Biochemical characterization of beta-glucosidase BglX from *Escherichia coli*

by

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Biochemical characterization of beta-glucosidase BglX from *Escherichia coli*

Lorna Ngo

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Abstract

Beta-glucosidase BglX from *E. coli* is an enzyme that hydrolyzes the $\beta$(1→4) glycosidic bonds. Unlike other beta-glucosidases produced by *E. coli*, this enzyme is found in the periplasm of the cell rather than the cytoplasm. BglX is a suitable target for drug therapy since this enzyme is produced solely by bacteria. Identification of key residues in the active site is important in the elucidation of the mechanism of BglX and for rationalization of inhibitor design. The objective of this research is to develop the purification procedure for BglX, characterize BglX, and identify the important residues in the active site of BglX. Purification of BglX was performed with ammonium sulfate precipitation, ion exchange and hydrophobic interaction chromatographies. The kinetic parameters of BglX were analyzed; the enzyme has a $K_M$ of 0.35 mM, a $k_{cat}$ of 1.19 s$^{-1}$, and a catalytic efficiency of $3.4 \times 10^3$ M$^{-1}$s$^{-1}$ for the chromogenic substrate, para-nitrophenol-$\beta$-D-glucopyranoside. Aspartate 287 was selected for mutation based on homology with other glycosidases. BglX mutants were constructed through PCR-based site-directed mutagenesis. The activity of the BglX D287N mutant protein was compared to the wild type BglX protein. Replacement of aspartate 287 with asparagine resulted in significantly reduced glucosidase activity, indicating the importance of this residue in catalysis.
Acknowledgements

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<td>Aspartate</td>
</tr>
<tr>
<td>BglX</td>
<td>Beta-glucosidase with unknown biological function</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAZy</td>
<td>Carbohydrate-Active Enzyme</td>
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<tr>
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<tr>
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<td>Tris-acetate-EDTA</td>
</tr>
<tr>
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Chapter 1: Introduction

*Escherichia coli*

*Escherichia coli* (*E. coli*) are large group of bacteria belonging to the family Enterobacteriacea. *E. coli* is currently the most studied and well understood organism. This bacterium commonly resides in the gastrointestinal tract of mammals, where it forms a mutualistic relationship with the host, but can also be found at sites outside of the intestine. *E. coli* obtains nutrients, consisting of a mixture of glycoconjugates, from the intestinal lining of the host (1). Intestinal strains of *E. coli* become pathogenic when presented access to extraintestinal habitats (2). Colonization at sites outside of the intestine results in serious infections such as urinary tract infections, neonatal meningitis, and septicemia (3).

*E. coli* is a model organism for laboratory research considering its rapid growth rate, ease of genetic modification, and most strains are safe to handle. For instance, *E. coli* cells duplicate every 30 minutes and can grow aerobically or anaerobically (4). Since the completion of the genome sequence of several *E. coli* strains, *E. coli* is a favorable organism to use in molecular cloning and is often used, for example, for the study of protein expression. *E. coli*, therefore, is often used for comprehension of biological processes.
**Glycosidases**

Glycosides are molecules containing sugars that are attached to other carbohydrate or non-carbohydrate molecules. Glycosides are linked by covalent bonds known as glycosidic bonds. More specifically, they are formed between the anomeric carbon, called a hemiacetal, and a hydroxyl group of an organic molecule; therefore, glycosidic bonds can link monosaccharides to produce complex carbohydrates. When referring to the cyclic structure of sugars, glycosidic bonds exhibit stereochemistry at the anomeric carbon, called anomers. For an α anomer, the hydroxyl group bound to the anomeric carbon is opposite to the CH$_2$OH group of the sugar while for a β anomer, the hydroxyl group bound to the anomeric carbon is on the same side as the CH$_2$OH group. Hydrolysis of glycosidic bonds is catalyzed by a large class of enzymes referred to as glycosidases (5).

Carbohydrates display a broad variety of stereochemistry and, as a result, there is also a wide array of enzymes that hydrolyze them. Glycosidases can be classified into groups according to Enzyme Commission (EC) numbers via the type of bond hydrolyzed or by amino acid sequence similarities. EC numbers specify enzymes based on enzyme-catalyzed reactions and their substrate specificity. For example, glycosidases with EC numbers 3. 2. 1. x are enzymes that hydrolyze O-glycosyl linkages, which are designated by the first three numbers and the last digit signifies the substrate or oftentimes the molecular mechanism (6). This type of classification is practical in avoiding trivial names and offers a distinct classification; however, it faces some problems. For instance, it does not consider evolutionary events that could cause a change in substrate specificity.
EC classification also faces a problem with enzymes that act on numerous substrates. Substrate specificity is characterized by several factors (5). Anomeric specificity identifies the enzyme as either α or β. The site of substrate hydrolysis by the enzyme is also considered. For example, if the monosaccharides are cleaved from the terminal end of the polysaccharide chain, then the enzyme is termed an exoglycosidase. Endoglycosidases cleave the polysaccharide within the chain.

Another factor of substrate specificity depends on the stereochemistry of the hemiacetal formed following hydrolysis. Hydrolysis of glycosidic bonds occurs via two mechanisms that result in either inversion or retention of the anomeric configuration. Inverting glycosidases use two carboxylate residues (usually a glutamate or aspartate) that act as general acids-base catalysts. This group hydrolyzes glycosides by a direct nucleophilic displacement of the aglycone moiety, resulting in a product with an anomeric configuration opposite to the substrate. Retaining glycosidases also use two carboxylate residues, although they hydrolyze glycosides by a double-displacement reaction. One residue acts as a nucleophile by attacking the anomeric carbon to produce a glycosyl-enzyme intermediate. The other residue acts as an acid by protonating the glycosidic oxygen and then as a base to deprotonate a water molecule while it hydrolyzes the glycosyl-enzyme intermediate. The product generated from this reaction shares the same stereochemistry as the substrate (7). Lastly, the sugar type that is hydrolyzed also defines glycosidase substrate specificity. For example, a retaining endo-β-galactosidase cleaves β-galactose constituents from the reducing end of the polysaccharide containing β-galactoside bonds, while maintaining the same anomeric configuration as the substrate.
The most effective type of classification is the comparison of the enzymes’ amino acid sequences since it is known that there is a direct correlation between protein sequence and resemblances in folding (6). Consequently, glycosidases can be assigned to the same family if the sequences could be aligned over a domain. Additionally, this type of classification can be helpful in predicting the three-dimensional structure of a protein if the structure of at least one of the member’s in the family is known (8). According to the Carbohydrate-Active Enzyme (CAZy) database, there are currently 113 glycosidase families and they represent the best characterized group of enzymes in the database (9).

Glycosidases are present in all living organisms and carry out several functions. First, they are essential enzymes for carbohydrate metabolism (6). As previously mentioned, glycosidases hydrolyze glycosidic bonds, thereby degrading oligo- and polysaccharides. Starch, a polysaccharide produced by all green plants, is degraded by glycosidases. Degradation of polysaccharides such as starch provides organisms with a source of energy. Glycosidases also play a role in the turnover of carbohydrates at the cell surface (8). Another function of glycosidases is that they have the ability to guard organisms from pathogens. For example, lysozyme is well known for its antibacterial protection. It defends against pathogens by inhibiting their growth or by destroying the bacteria by lysis (10). For that reason, lysozymes are a useful application in the food industry. In contrast, glycosidases can also play a role in pathogenesis. Viral neuraminidase has been shown to act as the initiator of the influenza virus (11).

Deficiency of some glycosidases may also cause health problems. Studies have shown that deficiencies in glycosidases can result in lactose intolerance or lysosomal
storage diseases. Lactose intolerance occurs due to the inability to digest lactose (milk sugar) because of a deficiency in the enzyme lactase. This results in a person feeling discomfort, with symptoms ranging from mild to severe. The most common lysosomal storage disease is called Gaucher’s disease and is caused by a deficiency in glucocerebrosidase. A person lacking this enzyme will accumulate glucosylceramide, a fatty substance, in their cells and certain organs (12). Symptoms for Gaucher’s disease include anemia, liver/spleen enlargements, progressive brain damage, and seizures.

**β-glucosidases**

β-glucosidases (EC 3. 2. 1. 21) are a family of glycosidases that specifically cleave β(1→4) linked glycosidic bonds. Furthermore, these enzymes have been classified into the glycosidase families: 1, 3, 5, 9, and 30 (13). β-glucosidases are frequently studied for their practical applications in biotechnological processes (14). Examples consist of the degradation of cellulose, the release of aromatic compounds with antioxidant activity from their glucosidic precursors, and the conversion of cellobiose to glucose that could be beneficial in producing renewable energy sources (14, 15). Glucose released by the hydrolysis of cellobiose (the last step in the complete hydrolysis of cellulose) can be fermented into ethanol (16). Cellulose, a polysaccharide that is the main component in the cell walls of plants, contains these types of bonds that can be degraded by β-glucosidases. Various bacteria and fungi produce β-glucosidases in order to consume cellulose and other beta-linked saccharides.
E. coli possess at minimum three operons, bgl, asc, and cel, which encode proteins that allow the bacteria to grow on and use β-glucoside sugars (17). These operons encode phosphoenolpyruvate-dependent phosphotransferase system proteins that simultaneously transport and phosphorylate β-glucosides from the periplasm to the cytoplasm (18). The phosphorylated β-glucosides are hydrolyzed by cytoplasmic phospho-β-glucosidases, also encoded by the operons, yielding glucose-6-phosphate (17). In most E. coli strains, these operons are defined as cryptic, meaning they are not normally expressed but can be activated by genetic mechanisms such as, mutation, insertion sequences, and recombination (19). Expression of the operons provides E. coli the ability to utilize the β-glucosides, cellobiose, arbutin, and salicin (20-22).

