This is to certify that the

dissertation entitled

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION

OF GLUCOSE ISOMERASE FROM THERMOANAEROBES

presented by

Chanyong LEE

has been accepted towards fulfillment
of the requirements for

ph.D. degree in Biochemistry

J. Gregory Zeikus
Major professor

Date October 31, 1989

DATE DUE	DATE DUE	DATE DUE

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF GLUCOSE ISOMERASE FROM THERMOANAEROBES

By

Chanyong Lee

A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1989

ABSTRACT

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF GLUCOSE ISOMERASE FROM THERMOANAEROBES

By

Chanyong Lee

Biochemical and molecular structural properties of glucose isomerase were investigated in *Clostridium thermosulfurogenes* and *Thermoanaerobacter* strain B6A. Both organisms produced intracellular thermostable glucose isomerase, and synthesis of the enzyme was induced by xylose. In addition, *Thermoanaerobacter* produced intracellular glucogenic amylase and ß-galactosidase activities constitutively, which were environmentally compatible with glucose isomerase activity (i.e. active at the same pH and temperature). The xylose grown *Thermoanaerobacter* cells produced these three enzymes simultaneously, and converted starch or lactose directly into a fructose syrup mixture in a single step process. Glucose isomerases were purified to homogeneity from *C. thermosulfurogenes* and *Thermoanaerobacter*, and both enzymes displayed very similar physicochemical and enzymatic properties. Long and flat crystals were formed from glucose isomerase purified from *C. thermosulfurogenes*. The gene (*xyl*A) encoding thermostable glucose isomerase of *C. thermosulfurogenes* was cloned and expressed constitutively in both *Escherichia coli* and *Bacillus subtilis*. The recombinant glucose isomerase produced in these mesophilic hosts retained the

To

My parents, wife, and sons

with love

ACKNOWLEDGMENT

I have been extremely fortunate to meet the right people at the right place and time that have made the completion of this dissertation possible. I would like to thank all of those and only a few are named here due to the limited space.

I would like to express my sincere appreciation to Professor J. Gregory Zeikus, my scientific advisor, philosophical mentor, and competitive racket ball partner, for his guidance, encouragement, and patience through my graduate school years. I would also like to thank all of the members of my guidance committee, Drs. John Wilson, Arnold Revzin, Robert Hausinger, and Michael Bagdasarian, who taught me molecular genetic techniques, for their advice and invaluable time.

I would like to acknowledge the support of my co-workers and scientists who shared techniques, ideas, and friendship; Drs. R. Lamed, S. Lowe, B. Saha, V. Morales, Mr. J. Kemner, S. Mathupala, Y. Lee, W. Wu, M. Meng, and former and all present lab members from the time in Madison, Wisconsin till now.

I would like to thank Michigan Biotechnology Institute for providing me with nice facilities, scientific equipment, and financial support.

Finally, I am most grateful to my parents, brother, and sister for their dedication and encouragement, and to Eunha, Maximilian, and Edmond who shared the happiness and frustration, and strengthened me with love and smile. I owe this dissertation to all of them, "My Big Family".

TABLE OF CONTENTS

LIST OF TABLES

page

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

LIST OF FIGURES

page

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

ABBREVIATIONS

bp	base pair
EDTA	ethylenediamine tetraacetic acid
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
IPTG	isopropyl-B-D-thiogalactopyranoside
MOPS	3-[N-morpholino]propanesulfonic acid
DEPC	diethylpyrocarbonate
PEG	polyethyleneglycol
Mr	molecular weight
KDa	kilo-dalton
D.E.	dextrose equivalence
O.D.	optical density
FPLC	fast performance liquid chromatography
HPLC	high pressure liquid chromatography

INTRODUCTION

Use of microbial enzymes in human life is an ancient art. By experience and empirical methods, it has developed to a highly sophisticated state; wine processing, cheese manufacture, and preparation of fermented oriental food are good examples. Recently, microbial and biochemical science with modern technology has made it possible to develop the enzyme industry at a larger scale. At present, the two major industrial enzymes are saccharidases and proteases which are used in starch processing and detergent manufacturing industries, respectively. Glucose isomerase is the most important commercial saccharidase and it is utilized in conversion of glucose into fructose to produce high fructose corn syrup as a food sweetener. The industrial process for sweetener production requires a glucose isomerase that is stable at high temperature and low pH, and available at low cost. Many studies have been made on glucose isomerase from mesophilic microbial sources for enzyme production, its physicochemical and enzymatic properties, and three dimensional structure.

Essentially, very little is known about the physiological, biochemical, and molecular genetic features of glucose isomerases from thermophilic bacteria that are known to produce intrinsically thermostable and thermoactive enzymes (e.g., amylase, cellulase, protease, or alcohol dehydrogenase). Therefore, an interest was generated from the concept that certain thermoanaerobes may possess enzymatic features unique for glucose isomerase activity. Furthermore, the molecular mechanism for high thermostability of glucose isomerase remains to be identified and understood.

The research presented in this thesis was initiated to understand the biochemical

and physiological properties of glucose isomerase from thermoanaerobic bacteria; to investigate the molecular mechanisms that account for enzymatic catalysis and high thermostability of the enzyme in comparison with thermolabile glucose isomerases; and to assess the biotechnological potential of the thermostable glucose isomerase from thermoanaerobes for novel sweetener production processes.

This thesis is composed of six chapters including a literature review, two manuscripts that were submitted for publication in *Applied and Environmental Microbiology*, a manuscript which was submitted for publication in the *Journal of Bacteriology*, a paper which was submitted for publication in the *Journal of Biological Chemistry*, and a final chapter that deals with concluding remarks and direction of future research.

The literature review in Chapter I describes previous and present knowledge about glucose isomerase in relation to: general biochemical properties, genetic organization, structure-function relationships and the industrial process for fructose production. In addition, the general properties and molecular mechanisms employed by thermophilic enzymes; and, the general biochemical features of other microbial saccharidases that are involved in industrial sweetener production are described. This chapter provides the rationale for detailed studies on both fundamental and applied aspects of thermophilic glucose isomerase from thermoanaerobic bacteria.

Chapter II, "Regulation and characterization of *Thermoanaerobacter* glucose isomerase in relation to thermostable saccharidase synthesis and development of a single step process for sweetener production", describes the general physicochemical properties and regulation of glucose isomerase from *Thermoanaerobacter* strain B6A.

Furthermore, these studies represent the first demonstration that a saccharidase mixture produced by a single microorganism can be used to directly process starch or lactose into a fructose sweetener.

Chapter III, "Purification and characterization of thermostable glucose isomerase from *Clostridium thermosulfurogenes* and *Thermoanaerobacter*", compares the biochemical and physicochemical properties of glucose isomerases purified from two different thermoanaerobic bacteria in detail. The results indicate that these microorganisms produce highly thermophilic glucose isomerases which display very similar enzymatic and physicochemical properties. Crystals were formed from *C. thermosulfurogenes* glucose isomerase which can be used for future X-ray crystallographic studies to identify the three dimensional structure of this enzyme. These findings advance fundamental understanding of the biochemical properties of thermostable glucose isomerases and of the evolutionary relationship between these two different species of thermoanaerobic bacteria.

Chapter IV, "Cloning and expression of the *Clostridium thermosulfurogenes* glucose isomerase gene in *Escherichia coli* and *Bacillus subtilis*", demonstrates cloning of the gene encoding thermophilic glucose isomerase from *C. thermosulfurogenes*. This is the first glucose isomerase gene from a thermophilic bacterium that was cloned and over-expressed in *E. coli* and *B. subtilis*. This chapter also describes a new screening method for thermostable glucose isomerase, based on a specific assay that detects conversion of fructose to glucose on agar plates. The recombinant glucose isomerase expressed in the mesophilic hosts displayed identical physicochemical (i.e., thermophilicity and molecular weight) and enzymatic properties

to those of the native enzyme of *C. thermosulfurogenes*, and this unique feature made it possible to use a simple heat treatment of crude cell extract as one of the most efficient purification steps when the thermophilic glucose isomerase was produced in *B. subtilis* a mesophilic and food-safe host. Finally, the potential of the recombinant glucose isomerase for industrial application was assessed.

Chapter V, "Molecular characterization of the xylose (glucose) isomerase gene from *Clostridium thermosulfurogenes*: Role of His_{101} in enzymatic catalysis", determined the nucleotide sequence of *C. thermosulfurogenes xyl*A gene and compared the deduced amino acid sequence with those of other thermostable and thermolabile glucose isomerases. To understand the molecular mechanism of enzymatic catalysis and thermophilicity of the enzyme, several key amino acids were changed by site directed mutagenesis. The experimental results indicated that His_{101} is the catalytic residue and functions as a base catalyst during glucose (xylose) isomerization; and that cystine disulfide bonds are not used in the protein molecule to achieve enzyme thermostability. In the final chapter the utility of these findings are discussed in relation to future research aimed at understanding structure function relationships of glucose isomerase, and at protein engineering to redesign the molecule in order to achieve desired properties (i.e., higher acid stability, turnover number, and substrate specificity).

Chapter I.

LITERATURE REVIEW

LITERATURE REVIEW

A. Glucose Isomerase

Xylose isomerase (D-xylose ketol isomerase, EC 5.3.1.5) is an intracellular enzyme that catalyzes the isomerization reaction between D-xylose and D-xylulose during the xylose metabolism in various microorganisms. Because this enzyme can also convert D-glucose to D-fructose, the enzyme is often referred to as glucose isomerase (1).

[1] Physiological and Biochemical Properties

Production of glucose isomerase has been reported from a large number of bacteria and actinomycetes that can grow on xylose as an energy source. Among these, *Streptomyces* species have been studied most extensively and used as a source of industrial enzymes (1-5). Production and properties of glucose isomerases from *Actinoplanes missouriensis*, *Bacillus coagulans*, *Lactobacillus* (an anaerobic species), *Norcardia*, and *Arthrobacter* species are also well characterized and some of them are used in the sweetener industry (6-9). However, due to the commercial importance of this enzyme, most of the industrial strains used as glucose isomerase producers have not been disclosed or the information available is brief and concentrated in the patent literature. The regulation of glucose isomerase synthesis and the final yield of enzyme depends on the selected microorganism and its chosen growth conditions (i.e. temperature, pH, mineral salts, and nutrients) (9). Most microorganisms require xylose to induce glucose isomerase production and enzyme synthesis is catabolite

repressed by the presence of glucose in the fermentation medium. However, because of the relatively high cost of xylose in industrial fermentation processes, isolation of constitutive mutants have been attempted and culture conditions which allow glucose isomerase production on cheaper xylan or hemicellulose substrates have been investigated (11,12). Most glucose isomerase producing organisms characterized are mesophiles except for reports on two aerobic, moderate thermophilic, *Bacillus stearothermophilus* and *B. coagulans* (2,90). Therefore, by further screening of thermophilic organisms more thermostable glucose isomerases may be identified. In the last few years many new species of extremely thermophilic bacteria, predominately anaerobic species have been isolated (65).

General biochemical properties of glucose isomerase from representative microbial sources are summarized in Table 1. Although numerous glucose isomerases have been characterized for their enzymatic properties, very few of them have been purified to homogeneity. Most purified glucose isomerases have molecular weights ranging from 80,000 to 190,000 and are composed of 2 or 4 identical subunits (10,13). The pH optima for different glucose isomerases reported lie in the range of 6.5 to 9.5 (10). Temperature optima for enzyme activity and stability vary greatly depending on the source of glucose isomerase. Glucose isomerase is a metalloenzyme, and it requires a divalent cation such as Mn^{++}, Co^{++}, or Mg^{++} for both catalytic activity and enzyme stability. Structural and catalytic roles of metal ions have been proposed for enzymatic activity of various glucose isomerases (14-16). The reaction catalyzed by glucose isomerase follows Michelis-Menten kinetic mechanism over a wide range of substrate concentrations. This enzyme

Table 1. Some Properties of Glucose Isomerase

Microorganism	Temp. Optimum (°C)	pH Optimum	Metal Ion Requirement	Molecular Weight	No. of Sub-units	Reference
Escherichia coli	45	6.0	---	96,000	2	23,91
Bacillus subtilis	≈50	7.5	---	---	---	This study
Lactobacillus brevis	50	6.0-7.0	Mn^{++},Co^{++}	195,000	---	15
Arthrobacter strain B3728	≈80	8.0	Mg^{++}	180,000	4	29
Ampullariella sp. strain 3876	≈75	---	---	170,000	4	28
Streptomyces violaceoniger	>70	7.5	Mg^{++}	160,000	4	26
Streptomyces strain YT-5	80	8.0-8.5	Mg^{++},Co^{++}	157,000	4	1
Streptomyces olivochromogenes	80	8.0-10.0	Mg^{++},Co^{++}	120,000	2	2,37
Streptomyces flavogriseus	70	7.5	Mg^{++},Co^{++}	171,000	4	12
Streptomyces griseofuscus	85	8.5	Mg^{++},Co^{++}	180,000	4	93,94
Streptomyces violaceruber	80	7.5-9.5	Mg^{++},Co^{++}	---	---	5
Bacillus coagulans	75	7.0	Co^{++}	160,000	4	90
Bacillus stearothermopillus	80	7.5-8.0	Co^{++}	130,000	---	2
Actinoplanes missouriensis	90	7.0-7.5	Mg^{++},Co^{++}	80,000	2	6,38,92

displays a lower K_m and higher V_{max} for xylose than for glucose indicating that the physiological function for this enzyme is in xylose isomerization. The concentration that between D-glucose and D-fructose is about 50:50 at 60°C. The equilibrium ratio is more favorable towards fructose at temperatures higher than 60°C (13).

[2] Genetic organization and Primary Structure of *xyl*A gene

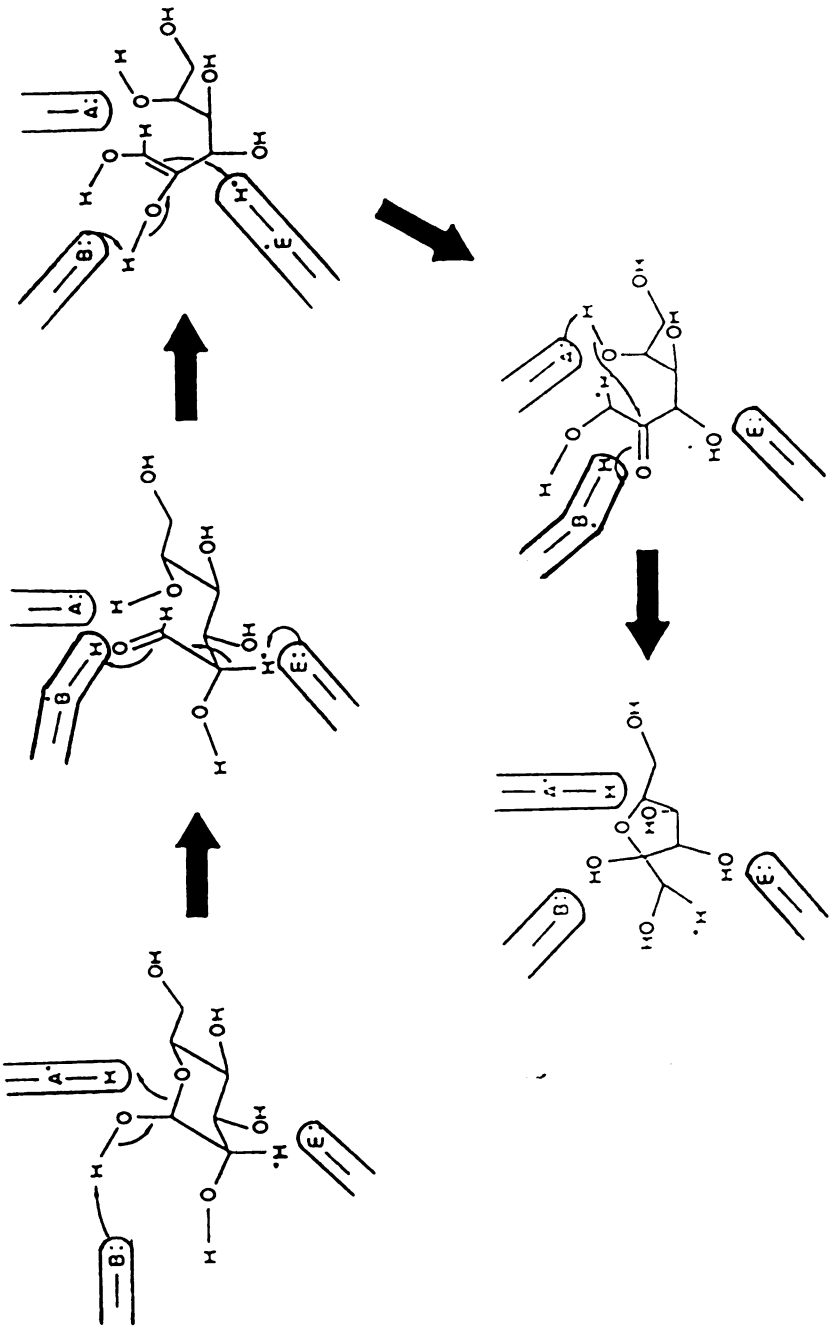
In spite of the industrial importance of the enzyme, few glucose isomerase genes have been cloned and characterized for their primary structure and genetic organization. Regulation of xylose catabolism in *Salmonella typhimurium* and *E. coli* involves an operon consisting of three structural genes; *xyl*T, *xyl*B, *xyl*A, responsible for xylose transport, xylulose kinase, and xylose isomerase, respectively (17-19). Expression of these structural genes are under the positive control of a regulatory gene, *xyl*R. The structural gene coding for xylose isomerase from *E. coli* and *B. subtilis* have been cloned and sequenced (20,21); and, a comparison of their nucleotide sequence showed about 50% homology (22). Overexpression of *E. coli* xylose isomerase by using *tac* or *lac* promoters in *E. coli* strains has been reported (23-25). The glucose isomerase gene of *Streptomyces violaceoniger* and *Ampullariella* species has been cloned in a *Streptomyces* host and in *E. coli*, respectively, and their deduced amino acid sequences have been reported (26-28). Although nucleotide sequences are not available, the amino acid sequences of *Arthrobacter* species have been published (29).

[3] Catalytic Mechanism and Three Dimensional Structure

The proposed catalytic mechanism for glucose isomerization involves formation of a cis-enediol intermediate via an intramolecular hydrogen transfer without proton exchange with water (30,31). A schematic diagram of the proposed mechanism is illustrated in Figure 1 (32). In the first step a base group of the enzyme initiates ring opening of the α-pyranose form of glucose which is known to be the preferred substrate to the ß-anomer (33,34). This process may be assisted by a neighboring acidic group. In the second step, another basic group in the active site abstracts the proton from the C_2 atom of the substrate to facilitate the formation of a cis-enediol intermediate. In the final step, the proton is transferred back to the C_1 atom of the substrate , and the first base and acidic groups again cooperate in the ring closing of the intermediate to form the furanose product. Although the exact amino acid residues which take part in catalysis at the active site are not proven, inhibition studies on *Streptomyces* glucose isomerase activity by chemical modification suggested the involvement of a histidine residue for catalytic activity of the enzyme (35).

The three dimensional structure of xylose isomerases have been derived from general enzyme sources including *Streptomyces rubiginosus*, *S. olivochromogenes*, *Actinoplanes missouriensis*, *S. violaceoniger*, *S. albus*, and *Arthrobacter* by X-ray crystallographic studies at different range of resolutions (36-39,29). Structure analysis of *S. rubiginosus* enzyme crystals at 4Å resolution indicated that the polypeptide chain consists of two structural domains containing an eight stranded ß-sheet and α-helix configuration for the larger domain and a loop structure for the smaller domain which overlaps with a larger domain in another subunit to form a tightly bound enzyme

Figure 1. Schematic illustration of the proposed catalytic mechanism of glucose isomerase (32).



dimer. The final tetrameric structure of native enzyme consists of two such dimers (36). Recent determination of the fine three dimensional structure of this enzyme complex with substrate (xylose) or designed-inactivator at 1.9Å (40) suggested specific amino acid residues for the putative active site and metal ion binding site (Figure 2). The histidine residue at position 54 was suggested as the catalytic residue and base catalyst, and this histidine is correctly placed to abstract a proton from the C_1 or C_2 atom of the substrate. The predicted structure of the active site in *Arthrobacter* xylose isomerase also indicated that the histidine residue at the same position 54 of the enzyme may be the catalytic residue (29). However, no biological or functional evidence for the proposed mechanism has been established nor have site directed mutants been used to provide proof that a specific histidine is involved in catalysis.

[4] Industrial applications

Currently, glucose isomerase is one of the most important industrial enzymes used in the food industry, as it is employed to convert glucose to fructose for the production of high fructose corn syrup (HFCS) as a nutritive sweetener (41). In 1982, more than 200 tons of glucose isomerase were used in the sweetener business and 8 billion pounds of HFCS were produced in the United States alone (42).

The overall process for HFCS production starts with starch processing steps, including liquefaction and saccharification, and ends with the isomerization step (43) (Figure 3). In the first stage, α-amylase converts raw starch into malto dextrin (D.E. 10-15) by catalyzing a random hydrolysis of the α-1,4 glycosidic linkages in starch. Then these D.E. 10-15 oligo-saccharides are hydrolyzed to produce glucose with 90%

Figure 2. Schematic diagram of the three dimensional structure of the the active site in *Streptomyces rubiginosus* xylose isomerse (40).

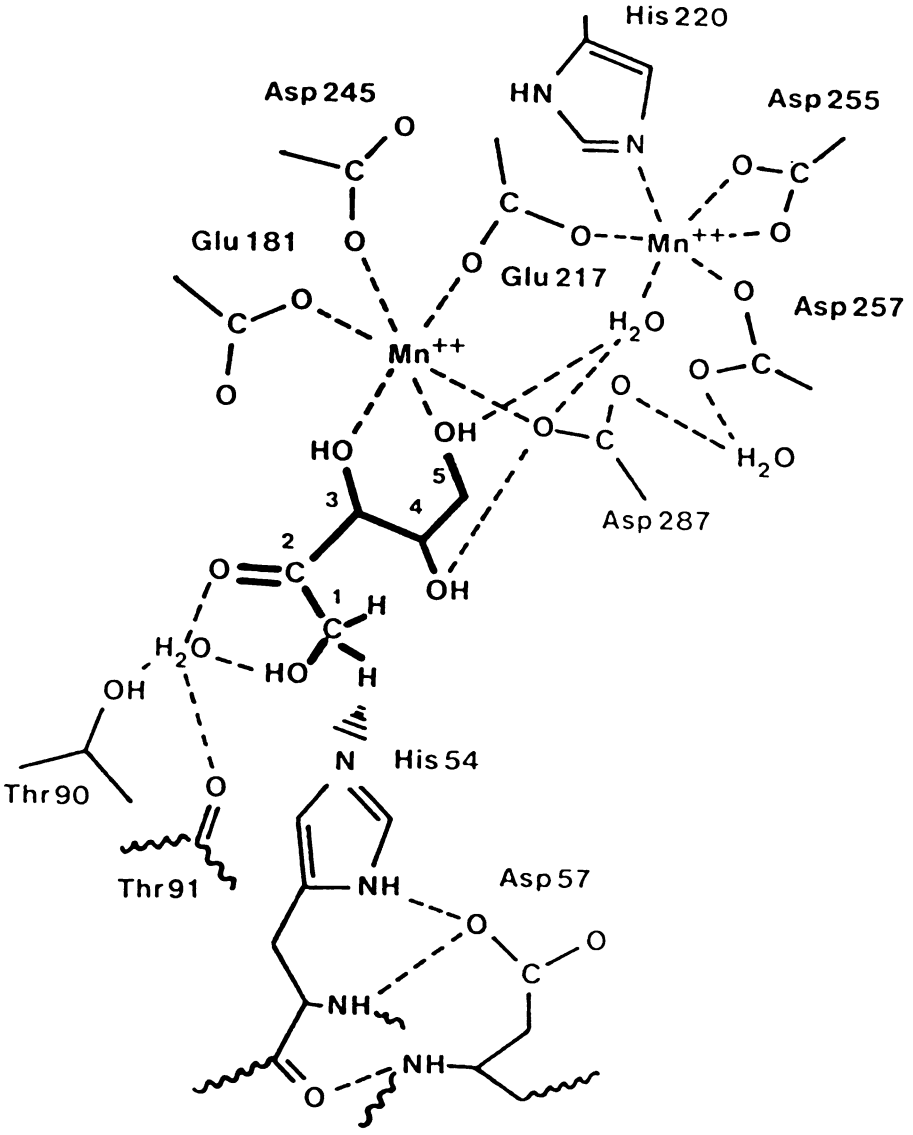

His220
Asp245
HN
Asp255
Glu181
Glu217
Asp257
Mn++
H2O
Asp287
HO
OH
Thr90
Thr91
His54
Asp57
NH

Figure 3. Schematic process biochemistry of enzymatic fructose production from starch.

Process	Stage	Enzyme	pH	Temperature	Metal Ions
Starch					
⇩	Liquefaction	Bacterial Alpha-Amylase	6.0 - 7.0	80 - 120°C	Ca^{++}
Malto Dextrin (D.E. 10 -15)					
(pH Adjustment with Acid)					
⇩	Saccharification	Glucoamylase	4.0 - 5.0	55-60°C	
Glucose					
(Filter, pH Adjustment Addition of Metal Cofactor)					
⇩	Isomerization	Glucose Isomerase	7.0	58-60°C	Mg^{++} Mn^{++} Co^{++}
Glucose and Fructose Mixture (58:42)					

conversion from starch by glucoamylase, which liberates ß-glucose consecutively from the non-reducing ends of the oligo-saccharides. Prior to the saccharification step, pH and temperature are adjusted to optimize the activity and stability of glucoamylase. Glucose syrup produced in the second stage is filtered and refined to remove Ca^{++} ions and other by-products which are inhibitory to glucose isomerase activity. Isomerization of glucose syrup by glucose isomerase produces a fructose/glucose mixture (42:58%), and this final step also requires a re-adjustment of pH and the addition of metal cofactors. To generate the final sweetener product containing 55% fructose, which was judged to be acceptable to replace the sucrose used in food and beverage industry, the 42% fructose syrup is separated by large-scale chromatographic columns, and then blended with an appropriate amount of original feedstock (44). Industrial glucose isomerases are the most expensive starch processing enzymes and they are used in the immobilized form to both prolong enzyme half-life by enhancing thermostability and to allow use of a continuous flow reactor system (45,46). Thus, the high process cost for glucose isomerase might be lowered by using a new source of enzyme with high temperature stability and activity. The current industrial process is limited to 60°C because of undesirable by-products and color formation during the glucose isomerization reaction at high temperature and the alkaline pH required for enzymatic stability. If the pH optimum of the enzyme activity could be lowered below 6.5 and temperature optimum raised to 70°C, while enzyme stability is retained, faster reaction rates during isomerization and higher final fructose concentrations at equilibrium could be attained. Furthermore, these novel enzymatic process improvements could reduce the viscosity of the glucose feedstock

and eliminate pH re-adjustments made after the saccharification step.

B. Other saccharidases used in food industry

An extensive variety of microbial enzymes are used in the food industry, and the principal enzymes include amylase for starch processing, glucose isomerase for sweetener production, ß-galactosidase for milk products and whey processing, pectinase for wine or fruit juice clarification, and protease for meat processing and cheese manufacturing (47). Saccharidases are the most important food-industrial enzymes employed to degrade polysaccharide to oligosaccharides or monosugars, and to convert these sugars into the final desirable form of saccharides in food products. This section will review only the enzymatic aspect of microbial amylases and ß-galactosidase.

[1] Amylase

Amylases are starch degrading enzymes that are widespread in microorganisms, plants, and animals. The term amylase was used to designate enzymes that catalyze the hydrolysis of α-1,4 glucosidic linkages of polysaccharides such as starch or glycogen (48). Starch is the principal storage carbohydrate of plants and its major commercial source in the U.S. is corn. Starch consists of two high molecular weight components: amylose, a linear glucose polymer which contains α-1,4 glucosidic linkages; and, amylopectin, a branched polymer which contains α-1,6 glucosidic

linkages between linear α-1,4 glucose chains (49). Microbial amylases such as α-amylase, glucoamylase, and starch debranching pullulanase have been widely used in starch processing industries.

(a) α-amylase: α-amylases catalyze a random hydrolysis of the α-1,4 glucosidic linkages in amylose, amylopectin, and glycogen in an endo-fashion. The end products of enzymatic hydrolysis are oligosaccharides of varying chain lengths with or without branched points with the α-configuration at the reducing glucose unit. α-amylases are able to by-pass α-1,6 branch points in starch and the enzyme action results in a rapid decrease in viscosity of starch solution and in the iodine staining power (50). α-amylases are generally stable in the pH range 5.5-8.0, and optimal activity of the enzymes occurs between pH 5.5 and 6.5 (51). These enzymes have been classified as metalloenzymes and they are stabilized by the presence of calcium ions (48). There are two different types of enzymes, saccharifying α-amylase and liquefying α-amylase which differ in their hydrolysis limits on soluble starch (52,53). Thermostable liquefying α-amylases isolated from *Bacillus* species are used in the first liquefaction step during starch processing for sweetener production (Figure 2).

(b) Glucoamylase: Glucoamylase is an exo-acting enzyme that yields ß-D-glucose from the non-reducing ends of amylose, amylopectin, and glycogen by hydrolysis of α-1,4 glycosidic linkages in a consecutive manner. It also hydrolyzes α-1,6 and α-1,3 linkages at a much slower rate than α-1,4 linkages. Glucoamylases are found in fungi and the enzymes used commercially originate from either

Aspergillus niger or *Rhizopus* species (54), where they are used for the conversion of malto-oligosaccharides into D-glucose (Figure 2). The pH and temperature optima of most glucoamylases are in the range of 4.5-5.0 and 40-60°C, respectively (55). All fungal glucoamylases are glycoproteins containing 5-20% carbohydrate in the enzyme molecule. Glucoamylases differ from α-glucosidases in their substrate specificity and the stereo configuration of the glucose product (56). α-glucosidase catalyzes the hydrolysis of α-1,4 linked α-D-glucose residues from the non-reducing ends in short chain oligosaccharides or maltose to release α-D-glucose as a final product.

(c) Pullulanase: Pullulanase is a starch debranching enzyme that hydrolyzes α-1,6 glucosidic linkages in pullulan, amylopectin or glycogen (57). The enzyme converts pullulan into maltotriose in an endo-fashion (58), and isomaltose, panose, or amylose are not degraded by pullulanase (59). The pH optima for pullulanases were reported to be 5.9-7.0, and the temperature optima of the enzyme ranges between 45-60°C (55). When starch is hydrolyzed with α-amylase, the branch points of amylopectin are resistant to attack, and the prolonged action of α-amylase on starch results in the formation of α-limit dextrin. Glucoamylases, on the other hand, are able to hydrolyze the α-1,6 linkages of starch, but the reaction proceeds relatively slowly due to their low affinity for the branch points. Therefore, pullulanases are generally used in combination with the saccharifying α-amylase, or glucoamylase to improve the saccharification and glucose yields from starch. It is interesting to note that recent studies on new pullulanases purified from thermoanaerobic bacteria

indicated that both α-1,6 linkages in pullulan and α-1,4 linkages in amylose could be cleaved by a new enzyme type, amylopullulanase (60).

[2] ß-galactosidase

ß-galactosidase hydrolyzes the ß-D-galactosidic linkage of lactose to generate an equimolar mixture of glucose and galactose. In practice, depending on the conditions, the equimolar pattern is sometimes not followed because ß-galactosidase displays reversion activity and can form allolactose and other oligosaccharide from galactose (61). Temperature and pH optima for this enzyme activity differs according to the enzyme source. In general, fungal ß-galactosidases have pH optima in the acid range (2.5-4.5) and bacterial enzymes are in the neutral region (6.5-7.5) (62). Although the temperature optima of most ß-galactosidases examined range between 50-60°C, one thermostable enzyme from *Thermus aquaticus*, an aerobic species, was reported to have an optimal temperature at 80°C (63). Lactose is a disaccharide found in milk products and by-products (whey), and is an important dietary carbohydrate. Hydrolysis of lactose during the processing of milk based products can potentially solve the digestion problems of lactase deficient adults, improve the solubility and decrease the tendency of crystallization during ice cream and yogurt production, and if further converted to fructose by other enzymes require less additive sugars in these dairy products (64).

C. Thermostable Biocatalysts

The recent interest in biotechnology coupled with the discovery of new, novel thermophiles has prompted studies on the general properties of their enzymes, understanding molecular mechanisms of enzyme thermophilicity, and the utilization of them for industrial purposes (65). As thermostable biocatalysts, thermophilic bacteria and/or their enzymes have potential for industrial applications due to their unique kinetic and stability properties, and because operation at high temperature often minimizes the risk of microbial contamination and lowers capital cost for large scale production with improved mass transfer rates for certain substrates and products (66,67). In this section, the microbial and enzymatic aspects of thermophilicity will be reviewed as it relates to the research presented in this thesis.

[1] Thermophilic bacteria

Extremely thermophilic bacteria display temperature optima for growth at 60°C to above 100°C, but do not proliferate at temperature below 40°C. Extreme thermophiles often possess faster metabolic rates and produce more active and stable enzymes than those found in either mesophilic bacteria, yeasts, fungi, plants, or animals (66). A variety of thermophilic saccharolytic anaerobes have been isolated from self-heating (manure piles and wet soils) or volcanic features in nature (68,69,70,71). These thermoanaerobic species display diverse properties in terms of growth characteristics, substrates utilized, metabolic pathways, enzymes, and fermentation products.

Clostridium thermosulfurogenes is a Gram-negative, spore forming rod that has a double-layered wall without an outer membrane layer (72). This novel species can utilize pectin as an energy source and transforms thiosulfate into elemental sulfur which deposits in the culture medium and on the cells. The organism ferments a variety of carbohydrates at 60°C such as xylose, galactose, glucose, starch, maltose, cellobiose, and sucrose; and it forms ethanol, acetate, lactate, and H_2/CO_2 as fermentation products. It also produces a highly thermostable and active ß-amylase when grown on starch (73).

Thermoanaerobacter **strain B6A** is a Gram-negative, non-spore forming rod that utilizes a very wide range of carbohydrates as a carbon source including xylan, xylose, starch, and glucose, but not cellulose (74). It can grow in a chemically defined medium at pH 3.5 and 60°C. Fermentation products from either glucose or xylan include ethanol, acetate, lactate, and H_2/CO_2.

Clostridium thermohydrosulfuricum is a spore forming rod and its endospore displays extreme heat resistance (75). This species grows optimally at temperature of 65°C, and utilizes a broad range of substrates as energy sources including xylose, starch, galactose, and cellobiose. Starch grown cells produce an amylopullulanase that can cleave both α-1,4 and α-1,6 glucosidic linkages of amylose and pullulan, respectively (60).

