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BIODEGRADATION OF POLYMERS AT TEMPERATURES UP TO 130°C

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ABSTRACT

Extreme thermophilic and hyperthermophilic microorganisms are those which are adapted to grow at temperatures from 70 to 110°C. Most of these exotic microorganisms are heterotrophic and are capable of attacking various polymeric substrates such as starch, hemicellulose, and proteins. Only recently, a number of novel extracellular enzymes like α-amylase, pullulanase, xylanase, and proteinase have been purified and studied in detail. By applying gene technology it was also possible to purify heat-stable enzymes after expression of their genes in mesophilic hosts. These novel enzymes are in general characterized by temperature optima around 90–105°C and a high degree of thermostability. Enzymic activity is still detectable even at 130°C and in the presence of detergents. Due to the remarkable properties of these enzymes, they are also of interest for biotechnological applications.

INTRODUCTION

A number of thermophilic microorganisms were found to produce extracellular enzymes that are capable of hydrolyzing polymers such as starch, cellulose, hemicellulose, and proteins. The majority of these organisms grow optimally between 60 and 75°C. Little, however, is known on the enzymology of hyperthermo-

philic microorganisms which grow optimally above 85°C. Only in the last decade has it been possible to isolate microorganisms which can grow optimally even above 100°C [1, 2]. The majority of this group that thrive above the boiling temperature of water belong to the archaea. However, some of these microorganisms also belong to the bacterial kingdom. The thermophilic representatives of the bacteria that optimally live above 65°C comprise four genera, namely, *Thermotoga*, *Thermosipho*, *Fervidobacterium* (Thermotogales order) and *Aquifex* (Aquificales order). The temperature optimum for growth of these microorganisms ranges between 65 and 90°C. On the other hand, the thermophilic representatives of the archaea comprise more than 19 genera which belong to the following orders: Sulfolobales, Pyrodictiales, Thermoproteales, Thermococcales, Archaeglobales, Thermoplasmales, and the methanogens Methanobacteriales and Methanococcales. The majority of the microorganisms are heterotrophic and anaerobic; only a few are strict autotrophes (Table 1). Most of these exotic microorganisms have been isolated from various geothermal habitats like hot springs, sulfataric fields, and deep-sea hydrothermal vents. Of great interest are the enzymes that are formed by extreme thermophilic and hyperthermophilic microorganisms. Some of the enzymes that have been recently studied are even active at 130°C [3]. Polymeric substrates are abundant in nature and provide a valuable and renewable source of carbon as well as of energy for various microorganisms that sustain life at high temperatures. These organisms are capable of attacking complex polymeric substrates by producing enzymes with a wide range of specificity.

STARCH DEGRADATION

Starch is composed of amylose (15–25%) and amylopectin (75–85%). Amylose is a linear macromolecule consisting of 1,4-linked α-D-glucopyranose residues. The chain length varies from several hundred to 6000 residues. The direction of the chain is characterized by the reducing and the nonreducing end. The reducing end is formed by a free C1 hydroxyl group. Like amylose, amylopectin is composed of α-1,4-linked glucose molecules, but in addition branching points with α-1,6-linkages occur. The branching points occur at every 17–26 glucose molecules, so that the content of α-1,6-linkages in amylopectin is about 5%. Due to the molecular mass of 10^6 to 10^9, amylopectin is one of the largest biological molecules.

The enzymes involved in the conversion of this substrate to low molecular weight compounds like glucose, maltose, and oligosaccharides with various chains are α-amylase, β-amylase, glucoamylase, debranching enzymes (pullulanases), and α-glucosidase. Enzymes that are capable of hydrolyzing α-1,6-glycosidic linkages in pullulan and amylopectin are defined as debranching enzymes or pullulanases. The coordinate action of many enzymes is usually needed for the efficient conversion of macromolecules.

