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Stabilization of Enzymes in Silk Films

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Abstract

Material systems are needed that promote stabilization of entrained molecules, such as enzymes or therapeutic proteins, without destroying their activity. We demonstrate that the unique structure of silk fibroin protein, when assembled into the solid state, establishes an environment that is conducive to the stabilization of entrained proteins. Enzymes (glucose oxidase, lipase and horseradish peroxidase) entrapped in these films over ten months retained significant activity, even when stored at 37°C, and in the case of glucose oxidase did not lose any activity. Further, the mode of processing of the silk protein into the films could be correlated to the stability of the enzymes. The relationship between processing and stability offers a large suite of conditions within which to optimize such stabilization processes. Overall, the techniques reported here result in materials that stabilize enzymes to a remarkable extent, without the need for cryoprotectants, emulsifiers, covalent immobilization or other treatments. Further, these systems are amenable to optical characterization, environmental distribution without refrigeration, are ingestible, and offer potential use in vivo, since silk materials are biocompatible and FDA approved, degradable with proteases and currently used in biomedical devices.

Keywords

enzyme; silk; fibroin; stability

Introduction

Enzyme stability is a critical feature for many applications. Even if an enzyme is identified to be useful for a given reaction, its application is often hampered by a lack of long-term stability under process conditions [1]. Therefore, it is important for many biomedical and industrial applications with enzymes and protein therapeutics to select proper stabilization or immobilization methods to improve enzyme activity to preserve function for the intended use. Therefore, extensive studies have been conducted into modes to stabilize enzymes and therapeutic proteins for a range of applications. In general, improved enzyme stability offers: (1) improved effectiveness of biomedical devices for controlled release of protein therapeutics; (2) stable enzyme-based devices for analytic and biomedical applications; (3) lowered costs through efficient recycling and control of the biocatalysis process for industrial applications; (4) fundamental tools for solid-phase protein chemistry [2].

Many modes of enzyme stabilization have been studied, including lyophilization [3], covalent immobilization [4], chemical and genetic modification of enzymes [5,6], and using nonaqueous

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organic solvents or ionic liquids as reaction medium [7,8]. In general, many immobilized enzymes demonstrate improved stability, likely due to reduced mobility to prevent changes in hydrophobic hydration and thus aggregation and loss of activity [9]. Traditionally used immobilization techniques fall into four categories: (1) noncovalent adsorption of enzymes to carrier material surfaces; (2) covalent attachment to material surfaces; (3) physical entrapment into a material matrix; and (4) crosslinking of an enzyme to "lock" the structure [10]. All these approaches are a compromise between maintaining high catalytic activity while achieving the advantages listed above. The lack of materials that provide specific surface binding sites or relative hydrophilic/hydrophobic microenvironments for the retention of high enzyme loading and activity limits the application of the above carrier-based immobilization approaches. Further, for biomedical applications, carrier materials need to be biodegradable and biocompatible, which rules out the use of most synthetic polymer materials.

Recently, new immobilization approaches have been developed to improve enzyme stability and activity [11]. For example, the microenvironment of the carrier material can be engineered by using blocking agents to reduce non-specific binding sites [12,13]. Hydrophilic macromolecules can be introduced proximal to the enzyme [14] or hydrophilic spacers used between the enzyme and the material surface [15]. Sol-gel materials have been used for immobilization and found to enhance the activity of lipases up to 88-fold due to the effects of microenvironmental confinement [16]. Enzyme cross-linking methods have been combined with protein crystallization to generate cross-linked enzyme crystals (CLECs) with increased enzyme stability and selectivity when compared to the native enzyme [17,18]. This method has also been used by pharmaceutical companies to formulate therapeutic protein drugs. However, the crystallization of proteins is not straightforward and often is unpredictable. Cross-linked enzyme aggregates (CLEAs) can be obtained by precipitation of proteins followed by cross-linking with glutaraldehyde. The method was used for stabilizing penicillin acylase in antibiotic synthesis and the result showed large improvements over other types of biocatalysts [19]. An alternative strategy to stabilize enzymes is using a carrier material, usually micro- or nanoparticles. Enzyme molecules are either noncovalently coated to the particles (so called protein coated micro crystals technique [20]) or covalently bound to the particles [21]. Thus, overall, there are a number of options today to improve enzyme stabilization. However, few of these methods are widely applicable, few are simple to use as a process, few generate enzyme systems that retain significant function at ambient storage conditions (e.g., room temperature) for long periods of time, and most of them lead to significant increased costs for the enzyme.

Silk is a unique protein biopolymer, with a block copolymer structure dominated by large hydrophobic domains and small hydrophilic spacers. This primary sequence, upon folding into assembled silk structures, leads to organized crystalline domains (beta sheets) and less organized more flexible domains (more hydrated) [22,23]. This assembly leads to localized nanoscale pockets where other proteins may be entrapped with limited but sufficient hydration. Further, silk biomaterials are inherently very stable to changes in temperature and moisture, along with being mechanically robust, due to the extensive network of physical crosslinks (beta sheets) formed during the assembly process [24,25]. Thus, as a starting point as a protein immobilization matrix, this biomaterial offers some important features that suggest utility as a stabilization matrix. Further, since the protein can be prepared and treated under ambient conditions in water, this serves as an important processing platform for retention of bioactivity of components incorporated in the process, since no harsh chemicals, temperature or pressure are needed to form stable material systems.

A number of prior studies have demonstrated that silk fibroin can be used as an immobilization matrix for enzymes, including use in biosensors. Glucose oxidase (GOx) was prepared in silk fibroin films that were physically treated by stretching, compression and with methanol

immersion to induce water-insolubility [26]. Enzyme stability improved with these treatments and the structural changes were characterized and related to the silk crystalline beta-sheet structure. Residual activity of GOx in these silk membranes was more than 90% when maintained at 4°C for 4 months [26]. GOx-immobilized silk fibroin films after methanol treatment were studied as amperometric glucose biosensors for the analysis of whole blood or serum and they retained activity over two years when stored at 4°C [27]. Silk fibroin protein was also evaluated for the immobilization of tyrosinase. Immobilized tyrosinase showed a higher degree of stability during 10 days storage and retained 80% of its original activity after repeated reuses [28]. In another study, heme-proteins, such as hemoglobin and myoglobin, were embedded in a silk fibroin film on graphite electrode surfaces that were treated with 80% methanol. After drying, the films exhibited nearly reversible cyclic voltammetric peaks, indicating that proteins retained their activities in the film [29]. The experiment was performed at room temperature, but protein stability versus time and temperature was not described.

In the present study, three enzymes with diverse physicochemical properties were studied with respect to long-term stability (up to ten months) in both water-soluble and insoluble silk fibroin films. The goal was to evaluate the impact of silk processing conditions (water vs. solvent) and environmental storage conditions (time and temperature) on the enzyme stability in the films. The mechanisms underlying enzyme stability in these systems were investigated in terms of silk structural changes, enzyme spatial distribution in the film, and enzyme denaturation/renaturation. The study provides baseline insight into a broader technological approach for the use of these novel silk protein material systems for the sustained stabilization of protein-based compounds, with applicability for silk-based biosensors as well as a range of biomedical and environmental uses.

Experimental Procedures

Materials

All enzymes (peroxidase from horseradish (HRP) (EC 1.11.1.7, Type VI-A, 1080 units/mg), glucose oxidase (GOx) from *Aspergillus niger* (E.C.1.1.3.4, Type II, 21 units/mg), and Lipase from *Candida rugosa* (EC 3.1.1.3, Type VII, 700 units/mg)) were purchased from Sigma and used without further purification. The standard substrate of HRP, 3,3'5,5' Tetramethylbenzidine (TMB) solution, was purchased from BioFX laboratories (Owing Mills, MD). Firefly Luciferase Assay Kit (30003-1) from Biotium, Inc., Amplex® Red Hydrogen Peroxide Assay Kit (A22188), Amplex® Red Glucose Oxidase Assay Kit (A22189), EnzChek® lipase substrate green fluorescent (E33955) and Alexa Fluor 594 Protein Labeling Kit were purchased from Invitrogen Corporation. All other chemicals used in the study were purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water from the Milli-Q system (Millipore) was used throughout this research.

Silk fibroin purification

Silk fibroin aqueous stock solutions were prepared as previously described [30]. Briefly, cocoons of *B. mori* were boiled for 20 min in an aqueous solution of 0.02 M sodium carbonate, and then rinsed thoroughly with pure water. After drying, the extracted silk fibroin was dissolved in 9.3 M LiBr solution at 60°C for 4 hours, yielding a 20% (w/v) solution. This solution was dialyzed against distilled water using Slide-a-Lyzer dialysis cassettes (MWCO 3,500, Pierce) for 3 days to remove the salt. The solution was optically clear after dialysis and was centrifuged to remove the small amounts of silk aggregates that formed during the process, usually from environment contaminants that are present on the cocoons. The final concentration of silk fibroin aqueous solution was approximately 8% (w/v). This concentration was determined by weighing the residual solid of a known volume of solution after drying.

