Bacterial Hormone-Sensitive Lipases (bHSLs): Emerging Enzymes for Biotechnological Applications

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Introduction

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) catalyze the hydrolysis of lipids or lipid-related compounds, which could release free fatty acids (FFAs), diacylglycerols, monoacylglycerols, and glycerols in the mobilization process of fats or oils [1, 2]. In addition, these enzymes, which are widespread in all three domains of life, are also involved in synthetic reactions such as esterification, transesterification, alcoholysis, or acidolysis [3, 4]. Most lipases belong to the \( \alpha/\beta \)-hydrolase superfamily, which is supposed to have a reaction mechanism similar to that of serine proteases [5, 6]. In addition to their physiological roles, lipases are highly useful for industrial applications in dairy, food, pharmaceutical, cosmetic, biofuel, and fine chemical industries owing to their high stability, broad substrate specificity, high regio-/-stereo-selectivity, and remarkable stability [7–9]. Moreover, there are still great demands for new lipases with great stability, high selectivity, and strong activity, which could be applied to a wide range of industrial processes.

Hormone-sensitive lipase (E.C. 3.1.1.79, HSL) is an intracellular lipase that can be stimulated by several hormones, such as catecholamine, adrenocorticotropic hormone (ACTH), and glucagon [10, 11]. The activity of HSL is regulated by reversible cAMP-dependent phosphorylation [12]. In adipose tissue, HSL catalyzes the breakdown steps of triacylglycerol and diacylglycerol, which promotes the release of FFAs for transport to other organs as a major source of energy. In addition, HSL has been shown to hydrolyze or synthesize a broad range of substrates containing ester linkages, which include cholesterol esters, esters of steroid hormones, retinyl esters, and other lipid-related substrates [13, 14]. This broad substrate specificity makes them highly attractive for industrial applications.

HSL has been comprehensively studied in mammalian species, and three isoforms are known, ranging in size from 84 to 130 kDa [15]. The human HSL enzyme is composed of a 786-amino-acid polypeptide without any membrane-spanning region. This enzyme could form functional aggregates at high concentrations as well as bind to phospholipid vesicles for its function [11, 13]. Using biochemical and biophysical methods, including sedimentation analyses, denaturation studies, and limited proteolysis, HSL has been shown to be composed of two domains; an N-terminal binding domain and an identical C-terminal catalytic domain [15–17]. The N-terminal domain has been implicated in mediating protein-protein interactions, whereas the C-terminal domain...
harbors a catalytic triad composed of Ser-Asp-His with several phosphorylation sites. Specifically, the N-terminal domain could interact with adipocyte lipid binding protein [18, 19]. The C-terminal domain has an α/β-hydrolase fold, which has a highly conserved Ser residue in the Gly-x-Ser-x-Gly sequence [20]. In addition, a regulatory module of ~150 amino acids, which is a main target of protein kinase A, is located in the C-terminal domain [21]. During hydrolysis, this catalytic Ser could work as a nucleophile, which is assisted by the other two residues in close spatial proximity.

Bacterial Hormone-Sensitive Lipases

Besides the highly conserved catalytic motif (Gly-Xaa-Ser-Xaa-Gly) that is observed in most lipases/esterases, the HSL family shows no sequence homology with other previously known mammalian lipases such as pancreatic lipase or hepatic lipase [22]. Specifically, the N-terminal domain lacks any similarity with any other known proteins, which is a distinctive feature of HSL. However, several bacterial proteins, which are collectively called bacterial hormone-sensitive lipases (bHSLs), are homologous to the C-terminal domain of HSL [22]. Initially, lipase 2 of Moraxella TA144 was noticed to be homologous to the C-terminal domain of HSL [22, 23]. Then, other bacterial proteins related to the HSL family have been found, including an esterase from Acinetobacter calcoaceticus, and N-acetyl hydrolases from Streptomyces hygroscopicus [24–26]. Over the last few years, there has been a substantial increase in the number of publications dealing with the isolation and characterization of bHSLs.

