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Hai Long Tran   April 8, 2010

Widening the Active Site of *Bacillus circulans* Xylanase by Amino Acid Insertion

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Abstract

Widening the Active Site of *Bacillus circulans* Xylanase by Amino Acid Insertion

By Hai L. Tran

Bacillus circulans xylanase (Bcx) is an enzyme of the glycoside hydrolase family which natively binds and degrades xylan, a polysaccharide abundant in plant cell walls. This characteristic makes it a highly desirable enzyme for industrial purposes particularly in papermaking, animal feed, and more recently the biofuel industry. Bcx adopts a β-jellyroll structure resembling a human right hand whereby the active site is located in the resulting cleft. Bridging the cleft, a loop region holds the finger and thumb regions in place to stabilize the protein. I hypothesize that the cleft width is an important determinant of the rate of hydrolysis and substrate specificity for glycoside hydrolase family members. By inserting 1-4 amino acids in the peptide bridge, the Bcx finger and thumb domains will spread open which could translate into new and broader substrate specificity and also an increased rate of catalysis. Here, we have created five Bcx mutants with glycine and proline residues inserted into the bridge to compare and contrast the effects of flexible and rigid residues. Our results suggest that out of the six variants tested, WT-Bcx has the highest activity for xylan substrate. Although the activity level for cellulose was low, G1-Bcx exhibited approximately a 35% increase in activity over wild-type. Circular dichroism data suggests that inserting flexible glycine residues decrease thermostability while inserting rigid proline residues maintain or improve it.

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Introduction

Biofuels

With the depletion of fossil fuel reserves and recent concerns of global climate change, it is important to look to new and renewable sources to suit our energy needs. By using fuels derived from plant biomass, we essentially have a carbon neutral system whereby the carbon dioxide absorbed by the plants are released back into the environment.[1] Current biofuel production relies heavily upon food sources such as corn and sugarcane due to their high level of freely available sugars and starches.[2] By using non-food or "waste" crop sources such as corn stover, sugarcane bagasse, wheat straw, poplar, and switchgrass, the price competition for food is eliminated.[3,4,5]

One major problem in biofuel production is the conversion efficiency from plant biomass to usable fuel. Plant cell walls are made up of three major components: cellulose, hemicelluloses, and lignin.[6] Cellulose is the most abundant component. Cellulases which degrade cellulose into glucose have been studied extensively and are currently used in the production of cellulosic ethanol. Hemicellulose is the second most abundant polysaccharide, accounting for approximately one-third of earth's renewable organic carbon source.[7] Hemicellulose is a heterogeneous polysaccharide composed mainly of xylose sugars in the form of a xylan backbone with mannose, galactose, rhamose, and arabinose attached. Being able to degrade xylan efficiently into its xylose will greatly improve the alternative fuel industry. Lignin is a highly complex polymer made up of random linkages between three primary lignin monomers: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol.[8] This makes it incredibly difficult to use

lignin as an energy source. Luckily, there are mechanical and chemical methods to remove lignin.[9] The pretreatment of plant biomass (Figure 1) generally involves the pulping and separating of plant components by mechanical and chemical processes.
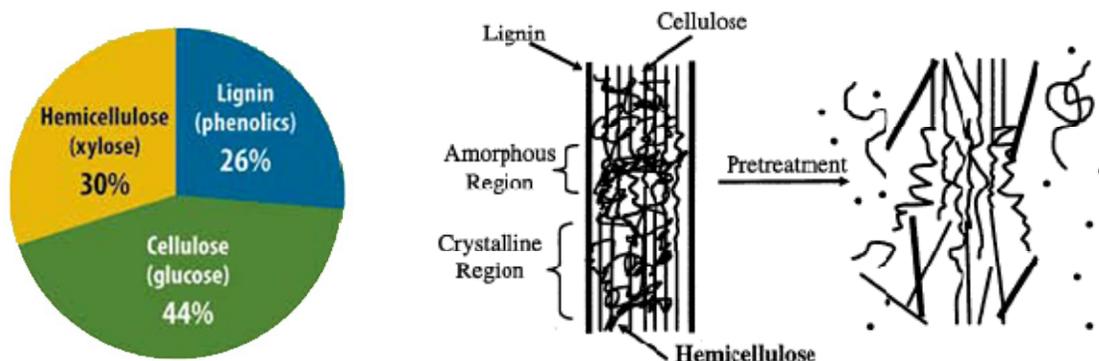

Figure 1: Left: Plant cell wall composition.[6] Right: The effect of pretreatment.[10]

Glycoside Hydrolases

At room temperature, glycosidic bonds have a half-life of approximately 4.7 million years.[11] Enzymes play a crucial role in catalyzing the hydrolysis of cellulose and hemicellulose components. Endo-glycoside hydrolases break the polysaccharides into many shorter fragments, greatly increasing the number of non-reducing ends.[12] This allows exo-glycoside hydrolases to efficiently cleave these fragments into glucose and xylose monomers. One stumbling block in using xylose as a source for bioethanol fermentation is the fact that the industrial alcohol fermenting yeast, *Saccharomyces cerevisiae*, cannot use it as an energy source. One solution would be to use a natural fermenter such as *Pichia stipitis* which can coferment glucose and xylose.[13] Another would be to introduce a xylose metabolizing pathway into *S. cerevisiae* containing the genes for xylose reductase, xylitol dehydrogenase, and xylulose kinase.[14] This would

alow xylose to eventually enter glycolysis by way of the pentose phosphate pathway

(Figure 2). A third option would be to use an engineered bacteria such as *Zymomomas*

