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Influence of antioxidant rich mulberry peptides on the growth rate pattern and economic attributes of silkworm

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ABSTRACT

Bombyx mori L. is a monophagous insect and it totally depends on the mulberry leaves at their larval stage. It was recognized that dietary proteins had an effect on silkworm growth and silk production. But until now, no work was achieved on the role of the peptide(s) isolated from mulberry leaves on silkworm rearing. In present work an effort was made to investigate the role of low molecular weight (LMW, 0.5-3 KDa) peptide(s) on silkworm rearing. Role of antioxidant activities of isolated peptide(s) on silkworm rearing system also was evaluated. For that purpose, mulberry leaves (S1 cultivar) was introduced in different peptide(s) solution and fed by silkworm larvae (Nistari variety). Larval growth rate at 5th instar stage was found effective under the influence of peptide(s). In peptide(s) treatment consumption rate of the larvae was significantly high over control. Economical parameters like shell weight and shell ratio was enhanced by peptide treatment than control. Apart from this, LMW peptide(s) exhibited high scavenging activity than high molecular weight (HMW, 3-10 KDa) peptide(s). HPLC analysis of bioactive peptide fraction was performed and the peptide with maximum abundance was sequenced. The findings from our study may perhaps assist the sericulture industry for enhancing silk productivity.

Keywords: *Bombyx mori (L.), HPLC, DPPH, Nitric oxide, Low molecular weight peptide.*

INTRODUCTION

The physiology of *Bombyx mori* (L.), a monophagous lepidopteran insect has been studied comprehensively due to economically valuable silk production. Optimal nutrient and favorable physical features are required for growth and silk production. Mulberry leaves is a traditional food for silkworm larvae as a source of nutrition. Mulberry leaves contain several chemicals like 80% water, 27% proteins, 11% carbohydrates, vitamins and different minerals[1]. Recently many works were completed on the supplemented diet of mulberry leaves for silkworm. Several reports stated that protein acts as an essential ingredient in silkworm diet for their growth and silk production. Numerous studies have clearly described that soybean meal as a protein source in silkworm diet can increase the larval weight and fresh silk glands[2,3].

Since smaller proteins have also been considered as peptides, therefore it can be predicted that these peptides may also have significant effect on the growth and development of silkworm[4]. In present study, a scientific attempt was made to figure out the effect of peptide (s) of two different molecular ranges (0.5-3 kDa and 3-10 kDa) isolated from mulberry (S1 cultivar) leaves of different maturation stages on silkworm growth and silk production.

MATERIAL AND METHODS

Plant sample collection

Leaves of S1 cultivars of mulberry were collected from sericulture farm of Malda Town and Matigara, Siliguri, West Bengal, India at same season and same time. On the basis of biological and morphological attributes, maturity status e.g. young, mature and senescence leaves were categorized and weighed (1 kg each).

Isolation and purification of low molecular weight peptide(s)

Mulberry leaves of different maturation stages were surface sterilized, separately cryo-crushed and extracted with measured amount of chilled distilled water by blender. 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 and lipids. It was then passed through separate cation exchange (Dowex-50; 900 meq. in glass column 60 × 2.9 cm) and anion exchange resins (Dowex-2; 700 meq. in glass column 60 cm × 2.9 cm) for trapping amphoteric molecules like proteins, peptides and amino acids. Then concentrated aqueous acidic column eluents were washed four times with equal volume of peroxide free ether to remove traces of IAA, ABA, and GA. After discarding of anionic hormones, the extracts were filtered through Millipore ultra filtration system with Amicon filters 10 kDa (YM 10), 3 kDa (YM3) and 0.5 kDa (YC 05) cut off with 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 and Sequence analysis of peptide

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

Feeding trail

Experimental insect and rearing method

Nistari silkworm variety was reared in the laboratory through the well established methods[5]. The fifth instar larvae were utilized for the treatment under standard temperature (27˚-29˚ C) and optimum humidity (70±5%). Healthy, fresh S1 mulberry leaves were collected from the nearest sericulture farm and stored cool to maintain its freshness and used for larval feeding.