The capability to utilize starch as carbon and energy source is widely distributed among microorganisms like bacteria, fungi, and yeasts [4]. It has been also shown that starch stimulates growth of a number of extreme thermophilic and hyperthermophilic microorganisms. Very recently a number of extreme thermophilic and hyperthermophilic microorganisms were found to produce novel thermoactive amylolytic enzymes with peculiar properties. These microorganisms belong to

TABLE 1. Taxonomy and Some Biochemical Features of Bacteria and Archaea Growing at High Temperatures[a]

Order	Genus	Optimal growth temperature, °C	Heterotrophic (het) Autotrophic (aut) Facultative autotrophic (f)	Anaerobic (an) Aerobic (ae)
		Bacteria		
Thermotogales	*Thermotoga*	70–80	het	an
	Thermosipho	70–75	het	an
	Fervidobacterium	65–70	het	an
Aquifecales	*Aquifex*	90	het	ae/an
		Archaea		
Sulfolobales	*Sulfolobus*	65–80	f	ae/an
	Metallosphaera	75	f	ae
	Acidianus	88	aut	ae/an
	Desulfurolobus	80	het	ae/an
Pyrodictales	*Pyrodictium*	100–105	het, aut	an
	Thermodiscus	88	f	an
	Hyperthermus	100	het	an
Thermoproteales	*Thermoproteus*	88	het, f, aut	an
	Thermofilum	88	het	an
	Desulfurococcus	85	het	an
	Staphylothermus	92	het	an
	Pyrobaculum	100	het, f	ae, an
Thermococcales	*Thermococcus*	70–87	het	an
	Pyrococcus	100	het	an
Archaeoglobales	*Archaeoglobus*	83	f	an
Thermoplasmales	*Thermoplasma*	60	het	ae/an

[a]Methanogenic microorganisms (Methanobacteriales and Methanococcales) with thermophilic representatives are not shown.

the genera *Thermotoga*, *Desulfurococcus*, *Staphylothermus*, *Thermococcus*, *Pyrococcus*, and *Sulfolobus* [3]. Detailed studies could be performed after purification of a number of these enzymes to homogeneity. The most thermostable amylase known so far has been purified from *Pyrococcus woesei* and *P. furiosus* [3, 5]. *P. woesei* and *P. furiosus*, which grow optimally on starch at 100°C, possess α-amylase, pullulanase, and α-glucosidase [5, 6].

In order to purify α-amylase from *P. woesei*, cell-free supernatant was concentrated and the enzyme was adsorbed onto soluble starch at 4°C. Release of the enzyme from starch was achieved by boiling and final desorption by electroelution. The native enzyme is composed of a single polypeptide chain with a molecular mass of 68 kDa. Enzymic activity is detected even after treatment of the purified enzyme with SDS (1%) and mercaptoethanol (2%) for 10 minutes at 100°C. This enzyme is capable of hydrolyzing randomly the α-1,4-glycosidic linkages in various glucose polymers such as amylopectin, glycogen, and amylose, forming various oligosaccharides. In addition to soluble polysaccharides, the α-amylase of *P. woesei* can hydrolyze native starch efficiently. Unlike the enzyme from other sources, glucose is not formed as an end product. The smallest substrate that can be attacked by the enzyme is maltoheptaose (DP7) which is converted to DP2 and DP5. Since this organism is unable to utilize glucose as the carbon source, it is highly likely that the products of starch degradation (α-limit dextrins) are directly transported into the cell and further hydrolyzed intracellularly. This novel enzyme displays a temperature optimum of 100°C and is active from 40 to 140°C. Almost 20% of α-amylase activity was detected even at 130°C. For the complete inactivation of the α-amylase, 10 hours of autoclaving at 120°C is necessary. The pH optimum for the enzymatic activity is 5.5; around 50% of activity is measured at pH 4.5 and 7.0. From these data it is evident that the prevailing growth conditions in geothermal habitats are optimal for this extracellular enzyme released into the environment. Additional in-vitro studies have shown that the addition of metal ions is not required for the catalytic activity of the purified enzyme.

The addition of 1 to 5 mM of Cr^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+} cause enzyme inhibition; Ca^{2+} ions (up to 7 mM), however, cause slight stabilization of the enzyme. Analysis of the amino acid composition of α-amylase shows no unusual features when compared with the enzymes from mesophilic and thermophilic bacteria.

