Abstract

HARRIS, JAMES MORGAN. Biochemical and Biophysical Characterization of Hyperthermophilic Sugar Isomerases and Epimerases. (Under the direction of Dr. Robert M. Kelly.)

The availability of enzymes with optimal functional temperatures above 70°C has had considerable impact on industrial biocataysis. Extremely thermophilic enzymes have expanded the known thermal range of biological systems and ushered in a new era in applied biocatalysis less restricted by limitations related to thermoactivity and thermostability. While advances have been in the made in understanding enzyme stabilization at high temperatures, much is to be learned of this complex biomolecular trait. Nonetheless, extremely thermophilic enzymes are being investigated as biocatalysts in a variety of bioprocesses. Here, the biochemical and biophysical properties of hyperthermophilic sugar isomerases and epimerases were examined with respect to their potential to mediate the biosynthesis of monosaccharides with nutritional and medical significance, also know as “rare sugars”.

D-xylose isomerase from *Thermotoga neapolitana* 5068 containing an N-terminal fusion with a chitin-binding domain (ChiBD) from a hyperthermophilic chitinase from *Pyrococcus furiosus* was examined incomparison to the wild type TNXI. The IM-ChiBD-TNXI half-life (19.9 h) was approximately three times longer than the soluble wild-type TNXI (6.8 h). Furthemore, the unbound soluble ChiBD-TNXI had a longer life-life (56.5 h) than the immobilized enzyme. Both unbound and immobilized ChiBD-TNXI not only had higher turnover numbers for glucose to fructose than the wild-type enzyme, but also for any known enzyme of this type. Molecular modeling, based on structural information on the wild-type TNXI and *Pf*ChiBD, showed that the N-terminal fusion likely impacted subunit interactions, thereby contributing to the enhanced thermostability of the unbound ChiBD-TNXI. These

results illustrate that substantial changes- in thermostability and reaction kinetics can result from affinity tags for hyperthermophilic proteins.

Sugar isomerases and epimerases (L-fucose isomerase (TMFI), L-arabinose isomerase TMAI), L-rhamnose isomerase (TMRI), D-tagatose 3 epimerase (TMTE), and D-xylose isomerase (TNXI)) from the hyperthermophilic bacterial genus *Thermotoga* were examined as biocatalysts for rare sugar synthesis. Single and multi step-reactions involving each isomerase with TMTE produced both expected and unexpected products, based on similar experiments with homologous mesophilic enzymes. The recently reported TMTE three-dimensional structure revealed a non conserved active site and hydrophobic binding pocket compared to mesophilic epimerases, likely responsible for the biocatalyic results observed in this study.

Biochemical and Biophysical Characterization of Hyperthermophilic Sugar Isomerases and Epimerases

by
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DEDICATION

I would like to dedicate this work to GOD, my grandparents (James and Mabel Hines), my parents (James and Lorna Harris), and to my brothers (Victor and Langston Harris). Withoutyour constant support and inspiration, none of my achievements would be possible. I would also like to dedicate this work to D.S., T.H., and C.E.

BIOGRAPHY

James Morgan Harris was raised in the town of Chapel Hill, NC. He attended North Carolina A&T State University (Greensboro, NC) and received a Bachelor of Science degree in chemical engineering and was one of the first North Carolina A&T State University honor program graduates to complete the thesis track in chemical engineering in May 2002. After graduation, James took courses at the University of North Carolina at Chapel Hill for Biophysics and Biochemistry for one year before starting the Ph.D. program in the department of chemical and biomolecular engineering at North Carolina State University in order to develop an expertise in the field of biotechnology. He obtained a non-thesis Masters of Science degree in chemical engineering in 2006. Following completion of his doctoral degree in chemical and biomolecular engineering with a minor in biotechnology in 2009, James plans to either work in industry (BD, Biogen Idec), obtain a postdoc (NIEHS, NCCU), or obtain a faculty position (NC A&T SU).

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Enzymes, Extremely Thermophilic

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ABSTRACT

The availability of enzymes with optimal functional temperatures above 70°C has had considerable impact on basic and applied elements of biocataysis. Not only have extremely thermophilic enzymes expanded the known thermal range of biological systems but they have also ushered in a new era in applied biocatalysis that is less restricted by limitations related to thermoactivity and thermostability. While much effort has been directed at understanding the intrinsic basis for enzyme stabilization at high temperatures, this is still much to be learned to appreciate this complex biomolecular trait. Nonetheless, strategic uses of extremely thermophilic enzymes continue to emerge and prospects for expanded use of biocatalysts in a variety of bioprocess settings are promising. As genome sequence information from extremely thermophilic microorganisms is mined for novel biocatalysts and molecular biological tools for improving enzyme function are refined, the future is bright for biologically-based catalysis.

I. INTRODUCTION

On earth, and perhaps on other planets and moons yet to be explored, extreme environments (e.g., temperature, pH, ionic strength, radiation, pressure) harbor microorganisms capable of growth and survival under otherwise biologically debilitating conditions [1-4]. Within this group of the so-called “extremophiles” are thermophilic (or “heat-loving”) bacteria and archaea that can be more precisely classified, according to their growth temperature, as extremely thermophilic if their optimal growth temperature (T_{opt}) is above 70°C. Hyperthermophiles are a subset of extreme thermophiles with T_{opt} values ≥ 80°C. For the purposes of this review, extremely thermophilic enzymes will be those that function optimally at 70°C or higher, regardless of their natural source. Enzymes with this functional temperature range can be derived from less thermophilic microorganisms than extreme thermophiles, or result from recombinant DNA manipulations that result in the stabilization of thermally labile enzymes.

Extremely thermophilic enzymes are highly desirable for a wide range of industrial uses because of their efficacy as biocatalysts at elevated temperatures and prolonged lifetimes under biologically-adverse processing conditions [1, 2, 5]. Even when operating temperatures are biocatalytically sub-optimal for a particular thermophilic enzyme, the trade-off between slower kinetics and longer operational half-life can still favor the heat-tolerant biomolecule. Extremely thermophilic enzymes can be obtained from natural sources, either directly from microbial biomass [6], or through the cloning and heterologous expression of genes encoding extremely thermophilic enzymes in mesophilic hosts, e.g., *Escherichia coli* [7]. Extremely thermophilic enzymes can also be created through thermostabilizing modifications of thermolabile enzymes using strategic recombinant DNA techniques, such as directed evolution [8, 9]. Furthermore, chemical and biochemical adjuvants and

compatible solutes can be employed to extend the period of useful biocatalysis [10, 11]. The utilization of such approaches over the past several decades have created new and expanded opportunities for industrial biocatalysis, such that bioconversions requiring elevated temperatures or extended periods of operation can make strategic use of naturally-occurring or recombinantly-created extremely thermophilic enzymes [12].

The discovery of extremely thermophilic microorganisms (growth $T_{opt} \geq 70°C$) and the subsequent purification and characterization of their extremely thermostable and thermoactive enzymes, has brought into focus the complex nature of protein stability. It is clear now that differences of 100°C or more can separate the temperature optima of enzymes carrying out identical biocatalytic functions. In cellular environments, enzyme stability is a property that has important physiological implications: protein stability must be consistent with *in vivo* functions. The free energy of stabilization of an enzyme, a balance between large but opposing entropic and enthalpic forces, is equivalent to only a few, weak, non-covalent bonds [13]. Once an enzyme has performed a needed cellular function, which is no longer necessary or becomes detrimental, it may denature, be de-attenuated through a regulatory response, or be proteolytically degraded. Clearly, the maintenance of an enzyme's structural integrity and catalytic efficiency both need to be considered for biocatalysis in extreme environments.

Even though site-directed mutagenesis and directed evolution have become routine approaches for conferring thermostability on an enzyme, success in such ventures is by no means assured. Nature has fortunately provided a source of thermostable biocatalysts that are produced by extremely thermophilic microorganisms. Genome sequences of extreme thermophiles completed to date indicates most enzymes from mesophilic sources have high

temperature, and presumably intrinsically thermostable, counterparts, many of which have technological potential [1, 14].

II. EXTREMELY THERMOPHILIC ENZYME DISCOVERY AND PRODUCTION

The methodology for discovering and producing enzymes with technological importance has advanced considerably in recent years with the ever-expanding use of molecular biology tools. Concomitantly, the search for enzymes from microorganisms in extreme environments has benefited from advances in modern biology and genomics [15]. Gone are the days when industrial enzymes were obtained exclusively in their native forms from soil microorganisms grown in pure culture. Now, only the gene encoding an enzyme of interest is, in principle, necessary, if it can be overexpressed in an appropriate recombinant host. As mentioned above, genome sequences have provided an expansive list of potentially important enzymes from biologically diverse organisms and cells. The key now is to match the technological need with the appropriate biocatalyst.

A. Direct purification of enzymes from extreme thermophile biomass

Expeditions to geothermal habitats, as accessible as hot springs in Yellowstone National Park [16] and as inaccessible as deep sea geothermal vents [17], have led to the isolation of an array of high temperature microorganisms covering a diversity of physiological types [4]. Typically, isolates are obtained through serial dilution techniques, since high temperatures preclude cultivation on solid media in many cases. Most extreme thermophiles are anaerobic, which is not surprising considering the low solubility of O_2 at elevated temperatures. Notable exceptions are members of the order *Sulfolobales*, many of

which are extreme thermoacidophiles since they typically grow best at pH less than 3 [18]. Hyperthermophiles, capable of growth at 90°C or higher, are almost exclusively anaerobic and many reduce sulfur species to sulfide during growth [19]. Additional details on extreme thermophiles can be found elsewhere in this volume (see *Microorganisms, Extremely Thermophilic*).

Cultivation of extreme thermophiles on a scale sufficient to provide biomass for enzyme purification efforts presents many challenges [6]. In short, lack of knowledge of microbial physiology, low biomass yields, generation of copious amounts of hydrogen sulfide, explosive substrate and product gases, salt-laden media, anaerobic conditions and cell sensitivity to shear are all potential obstacles. A few heterotrophic hyperthermophiles, e.g., *Pyrococcus furiosus* [20], *Sulfolobus solfataricus* [21], and *Thermotoga maritima* [22], can be cultivated without sulfur and, thus, have been the primary focus of direct purification efforts for native proteins. *P. furiosus* and *T. maritima* have been grown to biomass yields of 1 kg (wet) or higher in 600 liter fermentation systems, from which numerous enzymes can be purified [6].

Once sufficient biomass has been generated, enzyme purification can proceed along conventional lines using multi-step protocols involving various types of liquid chromatography. Cells can be broken in several of ways, including freeze-thaw cycles, French pressing or sonication. Because many extreme thermophiles are members of the domain Archaea, which have unusual cell membrane features including a lack of peptidoglycan, lysozyme treatments are typically not effective [23]. Most types of prep-scale chromatography have been used in the purification of these enzymes. These include ion exchange, hydrophobic interaction, gel filtration and affinity methods. The extreme thermostability of these enzymes does provide a convenience: purification can be carried

out at ambient temperatures rather than in refrigerated environments and sample storage conditions are less critical for maintaining enzyme activity. On the other hand, thermostability does present some difficulties in assessing the homogeneity of products of the purification process. Conventional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) can be misleading since extremely thermostable enzymes may not be sufficiently denatured in the conventional preparation steps for this assay. The result is that both the native and partially denatured forms of multimeric proteins may appear on SDS-PAGE, making assessment of molecular weight and homogeneity difficult. Unfortunately, extended periods of heating to ensure complete denaturation may also lead to covalent modifications of labile amino acid residues. With the advent of molecular biology techniques for cloning and expressing genes encoding extremely thermophilic enzymes in mesophilic hosts, purification of such enzymes from their native hosts is not the preferred method. However, inability to express soluble, functional version of certain enzymes may leave direct purification as the only possible approach, and this tact has been taken in many cases.

B. Production of recombinant versions of extremely thermophilic enzymes

Although not specific to extremely thermophilic enzymes, molecular biology has provided an array of techniques that circumvent the tedious process of obtaining biomass from large fermentations using pure cultures. Heterologous expression methods utilize the expanding genome sequence databases to identify genes encoding enzymes of interest, which are then produced in recombinant form [24]. Incompatibilty in codon usage patterns can create a significant challenge to the recombinant expression of many proteins of thermophilic origin in mesophilic hosts, arising from evolutionary and biogeographical

differences [25]. Selective pressure in high temperature biotopes are believed to create preferences for certain codons and amino acids [25, 26] [27]. Solutions to this problem center around replacing genes with rare codons with optimal synonymous reading frames. In many cases, an affinity system (e.g., poly-His tags on the amino or carboxy terminus used in conjunction with metal affinity chromatography) can simplify purification. Expression libraries containing DNA from an organism of interest can be screened using a specific high throughput assay to discover new enzymes and encoding genes. This same approach has been used to mine enzymes directly from environmental samples for which no effort is made to obtain individual organisms in pure culture [28]. For extreme thermophiles, the intrinsic thermostability of their enzymes facilitates partial purification from mesophilic expression host. A heating step can serve to denature the less stable enzymes produced by a mesophilic host such that the thermostable enzyme is enriched in the resulting mixture. Unfortunately, as mentioned above, expression cloning will not always work since some genes are difficult to express as soluble, active enzymes or a suitable high-throughput screening assay may not be readily available. However, recombinant methods can be a particularly attractive alternative to large-scale production of extremely thermophilic enzymes from native biomass and can also be subsequently used as the basis for high through-put screening for directed evolution efforts [28].

C. Sequence analysis of genomic DNA

Over the past decade, the availability of genome sequence data has profoundly impacted enzyme discovery efforts [29-32]. Hundreds of completed microbial genomes are now publicly available at www.genomesonline.org. Given some of the new DNA sequencing technologies, microbial genome sequences will soon be assessable in a matter of hours at

very reasonable costs [33]. Bioinformatic methods to tease out genes encoding heat-stable and thermoactive enzymes of interest have become the preferred approach for biocatalyst discovery [15]. Once the target gene has been identified, recombinant methods, as discussed above, can be employed to produce the enzyme of interest for further biochemical, biophysical or biotechnological evaluation. The fact that approximately half of the ORFs in microbial genomes lack confident annotation presents both a challenge and opportunity. Many more interesting and, perhaps, novel biocatalysts remain to be discovered but this will require innovative methods for discovery and annotation.

D. Functional genomics approaches to enzyme discovery

The use of functional genomics approaches for enzyme discovery has been reported for several extremely thermophilic microorganisms [7, 15]. The transcriptome of a given extreme thermophile exposed to specific environmental or nutritional conditions can reveal clues to specific responsive ORFs encoding enzymes of interest. For example, the hyperthermophilic bacterium *T. maritima*, cultured on 14 different carbohydrates, was found to respond to glycoside linkages and side-chain sugars in specific ways. This led to the identification of sugar transporters and glycoside hydrolases implicated in the processing of specific carbohydrates [34, 35]. Galactomannanases useful for oil and gas well stimulation processes were discovered by examining transcriptional response of *T. maritima* grown on guar [36]. A similar approach was used to examine the starch degradation pathways in the hyperthermophilic archaeon *P. furiosus* [37]. The key here is to infer from transcriptional response the cellular strategy to process particular substrates of interest.

E. Structural genomic approaches to enzyme discovery

Extremely thermophilic enzymes and clones of the genes that encode them have also become available through structural genomics approaches whereby attempts are made to obtain three-dimensional structures of all proteins within a given organism. This involves recombinant production of each protein and structural determination typically using X-ray crystallography. The structural genomics effort was not driven by biological or biotechnological considerations, but simply the desire to fill protein sequence-protein structure space. Extremely thermophilic organisms have been the targets of several of these efforts, due to the belief that their enzymes are more amenable to crystallization [38]. The organisms that have been studied have included the hyperthermophiles *P. furiosus* [39] and *T. maritima* [40]. With *T. maritima,* close to 1,000 recombinant proteins have been produced (from 1,877 cloned genes) and this resulted in more than 200 structures. With *P. furiosus,* almost 400 proteins were obtained (from 1,008 attempted) and this effort generated 36 structures. These statistics nicely illustrate the power of structural genomic approaches and the resources that they have provided to the scientific community at large. These include a large number of recombinant proteins and cloned genes from extreme thermophiles [38].

III. MECHANISMS OF EXTREME THERMOSTABILITY

The survival and growth of extremely thermophilic microorganisms hinge on the capacity of their constituent biomolecules to withstand the biologically debilitating effects of high temperature. The discovery of enzymes functioning in excess of 100°C for extended periods of time has driven extensive efforts to understand the intrinsic basis of protein thermostability. Extraordinary levels of thermostability have been attributed to many factors,

including strategic local and global networks of hydrogen bonds and salt bridges that arise from the structural realization of primary amino acid sequence information [41]. However, the relative importance of any particular non-covalent interaction in overall protein stabilization is usually not apparent. Many other intrinsic factors have been cited, including: surface loop deletions, better atomic packing [42], increased hydrophobicity [43-45], smaller and less numerous cavities, increased surface area buried upon oligomerization [46, 47], residue substitution within and outside the secondary structures [42, 48], increased occurrence of proline residues in loops [49-51], substitution of thermolabile residues for thermostable residues [42, 43, 52, 53], increased helical content, and increased polar amino acids along the surface of the thermozyme [43-45, 54]. Protein thermostabilization has been achieved in some cases through metal ion addition [53] or by incubation at elevated pressures [55]. Global analysis of protein databases has suggested that thermostability correlates with decreased protein size, higher compactness ratio [56-58], decreased unfolding rates [59], and efficient re-folding pathways [60]. Consequently, it is readily evident that no one strategy is universal in achieving enhanced protein thermostability [61].

Recently, it was proposed that there are two evolutionary mechanisms underlying extreme protein thermostability: one “sequence-based” and one “structure-based” [62]. Extreme thermophiles indigent to high temperature niches produce proteins that are more compact and hydrophobic (“structure-based” strategy) than their mesophilic counterparts. Extreme thermophiles that evolved first as mesophiles and later adapted to a hot environment through a combination of lateral gene transfer [63] with local extreme thermophiles and sequence mutations (sequence-based” strategy) produce proteins that have modifications counteracting the adverse effects of temperature on proteins essential to vital structural, catalytic, transport, regulatory, and protective roles within the cell [64]. As

more and more data become available, it will be interesting to see how this perspective on how proteins function at high temperatures is reconciled with recombinant-based, directed evolution methods to create extremely thermophilic enzymes from less stable counterparts.

A. Sequence Adaptation

Extensive efforts have been directed at understanding the basis of protein thermostability at the nucleotide and amino acid sequence level. Comparison of homologous mesophilic and thermophilic enzymes with similar biocatalytic functions has shown that key catalytic amino acid residues and domains are highly conserved, such that thermosensitivity likely resides in the regions of structure not conserved [43, 65]. This, for example, was the case for the β-glucosidases from the mesophilic soil bacterium *Agrobacterium faecalis* and the hyperthermophilic archaeon *Pyrococcus furiousus*. The enzymes from these two microorganisms had temperature optima more than 70°C apart, yet catalyzed the hydrolysis of β-linked disaccharides in similar ways [66]. While it is not yet possible to predict the level of thermostability of a given protein based on nucleotide or amino acid sequence information, many interesting observations and hypotheses have been developed along these lines.

Initial studies on the relationship between nucleotide sequence and protein thermostability suffered from a lack of gene sequence data, which is no longer the case given the expanding genome databases. A positive correlation between high G+C content in tRNA and rRNA genes and high growth temperatures has been identified [57, 67, 68]. Genes encoding thermophilic proteins in extreme thermophiles also appear to have higher G+C content [67, 69] in comparison to genes for thermolabile proteins [70-72]. This increased G+C content can be related to amino acid composition [57, 73, 74], since the

frequency of G+C-rich codons, such as Ala, Pro, Trp, Met, Gly, Glu, Arg, and Val may be higher. Increases in codons containing AAG, AGG, ATA, and AGA, along with fewer CAA and CGT codons, are noted in extremely thermophilic proteins [75]. Protein thermostabiliy may originate from a bias towards purine-rich codons in extreme thermophiles [73, 74], corresponding to adenine in mRNA, and manifested in variation of charged to polar amino acid ratios in thermostable proteins.

B. Effect of the ratio of charged to polar amino acid residues

Higher concentrations of charged amino acids in proteins positively correlate with ionic interactions and, hence, have been proposed to impact thermostability [45, 69, 76-78]. Comparative studies between mesophilic and thermophilic proteins revealed that certain amino acids, such as Glu, Arg, Tyr, Asp, and Lys, are more abundant in extreme thermophiles than Ala, Asn, Gln, Thr, Ser and Val [45, 73]. Additional sequence comparisons between mesophilic and thermophilic homologs showed an increase in Ala, Arg and Tyr [43, 65], and a decrease in Asn, Gln, and Cys, likely due to their susceptibility to deamination and oxidation at high temperatures [43, 79].

Protein conformational flexibility is also dependent upon amino acid content [80], and the corresponding impact on salt bridge and subunit assembly. Certain amino acids (i.e., Glu, Arg, Tyr, Asp, and Lys) could contribute to thermostability by promoting subunit tetramerization within the protein [81]. Sequence comparisons between thermophiles and hyperthermophiles indicated that charged amino acids, like Lys, occur at high frequency in hyperthermophiles whereas thermophiles prefer Arg over Lys [45]. Asp and Met are unstable at high temperatures and have a diminished presence in hyperthermophilic proteins. However, Tyr and Asp may be key to the thermostability of hyperthermophilic

proteins [81]. Even though Asp residues predominantly form Asp-Pro pairs, where the peptide bond is vulnerable to hydrolysis, these pairs can be protected by amino acid substitution or by increased conformational rigidity of neighboring protein residues [53]. Temperature-influenced deamination works against selection of polar or non-charged amino acids (i.e., Ser, Thr, Gln, and Asp) and favors the selection of charged amino acid residues (i.e., Lys, Arg and Glu) in thermostable proteins [53, 56]. Since Ser and Thr can catalyze deamination and backbone cleavage of Asp and Gln, reduction in all four of these residues favors high temperature function and stability. Certain mechanisms that are undetected *in vitro*, but important *in vivo*, could impact the charged state of amino acids and impact the ratio of charged to polar amino acids between thermophiles and mesophiles [61].