BglX

Yang et al. discovered an additional gene in E. coli encoding a β-glucosidase, named bglX. BglX, the gene product of bglX, is a β-glucosidase that differs from others produced by E. coli in sequence and location. BglX is located in the periplasm, the area between the inner cytosolic membrane and the outer membrane of the cell, rather than the cytoplasm (17). However, BglX shares significantly identical sequences to over 30 other β-glucosidases in various bacteria and fungi and exhibits similar kinetic activities as many other β-glucosidases (17).

Few studies on the bglX gene and BglX have been published. Though, data regarding the cloning, DNA sequence, chromosomal mapping of bglX, and characterization of BglX has been reported by Yang et al. The bglX gene is 2.3 kb in
length and is located at 47.8 min on the *E. coli* chromosome. The 765 residue amino acid sequence of BglX was determined from the nucleotide sequence. Yang *et al.* purified BglX from the periplasm and stated that the mature protein is derived from cleavage of a signal peptide 20 residues in length. The enzymatic activity of BglX was analyzed with the substrate *o*-nitrophenyl-β-D-glucopyranoside. BglX was reported to have a $K_M$ of 18 mM and a $V_{max}$ of 3 μM min$^{-1}$. Other observations by Yang *et al.* indicate that there was no apparent effect on the deletion of the *bglX* gene and overexpression of BglX does not assist growth on cellobiose, arbutin, or salicin. Also, the natural substrates for BglX remain unknown. The reason *E. coli* produce a periplasmic β-glucosidase is unknown as well; however, Yang *et al.* speculate that it may aid the bacterium to live in the gut or between hosts by producing glucose for energy through β-glucoside hydrolysis.

*Barley β-D-Glucan Exohydrolase as a Model for Structure Prediction of BglX*

The three-dimensional (3-D) structure of BglX has yet to be determined. A sequence alignment performed on the Phyre server using the amino acid sequence of BglX indicates that the enzyme has a 30% match to barley β-glucan exohydrolase isoenzyme ExoI (23). According to the CAZy, BglX would be classified as a family 3 glycosidase. Since members of the same family exhibit similar structures, β-glucan exohydrolase isoenzyme ExoI was used as a template for predicting the 3-D structure and active site of BglX.

The 3-D structure of barley β-D-glucan exohydrolase isoenzyme ExoI was the first enzyme of the family 3 glycosidases to be discovered (24). Joseph Varghese *et al.*
determined the 3-D structure of the enzyme by X-ray crystallography (24). The enzyme consists of 605 amino acid residues and has two domains. One domain, consisting of the first 357 residues, features an \((\alpha/\beta)_8\) TIM-barrel. Residues 374-559 constitute a six-stranded \(\beta\)-sandwich with three \(\alpha\) helices on either side. Varghese et al. observed a glucose molecule in a deep pocket at the interface of the two domains, suggesting that the interface of the domains serves as a pocket for substrate binding. Investigation of the bound glucose, revealed the active site of the enzyme. The C1 hydroxyl group of the bound glucose is situated near the acidic amino acid residues Asp285 and Glu491, which are believed to be involved in catalysis (24). The role of Asp285 as a nucleophile is conserved in the Ser-Asp-Trp (SDW) motif of family 3 glycosidases has been accepted via active-site affinity labeling, but has not been established by site-directed mutagenesis (25).

**Active Site Prediction in BglX**

The mechanism of family 1 \(\beta\)-glucosidases have been frequently studied, however, few studies have been done on family 3 \(\beta\)-glucosidases (25). Yaw-Kuen et al. have cloned and expressed a family 3 \(\beta\)-glucosidase from *Flavobacterium meningosepticum* to gain perspective on the mechanism and active site topology of enzymes from this family. Yaw-Kuen et al. proposed Asp247 as the necessary nucleophile for catalysis due to sequence alignment among other family 3 glycosidases. Although the \(\beta\)-glucosidase from *F. meningosepticum* does not contain the highly conserved SDW sequence, the analogous Thr-Asp-Tyr (TDY) sequence is
characteristically similar (25). For instance, serine is often substituted for threonine because of the similarities in polarity and size. Furthermore, tryptophan and tyrosine are both aromatic and non-polar amino acids.

Yaw-Kuen et al. replaced Asp247 with asparagine, glutamate, or glycine. The mutant enzymes were purified and kinetic assays were performed with 2, 4-dinitrophenyl β-D-glucopyranoside. The mutant enzymes showed similar $K_M$ values while the catalytic efficiencies for D247N and D247G were significantly decreased, by factors of $2 \times 10^5$ and $3 \times 10^4$ respectively. The D247E mutant enzyme maintained activity resembling the wild-type enzyme since the aspartate was replaced with glutamate, another negatively charged residue. These results indicated that Asp247 is essential in the enzymatic reaction catalyzed by the β-glucosidase. The mechanism for family 3 β-glucosidases has also been proposed and is illustrated in Figure 1-1.
In Figure 1-1, the carboxylate group of the aspartate acts as a nucleophile by attacking the anomeric carbon of the sugar in the glucosylation step. A nearby residue acts as an acid by protonating the glycosidic oxygen to form a glucosyl-enzyme intermediate. The same residue then acts as a base to deprotonate a water molecule. The resulting hydroxide ion hydrolyzes the glycosyl-enzyme intermediate in the deglucosylation step.

Based on the results of Varghese et al. and Yaw-Kuan et al., predictions on the active site of BglX can be formulated. According to Table 1-1, BglX has a partial alignment with the conserved SDW sequence in other family 3 glycosidases. Since
histidine is also aromatic and is polar, this amino acid may be an aid in protein active or binding sites. Thus, the proposed active site residue for BglX is Asp287. In addition, the predicted structure of BglX, depicted as Figure 1-2, shows Asp287 in a shallow pocket, resembling the active site pocket in barley β-D-glucan exohydrolase ExoI (Figure 1-3).

Table 1-1: Conserved sequence in family 3 glycosidases

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
<th>GenBank accession no.</th>
<th>Ref.</th>
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<tr>
<td>Barley Barley</td>
<td>K279GFVISDWEGL289</td>
<td>AF102868</td>
<td>24</td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em></td>
<td>D216GVVMDSWFGS226</td>
<td>M59852</td>
<td>26</td>
</tr>
<tr>
<td><em>Kluyveromyces fragilis</em></td>
<td>D219GMLMSDWFGT229</td>
<td>X05918</td>
<td>27</td>
</tr>
<tr>
<td><em>E. coli</em> E. coli</td>
<td>K271GITVSDHGA1291</td>
<td>AAC75193.1</td>
<td>17</td>
</tr>
</tbody>
</table>

**Figure 1-2:** Predicted 3-D structure and active site residue of BglX. (Acquired from Ref. 23)

**Figure 1-3:** 3-D structure of barley β-D-glucan exohydrolase ExoI. (Acquired from Ref. 23)
Protein Overexpression System

The overexpression of recombinant proteins is a valuable technique in the life sciences that generates large amounts of protein for study. Various expression systems are available for use including *E. coli*, yeast, insect cells, mammalian cells, and cell-free systems. BL21(DE3) *E. coli* cells are the most commonly used host and are used in conjunction with vectors that are controlled by the T7 promoter, such as pET, for high-level protein expression.

Protein overexpression using BL21(DE3) cells with pET vectors utilizes the *lac* operon of *E. coli*. When lactose or a lactose analog such as isopropyl-β-D-thiogalactoside (IPTG) is present in the cell, it acts as an inducer of the *lac* operon (28). An inducer functions by displacing a repressor protein bound to an operator. It is then possible for RNA polymerase to bind to the promoter, ultimately resulting in gene expression.

BL21(DE3) cells are designed to produce T7 RNA polymerase to conduct selective, high-level expression of cloned genes (29). T7 RNA polymerase is under the control of the *lac* promoter and would therefore be produced upon induction with IPTG. IPTG is advantageous to use because it cannot be metabolized by *E. coli* so it retains a constant concentration (30). The gene encoding the protein of interest is cloned into the pET-20b(+) vector (illustrated in Figure 1-4) in which gene expression is controlled by the T7 promoter. When the pET-20b(+) vector is introduced into BL21(DE3) cells, T7 RNA polymerase specifically recognizes and binds to the T7 promoter and can transcribe DNA that has been cloned into the pET-20b(+) vector. After transcription, the RNA is
translated to produce the protein of interest. The vector also contains a gene encoding for ampicillin resistance. Consequently, ampicillin included in the culture medium acts as a selective method of cell growth.

Figure 1-4: Plasmid map of pET-20b(+). (Acquired from BVTech, Inc.).

**Mutagenesis**

Mutagenesis is the process in which the DNA sequence of an organism is altered, whether naturally or experimentally. Mutations can have advantageous, harmful, or neutral effects on the organism (31). Naturally occurring mutations can arise from errors in DNA replication, where nucleotide bases may be mispaired, inserted, or deleted.
Mutations can also occur from exposure to agents known as mutagens. Mutagens include exposure to ultraviolet (UV) radiation, interaction with chemicals such as reactive oxygen species, nitrous acid, sodium azide, etc. (32-34). Errors in DNA that are not repaired can be transferred from one cellular generation to the next. However, mutations offer genetic diversity and serve as the driving force of evolution. The experimental introduction of mutations is extensively used by researchers to investigate the function of genes, examine phenotypes of an organism, or to design instrumental proteins, in a process known as protein engineering (35). Random mutagenesis and site-directed mutagenesis are the primary techniques used for designing proteins (36).

