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Thermal behavior of regenerated *Antheraea pernyi* silk fibroin film treated with aqueous methanol

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Abstract

The regenerated *Antheraea pernyi* silk fibroin films prepared from calcium nitrate solution were treated with 80% aqueous methanol. The thermal and dynamic thermomechanical behaviors of the films were investigated through TGA, DSC and DMTA. IR spectroscopy was used for the conformational changes with methanol treatments. The treatment time of aqueous methanol highly influenced the thermal behavior of regenerated films. As the methanol treatment time increased up to 60 min, the thermal decomposition behavior did not differ significantly compared with untreated films while the *endo*/*exo* transition was gradually decreased, with the exotherm finally disappearing. The dynamic mechanical thermal behavior was also affected by the treatment time of aqueous methanol. The onset temperature of storage modulus drop or damping peak shifted to a higher temperature due to the increase in crystallinity induced by the methanol treatment. The thermal transition was strongly dependent on the formation of β-sheet crystal conformation of the regenerated films upon methanol treatment.

Keywords: *Antheraea pernyi* silk fibroin; Methanol treatment; Thermal transition

1. Introduction

In recent years, growing interests on silk polymers as sources of textile fibers and biomaterials have promoted numerous studies on their structure and properties including enzyme immobilization, oxygen permeable membranes, and matrix for mammalian cell culture [1–6]. The *Bombyx mori* silk fibroin was of a special interest with many applications in biological and biomedical fields. The structure and physical properties of the fibroin films could be changed with simple physico-chemical treatments such as stretching [7], thermal treatment [8–10], and immersion in selected organic solvents [11–13].

The silk polymer produced by *Antheraea pernyi*, a wild-type silkworm, has been used as a textile fiber, and demands on its biomaterial applications including enzyme immobilization [14] and matrix for cell culture [15] are increasing. The molecular conformations of native *A. pernyi* fibroin films, taken from the posterior silk gland of a full-grown silkworm larvae, were reported by Freddi et al. [10,12]. The influences of casting temperature [13], drying rate [9], and aqueous methanol [12] and heat treatments [10] on the molecular conformation of the fibroin were extensively studied. The silk fibroin, once solidified through a spinning process of the silkworm, became a well-oriented and highly crystallized polymer. A silk fiber behaves like a thermoset polymer, although it is not completely crosslinked. Since the dissolution of *A. pernyi* silk fiber is difficult, the structural characteristics of regenerated form have been little investigated [16,17].

The applications of regenerated *A. pernyi* silk fibroin are somewhat restricted due to the difficulties in controlling the shape. Dissolution of silk fibroin is often required when nontextile applications are demanded in forms of film, porous membrane, powder, gel, and among others. *A. pernyi* silk fibroin film, containing several basic amino acids and the tripeptide sequence arg-gly-asp, can be highly attractive for several biotechnological and biomedical applications not only on enzyme immobilization and matrix for mammalian cell culture but also on wound covering and artificial skin.

α-Helix is the main conformation of *A. pernyi* silk fibroin regenerated from lithium thiocyanate [16] and calcium nitrate [17]. The conformational transition of fibroin film regenerated from calcium nitrate was dependent on the methanol concentration and immersion time [17]. The conformational changes from a random coil to β-sheet

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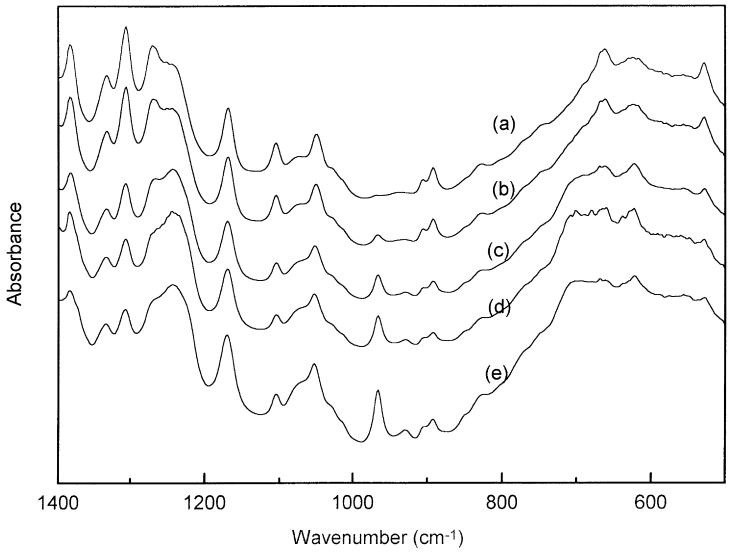


