CHAPTER- 3

MATERIALS AND METHODS

MATERIALS

Fresh cocoons of *Antheraea assamensis* and fresh leaves of *Machilus bombycina* (som) plant were collected from IASST som plant garden.

Natural agents like *Musa balbisiana* (banana), *Citrus limon* (lemon) and *Dillenia indica* (elephant apple) have been collected from the local markets of Assam and the methods of preparation of working solution, juice and extract has been described in the methodology part in the following pages. Chemicals like bovine serum albumin (BSA), casein and lithium thiocyanate and dialysis tubing cellulose membrane were obtained from Sigma Aldrich (St. Louis, MO USA) while 2’ 2’ azobisisobutyronitrile (AIBN), sodium carbonate (Na$_2$CO$_3$), potassium carbonate (K$_2$CO$_3$), citric acid (C$_6$H$_8$O$_7$), ethylenediaminetetraacetic acid (EDTA), sodium hydroxide (NaOH), methanol (MeOH), hexane, ethyl acetate and silica gel were obtained from Merck Co. Ltd.

METHODS

DEGUMMING

Preparation of banana plant extract (‘kolakhar’)

The alkaline extract of banana, locally known as ‘kolakhar’ in Assam, has been prepared by the method of Deka and Talukdar (2007). The trunk, rhizome and peels of banana plant were slashed into pieces and sun dried for several weeks. After drying, the
materials were burnt into ashes and later extracted with normal tap water to form the desired kolakhar extract.

**Preparation of lemon juice**

The pulps of lemon were squeezed in a beaker to obtain pure concentrated lemon juice.

**Preparation of the jelly like substance from the seed sac of elephant apple**

The jelly like content were taken out squeezing the seed sac of elephant apple and mixed with distilled water to form a slimy solution.

The raw materials used for bio-degumming are shown in Plate 1.

**Cocoon weight**

The weights of the cocoons— with pupa and without pupa were measured respectively with the help of a single pan digital micro-balance (Mettler Toledo, PB153-S).

Muga cocoons (without pupa) were degummed using the following methods—

**Degumming with sodium carbonate solution (control)**

5 g of cut cocoons were degummed with sodium carbonate (Na$_2$CO$_3$) using the standard method. The cocoons were boiled in 500 ml of aqueous solution of 0.3% sodium carbonate (pH 11), with material to liquid ratio (MLR) 1:100 for 30 min at 90 °C.
**Degumming with kolakhar solution**

Five beakers were taken with 5 g of cocoons each and added 500 ml of distilled water, (MLR 1:100). Then kolakhar was added drop-wise to obtain pH solutions of 9, 9.5, 10, 10.5, 11 and 11.5 respectively. The experiments with the above pH solutions were repeated for seven times from 15 to 45 min at the interval of 5 min to observe the effect of different pH concentrations on degumming loss percentage, at different time intervals. The experiments were conducted at a temperature of 90 °C.

**Degumming with lemon juice solution**

The experimental set up as above has been followed for degumming the cocoons with lemon juice solution (MLR 1:100) at pH 2, 2.5, 3, 3.5, 4 and 4.5 respectively for seven times from 15 to 45 min at 90 °C.

**Degumming with slimy solution of elephant apple**

5 g of cocoons were treated with the slimy solution of elephant apple (MLR 1:100) at pH 4, 4.5, 5, 5.5 and 6 respectively at 60 °C. The experiments were repeated for four times from 24 to 96 hours at the interval of 24 hours to observe the effect of different pH concentrations on degumming loss at different time intervals.
Plate 1: Raw materials for bio-degumming of muga silk, a- lemon, b- banana plant and c- elephant apple
Degumming under high temperature and pressure condition (autoclave)

5 g of cocoons were degummed in 500 ml of distilled water under universal autoclave condition (temperature 121 °C and pressure 15psi) for 20 min (MLR 1:100).

After degumming, the cocoons were washed thoroughly with tap water and subsequently dried in room temperature to study the amount of degumming loss percentage and later stored in desiccator for further use.

