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Thermostable carboxylesterases from hyperthermophiles

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Abstract—This report focuses on the lipolytic enzymes from hyperthermophiles. Most of the enzymes characterized to date are carboxylesterases that are structurally related to the hormone-sensitive lipase family, and prefer medium chain (acyl chain length of 6) *p*-nitrophenyl substrates. The presence of a GGGX motif in these carboxylesterases suggest the ability of these enzymes to catalyze the hydrolysis of tertiary alcohol esters. We will also introduce studies that have examined the effects of temperature and organic solvents on the catalytic efficiency and enantioselectivity of the thermostable carboxylesterase from *Sulfolobus solfataricus*. Finally, a BLAST search of the hyperthermophile genome sequences reveal candidate genes that may encode novel, thermostable esterases.

Contents

1. Introduction

The application of enzymes in organic synthesis is now a routine alternative for the organic chemist and process engineer. The native or engineered enzyme provides the selectivity, whether it be substrate selectivity, regioselectivity, or stereoselectivity, which is desired in the reaction. Unfortunately, the use of enzymes in many cases also brings about constraints in the conditions under which the reaction must be performed. In terms of stability, not to mention selectivity, the usual enzyme is far from the ideal catalyst, and in many cases the enzyme is more labile than the substrate and product of the reaction. Enzymes with enhanced stability would not only allow prolonged usage, but would enable us to explore a broader range of reaction conditions aimed to enhance further the selectivity and/or efficiency (turnover) of the enzyme reaction. Indeed, much effort has been spent in order to enhance the stability of enzymes, through modifying the enzyme itself or its immediate environment.[5] The dramatic increase in structural information of enzymes, along with recently developed techniques (DNA shuffling, high throughput screening technology, directed evolution), have led to great advances in enzyme engineering and technology.[4,18,19,24]

Another development that has provided valuable clues as to how proteins can be made more thermostable or thermotolerant is the discovery of hyperthermophiles and studies on their proteins. Hyperthermophiles are organisms that grow at temperatures above 90 °C,[1] or optimally grow at temperatures above 80 °C.[38] Many have been found to grow at temperatures above the boiling point of water.[39] Unlike chemical parameters such as

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pH, heat cannot be removed or pumped out of the cell, and consequently, all the biomolecules within a hyperthermophilic cell must endure and function at high temperature. Therefore, a single hyperthermophile provides well over a 1000 different proteins with extreme thermotolerance. This, along with the possibility that hyperthermophiles may represent the most primitive forms of present-day life, has led many to study the protein structure, physiology, and genome structure of hyperthermophiles. Hyperthermophiles have been found to constitute a diverse group of organisms in terms of energy and carbon metabolism.[2] Both chemoautotrophs and heterotrophs are present, with the latter group capable of utilizing a variety of organic compounds; disaccharides or polysaccharides with α- or β-1,4-glycosidic bonds, peptides, amino acids, and organic acids. This indicates the presence of various enzymes that can convert or degrade these compounds. As expected, a vast scope of enzymes with an application potential have been identified from these organisms in the past years.[15,17,25,29,40]

Carboxylesterases (EC 3.1.1.1) are a class of lipolytic enzymes that hydrolyze water-soluble, ester-containing molecules. Taking into account this substrate selectivity, carboxylesterases are distinguished from lipases (EC 3.1.1.3), which prefer water-insoluble long-chain triglycerides and display activation at lipid–water interfaces, and arylesterases (EC 3.1.1.2), which hydrolyze esters with aromatic moieties. Phospholipase A2 (EC 3.1.1.4), lysophospholipase (EC 3.1.1.5), and acetylcholine esterase (EC 3.1.1.7) are also representatives of the abundant number of ester bond hydrolyzing enzymes. On the other hand, the rapid accumulation of sequence data in recent years has made possible the classification of these enzymes in terms of primary structure.[3] Although this structural classification in general agrees well with the classification based on substrate selectivity, there are some structurally-related families of enzymes that include both the traditionally named lipases and carboxylesterases. This report will focus on the lypolytic enzymes identified from hyperthermophiles and their biochemical properties. At present, a lipase has not been identified from hyperthermophiles, and most of the enzymes characterized up till now are carboxylesterases. Although the number is still very limited, we will also introduce some initial examples where the application of hyperthermophilic esterases in organic synthesis has been explored.

