STUDIES ON THE ENHANCEMENT OF SILK PROTEINS OF SILKWORM (*Bombyx mori* L.) USING LOW-DOSE GAMMA IRRADIATION AND THEIR THERMOSTABILITY PROPERTIES

A Thesis submitted

By

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Examination Roll No. MS-070704
Session: 2006-2007

Submitted to

Biotechnology and Genetic Engineering Discipline, life science school, Khulna University, Khulna, in partial fulfillment of the requirements for the degree of 1year 'Master of science (M.Sc.) in Biotechnology and Genetic Engineering'

Biotechnology and Genetic Engineering Discipline,
Life Science School, Khulna University,
Khulna-9208, Bangladesh
November 2008.

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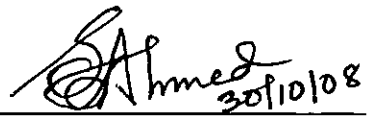

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Declaration

This thesis reports the original research work of the author, except as otherwise stated. The material presented has not been submitted either in whole or in part for a degree from this or any other university. The findings of the research has not been or will not be published anywhere without the concurrence of the concerned supervisor or co-supervisor.

Md. Rahul Amin

Student no: MS-070704
Session: 2006-2007
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Dedicated

To

My Beloved Parents

Acknowledgements

First of all, all praises be to the supreme of this universe, the Almighty Allah, who enabled me to undertake and complete this research work and finally write up the outcome research work leading towards the fulfillment of the degree of Master of Biotechnology and Genetic Engineering.

I wish to express my profound sense of gratitude to my most respected teacher, Professor Md. Raihan Ali, Head, Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh, for his inspiration, constant guide, valuable suggestions, sympathetic advice and unparalleled encouragement made throughout the course of study, without which this piece of work would not have taken its present shape.

It is my pleasure to express my greatest and profound gratitude, deep appreciation and heartfelt indebtedness with best regards to my honourable supervisor, Principal Scientific Officer, Insect Biotechnology Division, Institute of Food and Radiation Biology, Bangladesh Atomic Energy Commission, Savar, Dhaka, Bangladesh, for suggesting the topic of the research, active guidance, keen interest, continuous inspiration and constructive criticism for which this work comes to light.

I express my deepest gratitude to honorable sir, Md. Saidul Islam, Cheif Scientific Officer and Head, Insect Biotechnology Division, Institute of Food and Radiation Biology, Bangladesh Atomic Energy Commission, Savar, Dhaka. Bangladesh, for giving permission and extending full laboratory facilities, affectionate suggestion and guidance to perform this research work.

I am grateful to A. Y. K. M. Masud Rana, Scientific Officer, Insect Biotechnology Division, Institute of Food and Radiation Biology, Bangladesh Atomic Energy Commission, Savar, Dhaka. Bangladesh, for his active guidance, assistance and encouragement to perform the research work.

I am thankful to Md. Aftab Hossain Scientific Officer, Insect Biotechnology Division, Institute of Food and Radiation Biology, Bangladesh Atomic Energy Commission, Savar, Dhaka. Bangladesh, for constant assistance during preparing my thesis paper.

My cordial thanks and gratitude to Mr. Salam , Mr. Hamid, Mr. Zosim, Lab Assistant,, Insect Biotechnology Division, Institute of Food and Radiation Biology, Atomic Energy Commission, Savar, Dhaka, for their kind and friendly cooperation.

My special heartfelt thanks extend to all my friends, Imran, Rubel, Subrata, Modhu for their friendly cooperation and occasional help.

Finally, I would like to express my gratitude to my parents, brother and sister for their moral support, inspiration and encouragement throughout the work.

I also extend my gratitude towards those who helped me in one way or other during tenure of my study.

The Author

November.

ABSTRACT

This study was aimed to perceive whether low dose gamma irradiation could enhance the silk proteins of the silkworm, *Bombyx mori* and to understand their thermal stability properties. At initial stage of this study different extraction procedures of silk proteins were also tried to choose the best extraction procedures. The extraction of silk proteins from the silk gland was conducted in water and in sodium phosphate buffer at pH 7.4. Extraction of silk proteins in water was found to be suitable than the extraction in alkaline sodium phosphate buffer solution. Silk proteins became gelatinized very quickly when extracted in alkaline buffer solution. On the other hand, silk proteins extracted in water remained liquid for at least a week in cold (4°C). This study confirmed that low dose gamma irradiation (1.5 Gy) to embryonic stage could enhance the production of silk proteins. Comparing the proteins from the unirradiated silkworm, significant increase (48.97%) of silk proteins was obtained from the silk gland of irradiated silkworm. Results of thermal stability study showed that both Heavy (~350 kDa) and Light (~30 kDa) chain of fibroin protein were more stable than the sericin protein. Both the chains of fibroin protein could withstand at least 90°C for 10 minutes when extracted in water. But at 90°C, the H-chain of fibroin was found degraded when silk proteins were extracted in alkaline buffer solution. Silk fibroin (both H and L chain) was found quite stable up to 30 minutes at 90°C when extracted from the irradiated silkworm. But L-chain of the fibroin was lost completely at 90°C in 30 minutes. This study suggests the probable presence of hot spot in the genome of silkworm *B. mori* which is prone to be mutated at low dose gamma irradiation. The increased production of 48.97% silk proteins and the increased thermostability of the L-chain of fibroin from the irradiated silkworm larvae are probably the results of such mutation. This study also cautiously suggests the presence of a dimmer of H-chain of silk fibroin.

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Abbreviations Used in the Text

ASG	Anterior Silk Gland
MSG	Middle Silk Gland
PSG	Posterior Silk Gland
rpm	Rotation per minute
FCR	Folin-Ciocalteu phenol reagent
BSA	Bovine Serum Albumin
SDS	Sodium Dodecyl Sulfate
PAGE	Polyacrylamide Gel Electrophoresis
APS	Ammonium per sulfate
TEMED	N,N,N,N-tetremethylene-ethylenediamine
et al.,	*et alia* =and other people
etc.	*et cetra*= and the others
gm	Gram(s)
gm/l	Gram(s) per liter
i.e.	*id est* = that is
mg	Milligram(s)
mg/l	Milligram(s) per liter
ml	Milliliter(s)

nm	Nanometer(s)
µl	Micro liter
µg	Micro gram
Viz.	Videlicet = namely
Conc	Concentration (s)

Chapter One

Introduction

Chapter One

Introduction

Silk is the secretion from modified salivary glands by pupating larvae (caterpillar) of the insect families Lasiocampidae, Bombycidae, and Saturnidae. All the three families belong to insect order Lepidoptera. Silk from certain groups has commercial value .The economic importance of silk was first recorded in China in 2600 BC. Raw silk was one of the first commodities traded between China and Europe . The manufacture of silk was kept secret by Chinese for more than 2700 years from the out side world. The legendary history of silk dates back to 5000 years from now. The unraveling of silk from the cocoon discovered when a cocoon fell from a mulberry tree into the hot water, while princess Xi Ling Shi was preparing a cup of tea.

It is thought that silkworm eggs were taken to India in about 150 BC which started the Indian sericulture industry. Now a day, silk is cultivated commercially in almost parts of the world but the main production countries are China, Japan, Korea, Russia and India.

Bombyx Mori is the main species of moth used in commercial silk production. This moth is called as mulberry silkworm, oriental silkworm or just the silkworm. Other commercial species include: *Samia cynthia ricini*, *Antheraea assama*, *A. paphia*, *A. mylitta*, etc. Among these silk-producing insects, the mulberry silkworm (*B. mori*) is the most widely utilized, intensively cultured, and its rearing techniques are the most improved. *B. mori* is of Asian origin, and its larva produces the finest silk. It is widely raised in Bangladesh, particularly in the districts of Rajshahi and Nawabganj.

It is estimated that out of about 1 million tons (fresh weight) of cocoons produced world wide approximately 4, 00,000 tons of dry cocoon are generated, that have 50,000 tons of recoverable sericin (Gulrajani, 2005). Bangladesh produces about 2,8000 lbs (12,700 kg) of silk yarn annually.

Silk is the lustrous fiber secreted by the silkgland of the worm. Silk possesses a few unique properties: it is the finest animal fibre (diam.10-12 *u*m).The silkworm synthesized natural silk and spun in the form of a silk cocoon and actually synthesized in the silk gland. *B. mori* Silk gland is a typical exocrine gland secreting large amount of silk proteins. It is a paired organ consisting of modified salivary glands located at the two lateral sides under the alimentary canal. Each gland is tube like made of glandular epithelial cells surrounding the lumen. The cells of the gland are huge polyploid cells with extremely ramified nucleus containing numerous nucleoli. Nuclear ramification develops gradually as the larva grows and reaches conspicuous size in the 4th and 5th instars. Ramification considerably enlarges the nuclear surface and apparently facilitates the transfer of materials related to the silk synthesis between the nucleus and the cytoplasm. According to its morphology and function, the silk gland can be divided into three distinct regions. The posterior part, about 15 cm long and is composed of about 500 secretary cells, which synthesize silk fibroin. The middle silk gland of the lumen in which silk proteins are stored until spinning, is about 7 cm long and contains about 300 secretory cells producing silk sericin, the protein which cements the fibroin thread of the cocoon. The anterior part about 2 cm long is a thin duct composed of about 250 cells with no known secretory function. Fibroin is secreted in the Posterior and transferred by peristalsis to the Middle section, which acts as a reservoir. The majority of the sericin is created within the walls of the Middle section. In fact, these two proteins are reserved side by side in the Middle section without mixing one into the other (Akai *et al.*, 2005).

Raw silk consists of fibroin, about 65 per cent, sericin, about 22 per cent, moisture, about 11 per cent, with about 1 per cent of mineral and colouring substances. The sericin, an amorphous protein, is removed by boiling in soap solution; the process is known as "degumming." The silk thread has an inner core of 75-80% fibroin and an outer envelop of 20- 25% sericin. The silk, polymer is composed of a 350 kDa fibroin heavy chain (H-fibroin), a 25 kDa fibroin light chain (L-fibroin), and a family of proteins called sericins that bind the two threads together as they emerge from the glands and harden in contact with air.

Due to the commercial value of silkworm in textile business and in recent discoveries of the importance of sericin in many biotechnological applications including the drug delivery system demands more research in silkworm proteomics.

Silk has been widely studied in *Bombyx mori* for its protein composition and the configuration of its compound (Akai *et al.*, 1987). The silk fibers are composed of fibrous proteins, characterized by being water insoluble and quite strong. In Lepidoptera the secreted protein present a beta-sheet arrangement, with large amount of glycine residues (Ruadal, 1953; Flower and Kenchington, 1967; Lucas and Rudal, 1968). Although the composition of silk has been well studied, the degree of orientation and folding of their proteins and the level of its macromolecular organization are poorly understood.

Considerable amount of research on mutational analysis were conducted in early 20th century. In those studies X-ray and gamma ray were used (Katuski, 1925; Astaurov, 1925 and Ruiying *et al.*, 1985). Some authors reported that exposing the silkworm developmental stage to irradiation resulted derimental effect on fertility, fecundity and silk production (Park *et al.*,1968; Faruki and Kundu, 2005; Shamitha and Rao, 2006). Conversely, many reports also indicated that radiation could induce benifical effects to silkworm (Ruiying *et al.*, 1985; Sing *et al.*, 1990).