Since the discovery of the bHSL family, many bHSLs have been identified in a wide range of bacteria, such as Pseudomonas sp. B11-1 [27], Bacillus acidocaldarius [28], oil-degrading bacterium strain HD-1 [29], Escherichia coli [30], Archaeoglobus fulgidus [31], Psychrobacter sp. Ant300 [32], Mycobacterium tuberculosis [33–35, 42–44], Streptomyces coelicolor [36], Oenococcus oeni [37], Rhodococcus sp. CR-53 [38], Rheinheimera sp. [39], Lactobacillus plantarum WCFS1 [40, 41], and Bacillus halodurans [45]. Furthermore, many bHSL genes were identified through metagenomic approaches directly from environmental DNA samples [46–64]. These metagenomic bHSLs were isolated from soil [46, 53, 55, 56], mud and sediment-rich water [47], environmental soils [48], forest soil [49, 60, 64], marine sediments [50, 51, 58], activated sludge [52, 54], mountain soil [57], tidal flat sediment [59], surface sediment [61], and permafrost [62, 63]. Interestingly, bHSL family members were shown to be selectively inhibited by 5-methoxy-3-(4-phenoxyphenyl)-3H-[1,3,4]oxadiazol-2-one [65].

Although the number of bHSLs being discovered is...
largely increasing, there is still limited information about this protein family, and only a small number of them have been characterized. The consensus amino acid sequences of the representative members of the bHSL family members are shown in Fig. 1. For biochemical characterizations of bHSLs, heterologous expression of the bHSL family members were achieved by using *E. coli* as a host [36-39]. In addition, gene products from uncultured microorganisms were successfully expressed and purified [58-61, 63]. The products of Rv1399c (LipH), Rv3097c (LIPY), (Rv2970 (LipN), or Rv1076 (LipU) from *M. tuberculosis* were overexpressed as inclusion bodies, and proteins were refolded with several modifications [33, 34, 42, 43]. Interestingly, PsyHSL from *Psychrobacter* sp. TA144 has been expressed in a soluble form in the presence of trehalose or benzyl alcohol [66]. Moreover, an N-terminally his-tagged Cest-2923 was marginally stable in solution, and a C-terminally his-tagged protein showed an oligomeric conformation suitable for crystallization [40, 67]. After purification, molecular features of the bHSL family members were studied in terms of crystal structures, thermostability, substrate specificities, enantioselectivity, and kinetic parameters.

**Structures of bHSLs**

To date, several members of the bHSL family have been structurally described, including brefeldin A esterase (BFAE) from *Bacillus subtilis* [68], EST2 from *Alicyclobacillus acidocaldarius* [69], AFEST from *A. fulgidus* [70], EstE1 from a metagenomic library [71], EstE5 from a metagenomic library [72], Sto-Est from *Sulfolobus tokodaii* [73], EstE7 from a metagenomic library [74], PestE from *Pyrobaculum caldofontis* VA1 [75], Rv0045c from *M. tuberculosis* [76], Est25 from a metagenomic library [77], Cest-2923 from *L. plantarum* WCFS1 [40, 41], E40 from a marine sedimental metagenomic library [78], and Est22 from a deep-sea metagenomic library [79]. Although these bHSL family members showed relatively low primary sequence identity, all these crystal structures are composed of an α/β-hydrolase fold that is found in many hydrolases, including proteases, esterases, peroxidases, and dehalogenases [80, 81]. The α/β-hydrolase fold has a left-handed β-sheet containing parallel β-strands, although one strand (β2) strand is antiparallel to the other strands. These strands are connected by a bundle of helices in both concave and convex sides [82].