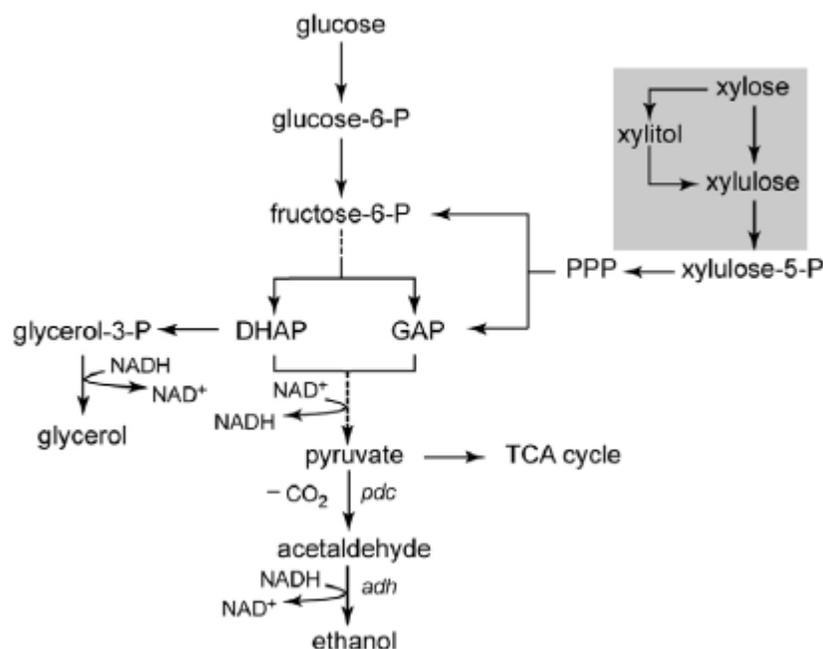*mobilis* which could perform the cofermentation with higher yields.[15,16]

Figure 2: Metabolic pathway in yeast for the fermentation of ethanol. Xylose can be converted to xylulose-5-P and enter into the pentose phosphate pathway.[17]

The enzyme studied in this project is *Bacillus circulans* xylanase (Bcx), an endo-1,4-xylanase. Bcx is a member of the glycoside hydrolase family 11 known only to degrade xylan polysaccharides.[18] Hemicellulose often forms a sheath around bundles of cellulose. Endo-1,4-xylanases are important because they can cleave the xylan backbone subjecting more cellulose to degradation. Xylanases along with other glycoside hydrolases are used in hydrolysis cocktails to break down cellulosic biomass. Due to advances in enzyme engineering and production over the past decade, the price of enzymes per gallon of ethanol produced has decreased from about $5.00 to $0.50 (Figure

3).[19] The cost is now approaching commercial feasibility. However, it is still beneficial

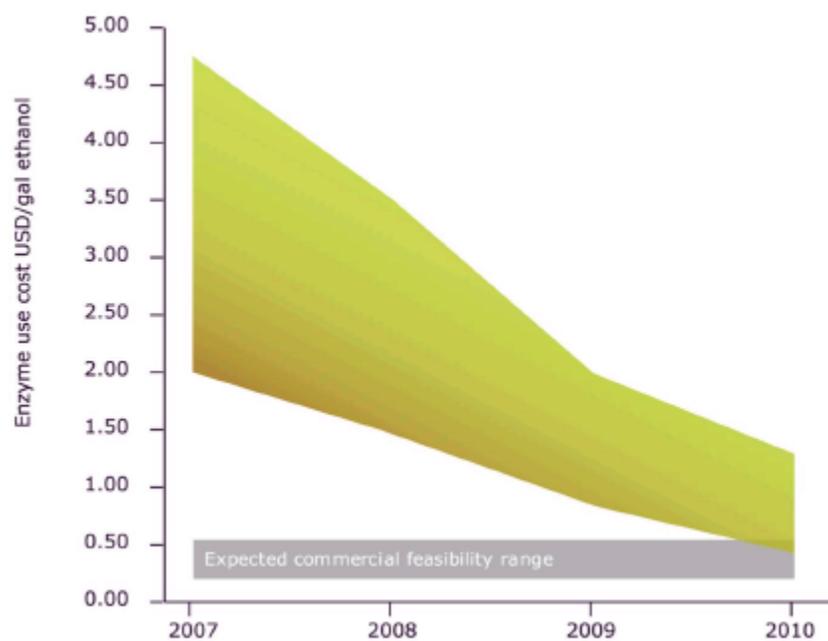to lower costs even further which can be done by engineering enzymes for improved

performance.

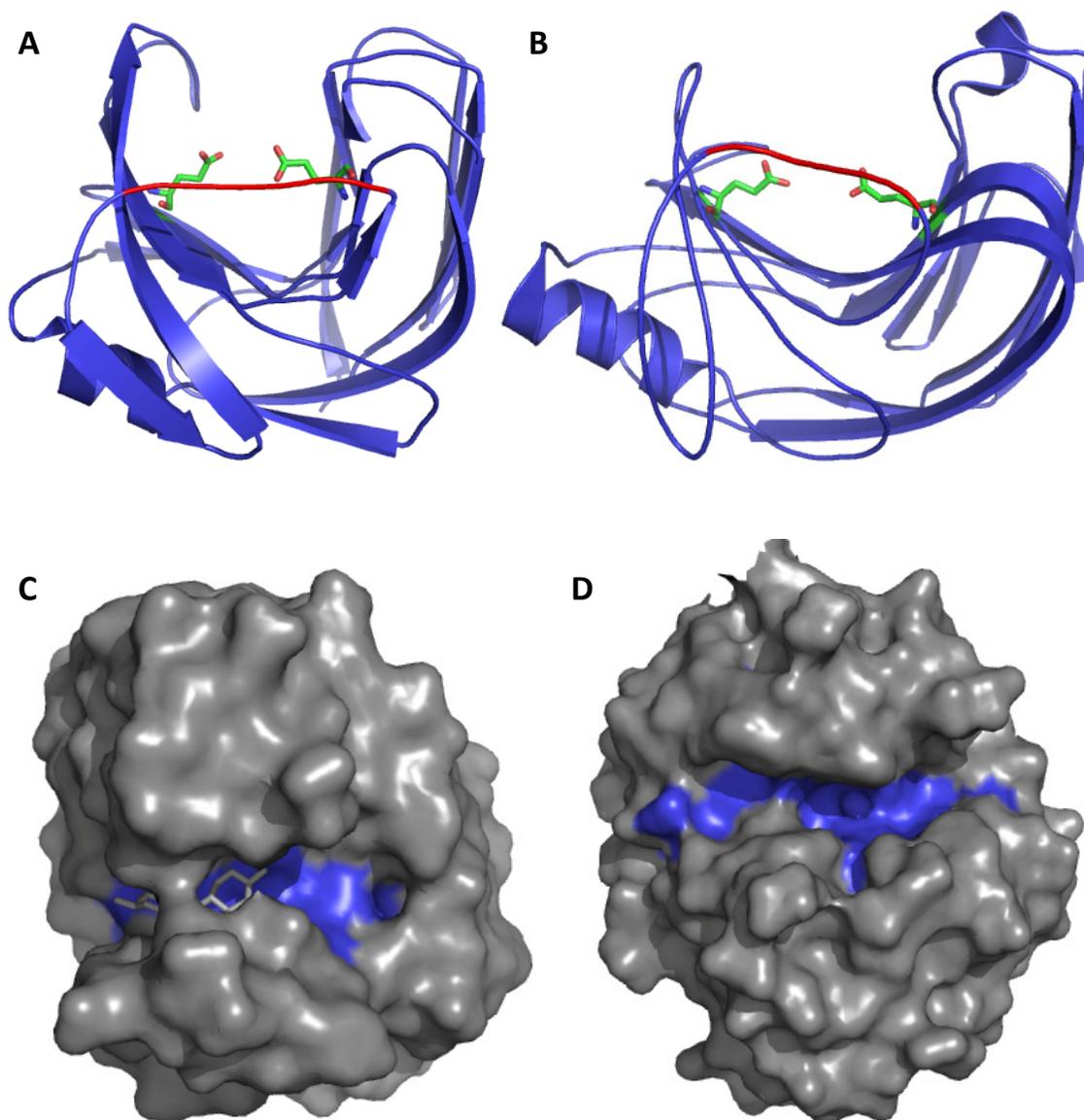**Figure 3:** Enzyme cost range for the production of ethanol.[20]