Enzyme entrapment in silk films

Dry enzymes were weighed and dissolved in PBS buffer then mixed with 6 wt% silk solution to generate a weight ratio of silk:enzyme = 100:1 and 1,000:1(ie enzymes concentration in silk films were 1 wt% and 0.1 wt%). Twelve milliliters of mixed solution was added to Petri dishes (90 × 15 mm) which were placed in a fume hood overnight with the cover off. The dried films were either treated or not treated with methanol. For the methanol treatment, the films were immersed in 90% methanol solution for 5 min and then dried in the fume hood. For the non-methanol treated films, the films were used after drying in the fume hood overnight. The films (methanol treated and untreated) were cut into small squares (7×7 mm^2) and each piece was weighed (about 8 mg) and placed into Eppendorf tubes. The tubes were tightly sealed and stored in tightly sealed plastic bags in a refrigerator (4°C), room temperature, or an incubator (37°C) for stability studies.

Enzyme activity measurement

Enzyme activity was measured right after film preparation, to establish baseline activity (time 0) with the various treatments. Five samples (N=5) were used for each point of analysis. For non-methanol-treated films, the sample was immersed in 0.5 ml of PBS buffer, pH 7.4, in an Eppendorf tube and incubated at room temperature for 30 min until the film was dissolved (since it had not been treated with methanol). The solution was then subjected to enzyme activity measurements as described below. For the methanol-treated samples, 0.5 ml of enzyme substrate solution was directly added to the Eppendorf tube to immerse the film, and activity was determined for each of the different enzymes as described below. As controls, enzyme activities were also measured for the non-methanol treated and methanol treated silk films that had no enzyme loaded. The enzyme activity determined was then compared to that originally added into the film (data taken from manufacture's data sheet, assuming no activity was lost prior to use), and the result was presented as residual activity after casting film (activity loaded, % in Table 1). To indicate the enzyme activity change after storage of films (activity measured, % in Table 1), the determined enzyme activity in a film at a certain time point was compared to that after casting film (activity loaded).

HRP activity—For free HRP in solution or non-methanol-treated samples dissolved in solution, 5 μl of enzyme solution was mixed with 100 μl of TMB (HRP substrate containing 0.02% hydrogen peroxide) in 96-well standard microplate wells for 1 min at room temperature and the reaction was stopped by the addition of 100 μl 0.1 M sulfuric acid. TMB was oxidized during the enzymatic degradation of H_2O_2 by HRP and the oxidized product of TMB has a deep blue color which turns to yellow after addition of the acidic stop solution. Absorbance was detected at 450 nm using a VersaMax microplate reader (Molecular devices, Sunnyvale, CA). For the methanol-treated samples, the reaction was initiated by adding 0.5 ml of TMB solution directly to the Eppendorf tube. Preliminary time-dependent experiments as well as Confocal laser scanning analysis, described later, indicated that substrate diffused to the middle of the film and reacted with HRP within 5 min. Therefore, the reaction mixture was incubated for 5 min before being stopped by the addition of 0.5 ml of 0.1 M sulfuric acid. The HRP activity was obtained using a standard curve generated under the same conditions, and further normalized by the film weight.

GOx activity—GOx activity was determined by a peroxidase coupled assay [31]. For free GOx in solution or non-methanol-treated samples dissolved in solution, 100 μl of 1% (w/v) *o*-dianisidine solution was added to 12 ml phosphate buffer, pH 6.0, and the solution was saturated with oxygen gas for 10 min. Immediately after gas saturation, the substrate solution was prepared by mixing 12 ml of *o*-dianisidine solution with 1.5 ml of 18% (w/v) glucose and 0.5 ml of 200 μg/mL of peroxidase in PBS buffer. Aliquots of 145 μl of substrate solution were added to a 96-well plate followed by addition of 5 μL of GOx in dissolved silk film solution.

The plate was incubated at room temperature for 5 min before being subjected to absorbance measurement at 460 nm using a VersaMax microplate reader (Molecular devices, Sunnyvale, CA). The GOx activity was obtained using a standard curve generated under the same conditions and further normalized by the film weight.. For methanol-treated samples, all steps were the same as above except that 0.5 ml of substrate solution was added directly into the tube containing silk film, and after 5 min reaction, 150 μl of mixed solution was pipetted into a 96-well plate for absorbance measurement at 460 nm. Similarly, preliminary experiments and Confocal laser scanning analysis (Figure 8a) confirmed that 5 min was sufficient time for substrate diffusion into the film for reaction with GOx.

Lipase activity—5,5'-Dithiobis(2-nitro benzoic acid) (DTNB) stock solution (40 mM) was prepared by adding 0.1584 g of DTNB to 10 mL of isobutanol. 2,3-Dimercapto-1-propanol tributyrate (DMPTB) was dissolved in 6% Triton X-100, 50 mM Tris-Cl, pH 7.2 to obtain a 10 mM stock solution [32]. The two stock solutions were stored at −20°C. *Candida rugosa* lipase was dissolved in or diluted with a buffer of 10 mM KCl, 10 mM Tris-Cl, pH 7.5. The standard substrate solution contained 0.2 mM DMPTB, 0.8 mM DTNB, 1 mM EDTA, 0.05% Triton X-100, and 50 mM Tris-Cl, pH 7.5. For the preparation of reaction mixtures, 20 μl of 10 mM DMPTB, 20 μl of 40 mM DTNB, 2 μl of 0.5M EDTA, 5 μl of 10% Triton X-100, and 50 μl of 1 M Tris-Cl, pH 7.5 were mixed in a microcentrifuge tube and 803 μl of deionized water was added to generate a final volume of 900 μl. For free lipase in solution or non-methanol-treated samples dissolved in solution, 20 μl of the solution was mixed with 180 μl of the substrate solution in a 96-well plate. The microplate was immediately transferred to a 37°C incubator to initiate the reaction. After 30 min incubation, the absorbance at 405 nm was measured using a VersaMax microplate reader (Molecular devices, Sunnyvale, CA). The lipase activity was obtained using a standard curve generated under the same conditions and further normalized by the film weight.. For methanol-treated samples, all steps were the same as above except that 0.5 ml of substrate solution was added directly into the tube containing silk film, and after 30 min reaction at 37°C, 200 μl of mixed solution was pipetted into a 96-well plate for absorbance measurement at 405 nm. Preliminary experiments and Confocal laser scanning analysis (Figure 8b) confirmed that 30 min was sufficient for substrate diffusion into the film to react with the lipase.

Fourier transform infrared (FTIR) spectroscopy

The fractions of secondary structural components including random coils, alpha-helices, beta-pleated sheets, turns were evaluated using Fourier self-deconvolution (FSD) of the infrared absorbance spectra. FTIR analysis of treated samples was performed in a Bruker Equinox 55/S FTIR spectrometer, equipped with a deuterated triglycine sulfate detector and a multiple-reflection, horizontal MIRacle ATR attachment (using a Germanium (Ge) crystal, from Pike Tech.). A slice of silk film was added to the Ge crystal cell and examined with the FTIR microscope in the reflection mode. Background measurements were taken with an empty cell and subtracted from the sample reading. For each measurement, 64 scans were coadded with resolution 4 cm^{-1}, and the wavenumber ranged from 400 to 4000 cm^{-1}. FSD of the infrared spectra covering the amide I region (1595–1705 cm^{-1}) was performed by Opus 5.0 software as described in previous studies [33]. The curves that had absorption bands at the frequency range of 1616 – 1637 cm^{-1} and 1695 – 1705 cm^{-1} represented enriched β-sheet structure in silk II form [34]. The curves that had absorption bands at the frequency range of 1638 – 1655 cm^{-1} corresponded to random coils structure, 1656 – 1663 cm^{-1} corresponded to alpha-helices structure and 1663 – 1695 cm^{-1} corresponded to turn structure [33].

Confocal laser scanning microscopy

To determine the overall enzyme distribution in films, the enzymes were labeled with Alexa Fluor 594 according to manufacturer's protocol. The labeled enzyme was mixed with silk

solution as described above and 1 ml of the mixture was cast on a tissue culture dish (3.5 cm diameter with glass bottoms). The dish was placed under a confocal laser scanning microscope (TCS Leica SP2, Welzlar, Germany), and the film was scanned from bottom to top (50 scans). Average fluorescence intensity in the scanned area was plotted against the scanning distance (film thickness). To determine the active enzyme distribution in the films after storage, enzymes were cast in the same way on tissue culture dishes and then stored for 3 months. The dishes were then placed under the Confocal laser scanning microscope, and 20 μl substrate solution was dropped on top of the film. The substrate compound used was designed in such a way that the product was fluorescent after reaction. For HRP, the substrate used was Amplex® Red which emitted red fluorescence after reaction when excited at 543 nm. For GOx, it was Amplex® Red which also emitted red fluorescence after reaction. For lipase, it was EnzChek® which emitted green fluorescence after reaction when excited at 514 nm. To monitor the overall process of substrate diffusion through the film, the microscope was set at certain excitation and emission wavelength ranges, and the film was scanned from bottom to top (50 scans) after 0, 5, 15, 30 minutes of adding substrate. Average fluorescence intensity in the scanned area was plotted against the scanning distance (film thickness). To detect the substrate diffusion through the film in a real time, the microscope was focused and fixed at the middle plane of the film after determining the full thickness of the film, and then the fluorescence intensity was followed as a function of time using the “xyt” mode with 2 seconds/frame scanning for 500 seconds. Based on the substrate diffusion rate in silk films, the incubation time was determined for the enzymatic reaction for the methanol-treated silk films (see above).

