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BIOCHEMICAL CHARACTERIZATION OF
AMYLOPULLULANASE FROM
CLOSTRIDIUM THERMOHYDROSULFURICUM 39E

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BIOCHEMICAL CHARACTERIZATION OF AMYLOPULLULANASE FROM CLOSTRIDIUM THERMOHYDROSULFURICUM 39E

By

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ABSTRACT

BIOCHEMICAL CHARACTERIZATION OF AMYLOPULLULANASE FROM CLOSTRIDIUM THERMOHYDROSULFURICUM 39E

By

Saroj Priyantha Mathupala

A novel pullulanase, which hydrolyzes both α-1,6 bonds in pullulan and α-1,4 bonds in amylose, was characterized from *Clostridium thermohydrosulfuricum* 39E, a thermophilic, anaerobic bacterium. Conditions were optimized for overproduction and secretion of the enzyme by using a maltose limited chemostat culture. The enzyme was purified by using affinity chromatography on an inhibitor-linked matrix, and the biochemical properties determined. Activity staining of PAGE gels, and inhibition kinetics using dual alternate substrates showed that both α-1,4 and α-1,6 glucosidic bond cleavage resided on the same enzyme. This amylopullulanase had higher affinity for pullulan than amylose. Both α-amylase and pullulanase activities were inhibited by β-cyclodextrin, a known inhibitor for both pullulanases and α-amylases. Amylose, glycogen, and amylopectin were hydrolyzed by the enzyme to maltose, maltotriose, and maltotetraose. ^{13}C NMR spectroscopy showed that the enzyme was capable of hydrolyzing both α-1,6 and α-1,4 bonds in glycogen. The gene encoding amylopullulanase was identified in a 6.1 kbp chromosomal DNA fragment and cloned into *Escherichia coli* and subcloned into *Bacillus subtilis*. The cloned enzyme was processed to the periplasmic space of *E. coli*, while in *B. subtilis* it was extracellular. The cloned enzyme from *E. coli* expressed both α-amylase and pullulanase activities and maintained thermostability and thermophilicity. The 4.4 kbp amylopullulanase (*apu*) gene was sequenced. Nested

deletion mutants and fusion proteins constructed from the gene allowed the identification of a 2.9 kbp segment in the middle of the coding region, that encoded a M_r 100,000 protein which maintained both α-amylase and pullulanase activities and thermostability, indicating that a single active site was probably involved in both α-1,6 and α-1,4 hydrolytic activities. Chemical modification of the enzyme with group specific reagents enabled the putative identification of either aspartate or glutamate, to be involved in catalysis. Site directed mutagenesis determined that Asp_{597}, Glu_{626}, and Asp_{703} were involved in catalysis, with each amino acid substitution resulting in loss of both α-1,4 and α-1,6 glucosidic bond cleavage.

Dedicated to my Parents

in gratitude

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Finally, I wish to thank my father, sister, and brothers for their encouragement and support "from across the globe," and I am specially grateful to my wife Lathika, for her support and encouragement during all these years of my "graduate-student life."

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ABBREVIATIONS

kbp	kilo base pair
DEPC	diethylpyrocarbonate
DEAC	1-(3-dimethylaminopropyl)-3-ethyl carbodiimide.HCl
M_r	molecular weight
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-*bis*-N,N,N',N'-tetraacetic acid
TLC	thin layer chromatography
GLC	gas-liquid chromatography
HPLC	high performance liquid chromatography
NMR	nuclear magnetic resonance spectroscopy
pCMB	p-chloro mercury benzoate
PEG	poly ethylene glycol
LB	Luria-Bertani medium
PAGE	polyacrylamide gel electrophoresis
CAP	catabolite gene activator protein
cAMP	cyclic AMP
OD	optical density
FPLC	fast protein liquid chromatography
SDS	sodium dodecyl sulfate

INTRODUCTION

Most natural products are biodegradable, due to the action of the enzymes inherent in microorganisms present in the environment. Microorganisms and their enzymes have been used by society due to their ability to alter the chemical nature and composition of food material, especially in fermentative processes. More recently, enzymes have been used in more chemically-specific processes such as chiral synthesis of natural products, for example in the production of optically pure pharmaceuticals. Most industrial processes using enzymes have relied on simple hydrolases, often carbohydrases or proteases, to hydrolyze macromolecules, since expensive and often unstable co-factors are not used in these enzymatic conversions. Enzymes are of value in manufacturing because of their rapid and efficient action at low concentrations and under milder conditions when compared to chemical mediated processes, and due to their high degree of substrate and product specificity and their low residual toxicity.

Thermophilic bacteria have tremendous potential in microbial and enzymatic technology because of their ability to function and maintain stability at higher temperatures, which enables the development of improved or new biotechnological processes. In addition, the biochemical properties of these enzymes are of great interest, since unique structural features might be expected to account for their high degree of thermostability in comparison to labile enzymes that function at mesophilic temperatures. Thermostable starch hydrolyzing enzymes play an important role in the starch processing industry. Several anaerobic bacteria have been investigated with respect to

their ability to produce extracellular saccharidases, which might be of applied importance.

Most the thermostable starch-processing enzymes that are available for industrial application, however, are α-amylases, glucoamylases, and pullulanases that are produced by aerobic bacteria and fungi. These enzymes catalyze the cleavage of α-1,4 or α-1,6 glucosidic linkages of starch.

We chose to study the pullulanase activity reported in *C. thermohydrosulfuricum* 39E, that cleaved both α-1,4 and α-1,6 glucosidic bonds, for two reasons. First, because of the potential insights that structural and functional biochemical analysis of the enzyme would give with regard to the mode of catalysis and nature of thermostability, and secondly because of it's potential use as both a solubilizing and debranching enzyme working at high temperatures during starch liquefaction or saccharification.

We chose to characterize further the dual pullulanase and α-amylase activities of this enzyme using kinetic analysis and molecular biological techniques. These studies enabled us to analyze this enzyme at the molecular level and to identify specific characteristics of the enzyme, including structural similarity to other amylases, factors responsible for structural intergrity, and the putative structure of the catalytic center.

The thesis is divided into six chapters: a literature review, a chapter on amylopullulanase production, purification and general biochemical characterization, a chapter characterizing the substrate kinetics and dual specificity of the enzyme, which has been published in *Biochemical and Biophysical Research Communications*, a chapter describing the amylopullulanase gene and the putative catalytic and thermal stability domain of the gene product, a chapter identifying the active site and mechanism of catalysis by site directed mutagenesis, a summary of the

research and future research directions, and an appendix that deals with preliminary characterization of cell surface microstructures, with respect to enzyme localization and cultural conditions.

Chapter I reviews the literature describing α-amylases, pullulanases, and of amylases harboring dual activities, with special emphasis on those enzymes isolated from microbes. The biochemical properties of the individual enzymes, cloning, sequencing, and expression of the genes encoding these enzymes, and structural information, where available, are also described in this chapter.

Chapter II, "Improved purification and biochemical characterization of extracellular amylopullulanase from *Clostridium thermohydrosulfuricum* 39E," describes optimization of the levels of enzyme production by *C. thermohydrosulfuricum* 39E, and release of the cell-bound enzyme into the culture medium, simplifying subsequent purification procedures. Purification of the enzyme from extracellular fractions of the culture, and the biochemical properties of the enzyme are described.

Chapter III, "Substrate competition and specificity at the active site of amylopullulanase from *Clostridium thermohydrosulfuricum* 39E," details an inhibition kinetic study with the purified enzyme using dual alternate substrates, which showed that both α-amylase and pullulanase activities reside on the same enzyme, and suggested the presence of a single active center. Product analyses are described that show the catalytic pattern of this enzyme on branched and unbranched starch related polysaccharides and on linear oligosaccharides, hydrolyzing both α-1,4 and α-1,6 linkages.

Chapter IV, "Cloning, identification of thermostability and catalytic regions and characterization of α-1,4 and α-1,6 bond specificity of amylopullulanase from *Clostridium thermohydrosulfuricum* 39E" describes

the cloning and subcloning strategies used to identify the gene encoding for amylopullulanase activity. By constructing nested deletion mutants of the amylopullulanase gene, gene products were isolated that possessed both α-1,4 and α-1,6 activities and thermostability, but were of a smaller molecular weight compared to the native enzyme. A structural study using NMR to characterize the ability of the cloned enzyme to debranch polysaccharides is described.

Chapter V, "Sequencing of the amylopullulanase (*apu*) gene of *Clostridium thermohydrosulfuricum* 39E, and identification of the active site by site directed mutagenesis" describes mutants constructed by creating nested deletions and single point base mutants, to identify the catalytic site of the enzyme. The experimental results provided supporting evidence of the dual specificity of amylopullulanase being due to a single enzyme with one active site, and identified within the gene, the regions that encoded for thermostability and catalysis.

The final chapter summarizes the findings of this study and the conclusions that can be made.

OBJECTIVES

This thesis research was undertaken to obtain a better understanding of the biochemistry and molecular biology of the novel pullulanase produced by *C. thermohydrosulfuricum* 39E. The enzyme is novel because it has high thermostability and can degrade both α-1,4 and α-1,6 glucosidic bonds, suggesting a new enzyme class.

To elucidate the biochemical features of the pullulanase from *C. thermohydrosulfuricum* 39E, including characterization of the enzyme and the gene encoding it, the following research objectives were undertaken.

(i) Enhancement/overexpression of this novel amylase in *C. thermohydrosulfuricum* 39E by altering the physiological growth conditions, to induce the release of the cell-bound enzyme into the growth medium.

(ii) Development of an effective purification scheme for the purification of pullulanase from *C. thermohydrosulfuricum* 39E.

(iii) Biochemical characterization of the purified enzyme from *C. thermohydrosulfuricum* 39E, in terms of activity against α-1,6 and α-1,4 linkages.

(iv) Determination of substrate specificity and product formation by the enzyme, and characterization of the active center by group specific chemical modification and inhibition kinetics, to obtain a preliminary understanding of the mode of action of the enzyme.

(v) Cloning, sequencing and expression of the gene encoding for amylase activity, into *Escherichia coli* and *Bacillus subtilis*.

(vi) Construction of fusion proteins, and deletion mutants of the gene, to obtain a better understanding of the various encoded regions of the primary sequence in enzyme function, and stability.

(vii) Site directed mutagenesis of the putative active site residues to identify the residues involved in catalysis, and to confirm the dual activity-single active site nature of the enzyme.

CHAPTER I.

LITERATURE REVIEW.

1. Starch composition

Starch is abundant in nature and is present at high concentrations in a variety of plants such as maize, potato, rice and wheat. This polysaccharide is made up of glucose molecules and composed of about 80% amylopectin and 20% amylose. The latter is a linear polysaccharide in which the glucose units are exclusively bound by α-1,4-glycosidic linkages. Amylopectin on the other hand, is a branched polysaccharide and contains α-1,6-glycosidic linkages in addition to the α-1,4 -glycosidic linkages. Every 20 to 25th glucose molecule in amylopectin is linked via an α-1,6-bond. A variety of yeast, fungi and bacteria are capable of degrading starch by the formation of extracellular enzymes (Fogarty and Kelly, 1979; Ingle and Erickson, 1978; Priest, 1977). Such enzymes include, α-amylase, β-amylase, glucoamylase, and α-glucosidase (Fig. 1, and Table 1).

2. Pullulan composition

Pullulan is a linear α-glucan produced by *Aureobasidium pullulans* (Bender and Wallenfels, 1961), and consists of maltotriose units linked by α-1,6 glucosidic bonds. Pullulan cannot be degraded by α-amylases or β-amylases. Isoamylase, which can hydrolyze the α-1,6 linkages in amylopectin, cannot hydrolyze pullulan (Yokobayashi *et al.*, 1970).

3. **α-Amylase** (1,4-α-D-glucan glucanohydrolase EC 3.2.1.1)

(i) Biochemical properties

α-Amylase, which catalyzes the hydrolysis of α-1,4-glucosidic linkages in starch and related polysaccharides, is an endoacting amylase that liberates

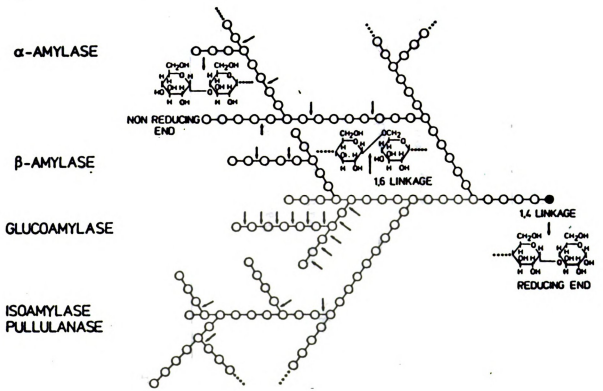


Figure 1. Schematic structure of amylopectin and the action pattern of some amylolytic enzymes.

The circles represent glucose units, and the arrows represent sites which the amylolytic enzymes can hydrolyze (Adapted from Vihinen and Mantsala, 1989).

Table 1. Substrate and bond specificity of various amylolytic enzymes*

Enzyme	Bond/s cleaved	Substrate	Products	Action	Metal ion requirement
α-amylase	α-1,4	amylose	maltose α-anomer	endo	Ca^{2+}
β-amylase	α–1,4	amylose	maltose β-anomer	exo	none
α-glucosidase	α–1,4	maltose maltooligosaccharides	glucose α-anomer	–	none
isoamylase	α–1,6	amylopectin	amylose	endo	none
pullulanase	α–1,6	pullulan amylopectin	maltotriose amylose	endo	Ca^{2+}
glucoamylase	α–1,4	maltooligosaccharides	glucose β-anomer	exo	none
cyclodextrinase	α–1,6 α–1,4	cyclodextrins	glucose maltose	–	none

* Adapted from Handbook of Amylases and Related Enzymes; The Amylase Research Society of Japan, 1988.

oligosaccharides of various chain lengths. The released end products have an α-configuration at the C1 position (reducing end) of the terminal glucose.

Some physical-chemical properties of α-amylases of bacterial and fungal origin are given in Table 2. Since starch cannot penetrate into cells, most of the enzymes are extracellular, being released into the culture medium, or associated with the outside of the cell. Membrane bound α-amylases are found in the fungus *Lipomyces starkeyi* (Moulin and Galzy, 1979) and in *Bacillus* sp. (Srivastava *et al.*, 1981).

pH optima of α-amylases ranges from 2.0 to 10.5, which reflects the ecological niche and optimal growth conditions of these organisms, which also varies from 3.0 to 10.5. Similarly, the temperature optima for activity of the α-amylases are reflected in the growth temperature of the microorganisms in their natural environment. The lowest temperature optima reported are 25°C to 30°C, while temperatures of 95°C to 100°C have been reported to be optimal for α-amylases from hyperthermophiles isolated from deep sea vents and fumaroles (Brown *et al.*, 1990; Schumann *et al.*, 1991). While temperature stability of most α-amylases is Ca^{2+} dependent, those from halophiles were dependent on NaCl for stability (Nachum and Bartholomew, 1969), due to the high salt environment these organisms occupy.

Molecular weights of α-amylases vary from 10,000 to 140,000 (Table 2), while most microbial α-amylases have a molecular weight between 50,000 to 60,000. Carbohydrate moieties increase the molecular weight of some α-amylases, and glycosylated α-amylases have been reported from *Aspergillus oryzae* (McKelvy and Lee, 1969), *Bacillus stearothermophilus* (Srivastava, 1984), and *Bacillus subtilis* (Matsuzaki, 1974) with the proportion of the carbohydrate in these enzymes being about 10% (w/w).

Table 2. General biochemical properties of α-amylase from bacteria and fungi

Organism	Strain	M_r	Optimum pH	Optimum Temp. (°C)	pI	Km (mg/ml)	Reference
Acinetobacter sp.	204-1	55,000	7.0	55	ND	ND	Onishi and Hikada, 1978
Aspergillus awamori	ATCC 22342	54,000	5.0	50	4.2	1.0	Bhella and Altosaar, 1985
Aspergillus oryzae	EI212	56,000	5.0	55	ND	3.35	Kundu and Das, 1970
Bacillus sp.	11-1S	54,000	2.0	70	ND	1.64	Uchino, 1982
Bacillus sp.	27-1	ND	10.5	50	ND	ND	Yamamoto *et al.*, 1972
Bacillus acidocaldarius	A-2	66,000	5.0	70	ND	1.6	Kanno,1986
Bacillus amyloliquefaciens	F	50,000	5.9	65	5.19	3.57	Borgia and Campbell, 1978
Bacillus caldolyticus	DSM405	10,000	5.4	70	ND	ND	Heinen and Heinen, 1972
Bacillus cereus	NY-14	55,000	6.0	55	6.13	ND	Yoshigi *et al.*,1985
Bacillus coagulens	109	62,000	6.2	50	5.0	1.5	Kitahata *et al.*, 1983

Table 2. General biochemical properties of α-amylases from bacteria and fungi (cont.).

Organism	Strain	M_r	Optimum pH	Optimum Temp. (°C)	pI	Km mg /ml	Reference
Bacillus licheniformis	NCIB 6346	62,600	8.0	90	ND	ND	Morgan and Priest,1981
Bacillus macerans	ATCC8514	140,000	6.3	ND	ND	1.33	DePinto and Campbell,1964
Bacillus stearothermophilus	Donk BS-1	44,000	5.5	70	4.4	0.77	Tsukagoshi *et al.*,1984
Bacillus subtilis	G63	25,000	6.5	50	ND	1.22	Takasaki, 1985
Fusarium oxysporum	Schl	ND	4.0	25	ND	ND	Chary and Reddy, 1985
Lactobacillus cellobiosus	D-39	22,500	7.3	50	ND	ND	Sen and Chakrabarty, 1984
Pyrococcus furiosus	--	ND	ND	>100	ND	ND	Brown *et al.*, 1990
Streptomyces aureofaciens	BM-K	40,000	5.0	40	ND	ND	Hostinova and Zelinka,1978
Thermotoga maritima	--	60,000	5.5	95	ND	ND	Schumann *et al.*, 1991

α-Amylases are metalloenzymes that contain at least one activating and stabilizing Ca^{2+} ion (Valee *et al.*, 1959). Heavy metal ions, sulfhydryl group reagents, and metal chelating reagents EDTA and EGTA inhibit α-amylases (Table 2). EDTA and EGTA are thought to destabilize α-amylase due to their chelating effect on the Ca^{2+} ions. The amount of bound Ca^{2+} varies from 1 to about 10 per mole of enzyme. Crystalline α-amylase from *A. oryzae* contains 10 bound Ca^{2+} ions, of which only one is tightly bound (Oikawa and Maeda, 1957). α-Amylase from *B. subtilis*, *B stearothermophilus*, or *B. amyloliquifaciens* contain four Ca^{2+} ions (Yutani, 1975) which are necessary for stability and conformation, although in other systems usually one Ca^{2+} is sufficient to stabilize the enzyme. Recent X-ray crystallographic studies have revealed that the essential Ca^{2+} is bound between the domains of α-amylase (Fig. 2) (Vihinen and Mantsala, 1990; Boel *et al.*, 1990).

Almost all α-amylases contain and require Ca^{2+}, and it is essential for the folding of the enzyme in *A. oryzae* (Matsuura *et al.*, 1984). It has been reported that Ca^{2+} inhibits the activity of α-amylases from *A. oryzae* strain EI 212 (Kundu and Das, 1970), though this effect may be due to excess calcium, which has been shown to bind within the catalytic cleft at concentrations above 5 mM, and inhibit the enzyme (Boel *et al.*, 1990).

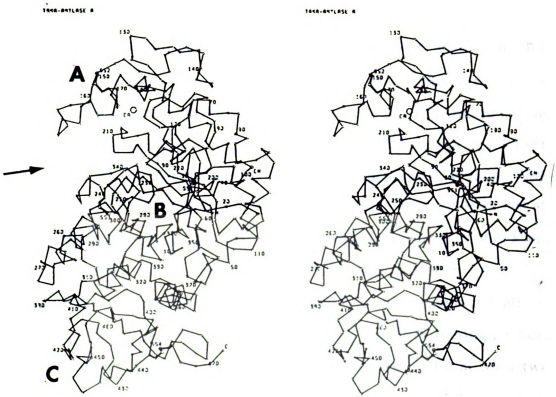
(ii) Catalytic mechanism and structure

Three-dimensional structures are available for α-amylase of *A. oryzae* (Taka-amylase A; TAA) (Matsuura *et al.*, 1984), and porcine pancreatic α-amylase (Buisson *et al.*, 1987). α-Amylases are multi-domain proteins belonging to a structural subfamily of those enzymes that contain an $(\alpha/\beta)_8$-barrel, described first in triose phosphate isomerase (Banner *et al.*, 1975).

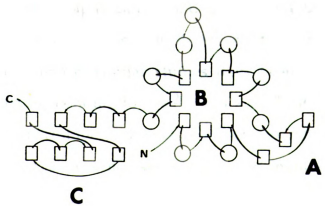
Fig. 2A **Stereo view of the three dimensional structure of α-carbon chain of α-amylase from *Aspergillus oryzae* (Adapted from Matsuura *et al.*, 1984).** Individual domains are denoted as A,B, and C. Active site and substrate binding cleft is indicated by arrow. A single Ca^{2+} atom is located between domains A and B.

Fig. 2B **Topological diagram of the secondary and super-secondary structures of α-amylase from *A. oryzae* (Adapted from Matsuura *et al.*, 1984).** Individual domains are denoted A,B, and C. The $(\alpha/\beta)_8$ barrel is denoted by domain B. The open circles represent α-helices while the open squares represent β-sheets.

2A



2B



Several amino acid side chains in the loops that connect the eight β-sheets to the succeeding α-helices constitute the extended substrate binding cleft (Matsuura *et al.*, 1984; Klein and Schulz, 1991; Farber and Petsko, 1990). A characteristic long loop at the C-terminus of the third β-sheet is grafted on to the $(\alpha/\beta)_8$-barrel (domain A) in α-amylases. This separate structural unit, termed domain B, participates in substrate binding, and is linked to domain A via an essential Ca^{2+} ion (Vihinen and Mantsala, 1990). Depending on the enzyme, domain B has 48 to 133 amino acid residues (Matsuura *et al.*, 1984; Buisson *et al.*, 1987; Klein and Schulz, 1991; MacGregor and Svensson, 1989; Svensson *et al.*, 1991). Domain C, succeeding the $(\alpha/\beta)_8$-barrel domain, has an immunoglobin type of fold (Matsuura *et al.*, 1984; Buisson *et al.*, 1987; Klein and Schulz, 1991), and has not been assigned to a specific function.

Based on X-ray crystal structures of *A. oryzae* and porcine pancreatic α-amylase, a subsite model has been proposed for substrate binding, where seven consecutive glucose units of the substrate are bound by at least two amino-acids on the substrate binding region of the enzyme (Matsuura *et al.*, 1984) (Fig.3). The essential Ca^{2+} is bound near the active center and appears to stabilize the two domains (A and B) forming the active site/substrate binding cleft. The catalytic mechanism of α-amylase has been modeled after the general acid-base catalytic mechanism proposed for lysozyme (Vernon, 1967). Using X-ray crystallography Glu^{230} and Asp^{297} have been proposed to be the catalytic residues in α-amylase from *A. oryzae* (Matsuura *et al.*, 1984). In porcine pancreatic α-amylase, similar studies have been used to propose Asp^{197} and Asp^{300} (corresponding to Asp^{206} and Asp^{297} of α-amylase from *A. oryzae*) as the catalytic residues, while Glu^{233} (corresponding to Glu^{230} of α-amylase from *A. oryzae*) was not suggested to be involved in catalysis (Buisson *et al*, 1987). In α-amylase from *A. oryzae*, the substrate is bound by

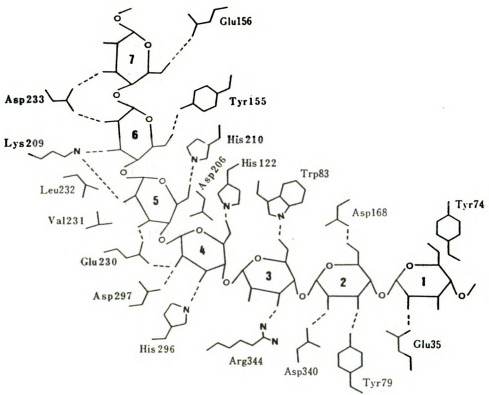


Fig. 3 Proposed substrate binding and catalytic site for α-amylase of *A. oryzae* (Adapted from Matsuura *et al.*, 1984).

Proposed catalytic residues; **Asp_{206}, Asp_{297} (Buisson *et al.*, 1987)**
Glu_{230}, Asp_{297} (Matsuura *et al.*, 1984)

the residues located on the walls of the active site cleft (Matsuura *et al.*, 1984). The residues predicted to be involved in either catalysis or substrate binding are highly conserved among the α-amylases from various mammalian, plant, fungal, and bacterial sources, according to amino acid sequence data analysis (Svensson, 1988).

(iii) Molecular biological studies

The structural genes encoding α-amylase have been cloned from various *Bacillus* spp. (Palva, 1982; Aiba *et al.*, 1983; Joyet *et al.*, 1984), *Aeromonas hydrophila* (Gobius and Pemberton, 1988), *Dictyoglomus thermophila* (Fukusumi *et al.*, 1988), *Streptomyces limosus* (Long *et al.*, 1987), and *Aspergillus oryzae* (Tada *et al.*, 1989). Two *B. stearothermophilus* strains have been found to harbor plasmid borne α-amylase genes (Mielenz, 1983). Host organisms most frequently used have been *Escherichia coli* and *Bacillus subtilis*, and to a much lesser extent, *B. brevis* (Tsukagoshi *et al.*, 1985), *B. stearothermophilus* (Aiba *et al.*, 1983), *Brevibacterium lactofermentum* (Smith *et al.*, 1986), *Pseudomonas aeruginosa* (Filloux *et al.*, 1985), *Saccharomyces cerevisiae* (Kunze *et al.*, 1988), and several *Streptomyces* strains (Thudt *et al.*, 1985). Sequences of the α-amylase genes have been determined from all the microbes mentioned above.

The sequenced α-amylase genes contain signal peptide coding regions preceded by sequences similar to the *E. coli* and *B. subtilis* consenses promoter sequences (Rosenberg and Court, 1979; McConnell *et al.*, 1986). For the sequenced α-amylases, the length of the signal peptide varies from 29 to 41 amino acids (Gray *et al.*, 1986; Yamane *et al.*, 1984). Signal peptides of cloned *B. stearothermophilus* α-amylase are processed at the same site in *E. coli* and the donor strain (Nakajima *et al.*, 1985; Ihara *et al.*, 1985).

	I	II
TAA	117 DVVANH 122	202 GLRIDTVKH 210
BStA	101 DVVFDH 106	230 GFRLDAVKH 238
BAA	98 DVVLNH 103	227 GFRIDAAKH 235
BLA	100 DVVINH 105	227 GFRLDAVKH 235
BME	109 DLVVNH 114	202 GFRLDAAKH 210
BSP	103 DVVMNH 108	233 GFRIDAVKH 241
BSU	107 DAVINH 112	181 GFRFDAAKH 190
SHY	88 DAVVNH 93	170 GFRIDAAKH 178

	III	IV
TAA	229 GEVLD 233	292 FVENHD 297
BStA	263 GEYWS 267	326 FVDNHD 332
BAA	260 AEYWQ 264	323 FVENHD 328
BLA	260 AEYWQ 264	323 FVDNHD 328
BME	246 GEVWD 250	308 FLTNHD 313
BSP	265 VEFWK 270	329 FVDNHD 334
BSU	217 GEILQ 221	274 WVESHD 279
SHY	199 QEVIY 203	257 FVDNWD 262

FIG. 4 **Conserved regions (I to IV) of some microbial amylases.**
TAA = ***Aspergillus oryzae***
BSta = ***Bacillus stearothermophilus***
BAA = ***B. amyloliquefaciens***
BLA = ***B. licheniformis***
BME = ***B. megaterium***
BSP = ***Bacillus* sp.**
BSU = ***B. subtilis***
SHY = ***Saccharomycopsys hygroscopicus***

α-Amylase sequences contain three (Rogers, 1985) or four (Nakajima *et al.*, 1986) conserved regions (Fig 4), and these regions have been proposed to be essential for the function of α-amylase because they are aligned and spaced at similar intervals in all the α-amylases studied so far. These regions form the active center, the substrate binding site, and the site for binding the stabilizing calcium ion, according to comparison with the refined 3-dimensional structure of α-amylases from *A. oryzae* (Matsuura *et al.*, 1984; Boel *et al.*, 1990) and porcine pancreas (Buisson *et al.*, 1987).

4. Pullulanase (α-dextrin 6-glucanohydrolase EC 3.2.1.41)

Pullulanase hydrolyzes α-1,6 linkages of pullulan and other branched oligosaccharides. The α-1,6 linkages are considered to mimic partially the α-1,6 branch points of amylopectin, and pullulan has been widely employed as a model substrate for starch debranching enzymes (Plant *et al.*, 1986). A specific pullulanase is an enzyme that can hydrolyze the α-1,6 linkages in pullulan, forming maltotriose as the sole reaction product. Pullulanase has been isolated from relatively few microorganisms (Table 3) compared to the number of α-amylases identified from various microorganisms. *Aerobacter aerogenes*, in which the enzyme was initially found (Bender and Wallenfels, 1961), is now classified as *Klebsiella aerogenes* (*Enterobacter aerogenes*). Pullulanase is also produced by mesophilic organisms such as *Bacillus acidopullulyticus*, *B. cereus* var. *mycoides*, *Streptomyces mitis*, and *Bacteroides thetaiotaomicron* (Table 3). Recently, thermostable pullulanase activities have been reported from several thermophilic microorganisms, including, *Clostridium thermosulfurogenes* (Burchhardt *et al.*, 1991), *Thermus aquaticus* YT-1(Plant *et al.*, 1986), *T. finii* (Koch *et al.*, 1987), *T. ethanolicus* (Koch *et al.*, 1987), *Thermobacteroides acetoethylicus* (Koch *et al.*,

Table 3. General biochemical properties of bacterial pullulanases.

Organism	Strain	M_r	Optimum pH	Optimum Temp.	pI	Km	Reference
Bacillus sp.	202-1	92,000	9.0	55	2.5	ND	Nakamura *et al.*,1975
Bacillus acidopullulyticus	--	100,000	5.0	60	5.0	0.37	Schulein *et al.*,1985
Bacillus cereus var. mycoides	--	110,000	6.5	50	ND	0.55	Takasaki, 1976
Bacillus subtilis	TU	450,000	7.0	60	ND	0.33	Takasaki,1987
Bacteroides thetaiotamicron	--	77,000	6.5	37	ND	ND	Smith and Salyers,1989
Clostridium thermosulfurogenes	--	130,000	7.0	70	ND	ND	*Buchardt et al.,1991*
Klebsiella pneumoniae	105	90,000	6.0	50	3.9	ND	Ohba and Ueda, 1975
Klebsiella pneumoniae	ATCC15050	143,000	5.0	47	ND	ND	Eisele *et al.*, 1972
Micrococcus sp.	--	120,000	10.0	50	ND	1.8	Kimura and Horikoshi, 1990
Streptomyces sp.	280	ND	5.5	50	ND	ND	Yagisawa,1971
Thermus aquaticus	AMD33	83,000	8.0	85	ND	6.3	Plant *et al.*,1986

1987), *Thermoactinomyces thalophilus* (Odibo *et al.*, 1988) and *B. stearothermophilus* (Kuriki *et al.*, 1988).

(i) Biochemical properties

pH optima of pullulanases vary from 4.9 to 8.5, while the temperature optima range from 30°C to 85°C. The biochemical properties of these pullulanases reflect the growth environment of the individual organisms from which the enzyme was isolated. Molecular weights reported are from 58,000 to 450,000. pCMB, heavy metal ions, α-,β-,and γ-cyclodextrins have been found to inhibit pullulanase activity.

(ii) Molecular biology studies

The gene responsible for pullulanase activity have been cloned from *K. aerogenes* (Takizawa and Murooka, 1985), *K. pneumoniae* (Michaelis *et al.*, 1985), *B. thetaiotamicron* (Smith and Salyers, 1989) and *B. stearothermophilus* (Kuriki *et al.*, 1988). Pullulanase from *K. aerogenes* and *K. pneumoniae* have been the most studied, and the nucleotide sequences of the genes are available. However, no X-ray crystallographic data with regard to the residues involved in catalysis or substrtae binding, or the catalytic mechanism, are yet available for any pullulanase.