Figure 4: A) Crystal structures of *Bacillus circulans* xylanase and **B)** *Trichoderma reesei* cellulase with peptide bridge shown in red. PDB code 1BCX and 1OLQ, respectively.[21,22] **C)** Space filling model of Bcx and **D)** *Rhodothermus marinus* cellulase (PDB code 2BW8) where the active site cleft is shown in blue.[23]

Protein Engineering

The crystal structure of Bcx (Figure 4A) reveals a β-jellyroll structure folded around the catalytic site.[24] Bcx contains a peptide bridge (red) stabilizing and pre-orienting the two halves of the protein for optimal substrate binding by the active site cleft. The space filling model of Bcx shows a narrow opening in the active cleft (Figure 4C) allowing only small pentose polysaccharides such as xylan (Figure 6) to enter the active site. The active residues, Glu78 and Glu172, are located inside the cleft. The mechanism (Figure 5) for glycosidic cleavage with these two residues involves two bimolecular S_N2 substitution reactions.[12] Cellulase from *Trichoderma reesei* (Figure 4B), a family 12 glycoside hydrolase, has a very similar structure to Bcx with a β-jellyroll fold containing a cleft and a bridge.[25] However, cellulase has a noticeably wider cleft (Figure 4D) which allows larger hexose polysaccharides such as cellulose (Figure 6) to reach the active site. The active residues of cellulase are Glu116 and Glu200.
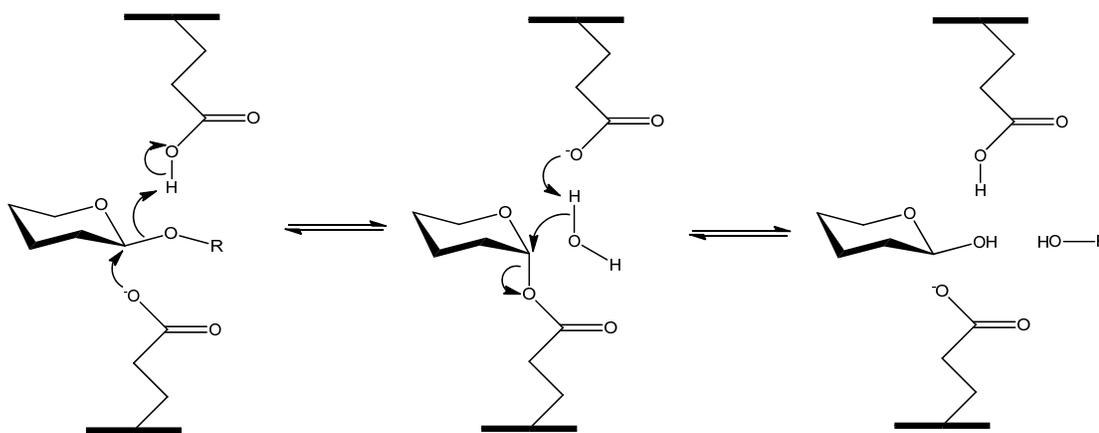

Figure 5: Mechanism for the hydrolysis of polysaccharides using two glutamate residues.
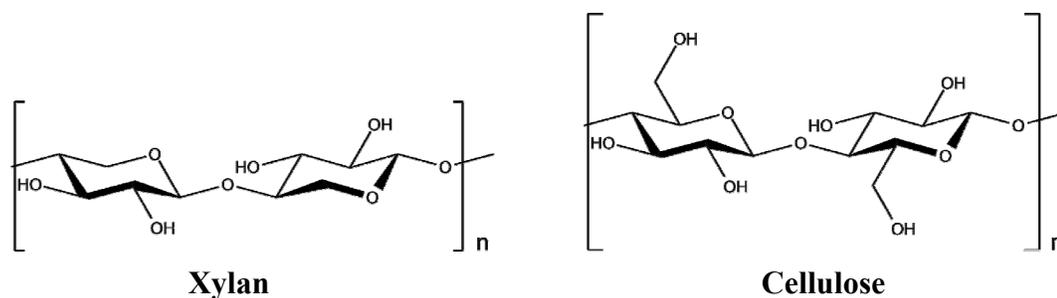
Figure 6: Structures of the polysaccharides xylan and cellulose.

