Engineering melting temperatures of carbohydrate binding modules through site-directed mutagenesis

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ABSTRACT

Biofuels are a promising alternative to environmentally harmful fossil fuels. However, in order to displace fossil fuels, biofuels must be competitive in cost. Currently, the production process is the most significant issue regarding their cost, despite feedstock material being fairly inexpensive. One of the largest contributors to this cost is the use of enzymes to breakdown the biomass into simple sugars for subsequent use. If the enzymes used in this process could be easily recycled, the total cost of production would be significantly reduced. This project targets carbohydrate binding modules (CBMs) in an attempt to lower their melting temperature (T_m) through mutagenesis. These modules allow the holoenzyme (CBM with the catalytic domain) to bind to the substrate. Engineering a T_m of the CBM that was below the T_m of the catalytic domain would allow binding to be turned "off" for enzyme recovery, and later turned back "on" for future reactions. T_m values were established for three CBMs as well as refolding capabilities after heat treatment. Assays were developed for CBMs fused with green fluorescent protein by evaluating fluorescence signal on cellulose substrates in order to assess binding activity. Potential amino acid substitutions were identified in CBMs 11 and 44 from *Ruminoclostridium thermocellum* that were predicted to lower the T_m. Two of these mutations in CBM 44 that specifically interfere with Ca^+ stabilization were created and sequenced. These mutants will be tested for changes in T_m and binding capabilities.

INTRODUCTION

Fossil fuels are currently the most widely used fuel source in the world. This is problematic for a number of reasons. Fossil fuels are derived from prehistoric organic matter that over time has been converted into fuel sources such as oil or natural gas. These fuels contribute to significant CO_2 emissions into the atmosphere which have a detrimental effect on the environment. The main reason fossil fuels are so universal is that they are easily accessible and relatively inexpensive sources of energy. If alternative, carbon neutral energy sources could be lowered in cost, they would be able to compete with fossil fuels. This would result in a significant reduction of fossil fuel usage and thus help to reduce the effects of global climate change.

Biofuels are one of the most promising alternatives to environmentally harmful fossil fuels. Particularly, second generation biofuels that use agricultural waste products rather than commodities that are also important in food production (i.e. corn starch, soybean oil) offer a currently underutilized potential fuel source. There is an enormous supply of biological waste products available that can be converted into bioethanol to be used as fuel.[1] This biorefinery is a wonderful opportunity to lessen society's dependence on oil. Lignocellulosic biomass in particular is considered the most abundant under-used resource available.[1] One of the only downsides to biofuels is the imperfection of the manufacturing process.[2] New technologies are constantly being developed in order to integrate biofuels as preferential to fossil fuels.[1]

The reason lignocellulosic biomass is so abundant is because it can be obtained from a large variety of sources. All agricultural processes produce some amount of plant waste in addition to food or other industrial products. It is this waste that has the potential to provide feedstock for biofuel production because it is so cheap. In some countries, sugar cane is now

being grown not only as a food source, but also specifically to provide fuel.[3] Fuel security in the modern economy is becoming just as important as food security because transportation is now a necessity of everyday life.[3] Additionally, more popular food crops such as cereals and grains produce enormous amounts of waste, the stalks and leaves of the plants, that can be converted into fuel. However, relying on these is not ideal because it could contribute to environmental monoculture that is already a threat in many regions.[4] One goal is to design methods of manufacturing biofuels that can be applied to all types of plant based waste products.

In the production of biofuels from plant based biomass, the most prominent obstacle is the cost of cellulases which are required for the saccharification of lignocellulosic biomass.[2] The structure of the plant cell wall makes it recalcitrant to normal digestion. Figure 1 shows a schematic diagram of the typical structure of a plant cell wall. Both cellulose and hemicellulose are insoluble in water making them difficult for most organisms to digest.[5,6] Pectin is soluble, but difficult to access due to its location inside the cell wall. Lignin (not shown), is a complex organic polymer within some plant cell walls that causes them to be rigid and woody.[6] Components of plant cell walls can only be broken down by specific enzymes that are required for digestion. These enzymes are responsible for 18-23% of the total cost of biofuel production.[5]

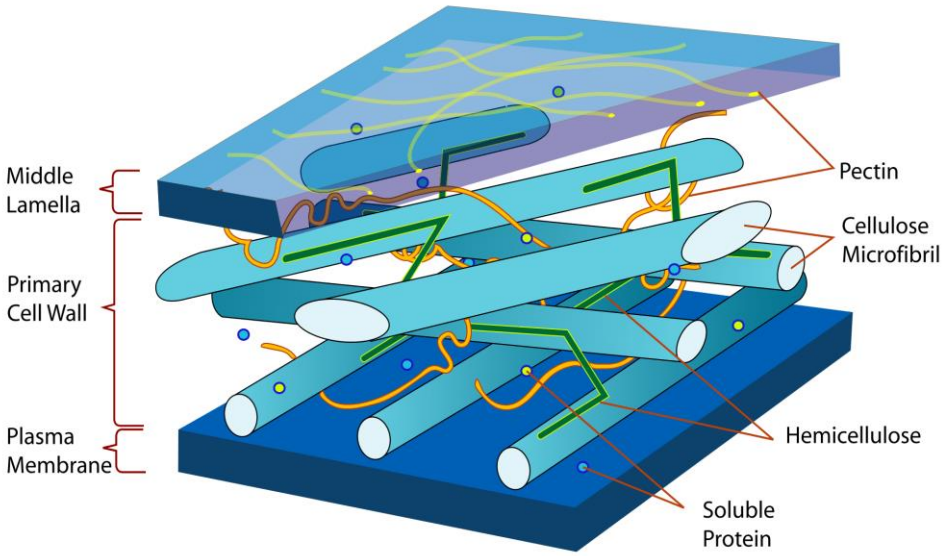

Figure 1. Normal structure of a plant cell wall
Credit: Public domain via. Wikimedia Commons

One convenient property of biological enzymes is that they are reusable, returning to their initial conformation after the reaction is complete. This means that recycling enzymes used in production should be fairly simple, as long as they can be recovered. It has been hypothesized that adsorption to the substrate is the most significant obstacle in the ability to recover free, soluble enzymes.[7] If some method could be developed to remove enzymes post production, without affecting the catalysis itself, then the enzymes could be recovered and reused. This would greatly reduce the overall cost of producing biofuels, making them more competitive with the cost of traditional fuel sources. While chemical pretreatments to help enzymes desorb from lignocellulosic biomass exist, they also contribute to this overall cost of production.[8,9] A more cost effective option would be to use enzymes that can be detached from the substrate without added chemicals. Genetically modified enzymes are expected to play an important role in the solution to this problem.[6]