C. Structural adaptations

Proteins are also stabilized at high temperature by structural features involving charge-to-charge [56, 82] and cation-π bond interactions [43, 83-86], created by protruding amino acid chains that create these interactions [56, 74, 87, 88]. Thermostable proteins can also have extensive hydrogen bonding networks in addition to more contacts between polar and non-polar residues [43, 89]. Proteins from hyperthermophiles also have a tendency to exist in multimeric forms and this has been proposed to be a thermostabilizing factor [65]. The salt and disulfide bridges responsible for the multimerization contribute significantly to this structural feature [43, 53, 90-92]. In addition, aromatic-aromatic interactions and disulfide bonds have been observed at a higher frequency in proteins from hyperthermophiles in comparison to mesophilic homologs [92-95]. The frequency of disulfide and salt bridges varies between different domains as well as within the same domain. For example, proteins from archaeal thermophiles and hyperthermophiles contain more disulfide

bridges compared to bacterial thermophiles and hyperthermophiles whereas archaeal hyperthermophiles and thermophiles still have a higher number than archaeal mesophiles [92].

D. Solute effects

Cellular localization can play a role in protein thermostability through certain extrinsic factors. For example, growth temperature has been positively correlated with intracellular salt (particularly K^+) concentration, possibly an adaptive response involving pI adjustment to minimize aggregation of intracellular proteins [96]. Proteins from extreme thermophiles do have a higher frequency of charged residues in support of this proposal [84]. It is unclear whether this pI adaptation is accomplished directly through the number of salt bridges [84], or indirectly by maintaining the same conformational stability at high salt concentrations. Other extrinsic factors can also contribute to protein stability. Divalent metal cations can play a key role in protein stabilization [53, 97]. Thermostabilizing compatible solutes, such as mannosylglycerate and di-*myo*-inositol-phosphate, are present at high levels in the cytosols of some extreme thermophiles [98]. Di-mannosyl-di-*myo*-inositol-phosphate and di-glycerol-phosphate, only found in certain extreme thermophiles, can also provide osmoprotection and thermoprotection.

E. Distribution of amino acids in protein structure

While there are many structural features that differentiate thermostable and thermolabile proteins, thermostability can also be impacted through subtle adjustments in amino acid distribution [53, 65]. Surface amino acid composition [76, 99-101], the presence or absence of certain residues at the N-terminus or C-terminus, shortening of surface

exposed loops, and explicit associations or avoidance of amino acids in loops or in the protein core are factors that can all play a role [43, 58, 81, 102, 103]. In the case of the L-isoaspartyl-O-methyltransferase from *Sulfolobus tokodaii,* Asp and Tyr residues are contained within the core structure of the transferase. These residues assist in the formation of unique tertiary structural folds that are essential for the added thermostability found in extremely thermophilic enzymes [81].

IV. USES OF EXTREMELY THERMOSTABLE ENZYMES

A. General considerations

Genome sequencing has provided the genetic basis for identifying and producing extremely thermophilic enzymes for a wide variety of uses. In some situations, existing enzyme-based applications could benefit from having a substantially more thermostable version of the same enzyme type. Operations at elevated temperatures reduce the risk of contamination by common mesophilic organisms (especially in food and medical applications). Elevated temperatures also facilitate higher overall reaction rates due to a decrease in viscosity, increased mass transfer of substrates and products, and possible shifts in reaction equilibria to favor the product of choice [104-107]. Bioprocesses that make use of improved biocatalyst thermostability and advantageously utilize higher operating temperatures have been considered. The most intriguing opportunities that make use of extremely thermophilic enzymes are those that catalyze reactions that otherwise are not amenable to biocatalysis.

B. Polymerase chain reaction (PCR)

The revolution in molecular biology has relied on the ability to amplify small amounts of DNA by the Polymerase Chain Reaction (PCR) to levels that facilitate cloning, sequencing, expression and detection of particular genes of interest. This can be accomplished through the use of thermostable DNA polymerases that are produced by extremely thermophilic microorganisms [108]. To be effective in catalyzing DNA polymerization, a DNA polymerase must be able to withstand repeated thermal swings from low to high temperatures so that the DNA template can denature to be copied and then re-annealed prior to the next cycle. Although the DNA polymerase from a moderately thermophilic bacterium, *Thermus aquaticus*, was initially used in PCR applications, a host of polymerases from bacterial and archaeal sources are now available [108]. Replication of long nucleotide sequences (20-40 kb) is possible by adding to the reaction mixture an additional DNA polymerase (usually from an extremely thermophilic archaeon) with proofreading (3' to 5' exonuclease activity) capability to repair mistakes that might otherwise terminate the replication process [109, 110]. PCR is an excellent example of the strategic use of protein thermostability and thermoactivity, since thermally labile DNA polymerases would not survive repeated exposures to high temperatures, thus requiring addition of this enzyme at each cycle.

In addition, thermostable DNA polymerases play an important role in making DNA copies (cDNA) of RNA. The poly-A tail of eukaryotic RNAs can be hybridized by a poly-T RNA primer which allows an enzyme termed reverse transcriptase to copy the mRNA into a complementary DNA chain forming a cDNA/RNA hybrid complex. The RNA portion of the hybrid is degraded by RNase H, leaving many RNA fragments attached to the cDNA. These fragments serve as primers for the DNA polymerase to come in and synthesize the

complementary DNA strand. cDNA libraries are made up of distinct sets of cDNA transcribed from distinct sets of mRNA molecules. The technology initially used mesophilic reverse transcriptase, but at low temperatures there was an issue with the formation of stable secondary structures in the RNA fragment and the inability of mesophilic DNA polymerase to utilize these fragments as primers [111]. Fortunately, numerous thermostable DNA polymerases, such as *Thermus thermophilus* HB8, can use RNA regardless of secondary structure formation. This adjustment has made cDNA libraries into inexhaustible resources that are widely shared among investigators and are commercially available.

Finally, new DNA sequencing technologies have made use of thermostable DNA polymerases leading to the development of the cycle sequencing method. This utilizes the same principles as PCR for the amplification of sequencing products. The advantages to using thermostable DNA polymerases here are that less template DNA is required, no primer annealing step is needed, and elimination of secondary structures associated with the template can be accomplished by high-temperature elongation [112].

C. Replacements for existing industrial enzymes

In some cases, industrial bioprocesses can advantageously use the more thermally stable version of existing biocatalysts to improve production rates or yields. Several such examples are provided below.

1. Glucose isomerase

The biocatalyzed conversion of corn starch to glucose and then to fructose for the production of high fructose corn syrup (HFCS) by xylose (glucose) isomerase represents the

largest existing industrial application of an immobilized enzyme (see Figure 1.1). Because of the limits of enzyme stability, this process is currently operated at approximately 60°C, at which temperature the equilibrium conditions limit the HFCS concentration of fructose to a maximum of 40-42% [113]. Since 55% fructose concentration is desired for sweetening properties, a chromatographic separation step is subsequently used to enrich the mixture to this level by the removal of unreacted glucose. By increasing the operating temperature from the current 60^{o}C to around 100^{o}C, the fructose yield reached 55% and eliminating the need of an expensive chromatographic fructose enrichment step [114]. Several issues must be overcome before increasing the operating temperature of the process, such as developing thermostable and thermoactive biocatalysts, and maintaining the stability of the substrate-product and enzyme [115]. The pH optima for commercially available glucose isomerases are between 7.5 and 9.0. For operations at neutral or basic pH and high temperature, the substrate/product would be vulnerable to non-enzymatic browning Maillard reactions [116]. Therefore, an HFCS process benefits by using a thermostable and thermoactive glucose isomerase with acidic pH optima to increase the processing rates, high fructose yields, process stability, and decrease the viscosity and by-product formation. Wild-type, recombinant, and engineered glucose isomerases (GI) have been investigated [117]. GIs have been sorted into two groups, Classes I and II. Class I GIs are missing a fifty-residue N-terminal sequence found in class II GIs [118]. GI's stability and activity are dependent on divalent metals [119-126] but the role the divalent metal plays varies between Class I and Class II GIs [117].

Class II GIs have been cloned and characterized from mesophilic, thermophilic, and hyperthermophilic bacteria [127-132]. Even though Class I and II GIs vary in cation specificities [133], the cation-binding residues and active site are highly conserved in both

enzyme classes. Thermophilic xylose isomerases have been characterized from *Thermoanaerobacterium* strain [134], *Thermus flavus* [135], *Thermoanaerobacterium thermosulfurigenes* [130, 136], *Thermotoga neapolitana* [127], *Clostridium thermosulfurigenes* [130], and *Thermotoga maritima* [131]. Brown et al. [131] discovered xylose isomerases in members of the hyperthermophilic eubacterial genus *Thermotoga*. For example, *T. neapolitana* produces a version of GI with an optimal temperature of at least 95°C, which was also found to be highly active over a broad pH range [127]. Compared to many other commercially utilized GIs, the *T. neapolitana* class II GI possesses a 50 amino acid insert at the N-terminus, the function of which is unknown. The catalytic efficiency of this enzyme was superior to those determined for other GIs from less thermophilic sources at their respective optimal temperature ranges.

The GI from *T. neapolitana* again illustrates the potential strategic benefits that arise from the availability of thermostable versions of enzyme used in current industrial applications. Operating at elevated temperatures eliminates costly separation steps needed for fructose enrichment. However, consideration of many other issues must be resolved before this enzyme replaces the commercially popular enzyme used in existing processes. For example, one must consider the levels of over-expression attained in foreign hosts, the impact of and approach used for immobilization, problems with potential side reactions of sugars at high temperatures, and regulatory approvals that might be required in the production of a substance for human consumption.

2. Hydrolysis of natural polymers

Interest in renewable resources has driven efforts to identify enzyme systems capable of hydrolyzing natural biopolymers, such as cellulose and hemicellulose, to more

readily usable saccharides. This may be done in a non-specific way such that the biopolymer is extensively hydrolyzed to simple sugars or in a specific way such that the properties of the biopolymer are systematically modified [137]. Solutions containing natural polymers are less viscous at high temperatures compared to ambient conditions such that enzyme accesses to specific sites for hydrolysis are facilitated. Therefore, extremely thermophilic enzymes may hydrolyze natural polymers more readily.

In the stimulation of oil and gas wells by hydraulic fracturing, aqueous solutions of natural polymers, such as guar gum (a molecule consisting of a mannan backbone substituted with galactose through α-1,6 linkages), are used to transport particles to the site of the fracture (see Figure 1.2) [138]. The objective is to prop open fissures in the bedrock perpendicular to well-bore and, hence, promote the flow of oil and/or gas to the point where it is readily recovered. The viscous properties of the guar gum solution used advantageously to transport the particles become problematic once the particles are in place. Hemicellulases are used to overcome the increase in viscosity by hydrolyzing the guar gum which in turn reduces viscosity. The high temperatures encountered in deep wells reduce the effectiveness of conventional enzyme systems but create an opportunity for extremely thermophilic enzymes, which can withstand temperatures above 100°C [139]. An additional advantage of using thermostable enzymes is their lower activity at ambient conditions which otherwise can lead to premature hydrolysis of the guar [140]. Such an enzyme system, consisting of β-mannanase, α-galactosidase and β-mannosidase from the hyperthermophile *T. neapolitana*, has been shown to work to hydrolyze guar at high temperatures but a lower activity was observed at moderate temperatures [140].

3. Production of rare sugars

"Rare sugars" are those that exist in extremely small amounts in nature, usually because they are the products of intermediary cellular metabolism. Rare sugars are converted within the cell to another form as soon as they are created, and include D/L-allose, D/L-altrose, L-glucose, D/L-psicose, D/L-tagatose, L-fructose, and D-sorbose [141, 142]. Rare sugars have found application in the food industry, as reduced calorie sweeteners, microbial growth inhibitors, and bulking agents, and in the pharmaceutical industry, as antiviral agents and glycosidase inhibitors [143]. Despite the promising potential of these rare sugars, scarcity and high production costs have limited their commercial utilization. Traditional chemical synthesis of rare sugars is costly and often results in a mixture of products, which are difficult to separate. Using enzymes to produce rare sugars from cheaper feedstocks would provide an inexpensive method of production. Since many of these sugars exist at extremely low levels in the cytosol, they need to be produced *in vitro*. Temperature impacts the chemical equilibrium between aldose and ketose, with higher temperatures often favoring the ketose form. Currently, extremely thermophilic arabinose isomerases (AIs) have been utilized to produce rare sugars from inexpensive substrates: e.g., D-tagatose from D-galactose and L-ribulose from L-arabinose [144-148]. AIs catalytic fidelity has also been exploited industrially for the production of D-tagatose, a novel and natural sweetener [144, 149]. Currently, extremely thermophilic AIs have been incorporated into biological processes for production of D-tagatose from D-galactose, obtained form the degradation of lactose-rich whey by β-galactosidase [150]. These isomerization reactions can be conducted at temperatures above 70^{o}C, which offers many advantages, including higher conversion yield, faster reaction rate, and the expected decreased viscosity of the substrate in the product stream [151].

D. New opportunities

To date, extremely thermophilic enzymes have been considered mostly as replacement enzymes to improve existing bioprocesses. However, their high degree of stability to thermal and other denaturing forces makes them candidates for catalyzing reactions not previously amenable to biocatalysis [14]. This includes reactions conducted in non-aqueous environments, such as organic solvents and supercritical fluids. The fact that their operational ranges are consistent with conditions used in chemical processing environments presents some intriguing possibilities for biocatalysis.

1. Esterases and Lipases

Esterases and lipases are of great biotechnological interest due to their ability to biosynthesize optically pure compounds. Esterases/lipases can be used to modify tri-, di-, and monoglycerides and act only at an oil-water interface [152]. The ability of these enzymes to remain active and stable in organic solvents in the presence of small amounts of water increases their utility [153]. Current lipase processing schemes, operating at temperatures around 70^{o}C for prolonged periods, are limited by the lack of thermostability exhibited by commercially available mesophilic esterases. Extremely thermostable and thermoactive lipases and esterases with the proper stereoselectivity could dramatically improve these processes [154, 155]. (Hyper)thermophilic esterases and lipases have been isolated and characterized from *Sulfolobus solfataricus [156], Archaeoglobus fulgidus [157],* and *Pyrobaculum calidifontis [158].* These esterases show high thermoactivity and thermostability in buffer/organic solvent mixtures, which include solvents, such as acetonitrile and dimethyl sulfoxide. *P. furiosus* produces an esterase that has a half-life of

50 min at 126^{o}C and has a temperature optimum of 100^{o}C. This is the most thermostable and thermoactive esterase know to date [159]. Hyperthermophilic carboxylesterases, structurally similar to hormone-sensitive lipase family (active site GGGX motif), prefer medium chain *p*-nitro phenyl substrates and can also hydrolyze tertiary alcohol esters [160]. Esterases/lipases process improvements have the potential to bring new and innovative products to the medicine, food, synthetic chemistry, and detergent industries.

2. Glycosyl hydrolases from extreme thermophiles

The natural polymer-processing industry, which converts saccharide polymers into valuable products such as trehalose, dextrins, fructose, and glucose, can benefit from the use of extremely thermostable enzymes [161]. Thermostable enzymes are beneficial due to their ability to hydrolyze natural polymers at elevated temperatures because of increased substrate solubility and lowered solution viscosity, which improves enzyme access to susceptible bonds [162]. Extreme hyperthermophiles and thermophiles are an excellent source of glycoside hydrolases for use as commercial enzymes [162, 163]. Because these enzymes are often capable of hydrolyzing a range of glycosidic bonds, classification in terms of substrate specificity can be confusing. Amino acid sequence information fortified traditional classifications schemes of glycoside hydrolases into over 110 Families [164, 165]. The synergistic catalytic effect of various thermostable glycoside hydrolases, such as amylases, glucoamylases, pullulanases, β-glycosidases, and xylanases, are advantageous to overall process efficiency and ultimately lowers the processing costs. There are many promising extremely thermophilic candidates for optimizing starch bioconversion that function at low pH and high temperatures.

α-Amylase. Amylases are enzymes that hydrolyze the α-1-4 bonds in starch to yield dextrins and smaller oligosaccharides [166]. They comprise approximately 25% of the enzyme market [167-169] and are used in production of bread, food, textiles, and paper [170]. Thermostable and thermoactive α-amylases are highly desirable, since some steps in starch hydrolysis, i.e., gelatinization (100-110^{o}C) and liquefaction (80-90^{o}C), occur at elevated temperatures (see Figure 1.1) [167]. Several thermostable α-amylases have been characterized [171-177], including one from *Pyrococcus woesei*, which retains activity after autoclaving for 4 hours at 120^{o}C [178]. The optimal temperature range for many extremely thermophilic α-amylases is 80-100^{o}C [161]. Currently, extremely thermostable α-amylases are commercially available, e.g., Termamyl®LC, and Termamyl®SC (Novozymes) and Amylase D45® (Diversa) [179]. Mesophilic amylases often require Ca^{++} for maximal thermostability. These enzymes are usually less active at low pH, such that adjustments are needed from the low pH of the natural polymer (i.e. starch) slurry to an optimum pH for the commercial amylase and then back down to a low pH for further processing. The combination of adjusting the pH and removing the leaching Ca^{++} from the product hydrolysate is costly and impacts product efficiency. Directed evolution methods have been used to create an α-amylase that does not require metal ions for thermostability and operates optimally at a low pH (Amylase D45® by Diversa (now, Verenium)). The superior thermostability and thermoactivity and absence of a metal cation requirement exhibited by extremely thermophilic α–amylases makes these enzymes ideal candidates for incorporation into the next generation of industrial applications.

Glucoamylases. Glucoamylases are exo-acting enzymes that cleave both $\alpha-1-6$ and α-1-4 bonds that find use in starch processing following α-amylase hydrolysis in high fructose corn syrup production (see Figure 1.1). Currently, the glucosamylase from *Aspergillus niger* is the industrial standard [83], but it functions at a different pH than commercially available α-amylases. Glucoamylases from thermoacidophilic archaea are active and stable at low pH and high temperature and offer a potential improvement to current processing schemes. Recently, a thermostable glucoamylase has been characterized from *Thermoplasma acidophilum, Picrophilus torridus* and *Picrophilus oshimae* [161]. These enzymes are optimally active at pH 2 and 90^{o}C and retain activity at pH 0.5 and 100^{o}C. In addition, thermoacidophilic glucoamylases act to release β-D-glucose from non-reducing ends of polysaccharides and could be utilized to improve glucose production in other processes.

Pullulanases. Type I pullulanases are de-branching enzymes that cleave α-1-6 bonds in starch. Current industrial processes utilize the synergetic action of pullulanase from *Bacillus acidopullulyticus* and glucoamylase from *A. niger* to optimize the saccharification step in the starch bioconversion process (see Figure 1.1)[180]. Several pullulanases have been purified and characterized from extreme thermophiles [181-183]. Most are type II pullulanases (also referred to as amylopullulanases [184]). Besides cleaving α-1-6 bonds, type II pullulanses can also cleave α-1-4 bonds and can convert starch directly to maltobiose, maltotriose, and maltotetraose. There have been numerous thermostable type II pullulanases reported [171, 185-187], including versions from *P. woesei* [183] and *P. furiousus* [188], that have optimal temperatures between 90°C and 105°C, and are remarkably thermostable even in the absence of substrate and calcium ions [161]. Archaeal

type II pullulanases are promising candidates for starch bioconversion since they could be used for saccharification at lower pHs and higher temperatures than currently used enzymes.

β-Glycosidase. β-glycosidases are exo-acting enzymes that have broad substrate specificity, making them potential candidates for numerous industrial applications [189]. These enzymes hydrolyze β–linked sugars converting, for example, lactose into glucose and galactose. β-glycosidases from extreme thermophiles have important biotechnological advantages that have been considered. Bauer et al. [66] reviewed the potential of several glycosidases from hyperthermophiles, including those from *P. furiousus* [190] and *S. solfataricus* [191]. β-Glycosidases are also utilized in oligosaccharide synthesis. The *P. furiosus* β-glycosidase was shown to produce tetra- and tri- saccharides with lactose as the substrate, and could be useful in commercial processes for prebiotics in yogurt and other dairy goods.

Xylanases. Xylanases catalyze the degradation of xylan, a major component of many hemicelluloses, and are used by microorganisms to process material as carbon and energy sources [192]. Xylanases are important commercial enzymes in the food and animal feed industries [193, 194] and in pulp and paper processing [195]. Xylanases for bio-bleaching of wood or bagasse pulp hold promise for reducing the amount of chlorine-based chemicals needed in pulp-bleaching process, thereby minimizing environmental concerns. While chlorine-free bleaching is carried out a very low pH, the step preceding this step is thermal caustic treatment of the wood. Thermostability and thermoactivity at elevated temperatures and extreme pH values are desirable characteristics of biotechnologically useful xylanases.

There are two commercially available xylanases, Pulpzyme [196] and Cartazyme [197], that are not thermostable. As a result, pulp must be cooled before enzymatic treatment and re-heated for subsequent steps [198]. The use of thermostable xylanases would eliminate the downstream processing step, and thus the lower processing costs.

3. Proteases from extreme thermophiles

Proteolysis (hydrolysis of peptide bonds) is essential to cellular function, and is implicated in nutrition, heat shock response, immune response, and certain housekeeping roles [199]. Historically, proteases have found important industrial uses in detergents, leather bating, food processing, among other applications, and, collectively, comprise the largest volume use of commercial enzymes [200]. In the last twenty years, these enzymes have also been shown to be active in organic solvents for peptide synthesis, and are often selective enough for pharmaceuticals and biotechnological applications [201]. Recently, protease inhibitors have received much attention in medical applications for combating the HIV virus and some cancerous tumors [202, 203].