Statistics

All experiments were performed with a minimum of N = 3 for each data point. Statistical analysis was performed by one-way analysis of variance (ANOVA) and Student-New-man-Keuls Multiple Comparisons Test. Differences were considered significant when $p \leq 0.05$, and very significant when $p \leq 0.01$.

Results

1. Enzyme activity change after immobilization in silk films

Silk films were prepared under four different conditions: high (1.0 wt%) and low (0.1 wt%) enzyme loading, and with and without methanol treatment. All three enzymes (GOx, lipase, HRP) were entrapped in the films with these four conditions to assess long term stability at three different temperatures (4°C, room temp, 37°C). Enzyme activities were determined after entrapment in the silk films and the results were compared with the original amount of activity that was taken from manufacturer’s data sheet (Table 1). For non-methanol treated films containing GOx, approximately 75% of the activity remained when higher enzyme loading (1 wt%) was used, while approximately 130% activity was obtained when the lower enzyme loading (0.1 wt%) was used. For the methanol treated films, about 14% and 1.3% activity remained for the low and high GOx loading, respectively, at this initial film formation step. For non-methanol treated films containing lipase, approximately 43% and 83% activity remained for high and low enzyme loading, respectively, while for the methanol treated films, approximately 10% and 54% activity remained for the high and low enzyme loading, respectively. For non-methanol treated films containing HRP, the enzyme activity was approximately 237% and 122% of its original value for the high and low enzyme loading, respectively, while for the methanol treated films, approximately 32% activity remained for the low enzyme loading. In general, GOx and lipase retained more activity in silk films when the low enzyme loading (0.1 wt%) was used, whereas HRP retained more activity when high enzyme loading (1.0 wt%) was used. A separate experiment showed that almost no enzyme was released from the methanol treated films to the medium when the film was incubated for 2 hours in PBS buffer, pH 7.4, indicating that the immobilized enzymes retained certain amount

of activity in methanol treated silk films and were able to react with the substrate and release the product (data not shown). Control silk films with no enzyme loaded showed no catalytic activity in all three cases.

2. Enzyme stability in silk films and solution

(1) GOx—The enzyme activity after storage was measured and compared with the initial residual activity measured in silk films (see column of Activity Measured in Table 1) in order to demonstrate enzyme stabilities. For non-methanol treated silk films containing 1 wt% GOx, residual activity decreased slightly or did not change during the first 3 months of storage. This trend was followed by a slow increase in activity over the remaining five months, to 167%, 140% and 103% (Figure 1a) of the original activity then a slow decrease to 118%, 104% and 72% of the original activity for the samples stored at 4°C, room temperature, and 37°C, respectively. For the same films but treated with methanol, GOx activity decreased to about 40% of original activity after one month and stayed at this level for the rest of storage time, regardless of different storage temperatures (Figure 1b). For the nonmethanol-treated samples with low GOx loading (0.1 wt%), GOx activity decreased 40% to 60% of the original activity during the first 3 months and then slightly increased during the remaining five months (Figure 1c). Similar to the films with high enzyme loading, after methanol treatment, GOx activity quickly decreased to about 40% of the original activity within one month and then stayed at this level for the rest of storage time (Figure 1d). For all samples tested, storage temperature did not have a significant effect on GOx activity in the silk films over the ten months of storage ($p > 0.05$, # in Figure 1).

GOx stability in solution with soluble silk, as a control, was assessed in parallel with the silk films. GOx was dissolved in PBS buffer, pH 7.4, and silk fibroin solutions at 1 and 10 mg/ml to reach a concentration of 10 μg/ml. The samples were stored at room temperature and the GOx activity was assayed over four months (Figure 2). GOx had the best stability in 10 mg/ml silk solution and the worst stability in the PBS buffer ($p<0.01$, ** in Figure 2).

The activity decreased as a first order kinetic process, with half-lives of 877, 74 and 14 days (Table 2) for the samples stored in 10 mg/ml silk solution, 1 mg/ml silk solution and PBS buffer, respectively. When the above GOx/PBS solution was stored at −20°C for 8 months, the activity decreased 40%, indicating that lower temperature improved the stability of GOx in solution, but still less effectively than when stored in the silk solution at room temperature.

(2) Lipase—For nonmethanol treated silk films containing 1 wt% lipase, lipase activity remained at 93% of the original activity after 7 months of storage at 4°C. Seventy percent and 75% remained for the samples stored at room temperature and 37°C, respectively (Figure 3a). After methanol treatment of the films, lipase activity decreased to about 20%, 40% and 30% in the first 2 months for the samples stored at 4°C, room temperature, and 37°C, respectively, and then remained stable at this level for the rest of time (Figure 3b). This observation is similar to that of GOx (Figure 1b). For the samples with low lipase loading (0.1 wt%), enzyme activity slowly decreased in the first three months and then residual activity for the rest of storage time was 65%, 30% and 50% of the original activity for the samples stored at 4°C, room temperature, and 37°C, respectively (Figure 1c). After methanol treatment of the films, lipase stability changed in a similar fashion to the samples with high loading density, with about 27%, 35% and 36% remaining for samples stored at 4°C, room temperature, and 37°C, respectively (Figure 3d). For all the samples tested, lipase stability did not change significantly at the various storage temperatures ($p > 0.05$, # in Figure 1), consistent with literature reports that lipase is not sensitive to temperature over reasonable ranges [35].

Lipase stability in PBS buffer, pH 7.4, was determined at room temperature for 4 months. Lipase activity decreased faster than that in silk films, with a first-order kinetic rate loss with a half-life of 15.7 days (Table 2).

(3) HRP—For nonmethanol treated silk films containing 1 wt% HRP, enzyme activity significantly decreased in the first month and then remained at about 25%, 20% and 18% of the initial residual activity for the samples stored at 4°C, room temperature, and 37°C, respectively (Figure 4a).

For methanol treated films containing 1 wt% HRP, enzyme activity could not be measured due to high enzyme concentration entrapped in a weighable small piece of silk film, even after the film size was minimized and volume of substrate solution was increased. More enzyme activity was lost after storage in non-methanol treated silk films containing 0.1 wt% HRP as compared to the non-methanol treated films containing 1 wt% HRP, regardless of the storage temperatures (compare Figure 4a and Figure 4b). Methanol treated silk films containing 0.1 wt % HRP showed a rapid initial decrease followed by long-time retention of activity. Activity decreased to 0.08%, 0.03% and 0.02% of the initial residual activity for samples stored at 4°C, room temperature, and 37°C after 5 months storage, respectively (Figure 4c).

All HRP activity was lost within 16 hours at room temperature when dissolved in PBS buffer, pH 7.4, at a concentration lower than 1 μg/ml. Once the concentration was above 1 μg/ml, HRP was more stable (compare Figure 5 a and b).

This finding indicates that HRP stability in solution is highly dependent on enzyme concentration [36]. Enzyme stability was further improved when silk solution was used instead of the PBS buffer to dissolve HRP. After 90 days storage at room temperature, the solution containing 100 μg/ml silk and 0.1 μg/ml HRP (weight ratio between silk and HRP = 1000:1) retained about 18% of the enzyme activity, and the solution containing 10 μg/ml silk and 0.1 μg/ml HRP (weight ratio between silk and HRP = 100:1) had about 15% activity remaining (Figure 5b). HRP activity decreased by first order kinetics with a half-life of 0.11, 30 and 37 days for samples stored in PBS buffer, 10 μg/ml and 100 μg/ml silk, respectively (Table 2). For a solution containing 60 mg/ml silk and 60 μg/ml HRP, enzyme activity did not decrease after 10 days stored at room temperature but the solution gelled afterwards (data not shown). For the same solution stored at 4°C, the enzyme activity did not decrease during the first 10 days and then slowly decreased, with about 20% of the activity remaining after 90 days storage (Figure 5b). Except for a short initial lag phase, HRP showed similar stabilities in PBS buffer and silk solution in this case (Figure 5b). Therefore, HRP stability in solution is dependent on both enzyme and silk concentrations.

3. Silk fibroin structural changes

The silk films showed significant changes ($p < 0.05$, * in Figure 6a) in fibroin secondary structure after methanol treatment, consistent with literature reports [37]. Figure 6a shows an example of fibroin structural changes in GOx-loaded silk films. Despite the different levels of GOx loading, the films demonstrated a large increase in β-sheet structure content from about 10% to above 50%, with a corresponding decrease of β-turns and random coils after methanol treatment (Figure 6a).

Due to the high content of beta-sheet, the methanol-treated silk films became water-insoluble. Fibroin structure did not change after long term storage of the films, regardless of treatment or enzyme loading. Figure 6b shows an example of fibroin structure stability in the GOx-entrapped films, 1 wt% non-methanol treated films stored at 37°C for 7 months. No significant structural changes could be observed by FTIR.