Every reports from this laboratory also indicated that when low dose gamma ray was applied at early embryonic developmental stage , an increase of silk production (corresponding increase of cocoon shell weight and filament length) could be achieved (Hossain *et al.*, 2005a and 2005b). Hossain *et al.*, 2005b reported an increase of cocoon shell weight of about 32.865 using 1.5 Gy gamma irradiation applied at early embryonic developmental stage. Though considerable amount of reports on irradiation effect to silkworm biology or silk production were published but virtually no reports of irradiation effect on major silk proteins like fibroin and sericin is available.

1.1 Objectives:

Therefore, this present research work was taken-

1. To unravel whether irradiation to silkworm eggs could induce more silk proteins and if so, which protein (s)?
2. To determine the thermal stability properties of silk proteins particularly fibroin and sericin.
3. Different extraction procedure of silk proteins from the 5^{th} instars larvae would be tried to choose the best extraction method.

In the results and discussion section of this thesis, analysis of extraction procedure, thermal stability of silk proteins and the effect of irradiation to silk proteins would be explained and discussed.

Chapter Two

Literature Review

Chapter Two

Literature Review

2.1 Effect of low dose irradiation on the quality of produced Silk:

In silkworm, studies on radiation were first carried out by Katsuki (1925) in the early days of radiation experiments. Since then a large number of works have been done with radiation on every aspect of silkworm, such as biology, physiology, pathology, genetics etc. The earlier work up to 1950 dealt mainly with the X-ray and ultraviolet ray. However, Astaurov (1925) used gamma ray for the first time for induced an artificial mutation in silkworm. During the last two decade the use of gamma ray to some extent is more extensive and quite a number of satisfactory results have been obtained with silkworm.

The application of irradiation technique to sericulture have begun in China since the late fifties (Ruiying *et al.*, 1985). They using gamma ray and electron beam on silkworm egg to produce genetic variation and to breed new varieties. They also use low dose gamma ray to promote growth and use electron beam to kill chrysalis to reduce silk loss. Using gamma ray quite a number of amazing result have been obtained with the silk worm (Rahman *et al.*,. 1984, Sutadis *et al.*, 1987, Meesilp *et al.*, 1987, Iusifov *et al.*, 1988, kotani *et al.*, 2002, Hoassain *et al.*, 2005a), have been described follows. Except gamma radiation on tasar silkworm, *Antheraea mylita* , made quite different result (Shamitha and Rao, 2006). They reported a clear mark of dent on filament, fragmentation and clear breakage were reported at 500 Rad (5 Gy) 700 Rad (7 Gy) and 1000 Rad (Gy) respectively.The authors also reported a dose dependent gradual decline of length compared to normal fibre.

Swelling behaviour of silk fibre due to X-ray scattering have been reported (Kawahara *et al.*, 1994). The authors also reported UV-irradiation and heat treatment made changes in the hue in raw silk fibre of *Antherea yamamai.*

Faruki and Kundu (2005) have reported the effect of UV-radiation on some commercially relevant traits of two multivoltine strains named Nistari-M and Urboshi-1 of silkworm. The authors applied UV-irradiation which resulted reduced weight of larvae, pupae and adults of both the strains of silkworm. The cocoon weight, shell weight and shell ratios were also reduced due to UV –irradiation. Increased larval mortality was also recorded at all the doses of UV rays.

Growth and other quantitative characters of silkworm are changed by gamma-irradiation depending upon dose rate, total dosage, developmental stage, temperature, moisture and other environmental conditions. Many authors reported benificial effect of low dose irradiation on silkworm at egg stage and larval stage(Khan *et al.*, 1990; Ruiying *et al.*, 1985; sing *et al.*,1990; Hossain *et al.*, 2005 a and 2005 b). Hatching rate of irradiated silkworm was reported to be increased by 2-4% compared to non-irradiated eggs (Ruiying *et al.*, 1985).

Mallik *et al.*, (1968) irradiated the larvae of mulberry silkworm(Nistari variety) at various larval stages namely third, fourth, fifth instar with a dose rate of 100-400 Rad. They reported irradiated larvae increased in weight. The author also reported that the cocoon weight of silkworm also increased by 10-15% when eggs were exposed to gamma ray. A dose of 100-200 Rad was reported to be beneficial.

Iusifov *et al.*, (1988) studied the effect of low dose gamma radiation on the eggs of mulberry silkworm(*Bombyx mori*). They reported that development might be stimulated by a single exposure of the egg to 2 Gy radiation. The acceleration of the larval growth promotes the increase in the cocoon weight and the raw- silk mass.

Hossain, *et al.*, (2006) also reported that the highest 32.86% increased cocoon shell weight at dose 1.5 Gy. About 22-55% (average 32.50%) more silk production reported from gamma irradiation silkworm egg(Ruiying, *et al.*, 1985). Meesilp *et al.*, (1987), Sutadis *et al.*, (1987) and Hossain *et al.*, (2005 b) reported that low dose gamma irradiation increased the cocoon yeild, shell ratio and cocoon percentage. The gamma radiation dose suggested 2.5 Gy (Sutadis, *et al.*, 1987), 2 Gy (lusifov *et al.*, 1988), and 1.5-2.5 Gy in case of Urboshi variety (Hossain *et al.*, 2005 a). They reported irradiated larvae showed an increase in cocoon shell weight compared to

unirradiated ones. The highest percentage (17.50%) of silk increased was recorded at 5 Gy in fourth instar larvae and the lowest wasd 0.62% at 6 Gy in the third instar larvae of silkworm The average highest shell weight (0.141 g) and filament length (527.09 metre) were also observed at 5 Gy in fourth instar larvae (Hossain *et al.*, 2005 b).

Park *et al.*, (1968) described an experiment of the effect gamma ray on the biology of the silkworm larvae. They irradiated the 1 day larvae of fifth instar at 4000R to 20000R and noted decrease the fertility and fecundity.

Babos (1962) irradiated silkworm pupae with gamma irradiation from 30000 rad to 400000 rad. All the spring pupae that received 300000 rad died within 10-12 days after their metamorphosis. Nine to eleven percent pupae were liquefied in the cocoons. The weight of the irradiated pupae was less then that of the controls.

Ariffov *et al.*, (1958) described the preservation of silk cocoon with the help of gamma ray treatment for the first time. From their result it was evident that irradiated cocoons could be preserved for longer time (104 days) than those treated with heat or vaccum. Moreover, gamma ray has a beneficial effect on the thread properties.

2.2 Silk Protein

Silk proteins are namely two types fibroin and sericin (Zurovec and Sehnal, 2004) and a third category protein named seroins (Boman, 1996) are synthesized from silk gland(SG). Silk gland is divided into the following three sub-organs (Fig-1): Posterior silk gland (PSG), Middle silk gland (MSG) and Anterior silk gland (ASG) (Yutaka and Eiichi 1970; Pingbo, 2005). The posterior section of the silk gland (PSG) generate heavy chain fibroin (H-fibroin), light chain fibroin (L-fibroin), and P25 glycoprotein (fibrohexamerin) (Couble, *et al.*, 1983; Tanaka, *et al.*, 1999; Michal, 1998).

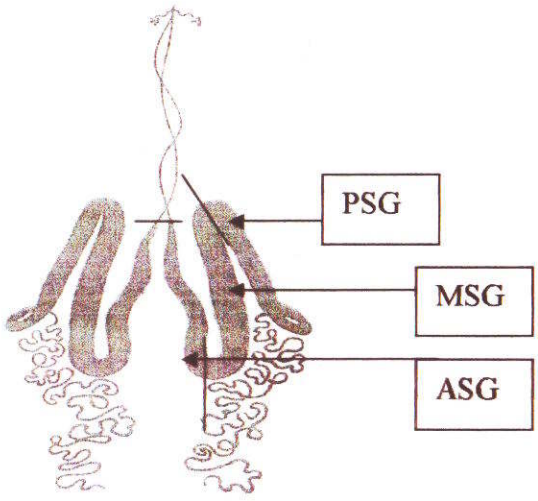


Fig.1. Schematic representation of silk gland of silkworm (after Mondal *et al.*, 2007). ASG = Anterior Silk Gland, MSG = Middle Silk Gland, PSG = Posterior Silk Gland.

Gulrajani (1988) also reported that the silk fiber is composed of two types of proteins viz., sericin and fibroin.Raw silk also contain other natural impurities namely, fat and waxes, inorganic salts and colouring mater .

Table-1: Composition of silk in Bombyx mori (Gulrajani, 1988).

Composition	Amount (%)
Fibroin	70-80
Sericin	20-30
Wax matter	0.4-0.8
Carbohydrates	1.2-1.6
Inorganic matter	0.7

Pigment	0.2
Total	100

Though Yutaka and Eiichi (1970) reported fibroin secreted in posterior section of silk gland but stored in middle silk gland as fibroin core. Sericin secreted in the middle silk gland and rounded the fibroin core in inner, middle and outer layer (Shimizu *et al.*, 1957 and Lucas *et al.*, 1958) and are synthesized in posterior, middle and anterior part of silk gland respectively (Yutaka and Eiichi, 1970; Yamanouchi, 1992; Machida, 1926).

Recent reports by Mondal *et al.*, (2007) also noted that Silk fibroin secreted in the lumen of posterior silk gland (PSG) of *B. mori* consists of three protein component: High (H)-chain 350 KDa (Shimura *et al.*, 1983, Zhou *et al.*, 2000), Low (L) - chain 26 KDa (Yamaguchi *et al.*, 1989), and Glycoprotein P25 30 KDa (Chevillard *et al.*, 1986a, 1986b). Although Molecular weight of H-chain and L-chain as 390 KDa and 30 KDa respectively described by Zurovec and Sehnal (2002). These three types of fibroin (H-chain, L-chain and P 25) are common among different silk producing insects in Lepidoptera, although the fibroin of Saturnidae species secreted as dimer of H-chain (Mondal *et al.*, 2007). Interspecific diversity of the H-fibroin size is due to differences in the large central protein region that is composed of repeats, whereas the terminal non-repetitive sequences are of similar length in all species. In *G. mellonella*, the non-repetitive N terminus encompasses 173 amino acid residues, of which a stretch of more than 100 is clearly homologous with the H-fibroins of *B. mori* and *A. pernyi*. Tussah silk fibroin composed of H-chain homodimer (Zurovec and Sehnal., 2002). Inoue *et al.*, (2000) described quantitative enzyme linked immuneosorbent assay (ELISA) with specific antibody for each protein component showed that the molar ratio of H-chain, L-chain, and P 25 is 6:6:1 for the fibroin secreted into the posterior silk gland (PSG). Fibrohexamer was proposed as a functional name of P25 and it has crucial role in maintaining the structure of elementary unit. They also reported that the disulfide linkage is not responsible for the maintenance of the once formed elementary unit that exist between Cys-172 of the L-chain and Cys-c20 of the H-chain (Tanaka *et al.*, 1999).

But Mori *et al.*, (1995) reported that the H-L linkage is essential for the large-scale production of fibroin, because fibroin is retained in endoplasmic reticulum in the absence of disulfide linkage between the H and L-chains (Gamo and Sato, 1985).