[2] Thermophilic enzymes

There has been increasing research interest in enzymes from thermophilic microorganisms during the last decade. Thermophilic enzymes are very active and

display long half-life at elevated temperature (> 60°C), and are less active but more resistant to most common protein denaturants than their counterparts from mesophilic sources at lowered (< 40°C) temperatures (76,77). A variety of thermostable enzymes have been studied and characterized for their physicochemical and catalytic properties, and many of them are now used extensively in industrial processes (78-80). In general, high thermostability of enzyme from extreme thermophiles is an intrinsic property, specified by the amino acid sequence and certain key amino acid residues in the protein secondary and tertiary structure (81-84). A major molecular mechanism proposed for thermophilicity of enzymes is explained on the basis of higher free energy of stabilization (ΔG) within the protein molecule, which represents a delicate balance between the stabilizing forces and the conformational entropy of the protein required to maintain the tertiary structure of the enzyme (85,86). Small percentage changes in either the stabilizing or destabilizing forces can result in large changes in the net free energy of stabilization, which explains how high thermostability of a protein can be conferred by small differences in the amino acid sequence that give rise to a few additional intramolecular interactions without any "obvious" structural alterations (82,87). Recently, protein engineering approaches have used site directed mutagenesis techniques to insert amino acid changes into enzymes in order to demonstrate the effect of protein chemical structure on enzyme thermostability (88,89). However, a limitation to the successful design of a thermophilic-thermostable protein by engineering techniques is that the effects of amino acid replacements are not easy to predict and thus it is difficult to decide which amino acid substitutions should be made. Therefore, an understanding of the

exact structure-function mechanisms that account for activity and stability relationships in thermophilic enzymes could be essential for engineering high thermostability and activity in proteins. Unfortunately, no specific explanations on the molecular mechanism of enzyme thermophilicity can be made at this time because only a handful of reports exist on understanding how to make a thermolabile enzyme stable at high temperature or why a thermophilic enzyme is active and stable at high temperature.

REFERENCES

1. Takasaki, Y., Kosugi, Y., and Kanbayashi, A. (1969) Agric. Biol. Chem. 33, 1527-1534

2. Suekane, M., Tamura, M., and Tomimura, C. (1978) Agric. Biol. Chem. 42, 909-917

3. Chen, W.P., and Anderson, A.W. (1979) Appl. Environ. Microbiol. 38, 1111-1119

4. Kasumi, T., Hayashi, K., and Tsumura, N. (1981) Agric. Biol. Chem. 45, 619-627

5. Callens, M., Kersters-Hilderson, H., Opstal, O.V., and De Bruyne, C.K. (1986) Enzyme Micro. Technol. 8, 696-700

6. Scallet, B.L., Shieh, K.,Ehrenthal, I., and Slapshak, L. (1974) Die Stärke 26, 405-444

7. Danno, G. (1970) Agric. Biol. Chem. 34, 1795-1804

8. Yamanaka, K., and Takahara, N. (1977) Agric. Biol. Chem. 41, 1909-1915

9. Chen, W. (1980) Process Biochem. 15, 30-35

10. Chen, W. (1980) Process Biochem. 15, 36-41

11. Srinivasan, M.C., Vartak, H.G., Powar, V.K., and Khire, J.M. (1983) Biotech. Lett. 5, 611-614

12. Chen, W.P., Anderson, A.W., and Han, Y.W. (1979) Appl. Environ. Microbiol. 37, 324-331

13. Antrim, R.L., Collila, W., and Schnyder, B.J. (1979) in Applied Biochemistry and Bioengineering (Wingard, L.B., ed) vol. 2, pp. 97, Academic Press, New York

14. Dano, G. (1971) Agric. Biol. Chem. 35, 997-1006

15. Yamanaka, K. (1969) Archiv. Biochem. Biophys. 131, 502-506

16. Young. J.M., Schray, K.J., and Mildvan, A.S. (1975) J. Biol. Chem. 250, 9021-9027

17. Lawlis, V.B., Dennis, M.S., Chen, E.Y., Smith, D.H., and Henner, D.J. (1984) Appl. Environ. Microbiol. 47, 15-21

18. Shamanna, D.K., and Sanderson, K.E. (1979) J. Bacteriol. 139, 71-79

19. Ghangas, G.S., and Wilson, D.B. (1984) J. Bacteriol. 157, 158-164

20. Schellenberg, G.D., Sarthy, A., Larson, A.E., Backer, M.P., Crabb, J.W., Lidstrom, M., Hall, B.D., and Furlong, C.E. (1984) J. Biol. Chem. 259, 6826-6832

21. Wilhelm, M., and Hollenberg, C.P. (1984) EMBO Journal 3, 2555-2560

22. Wilhelm, M., and Hollenberg, C.P. (1985) Nucleic Acids Res. 13, 5717-5723

23. Batt, C.A., O'Neil, E., Novak, S.R., Ko, J., and Sinskey, A. (1986) Biotechnology Progress. 2, 140-144

24. Panagiotis, E.S., and Ho, N.W.Y. (1985) Enzyme Micro. Technol. 7, 592-596

25. Wovcha, M.G., Steuerwald, D.L., and Brooks, K.F. (1983) Appl. Environ. Microbiol. 45, 1402-1404

26. Marcel, T., Drocourt, D., and Tiraby, G. (1987) Mol. Gen. Genet. 208, 121-126

27. Drocourt, D., Bejar, S., Calmels, T., Reynes, J.P., and Tiraby, G. (1988) Nucleic Acids Res. 16, 9337

28. Saari, G.C., Kumar, A.A., Kawasaki, G.H., Insley, M.Y., and O'Hara, P.J. (1987) J. Bacteriol. 169, 612-618

29. Henrick, K., Collyer, C.A., and Blow, D.M. (1989) J. Mol. Biol. 208, 129-157

30. Rose, I.A., O'Connell, E.L., and Mortlock, R.P. (1969) Biochem. Biophysic. Acta 178, 376-379

31. Ramchander, S., and Feather, M.S. (1977) Arch. Biochem. Biophys. 178, 576-580

32. Suckling, K.E. (1985) in Comprehensive Biotechnology (Moo-Young, M., Bull, A., and Dalton, H. ed) vol.1, pp. 539-551, Pergamon Press, Oxford

33. Schray, K.J., and Rose, I.A. (1971) Biochemistry 10, 1058-1062

34. Makkee, M., Kieboom, A.P.G., and van Bekkum, H. (1984) Recl.: J. R. Neth. Chem. Soc. 103, 361-164

35. Gaikwas, S.M., More, M.W., Vartak, H.G., and Deshpande, V.V. (1988) Biochem. Biophys. Res. Commun. 155, 270-277

36. Carrell, H.L., Rubin, B.H., Hurley, T.J., and Glusker, J.P. (1984) J. Biol. Chem. 259, 3230-3236

37. Farber, G.K., Petsko, G.A., and Ringe, D. (1987) Protein Engineering 1, 459-466

38. Rey, F., Jenkins, J., Janin, J., Lasters, I., Alard, P., Claessens, M., Matthyssens, G., and Wodak, S. (1988) Proteins 4, 165-172

39. Dauter, Z., Dauter, M., Hemker, J., Witzel, H., and Wilson, K.S. (1989) FEBS Letters 7, 1-8

40. Carrell, H.L., Glusker, J.P., Burger, V., Manfre, F., Tritsch, D., and Biellmann, J.-F. (1989) Proc. Natl. Acad. Sci. USA 86, 4440-4444

41. Lambert, P.W., and Meers, J.L. (1983) Phil. Trans. R. Soc. Lond. 300, 263-282

42. Coker, L.E., and Venkatasubramanian, K. (1985) in Biotechnology Applications and Research (Cheremisinoff, P.N., and Ovellette, R.P., eds) pp. 165-171, Technomic Publishing Co., Lancaster, Basel

43. Reichelt, J.R. (1983) in Industrial Enzymology (Godfrey, T., and Reichelt, J., eds) pp. 375-396, The Nature Press

44. Bucke, C. (1983) Trends in Biotechnol. 1, 67-69

45. Verhoff, F.H., Boguslawski, G., Lantero, O.J., Schlager, S.T., and Jao, Y.C. (1985) in Comprehensive Biotechnology (Moo-Young, M., ed), vol.3, pp. 837-859, Pergamon Press. Oxford, New York

46. Schnyder, B.J. (1974) Die Stärke 26, 409-412

47. Peppler, H.J., and Reed, G. (1987) in Biotechnology (Kennedy, J.F. ed), vol. 7a, pp. 547-603, VCH Publishers, NY

48. Fischer, E.H., and Stein, E.A. (1960) in The Enzymes (Boyer, P.D., Lardy, L., and Myrback, K. eds), vol. 4, pp.313-343, Academic Press, NY

49. French, D. (1975) in Biochemistry of Carbohydrates (Kornberg, H.L., Phillips,

D.C., and Whelan, W.J. eds), vol. 5, pp. 267-335

50. Fogarty, W.M., and Kelly, C.T. (1980) in Microbial Enzymes and Bioconversions (Rose, A.H. ed), pp. 116-158, Academic Press

51. Manning, G.B., and Campbell, L.L. (1961) J.Biol. Chem. 236, 2952-2957

52. Fukumoto, J., and Okada, S. (1963) J. Ferment. Technol. 41, 427-434

53. Pazur, J.H., and Okada, S. (1966) J. Biochem. 241, 4146-4151

54. Underkofler, L.A., Denault, L.J., and Hou, E.F. (1965) Die Stärke 17, 179-184

55. Fogarty, W.M. (1982) in Microbial Enzymes and Biotechnology (Fogarty, W.M. ed), pp. 1-92, Applied Sciece Publishers, NY

56. Reese, E.T., Maguire, A.H., and Parrish, F.W. (1968) Can. J. Biochem. 46, 25-34

57. Lee, E.Y.C., and Whelan, W.J. (1971) in The Enzymes (Boyer, P.D. ed), vol. 5, pp. 191-234, Academic Press, NY

58. Drummond, G.S., Smith, E.E., and Whelan, W.J. (1969) FEBS Lett. 5, 85-88

59. Bender, H., and Wallenfels, K. (1966) Methods in Enzymology 8,555-559

60. Saha, B.C., Mathupala, S.P., and Zeikus, J.G. (1988) Biochem. J. 252, 343-348

61. Shukla, T. (1975) CRC Critical Reviews in Food Technology 1, 325-347

62. Gekas, V., and Lopez-Leiva, M. (1985) Process Biochem. February, 2-12

63. Ulrich, J.T., McFeters, G.A., and Temple, K.L. (1972) J. Bacteriol. 110, 691-698

64. Pedersen, S., and Norman, B.E. (1986) in Chemical Aspecs of Food Enzymes (Andrews, A.T. ed), pp.156-187, Royal Society of Chemistry

65. Brock, T.D. (1986) in Thermophiles; General, Molecular, and Applied Microbiology. (Brock, T.D. ed), pp. 1-16, John Wiley and Sons, New York

66. Zeikus, J.G. (1979) Enzyme Microb. Technol. 1, 243-252

67. Zeikus, J.G. (1980) Ann. Rev. Microbiol. 34, 423-464

68. Ben-Bassat, A., and Zeikus, J.G. (1981) Arch. Microbiol. 128, 365-370

69. Weimer, P.J., Wagner, L.W., Knowlton, S., and Ng, T.K. (1984) Arch. Microbiol. 138, 31-36

70. Ljungdahl, L.G., Bryant, F., Carreira, L., Saiki, T., and Weigel, T. (1981) in Trends in the Biology of Fermentation for Fuels and Chemicals. (Lollaender ed.), pp. 397, Plenum Press, New York

71. Zeikus, J.G., Ben-Bassat, A., and Hegge, P.W. (1980) J. Bacteriol. 143, 432-440

72. Schink, B., and Zeikus, J.G. (1983) J. Gen. Microbiol. 129, 1149-1158

73. Hyun, H.H., and Zeikus, J.G. (1985) Appl. Environ. Microbiol. 49, 1162-1167

74. Weimer, P.J. (1985) Arch. Microbiol. 143, 130-136

75. Hyun, H.H., Zeikus, J.G., Longin, R., Millet, J., and Ryten, A. (1983) J. Bacteriol. 156, 1332-1337

76. Zuber, H. (1978) in Biochemistry of Thermophily (Friedman, S.M. ed), pp. 267-285, Academic Press, New York.

77. Amelunxen, R.E., and Murdock, A.L. (1978) in Microbial Life in Extreme Environments (Kushner, D.J. ed), pp. 215-278, Academic Press, London.

78. Ng. T.K., Kenealy, W.R. (1986) in Thermophiles; General, Molecular, and Applied Microbiology. (Brock, T.D. ed), pp. 197-215, John Wiley and Sons, New York

79. Klibanov, A.M. (1983) in Applied Microbiology (Laskin, A.I. ed), vol.29, pp. 1-28, Academic Press, New York.

80. Schmid, R.D. (1979) in Advances in Biochemical Engineering (Fiechter, A. et al. eds), vol.12, pp. 41-118, Spring-Verlag, Berlin.

81. Frank, G., and Tratschin, J.D. (1979) Biochemistry 18, 5698-5703

82. Daniel, R.M. (1986) in Protein Structure, Folding, and Design (Oxender, D.L. ed), pp. 291-296, Alan R. Liss, Inc., New York.

83. Fontana, A. (1988) Biophys. Chem. 29, 181-193

84. Vihinen, M. (1987) Protein Engineering 1, 477-480

85. Creighton, T.E. (1984) in Protein; Structure and Molecular Properties, pp. 133-158, W.H. Freeman and Co., New York.

86. Dill, K.A. (1987) in Protein Engineering (Oxender, D.L., Fos, C.F. eds), pp. 187-192, Alan R. Liss, Inc., New York.

87. Holmes, M.A., and Matthews, B.W. (1982) J. Mol. Biol. 160, 623-639

88. Fersht, A.R., Leatherbarrow, R.J., and Wells, T.N. (1986) Nature 322, 284-286

89. Beasty, A.M., Hurle, M., Manz, J.T., Stackhouse, T., and Matthews, C.R. (1987) in Protein Engineering (Oxender, D.L., Fox, C.F. eds), pp. 91-102, Alan R. Liss, Inc., New York.

90. Danno, G., Yoshimura, S., and Natake, M. (1967) Agric. Biol. Chem. 3, 284-292

91. Tucker, M.Y., Tucker, M.P., Himmel, M.E., Grohmann, K., and Lastick, S.M. (1988) Biotechnol. Lett. 10, 79-84

92. Gong, C.S., Chen, L., and Tsao, G.T. (1980) Biotechnol. Bioeng. 22, 833-845

93. Kasumi, T., Hayashi, K., and Tsumura, N. (1981) Agric. Biol. Chem. 45, 619-627

94. Kasumi, T., Hayashi, K., Tsumura, N., and Takagi, T. (1981) Agric. Biol. Chem. 45, 1087-1095

Chapter II

Regulation and Characterization of *Thermoanaerobacter* Glucose Isomerase in Relation to Thermostable Saccharidase Synthesis and Development of a Single Step Process for Sweetener Production

ABSTRACT

Regulation of glucose isomerase synthesis was studied in *Thermoanaerobacter* strain B6A which fermented a wide variety of carbohydrates including glucose, xylose, lactose, starch, and xylan. Glucogenic amylase activities and ß-galactosidase were produced constitutively, whereas the synthesis of glucose isomerase was induced by either xylose or xylan. Production of saccharidase was not significantly repressed by the presence of glucose or 2-deoxy-glucose in the growth media. In order to maximize glucose isomerase production, the culture pH was controlled at 5.5 during xylose fermentation. The apparent temperature and pH optima for these cell-bound saccharidase activities were: glucose isomerase 80°C, 7.0-7.5; glucogenic amylases 70°C, 5.0-5.5; and ß-galactosidase 60°C, 6.0-6.5. Glucose isomerase, glucogenic amylases, and ß-galactosidase activities were produced in xylose grown cells that were active and stable at 60°C and pH 6.0-6.5. Under single step process conditions, these enzyme activities in whole cells or cell extracts converted starch or lactose directly into fructose mixtures. Starch was 96% converted into a 49:51 mixture of glucose and fructose; whereas lactose was 85% converted into a 40:31:29 mixture of galactose, glucose, and fructose.

INTRODUCTION

The production of sweetener from corn starch by microbial saccharidases is an important application of enzyme technology in the food industry (7). The current process for high fructose corn syrup production involves several separate enzymatic steps including liquefaction by α-amylase, saccharification by glucoamylase, and isomerization by glucose isomerase. These steps require different reaction conditions (i.e. temperature, pH, and metal cofactors) (1,3). Most industrial saccharidases used in sweetener production require high thermostability and low production costs, and they are produced by mesophilic microbes. Many studies have been made on searching for more thermostable saccharidases and on immobilization of glucose isomerase to prolong enzyme half-life in the reactor (14,19,28). Also, many efforts have been made on strain improvement for hyper-producers of saccharidases and on selection for the use of catabolite resistant or constitutive mutants to lower enzyme production costs (12,24).

Recently, our laboratory reported *Clostridium thermohydrosulfuricum* produce highly thermostable amylolytic enzymes including glucogenic amylase, pullulanase, and glucosidase activities (14-16). When purified to homogeneity, this pullulanase was shown to cleave both α-1,6 bonds in pullulan and α-1,4 bonds in amylose and has been named amylopullulanase (23), and *Thermoanaerobacter* strain B6A has the same enzyme activities (B.C. Saha, R. Lamed, C. Lee, S.P. Mathupala, J.G. Zeikus, manuscript submitted to Appl. Environ. Microbiol.). Although glucose isomerase and ß-galactosidase have been detected in thermophilic microorganisms (6,11,20), little

is known about the biochemical properties and regulation of these enzymes in thermoanaerobic bacteria. In this paper, we report on the regulation and general biochemical properties of glucose isomerase in relation to other saccharidase activities from *Thermoanaerobacter* strain B6A. Notably, growth conditions were discovered that enabled glucose isomerase to be produced in conjunction with glucogenic amylases and ß-galactosidase activities. Thus, a single step process was developed to produce fructose mixtures from starch or lactose with *Thermoanaerobacter* saccharidases.

MATERIALS AND METHODS

Chemicals and Gases

Medium components and all chemicals were reagent grade. Larchwood xylan (Lot 113F-0003) was purchased from Sigma Chemical Co. (St. Louis, Mo.). The N_2/CO_2 (95:5) gas was obtained from Linde, Union Carbide Corporation (East Lansing, MI) and passed through heated copper columns to remove O_2 prior to use.

Organism

Thermoanaerobacter strain B6A isolated from a volcanic hot spring in Themopolis, Wyoming (27) was obtained from Dr. Paul Weimer (USDA Dairy Forage Lab, Madison, WI) and was maintained by stringent anaerobic culture techniques (29) in CM5 medium containing 0.5% xylan.

Culture Conditions, and Growth Measurement

Experimental cultures were grown at 60°C without shaking in either 125 ml serum bottles or in 26 ml anaerobic pressure tubes that contained 50 ml or 10 ml of TYE medium (29) supplemented with 1% carbon source and with a N_2/CO_2 (95:5) gas headspace. Enzyme production time course studies during xylose fermentation were conducted in a New Brunswick (Edison, N.J.) Multigen fermentor that contained 500 ml of TYE medium. The fermentor cultures were incubated at 60°C and gassed continuously with N_2/CO_2 (95:5) gas and mixed at 100 rpm. For the constant pH experiments, 0.5N NH_4OH was added during the fermentation by a feeding pump

which was connected to a pH controller. Cells used for crude enzyme extract preparations were cultured in a 14 liter New Brunswick fermentor containing 10 L of TYE medium at pH 6.8 with 1% xylose that was stirred at 60°C under a N_2/CO_2 (95:5) gas stream.

Cell growth in media containing soluble substrates was determined by measuring the optical density of the culture broth at 660 nm. When xylan was present in culture medium, ethanol concentration in culture supernatant was used to measure growth. Ethanol was measured in acidified samples by gas chromatography using a flame ionization detector with N_2 as the carrier gas and methods described elsewhere (16). Experimental ethanol production was related to growth by standard optical density (O.D.) versus ethanol plots determined with xylose medium.

Enzyme Preparations and Assays

For the preparation of the washed cells and culture supernatants, cultures were harvested during late exponential growth phase by centrifugation at 8,000 x g for 15 min. The supernatant was decanted, and the cells were washed with double distilled water and suspended in the appropriate amount of double distilled water.

Cell extract was prepared using fermentation grown cells recovered at the exponential phase (11 h) with a Millipore pellicon cell harvester (Bedford, MA.) and washed with double distilled water. Wet cell paste (1 g) was suspended in either 50 ml of 20 mM sodium phosphate buffer (pH 7.0) containing 10 mM $MgCl_2$ for glucose isomerase preparation, in 50 ml of 100 mM sodium acetate buffer (pH 5.5) for glucogenic amylase, or in 50 ml of 100 mM sodium phosphate buffer (pH 6.0)

for ß-galactosidase. The cells were disrupted by passage through a French pressure cell (American Instrument Co. Inc., Silver Spring, MD) at 20,000 lb/in^2 and the supernatant was collected after centrifugation at 15,000 x g for 30 min at 4°C. Protein concentration was determined by the Lowry assay method (18). The crude saccharidase preparation was stable upon incubation indicating the lack of significant protease activity. Xylanase activities were assayed by measuring the rate of reducing sugar formation from xylan; whereas, amylase is reported as glucose or reducing sugars produced from starch or pullulan (when indicated). The reaction mixture contained 1% substrate in 0.1 M sodium acetate buffer at pH 6.0, 5.5 or 5.0 with pullulan, soluble starch or xylan as respective substrates. After 30 min incubation at 65°C, the reaction mixtures were boiled in a steam bath for 5 min to stop the reaction. The samples were centrifuged and the amount of reducing sugars were quantified by the dinitrosalicylic acid method (21). Alternatively, glucose in the supernatant was determined by using either a glucose analyzer (Yellow Stone Instrument, model 27) or by a Sigma enzymatic glucose diagnostic kit. One unit of activity is defined as the amount of enzyme which released 1 µmol of reducing sugar per min under the assay conditions described above, with glucose as standard for amylase activity and with xylose as standard for xylanase activity.

Glucose isomerase activity was measured by incubating a reaction mixture that contained 0.8 M glucose in 0.1 M sodium phosphate or 0.1 M MOPS buffer (pH 7.0) and 10 mM $MgSO_4$, 1 mM $CoCl_2$, and the enzyme. After incubation at 65°C for 30 min, the amount of fructose formed was estimated by the cysteine carbazole sulfuric acid method (8). ß-galactosidase activity was assayed by measuring the

amount of phenoxide ion liberated from ortho-nitrophenol-ß-D-galactopyranoside (ONPG) at 420 nm after 20 min incubation at 60°C. The reaction mixture contained 10 mM KCl, 1 mM $MgSO_4$, 5 mM 2-mercaptoethanol, 2.7 mM ONPG with the enzyme in 100 mM sodium phosphate buffer (pH 6.0) (22).

One unit of amylase, glucose isomerase, and ß-galactosidase activity is defined as the amount of enzyme required to produce 1 µmol of glucose, fructose, and phenoxide ion, respectively, per min under the assay conditions described above. All the enzyme activities were determined at points where product formation was linear with time.

One Step Conversion of Starch or Lactose to Fructose

Starch (i.e., maltodextrin DE 10 and soluble starch) or lactose in 0.1 M sodium phosphate buffer containing 10 mM $MgSO_4$ and 1 mM $CoCl_2$ was incubated with cell extracts prepared from xylose grown cells at various temperatures (60-70°C) and pH's (6.0-6.8). Samples were taken during time course experiments performed in 50 ml serum vials containing 5 ml reaction mixtures that were sealed with rubber bungs, and were shaken at 100 rpm in a New Brunswick water bath shaker. Samples withdrawn from the reaction mixture were boiled in a steam bath for 5 min and centrifuged before sugar analysis. Quantitative and qualitative analyses were performed by high pressure liquid chromatography with saccharide analysis columns heated to 85°C. An Aminex HPX-87C and HPX-87P column (Bio-Rad Laboratories; Richmond, CA) were used for analysis of starch and lactose conversion, respectively.

RESULTS

Location and Types of Saccharidase Activities

Thermoanaerobacter strain B6A ferments starch and hemicellulose but not cellulose(27). In preliminary experiments, halos appeared around colonies grown on either Remazol Brilliant Blue-xylan agar plates or on starch agar plates stained with iodine indicating that the organism produced amylase and xylanase activities.

Experiments were initiated to determine the kinds of saccharidases and their cellular location when *Thermoanaerobacter* was grown on different saccharides as carbon and energy sources for growth. Table 1 shows that *Thermoanaerobacter* produces glucose isomerase, ß-galactosidase as well as glucogenic amylase and xylanase activities. Glucogenic amylase activity was extracellular and cell bound when starch or pullulan was used as substrate. It was not possible to discern whether the glucogenic amylase represented a mixture of α-amylase and glucoamylase or amylopullulanase and α-glucosidase by the assays used. However, About 70-80% of xylanase activity was excreted into the medium and the remaining activity was cell associated in xylan grown cultures. On the other hand, glucose isomerase, and ß-galactosidase activities were totally cell bound.

Regulation of Glucose Isomerase Production

Experiments were conducted to determine the regulation of glucose isomerase synthesis in relation to amylase and ß-galactosidase production. The results presented in Table 2 illustrate that glucose isomerase was only produced when either xylose or

Table 1. Cellular location of saccharidase activities in *Thermoanaerobacter* strain B6A.[a]

Saccharidase	Carbon Substrate	Final Growth (O.D. 660 nm)	Activity (unit/ml)	
			Supernatant	Washed Cell[b]
Glucose Isomerase	xylose	1.15	0.00	0.21
ß-galacto-sidase	lactose	0.98	0.00	0.14
Amylase				
	starch	1.07	0.04	0.19
	pullulan	1.07	0.73	0.43
Xylanase[c]	xylan	0.80	0.47	0.18

[a]Cultures were grown in TYE medium with 1.0% carbon substrate at 60°C.

[b]Cells were resuspended in 1/10 culture volume of double distilled water and activity was converted to the original culture volume.

[c]Due to the interference of xylan optical absorbance, cell growth was converted from ethanol production in relation to a standard growth curve.

Table 2. Effect of growth substrate on saccharidase synthesis in *Thermoanaerobacter* strain B6A.

Growth Substrate[a]	Final Growth[b] ($O.D._{660}$)	Specific Activity (U/mg cell protein)		
		Glucose Isomerase	Glucogenic Amylase	ß-galactosidase
Starch	1.36	0.00	0.61	0.41
Lactose	0.83	0.00	0.54	0.46
Maltose	0.75	0.00	0.43	0.38
Cellobiose	1.23	0.00	0.59	0.44
Glucose	1.60	0.00	0.42	0.31
Xylose	1.47	0.62	0.60	0.47
Xylan	0.68	0.39	0.58	0.33
Xylose + Glucose	1.46	0.36	0.48	0.34
Starch + Glucose	1.24	0.00	0.46	0.35
Lactose + Glucose	1.00	0.00	0.50	0.40
Xylose + 2-Deoxy-Glc	0.80	0.40	0.38	0.33
Starch + 2-Deoxy-Glc	0.95	0.00	0.48	0.42
Lactose + 2-Deoxy-Glc	0.46	0.00	0.30	0.33
Glucose + 2-Deoxy-Glc	0.98	0.00	0.33	0.25
2-Deoxy-Glc	0.11	n.d.	n.d.	n.d.
None	0.10	n.d	n.d.	n.d.

n.d. = Not Determined.

[a]Cultures were grown on TYE medium containing 0.5% main substrate and with or without 0.3% supplementing glucose or 0.1% 2-deoxy-glucose (2-Deoxy-Glc).

[b]Optical density was measured at the early stationary growth phase.

xylan was present as an inducer in the culture medium. Notably, glucogenic amylase and ß-galactosidase were produced constitutively on the wide range of growth substrates tested. The specific activities of glucogenic amylases from starch grown cultures and ß-galactosidase from lactose grown cultures were equal to those obtained from xylose grown culture. The presence of 0.1% 2-deoxy-glucose in culture media containing either 0.5% starch, xylose, or lactose did not repress synthesis of either glucogenic amylase, glucose isomerase, or ß-galactosidase, respectively.

In order to assess the mechanism of glucose isomerase synthesis, experiments were performed where glucose was added during xylan fermentation time courses and glucose isomerase activity was periodically assayed. Due to the turbidity of xylan, cell growth was monitored by measuring the concentration of ethanol in the culture broth. Ethanol was one of the major end products of *Thermoanaerobacter* fermentations (27) and its exponential production during growth is proportional to cell density measured by absorbance at 660 nm during xylose fermentation. These results shown in Figure 1 indicate that synthesis of glucose isomerase during exponential growth was not catabolite repressed by glucose, while xylanase production ceased after glucose addition during exponential growth on xylan (data not shown).

Figure 2 compares glucose isomerase and amylase activity levels during xylose fermentations in the absence (A) and presence (B) of pH control at 5.5. Synthesis of both enzyme activities were tightly growth coupled under either condition. Glucose isomerase activity, however, decreased rapidly in the stationary phase cultures that were not maintained at pH 5.5. Amylase and ß-galactosidase (data not shown) were quite stable throughout the stationary phase. Consequently, pH control at 5.5

Figure 1. Effect of glucose addition on glucose isomerase synthesis during xylan fermentation.

Cultures were grown on CM5 medium with 0.5% xylan at 60°C, and 0.3% glucose was added during the middle of exponential growth phase (⇩). Ethanol concentration in culture broth was measured as a growth indicator and 25 mM ethanol represents O.D. 1.0 at 660 nm in a standard growth curve. The closed symbols represent the culture growth whereas open symbols represent enzyme activity. The culture conditions indicated by symbols are: triangles, glucose only (control); circles, without glucose addition; squares, with glucose addition.

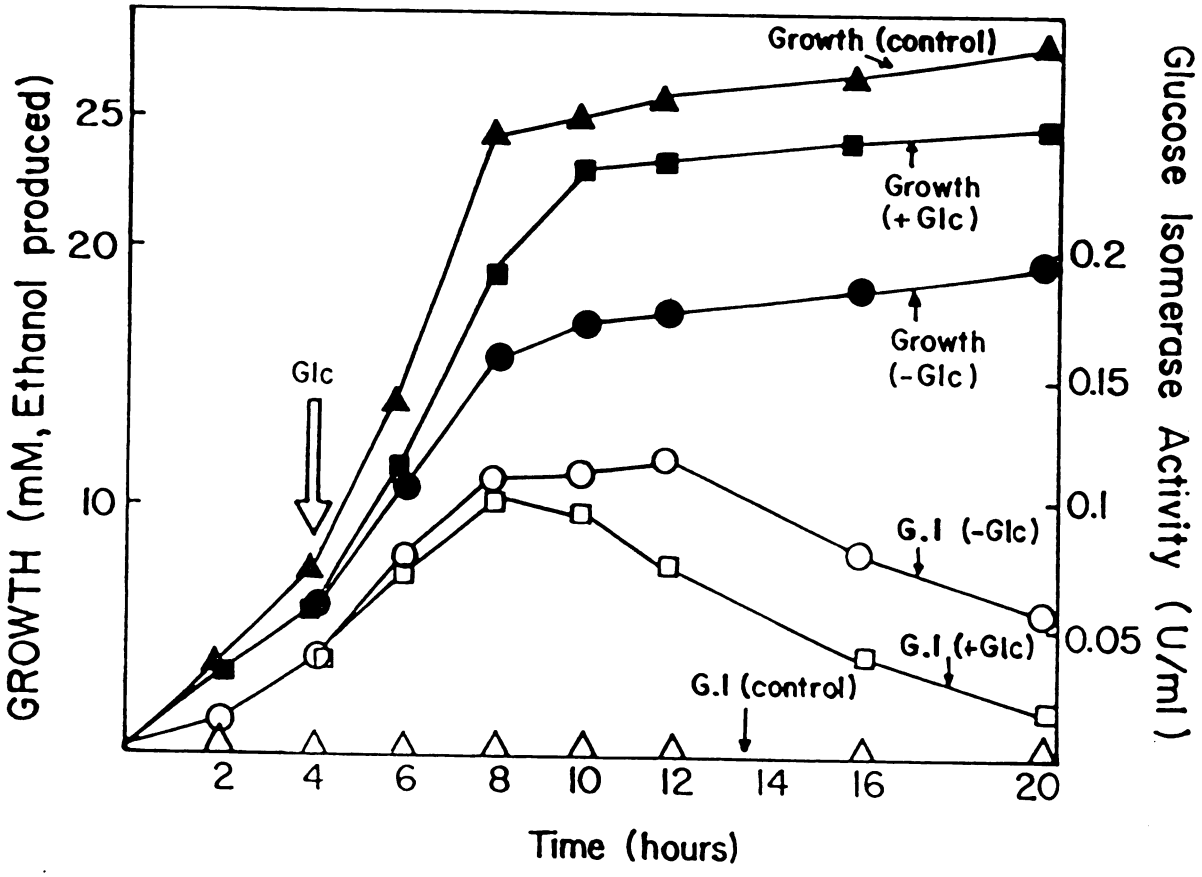
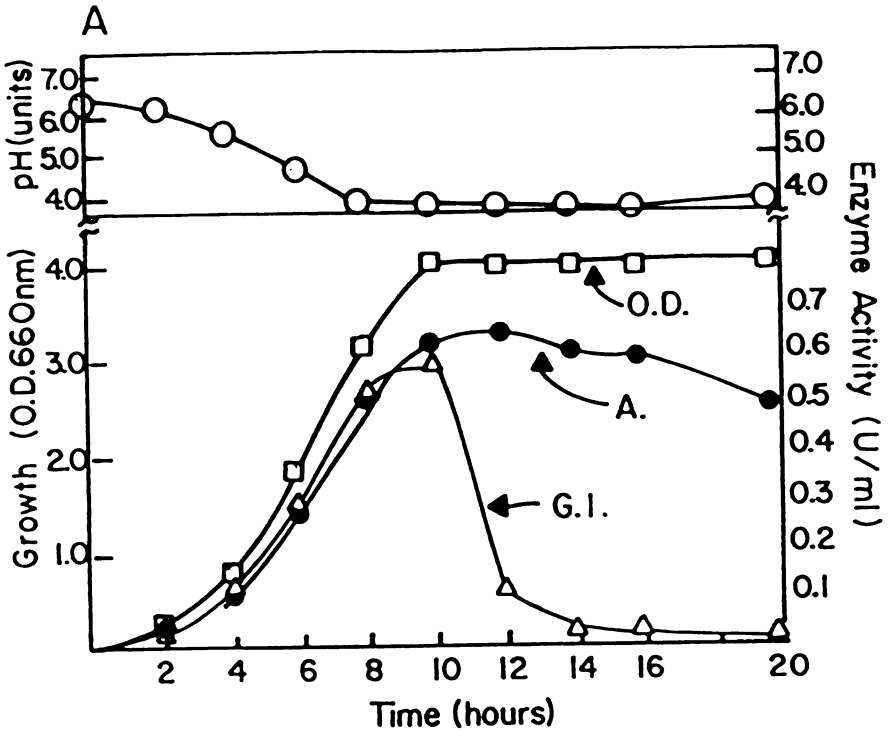

Growth (control)
Growth (+Glc)
Growth (-Glc)
Glc
G.I (-Glc)
G.I (+Glc)
G.I (control)
GROWTH (mM, Ethanol produced)
Glucose Isomerase Activity (U/ml)
Time (hours)
25
20
10
0.2
0.15
0.1
0.05
2
4
6
8
10
12
14
16
20

Figure 2. Effect of pH control on saccharidase production during xylose fermentation.