2. Properties of characterized carboxylesterases from hyperthermophiles

Thermostable carboxylesterases have been identified and characterized from *Archaeoglobus fulgidus*, *Pyrococcus abyssi*, *Pyrococcus furiosus*, *Aeropyrum pernix*, *Sulfolobus solfataricus*, and *Pyrobaculum calidifontis* (Table 1). Among these, the enzyme from *A. fulgidus* (AFEST) is the most characterized; its gene has been cloned, the recombinant enzyme has been purified and characterized (AAB89533),[22] and moreover, the crystal structure of the protein is available at 2.2 Å resolution.[8] The structure of AFEST should provide valuable information for future engineering of the enzyme, and for the modelling of other esterases from hyperthermophiles. AFEST is a member of the hormone sensitive lipase (HSL) family, or Family IV of the prokaryotic lipolytic enzymes proposed by Arpigny and Jaeger.[3] The HSL family also includes the carboxylesterase from the thermophile *Alicyclobacillus acidocaldarius* (EST2)[7] and Brefeldin A esterase from the mesophilic *Bacillus subtilis* (BFAE),[41] whose three-dimensional structures have been determined. The three structures thus allow a detailed structural comparison among closely related enzymes from mesophiles, thermophiles, and hyperthermophiles. As reported in other structural comparisons between mesophilic/hyperthermophilic proteins,[11,15,37,40] (i) an increase in the percentage of ion pairs, (ii) an increase in cationic-π aromatic interactions, (iii) a decrease in the surface area occupied by hydrophobic residues, and (iv) a reduction in the lengths

Table 1. Biochemical properties of thermostable esterases from hyperthermophiles

Organism	No of residues	T_{opt} (°C)	Substrate (examined temperature, °C)[a]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	Specific activity ($\mu mol\,min^{-1}\,mg^{-1}$)	Refs.
A. fulgidus	311	80	PNP-hexanoate (70)	11 ± 3	1014 ± 38	92.2	Ca. 3200[b]	19
S. solfataricus P1	305	95–100	4-Methylumbelliferyl acetate (80)	450	1000	2.2	1600[c]	28
S. solfataricus MT4	305	⩾90	PNP-valerate (60)	NR	NR	NR	747[d]	23
P. calidifontis VA1	313	90	PNP-caproate (70)	44.4 ± 5.9	2620 ± 90	59	4050[e]	13
A. pernix	582	90	PNP-caprylate (70)	NR	NR	NR	0.92[f]	9
P. furiosus	NR	100	NR	NR	NR	NR	Crude sample	15
P. furiosus	257	NR	NR	NR	NR	NR	NR	30
P. abyssi	NR	65–74	NR	NR	NR	NR	Crude sample	6

NR, not reported.

[a] Temperature at which kinetic analysis was performed, or specific activity measured.

[b] Measured with 0.2 mM PNP-hexanoate.

[c] Measured with 0.6 mM 4-methylumbelliferyl acetate.

[d] Measured with 0.3 mM PNP-valerate.

[e] Measured with 1 mM PNP-caproate.