These bHSLs have a cap domain, which is composed of two or three α-helices in the upward region of the main β-sheet. The cap domain is composed of 50–100 residues from the N-terminal residue and 30–50 residues between β6 and β7 in the central β-sheet. Specifically, in Est25, the first region of the cap domain is composed of 85 amino acids, which form two α-helices that cover the active site [77]. In EST2, this N-terminal region of the cap domain has been shown to play a significant role in maintaining enzyme activity, stability, and specificity [83]. Structural comparisons revealed that the most striking differences among the bHSL family are in the cap region (Fig. 2). The main variability resides in the different lengths and relative orientations of helices and connecting loops. In crystal structures of bHSLs, this region has high B factors compared with other regions [77-79]. The second region of the cap domain between β6 and β7 is clearly separated from the core β-sheets. This region (~150 residues) in the mammalian HSL family, which is notably longer than that of bHSL proteins, contains several post-translational phosphorylation sites for the regulation of these enzymes [84, 85]. The regulatory mechanism that mediates the movement of the cap domain toward efficient substrate binding is still not clearly understood. To date, in several attempts to understand the conformational changes that occur in bHSLs, X-ray crystallography as well as molecular...
dynamics simulations were performed [75, 77, 86].

Using site-directed mutagenesis, catalytic residues as well as the key residues in the substrate-binding region were identified [87–89]. The active site of the bHSL family members consists of a canonical catalytic triad, with Ser as the nucleophile, His as the proton carrier, and Asp as the acidic residue [80]. A tetrahedral intermediate, which could be stabilized by residues in the oxyanion hole, is formed when an alkoxide ion from the catalytic Ser attacks the electrophilic carbon of the ester linkage. The active site of these enzymes is negatively charged in their optimal pH range, which facilitates the separation of the products after the cleavage [90]. The crystal structure of the EstE5-PMSF complex showed a detailed atomic view around the catalytic triad of bHSLs [72]. Interestingly, a short sequences, motif of His-Gly-Gly-Gly, which is located 70–100 amino acids upstream of the catalytic Ser, is largely conserved within the members of the bHSL family. This motif has been reported to stabilize the oxyanion hole during the hydrolysis of tertiary alcohols [91, 92].

Most of the bHSL family members, including BFAE, Est25, EstE1, and PestE, exist as dimers or larger oligomers, which are stabilized mainly by hydrogen bonds and hydrophobic interactions between two antiparallel β8 strands from the α/β-hydrolase domain. These oligomers are further classified as subtype 1 and subtype 2 based on the differences in the dimeric interfaces [41]. In Aes, the two α-helices in the catalytic domain appear to be major factors in facilitating dimerization [93]. In EstE1, two residues of Val274 and Phe276 were identified to be important for its dimerization [71]. In addition, Tyr282 in the wall of the active site was largely responsible for the thermal stability of EstE1 [94]. In Sto-Est, Arg296 was shown to be critical for maintaining dimer conformation [73]. In E25, dimerization is essential for the catalytic function of Asp292 [61]. Interestingly, Cest-2923 exhibits a pH-dependent pleomorphic behavior, although a dimer is favored at neutral pH [40]. In E40, introduction of largely hydrophobic residues such as Trp or Phe in α7 significantly improved the half-life of this protein to several hundred folds, suggesting that hydrophobic interactions between the α/β-hydrolase fold and cap domain is a key element for thermostability [78]. As dimerization is closely related to enzyme stability, bHSL proteins are qualified for biotechnological and industrial applications at high temperatures [36, 62, 71].