Cultures were grown in 500 ml TYE medium containing 2% xylose in a Multigen fermentor at 60°C. A: without pH control; B: pH was controlled at 5.5 by feeding 0.5 N NH_4OH. Symbols represent glucose isomerase (G.I.) and amylase (A.).


A
pH (units)
7.0
6.0
5.0
4.0
Growth (O.D.660nm)
4.0
3.0
2.0
1.0
O.D.
A.
G.I.
Enzyme Activity (U/ml)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
2
4
6
8
10
12
14
16
20
Time (hours)

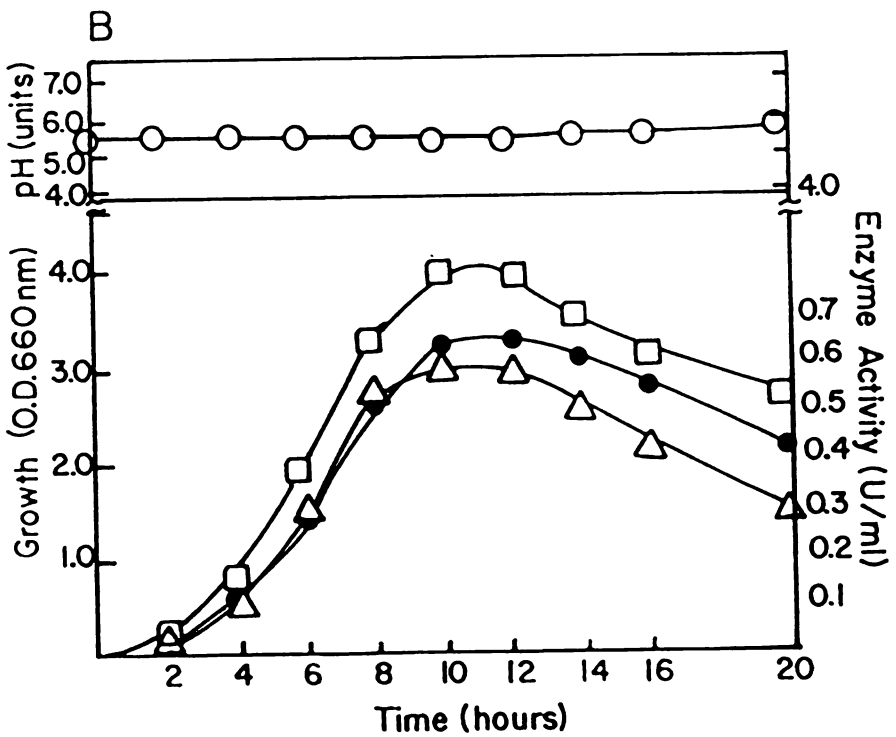
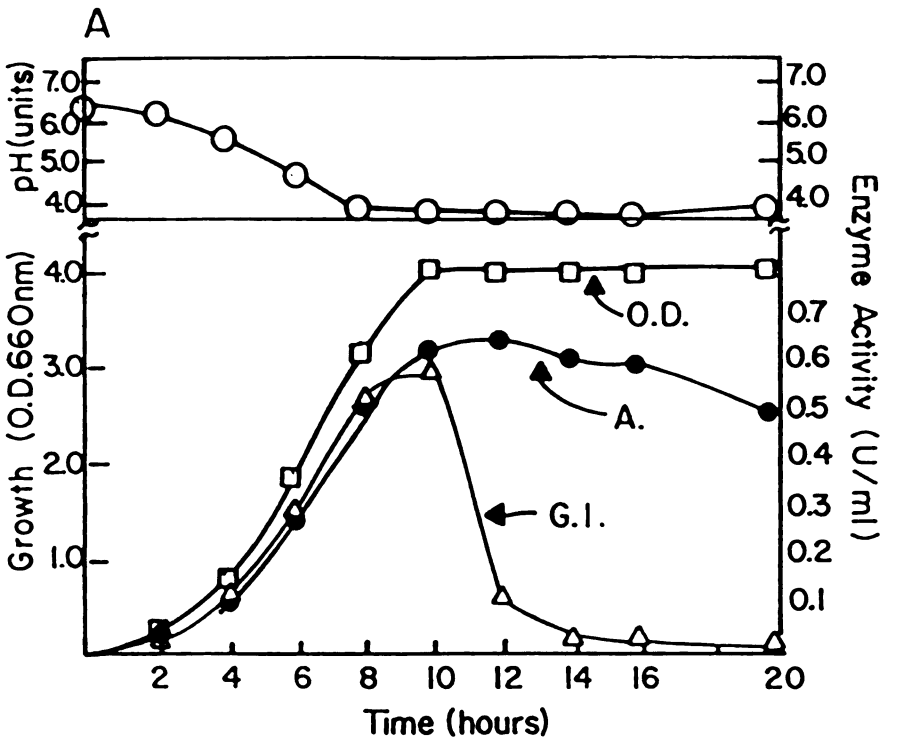
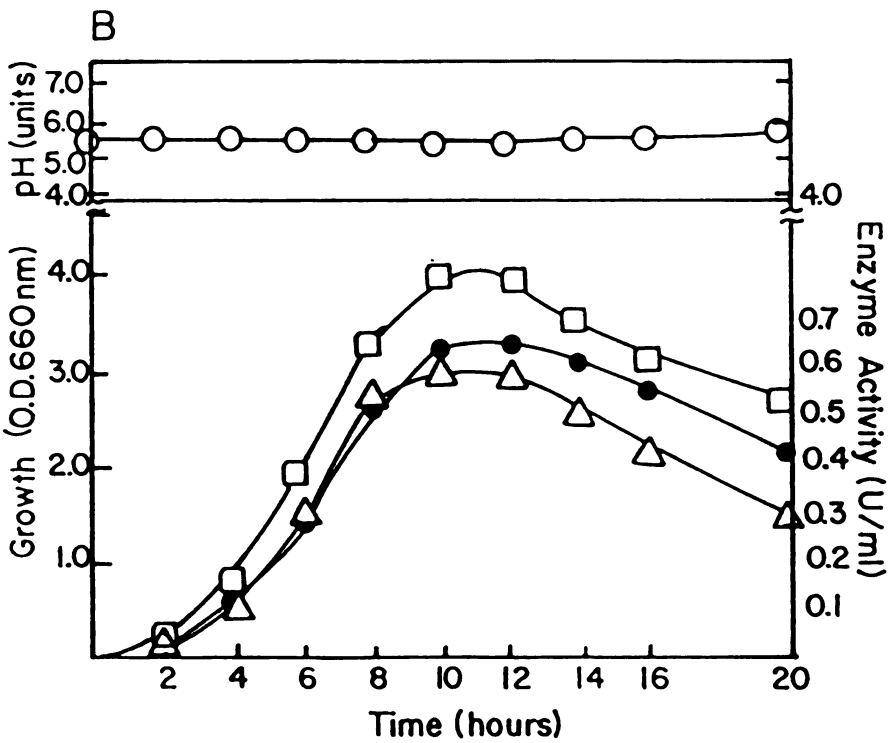

B
pH (units)
7.0
6.0
5.0
4.0
Growth (O.D.660nm)
4.0
3.0
2.0
1.0
Enzyme Activity (U/ml)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
2
4
6
8
10
12
14
16
20
Time (hours)

Figure 2. Effect of pH control on saccharidase production during xylose fermentation.

Cultures were grown in 500 ml TYE medium containing 2% xylose in a Multigen fermentor at 60°C. A: without pH control; B: pH was controlled at 5.5 by feeding 0.5 N NH_4OH. Symbols represent glucose isomerase (G.I.) and amylase (A.).


A
pH(units)
7.0
6.0
5.0
4.0
Growth (O.D.660nm)
4.0
3.0
2.0
1.0
Enzyme Activity (U/ml)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
O.D.
A.
G.I.
2
4
6
8
10
12
14
16
20
Time (hours)


B
pH (units)
7.0
6.0
5.0
4.0
Growth (O.D.660nm)
4.0
3.0
2.0
1.0
Enzyme Activity (U/ml)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
2
4
6
8
10
12
14
16
20
Time (hours)

was used in xylose fermentations to obtain *Thermoanaerobacter* enzyme preparations that contained active levels of amylase, glucose isomerase, and ß-galactosidase.

Physicochemical Properties of Saccharidases

The temperature and pH activity and stability profiles for glucose isomerase and ß-galactosidase were compared to the amylase activities in *Thermoanaerobacter* extracts prepared from washed whole cells grown on xylose. Glucose isomerase, amylase, and ß-galactosidase displayed an apparent temperature optimum for activity between 75-80°C, 70°C, and 65°C, respectively (Figure 3A). The effect of temperature on the stability of these enzyme activities is shown in Figure 3B. Cell extracts for enzyme assays were pre-incubated prior to measuring the residual a activities in 100 mM sodium phosphate buffer (pH 7.0) containing 10 mM $MgSO_4$ and 1 mM $CoCl_2$ for glucose isomerase, in 100 mM sodium acetate buffer (pH 5.5) for amylase, and in 100 mM sodium acetate buffer (pH 6.0) for ß-galactosidase. Under these conditions, glucose isomerase, amylase, and ß-galactosidase were stable up to 60 min at 85°C, 70°C, and 60°C, respectively.

Figure 4 illustrates the dependence of saccharidase activities on pH. Glucose isomerase displayed a broad pH range for activity from 5.5 to 9.0 with an apparent pH optimum of 7.0-7.5. During the enzyme assay at alkaline pH, chemical isomerization of glucose to fructose by alkali caused a high background reading and the amount of product formed by enzymatic isomerization was calculated by subtracting the control value from the total experimental value. The amylase and ß-galactosidase activities displayed a narrower pH range for activity than that of glucose

Figure 3. Comparison of temperature optima for activities (A) and stabilities (B) of glucose isomerase, amylase, and ß-galactosidase.

Enzymes were assayed with cell extract from xylose grown cells. 100% activity corresponds to 0.60 U/mg; 0.58 U/mg, 0.46 U/mg for glucose isomerase, amylase, ß-galactosidase, respectively. Cell extracts in 50 mM sodium phosphate buffer (pH 7.0) for glucose isomerase, in 100 mM sodium acetate buffer (pH 5.5) for amylase, and in 100 mM sodium phosphate buffer (pH 6.0) for ß-galactosidase were preincubated at the indicated temperatures prior to the assay for residual enzyme activities.

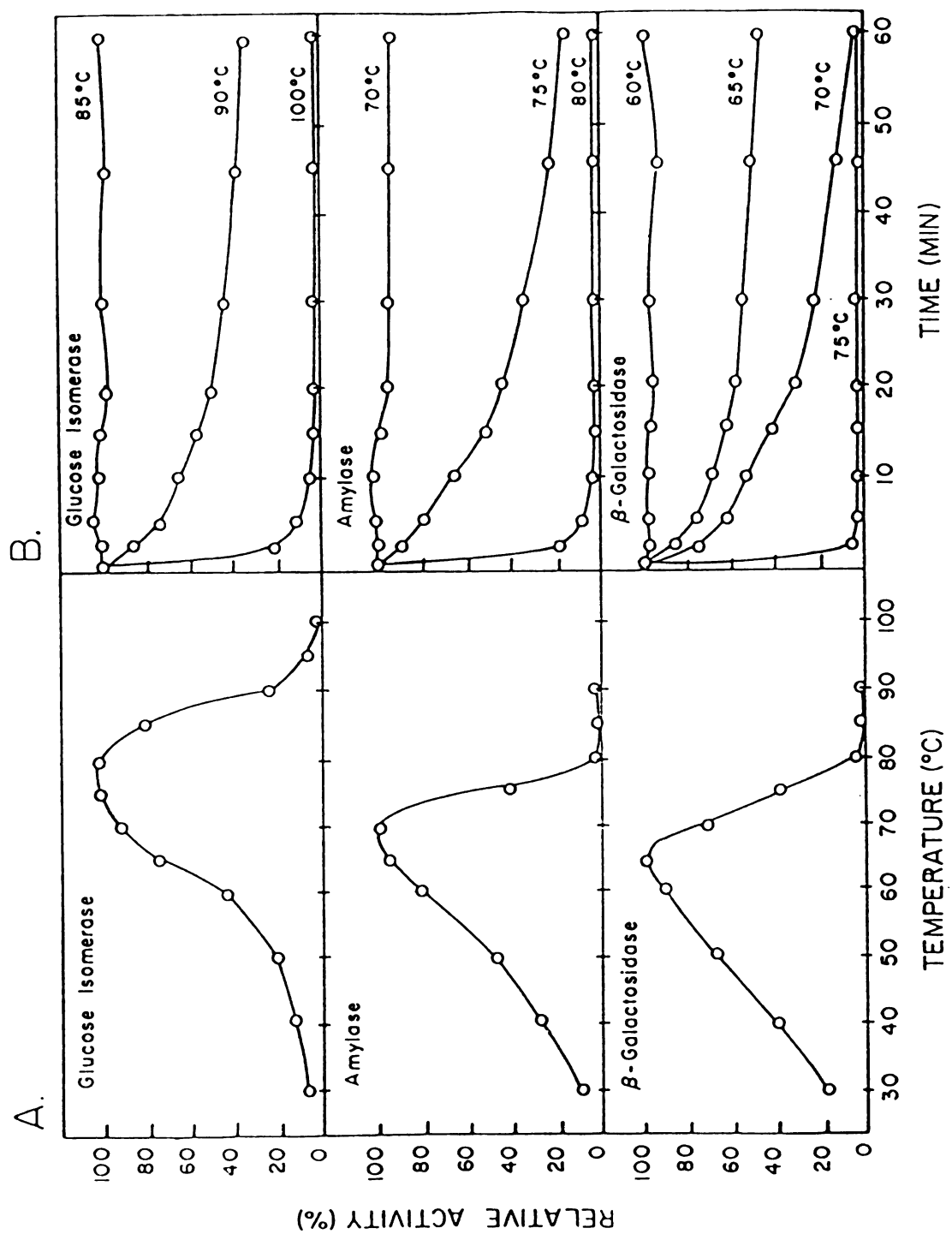
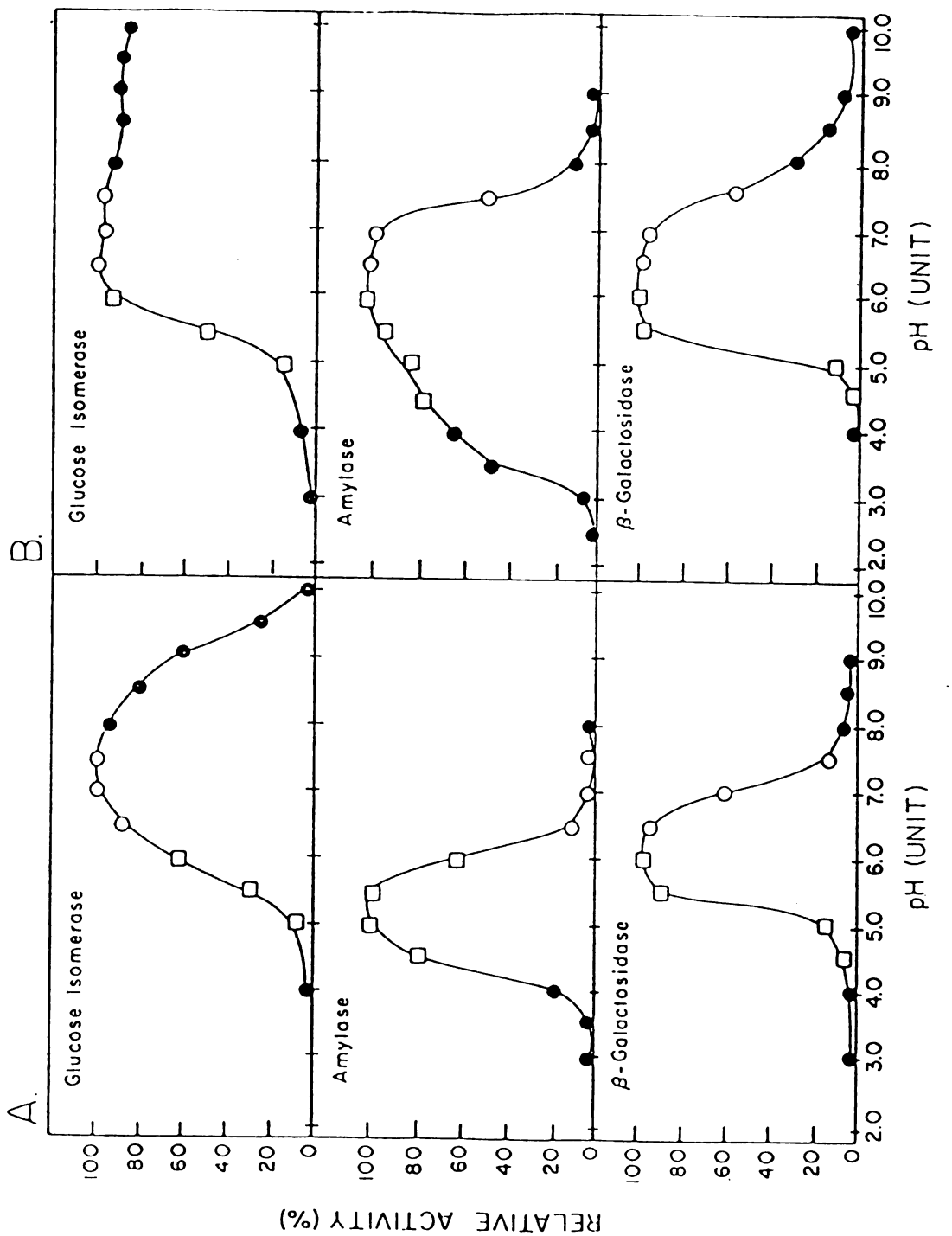

A.
B.
Glucose Isomerase
Amylase
β-Galactosidase
85°C
90°C
100°C
70°C
75°C
80°C
60°C
65°C
70°C
75°C
RELATIVE ACTIVITY (%)
TEMPERATURE (°C)
TIME (MIN)

Figure 4. Comparison of pH optima for activity (A) and stability (B) of glucose isomerase, amylase, and ß-galactosidase.

Enzyme activities were assayed with cell extracts in 100 mM glycylglycine hydrochloride (●—●), sodium acetate (□—□), and sodium phosphate (○—○) buffers. Residual activity was measured (B) after treatment at 60°C for 1 h.


A.
B.
Glucose Isomerase
Amylase
β-Galactosidase
RELATIVE ACTIVITY (%)
pH (UNIT)
0
20
40
60
80
100
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0

isomerase and the apparent pH optima were between 5.0-5.5 and 6.0-6.5, respectively. The stability of these enzymes in relation to pH was examined by measuring residual activities after incubation for 1 h at 60°C at different pH values (Figure 4B). Under these assay conditions, glucose isomerase, amylase, and ß-galactosidase activities were stable at pH 6.0-7.5, pH 5.5-7.0, and pH 5.5-7.0, respectively. All the saccharidase activities were stable and displayed at least 60% of maximal activity at pH 6.0.

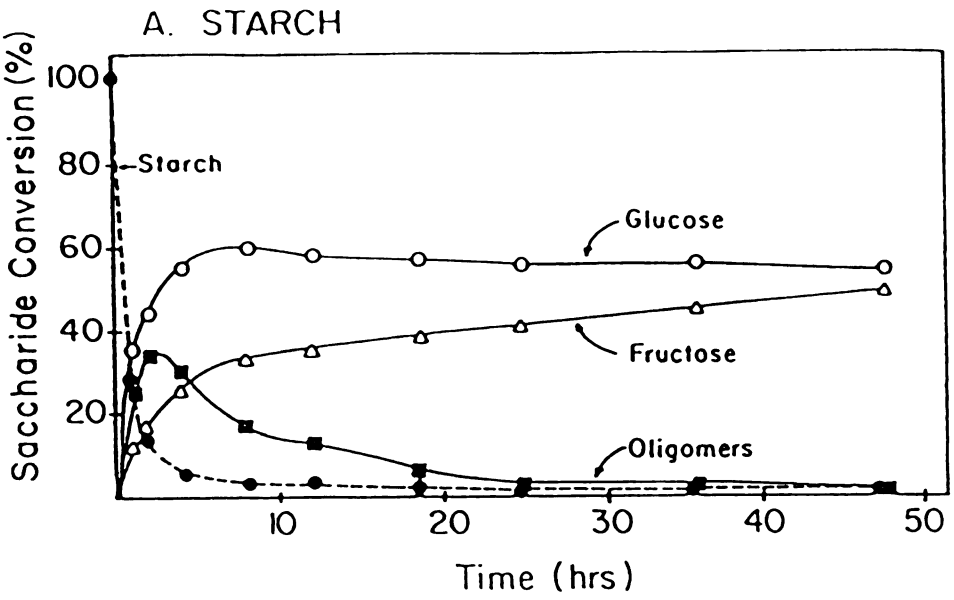
Single Step Conversion of Starch or Lactose into Fructose Syrups

The feasibility of using *Thermoanaerobacter* saccharidases in a single step enzymatic process for producing fructose syrups from liquefied starch or whey-lactose was examined using a crude extract of xylose grown *Thermoanaerobacter* cells which contained environmentally compatible saccharidases. Figure 5 depicts a typical time course for production of monosaccharides from starch or lactose using a single step process with *Thermoanaerobacter* saccharidases at 60°C. More than 90% of the starch or lactose was hydrolyzed into glucose mixtures within 4 h while the isomerization of glucose into fructose approached equilibrium by the end of incubation period (20-48 h). An unknown oligosaccharide, putatively allolactose, was produced at the early stages of lactose conversion and was slowly degraded into monomers. Allolactose is a reversion product formed by ß-galactosidase activity.

Table 3 compares the influences of specific reaction condition changes on the final saccharide product ratio achieved during single step conversion of starch and lactose by *Thermoanaerobacter* saccharidases. During enzymatic starch conversion,

Figure 5. Single step conversion of starch (A) and lactose (B) into a fructose mixture by a *Thermoanaerobacter* enzyme preparation.

5% maltodextrin (A) and 5% lactose (B) were incubated with cell extract at 60°C in 100 mM sodium phosphate buffer at pH 6.0 and 6.5, respectively.


A. STARCH
Saccharide Conversion (%)
100
80
60
40
20
Starch
Glucose
Fructose
Oligomers
10
20
30
40
50
Time (hrs)

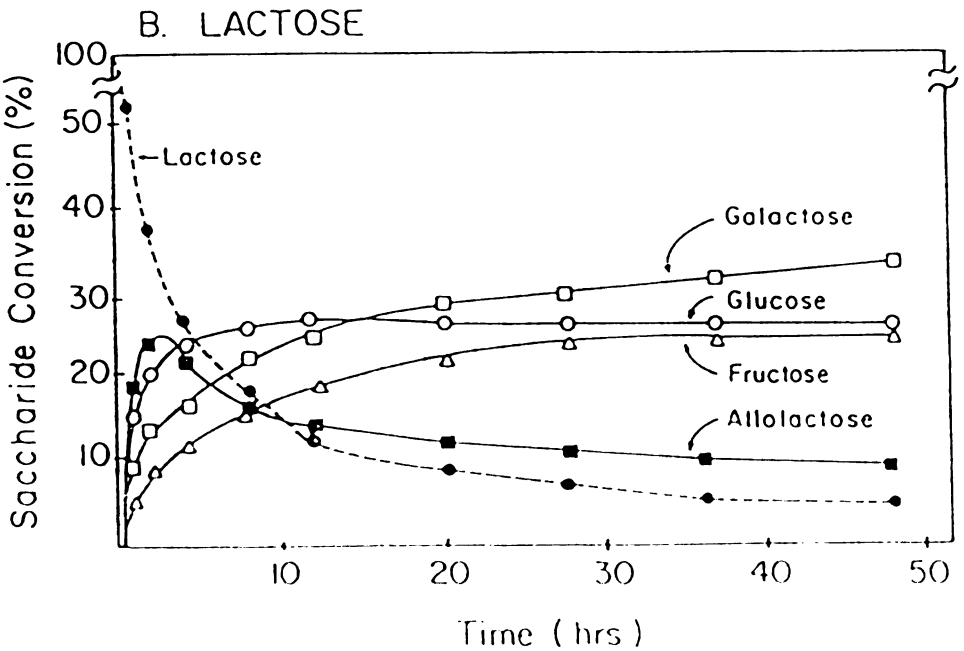

B. LACTOSE
Saccharide Conversion (%)
100
50
40
30
20
10
Lactose
Galactose
Glucose
Fructose
Allolactose
10
20
30
40
50
Time (hrs)

Table 3. Effect of reaction conditions on the final monosaccharide product ratio during a starch or lactose conversion process with a *Thermoanaerobacter* enzyme preparation.[a]

Substrate	Concentration (w/v) %	pH	Temperature (°C)	Conversion Yield (%)[b]	Final Product Ratio[c]
					(Glc:Frc)
Liquefied starch	5	6.0	60	92	53:47
	5	6.0	65	93	51:49
	5	6.0	70	96	49:51
	20	6.0	60	84	58:42
	20	6.0	70	91	51:49
					(Gal:Glc:Frc)
Lactose	5	6.4	60	85	40:31:29
	5	6.8	60	81	40:31:29
	5	6.8	65	76	35:33:32
	20	6.4	60	75	36:33:31

[a]Cell extract (5 mg and 10 mg/ml of reaction vol.) prepared from xylose grown cells was added into the reaction mixture containing 5% and 20% substrate, respectively. Maltodextrin (DE 10) was used as a liquefied starch.

[b]Total amount of monosaccharides hydrolyzed from the substrate.

[c]Relative ratio between monosaccharides was measured after 48 hours incubation under the given reaction conditions.

a higher sweetener concentration ratio for fructose to glucose and a higher yield of starch conversion into monosaccharides were achieved at 70°C than at 60°C. Liquefied starch was used here but equivalent results were obtained with soluble starch and the saccharidase preparation also hydrolyzed raw starch (data not shown). The highest sweetener conversion (fructose-51 to glucose-49) was achieved at pH 6.0 and 70°C from 5% (w/v) maltodextrin with a final conversion yield of 96%. Enzymatic hydrolysis of 5% lactose at pH 6.8 versus 6.4 lowered the total conversion yield of lactose, but did not affect the final product ratio between galactose, glucose, and fructose. Enzymatic hydrolysis of 20% lactose at 65°C versus 60°C lowered both the lactose conversion yield and the galactose concentration in the final products.

DISCUSSION

To our best knowledge, these findings represent the first reported studies on the general physicochemical properties and regulation of glucose isomerase from a thermophilic microorganism. Although thermophilicity is required in industrial glucose isomerase, thermostable enzymes produced by mesophilic bacteria have been examined as principal industrial sources (4). Furthermore, these studies represent the first demonstration that a saccharidase mixture produced by a single microorganism can be used to directly process starch or lactose into a fructose sweetener. Several previous studies have demonstrated that glucoamylase and glucose isomerase mixtures from different microbes can process oligodextrins into fructose syrups with marginal success because of enzyme pH and thermal incompatibilities (10, 13).

Regulation of amylase and xylanase activities in *Thermoanaerobacter* strain B6A is different from the inducible and glucose catabolite repressed synthesis of amylase activities reported in *C. thermosulfurogenes* and *C. thermohydrosulfuricum* (14-16). Thermostable ß-galactosidase has been previously reported in aerobic *Thermus* species; however, enzyme synthesis is regulated by induction and glucose catabolite repression. On the other hand, the ß-galactosidase of *Thermoanaerobacter* strain B6A is constitutive and non-catabolite repressible.

As expected of enzymes from thermophiles, the apparent optimum temperatures for glucose isomerase (75-80°C), glucogenic amylases (70°C), and ß-galactosidase (65°C) in crude extracts were relatively high. The reported temperature optima of glucose isomerases from other microorganisms vary with enzyme source, they range

from 45°C to 90°C (5). The temperature optima of glucogenic amylase activities reported in *Clostridium thermohydrosulfuricum* (13) was similar to those in *Thermoanaerobacter*. The temperature optima of ß-galactosidases range from 35°C to the high of 80°C reported in *Thermus* strains (6,9). Analysis of *Thermoanaerobacter* saccharidase thermostabilities showed that more than 95% of glucose isomerase, glucogenic amylases, and ß-galactosidase were retained after 60 min incubation at 85°C, 70°C, and 60°C.

The pH optima for glucose isomerase, glucoamylase, and ß-galactosidase are generally between 7.0 to 8.5, 4.5 to 5.5, and 4.5 to 7.5, respectively (5,14,9). Although the apparent pH optima for *Thermoanaerobacter* glucose isomerase, ß-galactosidase, and glucogenic amylase activities fall in this range, all three enzymes were stable and active at pH 6.0-6.5 and 60°C. The glucose isomerase displayed the least acid stability and activity of the saccharidases studied. In a separate report (manuscript submitted) we purified the *Thermoanaerobacter* glucose isomerase to homogeneity and showed that the 200,000 MW tetramer has pH and thermal properties identical to those reported here. Thus, we conclude here that the rapid destruction of glucose isomerase activity in non-pH controlled xylose fermentation is due to enzyme instability at the low pH values and high temperature of stationary phase cultures.

The difference in pH optima or temperature stability of these saccharidases has been a major problem in demonstrating the feasibility of a single step process for conversion of starch or lactose to fructose mixtures. The present study indicates that saccharidases simultaneously produced by *Thermoanaerobacter* are environmentally

compatible at pH 6.0 and 60°C and can be used coordinately in a single step conversion process for production of fructose sweetener from starch or milk derived substrates.

The final ratios between glucose and fructose during the single step conversion process from starch at various temperatures were very similar to the theoretical values at equilibrium of the glucose isomerization reaction (1,17). The maximum percent of starch conversion from liquefied starch during this process was also very similar to the values (94-96% of dextrose) after starch saccharification in the multistep commercial processes (1). The *Thermoanaerobacter* saccharidase preparation could be operated at 70°C to achieve the higher equilibrium concentrations of fructose and faster reaction rates than those practiced in industrial processes. If reduced to practice, a single step starch hydrolysis process at high temperature (>60°C) and acid pH (<pH 6.0) would lower costs for producing high fructose sweetener from corn starch. The *Thermoanaerobacter* saccharidases provide a first stage feasibility model for developments of such a process. Use of *Thermoanaerobacter* saccharidases can also directly enhance sweetness in milk containing products by converting lactose to fructose during the manufacturing process. Hydrolysis of lactose in these products can potentially solve the digestion problems of lactase deficient adults, improve the solubility during ice cream and yogurt production and require less additive sugars in dairy products. Further studies on long term stability of the immobilized enzymes and its food safety are required before any commercial value for *Thermoanaerobacter* saccharidases can be proposed.

LITERATURE CITED

1. Antrim, R. L., W. Colliala, and B. J. Schnyder. 1979. Glucose isomeraseproduction of high-fructose syrups, p. 97-155. In L.B. Wingard (ed.),Applied Biochemistry and Bioengineering, Academic Press, New York.

2. Aunstrup K., O. Andresen, E. A. Falch, and T. K. Nielsen. 1979. Production of microbial enzymes, p. 281-309. In Microbial Technology, 2nd ed., vol 1, Academic Press.

3. Buke, C. 1980. Enzymes in fructose manufacture, p. 51-72. In G.G. Birch, N. Blackebrough, and J.K. Parker (ed), Enzymes and Food Processing. Applied Science Publishers, London.

4. Chen, W. 1980a. Glucose isomerase (a review). Process Biochem. 15:30-35.

5. Chen, W. 1980b. Glucose isomerase (a review). Process Biochem. 15:36-41.

6. Cowan, D. A., R. M. Daniel, A. M. Martin, and H. W. Morgan. 1984. Some properties of a ß-galactosidase from an extremely thermophilic bacterium. Biotechnol. Bioeng. 26:1141-1145.

7. Crueger, W., and A. Crueger. 1982. Enzymes, p. 161-174. In W. Crueger and A. Crueger (ed.), Biotechnology: a textbook of industrial microbiology, Science Tech. Inc., Madison.

8. Dishe, Z., and E. Borenfreund. 1951. A new spectro-photometric method for the detection and determination of keto sugars and trioses. J. Biol. Chem. 192:583-587.

9. Gekas, V., and M. Lopez-Leiva. 1985. Hydrolysis of lactose: a literature review. Process Biochem. 20:2-12.

10. Guillaume, R., and P. Walon. 1977. Preparation of levulose from granular starch. U.S. Patent 4,009,074.

11. Goodman, R. E., and D. M. Pederson. 1976. ß-galactosidase from *Bacillus stearothermophilus*. Can. J. Microbiol. 22:817-825.

12. Hafner, E. W. 1985. Constitutive production of a thermostable glucose isomerase. U.S. Patent 4,551,430.

13. Horwath, R. O., and R. M. Irbe. 1986. Process for preparing fructose from starch. U. S. Patent 4,605,619.

14. Hyun, H. H., and J. G. Zeikus. 1985. General biochemical characterization of thermostable pullulanase and glucoamylase from *Clostridium thermohydrosulfuricum*. Appl. Environ. Microbiol. 49:1168-1173.

15. Hyun, H. H., and J. G. Zeikus. 1985. Regulation and genetic enhancement of glucoamylase and pullulanase production in *Clostridium thermohydrosulfuricum*. J. Bacteriol. 164:1146-1152.

16. Hyun, H. H., G. J. Shen, and J. G. Zeikus. 1985. Differential amylosaccharide metabolism of *Clostridium thermosulfurogenes* and *Clostridium thermohydrosulfuricum*. J. Bacteriol. 164:1153-1161.

17. Jorgensen, O. B., L. G. Karlsen, N. B. Nielsen, S. Pedersen, S. Rugh, and Bagsvaerd. 1988. A new immobilized glucose isomerase with high productivity produced by a strain of *Streptomyces murinus*. Starch/Starke 40:307-313.

18. Lowry, O. H., J. Rosebrough, A. I. Farr, and R. J. Randall. 1951. Protein measurement with the foline phenol reagent. J. Biol. Chem. 193:265-275.

19. Melasniemi, H. 1987. Characterization of α-amylase and pullulanase activities of *Clostridium thermohydrosulfuricum*: Evidence for a novel thermostable amylase. Biochem. J. 246:193-197.

20. Melasniemi, H. 1987. Effect of carbon source on production of thermostable α-amylase, pullulanase and α-glucosidase by *Clostridium thermohydrosulfuricum*. J. Gen. Microbiol. 133:883-890.

21. Miller, G. L. 1959. Dinitrosalicylic acid reagent for detection of reducing sugar. Anal. Chem. 31:426-428.

22. Miller, J. H. 1972. Purification of ß-galactosidase, p. 398-404. In J.H. Miller (ed.), Experiments in Molecular Genetics. Cold Spring Harbor Laboratory,

23. Saha, B. C., S. P. Mathupala, and J. G. Zeikus. 1988. Purification and characterization of a highly thermostable pullulanase from *Clostridium thermohydrosulfuricum*. Biochem. J. 252:343-348.