Degumming of cocoons for reeling

Another experimental set up has been run using muga cocoons (pupa inside) about 1 g each for degumming in solutions of sodium carbonate, kolakhar, lemon, elephant apple and under conditions of high temperature and pressure (MLR 1:100) at their optimum pH, time and temperature. After degumming, the cocoons were washed and subsequently reeled in tap water at room temperature to measure the amount of reeled fibers after every degumming procedure. The fibers were then dried in room temperature and stored in desiccator.

Degumming loss percentage

Degumming loss signifies a quantitative estimation of degumming efficiency by calculating the weight loss before and after degumming treatment. Degumming loss (%) is expressed with the help of following equation:

\[ D = \frac{W_0 - W_1}{W_0} \times 100 \]
Where $W_o$ is the weight of the original silk fiber (g) and $W_1$ is the weight of degummed silk fiber (g).

**DEMINERALIZATION**

**Treatment with EDTA solution**

For the demineralization procedure, the standard method described by Gheysens et al. (2011) were used with slight modification in temperature range. An aqueous 1 M solution of EDTA was adjusted to pH 10 with saturated sodium hydroxide solution. The cocoons (n=3) was immersed in this solution for 72 hours at 45 °C in a shaking incubator with MLR 1:50.

**Treatment with kolakhar solution**

Solution of kolakhar was prepared accordingly to the method described above. Muga cocoons (n=3) were treated in this alkaline solution of pH 10.5 for 72 hours at 45 °C in a shaking incubator with MLR 1:50.

**Treatment with lemon juice solution**

Muga cocoons (n=3) were immersed in lemon juice solution of pH 2.5 similarly to the above mentioned demineralizing conditions.

**Treatment with potassium carbonate and citric acid solutions**

Muga cocoons (n=3) were demineralized in solutions of commercial potassium carbonate (pH 10.5) and citric acid (pH 2.5) respectively, similarly to the above mentioned demineralizing conditions. This part of experiment was done to compare the results of biological agents i.e. kolakhar and lemon with commercially obtained potassium carbonate and citric acid chemicals since, kolakhar and lemon are chemically
known to contain potassium carbonate (Deka and Talukdar, 2007) and citric acid as their main constituent.

The cocoons taken for demineralization were about 1 g each. All the treated cocoons were then thoroughly washed and subsequently reeled in tap water, at room temperature. The reeled fibers were then air dried and stored in desiccator for further use.

**Degumming**

In another experimental part after reeling of demineralized cocoon, the reeled fibers were degummed following the commercial standard method of degumming with 0.3% sodium carbonate solution at 90 °C for 30 min. Afterwards, the samples were thoroughly washed with tap water and air-dried overnight at room temperature.

**Reeling**

As mentioned earlier, the cocoons after degumming and demineralization, were wet reeled in tap water at room temperature. The length of reeled silk fiber has been measured and the number of fiber breaks during reeling were counted manually. Reeling was stopped when the transparent layer of fiber surrounding the pupa appeared.

**GRAFTING**

**Grafting with BSA and casein**

Degummed muga fibers of 1 g each were treated with BSA/ casein in a double necked round bottom flask containing distilled water (MLR 1:250) by soxhlet extraction process by adding initiator AIBN. To find the optimum condition for maximum graft yield (%) different concentrations of BSA, casein and AIBN at different time and
temperature were used. The concentration of BSA were taken in the range of \(0.8 \times 10^{19}\) M to \(3.6 \times 10^{19}\) M and casein was taken in the range of \(4.5 \times 10^{-3}\) M to \(9 \times 10^{-3}\) M. On the other hand, AIBN concentration was taken in the range of \(1 \times 10^{-3}\) M to \(3 \times 10^{-3}\) M. The reaction time was set in the range of 1–6 hours and the temperature was maintained from 30–90°C. After the desired reaction had taken place the fibers were taken out and rinsed thoroughly with distilled water. Further, the silk fibers were oven dried and placed in a desiccator before measurements. The degummed fibers were used as control. The graft yield (G) % was calculated by the following equation,

\[
G = \frac{M_1 - M_0}{M_0} \times 100
\]

Where \(M_o\) is the weight of the control silk fiber (g) and \(M_i\) is the weight of grafted silk fiber (g).

