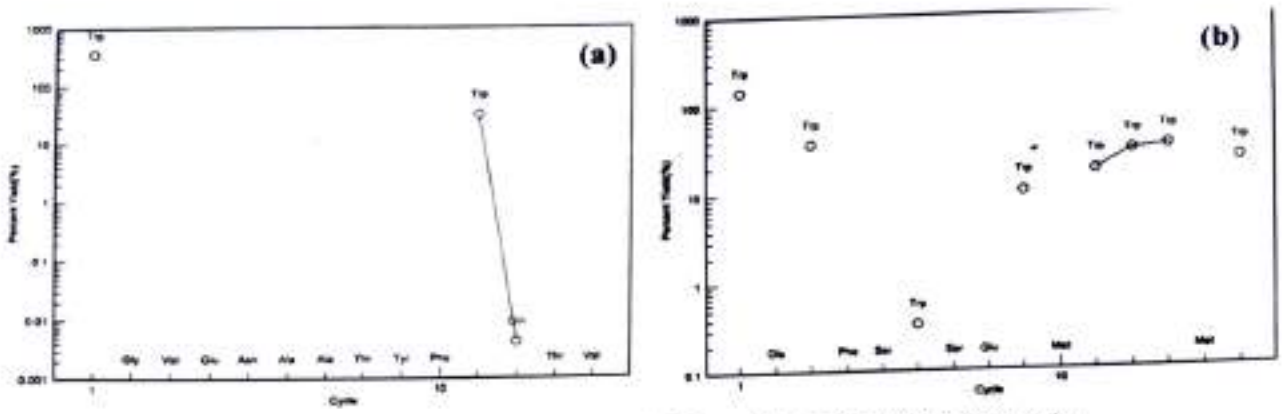


Figure 13: (a) and (b) shows the % of repetitive amino acid of S1 peptides and Dudhia respectively.

from S1 and Dudhia (Figure 11 and 12 respectively) were evaluated for determining the changes of HPLC peaks during maturation and senescence of mulberry leaves. At the time of maturation, non-overlapping peak 2 of HPLC of Dudhia genotype might be responsible for alteration of bioactivity, whereas during senescence, peptides isolated from Dudhia genotype comprising greater abundance might contribute inhibitory function as decoded from HPLC chromatogram.

The sequence of S1 mature peptides includes 14 amino acids which are "WGVENAATYFWQTV" with 100% reliability observed after 4th cycle of analysis in Try-His-Lys-Ala- followed by Ala-Try-Glu-Gly and Ala- Try-Pro-Asp as well as Try, Asp, Lys and Gly. On the other hand Dudhia mature peptides had 15 amino acids namely Trp-Glu-Trp-Phe-Ser-Trp-Ser-Glu-Trp-Met-Trp-Trp-Trp-Met-Trp with 87.7% reliability after 4th cycle. Figure 13 (a and b) shows the pattern of repetitive amino acids present in S1 and Dudhia mature peptides. From Figure 13 it was noted that Dudhia peptides contain repetitive units of tryptophan.

Conclusion

From our study it might be concluded that low molecular weight peptides especially at 0.5-3 k Da ranges could function as beneficial supplementary nutraceutical with mulberry leaves for silkworm rearing and could significantly improve economical attributes of rearing system. While Dudhia was refused by silkworm larvae, low molecular peptides isolated from mature leaves of Dudhia germplasm could influence larval growth. But the quantum of improvement of larval growth and economical attributes was comparatively lower than the bioactivity of S1 peptides. From this observation it can be stated that farmers would be potentially benefited if they use mulberry leaves treated with antioxidant enriched peptides as a food for silkworm rearing. However, more investigations will be required in this field for determining the exact mechanism of action.

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