Expression and Characterization of an Extremely Thermostable Beta Glycosidase (Mannosidase) from the Hyperthermophilic Archaeon *Pyrococcus Furiosus DSM3638* and Mutation Studies in the Active Site

By

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Suggested short title:

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF A β-GLYCOSIDASE FROM *PYROCOCCUS FURIOSUS DSM 3638*

DEDICATE TO

TO MY PARENTS WHO SUPPORT ME WITH LOVE AND CARE

TO MY WIFE WHO GIVE ME LOVE AND PEACE

ABSTRACT

Genomic analysis of the hyperthermophilic archaeon *Pyrococcus furiosus* revealed the presence of an open reading frame (ORF PF0356) similar to the enzymes in glycoside hydrolase family 1. This β-glycosidase, designated PFTG (*Pyrococcus furiosus* thermostable glycosidase), was cloned and expressed in *Escherichia coli*. The expressed enzyme was purified by heat treatment and Ni-NTA affinity chromatography. The gene was composed of 1452 bp encoding 483 amino acids for a protein with a predicted molecular mass of 56,326 Da. The temperature and pH optima were 100°C and 5.0 in sodium citrate buffer, respectively. The substrate specificity studies by conventional assays showed characteristics of both β-galactosidase and β-mannosidase activities. However, through kinetic studies by ITC (Isothermal Titration Colarimetry), this enzyme showed the highest catalytic activity in *p*-nitrophenyl-β-D-mannopyranoside (pNP-Man) with k_{cat}/K_m (3.02). The enzyme showed transglycosylation and transgalactosylation activities toward cellobiose, lactose and mannooligosaccharides, that could produce GOS (galactooligosaccharides) and MOS (mannooligosaccharides) which are the important prebiotic (bifidogenic) ingredients.

Sequence alignments and homology modeling of PFTG showed that the residue 150 is conserved as tryptophan in β−glycosidase and in other related enzymes such as β-mannosidase and β-galactosidase. To elucidate the relationship between the substrate size and geometric shape of the catalytic site of thermophilic β-glycosidase and category of PFTG, the Q77, the Q150 and the D206 located at the interface of the dimer were

replaced with Trp and Asn. Also, to confirm the role of active sites of PFTG, the Q77/150W double mutant was created through subcloning. The mutant enzymes were successfully cloned, expressed in *E. coli* and showed the same temperature and pH optima at 100 °C and pH 5.0, respectively.

By substituting Gln150 to Trp and Gln77/150Trp, the catalytic efficiency (k_{cat}/K_m) of the mutants by ITC_{200} on both synthetic and natural substrates were slightly changed. As compared with the wild-type enzyme, substrate specificities on the mutant enzymes were similar but with more affinity (K_m) to substrates and low turnover number (k_{cat}).

To confirm the category of PFTG, the protein structural studies were performed. After the wild-type enzyme was purified, the enzyme was set up for protein crystal screening under 200 different conditions. With several hit conditions, X-ray diffraction study was performed, but during the X-ray analysis, the data was too high to obtain the reasonable data. Through protein sequence analysis of PFTG, two Lys-Lys sequences which interfere the protein crystallization were revealed. After Lys-Lys amino acids were mutated to Ala-Ala by site-directed mutagenesis, the mutant enzymes were set up and incubated for crystallization. Clear protein crystals were not obtained, but the computer 3D model structure indicated that this enzyme was similar to that of β-glycosidase from *Thermosphaera aggregans*.

Keywords: β-glycosidase; β-galactosidase; β-mannosidase; hyperthermophile; *Pyrococcus furiosus*; hyperthermostable enzyme; ITC (Isothermal Titration Calorimetry); glycoside hydrolase; protein crystallography

RÉSUMÉ

L'analyse génomique de la *Pyrococcus furiosus*, hyperthermophile archaeon a révélé la présence d'un cadre de lecture ouvert (ORF PF0356) similaire aux enzymes de la famille glycoside hydrolase 1. L'enzyme β-glycosidase, désigné PFTG (*Pyrococcus furiosus* glycosidase thermostable), a été cloné et exprimé dans *Escherichia coli*. L'enzyme exprimée a subi un traitement thermique et a été purifié par une chromatographie d'affinité au nickel. Son gène a une taille de 1452 pb et code pour une protéine de 483 acides aminés. Sa masse moléculaire prédite est de 56,326 Da. Les conditions optimales de l'activité de cette enzyme ont été définies dans un tampon citrate de sodium à pH 5,0 et à une température de 100 °C. Les études de spécificité du substrat ont démontré des caractéristiques similaires aux activités enzymatiques de la β-galactosidase et de β-mannosidase. À partir des résultats obtenues de cinétique par ITC (titration isotherme colarimetry), cette enzyme a révélé avoir une activité catalytique la plus élevé pour le substrat p-nitrophényl-β-D-mannopyranoside (pNP-Man) avec un ratio K_{cat}/K_m de 3,02. L'enzyme a montré avoir des activités de transglycosylation et de transgalactosylation envers le cellobiose, le lactose ainsi que les mannooligo-saccharides.

Ce qui pourraient produire des GOS (galacto oligosaccharides) et des MOS (mannooligosaccharides) qui sont d'importants ingrédients prébiotiques (bifidogène).

Les alignements de séquences et de modélisation par homologie de la PFTG ont révélé que le résidu 150, qui est le tryptophane, est conservé dans la β-glycosidase ainsi que dans d'autres enzymes apparentés tels que la β-galactosidase et le β-mannosidase.

Pour élucider la relation entre la taille du substrat et la forme géométrique du site catalytique de l'enzyme thermophilique β-glycosidase ainsi que la catégorie de PFTG, les acides aminés Gln 77, Gln 150 et Asp 206 situées à l'interface du dimère ont été remplacés par mutagenèse dirigée. Ainsi, le tryptophane a été substitué aux résidus Gln 77 et 150 et l'asparagine a été substituée au résidu Asp 206. De plus, le double mutant Q77/150W a été créé par sous-clonage pour confirmer le rôle des sites actifs de PFTG. En effet, le gène PFTG de type sauvage a été muté, cloné puis exprimé dans *E. coli*. L'enzyme mutante exprimée à démontrer la même activité dans les mêmes conditions que l'enzyme natif, soit de tampon, de température et de pH.

En substituant Gln150 et Gln77 par des tryptophanes, l'efficacité catalytique (K_{cat}/K_m) du mutant mesuré par ITC_{200} a été légèrement modifié sur les substrats synthétiques et naturels. En comparant avec l'enzyme natif, la spécificité de l'enzyme mutant pour les substrats a été similaire mais avec une plus grande affinité (K_m) pour les substrats et un faible K_{cat}.

Pour confirmer la catégorie des PFTG, des études sur la structure de la protéine ont été effectuées. Après avoir purifié l'enzyme de type sauvage par chromatographie d'affinité au nickel et par chromatographie sur gel (GPC), l'enzyme purifiée a été préparée à des fins de dépistage de cristaux et mis à l'épreuve dans les 200 conditions différentes.

Au cours des analyses aux rayons X, les données du facteur de diffraction se sont avérées trop élevés pour donner des résultats significatifs. Par contre, l'analyse de séquence protéique de la PFTG native a révélé deux liaisons Lys-Lys qui ont entravé la cristallisation de la protéine. Après que la liaison d'acides aminés Lys-Lys a été

substituée par une liaison Ala-Ala par mutagenèse dirigée, l'enzyme mutant a été conduit à des analyses de cristallographie. Il n'a pas été possible d'obtenir des cristaux de protéine. Par contre, la modélisation par ordinateur de la structure 3D a indiqué que cette enzyme est similaire à celle de la β-glycosidase de *Thermosphaera aggregans*.

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most of my time in the lab has made all this possible. This work could not have been possible without her love.

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FOREWORD

This thesis is submitted in the form of original papers suitable for journal publication. A general introduction states the rationale and the objectives of this study, and it is followed by Chapter 1, a literature review, which presents the theory and background information on this subject. The next three chapters represent the body of the thesis, each being a complete manuscript with exception of Chapter 4 on crystallography (not completed). The last section is a summary of the major conclusions. This thesis format has been approved by the Faculty of Graduate and Postdoctoral Studies, McGill University, and follows the conditions outlined in the Guidelines concerning Thesis Presentation, which are as follows:

"Candidates have the option, subject to the approval of the Department, including as part of the thesis the text of an original paper, or papers suitable for submission to learn journals for publication. In this case, the thesis must still conform to all other requirements explained in the Guidelines Concerning Thesis Preparation. Additional materials (experimental and design data as well as descriptions of equipment) must be provided sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported. The abstract, full introduction and conclusion must be included, and where more that one manuscript appears, connecting texts and common abstract, introduction and conclusions are required. A mere collection of manuscripts is not acceptable; nor can reprints of published papers be accepted. While inclusion of manuscripts co-authored by the candidate and others is not prohibited by

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Although all the works reported here is the responsibility of the candidate, the project was supervised by Dr. Inteaz Alli, Department of Food Science and Agricultural Chemistry and Dr. Byong H. Lee, Department of Microbiology/ Immunology, McGill University.

CONTRIBUTION OF CO-AUTHORS FOR PUBLICATIONS

Dr. Inteaz Alli, my supervisor from Department of Food Science, McGill University contributed to part of this work by providing technical support and reviewing the manuscripts in a supervisory role in this research. He is a co-author on the publications presented in scientific meetings and on the manuscripts that have been published and submitted.

Dr. Byong H. Lee, my supervisor and co-supervisor later in the Department of Microbiology/Immunology, is the co-author on the publications presented in scientific meetings and on the manuscripts that have been published and submitted.

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A. Part of this work has been submitted for publication:

1. **S.H, Park**, B.C, Oh, I. Alli, K.H, Park and B.H, Lee. (2010). Expression and characterization of an extremely thermostable beta glycosidase (mannosidase) from the hyperthermophilic archaeon *Pyrococcus furious* DSM3638. *New Biotechnology* (submitted)

2. **S.H, Park**, B.C, Oh, I. Alli, K.H, Park and B.H, Lee. (2010). Mutation studies in the active site of β-glycosidase from *Pyrococcus furiosus* DSM 3638. *Biochimica et Biophysica Acta - Proteins and Proteomics* (submitted).

B. Part of this work has been presented at scientific meeting:

1. **S.H, Park**, I. Alli, K.H, Park and B.H, Lee. (2009). Biochemical and molecular characterization of a β-glydosidase from *Pyrococcus furiousus* DSM 3638. 14th World Congress, International Union of Food Science & Technology (IUFoST, Shanghai).

2. **S.H, Park**, I. Alli, K.H, Park and B.H, Lee. (2010). Expression and characterization of an extremely thermostable beta glycosidase (mannosidase) from the hyperthermophilic archaeon *Pyrococcus furious*. 110th General Meeting of the American Society for Microbiology (ASM, San diego).

3. **S.H, Park**, I. Alli, K.H, Park and B.H, Lee. (2010). Expression and characterization of an extremely thermostable beta glycosidase (mannosidase) from the hyperthermophilic archaeon *Pyrococcus furious*. 8th International Congress on Extremophiles, (Ponta Delgada, Portugal).

GENERAL INTRODUCTION

A hyperthermophile is an organism that thrives in extremely hot environments from 60 °C and an optimal temperature for the existence of hyperthermophiles is above 80°C. Hyperthermophiles are a subset of extremophiles, micro-organisms within the domain Archaea, although some bacteria are able to tolerate temperatures of around 100°C as well. Many hyperthermophiles are also able to withstand other environmental extremes such as high acidity or radiation levels. Due to these particular properties, enzymes from extremophiles (extremozymes) offer a high potential not only for basic research but also for biotechnological applications.

Extremozymes would enable us to expand the range of reaction conditions suitable for biocatalysis. This has proven to be the case and various applications in sugar chemistry, detergent production, lipid and oil chemistry, and food processing have been initiated or are being explored (van den Burg, 2003).

The heat-stable extremozymes not only serve as excellent models for understanding protein stability but also carry significant biotechnological potential. Application interest in thermophilic extremozymes is related to the fact that performing biotechnologically related processes at higher temperatures is often advantageous. For instance, in chemical reactions involving organic solvents, the decrease in viscosity and increase in diffusion coefficient that is achieved at elevated temperatures result in higher reaction rates (Krahe *et al*., 1996; Becker *et al*., 1997). Such considerations are relevant to a variety of processes including those using hydrophobic compounds with low solubility. High temperatures can also enhance the availability of such compounds for

biodegradation efforts. Furthermore, reactions at higher temperatures reduce the possibility of complications due to contamination. Due to their capability to function at such high temperatures, hyperthermophilic extremozymes have drawn the main focus of biotechnological interest.

These microorganisms, which belong to Archea and Bacteria, can facilitate the enzymatic degradation of carbohydrates by producing heat-stable enzymes that are catalytically active above 100°C. Such enzymes share the ability to hydrolyze the glycosidic bonds in sugar molecules or between a carbohydrate and noncarbohydrate moiety. Enzymatic hydrolysis and modification of polysaccharides are of great interest in the field of food technology. The potential exploitation of this natural source of sugars is not only useful for glucose syrup production, but also for the synthesis of non-fermentable carbohydrates, anticariogenic agents, and antistaling agents in baking (Cowan, 1996).

In most hyperthermophilic microorganisms, the activity levels of starch-hydrolyzing enzymes appear to be too low for biotechnological applications. The molecular cloning of the corresponding genes and their expression in heterologous hosts, however, circumvent the problem of insufficient expression in the host cell. The number of genes encoding amylolytic enzymes that have been cloned from hyperthermophiles and expressed in mesophiles has been increasing significantly. In most cases, the thermostable proteins expressed in mesophilic host maintain their thermostability, are correctly folded at low temperature, are resistant to host proteolysis, and can be purified by denaturing the mesophilic host proteins via heat treatment. The degree of enzyme purity obtained is generally suitable for most applications (Bertoldo *et al*., 2001).

The recent advent of genomic research has produced vast amounts of sequence information for many different Bacteria, Eukarya, and Archaea, now collected in databases such as GenBank; the full genome sequences of more than 699 different microorganisms have been completed. By a generally applicable combination of conventional genetic engineering and genomic research techniques, many extremely thermostable enzymes are being developed for biotechnological purposes. The genome sequences of some hyperthermophilic microorganisms such as *Thermotoga maritima*, *Pyrococcus furiosus*, and *Sulfolobus solfataricus* are of considerable biotechnological interest because they encode many highly heat-stable enzymes that are active under conditions previously regarded as incompatible with biological materials (Kuhad *et al*., 1997).

The glycoside hydrolases are an important group of enzymes that are responsible for cleaving a range of biologically significant carbohydrate compounds. Glycoside hydrolase family 1 belongs to a larger superfamily which contains at least 11 families and now comprises more than 250 members representing 18 different substrate specificities (Cantarel *et al*., 2009). However, as almost all carbohydrate enzymes including glycoside hydrolases had overlapping substrate specificity, it is very important to verify the true categories of the enzymes by kinetic studies and protein crystallography.

The overall objectives were biochemical and molecular characterizations of a β-glycosidase (mannosidase) from *Pyrococcus furiosus DSM 3638*.

The specific objectives of this study were:

i. To clone, express and purify β-glycosidase (mannosidase) from hyperthermophilic *Pyrococcus furiosus* DSM 3638.

ii. To characterize the β-glycosidase (mannosidase) for optimum temperature, pH, substrate specificity and thermostability.

iii. To determine the kinetic parameters and protein structure for elucidating the true category of the PFTG.

CHAPTER 1

LITERATURE REVIEW:

Glycoside hydrolase family1 (β-galactosidase and β-glycosidase) and hyperthermophilic enzyme

This chapter was summarized as “Glycoside hydrolase family 1 and hyperthermophilic enzyme” and will be submitted to *Critical Reviews in Biotechnology*, which was co-authored by Sung Hoon Park, Inteaz Alli and Byong H. Lee. This paper was written by Sung Hoon Park and supervised by Dr. Inteaz Alli and Dr. Byong H. Lee, who acted in an editorial capacity for the manuscript. This chapter serves as an introduction to the thesis, showing the background for the research that was done and the significance of this project.

1.1. ABSTRACT

The hydrolytic and transglycosylation mechanism of β-glycosidase (EC 3.2.1.21), carbohydrate active enzymes, and isothermal titration calorimetry are reviewed in this chapter.

Carbohydrate active enzymes (CAZymes) are a large class of enzymes, which build and breakdown the complex carbohydrates of the cell. On the basis of their amino acid sequences they are classified in families that show conserved catalytic mechanism, structure, and active site residues, but may conflict each other in substrate specificity. To solve the conflict each other in substrate specificity, kinetic studies are essential for categorized in glycoside hydrolase (GH). ITC (Isothermal Titration Calorimetry) can provide very accurate experimental results for enzyme kinetics. Among GH family 1 enzymes, some of them are from hyperthermophile origins (i.e. *Sulfolobus, Pyrococcos*, etc). Recently, the advantage of hyperthermophile enzyme has been studied and commercialized. The main advantage of hyperthermophilic enzyme is that in chemical reactions involving organic solvents, the decrease in viscosity and increase in diffusion coefficient that is achieved at elevated temperatures result in higher reaction rates and low contamination.

Part 1 describes carbohydrate active enzymes and its related information. Part 2 describes glycoside hydrolase family1 (GH1), Part 3 introduces other related glycoside hydrolase families. Part 4 is on a general introduction of extremozymes and application of thermophilic enzymes. Finally part 5 discusses on the isothermal titration calorimetry.

This chapter provides up-to-date information on β-glycosidase and the significance of this research.

1.2. INTRODUCTION

1.2.1. Carbohydrate active enzymes (CAZymes)

Carbohydrate active enzymes (CAZymes) are a large class of enzymes, which build and breakdown the complex carbohydrates of the cell. On the basis of their amino acid sequences they are classified in families that show conserved catalytic mechanism, structure, and active site residues, but may conflict each other in substrate specificity.

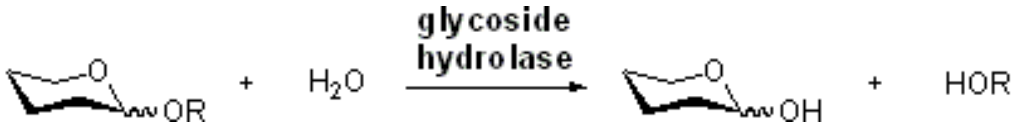
The CAZymes provides a continuously updated list of the glycoside hydrolase families, GHs (http://www.CAZY.org). This group of enzymes is classified based on functional similarity, but today they are classified into 108 GHs on the basis of amino acid sequence similarity. Despite their similarities to enzymes with known functions, their primary functions are still unclear. Based on these criteria, β-galactosidase activities are now divided into four different families: GH1, GH2, GH35 and GH42, among which the better studied GH2 includes β-galactosidases from *Escherichia coli*, *Aspergillus*, *Bacillus megatherium,* and *Sulfolobus solfataricus,* while those from thermophilic (Hirata *et al*, 1986; Moracci *et al*, 1992; Hidaka *et al*., 2002), psychrophilic (Gutshall *et al*., 1995; Skálová *et al*., 2005), and halophilic (Holmes *et al*., 1997) organisms belong to GH42. Lactase is often confused as an alternate name for β-galactosidase, but it is actually

simply a sub-class (small subunit) of β-galactosidase. (β-D-galactoside galactohydrolase, EC 3.2.1.23) that catalyses hydrolysis of the galactosyl moiety from non-reducing termini of oligosaccharides or from glycosides. Most genes encoding GH42 enzymes are from prokaryotes that are unable to grow on lactose as a sole carbon source (Daniel *et al*., 1997; Holmes *et al*., 1997), and at least two GH42 β-galactosidases do not cleave lactose *in vitro* (Holmes *et al*., 1997; Van Laere *et al*., 2000).

The determination of growth on lactose can be complicated by the multiple β-galactosidases because not all of the β-galactosidases are acting as lactases *in vitro* (Synowiecki *et al*., 2006). β-Galactosidase hydrolyses the β-1, 4-D-galactosidic linkage of lactose, as well as those of related chromogens, *o*-nitrophenyl-β-d-galactopyranoside (ONPG), *p*-nitrophenyl-β-d-galactopyranoside (PNPG) and 6-bromo-2-naphthyl-galactopyranoside (BNG). This enzyme has been purified and characterized from various sources, including plants (Ogasawara *et al*., 2007), animals (Taniguchi & Takano, 2004), and many microorganisms (Hung *et al*., 2001; Di Lauro *et al*., 2008). In humans, lactase is present predominantly along the brush border membrane of the differentiated enterocytes lining the villi of the small intestine. Lactase is essential for digestive hydrolysis of lactose in milk. Deficiency of the enzyme causes lactose intolerance. Several 3D structures are available for the GH1 and GH2 β-galactosidases for which the catalytic residues have experimentally been determined (Matthews, 2005), while three 3D structures of β-galactosidases from *E. coli* (Jacobson *et al*., 1994), *Penicillium* sp. (Rojas *et al*., 2004) and *Thermus thermophilus* A4 (Hidaka *et al*., 2002) are available for either the GH35 and GH42 families.

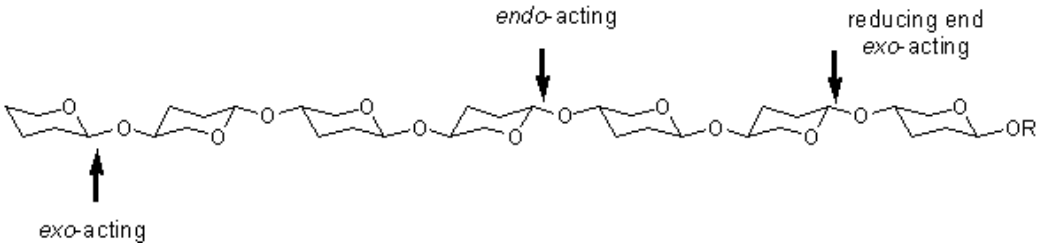
1.2.2. Glycoside Hydrolases

Glycoside hydrolases are enzymes that catalyze the hydrolysis of the glycosidic linkage of glycosides, leading to the formation of a sugar hemiacetal or hemiketal and the corresponding free aglycon. Glycoside hydrolases are also referred to as glycosidases, and sometimes also as glycosyl hydrolases. Glycoside hydrolases can catalyze the hydrolysis of O-, N- and S-linked glycosides.



1.2.2.1. Endo/exo

Exo- and *endo*- represents the ability of a enzyme to cleave a substrate at the end of non-reducing end or the middle of a chain. For example, most cellulases are *endo*-acting, whereas LacZ β-galactosidase from *E. coli* is *exo*-acting.

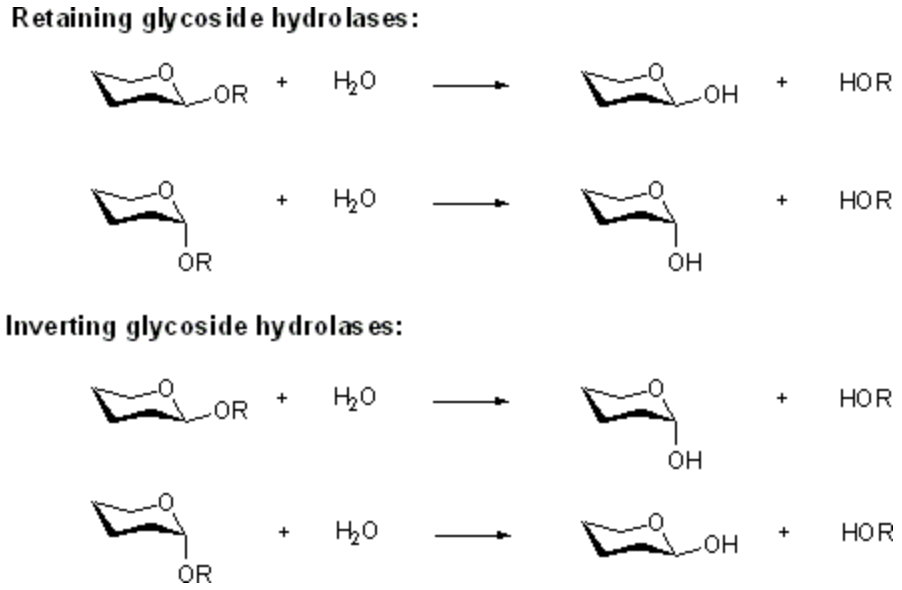


1.2.2.2. Enzyme Commission (EC) number

EC (Enzyme Commission Number) number is a numerical classification for enzyme that refers to the chemical reactions they catalyze. Every EC number is related with a recommended name for the respective enzyme. EC numbers do not specify enzymes, but enzyme-catalyzed reactions. If different enzymes catalyze the same reaction, then they get the same EC number. A necessary consequence of the EC classification scheme is that codes can be applied only to enzymes for which a function has been biochemically identified.

1.2.2.3. Mechanistic classification

Two reaction mechanisms are most commonly found for the retaining and inverting enzymes, as first outlined by Koshland and as described below (Gebler *et al*., 1992). However some variations on these mechanisms have been found, and one basically different mechanism, catalyzed by an NADH cofactor, has been discovered in recent years.



1.2.2.4. Sequence-based classification

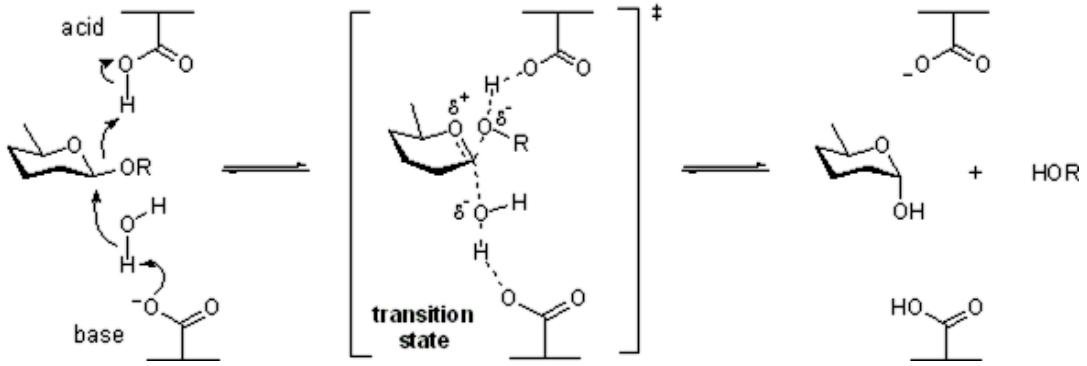
Sequence classification methods require knowledge of part of the amino acid sequence for an enzyme. Algorithmic methods are then used to compare sequences. Using a combination of comparison algorithms the glycoside hydrolases have been classified into more than 100 families, that are available at the Carbohydrate Active enZyme database (Cantarel *et al.*, 2009). Each family (GH family) contains proteins that are associated by sequence, and by corollary. An obvious shortcoming of sequence-based classifications is that they can be applied to enzymes for which sequence information is available. But sequence-based classification allow classification of proteins for which no biochemical evidence has been obtained like the huge amount of not characterized glycosidase-related sequences that come from genome sequencing efforts worldwide.