There are many potential benefits to be derived from the use of proteases from extreme thermophiles. At elevated temperatures, mesophilic proteins in their denatured state are susceptible to specific peptide bonds by thermostable proteases that mesophilic protease cannot access because of temperature limitations. Also, at higher temperatures, many substrates are more soluble, solution viscosity is reduced, and diffusion rates are higher, which can lead to higher reaction rates [204]. Since these enzymes appear to also be more stable to denaturing forces other than heat, proteases from extreme thermophiles should be amenable to use in organic solvents for synthetic reactions.

Many proteases have been purified and characterized from extreme thermophiles. These proteases include, but are not limited to, serine proteases from *P. furiosus* and *Aquifex pyrophilus* [205, 206], thiol protease from *Pseudomonas sp.* [207, 208], *Desulfurococcus* proteinase from *Desulfurococcus mucosus* [209], and pernilase from *Aeropyrum pernix* K1 [210]. Compared to mesophilic proteases, hyperthermophilic proteases are more thermostable and are more resistant to chemical denaturants and industrial solvents [211].

V. SUMMARY

Extremely thermophilic microorganisms have proven to be a rich source of thermostable enzymes. The diversity of such enzymes, and their associated biochemical properties, are only beginning to be understood. The discovery process has made good use of molecular biology tools and genomics. The challenge now is to steer the use of these enzymes to strategic applications. The promise that these enzymes hold for the expansion of uses of biocatalysis is considerable. It is clear that biocatalysis has added a new dimension, which bodes well for the development of more efficient and effective bioprocesses.

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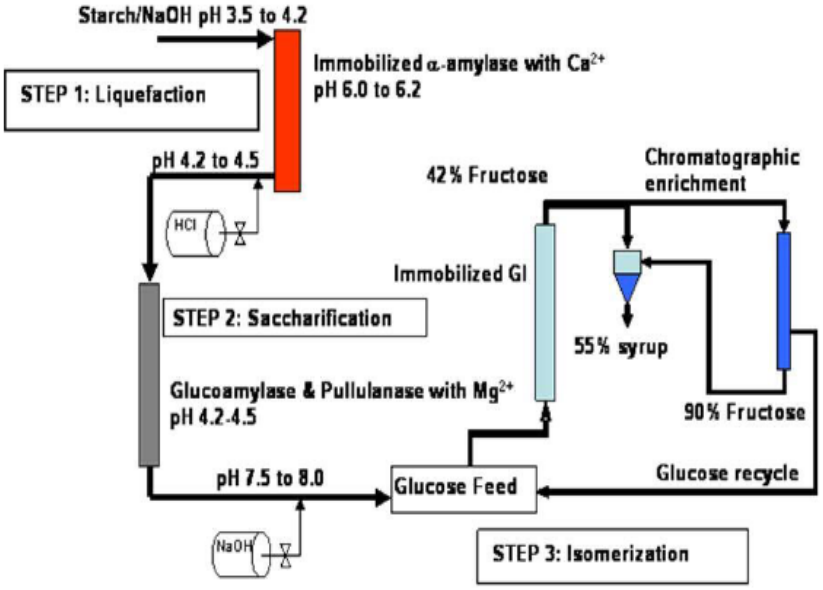

Figure 1.1. High Fructose Corn Syrup (HFCS) production. Step 1: Liquefaction starts at 105ºC for 5-8 min and concludes with a holding step at 95ºC for 1-2 hr. Step 2: Saccharification is carried out at 60ºC for 36-96 hrs. Step 3: Isomerization occurs at 60°C for 0.3-3 hours, yielding 55% conversion of glucose to fructose. Thermostable, thermoactive glucose isomerase could be used to improve the fructose yield exiting the immobilized GI column and eliminate or at least minimize the need for an enrichment step.

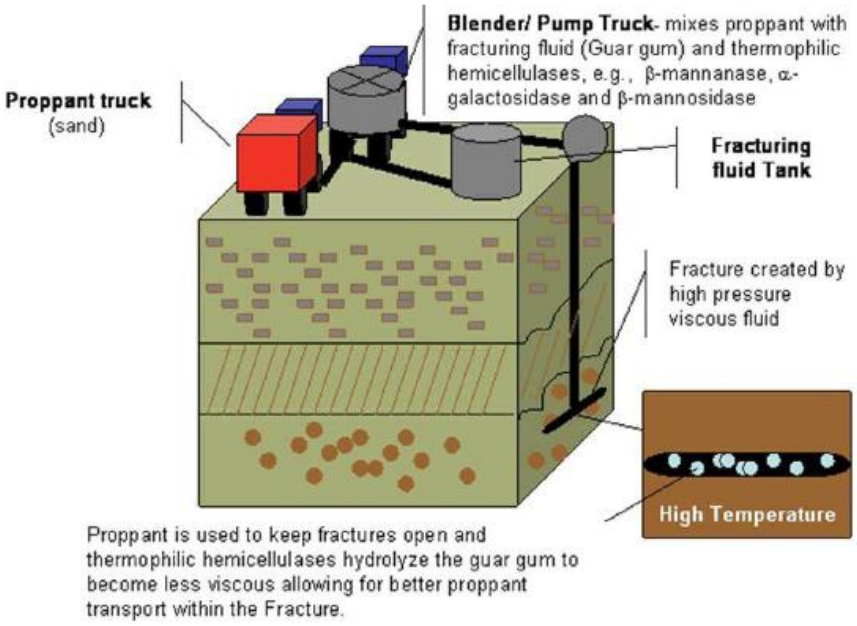

Figure 1.2. Stimulation of oil and gas wells by hydraulic fracturing utilizing extremely thermophilic hemicellulases to improve proppant transport within the fracture and increase thermal range (depth) for oil/gas recovery.

Table 1.1 Extremely thermophilic enzymes of technological interest

Enzyme	M_r (kDa) (structure)	T_{opt} (°C)	Application	Source	Thermostability ($t_{1/2}$)	Ref
Sugar Isomerases						
L-arabinose isomerase	55 (α_4)	90	rare sugar synthesis, prescription drug additive to mask unpleasant tastes, sweetener in toothpaste, mouthwash, and cosmetics (flavored lipstick)	*Thermotoga maritima*	3 h @ 90°C	**[212]**
D-xylose isomerase	50 (α_2 ,α_4)	98	rare sugar synthesis, high fructose corn syrup	*Thermotoga neapolitana*	2.3 h @ 95°C	**[213]**
Hydrolases						
α-galactosidase	41 ($\alpha_?$)	110	animal feed processing	*Pyrococcus furiosus*	9 h @ 85°C	**[214]**
β-glucosidase	81 (α_1)	105	polymer degradation, synthesis of sugars, color brightening	*Thermotoga neapolitana*	3.6 h @ 100°C	**[215]**
endoglucanase	36 (α))	100	glucan hydrolysis, laundry detergents	*Pyrococcus furiosus*	40 h @ 95°C	**[216]**
α-glucosidase	125 (α)	115	maltose hydrolysis, polymer degradation, synthesis of sugars	*Pyrococcus furiosus*	48 h @ 98°C	**[182]**
β-galactosidase	59 ($\alpha_?$)	90	oligosaccharide synthesis, lactose transformation	*Pyrococcus woesei*	3.5 h @ 100°C	**[217]**
β-mannosidase	59 (α_4)	105	mannobiose hydrolysis	*Pyrococcus furiosus*	17 h @ 85°C	**[66]**
β-mannanase	65 (α_2)	92	mannan hydrolysis	*Thermotoga neapolitana*	34 h @ 85°C	**[218]**
α-amylase	56 (α_3)	120	bread and baking applications, starch liquefaction and saccharification, high fructose corn syrup production	*Methanocaldococcus janaschii*	4 h @ 90°C	**[219]**
amylomaltase	76 (α_2)	100	starch hydrolysis	*Pyrococcus kodakaraensis*	8.4 h @ 87°C	**[220]**
glucoamylase	140, 85 ($\alpha\beta$)	90	enhanced ethanol production, production of glucose, which is a feed stock for high fructose syrup	*Picrophilus oshimae*	24 h @ 90°	**[221]**
pullulanase	90 (α)	100	synthesis of novel heterobranched β-cyclodextrins	*Pyrococcus woesei*	6.7 h @ 90°C	**[222]**
chitinase	134 (α)	90	chitin hydrolysis, binding domains used for enzyme immobilization	*Thermococcus kodakaraensis*	1 h @ 120°C	**[223]**
Proteases						
proteasome	700 ($\alpha_{14}\beta_{14}$)	119	protein turnover	*Methanocaldococcus janaschii*	24 h @ 95°C	**[224]**
serine protease	150 (α)	115	peptide hydrolysis	*Pyrococcus furiosus*	33 h @ 98°C	**[225]**
thiol protease	45 (α)	110	peptide hydrolysis	*Thermococcus kodakaraensis*	1 h @ 100°C	**[207]**

Table 1.1 continued.

acidic protease		90	peptide hydrolysis	*Sulfolobus acidocaldarius*		**[226]**
metalloprotease	52 (α)	100	peptide hydrolysis	*Aeropyrum pernix*		**[227]**
DNA-processing						
DNA polymerase	152 (α)	80	molecular biology	*Pyrococcus. sp. GB-D*	12 h @ 95^{o}C	**[228]**
phosphatase	34 (α_2)	90	molecular biology	*Thermotoga maritima*	18 h @ 100^{o}C	**[229]**
ligase	66 (α)	90	molecular biology	*Pyrococcus horikoshi*	1 h @ 95^{o}C	**[230]**
Esterase						
	33 (α)	100	biotransformations in organic solvent, scent formulation	*Pyrococcus furiosus*	34 h @ 100^{o}C	**[159]**

N-terminal fusion of a hyperthermophilic chitin-binding domain to xylose isomerase from *Thermotoga neapolitana* enhances kinetics and thermostability of both unbound and immobilized enzymes

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Abstract

Immobilization of a hyperthermophilic D-xylose isomerase (EC 5.3.1.5) from *Thermotoga neapolitana* 5068 (TNXI) on chitin beads was accomplished through a N-terminal fusion with a chitin-binding domain (ChiBD) recruited from a hyperthermophilic chitinase produced by *Pyrococcus furiosus* (PF1233). Loss of enzyme activity at 100°C, determined by a bi-phasic inactivation model, showed that the ChiBD-TNXI bound to chitin (IM-ChiBD-TNXI) had a half-life (19.9 h) approximately three times longer than the soluble wild-type TNXI (6.8 h). Surprisingly, the unbound soluble ChiBD-TNXI had a significantly longer half-life (56.5 h) than the immobilized enzyme. Both unbound and immobilized ChiBD-TNXI had higher turnover numbers for glucose to fructose than the wild-type enzyme; k_{cat} (min^{-1}) was 971, 3798, and 5776 for the wild-type, unbound, and immobilized enzymes at 80°C, and 1139, 10353, and 6961 at 90°C, respectively. These k_{cat}s for the glucose to fructose isomerization are among the highest reported to date at any temperature for this biotransformation. Molecular modeling, based on structural information on the wild-type TNXI and *P. furiosus* ChiBD, showed that the N-terminal fusion likely impacted subunit interactions, thereby contributing to the enhanced thermostability of the unbound ChiBD-TNXI. These interactions could also have played a role in modifying active site structure, consequently diminishing substrate-binding affinities and generating higher turnover rates. The additional subunit interactions were confirmed through Native gel electrophoresis of ChiBD-TNXI. Taken together, the results illustrate that substantial changes in thermostability and reactions kinetics can result from affinity tags for hyperthermophilic proteins.

INTRODUCTION

Enzymes from hyperthermophilic microorganisms have been considered for improving existing biocatalytic processes, as well as for generating novel routes for producing biotechnological products [1-5]. In either case, the need can arise to immobilize the hyperthermophilic enzyme of interest to avoid contaminating the product stream, as well as to recycle the biocatalyst for process economics reasons.

High temperature bioprocessing conditions present certain challenges for enzyme immobilization. Entrapment in a porous solid phase necessitates that the matrix be impervious to thermal conditions. Of course, entrapment can also diminish overall process efficiency at any temperature because of mass transfer limitations [6-8]. Non-covalent and covalent attachment strategies are options for hyperthermophilic enzyme immobilization [9-13]. However, physical adsorption of hyperthermophilic enzymes, based on electrostatic and hydrophobic multipoint interactions between the protein and immobilization surface, may not be sufficiently strong at elevated temperatures, such that the protein is easily displaced. Immobilization by covalent attachment often involves using toxic chemicals, such as carbodiimides and succinic anhydride [14, 15], to modify the support surface and bind the enzyme. The concern is that substantial loss of enzyme activity can result during the immobilization process, thus impacting the biocatalytic capacity of the process at the outset and accelerating enzyme inactivation.

An attractive method for enzyme immobilization involves creating a fusion protein containing an affinity tag to bind the enzyme to a support matrix; many examples of this strategy have been reported [16]. Metal affinity tags, based on poly-His residues at the N- or C-terminus, are commonly used for purifying recombinant proteins on small scale. This

approach is inappropriate for food and pharmaceutical applications and may not be sufficiently strong for elevated temperatures. Carbohydrate-binding domains (CBDs), discrete protein folding domains responsible for binding insoluble carbohydrate substrates such as chitin and cellulose, have been used as affinity tags for protein purification, as well as for enzyme immobilization [17]. In addition to their identification through the characterization of cellulases and chitinases, many CBDs have been found from genome sequence data (for a complete listing, see the CAZY database at http://afmb.cnrs-mrs.fr/CAZY/). However, for enzyme immobilization at elevated temperatures, the thermostability of the CBD is an important consideration. Unfortunately, CBDs are rare in the sequenced genomes of hyperthermophilic microorganisms. Those that have been characterized from less thermophilic sources may not be sufficiently thermostable to service high temperature bioprocessing applications. For instance, the denaturation temperature of the CBD from the mesophilic soil bacterium *Cellulomonas fimi* was 78ºC when bound to crystalline, bacterial cellulose [18]. Although rare in hyperthermophiles, chitin-binding domains (ChiBDs) have been identified in multi-domain chitinases produced by hyperthermophilic archaea, ChiA in *Thermococcus kodakarensis* [19-22] and ChiA-ChiB (PF1233-PF1234) in *Pyrococcus furiosus* [23, 24]. The three-dimensional structure of a ChiBD from *P. furiosus* has been recently reported [25], providing some insights into how these polypeptides function at elevated temperatures. ChiBDs from *P. furiosus* have been used to create cellulose-degrading fusion proteins [26, 27], yet have not been used to immobilize enzymes on chitin. Chitin, a β-1,4-linked N-acetyl-D-glucosamine homopolymer and the main structural component of fungal cell walls and arthropod exoskeletons [28], is an attractive affinity-based enzyme immobilization support [29-31] since it has low non-

specific binding affinity for most proteins [18]. Its use for hyperthermophilic enzyme immobilzation should be considered.

Xylose isomerases (XI), which find wide industrial use as “glucose isomerases” in the production of high fructose corn syrup [32] represents the largest bulk application of an immobilized enzyme. XIs can be separated into two groups (class I and class II) based on primary amino acid sequence homology. While crystal structures for class I and class II XIs reveal that large parts of their assemblies are virtually superimposable [33], structural differences do exist, localized to the C-terminal loop and N-terminus. The C-terminal loop in both XI classes makes many inter-subunit contacts in the assembled tetrameric enzyme, although none of these contacts appear to be involved with the interface that contains the active site [33, 34]. At the N-terminus, class II XIs contain a 30-40 amino acid insert that is not present in the class I enzymes [35]; the functional role of this N-terminal configuration in class II XIs is not known. Although existing commercial processes for HFCS production use class I XIs, thermostable and thermoactive class II XIs have been identified in hyperthermophilic bacteria of the genus *Thermotoga* [36-40]. These enzymes offer the prospect of operation at elevated temperatures for extended periods of time, taking advantage of favorable equilibrium characteristics of ketose over the aldose [38]. The inactivation behavior of the XI from *Thermotoga neapolitana* (TNXI) when immobilized by covalent linkage to glass beads has been examined [13], but this approach would not be appropriate for bioprocess applications. There have been no reports of TNXI immobilized through fusion protein affinity tags. Although chitin has previously been used as an immobilization support for XIs from mesophilic bacteria, this required cross-linking with glutaraldehyde [41, 42]. Here, we explore the prospect of affinity immobilization of the class

II XI from *T. neapolitana* to chitin by means of a fusion to a CBD from the hyperthermophile *P. furiosus*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli strains XL1-Blue (Novagen, Madison, WI) and BL21(DE3) (Novagen) were used for cloning and expression of the *T. neapolitana* 5068 *xlyA* gene (TNXI), respectively. The wild-type and N-terminal deletion mutants were cloned as an *Nde*I-*Hind III* insert into pET22b(+) (pTNXI) [37]. The ChiBD-TNXI fusion plasmid (pTNXICBD) was created from the pTNXI and the construction of the plasmid, as described below.

Production of *xylA* N-terminus Deletion Mutants

N-terminal truncations of *xylA* were introduced via PCR. Forward primers were designed to introduce an *NdeI* restriction site before the desired truncation. The plasmid pTNXI was used a template and each forward primer was designed to begin at various intervals along the N-terminus of *xylA* in pTNXI. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). PCR products were treated with *DpnI* to degrade the pTNXI template prior to cloning into pET22b(+). Plasmids with N-terminal truncations are listed in Supplementary Table 2.1.

Assembly of the ChiBD-TNXI Fusion Construct into pChiBD-TNXI

The *xylA* gene was cloned as *Nde*I-*Hind*III inserts into plasmid pET22b(+), thereby creating pTNXI and its derivatives [37]. Based on sequence analysis of *chiB* (PF1233) in *P. furiosus*

(DSM3638) [23], primers were designed to clone the chitin-binding domain for insertion into pTNXI. The pChiBD-TNXI primer set was used to clone a ~350 base pair fragment of the *chiB* gene containing the ChiBD. Both primers included an *Nde I* restriction site to insert the ChiBD at the N-terminus of pTNXI. Plasmid mini-preps were prepared from 3-5 ml overnight cultures (Qiagen, Valencia, CA). Positive results from digests were also confirmed via PCR, using the *chiB* forward primer and the pTNXI C-terminus primer.

Purification of Recombinant TNXI

Wild-type recombinant TNXI was purified from 1-L cultures grown on LB medium. After centrifugation for 10 min at 4,000 x *g*, the cells were re-suspended in 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS) (pH 7.0), containing 5 mM $MnSO_4$ and 0.5 mM $CoCl_2$ (i.e., buffer A). Cells were disrupted by two consecutive passes through a French Pressure cell (Thermo Spectronic, Walthum, MA) while using a pressure drop of 11,000 psi. After centrifugation at 5,000 x *g*, the supernatant was heat-treated for 15 min at 65ºC. The precipitated material was separated by centrifugation at 5,000 x g for 30 min. The soluble fraction was filtered by a 0.45 μm filter and loaded on a DEAE-Sepharose Fast-Flow column equilibrated with buffer A. The protein was eluted with a linear 0-0.5 M NaCl gradient in buffer A, and the active fractions were analyzed by SDS-PAGE. Partially purified enzymes were loaded onto a Q-Sepharose column and eluted with a linear 0-0.5 M NaCl gradient in buffer A. Active fractions were combined and concentrated in a stirred ultrafiltration cell (Amicon, Beverly, MA), dialyzed against buffer A, and stored at 4ºC. Protein concentrations were assayed by the method of Bradford [43], using bovine serum albumin as the standard.

Determination of Kinetics for TNXI, Unbound ChiBD-TNXI and Immobilized ChiBD-TNXI

Michaelis-Menten kinetics were determined based on time-course samples of D-glucose conversion to D-fructose at 80 and 90°C. Briefly, 50 μL of sample containing 10-500 mM D-gluose, 5 mM $MnCl_2$ and 0.5 mM $CoCl_2$ was pre-heated for 15 min at 80 or 90°C and then added to 50 μL of enzyme solution (~1 mg/ml). After 0.5 min, the solution was placed on ice to stop the reaction. Abiotic isomerization was accounted for with a negative control containing only the reaction solution without the enzyme. Prior to analysis, samples were processed by micro-centrifugation on 3 kDa membrane filters (Microcon). The reaction products were measured using a resorcinol assay for the determination of ketoses [44]. To test the amount of fructose produced, 50 μl of the reaction solution was aliquoted into 96 well microplate and 150 μl of a 1:1 mixture of 0.05% resorcinol in ethanol and $FeNH_4(SO_4)_2 \cdot 12 \cdot H_2O$ in concentrated HCl were added. For color development, the plate was incubated at 80°C for 40 min. The absorption was measured with a microplate reader at 490 nm for D-fructose. One unit of D-xylose isomerase catalyzed the formation of 1 μmol ketose (fructose) min^{-1} at 80°C in this assay system.

Temperature Optimum

The effect of temperature on ChiBD-TNXI activity was determined in 100 mM Mops buffer, containing 1 mM $CoCl_2$. The $\Delta pK_a/\Delta T$ of Mops (-0.011) was taken into account to ensure a pH of 7.0 at all temperatures assayed. The enzyme (0.10 mg/mL) and substrate (300 mM fructose) were pre-incubated separately for one minute at the temperature of interest in a Perkin-Elmer Cetus PCR system (Perkin-Elmer) (temperatures ≤ 100ºC) or a heat block (temperatures > 100ºC). Then the enzyme and substrate were combined in equal volumes

(50 µl each) and incubated for 5 minutes. The reaction was stopped by submersion in an ice bath. Glucose produced was determined as previously described. One unit of isomerase activity is defined as the amount of enzyme that produces 1 µmol of product per minute under the assay conditions.