Prior to the initiation of silkworm rearing the rearing room, plastic tray, and other materials used for rearing was carried out as preventive measure against pathogens. In a plastic tray, rearing of ten caterpillars was conducted by feeding with S1 mulberry cultivars as a control treatment. In parallel ways six different sets were established for different peptide(s) treatment. All trays were placed under adequate ventilation. At rearing time, disinfection of the room was strictly maintained. Hands were sterilized with antiseptic solution before handling the worms. The grass of larvae was continuously discarded from the tray. If any larvae were died, dead larvae were removed immediately during the rearing period.

Leaf treatment

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 20 times diluted by distilled water. Leaves were soaked in peptide(s) for 30 minutes before feeding them to the larvae and air-dried for 15 minutes and given to silkworm. Six separate groups, with 10 larvae were kept and fed with different peptide(s) treated leaves in separate plastic tray.

Data collection

The weight of larvae in each tray was monitored by weighing them on weighing balance daily and the growth rate pattern of caterpillar was calculated. When larvae started spinning they were left uninterrupted for four to five days to form the cocoon. After complete cocoon formation, the weight of cocoon of each set was measured.

Cocoon shell weight was also measured after release of the moth from cocoon shell. Growth index, shell ratio, effective rearing rate % (ERR%) were calculated by formulae (given below). The collected data was subjected for graphical and statistical analysis[6].

Shell ratio (%) = (Single shell weight / Single cocoon weight) × 100

ERR % = (Total no. of cocoons harvested / Total no. of larvae brushed) × 100

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

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

Determination of Antioxidant activity of isolated peptide (s)

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[7]. In this assay ascorbic acid was used as a standard compounds. The absorbance was measured at 517 nm.

$ABTS^+$ scavenging activity

The spectro-photometric analysis of $ABTS^+$ radical cation(s) scavenging activity was determined according to Re *et al.* method with some modifications[8]. 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.

Reducing power

The assay was performed according to the method of Oyaizu with some modifications[9]. 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[10]. 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% napthylethylenediamine 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.* with slight modification[11]. The reaction mixture contained 1 mL of NBT solution, 1 mL of NADH solution and 1mLof methanolic extract of different concentration. 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.

RESULTS

Peptide(s) effects on silkworm rearing

The development of silkworm larvae depends on the essential nutrients in exact ratio in silkworm food which become easily digested and assimilated in the body and helps in silk synthesis[12]. In our present work growth rate of 5th instar larval was noted under the influence of S1 peptide(s). Consumption rate of the larvae under peptide(s) treatment was recognized at 24 hrs interval which increased significantly over control. In both range of peptide(s) treatment and as well as in control set, highest larval growth rate was observed during 96 hrs.

From our observation, it was clearly noted that from 24h to 72h, larval weights was gradually increased in each treatment set and control. But after 72h to 96h, it was slightly decreased. Highest growth rate was found in case of Py^{3-10} followed by Pm^{3-10}, $Py^{0.5-3}$, $Pm^{0.5-3}$, Ps^{3-10} and $Ps^{0.5-3}$. So, it is clear that the peptide(s) had an effect on silkworm growth.

Weight of cocoon was highly influenced by feeding the silkworm with mulberry peptide(s). Weight of single cocoon (g) and single cocoon shell weight was higher in $P_y{}^{3-0.5}$ than other peptide treatment and also over control (**Table 1).**

Table 1: effect of peptides on various attributes of silkworm rearing system

Treatment	Growth rate	ERR%	Weight of single cocoon (g)	Weight of single shell (g)	Shell ratio (%)
Control	612.24±0.02^{g}	50.00	0.68±0.57^{a}	0.12±0.07^{a}	17.65
$Py^{0.5-3}$	692.48±0.025^{c}	80.00	0.84±0.57^{a}	0.19±0.07^{a}	22.62
$Pm^{0.5-3}$	673.08±0.019^{d}	70.00	0.80±0.57^{a}	0.15±0.07^{a}	18.75
$Ps^{0.5-3}$	645.67±0.026^{f}	50.00	0.79±0.35^{a}	0.13±0.07^{a}	16.46
Py^{3-10}	762.72±0.035^{a}	60.00	0.78±0.57^{a}	0.17±0.07^{a}	21.79
Pm^{3-10}	732.62±0.02^{b}	50.00	0.76±0.71^{a}	0.14±0.11^{a}	18.42
Ps^{3-10}	666.82±0.02^{e}	40.00	0.71±0.78^{a}	0.12±0.14^{a}	16.90

