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Protein Banding Pattern and Major Amino Acid Component in De-Oiled Pupal Powder of Silkworm, *Bombyx mori* Linn.

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Abstract: Quantitatively *Bombyx mori* pupal powder contains significantly higher amount of protein in female as compared to male in both the hybrids of PM × CSR2 and CSR2 × CSR4 studied. On an average, in the former hybrid 14.81% and in the later hybrid 14.58% soluble protein was present as compared to total protein 60.81 and 63.66%, respectively. Qualitatively, no difference was found in amino acid content between the male and female of both hybrids. Five major amino acids found in PM × CSR2 were nor leucine, methionine, glutamic acid, hydroxyl proline, Aspartic acid and in CSR2 × CSR4 were isoleucine, valine, amino butyric acid, hydroxyl proline and leucine. SDS PAGE results reveled that there was no polymorphism between the sexes of two hybrids. Two polypeptides were identified in the range at 43 and 14.3 KD. Of the two polypeptides identified, the banding pattern of 43 KD was prominent in both the sexes of the two hybrids.

Key words: Amino acids, *Bombyx mori*, gel electrophoresis, protein, pupal powder

INTRODUCTION

The Silkworm, *Bombyx mori* L. is an economically important insect, which produces large quantity of silk. In silk reeling process, large quantity of waste accumulates in the form of pupae and left over unreelable silk, which could be utilized in a better way by producing value added products with the adoption of improved technology. By this, the cost of production of silk can be reduced either by standardization of processing methods or by utilizing the waste produced by the silk industry (Majumder, 1992). By-products of an industry are often brought to purposeful utilization to augment the profits (Sahay *et al*., 1997). This vital aspect so far seems to have not been taken proper care in sericulture industry. It is a well-known fact that pupae, which are obtained after reeling of silkworm cocoons are generally thrown away which is very rich in protein, oil, carbohydrate and minerals. The pupa, which is available abundantly in the reeling industry as a waste, can be utilized as a high potential raw material for various industries including pharmaceuticals. The oil extracted from the pupa is used in paints, varnishes soaps, candles, pharmaceuticals, bio-diesel and plastic industries (Basavanna *et al*., 1967; Datta *et al*., 1993; Chavan *et al*., 1999; Chaudhury, 2003). Sarker and Quader (1990) have studied extractability and properties of pupal oil. Pupal powder is generally used as feed for fowl, fish and swine as it is rich in protein (Nagaraj and Basavanna, 1969; Shiva Prakash, 1988; Mathur *et al*., 1988; Saratchandra, 1988; Bose *et al*., 1993). In fact the protein of pupae is better than the protein of soyabean, fish or beef. Silkworm pupae is a nutritious food for human diet as it contains a balanced amount of moisture, chitin, water soluble proteins, carbohydrates, amino acids and vitamin

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C besides crude protein as the major constituent (Majumder, 1992; Majumder *et al*., 1994; Ashok Kumar *et al*., 2000). Pupae protein has a well-balanced amino acid composition. Silkworm meal made of silkworm/pupa has been used in feeding monogastric and ruminant species for many years in Asian countries (Lakshminarayana and Thirumalarao, 1971; Majumder *et al*., 1994; Roychoudhury and Joshi, 1995). The protein is one of the important macromolecule found in all living cells, which is nothing but polymer of different amino acids. There are about 20 amino acids, which make these proteins. These amino acids are organic, amphoteric molecules having an amino group at one end and a carboxylic group at the other end. It was thought worthwhile to obtain few byproducts from the pupae and find out the economic utilization of these by-products for commercial exploitation. Therefore, the present investigation was taken up to study the protein, amino acid contents and protein quality in the de-oiled pupal powder of commercially available hybrids.

MATERIALS AND METHODS

Two hybrids viz., PM × CSR2 (multivoltine × bivoltine) and CSR2×CSR4 (bivoltine × bivoltine) were reared following standard procedure of Rajan and Himantharaj (2005). Both male and female pupae were separated and weighed separately. The fresh pupae were dried in hot air drier at 50°C for 4 days. The dried pupae were ground in mortar and pestle. Oil was removed by solvent soxhlet extraction process using chloroform: methanol = 2:1 (v/v) (Shreekantaswamy and Siddalingaiah, 1980). Total protein (Lowry *et al*., 1951) and total nitrogen content (Kjeldhal method) (Wilson and Walker, 2000) of the de-oiled pupal powder were estimated.