Many enzymes used in the breakdown of lignocellulosic biomass contain carbohydrate binding modules (CBMs). These are the portions of the protein that physically bind it to the substrate because plant cell walls are comprised mainly of carbohydrates.[7]

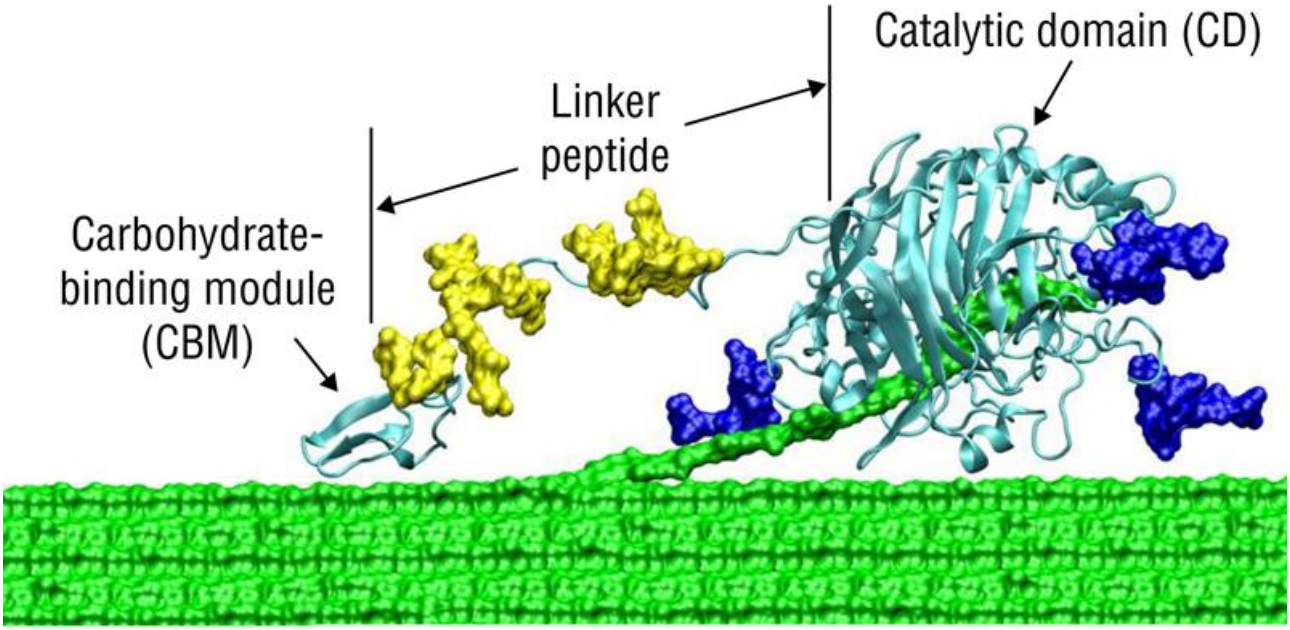

Figure 2. Schematic diagram of the modular structure of a typical cellulase enzyme.
Credit: National Renewable Energy Laboratory

As mentioned above, the attachment of enzymes via these CBMs is a major issue in biofuel production because it prevents the recyclability of the enzymes. CBMs target carbohydrate ligands with high specificity so attempting to remove them by introducing a different potential substrate would likely not work.[10] The most promising approach would be to desorb the enzyme by specifically altering the CBM.

In previous studies, lignocellulosic enzymes have been altered by removing the CBM altogether to prevent adsorption entirely.[11] This method greatly reduced the efficiency of the enzyme making it essentially useless in biofuel production. Alternatively, some CBMs were

replaced with similar modules that were thought to have lower affinity for the substrate. However, these replacements still had strong affinity for other compounds and adsorption remained an issue.[12] It is clear that the presence of some type of CBM is necessary for proper enzymatic activity. However, it is possible that some aspect of that CBM could be changed without entirely removing it from the enzyme.

Some desorption techniques have been attempted by changing the conditions of the reaction. For instance, increasing the pH of the solution would hypothetically encourage the CBM to un-bind by weakening its hold on the substrate. However, this also interferes with the activity of the enzyme as it operates at the same optimal pH level as the CBM.[13] Other chemical methods include the addition of detergents to inhibit strong binding of the CBM. This has a similar issue in that it interferes with the activity of itself or other enzymes, further adding to the total expense of biomass processing.[13] These attempts support the idea that changing some aspect of the CBM (such as melting temperature) is preferential to changing the total reaction because any effect on the catalytic domain will decrease the efficiency of the reaction.

Protein structures such as CBMs are essentially completely folded below their melting temperature (T_m), and completely unfolded and non-functional above their T_m. One possible avenue of CBM alteration would be to decrease its T_m relative to the catalytic domain. This would allow CBM binding to be "turned off" by raising the temperature of the reaction by only a few degrees. The benefit of this approach is that the catalytic domain of the enzyme should remain unaffected by such a small temperature change. In order for this method to be successful though, the CBM would have to spontaneously refold upon cooling. If the desorption of the enzyme were irreversible, then the enzyme recovery would be worthless. It must first be established that CBMs are still functional after a treatment of heating and subsequent cooling.

The melting point of a protein is a measure of its overall stability. A more stable molecule will have a higher stability because it has stronger intramolecular forces that allow it to resist unfolding with increased temperature. Several factors influence the stability of the protein including hydrophobic interactions and ion stabilization. Proteins have a hydrophobic core that does not want to be exposed to the water-based environment which encourages proper folding. More hydrophobic residues will have a stronger effect on this interaction than non-hydrophobic residues.[14] Ion stabilization refers to a charged atom that binds to one area of the protein and holds those residues in place, also encouraging proper folding. Interfering with one or more of these factors would decrease the overall T_m of the protein.[14] These are certainly not the only interactions that impact T_m, but they are the two focused on in this project.

The first requirement to pursue a recycling strategy of selectively and reversibly denaturing the CBM is to identify CBMs with the ability to refold spontaneously after denaturation.[14] A potential issue that arises from the approach of lowering the temperature of CBMs, it the possibility that they will not refold properly after denaturation. The refolding capabilities of CBMs 11, 30, and 44 have been evaluated in a previous stage of this study.[15] This experiment involved creating CBMs that were fused to fluorescent proteins. The fluorescent signal for heat treated and non-heat treated CBMs was then measured as an indication of proper folding. Ultimately, the CBMs studied maintained similar binding behavior before and after being heated above their melting temperatures.