Results are represented as Mean ± SEM, n=3. Values with different letters (a, b, c, d, e & f) are significantly ($p < 0.05$) different from each other by Duncan's Multiple Range Test (DMRT). Py, Pm and Ps represents Mulberry peptides isolated from young, mature and senescent leaves respectively. 0.5-3 and 3-10 indicates the molecular weight ranges of peptides between 0.5-3 kDa and 3-10 kDa respectively.

Table 2: expressed correlation in between scavenging potency of different free radicals by peptide(s) over different parameters of silkworm rearing system

	Growth rate	ERR %	Weight of single cocoon	Weight of single shell	Shell ratio (%)
DPPH	-0.105	-0.697	-0.755	-0.674	-0.583
$ABTS^{+}$	-0.182	-0.372	-0.249	-0.482	-0.527
Nitric oxide	0.119	-0.697	-0.824*	-0.440	-0.246
Superoxide	-0.498	-0.852*	-0.779	-0.813*	-0.753
Reducing power	0.429	0.947**	0.801	0.956**	0.914*
Metal chelating	0.021	0.173	0.147	-0.128	-0.197

*correlation is significant at the 0.05 level (2-tailed)

**correlation is significant at the 0.01 level (2-tailed)

Different commercial characters like Shell Ratio (%), ERR % (Effective Rearing Rate) was calculated from cocoon weight. Our results (**Table 1**) stated that a significant difference in the shell ratio (%) was achieved with P_y peptide(s) application followed by P_m and P_S in both molecular weight range over control.

Scavenging activity of S1 peptide

Antioxidants are the agents that are competent of successfully neutralizing the injurious effect of free radicals. Different antioxidant activity of peptide(s) at their both molecular ranges is shown in **Table: 3.** High scavenging activity was found in case of $P_y{}^{0.5-3}$ followed by $P_m{}^{0.5-3}$, $P_s{}^{0.5-3}$ in $ABTS^{+}$, DPPH, super

oxide scavenging assay and in reducing power activity. Our experiments revealed that P_m had shown higher scavenging activity rather than P_y and P_s in case of nitric oxide scavenging assay only. The IC_{50} values of $P_m^{0.5\text{-}3}$ and $P_m^{3\text{-}10}$ were 5.11 mg/mL and 414.00 mg/mL respectively. The results signify that smaller size peptide(s) exhibited better scavenging activity in all assays than high molecular weight fractions i.e. the antioxidant activity of peptides were acted according to molecular weight dependent manner. In case of metal chelating activity, only $Py^{0.5\text{-}3}$ exhibited better scavenging activity than $Py^{3\text{-}10}$. But in case of Pm and Ps, HMW peptides showed greater activity than LMW.

Table 3: antioxidant activities of isolated peptide(s) at two different molecular weight ranges

Sample	DPPH	ABTS	Nitric oxide	Super oxide	Reducing power	Metal chelating
$Py^{0.5\text{-}3}$	570.21±9.51b	131.01±4.66b	63.08±6.38b	101.87±3.54a	317.74 ± 3.07a	950 ± 0.41b
$Pm^{0.5\text{-}3}$	698.75±1.92c	147.55±1.30c	5.11 ± 1.86a	113.06±1.95b	258.99±29.01b	4266 ± 0.59f
$Ps^{0.5\text{-}3}$	783.63±3.05d	191.52±8.10e	453.41±6.62d	311.68±1.43e	147.85 ± 8.1d	1866.41±0.18d
$Py^{3\text{-}10}$	261.88±13.95a	14.25 ± 3.37a	1312 ± 3.32e	151.35±6.87c	258.99±29.01b	1622 ± 0.94c
$Pm^{3\text{-}10}$	2181.71±2.72f	345.13±4.60d	414 ± 6.41c	172.25±6.51d	199.6 ± 27.49c	3050.83±0.39e
$Ps^{3\text{-}10}$	2123.75±2.52e	165.8 ± 6.23f	2061.59±0.71f	423.13±0.85f	131.06 ± 4.46d	825 ± 0.13a

