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REVIEW

The production, properties, and applications of thermostable steryl glucosidases

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Abstract
Extremophilic microorganisms are a rich source of enzymes, the enzymes which can serve as industrial catalysts that can withstand harsh processing conditions. An example is thermostable β-glucosidases that are addressing a challenging problem in the biodiesel industry: removing steryl glucosides (SGs) from biodiesel. Steryl glucosidases (SGases) must be tolerant to heat and solvents in order to function efficiently in biodiesel. The amphipathic nature of SGs also requires enzymes with an affinity for water/solvent interfaces in order to achieve efficient hydrolysis. Additionally, the development of an enzymatic process involving a commodity such as soybean biodiesel must be cost-effective, necessitating an efficient manufacturing process for SGases. This review summarizes the identification of microbial SGases and their applications, discusses biodiesel refining processes and the development of analytical methods for identifying and quantifying SGs in foods and biodiesel, and considers technologies for strain engineering and process optimization for the heterologous production of a SGase from *Thermococcus litoralis*. All of these technologies might be used for the production of other thermostable enzymes. Structural features of SGases and the feasibility of protein engineering for novel applications are explored.

Keywords Extremozymes · Biofuels · Steryl glucosidases

Introduction

Enzymes are biocatalysts with a great diversity of industrial applications, having constantly evolved since the 1960s to form a multibillion dollar global market. Not only have enzymes replaced traditional chemical processes, but they have found novel uses in feed and food production, biofuel and energy generation, and the production of bulk chemicals and pharmaceuticals. The intrinsic biodegradability of enzymes confers upon them a low environmental impact (Rozzell 1999), another value in addition to their fast, efficient, and selective catalytic activities. However, conditions in current industrial processes are often far from those required by standard enzymes, commonly involving extremely high or low temperatures, acidic or basic pHs, and elevated salinity and the presence of solvents. Such working conditions normally denature enzymes, making traditional chemical processes the only option.

In the search for enzymes capable of catalyzing reactions under more extreme conditions, extremophilic microorganisms have become a promising source of novel biocatalysts. Enzymes found in extremophilic bacteria and archaeal microorganisms, called extremozymes, can catalyze their natural reactions at extreme pH, temperatures, or pressures, in water/solvent mixtures, or even in nonaqueous environments (Adams et al. 1995). Yet in spite of their many potential industrial applications, only a few extremozymes have found their way to the market (Elleuche et al. 2014; Sarmiento et al. 2015). In fact, the majority of enzymes that are currently used in industry come from fungi or mesophilic bacteria. Difficulties related to working with extremophiles

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and/or extremozymes (i.e., the requirement of new methodologies, assays, and techniques that operate under non-standard conditions), and many technical challenges that involve the production of extremozymes at an industrial scale, still need to be solved in order to fully realize the potential of these biocatalysts.

Recently, extremozymes solved a very complex industrial problem. The need for an efficient and inexpensive method to remove insoluble precipitates in biodiesel, the precipitates being mainly composed of steryl glucosides (SGs), guided the discovery of thermostable steryl glucosidases (SGases) (Aguirre et al. 2014). Not only can this group of thermostable β-glucosidases catalyze the hydrolysis of SGs at temperatures up to 80 °C, but the enzymes can tolerate the presence of organic solvents such as biodiesel. The identification of an enzyme with SGase activity was followed by characterization and process improvements that overcame high production costs.

Steryl glucosides in biodiesel: an enzymatic solution

SGs and acylated SGs (ASGs) are present naturally in plant tissues and vegetable oils, being derivatives of sterols that are the main unsaponifiable components of plants (Sugawara and Miyazawa 1999). In ASGs, the 6-position of the sugar is esterified to a long chain fatty acid. Biodiesel [fatty acid methyl esters (FAME)] is synthesized via chemical transesterification from vegetable oils. Under the alkaline conditions employed during transesterification, the ester bond between glucose and the fatty acid is broken, and the ASGs are converted to SGs. This reaction increases the concentration of SGs in biodiesel compared to their initial concentrations in the oil feedstock (Van Hoed et al. 2008). The loss of the aliphatic chain increases the polarity of SGs relative to ASGs, causing the formation of insoluble precipitates (Lacoste et al. 2009). If not removed from the biodiesel, SGs can clog oil filters and cause engine failures. Particles of clumped SG molecules can also promote crystallization, aggregation, or precipitation of other compounds in biodiesel. These phenomena further reduce the flowability of biodiesel and increase the likelihood of clogging. SGs typically have a high melting point and thus cannot be heated in order to pass them through an oil filter. Consequently, they form crystals in biodiesel, especially at low temperatures, creating cold-flow problems and blockages in fuel lines under cold conditions. Additionally, the formation of these precipitates can cause problems during the production of biodiesel, increasing production costs (Menzella et al. 2012; Soe 2010).

