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Uptake of Azo Dyes into Silk Glands for Production of Colored Silk Cocoons Using a Green Feeding Approach

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Supporting Information

ABSTRACT: Dyeing of textile fabrics is considered to be one of the most polluting industries today, and there is a need to develop green processes that can reduce this pollution. A promising technology that can potentially cleanup the dyeing of silk fibers that are widely used for textile applications would involve the generation of intrinsically colored silk cocoons. This can be achieved by feeding of *Bombyx mori* silkworm larvae with a modified feed of mulberry leaves containing a sprayed dye solution. This process significantly reduces the need for treating toxic dye effluents that are generated in traditional dyeing processes. In this report, we have evaluated a set of seven different azo dyes that are used in the textile industry for dyeing to produce intrinsically dyed silk. The dyes used in the study had similar chemical structures with systematically varying partition coefficients. The results suggest that while some dyes produced intrinsically colored silk other did not. Careful evaluation of the physical properties of these related azo dyes suggest that the balance of hydrophobic and hydrophilic character is necessary for diffusion of the dye from the alimentary canal of the silkworm larva into the hemolymph and later into the silk glands. The partition coefficient of the dye also determines the preferential association of the dye with either sericin or fibroin protein in the silkworm gland and finally into the cocoon. These insights are extremely important in development of novel dye molecules that can be successfully fed to *Bombyx mori* silkworm larvae for producing intrinsically colored silk of various colors and shades.

KEYWORDS: *Color silk, "Green" silk, Azo dyes, Dye uptake, Biochemical pathways*

INTRODUCTION

Textiles and their end products constitute the world's second largest industry, only ranking below food products.[1] Textile production involves several wet processes that include scouring, bleaching, dyeing, and finishing of textile fibers. Because the largest use of textiles is in the retail apparel market, most textile fibers (both natural and synthetic) undergo a dyeing process to obtain colored fabric. Dyeing is the application of color, mostly with aqueous solutions of synthetic organic dyes, to fiber, yarn, or fabric. Despite a century of process improvements, dyeing remains one of the most polluting chemical processes because it produces large volumes of toxic wastewater as a byproduct, which needs efficient and cost-effective effluent treatment before the water can be safely released back to nature.[2−4] Hence, new "green" dyeing methods have to be developed to solve this enormous problem.

Among natural fabrics, silk has been used as a textile fabric for thousands of years. Silk is a fine, smooth, lustrous fiber produced by a variety of insects, moths, and worms. Silk fiber consists of two proteins: sericin and fibroin. Sericin forms the outer protective coat, while fibroin is the main structural protein. Sericin is hydrophilic in nature and can be easily removed by boiling the silk in alkaline solution. This process is called degumming. The fibroin protein gives the silk its characteristic luster and feel. The fibroin molecule is amphiphillic in nature; it consists of alternating hydrophilic and hydrophobic domains.[5] Silk used in textile applications is produced in variety of qualities, which include raw silk or undegummed silk (sericin and fibroin) and either partially or fully degummed silk (only fibroin).

Commercial silk fiber produced by the mulberry silkworm is generally white. However, there are other strains of silkworms that can produce cocoons that are pink, yellow, brown, or green in color. Recent studies have focused on understanding the

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coloration of these cocoons by correlating the genes required for transport of certain pigments from the mulberry leaves into the silkworm cocoon, but this color is generally lost upon degumming.[6,7] The modern textile industry demands that the original silk fiber be dyed into a variety of colors so as to later spin it into attractive textiles. There have been attempts to develop greener methods to produce colored silk fabrics. These include using nontoxic natural dyes so that the use of hazardous chemicals is minimized.