Results are represented as Mean ± SEM, n=3. Values with different letters (a, b, c, d, e & f) are significantly ($p < 0.05$) different from each other by Duncan's Multiple Range Test (DMRT). Py, Pm and Ps represents Mulberry peptides isolated from young, mature and senescent leaves respectively. 0.5-3 and 3-10 indicates the molecular weight ranges of peptides between 0.5-3 kDa and 3-10 kDa respectively.

HPLC and Peptide sequencing

The HPLC chromatogram profile of derived peptide(s) extract from different maturity status of mulberry leaf clearly exhibited the different segmental appearance according to desired molecular weight separation (**Figure 1, 2** and **3**). However, this separation based on the retention time also helped us to purify the exact peptide(s) fractions and concentrated it for repeated cycles.

Fig. 1: HPLC generated auto-scaled chromatogram of S1 young leaves' peptide(s)

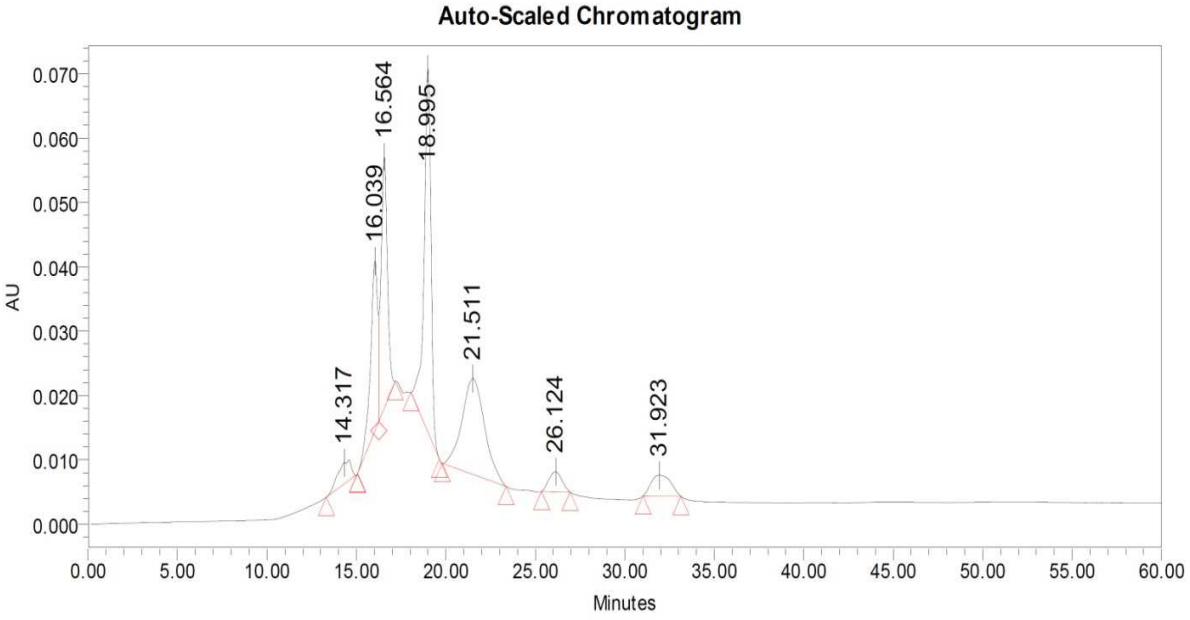


Fig. 2: HPLC generated auto-scaled chromatogram of S1 mature leaves' peptide(s)