Amylases with dual activity

Amylases that harbor dual activities are a recent addition to the repertoire of starch-hydrolyzing enzymes, and have yet to be categorized by the Enzyme Commission. Almost all the enzymes with dual activity reported so far are from anaerobic thermophiles. Initial reports of an enzyme containing dual amylase activities (α-amylase and pullulanase) were reported

from *B. subtilis* (Takasaki, 1987), where it was inferred that the two activities are due to two individual enzymes forming a complex dimer of 450,000 molecular weight.

A chromosomal DNA segment encoding both α-amylase and pullulanase activities was cloned from *T. brockii* into *E. coli* and *B. subtilis* (Coleman *et al.*, 1987), although no further studies were carried out to determine whether the DNA insert encoded a single protein imparting the dual activities.

An amylase-pullulanase enzyme of molecular weight 220,000, produced by *B. circulans* F-2, which can hydrolyze soluble starch (α-amylase activity) and pullulan (pullulanase activity) at equivalent rates, has been characterized using inhibition kinetics to show that the enzyme has two distinct and separate sites, each site having either α-amylase or pullulanase activity (Sata *et al.*, 1989). However, using the same approach, a similar enzyme was isolated from *Thermoanaerobium* Tok6-B1 and has been suggested to contain a single active site for both activities (Plant *et al.*, 1987).

An amylase with dual activity, denoted α-amylase-pullulanase, has been reported from *C. thermohydrosulfuricum* strain E101. This enzyme is reported to be a dimer with a subunit molecular weight of 190,000, containing a M_r 20,000 satellite protein that is necessary for structural and functional stability (Melasniemi, 1988). This differed markedly from the subunit structure and molecular weight of the amylase with dual activities reported from *C. thermohydrosulfuricum* 39E (Saha *et al.*, 1988; Mathupala *et al.*, 1990), where the enzyme is a monomer of mw 136,000. Based on sequence analysis, it has been suggested that the *C. thermohydrosulfuricum* E101 α-amylase-pululanase may contain two distinct sites, one for each activity (Melasniemi *et al.*, 1990), and that the enzyme is encoded such that the initial segment of

the gene encodes a pullulanase, while the subsequent half encodes the α-amylase activity.

Therefore, detailed biochemical characterization with regard to gene organization, subunit structure, catalytic and substrate binding sites, mechanism of action, is necessary to obtain a better understanding of these enzymes with dual activities.

Other enzymes that hydrolyze starch

β-Amylase (EC 3.2.1.2) attacks the α-1,4 glycosidic linkages in polysaccharides from the non-reducing ends and forms maltose as the major product. This enzyme does not process debranching activity, and therefore is not capable of hydrolyzing α-1,6-linkages in branched polysaccharides like amylopectin or glycogen. Glucoamylase (EC 3.2.1.3) attacks α-1,4 linkages in polymers from the non-reducing end forming one glucose at a time. This enzyme also has the capability of splitting the α-1,6-linkages, but at a slower rate (Saha and Zeikus, 1989). The enzymatic action of α-glucosidase (EC 3.2.1.20) is similar to glucoamylase, but it attacks preferentially α-1,4 linkages in short chain polysaccharides (Kelly and Fogarty, 1983).

Other enzymes that hydrolyze pullulan

Isoamylase is capable of hydrolyzing α-1,6 linkages in branched polysaccharides, but not in pullulan (Yokobayashi *et al.*, 1970). Isoamylases are usually secreted by fungi and are also classified as debranching enzymes.

Four different types of enzymes hydrolyze pullulan and produce different end products (Fig. 5). Many glucoamylases hydrolyze pullulan by liberating glucose units from the non-reducing end. Isopullulanase cleaves the first α-1,4 linkage following the α-1,6 bond, yielding isopanose (Sakano *et*

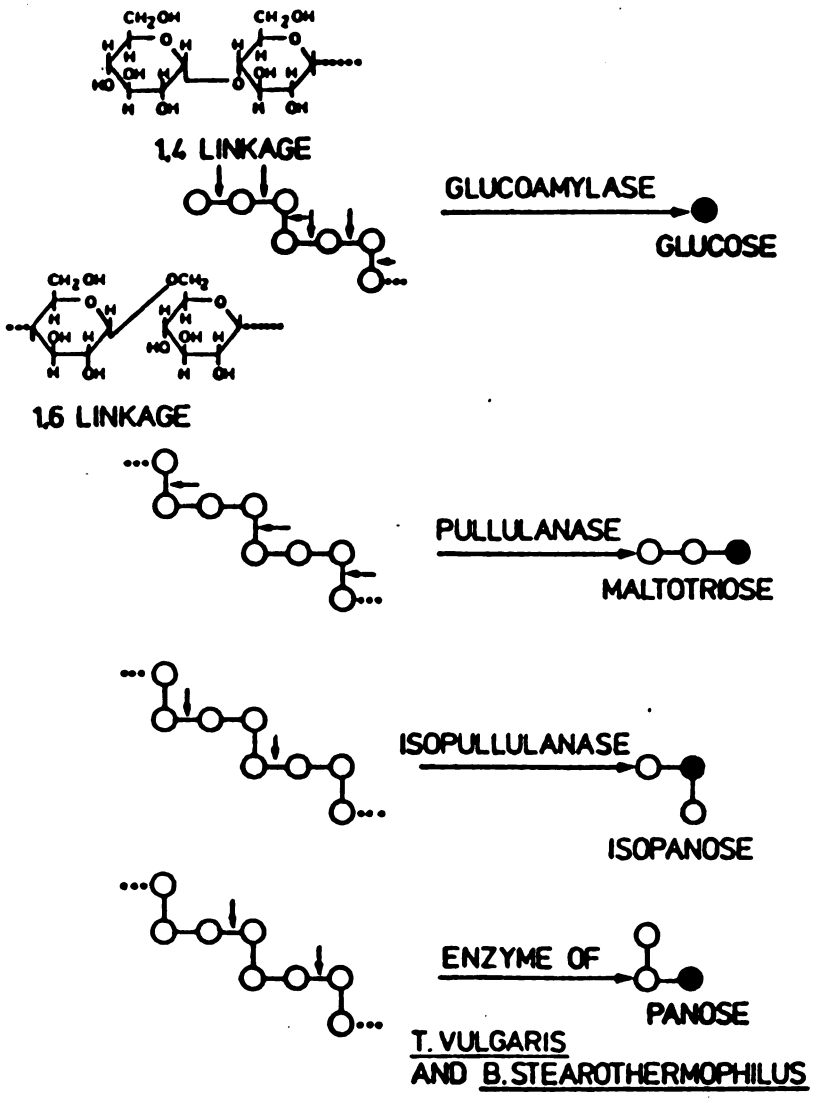


Fig. 5 Action pattern of pullulan degrading enzymes. The circles represent glucose units, the horizontal lines α-1,4 linkages, and the vertical lines α-1,6 linkages (Adapted from Vihinen and Mantsala, 1989).

al., 1971). Neopullulanase of *B. stearothermophilus* cleaves the α-1,4 glucosidic bond preceding the α-1,6 linkage liberating panose from pullulan, but cannot hydrolyze starch or any other polysaccharide efficiently (Kuriki *et al.*, 1988).

The formation of debranching enzymes besides α-amylase, β-amylase or glucoamylase enables microorganisms to convert branched complex polysaccharides completely and efficiently into small utilizable sugars. The ability to produce such enzymes is distributed among aerobic and anaerobic microorganisms. Compared to aerobes, little information, is available concerning the physiology and enzymology of starch hydrolyzing anaerobic, thermophilic, bacteria.

Thermostable enzymes

Thermophilic microorganisms, capable of growth at temperatures of 75°C and higher, were first isolated from hot springs at Yellowstone National Park (Brock, 1986). Thermostable as well as thermophilic enzymes have been isolated from many of these organisms, having temperature optima for stability and activity comparable to the physiological or natural growth temperature of the respective microorganisms from which they were isolated from, and in some cases surpassing the temperature optimum found in nature (Zeikus *et al.*, 1977; Lamed and Zeikus, 1980; Saha *et al.*, 1991; Zeikus *et al.*, 1991). Current evidence suggests that the upper limit of temperature for growth of thermophilic bacteria is about 110°C (Stetter, 1982; Stetter *et al.*, 1987; Costantino *et al.*, 1990; Brown *et al.*, 1990). Three enzymes from the hyperthermophilic archaebacterium *Pyrococcus furiosus*, have the highest temperature optima reported so far of 105° to 115°C, for an α-glucosidase (Costantino *et al.*, 1990), an α-amylase with an optimum greater than 108°C

(Brown *et al.*, 1990), and a serine protease with an optimum of 115°C (Eggen *et al.*, 1990).

Thermophilic enzymes are ideal for studying the structural basis of protein stability. Since the initial discovery of these thermophilic enzymes, many attempts have been made to identify and characterize any special molecular properties that could impart such stability at higher temperatures. These studies are important for basic research into protein structure and folding (Dill, 1985; Schellman, 1987), and because of the possibility of using these enzymes as practical catalysts under different experimental conditions (Torchillin and Martinek, 1979; Mozhaev and Martinek, 1984; Shami *et al.*, 1989). Biotechnological applications of mesophilic enzymes are often hindered by their intrinsic lability toward environmental factors such as heat, organic solvents, detergents, proteolytic enzymes, etc., which may be overcome by using thermostable enzymes due to their greater stability against these environmental parameters (Zeikus, 1979). However, detailed structural studies carried out on thermostable enzymes, in comparison with their mesophilic counterparts, has therefore failed to identify any unifying properties accounting for thermostability. Instead, current evidence shows that the intrinsic stability of thermophilic enzymes cannot be attributed to any common determinant, but is due to the overall contribution of a variety of stabilizing effects including hydrophobic interactions, ionic and hydrogen-bonding, disulfide bonds, metal ion binding etc., similar to those effects that have already been observed for mesophilic proteins (Mathews *et al.*, 1974).

Clostridium thermohydrosulfuricum 39E

C. thermohydrosulfuricum 39E (ATCC 33223) is an obligate spore forming anaerobe obtained from glucose and xylose enrichment cultures

from bacterial-algal mats associated with Octopus hot spring in Yellowstone National Park (Zeikus *et al.*, 1980). The optimum temperature of growth of this organism is 65°C. The organism is capable of catabolizing starch, hexoses and pentoses to form ethanol as the only significant fermentation product. Highly thermostable cell bound amylase activities have been reported from this organism (Hyun and Zeikus, 1985a), and enzyme synthesis was inducible and subject to catabolite repression (Hyun and Zeikus, 1985b). The enzyme responsible for pullulanase activity has been purified and initial characterizations carried out (Saha *et al.*, 1988; Mathupala *et al.*, 1990; this study). Recent studies on C. *thermohydrosulfuricum* 39E using DNA-DNA hybridization has showed that strain 39E has only 79% homology to C. *thermohydrosulfuricum* neotype (Lee *et al.*, manuscript submitted), while 97% homology is shown toward *Thermoanaerobacter ethanolicus*. Therefore, a new name *Thermoanaerobium ethanolicus* has been proposed for this organism (Lee *et al.*, manuscript submitted). In this thesis, the organism will be referred to as C. *thermohydrosulfuricum* 39E.

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CHAPTER 2

IMPROVED PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF EXTRACELLULAR AMYLOPULLULANASE FROM *Clostridium thermohydrosulfuricum* 39E

ABSTRACT

Maltose-limited chemostat culture was used to investigate the overexpression and excretion of amylopullulanase by *C. thermohydrosulfuricum* 39E. In maltose limited continuous culture, amylopullulanase was secreted and produced at 10 fold higher levels than in batch culture. The extracellular amylopullulanase was purified to homogeneity by using an inhibitor linked affinity column matrix. The purified amylopullulanase had a specific activity of 480 U/mg protein for pullulanase and 175 U/mg protein for α-amylase. β-Cyclodextrin inhibited both α-amylase and pullulanase activities, with a K_i of 0.065 mg/ml. Amylopullulanase had a M_r of 140,000 on SDS-PAGE analysis and a M_r of 133,000 using gel-filtration chromatography. The N-terminal sequence of the enzyme was Glu-Thr-Asp-Thr-Ala-Pro-Ala. The purified enzyme displayed K_m values of 0.35 mg/ml for pullulan and 1.00 mg/ml for amylose. The enzyme had a pI of 4.0, and displayed an optimum pH for stability and activity of 6.2 and 5.5 respectively. The enzyme was stable up to 85°C in the presence of Ca^{2+}, and had a half life of 40 min at 90°C (pH 6.2). Ca^{2+} was required for thermal stability, but not for activity. Amylose, glycogen, and amylopectin were degraded to maltose, maltotriose, and maltotetraose, whereas only maltotriose was formed from pullulan.

INTRODUCTION

α-Amylase (1,4-α-D-glucan glucanohydrolase EC 3.2.1.1) and pullulanase (α-dextrin 6-glucanohydrolase EC 3.2.1.41) are endo-amylases, which split glucosidic linkages in the interior of the starch molecule in a random fashion. α-Amylase hydrolyzes internal α-1,4 bonds, and can bypass α-1,6 linkages (or branch points) in the starch molecule. Pullulanase is known as a debranching enzyme, which hydrolyzes α-1,6 linkages (branch points) in starch molecules. Pullulanase but not α-amylase can degrade the α-1,6 linkages of pullulan (poly α-1,6 maltotriose), a polysaccharide composed of α-1,6 linked maltotriose units, into maltotriose (Bender and Wallenfels, 1961).

Both α-amylase and pullulanase are industrially important enzymes due to their hydrolytic activity on starch, and derivatives of starch, to produce conversion and glucose syrups (Allen and Dawson, 1975). For enzymatic processing of starch, temperatures of 95°C to 105°C are required for periods ranging from 5 min (105°C) to 2 h (95°C). The current technology uses two enzymes, in a two stage system, a liquefaction step in which starch granules are dispersed or gelatinized in an aqueous solution and then partially hydrolyzed by a thermostable fungal α-amylase, and a saccharification step in which liquefied starch is converted into glucose or low molecular weight saccharides by a pullulanase in the presence of either glucoamylase or β-amylase. Pullulanase is used in combination with saccharifying amylases such as glucoamylase, fungal α-amylase or β-amylase for the production of various sugar syrups because it improves saccharification and yield (Norman, 1982). In addition pullulanase has gained significant attention as a tool for structural studies of carbohydrates (Whelan, 1971).

Enzymes isolated from thermophilic bacteria, when compared to similar enzymes of mesophilic microorganisms, have higher heat stability, while usually possessing similar catalytic properties. Predictably, these thermostable enzymes show catalytic activity only at high temperature (50°C to 100°C), and are almost inactive at room temperature. The enhanced stability of these enzymes is reflected in their greater stability against other extremes, such as extremes of pH, solvent, salt concentrations, and in their resistance to the action of certain proteases and strong denaturants such as urea and guanidium hydrochloride (Brock, 1987).

Hyun and Zeikus (1985a) reported a highly thermostable and thermoactive pullulanase activity from *C. thermohydrosulfuricum* 39E. They also studied the regulation of the synthesis of the pullulanase in this organism (Hyun and Zeikus, 1985b), with the finding that enzyme synthesis was inducible and was subject to catabolite (glucose) repression. A cell bound pullulanase that cleaved starch and pullulan was purified and partially characterized from this organism (Saha *et al.*, 1988).

Several studies have been conducted to determine the importance of culture conditions on cellular localization of hydrolytic enzymes. In *Klebsiella aerogenes*, pullulanase activity was detected in the culture medium in batch culture with excess maltose, which acted as an inducer for the enzyme, while under substrate limited chemostat culture, the enzyme activity remained firmly cell-bound (Hope and Dean, 1974).

In Clostridia producing extracellular amylases, continuous culture has been used to overproduce and increase the yield of amylases in the culture medium (Antranikian *et al.*, 1987a; Antranikian *et al.*, 1987b; Madi *et al.*, 1987). In *Clostridium thermohydrosulfuricum* 39E, however, the amylolytic activities

remained mainly cell-associated during growth up to late exponential phase under batch culture conditions (Hyun and Zeikus, 1985a,b).

The purpose of this study was three fold: first, to maximize conditions for production and purification of amylopullulanase from *C. thermohydrosulfuricum* 39E, secondly, biochemical comparison of the extracellular pullulanase purified here, with the cell-bound pullulanase (Saha *et al.*, 1988); and thirdly, to provide more detailed biochemical characterization of amylopullulanase with respect to inhibitors, stability, amino acid composition and N-terminal sequence, carbohydrate content, substrate and product hydrolysis rates and yields, and amino acids involved in catalysis.

MATERIALS AND METHODS

Chemicals and gases

All chemicals used were reagent grade and obtained from either Sigma Chemical Company (St. Louis, MO) or Aldrich Chemical Company (Milwaukee, WI). Nitrogen was 99.9% pure and was made free of oxygen by passage over heated (370°C) copper filings.

Chromatographic gel material were obtained from Pharmacia Limited (Piscataway, NJ). The columns for standard chromatography were obtained from Bio-Rad (Melville, NY).

Organism and cultural conditions

C. thermohydrosulfuricum 39E (ATCC 33223) was used as the source of enzyme. The organism was grown at 60°C under anaerobic conditions on TYE medium (Zeikus *et al*, 1980), supplemented with 0.2% to 0.5% (w/v) soluble starch, maltose or glucose as the substrate. Batch culture studies were performed using pressure tubes containing 10 ml of medium, and samples were taken at mid to late exponential phase of growth.

Enzyme production and continuous culture studies

For continuous culture of *C. thermohydrosulfuricum* 39E, a 300 ml continuous culture vessel (Multigen, New Brunswick Scientific Co., Edison, NJ) equipped with pH, temperature, and media flow controllers and a stirrer was used, and the vessel was continuously gassed with nitrogen. Complex media modified from Antranikian *et al.*, (1987) contained 0.2% (w/v) maltose as the carbon source; and the following components in % (w/v) : Tryptone, 0.25; Yeast extract, 0.1; $MgCl_2.6H_2O$, 0.016; KH_2PO_4, 0.167; $Na_2HPO_4.7H_2O$, 0.167;

Cysteine.HCl, 0.05; $CaCl_2.2H_2O$, 0.0008. Vitamin solution (Zeikus *et al.*, 1980) and trace mineral solution (Zeikus *et al.*, 1980) were added at 1% (v/v). Cysteine.HCl, maltose, and vitamin solution were filter sterilized together and added to the autoclaved medium. The medium pH was varied from 5.5 to 7.5 using 2.0 M KOH, and the temperature was maintained at 60°C. The culture medium was monitored for extracellular α-amylase and pullulanase activity, by collecting the outflow into vessels maintained at 4°C under anaerobic conditons, and then assaying for enzyme activity.

For assay of enzyme activity, 1.0 ml of the cell culture was withdrawn from the chemostat and centrifuged at 14,000 rpm for 1 min in an Eppendorf microcentrifuge at room temperature. The supernatant was removed and adjusted to 1.0 ml by using 1.0 M sodium acetate (pH 6.0) containing 0.1 M $CaCl_2$ stock buffer solution to give a final concentration of 50 mM sodium acetate, 5 mM $CaCl_2$, pH 6.0, and assayed for extracellular enzyme activity. The cell pellet was resuspended in 100 µl of 50 mM sodium acetate buffer containing 5 mM $CaCl_2$ (pH 6.0), and assayed for cell bound enzyme activity.

Enzyme assays

For assay of pullulanase activity, 160 µl of 1.25% (w/v) pullulan in 50 mM acetate buffer, pH 6.0, containing 5 mM $CaCl_2$ and 40 µl of enzyme solution were mixed and incubated at 60°C for 30 min. The reaction was stopped by adding 0.8 ml of dinitro salicylate (DNS) solution (Miller, 1959), and heated in a boiling water bath for 15 min to develop the color reaction. The samples were cooled on ice and centrifuged in an Eppendorf microcentrifuge (14,000 rpm x 10 min). The supernatant was recovered and measured at 640 nm, for measurement of pullulanase activity. One unit of pullulanase activity is defined

as the amount of enzyme that produces 1 μmol of reducing sugar (with glucose as the standard) per min under the assay conditions.

For assay of α-amylase activity, 160 μl of 1.25% (w/v) soluble starch in 50 mM acetate buffer, pH 6.0, containing 5 mM $CaCl_2$ and 40 μl of enzyme solution were mixed and incubated at 60°C for 30 min. The reaction was stopped by adding 0.8 ml of DNS, and heated in a boiling water bath for 15 min to develop the color reaction. The samples were cooled on ice and centrifuged in an Eppendorf microcentrifuge (14,000 rpm x 10 min). The supernatant was recovered and measured at 640 nm, for measurement of α-amylase activity. One unit of α-amylase activity is defined as the amount of enzyme that produces 1 μmol of reducing sugar (with glucose as the standard) per min under the assay conditions.

For assay of glucogenic amylase activity, 160 μl of 1.25% (w/v) soluble starch in 50 mM acetate buffer, pH 6.0, containing 5 mM $CaCl_2$ and 40 μliters of enzyme solution were mixed and incubated at 60°C for 30 min. The sample was boiled for 10 minutes to destroy enzyme activity and centrifuged (14,000 rpm x 10 min) at 4°C. The supernatant was assayed for released glucose with a glucose analyzer (Model 27, Yellow Spring Instrument Co., Yellow Springs, OH), or by the hexokinase method (Sigma Glucose Diagnostic Kit 115-A). One unit of glucogenic amylase activity is defined as the amount of enzyme that produces 1 μmol of glucose per min under the assay conditions described.

Affinity column coupling

Sepharose CL-6B was activated with epoxy groups using bisepoxirane (Sundberg and Porath, 1974; Janson and Ryden, 1989), or using epichlorohydrin (Porath and Fornstedt, 1970; Janson and Ryden, 1989). Epoxy activation was carried out as follows; 20 grams of Sepharose CL-4B was washed with water and

suction dried. The moist gel was resuspended in 20 ml of 1,4-butanediol diglycidyl ether, and put under vacuum briefly to remove the air entrapped in the gel matrix. 20 ml of 0.6 M NaOH containing 2 mg/ml sodium borohydride was added to the gel suspension while shaking on a rotary water bath (200 rpm) and mixed for 8 h at room temperature. The reaction was stopped by washing the gel with 2000 ml of water by suction filtration.

20 g of epoxy-activated Sepharose CL-4B was washed with 125 ml of 0.1 M NaOH, and the moist gel transferred to a solution of 1.5 g of β-cyclodextrin in 60 ml of 0.1 M NaOH. Coupling of β-cyclodextrin proceeded for 16 h at 45°C in a rotary shaker at 200 rpm. After completion of the coupling, the gel was washed serially in 500 ml each of glucose (25 mg/ml), 0.1 M borate buffer (pH 8.0) containing 0.5 M NaCl, and 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl. Between each addition, the gel was washed with 500 ml of water. The final product was stored at 4°C in water.

Purification of the extracellular enzyme:

(i) Concentration and ion-exchange chromatography: 1000 ml of the supernatant from the continuous culture outflow was adjusted to 50 mM acetate and 5 mM $CaCl_2$, pH 6.0 and was concentrated to 10 ml by ultrafiltration using an Amicon YM-100 membrane (Amicon Co., Danvers, MA). The concentrate was passed through a Q-Sepharose FF column (Pharmacia) (2.5 x 50 cm) pre-equilibrated with 50 mM acetate buffer with 5 mM $CaCl_2$ (pH 6.0) at a flow rate of 1 ml min^{-1}. The column was then washed with 50 mM NaCl in the same buffer until no significant amount of protein eluted. The active enzyme was eluted with a NaCl gradient from 0.05 M to 0.45 M in the same buffer.

(ii) Affinity purification: The active fractions from the Q-Sepharose FF column were pooled and concentrated by ultrafiltration by using a YM 100 membrane

(Amicon). The concentrate was then applied to a β-cyclodextrin coupled Sepharose 4B affinity column (10 ml bed volume), pre-equilibrated with 50 mM acetate buffer with 5 mM $CaCl_2$ (pH 6.0). The column was washed with the same buffer and then washed with buffer containing 0.5 M NaCl. The enzyme was eluted with 1% (w/v) β-cyclodextrin at 4°C. The eluted peak, detected at 280 nm, was collected and washed extensively using ultrafiltration to remove β-cyclodextrin.

Characterization of the enzyme

Molecular weight and pI determination

Molecular weight was determined by gel-filtration chromatography on a Superose-12 column (in acetate buffer containing 50 mM NaCl) calibrated with known protein standards, and from SDS-polyacrylamide gel electrophoresis (7.5%) by the method of Laemmli (1970) using a Bio-Rad Mini-Protean II gel apparatus (Bio-Rad). For calibration of the gel filtration column, molecular weight standards (MW-GF-200; Sigma) containing blue dextran (M_r >2,000,000), β-amylase (M_r 200,000), alcohol dehydrogenase (M_r 150,000), bovine serum albumin (M_r 66,000), bovine carbonic anhydrase (M_r 29,000), and horse heart cytochrome C (M_r 12,400) were used. For SDS-PAGE electrophoresis, molecular weight standards (high range) (Bio-Rad) containing myosin (M_r 200,000), *E. coli* β-galactosidase (M_r 116,250), rabbit muscle phosphorylase b (M_r 97,400), bovine serum albumin (M_r 66,200), and hen egg white ovalbumin (M_r 42,699) were used. The isoelectric point was determined using a Servalyt Precote isoelectric focusing gel (pH 3-10) (Serva Biochemicals Co., Westbury, NY), with an LKB Multiphore II isoelectric focusing apparatus cooled to 10°C. Serva protein test mixture 9 was used for calibration, and the gel was stained with Serva Blue W.

Amino acid composition and N-terminal analysis

Purified enzyme was desalted by using double distilled water with a Centricon-30 (Amicon) ultrafiltration device. Amino acid composition was determined by reversed phase HPLC using the PICO-TAG method (Waters Div., Millipore Co., Milford, MA), after modification of the enzyme with iodo-acetic acid (for S-carboxymethylation) and with performic acid. N-terminal analysis of the enzyme was carried out with a Beckman Model 890M sequencer at the Macromolecular Facility, Department of Biochemistry, Michigan State University.

Active site titration and chemical modification

Initial velocity studies were performed within the pH range of 3.5 to 9.9 at 0.5 pH intervals, using glycine, acetate, MES, and Tris buffers. K_m^{app} and V_{max}^{app} were determined at each pH value. 1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide.HCl (DEAC) was used to modify aspartate and glutamate residues (Carraway and Koshland, 1972). Diethylpyrocarbonate (DEP) was used to modify histidine (Miles, 1977).

For asp or glu modification, ethanolamine or glycine methyl ester was used as the nucleophilic reagent, and the reaction was performed in 50 mM phosphate buffer, pH 6.0 at 25°C. DEAC concentrations of 0 to 100 mM in 25 mM intervals were used, in the presence of 200 mM nucleophile. The reactions were quenched in 1.0 M acetate buffer, pH 6.0 and he residual activity assayed.

Histidine residues were modified with DEP at 0 to 40 mM concentrations, at 10 mM intervals in 50 mM acetate buffer (pH 6.0) at 25°C. The reactions were monitored at 241.5 nm for 1 h, and quenched with 0.5 M imidazole buffer pH 6.0 before assay of residual activity.

Analysis of glycan component in amylopullulanase

Amylopullulanase was hydrolyzed with HCl and subsequently reduced and acetylated (Chaplin and Kennedy, 1986; Knapp, 1986). The acetylated hydrolysate was analyzed by capillary GC (Restec Rtx 2330; 90% biscyanopropyl-10% phenylcyanopropyl polysiloxane) in a temperature gradient of 140°C to 200°C (2°C/ min), with nitrogen used as carrier gas at 0.5 ml/min. Acetylated monosaccharide standards (alditol acetates) were used for calibration and identification of carbohydrate components.

HPLC analysis of product formation by action of enyme on polysaccharides

For determination of rate of product formation by enzyme action on polysaccharides, 1.0% (w/v) solutions of pullulan, soluble starch, amylose, amylopectin, mammalian glycogen, and oyster glycogen were incubated with affinity-purified enzyme (0.05 U/ml). 100 µl samples were withdrawn and boiled for 10 min to destroy the enzymatic activity, and centrifuged. The supernatant was analyzed by HPLC using an Aminex HPX-42A saccharide analysis column (Bio-Rad Laboratories) and a refractive index detector (Model 410 differential refractometer, Waters, Danvers, MA). Analysis was carried out at 85°C, using water as the eluant.

Thin Layer Chromatographic analysis of product formation of enzyme action on oligosaccharides

2 µl samples of the reaction mixture were applied to Whatman HP-K high performance silica gel plates (4.5 m particle size; 10 x 10 cm). The plates were developed with a n-butanol:ethanol:water (3:2:2 v/v) eluant mixture at room temperature. The resolved sugars were detected by using a 1:1 mixture of

0.2% (w/v) orcinol in methanol and 20% (v/v) sulfuric acid in methanol (1:1, v/v) and the plates heated at 100°C for color development.

Activity staining by native PAGE

The affinity-purified native enzyme was electrophoresed at a concentration of 1 μg/well 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 the polymerization of the gels, pullulan or soluble starch were added to a final concentration of 1% (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 pullulan embedded gels, the gel was processed as for determination of glycoprotein in SDS-PAGE gels (Pharmacia), and developed using Schiff's reagent. For activity staining of soluble starch embedded gels, a solution of 0.15% iodine:1.5% KI was added as an overlay. Pullulanase activity could be detected as a clear band against the dark red background of the pullulan embedded gel, while α-amylase activity could be detected as a clear band against the dark blue background.

RESULTS

Cellular location and production of amylopullulanase

In order to determine the effect of culture conditions on the cellular location and level of amylopullulanase produced, chemostat cultures of *C. thermohydrosulfuricum* 39E were compared with batch cultures of the organism with the aim of producing higher levels of predominantly extracellular activity that would simplify purification of the enzyme.

For comparison of enzyme activity and localization under different growth conditions, cell bound and extracellular pullulanase and α-amylase activities were analyzed during growth on maltose and soluble starch (Table 1). Under batch culture conditions low levels of amylase and pullulanase were produced during growth of *C. thermohydrosulfuricum* 39E on starch, while higher levels were detected during growth on maltose. With maltose, higher levels of both α-amylase and pullulanase activities occured in late exponential phase, with activities being predominantly extracellular. Under maltose limited continuous culture conditions (0.2% w/v), a 12 fold increase in extracellular amylopullulanase activity occurred when compared to cells grown under batch culture conditions (0.2% w/v maltose).

Purification and comparison of excreted versus cell bound amylopullulanase

The scheme used for the purification of extracellular amylopullulanase is given in Table 2. The purification of the cell-free enzyme from the culture supernatant was simplified due to the higher specific activity of the starting crude extract containing the pullulanase activity, and due to the approximately 12 fold higher yield of enzyme in the culture medium under maltose limited chemostat culture. The final affinity chromatographic step resulted in purified

Table 1. Effect of cultural conditions on α-amylase and pullulanase activities of *C. thermohydrosulfuricum* 39E.

Cultural condition	Substrate	Concentration (w/v)	Cell Density (OD 660)	Pullulanase activity * (U/ml)		α-amylase activity* (U/ml)	
				extracellular	cell bound	extracellular	cell bound
batch culture	maltose	0.2%	0.36#	0.286	0.011	0.255	0.011
batch culture	maltose	0.2%	0.49@	0.150	0.056	0.160	0.037
batch culture	maltose	0.5%	0.50#	0.100	0.014	0.160	0.012
batch culture	maltose	0.5%	0.95@	0.48	0.14	0.26	0.080
continuous culture	maltose	0.2%	0.47	1.87	0.11	0.68	0.10
batch culture	soluble starch	0.5%	1.30@	0.14	0.11	0.07	0.05

assayed at mid exponential phase of growth (~ 8 hours)

@ assayed at late exponential phase of growth (~ 24 hours)

* relative enzyme activity when cell density is adjusted to OD 660 = 1.0

Table 2. Purification scheme of extracellular amylopullulanase from *C. thermohydrosulfuricum* 39E

Purification step	Total activity (Units)	Total protein (mg)	Specific activity[a] (U/mg)	Recovery (%)	Purification (fold)
Supernatant	500	2500	0.2	100	1
Q Sepharose	200	134	1.5	40	7.5
β-cyclodextrin Sepharose	144	0.3	480	29	2400

[a] Pullulanase activity was monitored during the purification

enzyme with a specific activity of 480 U/mg for pullulanase activity, and 175 U/mg for α-amylase activity, using soluble starch as the substrate. The purified excreted enzyme gave a M_r of 140,000 on SDS-PAGE gels, and had a pI of 4.0 on Serva Précote isoelectric focusing gels (pH 3-10) (Fig. 1). Native gel electrophoresis on soluble starch or pullulan embedded gels showed that the same protein band contained both α-amylase and pullulanase activities. The molecular weight, temperature and pH optima for activity for the excreted amylopullulanase were identical with that reported for cell bound enzyme (Saha *et al.*, 1988).