In yet another approach, researchers have recently developed a green technique to produce color silk in which the silkworm directly spins a colored silk cocoon after being fed with a diet that is modified with a dye formulation.[8−17] Figure 1 depicts

Figure 1. (a) 5th Instar *Bombyx mori* larvae feeding on mulberry leaves sprayed with Direct Acid Fast Red dye solution. (b) Silkworm larvae spinning cocoon on a mesh (Chandrike). (c) Colored cocoon shells after the larvae have been taken out. (d) Twists of Direct Acid Fast Red threads after degumming.

this green method for producing colored silk twists. The silkworms, during the fifth instar stage, are fed with a modified feed comprising mulberry leaves that are dipped into or sprayed with the dye solution or alternatively powdered mulberry leaf feed in which the dye solution is mixed. In either embodiment, the process does not produce large quantities of dye containing effluent water and hence is a green alternative. The dye is transported along the biochemical pathways of the silkworm to produce a colored cocoon or colored silk fiber. The intensity of color in the silkworm cocoon can be easily controlled by controlling the concentration of dye in the feed. These dyes do not harm the silkworm, and no adverse effects on the silkworm growth have been reported. However, all the reported studies[10,11] on color silk have so far focused on a single model compound, namely, derivatives of the fluorescent dye Rhodamine. For large-scale commercial synthesis of colored silk, the usage of common cheap textile dyes is necessary. Although some azo dyes are known to have human and ecological hazards, even today they account for more than 50% of the textile dyes produced annually.[18] We have used a series of azo dyes as "model compounds" to establish the important molecular structural parameters that decide the efficacy of coloration to produce colored silk.

It has been suggested that the physical property of the dye and its self-assembly in water are crucial parameters that control the uptake of the dye into the silk gland.[10,11] Hence, we envisioned that if we systematically vary the hydrophobicity of a class of functionally similar azo dyes, we would be able to correlate the production of intrinsically colored silk to the physiochemical property of these dyes. We hereby report a systematic study in which a group of synthetic azo dyes are fed to the silkworm in an effort to produce intrinsically colored silk.

MATERIALS AND METHODS

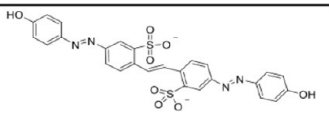
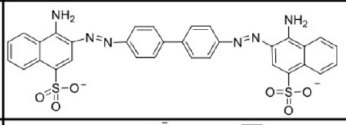
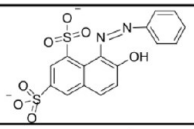
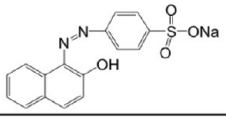
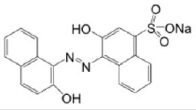
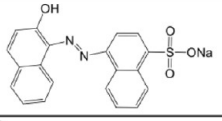
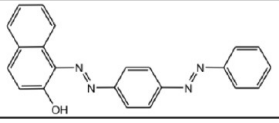
Chemicals. Seven different dyes viz. Brilliant yellow (Loba Chemie ART 2257), Congo Red (SD. Fine Chemicals 30025), Acid Orange G (Jostar Orgotech Pvt. Ltd. Mumbai), Acid Orange II (Sigma Aldrich), Mordant Black 17 (TCI Chemicals), Direct Acid Fast Red (Jostar Orgotech Pvt. Ltd. Mumbai), and Sudan III (Himedia RM 991) were used for the experiments as supplied. These dyes will be referred to in the paper using the codes D1 to D7 as listed in Table 1 with higher number indicating higher hydrophobicity.

Measurement of Partition Coefficient. Aqueous and organic solutions of equal concentrations of a dye were prepared in MES buffer and in octanol, respectively. The molar extinction coefficient (ε) was determined for both solutions by Beer−Lambert's law using UV spectrophotometer. Five milliliters of MES solution of a dye was taken into the separating funnel, and 5 mL of octanol was added to it. The separating funnel was shaken vigorously and was kept undisturbed for 15 min to attain equilibrium. Aqueous and organic layers were collected separately. Concentration of dye in each layer was measured using a UV spectrophotometer. The partition coefficient of the dye was calculated by taking the log of the ratio of these concentrations values.

Modified Feed Experiments. The silkworm larvae in their fifth instar stage were fed with fresh mulberry leaves. On the fourth and fifth days of the fifth instar stage, the larvae were fed with a modified feed. This feed was prepared by dissolving a measured amount of dye in water and dipping the mulberry leaves in this dye solution or spraying the dye solution on the leaves. A picture of dye solutions ranging from D1 to D7 is shown in Figure S1 of the Supporting Information. The air-dried leaves were then fed to the silkworm. In another experiment, the silkworms were also fed with commercial grade mulberry feed powder, which was modified by mixing a measured quantity of dye into the same. However, either method of feeding gave similar results in terms of the final cocoon color obtained, and hence, here we report the results on the silkworm larvae fed with fresh mulberry leaves dipped in colored solution.