Numerous methods of enzyme desorption have been attempted in the past, however these have not been successful. Chemical desorption has too strong of an effect on the catalytic domain and may be cost-prohibitive. Removal of the CBM prevents the protein from binding the substrate in the first place which is essential. This project attempts to deal with desorption by

subtly decreasing the stability of the CBM through site directed mutagenesis. If successful, this would produce CBMs with a lower melting point than that of the catalytic domain. The temperature of the reaction could be increased slightly to unbind the CBM with minimal effect on enzymatic activity, and then subsequently lowered following enzyme recovery in order to be used in additional reaction cycles.

METHODS

Preliminary work performed by Jose Sanchez [15]

Initial T_m values for CBMs 11, 30, and 44 were determined using a SPYRO orange assay. This method involves a hydrophobic fluorescent orange dye that only produces signal when bound to a hydrophobic region of a protein. The percent denaturation of the CBMs was determined by measuring the increase in fluorescence signal over time. The inflection of the slope of fluorescence as a function of temperature indicated the melting temperature of the CBMs.

After T_m values were determined, refolding capabilities were determined using a Fold-N-Glow complementation assay by Cabantous. This assay works by using a small peptide tag from the green fluorescence protein attached to the CBM that is only exposed when the CBM is properly folded. When the tag is exposed, it is free to bind Green Fluorescent Protein and a fluorescence signal will be observed. If the protein is improperly folded, the tag will be hidden in the hydrophobic region of the CBM and no fluorescence will occur. The CBMs were each heated to 5 ℃ above their established T_m and cooled before this assay. They were compared to an identical assay of non-heat treated CBMs to determine if folding capability was retained.

Preparation of GFSP-CBM fusion plasmids

The genetic sequence for Green Fluorescent Superfolder Protein (GFSP) was cloned using polymerase chain reaction (PCR). PCR products were purified using QIAprep Spin Miniprep Kits by Qiagen. Sequences for CBMs 11, 44, and 30 from *Clostridium thermocellum* were likewise cloned and purified. The GFSP plasmids were digested with NedI and phosphate groups were removed with shrimp alkaline phosphatase (SAP). Prepared GFSP plasmids were then ligated with each of the CBM sequences to create GFSP-CBM fusions. These plasmid fusions were ultimately transformed into early log TOP10 competent *E. coli* cells. Colonies were grown on LB plates with 0.1μg/mL added kanamycin at 37 ℃ overnight. The plasmids were purified as before with QIA kits and screened for properly oriented plasmid inserts. The screening process involved performing PCR using the forward primer of the CBM with the reverse primer of GFP so that only plasmids with both regions running in the correct direction would be amplified. Those that appeared successful were transferred to a master plate as candidates for protein expression.

Protein expression

DNA from successful plasmid containing colonies was transformed into BL21 *E. coli* expression cells. These were grown overnight at 37 ℃ in LB broth with 0.1μg/mL added kanamycin and protein expression was then induced with 10x Isopropyl β-D-1-thiogalactopyranoside (IPTG) while shaking vigorously now at 25 ℃ to reduce the formation of inclusion bodies. Fluorescence was observed under ultraviolet light to evaluate successful GFSP expression. A positive fluorescence signal was interpreted as an indication that the CBM-GFSP fusions were successful. These proteins were extracted from the expression cells first chemically using 1μL lysozyme to degrade cell walls. They were further lysed mechanically using a Bead

Ruptor from Omni International using 0.75g of beads with 0.5mL or protein solution in a 1.5mL tube. The Bead Ruptor was set to 300rpm for 5 cycles of 30s each. The fluorescence signal for each extraction sample differed in intensity. The fluorescence was quantified using a fluorometric plate reader and appropriate dilutions were made so that fluorescence signals were as similar as possible across all samples. This was done for easier comparison of binding capabilities in later assays.

Binding Assay

Assays were developed to determine optimal binding and wash conditions for each wild type CBM. The goal was to identify one condition where the GFSP control with no CBM would be washed off to show that CBM-GFSP fusions expressed preferential binding. Additionally, a second set of conditions were identified that could successfully remove the CBMs from the substrate. Cellulose based filter paper from Fisher Scientific was used as a substrate and spotted with 2-5μL of diluted samples of CBM-GFSP fusion protein for CBM's 11, 30 and 44 in the binding stage. The binding stage lasted for 20-60min at various temperatures from 20-80 ℃. The paper was imaged under UV light to quantitate fluorescence signal. Then various conditions were altered for the wash stage to unbind the protein before imaging again. Conditions that were altered were temperature, duration, salt ion concentration, and buffer pH.

Alternatively, binding assays were developed using a variety of carbohydrate based powders In this case, the binding stage involved mixing the protein solution with the powder and allowing the protein to bind for a certain amount of time. Then the mixture was spun down and the supernatant was evaluated using a fluorometric plate reader. Binding capability was determined using the difference between initial fluorescence and fluorescence after binding.

Mutagenesis

Point mutation targets were determined using PoP MuSiC mutation prediction software with an objective destabilization of around -0.5 ΔG. Possible candidates were found for CBM 11 at amino acids 116 (isoleucine to leucine) and 165 (isoleucine to leucine). Possible sites for CBM 44 were taken from Najmudin, S., Guerreiro (2005), at amino acids 103 and 96. Site directed mutagenesis was performed on plasmids previously prepared using the Quikchange Lightning Site Directed Mutagenesis Kit from Agilent Technologies with appropriate primers shown in Table 1. Additionally, a double mutant containing both 103 and 96 point mutations was created by using the 96 primers on 103 mutant plasmids. Mutated DNA was transformed into 10XL Gold *E. coli* cells and were grown on LB kan plates.