**Characterization of silk fiber after degumming, demineralization and grafting**

**Tensile properties**

The tensile properties of treated silk fibers were measured using Instron 4301(3343) universal tensile tester (interfaced with a computer) at atmospheric temperature of 27±2°C and humidity 65±2% RH. A gauge length of 5 cm, strain rate of 1 cm/min and cross head speed of 10 mm/min was taken for conducting the tests. 20 numbers of specimens were tested for each treatment to obtain average values of tenacity, strain percent, Young’s modulus and toughness.
**Denier**

In textile terminology, denier of a fiber is defined as the weight in grams of 9000 meters of the fiber (Booth, 1996). The denier is used to correlate the density and tensile strength because it is proportional to the density of a fiber and its cross-sectional area. When the denier decreases the filament becomes thinner or finer (Shenai, 1980).

To determine the denier of the fiber, a few pieces, each of 9 cm long, were cut from a finely prepared bundle of the fiber. The total number of the filaments contained in the lot was accurately counted from which the effective length of the fiber filaments was measured.

The weight of the fiber filaments already counted was measured with the help of an electronic single-pan balance (Mettler). Knowing the weight and length of the fiber filaments, the denier values were calculated for different fiber samples with the help of the following formula-

\[
\text{Denier} = \frac{\text{Weight (g)} \times 9000}{\text{Length (m)}}
\]

**Surface morphology by Scanning Electron Microscopy (SEM)**

Morphological view of the surface of the treated muga silk fibers were imaged by scanning electron microscopy (SEM) (Carl Zeiss Sigma VP). Samples were observed with incident electron beam energy of 5 kV, after gold sputtering.
Elemental analysis by Energy Dispersive X-Ray Spectroscopy (EDX)

Along with SEM an ISIS 300 EDX unit (Oxford Instruments) is operated, where the energy dispersive X-ray spectroscopy (EDX) were observed with electron beam energy of 20kV in SE2 detector mode for elemental analysis.

Elemental analysis by Atomic Absorption Spectrophotometry

The extracted metals present in the demineralized water obtained by the above methods were determined by Atomic Absorption Spectrophotometer (Shimadzu AA 7000) equipped with a deuterium lamp for background correction. The hollow cathode lamps for Ca, Cu, Fe, Al, Mn, Mg, Zn, Ni, Cd were employed as the radiation source. The source of flames used were N$_2$O and /acetylene and nitrogen was used as the carrier gas.

Chemical property by Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectra (FTIR) of the treated muga silk fibers were recorded by a Bruker Vector 22 FTIR spectrophotometer in the spectral range of 4000–400 cm$^{-1}$ in the absorption and ATR mode using 32 scans. For the absorption mode, silk fibers were cut into small pieces and were pressed into a dry press using appropriate binder (KBr) in the form of a pellet. In case of ATR mode the samples were analyzed directly without further sample preparation.

Thermal properties by Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC)

The thermal degradation of the silk fibers was studied using a Perkin-Elmer thermal analyzer, TGA 4000 (temperature accuracy: 0.25%, weighing precision: 0.01%).
Small pieces of fibers (3-4 mg) were kept in the aluminium sample pan of the TGA cell under nitrogen atmosphere at flow a rate 20 ml/min. A heating rate of 10 °C/min was maintained over a temperature range of 40-800 °C.

The DSC thermograms were recorded with a Perkin-Elmer thermal analyzer, DSC 6000 (temperature accuracy: 0.25%, weighing precision; 0.01%) coupled with thermo analyzer (TA) processor. Small pieces of fibers (2.1-3.3 mg) were kept in the aluminium sample pan of the DSC cell under nitrogen atmosphere at a rate 20 ml/min. A heating rate of 10 °C/min was maintained to get the DSC thermograms with temperature accuracy within the temperature range of 30-440 °C.