Quantification of Dye in Sericin and Fibroin. The colored cocoon was finely chopped and separated into different fiber layers using a pair of sharp forceps, and 20 mg of this was immersed in 1 mL of dimethyl sulfoxide (DMSO) at room temperature for 24 h to dissolve the dye completely. The quantity of dye in DMSO was estimated by doing UV−vis measurements on the solution. A few colored cocoons were also enzymatically degummed separately with fungal alkaline protease enzyme. Enzyme was produced by submerged fermentation using a fungal strain *Conidiobolusbrefeldianus* MTCC 5185 (PCT/IB2001/000516). Fermentation was carried out at 28 °C for 48−72 h, and the cell free broth was used for enzymatic degumming. Colored cocoons were soaked in 0.5% $NaHCO_3$ for 1 h at 50 °C. Fiber layers were separated by a pair of forceps, and enzymatic degumming was carried out at 50 °C for 1 h using 400 units of protease per gram of cocoon.

Table 1. Azo Dyes, Chemical Structure, And Properties

Dye	Code	Mol. wt. (g/mol)	Log P/Po	Conc in feed (wt %)	Structure	Cocoons
Brilliant yellow	D1	624.55	-1.71	0.08		
Congo red	D2	696.66	**-1.46**	1		
Acid Orange G	D3	452.37	**-0.76**	0.2		
Acid Orange II	D4	350.32	**+0.31**	0.2		
Mordant black 17	D5	416.39	**+0.49**	1		
Direct Acid fast red	D6	400.38	**+0.59**	0.08		
Sudan III	D7	352.39	+++	0.08		

After degumming, the liquor was separated, and weight loss of degummed silk was determined after washing and drying the samples to constant weight in hot air oven. The weight loss obtained by this method was 25−27%. The fibroin so obtained was again immersed in DMSO at room temperature for 24 h to extract all the color. An UV−vis measurement on the DMSO−dye solution was done to quantify the amount of dye in fibroin. These measurements were done for only the colored cocoons, viz. D3−D6.

RESULTS

Bombyx mori silkworm larvae were fed with fresh mulberry leaves until the third day of the fifth instar stage. Fresh mulberry leaves were replaced with dye-sprayed mulberry leaves from the fourth day until they started spinning the cocoons. This modified feed was prepared by spraying aqueous azo dye solutions of predetermined concentrations of seven different dyes on fresh mulberry leaves. Initially, all dyes were tested for a uniform concentration of 0.08 wt %. If no significant color was evident in the cocoons, higher concentrations up to 1 wt % were evaluated. The experiments using the highest concentration for each dye have been tabulated here. No visible difference in terms of food consumption or growth was evident for any of the dyes used in this work. The silkworms continued to grow normally in size without any differences in mortality rate. For all the dyes used in the experiment, the body of the silkworm larvae showed staining on the external tissue after about 30 min. The larvae being fed with dye Direct Acid Fast Red showed the highest staining on the external body surface.

Table 1 tabulates the seven azo dyes (D1−D7) used in this work. Also shown in Table 1 are their chemical structures, molecular properties, and the cocoons produced using the modified feed experiment. The azo dyes were chosen such that they have similar chemical structures but varying molecular weights and lipophilicity. All these dyes contain either one or two aromatic rings on either sides of the azo bond bearing sulfonate (SO_3), amine (NH_2), or hydroxyl (OH) group as substituents. These aromatic groups impart hydrophobicity, while the polar groups gives the molecule its hydrophilic character making the overall nature of this dyes lipophilic. These dyes contain a varying number of aromatic and polar moieties and hence display systematic differences in lipophillicity. The lipophillicity is measured as the partition coefficient of these dyes in octanol:water mixtures, which varies systematically from −1.71 to about +0.6. The molecular weight of these azo dyes also varies between 624 and 325 g/mol.