H-fibroin (350 KDa) is linked by disulfied bond to L- fibroin (25 KDa) and this is associated with P25 glycoprotein(27 KDa and 30 KDa) (Tanaka *et al.*, 1999; Michal, 1998; Oyama *et al.*, 1984). P25 has N-linked oligosaccharide chains, and its molecular size vary depending on the degree of glycosylation (Pingbo *et al.*, 2005; Inoue *et al.*, 2000; Inoue, 2004). Pingbo *et al.*, (2005) also reported that fibroin formed as following manner: a disulfide bond links a heavy chain with a light chain yielding a dimmer; six set of the dimmer are non covalently attached to a molecule of P 25 glycoprotein and form a [(H-L)6-P25] (Inoue *et al.*, 2000).

The molecular weight of fibroin has been determined by various methods, 33KDa by osmotic pressure (Coleman and Howitt, 1947); 60 KDa by sedimentation-viscosity measurement (Holmes and Smith, 1952); 250-450 KDa (average 300KDa) (Hyde and Wipple, 1962); 290 KDa (Iizuka, 1963) by light scattering measurement and 200-300KDa by amino acid analysis (Braunitzer and Wolff, 1955). The fibroin solution used for this purpose prepared by rather violent procedures and it is probable that the fibroin may have suffered more or less extensive degradation during these procedures (Yutaka and Eiichi, 1970).

It has been proven that the gene for H-fibroin (Suzuki *et al.*, 1972); L-fibroin (kimura, 1985) and P25 protein are expressed in posterior silk gland. The mutant variety of silk is Nd-sD which is fibroin secretion deficient. In this mutant, a disulfide linkage between the heavy (H) chain and light (L) chain is not formed because of partial deletion of the L-chain gene, which is essential for the intercellular transport and secretion of fibroin. Inoue *et al.*, (2005) investigated the possibility of using the NdsD mutant for the efficient production of recombinants in the silkworm. The posterior silk glands are not sufficiently developed and the liquid fibroin is scarcely secreted, but there is no such disorder in the middle silk glands. Yamamoto *et al.*, (2002) have introduced a new silkworm race which produces only sericin in Japan in the name of "Sericin Hope" introducing Nd-sD mutant. Feiying *et al.*, (2005) studied the analysis of protein

variety of middle silk gland cells of the fifth instar larvae of silkworm, *B. mori* at different developmental stages.

Fibroin contain high proportions of alanine, glycine and serine. A small amount of cystine residues give a very small amount of sulphar in the fibre. Fibroin contains only a small amount of amino acids, which have acid side chains. H-fibroin makes up the bulk of the lepidopteran silk fiber, and its structure determines the physical properties of the silk. On the basis of x-ray diffraction studies and amino acid analyses five structural silk categories were recognized (Warwicker, 1960). The silk of *B. mori* was classified as group 1, which is characterized by dense molecule packing corresponding to high glycine content (Zurovec and Sehnal, 2002).

Table-2: Amino acid composition of silk fibroins (residues/ 1000 residues) (Robson, 1985).

Amino acids	*B. mori* fibre
Glycine	446.0
Alanine	294.0
Valine	22.0
Leucine	5.3
Isoleucine	6.6
Serine	121.0
Threonine	9.1
Aspartic acid	13.0

Glutamic acid	10.2
Lysine	3.2
Arginine	4.7
Histidine	1.4
Tyrosine	51.7
Phenylalanine	6.3
Proline	3.6
Tryptophan	1.1
Methionine	1.0
(Cysteine)	2 2.0

Sericins from middle silk gland include several glycoproteins ranging from 65 to 400 KDa (Gamo, *et al.*, 1977; Sinohara, 1979). Sericins are characterized by unbalanced amino acid composition (Prudhomme, 1985) and two sericin genes Ser1 (Michaille, 1983) and Ser2 (Couble, 1987) are active in middle silk gland (Michal, 1998). Mondal *et al.*, (2007) noted that the part of the liquid sericin in the middle silk gland easily crystallized by drying is possibly made of amino acid residues with short side-chains and is folded into the globular matrix made of stretches with longer side-chains, become film of unoriented crystal structure. After the film is swollen in water, stretched and dried, it changes to oriented fiber structure. There is a reversible relationship between the orientation and non-orientation as the orientation is unstable by hot water treatment .

Iizuka (1969) extracted sericin from the liquid silk and fresh cocoon using mutant silkworm which secretes only sericin and reported that sericin secretd as random coil with 5-10% beta-structure and no alpha-helix. Komatsu (1975) also reported that sericin, in aqueous solution, displays both random coil and b- structure, but lacks a-helix. Though Tsukada (1983) based on analysis of circular dichroism spectrum, which, repoted sericin extracted from liquid silk for 45 minutes with water contains a small fraction (10%) of alpha-helix. Komatsu (1982) also confirmed the relationship between solubility in hot water and molecular conformation. To date, most experimental evidences indicate that sericin exists mainly in the random coil or beta-structure. It is believed that beta structure is intrinsic to liquid silk (Mondal *et al.*, 2007).

Komatsu (1980) reported part of the sericin become a white suspension due to the coexisting cocoon yarn wax during dissolution of the liquid silk in water, but does not coagulate and the b structure is originally present in the liquid silk. Beta-structure sericin is more insoluble than random coil sericin (Mondal *et al.*, 2007). The structural changes of sericin from its random coil to b- structure takes place by repeated absorption and de-absorption of moisture and by heating during the absorbed state i.e., hygroscopic conditions (Komatsu, 1980). Ishikawa and Hirabayashi (1968) reported that sericins have a cross b structure when a water solution of sericin containing 50% dioxane (Iizuka, 1969) or methanol (Ishikawa *et al.*, 1987).

Komatsu (1975) explained how sericin transformed from to beta structure, he belived that the sericin chain closest to fibroin in the cocoon has arranged in cross beta structure. The intra-molecular bonds of sericin having random coils are broken by the absorption of water molecules and the folded structure becomes unfolded into an extended structure and is transformed into a beta structure. Regarding energy beta Structure is more stable. Part of the sericin thus transformed into beta structure is fixed by its new intermolecular hydrogen bonds and remains crystallized even when the water molecules are removed by drying (Mondal *et al.*, 2007)

Mondal *et al.*, (2007) noted sericin of cocoon shell have two proportions: a-sericin and b-sericin. The a- Sericin is present in the outer layer of cocoon shell, having more solubility than b-sericin that present in the inner layer. The a-sericin contains lesser C and H and somewhat more N and

O than the b-sericin (Bose *et al.*, 1989). Sadov *et al.*, (1987) have described that native sericin is mixture of two substances, sericin A and Sericin B. Komatsu (1975) investigated four fractions of sericin viz., I, II, III and IV having different solubilities. On the basis of the degree of solubility by UV absorption Robson (1985) reported that sericin may be separated into sericin I, II, III and IV by their different solubilities in hot water. Based on the different degree of solubility, sericin layers can be classified as Sericin A and Sericin B or Sericin a and b or Sericin I, II, and III. The sericin A is more soluble than the others as the sericin I on the cocoon filament is amorphous where as II and III are crystalline. The greatest sericin content is present in the outer layer of cocoon whereas the lowest sericin in the innermost layer of a cocoon. Fibroin is the principal water insoluble protein (Mondal *et al.*, 2007).

The sericin of wild silkworm, *Bombyx mori mandarina*, and domesticated silkworm, *B. mori* contain the same kind of amino acids. Though the wild silkworm sericin contained serine, proline, methoinine, glucosamine, galactosamine and histidine in lower amount and throeonine, glutamic acid, cystine and phenyl amine in higher amount (Yamada, 1978).Heterogeneity of sericin molecules suggested by a number of authors, for eample Shelton and Johnson (1925) fractinated sericin into two parts by farctional precipitation. Later Mosher (1932) and Bryant (1948) concluded that there are three distinct sericins (Yutaka and Eiichi, 1970).

Fibroin and sericin can be separated in the solution of protein by precipitation, and the supematant fraction known as fibroin which is rich in glycine ,alanine and Serine, and those with bulky apolar side chains (Zurovec and Sehnal, 2002) together accounted for 78%.On the other hand, the precipitate fraction is known as sericin, which was rich in glycine, serine, alanine and aspertic acid, the combined contents of which amounted to 72%. Electrophoresis results suggest that components of sericin have define molecular weights but those of fibroin do not (Mondal *et al.*, 2007).

Recent studies describe a third category protein that is named seroins (22.5 and 23.5 KDa) and both sericin and fibroin producing cell participate in seroin secretion. Seroin is distinguished from the other silk protein by high proline content (34 residues) lack of cysteins. And seroin gene is expressed in both posterior and middle silk gland section. The relatively high polar amino

acids and the glycosylation make seroin water soluble. High proline content is reminiscent of the proline-rich antimicrobial peptides (Boman, 1996) and seroin may be responsible for silk reisstance to molds and bacteria (Michal *et al.*, 2002).

2.3 Stability of Silk Proteins:

Fibroin extracted in water is quite stable as long as it is kept in cold, though partially gelled by prolonged ageing in cold but fibroin still in solution sediment revealed by ultracentrifugation analysis (Yutaka and Eiichi 1970).

This protein is quite unstable in alkaline buffer and degraded easily. Degradation depends on PH, temperature and concentration of fibroin. Degradation is faster in more alkaline solution and accelerated by rise in temperature. At PH 9.0 and even the temperature is kept at 370C fibroin is degraded rapidly. In warm alkaline solution some sort of changes of fibroin molecule may take place. Unfolding and conformational changes of the fibroin molecule might occur in warm alkaline solution. The other might take place is that the fibroin molecule may dissociate into subunits in alkaline buffer solution (Yutaka and Eiichi, 1970). It has been also reported that some disulfide bonds of certain protein molecules are unstable in warm alkaline solution (Cecil,1963 and Tanford, 1968).

Yutaka and Eiichi (1970) also suggested that native fibroin molecule is composed of the two subunits, approximately equal in molecular weight which are connected by one or several disulfide bonds.

Singhvi and Bose (1991) reported that the sericin content is being the deciding factor in the quality of the cocoon, however filament length and quality of the shell are based on the fibroin content. Some of the amino acids named glycine, alanine and serine are abundantly found in fibroin (Shimura *et al.*, 1958).

Gulrajani (1988) reported that sericin is insoluble in cold water, however, it is easily hydrolyzed, where by the long protein molecules brakes down to smaller fractions, which are easily dispersed, or solubilised in hot water. Hidetoshi *et al.*,(2005) repoted SDS-PAGE pattern of boiled sericin solution for 20 minutes still exihibited clear but slighty fuzzy protein bands which suggested slight decomposition of the protein molecules. When sericin was autoclaved for 121oC for 20 minutes the protein bands of sericin component disappeared showing significant decomposition of sericin molecules.

2.4 Properties of silk protein:

Silk protein is a biodegradable natural polymers. This polymer could be cross linked by its reactive functional groups with other polymers. Therefore, this property of silk protein might be useful in controlled drug delivery system (Mondal *et al.*, 2007).

As the silk gland produces large amount of silk proteins, Chavancy (2005) developed piggyBac vectors to express transgenes in the silk gland using silk gene promoters to express the integrated foreign genes. Several promoters like procollagen III or globular ones were tested and successfully produced in the silk glands of *Bombyx mori.*

Annamaria *et al.*, (1998) reported blending sericin with other resins environment- friendly biodegradable polymers can be produced . Polymer films, foams, molding resins, and fibers containing sericin (0.01-50% w/w) can be produce by reacting a composition comprising a polyol, tolylene, di- isocyanet, di-butyltin di-laurate (catalyst) and trichloromonofluoromethane (a blowing agent) in the presence of sericin (Mondal *et al.*, 2007). The Polyurethane foams incorporating sericin have excellent moisture absorbing and desorbing properties (Minoura *et al.*, 1995).

