Rational Design of Spider Silk Materials Genetically Fused with an Enzyme

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Enzyme immobilization is an attractive route for achieving catalytically functional surfaces suitable for both continuous and repeated use. Herein, genetic engineering is used to combine the catalytic ability of a xylanase with the self-assembly properties of recombinant spider silk, realizing silk materials with enzymatic activity. Under near-physiological conditions, soluble xylanase-silk fusion proteins assembled into fibers displaying catalytic activity. Also, a xylanase-silk protein variant with the silk part miniaturized to contain only the C-terminal domain of the silk protein formed fibers with catalytic activity. The repertoire of xylanase-silk formats is further extended to include 2D surface coatings and 3D foams, also being catalytically active, showing the versatile range of possible silk materials. The stability of the xylanase-silk materials is explored, demonstrating the possibility of storage, reuse, and cleaning with ethanol. Interestingly, fibers can also be stored dried with substantial residual activity after rehydration. Moreover, a continuous enzymatic reaction using xylanase-silk is demonstrated, making enzymatic batch reactions not the sole possible implementation. The proof-of-concept for recombinantly produced enzyme-silk, herein shown with a xylanase, implies that also other enzymes can be used in similar setups. It is envisioned that the concept of enzyme-silk can find its applicability in, for example, multienzyme reaction systems or biosensors.

1. Introduction

There are numerous reasons for immobilizing enzymes on solid supports. In many cases immobilization improves stability of the enzyme,[1] or even enhances the kinetic parameters of the enzymatic reaction.[2] Another incentive for enzyme immobilization, especially in industrial processes, is the possibility to retain and reuse enzymes, which otherwise constitute a substantial cost for the whole conversion process where the enzyme is used.[3] Moreover, enzyme immobilization allows for convenient separation of the product from the enzymes used, thus avoiding the often costly downstream separation steps in conventional processes with soluble enzyme/product mixtures.[3] Several techniques have traditionally been used for immobilization of enzymes (and other proteins), e.g., adsorption, entrapment, encapsulation, and covalent attachment.[4] Immobilization using adsorption is a simple and inexpensive procedure that does not alter the enzyme chemically, but problems with leaching can be an issue.[4] By both entrapment and encapsulation the enzyme is somewhat protected from the surrounding environment, but on the other hand, such systems can suffer from mass transfer limitations.[4] Covalent attachment of the enzyme to a support or surface is the method that provides the most stable bond to prevent release of the enzyme into the reaction solution, but on the other hand, this can cause decreased activity[5] if the site for attachment is not chosen carefully.

Immobilization of enzymes on silkworm silk has previously been achieved mainly by noncovalent methods,[6–8] but examples using covalent attachment are also reported and concerns enzymes such as horseradish peroxidase,[9] lipase,[10] cholesterol oxidase,[11] L-asparaginase,[12] and protease.[13] In some of these reports an advantage of using silk as support has been ascribed to the overall stabilization of the enzyme under study by the silk protein. Regarding covalent attachment of enzymes to recombinant spider silk there are previous reports on chemical coupling of the enzyme β-galactosidase to silk films via exposed carboxyl[14] or thiol groups.[15] The retained activity of β-galactosidase coupled to the silk materials was then demonstrated by a colorimetric assay. In this paper, a new principle for enzyme immobilization to recombinant silk is presented, in which the DNA sequences for an enzyme and silk are combined at gene level. This allows combined production and immobilization, as the silk fusion protein assembles into a silk material densely populated with the target enzyme. To our knowledge there are up to this date no other reports on enzymes genetically fused to recombinant spider silk.

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Spider silk proteins, so-called spidroins, are in their native state of high molecular-weight (200–350 kDa),[16] and composed of three distinct entities: a conserved N-terminal[17] and C-terminal[18] domain (NT and CT, respectively) flanking a long stretch of alternating poly-alanine and glycine-rich moieties.[16] These highly specialized spider silk proteins have the ability to transform from a soluble state to solid fibers under the influence of simultaneous changes in several physiochemical parameters such as pH, ion concentrations, and shear forces.[19] The use of DNA technology has proven the combination of four consecutive repeats of the poly-alanine/glycine-rich moiety with the nonrepetitive C-terminal domain (partial spider silk protein, denoted 4RepCT) as sufficient for self-assembly into macroscopic silk fibers.[20,21] After recombinant production of soluble 4RepCT in *Escherichia coli* (*E. coli*), the protein can be further converted into various formats, such as film, foam and mesh,[22] apart from forming fiber. The entire process from purification of the silk protein to formulation into different formats is performed under physiological-like conditions, as opposed to commonly used procedures for silkworm silk extracted from, for example, *Bombyx mori* cocoons.[23] Performing silk processing solely under conditions not compromising protein fold can be of immense value, especially if fold-dependent protein domains are to be combined with the silk protein.

To extend the applicability of recombinant spider silk materials, genetic engineering approaches have previously been used with 4RepCT to incorporate small (≈1 kDa) cell-binding peptides,[24] but also protein domains (5–17 kDa) with fold-dependent affinity properties.[25] Hitherto, the type of peptide or protein domain genetically linked to the N-terminus of 4RepCT has not interfered with the ability of the silk part to form fibers from soluble silk-fusion proteins. In addition, the silk-linked affinity domains showed retained fold-dependent ability to bind their respective target molecules.[25]

The schematic picture in **Figure 1**a outlines a hypothetical description of the route from soluble silk-fusion protein to solid fiber. In solution, the silk module (4RepCT or CT) of the silk-fusion protein will adopt a mainly random/alpha-helical fold, whereas the added domain attains its own natural fold. Next, the dimeric silk-fusion proteins will simultaneously self-assemble and structurally rearrange from random/alpha-helical fold to beta-sheet structure, i.e., formation of silk. The formed silk fiber will then continuously interact with many more structurally converted silk-fusion proteins, causing a continuous build-up of a solid fiber with the added domains exposed outward, keeping their natural fold. Combining a protein domain of interest with silk already at the genetic level leads to expression of silk fusion proteins in which each domain molecule is

