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REVIEW

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Thermozymes: biotechnology and structure–function relationships

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Abstract Recent findings on the biochemical and molecular features of the following thermozymes are presented, based on their biotechnological use: α-amylase and amylopullulanase, used in starch processing; glucose isomerase, used in sweetener production; alcohol dehydrogenase, used in chemical synthesis; and alkaline phosphatase, used in diagnostics. The corresponding genes and recombinant proteins have been characterized in terms of sequence similarities, specific activities, thermophilicity, and unfolding kinetics. Site-directed and nested deletion mutagenesis were used to understand structure–function relationships. All these thermozymes display higher stability and activity than their counterparts currently used in the biotechnology industry.

Key words Industrial enzymes · Protein stability · Thermophilicity

Introduction

Thermozymes are a hot research topic because they are remarkable tools for developing commercial biotechnologies and for studying protein stability. Thermozymes are enzymes that evolved in thermophilic (growth at temperatures above 60°C) and hyperthermophilic (growth at temperatures above 80°C) microbes. Thermozymes are resistant to irreversible denaturation and are optimally active at very high temperatures (from >60°C to 120°C). Thermozymes share the same catalytic mechanisms as their mesophilic counterparts. Thermozymes generally retain their thermal properties when they are expressed in mesophilic hosts, indicating that their unique thermal properties are genetically encoded. The specific molecular mechanisms that account for enzyme thermophilicity and thermostability vary from enzyme to enzyme. Recent structural comparisons between enzymes from mesophiles (or mesozymes) and thermozymes have validated numerous protein stabilizing mechanisms including hydrophobic interactions, packing efficiency, salt bridges, hydrogen bonds, reduction of conformational strain, loop stabilization, and resistance to covalent destruction. Our lab has been researching the molecular determinants for thermozyme activity and stability using enzymes from model thermophilic (i.e., *Thermoanaerobacter* and *Thermoanaerobacterium*) and hyperthermophilic (i.e., *Pyrococcus* and *Thermotoga*) genera (Hollenbeck-Petersen 1996; Vieille et al. 1996; Vieille and Zeikus 1996).

In general, thermozymes are more rigid than mesozymes and are more resistant to thermal and chemical denaturation. The business markets for industrial and specialty enzymes are growing at more than 8% and 15%, annually, respectively. Thermozymes are emerging as biocatalysts in both specialty markets such as Taq polymerase and industrial markets such as α-amylase. We review our recent findings on the industrial and specialty thermozymes that are developed in our laboratory, in relation to their biotechnological importance (Hollenbeck-Petersen 1996; Vieille et al. 1996; Vieille and Zeikus 1996).

Industrial thermozymes

We targeted the improvement of α-amylase and glucose isomerase used in starch processing and fructose manufacturing. Industry wanted a more acid-stable and non-calcium-requiring α-amylase to improve the starch liquefaction process at 100°C. Industry also wanted a more stable glucose isomerase that worked at 95°C instead of 60°C to avoid the concentration step required for making a 55% fructose syrup. Our approach was to screen thermophiles

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and hyperthermophiles to identify the target enzymes. We then cloned their respective genes into mesophilic hosts to achieve a protein purification process by simple heat treatment to denature the host proteins.

Amylolytic enzymes

When we screened thermophiles for α-amylase, we identified a new enzyme, amylopullulanase (Apu), present in most amylolytic species, which both solubilized and debranched starch. We discovered that thermophilicity and thermostability were controlled by separate molecular determinants in *Thermoanaerobacter ethanolicus* 39E Apu. A deletion of residues I–293 (mutant ApuN293) decreased the enzyme's temperature optimum from 90°C to 65°C (Fig. 1) without affecting its half-life at 85°C. While the Arrhenius plot of the wild-type enzyme was linear, the Arrhenius plot of mutant ApuN293 was biphasic (Fig. 2), with a discontinuity at 50°C (Hollenbeck-Petersen 1996). With a higher Km_{app} for pullulan at 40°C than at 60°C, we believe that this mutant adopts two catalytically significant different conformations below and above 50°C. This result suggests that certain enzymes can be designed to be highly stable but to be optimally active at moderate temperatures (37°–50°C). We are currently using *T. ethanolicus* Apu as a model enzyme to understand how the stereochemistry of its active site determines its ability to solubilize versus to debranch starch.