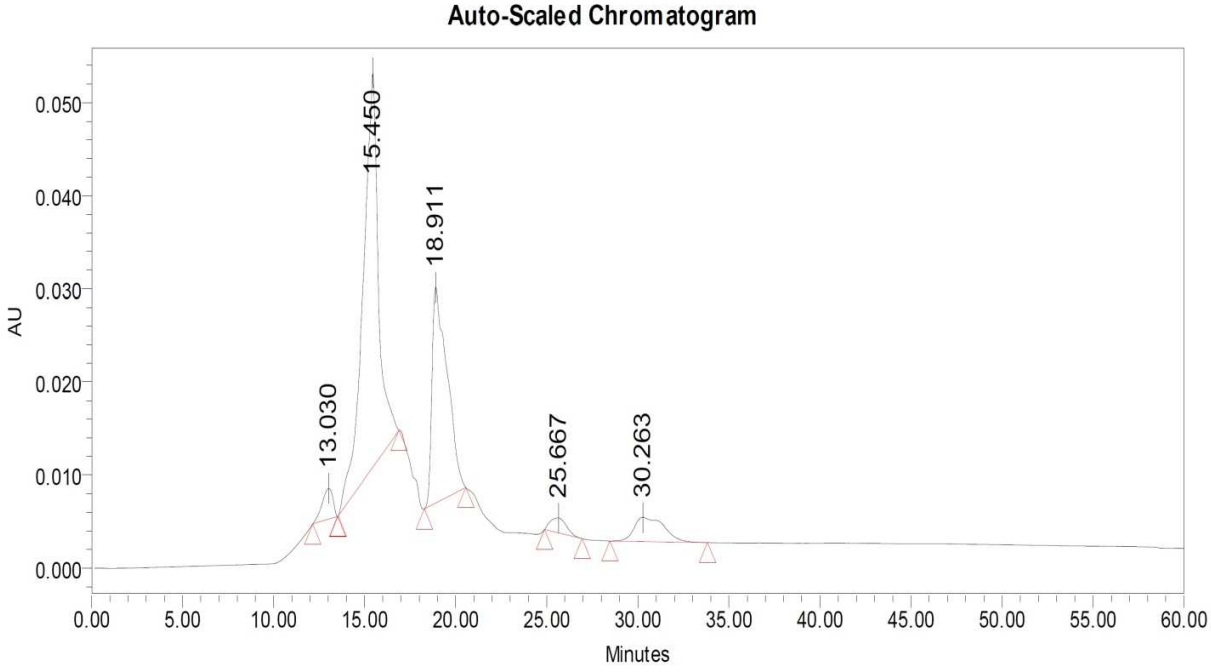
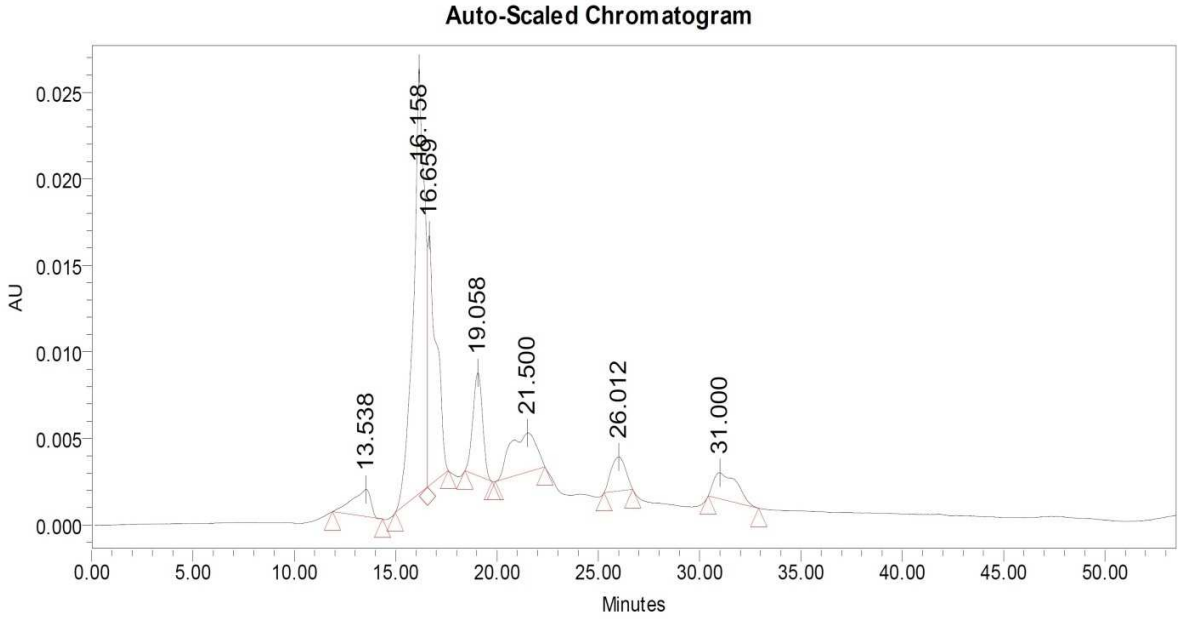