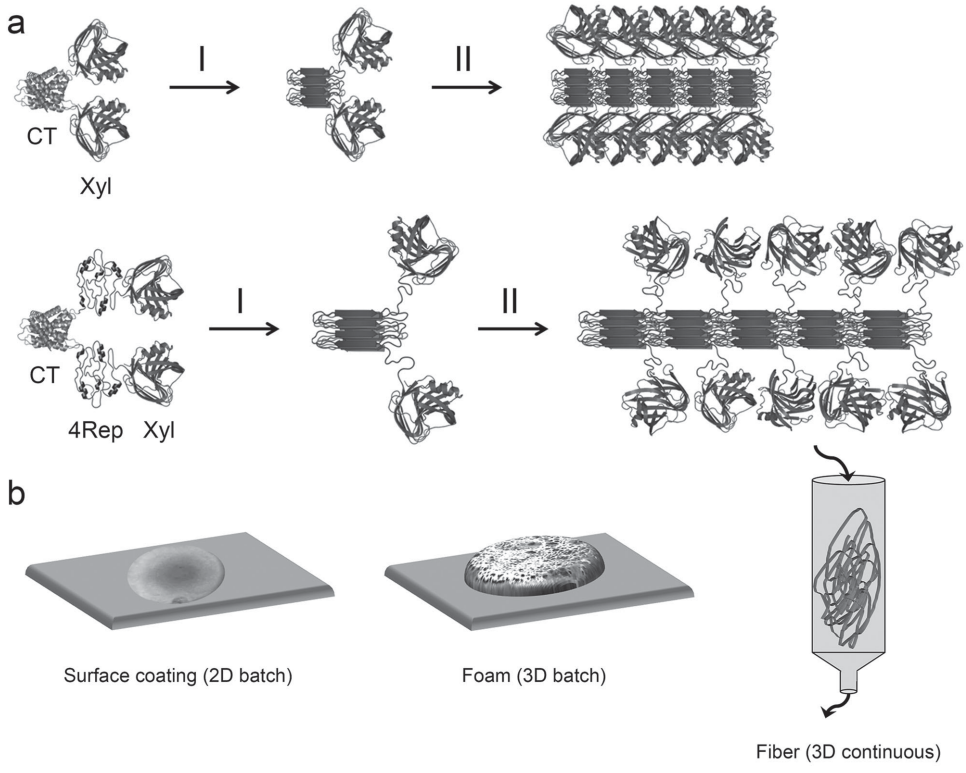

Figure 1. Conversion of soluble silk-fusion proteins into silk-like materials. a) Schematics outlying the hypothetical process for conversion of soluble silk-fusion proteins into silk-like fibers. An example with CT is shown in the upper panel, while a 4RepCT fusion is shown in the lower panel. The added protein domain is represented by the enzyme xylanase (xyl, Protein Data Bank ID: 2QZ3), arranged in mainly beta-structure. In solution, the silk module will attain a mainly random/alpha-helical fold. The silk modules of dimeric silk-fusion proteins will be spontaneously converted to beta-sheet structure (I), characteristic for silk formation. Transformed silk-fusion proteins will interact with each other (II), and as constantly more silk-fusion proteins are attracted, a macroscopic fiber will eventually appear with the added protein domains exposed outward. b) From soluble silk-fusion proteins, not only fibers can be made, but also 2D surface coatings and 3D foams. With an enzyme fused to silk, these different materials give the possibility of enzymatic batch processing (surface coating and foam), while the fiber format are best suited for continuous processing.

covalently attached to a silk molecule (i.e., 1:1 ratio). This relationship between domains and silk molecules will also apply when the silk material has self-assembled, thus resulting in a dense immobilization of the domain of interest. Moreover, the herein presented process to produce the domain (e.g., enzyme) and the material (silk) simultaneously as one entity (i.e., as a silk fusion protein) is not markedly changed from the recombinant production process of the domain alone.

In the present study, the complexity of silk-linked proteins has been extended to domains with an active site which allow catalysis of enzymatic reactions. The principle of enzyme-silk is shown for a 20 kDa large xylanase enzyme[26] genetically fused to two different silk modules, 4RepCT (23 kDa) and the even further miniaturized CT (12 kDa) lacking the repetitive part. Flat surface coatings as well as foam coatings (offering larger surface area) were explored for their suitability in enzymatic batch reactions (Figure 1b). Moreover, fibers packed in a column were investigated for enzymatic processing using a continuous substrate flow, to show the versatility offered in the types of silk formats available.

2. Results

2.1. Expression and Purification of Soluble Xylanase-Silk Fusion Proteins

A DNA sequence encoding the glycosyl hydrolase family 11 enzyme xylanase (XynA) from *Bacillus subtilis* (from now on denoted Xyl) was fused to a gene encoding the partial spider silk protein 4RepCT and the CT domain thereof, respectively (**Figure 2**a). Both fusion proteins, Xyl-4RepCT and Xyl-CT, were efficiently expressed in *E. coli*, and could be recovered as soluble silk fusion proteins after purification using immobilized metal ion affinity chromatography (IMAC). By SDS-PAGE analysis of recovered proteins the two silk fusion proteins were identified (Figure 2b, highlighted with squares). Moreover, mass spectrometry analysis of tryptic digests of these bands revealed the presence of peptide fragments from the xylanase domain as well as a peptide fragment from the CT domain, confirming the identity of the fusion proteins (data not shown). Besides bands for the full-length fusion protein Xyl-4RepCT and Xyl-CT, there were also pronounced protein bands at a molecular size of approximately 30 and 25 kDa, respectively (Figure 2b, encircled protein bands). Mass spectrometry analysis of these two shorter proteins revealed tryptic peptide fragments from xylanase, but only the N-terminal part of the silk molecule (data not shown). However, these truncated proteins are not incorporated into the respective Xyl-4RepCT and Xyl-CT silk, as the bands still appear at comparable intensities when analyzing supernatant fractions after fiber formation (Figure S1, Supporting Information), and will thus not contribute to the measured xylanase activity of the fibers.

2.2. Xylanase-Silk Fusion Proteins form Silk-like Fibers

After purification, the soluble Xyl-4RepCT and Xyl-CT fusion proteins were able to self-assemble into macroscopic fibers

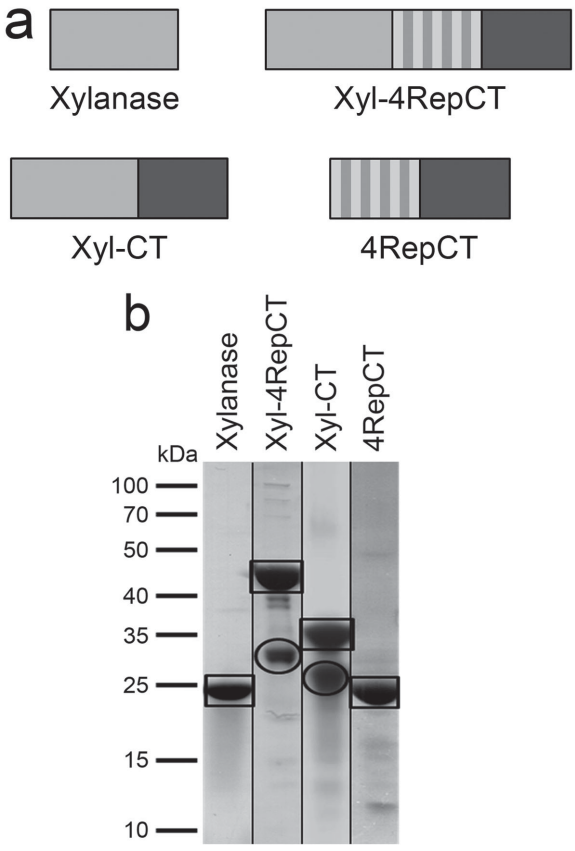

Figure 2. Design and production of soluble xylanase-silk fusion proteins. a) Schematic block representations of two enzyme-silk fusion proteins, Xyl-4RepCT (45 kDa) and Xyl-CT (34 kDa). The silk fusion constructs were designed at the genetic level by N-terminal linkage of a gene encoding the enzyme endo-1,4-beta-xylanase A (herein denoted Xyl) to a gene encoding a recombinant partial spider silk protein, 4RepCT, and to the C-terminal (CT) silk domain thereof. Shown are also free xylanase (20 kDa) and nonfunctionalized 4RepCT silk (23 kDa). All four proteins are designed to also contain an N-terminal His_6-tag (not shown). b) SDS-PAGE analysis of eluted fractions of soluble proteins after IMAC purification. Full-length fusion protein is highlighted with a black square, while truncated protein versions thereof are encircled. Free xylanase and 4RepCT silk are also indicated by black squares.