Qualitative Analysis

To analyze the banding pattern of de-oiled pupal powder, the standard procedure for SDS Polyacrylamide gel of Laemmli (1970) was followed. In the present study 10% separating and 4% stacking gel was used. The samples were taken out of -80°C and allowed to thaw on ice. Five gram of de-oiled protein powder was dissolved in tris buffer of pH 7. The grounded mixture was centrifuged for 15 min at 8000 rpm. The supernatant was used for further analysis of protein. The samples were mixed with PBS (Phosphate buffer solution) and sample buffer, boiled for 4 min at 96°C. The protein marker of 3.5-205 KD was used as standard (GENEI Industries, Inc., Saginaw, MI 48601).

Thin Layer Chromatography

All standard amino acid samples (Amino acid reference collection kit, S.D. Fine Chem. Ltd., India) were dissolved in citrate buffer and water (100 μg mL^{-1}) depending upon their solubility. Four gram of de-oiled pupal power was added to 50 mL of 6N HCl and kept in an oven at 75-80°C for 12 h or 36-40°C for 36 h to hydrolyze the pupal protein. The hydrolyzed samples were centrifuged at 10,000 rpm for 15 min, filtered through Whatman No. 1 filter paper and the residue was mixed with known amount of water, centrifuged again at 10,000 rpm. This procedure was repeated for 3-4 times until supernatant gives negative Ninhydrin test. All the supernatants were collected and concentrated to remove HCl. The absence of HCl solution in the supernatant was confirmed by pH paper test. Later 4 g of activated charcoal powder was added to each sample to remove undesired colour and the sample was kept in refrigerator for over night and filtered. The process was repeated to obtain the colourless solution. Amino acids extracted from silkworm pupae are in yellowish powder form. Standard TLC procedure was followed (Palanivelu, 2004) to identify the major amino acid content. Sample and standard were run on the plates coated with silica G and mobile phase used was n butyl alcohol, acetic acid and water in the ratio of 8:2:2. After developing, plates were sprayed with 0.3% ninhydrin solution and retention factor (Rf) values were calculated.

RESULTS AND DISCUSSION

Estimation of Oil, Crude-Protein and Others

23.6 and 37.8 g de-oiled pupal powder each was obtained from 100 g fresh male and female pupae in PM × CSR2 whereas 21.5 and 36.5 g in male and female pupae in CSR2 × CSR4 (Table 1). Statistical analysis (CD at 5%) showed there is significant difference in de-oiled pupa powder recovery between the male and female pupae of both the hybrids. Female pupae yielded more pupa powder than male in both the hybrids. Similarly oil and crude protein with other constituents were estimated as 40.02 and 59.98% in male pupae and 25.17 and 74.67% in female pupae of PM × CSR2, whereas in CSR2 × CSR4 these were 40.00 and 60.00% in male and 24.0 and 76.00% in female, respectively. It was found that in pupa powder oil is more in male than female of both the hybrids, whereas, crude protein was significantly more in female of both the hybrids as compared to male. Choudhury and Kumar (1979) reported that *Antheraea mylitta* pupae contain 20-25% oil. The oil content in Eri Silkworm pupae was estimated to be in the range of 18-20% (dry basis) by Shanker *et al*. (2006). Mishra *et al*. (2003) found the proximate composition (%) of total protein ranges between 12 to 16%, total fat between 11 to 20%, carbohydrate between 1.2 to 1.8%, moisture between 65 to 70% and ash between 0.8 to 1.4% for non-mulberry and mulberry silkworm pupae.

Quantification of Protein

The results of the present study showed 9.08% nitrogen in male pupa and 10.38% in female pupa of PM × CSR2. Similarly, 9.85% nitrogen in male pupa and 10.52 % in female pupa of CSR2 × CSR4. In practice the nitrogen content of protein is generally assumed to be 16% by weight (Wilson and Walker, 2000). Therefore, for calculating protein percentage in tissue, nitrogen content is multiplied by the factor 6.25. It revealed that the pupae are rich source of protein. Average protein content in PM × CSR2 pupae is 60.80%. It was observed that male pupa contain 56.75% whereas female pupae contain 64.88% of total protein. Similarly the average protein content in CSR2 × CSR4 pupa is 63.66 %. The male pupae contain 61.56% and female pupae contain 65.76% of total protein (Table 2).