Fig. 1. FTIR spectra of regenerated *Antheraea pernyi* silk fibroin films treated with 80% aqueous methanol for: (a) 0; (b) 10; (c) 30; (d) 60; and (e) 90 min.

structure occurred within 5 min after the treatment of 40–60% aqueous methanol. As the methanol concentration increased up to 100%, the transition time was delayed.

Our present work focuses on the study of thermal behavior of regenerated *A. pernyi* silk fibroin films treated with aqueous methanol for crystallization. The thermal and dynamic thermomechanical behaviors of these films, revealed through DSC, TGA, and DMTA, will be discussed along with the conformational changes of silk fibroin film.

2. Experimental

2.1. Materials

The *A. pernyi* silk fibers were degummed using enzymatic degumming method and dissolved in calcium nitrate solution. They were first treated with degumming solution (Alcalase 2.5 l from Novo Industri Co. 1 g/l, sodium bicarbonate 5 g/l, and nonionic surfactant 1 g/l) at 55°C for 60 min. The degummed fibers were washed in a mixture solution of nonionic surfactant (2 g/l) and sodium hydrosulfite 5% on the weight of fiber and thoroughly rinsed in warm distilled water. They were then dried at room temperature and stored in a desiccator prior to use.

The dissolution of degummed fibers were carried out in the melt of calcium nitrate 4 hydrate for 5 h at 105°C. The solution was then dialyzed in a cellulose tube (molecular cut-off = 3500) against distilled water for 4 days at room temperature. The regenerated *A. pernyi* silk fibroin solution (0.3%), filtered through Whatman filter paper, was cast on a polystyrene plate as the substrate at 20°C and 40% relative humidity.

According to our preparatory experiment, the dissolution of *A. pernyi* silk fibroin required a high concentration of chaotropic salts, high temperature, and somewhat long treatment time. The silk fiber could not be dissolved in less than 6 M calcium nitrate solution at 100°C for prolonged times. When dissolution conditions are severe, decomposition occurs with a decrease in molecular weight, in which case regenerated film cannot be formed. Therefore, the preparation conditions of the film are fixed to avoid a variance of sample which affects the thermal behavior.

2.2. Measurements

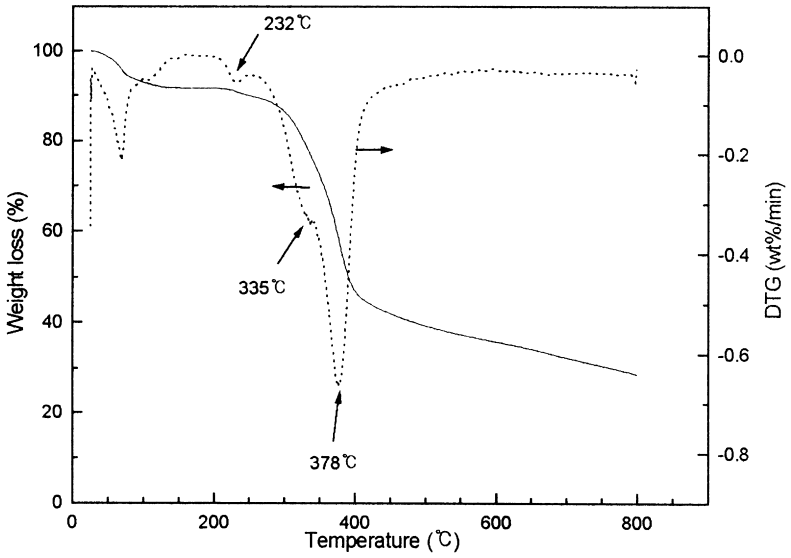
Fourier transform infrared spectra were obtained using a Midac M series spectrometer (USA) in the spectral region of 1400–500 cm^{-1}. Thermogravimetric Analysis (TGA) was run under the flow of nitrogen gas at a scanning speed of 20°C/min using Rheometric Scientific TGA 1000 (USA). Differential Scanning Calorimeter (DSC) curves were obtained through a Thermal Analysis Instrument (TA 2910, USA) at a heating rate of 10°C min^{-1} and nitrogen gas flow rate of 50 ml min^{-1}.

