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Research review paper

Structural traits and catalytic versatility of the lipases from the *Candida rugosa*-like family: A review

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ABSTRACT

Lipases and sterol esterases are enzymes with broad biotechnological applications, which catalyze the hydrolysis or synthesis of long-chain acylglycerols and sterol esters, respectively. In this paper, we review the current knowledge on the so-called *Candida rugosa*-like family of enzymes, whose members display in most cases affinity against the two substrates mentioned above. The family includes proteins with the α/β-hydrolase folding, sharing conserved motifs in their sequences, and common structural features. We will go through their production and purification, relate their described structures and catalytic activity, and discuss the influence of the hydrophobic character of these lipases on their aggregation state and activity. On the basis of the few crystal structures available, the role of each of the functional areas in catalysis will be analyzed. Considering the particular characteristics of this group, we propose their classification as "Versatile Lipases" (EC 3.1.1.x).

Contents

1. Introduction

Lipases, also known as triacylglycerol lipases (EC. 3.1.1.3), act on ester bonds of several compounds, with acylglycerols as their natural substrates. These enzymes catalyze the hydrolysis of triglycerides to produce free fatty acids, diglycerides and/or monoglycerides under aqueous conditions, but they can also carry out synthesis reactions, such as esterification and transesterification, in the presence of organic solvents (Houde et al., 2004; Reis et al., 2009).

On the other hand, sterol esterases (EC 3.1.1.13) are defined as enzymes that hydrolyze sterol esters releasing free sterols and fatty acids in aqueous media, being also able to perform synthesis reactions

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in the presence of organic solvents (Barba Cedillo et al., 2013; Morinaga et al., 2011). Most of the known sterol esterases have been reported to have both lipase and sterol esterase activity (Calero-Rueda et al., 2002; Maeda et al., 2008; Vaquero et al., 2016).

Lipases and sterol esterases are carboxylic ester hydrolases (EC 3.1.1) that share the α/β-hydrolase fold (Grochulski et al., 1993; Holmquist, 2000; Nardini and Dijkstra, 1999). Hence, their catalytic machinery includes the residues of the catalytic triad (serine, histidine and aspartic or glutamic acid) and the oxyanion hole, a pocket in the active site involved in catalysis. They compose a very diverse group of ubiquitous enzymes in nature and are represented from microbes to plants and animals. Nevertheless, bacterial and fungal lipases are of special interest as they are easily produced and applicable for industrial processes due to their versatility and stability to harsh conditions (Gupta et al., 2015; Jaeger and Eggert, 2002; Schmid and Verger, 1998; Singh and Mukhopadhyay, 2012).

These enzymes can perform a variety of reactions, have wide substrate specificity and good selectivity, and are regio- and stereoselective. The most important lipases from a commercial point of view belong to yeasts, such as *Candida rugosa* (synonym *Candida cylindracea*) and *Candida antarctica*, or filamentous fungi, such as *Aspergillus niger*, *Humicola lanuginosa*, *Mucor miehei*, and *Rhizopus* species (Bornscheuer, 2002; Domínguez de María et al., 2006; Gupta et al., 2015; Hasan et al., 2006; Singh and Mukhopadhyay, 2012). Among them, some of the characterized lipase isoenzymes from *C. rugosa* show also sterol esterase activity, as those from other microorganisms, still not produced at commercial level, which we will see across this review.

Esterol esterases and lipases are included in ESTHER (http://bioweb.ensam.inra.fr/esther), a broad database that collects very complete information on the members of the α/β-hydrolase fold superfamily (Lenfant et al., 2013). In addition, a sequence-based comparatively simplified version is available in the Lipase Engineering Database (LED) (http://www.led.uni-stuttgart.de/). LED lists sequences of all the available microbial enzymes with lipase activity, putative or not, and provides links to 22 published lipase structures. This database serves as a bioinformatics tool for the systematic analysis of sequence, structure and function of diverse lipases, and for designing variants with optimized properties (Fischer and Pleiss, 2003). Based on different characteristics, such as the presence of specific conserved motifs in their amino acidic sequence, microbial enzymes with lipase activity are grouped under several classes or subclasses in these databases. Yeast and fungal lipases fall into five different subclasses: *Yarrowia lipolytica*-like lipase, *C. rugosa*-like lipase, filamentous fungi lipases, *C. antarctica* lipase B-like and *C. antarctica* lipase A-like. Moreover, on the basis of their sequence, structure, and function, these enzymes are classified in LED into the GX, GGGX and Y classes.