Table 1. Primer sequences used for site-directed mutagenesis of CBMs 11 and 44 with mutated base pairs highlighted in red

Mutant Target	Primer sequences
CBM 11 Res. 116	Fwd. CATTGGGTGTATTCCCTTACCCCGGATTCCTCC Rev. GGAGGAATCCGGGGTAAGGGAATACACCCAATG
CBM 11 Res. 165	Fwd. AAAATTTCCTTTGATCTTAAATCCGTGGATGGC Rev. GCCATCCACGGATTTAAGATCAAAGGAAATTTT
CBM 44 Res. 96	Fwd. CCCAGCGTGCCGTCTTCAAAGTTGAATTTGGAA Rev. GGCCAAATTCAACTTTGAAGACGGCACGCTGGG
CBM 44 Res. 103	Fwd. TTGAATTTGGAATTGTCCCCGGTAGCTGAGGTT Rev. AACCTCAGTACCGGGGACAATTCCAAATTCAA

Plasmid DNA extracted from these cells with Qiagen mini rapid plasmid kit then was sent to Frederick National lab, Lab of Molecular Technology for sequence determination. Sequence results were further evaluated using the Clustal Omega multiple sequence alignment program. This was used to compare mutated sequences to the wild type and determine if the mutations were successful.

RESULTS AND DISCUSSION

Preliminary results [15]

Melting point assays identified denaturation temperatures of all three CBMs studied (Table 2). This established the original T_m as a baseline comparison for later T_m lowering attempts. Additionally, refolding assays were relatively promising for all three CBMs. Heat-treated and non-heat-treated samples produced nearly identical results in the GFP complementation assay as demonstrated by CBM 44 in Figure 3. Data for CBMs 11 and 30 shows similar trends. This data suggests that CBM binding should be able to be turned "off" at a certain temperature as expected, and then turned back "on" when the reaction is cooled. These are both important capabilities to consider moving forward.

Table 2. Results of preliminary melting point research by Jose Sanchez.[15]

	CBM 11	CBM 30	CBM 44
Melting point determination	T_m = 66 ℃	T_m = 75 ℃	T_m = 75 ℃

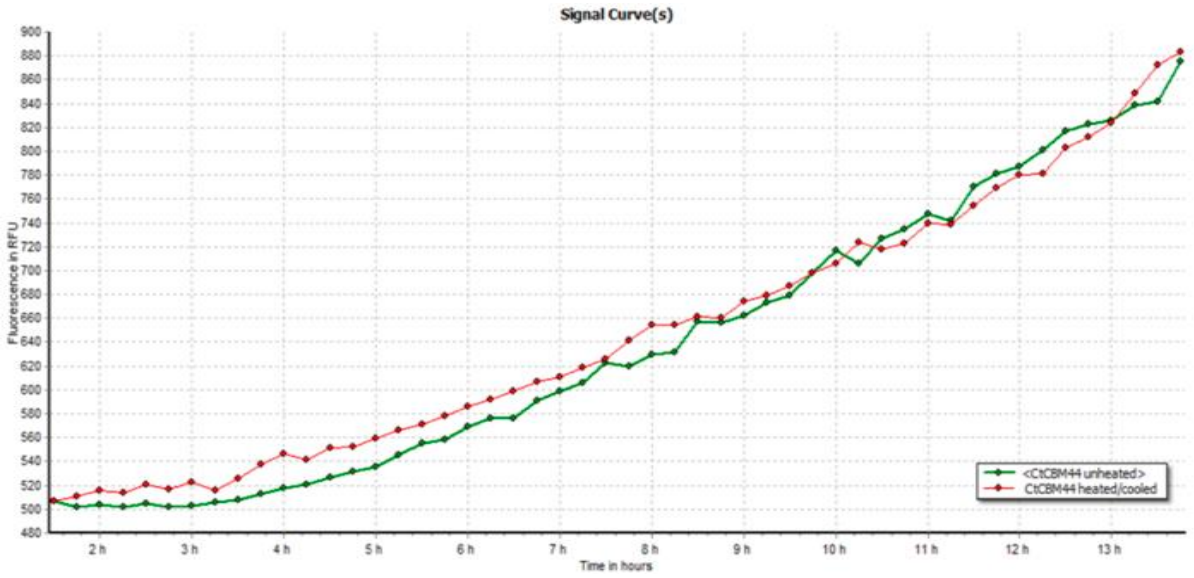

Figure 3. Fluorescence vs. time of example GFP complementation refolding assay of CBM 44. [15]

Results

In order to readily determine CBM binding properties, the coding regions from *R. thermocellum* CBM families 11, 30 and 44 were fused to the coding region for super folder green fluorescent protein (GFSP). This provides a means for cellulose binding to be easily monitored by measuring the fluorescence signal from the GFSP portion of the resulting protein. Figure 4 shows a gel with GFSP-CBM fusions alongside lone GFSP with no CBM added. This demonstrates that fusion samples are larger than the original plasmid suggesting that the added DNA sequences for each CBM are present.