Mondal *et al.*, (2007) proposed sericin protein has special properties viz., resists oxidation, antib acterial, UV resistant and absorbs and release moisture easily, inhibitory activity of tyrosine and kinase etc. and fibroin is well known for its water absorbency, dyeing affinity, thermo

tolerances, insulation properties and luster. Moreover it is the raw material for producing parachutes, precious fabrics, tyre lining materials, surgical sutures and artificial blood vessels.

Chisti (1998) repoted the insolubilized silk fibroin membrane could be used to separate the mixture of water and alcohol. Sericin cross-linked, blended, or copolymerized membranes with other substance are made easilly, because sericin contains large amount of neutral polar amino acid with functional groups. Sericin and fibroin can be used to make membranes for use in separation processes, Membrane based separations such as reverse osmosis, dialysis, ultra filtration and micro filtration are used in process such as desalination of water, production of extremely pure water, the bioprocess industry and some chemical processes (Mondal *et al.*, 2007). Mizoguchi *et al.*, (1991) describe a cross- linked thin film made of sericin for use as a separating membrane for water and ethanol. Yamada and Fuwa (1994) and Yamada *et al.*, (1993) reported Sericin with acrylonitrile to prepare a protein containing synthetic polymer film for separating water from organics.

Tanaka and Mizuno (2001) reported sericin- coated film is used on the surface of refrigeration equipment because of its antifrosting action. Moreover use of the coated film on roads and roof can prevent frost damage. Li (1996) described sericin protein can be coated on surfaces of various durable materials to enhance functionality. Sericin can be used in preparation of art pigments and for surface protection of articles. The material coated the sericin have excellent weather ability, good permeability and do not wrap on drying (Yoshii *et al.*, 2000). Miyairi and Sugiura (1978) reported a cross-linked sericin film for enzyme immobilization.

Silk fibroin based wound dressing that accelerate healing and be peeled off without damaging the newly formed skin have been reported (Tasubouchi, 1999 a). Fibroin film used in wound healing composed of a mixture of both fibroin and sericin had 3-16% water content and a thickness of 10-100 micrometer (Tasubouchi, 1999 b).

Mondal *et al.*, (2007) noted that membrane composed of sericin and fibroin can be used as substitute for collagen and is an effective substrate for the proliferation of adherent animal cells. Growth of animal cells on the film also reported by Mnoura *et al.*, (1995) and Tsukada *et al.*,

(1999) and the cell growth or attachment dependent on maintaining a minimum of around 90 percent sericin in the composite membrane.

Film made of sericin and fibroin has excellent oxygen permeability and is similar to human cornea in its functional properties. This Sericin-fibroin blended film could be used to form article corneas (Murase, 1994).

Sericin suppressed in vitro lipid peroxidation follows the antioxidant action of the silk protein. Furthermore, sericin also found to inhibit tyrosinase activity, these suggests that sericin is the valuable natural ingredient for food and cosmetics. The biopolymer sericin has a strong affinity to keratin (Kato *et al.*, 1998).

The silk sericin has the natural moisturizing factor. Sericin gel is prepared by using sericin solution to prevent water loss from the upper layer of the skin forming a moisturizing, semi-occlusive, protective, antiwrinkle film on the skin surface giving an immediate, long lasting, smooth, silky feeling (Padamwar *et al.*, 2005).

Mondal *et al.*, (2007) noted that sericin can naturally saturate into skin and revitalize cells and also restrain the functions of active-oxygen which is major factor of aging that brings wrinkles and dark spots as the configuration of sericin is very close to the one of human beings.

Gulrajani (2005) reported sericin could be used in other purposes includes, as a soil conditioner, coagulant for purification of waste waters, additives to rice cooking, fabric care compositions, light and sunscreen compositions, foam-forming aerosol shaving gels, sericin-coated powders for cosmetics, as dermatitis inhibitor, as wound protection film, nail cosmetics, and chewing gums.

Fibroin has been using as a biomedicine for various applications. Fibroin powder with its natural, optical beauty used to hold and release moisture depending on the temperature and humidity of the surroundings and is particularly ideal for applications in pressed powders, blusher, eye make up, lipstick and nail enamel (Mondal *et al.*, 2007).

Wu *et al.*, (1996) studied the properties and application of wound protective membrane made by silk fibroin and concluding that the fibroin membrane has good wound healing properties. Matta *et al.*, (2004) reported the fibroin hydrogels used as scaffolds able to promote in situ bone regeneration .

Gotoh *et al.*, (2000) reported the sulfated silk fibroins have anti-HIV-1 activity in vitro, apparently due to interference with the adsorption of virus particles to CD4+ cells, and completely blocked virus binding to the cells. Inouye *et al.*, (1998) reported that silk fibroin can be used as the substratum for the culture of animal cells in place of collagen. Aslani and Eral (1994) investigated the uranium recovery from dilute aqueous solutions using silk fibroin.

Drug release kinetics were evaluated of silk fibroin (SF) materials as a function of dextran molecular weight, and film crystallinity and concluding silk fibroin is an interesting polymer for drug delivery of polysaccharides and bioactive proteins due to the controllable level of crystallinity and the ability to process the biomaterial in biocompatible fashion under ambient conditions to avoid damage to labile compounds to be delivered (Hofmann *et al.*, 2006). Michael *et al.*, (2007) reported on the development of silk fibroin microtubes for blood vessel repair with several advantages over existing scaffold materials.

Chapter Three

Materials and Methods

Chapter Three

Materials and Methods

The silk worm, *Bombyx mori*, was used in this present experiment. The experiments were conducted in the Insect Biotechnology Division, Institute of Radiation Biology, Atomic Energy Research Establishment, Savar, Dhaka.

3.1. Materials:

3.1.1. Equipments:

Equipments for Protein concentration Estimation

1. Spectrophptometer (model: UV-1201).
2. Cuvettes (glass)
3. Pipettes
4. Pasteut pipettes and pipettes bulbs
5. Test tubes
6. Test tube rack
7. Vortex mixer

Equipments for sodium dodicyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

1. Power supply
2. Boiling water bath
3. Eppendrof centrifuge
4. Normal syringes
5. Eppendrof tubes
6. Rotary shaker, etc.

3.1.2. Reagents

Reagents for Protein concentration Estimation

1. $CuSo_4$. 5 H_2O
2. $Na_3C_6H_5O_7$ ($2H_2O$) (Sodium Citrate)
3. Na_2CO_3
4. NaOH
5. Folin- Ciocalteu phenol reagent (FCR)

Reagents for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

1. Acrylamide,electrophoresis grade.
2. Bis- acrylamide (N,N-methylenebisacrylamide).
3. Tris (2-hydroxymethyl-2-methyl-1,3-propanediol)
4. SDS(Sodium dodecyl sulfate or Sodium lauryl sulfate)
5. TEMED (N,N,N,N-tetremethylene-ethylenediamine)
6. Amonium per sulfate
7. 2-mercaptoethanol
8. Glycerol
9. Bromophenol blue
10. Glycine
11. Hydrocloric acid

3.2. Silkworm Rearing:

Egg strips were placed in Petri dishes and small vial filled with water clogged with cotton ball was also placed inside the Petri dishes to prevent the eggs from desiccation. The eggs were hatched between 9 and 10 days at ambient temperature. After hatching, the larvae were fed chopped young mulberry leaves. The larvae were reared in plastic box covered with fine meshed netted lid. Larvae were feed four times a day. There are five larval instars. Larvae were reared up to the 5^{th} instars (20-25 days) and then the silk glands were dissected out carefully.

3.2.1. Irradiation of eggs

The eggs of the silkworm were exposed to irradiation at a dose of 1.5 Gy and the eggs were allowed to hatch. After hatching, larvae were reared as described above. When the larvae reached to 5^{th} instar, the silk glands were dissected out carefully.

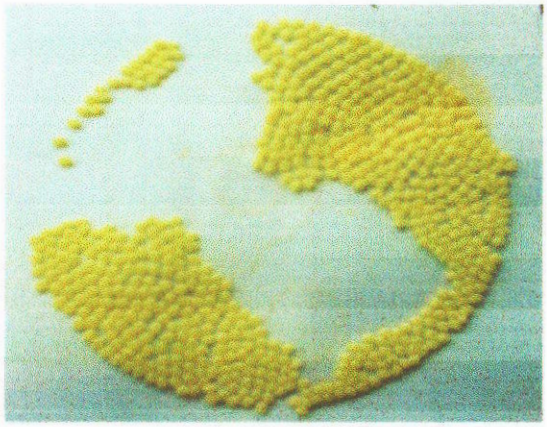
3.3. Methods

3.3.1. Extraction of native fibroin:

The silk proteins from the silk gland was extracted according to Yutaka and Eiichi (1970). The procedures of extraction described below:

1. The silk glands of both sides were dissected out and washed briefly in distilled water. Middle and posterior portion are cut into parts and put into a small Petri dish containing a small amount of distilled water.
2. After severals minutes in distilled water the luminar silk protein columns were softened and partially extruded out of the glandular lumina and the swollen glandular tissue became easily seperable by fine forceps from the protein columns.

(a)



(b)

(c)

(d)

(e)

(f)

Fig. 2. Silkworm rearing. (a), the eggs strips of *Bombyx mori*; (b),the egg strip placed in a perti dishes with one vial filled with water to ensure moisture condition; (c) the 1st instar larvae; (d) the 2nd instar larvae; (e) the 4th instar larvae; (f) the fifth instars 1st day larvae;

3. These Petri dishes with silk protein columns were then shaken very gently overnight in the cold (2-3°c) vigorous shaking was avoided strictly because it could include extensive surface denaturation and gelation of the silk protein.

4. The water extract was then collected by decantation and a small amount of distilled water added again to the dishes and the second extraction continued for several hours more.

5. The first and the second extracted protein were pooled together and then combined extraction centrifuged at 15000 rpm and the clear supernatant was collected. The proteins were stored in the cold (2-3°c) until use.

3.3.2. Extraction of silk proteins in buffer solution

Instead of water, a sodium phosphate buffer of pH 7.4 was also used to extract the silk proteins from the silk gland. The extraction procedures were the same used in water extraction method of Yutaka and Eiichi (1970).

3.3.3. Protein Concentration Estimation

Protein Concentration Determination can be determined by the various methods namely Bradford method and Lowry method. In this experiment the Lowry method (Lowry *et al.*, 1951) for protein concentration estimation was used. It is widely used quantitative assay for determining protein content in a solution. Most interfering substrate can be removed by precipitating the proteins from solution prior to running the assay.

3.3.3.1.Working Reagents:

- **Solution A:** 2% Na_2CO_3 solution in 0.1 N NaOH solution, that is 2 gm Na_2CO_3 was dissolved in 100ml 0.1 N NaOH solution.

- **Solution B:** 0.5% $CuSo_4$ solution in 1% sodium-potasium-tartrate, that is 500gm $CuSo_4$ was dissolved in 100ml 1% Na-K tartrate.

- **Solution C:** Mixture solution A and solution B in a ratio 50:1 just before use.