24. Sanchez, S., and C. M. Quinto. 1975. D-Glucose isomerase: constitutive and catabolite repression-resistant mutants of *Streptomyces phaeochromogenes*. Appl. Microbiol. 30:750-754.

25. Ulrich, J. T., G. A. McFeters, and K. L. Temple, 1972. Induction and Characterization of ß-galactosidase in an Extreme Thermophile. J. Bacteriol. 110:691-698.

26. Wiegel, J.W.K. 1986. Genus Thermoanaerobacter, p. 1379-1383. In J.G. Holt, P.H.A. Sneath, N.S. Mair, and M.E. Sharpe (ed), Bergy's Manual of Systematic Bacteriology, vol 2, 9th ed. Williams and Wilkins, Baltimore.

27. Weimer, P. J., L. W. Wagner, S. Knowlton, and T. K. Ng. 1984. Thermophilic anaerobic bacteria which ferment hemicellulose: characterization of organisms and identification of plasmids. Arch. Microbiol. 138:31-36.

28. Yokote, Y., K. Kimura, and H. Samejima. 1975. Production of high fructose syrup by glucose isomerase immobilized on phenol-formaldehyde resine. Die Starke. 27:302-306.

29. Zeikus, J. G., A. Ben-Bassat, and P. W. Hegge. 1980. Microbiology of methanogenesis in thermal, volcanic environments. J. Bacteriol. 143:432-440.

30. Zeikus, J. G. 1979. Thermophilic bacteria: ecology, physiology, and technology. 1979. Enzyme Microb. Technol. 1:243-252.

Chapter III

Purification and Characterization of Thermostable Glucose Isomerase from *Clostridium thermosulfurogenes* and *Thermoanaerobacter*

ABSTRACT

Glucose isomerases produced by *Thermoanaerobacter* strain B6A and *Clostridium thermosulfurogenes* strain 4B were purified 10- to 11-fold to homogeneity and their physicochemical and catalytic properties were determined. Both purified enzymes displayed very similar properties (native M_r= 200 kDa, tetrameric subunit composition, and apparent temperature and pH optima at 80°C and pH 7.0-7.5). The enzymes were stable at pH 5.5-12.0, and maintained more than 90% activity after incubation at high temperature (85°C) for 1 hr in the presence of metal ions. The N-terminal amino acid sequence of both thermostable glucose isomerases were Met-Asn-Lys-Tyr-Phe-Glu-Asn and were not homologous to that of the thermolabile *Bacillus subtilis* enzyme. The glucose isomerase from *C. thermosulfurogenes* and *Thermoanaerobacter* displayed isoelectric pH's of 4.9 and 4.8, and their K_{cat} and V_{max} values for D-glucose were 1040, 1260 min^{-1} and 140, 120 mM, respectively. Both enzymes displayed higher K_{cat} and lower K_m values for D-xylose than those for D-glucose. *C. thermosulfurogenes* enzyme required Co^{++} or Mg^{++} for thermal stability and glucose isomerase activity, and Mn^{++} or these metals for xylose isomerase activity. Crystals of *C. thermosulfurogenes* glucose isomerase were formed at room temperature by the hanging drop method using 16%-18% PEG 4000 in 0.1 M citrate buffer.

INTRODUCTION

D-Glucose (D-xylose) isomerase (EC 5.3.1.5) is an intracellular enzyme found in a number of bacteria which can utilize xylose as a carbon substrate for growth (8). Although the physiological function of the enzyme *in vivo* is isomerization of D-xylose to D-xylulose, this enzyme also converts D-glucose to D-fructose ***in vitro*** (32). The latter activity of the enzyme is being used in industry for production of high fructose corn syrup, and glucose isomerase is one of the largest volume commercial enzymes used today (2,6). Due to the industrial significance of the enzyme, glucose isomerases have been studied from various microorganisms and their catalytic and physicochemical properties have been reviewed (9). Immobilization techniques and a continuous isomerization process with the enzyme have also been described (18,29).

Most commercially available glucose isomerases are isolated from mesophilic microorganisms including those from *Streptomyces, Actinoplanes, Flavobacterium*, and *Bacillus* species. These enzymes are generally thermostable, and are utilized in the immobilized form to enhance enzyme half-life (35). These enzymes require metal ions for their activity and stability, and the pH optima for enzyme activity fall in the range of 7.5-9.0. The reaction temperature used in the current industrial process for sweetener production is limited to 60°C because of by-product and color formation during reaction at high temperature and alkaline pH (7). However, operation of the reaction at higher temperature than 60°C has several advantages to achieve faster reaction rates, higher equilibrium concentrations of fructose, and reduced viscosity of the substrate and product. Therefore, a novel glucose isomerase with a lower

optimum pH (neutral or slightly acidic) and high thermal stability might have practical value for industrial application.

Thermophilic microorganisms are known to produce intrinsically thermostable enzymes which have been evolved and adapted to the extreme environment of their natural habitat (1). Advantages of using these thermostable enzymes in industrial processes were proposed (24, 40). Our laboratory has recently reported on purification and biochemical characterization of extremely thermostable ß-amylase and pullulanase from *Clostridium thermosulfurogenes* and *C. thermohydrosulfuricum*, respectively (25, 30). In spite of the large amount of studies on glucose isomerases from various enzyme sources, nothing is known about the detailed biochemical or molecular genetic properties of glucose isomerases from thermoanaerobic bacteria.

Recently we demonstrated that a new *Thermoanaerobacter* species contained an inducible xylose/glucose isomerase activity (C. Lee, B. Saha, and J. G. Zeikus, manuscript submitted to Appl. Environ. Microbiol.). We demonstrate here that thermostable glucose isomerases are also present in *C. thermohydrosulfuricum* and *C. thermosulfurogenes*; and, report on the biochemical characterization of similar glucose isomerases purified from *C. thermosulfurogenes* strain 4B and *Thermoanaerobacter* strain B6A.

MATERIALS AND METHODS

Chemicals, Organisms, and Growth Conditions

Medium components and all other chemicals were reagent grade. *C. thermosulfurogenes* strain 4B (28) and *Thermoanaerobacter* strain B6A (36) were routinely grown at 60°C, and *C. thermohydrosulfuricum* strain 39E (39) was grown at 65°C in anaerobic 26 ml pressure tubes or in 1 liter round bottom flasks that contained TYE medium (39) supplemented with 1% xylose. For large-scale enzyme preparation, cultures were grown at 60°C in a 14 liter Braun fermentor (West Germany) containing 10 liter TYE medium with 2% xylose, and the culture pH was maintained at 5.5 on line by control with 1 N NaOH. Cells were harvested at the late exponential growth phase using a Pellicon cell harvester (Millipore Corp., Bedford, MA) and washed with 50 mM MOPS buffer (pH 7.0) containing 10 mM $MgSO_4$ and 1 mM $CoCl_2$ prior to freezing.

Enzyme Assays

Glucose isomerase activity was measured by incubating a 1 ml reaction mixture that contained 0.8 M glucose, 10 mM $MgSO_4$, 1 mM $CoCl_2$, and an enzyme preparation in 100 mM MOPS buffer (pH 7.0). For the assay of xylose isomerase activity, the reaction mixture (1 ml) contained 70 mM xylose, 10 mM $MnSO_4$, and the enzyme preparation in 100 mM MOPS buffer (pH 7.0). After 30 min incubation at 65°C, 1 ml of 0.5 M perchloric acid was added to stop the reaction and the mixture was further diluted 50- and 10-fold with double distilled water before the

quantitative analysis for fructose and xylulose, respectively. The amount of fructose and xylulose was estimated by the cysteine-carbazole sulfuric acid method (14). Fructose isomerizing activity was assayed under the same reaction conditions as the glucose isomerase assay, except 0.4 M fructose instead of 0.8 M glucose was used in the reaction mixture. After 30 min incubation, the reaction was terminated by placing the assay tubes on ice, and the amount of glucose formed was estimated with a glucose analyzer (Yellowstone Instrument, model 27). One unit of glucose isomerase, xylose isomerase, and fructose isomerase activity is defined as the amount of enzyme that produced 1 μmol of fructose, xylulose, and glucose, respectively, per min under the assay condition. Protein concentration was determined by the method of Lowry (23) with bovine serum albumin used as the standard.

Purification of Glucose Isomerase

All the procedures were performed under aerobic conditions at 4°C.

(i) Preparation of cell extract: 50 g of cells of *Thermoanaerobacter* or *C. thermosulfurogenes* were suspended in 200 ml of 50 mM MOPS buffer (pH 7.0) containing 10 mM $MgSO_4$ and 1 mM $CoCl_2$. The cells were disrupted by two passages through a French pressure cell at 18,000 p.s.i. The cell debris was removed by centrifugation at 12,000 x g for 20 min, and the supernatant was used as the crude enzyme preparation.

(ii) Heat treatment: The cell extracts of *Thermoanaerobacter* and *C. thermosulfurogenes* were heated for 15 min at 85°C and 80°C, respectively, and cooled to 4°C. The soluble fractions were recovered after centrifugation at 12,000

x g for 20 min.

(iii) Ammonium sulfate fractionation: Solid ammonium sulfate was added to the heat-treated extracts to give 65% saturation and the precipitate was removed by centrifugation. More ammonium sulfate was added to the supernatant to give 85% saturation. This precipitate was collected, and dissolved in 50 mM MOPS buffer (pH 7.0) containing 5 mM $MgSO_4$ and 0.5 mM $CoCl_2$, and dialyzed overnight against the same buffer.

(iv) Column chromatography: The above enzyme preparations were loaded onto DEAE-Sepharose CL-6B columns (4.0 cm x 32 cm), previously equilibrated with 50 mM MOPS buffer (pH 7.0) containing 5 mM $MgSO_4$ and 0.5 mM $CoCl_2$. The columns were washed with same buffer and then eluted with NaCl salt gradient (0.0 to 0.5 M) in the same buffer. The activity peak fractions were pooled and concentrated by ultrafiltration (YM 30 membrane, Amicon Co.). The enzyme solutions were divided into three portions and each portion was applied onto a Superose-12HR (Pharmacia, Inc.) gel filtration column, and were eluted with the same buffer with a Pharmacia FPLC System.

Electrophoresis and molecular weight determination

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (22). Native-PAGE was performed without SDS, and Tris-HCl buffer (pH 8.6) was used during polyacrylamide gel preparation. Protein bands were visualized by Coomassie Brilliant Blue G-250 staining.

Molecular weights of purified glucose isomerases were determined by gel

filtration on a Superose-12HR column in a FPLC system, and blue dextran (Mr 2,000,000); apoferritin (443,000); alcohol dehydrogenase (150,000); and bovine serum albumin (66,000) were used as molecular weight standards. The subunit molecular weights were estimated by SDS-PAGE with low range protein molecular weight standards (Bio Rad Laboratories) : phosphorylase (Mr 97,400); bovine serum albumin (66,200); ovalbumin (42,700); carbonic anhydrase (31,000); and soybean trypsin inhibitor (21,500).

Two different pH ranges of Servalyt-Precotes isoelectric focusing gels (Serva Co.; pH 3-10 and pH 3-6) were used for isoelectric pH determination. An LKB ultrapore isoelectric focusing apparatus was used and the gels were stained with Serva Blue W.

Amino acid compositions and sequence determination

Samples were prepared by washing the purified glucose isomerase preparations with double distilled water five times with a Centricon-30 (Amicon Co.) filtration device to remove metal salts in the enzyme solution. Amino acid composition analysis was performed with Water's Pico-Tag amino acid analyzer, and the N-terminal amino acid sequences were identified with a Beckman Model 890M phase sequencer in the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University.

Metal ion effects on enzyme activity and stability

To prepare a metal ion-free enzyme solution, glucose isomerase purified from *C.*

thermosulfurogenes was treated with 5 mM EDTA at 60°C for 1 hr, and then washed with double distilled water five times by an Amicon Centricon-30 filtration device. The effect of metal ions on enzyme thermal stability was determined by measuring residual glucose isomerase activity under optimum assay conditions after a 15 min preincubation at 80°C in the presence of various metal ions (1 mM). The effect of metal ions on enzyme activity was performed at 55°C where the enzyme was stable without metal cofactors during the reaction period.

Protein crystallization

The hanging drop vapor diffusion method was used to crystallize the purified glucose isomerase from *C. thermosulfurogenes* in Linbro tissue culture multi-well plates (Flow Laboratories, Inc., Virginia). The protein solution used to prepare crystals contained glucose isomerase (15 mg/ml) in 50 mM MOPS buffer (pH 7.0) with 10 mM $MgSO_4$ and 1 mM $CoCl_2$. The reservoir solution (1 ml) in each well contained a series of different PEG 4000 concentrations (5%-30%) in 0.1M citrate buffer (pH 5.5) containing 0.2 M Li_2SO_4. Equal volume (6 µl) of protein and reservoir solutions were mixed to make a droplet on a siliconized glass microscope clover slip and then the cover slips were sealed onto the corresponding well with high vacuum grease. Duplicated samples of the plates were incubated at 4°C and 20°C until crystals appeared.

RESULTS

Comparison of glucose isomerase activity in thermoanaerobes

The general biochemical properties of glucose isomerase in crude cell extracts of three different thermoanaerobes were compared (Table 1). Glucose isomerase activity from *C. thermohydrosulfuricum* differed from the other species by displaying a slightly higher thermostability and apparent temperature optimum and a basic (8.5) pH optimum for enzyme activity. All three glucose isomerases required metal ions (Co^{++}, Mg^{++}) for their activity and stability. Glucose isomerase was purified from *C. thermosulfurogenes* and *Thermoanaerobacter* because of their similar neutral pH optima for activity and thermal properties.

Purification and molecular properties

The protocols used for glucose isomerase purification from *C. thermosulfurogenes* and *Thermoanaerobacter* strain B6A achieved a 11- to 10-fold purification and 19-13% yield from cell extracts (see Table 2). An ammonium sulfate fractionation was used to purify the enzyme from *Thermoanaerobacter* because it removed a certain protein that was not well separated by the following steps. In the final purification step, only a single major protein peak was detected by Superose-12 gel filtration with fast performance liquid chromatography. The purified enzymes were considered to be homogeneous by the detection of single bands on SDS-PAGE, native PAGE, and Serva Precoat isoelectric focusing gels (Figure 1).

The molecular weight of purified glucose isomerase was determined by

Table 1. Biochemical properties of glucose isomerase activity in crude cell extracts of thermoanaerobes.

Property	*C. thermosulfurogenes* strain 4B	*Thermoanaerobacter* strain B6A	*C. thermohydrosulfuricum* strain 39E
Optimum pH	7.0-7.5	7.0-7.5	8.5
Optimum temperature	75-80°C	80°C	80-85°C
Temperature stability[a]	80°C	85°C	90°C

[a]Indicates the highest temperature where more then 90% of original activity was retained after a 1 h incubation.

Table 2. Summary of glucose isomerase purification steps

A. *Thermoanaerobacter*

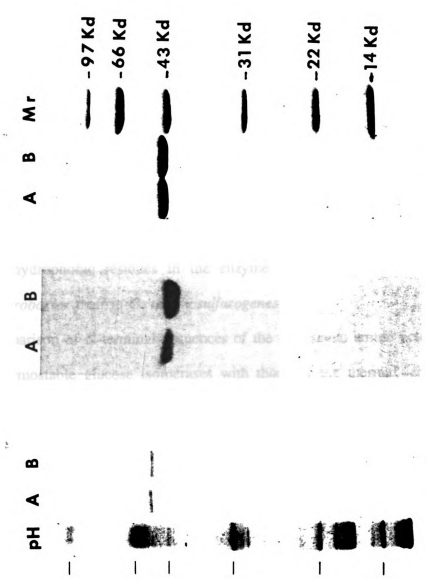
Step	Total Protein (mg)	Total Activity (unit)	Specific Activity (unit/mg)	Purification
Cell Free Extract	2820	1410	0.5	1.0
Heat Treatment (85°C, 15 min)	725	1310	1.8	3.6
Ammonium Sulfate Fractionation	254	585	2.3	4.6
DEAE-Sepharose Anion Exchange	94	385	4.1	8.2
Superose-12 Gel Filtration	38	181	4.8	9.6

B. *C. thermosulfurogenes*

Step	Total Protein (mg)	Total Activity (unit)	Specific Activity (unit/mg)	Purification
Cell Free Extract	2640	820	0.3	1.0
Heat Treatment (80°C, 10 min)	920	690	0.8	2.7
DEAE-Sepharose Anion Exchange	118	342	2.9	9.7
Superose-12 Gel Filtration	46	156	3.4	11.3

Figure 1. Electrophoretic analysis of glucose isomerases purified from *Thermoanaerobacter* (A) and *C. thermosulfurogenes* (B).

pI: isoelectric focusing gel electrophoresis with Servalyt-Precotes gel (pH range; 3-10), Native: native-PAGE with Tris-HCl buffer (pH 8.6), SDS: SDS-PAGE with a 12% gel, pH represents pH separations of protein standards, Mr represents MW separation of protein standards.


pH A B
3.5
4.6
5.1
5.9
6.9
8.3
pI
A B
Native
A B Mr
-97 Kd
-66 Kd
-43 Kd
-31 Kd
-22 Kd
-14 Kd
SDS

Superose-12 gel filtration with FPLC. Glucose isomerase from *C. thermosulfurogenes* and *Thermoanaerobacter* strain B6A displayed an identical molecular weight of 200,000. SDS-PAGE analysis displayed a single band for both enzymes with a molecular weight of 50,000 indicating that both enzymes were composed of homo-tetrameric subunits. Isoelectric points of *C. thermosulfurogenes* and *Thermoanaerobacter* glucose isomerase were pH 4.9 and 4.8, respectively.

The amino acid composition of the purified glucose isomerases are compared in Table 3. These enzymes contained similar amino acid molar ratios except for glutamine and glutamate. Alanine was the most abundant residue and histidine, the proposed catalytic residue (15) was present in both enzyme molecules. The total amount of hydrophobic residues in the enzyme molecule was slightly higher in *Thermoanaerobacter* than in *C. thermosulfurogenes.*

Comparison of N-terminal sequences of the first seven amino acid residues in the two thermostable glucose isomerases with those of the thermal labile enzymes from *Escherichia coli* (27) and *Bacillus subtilis* (37) indicated that the N-terminal sequences of the two thermophile glucose isomerases were identical but they displayed lower homology with mesophile enzymes (Figure 2).

Physicochemical properties

The purified glucose isomerases displayed very similar apparent temperature and pH optima for enzyme activity at 80°C and pH 7.0-7.5 (Figure 3,4). Both glucose isomerases were stable in the broad pH range of 5.5 to 12.0, and were readily denatured at pH values lower than 5 (Figure 4).

Table 3. Amino acid compositions of thermostable glucose isomerase from *C. thermosulfurogenes* and *Thermoanaerobacter*.

Amino Acid	Molar Ratio (%)	
	C. thermosulfurogenes	*Thermoanaerobacter*
Asx	9.5	7.9
Glx	7.3	3.6
Ser	4.0	3.6
Gly	9.1	8.5
His	2.0	2.3
Arg	5.9	6.0
Thr	4.4	4.9
Ala	13.9	15.4
Pro	5.3	4.7
Tyr	4.3	4.9
Val	4.3	3.7
Met	1.7	2.7
Ill	4.3	3.9
Leu	7.1	7.9
Phe	8.8	11.3
Lys	7.6	8.1
Trp	n.d.	n.d.
Cys	n.d.	n.d.
Total	100	100
Hydrophilic	50.3	45.5
Hydrophobic	49.7	54.5

Asx: Aspartic acid and asparagine; Glx: Glutamic acid and glutamine; n.d.: Not determined.

Figure 2. Comparison of N-terminal amino acid sequence of purified glucose isomerases.

Abbreviations are: 4B, *C. thermosulfurogenes*; B6A, *Thermoanaerobacter*; E.C., *E. coli*; B.S., *Bacillus subtilis*. Experimentally determined sequence of 4B and B6A enzymes were aligned with published data for *E. coli* (25) and *B. subtilis* (35).

		1										5		
4B	:	Met	-	Asn	-	Lys	-	Tyr	-	Phe	-	Gln	-	Asn
B6A	:	Met	-	Asn	-	Lys	-	Tyr	-	Phe	-	Gln	-	Asn
E.C.	:	Met	-	Gln	-	Ala	-	Tyr	-	Phe	-	Asp	-	Gln
B.S.	:	Met	-	Ala	-	Gln	-	Ser	-	His	-	Ser	-	Ser

Figure 3. Effect of temperature on glucose isomerase activity.

100% activity corresponds to 7.1 unit/mg, 8.8 unit/mg for *C. thermosulfurogenes* (●—●) and *Thermoanaerobacter* (△—△) glucose isomerases, respectively.

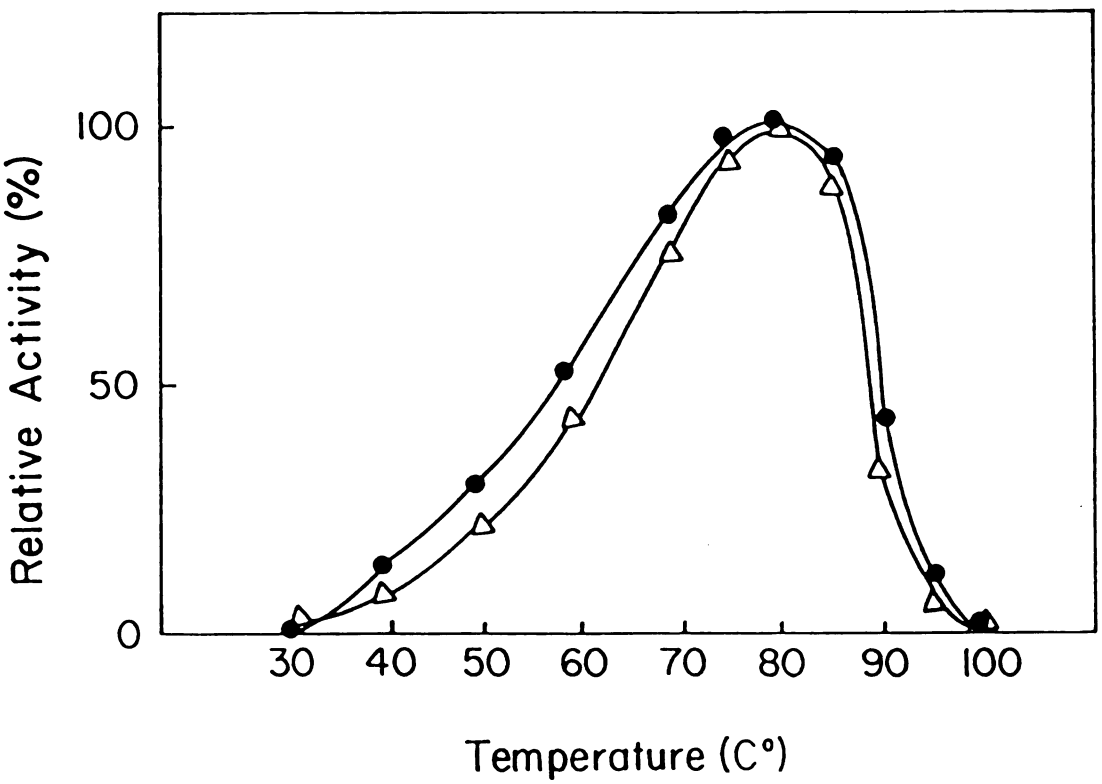
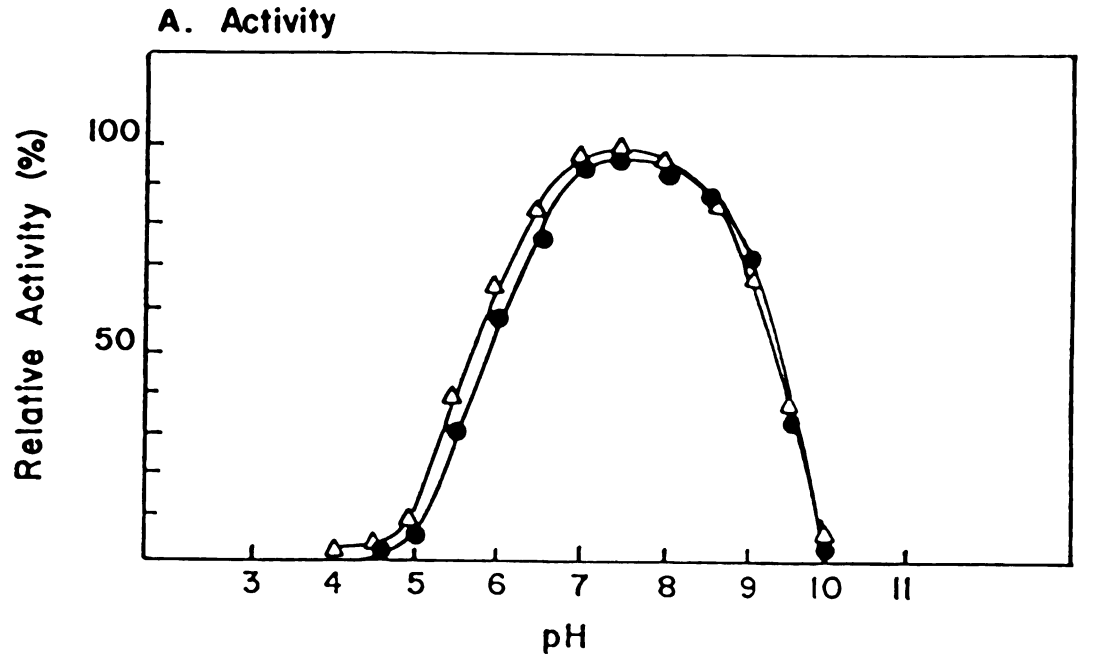
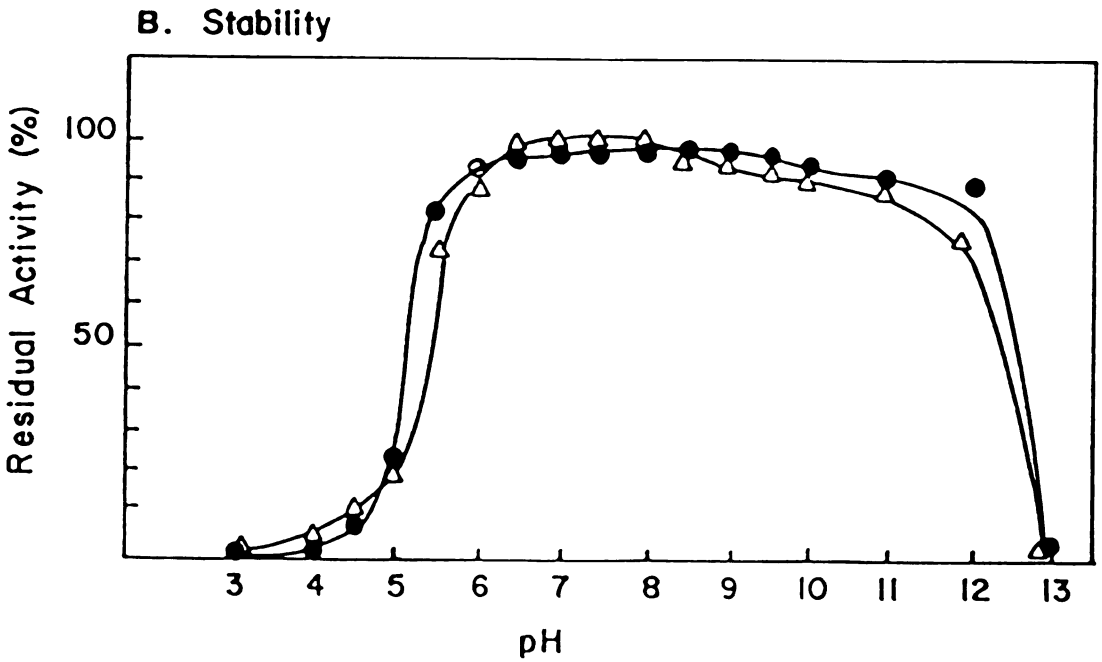

Relative Activity (%)
100
50
0
30
40
50
60
70
80
90
100
Temperature (C°)

Figure 4. Effect of pH on thermostable glucose isomerase activity (A) and stability (B).

The symbols represent: ●—● , *C. thermosulfurogenes*; △—△ , *Thermoanaerobacter.* Enzyme activities were assayed in either glycylglycine buffer at pH 3.0-4.5 and 8.5-13.0, sodium acetate buffer at pH 4.5-6.5 or MOPS buffer at pH 6.5-8.5. Enzyme stabilities were detected by assaying the residual activities after preincubation of the enzyme in these buffer solutions with 5 mM $MgSO_4$ and 0.5 mM $CoCl_2$ at 60°C for 1 h. 100% activity corresponds to 4.5 unit/mg and 5.3 unit/mg, for *C. thermosulfurogenes* and *Thermoanaerobacter*, respectively.


A. Activity
Relative Activity (%)
100
50
3
4
5
6
7
8
9
10
11
pH


B. Stability
Residual Activity (%)
100
50
3
4
5
6
7
8
9
10
11
12
13
pH

The effect of temperature on the stability of *C. thermosulfurogenes* glucose isomerase is shown in Figure 5. EDTA treated enzyme was stable for 1 hr at 60°C but it was readily denatured at 70°C. In the presence of metal ions (5 mM $MgSO_4$ and 0.5 mM $CoCl_2$), the enzyme was stable for 1 hr at 85°C. The half-life of the glucose isomerase in 50 mM phosphate buffer (pH 7.0) containing 5 mM $MgSO_4$ and 0.5 mM $CoCl_2$ was 198 hr at 60°C and 42 hr at 70°C (data not shown). The effect of various metals on thermal stability of EDTA treated glucose isomerase from *C. thermosulfurogenes* is shown in Table 4. The enzyme required Co^{++} and/or Mg^{++} for high thermal stability, and Mn^{++} was less effective to protect the enzyme from heat denaturation at 80°C. Other metal ions examined in this study did not enhance thermostability of the enzyme, and Cu^{++} and Zn^{++} showed an inhibitory effect.

Crystals of glucose isomerase from *C. thermosulfurogenes* usually appeared on the next day in a hanging drop in the well with the reservoir solution containing 16%-18% PEG 4000 at room temperature. Figure 6 shows crystals of glucose isomerase which exhibited a long and flat shape with a size of 0.7 x 0.1 mm in length and width. The preliminary results of X-ray diffraction analysis confirmed that the crystal is a protein crystal and is large enough for detailed analysis for future studies on the three dimensional structure of the enzyme.

Catalytic properties

The kinetic features of the purified glucose isomerases on three different substrates are compared in Table 5. K_m and V_{max} values were obtained from Lineweaver-Burk plots of specific activities at various substrate concentrations.

Figure 5. Thermostability of EDTA-treated glucose isomerase of *C. thermosulfurogenes* in the presence (○—○) or absence (●—●) of metal ions.

Residual activities were assayed after preincubation of the DEAE-treated enzyme in 50 mM MOPS buffer (pH 7.0) with or without 5 mM $MgSO_4$ and 0.5 mM $CoCl_2$ at indicated temperatures for various time periods. 100% activity corresponds to 4.9 unit/mg of the pure enzyme.

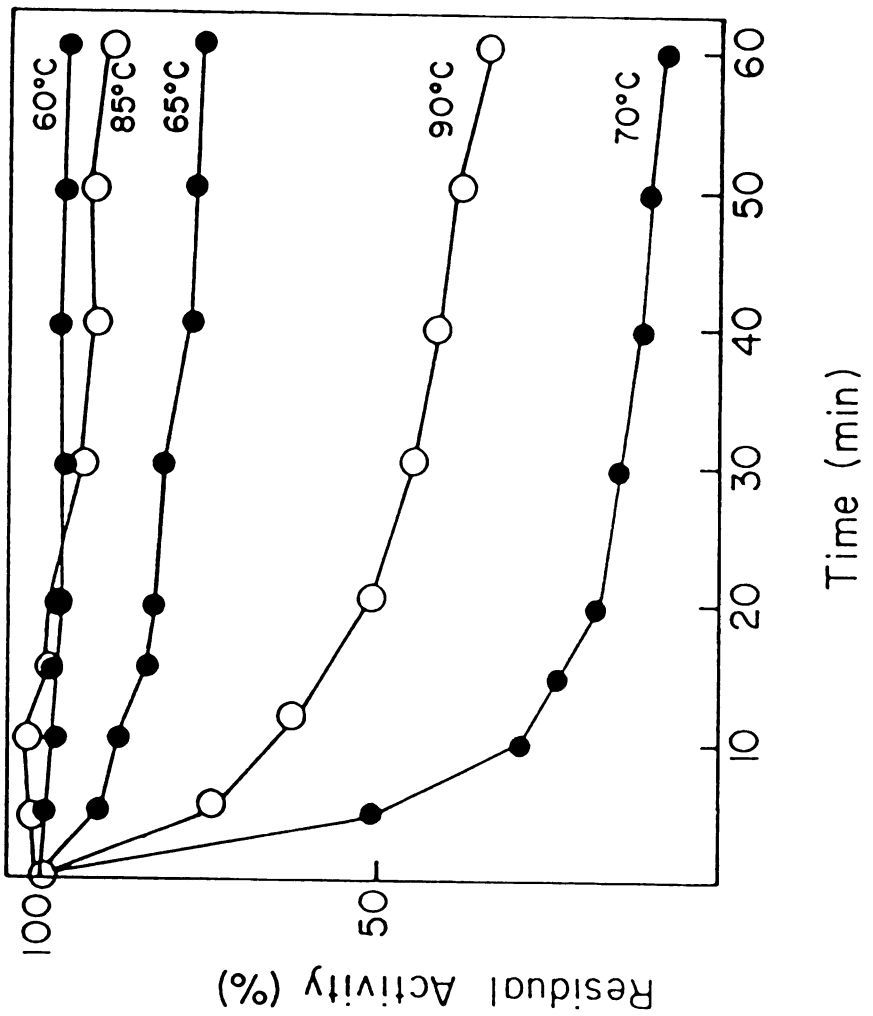

Residual Activity (%)
100
50
10
20
30
40
50
60
Time (min)
60°C
85°C
65°C
90°C
70°C

Table 4. Comparison of thermostable glucose isomerase kinetic properties from thermoanaerobes.

	K_m (mM)		V_{max} (unit/mg)		k_{cat} (1/min)	
Substrate	4B	B6A	4B	B6A	4B	B6A
Glucose	140	120	5.2	6.3	1040	1260
Fructose	60	50	2.5	2.8	500	560
Xylose	20	16	15.7	17.6	3140	3520

4B: *C. thermosulfurogenes;* B6A: *Thermoanaerobacter.*

Figure 6. Crystals of glucose isomerase from *C. thermosulfurogenes*.

0.1 mm

Table 5. Effect of metals on activity and thermal stability of EDTA-treated glucose isomerase from *C. thermosulfurogenes*.