The levels of SGs in finished biodiesel can differ depending on the processing method used for vegetable oil and on the biodiesel production process (Lee et al. 2007), ranging from 20 to 500 ppm (Ringwald 2007). The only method capable of completely removing SGs from biodiesel is distillation, which is energy-intensive and therefore reduces the cost efficiency and net energy gain of biodiesel production (Tang et al. 2010). Filtering, such as through diatomaceous earth, or adding absorbents, is expensive, time consuming, and not easily scalable (Brask and Nielsen 2010).

A glucosidase capable of hydrolyzing SGs in biodiesel would produce sterols (which are soluble in biodiesel) and a free sugar molecule (which can be removed by a water-washing step typically used in the biodiesel production process) (Fig. 1). In a scheme for producing biodiesel, the optimal timing for an enzymatic treatment would be after the aqueous washing step that follows the transesterification process, when the concentration of the denaturant, methanol, and methylate are relatively low and, importantly,

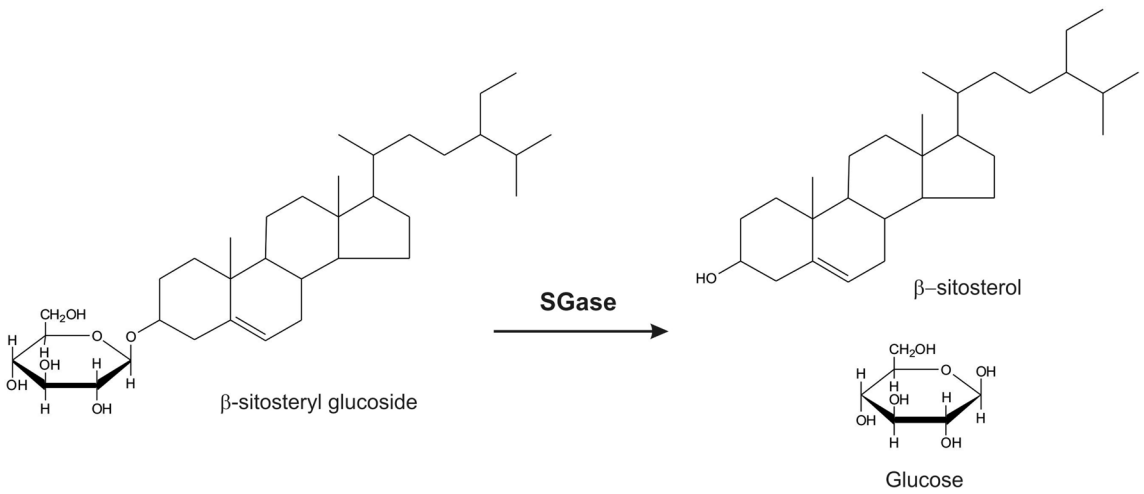

Fig. 1 Schematic representation of the SGase reaction, showing the hydrolysis of β-sitosteryl glucoside, the most abundant SG in soybean oil

temperatures around 70 °C keep SGs soluble (Leung et al. 2010). Thus the search for SGases focused on thermostable extremozymes, which are naturally adapted to high temperatures and are also often tolerant to solvents (Elleuche et al. 2014), a critical feature considering the intended interaction with biodiesel.