(**Figure 3**a). These results demonstrate that the partial spider silk module 4RepCT (23 kDa) preserves its ability to self-assemble even when fused with the relatively large xylanase domain (20 kDa). Moreover, the fiber forming ability of the CT domain (12 kDa) is also retained when fused with the same protein.

One noticeable difference in the fiber formation process for both Xyl-4RepCT and Xyl-CT, compared to nonfunctionalized 4RepCT and CT, was the decreased rate at which fibers were formed from protein solutions. For fiber formation of 4RepCT, macroscopic fibers are visible in less than one hour, with no apparent fiber build-up after 5 h.[21] In the case of Xyl-4RepCT and Xyl-CT, no fibers were visible until several hours of fiber formation, and both types of fibers continued growing for about one week.

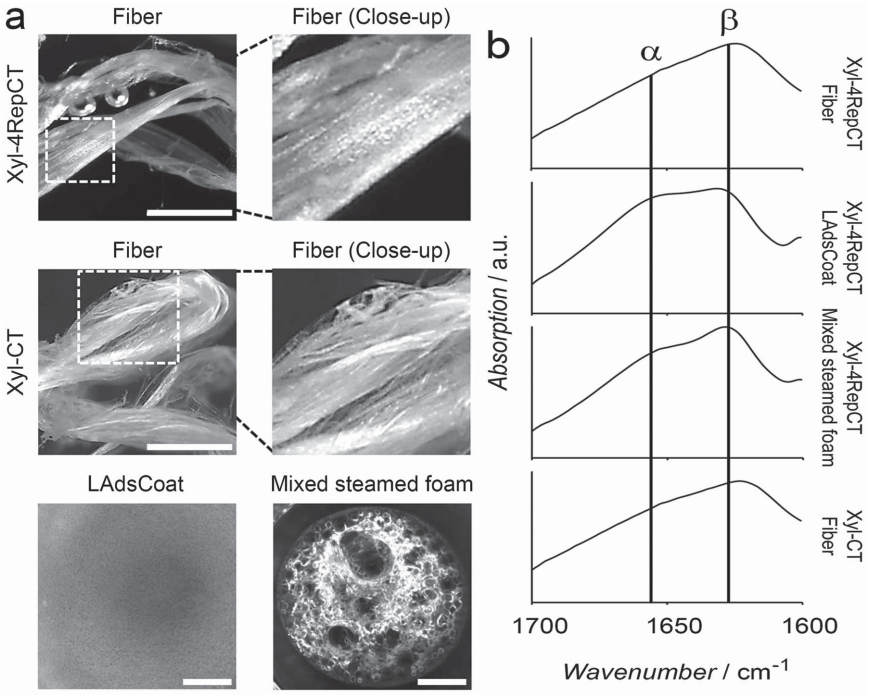

Figure 3. Various xylanase-silk materials. a) The different panels show light microscopy appearances of xylanase-silk materials in the form of Xyl-4RepCT and Xyl-CT fibers in water (top and middle panel, respectively), LAdsCoat and mixed steamed foam (bottom panel). Fibers are also complemented with close-up pictures of selected fiber areas, highlighting the more loose packing of fiber bundles in Xyl-CT fibers compared to fibers of Xyl-4RepCT. Scale bars indicate 1 mm. b) ATR-FTIR spectra from fibers of Xyl-4RepCT and Xyl-CT, and LAdsCoat, and mixed steamed foam of Xyl-4RepCT. Peaks for alpha-helix and beta-sheet are indicated by lines.

Light microscopy revealed resembling macroscopic appearances of Xyl-4RepCT and Xyl-CT fibers, compared to fibers of 4RepCT, all composed of fiber bundles (Figure 3a, upper and middle panel). However, the Xyl-CT fibers differed slightly in their macroscopic structure in that they were more ragged, with individual fibers sometimes partly detached from the fiber bundle (Figure 3a, close-up images). Also, Xyl-CT fibers were more prone to disassemble into individual fibers from the fiber bundle upon physical handling.

2.3. Both Soluble Xylanase-Silk Protein and Xylanase-Silk Fiber Display Enzymatic Activity

The activity of xylanase in a solution of xylanase-silk fusion protein and in solid xylanase-silk fibers was determined using a colorimetric assay (**Figure 4**a). In this assay a substrate is added to the enzyme, after which the enzymatic reaction is terminated with a stop solution before the product is visualized in a color-development procedure. Soluble Xyl-4RepCT showed a xylanase activity comparable to pure xylanase (i.e., positive assay control), when comparing the same molar amount of the respective protein (Figure 4a, left panel). As expected, nonfunctionalized soluble 4RepCT protein gave background signal comparable to buffer lacking xylanase (i.e., negative assay control).

Both Xyl-4RepCT and Xyl-CT also showed activity in fiber format, as opposed to nonfunctionalized 4RepCT control fibers (Figure 4a, right panel). These results demonstrate that the xylanase can preserve its activity although produced in fusion with the silk modules 4RepCT or CT, and also after further processing into solid fibers.

2.4. Other Xylanase-Silk Formats also Display Enzymatic Activity but Have to be Stabilized

An easy way of adding new protein functions to an already existing material is to perform surface coating by plain adsorption. A large surface-to-volume ratio is often preferred in order to increase the efficiency in, for example, chemical and biological reaction systems. Therefore, in addition to fibers, also 2D coatings and 3D foams were prepared from Xyl-4RepCT (Figure 3a, lower panels) and analyzed for xylanase activity.