Metal	Isomerase Activity[a] (% maximum)		Thermal Stability[b] (% Residual)
	Glucose	Xylose	
None	2	13	14
$MgSO_4$ (1 mM)	21	89	94
$MgSO_4$ (5 mM)	97	90	94
$CoCl_2$ (1 mM)	100	98	100
$MnSO_4$ (1 mM)	6	100	61
$FeSO_4$ (1 mM)	4	15	15
$NiSO_4$ (1 mM)	2	8	20
$BaCl_2$ (1 mM)	2	14	16
$CaCl_2$ (1 mM)	2	16	22
$ZnSO_4$ (1 mM)	0	0	0
$CuSO_4$ (1 mM)	0	0	0
$MgSO_4$ + $CoCl_2$ (1 mM)	98	117	111
$MgSO_4$ + $MnSO_4$ (1 mM)	16	113	86
$MnSO_4$ + $CoCl_2$ (1 mM)	35	120	78

[a]Enzyme activity was assayed at 55°C where the enzyme was stable during the reaction period without metal cofactors.

[b]Residual activity was measured at 65°C after incubation of these enzymes in the solution containing metal cofactor at 80°C for 15 min.

Glucose isomerases from two different thermoanaerobic bacteria had very similar apparent K_m, V_{max}, and K_{cat} for glucose, fructose, and xylose. Both enzymes displayed lower K_m values for xylose than for glucose or fructose, and the K_m values for fructose were about 2-fold lower than those for glucose. The apparent V_{max} and k_{cat} values of these enzymes with xylose as substrate were approximately 3-fold higher than those with glucose, and were approximately 6-fold higher than those with fructose.

The effect of various metal ions on the activity of EDTA treated enzymes from *C. thermosulfurogenes* was investigated (see Table 4). Co^{++} or Mg^{++} was required for glucose isomerase activity, whereas Mn^{++} did not enhance enzyme activity. The addition of Co^{++} and Mg^{++} did not show a synergistic effect on glucose isomerase activity and, Mn^{++} reduced the activation effect of Co^{++} or Mg^{++}. A minimum concentration of 5 mM $MgSO_4$ or 0.5 mM $CoCl_2$ was required to achieve maximum glucose isomerase activity (data not shown). For xylose isomerase activity, Mn^{++} (1 mM), Co^{++} (1 mM), or Mg^{++} (1 mM) were required and any combination of these metal ions showed a synergistic activation effect. Other metal ions had very little effect on both glucose isomerase and xylose isomerase activities, and Zn^{++} (1 mM) and Cu^{++} (1 mM) totally inhibited enzyme activity on glucose and xylose.

DISCUSSION

To our best knowledge, this study represents the first detailed characterization of glucose isomerases purified from thermoanaerobic bacteria. As expected, the glucose isomerases in these microbes were very thermostable and two different types of activity were identified based on their pH optima. The glucose isomerases with a neutral pH optimum were purified from *C. thermosulfurogenes* and *Thermoanaerobacter* because of enzyme requirements in industry (2,7) and the desire to compare similar enzyme activities from different species.

The enzymes from both microorganisms were very stable under the conditions used during purification. Glucose isomerase was one of the major proteins (approximately 10% of total protein) in the cell extract, and purification of the enzymes was relatively simple. The abundance of this enzyme in the cell extracts may be due to the fact that the enzyme had a relatively low k_{cat} and high K_m which is typical of xylose isomerase (2,31), and perhaps the organism needed to overproduce this enzyme which may be rate limiting for growth on xylose. The growth rate of these bacteria is faster on glucose than xylose (28,36).

The overall biochemical and physicochemical properties of the glucose isomerases purified from these two thermoanaerobic bacteria were similar yet these species differ in sporulation and in saccharidase activities (unpublished results). Different microbial glucose/xylose isomerases characterized previously vary in molecular weight from 80-195 KDa and are composed of two or four identical subunits. Molecular weights (200 KDa) and tetrameric subunit composition of both

thermoanaerobic glucose isomerases are similar to the 195 KDa enzyme present in *Lactobacillus brevis* (38). Glucose isomerases characterized from *Streptomyces* species (32,20), *Arthrobacter* (17), *Bacillus coagulans* (12), and *Flavobacterium arborescens* (5) display smaller molecular weights (157-183 KDa) with tetrameric subunit compositions. The size and shape of *C. thermosulfurogenes* glucose isomerase crystals was very different from those reported previously from other enzyme sources (12,31,32,38). Glucose isomerases from *E. coli* (34), alkalophilic *Bacillus* (21), *Actinoplanes missouriensis* (16), and *Streptomyces olivochromogenes* (31) are dimers with molecular weights of 80-120 KDa.

The optimum pH for the glucose isomerizing activity of *C. thermosulfurogenes* and *Thermoanaerobacter* was 7.0-7.5, which was similar to those of the enzymes from *B. coagulans* (13) and *A. missouriensis* (26), and was lower than those (pH 8.0-10.0) of enzymes from *S. phaechromogenes* (33), *S. griseofuscus* (19), and *S. olivochromogens* (31). The enzymes from both thermoanaerobes were stable within the pH range of 5.5-11.0 and displayed isoelectric points of pH 4.9 and 4.8.

Glucose isomerases are generally classified into two types of enzymes according to their thermal stability and temperature optima for enzyme activity (8,9). *Lactobacillus brevis* glucose isomerase (38), *B. subtilis* (C. Lee, et al. manuscript submitted to Appl. Environ. Microbio.), and *E. coli* xylose isomerase (4) are thermolabile and active at 37°-50°C. Glucose isomerases produced from *C. thermosulfurogenes* and *Thermoanaerobacter* were highly thermostable, and displayed one of the highest optimum temperature for activity among reported glucose isomerases (8,9). Glucose isomerases from certain mesophilic microbial sources [e.g.,

S. phaeochromogenes (32) and *A. missouriensis* (25)], however, can display quite high temperature optima and stability (9).

The requirement of Co^{++} or Mg^{++} for glucose isomerizing activity and the requirement of Mn^{++} for xylose isomerizing activity of *C. thermosulfurogenes* enzyme was similar to that reported for *B. coagulans* enzyme (11). Although *C. thermosulfurogenes* glucose isomerase required Co^{++} or Mg^{++} for its optimal thermostability, EDTA treated enzyme was more thermostable than the enzymes from *E. coli* or *B. subtilis* in the presence of these metals (4, unpublished result).

These findings demonstrate that the two distinct thermoanaerobic bacteria produce highly thermostable glucose isomerases with close similarity in physicochemical and catalytic properties. Because these bacteria have evolved in thermal hot spring ecosystems (28,36) and are thought to have a common phylogenic origin (3), one might expect that their glucose isomerases have similar properties yet represent distinct species. At present, the molecular mechanism of thermostability in these enzymes is not clear and remains to be solved. The gene coding for thermostable glucose isomerase of *C. thermosulfurogenes* has been cloned and its amino acid sequence displays higher homology to the thermolabile enzyme of *B. subtilis* than the thermostable enzyme of *Streptomyces* (C. Lee et al., manuscript submitted to J. Biol. Chem.). Further studies on the three dimensional structure of the enzyme and protein modification via site directed mutagenesis are required to explain the molecular mechanism of high thermostability in this glucose isomerase.

LITERATURE CITED

1. Amelunxen, R. E., A. L. Murdock. 1978. Microbial life at high temperatures: Mechanisms and molecular aspects, p. 217-277. In D.J. Kushner (ed.) Microbial life in extreme environments, Academic Press, San Francisco.

2. Antrim, R. L., W. Colliala, and B. Schnyder. 1979. Glucose isomerase production of high fructose syrups, P. 97-155. In L.B. Wingard (ed.) Applied Biochemistry and Bioengineering, Academic Press, New York.

3. Bateson, M. M., Wiegel, J., and D. M. Ward. (1989) Comparative analysis of 16S ribosomal RNA sequences of thermophilic fermentative bacteria isolated from hot spring cyanobacterial mats. Syst. Appl. Microbiol. 12:1-7.

4. Batt, C.A., e. O'Neil, S.R. Novak, J. Ko, and A. Sinskey. 1986. Hyperexpression of *Escherichia coli* xylose isomerase. Biotechnol. Prog. 2:140-144.

5. Boguslawski, G. 1983. Purification and characterization of glucose isomerase from *Flavobacterium arborescens*. J. Appl. Biochem. 5:186-196.

6. Bucke, C. 1980. Enzymes in fructose manufacture, p. 51-72. In G.G. Birch, N. Blackebrough, and J.K. Parker (ed.), Enzymes and Food Processing. Applied Science Publishers, London.

7. Bucke, C. 1977. Industrial glucose isomerase, p. 147-171. In A. Wiseman (ed.) Topics in Enzymes and Fermentation Biotechnology, part b, Halstead Press, Chichester.

8. Chen, W. 1980a. Glucose isomerase (a review). Process Biochem. 15:30-35.

9. Chen, W. 1980b. Glucose isomerase (a review). Process Biochem. 15:36-41.

10. Chen, W. P., A. W. Anderson, and Y. W. Han. 1979. Extraction of glucose isomerase from *Streptomyces flavogrisius*. Appl. Environ. Microbiol. 37:785-787.

11. Danno, G., S. Yoshimura, and M. Natake. 1967. Studies on D-glucose isomerizing activity of D-xylose-grown cells from *Bacillus coagulans*, strain HN-68. Agric. Biol. Chem. 31:284-292.

12. Danno, G. 1970. Studies on D-glucose-isomerizing enzyme from *Bacillus coagulans*, strain HN-68 Part IV. Purification, crystallization and some physicochemical properties. Agr. Biol. Chem. 34:1795-1804.

13. Danno, G. 1970. Studies on D-glucose-isomerizing enzyme from *Bacillus coagulans*, strain HN-68 Part V. Comparative study on the three activities of D-glucose, D-xylose, and D-ribose isomerization of the crystalline enzyme. Agric. Biol. Chem. 34:1805-1814.

14. Dishe, Z., and E. Borenfreund. 1951. A new spectrophotometric methods for the detection and determination of keto sugars and trioses. J. Biol. Chem. 192:583-587.

15. Gaikwad, S. M., M. W. More, H. G. Vartak, and V. V. Deshpande. 1988. Evidence for the essential histidine residue at the active site of glucose/xylose isomerase from *Streptomyces*. Biochem. Biophys. Res. Commun. 155:270-277.

16. Gong, C.-S., L. F. Chen, and G. T. Tsao. 1980. Purification and properties of glucose isomerase of *Actinoplanes missouriensis*. Biotechnol. Bioeng. 12:833-845.

17. Henrik, K., C. A. Collyer, and D. M. Blow. 1989. Structures of D-xylose isomerase from *Arthrobacter* strain B3728 containing the inhibitors xylitol and D-sorbitol at 2.5 A and 2.3A resolution, respectively. J. Mol. Biol. 208:129-157.

18. Jorgensen, O. B., L. G. Karlsen, N. B. Nielsen, S. Pedersen, and S. Rugh, Bagsvaerd (Denmark). 1988. A new immobilized glucose isomerase with high productivity produced by a strain of *Streptomyces murinus*. Starch/Stärke. 40:307-313.

19. Kasumi, T., K. Hayashi, and N. Tsumura. 1981. Purification and enzymatic properties of glucose isomerase from *Streptomyces griseofuscus*, S-41. Agric. Biol. Chem. 45:619-627.

20. Kasumi, T., K. Hayashi, N. Tsumura, and T. Takagi. 1981. Physicochemical characterization of glucose isomerase from *Streptomyces griseofuscus*, S-41. Agric. Biol. Chem. 45:1087-1095.

21. Kwon, H., M. Kitada, and K. Horikoshi. 1987. Purification and properties of D-xylose isomerase from alkalophilic *Bacillus* No. KX-6. Agric. Biol. Chem. 51:1983-1989.

22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London). 227:680-685.

23. Lowry, O. H., J. Rosebrough, A. I. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

24. Ng, T. K., W. R. Kenealy. 1987. Industrial applications of thermostable enzymes, p. 197-215. In T.D. Brock (ed.) Thermophiles; General, molecular and applied microbiology. John Wiley and Sons, New York.

25. Saha, B. C., S. Mathupala, and J. G. Zeikus. 1988. Purification and characterization of a highly thermostable pullulanase from *Clostridium thermohydrosulfuricum*. Biochem. J. 252:343-348.

26. Scallet, B. L., K. Shieh, I. Ehrenthal, and L. Slapshak. 1974. Studies in the isomerization of D-glucose. Die Stärke. 26:405-408.

27. Schellenberg, G. D., A. Sarthy, A. E. Larson, M. P. Backer, J. W. Crabb, M. Lindstrom, B. D. Hall, and C. E. Furlong. 1984. Xylose isomerase from *Escherichia coli*: characterization of the protein and the structural gene. J. Biol. Chem. 259:6826-6832.

28. Schink, B., and J. G. Zeikus. 1983. *Clostridium thermosulfurogenes* sp. nov., a new thermophile that produces elemental sulphur from thiosulfate. J. Gen. Microbiol. 129:1149-1158.

29. Schnyder, B. J., 1974. Continuous isomerization of glucose to fructose on a commercial basis. Die Stärke. 26:409-412.

30. Shen, G., B. C. Saha, Y. E. Lee, L. Bhatnagar, and J. G. Zeikus. 1988. Purification and characterization of a novel thermostable B-amylase from *Clostridium thermosulfurogenes*. Biochem. J. 254:835-840.

31. Suekane, M., M. Tamura, and C. Tomimura. 1978. Physico-chemical and enzymatic properties of purified glucose isomerases from *Streptomyces olivochromogenes* and *Bacillus stearothermophilus*. Agric. Biol. Chem. 42:909-917.

32. Takasaki, Y., Y. Kosugi, and A. Kanbayashi. 1969. Studies on sugar-isomerizing enzyme purification, crystallization and some properties of glucose isomerase from *Streptomyces* sp. Agric. Biol. Chem. 33:1527-1534.

33. Tsumura, N. and T. Sato. 1965. Enzymatic conversion of D-glucose to D-fructose Part VI. Properties of the enzyme from *Streptomyces phaeochromogenus*. Agric. Biol. Chem. 29:1129-1134.

34. Tucker, M. Y., M. P. Tucker, M. E. Himmel, K. Grohmann, and S. M. Lastick. 1988. Properties of genetically overproduced *E. coli* xylose isomerase. Biotechnol. Lett. 10:79-84.

35. Verhoff, F. H., G. Boguslawski, O. J. Lantero, S. T. Schlager, and Y. C. Jao. 1985. Glucose isomerase, p. 837-859. In H.W. Balanch, S. Drew, and D.I.C. Wang (eds.), Comprehensive Biotechnology , vol. 3, Pergamon Press, Oxford.

36. Weimer, P. J., L. W. Wagner, S. Knowlton, and T. K. Ng. 1984. Thermophilic anaerobic bacteria which ferment hemicellulose: Characterization of organisms and identification of plasmids. Arch. Microbiol. 138:31-36.

37. Wilhelm, M., and C. P. Hollenberg. 1985. Nucleotide sequence of the *Bacillus subtilis* xylose isomerase gene: extensive homology between the *Bacillus* and *Escherichia coli* enzyme. Nucleic Acids Res. 13:5717-5723.

38. Yamanaka, K. 1968. Purification, crystallization and properties of the D-xylose isomerase from *Lactobacillus brevis*. Biochim. Biophys. Acta. 151:670-680.

39. Zeikus, J. G., A. Ben-Bassat, and P. W. Hegge. 1980. Microbiology of methanogenesis in thermal, volcanic environments. J. Bacteriol. 143:432-440.

40. Zeikus, J. G. 1979. Thermophilic bacteria: ecology, physiology, and technology. Enzyme Microb. Technol. 1:243-252.

Chapter IV

Cloning and Expression of the *Clostridium thermosulfurogenes* Glucose Isomerase Gene in *Escherichia coli* and *Bacillus subtilis*

ABSTRACT

The gene encoding thermostable glucose isomerase in *Clostridium thermosulfurogenes* was cloned by complementation of glucose isomerase activity in a *xyl*A mutant of *Escherichia coli*. A new assay method for thermostable glucose isomerase activity on agar plates, using a top agar mixture containing fructose, glucose oxidase, peroxidase and benzidine, was developed. One positive clone, carrying plasmid pCGI38 was isolated this from a cosmid library of *C. thermosulfurogenes* DNA. The plasmid was further subcloned into a *Bacillus* cloning vector, pTB522, to generate a shuttle plasmid pMLG1, able to replicate in both *E. coli* and *Bacillus subtilis*. Expression of thermostable glucose isomerase gene in both species was constitutive whereas synthesis of the enzyme in *C. thermosulfurogenes* was inducible by D-xylose. *B. subtilis* and *E. coli* produced higher levels of thermostable glucose isomerase (1.54 and 0.46 units/mg protein, respectively) than *C. thermosulfurogenes* (0.29 units/mg protein). The glucose isomerase synthesized in *E. coli* and *B. subtilis* was purified to homogeneity and displayed identical properties (subunit M_r=50 kDa, tetrameric molecular structure, thermostability, metal ion requirement, apparent temperature and pH optima) to those of the native enzyme purified from *C. thermosulfurogenes*. Simple heat treatment of crude extracts from *E. coli* and *B. subtilis* cells, carrying the recombinant plasmids, at 85°C for 15 min generated 80% pure glucose isomerase free of protease activity. The maximum conversion yield of glucose (35%, wt/wt) to fructose with the thermostable glucose isomerase (10.8 units/g dry substrate) was 52% at pH 7.0 and 70°C.

INTRODUCTION

Xylose isomerase (D-xylose ketol isomerase, EC 5.3.1.5) which reversibly catalyzes the isomerization reaction between D-xylose and D-xylulose can also convert D-glucose into D-fructose, hence the enzyme is often referred to as glucose isomerase (27). Glucose isomerase is one of the largest volume commercial enzymes used today, and it is employed for the industrial production of high fructose corn sweetener (9). Improvement in the enzyme process used by the sweetener industry requires higher thermal and acid stability of glucose isomerase, higher specific activity and over-production of the enzyme, and a lower cost enzyme recovery process from the host cell after fermentation (9,19).

In spite of the industrial importance of microbial glucose isomerase, few reports exist on molecular comparisons of the enzyme in different species (especially in thermophiles) or on molecular understanding of enzyme thermal and acid stability. The D-xylose isomerase genes from both *E. coli* and *B. subtilis* have been cloned and sequenced (25,28), and over-expression of *E. coli* xylose isomerase by using *tac* or *lac* promoters in *E. coli* strains has been reported (2,24,29). Cloning of the glucose isomerase gene from *Streptomyces violaceoniger* in a *Streptomyces* host has been reported (22).

Recently, we have purified and characterized thermostable glucose isomerases from thermoanaerobic bacteria (C. Lee and J.G. Zeikus, manuscript submitted to J. Bacteriol.). At present, nothing is known about the molecular structure or organization of glucose isomerase genes from thermophilic microorganisms.

Therefore, we undertook studies aimed at cloning of a gene coding for thermostable glucose isomerase from an anaerobic thermophile and its over-expression in a food-safe *B. subtilis* host. In this paper we report on a new plate assay method for detection of thermostable glucose isomerase, on cloning and over-expression of the thermophile gene and enzyme, and on biochemical and biotechnological features of the recombinant enzyme.

MATERIALS AND METHODS

Bacterial strains and plasmids

C. thermosulfurogenes strain 4B was used as the source of the glucose isomerase gene (26). *E. coli* strain W595 [F⁻ malA1 *xyl*-7 *ara*-13 *mtl*-2 *ton*A2 *gal*T1] (1), obtained from Dr. Bachmann, *E. coli* Genetic Stock Center, Yale University, and HB101 [F⁻ *hsd*S20 *ara*-14 *rec*A13 *pro*A12 *lac*Y1 *gal*K2 *vpo*L20 *mtl*-1 *xyl*-5] (21) were used for DNA manipulation and complementation test. *B. subtilis* strain NA1 [*arg*-15 *hsd*R *hsd*M *amy*⁻ *npr*⁻] (18) which does not exhibit any glucose isomerase activity at 65°C was used as a host strain for subcloning of the glucose isomerase gene. Cosmid vector pHSG262(4) was used for the construction of a genomic library of *C. thermosulfurogenes* in *E. coli* HB101. *Bacillus* cloning vector pTB522 (16) was used during the subcloning procedure.

Chemicals, media, and growth conditions

All chemicals were of reagent grade. *C. thermosulfurogenes* strain 4B was grown under anaerobic conditions at 60°C in TYE medium containing 1% xylose or maltose as a carbon source (31). For growth of *E. coli* and *B. subtilis* strains, Luria broth (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter at pH 7.5) was used for liquid culture and solid media contained 1.5% agar (Difco Co., Detroit, MI). Kanamycin (50 μg/ml) or tetracycline (25 μg/ml) were used for selection of pHSG262 or pTB522 carrying strains, respectively.

Plate assays for detection of thermostable glucose isomerase

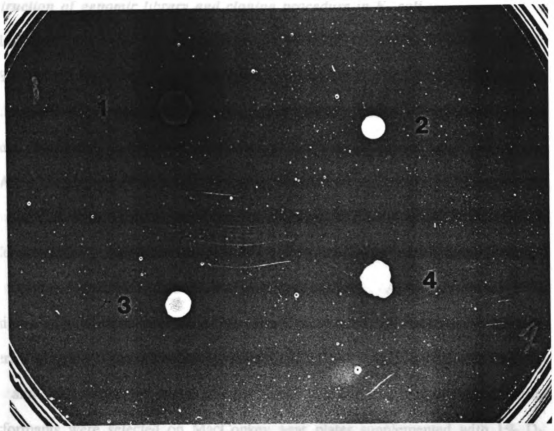
Fructose (2%), $MgSO_4$ (5 mM), $CoCl_2$ (0.5 mM), glucose oxidase (20 unit/ml), peroxidase (4 unit/ml), and benzidine (0.4 mg/ml) in 100 mM MOPS buffer (pH 7.0) were mixed with top agar (0.7%) at 50°C, and poured on the colonies grown overnight on LB agar plates with 1% xylose. After soft agar had solidified the plates were incubated overnight at 50°C. Positive clones showed a dark brown halo around the colonies (see Figure 1). One positive colony was found after screening 1000 transformants by this method. MacConkey agar plates containing 1% xylose were also used to detect complementation of xylose utilization in *E. coli xyl-5* mutants at 37°C.

Extraction and manipulation of DNA

Chromosomal DNA was isolated from *C. thermosulfurogenes* cells harvested at mid-logarithmic growth phase by a modification of the Marmur procedure (23). Cells suspended in 0.25 M sucrose were treated with lysozyme (0.5 mg/ml) and EDTA (50 mM, pH 8.0) for 5 min followed by SDS (0.2%) at 60°C. The lysate was treated with proteinase K (0.1 mg/ml) and further deproteinized with repeated phenol/chloroform extraction. DNA was precipitated with 0.6 volumes of isopropanol, collected by "spooling" on a glass rod, and dissolved in 20 mM Tris, 1 mM EDTA. Plasmid DNA was purified by the procedure described by Clewell (6) followed by centrifugation in a cesium chloride-ethidium bromide density gradient. The alkaline denaturation method was used for rapid extraction of plasmid DNA (3). Digestion of DNA with restriction endonucleases, separation of fragments by

Figure 1. Indicator plate for thermostable glucose isomerase activity.

1: *E. coli* HB101/pCGI38, 2: *E. coli* HB101/pHSG262, 3: *E. coli* JM107, 4: *B. subtilis* NA1.


1
2
3
4

electrophoresis, recovery of DNA from agarose gels, dephosphorylation with calf intestine phosphatase, ligation of DNA fragments, and transformation of *E. coli* with plasmid DNA were performed as described by Maniatis et al. (21) or as recommended by the manufacturers of enzymes.

Construction of genomic library and cloning procedure in E. coli

Left and right arms of the cosmid vector, pHSG262, dephosphorylated at *Hin*cII and *Eco*RI sites were prepared as described by Brady et al. (4). *Hin*cII-*Bam*HI and *Eco*RI-*Bam*HI fragments were then ligated with DNA fragments (30-40 kb in length) prepared by partial digestion of chromosomal DNA of *C. thermosulfurogenes* with *Sau*3AI. The ligated mixture was packaged *in vitro* into bacteriophage lambda heads (10), and the recombinant cosmids were introduced into cells of HB101 strain by transfection (12). Approximately 1,000 colonies containing recombinant plasmids were replicated onto LB agar plates with kanamycin. After growth at 37°C overnight, colonies were screened by the direct plate assay for thermostable glucose isomerase activity. The recombinant plasmid pCGI38 was isolated from one positive clone and introduced by transformation into different *xyl*⁻ mutants of *E. coli*. Transformants were selected on MacConkey agar plates supplemented with 1% D-xylose and kanamycin.

Subcloning of a DNA fragment containing glucose isomerase gene into B. subtilis

EcoRI digested pCGI38 was ligated with pTB522 that had been cut with EcoRI and dephosphorylated. The ligation mixture was used to transform competent cells

of *B. subtilis* as described earlier (15). Transformants were selected on LB agar plates with tetracycline and recombinant clones were identified by the restriction pattern of the plasmid DNA isolated by rapid alkaline extraction.

Enzyme assays and protein determination

Washed whole cells, cell-free extracts or purified preparations were used as enzyme sources. Cells grown to late-logarithmic phase in LB media with or without appropriate antibiotics and 1% xylose were harvested by centrifugation at 5,000 x g for 10 min, and washed with 50 mM MOPS buffer (pH 7.0) once and resuspended in 1/10 of the original volume of the same buffer. Cell-free extract was prepared by sonication of the washed cell suspensions on ice. The activity of glucose isomerase was measured by incubating a reaction mixture that contained 10 mM $MgSO_4$, 1 mM $CoCl_2$, 0.8 M glucose, and the enzyme in 100 mM MOPS buffer (pH 7.0) at 65°C. For the assay of xylose isomerase activity, the reaction mixture was incubated at 37°C and contained 70 mM xylose, 10 mM $MnSO_4$, and the enzyme preparation in the same buffer. The amount of fructose or xylulose formed after the enzyme reaction was estimated by the cysteine-carbazole sulfuric acid method (11). One unit of activity is defined as the amount of enzyme which released 1μmol of ketose per min under the assay conditions described above. Protein concentration was determined by the method of Lowry et al. (20). Bovine serum albumin was used as a standard.

Purification of glucose isomerase

Cell extract preparation: 10 g of wet weight *E. coli* W595/pCGI38 cells or 3 g of wet weight *B. subtilis* NA1/pMLG1 cells were suspended in 4 volume of 50 mM MOPS buffer (pH 7.0) containing $MgSO_4$ (10 mM) and $CoCl_2$ (1 mM). The cells were broken by two passages through a French pressure cell (American Instrument Co. Inc., Silver Spring, MD) at 18,000 p.s.i. The debris was removed by centrifugation at 12,000 x g for 20 min.

Heat treatment step: The cell extracts obtained from the broken cells were heated at 85°C for 15 min, and cooled to 4°C. The precipitated protein was removed by centrifugation at 12,000 x g for 20 min.

Anion exchange column chromatography: DEAE-sepharose CL-6B columns were equilibrated with 50 mM MOPS buffer (pH 6.8) containing 5 mM $MgSO_4$ and 0.5 mM $CoCl_2$. The soluble fractions from the heated cell extracts were loaded onto the columns and eluted with a NaCl salt gradient (0.0-0.5M) in the same buffer. The fractions containing significant glucose isomerase activity were pooled.

Gel filtration chromatography: A prepacked gel filtration column, Superose 12 HR 10/30 (Pharmacia, Inc., Picataway, N.J.) was used with the Phamacia FPLC System. The pooled fractions from the previous step were concentrated into 0.2 ml of enzyme solution by using ultrafiltration membrane YM30 with 30 KDa molecular weight cut-off (Amicon; Division of W.R. Grace and Co., Danverse, MA) prior to the loading. Blue dextran (2000 KDa), alcohol dehydrogenase (150 KDa), and bovine serum albumin (66 KDa) were used as molecular weight standards.

Time course of glucose conversion into fructose

Glucose solution (35%, wt/wt) was incubated with the heat treated cell extract from *B. subtilis* NA1/pMLG1 as a glucose isomerase source (10.8 unit/g of dry substrate) in 50 mM MOPS buffer (pH 7.0) containing $MgSO_4$ (5 mM) and $CoCl_2$ (0.5 mM) at 70°C. Quantitative and qualitative analyses for glucose and fructose were performed by HPLC as described elsewhere (C. Lee, B.C. Saha, and J.G. Zeikus, manuscript submitted to Appl. Environ. Microbiol.).

RESULTS

Expression of recombinant glucose isomerase

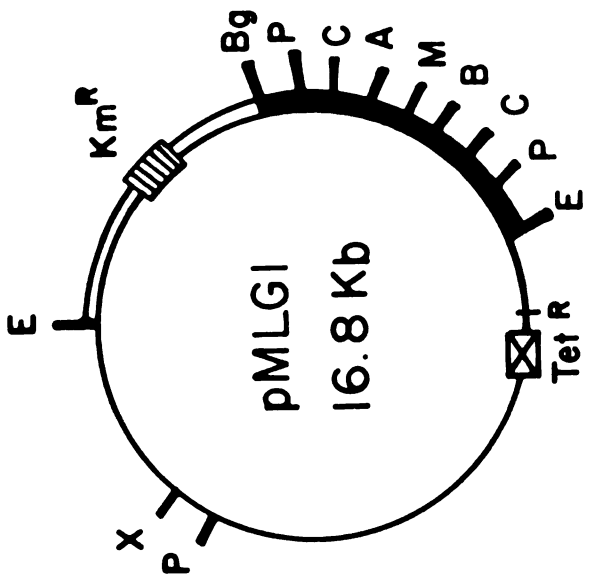
The recombinant plasmid pCGI38 was able to complement the *xyl*-5 mutation in *E. coli* strain HB101. It contained the vector pHSG262 and an insert fragment of 4.0 kb (Figure 2). Since pCG38 originated from a cosmid library we must conclude that the insert had undergone a partial deletion during propagation in the *E. coli* host. Upon introduction into different *xyl*A mutants of *E. coli*, pCGI38 conferred the *xyl*$^+$ phenotype to the hosts and the ability to express a thermostable xylose and glucose isomerase (Table 1).

A new recombinant plasmid, pMLG1, was constructed by ligation of pCGI38 with a *Bacillus* vector pTB522 at the *Eco*RI site. This double-replicon could be maintained in both *E. coli* and *B. subtilis*. As shown in Table 2 this plasmid conferred the ability to express thermostable glucose isomerase to both organisms. The orientation of the insert DNA fragment in the vector did not affect the level of glucose isomerase in either of the two hosts (results not shown). We conclude, therefore, that the cloned *xyl*A gene from *C. thermosulfurogenes* was still under the control of its own promoter and this promoter (or promoter region) was expressed well in each of the three bacterial hosts tested.

In *C. thermosulfurogenes*, the glucose isomerase gene was not expressed unless D-xylose was present in the medium (Table 3). The gene present on the cloned DNA fragment was expressed constitutively in both *E. coli* and *B. subtilis* and the addition of D-xylose to the medium did not increase its production (Table 3).

Figure 2. Physical and genetic maps of plasmids pCGI38 and pMLG1, which carry the *C. thermosulfurogenes* DNA insert expressing thermostable glucose isomerase.

Derivation of DNA fragments represent ▭, pHSG262; ——, pTB523; ▬, *C. thermosulfurogenes*. Abbreviations for the restriction sites are: A, *Acc*I; B, *Bal*I; Bg, *Bg*1II; C, *Cla*I; E, *Eco*RI; M, *Mlu*I; P, *Pst*I; S, *Sma*I; X, *Xba*I. Kanamycin and tetracycline resistance genes are indicated by ▯▯▯▯ and ⊠, respectively.


pMLG1
16.8 Kb
Km^R
Bg
P
C
A
M
B
C
P
E
Tet^R
E
X
P

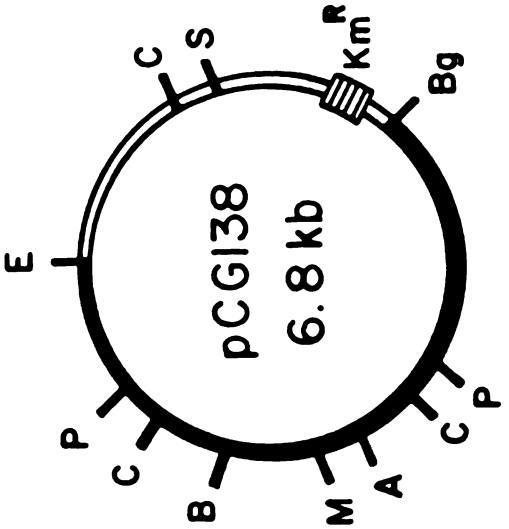

pCG138
6.8 kb
C
S
Km^R
Bg
E
P
C
B
M
A
C
P

Table 1. Comparison of glucose/xylose isomerase activities in *E. coli* xyl⁻ mutant strains carrying the recombinant plasmid pCGI38.[a]

		Specific activity (unit/mg protein)	
E. coli Strain	Plasmid	Glucose (65°C)	Xylose (37°C)
C. thermosulfurogenes (control)	--	0.29	0.07
JM107 (xyl⁺)	--	0.00	0.08
HB101	pHSG262	0.00	0.00
HB101	pCGI38	0.21	0.05
W595	pHSG262	0.00	0.00
W595	pCGI38	0.46	n.d.

[a] *E. coli* transformants carrying pCGI38 or pHSG262 were cultured in LB media with 1% xylose and kanamycin. Strain JM107 was grown in LB medium with 1% xylose. *C. thermosulfurogenes* was grown anaerobically in TYE medium with 1% xylose at 60°C.

n.d.: not determined.

Table 2. Expression of cloned glucose isomerase by recombinant shuttle plasmid pMLG1 in *E. coli* and *B. subtilis*[a]

Host	Plasmid	Antibiotics resistance	Specific activity (unit/mg protein)
E. coli W595	pHSG262	Kan^r	0.00
E. coli W595	pMLG1	Kan^r, Tet^s	0.50
B. subtilis NA1	pTB522	Tet^r	0.00
B. subtilis NA1	pMLG1	Tet^r, Kan^s	1.54

[a] Cells were cultured in LB medium with 1% xylose and appropriate antibiotics. Cells were harvested and washed in 50 mM MOPS buffer (pH 7.0) before enzyme assay at 65°C and protein determination.

Table 3. Effect of D-xylose and D-glucose on synthesis of recombinant thermostable glucose isomerase in *E. coli* and *B. subtilis*.[a]

Strain[a]	Plasmid	Specific Activity (unit/mg protein) Carbohydrates added: xylose	glucose	none
C. thermosulfurogenes (control)	--	0.29	0.00	--
E. coli W595	pHSG262	0.00	0.00	0.00
E. coli W595	pCGI38	0.46	0.24	0.43
B. subtilis NA1	pTB522	0.00	0.00	0.00
B. subtilis NA1	pMLG1	1.54	1.29	1.47

[a] *E. coli* and *B. subtilis* were grown in LB media with 1% carbohydrate as indicated and *C. thermosulfurogenes* was grown in TYE medium with 1% carbohydrate prior to assay of activity at 65°C.

A reduction of specific activity upon addition of glucose might be a result of catabolite repression.

