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Molecular Biochemistry of Thermoanaerobacter ethanolicus 39E Amylopullulanase: Analysis of Substrate Cleavage Specificity and Thermophilicity by Site-Directed and Deletion Mutagenesis

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MOLECULAR BIOCHEMISTRY OF *Thermoanaerobacter ethanolicus* 39E AMYLOPULLULANASE: ANALYSIS OF SUBSTRATE CLEAVAGE SPECIFICITY AND THERMOPHILICITY BY SITE-DIRECTED AND DELETION MUTAGENESIS

By

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ABSTRACT

MOLECULAR BIOCHEMISTRY OF *Thermoanaerobacter ethanolicus* 39E AMYLOPULLULANASE: ANALYSIS OF SUBSTRATE CLEAVAGE SPECIFICITY AND THERMOPHILICITY BY SITE-DIRECTED AND DELETION MUTAGENESIS

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The need to identify starch-hydrolyzing enzymes with increased thermal stability has led researchers to screen thermophilic bacteria for enzymes with these characteristics. Amylopullulanase activity has been detected in various thermophiles and hyperthermophiles. The enzyme's thermostability and ability to attack both alpha-1,4 and alpha-1,6 glucosidic linkages found in starch may lead to improvements in bioprocessing industrial starches. It has been previously shown that four highly conserved regions exist in the active center of various amylolytic enzymes. The active center of amylopullulanase from *Thermoanaerobacter ethanolicus* 39E was analyzed by means of site-directed mutagenesis. A loss of catalytic activity was observed when each of the three conserved catalytic residues in regions II, III and IV was mutated. A change in hydrolysis pattern was evident when mutating three of the four conserved substrate binding residues in regions I, II, and IV. Transglycosylation activity was not detectable in the wild type or mutant constructs of amylopullulanase. This is the first report of altered binding characteristics with amylopullulanase. Native amylopullulanase and deletion mutants lacking the N or C-terminus have been expressed in *E. coli* and analyzed for possible roles in enzyme thermostability and thermophilicity. A putative thermophilicity region (TPR)

has been identified at the N-terminal end of the protein which is important for maintaining the optimal activity of the enzyme. The TPR is shown to control the temperature activity optimum without changing enzyme thermostability. The Arrhenius plot for the wild type enzyme was linear, unlike the discontinuous plot observed for the ApuN324 deletion construct. The biphasic Arrhenius plot appears to be due to a structural change in ApuN324 that does not alter the binding characteristics of the enzyme relative to wild type. To our knowledge this is the first report to show that enzyme stability and activity are controlled by separate protein features. It is hypothesized that the flexibility of the mutant protein was increased to account for a 20°C lower temperature optimum because the Km_{app} value was not altered relative to the wild type enzyme.

To my Grandma and Grandpa Hollenbeck

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I owe many thanks to those who have helped me succeed in the completion of this dissertation. First, is my advisor, Dr. Greg Zeikus, who accepted me into his lab at a time when I was searching for appreciation. He believed in me every step of the way, was a great mentor, and helped complete my development as a scientist. I never thought I would find the area of biotechnology so interesting, but the enthusiasm Dr. Zeikus had for the lab and the research was contagious. I would also like to thank the members of my guidance committee; Drs. Zach Burton, Lee Kroos, Loren Snyder and John Wang for their continued support and encouragement over the years. My appreciation also extends to everyone in the Macromolecular Structure Facility, the Computer Facility, the administrative and secretarial staff for all of their help and friendly smiles.

Graduate school did not only provide me with scientific development, but rewarded me with many friendships that I will charish over my lifetime. I especially thank Marty (Dr. Marty Regier) who is a friend like no other. Her support and encouragement, which has carried me to the end, has been undying and unconditional. I am glad that our 'post-graduate' endeavors have left us in close proximity to continue many life experiences including Friday morning coffee/journal club. This brings me to Carla Margulies who, although sometimes late, has also been involved in the coffee/journal club experience. Her love for science and worldly experiences is like no other I have seen. I was glad to have had the opportunity to experience a worldly endeavor with Carla, even though the hills *were* definitely much bigger than in Michigan! I have enjoyed listening

to Carla's views and opinions that have created great debate with many, including two of my colleagues and friends Kevin Carr and Mark Sutton. I would like to thank Kevin for always being there 24/7. Whether it was a trip to The Peanut Barrel or computer assistance, I could always count on Kevin. Mark taught me the importance of constructing a well balanced lunch (corn dogs, a brownie and beer) and that the best football team *is* the Chicago Bears.

I also thank the Zeikus lab members, past and present, for their friendship and support over the years: Drs. Doug Burdette, Guoqiang Dong, Jong-Hyun Park, Vladimir Tchernajenko, Claire Vieille, Mariet van der Werf, and almost doctors Eric and Greg Zeikus and last but not least Maris Laivenieks.

Finally, my appreciation for the support and encouragement I received from each and every member of my family, my husband, and Duke.

The potential of the Big M has yet to be utilized.

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ABBREVIATIONS

Adh	alcohol dehydrogenase
Apu	amylopullulanase
BSA	bovine serum albumin
BLMA	*Bacillus licheniformis* maltogenic alpha-amylase
bp	base pair
G1-G7	glucose, maltose, maltotriose, maltotetraose, maltoheptaose maltohexaose, and maltoheptaose
GuHCl	guanidine hydrochloride
HCA	hydrophobic cluster analysis
HFCS	high fructose corn syrup
HPIC	high performance ion chromatography
IPTG	isopropyl β-D-thiogalactopyranoside
MW	molecular weight
PAD	pulsed amperometric detector
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulfate
TB	Terrific broth
TPR	thermophilicity related region

OVERVIEW

This dissertation is divided into four chapters: a literature review, a chapter on substrate cleavage characteristics and characterization of the dual specificity (both alpha-1,4 and alpha-1,6 cleavage activity) of the enzyme by site-directed mutagenesis, a chapter describing thermophilicity and identification of a determinant responsible for maintenance of the enzyme's temperature activity optimum by deletion mutagenesis, and a summary of the research and future research directions.

Chapter I reviews the literature on saccharolytic enzymes with an emphasis on those enzymes isolated from thermophilic organisms. The biochemical properties of the enzymes and sequence conservation as it relates to substrate cleavage specificity will be examined, as well as thermophilic organisms and their industrial application.

Chapter II, "Biochemical and Enzymatic Characterization of Catalytic Activity and alpha-1,4/alpha-1,6 Cleavage Specificity of Recombinant Amylopullulanase from *Thermoanaerobacter ethanolicus* 39E," examines mutant constructs created by single point mutations to study substrate cleavage specificity and activity. Product analyses by HPIC are described that show the catalytic specificity of this enzyme hydrolyzing both alpha-1,4 and alpha-1,6 linkages on branched and unbranched starch, related polysaccharides and linear oligosaccharides.

Chapter III," Molecular analysis of thermophilicity and thermostability of recombinant amylopullulanase from *Thermoanaerobacter ethanolicus* 39E," describes experiments on a series of nested deletion mutants of the amylopullulanase gene. Gene products were isolated that possessed both

alpha-1,4 and alpha-1,6 activities. A mutant was identified having a lower optimal temperature range relative to wild type and other deletion constructs. The mutant also displays a discontinuous Arrhenius plot indicative of a conformational change required for optimal activity. The final chapter summarizes the findings of this study, conclusions, and directions for future research.

CHAPTER I

LITERATURE REVIEW

I. Introduction

A. Enzymes Involved in Conversion of Polysaccharides to Sugars.

Commonly used enzymes in starch processing are alpha-amylase, beta-amylase, glucoamylase, cyclodextrin glucotransferase and debranching enzymes. These are collectively referred to as amylosaccharidases due to their ability to cleave sugar units. Two general types of activities present in these enzymes are alpha-1,4 and alpha-1,6 hydrolysis, and alpha-1,4 and alpha-1,6 transfer reactions. The hydrolysis reaction results in the cleavage of glucosidic bonds in a polysaccharide producing linear or branched low molecular weight oligosaccharides. The transfer, or transglycosylation, reaction is the addition of glucose to glucose or maltose produced from the hydrolysis reaction producing small branched oligosaccharides like isopanose, isomaltose and panose.

Alpha-amylases cleave alpha-1,4 glucosidic linkages in an endo, or random, fashion and are used as liquefying agents in the starch-processing industry. Alpha-amylases have been identified in various bacteria, fungi, plants, and animals. It is the only amylosaccharidase whose three-dimensional structure (from *Aspergillus oryzae* and swine) has been determined (Matsuura *et al.*, 1984 and Buisson *et al.*, 1987). Alpha-amylase from *A. oryzae* is widely used in starch processing due to its thermal (90°C) and pH (up to pH 11) stability (Saito, 1973). Beta-amylase, like alpha-amylase, cleaves alpha-1,4 glucosidic linkages. Its pattern of action is an exotype, acting from the nonreducing end of starch, producing maltose which is used in industry as a sweetner. Beta-amylases are produced by plants and some bacteria. Thermophilic beta-amylase from *Clostridium thermosulfurogenes* cloned into *Bacillus brevis* may be useful for starch processes due to its activity and stability at high temperature (Mizukami *et al.*, 1992). Glucoamylase has been identified in yeast and fungi and functions as an exotype enzyme removing glucose in an ordered fashion from the nonreducing

end of starch. In industry, this enzyme functions after liquifaction by alpha-amylase and in conjuction with debranching enzyme to produce glucose from starch. The enzyme from *Aspergillus awamori* (Nunberg *et al.*, 1984) is most widely used in industrial processes. The enzyme cyclodextrin glucotransferase (CGTases) catalyzes both transglycosylation and hydrolysis reactions on starch to produce alpha, beta, and gamma cyclodextrins. CGTases have been identified in *Bacillus* species and *Klebsiella pneumoniae* (Fogarty *et al.*, 1980). Debranching enzymes include, but are not limited to, isoamylase, pullulanase, amylopullulanase, and neopullulanase. All of these enzymes are capable of cleaving alpha-1,6 glucosidic linkages as well as other hydrolytic and transferring activities. This group of enzymes is present in higher plants and microorganisms with isoamylase and pullulanase currently the most industrially used. Isoamylase in combination with glucoamylase, and pullulanase in conjunction with beta-amylase function to produce glucose and maltose, respectively, from starch after action by alpha-amylase.

A more detailed review of amylosaccharidases whose substrate cleavage specificity has been studied will be presented later as these enzymes are relevent to this thesis project and the potential design of altered enzymes for use in industrial starch degradation.

B. Starch Bioprocessing

In the United States, 18 billion pounds of sweetners are produced per year demonstrating the importance of the industrial starch conversion process (Hebeda, 1987). Starch, a branched polymer containing alpha-1,4 and alpha-1,6 glucosidic linkages is used to manufacture sugars in a multistep process utilizing different amylosaccharidases. Three classes of enzymes are involved in the production of sugars from starch: 1) endo-amylase (alpha-amylase),

2) exo-amylases (beta-amylase, glucoamylase); and, 3) debranching enzymes (pullulanase, isoamylase) (Ramesh *et al.*, 1992). Starch bioprocessing normally involves two steps, liquifaction and saccharification, which run at high temperatures. Liquefaction is a process in which starch granules are gelatinized in aqueous solution and partially hydrolyzed at alpha-1,4 branch points. Saccharification results in conversion of liquefied starch to low molecular weight saccharides by various debranching enzymes. Alpha-amylase is used in the liquefaction process which occurs at pH 6-7 and 80°C-150°C over a period of up to 3 hours. The pH is then adjusted to 4.0-4.5 and the temperature is lowered to 55-60°C for the saccharification step which occurs in the presence of glucoamylase and the proper debranching enzyme over 24-90 hours (Saha *et al.*, 1989). Production of high fructose syrup is then achieved by the use of glucose isomerase (Table 1). Amylosaccharidases from thermoanaerobes such as amylopullulanase may be suitable for application in starch conversion biotechnologies because of their novel activity, extreme thermostability, thermoactivity, and acidic pH compatibility. Industry is seeking to design engineered enzymes with specificity for alpha-1,4 glucosidic linkages only, specificity for alpha-1,6 glucosidic linkages only, and with various end-product specificities. This would improve the starch degradation process by development of an acid thermostable debranching enzyme or use it directly to make conversion syrup for fermentation.

Table 1. Starch conversion process producing glucose and fructose (Adapted from Zeikus, J.G., 1990)

Substrate/ Product and Process	Stage	Enzyme	pH	Temp.	Metal
STARCH					
↓	Liquefaction	Alpha-amylase	6.0-7.0	80-120°C	Ca^{++}
MALTODEXTRIN (D.E. 10-15) [pH adjustment with acid]					
↓	Saccharifaction	Glucoamylase and Isoamylase	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					

Identification or design of a thermostable amylopullulanase, an amylosaccharidase with alpha-1,4 and alpha-1,6 hydrolytic activity, would allow a shift to a higher temperature in the saccharification process which would have many industrial benefits: a) increased reaction rates with decreased operation time, b) lower costs for enzyme purification, c) higher substrate concentrations facilitating enzymatic degradation due to increased starch solubility, and d) minimal risks of bacterial contamination (Antranikian, 1990).

II. Thermophiles and Thermozymes

Over 100 enzymes and proteins have, to date, been purified to homogeneity from thermophilic micro-organisms. The discovery that many enzymes from thermophilic organisms (thermozymes) have higher thermostability than homologous proteins from mesophiles has inspired a search for the molecular basis of this higher thermostability. Thermostability, measured by the protein's ability to resist irreversible thermal inactivation, and is expressed as the enzyme's half-life at a given temperature (Vieille *et al* , 1996). Enzyme thermophilicity is the temperature at which the enzyme has its highest activity (Vieille *et al,* 1996).

Thermophilic organisms can be classified as moderate thermophiles with a growth optima of 60-80°C and hyperthermophiles with a growth optima above 80°C. Habitats suitable for growth of moderate thermophiles are widespread. They include geothermally heated springs, ground water and sea water as well as marine thermal vents and solar heated soils (Lowe *et al.,* 1993). Environments that exist at temperatures appropriate for the growth of hyperthermophiles are less common. The most extremely thermophilic organisms have been isolated from continental volcanic areas (Brock *et al.*, 1972).

Initial comparison of mesophilic and thermophilic proteins has identified features unique to thermophilic proteins. It has been observed that thermophilic enzymes are smaller, have less ordered structure, contain more hydrophobic interactions, and less beta-structure, among other features (Amelunxen and Murdock, 1978). However, it appears that the key to thermostability will be determined by comparing thermodynamic properties, amino acid composition and sequence of homologous mesophilic and thermophilic proteins; not by their molecular architecture (Sundaram 1986). Site directed mutagenesis has become a useful tool in the analysis of protein thermostabilization. Small changes in the stabilizing forces caused by only one or two amino acid changes can raise the relative stability of an enzyme by several degrees centigrade (Coolbear *et al.*, 1992). Other factors leading to increased thermostability include: increased protein rigidity at mesophilic temperatures, location of proline residues in the loop regions of thermophilic proteins, and interaction of the protein with its surroundings (Vihinen, 1987 and Watanabe *et al.*, 1991).

Enzymes from hyperthermophiles have a higher optimum temperature (more thermophilic) and increased thermostability in comparison to moderate thermophiles or mesophiles (growth optimum of 30-45°C), which is an important rationale for use of thermostable enzymes in industrial starch processing applications. A number of extremely thermostable enzymes of potential industrial utility have been purified and/or cloned from anaerobic thermophiles. A number of hyperthermophiles are characterized by their ability to utilize complex saccharides which are metabolized to meet carbon and energy requirements.

III. Amylosaccharidases

Amylosaccharidases have been identified in various mesophilic, thermophilic and hyperthermophilic bacteria (Table 2), and Archae. They are classified based on their enzymatic activity, substrate specificity, and products of hydrolysis (Table 3). The two main catagories are endo-acting enzymes and exo-acting enzymes which cleave internally and from the end of the polysaccharide, respectively. Endo-acting amylosaccharidases produce a mixture of malto-oligosaccharidases due to their random cleavage pattern. The exo-acting enzymes cleave sugar units from the non-reducing end to the reducing end of the substrate in an ordered, processive fashion (Fogarty *et al.*, 1979).

Amylosaccharidases are broadly grouped into amylases and pullulanases. Pullulanases can be separated into four groups: pullulanase, isopullulanase, amylopullulanase, and neopullulanase, depending on the cleavage products produced from action on pullulan (Figure 1). Enzymes in this class have been purified and characterized from a wide range of bacterial species. Pullulanase from *Thermoanaerobium* Tok6-B1 (Plant *et al.*, 1987), and amylopullulanase from *Clostridium thermohydrosulfuricum* (Mathupala *et al.*, 1990) hydrolyze the alpha-1,6 linkages in pullulan to produce maltotriose. Isopullulanase from *Aspergillus niger* (Sakano *et al.*, 1971) hydrolyzes alpha-1,4 linkages producing isopanose. Neopullulanase from *Bacillus stearothermophilus* (Kuriki *et al.*, 1988a) produces panose by cleavage of alpha-1,4 linkages and a small amount of glucose and maltose by the limited alpha-1,6 cleavage activity on pullulan. Amylopullulanases and neopullulanases are also capable of cleaving alpha-1,4 linkages in starch producing low molecular weight oligosaccharides. Isopullulanase and pullulanase can not hydrolyze starch.

Table 2. Amylosaccharidases from thermophilic and hyperthermophilic organisms

Enzyme	Organism (enzyme optimal temperature)	Reference (s)
THERMOPHILES		
α-Amylase	*Bacillus caldovelox* (90°C)	Bealin-Kelly *et al.*, 1991
α-Amylase	*Bacillus stearothermophilus* (90°C)	Brosnan *et al.*, 1991 Gray *et al.*, 1986
α-Amylase (AmyA)	*Dictyoglomus thermoophilus* (90°C)	Fukusumi *et al.*, 1988
α--Amylase (AmyB)	*Dictyoglomus thermophilum* (80°C)	Horinouchi *et al.*, 1988
α-Amylase (AmyC)	*Dictyoglomus thermophilum* (70°C)	Horinouchi *et al.*, 1988
β-Amylase	*Thermoanaerobacterium thermosulfurigenes* 4B (75°C)	Hyun and Zeikus 1985
Amylopullulanase	*Clostridium thermohydrosulfuricum* E101 (85°C)	Melasniemi 1987
Amylopullulanase	*Thermoanaerobacter ethanolicus* 39E (85°C)	Mathupala and Zeikus 1993
Amylopullulanase	*Thermoanaerobacterium sp.* Tok6-B1 (80°C)	Plant *et al.*, 1987
Amylopullulanase	*Thermoanaerobacterium saccharolyticum* (75°C)	Saha *et al.*, 1990
Amylopullulanase	*T. thermosulfurigenes* EM1 (70-75°C)	Wind *et al.*, 1990
Cyclodextrinase	*T. ethanolicus* 39E (65°C)	Saha and Zeikus 1990
Cyclodextrin glycosyltransferase	*T. thermosulfurigenes* EM1 (90-95°C)	Wind *et al.*, 1990
α-Glucosidase	*Bacillus sp.* (75°C)	Nakao *et al.*, 1994
α-Glucosidase	*T. ethanolicus* 39E (75°C)	Saha and Zeikus 1991
β-Glucosidase	*Clostridium saccharolyticus*	Coolbear *et al.*, 1992
β-Glucosidase	*C. thermocellum*	Margaritis and Merchant 1986
Neopullulanase	*B. stearothermophilus* (60-65°C)	Kuriki *et al.*, 1988
Pullulanase	*B. stearothermophilus* (65°C)	Kuriki *et al.*, 1988
Pullulanase	*Bacillus sp.* (75°C)	Shen *et al.*, 1990
Pullulanase	*Thermus sp.* AMD-33 (70°C)	Aubert *et al.*, 1993 Nashihara *et al.*, 1988

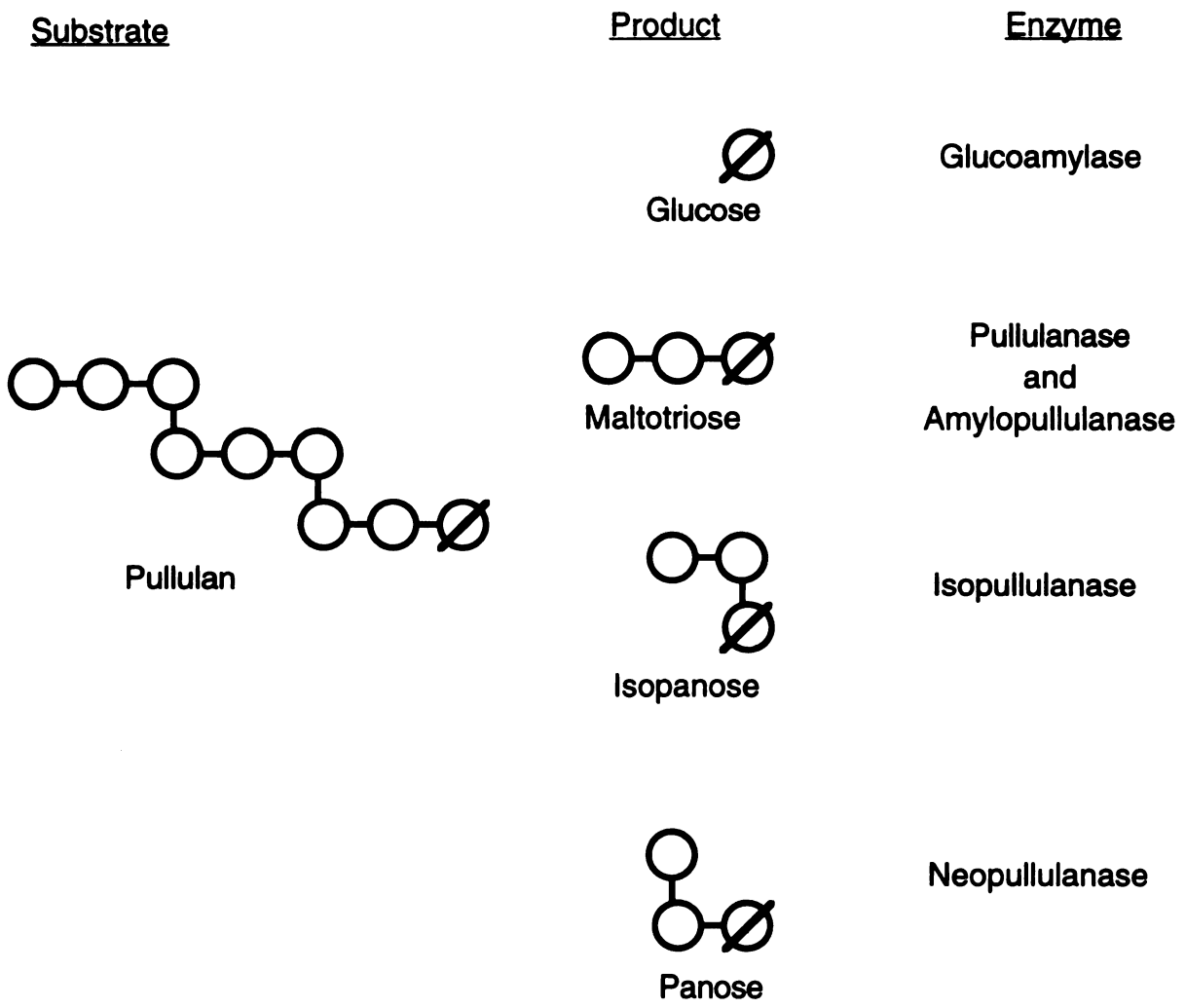
HYPERTHERMOPHILE		
α-Amylase (extracellular)	*Pyrococcus furiosus* (100°C)	Koch *et al.*, 1990
α-Amylase (intracellular)	*P. furiosus* (100°C)	Laderman *et al.*., 1993
α-Amylase	*P. woesi* (100°C)	Koch *et al.*, 1991
α-Amylase	*Thermococcus profundus* (80°C)	Chung *et al.*, 1995
β-Amylase	*T. maritima* (95°C)	Schumann *et al.*, 1991
Amylopullulanase	*P. furiosus* (125°C)	Brown and Kelly 1993
Amylopullulanase	ES4 (110-125°C)	Schuliger *et al.*, 1993
Amylopullulanase	*T. litoralis*	Brown and Kelly 1993
β-Glucosidase	*Thermotoga sp.*	Ruttersmith and Daniel 1993
α-Glucosidase	*P. furiosus* (110°C)	Constantino *et al.*, 1990

Table 3. Amylosaccharidases: Cleavage action pattern and product formation.

