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Propionibacterium acnes GehA lipase, an enzyme involved in acne development, can be successfully inhibited by defined natural substances

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Abstract

Propionibacterium acnes, a usual inhabitant of human skin, plays an important role in acne development, related to the production of numerous enzymatic activities involved in the degradation of host molecules. Among these enzymes, *P. acnes* lipase (GehA, glycerol-ester hydrolase A) has been recognized as one of the major factors in the pathogenesis of acne, being responsible for the hydrolysis of sebum and the release of inflammatory compounds. Anti-acne treatments are based on the use of retinoids or benzoyl peroxide, frequently in combination with antibiotics. However, the low effectiveness of such treatments and the increasing antibiotic resistance has led to the development of alternative therapies such as Kampo formulations, containing traditional herbal drugs. Search for new anti-acne treatments led us to perform the cloning, characterization and inhibition of *P. acnes* GehA, considered an interesting pharmaceutical target for anti-acne therapies. The genetic, molecular and biochemical properties of the cloned lipase were analysed, and several inhibitor agents were tested, including natural substances like saponins, alkaloids or flavonoids. Among these, the flavonoids (±)-catechin and kaempferol were the most promising candidates for acne treatment, whereas saponins like glycyrrhicic acid and digitonin produced a lower inhibition of the enzyme. No inhibition by alkaloids was found. Therefore, the inhibition caused by (±)-catechin and kaempferol on GehA, together with their wide anti-acne properties and low toxicity, make them very suitable candidates for the treatment of acne and other *P. acnes*-related diseases.

Keywords: Lipase; Saponins; Flavonoids; Alkaloids; *Propionibacterium acnes*

1. Introduction

Propionibacterium acnes is a common resident of human skin sebaceous follicles, usually as a harmless commensal but occasionally involved in acne development through colonization of human skin surface [1,2]. Acne vulgaris is the most common disease associated to *P. acnes*, affecting 80% of the population at least once during life. Acne develops chiefly in 10–30 years people, although it can be present in some patients up to 50 years, or during menstruation, drug treatments, or stress. Many patients undergo spontaneous and complete resolution of their lesions, whereas others have continuous acne or long-term consequences such as disfigurative scarring and keloids that can lead to psychological disorders. Thus, an extensive research on this disease has been done during the last decades [2].

Acne is an inflammatory chronic disease of multifactorial aetiology [3,4]. Overproduction of sebum, ductal hypercornification, multiplication of *P. acnes*, and inflammation are the main causes for acne development [2,3], being all these factors potential therapy targets. Anti-acne treatments are based on the use of retinoids or benzoyl peroxide, frequently in combination with antibiotics. However, the low effectiveness of such treatments and the increasing antibiotic resistance has led to the development of alternative therapies such as Kampo formulations, containing traditional herbal drugs, used alone or in combination with western therapies [5]. Severe acne usually requires the use of oral isotreonin (13-*cis* retinoic acid), a very effective compound with sebostatic, keratolytic, anti-*P. acnes*, and anti-inflammatory activities, but associated to many secondary effects and forbidden in some countries like Japan [6].

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The role of *P. acnes* in acne development seems to be related to the production of numerous enzymatic activities involved in the degradation of host molecules, including lipase, protease, hyaluronidase, and acid phosphatase activities. Moreover, *P. acnes* produces surface-associated and secreted immunogenic and chemotactic factors which seem to be involved in triggering inflammation [2,3]. Complete sequencing of *P. acnes* genome has revealed the existence of additional putative enzymes and immunogenic factors that could also have a pathogenic role [7].

Among the enzymatic activities, *P. acnes* lipase (GehA, glycerol-ester hydrolase A) has been recognized as one of the virulence factors involved in the pathogenesis of acne [5]. GehA enzyme is the main responsible for the hydrolysis of sebum triacylglycerides, thus releasing glycerol and free fatty acids [2]. Glycerol is a source of nutrients for *P. acnes*, whereas fatty acids are highly inflammatory, chemotactic, and irritating for the sebaceous follicle cells [2,4,8]. Moreover, fatty acids favour ductal hypercornification by adhesion and packaging between keratinocytes [4], and increase adhesion between *P. acnes* cells and between *P. acnes* cells and follicle cells, which favours *P. acnes* colonization and biofilm formation [9,10]. Furthermore, GehA itself is a strong chemotactic and pro-inflammatory antigen [11].

Therefore, GehA has recently generated a great interest as a pharmacological target. The enzyme was previously characterized and cloned in *Escherichia coli* [12–16] and consists of a secreted serine lipase of 36 kDa, that shows an optimum pH of 6.8, and stability in a pH range of 5–6, becoming completely inactivated after 30 min incubation at 60 °C. GehA can hydrolyse a wide variety of substrates, displaying non-linear kinetics and acting equally at positions α and β of glycerol (non-regiospecific), but does not exhibit phospholipase or other enzymatic activities [15]. The recombinant enzyme produces insoluble aggregates that can be overcome by incubation of the overexpressing strain at a reduced temperature in the presence of saccharose [16].