I hypothesize that elongating the peptide bridge in Bcx should widen the active site cleft closer to the size of cellulase which may allow the binding of larger substrates and increase the rate of catalysis. To widen the active site, glycine and proline residues (Figure 7) will be inserted into the peptide bridge. These two amino acids were selected because their level of backbone flexibility is on opposite extremes.   Glycine has no side chain which allows for maximum conformational flexibility. Inserting glycines may widen the active site and also give the protein the flexibility it needs to open and close during catalysis. Proline, on the other hand, has a cyclic side chain which inhibits the rotation and bending of the amide bond which gives rise to its rigid structure. The rigidity of proline will help incrementally force the active site cleft of Bcx wider and possibly increase stability in the structure. However, the increased structural rigidity may hinder protein flexibility. Using glycine and proline insertions will allow us to compare and contrast the effects of lengthening the peptide bridge with highly flexible or highly rigid residues.
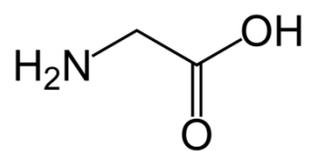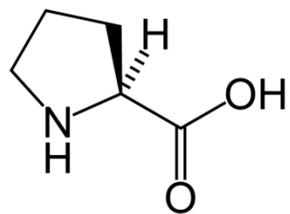
Glycine   **Proline**

Figure 7: Structures of glycine and proline.

Materials and Methods

Materials

WT-Bcx gene was obtained from the Withers lab (University of British Columbia, Vancouver, Canada). Enzymes used were purchased at New England Biolabs (Ipswich, MA). Reagents and substrates were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher (Pittsburgh, PA). QIAquick purification kit (Qiagen, Valencia, CA) was used to purify all DNA samples according to the manufacturer's protocols. DNA extractions were completed using QIAprep miniprep purification kit (Qiagen, Valencia, CA). Primers used in DNA sequencing and in mutagenesis to create restriction sites were purchased through Integrated DNA Technologies (Coralville, IA). DNA samples were sequenced by GENEWIZ Inc. (South Plainfield, NJ). Proteins were purified by Amersham Pharmacia Biotech ÄKTAdesign FPLC (GE Healthcare). A Sepharose Fast Flow Cation Exchange and a Superdex^{TM} 200 gel filtration column were purchased from GE Healthcare (Piscataway, NJ). Circular Dichroism analysis was performed on a Jasco J-810 spectropolarimeter (Easton, MD).

Mutagenesis

Site-directed mutagenesis was performed on WT-Bcx to create five variants with a number of glycine or proline residues inserted into position 90 (Figure 4A). The primers listed in Table 1 include the corresponding mutations. The names G1, G2, P2, P3, and P4 refer to the number of glycine or proline residues inserted. The WT primers were designed to include restriction sites for NdeI and HindIII (NEB). With the primers

and WT gene, an overlap extension PCR technique was used to incorporate the glycine

and proline insertions, respectively.

Primer	Sequence
WT-FOR	5′- CTT <u>CAT ATG</u> GCT AGC AGA GAT TAC TGG CAA AAC -3′
WT-REV	5′- GAA <u>AAG CTT</u> CCA CAC TGT AAC GTT GGA AGA ACC -3′
G1-FOR	5′- ACG TAC CGT **GGA** CCG ACT GGA ACC TAC AAA GGC -3′
G1-REV	5′- TCC AGT CGG **TCC** ACG GTA CGT TCC CCA AGA GTC -3′
G2-FOR	5′- ACG TAC CGT **GGA GGA** CCG ACT GGA ACC TAC AAA GGC -3′
G2-REV	5′- TCC AGT CGG **TCC TCC** ACG GTA CGT TCC CCA AGA GTC -3′
P2-FOR	5′- ACG TAC CGT **CCG CCG** CCG ACT GGA ACC TAC AAA GGC -3′
P2-REV	5′- TCC AGT CGG **CGG CGG** ACG GTA CGT TCC CCA AGA GTC -3′
P3-FOR	5′- ACG TAC CGT **CCG CCG CCG** CCG ACT GGA ACC TAC AAA GGC -3′
P3-REV	5′- TCC AGT CGG **CGG CGG CGG** ACG GTA CGT TCC CCA AGA GTC -3′
P4-FOR	5′- ACG TAC CGT **CCG CCG CCG CCG** CCG ACT GGA ACC TAC AAA GGC -3′
P4-REV	5′- TCC AGT CGG **CGG CGG CGG CGG** ACG GTA CGT TCC CCA AGA GTC -3′

Table 1: Primers with glycine and proline residues in bold and restriction sites underlined.

Cloning and Sequencing

Each Bcx variant was cloned into the multiple cloning site of pET-21a(+) vector

(Novagen, Madison, WI). This was done by digesting the vector and each insert with

HindIII and NdeI restriction enzymes and performing a ligation reaction. Aliquots of

each ligation mixture was transformed into *E. coli* DH5α strain (Invitrogen, Carlsbad,

CA) and plated on LB-agar plates containing 0.1 mg/mL of ampicillin. The plates were

incubated for 16 hours at 37°C. Plasmids were extracted and sequenced to verify that the

mutations were properly introduced.

Protein Expression

The mutant and WT plasmids were transformed into *E. coli* BL21 (DE3) strain (Invitrogen) for overexpression. A culture for each variant was grown in LB medium with 0.1 mg/mL ampicillin to an optical density of 0.5 - 0.8 at 600 nm. Expression was induced with the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture was incubated in an orbital shaker at 250 rpm for 12-16 hours at 20°C. Afterwards, the main culture was aliquoted into 50 mL tubes and centrifuged at 4°C for 15 minutes at 3,200 rpm. Supernatant was poured off and cell pellets were stored at -20°C for later purification. Before aliquoting the culture, 1 mL samples were taken and analyzed for expression levels of soluble protein. Culture samples were pelleted and the soluble and insoluble proteins were separated with BugBuster™ Protein Extraction Reagent (Novagen) following retail protocols. The soluble and insoluble proteins were analyzed via SDS-PAGE.