The dynamic mechanical thermal properties were measured using Rheometric Scientific DMTA Mark 4 (USA). The frequency of oscillation was adjusted to 1 Hz. The temperature range studies was from 100 to 250°C, and the heating rate of the sample was 4°C/min.

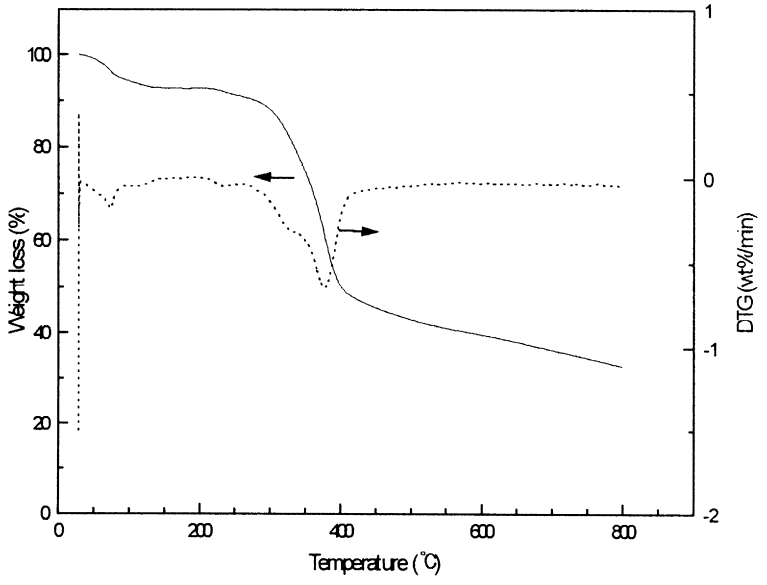
3. Results and discussion

3.1. IR spectra

IR spectroscopy is used by many researchers to study the conformation of silk fibroin as the IR spectrum represents typical absorption bands sensitive to the molecular conformation of silk fibroin. The IR spectra of the regenerated *A. pernyi* silk fibroin films treated with 80% aqueous methanol are shown in Fig. 1. The methanol treatment time was varied to discover the effect of treatment time on the



(a)