Previous reports of SGase activity were scarce, involved crude or partially purified extracts from mesophilic organisms, and had no known protein sequences (Kalinowska and Wojciechowski 1978; Nystrom 2008). The clue came from investigating β-glucosidases from glycoside hydrolase family 1, a family of enzymes that recognizes the glucose moiety and displays a relaxed specificity with regard to the aglycone portion of the molecule. Narrowing the search to thermostable enzymes that were previously reported to hydrolyze ginsenosides, a group of molecules structurally analogous to SGs (An et al. 2010; Noh and Oh 2009; Noh et al. 2009), permitted the determination of SGase activity in LacS from *Sulfolobus solfataricus* and seven closely related homologs (Aguirre et al. 2014). In this work, SGase activity was initially tested with SGs dissolved in aqueous media at 70 °C, using LacS obtained by heterologous expression of a codon-optimized gene in *Escherichia coli*. LacS also possessed SGase activity in reactions performed with distilled biodiesel supplemented with SGs and 13% aqueous buffer at 80 °C. The performance of LacS was evaluated in different reaction conditions in order to improve the efficiency of treating biodiesel. A critical factor for increasing the hydrolysis of SGs was the use of polyglycerol polyricinoleate (PRPG), a powerful water-in-oil emulsifier, to overcome mass-transfer limitations of SGs across the biodiesel/water interface. Maximum activity was achieved at 87 °C and pH's between 5 and 6.5, when the total amount of hydrolyzed SGs was 81%. SGs used in these experiments consisted of a mixture of β-sitosteryl glucoside, campesteryl glucoside, and stigmasteryl glucoside (50:25:25). It was observed that the three SGs species decreased at the same rate, indicating no preference by LacS between these substrates.

The requirement by LacS for an emulsifier limited the applicability of the enzyme for treating biodiesel at an industrial scale, requiring a search for new thermostable SGases. A new group of β-glucosidases that naturally function at water/lipid interfaces was evaluated (Peiru et al. 2015). These enzymes are related to BGPh from *Pyrococcus horikoshii*, a membrane-associated protein which catalyzes the hydrolysis of glucosides with long alkyl chains (Matsui et al. 2000). A SGase named BGTl, obtained from the archaeon *Thermococcus litoralis*, was selected from this group because it efficiently catalyzed the hydrolysis of SGs in biodiesel without needing an emulsifier. GC-FID analysis showed that the enzyme was an equally efficient catalyst with the three main types of SGs present in biodiesel derived from soybean oil (Fig. 2a). In that work, process optimization allowed a reduction of the amount of water used for hydrolysis to 4.5% of the volume of treated biodiesel. This reduction also decreased the concentration of ions present in the hydrolysis buffer to levels that meet the requirements of ASTM standard D6751 and CEN standard EN14214. Treatment of biodiesel at a 20-ton scale was successfully achieved by using a stirred-tank reactor. Within approximately 2 h, 7 g of BGTI per ton of biodiesel completely eliminated the 75 ppm of SGs present (Fig. 2b).

The presence of SGs in biodiesel strongly impacts tests for quality, such as the Total Contamination (TC) test, the Cold Soak Filtration Test (CSFT), the Filter Blocking Tendency (FBT) test, and the Cold Soak Filter Blocking Tendency (CSFBT) test (Camerlynck et al. 2012; Na-Ranong and Kitchaiya 2014; Pfalzgraf et al. 2007; Plata et al. 2014). Treatment by SGase greatly improved the performance of biodiesel in all of the tests, reaching results far below the limits specified for each test. The values observed in all of the cases were comparable to those of distilled biodiesel, indicating the outstanding efficiency of the enzymatic process in generating biodiesel of superior quality.

Other applications of SGases

Highly efficient degradation of SGs in biodiesel catalyzed by BGTI enabled the development of a sensitive and reproducible assay to quantify SGs in samples of biodiesel (Aguirre et al. 2015). The assay is based on measuring the glucose released by the interaction of BGTI with SGs by means of a fluorometric-enzymatic assay. Glucose quantitatively partitions to the water phase, after which a coupled reaction with hexokinase and glucose-6-phosphate dehydrogenase produces one mole of NADPH per mole of glucose. Quantification of SGs is thus performed by measuring the fluorescence of NADPH at 460 nm upon excitation at 360 nm.

In contrast to GC/HPLC/MS analyses, this assay can be performed by using low-cost instrumentation such as a standard fluorimeter. A plate reader can also be used, making high-throughput formats possible. With a plate reader, the content of SGs in 96 samples can be quantified in just 3 h. Because the assay functions in complex heterogeneous matrices such as crude biodiesel, it can be used routinely for the quality control of samples of biodiesel. Similarly, the method has been used to detect SGs in crude vegetable oils (Aguirre, unpublished results). This application is particularly relevant for the olive oil industry, where the content of SGs can be used as an identity parameter and a blend indicator (Gómez-Coca et al. 2012).