[f] Measured with 0.2 mM PNP-caprylate.

of loops connecting secondary structures, was observed.[8] Further statistical analyses of single amino acid replacements among the three aligned proteins have revealed particular trends in residue exchange in the direction mesophilic to hyperthermophilic.[23] In terms of the biochemical performance of the enzyme, AFEST was thermostable with $t_{1/2}$ values of 30 h (58 °C), 7.5 h (70 °C), 60 min (85 °C), 28 min (90 °C), and 26 min (95 °C). The optimal temperature of the enzyme under the conditions examined was 80 °C. The thermostability and optimal temperature of the enzyme may seem relatively low, as *A. fulgidus* grows at temperatures up to 95 °C. There are some examples in which the in vitro thermostability of an enzyme from a hyperthermophile is lower than one would expect.[9] There is a possibility that these intracellular enzymes are further stabilized in vivo by small intracellular molecules such as polyamines.[21]

Kinetic analyses of AFEST toward various *p*-nitrophenyl (PNP) esters revealed maximum k_{cat}/K_m values toward PNP-hexanoate ($92.2\,s^{-1}\,\mu M^{-1}$). Activities toward long PNP esters were very low, and hydrolysis of trioleoylglycerol could not be detected. Enantioselectivity of AFEST was examined with several compounds, and although significant conversion was observed in short reaction times with high substrate/enzyme ratios, only moderate enantioselectivity was observed (Fig. 1, 60% enantiomeric excess of (*R*)-6-methyl-5-hepten-2-ol with hydrolysis of (±)-6-methyl-5-hepten-2-yl butanoate).[22]

Figure 1.

Carboxylesterases have been examined from two strains of *S. solfataricus*, strains P1[32] and MT4.[26] The strain whose genome has been sequenced is *S. solfataricus* strain P2.[36] In order to avoid misunderstanding, the enzyme from strain MT4 (EstA) is 99% identical with the enzyme from strain P1 (Sso EST1), and both are 91% identical to a gene on the P2 genome annotated as lipP-1 lipase. Sso EST1 and EstA (along with lipP-1) are also members of the HSL family. Sso EST1 exhibits a surprisingly high optimal temperature between 95 and 100 °C compared to the optimal growth temperature of its host (75 °C). The enzyme prefers PNP-caproate among the PNP-esters, and displays k_{cat}/K_m values of $2.2\,s^{-1}\,\mu M^{-1}$ at 80 °C with 4-methylumbelliferyl acetate.[32]

The effects of temperature and various organic co-solvents on the structure and catalytic activity of Sso EST1 have been examined in detail.[33–35] The conformational state of hyperthermophilic enzymes at moderately high temperatures, such as in the range of 50–80 °C, can be expected to differ from those of mesophilic enzymes. This is due to the fact that these temperatures are still below the optimal temperature of hyperthermophilic enzymes, and therefore these enzymes can be considered to be in a structurally rigid state, while mesophilic enzymes, at temperatures above their optimum, are already in a highly flexible state.[35] In order to examine the possibilities of enhancing the function of Sso EST1 at suboptimal temperatures by increasing enzyme flexibility, various co-solvents were added to the reaction mixture using 4-methylumbelliferyl acetate as the substrate. Dimethyl sulfoxide (DMSO) was found to have an activating effect at concentrations between 1.2% and 10% (v/v), and the effect was more striking at lower temperatures. Structural and biochemical analyses at various temperatures in the presence of co-solvent suggested that the activating effect of DMSO at relatively lower temperatures could be attributed to an increase in the structural flexibility of the enzyme at suboptimal temperatures. The results point out the fact that the presence of co-solvent, in some cases, may compensate for the activating effect of temperature, and provide an alternative to reaction systems with highly stable enzymes and thermolabile substrates.[35]