Enzyme	Action	Product Formation	Bond Cleavage
alpha-amylase	endo	maltosaccharides, maltose	alpha-1,4
beta-amylase	exo	maltose	alpha-1,4
amyloglucosidase	exo	glucose	alpha-1,4/alpha-1,6
alpha-glucosidase	exo	glucose	alpha-1,4/alpha-1,6
pullulanase	endo	maltotriose from pullulan	alpha-1,6
isoamylase	endo	maltodextrin from starch and glycogen	alpha-1,6
cyclodextrin glucosyl transferase	endo	maltosaccharides, cyclodextrins	alpha-1,4
isopullulanase	endo	isopanose from pullulan	alpha-1,4
oligo-1,6 glucosidase	exo	glucose from maltose	alpha-1,6
neopullulanase	endo	panose from pullulan	alpha-1,4/alpha-1,6
amylopullulanase	endo	maltose, maltotriose, and maltotetrose from starch	alpha-1,4/alpha-1,6

Figure 1 Enzymatic hydrolysis of pullulan by enzymes having alpha-1,4, alpha-1,6, or alpha-1,4 and alpha-1,6 hydrolytic activity. Open circles and slashed circles denote non-reducing glucopyranosyl residues and reducing glucose residues, respectively. Horizontal lines and vertical lines connecting circles indicate alpha-1,4 and alpha-1,6 glucosidic linkages respectively.

Figure 1. Enzymatic hydrolysis of pullulan by enzymes with alpha-1,4, alpha-1,6, or alpha-1,4 and alpha-1,6 hydrolytic activity.



Classically, amylases are separated into three groups: alpha-amylases, beta-amylases, and glucoamylases and catalyze the cleavage of starch, glycogen, and related glucans. Alpha-amylase is an endo-acting enzyme responsible for the random cleavage of alpha-1,4 glucosidic linkages, whereas, beta-amylases and glucoamylases are exo-acting enzymes that hydrolyze alpha-1,4 linkages, or alpha-1,4 and alpha-1,6 linkages, respectively. The most widely studied alpha-amylase is from *Aspergillus oryzae* (Taka amylase-A) (Matsuura *et al.*, 1983).

Cleavage action pattern has been most extensively studied in neopullulanase from *B. stearothermophilus* (Kuriki *et al.*, 1991; Imanaka *et al.*, 1989; and Takata *et al.*, 1989), alpha-amylase from *B. licheniformis* (Kim *et al.*, 1992; Kim *et al.*, 1994; and Lee *et al.*, 1995) (Figure 2,3), and amylase-pullulanase from *Bacillus circulans* F-2 (Kim *et al.*, 1995). Cleavage activity of amylopullulanase from *T. ethanolicus* 39E will be investigated in this dissertation and compared to the cleavage patterns seen with the above enzymes.

In contrast to hydrolysis (break down) reactions, transglycosylation (build up) reactions have been identified and extensively studied in neopullulanase. This enzyme can catalyze the transglycosylation reaction to form alpha-1,4 and alpha-1,6 glucosidic linkages producing highly branched oligosaccharides (Kuriki *et al.*, 1991 and Takata *et al.*, 1992). Hydrolysis and branching activities are present in one active center of neopullulanase as evidenced by results of site-directed mutagenesis of conserved amino acids in regions I-IV (Kuriki *et al.*, 1991). The presence of transglycosylation activity will be examined with amylopullulanase.

Figure 2 Proposed action pattern of neopullulanase. Open circles and slashed circles denote non-reducing glucopyranosyl residues and reducing glucose residues, respectively. Horizontal lines and vertical lines connecting circles indicate alpha-1,4 and alpha-1,6 glucosidic linkages respectively.

Figure 2. Proposed action pattern of neopullulanase from *B. stearothermophilus* on pullulan (Adapted from Iminaka, I. and T. Kuriki 1989).

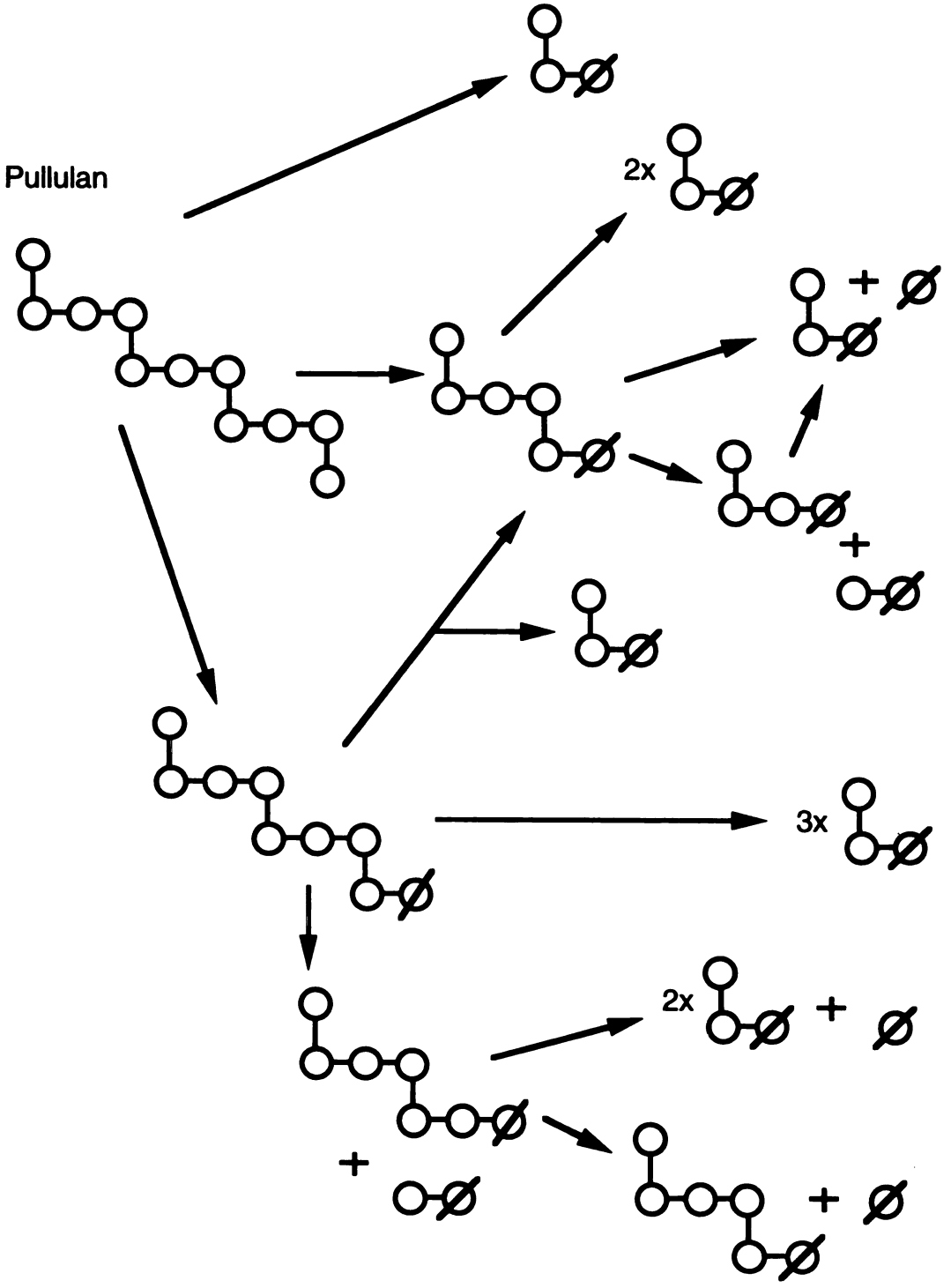
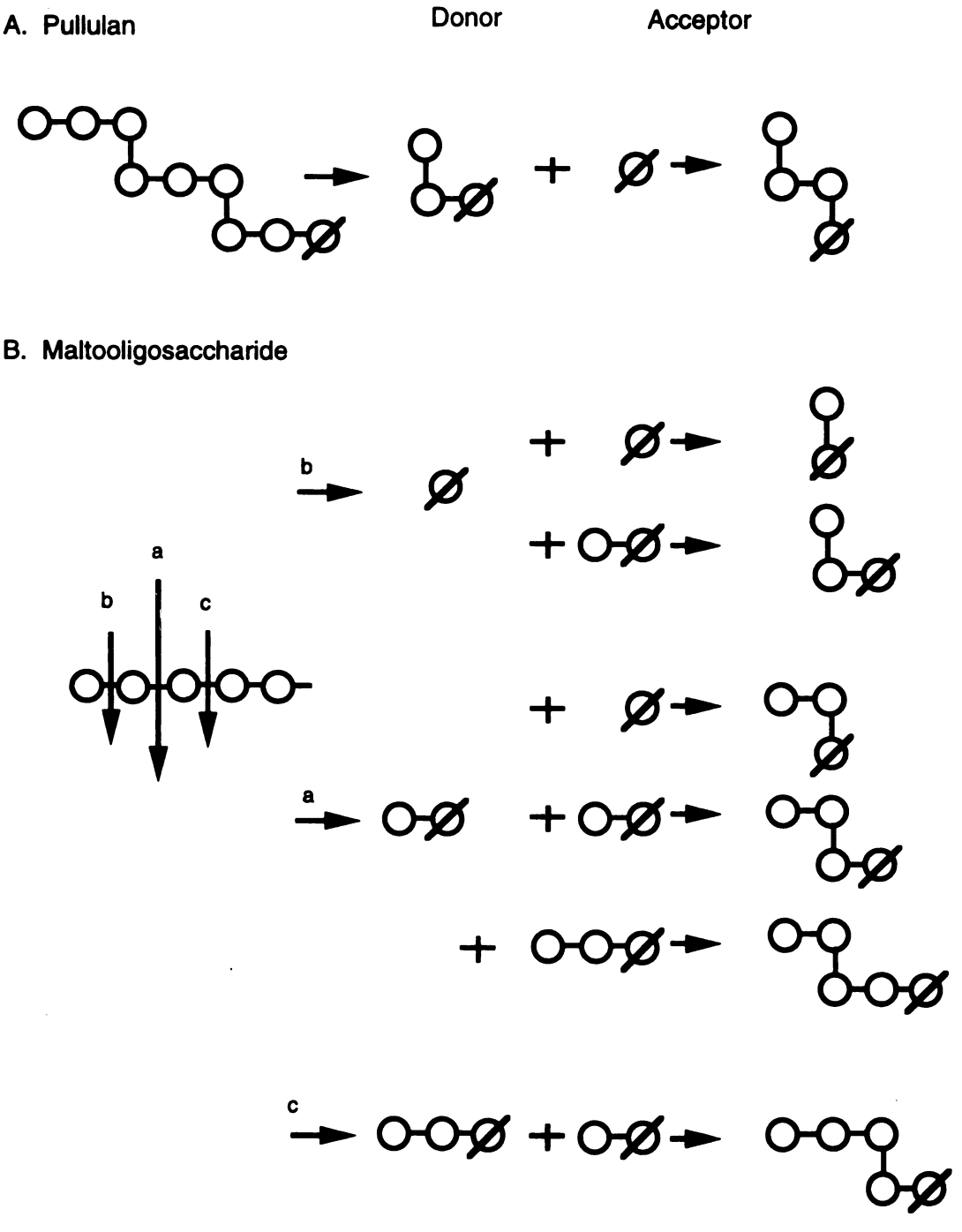


Figure 3. Proposed model of hydrolysis and transfer reactions of alpha-amylase from *B. licheniformis* on pullulan and maltooligosaccharide (Adapted from Lee, S.J. *et al.*, 1995). (A) Alpha-amylase hydrolyzed pullulan producing panose which, in the presence of excess glucose, results in the formation of an alpha-1,6 linkage producing 6^1-O-α-(6^2-O-α-glucosyl-maltosyl)-glucose. (B) Hydrolysis of maltooligosaccharides produces mainly maltose (path a) but also to glucose and maltotriose (paths b and c). These products undergo transfer reactions using additional glucose, maltose and maltotriose to produce isomaltose, panose, isopanose, 6^2-O-α-maltosyl-maltose, 6^3-O-α-maltosyl-maltotriose, and 6^2-O-α-maltotriosyl-maltose. Longer branched products, if produced, are likely to be hydrolyzed by the enzyme. Open circles and slashed circles denote non-reducing glucopyranosyl residues and reducing glucose residues, respectively. Horizontal lines and vertical lines connecting circles indicate alpha-1,4 and alpha-1,6 glucosidic linkages respectively.

Figure 3. Proposed model of hydrolysis and transfer reactions of alpha-amylase from *B. licheniformis* on pullulan and maltooligosaccharide.



B. Classes of Amylosaccharidases used to Study Catalytic Activity and Substrate Cleavage Specicifity

1). Alpha-amylase

The hydrolysis of starch is a common first step in the conversion of starch into a utilizable substrate for fermentation or for the conversion to dextrose and high fructose syrups. The enzyme alpha-amylase catalyzes the cleavage of the alpha-1,4-glucosidic linkages in starch, glycogen and related glucans. This enzyme was among the earliest to be purified and its mechanisms of action and substrate specificities have been widely studied (Thoma *et al.*, 1971). Although amylases share high homology in the four conserved regions, their optimum temperature, pH, thermostability, and major product formation from starch vary significantly among species (Fogarty *et al.*, 1980). Alpha-amylases in general cleave only the alpha-1,4 linkages of starch and can hydrolyze past alpha-1,6 branch points due to their random attack pattern with the substrate.

All alpha-amylases are metalloenzymes and tightly bind one calcium ion per enzyme molecule (Thoma *et al.*, 1971). Almost all alpha-amylases contain and require Ca^{2+} for optimal activity, and it is essential for the folding of the enzyme in *Aspergillus oryzae* (Matsuura *et al.*, 1984) and porcine pancreatic alpha-amylase (Buisson *et al.*, 1987). Alpha-amylases of different origins have similar enzymatic properties and calcium requirements (Yang *et al.*, 1993). Alpha-amylase is most active in the lower pH range (pH 4.5-7.0) with an optimum pH near 5.5. The optimal temperature for activity is dependent on the thermophilicity of the organism from which the enzyme was isolated. The lowest temperature optimum reported is 30°C, while temperatures of 95°C to 100°C have been reported from hyperthermophiles isolated from deep sea vents and fumaroles (Brown *et al.*, 1990). Molecular weights of alpha-amylase ranges from 10,000 to 140,000, while most microbial alpha-amylases have a molecular

weight between 50,000 to 60,000 (Table 4). On the basis of sequence comparisons a calcium binding site is suggested to be a common structural feature of all alpha-amylases (Matsuura *et al.*, 1984).

Among the amylolytic enzymes, 3-dimensional structures have been determined only for the alpha-amylases from *Aspergillus oryzae* (Matsuura *etal.*, 1984) and from porcine pancreas (Buisson *et al.*, 1987) by X-ray crystallographic studies. Initial identification of substrate binding site and catalytic residues was by difference Fourier analysis with Taka amylase-A from *Aspergillus oryzae.* A model fitting of an amylose chain in the catalytic site showed a possible binding interaction between substrate and enzyme (Figure 4). Glu230 and Asp297 were shown to be catalytic residues, acting as a general acid and a general base, respectively; His122, Lys209, His210, and His296 were proposed as substrate-binding site residues (Matsuura *et al.*, 1984).

Detailed investigation of the function of amino acids in four conserved regions has been done on maltogenic alpha-amylase from *Bacillus licheniformis* (BLMA). BLMA has both alpha-1,4 hydrolytic activity and alpha-1,6 transferring activity of glucose or maltose to oligosaccharides (Cheong *et al.*, 1995). Site-directed mutagenesis of amino acid residues His250, Asn330, Glu331, and Asp422 revealed their importance as putative substrate-binding site residues (Cheong *et al*, 1995). His250, when substituted with Gln, resulted in an increased hydrolysis activity on soluble starch, slightly decreased activity on pullulan, and increased alpha-1,6 transferring activity as compared with the wild type BLMA enzyme. Substitution of Asn300 and Glu331 with Ser and Val respectively resulted in slightly better hydrolysis activity on pullulan and soluble starch with no significant difference in alpha-1,6 transferring activity. More interestingly, this mutation endowed the enzyme with alpha-1,6 cleavage activity producing a similiar enzyme cleavage activity on pullulan as neopullulanase. Wild type

Table 4. General properties of alpha-amylases from bacteria and fungi

Organism	MW	Optimum pH	Optimum Temp (°C)	Reference
Acinetobacter sp.	55,000	7.0	55	Onishi *et al.*, 1978
Aspergillus awamori	54,000	5.0	50	Bhella *et al.*, 1985
Aspergillus oryzae	56,000	5.0	55	Kundu *et al.*, 1970
Bacillus sp.	54,000	2.0	70	Uchino, 1982
Bacillus sp.	N.D.	10.5	50	Yamamoto *et al.*, 1972
Bacillus acidocaldarius	66,000	5.0	70	Kanno, 1986
Bacillus amyloliquefaciens	50,000	5.9	65	Borgia *et al.*, 1978
Bacillus caldolyticus	10,000	5.4	70	Heinen *et al.*, 1972
Bacillus cereus	55,000	6.0	55	Yoshigi *et al.*, 1985
Bacillus coagulens	62,000	6.2	50	Kitahata *et al.*, 1983
Bacillus licheniformis	62,600	8.0	90	Morgan *et al*, 1981
Bacillus macerans	140,000	6.3	N.D.	DePinto *et al.*, 1964
Bacillus stearothermophilus	44,000	5.5	70	Tsukagoshi *et al.*, 1984
Bacillus subtilis	25,000	6.5	50	Takasaki, 1985
Fusarium oxysporum	N.D.	4.0	25	Chary *et al.*, 1985
Lactobacillus cellobiosus	22,500	7.3	50	Sen *et al.*, 1984
Pyrococcus furiosus	76,300	5.6	98	Brown *et al.*, 1990
Streptomyces aureofaciens	40,000	5.0	40	Hostinova *et al.*, 1978
Thermotoga maritima	60,000	5.5	95	Schumann *et al.*, 1991

BLMA alpha-amylase from *B. licheniformis* does not hydrolyze alpha-1,6 linkages of pullulan (Lee, 1993), while neopullulanase from *B. stearothermophilus* hydrolyzes both alpha-1,4 and alpha-1,6 glucosidic linkages (Kuriki *et al.*, 1991). The double mutant, Asn330Ser/Glu331Val, hydrolyzed pullulan to glucose (10%), maltose (28%), and panose (62%), while the wild type BLMA produced panose only from hydrolysis of pullulan (Cheong *et al*, 1995). These results indicate potential importance of Asn330 and/or Glu331 to alpha-1,6 hydrolytic activity.

2). Pullulanase

The enzyme used following liquifaction by alpha-amylase in the bioprocessing of starch is the debranching enzyme pullulanase, which is capable of cleaving alpha-1,6 glucosidic linkages in starch and pullulan (Fogarty *et al.*, 1979; Fogarty *et al.*, 1980; Lee *et al.*, 1971; and Price, 1968). This enzyme improves the saccharification rates and yields, and decreases reaction times in the production of glucose or conversion syrups and is used in combination with liquifying alpha-amylase (Norman, 1979; Reilly, 1979; and Saha *et al*, 1987). Compared to the alpha-amylases, pullulanases have been isolated from relatively few microorganisms, mostly mesophiles. The pH optima of pullulanases range from 5.0 to 9.0, with optimum temperatures ranging from 30°C to 85°C. Molecular weights reported are from 55,000 to 450,000 (Table 5). Calcium is important for maintaining thermostability as with the alpha-amylase, but there is no evidence to indicate a role for calcium in the folding of the protein (Fogarty *et al.*, 1979 and Fogarty *et al.*, 1980). A calcium binding site has yet to be identified. Detailed experiments have yet to be done to study substrate cleavage specificity in this group of enzymes. It has been shown that the cleavage pattern of recombinant pullulanase from *P. woesei* on pullulan produces 100% conversion to maltotriose (Rudiger *et al.*, 1995).

Figure 4 Proposed substrate binding and catalytic sites for alpha-amylase from *A. oryzae* (Adapted from Matsuura *et al*, 1984). The corresponding amino acid residues of amylopullulanase are indicated by rectangles.

Figure 4. Proposed substrate binding and catalytic sites for Taka amylase-A from *A. oryzae*.