- **Folin-Ciocalteu phenol reagent (FCR):**

To a 2000ml flat bottom flask 100gm of sodium-tungstate (Na_2WO_3. 2 H_2O), 25 gm sodium molybdate (Na_2 MoD_4. $2H_2O$), 700ml distilled water, 50 ml 85% phosphoric acid, 100ml conc. HCl were added and reflaxed gentlyfor 10 hours with a condencer. After cooling 250gm Li2SO4, 50 ml distilled water and a few drops of bromine were added to it and boiled for 15 minutes. It was cooled and diluted to 1 litre and filtered and was stored in cold in an amber color bottle.

- **Standard protein solution (100 micro grams in distilled water):** 10 *u*g of protein BSA (Bovine Serum Albumin) was dissolved in 100ml distilled water.

3.3.3.2. Procedure:

- **Preparation of calibration curve:**

1. To prepare calibration curve 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 ml of standard protein solution were taken in different test tubes and made up to 1ml with distilled water.
2. To each of the test tubes 5ml of solution C was added and the test tubes were kept in room temperature for 10 min.
3. 0.5 ml of diluted FCR was added to each of the test tubes and mixed well.
4. The solution was allowed to stand for 30 min. in dark and the absorbency was measured at 650 nm.
5. A standard curve was made by plotting concentration against absorbance.

- **Assay of silkworm protein:**

1. 10,20,40,50 and 100 micro litre protein samples was taken in a test tube and the volume was made 1 ml with distilled water.
2. In each test tube 5 ml of solution of C added and the test tubes kept in room temperature for 10 min.

3.0.5 ml of diluted FCR was added to each test tubes and kept in dark for 30 min.

4. Absorbency was measured at 650 nm against the blank or control.

The amount of protein present in the sample was calculated from the standard curve shown in the fig. 3 and expressed as gm/100ml solution

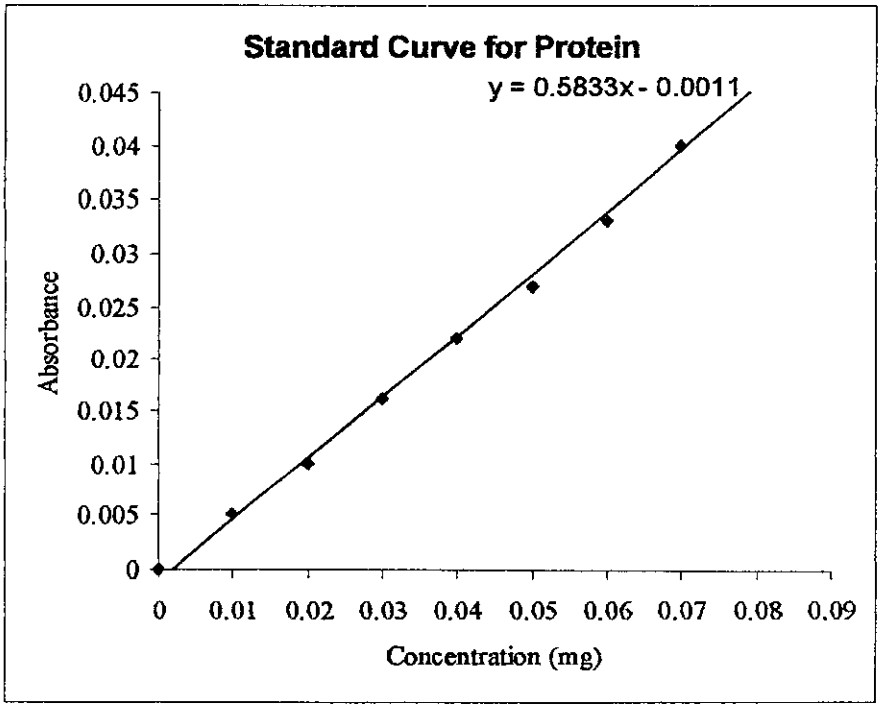


Fig.3. Standard curve for protein estimation of *Bombyx mori*.

3.3.4. Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis is low cost, reproducible and rapid method for quantifying, comparing and characterizing proteins. This method separates protein based primarily on their molecular weights (Laemmli, 1970). SDS binds to hydrophobic portion of a protein, disrupting its folded structure and allowing it to exist stably in solution in an extended conformation. As a result the length of the SDS-protein complex is proportional to its molecular weight.. Our description of SDS-PAGE will include protocols for linear slab gels, i.e., gels formed as slab between two sheets of supporting glass. Slab gels have become more widely used than the tube gels (formed with glass tube supports), since many samples can be run on the same gel, thereby providing uniformity during polymerization, staining and destaining. For most analytical application the mini slab gel has gained considerable popularity due to increased resolution and reduced time and materials required for electrophoresis. Coomassie blue was used as staining agent of protein gels.

3.3.4.1. Working solution:

Electrophoresis Buffer (Tris-glycin) P^H -8.3: (25mM Tris; 192mM glycin; o.1% SDS) - 500ml
Concentration: 10X

Name	amount
Tris- Base	15.1 g
Glycin	72 g

420 ml water was added and P^H adjust to 8.3 and then SDS 50 ml or 5 g SDS and make volume 500ml and keep at room temperature indefinitely.

4X Gel Buffer or separating buffer -100ml (sol^n B)

Tris (1.5M) 16.95g

90 ml was added and adjusted P^H to 8.8 (by Hcl) and 4 ml of 10% SDS or 0.4 g SDS and bring the volume to 100ml.

Acrylamide Sol^n (A) -100ml

Acrylamide-29.2g

Bis- acrylamide- 0.8g

Water was added to make 100 ml and stirred until dissolve under hood and covered with parafilm and keep at 4^0 C until use.

Preparation of gel

Staining with 7.5% gel

Sol^n A	2.5ml
Sol^n B	2.5ml
Water	5.0ml
APS(10%)	50µl
TEMED	5 µl

X% gel Preparation

Sol^n B	2.5ml
Water	7.5-X/3ml
APS(10%)	50µl
TEMED	5 µl
Total	10ml

Staining sol^n -1 litre

Comasine blue R-250	1g
MeOH	450ml
H_2O	450ml
Glacial acetic Acid	100ml

Destaining sol^n-1 litre

MeOH	100ml
Glacial acetic Acid	100ml
H^2O	800ml

3.3.4.2.Procedures:

Apparatus were assembled according to the instruction. Then a small beaker was washed and dried. The solution A and B and water are Combined in that beaker. Ammonium per sulfate and N,N,N,N-tetremethylene-ethylenediamine (TEMED) are added and mixed by gently swirling or inverting the container. Gel solution pipetted by using normal syringes into separating gel until the solution reaches top of the front plate. Then the combs were placed carefully and allowed 30-45 minutes to polymerize the gel. After polymerization of the gel comb carefully removed, making sure not to tear the well slots and the gel placed into electrophoresis chamber. Electrophoresis buffer added to inner and outer reservoir, making sure that both top and bottom of gel were immersed in buffer. A pre-run of 30 minutes was conducted and the well slots were rinse with syringe to remove the unpolymerized acrylamide and any contaminants.

Preparing and loading samples:

Combine the even amount protein samples and loading buffer in an eppendrof tube The samples including the protein marker were loaded in the slots with micropipettes carefully.

Running a Gel:

Electrode plugs attached to the proper electrode, current would follows towards the anode. The power supply was turned on to 100 V for one hour and 45 minutes or long time as required. After finish the run, gels were separated from the plates, washed briefly in distilled water and placed in a staining box.

Satining the Gel with coomassie Blue:

The solution of coomassie brilliant blue was added in the staining box and was placed at 37°C with slight shaking for approximately an hour. This staining method is simple, fast and can detect as little as 0.1 micro gram of protein in a single band. After staining the gel, the staining solution was poured out and rinsed the gel with water for few changes.

Table 5. Concentration of silk protein from posterior silk gland (PSG) of both irradiated and unirradiated silkworm, *Bombyx mori* larvae

Samples	**Protein concentration mg/ml(±SD)**
Unirradiated silkworm larvae	2.82 (± 0,14)
Irradiated silkworm larvae	3.17 (± 0.17)

Concentration of proteins of irradiated silkworm larvae were increased at varying degrees to different parts. The calculated percent increase of the silk proteins is presented in Table 6.

Table 6. Percent increase of silk protein of irradiated silkworm

Part of the silk gland	**% increase**
Anterior Silk Gland	15.3
Median Silk Gland	21.25
Posterior Silk Gland	12.41

4.2 Proteins extracted from whole silk gland in sodium phosphate buffer solution

Proteins from whole silk gland of silkworm *Bombyx mori* were extracted in buffer solution at pH 7.4. Extracted proteins were analysed by 7.5% polyacrylamide gel and the results are presented in Fig. 5. A total of 5 protein bands of 30 KDa, 45 KDa, 60 KDa, 70 KDa, 110 KDa and 220 KDa were obtained.

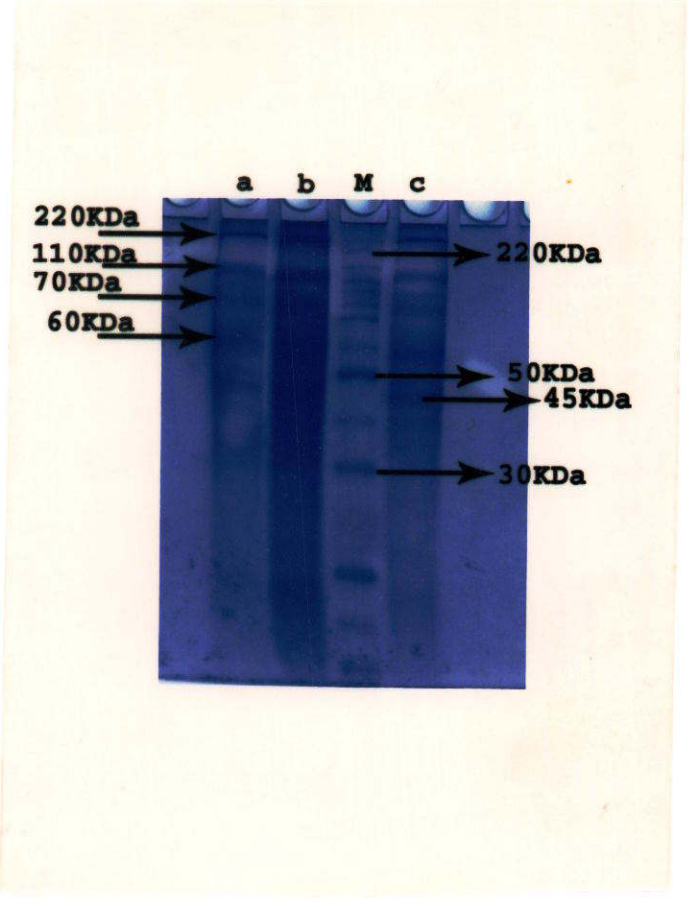


Fig 5 Buffer extracted protein of whole silk gland. a (2x diluted), b (non-diluted), c (3x diluted) and M (marker).

4.3 Proteins extracted from anterior silk gland (ASG)

After removing the whole silk gland of *Bombyx mor*, the anterior part of silk gland was carefully excised. Proteins of this part of the gland were allowed to liberate in water according to Yutaka and Eiichi (1970). Extracted proteins were analyzed in 7.5% polyacrylamide gel and the results are presented in Fig. 6.

A total of three protein bands of sizes 350, 220 and 110 kDa were detected by polyacrylamide gel electrophoresis analysis.