Another method for detecting SGs based on the enzymatic hydrolysis of SGs was described by Münger and Nyström (2014). This method was developed to accurately determine the sterol profile of SGs in extracts of lipids from

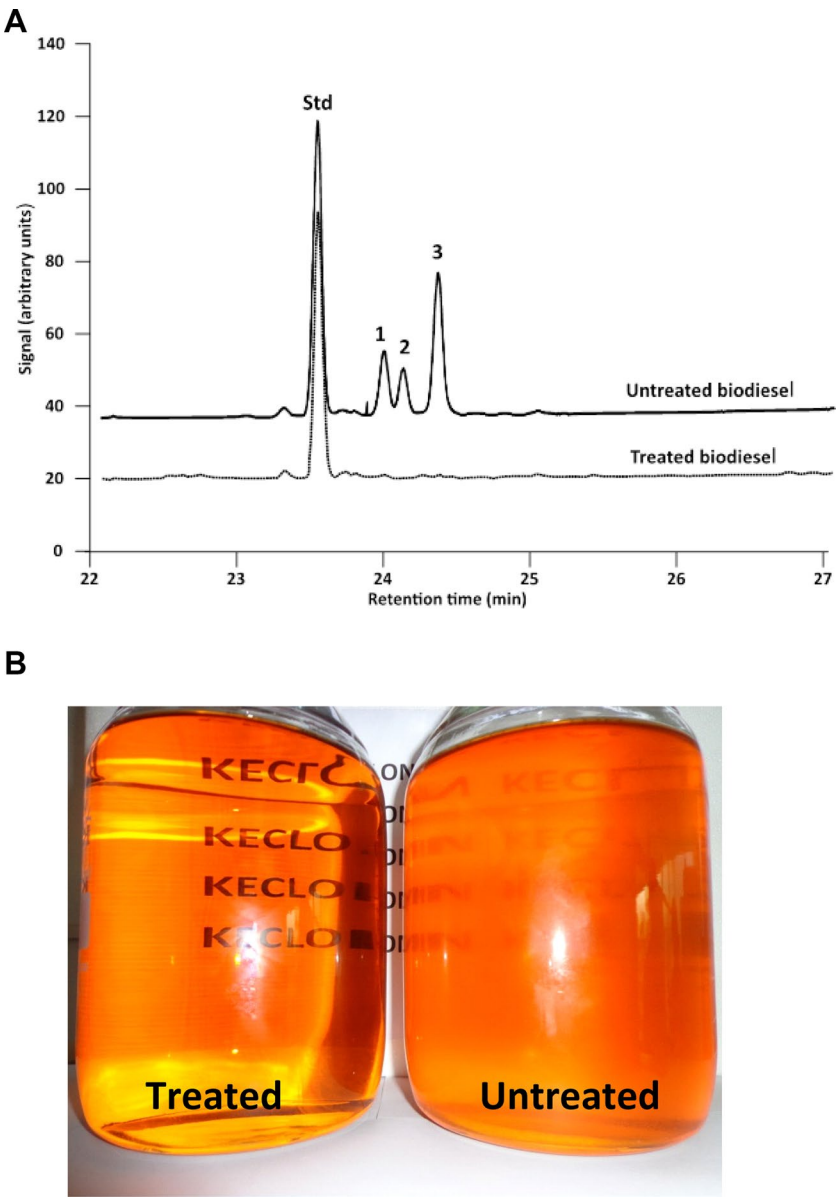

Fig. 2 BGTl-mediated removal of SGs from soybean biodiesel in a 20 ton industrial reactor. **a** Samples of biodiesel before and after enzymatic treatment. **b** SPE-GC-FID analysis of both biodiesel samples. Traces are shifted on y axe for clarity. Peaks are labeled as follows: Std, cholesteryl glucoside standard; 1, campesteryl glucoside; 2, stigmasteryl glucoside and 3, β-sitosteryl glucoside

different foods, information which had been difficult to obtain due to analytical constraints. Several reports indicate that phytosterols, when consumed regularly, can decrease total and low-density lipoprotein cholesterol levels in serum. These and other health benefits, such as the reduction of benign prostate hyperplasia and the enhancement of T-cell proliferation, have been demonstrated when free and glycosylated phytosterols are part of the human diet (Bouic et al. 1996; Lin et al. 2009). The amphiphilic nature of SGs imply better solubility in more polar solvents when compared to other sterols, such that SGs might be introduced into foods with a higher water content than sterol-enriched functional foods (Oppliger et al. 2014).