We cloned the gene encoding *Pyrococcus furiosus* Apu and characterized the recombinant enzyme (Dong et al. 1997b). This enzyme does not belong to the α-amylase family; instead, it shows similarity to the *P. furiosus* intracellular α-amylase. With a half-life of 44 h at 90°C and an optimal activity at 105°C, *P. furiosus* Apu is an extremely thermostable and thermophilic enzyme. It is also highly resistant to diverse chemical denaturing reagents (Table 1); surfactant addition even increased its activity as much as twofold.

We recently discovered that *P. furiosus* produces a true extracellular α-amylase (PfuAMY), in addition to an intracellular α-amylase and amylopullulanase. We have cloned the corresponding gene and characterized the recombinant enzyme (Dong et al. 1997a). The recombinant enzyme was compared to *Bacillus licheniformis* α-amylase (or Taka-therm), the most widely used industrial amylase (Table 2). PfuAMY is almost twice as active at 98°C as is Taka-therm at 90°C. It did not require calcium and was optimally active at a lower pH than Taka-therm. In addition, PfuAMY was more than 13 times more stable at 98°C than the industrial enzyme. Unlike *P. furiosus* intracellular α-amylase, PfuAMY shows significant similarity to other amylolytic enzymes of the α-amylase family and contains the four consensus regions characteristic of that enzyme family. It is most similar to Taka-therm, with which it shares 56% similarity and 36% identity. Detailed comparisons of the sequences and amino acid compositions of the two enzymes point to a few differences that could account for PfuAMY's extreme stability. PfuAMY contains bulkier hydrophobic residues (i.e., 5% more isoleucines and 5% fewer alanines), 16 (5%) more aromatic residues, shorter loops (PfuAMY is

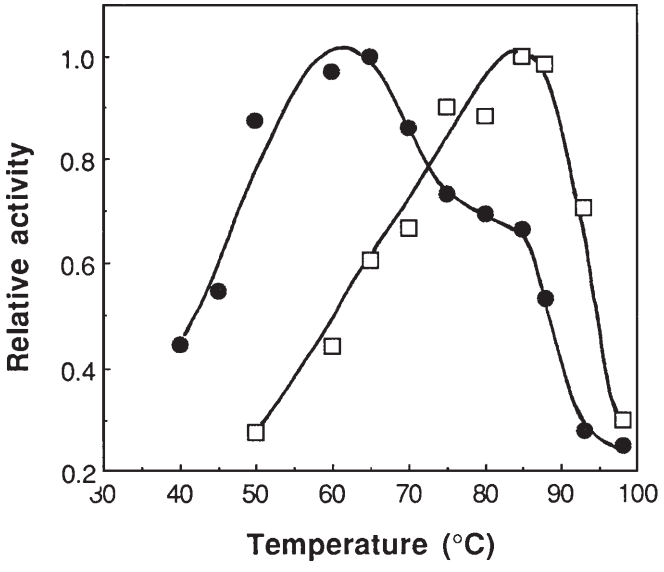

Fig. 1. Effect of temperature on the activity of *Thermoanaerobacter ethanolicus* wild-type and ApuN293 mutant amylopullulanases. Assays were performed in triplicate with pullulan as the substrate at temperatures ranging from 40° to 100°C. *Squares*, wild-type Apu; *circles*, ApuN293. (Adapted from Hollenbeck-Peterson 1996)