Random mutagenesis is generally employed to improve or modify enzyme characteristics such as alkaline stability, thermal stability, and substrate specificity (37). Methods of random mutagenesis include cell exposure to UV radiation, chemical treatment, and the most broadly used, error-prone polymerase chain reaction (PCR) (38). Most applications of PCR are for the accurate amplification of DNA sequences. However, with modifications to the reaction mixture, PCR can be used to alter genetic information. Modifications may consist of an increased concentration of Taq DNA polymerase, the addition of Mn$^{2+}$ or Mg$^{2+}$, an increased concentration of deoxyribonucleotide triphosphates, and an increased polymerase extension time (38, 39). Under these conditions, the fidelity of Taq DNA polymerase decreases, leading to the introduction of random mutations caused by mistakes in base pairing (38). As a result, mutant libraries, consisting of various versions of the DNA sequence, can be created.
Figure 1-5: Schematic of random mutagenesis by error-prone PCR with a mutation denoted as “X”. (Ref. 38).

Figure 1-5 is a depiction of the design for error-prone PCR. Mutations that are introduced under atypical conditions increase in number with each successive cycle of replication. The PCR products obtained are cloned into an expression vector and transformed into competent cells in order to screen the mutant library for desired phenotypes.

In site-directed mutagenesis, a mutation is incorporated into the DNA sequence at a specific location due to insertions, deletions, or substitutions. Site-directed mutagenesis is valuable in studies concerning protein function, identification of enzyme active sites, and inhibitor design (40). PCR-based site-directed mutagenesis is the most common approach and can be accomplished efficiently through the use of commercial kits.
Figure 1-6: Overview of PCR-based site-directed mutagenesis with Quikchange II site-directed mutagenesis kit from Stratagene. (Adapted from QuikChange® II Site-Directed Mutagenesis Kit Instruction Manual).

Figure 1-6 illustrates the process of PCR-based site-directed mutagenesis using the Quikchange II kit from Stratagene. The procedure requires the design of primers containing the desired change, which otherwise are complementary to the template DNA. In the first step, the primers and template DNA are added to a reaction mixture and subjected to thermal cycling. Following thermal cycling, the reaction mixture now contains the parental DNA and newly synthesized mutant DNA. The restriction enzyme, Dpn I which is specific for methylated and hemimethylated DNA, is added to the reaction to digest the parental DNA (41). Therefore, it is important in using parental DNA that is purified from bacterial strains that methylate plasmid DNA. Since the mutant DNA was synthesized in vitro, the DNA is unmethylated and is selective with Dpn I treatment. The mutant DNA obtained after digestion is transformed into competent cells for nick repair.
Upon the confirmation of a successful mutation via DNA sequencing, the desired product is applicable for analytical purposes.

**Statement of Purpose**

The objective of this project is to develop a purification procedure for BglX, characterize BglX, and identify the important amino acid residues in the active site of BglX. This enzyme is considered a suitable target for drug therapy as BglX is produced solely by bacteria. Inhibition of BglX would deprive *E. coli* of a source of energy, resulting in cell death. Identification of essential residues in the active site would help to elucidate the mechanism of the enzyme and could be used as targets for inhibition.

Purification of BglX will consist of protein precipitation with ammonium sulfate and chromatography techniques. The presence and purity of BglX in a sample can be verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Once BglX is purified to homogeneity, kinetic parameters can be determined. BglX activity will be analyzed using the model substrate *p*-nitrophenol-β-D-glucopyranoside (PNPG). The purification of BglX will be performed using a similar method to Yang et al. which consisted of cell resuspension in 10 mM Tris buffer, pH 7.0 containing 2 mM EDTA, desalting of the sample with a PD-10 column, and protein purification with a HiTrap Q column (17).

In addition, BglX mutants will be constructed for analysis. Mutagenesis will be carried out using a PCR-based method. Upon successful results, the plasmids will be transformed into BL21(DE3) competent *E. coli* cells. Colonies yielding high protein
expression will be used for protein purification to determine the activity of the mutant enzyme. Based upon the studies of Varghese et al. and Yaw-Kuan et al., mutation of the amino acid residue Asp287 of BglX to asparagine will result in the decrease of BglX activity (24, 25). Protein purification and kinetic assays will be performed under the same conditions as the recombinant wild type BglX.
Chapter 2: Materials and Methods

Materials

LB broth, SOB broth, bacteriological agar, IPTG, Acryl/Bis™ 29:1, 40% solution, glycerol 99%, streptomycin sulfate, ampicillin sodium salt, ammonium sulfate, tris hydrochloride, EDTA, sodium acetate trihydrate, TEMED, MES free monohydrate, EZ-vision loading dye, and TG-SDS 10x liquid concentrate, sodium dodecyl sulfate, coomassie brilliant blue G-250, were purchased from Amresco (Solon, Ohio). DEAE-Cellulose, SP-Sephadex, CM-Sephadex, and Q Sepharose Fast Flow were obtained from GE Healthcare (Pittsburgh, Pennsylvania). Sephacryl S-200 was obtained from Sigma (St. Louis, Missouri). Toyopearl Butyl-650m was from Tosch Biosciences (San Francisco, California). Protein molecular weight markers were purchased from Amresco and Bio-Rad (Hercules, California). SDS-PAGE Mini Protean II apparatus was purchased from Bio-Rad. Go Taq Green Master Mix was purchased from Promega (Madison, Wisconsin). MUG and PNPG were purchased from Acros Organics (Pittsburgh, Pennsylvania). A Hewlett Packard (Agilent) 8453 Diode array spectrophotometer was used for kinetic analysis. Amicon ultra centrifugal filters: Ultracel-30k cellulose and Amicon ultrafiltration cell model 8050 were used for protein concentration. QIAprep Spin Miniprep Kit was purchased from Qiagen. QuikChange II Site-Directed Mutagenesis Kit was purchased from Agilent (Santa Clara, California). Primers for mutagenesis were ordered from Invitrogen (Carlsbad, California).


Methods

Production of BglX

The cloning of the bglX gene and transformation of the gene into BL21(DE3) competent E. coli cells were completed by former students, Devin Kelly and Carol Ann Pitcairn. BL21(DE3) cells containing the pET20-bglX were used to inoculate 100 mL of LB media with 100 mg/mL of ampicillin. The culture was grown overnight in an incubator at 37 °C with shaking at 150 rpm. 72 mL of overnight culture was diluted into 7.2 L of LB media with 100 mg/mL of ampicillin. The diluted culture was grown at 37 °C with shaking at 200 rpm until an OD600 of ~0.6 was reached, indicating the exponential phase of cell growth. Once the cells reached the exponential phase, the culture was induced with 1 M isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 0.3 mM IPTG. The culture was grown overnight at 37 °C with shaking at 200 rpm. The cells were harvested by centrifugation at 6,000 × g at 4 °C for 10 minutes. The cell pellets were stored at -20 °C.

Initial Protein Purification with Streptomycin Sulfate Treatment and Precipitation by Ammonium Sulfate

All initial purification steps were performed on ice with stirring. Centrifugation of solutions was performed at 11,000 × g.

Cell pellets were resuspended in 10 mM Tris buffer, pH 7.0 with 2 mM EDTA and allowed to stir for one hour to break the outer membrane of the cells. The mixture
was centrifuged for 20 minutes. The lysate stirred on ice while the pellets were dissolved in 10 mM Tris buffer, pH 7.0 with 2 mM EDTA. The redissolved pellet was sonicated for 30 seconds and cooled for 1 minute for a total of six cycles to lyse remaining cells. The lysates were combined and centrifuged once again. The supernatant was treated with 1% (w/v) of streptomycin, added dropwise as a solution of streptomycin sulfate in water. The solution was stirred for 10 minutes and was centrifuged for 20 minutes. Ammonium sulfate was added to the supernatant to 50% saturation. The solution was stirred for 20 minutes and was centrifuged for 30 minutes. The pellet was stored at 4 °C. Additional ammonium sulfate was added to the supernatant to 75% saturation. The mixture stirred for 10 minutes and was centrifuged for 25 minutes. The pellet was stored at 4 °C. 20 μL samples were collected after each centrifugation and trace amounts of the pellets resulting from ammonium sulfate precipitation were dissolved in 10 mM Tris buffer, pH 7.0 containing 2 mM EDTA. The samples were mixed with 2× SDS loading buffer in a 1:1 ratio, heated at 95 °C for 5 minutes, and analyzed using SDS-PAGE. Purification of BglX was continued based on the results obtained from SDS-PAGE.

Protein Purification with Column Chromatography

Different chromatographic techniques were utilized for protein purification in order to establish the best method for purifying BglX. The chromatographic techniques used included ion exchange, gel filtration, and hydrophobic interaction chromatography.
Ion Exchange Chromatography

Ion exchange chromatography was the first technique used in several sequences of protein purification with column chromatography. Four ion exchange columns were utilized: DEAE-Cellulose, SP-Sephadex, CM-Sephadex, and Q Sepharose FF. Following the elution of proteins from each column, the fractions were measured for their absorbances at 280 nm and pooled according to high absorbance readings and a band of 81 kDa on SDS-PAGE. All protein solutions were stored at 4 °C.

A DEAE-Cellulose column (3 × 11.2 cm) was equilibrated with 500 mL of 20 mM Tris buffer, pH 7.0. Pellets from ammonium sulfate precipitation were resuspended in 20 mM Tris buffer, pH 7.0 and dialyzed against 2 L of the same buffer overnight at 4 °C. The dialyzed sample was centrifuged at 10,000 × g for 5 minutes to remove visible precipitate. The protein sample was loaded onto the column and the flow through was collected. The column was washed with 500 mL of 20 mM Tris buffer, pH 7.0 to remove unbound proteins. The proteins bound to the column were eluted with a 200 mL linear gradient of 0-400 mM NaCl in 20 mM Tris buffer, pH 7.0 and 4.5 mL fractions were collected.

An SP-Sephadex column (1.9 × 10.7 cm) was equilibrated with 200 mL of 20 mM MES buffer, pH 6.0. Protein solution was dialyzed against 2 L of 20 mM MES buffer, pH 6.0 overnight at 4 °C. The dialyzed sample was centrifuged at 10,000 × g for 10 minutes. The sample was loaded onto the column and the flow through was collected. The column was washed with 200 mL of 20 mM MES buffer, pH 6.0. The proteins...
bound to the column were eluted with a 100 mL gradient of 0-400 mM NaCl in 20 mM MES buffer, pH 6.0 and 4.5 mL fractions were collected.