The enzymatic hydrolysis of this method aims to avoid the disadvantages of the acid hydrolysis needed to cleave the acetal bond in SGs, the cleavage which permits further derivatization for analysis by gas chromatography coupled to a flame ionization detector (GC-FID). Acid hydrolysis isomerizes some sterols due to protonation of their alkane side chain at low pH, altering the apparent sterol profile for certain plant foods such as nuts and seeds. Münger and Nyström tested several enzymes and discovered that inulinases obtained from *Aspergillus niger* exhibit SGase activity. The application of these SGases established a method to determine the content of SGs in previously uncharacterized foods. Although the enzymes tested are mesophilic, some thermophilic inulinases have been reported and should be evaluated for their potential SGase activity. Conversely, the well-known catalytic properties of BGTl indicate that this enzyme could also be used in this method.

Optimization of the production of thermostable SGases

Cultivation of hyperthermophiles is usually problematic because of low growth yields, heating requirements, and the generation of H_2S when elemental sulfur is used as an electron acceptor. Although several developments have been reported for the production of biomolecules by extremophiles at the bioreactor scale (Deive et al. 2012), the large-scale production of extremozymes typically relies on heterologous mesophilic hosts. *E. coli* is by far the most widely used expression host for the production of recombinant proteins. However, hyperthermophilic proteins are frequently expressed as inclusion bodies in this host, due to the difference in the optimal temperature for growth between the host cell and hyperthermophilic archaeon (Umetsu et al. 2004). This was the case for some of the thermophilic SGases expressed in *E. coli* (Peiru et al. 2015), such that several approaches were employed to overcame this problem and to avoid the need to use refolding strategies.

BGTl and its homologs exhibit an exposed hydrophobic patch, which indicates that they are membrane-bound proteins (Akiba et al. 2004; Peiru et al. 2015). It is well known that the heterologous expression of membrane proteins represent a technical challenge, because the proteins are frequently (i) toxic for the host, (ii) expressed at very low levels in a spatially delimited membranous environment, and (iii) mis- or unfolded (and thus inactive) (Bernaudat et al. 2011; Miroux and Walker 1996). Several hosts have been described for the heterologous production of membrane proteins, *E. coli* being the most commonly used system (Junge et al. 2008). Moreover, a comparative study in which a set of 20 different membrane proteins (including both peripheral and integral membrane proteins) were expressed in different heterologous hosts also indicated that the highest production yields were obtained with *E. coli* (Bernaudat et al. 2011). This host also provides several alternatives for protein expression, such as strains C41, C43, or Lemo21, which are more tolerant to toxic membrane proteins, or to the introduction of tags such as GFP, MBP, GST, NusA, or Mistic, that can facilitate and improve the production of membrane proteins (Bernaudat et al. 2011).

The potential use of SGases in a process for treating biodiesel demanded the development of a robust and cost-effective procedure for their industrial production. When a cost analysis of soybean biodiesel production and refining are considered, the manufacturing process for producing a SGase should cost below $1 per dose of enzyme (defined as the amount required to eliminate SGs from a ton of biodiesel), in order to make possible a cost-effective alternative to distillation for producing high quality biodiesel. To reach this objective, which implies the unprecedented goal of producing a membrane-bound extremozyme at titers of multiple grams per liter, three aspects of the production process were targeted: strain engineering, fermentation media, and protein purification.

The expression of SGases in *E. coli* is normally achieved by standard means, such as codon-optimization, gene expression controlled by the T7 promoter in a pET28a vector, and the use of the strain BL21(DE3) as the host. The development of an improved expression system for BGTl was accomplished by two major modifications to these standard strategies (Eberhardt et al. 2017). First, the most effective promoter from different induction systems was determined. Tests with promoters with different regulation and strengths identified the *araBAD* promoter system in the strain *E. coli* BL21AI as the most effective. A large amount of insoluble aggregates necessitated the second modification. Different combinations of chaperones were expressed from plasmids compatible with the BGTl expression vector and analyzed in co-expression experiments. Overexpression of GroES/EL increased the activity of the SGase significantly, overexpression correlating with enhanced solubility of BGTl.