4. Enzyme distribution and substrate diffusion in silk films

Confocal scanning microscopy was utilized to assess the enzyme distribution and substrate diffusion due to enzyme-substrate reaction in the silk films. The diffusion rates of substrate and the spatial distribution of active enzymes across the thickness of the film were estimated based on the scanned spectra. Silk films after 3 months storage time were used in the experiment. GOx showed a relatively homogeneous enzyme distribution in the films, for both overall distribution and active enzyme distribution based on substrate diffusion (Figure 7). The substrate diffused through the film in about 2 min. Lipase showed non-homogeneous distributions in the films, probably due to selective partitioning at the air side during film preparation (Figure 7). It took less than 1 min for the substrates to diffuse through the lipase-silk film. HRP, unlike the GOx or Lipase, showed higher concentration at the two sides of the film. From the active enzyme distribution data, the HRP immobilized near the surface of the film was inactive, as the distribution pattern did not show two peaks at the sides of the film (Figure 7). This might be caused by the fact that the HRP near the surface was irreversibly denatured due to oxidation or less water content (assuming interior film had higher water content than the surface area). It took about 5 min for the substrates to diffuse through the film in this case.

Discussion

Enzyme stability during storage and transport of proteins, such as in biosensor systems or for pharmaceutical distribution, is important for retention of function of devices and for therapeutic benefit, respectively. Issues of shipping, distribution and storage of enzymes raise questions about the best modes to preserve activity, with consideration for the costs involved in accomplishing this goal. Often refrigeration is needed to maintain sufficient shelf life. Even when lyophilized, loss of activity ensues unless the preparation is kept frozen or cold. Further, reconstitution on site is required with lyophilized preparations. In the case of pharmaceutically important proteins and vaccines, shipment and distribution to developing countries, where low temperature storage is not an option, suggests that new modes of simple yet effective stabilization of bioactive proteins over a range of environmental conditions would be of significant benefit.

Although a number of immobilization approaches have been developed to improve the enzyme stability and many of them have been successfully used in industries, such as protein coated micro crystals (PCMC), CLECs and CLEAs, alternative protein stabilization methods, especially those with features such as ease of preparation, safety, cost - efficiency and reproducibility, are still needed for the rapidly expanding market. The novel chemistry, structure and assembly of silk fibroin protein offers a unique environment in which enzymes can be stabilized and remain active over extended periods of time. Without the use of chemical reagents, enzymes can be easily entrapped in silk films, and their activities retained in the nano- and micro-environments formed during silk chain self-assembly. The resulting structures consist of hydrophobic domains (crystalline β-sheet) on the order of tens of nanometers in the x,y,z orientations [38], with additional nano- to micro-domains formed consisting of the less hydrophobic domains, where sufficient water molecules are present [39]. The water content of methanol-treated silk films is about 5% [40] while that of non-methanol-treated silk films is about 10%. Due to the high glass transition temperature of silk fibroin at about 175°C [41], silk fibroin protein is thermodynamically stable once self-assembled into the β-sheet structure. Therefore, these features provide a suitable stable water-containing matrix to stabilize bioactive proteins.

Enzyme molecules exist in native form and the silk fibroin chains are present mainly in random coils in solution. After casting the films, some limited conversion of the silk fibroin to β-sheets occurs, which contains the most hydrophobic regions of the material. The remaining random

coils encompass the more hydrophilic regions. The enzymes appear to be stabilized in these nano-environments, perhaps due to the limited water content, interactions with the fibroin chains, or constraints on chain mobility. When the films are treated with methanol, most random coil structures are converted to β- sheets. As a result, the detectable enzyme activities were largely reduced, probably due to stronger interaction of enzyme molecules with the fibroin chains and more constraints on chain mobility. This difference also suggests a role for the limited water in the nano-environments in facilitating stabilization. GOx activity increased after long term storage in the films (Figure 1). One hypothesis for this outcome is that denatured GOx renatured over time in the film structure. The denaturation of enzymes discussed here refers to spontaneous denaturation process which might still be reversible under certain conditions and does not determine proteolytic, peroxide-mediated, or other deactivation mechanisms. Protein renaturation in material matrices has been previously reported. For example, soybean protease inhibitors can renature after thermal denaturation by binding to polysaccharides [42]. Altamirano et al [43] attached the enzymes (glucosamine 6-phosphate deaminase, indole 3-glycerol phosphate synthase, cyclophilin A, firefly luciferase) to agarose gels and found this strategy could be used for efficient renaturation of the proteins. In the nonmethanol treated silk films about 10 wt% water is present, and presumably the mobility of the enzyme molecules allows for retention of activity as well as refolding/renaturation in the case of GOx. For methanol treated films, the more rigid hydrophobic β-sheets, lock in the structures and the enzyme molecules have limited mobility and thus less options for refolding/renaturation. We have tested the water content in silk films before and after storage, and no significant change in water content was observed, so that the increase of GOx activity was unlikely due to the change of water content. An increase of GOx activity was also observed when GOx powder was dissolved in buffer and the enzyme activity was measurement in time, and the activity increase lasted for more than one hour before leveling off (data not shown). This indicates that similar to that in silk films, GOx renaturation might have also occurred in silk solution, but at a much shorter time scale. Similar activity increase was not observed for lipase and HRP in solution (data not shown). The hypothesized denaturation/renaturation processes of GOx in solution and silk films are schematically depicted in Figure 8.

Interestingly, when compared to silk films, silk solution also showed significant stabilization effect on enzymes that were stored in it for more than 3 months (Figure 2). Such a stabilization effect was dependent on silk concentration, suggesting that the interaction of enzyme with silk molecules, through either the hydrophobic (crystalline beta sheet) or hydrophilic regions, plays an important role in stabilizing enzymes. For applications, however, silk solution is less favorable than silk films because it is less stable. At an optimal condition (temperature, pH, salt concentration, etc) silk solution can be stored from several weeks to several months before gelation, but silk films can be stored from months to years without changing morphologies and structures. In addition, silk films also provide benefits such as a) small volume for storage, b) ease of manipulation, and c) ease of sterilization. Therefore, the present study is more focused on enzyme stabilities in silk films whereas the enzyme solution stabilities are used as controls.

Glucose oxidase from *A. niger* is a 160 KDa homodimer containing two disulfide bonds and 16% v/v carbohydrate [44,45]. The active form of the enzyme is associated with two flavin adenine dinucleotide (FAD) moieties which are responsible for the oxidation-reduction properties of the enzyme [46]. GOx immobilization and stabilization in various carrier matrices has been extensively studied. Once covalently immobilized on polyacrylonitrile films, 80% of the original activity remained after 50 days of storage at 4 °C [47]. When GOx was entrapped in a photosensitive crosslinking film, enzyme activity decreased to 23% of the original level after 6 months storage at 4°C [48]. GOx immobilized on chitosan-SiO_2 exhibited 56% residual activity after 15-days of storage at 30°C [49]. The immobilization of GOx in silk films and its potential application for glucose biosensors has also been studied [50]. In the present study, after immobilized in silk films, GOx activity increased when compared to the initial residual

activity in the film (Table 1). From literature studies, GOx activity increased once immobilized in two hydrophobically modified micellar polymers, chitosan and Nafion, and the activity increase was attributed to the increase in hydrophobicity of the polymers [51]. Similar phenomena were also observed with lipase immobilization on decaoctyl sepharose [52]. The authors claimed that during the immobilization procedure the lipase underwent a conformational change from the closed to an open structure, as a hydrophobic “lid” moves aside by an intermolecular hydrophobic interaction, providing enhanced substrate access to the active-site residues. With a similar strategy, the same group also reported modulation of the properties of penicillin acylases from three different species which also undergo conformational changes upon binding of the acyl donor substrate [53]. Therefore, the GOx activity increase upon immobilization in silk films in the present study can be attributed to the intermolecular hydrophobic interaction between silk and GOx molecules.

GOx stability was monitored up to 10 months in different silk films, nonmethanol treated (water-soluble, low crystallinity and low β-sheet structure) and methanol treated (water-insoluble, high crystallinity and high β-sheet structure). The highest stability was found in the nonmethanol treated films with high GOx loading (1 wt%). Following an initial decrease, GOx activity eventually increased to 118% of its original residual activity in silk films (Figure 1a). To our knowledge, this is the first time that increased GOx activity after long-term storage has been reported. Interestingly, GOx showed a similar trend of increasing activity, but at a lower level, in the case of high GOx loading in methanol-treated films (Figure 1b) and low GOx loading in nonmethanol-treated silk films (Figure 1c). There was no increase in activity in the case of low GOx loading in methanol-treated silk films (Figure 1d). Hydrophobic interactions between GOx and immobilizing matrices may result in a GOx more compact dimeric GOx structure through which enzyme stability can be improved due to restricted chain mobility [54]. This stabilization mechanism might account for the improved stability in silk films in which GOx likely interacts with silk molecules via hydrophobic interactions. This assumption is supported by the control experiment in which GOx stability in solution was dependent on silk concentration in solution (Figure 2). The degree of hydrophobic interaction between GOx and silk might be optimal in the case of nonmethanol treated films so that GOx molecules still have sufficient mobility to interact with silk chains and to undergo structural changes related to activity (Figure 8). In the case of methanol treated films, highly hydrophobic and stacked β-sheet structures may restrict GOx conformational changes so that those inactive (misfolded) GOx molecules are “locked” as is the case during long term storage. From the literature [55], GOx activity can be increased by introducing more oxygen to the enzyme. GOx also had a stable and homogenous distribution throughout the silk film (Figure 7a). Both of these factors, distribution and oxygen response may help to improve the stability of GOx in silk films, given the fact that silk films are gas permeable [56].