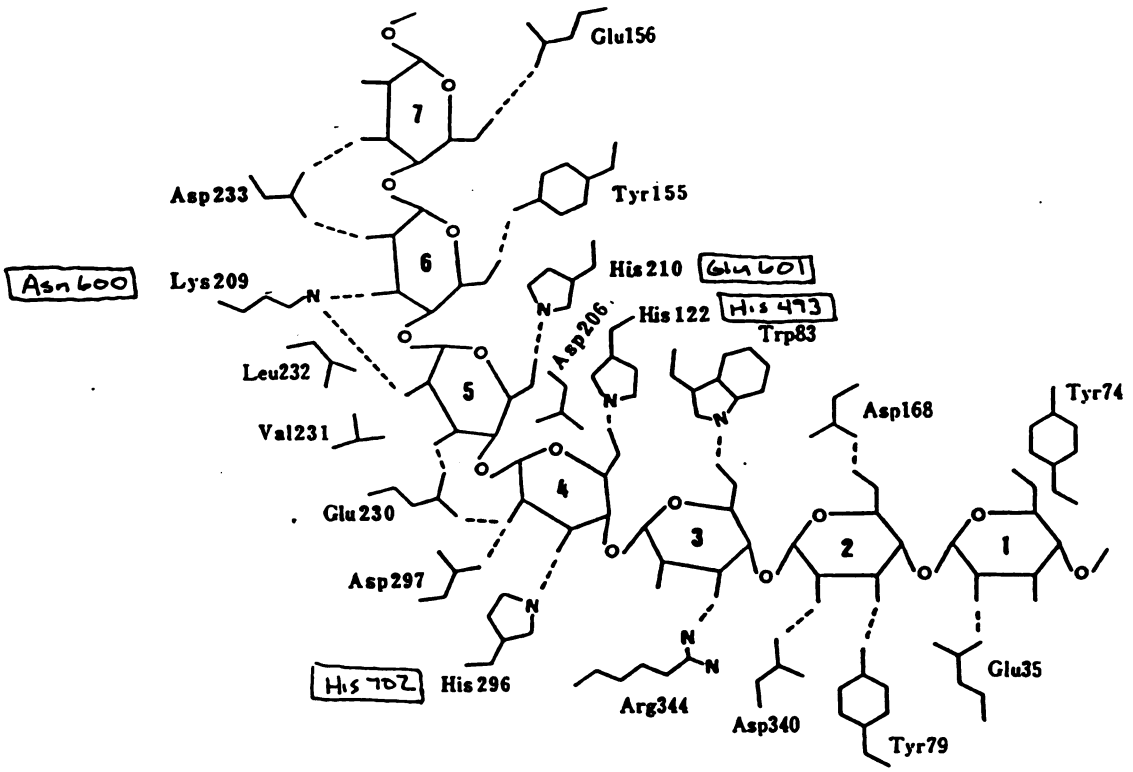


Table 5. General properties of bacterial pullulanases.

Organism	MW	pH	Optimum Temp(°C)	Reference
Bacillus sp.	92,000	9.0	55	Nakamura *et al.*, 1975
Bacillus acidopullulyticus	100,000	5.0	60	Schulein *et al.*, 1985
Bacillus cereus var. myc.	110,000	6.5	50	Takasaki, 1976
Bacillus subtilis	450,000	7.0	60	Takasaki, 1987
Bacteroides thetaiotamicron	77,000	6.5	37	Smith *et al.*, 1989
Clostridium thermosulf.	130,000	7.0	70	Buchardt *et al.*, 1991
Klebsiella pneumoniae	143,000	6.0	50	Ohba *et al.*, 1975
Klebsiella pneumoniae	143,000	5.0	47	Eisele *et al.*, 1972
Micrococcus sp.	120,000	10.0	50	Kimura *et al.*, 1990
Streptomyces sp.	N.D.	5.5	50	Yagisawa, 1971
Thermus aquaticus	83,000	8.0	85	Plant *et al., 1986*

3). Amylopullulanase

A new class of amylosaccharidase which cleaves both alpha-1,4 and alpha-1,6 glucosidic linkages has been identified and termed amylopullulanase or neopullulanase, depending on the products produced from action on pullulan. Amylopullulanase, which produces maltotriose from pullulan, was initially reported as an enzyme containing dual activities (both alpha-amylase and pullulanase) (Mathupala *et al.*, 1990). Analysis of the enzyme from *Bacillus circulans* F-2 showed the presence of separate active sites for the two activities (Sata *et al.*, 1989), and from *Clostridium thermohydrosulfuricum* E101 which reported a cassette model, where half of the gene encodes alpha-amylase activity while the other half encodes pullulanase activity (Melasniemi *et al.*, 1990). Site directed mutagenesis results, from the monomeric amylopullulanase from *Thermoanaerobacter ethanolicus* 39E, identified a single active site involved in the dual activity (Mathupala *et al.*, 1993). The pH optima of these enzymes varies from 5.5 to 6.0 with a temperature optimum range of 70°C to 100°C. The molecular weight varies from 110,000 to 162,000 (Table 6), with a calcium requirement for thermostability but not enzymatic activity (Mathupala and Zeikus 1993). Hydrolysis products produced from cleavage of starch include maltotetraose, maltotriose, maltose and glucose; a single cleavage product, maltotriose, is produced from action of amylopullulanase on pullulan (Mathupala *et al.*, 1990). Amylopullulanase has a higher activity on pullulan than starch (3:1) and is incapable of further hydrolysis of maltotriose (Mathupala *et al.*, 1990). This report will be the first to conduct a detailed investigation into the cleavage pattern profile of wild type and site-directed mutants of *T. ethanolicus* 39E amylopullulanase.

Table 6. General properties of bacterial enzymes with alpha-1,4 and alpha-1,6 cleavage activity (neopullulanases and amylopullulanases).

Organism	MW	pH	Optimum Temp (°C)	Reference
Pyrococcus furiosus	110,000	5.5	100	Brown *et al.* 1993
Pyrococus litoralis	119,000	5.5	90	Brown *et al.* 1993
Thermoanaerobacter ethanolicus 39E	162,000	6.0	90	Mathupala *et al.*, 1993
Thermoanaerobacterium saccharolyticum B6A-RI	142,000	6.0	70	Ramesh *et al.*, 1994
Bacillus stearothermophilus	62,000	6.0	65	Kuriki *et al.*, 1988

4). Neopullulanase

Neopullulanase, which produces panose from pullulan, was identified and shown to contain one active center that is involved in the dual amylase (alpha-1,4 cleavage) and pullulanase (alpha-1,6 cleavage) activities as well as alpha-1,4 and alpha-1,6 transferring activity (Kuriki *et al.*, 1991; Imanaka and Kuriki, 1989). This enzyme from *Bacillus stearothermophilus* has a molecular weight of 62,000 and is fairly thermostable. The optimum temperature is 65°C with an optimum pH for the enzyme of 6.0 (Table 6) (Kuriki *et al.*, 1988). Detailed biochemical characterization with regard to gene organization, subunit structure, catalytic and substrate binding sites, and mechanism of action is necessary to obtain a better understanding of these enzymes with dual activities. The dual activity of neopullulanase results in production of panose, maltose, and glucose (3:1:1) from hydrolysis of pullulan (Imanaka and Kuriki, 1989). Similiar to amylopullulanase, neopullulanase hydrolyzes pullulan more efficiently than starch. This enzyme is also capable of transglycosylation which was analyzed using maltotriose. The by-products of the reaction were glucose and maltose from the hydrolysis reaction, in addition to the formation of branched oligosaccharides (isomaltose, isopanose, and panose) by the transfer reaction (Kuriki *et al.*, 1993). One active site with dual activities was identified, and the enzyme, catalytic activity, cleavage specificity, and transferring activity can be altered by mutating residues in the four conserved regions (Kuriki *et al.*, 1993).

Replacement of amino acid residues in neopullulanase corresponding to the putative catalytic sites resulted in loss of enzyme activity toward alpha-1,4 and alpha-1,6 glucosidic linkages. Asp328, Glu357, and Asp424 of neopullulanase correspond to catalytic residues, Asp206, Glu230 and Asp297, of Taka amylase-A. Substitution of these amino acids in Taka amylase-A with His,

His and Gln, respectively, resulted in complete loss of catalytic activity (Kuriki *et al.*, 1991).

When amino acid residues corresponding to putative substrate binding sites (His247, His423, Asn331, and Glu332 of neopullulanase correspond to His122, His296, Lys209, and His210 of Taka amylase-A) were replaced, a change in alpha-1,4 and alpha-1,6 hydrolytic and transferring specificity was observed (Kuriki *et al.*, 1991). The production ratio of panose from pullulan was used as an indicator for changed specificity in alpha-1,4 and alpha-1,6 cleavage activity. The production of panose is increased when the specificity toward alpha-1,4 linkages increases. In contrast, the production of panose decreases when the specificity toward alpha-1,6 linkages is higher. When His247 was mutated to Glu and His423 to Glu the mutant enzymes exhibited a higher specificity toward alpha-1,4 glucosidic linkages producing more panose from pullulan than the wild type enzyme (Kuriki *et al.*, 1992). The double mutant Asn331Ser/Glu332Val had an opposite effect, producing less panose from pullulan due to decreased specificity toward alpha-1,4 glucosidic linkages (Kuriki *et al.*, 1992). These results suggest that amino acids His247, His423, Asn331, and Glu332 are involved in substrate cleavage specificity.

C. Sequence Conservation

Amylosaccharidases from different organisms generally display low homology, but share significant homology in four conserved regions (Kuriki and Imanaka 1989; Kuriki *et al.*, 1990; Nakajima *et al.*, 1986; and Mathupala *et al.*, 1993). Alpha-amylase, pullulanase, neopullulanase and amylopullulanase sequences contain these four conserved regions, and these regions have been proposed to be essential for the commonality of enzymatic function by amylosaccharidases. The conserved regions form an active center,

and the substrate binding site by comparison to the refined 3-dimensional structure of alpha-amylase from *A. oryzae* Taka amylase-A (Matsuura *et al.*, 1984). The dual activity of amylopullulanase, which cleaves both alpha-1,4 and alpha-1,6 linkages, functions by similiar mechanisms. In enzymes that cleave both alpha-1,4 and alpha-1,6 glucosidic linkages there has been identified a duplicated region II (II') which is located between regions III and IV (Matuschek *et al.*, 1994). It has not yet been determined whether this is significant for the specificity of dual activity enzymes. A catalytic triad comprised of two aspartate residues and one glutamate residue, in regions II, III, and IV, is required for both catalytic activities. Substrate binding is governed by four amino acids present in regions I, II, and IV; three histidines, and a lysine residue (Mathupala *et al.*, 1993).

Amino acid substitutions in neopullulanase and Taka amylase-A by site-directed mutagenesis confirmed one active center involved in substrate cleavage specificity (Kuriki 1992 and Matsuura *et al.*, 1984). The specificity for alpha-1,4 versus alpha-1,6 cleavage action was altered by replacing amino acids that are involved in substrate recognition. For example, a mutated neopullulanase was obtained which exhibited higher cleavage activity for alpha-1,4 glucosidic linkages producing a higher yield of panose from pullulan (Kuriki *et al.*, 1991). The difference in cleavage specificity may be due to a difference in the binding interaction between the substrate and the enzyme. The enzyme activity can be altered by manipulating the binding specificity for the substrate. Similiar experiments are proposed for amylopullulanase from a hyperthermophile in an effort to design an enzyme with altered cleavage activity for biotechnological production of high fructose syrups.

IV. Dissertation objectives and significance

This disssertation focuses on characterization of amylopullulanase from the thermophile *Thermoanaerobacter ethanolicus* 39E with regard to catalytic activity, substrate cleavage specificity, and thermophilicity. Structure-function relationships were analyzed in relation to catalysis and thermophilicity of this enzyme. Catalysis was analyzed in four ways. First, the protein sequence was compared with homologous mesophilic and thermophilic amylases, pullulanases, amylopullulanases, and neopullulanases. Second, deletion mutagenesis experiments were performed to determine regions of the gene required for the maintenance of thermostability and thermophilicity. Finally, mutations at amino acid residues important for substrate binding specificity and catalytic activity were constructed to analyze their affect on enzyme activity and the regulation of alpha-1,4 and alpha-1,6 glucosidic cleavages. Studies into the molecular basis for enzyme activity, substrate specificity, and thermostability will provide insight for designing enzymes of industrial importance. Specific aims in this project included:

(i) Design of mutated amylopullulanase with altered catalytic activity and specificity.

(ii) Identification of a specific protein region responsible for thermophilic characteristics of amylopullulanase.

To better control the various steps of starch conversion, industry needs a variety of monospecific enzymes which are optimally active at high temperatures (90°C-100°C) and low pH (5.5). Biochemical and structural analysis of regions related to thermostability and thermophilicity, as well as defining the amino acid residues responsible for cleavage specificity, may allow design of mutated amylopullulanase engineered to act as a true alpha-amylase or pullulanase in starch bioprocessing.

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CHAPTER II

Biochemical and Enzymatic Characterization of Catalytic Activity and alpha-1,4 / alpha-1,6 Cleavage Specificity of Recombinant Amylopullulanase from *Thermoanaerobacter ethanolicus* 39E

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ABSTRACT

The active center of amylopullulanase from *Thermoanaerobacter ethanolicus* 39E was analyzed by means of site-directed mutagenesis using the crystal structure of Taka-amylase A as a model. It was previously shown that four highly conserved regions exist in the active center of various amylolytic enzymes. When the active site residues Asp597, Glu626, and Asp703 were mutated, hydrolysis of alpha-1,4 and alpha-1,6 glucosidic linkages were not detectable. Mutation of the individual substrate binding site residues altered the specificity as either an increased or decreased alpha-1,4 cleavage activity, or resulted in complete loss of both alpha-1,4 and alpha-1,6 cleavage activity. Pullulan, amylose, soluble starch, and beta-limit dextrin were used as substrates to analyze substrate cleavage specificity and the rate of catalytic activity. The His493Gln and Asn600Ser/Glu601Val mutants increased the rate of alpha-1,4 and alpha-1,6 cleavage activity relative to the wild type amylopullulanase. The His493Gln mutant showed increased cleavage specificity for alpha-1,4 linkages on soluble starch; while, the Asn600Ser/Glu601Val mutant resulted in decreased alpha-1,4 cleavage specificity on soluble starch when compared to the wild type enzyme. Here we show that the rate of hydrolysis and end product formation can be altered by manipulating the putative substrate binding residues of amylopullulanase without significantly changing the kinetic parameters. This is the first report of changing the binding characteristics of amylopullulanase.

INTRODUCTION

Amylopullulanase is an enzyme capable of hydrolyzing both alpha-1,4 and alpha-1,6 glucosidic linkages. It is distinguished from its counterparts alpha-amylase or pullulanase, which have the ability to hydrolyze only alpha-1,4 or alpha-1,6 linkages, respectively.

Amylosaccharidases, broadly grouped into amylases and pullulanases, are classified based on substrate cleavage specificity and have been identified in various mesophilic, thermophilic, hyperthermophilic bacteria, and Archae. Pullulanases can be separated into four groups: pullulanase, isopullulanase, amylopullulanase, and neopullulanase, depending on the products of cleavage on pullulan. Examples include, but are not limited to, the following: pullulanase from *Thermoanaerobium* Tok6-B1 (Plant *et al.*, 1987), and amylopullulanase from *Thermoanaerobacter ethanolicus*, formerly *Clostridium thermohydrosulfuricum* (Mathupala *et al.*, 1990) which hydrolyze the alpha-1,6 linkages in pullulan to produce maltotriose; isopullulanase from *Aspergillus niger* (Sakano *et al.*, 1971) hydrolyzes alpha-1,4 linkages producing isopanose; and neopullulanase from *Bacillus stearothermophilus* (Kuriki *et al.*, 1988a) produces panose by cleavage of alpha-1,4 linkages and a small amount of glucose and maltose by the limited alpha-1,6 cleavage activity on pullulan. Amylopullulanases and neopullulanases are also capable of cleaving alpha-1,4 linkages in starch, producing low molecular weight oligosaccharides (Mathupala *et al.*, 1990; Kuriki *et al.*, 1988a). Isopullulanase and pullulanase can not hydrolyze starch.

Amylases are separated into three groups: alpha-amylases, beta-amylases, and glucoamylases. These enzymes catalyze the cleavage of starch, glycogen, and related glucans. Alpha-amylase is an endo-acting enzyme responsible for the random cleavage of alpha-1,4 glucosidic linkages (Matsuura *et al.*, 1983),

whereas, beta-amylases and glucoamylases are exo-acting enzymes that hydrolyze alpha-1,4 linkages, or alpha-1,4 and alpha-1,6 linkages, respectively (Fogarty and Kelly 1979). The most widely studied alpha-amylase is from *Aspergillus oryzae* (Taka amylase-A) (Matsuura *et al.*, 1983). While no crystal structure is available for an enzyme with both alpha-1,4 and alpha-1,6 activity, Taka amylase-A has been crystallized and the alignment of amino acids in homologous regions has been used for analysis of other alpha-amylases, neopullulanases, isoamylases, pullulanases, cyclodextrin glucotransferases, and amylopullulanases.

Cleavage action pattern has been most extensively studied in neopullulanase from *B. stearothermophilus* (Kuriki *et al.*, 1991; Imanaka and Kuriki 1989; Takata *et al.*, 1992), alpha-amylase from *B..licheniformis* (Kim *et al.*, 1994; Lee *et al.*, 1995; Kim *et al.*, 1995), and amylase-pullulanase from *Bacillus circulans* F-2 (Kim and Kim 1995). Cleavage activity of amylopullulanase from *T. ethanolicus* 39E will be compared to the cleavage patterns seen with the above enzymes. Amylopullulanase described in this report showed alpha-1,4 cleavage activity against soluble starch, amylose, beta-limit dextrin, and low molecular weight oligosaccharides; and alpha-1,6 cleavage activity against pullulan and beta limit dextrin. The catalytic activity on pullulan was 2-3 fold higher then that detected on amylose, soluble starch, and beta-limit dextrin. Kinetic analysis of wild type amylopullulanase established similiar Km_{app} values for soluble starch, amylose, and beta-limit dextrin (0.87 mg/ml), while Km_{app} for pullulan was 2-fold lower (0.36 mg/ml). The substrate affinity for the mutant enzymes was similiar to that reported above for wild type amylopullulanase. A model has been proposed for enzymatic activity on the above mentioned substrates. This paper describes characterization of specific

amino acid residues important for catalytic activity and cleavage properties of wild type and mutant amylopullulanase.

EXPERIMENTAL PROCEDURES

Chemicals and reagents - All chemicals were of molecular biology or analytical grade and were obtained from Aldrich Chemical Co., or Sigma.

Bacterial strains, plasmids, and culture conditions - *E. coli* strain TG-1 {F'*traD36 lacI*^{q} [*lacZ*]M15 *proA*$^+$*B*$^+$/*supE* [*hsdM-mcrB*]5[$r^{k-}m^{k-}$McrB$^-$] *thi lac-proAB*]} was obtained from Amersham Corp. *E. coli* DH5αF' {F'*hsdR17* ($r^{k-}m^{k+}$) *supE44 thi-1recA1gyrA* (Nal^r) *relA1* (*lacZ-argF*)U169 *deoR* (80*dlac* (*lacZ*)M15} was obtained from Bethesda Research Laboratories (Gaithersburg, MD). *E. coli* CJ236 {*dut, ung, thi, relA;* pCJ105 [Cm^r]} was obtained from BIO-RAD (Richmond, CA). Plasmids M13mp19 (New England Biolabs, Beverly, MA) and pUC18 (Gibco BRL, Grand Island, NY) were used as cloning vectors. The plasmid constructed in this study is shown in Fig. 1.

Media and growth conditions - *E. coli* cultures were grown in LB (Sambrook *et al.*, 1989) medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter). Ampicillin was used at 100 μg/ml for strains harboring plasmid.

Manipulation of DNA - Plasmid DNA purification, restriction analysis, ligations, PCR, and transformations were performed by conventional techniques (Sambrook*et al.*, 1989).

Oligonucleotides - Oligonucleotides were synthesized in an Applied Biosystems model 380A DNA synthesizer at the Macromolecular Structure, Synthesis and Sequencing Facility, Department of Biochemistry, Michigan State University. The oligonucleotides were subsequently 5'-phosphorylated using T4 polynucleotide kinase, for use in site directed mutagenesis (Table 1).

Enzyme purification - *E. coli* DH5αF' harboring the prAP164-UC wild type or recombinant plasmids containing the specific amino acid mutations were

Figure 1 Physical map of the pUC18 clone (prAP164-UC) containing the *apu* gene. A fragment containing the *apu* gene with *Eco* RI and *Bam* HI ends engineered by PCR was inserted into the multiple cloning site of the vector pUC18. Other loci are *bla,* β-lactamase gene conferring resistance to ampicillin; *lacI,* the lacIq repressor protein which binds downstream of the promoter to downregulate expression in the absence of induction; *ori,* pUC18 origin of replication.

Figure 1. Physical map of the pUC18 clone (prAP164-UC) containing the *apu* gene.

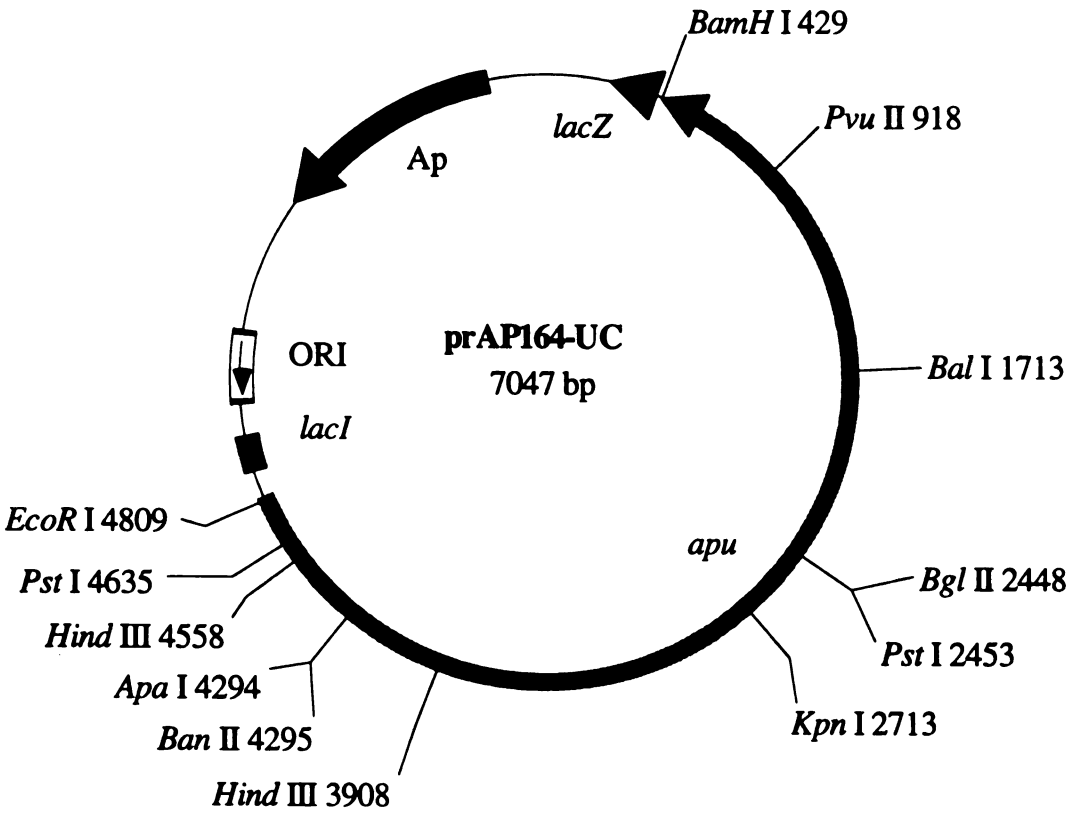


Table 1. Oligonucleotides used in this study

MUTATION	OLIGONUCLEOTIDE SYNTHESIZED
His493Gln	5' CTATCATCACTTGTCTGATTGAAGACGCC 3'
Asp597Leu	5' GAGCAATTTCATTTGCAACAAGCAATCTCCAG 3'
Asn600Ser/ Glu601Val	5' CGTGAGCAATTACACTTGCAACATCC 3'
Glu626Asp	5' GAAGCATCTCCCCAAAGGTCTGCAATCATTGG 3'
His702Arg	5' CTCATGGTGTCACGACAACCTAAAAGG 3'
His702Asp	5' CTCATGGTGTCATCACAACCTAAAAGG 3'
His702Glu	5' CTCATGGTGTCTTCACAACCTAAAAGG 3'
His702Lys	5' CTCATGGTGTCTTTACAACCTAAAAGG 3'
Asp703Gly	5' CTCATGGTGCCATGAGAACCTAAAAGG 3'
Asp672Asn	5' GTCAAGTTTTGCTGCATTTATAGGATTGTGAAC 3'
Asp672Glu	5' GTCAAGTTTTGCTGCTTCTATAGGATTGTGAAC 3'
Asp672Phe	5' GTCAAGTTTTGCTGCAAATATAGGATTGTGAAC 3'
Lys675Arg	5' GCCTTTGGTCAAGTCTTGCTGCATCTATAG 3'
Lys675Asp	5' GCCTTTGGTCAAGATCTGCTGCATCTATAG 3'
Lys675Ala	5' GCCTTTGGTCAAGTGCTGCTGCATCTATAG 3'
Leu676His	5' CATAAGCCTTTGGTCATGTTTTGCTGCATC 3'
Phe382Asn	5' GATATTCAACAGGATCATTACCTCTACTGTATTC 3'
Lys401Ala	5' GTATATCCAGGTTTATCTGCATCATTCGGATTG 3'
Asp402Gly	5' GTATATCCAGGTTTACCTTTATCATTCGGATTG 3'
Lys525Leu	5' CACCGTATGGAGATAATGACTGATCTCCCTG 3'

underlined: altered nucleotides in the primer to produce the desired amino acid change.

grown at 37°C in 5x 10.0 ml LB medium containing ampicillin (100 μg/ml). Cells were harvested by centrifugation in a microcentrifuge (12,000 rpm x 5 min) and the cell pellet resuspended in 0.5 ml 50 mM Na-acetate buffer (pH 6.0) containing 5 mM $CaCl_2$ and 150 μg lysozyme. The suspension was incubated on ice for 30 minutes followed by freeze-thaw. The cell lysate was centrifuged (14,000 rpm x 5 min) and the supernatant heat treated at 85°C for 5 min. After centrifugation (14,000 rpm x 5 min), the supernatant was recovered and tested for amylase and pullulanase activity. The purity of the various samples of Apu protein, judged by coomassie stained SDS-PAGE, was ~15%. The host cell contained no amylosaccharidase activity.