Fig. 3: HPLC generated auto-scaled chromatogram of S1 senescence leaves' peptide(s)



HPLC analysis of most effective bioactive peptide fraction at 16 minutes retention time was selected for amino acid sequencing. The sequence includes 14 amino acid 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. Psi-Blast analysis from the protein database (NCBI) of isolated peptide sequences showed partial identity especially with developmental proteins of *Bombyx mori* and other plant species like *Lycopersicon esculentum*, *Fragaria vesca* etc as demonstrated in **Table 4**. The S1 peptide fraction exhibited sequence similarity with the Cathepsin-B precursor and Bombyxin-B8 precursor, both obtained from *B. mori* and in case of Cathepsin B the sequence similarity was identical at 64% with an appearance bit source of 22.3 and a possible gap in amino acid was 9%. Bombyxin B precursor on the other hand showed 83% identical sequences.

Table 4: isolated peptide sequence similarity with other peptide or protein sequence

S.No	Name of the Protein	Source	Sequence similarity	Score	Identity	Positive	Gap	Expected function
1.	Cathepsin B precursor	*Bombyx mori*	WGVENAATYFW WGVEN-------Y-W WGVENDNKY-W	22.3 bits (45)	7/11 (64%)	7/11 (63%)	1/11 (9%)	Involved in the bulk degradation of intracellular and endocytosed proteins
2.	Bombyxin B-8 precursor	*Bombyx mori*	ATYFWQ A-YFWQ APYFWQ	21.0 bits (42)	5/6 (83%)	5/6 (83%)	0/6 (0%)	Helps in insect molting and metamorphosis. It plays important roles in sugar metabolisms, ovarian development and growth of insects
3.	Ribose-phosphate pyrophosphokinase-3	*Solanum tuberosum*	ENAATYFW----------QTV EN----TYFW----------QTV ENGMTYFWITDSCPQTV	26.5 bits (55)	9/17 (53%)	9/17 (52%)	6/17 (35%)	Involved in the synthesis of purine and pyrimidine, Co-factor likes NAD, NADP and amino acid *His* and *Try*
4.	Ribose-phosphate pyrophosphokinase-3	*Lycopersicon esculentum*	ENAATYFW----------QTV EN-----TYFW----------QTV ENGMTYFWITDSCPQTV	26.5 bits (55)	9/17 (53%)	9/17(52 %)	6/17 (35%)	Involved in the synthesis of purine and pyrimidine, Co-factor likes NAD, NADP and amino acid *His* and *Try*
5.	Methyltransferase-like protein	*Fragaria vesca* subsp. *vesca*	WGVENAATYFW WGV----------AT--FW WGVTHVATFFW	26.5 bits (55)	7/11 (64%)	8/11 (72%)	0/11 (0%)	Has a regulatory role in protein-protein interaction, protein-DNA interaction and protein activation