This allows a number of useful predictions to be made since it has long been noted that the catalytic machinery and molecular mechanism is conserved for the vast majority of the glycosidase families (Gebler *et al*., 1992) as well as the geometry around the glycosidic bond. Classification of families into larger groups, termed 'clans' has been mentioned (Henrissat & Bairoch, 1996). A clan is a group of families that has similarity in their tertiary structure, catalytic residues and mechanism. Families in clans are thought to have a common evolutionary origin.

1.2.2.5. Inverting glycoside hydrolases

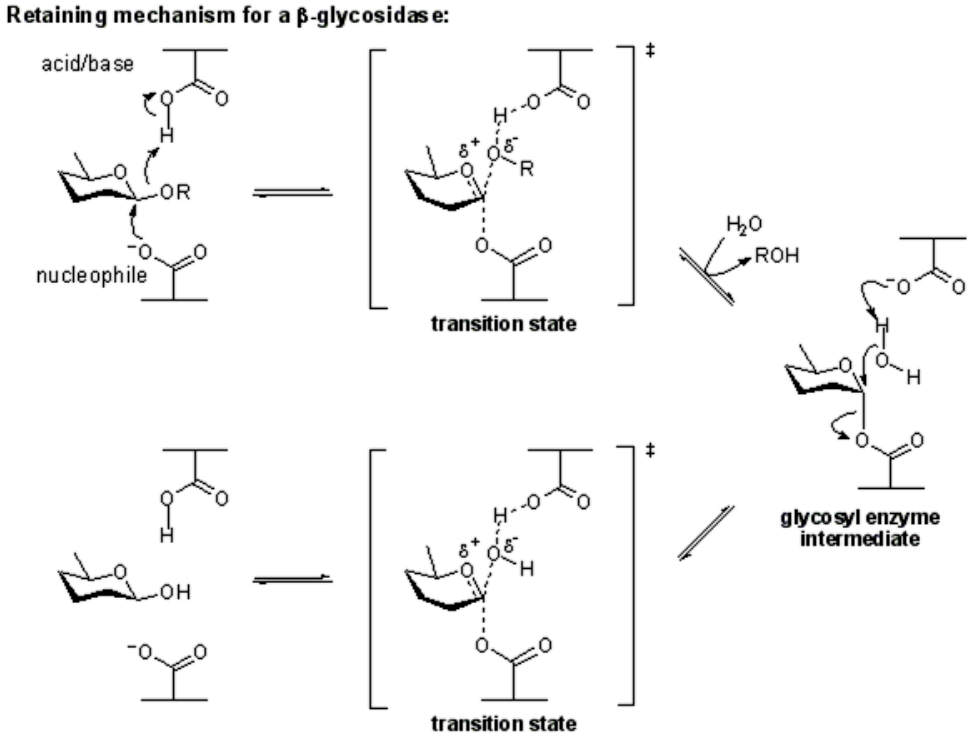
Hydrolysis of a glycoside with net inversion of anomeric configuration is generally achieved through one step, single-displacement mechanism involving oxocarbenium ion like transition states, as shown below. The reaction occurs with acid/base assistance from two amino acid side chains, normally glutamic or aspartic acids (McCarter & Withers, 1994).

Inverting mechanism for a β-glycosidase:



1.2.2.6. Retaining glycoside hydrolases

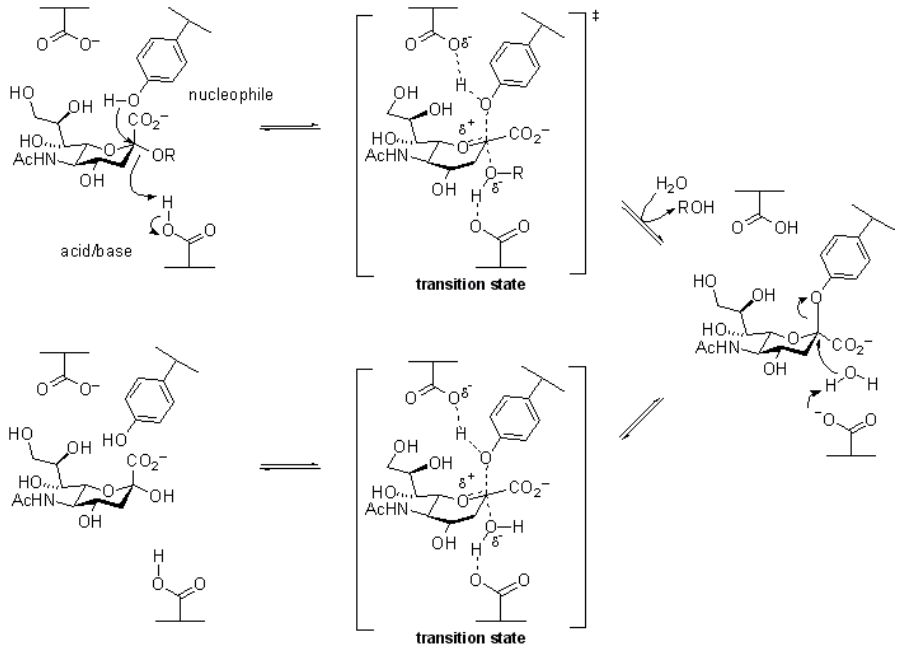
Hydrolysis with net retention of configuration is most commonly achieved via a two steps, double-displacement mechanism involving a covalent glycosyl-enzyme intermediate, as is shown in the figure below. Each step passes through an oxocarbenium ion like transition states. Reaction occurs with acid/base and nucleophilic assistance provided by two amino acid side chains, typically glutamate or aspartate. In the first step, one residue plays the role of a nucleophile, attacking the anomeric center to displace the aglycon and form a glycosyl enzyme intermediate. At the same time the other residue functions as an acid catalyst and protonates the glycosidic oxygen as the bond cleaves. In the second step, the glycosyl enzyme is hydrolyzed by water, with the other residue now acting as a base catalyst deprotonating the water molecule as it attacks. The pK_a value of the acid/base group cycles between high and low values during catalysis to optimize it for its role at each step of catalysis (McIntosh *et al*., 1996).



1.2.2.7. Alternative nucleophiles

Sialidases hydrolyze glycosides of sialic acids. Closely related enzymes termed trans-sialidases catalyze the transglycosylation of sialisides. The sialidases and trans-sialidases of glycoside hydrolase families 33 and 34 utilize a tyrosine as a catalytic nucleophile, which is activated by an adjacent base residue. A rationale for this unusual difference is that the use of a negatively charged carboxylate as a nucelophile will be disfavoured as the anomeric center is itself negatively charged, and thus charge repulsion interferes. A tyrosine residue is a neutral nucleophile, but requires a general base to enhance its nucleophilicity. This mechanism was implied from X-ray structures, and was supported by experiments involving trapping of the intermediate with fluoro sugars

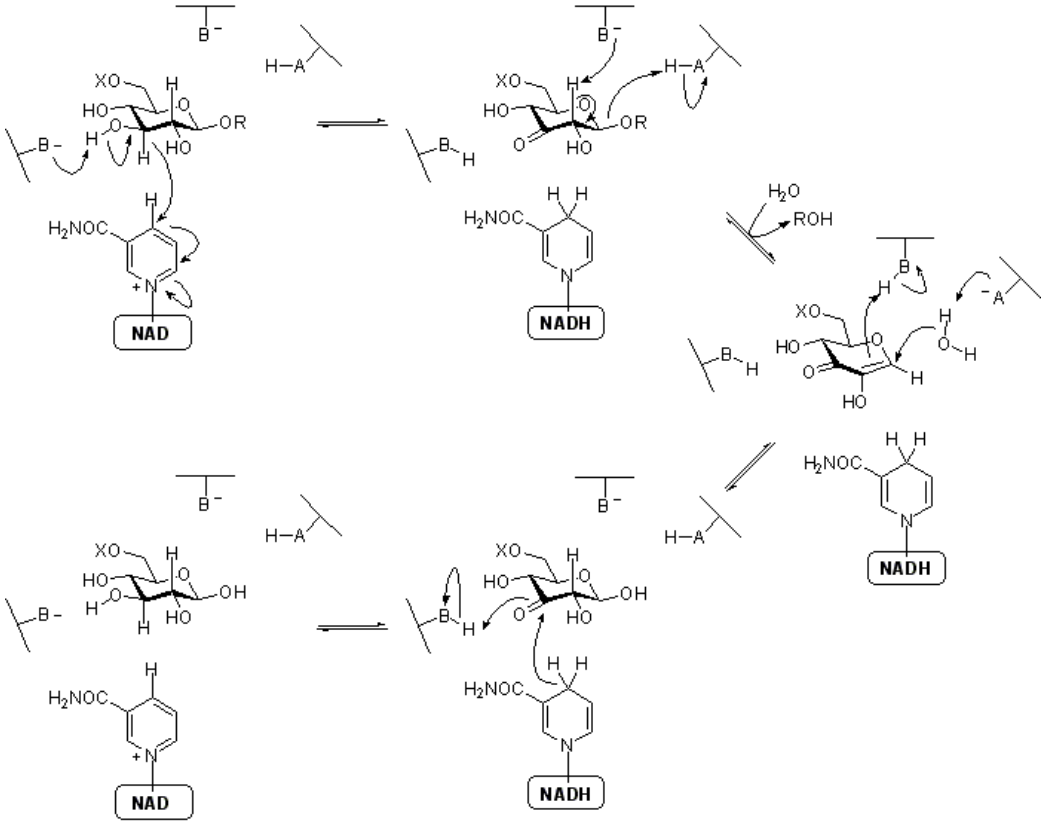
followed by peptide mapping and then crystallography (Watts *et al*., 2003; Amaya *et al*., 2004), and also through mechanism studies on mutants (Watson *et al*., 2003).



1.2.2.8. NAD-dependent hydrolysis

The glycoside hydrolases of family 4 and 109 use a mechanism that requires an NAD cofactor, which remains tightly bound throughout catalysis. The mechanism proceeds through anionic transition states with elimination and redox steps rather than the classical mechanisms proceeding through oxocarbenium ion-like transition states. As shown below for a 6-phospho-beta-glucosidase, the mechanism involves an initial oxidation of the 3-hydroxyl of the substrate by the enzyme-bound NAD cofactor. This increases the acidity of the C2 proton such as an E1. Elimination can occur with assistance from an enzymatic base. The alpha-beta unsaturated intermediate formed then

undergoes addition of water at the anomeric centre and finally the ketone at C3 is reduced to generate the free sugar product. Thus, even though glycosidic bond cleavage occurred via an elimination mechanism, the overall reaction is hydrolysis. This mechanism was elucidated through a combination of stereochemical studies by NMR, kinetic isotope effects, linear free energy relationships, X-ray crystallography and UV/Vis spectrophotometry (Rajan *et al*., 2004; Yip *et al*., 2004).



1.3. Glycoside Hydrolase Family 1

Glycoside hydrolases (GHs), including glycosidases and transglycosidases constitute 113 protein families that are responsible for the hydrolysis and/or transglycosylation of glycosidic bonds. GH-coding genes are abundant and present in the vast majority of genomes corresponding to almost half-presently about 47% - of the enzymes classified in CAZy. Because of their importance for biotechnological and biomedical applications, GHs constitute so far the best biochemically characterized set of enzymes present in the CAZy database (Cantarel *et al*., 2009).

1.3.1. Substrate specificities

The most common known enzymatic activities for glycoside hydrolases in this family, at the current time, are β-glucosidases and β-galactosidases: typically both activities are found within the same active site, often with similar k_{cat} values, but with substantially higher K_m values for the galactosides. However, other commonly found activities are 6-phospho- β-glucosidase and 6-phospho- β-galactosidase, β-mannosidase, β-D-fucosidase and β-glucuronidase. Family GH1 enzymes are found a broad spectrum of life forms. Enzymes of medical interest include the human lactase/phlorizin hydrolase whose deficiency leads to lactose intolerance. In plants Family GH1 enzymes are often involved in the processing of glycosylated aromatics such as saponins and some plant

hormones stored in inactive glycosylated forms. Indeed some have been identified as plant oncogenes due to aberrant control of auxin levels. Some plants also use Family GH1 enzymes as part of their defense system in order to release toxic aglycons, the most known examples being *Trifoliumrepens* β-glucosidase and *Sinapisalba* myrosinase, which respectively hydrolyse linamarin and glucosinolates. One of the work horses of glycosidase enzymology, the almond emulsin β-glucosidase, even if not fully sequenced, is deduced to belong to Family GH1 by limited sequence analysis (He & Withers, 1997).

1.3.2. Catalytic residues

He &Withers (1997) was first identified β-glucosidase of *Agrobacterium* species as Glu358 in the sequence YIT**E**NG through trapping of the 2-deoxy-2-fluoroglucosyl-enzyme intermediate and subsequent peptide mapping. The general acid/base catalyst was first identified as Glu170 in this same enzyme through detailed mechanistic analysis of mutants at that position, which included azide rescue experiments (Wang *et al*., 1995).

The GH1 enzymes, as a typical of Clan GH-A, have an asparagine residue preceding the general acid/base catalyst in a typical NEP sequence. The asparagine engages in important hydrogen bonding interactions with the substrate 2-hydroxyl. Interestingly, the plant myrosinases cleave thioglycosides bearing an anionic aglycone (glucosinolates), and have evolved an active site in which the acid/base glutamate is replaced by glutamine. Substrates are sufficiently reactive not to require the acid catalyst,

while the role of base catalyst is played by exogenous ascorbate, which binds to the glycosyl enzyme (Burmeister *et al*., 2000).

1.3.3. Three-dimensional structures of GH1

Three-dimensional structures are available for a large number of Family1 enzyme, the first solved being that of the white clover (*Trifolium repens*) cyanogenic β-glucosidase (Barrett *et al*., 1995). As members of Clan GH-A they have a classical $(\alpha/\beta)_8$ TIM barrel fold with the two key active site glutamic acids being approximately 200 residues apart in sequence and located at the C-terminal ends of β-strands 4 (acid/base) and 7 (nucleophile) (Henrissat *et al*., 1995).

1.3.3.1. Structures of GH-1 β-galactosidases from the hyperthermophilic archeon *Sulfolobus solfataricus* (Ss-β-gal) and *Pyrococcus furiosus* (Pf- β-gal)

In recent years, several thermostable β–galactosidases have been reported (Wanarska *et al*., 2005; Synowiecki *et al*., 2006). *Sulfolobus solfataricus* MT4, a hyperthermophilic archaeon first isolated from hot mud in the Solfatara crater north of Naples grows optimally at 87°C and expressed a GH activity, initially characterized as a β-galactosidase on the basis of hydrolysis of the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Subsequent enzymatic analysis showed

that this enzyme had several exo-β-glycosidase substrate specificity (Grogan, 1991). The amino acid sequence derived from the *lacS* gene put the enzyme in family1 of the β-glycosyl hydrolases, along with bacterial β-glycosidases, 6-phospho-β-galactosidases, cyanogenic β-glycosidases, plant myrosinases and mammalian gut lactases. The Ss-β-gal that had optimal activity with a half-life of 48h at 85°C was not thermally denatured under 100°C (Moracci *et al*., 1992) and resistant to denaturation by organic solvents that can be very useful to synthesize a variety of glycosides by transglycosidation and condensation (Trincone *et al*., 1994). Aguilar *et al.* (1997) reported the structure of the native tetrameric enzyme, and site-directed mutagenesis and homology with other GH-1 glycosidases have allowed to identify the active site of the enzyme and to define the substrate binding site. From analysis of the refined structure, the main feature that distinguishes this enzyme from mesophilic proteins was the presence of a large number of ion-pair networks which crosslink the surface of the protein. This feature, coupled with the observation of substantial numbers of buried water molecules, suggests that this enzyme (possibly other hyperthermophile proteins) may be achieved its hyperthermostability by resilience rather than rigidity. Hyperthermostable β-galactosidases from *Pyrococcus furiosus* (EP 0687732, 2002; EP 0606008, 2004) and *Pyrococcus woesei* (Dąbrowski *et al*., 1998, 2000) have also cloned and sequenced, but the structures are not known. Their hydrolytic activities on lactose have not been studied, despite the high chromogenic activities on ONPG or X-gal.

1.4. OTHER GLYCOSIDE HYDROLASE FAMILIES

1.4.1. Glycoside hydrolase family 42 (β-galactosidase)

1.4.1.1. Substrate specificity

The most common activity for glycoside hydrolases of this family are β-galactosidases (EC 3.2.1.23), however, other commonly found activities are α-L-arabinosidase (EC 3.2.1.55) and β-D-fucosidase (EC 3.2.1.38) with both K_m and k_{cat} values being of the same order of magnitude for the different substrates (Kosugi *et al*., 2002; Di Lauro *et al*., 2008).

Family GH42 enzymes have been identified only in unicellular organisms, mainly from prokaryotes (bacteria), and with a few examples from archaea and fungi. GH42 enzymes are active on lactose (Kang *et al*., 2005; Yuan *et al*., 2008) and transgalactosylation was observed with production of galactooligosaccharides (Møller *et al*., 2001). However, some GH42 enzymes are extracted from diverse habitats where lactose would not be present and they are active on galactooligosaccharides, suggesting that these enzymes would be involved *in vivo* in plant cell wall degradation. This feature could be performed in cooperation with family GH53 galactanases, often encoded from genes adjacent to GH42 genes (Shipkowski & Brenchley, 2006), and with cellulosome (Kosugi *et al*., 2002).

The activity of GH42 enzymes on lactose and also lactulose (Di Lauro *et al*., 2008) has interesting potential for the removal of the former from dairy products and to monitor lactulose concentration during heat treatment leading to UHT milk.

1.4.1.2. Catalytic residues

The catalytic nucleophile was first identified in the *Bacillus subtilis* YesZ β-galactosidase as Glu295 through the use of a mechanism-based inhibitor that allowed trapping of the 2-deoxy-2-fluorogalactosyl-enzyme intermediate and subsequent peptide mapping. These experiments were performed on the mutant of the inferred acid/base, which was more sensitive to the inhibitor (Shaikh *et al*., 2007). The acid/base catalyst was first identified as Glu157 in the β-galactosidase from *Alicyclobacillus. acidocaldarius* through detailed mechanistic analysis and azide rescue experiments of a mutant in that position (Di Lauro *et al*., 2008).

1.4.2. β- Mannosidase

β-Mannan and its heteropolysaccharides are found in endosperms of copra, ivory nuts, guar beans, locust beans, coffee beans, the roots of konjak, and hemicellulose of soft and hardwoods. Some β-mannans from agricultural crops were used as food additive for maintaining desired consistency or non-caloric dietary fiber. Oligosaccharides from these polysaccharides by an enzymatic hydrolysis are expected to be healthy material, such as fructooligosaccharide. The hydrolysis of β-mannan is catalyzed by β-mannanase (endo-type, EC 3.2.1.78) and β-mannosidase (exo-type, EC 3.2.1.25). These enzymes were produced by various microorganisms (Kurakake & Komaki, 2001).

Hemicellulases are key components in the degradation of plant biomass and carbon flow in nature. The substrates of these enzymes, the hemicelluloses, are a heterogeneous group of branched and linear polysaccharides that are bound via hydrogen bonds to the cellulose microfibrils in the plant cell wall, crosslinking them into a robust network. Hemicelluloses are also covalently attached to lignin, forming together with cellulose a highly complex structure. The degradation of cellulose and hemicellulose is carried out by microorganisms that can be found either free in nature or as part of the digestive tract of higher animals. The variable structure and organization of hemicellulose require the concerted action of many enzymes for its complete degradation (Shallom & Shoham, 2003).

The catalytic modules of hemicellulases are either glycoside hydrolases (GHs) that hydrolyze glycosidic bonds, or carbohydrate esterases (CEs), which hydrolyze ester linkages of acetate or ferulic acid side groups. These catalytic modules, based on homology of their primary sequence, can be assigned into families marked by numbers (e.g. GH7 for glycoside hydrolase family 7). Some families, with overall similar fold, can be further grouped into clans, marked alphabetically (e.g. GH-A).

The various chemical bonds hydrolyzed by hemicellulases and the classifications of these enzymes into families are xylanases (EC 3.2.1.8), β-mannanases (EC 3.2.1.78), β-mannosidases (EC 3.2.1.25), α-L-arabinofuranosidases (EC 3.2.1.55) and β-xylosidases (EC 3.2.1.37) (Shallom & Shoham, 2003).

β-Mannosidase (EC 3.2.1.25) is an enzyme that catalyzes the hydrolysis of terminal, non-reducing beta-D-mannose residues in beta-D-mannosides. The enzyme plays a role in the lysosomal degradation of the N-glycosylprotein glycans. β-

mannosidases are also found in higher animals and are involved in lysosomal degradation of glycoproteins.

In another study, the substrate specificities of two GH1 enzymes, β-mannosidase and β-glycosidase, were analyzed by constructing several reciprocal replacements of two active-site-conserved residues (Kaper *et al.*, 2002): in the β-glycosidase, both substitutions increased the specificity for mannosides, whereas in the β-mannosidase, one of the replacements resulted in improved catalysis towards glucosides. In combination with inhibition studies, it was concluded that the mutated residues are directly involved in the stabilization of the transition states, and also participate in the ground-state binding of substrates with the equatorial C2-hydroxyl (Shallom & Shoham, 2003). Fungi and bacteria that are able to degrade hemicellulose also secrete β-mannosidase. Fungal and bacterial β-mannosidases hydrolyze β(1-4)-D-mannosyl groups from manno-oligosaccharides and mannose-containing glycopeptides that are produced from the hemicellulose pulp by endoenzymes (Kuhad *et al.*, 1997). In seeds that have galactomannans as storage carbohydrates, the enzyme converts mannooligosaccharides to monosaccharides (Dey & Del Campillo, 1984).

Defects in the lysosomal form of the enzyme in humans result in a buildup of mannoside intermediate metabolites and the disease beta-mannosidosis. Deficiency in these enzymes in humans and ruminants (termed β-mannosidosis) leads to mental retardation and skeletal abnormalities. Beta-mannosidosis is an autosomal recessive lysosomal storage disease of glycoprotein catabolism caused by a deficiency of lysosomal beta-mannosidase activity (Bedilu *et al.*, 2002). The disorder was first

described in goats (Jones & Dawson, 1981), who have a more severe neurodegenerative disorder than that seen in humans. In the case of man, clinical manifestations are heterogeneous and include mental retardation, peripheral neuropathy, and skeletal abnormalities (Rodriguez *et al*., 1996).

1.5. Hyperthermophilic Extremozymes

Extremophiles are a group of microorganisms that can survive and even thrive in extreme environments. They are classified according to the conditions under which they grow at the extremes of physical parameters as (hyper)thermophiles (at high temperature), psychrophiles (at low temperature), acidophiles (at low pH), alkaliphiles (at high pH), piezophiles (at high pressure) and halophiles (at high salinity). Therefore, their biomolecules such as proteins, nucleic acids, and lipids have been evolved to function properly under these severe conditions (Schiraldi *et al*., 2000).

Due to these particular properties, enzymes from extremophiles (extremozymes) offer a high potential not only for basic research but also for biotechnological applications. Potentially, extremozymes would enable us to expand the range of reaction conditions suitable for biocatalysis. This has proven to be the case and various applications in sugar chemistry, detergent production, lipid and oil chemistry, and food processing have been initiated or are being explored (Demirjian *et al*., 2001; van den Burg, 2003).

Thermophilic microorganisms have attracted most attention, among which the most studies are toward the extremophile. Thermophiles can be generally classified into moderate thermophiles (growth optimum temp 50–60 °C), extreme thermophiles (growth optimum temp 60–80 °C) and hyperthermophiles (growth optimum temp 80–110 °C). Of all extremozymes, thermophilic enzymes have attracted most attention during the past four decades. Such enzymes are of great industrial and biotechnological interest due to the fact that the enzymes are better suited for harsh industrial processes. There are many advantages of conducting industrial processes at high temperature, such as the increased solubility of many polymeric substrates, resulting in decreased viscosity, increased bioavailability, faster reaction rate and the decreased risk of microbial contamination (Haki & Rakshit, 2003). These enzymes have also been used as models for the understanding of thermostability and thermoactivity, which is useful for protein engineering. Structural features of thermophilic extremozymes have attracted much attention. Several three-dimensional structures have been resolved and compared with those of mesophilic counterparts, with the ultimate goal of elucidating the mechanisms underlying thermostability (Gomes & Steiner, 2004).

A large number of sequence and structural factors are thought to contribute toward higher intrinsic thermal stability of proteins from thermophiles and hyperthermophiles. Thermophiles produce special proteins called 'chaperonins', which are thermostable and resistant to denaturation and proteolysis. Proteins of thermophiles, denatured at high temperature, are refolded by the chaperonins, thus restoring their native form and function. The cell membrane of thermophiles also consists of saturated fatty acids, which increase protein core hydrophobicity and keep the cell rigid enough to

survive at high temperatures. Moreover, hyperthermophiles have membranes containing lipids linked with ether to their cell walls. This layer is much more heat resistant than a membrane formed of fatty acids. In addition, proteins of thermophiles have increased surface charge and less exposed thermolabile amino acids. Thus, increased ionic interaction and hydrogen bonds, increased hydrophobicity, decreased flexibility and smaller surface loops confer stability on the thermophilic protein (Gomes & Steiner, 2004).