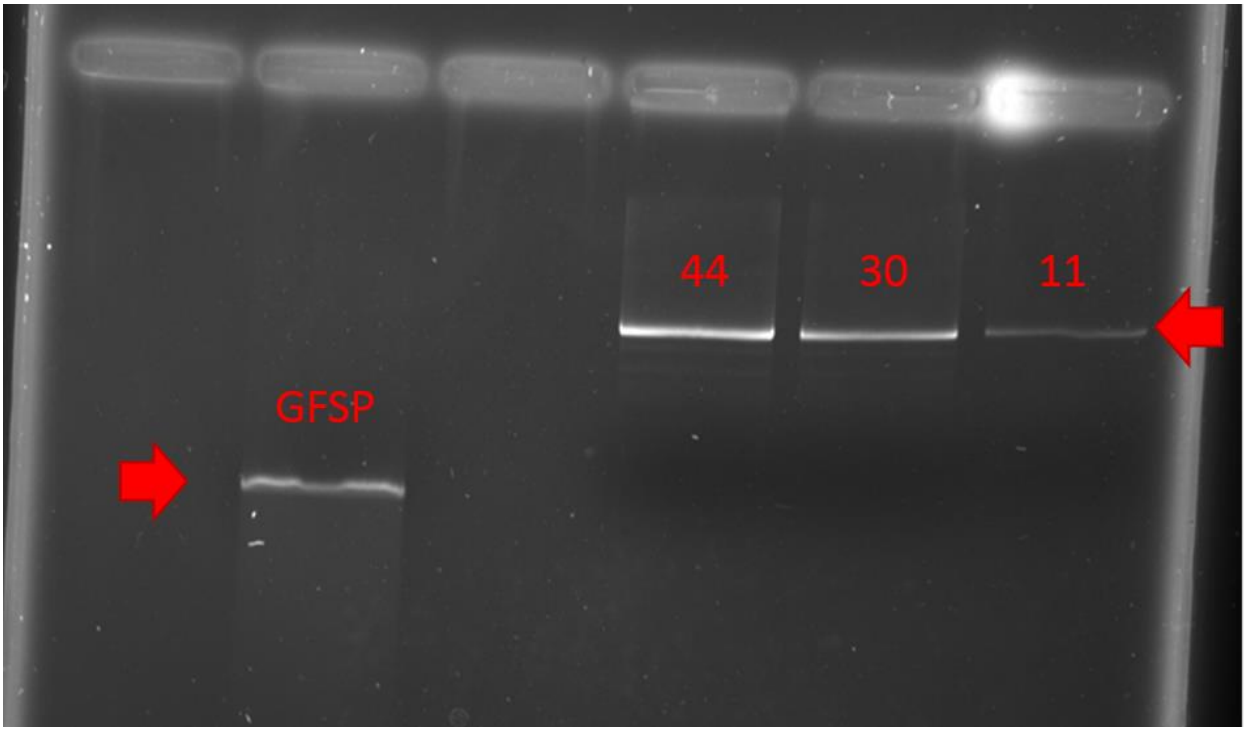

Figure 4. Electrophoresis gel of initial CBM-GFSP fusions with unaltered GFSP as a reference

Following the cloning reaction candidate recombinant plasmids were isolated and analyzed for correct insert orientation. Correct orientation was confirmed by performing PCR with the forward primer of GFSP and the reverse primer of the relevant CBM. Any fusions that

formed in the wrong orientation did not amplify in this test and were rejected as shown in figure 5.

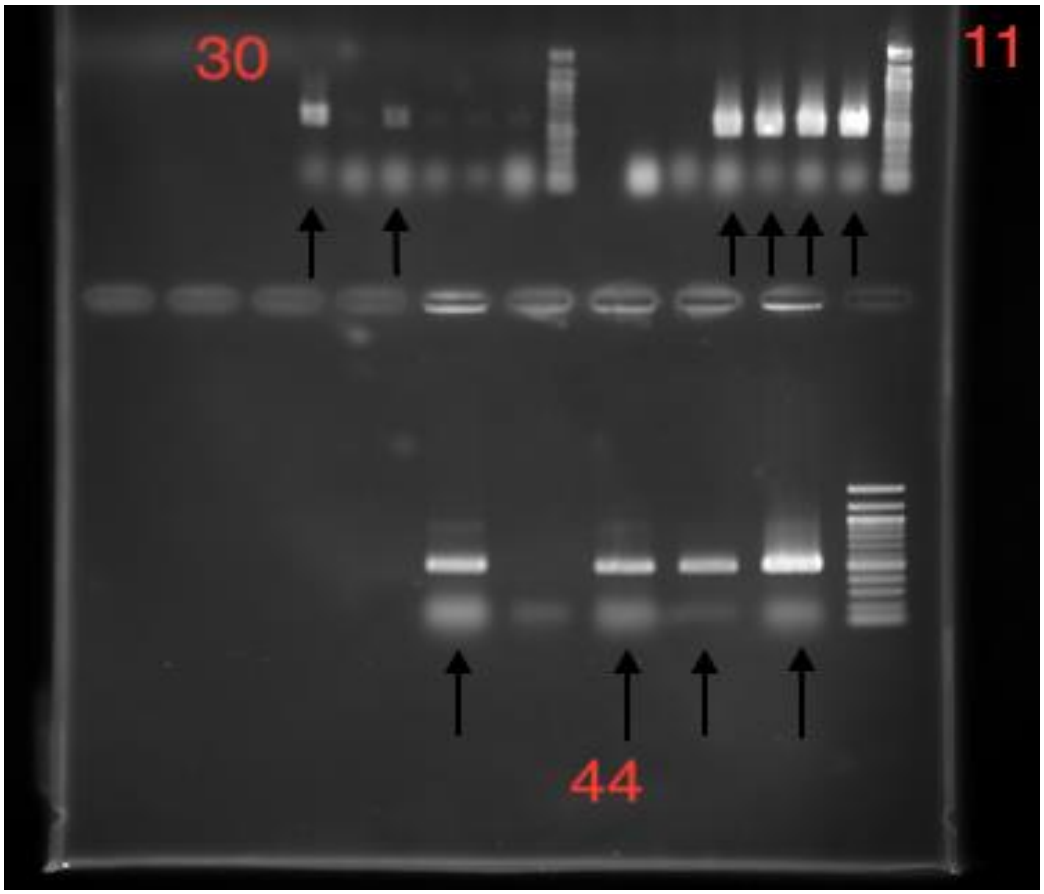

Figure 5. CBM-GFSP plasmid fusion DNA after screening for proper orientation. CBM 30 (top row left) shows two successful candidates, CBM 11 (top right) and CBM 44 (bottom row) each show four successful candidates.

The gel electrophoresis of CBM-GFSP fusion plasmids shown in figure 4 demonstrates the success of the fusion preparations. Lanes with the expected size PCR product are observed for all three CBMs. CBM 30 was slightly less successful with only two candidates as opposed to four for CBMs 11 and 44.

The CBM-GFSP fusion plasmids in the correct orientation were transformed into BL21 DE3 (pLysS) competent cells and plated on LB agar containing kanamycin and chloramphenicol.

This *E. coli* strain produces T7 RNA polymerase upon IPTG induction and can drive expression of the fusion protein. Finally, protein extraction was performed on cells containing fusion DNA. Resulting protein fluorescence under UV light (Fig 6.) indicated that the sequence for GFSP was both present and functional in the cells. The three samples on the right-hand side of the image did not fluoresce indicating that GFP-CBM 30 proteins had not been properly expressed.