Properties of recombinant glucose isomerase

The general biochemical properties of glucose isomerase, synthesized in *E. coli* and *B. subtilis* cells, containing the gene from *C. thermosulfurogenes*, were very similar to those determined for the enzyme produced by its original host. The enzyme maintained 80 - 95% of the original activity after preincubation of cell extracts at 80°C (Figure 3). The apparent pH and temperature optima for the activity of the recombinant glucose isomerase isolated from the mesophilic bacterial hosts were 80°C and pH 7.0.

The usefulness of the heat treatment step in purification of thermostable glucose isomerase from either *E. coli* or *B. subtilis* is shown in Table 4. After the heat treatment of crude cell extracts the enzyme was obtained at 70 - 80% purity. After additional steps of purification by anion exchange chromatography and gel filtration, preparations of homogeneous glucose isomerase were obtained on SDS-polyacrylamide gel electrophoresis. The enzyme isolated from either *C. thermosulfurogenes* or the mesophilic organisms consisted of one type of subunit with a molecular weight of 50 kDa (Figure 4). Upon gel filtration of the native enzyme on Superose-12 a molecular weight of 200 kDa was observed indicating that the native enzyme molecule is a tetramer of identical subunits.

As indicated by the results in Figure 4, the *C. thermosulfurogenes* glucose isomerase gene was expressed at high levels from the recombinant plasmids in both

Figure 3. Comparison of native versus cloned glucose isomerase thermal stabilities.

Symbols represent: ▲—▲, *C. thermosulfurogenes* (CT); ●—●, *E. coli* (EC) W595/pCGI38; ○—○, (BS) *B. subtilis* NA1/pMLG1; ■—■, *E. coli* JM107; and, □—□, *B. subtilis* NA1. Residual activities were assayed at 65°C (except controls, *E. coli* JM107 and *B. subtilis* NA1, which were assayed at 37°C) after preincubation of cell extracts at 80°C for various time periods.

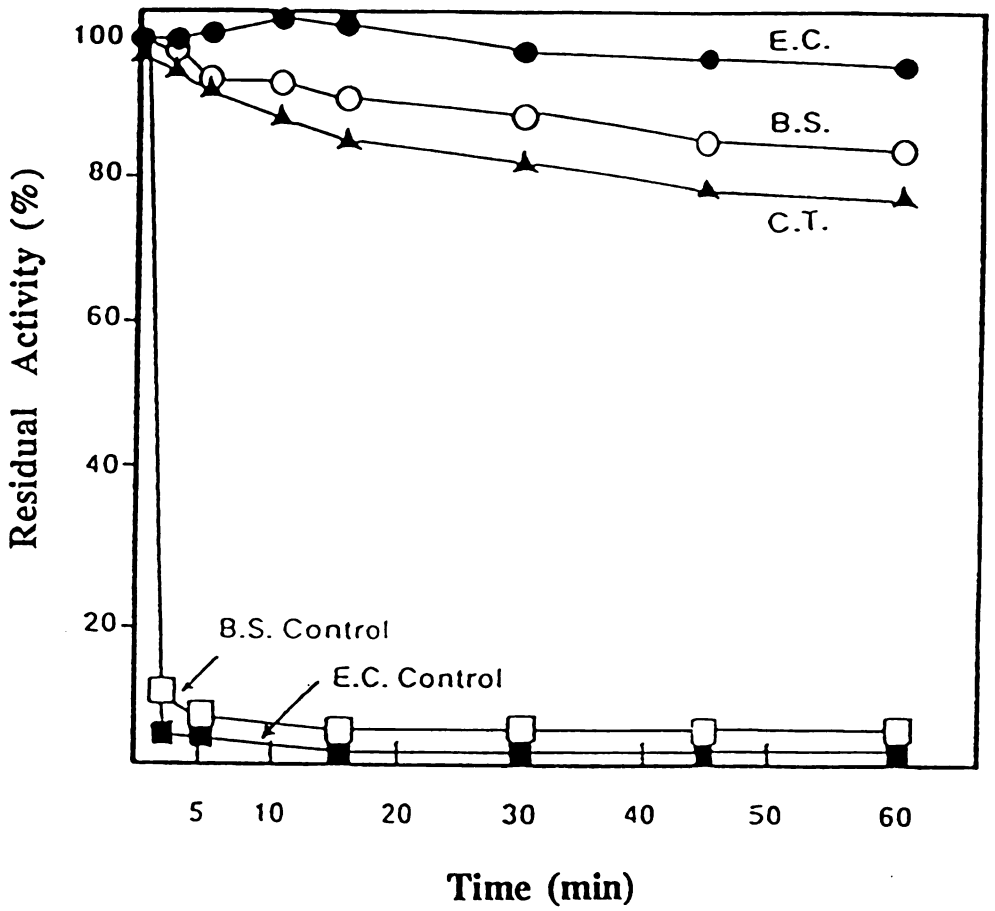
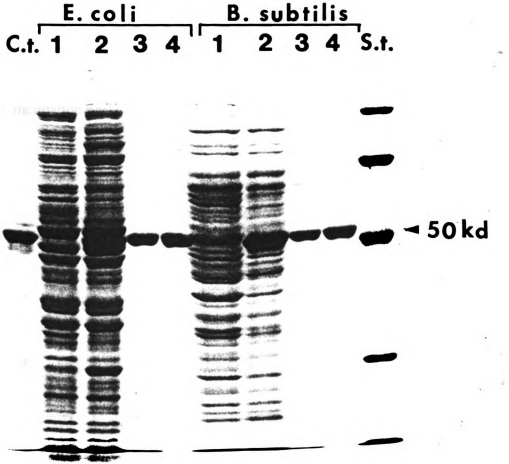

100
80
60
40
20
Residual Activity (%)
E.C.
B.S.
C.T.
B.S. Control
E.C. Control
5
10
20
30
40
50
60
Time (min)

Figure 4. SDS-PAGE analysis of glucose isomerase activity fractions.

Various fractions obtained during purification of native and recombinant glucose isomerase were analyzed by 12% SDS-PAGE, and visualized by Coomassie blue staining. Lane C.t., glucose isomerase purified from *C. thermosulfurogenes*; 1, whole cell preparation from *E. coli* W595/pHSG262 or *B. subtilis* NA1/pTB522 that did not carry the DNA insert; 2, whole cell preparation from *E. coli* W595/pCGI38 or *B. subtilis* NA1/pMLG1; 3, soluble fraction from heat-treated cell extract of *E. coli* W595/pCGI38 or *B. subtilis* NA1/pMLG1; 4, glucose isomerase purified from *E. coli* W595/pCGI38 or *B. subtilis* NA1/pMLG1; S.t., molecular weight standards (97,400. phosphorylase; 66,200. bovine serum albumin; 42,700. ovalbumin; 31,000. carbonic anhydrase; 21,500. soybean trypsin inhibitor).


E. coli
B. subtilis
C.t. 1 2 3 4 1 2 3 4 S.t.
50kd

E. coli and *B. subtilis.* This was confirmed by the fact that 7-fold purification was required to generate a homogeneous enzyme preparation from crude extracts of *E. coli* and only 3-fold purification from the extracts of *B. subtilis* (Table 4).

To test the practical utility of recombinant thermostable glucose isomerase produced by food-safe strains of *B. subtilis*, the time course of fructose formation from concentrated glucose syrups were tested at temperatures higher than those used in current industrial practice. As shown in Figure 5, the isomerization reaction reached equilibrium and achieved a yield of 52% fructose at 70°C and pH 7.0 after 8 hours of incubation.

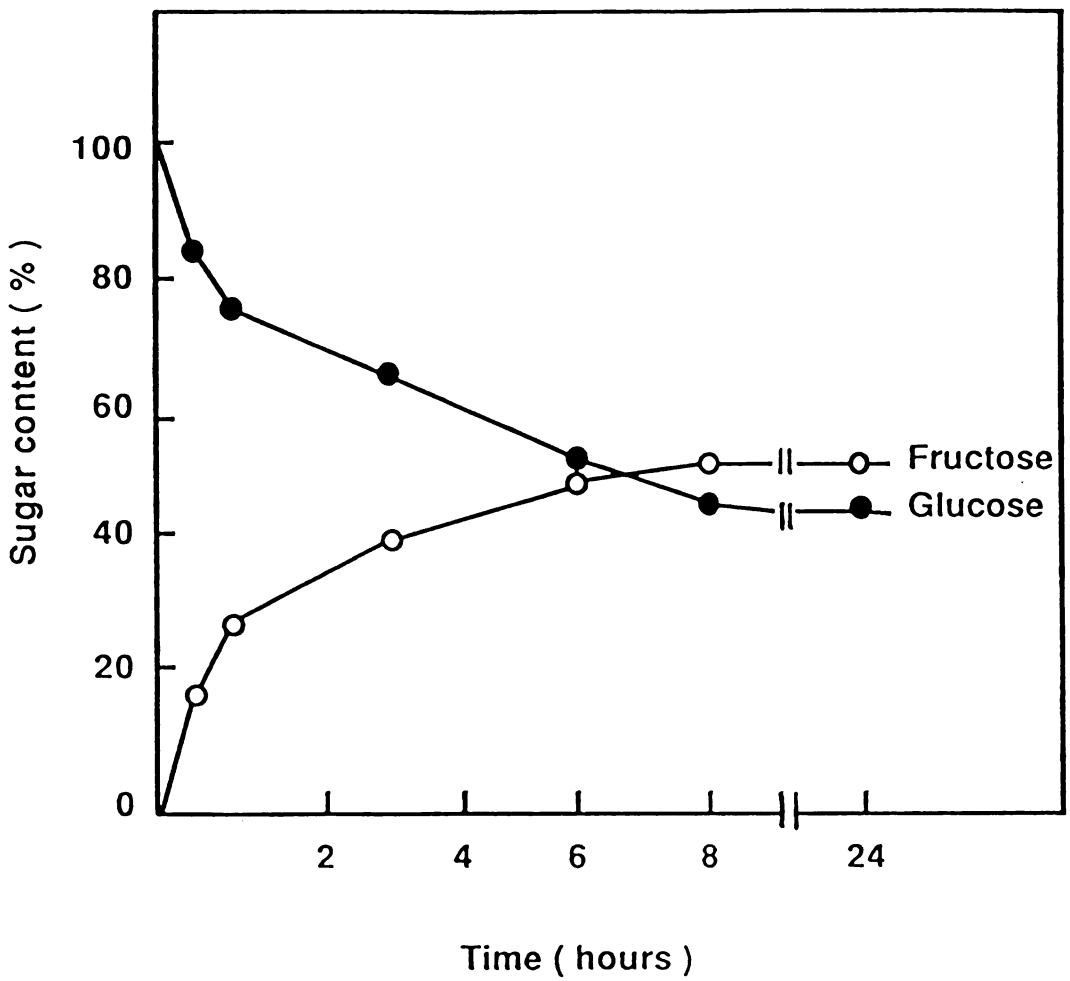
Table 4. Purification scheme of cloned thermostable glucose isomerase from *E. coli* and *B. subtilis*[a]

Step	Total protein (mg)		Total activity (units)		Specific activity (units/mg)		Purification (fold)	
	E.C.	B.S.	E.C.	B.S.	E.C.	B.S.	E.C.	B.S.
Crude cell extract	800	122	400	159	0.5	1.3	1	1
Heat treatment (85°C, 15 min)	144	37	331	96	2.4	2.6	5	2
DEAE-sepharose anion exchange	88	28	282	84	3.2	3.5	6.4	2.8
FPLC-Superose 12 gel filtration	39	15	136	56	3.5	3.7	7	2.9

[a] Abbreviations: E.C., *E. coli* W595 containing the plasmid pCGI38; and, B.S., *B. subtilis* NA1 containing the plasmid pMLG1.

Figure 5. Time course of glucose conversion into fructose using heat-treated glucose isomerase obtained from recombinant *B. subtilis*.

Glucose isomerization was performed at 70°C under conditions described in Methods.


100
80
60
40
20
0
Sugar content (%)
2
4
6
8
24
Time (hours)
Fructose
Glucose

DISCUSSION

This work demonstrates cloning of the gene for thermostable xylose and glucose isomerase from *C. thermosulfurogenes.* As far as we are aware, this is the first glucose isomerase gene from a thermophilic bacterium that has been cloned and over-expressed in *E. coli* and *B. subtilis.* A new screening method, based on a specific assay that detected conversion of fructose to glucose on agar plates facilitated the isolation of positive clones. In *E. coli xyl*A mutants, detection of over-produced xylose isomerase was also possible on McConkey/xylose agar plates. However, the direct assay of fructose to glucose conversion is not only more specific, it is also applicable to non-fermenting, mesophilic or thermophilic hosts.

When the cloned *xyl*A gene of *E. coli*, containing its own promoter, was intoduced into *B. subtilis*, it was not expressed unless a *B. licheniformis* promoter was inserted upstream (13). However, the deduced amino acid sequence of xylose isomerase gene from *C. thermosulfurogenes* exhibited a high degree of homology to those of both *E. coli* and *B. subtilis xyl*A genes, and it was expressed well from a putative promoter region located upstream of the structural gene, in each of the three organisms used in this study (Table 2, 3, and C. Lee, et al., manuscript submitted to J. Biol. Chem.).

It is known that single amino acid substitutions in the primary structure of an enzyme may alter dramatically its biochemical properties including thermostability, turnover number or substrate specificity (8,30). The identical biochemical properties of thermostable glucose isomerase produced in its native host and in *E. coli* and *B.*

subtilis indicates that the cloned gene did not undergo significant mutational changes upon manipulation and propagation in the new hosts.

High thermostability of *C. thermosulfurogenes* glucose isomerase made it possible to use heat treatment of crude cell extracts as one of the most efficient purification steps when the enzyme was produced in mesophilic hosts (Figure 4). The presence of metal ions (Mg^{++} and Co^{++}) and high protein concentration in cell extracts were essential for optimal recovery of the enzyme during the heat treatment step. The *Bacillus subtilis* host strain used here lacked neutral protease but contained alkaline protease. The heat treated recombinant glucose isomerase preparation recovered from *B. subtilis* lacked detectable protease activity.

Analysis of crude extracts and of the purified preparations by SDS-PAGE and by specific activity determinations indicated that the thermostable glucose isomerase specified by the recombinant plasmids used in this study was one of the most abundant proteins in the cell extracts and acounted for approximately 14% of total cell extract protein in *E. coli* and 34% in *B. subtilis*. Heterologous proteins over-produced in *E. coli* often form insoluble "inclusion bodies" (7). Whether a protein precipitates in the cytoplasm or remains soluble, however, depends also on the nature of the protein being over-produced (5). In the case of the recombinant thermostable glucose isomerase, the enzyme remained soluble which may be an important factor in attempts to scale-up its production.

Findings of this study also suggest potential of utilizing the recombinant thermostable glucose isomerase over-expressed in *B. subtilis* for sweetener production. The neutral pH optimum for the activity of the cloned glucose isomerase is suitable

for the neutral or slightly acidic reaction conditions which reduce by-products and color formation during fructose production. The high thermostability of the enzyme allowed us to operate the isomerization reaction at high temperature and hence to shift the equilibrium towards higher fructose (17). Since, *B. subtilis* is a mesophilic and non-pathogenic host, it is feasible to produce a food-safe and thermostable glucose isomerase with a practical grade of purity (80%) by a simple heat treatment step. Also, constitutive production of the enzyme in *B. subtilis* will reduce the enzyme cost by saving on expensive inducers. Further studies on the long-term thermostability, metal ion requirements, and safety of the enzyme are needed before practical industrial utility of the recombinant thermostable glucose isomerase in *B. subtilis* can be assessed.

LITERATURE CITED

1. Bachmann, B. J. 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. 36:525-557.

2. Batt, C. A., E. O'Neill, S. R. Novak, J. Ko, and A. Sinskey. 1986. Hyper-expression of *Escherichia coli* xylose isomerase. Biotechnology Progress. 2:140-144.

3. Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.

4. Brady, G., H. M. Jantzen, H. U. Bernard, R. Brown, T. Hashimoto-Gotoh, and G. Schüetz. 1984. New cosmid vectors developed for eukaryotic DNA cloning. Gene 27:223-232.

5. Buell, G., and N. Panayotatos. 1986. Mechanism and practice, p. 345-363. In W. Reznikoff and L. Gold (ed.), Maximizing gene expression. Butterworths Publishers. Boston.

6. Clewell, D. B. 1972. Nature of Col E1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. 110:667-676.

7. Craik, C. S. 1987. Expression and overexpression of proteins discussion group summary, p. 467-476. In D. L. Oxender (ed.), Protein structure, folding, and design 2. Alan R. Liss, Inc. New York.

8. Craik, C. S., C. Largman, T. Fletcher, S. Roczniak, P J. Barr, R. Fletterik, and W. J. Rutter. 1985. Redesigning trypsin: Alteration of substrate specificity. Science. 228:291-297.

9. Crueger, W., and A. Crueger. 1982. Enzymes, p. 161-174. In W. Crueger and A. Crueger (ed.), Biotechnology: a textbook of industrial microbiology, Science Tech. Inc., Madison.

10. Dilella, A. G., and S. L. C. Woo. 1987. Cloning large segments of genomic DNA using cosmid vectors. Methods Enzymol. 152:199-212.

11. Dishe, Z., and E. Borenfreund. 1951. A new spectrophotometric methods for the detection and determination of keto sugars and trioses. J. Biol. Chem. 192:583-587.

12. Frischauf, A.-M. 1987. Construction and characterization of a genomic library in lambda vectors. Methods Enzymol. 152:190-199.

13. Huang, J. J., and N. W. Y. Ho. 1985. Cloning and expression of the *Escherichia coli* D-xylose isomerase gene in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. 126:1154-1160.

14. Hyun, H. H., and J. G. Zeikus. 1985. General biochemical characterization of thermostable extracellular ß-amylase from *Clostridium thermosulfurogenes*. Appl. Environ. Microbiol. 49:1162-1167.

15. Imanaka, T., M. Fujii, and S. Aiba. 1981. Isolation and characterization of antibiotic resistance plasmids from thermophilic bacilli and construction of deletion plasmids. J. Bacteriol. 146:1091-1097.

16. Imanaka, T., T. Himeno, and S. Aiba. 1985. Effect of *in vitro* DNA rearrangement in the NH_2-terminal region of the penicillinase gene from *Bacillus licheniformis* on the mode of expression in *Bacillus subtilis*. J. Gen. Microbiol. 131:1753.1763.

17. Jorgenson, O. B., L. G. Karlsen, N. B. Nielson, S. Pedersen, and S. Rugh. 1988. A new immobilized glucose isomerase with high productivity produced by a strain of *Streptomyces murinus*. Starch. 40:307-313.

18. Kuriki, T., S. Okada, and T. Imanaka. 1988. New type of pullulanase from *Bacillus stearothermophilus* and molecular cloning and expression of the gene in *Bacillus subtilis*. J. Bacteriol. 170:1554-1559.

19. Lambert, P. W., and J. L. Meers. 1983. The production of industrial enzymes. Phil. Trans. R. Soc. Lond. B300:263-282.

20. Lowry, O. H., N. J. Rosebrough, A. I. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

21. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

22. Marcel, T., D. Drocourt, and G. Tiraby. 1987. Cloning of the glucose isomerase (D-xylose isomerase) and xylulose kinase genes of *Streptomyces violaceoniger*. Mol. Gen. Genet. 208:121-126.

23. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3:208-218.

24. Panagiotis, E. S., and N. W. Y. Ho. 1985. Over-production of D-xylose

isomerase in *Escherichia coli* by cloning the D-xylose isomerase gene. Enzyme Micro. Technol. 7:592-596.

25. Schellenberg, G. D., A. Sarthy, A. E. Larson, M. P. Backer, J. W. Crabb, M. Lidstrom, B. D. Hall, and C. E. Furlong. 1984. Xylose isomerase from *Escherichia coli*: characterization of the protein and the structural gene. J. Biol. Chem. 259:6826-6832.

26. Schink, B., and J. G. Zeikus. 1983. *Clostridium thermosulfurogenes* sp. nov., a new thermophile that produces elemental sulphur from thiosulphate. J. Gen. Microbiol. 129:1149-1158.

27. Takasaki, Y., Y. Kosugi, and A. Kanbayashi. 1969. Studies on sugar-isomerizing enzyme; purification, crystallization and some properties of glucose isomerase. Arg. Biol. Chem. 33:1527-1534.

28. Wilhelm, M., and C. P. Hollenberg. 1985. Nucleotide sequence of the *Bacillus subtilis* xylose isomerase gene: extensive homology between the *Bacillus* and *Escherichia coli* enzyme. Nucleic Acids Res. 13:5717-5723.

29. Wovcha, M. G., D. L. Steuerwald, and K. E. Brooks. 1983. Amplification of D-xylose and D-glucose isomerase activities in *Escherichia coli* by gene cloning. Appl. Environ. Microbiol. 45:1402-1404.

30. Yutani, K., K. Ogasahara, Y. Sugino, and A. Matsushiro. 1977. Effect of a single amino acid substitution on stability of conformation of a protein. Nature. 267:274-275.

31. Zeikus, J. G., A. Ben-Bassat, and P. Hegge. 1980. Microbiology of methanogenesis in thermal, volcanic environments. J. Bacteriol. 143:432-440.

Chapter V

Molecular Characterization of the Xylose (Glucose) Isomerase Gene from *Clostridium thermosulfurogenes*: Role of His_{101} in Enzymatic Catalysis

ABSTRACT

The gene coding for thermophilic xylose (glucose) isomerase of *Clostridium thermosulfurogenes* was isolated and its complete nucleotide sequence was determined. The structural gene (*xyl*A) of the xylose isomerase encodes a polypeptide of 439 amino acids with an estimated molecular weight of 50,474. The deduced amino acid sequence of thermostable *C. thermosulfurogenes* xylose isomerase showed 50% and 70% homology to those of thermolabile xylose isomerases from *Escherichia coli* and *Bacillus subtilis*, respectively, whereas, it displayed much lower homology (22-24%) to those of thermostable xylose isomerases from *Ampullariella, Arthrobacter*, and *Streptomyces violaceoniger.* However, several discrete regions which are highly conserved throughout amino acid sequences of all six xylose isomerases could be detected. To elucidate the catalytic mechanism and the role of histidine in the active site of xylose isomerase, four histidine residues at different positions in the *C. thermosulfurogenes* enzyme were individually changed to phenylalanine by site-directed mutagenesis. Substitution of His_{101} by phenylalanine completely abolished enzyme activity whereas the other histidine substitutions had no effect on enzyme activity. When His_{101} was changed to glutamine, approximately 10% of wild-type enzyme activity was retained without appreciable change in the apparent K_m for glucose or xylose. The Gln_{101} mutant enzyme was not inactivated by treatment with 10 mM diethylpyrocarbonate while the xylose isomerases containing histidine at position 101 were totally inactivated. In addition, the log $Vmax_{app.}$ values of the Gln_{101} mutant enzyme at different pH values over the range of 5.0-8.0 remained

constant, whereas those of the wild-type (His_{101}) enzyme decreased markedly at pH 6.5-5.0. These findings are consistent with the proposed catalytic mechanism for glucose or xylose isomerization that is accomplished by histidine in the active site acting as a general base catalyst.

Introduction

Xylose isomerase (D-xylose ketol isomerase, EC 5.3.1.5) is an intracellular enzyme that catalyzes the isomerization reaction of D-xylose to D-xylulose. Its practical significance and commercial importance stems from its ability to isomerize D-glucose and convert it to D-fructose. It is, therefore, often referred to as glucose isomerase and is widely used in industry for production of high fructose syrups (1-3). Practical use of the enzyme has stimulated research on the physicochemical properties, function and structure of the enzyme from different sources and on the organization and regulation of its gene in different microorganisms (for review see 3-9). Thus, organization of the *xyl* operon in *Escherichia coli* (10, 13), *Bacillus subtilis* (11,14) and *Salmonella typhimurium* (12) has been established by genetic and nucleotide sequence analysis. In addition, the complete nucleotide sequence of the *xyl*A gene from *Ampullariella sp* (15) and *Streptomyces violaceoniger* (16) were reported.

It has been proposed that enzymatic isomerization of xylose to xylulose and glucose to fructose involves a cis-enediol intermediate via intramolecular hydrogen transfer and is accomplished by general base catalysis (17,18). Inhibition studies on *Streptomyces* glucose isomerase by chemical modification suggest the involvement of a histidine residue as the general base in the catalytic center of the enzyme (19). Recent crystallographic studies on xylose isomerases from *S. rubiginosus*, as well as on the enzyme from *Arthrobacter*, have indicated a putative structure of the active site and suggested that histidine residue at position 54 is involved in initial abstraction of

the proton from the first carbon of the substrate and thus assisted the formation of enediol intermediate (20,21). However, no biochemical evidence in support of the proposed mechanism and the involvement of His_{54} in the catalysis has been published to date.

In our laboratory, xylose isomerases from thermoanaerobic bacteria have been characterized for their biochemical properties (Lee, C. et al. manuscript submitted to J. Bacteriol.), and the gene coding for xylose isomerase of *Clostridium thermosulfurogenes* has been cloned and over-expressed in both *E. coli* and *B. subtilis* (Lee, C. et al. manuscript submitted to Appl. Environ. Microbiol.). Xylose isomerase of *C. thermosulfurogenes* purified from its original host or from either of the two recombinant mesophilic organisms exhibited high thermostability up to 85°C and was crystallized by the vapor diffusion method.

In this report we have isolated the gene coding for the *Clostridium thermosulfurogenes* xylose isomerase, which we propose to call *xyl*A in accordance with the nomenclature used for *E. coli*. We have determined the complete nucleotide sequence of this gene and demonstrated, by site-directed mutagenesis, the role of $histidine_{101}$ in catalysis and a $glutamine_{101}$ mutant enzyme that displays acid stable isomerase activity.

MATERIALS AND METHODS

Bacterial strains, enzymes, and chemicals

E. coli strain HB101 [F⁻*hsd*S20 *ara*-1 *rec*A13 *pro*A12 *lac*Y1 *gal*K2 *vpo*L20 *mtl*-1 *xyl*-5] (22) was used for expression of the cloned *C. thermosulfurogenes* xylose isomerase gene and *E. coli* strain JM107 [*end*A1 *gyr*A96 *thi hsd*R17 *sup*E44 *rel*A1 Δ(*lac-pro*AB) / F' *tra*D36 *pro*AB *lac*I^q ZΔM15] (23) was used as a host cell and lawn cells during nucleotide sequence determination and site-directed mutagenesis. *E. coli* strain GM33 [*dam*-3] (24) was used to confirm a methylated ClaI restriction site in the cloned DNA. Restriction endonucleases and enzymes for subcloning experiments were obtained from Bethesda Research Laboratories, Gaithersburg, MD. and New England Biolabs, Inc., Beverly, MA.. [α-^{35}S]dATP (500 Ci/mmol) was obtained from Du Pont-New England Nuclear, Wilmington, DE.. All chemicals were of reagent grade and of the highest purity available.

Subcloning and manipulation of DNA

A 5.2 kilobase *Eco*RI/*Sma*I DNA fragment containing the *C. thermosulfurogenes* xylose isomerase gene was isolated from plasmid pCGI38, and subcloned between the *Eco*RI and *Sma*I sites of expression vectors pMMB67EH and pMMB67HE (25). The resulting recombinant plasmids contained the *xyl*A gene in both orientations with respect to the inducible *tac* promoter of each vector. Determination of glucose isomerase activity, after induction with IPTG, in *E. coli* cells containing these recombinant plasmids, indicated that the transcriptional orientation of the *xyl*A gene

is in the direction from the *Acc*I site towards the *Bgl*II site as shown in the physical map of pCMG1-2 plasmid in Figure 1. A series of derivatives, containing deletions at each end of the insert DNA, were generated by *Bal*31 nuclease treatment of pCMG1-2, after linearization with either *Bam*HI or *Eco*RI. Shortened DNA fragments were blunt ended by treatment with T4 DNA polymerase and reinserted as *Eco*RI-blunt-end or *Bam*HI-blunt-end fragments into the vector pMMB67EH (26). Complementation of xylose isomerase activity in the strain HB1010 by the deletion plasmids was scored on MacConkey agar plates containing 1% xylose in the presence or absence of IPTG (0.2 mM).

The alkaline denaturation method was used for rapid extraction of plasmid DNA (27). Recovery of DNA from agarose gels was performed by using an electroelution apparatus, Elutrap (Schleicher and Schuell, Inc., Keene, NH). Digestion of DNA with restriction endonucleases, separation of fragments by electrophoresis, fill-in reaction of recessed ends in DNA fragments, ligation of DNA fragments, and transformation of *E. coli* with plasmid DNA were performed as described by Maniatis et al. (28) or as recommended by the manufacturers of enzymes.

Nucleotide sequence determination

DNA inserts (see Figure 1) of pCMG11-3 and pCMG11-9 were digested with restriction endonucleases, and the resulting DNA fragments were isolated and subcloned into M13mp18 or M13mp19 bacteriophage vectors. Nucleotide sequences of the subcloned inserts (see Figure 2) were determined by the dideoxy chain

Figure 1. Deletion mapping of the xylose isomerase gene (*xyl*A) of *C. thermosulfurogenes.*

The partial restriction map around the *xyl*A gene is shown at the top. Open bars (▭) represent *C. thermosulfurogenes* DNA, and the closed bar (▬) and the lines (——) represent the DNA of pHSG262 (41) and pMMB67EH vector plasmids, respectively. The triangles indicate an inducible *tac* promoter of pMMB67EH. Gene expression by each deletion plasmid were detected by complementation of xylose isomerase activity of *E. coli* HB101 on MacConkey agar plates containing 1% xylose. The closed bar marked *xyl*A denotes the approximate position of the structural gene *xyl*A. Restriction sites: E, *Eco*RI; P, *Pst*I; C, *Cla*I; A, *Acc*I; Bg, *Bgl*II; Hc, *Hin*cII; S, *Sma*I; B, *Bam*HI; Ha, *Hae*III.

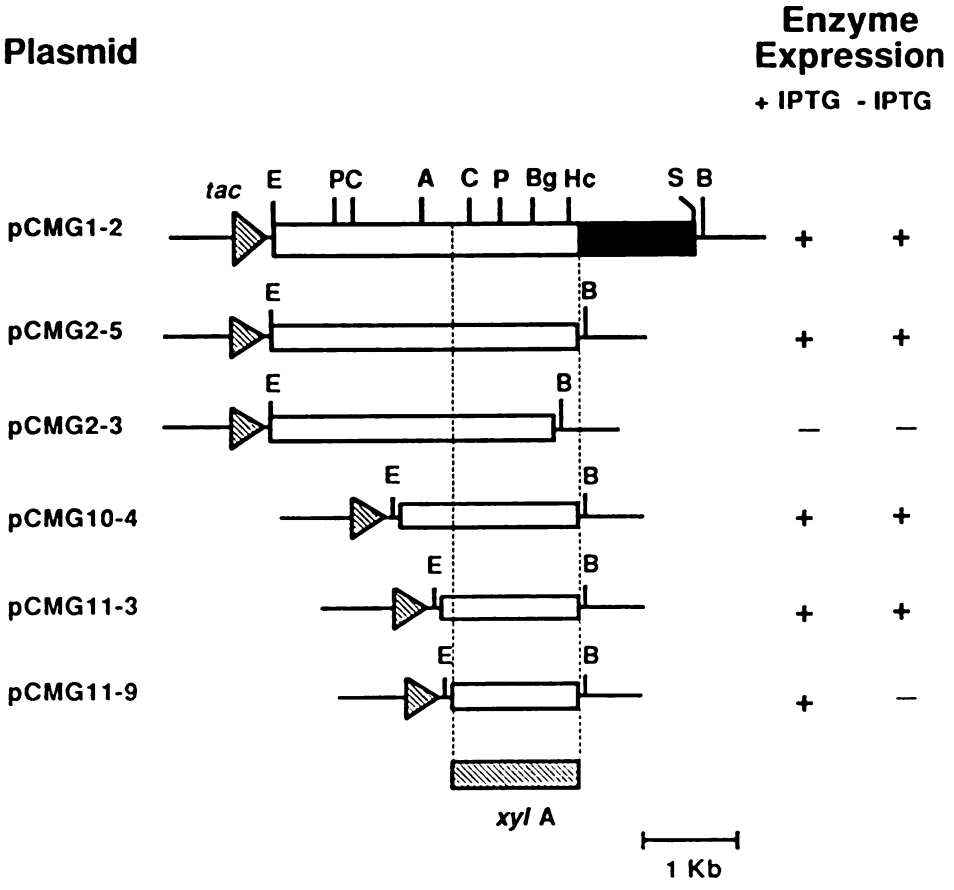
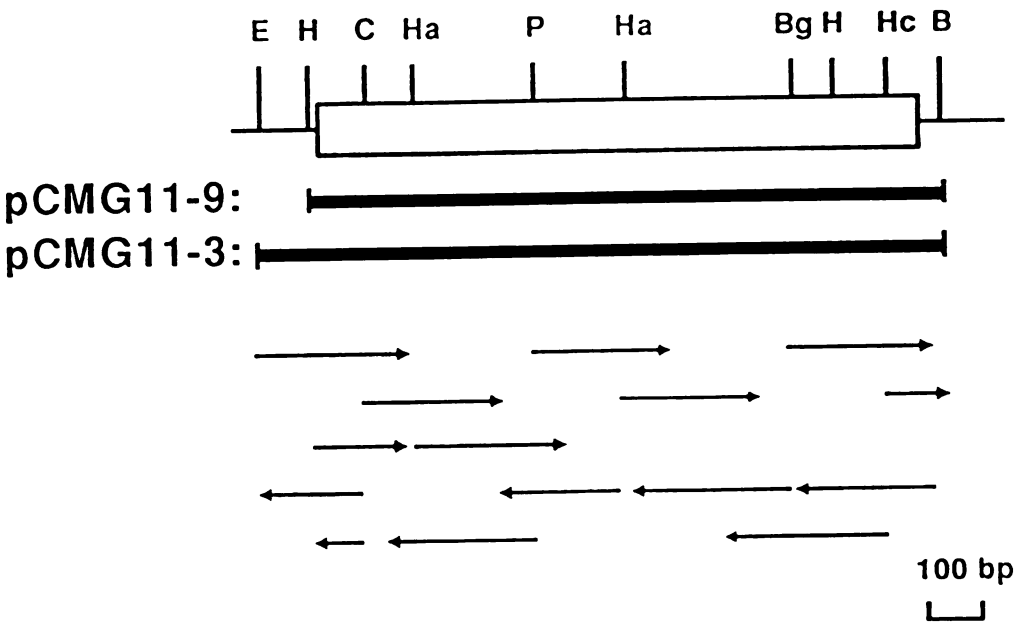

Plasmid
Enzyme Expression
+ IPTG - IPTG
tac
E
PC
A
C
P
Bg
Hc
S
B
pCMG1-2
pCMG2-5
pCMG2-3
pCMG10-4
pCMG11-3
pCMG11-9
+ +
+ +
– –
+ +
+ +
+ –
xyl A
1 Kb

Figure 2. Sequencing strategy for DNA encoding *C. thermosulfurogenes* glucose isomerase.