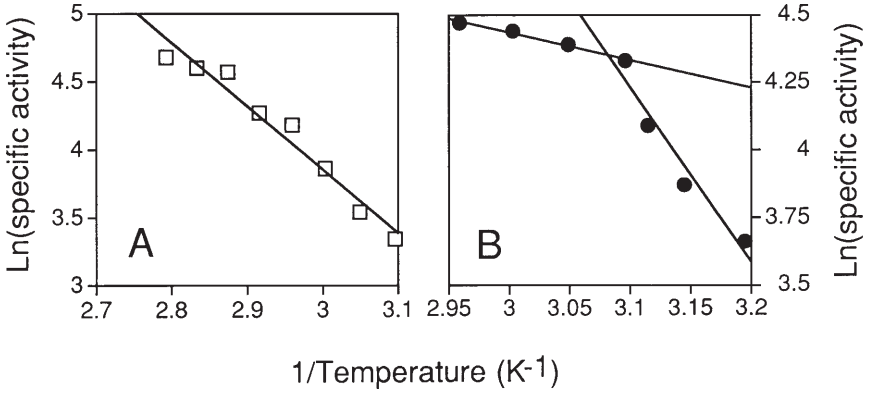

Fig. 2A,B. Arrhenius representation of the activity of *T. ethanolicus* wild-type (**A**) and ApuN293 mutant (**B**) amylopullulanases between 40° and 85°C. (Adapted from Hollenbeck-Peterson 1996)

Table 1. Effect of different reagents on *Pyrococcus furiosus* recombinant amylopullulanase stability

Reagents	Concentration	Residual relative activity (%)
None	–	100
SDS	1 mM	177
	5 mM	75
	10 mM	74
	50 mM	16
Urea	1.0 M	114
	3.0 M	114
	5.0 M	111
Guanidine HCl	0.1 M	109
	0.5 M	102
	1.0 M	93
	2.0 M	47
Triton X-100	0.1%	221
	1.0%	229
	5.0%	225

Adapted from Dong et al. (1997b).

Table 2. Comparison of some biochemical properties of PfuAMY and Taka-therm

Properties	PfuAMY	Taka-therm
Specific activity (U/mg)	3900 at 98°C	2000 at 90°C
Optimal pH	5.5–6.0	7.0–8.0
Optimal temperature	100°C	90°C
Ca^{2+} requirement	No	Yes
Half-life at 98°C	13 h	<1 h
End products	G_2–G_7	G_1–G_6

Adapted from Dong et al. (1997a).

overall 10% shorter), and 5 more prolines than Taka-therm. Unlike many enzymes isolated from hyperthermophiles, however, PfuAMY contains 5 cysteines, which are absent in the *B. licheniformis* enzyme (Dong et al. 1997a). Whether some or all of these differences are responsible for PfuAMY's higher stability is under study in my laboratory.

Glucose isomerases

Enzymes from hyperthermophiles were originally thought to be inefficient when compared to mesophilic enzymes. The contention that thermozymes lost catalytic efficiency to gain stability was shown not to be the case for xylose isomerase (XI) (also called glucose isomerase), whose gene we cloned and expressed from *Thermotoga neapolitana* (Vieille et al. 1995). The *T. neapolitana* XI (TNXI) displays a K_{cat}/K_m at 90°C that was 2 to 14 times higher than any other thermophilic XIs at temperatures between 60° and 90°C (Vieille et al. 1995). A type II XI, the *T. neapolitana* enzyme is highly similar (82% and 78%) to the less thermophilic type II XIs from *Thermoanaerobacterium thermosulfurigenes* (TTXI) and *B. licheniformis* (BLXI), respectively. Still, the *T. neapolitana* enzyme is optimally active at 95°C, which is 15° and 28°C higher than TTXI and BLXI, respectively.