Protein Purification

The *E. coli* pellets were resuspended in 2 mL of 0.01 M sodium phosphate buffer (pH 6.0) with 20 µL protease inhibitor cocktail and 2 µL benzonase endonuclease (Novagen). The solution was sonicated (6 times for 10 sec with 20 sec rest on ice) and centrifuged (10,000 rpm, 4°C, 20 min). The supernatant was purified in two steps via fast protein liquid chromatography (FPLC) as described below:

Step 1: Cation Exchange

The 1 mL Sepharose Fast Flow Cation Exchange column was pre-equilibrated with 20 mL 0.01 M sodium phosphate buffer (pH 6.0) at room temperature at a rate of 0.4 mL/min. 500 µL of the soluble protein was injected onto the column. To remove unspecific non-binding protein, 4 mL of 0.01 M sodium phosphate buffer (pH 6.0) was flowed through the column at a rate of 0.4 mL/min. A gradient of 40% 1.0 M sodium chloride and 60% 0.01 M sodium phosphate buffer (pH 6.0) was introduced at 0.4 mL/min for a volume of 6 mL through the column to elute the target protein. The fraction containing the target protein, as shown by the UV detector at 280 nm, was collected. The process was repeated for a total of 4 times and the fractions were combined and concentrated to a volume of 500 µL via an Amicon Ultra-4 ultrafiltration unit (Millipore, Bedford, MA; MWCO: 10kDa, 3,600rpm at 4°C)

Step 2: Gel Filtration

For this purification step, a 24 mL Superdex^{TM} 200 gel filtration column was used to separate the sample by molecular weight. The column was pre-equilibrated with 30 mL of 50 mM potassium phosphate (pH 7.0), 150 mM NaCl buffer. 500 µL of the concentrated sample from step 1 was injected onto the column. 30 mL of the standard buffer was flowed through the column at 0.6 mL/min to elute the protein. The corresponding fraction was collected and concentrated to a volume of 100 µL via an Amicon Ultra-4 ultrafiltration unit using specifications described above. Enzyme concentration was measured at 280 nm ($\varepsilon = 81,790$ M$^{-1} \cdot$ cm^{-1}). A sample from each step of purification was analyzed on SDS-PAGE to examine the purity of the product.

Enzyme Activity Experiment

To test the enzymes for xylanase activity, a soluble chromogenic substrate, Remazol Brilliant Blue-Xylan (RBB-Xylan) (Sigma), was used.[26] A 50 µL reaction mixture was prepared in 0.1 M sodium phosphate buffer (pH 6.0) with a substrate concentration of 5 mg/mL and an enzyme concentration of 0.5 µM. A control experiment containing only buffer and substrate was run in parallel. The reaction mixtures were mixed and incubated for 10 minutes in a water bath at 40°C. The reaction was terminated by the addition of 125 µL 96% ethanol and vigorous mixing. The mixtures were incubated for 10 minutes at room temperature followed by centrifugation at 1.5 g for 10 minutes at room temperature. The absorbance of the supernatant was measured at 590 nm. Samples were diluted with 96% ethanol if necessary to keep absorption below 1 absorption unit. The measurement for the control experiment was subtracted from the reading.

A similar procedure was used to test the enzymes for cellulase activity. The substrate, Ostazin Brilliant Red-hydroxyethyl cellulose (H3B-Cellulose) (Sigma), is a soluble chromogenic substrate.[27] A 40 µL reaction mixture was prepared in 0.1 M sodium phosphate buffer (pH 6.0) with a substrate concentration of 5 mg/mL and a high enzyme concentration of 7.5 µM. A control experiment with no enzyme present was run in parallel. The reaction mixtures were mixed and incubated for 1 hour at 40°C in a water bath. The reaction was terminated by the addition of 120 µL of 2:1 ethanol:acetone with vigorous mixing. The mixtures incubated for 30 minutes at room temperature to

allow precipitation of unused substrate. Reaction vessels were mixed and centrifuged at 2.0 g for 2 minutes at room temperature. The absorbance of the solution was measured at 550 nm with compensation for the control experiment.

Circular Dichroism Analysis

Far-UV spectra was recorded at room temperature from 260-190 nm (0.5 nm increments) with a 0.1 mm path-length cell, 20 nm min^{-1} scan rate, 4 sec response time, and 2 nm bandwidth. Proteins were analyzed at concentrations of 450-500 mg mL^{-1} in potassium phosphate buffer (50 mM, pH 7.0). The recorded data represents the mean of three scans minus a correction for the buffer. Thermal denaturation was monitored by following the ellipticity at 218 nm from 30 to 85°C in increments of 0.5°C.

Results

Gene Construction

One and two glycine codons and also two, three, and four proline codons were successfully inserted into the wild type gene. The sequences of the mutant constructs were verified by GENEWIZ, Inc. sequencing service.

Protein Expression

WT-Bcx was originally expressed under conditions of 1.0 mM IPTG for 4 hours at 37°C. However, due to very low levels of soluble protein, conditions needed to be further optimized. We slowed the expression process by lowering the temperature and reducing the concentration of the inducing agent. This should give the chaperones more time to properly fold the proteins. It was found that the low temperature of 20°C with 0.1 mM IPTG was optimal in obtaining soluble protein. Figure 8 shows the results of WT-Bcx expression. WT-Bcx has a molecular weight of 20.2 kDa and can be seen in the appropriate location on the gel. Similar results were observed among the five mutants.
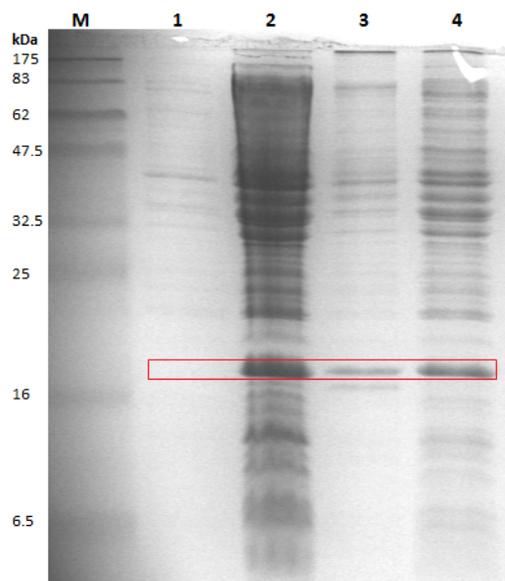
Figure 8: Expression of WT-Bcx at 20°C

Lane 1: Total Bcx protein at 0 hour

Lane 2: Total Bcx protein at 12 hours

Lane 3: Insoluble Bcx protein at 12 hours

Lane 4: Soluble Bcx protein at 12 hours

Red box indicates location of target protein at approximately 20.2 kDa.