Differential scanning calorimetry (DSC)

Melting temperatures for free and immobilized enzymes were determined with a Nano-Cal differential scanning calorimeter (Calorimetry Sciences Corp., Provo, UT), using a scan rate of 1 ºC/min. All TNXI variants (TNXI conc. ≥ 1.0 mg/mL) were dialyzed overnight at 4ºC against 2 L of 50 mM Mops, pH 7.0, containing 5mM $MgSO_4$ and 0.5 mM $CoCl_2$. The ChiBD-TNXI (1.1 mg/mL) fusion protein was immobilized onto chitin following dialysis. ChiBD-TNXI was dialyzed overnight at 4^{o}C against 2 L of 50 mM Mops, pH 7.0, containing 5 mM $MnCl_2$ and 0.5 mM $CoCl_2$ or 50 mM Mops, pH 7.0, containing 10 mM EDTA for metal-bound and apo enzymes, respectively. Dialysis buffer was used as a baseline for the free enzymes whereas the dialysis buffer containing chitin beads was used as the baseline for the immobilized enzyme. Each sample was scanned from 25ºC to 125ºC and the data was analyzed by vendor-provided software.

TNXI immobilization on chitin and thermoactivity/thermostability assays

Crude cell extract containing ChiBD-TNXI (1.5mg/mL) or purified native TNXI (1.1 mg/mL) were mixed with chitin beads (New England Biolabs, Beverly, MA) and incubated overnight at 4ºC. The weight of the chitin beads was measured before and after immobilization, and at least 1 mg of protein was loaded onto the beads for each experiment. The enzyme-chitin

mixture was then heated to 55ºC for 15 minutes and poured into jacketed, fritted glass columns (0.7 x 15 cm) (Sigma Chemical Co., St. Louis, MO) to drain. The chitin beads were re-suspended in 40 mL of buffer A and centrifuged at 8,000 x *g* for 10 minutes; the supernatant was discarded to remove any unbound enzyme. The beads were re-suspended for a final wash in 5 mL of buffer A and spun for 5 minutes at maximum speed in a Denville 260D at 14,000 x g. The supernatant was discarded and the beads were re-suspended in 5 mL of a 600 mM fructose solution in buffer A, which was incubated in a water bath at 75ºC for 15 minutes and then placed on ice. A 5 mL sample of the fructose solution was incubated along with the samples to correct for non-enzymatic isomerization. A 2 mL sample was taken from each of the chitin bead solutions and spun for 5 minutes at maximum speed in a Denville 260D centrifuge (14,000 x g) to remove the beads from the suspension. The supernatant was analyzed for glucose concentration, as described previously.

To determine the effects of temperature on the immobilized enzyme, the beads were suspended in buffer A to achieve a concentration of 0.5 mg/mL. The substrate (600 mM) was prepared in 50 mM Mops buffer with the pH adjusted to 7.0 at the incubation temperature. The reaction was initiated by combining 100 μL of each solution, and then incubating for 15 min, as described previously, at the temperature of interest. A control with buffer and no enzyme was also incubated to account for non-enzymatic isomerization. The reaction was stopped, by transferring the tubes to an ice bath; the amount of glucose formed was quantified, as previously described.

To determine the thermostability of ChiBD-TNXI, the fusion protein was immobilized onto chitin beads in buffer A, with the resulting wet bead weight of ~ 1.0 mg. Substrate was added to 50 mM Mops buffer (pH 7.0) containing 5mM $MnCl_2$ and 0.5mM $CoCl_2$ at pH 7.0 to a final concentration of 100 mM at the incubation temperature. The reaction was initiated

began by combining 100 μL of each solution and incubating the reaction at 100°C and 90°C for 17 h. Time course samples were taken and analyzed using the resourcinol assay method described above.

Structural Modeling

The *P. furiosus* ChiBD amino acid sequence was added to the N-terminus of TNXI at the NdeI site so that the fusion protein could be examined using to the protein modeling program 3D-jigsaw (http://bmm.cancerresearchuk.org/~3djigsaw/) [45]. This program was used to predict a three-dimensional structure, based on homology to the existing structures of TNXI (1A0E) and *P. furiosus* ChiBD (2crw) in the Protein DataBase (PDB). The predicted 3D structure of ChiBD-TNXI was visualized and compared to the native TNXI structure using the UCSF Chimera molecular graphics program (http://www.cgl.ucsf.edu/chimera/) [46].

RESULTS

Location of ChiBD Fusion and Role of N-terminus in the Structure and Function of TNXI

ChiBDs can be engineered into fusion proteins to serve as N-terminal, internal, or C-terminal affinity tags [47-49]. Since the □ $\alpha\beta_8$ barrel motif predominates in the TNXI structure and includes the catalytic site, an internal ChiBD was ill-advised. The C-terminus of a class I XI from an *Arthrobacter* species could be removed post–translationally without affecting the stability or activity of the enzyme [34]. However, the proximity of the C-terminus to the other subunits in class II XIs makes it a less ideal location than the N-terminus which extends away from the rest of the enzyme. In fact, TNXI structural data suggest possible

complications with formation of subunit interactions, if a C-terminal fusion of the *P. furiosus* CBD was used for TNXI immobilization.

While it appears that the N-terminus of TNXI and other class II XIs is not implicated in a critical structural role, no experimental information is available on this issue. Amino acid sequence information suggests that the class II N-terminus may play a role in protein structure (see Figure 2.1). Thus, this question was investigated through creation of a series of N-terminal deletion mutants, created by systematically eliminating sets of residues after the initial methionine. A 41-amino acid (aa) deletion mutant (TNXI41) completely eliminated most of the class II N-terminal insert from the enzyme (based on comparison to the class I XI from *Streptococcus murinus* [38], truncating the enzyme at the start of an $\alpha\beta_8$ barrel fold (Figure 2.2). A 34-aa deletion mutant (TNXI34), on the other hand, confers on TNXI an N-terminus that corresponded in length to class I XIs. Neither mutation produced a soluble or active enzyme, indicating the importance of at least some of the N-terminus in the class II TNXI. Mutants TNXI12 and TNXI24 were then created to help determine the length of N-terminus needed to produce an active TNXI. TNXI24 was inactive, while the TNXI12 mutant isomerized fructose to glucose at normal assay temperatures. The TNXI12 mutant behaved exactly as wild-type TNXI during purification, and had a comparable temperature optimum (90-95ºC). However, the TNXI12 mutant did not exhibit the characteristic two melting transitions (99.5ºC and 110ºC) observed for the native enzyme in the presence of two activating metals [13, 37, 40]. Instead, the TNXI12 had only a single melting transition at 105.4ºC (data not shown). To further examine the extent that the N-terminus was needed for active enzyme, mutants covering the range from 13-23 aa were created. The mutants up to TNXI18, with the exception of TNXI16, tested positive for fructose isomerization, indicating that some of the N-terminal insert is critical for a properly folded and active enzyme. A

further examination of the crystal structure revealed that residues 1-12 are situated outside the core structure and not associated with subunit interactions. In the region around residues 16-20, there are more hydrophobic interactions with the surface of the $\alpha\beta_8$ fold and the surface of other subunits; deletions TNXI19 and higher produced inactive, insoluble enzyme. These results indicated that an N-terminal fusion of *P. furiosus* CBD was a plausible basis for an immobilization strategy.

ChiBD-TNXI Immobilization

CBDs are classified into 14 different families based on amino acid sequence, binding specificity, and structure [17]. While the binding of some CBDs to cellulose is reversible (such as the Family 1), the Family 2 CBDs bind irreversibly and exhibit little or no enzyme leakage [50]. In fact, several Family 2 CBDs have been used commercially as affinity tags for purification [51]. In view of this, the Family 2 CBD from *P. furiosus* chitinase ChiB (PF1233) [23], previously used to create a fusion protein with a *Thermotoga maritima* endoglucanase [27], was selected to create a fusion protein at the N-terminus of TNXI.

To test for immobilization efficacy and non-specific binding, both recombinant *E. coli* crude extract containing TNXI-CBD and purified recombinant TNXI without the CBD were incubated in a solution containing chitin beads at 4°C overnight. Initially, both enzyme samples decreased in protein concentration by about 0.7 mg/mL. However, after washing the chitin with buffer, the activity of the TNXI-CBD beads was over 20 times greater than the beads with TNXI (430 U/mg vs. 15 U/mg specific activity, respectively). When the beads were treated with protein dye, the TNXI-CBD bound beads turned blue indicative of protein, while the wild-type TNXI beads remained white.

Thermostability of TNXI, Unbound ChiBD-TNXI and Immobilized ChiBD-TNXI

Differential scanning microcalorimetry was used to examine the melting behavior of the wild-type TNXI and the unbound and immobilized ChiBD-TNXI. Previous results with TNXI had shown that wild-type enzyme exhibited two thermal transitions that were associated with the binding of divalent cations Co^{2+} and Mn^{2+}; the apo-enzyme was characterized by a single melting transition [40]. Similar results were observed in this study, although the temperatures for the two transitions of the halo-enzyme varied. TNXI-apo melted at 97.5°C, while in the presence of divalent cations Co^{2+} (0.5 mM) and Mn^{2+} (5.0 mM), transitions at 100°C and 112°C were observed (see Table 2.2). The apo version of the immobilized enzyme melted at 104°C, with transitions at 87°C and 110°C for the halo enzyme. The unbound ChiBD-TNXI showed a lower melting transition temperature for both the metal and apo version of the soluble enzyme (60°C/110°C and 71°C respectively) this is likely due to the added subunit interactions made by the chitin binding domain. A native gel showed additional quaternary structures for the ChiBD-TNXI (Dimer and Tetramer form) compared to the native TNXI (Dimer form mostly). This results in a large population of ChiBD-TNXI active tetramers (probably formed by many weak ChiBD and subunit interactions) that readily disassociate into the active dimer form. As noted before, the release of the metals from the metal binding site probably accounts for the additional metal transition temperature peak seen in the metal bound case compared to the apo case.

Enzyme inactivation was also determined for the three versions of TNXI. Using the bi-phasic model described previously [40], the wild-type enzyme had a half-life of 6.8 $\pm$ 0.6 min at 100°C, compared to 19.9 $\pm$ 1.5 min for the immobilized enzyme (see Table 2.1). Surprisingly, the unbound ChiBD-TNXI had a significantly longer half-life (56.5 $\pm$ 9.0 min) than either of the other two versions.

Kinetics of TNXI, Unbound ChiBD-TNXI and Immobilized ChiBD-TNXI

The kinetics of the wild-type TNXI was compared to the unbound and immobilized ChiBD-TNXI. The time-dependent conversion of glucose to fructose was tracked at 80°C, the growth temperature optimum for *T. neapolitana*, and 90°C, closer to the temperature optimum for the enzyme. Data were fit to a Michaelis-Menten formulation, accounting for substrate inhibition for only the 80°C case for the fusion protein[1], and assuming that TNXI is all cases was dimeric and contained one active site per monomer [37]. Turnover numbers (k_{cat}) for both TNXI and the immobilized ChiBD-TNXI increased approximately 25% from 80 to 90°C, although the immobilized enzyme was 6-fold more active at both temperatures (see Table 2.3). K_i's for both immobilized and unbound ChiBD-TNXI were determined for the 80°C case, 16.9 $\pm$ 1.6 and 27.5 $\pm$ 1.7 mM, respectively. Due to these large values for the K_i's, the effect of substrate inhibition on the reaction velocity was negligible. K_M's for both immobilized and wild-type TNXI also increased between 80 and 90°C, such the effect of temperature on the catalytic efficiencies in both cases was minimal. In contrast, k_{cat} for the unbound ChiBD-TNXI increased from 3798 min^{-1} at 80°C (4 times that for TNXI) to 10,353 min^{-1} at 90°C (9 times that for TNXI at 90°C and 50% higher than the immobilized ChiBD-TNXI at 90°C). Furthermore, the K_M for the unbound ChiBD-TNXI (158 mM) was comparable to the immobilized ChiBD-TNXI (122 mM) at 80°C but nearly 4-fold higher (536 mM vs. 149 mM) at 90°C. Thus, the effect of adding the ChiBD at the N-terminus significantly increased conversion rates of glucose to fructose, with the immobilized version being more active at 80°C and the unbound version most active of all at 90°C.

[1] K_i was calculated for both 90°C and 80°C for the fusion protein, but the $(1/K_i) \rightarrow 0$ since K_i is large for the 90°C case. Therefore only the 80°C K_i's are reported.

DISCUSSION

Many strategies have been proposed and implemented for immobilizing enzymes seeking to extend the lifetime of the biocatalyst for a particular application and to improve bioprocess economics. The biomolecular consequences of immobilization are usually difficult to predict – whether or not the enzyme is a better and/or more stable biocatalyst in a constrained state needs to be determined experimentally. Enzymes from high temperature microorganisms present both interesting challenges and opportunities when it comes to their immobilization, as mentioned previously. Choice of immobilization method must take into consideration adverse thermal effects on the matrix or support although it is possible that the hyperthermophilic enzyme will be more robust and less impacted by the immobilization process.

Here, we show that the xylose isomerase from the hyperthermophilic bacterium, *T. neapolitana* (TNXI), was both significantly stabilized and activated for glucose to fructose conversion through the N-terminal addition of a hyperthermophilic chitin binding domain from another hyperthermophilic microorganism, *P. furiosus*. It was first determined that the N-terminal fusion was a plausible immobilization strategy through deletion mutations of amino acid residues from the N-terminus. However, there was no indication that any enhancement in activity and stability would result from the construction of the fusion protein. Note that the k_{cat}s observed here for the unbound and immobilized versions of TNXI represent the highest ever reported for the biocatalytic conversion of glucose to fructose for any enzyme at any temperature [36].

In hindsight, molecular modeling tools provide some insights into the reasons for the improvements observed. A comparison of the dimer forms of ChiBD-TNXI and TNXI (Figures 2.3 and 2.4) shows a more flexible active site in ChiBD-TNXI. Upon closer

observation, the residue displacements were measured (Table 2.5, Figure 2.5, and Figure 2.6), and the ChiBD-TNXI and TNXI active site residues were not superimposable. Site-directed mutagenesis studies on the xylose isomerases from *Thermotoga neapolitana* and *Thermus thermophilus* found the of TRP138PHE and VAL185THR mutations for *T. neapolitana* and *T. thermophilus* xylose isomerases, respectively, optimized the catalytic efficiency of enzymes on glucose [52]. In the TNXI mutant case, the VAL185THR mutation contributed to additional hydrogen bonding to the glucose's C6-OH and the increased catalytic efficiency. The TRP138PHE mutation improved the thermal stability. For TTXI, the TRP138PHE mutation doubled the half-life of the enzyme at 85^{o}C [53]. Here, the ChiBD could have enabled hydrogen bonding to produce the same effect (Figure 2.7). The proximity of ChiBD residues GLU47 (15.68 Á), GLY48 (13.95 Á), TYR51 (16.01 Á), GLY50 (14.13 Á), and ASN49 (12.71 Á) to VAL185/253 could create favorable interactions to stabilize glucose in the active site. This local shift in active site residue orientation could also affect the enzyme-substrate binding properties and/or the reactivity of the residues during catalysis. This would explain the increased catalytic efficiency and increased K_M. In addition, the displacement of the TRP138/206 mutation had a similar affect onimproving the thermostability via the increase in the half-life of the ChiBD-TNXI compared to TNXI. Taken together, the increased conformational flexibility in the active site in the bound and unbound immobilized enzymes seems to have not only improved substrate to product turnover but also enhanced thermostability. This fortuitous result led to perhaps the most thermostable and thermoactive xylose isomerase yet reported. Detailed structural analysis is needed to more definitively identify the active site structure in the fusion protein and possible implications away from the catalytic center.

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TABLE 2.1 Thermostability of Soluble and Immobilized TNXI		
Enzyme	**Format**	**Half-life (min) at 100°C**
TNXI	Soluble	6.8 ± 0.6
TNXI-PFChiBD	Soluble	56.5 ± 9.0
IM-TNXI-PFChiBD	Immobilized	19.9 ± 1.5

TABLE 2.2 Differential Scanning Calorimetry of TNXI and TNXI-PFChiBD			
Enzyme	**Format**	**Melting Transition(s)**	**Added to Reaction Buffer***
TNXI	Soluble	100°C, 112°C	5 mM Mn^{2+}, 0.5 mM Co^{2+}
TNXI (apo)		97.5°C	10 mM EDTA
ChiBD-TNXI	Unbound	60°C, 110°C	5 mM Mn^{2+}, 0.5 mM Co^{2+}
ChiBD-TNXI (apo)		71°C	10 mM EDTA
ChiBD-TNXI	Immobilized	87°C, 110°C	5 mM Mn^{2+}, 0.5 mM Co^{2+}
ChiBD-TNXI (apo)		104°C	10 mM EDTA
*50 mM MOPS, pH 7.0			

TABLE 2.3 Kinetic Parameters for Wild-type, Unbound and Immobilized TNXI							
Enzyme	**Format**	**T (°C)**	**V_{max} (U/mg)**	**K_M (mM)**	**Ki (mM)**	**k_{cat} (min^{-1})**	**k_{cat}/K_M (mM^{-1} min^{-1})**
TNXI	Soluble	80	19.1 ± 3.4	65.2 ± 9.0	--**	971	14.9
		90	22.4 ± 1.3*	88.5 ± 16.5*	--**	1139	12.9
TNXI-ChiBD	Unbound	80	74.7 ± 17.0	157.8 ± 0.4	27.5 ± 1.7	3798	50.8
		90	203.6 ± 15.9	536.1 ± 17.3	--**	10353	19.3
TNXI-ChiBD	Immobilized	80	113.6 ± 11.9	121.5 ± 15.3	16.9 ± 1.6	5776	47.5
		90	136.9 ± 11.0	148.9 ± 4.4	--**	6961	46.7
*Data from Vielle et al. (1995) **substrate inhibition not observed							

TABLE 2.4 Primers used for construction of deletion mutants of TNXI	
# Residues Deleted from N-terminus	**Oligonucleotide primer for N-terminus**
12	5’ – CCG AAA GTA **CAT ATG** GAA GGC AAA G – 3’
13	5’ – GAA GTG AT**C ATA TG**G GCA AAG AAA GCA C – 3’
14	5’ – GAC CAG AAC **CAT ATG** AAA GAA AGC AC – 3’
15	5’ – CAG GTC GAA **CAT ATG** GAA AGC ACA AAT C – 3’
16	5’ – GTA GCA AGG C**CA TAT G**AG CAC AAA TCC – 3’
17	5’ – GAA GCG AAA **CAT ATG** ACA AAT CCA CTT GC – 3’
18	5’ – GCC AAT GAA **CAT ATG** AAT CCA CTT GCG – 3’
19	5’ – CAA AGA AAC C**CA TAT G**CC ACT TGC GTT C – 3’
20	5’ – GTT GTC ACA **CAT ATG** CTT GCG TTC – 3’
21	5’ – GAA GGA CAA AT**C ATA T**GG CGT TCA AGT TC – 3’
22	5’ – CTA AGA CGC **CAT ATG** TTC AAG TCC TAC G – 3’
23	5’ – GAC TCC GAG A**CA TAT G**AA GTT CTA CGA TC – 3’
24	5’ – CAC TTG CA**C ATA TG**T TCT ACG ATC CAG – 3’
34	5’ – GTC TAG TAG **CAT ATG** AAA CCC CTC AAG – 3’
41	5’ – GTA TGT G**CA TAT G**AA GTT CTC CGT TG – 3’
	Oligonucleotide primer for C-terminus
	5’ – CGC **AAG CTT** CAC ACT CTG – 3’

Table 2.5	Displacement of Active Site between TNXI and TNXI-CBD		
Residue	**Residue # TNXI**	**Residue # TNXICBD**	**Distance Á**
ASP	103	171	2.62
HIS	100	168	2.23
LEU	143	211	2.81
GLU	236	304	2.43
THR	140	208	2.03
VAL	185	253	1.58
TRP	138	206	1.74
ASP	338	406	1.43
ASP	308	376	2.26
ASP	295	363	1.80
HIS	270	338	1.96
GLU	231	299	1.68
LYS	233	301	2.28
GLU	267	335	1.85
GLU	236	304	2.43
ASP	306	377	2.57

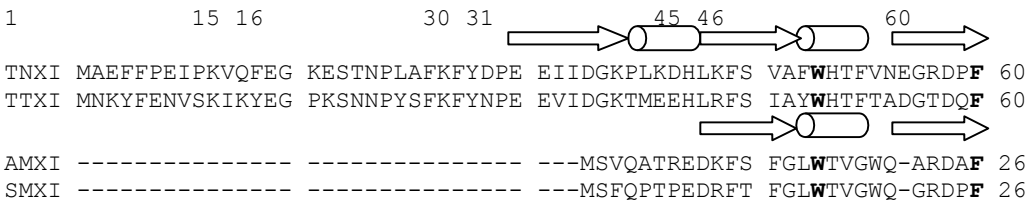
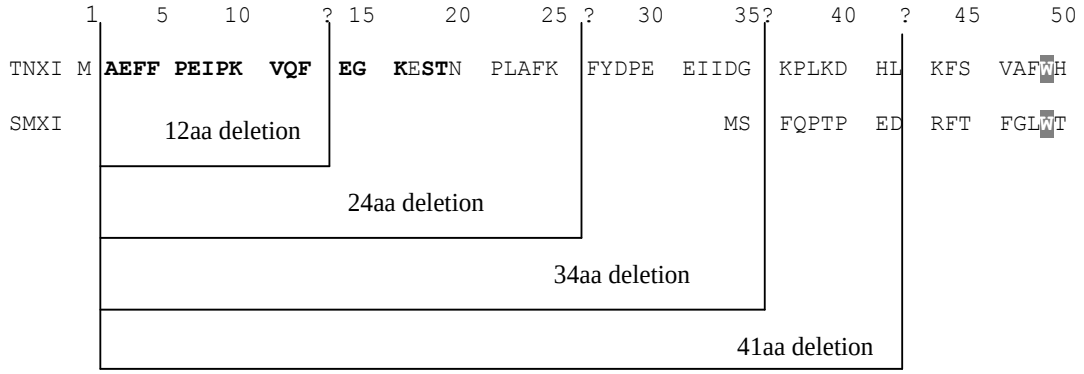


Figure 2.1. N-terminal sequences of representative Class I (*Arthrobacter missouriensis* – AMXI and *Streptomyces murinus* – SMXI) and Class II (*Thermotoga neapolitana* - TNXI and *Thermus thermophilus* - TTXI) xylose isomerases showing additional amino acid residues for the Class II enzymes. Conserved tryptophan (W) and phenylalanine (F) residues shown in bold. α-helices represented as ⬭ , β-sheets as ⟹ .