Amino acid composition

The amino acid composition of the purified enzyme is given in Table 3. Analysis of amino acid composition showed that the enzyme is composed of 30% hydrophobic amino acids, while the remainder consists of polar and acidic amino acids, and 10% of basic amino acid residues. Comparison of the N-terminal sequence of the initial seven amino acid residues in thermostable and mesophilic pullulanses and α-amylases of microbial origin are given in Fig. 2. There were marked differences in the amino acid sequences of the N-terminal end of these enzymes, with α-amylase and glucoamylase from *Aspergillus oryzae* being the most similar.

Carbohydrate analysis

GLC analysis showed mannose and N-acetyl galactosamine as the major carbohydrate components in amylopullulanase (Fig. 3). Glucose, rhamnose and fucose were present in minor amounts. Based on GLC analysis, amylopullulanase had a carbohydrate content of 9-10% (w/w). Recently, galactose, rhamnose, mannose and glucose have been found to be the

Fig. 1A. SDS-PAGE (7.5% cross-linked) of affinity purified amylopullulanase.
lane 1 = high molecular weight standards
lane 2 = purified amylopullulanase

Fig. 1B. Native-PAGE (7.5% cross-linked) of affinity purified amylopullulanase.
lane 1,3 = Purified amylopullulanase after Coomassie Blue staining
lane 2 = Staining for α-amylase activity, after overlay of starch embedded gels with iodine.
lane 4 = Staining for pullulanase activity, after overlay of pullulan embedded gels with Schiff's Reagent.

Fig. 1C. Isoelectric focusing gel electrophoresis of purified amylopullulanase (pH range 3-10).
lane 1 = Purified amylopullulanase
lane 2 = Isoelectric focusing protein standards (Protein Test Mix 9 ; Serva).

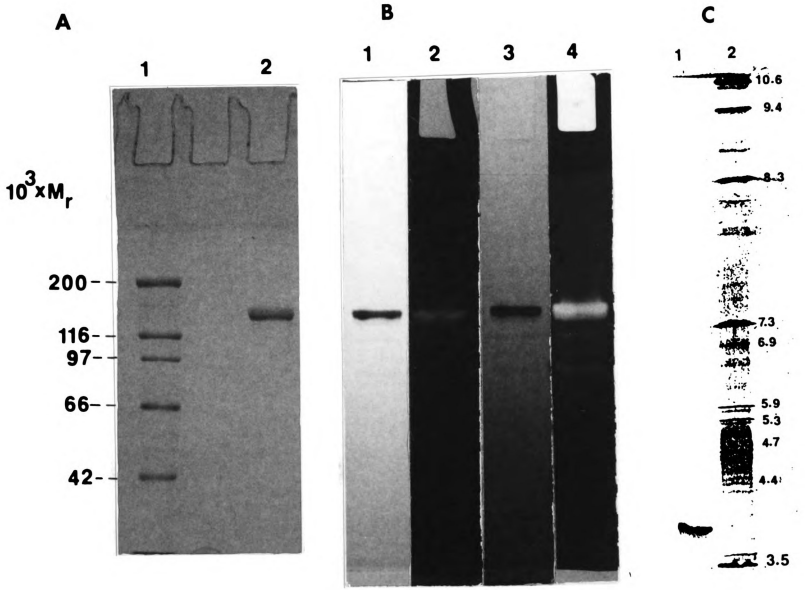

A
1
2
$10^3 \times M_r$
200
116
97
66
42
B
1
2
3
4
C
1
2
10.6
9.4
8.3
7.3
6.9
5.9
5.3
4.7
4.4
3.5

Table 3. **Amino acid composition of amylopullulanase from *C. thermohydrosulfuricum* 39E.**

Amino Acid	Molar Ratio (%)
Asp, Asn	15.1
Glu, Gln	10.3
Ser	8.6
Gly	10.7
His	1.4
Arg	3.6
Thr	7.7
Ala	7.2
Pro	5.9
Tyr	4.5
Val	7.5
Met	1.1
Ile	0.9
Leu	5.8
Phe	3.6
Lys	5.8
Trp	not determined
Cys	not detected
Total	100

39E	Glu - Thr - Asp - Thr - Ala - Pro - Ala
Kaepula	Asn - Lys - His - Ile - Arg - Asp - Tyr
Kpnpula	Arg - Val - Tyr - Asn - Thr - Ser - Tyr
TAA	Ala - Thr - Pro - Ala - Asp - Trp - Leu
Asagai	Ala - Thr - Leu - Asp - Ser - Trp - Leu
Angglu	Met - Ser - Phe - Arg - Ser - Leu - Leu

Figure 2. **Comparison of N-terminal sequences of various amylases of microbial origin.** Abbreviations are: 39E, *Clostridium thermohydrosulfuricum* 39E amylopullulanase; Kaepula, *Klebsiella aerogenes* pullulanase; Kpnpula, *Klebsiella pneumoniae* pullulanase; TAA, *Aspergillus oryzae* α-amylase; Asagai, *A.spergillus oryzae* glucoamylase; Angglu, *Aspergillus niger* glucoamylase

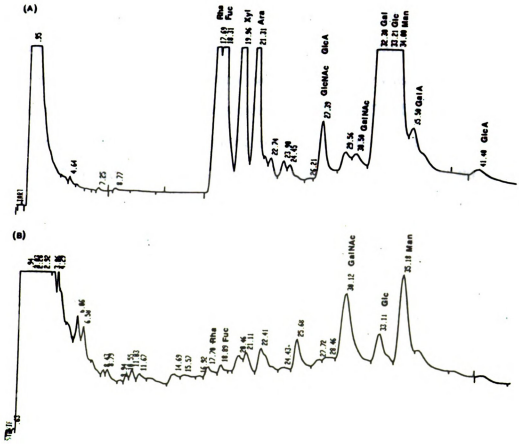


Fig. 3. GLC Analysis of carbohydrate composition of amylop**ullulanase.**

A = Monosaccharide standards

B = Monosaccharides released after acid hydrolysis of amylopullulanase

Rha = Rhamnose; Fuc = Fucose; Xyl = Xylulose; Ara = Arabinose; GlcA = Glucosamine; GlcNAc = N-acetyl glucosamine; GalNAc = N-acetyl galactosamine; Gal = Galactose; Glc = Glucose; Man = Mannose; GalA = Galactosamine; GlcA = Glucosamine.

components of glycoproteins isolated from the outermost cell surface layer (S-layer) of C. *thermohydrosulfuricum* (Messner *et al.*, 1992).

Enzyme stability

The Ca^{2+} requirement for high thermal stability of amylopullulanase is shown in Fig. 4. The purified pullulanase was thermostable up to 90°C, with a half life of 40 min at that temperature. No loss of activity was detected at 85°C for a period up to 60 min in the presence of 5 mM $CaCl_2$. However, when the enzyme was initially incubated at 60°C for 10 min with 25 mM EGTA or 25 mM EDTA (pH 7.0) and then extensively dialyzed in the presence of the chelating agent, the resultant enzyme solution rapidly lost activity at 85°C, with a half life of <5 min, indicating the importance of Ca^{2+} in maintaining thermostability of the enzyme. At 25°C, the enzyme was stable at pH 3.5 to 9.5 in the presence of 5 mM Ca^{2+}. However, at 60°C, over the same period of time, the range narrowed to pH 3.5 to 7.0.

Inhibition and substrate-product specificity

In order to identify any similarity between the catalytic site of C. *thermohydrosulfuricum* 39E amylopullulanase and other characterized α-amylases and pullulanases, the effect of β-cyclodextrin on activity was determined. Kinetic analysis showed the ability of β-cyclodextrin to inhibit both pullulanase and α-amylase activities completely, with a K_i of 0.065 mg/ml (Fig. 5). This showed the similarity of the active site of this enzyme to the active site of pullulanases and of α-amylases, which are inhibited by β-cyclodextrin.

In order to determine the activity of the enzyme on various polysaccharides, product formation on linear chain polysaccharides (i.e., amylose and pullulan) was compared to glycogen (Fig. 6). For glycogen, there

Figure 4. **Effect of temperature and Ca^{2+} on stability of *C. thermohydrosulfuricum* 39E amylopullulanase in the absence of starch.** Determination of enzyme stability in the absence of metal ions, was achieved by incubating samples of the enzyme in the presence of 25 mM EGTA or EDTA (pH 7.0) at 60°C, and desalted by ultrafiltration initially with the chelating agent, and finally with distilled water. The desalted enzyme (at a concentration of 0.05 mg/ml), was used to determine the stability of the enzyme in the presence and absence of Ca^{2+}.

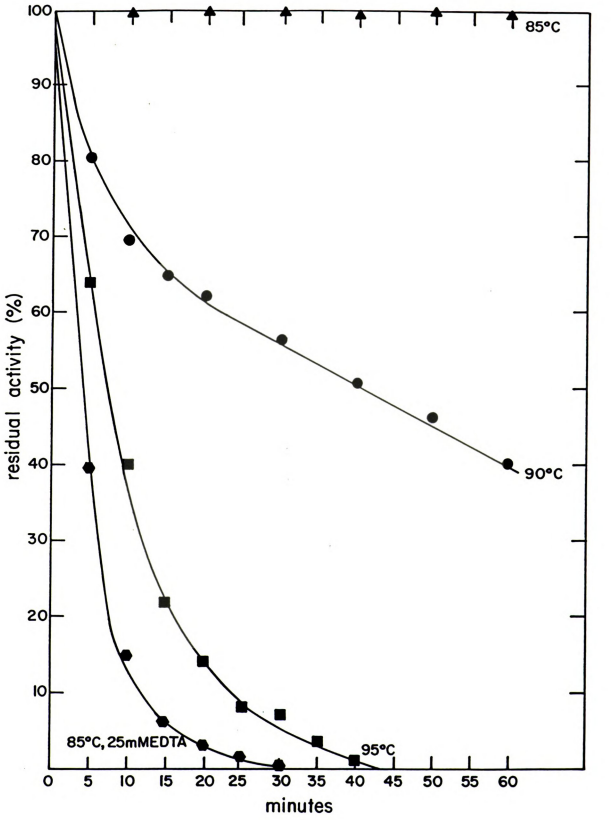
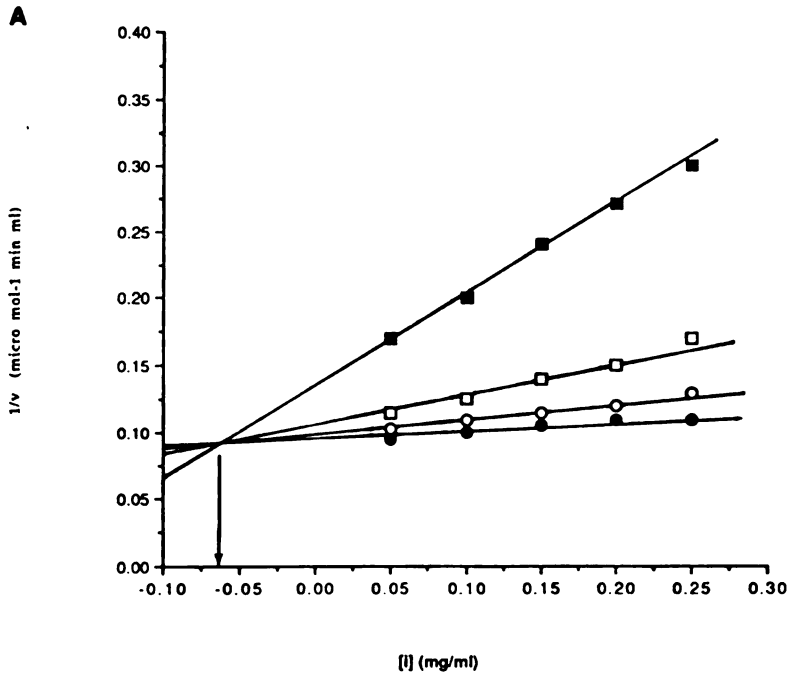

100
90
80
70
60
50
40
30
20
10
residual activity (%)
85°C
90°C
95°C
85°C, 25mMEDTA
0
5
10
15
20
25
30
35
40
45
50
55
60
minutes

Figure 5A. **Inhibition of pullulanase activity of *C. thermohydrosulfuricum* amylopullulanase by β-cyclodextrin.** Final pullulan concentrations of 0.4 (■), 0.8 (□), 2.0 (○), and 4.0(●) mg/ml were used, β-cyclodextrin concentrations were 0.05, 0.1, 0.15, and 0.2, 0.25 mg/ml. All assays were performed in 50 mM acetate buffer, pH 6.0, containing 5 mM $CaCl_2$ at 60°C and samples were taken at 15 minute intervals.

Figure 5B. **Inhibition of α-amylase activity of *C. thermohydrosulfuricum* amylopullulanase by β-cyclodextrin.** Final amylose concentrations of 0.25 (■), 0.5 (□), 1.0 (●), and 2.0 (○) mg/ml were used, β-cyclodextrin concentrations were 0.05, 0.1, 0.15, 0.2, 0.25 mg/ml.


A
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1/v (micro mol-1 min ml)
-0.10
-0.05
0.00
0.05
0.10
0.15
0.20
0.25
0.30
[I] (mg/ml)

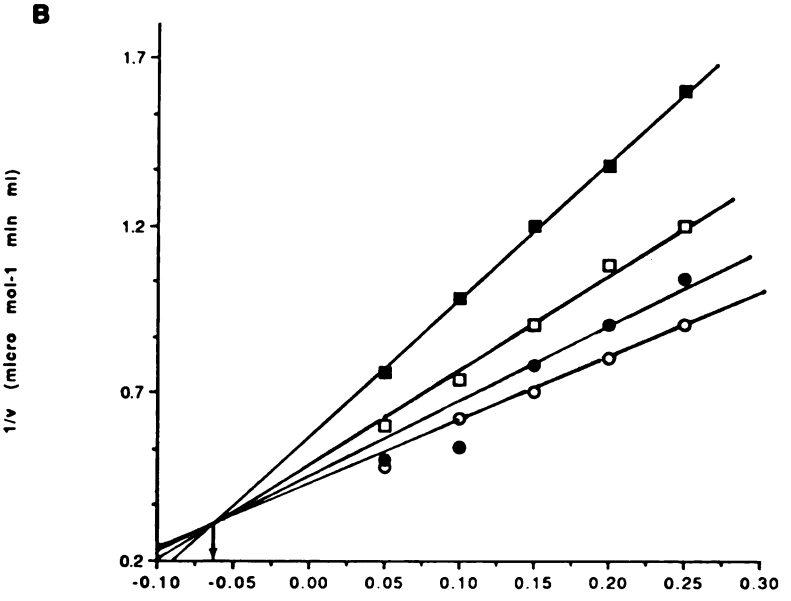

B
1.7
1.2
0.7
0.2
1/v (micro mol-1 min ml)
-0.10
-0.05
0.00
0.05
0.10
0.15
0.20
0.25
0.30

Figure. 6. **HPLC analysis of the product formation profile of amylopullulanase on pullulan (A), glycogen (B), and amylose (C).** The products formed include maltose (▽), maltotriose (△), maltotetraose (⬢).

A
PULLULAN
product formation (%)
h
B
MANNITOL
product formation (%)
h
C
AMYLOSE
product formation (%)
h

was a significant lag period of approximately 24 h, before rapid and efficient hydrolysis of the remaining polysaccharides occurred. This was not observed with pullulan or amylose. Glycogen and amylose were hydrolyzed to maltose, maltotriose and maltotetraose, whereas pullulan hydrolysis yielded only maltotriose.

Thin layer chromatographic analysis identified what hydrolysis products were obtained upon action of amylopullulanase on low molecular weight oligosaccharides containing α-1,4 linkages (Fig. 7). Maltoheptaose was hydrolyzed to maltotriose and glucose, with a small amount of maltose. Maltohexaose was cleaved to maltotriose. Maltose and maltotriose were the products after hydrolysis of maltopentaose and maltotetraose was cleaved to maltotriose and glucose. There was no enzyme activity against maltotriose.

Active site titration and chemical modification

In order to determine the amino acids involved in activity, an active site titration of amylopullulanase, and chemical modification with group specific reagents, were performed. The active site titration curve showed inflection points at pH values of 3.9 and 6.1, which are approximate pK_a values for aspartate or glutamate residues, and for histidine, respectively. However, chemical modification of histidines in amylopullulanase using DEP, which was monitored spectrophotometrically, did not cause a loss in activity (data not shown), while aspartate/glutamate modification with DEAC resulted in loss of activity (Fig. 8). Kinetic analysis of the rate of loss of activity with respect to the concentration of DEAC showed the involvement of at least a single acidic amino acid residue in catalysis.

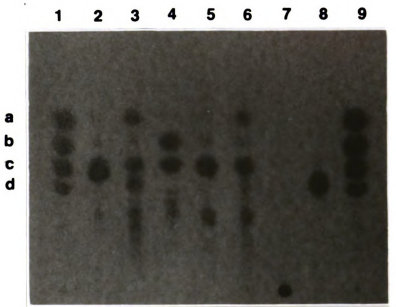


Figure 7. Thin layer chromatographic analysis of product hydrolysates upon action by amylopullulanase from *C. thermohydrosulfuricum* 39E on oligosaccharides. 1.0 ml aliquotes of 1.0% (w/v) maltotriose (lane 2), maltotetraose (lane 3), maltopentaose (lane 4), maltohexaose (lane 5), and maltoheptaose (lane 6) were incubated with the affinity purified enzyme (0.05 U) at 60°C for 72 hours. lanes 1,9 = standards (a=glucose; b=maltose; c=maltotriose; d=maltotetraose)

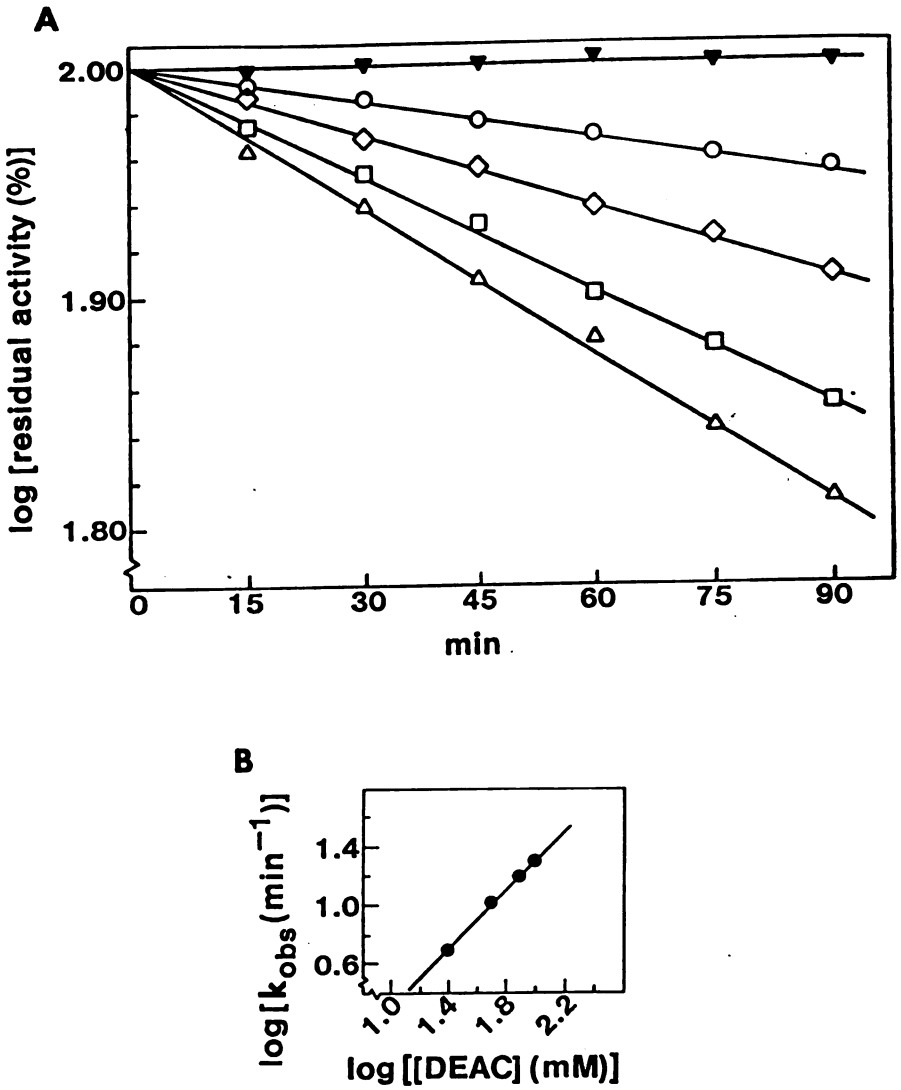


Figure 8 **Chemical modification of aspartate and glutamate residues of amylopullulanase from *C. thermohydrosulfuricum* 39E using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide.**

A : Plot of Log [residual activity (%)] vs time at different concentrations of DEAC; 0 (▼), 25 (○), 50 (◇), 75 (□), 100 (△) mM.

B : Plot of rate of loss of activity vs log of DEAC concentration (Eyzaguirre, 1987)

$$\log k_{obs} = n \log [DEAC] + \log k_1$$

DISCUSSION

This is the first report on the use of maltose-limited chemostat conditions for overexpression and secretion of amylopullulanase activity in thermoanaerobes. Prior studies on continuous culture using substrate limitation had centered on the use of starch for amylase overexpression (Antranikian *et al.*,1987; Madi *et al.*,1987; Koch *et al.*,1987), where optimal conditions were found to be 0.4% (w/v) starch, with the dilution rate being 0.05 h^{-1}. With *C. thermohydrosulfuricum* 39E grown under maltose limitation, it was possible to obtain optimal overexpression of enzyme in 0.2% (w/v) maltose, at a dilution rate of 0.03 h^{-1}. The findings in this study of release of cell-bound amylopullulanase into the culture medium during maltose limited continuous culture contrasted with the effect of the same conditions on the gram negative organism *Klebsiella aerogenes*, where extracellular pullulanase became cell-bound (Hope and Dean, 1984).

The recovery of enzyme activity from *C. thermohydrosulfuricum* 39E was improved by using cell-free supernatant from maltose limited continuous culture, since no residual starch related material was present in the starting crude extract, which would tend to lower the efficiency of purification during the column chromatography steps.

In all the purification steps, where the chromatographic fractions were monitored with pullulan (for pullulanase activity) and with soluble starch (for α-amylase activity), both activities responded almost identically to all parameters tested, suggesting that both activities are the function of a single, novel thermostable saccharidase.

The purified secreted amylopullulanase had a similar molecular weight, pI, and activity as the purified cell-bound enzyme from *C. thermohydrosulfuricum* 39E (Saha *et al*, 1988).

The purified enzyme was able to hydrolyze α-1,6 branched and α-1,4 unbranched polysaccharides. It lacked the ability to hydrolyze starch into glucose, a characteristic of glucoamylase; although it was capable of producing maltose, maltotriose and maltotetraose from starch, an activity typical of α-amylase, and it produced maltotriose from pullulan, a characteristic of pullulanase.

Similar enzymes with dual activties have been isolated from several anaerobic thermophilic bacteria including *C. thermohydrosulfuricum* E101 (Melasniemi, 1988) where the enzyme is a dimer with 190,000 subunit molecular weight. The enzyme was reported to contain 11-12% (w/w) carbohydrate content, containing rhamnose, mannose, galactose, and glucose. A monomeric pullulanase with dual activties isolated from *C. thermosulfurogenes* (Spreinat and Antranikian, 1990) had a molecular weight of 102,000, with a higher affinity for amylose than for pullulan. Amylase-pullulanase of *Bacillus circulans* F-2 (Sata *et al.*, 1989) is a monomer of 220,000 molecular weight and had similar affinity toward both amylose and pullulan.

Inhibition of both α-amylase and pullulanse activities showed the similarity of the substrate binding sites in amylopullulanase to those of known pullulanase and α-amylase (Marshall, 1973).

The results from chemical modification studies showed that aspartates or glutamates are involved in catalysis and the possibility exists for the second titration point at pKa 6.1 to be due to another aspartate or glutamate in a hydrophobic environment in the catalytic center, similar to the pKa value observed for Glu^{35} of lysozyme in it's catalytic center (Walsh, 1979).

Although kinetic analysis of modification of amylopullulanase by DEAC showed the involvement of only a single acidic amino acid in catalysis, involvement of more than one acidic residue or histidine, in catalysis or substrate binding cannot be ruled out, since the thermostable amylopullulanase may be folded in to a rigid structure at 25°C, used during the chemical modification studies. This may effectively make the residues in catalysis or substrate binding inaccessible to the modifying reagents.

Further studies are required to identify whether both pullulanase and α-amylase dual activities reside in the same enzyme, and to identify whether both activities are located at the same active center.

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CHAPTER 3

SUBSTRATE COMPETITION AND SPECIFICITY AT THE ACTIVE SITE OF AMYLOPULLULANASE FROM *Clostridium thermohydrosulfuricum*.

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SUBSTRATE COMPETITION AND SPECIFICITY AT THE ACTIVE SITE OF AMYLOPULLULANASE FROM *CLOSTRIDIUM THERMOHYDROSULFURICUM*

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A highly thermostable pullulanase purified from *Clostridium thermohydrosulfuricum* strain 39E displayed dual activity with respect to glycosidic bond cleavage. The enzyme cleaved α-1,6 bonds in pullulan, while it showed α-1,4 activity against malto-oligosaccharides. Kinetic analysis of the purified enzyme in a system which contained both pullulan and amylose as the two competing substrates were used to distinguish the dual specificity of the enzyme from the single substrate specificity known for pullulanases and α-amylases.

Clostridium thermohydrosulfuricum strain 39E produces a highly thermostable pullulanase activity when grown on starch (1). This enzyme was homogeneously purified and partially characterized in terms of MW, pI, pH stability, and thermal stability (2).

Pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) is a debranching enzyme that specifically cleaves α-1,6 links in starch, amylopectin, pullulan and related oligosaccharides (3), while α-amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) hydrolyse the α-1,4 linkages (4). Pullulanases do not show activity against linear (α-1,4-linked) oligosaccharides, and α-amylases show no activity against pullulan.

The novel pullulanase of *C. thermohydrosulfuricum* strain 39E described in this communication showed α-1,4 as well as α-1,6 cleavage activity against amylose and pullulan, respectively. Detailed kinetic studies with the homogeneously purified enzyme were performed with pullulan, amylose, and linear low MW oligosaccharides in order to biochemically evaluate the active site and to propose a name for this newly recognized enzyme activity.

METHODS

Materials. All chemicals used were obtained from either Sigma Chemical Company (St. Louis, MO) or Aldrich Chemical Company (Milwaukee, WI).

Bacterial strains and growth conditions. *Clostridium thermohydrosulfuricum* strain 39E (ATCC 33223) was used as the source of pullulanase. The organism was grown at 60°C under

anaerobic conditions in TYE medium (5) with 1% (w/v) soluble starch as the substrate. Isolation and purification of the enzyme was performed as described previously (2).

Enzyme assay. 160 microliters of pullulan solution (1.25% w/v in 50 mM NaOAc buffer, pH 6.0, containing 5 mM $CaCl_2$) and 40 microliters of enzyme solution were mixed and incubated at 60°C for 30 min. The reaction was stopped by adding 0.8 ml of DNS solution (6) and heated in a boiling water bath for 15 min. The samples were cooled in ice and the absorbance of the reaction solution was measured at 640 nm. One unit of pullulanase activity is defined as the amount of enzyme which produced 1 micromole of reducing sugar (with glucose as standard) per minute under the above assay conditions.

Competitive inhibition kinetics with mixed alternative substrates. The enzyme recognizes both pullulan and amylose as substrates. It cleaves essentially the α-1,6 bonds in pullulan, while in amylose, the α-1,4 bonds are cleaved. In this experiment both substrates were present as mixed alternative substrates (as outlined by Segel [7]), and was used to investigate the following possibilities:

1. If both activities are due to an enzyme complex of a pullulanase and an α-amylase, or if both activities occurred within the same enzyme, but at two individual active centers non-interacting with each other, then the initial velocity obtained for product formation with both substrates present should be approximately the sum of the individual initial velocities when either of the substrates is present.

$$v = v_P + v_A$$

$$v = \frac{V_A \cdot [A]}{Km_A + [A]} + \frac{V_P \cdot [P]}{Km_P + [P]}$$

where [A] = amylose concentration
[P] = pullulan concentration
V, maximum velocity for each substrate
v, initial velocity
Km, apparent Michaelis constant for each substrate

2. If both activities are due to a single enzyme having a single active center, or two active sites negatively interacting with each other, then the initial velocity obtained with both substrates present will be less then the sum of the individual velocities.

$$v < v_P + v_A$$

$$v = \frac{V_A \cdot [A]}{Km_A\,(1+[P]/Km_P)+[A]} + \frac{V_P \cdot [P]}{Km_P(1+[A]/Km_A)+[P]}$$

RESULTS AND DISCUSSION

Incubation of the enzyme with low MW oligosaccharides, (maltotetraose to maltoheptaose) resulted in the substrates being degraded into units of maltotriose and residual sugars (i.e., glucose or maltose), depending on the parent oligosaccharide subjected to enzymatic action (Table 1), demonstrating that the enzyme had no activity against maltotriose. Maltotetraose was a poor substrate for the enzyme and maltose was not detected as its hydrolysis product. These results reflect an important deviation from known α-amylase activity and pullulanase activity, as maltotriose, as well as other oligosaccharides, would have been

TABLE 1. Reaction products of amylopullulanase from *C. thermohydrosulfuricum* strain 39E on low MW oligosaccharides[a]

Substrate	End Products					
	G_1	G_2	G_3	G_4	G_5	G_6
Maltotriose	--	--	++	--	--	--
Maltotetraose[b]	++	--	++	++	--	--
Maltopentaose	--	++	++	--	--	--
Maltohexaose	--	--	++	--	--	--
Maltoheptaose	++	--	++	--	--	--

Major products observed are shown by the positive sign.

[a]Solutions of 1% (w/v) maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were incubated at 60°C with purified enzyme (0.05 U/ml). Products were analyzed after 72 hours by HPTLC (Whatman HP-K). Plates were developed with n-BuOH:EtOH:H_2O (3:2:2, v/v) at 25°C and the products detected with a mixture of 0.2% (w/v) orcinol in MeOH and 20% (v/v) H_2SO_4 in MeOH (1:1, v/v).

[b]Maltotetraose is a very poor substrate for the enzyme and the products were observed only after long-term reaction.

finally degraded into glucose and maltose by saccharifying α-amylase, and no activity would have been shown towards any of these α-1,4-linked oligosaccharides by pullulanase.

The enzyme from *C. thermohydrosulfuricum* strain 39E showed broad range substrate specificity with regard to high MW polysaccharides. The final reaction products obtained were maltose, maltotriose, and maltotetraose (Table 2). The only product obtained upon incubation of the enzyme with pullulan was maltotriose, demonstrating that the enzyme shows typical pullulanase activity.

The present data indicate that the pure enzyme has both "α-amylase" and "pullulanase-like" activity, and its own unique mode of action. In general, these data show that the mode of action of the enzyme from *C. thermohydrosulfuricum* strain 39E is different from that

TABLE 2. Reaction products of amylopullulanase from *C. thermohydrosulfuricum* strain 39E on high MW polysaccharides[a]

Substrate	End Products					
	G_1	G_2	G_3	G_4	G_6	G_9
Pullulan	--	--	100	--	--	--
Amylose	--	37	47	16	--	--
Amylopectin	--	36	36	28	--	--
Soluble starch	--	39	39	22	--	--
Mammalian glycogen	--	22	47	31	--	--
Oyster glycogen	--	17	50	33	--	--

[a]Solutions of 1% (w/v) pullulan, amylose, amylopectin, mammalian glycogen, oyster glycogen and soluble starch (pH 6.0) were incubated at 60°C with purified enzyme (0.05 U/ml). Samples were withdrawn after 216 hrs and heated at 100°C for 15 min for enzyme inactivation. The reaction products were analyzed by HPLC for sugars (2).