Another strategy for the purification of heat stable enzymes is the application of gene technology. Attempts were made to clone genes, encoding thermostable enzymes in a mesophilic host. Recently, the DNA encoding of the extremely thermostable pullulanase from *P. woesei* was cloned and expressed in *Escherichia coli*. Due to the thermostability of the pullulanase, it was possible to purify the enzyme in a most remarkable way. Purification of the expressed enzyme was achieved by boiling the fermentation broth, denaturing of the host proteins, and recovery of the thermostable enzyme in the supernatant (Table 2). The activity of the enzyme is highest at 100°C, and 40% of enzymatic activity is detected at 120°C; the pH optimum is 5.5 (Fig. 1). No difference was observed in the physiochemical properties of the native and the cloned enzyme. Unlike the α-amylase from the same organism, the pullulanase activity was stimulated by the addition of calcium ions. The addition of 0.2 mM of Ca^{2+} causes an increase in the enzyme activity of up to fourfold. The pullulanase of *P. woesei* is capable of hydrolyzing α-1,6-linkages and α-1,4-linkages

TABLE 2. Purification of the Cloned Pullulanase from *Pyrococcus woesei*

Step	Total protein, mg	Total activity, U	Specific activity, U/mg	Recovery, %
Cell-free extract	1530	230	0.15	100
Heat treatment	240	180	0.75	79
Maltotriose-Sepharose	9.2	80	8.6	34
Mono Q	2.5	34	13.5	15

in branched oligo- and polysaccharides and hence can be classified as pulluanase type II (also named amylopullulanase). The hydrolysis products are glucose, maltose, and various linear oligosaccharides. HPLC analysis also shows that the pullulanase is able to attack α-1,6-linkages of pullulan in an *endo* fashion, forming a mixture of DP3, DP6, DP9 and DP12 (DP is degree of polymerization). Unlike pullulanases known so far, the thermoactive enzyme from *P. woesei* is also able to attack the α-1,6-linkage in panose, forming maltose and glucose as final products. Due to the multiple specificity of this enzyme, its action causes the complete and efficient conversion of starch to small sugars without the requirement for other amylolytic enzymes. Since the above-mentioned hydrolytic enzymes are optimally active under the same condition, they can be applied in a one-step process for the industrial bioconversion of starch. The improvement of the starch conversion process by finding new efficient and thermoactive enzymes would therefore significantly lower the cost of sugar syrup production.

XYLAN DEGRADATION

Hemicelluloses are noncellulosic low molecular weight polysaccharides that are found together with cellulose in plant tissues. In the cell walls of land plants,

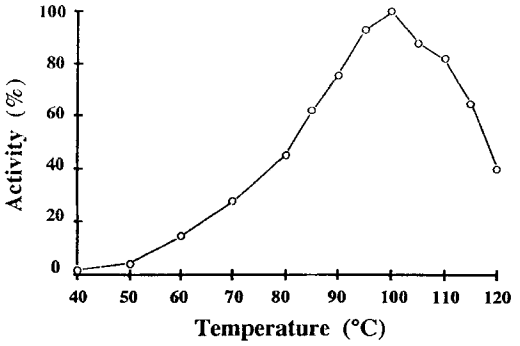

FIG. 1. Influence of temperature on the activity of the cloned pullulanase from *Pyrococcus woesei*; incubation time was 30 minutes.

xylan is the most common hemicellulosic polysaccharide, representing more than 30% of the dry weight. Most xylans are heteropolysaccharides which are composed of 1,4-linked β-D-xylopyranosyl residues. This backbone chain is substituted with acetyl, arabinosyl, and glucuronosyl residues. Due to the heterogeneity of xylan, its hydrolysis requires the action of a xylanolytic enzyme system which is composed of β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, and acetyl xylan esterase activities. The concerted action of these enzymes converts xylan to its constituent sugars. Xylan degrading enzymes have been reported to be present in marine and terrestrial bacteria, rumen and ruminant bacteria, fungi, marine algae, protozoa, snails, crustaceans, insects, and seeds of terrestrial plants.