A CM-Sephadex column (1.3 × 14.5 cm) was equilibrated with 100 mL of 50 mM sodium acetate buffer, pH 6.0. Protein solution was dialyzed against 1 L of 50 mM sodium acetate buffer, pH 6.0 overnight at 4 °C. The dialyzed sample was loaded onto the column and the flow through was collected. The column was washed with 100 mL of 50 mM sodium acetate buffer, pH 6.0. The bound proteins were eluted with 100 mL gradient of 0-400 mM NaCl in 50 mM sodium acetate buffer, pH 6.0 and 2 mL fractions were collected.

A Q Sepharose FF column (3 × 11 cm) was equilibrated with 500 mL of 20 mM Tris buffer, pH 7.4. Protein solution was dialyzed against 2 L of the same buffer overnight at 4 °C. The dialyzed sample was centrifuged at 10,000 × g for 5 minutes. The sample was loaded onto the column and the flow through was collected. The column was washed with 500 mL of 20 mM Tris buffer, pH 7.4. The proteins bound to the column were eluted with a 400 mL linear gradient of 0-400 mM NaCl in 20 mM Tris, pH 7.4 and 4.5 mL fractions were collected.

**Gel Filtration Chromatography**

A Sephacryl S-200 column (1.2 × 41 cm) was equilibrated with 50 mL of 50 mM Tris buffer, pH 7.0 containing 150 mM NaCl. Protein solutions were concentrated to 1 mL using an Amicon ultra centrifugal filter (molecular weight cutoff of 30 kD) at
4,000 × g. 500 μL of sample was loaded onto the column. Proteins were eluted with 50 mL of 50 mM Tris buffer, pH 7.0 with 150 mM NaCl and 1.5 mL fractions were collected.

**Hydrophobic Interaction Chromatography**

Pooled fractions from Q Sepharose FF were concentrated to 1.5 mL with an Amicon ultra centrifugal filter (molecular weight cutoff of 30 kD) at 4,000 × g. The concentrated protein solution was dialyzed against 1 L of 50 mM Tris buffer, pH 7.0 overnight at 4 °C. Ammonium sulfate was added to the dialyzed protein solution to a final concentration of 1.2 M. A Toyopearl Butyl-650M column (2.8 × 3.4 cm) was equilibrated with 150 mL of 50 mM Tris buffer, pH 7.0 containing 1.2 M ammonium sulfate. The sample was loaded onto the column and the flow through was collected. The column was washed with 125 mL of 50 mM Tris buffer, pH 7.0 with 1.2 M ammonium sulfate. The proteins were eluted with a 100 mL gradient of 1.2-0 M ammonium sulfate in 50 mM Tris buffer, pH 7.0 and approximately 1 mL fractions were collected.

**Zymography**

Native-PAGE was performed in a 10% polyacrylamide gel. The concentrated enzyme purified from a Q Sepharose column was mixed in a 1:1 ratio with sample buffer and the sample was loaded into two lanes of the same gel. After native electrophoresis, the gel was cut in half. One half was stained with coomassie brilliant blue while the other
was used for zymography. The part of the gel to be used for zymography was cut into lanes containing sample and subsequently submerged in 100 mM sodium acetate buffer, pH 5.0 for 15 minutes. The buffer was replaced with the 10 mM solution of 4-methylumbelliferyl-β-D-glucopyranoside (MUG) in 100 mM sodium acetate buffer, pH 5.0 and the gel was incubated at 37 °C for 30 minutes. The zymogram was analyzed under UV light for detection of enzyme activity. Enzyme activity was indicated by a fluorescent spot due to the release of methylumbelliferone. The gel stained with coomassie brilliant blue was placed alongside the zymogram for protein band position comparison.

Protein Concentration Determination

The concentration of BglX was determined by the Bradford protein assay and bovine serum albumin (BSA) was used as the standard. Four standard solutions were prepared with Bradford reagent, water, and BSA at varied concentrations (10-70 μg). The concentrated protein solution after purification was diluted five times in Bradford reagent and water. The solutions were mixed and incubated at room temperature for 10 minutes. The absorbance of each solution was recorded at 595 nm. A standard curve was constructed from which the concentration of BglX was calculated.

Analysis of BglX Kinetics

p-Nitrophenol-β-D-glucopyranoside (PNPG) was used as the model substrate for determining BglX activity. Enzyme activity was monitored by the change of absorbance
at 405 nm at room temperature (25 °C) due to the production of the p-nitrophenolate anion. The reaction assay consisted of 800 μL 100 mM sodium acetate buffer, pH 5.0, 100 μL PNPG, and 100 μL of purified enzyme (82 μM). Final concentrations of PNPG varied from 0.01 to 2 mM. Assays were duplicated and the results were averaged for each concentration of PNPG used. The parameters $V_{\text{max}}$, $K_M$, and $k_{\text{cat}}$ were calculated using the Michaelis-Menten plot.

Construction of BglX Mutants

pET20-\textit{bglX} plasmids from BL21(DE3) cells were isolated using the QIAprep spin miniprep kit following the protocol described by the manufacturer. The concentration of the purified plasmid was determined using the absorbance at 260 nm and Beer’s Law. Purified pET20-\textit{bglX} (58 ng/μL) was used as the template for mutagenesis. The primers used to generate the mutant D287N consisted of the forward primer 1: 5’-GGCATCACC\underline{T}TTCC\underline{A}TCACGGTGCAATCAAAG-3’ and the reverse primer 1: 5’-CTTTGATTGCACCC\underline{G}TGGATTGGAACGGTGATGCC-3’. The primers used to create the mutant D287G included the forward primer 2: 5’-GGCATCACC\underline{G}T\underline{T}TCC\underline{G}TCACGGTGCAATCAAAG-3’ and the reverse primer 2: 5’-CTTTGATTGCACCGTG\underline{A}CCGGAAACGGTGATGCC-3’ (underlining indicates the location of the mutation).
**Mutagenesis of bglX**

Mutations in the *bglX* gene were introduced using the QuikChange II site-directed mutagenesis kit. The 50 μL mutagenesis reaction contained 5 μL 10× reaction buffer, 1 μL pET20-*bglX* dsDNA template (58 ng), 1 μL forward primer (100 ng), 1 μL reverse primer (100 ng), 1 μL dNTP mix, 44 μL sterile water, and 1 μL *PfuUltra* HF DNA polymerase (2.5 U/μL). The cycling parameters consisted of an initial denaturing temperature at 95 °C for 30 seconds proceeded by 12 cycles of a denaturing temperature of 95 °C for 30 seconds, an annealing temperature of 55 °C for 1 minute, and a primer extension temperature of 68 °C for 6 minutes. The reaction was placed on ice for 2 minutes following thermal cycling. A control reaction was prepared for digestion with *Dpn* I restriction enzyme. The control reaction contained 5 μL 10× reaction buffer, 1 μL pET20-*bglX* dsDNA template (58 ng), and 44 μL sterile water. 1 μL of *Dpn* I was added to both the mutagenesis and control reactions. The reactions were gently mixed, centrifuged for 1 minute, and incubated at 37 °C for 1 hour.

Following *Dpn* I digestion, DNA from the mutagenesis and control reactions were used to transform XL1-Blue supercompetent cells. XL1-Blue supercompetent cells thawed on ice and 50 μL were aliquotted to prechilled sterile culture tubes. 1 μL of DNA from the mutagenesis and control reactions was transferred to separate aliquots of the supercompetent cells. The reactions were gently swirled and incubated on ice for 30 minutes. The cells were heat shocked for 45 seconds at 42 °C and placed on ice for 2 minutes. 500 μL of SOC media preheated to 42 °C were added to each reaction. The tubes incubated at 37 °C for 1 hour with shaking at 200 rpm. 150 μL of each culture was
spread onto separate LB/agar plates containing 100 mg/mL of ampicillin and incubated overnight at 37 °C.

Plasmid Isolation and DNA Sequencing of Mutants

As a result of successful transformation, five colonies were selected from the mutagenesis plate. Each colony was inoculated into 10 mL of LB media with 100 mg/mL of ampicillin and incubated overnight at 37 °C with shaking at 150 rpm. Plasmids from the overnight cultures were purified using the QIAprep spin miniprep kit. The concentrations of the purified plasmids were calculated using the absorbance at 260 nm and Beer’s Law. The purified plasmids were sequenced at the DNA Sequencing laboratory at Vanderbilt University, Nashville, TN.

Transformation of Competent Cells and Preparation of Glycerol Stock of Mutant Cells

Plasmids that were successfully mutated were used to transform BL21(DE3) competent cells. Six colonies of transformants from BL21(DE3) plates were selected. Each colony was inoculated into 3 mL aliquots of LB with 100 mg/mL of ampicillin and incubated overnight at 37 °C with shaking at 150 rpm. 50 μL from each overnight culture were diluted into 5 mL of LB media with 100 mg/mL of ampicillin. The diluted cultures were grown at 37 °C with shaking at 200 rpm to an OD₆₀₀ of ~0.6. 500 μL of each culture were aliquotted. The remaining cells were induced with 1 M IPTG for a final concentration of 1 mM and grown for 3 hours. 500 μL samples before and after IPTG induction were collected and centrifuged at 10,000 rpm for 1 minute. Cell pellets
obtained after centrifugation were used for SDS-PAGE analysis to determine the effect of IPTG induction on protein expression. Cultures that exhibited better expression of mutant BglX after SDS-PAGE analysis were aliquotted in a 1:1 ratio with 75% sterile glycerol in cryogenic vials, flash frozen in dry ice and stored at -80 °C.