In this work we describe the isolation, cloning and expression in *E. coli* of *P. acnes* lipase GehA, considered an interesting pharmaceutical target for anti-acne therapies because of its implication in acne development [5]. Our efforts have focussed on the search for new anti-acne treatments based on the inhibition caused by selected natural substances like saponins, alkaloids and flavonoids on *P. acnes* lipase GehA.

2. Experimental

2.1. Cloning of P. acnes P-37 gehA

Strain *P. acnes* P-37, kindly provided by Dr. M.D. Farrar and Dr. K.T. Holland, was cultured on Reinforced Clostridial Agar plates incubated for 24 h at 34–37 °C in an anaerobic jar under an atmosphere of $N_2/CO_2/H_2$ (80:10:10, by volume), achieved by using Anaero *Gen*^{TM} system (Oxoid), and confirmed with the anaerobic indicator BR35 (Oxoid). The resulting colonies were suspended in distilled water and used as template for PCR amplifications using primers PALIPFW (5′-TTT CTG CAG GCT ACC CTT TTC G-3′; *Xba*I site underlined) and PALIPBW (5′-GGA TCT AGA ACT GTT CGT TGT CAC C-3′; *Pst*I site underlined), designed for the specific isolation of *P. acnes gehA*, including the upstream (76 bp) and downstream (84 bp) regions of the gene, and bearing the sequences for the restriction nucleases *Xba*I and *Pst*I, respectively. Amplification by PCR was performed using *Pfu* polymerase, and the resulting fragment was purified and sequenced to confirm its nucleotide sequence. The isolated DNA fragment was then digested with *Xba*I and *Pst*I, and subsequently ligated to *Xba*I–*Pst*I-digested pUC19 plasmid. The resulting recombinant plasmid pUC-GehA was transformed into *E. coli* XL1-Blue to obtain recombinant clone *E. coli* XL1-Blue–pUC-GehA. The nucleotide sequence of the cloned insert was then re-confirmed, and studied by computational analysis [17].

2.2. Production of GehA in E. coli

Crude cell extracts of recombinant *E. coli* XL1-Blue–pUC-GehA, prepared as described before [18], were used for molecular and biochemical characterization of GehA. Although the recombinant clone displayed activity on lipid-IPTG-supplemented plates, no activity could be detected on liquid assays due to the formation of inactive, insoluble aggregates when expressed in *E. coli* [16]. Thus, active cell extract preparation required the cultivation of *E. coli* XL1-Blue–pUC-GehA at 25 °C in LB-Ap medium supplemented with 0.45 M saccharose [16]. At $OD_{600\,nm}$ = 0.6–1, 1 mM IPTG was added, and the culture was incubated for two more hours at 25 °C before preparing 50-fold concentrated cell extracts in 50 mM phosphate buffer pH 7.0 [18].

2.3. Activity assays

Lipolytic activity was detected on agar plates supplemented with 1% tributyrin, olive oil or triolein, and 0.002% Rhodamine B [19], or by 4-methylumbelliferone (MUF) release from MUF-derivative substrates [20]. Activity determination and zymogram assays were routinely performed using crude cell extracts, prepared as described before [18]. The release of *para*-nitrophenol (*p*-NP) or 4-methylumbelliferone (MUF) from *p*-NP or MUF-derivative substrates was measured as described [21,22]. One unit of activity was defined as the amount of enzyme that released 1 μmol of *p*-NP or MUF per minute under the assay conditions used. Electrophoresis and isoelectric focusing were performed as previously described [21,23]. After protein separation, activity was detected by zymogram, and gels were subsequently stained with Coomassie Brilliant Blue R®-250 for protein band visualization [20].

2.4. Inhibition assays

Lipase inhibition or activation experiments were performed according to a previously described colorimetric microassay, using *p*-NP laurate (*para*-nitrophenyl laurate) as a substrate [22]. Lipase inhibition was calculated from the residual activ-

ity detected in the presence of the compound under assay with respect to that of untreated samples (without inhibitor but prepared and analysed under the same conditions, including the inhibitor's solvent to take into consideration the effect of each solvent on lipase activity). The concentrations yielding a lipase inhibition of 16% (IC_{16}) and 50% (IC_{50}) were calculated from the inhibition rate versus inhibitor concentration curves by regression analysis performed using the Sigma-Plot 8.0 software (SPSS). Three or more replicates of regression curves with Rsquare coefficients higher than 0.99 were used for IC calculations, being each replicate the result of an independent assay performed in duplicate.

3. Results and discussion

3.1. Cloning and analysis of GehA

P. acnes lipase GehA is considered a major etiological agent in the pathogenesis of acne [5]. For this reason, *P. acnes* P-37 GehA lipase, an enzyme previously cloned and overexpressed [16], belonging to subfamily I.7 of bacterial lipases [24], was cloned and characterized in more detail in this work.