Lipase from *C. rugosa* is a 58 KDa monomer [57] that has been widely used in biochemical and fine chemical industries as a catalyst for hydrolysis and esterification [58]. Immobilization of lipase on solid supports improves enzyme stability, activity and reusability [59,60]. In the present study, in a similar response to that of GOx, lipase was most stable in non-methanol treated silk films at high enzyme loadings, as compared to its initial residual activity in silk films (Figure 3a). For the other groups, lipase activity slowly decreased over seven months of storage, but even in the worst case, methanol-treated films with low enzyme, there was still about 20% of the original activity retained (Table 2), much higher than that measured for the lipase stored in solution. Remarkably, lipase stability in silk films was not significantly influenced by storage temperatures, which was distinguishing versus the other enzymes studied. It has been known from the literature that lipase activity is not sensitive to temperature change and found to be thermostable up to 70°C [35], consistent with the present results.

HRP is a monomeric heme-containing plant enzyme with a molecular weight of 44 kDa that has been widely used as indicator enzyme in enzyme immunoassays [61], enzyme electrodes [62], and nucleic acid detection systems [63]. It is a hydrogen peroxide oxidoreductase which catalyses the oxidation of a variety of organic and inorganic substances with H_2O_2 as the electron acceptor [64]. Previous studies showed that HRP stability was dependent on enzyme concentration in solution [36]. For example, HRP at a concentration of 0.5 μg/ml lost almost 50% of the activity within 14 days at 4°C [65]. We observed that HRP at 60 μg/ml in PBS buffer, pH 7.4, retained more than 20% of original activity after three months of storage at 4° C (Figure 5b). Moreover, the half-life of HRP activity in silk solution was 300 folds longer than that in PBS buffer, Similar to the results with GOx, the addition of silk solution into the HRP solution dramatically reduced enzyme deactivation, and the effect was more pronounced for HRP at low concentrations (Figure 5). It has been known that histidine, tyrosine and cysteine are good stabilizing reagents for HRP [66]. However, no stabilizing effect was observed with additives like BSA or Tween 20, which are often proposed as stabilizers in literatures [67]. Thus, compared to other commonly used biomaterials, silk material provides a unique protein-based matrix for stabilizing HRP, and this might be due to the following reasons. First, HRP might form more β-sheet structure upon interacting with silk fibroin molecules, especially those crystalline β-sheet domains. It has been reported that the secondary structure of HRP was different under different conditions. The β-sheet content was about 23% in HRP powder versus 10% in solution, resulting in higher HRP stability in a powder form [68]. Second, since HRP is cationic at neutral pH, both hydrophobic and electrostatic interaction between silk and HRP might have contributed to the stabilization. It has been shown that one HRP molecule contains ten α-helixes and one β-sheet, and the β-sheet structure is more buried in the molecule at high protein concentrations, while more exposed at low protein concentrations [69]. The exposure of β-sheet structure may significantly destabilize HRP. The possible interaction between HRP and silk fibroin might have prevented the exposure of HRP β-Sheet structure.

After immobilized in silk films, HRP had residual activity of 237% and 122%, at the different loadings, respectively, compared to the original activity. A similar activity increase was also reported when HRP was immobilized in silk scaffolds and the samples were incubated at 30° C and 37°C and compared with free enzyme in solution [70]. The HRP stability in silk films depended on both enzyme loading and storage temperature. After 5 months storage, nonmethanol treated films with low HRP loading still had 5% residual activity stored at 4°C, while stored at room temperature and 37°C less than 0.3% of the original activity remained (Figure 4). Comparing GOx and HRP stability in silk films, in general, the latter was less stable. The difference could be due to different responses to environmental oxygen, activation vs. deactivation, or different interactions with the silk scaffolding, or aggregation phenomena. Based on crystallographic structure, the surface of a folded GOx molecule is negatively charged [71], and that of a HRP molecule is positively charged [72]. Since silk molecules are negatively charged, electrostatic interactions might also play a role in determining enzyme stability in silk matrices.

With the relatively simple and straightforward approach to enzyme stabilized film preparation described here, the results suggest further inquiry into mechanisms and further options to enhance the system. For example, initial loss of enzyme during film preparation could be addressed in many ways, such as pre-encapsulation, other pretreatments, other modes of solubilization, use of surfactants and related approaches. Additional studies at temperatures above 37°C are also warranted based on the results presented here. Finally, the proteolytic degradability of the silk films as been well documented and would suggest some interesting strategies for controlled release of the entrapped enzymes or therapeutic proteins, from these systems.

Conclusion

Self-assembled silk fibroin processed into film formats provided efficient and highly effective carriers for the long-term stabilization of entrapped enzymes. Among the three enzymes studied, GOx showed almost no activity decrease in silk films over ten months of storage at temperatures up to 37°C. The enzymes were more stable in silk films without methanol treatment than those treated with methanol, and more stable in the films with higher enzyme loading than low loading. GOx and HRP activity was also significantly stabilized in solution in the presence of soluble silk fibroin, when compared to activity in plain buffer solution without silk. The degree of intermolecular interaction between silk fibroin chains and enzyme molecules, together with enzyme sensitivity to oxidation and hydrophobic-hydrophilic interfaces, are suggested mechanisms that impact the relative stabilization results among the three enzymes studied.

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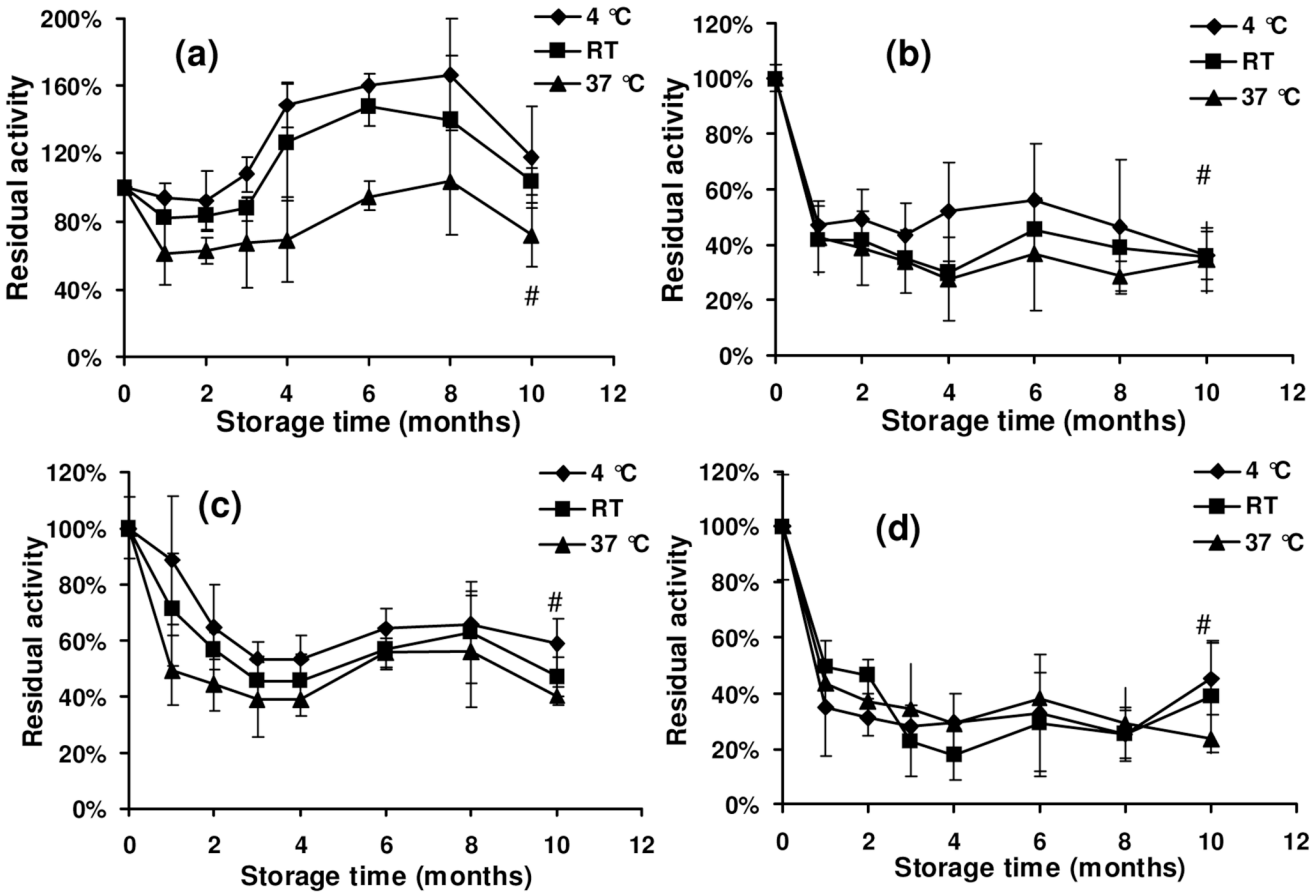

Figure 1.

GOx stability in silk films stored at different temperatures for 10 months. (a) non-methanol treated film containing 1 wt% GOx. (b) methanol treated film containing 1 wt% GOx. (c) non-methanol treated film containing 0.1 wt% GOx. (d) methanol treated film containing 0.1 wt% GOx. Values are average ± standard deviation of a minimum of N=5 samples for each group. # indicates no significant difference between samples ($p > 0.05$).