Protein determination and gel electrophoresis - Protein concentrations were determined using bicinchroninic acid (BCA Assay Kit, Pierce Chemical Co.), using bovine serum albumin as the standard. SDS-PAGE was performed according to the method of Laemmli (Laemmli 1970) using 10.0% polyacrylamide gels in a Mini-Protean II apparatus (Bio-Rad), and proteins were visualized by staining with Coomassie Brilliant Blue R-250. The molecular weights of the recombinant proteins were estimated by comparison to high range molecular weight standards (Bio-Rad).

Activity staining by native PAGE - Partially purified enzyme was electrophoresed as for SDS-PAGE, on a Bio-Rad Mini Protean II electrophoresis apparatus, except for the absence of SDS in the buffer systems and in the gel. Prior to polymerization of the gels, soluble starch was added to a final concentration of 1.0% (w/v). After electrophoresis, the gels were washed with acetate buffer (pH 6.0), and incubated at 60°C for 5 to 10 min. For activity staining of soluble starch embedded gels, a solution of 0.15% iodine: 1.5% KI was added as an overlay. Alpha-amylase activity could be detected as a clear band, indicating absence of starch, against the dark black background.

Enzyme assays - Cell extracts prepared as described above were used as enzyme sources. Alpha-amylase activity was determined in a reaction mixture that contained 1.0% soluble starch (w/v) in 50 mM Na-acetate buffer (pH 6.0) with 5 mM $CaCl_2$, and enzyme. After incubation at 60°C for 30 min., the reaction was stopped by adding 0.8 ml of dinitrosalicylate solution [0.25 M NaOH, 71.0 mM sodium potassium tartrate, 4.0 mM Na_2SO_3, 5.0 mM phenol, and 44.0 mM 3,5 dinitrosalicylic acid] (Bernfeld 1955) and heated in a 100°C oil bath for 15 min. The samples were cooled on ice and the absorbance at 640 nm was measured. One unit of amylase activity was defined as the amount of enzyme which produces 1 μmol of reducing sugar/min with glucose as the standard (Miller 1959). Pullulanase activity was determined as above except that 1.0% pullulan was used in place of starch.

Kinetic analysis - Assays to determine Km_{app} were conducted at 60°C with substrate concentrations between 20 x Km_{app} and 0.2 x Km_{app}. Kinetic parameters were determined using nonlinear curve fits of the Michaelis-Menten equation to the data. Calculations were done on an IBM personal computer using Kinzyme.

Oligonucleotide-directed mutagenesis of the amylopullulanase gene and sequencing - Mutagenesis was performed using an oligonucleotide-directed *in vitro* mutagenesis system (BIO-RAD, Richmond, CA). Chemically synthesized oligonucleotides (18-mer to 28-mer) were used; the sequences are shown in Table 1. The *Apa* I-*Bgl* II segment of the prAP164-UC was ligated into M13mp19 using *Xba* I and *Bam* HI restriction sites. (The *Xba* I site was end filled with Klenow, producing a blunt end to complement the blunt end produced by *Apa* I, and the *Bam* HI overhang is compatible with *Bgl* II). Uracil-containing single strand DNA was synthesized and used as the template. An oligonucleotide complementary to the region to be altered, except for the mismatch, was

hydridized to the single-strand uracil DNA. The complementary strand was then synthesized by T4 DNA polymerase using the oligonucleotide as primer. Ligase was used to seal the new strand. The double-stranded DNA containing the mutation of interest was transformed into *E. coli* TG-1. The mutations were confirmed by DNA sequencing, by the dideoxy chain termination technique of Sanger et al. (Sanger *et al.*, 1977), using the Sequenase Version 2.0 kit (U.S. Biochemical Corp., OH). The sequencing reaction was primed by annealing 17-mer synthetic oligonucleotides. The double stranded mutant DNA was then introduced back into prAP164-UC using *Apa* I and *Bgl* II restriction enzyme sites.

Analysis of hydrolysis products - Enzyme samples (0.05 Units) were incubated in the presence of various oligo- or polysaccharide substrates (1.0% w/v) in 50 mM acetate buffer (pH 6.0) with 5 mM $CaCl_2$ at 60°C for 16 hours (unless otherwise indicated), and the products were analyzed by High Performance Ion Chromatography (HPIC) using a CarboPac PA1 column (Dionex BioLC4500i) and a pulsed amperomeric detector (PAD, Dionex). Solution A (100 mM NaOH in water), solution B (1.0 M Na-acetate in solution A), and solution C (water) were used for elution. All the solvents were prepared with Milli-Q water, and filtered through a polyvinylidene difluoride membrane filter (0.22μm) (Gelman Sciences, Inc.). The samples were eluted at a flow rate of 1.0 ml/min with solution A (30%-10%), solution B (0-20%), and solution C (70%) over 25 minutes. Twenty five microliters of 0.02% sample solution was injected into the column for analysis.

RESULTS

Subcloning and reconstruction of recombinant amylopullulanase - In order to preserve stability of the enzyme during purification, a new subclone (prAP164-UC) was constructed from the original clone (pAPZ72) (Mathupala *et al.*, 1993). Elimination of the 331 nt upstream from the GTG start codon and the 31 amino acid signal sequence present in pAPZ72 resulted in increased stability of the enzyme (data not shown). An *Eco* RI site was introduced immediately preceeding the first coding amino acid, and a *Bam* HI site immediately following the TGA stop codon by PCR amplification from *Thermoanaerobacter ethanolicus* 39E chromosomal DNA. The PCR product was introduced into the polylinker of pUC18 and the validity of the construct confirmed by sequence analysis and restriction enzyme mapping.

Identification of conserved amino acid residues constituting the active center of amylopullulanase - Four highly conserved regions present in amylosaccharidases, which are essential for the function of these enzymes, have been identified (Jespersen *et al.*, 1993; Kuriki and Imanaka 1989; Matuschek 1994; Nakajma *et al.*, 1986). Alignment of amino acid residues in the highly conserved regions of Taka amylase-A (Matsurra *et al.*, 1983) and other saccharolytic enzymes (Table 2) was used for analysis of amylopullulanase. Asp206, Glu230 and Asp297, located in consensus regions II, III, and IV respectively, were proposed as catalytic residues of Taka amylase-A. His-122, Asp206, Lys209, His210, and His296, located in consensus regions I, II, and IV respectively, were proposed as substrate-binding residues (Matsurra *et al.*, 1983).

It has been suggested previously (Mathupala *et al.*, 1993) and reported here that Asp597, Glu626, and Asp703 of amylopullulanase, which correspond to Asp206, Glu230, and Asp297 of Taka amylase-A, might act as catalytic sites.

Table 2 Consensus sequences in the alpha-amylase family. ***italicized*****: catalytic residues, bold: substrate specificity residues. Numbering of the amino acid residues starts at the N-terminus of the mature protein. Underlined residues correspond to the split consensus region II, in enzymes with dual activity, relative to the consensus for alpha-amylase.**

Table 2. Consensus sequences in the alpha-amylase family (adapted from Matuschek et al., 1994)

	CONSENSUS SEQUENCE			
	Region I	Region II/ Region II'	Region III	Region IV
α 1.4 activity AMYLASE				
α-amylase consensus	DAVINH	GFRLDAAKH	EVID	FVDNHD
TAKA α-amylase	117 DVVANH	202 GLRIDTVKH	230 EVLD	292 FVENHD
B. licheniformis BLMA	245 DAVFNH	323 GWRLDVAKH	356 EIWH	418 LLDSHD
α-1.6 activity PULLULANASE				
K. aerogenes	620 DVVYNH	691 GFRFDLMGY	723 EGWD	846 YVSKHD
K. pneumoniae	610 DVVYNH	681 GFRFDLMGY	713 EGWD	836 YVSKHD
Thermus AMA-33	339 DAVYNH	406 GFRFDLMGV	439 EGWD	522 YVECHD
ISOAMYLASE				
P. amyloderamosa	291 DVVYNH	370 GFRFDLASV	416 EFTV	502 FIDVHD
α-1.4 and α-1.6 activity AMYLOPULLULANASE				
39E	488 DGVFNH	593 GWRLDVAKH/ 668 HNPIDAAKL	626 ELWND	698 LLGSHD
E101	488 DGVFNH	595 GWRLDVAKH/ 669 HNPIDAAKL	627 ENWND	699 LLGSHD
B6A-RI	487 DGVFNH	590 GWRLDVEKH/ 665 HNPIDAAKL	623 ENWGD	695 LLGSHD
EM1	488 DGVFNH	589 GWRLDVEKH/ 664 HNPIDAAKL	622 ENWGD	694 LLGSHD
NEOPULLULANASE				
B. stearothermophilus	242 DAVFNH	324 GWRLDVAKH/ 382 GVLRFFAKE	357 EIWHHD	419 LLGSHD

Identification of His493, Asn600, Glu601, and His702 of amylopullulanase, corresponding to His122, Lys209, His210, and His296 of Taka amylase-A, may be responsible for substrate binding specificity.

Identification of a duplicated region II (region II') in those enzymes with both alpha-1,4 and alpha 1-6 hydrolytic activity (Matuschek *et al.*, 1994) may be significant for the dual specificity of these enzymes. Mutational experiments with Asp672 (region II') of amylopullulanase, corresponding to Asp206 (region II) of Taka amylase-A, and Asp675 and Lys676, corresponding to Lys209, His210 of Taka amylase-A will be conducted to analyze their effect on catalytic activity and substrate binding specificity respectively.

Based on amino acid comparison, we identified amino acid residues Asp597, Glu626, Asp703, His493, Asn600, Glu601, His702, Asp672, Lys675, and Leu676 as targets for mutagenesis to analyze the active center of amylopullulanase (Table 3).

Time course of substrate hydrolysis by wild type amylopullulanase - The hydrolysis pattern of amylopullulanase from *Thermoanaerobacter ethanolicus* 39E was tested on many low and high molecular weight oligosaccharides. Maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, pullulan, and soluble starch were digested with amylopullulanase, and the products analyzed by HPIC (Dionex). A 200 µl reaction mixture containing 1.0% (w/v) the indicated substrate in 50 mM Na-Acetate buffer (pH 6.0) with 5 mM $CaCl_2$ and 0.05 Units enzyme was incubated at 60°C for 30 minutes. The reaction was stopped by incubating at 100°C for 15 minutes and diluted 1:1500 before HPIC analysis. HPIC profile of glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose (G1-G7) standard mixture is shown in Figure 2.

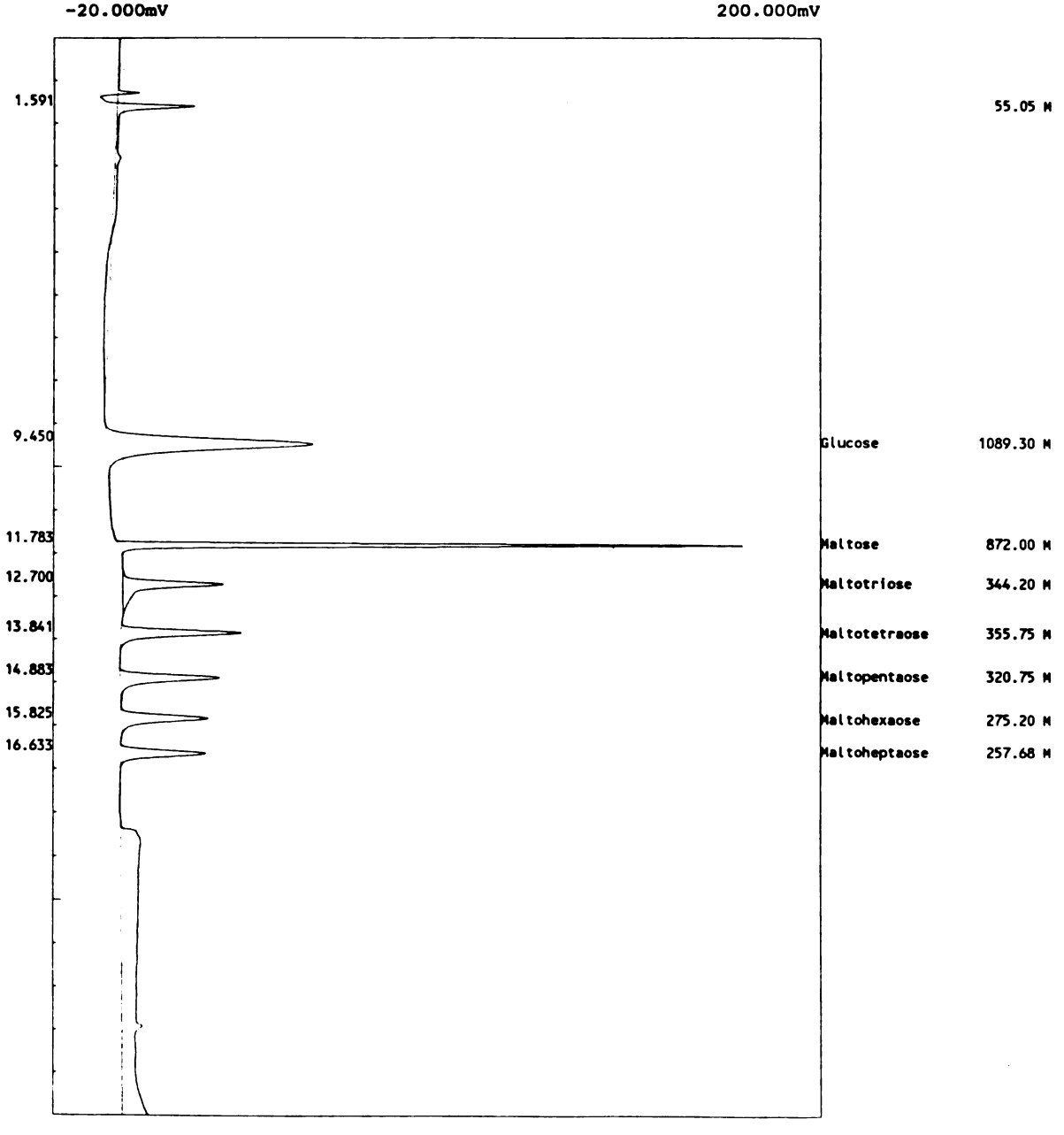
Table 3. Amylopullulanase mutants generated by site directed mutagenesis

	CONSERVED REGIONS			
	I	II	III	IV
Wild Type	**488 DGVFNE**	**593 GWRLDVAME**	**626 ELWND**	**698 LLGSHD**
His493Gln	——Q			
Asp597Leu		——L——		
Asn600Ser/ Glu601Val		——SV		
Glu626Asp			D——	
His702Arg				——R
His702Asp				——D
His702Glu				——E
His702Lys				——G
Asp703Gly				——G
		II'		
Wild Type	**488 DGVFNE**	**668 HNIPDAAKL**	**626 ELWND**	**698 LLGSHD**
Asp672Asn		——N——		
Asp672Glu		——E——		
Asp672Phe		——F——		
Lys675Arg		——R		
Lys675Asp		——D		
Leu676Ala		——A		

underlined: Active site amino acid residues
bold: Substrate binding site amino acid residues

Figure 2 HPIC profile showing standard mixutre of G1-G7. A carbopak-column (0.4 x 25 cm, 10mm of particle size, Dionex) and a PAD detector were used. A buffer (100 mM NaOH in water) and B buffer (1.0 M Na-acetate in A buffer) were used for elution as described in Materials and Methods.

Figure 2. HPIC profile showing standard mixture of G1-G7.



Degradation of 1.0% soluble starch by wild type Apu was monitored over a period of 96 hrs (Table 4). The major hydrolysis products were maltose and maltotriose. As the reaction proceeded maltotetraose was completely degraded, maltose and maltotriose remained unchanged, and the levels of glucose accumulated. This end product profile resembles that exhibited by thermophilic alpha-amylases. The percent of maltotriose remained constant throughout the time course which is consistent with the hydrolysis results of maltotriose, maltohexaose, and pullulan producing maltotriose as the only product by action of alpha-1,4 (maltotriose and maltohexaose as substrates), and alpha-1,6 (pullulan as substrate) hydrolytic activity.

Time course of maltoheptaose hydrolysis revealed maltotriose and glucose as the only hydrolysis products present at 80% and 20% respectively throughout the reaction (Table 4). Maltoheptaose is hydrolyzed producing two maltotriose and one glucose. Another posibility for cleavage activity on maltoheptaose would be production of maltotetraose and maltotriose . This is not likely due to the absence of maltotetraose as an end product after the 16 hr reaction.

Other substrates examined include maltotriose, maltohexaose, and pullulan, all of which produced maltotriose as the only hydrolysis product (Table 5). Amylopullulanase has no cleavage activity on maltotriose. The activity on maltohexaose is amylase like, while that on pullulan is characteristic of a pullulanase, both yielding maltotriose as the sole product which can not be further hydrolyzed. Maltopentaose produced maltotriose and maltose each present at roughly 50% after a 16 hr. reaction (Table 5). Hydrolysis of beta-limit dextrin produced maltose, maltotriose, maltotetraose, maltopentaose, and maltoheptaose as end products (Table 5). The maltopentaose and maltoheptaose

Table 4 Time course of reaction products produced from wild type amylopullulanase on various low and high MW oligosaccharides. Solutions of 1.0% pullulan, soluble starch, maltotriose (G3), maltohexaose (G6), and maltoheptaose (G7) were incubated at 60°C with 0.05 U/ml partially purified enzyme. Samples were withdrawn at various time points and heated at 100°C for 15 minutes for enzyme inactivation. The reaction products were analyzed by HPIC for sugars (Dionex). Abbreviations; glucose (G1), maltose (G2), and maltotetraose (G4). Numbers indicate percent molar equivalents relative to standards and results after triplicate analysis.

Table 4. Time course of reaction products produced from wild type amylopullulanase on various low and high MW oligosaccharides.

SUBSTRATE	PRODUCT	PERCENT OF TOTAL				
		1 hr	7 hr	16 hr	24 hr	96 hr
Soluble Starch	G1	2.7	6.4	10.1	15.6	17.6
	G2	53.0	57.5	56.4	52.7	51.0
	G3	33.2	28.1	29.2	28.7	30.7
	G4	11.1	7.9	4.3	3.0	0
Maltoheptaose	G1	20.3	18.7	18.5	17.4	20.2
	G3	79.7	81.3	81.5	82.6	79.8
Pullulan	G3	100	100	100	100	100
Maltotriose	G3	100	100	100	100	100
Maltohexaose	G3	100	100	100	100	100

Table 5 Comparison of end products from enzymatic action of His493Gln, Asn600Ser/Glu601Val, and wild type enzymes on low and high MW oligosaccharides. Solutions of 1% pullulan, soluble starch, beta-limit dextrin, maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), and maltoheptaose (G7) were incubated at 60°C with 0.05 U/ml partially purified enzyme. Samples were withdrawn after 16 hours and heated at 100°C for 15 minutes for enzyme inactivation. The reaction products were analyzed by HPIC for sugars (Dionex). Numbers indicate percent molar equivalents relative to standards and results after triplicate analysis.

Table 5. Comparison of end products from enzymatic action of His493Gln, Asn600Ser/Glu601Val, and wild type enzymes on low and high MW oligosaccharides.

A. Wild Type

Substrate	End Products (%)					
	G1	G2	G3	G4	G5	G7
Maltotriose			100.0			
Maltotetraose	20.3		54.3	25.4		
Maltopentaose		48.1	51.9			
Maltohexaose			100.0			
Maltoheptaose	18.5		81.5			
Pullulan			100.0			
Soluble Starch	10.1	56.4	29.2	4.3		
Beta-Limit Dextrin		27.0	16.4	16.5	23.0	17.2

B. His493Gln

Substrate	End Products (%)					
	G1	G2	G3	G4	G5	G7
Maltotriose			100			
Maltotetraose	49.4		40.5	10.2		
Maltopentaose		49.6	50.4			
Maltohexaose			100			
Maltoheptaose	20.4		79.6			
Pullulan			100			
Soluble Starch	4.0	23.1	58.2	14.7		
Beta-Limit Dextrin		24.7	9.5	18.9	27.5	19.4

C. Asn600Ser/Glu601Val

Substrate	End Products (%)					
	G1	G2	G3	G4	G5	G7
Maltotriose			100			
Maltotetraose	30.0		46.7	23.3		
Maltopentaose		49.3	50.7			
Maltohexaose			100			
Maltoheptaose	14.1		85.9			
Pullulan			100			
Soluble Starch		46.8	35.2	18.0		
Beta-Limit Dextrin		21.2	19.2	16.1	27.7	15.7

produced is indicative of debranching at alpha-1,6 branch points. A model of Apu action pattern is proposed in Figure 3.