The open bar (▭) represents the coding region. The solid lines (▬) represent the *Eco*RI/*Bam*HI fragments from the recombinant plasmids, pCMG11-9 and pCMG11-3 (Figure 1). The arrowes indicate the direction and extent of the nucleotide sequence determined by the dideoxy chain termination method (29). For the abbreviation of restriction sites see the legend to Figure 1 except Ha for *Hae*III. bp, base pairs


E H C Ha P Ha Bg H Hc B
pCMG11-9:
pCMG11-3:
100 bp

termination method (29), using a Sequenase Version 2.0 Kit (United States Biochemical Co.). The sequence information was analyzed using the Sequence Analysis Software Package of the Genetics Computer Group, version 5, (University of Wisconsin) (30).

Site-directed mutagenesis

Plasmid pCMG11-3 was digested with *Eco*RI and *Bam*HI. The 1.4 kilobase DNA fragment, containing the entire *C. thermosulfurogenes xyl*A gene and its promoter region was isolated and subcloned into the *Eco*RI/*Bam*HI sites of M13mp19. The oligonucleotide primers, obtained from Genetic Designs, Inc., (Houston, TX), were designed to be complementary to the single-strand template DNA and to contain appropriate mismatches as indicated in Figure 3. Synthesis of mutant genes and selection were performed by the method of Eckstein and co-workers (31), using a kit of Oligonucleotide-directed Mutagenesis System, version 2 (Amersham Co., Arlington Heights IL). Nucleotide sequences of the mutant genes obtained were confirmed by the di-deoxy sequencing method as described above. The 1.4 kilobase *Eco*RI/*Bam*HI fragments, containing the mutant genes were subcloned from each M13mp19 recombinant into the *Eco*RI and *Bam*HI sites of pMMB67EH vectors, and introduced into *E. coli* strain HB101.

Enzyme purification

E. coli cells, carrying appropriate recombinant plasmids that specified mutant or wild-type xylose isomerase were cultivated overnight in LB media (28), containing

Figure 3. Synthetic oligonucleotide primers for site-directed mutagenesis.

The template DNA represents the coding strand of the *xyl*A gene inserted in M13mp19.

```
                                        40                  45
Template DNA   : 3' - TTC TGC TAC CTC CTC GTA GAA GCG AAA AGA TAT CGA - 5'
(Wild type)          Lys Thr Met Glu Glu His Leu Arg Phe Ser Ile Ala

   Primer I    : 5' -- AG ACG ATG GAG GAG TTT CTT CGC TTT TCT ATA GC -- 3'
   (for Phe41)                           Phe
```

```
                 65                  70                  75
Template DNA   : 3' - TAC GTT TCC GGT ACC TTA GTG ATA TGT CTA GGA TAC CTG - 5'
(Wild type)          Met Gln Arg Pro Trp Asn His Tyr Thr Asp Pro Met Asp

   Primer II   : 5' --- G CAA AGG CCA TGG AAT TTC TAT ACA GAT CCT ATG G --- 3'
   (for Phe71)                               Phe
```

```
                       95                  100                 105
Template DNA   : 3' - TAT TTA CGT GGC ATA AAG ACG AAG GTA CTA TCT CTA TAA CGG GGA - 5'
(Wild type)          Ile Asn Ala Pro Tyr Phe Cys Phe His Asp Arg Asp Ile Ala Pro

   Primer III  : 5' --------- GCA CCG TAT TTC TGC TTC TTT GAT AGA GAT ATT GCC CC -- 3'
   (for Phe101)                                    Phe

   Primer IV   : 5' ---------- A CCG TAT TTC TGC TTC CAA GAT AGA GAT ATT GCC ----- 3'
   (for Gln101)                                    Gln

   Primer V    : 5' -- TA AAT GCA CCG TAT TTC GCC TTC CAT GAT AGA GAT A ---------- 3'
   (for Ala99)                               Ala
```

```
                                   150                 155
Template DNA   : 3' - AGG TTA GGT TCT AAA CAC GTA CCA CGT AGT TGC AGA - 5'
(wild type)          Ser Asn Pro Arg Phe Val His Gly Ala Ser Thr Ser

   Primer VI   : 5' -- CC AAT CCA AGA TTT GTG TTT GGT GCA TCA ACG TC -- 3'
   (for Phe152)                          Phe
```

100 μg/ml Ampicillin, at 37°C with vigorous shaking. Cells harvested by centrifugation at 5,000 x g were washed and suspended in 50 mM MOPS buffer (pH 7.0) containing 10 mM $MgSO_4$ and 1 mM $CoCl_2$. For preparation of cell extracts, cells were broken by two passages through a French pressure cell at 18,000 p.s.i., and the debris was removed by centrifugation at 12,000 x g for 20 min. The cell extracts were stirred at 85°C for 20 min and centrifuged at 12,000 x g for 30 min. In the case of the His-Phe_{152} mutant enzyme, 60°C was used during the heat treatment because this mutant enzyme exhibited lower thermostability than the wild-type enzyme. The soluble fractions from the heated cell extracts were loaded onto a DEAE-Sepharose CL-6B columns (4.0 cm x 32 cm), pre-equilibrated with 50 mM MOPS buffer pH 7.0, and proteins were eluted with linear NaCl salt gradients (0.0-0.5M) in the same buffer. Fractions containing significant glucose isomerase activity were pooled. For the His-Phe_{101} mutant protein, which lacks enzyme activity, the protein was identified by SDS-PAGE analysis from the fractions.

Enzyme assays

Reaction mixtures (1 ml) contained: 0.8 M glucose, 10 mM $MgSO_4$, 1 mM $CoCl_2$, and the enzyme in 100 mM MOPS buffer (pH 7.0). For the assay of xylose isomerase activity, the reaction mixture (1 ml) contained: 70 mM xylose, 10 mM $MnSO_4$, and the enzyme in 100 mM MOPS buffer (pH 7.0). After 30 min incubation at 65°C, 1 ml of 0.5 M perchloric acid was added to stop the reaction and the mixture was further diluted 10- and 50-fold with double distilled water before the addition of cysteine-carbazole-sulfuric acid reagent (32). Fructose isomerizing

activity was assayed under the same reaction conditions as in the glucose isomerase assay except for the substitution of 0.4 M fructose as a substrate in the reaction mixture. After 30 min incubation at 65°C, the reaction was terminated by placing the reaction tubes in ice water, and the amount of glucose formed was determined with a glucose analyzer model 27 (Yellowstone Instrument Co. Inc., Yellow Springs, OH). One unit of activity is defined as the amount of enzyme that produced 1 μmol of product per min under the assay conditions described above. Apparent K_m and V_{max} values were obtained from Lineweaver-Burk plots of specific activities at various substrate concentrations. Protein concentration was determined by the method of Lowry et al. (33) with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Expression of C. thermosulfurogenes xylA gene

Physical maps of the DNA fragment containing the *C. thermosulfurogenes xyl*A gene and the deletion fragments generated from it are shown in Figure 1. Expression of glucose isomerase by *E. coli* HB101, harboring recombinant plasmids containing these DNA fragments is presented in Table 1. The *xyl*A gene in plasmid pCMG11-3 (and in all recombinant plasmids carrying bigger DNA inserts; results not shown) was expressed constitutively, whereas the expression from the smaller DNA fragment, present in the plasmid pCMG11-9 occurred only in the presence of IPTG. These results indicate that the region of approximately 0.1 kilobase upstream of the *xyl*A gene, present in the plasmid pGM11-3, contains promoter sequences functional in *E. coli*, presumably the original promoter of the *C. thermosulfurogenes xyl*A gene, whereas the 1.2 kilobase insert of the plasmid pCMG11-9 contains the structural gene of xylose isomerase that is expressed from the *tac* promoter of the vector.

Nucleotide sequence of C. thermosulfurogenes xylA gene

The nucleotide sequence of the insert in the recombinant plasmid pCMG11-3 was 1434 base pairs in length and contained an open reading frame starting with the ATG codon at nucleotides 79-81 and ending with two termination codons TAA and TGA at nucleotides 1396-1401 (Figure 4). It was confirmed as the correct reading frame for the *C. thermosulfurogenes xyl*A gene by comparison of the N-terminal amino acid sequence (7 amino acids underlined in Figure 4), determined on the xylose

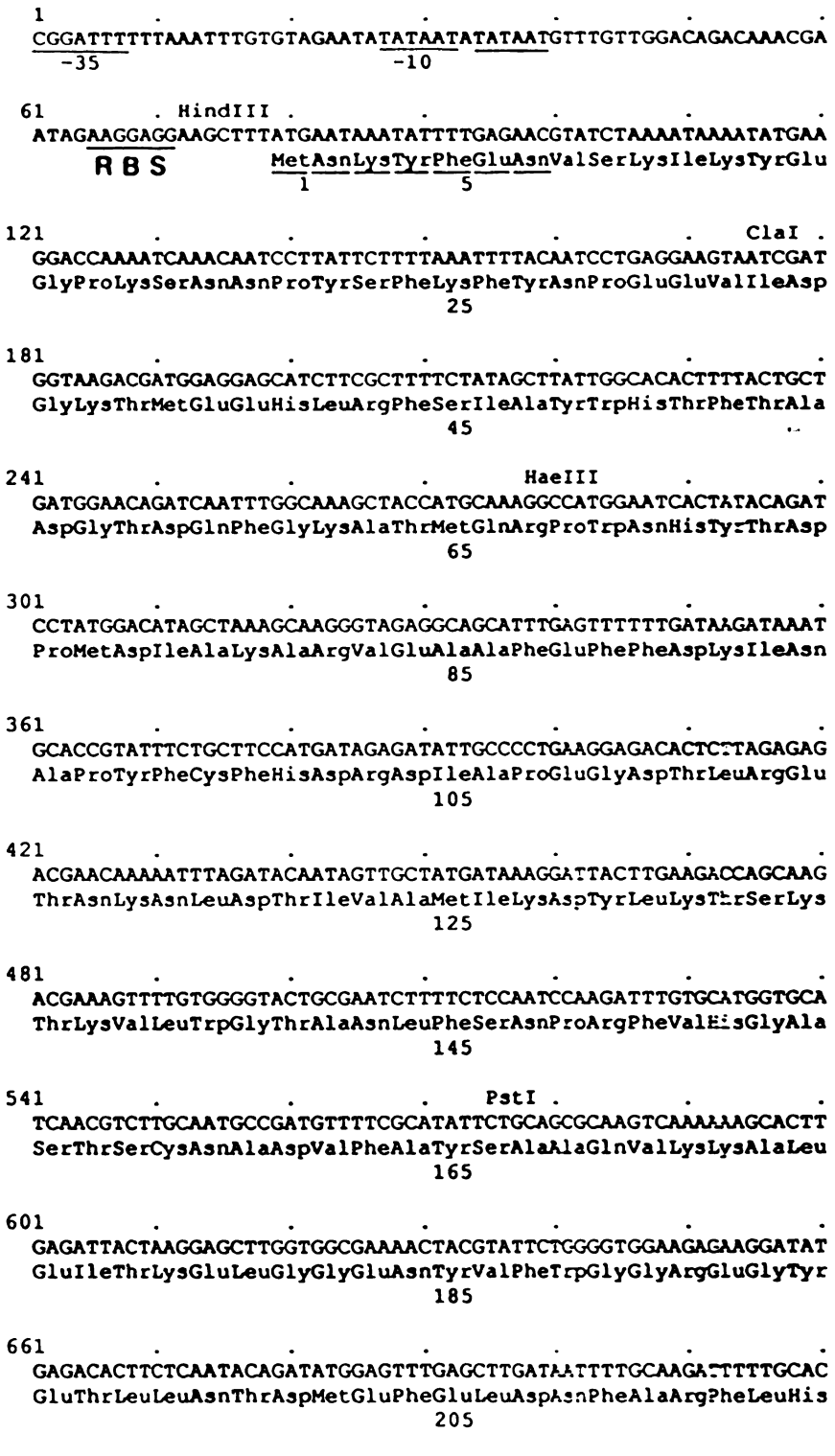
Table 1. Expression of *Clostridium xyl*A gene by recombinant plasmids in *E. coli*

Specific activity of glucose isomerase were determined as described under Methods and Materials from cell extracts which contained a recombinant plasmid with different size of insert DNA (see, Figure 2). Cultures of *E. coli* HB101, containing recombinant plasmids described in Fig. 2, were grown in LB media containing no inducers, 1% D-xylose, 0.2 mM IPTG, or 1% xylose and 0.2 mM IPTG, at 37°C in the presence of ampicillin(100 μg/ml). Cells were harvested at late exponential growth phase, washed with 50 mM MOPS buffer, resuspended in the same buffer and disrupted by sonication. Cell debris was removed by centrifugation at 12,000 x g and the extracts were used for activity determination as described in Materials and Methods.

Plasmids	Glucose isomerase activity (unit/mg protein)			
	Inducer			
	None	D-xylose	IPTG	D-xylose + IPTG
pMMB67EH	0.00	0.00	0.00	0.00
pCMG11-3	0.29	0.26	0.56	0.55
pCMG11-9	0.00	0.00	0.12	0.10

Figure 4. Nucleotide and deduced amino acid sequence of *C. thermosulfurogenes xylA* gene.

The numbers on the left refer to the nucleotide sequence starting at the first base of the insert DNA of pCMG11-3. The deduced amino acid sequence is shown below the nucleotide strand. The ATG initiation codon of the correct open reading frame was identified by comparison of the deduced amino acid sequence with the seven NH_2-terminal amino acid sequence (underlined) determined from the purified *C. thermosulfurogenes* xylose isomerase. The putative -35 and -10 sequences in the promoter region and the putative ribosome-binding site (RBS) are underlined. The first base of the insert DNA of pCMG11-9 begins at *Hin*dIII site (AGCTT) immediately downstream from the ribosome-binding site. The restriction sites used in this study are shown above the nucleotide sequence. The *Cla*I site in parenthesis could be detected only in a methylation deficient (*dam*) *E. coli* strain (24) (see Methods and Materials).


1
CGGATTTTTTAAATTTGTGTAGAATATATAATATATAATGTTTGTTGGACAGACAAACGA
-35
-10
61
HindIII
ATAGAAGGAGGAAGCTTTATGAATAAATATTTTGAGAACGTATCTAAAATAAAATATGAA
RBS
MetAsnLysTyrPheGluAsnValSerLysIleLysTyrGlu
1
5
121
ClaI
GGACCAAAATCAAACAATCCTTATTCTTTTAAATTTTACAATCCTGAGGAAGTAATCGAT
GlyProLysSerAsnAsnProTyrSerPheLysPheTyrAsnProGluGluValIleAsp
25
181
GGTAAGACGATGGAGGAGCATCTTCGCTTTTCTATAGCTTATTGGCACACTTTTACTGCT
GlyLysThrMetGluGluHisLeuArgPheSerIleAlaTyrTrpHisThrPheThrAla
45
241
HaeIII
GATGGAACAGATCAATTTGGCAAAGCTACCATGCAAAGGCCATGGAATCACTATACAGAT
AspGlyThrAspGlnPheGlyLysAlaThrMetGlnArgProTrpAsnHisTyrThrAsp
65
301
CCTATGGACATAGCTAAAGCAAGGGTAGAGGCAGCATTTGAGTTTTTTGATAAGATAAAT
ProMetAspIleAlaLysAlaArgValGluAlaAlaPheGluPhePheAspLysIleAsn
85
361
GCACCGTATTTCTGCTTCCATGATAGAGATATTGCCCCTGAAGGAGACACTCTTAGAGAG
AlaProTyrPheCysPheHisAspArgAspIleAlaProGluGlyAspThrLeuArgGlu
105
421
ACGAACAAAAATTTAGATACAATAGTTGCTATGATAAAGGATTACTTGAAGACCAGCAAG
ThrAsnLysAsnLeuAspThrIleValAlaMetIleLysAspTyrLeuLysThrSerLys
125
481
ACGAAAGTTTTGTGGGGTACTGCGAATCTTTTCTCCAATCCAAGATTTGTGCATGGTGCA
ThrLysValLeuTrpGlyThrAlaAsnLeuPheSerAsnProArgPheValHisGlyAla
145
541
PstI
TCAACGTCTTGCAATGCCGATGTTTTCGCATATTCTGCAGCGCAAGTCAAAAAAGCACTT
SerThrSerCysAsnAlaAspValPheAlaTyrSerAlaAlaGlnValLysLysAlaLeu
165
601
GAGATTACTAAGGAGCTTGGTGGCGAAAACTACGTATTCTGGGGTGGAAGAGAAGGATAT
GluIleThrLysGluLeuGlyGlyGluAsnTyrValPheTrpGlyGlyArgGluGlyTyr
185
661
GAGACACTTCTCAATACAGATATGGAGTTTGAGCTTGATAATTTTGCAAGATTTTTGCAC
GluThrLeuLeuAsnThrAspMetGluPheGluLeuAspAsnPheAlaArgPheLeuHis
205

```
721          .          .          .       HaeIII     .          .
   ATGGCTGTTGATTATGCAAAGGAAATCGGCTTTGAAGGCCAGTTCTTGATTGAGCCGAAG
   MetAlaValAspTyrAlaLysGluIleGlyPheGluGlyGlnPheLeuIleGluProLys
                                    225

781          .          .          .          .          .          .
   CCAAAGGAGCCTACAAAGCATCAATACGACTTTGACGTGGCAAATGTATTGGCATTCTTG
   ProLysGluProThrLysHisGlnTyrAspPheAspValAlaAsnValLeuAlaPheLeu
                                    245

841          .          .          .          .          .          .
   AGAAAATACGATCTTGACAAATATTTCAAAGTTAATATCGAAGCAAATCATGCAACATTA
   ArgLysTyrAspLeuAspLysTyrPheLysValAsnIleGluAlaAsnHisAlaThrLeu
                                    265

901          .          .          .          .          .       (ClaI)
   GCATTCCATGATTTCCAGCATGAGCTAAGATACGCCAGAATAAACGGTGTATTAGGATCG
   AlaPheHisAspPheGlnHisGluLeuArgTyrAlaArgIleAsnGlyValLeuGlySer
                                    285

961          .          .          .          .          .          .
   ATTGACGCAAATACGGGTGATATGCTATTAGGATGGGATACAGATCAGTTCCCTACAGAT
   IleAspAlaAsnThrGlyAspMetLeuLeuGlyTrpAspThrAspGlnPheProThrAsp
                                    305

1021         .          .          .          .          .          .
   ATACGCATGACAACACTTGCTATGTATGAAGTTATAAAGATGGGTGGATTTGACAAAGGC
   IleArgMetThrThrLeuAlaMetTyrGluValIleLysMetGlyGlyPheAspLysGly
                                    325

1081         .          .          .          .       BglII      .
   GGACTCAACTTCGATGCGAAAGTAAGACGTGCTTCATTTGAGCCAGAAGATCTTTTCTTG
   GlyLeuAsnPheAspAlaLysValArgArgAlaSerPheGluProGluAspLeuPheLeu
                                    345

1141         .          .          .          .          . HindIII .
   GGTCACATAGCAGGAATGGATGCTTTTGCAAAAGGCTTCAAAGTGGCTTACAAGCTTGTA
   GlyHisIleAlaGlyMetAspAlaPheAlaLysGlyPheLysValAlaTyrLysLeuVal
                                    365

1201         .          .          .          .          .          .
   AAAGATAGGGTATTTGACAAGTTCATCGAAGAAAGATATGCAAGCTACAAAGATGGCATA
   LysAspArgValPheAspLysPheIleGluGluArgTyrAlaSerTyrLysAspGlyIle
                                    385

1261         .          .          .          .          .          .
   GGTGCAGATATTGTAAGTGGGAAAGCTGATTTTAGAAGTCTTGAAAAGTATGCATTAGAG
   GlyAlaAspIleValSerGlyLysAlaAspPheArgSerLeuGluLysTyrAlaLeuGlu
                                    405

1321       . HincII .          .          .          .          .
   CGCAGCCAGATTGTCAACAAATCAGGAAGACAAGAGCTATTAGAGTCAATCCTAAATCAG
   ArgSerGlnIleValAsnLysSerGlyArgGlnGluLeuLeuGluSerIleLeuAsnGln
                                    425

1381         .          .          .          .          .
   TATTTGTTTGCAGAATAATGAAACATGAGGGCAGCTTCATGCTTCATTAAAGCG
   TyrLeuPheAlaGlu******
                439
```

isomerase purified from *C. thermosulfurogenes*, to the amino acid sequence deduced from the nucleotide sequence presented in Figure 3. Thus, the predicted polypeptide encoded by this reading frame contains 439 amino acids and has a calculated molecular weight of 50,474, a value that is in close agreement with the subunit molecular weight estimated by SDS-polyacrylamide gel electrophoresis on xylose isomerase purified from the original host. The ATG initiation codon is preceded, with a spacing of 7 base pairs, by a sequence AAGGAGG which is complementary to the 3' end of both the *E. coli* and *B. subtilis* 16S ribosomal RNA (34,35). This sequence presumably acts as ribosome-binding site during the expression of the *C. thermosulfurogenes xyl*A gene in these organisms. Upstream of the ribosome binding site a sequence CGGATT, at nucleotides 1-6 and TATAATATATAAT, at nucleotides 27-39, are homologous to -35 and -10 region, respectively, of promoters found in *E. coli* and *B. subtilis* (36,37).

It has been reported that *xyl*A gene of *E. coli* could not be expressed from its own promoter in *B. subtilis* cells unless a promoter from *B. licheniformis* was inserted upstream of the structural gene (38). In the case of the *C. thermosulfurogenes xyl*A gene the region of the first 81 base pairs, shown in Figure 4, was sufficient for high level expression in both *E. coli* and *B. subtilis* (Table 1 and unpublished data). It is not clear at present whether the same sequences are functional as promoters and ribosome binding sites in each of three organisms (*C. thermosulfurogenes, E. coli*, and *B. subtilis*) or whether different sequences present in the upstream region function in different hosts.

Comparison of amino acid sequences in different xylose isomerases

Comparison of the amino acid sequence, deduced from the nucleotide sequence of *C. thermosulfurogenes xyl*A gene, to amino acid sequences of xylose isomerases from *E. coli* (13), *B. subtilis* (14), *S. violaceoniger* (16), *Ampullariella* sp. 3876 (15) and *Arthrobacter* strain 3728 (21) are presented in Figure 5. The length of the polypeptide chain encoded by the *C. thermosulfurogenes xyl*A gene (439 amino acid residues) is similar to those of *E. coli* and *B. subtilis* enzymes (440 residues), whereas the enzymes from *S. violaceoniger*, *Ampullariella*, and *Arthrobacter* have polypeptides shorter by 47 amino acid residues at the N-terminal end. The deduced amino acid sequence of thermostable *C. thermosulfurogenes* xylose isomerase exhibits a higher degree of homology to the sequences of thermolabile xylose isomerases from *E. coli* and *B. subtilis* (50-70%) than to those of thermostable enzymes from *S. violaceoniger*, *Ampullariella* and *Arthrobacter* (22-24%; see Figure 6). On the other hand, the sequences of thermostable xylose isomerases of the mesophilic microorganisms, *S. violaceoniger*, *Ampullariella*, and *Arthrobacter*, display a high degree of homology among themselves (65-68%; Figure 5 and 6). These comparisons indicate that there are three distinct classes of xylose isomerases, thermolabile of the *E. coli* and *B. subtilis* type, thermostable of the *C. thermosulfuricum* type, and thermostable of the *S. violaceoniger* type.

To understand the molecular mechanism of thermostability in these enzymes, the possibility that disulfide bond formation and composition of hydrophobic regions within each protein molecule could contribute to the compactness of the secondary and tertiary structure was considered. The sequence of *C. thermosulfurogenes* xylose

Figure 5. Comparison of the amino acid sequences of different xylose isomerases.

The abbreviations are: E.c., *Escherichia coli* (13); B.s., *Bacillus subtilis* (14); C.t., *Clostridium thermosulfurogenes* (this study); S.v., *Streptomyces violaceoniger* (16); Amp., *Ampullariella* sp. strain 3876 (15); Art., *Arthrobacter* strain B3728 (21). The sequences of thermolabile enzymes are placed above, and the sequences of thermostable enzymes below that of *C. thermosulfurogenes* enzyme. The sequences are aligned to give a maximum homology by using the computer program, University of Wisconsin-Genetics Computer Group, version 5 (30). Dots in the amino acid sequences represent the gaps introduced to obtain the best fit. Identical residues between *C. thermosulfurogenes* and other species amino acid sequences are denoted by asterisks, and highly conserved regions in all xylose isomerases are boxed. The arrow indicates the position of the His_{101} residue in *C. thermosulfurogenes* xylose isomerase.

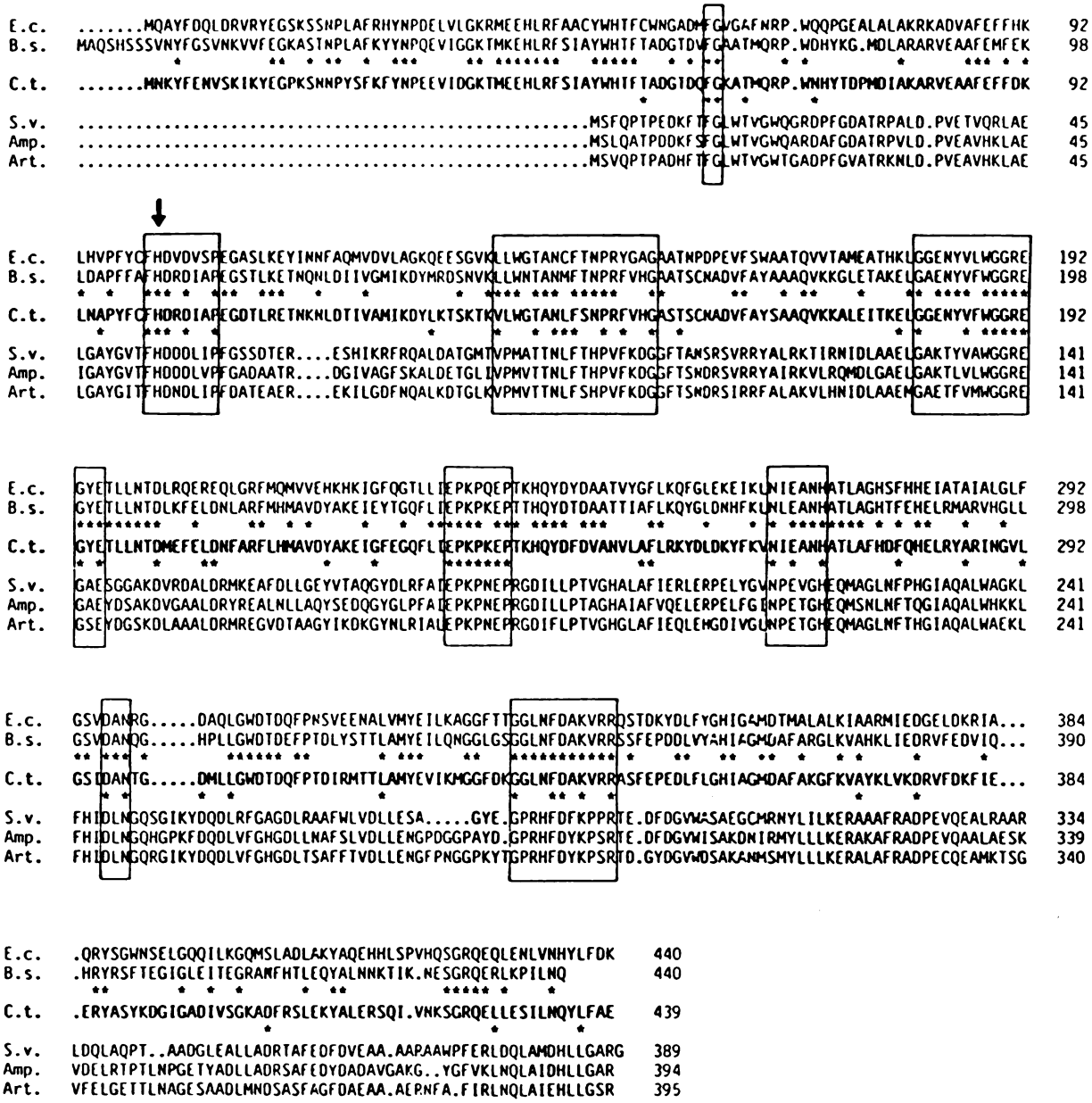
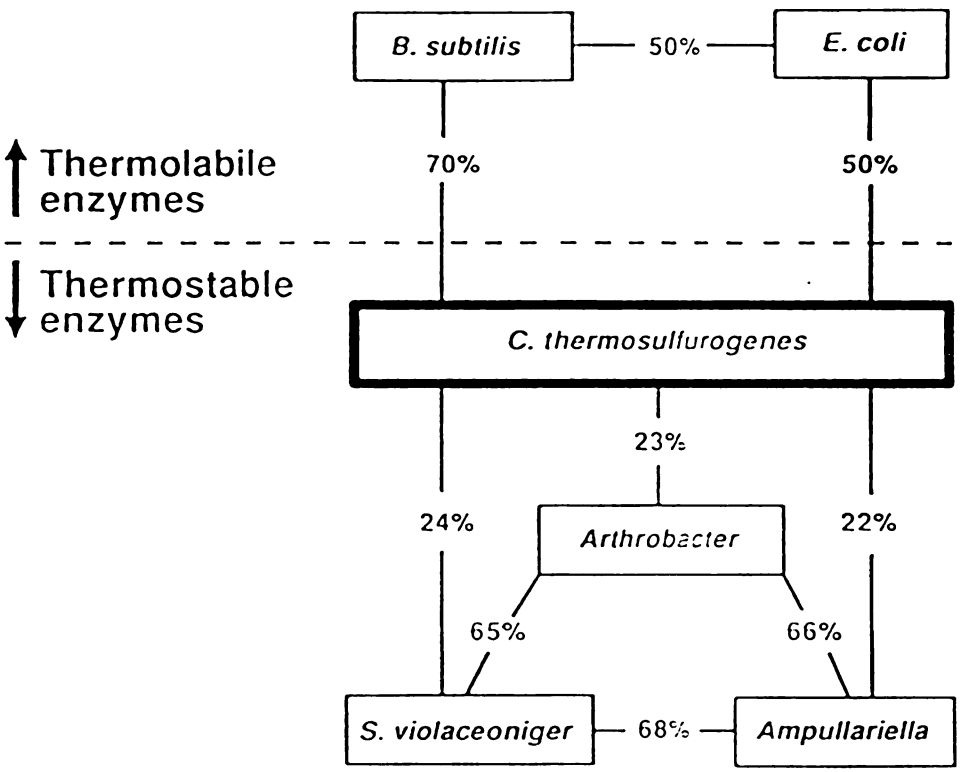

E.c. .....MQAYFDQLDRVRYEGSKSSNPLAFRHYNPDELVLGKRMEEHLRFAACYWHTFCWNGADMFGVGAFNRP.WQQPGEALALAKRKADVAFEFFHK 92
B.s. MAQSHSSSVNYFGSVNKVVFEGKASTNPLAFKYYNPQEVIGGKTMKEHLRFSIAYWHTFTADGTDVFGAATMQRP.WDHYKG.MDLARARVEAAFEMFEK 98
C.t. .....MNKYFENVSKIKYEGPKSNNPYSFKFYNPEEVIDGKTMEEHLRFSIAYWHTFTADGTDQFGKATMQRP.WNHYTDPMDIAKARVEAAFEFFDK 92
S.v. .....MSFQPTPEQKFTFGLWTVGWQGRDPFGDATRPALD.PVETVQRLAE 45
Amp. .....MSLQATPDDKFSFGLWTVGWQARDAFGDATRPVLD.PVEAVHKLAE 45
Art. .....MSVQPTPADHFTFGLWTVGWTGADPFGVATRKNLD.PVEAVHKLAE 45
E.c. LHVPFYCFHDVDVSPEGASLKEYINNFAQMVDVLAGKQEESGVKLLWGTANCFTNPRYGAGAATNPDPEVFSWAATQVVTAMEATHKLGGENYVLWGGRE 192
B.s. LDAPFFAFHDRDIAPEGSTLKETNQNLDIIVGMIKDYMRDSNVKLLWNTANMFTNPRFVHGAATSCNADVFAYAAAQVKKGLETAKELGAENYVFWGGRE 198
C.t. LNAPYFCFHDRDIAPEGDTLRETNKNLDTIVAMIKDYLKTSKTKVLWGTANLFSNPRFVHGASTSCNADVFAYSAAQVKKALEITKELGGENYVFWGGRE 192
S.v. LGAYGVTFHDDDLIPFGSSDTER....ESHIKRFRQALDATGMTVPMATTNLFTHPVFKDGGFTANSRSVRRYALRKTIRNIDLAAELGAKTYVAWGGRE 141
Amp. IGAYGVTFHDDDLVPFGADAATR....DGIVAGFSKALDETGLIVPMVTTNLFTHPVFKDGGFTSNDRSVRRYAIRKVLRQMDLGAELGAKTLVLWGGRE 141
Art. LGAYGITFHDNDLIPFDATEAER....EKILGDFNQALKDTGLKVPMVTTNLFSHPVFKDGGFTSNDRSIRRFALAKVLHNIDLAAEMGAETFVMWGGRE 141
E.c. GYETLLNTDLRQEREQLGRFMQMVVEHKHKIGFQGTLLIEPKPQEPTKHQYDYDAATVYGFLKQFGLEKEIKLNIEANHATLAGHSFHHEIATAIALGLF 292
B.s. GYETLLNTDLKFELDNLARFMHMAVDYAKEIEYTGQFLIEPKPKEPTTHQYDTDAATTIAFLKQYGLDNHFKLNLEANHATLAGHTFEHELRMARVHGLL 298
C.t. GYETLLNTDMEFELDNFARFLHMAVDYAKEIGFEGQFLIEPKPKEPTKHQYDFDVANVLAFLRKYDLDKYFKVNIEANHATLAFHDFQHELRYARINGVL 292
S.v. GAESGGAKDVRDALDRMKEAFDLLGEYVTAQGYDLRFAIEPKPNEPRGDILLPTVGHALAFIERLERPELYGVNPEVGHEQMAGLNFPHGIAQALWAGKL 241
Amp. GAEYDSAKDVGAALDRYREALNLLAQYSEDQGYGLPFAIEPKPNEPRGDILLPTAGHAIAFVQELERPELFGINPETGHEQMSNLNFTQGIAQALWHKKL 241
Art. GSEYDGSKDLAAALDRMREGVDTAAGYIKDKGYNLRIALEPKPNEPRGDIFLPTVGHGLAFIEQLEHGDIVGLNPETGHEQMAGLNFTHGIAQALWAEKL 241
E.c. GSVDANRG.....DAQLGWDTDQFPNSVEENALVMYEILKAGGFTTGGLNFDAKVRRQSTDKYDLFYGHIGAMDTMALALKIAARMIEDGELDKRIA... 384
B.s. GSVDANQG.....HPLLGWDTDEFPTDLYSTTLAMYEILQNGGLGSGGLNFDAKVRRSSFEPDDLVYAHIAGMDAFARGLKVAHKLIEDRVFEDVIQ... 390
C.t. GSIDANTG.....DMLLGWDTDQFPTDIRMTTLAMYEVIKMGGFDKGGLNFDAKVRRASFEPEDLFLGHIAGMDAFAKGFKVAYKLVKDRVFDKFIE... 384
S.v. FHIDLNGQSGIKYDQDLRFGAGDLRAAFWLVDLLESA.....GYE.GPRHFDFKPPRTE.DFDGVWASAEGCMRNYLILKERAAAFRADPEVQEALRAAR 334
Amp. FHIDLNGQHGPKFDQDLVFGHGDLLNAFSLVDLLENGPDGGPAYD.GPRHFDYKPSRTE.DFDGVWISAKDNIRMYLLLKERAKAFRADPEVQAALAESK 339
Art. FHIDLNGQRGIKYDQDLVFGHGDLTSAFFTVDLLENGFPNGGPKYTGPRHFDYKPSRTD.GYDGVWDSAKANMSMYLLLKERALAFRADPECQEAMKTSG 340
E.c. .QRYSGWNSELGQQILKGQMSLADLAKYAQEHHLSPVHQSGRQEQLENLVNHYLFDK 440
B.s. .HRYRSFTEGIGLEITEGRANFHTLEQYALNNKTIK.NESGRQERLKPILNQ 440
C.t. .ERYASYKDGIGADIVSGKADFRSLEKYALERSQI.VNKSGRQELLESILNQYLFAE 439
S.v. LDQLAQPT..AADGLEALLADRTAFEDFDVEAA.AAPAAWPFERLDQLAMDHLLGARG 389
Amp. VDELRTPTLNPGETYADLLADRSAFEDYDADAVGAKG..YGFVKLNQLAIDHLLGAR 394
Art. VFELGETTLNAGESAADLMNDSASFAGFDAEAA.AEPNFA.FIRLNQLAIEHLLGSR 395

Figure 6. Summary of amino acid sequence homology between different xylose isomerases.