#Amino acids Deleted	12	13	14	15	16	17	18	19	20	21	22	23	24	34	41
Active?	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No	No	No	No	No	No	No

Figure 2.2. Consequence of TNXI N-terminal mutations. Bolded portion represents the maximum number of amino acid residue deletion that still retained activity. Class I *Streptomyces murinus* (SMXI) N-terminus shown for comparison.

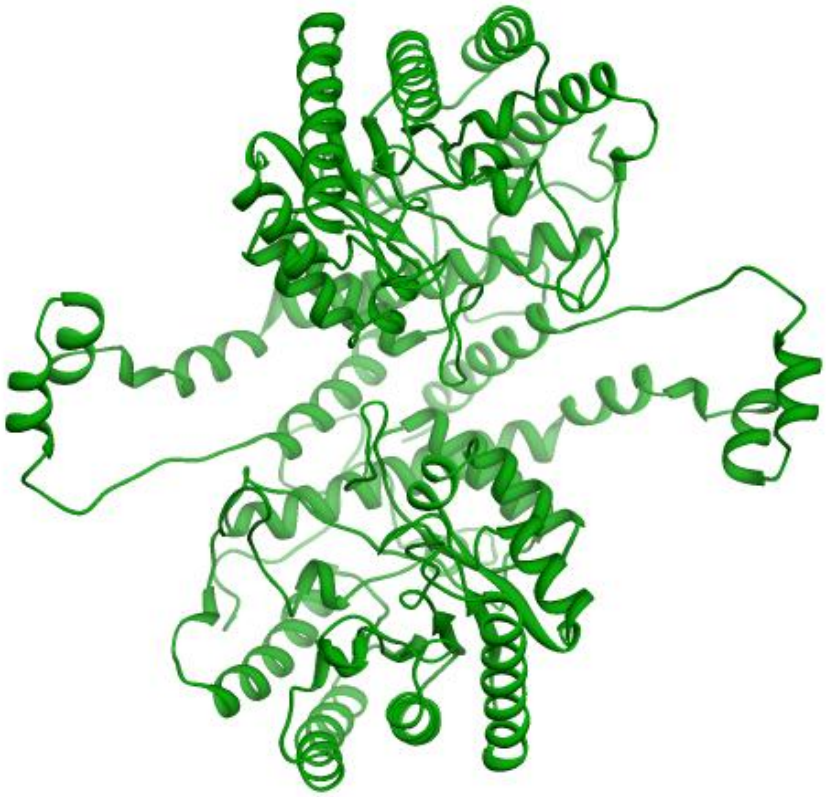


Figure 2.3. *Thermotoga neapolitana* Xylose Isomerase (TNXI) – dimeric form. The PDB code for TNXI is 1a0e and the modeling software Chimera was used to construct the dimer.

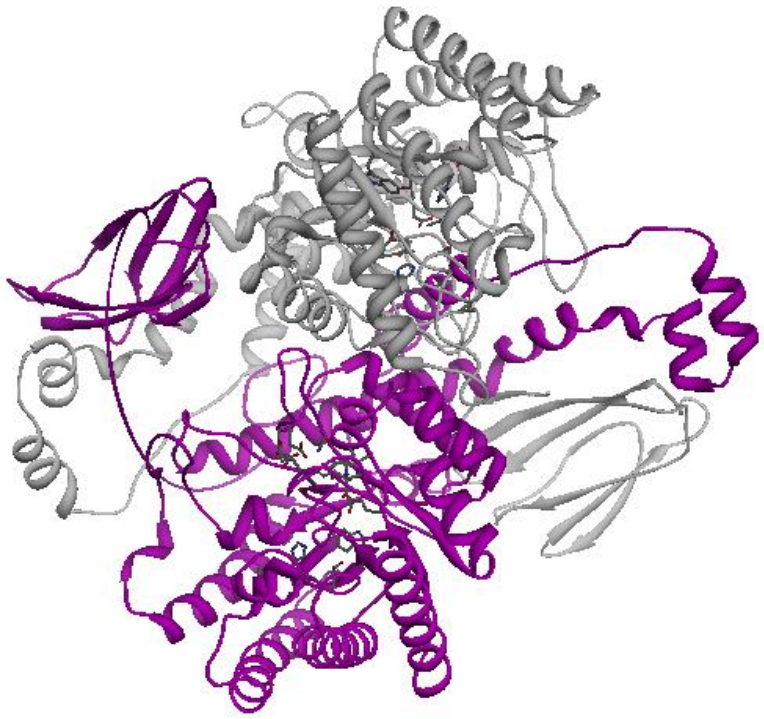


Figure 2.4. Dimer of *Thermotoga neapolitana* Xylose Isomerase with a fused chitin binding domain from *P. furiosus*. 3D-jigsaw [45] was used to thread the structure of ChiBD-TNXI using the TNXI PDB (1a0e) and Chitin binding domain from *P. furiosus* (2cwr) as a template. The modeling program Chimera was used to create the dimer of the threaded structure.

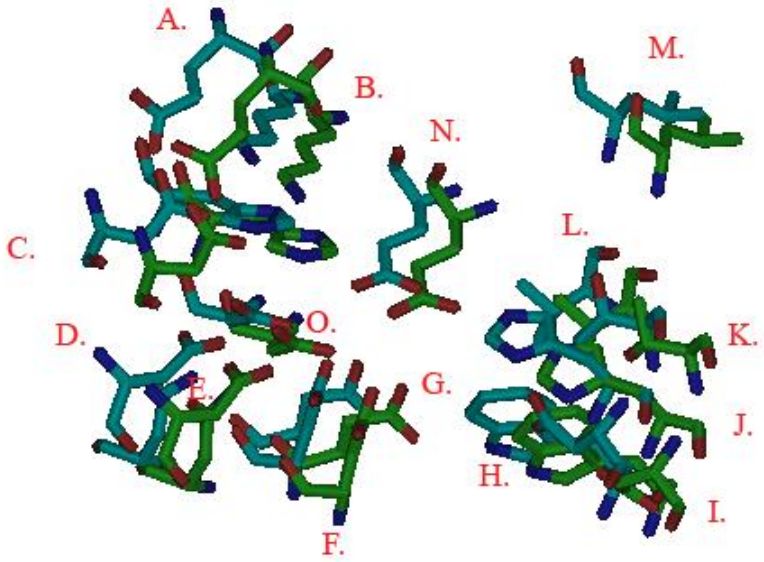


Figure 2.5. Superimposition of ChiBD-TNXI (green) and TNXI (teal) active site residues. A. GLU304/236, B. LYS301/233, C. ASP374/306, D. ASP376/308, E.ASN365/297, F. ASP406/338, G. ASP363/295, H. TRP206/138, I. ASP171/103, J. HIS168/100, K. THR208/140, L. VAL253/185, M. LEU211/143, N. GLU299/231, O. GLU335/267.

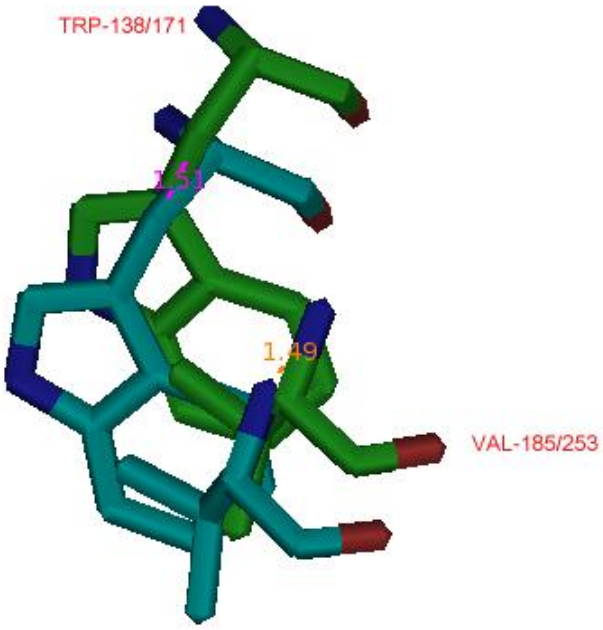


Figure 2.6. Key catalytic and structural residues in TNXI compared to ChiBD-TNXI. The displacement of VAL 185 to ChiBD-TNXI's VAL 253 and TRP 138 to ChiBD-TNXI's TRP 171 was 1.49 and 1.51Å respectively.

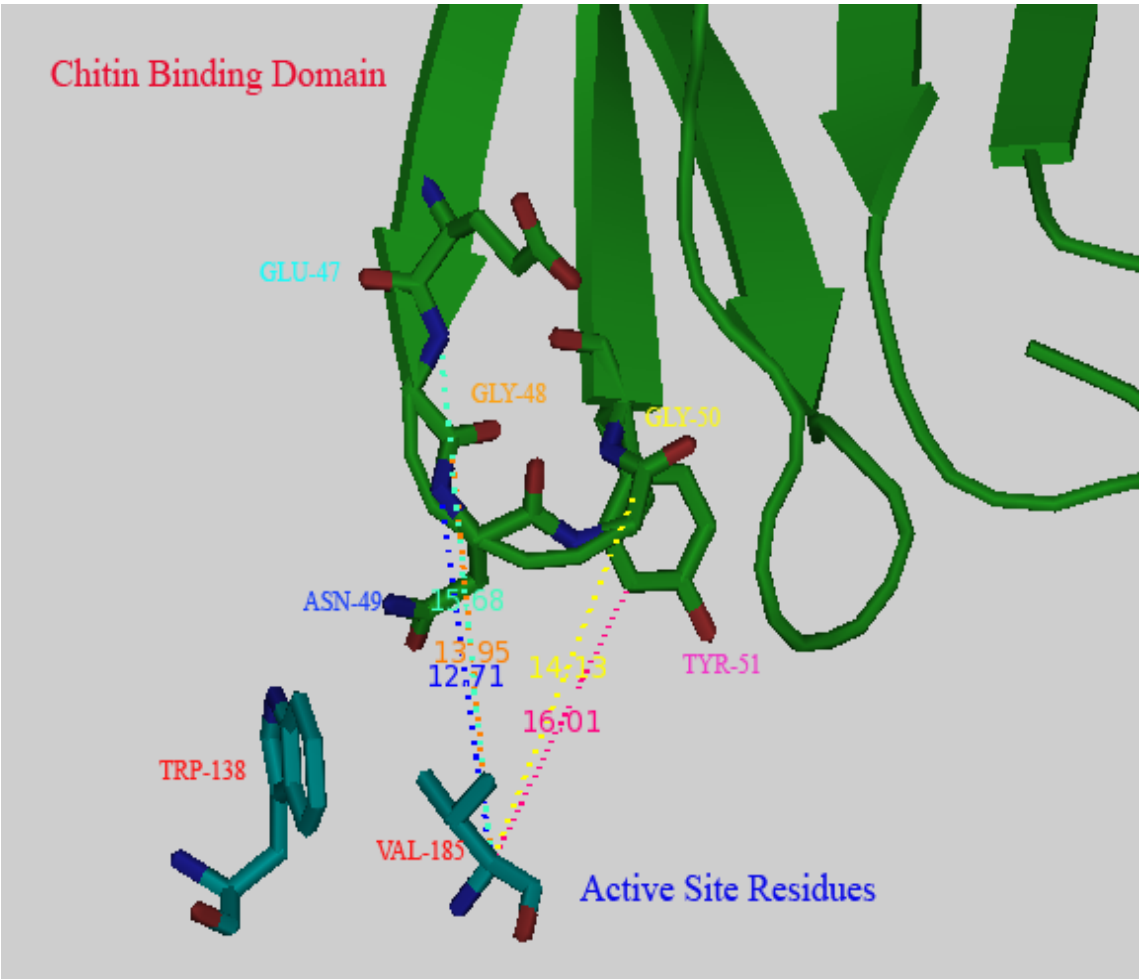


Figure 2.7. Influence of Chitin Binding residues on H-bonding near Val 185. The displacement distances from VAL 185 to GLY 48 (13.95Å), GLU47 (15.68Å), to ASN 49 (12.71Å), to GLY 50 (14.13Å), and to TYR 51 (16.01Å) were determined.

Biocatalytic versatility of the sugar isomerases and epimerases from hyperthermophilic bacterium *thermotoga maritima* for production of rare sugars

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ABSTRACT

Several sugar isomerases and epimerases from the hyperthermophilic bacteria *Thermotoga maritima* and *Themotoga neapolitana* have mesophilic homologs that can produce rare sugars, carbohydrates of technological importance that typically occur in intermediate steps in metabolic pathways. *Thermotoga* versions of L-fucose isomerase (TM0307), L-arabinose isomerase (TM0276), L-rhamnose isomerase (RI) (TM1071), D-tagatose-3-epimerase (TM0416), and D-xylose isomerase (TN1667) were identified in various monosaccharide utilization operons using bioinformatic analysis and functional genomics. Recombinant versions of these enzymes were expressed in *Escherichia coli* to facilitate efforts to define the biocatalytic versatility of these enzymes for a range of monosaccharide substrates. TMRI (T_{opt} of 85°C and pH_{opt} of 7.3) occurred in both dimeric and tetrameric forms and exhibited a K_m and V_{max} of 27.95 ± 0.91 mM and 494.0 ± 4.2 U/mg, respectively, for the conversion of L-rhamnose to L-rhamnulose. Furthermore, TMRI also catalyzed the conversion of xylose to xylulose, glucose to fructose, D-psicose to D-allose (13:87 D-psicose:D-allose), and L-lyxose to L-xylulose (37:63 L-lyxose:L-xylose) at yields comparable to those that been reported for mesophilic L-rhamnose isomerases. TMFI (T_{opt} of 85°C and pH_{opt} of 8.0) was tetrameric, with K_m and V_{max} of 6.0 ± 0.2 mM and 612.7 ± 21.2 U/mg, respectively, for the conversion of fucose to fuculose. TMFI converted D-psicose to D-altrose with high efficiency (54:50 D-psicose:D-altrose), while also producing several side products that could not be identified. TMAI, previously characterized elsewhere (T_{opt} 90^{o}C, pH 7.5), not only converted L-arabinose to L-ribulose, but also D-galactose to D-tagatose. TMTE, present in both dimeric and trimeric forms, exhibited a K_m and V_{max} of 7.9 ± 0.8 mM and 192.2 ± 18.1 U/mg, respectively, on D-tagatose. These four isomerases were coupled with TMTE to explore multi-step sugar biotransformations to form rare sugars, similar to what has been

reported for the mesophilic homologs of these enzymes. However, TMTE yielded significantly different products from sugar substrates than reported for its mesophilic homologs, thus altering the expected product distribution in multi-step biotransformations. To this point, the recently solved three-dimensional crystal structure of TMTE revealed a completely altered binding pocket, compared to mesophilic TE homologs, which likely alters the specificity of TMTE to natural and unnatural substrates. Further work is needed to resolve the many unknown products produced from multi-step reactions coupling TMTE with *Thermotoga* sugar isomerases, but it is possible that novel, rare sugars could be produced by these hyperthermophilic enzymes.

INTRODUCTION

Monosaccharides that arise from intermediate steps in cellular metabolism, referred to as “rare sugars”, can have technological importance as food additives and therapeutic compounds [1]. These rare sugars have been produced from inexpensive natural resources, such as wood, starch, and whey, through the coupled use of single or multi-step biotransformations involving natural or recombinantly modified sugar isomerases and epimerases. Certainly, many opportunities exist for adding value through rare sugar reactions, as indicated in Table 3.1. For example, conversion of D-tagatose to D-gulose improves the product value by 10,000-fold [2]. However, much needs to be done to determine the best biocatalytic routes for the production of specific rare sugars to ultimately produce them in optimal bioprocessing scheme.

Rare sugars are used as drug formulation agents and even directly as anti-cancer therapeutics, although their chemical synthesis on a large-scale is prohibitively expensive and technically difficult [3]. Enzymatic approaches to rare sugars typically have low equilibrium yields and require long reaction times, such that enzyme stability can compromise processing objectives [4]. Among the most promising enzymes types to be considered for rare sugar synthesis are D-tagatose-3-epimerase, various oxidoreductases, polyol dehydrogenases, and aldose isomerases. Some background on sugar isomerases and epimerases is provided below.

D-Xylose Isomerase. Xylose isomerase (D-xylose ketol isomerase, XI) is the most utilized industrial sugar isomerase for an unnatural substrate [5]. XI is used to convert glucose to fructose for industrial scale production of the high fructose corn syrup (HFCS) [6]. The industrial utility of XIs has made these enzymes the target of extensive biochemical,

biophysical and bioprocessing efforts [7-9]. Several studies have focused on improving XI activity on glucose, through site directed mutagenesis, to create a true “glucose isomerase” [10-13]. X-ray crystallography and biochemical analysis have shown the importance of interactions at the substrate’s hydroxyl oxygen bond to backbone carbon 1 (O1) and O2, coupled with either O3 and O4 or O4 and O5 for catalysis. XI reacts with many substrates (native and non-native) through substitutions at C3, C5, and C6 (reviewed in [4]). This versatility has thus far received little attention, relative to its industrial application for HFCS production, despite its potential for applications in bioorganic synthesis [14]. Sweetzyme [15], among other commercial XIs, have been examined with respect to substrate specificity and reaction mechanism. With the exception of L-arabinose isomerase from *Geobacillus stearothermophilus* that was engineered to be a more efficient D-galactose isomerase [16], no attempts to metabolically engineer sugar isomerases to increase their efficiency in the production of rare sugars. Although efforts have been made to improve enzyme/substrate ratios (e.g., using cross-linked *Streptomyces rubiginosus* XI crystals in bioreactors and effluent recycling), yields in rare sugar reactions remain extremely low [17]. Two different parallel reaction pathways, a 1,2 hydride shift and a Lobry de Bruyn-Alberda van Ekenstein re-arrangement through an ene-diol intermediate for (hexoses/pentoses), have been proposed to account for the formation of epimeric aldoses from aldopentoses, aldohexoses, and ketoses (Figure 3.1) [5]. For XIs, however, there is no base position to allow proton transfer between C_1 and C_2 for an ene-diol mechanism, which suggests a 1, 2-hydride shift mechanism. XIs have been reported to catalyze several different rare sugar reactions. For example, at 60°C, the XI from *Streptomyces rubiginosus* catalyzes other isomerization reactions, including D-arabinose↔D-ribulose, L-arabinose↔L-ribulose, D-ribose↔D-ribulose, L-ribose↔L-ribulose, and D-lyxose↔D-xylulose [18]. This XI also accepts D-

altrose, D-gulose, D-sorbose, and D-idose as substrates [18]. The use of divalent ions (e.g., Mg^{2+}, Co^{2+} and Mn^{2+}) is required for activity in most XIs. The use of Co^{2+}, in particular, often leads to optimal catalytic efficiency. However, due to potential toxicity of this cation in foods and pharmaceuticals, investigations of XIs for rare sugar synthesis have focused on the Mg^{2+}-dependent, commercially available *Streptomyces* XIs. The reactivity with non-natural substrates makes XI an interesting candidate for the production of rare and valuable monosaccharides, an elusive synthetic target for carbohydrate chemistry [4]. Rare sugars are expensive to produce, especially when including a chromatography step is needed to remove a toxic metal. Highly thermostable XIs from hyperthermophiles, such as *Thermotoga maritima* ([19]) and *Thermotoga neapolitana* ([12, 13, 20-24]), are candidates for rare sugar production, especially if protein-engineering techniques can improve their efficacy in this regard. Recently, Vieille et al. [25] discovered a manitol dehydrogenase from *T. maritima* that could be used in conjunction with *T. neapolitana* xylose isomerase mutant (TNXI 1F1) for the production of mannitol from D-glucose at 60^{o}C. The final concentration of mannitol was 19mM, achieved in the first 10 minutes of the experiment from a 200 mM D-glucose solution.

L-Rhamnose Isomerase. L-rhamnose isomerase was first discovered in the context of L-rhamnose metabolism in *E. coli*; the enzyme reversibly isomerizes L-rhamnose to its corresponding ketose, L-rhamnulose [26-28]. A mutant strain of *Pseudomonas aeruginosa* (sp.) strain LL172, containing L-rhamnose isomerase, was grown on a L-lyxose based medium [29]. This L-rhamnose isomerase showed a broad specificity to various rare aldoses and ketoses . Maximum activity was observed at 50^{o}C and pH 9.0, and the enzyme was stable over a range of pH 5.0-11.0 at 4^{o}C for 24 hours. The optimal temperature for

activity was approximately 60^{o}C at pH 9.0, but the enzyme was stable at 60^{o}C for only 10 min, thus making its use for lengthy reactions at this temperature problematic. With respect to substrate specificity of RI, the purified enzyme had broad substrate specificity for various aldoses. The highest activity was detected against L-rhamnose (100%), followed by L-lyxose (97%). However, this enzyme was active on L-mannose, D-gulose, D-ribose, D-allose, and L-talose. This L-rhamnose isomerase was also immobilized on chitopearl beads and produced L-mannose from L-fructose at 60^{o}C, with a 30% yield [30]. This same immobilization scheme was used to produce D-allose from D-psicose [31], L-lyxose from L-xylulose [32], L-talose from L-tagatose, and D-gulose from D-sorbose [3].