Thermoactive xylanases, arabinofuranosidase, and β-xylosidase have been recently characterized from extreme thermophilic bacteria, namely from *Thermotoga maritima*, *T. neapolitana*, *T. thermarum*, and *Thermotoga sp.* FjSS3-B.1 [11; Sunna et al., submitted]. Xylanase which was isolated from the latter strain has a molecular mass of 31 kDa and is optimally active at pH 5.0–5.5 and 100°C. The xylanase of *T. neapolitana*, *T. thermarum*, and *T. maritima* and β-xylosidase from *T. thermarum* show optimal activity at 90°C. Above 80% of enzymatic activity is still detectable at 105°C. The arabinofuranosidase from the latter organism is less thermoactive and shows maximal activity at 70°C. Substrate specificity tests with the enzyme system of *T. thermarum* indicated that the commercial birchwood xylan as well as xylan from an industrial process (Lenzing AG) were the most suitable substrates. Oat spelt and larchwood xylan were less suitable. When incubated with hydroxyethyl cellulose or filter paper, the xylanolytic enzyme system did not show cellulase activity. The xylanase from *T. thermarum* is capable of hydrolyzing insoluble beech xylan as well as partially soluble birchwood xylan to yield mainly xylobiose, xylotriose, xylotetraose, and longer chains of xylooligosaccharides. This therefore proves the presence of a depolymerizing endo-1,4-β-xylanase. The molecular mass of the major protein band with xylanolytic activity is 40 kDa. An attractive application of such enzymes would be enzyme-assisted pulp bleaching.

PROTEIN DEGRADATION

Proteins are the most abundant organic molecules in living cells and constitute more than 50% of their dry weight. The molecular weight of proteins that are made up of one or more polypeptide chains can vary from a few thousands to more than one million. The three-dimensional conformation of proteins may vary. Globular proteins (spherical or globular) are soluble and usually have dynamic function. Fibrous proteins, on the other hand, occur as sheets or rods, are insoluble, and serve as structural elements. The enzymes which hydrolyze the peptide bonds in proteins are defined as proteinases. They are classified into four groups depending on the nature of their active center.

I. Serine proteinases have a serine residue in their active center and are inhibited by DFP (diisopropylphosphofluoride) and PMSF (phenylmethylsulfonylfluoride).

II. Cysteine proteinases have a SH groups in their active center and are inhibited by thiol reagents, heavy metal ions, alkylating agents, and oxidizing agents.
III. The activity of metal proteinases depends on tightly bound divalent cations. They are inactivated by chelating agents.
IV. Aspartic proteinases (acid proteinases) are rare in bacteria and contain one or more aspartic acid residues in their active center.

Inactivation of the enzyme can be achieved by alkylation of the aspartic acid residues with DAN (diazoacetyl-DL-norleucine methyl ester) [7].

Several extreme thermophilic and hyperthermophilic microorganisms which were isolated from submarine hydrothermal vents, solfataric fields, and alkaline springs grow preferentially on complex media containing proteins and peptides, and hence produce thermoactive proteinases. Thermoactive proteinases have been identified very recently from a number of extreme thermophilic and hyperthermophilic archaea like *Pyrococcus furiosus*, *P. woesei*, *Desulfurococcus mucosus*, *Thermococcus celer*, *T. stetteri*, *T. litoralis*, *Thermococcus* sp. AN1, *Staphylothermus marinus*, *Sulfolobus acidocaldarius*, and *S. solfataricus*. Most of the enzymes formed seem to be associated to the cells. From *P. furiosus* and *P. woesei* a serine-type proteinase (pyrolysin) has been identified and characterized [8–10]. Pyrolysin is a cell-envelope associated proteinase and displays high activity at 110°C; the half-life at 100°C is 4 hours. The enzyme system is active between pH 6.5 and 10.5. Zymogram staining shows the presence of multiple protease bands ranging from 65 to 140 kDa. Further experiments with cell extracts of *P. furiosus* have shown that at least two proteinases are resistant to SDS denaturation. Enzyme resistance to detergents and the appearance of multiple bands in polyacrylamide gels seems not to be unusual for thermoactive enzymes. Other serine proteases were detected in the archaea *T. celer*, *T. stetteri*, *Thermococcus* sp. AN1, *T. litoralis*, and *S. marinus* [12]. These enzymes demonstrate optimal activities between 80 and 95°C. The pH optimum is either neutral (*T. celer*, *T.* AN1) or alkaline. These proteinases exhibited preference to phenyl alanine in the carboxylic site of the peptide. Zymogram staining also shows multiple bands for all strains investigated.