Upon feeding dye D1 to the silkworm larvae, a white color cocoon was obtained. The cocoon is analogous in terms of its color, size, and weight to that produced by a silkworm larva fed on a control feed (only mulberry leaves). Similar results were observed for dyes D2, D3, and D7. However, for dyes D4, D5, and D6, intrinsically colored bright orange, light violet, and pink cocoons, respectively, were observed. It should be noted that dyes D1, D2, and D3 are relatively more hydrophilic as is evident from their negative partition coefficient values. This hydrophilicity is due to the presence of two sulfonate

substituents on the aromatic ring. On the other hand, dyes D4, D5, and D6 are less hydrophilic as can be seen from their positive partition coefficient values. This decreased hydrophilic character can be well correlated with the fact that these dyes have a single sulfonate group present on the aromatic ring. Dye D7 is extremely hydrophobic and is practically insoluble in water.

It was found that treatment of the colored cocoon with DMSO led to complete extraction of the dyes from the cocoon. Hence, DMSO was used for dye extraction from the cocoon, and the amount of dye extracted was quantified by UV−vis spectroscopy. As shown in Figure 2, the quantity of dye in the

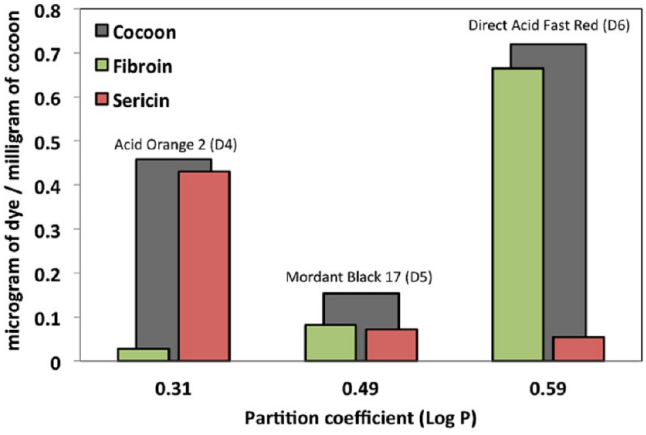


Figure 2. Quantification of dye in cocoon, sericin, and fibroin.

cocoon was highest in case of D6, i.e., the Direct Acid Fast Red dye. A significant amount of dye D4 (Acid Orange II) was also present in the cocoon. However, the amount of D5 (Mordant Black 17) dye in the cocoon was less than 50% of the amount of D4 or D6. Thus, there are probably other factors in the biochemical pathways of a silkworm in addition to hydrophilicity (as characterized by the partition coefficient) that decides transfer of dye to the silk fiber. A probable explanation for these observations is given later in the Discussion section.

Because silk fiber consists of sericin and fibroin where sericin forms the outer protective coat while fibroin is the main structural protein, we proceeded to quantify the amount of dye preferentially associated with sericin and fibroin. To achieve this, the cocoons were degummed using a protease enzyme to remove the sericin, and the dye in the left over fibroin was extracted with DMSO for quantification. It was observed that although the absorption of D4 dye into the cocoon was significant, more than 95% of this dye was associated with sericin protein. Thus, the cocoon was found to be completely colorless after a typical degumming process. However, dye D5 (Mordant Black 17) was more or less equally split between the sericin and fibroin components. For dye D6 (Direct Acid Fast Red), a higher concentration of the dye was found in fibroin as compared to that in the sericin protein. For textile applications, it is highly desirable that the dye associates with the hydrophobic protein fibroin, as this protein is responsible for the desirable properties of silk such as feel and luster. Thus, dye D6 is a viable candidate for evaluation in textile applications. The quality of the silk thread and finally the cloth produced by using this technique needs to be compared with a conventional dyeing process.

In order to further understand the mechanism of cocoon coloration, we dissected the silkworms fed with both control and modified feed on day 5 of the fifth Instar stage. The images of the dissected silkworm glands for day 5 for the control and modified feed experiment (D4−D6 dyes) are compiled in Figure 3.