Protein Purification

In the first step of purification, a cation exchange column was used to exploit the net positive charge of the Bcx variants. The protein bound to the resin and eluted soon after the NaCl gradient was introduced. The target protein eluted between 5.5 mL and 7.0 mL (Figure 9).

As shown in Lane 3 of Figure 10, impurities still exist after the first step of purification. To further purify the protein, the samples were loaded onto a size exclusion column in order to eliminate impurities of higher molecular weight. Bcx is a monomeric protein and was eluted between 17 and 21 mL (Figure 10). SDS-PAGE analysis (Figure 11) revealed that the two-step purification process was successful in purifying the Bcx variants.   Typical expression yields are approximately 2 mg of purified protein per liter of culture.
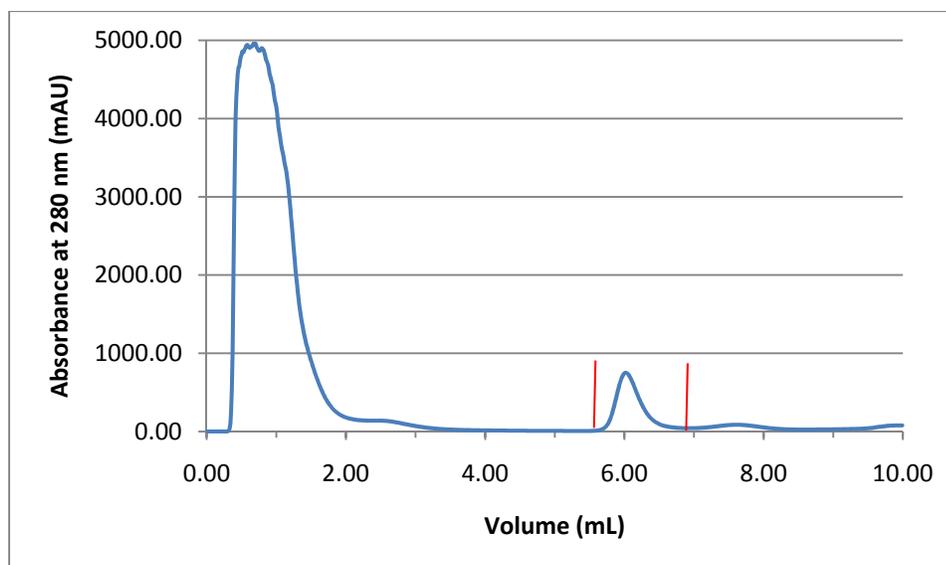
Figure 9: Purification with cation exchange column. Red lines indicate the collected fraction.
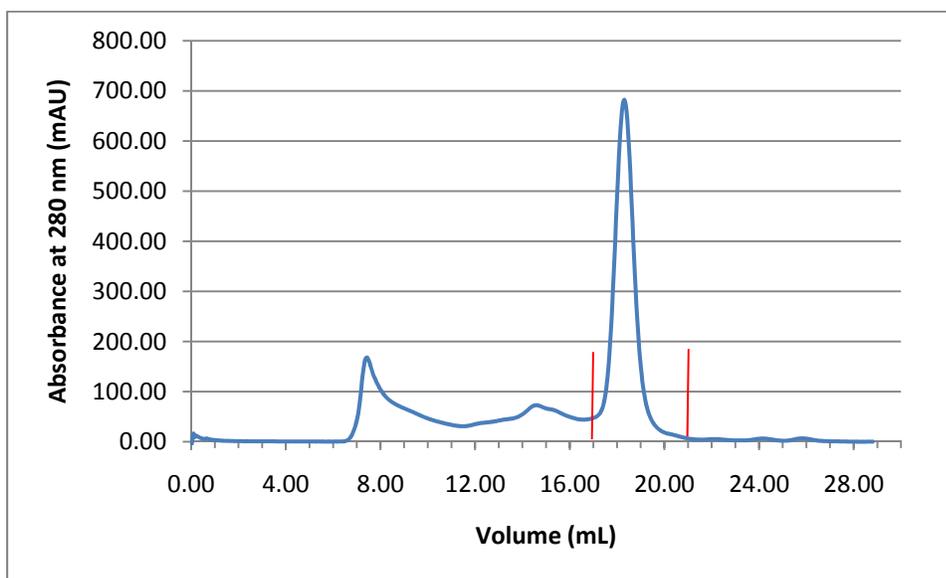

Figure 10: Purification with size exclusion column. Red lines indicate the collected fraction.
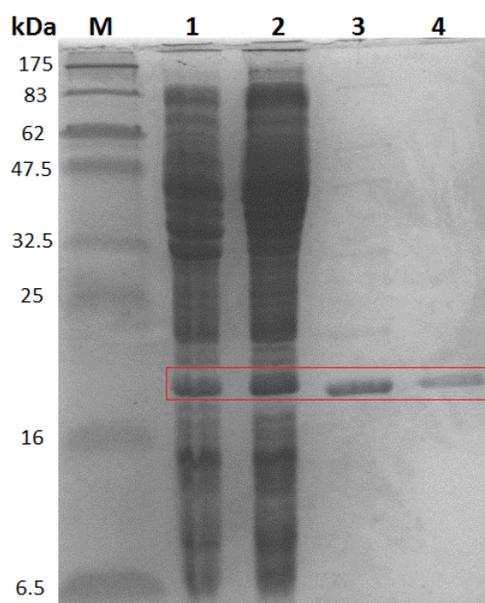
Figure 11: SDS-PAGE analysis of purification process

Lane 1: Insoluble protein

Lane 2: Soluble protein

Lane 3: Purification step 1

Lane 4: Purification step 2

Purification process is shown for P2-Bcx. All variants showed similar results. Red box indicates location of target protein.