*Production of BglX Mutants*

BL21(DE3) cells containing the mutated *bglX* gene were grown using the same method as the cloned wild type *bglX* gene.

*Initial Purification of BglX Mutants*

Initial purification of BglX mutants were performed similarly to the recombinant wild type BglX.

*Purification of BglX Mutants with Column Chromatography*

The BglX mutants were purified using a Q Sepharose FF column as previously described for the wild type BglX. Enzyme activity was monitored using the procedure stated above.
Chapter 3: Results

*Initial Protein Purification with Streptomycin Sulfate Treatment and Precipitation by Ammonium Sulfate*

Initial protein purification of BglX involved cell lysis, streptomycin treatment, and precipitation by ammonium sulfate. Samples at each step of purification were analyzed by SDS-PAGE. BL21(DE3) *E. coli* cells containing the pET20-bglX plasmid were grown with the addition of IPTG to overexpress BglX. Harvested cells were resuspended in 10 mM Tris buffer, pH 7.0 containing 2 mM EDTA, leading to the rupturing of the outer membrane. The mixture was centrifuged; however, the band for BglX, near 81 kDa was absent in the supernatant (Figure 3-1, Lane 1). The pellet (not shown) was resuspended in buffer and the cells were lysed by sonication. The supernatant after sonication (Lane 2) was treated with 1% streptomycin sulfate. Streptomycin sulfate was used to precipitate DNA, leaving BglX to remain in solution (Lane 4). Lane 7 shows that most proteins precipitated at 50% saturation with ammonium sulfate. Proteins remaining in the supernatant (Lane 6) were precipitated at 75% ammonium sulfate saturation. The band for BglX is shown in Lanes 7 and 9. The brightness and the thickness of a band correlate with the amount of protein loaded. The pellets obtained after ammonium sulfate precipitation were used for further purification.
Protein Purification with Column Chromatography

Various chromatographic approaches were undertaken to attain the most efficient purification of BglX. This included the use of a combination of chromatography resins, buffers with various pH values, and elution of proteins with different concentration ranges of NaCl.
Purification of BglX with DEAE-Cellulose and SP-Sephadex Ion Exchange and Sephacryl S-200 Gel Filtration Chromatographies

The pellets obtained at 50% ammonium sulfate saturation were resuspended in 20 mM Tris buffer, pH 8.2 and dialyzed against the same buffer overnight at 4 °C. The dialyzed sample was loaded onto a DEAE-Cellulose ion exchange column. The proteins were eluted with a 0-400 mM gradient of NaCl in 20 mM Tris buffer, pH 8.2. Figure 3-2 shows a plot of the absorbance values measured at 280 nm for each fraction collected. Fractions with a high absorbance were tested for enzyme activity. BglX catalyzes the hydrolysis of the substrate PNPG to form glucose and the p-nitrophenolate anion (Figure 3-3). Enzyme activity was analyzed following an increase in absorbance at 405 nm due to p-nitrophenolate anion production. Fractions 12-15 showed the highest enzymatic activity.

**Figure 3-2:** Graph of absorbance at 280 nm and activity of fractions from DEAE-Cellulose chromatography.
Figure 3-3: Hydrolysis of PNPG catalyzed by BglX.

Figure 3-4 shows the SDS-PAGE analysis of fractions collected from the DEAE-Cellulose column. BglX can be seen in Lanes 6-9 in small quantities. Purification of BglX with DEAE-Cellulose chromatography was ineffective.

Figure 3-4: BglX purification with DEAE-Cellulose ion exchange chromatography. Lane 1: protein molecular weight marker, a: 116 kDa, b: 97 kDa, c: 66 kDa; Lane 2: load; Lane 3: flow through; Lane 4: wash; Lane 5: fraction 7; Lane 6: fraction 9; Lane 7: fraction 11; Lane 8: fraction 13; Lane 9: fraction 15; Lane 10: fraction 18; Lane 11: 20; Lane 12: fraction 22; Lane 13: fraction 26; Lane 14: fraction 28.
Purification of fractions with high enzyme activity from the DEAE-Cellulose column was continued with SP-Sephadex ion exchange chromatography. Those fractions were pooled and dialyzed against 20 mM MES buffer, pH 6.0 overnight at 4 °C. The dialyzed sample was loaded onto an SP-Sephadex column. The SDS-PAGE analysis of samples from the column is shown in Figure 3-5. The flow through (Lane 3) contains BglX. Several distinct bands are visible in the flow through; however, BglX is not the dominant protein in this sample.

**Figure 3-5**: BglX purification with SP-Sephadex ion exchange chromatography. Lane 1: protein molecular weight marker, a: 97 kDa, b: 66 kDa; Lane 2: load; Lane 3: flow through; Lane 4: wash; Lane 5: fraction 7; Lane 6: fraction 12; Lane 7: fraction 15; Lane 8: fraction 20; Lane 9: fraction 23; Lane 10: fraction 40.

The flow through collected from the SP-Sephadex column was tested for BglX activity with PNPG. Figure 3-6 represents a time trace of p-nitrophenolate anion formation at 405 nm. The rate of reaction is given by the initial slope of the curve and confirms enzyme activity in the flow through.
The flow through from the SP-Sephadex column was concentrated and applied to a Sephacryl S-200 gel filtration column. Proteins were eluted with 50 mM Tris buffer, pH 7.0 containing 150 mM NaCl. The band for BglX is very faint but visible in Lanes 8-14 (Figure 3-7). These fractions were combined and concentrated for examining enzyme activity.
Figure 3-7: BglX purification with Sephacryl S-200 gel filtration chromatography. Lane 1: protein molecular weight marker, a: 97 kDa, b: 66 kDa; Lane 2: load; Lane 3: fraction 8; Lane 4: fraction 9; Lane 5: fraction 10; Lane 6: fraction 11; Lane 7: fraction 12; Lane 8: fraction 13; Lane 9: fraction 14; Lane 10: fraction 15; Lane 11: fraction 16; Lane 12: fraction 17; Lane 13: fraction 18; Lane 14: fraction 19; Lane 15: fraction 20.

Figure 3-8 demonstrates the analysis of enzyme activity in the concentrated sample. No activity for BglX was observed in this sample.

Figure 3-8: Spectrophotometric assay of BglX sample from Sephacryl S-200 gel filtration chromatography.
Purification with DEAE-Cellulose and CM-Sephadex Ion Exchange Chromatographies

Protein elution from a DEAE-Cellulose column using a wider concentration of NaCl was carried out in order to improve protein separation. Proteins that precipitated with 50% saturation of ammonium sulfate were prepared for application onto a DEAE-Cellulose column as described previously. Proteins were eluted with a gradient of 0-500 mM NaCl in 20 mM Tris buffer, pH 8.2. BglX is present in low quantities in Lane 4 and trace amounts in Lane 5, according to Figure 3-9. BglX is dominated by several proteins with lower molecular weights.

**Figure 3-9:** BglX purification with DEAE-Cellulose chromatography with (0-500 mM) NaCl gradient. Lane 1: load; Lane 2: flow through; Lane 3: wash; Lane 4: fraction 17; Lane 5: 19; Lane 6: 20; Lane 7: protein molecular weight marker, a: 97 kDa, b: 66 kDa; Lane 8: fraction 21; Lane 9: fraction 22; Lane 10: fraction 23; Lane 11: fraction 24; Lane 12: fraction 25; Lane 13: fraction 27.

Fractions 15-18 collected from the DEAE-Cellulose column were concentrated and assayed for BglX activity using PNPG. Glycosidase activity was observed in the concentrated sample (Figure 3-10).
The concentrated sample from the DEAE-Cellulose column was used for further purification with CM-Sephadex ion exchange chromatography. The SDS-PAGE gel (Figure 3-11) after purification reveals a very faint band for BglX in the flow through (Lane 2). In comparison of the flow through with the sample that was loaded onto the column (Lane 1), the band for BglX is also weak. Purification of BglX with CM-Sephadex was unsuccessful. The flow through collected from the CM-Sephadex column was concentrated and BglX activity was analyzed. The kinetic assay with PNPG did not show any enzyme activity.
Purification with Q Sepharose FF Ion Exchange and Toyopearl Butyl-650M Hydrophobic Interaction Chromatographies

Purification of BglX from the pellets obtained with 50% saturation of ammonium sulfate was performed with Q Sepharose FF chromatography. The pellets were resuspended in 20 mM Tris buffer, pH 8.2 and dialyzed against the same buffer overnight at 4 °C. The dialyzed sample was loaded onto a Q Sepharose FF column. The proteins bound to the column were eluted with a gradient of 0-400 mM NaCl in 20 mM Tris buffer, pH 8.2. The band for BglX is visible in Lanes 4-6 of Figure 3-12. Lane 5 contains the largest amount of BglX. Fractions 9-13 collected from the Q Sepharose FF
column were combined and concentrated. The concentrated sample failed to show BglX activity.

Figure 3-12: BglX purification with Q Sepharose FF chromatography. Lane 1: load; Lane 2: flow through; Lane 3: wash; Lane 4: fraction 9; Lane 5: fraction 13; Lane 6: fraction 17; Lane 7: fraction 21; Lane 8: protein molecular weight marker, a: 97 kDa, b: 66 kDa; Lane 9: fraction 25; Lane 10: fraction 29; Lane 11: fraction 33; Lane 12: fraction 37; Lane 13: fraction 41; Lane 14: fraction 45.