Leang et al. [33] discovered a more promiscuous version of L-rhamnose isomerase from *Pseudomonas stutzeri*. This enzyme exhibited a temperature optimum around that of *P. aeruginosa* (sp.) strain LL172, which was 60^{o}C, with a pH range from 5.0 to 11.0 and an optimum pH of 9.0. The broad specificity of this L-rhamnose isomerase was demonstrated by its activity on L-mannose, L-lyxose, L-glucose, L-tagatose, D-allose, and D-ribose [34]. The basis for this broad specificity was later uncovered when L-rhamnose isomerase from *P. stutzeri* was crystallized in the presence of L-rhamnose and D-allose [35, 36]. These studies showed that this L-rhamnose isomerase lacked a Phe66 in the active site, thus eliminating the hydrophobic interaction in the active site to the 4, 5, and 6 positions of the substrate. This missing interaction led to reduced recognition at the 4, 5, and 6 positions and, thus, broader substrate specificity. It is interesting that the L-RI expressed from *P. stutzeri* requires Mn^{2+} and Zn^{2+}, but this RI requires only Mn^{2+} for maximum activity. Large-scale production of D-allose from D-psicose using a continuous bioreactor containing immobilized L-rhamnose isomerase from *P. stutzeri* has also been reported [37]. A 10% conversion rate of D-psicose to D-allose was achieved, following the completion of a 17-day

reaction period. To date, no L-rhamnose isomerase has been characterized from a hyperthermophilic microorganism, although a thermostable L-rhamnose isomerase from the mesophilic bacterium *Bacillus pallidus* Y25 was reported [38]. This L-rhamnose isomerase was optimally active at 65^{o}C, which is the highest optimum temperature to date for L-rhamnose isomerases. Although no structural studies have been performed on the enzyme, it is worth noting that this version has a more rigid active site than L-rhamnose isomerase from *P. stutzeri,* as indicated by the reduction in side products. At 50°C, the L-rhamnose isomerase from *B. pallidus* Y25 converted D-psicose to D-allose, L-fructose to L-mannose, D-ribose to D-ribulose, and L-tagatose to L-talose, with yields of 35, 25, 16 and 10%, respectively.

L-Arabinose isomerase. L-Arabinose isomerase has been examined in detail for rare sugar synthesis [39-43]. Initially, L-arabinose isomerases from *E. coli*, *Bacillus subtilis* and *Salmonella typhimurium* were compared to determine which enzyme produces D-tagatose from D-galactose most efficiently. Three plasmids were constructed: pTC101, pTC105 and pTC106, containing *araA* from *E. coli*, *B. subtilis* and *S. typhimurium*, respectively. From recombinant *E. coli* cultures containing pTC101, pTC105 and pTC106, tagatose was produced from galactose in 9.9, 7.1 and 6.9% yields, respectively. The enzyme extract of *E. coli* containing pTC101 alone converted galactose into tagatose with a 96.4% yield [43]. The Kim group continued their investigation of L-arabinose isomerase from *E. coli* by optimizing the conversion of galactose into tagatose. L-arabinose isomerase, partially purified 15-fold with a specific activity of 70 U mg^{-1}, produced 50 g galactose/L from 17.7 g tagatose/L in 168 hours (34% equilibrium) [39]. Continuing a previous study, L-arabinose isomerase conversion of L-arabinose to L-ribulose was compared to the galactose conversion to

tagatose. The Michaelis constant, K_m, for galactose was 1480 mM, which is 25-fold higher than that for arabinose. The addition of Fe^{2+} ion enhanced the conversion of galactose to tagatose by decreasing the K_m for galactose to 300 mM [40]. The Kim group followed up the previous efforts with L-arabinose isomerase through a study that utilized immobilized L-arabinose isomerase of *E. coli*, bound covalently to agarose, to produce D-tagatose. The immobilized L-arabinose isomerase produced an average of 7.5 g-tagatose/L-day for 7 days with a productivity exceeding that of the free enzyme (0.47 vs. 0.30 mg/U-day) [42]. This group also studied immobilized L-arabinose isomerase using alginate beads crosslinked with glutaraldehyde, which produced an average of 30 g tagatose/day from 100 g galactose/L for 8 days [41]. A mesophilic D-arabinose isomerase from *Klebsiella pneumoniae* 40bXX has also been characterized [44]. A *K. pneumoniae* mutant, that constitutively expressed this D-arabinose isomerase, was produced through a series of sub-cultures grown on mineral salt medium with L-xylose as the sole source of carbon. The D-arabinose isomerase was active at 40^{o}C and achieved equilibrium ratios for D-arabinose and D-ribulose, L-fucose and L-fuculose, D-altrose and D-psicose, and L-galactose and L-tagatose of 90:10, 90:10, 13:87 and 25:75, respectively. In addition, It was shown that this D-arabinose isomerase could convert L-fructose to L-glucose at a 35% yield [45]. Recently, a D-arabinose isomerase gene was cloned from *Bacillus pallidus* and the resulting protein was crystallized to a resolution of 2.3Å [46]. It was noted that this D-arabinose isomerase has broad substrate specificity and reacted with D-arabinose, L-fucose, L-xylose, L-galactose and D-altrose.

L-Fucose Isomerase. L-Fucose Isomerase (FI), isolated from *E. coli*, was tested in conjunction with other enzymes for synthesizing L-fucose derivatives and analogs [5, 47,

48]. The enzyme catalyzes reversible ketol isomerization between sugars with stereo-specific C_2 and C_3 centers, but tolerates modifications at other centers, thus permitting the use of epidemic or decoy sugars [49]. FI, a homohexamer, required divalent ions (Mn^{2+} and Co^{2+}) for activity. Its structure suggests an ene-diol mechanism rather than a hydride shift. D-tagatose-3-epimerase from *Pseudomonas cichorii* ST-24 was combined with L-fucose isomerase from *Klebsiella pneumoniae* 40bXX to produce D-altrose from D-fructose at an overall yield of 6% [44]. This work was significant as a proof of concept of producing D-altrose from an inexpensive substrate, D-fructose.

D-tagatose-3-epimerase. D-tagatose-3-epimerases from *Pseudomonas cichorii* ST-24 [50, 51] and *Pseudomonas* sp. [52] have been characterized. Further efforts on D-tagatose-3-epimerase have focused on the conversion of L-sorbose and L-psicose to L-tagatose and L-fructose [53], D-fructose to D-psicose [52], and D-tagatose to D-sorbose [52]. Conversion of L-tagatose to L-sorbose L-tagatose and L-psicose produced yields of 20% and 65%, respectively. Immobilized D-tagatose-3-epimerase (chito pearl beads) produced D-psicose from D-fructose with a yield of 25%, compared to the unbound form of D-tagatose-3-epimerase that yielded 20%. This immobilized form of D-tagatose-3-epimerase also produced D-sorbose from D-tagatose with a yield of 70%. D-tagatose-3-epimerase from *Rhodobacter sphaeroides* converted D-fructose to D-psicose (17% conversion) [54]. This study compared the relative activities of the *P. cichorii* D-tagatose-3-epimerase and *Agrobacterium tumefaciens* D-psicose-3-epimerase with the D-tagatose-3-epimerase from *Rhodobacter sphaeroides* and showed the *R. sphaeroides* enzyme was more active on D-fructose at 40^{o}C; all of these enzymes required Mn^{2+} as a metal cofactor. In addition, *A. tumefaciens* D-psicose-3-epimerase produced D-psicose from D-fructose with a yield of

32.9% at 50^{o}C [55]. The addition of borate to *A. tumefaciens* D-psicose-3-epimerase increased the D-psicose yield from fructose with increasing molar ratios of borate: D-fructose up to 0.6 [56]. The shift in equilibrium related to borate complexes that form with the D-psicose product that exclude the D-psicose from being included in the equilibrium; this forces the equilibrium to favor additional D-psicose formation. Once a 0.6 molar ratio of borate to D-fructose is reached, borate complexes with D-fructose shift the equilibrium to form less product.

Multi-enzymatic reaction pathways for rare sugars. A combinatorial process was created to produce allitol from D-fructose by coupling reactions typically catalyzed by D-tagatose-3-epimerase, and ribitol dehydrogenase [51]. *Mycobacterium smegmatis*, in combination with *Acinetobacter* sp. strain DL-28 and *Acetobacter aceti* IFO 3281, produced L-ribose and L-arabinose from ribitol [57]. Three immobilized enzymes, D-xylose isomerase (*Saccharomyces cerevisiae*), D-tagatose-3-epimerase and D-arabinose isomerase (*Klebsiella pneumoniae* 40bXX), were used for the preparation of D-arabinose from D-xylose in a coupling reaction that produced 2.0 g of D-arabinose from 5 g of the substrate [58]. L-Glucose was produced from L-psicose using immobilized D-tagatose-3-epimerase and D-arabinose isomerase from the mutant strain *Klebsiella pneumoniae* 40bXX [45]. From this process, 0.35g L-glucose (as mentioned above) obtained from 1.0 g of L-fructose provided an overall yield of 35%.

Current bioprocessing technology for rare sugar synthesis has been developed exclusively with mesophilic enzymes. These processing systems inherit the fundamental flaws associated with mesophilic enzymes used for industrial processes including: lack of thermostability, limited half-lives under bioprocessing conditions, and limited reactor

operating periods. However, enzymes from hyperthermophilic microorganisms may be more suitable for rare sugar biosynthesis. In fact, high temperature enzymes have already been shown to be effective for single and multiple step rare sugar biosynthesis schemes: L-arabinose Isomerase for D-tagatose production [39, 40, 43, 59], and L-arabinose Isomerase for conversion of L-arabinose into L-ribulose [40].

MATERIALS AND METHODS

PCR amplification of genes encoding sugar isomerases and epimerases. Purified chromosomal DNA from *T. maritima* was used as a template for PCR. In order to amplify the L-rhamnose isomerase gene (-*rhI*), the following oligonucleotide primers were designed: Forward primer 5' - GCCGCCCATATGATAAACATGGAAAGGATTTT- 3'**,** Reverse primer 5' - GATGCCCTCGAGTCATCGTCTCTCTCTCCTTCT - 3'. In addition, the D-tagatose-3-epimerase gene (*tgE*) was amplified using the following oligonucleotide primers: Forward primer 5' - GCGATACATATGAAGCTATCTCTGGTGATCAGTACTTC – 3', and Reverse primer 5' – GCGATACTCGAGTGTAAGTTTAATAATCAGTT – 3'. The amplification reactions were performed in a 50-μl reaction volume containing PCR buffer, 0.25 mM concentration of each deoxynucleotide triphosphate, 2.5 mM $MgCl_2$, 100 pmol of each primer, and 1.25 U of PFU polymerase. The DNA sequences for the specific 1152 bp and 813 bp fragments were obtained for *rhI* and *tgE* genes, respectively.

Construction of pTMRI and pTMTE. The primers for the *rhI* and *tgE* genes were designed to incorporate an NdeI site in the forward primer and an XhoI site in the reverse primer. These gene fragments containing the complete coding sequence of *rhI* and *tgE* were

prepared by digesting the PCR product with restriction enzymes NdeI and XhoI. The pET-15b was also digested with the restriction enzymes and de-phosphorylated with 5 units of antarctic phosphatase (Promega). The resulting vector and PCR fragments were ligated together using a standard ligation reaction. The pTMRI and pTMTE plasmids were created from the ligation of the digested pET-15b vector and the digested genes *rhI* and *tgE*, respectively. The ligated fusion plasmids were transformed into XL1-Blue cells. The insert was confirmed by PCR amplification using the previously mentioned primers. The pTMRI and pTMTE plasmids were transformed into BL21 cells, which were transformed in a 2L standard LB broth at 37°C for 12 hours. TMRI and TMTE expression was induced by the addition of IPTG at a cell optical density of 0.7 (600 nm) and cell growth continued for 4 hours afterwards. The plasmid pTMAI was obtained from Diversa Corp., San Diego, CA. The L-arabinase gene (*araA*) from *Thermotoga maritima* was subcloned into the overexpression vector pSE420 (Invitrogen) by inserting a PCR amplified *araA*. The host vector was XL1 blue MRF' (Stratagene). The expression plasmid pTMAI was transformed into 500 ml LB (100 μg/ml ampicillin) and grown in a shaking bath at 30°C to an OD of ~1. IPTG was then added to 1 mM and growth was continued at 30°C overnight. PTMFI was obtained from Scott Lesley (Joint Center for Structural Genomics, La Jolla, CA). TMFI was sub-cloned into pMH1 vector with an N-terminal His tag with amp resistance. The TMFI plasmid (pTMFI) was transformed into an L-methionine auxotrophic *E. coli* strain D41. The expression plasmid pTMFI was transformed into 1L LB (100 mg/ml ampicillin) and grown in a shaking bath at 37°C to an OD of ~0.7. Expression was induced by the addition of 0.15% arabinose for 3 hr.

For all protein expression trials, cells were collected by centrifugation at 4^{o}C and re-suspended in 10 ml of 50 mM Mops with a pH of 7.0. The cells were then lysed by three

passes through a French press (Neose Technologies, Horsham, Pennsylvania) at 1100 psi. The lysate was then heated in a water bath at 65°C for 20 minutes. Precipitated proteins were centrifuged out at 5,000 g for 30 minutes, following which the supernatant was collected.

Purification of recombinant TMRI, TMAI, TMFI, and TMTE. TMRI, TMTE, TMFI, TNXI, and TMAI supernatants were purified on a FPLC chromatography system (Biorad) equipped with a DEAE column (GE Healthcare Life Sciences, Piscataway, New Jersey), using a mobile phase of 50mM Mops pH 7.0 and 50mM Mops pH 7.0/0.5 M sodium chloride. Column fractions were collected and tested for activity and protein concentration. The fractions containing the enzyme of interest were pooled and visualized on a 7% SDS-PAGE gel.

Enzyme Assay and Protein Determination. TMRI, TMAI, and TMFI activities were determined, as described in [33]. TMTE activity was measured using a Waters HPLC and a Waters 410 refractometer. The pH range for maximal enzyme activity was determined using acetic acid, citrate, sodium phosphate, and tris-HCl buffers, which spanned from pH 3.7 to 10.0. For the reaction, 450 μl of the master mix containing 100 μM of the buffer, 1 mM of Magnesium chloride, and 100 mM of the monosaccharide of interest combined with 50 μl of the protein solution and underwent incubation an 80^{o}C. Once the buffer that conferred the maximum activity was identified, then optimum temperature was determined. Reactions were performed at temperatures ranging from 45°C to 100°C. The metal ion effect on enzyme activity was measured at optimum pH and temperature, using one of the following cations, $MgCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$, $CaCl_2$, $ZnSO_4$, or $BaCl_2$. The enzyme was dialyzed in 2

mM EDTA in sodium phosphate buffer at pH 7.0 to remove any pre-existing metal ions. The metal ions were added afterwards. Sugar isomerase activity was measured for all of the reaction, as described elsewhere [60]. All of the reactions were initiated by placing the samples in a PCR machine at a constant temperature for a specific amount of time, determined by the dilution of the protein concentration. This solution was combined with a freshly prepared 1:1 mixture (v/v) of a solution of 0.05% resorcinol in ethanol and a solution of 0.216 g $FeNH_4(SO_4)_2 \cdot 12 \cdot H_2O$ in 1L of concentrated HCL. Color development indicated the presence of ketose, which was measured at 419 nm. One unit of isomerase activity was defined as the amount of enzyme that produced 1 µmol of product per min from the substrate under the assay conditions. Protein concentration was determined by the Bio-Rad protein assay kit using a standard protocol [61]. Samples (0.2 ml) were taken from the reactions and visualized by HPLC and confirmed, as needed, by H^1 NMR. For epimerase activity, the reaction products were determined using HPLC. The amount sugar product (µmol/µl) was determined through use of a calibration curve that related the peak area to the sugar concentration (using sugar standards). One unit of epimerase activity was defined as the amount of enzyme that produced 1 µmol of product per min from the substrate.

Rare Sugar Detection and Quantification. Substrates and products of carbohydrate conversions were initially visualized by HPLC using sugar standards. Prior to analysis, proteins were removed by heat treatment and ultra-centrifugation. Products were analyzed on a Waters 2690 HPLC equipped with a Shodex SP0810 sugar analysis column and a refractive index detector. The SP0810 is an ion-exchange column that uses sulfonated styrene divinylbenzene resin with Pb^{2+} cations to resolve monosaccharides and sugar alcohols by hydroxyl coordination to the metal ion. This column (Waters Inc., Reno, Nev)

was linked to a refractive index detector (Model 156; Beckman, Frankfurt, Germany), and water was used as the mobile phase at a column temperature of 80°C and a flow rate of 1.00 ml/min. H^1 NMR was used as needed for product identification. Fractions were collected for the peaks observed on the HPLC and evaporated until a dry crystal residue remained. The sample was re-solubilized in D_2O to an appropriate concentration (5-10 mg/ml). The NMR spectra were recorded on a Varian Mercury 300 NMR at 300 MHz for H^1 at 300°K. Chemical shifts were referenced to the standard H^1 spectrum of the Sigma Aldrich Library of ^{13}C and ^1H FT-NMR Spectra [62].

Effect of Temperature on Product Formation. The effect of temperature on product formation and yield was tested by running parallel reactions for the conversion of D-fructose to D-allose, utilizing the TMTE and TMRI. The Pierce ReactiTherm Heating/Stirring Module was used for 90°C, the Hybaid Incubator was used for 60°C, and the Fisher Scientific Isotemp Incubator was used for 37°C incubations. A reaction buffer of 50 mM Mops pH 7.0, 500 mM D-Fructose, 5 mM $MnCl_2$ and 3 mM $CoCl_2$ was passed through a Millex GP 0.22 μm syringe filter to sterilization the solution prior to use. An aliquot of 50 μg/ml of total enzyme concentration of TMRI and TMTE was added to begin the reaction. A reaction time of 48 hours was typically used for a 2 ml sample. The Amincon micron centrifugal filtration devices (Millipore, Billerica, Massachusetts) were prepared for use by first removing the glycerol residue in the filter (by applying 500 ml of 0.1 M NaOH and three washes with 500 ml of DiH2O). The reaction product was filtered from the enzymes by applying the solution to the Amincon micron centrifugal filtration device (10kDa). The filtrate was collected and run on a Waters 2690 HPLC with a SP0810 Shodex sugar column at 80°C to separate the individual products. Dosing in the expected sugar standard into the sample and analyzing

with the Waters 2690 HPLC assisted in identifying reaction products. The resulting chromatographs were compared and the peaks that increased in size due to the dosing allowed for the identification of the sugar.

Single and Multi-step Rare Sugar Biotransformations. Reactions were performed in a 2 ml glass vial, sealed with a Teflon rubber stopper. All reactions were initiated by inserting the vial containing the enzyme(s) and substrate of interest into the Pierce ReactiTherm Heating/Stirring Module set at a specific temperature.

TMTE conversion of D-fructose. To a reaction buffer of 50 mM Mops pH 7.0, 500 mM D-fructose, 5 mM $MnCl_2$ and 3 mM $CoCl_2$, 227 μg/ml of total enzyme concentration of TMTE was added and the reaction was run for 12 hours at 80°C.

TMRI conversion of D-psicose. To a reaction buffer of 50 mM Mops pH 7.0, 100 mM D-psicose, 5mM $MgCl_2$, 50 μg/ml of TMRI was added to the reaction solution and the reaction was run for 12 hours at 80°C.

TMTE conversion of D-tagatose to D-sorbose. To a reaction buffer of 50 mM Mops pH 7.0, 500 mM D-tagatose, and 5 $MnCl_2$, 227 μg/ml of TMTE was added to the reaction solution and the reaction was run for 12 hours at 80°C.

TMTE and TMRI conversion of D-fructose. To a reaction buffer containing 50 mM Mops pH 7.0, 500 mM D-Fructose, 5 mM $MgCl_2$, 50 μg/ml of total enzyme concentration of TMTE and TMRI were added and the reaction was run for 120 hours at 60°C

TMTE and TMFI conversion of D-fructose. To a reaction solution containing 50 mM Mops pH 7.0, 500 mM D-Fructose, 5 mM $MnCl_2$, 50 μg/ml of total enzyme concentration of TMTE and TMRI were added and the reaction was run for 48 hours at 80°C for the first attempt and 120 hours at 60°C for the second attempt.

TNXI and TMTE conversion of D-glucose. To a reaction buffer containing 50 mM Mops pH 7.0, 500 mM D-Glucose, 5 mM $MnCl_2$, and 3 mM $CoCl_2$, 50 μg/ml of total enzyme concentration of TMTE and TMXI were added and the reaction was run for 120 hours at 60°C.

TNXI and TMTE conversion of L-sorbose. To a reaction buffer containing 50 mM Mops pH 7.0, 100 mM L-sorbose, 5mM $MnCl_2$, and 3 mM $CoCl_2$, 50 μg/ml of total enzyme concentration of TMTE and TMXI were added and the reaction was run for 120 hours at 60°C.

TMRI and TMTE conversion of L-sorbose. To a reaction buffer containing 50 mM Mops pH 7.0, 100 mM L-sorbose, 5mM $MnCl_2$, and 5 mM $MgCl_2$, 50 μg/ml of total enzyme concentration of TMTE and TMXI were added and the reaction was run for 120 hours at 60°C.

TMAI and TMTE conversion of D-galactose. A reaction buffer of 50 mM Mops pH 7.0, 100 mM D-galactose, 5mM $MgCl_2$, and 5 mM $MnCl_2$, 50 μg/ml of total enzyme concentration of TMTE and TMXI were added and the reaction was run for 120 hours at 60°C.