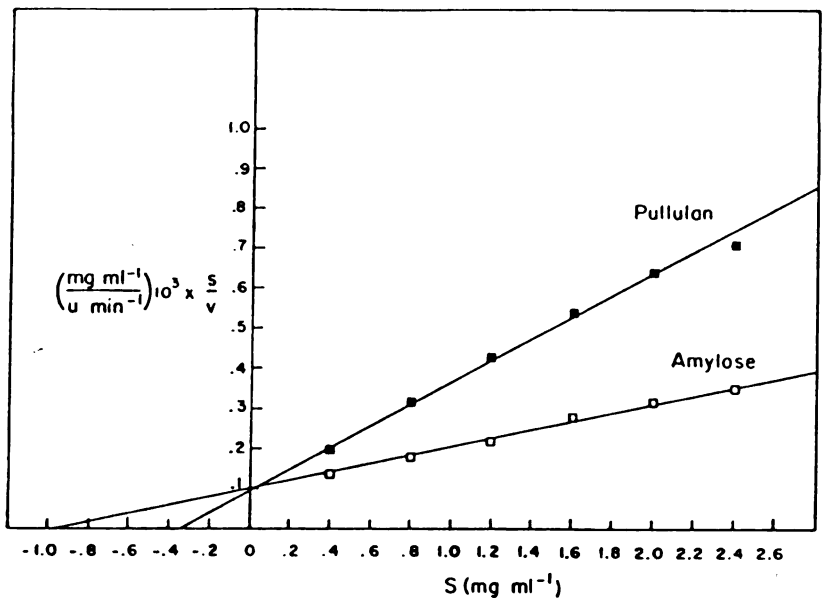

Fig. 1. Km^{app} determination. Pullulan (■) and low MW amylose (□) at the concentrations indicated were incubated with purified enzyme at 60°C.

previously reported for pullulanase and α-amylase, in terms of bond cleavage specificity as well as product formation.

The Km^{app} for pullulan (average MW 50,000) and low MW amylose (MW 4,100) were obtained at substrate concentrations between 0.4 mg/ml to 2.4 mg/ml at 0.4 mg/ml increments (Fig. 1). The dependence of the rate of pullulan and amylose hydrolysis on the substrate concentration followed Michaelis-Menten kinetics. The apparent Km for pullulan (average MW 50,000) and amylose (MW 4,100) as determined from the Hanes-Woolf plot were 0.35 mg/ml and 1.00 mg/ml, respectively (Fig. 1). The apparent k_{cat} for pullulan was 16,000 min^{-1}.

Kinetic experiments on competitive inhibition with mixed alternative substrates were performed at pullulan concentrations of 0.4 to 2.4 mg/ml at 0.8 mg/ml increments, while amylose concentrations were varied from 0.6 to 3.0 mg/ml at 0.6 mg/ml increments. Initial velocities were determined for each combination of pullulan and amylose at the different concentrations. The initial velocity was plotted against the total substrate concentration in a S/V versus S plot (where S is the total substrate concentration), and is shown in Fig. 2. For clarity, only two sets of data with amylose at 0.4 mg/ml and 2.4 mg/ml with varying pullulan concentrations are shown. The initial velocities obtained closely followed the theoretical plot for case 2 (as described in Methods), where the observed velocity was less than the cumulative value of the individual initial velocities obtained for the two substrates. This demonstrates competition between the two substrates used, indicating that the enzyme possesses an active

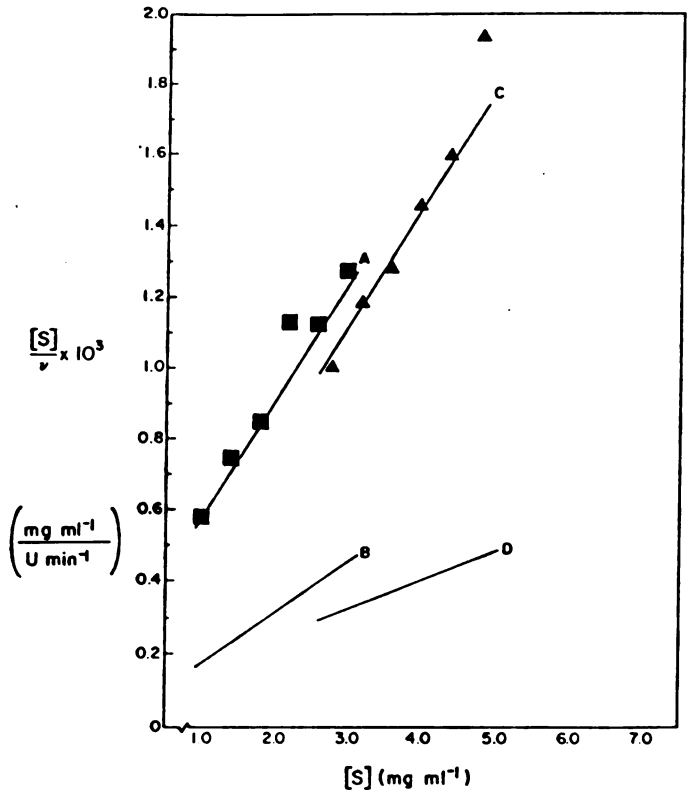

Fig. 2. Kinetics of competitive inhibition with mixed substrates. The solid lines A and C indicate the theoretical plots for competitive inhibition at amylose concentrations of 0.6 and 2.4 mg/ml, respectively. Lines B and D are the theoretical plots for absence of inhibition at the same respective amylose concentrations. Pullulan was used at concentrations of 0.4, 0.8, 1.2, 1.6, 2.0, 2.4 mg/ml. For clarity, only two sets of data points were used in the above plot. (■) and (▲) are the practical data points obtained at 0.6 and 2.4 mg/ml amylose concentrations. All reaction mixtures contained 5% (v/v) dimethyl sulfoxide for solubility of amylose.
[S] = [A] + [P], where S is the total substrate concentration. A and P are the concentrations of amylose and pullulan, respectively.

site for cleavage of both α-1,6 and α-1,4-linked substrates. This apparent competition between two substrates has been used by several authors as proof of a given enzyme having a single active center with two different modes of activity (8-12). However, as the same result can occur under certain conditions for an enzyme with two active centers (8), the results obtained for the enzyme from *C. thermohydrosulfuricum* strain 39E can only be used as positive proof for both activities belonging to the same enzyme.

The first description of pullulanase activity was an enzyme from *Klebsiella pneumoniae* (13), which specifically hydrolyses α-1,6 glycosidic linkages of pullulan to yield maltotriose. Other pullulan degrading enzymes (e.g., neopullulanase) which specifically cleave α-1,4 glycosidic bonds in pullulan to yield panose (14) and isopanose (15) have been reported.

This is the first detailed kinetic and substrate hydrolysis studies reported on novel pullulanases that cleave α-1,6 and α-1,4 bonds. Pullulanases possessing both α-1,6 and α-1,4 activity have been isolated from *Thermoanaerobium* Tok 6-B1 (16, 17), *T. brockii* (18), *Clostridium thermosulfurogenes* (19), and *C. thermohydrosulfuricum* strain E101-69 (20, 21). The enzyme from *T. brockii* was reported to hydrolyze starch into various sized oligomers, while the *Thermoanaerobium* strain Tok 6-B1 enzyme acted upon starch, amylopectin and amylose to yield predominantly maltose and maltotriose. Maltotetraose was completely hydrolysed to maltose by *Thermoanaerobium* Tok 6-B1 enzyme (16), and the enzyme was found to hydrolyse low MW oligosaccharides at two glucose residues away from a terminal, and release maltose as the product (17). The enzyme isolated from *C. thermohydrosulfuricum* strain E101-69 was in two forms of MW over 300,000, and the possibility of the presence of a third form was suggested (21). Each form was capable of hydrolysing both pullulan and amylose but oligosaccharide hydrolysis studies were not reported. In contrast, the pullulanase described in this paper was of monomeric form with a MW of 136,500.

In comparison to the enzyme from *Thermoanaerobium* Tok 6-B1, *C. thermohydrosulfuricum* strain 39E pullulanase produced maltose, maltotriose, and maltotetraose upon reaction with high MW polysaccharides. Also, it hydrolysed low MW oligosaccharides three glucose units away from a terminal, released maltotriose as the product, and did not form maltose from maltotetraose. These results represent significant differences between the pullulanases from *Thermoanaerobium* Tok 6-B1 and *C. thermohydrosulfuricum* strain 39E.

Kinetic studies of the homogeneous enzyme from *C. thermohydrosulfuricum* strain 39E on low MW amylose and pullulan as competing substrates, gave further proof that both activities resided on the same enzyme and putatively within the same active site. Therefore, the enzyme described here as amylopullulanase higher affinity and activity toward pullulan than amylose; and, it shows unique characteristics with regard to substrate utilization as well as product formation. In light of these results, it is now necessary to distinguish pullulanases which possess only α-1,6 cleavage activity from amylopullulanases that contain both α-1,6 and α-1,4 cleavage activity. Thus, we propose the enzyme commission use the term amylopullulanase to describe those enzymes which act on starch and cleave both α-1,6 bonds in pullulan and α-1,4 bonds in amylose.

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CHAPTER 4

CLONING, IDENTIFICATION OF THERMOSTABILITY AND CATALYTIC REGIONS; AND, CHARACTERIZATION OF α-1,4 AND α-1,6 BOND SPECIFICITY OF AMYLOPULLULANASE FROM *Clostridium thermohydrosulfuricum* 39E.

ABSTRACT

A gene (*apu*), encoding amylopullulanase, which exhibits both α-1,4 cleaving amylase and α-1,6 cleaving pullulanase activities, was cloned from the thermophilic anaerobe *Clostridium thermohydrosulfuricum* strain 39E and expressed in both *Escherichia coli* and *Bacillus subtilis*. In both host strains the cloned enzyme was expressed constitutively and was located intracellularly or within the periplasm of *E. coli* cells, and secreted by *B. subtilis*. The recombinant amylopullulanase in *E. coli* displayed a molecular weight of 160,000, a half-life of 40 min at 90°C, and hydrolyzed glycogen, soluble starch, amylopectin, and amylose to maltose, maltotriose, and maltotetraose. The 6.1 kbp DNA insert containing the *apu* gene was restricted to 2.9 kbp resulting in a M_r 100,000 expressed protein, which did not show any quantitative loss of activity or thermostability. Nested deletion mutants from both 3′ and 5′ ends of the 2.9 kbp DNA fragment were prepared that lost thermostability but retained activity. The Mr 100,000 protein was used to prove by ^{13}C NMR analysis the ability of the enzyme to cleave α-1,6 bonds and α-1,4 bonds in glycogen.

INTRODUCTION

α–Amylases are endoglucanases which randomly hydrolyze the α–1,4 linkages found in amylose, amylopectin, glycogen, and related polysaccharides. Pullulanase, also known as a debranching enzyme, is capable of attacking specifically the α–1,6 linkages found in the linear polysaccharide pullulan, and in other branched polysaccharides such as glycogen and amylopectin.

Amylopullulanase of *Clostridium thermohydrosulfuricum* 39E (Saha *et al.*, 1988; Mathupala *et al.*, 1990), α–amylase-pullulanase of *C. thermohydrosulfuricum* E101-69 (Melasniemi, 1988), pullulanase of *Thermoanaerobium* Tok6-B1 (Plant *et al.*, 1987), debranching enzyme of *T. brockii* (Coleman *et al.*, 1987), and amylase-pullulanase of *B. circulans* F-2 (Sata *et al.*, 1989) are thermophilic enzymes exhibiting α–amylase activity against α–1,4 bonds in amylose and related polysaccharides as well as pullulanase activity against α–1,6 bonds in pullulan. These proteins may represent a new class of enzymes capable of cleaving both α-1,4 and α-1,6 bond in glycogen and starch, but this remains to be proven.

C. thermohydrosulfuricum 39E (ATCC 33223), a thermophilic, obligately anaerobic bacterium isolated from Octopus Spring in Yellowstone National Park (Zeikus *et al.*, 1980) can ferment a wide variety of starch related polysaccharides, including amylose, amylopectin, glycogen, and pullulan (Hyun and Zeikus, 1985). We have identified and characterized a specific amylase from this organism (Saha *et al.*, 1988; Mathupala *et al.*, 1990), tentatively named amylopullulanase, because of it's hydrolytic activity against all the above mentioned polysaccharides. Our previous study had centered on the biochemical characterization of this amylopullulanase to

demonstrate that the dual activities are due to the action of a single enzyme (Mathupala *et al.*, 1990).

The purpose of this study was to further substantiate these findings by cloning, expressing, and characterizing the *apu* gene, followed by biochemical characterization of the recombinant enzymes to demonstrate that the a-amylase and pullulanase activities are encoded by a single gene, and to test the ability of the enzyme to cleave both α-1,6 and α-1,4 linkages in glycogen, to identify thermostability regions of the gene and, to demonstrate that the active site of this enzyme is encoded by a single region within the gene.

MATERIALS AND METHODS

Reagents

All chemicals were of molecular biology or analytical grade and were obtained from Aldrich Chemical Co. (Milwaukee, WI), or Sigma Chemical Co (St. Louis, MO).

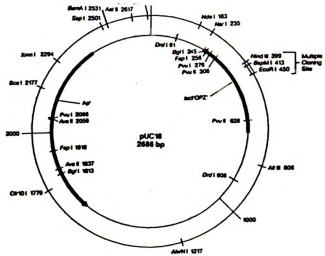
Bacterial strains, plasmids, and transformation

C. thermohydrosulfuricum 39E (ATCC 33223) was grown anaerobically at 60°C in TYE medium (Zeikus *et al.*, 1980) containing 0.5% (w/v) glucose, and was used for chromosomal DNA isolation. The bacterial strains used as the recipient hosts for the recombinant plasmids are listed in Table 1. Plasmid pUC 18 (Yanisch-Perron *et al.*, 1985), was used as the initial cloning vector. pUC 19 (Yanisch-Perron *et al.*, 1985) and pUB 110 (Gryczan *et al.*, 1978) were used in subsequent subcloning experiments (Fig. 1). Plasmid pCPC 902, containing a chromosomal DNA insert encoding for the debranching enzyme of *T. brockii* (Coleman *et al.*, 1987) was obtained from the American Type Culture Collection (ATCC 53114) and was used in Southern hybridization experiments to isolate and identify the chromosomal DNA fragment of *C. thermohydrosulfuricum* 39E expressing amylopullulanase activity. *Escherichia coli* strains used in DNA manipulations were made competent by the Hanahan method as described by Perbal (1988) while recombinant vectors were introduced into *E. coli* strains by heat-shock treatment (Hanahan, 1983). *Bacillus subtilis* was transformed by PEG mediated protoplast transformation (Chang and Cohen, 1979; Puyet *et al.*, 1987).

TABLE 1. ***E. coli*** **and** ***B. subtilis*** **strains used in cloning and subcloning experiments.**

Strain	Genotype	Source or Reference
E. coli HB101	F⁻ *mcr*B *mrr sup*E44 *hsd*S20(rb⁻ mb⁻) *rec*A13 *ara*14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20(Sm^R)*xyl*5 l- *leu mtl*1	Bethesda Research Labs.
E. coli DH5α	F⁻ ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rK⁻mK⁺) *sup*E44 l⁻*thi*-1 *gyr*A96 *rel*A1	Bethesda Research Labs.
E. coli TG-1	*sup*E *hsd*D5 *thi* Δ(*lac-pro*AB) F' (*tra*Δ36 *pro*AB⁺lacI^q lacZΔM15)	Amersham International.
E. coli SURE	e14-(*mcr*A) Δ(*mcr*CB-*hsd*SMR-*mrr*)171 *end*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *lac rec*B *rec*J *sbc*C *umu*C:Tn5(kan^R) *uvr*C [F' *pro*AB *lac*I^{q}ZΔM15 Tn10(tet^R)]	Stategene Co.
B. subtilis NA-1	*arg*-15 *hsd*R *hsd*M AmyNpr⁻	Nakajima *et al.*, 1985

Figure 1. **Plasmids used in cloning of amylopullulanase (*apu*) gene from *C. thermohydrosulfuricum* 39E.** Plasmid pUC 18 was used as the initial cloning vector (A), and pUB 110 was used for subcloning into *B. subtilis* NA-1 (B). Phagemid vector pUC 119 (C) was used for sequencing.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Sap I 2501
Ahl I 163
Ner I 235
Dra I 81
Sgr I 245
Fsp I 256
Pvu I 276
Pvu II 309
Hind III 399
BspM I 413
EcoR I 420
Multiple Cloning Site
Xmn I 2294
Sca I 2177
lacI^Q
lacZ'
Pvu I 2066
Ava II 2066
pUC18
2686 bp
Pvu II 626
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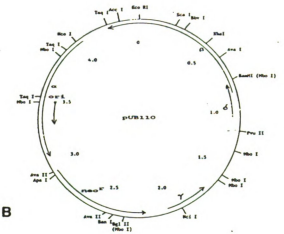
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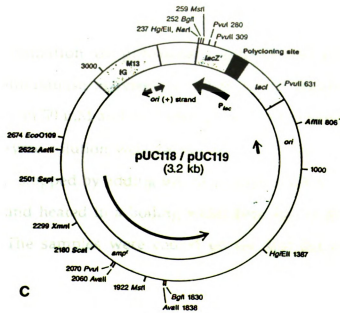
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252 Bgl I
237 Hgi EII, Nar I
Pvu I 280
Pvu II 309
Polycloning site
lacZ'
M13 IG
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lacI
Pvu II 631
Aft III 806
2674 Eco O109
2622 Ase II
pUC118 / pUC119
(3.2 kb)
ori
1000
2501 Sap I
2299 Xmn I
2180 Sca I
sing'
Hgi EII 1357
2070 Pvu I
2060 Ava II
1922 Mst I
Bgl I 1830
Ava II 1838
C

Growth media and preparation of cell extracts

E. coli and *B. subtilis* were grown at 37°C in modified LB broth containing tryptone (10g/liter), yeast extract (5g/liter), and NaCl (5g/liter) without pH adjustment. The medium was supplemented with ampicillin or kanamycin at a final concentration of 50 μg/ml, for appropriate *E. coli* or *B. subtilis* strains carrying a vector or a recombinant plasmid. For agar plates, 1.5% (w/v) Bacto-Agar (Difco Laboratories, Detroit, MI) was added. For soft agar overlay, 0.7% (w/v) agar was used. For preparation of cell extracts, cultures were grown to late exponential phase and harvested by centrifugation at 8,000 x g for 10 min and washed twice with 50 mM acetate buffer (pH 6.0) containing 5 mM $CaCl_2$. The periplasmic enzyme fraction was isolated by osmotic-shock treatment of the cells (Willis, 1984; Neu and Heppel, 1965). The intracellular enzyme fraction was isolated either by passing the cells through a French-pressure cell at 20,000 lb/in^2 (American Instrument Co., Inc., Silver Spring, MD) for large scale enzyme isolation, or for small volumes, cell samples (1.0 ml) were sonicated using a microsonicator for 5 s (Kontes, Vineland, NJ). The disrupted cells were centrifuged and the supernatant used as the source of amylopullulanase.

Enzyme assays

For determination of pullulanase activity, 160 μl of 1.25% (w/v) pullulan (for pullulanase activity) or 1.25% (w/v) soluble starch (for α-amylase activity) in 50 mM acetate buffer, pH 6.0, containing 5 mM $CaCl_2$ and 40 μliters of enzyme solution were mixed and incubated at 60°C for 30 min. The reaction was stopped by adding 0.8 ml of dinitro salicylate (DNS) solution (Miller, 1959), and heated in a boiling water bath for 15 min to develop the color reaction. The samples were cooled on ice and the absorbance of the

reaction solution measured at 640 nm. One unit of pullulanase or α-amylase activity is defined as the amount of enzyme which produces 1 μmol of reducing sugar (with glucose as the standard) per min under the assay conditions.

For determination of α-amylase activity, 160 μl of 1.25% (w/v) soluble starch in 50 mM acetate buffer, pH 6.0, containing 5 mM $CaCl_2$ and 40 μl of enzyme solution were mixed and incubated at 60°C for 30 min. The reaction was stopped by adding 0.8 ml of DNS solution, and heated in a boiling water bath for 15 min to develop the color reaction. The samples were cooled in ice and the absorbance of the reaction solution measured at 640 nm. One unit of α-amylase activity is defined as the amount of enzyme that produces 1 μmol of reducing sugar (with glucose as the standard) per min under the assay conditions described.

DNA manipulation

Enzymes and kits for DNA manipulations were obtained from Bethesda Research Laboratories (Gaithersburg, MD), United State Biochemical Co. (Cleveland, OH), or Boehringer Mannheim Biochemicals (Indianapolis, IN). Chromosomal DNA from *C. thermohydrosulfuricum* 39E was isolated and purified by a modification of the procedure by Doi (1983) as described for *B. subtilis*, where the cell lysate was treated with proteinase K (0.1 mg/ml, 37°C, 1 h) prior to deproteinizing with buffer equilibrated phenol. Plasmid DNA was routinely prepared by the alkaline lysis method (Sambrook *et al.*, 1989), while large scale plasmid DNA isolation was performed by the method of Clewell and Helinsky (1969) followed by ultracentrifugation in a CsCl density gradient as described previously (Sambrook *et al.*, 1989). Enzymatic manipulations of DNA, and separation of DNA fragments by agarose gel

electrophoresis were carried out as described by Sambrook *et al.*, (1989) or according to the manufacturer's instructions. A 2.2 kbp gene fragment isolated from plasmid pCPC 902 after restriction with *Pst*I, and by electroelution using an electroelution apparatus, the Elutrap (Schleicher and Schuell Inc., Keene, NH) was labelled with α-^{32}P dATP (400 Ci/mmol, New England Nuclear, Wilmington, DE), by nick translation (Nick Translation Reagent Kit, Bethesda Research Laboratories, Gaithersburg, MD). This probe was used in Southern hybridization experiments to identify the amylopullulanase (*apu*) gene.

Southern hybridization

Chromosomal DNA from *C. thermohydrosulfuricum* 39E was completely restricted by single or double digestion with SstI, BamHI, EcoRI, and XbaI. The digested DNA was electrophoresed through 0.8% (w/v) agarose gels and visualized by staining with ethidium bromide. For hybridization with the 2.2 kbp radiolabelled probe, the DNA was transferred onto Zeta-Probe membranes (Bio-Rad, Richmond, CA) by the method of Southern, as described by Sambrook *et al.*, (1989).

Cloning of the amylopullulanase (*apu*)gene into *E. coli*

Chromosomal DNA of *C. thermohydrosulfuricum* 39E was double digested with *Eco*RI and *Xba*I. 5 to 8 kbp DNA fragments were isolated from an agarose gel (0.8% w/v) by electroelution. The plasmid vector pUC 18 was linearized by double digestion with *Eco*RI and *Xba*I. The 5 to 8 kbp chromosomal DNA fragments were ligated with linearized pUC 18 at a molar ratio of 1:3. The ligated DNA was transformed into competent *E. coli* SURE cells by the Hanahan method (1983).

Identification of amylopullulanase positive transformants

E. coli SURE transformants harboring recombinant pUC 18 plasmids were selected by plating the transformation sample on LB agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) as described by Rodriguez and Tait (1983). For detection of *E. coli* SURE transformants expressing amylopullulanase activity, individual transformant colonies were replica plated onto LB agar plates containing 0.5%(w/v) soluble starch or 0.5%(w/v) pullulan coupled to Reactive Red dye (Yang and Coleman, 1987). Upon growth of colonies, the plates were transferred to 60°C for 24 h. The starch containing plates were overlayed with 0.15% iodine/1.5% potassium iodide solution to visualize colonies containing α-amylase activity.

Positive clones showed a colorless halo around the colonies, against the dark blue background of the starch-iodine complex. Pullulanase positive clones gave a pale diffusion ring around the colonies against the dark red background of Reactive Red-pullulan plates.

Subcloning of amylopullulanase (*apu*) gene into *B. subtilis*

Recombinant plasmids expressing amylopullulanase actvity in *E. coli* SURE were double digested with *Eco*RI and *Xba*I. The *Eco*RI-*Xba*I chromosomal DNA insert was recovered by agarose gel electrophoresis followed by electroelution, and ligated into pUB 110 double digested and linearized with *Eco*RI and *Xba*I at a molar ratio of 1:3. For PEG mediated transformation of *B. subtilis* cells, protoplasts were prepared from cells grown to exponential phase in SMMP medium (Chang and Cohen, 1979) using lysozyme. The ligation mixture was used in PEG mediated transformation of *B. subtilis* NA-1 protoplasts (Chang and Cohen, 1979). The transformants

were plated onto cell wall regeneration media (Puyet *et al.*, 1987) and the resultant colonies were replica plated onto LB agar containing kanamycin. α-amylase and pullulanase positive colonies were identified as indicated above for *E. coli* transformants.

Construction of lac Z fusion proteins

Recombinant plasmid DNA isolated from transformants expressing amylopullulanase activity, was partially digested with *Hind*III. The DNA fragments were isolated by electrophoresis and subsequent electroelution. The plasmid vector pUC 18 was linearized with *Hind*III and dephophorylated with calf intestine alkaline phosphatase. The partially digested DNA fragments were ligated with linearized and dephosphorylated pUC 18 at a molar ratio of 1:3. The ligated DNA was used to transform *E. coli* HB101, TG-1, and DH5α. Transformants were tested for α-amylase and pullulanase activities, as described previously.

Construction of nested deletion mutants

Recombinant plasmid DNA was double digested with *Aat*II and *Nde*I restriction sites available on the pUC 18 vector, creating an exonuclease III sensitive restriction site (*Nde*I) towards the DNA insert, which was used to construct nested deletion mutants of the DNA insert from the 3′ direction. To construct nested deletion mutants from the 5′ direction of the DNA insert, double digestion was carried out with *Sst*I and *Bam*HI restriction sites available on the multicloning site of the pUC 18 vector, creating an exonuclease III sensitive site (*Bam*HI). Nested deletion mutants were constructed with exonuclease III and nuclease S1 (Ausubel *et al.*, 1988) at a reaction concentration of 150 units of exonuclease III per pmol of susceptible

3′ ends, at 30°C. Each deletion mutant was transformed into *E. coli* DH5α, and tested for activity and thermostability. *E. coli* DH5α harboring the deletion mutants were grown at 37°C in 5.0 ml of LB media containing ampicillin (50 μg/ml). Cells were harvested from 1.0 ml of culture by centrifugation in a microcentrifuge (14,000 rpm x 1 min) and the cell pellet sonicated in 150 μl of 50 mM acetate buffer (pH 6.0) containing 5 mM $CaCl_2$. To test for thermostability, the cell lysate was centrifuged (14,000 rpm x 5 min) and the supernatant heat treated at 85°C for 5 to 30 min and centrifuged (14,000 rpm x 5 min). The supernatant was recovered and tested for α-amylase and pullulanase activity. To test for activity in deletion mutants which lost thermostability, the cell lysate was assayed directly without heat treatment.

Purification of recombinant amylopullulanase

(i) Preparation of cell extract: Unless otherwise noted, all operations were performed at 25°C. *E. coli* DH5α carrying pAPZ72 were grown at 37°C for 12 h (mid-exponential phase) in LB medium (1 L) containing ampicillin (50 μg/ml), and harvested by centrifugation (8,000 x g, 10 min). The cells were resuspended in 1/25 of the original volume in 50 mM acetate buffer (pH 6.0), containing 5 mM $CaCl_2$, and then disrupted by passing through a French Pressure cell (American Instrument Co., Silver Springs, MD) operating at 20,000 lb/in^2.

Recombinant amylopullulanase from *B. subtilis* NA-1, harboring pAPZ 74 was recovered from the culture supernatant, by repeated dilution (with 50 mM acetate buffer, pH 6.0, containing 5 mM $CaCl_2$) and reconstitution, using an Amicon ultrafiltration cell, with a 100,000 molecular weight cut off membrane (YM-100) (Amicon Co., Danvers, MA).

The periplasmic recombinant enzyme fraction of *E. coli* SURE (from pAPZ 71), was recovered by subjecting the cells to osmotic shock for release of periplasmic proteins (Neu and Heppel, 1965; Willis, 1984). The cells were then centrifuged and the supernatant recovered as the periplasmic enzyme fraction, and processed in a manner similar to enzyme recovery from *B. subtilis*. The cell pellet was suspended in 50 mM acetate buffer (pH 6.0) containing 5 mM $CaCl_2$, and lysed for recovery of the intracellular enzyme.

(ii) Heat treatment: After centrifugation of the cell lysate (38,000 x g, 30 min), the supernatant was heat treated at 85°C for 15 min and chilled on ice for 10 min. The resultant suspension was centrifuged (38,000 x g, 30 min) to remove the denatured proteins, and the supernatant containing the thermostable protein fraction, was concentrated and desalted by repeated ultrafiltration through an Amicon YM-100 membrane equipped cell (Amicon), using 50 mM acetate buffer, pH 6.0, containing 5 mM $CaCl_2$.

(iii) Anion exchange chromatography: The concentrate (5.0 ml) was applied to a Q-Sepharose column (30 cm x 2.5 cm) equilibrated with 50 mM acetate buffer at pH 6.0 containing 5 mM $CaCl_2$, and washed with the same buffer containing 0.05 M NaCl and then eluted with a 500 ml linear gradient of 0.05 M NaCl to 0.45 M NaCl at a flow rate of 1.0 ml/min.

(iv) Gel filtration chromatography: The amylopullulanase fractions collected from anion exchange chromatography were pooled and concentrated in Centricon-30 filter cartridges (Amicon) to 200 µl. 50 µl aliquotes were then applied to a Superose-12 column (Pharmacia, Piscataway, NJ) column equilibrated with 50 mM acetate buffer (pH 6.0) containing 50 mM NaCl and 5 mM $CaCl_2$.

(v) Affinity column chromatography: Amylopullulanase fractions obtained from the Superose-12 column were applied directly to a β-cyclodextrin-

coupled Sepharose CL-4B affinity column (10 ml bed volume) (prepared as described by Mathupala and Zeikus, manuscript submitted), at 25°C and washed sequentially with 50 mM acetate buffer (pH 6.0) containing 0.5 M NaCl, and then with buffer containing 1%(w/v) maltose. Purified amylopullulanase was eluted from the column using 1%(w/v) β-cyclodextrin in 50 mM acetate buffer (pH 6.0). The eluate, detected at 280 nm, was concentrated and reconstituted in acetate buffer (pH 6.0) containing 5 mM $CaCl_2$, by using ultrafiltration to remove β-cyclodextrin.

Protein determination and gel electrophoresis

Protein was determined by using either the dye binding assay (Bradford, 1976) with the Bio-Rad protein assay kit (Bio-Rad), or by using bicinchoninic acid with the BCA Assay Kit (Pierce Co., Rockford, IL), using bovine serum albumin as the standard. SDS-PAGE was performed according to the method of Laemmli (1970) using 7.5% 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 determined by using high range molecular weight standards (Bio-Rad) containing myosin (200,000), β-galactosidase (116,250), phosphorylase b (97,400), bovine serum albumin (66,200), and ovalbumin (42,700).

Amino acid analysis and N-terminal sequence analysis

Purified recombinant enzymes were desalted using double distilled water with a Centricon-30 ultrafiltration device (Amicon). Amino acid composition was determined by reversed phase HPLC using the PICO-TAG method (Waters Div., Millipore Co., Milford, MA) in a Beckman 7300 HPLC

analyzer. The N-terminal amino acid sequence of the recombinant enzyme was identified by using a protein sequencer Model 477A (Applied Biosystems, Foster City, CA) at the Macromolecular Facility, Department of Biochemistry, Michigan State University.