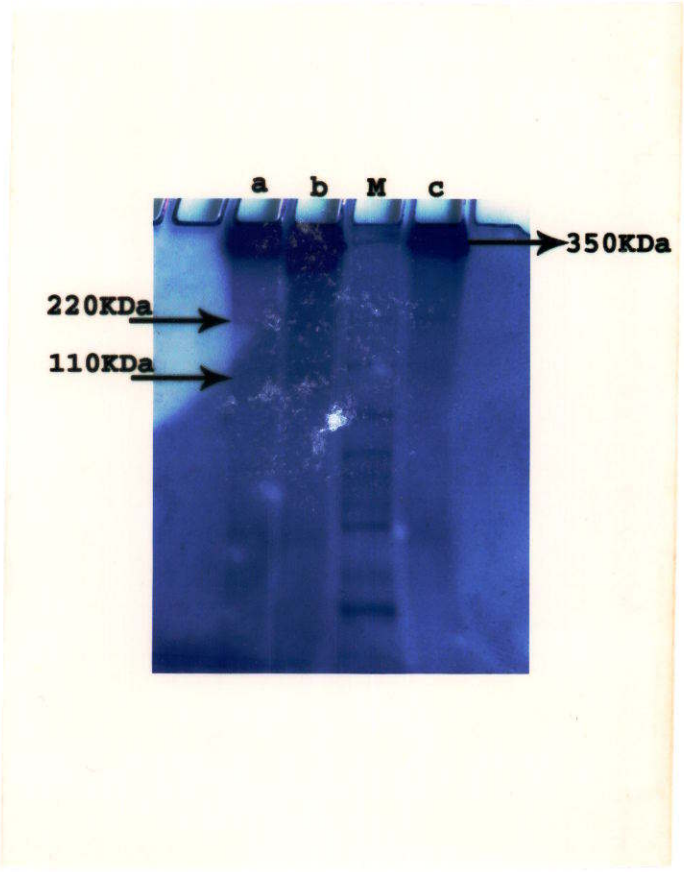


Fig. 6 Water extracted protein of anterior portion of silk gland; a (2x diluted); b (non-diluted); M (Marker); A (3x diluted).

4.4 Proteins extracted from middle silk gland (MSG)

The median part of the silk gland was carefully excised from the rest of the silk gland and the proteins were extracted in water followed by the methods of Yutaka and Eiichi (1970). Polyacrylamide gel electrophoresis analysis was carried out to analyse the proteins of MSG and the results are presented in fig. 7.

A total of 4 protein bands of sizes 350, 60, 50 and 30 kDa were detected by gel electrophoresis analysis.

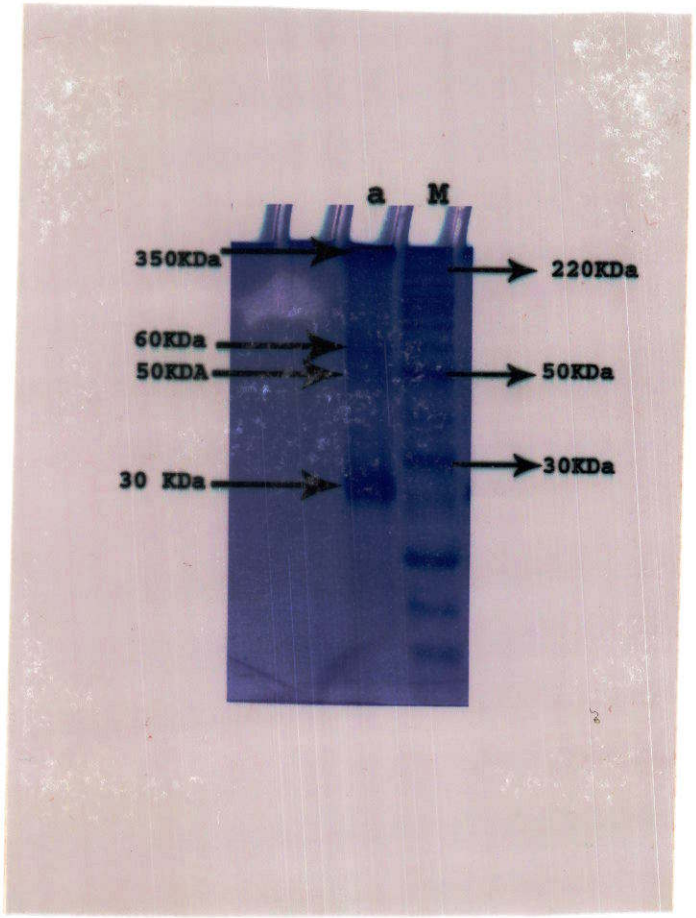


Fig. 7 Water extracted protein sample of Middle silk gland. a (MSG protein) and M (marker).

4.5 Proteins extracted from posterior silk gland (PSG)

The posterior part of the silk gland was carefully excised from the rest of the silk gland and the proteins were allowed to liberate in water accordingly to Yutaka and Eiichi, (1970). Polyacrylamide gel electrophoresis analysis was carried out to analyze the proteins.

Two protein bands of sizes 350 and 30 kDa were detected by gel electrophoresis analysis (figure not shown, as the gel was damaged during photography).

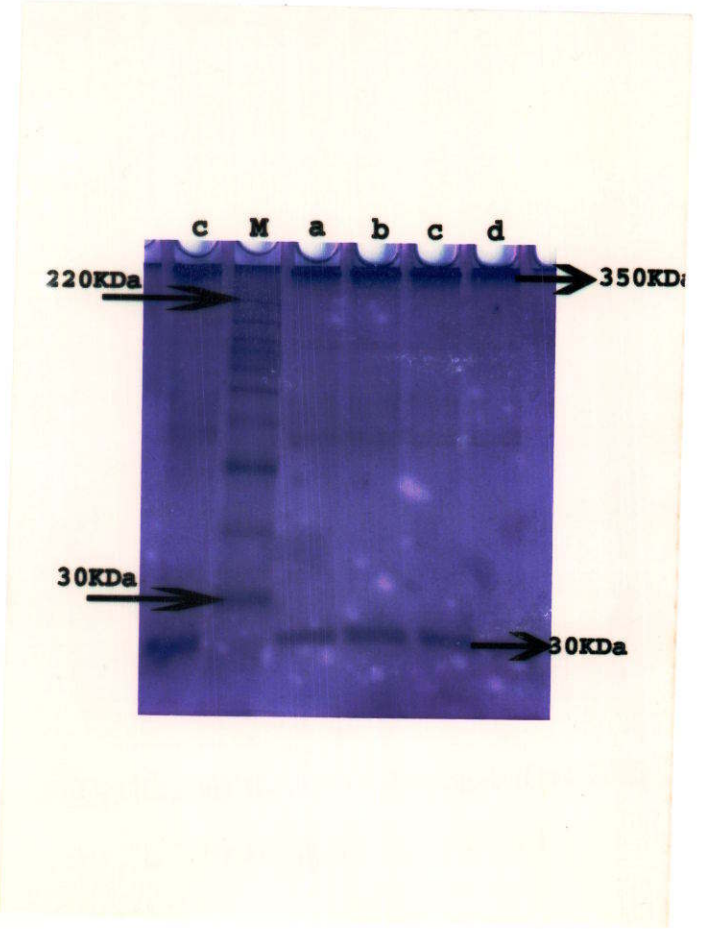


Fig 8 Heat treated MSG protein samples. a (40^0C),b (50^0C),c (60^0C), d (70^0C) , M (marker) and C (control)

4.6 Thermal stability of silkworm proteins

4.6.1 Stability of silk proteins extracted in water

Water extracted protein samples of Middle silk gland (MSG) were incubated at 40^0 C, 50^0 C, 60^0C and 70^0C for 10 minutes. The results showed no degradation or changes in protein bands up to 70^0C for up to 10 minutes incubation (Fig.8). Therefore, additional middle silk gland proteins were incubated at higher temperatures of 80^0 C and 90^0 C for 10 minutes. An SDS PAGE was used to analyze the protein. The results are presented in Fig. 9. The results indicated that the fibroin heavy chain of 350 kDa started to be degraded at 90^0C resulting fuzzy bands.

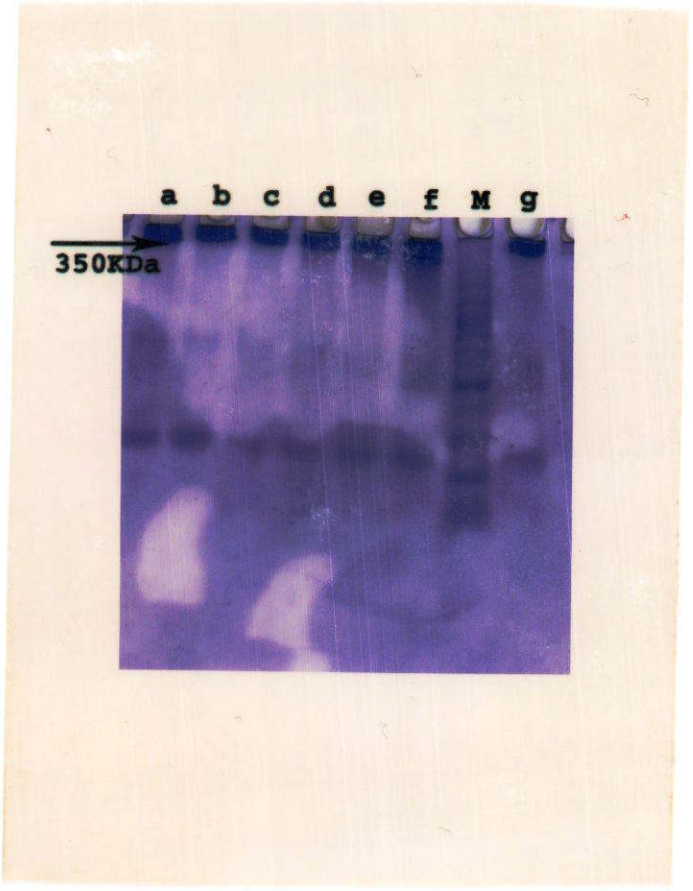


Fig 9 Water extracted protein samples of MSG treated with several temperature treatment .a, b, c, d, e , f, g and M describe heat treated protein at 40^0C, 50^0C, 60^0C, 70^0 C· 80^0C, 90^0C and marker

4.6.2 Stability of silk proteins extracted in buffer solution at pH 7.4

Buffer extracted proteins from MSG were incubated at 40^{0}C, 50^0 C, 60^0 C, 70^0 C, 80^0 C and 90^0 C for 30 minutes. Heat treated proteins were analyzed in SDS-PAGE gel. The results are presented in Figs 10 (a) and (b). At 80^{0}C the fibroin heavy chain of 350 kDa was started to be degraded resulting slightly fuzzy bands. On the other hand, when proteins were heat treated at 90^{0}C for 30 minutes, the heavy chain of fibroin was disappeared. This result showed that proteins of MSG extracted in buffer solution are stable up to 70^{0}C. Comparing the results obtained from the proteins extracted in water and buffer solution at pH 7.4 showed slightly different banding pattern. The bands those are not found in proteins extracted in water are shown by arrow in figures 10 (a) and 10 (b).