Amino acid replacement by site-directed mutagenesis and construction of mutated amylopullulanases - The *Apa* I-*Bgl* II fragment of prAP164-UC (Figure 1) contains the four highly conserved regions which most likely constitute the active center of amylopullulanase. Subcloning into the M13mp18 multiple-cloning site, propagation of single stranded DNA, and subcloning back into pUC18 was performed out as described in the experimental procedures. The 1900 bp fragment was sequenced to verify the presence of the desired amino acid change and confirm that no second-site mutations were present.

E. coli TG-1 carrying the wild type or mutant plasmids was grown in LB and the partially purified enzyme sample was prepared. Kinetic experiments were performed using the standard assay at a variety of substrate (pullulan, soluble starch, amylose, and beta-limit dextrin) concentrations. Values for Km_{app} and $Vmax_{app}$ determined from Lineweaver-Burk plots showed no significant difference between those mutants which were active when compared to the wild type enzyme (Table 6).

Activity of mutated amylopullulanases - In a previous study, three amino acids important for catalytic activity were identified by sequence alignment with alpha-amylase (Taka-amylase) from *Aspergillus oryzae* (Mathupala *et al.*, 1993; Matsurra *et al.*, 1983). Due to the reconstruction of recombinant amylopullulanase mentioned earlier, mutational analysis of each of the three catalytic amino acid residues was repeated. Single mutants of Asp597 (in conserved region II), Glu626 (in conserved region III), and Asp703 (in conserved region IV), resulted in complete loss of enzymatic activity as seen previously (Table 7).

Figure 3. Proposed action pattern of amylopullulanase. Open circles and slashed circles denote non-reducing glucopyranosyl residues and reducing glucose residues, respectively. Horizontal lines and vertical lines connecting circles indicate alpha-1,4 and alpha-1,6 glucosidic linkages respectively.

Figure 3. Proposed action pattern of amylopullulanase

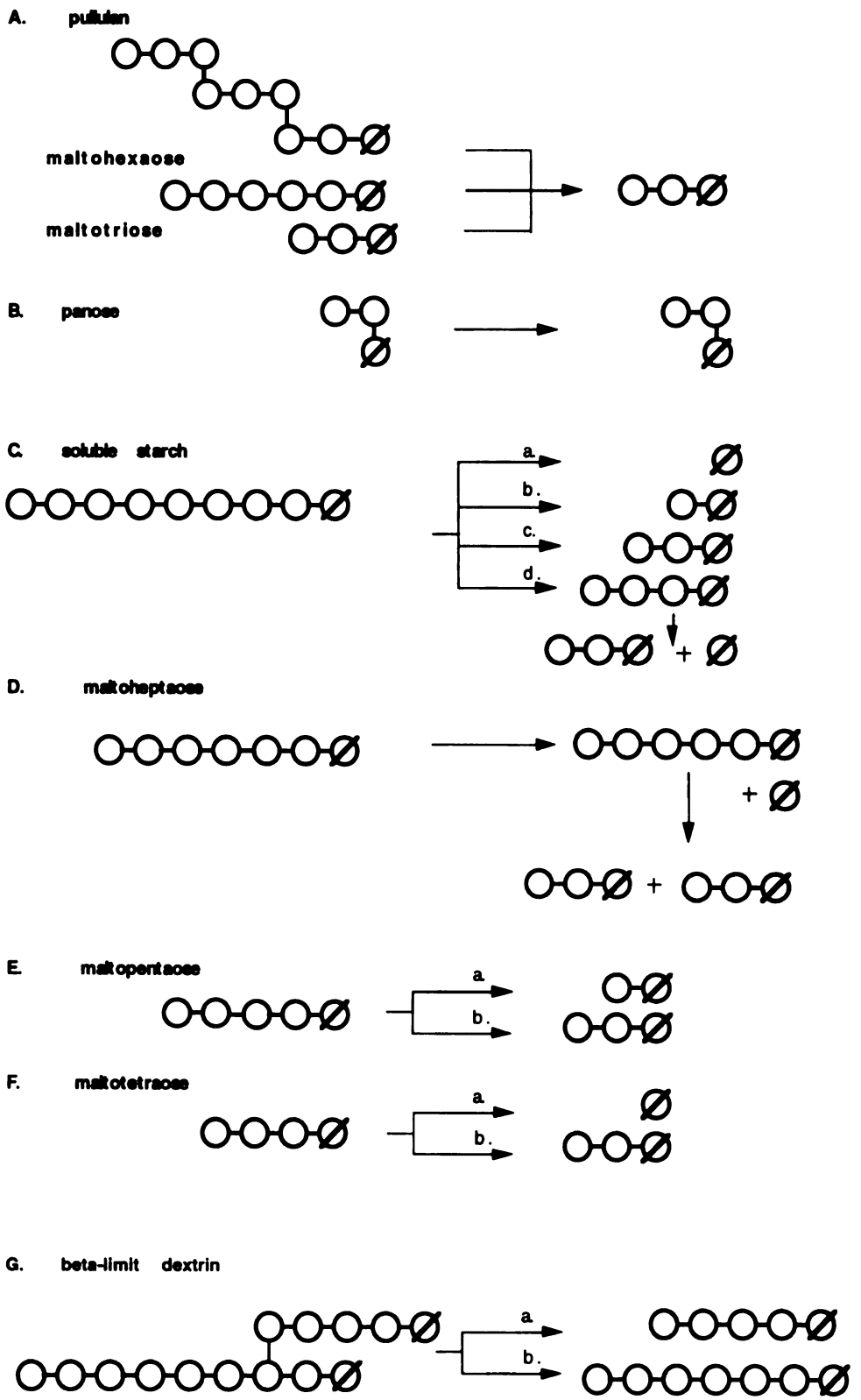


Table 6 Kinetic analysis of wild type and mutant amylopullulanase on various substrates. Assays to determine Km_{app} and $Vmax_{app}$ were conducted at 60°C with substrate concentrations between 20 x Km_{app} and 0.2 x Km_{app}. All assays were done in triplicate.

Table 6. Kinetic analysis of wild type and mutant amylopullulanase on various substrates.

Enzyme Km_{app} (mg/ml)/$Vmax_{app}$ (Units/mg)

	Pullulan	Soluble Starch	Amylose	Beta-Limit Dextrin
Wild Type	0.36/32.76	0.87/25.20	0.90/28.14	0.92/27.98
His493Gln	0.32/35.96	0.93/25.26	0.88/36.14	1.05/32.17
Asn600Ser/ Glu601Val	0.40/38.74	1.01/27.02	0.97/21.28	1.00/30.61
Asp672Asn	0.30/33.63	0.92/13.07	0.91/14.43	1.20/19.31
Asp672Glu	0.33/25.04	0.90/14.19	0.97/16.21	1.00/19.03

Table 7 Activity comparisons of Asp597Leu, Glu626Asp, and Asp703Gly.

Rate of product formation represents the Units/mg on pullulan, amylose, soluble starch, and β-limit dextrin under standard assay conditions. One unit is defined as the amount of enzyme which produces 1.0 μmol of reducing sugar/min with glucose as the standard. All assays were done in triplicate. P/A ratio, pullulanase/amylase activity ratio.

Table 7. Activity comparisons of Asp597Leu, Glu626Asp, and Asp703Gly.

	Rate of Product Formation Substrate:				
Enzyme	Pullulan	Amylose	Soluble Starch	β-Limit Dext.	P/A Ratio
Wild Type	28.83	13.17	13.68	12.53	2.54
Asp597Leu	< 0.001	< 0.001	< 0.001	< 0.001	-
Glu626Asp	< 0.001	< 0.001	< 0.001	< 0.001	-
Asp703Gly	< 0.001	< 0.001	< 0.001	< 0.001	-

When the putative substrate binding site residues of amylopullulanase were mutated, the rate of product formation increased relative to wild type with mutants His493Gln and Asn600Ser/Glu601Val. By contrast the mutants His702Arg, His702Asp, His702Lys were void of cleavage activity on pullulan, amylose, soluble starch, and beta-limit dextrin (Table 8). Mutating His702 (in conserved region IV) to Arg, Asp, and Lys resulted in complete loss of activity. The His493Gln mutant and the Asn600Ser/Glu601Val (corresponding to putative substrate binding residues His247 and Asn331/Glu332 respectively in neopullulanase), both resulted in an increased rate of alpha-1,4 and alpha-1,6 cleavage activity.

End product formation from low and high MW oligosaccharides using wild type and mutated amylopullulanases - Incubation of wild type and mutant enzymes with low-molecular weight oligosaccharides, pullulan and soluble starch resulted in production of various oligosaccharide end products, depending upon the substrate used. Amylopullulanase does not hydrolyze maltotriose. The only product obtained upon incubation with pullulan was G3, demonstrating that the enzyme shows typical pullulanase activity. Activity toward alpha-1,4 linkages is seen as well, demonstrating amylase activity. The percent of each end product produced differs when comparing wild type and mutant enzymes (Table 5).

Twenty-five percent of the maltotetraose substrate remained unhydrolyzed after 16 hour incubation with wild type Apu, by contrast with the His493Gln mutant only 10.2% maltotetraose remaining. The further cleavage to produce G1/G3 products are 20.3%/54.3% and 49.4%/40.5%, respectively, for wild type and mutant enzymes. Maltoheptaose as the substrate produces 18.5% G1 and 81.5% G3 for wild type enzymatic activity and 20.4% G1 and 79.6% G3 for the His493Gln enzyme. The His493Gln enzyme has a higher breakdown to G1 and G3 when maltotetraose is used as the substrate indicating an increased

Table 8 Activity comparisons of His493Gln, Asn600Ser/Glu601Val, His702Arg, His702Asp, and His702Lys. Rate of product formation represents the Units/mg on pullulan, amylose, soluble starch, and β-limit dextrin under standard assay conditions. One unit is defined as the amount of enzyme which produces 1.0 μmol of reducing sugar/min with glucose as the standard. All assays were done in triplicate. P/A ratio, pullulanase/amylase activity ratio.

Table 8. Activity comparisons of His493Gln, Asn600Ser/Glu601Val, His702Arg, His702Asp, and His702Lys.

	Rate of product formation Substrate:				
Enzyme	Pullulan	Amylose	Soluble Starch	β-Limit Dext.	P/A ratio
Wild type	28.83	13.17	13.68	12.53	2.54
His493Gln	39.99	20.61	20.36	17.41	2.10
Asn600Ser/ Glu601Val	50.90	15.61	13.30	16.64	3.81
His702Arg	< 0.001	< 0.001	< 0.001	< 0.001	–
His702Asp	< 0.001	< 0.001	< 0.001	< 0.001	–
His702Lys	< 0.001	< 0.001	< 0.001	< 0.001	–

alpha-1,4 cleavage specificity. In contrast, the Asn600Ser/Glu601Val enzyme revealed a decreased alpha-1,4 cleavage specificity on maltoheptaose due to the lower production of G1 and G3 from hydrolysis activity. G1 and G3 produced from action of Asn600Ser/Glu601Val on maltoheptaose are 14.1% and 85.9%, respectively. Activity on maltotetraose is slightly higher than the wild type enzyme but lower then the His493Gln enzyme, yielding 30.0% G1, 46.7% G3, and 23.3% remaining maltotetraose that was not broken down after the 16 hour reaction (Table 5).

End product formation from cleavage activity on soluble starch provided the most interesting results. Wild type amylopullulanase produced 10.1% G1, 56.4% G2, 29.2% G3, and 4.3% G4 yielding a higher conversion to smaller oligosaccharides than the His493Gln or Asn600Ser/Glu601Val mutant enzymes. His493Gln produced 4.0% G1, 23.1% G2, 58.2% G3, and 14.7% G4; while the double mutant produced 46.8% G2, 35.2% G3, and 18.0% G4 with no detectable production of G1 before the completion of the reaction (Table 5).

Action of the wild type enzyme, the His493Gln enzyme, and the Asn600Ser/Glu601Val enzyme produced an equal distribution of G2 (50%) and G3 (50%) from hydrolysis of maltopentaose (Table 5). Maltotriose was produced from activity of the above enzymes on maltohexaose and pullulan with no further breakdown of maltotriose to maltose or glucose (Table 5).

The use of beta-limit dextrin as substrate produced no significant change in hydrolysis products when comparing His493Gln and Asn600Ser/Glu601Val mutants to wild type amylopullulanase. All produced statistically equivalent amounts of maltose, maltotriose, maltotetraose, maltopentaose, and maltoheptaose (Table 5). The rate of activity of His493Gln, Asn600Ser/Glu601Val and wild type enzyme on beta-limit dextrins was similiar to that observed for amylose or soluble starch (Table 8).

Analysis of region II' for altered catalytic or cleavage activity - The mutations we have constructed in the duplicated region II (region II' - Table 2) have yet to be analyzed in any amylolytic enzyme system. In enzymes with dual specificity, the separation of region II into two discrete regions (II and II') might allow for hydrolysis of both alpha-1,4 and alpha-1,6 linkages due to increased flexibility of the active center. Site-directed mutants were constructed to determine if the separation of region II is important for catalytic activity and substrate cleavage specificity.

We have shown previously (Table 7), that Asp597 (region II) was essential for catalytic activity of amylopullulanase. Asp672 (region II') in amylopullulanase corresponding to catalytic residue Asp206 (region II) of Taka alpha-amylase was mutated to Asn and Glu with similiar reductions in rate of activity on pullulan, amylose, soluble starch, and beta-limit dextrin; 5.7x, 7.9x, 7.5x, and 7.4x, respectively (Table 9).

We further examined the role of Lys675 and Leu676 of amylopullulanase in region II' to determine if these residues provide the same function in substrate binding as Lys209 and His210 in region II of Taka alpha-amylase. Lys675 has been substituted with Arg, Asp and Ala; and Leu 676 was mutated to His, similiar to that present in position 210 of Taka alpha-amylase. There were no differences observed in product formation or rate of activity with various substrates when compared to wild type amylopullulanase (data not shown).

Table 9 Activity comparisons of Asp672Asn and Asp672Glu. Rate of product formation represents the Units/mg on pullulan, amylose, soluble starch, and β-limit dextrin under standard assay conditions. One unit is defined as the amount of enzyme which produces 1.0 μmol of reducing sugar/min with glucose as the standard. All assays were done in triplicate. P/A ratio, pullulanase/amylase activity ratio.

Table 9. Activity comparisons of Asp672Asn and Asp672Glu.

	Rate of product formation Substrate:				
Enzyme	Pullulan	Amylose	Soluble Starch	β-Limit Dext.	P/A ratio
Wild type	28.83	13.17	13.68	12.53	2.54
Asp672Asn	7.52	4.32	4.57	4.32	3.68
Asp672Glu	7.52	4.32	4.44	4.70	3.85

DISCUSSION

We tested the effect on activity, substrate preference, and end product formation by site-directed mutagenesis of conserved amino acid residues in consensus regions I - IV and duplicated region II' of amylopullulanase from *T. ethanolicus* 39E. Catalytic activity and end product formation by alpha-1,4 and/or alpha-1,6 hydrolytic activity of various mutants were altered when compared to the wild type amylopullulanase. Substrates used to analyze hydrolytic activity included: pullulan, amylose, soluble starch, and beta-limit dextrin.

Glu626, Asp703, and Asp597 of amylopullulanase correspond to the catalytic residues Glu230, Asp297, and Asp206 of Taka amylase-A (Matsuura *et al.*, 1983). When these three residues were singly replaced with Asp, Gly, and Leu, respectively, catalytic activity on pullulan, amylose, starch and beta-limit dextrin was not detectable (Table 6). Therefore, amino acid residues Asp597, Glu626, and Asp703 are absolutely required for catalytic activity.

Several mutated amylopullulanases were engineered by substituting amino acid residues corresponding to the substrate-binding residues of Taka amylase-A. Some of the mutants showed altered alpha-1,4 and/or alpha-1,6 cleavage specificity when compared to the wild type amylopullulanase. Mutating His702 (in conserved region IV) to Arg, Asp, and Lys resulted in complete loss of activity. The same result was seen when the corresponding amino acid (His423) from neopullulanase was mutated to Arg, Asp, and Lys (Kuriki *et al.*, 1991). Mutating His702 to Arg, Asp, and Lys may abolish catalytic activity due to its potential interference in the acid-base mechanism of one of the amino acid residues (Asp703) absolutely required for catalysis. The His493Gln

mutant and the Asn600Ser/Glu601Val (corresponding to His247 and Asn331/Glu332 respectively in neopullulanase), both resulted in an increased rate of alpha-1,4 cleavage activity. Similiar results were seen with the above neopullulanase mutants (Kuriki *et al.*, 1991) and the His250Gln mutant in *B. licheniformis* alpha-amylase (Cheong 1995).

The His493Gln Apu shows more efficient cleavage activity when G4 was used as the substrate, yielding higher percentages of G1/G3 after a 16 hr reaction relative to wild type. The efficiency is reduced when soluble starch is used as the substrate yielding lower percentages of G1, G2,and G3 with more G4 remaining after the reaction was terminated. The neopullulanase mutant (His423Gln) showed an increased production of panose (Kuriki *et al.*, 1991), while the alpha-amylase mutant (His250Gln) showed similiar percentages of panose production (Cheong 1995) relative to the wild type enzymes with pullulan as the substrate.

The amylopullulanase double mutant Asn600Ser/Glu601Val enzyme had decreased cleavage specificity for alpha-1,4 linkages on soluble starch as evidenced by the lack of G1 production and more G4 remaining after the hydrolysis reaction (Table 5). The active center of amylopullulanase is similiar to that of neopullulanase, but their cleavage pattern on pullulan is completely different. Mutational analysis of Asn331Ser/Glu332Val from neopullulanase indicated a decrease in alpha-1,4 cleavage specificity and an increase in alpha-1,6 specificity as evidenced by the production of less panose from pullulan than the wild type enzyme (Kuriki *et al.*, 1991). The mutant Asn330Ser/Glu331Val from alpha-amylase increased the alpha-1,4 hydrolytic specificity and endowed the amylase with alpha-1,6 activity similar to that of neopullulanase on pullulan. These two amino acids were thought to be related to alpha-1,6 hydrolytic activity and specificity. A dramatic increase in the rate of alpha-1,4 and alpha-1,6 catalytic activity was seen when mutating amino acid residues 493 and 600/601

of amylopullulanase. It is difficult to examine the specificity with amylopullulanase using pullulan as the substrate due to maltotriose being the only product produced from hydrolysis which can not be further cleaved.

Mutations constructed from amino acid residues present in the duplicated region II (II') showed no difference in their end product formation on low and high molecular weight oligosaccharides. There was a significant decrease in the rate of product formation using pullulan, amylose, soluble starch, and beta-limit dextrin as the substrates with the Asp672Asn and Asp672Glu enzymes when compared to wild type amylopullulanase. Both mutants had a 6x and 8x reduction in activity on pullulan and amylose, respectively. The rate of product formation on soluble starch and beta-limit dextrins decreased similiarly to that seen with amylose for both Asp672Asn and Asp672Glu.

In summary (Table 10), our results show: 1) a loss of catalytic activity when each of the three conserved catalytic residues are mutated, and 2) a change in hydrolysis pattern when mutating three of the four conserved substrate binding residues. We took this analysis one step further and identified a duplicated region II (II') in dual activity enzymes which is similiar to region II in alpha-amylase. Our hypothesis was that duplication of region II may be important for catalytic activity and substrate specificity. Preliminary research presented here indicates that individual amino acids in region II' may be important for catalytic activity due to the decrease in rate of activity on all substrates tested, but no difference was seen in substrate cleavage specificity.

Table 10. Summary of amino acid alteration in the four conserved regions with amylopullulanase, neopullulanase, and alpha-amylase.

	Consensus Sequence				
	Region I	Region II	Region II'	Region III	Region IV
	DAVINH	GFRL*D*AAKH	HNPI<u>D</u>AAKL	*E*VID	FVDNH*D*
Enzyme	Amino Acid Altered/Result				
Apu	H/increase alpha-1,4	*D*/catalytically inactive	KH/increase alpha-1,4	<u>D</u>/reduction in catalytic activity	<u>K</u>/same rate and cleavage activity
				E/catalytically inactive	
					D/catalytically inactive
Neo	H/increase alpha-1,4	*D*/catalytically inactive	KH/increase alpha-1,6		
				E/catalytically inactive	
					H/increase alpha-1,4
					D/catalytically inactive
BLMA	H/increase alpha-1,4	*D*/catalytically inactive	KH/increase alpha-1,4 endowed alpha-1,6		
					D/catalytically inactive

Future experiments will focus on a strategy to convert the dual activity enzyme, amylopullulanase, to an enzyme with only alpha-1,4 or alpha-1,6 hydrolytic activity. The main limitation in the design of an industrially significant enzyme is the inability to overexpress the recombinant protein. Thus far, our attempts at overexpression of amylopullulanase resulted in cell toxicity and loss of the insert from the cloning vector. A yeast overexpression system is currently being pursued and, if successful, may allow us to overexpress the protein and obtain crystals for three-dimensional structure analysis. We will then confirm the results presented in this report by model fitting oligosaccharides into the catalytic site of the enzyme. This will provide detailed information of the binding mode between substrate and enzyme, and aid in the production of mutant monospecific, thermophilic amylopullulanases for industrial starch degradation.

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Chapter III

Molecular analysis of thermophilicity and thermostability of recombinant amylopullulanase from *Thermoanaerobacter ethanolicus* 39E.

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ABSTRACT

Thermophilicity and thermostability of amylopullulanase from *Thermoanaerobacter ethanolicus* 39E were analyzed by N- and C-terminal deletion mutagenesis. Sequence and HCA comparison of amylopullulanase from *T. ethanolicus* 39E to organisms with lower thermostability showed high sequence homology and secondary structure similarity. Deletion mutant construction has identified a region related to thermophilicity at the N-terminus of *T. ethanolicus* 39E amylopullulanase. The region corresponding to amino acid residues 107-324 is important for maintaining activity at the enzyme's optimal temperature. The wild type has an optimum temperature of about 85°C while the ApuN324 deletion construct has an optimum temperature of about 65°C with a broad temperature range from 45°C to 93°C. An Arrhenius plot for wild type amylopullulanase was linear; however, the observed plot for ApuN324 was discontinuous, probably due to a temperature dependent structural change occurring at 50°C. The Km_{app} values of the wild type and ApuN324 enzymes were similar at both 40°C and 60°C; while the $Vmax_{app}$ was lower for both enzymes at 40°C relative to that at 60°C. To our knowledge, this report is the first to identify a change in thermophilic characteristics of an enzyme. It is hypothesized that the flexibility of the mutant protein was increased to account for a 20°C lower temperature optimum because the Km_{app} value was not altered relative to the wild type enzyme.

INTRODUCTION

Amylopullulanases from thermophiles are as active and stable above 90°C as commercial *Aspergillus oryzae* alpha-amylase, thus having great potential for a single step liquifaction and saccharifaction process producing high oligosaccharide syrups from starch. Attempts have been made to understand the property of enhanced thermal stability inherent in the structures of thermophilic enzymes in comparison to homologous mesophilic enzymes.