^{13}C NMR analysis

Natural abundance ^{13}C NMR spectra were obtained on maltose and iso-maltose, which were used as standards to identify the spectral positions for α-1,4 and α-1,6 linkages respectively, and on glycogen, prior to, and following hydrolysis by amylopullulanase. For preparation of the product hydrolysate, 60 units of recombinant enzyme were incubated for 48 h at 60°C with 25 ml of mammalian glycogen (10 mg/ml) in acetate buffer, pH 6.0. Completion of hydrolysis was determined using TLC, and the product hydrolysate from glycogen was desalted and deproteinized by anion exchange (Amberlite IR-118H) (5 ml bed volume) and cation exchange (Amberlite IRA-400OH) (5 ml bed volume) mini-column chromatography. All samples were lyophilized twice in the presence of D_2O prior to NMR analysis. The proton decoupled ^{13}C nuclear magnetic resonance spectra (NMR) were recorded at 75.429 MHz on a Varian VXR-300 spectrometer with 5 mm (diameter) NMR tubes. The solvent deuterium resonance was used as a field-frequency lock and chemical shifts are expressed relative to tetramethylsilane. Maltose, isomaltose, and glycogen were analyzed at a concentration of 100 mg/ml, while the product hydrolysate was analyzed at a concentration of 250 mg/ml. Glycogen was analyzed for 5000 transients, while maltose, isomaltose, and the product hydrolysate were analyzed for 1500 transients. NMR spectra of maltose and iso-maltose standards were assigned carbon numbers using published data (Colson *et al.*, 1974; Nunez *et al.*, 1977). C_7 position carbon of

maltose and isomaltose, were assigned signals at 100.1 ppm (for α-1,4 glucosidic bond) and 98.5 ppm (for α-1,6 glucosidic bond) respectively. C_1 position carbon of maltose and isomaltose, was assigned signals at 96.3 ppm and 96.6 ppm respectively.

Thin Layer Chromatography

2 µl aliquots of the samples prepared for NMR analysis were applied to Whatman HP-K High Performance Silica gel plates (4.5 µM partcle size; 10 x 10 cm). The plates were developed with a n-butanol:ethanol:water (3:2:2 v/v) eluant mixture at room temperature. The resolved sugars were detected by using a 1:1 mixture of 0.2% (w/v) orcinol in methanol and 20% (v/v) sulfuric acid in methanol (1:1, v/v) and heating the plates at 100°C for color development.

RESULTS

Cloning of amylopullulanase (*apu*) gene into *E. coli* and *B. subtilis*

In order to identify the *apu* gene of *C. thermohydrosulfuricum* 39E, Southern analysis was performed on chromosomal DNA digested with *Eco*RI, *Xba*I, and *Sst*I, or double digested with *Eco*RI/*Xba*I and *Sst*I/*Xba*I. The results are shown in Fig. 2. An approximately 6 kbp chromosomal DNA fragment, resulting from the *Eco*RI/*Xba*I double digestion, which was the smallest DNA fragment that hybridized to the radiolabelled 2.2 kbp DNA probe upon Southern analysis, was selected for cloning experiments to isolate the *apu* gene, in *E. coli* SURE cells using pUC18 vector. The cloning strategy is summarized in Fig. 3. Approximately 600 *E. coli* SURE transformants were screened for amylopullulanase activity on the starch-iodine and Reactive Red-pullulan plates. A transformant colony which produced a clear halo on both pullulan and starch plates was identified. Plasmid DNA was isolated from the positive transformant clone, and subjected to restriction enzyme analysis. The recombinant plasmid designated pAPZ 71, contained a DNA insert of 6.1 kbp. The physical map of pAPZ 71 is shown in Fig. 4.

In order to express the amylopullulanase activity in *B. subtilis*, the 6.1 kbp DNA insert was recovered from pAPZ 71, and ligated to plasmid pUB110. Transformation experiments were performed initially, using $CaCl_2$ mediated transformation of *B. subtilis* (Kuriki *et al.*, 1988). Although transformants could be selected on kanamycin/LB agar plates by colony hybridization using the 2.2 kbp radiolabelled probe DNA, none of the transformants showed amylopullulanase activity. Restriction enzyme analysis of the recombinant pUB110 plasmid DNA indicated spontaneous partial deletions of the 6.1 kbp chromosomal DNA insert upon transformation into *B. subtilis*. However,

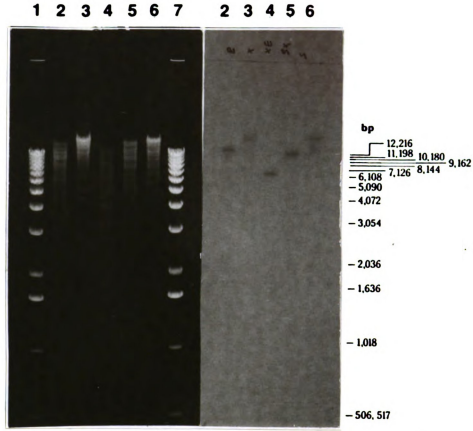


Figure 2. Southern hybridization analysis of *C. thermohydrosulfuricum* 39E chromosomal DNA for cloning of the *apu* gene. A 2.2 kbp DNA containing a fragment of the debranching enzyme gene of *T. brockii* was used as the probe. lanes 1,7 = 1 kb DNA size markers; lane 2 = *Eco*RI digest; lane 3 = *Xba*I digest; lane 4 = *Eco*RI/*Xba*I double digest; lane 5 = *Sst*I/*Xba*I double digest; lane 6 = *Sst*I digest

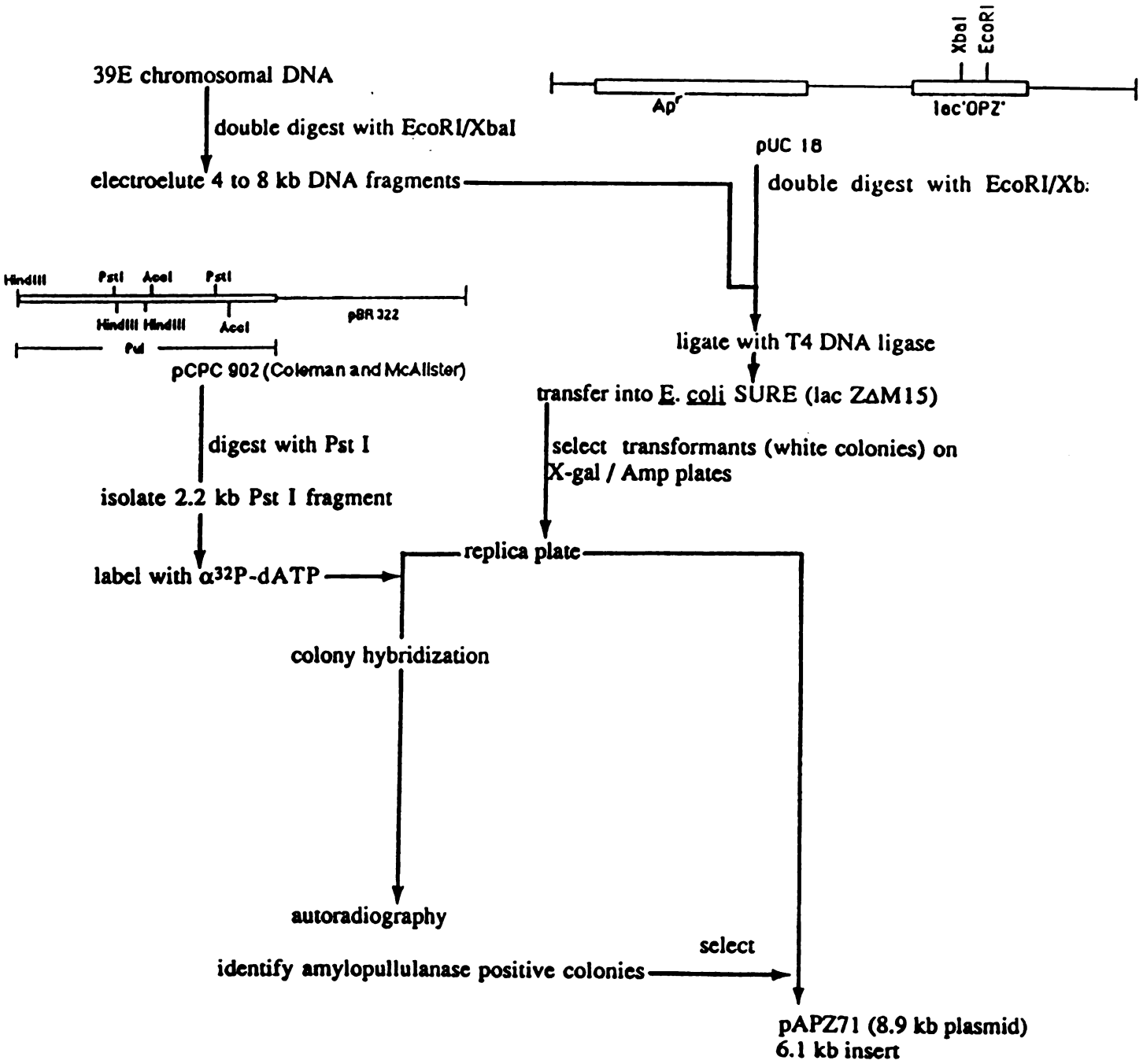


Figure 3. Cloning strategy for the amylopullulanase (*apu*) gene from *C. thermohydrosulfuricum* 39E into *E. coli* SURE

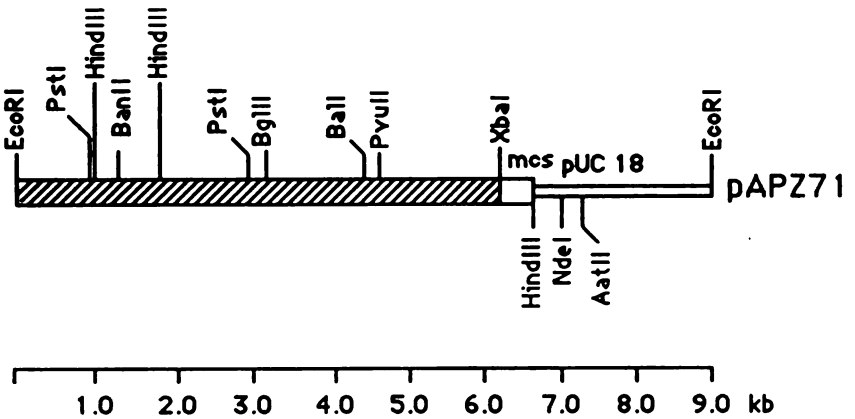


Figure 4. **Physical map of the pUC 18 clone (pAPZ 71) containing the *apu* gene.**
The hatched area represents *C. thermohydrosulfuricum* DNA, and the open region represents the plasmid DNA. mcs = multicloning site

after PEG mediated transformation of *B. subtilis* protoplasts, expression of α-amylase and pullulanase activity was observed for the recombinant *B. subtilis* subclones. A plasmid recovered from a colony expressing amylopullulanase activity was denoted pAPZ 74 (Fig. 5).

Expression and location of recombinant enzyme in *E. coli* and *B. subtilis*

Recombinant *E. coli* SURE containing pAPZ 71 expressed amylopullulanase constitutively. The 6.1 kbp insert, when subcloned into pUC 19 vector in the opposite orientation, expressed amylopullulanase in quantitatively similar amounts to that in pUC 18 (data not shown). Since the DNA insert is in opposite orientation to the *lac*Z promoter in pUC 19, and the quantitative expression of the recombinant enzyme was not affected, it can be inferred that the *apu* gene contains an indigenous promoter sequence recognized by *E. coli*, allowing for efficient transcription. In *E. coli* SURE transformants harboring pAPZ 71, approximately a third of the recombinant enzyme was localized in the periplasmic space, while the remainder was located intracellularly. The occurrence of amylopullulanase in the periplasmic space indicates the presence of a putative signal sequence on the *apu* gene, identified and processed by *E. coli*, although at low efficiency.

The molecular weight of the expressed recombinant enzyme from pAPZ 71 as determined by SDS-PAGE analysis, was 160,000, which was 20 kDa higher than the molecular weight observed for the native enzyme from *C. thermohydrosulfuricum*. Therefore, a minimum coding region of 4.4 kbp, within the 6.1 kbp DNA insert is necessary for the expression of the enzyme. The expressed enzyme maintained amylopullulanase activity, and thermostability at 90°C with a half-life of 40 min.

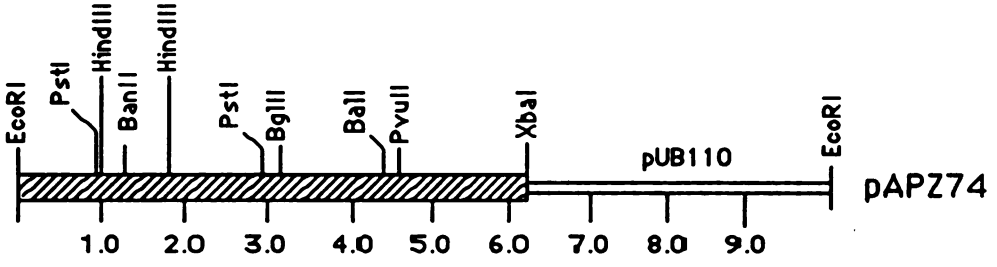


Figure 5. **Physical map of the pAPZ 74 subclone in *Bacillus subtilis* NA-1.** pUB 110 was used as the subcloning vector. The DNA insert is depicted by the hatched area.

B. subtilis NA-1 harboring pAPZ 74 expressed amylopullulanase constitutively and secreted the enzyme into the growth medium. Growth of the host into the late stationary phase was necessary to obtain optimal yields of the recombinant enzyme. Secretion of amylopullulanase by *B. subtilis* suggests the recognition of the putative secretion signal sequence of *apu* by the host. The quantitative amounts of recombinant enzyme expressed by individual subclones are given in Table 2.

Table 2. Activity[a] and location of recombinant amylopullulanase isolated from *E. coli* and *B. subtilis* .

Plasmid construct and host	Activity (U/ml)		
	supernatant	periplasmic	intracellular
pAPZ71 in *E. coli* SURE	<0.01	0.17	0.8
pAPZ74 in *B. subtilis* NA-1	4.2	--	0.23

[a] pullulanase activity is shown

The native and recombinant amylopullulanase in *E. coli* and *B. subtilis* displayed similar pH stability (pH 6.0) and activity (pH 5.5) optima (data not shown). Ca^{2+} (up to 5 mM) was necessary for the stability of both recombinant enzymes, which was similar to that observed for the native enzyme. Product analysis by thin-layer chromatography for polysaccharides

and oligosaccharides were similar for both native and cloned enzymes, with maltose, maltotriose, and maltotetraose being the products formed upon hydrolysis of glycogen, soluble starch, amylopectin, and amylose. Glucose was not observed in any of the polysaccharide product hydrolysates (data not shown).

When pAPZ 71 plasmid DNA was subcloned into *E. coli* strains HB101, DH5α, and TG-1, spontaneous deletions occurred within the recombinant plasmid, resulting in loss of activity. Restriction enzyme analysis of the deletion mutants identified an approximately 2.5 kbp *Eco*RI-*Pst*I region within pAPZ 71, which was prone to deletion upon introduction into *E. coli* HB101, DH5α, or TG-1.

In order to stabilize the *apu* gene within these hosts for further DNA manipulations, construction of fusion proteins with the β-galactosidase N-terminal encoded by the lacZ region in pUC18 vector was investigated. Several subcloning experiments were performed where pAPZ 71 DNA was partially digested with *Hind*III, and ligated into pUC18. Two subclones harboring DNA inserts of 5.3 kbp and 4.5 kbp and expressing amylopullulanase activity were isolated (Fig. 6). Recombinant plasmids pAPZ 72 and pAPZ 73, with 5.3kbp and 4.5 kbp DNA inserts respectively, were expressed constitutively in hosts *E. coli* SURE, HB 101, and DH5α. However, in *E. coli* TG-1, which overexpresses lac I protein, the presence of IPTG (1 mM) in the growth medium was necessary for expression of both pAPZ 72 and pAPZ 73 amylopullulanase fusion proteins, suggesting that in pAPZ 72 and pAPZ 73, transcription is under the control of the *lac*Z promoter of the pUC 18 vector (Table 3). In *E. coli* harboring either pAPZ72 or pAPZ73, amylopullulanase activity was located intracellularly. These data enabled identification of the orientation of the *apu* gene within the recombinant

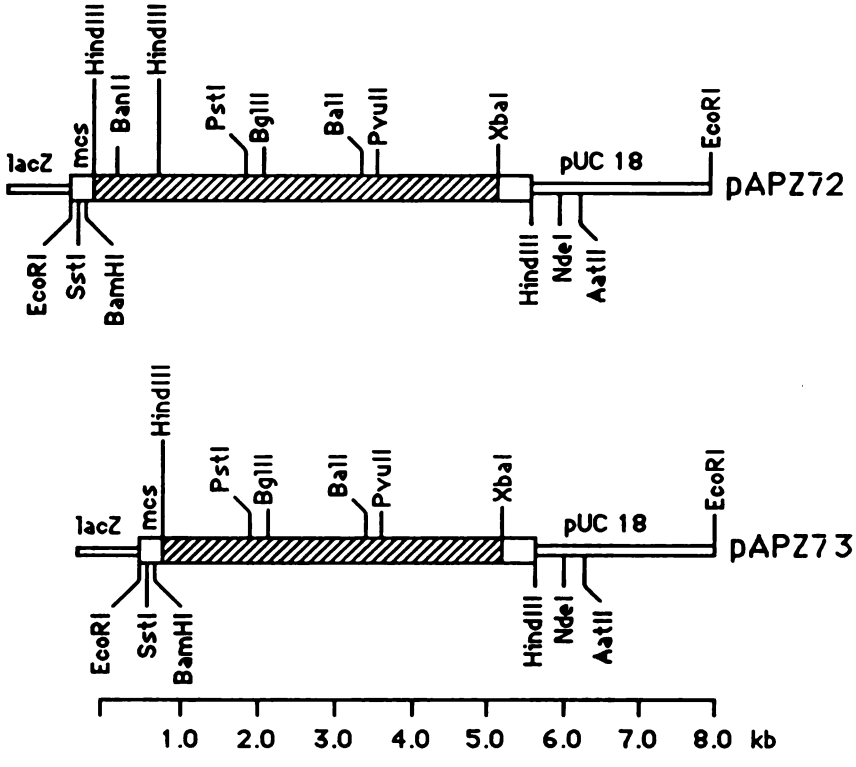


Figure 6. **Construction of fusion proteins containing the amylopullulanase gene from *C. thermohydrosulfuricum* 39E.**
The DNA insert is depicted by the area of shading, and the open region represents the plasmid DNA. pAPZ72, and pAPZ73 are plasmids used to transform *E. coli* HB 101, DH5α and DH5αF'. mcs = multicloning site. Note, the lac Z promoter region and multicloning site are not drawn to scale

Table 3. Activity*, location, induction and thermostability of recombinant constructs.

Plasmid construct	*E. coli* strain	Activity (U/ml)			IPTG (mM)	t1/2 (min) 85°C
		Intracellular	Periplasmic	Extracellular		
pAPZ 71	SURE	0.8	0.17	<0.01	0	>30
pAPZ 72	HB101	4.6	<0.01	<0.01	0	>30
pAPZ 72	TG-1	0.15	<0.01	<0.01	0	-
pAPZ 72	TG-1	2.9	<0.01	<0.01	1.0	>30
pAPZ 73	HB101	0.3	<0.01	<0.01	0	<5
pAPZ 73	TG-1	<0.01	<0.01	<0.01	0	-
pAPZ 73	TG-1	0.12	<0.01	<0.01	1.0	<5

*Pullulanase activity is shown.

plasmids, and the location of the putative promoter region of *apu* gene and the putative secretion signal sequence encoded by the gene, within the *Eco*RI-*Hind*III region of the 6.1 kbp chromosomal DNA insert.

Purification and characterization of recombinant amylopullulanase

Recombinant amylopullulanase was purified from *E. coli* and *B. subtilis* and characterized with respect to physical and biochemical properties. A summary of the purification procedure is shown in Tables 4 and 5. Amylopullulanase was purified 16-fold from *E. coli* and 14-fold from *B. subtilis*.

Analysis of catalytic and thermostability regions

In order to further characterize the 6.1 kbp DNA insert containg the *apu* gene, specifically to identify the gene region encoding peptide regions necessary for thermostability and catalysis, nested deletion mutants were constructed from 5′ and 3′ directions of the 5.3 kbp DNA insert of pAPZ72. Nested deletion mutants obtained from pAPZ 72 were tested for their expression of amylopullulanase activity, as well as for thermostability. Under the conditions used, deletion of approximately 217 bp could be obtained per min (Fig. 7). It was possible to restrict the 5.3 kbp gene insert to a size of 2.9 kbp without any quantitative loss of activity or thermostability. The mutant plasmid containing the 2.9 kbp residual insert was denoted pAPZ 75 (Fig. 8). The enzymes purified from individual subclones are shown in Fig. 9. The thermostability of individual subclones are given in Table 3. These results allowed location of the *apu* gene segment within the 6.1 kbp DNA insert which encoded the thermostable domain of amylopullulanase involved in catalysis. Further deletions in to the gene, enabled the identification of a 2.0

Table 4. Purification of recombinant amylopullulanase from *E. coli* SURE[a].

Step	Activity (U)	Specific activity (U/mg)	Yield	Purification (fold)
cell free extract	46,300	29	100	1.0
heat treat (85°, 15 min)	37,170	53	81	1.8
Q Sepharose anion exchange	21,580	124	46	4.3
Superose 12 gel filtration	14,640	212	31	7.3
β-cyclodextrin affinity	9,110	468	20	16.1

[a]Pullulanase activity was monitored during the purification scheme

Table 5. Purification of recombinant amylopullulanase from *B. subtilis* NA-1[a].

Step	Activity (U)	Specific activity (U/mg)	Yield	Purification (fold)
culture supernatant	38,440	31	100	1.0
Amicon YM30 ultrafiltration	24,290	46	63	1.5
heat treat (85°, 15 min)	10,250	71	27	2.3
Q Sepharose anion exchange	5,860	102	15	3.3
Superose 12 gel filtration	3,670	190	10	6.1
β-cyclodextrin affinity	2,240	435	6	14.0

[a]pullulanase activity was monitored during the purification

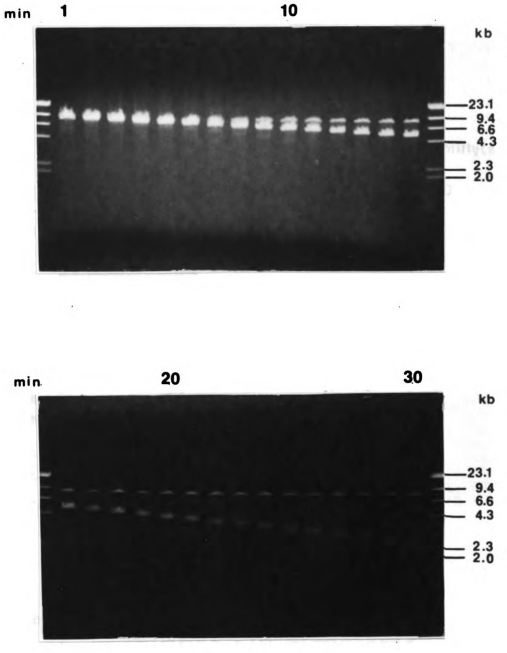


Figure 7. **Agarose gel electrophoresis pattern of nested deletion mutants constructed from the 3' direction.** The reaction time is indicated above the individual lanes in minutes. lambda/ *Hind*III DNA size markers are indicated on either side of the agarose gels.

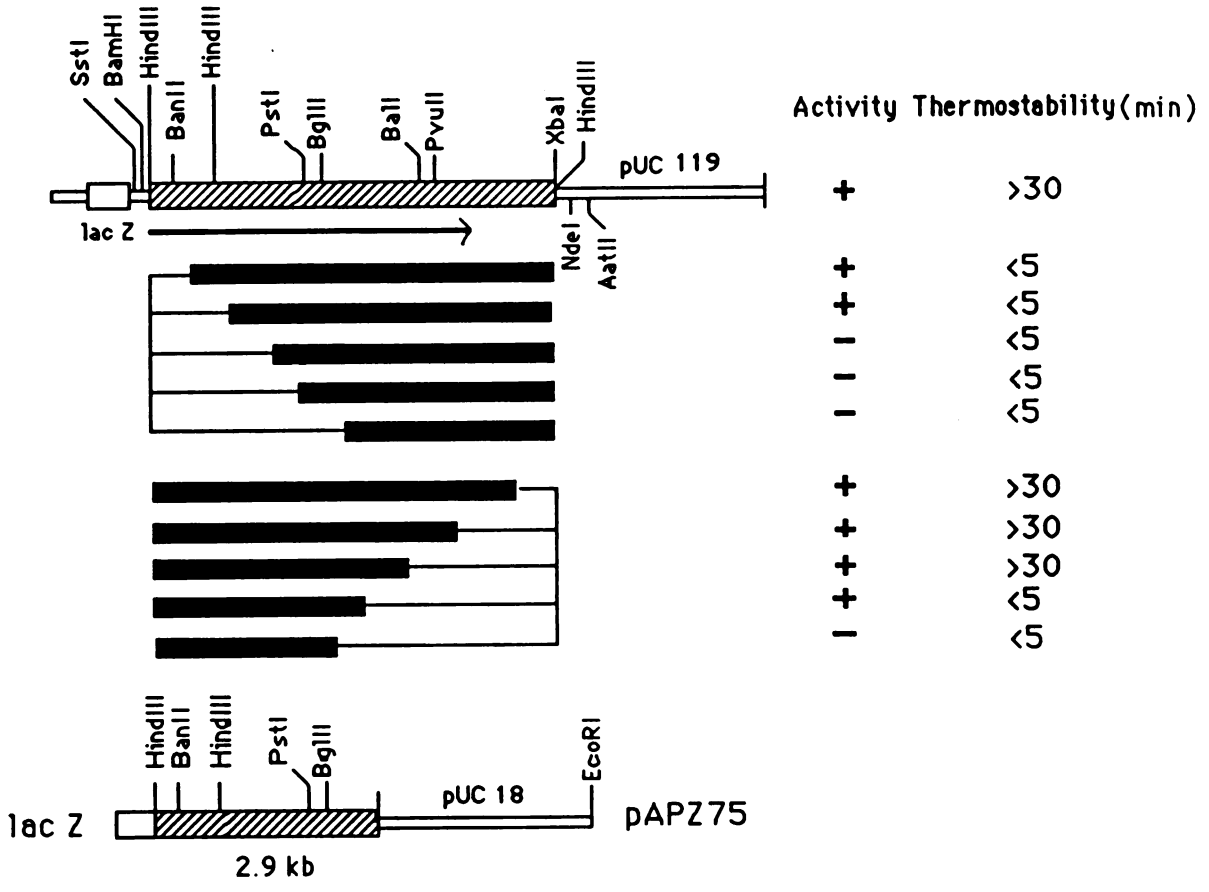


Figure 8. **Construction of nested deletion mutants of lacZ construct of pAPZ 72.** For deletion from the 5' direction, double digestion with *Sst*I and *Bam*HI was used. For deletion from 3' direction, double digestion with *Aat*II and *Nde* I was used. Amylopullulanase activity and thermostability was determined as described in the Materials and Methods. Thermostability was tested at 85°C.

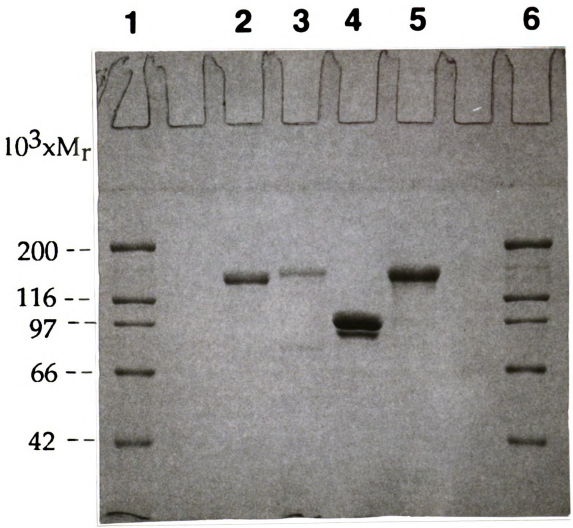


Fig. 9. SDS-PAGE (7.5% cross-linked) analysis of recombinant amylopullulanase purified from *E. coli* and *B. subtilis*.

lane 1,6 = high molecular weight standards
lane 2 = native amylopullulanase (M_r 140,000)
lane 3 = recombinant amylopullulanase (M_r 160,000) isolated from the periplasmic space of *E. coli* SURE harboring pAPZ71.
lane 4 = recombinant amylopullulanse (M_r 100,000) isolated from *E. coli* DH5α harboring pAPZ 75. The presence of a minor protein band below the Mr 100,000 protein is due to *in vivo* protease digestion of the recombinant enzyme.
lane 5 = recombinant amylopullulanase secreted by *B. subtilis* harboring pAPZ 74.

kbp region within the *Hind*III-*Bal*I sites of the gene, which imparted amylopullulanase activity, but not thermostability.

^{13}C NMR analysis

In order to determine the ability of amylopullulanase to hydrolyze α-1,6 bonds in branched polysaccharides, ^{13}C NMR analysis of product hydrolysates upon action by the Mr 100,000 recombinant thermostable enzyme encoded by the 2.9 kbp insert in pAPZ75, was performed. Prior studies (Mathupala *et al.*, 1990) had shown the ability of the native enzyme from C. *thermohydrosulfuricum* 39E to hydrolyze branched polysaccharides, i.e. mammalian and oyster glycogen, and amylopectin. To test for catalytic action by amylopullulanase against α-1,6 glucosidic bonds, mammalian glycogen was chosen, due to it's higher solubility in D_2O. ^{13}C NMR of maltose and isomaltose are included as standards (Fig. 10).

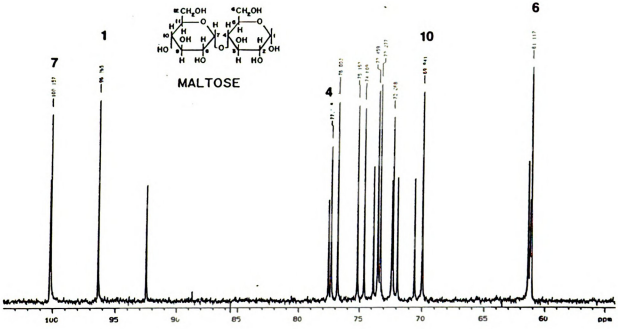
^{13}C NMR was used to clearly identify α-1,6 bond and α-1,4 bond cleavage in glycogen (Fig. 11). The C_7 of α-1,4 and α-1,6 bonds in glycogen were assigned signals at 101.0 ppm and 99.8 ppm respectively. The signal for C_1 position was absent in glycogen since reducing ends are present in minute quantities in the unhydrolyzed polymer. Upon hydrolysis by recombinant amylopullulanase, the reaction products gave signals at 100.4 ppm (C_7 of α-1,4 bond) and 96.4 ppm (C_1 of reducing carbon)(Fig. 11). A signal corresponding to C_7 of α-1,6 bond was not present in the spectrum (98 ppm), indicating efficient hydrolysis of the branch points of glycogen by the recombinant amylopullulanase.