A second strategy for reducing the cost of manufacturing SGase was to test a panel of inexpensive carbon and energy sources for a fed-batch fermentation process (Eberhardt et al. 2018). Feedstocks such as sucrose, glycerol, and industrial products containing these compounds, such as molasses and biodiesel-derived raw glycerol, were examined. The case of sucrose required introducing a sucrose utilization cassette into the *E. coli* BL21-based producing strain. The production of BGTl was first analyzed in shake-flask cultures and subsequently validated in fed-batch fermentations at high cell densities. The observed activities and the economic advantage of using a waste product made crude glycerol the preferred carbon source. Considering different strategies to feed nutrients post-induction improved our manufacturing process further. A constant feeding rate of 12 g/L h achieved optimal productivity. Not only did this change increase the production of the SGase by 50%, but the duration of the fermentation decreased. A large-scale bioreactor (1300 L) validated this manufacturing process, in which the final cell density, the yield of substrate, and the productivity of BGTl resembled those of laboratory scale fermentations.

A third improvement to the process for manufacturing the SGase was to develop an inexpensive method for recovering the enzyme from the broth of *E. coli* fermentations (Eberhardt et al. 2018). Because BGTl is produced as an intracellular protein in *E. coli*, a mechanical method for disrupting cells is required to release the protein after fermentation. Typical industrial-scale methods include high-pressure homogenizers and bead mills (Middelberg et al. 1991). However, these methods have disadvantages,

such as high capital costs of the equipment and the inherent contamination by intracellular proteins and DNA in the extracts. Taking advantage of the thermal stability of BGTl, a thermolysis method was developed to isolate the enzyme. Because this enzyme is physically associated with the cell membrane, the method also requires the use of a detergent. An examination of different incubation temperatures and ionic and non-ionic detergents produced a final procedure that consists of incubating the broth at 70 °C for 30 min in the presence of 0.2% lauryl alcohol ethoxylate (7 mol). This method recovers SGase in the soluble fraction of cell extracts at yields greater than 90%. Solids are easily removed by using continuous centrifugation. Not only does this process of thermolysis reduce capital expenditures and simplify the recovery of the target protein, but it also kills the host cell and inactivates proteases.

Activity of enzymes in heterogeneous reaction media: SGases in biodiesel

Hydrolysis of SGs present in biodiesel requires strategies to disperse SGase in a hydrophobic medium. The use of enzymes in organic media has a long history, as it has been an approach to catalyze organic reactions with extraordinary stereoselectivity and regioselectivity, allowing the production of compounds that are insoluble in aqueous media (Gupta and Khare 2009). Typically the enzymes are directly added to the organic reaction medium as a solid, in a lyophilized form, immobilized on insoluble support matrices, or modified to improve their dispersive properties (Doukyu and Ogino 2010). Alternatively, an enzyme in water is dispersed in the organic medium, either by forming stable emulsions or by enhancing the distribution of the enzyme in water droplets by means of high shear rate mixing.

Interfacial enzyme reactions have been extensively studied for lipases. These enzymes are employed in different biochemical reactions, including esterification, transesterification, interesterification, acidolysis, aminolysis, alcoholysis, acylation, and the resolution of racemates. Lipases are active in heterogeneous environments where substrates, products, and enzymes partition themselves among the bulk and surface phases. The physical complexity of the environment has made the understanding of the regulation of lipolysis less complete than that of homogeneous catalysis (Reis et al. 2009). Whereas most enzymes irreversibly denature at interfaces, lipases can be part of the interface and remain fully active at even very low interfacial tensions.

From structural analyses, two kinds of interfacial binding modes were identified in lipases. In most cases, a "lid" domain, which closes the active site in the free enzyme, adopts a different conformation in the presence of phospholipids and bile salts, generating a large hydrophobic surface surrounding the active site that is the main site of interaction with the interface. Active site lid opening appears to be the main binding mode of single domain lipases. However, human pancreatic lipase presents a beta-sandwich C-terminal domain that binds to a triglyceride:water interface. A truncated form of this lipase, lacking the C-terminal domain, is not active towards emulsions of insoluble triglycerides, indicating that interfacial binding of human pancreatic lipase is governed by this domain (Aloulou et al. 2006). In the case of BGTl, interfacial binding and the active site appear to be located separately within a single domain (see below).