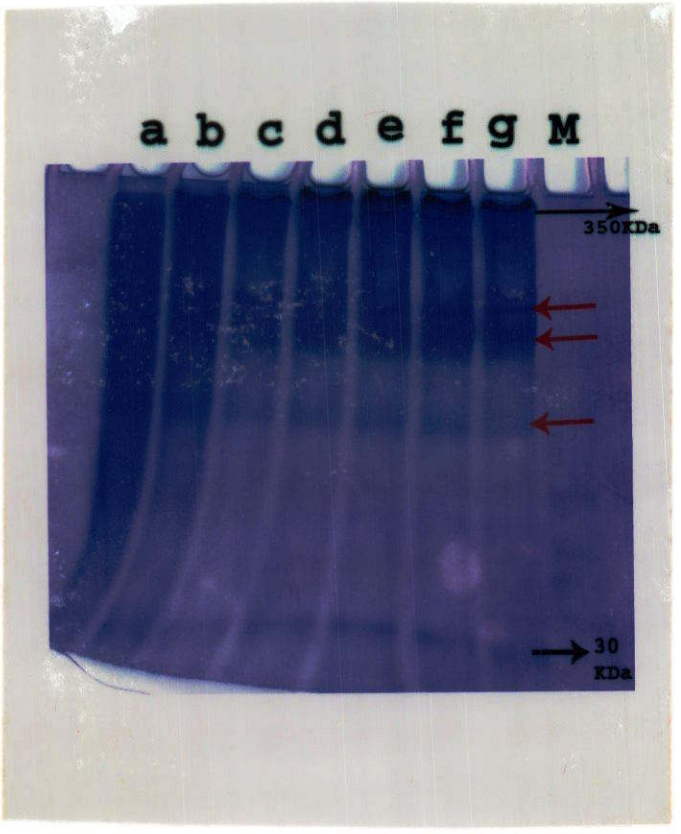


Fig 10 (a) Buffer Extracted Protein Sample of MSG and all the sample treated for 30 minutes. **a** (90^{0}C), **b** (80^{0}C), **c** (70^{0}C), **d** (60^{0}C), **e** (50^{0}C), **f** (40^{0}C), **g** (control) and **M** (marker) describes respectively.

Fig 10 (b) Buffer Extracted Protein Sample (dilluted) of MSG and all the sample treated for 30 minutes. **a** (50^0C) , **b** (60^0C) , **c** (70^0C) , **d** (80^0C) , **e** (90^0C), **f** (control) and **M** (marker) describes respectively.

4.6.3 Stability of silk gland proteins of irradiated silkworm

Silk gland proteins **(MSG)** from irradiated silkworm were extracted (in water) and the proteins were incubated at 80^0 C and 90^0 C for 15 and 30 minutes. Proteins from unirradiated silkworm

were also incubated as control simultaneously. Both heat treated proteins were analyzed by SDS-PAGE and the results are presented in Figs. 11 (a) and 11 (b). The results showed that the light chain of fibroin (~30 kDa) proteins of irradiated silkworm is more stable than the same proteins of unirradiated ones.

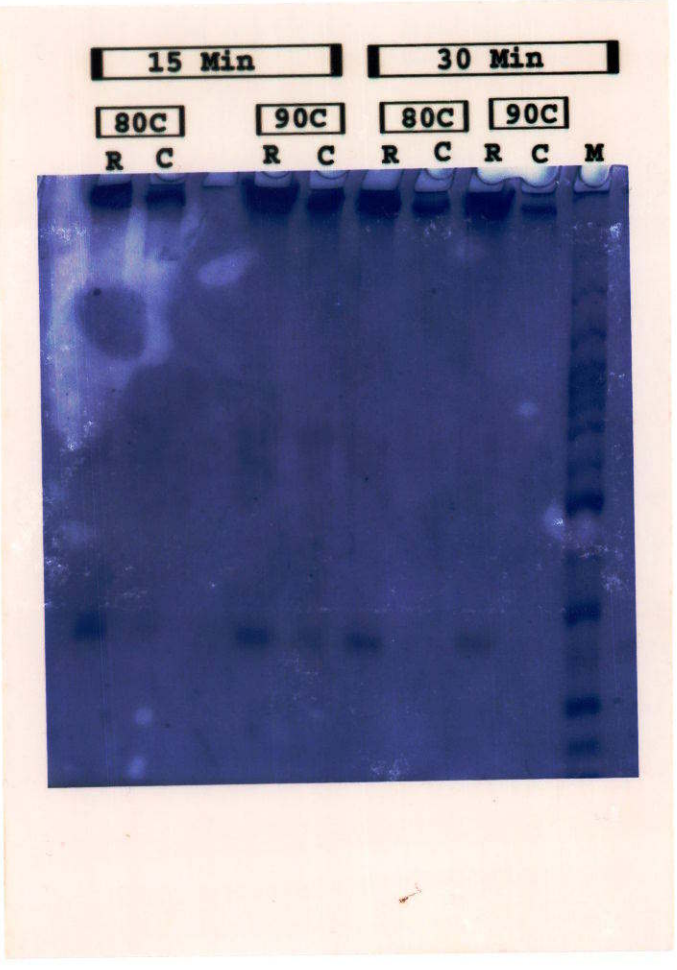


Fig 11 (a) Irradiated and non-irradiated protein samples treated with 15min and 30 min treatment and run in SDS-PAGE.

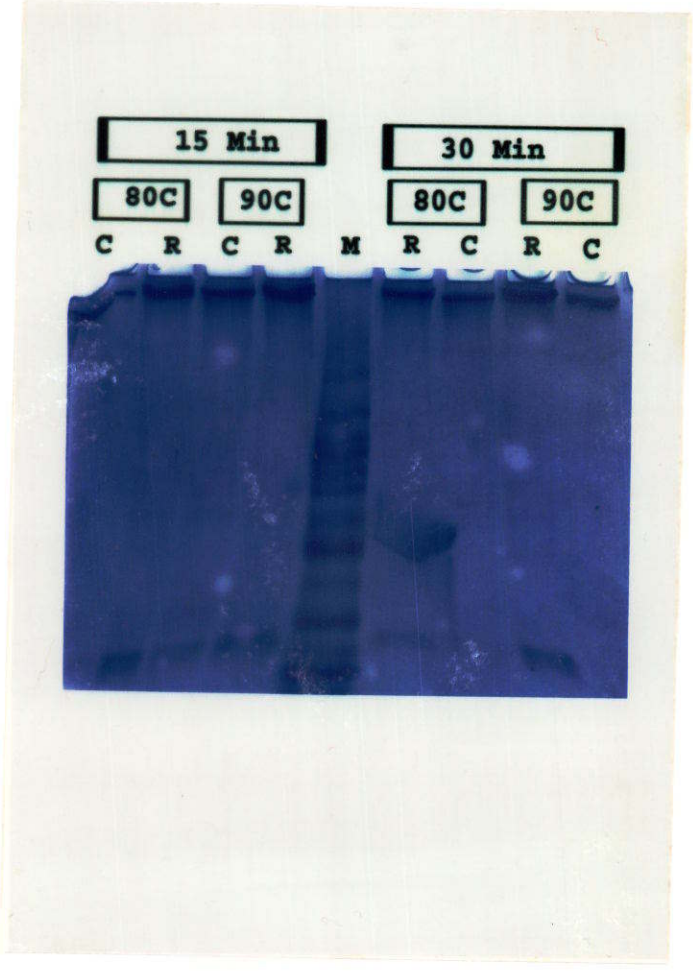


Fig 11 (b) Samples loaded with 80^0 C and 90^0 C for 15 minutes and 30 minutes treatment (even concentration)

Fig 12 Irradiated and non-irradiated proteins from ASG and MSG treated with heat at 80^0 C. a (irradiated) and b (non-irradiated) from ASG; c (irradiated) and d (non-irradiated) from MSG.

Chapter Five

Discussion

Chapter Five

Discussion

5.1 Silk proteins extracted from whole silk gland of *Bombyx mori* in buffer solution

A total of six protein bands were detected from whole silk gland extracted in buffer at P^H 7.4. The sizes of the proteins were approximately 30 KDa, 45 KDa, 60 KDa, 70 KDa, 110 KDa and 220 KDa. The fibroin protein of 350 KDa could not be detected from the buffer extracted proteins of whole gland (Fig. 5). The absence of H- chain of ~350 kDa fibroin is unexpected. The possible reasons behind this unexpected result could be the early extraction methods for the silkworm. Initially, the method of Yutaka and Eiichi (1970) was not followed strictly for the protein extraction. Therefore, H-chaion of 350 kDa of fibroin might be damaged or lost. Yutaka and Eiichi (1970) reported that crashing the whole gland might remove the fibroin portion from samples as it is easily coagulated when force applied even faster rotation per minutes in extraction procedure. Later when we extracted silk proteins using buffer and water followed by the methods of Yutaka and Eiichi (1970), ~350 KDa protein was obtained from the ASG, MSG and PSG of *B. mori* (Figs. 6, 7 and 8 respectively).

The 30 KDa protein band were obtained from both the whole gland (buffer extraction) and MSG (water exatraction), which is presumably the chain of light chain of fibroin. It has been reported that the fibroin of *B. mori* is composed of heavy chain (350 KDa), light chain (26 KDa) and a glycoprotein P25 of 30 KDa (Chevillard *et al.*, 1986a, 1986b).

In this study, we presume that both the glycoprotein of 30 KDa and light chain of fibroin of 26 KDa could not be separated well by using mini gel electrophoresis system. It is possible that both the 26 KDa (light chain) and 30 KDa (P25 glycoprotein) might be present but are not separated enough to distinguish from each other. A long SDS-PAGE might be useful to separate these two close molecular weight proteins.

Note that different extraction procedures of silk protein of silk gland could generate different results. Muthamani *et al.*, (1995) obtained five protein bands ranging from 14.2 to 97 KDa in phosphate buffer at pH 7.0. On the other hand, Raman *et al.*, (2007) obtained 10-15 protein bands from the silk gland of *B. mori* in tris-cl buffer at pH 6.8. The authors also reported the presence of *B. mori* fibroin heavy chain as a streak of multiple bands from 350 to 150 KDa.

Mondal *et al.*, (2007) reported that three types of fibroin (heavy chain, light chain and P25 glycoprotein) are common in Lepidoptera though Saturnidae species secreted fibroin as a dimer of heavy chain. In this study two close protein bands of ~350 KDa (fig. 12) also obtained. Raman *et al.* (2007) also reported two close protein bands of 350 KDa but they did not give any further explanation of those two close protein bands. The authors only mentioned the presence of 350 KDa as fibroin heavy chain. We conclude that these two protein band of 350 KDa might be a dimer of heavy chain of fibroin. But to confirm this hypothesis, further analysis would be required.

Besides 350 and 30 KDa proteins another five protein bands of 45, 60, 70, 110 and 220 KDa were also obtained. Those proteins are probably the types or component of sericin, another important silk protein. Similar results were reported by Teramoto *et al.* (2005). They identified three distinct bands of sericin components were less than 250, 180 and 100 KDa. Presence of several sericins of *Bombyx mori* ranging from 65 to 400 KDa reported by Gamo *et al.* (1997). Mondal *et al.* (2007) classified the sericin of *B. mori* on the basis of their solubility. The authors classified a total of four sericins as sericin I, II, III, and IV. Michaille *et al.* (1986) and Couble *et al.* (1987) identified two sericin genes as *ser I* and *ser II*. This implies that at least two distinct types of sericin are present in *Bombyx mori*.