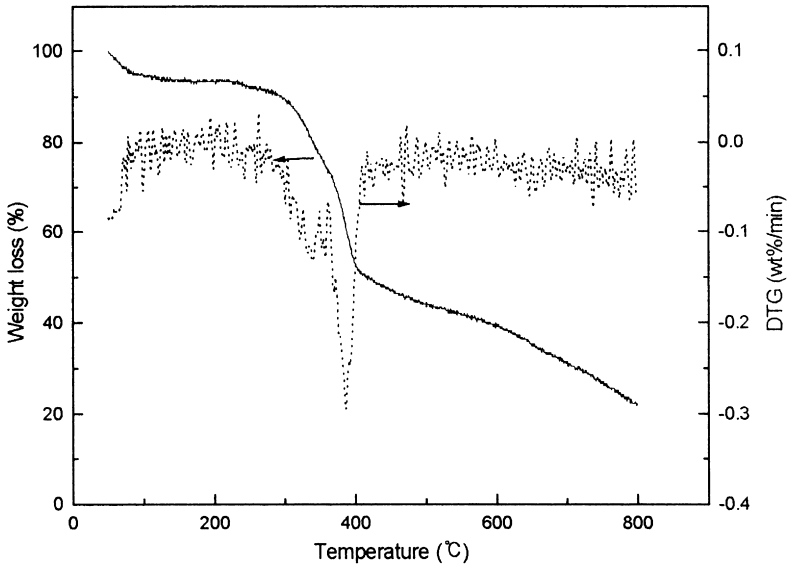


(b)

Fig. 2. Weight loss and differential weight loss curves of regenerated *Antheraea pernyi* films treated with 80% aqueous methanol for: (a) 0; (b) 30; and (c) 60 min.

conformational changes, which affect the crystallinity of the fibroin film. The untreated regenerated film showed strong absorption bands at 1270 cm^{-1} (amide III), 896 cm^{-1} (amide IV) and 625 cm^{-1} (amide V), attributed to the α-helix conformation, and 660 cm^{-1} (amide V), to a random coil conformation. As the immersion time is increased up to 90 min, the strength of the absorption bands at 660 and 896 cm^{-1} decreased while those at 1240 cm^{-1} (amide III), 965 cm^{-1} (amide IV), and 700 cm^{-1} (amide V), attributed to β-sheet conformation, increased. In particular, the IR spectrum of fibroin film treated with methanol for 30 min was drastically different from that of the untreated film.

The conformational transition of regenerated [17] and native [11,12,18] *A. pernyi* fibroin films induced by aqueous methanol has been studied extensively. The results revealed the concentration of aqueous methanol significantly affected the conformational changes of the fibroin film. It was also reported that the transition time of conformation change was



(c)

Fig. 2. (*continued*)

delayed as the methanol concentration was increased. Especially, when absolute methanol was used in the crystallization of regenerated film, the conformational change to β-sheet structure did not occur. In the case of 80% aqueous methanol (Fig. 1), complete conformational change to β-sheet structure could be obtained with a prolonged time (more than 60 min), indicating the delay of crystallization.

3.2. Thermogravimetry

The thermogravimetric curves of regenerated fibroin films are shown in Fig. 2. Weight loss, which occurred below 150°C, was due to the evaporation of water. The regenerated film showed a distinct weight loss (%) in three decomposition stages. According to TGA curves, the regenerated *A. pernyi* fibroin film underwent three main thermal decomposition stages: the first step, slight thermal decomposition of silk fibroin molecules which occurs from 220 to 300°C; the second, abrupt decomposition step from 300 to 370°C; and the third, from 370 to 400°C.

The differential weight loss (DTG) curves support a clear evidence for the three degradation steps. The maximum degradation temperatures of the three steps in regenerated fibroin film were 232, 355, and 378°C, totally different from the result of *B. mori* silk fibroin [19,20]. Weight loss and DTG curves did not significantly change although the treatment time of methanol was increased.

Thermal decomposition of *B. mori* fibroin film took place in a single step, but those of *A. pernyi* silk fibers and other silks belonging to the family Saturniidae underwent several steps [20,21]. Polymorphs of crystalline structure and amino acid composition of *A. pernyi* silk fibroin are different from those of *B. mori* silk fibroin. Therefore, the thermal degradation behaviors of *B. mori* and *A. pernyi* silk fibroin are expected to be different from each other. According to the previous results [17], the total crystallinity was about 76% when the regenerated film was immersed in 80% aqueous methanol for 60 min. In spite of a long methanol treatment time on the regenerated films, the thermal decomposition and DTG curves of the films did not change significantly from those of the untreated films, indicating that the change of conformation seems to little affect the thermal decomposition behavior.