To prevent the loss of activity, buffer with a different pH was used. The additional pellets from 50% ammonium sulfate saturation were resuspended and dialyzed in 20 mM Tris buffer, pH 7.4. The sample was loaded onto a Q Sepharose FF column and proteins were eluted with a gradient of 0-400 mM NaCl in 20 mM Tris buffer, pH 7.4. Figure 3-13 shows the fractions collected from the Q Sepharose FF column analyzed by SDS-PAGE. Lane 4 consists of a nearly pure fraction of BglX, which is shown as a bright band near 81 kDa.
**Figure 3-13**: BglX purification with Q Sepharose FF ion exchange chromatography. Lane 1: load; Lane 2: fraction 8; Lane 3: fraction 16; Lane 4: fraction 24; Lane 5: protein molecular weight marker; Lane 6: fraction 32; Lane 7: fraction 40; Lane 8: fraction 48; Lane 9: fraction 56; Lane 10: fraction 64; Lane 11: fraction 72.

Figure 3-14 is a graph of the absorbance at 280 nm of the fractions collected from the Q Sepharose FF column. Fractions showing glycosidase activity are consistent with the fractions containing BglX in Figure 3-13.
In order to recover BglX that precipitated at 75% ammonium sulfate saturation, the pellets were also applied onto the Q Sepharose FF column and eluted with a gradient of 0-400 mM NaCl in 20 mM Tris buffer, pH 7.4. Figure 3-15 shows the SDS-PAGE analysis of fractions collected from the column. BglX can be seen in Lanes 4, 5, 7, and 8 as a band near 81 kDa. The largest amount of BglX is present in Lanes 4 and 5 with fewer proteins as compared to fractions containing proteins that elute at higher salt concentrations.
Fractions containing BglX obtained from a Q Sepharose FF ion exchange column underwent further purification with hydrophobic interaction chromatography. The fractions were concentrated and dialyzed against 50 mM Tris buffer, pH 7.0 overnight. 1.2 M ammonium sulfate was added to the sample and loaded onto a Toyopearl Butyl-650M column. Protein elution was achieved with a gradient of 1.2-0 M ammonium sulfate in 50 mM Tris buffer, pH 7.0. Figure 3-16 represents the SDS-PAGE analysis of BglX after using the hydrophobic interaction column. BglX is the major protein in the sample loaded onto the column (Lane 1). Lanes 13-15 show highly purified BglX.
Fractions 57-70, containing BglX, were pooled and concentrated. The activity of the concentrated sample was analyzed. Figure 3-17 illustrates an increase in $p$-nitrophenolate anion formation as detected by the change of absorbance at 405 nm.
Figure 3-17: Spectrophotometric assay of BglX sample from Toyopearl Butyl-650M hydrophobic interaction chromatography.

Zymography

A zymographic assay was performed with the substrate 4-methylumbelliferyl-β-D-glucopyranoside (MUG) to analyze BglX activity. Zymography is a technique that involves electrophoretic separation of proteins under non-denaturing conditions (native-PAGE) proceeded by the reaction with substrate within the gel. Concentrated BglX (82 μM) from purification with Q Sepharose FF was loaded into two lanes of the same gel for native-PAGE. Following electrophoresis, the gel fragment containing BglX (Figure 3-18, Lane 1) was submerged in a 10 mM solution of MUG in 100 mM sodium acetate buffer, pH 5.0 and incubated at 37 °C for 30 minutes. Hydrolysis of MUG by BglX produces methylumbelliferone, which is detectable under UV light and is demonstrated in Figure 3-18. The fluorescent band present in Lane 1 indicates enzymatic activity. Lanes 2 and 3 were stained with coomassie brilliant blue dye and destained afterward. BglX
was identified as the darkest high molecular weight protein in Lane 2. The alignment of the fluorescent band in Lane 1 and BglX in Lane 2 confirms the cleavage of MUG by BglX.

![Zymogram of BglX](image)

**Figure 3-18**: Zymogram of BglX. Lane 1: Gel fragment containing BglX incubated in 4-methylumbelliferyl-β-D-glucopyranoside solution, Lane 2: gel fragment containing BglX stained with coomassie brilliant blue dye, Lane 3: protein molecular weight marker, a: 97 kDa, b: 66 kDa.

*Protein Concentration Determination*

The concentration of purified BglX protein was determined by the Bradford assay using BSA as a standard. The absorbance of the samples with known concentrations of BSA was measured at 595 nm and then plotted against the amount of BSA (Figure 3-19). A calibration curve was generated from the data points (Table 3-1), from which an equation and the R² value were obtained. The amount of purified BglX was determined using the equation from Figure 3-19. The concentration of BglX...
corresponds to 23 μM after calculating in the volume of protein added to the sample and the molecular weight of the protein.

![Bradford Assay Standard Curve](image)

**Figure 3-19**: Standard curve for determining protein concentration using the Bradford assay.

**Table 3-1**: Amount of BSA and absorbance of samples at 595 nm

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount of BSA (μg)</th>
<th>Absorbance at 595 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>0.11183</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>0.24918</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>0.38576</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>0.49976</td>
</tr>
</tbody>
</table>

**Analysis of BglX Kinetics**

BglX acquired after purification with Q Sepharose FF and Toyopearl Butyl-650M columns was used for kinetic analysis. Concentrations of the substrate PNPG were varied between 0.01-2 mM. To analyze enzyme activity, the production of the
The $p$-nitrophenolate anion was followed as a change in absorbance at 405 nm. For each concentration of substrate, the assay was repeated twice and the rates of the reaction were averaged. The initial velocity was calculated using the change of absorbance per second obtained from each assay and the extinction coefficient of the $p$-nitrophenolate anion $0.2181 \text{ mM}^{-1}\text{cm}^{-1}$ (determined by former YSU student, Erin Schuler). A Michaelis-Menten plot showing the dependence of the initial velocity of the enzyme against substrate concentration was generated (Figure 3-20). The best fit curve was constructed to include five data points. From Figure 3-20, the maximum velocity of the reaction ($V_{\text{max}}$) and the substrate concentration at half of $V_{\text{max}}$, known as the Michaelis constant ($K_M$), were determined.

![Average Reaction Rate vs [PNPG]](image)

**Figure 3-20**: Michaelis-Menten plot of BglX.
Enzymatic characteristics of BglX are listed in Table 3-2. $k_{\text{cat}}$ is a number of substrate molecules that are converted to product by the enzyme per of unit time. $k_{\text{cat}}$ was determined to be $1.19 \text{ s}^{-1}$. The catalytic efficiency ($k_{\text{cat}}/K_M$) of BglX was calculated to be $3.4 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$.

**Table 3-2: Kinetic parameters of BglX**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$V_{\text{max}}$</th>
<th>$K_M$</th>
<th>$k_{\text{cat}}$</th>
<th>$k_{\text{cat}}/K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BglX</td>
<td>0.275 μM/s</td>
<td>0.35 mM</td>
<td>1.19 s$^{-1}$</td>
<td>$3.4 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$</td>
</tr>
</tbody>
</table>

(23 μM)

*Mutagenesis of bglX*

Site-directed mutagenesis of wild type BglX was performed to determine essential residues involved in catalysis. BL21(DE3) cells containing the pET20-\textit{bglX} plasmid were grown overnight at 37 °C. The plasmid was purified from the overnight culture and used as the template for mutagenesis. Primers used to generate the BglX mutants were designed to modify the amino acid residue at position 287 of the wild type BglX protein. The amino acid at position 287 is aspartate (single letter code D) and is coded by the codon GAT in wild type \textit{bglX}. The primer used to generate the BglX D287N mutant was designed to alter the codon GAT to AAT, which codes for the amino acid asparagine (single letter code N). The primer used to generate the BglX D287G mutant was designed to modify GAT to GGT, which codes for the amino acid glycine (single letter code G).
The mutations in the \textit{bglX} gene were introduced by PCR-based site-directed mutagenesis. The PCR reaction mixtures were treated with \textit{Dpn} I restriction enzyme to digest the template plasmid. Following digestion, the pET20\textit{-bglX} D287N and pET20\textit{-bglX} D287G plasmids were introduced into XL1-Blue supercompetent cells via a heat shock transformation. The cells were spread on an LB/ampicillin plates with one designated for cells transformed with pET20\textit{-bglX} D287N and another plate for cells transformed with pET20\textit{-bglX} D287G. Both plates were incubated at 37 °C overnight. Colony growth can be seen on the plate containing cells transformed with pET20\textit{-bglX} D287N (Figure 3-21) and on the plate containing cells transformed with pET20\textit{-bglX} D287G (Figure 3-22). No growth was observed on the control plates containing cells transformed with the digested pET20\textit{-bglX} plasmid.

\textbf{Figure 3-21}: Colonies of XL1-Blue cells transformed with pET20\textit{-bglX} D287N.

\textbf{Figure 3-22}: Colonies of XL1-Blue cells transformed with pET20\textit{-bglX} D287G.

The success of the mutations was verified by DNA sequencing. Several colonies were selected from each plate (Figures 3-21 and 3-22) and grown in LB media overnight.
at 37 °C. The plasmids from the overnight cultures were purified and sequenced. Figure 3-23 displays fragments of the DNA sequence chromatograms for wild type \( bglX \), \( bglX \) D287N, and \( bglX \) D287G. The replacement of GAT with AAT in the \( bglX \) D287N mutant is shown in the left column of Figure 3-23 while the right column shows the replacement of GAT with GGT in the \( bglX \) D287G mutant. Figure 3-23 confirms the success of the mutations in wild type \( bglX \).

\[
\begin{align*}
\text{D287N: GAT } & \rightarrow \text{ AAT} \\
\text{D287G: GAT } & \rightarrow \text{ GGT}
\end{align*}
\]

**Figure 3-23**: Fragments of DNA sequence chromatogram showing mutations.

*Expression of BglX Mutants*

Expression of the BglX mutants was examined for several colonies of BL21(DE3) cells containing the mutant plasmids. Figure 3-24 represents the SDS-PAGE analysis of protein expression after induction with IPTG. Lanes 10 and 11 show a better expression of BglX D287N with IPTG compared to the other mutants. Lane 10 shows that before
the addition of IPTG, the cells lack a band for BglX D287N near 81 kDa. However, a band near 81 kDa appears after the addition of IPTG (Lane 11).