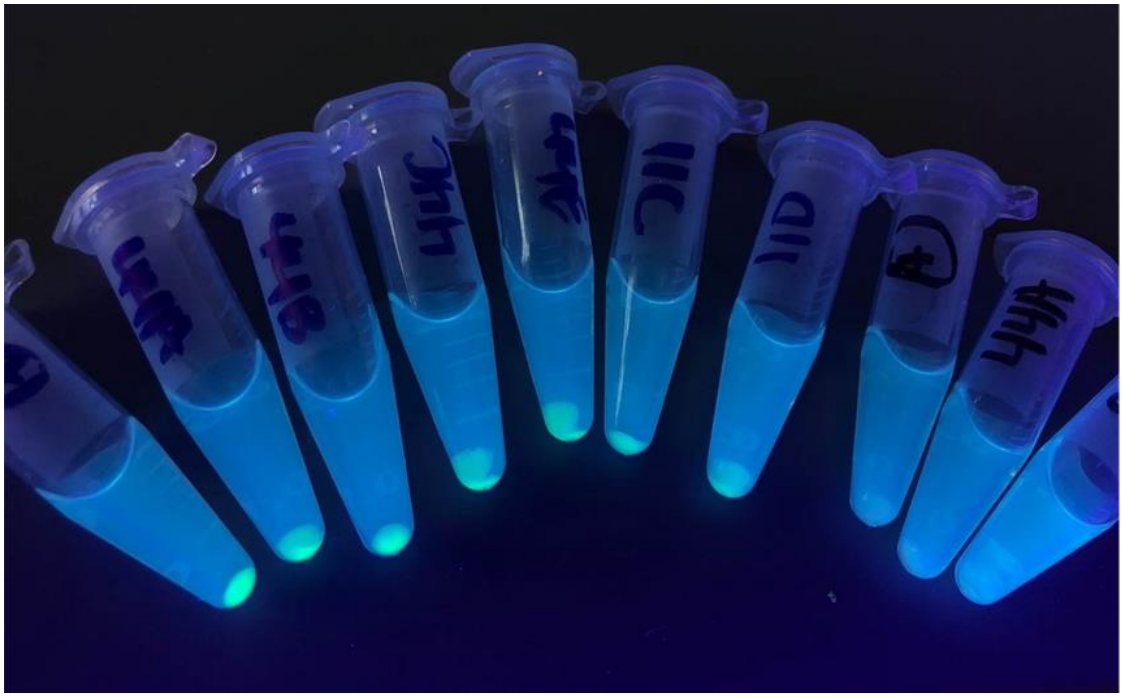

Figure 6. CBM-GFP fusions from CBM 11 and CBM 44 showing fluorescence signal under UV light

With newly cloned CBM-GFSP fusion plasmids, a significant amount of protein could be extracted for analysis. The goal was to develop an assay which measured CBM binding by looking at GFSP fluorescence. The attempted assays involved allowing the protein to bind to some cellulose based substrate, then washing the substrate under various conditions to test what would cause the CBM to unbind. Ideally, after the binding stage all fluorescence signal would be on the substrate then, after washing, all fluorescence would be in the solvent. These assays were

important in determining the baseline binding conditions of the wild-type CBMs so that mutated CBMs later on in the process could be properly compared.

One type of assay used cellulose based powders as substrates for the proteins to bind. . In this case, the supernatant was measured after both the bind and wash stages to evaluate fluorescence. This approach was problematic for several reasons. The fluorimeter used was inconsistent and data analysis was technically demanding. One reason this assay may seem less intuitive is because instead of measuring GFSP signal directly on the substrate, it measured GFSP that did not bind the substrate. Ultimately this should give the same result, but it is less confusing to measure fluorescence from protein directly bound to the substrate.

The second method of developing a binding assay involved pipetting the protein directly onto cellulose based filter paper and evaluating fluorescence under UV light. This process was much less time consuming and easier to analyze. The fluorescence was quantified using computer software allowing the values to easily be compared from one image to another. In this assay the conditions found to wash off the GFSP control without removing CBM-GFSP fusions were at room temperature for one hour in sodium phosphate buffer pH 8. Conditions that successfully unbound the protein were the same buffer, this time with 200 mM NaCl. The CBM detached from the filter paper after washing in this solution at 80 ℃ for one hour.

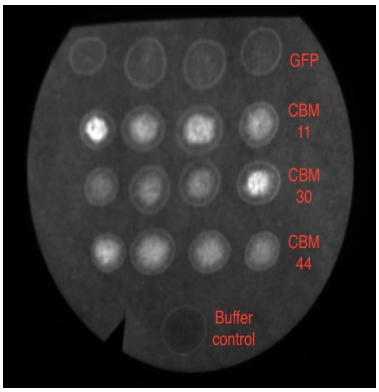

Figure 7. Initial wash stage in sodium phosphate buffer pH 8, at room temperature 1 hour

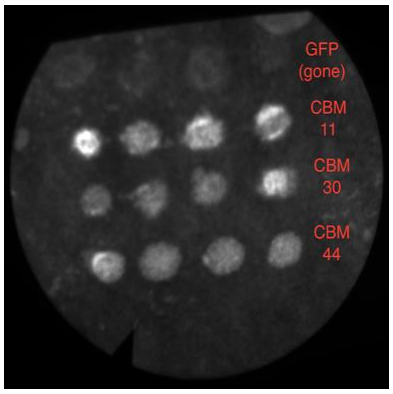

Figure 8. Final wash stage in sodium phosphate buffer pH 8 with 200mM NaCl at 80 ℃ for 1 hour

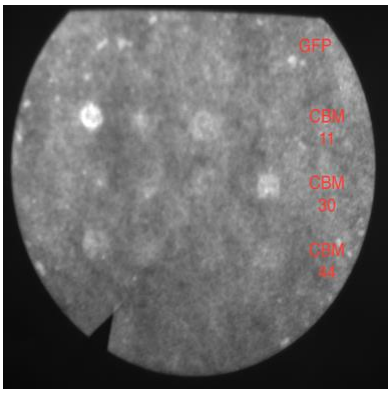

Figure 9. CBM-GFSP fusions after dry binding at room temperature for 20 min

Initial protein solutions were diluted such that they provided approximately the same fluorescence signal. Figure 7 shows the binding stage in which each protein was spotted four times onto one filter paper and allowed to bind. In figure 8, only pure Green Fluorescent Protein was washed off because it contains no CBM to preferentially bind it to the paper. Figure 9 shows the remaining CBM-GFSP proteins washed off indicating that 80 ℃ is above the wild type denaturation point for all CBMs used which agrees with preliminary data (Table 1). It should be noted that the washing off of GFSP with no CBM (from Fig. 8 to Fig. 9) is difficult to see qualitatively because it is initially much more dilute than the other samples and so the difference in fluorescence is not readily apparent. However, using computerized methods of quantifying fluorescence it was found that the difference was in fact significant.

After binding assays were developed, the project could move forward to actual mutagenesis. The first set of mutant predictions were made using computer software. The intent was to find highly hydrophobic residues on the interior of the module, and mutate them to residues with lower hydrophobicity. Two specific amino acid mutations were identified on CBM 11 that fit these criteria. Both mutations happened to be changing an isoleucine to a leucine at

sites 116 and 165 (Figure 10.). Isoleucine and leucine are both hydrophobic residues, however leucine is smaller and so will have a slightly lower hydrophobic effect. These were predicted to destabilize the CBM by interfering with hydrophobic interactions within the protein.