Heat-tolerant enzymes are investigated currently the most of all extremozymes. Such extremozymes are obtained from either extreme thermophiles that grow optimally at temperatures above 60°C or from hyperthermophiles with optimal growth temperatures above 90°C. Extreme thermophiles include both archaeal and bacterial species, while the vast majority of hyperthermophiles are members of the domain Archaea (Adams, 1993). Species found at the highest temperatures (103–113°C) are exclusively archaea. Most of hyperthermophiles encompassing more than 70 species, 29 genera, and 10 orders are archaea among which *Thermotogales* and *Aquificales* are the only bacteria (Stetter, 1996).

Application interest in thermophilic extremozymes is related to the fact that performing biotechnologically related processes at higher temperatures is often advantageous. For instance, in chemical reactions involving organic solvents, the decrease in viscosity and increase in diffusion coefficient that is achieved at elevated temperatures result in higher reaction rates (Krahe *et al*., 1996). Such considerations are relevant to a variety of processes including those using hydrophobic compounds with low solubility. High temperatures can also enhance the availability of such compounds for biodegradation efforts. Furthermore, reactions at higher temperatures reduce the

possibility of complications due to contamination. Because of their capability to function at such high temperatures, hyperthermophilic extremozymes have been the main focus of biotechnological interest (Eichler, 2001).

The recent advent of genomic research has produced vast amounts of sequence information for many different taxa of Bacteria, Eukarya, and Archaea, now collected in databases such as GenBank; the full genome sequences of more than 699 different microorganisms have been completed [www.ncbi.nlm.nih.gov/sutils/genometable.cgi, 2010]. By a generally applicable combination of conventional genetic engineering and genomic research techniques, many extremely thermostable enzymes are being developed for biotechnological purposes. The genome sequences of some hyperthermophilic microorganisms such as *Thermotoga maritima, Pyrococcus furiosus,* and *Sulfolobus solfataricus* are of considerable biotechnological interest because they encode many highly heat-stable enzymes that are active under conditions previously regarded as incompatible with biological materials (Nelson *et al*., 1999; Schiraldi *et al*., 2000).

In the genome sequences of hyperthermophilic Archaea and Bacteria, number of gene sequences in glycoside hydrolase family is summarized based on the web accessible CAZY (Carbohydrate-Active Enzymes) database (Table 1) [http://www.cazy.org/CAZY/, 2010]. Amylolytic enzymes are of great significance in many industrial processes including those for foods, textiles, and detergents. Many hyperthermophilic microorganisms possess starch-hydrolyzing enzymes, such as α-amylase, α-glucosidase, pullulanase, and cyclodextrinase, in their genomes even though they live in environments where starch is rare (Bibel *et al*., 1998).

Analysis of the full genome of *Pyrococcus furiosus*, a hyperthermophilic archaeon, has revealed that this microorganism has several amylolytic enzymes. An amylopullulanase and two distinct α-amylase genes of *Pyrococcus furiosus* were identified and expressed in *E. coli* (Laderman *et al*., 1993; Dong *et al*., 1997). These enzymes can hydrolyze a wide variety of substrates such as soluble starch, amylose, amylopectin, glycogen, and oligosaccharides. However, α-amylase does not hydrolyze pullulan and CD (cyclodextrin), whereas amylopullulanase can degrade pullulan.

1.5.1. Lactose hydrolyzing enzymes from hyperthermophiles

1.5.1.1. β-galactosidase

Lactose is a primary carbohydrate contained in mammal's milk and dairy products (at a concentration between 5 and 10 %, depending on the source of milk) and is also called milk sugar. The consumption of lactose or lactose containing foods can cause digestion problem for almost a 70 % of the world population, as the enzyme naturally present in human intestine loses its activity during lifetime. Moreover, low solubility and sweetness of lactose lead to huge interest in industrial development of processes to hydrolyze lactose and lactose contained dairy foods. Lactose, a natural disaccharide, is consists of monosaccharides, glucose and galactose bonded by a beta-1, 4 linkages. Lactose can be hydrolyzed to monomers, glucose and galactose by enzyme (β-galactosidase) or acids.

β-Galactosidase (EC 3.2.1.23) is a common name of β-D-galactoside galactohydrolase and also called lactase (EC 3.2.1.108). Lactase cleaves the β-1, 4-glycosidic linkage of lactose and gives rise to its component monosaccharides, glucose and galactose. The hydrolysis of lactose in dairy products by lactase can be beneficial in many regards; (1) the hydrolysis of lactose alleviates problems and improves processes for dairy products, (2) the low solubility and lack of sweetness that are often experienced in concentrated milk products and ice cream could be overcome by lactose hydrolysis, (3) cheeses that have been manufactured from hydrolyzed milk ripens more quickly than that made from normal milk (Kim *et al*., 1997), and (4) the use of lactase could also reduce the amount of the lactose in whey, which can cause environmental pollution when discharged in large quantities (Gekas & Lopez-leiva, 1985).

Lactose hydrolyzed (lactose free) milk can reduce lactose intolerance problem and lactose hydrolyzed whey syrup can be utilized in frozen desserts, confectionary, bakery, fermentation products, and beverages. The galacto-oligosaccharides can also be employed as probiotic food ingredients, humectants, and emulsifiers (Lee, 2008).

Table 1.1. Carbohydrate-Active Enzymes (CAZYs) in glycoside hydroase family (GHF), http://www.cazy.org/CAZY/. Archaea are indicated by the shaded box.

Hyperthermophilic microorganisms	Completed	Genome size (bp)	Growth temp. (°C)	Putative CAZYmes
Aeropyrum pernix	2001	1,669,695	95	0
Archaeoglobus fulgidus DSM 4304	1997	2,178,400	83	0
Methanococcus jannaschii DSM 2661	2001	1,664,970	85	2
Methanopyrus kandleri AV19	2002	1,694,969	98	8
Nanoarchaeum equitans Kin4-M	2003	490,885	90	0
Pyrobaculum aerophilum IM2	2001	2,222,430	100	6
Pyrococcus abyssi GE5 Orsay	2001	1,765,118	103	7
Pyrococcus furiosus DSM 3638	2002	1,908,256	100	15
Pyrococcus horikoshii OT3	2001	1,738,505	98	8
Sulfolobus solfataricus P2	2001	2,992,245	85	22
Sulfolobus tokodaii 7	2001	2,694,756	80	14
Thermococcus kodakarensis KOD1	2005	2,088,737	85	13
Aquifex aeolicus VF5	2001	1,551,335	96	7
Thermoanaerobacter tengcongensis MB4	2001	2,689,445	75	24
Thermotoga maritima MSB8	2001	1,860,725	80	41
Total				**167**

http://afmb.cnrs-mrs.fr/CAZY/

1.5.1.2. Galacto-oligosaccharide formation by β-galactosidase-transgalactosylation

β-Galactosidase hydrolyzes terminal, non-reducing β-D-galactose residues in β-D-galactosides or lactose, but some of this enzyme catalyzes both hydrolytic and reverse transgalactosylation (EC 2.4.1.22: galactosyl transferase) reaction (Bourne & Henrissat, 2001). The transferase activity by a β-galactosidase that produce oligosaccharides was reported in the early 1950s (Pazur, 1953). Apart from theoretical aspects, early research was prompted by nutritional concerns about the presence of these compounds in low-lactose milk (Burvall *et al*., 1980). Later studies were based on the need to consider oligosaccharide formation when modelling lactose hydrolysis (Prenosil *et al*., 1987). More recently, interest in the reaction has been raised by observation that oligosaccharides may have beneficial effects as 'bifidus factors'-promoting the growth of desirable intestinal microflora. Also, the transferase reaction can be used to attach galactose to other chemicals and consequently has potential applications in the production of food ingredients, pharmaceuticals and other biologically active compounds (Mahoney, 1998). Lactose hydrolysis catalysed by β-galactosidases has proven to be a very complex reaction. Apart from the actual hydrolysis product, glucose and galactose, many newly formed β-glycoside, mainly di, tri, and tetrasaccharide, occur as kinetic intermediates, derived from so-called transgalactosylation reaction (Nakayama & Amachi, 1999). Because transgalactosylation products (galacto-oligosaccharides) are substrate of β-galactosidases-catalyzed hydrolysis, the composition of the product mixture changes quite significantly with progressing reaction time (Nakayama & Amachi, 1999).

The specific properties of oligosaccharides are very different depending on the formation of oligosaccharides, but some properties are common to almost all oligosaccharides. The sweetness of the oligosaccharide depends on structure and molecular mass of the oligosaccharides (Crittenden & Playne, 1996). Oligosaccharides are normally water soluble and mildly sweet, typically lower than sucrose and this low sweetness is useful in food production when reduced sweetness is desirable to enhance other food flavors. Compared with mono- and disaccharides, the higher molecular weight of oligosaccharides provides increased viscosity, leading to improved body and mouthfeel. They can also be used to alter the freezing temperature of frozen foods, and to control the amount of browning due to Maillard reactions in heat-processed foods. Oligosaccharides provide a high moisture-retaining capacity, preventing excessive drying, and a low water activity, which is convenient in controlling microbial contamination.

Although oligosaccharides possess these useful physicochemical characteristics, most of the interest in their use as food ingredients stems from their many beneficial physiological properties. Unlike starch and simple sugars, the currently available food-grade oligosaccharides are not utilized by mouth microflora to form acid or polyglucans. Hence, they are used as low-cariogenic sugar substitutes in confectionery, chewing gums, yogurts and drinks. Many oligosaccharides are not digested by humans (Tomomatsu, 1994). Oligosaccharides have recently been described as one of several ‘prebiotics’, which can stimulate the growth of beneficial microflora (Gibson & Roberfroid, 1995).

Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon". They are recognized for their ability to increase levels of health promoting bacteria in the intestinal tract of humans or animals. Some studies have shown that prebiotics target the activities of

bifidobacteria and/or lactobacilli (Tannock, 2002). Both the volume and diversity of oligosaccharide products are increasing very rapidly as their functional properties become further understood. Detailed production methods for various oligosaccharides have been reviewed by Playne (1994).

1.5.2. Mannose hydrolyzing enzymes from hyperthermophiles

The complexity of cell wall-degrading enzyme systems is a consequence of the complex nature of plant cell wall. Hemicelluloses act as linkers between lignin and cellulose. The high percentage of hemicellulose fraction in the cell wall of higher plants makes this material the second most abundant biopolymer in nature. Besides xylan, mannan is the other major hemicelluloses constituent. Galactomannan, found in large quantities in seeds of leguminous plants, is composed of a homogeneous backbone of β-1, 4-linked mannose residues, whereas acetylated galactoglucomannan, a main constituent of softwoods, has a heterogeneous backbone of β-1, 4-linked glucose and mannose residues (Bacic *et al*., 1988). The complete conversion of galactomannan into galactose and mannose requires the activity of three types of enzymes, namely, endomannanases (EC 3.2.1.78), β-galactosidases (EC 3.2.1.22), and β-mannosidases (β-D-mannopyranoside hydrolase [EC 3.2.1.25]). Endomannanases catalyze the random hydrolysis of the β-1, 4-mannosidic backbone of the main mannan chain, β-galactosidases cleave the terminal β-1, 6-linked D-galactosyl residues, and β-mannosidases hydrolyze β-1, 4-linked mannose residues from the nonreducing ends of various oligosaccharides (Bacic *et al*., 1988).

Almost every β-mannosidase belongs to family 2 of glycosyl hydrolases (GHs), except for the *Pyrococcus* sp. β mannosidase, which has been assigned to family 1 (Bauer *et al*., 1996). Hemicellulases are widely used in coffee bean fermentation to promote the hydrolysis of β-mannan-based oligosaccharides (Sachslehner *et al*., 2000).

In general, mannosidases constitute only a small percentage of the proteins secreted by hemicellulose degrading organisms; their purification is therefore rather difficult. This problem could be solved by cloning and heterologous expression of mannosidase-encoding genes.

Given the natural abundance of hemicellulose (heteroglycans), it is not surprising that many microorganisms have enzyme systems for its hydrolysis (Hazlewood & Gilbert, 1993). Moreover, given the variety and complexity of hemicellulases, several biocatalytic steps are typically required to hydrolyze specific polysaccharides completely into simpler sugars that can be readily used as carbon and/or energy sources by particular microorganisms. Hetero-1, 4-β-D-mannans, one of the major constituents of hemicellulose, are hydrolyzed to mannose through endo-acting β-mannanases (1, 4-β-D-mannan mannanohydrolase [EC 3.2.1.78]) (McCleary, 1988a), and exo-acting β-mannosidases (β-D-mannopyranoside hydrolase [EC 3.2.1.25]) (McCleary & Matheson, 1983; Mccleary & Matheson, 1987). Additional enzymes are required to remove side chain sugars that are attached at various points on mannans. For example, galactomannans have galactose residues bound to the mannan backbone, and their removal is effected through the action of α-galactosidases (α-D-galactoside galactohydrolase [EC 3.2.1.22]) (McCleary, 1988b). β-Mannan-based natural polymers have wide-ranging industrial applications, such as those used in the processing of foods and the massive hydraulic fracturing of oil and gas wells. Recent developments in the oil and gas industries have established a need for the in situ enzymatic hydrolysis of galactomannans used in well stimulation (McCutchen *et al*., 1996). In these applications, enzyme

thermostability and thermoactivity are factors. In food processing, for reasons of asepsis and the need to process viscous materials, elevated temperatures provide obvious advantages. In oil and gas well stimulation, temperatures exceeding 100 °C are characteristic of the deeper reaches of well bores (McCutchen *et al*., 1996). Thus, the availability of thermostable and thermoactive versions of enzymes involved in the hydrolysis of β-mannan-based natural polymers is necessary.

1.6. Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) is the only technique that can directly measure the binding energetics of biological processes, including protein-ligand binding, protein- protein binding, DNA-protein binding, protein-carbohydrate binding, protein-lipid binding, and antigen-antibody binding. ITC has the ability to precisely determine the Gibbs energy, enthalpy, entropy, and heat capacity changes associated with binding.

1.6.1. Principles of the ITC

The ITC instrument is a heat-flux calorimeter operating according to the dynamic power compensation principle, i.e., it measures the amount of power (μcal/sec) required to maintain a constant temperature difference (close to zero) between the sample and the reference cell. Each injection of the syringe solution (usually termed as ligand) triggers the binding reaction and, depending on the binding affinity and the concentration of reactants (macromolecule, M, and ligand,

L) in the cell, a certain amount of macromolecule/ligand (ML) complex is formed. The formation of complex is accompanied by the release (exothermic reaction) or the absorption (endothermic reaction) of heat that causes a difference in temperature between the two cells. Then, the feedback system either lowers or raises the thermal power applied to compensate such temperature unbalance.

After each injection, the system reaches equilibrium and the temperature balance is restored. Integrating the area under the peak, assuming the baseline as reference, provides the amount of heat associated with the injection. As the reactant in the cell becomes saturated, the heat signal diminishes until only the background heat is observed (Freire, 2004).

1.6.2. Instrumentation of ITC

Two identical coin-shaped cells, sample and reference, are enclosed in a jacket. The temperature difference between the reference cell and the jacket is continuously monitored to maintain a constant temperature. A feedback control system monitors the difference in temperature between the two cells through a semiconductor Peltier sensor device sandwiched between them. This temperature difference is kept constant and as close to zero as possible at any time.

One of the samples is placed in the sample cell and the other one in the injection syringe. During the course of an experiment, the syringe is used to add the titrant reactant to the sample cell in a stepwise fashion and, at the same time, stir the solution in the sample cell to achieve fast mixing. The reference cell serves only as a temperature reference (Freire, 2004).

1.6.3. Enzyme kinetics using ITC

ITC is well-established in the study of affinity of molecular interactions, and is now becoming a popular tool in the study of enzyme kinetics. Traditional enzyme assays utilize a probe to monitor either substrate depletion or product formation. These probes are system-dependent and must be optimized for each reaction under specific conditions. Also, the substrate may need to be modified which could interfere with the catalysis reaction. For optical methods, the experimental conditions can affect the detection system, preventing accurate measurements. This means that with traditional assay methods, many enzymes do not have practical assays.

ITC uses heat as a probe, and since every reaction generates or absorbs heat, there is no need for lengthy method development each time when a new enzyme is assayed. ITC directly measures the heat change as catalysis proceeds, which is proportional to the rate of reaction. K_m and k_{cat} from ITC experiments agreed favorably with traditional enzyme kinetics methods, and can be used with every class of enzyme, including those with no other direct assay methods (Todd & Gomez, 2001).

The use of ITC to monitor the rate of enzymatic reactions is a non-destructive, sensitive, and direct assay. Multiple injections of substrate can be done in a single experiment, so K_m and k_{cat} can be determined in a single ITC experiment. With ITC, it is straightforward to vary experimental conditions such as pH and ionic strength, and one can get a complete analysis of catalysis and kinetics.

Chapter 2

Expression and Characterization of an Extremely Thermostable Beta Glycosidase (Mannosidase) from the Hyperthermophilic Archaeon *Pyrococcus Furiosus* DSM3638

Glycoside hydrolases are enzymes catalyze that hydrolysis of the glycosidic linkage of glycosides, leading to the formation of a sugar hemiacetal or hemiketal and the corresponding free aglycon. Glycoside hydrolases are also referred to as glycosidases, and sometimes also as glycosyl hydrolases. Glycoside hydrolases can catalyze the hydrolysis of O-, N- and S-linked glycosides. Cloning glycoside hydrolases from hyperthermophiles will be useful in sugar industries due to its fast reaction rate. This chapter addressed the first research objective on the expression and characterization of hyperthermophilic β-glycosidase (mannosidase).

2.1. ABSTRACT

Genomic analysis of the hyperthermophilic archaeon *Pyrococcus furiosus* revealed the presence of an open reading frame (ORF PF0356) similar to the enzymes in glycoside hydrolase family 1. This β-glycosidase, designated PFTG (*Pyrococcus furiosus* thermostable glycosidase), was cloned and expressed in *Escherichia coli*. The expressed enzyme was purified by heat treatment and Ni-NTA affinity chromatography. The gene was composed of 1452 bp encoding 483 amino acids for a protein with a predicted molecular mass of 56,326 Da. The temperature and pH optima were 100°C and 5.0 in sodium citrate buffer, respectively. The substrate specificity of PFTG suggests that it possesses characteristics of both β-galactosidase acitivity and β-mannosidase activity. However, through kinetic studies by ITC (Isothermal Titration Colorimetry) which is very sensitive method for enzyme kinetics, PF0356 enzyme revealed the highest catalytic efficiency toward *p*-nitrophenyl-β-D-mannopyranoside (pNP-Man) with k_{cat}/K_m (3.02). The enzyme showed transglycosylation and transgalactosylation activities toward cellobiose, lactose and mannooligosaccharides, that could produce GOS (galacto oligosaccharides) and MOS (maltooligosaccharides). This novel hyperthermostable β−glycosidase may be useful for food and pharmaceutical applications.

Keywords: β-glycosidase; β-mannosidase; hyperthermophile; *Pyrococcus furiosus*; hyperthermostable enzyme; ITC (Isothermal Titration Calorimetry)

2.2. INTRODUCTION

Extremophiles are a group of microorganisms that can survive and even thrive in extreme environments. They are classified according to the conditions under which they grow at the extremes of physical parameters as (hyper)thermophiles (at high temperature), psychrophiles (at low temperature), acidophiles (at low pH), alkaliphiles (at high pH), piezophiles (at high pressure) and halophiles (at high salinity) (Gunde *et al*., 2005). Therefore, their biomolecules such as proteins, nucleic acids, and lipids have been evolved to function properly under these severe conditions. Due to these particular properties, enzymes from extremophiles (extremozymes) offer a high potential not only for basic research but also for biotechnological applications. Potentially, extremozymes would enable us to expand the range of reaction conditions suitable for biocatalysis. This has proven to be the case and various applications in sugar chemistry, detergent production, lipid and oil chemistry, and food processing have been initiated or are being explored (Demirjian *et al*., 2001; van den Burg, 2003).

Heat-tolerant enzymes are obtained from either extreme thermophiles that grow optimally at temperatures above 60°C or from hyperthermophiles with optimal growth temperatures above 90°C. Extreme thermophiles (or hyperthermophiles) including both archaeal and bacterial species are members of the domain Archaea. Species found at the highest temperatures (103–113°C) are exclusively archaea. Among hyperthermophiles encompassing more than 70 species, 29 genera, and 10 orders are archaea, *Thermotogales* and *Aquificales* are the only bacteria (Stetter, 1996). Studies on the extremozymes from hyperthermophilic archaea have been mainly focused on the strains belonging to the genera *Pyrococcus* and *Thermococcus* (Adams, 1993). The heat-stable extremozymes not only serve as excellent models for understanding protein stability but also carry

significant biotechnological potential. Application interest in thermophilic extremozymes is related to the fact that performing biotechnologically related processes at higher temperatures is often advantageous. Such considerations are relevant to a variety of processes including those using hydrophobic compounds with low solubility. High temperatures can also enhance the availability of such compounds for biodegradation efforts. Furthermore, reactions at higher temperatures reduce the possibility of complications due to contamination. Because of their capability to function at high temperatures, hyperthermophilic extremozymes have been focused of biotechnological interest.

Applications of hyperthermophilic beta-galactosidase for lactose hydrolysis and galactooligosachrides as a prebiotic bifidogenic factor (Lee, 2008) and beta-mannosidase as one of hemicellulases for abundant galactomannan or hemicellulose (Duffaud *et al*., 1997) are well known. The aim of this research was to overexpress and characterize β-glycosidase (β-mannosidase) of *Pyrococcus furiosus DSM3638*.

2.3. MATERIALS AND METHODS

2.3.1. Bacterial strains, plasmids, and culture conditions

E. coli MC1061 [F_*araD139 recA13* (*araABC-leu*) *7696 galU galK lacX74 rpsL thi hsdR2 mcrB*] and BL21(DE3) (Novagen Inc., Gibbstown, NJ) were used as hosts for gene cloning and expression, respectively. Plasmid pET-28b(+) (Novagen) was used as a cloning and expression vector. *E. coli* cultures were grown at 37°C either on Luria-Bertani (LB) agar containing 1% (wt/vol)

Bacto-tryptone, 0.5% (wt/vol) yeast extract, and 1% (wt/vol) NaCl, or in LB broth supplemented with kanamycin (100 μg /ml) or chloramphenicol (34 μg /ml) if needed.

2.3.2. Chemicals and media

4-nitrophenyl-β-D-mannopyranoside(ManpβNp), 4-nitrophenyl-β-D-galactopyranoside(GalpβNP), 4-nitrophenyl-β-D-glucopyranoside(GlcpβNp),4-nitrophenyl-β-D-xylopyranoside(XylpβNP), cellobiose and lactose were purchased from Sigma (St. Louis, MO). Mannobiose and mannotriose were purchased from Megazyme (Wicklow, Ireland). Bacto yeast extract and Bacto tryptone were purchased from BD Diagnostics (Sparks, MD). All restriction enzymes and calf intestine alkaline phosphatase were purchased from New England Biolabs, Inc. (Ipswich, MA). *Pfu* Turbo DNA polymerase, T4 DNA ligase, and protein molecular weight standards were supplied by Stratagene (La Jolla, CA). All other chemicals were of reagent grade and were purchased from Fisher scientific (Ottawa, ON).

2.3.3. General DNA manipulation, DNA sequencing, and computer analysis

The genomic DNA of *P. furiosus* DSM 3638 was kindly provided by A. M. Grunden of North Carolina State University (Raleigh, N.C.). In order to obtain the recombinant enzyme, the PF0356 gene was amplified with PCR with the *Pfu* DNA polymerase and the genomic DNA of *P. furiosus* as a template. The PF0356 gene-specific oligonucleotide primers flanking the 5′ and 3′

gene ends were designed from the known PF0356 gene sequence. The forward primer (PFTG-Fwd, 5′-CAATTGCTACA*CATATG*TATAAGCTCG-3′) and the reverse primer (PFTG-Rev, 5′-GCGGATGGGTA*CTCGAG*GAGGCACCACCT-3′) contained *Nco*I and *Xho*I restriction sites, respectively (italic). Amplification was performed at an annealing temperature of 55°C. The DNA fragment (1.5 kb) amplified by PCR was digested with *Nco*I and *Xho*I and ligated into the expression vector pET-28b (+) to finally create pET PFTG0356 - 6h. The nucleotide sequence of the PCR-generated gene was determined with the BigDye terminator cycle sequencing kit for the ABI 377 Prism (Perkin- Elmer, Norwalk, CT). The other genetic manipulations were performed as described by Sambrook & Russel, 2001. The initial similarity search was carried out with the BLAST program.

2.3.4. DNA preparation and gene structure analysis

Small scale preparation of plasmid DNA was carried out by following the procedure of Sambrook & Russell (2001). Plasmid DNA samples were isolated and purified using QIAprep Spin Miniprep Kit (Qiagen, Mississauga, ON) following the instruction of manufacturer.

The initial similarity search was carried out with the BLAST program (Altschul *et al*., 1990). Detailed analysis of the DNA and the deduced amino acids of various genes were performed with the DNASIS, PROSIS (v7.0, Hitachi Software, Tokyo, Japan), and CLUSTAL programs (Thompson *et al*., 1994).

2.3.5. IPTG induction and SDS-PAGE analysis

Prepared *E. coli* BL21(DE3) cell containing pET28PFTG plasmid was grown at 37°C in 50 ml of 2X YT medium (1.6 % tryptone; 1.0 % yeast extract; 0.5 % NaCl, pH 7.0) containing 40 µg/ml kanamycin until OD_{600} of 0.8 was reached. A portion (1 ml) of seed culture was inoculated in 50 ml 2X YT medium containing 40 µg/ml kanamycin with shaking at 250 rpm. Final concentration of 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside, invitrogen, Carlsbad, CA) was added into culture for further incubation for 7 h at 37°C. After IPTG was added, 5 ml of culture was withdrawn at 1 h intervals, that was assayed for the enzyme activities and growth rates. Samples (100 µl) were serially diluted and plated on LB agar plates in duplicate and incubated at 37°C for 16 h. The colonies were counted and expressed as colony forming units per ml (CFU/ml). Samples (4 ml) were centrifuged (8,000 g; 10 min), washed twice with sodium phosphate buffer (50 mM, pH 7.0) and resuspended in 500 µl of the same buffer. Cells were disrupted with sonicator (Fisher Scientific, Mississauga, ON) under ice bath. The disrupted cells were centrifuged (12,000 g, 20 min, 4°C) and the supernatants (cell free extracts) were assayed for the enzyme activities. The molecular mass of the recombinant enzyme was estimated by sodium-dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli *et al* (1970).