The difference in behavior between hyperthermophilic and mesophilic enzymes can also be observed through the effects of temperature on their enantioselectivity.[19,28,33] The enantiomeric ratio of an enzyme reaction is related to the difference in the free energy of activation of the paths of the two enantiomers ($\Delta\Delta G^{\ddagger}$) as $\Delta\Delta G^{\ddagger} = -RT\ln E$. $\Delta\Delta G^{\ddagger}$ can also be expressed by the differences in activation enthalpy ($\Delta\Delta H^{\ddagger}$) and entropy ($\Delta\Delta S^{\ddagger}$) as $\Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger}$. When there is no enantiomeric discrimination, $E = 1$, and hence $\Delta\Delta G^{\ddagger} = 0$, or $\Delta\Delta H^{\ddagger} = T\Delta\Delta S^{\ddagger}$. The temperature at which enantiomeric discrimination is absent is defined as the racemic temperature, T_r.[28] At temperatures below T_r, the $\Delta\Delta G^{\ddagger}$ is dominated by $\Delta\Delta H^{\ddagger}$ (under enthalpic control), and the E value will decrease as temperature is elevated until it reaches 1 at T_r. At temperatures above T_r, the $\Delta\Delta G^{\ddagger}$ is dominated by $T\Delta\Delta S^{\ddagger}$ (under entropic control), and the E value will increase with the increase in temperature. $\Delta\Delta H^{\ddagger}$ is due to differences in the steric binding of the enantiomers to the substrate pocket of the enzyme through van der Waals or other noncovalent interactions, while $\Delta\Delta S^{\ddagger}$ most likely reflects differences in the rotational motion of the substrate and amino acid side chains lining the substrate binding pocket. When substrates bind to the enzyme pocket through strong interactions such as hydrogen bonds or ionic bonds, $\Delta\Delta H^{\ddagger}$ can be expected to be large, resulting in little or no effect of temperature on the enantioselectivity of the enzyme. As the substrates for carboxylesterases and lipases are in many cases lipophilic, and interact with the enzymes through relatively weak hydrophobic interactions, the effect of temperature on these enzyme reactions can be expected to be significant.[28]

The enantioselectivities of Sso EST1 and the mesophilic enzymes *Candida rugosa* lipase (CRL) and Palatase in the hydrolysis of (*RS*)-Naproxen methyl ester have been examined at various temperatures (Scheme of Fig. 2).[33]

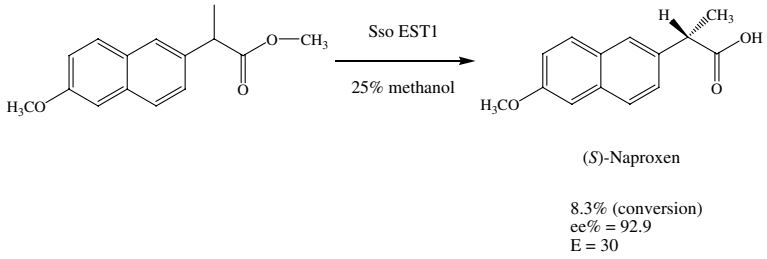

Figure 2.

The $\ln E$ versus $1/T$ (K^{-1}) plot revealed an inverse relationship between Sso EST1 and the mesophilic enzymes, the former displaying a decrease in the E value with higher temperature [>6-fold higher (*S*)-selectivity at 48.5 °C than at 70 °C], while the latter exhibited an increase in E values [>3-fold higher (*S*)-selectivity at 55 °C than at 4 °C]. The estimated T_r values were 88.1, −46.3, and 1.1 °C for Sso EST1, CRL, and Palatase, respectively. The results clearly reveal that the reactions are controlled by distinct thermodynamic features; the CRL and Palastase reactions are under entropic control, while the Sso EST1 reaction is under enthalpic control.[33] This difference can be related to the different conformational states of the enzymes mentioned above; at the examined temperatures the flexibility of the mesophilic enzymes is sufficient to encourage entropic control, while the rigidity of thermostable enzymes give rise to enthalpic control.