The inactivation characteristics of these three XIs were compared. TTXI and BLXI inactivations rates follow first-order kinetics, are independent of enzyme concentration, and have high activation energies (120 and 217 kcal/mol, respectively), indicating that these two enzymes inactivate by unfolding, followed by molecular scrambling and aggregation (Meng et al. 1993; Vieille, unpublished results). The temperature for TTXI 50% unfolding (94°C) is significantly higher than the temperature at which it starts inactivating (85°C), suggesting that the unfolding involved in its inactivation is only partial. The limiting step in TNXI inactivation remains unclear. TNXI inactivation does not follow first-order kinetics, is dependent on enzyme concentration, and has a low activation energy (11–29 kcal/mol, Vieille, unpublished results). This low activation energy suggests that TNXI inactivation proceeds via a covalent step such as deamidation, a mechanism that would be expected to be independent of enzyme concentration. TNXI melting experiments in the presence of increasing concentrations of GuHCl indicate that TNXI shows two unfolding transitions at 95° and 110°C (Vieille, unpublished results). The 95°C transition corresponds to the temperature at which TNXI starts inactivating and aggregating, suggesting that TNXI partially unfolds as it starts inactivating. This result is unexpected in view of the low activation energy calculated for TNXI inactivation.

To identify the molecular determinants responsible for TNXI's higher thermostability, TNXI and TTXI crystal structures were compared (Gallay et al., in preparation). With the exception of a few additional prolines present in TNXI, none of the molecular features often associated with stabilization (Vieille and Zeikus 1996) could be identified in TNXI (Gallay et al., in preparation). Among the additional prolines found in TNXI, Pro58 and Pro62 are present in a large loop (Fig. 3) where 14 hydrophilic residues surround Phe59. This loop is observed in crystallographic studies, with the Phe60 (corresponding to Phe26 in the *Actinoplanes* enzyme) involved in surface interaction with another subunit. The 2 additional Pro probably help expose the Phe residue at the surface of the subunit, improving the intersubunit interaction.

Specialty thermozymes

Secondary alcohol dehydrogenase

The *adhB* gene encoding *T. ethanolicus* 39E secondary alcohol dehydrogenase (2°ADH) was cloned and expressed in *E. coli* (Burdette et al. 1997). Hydrophobic cluster analysis comparisons of primary (1°) and 2°ADHs predicted that the catalytic Zn^{2+}-binding motifs were different in 1°ADHs (two Cys and one His) and in 2°ADHs (one Cys, one His, and one Asp), and that 2°ADHs bind the nicotinamide cofactor in a Rossmann fold (Burdette et al. 1997). These predictions were confirmed by site-directed mutagenesis of *T. ethanolicus adhB* (Burdette et al. 1996). Mutants Cys37Ser, His59Asn, and Asp150Asn were inactive (Table

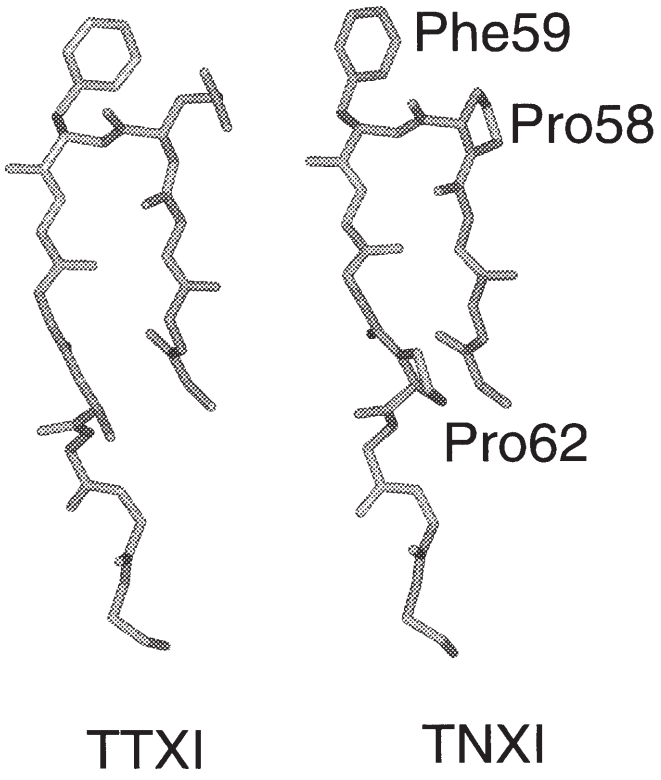

Fig. 3. Comparison of the structures of TTXI and TNXI "Phe59" loops. TNXI Pro58 and Pro62 replace TTXI Gln58 and Ala62, respectively