Biotechnological Perspectives

Most of the bHSL family members function optimally at mesophilic temperatures, although a systematic approach was carried out to identify HSL genes from glacier soil [95]. However, Blip from Bacillus halodurans C-125 showed high thermal stability with a $t_{1/2}$ of 35 min at 100°C, and complete refolding to its native conformation after 30 min at 90°C was observed [45]. In addition, EstE1 and EST2 were active at high temperatures of up to 95°C [96, 97]. A structural comparison of AFEST, BFAE, and EST2 has demonstrated that an increase in the number of electrostatic interactions and hydrogen bonds as well as a decrease in loop sizes and hydrophobic areas seemed to be a main reason for their thermostability [98]. Important residues in AFEST, such as Arg48 and Arg448, for thermostability were identified mostly in loops regions or α-helices [97]. In contrast, PsyHSL, Lipo1, and EstIM1 are cold-active enzymes with high catalytic activity at low temperatures [52, 57, 99]. Interestingly, in PsyHSL, osmolytes were shown to increase its thermal stability and its specific activity. In the presence of 3 M trimethylamine N-oxide (TMAO), the specific activity of PsyHSL increased by ~2.5 fold at 50°C, whereas the addition of 1 M TMAO increased its thermostability by 5-fold at 45°C [66]. In addition, the activity of PMGL2 from a permafrost metagenomic library was reported to be stimulated in the presence of 0.25–1.5 M NaCl [62]. Interestingly, immobilization increased the thermal stability and half-life of LipN [42]. The increased stability and recyclability of cross-linked Est25 aggregates (CLEA-Est25) were also reported [77].

It is widely known that bHSLs are promiscuous by nature, and this is related to their physiology, evolution, and metabolism [81]. Most of the bHSL family members preferentially hydrolyze short-chain p-nitrophenyl esters and tributyrin. Specifically, AFEST and EST2 hydrolyzed p-nitrophenyl hexanoate with $K_m$ and $k_{cat}$ values of 11 μM and 220 μM, and 1,014 s$^{-1}$ and 3,420 s$^{-1}$, respectively [100, 101]. In addition, PsyHSL was highly active against pNP-pentanoate, with $k_{cat}/K_m$ of 215 mM$^{-1}$s$^{-1}$ [99]. The $K_m$ and $V_{max}$ of LipU were calculated to be 1.73 μM and 62.24 μM/min, respectively [102]. LipN was able to synthesize methyl butyrate as well as hydrolyze 4-hydroxyphenylacetate to hydroquinone [42]. EstDL26 and EstDL136 reactivated chloramphenicol from its acetyl derivatives by countering the chloramphenicol acetyltransferase activity in E.coli [56]. Substantial enantioselectivity of Est25 and EST2 was observed with the preference for (R)-product [103, 104]. Furthermore, Est4 and Est25 successfully hydrolyzed the tertiary alcohol esters such as linalyl acetate and α-terpinyl acetate [38, 77]. In addition, PestE showed high enantioselectivity in the kinetic resolution of racemic esters, which could be explain
by its structural features [75]. In a recent report, EST2 was used as a biosensor for the detection of organophosphate pesticides such as paraoxon [105] or foodborne bacteria [106]. Furthermore, EST2 was immobilized on a nitrocellulose membrane for longer stability, good reproducibility, and high sensitivity [105]. In this respect, HerE was shown to hydrolyze the acetyl groups from heroin to yield morphine, which could be exploited to develop a heroin biosensor [107].

In conclusion, bHSLs from different bacteria and metagenomic libraries exhibit a wide range of thermal and chemical stabilities, substrate specificities, and kinetic properties. These varying properties allow bHSLs to be employed in a large spectrum of biotechnological applications, such as in detergents, food additives, bioremediation, biofuels, and biotransformation. This review aimed to compile recent advances in the study of bHSLs, which could pave a way for the preparation of more active and selective biocatalysts. Further studies on the binding mechanisms, interaction modes, cap domain movements, and in vivo substrates of the bHSL family remain to be investigated. In addition, crystallographic studies of the bHSL family members are also required to comparatively elucidate the differences in structures, substrate-binding mechanisms, and catalysis between them and other related lipases. Moreover, protein engineering strategies could also be employed in order to design enzymes that are more suitable and efficient for biotechnological applications. In summary, the study of bHSLs and their use in industrial applications are exciting fields of research, and various new applications are to be expected in many areas of biotechnology.

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