2.3.6. Differential scanning calorimetry (DSC)

Thermal inactivation of PFTG was analyzed by DSC using a microcalorimeter (MicroCal VP-DSC, Piscataway, NJ) operating over a temperature range from 20 to 130°C. The sample cell was pressurized to 219.59 kPa to prevent boiling at 100°C. The purified enzyme was dialyzed against 50 mM Tris-HCl buffer (pH 7.5) and concentrated to 0.78 mg/mL in 50 mM Tris-HCl buffer (pH 7.5) with a 0.45 μm Microcon filter (Millipore Co., Billerica, MA). The equilibrated enzyme was scanned at a rate of 1.0°C/min. The enzyme scan was corrected using a buffer-buffer baseline.

2.3.7. Assays of enzymes and protein

Enzyme activity was measured by modified procedure of Craven *et al*. (1965). Briefly, enzyme was reacted with 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma Chemical Co., St. Louis, MO) in 50 mM sodium citrate buffer (pH 5.0) at 100 °C for 10 min, and reaction was stopped by adding equal volume of 1.0 M Na_2CO_3. The released *o*-nitrophenyl was quantitatively determined by measuring the optical density at 420 nm. One unit of enzyme activity was defined as that amount of enzyme liberating one μmol of *o*-nitrophenyl per minute. Specific activity was defined as units per mg of protein. Each value is the mean of at least three independent determinations. Glycosides of *p*-nitrophenyl are frequently used as substrates in the analysis of β-glycosidase activities, because their enzymatic hydrolysis releases *p*-nitrophenol, which is readily detected by spectroscopic analysis at 405 nm (Chadwick *et al*., 1995).

The protein concentration was determined according to the Bradford method (Bradford *et al*., 1976), using Bio-Rad protein assay reagent (Bio-Rad, Mississauga, ON) and bovine serum albumin as a standard.

2.3.8. Effects of pH and temperature on the activity and stability of PFTG

For determining the optimal pH of PFTG, the relative activities against ONPG in various pH ranges from 3 to 8 were examined. The buffers used were 50 mM sodium citrate buffer (pH 3.0 to 5.0); 50 mM sodium acetate buffer (pH 4.0 to 6.0); 50 mM sodium phosphate buffer (pH 6.0 to 7.5); and 50 mM Tris-HCl buffer (pH 7.5 to 8.0). To examine the pH stability, the enzyme (0.1 mg/ml) was incubated at various pH solutions of 0.1 M Britton-Robinson buffer. After incubation at 37°C for 24 h, the remaining activity was measured at the optimal temperature under the standard conditions. The optimal temperature of PFTG activity was determined in 50 mM sodium citrate buffer (pH 5.0) in a range of 75 to 110°C. The thermostability of the purified enzyme dissolved in 50 mM sodium citrate buffer (pH 5.0) was analyzed by incubating the enzyme solution (0.1 mg/ml) at different temperatures (85, 90, 95, and 100°C), from which aliquots were taken at different time intervals and placed immediately in ice-water bath. The residual ONPG hydrolyzing activities of the aliquots were measured at the optimal temperature condition.

2.3.9. Production and identification of oligosaccharides by HPLC

The HPLC analyses were carried out in high performance liquid chromatography, HPLC (Millipore, Waters, maple street, Milford, MA), equipped with a 600 E system controller, a differential refractometer R401, a Millenium 2010 chromatography manager. Chromatographic separation of sugars was achieved with a cation-exchange column ION-300 (300mm x 7.8mm, Transgenomic Inc. CA, USA). The flow rate was adjusted to 0.25 ml/min with 0.02 N H_2SO_4 as mobile phase and column temperature was maintained at 55°C. Running time was set to 18 min. Peaks were identified by comparing retention times with sugar standards. The sugars were eluted with the sequence of oligosaccharides, lactose, glucose, and galactose. The respective peak areas were used for the quantitative analysis.

2.3.10. Determination of kinetic parameters by ITC (Isothermal Titration Calorimetry)

Calorimetric assays were carried out with ITC_{200} system instruments (MicroCal Inc, Piscataway, NJ). Reaction cells (200 µl) were filled with degassed solutions and equilibrated at the indicated temperatures. Stirring speed was 400 rpm, thermal power was recorded at every 2 sec, and instrumental feedback was 10–50%. Enzyme reaction rates are determined by measuring the change in instrumental thermal power supplied to the sample cell after addition of the substrate or enzyme through a stirred injection syringe. If the reaction in the cell generates heat (exothermic), the calorimeter must supply less power to maintain constant temperature, and a negative deflection in the

observed thermal power is observed. Conversely, if a reaction consumes heat (endothermic), the calorimeter must supply additional power to maintain isothermal conditions, and a positive deflection in the observed thermal power is observed (Todd & Gomez, 2001).

2.4. RESULTS AND DISCUSSION

2.4.1. Cloning of the glycoside hydrolase gene from *Pyrococcus furiosus*

2.4.1.1. Genomic analysis o*f Pyrococcus furiosus* DSM 3638

Genomic analysis of the hyperthermophilic archeon, *Pyrococcus furiosus* DSM 3638 (Maeder *et al*., 1999) revealed the presence of an open reading frame (ORF, PF0356) similar to the enzymes in glycoside hydrolase family 1 (GH1). As shown in Fig. 2.1, this glycosidase, designated as PFTG (*Pyrococcus furiosus* thermostable β-glycosidase), was flanked by genes encoding hypothetical proteins (PF0353) and di-peptide binding proteins (PF0357).

2.4.1.2. Cloning of the PFTG gene

To isolate the gene for PF0356, the *pf0356* gene was amplified using PCR with the *Pfu* DNA polymerase and the genomic DNA of *P. furiosus* as a template. The *pf0356* gene-specific oligonucleotide primers flanking the 5'- and 3'-gene ends were designed from the known *pf0356* sequence. The forward primer (PFTG-Fwd, 5'-CAATTGCTACA*CATATG*TATAAGCTCG-3') and the reverse primer (PFTG-Rev, 5'GCGGATGGGTA*CTCGAG*GAGGCACCACCTCCT-3') contained *Nco*I and *Xho*I restriction sites, respectively (italic). After amplication was performed at

an annealing temperature of 55°C, the DNA fragment (1.5 kb) amplified by PCR (Fig. 2.2) was digested with *Nco*I and *Xho*I and ligated into the expression vector pET-28b (+) to finally create pET28β-gal. The nucleotide sequence of the PCR-generated gene was determined with the BigDye terminator cycle sequencing kit for the ABI 377 Prism (Perkin- Elmer, Norwalk, CT). The other genetic manipulations were performed as described by (Sambrook & Russell, 2001). The initial similarity search was carried out with the BLAST program.

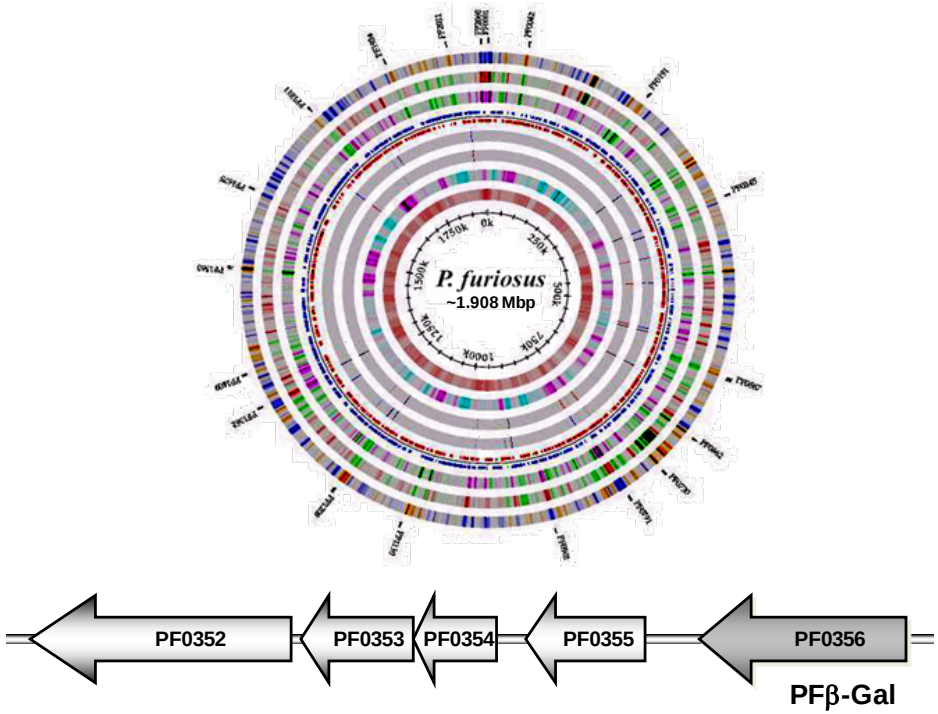


Figure 2.1. Schematic representation of annotated open reading frames in the *P. furiosus* genome sequence between positions 1783800 and 1792400 (Maeder *et al*., 1999). The PFTG-encoding gene (PF0356) is indicated in black. Genes encoding beta galactosidase precursor and hypothetical proteins (PF0353 to PF0355) are indicated in gray.

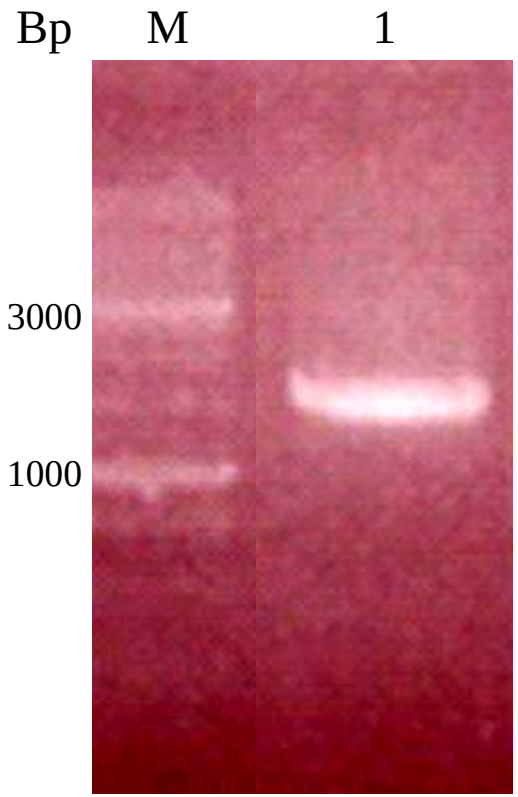


Figure 2.2. Agarose gel electrophoresis of the PCR products. Lane M was loaded with size marker; lane 1, the DNA fragment (~1.5 kb) amplified from the *P. furiosus* DSM 3638 genomic DNA.

2.4.1.3. Nucleotide sequence analysis of the PFTG gene

The nucleotide sequence of the gene amplified by PCR was determined using the BigDye terminator cycle sequencing kit for ABI 377 PRISM (Perkin-Elmer, Norwalk, CT). The nucleotide sequence and deduced amino acid sequence of PFTG were shown in Fig. 2.3. The putative translational initiation site was determined by comparison with the sequences of other glycoside hydrolase genes and by the structural features. An open reading frame of PFTG initiating from an ATG at nucleotide 1 and ending with a TAA at nucleotide 1,452 would encode a single polypeptide of 483 amino acids with an estimated molecular mass of 56,326 Da. The A/T enrichment of archaeal intergenic regions can be attributed to the presence of TATA box and transcription factor B recognition elements, BRE (Verhees *et al*., 2002) in which the consensus sequence of 19 mapped promoters of *P*. *furiosus* has a similar A/T bias position.

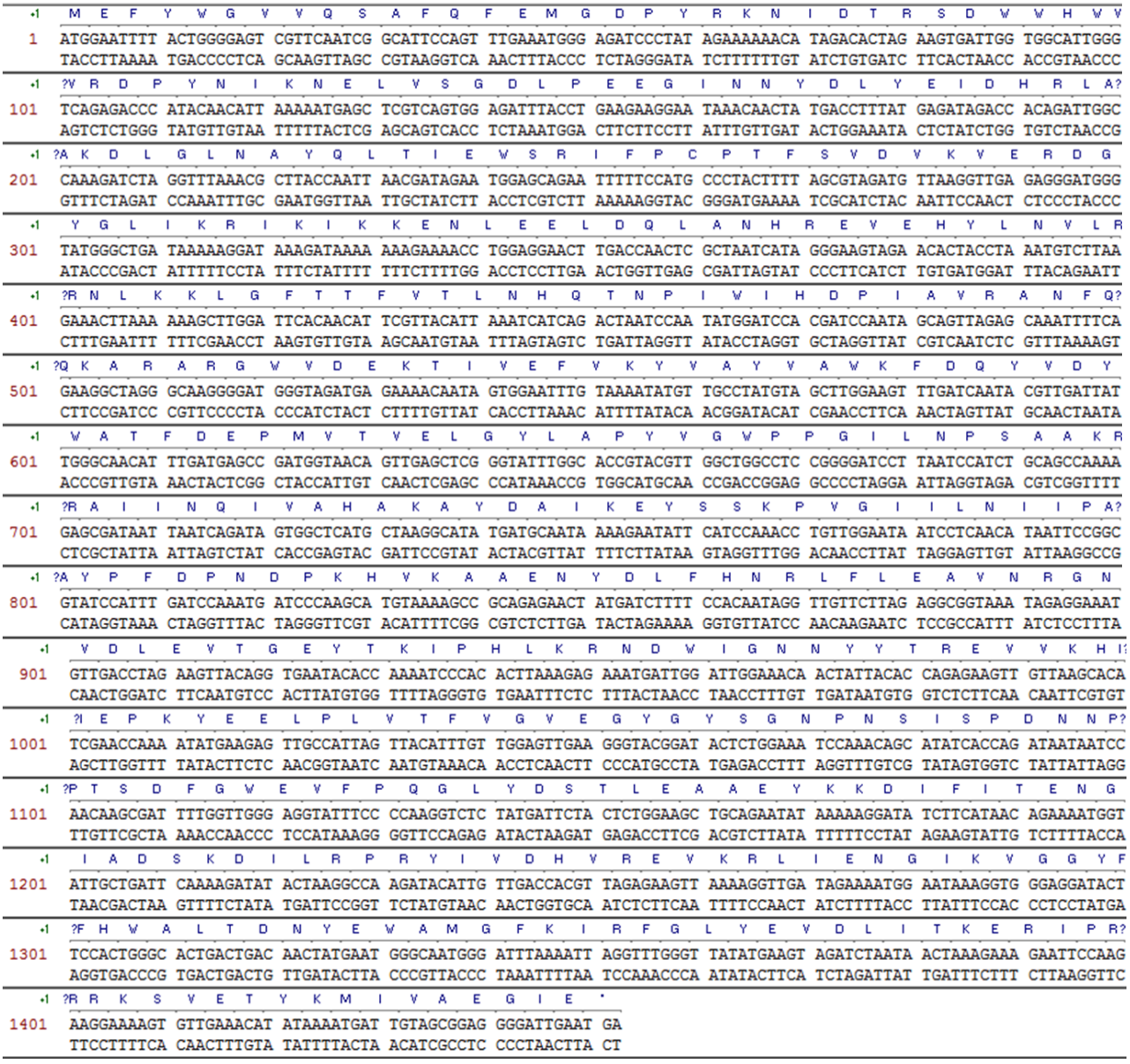


Figure 2.3. Deduced amino acid sequence of PFTG and nucleotide sequence.

2.4.1.4. Comparison of PFTG with glycoside hydrolase family1 (GH1)

The analysis of the PFTG0356 gene showed that it was highly homologous to the genes encoding glycoside hydrolases such as β-galactosidase, β-mannosidase, and β-glycosidase. As shown in Table 2.1, the predicted amino acid sequence of PFTG shared 88% identity with that of β-mannosidase from *Pyrococcus horikoshii OT3,* 84% with that of β-galactosidase from *Pyrococcus abyssi GE5*, and 74% with that of β-glycosidase of *Thermococcus kodakarensis* KOD1 (Table 2.1).

Based on both substrate specificity and amino acid sequence, the β-mannosidase and β-glycosidase are the most closely related. Both of these enzymes are exo-acting, β-specific glycosyl hydrolases that release the terminal, non-reducing sugars from β-glycosidic bonds. The β-mannosidase is most active with mannose as the terminal non-reducing sugar, while the β-glycosidase has its highest specific activity with glucose in this location (Kengen *et al*., 1993). This suggests a difference in the way that the two enzymes interact with the hydroxyl on C-2 of the terminal, non-reducing sugar. Although the presence of two similar enzymes within *P.furiosus* might appear to be an unnecessary metabolic burden, several other organisms including some thermophiles contain the genes for two or more exo-acting, β-specific glycosyl hydrolases (Bauer *et al*., 1996). For example, two β-glycosidase-encoding genes have been sequenced from the thermoacidophilic archaeon *S. solfataricus.* Although these genes may have come from different strains of *S. solfataricus* (Grogan, 1991), they are both homologous to family 1 glycosyl hydrolases.

The high degree of homology between the sequences of the β-mannosidase and β-glycosidase from *P. furiosus* suggests that the enzymes may be evolutionarily related. Gene duplication is frequently observed among glycosyl hydrolases (Henrissat, 1991). It has been proposed that the enzyme produced from the original gene copy would continue hydrolyzing the

original substrate, while duplicate gene copies could constitute templates for constructing enzymes with activity directed to a new, but stereochemically similar substrate (Henrissat, 1991). The divergence of glycosyl hydrolases to acquire new specificities is not unexpected, given the stereochemical resemblance among pyranoside substrates (Bauer *et al*., 1996).

Table 2.1. Identity of protein sequence among PFTG and related glycoside hydrolases family1.

Enzyme	% Identity										
	PFTG	PHbm	PAbg	TKbGGH1	TKbG	PFbm	PWbg	PAbm	PFbG	SSbg	SAbg	SSbG
PFTG		88	84	74	72	57	57	56	42	41	41	41
PHbm			85	74	72	58	58	56	38	42	42	42
PAbg				73	71	58	58	57	32	41	41	41
TKbGGH1					96	56	56	55	41	41	41	42
TKbG						55	55	54	41	40	40	41
PFbm							99	77	41	41	41	44
PWbg								77	41	41	41	44
PAbm									42	42	42	43
PFbG										52	52	52
SSbg											99	72
SAbg												72
SSbG												

PFTG : β-galactosidase [Pyrococcus furiosus DSM 3638], PHβm:β-mannosidase [Pyrococcus horikoshii OT3], Paβg :β-galactosidase [Pyrococcus abyssi GE5], TKβGGH1:β-glycosidase, GH1 family [Thermococcus kodakarensis KOD1], TKβG: β-glycosidase [Thermococcus kodakaraensis], PFβm: β-mannosidase [Pyrococcus furiosus DSM 3638], PWβg:β-galactosidase [Pyrococcus woesei], Paβm: β-mannosidase [Pyrococcus abyssi GE5], PFβG: β-glucosidase [Pyrococcus furiosus DSM 3638], SSβg: β-galactosidase (EC 3.2.1.23) - Sulfolobus solfataricus , Saβg: β-galactosidase [Sulfolobus acidocaldarius DSM 639]

2.4.2. Expression and purification of the recombinant PFTG

2.4.2.1. Expression of the recombinant PFTG

For efficient expression in *E. coli*, the PCR-amplified DNA fragment containing the gene for PFTG was subcloned into pET28 (b) under the control of the promoter for T7 RNA polymerase. The expression of PFTG in the crude extract of the recombinant *E. coli* cells was analyzed by SDS-PAGE at different time intervals. Proteins of *E. coli* in the crude extract were denatured at 75°C for 20 min. After the IPTG induction for 6 h, the enzyme was found to be expressed within 20 h (Fig. 2.4). The purification was improved by attaching eight additional amino acids (Leu, Glu, and six His residues) at the C-terminus rather than at the N-terminus. SDS-PAGE analysis indicated that PFTG was purified to an apparent homogeneity with an estimated molecular mass of 57 kDa.

2.4.2.2. Purification of recombinant PFTG

The *E. coli* BL21 (DE3) transformant harboring PFTG0356 was grown in LB broth supplemented with 100 μg of kanamycin per ml at 37°C until the optical density at 600 nm reached 0.8 and then induced with 1 mM IPTG for 6 h. The harvested cells by centrifugation (7,000 x g; 30 min; 4°C) and resuspended in lysis buffer [50 mM Tris-HCl buffer (pH 7.0) containing 300 mM NaCl, and 10 mM imidazole]. After the cells were sonicated (5 min, output control 4, 50% duty cycle), the crude extracts were denatured by heat treatment at 75°C for 20 min to eliminate endogenous heat-labile proteins of *E. coli*. When the supernatant was collected and the recombinant

enzyme was purified with nickel-nitrilotriacetic acid (Ni-NTA) affinity column chromatography (Qiagen, Hilden, Germany), SDS-PAGE showed a single band of the purified 57-kDa protein (Fig. 2.5, lane 2).

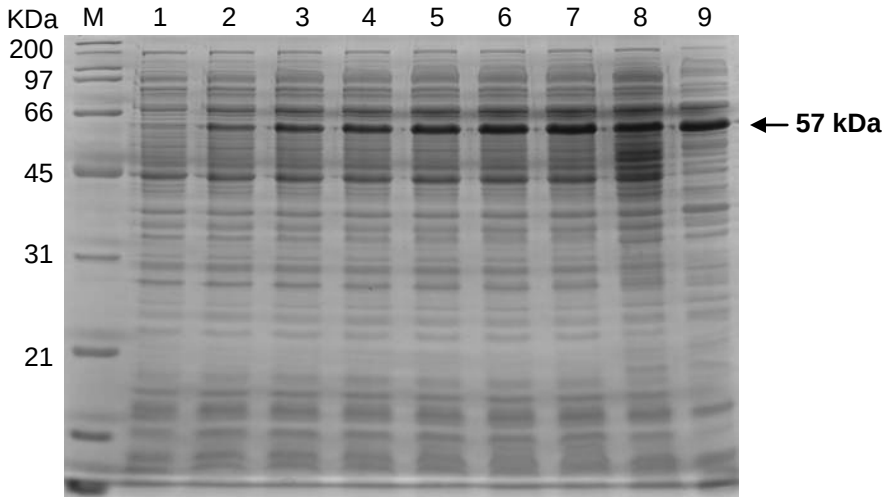


Figure 2.4. The expression rate of PFTG harboring *E. coli* cell during growth.

Lane 1: before IPTG induction, lane 2: after 1h induction, lane 3: after 2h, lane 4: after 3h, lane 5: after 4h, lane 6: after 5h, lane 7: after 8h, lane 8: after 20h, lane 9: after 20h then heating 75°C (20 min).

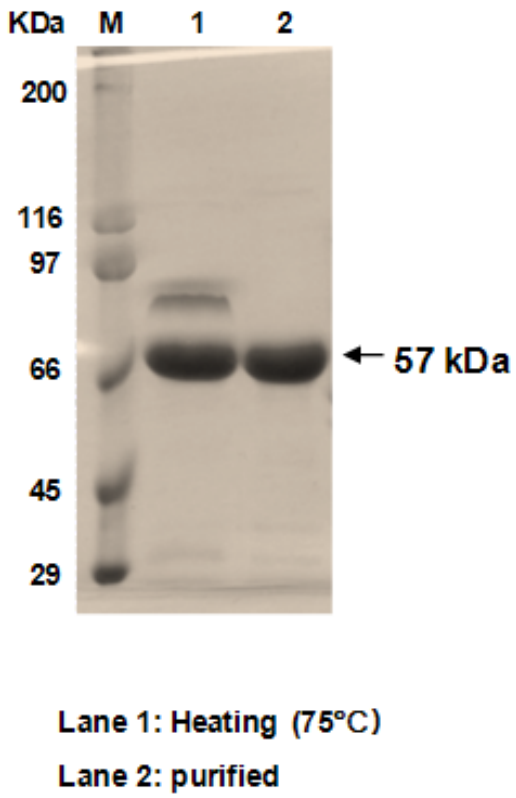


Figure 2.5. SDS-PAGE analysis of recombinant PFTG at different stages of purification. Lane M, protein size standards; lane 1, proteins after heat treatment; lane 2, purified PFTG after Ni-NTA column chromatography purification.

2.4.3. Characterization of the recombinant PFTG

2.4.3.1. Effect of temperature on PFTG activity and stability

The optimal temperature of PFTG was about 100°C on ONPG (Fig. 2.6). As expected, PFTG exhibited a remarkable thermal stability by retaining its full activity after 120 min of incubation at 85°C (Fig. 2.7). The half-life of PFTG was determined to be 398 and 114 min at 90 and 95°C, respectively. More than 27% of the enzyme activity remained after 1 h of incubation at 100°C. PFTG showed higher thermal stability than other glycoside hydrolases (Shockley *et al*., 2003).

DSC analysis of PFTG was performed to determine the thermal transition midpoint (T_m) of PFTG (Fig. 2.8). The result showed that the T_m of PFTG was 109.9°C. This indicated that the recombinant enzyme from *P. furiosus* was successfully expressed in *E. coli,* that was extremely thermostable. Among archaeal glycoside hydrolytic enzymes, *P. furiosus* had highest optimal temperatures of 95-105°C (Chhabra *et al*., 2002). For other glycoside hydrolases, however, β-galactosidase from *Sulfolobus sofataricus* showed the highest temperature optimum of 90°C (Moracci *et al*., 1992).

2.4.3.2. Effect of pH on PFTG activity and stability

The range of pHs at which the recombinant PFTG was active and stable was determined using ONPG as a substrate. As shown in Fig. 2.9, the maximal activity of PFTG was observed at pH 5.0. When compared with other glycoside hydrolases from

bacterial and archaeal origins on the optimal pHs on ONPG (Chhabra *et al*., 2002), PFTG showed an optimal activity at more acidic pH. More than 65% of the maximal activity was retained in the range between pH 4.5 and pH 5.5. In addition, PFTG was shown to be stable in a broad range between pH 4 and pH 7 (Fig. 2.9).