3) and bound significantly less Zn per subunit than the wild-type enzyme. Substituting Gly198 with Asp in the putative Rossman fold increased the enzyme K_m for $NADP^+$ by 225 fold and decreased its K_m for NAD^+ by 3 fold (Burdette et al. 1996), making the Gly198Asp mutant more specific for NAD^+ than for $NADP^+$ (Table 3). This mutant has the advantage over the wild-type enzyme of using the more stable cofactor NAD instead of NADP. Secondary ADH shows a temperature-dependent enantiospecificity: on the basis of k_{cat}/K_m values, (S)-2- and (R)-2-butanol are the preferred substrates at temperatures below and above 26°C, respectively (Phan and Phillips 1990). The Ser40Thr mutation strongly enhances the enantiospecificity of *T. ethanolicus* 2°ADH toward (R)-2-alcohols (Table 3), shifting its racemic temperature (i.e., temperature at which the enzyme has no preference for the R- or the S- form) for 2-butanol from 26°C to −30°C (Tripp et al., in manuscript).

Alkaline phosphatase

A hyperthermophilic alkaline phosphatase (AP) was purified from *T. neapolitana* by heat treatment at 100°C in the presence of Co^{2+}, followed by a combination of ion-exchange and affinity chromatographies (Dong and Zeikus 1997). This enzyme is optimally active at 85°C and pH 9.9. It is as active as the calf intestine enzyme on *p*-nitrophenol phosphate, but it uses Co^{2+} instead of Mg^{2+} as cofactor.

Table 3. Phenotype of the different *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase (2°ADH) mutants constructed

Mutation	Residue function	Mutant phenotype
Cys37Ser	Catalytic Zn^{2+}-binding site	Inactive
His59Asn	Catalytic Zn^{2+}-binding site	Inactive
Asp150Asn	Catalytic Zn^{2+}-binding site	Inactive
Gly198Asp	NADP-binding site	Cofactor specificity changed from NADP to NAD
Ser40Thr	Enantiospecificity toward (S)-2-alcohols	Reversed enantiospecificity

Source: Burdette et al. (1997); Tripp et al. (in manuscript).

Table 4. Comparison of *Thermotoga neapolitana* and calf intestine alkaline phosphatases (APs) thermostabilities

Enzyme source	Temperature (°C)	Metal ion added	Half-life (min)
T. neapolitana	90	Co^{2+}	238
	90	Mg^{2+}	179
	65	Co^{2+}	1125
Calf intestine	65	Mg^{2+}	60

Adapted from Dong and Zeikus (1997).

T. neapolitana AP is also significantly more stable than the calf intestine enzyme (Table 4). The *T. neapolitana* AP (*phoA*) gene was cloned in *E. coli* and sequenced (Savchenko, unpublished results). *T. neapolitana* AP is most closely related to the *Bacillus subtilis* enzyme, with 43% similarity and 33.5% identity. All primary metal ligands and catalytic residues are conserved in the *T. neapolitana* enzyme. The molecular determinants for this enzyme's thermostability are under study.

Conclusions

Recent findings on thermozymes may expand the following horizons on new frontiers of microbiology, biochemistry, and biotechnology. (i) Molecular determinants that account for protein thermostability at temperatures above 100°C are now being recognized. (ii) The discovery of thermophilicity domains in enzymes suggests that stability and activity can be encoded by separated molecular determinants. (iii) Thermozymes are becoming tools for protein chemistry because they are now easy to produce, purify, and crystallize. (iv) Thermozymes may become the catalysts of choice for developing industrial enzymes because they are intrinsically very stable and genetic techniques can be used to alter their optimum temperature and specific activity. (v) Thermozymes used in diagnostics and reagents may expand beyond DNA polymerase and ligase because reagent stability and extended shelf life are important features. Thermozymes can be stored at room temperature instead of in the deep freezer, making them more convenient to use. Future genetic engineering of thermozymes may yield cata-

lysts with high stability and temperature activity optima designed to meet the exact reaction temperature desired.

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