Enzyme Activity

The activities of the enzymes were tested with xylan, the native substrate, and also with cellulose, a substrate which Bcx does not naturally degrade. Data acquisition was performed in triplicate. With the xylan substrate, it can be seen from Figure 12 that the activity of Bcx variants decline with the glycine mutants having a greater loss. With the cellulose substrate, the G1-Bcx mutant had a 35% increase in activity over the wild-type. However, the significance of this data is questionable due to the large errors associated.
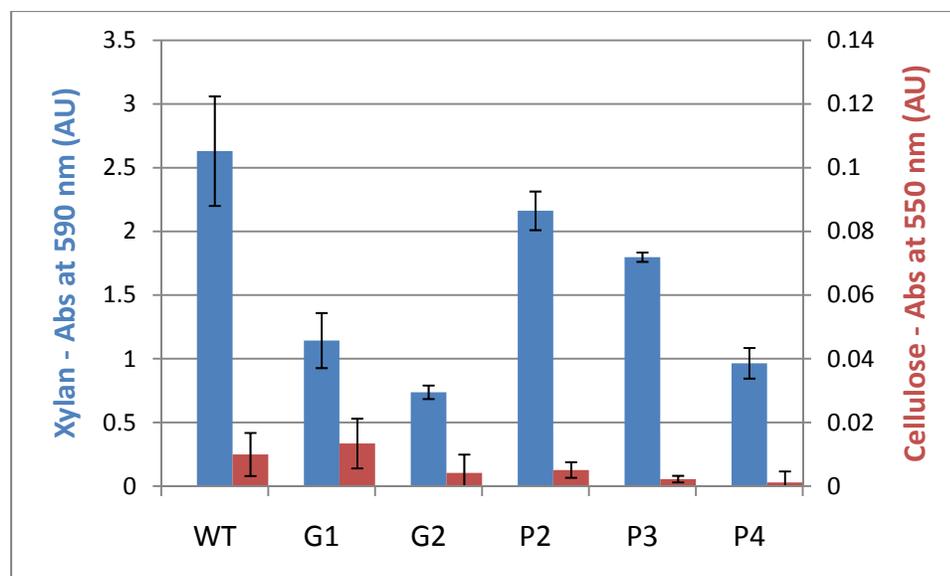
Figure 12: Enzyme activity against xylan and cellulose substrates. Error bars show ± 1 standard deviation.