3.3. Differential scanning calorimetry

The DSC thermograms of regenerated film treated with absolute methanol are shown in Fig. 3. The regenerated silk fibroin exhibited sharp endothermic and exothermic peaks at 228 and 232°C, respectively. The endotherm at 228°C is attributed to the strong molecular motion within α-helix crystals, while the sharp exotherm at 232°C could be attributed to the crystallization during heating by forming β-sheet structure from a random coil conformation [22]. Moreover, another major endotherm appeared at ca 360°C with a minor weak endotherm at 286°C. It is known that this endotherm is due to the decomposition of fibroin molecules with unoriented β-sheet conformations [8,11,16]. When absolute methanol was used, DSC curves of regenerated films were similar regardless of treatment times, which indicates that absolute methanol does not induce the conformational changes of *A. pernyi* silk fibroin film.

Contrary to the DSC thermograms of fibroin film treated with methanol, in 80% aqueous methanol treatment, the thermal behavior was significantly dependent on the treatment time. Although main decomposition peak at ca 360°C

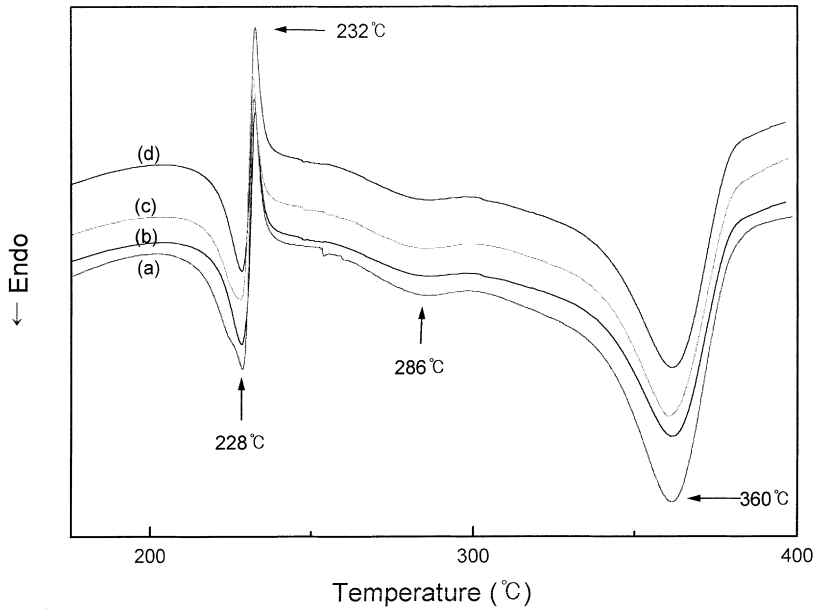


Fig. 3. DSC thermograms of regenerated *Antheraea pernyi* films treated with absolute methanol for: (a) 0; (b) 30; (c) 60; and 120 min.

did not change, Fig. 4 shows that the intensity ratio of *endo*/*exo* transition peak, appearing around 230°C, decreased as the methanol treatment time increased up to 60 min. Moreover, the crystallization exotherm at 232°C completely disappeared when the regenerated film was treated with 80% aqueous methanol for over 60 min, which could be explained by the result of the IR spectra (Fig. 1), showing the conformational transition from a random coil to β-sheet structure varied with the treatment time of 80% aqueous methanol. According to the calculated values of crystallinity index through IR spectra reported previously [17], the β-sheet content of regenerated *A. pernyi* fibroin can be increased by the treatment of aqueous methanol. As such the decrease in *endo*/*exo* transition on DSC curves is attributed to the previous crystallization of regenerated film induced by aqueous methanol treatment. However, recrystallization did not occur in the crystal form of β-sheet structure.