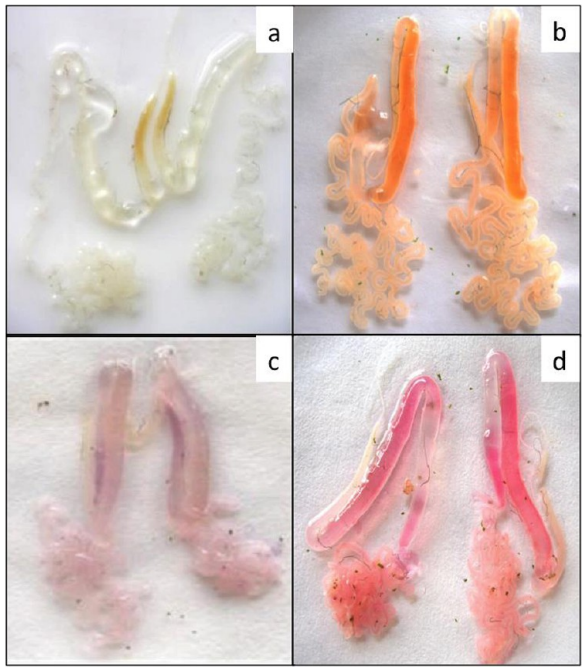


Figure 3. Dissected silkworm glands for (a) control, (b) dye D4, (c) dye D5, and (d) dye D6.

As shown in Figure 3a, the silk glands for larvae fed with control feed are white or colorless. For dyes D1 and D2, we observed that a significant concentration of the dye was accumulated in the peritoneal membrane of the alimentary canal. (Figure S1, Supporting Information). The peritoneal membrane presumably acts like a filter—screening out dye molecules and not enabling their transfer out of the alimentary canal. For dye D3, we did not observe any accumulation of the dye in the peritoneal membrane. Also, the silk glands appear very similar to those of the control silkworm. This suggests that although the dye permeates out of the alimentary canal into the hemolymph, the dye is excreted out of the silkworm body, as it does not associate with the silkworm glands or any other tissue. The silkworm larvae that are fed with modified feed of dyes D4−D6 are shown in Figure 3b−d. It is evident that the silk glands have taken up the dye color. Interestingly, the intensity of the color D5 (Mordant Black 17) in the silkworm gland is lower as compared to the colors observed for dyes D4 (Acid Orange 2) and D6 (Direct Acid Fast Red). This observation is agreement with the measurement of dye uptake reported in Figure 2, despite the fact that dye D5 was fed in higher concentrations as compared to dyes D4 and D6. The silkworm has an open circulatory system, i.e., the hemolymph surrounds all the tissues. Thus, the dyes must diffuse out of the alimentary canal into the silkworm hemolymph and then from the hemolymph into the silkworm glands and other tissues. The solubility of the dye in the hemolymph and the differential permeation through the linings of the various tissues are likely to control the transfer of the dye to the silk gland.

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We also collected the hemolymph from the control and modified feed experiment larvae on day 5 from a surgical cut at the rear leg of the larvae. No visible difference in the hemolymph color could be observed when compared with control feed hemolymph color (Figure S3, Supporting Information). The objective of this experiment was to measure the concentration of different dyes in the hemolymph using UV spectroscopy and correlate these results with the ability of the dye to produce colored cocoons. None of the dyes showed measurable quantities of dye in the hemolymph. Also, dyes D4−D6 did not significantly stain other tissues in the silkworm body. These results suggest that the dyes preferentially permeate into the silkworm glands as shown in Figure S3 of the Supporting Information. Some concentration of dyes may also be excreted out of the silkworm body and hence cannot be measured.

To probe further, we injected 250 μL of dye solutions into the hemolymph from the second abdominal spiracle of the silkworm larvae feeding on control feed on the fourth day of the fifth instar stage. Dye D2 was injected directly into the hemolymph, and the larva was sacrificed after 30 min. It was observed that only a small concentration of the dye did diffuse into the silkworm gland. Some of the dye may also be excreted out, while a slight coloration of other tissues was also observed. However, dyes D4, D5, and D6 when injected into the hemolymph showed significantly colored silkworm glands. These interesting results suggest that further experiments need to be done to understand the diffusion of dyes from silkworm hemolymph into the silkworm gland.