2.4.3.3. Hydrolysis on various substrates and substrate specificity of PFTG

The enzymatic activity of PFTG was examined using various substrates (1%, wt/vol) typically used for analyzing the enzymatic activity of glycoside hydrolase. Those include mannooligosaccharides, cellobiose and lactose. Lactose hydrolysis pattern are shown in Fig. 2.10. PFTG hydrolyzed mannooligosaccharides and cellobiose, typical substrates for β-glycosidase (4NP substrates). In order to understand the action mode of PFTG in detail, the hydrolysis pattern was investigated as a function of time using *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-β-D-xylopyranoside as substrates (Table 2.2). PFTG showed the activities of both glycoside hydrolase and β-mannosidase. PFTG demonstrated the ability to hydrolyze the typical substrates of glycosidic bonds without showing transglycosylation activity, but the degradation products were similar to those typical products of β-glycosidase.

When the enzymatic activity was examined using various substrates for glycoside hydrolases at 100°C for 30 min, PFTG hydrolyzed mannooligosaccharides (M2-M4) and cellobiose and lactose (Table 2.3). However, PFTG could not hydrolyze α-form

substrates and bulk polymers like mannan and starch (data not shown). The relative activities of PFTG toward various small substrates such as 4-nitrophenyl glucopyranoside, mannopyranoside, xylopyranoside and galactopyranoside were analyzed (Table 2.2). The catalytic properties of the enzyme were most closely related to those of β-glycosidases, which were reported to have multi-substrate specificity and produced mannose and glucose mainly from mannooligosaccharides and cellobiose by hydrolyzing them from the reducing end.

2.4.3.4. Determination of kinetic parameters by ITC

To determine kinetic parameters of PFTG, 4 synthetic substrates (*p*NP substrates) and 5 natural substrates (lactose, mannobiose, mannotriose, mannotetraose and cellobiose) were used. Each analysis was done in single, multiple injection that were triplicated (Figs. 2.11 & 2.12). Among these synthetic substrates, *para* nitrophenyl β-D mannopyranoside and natural substrates, mannobiose showed highest catalytic efficiency (k_{cat}/K_m) in Table 2.3. Three different 4-nitrophenyl substrates also showed the similar trend on the catalytic efficiency. In case of natural substrates, lactose and mannobiose showed low K_m value, indicating that the PFTG has high affinity towards those substrates. According to ITC results, this PFTG enzyme should be renamed as β-glycosidase or β-mannosidase than β-galactosidase. It is well know that β-glycosyl hydrolases have substrate overlapping problems (Opassiri *et al*., 2006), and thus this enzyme appears to be a member of β-glycosidase.

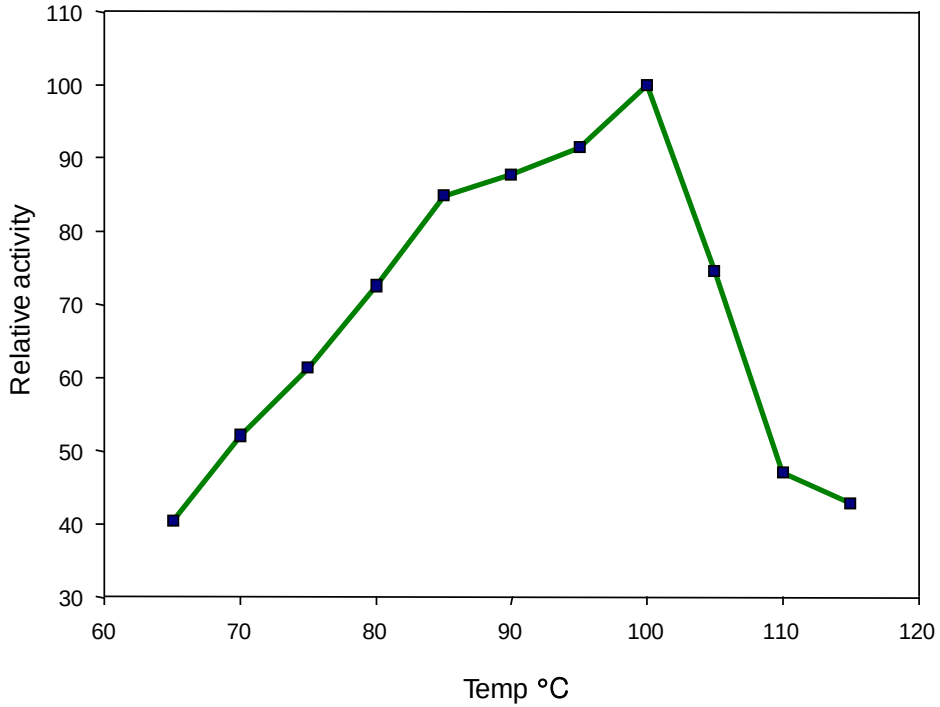


Figure 2.6. Temperature profile of PFTG.

For determination of temperature profile of PFTG, the enzyme was assayed from 65°C to 115°C in water bath overlayed with oil. After 30 min incubation, samples were withdrawn, and the residual activity was measured under the standard conditions of the assay.

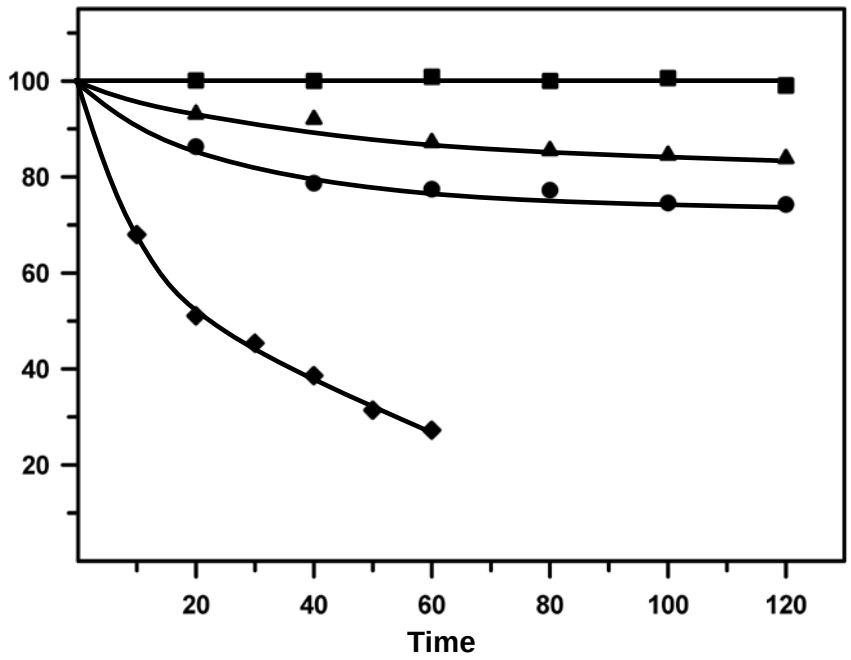


Figure 2.7. Effect of temperature on the stability of PFTG.

For determination of the thermostability of PFTG, purified enzyme (0.1 mg/ml) was incubated at 85°C (■), 90°C (▲), 95°C (●), and 100°C (◆) in 50 mM sodium citrate buffer (pH 5.0). After various time intervals indicated on the plot, samples were withdrawn, and the residual activity was measured at 90°C under the standard assay conditions.

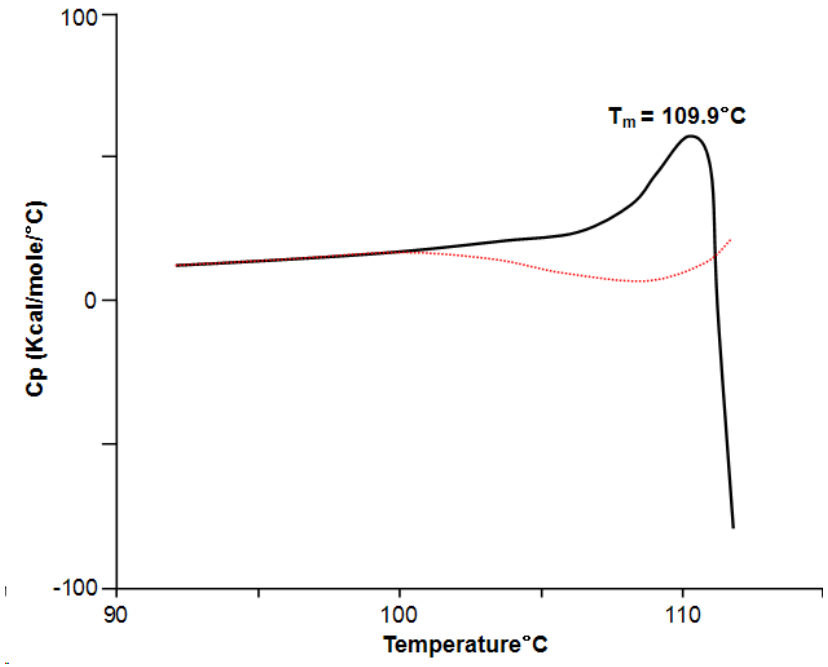


Figure 2.8. DSC (Differential Scaning Calorimetry) analysis of PFTG.

The enzyme was concentrated to 0.80 mg/mL in 50 mM Tris–HCl buffer (pH 7.5) using a 0.45- mm Microcon filter. The equilibrated enzyme was scanned from 20 to 130 °C at a rate of 1.0 °C per min. The enzyme scan was corrected using a buffer–buffer baseline.

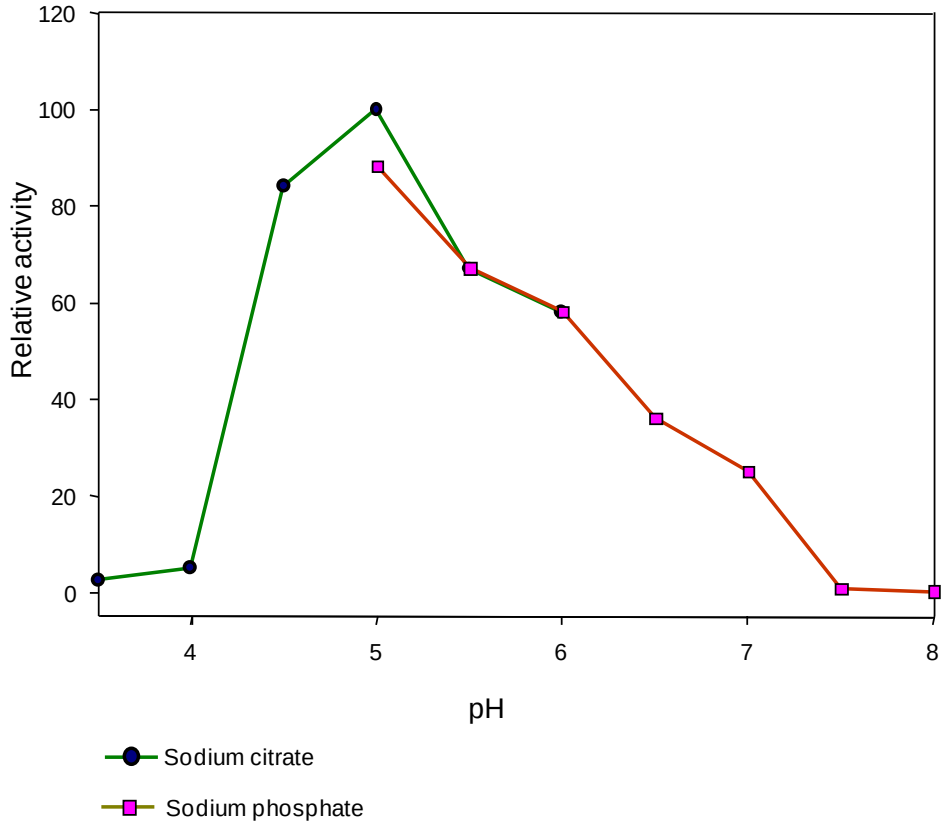


Figure 2.9. pH profiles of PFTG.

For determination of the optimum pH of PFTG, the purified enzyme (0.1 mg/ml) was incubated at 95°C. (●): 50mM of sodium citrate buffer; (■): 50mM of sodium phosphate.

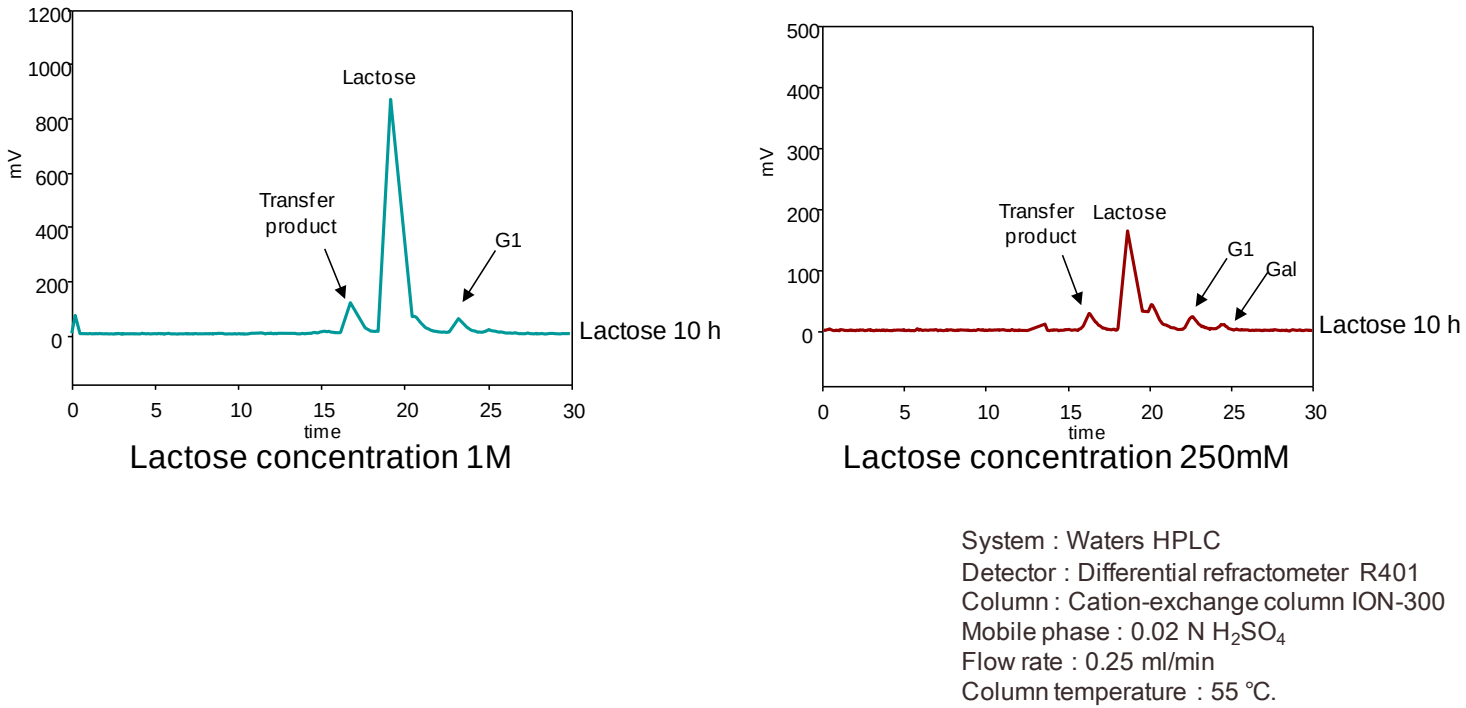


Figure 2.10. Transgalactosylation activity of PFTG with various concentration of lactose. Different concentration of lactose (1M-250mM) was reacted and samples were analyzed by HPLC after 10 h.

Table 2.2. Relative activity of PFTG with 5 synthetic substrates.

Substrates	Relative activity (%)
4-Nitrophenyl β -D-mannopyranoside (ManpβNp)	100
4-Nitrophenyl β -D-galactopyranoside (GalpβNP)	37
4-Nitrophenyl β -D-glucopyranoside (GlcpβNp)	114
4-Nitrophenyl β -D-xylopyranoside (XylpβNP)	31
4-Nitrophenyl α -D-glucopyranoside (GlcpαNp)	0

Table 2.3. Determination of kinetic parameters of PFTG with various substrates.

	K_m(mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
*P*nβman	0.09	0.30	3.02
*P*nPG	0.09	0.26	2.90
*O*nPG	0.12	0.23	1.89
Cellobiose	0.07	0.16	2.28
Lactose	0.04	0.08	1.82
Mannobiose	0.03	0.14	4.32
Mannotriose	0.07	0.28	4.21
Mannotetrose	0.07	0.16	2.21

ITC : Isothermal Titration Calorimetry

*P*nβman(*para-nitro*- phenyl β-d-mannopyranoside), *P*nPG(*p*-nitrophenyl glucopyranosides), *O*NPG(*o*rtho-Nitrophenyl-β-galactopyranoside)

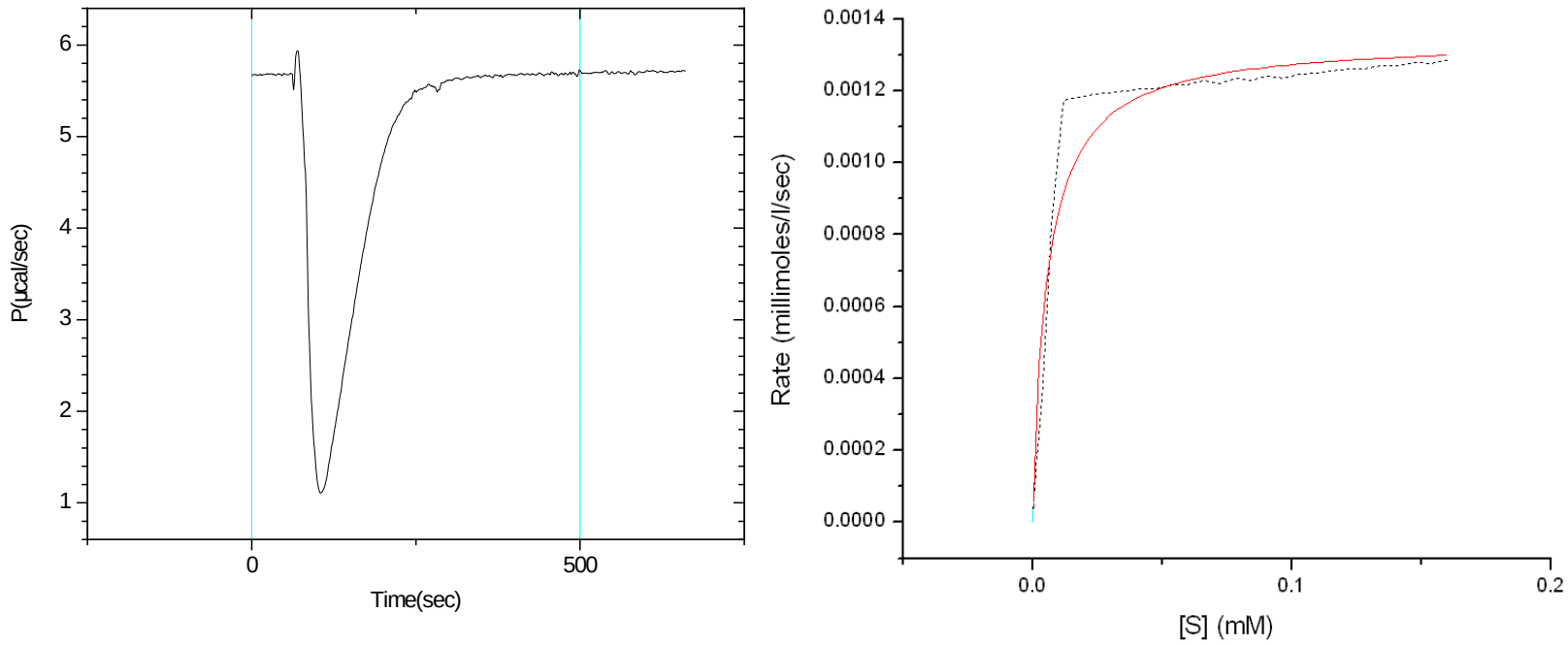


Figure 2.11. Single injection of ITC (Isothermal Titration Calorimetry) that was converted to [S] vs [V].

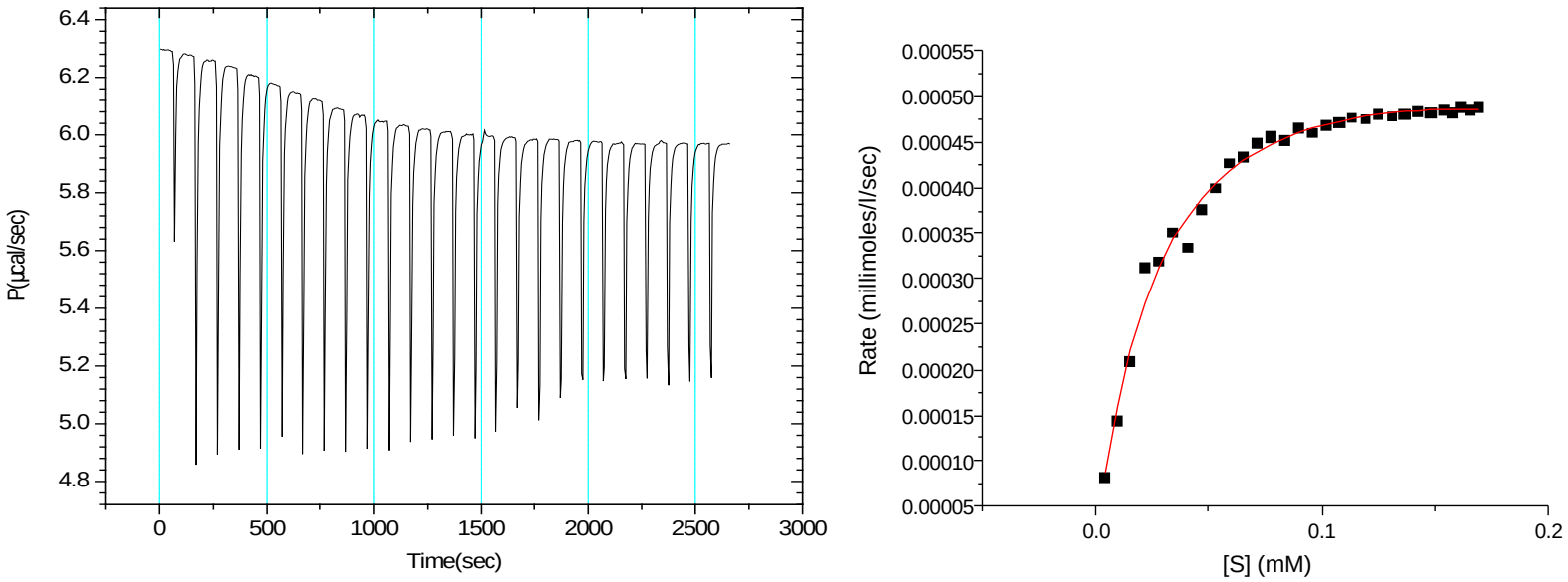


Figure 2.12. Multiple injection of ITC (Isothermal Titration Calorimetry) that was coverted to [S] vs [V].

2.5. CONCLUSIONS

An extremely thermostable glycoside hydrolase was isolated from the genome of hyperthermophilic archaeon *Pyrococcus furiosus*. The *P. furiosus* thermostable β−glycosidase gene (*pftg*) encoded a single polypeptide of 483 amino acids with an estimated molecular mass of 56,326 Da. The deduced amino acid sequence of this PFTG was highly homologous with various β−glycosidases. The purified PFTG was extremely thermostable, with an optimum temperature of 100°C at pH 5.0 and the T_m of the enzyme was 109.9°C, confirming that this enzyme is highly thermostable. The PFTG hydrolyzed mannooligosaccharides, synthetic substrates (*para* nitrophenyl β-D-glucopyranoside, galactopyranoside, xylopyranoside and mannopyranoside), lactose and cellobiose to produce glucose and galactose, like typical β-glycosidase. This enzyme showed highest catalytic efficiency (k_{cat}/K_m) towards para-nitrophenyl mannopyranoside, with more affinity toward β-mannosidase than that of β-galactosidase.

Chapter 3

Mutation Studies in the Active Site of β-Glycosidase from *Pyrococcus Furiosus* DSM 3638

The Glycoside hydrolase from hyperthermophilic *Pyrococcus furiosus* (PFTG) were sucessfully cloned, expressed and characterized in Chapter 2.

Through the kinetic studies of the PFTG by ITC, this enzyme showed more similarity to β-glycosidase (β-mannosidase) than β-galactosidase. This chapter describes the mutational studies in the active sites of the PFTG to determine the exact catagory.

3.1. ABSTRACT

Sequence alignments and homology modeling of PFTG showed that the residue 150 is conserved as tryptophan in β-glycosidase and in other related enzymes such as β-mannosidase and β-galactosidase. To elucidate the relationship between the substrate size and geometric shape of the catalytic site of thermophilic β-glycosidase and category of PFTG, the Q77, Q150 and D206 located at the interface of the dimer were replaced with Trp and Asn. Also, to confirm the role of active sites of PFTG, the Q77/150W double mutant was created through subcloning. The mutant enzymes identified in the wild-type enzyme were cloned and expressed in *E. coli*. When the expressed enzyme was purified by heat treatment and Ni-NTA affinity chromatography, the temperature and pH optima of the mutants and native enzyme were same at 100 °C and pH 5.0 in sodium citrate buffer, respectively. The catalytic efficiency (k_{cat}/K_m) of the mutant both on synthetic and natural substrates by ITC was slightly changed, but indicated the characteristics of β-glycosidase activity. Kinetic parameters of the mutant enzymes by ITC_{200} indicated that they possess characteristics of both β-galactosidase acitivity and β-mannosidase activity. Although the mutant enzymes showed same substrate specificities compared to the wild-type enzyme, they had more affinity (K_m) to substrates with low turnover number (k_{cat}).