**Figure 3-24**: Expression of BglX D287N with IPTG. Lanes 1-6, 8-9: BglX D287N mutants from BL21(DE3) cells without (-) and with (+) IPTG; Lane 7: protein molecular weight marker, a: 97 kDa, b: 66 kDa.

*Initial Purification of BglX D287N with Streptomycin Sulfate Treatment and Precipitation by Ammonium Sulfate*

Figure 3-25 shows the SDS-PAGE analysis of the initial purification of BglX D287N, which was done similarly to wild type BglX. BglX D287N can be seen in Lane 9 after 75% ammonium sulfate saturation, however it is not the dominant protein in the sample.
Figure 3-25: Initial purification of BglX D287N. Lane 1: crude supernatant; Lane 2: supernatant after sonication; Lane 3: pellet after sonication; Lane 4: supernatant after streptomycin treatment; Lane 5: protein molecular weight marker, a: 97 kDa, b: 66 kDa; Lane 6: supernatant after 50% ammonium sulfate saturation; Lane 7: pellet after 50% ammonium sulfate precipitation; Lane 8: supernatant after 75% ammonium sulfate saturation; Lane 9: pellet after 75% ammonium sulfate saturation.

The pellets obtained from 75% ammonium sulfate saturation were used for purification with Q Sepharose FF ion exchange chromatography. The pellets were resuspended in 20 mM Tris buffer, pH 7.4 and dialyzed against the same buffer overnight. The dialyzed sample was loaded onto a Q Sepharose FF column. Proteins were eluted with a gradient of 0-400 mM NaCl in 20 mM Tris buffer, pH 7.4. BglX D287N is shown in Figure 3-26 as a nearly pure and thick band close to 81 kDa in Lanes 10 and 11.
**Figure 3-26:** BglX D287N purification with Q Sepharose FF chromatography. Lane 1: load; Lane 2: flow through; Lane 3: wash; Lane 4: fraction 10; Lane 5: fraction 14; Lane 6: fraction 18; Lane 7: fraction 22; Lane 8: fraction 26; Lane 9: protein molecular weight marker, a: 97 kDa, b: 66 kDa; Lane 10: fraction 30; Lane 11: fraction 34; Lane 12: fraction 38; Lane 13: fraction 42; Lane 14: fraction 46; Lane 15: fraction 50.

**Analysis of BglX D287N Activity**

BglX D287N activity after purification with a Q Sepharose FF column was analyzed via a spectrophotometric assay using PNPG. The final concentration of enzyme (4.1 μM) was kept consistent with respect to wild type BglX. BglX D287N exhibits reduced enzyme activity (Figure 3-27) compared to the activity displayed by wild type BglX (Figure 3-28).
The activity of BglX D287N was also studied by a zymographic assay using MUG. Zymography was performed on wild type BglX and BglX D287N using 82 μM enzyme concentrations. Figure 3-29 demonstrates the ability of the enzymes to hydrolyze MUG. The product formed from cleavage of MUG is detectable under UV
light as a fluorescent band. The intensity of brightness displayed by a band indicates the activity level of the enzyme. Wild type BglX exhibits greater activity than BglX D287N.

Figure 3-29: Zymogram of wild type BglX and BglX D287N.
Chapter 4: Discussion

Glycosides are sugars that are attached to other carbohydrate or non-carbohydrate molecules by glycosidic bonds. Furthermore, polymerization of monosaccharides can produce complex carbohydrates. Glycosidases are a large class of enzymes that hydrolyze glycosidic bonds. Glycosidases are present in all living organisms and are involved in carbohydrate metabolism, protection of organisms from pathogens, and promoting infections.

β-glucosidases are a family of glycosidases that cleave β(1→4) linked glycosidic bonds formed by glucose. β-glucosidases are commonly studied for their applications in biotechnological processes consisting of the degradation of cellulose, the release of aromatic compounds with antioxidant activity from their glucosidic precursors, and their potential in producing renewable energy sources by converting cellobiose to glucose. Various bacteria and fungi produce β-glucosidases in order to degrade cellulose and other beta-linked saccharides as an energy source.

The bglX gene in E. coli encodes a β-glucosidase named BglX. BglX is located in the periplasm of the cell unlike other β-glucosidases produced by E. coli which are found in the cytoplasm. Few studies on the bglX gene and BglX protein have been published aside from cloning, DNA sequence and chromosomal mapping of bglX, and preliminary characterization of BglX (17). This enzyme is a suitable target for drug therapy as BglX is produced only by bacteria. Inhibition of BglX would prevent E. coli from obtaining energy, leading to cell death.
Purification of BglX

In order to study BglX for the purpose of developing inhibitors, purification of the protein must be achieved. *E. coli* cells containing the pET20-bglX plasmid were grown and protein expression was induced with IPTG. The purification method utilized by Yang et al. included resuspension of the harvested cells in 10 mM Tris buffer, pH 7.0 containing 2 mM EDTA, desalting with a PD-10 column, and a HiTrap Q column (17). Purification of BglX was performed similarly to Yang et al. although with slight deviations. Since BglX is a periplasmic protein, cell pellets were initially resuspended in hypotonic buffer to break the outer membrane. This would result in the release of the proteins located in the periplasm. However, it was shown that a significant amount of BglX was still remaining in the cells. In order to release the rest of the protein, sonication was implemented. BglX was purified following three steps: ammonium sulfate precipitation, Q Sepharose FF anion exchange and Toyopearl Butyl-650M hydrophobic interaction chromatographies. Initial purification with ammonium sulfate precipitation at 50% and 75% saturation showed an improvement in purifying BglX as opposed to precipitation at only 75%. Studies on β-glucosidases from other organisms have used ammonium sulfate precipitation without fractionation and have had success with protein purification (42, 43). In our studies, ammonium sulfate at 50% saturation precipitates most proteins, including a portion of BglX. The remainder of BglX precipitated at 75% saturation along with a mixture of fewer proteins (Figure 3-1).

Purification of BglX with column chromatography involved the use of ion exchange, gel filtration, and hydrophobic interaction chromatographies. Ion exchange
chromatography separates molecules based on charge. Proteins carry charges due to various amino acid functional groups and are separated depending on the interaction of the proteins with the charged column resin. For example, DEAE-Cellulose is an anion exchanger because the column resin is positively charged. In a sample mixture, proteins with a negative charge will bind to the column, while positively charged proteins will pass through. Bound proteins are eluted with a gradient of an increasing concentration of salt, such as NaCl. The ions compete with proteins for binding sites on the column. Therefore, proteins with weak ionic interactions are eluted first while proteins with stronger ionic interactions elute later at a higher salt concentration. Adjusting the pH of the sample buffer can affect ionic interactions. The isoelectric point (pI) of a protein is the pH at which the net charge of a protein is zero. For instance, at a pH that is greater than the pI of a protein, that protein carries a net negative charge. BglX has a pI of 5.77 and has a net negative charge in a buffer of pH 7.4. Thus, it was observed that BglX binds to the anion exchange columns, DEAE-Cellulose and Q Sepharose FF and does not interact with the cation exchangers, SP-Sephadex and CM-Sephadex.

DEAE-Cellulose is a weak anion exchanger that uses the weak base, diethylaminoethyl as the ion exchange group. The results from the use of DEAE-Cellulose chromatography revealed minimal purification of BglX (Figure 3-4) as many other proteins are visible in fractions that contain BglX (Lanes 6-9). BglX was estimated to elute with 200 mM NaCl. To improve separation using DEAE-Cellulose chromatography, the elution gradient range was extended from 0-400 mM to 0-500 mM NaCl. The SDS-PAGE analysis of fractions collected under this condition is shown in
Figure 3-9. Lanes 4 and 5 contain BglX and there are fewer proteins in these fractions as opposed to fractions shown in Figure 3-4.

Another type of anion exchange chromatography is Q Sepharose FF, which is a strong anion exchanger that uses a quaternary amine group. Purification with Q Sepharose FF displayed increased purification of BglX. Lane 4 of Figure 3-13 shows a nearly pure fraction of BglX. BglX eluted at approximately 170 mM NaCl. The purification method for BglX used by Yang et al. also consisted of a Q Sepharose FF column (17). Yang et al. stated that BglX was the first peak to elute and that protein was estimated to be 80% pure via SDS-PAGE (17). Our results are consistent with the work of Yang et al., where Figure 3-14 shows BglX as the first peak to elute and the purity of the protein is approximate to their findings.

Cation exchange columns were used as well for purifying BglX. Cation exchange columns have a negatively charged resin and since BglX is negatively charged at pH 6.0, the protein did not bind to either SP-Sephadex or CM-Sephadex columns. Purification with SP-Sephadex (strong cation exchanger) showed more success than with CM-Sephadex (weak cation exchanger). The SDS-PAGE analysis of purification after SP-Sephadex (Figure 3-5) shows BglX in a mixture of a smaller quantity of proteins than what was loaded onto the column. In regards to the SDS-PAGE analysis of purification by CM-Sephadex chromatography (Figure 3-11), purification of BglX was unsuccessful.

Another type of chromatography used to purify BglX was gel filtration chromatography which separates proteins on the basis of size. The resin of a gel filtration column is composed of porous beads whose pores are of a particular size. Molecules
small enough to diffuse through the pores move through the column more slowly, while larger molecules are excluded from the pores and move through the column more quickly. Thus, gel filtration chromatography is a common technique used in separating high and low molecular weight proteins. On the SDS-PAGE analysis of fractions collected from Sephacryl S-200 gel filtration chromatography (Figure 3-7), low and high molecular weight bands are visible in Lanes 7-15. This indicates inefficient separation of proteins by the column.