RESULTS

Thermotoga species have the ability to grow on wide variety of carbohydrates, including several monosaccharides [63], which can trigger transcription of genes encoding sugar isomerases and epimerases. *T. maritima*'s genome encodes L-arabinose isomerase, AraA (TM0276), L-fucose isomerase, FucI (TM0307), D-xylose isomerase, XI (TM1667), in addition to several putative sugar isomerases (e.g., TM0283 and TM1071). Previous work had shown that the operon containing TM1071 was responsive to L-rhamnose as a growth substrate (Figure 3.2; [63]). Upon further analysis, TM1071 appeared to be an L-rhamnose isomerase; at the amino acid level, the putative protein is 55% identical to *Bacillus licheniformis ATCC 14580* L-rhamnose isomerase and 52% identical to a *Streptomyces avermitilis* MA-4680 putative sugar isomerase. Figure 3.2 shows the proposed pathway for rhamnose uptake and utilization by *T. maritima*. In bacteria, rhamnose plays a common structural role in cell wall synthesis and maintenance, but is capable of being an efficient energy source as seemed to be the case for *T. maritima* (Conners et al., 2005).

TMRI. L-rhamnose isomerase from *T. maritima* (TMRI) was cloned and expressed as a 42 kDa monomer, as shown in the Figure 3.3. The optimal pH of TMRI is 7.3 (range of 3.7-10.0), with a temperature optimum of 95°C (range of 45°C to 100°C). The kinetic parameters for TMRI on rhamnose (Figure 3.4A) were K_m = 28.0 ± 0.9 mM and V_{max} = 494.0 ± 4.2 U/mg, which are comparable to and higher than, respectively, the same parameters reported for the *P. stutzeri* RI [64].

TMRI activity was impacted by covalent cations, as shown in Table 3.2, with Mg^{2+} conferring the highest activity followed by (ranked highest to lowest) Ca^{2+}, Ni^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+}. The apo- form of the enzyme had a nominal activity of 5.4% of the maximum. The effect of temperature on the equilibrium of D-psicose and D-allose upon conversion by TMRI was also determined. Over 40-70°C, a linear relationship was observed, favoring D-allose with increasing temperature such that the yield at 70°C was 15% higher than at 40°C. TMRI converts L-rhamnose to L-rhamnulose at 80°C with a yield of ~40%, whereas TMRI converts D-psicose to D-allose at 80°C with a yield of ~13% ratio at this temperature (Figures 3.5 and 3.6, respectively).

TMFI. In *T. maritima*, L-fucose isomerase (TMFI) anchors pathways that synthesize and utilize D-fucose and L-fucose to maintain cellular processes. TMFI was expressed in *E. coli* as an apparent homotetramer based on a subunit M_r of 54 kDa (Figure 3.7)). TMFI (pH_{opt} = 8.0, T_{opt} = 85°C) had a K_m and V_{max} of 6.0 ± 0.2 mM and 612.7 ± 21.2 U/mg, respectively (Figure 3.8). Figure 3.5 and 3.6 illustrate TMFI's ability to convert natural and unnatural substrates, respectively. TMFI conversion of L-fucose, the natural substrate of TMFI, has a low yield, and several unknown by-products were noted. In contrast, TMFI conversion of D-psicose to D-altrose (54:50 ratio) generated only D-altrose with no by-products.

TMTE. D-Tagatose-3-epimerase (TMTE) is involved in *myo*-inositol degradation during exopolysaccharide production in *T. maritima*. In the *T. maritima* genome sequence, TM0416 is classified as a "putative D-tagatose-3-epimerase-like protein", with significant homology to D-tagatose-3-epimerase from *P. cichorii* and D-psicose-3-epimerase from *A. tumefaciens*. The recently reported three-dimensional structure of TMTE revealed the unique nature of

the active site of this hyperthermophilic enzyme. Upon comparison with the epimerase homologs, clear topological differences between TMTE and the other two enzymes exist [65]. The α 8'helix that covers the active site clef in *P. cichorii* D-tagatose-3-epimerase and *A. tumefaciens* D-psicose-3-epimerase is absent in TMTE. Accordingly, the substrate-binding pocket of TMTE appears to be uniquely solvent-accessible, such that substrate specificity could be dramatically altered. Here, TMTE function was found to differ significantly from that reported for the mesophilic homologs from *P. cichorii* and *A. tumefaciens*. This was evident from determination of TMTE kinetics on D-tagatose, the enzyme's presumed natural substrate. The K_m and V_{max} for D-tagatose was determined to be 7.9 ± 0.8 mM and 192.2 ± 18.1 U/mg, respectively (Figure 3.9), based on the disappearance of the substrate. However, the product formed by this reaction was not just D-sorbose, as shown by the multiple peaks observed in Figure 3.5. Reports on mesophilic D-tagatose-3-epimerase homologs show a single peak of D-sorbose as product of the reaction with D-tagatose. To further examine the TMTE, the conversion of D-glucose to D-psicose was attempted using TMRI and TMTE at 80°C for 12 hours. While a small amount of D-fructose was formed, no D-psicose was noted and significant amounts of other unknown sugar products were observed.

Multiple step reactions. First efforts multiple step reaction (D-glucose to D-psicose using TMRI and TMTE) were unsuccessful, yielding no product beyond the first intermediate product. In retrospect, this result was not unexpected because of the low concentration of metal cofactor used and the evidence of a "Maillard reaction" was occurring (solution discoloration was observed). Maillard reactions have been cited as a cause of inactivation of sugar-modifying enzymes [66]. In addition, the initial reaction time was much too short (1

hour). The concentration of the metal cofactors (5 mM $MgCl_2$, 5 mM $MnCl_2$, 3 mM $CoCl_2$) was subsequently increased and the reaction time was extended but at a lower temperature or a lower pH to avoid Maillard reactions. But before these changes were made, the best course of action was to optimize the production of one reaction through the use of varying temperatures and different reaction configurations. First, the right reaction needed to be identified as a test case and for this reaction the conversion of D-fructose to D-psicose was used.

In *T. maritima* and other bacteria, D-fructose forms D-psicose as a metabolic intermediate via D-tagatose-3-epimerase, before being converted to D-allose via an isomerization reaction catalyzed by L-rhamnose isomerase. The conversion of D-fructose to D-psicose by TMRI and TMTE was examined. The reaction was conducted at 80°C for 48 hours. Figure 3.10 shows that D-fructose was not converted by TMRI. However, the single step reaction of D-fructose with TMTE generates several side products. As mentioned above, the structure of TMTE, compared to the D-tagatose-3-epimerase homologs, reveals that the active site is considerably different, suggesting that the natural substrate of TMTE may not be tagatose. Furthermore, a minimum of ~28 mM D-psicose is needed for TMRI to produce D-allose. D-allose could not be resolved with the chromatography column used since it co-elutes with fructose. However, based on the low yields for D-psicose, it is unlikely that much D-allose is formed.

An alternative approach was used to generate rare sugar products from multi-enzyme transformations. Single step reactions were carried out initially, to which the second enzyme was added. For the production of D-allose from D-fructose using TMRI and TMTE, this configuration produced the highest yields of D-psicose and D-allose. Figure 3.11 shows the formation of D-psicose and Figure 3.12 shows the formation of D-psicose and D-allose.

D-psicose and D-allose production also showed an improvement with the reaction temperature being set at 90^{o}C. Therefore, maillard reactions must not adversly effect the enzymes used in this process (TMRI and TMTE) and the lack conversion was solely based on the lack of D-psicose produced. Once D-psicose was optimally produced, then D-allose production commenced. Other multiple step reactions were attempted such as: D-tagatose conversion to D-gulose via TMTE and TMRI, D-fructose conversion to D-altrose via TMTE and TMFI, D-galactose conversion to D-sorbose via TMTE and TMAI, and L-sorbose to L-galactose conversion via TMTE/TMRI and TMTE/TNXI. These reactions (not shown) were conducted at a lower temperature (60°C) and for a longer period of time (120 hours). Unlike the conversion of D-fructose to D-allose at 90°C, these reactions made either completely different products or final product that could not be identified. Therefore temperature proved to be an effect parameter to influence of regioselectivity for our rare sugar products.

DISCUSSION

Two of the Thermotoga enzymes (TMTE and TMRI) studied here clearly have different biochemical and biophysical properties than their mesophilic homologs, leading to unexpected results for the in vitro single and multiple step reactions studied here.

TMTE substrate specificity. *P. cichorii* D-tagatose-3-epimerase and *A. tumefaciens* D-psicose-3-epimerase were shown to convert D-fructose to D-psicose (with 6% and 32.5% yields, respectively) and D-tagatose to D-sorbose (with 70% yield for the *P. cichorii* enzyme; not reported for the *A. tumefaciens* enzyme). Both D-tagatose-3-epimerase and D-psicose-3-epimerases from *P. cichorii* and *A. tumefaciens* thus convert the *cis* configuration ketoses at carbons 3 and 4 (D-psicose and D-tagatose, D-ribulose) to their carbon 3 epimers, *trans*

ketoses (D-fructose, D-sorbose, and D-xylulose). For the two epimerase from *P. cichorii* and *A. tumefaciens*, the key catalytic residues are conserved (Trp13/Trp113/Phe248 and Trp15/Trp112/Phe246 for *P. cichorii* and *A. tumefaciens* epimerases, respectively). The hydrophobic binding pocket is less conserved (Tyr6/Gly65/Ala107 to Phe7/Cys66/Leu108 for *P. cichorii* and *A. tumefaciens* epimerases), which leads to a similar catalytic mechanism but with different substrate specificity. In the case of TMTE (TM0416), the key catalytic residues and the hydrophobic pocket residue are not conserved with respect to either of the previously mentioned epimerases (catalytic residues (---/Leu113/Leu245 and hydrophobic binding pocket residues-Val16/Gly66/Gly112). Thus, TMTE appears to have a substrate specificity that is different from the other epimerases. Additional work is needed to determine the physiological substrate of this enzyme.

TMRI active site. A multiple sequence alignment was doen for the L-rhamnose isomerases from *T. maritima, B. stearothermophilus, and P. stutzeri* (Figure 3.13). BSRI is the only previously known L-rhamnose isomerase which functions at elevated temperatures [68], with a maximum temperature of 80^{o}C and half-life of 1 hour at 50°C. PSRI was found to have a wide substrate specificity attributed to its altered binding pocket when compared to other rhamnose isomerases. The key roles of residues within the catalytic pocket for PSRI have been identified [35, 36]. The substrate-binding site consists of residues Glu219, Asp254, His281, Asp327, His257, and Asp289. These residues also have structural and mechanistic roles. Glu219 forms a hydrogen bond with the O2 of the L-rhamnose to properly orient the substrate for catalysis. Asp254 forms a hydrogen bond with the catalytic W5 to assist in the hydride shift mechanism. Finally, Asp327 along with the peripheral residue His101 forms hydrogen with the O4 and O5 of the substrate respectively to help

recognize –OH groups and confer substrate specificity at the 4 and 5 positions of the substrate. The other two peripheral residues, Lys221and Asp281 form hydrogen bonds with the O1 for proper substrate configuration and with W4 to assist in the hydride shift mechanism, respectively. In comparison to TMRI, all of the substrate binding and peripheral residues are conserved, except for the Asp289 counterpart, which is an Ala248. This variation impacts the hydrophobic environment, and opening up the possibility that a wider variety of substrates could be processed, compared to PSRI. BSRI substrate binding and peripheral residues are all conserved in TMRI, except for the Asp327 and Asp289 counterparts, which are deleted for the Asp327 residue and is Trp292 instead of Asp289. The aspect of TMRI specificity toward D-glucose is more due to the altered hydrophobic binding pocket identified from this comparison. The mutations within the binding pocket make the pocket wider allowing for less sterically restrictive substrates to enter. Coupling that fact with the predicted dual role TMRI determined annotatively from microarray as sharing the same reaction pathways as TMXI, then the noted enhanced ability toward D-glucose comes at no surprise.

Summary. Here we report information on three previously uncharacterized sugar modifying enzymes from *T. maritima* - TMRI, TMTE, and TMFI. These enzymes were found to catalyze reactions with their natural and unnatural substrate to different extents and at different rates. Multiple step reactions, all of which utilized TMTE, were unsuccessful because of low metal cofactor concentrations, possible Maillard reactions at high temperatures, and short reaction times. At 90^{o}C and after 48 hours D-psicose and D-allose production from D-fructose was noted with TMRI and TMTE; however, each of these enzymes have different structural (and hence biocatalytic) features than their mesophilic counterparts so that this reaction

sequence may not be optimal. Much more needs to be done to determine the best opportunities for high temperature enzymes for rare sugar synthesis, but the capacity of these enzymes to function over extended periods of time need for the conversion of unnatural sugar substrates is an attractive characteristic.

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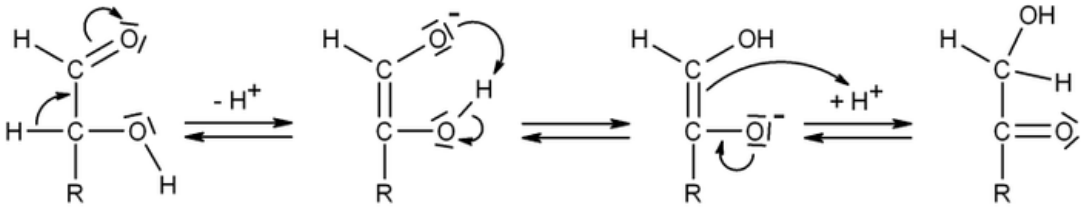
Table 3.1- A listing of interesting rare sugars, their applications, the cost of their cheapest starting substrate, and market value of the sugar.

[A]Prices were obtained through the Sigma Aldrich website.

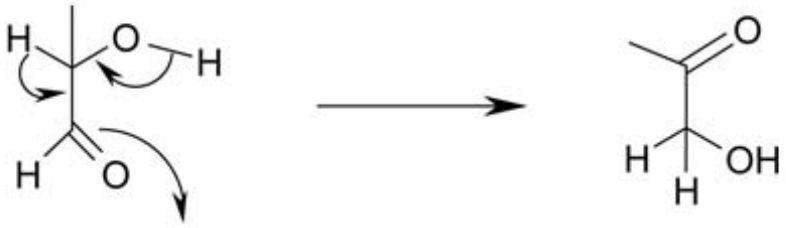
Conversion	Applications	Cost of Sugar ($/g)	Value of Product ($/g)
D-tagatose to D-gulose	Drug formulation agents	0.64	9320
galactitol to L-glucose	Starting material for glycoconjugate vaccines against dysentery	0.64	112
galactitol to D-tagatose	Pharmaceutical formulations	0.64	83
xylitol to D-lyxose	Production of anti-tumoral agents; cancer therapy	0.11	6
xylitol to L-ribose	Pharmaceutical applications	0.11	218
xylitol to L-xylulose	Potential inhibitors of various glycosidases	0.11	N/A
xylito to L-xylose	Antiviral agents such as nucleoside analogues	0.11	8

Table 3.2. Effect of metal ion on the activity of *Thermotoga maritima* RI

Metal Ion Effect	(Dialyzed in 1mM EDTA)
Mg	100.00%
Mn	36.46%
Ni	45.32%
Co	33.96%
Ca	47.86%
Zn	32.43%
EDTA	5.37%
Ba	40.02%



A. Aldose Endiol (deprotonierte Form) Ketose



B. Hydride Transfer Mechansim

Figure 3.1. Representative reaction mechanisms:

A. Lobry de Bruyn-Alberda van Ekenstein re-arrangement

B. 1,2 hydride shift.

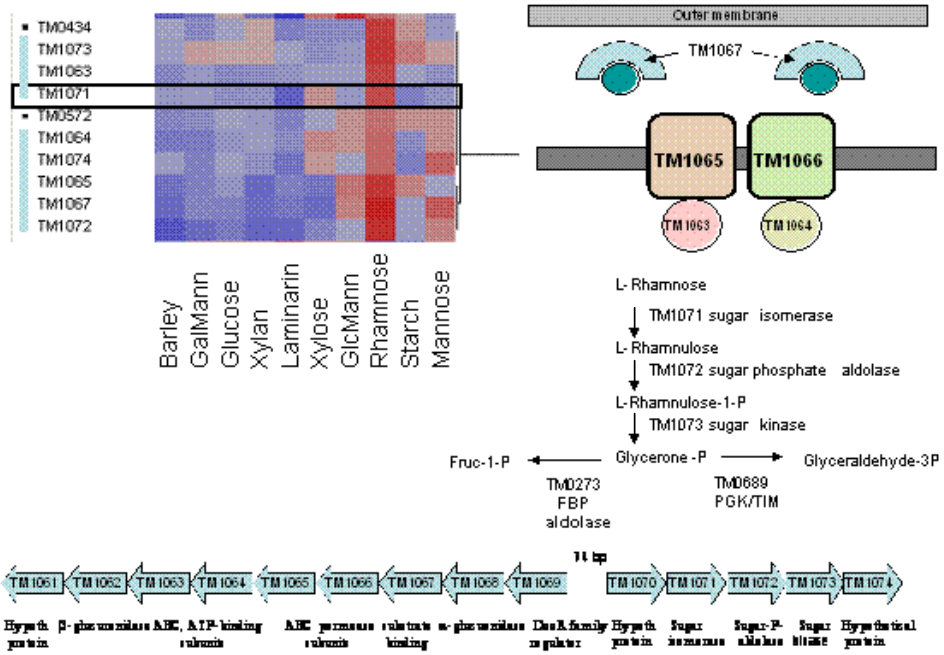


Figure 3.2. Functional genomics-based identification pathways for L-rhamnose utilization by *T. maritima* on L-rhamnose [63]. Heat plot shows up-regulation of genes on L-rhamnose; TM1071 corresponds to the L-rhamnose isomerase studied here.

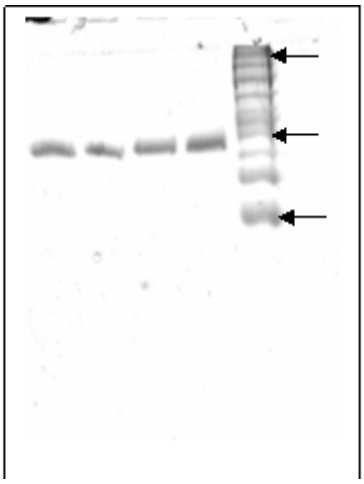
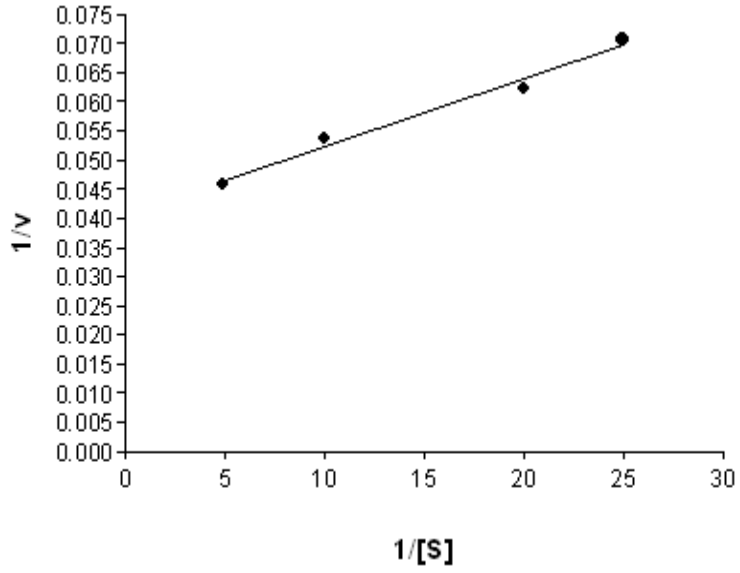


Figure 3.3. SDS page gel (7%) of TMRI. Benchmark protein ladder (far right) and TMRI (lanes 1-4). M_r of TMRI was determined to 42 kDa, consistent with amino acid sequence. The arrow from the top to the bottom are 100 kDa, 50 kDa, and 20 kDa.

A



B

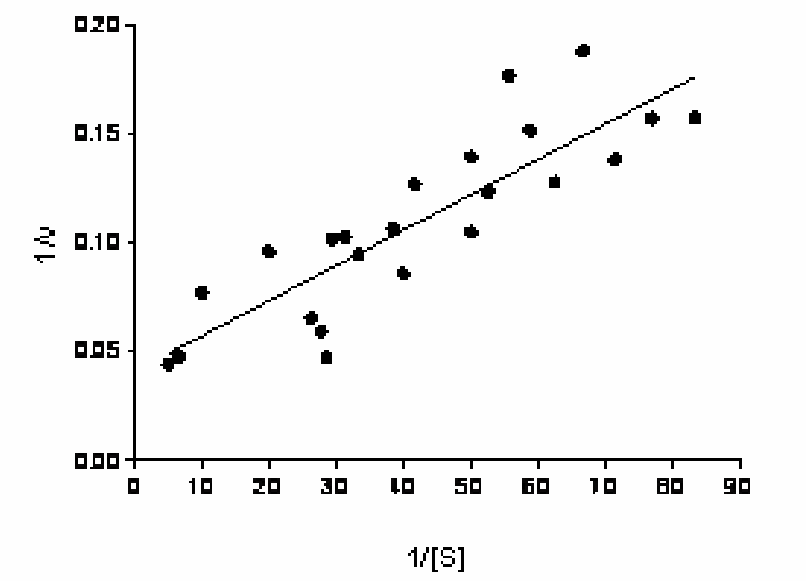


Figure 3.4. TMRI Michaelis-Menten kinetics determined at 80°C on L-rhamnose and D-glucose. **A.** TMRI had a K_m and V_{max} of 28.0 ± 0.9 mM and 494.0 ± 4.2 U/mg, respectively, on L-rhamnose. **B.** TMRI had a K_m and V_{max} of 26.2 ± 15.3 mM and 242.1 ± 13.4 U/mg on D-glucose.