Possibilities for the application of Sso EST1 in chiral separations of racemic esters have also been explored. A strategic selection of esterases from hyperthermophiles was carried out for the resolution of 2-arylpropionic esters.[34] The abundant sequence information available from hyperthermophile genomes was searched with the sequences of two mesophilic esterases that have been experimentally proven to exhibit high enantioselectivity in the resolution of Naproxen ester derivatives (CRL and Carboxylesterase NP from *Bacillus subtilis* ThaiI-8). Sso EST1, along with a putative lysophospholipase from *P. furiosus*, was identified as a potential candidate. Sso EST1 proved to be the more effective enzyme, hydrolyzing the (*S*)-Naproxen methyl ester with an enantiomeric excess of over 90 and an enantiomeric ratio of 24 at 50 °C. Addition of 25% methanol led to an increase in the E value from 24 to 30 (Fig. 2). The effects of other co-solvents were also examined and revealed an inverse relationship between the denaturation capacity of the solvent[20] and the observed enantiomeric ratio. This can also be attributed to the increase in flexibility of the enzyme brought about by the solvent, counteracting with the enantioselectivity of the enzyme under enthalpic control.

Another HSL carboxylesterase has been characterized from *P. calidifontis* (*Pc*-Est).[14] *Pc*-Est is extremely thermostable, with a $t_{1/2}$ value of ca. 1 h at 110 °C, with no apparent decrease in activity after 2 h at 100 °C. The optimal temperature of the enzyme under the applied conditions was 90 °C. The enzyme also retained activity in the presence of various co-solvents; 2100 μmol mg^{-1} min^{-1} with 50% (v/v) DMSO, 560 μmol mg^{-1} min^{-1} with 50% methanol and 300 μmol mg^{-1} min^{-1} with 50% dimethylformamide (4000 μmol mg^{-1} min^{-1} with no co-solvents). *Pc*-Est preferred PNP-valerate, PNP-caproate, and PNP-caprylate among the examined PNP-esters, and displayed only little activity against PNP-palmitate. One interesting property of *Pc*-Est is its activity toward esters with branched alcohols. The enzyme hydrolyzed *sec*-butyl acetate and moreover *tert*-butyl acetate with specific activities of 880 and 270 μmol mg^{-1} min^{-1}, respectively. Carboxylesterases that hydrolyze tertiary alcohol esters are limited in number; the lipase from *Candida rugosa* and the lipase A from *Candida antarctica* have been shown to exhibit this activity.[12,13] These enzymes, as well as *Pc*-Est, harbor a GGGX motif located in the active site that contributes to the oxyanion hole. Along with a systematic examination of the enzyme activities of various GGGX-type α/β hydrolases, the importance of this motif structure in allowing the hydrolysis of tertiary alcohol esters has been revealed by computer modelling,[12,13] indicating that the GGGX motif creates a larger active site, providing more space for the alcohol. The enzymes mentioned above from *A. fulgidus* and *S. solfataricus* also harbor this motif, and are therefore also likely to hydrolyze tertiary alcohol esters.

While the enzymes mentioned above are all members of the HSL family of α/β hydrolases, a structurally distinct protein with both esterase and acyl amino acid-releasing enzyme (AARE) activity has been identified and characterized from *A. pernix*[10] The enzyme was 29% identical to the AARE from pig liver and 27% identical to the carboxylesterase from mouse liver. The pentapeptide motif was found with the sequence G-Y-S-Y-G. The recombinant enzyme was extremely thermostable, retaining 60% activity after incubation at 90 °C for 160 h. Among PNP-esters at a fixed concentration of 2 mM, PNP-caprylate was the most hydrolyzed substrate. The enzyme also hydrolyzed *N*-acetylamino acid *p*-nitroanilide derivatives as well as dipeptides.