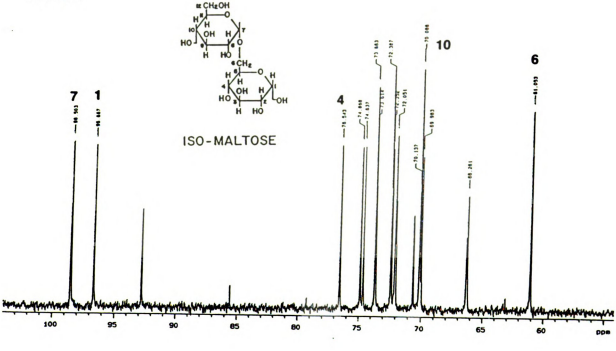
The results from NMR analysis, where the signals corresponding to the C_7 of α1,6 bonds were removed upon enzymatic action, indicates the ability of amylopullulanase to hydrolyze α-1,6 bonds efficiently, and identifies the 2.9

Figure 10. **^{13}C NMR spectra of maltose (A), and iso-maltose (B) standards.** The samples were scanned for 1500 transients. The individual carbon numbers assigned to the NMR signals are given in bold face.
7 = non reducing C1 position of the α-1,4 or α-1,6 glucosidic bond; 1 = C1 reducing carbon position

C_7 of α-1,4 bond ~ 100 ppm

C_7 of α-1,6 bond ~ 98 ppm


MALTOSE
7
1
4
10
6
100
95
90
85
80
75
70
65
60
ppm


ISO-MALTOSE
7
1
4
10
6
100
95
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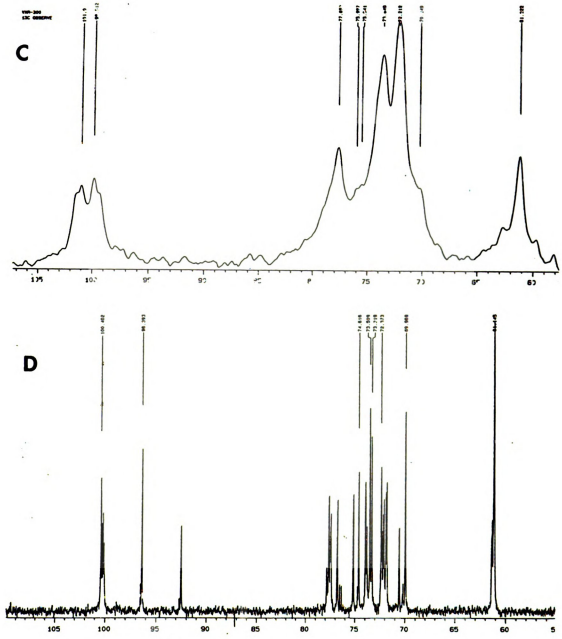
85
80
75
70
65
60
ppm

Figure 11. **^{13}C NMR spectra of glycogen (C), and hydrolysate of glycogen (D).** The glycogen standard was scanned for 10,000 transients, while the product hydrolysate of glycogen were scanned for 1500 transients. 25.0 ml of a glycogen solution (10 mg/ml in D_2O) in 50mM acetate buffer containing 5mM $CaCl_2$ was hydrolyzed with 80 Units of purified amylopullulanase from pAPZ 75 for 48 h at 60°C. Completion of the hydrolysis was inferred by thin-layer chromatography of reaction products, where no retentate was observed for the starting substrate glycogen.


C
D
105
100
95
90
85
80
75
70
65
60
5

kbp region within the *apu* gene as the essential gene motif that encodes the thermostable catalytic domains responsible for the α-1,4 and α-1,6 activities. The presence of α-1,4 bonds in the NMR spectrum of the product hydrolysate is due to the formation of maltose, maltotriose, and maltotetraose upon action by amylopullulanase on glycogen, and the sharper peaks indicate the total hydrolysis of glycogen to maltose, maltotriose, and maltotetraose, as corroborated by TLC.

DISCUSSION

To our knowledge, this represents the first reported study on analysis of activity and thermostability of an amylase by construction of nested deletion mutants into the gene encoding for the enzyme, in order to test the effects of progressive deletion of the gene from both 5′ and 3′ directions, and hence the progressive truncation of the enzyme from both the N- and C-terminal ends. Using this method, we were able to localize a 2.9 kbp region within the 6.1 kbp DNA insert, which translated into a mature peptide of approximately 100,000 molecular weight, that was thermostable and maintained dual activities.

N-terminal analysis of the enzyme purified from pAPZ 71 was identical to that of the native enzyme. The disparity in molecular weight between the native enzyme (M_r 140,000) and the recombinant enzyme in *E. coli* (M_r 160,000) indicates post-translational processing of the C- terminal region of the mature peptide in *C. thermohydrosulfuricum* 39E, or the possibility of specific protease action against the native enzyme under the culture conditions used for isolation of the native enzyme. Although the molecular weight of the mature peptide of amylopullulanase denotes a minimum coding region of 4.4 kbp, the result from nested deletion mutants indicated that the major part of thermostability and activity of the enzyme was provided by a peptide region corresponding to approximately 2.9 kbp. Within this 2.9 kbp region, the DNA motif encoding for the dual activities could be further narrowed to 2.0 kbp. Thus, this starch-degrading enzyme is not a fusion of an α-amylase and a pullulanase as proposed for amylase-pullulanase from *B. circulans* F-2 (Sata *et al.*, 1989) or structured according to a

casette model, as described for α-amylase-pullulanase from *C. thermohydrosulfuricum* E101 (Melasniemi *et al.*, 1990; Sata *et al.*, 1989).

Thermostability of the native and recombinant enzymes were similar, suggesting that the glycan moiety reported for the native enzyme (Saha *et al.*, 1988) is not a contributing factor towards the thermostability of amylopullulanase. Molecular weights of 220,000 (Sata *et al.*, 1989), 165,000 (Melasniemi *et al.*, 1989), 102,000 (Spreinat and Antranikian, 1990), and 105,000 (Coleman *et al.*, 1987) have been reported for thermophilic enzymes exhibiting dual activities isolated from different thermophilic microorganisms. It will be of interest to identify whether these enzymes too could be truncated from N- and C- termini to a smaller molecular weight enzyme product without loss of thermostability or dual activities.

Construction of fusion proteins of amylopullulanase to the lac Z promoter in pUC vectors enabled the overexpression of amylopullulanase activity in *E. coli*, in addition to stabilizing the gene within the *E. coli* hosts. The yield of secreted recombinant amylopullulanase from *B. subtilis* was low, due to the secretion of highly thermostable protease into the culture medium by the host, indicated by the substantial degradation of the recombinant amylopullulanase detected by SDS-PAGE analysis.

NMR analysis indicating that α-1,6 bonds in glycogen are efficiently hydrolyzed by the M_r 100,000 enzyme expressed by the 2.9 kbp truncated gene region, verified the ability of amylopullulanase to cleave α-1,6 linkages in branched polysaccharides, in contrast to the debranching enzyme of *T. brockii*, which could only cleave α-1,4 bonds in starch, although it was capable of hydrolyzing α-1,6 bonds in pullulan (Coleman *et al.*, 1987).

Further studies (Mathupala and Zeikus, manuscript in preparation) in sequencing the 2.9 kbp gene region have enabled the identification of four

conserved peptide regions common to α-amylases which are involved in catalysis and substrate binding. This 2.9 kb region may code for two major domains, similar to the domain structure of α-amylase of *Aspergillus oryzae*, for which the active site and substrate binding subsites are located in the inter-domain region (Matsuura *et al.*, 1988; Boel *et al.*, 1987).

Since the catalytic activity was maintained in nested deletion mutants that lost thermostability, it can be inferred that the loss of thermostability is not due to loss of peptide regions essential for calcium binding, which in α-amylase are located at the catalytic and substrate binding center (Matsuura *et al.*, 1984). The loss of thermostability is possible due to general destabilization of the catalytic domain of amylopullulanase, where part of the peptide contributing to it's tertiary structure is deleted.

Findings in this study suggest the possible use of amylopullulanase in the starch industry, to replace the current technology involving α-amylase and pullulanase in the stepwise process for the production of conversion syrups. Similar studies for determination of the gene regions encoding the domains necessary for thermostability and activity within glucoamylase, an industrially important amylase for conversion of oligosaccharides to glucose, may enable the construction of a multifunctional fusion protein using the thermostable catalytic domains from both glucoamylase and amylopullulanase, for single step conversion of raw starch to glucose, for use in bioprocessing.

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CHAPTER 5

SEQUENCING OF THE AMYLOPULLULANASE (*apu*) GENE OF *Clostridium thermohydrosulfuricum* 39E, AND IDENTIFICATION OF THE ACTIVE SITE BY SITE DIRECTED MUTAGENESIS.

ABSTRACT

The complete nucleotide sequence of the gene encoding the dual active amylopullulanase of *C. thermohydrosulfuricum* 39E was determined. The structural gene (*apu*) contained a single open reading frame 4443 bp in length, which corresponds to 1481 amino acids, with an estimated molecular weight of 162,780 for the deduced amino acid sequence. The 5' region preceding the structural gene contained sequences similar to the *E. coli* consensus promoter sequences, and a sequence similar to the consensus sequence for binding of the cAMP-CAP complex of *E. coli*. The N-terminal of the deduced primary sequence contained a putative 31 amino acid signal sequence, which may be involved in cell-surface localization of the mature peptide in *C. thermohydrosulfuricum* 39E. Analysis of the deduced sequence of *apu* gene with sequences of α-amylases and α-1,6 debranching enzymes enabled the identification of four conserved regions putatively involved in substrate binding and in catalysis. The conserved regions were localized within a 2.9 kbp gene fragment, which encoded a M_r 100,000 protein product that maintained the dual activities and thermostability of the native enzyme. Oligonucleotide directed mutants constructed within the 2.9 kbp gene segment enabled the identification of three acidic amino acid residues, located within the second, third and fourth conserved regions, that were involved in α-1,4 and α-1,6 glucosidic bond cleavage. Asp_{597}, Glu_{626}, and Asp_{703} were individually modified to their respective amide form, or the alternate acid form, and in all cases both α-amylase and pullulanase activities were lost, suggesting the involvement of three residues in catalysis, and the presence of a single catalytic site within the enzyme and substantiated amylopullulanase as a new enzyme class.

INTRODUCTION

Enzymes harboring pullulanase and α-amylase activity have recently been reported from various bacteria (Sakano *et al.*,1982; Coleman *et al.*, 1987; Plant *et al.*, 1987; Takasaki, 1987; Melasniemi, 1988; Saha *et al.*, 1988; Sata *et al.*, 1989; Spreinat and Antranikian, 1990). The dual activity of an amylase from *B. subtilis* is reported to be due to two individual enzymes forming a complex dimer of 450,000 (Takasaki, 1987). An amylase-pullulanase enzyme of M_r 220,000 produced by *B. circulans* F-2, which has α-amylase and pullulanase activities at equivalent rates, has been shown to contain two active sites for the individual activities (Sata *et al.*, 1989). α-amylase-pullulanase from *C. thermohydrosulfuricum* E101 has been reported to be structured similar to a cassette model, where one half of the enzyme codes for α-amylase activity while the other half encodes pullulanase activity, based on sequence analysis studies (Melasniemi *et al.*, 1990).

The amylopullulanase of *C. thermohydrosulfuricum* 39E has been purified to homogeneity, and is a monomer (M_r 140,000), with a catalytic optimum of 90°C (Saha *et al.*, 1988). Ca^{2+} is required for the stability of the enzyme, which has a half-life of 40 minutes at 90°C (pH 6.0). Amylopullulanase is an enzyme exhibiting both α-amylase and pullulanase activities, giving rise to α-1,4 and α-1,6 glucosidic bond cleavage in starch and related polysaccharides (Mathupala *et al.*, 1990). The gene encoding amylopullulanase, designated *apu*, has been cloned and expressed in *Escherichia coli* and *Bacillus subtilis* (Mathupala and Zeikus, manuscript submitted for publication). In *E. coli*, the enzyme was located in the intracellular and periplasmic spaces, while in *B. subtilis* the recombinant enzyme was secreted to the culture supernatant. The expressed recombinant

protein in *E.coli* had a M_r of 160,000. The identity of the recombinant protein was verified by N-terminal amino acid sequencing, which was identical with the native enzyme. Nested deletion mutants constructed from the chromosomal DNA insert containing the *apu* gene enabled restriction of the putative coding region to a size of 2.9 kbp, with concomitant decrease of the molecular weight of the expressed gene product from 160,000 to 100,000, without loss of dual activities or thermostability.

The purpose of this study was to sequence the *apu* gene and compare the deduced amino acid sequence with known amino acid sequences of α-amylases and debranching enzymes, in order to identify the conserved regions and the catalytic residues. In order to test the hypothesis that a single active site imparts dual activity, site directed mutagenesis of the amino acids putatively identified as being involved in catalysis was performed and the effect on amylopullulanase activity determined.

MATERIALS AND METHODS.

Reagents

All chemicals were of molecular biology or analytical grade and were obtained from Aldrich Chemical Co. (Milwaukee, WI), or Sigma Chemical Co. (St. Louis, MO).

Bacterial strains and plasmids

E. coli strain DH5αF' [F^- φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK^- mK^+) supE44 l^- thi-1 gyrA96 relA1] was obtained from Bethesda Research Laboratories (Gaithersburg, MD). *E. coli* TG-1 [supE hsdD5 thi Δ(lac-proAB) F' (traΔ36 $proAB^+$ $lacI^q$ lacZΔM15)] was obtained from Amersham Co. (Arlington Heights, IL). Phagemid vector pUC 119 and helper phage M13KO7 were obtained from Dr. T. Freidman of Michigan State University. Plasmids pAPZ 71 (containing the complete *apu* gene) in *E. coli* SURE, and pAPZ 72 (containing a fusion construct of *apu* gene to the lac Z promoter in pUC 18) in *E. coli* DH5α, are the parental plasmids used in this work (Mathupala and Zeikus, manuscript submitted for publication).

Enzymes

Restriction enzymes were obtained from Bethesda Research Laboratories, United States Bochemical Co. (Cleveland, OH), or Boehringer Mannheim Biochemicals (Indianapolis, IN).

Oligonucleotides

Oligonucleotides were synthesized in an Applied Biosystems model 380A DNA synthesizer at the Macromolecular Facility, Department of

Biochemistry, Michigan State University. Purification of the oligonucleotides used in site directed mutagenesis was performed using thin layer chromatography (Sure Pure oligonucleotide purification kit; United States Biochemicals, Cleveland, OH) and subsequently 5' phosphorylated using T4 polynucleotide kinase, for construction of oligonucleotide directed mutants (Oligonucleotide-directed *in vitro* mutagenesis system version 2.1, Amersham Co, Arlington Heights, IL).

Enzyme assays

For assay of pullulanase activity, 160 µl of 1.25% (w/v) pullulan (for pullulanase activity) in 50 mM acetate buffer, pH 6.0, containing 5 mM $CaCl_2$ and 40 µl of enzyme solution were mixed and incubated at 60°C for 30 min. The reaction was stopped by adding 0.8 ml of dinitro salicylate (DNS) solution (Miller, 1959), and heated in a boiling water bath for 15 min. The samples were cooled on ice and the absorbance of the reaction solution measured at 640 nm. One unit of pullulanase activity is defined as the amount of enzyme which produces 1 µmol of reducing sugar (with glucose as the standard) per min under the assay conditions.

For the assay of α-amylase activity, 160 µl of 1.25% (w/v) soluble starch in 50 mM acetate buffer, pH 6.0, containing 5 mM $CaCl_2$ and 40 µl of enzyme solution were mixed and incubated at 60°C for 30 min. The reaction was stopped by adding 0.8 ml of DNS solution, and heated in a boiling water bath for 15 min. The samples were cooled on ice and the absorbance of the reaction solution measured at 640 nm. One unit of α-amylase activity is defined as the amount of enzyme that produces 1 µmol of glucose per min under the assay conditions described.

Protein determination and gel electrophoresis

Protein concentrations were determined using the Bio-Rad protein assay kits based on the dye binding assay of Bradford (1976) (Bio-Rad Laboratories, Richmond, CA), or using bicinchoninic acid (BCA Assay Kit, Pierce Co., Rockford, IL), using bovine serum albumin as standard. SDS-PAGE was performed according to the method of Laemmli (1970) using 7.5% polyacrylamide gels in a Mini-Protean II apparatus (Bio-Rad Laboratories), and protein bands were visualized by staining with Coomassie Brilliant Blue R-250. The molecular weights of the recombinant proteins were determined using high range molecular weight standards (Bio-Rad Laboratories, Richmond, CA) containing myosin (200,000), β-galactosidase (116,250), phosphorylase b (97,400), bovine serum albumin (66,200), and ovalbumin (42,700).

Preparation of *E. coli* competent cells and transformation

E. coli cells were made competent by the method by Hanahan (1983), as described by Perbal (1988). Transformation of competent cells were carried out as follows: 1 µl of the ligation reaction (10 ng of DNA) was mixed with 20 µl of competent cells and incubated on ice for 30 min in 1.5 ml Eppendorf microcentrifuge tubes. The mixture was heat shocked for 40 s at 42°C to 44°C and placed on ice for 2 min. 80 µl of cold SOC medium (Hanahan, 1983) was added and the transformation mixture incubated at 37°C for 1 h in a rotary shaker at 225 rpm. Transformants harboring recombinant pUC 119 plasmids were selected by plating the transformation sample onto 2 x YT agar (Sambrook *et al.*, 1989) containing 50 µg/ml ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-gal) and isopropyl-β-D-thiogalacto pyranoside (IPTG) as described by Rodriguez and Tait (1983).

Construction of nested deletion mutants for sequencing

pAPZ 72 DNA, which contained a 5.4 kbp *apu* gene fragment (Mathupala and Zeikus, manuscript submitted for publication), was linearized with *Xba*I and the 5.4 kbp fragment isolated from an agarose gel (0.8% w/v) by electroelution. The 5.4 kbp DNA fragment was ligated into phagemid vector pUC119 linearized with *Xba*I and transformed into *E. coli* DH5αF' competent cells. Directional subclones of the insert within pUC119 were identified by restriction mapping using the unique *Sal*I and *Sph*I restriction enzyme sites (originating from the respective multicloning sites on the pUC18 donor plasmid and pUC119 recipient plasmid) within the recombinant pUC119 vector. Inserts in opposite orientations were denoted pAPZ118 and pAPZ119. The individual plasmids were double digested with *Sst*I and *Bam*HI and used to create nested sets of deletion mutants (Ausubel *et al.*, 1988) (Fig. 1). Single stranded DNA generated from the deletion mutants by superinfection of phagemid containing DH5αF' cells with helper phage M13KO7, was used to deduce the DNA sequence of the amylopullulanase gene by the dideoxy chain termination method (Sanger, 1977). The regions external to the 5.4 kbp *apu* DNA fragment were sequenced by double stranded DNA sequencing of pAPZ 71 plasmid DNA using synthetic oligonucleotides.

Production of single stranded DNA from phagemid vectors

Helper phage M13KO7 was grown and purified as described previously (Sambrook *et al.*, 1989). For production of single stranded DNA, a fresh bacterial colony (grown on 2 x YT agar containing 50 µg/ml ampicillin) harboring pUC 118 or pUC 119 recombinant phagemid containing the 5.4 kbp *apu* gene insert (from pAPZ 72) was suspended in a 15 ml culture tube (Corning, New York, NY) containing 3 ml of 2YT (Sambrook *et al.*, 1989)

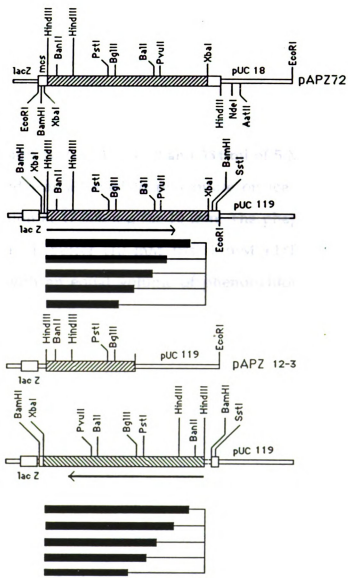


Figure 1. **Nested deletion mutant constructs used for sequencing the *apu* gene.** The hatched area represents *C. thermohydrosulfuricum* 39E DNA insert; mcs = multicloning site. The solid areas represent part of the *C. thermohydrosulfuricum* 39E DNA insert after nested deletion mutations.

media. The medium was adjusted to 100µg/ml with ampicillin and 30µl of helper phage M13KO7 (2 x 10^9 pfu/ml) was added to a final concentration of 2 x 10^7 pfu/ml. The tubes were incubated at 37°C, at 250 rpm for 1.5 h, or until slightly turbid. Kanamycin was added to a final concentration of 70µg/ml and incubation continued for a further 12 to 14 h more at 300 rpm, at 37°C. The culture was centrifuged at 17,000 x g for 10 min at 4°C, and the supernatant recovered. The phagemid particles were precipitated by adding to the 3 ml of supernatant, 334 ml of 40% (w/v) PEG-8000 and 334 ml of 5 M sodium acetate (pH 7.0). The phagemid was allowed to precipitate on ice for 15 min, and collected by centrifugation at 17,000 x g for 10 min. The phagemid pellet was resuspended in 0.3 ml TE buffer (10 mM tris, 1 mM EDTA, pH 8.0) and extracted three times with an equal volume of phenol-chloroform (3:1 v/v) by gentle mixing for 5 min. After a final extraction with an equal volume of chloroform-isoamyl alcohol (24:1 v/v), the single stranded DNA was precipitated with 0.1 volume of 3M sodium acetate and 2.5 volume of ethanol, by chilling at -70°C for 30 min, and subsequent centrifugation for 30 min. The DNA was redissolved in 10 µl of water and used for DNA sequencing or site directed mutagenesis.

DNA sequencing

Sequenase V.2.0 DNA polymerase and Sequenase V.2.0 sequencing kit from United States Biochemicals were used for DNA sequencing. For single stranded DNA sequencing, the protocol as described by the manufacturer was used (Instruction manual, Sequenase V.2.0, United State Biochemicals). For double stranded DNA sequencing, denaturation of double stranded plasmid DNA was performed as described by Zhang *et al.*, (1988). For sequencing reactions where lac Z fusion constructs were involved (pAPZ 119), universal

M13/pUC forward sequencing primer was used. For double stranded DNA sequencing, within the 5′ EcoRI-HindIII region of the 6.1 kb DNA insert of the original recombinant plasmid pAPZ 71, synthetic oligonucleotides were used as primers.

Sequence analysis

The amino acid sequence inferred from the amylopullulanase DNA sequence was compared with the primary structures of α-amylases and pullulanases available through GenBank (IntelliGenetics Inc., Mountain View, CA). GCG Sequence Analysis Software Package V.7.0 (Devereux *et al.*, 1984) was used in the analysis and multiple sequence alignment and subsequent data manipulations.

Oligonucleotide directed mutagenesis of amylopullulanase gene

Oligonucleotide-directed in vitro mutagenesis system V.2.1 from Amersham Co. (Arlington Heights, IL) was used in constructing single point base mutants (Nakamaye & Eckstein, 1986). *E. coli* TG-1 was transformed with a nested deletion mutant, containing an *apu* gene fragment of 2.9 kb, denoted pAPZ 12-3 (in pUC 119), which maintained both α-amylase and pullulanase activities, as well as thermostability. This was used to generate single stranded DNA, using M13KO7 helper phage, for use as the template for oligonucleotide directed mutagenesis. The synthetic oligonucleotide primers were designed to be complementary to the single stranded template DNA and contained the appropriate single point base mismatches as shown in Fig. 2.

Following in vitro mutagenesis, plasmid DNA was isolated from *E. coli* TG-1 transformants which did not express amylopullulanase activity. The 0.9 kbp *Kpn*I-*Bgl*II DNA fragment was isolated from the plasmids by double

		597	
Template DNA	5′	**C TGG AGA TTG GAT GTT GCA AA- --- -**	3′
pAPZ 76		**Trp Arg Leu Asp Val Ala**	
Primer Asp-Asn	3′	**G ACC TCT AAC TTA CAA CGT TT- --- -**	5′
GZ 52			
Primer Asp-Glu	3′	**- -CC TCT AAC CTT CAA CGT TTT AC- -**	5′
GZ 69			
		626	
Template DNA	5′	**A ATG ATT GCA GAA CTT TGG GGA GA**	3′
pAPZ 76		**Met Ile Ala Glu Leu Trp Gly**	
Primer Glu-Gln	3′	**T TAC TAA CGT GTT GAA ACC CC- --**	5′
GZ 56			
Primer Glu-Asp	3′	**- TAC TAA CGT CTA GAA ACC CCT CTA CGA**	5′
GZ 88			
		703	
Template DNA	5′	**TTA GGT TCT CAT GAC ACC ATG AGA ATA**	3′
PAPZ 76		**Leu Gly Ser His Asp Thr Met Arg Ile**	
Primer Asp-Asn	3′	**--T CCA AGA GTA TTG TGG TAC TC- ---**	5′
GZ 55			
Primer Asp-Glu	3′	**--- -CA AGA GTA CTC TGG TAC TCT T--**	5′
GZ 68			

Figure 2. **Synthetic oligonucleotide primers used in oligonucleotide directed mutagenesis of active site amino acids.** Plasmid pAPZ 76 is identical to pAPZ 12-3 (in pUC 119) except for growth in *E. coli* TG-1

digestion with *Kpn*I and *Bgl*II, followed by agarose 1.2% (w/v) gel electrophoresis and electroblotting onto NA-45 membranes (Schleicher & Schuell, Inc., Keene, NH). pAPZ75 plasmid DNA lacking the 0.9 kbp *Bgl*II-*Kpn*I fragment was prepared by digestion with *Bgl*II and *Kpn*I, followed by agarose gel electrophoresis and electroelution (Elutrap, Schleicher & Schuell). The individual 0.9 kbp DNA fragments recovered from the site directed mutants were ligated to the linearized vector pAPZ75 lacking the 0.9 kbp DNA region, and transformed into *E. coli* TG-1. Single stranded DNA was recovered from the transformants by infection with M13KO7 helper phage as described previously. Nucleotide sequence of the single point base mutants were confirmed by sequencing the 0.9 kbp *Kpn*I-*Bgl*II region using synthetic oligonucleotides.

Enzyme purification

E. coli TG-1 harboring the pAPZ 12-3 recombinant plasmids containing the specific single point base mutations were grown at 37°C in 5.0 ml of LB media containing ampicillin (50 μg/ml) and IPTG (1 mM). Cells were harvested by centrifugation in a microcentrifuge (14,000 rpm x 1 min) and the cell pellet sonicated in 1.0 ml of 50 mM acetate buffer (pH 6.0) containing 5 mM $CaCl_2$. 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 α-amylase and pullulanase activity.

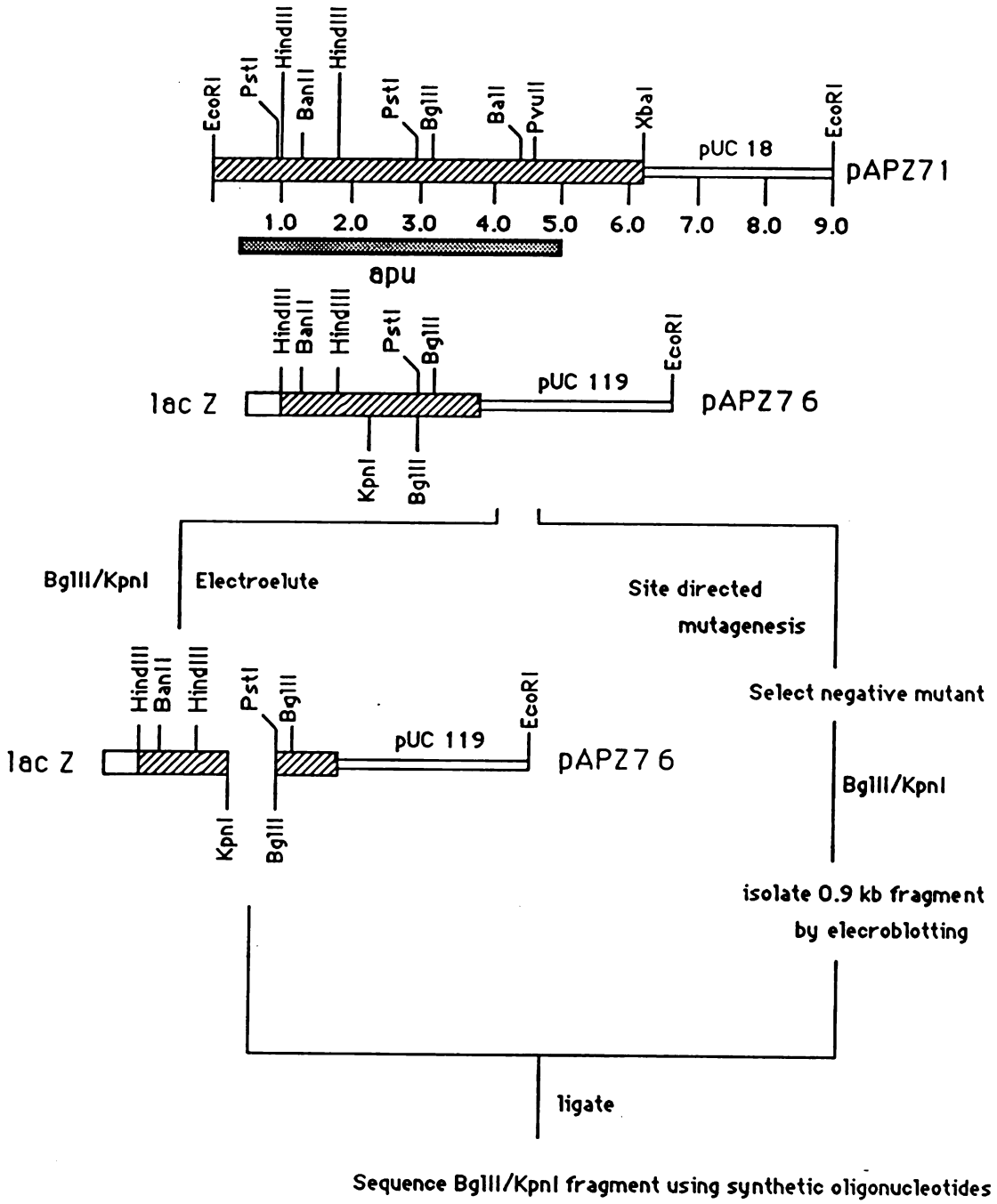


Figure 3. **Strategy for constructing oligonucleotide directed mutants.** The hatched area represents *C. thermohydrosulfuricum* 39E DNA insert; apu represents the gene encoding amylopullulanase activity.

RESULTS

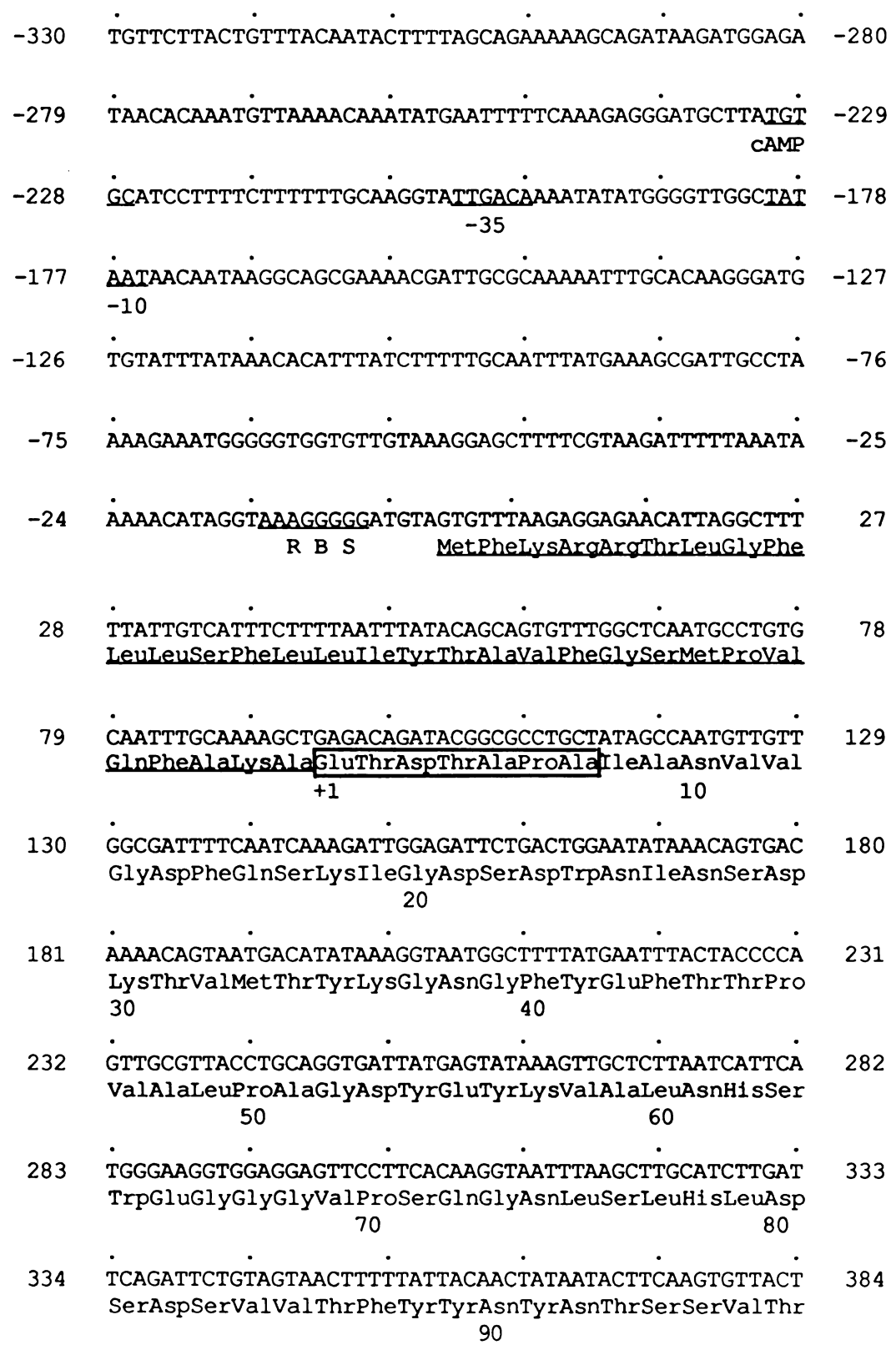
Subcloning and construction of nested deletion mutants

In order to deduce the primary sequence of the amylopullulanase by sequencing of the 6.1 kbp DNA insert containing the *apu* gene, nested deletion mutants were constructed from the 5′ and 3′ ends of the gene. The nested deletion mutants constructed from subclones pAPZ118 and pAPZ119 are shown in Fig. 1. Since the open reading frame of 5.3 kbp *Hind*III/*Xba*I fragment of the *apu* gene is in the correct orientation to the lac Z promoter of the pUC119 vector, and in the correct reading frame, amylopullulanase was expressed by *E. coli* harboring the pAPZ119 or harboring mutants constructed by deletion from the 3′ end of the gene. In pAPZ118 however, the gene was in the opposite orientation to the lac Z promoter, and therefore, the enzyme was not expressed.