**Crystalline property by X-ray Diffraction (XRD)**

Diffraction curves were recorded with Bruker D8 Advance X-ray Diffractometer at a scan speed of 1 sec/step and increment of 0.02°. Voltage and current of the X-ray source were 40 kV and 30 mA, respectively.

Silk fibers were cut into small pieces and were pressed into a dry press in the form of a pellet. The pellet was put in the X-ray diffractometer and X-ray spectra were recorded for 2θ of 10-50°.

**Chemical resistance measurement**

The chemical resistance of grafted and control silk fibers was measured using acetic acid, benzene and toluene as the chemicals (Deka and Kakati, 2012). Each fiber was dipped in the respective chemical for 24 hours. The fiber was then removed from the respective chemical and washed with distilled water. The samples were dried in a
vacuum oven at 65±2°C for 2 hours and then weighed. The loss in weight was calculated in terms of percentage.

**Measurement of Water sorbency (Water Retention Value)**

For determination of water sorbency, the control and grafted silk fibers (of known weight) were immersed in distilled water for 2 hours. The fibers were then taken out and excess water was removed by putting the fibers inside two filter papers under gentle pressure. The fibers were weighed and recorded (Wet). The fibers were then dried in an oven at 65±2°C, weighed and recorded (Dry). The water sorbency is expressed as the water retention value (WRV) in grams of water per grams of the fiber and was calculated as follows-

\[
WRV \ (g/g) = \frac{Wet - Dry}{Dry}
\]

**Dynamic Contact Angle Measurement**

The dynamic contact angle of the control and grafted fibers were measured in tensiometer (DCAT-11, Dataphysics, San Jose CA, USA) using dynamic Wilhelmy technique (Sudit and Chen, 1994) at ambient conditions. The wetting properties of the fibers were studied using distilled water as the medium. The muga fibers were immersed in the liquid (distilled water) at a speed of 0.1mm/sec with the help of a computer controlled motor at an immersion depth of 2.5 mm. The instrument provided a measuring contact angle accuracy of ±0.01° with a position resolution of 0.1 µm.
**Statistical Analysis**

Results were expressed as mean ± standard deviation for n=3, unless indicated otherwise. Statistical analysis of data were performed by one-way analysis of variance (ANOVA) using Origin 6.1 software. Differences between experimental groups at a level of p ≤0.05 were considered as statistically significant and those at p ≤0.001 as highly significant.

**PIGMENTATION PROFILE**

**Extraction of som leaf**

The leaves of som plant were collected and washed with normal tap water to remove the dirt particles and then subsequently air dried at room temperature. The dried leaves were finely powdered (20 g) in a grinder and extracted in 100% methanol in the ratio (MLR) 1:25 under oven condition at 70 ºC for 48 hours. After that the extracts were filtered and the filtrate were concentrated under reduced pressure at 40 ºC using rotary evaporator to obtain a viscous semi solid mass.

**Extraction of muga cocoon**

For muga fiber extraction, the non-degummed cocoons (40g) were cut into small pieces (pupa removed) and extracted with 100% methanol (MLR 1:25) under oven condition as above and filtered. The filtrate was concentrated under reduced pressure at 40 ºC using rotary evaporator to obtain a concentrated extract solution. During the process the sericin protein contained in the extract were precipitated out, which were collected separately and kept in 4 ºC for further use. Plate 2 shows the flowchart of extraction of muga cocoons and som plant leaves.
**Dissolution of fiber**

Dried muga cocoons (pupa removed) were cut into small pieces and degummed with boiling aqueous solution of 0.3% sodium carbonate for 1 hr at 100° C. After degumming, the whole mass was repeatedly washed with distilled water to remove the glue-like sericin protein and eventually dried in hot air oven. Silk fibroin solution was prepared by dissolving 0.5 gm of degummed silk in 9.3M lithium thiocyanate (LiSCN) solution at 70°C for 7 hrs. The fibroin solution was dialyzed in a cellulose membrane based dialysis cassette (molecular cutoff 12,400 da) against distilled water for 3 days, changing water every 6 hours in order to remove LiSCN. After dialysis, silk fibroin solution was centrifuged at 5-10 °C and 9000 rpm for 20 min. The concentrated solutions were stored at 4 ºC for further study. The flowchart for dissolution of muga fiber is shown in Figure 1.