Other than the enzymes mentioned above, a thermostable protein with esterase activity has been cloned from *P. furiosus*.[16] Unfortunately, sequence information is not available. The enyzme displayed maximum activity at 100 °C under the conditions employed, with a $t_{1/2}$ value of 34 h at 100 °C. At a substrate concentration of 625 μM, 4-methylumbelliferyl acetate was hydrolyzed 2-fold faster than 4-methylumbelliferyl butyrate. This enzyme did not hydrolyze peptide substrates. Another

study reports the screening of 160 thermophilic or hyperthermophilic microorganisms for esterase activity.[6] Forty seven strains were esterase positive, and electrophoretic profiles suggested at least three different classes of esterases were present. Interestingly, the percentage of esterase-positive microorganisms increased with the increase in isolation temperature. The thermostable esterase from *P. abyssi* was selected for further examination. At a fixed concentration of PNP-esters, C4–C6 acyl moieties were hydrolyzed the most efficiently. This esterase was also extremely thermostable, but sequence information is not available.

3. Other candidate carboxylesterase orthologues on the hyperthermophile genomes

We performed a BLAST search for serine esterases against the genome sequences of *A. pernix* K1, *S. solfataricus* P2, *Pyrobaculum aerophilum* IM2, *Sulfolobus tokodaii* 7, *A. fulgidus* DSM4304, *Methanococcus jannaschii* DSM2661, *Methanopyrus kandleri* AV19, *P. abyssi* GE5, *P. furiosus*, *P. horikoshii* OT3, *Aquifex aeolicus* VF5, and *Thermotoga maritima* MSB8 (Table 2). The sequences applied to the BLAST search were representatives of each of the (sub)families of lipolytic enzymes classified by Arpigny and Jaeger.[3] As we intended to identify as many candidate genes as possible that may encode proteins with esterase activity, we did not exclude genes that were annotated with a different function, such as a peptidase. Candidates were excluded only when the GXSXG motif was absent. We would also like to note that a sequence identified from a BLAST search is not necessarily a member of the same Family as the template sequence. A more detailed structural examination and alignment is recommended before one initiates experiments with a particular candidate.

With the Family I-2 lipase from *Burkholderia glumae*, an open reading frame with notable similarity was found on the *A. fulgidus* genome (annotated as 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase). Interestingly, a further Blast using this sequence did not lead to genes from other hyperthermophiles, but to mesophilic sequences. The sequence was 26% identical to the β-ketoadipate enol–lactone hydrolase from *Agrobacterium tumefaciens*.[27] Using the Family I-4 sequence of the lipase from *Bacillus subtilis*, a second, rather long (474 amino acid residues) open reading frame from *A. fulgidus* (annotated as putative lipase) was identified. The Family IV enzymes (HSL) are found in multiple hyperthermophiles, and besides the specific enzymes described in the previous section, orthologues can also be found on the *S. tokodaii* and *T. maritima* genomes. Using the Family V sequence from *Pseudomonas oleovorans*, multiple open reading frames from *A. fulgidus* were

Table 2. BLAST search against hyperthermophile genome sequences using members of the Family I–VIII lipolytic enzymes

BLAST template	Hits			GXSXG	Protein characterization
	Organism	Accession No	No of residues		
Family I-2 lipase from *Burkholderia glumae* (CAA49812)	*A. fulgidus*	AAB89544[a]	238	98-GLSMG-102	No
	T. maritima	AAD35147	364	160-AHSMG-164	No
	A. aeolicus	BAA80234	570	186-GVSMG-190	No
Family I-4 lipase from *Bacillus subtilis* (AAA22574)	*A. fulgidus*	AAB89488	474	134-GHSMG-138	No
Family IV esterase from *Alicyclobacillus acidocaldarius* (1EVQ_A)	*A. fulgidus*	AAB89533[b]	311	158-GDSAG-162	Yes
	S. solfataricus	AAK42652[b]	311	154-GDSAG-158	No
	S. tokodaii	BAB65028[b]	303	148-GDSAG-152	No
	S. solfataricus	AAK42629[b]	305	149-GDSAG-153	No[c]
	S. solfataricus	AAK42648[b]	251	97-GISAG-101	No
	T. maritima	AAD36236	306	158-GLSAG-162	No
Family V PHA-depolymerase from *Pseudomonas oleovorans* (AAA25933)	*A. fulgidus*	AAB88916	247	86-GHSLG-90	No
	A. fulgidus	AAB90371	266	93-GHSFG-97	No
	A. fulgidus	AAB89709	251	87-GHSLG-91	No
	S. solfataricus	AAK40458	231	69-GHSIG-73	No
	A. aeolicus	AAC07858	207	60-GWSLG-64	No
	P. abyssi	CAB50498	259	86-GHSLG-90	No
	T. maritima	AAD36421	259	84-GHSLG-88	No
	S. tokodaii	BAB67203	193	92-GASMG-96	No
	S. solfataricus	AAK43219	310	114-GHSYG-118	No
	P. furiosus	AAL80604	257	86-GHSLG-90	Yes
Type VI esterase from *Pseudomonas fluorescens* (AAC60403)	*T. maritima*	AAD35127	395	284-GLSMG-288	No
	A. pernix	BAA81456	591	449-GGSYG-453	No