5.2 Proteins extracted from different parts of the silk gland

Three protein bands of sizes ~350, ~220 and ~110 kDa were detected from the proteins of anterior silk gland (Fig. 6). In the middle part of the silk gland four proteins of ~350, ~60, ~50 and ~30 kDa were obtained (Fig. 7). The 220 and 110 kDa proteins were not obtained from the

middle gland. Only two protein bands of 350 kDa and 30 kDa were obtained from the posterior silk gland (figure not shown, as the gel was damaged during photography). Akai *et al.* (2005) reported that *Bombyx mori* silk gland secretes one fibroin and three layers of sericin from the each posterior and middle silk gland. On the other hand, the anterior part of the silk gland is composed of 250 cells with no known secretory function (Mondal *et al.*, 2007). During spinning the silk, the liquid silk protein passes through the anterior silk gland and expelled out through the spinneret opening (Shimizu, 2000). Mondal *et al.* (2007) reported that middle silk gland is composed of about 300 secretory cells producing sericin, which is used to cement the fibroin thread of the cocoon. The authors also reported that the middle part of the silk gland is also used for storing the silk gland proteins. The results of the present findings confirm that the middle silk gland producing sericin proteins (Fig. 7). In this present work, only fibroin proteins of H-cian ~350, L-chain and p25 glycoprotein of ~30 kDa were recorded from the posterior part of the silk gland. Theses results are in good agreement with that of the reports of Akai *et al.* (2005) and Mondal *et al.* (2007). Suzuki *et al.* (1972) and Kimura (1985) also reported that the gene for H-fibroin, L-fibroin and P25 protein are expressed in posterior silk gland. As expected, we did get only fibroin proteins from the posterior silk gland but not the sericin. The middle silk gland was reported to be used for storing the silk proteins as well. Therefore, as expected, fibroin proteins in addition to the sericin proteins were also obtained in the middle silk gland (Fig 7). Tables 3, 4 and 5 further supports our findings that middle silk gland of *B. mori* serve as storing lumen as well. The proteins that were obtained from the anterior part of the silk gland were ~350, ~220 and ~110 kDa. It is expected that both fibroin and sericin would pass through the anterior silk gland before expelling out of the gland. Therefore, presence of both fibroin and sericin in the anterior part of silk gland is expected. The presence of ~220 and 110 kDa proteins in the anterior part of the gland is unique as both the proteins were not detected in either MSG or PSG. Further works are needed to clarify about these two proteins.

5.3 Effect of low-dose irradiation on the production of silk proteins

Exposing the eggs at their early embryonic stage clearly shows that low dose gamma irradiation (1.5 Gy) indeed increased the production of proteins in all the parts of the silk gland with varying degrees (Tables, 3, 4, and 5). The highest concentration of proteins were obtained in MSG (5.42

mg/ml) followed by PSG (3.16 mg/ml) and the lowest being recorded from ASG (2.26 mg/ml). The protein concentrations from unirradiated silk worms were recorded as 4.47 mg/ml, 2.82 mg/ml and 1.96 mg/ml in MSG, PSG, and ASG respectively. Comparing the proteins produced from unirradiated silkworm, 21.25%, 15.3%, and 12.05% increased amount of proteins were obtained from MSG, PSG and ASG of the irradiated silkworm respectively (Table 6). A total of 48.97% protein was found to increase in the irradiated silkworm when combine the amount of proteins obtained from ASG, MSG and PSG of *B. mori*.

The present findings are in good agreement with the previous reports from this laboratory that low dose gamma irradiation could increase cocoon shell weight and filament length (Hossain *et al.*, 2005a; Hossain *et al.*, 2005b).

Similar observation was also reported that exposure of the egg of *B. mori* to 2 Gy radiation accelerates the larval growth, increase in the cocoon weight and the raw- silk mass (Iusifov *et al.*, 1988). Many authors were also reported that low dose gamma irradiation (1.5 to 2.5 Gy) to egg could increase cocoon yeild, shell ratio and cocoon percentage (Ruiying *et al.*, 1985; Meesilp *et al.*, 1987 and Sutadis *et al.*, 1987). Hossain *et al.* (2005) also reported that irradiation to 5th instar larvae could also increase the cocoon shell weight albeit to a lesser amount (17.5%).

Recent reports from this laboratory showed that a dose of 1.5 Gy could increase up to 32.68% cocoon shell weight (Hossain *et al.*, 2006). In the present findings, total increased proteins (proteins from ASG, MSG and PSG) of the irradiated silkworm were calculated to be 48.97% compared to that of unirradiated ones. Obtaining the much higher amount of proteins (48.97%) than the reports of increased cocoon shell weight (32.68%) (Hossain *et al.*, 2006) could be explained that it might be the variations of experimental results conducted in different seasons. Another possibility might be that the proteins produced in the silk gland are not used totally for cocoon formation but retain in the silk gland for other purposes.

This study clearly reveals that low dose gamma irradiation (1.5 Gy) to embryonic stage could enhance the production of silk gland proteins of silkworm, *Bombyx mori*. But this study could not establish which specific protein (s) was increased due to irradiation effect.

Based on the results of this study and the early reports from this laboratory, a conclusion can be drawn that silkworm, *Bombyx mori* genome has specific hot spot which is very prone to be mutated by low dose irradiation. This mutation is not detrimental to the silkworm but beneficial in terms of the production of increased silk proteins. To proof this hypothesis, further works are required.

5.4 Thermal stability of silkworm proteins

Thermal stability of proteins from posterior silk glands was analyzed after incubating up to 90^0C. Comparing the proteins extracted in water with that of the proteins extracted in sodium phosphate buffer solution at pH 7.4, it was evident that the proteins extracted in water is slightly thermostable. The results showed no degradation or changes in protein bands up to 80^0C for 10 minutes incubation. But at 90^0C, the fibroin heavy chain of 350 kDa was started to be degraded which resulted fuzzy bands (Fig. 9). On the other hand, the fibroin heavy chain of 350 kDa proteins, extracted in buffer solution at pH 7.4, started to be degraded at 80^0C. At 90^0C, the heavy chain of fibroin 350 kDa was disappeared Fig 10 (a) and (b). Another observation is that some protein bands were also disappeared in buffer extracted proteins. These disappeared protein bands are presumably the components or type of sericin protein.

The present findings are in good agreement with that of Yutaka and Eiichi (1970). The authors reported that the fibroin is quite stable if extracts in water. In the present findings, the fibroin heavy chain was found to be less thermostable when extracted in alkaline buffer solution. The reports of Cecil, (1963) and Tanford, (1968) and Yutaka and Eiichi (1970) also supported our findings. These authors reported that at alkaline fibroin molecule may dissociate into subunits in alkaline buffer solution or some disulfide bonds of certain protein molecules become unstable in warm alkaline solution.

In the present results, it is evident that fibroin protein (both H and L chain) are more thermostable than the components of sericin proteins. This present findings are supported by Gulrajani (1988) and Hidetoshi *et al.*, (2005).

Gulrajani (1988) reported that sericin is insoluble in cold water, but it is easily hydrolyzed, where by the long protein molecules break down to smaller fractions, which are easily dispersed, or solubilised in hot water.

Hidetoshi *et al.*, (2005) reported that SDS-PAGE pattern of boiled sericin solution for 20 minutes still exihibited clear but slighty fuzzy protein bands which suggested slight decomposition of the protein molecules. When sericin was autoclaved for 121oC for 20 minutes the protein bands of sericin component disappeared showing significant decomposition of sericin molecules.

In this present study the light chain of fibroin (~30 kDa) proteins of irradiated silkworm is found to be more thermostable than the same proteins of unirradiated ones [Fig 11 (a) and (b)] . One possible explanation could be that irradiation might have changed the conformation of light chain of the fibroin protein resulted the L-chain more stable at high temperature. Unfolding and conformational changes of the fibroin molecule might occur in warm alkaline solution (Cecil, 1963 and Tanford, 1968).

APPENDICES

Appendix I

Table 7 optical densities (O. D) of irradiated posterior gland

Treatment	measured amount	O.D	X= Y-C/m mg/ml	average mg/ml	average of RP1 and RP2 mg/ml
RP1	0	0.088		3.30	3.16
	10	0.126	6.51		
	20	0.155	3.49		
	40	0.235	3.43		
	50	0.271	1.23		
	100	0.380	1.87		
RP2	0	0.088		3.05	
	10	0.124	5.17		
	20	0.158	2.91		
	40	0.250	3.94		
	50	0.298	1.65		
	100	0.301	1.59		

Table 8 optical densities (O.D) of non-irradiated posterior gland

Treatment	measured amount	O.D	X= Y-C/m mg/ml	average mg/ml	average of CP_1 and CP_2 mg/ml
CP_1	0	0.087		2.72	2.82
	10	0.107	3.42		
	20	0.137	3.57		
	40	0.181	3.31		
	50	0.224	1.47		
	100	0.311	1.83		
CP_2	0	0.086		2.92	
	10	0.111	4.29		
	20	0.139	3.40		
	40	0.196	3.40		
	50	0.228	1.88		
	100	0.322	1.61		

Table 9 optical densities (O.D) of irradiated middle gland

Treatment	measured amount	O.D	X= Y-C/m mg/ml	average mg/ml	average of CP_1 and CP_2 mg/ml
RM_1	0	0.087		5.75	5.42
	10	0.147	10.29		
	20	0.197	4.29		
	40	0.312	4.93		
	50	0.367	1.89		
	100	0.617	7.34		
RM_2	0	0.088		5.09	
	10	0.146	9.9.4		
	20	0.201	4.71		
	40	0.307	4.54		
	50	0.373	2.26		
	100	0.607	4.01		

Table 10 optical densities (O.D) of non- irradiated middle gland

Treatment	measured amount	O.D	X= Y-C/m mg/ml	average mg/ml	average of CM_1 and CM_2 mg/ml
CM_1	0	0.085		4.64	4.47
	10	0.140	9.42		
	20	0.170	3.94		
	40	0.260	3.86		
	50	0.306	1.58		
	100	0.565	4.44		
CM_2	0	0.085		4.30	
	10	0.149	9.60		
	20	0.183	3.60		
	40	0.270	3.73		
	50	0.318	1.65		
	100	0.580	3.92		

Table 11 optical densities (O.D) of irradiated anterior gland:

Treatment	measured amount	O.D	X= Y-C/m mg/ml	average mg/ml	average of RA_1 and RA_2 mg/ml
RA_1	0	0.082		2.10	2.26
	10	0.099	2.91		
	20	0.137	3.26		
	40	0.170	1.42		
	50	0.201	1.06		
	100	0.310	1.87		
RA_2	0	0.082		2.41	
	10	0.101	3.26		
	20	0.131	2.57		
	40	0.198	2.87		
	50	0.240	1.61		
	100	0.347	1.75		

Table 12 optical densities (O.D) of non-irradiated anterior gland

Treatment	measured amount	O.D	X= Y-C/m mg/ml	average mg/ml	average of RA_1 and RA_2 mg/ml
CA_1	0	0.082		1.86	1.96
	10	0.096	2.40		
	20	0.115	1.63		
	40	0.176	2.61		
	50	0.211	1.20		
	100	0.296	1.46		
CA_2	0	0.082		2.06	
	10	0.096	2.40		
	20	0.125	2.46		
	40	0.198	3.12		
	50	0.234	1.23		
	100	0.298	1.09		

Appendix II

1. % of increase = (increased value / control value) X 100

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