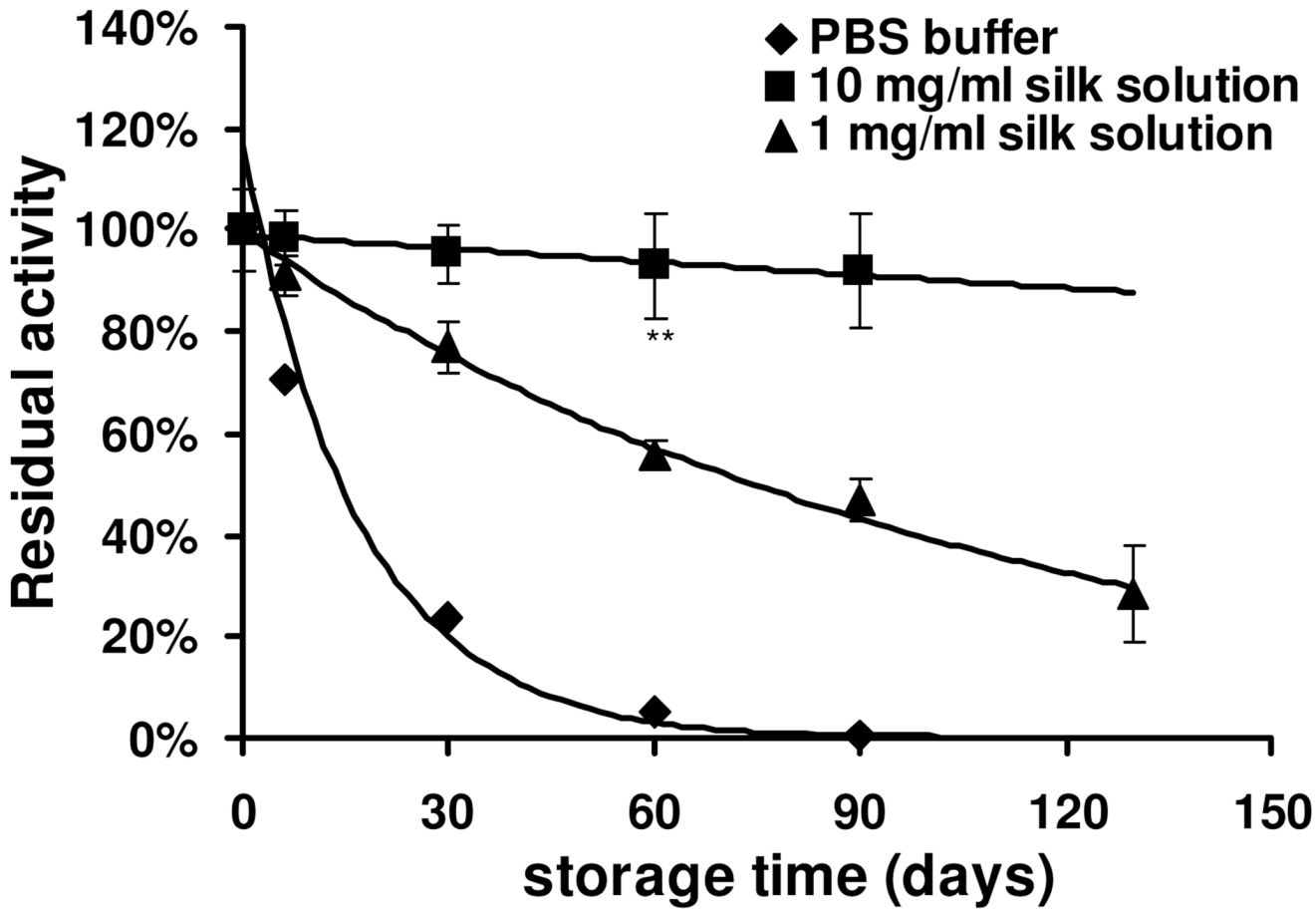

Figure 2.

GOx stability in different solutions. GOx was dissolved in PBS buffer, 10mg/ml silk solution and 1 mg/ml silk solution to reach a concentration of 10μg/ml. The solutions were stored for 4 months at room temperature. Values are average ± standard deviation of a minimum of N=4 samples for each group. ** indicates very significant difference between samples ($p < 0.01$)

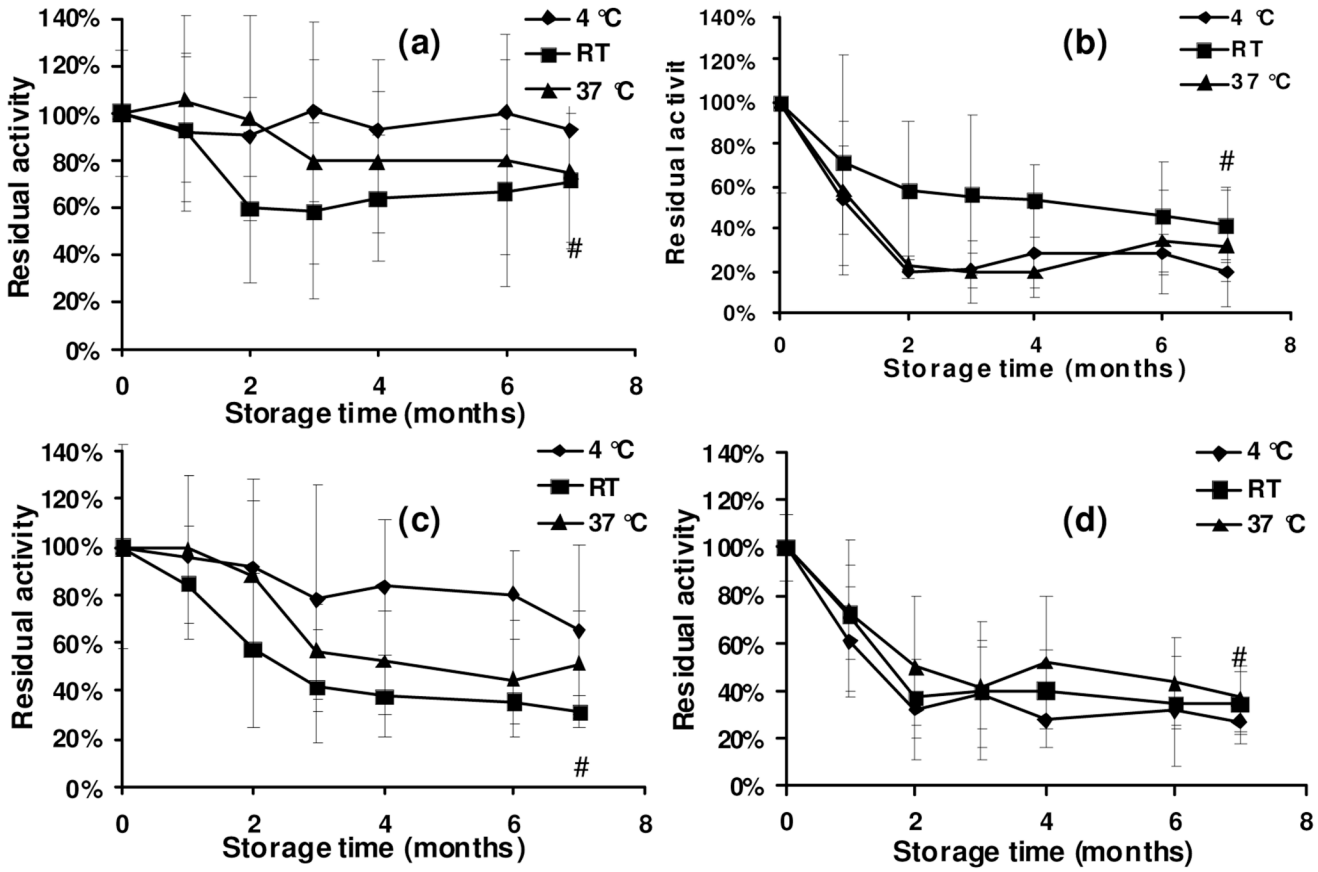

Figure 3.

Lipase stability in silk films stored at different temperatures for 7 months. (a) non-methanol treated film containing 1 wt% Lipase. (b) methanol treated film containing 1 wt% Lipase. (c) non-methanol treated film containing 0.1 wt% Lipase. (d) methanol treated film containing 0.1 wt% Lipase. Values are average ± standard deviation of a minimum of N=5 samples for each group. # indicates no significant difference between samples ($p > 0.05$).