Keywords: *Pyrococcus furiosus*; Hyperthermostable enzyme; ITC (Isothermal Titration Calorimetry); Glycoside hydrolase

3.2. INTRODUCTION

The glycoside hydrolases are an important group of enzymes that are responsible for cleaving a range of biologically significant carbohydrate compounds. Glycosyl hydrolases are a large group of enzymes that hydrolyze the various stereochemical conformations of the carbohydrate glycosidic bond. These enzymes are found in almost all organisms and have diverse biological functions, ranging from the essential processing of glucosides in the mammalian brain to the cleavage of cellobiose, a biomass degradation product (ChiI *et al*., 1999). Glycosidase and related enzymes have been classified into more than 100 families on the basis of amino acid sequence homology and structural similarities rather than substrate selectivities (ChiI *et al*., 1999; Cantarel *et al*., 2009). Glycoside hydrolase family 1 (GH 1) belongs to a larger superfamily called 'clan GH-A' which contains at least 11 families and now comprises more than 250 members representing 18 different substrate specificities. However, almost all carbohydrate enzymes including glycoside hydrolases had overlapping substrate specificities and thus it is very important to identify the enzymes.

Isothermal titration calorimetry (ITC) is the technique that can directly measure the binding energetics of biological processes, including protein-ligand binding, protein- protein binding, DNA-protein binding, protein-carbohydrate binding, protein-lipid binding, and antigen-antibody binding. ITC has the ability to precisely determine the Gibbs energy, enthalpy, entropy, and heat capacity changes associated with binding (Freire, 2004). For enzymatic kinetic study, ITC is well-established in the study of affinity of molecular interactions, and is now becoming a popular method in the study of enzyme kinetics. Traditional enzyme assays utilize a probe to monitor either substrate depletion or product formation. These probes are system-dependent and must be optimized for each

reaction under specific conditions. Also, the substrate may need to be modified which could interfere with the catalysis reaction. ITC uses heat as a probe, and since every reaction generates or absorbs heat, there is no need for lengthy method development each time a new enzyme is assayed.

ITC directly measures the heat change as catalysis proceeds, which is proportional to the rate of reaction. The K_m and k_{cat} from ITC experiments agreed favorably with traditional enzyme kinetics methods, and can be used with every class of enzyme, including those with no other direct assay methods (Todd & Gomez, 2001). The advantage of ITC is to monitor the rate of enzymatic reactions is a non-destructive, sensitive, and direct assay. Multiple injections of substrate can be done in a single experiment, thus K_m and k_{cat} can be determined in a single ITC experiment.

The objective of this research was to elucidate substrate specificities of the PFTG with the mutant enzymes by protein engineering and ITC.

3.3. MATERIALS AND METHODS

3.3.1. Chemicals and media

4-nitrophenyl-β-D-mannopyranoside(ManpβNp),4-nitrophenyl-β-D-galactopyranoside (GalpβNP), 4-nitrophenyl-β-D-glucopyranoside (GlcpβNp), 4-nitrophenyl-β-D-xylopyranoside (XylpβNP), cellobiose and lactose were purchased from Sigma (St. Louis, MO). Mannobiose and mannotriose were purchased from Megazyme (Wicklow, Ireland). Bacto yeast extract and Bacto tryptone were purchased from BD Diagnostics (Sparks, MD). All restriction enzymes and calf intestine alkaline phosphatase were purchased from New England Biolabs, Inc. (Ipswich, MA).

Pfu Turbo DNA polymerase, T4 DNA ligase, and protein molecular weight standards were supplied by Stratagene (Santa Clara, CA). All other chemicals were of reagent grade and were purchased from Fisher scientific (Ottawa, ON).

3.3.2. Transformation of *E. coli* and DNA preparation

Transformation of *E. coli* was carried out as described by Sambrook & Russel (2001). *E. coli* MC1061 or BL21 (DE3) was used as a host. Small-scale preparation of plasmid DNA was also carried out by following the procedure of Sambrook & Russel (2001). *E. coli* MC1061 or BL21 (DE3) containing recombinant DNA was cultured for 16 h in 5 ml LB broth in the presence of appropriate antibiotics and plamid DNA samples were isolated and purified using a QIAprep Spin Miniprep kit as the manufacturer recommended (Qiagen, Mississauga, ON).

3.3.3. Site-directed mutagenesis

For more efficient mutagenesis, the protocol for QuikChange^{TM} site-directed mutagenesis kit (Stratagene, USA) was modified slightly in this study. Two complementary oligonucleotide primers with appropriate mutation flanked by unmodified nucleotide sequence were synthesized. About 125 ng of each synthetic oligonucleotide primer was added to the reaction mixture (50 μl) containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% (vol/vol) Triton X-100, 0.1 mg/ml of nuclease-free bovine serum albumin, about 5~50 ng of dsDNA template and 100 μM dNTP mixture (25 μM each of dGTP, dCTP, dATP, and dTTP). After 2.5 units of *Pfu* polymerase (Stratagene, USA) was added, PCR was performed as follows;

one initial denaturation step for 30 sec at 95°C followed by 12–18 cycles of 1 min at 95°C, 1 min at 55°C, 6 min at 68°C. When the cycling reaction was completed, 10 units of *Dpn*I restriction enzyme was added directly to the amplification reaction and mixed thoroughly by pipetting the solution up and down several times. The mixture was spun down in a microcentrifuge for 1 min and incubated at 37°C for 1h to digest the template DNA. The reaction mixture was extracted with phenol-chloroform and DNA was recovered by ethanol precipitation. The pellet was resuspended in 20 μl of kinase buffer [70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithio-threitol, 1 mM ATP] and 10 units of kinase were added. After kinasing at 37°C for 2 h, 3~5 μl of the mixture was taken and self-ligated at 12~16°C for 2 h by adding 5 units of T4 DNA ligase. The ligation mixture was transformed into *E. coli* MC1061 and the mutation carried by the transformants was confirmed by DNA sequencing.

3.3.4. DNA sequencing and analysis of the gene structure

The nucleotide sequence of the PCR-generated gene was determined with the BigDye terminator cycle sequencing kit for the ABI 377 Prism (Perkin-Elmer, Norwalk, CT). The initial similarity search was carried out with the BLAST program (Altschul *et al*., 1990). Detailed analyses of the DNA and the deduced amino acids of various genes were performed with the DNASIS, PROSIS (v7.0, Hitachi Software, Tokyo, Japan), and CLUSTAL programs (Thompson *et al.*, 1994).

3.3.5. Modeling studies

The three-dimensional structure model of PFTG and variants were constructed based on the

atomic coordinate of a β-glycosidase 1QVB from *Thermococcus kodakarensis* KOD1 using swiss-model (http://swissmodel.expasy.org/), a model with overall structure but not very high sequence homology identity (44%).

3.3.6. Enzyme assays and hydrolytic patterns by spectrophotometric methods

The hydrolytic activities of PFTG and mutant enzymes were assayed at 95°C in 50 mM sodium citrate buffer (pH 5.0) with 4% (w/v) 4-nitrophenyl-β-D-mannopyranoside (ManpβNp), 4-nitrophenyl-β-D-galactopyranoside (GalpβNP), 4-nitrophenyl-β-D-glucopyranoside (GlcpβNp), 4-nitrophenyl-β-D-xylopyranoside (XylpβNP). β-Glycosidase (mannosidase) activity was assayed routinely using 1.0 mM *para* nitrophenol (*p*Np) substrate equilibrated to the desired temperature and pH. After equilibrating the sample to the desired temperature and pH in the water or oil bath, the reaction was initiated by adding equilibrated substrate to the sample in Eppendorf tubes. The reaction was stopped at 10 min by chilling on ice. After cooling, the increase of the sample in absorbance at 405 nm as a result of *p*Np liberation was measured using an EL 340 Microplate Bio-Kinetics Reader (Bio-Tek™ Instruments, Winooski, VT). All activities were corrected for thermal degradation of the *p*Np substrate, which was below 0.5% of the enzyme hydrolysis rate. Absorbances were converted to concentrations of *p*Np using standards of known concentration. All assays were performed in triplicate. One unit of glycosidase activity was defined as that amount of enzyme required to catalyze the release of 1.0 mmol of *p*Np/min.

In order to examine the hydrolytic pattern of PFTG and mutant enzymes, 0.5 ml of purified enzyme was incubated with 0.5 ml of 1.0% (wt/vol) substrate in 50 mM sodium citrate buffer (pH 5.0) at 100°C for various lengths of time. The substrates used were 4-nitrophenyl-β-D-

mannopyranoside (ManpβNp), 4-nitrophenyl-β-D-galactopyranoside (GalpβNP), 4-nitrophenyl-β-D-glucopyranoside (GlcpβNp), 4-nitrophenyl-β-D-xylopyranoside (XylpβNP), mannooligosacchrides (mannose to maltotetraose), cellobiose and lactose. A 100-μL aliquot of the reaction mixture, which contained 2.8 μL of diluted PFTG and 1 mM substrate in 50 mM sodium citrate buffer (pH 5.0) at 95°C, was collected at 10 min, and the reaction was immediately stopped by the chilling in ice.

For relative activity of wild-type and mutant enzymes, the amount of glucose released from substrates was assayed by the glucose oxidase-peroxidase method (Sigma, St.Louis, USA). Enzyme reaction mixture was composed of 1.8 ml of the substrates (cellobiose or lactose) in 50 mM sodium citrate buffer (pH 5.0) at the reaction temperature. The reaction was initiated by adding 0.2 ml of diluted enzyme solution; 140 μl aliquots of the enzyme digest were taken at various time intervals and immediately added to 80 μl of 0.1 M HCl on ice. After neutralizing by adding 80 μl of 0.1 N NaOH, aliquots of the reaction mixture were boiled for 10 min and mixed with 700 μl of glucose oxidase and peroxidase mixture for the quantification of glucose produced. The reaction mixture was incubated at 37℃ for 30 min and its absorbance was measured at 570 nm using a microplate reader.

3.3.7. Enzyme assays with Isothermal Titration Calorimetry (ITC)

Calorimetric assays were done on ITC_{200} system instruments (MicroCal Inc., Piscataway, NJ). Reaction cells (200 μl) were filled with degassed solutions and equilibrated at the indicated temperatures. Stirring speed was 400 rpm, thermal power was recorded every 2 sec, and

instrumental feedback was 10–50%. All solutions and buffers were degassed at 25°C immediately prior to use. All reactions were carried out at 75°C in 50 mM citrate buffer, pH 5.0. The reference cell was filled with distilled water, the sample cell was filled with the enzyme solution and the syringe was filled with each substrate. The reaction rates achieved at each steady state are plotted against the corresponding total substrate concentration in the cell to produce a curve (Figs. 3.6 & 3.7). The data were then fit to obtain estimates of the half saturation constant (K_m), and the reaction rate constant (k_{cat}). Curve fitting was conducted in KaleidaGraph ver. 3.6 (Synergy Software), which utilizes the Levenberg–Marquardt algorithm (Jeoh *et al*., 2005). Single injection methods were used to obtain enthalpy and multiple injection methods were used to obtain kinetic parameters. All assays were triplicated.

3.4. RESULTS AND DISCUSSION

3.4.1. The sequence comparison of PFTG with other GH1 Enzymes

To clarify the real category of PFTG, the sequence of PFTG was compared to other GH1family through BLAST search. The PFTG was highly homologous to the genes encoding GH such as β-galactosidase, β-mannosidase and β-glucosidase. Sequence alignment studies showed that Gln77, Gln150 and Asp206 residues are around the active site of most of GH1 family. To elucidate the role of these amino acid residues which located around the active site, we decided to mutate each amino acid to Arg, Trp and Asn. The different members of GH1 catalyze hydrolysis to release a glucose molecule from a number of different substrates, including some hydrophobic substrates linked to glucose. Only few members of the family are however commercially available,

and many different analytical assays have thus utilized β-glucosidase from almond, which has been classified under GH1 (He & Withers, 1997), and which is often available in heterogeneous preparations.

3.4.2. Mutation of active sites by site-directed mutagenesis

In order to mutate specific amino acids of PFTG, the PFTG whole gene was amplified using PCR with the *Pfu* DNA polymerase and native PFTG as a template. The *pftg* gene-specific oligonucleotide primers flanking the 5'- and 3'-gene ends were designed from the known *pf0356* sequence (Tables 3.1 & 3.2). Mutagenesis was performed to introduce the following designed changes: Q77R, Q150W, D206N and Q77/150W, respectively. All mutated genes were purified with QIAEXII Gel Extraction kit (Qiagen) after gel separation. The mutated nucleotides were confirmed by agarose gel electrophoresis using restriction enzyme (Fig. 3.1). Each mutant enzyme (Q77R, Q150W, D206N and Q77/150W) were subcloned by restriction enzymes (*NcoI* & *XhoI*). The mutant enzymes were successfully substituted, expressed and purified. In the final construction, the mutant enzymes were extended by eight additional amino acid residues (Leu, Glu, and six His residues), which included six histidine tags.

Table 3.1. Bacterial strains and plasmids used or constructed in this study.

Bacterial strains and plasmids	Characteristics and Usages	References
E. coli BL21(DE3)	Expression host	Sambrook
E. coli MC1061	Cloning or expression host	Sambrook
pET-28b(+)	C-terminal 6xHis tag, T7 promoter, Kan^r	Novagen
pTkNd6xH	C-terminal 6xHis tag, *B. licheniformis* MAase (BLMA) promoter, Kan^r	Kim, 2005
pETPFTG-6h	Expression of C-terminally 6xHis tagged PFTG, T7 promoter, Kan^r	This study

Table 3.2. Nucleotides used in this study. Each designated forward and reverse primers were used for substitution of active sites.

Name	Sequence of oligonucleotide
Q77R(Fwd)	5’- GGTTTAAACGCTTAC***AGA***TTAACGATAGAATGGAG-3’
Q77R(Rev)	3’-CTCCATTCTATCGTTAA***TCT***GTAAGCGTTTAAACC-5’
Q150W(fwd)	5’-TTCGTTACATTAAATCAT***TGG***ACTAATCCAATATGG-3’
Q150W(Rev)	3’-CCATATTGGATTAGT***CCA***ATGATTTAATGTAACGAA-5’
D206N(fwd)	5’-TGGGCAACATTT***AAC***GAGCCGATGGTAACAGTT-3’
D206N(Rev)	3’-AACTGTTACCATCGGCTC***GTT***AAATGTTGCCCA-5’

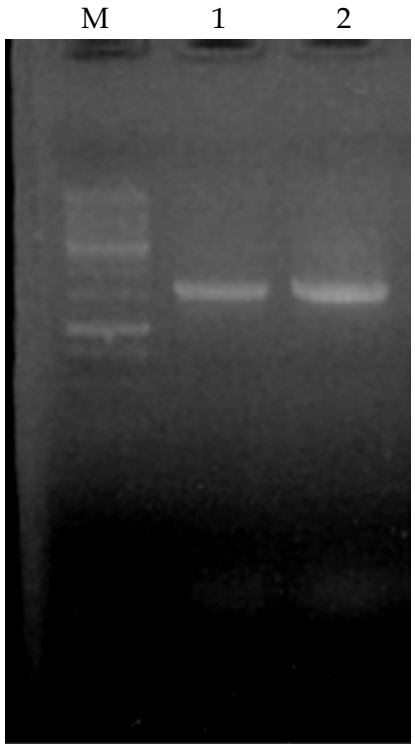


Figure 3.1. Agarose gel electrophoresis of the PCR products. Lane M was loaded with size marker; lane 1(Q77R), 2(Q150W) the mutant DNA fragment amplified from the PFTG.

3.4.3. Expression, purification and mutational studies on the role of Gln77 and Gln150

After the wild-type and mutated enzymes were produced in *E. coli* BL21 (DE3) and the expression level was analyzed by SDS-PAGE, all enzymes showed very similar patterns. All mutated enzymes were screened for activity using ONPG as a substrate (data not shown). After the cell free extracts were heated at 70°C for 20 min to remove all thermolabile proteins, the crude enzyme was further purified using with an AKTA FPLC system (GE Healthcare Life Sciences, Amersham, UK). The active fractions in elution buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 200 mM imidazole) were dialyzed against 50 mM Tris-HCl buffer (pH 7.5). The purity and molecular weight range of the protein were analyzed by discontinuous SDS-PAGE. SDS-PAGE showed a single band of the purified 57kDa protein (Fig. 3.2, lane 1; Fig. 3.3). Most of the glycosyl hydrolases such as β-galactosidase, β-glucosidase and β-mannosidase contained the highly conserved Asn and Glu residues within the conserved region (Zechel & Withers, 2000).

The variant amino acid residues in the conserved regions might play an important role in the unique properties of PFTG. Several mutants with a single or double amino acid substitution were constructed by site-directed mutagenesis to elucidate the role of the amino acid residues in details (Fig 3.3 & Fig. 3.4). The hydrolysis patterns and kinetic properties of the purified mutant enzymes were compared to those of wild-type enzyme.

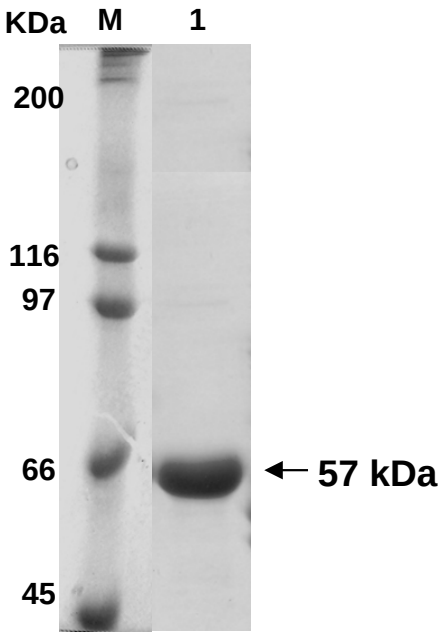


Figure 3.2. SDS-PAGE analysis of purified mutant enzyme of recombinant PFTG. Lane M, protein size standards; lane 1, purified PFTG mutant enzyme (Q77R) after Ni-NTA column chromatography.

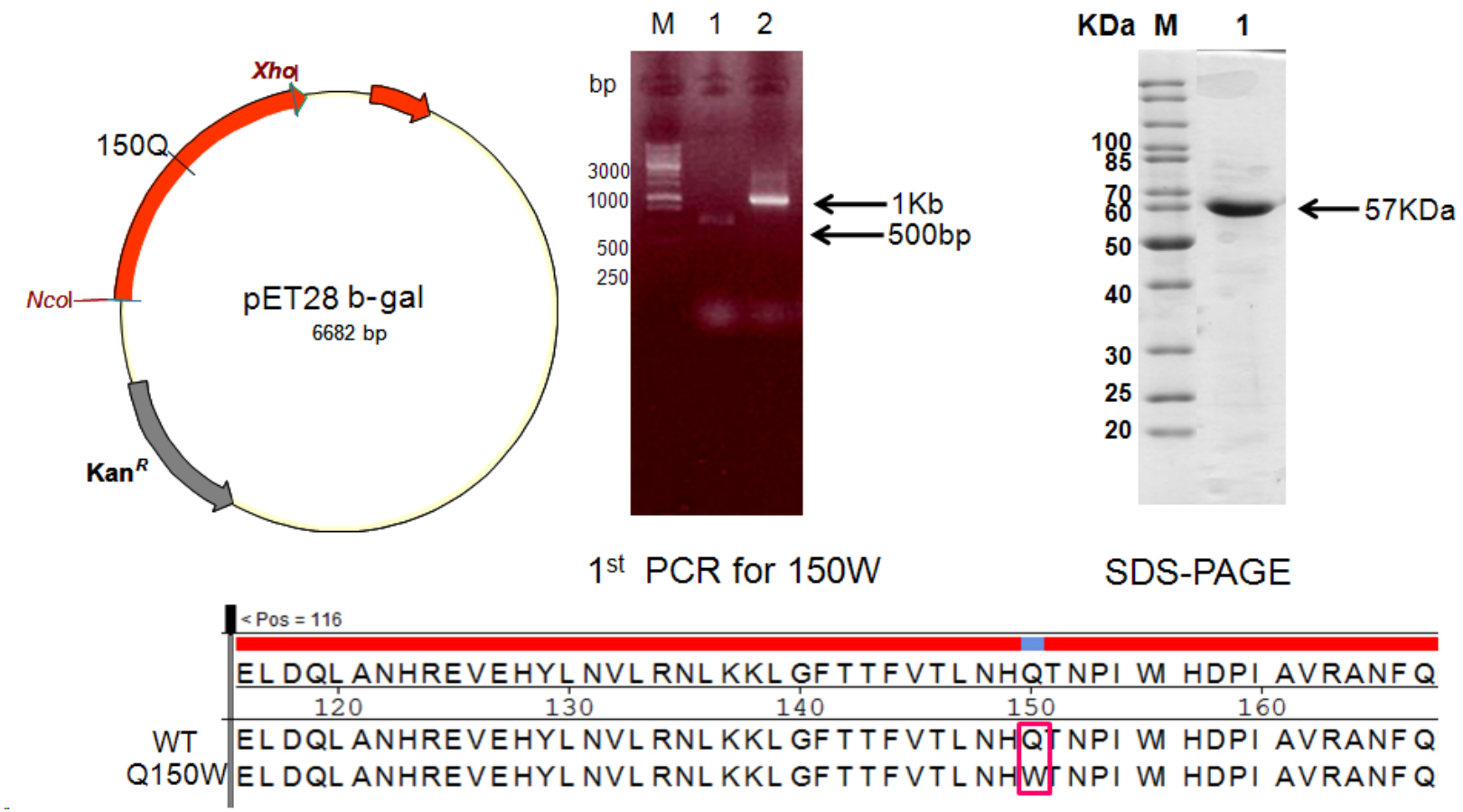


Figure 3.3. Cloning, expression, purification and sequencing results of Q150W mutant enzyme.

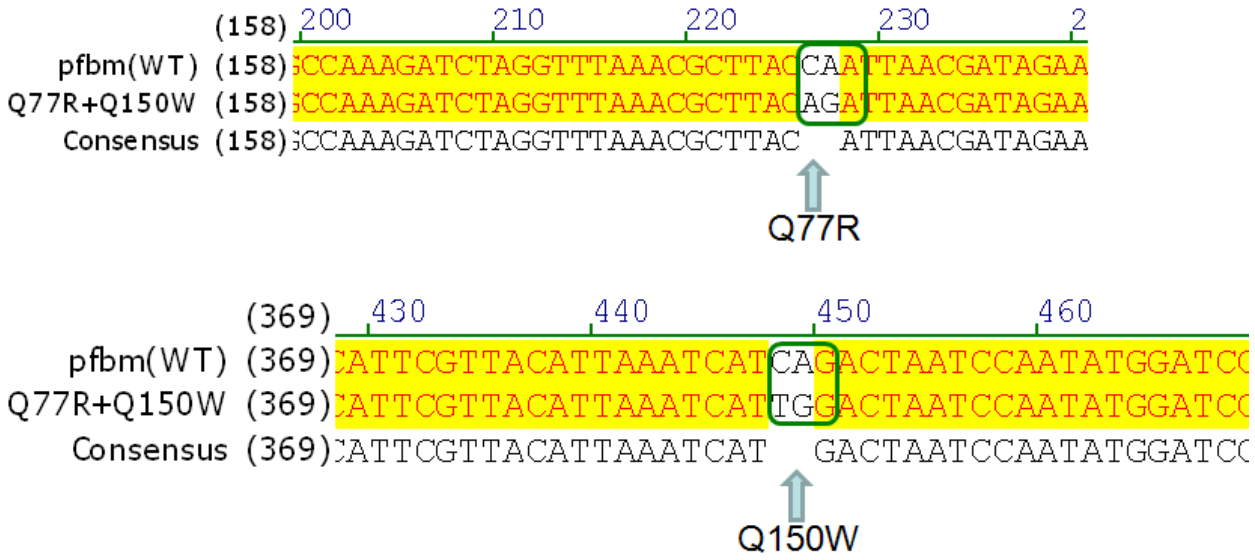


Figure 3.4. Sequencing results of Q77/150W double mutant enzyme (green boxes indicate mutated nucleotides).

The parallel stacking interactions (CH/π interaction) between the hydrophobic faces of carbohydrate residues and the aromatic amino acid residues (Trp, His, Phe, and Tyr) have been suggested to play an important role in the ligand-recognition function of carbohydrate recognizing enzymes such as glycosidases and glycosyltransferases as well as the conventional hydrogen bonding and van der Waals interactions (Vyas, 1991; Elgavish, 1997; Muraki, 2002).

Some amino acid residues in the conserved region of GH 1 enzymes are known to be important for structural and catalytic functions. These amino acid residues (Asn and Glu) in active site, which are strictly conserved among β-mannosidase, β-glycosidase and β-galactosidase, can be directly or indirectly involved in the interactions with substrates. Kim *et al* (1999) reported that Trp359 in the crystal structure of ThMA (*Thermus* maltogenic amylase) was in close contact with beta-cyclodextrin (β-CD). Ohtaki *et al* (2006) also reported that Trp356 in TVAII (*Thermoactinomyces vulgaris* R-47) changed the conformation of the side chain depending on the structure and size of the ligands (acarbose, α-, β-, and γ-CD). The Trp356 residue is thought to be responsible for the multiple substrate-recognition mechanism of this enzyme, providing the unique substrate specificity.

Through modeling studies of PFTG, we investigated Q77, Q150, D206 and E398 residues that were one of the active sites of PFTG (Fig. 3.5). To elucidate the role of Q77 and Q150 residues within the active site of PFTG in the transglycosylation activity or catalysis, mutational analysis of these positions was performed. Three PFTG mutants, Q77R (Gln77→Arg), Q150W (Gln150→Trp), and Q77R/Q150W (Gln77→Arg and Gln150→Trp), were constructed by site-directed mutagenesis. These mutations were confirmed by sequencing analysis. Each mutant enzyme was expressed in *E. coli* and purified to apparent

homogeneity using Ni-NTA affinity column chromatography.

Q150W and Q77/150W mutant enzymes hydrolyzed 4-nitrophenyl and β-glycosidic linkage and produce glucose, mannose and galactose as a major products as well as smaller galacto- and mannooligosaccharides. Their hydrolysis patterns seemed to be similar to that of wild-type enzyme.