3.4. Dynamic mechanical thermal analysis

Dynamic mechanical thermal analysis (DMTA) is known as a more sensitive method of monitoring the side/main-chain motion in specific regions and local mode relaxations

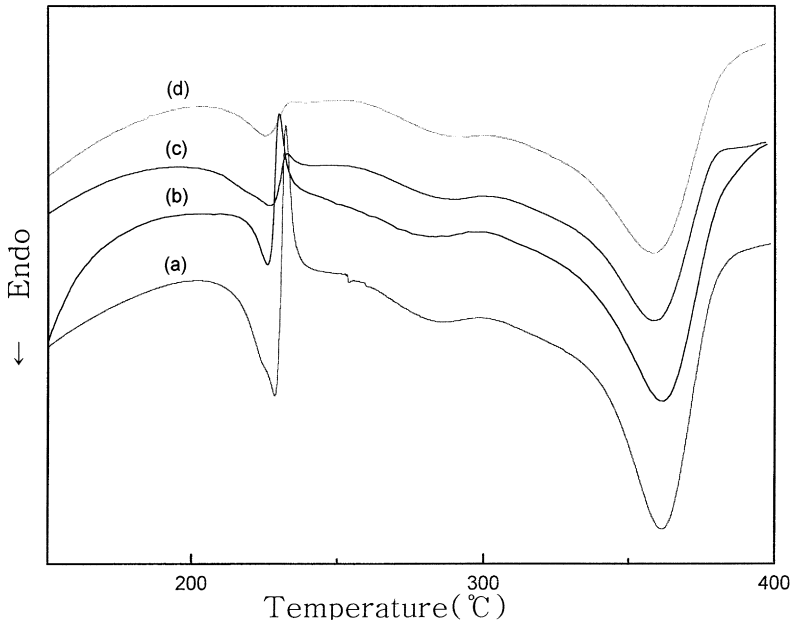


Fig. 4. DSC thermograms of regenerated *Antheraea pernyi* films treated with 80% aqueous methanol for: (a) 0; (b) 10; (c) 30; and 60 min.

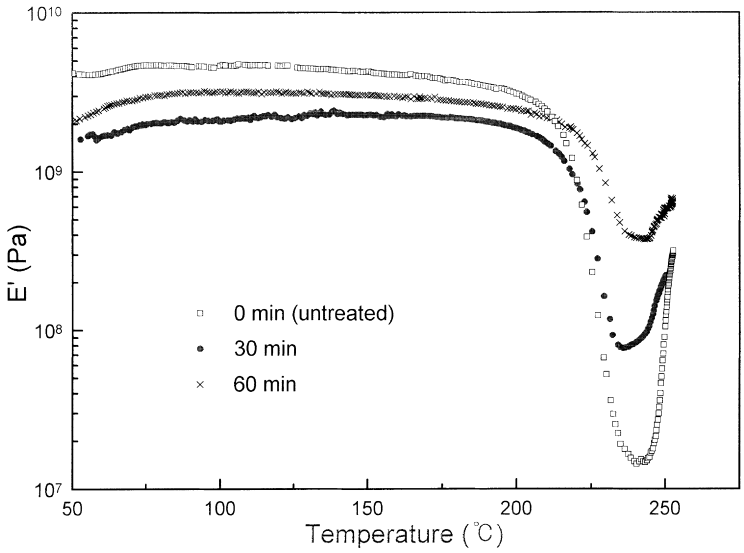


Fig. 5. Dynamic storage modulus (E') of regenerated *Antheraea pernyi* silk fibroin films treated with 80% aqueous methanol at different time periods.

of the silk protein. The dynamic storage modulus (E') and mechanical damping (tan δ) of regenerated *A. pernyi* silk fibroin films were examined. Fig. 5 shows the storage modulus curves of regenerated films treated with 80% aqueous methanol at different time periods. For untreated regenerated film, the storage modulus began to drop remarkably around 210°C, reached a minimum around 240°C, and then finally increased. As the treatment time of aqueous methanol increased, the onset temperature of the storage modulus drop shifted to somewhat higher temperature and the height of minimum appearing at ca 240°C markedly decreased. The onset temperature of abruptly decreased dynamic storage modulus was calculated from the point of intersection between the two extension lines.