Two types of 2D surface coatings of Xyl-4RepCT (AdsCoat and LAdsCoat) were investigated and compared for xylanase activity. The two coatings differ in that AdsCoat consists of an adsorbed layer of Xyl-4RepCT, while the LAdsCoat instead combines first an adsorbed layer of 4RepCT with a second layer of Xyl-4RepCT. Both coating variants showed preserved xylanase activity at comparable levels, whereof the activity of LAdsCoat is presented in Figure 4b (left panel). Furthermore, neither of the materials

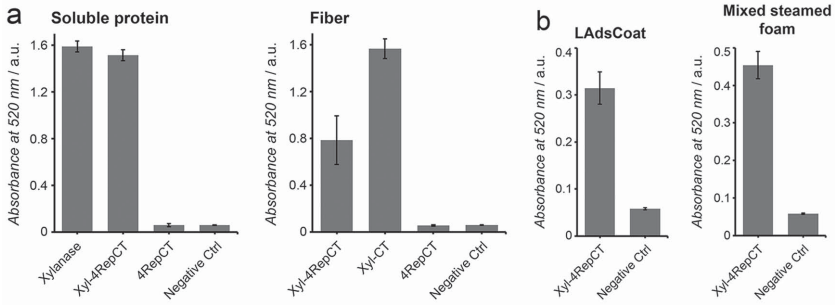

Figure 4. Enzymatic activity of xylanase-silk materials. a) The graphs in the left panel show xylanase activity of soluble xylanase and xylanase-silk fusion protein Xyl-4RepCT, respectively, at equal molar amounts. In the right panel, the enzymatic activity of Xyl-4RepCT and Xyl-CT fibers is presented. For comparison, both panels are also showing the activity of nonfunctionalized 4RepCT silk and a negative assay control (buffer without xylanse). b) Graphs showing the xylanase activity of two additional Xyl-4RepCT materials, a 2D surface coating (LAdsCoat), and a 3D foam (mixed steamed foam). ($n \geq 2$, in triplicates.)

displayed any activity in wash fractions after initial washing, indicating good rigidity of the formed materials. The LAdsCoat variant was chosen for further analysis, based on the fact that combining Xyl-4RepCT with nonfunctionalized 4RepCT silk showed increased stability for steamed foam (see next section).

Activity measurement of 3D foams of Xyl-4RepCT revealed preserved xylanase activity (data not shown), but also showed substantial amount of activity in wash fractions of the foam. In addition, the foam sometimes partly disintegrated into smaller pieces when liquid was applied. In attempt to stabilize the Xyl-4RepCT foam format, two new foam variants were produced; steamed foam and mixed steamed foam. The steamed foam gets more flattened than the standard foam by the steaming process. This indeed turned out to prevent later disintegration of the material. Furthermore, the colorimetric assay revealed active xylanase domains within the steamed foam (data not shown), although still some signal was observed for the wash fractions. Therefore, mixed steamed foam was prepared from a mixture of soluble Xyl-4RepCT and 4RepCT. This foam variant also showed improved mechanical stability, with no apparent material disintegration when immersed in liquid. More importantly, after initial material washing no activity was observed in following wash fractions and the final foam showed preserved xylanase activity (Figure 4b, right panel). This more stable mixed steamed foam format was therefore used for further investigation.

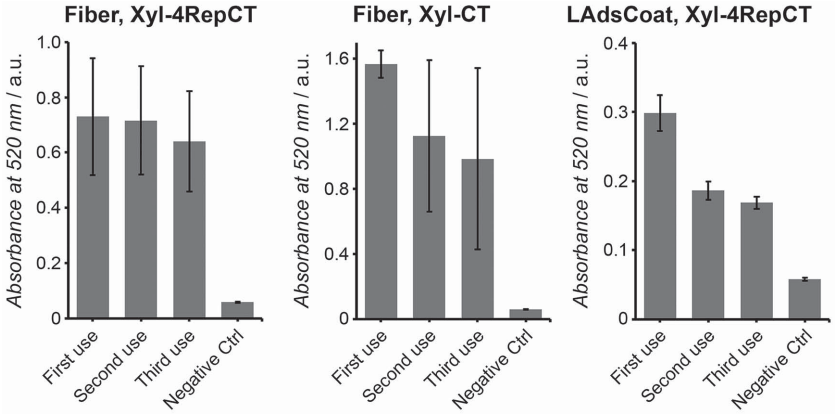

Figure 5. Enzymatic activity of xylanase-silk materials after reuse. Graphs showing the xylanase activity for fibers of Xyl-4RepCT (left panel), Xyl-CT (middle panel), and for LAdsCoat (right panel) after reuse of the materials. Each xylanase-silk material was subjected to three consecutive activity measurements with extensive washing in between the measurements. (n = 1–3, in triplicates).

2.5. IR Spectroscopy Reveals Typical Beta-Sheet Signal in all Silk Materials

As a measure of the secondary structure content in materials made from enzyme-silk fusion proteins, attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) was utilized. This method makes it possible to distinguish beta-sheet structure (band position: 1623–1641 cm^{-1}) from alpha-helical fold (band position: 1648–1657 cm^{-1}),[27] and thereby determine the degree of conversion from soluble enzyme-silk proteins (alpha/random) to silk-like materials (beta-sheet). Fibers of both Xyl-4RepCT and Xyl-CT showed a clear beta-sheet signal (Figure 3b) typical of silk,[28] as well as previously seen for 4RepCT fibers.[25] Also the stabilized versions of the other formats, LAdsCoat and mixed steamed foam, showed a clear beta-sheet signal, although with a pronounced peak also in the alpha-helical region (Figure 3b), indicating partial but incomplete silk transformation from alpha-helical to beta-sheet structure.

2.6. Xylanase-Silk Materials are Active after Storage

The remaining functionality of various xylanase-silk materials after prolonged storage at wet and dried conditions was investigated (Figure S2, Supporting Information). For storage under wet conditions (Tris-HCl buffer), fibers of Xyl-4RepCT showed comparable activities after both one and three months of storage. A Xyl-CT fiber had a residual activity after 11 months of storage that was comparable to that after 1 month storage. Moreover, a fiber of Xyl-CT stored dried for 3 d displayed the same activity as before drying. AdsCoat and mixed steamed foam showed comparable xylanase activities before and after dry storage at room temperature for 1.5 months and 1 month, respectively. Taken together, these results show the possibility to store xylanase-silk materials in both wet and dried conditions with maintained activity.

2.7. Xylanase-Silk Materials can be Reused

To investigate the possibility to reuse xylanase-silk materials, fibers along with LAdsCoat were analyzed. For Xyl-4RepCT fibers, the activities measured for all three consecutive reactions were approximately at the same level (**Figure 5**, left panel), whereas reuse of Xyl-CT fibers showed a trend (not significant) to decrease to ≈70% of the initial activity at the second use (Figure 5, middle panel).

Reuse of Xyl-4RepCT LAdsCoat showed that the material was active at the second and third use (Figure 5, right panel), although the activity decreased to ≈60% of the initial activity after the first use. Moreover, a previously used Xyl-4RepCT AdsCoat subjected to drying and storage for 1.5 months at room temperature showed an activity comparable to that before drying (data not shown). Thus, the investigated xylanase-silk materials can be reused, and all show substantial xylanase activity for at least three consecutive rounds of enzymatic reaction.