[a] Also identified in the Family V BLAST.
[b] Also identified in the Family VII BLAST (not shown due to redundancy).
[c] LipP-1 mentioned in the text.

identified. One was the sequence found in the search with Family I-2 lipase and three other open reading frames annotated as est-1 (AAB90371), est-2 (AAB89709), and est-3 (AAB88916) were identified. Predicted hydrolases and lysophospholipases from various sources, including the lysophospholipase from *P. furiosus*, were also found here. A search with the lysophospholipase sequence from *P. furiosus* identified yet another putative lysophospholipase gene on the *A. fulgidus* genome (AAB89497, not shown in Table 2). A search with the Family VII enzyme from *A. oxydans* led to results similar to those obtained with the Family IV enzymes. Open reading frames with notable similarity with members of Families I-1, I-3, I-5, I-6, II, III, and VIII could not be identified.

4. Practical advantages in the use of enzymes from hyperthermophiles

Besides their thermostability, enzymes from hyperthermophiles exhibit several practical advantages compared to their counterparts from mesophiles. After gene expression in *E. coli*, or any other mesophilic host cell, purification of the protein can be performed to a relatively high degree simply by heating the cell-free extract (e.g., 85 °C, 15 min). Nearly all of the proteins deriving from the host cell precipitate after this treatment, while the recombinant thermostable protein remains in a soluble form. It is often the case that the enzymes from hyperthermophiles are 'activated' during this heat treatment to an optimal protein conformation.[17] Another important fact is that the hydrophilicity of the solvent accessible surface of a hyperthermophilic protein is generally higher than that of a mesophilic protein, with less hydrophobic patches. This allows use of the enzymes at higher concentrations in aqueous environments, and can also be supposed to increase the chances of obtaining protein crystals for structural studies. A further advantage can be easily imagined when one considers the high stability of the protein structure; introduction of a greater extent of mutations should be possible without fatally disturbing the protein fold itself. As these features generally apply for all proteins from hyperthermophiles, it is certainly worth the effort to search the hyperthermophile genome sequences for a candidate protein of one's interests.

5. Conclusions

An overwhelming number of lipases/esterases have been identified from mesophilic organisms and are being used as starting material for further improvement in performance. As described in this report, the number of proteins from hyperthermophiles that have been experimentally shown to exhibit esterase activity is still very limited, and those that have mostly belong to the Family IV (HSL) group of enzymes. The results of the BLAST analyses shown in Table 2 suggest the presence of other structurally distinct serine esterase candidates on the hyperthermophile genomes. There have also been reports where enzymes from hyperthermophiles, although exhibiting identical enzyme activities to their mesophilic counterparts, display entirely different primary structure.[30,31] Biochemical examination of the candidate esterase genes in the genome sequences, along with classical activity screening methodology, should lead to the discovery of novel thermostable esterases or possibly other hydrolases that can be used as thermostable starting material for protein engineering.

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