Initial studies on increased temperature optima for enzyme activity from thermophilic organisms, when compared to similar mesophilic enzymes, focused on differences in the amino acid composition. A recent report by Jaenicke and Bohn (1994) showed that a shift in the amino acid composition was not enough to explain the mechanism of thermophilic enzyme adaptation. Extensive comparison of the tertiary structures (Blake *et al.*, 1993 and Bradley *et al.*, 1993) of mesophilic and thermophilic enzymes indicated that there is no large detectable structural difference (Tomazic and Klivanov 1988). It has also been shown that hydrophobicity is the dominant force of protein folding, whereas ion pairing, hydrogen bonding, and Van der Waals interactions are of less importance but may affect stability (Dill *et al.*, 1990).

Hydrophobicity and increased packing of residues in the protein core are generally accepted to be a major stabilizing factor in thermophilic proteins (Vieille *et al.*, 1996). For increased stability, the positioning of the residues within the interior of the protein is more important, due to geometrical considerations (Sandberg and Terwilliger 1989) yielding increased protein rigidity (Jaenicke 1991; Eijsink *et al.*, 1992), than the total content of hydrophobic residues.

Many properties of proteins arise from two extremes in their folded conformation, flexibility (increased molecular motion) and rigidity (resistance to

unfolding). The thermophilicity of enzymes may be closely related to flexibility and rigidity of the protein's folded conformation that is essential for preserving their catalytically active structure. Flexibility is essential for enzyme function while rigidity is necessary for maintaining the globular structure. Thermostable enzymes are thought to have a more rigid structure than that of mesophilic enzymes. This has been addressed by utilizing hydrogen (deuterium) exchange experiments. Experiments done by Tsuboi *et al* (1978) with elongation factor Tu showed a reduced rate of exchange with the enzyme from *Thermus thermophilus* relative to the enzyme from *E. coli* (Fontana 1990). The higher the exchange rate the higher the flexibility of the protein. The flexibility of thermophilic and mesophilic enzymes was equivalent at their respective optimum temperatures (Tsuboi *et al.*, 1978). The rigidity of thermozymes has also been demonstrated by lower susceptibility to proteolytic degradation and chemical or thermal denaturant unfolding (Wrba *et al.*, 1990; Kanaya and Itaya 1992).

To examine features responsible for thermophilicity, we constructed and analyzed deletion mutants of *T. ethanolicus* 39E amylopullulanase expressed in *E. coli*. Information presented here provides evidence to support the hypothesis that the temperature activity optimum is related to optimal enzyme flexibility.

EXPERIMENTAL PROCEDURES

Reagents, Enzymes, and oligonucleotides - All chemicals were of molecular biology or analytical grade and obtained from Aldrich Chemical Co., or Sigma. Restriction enzymes and ligase were obtained from Bethesda Research Laboratories, United States Biochemical Co., or Boehringer Manheim.

Bacterial strains, plasmids, and transformations - *E. coli* strain SURE {e14-(*mcr*A) (*mcrCB-hsdSMR-mrr*)171 *endA1 supE44 thi*-1 *gyrA96 relA1 lac recB recJ sbcC umu*C:Tn5(kan^r)*uvr*C [F' *proAB lacI*^{q}Z M15Tn10(tet^r)]} from Strategene Co. and DH5α-F' {F' 80*dlac* (*lacZYA-argF*)U169 *deoR recA1 end*A1 *hsdR17*(r_K-m_K+)*supE44* I- *thi*-1 *gyrA96 relA1*}, from Bethesda Research Labs.) were used as hosts for cloning, and *E. coli* BMH 71-18 *mut*S, JM109 and ES1301 *mut*S from Promega Corporation were used. *E. coli* strains were made competent by the Hanahan method as described by Perbal (Perbal 1988), while recombinant vectors were introduced in *E. coli* strains by heat-shock treatment (Hanahan 1983).

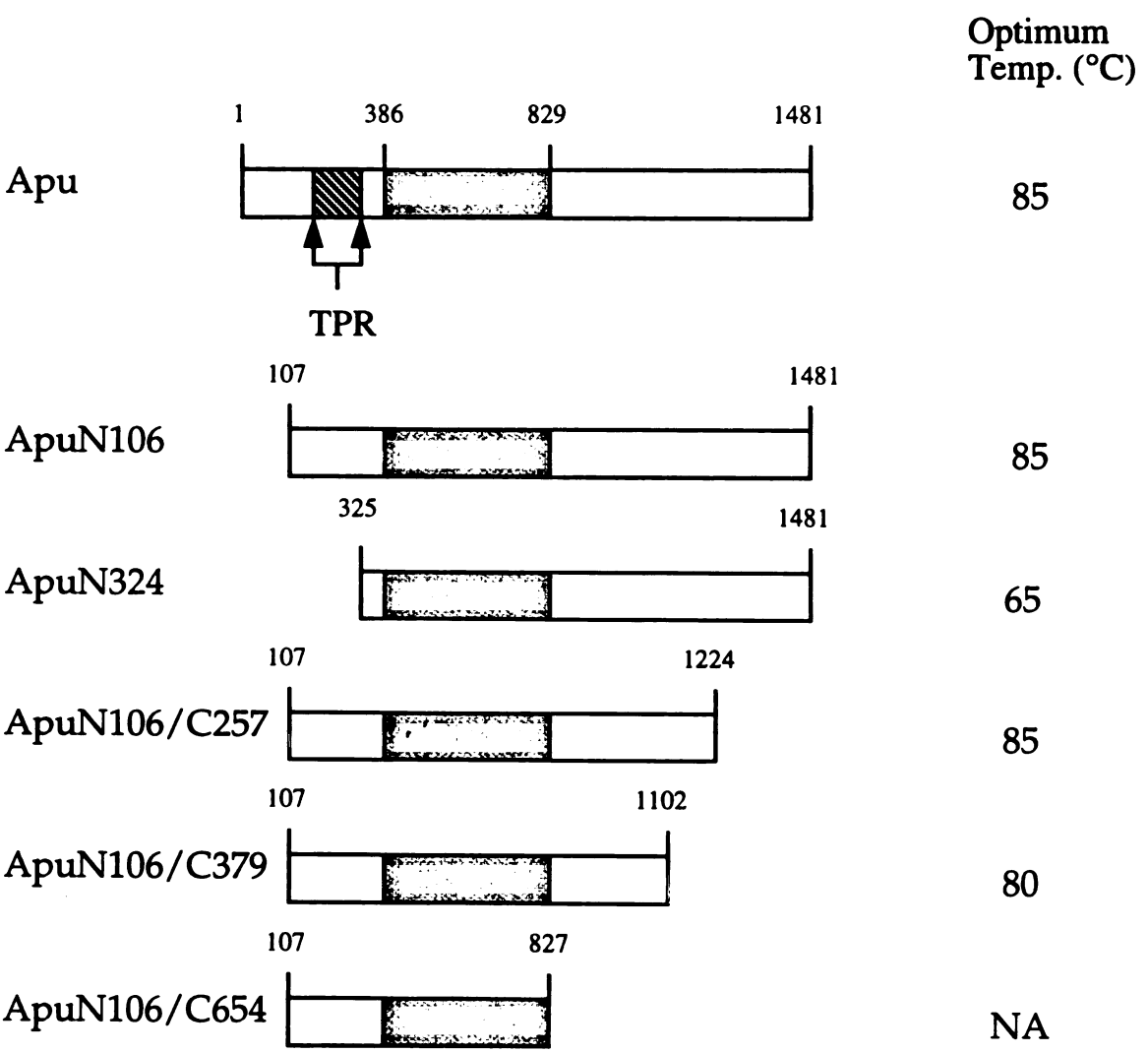
Enzyme assays - For determination of amylopullulanase activity, 160 μl of 1.25% (w/v) pullulan in 50 mM acetate buffer (pH 6.0) containing 5 mM $CaCl_2$ and enzyme (heat treated culture supernatant) to a total volume of 200 μl were mixed and incubated at 60°C for 30 min. The reaction was stopped by adding 0.8 ml of dinitrosalicylate solution [0.25 M NaOH, 71.0 mM sodium potassium tartrate, 4.0 mM Na_2SO_3, 5.0 mM phenol, and 44.0 mM 3,5 dinitrosalicylic acid] (Miller 1959) and heated in an oil bath (100°C) for 15 min. The samples were cooled on ice and the absorbance of the solution measured at 640 nm. One unit of amylopullulanase activity was defined as the amount of enzyme which produced 1 μmol of reducing sugar/min (with glucose as the standard) under standard assay conditions.

Sequence analysis and DNA sequencing - The amino acid sequence deduced from the amylopullulanase DNA sequence was compared with the primary structures of less thermostable amylopullulanases, alpha-amylases and pullulanases available through GenBank (IntelliGenetics Inc., Mountain View, CA). GCG Sequence Analysis Software Package ver. 8.0 (1994) was used in the analysis and multiple sequence alignments and subsequent data manipulations. Hydrophobic cluster analysis (HCA) of the amino acid sequences was performed as described by Lemesle-Varloot *et al* (1990). Sequenase V.2.0 T7 DNA polymerase and Sequenase V.2.0 sequencing kit from United States Biochemicals were used for verifying the mutated gene fragment according to Sanger's dideoxy chain termination method (Sanger *et al.*, 1977). For double-stranded DNA sequencing, denaturation of double-stranded plasmid DNA was performed as described by Zhang *et al* (1988). For sequencing reactions where *lacZ* fusion constructs were involved, universal M13/pUC forward and reverse sequencing primers were used. The synthetic oligonucleotides listed in Table 1 were also used as sequencing primers.

Deletion mutant construction - Several mutants were constructed by deletion from 5'- and/or 3'-ends of the *apu* gene and expressed in *E. coli* (Fig. 1). Recombinant plasmid pAPZ72 (Mathupala *et al.*, 1993) was digested with *Aat* II and *Nde* I, creating an exonuclease III sensitive restriction site (*Nde* I) towards the DNA insert, which was used to construct deletion mutants of the DNA insert from the 3' to 5' direction (Deletion kit of New England BioLabs Inc.). Six mutants, which were deleted at the C-terminal end, were constructed. To construct deletion mutants in the 5' to 3' direction, restriction sites *Hind* III, *Ban* II, *Hind*III, and *Kpn*I at the 5' end of the insert were used. *Ban* II-*Hind* III (5.4 kb), *Hind* III-*Hind* III (4.8 kb) and *Kpn* I-*Hind* III (4.2 kb) fragments from pAPZ72 were obtained and ligated into pUC vectors. Four deletion

Figure 1 Effect of N and C-terminus deletions of amylopullulanase gene from *Thermoanaerobacter ethanolicus* 39E on optimum temperature for enzyme activity. The Apu deletion constructs were expressed in *E. coli* and partially purified by heat treatment at 85°C for 5 min. Optimum temperature analysis was done in triplicate under standard assay conditions (similiar specific activities) with pullulan as the substrate at temperatures ranging from 45-98°C. The figure shows the structural gene of each construct and the number of deleted residues. The dotted box depicts the proposed catalytic domain of Apu. NA: not active; TPR (proposed thermophilicity region) spans amino acids 195-324.

Figure 1. Effect of N and C-terminus deletions of amylopullulanase gene from *Thermoanaerobacter ethanolicus* 39E on optimum temperature for enzyme activity.



mutants at the N-terminal end were constructed and characterized. Each deletion mutant was transformed into *E. coli* DH5α, and tested for activity and thermocharacteristics. The deletion constructs were grown at 37°C in 5.0 ml of LB media (Sambrook *et al.*, 1989) containing ampicillin (50 μg/ml). Cells were harvested from 1.0 ml of the culture by centrifugation (14,000 rpm x 1.0 min) and the cell pellet lysted in 50 mM Na-acetate buffer (pH 6.0) containing 5 mM $CaCl_2$ and lysozyme. To test for thermal characteristics, the cell lysate was centrifuged and the supernatant heat-treated at each temperature and centrifuged. The supernatant was used for activity analysis.

Thermal denaturation and temperature optimum - Samples of amylopullulanase and mutant constructs were incubated in 50 mM Na-acetate/ 5 mM $CaCl_2$ in a thermally controlled oil bath at 85°C for 0-210 minutes. The time course of inactivation was followed by withdrawing samples every 10 min. The samples were then cooled on ice. Substrate was added, and the samples processed under standard assay conditions. Optimum temperature analysis was done by incubating the enzyme reaction at various temperatures under standard assay conditions.

Kinetic analysis - Assays to determine Km_{app} were conducted at 60°C with substrate concentrations between 20 x Km_{app} and 0.2 x Km_{app}. Kinetic parameters were determined using nonlinear curve fits of the Michaelis-Menten equation to the data. Calculations were done on an IBM personal computer using Kinzyme.

Protein determination and gel electrophoresis - Protein concentrations were determined using bicinchroninic acid (BCA Assay Kit, Pierce Chemical Co.), using bovine serum albumin as the standard. SDS-PAGE was performed according to the method of Laemmli (Laemmli 1970) using 10.0% polyacrylamide gels in a Mini-Protean II apparatus (Bio-Rad), and protein bands were visualized

by staining with Coomassie Brilliant Blue R-250. The molecular weights of the recombinant proteins were estimated by comparison to high range molecular weight standards (Bio-Rad). The purity of the various samples of Apu protein, judged by coomassie stained SDS-PAGE, was ~15%. The host cell contained no detectable amylosaccharidase activity.

RESULTS

Identification of a thermophilicity region in T. ethanolicus 39E amylopullulanase (Apu). - The secondary structures (16) of *T. ethanolicus* 39E Apu and those of less thermophilic amylopullulanases from *Thermoanaerobacter thermohydrosulfuricus* E101 (optimal temp. 80°C), *Thermoanaerobacterium thermosulfurigenes* EM1 (optimal temp. 60°C), and *Thermoanaerobacterium saccharolyticum* B6A-RI (optimal temp. 70°C) were compared by Hydrophobic Cluster Analysis (HCA) and reveal high secondary structure similarity.

Five deletion mutants were constructed from both the C-terminal and N-terminal ends to identify their potential contribution to thermophilicity and thermostability. The constructs were expressed in *Escherichia coli* and characterized after partial purification. ApuN106 showed a similar optimum temperature with respect to wild type Apu (Figure 1). ApuN324 showed similar half-life to wild type, however, the optimal temperature for enzyme activity was 65°C versus 85°C for the wild type enzyme (Figure 2). Due to the shift in optimum temperature seen with the ApuN324 mutant, we have designated the region between amino acid 194 to 324 important for maintenance of the optimum temperature for enzyme activity. There was no difference in the thermostability of the ApuN324 deletion construct relative to wild type (Figure 3). Deletion constructs ApuN106/C257 and ApuN106/C379 maintained an optimum temperature profile similar to that of the wild type enzyme. Deleting 654 amino acids from the C-terminus (ApuN106/C654) resulted in complete loss of activity.

Figure 2. Optimal temperature profiles of wild type amylopullulanase and deletion construct ApuN324. Optimum temperature analysis was done in triplicate under standard assay conditions with pullulan as the substrate in 50 mM Na-Acetate buffer (pH 6.0) containing 5 mM $CaCl_2$ at temperatures ranging from 40-100°C.

Figure 2. Optimal temperature profiles of wild type amylopullulanase and deletion construct ApuN324.

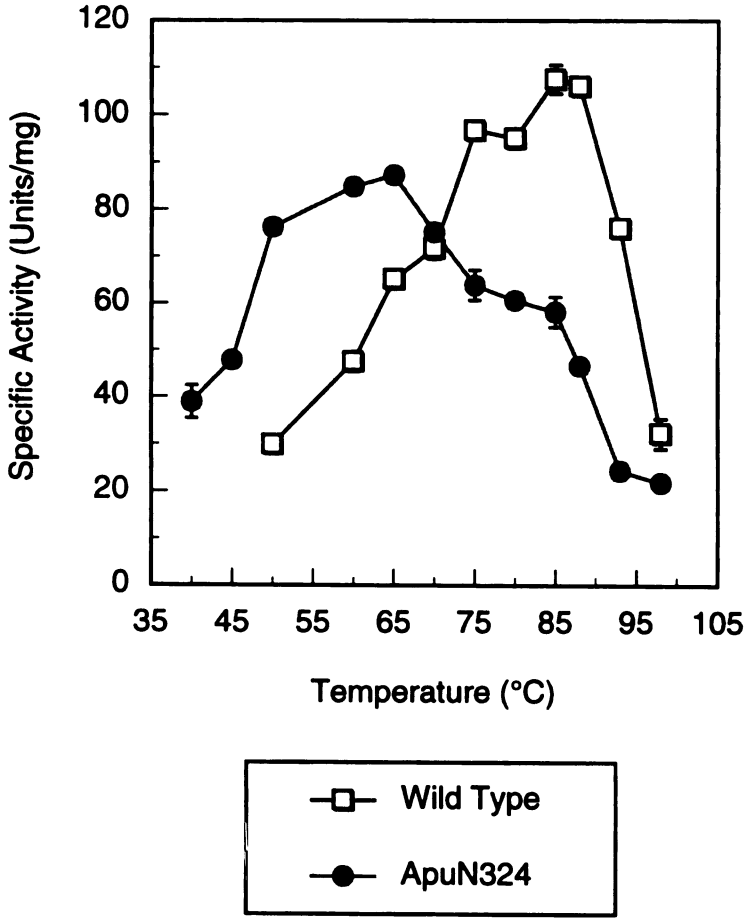
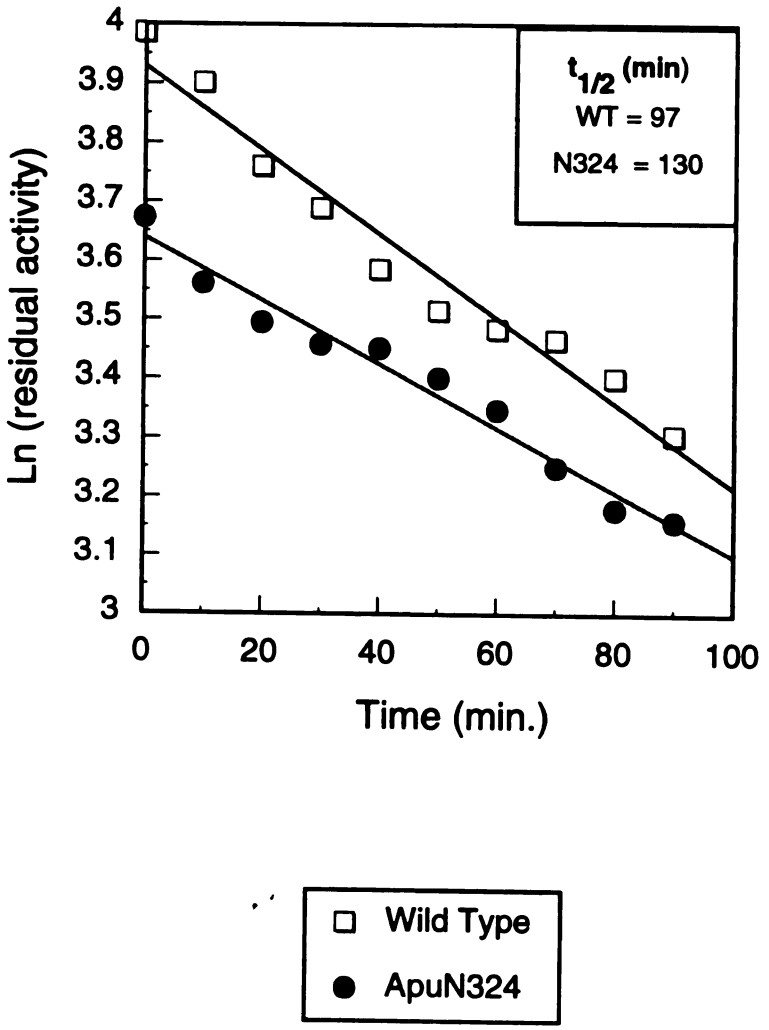


Figure 3. Thermostability profiles of wild type and ApuN324 deletion construct of amylopullulanase after enzyme pre-incubation at 85°C for 0-90 minutes. Aliquots of amylopullulanase and the mutant construct were incubated in 50 mM Na-acetate/5 mM $CaCl_2$ in a thermally controlled oil bath at 85°C for 0-90 min. The time course of inactivation was followed by withdrawing samples every 10 min. The samples were then cooled on ice. Substrate was added, and the samples processed under standard assay conditions. All experiments were done in triplicate.

Figure 3. Thermostability profiles of wild type and ApuN324 deletion construct of amylopullulanase after enzyme pre-incubation at 85°C for 0-90 min.



Comparison of thermal and kinetic properties of the wild type and ApuN324 deletion construct of amylopullulanase. - The kinetic properties of wild type amylopullulanase and ApuN324 were determined under standard assay conditions at 40°C and 60°C. The Km_{app} value for the recombinant enzyme (0.36 mg/ml) is similar to that reported for native amylopullulanase from *T. ethanolicus* 39E (0.35 mg/ml); Km_{app} for ApuN324 was 0.39 mg/ml. $Vmax_{app}$ were 32.7 Units/mg and 78.7 Units/mg for the wild type and ApuN324 enzymes at 60°C, respectively (Table 1). Km_{app} and $Vmax_{app}$ calculations at 40°C were 0.36 mg/ml and 19.3 Units/mg for the wild type enzyme, and 0.46 mg/ml and 32.2 Units/mg for ApuN324 (Table 1)

The Arrhenius plot showing temperature-activity data for pullulan hydrolysis was linear from 50°C to 85°C for the wild type enzyme, by contrast the analysis shows a discontinuity for ApuN324 from 40°C to 65°C (Figure 5). The discontinuity was observed at 50°C for the deletion construct. The slopes of the best fit regression line were different above (10 kJ mol^{-1}) and below (56 kJ mol^{-1}) the discontinuity for ApuN324 (Table 2). The slope of Arrhenius analysis for the wild type enzyme revealed an activation energy of 39 kJ mol^{-1} (Table 2). The activation energies above and below the discontinuity for ApuN324 were significantly different from that observed for the wild type enzyme.

Table 1 Kinetic analysis of wild type and ApuN324 amylopullulanase on pullulan at 40°C and 60°C. Km_{app} and $Vmax_{app}$ determinations in triplicate on pullulan at various substrate concentrations assayed at 40°C and 60°C for
30 minutes.

Table 1. Kinetic analysis of wild type and ApuN324 amylopullulanase on pullulan at 40°C and 60°C.

	Wild Type		ApuN324	
	Km_{app}	$Vmax_{app}$	Km_{app}	$Vmax_{app}$
40°C	0.36 mg/ml	19.3 Units/mg	0.46 mg/ml	32.2 Units/mg
60°C	0.35 mg/mg	32.7 Units/mg	0.39 mg/ml	78.7 Units/mg

Figure 4. Arrhenius plots for the recombinant *T. ethanolicus* 39E wild type and ApuN324 deletion construct of amylopullulanase between 40°C and 85°C. Temperature-activity data done in triplicate for pullulan hydrolysis. (A) Wild Type (B) ApuN324 deletion construct.

Figure 4. Arrhenius plots for the recoombinant *T. ethanolicus* 39E wild type and ApuN324 deletion construct of amylopullulanase between 40°C and 85°C.