Nucleotide sequence of *Clostridium thermohydrosulfuricum* 39E *apu* gene.

In order to deduce the primary sequence of amylopullulanase, a 5 kbp region within the 6.1 kbp chromosomal DNA insert was sequenced (Fig. 4). The 5000 bp segment contained an open reading frame of 4443 bp, starting at nucleotides 331 to 333 with a GTG codon, and terminated at 4773 bp, followed by termination codons at 4774 bp (TGA) and at 4796 bp (TAA) positions. The reading frame deduced from the DNA sequence was confirmed by N-terminal amino acid sequencing, which was identical to the first seven amino acids of the native enzyme (Mathupala and Zeikus, manuscript submitted for publication). Therefore, the predicted primary sequence encoded by the open reading frame corresponds to 1450 amino acids with an estimated molecular weight of 162,780, which agrees closely with the M_r of 160,000 obtained by

Figure 4. **Nucleotide sequence and the deduced aminoacid sequence of *apu* gene of *C. thermohydrosulfuricum* 39E.** cAMP = sequence resembling the consensus sequence for binding of cAMP-CAP complex of *E. coli*. RBS = putative ribosome binding site. -35 = sequence similar to -35 consensus promoter sequence of *E. coli*. -10 = sequence similar to -10 consensus promoter sequence of *E. coli*. The initial underlined deduced amino acid sequence refers to the putative signal sequence. Box represents the N-terminal amino acid sequence of the processed protein.


-330 TGTTCTTACTGTTTACAATACTTTTAGCAGAAAAAGCAGATAAGATGGAGA -280
-279 TAACACAAATGTTAAAACAAATATGAATTTTTCAAAGAGGGATGCTTATGT -229
cAMP
-228 GCATCCTTTTCTTTTTTGCAAGGTATTGACAAAATATATGGGGTTGGCTAT -178
-35
-177 AATAACAATAAGGCAGCGAAAACGATTGCGCAAAAATTTGCACAAGGGATG -127
-10
-126 TGTATTTATAAACACATTTATCTTTTTGCAATTTATGAAAGCGATTGCCTA -76
-75 AAAGAAATGGGGGTGGTGTTGTAAAGGAGCTTTTCGTAAGATTTTTAAATA -25
-24 AAAACATAGGTAAAGGGGGATGTAGTGTTTAAGAGGAGAACATTAGGCTTT 27
R B S MetPheLysArgArgThrLeuGlyPhe
28 TTATTGTCATTTCTTTTAATTTATACAGCAGTGTTTGGCTCAATGCCTGTG 78
LeuLeuSerPheLeuLeuIleTyrThrAlaValPheGlySerMetProVal
79 CAATTTGCAAAAGCTGAGACAGATACGGCGCCTGCTATAGCCAATGTTGTT 129
GlnPheAlaLysAlaGluThrAspThrAlaProAlaIleAlaAsnValVal
+1 10
130 GGCGATTTTCAATCAAAGATTGGAGATTCTGACTGGAATATAAACAGTGAC 180
GlyAspPheGlnSerLysIleGlyAspSerAspTrpAsnIleAsnSerAsp
20
181 AAAACAGTAATGACATATAAAGGTAATGGCTTTTATGAATTTACTACCCCA 231
LysThrValMetThrTyrLysGlyAsnGlyPheTyrGluPheThrThrPro
30 40
232 GTTGCGTTACCTGCAGGTGATTATGAGTATAAAGTTGCTCTTAATCATTCA 282
ValAlaLeuProAlaGlyAspTyrGluTyrLysValAlaLeuAsnHisSer
50 60
283 TGGGAAGGTGGAGGAGTTCCTTCACAAGGTAATTTAAGCTTGCATCTTGAT 333
TrpGluGlyGlyGlyValProSerGlnGlyAsnLeuSerLeuHisLeuAsp
70 80
334 TCAGATTCTGTAGTAACTTTTTATTACAACTATAATACTTCAAGTGTTACT 384
SerAspSerValValThrPheTyrTyrAsnTyrAsnThrSerSerValThr
90

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                .         .         .         .         .         .
385   GATTCTACAAAATATACACCAATTCCGGAAGAAAAACTTCCAAGAATTGTA   435
      AspSerThrLysTyrThrProIleProGluGluLysLeuProArgIleVal
            100                                 110
                .         .         .         .         .         .
436   GGTACTATACAATCAGCAATAGGAGCAGGTGATGATTGGAAACCTGAAACA   486
      GlyThrIleGlnSerAlaIleGlyAlaGlyAspAspTrpLysProGluThr
                      120                                 130
                .         .         .         .         .         .
487   TCGACAGCTATAATGAGAGACTATAAGTTTAACAATGTTTACGAATACACT   537
      SerThrAlaIleMetArgAspTyrLysPheAsnAsnValTyrGluTyrThr
                                140
                .         .         .         .         .         .
538   GCAAATGTTCCAAAAAGGTATTATGAGTTTAAAGTAACTTTAGGGCCCTCA   588
      AlaAsnValProLysArgTyrTyrGluPheLysValThrLeuGlyProSer
         150                                 160
                .         .         .         .         .         .
589   TGGGATATAAATTATGGCTTAAATGGTGAACAAAATGGTCCAAATATTCCT   639
      TrpAspIleAsnTyrGlyLeuAsnGlyGluGlnAsnGlyProAsnIlePro
                   170                                 180
                .         .         .         .         .         .
640   TTGAATGTAGCCTATGATACTAAGATTACATTTTACTATGATTCGGTTTCA   690
      LeuAsnValAlaTyrAspThrLysIleThrPheTyrTyrAspSerValSer
                              190
                .         .         .         .         .         .
691   CATAATATATGGACAGATTACAATCCACCTCTCACAGGGCCTGATAATAAC   741
      HisAsnIleTrpThrAspTyrAsnProProLeuThrGlyProAspAsnAsn
      200                                 210
                .         .         .         .         .         .
742   ATATATTATGACGATTTAAAACATGACACCCATGACCCATTCTTCCGCTTC   792
      IleTyrTyrAspAspLeuLysHisAspThrHisAspProPhePheArgPhe
               220                                 230
                .         .         .         .         .         .
793   GCTTTCGGTGCAATAAAAACAGGTGATACAGTGACTTTGAGGATACAGGCT   843
      AlaPheGlyAlaIleLysThrGlyAspThrValThrLeuArgIleGlnAla
                            240                                 250
                .         .         .         .         .         .
844   AAAAATCATGACCTTGAGTCAGCTAAAATTTCTTATTGGGATGATATTAAA   894
      LysAsnHisAspLeuGluSerAlaLysIleSerTyrTrpAspAspIleLys
                                  260
                .         .         .         .         .         .
895   AAAACAAGAACAGAAGTCCCGATGTATAAAATTGGTCAAAGTCCTGACGGG   945
      LysThrArgThrGluValProMetTyrLysIleGlyGlnSerProAspGly
            270                                 280
                .         .         .         .         .         .
946   CAATATGAATACTGGGAAGTGAAGTTAAGCTTTGACTATCCCACAAGAATT   996
      GlnTyrGluTyrTrpGluValLysLeuSerPheAspTyrProThrArgIle
                      290                                 300
                .         .         .         .         .         .
997   TGGTATTACTTTATACTTAAAGACGGGACAAAAACTGCTTATTACGGAGAT   1047
      TrpTyrTyrPheIleLeuLysAspGlyThrLysThrAlaTyrTyrGlyAsp
                                  310
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                .         .         .         .         .         .
1048    AACGATGAACAATTAGGTGGAGTAGGTAAAGCCACAGATACGGTAAATAAA   1098
        AsnAspGluGlnLeuGlyGlyValGlyLysAlaThrAspThrValAsnLys
           320                         330
                .         .         .         .         .         .
1099    GACTTTGAACTTACTGTATACGATAAAAATTTAGACACCCCTGATTGGATG   1149
        AspPheGluLeuThrValTyrAspLysAsnLeuAspThrProAspTrpMet
                     340                         350
                .         .         .         .         .         .
1150    AAAGGGGCAGTAATGTATCAAATATTCCCAGATAGATTTTACAATGGTGAC   1200
        LysGlyAlaValMetTyrGlnIleProAspArgPheTyrAsnGlyAsp
                           360
                .         .         .         .         .         .
1201    CCTTTAAATGACCGCCTAAAGGAATACAGTAGAGGTTTTGATCCTGTTGAA   1251
        ProLeuAsnAspArgLeuLysGluTyrSerArgGlyPheAspProValGlu
        370                         380
                .         .         .         .         .         .
1252    TATCATGACGACTGGTATGACCTTCCCGACAATCCGAATGATAAAGATAAA   1302
        TyrHisAspAspTrpTyrAspLeuProAspAsnProAsnAspLysAspLys
                  390                         400
                .         .         .         .         .         .
1303    CCTGGATATACAGGGGATGGTATATGGAATAATGACTTCTTTGGTGGTGAT   1353
        ProGlyTyrThrGlyAspGlyIleTrpAsnAsnAspPhePheGlyGlyAsp
                           410                         420
                .         .         .         .         .         .
1354    TTACAAGGTATAAATGATAAATTGGATTATCTAAAAAACCTTGGAATATCA   1404
        LeuGlnGlyIleAsnAspLysLeuAspTyrLeuLysAsnLeuGlyIleSer
                                 430
                .         .         .         .         .         .
1405    GTTATTTATCTCAATCCAATTTTCCAATCACCTTCCAATCACCGATATGAT   1455
        ValIleTyrLeuAsnProIlePheGlnSerProSerAsnHisArgTyrAsp
              440                         450
                .         .         .         .         .         .
1456    ACAACCGATTACACAAAGATAGACGAGTTATTGGGAGATTTAGATACATTT   1506
        ThrThrAspTyrThrLysIleAspGluLeuLeuGlyAspLeuAspThrPhe
                        460                         470
                .         .         .         .         .         .
1507    AAAACACTTATGAAAGAAGCCCATGCAAGAGGAATTAAAGTAATACTTGAT   1557
        LysThrLeuMetLysGluAlaHisAlaArgGlyIleLysValIleLeuAsp
                                 480
                .         .         .         .         .         .
1558    GGCGTCTTCAATCATACAAGTGATGATAGTATTTATTTTGATAGATACGGG   1608
        GlyValPheAsnHisThrSerAspAspSerIleTyrPheAspArgTyrGly
           490                         500
                .         .         .         .         .         .
1609    AAGTACTTGGATAATGAATTAGGTGCTTATCAAGCCTGGAAACAGGGAGAT   1659
        LysTyrLeuAspAsnGluLeuGlyAlaTyrGlnAlaTrpLysGlnGlyAsp
                     510                         520
                .         .         .         .         .         .
1660    CAGTCAAAATCTCCATACGGTGACTGGTACGAAATTAAGCCTGACGGTACC   1710
        GlnSerLysSerProTyrGlyAspTrpTyrGluIleLysProAspGlyThr
                           530
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                .         .         .         .         .         .
1711  TATGAGGGCTGGTGGGGATTTGACAGCTTACCGGTAATAAGGCAGATAAAC  1761
      TyrGluGlyTrpTrpGlyPheAspSerLeuProValIleArgGlnIleAsn
      540                               550
                .         .         .         .         .         .
1762  GGAAGTGAGTACAATGTAAAAAGTTGGGCAGATTTTATCATAAATAATCCT  1812
      GlySerGluTyrAsnValLysSerTrpAlaAspPheIleIleAsnAsnPro
                560                               570
                .         .         .         .         .         .
1813  AATGCAATATCTAAGTATTGGTTAAATCCTGATGGGGATAAAGATGCAGGT  1863
      AsnAlaIleSerLysTyrTrpLeuAsnProAspGlyAspLysAspAlaGly
                          580                               590
                .         .         .         .         .         .
1864  GCAGATGGCTGGAGATTGGATGTTGCAAATGAAATTGCTCACGATTTCTGG  1914
      AlaAspGlyTrpArgLeuAspValAlaAsnGluIleAlaHisAspPheTrp
                                    600
                .         .         .         .         .         .
1915  GTTCATTTTAGAGCTGCAATTAATACTGTGAAACCAAATGCGCCAATGATT  1965
      ValHisPheArgAlaAlaIleAsnThrValLysProAsnAlaProMetIle
            610                               620
                .         .         .         .         .         .
1966  GCAGAACTTTGGGGAGATGCTTCATTAGATTTACTTGGAGATTCTTTTAAC  2016
      AlaGluLeuTrpGlyAspAlaSerLeuAspLeuLeuGlyAspSerPheAsn
                      630                               640
                .         .         .         .         .         .
2017  TCTGTTATGAACTATCTTTTTAGAAATGCAGTTATTGATTTTATACTCGAT  2067
      SerValMetAsnTyrLeuPheArgAsnAlaValIleAspPheIleLeuAsp
                                650
                .         .         .         .         .         .
2068  AAACAGTTTGATGATGGAAATGTGGTTCACAATCCTATAGATGCAGCAAAA  2118
      LysGlnPheAspAspGlyAsnValValHisAsnProIleAspAlaAlaLys
         660                               670
                .         .         .         .         .         .
2119  CTTGACCAAAGGCTTATGAGCATATATGAGAGATATCCTCTTCCAGTATTT  2169
      LeuAspGlnArgLeuMetSerIleTyrGluArgTyrProLeuProValPhe
                  680                               690
                .         .         .         .         .         .
2170  TATTCTACTATGAACCTTTTAGGTTCTCATGACACCATGAGAATATTGACA  2220
      TyrSerThrMetAsnLeuLeuGlySerHisAspThrMetArgIleLeuThr
                              700
                .         .         .         .         .         .
2221  GTATTTGGATATAACTCTGCTAATGAAAATCAAAATTCTCAAGAGGCGAAA  2271
      ValPheGlyTyrAsnSerAlaAsnGluAsnGlnAsnSerGlnGluAlaLys
      710                               720
                .         .         .         .         .         .
2272  GACCTTGCAGTTAAGAGGCTTAAACTTGCCGCAATATTGCAAATGGGCTAT  2322
      AspLeuAlaValLysArgLeuLysLeuAlaAlaIleLeuGlnMetGlyTyr
               730                               740
                .         .         .         .         .         .
2323  CCGGGAATGCCTTCTATTTACTATGGTGACGAGGCAGGACAATCTGGTGGA  2373
      ProGlyMetProSerIleTyrTyrGlyAspGluAlaGlyGlnSerGlyGly
                        750                               760
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                .         .         .         .         .         .
2374  AAAGACCCAGATAACAGGAGAACATTCTCTTGGGGAAGAGAAGATAAAGAT   2424
      LysAspProAspAsnArgArgThrPheSerTrpGlyArgGluAspLysAsp
                                   770
                .         .         .         .         .         .
2425  CTGCAGGATTTCTTTAAGAAAGTCGTAAACATAAGGAATGAAAATCAAGTT   2475
      LeuGlnAspPhePheLysLysValValAsnIleArgAsnGluAsnGlnVal
            780                               790
                .         .         .         .         .         .
2476  TTAAAAACAGGAGACCTTGAAACACTTTATGCAAATGGCGATGTTTATGCC   2526
      LeuLysThrGlyAspLeuGluThrLeuTyrAlaAsnGlyAspValTyrAla
                        800                               810
                .         .         .         .         .         .
2527  TTTGGAAGAAGAATTATAAATGGAAAAGATGTATTTGGTAATTCTTATCCT   2577
      PheGlyArgArgIleIleAsnGlyLysAspValPheGlyAsnSerTyrPro
                                  820
                .         .         .         .         .         .
2578  GACAGTGTAGCTATTGTTGTGATTAATAAAGGTGAGGCAAAGTCAGTACAA   2628
      AspSerValAlaIleValValIleAsnLysGlyGluAlaLysSerValGln
         830                               840
                .         .         .         .         .         .
2629  ATAGATACTACTAAATTTGTAAGAGATGGAGTTGCTTTTACAGATGCCTTA   2679
      IleAspThrThrLysPheValArgAspGlyValAlaPheThrAspAlaLeu
                   850                               860
                .         .         .         .         .         .
2680  AGTGGTAAGACATACACGGTTCGTGATGGACAAATTGTTGTAGAAGTTGTG   2730
      SerGlyLysThrTyrThrValArgAspGlyGlnIleValValGluValVal
                              870
                .         .         .         .         .         .
2731  GCATTGGATGGGGCTATACTCATTTCAGATCCAGGACAGAATTTGACGGCA   2781
      AlaLeuAspGlyAlaIleLeuIleSerAspProGlyGlnAsnLeuThrAla
      880                               890
                .         .         .         .         .         .
2782  CCTCAGCCAATAACAGACCTTAAAGCAGTTTCAGGAAATGGTCAAGTAGAC   2832
      ProGlnProIleThrAspLeuLysAlaValSerGlyAsnGlyGlnValAsp
               900                               910
                .         .         .         .         .         .
2833  CTTTCGTGGAGTGCAGTAGATAGAGCAGTAAGTTATAACATTTACCGCTCT   2883
      LeuSerTrpSerAlaValAspArgAlaValSerTyrAsnIleTyrArgSer
                           920                               930
                .         .         .         .         .         .
2884  ACAGTCAAAGGAGGGCTATATGAAAAAATAGCTTCAAATGTTACGCAAATT   2934
      ThrValLysGlyGlyLeuTyrGluLysIleAlaSerAsnValThrGlnIle
                                   940
                .         .         .         .         .         .
2935  ACTTATATTGATACAGATGTTACCAATGGTCTAAAGTATGTGTATTCTGTA   2985
      ThrTyrIleAspThrAspValThrAsnGlyLeuLysTyrValTyrSerVal
            950                               960
                .         .         .         .         .         .
2986  ACGGCTGTAGATAGTGATGGAAATGAAAGTGCTTTAAGCAATGAAGTTGAG   3036
      ThrAlaValAspSerAspGlyAsnGluSerAlaLeuSerAsnGluValGlu
                           970                               980
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                .         .         .         .         .         .
3037    GCATATCCAGCATTTTCTATTGGTTGGGCAGGAAATATGAACCAAGTTGAT    3087
        AlaTyrProAlaPheSerIleGlyTrpAlaGlyAsnMetAsnGlnValAsp
                                  990
                .         .         .         .         .         .
3088    ACCCATGTAATAGGCGTAAATAATCCAGTTGAAGTTTATGCTGAAATTTGG    3138
        ThrHisValIleGlyValAsnAsnProValGluValTyrAlaGluIleTrp
          1000                        1010
                .         .         .         .         .         .
3139    GCAGAAGGATTAACAGATAAACCTGGCCAAGGGGAAAATATGATTGCCCAG    3189
        AlaGluGlyLeuThrAspLysProGlyGlnGlyGluAsnMetIleAlaGln
                    1020                            1030
                .         .         .         .         .         .
3190    TTAGGATATAGGTATATTGGAGATGGTGGGCAAGATGCTACACGCAACAAA    3240
        LeuGlyTyrArgTyrIleGlyAspGlyGlyGlnAspAlaThrArgAsnLys
                          1040
                .         .         .         .         .         .
3241    GTAGAAGGTGTTGAAATAAATAAGGACTGGACATGGGTTGATGCACGGTAT    3291
        ValGluGlyValGluIleAsnLysAspTrpThrTrpValAspAlaArgTyr
        1050                        1060
                .         .         .         .         .         .
3292    GTAGGGGATTCTGGAAATAACGACAAATACATGGCTAAATTTGTACCTGAT    3342
        ValGlyAspSerGlyAsnAsnAspLysTyrMetAlaLysPheValProAsp
                  1070                            1080
                .         .         .         .         .         .
3343    ATGGTAGGCACATGGGAATATATTATGAGATTTTCCTCTAACCAAGGACAG    3393
        MetValGlyThrTrpGluTyrIleMetArgPheSerSerAsnGlnGlyGln
                            1090                        1100
                .         .         .         .         .         .
3394    GATTGGACGTATACAAAAGGGCCAGATGGGAAAACAGATGAAGCAAAACAG    3444
        AspTrpThrTyrThrLysGlyProAspGlyLysThrAspGluAlaLysGln
                                    1110
                .         .         .         .         .         .
3445    TTTATTGTCGTGCCATCAAATGATGTAGAACCACCTACAGCTCTAGGCTTA    3495
        PheIleValValProSerAsnAspValGluProProThrAlaLeuGlyLeu
              1120                            1130
                .         .         .         .         .         .
3496    CAACAACCAGGAATTGAATCCTCAAGAGTTACACTTAACTGGAGTCTCTCA    3546
        GlnGlnProGlyIleGluSerSerArgValThrLeuAsnTrpSerLeuSer
                          1140                        1150
                .         .         .         .         .         .
3547    ACTGATAATGTAGCTATCTATGGCTACGAAATATACAAATCTTTAAGTGAA    3597
        ThrAspAsnValAlaIleTyrGlyTyrGluIleTyrLysSerLeuSerGlu
                                    1160
                .         .         .         .         .         .
3598    ACAGGACCATTTGTAAAGATTGCAACTGTGGCTGACACTGTGTATAACTAC    3648
        ThrGlyProPheValLysIleAlaThrValAlaAspThrValTyrAsnTyr
           1170                            1180
                .         .         .         .         .         .
3649    GTAGATACAGATGTAGTAAATGGAAAAGTGTACTATTATAAAGTTGTAGCA    3699
        ValAspThrAspValValAsnGlyLysValTyrTyrTyrLysValValAla
                    1190                            1200
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                .         .         .         .         .         .
3700    GTTGATACTTCTTTTAACAGAACAGCATCAAATATAGTGAAAGCTACACCT    3750
        ValAspThrSerPheAsnArgThrAlaSerAsnIleValLysAlaThrPro
                                1210
                .         .         .         .         .         .
3751    GATATAATACCTATCAAAGTGATATTTAATGTAACAGTCCCTGATTATACT    3801
        AspIleIleProIleLysValIlePheAsnValThrValProAspTyrThr
        1220                            1230
                .         .         .         .         .         .
3802    CCTGATGACGGTGCAAATATTGCTGGAAACTTCCATGACGCTTTCTGGAAT    3852
        ProAspAspGlyAlaAsnIleAlaGlyAsnPheHisAspAlaPheTrpAsn
                1240                            1250
                .         .         .         .         .         .
3853    CCAAGTGCCCATCAGATGACAAAGACAGGACCTAACACTTACAGTATTACA    3903
        ProSerAlaHisGlnMetThrLysThrGlyProAsnThrTyrSerIleThr
                        1260                            1270
                .         .         .         .         .         .
3904    TTGACTTTAAATGAAGGAACACAGCTTGAATATAAATATGCAAGGGGCAGC    3954
        LeuThrLeuAsnGluGlyThrGlnLeuGluTyrLysTyrAlaArgGlySer
                                1280
                .         .         .         .         .         .
3955    TGGGATAAGGTAGAAAAAGGTGAATATGGAGAGGAAATTGCAAATAGAAAA    4005
        TrpAspLysValGluLysGlyGluTyrGlyGluGluIleAlaAsnArgLys
              1290                            1300
                .         .         .         .         .         .
4006    ATAACTGTTGTCAATCAAGGTTCAAATACCATGGTGGTAAATGACACAGTG    4056
        IleThrValValAsnGlnGlySerAsnThrMetValValAsnAspThrVal
                        1310                            1320
                .         .         .         .         .         .
4057    CAAAGATGGAGAGACTTACCAATATACATTTATTCTCCAAAAGATAATACT    4107
        GlnArgTrpArgAspLeuProIleTyrIleTyrSerProLysAspAsnThr
                                1330
                .         .         .         .         .         .
4108    ACAGTAGATGCAAATACAAACGAGATAGAGATTAAAGGCAATACCTATAAA    4158
        ThrValAspAlaAsnThrAsnGluIleGluIleLysGlyAsnThrTyrLys
           1340                            1350
                .         .         .         .         .         .
4159    GGTGCAAAAGTAACTATAAATGATGAATCTTTTGTACAACAAGAAAATGGC    4209
        GlyAlaLysValThrIleAsnAspGluSerPheValGlnGlnGluAsnGly
                        1360                            1370
                .         .         .         .         .         .
4210    GTATTTACAAAAGTAGTGCCTCTTGAATACGGTGTAAATACTACTAAAATA    4260
        ValPheThrLysValValProLeuGluTyrGlyValAsnThrThrLysIle
                                1380
                .         .         .         .         .         .
4261    CATGTGGAGCCGAGTGGTGACAAGAATAATGAACTCACAAAAGATATAACA    4311
        HisValGluProSerGlyAspLysAsnAsnGluLeuThrLysAspIleThr
        1390                            1400
                .         .         .         .         .         .
4312    ATAACTGTTATAAGAGAGGAGCCTGTCCAGGAAAAAGAACCAACTCCTACG    4362
        IleThrValIleArgGluGluProValGlnGluLysGluProThrProThr
                1410                            1420
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                .         .         .         .         .         .
4363    CCAGAGTCTGAGCCAGCACCAATGCCTGAACCACAACCGACACCAACACCA    4413
        ProGluSerGluProAlaProMetProGluProGlnProThrProThrPro
                                1430                    1440
                .         .         .         .         .         .
4414    GAACCACAGCCTTCTGCAATTATGGCATTGTGACTGCCTCAACACTTAATT    4464
        GluProGlnProSerAlaIleMetAlaLeu *
                                     1450
                .         .         .         .         .         .
4465    TAAGAGAAGGAGCAAGTATCACAAGTAAAATTATAGGTACTATCTGCTGGG    4515
         *
                .         .         .         .         .         .
4516    AAAGTTGTAAAATGGCTTGAGGAAGTGAATGGATGGTACAAAGTTGACTAT    4566

                .         .         .         .         .         .
4567    AACGGCAAAGTAGGATATGTTTCAACCAAATATGTTTCTTCAGTGCCAGAT    4617

                .         .         .         .         .         .
4618    CCATCAAAGGTAACCGTTGCAAAATCAGTGAAAGTTATAGTGAAGAGCGGA    4668

4669    TT  4670
```

SDS-PAGE analysis for the enzyme expressed in *E. coli*, although it is about 22 kDa higher than the 140,000 mw determined for the native enzyme (Saha *et al.*, 1988). The GTG initiation codon is preceded with a spacing of 5 bp, by a putative ribosomal binding site (5'-AAAGGGGG-3') exhibiting strong similarity to the 16S rRNA of *Bacillus subtilis* (McConnell *et al.*, 1986). The structural gene is preceded by a putative promoter sequence, similar to the consensus promoter sequences of *E. coli* and *B. subtilis* (Rosenburg and Court, 1979; McConell *et al.*, 1986). A region (5'-TATAAT-3') similar to the -10 consensus promoter sequence of *E. coli* can be identified 158 bp upstream of the ribosome binding site, preceded by a region (5'-TTGACA-3') similar to the -35 consensus promoter sequence of *E. coli*. Also recognizable upstream of these regions is a sequnce resembling the consensus sequnce for binding of the cAMP-CAP complex of *E. coli* (Crombrugghe *et al.*, 1984), with the sequence 5'-TGTGC-3'. The N-terminal of the deduced amino acid sequence contained a 31 amino acid signal sequence putatively involved in cell-surface localization of the enzyme in *C. thermohydrosulfuricum* 39E. Hydropathy profile analysis (data not shown) indicated the putative signal sequence as the most hydrophobic region within the deduced peptide sequence. The 31 amino acid sequence had strong similarity to typical secretion signal sequences (McConnell *et al.*, 1986).

Sequence analysis

In order to identify the conserved regions, and the putative catalytic residues of amylopullulanase, the deduced sequence was aligned with known amino acid sequences of α-amylases and debranching enzymes, available through GENBANK (Intelligenetics, Inc., Mountain View, CA). Amino acid sequence comparisons of the conserved regions between the

deduced sequence of amylopullulanase with the sequence of enzymes capable of hydrolyzing α-1,4 bonds in polysaccharides are shown in Fig. 5. Amino acid sequence comparisons of the deduced sequence of amylopullulanase with conserved regions within the sequence of enzymes hydrolyzing α-1,6 bonds in polysaccharides are presented in Fig. 6. It can be seen that the sequence of amylopullulanase contains four motifs, DGVFNH, DGWRLDVA, AELWG, LLGSHD, which are aligned with the consensus sequence motifs of enzymes capable of α-1,4 or α-1,6 glucosidic bond hydrolysis. Previous studies (Mathupala and Zeikus, manuscript submitted for publication) using group specific chemical modification of amylopullulanase had indicated the involvement of acidic amino acids in catalysis. Therefore, aspartate and glutamate residues within these regions were targeted for site directed mutagenesis studies to identify those involved in catalysis.

Identification and modification of the putative catalytic residues on amylopullulanase

In order to test the hypothesis for a single active site imparting dual activity, residues tentatively identified using the alignment data for α-amylase of *A. oryzae*, were modified by site directed mutagenesis.

Using the four conserved regions of α-amylases as a template, similar regions were identified on amylopullulanase from *C. thermohydrosulfuricum* 39E. Most of the residues identified in α-amylase of *A. oryzae* to be involved in substrate binding or catalysis were also conserved in amylopullulanase. The catalytic residues of *A. oryzae* α-amylase (taka-α-amylase; TAA) reported to be Glu^{230} in the third conserved region, and Asp^{297} in the fourth conserved region (Matsuura *et al.*,1984), or as Asp^{206} in the second conserved region and Asp^{297} in the fourth conserved region

Figure 5. **Multiple sequence alignment of deduced amino acid sequence of *apu* from *C. thermohydrosulfuricum* 39E with sequences of α-amylases using 'Pileup' computer program (Devereux *et al.*, 1984).** The legend at the bottom of the figure identifies the organisms from which the sequences are represented. The four regions indicated by Roman numerals represent sequence motifs identified by alignment with the four conserved regions of α-amylase. Putative catalytic residues targeted for site directed mutagenesis on amylopullulanase are represented by closed circles. The dots represent regions within the sequences where an overall alignment could not be found.

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                                                  I
39E        465   LGDLDTFKTLMKEAHARGIKVILDGVFNHT        494
Taa        094   YGTADDLKALSSALHERGMYLMVDVVANHM        123
Cloem1     138   FGSFTDFQNLINTAHAHNIKVIIDFAPNHT        167
Stmamyl    093   LGDRAAFKSMVDTCHAAGVKVVADSVINHM        122
Bacamyla   078   YGTKAQYLQAIQAAHAAGMQVYADVVFDHK        107

                                    II
39E        582   NPDG.DKDAGADGWRLDVANEIAHDFWVHF        610
Taa        190   WVGSLVSNYSSDGLRIDTVKHVQKDFWPGY        219
Cloem1     241   AIKVWLD.MGIDGIRLDAVKHMPFGWQKNF        269
Stmamyl    191   YLNDLLS.LGVDGFRIDAAKHMPAA.DLTA        219
Bacamyla   218   WGKWYVNTTNIDGFRLDGLKHIKFSFFPDW        247

                                        III
39E        611   RAAINTVKPNAPMIAELWGDASLD......        634
Taa        220   NKA....AG.VYCIGEVLDGDPAYTCPYQN        244
Cloem1     270   MDSILSYRP.VFTFGEWFLGTNEIDVNNTY        299
Stmamyl    220   IKAKVGNGS.TYWKQEAIHGAGEAVQPSEY        249
Bacamyla   248   SYVRSQTGKPLFTVGEYWSYDINKLHNYIT        278

                                    IV
39E        685   ERYPLPVFYSTMNLLGSHDTMRILTVFGYN        714
Taa        289   ..........LGTFVENHDNPRFASYTNDI        308
Cloem1     348   ..........MVTFIDNHDMDRFYNGGSTR        367
Stmamyl    290   ..........SAVFVDNHDTER...GGDTL        306
Bacamyla   324   ..........VTFVDNHDTNPAKRCSHGR        342
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39E *Clostridium thermohydrosulfuricum* 39E amylopullulanase

Taa *Aspergillus oryzae* α-amylase

Cloem1 *C.thermosulfurogenes* EM1 α-amylase

Stmamyl *Streptomyces limosus* α-amylase

Bacamyla *Bacillus stearothermophilus* liquefying α-amylase

Figure 6. **Multiple sequence alignment of the deduced amino acid sequence of *apu* with sequences of α-1,6 hydrolyzing enzymes using the 'Pileup' computer program (Devereux *et al.*, 1984).** The legend at the bottom of the figure identifies the organisms from which the sequences are represented. The four regions indicated by Roman numerals represent sequence motifs identified by algnment with the four conserved regions of α-amylases. Putative catalytic residues are represented by closed circles. The dots represent regions within the individual sequences where an overall alignment could not be found.