3.4.4. Kinetic parameters of PFTG wild-type, Q150W and Q77/150W

When the kinetic parameters were studied by ITC, the mutant enzymes showed same patterns compared to that of wild-type enzyme. 4-Nitrophenyl D-mannopyranoside (Q150W; 2.83$s^{-1}mM^{-1}$, Q77/150W; 3.63 $s^{-1}mM^{-1}$) showed highest catalytic efficiency among synthetic substrates and mannobiose (Q150W; 5.00 $s^{-1}mM^{-1}$, Q77/150W; 4.00 $s^{-1}mM^{-1}$) in Tables 3.3, 3.4 showed highest catalytic efficiency among natural substrates. The mutant enzymes had more affinity (K_m) towards each substrate but showed lower turnover number (k_{cat}) compared to the wild-type enzyme. Each experiment was done by single injection and multi injection methods and triplicated (Figs. 3.6 & 3.7).

The k_{cat} value of the mutant enzyme for both substrates decreased compared to the wild-type enzyme. However, the K_m value of the mutant enzyme on each substrate slightly decreased which means that the mutant enzymes have more affinity towards substrates, as compared with that of wild-type enzyme. These results showed that the Gln residue plays an important role for efficient binding of substrates to the catalytic site.

As demonstrated in this study, the ITC method offered significant advantage for obtaining a precise kinetic data for the native PFTG and mutant enzymes. The kinetic assays

used were continuous with real-time data acquisition, which allowed monitoring the progress of the reaction during the assay. In conventional kinetic enzyme assays, several control experiments have to be conducted to determine that the reaction is indeed at steady state in the assay reaction times. To be rigorous, the control experiments must be repeated for each mutant tested. For the ITC experiments, the steady state can be observed and verified during the experiment itself. ITC, therefore, is a novel tool for studying the kinetics of any soluble enzyme system (Jeoh *et al*. 2005). ITC kinetic data showed that this PFTG could be named as β-glycosidase having more affinity to β-mannosidase than that of β-galactosidase.

The effect of amino acid substitution might not be an effect of direct interactions with the substrate in this study, where changes in interactions lead to changes in the conformation of the active site. Mutations of Q150W and Q77/150W did not lead to any corresponding or significant changes in affinity or turnover. The effect of this change on the activity is, however not completely clear. It may lead to a minor decrease in activity compared to clones where this mutation is lacking, but its effect appears to be small.

The PFTG belongs to a GH family with diverse substrate specificity (including enzymes active on mannooligosaccharides), with many gene sequences available allowing comparison, and with necessary structural information available.

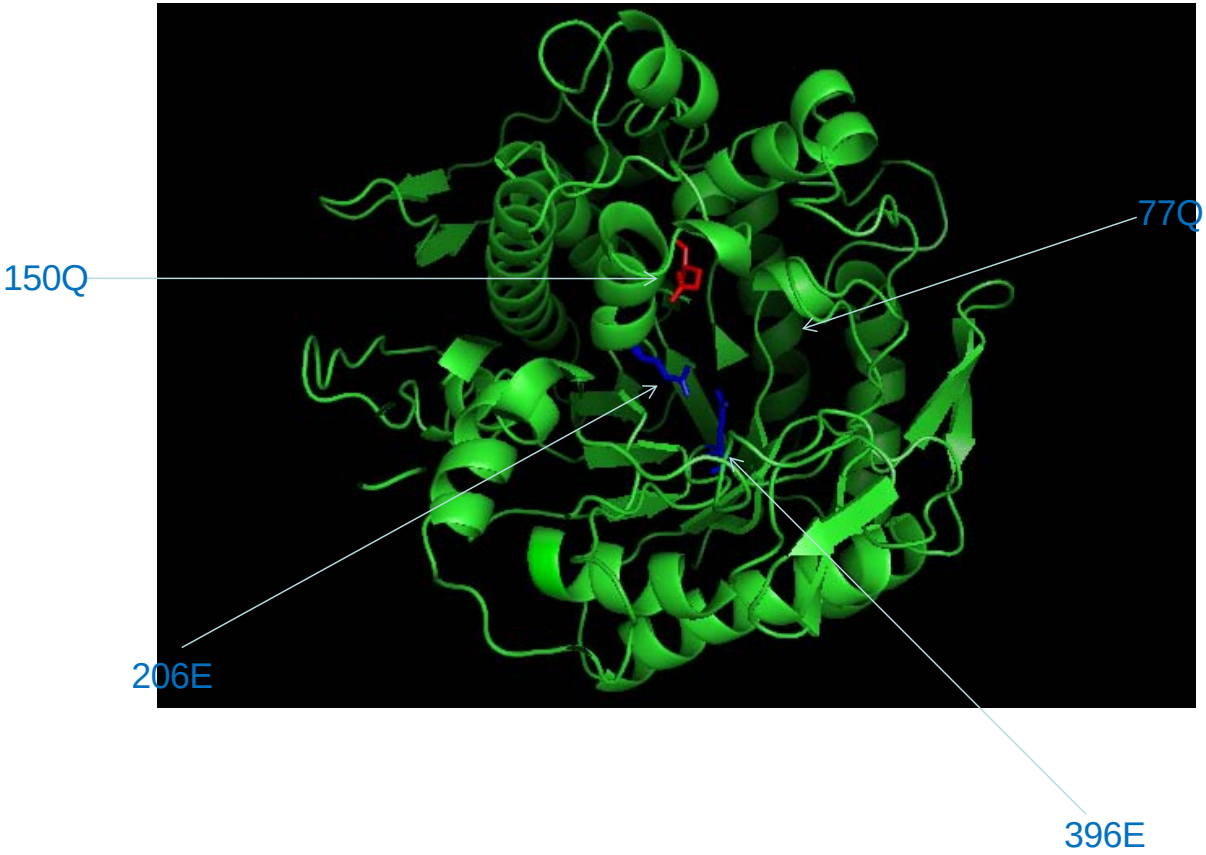


Figure 3.5. Ribbon diagram of 3D model of PFTG. Modeled structure comprised residues 1 to 483 of amino acid residues. N-terminal domain, $(\beta/\alpha)_8$-barrel domain, and C-terminal domain. Catalytic residues Gln77, Gln150, Glu206 and Glu396 are indicated with stick representation. The image was rendered with the program PyMOL (DeLano, 2002).

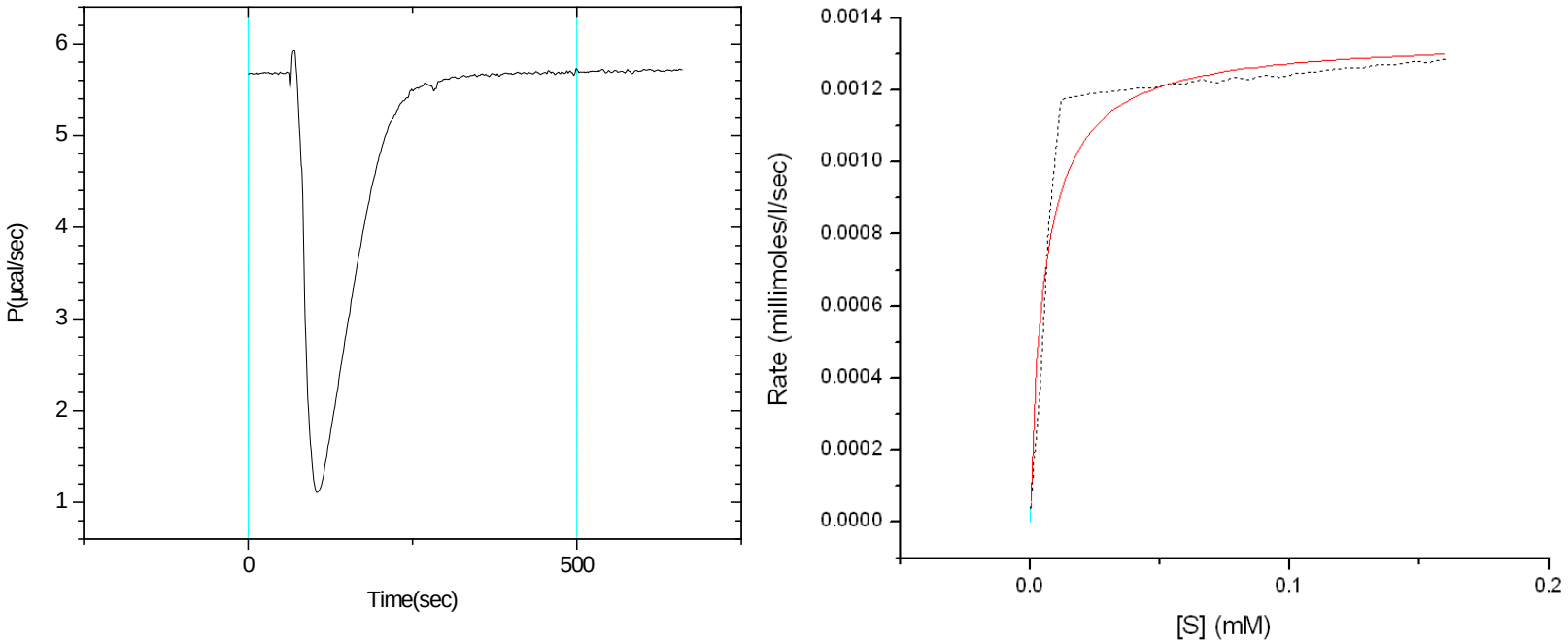
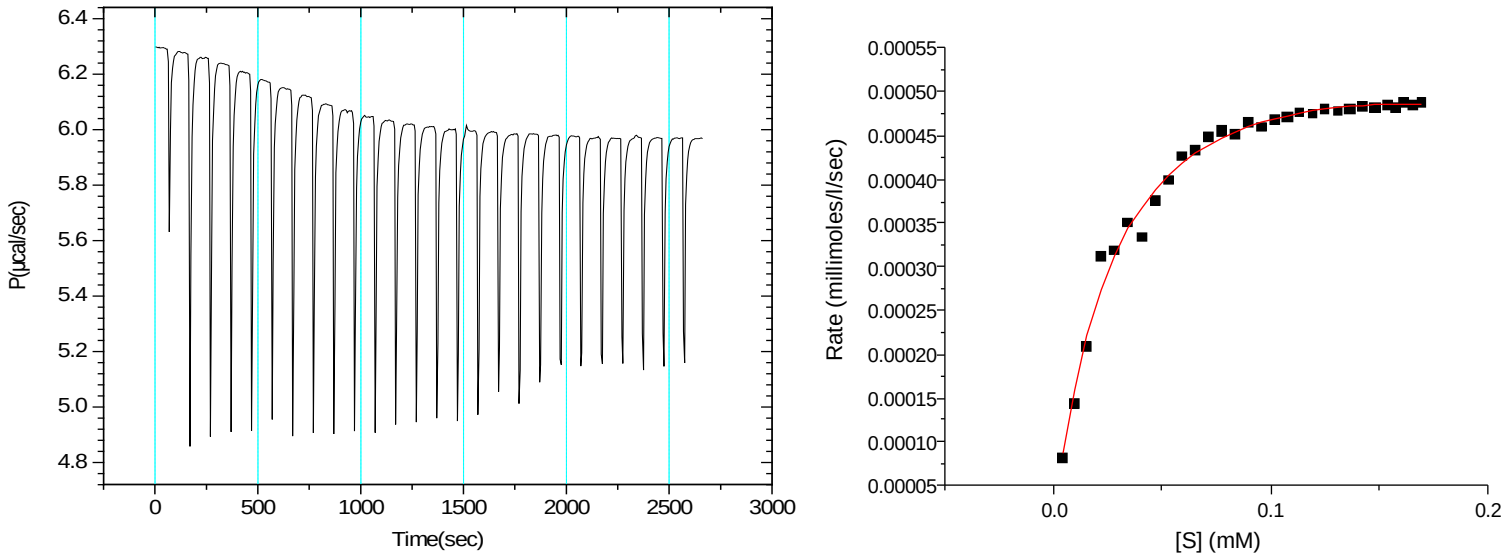


Figure 3.6. Single injection of ITC (Isothermal Titration Calorimetry) that was converted to [S] vs [V].



(a) (b)

Figure 3.7. Multiple injection of ITC (Isothermal Titration Calorimetry) that was converted to [S] vs [V]. (a) shows monitoring heat differences each injections and (b) shows the conversion to [S] vs [V].

Table 3.3. Determination of kinetic parameters of PFTG Q150W mutant enzyme with various substrates.

	K_m(mM)	k_{cat} (sec^{-1})	k_{cat}/K_m($s^{-1}mM^{-1}$)
*P*nβman	0.05	0.13	2.83
*P*nPG	0.05	0.13	2.44
*O*nPG	0.30	0.67	2.26
Cellobiose	0.38	0.74	1.92
Lactose	0.03	0.03	1.36
Mannobiose	0.01	0.05	5.00
Mannotriose	0.06	0.10	1.67
Mannotetrose	0.03	0.14	4.66

ITC : Isothermal Titration Calorimetry

*P*nβman(*para-nitro*- phenyl β-d-mannopyranoside), *P*nPG(*p*-nitrophenyl glucopyranosides), *O*NPG(*o*rtho-Nitrophenyl-β-galactopyranoside)

Table 3.4. Determination of kinetic parameters of PFTG Q77/150W double mutant enzyme with various substrates.

	K_m(mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
*P*nβman	0.03	0.11	3.63
*P*nPG	0.02	0.05	2.95
*O*nPG	0.10	0.22	2.17
Cellobiose	0.04	0.10	2.71
Lactose	0.01	0.03	2.21
Mannobiose	0.01	0.04	4.00
Mannotriose	0.10	0.16	1.64
Mannotetrose	0.01	0.04	2.90

ITC : Isothermal Titration Calorimetry

*P*nβman(*para-nitro*- phenyl β-d-mannopyranoside), *P*nPG(*p*-nitrophenyl glucopyranosides), *O*NPG(*o*rtho-Nitrophenyl-β-galactopyranoside)

3.5. CONCLUSIONS

The amino acids around the active site (Q77, Q150 and Q77/150W) of PFTG were successfully substituted by site-directed mutagenesis and the mutant enzymes were expressed.

Through kinetic study by ITC (Isothermal Titration Calorimetry) which is a very sensitive method, the mutant enzymes and wild-type enzyme showed the same patterns. However, the mutant enzymes showed more affinity (K_m) towards each substrate and lower turnover numbers (k_{cat}) than that of wild-type enzyme.

Transglycosylation and transgalactiosylation activity of the mutant enzyme (Q77/150W) showed a slightly different hydrolytic pattern, but produced same products compared with the native enzyme. To confirm the category of PFTG, the structure studies are essential, that were reported in Chapter 4.

CHAPTER 4

Crystal Structure Studies of *Pyrococcus Furiosus* Thermophilic β-Glycosidase

The characterization and mutational studies demonstrated that the PFTG should be renamed as β-glycosidase (Chapters 2 & 3). However, to confirm the category of the PFTG, the protein crystal structure and sequence aligmnent studies were studied in this chapter.

4.1. ABSTRACT

After the PFTG wild-type enzyme was purified through Ni-NTA affinity chromatography and gel permeation chromatography (GPC), the purified enzyme was set up for protein crystal screening in 200 different conditions. After incubation at 18°C, several small protein crystals were shown in some conditions.

With several hit conditions, X-ray diffraction study was performed. During the X-ray analysis, the data of diffraction factor was too high to obtain reasonable data. Through protein sequence analysis of PFTG, two Lys-Lys sequences were revealed. This Lys-Lys bond might have inhibited the protein crystallization because that sticked sequence cleaves the protein structure. After Lys-Lys amino acids were mutated to Ala-Ala by site-directed mutagenesis, the mutant enzymes were set up and incubated for crystallization.

Keywords : *Pyrococcus furiosus*, Thermostable glycosidase, Protein crystallography

4.2. INTRODUCTION

4.2.1. Protein structure studies

Protein crystallography is a method of determining the arrangement of atoms within a crystal, in which a beam of X-rays strikes a crystal and diffracts into many specific directions. From the angles and intensities of these diffracted beams, the tertiary structure of protein can produce a three-dimensional picture of the density of electrons within the crystal. From electron density, the positions of the atoms in the crystal as well as their chemical bonds, their disorder and various other information can be determined.

Since many materials can form crystals such as salts, metals, minerals, semiconductors, as well as various inorganic, organic and biological molecules, X-ray crystallography has been fundamental in the development of many scientific fields. The X-ray crystallography method revealed the structure and functioning of many biological molecules, including vitamins, drugs, proteins and nucleic acids such as DNA (Law, 1973). X-ray crystallography is still the major method for characterizing the atomic structure of new materials and in discerning materials that appear similar by other experiments. X-ray crystal structures can also account for unusual electronic or elastic properties of a material, or serve as the basis for designing pharmaceuticals against diseases.

In a X-ray diffraction measurement, a crystal is mounted on a goniometer and gradually rotated while being bombarded with X-rays, producing a diffraction pattern of regularly spaced spots known as *reflections*.

The two-dimensional images taken at different rotations are converted into a three-dimensional model of the density of electrons within the crystal using the mathematical method of Fourier transforms, combined with chemical data known for the sample. Poor resolution or even errors may result if the crystals are too small, or not uniform enough in their internal makeup.

The science of protein crystallization is an underdeveloped area, although interest is growing, especially by microgravity experiments in space flights (McPherson *et al*., 1996; Kundrot *et al*., 2001). Protein crystallization is mainly a trial-and-error procedure in which the protein is slowly precipitated from its solution. The aim of protein crystallization is to produce well-ordered protein mono-crystals without any inclusion and large enough to diffract X-ray beam. Despite wide knowledge about protein crystallization it is still impossible to predict any conditions for protein crystallization. The protein crystallization process is still empiric and the biggest part of success is hidden in the hand and experience of the scientist who performed the protein crystallization and depends on luck.

The impurity of protein, crystallization nuclei, and other unknown factors play a role in this process. The purity of protein should be extremely important. On the other hand, in order to achieve protein crystallization, not only other compounds absent, but all molecules of the protein should have the same surface properties, especially the same charge distribution on their surface, as this influences the packing of the molecules in the crystal. Mass spectrometry is a valuable tool in protein crystallization (Cohen & McDowall, 1997).

The crystallization of proteins contains important steps: First, the purity of the protein is very important to obtain a pure crystal. Then the protein is set up in a suitable solvent from which it should be precipitated by a salt or an organic compound. The commercial crystal screening kits are mostly used as a solvent recently. After that, the solution is brought to supersaturation. In this step, small aggregates are formed, which are the nuclei for crystal growth. For the crystallization of small molecules, which is understood much better than the crystallization of proteins, the spontaneous formation of nuclei requires a supply of surface tension energy. Once the energy barrier has been passed, crystal growth begins. The energy barrier is easy to overcome at a higher level of supersaturation. Formation of nuclei can be studied as a function of supersaturation and other parameters by several techniques (i.e. light scattering, fluorescence depolarization and electron microscopy). Finally, once crystal has formed, actual crystal growth can begin. The best crystals grow by reducing the supersaturation to a lower level; maintaining a high supersaturation would result in the formation of too many nuclei and, therefore, too many small crystals. Also, crystals should grow slowly to reach a maximum degree of order in their structure (Drenth, 1999).

Precipitation of the protein can be achieved by the addition of a salt, polyethyleneglycol (PEG), or an organic solvent. As a precipitating agent a salt acts in two ways (salting out). Its ions screen electric charges on the surface of the protein molecules, thereby weakening the repulsive forces between the molecules. For best results, the crystals should be grown at a lower level of supersaturation than is required for formation of nuclei.

In addition, part of the water is immobilized and not available to the protein because of the generation of a hydration layer around the salt ions. Ammonium sulfate which is highly

soluble and is not harmful to most proteins even in a high concentration and polyethyleneglycol (PEG) which has a high affinity for water and can immobilize water as well as salt is the most popular used as an immobilizing agent for protein crystal studies. Some proteins are poorly soluble in water but do dissolve if a small amount of salt (much smaller than for salting out) is added. By removing the salt, the protein precipitates. This "salting in" effect is explained as the result of a competition between charged groups on the protein molecular surface and the ions in the solution. In the absence of solvent ions, the protein molecules are not surrounded by an ionic double layer and can aggregate by Coulomb attraction between opposite charges on different protein molecules. If a small amount of ions is added, a double layer forms around the protein molecules. These layers do not penetrate and repel each other.

Other methods to decrease the protein solubility are changing the pH of the solution or the temperature. To obtain the best crystallization conditions, it is usually necessary to carry out many trials of experiments (Drenth & Mesters, 2007).

The strategy for membrane proteins is to use detergents to solubilize them in an aqueous solution and then follow one of the procedures for water-soluble proteins (Madhusudan *et al*., 1994). Landau & Rosenbusch (1996) introduced a new method that could be promising for the crystallization of membrane proteins. They applied lipidic cubic phases as crystallization matrices for a variety of compounds. Lipidic cubic phases are liquid crystals with a cubic symmetry. They can be formed in a mixture of lipids and water (Lindblom & Rilfors, 1989). Several types of lipidic cubic phases exist. Pebay-Peyroula *et al*. (1997) succeeded in growing well-ordered crystals of the membrane protein of bacterial rhodopsin in

a certain type of lipidic cubic phase. It remains to be seen whether this method will be successful in the crystallization of more membrane proteins.

4.2.2. Substitution of lysine residue for improving protein crystallization

Solvent-exposed amino acid side chains can be equally disruptive to a well-ordered crystal. One approach to this problem has been to mutate these residues to smaller hydrophobic residues (Lawson *et al*., 1991), where the diffraction quality of crystals was greatly improved by the mutation of an exposed lysine to a glutamine. Particular attention has been given to the mutation of large charged residues such as lysine (McElroy *et al*., 1992; Longenecker *et al*., 2001) and glutamate (Mateja *et al*., 2002). Site-directed mutagenesis is now an established technique. In addition, in the absence of structural knowledge, it is quite possible that key stabilizing salt bridges might be inadvertently targeted for destruction.

The most common approach to chemical modification has been the reductive methylation of free amino groups in which primary amines (i.e., lysine residues and the N terminus) are modified to tertiary amines. This has proven value for protein crystallization, allowing, in certain instances, the formation of structure diffraction-quality crystals for proteins that had previously proved refractory to crystallization (Rayment, 1997; Kobayashi *et al*., 1999; Kurinov *et al*., 2000; Schubot & Waugh, 2004; Saxena *et al*., 2005).

Many soluble proteins purified to homogeneity resist crystallization because of the intrinsic flexible nature of the surface residues. Lysine and glutamic acid residues found on the surface of the proteins have long side chains that are mobile. These side chains need to be

localized in space in order to promote homogenous intermolecular interactions necessary for formation of a crystal.

The concept of crystal engineering has suffered from the lack of a rational approach: mutations were clearly recognized as a means to change the solubility of proteins, but no rational protocol was ever designed to crystallize a new protein by surface mutagenesis. Mutagenesis of surface residues such as Lys and Glu to Ala or other smaller amino acids might systematically improve protein crystallization by creating epitopes more favorable for the formation of crystal contacts with negligible loss of conformational entropy (Longenecker *et al*., 2001).

Through modeling study of PFTG, all of the lysines are either completely or partially exposed on the surface. On average, Lys is the most solvent-exposed residue in proteins, that might be affected the crystallization. Thus, we decided to substitute Lys-Lys sticked residues to Ala-Ala in hoping that this might improve the PFTG crystal efficiency.

4.3. MATERIALS AND METHODS

4.3.1. Chemicals and media

Buffer kits for crystallization (MembFac, Crystal Screen Lite, Index, Crystal Screen, Crystal Screen 2, Crystal Screen Cryo, Crystal Screen 2 Cryo) were purchased from Hampton Research. (Journey Aliso Viejo, CA). Bacto yeast extract and Bacto tryptone were purchased

from BD Diagnostics (Sparks, USA). All restriction enzymes and calf intestine alkaline phosphatase were obtained from New England Biolabs, Inc. (Ipswich, USA). Ex-Taq DNA polymerase, T4 DNA ligase, and protein molecular weight standards were purchased from Takara Bio, Inc. (Shiga, Japan). All other chemicals of reagent grade were purchased from Fisher Scientific (Colonnade Road, Ottawa, ON).

4.3.2. Protein Purification

After *E. coli* cells carrying the *pftg* gene were cultured in LB medium containing kanamycin and harvested by centrifugation, the cells were resuspended in lysis buffer (50 mM Tris- HCl, pH 7.5, 300 mM NaCl, and 10 mM imidazole) and sonicated using a VC-600 sonicator (Sonics & Materials, Inc., Newtown, CT). After the crude cell extract was centrifuged (10,000x*g*; 15 min, 4°C), the supernatants were subjected to nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen).

4.3.3. Site-directed mutagenesis

Site-directed mutagenesis was performed using a Quik-Change kit (Stratagene, La Jolla, CA, USA). The recombinant DNA 6xH PFTG carrying the *pftg* gene with six histidines fused at the N-terminus was used as template DNA (Table 4.1). The synthetic oligonucleotide used for the mutation of KK111AA and KK138AA had the sequence in Table 4.2. After PCR, the amplified DNA fragment was phosphorylated by T4 polynucleotide kinase and ligated

with T4 DNA ligase. Transformation was carried out using the calcium chloride method (Sambrook & Russell, 2001). The specific nucleotide mutation was confirmed using the dideoxy chain termination sequencing method with an ABI3700 PRISM DNA sequencer (Perkin-Elmer, Wellesley, MA, USA).

4.3.4. Expression and purification of PFTG wild-type and mutant enzymes

Escherichia coli MC1061 [F⁻, araD139, recA13, Δ(araABC-leu)7696, galU, galK, ΔlacX74, rpsL, thi, hsdR2, mcrB] was used as a host for the expression of wild-type PFTG and its KK111AA/KK138AA mutants. The *E.coli* transformants were cultured in Luria–Bertani (LB) medium (1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl) containing kanamycin (100 μg/ml) at 37 °C. Six-His-tagged PFTG and its mutant were first purified using a Ni-NTA column (1×4cm, Ni- NTA Superflow™, Qiagen, Hilden, Germany) as previously described (Kim *et al*., 1999). The eluted enzymes were concentrated using Viva spin (Sartorius Mechatronics, Mississauga, ON), applied to a Superdex™200 column (Pharmacia Biotech, Uppsala, Sweden) and eluted with 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl. The fraction showing enzyme activity was dialyzed against 50 mMTris–HCl buffer (pH 7.5), and concentrated. The purity of the enzyme was confirmed by SDS-PAGE analysis (Laemmli *et al*., 1970).