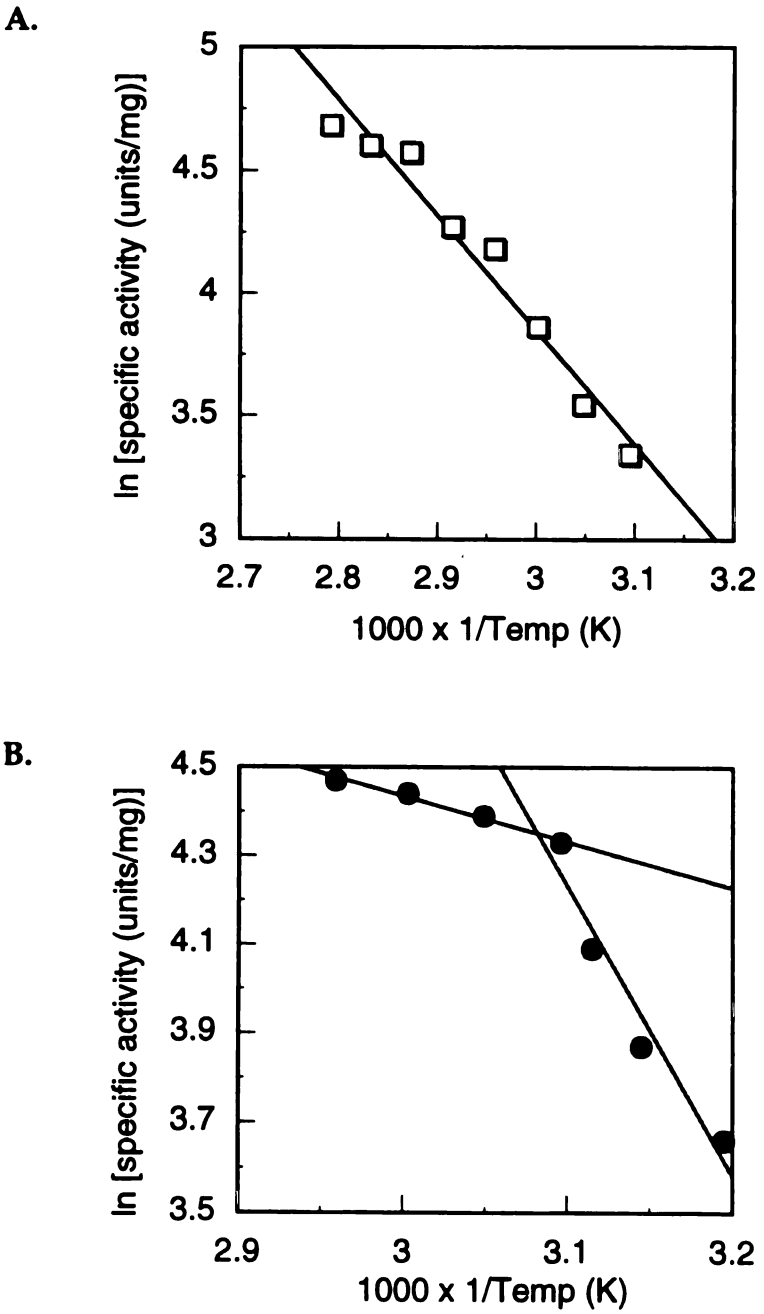


Table 2. Activation energy calculations from Arrhenius plots of wild type and ApuN324 amylopullulanase.

		Activation Energy (kJ mol^{-1})		
Enzyme	Discontinuity Temp. (°C)	above discontinuity	below discontinuity	^{b}D
Wild Type	^{a}ND	39	39	0
ApuN324	50°C	10	56	46

^{a}ND: not detectable

^{b}D = (activation energy below the discontinuity) - (activation energy above the discontinuity)

DISCUSSION

Amylopullulanase deletion mutant constructs were made and a region corresponding to the first 324 amino acid residues was found to be important for maintenance of enzyme activity at its optimal temperature (≈85°C). The first 106 amino acids are not necessary for optimal activity at 85°C, but deletion of an additional 218 amino acids (to amino acid 324) resulted in an altered optimum temperature for enzyme activity of about 65°C. Deleting 379 amino acids (ApuN106/C379) from the C-terminal end had no effect on enzyme activity, while deleting 654 amino acids (ApuN106/C654) from the C-terminus produced an inactive enzyme.

The structural similiarity of mesophilic and thermophilic enzymes and their enzymatic activity can be shown by the appearance of linear Arrhenius plots. If the structural integrity of an enzyme changes to maintain optimal activity at an elevated temperature it would result in atypical Arrhenius behavior. Biphasic Arrhenius plots would be seen and have been reported for both mesophilic and thermophilic enzymes
(Hartog and Daniel 1992; Hensel *et al.*, 1987; Steigerwald 1990). The discontinuities reported for thermozymes may indicate a structural change in the enzyme from a highly rigid to a less rigid structure needed for optimal enzymatic activity (Wrba *et al.*, 1990).

Arrhenius plots for mesophilic and thermophilic enzymes are typically linear revealing the maintenance of the molecular architecture of the enzyme in the temperature range for optimal activity. Biphasic Arrhenius plots have been observed for some mesozymes and thermozymes due to a significant structural change to accomodate the change in environmental temperature. For example,

Glyceraldehyde-3-phosphate dehydrogenase from *Thermoproteus tenax* has a biphasic Arrhenius plot (Hensel *et al.*, 1987). Similar observations were also seen in our lab with the *T. ethanolicus* 39E 2° Adh when ethanol was used as the substrate (Burdette and Zeikus 1996).

The Arrhenius plot for the wild type amylopullulanase was linear, unlike the discontinuous plot observed for the ApuN324 deletion construct. The discontinuity is due to the presence of two different enzyme forms with different activation energies, probably due to a temperature dependent structural change. This observation is more of a destabilization event over a wide range of temperatures than a catastrophic falling apart of the enzyme. This is a unique conformational change which is temperature dependent. Work by Burdette and Zeikus (1996) with 2°Adh also showed that if the temperature for Arrhenius analysis was increased there was no discontinuity. The conformational change was not detectable due to the appearance of one enzyme form at the higher temperature.

The Km_{app} for pullulan was obtained over a wide range of substate concentrations at both 40°C and 60°C. The temperatures chosen for analysis span both sides of the transition present in the Arrhenius plot for ApuN324. The dependence of the rate of pullulan hydrolysis on the substrate concentration followed Michaelis-Menten kinetics. The deletion construct and wild type amylopullulanase both have similar affinity toward pullulan at both temperatures; with ApuN324 having a higher maximal velocity at 40°C and 60°C relative to the wild type enzyme. The optimum temperature for ApuN324 was about 65°C, versus 85°C for the wild type enzyme, and accounts for the higher maximal velocity observed at 60°C. The similiar Kmapp values for the mutant and wild type enzymes suggest that increased flexibility accounts for the

difference in optimum temperature. This observation supports the work by Jaenicke (1991) using deuterium exchange studies to examine protein flexibility.

We believe we have provided evidence supporting the hypothesis that protein flexibility controls the temperature optimum of enzymes. Previously, Jaenicke (1991) has provided evidence that the flexibility of mesophilic and thermophilic enzymes is equivalent at their respective temperatures for optimal activity. We show by nested deletions that we can lower the optimum temperature of a thermozyme by 20°C and not effect the catalytic site or thermostability.

To our knowledge, this report is the first to identify a region important for the temperature activity characteristics of an enzyme that is distinct for what controls catalysis or thermal stability. Flexibility measurements were limited by the inability to overexpress and purify the recombinant protein. Current attempts at overexpression of amylopullulanase resulted in cell toxicity and loss in the insert from the cloning vector. A yeast overexpression system is currently being pursued and, if successful, will allow us to overexpress the protein and obtain crystals for three-dimensional structure analysis. Further studies into flexibility measurements, the three dimensional structure, physical biochemical properties, and folding properties of enzymes will help elucidate the characteristics unique to thermozymes.

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Chapter IV

Summary and Directions for Future Research

Thermophilic micro-organisms can be classified either as moderate thermophiles with growth optima of 60-80°C or hyperthermophiles having growth optima above 80°C. Habitats suitable for growth of thermophiles are widespread. Enzymes studied from hyperthermophiles have a higher temperature optimum and increased thermostability over moderate thermophiles or mesophiles (growth optima of 25-45°C) (Vieille *et al.*, 1996). This is an important rationale for use of thermophilic enzymes in industrial starch processing applications that require high temperature operation. A number of extremely thermostable enzymes of potential industrial utility have been purified or their genes cloned from anaerobic thermophiles. A characteristic of a number of hyperthermophiles is the capability to utilize complex saccharides which are metabolized to meet carbon and energy requirements. Starch, which is used in many industrial processes (e.g. production of fructose fermentation syrup), is composed of glucose linked by both alpha-1,4 and alpha-1,6 linkages which can be hydrolyzed into sugar syrups by various solubilizing and debranching enzymes; known collectively as amylosaccharidases.

An interesting and novel enzyme, amylopullulanase, has been identified in many thermophiles (Hyun and Zeikus 1985; Ramesh *et al.*, 1994; Melasniemi 1988; Saha *et al.*, 1988; Coleman *et al.*, 1987; Antranikian 1990; Spreinat and Antranikian 1990). Amylopullulanase is a debranching and solubilizing enzyme capable of hydrolyzing both alpha-1,4 (alpha-amylase activity) and alpha-1,6 (pullulanase activity) linkages in starch and related sugars
(Hyun and Zeikus 1985; Ramesh *et al.*, 1994; Melasniemi 1988; Saha *et al.*, 1988; Coleman *et al.*, 1987; Antranikian 1990; Spreinat and Antranikian 1990). Cloning and purification of amylopullulanase (a maltotriose producing enzyme) and neopullulanase (a panose producing enzyme) from moderate thermophilic

organisms has revealed four highly conserved regions important for enzymatic activity (Takata *et al.*, 1992). Hydrolysis of alpha-1,4 and alpha-1,6 linkages are catalyzed by the same active site (Svensson 1991).

Identification of an acid stable amylopullulanase in a thermophile will have important biotechnological application because both elevated temperature and low pH facilitate their use in industrial starch processing. Currently, starch is solubilized and saccharified at neutral pH and high temperature due to limitations of current alpha-amylases and pullulanases. Amylopullulanase offers the capacity for a one-step process to produce new sugar syrups that can be used as feedstock for the production of high-value products for yeast, fungal, or bacterial fermentations. This dissertation describes experiments on the biochemical, biophysical, and molecular biological characterization of amylopullulanase from *Thermoanaerobacter ethanolicus* 39E with respect to substrate cleavage characteristics and thermophilicity.

The recombinant gene was expressed in *E. coli* and partially purified by cell lysis and heat treatment. Analysis of the deduced amino acid sequence of many amylosaccharidases in relation to amylopullulanase identified four highly conserved regions within these enzymes important for catalysis and substrate cleavage specificity. Genetic engineering techniques were used to construct altered *apu* gene products with different catalytic activities and substrate cleavage specificities. This suggests the possibility for protein-engineering of the substrate binding sites in amylopullulanase to alter the function of this dual activity enzyme to that of either alpha-amylase (alpha-1,4) or pullulanase (alpha-1,6).

A thermophilicity region (TPR) was also identified by deletion mutagenesis at the N-terminal end of the protein resulting in a shift of the optimum temperature for enzyme activity. Arrhenius plot for wild type

amylopullulanase was linear, however, the observed plot for the ApuN324 deletion was discontinuous due to a temperature dependent structural change necessary for optimal catalytic activity. Further experiments into the flexibility and rigidity of the protein (deuterium exchange, proteolysis analysis, and exposure to denaturants) may identify a correlation between the observed optimal temperature shift and enzyme flexibility.

More detailed analysis of cleavage specificity and protein flexibility will be aided by the ability to overexpress amylopullulanase. Currently, overexpression has been inhibited by cell toxicity and plasmid instability. A yeast overexpression system is currently being pursued and, if successful, may allow us to overexpress the protein and obtain crystals for three-dimensional structure analysis. This will allow us to confirm the catalytic activity and cleavage specificity results presented in Chapter 2 by model fitting starch, and other substrates, into the catalytic site of the enzyme. Detailed information into the binding mode between substrate and enzyme will be obtained, and aid in the production of a monospecific, thermophilic amylopullulanase for industrial starch degradation. Examination of protein flexibility characteristics of the ApuN324 nested deletion of amylopullulanase will also be possible; as well as pullulan binding to the active site of wild type amylopullulanase versus ApuN324.

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APPENDIX A

Construction and Characterization of *Thermoanaerobacter ethanolicus* 39E Amylopullulanase Proline Deficient Mutants

INTRODUCTION

Initial comparison of mesophilic and thermophilic enzymes has identified features unique to thermozymes. It has been observed that thermophilic enzymes are smaller, have less ordered structure, contain more hydrophobic interactions, and less beta-structure, among other features (Vieille *et al.*, 1996). However, it appears that the key to thermostability will be determined by comparing thermodynamic properties, amino acid composition and sequence of homologous mesophilic and thermophilic proteins; not by their molecular architecture (Sundaram 1986). Site directed mutagenesis has become a useful tool in the analysis of protein thermostabilization. Small changes in the stabilizing forces caused by only one or two amino acid changes can raise the relative stability of an enzyme by several degrees centigrade (Coolbear *et al.*, 1992). Other factors leading to increased thermostability include: increased protein rigidity at mesophilic temperatures, location of proline residues in the loop regions of thermophilic proteins, and interaction of the protein with its surroundings (Vihinen 1987).

Thermophilicity and thermostability of enzymes are closely related to flexibility and rigidity of the proteins' folded conformation. Proline residues may play an important role in increasing the rigidity of the enzyme, and it has been reported that proline residues, present at a high frequency in beta-turns and within loop regions and binding adjacent secondary structures, are responsible

for thermostability (Watanabe *et al.*, 1991; Suzuki *et al.*, 1987). The data presented here examines the effect of mutating the 5 individual *T. ethanolicus* amylopullulanase proline residues (Pro195, Pro210, Pro213, Pro240, Pro244) (Mathupala *et al.*, 1993) not present in the less thermoactive*Thermoanaerobacterium saccharolyticum* B6A-RI and *Clostridium thermosulfurogenes* EM1 amylopullulanase enzymes based on sequence alignments (Table 1). The thermophilicities and thermostabilities of the wild type and proline mutant enzymes are reported.

Table 1 Amino acid alignment of the TPR of several thermophilic amylopullulanases. The legend at the bottom of the figure identifies the organisms from which the sequences are represented. The five unique proline residues within TPR of 39E are denoted as P1-P5. Proline residues present in TPR and depicted in bold.

Table 1. Amino acid alignment of the TPR of several thermophilic amylopullulanases.

39E amino acid 148

B6A	IQSAIGAGKD	WDPGTSTAIM	IDDNFDNVYS	YTAHIPKGDY	QYKVTLGNTW
EM1	IQSAIGAGND	WKPETSTAIM	TDDNFDNVYS	YTAHVPKGDY	QYKVTLGNTW
EM101	IQPAIGAGDD	WKPETSTAIM	RDYKFNNVYE	YTANVPKGNY	EFKVTLGPSW
39E	IQSAIGAGDD	WKPETSTAIM	RDYKFNNVYE	YTANVPKRYY	EFKVTLGPSW
B6A	AENYGANGVQ	DGSNIOLSVA	DNADITFFYD	ANTHNIWTNY	SPTLTGLDNN
EM1	DENYGANGVK	DGSNIQINVT	DNADITFYYD	ANTHNIWTNY	SPILTGLDNN
EM101	DINYGLNGEQ	NGPNIPLNVA	YDTKITFYYD	SVSHNIWTDY	NPPLTGPDNN
39E	DINYGLNGEQ	NGPNIPLNVA	YDTKITFYYD	SVSHNIWTDY	NPPLTGPDNN
B6A	IYYDDLKHDT	HDPFFRNPFG	AIKVGQTVTL	RIQAKNHDLE	SARISYWDDI
EM1	IYYDDLKHDT	HDSFFRNPFG	AVKVDQTVTL	RIQAKNHDLE	SARISYWDDI
EM101	IYYDDLRHDT	HDPFFRSPFG	AIKTGDTVTL	RIQAKNHDLE	SAKISYWDDI
39E	IYYDDLKHDT	HDPFFRFAFG	AIKTGDTVTL	RIQAKNHDLE	SAKISYWDDI
B6A	NKTRTELPMT	RIGESPDGNY	EYWEIKLSFD	HPTRIWYYFI	LKDGTKTAYY
EM1	NKTRTELPMT	RIGESPDGNF	EYWEIKLSFD	HPTRIWYYFI	LKDGTKTAYY
EM101	KKTRIEVPMY	RIGQSPDGKY	EYWEVKLSFD	HPTRIWYYFI	LKDGTKTAYY
39E	KKTRTEVPMY	KIGQSPDGQY	EYWEVKLSFD	YPTRIWYYFI	LKDGTKTAYY

39E = *Thermoanaerobacter ethanolicus* 39E
B6A = *Thermoanaerobacterium saccharolyticum* B6A-RI
EM1 = *Clostridium thermosulfurogenes* EM1
EM101 = *Clostridium thermosulfurogenes* EM101

MATERIALS AND METHODS

Reagents, Enzymes, and oligonucleotides - All chemicals were of molecular biology or analytical grade and obtained from Aldrich Chemical Co., or Sigma. Restriction enzymes and ligase were obtained from Bethesda Research Laboratories, United States Biochemical Co., or Boehringer Manheim. Oligonucleotides were synthesized in an Applied Biosystems model 380A DNA synthesizer at the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University. The oligonucleotides were subsequently 5'-phosphorylated using T4 polynucleotide kinase, for use in site directed mutagenesis (Table 2).

Bacterial strains, plasmids, and transformations - *E. coli* strain SURE {e14-(*mcrA*) (*mcrCB-hsdSMR-mrr*)171 *endA*1 *supE*44 *thi*-1 *gyrA*96 *relA*1 *lac recB recJ sbcC umuC*:Tn5(kan^r)*uvrC* [F' *proAB lacI*^{q}Z M15Tn10(tet^r)]} from Strategene Co. and DH5α-F' {F' 80*dlac* (*lacZYA-argF*)U169 *deoR recA*1 *endA*1 *hsdR*17 ($r^{k-}m^{k+}$)*supE*44 I- *thi*-1 *gyrA*96 *relA*1}, from Bethesda Research Labs.) were used as a host for transformation, and *E. coli* BMH 71-18 *mut*S, JM109, JA221 and ES1301 *mut*S from Promega Corporation were used. *E. coli* strains were made competent by the Hannan method as described by Perbal (Perbal 1988), while recombinant vectors were introduced in *E. coli* strains by heat-shock treatment (Hannan 1983).

Enzyme assays - For determination of amylopullulanase activity, 160 μl of 1.25% (w/v) pullulan in 50 mM acetate buffer (pH 6.0) containing 5 mM $CaCl_2$ and enzyme (heat treated culture supernatant) to a total volume of 200 μl were mixed and incubated at 60°C for 30 min. The reaction was stopped by adding 0.8 ml of dinitrosalicylate solution (Miller 1959) and heated in an oil bath (100°C) for 15 min. The samples were cooled on ice and the absorbance at 640 nm was

Table 2. Oligonucleotides used in this study.

Mutation	Oligonucleotide synthesized
Pro195Asn (P1)	5'- CCCATGAGTTCCCTAAAAGTTACTTTAAACTC -3'
Pro210Ser (P2)	5'- CAAAGGAATATTTGAACCATTTTGTTCAC -3'
Pro213Gln (P3)	5'- CATAGGCTACATTCAATTGAATATTTGGAC -3'
Pro240Ile (P4)	5'- GGCCCTGTGAGTATTGGATTGTAATCTG -3'
Pro244Leu (P5)	5'- CATAATATATGTTATTATCAAGCCCTGTGAGAG -3'
Pro210Ser/ Pro213Gln (P2/P3)	5'- CTACATTCAATTGAATATTTGAACCATTTTGTTC -3'
Pro240Ile/ Pro244Leu (P4/P5)	5'- GTTATTATCAAGCCCTGTGAGTATTGGATTGTAATC -3'

Table 2. The alanine mutant oligonucleotides for P1, P2, P3, P4, and P5 were synthesized substituting the underlined residues with 5'-CGC-3'. underlined: altered nucleotides in the primer to produce the desired amino acid change.

measured. One unit of amylopullulanase activity was defined as the amount of enzyme which produced 1 μmol of reducing sugar (with glucose as the standard)/min under standard assay conditions.

Sequence analysis and DNA sequencing - The amino acid sequence deduced from the amylopullulanase DNA sequence was compared with the primary structures of less thermostable amylopullulanases, alpha-amylases and pullulanases available through GenBank (IntelliGenetics Inc., Mountain View, CA). GCG Sequence Analysis Software Package ver. 8.0 (1994) was used in the analysis and multiple sequence alignments and subsequent data manipulations. Hydrophobic cluster analysis (HCA) of the amino acid sequences was performed as described by L. Lemesle-Varloot *et al* (Lemesle *et al.*, 1990). Sequenase V.2.0 T7 DNA polymerase and Sequanase V.2.0 sequencing kit from United States `iochemicals were used for verifying the mutated gene fragment according to Sanger's dideoxy chain termination method (Sanger *et al.*, 1977). For double-stranded DNA sequencing, denaturation of double-stranded plasmid DNA was performed as described by Zhang *et al* (Zhang, *et al.*, 1988).

Site-directed mutant construction - Mutagenesis was done with an oligonucleotide-directed *in vitro* mutagenesis system (BIO-RAD, Richmond, CA). Chemically synthesized oligonucleotides (18-mer to 28-mer) were used and the sequences are shown in Table 1. The *Apa* I -*Bgl* II segment of the prAP164-UC was ligated into M13mp19 using *Xba* I and *Bam* HI restriction sites. (The *Xba* I site was end filled with Klenow producing a blunt end to complement the blunt end produced by *Apa* I, and the *Bam* HI overhang is compatible with *Bgl* II). Uracil-containing single strand DNA was synthesized and used as the template. An oligonucleotide complementary to the region to be altered, except for the mismatch, was hydridized to the single-strand uracil DNA. The complementary strand was then synthesized by T4 DNA polymerase using the oligo as primer.

Ligase was used to seal the new strand to the 5' end of the oligo. The double-stranded DNA containing the mutation of interest was transformed into *E. coli* TG-1. The mutations were confirmed by DNA sequencing, using the Sequenase Version 2.0 kit (U.S. Biochemical Corp., OH). The sequencing reaction was primed by internally annealing 17-mer synthetic oligonucleotides.

Thermal denaturation and temperature optimum - Aliquots of amylopullulanase and mutant constructs were incubated in 50 mM Na-acetate/ 5 mM $CaCl_2$ in a thermally controlled oil bath at 85°C for 0-90 min. The time course of inactivation was followed by withdrawing samples every 10 min. The samples were then cooled on ice. Substrate was added, and the samples processed under standard assay conditions. Optimum temperature analysis was done by incubating the enzyme reaction at various temperatures under standard assay conditions.

Protein determination and gel electrophoresis - Amylopullulanase concentration in partially purified fractions was determined by scanning densitometry of Coomassie Blue stained SDS-PAGE (Laemmli 1970) gels. BSA served as a standard.