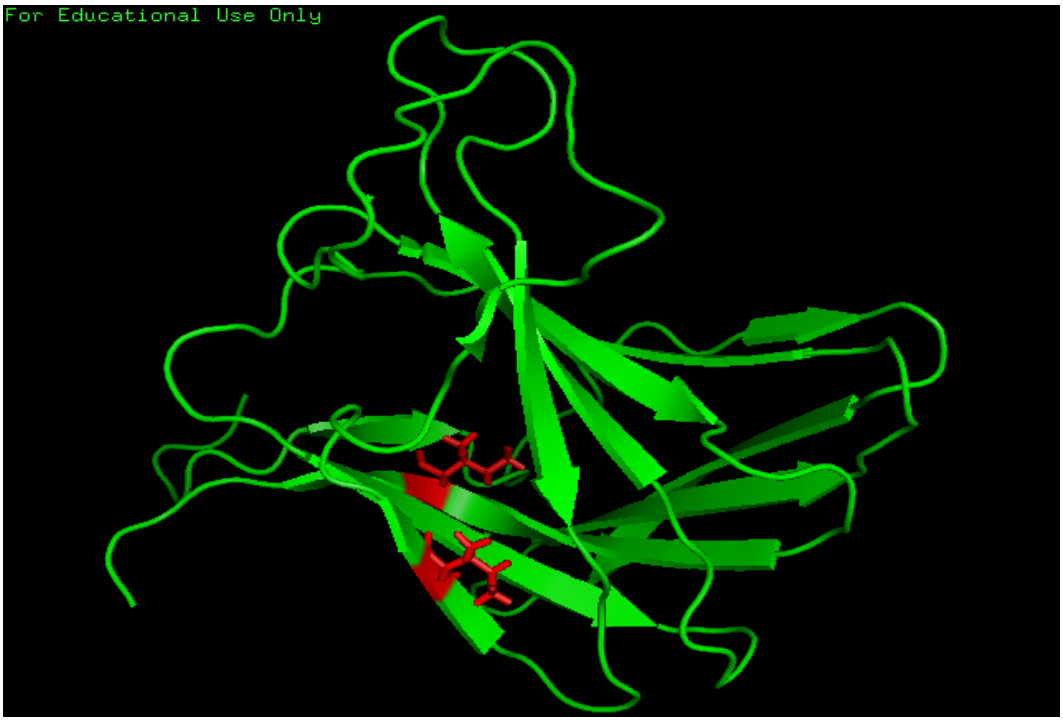

Figure 10. Visual representation of CBM 11 targeted mutations with specific residues 165 and 116 highlighted in red. Figure was made using Pymol.

The residues shown in Figure 9 are highlighted to indicate those targeted for mutagenesis. Both amino acids 165 and 116 are located on the interior of a □-sheet within the protein. This would imply that mutating either one will have a very similar effect on overall stability. Additionally, the fact that these residues are located within the secondary structure of a □-sheet means that these mutations would only destabilize the CBM by a small amount allowing normal folding to still occur at temperatures below the T_m. Unfortunately, the mutagenesis performed on CBM 11 were unsuccessful. Genetic sequencing results were inconsistent with the intended changes. This is likely due to an issue with the design of the original primers.

In addition to computer predicted residue changes, other potential mutation sites were found in the literature. One study examined the structure of a protein related to CBM 44 and estimated the destabilization levels of certain key structures.[14] This protein, like CBM 44 contains a Ca^+ ion which greatly stabilizes its folded structure (Fig 11). In that study, destabilized mutants with weaker Ca^+ binding was still able to bind to substrate. There are five residues that interact with this ion and removing only one of them could reduce the melting point of the CBM by up to 23 ℃. Of these five residues, three only interact with Ca^+ by their backbone and so mutating them to a different amino acid would make no difference because all common amino acids contain the same backbone. However, there are two key amino acids, aspartic acid 96 and glutamic acid 103, that interact with Ca^+ by their R group directly. By mutating one of these to alanine, the stability of the CBM should be reduced.

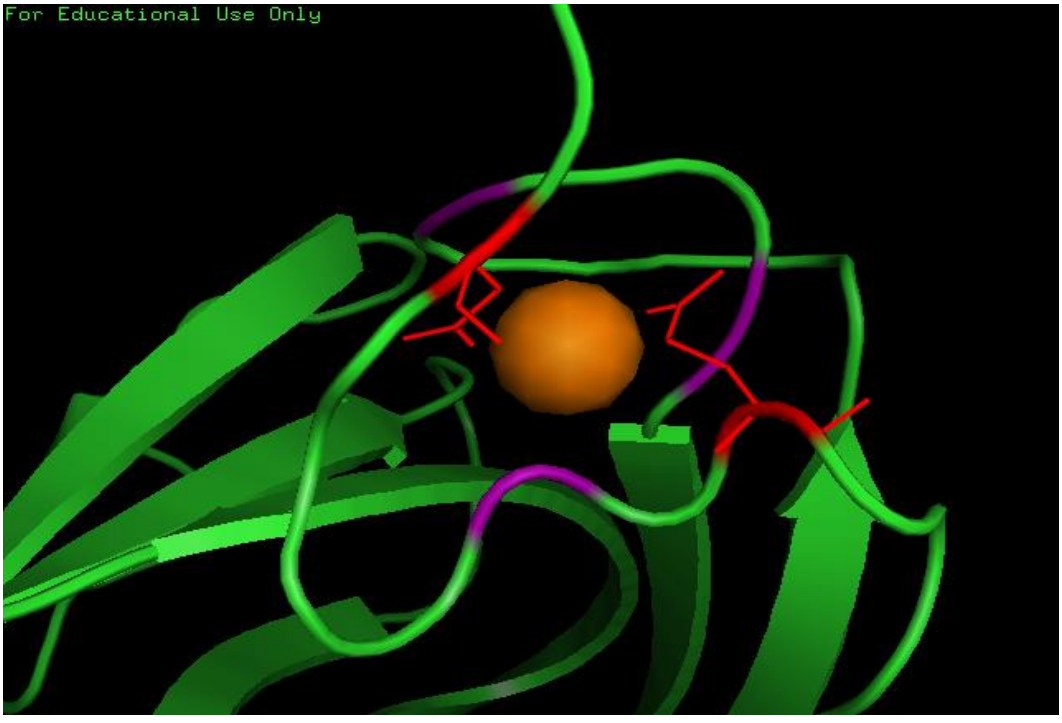

Figure 11. Visual representation of CBM 44 targeted mutations with specific residues 103 and 96 highlighted in red and additional Ca^+ ion stabilizing residues highlighted in purple. Figure was made using Pymol.