In terms of understanding the kinetics of the interfacial reaction, the simplest model is the one proposed by Verger and De Haas. Here, the water-soluble enzyme binds to the lipid–water interface via a reversible adsorption–desorption mechanism, then is activated, and binds a substrate molecule. This results in the formation and the subsequent decomposition of the complex on a two dimensional framework. The reaction products are soluble in the aqueous phase and instantaneously diffuse away (Verger and De Haas 1976). However, these simplifying assumptions do not hold for many interfacial reaction systems. Different kinetic models with varying degrees of complexity are proposed to describe the complex relationships between enzyme concentrations, degree of progress of the reaction, including substrate and product inhibition, the amounts of reagents (such as methanol in transesterification reactions), volume fractions of water and oil, and the rate of agitation.

Detailed information on lipase kinetics were obtained by using controlled systems with a constant interfacial area and triglycerides dissolved in isooctane (Hermansyah et al. 2006, 2010). Models proposed from these experiments describe the effect of the enzyme concentrations, the interfacial area, and the concentrations of substrate and product in the complete process. The effect of mass transport of product and substrate were also studied in a Lewis-cell type reactor, in which agitation of the two phases can be independently controlled. Whereas the rate of hydrolysis was shown to increase with the rates of stirring in both phases, pronounced velocity gradients in the interfacial layer of the water phase gave rise to a local shear stress, which in turn reduced the activity of the enzyme (Schröter et al. 2015). While this phenomenon applies to lipases activated at interfaces, studies of other enzymes bound to interfaces are needed to verify its generality.

Some generalizations can be drawn from the different models proposed. Catalysis of the hydrolysis of oil by lipases occurs at the interface between the aqueous phase containing the enzyme and the oil phase, the surface of the oil phase being where the enzyme must be adsorbed for the first step of the reaction. Lipase molecules are adsorbed and desorbed continuously at the surface of the substrate. In a mechanically stirred reactor, the interfacial area is affected

by the speed of agitation, the ratio of the aqueous to organic phases, and temperature. Because the total free interfacial area available is limited and enzymes are active in the interface, a critical concentration of enzymes exists at which the interfacial area becomes saturated with the adsorbed enzyme (Al-Zuhair et al. 2008; Badenes et al. 2011). A thorough understanding of these phenomena is necessary to optimize conditions for interfacial enzyme reactions in an industrial process.

In terms of reactions, the hydrolysis of SGs is comparable to systems in which triglycerides are dissolved in an inert organic phase. But while SGs remain soluble in biodiesel at high temperatures, their amphiphilic nature makes them also partially soluble in the aqueous phase. The substantial increase in activity of the enzyme LacS in the presence of surfactants can be interpreted in light of the evidence accumulated about interfacial reactions by lipases. Surfactants could simply provide a larger area for the exchange of substrate and product between the two phases. Alternatively, they could increase the solubility of SGs in water to allow the glycosidase to catalyze hydrolysis. Finally, surfactants could drive the enzyme to the interface, increasing the local concentration of the enzyme. Clearly, these three possibilities are not mutually exclusive and a combination of all of them could be necessary to explain the experimental observations. In any case, the results obtained with the enzyme BGTl (see below) show clearly that the reaction proceeds more readily at the biodiesel:water interface.

A structural rationale for the SGase activity of BGTl and the potential for improving the enzyme

All glycosidases described here belong to glycoside hydrolase family 1, as defined in the Carbohydrate-Active enZYmes Database (CAZY) (Lombard et al. 2013). These enzymes are distributed widely among living organisms. The enzymes catalyze the hydrolysis of the β-glycosidic linkage, while retaining the anomeric configuration (Davies and Henrissat 1995). Structurally, family 1 glycosidases fold as single-domain, eightfold beta barrels with a molecular mass of ca. 50 kDa, although their quaternary structures differ (Isorna et al. 2007). Catalysis by family 1 glycoside hydrolases is mediated by two active site amino acid residues, a nucleophile and a proton donor, via a double-displacement mechanism that involves the formation of a covalent glycosyl-enzyme intermediate (Isorna et al. 2007). Two conserved glutamic acid residues in these enzymes play the roles of nucleophile and proton donor (Akiba et al. 2004; Anbarasan et al. 2015).