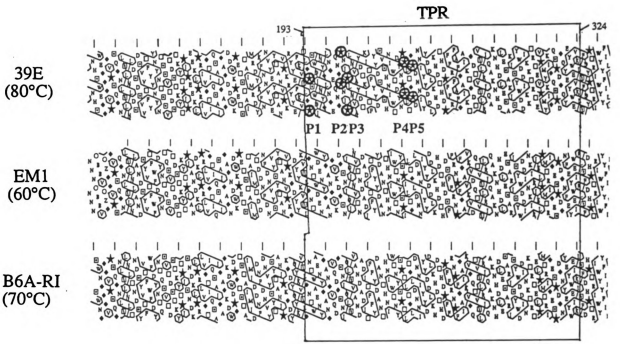
RESULTS

A thermophilicity region (TPR) was identified in *T. ethanolicus* and was compared by amino acid alignment and Hydrophobic Cluster Analysis (HCA) to amylopullulanases from *T. thermosulfurigenes* EM1 and *T. saccharolyticum* B6A-RI (Figure 1). In this region, there were five additional proline residues at positions 195, 210, 213, 240, and 244 (designated as P1, P2, P3, P4, and P5 respectively) in the *T. ethanolicus* enzyme (Table 1). The five proline residues were singly substituted with alanine, by site-directed mutagenesis. No change in optimum temperature or thermostability was detected (data not shown).

P1-P5 in *T. ethanolicus* were then substituted singly with the corresponding residues in the less thermophilic enzyme *T. thermosulfurigenes* EM1 (Asn, Ser, Gln, Ile, and Leu) (Figure 2). Similiar results were observed. Double mutants Pro210Ser/Pro213Gln and Pro240Ile/Pro244Leu were constructed and again, no change in optimum temperature or thermostability (Figure 3). The contribution of additional proline residues in the more thermophilic enzymes does not seem to give an individual positive effect on thermophilicity.

Figure 1 HCA comparison of thermophilic amylopullulanase at TPR. The legend at the bottom of the figure identifies the organisms represented in the HCA analysis.

Figure 1. HCA comparison of thermophilic amylopullulanases at TPR

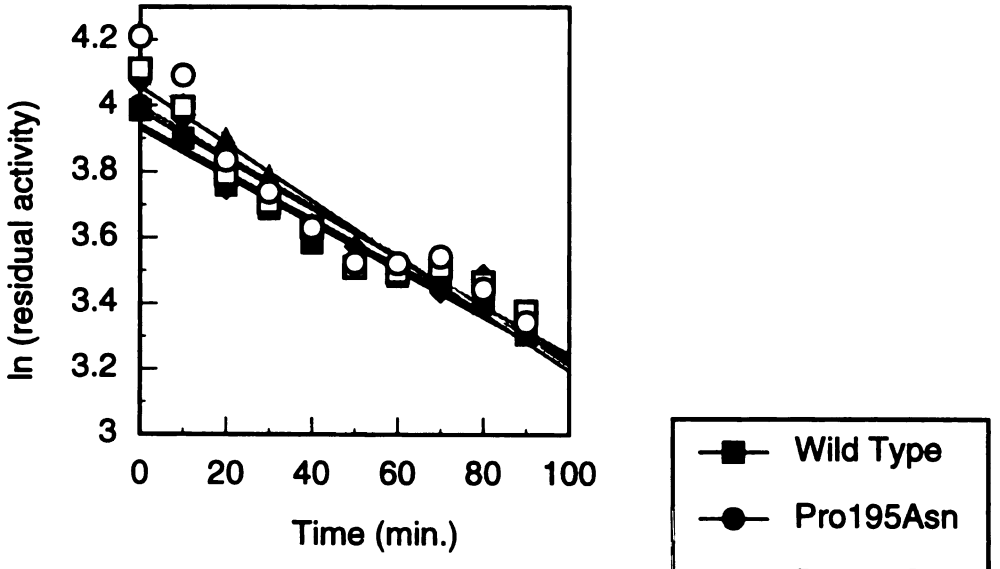


39E = *Thermoanaerobacter ethanolicus*39E
EM1 = *Clostridium thermosulfurogenes* EM1
B6A-RI= *Thermoanaerobacterium saccharolyticum* B6A-RI

Figure 2. Thermostability and optimum temperature analysis after single substitution of proline residues 1-5 from *T. ethanolicus* 39E with the corresponding residues of the less thermophilic enzyme from *T. thermosulfurigenes* EM1. Aliquots of amylopullulanase and the mutant constructs (similiar specific activities) were incubated in 50 mM Na-acetate/5 mM $CaCl_2$ in a thermally controlled oil bath at 85°C for 0-90 minutes. The time course of inactivation was followed by withdrawing samples every 10 minutes. The samples were then cooled on ice. Substrate was added, and the samples processed under standard assay conditions. Optimum temperature analysis was done in triplicate under standard assay conditions with pullulan as the substrate in 50 mM Na-Acetate buffer (pH 6.0) containing 5 mM $CaCl_2$ at temperatures ranging from 40-100°C.

Figure 2. Thermostability and optimum temperature analysis after single substitution of proline residues 1-5 from *T. ethanolicus* 39E with the corresponding residues of the less thermophilic enzyme from *T. thermosulfurigenes* EM1.

A. Thermostability Profiles



B. Optimum Temperature Profiles

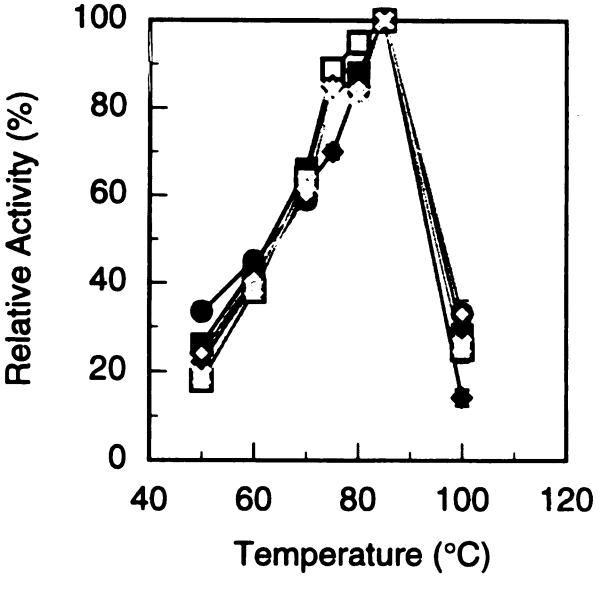
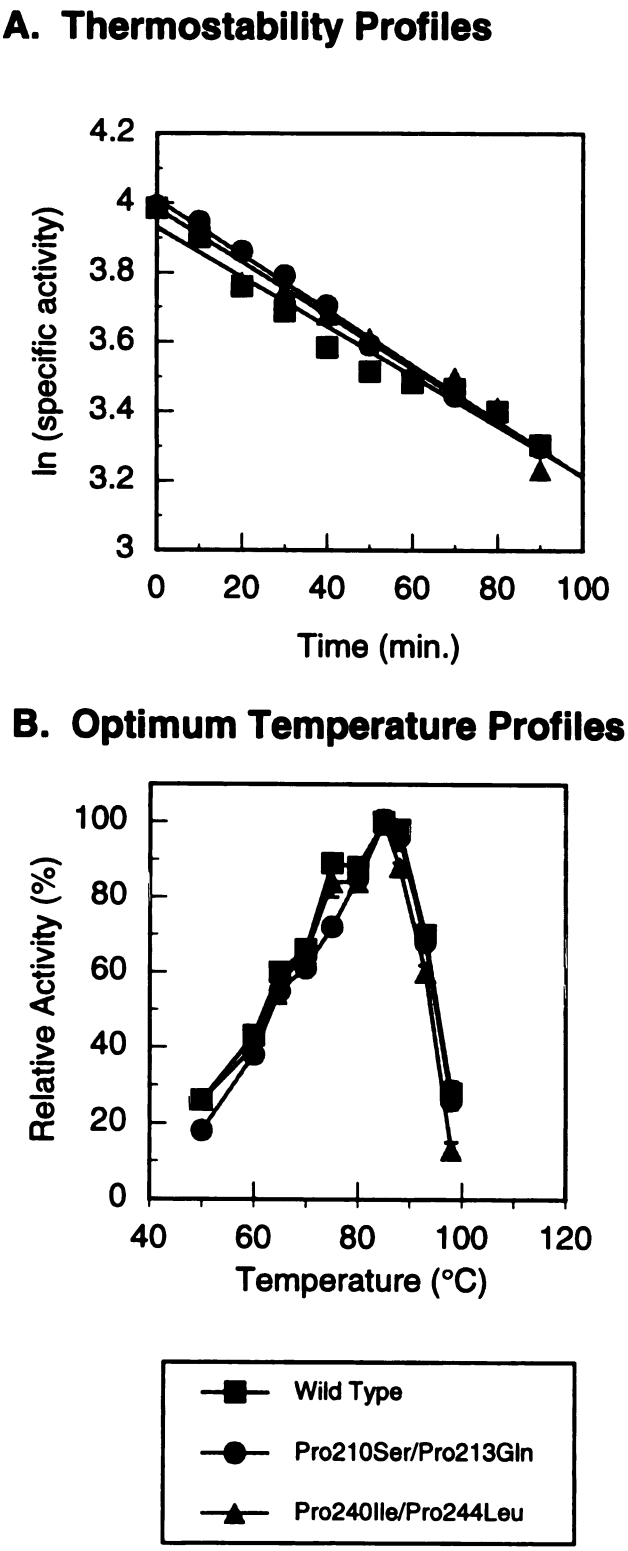


Figure 3. Thermostability and optimum temperature profiles of double proline mutants (Pro210Ser/Pro213Gln and Pro240Ile/Pro244Leu) relative to wild type amylopullulanase. Aliquots of amylopullulanase and the mutant constructs (similiar specific activities) were incubated in 50 mM Na-acetate/5 mM $CaCl_2$ in a thermally controlled oil bath at 85°C for 0-90 minutes. The time course of inactivation was followed by withdrawing samples every 10 minutes. The samples were then cooled on ice. Substrate was added, and the samples processed under standard assay conditions. Optimum temperature analysis was done in triplicate under standard assay conditions with pullulan as the substrate in 50 mM Na-Acetate buffer (pH 6.0) containing 5 mM $CaCl_2$ at temperatures ranging from 40-100°C.

Figure 3. Thermostability and optimum temperature profiles of double proline mutants (Pro210Ser/Pro213Gln and Pro240Ile/Pro244Leu) relative to wild type amylopullulanase.



DISCUSSION

Research in the area of the determinants of protein stability is still in an early stage. Site-directed mutants of T4 lysozyme in the form Xaa to Pro indicated no change in enzymatic activity or its three-dimensional structure relative to wild type (Matthews *et al.*, 1987). Specifically, when Ala82, present in the turn of an alpha-helix, was substituted with proline the stability of the T4 lysozyme was increased by 0.8 kcal/mol at 64.7°C (Matthews *et al.*, 1987). This initiated prospect that amino acid substitutions might be a general strategy for enhancing the stability of proteins.

An increase in proline content is seen in the more thermophilic enzymes (Table 3). The effect of proline residues on protein stabilization was studied by site-directed mutagenesis of the five proline residues (P1-P5) in the *T. ethanolicus* 39E amylopullulanase. Single substitutions to alanine, or the corresponding amino acid in the less thermophilic organism, *T. thermosulfurigenes* EM1, produced no significant change in activity, optimum temperature or thermostability. While none of the proline deficient mutants demonstrated a loss of thermostability relative to the wild type, a more detailed mutagenesis approach needs to be conducted. Multiple mutations up to all five proline residues should be done to confirm the lack of effect of prolines on protein stabilization.

Table 3 Proline profiles of amylopullulanase. Apu 39E is amylopullulanase from *Thermoanaerobacter ethanolicus* 39E; Apu B6A-RI is amylopullulanase from *Thermoanaerobacterium saccharolyticum* B6A-RI

Table 3. Proline profiles of amylopullulanase

Enzyme	Optimum Temperature	Proline Content
Apu 39E	90°C	5.24%
Apu B6A-RI	75°C	4.04%

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Appendix B

Attempts at Overexpression of Recombinant Amylopullulanase from *Thermoanaerobacter ethanolicus* 39E

General Procedure for Overexpression

Recombinant plasmids (pPROEX-1, pT7SC, and pIN-III-*ompA*) carrying the *apu* gene were transformed into *E. coli* strains TG-1, BL21, MC1061, HMS174(DE3)(pLysS), or JA221 for expression of the target protein. Cultures were grown at room temperature, 30°C, or 37°C in 20 x 10 ml cultures of LB + 100 μg/ml ampicillin, and 25 μg/ml chloramphenicol if a pLysS strain was being used. Cell growth was monitored by measuring the OD at 595 nm. When the OD_{595} was between 0.6 and 0.8, expression was induced. IPTG was added to a final concentration of 0.4 mM (unless otherwise indicated); expression of protein varied from 1-3 hours at the designated temperature. The cultures were chilled by placing the tubes in ice-water baths, and cells collected by centrifugation in an Sorvall GS-3 rotor at 5,000 rpm for 10 minutes at 4°C. The cleared supernatant was poured off and the cell pellet was resuspended in 5 ml of 50 mM Na-Acetate (pH 6.0)/5 mM NaCl and 0.3 mg/ml lysozyme. The resuspended cells were incubated on ice for 30 min, then frozen in liquid nitrogen. Lysed cells were thawed, centrifuged in a Sorval SS-34 rotor at 12,000 rpm for 30 min and the supernatant recovered. Protein was heat treated at 85°C for 15 min, chilled on ice, and the supernatant recovered by centrifugation in an SS-34 rotor at 8,000 rpm for 15 min. The protein was quantitated by SDS-PAGE. The activity of the protein was stable at 4°C for 2-3 months.

RESULTS

Amylopullulanase clone in pPROEX-1 vector (prAP164-EX).

This cloning vector (Polayes and Hughes 1994) is inducible by addition of IPTG and contains the 6xHis sequence allowing for ease of purification by Ni-NTA resin (Hochul *et al.*, 1987). *E. coli* host cells containing the verified recombinant plasmid were grown and induced under the various conditions listed below with the accompanying results.

1. Standard induction using *E. coli* TG-1 with 0.6 mM IPTG at 37°C removing aliquots at 1, 2 and 3 hrs post induction.
   Result: No overexpression and lower activity after induction indicating a stability or toxicity problem.
2. Introduced a Kan^r (Vieira and Messing 1982) cartridge in prAP164-EX to increase stability. Standard induction using *E. coli* TG-1 with 0.6 mM IPTG at 37°C removing aliquots at 1, 2 and 3 hrs post induction.
   Result: No overexpression and lower activity after induction.
3. Standard induction using strains void of some proteases (*E. coli* BL21 and *E. coli* MC1061) with 0.6 mM IPTG at 37°C removing aliquots at 1, 2 and 3 hrs post induction.
   Result: No overexpression and lower activity after induction. Analysis of the starter culture and subcultures after induction indicate that the activity is higher in the starter culture and decreases after subculturing.
4. Induction of starter culture (*E. coli* TG-1) with 0.6 mM IPTG at 37°C removing aliquots at 1, 2 and 3 hrs post induction.

Result: Activity is maintained but no overexpression.

5. Induction of starter culture (*E. coli* TG-1) titrating IPTG from 0.05 mM to 4.0 mM at 37°C removing aliquots at 1, 2 and 3 hrs post induction.

   Result: Activity is maintained but there is no overexpression. There is no relevant difference in activity at the different IPTG concentrations.

6. Induction of starter culture (*E. coli* TG-1) titrating IPTG from 0.05 mM to 4.0 mM at different temperatures (RT, 30°C, and 37°C) removing aliquots at 1, 2 and 3 hrs post induction.

   Result: No overexpression and no difference in activity at the different temperatures.

Amylopullulanase clone in pT7SC vector (prAP164-SC).

This vector (Brown and Campbell 1993) suppresses readthrough transcription from cryptic promoters and start points on the plasmid, in order to reduce expression in the absence of T7 RNA polymerase and improve use in the expression of highly toxic gene products. Protein expression was inducible by infection with lambda phage containaing the T7 RNA polymerase gene (lambdaCE6). Induction with IPTG and *E. coli* strain HMS174(DE3)pLysS was also used. In this strain, T7 lysozyme was provided by pLysS which down regulates activity of T7 RNA polymerase in the absence of expression.

E. coli host cells containing the verified recombinant plasmid were grown and induced under the various conditions listed below with the accompanying results.

1. Induction from starter culture with 0.4 mM IPTG in HMS174(DE3)pLysS [source of T7 RNA polymerase] at 37°C removing aliquots at 1, 2, and 3 hrs post induction.

Result: Active but no overexpression.

2. Induction from starter culture titrating IPTG from 0.05 mM to 4.0 mM in HMS174(DE3)pLysS [source of T7 RNA polymerase] at 37°C removing aliquots at 1, 2, and 3 hrs post induction.

   Result: Active but no overexpression. No difference in activity at the different IPTG concentrations.

3. Induction from starter culture with 0.4 mM IPTG in HMS174(DE3)pLysS [source of T7 RNA polymerase] at various temperatures (RT, 30°C, and 37°C) removing aliquots at 1, 2, and 3 hrs post induction.

   Result: Active but no overexpression. No difference in activity at the different temperatures.

4. Induction from starter culture using lambdaCE6 and *E. coli* TG-1 as host strain at 37°C removing aliquots at 0.5, 1, 2, and 3 hrs post induction.

   Result: Active but no overexpression.

5. Induction from starter culture with using lambda CE6 and *E. coli* host strains (MC1061 and BL21) at 37°C removing aliquots at 0.5, 1, 2, and 3 hrs post induction.

   Result: Active but no overexpression.

6. Induction from starter culture with using lambda CE6 and *E. coli* TG-1 as host strain at various temperatures (RT, 30°C, and 37°C) removing aliquots at 0.5, 1, 2, and 3 hrs post induction.

   Result: Active but no overexpression.

Amylopullulanase clone in pIN-III-*omp*A vector (prAP164-IN).

This cloning vector (Ghrayeb *et al.*, 1984)is inducible by addition of IPTG and results in the expressed protein being secreted into the periplasmic space

using the signal sequence of *ompA* (an *E. coli* outer membrane protein) in the cloning vector. *E. coli* host cells (JA221) containing the verified recombinant plasmid were grown and induced under the various conditions listed below with the accompanying results.

1. Induction at various temperatures (RT, 30°C, and 37°C with 0.05, 0.1, 0.05, 1.0, 2.0, and 4.0 mM IPTG removing samples at 1, 2, and 3 hrs post induction.

   Result: No activity and no overexpression.

2. Induction as indicated above using TB media instead of LB media.

   Result: No activity and no overexpression.

DISCUSSION

Our initial attempts using the pPROEX-1 vector were to optimize for purification following overexpression using the 6x His sequence added to the N-terminus of the protein. Lack of overexpression halted any subsequent attempts at purification. We then decided to try the pT7SC vector due to its ability to suppresss readthrough transcription from cryptic promoters on the plasmid and aid in the expression of highly toxic gene products. Overexpression was again not detected in this system. The last attempt at protein expression was done utilizing the pIN-III-*omp*A secretion vector. Upon induction of gene expression with IPTG, the gene product is secreted into the periplasmic space using the signal sequence of *omp*A in the cloning vector. The advantages of this system include: stability of the gene product from protease activity due to secretion into the periplasmic space; and maintenance of toxic enzymes due to secretion of the protein while being synthesized. The consistent result in the pIN-III-*omp*A vector was complete loss of activity. The validity of the construct was confirmed by DNA and protein analysis. The presence of the protein was verified by activity staining using native PAGE containing soluble starch (see experimental procedures Chapter 2).

Extensive examination of overexpression of amylopullulanase has been problematic in all systems tried in this report. Drs. Soroj Mathupala, Sue Lowe, and Jong-Hyun Park also tried overexpression of recombinant amylopullulanase with similiar results. Reasons for lack of overexpression may include, but are not limited to: 1) the large size of the protein (163,000 MW), 2) toxicity to cells, 3) protein degradation, and 4) presence of AGG and AGA Arg codons which may be problematic in the expression of large proteins in *E. coli*.. Personal

communication with other investigators attempting to overexpress and purify some thermozymes have encountered similiar problems. pET vector expression with overlapping *lac*O/P_{T7} may give better repression as a modified *E. coli* expression system. If overexpression is still an issue, a yeast secretion system may be useful in overcoming the toxicity problem inhibiting overexpression. The analysis of protein production by SDS-PAGE would be aided by the use of antibodies to amylopullulanase from the native organism. The current system, native starch PAGE, for analysis of protein abundance requires large amounts of protein for detection (1.0 ug) and is not quantitative.

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Appendix C

Transglycosylation Activity Analysis of Recombinant Mutant and Wild Type Amylopullulanase from *Thermoanaerobacter ethanolicus* 39E

No amylopullulanase has been studied for its transglycosylation activity. The presence of transglycosylation activity in other amylosaccharidases tempted us to study this enzymatic reaction with amylopullulanase. Transglycosylation activity is present and has been studied in *B. licheniformis* alpha-amylase (BLMA) and *B. stearothermophilus* neopullulanase. BLMA has alpha-1,6 transglycosylation activity (Kim *et al.*, 1992) while neopullulanase has both alpha-1,4 and alpha-1,6 transglycosylation activity (Kuriki *et al.*, 1993). Studies carried out with neopullulanase showed a single catalytic site (regions I-IV) is responsible for both hydrolysis and transfer activities. Results of experiments with amylopullulanase reveal an inability to catalyze alpha-1,4 or alpha-1,6 transfer activities.

General Procedure for Testing Transglycosylation Activity

Enzyme samples (0.05 Units) were incubated in the presence of 24.0% liquefied corn starch in 50 mM acetate buffer (pH 6.0) with 5 mM $CaCl_2$ at 60°C for 24 hours, and the products were analyzed by High Performance Ion Chromatography (HPIC) using a CarboPac PA1 column (Dionex BioLC4500i) and a pulsed amperomeric detector (PAD, Dionex). Buffer A (150 mM NaOH in water), buffer B (600 mM Na-acetate in buffer A), and buffer C (water) were used for elution. All the solvents were prepared with Milli-Q water, and filtered through a polyvinylidene difluoride membrane filter (0.22μm) (Gelman Sciences, Inc.). The samples were eluted at a flow rate of 1.0 ml/min with 0-40% solvent B

for 40 min. Twenty five microliters of 0.1% sample solution was injected into the column for analysis.

Results of Transferase Analysis

Transferase analysis was done in collaboration with Dr. Kwan Hwa Park (Seoul National University - Seoul, Korea) using partially purified extracts of *E. coli* expressing the mutant recombinant enzymes and wild type amylopullulanase. The time course reaction with wild type amylopullulanase resulted in hydrolysis of large oligosaccharides, accumulation of maltose, and the lack of increase in peak area of branched oligosaccharides, all evidence for lack of transglycosylation activity. No detectable transglycosylation activity was present in any of the active mutants constructed in this study.

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