DISCUSSION

The results of the experiments suggest that a balance of hydrophilicity and hydrophobicity is required to produce color in the cocoon. Extremely hydrophilic dyes (D1−D3) do not color the cocoon nor do highly hydrophobic dyes. Dyes D4, D5, and D6 have an optimum balance of hydrophobic and hydrophilic character on account of their molecular structures. Although Mordant black 17 (D5) is more hydrophobic than Acid Orange II (D4), the total amount of D5 incorporated into silk is much lower than more hydrophilic D4. A possible reason for this might be that D5 is known to bind ions like Ca^{2+} by using the two phenolic −OH groups and the azo group as a tridendate ligand. Possible interaction with metal ions can change its physical properties, which leads to lower uptake of the dye into the silk. Dye D7 is extremely hydrophobic and sparingly soluble in water. This dye again does not show any coloration in the cocoon.

It has been shown for Rhodamine dyes[10] that the partition coefficient of the dye is an important parameter that determines the amount of dye adsorbed into the silkworm gland. The authors reported a peak in the amount of dye absorbed in the cocoon for partition coefficients of 0.6. Further increase in hydrophobicity (partition coefficients between 0.6 and 2), resulted in lower absorption of dye into the cocoon. However, for Rhodamine B (partition coefficient greater than 2), the dye resulted in the highest concentration in the cocoon. The authors have shown that although Rhodamine B is a hydrophobic dye, it is also water soluble on account of its ability to self-assemble in water.[11] Thus, they concluded that hydrophobic dyes with good water solubility are promising candidates for producing naturally dyed silk. Our results are in agreement with these observations. Dyes with partition coefficients in the range of 0.3−0.6 are absorbed into the cocoon, and dye D6 with a partition coefficient of 0.59 shows the highest absorption. Dye D7 is extremely hydrophobic and also sparingly water soluble. This dye is not absorbed into the biochemical pathways in the silkworm.

Another factor that also affects the absorption of the dye into the cocoon is its molecular weight. It has been observed by authors Hamamoto et al.[19] that therapeutics drugs having molecular weights greater than ∼400 g/mol find it difficult to permeate through the silkworm alimentary canal into the hemolymph. This observation is in tune with our experiments where dyes D1 and D2 have molecular weights significantly greater than 400 g/mol. The higher molecular weight limits the absorption of these dyes into the biochemical pathways. The dye accumulates in the peritoneal membrane of the alimentary canal as shown in Figure S2 of the Supporting Information. Thus, molecular weights less than 400 g/mol are essential for effective transport of dye in the biochemical pathways of the silkworm body and hence in production of naturally dyed silk fibers.

Our experiments on dye quantification in cocoons indicate that lipophillicity is an important parameter governing association of the dye with the two different proteins present in the silk gland. This behavior may be attributed to the fact that sericin is the hydrophilic protein while fibroin is more hydrophobic. As the lipophillicity in the dye molecule is increased, it preferentially associates with the hydrophobic protein, i.e., fibroin. The interaction between the dye molecule and the protein is not well understood, and further studies are underway to determine and understand these associations. These associations will also help us further understand the utility of these dyes in textile applications.

CONCLUSION

Natural silk fibers have been used in textile applications for centuries. These silk fibers are typically dyed to increase their appeal in the retail apparel market. Dyeing of fabrics involves the use of hazardous chemicals, and recent efforts have hence focused on development of greener technologies. A promising technology reported recently involves feeding silkworm larvae with a modified feed containing a sprayed dye solution. We have evaluated a set of seven different azo dyes in this green method to produce dyed silk. The dyes used in the study had similar chemical structures with systematically varying partition coefficients. Our experiments suggest that a balance of hydrophobic and hydrophilic character is necessary for diffusion of the dye from the alimentary canal of the silkworm larva into the hemolymph and later into the silk glands. Dyes with molecular weights greater than 400 g/mol cannot diffuse out of the peritoneal membrane of the alimentary canal. The partition coefficient of the dye also determines the preferential association of the dye with either sericin or fibroin protein in the silkworm gland and finally into the cocoon. These insights are extremely important in development of novel dye molecules that can be successfully used in this green method of producing colored silk fabrics.

ASSOCIATED CONTENT

Supporting Information

Figures for all the dye solutions used and dissected silkworm larvae after modified feed experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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