**Analysis of cocoon and leaf extract by-**

**UV-Visible Spectroscopy**

The UV-Visible spectroscopy of crude methanolic extracts of muga cocoons and som leaves and dissolved fiber solution were done using a Shimadzu UV Spectrophotometer UV 1800. The scanning was performed in the UV-visible region from wavelength 190-800nm in the absorption mode.
Plate 2: Flowchart of methanol extraction of muga silk cocoons and som leaves
Thin Layer Chromatography (TLC)

Thin layer chromatography was performed in silica gel bound aluminum plates. Extracts of som leaves and muga cocoons, sericin solution and fibroin solution were spotted on the TLC plate using a capillary tube. The spots were allowed to dry by a dryer. The TLC plate was placed inside a beaker containing the mobile solvent system hexane and ethyl acetate in the ratio 4:1. The mobile organic solvents were allowed to move up the plate, potentially carrying with it the various compounds present in the
respective samples. The different compounds present in the samples are separated based on their affinities for the stationary phase (silica) and for the mobile phase. The TLC spots were visualized in an UV chamber. Rf (retention factor) value of the spots were calculated as follows-

\[
Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by the solvent system}}
\]

**Column chromatography**

The adsorbent (silica) was poured dry in a cylindrical tube that is plugged at the bottom by a porous disc. The samples i.e. extracts of cocoons and leaves were introduced at the top of the column and were allowed to pass through the same by pouring mobile solvent continuously through it. At first 100% hexane was used as the mobile phase but since compound separation was not seen in this solvent system the polarity of the solvent system was increased by adding ethyl acetate (5%). Latter, for further good separation of sample mixture the mobile system used was adjusted to the ratio of hexane: ethyl acetate (9: 1). The separated compounds were collected in different test tubes, which were concentrated in a rotary evaporator at 40 °C under reduced pressure like the above experiments. The column purified fractions obtained from the sample extracts were respotted on a TLC plate like above and visualized in the UV chamber for confirmation of their Rf values. The purified fractions obtained after column chromatography were used for our further analysis.
Gas Chromatography-Mass Spectroscopy (GC-MS)

The purified samples were filtered through 0.2 µm filter for GC-MS analysis performed in a Perkin Elmer Clarus 680/600C unit fitted with Elite 5 MS column (30m×0.25mm, 0.25 µm film thickness). The injector and detector temperatures were maintained at 300 ºC. The samples were injected in the split mode, using pressure controlled helium as carrier gas at a linear velocity 37.2 cm/s (at 50 ºC). The oven temperature was programmed from 50°C (after 2.5 min) to 150°C at 15°C/min. Again temperature was increased upto 200 ºC at 3° C/min. The final temperature was programmed to 300 ºC at 8 ºC/min and held for 8 min. The mass spectrometer (Clarus 600C; single quad) was operated in the electron ionization (EI) mode at 70 eV with a source temperature of 200ºC and a continuous scan from $m/z$ 50 to 600. The peaks were identified by matching the mass spectra with the National Institute of Standards and Technology (NIST) library, USA.

Nuclear Magnetic Resonance Spectroscopy (NMR)

$^1$H NMR spectra were obtained at 600 MHz on a Bruker DPX-300 spectrometer. Chemical shifts are given in $\delta$ values (ppm) using tetramethylsilane as the internal standard. Peak multiplicities are expressed as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublet; br, broad; br s, broad singlet; m, multiplet. The sample is dissolved in ca. 0.6-0.7 ml deuteriated CDCl3 and then filtered through a Pasteur pipette to remove the unwanted particles.