The biochemical analysis by Lowrys method also revealed that PM × CSR2 male pupae contained 141.6 mg g^{-1} and female pupae contain 154.60 mg g^{-1} of soluble protein (average 148.1 mg g^{-1}). Similarly in CSR2 × CSR4, male pupae contain 125.10 mg g^{-1} and female pupa contain

Table 1: Estimation of oil (%) and crude-protein and others in the pupae of two hybrids CSR2 × CSR4 and PM × CSR2

Hybrids	De-oiled pupa powder wt./fresh wt. (100 g)	Pupae oil % on dry wt basis	Crude protein and others % on dry wt basis
PM × CSR2♂♂	23.600	40.020	59.980
PM × CSR2♀♀	37.800	25.167	74.670
CSR2 × CSR4♂♂	21.500	40.000	60.000
CSR2 × CSR4♀♀	36.500	24.000	76.000
SE±	0.100	0.383	0.384
CD5%	0.200	1.327	1.328

Table 2: Estimation nitrogen and protein by Kjeldhal method in silkworm pupae, *Bombyx mori*

	Kjeldhal method					
	Male		Female		Lowry *et al*. (1951) methods	
Hybrids	% of nitrogen (A)	% of protein (A × 6.25)	% of nitrogen (A)	% of protein (A × 6.25)	Male (mg of soluble protein/ gm of dry wt.)	Female (mg of soluble protein/ gm of dry wt.)
PM × CSR2	9.08	56.75	10.38	64.88	141.60	154.60
SD±	0.01	0.06	0.00	0.00	0.10	0.56
CSR2 ×CSR4	9.85	61.56	10.52	65.76	125.10	166.47
SD±	0.01	0.06	0.01	0.08	0.10	0.15
T-test	-94.305**	-94.305**	-20.31	-20.306**	202.083**	-35.599**

**: Significant at 1% level

166.47 mg g^{-1} of soluble protein (average 145.78 mg g^{-1}). Data reveled that female pupae contains significantly higher amount of soluble protein compared to male pupae in both the hybrids (Table 2). Comparing the results, it is noted that, on an average 14.81 and 14.58% soluble protein was present in PM ×analysis is a Precise method for the determination of total nitrogen contains in the sample such as DNA, uric acid, enzymes and all the NO_2 and NO_3 etc. By Kjeldhal method, total protein present in the sample can be estimated through factor multiplication, Whereas by Lowry method, only soluble protein digested through 10% TCA can be determined. Other proteins insoluble in TCA could not be measured. Accordingly, there is difference in total protein content and soluble protein content. The result showed that female pupae contains significantly higher amount of protein as compared to male of both the hybrids. According to Rao (1994), spent silkworm pupae were analyzed for its nutrient composition and their protein quality was evaluated in weanling rats. Protein content of the pupae was found to be 48.7%. Defatted spent silkworm pupae meal contained 75.2% protein, which perhaps may be based on total nitrogen estimation. The pupae of *Antheraea mylitta* which contains 80% protein can be suitably utilized in baking industry for manufacturing protein rich biscuits (Agarwal *et al*., 1974). Bose and Majumder (1990) reported that the pupae powder contains 7.18, 29.57% fat, 48.98% protein (Kjeldhal method), 4.655% glycogen, 3.37% chitin, 2.19% ash and 3.7% vitamins etc. which shows that pupa is good source of protein and fat.