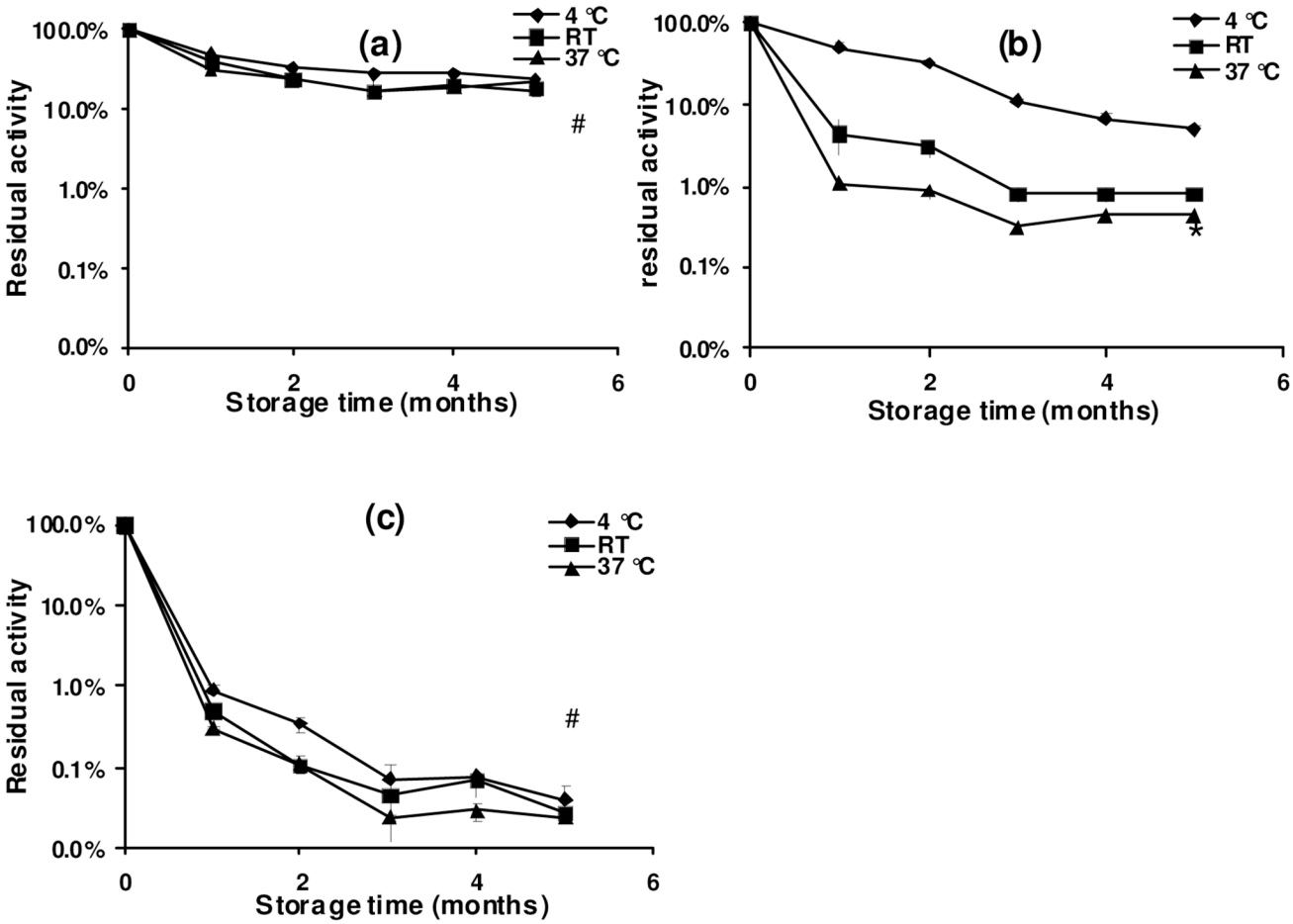

Figure 4.

HRP stability in silk films stored at different temperatures for 5 months. (a) non-methanol treated film containing 1 wt% HRP. (b) non-methanol treated film containing 0.1 wt% HRP. (c) methanol treated film containing 0.1 wt% HRP. The activity could not be measured in the case of methanol treated film containing 1 wt% HRP due to high enzyme concentration in a weighable silk film. Values are average ± standard deviation of a minimum of N=5 samples for each group. # indicates no significant difference between samples ($p > 0.05$). * indicates significant difference between samples ($p < 0.05$).

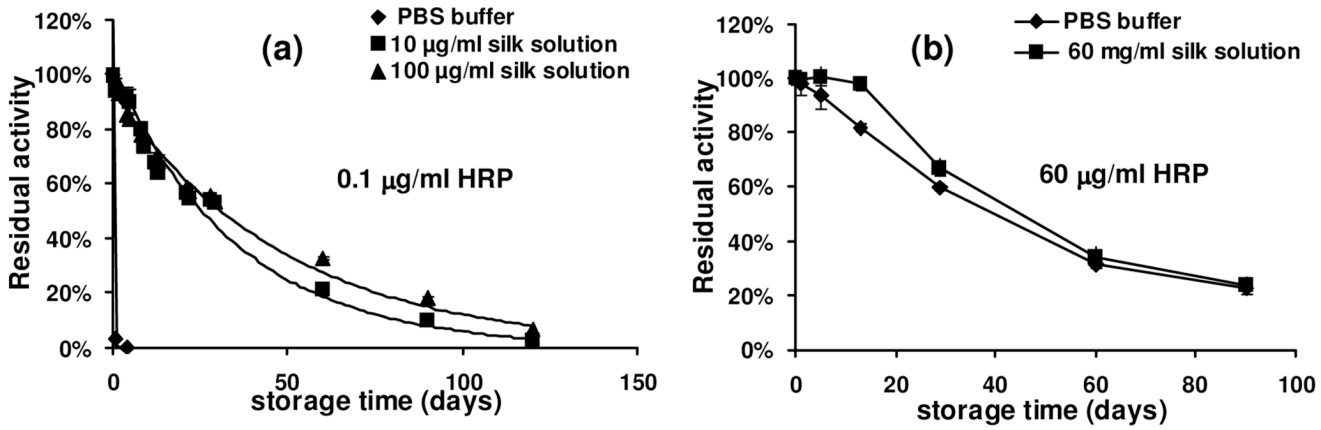

Figure 5.
HRP stability in different solutions. (a) low concentration (0.1 µg/ml) HRP stored at room temperature. (b) high concentration (60 µg/ml) HRP stored at 4°C. Values are average ±standard deviation of a minimum of N=4 samples for each group.

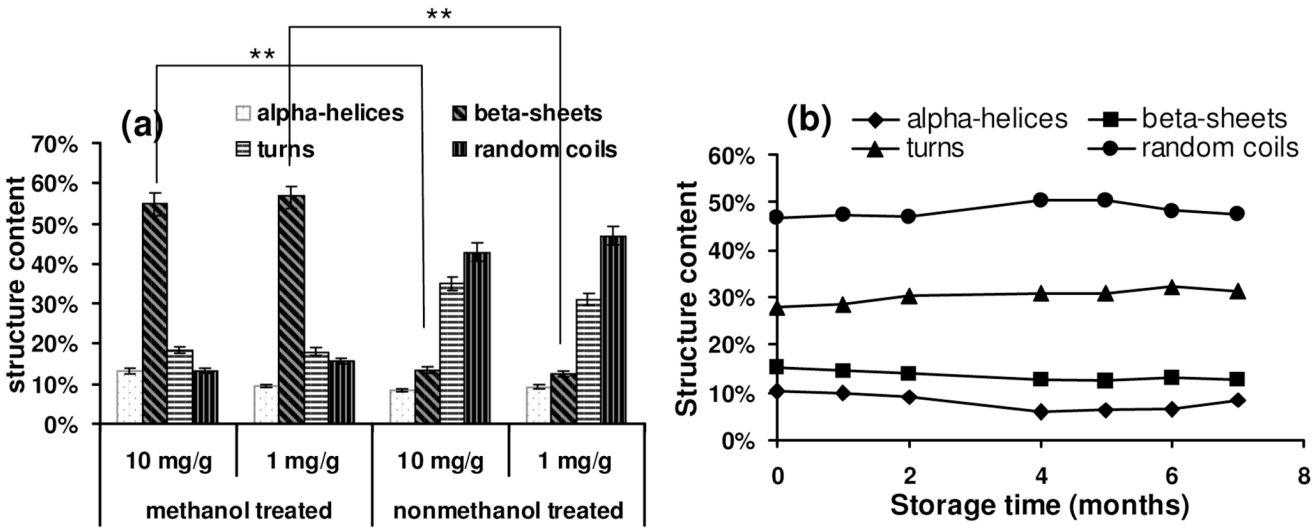

Figure 6.

FTIR analysis on silk fibroin structure during storage of silk films. (a) Silk film containing 1 wt% GOx. Data show the silk structures right after casting the film. (b) Silk structure changes in the same films during 7 months storage at 37°C. ** indicates very significant difference between samples ($p < 0.01$).