The percent of homology was calculated for the number of amino acid residues that match exactly between amino acid sequences from different sources by using the University of Wisconsin-Genetics Computer Group, version 5 program (30).

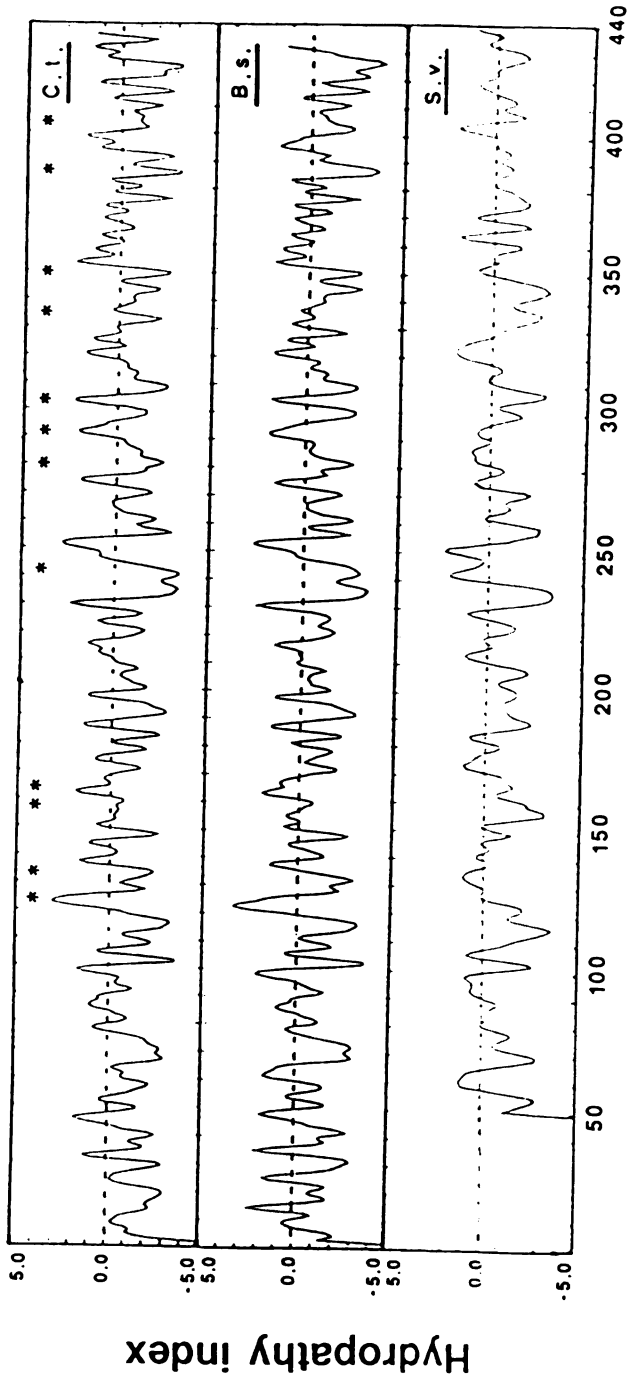

B. subtilis
50%
E. coli
Thermolabile enzymes
70%
50%
Thermostable enzymes
C. thermosulfurogenes
23%
24%
Arthrobacter
22%
65%
66%
S. violaceoniger
68%
Ampullariella

isomerase contains two cysteine residues, at positions of 99 and 158, whereas the thermolabile enzyme of *B. subtilis* type contains only one cysteine residue at amino acid position 158 of the enzyme (Figure 5). The Cys_{99} of *C. thermosulfurogenes* enzyme was, therefore, changed, by site-directed mutagenesis, to alanine which is present in at the corresponding site in the *B. subtilis* enzyme. This substitution, however, did not affect either the thermostability or specific activity of the *C. thermosulfurogenes* enzyme. Moreover, treatment with a reducing agent (25 mM dithiothreitol) did not affect the specific activity of the wild-type enzyme (Table 2, and results not shown). If we consider, in addition, the fact that thermoresistant enzymes from *S. violaceoniger* and *Arthrobacter* each contain only one cysteine residue and the *Ampullariella* enzyme contains no cysteine we have to conclude that, in these xylose isomerase molecules, disulfide bonds do not contribute significantly to the compactness of the molecule which is generally considered to be a requirement for protein thermostability.

Another feature believed to contribute significantly to thermostability of proteins is the number and distribution of hydrophobic regions in the molecule (40). We have, therefore, compared the predicted hydrophobicity profiles of the *Clostridium*, *B. subtilis* and *S. violaceoniger* enzymes using the method of Kyte and Doolittle (39). The results, presented in Figure 7, indicated that the thermostable enzyme from *S. violaceoniger* contained fewer hydrophobic regions than did the thermostable enzyme from *Clostridium*, which in turn, exhibited a very similar hydrophobicity profile to that of the thermolabile enzyme from *B. subtilis*. These results argue against the general idea that enzyme thermostability relates to the compactness of the native

Figure 7. Comparison of hydropathy profiles of xylose isomerases from *C. thermosulfurogenes* (C.t.), *B. subtilis* (B.s.), and *S. violaceoniger* (S.v.).

The deduced amino acid sequences were analyzed by the method of Kyte and Doolittle (39) with a computer program MSEQ, University of Michigan (42) using a window size of 7-residues. The hydrophobic regions have a positive hydropathy index score and appear above the central dotted line. Amino acid numbers refer to the sequence of *C. thermosulfurogenes* enzyme. Asterisks indicate the regions where the hydropathy profile is significantly different between *C. thermosulfurogenes* and *S. violaceoniger* enzymes.


C.t.
B.s.
S.v.
5.0
0.0
-5.0
50
100
150
200
250
300
350
400
440
Hydropathy index
Amino acid number

molecule which can be predicted alone from the number of disulfide and hydrophobic bonds that may be formed by the polypeptide chain. It is concluded here that regions actually involved in the formation and maintenance of the three dimensional structure are the key factors that determine the general resistance of protein molecule to thermal changes. In support of this conclusion, the site-directed mutant Phe_{152} enzyme, described below, was less thermal stable than the wild-type His_{152} enzyme by 20°C (data not shown). Xylose isomerases that share extensive homology, but differ widely in thermostability, such as that of *B. subtilis* and *C. thermosulfurogenes* seem to be excellent models for further investigation of this problem.

Despite the existence of profound differences in the amino acid sequences between the individual classes of xylose isomerase, outlined in Figures 5 and 6, several regions are highly conserved throughout all of the six xylose isomerase polypeptides (Figure 5). Amino acid residues suggested to be parts of the catalytic site, metal ion binding site and substrate binding site, by X-ray crystallographic studies reported (20) on the *S. rubiginosus* xylose isomerase are found as the most strictly conserved residues in these regions.

Modification of the xylose isomerase catalytic site

A complete amino acid sequence of *S. rubiginosus* xylose isomerase has not been published. However, a recent X-ray crystallographic study (20) of the enzyme proposed that His_{54} is an incipient proton abstracting residue acting as the general base during the isomerization reaction. Comparison of amino acid sequences in Figure 5 indicated that His_{101} of *C. thermosulfurogenes* xylose isomerase is part of a highly

conserved region and it corresponds by homology analysis to His_{54} of the enzyme from *S. violaceoniger* (16), an organism closely related to *S. rubiginosus*. To provide biochemical evidence for the involvement of histidine in the mechanism of catalytic isomerization of xylose and glucose we have changed several histidine residues in the *C. thermosulfurogenes* enzyme to phenylalanine by oligonucleotide-directed mutagenesis of the cloned *C. thermosulfurogenes xyl*A gene. Phenylalanine was chosen because it has a side chain comparable in size to histidine, but can not function as a base.

Mutant enzymes were expressed well in *E. coli* HB101 cells as indicated by the results of SDS-polyacrylamide gel electrophoresis of the cell lysates (Figure 8A). They could also be purified to near-homogeneity by the same procedures used for the wild-type enzyme (Figure 8B). Substitution of histidine residues at positions 41, 71 or 152 of the polypeptide chain did not affect enzymatic activity (measured as glucose isomerase) of the protein, whereas substitution of His_{101} completely destroyed both xylose and glucose isomerase activity of the *xyl*A product (Table 2 and 4).

To confirm the role of His_{101} as the hydrogen abstracting residue in the active site of the enzyme, two further experiments were performed. In the first experiment, the His_{101} residue was substituted by glutamine for several reasons. First, the resonance form of the oxygen atom in the carbonyl group of glutamine side chain might have some nucleophilic properties and abstract a hydrogen from C_2 atom of xylose or glucose. Second, the distance between the carbonyl group of the side chain to the appropriate atoms of the substrate may be similar to that of the imidazole of histidine. The results showed that Gln_{101} mutant enzyme was active as xylose and

Figure 8. SDS-polyacrylamide gel electrophoresis comparison of mutant xylose isomerases expressed by different alleles of *C. thermosulfurogenes xyl*A gene.

(A) Total protein (approximately 100-200 μg/lane) of crude cell lysates of *E. coli* HB101 carrying chimeric plasmids expressing different mutant *xyl*A genes and (B) purified mutant xylose isomerases (approximately 4-10 μg/lane), isolated from the *E. coli* HB101 as described in Materials and Methods, analyzed by 12% SDS-PAGE (43) at constant current of 25 mA using a 16 x 20 cm Protein II Multi-Cell (Bio-Rad) and visualized by Coomassie blue staining. Lanes : 1, His→Phe_{41}; 2, His→Phe_{101}; 3, His→Phe_{101}; 4, His→Phe_{152}; 5, His→Gln_{101}; wt, wild-type; Ctr, *E.coli* HB101 carrying the vector plasmid without inserts; Mr, molecular weight standards (97,400, phosphorylase; 66,200, bovine serum albumin; 42,700, ovalbumin; 31,000, carbonic anhydrase; 21,500, soybean trypsin inhibitor). The arrow indicates the polypeptide band corresponding to the wild-type xylose isomerase.

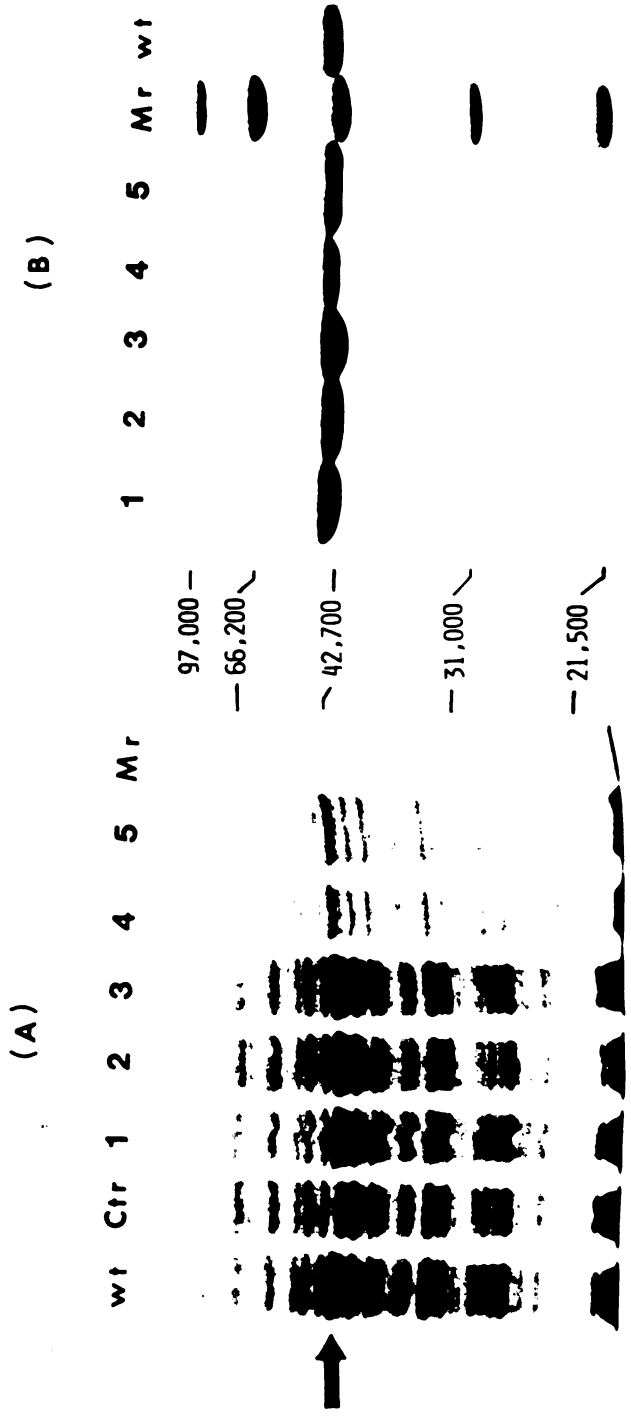

(A)
wt Ctr 1 2 3 4 5 Mr
(B)
1 2 3 4 5 Mr wt
97,000
66,200
42,700
31,000
21,500

Table 2. Comparison of site-directed mutant glucose isomerase activities in *E. coli* carrying different alleles of *C. thermosulfurogenes* xylA gene.

Preparation of cell extracts, enzyme purification, and glucose isomerase assay for each mutant and wild-type enzyme was performed as described in Materials and Methods.

	Specific Activity (unit/mg)[a]	
Enzyme	Cell Extract	Purified Enzyme
Wild-type	0.32 (1.00)	3.7 (1.00)
His - Phe_{41}	0.33 (1.03)	3.6 (0.97)
His - Phe_{71}	0.36 (1.12)	4.0 (1.08)
His - Phe_{152}	0.31 (0.97)	3.2 (0.86)
His - Phe_{101}	0.00 (0.00)	0.0 (0.00)
His - Gln_{101}	0.03 (0.09)	0.4 (0.11)
Cys - Ala_{99}	0.34 (1.06)	3.7 (1.00)

[a] Fraction of specific activity relative to wild-type is given in parenthesis.

glucose isomerase, although only a fraction of specific activity of the wild-type enzyme was retained (Table 2 and 4).

In the second experiment, the effect of diethylpyrocarbonate, known to inhibit enzymes containing histidine as a catalytic residue by covalent modification of the imidazole moiety (19), on specific activities of wild-type and mutant enzymes was determined. As shown in Table 3, the enzymes containing histidine at the position 101 were strongly inhibited by 1 mM DEPC, whereas the Gln_{101} mutant enzyme was not inhibited even by 10 mM DEPC. The results of these experiments indicate that His_{101} is essential for the catalytic activity of *C. thermosulfurogenes* xylose isomerase. Moreover, they are consistent with the proposed catalytic mechanism of the isomerization reaction that involves a general base catalysis mediated by imidazole moiety of a specific histidine of xylose isomerase (His_{101} in this enzyme).

Kinetic properties of the wild-type and mutant enzymes are compared in Table 4. The apparent K_m values for glucose did not change significantly upon substitution of His_{101} by glutamine. This indicated that this histidine residue is not essential for substrate binding. The apparent V_{max} of the Gln_{101} mutant enzyme was changed to a different extent for xylose, glucose, and fructose. However, the Phe_{101} mutant enzyme showed no activity with either of these substrates (Table 4). We conclude, therefore, that the same amino acid residue (His_{101}) is involved in the catalytic reaction for each of these three substrates. It should be noted that the ratio of apparent maximal velocities for the fructose to glucose conversion was 2.4-fold higher than that obtained with the wild-type enzyme. It is possible that the position of the functional group of glutamine, in the folded molecule of the enzyme, is closer to the C_1 atom

Table 3. Effect of diethylpyrocarbonate (DEPC) on glucose isomerase activity of the wild-type and site-directed mutant enzymes.

Purified enzymes (80 μg for wild-type and His-Phe_{71} mutant enzyme and 800 μg for His-Gln_{101} mutant enzyme) in 50 mM sodium phosphate buffer (pH 7.0) were incubated with DEPC at room temperature for 30 min. The residual glucose isomerase activity was assayed as described in Materials and Methods and expressed as percentage of specific activity found in the control without DEPC.

	Residual Activity (%)		
Enzyme	0 mM DEPC	1 mM DEPC	10 mM DEPC
Wild-type	100	8	1
His - Phe_{71}	100	10	3
His - Gln_{101}	100	100	100

Table 4. Comparison of kinetic properties of site-directed mutant and wild-type xylose isomerases

Enzyme activities with each substrate were determined with purified enzymes at various substrate concentrations as described under Materials and Methods. Apparent K_m and V_{max} values were obtained from Lineweaver-Burk plot.

	K_m (mM)	Vmax (unit/mg)			Ratio of V_{max}
Enzyme	Glucose	Xylose	Glucose	Fructose	(Frc/Glc)
Wild-type	142	14.0	5.3	2.9	0.55
His - Phe_{71}	130	14.9	5.5	3.3	0.60
His - Phe_{152}	152	16.2	5.2	2.9	0.56
His - Phe_{101}	n.d.	0.0	0.0	0.0	n.d.
His - Gln_{101}	138	1.6	0.6	0.8	1.33

n.d. Not Determinable

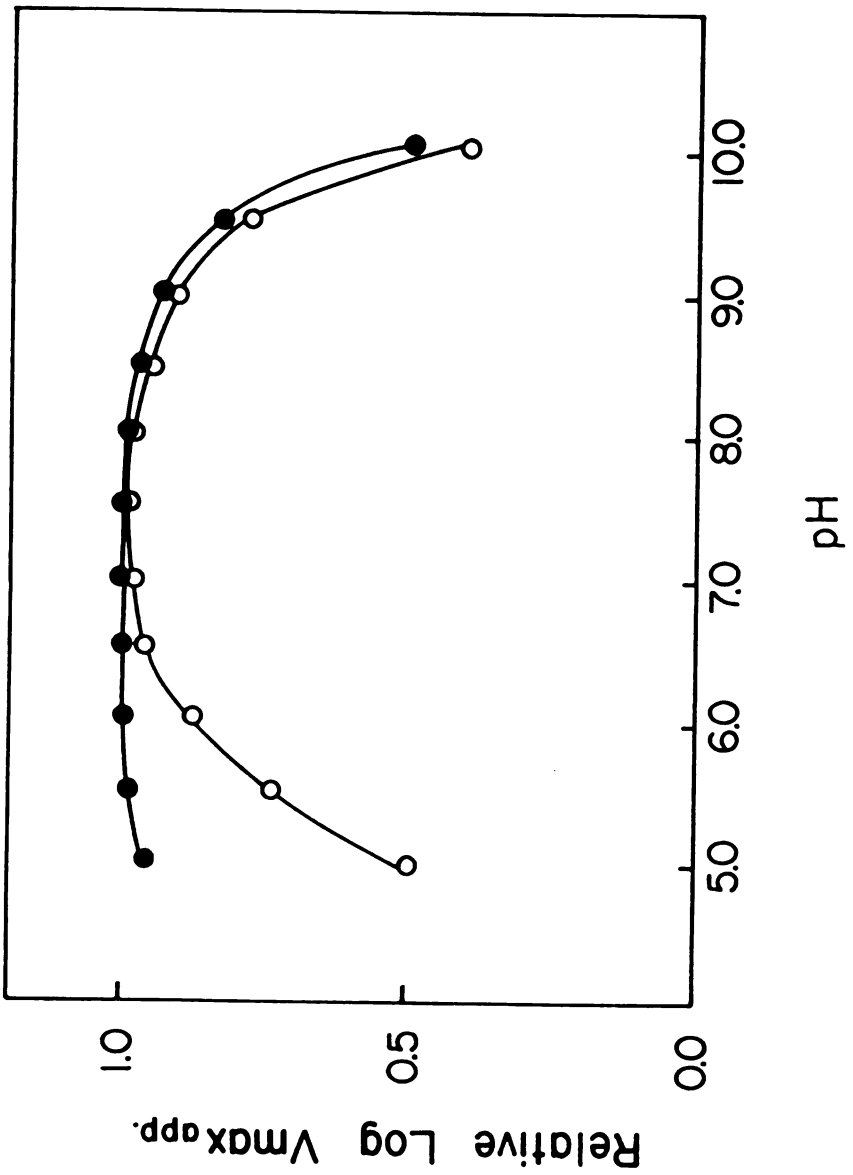
of the substrate than the imidazole residue of the His_{101}. This assumption may be verified when the information on three-dimensional structure of the Gln_{101} enzyme becomes available.

Dependence of kinetic parameters on pH for glucose isomerization of the wild-type and Gln_{101} mutant enzymes were determined over the pH range of 5.0 to 10.0. The plot of log $Vmax_{app.}$ versus pH indicated that the apparent pKa values of the catalytic residues in the wild-type enzyme were approximately 6.0 and 9.4, whereas the Gln_{101} mutant enzyme displayed only one detectible pKa value at 9.4 (Figure 9). Although the apparent pKa value of an amino acid residue in an enzyme may be affected by its micro-environment (e.g. neighboring amino acid residues or ions), the estimated pKa value of 6.0 may represent the apparent pKa of the imidazole moiety of the His_{101} of the wild-type enzyme. Therefore, the observed changes of the enzyme activity at pH below 7.0 may be due to the different status of protonation of the imidazole moiety in the His_{101}, which would affect the nucleophilicity of this functional group. On the other hand, the amino and carbonyl groups of the glutamine side chain are not protonable over the physiological pH range, and the potential nucleophilic property of these groups is not expected to be altered by the change of pH from 8.0 to 5.0.

We have not yet definitely ruled out the possibility that the changes in enzymatic activity, reported in this paper, might be due to structural changes in the catalytic site, resulting from the introduction of strongly hydrophobic residue, phenylalanine, in place of His_{101} and that glutamine simply played a role of a more hydrophilic substitution than phenylalanine. However, based on the existing

Figure 9. Plot of relative log of apparent V_{max} versus pH for Gln_{101} mutant (●—●) and wild type (○—○) xylose isomerases.

Apparent V_{max} values at different pH were determined from Lineweaver-Burk plots. The scale of relative log $Vmax_{app.}$ indicates the fraction of each experimental value at different pH relative to the maximal value.


Relative Log Vmax app.
1.0
0.5
0.0
5.0
6.0
7.0
8.0
9.0
10.0
pH

crystallographic data and on results on comparing the amino acid sequence and properties of six mutant xylose isomerases including their inhibition by DEPC and the change of activity at different pH values, we favor the interpretation forwarded in this paper that His_{101} is the amino acid that acts as a general base catalysts in the active site of the *C. thermosulfurogenes* xylose isomerase. Further studies are required to understand the mechanism of isomerase activity at acidic pH values including additional site specific amino acid changes and three-dimensional structure analysis of the modified molecules.

REFERENCES

1. Takasaki, Y., Kosugi, Y., and Kanbayashi, A. (1969) Arg. Biol. Chem. 33, 1527-1534

2. Antrim, R.L., Colliala, W., and Schnyder, B. (1979) in Applied Biochemistry and Bioengineering (Wingard, L.B., ed) pp. 97-155, Academic Press, New York

3. Bucke, C. (1980) in Enzymes and Food Processing (Birch, G.G., Blackebrough, N., and Parker, J.K., ed) pp. 51-72, Applied Science Publishers, London

4. Chen, W. (1980) Process Biochem. 15, 30-35

5. Chen, W. (1980) Process Biochem. 15, 36-41

6. Carrell, H.L., Rubin, B.H., Hurley, T.J., and Glusker, J.P. (1984) J. Biol. Chem. 259, 3230-3236

7. Farber, G.K., Petsko, G.A., and Ringe, D. (1987) Protein Engineering 1, 459-466

8. Rey, F., Jenkins, J., Janin, J., Lasters, I., Alard, P., Claessens, M., Matthyssens, G., Wodak, S. (1988) Proteins 4, 165-172

9. Dauter, Z., Dauter, M., Hemker, J., Witzel, H., and Wilson, K. (1989) FEBS Letters 7, 1-8

10. Batt, C.A., Claps, M.C., Bodis, M.S., Jamas, S., and Sinskey, A.J. (1985) Can. J. Microbiol. 31, 930-933

11. Wilhelm, M., and Hollenberg, C.P. (1984) EMBO Journal 3, 2555-2560

12. Shamanna, D.K., and Sanderson, K.E. (1979) J. Bacteriol. 139, 71-79

13. Schellenberg, G.D., Sarthy, A., Larson, A.E., Backer, M.P., Crabb, J.W., Lidstrom, M., Hall, B.D., and Furlong, C.E. (1984) J. Biol. Chem. 259, 6826-6832

14. Wilhelm, M., and Hollenberg, C.P. (1985) Nucleic Acids Res. 13, 5717-5723

15. Saari, G.C., Kumar, A.A., Kawasaki, G.H., Insley, M.Y., and O'Hara,

P.J. (1987) J. Bacteriol. 169, 612-618

16. Drocourt, D., Bejar, S., Calmels, T., Reynes, J.P., and Tiraby, G. (1988) Nucleic Acids Res. 16, 9337

17. Rose, I.A., O'Connell, E.L., and Mortlock, R.P. (1969) Biochem. Biophys. Acta 178, 376-379

18. Suckling, K.E. (1985) in Comprehensive Biotechnology (Moo-Young, M., Bull, A., and Dalton, H. ed) vol.1, pp. 539-551, Pergamon Press, Oxford

19. Gaikwad, S.M., More, M.W., Vartak, H.G., and Deshpande, V.V. (1988) Biochem. Biophys. Res. Commun. 155, 270-277

20. Carrell, H.L., Glusker, J.P., Burger, V., Manfre, F., Tritsch, D., and Biellmann, J.-F. (1989) Proc. Natl. Acad. Sci. USA 86, 4440-4444

21. Henrick, K., Collyer, C.A., and Blow D.M. (1989) J. Mol. Biol. 208, 129-157

22. Boyer, H.W., and Roulland-Dussoix, D. (1969) J. Mol. Biol. 41, 459-472

23. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene 33, 103-119. Marinus, M.G., and Konrad, E.B. (1976) Mol. Gen. Genet. 149, 273-277

24. Marinus, M.G., and Konrad, E.B. (1976) Mol. Gen. Genet. 149, 273-277

25. Fürste, J.P., Pansegrau, W., Frank, R., Blöcker, H., Scholz, P., Bagdasarian, M., and Lanka E. (1986) Gene 48, 119-131

26. Poncs, M., Solowiejczyk, D., Ballantine, M., Schwartz, E., and Surry, S. (1982) Proc. Natl. Acad. Sci. USA 79 4298-4302

27. Birnboim, H.C., and Doly, J. (1979) Nucleic Acids Res. 7, 1513-1523

28. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) in Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

29. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467

30. Devereux, I., Haeberli, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 387-395

31. Sayers, J.R., Schmidt, W., and Eckstein, F., (1988) Nucleic Acids Res. 16, 791-802

32. Dishe, Z., and Borenfreund, E. (1951) J. Biol. Chem. 192, 583-587

33. Lowry, O.H., Rosebrough, N.J., Farr, A.I., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

34. Stormo, G.D., Schneider, T.D., and Gold, L. (1982) Nucleic Acids Res. 10, 2971-2996

35. McLaughlin, J.R., Murray, C.L., and Rabinowitz, J.C. (1981) J. Biol. Chem. 256, 11283-11291

36. Harley, C.B., and Reynolds, R.P. (1987) Nucleic Acids Res. 15, 2343-2361

37. Moran, C.P., Lang, N., LeGrice, S.F., Lee, G., Stephens, M., Sonenshein, A.L., Pero, J., and Losick, R. (1982) Mol. Gen. Genet. 186, 339-346

38. Huang, J.J., and Ho, W.Y. (1985) Biochem. Biophys. Res. Commun. 126, 1154-1160

39. Kyte, J., and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105-132

40. Kitamoto, N., Yamagata, H., Kato, T., Tsukagoshi, N., and Udaka, S. (1988) J. Bacteriol. 170, 5848-5854

41. Brady, G., Jantzen, H.M., Bernard, H.U., Brown, R., Hashimoto-Gotoh, T., and Schütz, G. (1984) Gene 27, 223-232

42. Black, S.D., and Glorioso, J.C. (1986) Biotechniques 4, 448-460

43. Laemmli, U.K. (1970) Nature (London) 227, 680-685

Chapter VI

CONCLUSIONS AND PERSPECTIVES

CONCLUSIONS

The studies described in this thesis on thermostable glucose isomerases of the thermoanaerobes *Thermoanaerobacter strain B6A* and *Clostridium thermosulfurogenes* broaden knowledge and information on the following topics: physicochemical properties and regulation of glucose isomerase in thermoanaerobes; potential applications of these thermostable glucose isomerases in novel sweetener production processes; molecular and genetic features of the glucose isomerase gene from thermoanaerobic bacteria; catalytic function of histidine in the active site during glucose isomerization; and site directed alteration of kinetic and catalytic properties of glucose isomerase by protein engineering.

Most investigations on the production and biochemical characterization of glucose isomerase have concentrated on mesophilic, aerobic microorganisms. Glucose isomerase is the most expensive commercial saccharidase used in sweetener production from starch, and the current industrial process can be improved with an enzyme that possess higher thermostability and higher activity at acidic pH. Therefore, the present studies were initiated to advance fundamental understanding about the biochemical and physiological properties of glucose isomerase of thermoanaerobic bacteria and the molecular mechanisms that account for enzymatic catalysis and thermostability.

Initially, two different types of glucose isomerase activities were identified in diverse thermoanaerobic species based on differences in their apparent pH optima. *Thermoanaerobacter* and *C. thermosulfurogenes* which produced cell-bound,

thermostable glucose isomerases with neutral pH optima for activity were selected as model microorganisms for further studies. *Thermoanaerobacter* constitutively produced glucogenic amylase and ß-galactosidase activities which were environmentally compatible (i.e. active and stable at low pH and high temperature) with glucose isomerase activity. These findings made it possible to design a new sweetener production process that directly converted starch or lactose into a fructose sweetener by using a saccharidase mixture produced simultaneously by *Thermoanaerobacter.* This novel single step process represents a very efficient sweetener production system because it can eliminate the need for multiple steps and enzymes in the conventional starch process, and solve the digestion problems of lactase deficient individuals by hydrolysis of lactose into sweetener when used in dairy products. The utility of this single step process for industrial sweetener production requires further investigation on enzyme immobilization, continuous reaction system development, improvement of enzyme activity and stability at low pH, and other studies on enzyme production cost and safety.

Biochemical properties of glucose isomerases purified from *Thermoanaerobacter* and *C. thermosulfurogenes* were characterized in detail. The two distinct thermoanaerobic bacterial species produced highly thermostable glucose isomerases with close similarity in physicochemical and catalytic properties. These findings suggest that glucose isomerases from species with a common phylogenic origin have not diversified dramatically during their evolution under the similar conditions in thermal hot spring ecosystems. At present, the molecular mechanism of thermophilicity in their glucose isomerase is not clear and remains to be solved.

However, further studies on three dimensional structure of the enzyme and protein modification via site directed mutagenesis may be able to answer the questions.

The gene coding for thermostable glucose isomerase of *C. thermosulfurogenes* was cloned and overproduced in *Escherichia coli* and *Bacillus subtilis* using its original promoter. It is worth noting that a hyper-expression vector system by using the promoter of the *C. thermosulfurogenes* was developed in mesophilic *B. subtilis* or *E. coli* hosts. Production of thermostable glucose isomerase in a mesophilic host was of particular interest in simplifying large scale purification of the enzyme by a simple heat treatment procedure. Thus, these findings suggest the potential of utilizing the recombinant thermostable glucose isomerase over-expressed in a food microorganism *B. subtilis* as an industrial enzyme for sweetener production.

Nucleotide and deduced amino acid sequence analysis of the cloned glucose isomerase gene advanced insights on the primary structure of thermophilic glucose isomerases. Comparison of amino acid composition of glucose isomerases and substitution of several amino acid residues in the *C. thermosulfurogenes* enzyme molecule indicated that the factors which determine protein thermostability are the three dimensional coordination of key amino acids involved in the formation and maintenance of the protein structure; and, not simply the presence of disulfide bonds or the number of hydrophobic bonds which can be predicted from the primary structure of the native molecule. Glucose isomerases that share extensive homology, but differ significantly in thermostability, such as those from *B. subtilis* and *C. thermosulfurogenes* seem to be excellent models for further investigation in determining the molecular mechanisms of protein thermophilicity.

Moreover, the catalytic residue in the active site of *C. thermosulfurogenes* glucose isomerase was identified by homology analysis with other glucose isomerase amino acid sequences in relation to their three dimensional structure predicted from X-ray crystallographic studies. Histidine was demonstrated to function in catalysis by site directed mutagenesis, and by redesigning the catalytic site of the enzyme molecule to contain glutamine in lieu of histidine, a thermostable glucose isomerase with acid stable activity was obtained. Further studies including additional site specific amino acid changes and three-dimensional structure analysis of the modified molecules are required to understand the exact chemical mechanism of isomerase activity at acidic pH values or to further improve enzyme substrate specificity and activity.

In ending, the results on biochemical and molecular characterization of glucose isomerase completed in this thesis will serve as a basis to start assessing the industrial applications of both the native and site-specifically engineered glucose isomerases developed here. In addition, the glucose isomerase gene and the engineered enzyme production system can serve as a model system for future research aiming at understanding the molecular mechanism of glucose isomerase catalysis and thermostability, and also for providing a rationale basis to redesign the molecule for property improvements by protein engineering. Hopefully, the present report on cloning, crystallization, and determination of the primary structure of a thermostable glucose isomerase will also be useful for the continuation of basic studies on understanding enzyme thermophilicity in relation to its three dimensional structure.