```
                                   I
39E      473   LMKEA.HARGIKVILDGVFNHTSDDSIYFD      501
Neo      234   LIDRC.HEKGIRVMLDAVFNHC.........     254
A16glu   084   LLHEM.HERNMKLMMDLVVNHTSDEHNWFI      113
Isoam    304   MVQAF.HNAGIKVYMDVVYNHTAEGGTWTS      332
Kaepula  610   MIQAIKQDLGMNVIMDVVYNHTNAAGP.TD      638

                              II •
39E      585   G..DKDAGADGWRLDVANEIA.........      603
Neo      321   TYWIREFDIDGWRLDVANEID.........      340
A16glu   192   .....EKGIDGFRMDVINFISKEEGLPTVE      215
Isoam    387   AYWANTMGVDGFRFDLASVLGN....SCLN      412
Kaepula  686   AVWTTDYKIDGFRFDLMGYHPKAQILSAWE      715

                                 • III
39E      615   NT...VKPNAPMIAELWGDASLDLLGDSFN      641
Neo      353   KA...LKPDVYILGEIWHDAMPWLRGDQFD      379
A16glu   247   ....LSHYDIMTVGEMPGVTTEEAKLYTGE      272
Isoam    415   ...HASAPNCPNGGYFNDAADSNVAINRIL      441
Kaepula  718   ...KALNPDIYEFGEGWDSNQSD..REFIA      742

                                IV   •
39E      686   RYPLPVFYSTMN..LLGSHDTMRILTVFGY      713
Neo      415   SYPNNVNEAAFN..LLGSHDTSRILTVCGG      442
A16glu   312   KWQKALEHTGWNSLYWNNHDQPRVVSRFGN      341
Isoam    515   LFQSSGRSPWNSINFIDVHDGMTLKDVYSC      544
Kaepula  809   LGMAGN...LADFVLIDK.DGAVKRGSEID      834
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39E *Clostridium thermohydrosulfuricum* 39E amylopullulanase
Neo *Bacillus stearothermophilus* neopullulanase
A16glu *B. cereus* oligo 1,6 glucosidase
Isoam *Pseudomonas amylodermosa* isoamylase
Kaepula *Klebsiella aerogenes* pullulanase

(Buisson *et al.*, 1987), were used in identifying the putative catalytic residues of amylopullulanase from *C. thermohydrosulfuricum* 39E. Mutagenesis of targeted residues, Asp^{597}, Glu^{626}, and Asp^{703}, resulted in both α-amaylse and pullulanase activities being lost almost completely, indicating that Asp^{597} of amylopullulanase, corresponding to Asp^{206} of TAA in the second conserved region, Glu^{626} corresponding to the Glu^{230} in the third conserved region of TAA, and Asp^{703} corresponding to Asp^{297} of the fourth conserved region of TAA, are tentatively involved in catalysis (Table 1). The thermostability of the mutants was verified by heat treatment of the cell lysates and testing for the mutant proteins by SDS-PAGE.

Alignment of the sequence of amylopullulanase with those of α-amylases, pullulanases and glucoamylases is shown in Fig. 7. The *apu* sequence of *C. thermohydrosulfuricum* 39E displayed similarities to α-amylase-pullulanase of *C. thermohydrosulfuricum* E101 (82%), α-amylase of *A. oryzae* (48%), neopullulanase of *B. stearothermophilus* (60%), and pullulanases of *Klebsiella* spp. (44%). However, the overall similarity towards glucoamylases of *Aspergillus* spp. was much less, with a similarity of 40%.

It can be seen that the four conserved regions of all the amylases are located in the center of the encoded peptide. It is interesting to note that the M_r 100,000 peptide encoded by the 2.9 kbp nested deletion mutant constructed from the 4.4 kbp *apu* gene is located towards the center of amylopullulanase primary sequence, in alignment with the conserved sequence motifs of other amylases.

Table 1. Activity [b]of oligonucleotide directed mutant constructs of *apu* gene.

Enzyme	Mutation	Activity (U/ml)[a]
pAPZ75	none	4.200 ± 0.07 (100%)
D597N	Asp_{597}→Asn	0.006 ± 0.002 (0.14%)
D597E	Asp_{597}→Glu	0.008 ± 0.003 (0. 19%)
E626Q	Glu_{626}→Gln	0.004 ± 0.002 (0.10%)
E626D	Glu_{626}→Asp	0.006 ± 0.002 (0.14%)
D703N	Asp_{703}→Asn	0.005 ± 0.002 (0.12%)
D703E	Asp_{703}→Glu	0.004 ± 0.003 (0.10%)

[a]adjusted to OD_{660} = 1.0 for all subclones

[b]pullulanase activity is shown. In all mutants, concomitant loss of α-amylase activity was also observed.

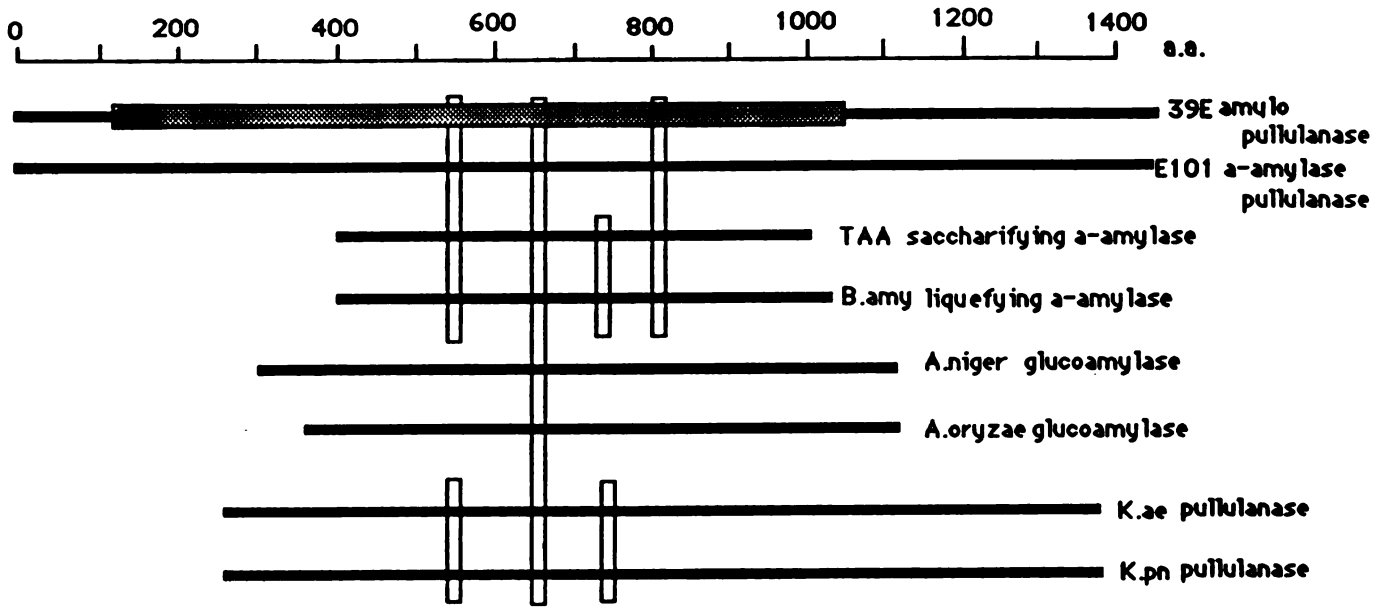

Figure 7. **Overall alignment of the deduced sequence of amylopullulanase of *C. thermohydrosulfuricum* 39E with amylases from microbial and fungal origin.** 39E = *C. thermohydrosulfuricum* 39E; E 101 = *C. thermohydrosulfuricum* E 101; TAA = *Aspergillus oryzae;* B.amy = *Bacillus amyloliquefaciens;* K. ae = *Klebsiella aerogenes;* K. pn = *K. pneumoniae* ; The open boxes represent regions putatively identified on all sequences based on the four conserved regions of α-amylase of *A. oryzae.* The 100 kDa thermostable amylopullulanase fusion protein encoded by the 2.9 kbp *apu* gene fragment, is shown by the shaded area on amylopullulanase.

DISCUSSION

This is the first report which identifies the catalytic amino acids of amylopullulanase, an enzyme capable of hydrolyzing α-1,6 and α-1,4 bonds of polysaccharides, as well as demonstrating categorically that a single active site is responsible for the dual activities. In this report, we present evidence for the true nature of the catalytic site of the dual substrate specific amylopullulanase type enzymes, indicating the involvement of an additional acidic residue in the general acid-base type catalytic mechanism proposed for α-amylase. The extra acidic residue may be involved in catalysis by either enhancing or modifying the ionization state of the two other residues involved in catalysis, or by being involved in stabilizing the transition state intermediate. Multiple sequence alignment of the deduced sequence of C. *thermohydrosulfuricum* 39E showed greatest similarity towards α-amylase-pullulanase of C. *thermohydrosulfuricum* DSM 3783 (87% identity) and pullulanase of C. *thermosulfurogenes* (69% identity). Analysis of the 5′ region of *apu* gene enabled the identification of a sequence motif for binding of the cAMP-CAP complex of *E. coli* (Crombrugghe *et al.*, 1984) for regulation of gene expression, which may be involved in catabolite (glucose) repression of pullulanase activity, reported previously for C. *thermohydrosulfuricum* 39E (Hyun and Zeikus, 1985).

In order to clarify the true nature of catalysis, and to locate the *apu* gene region encoding the catalytic domain, we proceeded to restrict the gene from the N- terminal end and the C- terminal end, testing each nested deletion mutant quantitatively for both α-amylase and pullulanase activities, as well as for thermostability. The smallest gene fragment, capable of expressing both activities and thermostability was 2.9 kbp in length and contained sequence

motifs similar to each of the four conserved regions identified on α-amylases, indicating that only one active site is present on *apu* gene. The 2.9 kbp gene fragment was identified within the *apu* gene by sequencing the 5′ and 3′ regions of the nested deletion mutant. This region corresponded to a polypeptide containing 955 amino acid residues, which was encoded by a region 2865 bp in length.

The catalytic mechanism of α-amylases (Matsuura *et al.*, 1984) has been modeled after the acid-base catalytic mechanism proposed for lysozyme (Walsh, 1979), where a glutamate, Glu^{35}, acts as a general acid catalyst while an aspartate, Asp^{52}, acts as a general base, stabilizing the glycosyl-oxocarbonium transition state intermediate. α-Amylases contain four highly conserved regions, which have been proposed to form the active site, the substrate binding sub-sites and the sites for calcium binding, based on X-ray crystallography data obtained for the α-amylase of *A. oryzae* (Matsuura *et al.*, 1984) and porcine liver pancreatic α-amylase (Buisson *et al.*, 1989).

In the present study, alignment of the sequences of amylopullulanase from *C. thermohydrosulfuricam* 39E with α-amylase of *Aspergillus oryzae* was used to identify the location of the catalytic residues in amylopullulanase. Asp^{597} and Asp^{703} of amylopullulanase were changed to Asn^{597} and Asn^{703} respectively, by single point base mutations of the *apu* gene, and Glu^{626} was changed to Gln^{626}. Although loss of activity is expected when only two of the three residues are individually modified, complete loss of α-1,6 and α-1,4 cleavage activity was detected when all three residues were mutated individually. Further resolution of which aspartate or glutamate is essential for catalysis and proof of the catalytic mechanism requires X-ray crystallographic structure analysis.

In the 3-D structure of α-amylase from *A. oryzae,* the residues Asp^{206}, Glu^{230}, and Asp^{297}, which align with Asp^{597}, Glu^{626}, and Asp^{703} of amylopullulanase, are located within close proximity to each other, within the catalytic center, indicating that the three catalytic residues identified in amylopullulanase are also closely positioned, forming a single active site for the dual activities. Therefore, the data suggest that amylopullulanase of *C. thermohydrosulfuricum* 39E contains a single active site for dual activities, in contrast to the dual active sites proposed for the α-amylase-pullulanase of *C. thermohydrosulfuricum* E101 (Melasniemi *et al.*, 1990) and amylase-pullulanase of *B. circulans* F-2 (Sata *et al.*, 1989).

The loss of activity upon change of Asp^{597} to Glu^{597}, Asp^{703} to Glu^{703} or Glu^{626} to Asp^{626} indicates that the geometric alignment of the catalytic residues within the active center of amylopullulanase is critical, in order to bring about catalysis. This contrasts with the flexibility shown by amylopullulanase towards a wide range of oligosaccharides and polysaccharides containing α-1,6 and α-1,4 linkages, which may be related to flexibility of the substrate binding residues rather than of the catalytic residues.

Amylopullulanase of *C. thermohydrosulfuricum* 39E is an enzyme with greater flexibility towards a wide variety of polymeric substrates, in contrast to the amylases reported from other prokaryotic or eukaryotic organisms, which are highly specific towards either α-1,4 or α-1,6 linkages for catalysis.

Amylopullulanase may be considered as a non-specific amylase which has the ability to cleave both α-1,4 and α-1,6 linkages, rather than as an α-amylase or a pullulanase which had acquired the ability to hydrolyze α-1,6 or α-1,4 bonds respectively. Since amylopullulanase was isolated from a

thermophilic anaerobe, which are believed to be among the first microorganisms to evolve on earth, the broad substrate specificity of this enzyme suggests that α-amylases and pullulanases may have evolved from an amylopullulanase type precursor enzyme.

Therefore this enzyme may be of great value in protein engineering studies to alter the substrate or product specificity of amylases, by changing the substrate binding or catalytic residues.

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CHAPTER VI

CONCLUSIONS AND PERSPECTIVES

CONCLUSIONS AND PERSPECTIVES.

The studies described in this dissertation on the biochemical characterization of an amylase from *Clostridium thermohydrosulfuricum* 39E has contributed to the information available on these unique thermostable enzymes found in thermophilic, anaerobic bacteria. The studies provided information on; the unique catalytic properties of this amylase, named amylopullulanase, from *C. thermohydrosulfuricum* 39E; the biochemical relationship of amylopullulanase to known microbial pullulanases and α-amylases; cloning of the amylopullulanase gene into *E. coli* to investigate the molecular biological features of the gene in relationship to amylases reported from thermophiles, and their gene organization; methods for overexpression and excretion of amylopullulanase from *C. thermohydrosulfuricum* 39E; recognition of regions within the amylopullulanase gene that encodes the peptide region of amylopullulanase essential for thermostability and catalysis; identification of aspartate and glutamate residues essential for catalysis; expression of the amylopullulanase gene in *B. subtilis* for potential use in biotechnological processes; identification of potential use of amylopullulanase in starch industry by biochemical manipulation of the enzyme using protein engineering for improved or altered catalytic properties.

Thermostable starch hydrolyzing enzymes, i.e., glucoamylase, pullulanase, and α-amylase play an important role in the starch processing industry. These enzymes, are used in solubilization and liquefaction of starch for the production of maltodextrin and conversion syrups. Several thermophilic anaerobic bacteria have been investigated with respect to their ability to produce extracellular saccharidases, and in the ability of such enzymes isolated from these organisms for their potential for use in applied biotechnological processes. The

present studies were initiated after investigations into a thermostable pullulanase produced by *C.thermohydrosulfuricum* 39E indicated the ability of this enzyme to cleave α-1,4 glucosidic bonds in starch. From a biochemical standpoint, the studies were important due to the potential insights the biochemical analysis of this unique enzyme would give with regard to the mode of catalysis and nature of thermostability.

For potential biotechnological applications, the study was of applied importance because of the potential for use of this enzyme, both as a solubilizing and debranching enzyme functioning at higher temperatures and at lower pH, in starch liquefaction and saccharification.

Initial investigations identified the high degree of thermostability, lower pH optimum for activity and the dual α-amylase and pullulanase activities of the purified enzyme. Subsequent characterization identified the biochemical relationship of amylopullulanase to pullulanase and α-amylase isolated from microbes, with respect to affinity for substrates, inhibition by cyclodextrins, product formation upon action against oligosaccharides and branched polysaccharides. For isolation and purification of amylopullulanase, an effective purification scheme was developed using an affinity matrix. Manipulation of the culture conditions with respect to substrate provided a method for overexpression and excretion of amylopullulanase from *C. thermohydrosulfuricum* 39E. Kinetic analysis of the purified enzyme verified the unique nature of amylopullulanase, and conclusively showed that the α-amylase and pullulanase activities are maintained in the same enzyme.

For detailed analysis of amylopullulanase, the gene encoding the enzyme was cloned and expressed in *E. coli*. For potential use of the enzyme in starch industry, the gene was subcloned and expressed extracellularly in *B. subtilis*. NMR analysis of the recombinant enzyme conclusively showed the ability of

amylopullulanase to hydrolyze α-1,6 branch points in polysaccharides. Overexpression of amylopullulanase in *E. coli* and *B. subtilis* provided a simplified purification process for large scale isolation of amylopullulanase.

Analysis of the deduced amino acid sequence of amylopullulanase with sequences from microbial α-amylases provided further insights into the primary structure and identified the regions within the enzyme involved in catalysis. Conservation of thermostability in the recombinant enzyme expressed in *E. coli*, and lack of cysteins in the deduced sequence of amylopullulanase indicated that the glycan moiety detected in the native enzyme or disulfide bonds are not involved in maintaining thermostability.

Identification of the residues essential for catalysis conclusively showed that a single active site is involved in both α-amylase and pullulanase activities of amylopullulanase. The identificationof a single active site supported the biochemical evidence obtained previously for this enzyme, and suggests the possibility of using this enzyme in structure, function studies in to the catalytic and substrate binding sites of amylases. Location of the esssential catalytic residues by sequence analysis identified the region within the enzyme involved in catalysis and substrate binding, giving potential insights for future research in to altering the catalytic and substrate specificity by using protein engineering approaches. Further studies using X-ray crystallography are necessary to identify the residues involved in catalysis, and to identify the residues involved in substrate binding to bring about the broad substrate specificity observed for this enzyme.

In perspective, the results obtained upon detailed biochemical characterization of this unique thermostable amylase from *Clostridium thermohydrosulfuricum* 39E, has indicated it's potential for use as a novel biocatalyst, for use in maltodextrin and conversion syrup production where this

acid stable thermophilic enzyme can be substituted for fungal α-amylase and bacterial pullulanase used at present. The process can be operated at higher temperatures, and thus enable the use of higher concentrations of raw starch in the process.

Using genetic engineering techniques, it was possible to restrict the amylopullulanase by a third, giving rise to a truncated but thermostable 100,000 molecular weight enzyme which maintained both α-amylase and pullulanase activities. This suggested the possibility for protein engineering of fusion proteins, where the 2.9 kbp gene region encoding the dual activities of amylopullulanase can be fused to a gene encoding thermostable glucoamylase, to engineer an amylase fusion protein with α-amylase, pullulanase, and glucoamylase activities, for single step conversion of raw starch into glucose. Also evident from this study is the possibility for nested deletion of genes from other thermostable amylases to identify truncated protein products that are thermostable and active, for use in protein engineering of fusion proteins.

Since this enzyme was isolated from a thermophilic anaerobe, which are believed to be among the first microorganisms to evolve on earth, the broad substrate specificity of this enzyme suggests that α-amylses and pullulanases may have evolved from an amylopullulanase type precursor enzyme. Possibilities exist for protein-engineering of the substrate binding and catalytic sites of amylopullulanase to generate unique amylases with novel substrate specificities and product profiles.

APPENDIX

PRELIMINARY CHARACTERIZATION OF CELL SURFACE MICROSTRUCTURES OF *Clostridium thermohydrosulfuricum* 39E, IN RELATION TO LOCATION OF AMYLOPULLULANASE.

ABSTRACT

Cationized ferritin was used to visualize the cell-surface of *C. thermohydrosulfuricum* 39E, using scanning electron microscopy. The cell-surface structure of the organism under continuous culture lacked any micro-structure, while under batch culture, cells showed significant cell-surface heterogeneity in the form of blebs or granular structures. The presence of these cell surface structures coincided with predominantly cell associated enzyme activity, in contrast to mainly extracellular activity found in cells lacking these protruberances.

INTRODUCTION

In *Clostridium thermocellum*, a cell surface glycoprotein complex, termed the cellulosome, containing enzymes necessary for the degradation of cellulose has been demonstrated. This complex exists in cell surface bound and cell-free forms, and has been shown to be responsible for cellular adherence to cellulose and for the degradation of cellulose to cellobiose by the intact organism (Bayer and Lamed, 1986; Lamed and Bayer, 1988). The cellulosome constitutes the majority of the endogluconase activity (~70%) and about one third of the total extracellular protein, and possesses the major proteins so far reported for the entire cellulolytic apparatus in this organism (Lamed *et al.*, 1987).

Cationized ferritin (Erdos, 1986) has been used for the visualization of exocellular structures and for general enhancement of the resolution of surface topology of single cells (Lamed *et al.*, 1987). With this technique,

negatively charged cell surface structures are labelled and can be observed using scanning electron microscopy.

The purpose of this study was to examine the ultrastructure of the cell surface of the cells grown under continuous culture and under batch culture, in order to identify any morphological differences which could be correlated with the cellular location of the enzyme, and to tentatively identify the presence of a multienzyme complex formed and released to the medium by C. *thermohydrosulfuricum* 39E, which may be involved in complete degradation of any starch related polysaccharide into glucose.

MATERIALS AND METHODS

Chemicals and gases

All chemicals used were reagent grade and obtained from either Sigma Chemical Company (St. Louis, Mo) or Aldrich Chemical Company (Milwaukee, Wi). Nitrogen used was 99.9% pure and was made free of oxygen by passage over heated (370°C) copper filings.

Organism and culture conditions

C. thermohydrosulfuricum 39E (ATCC 33223) was grown at 60°C under anaerobic conditions on TYE medium (Zeikus *et al*, 1980), supplemented with 1% (w/v) soluble starch, maltose or glucose as the substrate. Batch culture studies were carried out using pressure tubes containing 10 ml of medium and samples were taken at mid to late exponential phase for electron microscopy studies.

Affinity cytochemistry

C. thermohydrosulfuricum 39E was grown on glucose, maltose, or soluble starch to mid-exponential phase in anaerobic tubes (having previously subcultured twice in medium containing the same substrate). Cells grown under continuous culture were harvested directly from the chemostats grown in media containing maltose.

Cells were treated with cationized ferritin as described previously (Bayer *et al.*, 1985). 10 to 50 ml of cell culture was washed twice with 0.9% (w/v) NaCl, and centrifuged at 750 xg for 10 min at 4°C. The cells were resuspended in 5.0 ml of the same solution with 2.0 ml of 2.5% (v/v) glutaraldehyde in 0.1 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.2). After incubation for 20 min at room temperature, the cells were washed three times with 0.9% (w/v) NaCl and finally resuspended in 3.0 ml of saline. 5.0 ml of the glutaraldehyde-phosphate fixative was added to 1.5 ml of this cell suspension, and stored at 4°C, for use as negative controls for cationized ferritin staining. To the remaining 1.5 ml of the cell suspension, 0.25 ml of cationized ferritin (1 mg/ml in 0.9%(w/v) NaCl; Sigma) was added, and the solution incubated at room temperature for 1 hour. The cells were then centrifuged and washed twice with 0.9%(w/v) NaCl and resuspended in 6.5 ml of the glutaraldehyde-phosphate fixative.

Scanning electron microscopy

For scanning electron microscopy, the cells were deposited on 13mm diameter polyester membrane, pore size 0.4 μm (Nucleopore, Pleasenton, CA) by suction, immersed in fixative, and stored overnight at 4°C. Dehydration of the cells was carried out in an ethanol series of 25% to 100%(v/v), where the membranes with deposited cells were immersed sequentially in 25%, 50%,

75%, 90%, and 100% ethanol (v/v in water), for 10 minutes, and finally stored in 100%(v/v) ethanol. Critical point drying and gold coating of the cells were carried out as described previously (Klomparens *et al.*,1986), in an Emscope Sputter Coater model SC 500 to a thickness of 28 nm. Scanning electron micrographs were obtained in a JEOL JSM-35CF microscope at an accelerating voltage of 15 kV, and at a magnification of 20,000.

RESULTS

Scanning electron microscopy

As the cellular location of α-amylase and pullulanase activities varied according to the growth condition, cells were examined for cell surface integrity and for cell associated structures, using cationized ferritin and gold labelling to identify negatively charged structures, with scanning electron microscopy. In the absence of cationized ferritin, cell surface structures were not evident on cells of *C. thermohydrosulfuricum* 39E grown on glucose, maltose, or soluble starch, either under batch culture or under continuous culture conditions (Fig. 1 and 2). Under batch culture, the cell surface morphology of glucose grown cells treated with cationized ferritin appeared similar to non-treated cells, with the cell surface remaining smooth in appearance (Fig. 1). This differed from cells grown on maltose, or soluble starch. Treatment of these cells with cationized ferritin revealed the presence of protruberances on the cell surface (Fig. 1 and 2). The concentration of maltose in the growth medium appeared to influence the surface topology of the cell. Cells of *C. thermohydrosulfuricum* 39E grown at higher concentrations of maltose (0.5% w/v) in batch culture were covered with

protruberances, and such structures were almost absent on cells grown under maltose limitation (0.2% w/v) in continuous culture.

DISCUSSION

This study describes the influence of culture conditions on the presence or absence of cell surface structures (protruberances) visualized using scanning electron microscopy.

This study provides preliminary evidence to correlate the cell-surface structures to the possible presence of multienzyme complexes, on the cell surface of *C. thermohydrosulfuricum* 39E, using immuno-labelling methods. Since *C. thermohydrosulfuricum* 39E can efficiently degrade starch and related polysaccharides, these studies will indicate whether the organism harbors multienzyme complexes on the cell surface to ensure effective hydrolysis of starch, similar to the cellulosome, a multienzyme complex, that has been isolated from the cell-surface of *C. thermocellum* (Lamed *et al.*, 1987; Lamed and Bayer, 1988), for the efficient degradation of cellulose polymers. This multifunctional, multienzyme complex is capable of efficient degradation of cellulosic substrates. The localization of these enzymes in the form of a high molecular weight complex was envisaged as a major contributing factor in the synergistic action of the cellulase system of *C. thermocellum*, for the efficient degradation of cellulosic polymers.

An α-glucosidase (Saha and Zeikus, 1991) which can efficiently cleave small oligosaccharides into glucose, and a cyclodextrinase (Saha and Zeikus, 1990) which can hydrolyze cyclic dextrins, have been reported from *C. thermohydrosulfuricum* 39E. Therefore, an amylolytic system similar to the cellulosome may be present in *C. thermohydrosulfuricum* 39E, for the

efficient degradation of glucose polymers, since the enzymes isolated so far are synergistically capable of breaking down starch related polymers into glucose. Polyclonal antibodies raised against each enzyme will be used in immunolabelling studies to identify the location of each enzyme type under different growth stages.

The preliminary studies on *C. thermohydrosulfuricum* 39E cells from batch culture and continuous culture using scanning electron microscopy after treating with cationized ferritin have shown that the surface microstructure was structurally different between cells from batch culture and continuous culture. Cationized ferritin labelled structures were found on batch culture cells, while cells grown under continuous culture lacked any structures.

Another possible explanation for the release of cell-bound enzyme to the medium upon substrate limited chemostat culture may be disruption of the S-surface layer (Hollaus and Sleytr, 1972; Sleytr and Messner, 1983) of this organism. The S layer, found as the outermost surface layer of archaebacteria and most eubacteria, including *C. thermohydrosulfuricum* (Sleytr and Messner, 1983), is inferred to be directly involved in the interaction of these cells with their environment. It can be speculated that the porous glycoprotein (Messner *et al.*, 1992) meshwork of the S layer may entrap or anchor a segment (or domain) of the extracellular-but cell-bound enzyme, while the remainder of the enzyme containing the domains responsible for substrate binding and catalysis are exposed to the external medium so that large polymers such as polysaccharides are readily accessible and degradable by the organism. The S-layer may be disrupted or differentially synthesized under the physiologically stressed conditions present in the chemostat, releasing the enzyme to the medium, while the inner cell-wall and cell-

membrane layers remain intact. Therefore, evidence for the involvement of the S layer in the localization of the enzyme can be obtained by immuno-gold labelling studies using polyclonal antibodies raised against the S layer glycoprotein, for which isolation protocols are already available (Heckels and Virji, 1988).

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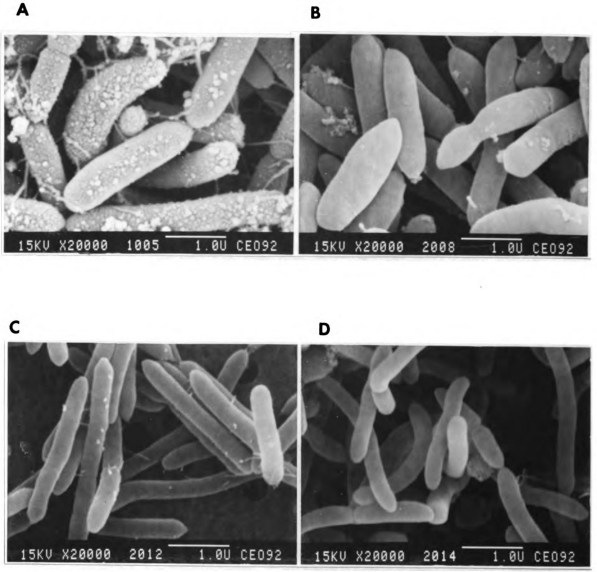

Fig. 1 Scanning electron microscopy of gold coated cells of *C. thermohydrosulfuricum* 39E, grown on maltose (0.5% w/v) in batch culture (A,B), and on maltose 0.2% (w/v) in continuous culture (C,D).

Figures A and C are of cells in the presence of cationized ferritin, and figures B and D are of untreated cells.

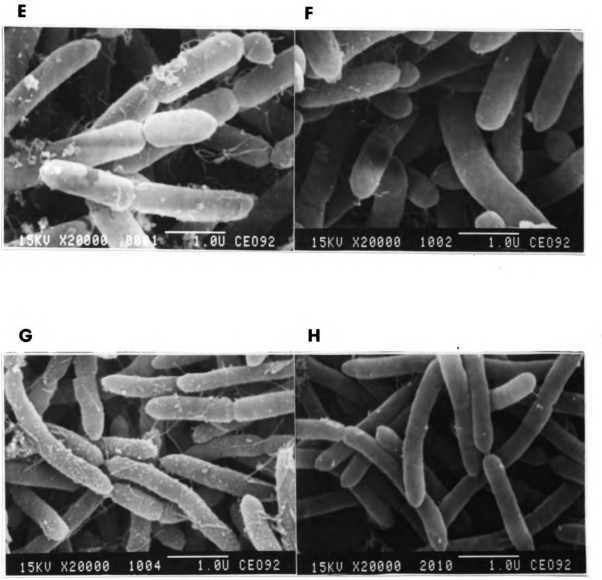

Fig. 2 Scanning electron microscopy of gold coated cells of *C. thermohydrosulfuricum* 39E grown on glucose (0.5% w/v) in batch culture (E,F), and on soluble starch (0.5% w/v) in batch culture (G,H).

Figures E and G are of cells in the presence of cationized ferritin, and figures F and H are of untreated cells.