Circular Dichroism

CD scans confirmed that the secondary structure of Bcx consists of mainly β-sheets (Figure 13). Since the scans for all variants adopted a similar curvature, it is safe to assume that all the mutants adopted the same conformational structure as wild-type. The proteins were then thermally denatured by gradually raising the temperature from 30 to 85°C and the ellipticity was monitored at 218 nm. The data points were fit to Boltzmann sigmoidal curves using Origin software and are shown in Figure 14.   The melting temperatures (T_m) were calculated by the curve fitting.
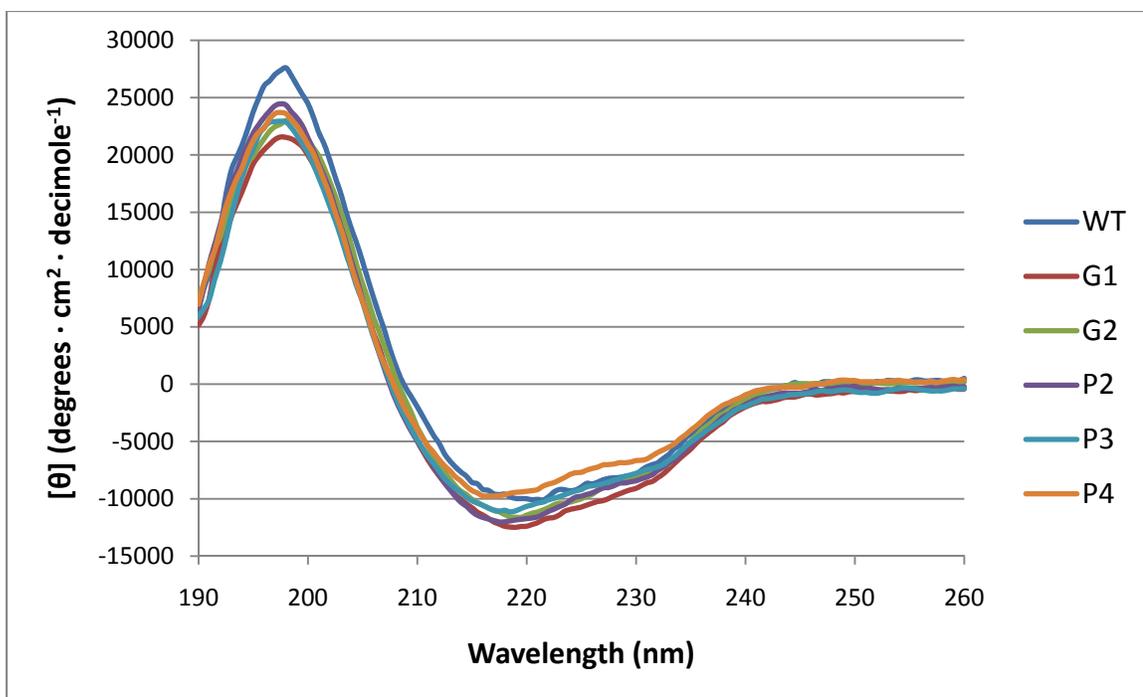
Figure 13: CD scans of Bcx variants. Each curve represents mean of three scans with buffer correction.
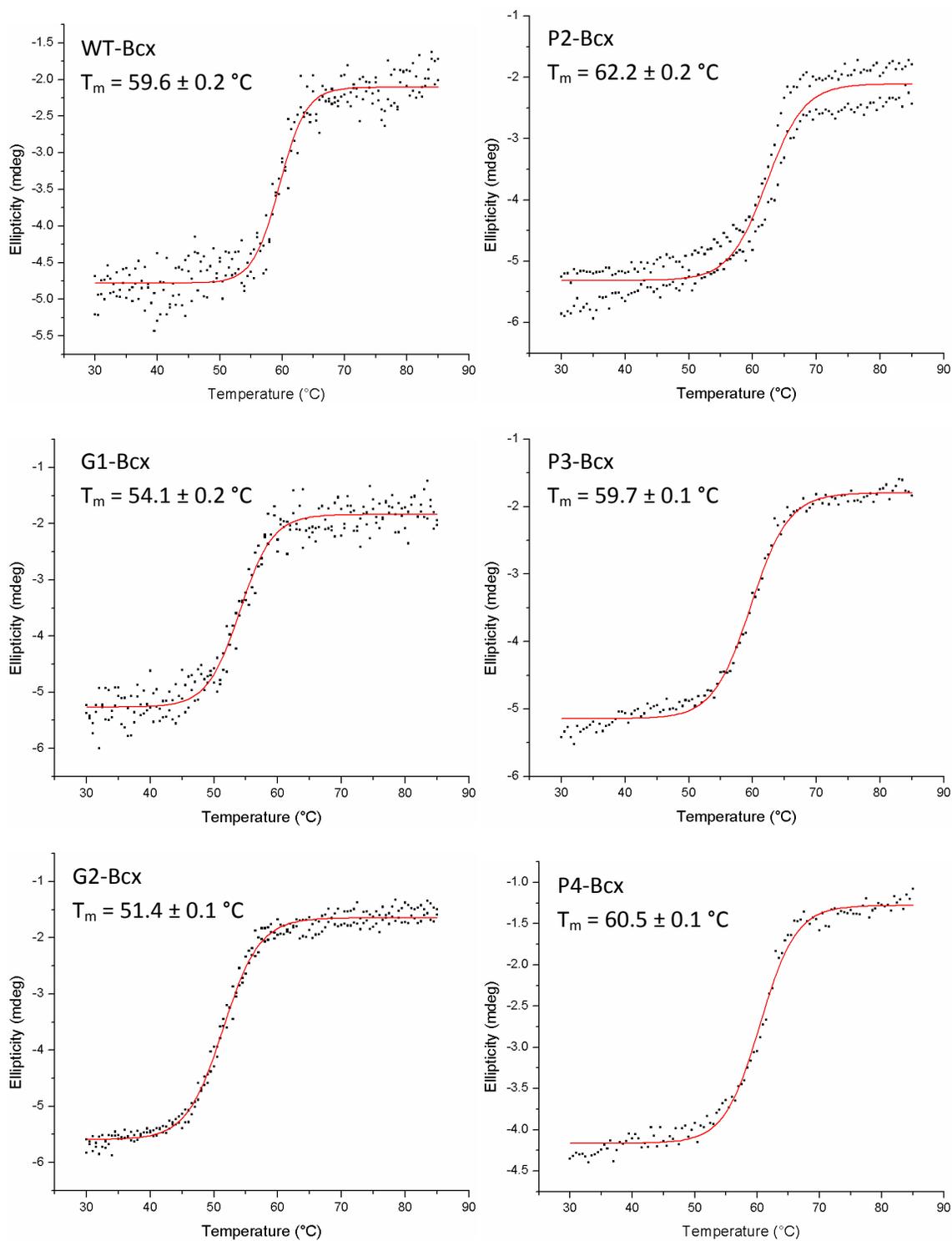
Figure 14: Thermal denaturation was performed in duplicates (P3 and P4 in singlicate). Curve fittings (red) were used to calculate T_m values.

Discussion

Our data suggests that widening the active site of Bcx did not increase its activity for xylan substrate and out of the six variants tested, WT-Bcx was the most optimal. A general trend can be seen that with more amino acids inserted into the bridge, the more the activity level begins to decline. Also, the activity of the proline mutants tends to retain its activity better than the glycine mutants. This is most likely due to the fact that proline is a much more rigid residue than glycine. In retrospect, it may be possible that the wild-type active site cleft is too wide and that the activity may be optimized by reducing the width of the active site. It will be an interesting experiment to delete one or more amino acids from the sequence and see the correlation in activity. Also, incorporating other amino acids with different properties may give interesting results. For example, incorporating hydrophobic residues such as phenylalanine or isoleucine may pull the peptide bridge in toward the center which may narrow the active site.

The cellulose activity assay showed no significant signs of increased binding or activity for the larger polysaccharide. Only the G1-Bcx mutant showed an increase in activity (~35% increase) over wild-type, but the significance of this data is not very reliable due to the large errors. It is important to note that an enzyme concentration of 7.5 μM was used which is 15-fold higher than in the xylan assay. Also, the incubation time for the reaction was increased from 10 to 60 minutes. The errors may be attributed to the fact that activity on the non-native substrate was very low and the measurements in activity had much more variability. However, if we compare the mutants with two amino acid insertions, G2-Bcx and P2-Bcx, we see that the activity P2-Bcx is about 21% higher. It is quite possible that if a P1-Bcx mutant were to be created, it may have activity more

than 35% over wild-type. It will also be interesting to see how the cellulase activities of these Bcx variants compare to a natural cellulase enzyme. The literature suggests that xylanases with natural cellulose activity tends to be several orders of magnitude lower.[28]

The CD data shows some interesting trends with the mutants. With the glycine mutants, we see that the T_m drops by about 4°C for every glycine inserted. This may be attributed to the increased flexibility introduced by the glycine residues. On the other hand, the proline mutants maintained or improved upon wild-type thermostability. This exemplifies the differences between using rigid and flexible residues in the peptide bridge. The decline in activity in the glycine mutants over the proline mutants may be attributed to the reduced thermostability.

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