In this review we will focus on a very versatile group of enzymes that generally show activity towards acylglycerols and sterol esters, the so-called *C. rugosa*-like or abH03.01 lipase family. Only a few members of this group have been characterized, although recent findings have shown their potential for engineering future industrial applications. The characterized proteins belong to ascomycetes and basidiomycetes, and little is known about their phylogenetic affiliation and evolution (Barriuso et al., 2013). According to LED, the 336 protein sequences currently assigned to this family belong to the class GGGX. However, most of them correspond to hypothetical proteins and, according to this sequence-based classification, some enzymes can be wrongly ascribed to this group. This was the case of the hypothetical protein EstA from *A. niger* that, once expressed, showed to have structural characteristics and substrate preferences different to those characteristic of the *C. rugosa*-like family (Bourne et al., 2004). The enzymes described within this group are glycoproteins with a common overall structure. Their active sites are hidden under a mobile region denominated lid or flap that, in a lipid-water interface or in the presence of substrates or inhibitors, rearranges its position leaving an open gate to the active center. Then, the position of the lid marks the difference between the open (active) or closed (inactive) forms of these proteins. Other interesting properties of these catalysts as their temperature and pH stabilities (T_{50} = 40–60 °C, and pH 4–10), or their stereo- and regioselectivity (Colton et al., 2011; Lee et al., 2007; Palocci et al., 2007; Vaquero et al., 2015a), make them very attractive, and the current or proposed application of these catalysts affect a wide range of industrial sectors, such as biofuels, oleochemical, food, detergents, cosmetics, pharmaceutical, textile and paper industry (Hasan et al., 2006; Houde et al., 2004; Reetz, 2002; Singh and Mukhopadhyay, 2012). Excellent reviews covering the biotechnological applications of lipases, including the best known enzymes from the *C. rugosa*-like family, have been recently published (Gupta et al., 2015; Stergiou et al., 2013). Here, we will revise their structural and catalytic properties, their production and characterization in different hosts, and the applicability of tools as genome mining for *in silico* search of novel catalysts or protein engineering for tailoring enzyme activity.

2. Ecophysiological role

Fungi producing *C. rugosa*-like enzymes share a plant-associated habitat, and most of them have been isolated from natural soils where plant material is frequent (Tomizuka et al., 1966). In these cases, the ecological role of the extracellular lipases can be related to the attack of esters from the epicuticular waxes and cuticle (Juniper and Jeffree, 1983), improving the accessibility to cell-wall polysaccharides that can serve as carbon sources for plant-associated fungi. In pathogenic species these enzymes may act also on such components, located in the surface of leaves, facilitating fungal colonization (de Vries et al., 1997; Doss, 1999).

The only studied lipase of this family from a basidiomycete is produced by *Pleurotus sapidus*, a white-rot ligninolytic fungus (Zorn et al., 2005), while several have been characterized form saprophytic ascomycetes, isolated from forest, agricultural, or composting soils. Among them, we can mention *C. rugosa*, a non-sporogenic imperfect hemiascomycete, a number of filamentous fungi, as the thermophilic *Melanocarpus albomyces* (Kontkanen et al., 2006a), isolates from *Trichoderma* (Schuster and Schmoll, 2010) or *A. niger* (Hu et al., 2011), and the dimorphic ascomycetous yeast-like fungi *Geotrichum candidum* and *Ophiostoma piceae*. The habitat of *G. candidum* is often associated with all kinds of soft plant tissues, but it is ubiquitous in soils and pupal galleries of bark beetles (de Hoog and Smith, 2004). The environment of the wood-staining fungus *O. piceae* is much more restricted. This species disseminates its spores using bark beetles as vectors and lives as saprobe in the superficial layers of conifers' sapwood. There, it metabolizes wood lipids, releasing hydrolysis products as glycerol that seem to play an important role in the formation of the dark pigments responsible for the "blue stain" (Eagen et al., 1997), which causes severe losses to the forestry industry (Calero-Rueda et al., 2002; Haridas et al., 2013). Some extracellular fungal lipases are also gaining attention for their potential role as virulence factors in relation to colonization, adhesion, biofilm formation and pathogenesis (Gupta et al., 2015). Plant pathogens as *Fusarium solani* are also producers of these enzymes (Vaquero et al., 2015a), and the causal agent of the bunch rot disease of grapes, *Botritys cinerea*, releases lipases during the early phases of parasitism induced by the components of the cuticle, being capable of entering its host directly through an undamaged plant cuticle (Comménil et al., 1999).

Many other hypothetical proteins from this family have been described in the genomes of fungi usually living as vegetal saprobes or parasites, such as ascomycetes from the genera *Alternaria*, *Pyrenophora* and *Neurospora*, or basidiomycetes belonging to *Postia*, *Laccaria* and *Puccinia*, although these enzymes have not yet been produced and characterized (Barriuso et al., 2013; Fischer and Pleiss, 2003).

3. Production, purification and biochemical characterization of the *C. rugosa*-like enzymes

Due to their wide applicability, enzymes from this family have been produced in a variety of hosts, from their native producers to recombinant