DISCUSSION

Peptide affects silkworm rearing

In our work, larval weight was gradually increased from one to three days in each peptide treatment which was already observed by Rahmathulla with folic acid administration where growth of larvae was increased by folic acid from 24 hours onward[13]. Nirwani and Kaliwal performed experiments on the effect of different vitamin supplementation and they found that larval weight was increased with different vitamin treated mulberry leaves[14]. Sridhar *et al.* got significantly higher larval weight when they fed mulberry leaves supplemented with soybean flour[15]. Cocoon weight is an economic character which was effectively improved by feeding the larvae with amino acids treated mulberry leaves[15]. Protein supplemented mulberry leaves have significant effect on larval growth and different economical parameters of silkworm[16]. Different nutrient formulation alters the cost benefit ratio in sericulture directly or indirectly. In the present work, peptide(s) isolated from mulberry leaves have a beneficial effect on the silkworm rearing system. LMW peptide(s) supplemented with mulberry leaves have a significant role on larval growth, cocoon weight, and ultimately silk production.

Antioxidant activity

In our recent work LMW peptide(s) showed better response in scavenging of DPPH, $ABTS^{+}$ and superoxide free radicals than HMW. Li *et al.* explained that LMW peptide(s) from chickpea protein hydrolysates exhibited strong superoxide radical scavenging activity[17].

In our study reducing power activity of LMW peptide(s) also appeared better. In contrast, Girgih *et al.* reported that the reducing power activity was improved with the increase in molecular weight of peptide(s) in hemp seed protein[18].

Biological activity of peptide depends on their amino acid composition[19]. Peptide containing aromatic amino acid (*Try* and *Phe*) had strong antioxidant activity[20]. Some amino acids in presence of their aromatic side chain like *Trp* and *Tyr* (indolic and phenolic group respectively) acts as strong free radical scavenger[21]. Saiga *et al.* stated that the presence of hydrophobic amino acids like *Phe, Ala*, in peptide shows higher free radical scavenging activity[22]. Our noble peptide contains *Phe* an aromatic amino acid, *Tyr* a heavy amino acid, and also hydrophobic (*Phe, Ala*) amino acid which are all responsible for high antioxidant activity of peptide. Thus, it can be stated that the existence of specific amino acids in peptides are responsible for enhanced antioxidant activity.

Antioxidant has a crucial role on larval growth and silk production[4]. Present study showed correlation between antioxidant activity with ERR%, weight of single cocoon, single shell and also with shell ratio (**Table: 2**). Cocoon shell weight depends on the weight of raw silk. Antioxidant acts as reducing agents for free radical ions presents in the silkworm larval body. It might happen that larvae could produce more silk proteins if the oxidative stress could be mitigated through antioxidant rich peptides. Economic attributes like cocoon weight and single shell weight might be increased with the elicitation of free-radical scavenging properties in mulberry leaves by absorbing these peptides. Other possibility may be that the antioxidants directly or indirectly affect silk protein synthesis in the larval body and subsequently increase shell weight.

CONCLUSION

Therefore, from our study it might be concluded that the farmers will be benefited by using mulberry leaves elicited with antioxidant enriched peptides as a food for silkworm rearing, as more silk production may possibly be expected by supplementing those nutrients with mulberry leaves. However, more investigation will be required on this field for clarification of the above statement.

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Abbreviations: LMW **(**Low Molecular Weight); HMW (High Molecular Weight); ERR (Effective Rearing Rate); $Py^{0.5\text{-}3}$, $Pm^{0.5\text{-}3}$ and $Ps^{0.5\text{-}3}$ (Peptides isolated from young, mature and senescence leaves respectively in between 0.5-3 kDa molecular weight range); $Py^{3\text{-}10}$, $Pm^{3\text{-}10}$ and $Ps^{3\text{-}10}$ (Peptides isolated from young, mature and senescence leaves respectively in between 3-10 kDa range); IAA (indole acetic acid); ABA (abscisic acid); GA_3 (gibberellic acid); DPPH (2,2-diphenyl-1-picrylhydrazyl); $ABTS^+$ (2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt); NBT (Nitroblue tetrazolium chloride); PMSF (Phenylmethylsulfonyl fluoride).

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