Qualitative Analysis

Each protein is unique and characterized by its amino acid composition and sequence. In any protein, many amino acids (usually more than hundred) are linked by peptide bonds to form polypeptide chain. The cleavage of peptide bonds is usually achieved by boiling the protein in 6N HCl, which hydrolyzes peptide bonds thereby releasing free amino acids. So in order to determine amino acid composition of a protein, it is necessary to break down the polypeptide chain into its constituent amino acids by hydrolysis (Geis, 1989). It was found that the nutritionally important amino acids were present in silkworm pupa of both the hybrids (PM × CSR2 and CSR2 × CSR4). Both essential and non-essential amino acids were identified by comparing their Rf values with standard amino acids. It was found that both male and female pupae of CSR2 × CSR4 contain five amino acids viz., nor leucine, methionine, glutamic acid, hydroxyl proline and aspartic acid. Whereas, in the pupae of both the sexes of PM × CSR2 contain iso-leucine, valine amino butyric acid, hydroxyl proline and leucine. There is no difference in amino acid content between the two sexes of the same hybrid whereas there is difference in amino acid content between the hybrids. Hydrolyzed pupae of silkworm were analyzed for amino acid content by Majumder *et al*. (1994) and found to contain lysine, leucine + isoleucine, valine + methionine, threonine, cystine, tyrosine, histidine, arginine, glutamic acid, glycine, serine, alanine, proline and cysteic acid. Amino acids extracted from silkworm pupa are in yellowish powder form. It is soluble in water with pH between 4 to 6.5 and positive to ninhydrin reaction. In the present study pupae hydrolysate contains all essential amino acids of group 1 (isoleucine, leucine, methionine and valine), group 2 (aspartic acid and glutamic acid) and semi essential amino acids (proline-could be produced in body) that are important for human beings and are getting only through food. These amino acids are necessary for human health. As per the quality standards of FAO/WHO, the nutritive value of silkworm pupae is superior to that of eggs and milk (Xiao, 1983). Its good solubility in water enables to produce transparent beverage. It has been widely used in health food industry, such as amino acid drink, granule, capsule, fruity beverage and kinds of good additives. It can also be used as raw material for the production of medicinal amino acids and single amino acid (www.alibaba.com/showroom/Amino_Acid_Powder.html). Methionine is the most important amino acid for the catalysis of the biotin enzyme (Shenoy *et al*., 1992), gamma-aminobutyric acid and glutamate which are neurotransmitters (Dodd *et al*., 1992). Rao (1994) reported that the essential

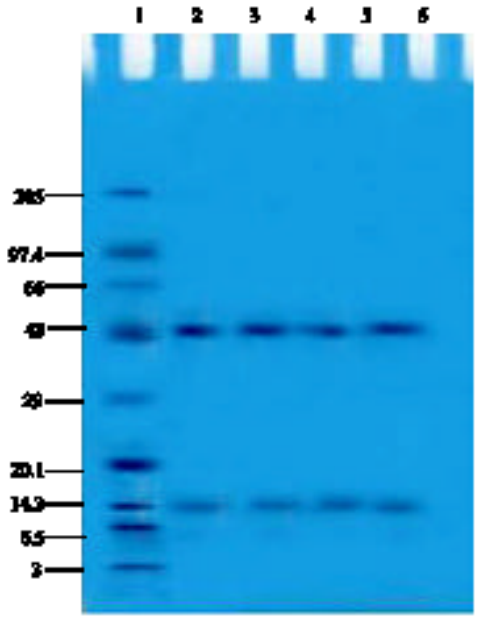

Fig. 1: Protein banding pattern of de-oiled pupae powder of silkworm *Bombyx mori*. 1. Maker, 2. PM × CSR2 ♂♂, 3. PM × CSR2 ♀♀, 4. CSR2 × CSR4 ♂♂, 5. CSR2 × CSR4 ♀♀ and 6. No sample

amino acid content of the pupal protein was similar to that of whole egg protein with the exception of tryptophan (0.9 16 g of N). Tryptophan is the limiting amino acid of pupal protein. The chemical score of the protein was found to be 60, as compared to 100 for whole egg protein.

The protein-banding pattern studied on SDS PAGE electrophoresis (Fig. 1) indicated that there was no difference in banding pattern of polypeptide between male and female de-oiled pupal protein. Thus the present study indicated the quantitative difference in pupal protein between the two sexes, which may help in identifying the functional significance of these proteins. Two bands were identified in the range of 43 and 14.3 KD. Of the two bands, the band with molecular weight of 43 KD was prominent in both the sexes. The electrophoretic separation of pupal proteins shows two prominent bands of similar molecular weight in male and female pupa of PM × CSR2 and CSR2 × CSR4. However, a significant band at 43 KD was more prominent in the female pupa suggesting that the quantity of this particular protein is relatively high. However, the electrophoretic separation of non-mulberry Eri silkworm pupal proteins showed six prominent bands of similar molecular weight in male and female and there was no difference in banding pattern between male and female. In both sexes six bright bands were observed in the range at 66, 45, 34, 24, 18 and 14.3 KD (Reddy *et al*., 2004), whereas, only two bands obtained in the present study may be due to the difference in species.

In brief, female pupae yielded more pupa powder than male in both the hybrids. Oil content was more in male pupae compared to female pupae, whereas the quantity of crude and soluble protein was significantly more in female pupae of both the hybrids compared to male pupae. There was no difference in protein banding pattern between the sexes and between the hybrids. However, there were differences in amino acids between PM × CSR2 and CSR2 × CSR4 whereas no difference was observed between the two sexes. In the same hybrid, qualitatively protein may be the same but there is difference in their quantities.

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