2.8. Xylanase-Silk Materials show Enzymatic Activity after Ethanol Treatment

The ability of xylanase-silk materials to withstand treatment with 70% ethanol was determined for Xyl-4RepCT and Xyl-CT fibers, and also for LAdsCoat (**Figure 6**). Fibers of Xyl-4RepCT were active after ethanol treatment, but showed a trend (not significant) toward about 30% reduction in activity compared to

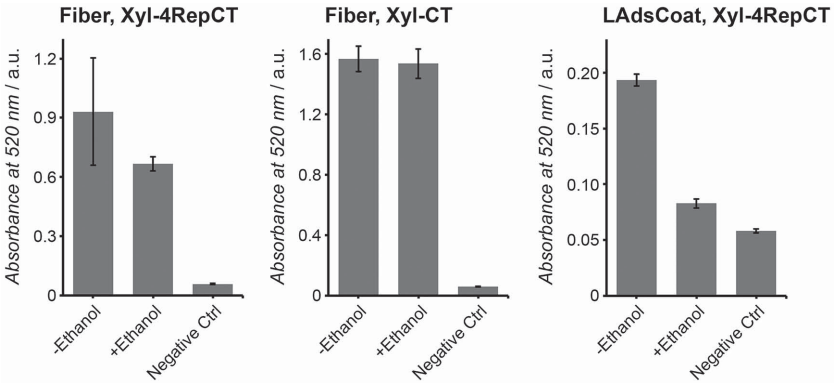

Figure 6. Enzymatic activity of xylanase-silk materials after ethanol treatment. Measured xylanase activity for Xyl-4RepCT and Xyl-CT fibers (left and middle panel, respectively), and for LAdsCoat (right panel), after ethanol treatment. The xylanase-silk materials were treated with 70% ethanol prior to determination of the xylanase activity (+Ethanol) and, in parallel, corresponding materials not subjected to ethanol were assessed for activity (–Ethanol). (n = 1–3, in triplicates).

nontreated fibers (Figure 6, left panel). In contrast, the xylanase activity of Xyl-CT fibers did not seem to be affected at all by the ethanol treatment (Figure 6, middle panel). The LAdsCoat also displayed activity after ethanol treatment, although the activity was reduced to about 40% of the activity for nontreated LAdsCoat (Figure 6, right panel).

2.9. Sodium Hydroxide and Autoclaving Irreversibly Destroy the Activity of Xylanase-Silk

Standard procedures for cleaning-in-place and sterilization of solid matrices are, for example, sodium hydroxide (NaOH) wash and autoclaving, respectively. The effect of these treatments on the activity and stability of xylanase-silk materials was therefore investigated.

After treatment with NaOH, a harsh chemical known to denature proteins although not breaking peptide bonds, neither Xyl-4RepCT fibers nor LAdsCoat displayed any xylanase activity, irrespective of the NaOH concentration (0.5 and 1 M) used (data not shown). Furthermore, all Xyl-4RepCT fibers seemed slightly softer and more loosened upon handling, compared to nontreated fibers, after NaOH treatment. To give the unfolded xylanase domains in NaOH treated Xyl-4RepCT fibers a chance to refold, 2 d of incubation of the fibers in Tris-HCl buffer was allowed. However, no measurable xylanase activity was determined for any of the fibers after 2 d in Tris-HCl (data not shown). In contrast to the Xyl-4RepCT fibers, fibers of Xyl-CT completely dissolved during treatment with 1 M NaOH and, thus, could not be assessed for xylanase activity.

The performance and functionality of steam sterilized xylanase-silk fibers was also explored. After autoclaving, measurement of the xylanase activity did not indicate any detectable activity for Xyl-4RepCT or Xyl-CT fibers (data not shown). Moreover, the fibers were considerably stiffer in texture than nonautoclaved ones. ATR-FTIR spectroscopy of the treated fibers could not reveal any reason for the change, since their signatures were essentially the same as for untreated fibers (data not shown).

2.10. Xylanase-Silk Fibers are Enzymatically Active under Continuous Substrate Flow

To test the enzymatic activity of xylanase-silk fibers in a continuous process, a substrate solution was circulated through a column packed with Xyl-4RepCT fibers (**Figure 7**a). Measurement of the Xyl-4RepCT fibers subjected to continuous substrate flow revealed xylanase activity already after one substrate cycle (2 min and 45 s) that continued for at least 20 cycles (55 min), as shown by the gradual increase in measured absorbance from accumulated product (Figure 7b). These absorbance

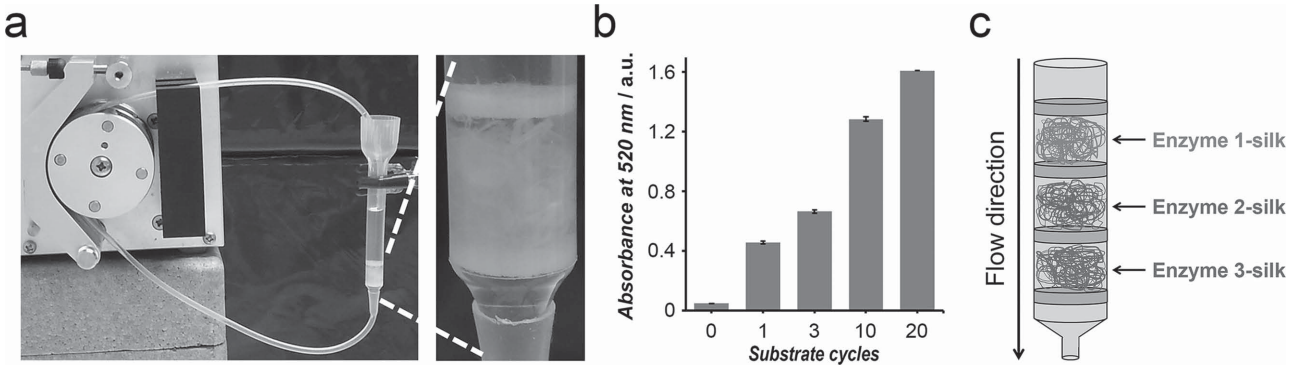

Figure 7. Enzymatic activity of xylanase-silk fibers subjected to a continuous substrate flow. a) The set-up used to investigate the activity of Xyl-4RepCT fibers in a continuous substrate flow. Fibers were placed inside a plastic column and fixed between two filters (right panel). Rubber tubing was attached to the outlet and inlet of the column, creating a loop, and the tubing was mounted to a peristaltic pump (left panel). The column and tubing system was filled with substrate solution that was allowed to be pumped through the fibers for a certain number of cycles. b) Absorbance of accumulated products from a substrate solution passing Xyl-4RepCT fibers for 1, 3, 10, and 20 cycles. Cycle 0 corresponds to the absorbance of substrate solution before passage through the fibers. c) Schematics showing a principle applicable for a multienzyme system using continuous flow and enzyme-silk. In this example system three different enzymes are used in series, i.e., the enzymatic product of Enzyme 1 acts as substrate for Enzyme 2, and the product of Enzyme 2 thus acts as substrate for Enzyme 3. The final product generated from Enzyme 3 can easily be collected at the column outlet.

values can be compared to cycle 0, corresponding to a substrate fraction that has not been in contact with the fibers and therefore serving as a negative control. From these results, it could be concluded that the covalently silk-linked xylanase domain is not only active in batch reactions, but also has the ability to retain enzymatic activity during a continuous substrate flow.