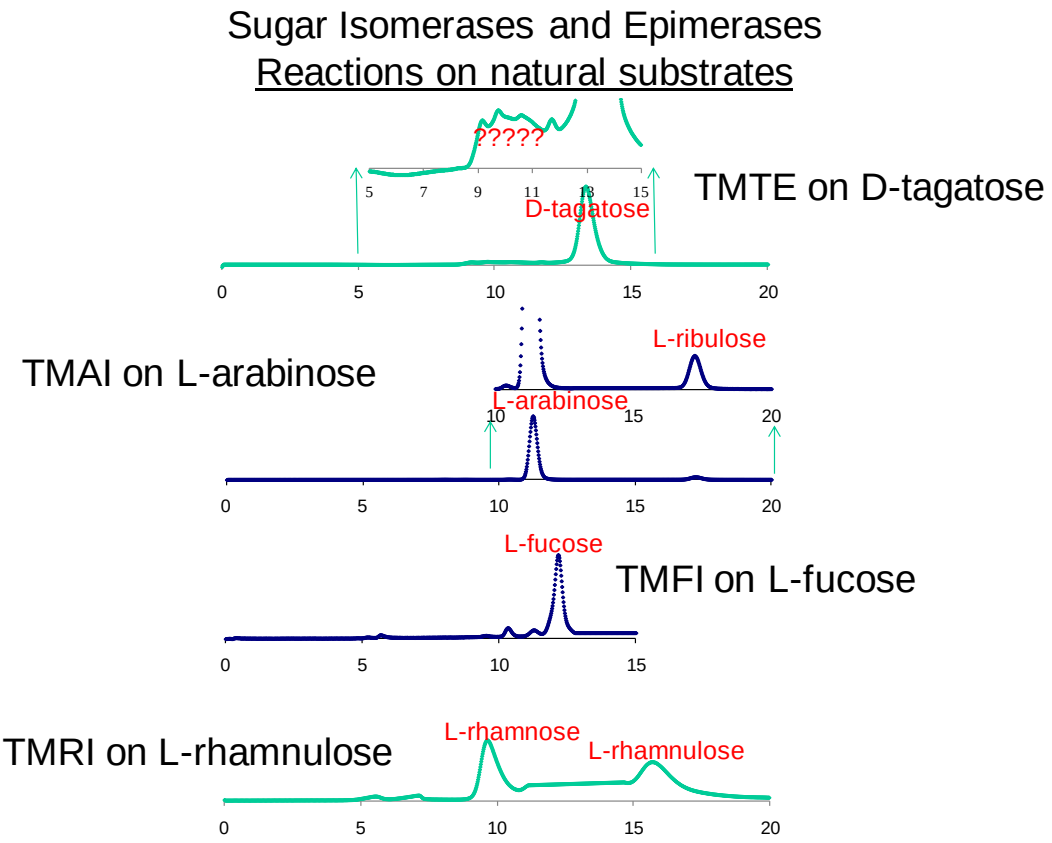


Figure 3.5. Thermotoga sugar isomerases and epimerases reactions on natural substrates: TMTE (D-tagatose), TMFI (L-fucose), TMRI (L-rhamnose), and TMAI (L-arabinose).

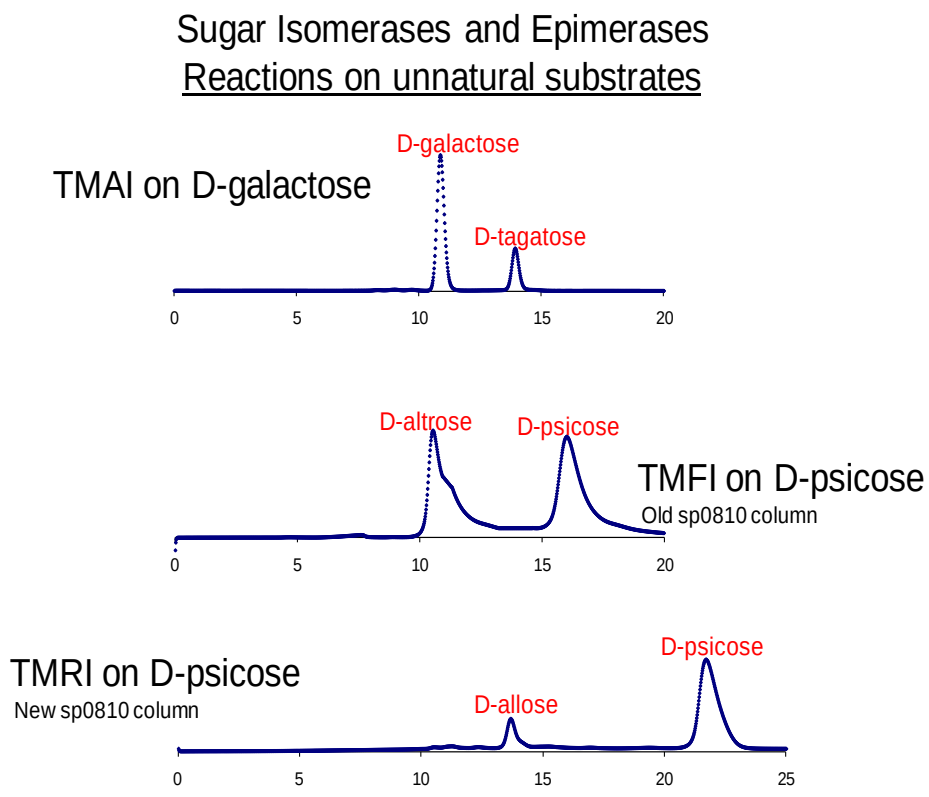


Figure 3.6. Thermotoga Sugar Isomerases and Epimerase Reactions on unnatural substrates: TMRI (D-psicose), TMAI (D-galactose), and TMFI (D-psicose).

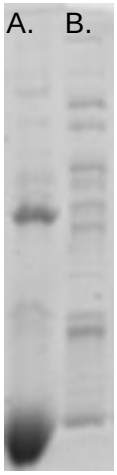


Figure 3.7. SDS page gel (7%) of TMFI. A. TMFI and B. Benchmark protein ladder. M_r of TMFI was determined to be 54 kDa, consistent with amino acid sequence.

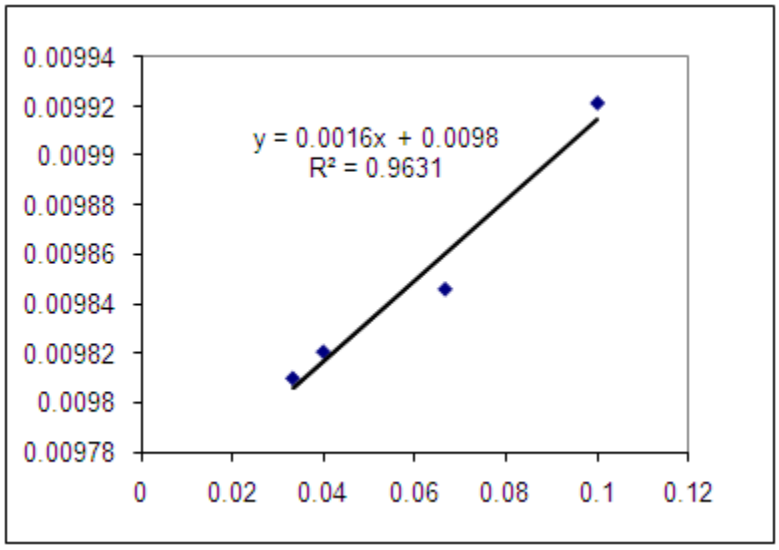


Figure 3.8. TMFI Michaelis-Menten kinetics determined at 80°C on L-fucose to L-fuculose. TMFI had a K_m and V_{max} of 6.0 ± 0.2 mM and 612.7 ± 21.2 U/mg, respectively, on L-fucose.

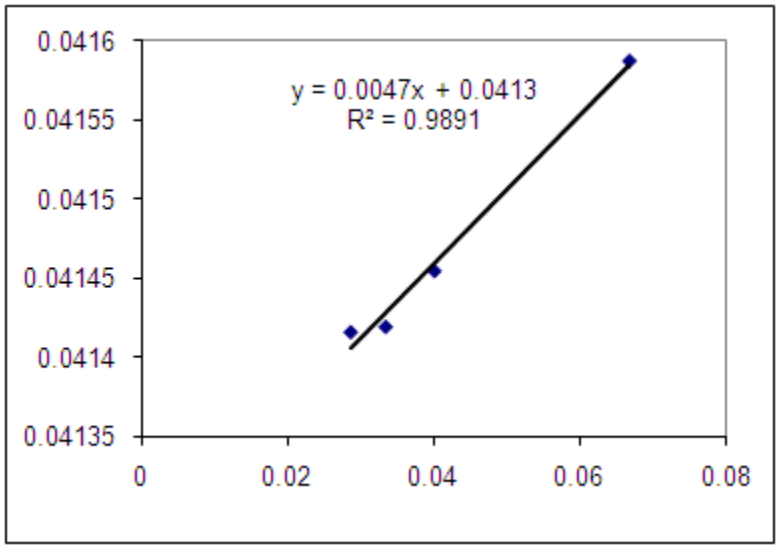


Figure 3.9. TMTE Michaelis-Menten kinetics determined at 80°C by L-tagatose disappearance. TMTE had a K_m and V_{max} of 7.9 ± 0.8 mM and 192.2 ± 18.1 U/mg, respectively, on D-tagatose.

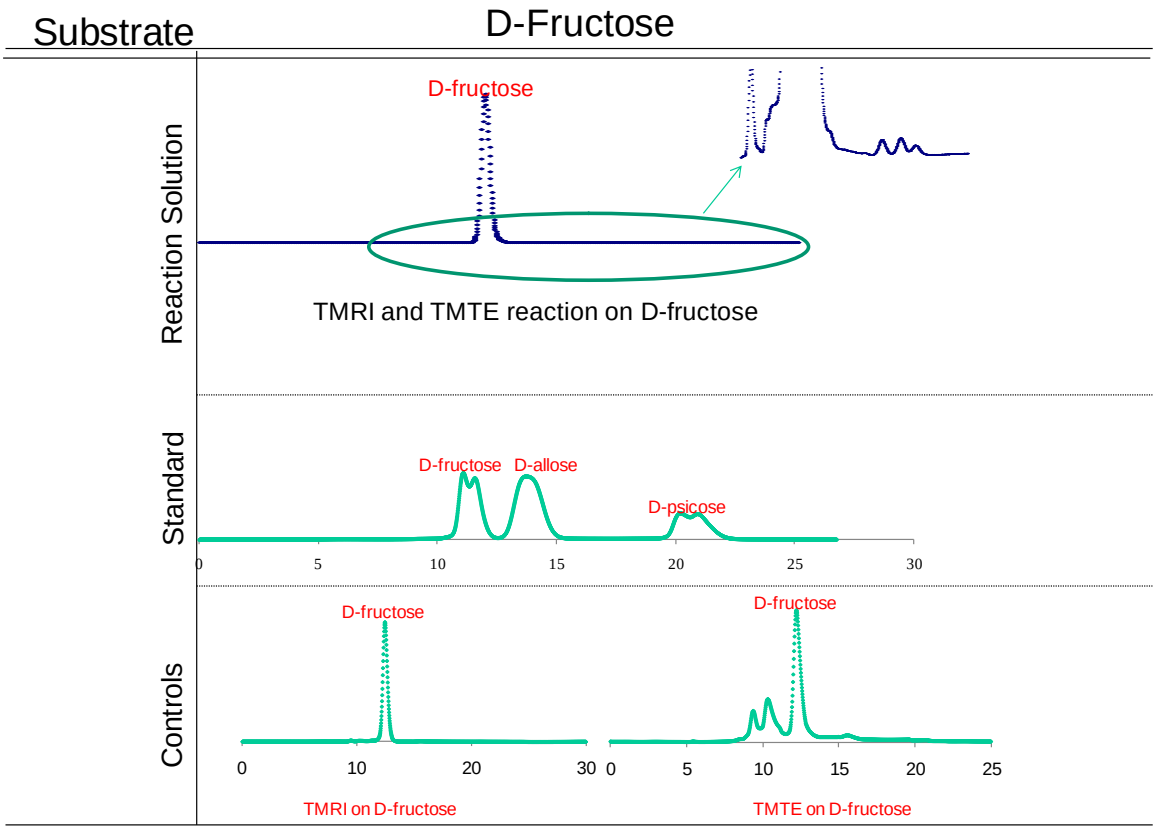


Figure 3.10. TMRI and TMTE conversion of D-fructose to D-allose. The multiple step reaction of TMRI and TMTE's conversion of D-fructose to D-allose (12 hours at 80^{o}C), the standard of a mixture of D-fructose, D-allose and D-psicose, and single step reactions of TMRI and TMTE on D-fructose is shown.

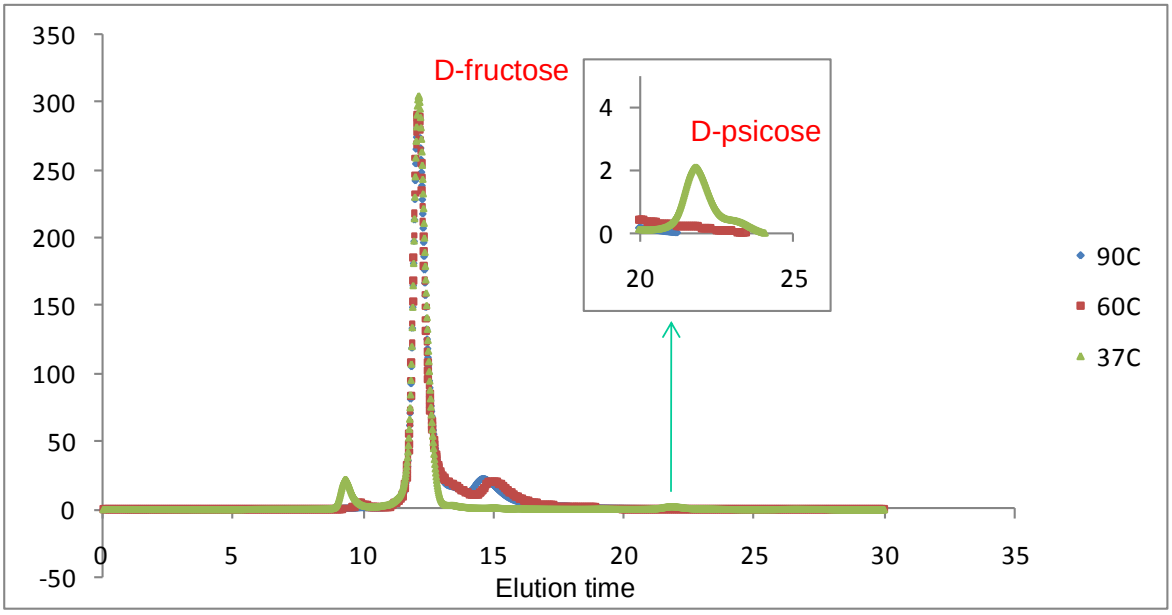


Figure 3.11. Optimization of TMTE and TMRI conversion of D-fructose varying temperature and reaction configuration. The temperatures used in this experiment was 37°C, 60°C, and 90°C. The enzymes were added in the same reaction for these results, the other condition (data not shown) had the enzymes added in separate reactions.

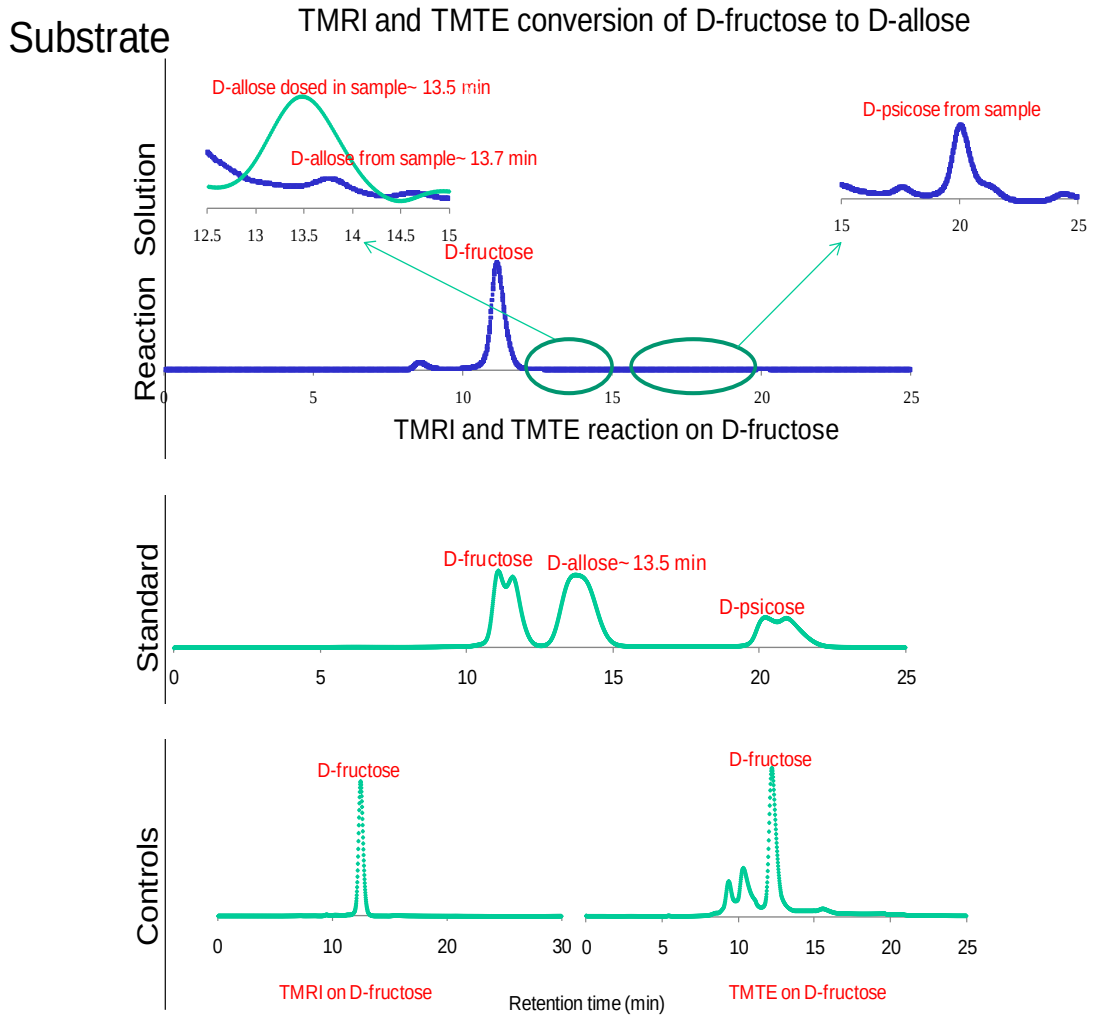


Figure 3.12. TMRI and TMTE conversion of D-fructose to D-allose (48 hours at 90°C). Mixture of sugar standards D-fructose, D-allose and D-psicose, and single step reactions of TMRI and TMTE on D-fructose are shown. D-allose (retention time of 13.5 to 13.7 min) was identified by adding D-allose (5 mM) to the multiple step reaction solution.

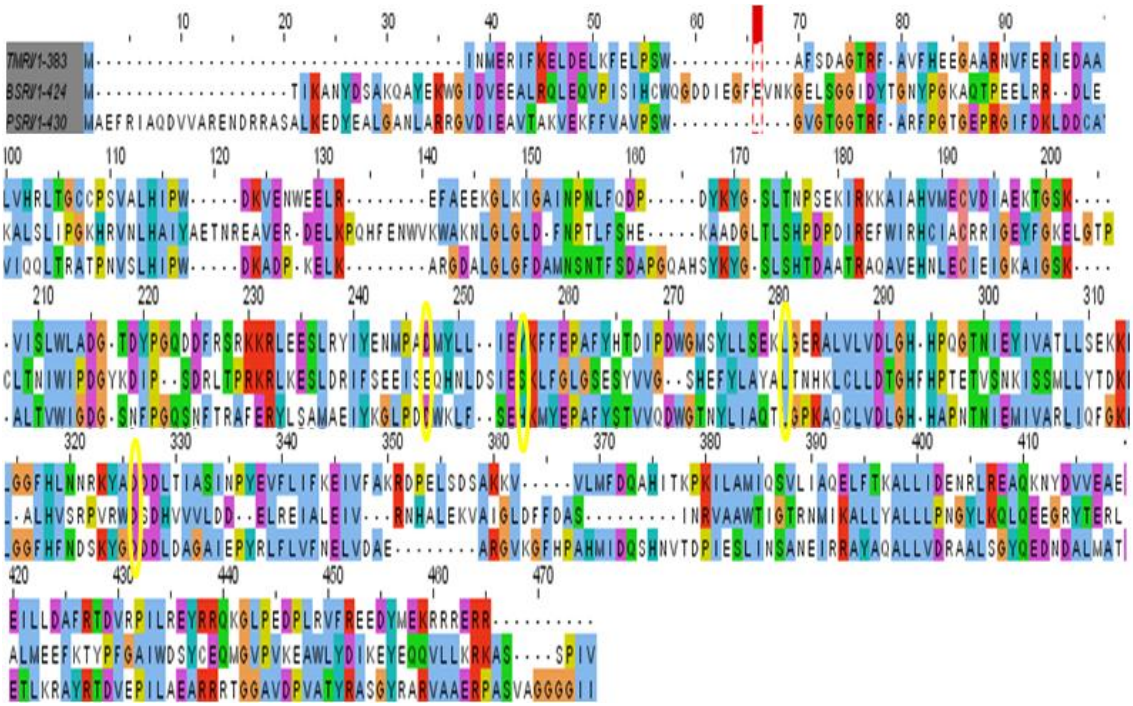


Figure 3.13 Multiple sequence alignment of *T. maritima*, *B. stearothermophilus*, and *P. stutzeri* L-rhamnose isomerase (TMRI, BSRI, and PSRI respectively).