Although the specificity for the glycone end of the substrate is well characterized in family 1 glycoside hydrolases, the enzymes possess broad specificities for the aglycone site and thus can possibly be steered towards functioning with a range of glycoside substrates. The initial finding of the SGase activity in LacS (Aguirre et al. 2014) showed the potential of this group of enzymes for removing SGs in biodiesel. However, LacS requires an emulsifier in its reaction medium, presumably to facilitate the transfer of SGs in biodiesel to the aqueous phase, where the enzyme resides. In contrast, BGTl is an efficient SGase (Eberhardt et al. 2017) because the enzyme associates with membranes. However, neither enzyme has potential transmembrane structures for insertion into a lipid bilayer. Analysis of the crystal structure of BGPh, a close homologue of BGTl, suggests how these enzymes localize to water/oil interfaces (Akiba et al. 2004). A structure-based sequence alignment between BGPh and other glycosidases of the same type shows two short insertions in BGPh that contain six aromatic and hydrophobic residues. Concomitantly, a modified distribution of charged residues that creates an asymmetric electrostatic potential around BGPh molecular surface is conserved in BGTl. The combination of the insertions that form an exposed hydrophobic patch and of the uneven charge distribution likely guides the enzymes towards the membrane of bacteria. These natural properties were exploited to perform the SGase reaction without emulsifiers.

The LacS enzyme has greater specific activity than BGTl does, but requires surfactants to function efficiently in the water/biodiesel system. The modeled structure of BGTl was based on the known structure of BGPh by using Modeller (Webb and Sali 2014). This modeling allowed the active sites of BGPh, BGTl, and LacS to be compared. In all three cases, the residues that form the cavity and the carbohydrate recognition site are fully conserved (Fig. 3). However, several substitutions are found in the region where the aglycone is located. These substitutions could be hindering the binding of SGs to BGTl, reducing its activity. Alternatively, limited flexibility in the highly thermally stable BGTl might be slowing the reaction. In any case, the presence of desirable features separated in either BGTl or LacS, namely interfacial location and high specific activity, respectively, suggest that these enzymes can be improved by protein engineering, optimizing the activity towards SGs in industrial conditions.

Concluding remarks

Research on thermostable SGases has led to the adaptation of a thermozyme to a novel industrial process. That research ranged from identifying thermostable SGases to optimizing the treatment of biodiesel at the ton scale. The outcome was a cost-effective strategy for manufacturing an enzyme that can readily be used by the biodiesel industry. Current knowledge also suggests improved approaches for producing

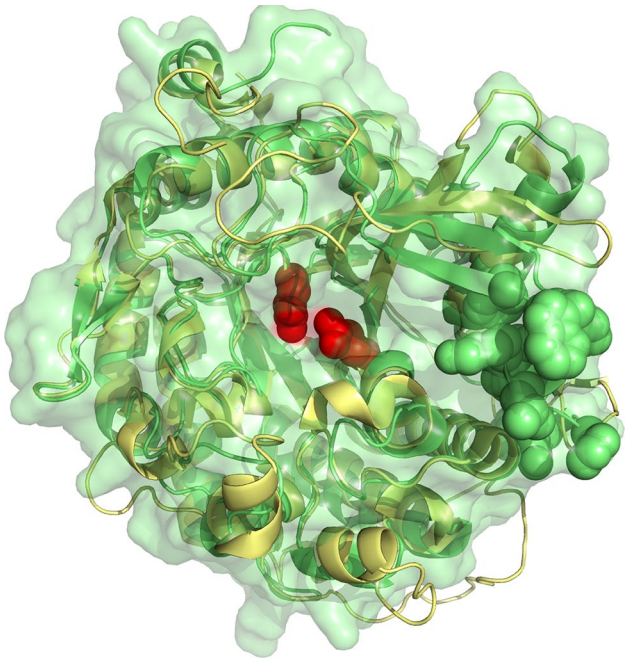

Fig. 3 Modeled structure of BGTl obtained with Modeller (green) and structure of the β-glycosidase from *Sulfolobus solfataricus* LacS (PDB 2CER, yellow). Catalytic glutamic acid residues in the active site are shown in red. Residues forming the surface hydrophobic patch in BGTl are shown in spacefill representation. Notice the distortion between both structures in the beta-hairpin where the hydrophobic patch is located

thermozymes in heterologous hosts as well as new applications of these enzymes, and that knowledge also provides some insights for engineering SGase proteins.

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