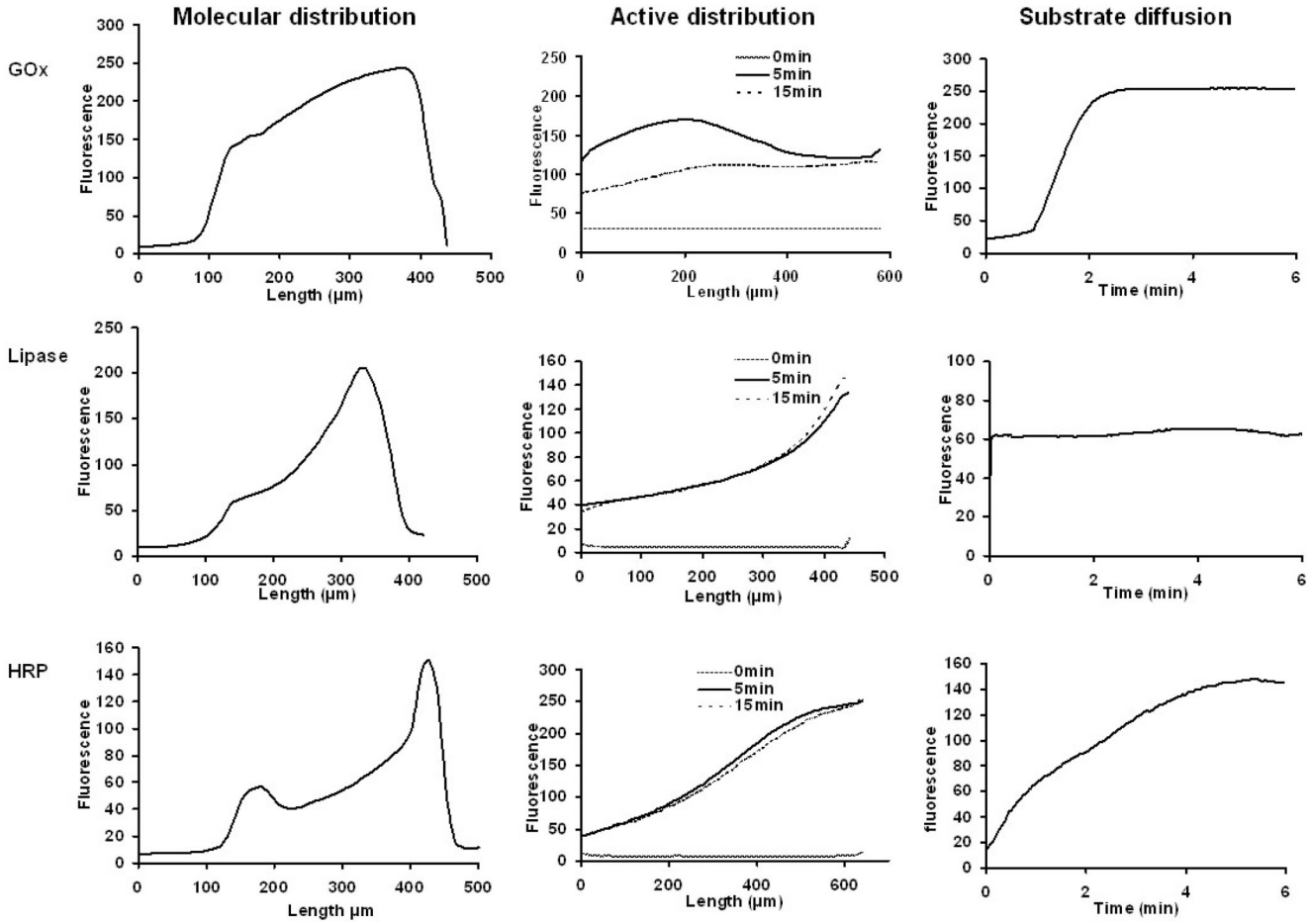

Figure 7.
Confocal scanning microscopic study on total and active enzyme distribution and substrate diffusion in silk films.

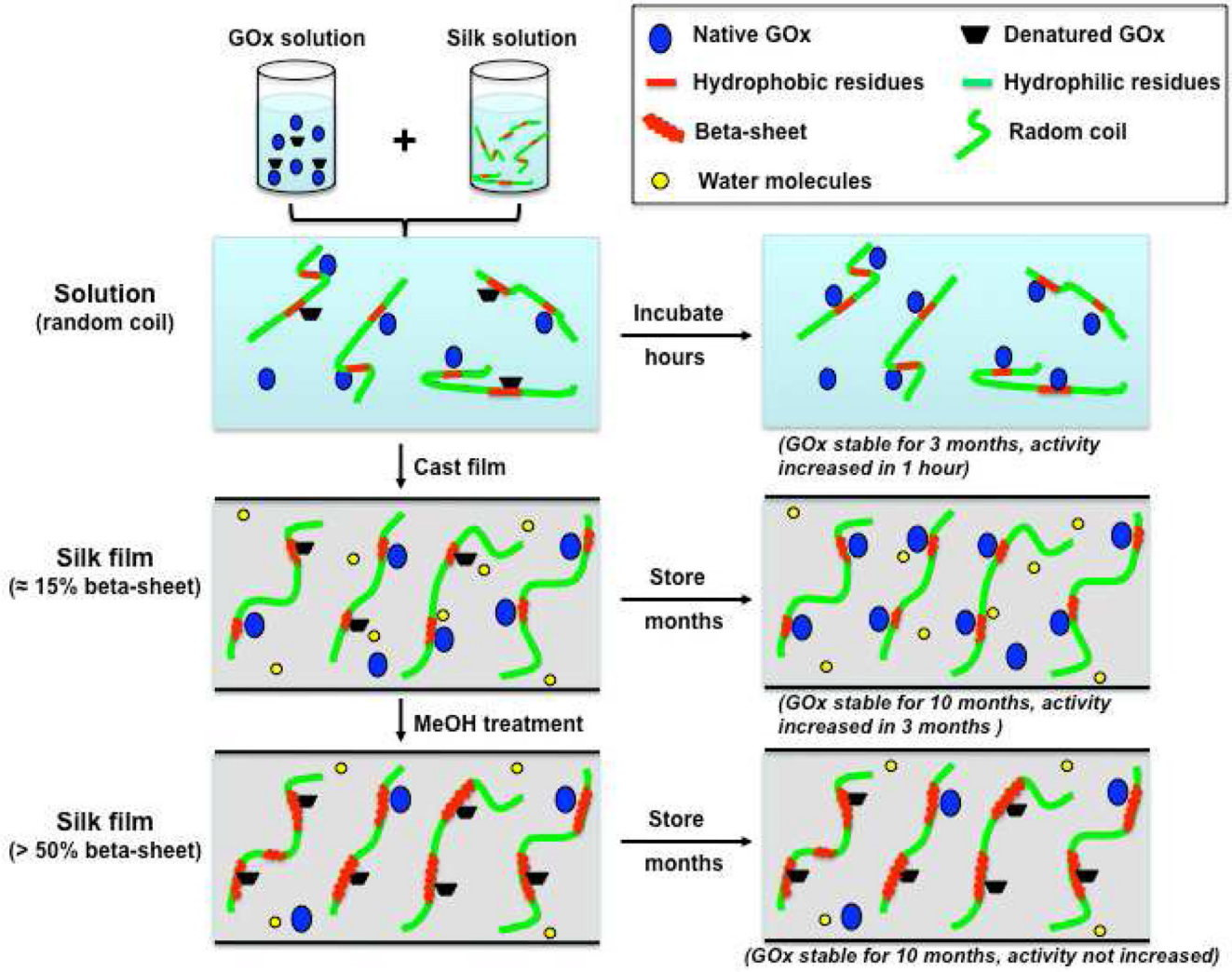

Figure 8.

Schematic illustration of hypothesized mechanism of GOx activity change in solution and silk films. The interaction between GOx and silk hydrophobic region (beta-sheet structure after methanol treatment) as well as limited chain mobility might account for GOx stabilization in silk solution and films. In addition, GOx activity was observed to increase in both the solution and films, probably due to the GOx reversible denaturation in the original solution and the renaturation taking place upon interaction with the silk material.

Table 1

Initial enzyme activity after loading enzymes and processing into silk films

Enzyme	Treatment	Total amount loaded (wt%)	Activity loaded[a] (unit/mg film)	Activity measured[b] (unit/mg film)	Activity retention[b] (%)
GOx	No MeOH	1	0.21	0.16±0.03	75.2±14.7
		0.1	0.021	0.028±0.002	131±14
	MeOH	1	0.21	0.0027±0.001	1.26±0.64
		0.1	0.021	0.0029±0.006	13.8±2.6
Lipase	No MeOH	1	7	3.01±0.84	43.0±11.5
		0.1	0.7	0.58±0.24	82.6±35.0
	MeOH	1	7	0.70±0.38	10.3±5.5
		0.1	0.7	0.38±0.07	53.9±7.6
HRP	No MeOH	1	10.8	25.6±2.6	237±52
		0.1	1.08	13.2±0.11	122±9
	MeOH[c]	0.1	1.08	0.33±0.11	31.9±8.4

[a] Enzyme activity values (units) were taken from manufacturer's data sheet and it was assumed that no activity was lost prior to use.

[b] Values are average ± standard derivation of N=5 samples.

[c] The activity could not be measured in the case of methanol treated film containing 1 wt% HRP because the enzyme concentration was too high to measure, even at the smallest film size and largest substrate volume.

Table 2

Enzyme activity in different solutions stored at room temperature

Enzyme	Solution	Concentration	Half-life (days)	R^2
GOx	−20°C in PBS buffer	10 mg/ml	366	0.971
	PBS buffer	10 μg/ml	14.4	0.933
	10 mg/ml Silk solution	10 μg/ml	877	0.991
	1 mg/ml silk solution	10 μg/ml	74.2	0.990
HRP	PBS buffer	0.1 μg/ml	0.115	0.902
	100 μg/ml Silk solution	0.1 μg/ml	25.4	0.982
	10 μg/ml Silk solution	0.1 μg/ml	25.4	0.983
	PBS buffer at 4°C	60 μg/ml	39.8	0.991
	60 mg/ml Silk solution at 4°C	60 μg/ml	44.1	0.982
Lipase	PBS buffer	250 μg/ml	15.8	0.941