Magoshi et al. [23] reported on the crystallinity dependence of dynamic modulus E' for the *B. mori* silk fibroin film. With the increase in crystallinity, the onset temperature of fibroin film shifted to higher temperature and the minimum at 200°C decreased in its height and became less apparent. In the case of PET, a synthetic polymer, a similar result was reported [24].

According to our previous report [17], the crystallinity index of *A. pernyi* silk fibroin treated with 80% aqueous methanol increased from 45 to 76% with increase in treatment time up to 60 min. Concerning the crystallinity differences measured with IR spectroscopy, the shift of onset temperature to a higher value and the decrease of minimum depth at 240°C could be interpreted by the increase in crystallinity of regenerated fibroin film. On the other hand, the increase in storage modulus starting ca 240°C are attributed to the recrystallization of amorphous regions accompanying the temperature-induced transition.

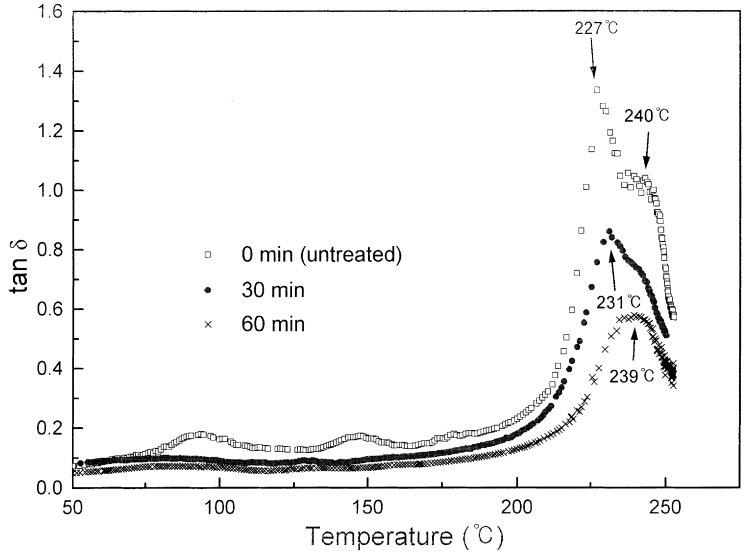


Fig. 6. Tan δ of regenerated *Antheraea pernyi* silk fibroin films treated with 80% aqueous methanol at different time periods.

In the glass transition region, some molecular chain segments are frozen while others are free to move around. The damping peak is associated with the partial loosening of the polymer structure enabling the groups and small chain segments to move freely. The mechanical damping dissipates energy as heat during deformation. Fig. 6 shows the mechanical damping of regenerated *A. pernyi* silk film treated with 80% aqueous methanol. The loss tangent increased abruptly at 210°C, reached a maximum at 227°C, and then decreased. After passing through a minor shoulder peak ca 240°C, the loss tangent again decreased. As the methanol treatment time increased up to 60 min, the maximum damping was shifted slightly to a higher temperature merging to the shoulder peak. The loss tangent peak is attributed to the segmental motion of molecular chains not only in the amorphous but also in the crystalline regions. According to our results, a lower damping peak is the main glass transition temperature related to amorphous regions. On the other hand, a higher peak appeared due to the crystallization during heating in thermal experiments. In the case of complete conformational changes which occurred to the β-sheet crystal structure (60 min), only single damping peak appeared at ca 240°C, while the damping peak affected by the crystallization was absent.

The magnitude of damping peak of untreated fibroin film is much higher than that of methanol-treated film because the chain segments of the untreated regenerated film are free from restraints imposed by the methanol treatment.

The regenerated *A. pernyi* silk fibroin films were cast from calcium nitrate solution and treated with 80% aqueous methanol. The conformation of these films changed with the aqueous methanol treatment at different time periods from a random coil and α-helix to β-sheet structure. As the treatment time of aqueous methanol was increased, the thermal characteristics of regenerated films, attributed to the crystallization of silk fibroin from a random coil to β-structure, diminished and finally disappeared.

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