The last type of chromatography used was hydrophobic interaction chromatography. In this technique, proteins are separated by the interaction experienced between the hydrophobic areas of the protein and the hydrophobic resin. A sample is loaded onto a hydrophobic column with a high concentration of ammonium sulfate. This aids hydrophobic interaction so proteins that have many hydrophobic residues exposed on the surface will bind to the column more tightly and will elute at low concentrations of ammonium sulfate. According to the SDS-PAGE analysis of fractions collected from Toyopearl Butyl-650M hydrophobic interaction chromatography (Figure 3-16), BglX elutes at an estimation of 0.5 M ammonium sulfate. This indicates the hydrophobic nature of the protein. Purification with this column was very effective since large amounts of BglX can be seen while other proteins are barely visible.

Loss of BglX activity was observed during the purification process. Purification with the anion exchange columns utilized a buffer of pH 8.2 to increase the affinity of BglX to the column in an effort for better separation. Separation was more effective in purification with Q Sepharose FF chromatography; however, loss of activity was noted.
Since purification with DEAE-Cellulose did not result in high separation of the proteins, activity observed could be due to other glycosidases in the sample. For instance, it has been reported that the glycosidase β-galactosidase can cleave the substrate PNPG as well (44). β-galactosidase in *E. coli* has a molecular weight of 116 kDa (45). Figure 3-4 shows the SDS-PAGE analysis of DEAE-Cellulose fractions and a large band is visible at 116 kDa in Lanes 8-14. To prevent the loss of activity, the pH of the buffer used with Q Sepharose FF chromatography was changed, following the work of Yang et al. The purification method for BglX used by Yang et al. consisted of a HiTrap Q Sepharose FF column using 20 mM triethanolamine buffer, pH 7.4 (17). The optimal pH for BglX activity has not been reported, although other β-glucosidases have been found to be optimally active in the pH ranges of 6.0-6.8 (43, 44). Extremely low or high pH values can lead to the loss of enzyme activity due to denaturation.

The loss of activity could also be due to poor recovery of BglX through multiple steps of purification with column chromatography. For instance, the sequence of purification using DEAE-Cellulose, SP-Sephadex, and Sephacryl S-200 chromatographies yielded a low recovery of BglX. Purification of BglX was improved by using Q Sepharose FF proceeded by Toyopearl Butyl-650M hydrophobic interaction chromatography as it retained much of the protein in nearly pure amounts, while decreasing the number of chromatographic steps. Results obtained from this purification method of BglX resembled that of Yang et al. despite minor deviations.
Analysis of BglX Kinetics

Purified BglX from Q Sepharose FF and hydrophobic interaction chromatographies was used to determine the kinetic parameters of BglX. The enzyme has a $K_M$ of 0.34 mM, a $k_{cat}$ of 1.19 s$^{-1}$, and a catalytic efficiency of $3.4 \times 10^3$ M$^{-1}$s$^{-1}$. Kinetic analysis of BglX by Yang et al. reports $K_M$ to be 18 mM (17). Our determination of $K_M$ is lower which indicates a higher affinity by the enzyme for the substrate. $k_{cat}$ represents the number of substrate molecules that are converted to product by the enzyme per unit of time and is used in determining the catalytic efficiency of an enzyme. BglX has a catalytic efficiency ($k_{cat}/K_M$) of $3.4 \times 10^3$ M$^{-1}$s$^{-1}$ for the substrate PNPG. Furthermore, in order to accurately assess the kinetic parameters of BglX, it is necessary for the protein to be purified to homogeneity.

Mutagenesis of BglX

Mutagenesis of BglX was performed to identify important residues in the active site of the enzyme. Identification of important catalytic residues would help to elucidate the mechanism of BglX.

Figure 4-1 portrays the predicted structure of BglX. The structure was created based on sequence similarities with β-glucosidases from other organisms using the Phyre server. Asp287 resembles the active site residue Asp285 in barley β-D-glucan exohydrolase isoenzyme ExoI. Asp287 (shown in red) is located in a shallow pocket which enables substrate binding. The predicted active site residue Asp287 of BglX was targeted for mutagenesis.
Asp287 is encoded by the codon GAT. Replacement of the nucleotide G with A will result in the coding of the amino acid asparagine. The amino acid glycine is encoded when GAT is modified to GGT. Primers were designed correspondingly to construct the BglX mutants, D287N and D287G. Mutagenesis of the bglX gene was performed by a PCR-based method. To eliminate the template plasmid, the PCR mixture was treated with Dpn I. Dpn I digests parental DNA that is isolated from bacteria and therefore is methylated. The pET20-bglX plasmid isolated from BL21(DE3) E. coli cells was digested, whereas the mutant DNA was not since it was synthesized in vitro. The mutant plasmids were transformed into XL1-Blue cells. Plasmids were isolated and were sequenced for verification of the mutation. DNA sequencing of the BglX mutations (Figure 3-23) revealed the success of mutagenesis. The codon GAT in wild type BglX was altered to AAT for the bglX D287N mutant and GGT for the bglX D287G mutant.
The peaks of the chromatogram correspond to nucleotides. The peaks for the codons AAT and GGT of the mutants are well resolved and do not overlap, indicating the accuracy of the results.

BglX D287N was expressed in BL21 (DE3) cells with the induction by IPTG. Results from the SDS-PAGE analysis of BglX D287N expression (Figure 3-24) show the mutant protein is not significantly overexpressed with IPTG induction. BglX D287N was isolated from the cells by cell lysis, streptomycin sulfate treatment, and ammonium sulfate precipitation. The mutant protein was partially purified with Q Sepharose FF chromatography.

Analysis of BglX D287N Activity

Studies by Yaw-Kuan et al. involved replacement of the proposed active site residue Asp247 of a β-glucosidase from Flavobacterium meningosepticum, with asparagine, glutamate, or glycine (25). Catalytic efficiencies of D247N and D247G mutants were $2 \times 10^5$- and $3 \times 10^4$-fold lower respectively than that observed for the wild type enzyme (25). Asp287 of BglX from E. coli and Asp247 of a β-glucosidase from F. meningosepticum are homologous to the active site residue Asp285 of barley β-D-glucan exohydrolase isoenzyme ExoI (25). Asp287 of BglX was replaced with asparagine by site-directed mutagenesis. To determine the effects of the mutation, enzymatic activity of BglX D287N was examined spectrophotometrically using the model substrate PNPG (Figure 3-27) and by zymography (Figure 3-29). BglX D287N displayed reduced activity in comparison with wild type BglX, which is in agreement with the work of
Yaw-Kuan *et al.* Replacement of Asp287 (negatively charged) with asparagine (neutral) supports the hypothesis that aspartate acts as the catalytic nucleophile through its carboxylate group. Asparagine is similar in size and structure to aspartate, therefore, the mutation resulted in only subtle changes, aside from a difference in charge. Asp287 is an important residue that is involved in catalysis as indicated by the consequences of its replacement.

*Future Work*

Future work will include the development of more efficient methods for purification of BglX. The purity of the protein should be verified by matrix-assisted laser desorption/ionization mass spectrometry. Once BglX is purified, kinetic parameters of the enzyme can be studied in more details. Further characterization of BglX will include determining the optimal pH and temperature for activity. In addition, enzymatic activity of the BglX D287G mutant should be analyzed following purification. Identification of other catalytically important residues should be pursued as well. After gaining a better understanding of the mechanism of BglX, inhibitors’ design will be possible.
Chapter 5: Conclusion

Beta-glucosidase BglX from *E. coli* is a suitable target for drug therapy since this enzyme functions to provide energy for the bacterium and is not produced in humans. Inhibition of BglX can be accomplished with a better understanding of the mechanism of the enzyme through identification of essential amino acid residues in the active site.

A purification method has been developed for BglX and preliminary characterization of the enzyme was accomplished. The periplasmic enzyme BglX was overexpressed in BL21(DE3) *E. coli* cells with IPTG induction. The cells were harvested and lysed by sonication. Purification of BglX was accomplished using ammonium sulfate precipitation, Q Sepharose FF anion exchange and Toyopearl Butyl-650M hydrophobic interaction chromatographies. Ammonium sulfate fractionation at 50% and 75% saturation provided efficient initial purification of BglX.

Purification of BglX was continued with Q Sepharose FF anion exchange chromatography. The enzyme was shown to elute in relatively pure fractions at approximately 170 mM NaCl. Enzyme activity was retained after purification with a Q Sepharose FF column using Tris buffer at pH 7.4 as opposed to pH 8.2. Additional purification of BglX was performed with a Toyopearl Butyl-650M column. BglX eluted in nearly pure fractions at about 0.5 M ammonium sulfate. The kinetic parameters of BglX were determined using purified enzyme. BglX has a $K_M$ of 0.35 mM, a $k_{cat}$ of $1.19 \text{ s}^{-1}$, and a catalytic efficiency of $3.4 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ for the chromogenic substrate PNPG.
An improved purification should be developed in order to accurately determine the kinetic parameters of BglX.

The predicted active site residue Asp287 of BglX was determined to play a significant role in the enzyme’s activity. Asp287 was modified to an asparagine and a glycine through PCR-based site-directed mutagenesis. The mutations were confirmed by DNA sequencing. The BglX D287N mutant was expressed in BL21(DE3) *E. coli* cells and partially purified with Q Sepharose FF anion exchange chromatography. The effect of the mutation on enzyme activity was investigated by spectrophotometric and zymographic assays. BglX D287N failed to cleave the substrate PNPG as effectively as the wild type enzyme. Zymography confirmed the decreased activity in the mutant enzyme which indicates the significance of Asp287 in catalysis. Further studies are required to determine the activity level of BglX D287G. In addition, other important amino acid residues in the active site should be identified.
Chapter 6: References


17. Yang, Maria *et al.*; The *bglX* Gene Located at 47.8 min on the *Escherichia coli* Chromosome Encodes a Periplasmic β-glucosidase. *Microbiology*. 1996, 142: 1659-1665.