Few proteinases from hyperthermophiles, however, have been purified and studied in detail. The enzyme (archaelysin) which was purified from *Desulfurococcus mucosus* has a molecular mass of 52 kDa and is optimally active at 100°C [13]. Substrate specificity studies of this serine-type enzyme suggest its preference for hydrolytic residues on the C-terminal side of the splitting point. Only recently a serine-type proteinase from *T. stetteri* was purified by preparative SDS-gel electrophoresis. The proteinase, with an apparent molecular mass of 68 kDa, was purified 67-fold. Maximal activity is measured at 85°C, and 40% of activity is measurable at 100°C. The enzyme is active at a broad pH range between 5 and 11. For mapping the P1 binding site, different *N*-protected *p*-nitroanilides were tested. The derivatives of glycine, alanine and aspartic acid were not hydrolyzed. The highest activity was obtained with the derivatives of arginine and phenylalanine. The determined kinetic constants are presented in Table 3; the enzyme has esterase activity. The K_{cat}/K_m value for Z-Phe-ONp is four orders of magnitude higher when compared to Suc-Phe-pNA. The amino acid anilides with nonprotected amino groups are not hydrolyzed. Interestingly, the enzyme at various temperatures is highly stereoselective and it hydrolyzes exclusively the L-forms of Bz-Arg-pNa, phenyl glycine amide,

TABLE 3. Kinetic Parameters for the Hydrolysis of Amino Acid Derivatives at 80°C, Catalyzed by the Purified Protease from *Thermococcus stetteri*[a]

Substrate, mM[b]	K_m, mM	k_{cat}, s^{-1}	k_{cat}/K_m, $M^{-1} \cdot s^{-1}$
Suc-L-Phe-pNA	0.9	0.1	110
H-L-Phe-pNA	0	0	0
Bz-L-Arg-pNA	0.06	0.013	215
Bz-D-Arg-pNA	0	0	0
Ac-L-Lys-pNA	1	0.018	18
Ac-L-Tyr-pNA	1.7	0.025	15
Z-Phe-ONp	0.02	15	750,000

[a]The derivatives of Gly, Ala, and Asp were not hydrolyzed.

[b]Abbreviations: pNA = *p*-nitroanilide; ONp = *p*-nitrophenyl ester.

phenyl alanine amide, and arginine amide. Furthermore, a thermoactive serine proteinase with maximal activity at 80°C was investigated from a newly isolated bacterium from the Azores islands. This organism grows optimally at 70°C and was identified as *Fervidobacterium pennavorans*. The enzyme system is capable of degrading insoluble proteins which are derived from chicken feather, hair, or wool. The keratin degrading enzyme(s) are active in a broad temperature and pH range; 50–100°C and pH 6.0–11.0.

Unlike the proteinases described above, the enzyme system of *Sulfolobus acidocaldarius*, which grows optimally at 70°C and pH 2.0, is not influenced by serine protease inhibitors. The proteinase of *S. acidocaldarius* is active under extremely low pH values and high temperatures, namely pH 2.0 and 90°C [14, 15].

CONCLUSION

The steady increase in the number of newly isolated thermophilic microorganisms documents the increased interest of the scientific community in hyperthermophiles. Although major advances have been made in the last decade, our knowledge on the physiology, metabolism, enzymology, and genetics of this fascinating group of organisms is still limited. In-depth information on the molecular properties of the enzymes and their genes, however, has to be obtained in order to analyze the structure and function of proteins that are functional even above 100°C. Future research has to reveal which strategies the evolutionary distinctive archaea have developed to ensure the remarkable thermostability of their enzymes. There is little doubt that this group of organisms will supply novel catalysts with unique properties that will also give a strong impetus to the development of new applications. Due to

the unusual properties of these enzymes, they are expected to fill the gap between biological and chemical industrial processes.

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