While there were major issues with the mutagenesis of CBM 11, CBM 44 was much more promising. Resulting mutant plasmids were sent out for sequencing and the results showed that the intended point mutations were successful. (Fig. 12)

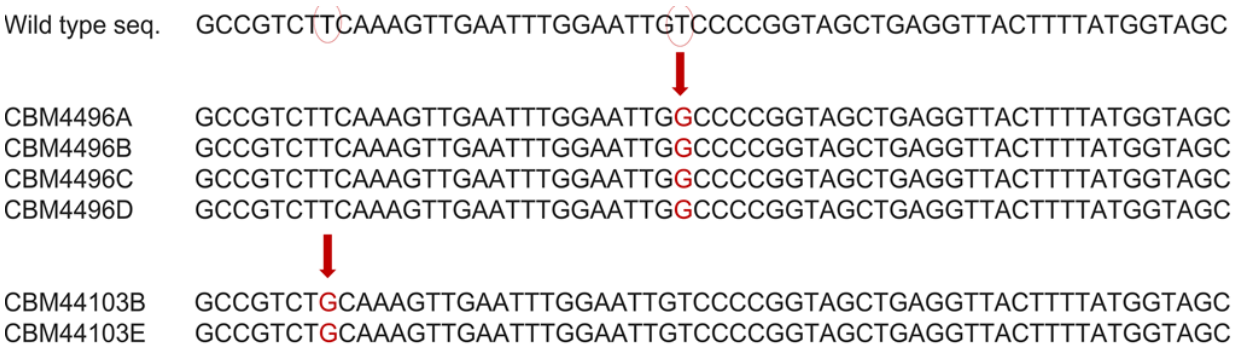

Figure 12. CBM 44 successful mutant sequences with mutant sites highlighted in red.

The above figure shows the most recent data collected in this project. Four candidates from the CBM 44 residue 96 mutation were identified that successfully changed a guanine to a thymine resulting in an aspartic acid being changes to an alanine. Similarly, two candidates for the CBM 44 residue 103 mutation likewise changes a guanine to a thymine resulting in a glutamic acid being changed to an alanine. In addition to these single point mutations, double mutations were made by using the aspartic acid 96 primers on the glutamic acid 103 mutant plasmids. The results of this double mutation are shown in Figure 13 along with the original single mutations as reference.

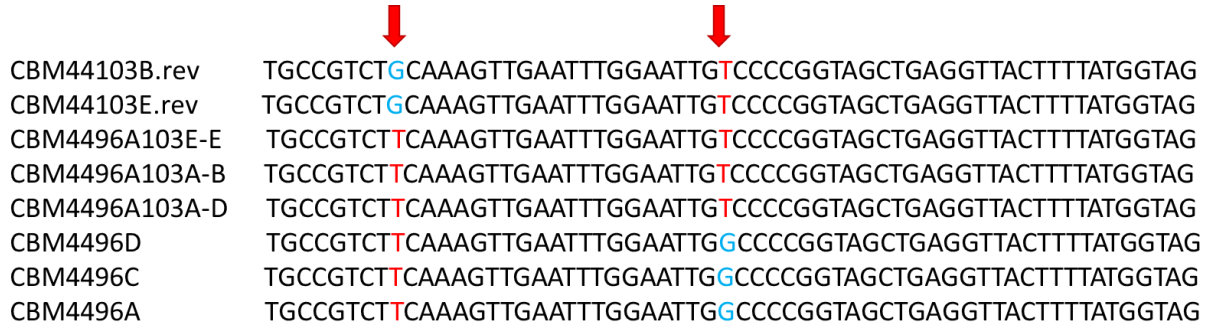

Figure 13. Double mutant sequences of CBM 44 with single mutation sequences as reference. Wild type nucleotides are shown in blue and mutations are shown in red.

The double mutant was also successful because it has a thymine where the wild type would have a guanine in both the 96 and 103 residue sites. This double mutant can be used to compare the relative stabilities of each mutation and evaluate how they depend on each other. Presumably, the stability decrease will be greater for the double mutant will be greater than the sum of the other two mutations alone. This is because they are both involved in ion binding and so it is very likely that they will affect one another. All three mutated sequences are currently in the process of being expressed so that the resulting proteins can be analyzed.

CONCLUSIONS

Overall, the work completed in this project has been promising. Although not all CBMs were able to be successfully mutated yet, CBM 44 has shown encouraging results. The issues surrounding CBMs 11 and 30 are likely minor and once resolved, mutagenesis can move forward for those as well. The mutation predictions for CBM 11 are still good candidates, the only problem was with the primer sequence itself. The CBM 44 candidates on the other hand, are ready to move forward in the process of becoming viable temperature tunable units.

The next step in this project will be to determine whether or not the mutants produced here are actually lower in T_m than the original CBMs. This can be done through the same

SPYRO orange assay used on the original samples. Melt curve plots can then be compared to the preliminary results to show if the melting point was decreased. This can be reaffirmed using the filter paper assays developed here by evaluating the conditions under which these new mutants desorb from the substrate. Based on predictions, both of these should show the mutants denaturing more easily than the wild type CBMs.

Once mutant T_m values are established, their refolding capabilities will also need to be assessed. Again, this can be done using an assay related to the preliminary work. The Fold-N-Glow complementation assay will be performed on the mutants just as it was to the original CBMs. Heat treated mutants can be compared to non-heat treated samples to ensure that the protein refolds properly after it has been denatured. It is important that refolding after heat treatment is spontaneous and does not result in any misfolded character.

Finally, the effect of these mutations on the holoenzyme will be evaluated. The CBMs will be re-expressed with the catalytic domain and their binding capabilities will be tested again. It also must be established that the original enzymatic activity is largely unaffected. If these alterations somehow prevent the catalytic domain from functioning properly, then this approach is not viable option for real life application. However, if temperature tunable CBMs in combination with the catalytic domain are successful, then this method has the potential to significantly reduce the overall cost of industrial biofuel production.

CITATIONS

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