The absorbance values increased gradually for up to 20 substrate cycles. However, for cycle 50 and 100, the measured absorbance leveled off to approximately the same value as for cycle 20. To confirm that this effect was not due to inactivated enzymes, the same fibers were washed and again subjected to a continuous substrate flow, showing a similar activity pattern (Figure S3, Supporting Information). The reason for leveling off is thus either due to lack of substrate, a limitation in the availability of other reagents used in the colorimetric assay, and/or product inhibition of the enzyme by accumulated end-product.

3. Discussion

The silk module 4RepCT has previously been shown to be capable of spontaneous assembly into silk-like fibers under near-physiological conditions.[21] In solution 4RepCT attains a random/alpha-helical structure,[20] that upon self-assembly into fibers will be converted into a mainly beta-sheet structure. Macroscopic fibers assembled from 4RepCT protein obtain strength and elasticity by the alternating poly-alanine/glycine-rich blocks within the 4Rep part, while the CT domain is thought to mainly order the silk assembly process. However, the CT domain alone is also capable of forming fibers,[20] although not as stable as 4RepCT fibers. In this study we report the first case of silk functionalization using only the CT domain, as we demonstrate that both of the enzyme-silk fusion proteins Xyl-4RepCT and Xyl-CT are able to spontaneously self-assemble into macroscopic fibers in spite of the relatively large xylanase domain that has been attached to the spider silk modules. Notably, the molecular size of xylanase is approximately the same as for 4RepCT, thus almost twice the size of the CT domain. Moreover, the displayed inherent catalytic ability of xylanase to cleave beta-1,4-glycosidic linkages of xylan shows that the enzymatic activity of xylanase is preserved in the investigated xylanase-silk materials. The retention of enzymatic activity also indicates that the predominant beta-structure of xylanase[29] is not destroyed or trapped by the random/alpha-helical to beta-sheet conversion of silk upon self-assembly.

However, the fiber formation process of 4RepCT and CT was not entirely unaffected by the covalent linkage to xylanase. A prolonged time for fibers to form from both soluble Xyl-4RepCT and Xyl-CT protein was noticed, compared to nonfunctionalized 4RepCT, and can most likely be attributed to the high solubility of the xylanase itself. Another potential rational for the delayed/slower fiber formation could be the relatively large size of the covalently linked xylanase. The xylanase domain may serve as a steric hindrance for the silk parts to interact with each other, which not completely prevents the interaction but just prolongs the time for silk assembly. This is in line with a previous finding, reporting on reduced assembly kinetics of hydrogels based on recombinant spider silk when functionalized with fluorescein molecules.[30] Furthermore, the more ragged macroscopic structure observed herein for Xyl-CT fiber, that lacks the repetitive silk part, might be due to decreased interaction between individual silk fibrils.

For all three types of enzyme-silk materials presented herein, the actual molecular content is not known in the samples used for activity measurement, and this constitutes a problem for direct comparison of the activity between different silk materials. For that reason, the activities of the different silk materials in Figures 4–6 are separated into individual graphs, and not directly compared (the negative control without xylanase was always approximately 0.06 absorbance units). For fibers there is no simple way of determining how many of the soluble silk molecules have been incorporated into the final silk fiber, or left in soluble form in the supernatant, respectively. Direct weighing of dried fibers could be performed but is a difficult task due to its light weight (the density of natural spider silk being 1.3 g cm^{-3},[31] and the small samples used (mg to sub-mg for fiber and µg for coating/foam). One should also keep in mind that the surface area will probably vary among individual fibers with similar molecular content, affecting the number of exposed enzyme domains. In the case of surface coatings it is difficult to determine how many silk molecules, or layers of molecules, that have been attached to the surface during the coating process. Work is in progress to investigate the possibility of using sensor technology such as quartz crystal microbalance (QCM) to monitor this process.[32] In contrast to the stable fibers, both foam and surface coating are suffering from release of silk molecules upon initial washing, further complicating the estimation of molecular content in the final silk sample.

Initially, the prepared 2D coatings and 3D foams displayed poor stability with release of soluble enzyme-silk proteins and material disintegration during washing. One can speculate that the large size of xylanase could, to some extent, hinder the tight beta-sheet organization of the 4RepCT modules, in line with what has been hypothesized for fluorescein-coupled silk.[30] This would make the silk material physically less stable, with material disintegration as a consequence. To facilitate silk–silk interactions when producing surface coatings and foams, Xyl-4RepCT was combined with nonfunctionalized 4RepCT. In the case of coating, a two-layer approach was adopted in which the first layer of 4RepCT attached to the surface, followed by a second layer of Xyl-4RepCT interacting with the 4RepCT layer. This resulted in a stable coating, LAdsCoat, with no activity in wash fractions. In addition to increased silk–silk interactions, a contribution to the stabilization of the LAdsCoat format could be due to a more stable initial adsorption of 4RepCT to the plastic surface, compared to Xyl-4RepCT. Production of stabilized foam was achieved in a similar way, from a 1:1 molar ratio of soluble Xyl-4RepCT and 4RepCT. The overall increase in stability of the foam is also most likely due to an increased probability of silk–silk interactions to occur, facilitated by the higher ratio of 4RepCT modules.

It should be pointed out that production of the silk materials described herein does not involve any denaturing post-treatment procedures with alcohol to render the materials water-insoluble. Treatment with alcohol is otherwise commonly used for increasing the beta-sheet content, and thereby the stability, for production of water-insoluble silkworm silk materials as

well as recombinant spider silk films.[15,33] If exposure of the silk material to the denaturing effect from alcohol is avoided, irreversible destruction of the protein domains can be prevented. The functionalized fibers produced herein were water-insoluble and stable without any denaturing treatment. To stabilize the enzyme-silk coatings and foams presented herein, we made sure to use other methods for stabilization instead, to avoid unwanted protein denaturation of the immobilized enzyme.

The ability to store a material without significant loss of function, the so-called shelf-life, is of great concern for functional materials. Data on storage of xylanase-silk materials presented here does not only confirm that the xylanase itself is active after storage, but more importantly to show the retained functionality of the combined xylanase-silk materials after storage. Previously, unchanged activity of glucose oxidase entrapped in silk films after ten months of storage has been reported.[8] The interaction between silk and glucose oxidase was then suggested to play an overall role in the reported stabilization. Xylanase-silk fibers were herein shown to not only maintain activity after storage in wet conditions, but also after drying and rewetting. Whether this ability resides in the properties of xylanase alone, or if the covalent linkage to silk is a contributing factor is currently not known.

Another issue concerning functional materials is the ability to reuse the material. Both types of xylanase-silk fibers and LAdsCoat could be reused for at least three times with retention of significant xylanase activity. However, a reduction in activity for the second use was obvious for LAdsCoat, and also indicative for the Xyl-CT fiber. For xylanase-silk fibers, the manual handling during washing could have caused the occasionally observed decrease in activity. The manual handling was avoided during the column experiment, where the fibers were trapped between two filters and activity was shown for more than 20 cycles of continuous substrate flow, even after extensive washing of the column. For LAdsCoat, the reduction in activity for the second reuse is most likely due to removal of xylanase-silk proteins that have not been properly converted into beta-sheet structure, similar to what was observed during material prewashing. The activity shown during the third reuse was similar to during the second reuse, thus suggesting that poorly incorporated xylanase-silk molecules then had been washed away.