Table 4.1. Bacterial strains and plasmids used or constructed in this study.

Bacterial strains and plasmids	Characteristics and Usages	References
E. coli BL21(DE3)	Expression host	Sambrook
E. coli MC1061	Cloning or expression host	Sambrook
pET-28b(+)	C-terminal 6xHis tag, T7 promoter, Kan^r	Novagen
pTkNd6xH	C-terminal 6xHis tag, *B. licheniformis* MAase (BLMA) promoter, Kan^r	Kim, 2005
pETPFTG-6h	Expression of C-terminally 6xHis tagged PFTG, T7 promoter, Kan^r	This study

Table 4.2. Oligonucleotides used in this study.

Name	Sequence of oligonucleotide
KK111AA(fwd)	5’-AAAAAGGATAAAGAT***GCCGCC***AGAAAACCTGGAGGAACTT-3’
KK111AA(Rev)	5’-AAGTTCCTCCAGGTTTTCT***GGCGGC***ATCTTTATCCTTTTT-3’
KK138AA(fwd)	5’-AATGTCTTAAGAAACTTA***GCCGCC***CTTGGATTCACAACATTCGTTA- 3’
KK138AA(Rev)	5’-TAACGAATGTTGTGAATCCAAG***GGCGGC***TAAGTTTCTTAAGACATT-3’

4.3.5. Computer modeling structure

The modeling structure of PFTG was based on the knowledge of the X-ray structures for β-glycosidase from *Thermosphaera aggregans* (1qvb), whose amino acid sequence was 44% identity to the PFTG. The software InsightII (Accerlys, San Diego, CA, USA) and pymol (DeLano, 2002) were used for the modeling and energy minimization on a Silicon Graphics Indigo workstation. The minimization was performed with the Discover Platform of the InsightII program using a consistent valence force field (CVFF) and conjugate gradient options.

4.3.6. Screening of protein crystallization

The purified PFTG (10 mg/ml) and KK111AA/KK138AA mutant enzymes (10 mg/ml) were prepared and assessed for crystallization. All samples were shown to crystallize using the MembFac, Crystal Screen Lite, Index, Crystal Screen, Crystal Screen 2, Crystal Screen Cryo, Crystal Screen 2 Cryo buffer kits (Hampton Research).

4.4. RESULTS AND DISCUSSION

4.4.1. Over-expression and purification of PFTG wild-type and mutant enzymes

For efficient expression in *E. coli*, the PCR-amplified DNA fragment containing the gene for PFTG was subcloned into pET28 (b) under the control of the promoter for T7 polymerase. Proteins of *E. coli* in the crude extract were denatured by incubating at 75°C for 20 min. After heat inactivation, the enzyme was purified using Ni-NTA affinity chromatography. Purifications of PFTG and mutant enzyme were improved by attaching eight additional amino acids (Leu, Glu, and six His residues) at the C-terminus rather than at the N-terminus. After the purified enzymes were concentrated, they were subjected to a Superdex™200 column and eluted with 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl. The fraction showing enzyme activity and the purity of the enzyme was analyzed by SDS-PAGE shown in Fig. 4.1 (a), (b).

4.4.2. Screening of protein crystallization and data collection Modeling study

The purified PFTG (10 mg/ml), KK111AA and KK138AA mutant enzyme (10 mg/ml) were prepared and assessed for crystallization and all were shown to crystallize using the Hampton Research buffers (MembFac, Crystal Screen Lite, Index, Crystal Screen, Crystal

Screen 2, Crystal Screen Cryo, Crystal Screen 2 Cryo). The crystal set up procedures were carried out at 18°C and incubated at 18 °C.

Crystallization trials of PFTG were set up using the microbatch and hanging drop method under oil at 18 °C. The native protein was crystallized in needle shape crystal forms. The crystal of the PFTG was grown from 0.1M Tris-HCl (pH 7.5) + 2M ammonium sulfate and 0.1M Tris-HCl (pH 8.0) + 2M ammonium sulfate (Fig. 4.2). Before data collection, the crystals were cryocooled to 95 K using a cryoprotectant consisting of mother liquor supplemented with 20% glycerol. The native and ligand-bound data sets were collected at the MX4II and MX6C beamlines of the Pohang Accelerator Laboratory (Pohang, Korea), respectively. However, the diffraction factor was too high to get the reasonable data. The protein crystal needed to grow more to obtain the good data.

By BLAST search PFTG had 44% homology identity with β-glycosidase from *Thermosphaera aggregans*. Through modeling study of PFTG, all of the lysines are either completely or partially exposed on the surface (Fig. 4.3). On average, Lys was the most solvent-exposed residue in proteins, that may have been a problem for the crystallization. Thus, we decided to substitute Lys-Lys sticked residues to Ala-Ala (Fig. 4.4). This substitution might be useful to increase the PFTG crystal efficiency.

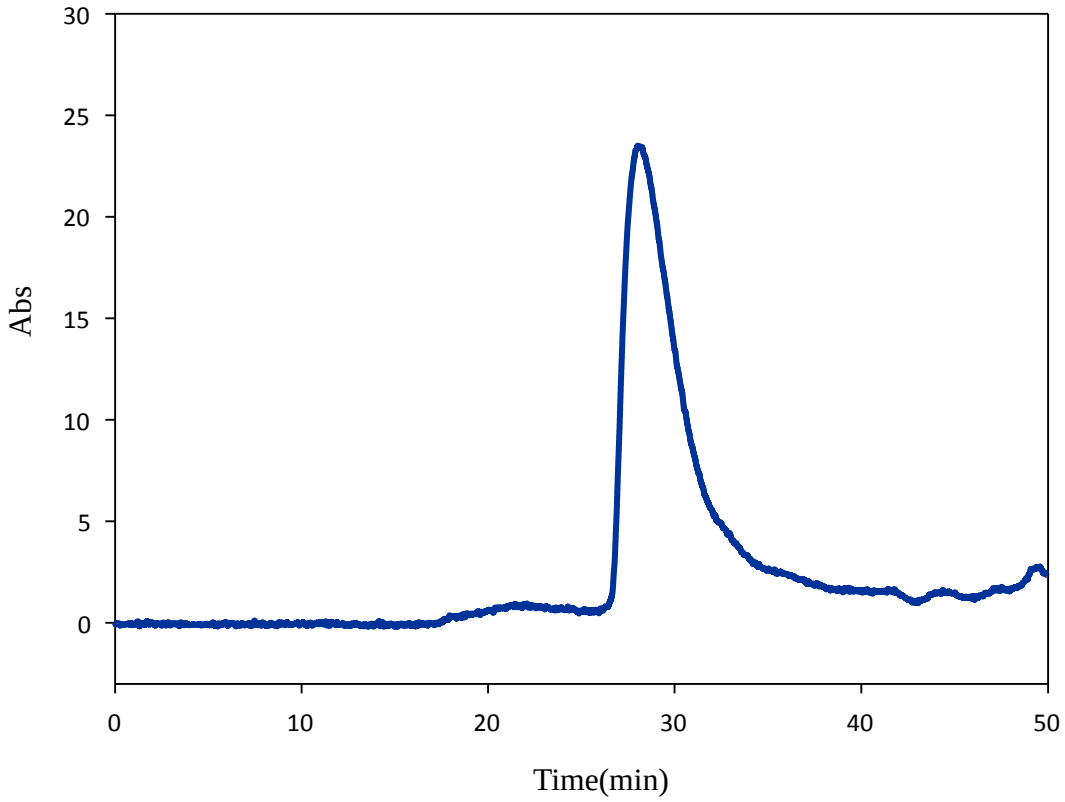
4.4.3. Mutagenesis of KK111AA and KK138AA

Mutagenesis was performed in order to introduce the following designed changes: KK111AA and KK138AA, respectively. All mutated genes were purified with QIAEXII Gel

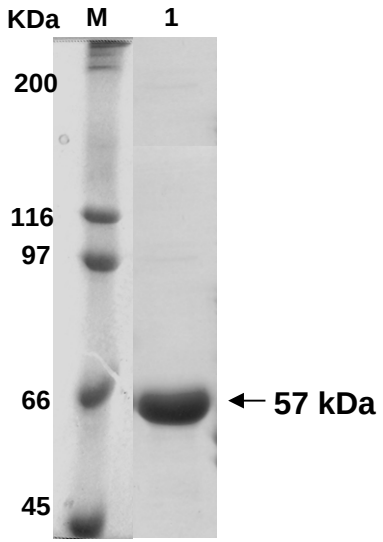
Extraction kit (Qiagen) after gel separation.

For more efficient mutagenesis, the protocol for QuikChange^{TM} site-directed mutagenesis kit (Stratagene, USA) was modified slightly in this study. The ligation mixture was transformed into *E. coli* MC1061 and the mutation carried by the transformants was confirmed by DNA sequencing. The mutated nucleotides were then confirmed by agarose gel electrophoresis and DNA sequencing (Figs. 4.5 & 4.6). Both mutant enzymes (KK111AA and KK138AA) were subcloned separately by digestion by restriction digestions and ligation of the plasmids.

The mutant enzymes KK111AA and KK138AA were also set to crystallize under incubation at 18°C.



(a)



M : size marker
1 : purified enzyme

(b)

Figure 4.1. (a) Gel permeation chromatography (buffers : Tris-HCl 7.5 + 150mM NaCl using Superdex^{TM}75 column) and (b) SDS-PAGE results of purified PFTG .

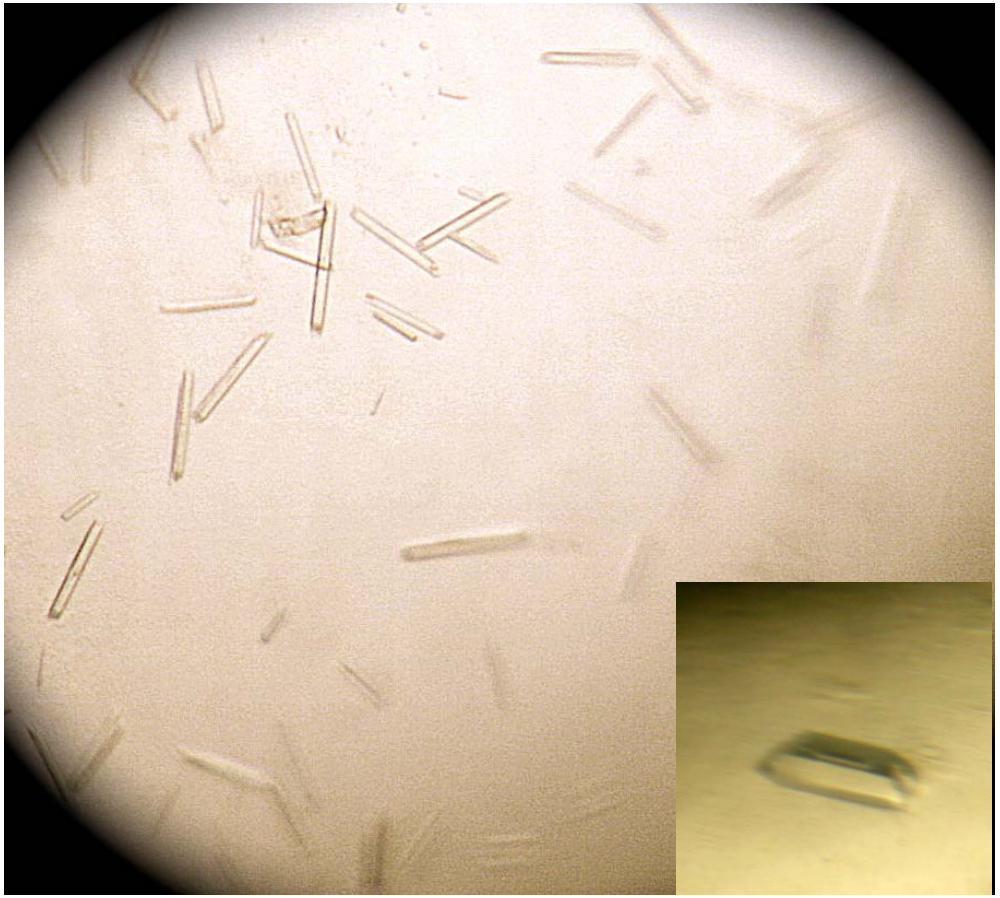


Figure 4.2. Protein crystals of PFTG wild-type (conditions: 0.1M Tris-HCl (pH 8.0) + 2M ammonium sulfate).

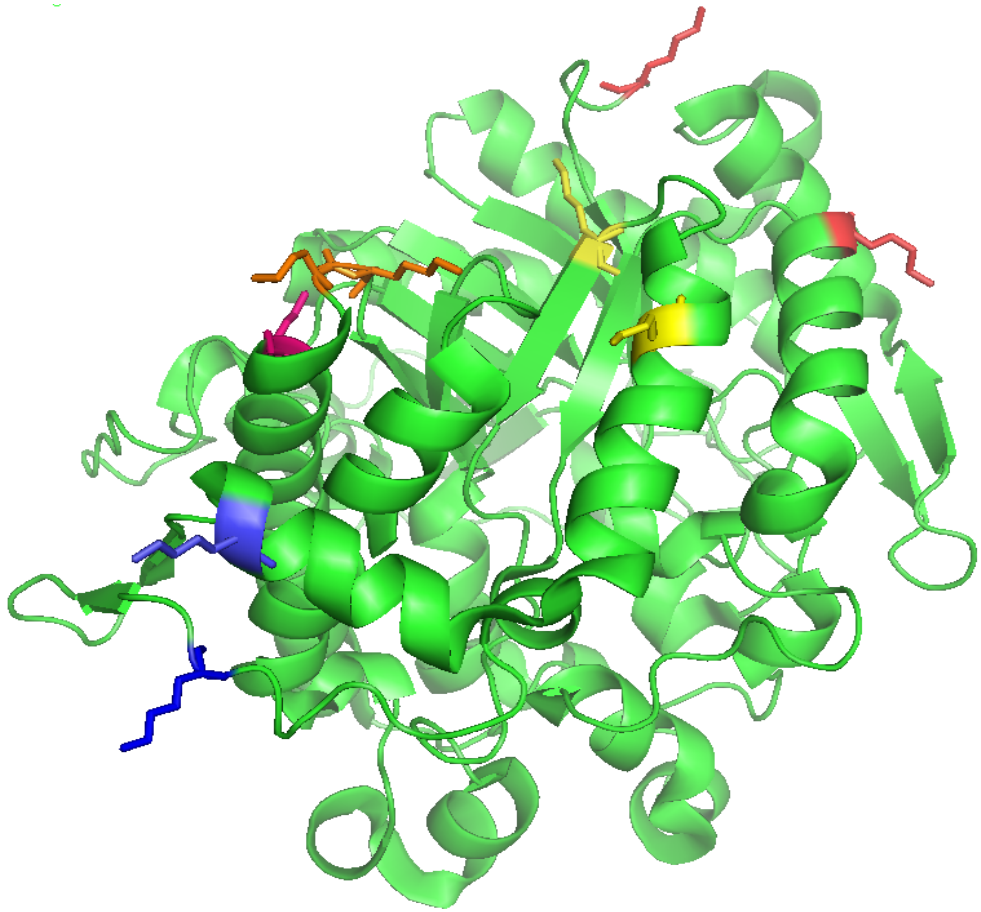


Figure 4.3. Crystal structure of the beta-glycosidase from the hyperthermophile *Thermosphaera aggregans* which has 44% homology identity with PFTG. Lysine molecules (shown in yellow, red, pink, orange, and blue sticks) exposed on the space.

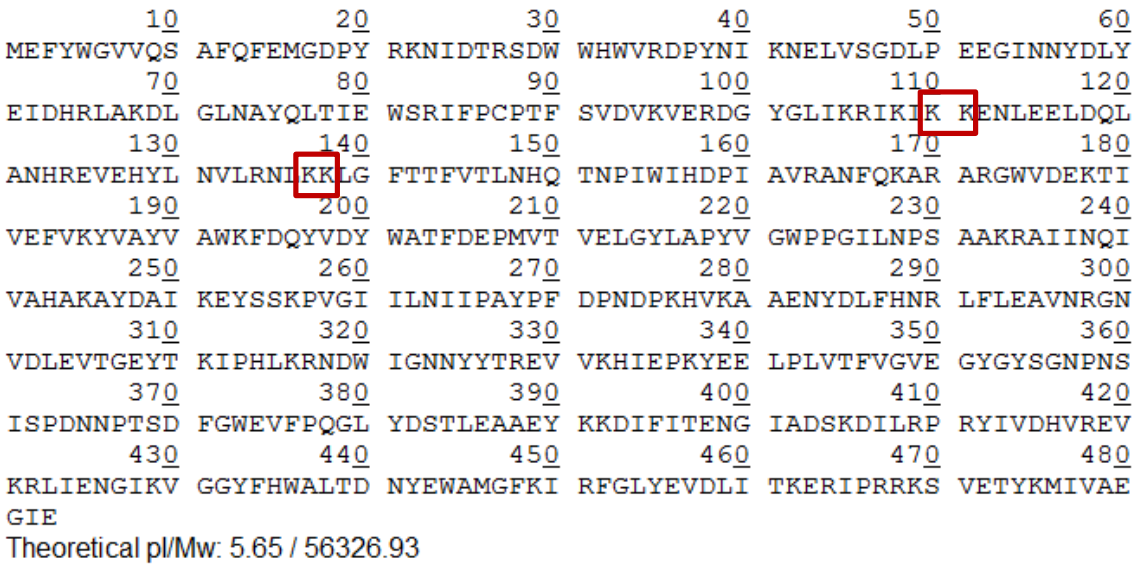


Figure 4.4. Protein sequence of PFTG indicated residue 111 to 112 and two lysines (137,138) have sticked together (http://ca.expasy.org/).

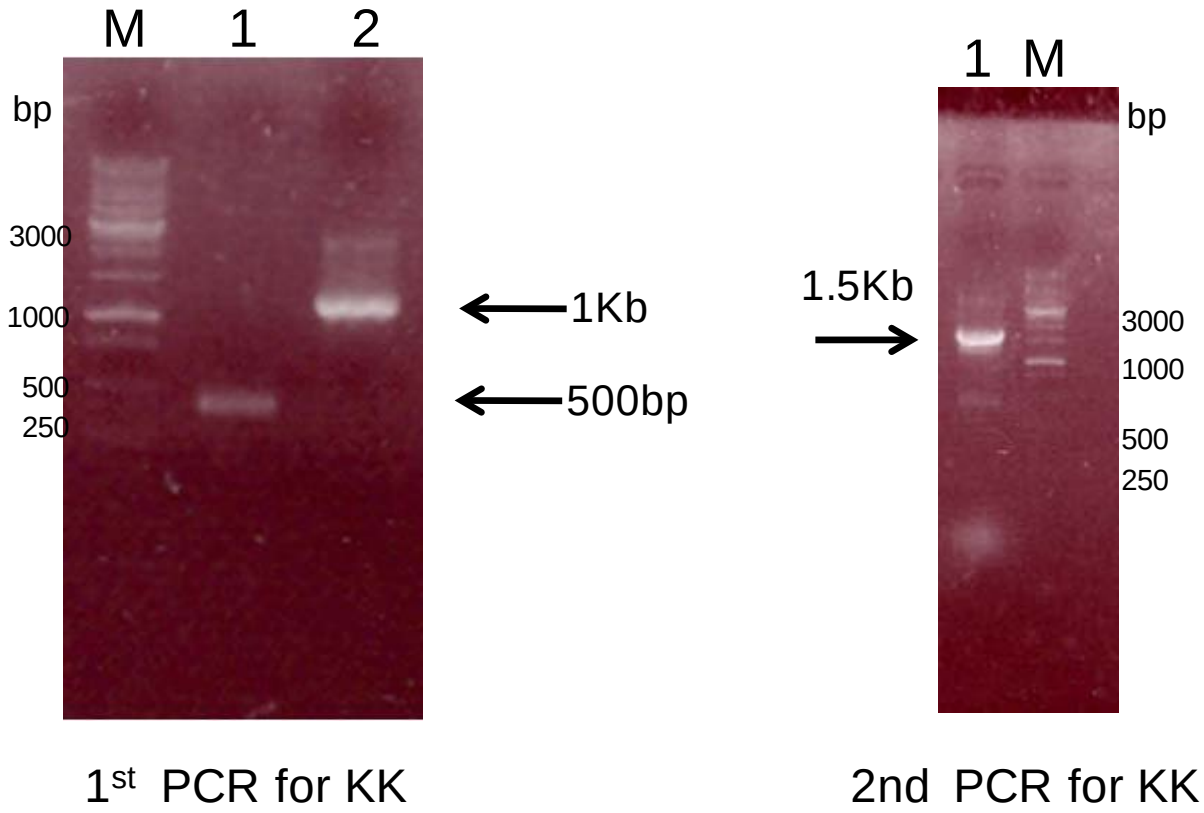


Figure 4.5. Agarose gel electrophoresis of the PCR products. Lane M was loaded with size marker; lane 1, the small DNA fragment (~500 bp) and lane2 is the large fragment (~1.0 kb) of PFTG DNA. Right figure showed ligated DNA fragments.

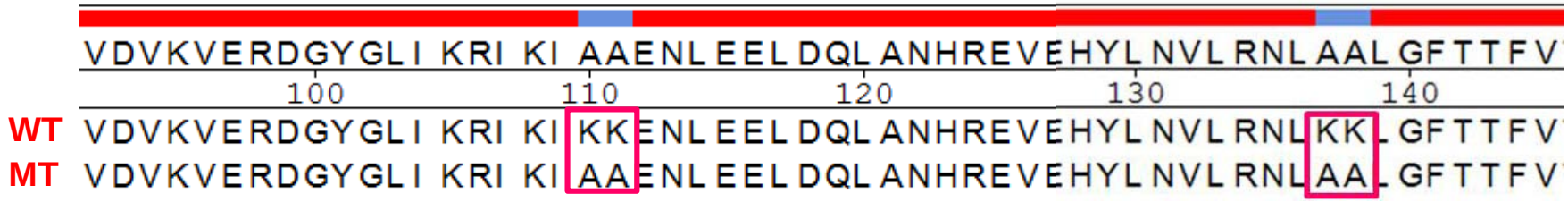


Figure 4.6. DNA sequence results of mutation KK111AA and KK138AA. Boxes indicated substituted amino acids.

4.5. CONCLUSIONS

The purified PFTG wild-type enzyme was set up for protein crystallization under over 200 different conditions with micro-batch methods. After incubation at 18°C, several hits were found for protein crystal of native enzyme in some conditions (0.1M Tris-HCl (pH 7.5) + 2M ammonium sulfate and 0.1M Tris-HCl (pH 8.0) + 2M ammonium sulfate).

The protein crystals were analyzed through X-ray (beam line), but the diffraction factor (~7Å) was too high to obtain the reasonable data. To enhance protein crystallization of PFTG, lysine-lysine residues which may interfere protein crystallization were substituted to alanine-alanine by site-directed mutagenesis. The mutant enzymes (KK111AA/KK138AA) were successfully created, purified and set up for protein crystallization. The crystal structure will eventually demonstrate the real identity of this hyperthermophilic β-glycosidase.

GENERAL CONCLUSION

An extremely thermostable glycoside hydrolase was isolated from the genome of hyperthermophilic archaeon *Pyrococcus furiosus*. The *P. furiosus* thermostable β–glycosidase gene (*pftg*) encoded a single polypeptide of 483 amino acids with an estimated molecular mass of 56,326 Da. The deduced amino acid sequence of this PFTG was highly homologous with various β-glycosidases.

The *pftg* gene was successfully expressed in *E. coli*, and the recombinant PFTG with eight additional amino acid residues (Leu, Glu, and six His residues) at the C-terminus was easily purified using Ni-NTA affinity chromatography. The recombinant PFTG was extremely thermostable with the optimum of 100°C at pH 5.0 that retained its full activity after 120 min of incubation at 85°C. Differential scanning calorimetry analysis showed that the T_m of the enzyme was 109.9°C, confirming that this enzyme is highly thermostable.

PFTG hydrolyzed mannooligosaccharides, synthetic substrates (*para* nitrophenyl β-D-glucopyranoside, galactopyranoside, xylopyroside and mannopyranoside), lactose and cellobiose to produce glucose and galactose, like a typical β-glycosidase. Through kinetic studies by ITC (isothermal titration calorimetry) this enzyme showed the highest catalytic efficiency (k_{cat}/K_m) toward para-nitrophenyl mannopyranoside, showing closer homology to β-mannosidase than β-galactosidase.

To elucidate the category and accurate substrate specificity of the PFTG, the amino acids around the active site (Q77, Q150 and Q77/150W) of PFTG were substituted by site-directed mutagenesis and expressed. Kinetic studies by ITC (Isothermal Titration Calorimetry) revealed

that the mutant enzymes and native enzyme showed same patterns, but the mutant enzymes showed more affinity (K_m) towards each substrate and lower turnover numbers (k_{cat}) than that of wild-type enzyme. Transglycosylation and transgalactiosylation activity of the mutant enzyme (Q77150W) showed slightly different hydrolyzing rate but produced same products compare to the wild-type enzyme.

To confirm the real category of PFTG, the purified wild-type enzyme was set up for protein crystallization under 200 different conditions with micro-batch methods. After incubation at 18°C, several hits for protein crystals of native enzyme were obtained in some conditions (0.1M Tris-HCl (pH 7.5) + 2M ammonium sulfate and 0.1M Tris-HCl (pH 8.0) + 2M ammonium sulfate. The protein crystals were analyzed through X-ray (beam line), but the diffraction factor (~7Å) was too high to obtain the reasonable data. To enhance protein crystallization of PFTG, lysine-lysine residues which might be interfere protein crystallization were substituted to alanine-alanine by site-directed mutagenesis. The mutant enzyme (KK111AA/KK138AA) was set up again for protein crystallization. Clear protein crystals were not obtained, but the computer 3D model structure indicated that this enzyme was similar to that of β-glycosidase from *Thermosphaera aggregans*. The crystal structure will eventually demonstrate the real identity of this hyperthermophilic β-glycosidase. This hyperthermostable β-glycosidase which has novel catalytic properties might be useful for food and pharmaceutical industries.

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