Immobilized enzymes give, except for the benefit of being reusable and ease of product separation, the possibility to operate an enzymatic reaction under continuous substrate flow, in addition to batch processing. The set-up explored herein showed a practical example of the possibility to use a continuous substrate flow with retained enzymatic activity of Xyl-4RepCT fibers. A continuous enzyme catalysis set-up as this could be useful in multienzymatic systems, where the product from one enzyme will serve as substrate for another enzyme, as previously investigated for various purposes.[34] In line with this, we do propose the use of enzyme-silk fibers in combination with a continuous substrate flow for sequential enzymatic reactions, such as the system outlined in Figure 7c. In this hypothetical system, three variants of enzyme-silk fibers are produced and packed inside a column in a suitable order. The continuous substrate flow will transport the first substrate to the first enzyme in the enzyme chain (Enzyme 1), after which its product will be further transported and act as substrate for the second enzyme in the chain (Enzyme 2). The final product from Enzyme 3 can finally be collected at the outlet of the column.

There are a few previous reports on the use of silkworm silk in combination with immobilized enzymes for the construction of biosensors. For example, silk has been combined with glucose oxidase for sensing of glucose,[6,35] peroxidase for hydrogen peroxide sensing,[36] and uricase for detection of uric acid.[37] The possibility to genetically combine these enzymes with recombinant spider silk using the strategy presented herein could pave the way for biosensors with increased sensitivity, as one enzyme is attached to every silk molecule, allowing precise control and possible high density. Moreover, a multienzymatic silk set-up could lead to more complex biosensors.

4. Conclusion

The research work presented in this paper demonstrates a proof-of-concept for genetically fused enzyme-silk materials, combining xylanase with recombinant spider silk. Xylanase-silk proteins were expressed and purified as soluble fusion proteins, and assembled into fibers displaying catalytic xylanase activity. Other formats of the xylanase-silk material, as 2D surface coating and 3D foam, also showed retained enzyme activity. The catalytic properties were also retained in the silk materials after storage, reuse, cleaning with ethanol as well as in a continuous substrate flow.

5. Experimental Section

Gene Constructions: A gene encoding glycoside hydrolase family 11 (GH11) endo-1,4-beta-xylanase (XynA) from *B. subtilis*, strain 168 (NCBI Gene ID: 939861, including a signal peptide sequence) was inserted into a pEXP5-CT/TOPO vector. To eliminate an internal *Nde*I restriction cleavage site within the xylanase gene, forward and reverse primers were designed followed by site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit, Agilent Technologies) creating a silent mutation (*ACA* to *ACG*). The sequence verified vector was then used as template for PCR (KAPA HiFi HotStart ReadyMix, Kapa Biosystems, Inc.) of the xylanase sequence (signal peptide sequence excluded), using forward and reverse primers containing an *Nde*I and an *Eco*RI restriction cleavage site, respectively. The amplified xylanase gene was inserted into vectors containing either a sequence for the partial spider silk, 4RepCT, or for the CT domain.[20] Both target vectors also contained a sequence for a His_6-tag under control of the T7 promoter.

Expression and Purification: The two xylanase-silk DNA constructs (denoted Xyl-4RepCT and Xyl-CT) were used to transform *E. coli* BL21(DE3) cells (Merck Biosciences). Protein expression in *E. coli* and purification using IMAC were then performed essentially as previously described,[25] with the exception that NaCl (200×10^{-3} M) was included in the buffers during protein loading onto the column and protein elution. Purified protein was obtained at concentrations of 0.5–3 mg mL^{-1}. Partial spider silk protein without attached xylanase (4RepCT) or RGE-4RepCT (with tripeptide RGE) was used as control and produced and purified as previously described.[20] Soluble xylanase was expressed and purified as described elsewhere.[38]

Mass Spectrometry: After running proteins on SDS-PAGE, gel slices were excised followed by tryptic digestion and mass spectrometry analysis of generated peptides by MALDI-TOF/TOF (WCN Proteomics Facility, Department of Medical Biochemistry and Microbiology, Uppsala University, and Division of Protein Technology, School of Biotechnology, KTH Royal Institute of Technology).

Formation of Xylanase-Silk Materials: Macroscopic fibers were self-assembled from soluble Xyl-4RepCT, Xyl-CT, and 4RepCT protein by gentle tilting of the respective protein solution at room temperature for 1–14 d. Coatings of Xyl-4RepCT in 96-well plates (Tissue Culture Plate, Suspension Cells, 83.1835.500, Sarstedt) were prepared in two different ways, denoted AdsCoat and LAdsCoat. For AdsCoat, soluble Xyl-4RepCT protein (50 µL, 1 mg mL^{-1}) was transferred to cover the bottom of the wells, for 2 h at room temperature. Next, remaining soluble protein was removed and the plates incubated overnight at 20 °C and 35% relative humidity (rh). LAdsCoat was produced by a first protein adsorption using 4RepCT (50 µL, 0.5 mg mL^{-1}) for 2 h at room temperature, followed by removal of remaining soluble protein prior to incubation of the plate with Xyl-4RepCT (50 µL, 1 mg mL^{-1}), also for 2 h at room temperature. Three different types of Xyl-4RepCT foams were produced (foam, steamed foam, and mixed steamed foam), all variants prepared in 96-well plates (Tissue Culture Plate, Suspension Cells, 83.1835.500, Sarstedt). For preparation of foam, soluble Xyl-4RepCT (5 µL, 3 mg mL^{-1}) was foamed by pipetting air into the protein solution, followed by overnight solidification (20 °C, 35% rh). Steamed foam was prepared in the same way, followed by foam steaming, a procedure in which the solid foams were subjected to high humidity for approximately 4–8 h by enclosing the foams in a box containing beakers with hot water. Mixed steamed foam was prepared in the same way as steamed foam, with one exception: the protein solution to be foamed (5 µL) consisted of equimolar (100 pmol) amounts of Xyl-4RepCT and 4RepCT.

Light Microscopy: Light microscopy was used for visual documentation of xylanase-silk materials. For Xyl-4RepCT fibers, light images were taken at two times magnification using an inverted Nikon Eclipse T*i* light microscope. To document Xyl-CT fibers, LAdsCoat and mixed steamed foam, a stereo light microscope (Nikon) with portable USB camera was used at two times magnification.

Xylanase Activity Assay: To measure xylanase activity a colorimetric reducing-sugar assay (Megazyme, Bray, Ireland) based on the Nelson/Somogyi method[39] was utilized. As substrate for enzymatic reactions with xylanase, a 1% (w/v) arabinoxylan (P-WAXYM, Megazyme) solution was used and prepared as described by Megazyme. The pH of the substrate solution was adjusted within the range of 6.0–7.0 by addition of sodium phosphate buffer (10 mL, 0.5 M). The xylanase activity for soluble silk proteins and for solid silk materials was determined by incubation with arabinoxylan (100 µL) for 1 h at room temperature, after which the reaction was terminated by addition of copper reagent (100 µL). After boiling for 20–25 min, the product was visualized through color development by addition of a sodium arsenate solution (600 µL) according to the protocol in the manual provided with the kit (Megazyme, Bray, Ireland). The absorbance of formed product at 520 nm was measured using a plate reader (Infinite M200, Tecan) for each sample (200 µL). All investigated samples were analyzed in triplicates. For each activity measurement, both a positive and a negative assay control were included. As positive control xylanase (20 µL, 0.4–0.3 mg mL^{-1}) was added to substrate solution (80 µL), followed by sample incubation for 1 h at room temperature and reaction termination and color development. As negative control, Tris-HCl (20 µL, 20 × 10^{-3} M, pH 8.0) was used. Positive and negative controls were also analyzed in triplicates.

Activity of Xylanase-Silk: For activity determination of soluble fusion proteins, Xyl-4RepCT (20 µL, 1 mg mL^{-1}) and 4RepCT or RGE-4RepCT (20 µL, 1 mg mL^{-1}) control protein was mixed with arabinoxylan substrate (100 µL), and the absorbance measured. For solid silk materials of Xyl-4RepCT (fiber, AdsCoat, LAdsCoat, foam, steamed foam, mixed steamed foam) and Xyl-CT (fiber), each material was washed 1–5 times in Tris-HCl (100 µL, 20 × 10^{-3} M, pH 8.0) prior to activity measurement.

Activity of Xylanase-Silk Materials after Storage: Fibers of Xyl-4RepCT were stored in Tris-HCl (20 × 10^{-3} M, pH 8.0) at +4 °C for up to three months, prior to measurement of residual xylanase activity. In addition, other Xyl-4RepCT fibers were first stored for 36 d in Tris-HCl buffer (+4 °C) and then used to determine the xylanase activity, after which the fibers were dried and stored for another 40 d in room temperature, followed by rehydration of the fibers in Tris-HCl buffer and activity measurement. One Xyl-CT fiber, stored for 11 months in Tris-HCl buffer at +4 °C, was used to measure xylanase activity. Next, the Xyl-CT fiber was dried and stored in room temperature for 3 d, whereupon it was re-hydrated and the xylanase activity was measured again.

AdsCoat and mixed steamed foam were stored dried at room temperature for 49 and 31 d, respectively, followed by determination of the residual xylanase activity. Also, AdsCoated plates, previously used one time for activity measurement were dried, stored at room temperature for 43 d and then again assessed for xylanase activity.

Activity of Xylanase-Silk Materials upon Reuse: Fibers of Xyl-4RepCT and Xyl-CT, as well as LAdsCoat were initially washed 3–5 times with Tris-HCl (100 µL, 20 × 10^{-3} M, pH 8.0). Substrate was subsequently added, and after 1 h of incubation at room temperature, the substrate solution was removed and xylanase activity measured. The solid materials were then immediately washed three times with Tris-HCl (100 µL, 20 × 10^{-3} M, pH 8.0), followed by two more rounds of substrate incubation. Residual xylanase activity after each set of washes was measured as stated previously.

Activity of Xylanase-Silk Materials after Ethanol Wash: Xyl-4RepCT and Xyl-CT fibers, and also LAdsCoat were washed 3–5 times with Tris-HCl (100 µL, 20 × 10^{-3} M, pH 8.0), prior to incubation in 70% ethanol (100 µL) for 20 min at room temperature. After ethanol removal and three rounds of washing with Tris-HCl (100 µL, 20 × 10^{-3} M, pH 8.0), substrate was added and xylanase activity determined according to the protocol previously stated. As controls, corresponding materials without ethanol treatment, instead immersed in Tris-HCl (20 × 10^{-3} M, pH 8.0), were used.

Activity of NaOH Treated Xylanase-Silk Materials: Fibers of Xyl-4RepCT and Xyl-CT, as well as LAdsCoat were initially washed once (fiber) or five times (LAdsCoat) in Tris-HCl (20 × 10^{-3} M, pH 8.0). All materials were then treated with NaOH (100 µL, 0.5 M (omitted for Xyl-CT fiber) and 1 M, respectively) for 20 min at room temperature. Also, all conditions included a nontreated control, instead immersed in Tris-HCl (20 × 10^{-3} M, pH 8.0). After three times of washing of Xyl-4RepCT fibers in Tris buffer (100 µL), the pH of the third wash was measured with a pH paper to ensure a pH close to pH 8. The xylanase activity of the functionalized spider silk material was measured according to previous description. After performing activity measurement on fibers of Xyl-4RepCT, the fibers were immersed in Tris-HCl (20 × 10^{-3} M, pH 8.0) for 2 d. Next, the fibers were washed three times in Tris-HCl buffer, followed by a new measurement of residual xylanase activity.

Activity of Xylanase-Silk Fibers after Autoclaving: Fibers of Xyl-4RepCT and Xyl-CT were sterilized by autoclaving (20 min, 121 °C) in Tris-HCl (20 × 10^{-3} M, pH 8.0) buffer. Prior to determination of xylanase activity for autoclave-treated and nontreated fibers, they were washed 1–4 times with Tris-HCl buffer (100 µL).

Fourier Transform Infrared Spectroscopy: Fibers of Xyl-4RepCT (nontreated, ethanol treated, NaOH (1 M) treated and autoclaved) and Xyl-CT (nontreated, ethanol treated, and autoclaved) previously assessed for xylanase activity were collected and air-dried at room temperature. LAdsCoat and mixed steamed foam of Xyl-4RepCT were prepared as previously stated. To record the absorbance from ATR-FTIR a platinum ATR unit from Bruker (Tensor 37) was used. The wavelength region investigated was 2000 to 850 cm^{-1}, with increments of 1 cm^{-1}.

Activity of Xylanase-Silk Fibers in a Continuous Flow: Fibers of Xyl-4RepCT were enclosed inside an empty plastic column and kept in place in the column by top and bottom filters. A loop through the column was created by attachment of tubing to the bottom outlet of the column and allowed to pass through a peristaltic pump, and then inserted into the top inlet of the column (for complete set-up, see Figure 7a). The loop system was filled with 3 mL of substrate solution, after which the pump immediately started to circulate the substrate through the fibers inside the column at a flow rate of approximately 1.1 mL min^{-1}. Around 100 µL of substrate solution was withdrawn from the system, through the tubing placed at the column inlet, for a total of seven times. The time points for substrate removal were 0 min (0 substrate cycles through the column), 2 min and 45 s (≈1 substrate cycle); 8 min and 15 s

(≈3 substrate cycles), 27 min and 30 s (≈10 substrate cycles), 55 min (≈20 substrate cycles), 2 h and 17 min (≈50 substrate cycles), 4 h and 35 min (≈100 substrate cycles). The enzymatic activity was measured for the withdrawn substrate solution for each selected substrate cycle. The column set-up was disassembled and the fibers washed and stored in Tris-HCl (20×10^{-3} M, +4 °C) overnight, after which the experiment was repeated again up to 50 substrate cycles.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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