Strain *P. acnes* P-37 was used for PCR amplification of lipase gene *gehA*, as described in Section 2. A DNA fragment of ca. 1.2 kbp was obtained, ligated into pUC19 and transformed into *E. coli* XL1-Blue to obtain recombinant clone *E. coli* XL1-Blue–pUC-GehA. The nucleotide sequence of the cloned gene was determined, confirming that it was identical to the *gehA* sequence (X99255) previously reported [16]. Analysis of the predicted GehA protein indicated that it was a protein of 339 amino acid residues of 35,995 Da with an N-terminal signal peptide of 26 residues, whose cleavage yielded a mature protein of 313 residues and 33,396 Da, as reported [16]. The deduced *p*I of the original and mature forms of the protein were 6.59 and 6.26, respectively. GehA showed also a high contents in short non-polar residues (42%), a feature described for enzymes acting on hydrophobic substrates that can be found in aggregated state [25].

GehA showed the highest identity (50%) to *Streptomyces cinnamoneus* lipase [26], the other member of subfamily I.7 of bacterial lipases [24,27], whereas much lower identity to other family I lipases was found. Study of the protein fold recognition using 1D and 3D sequence profiles coupled with secondary structure information [28] allowed predicting that GehA is a globular, compact serine-hydrolase with a single domain, displaying the typical α/β hydrolase fold of lipases [29]. The secondary structure of GehA (not shown) revealed the presence of 8β strands and 11α helices in mature GehA [28], in agreement with the typical 8β sheet of lipases [29]. However, the number of α helices obtained should be considered with care as the last four could correspond to just two α helices, since they are short and close, and the model shows a lower confidence in this region. Moreover, an additional α helix and another β strand were present in the signal peptide of GehA. Amino acid sequence alignment confirmed that the catalytic serine was located at position 169 of the non-processed protein, included in the pentapeptide Gly–His–Ser–Gln–Gly of the conserved Gly–Xaa–Ser–Xaa–Gly pentapeptide of lipases [25]. Further analysis of the secondary structure confirmed that the conserved pentapeptide containing the catalytic serine forms a turn between strand β5 and the following α helix, the so-called "nucleophile elbow", which is present in all known lipases and constitutes the most conserved structural arrangement of the α/β hydrolase fold [29]. Asp^{267} (located in a turn after strand β7) and His^{297} (located after β8) were assigned as the two other members of the catalytic triad of GehA, according to their position with respect to the prototypic α/β hydrolase fold [29].

3.2. Characterization of GehA

Production of active GehA was achieved by cloning *gehA* in *E. coli*, followed by overexpression of the enzyme at low temperature and using saccharose-supplemented culture media to avoid the formation of insoluble aggregates [16].

The lipolytic activity of recombinant *E. coli* XL1-Blue–pUC-GehA was detected on lipid-supplemented agar plates. In agreement with previously reported results [12–15], clear hydrolysis zones were observed using tributyrin and triolein as substrates, whereas low fluorescence emission [19] was found on plates containing olive oil (not shown). Cell extracts from IPTG-induced cultures of the recombinaant clone were assayed to determine the lipolytic activity of GehA on several *p*-NP- and MUF-derivatives (Table 1). GehA displayed an intermediate behaviour between "true" lipases and carboxylesterases, since it showed preference for acyl groups of medium-chain length and a lower activity on longer and shorter substrates. The highest activity (100%) was found on *p*-NP caprate ($4.7 \times 10^{-1} \pm 0.3 \times 10^{-2}$ mU mg^{-1} protein) and MUF-butyrate ($1.1 \times 10^{-2} \pm 0.1 \times 10^{-3}$ mU mg^{-1} protein). GehA also efficiently hydrolyzed *p*-NP laurate, whereas it showed low activity on other *p*-NP derivatives (residual activity 20–40% on the other $C_{2–16}$-derivatives). *p*-NP stearate and MUF-oleate were the poorer substrates, although their activity with respect to *p*-NP butyrate and MUF-butyrate was about 30–50% (Table 1).

Table 1
Sustrate profile of GehA lipase

Substrate	GehA activity	
	mU mg^{-1} GehA	%
p-NP acetate ($C_{2:0}$)	0.156	32.9
p-NP butyrate ($C_{4:0}$)	0.133	28.1
p-NP valerate ($C_{5:0}$)	0.187	39.5
p-NP caproate ($C_{6:0}$)	0.101	21.4
p-NP caprylate ($C_{8:0}$)	0.169	35.7
p-NP caprate ($C_{10:0}$)	0.473	100.0
p-NP laurate ($C_{12:0}$)	0.292	61.8
p-NP palmitate ($C_{16:0}$)	0.103	21.9
p-NP stearate ($C_{18:0}$)	0.038	8.0
MUF-butyrate ($C_{4:0}$)	0.011	100.0
MUF-oleate ($C_{18:1c\Delta9}$)	0.006	51.8

The standard deviations obtained ranged from 2% to 10% of the corresponding mean values. All the substrates were assayed at a concentration of 1 mM, at 37 °C and pH 7.

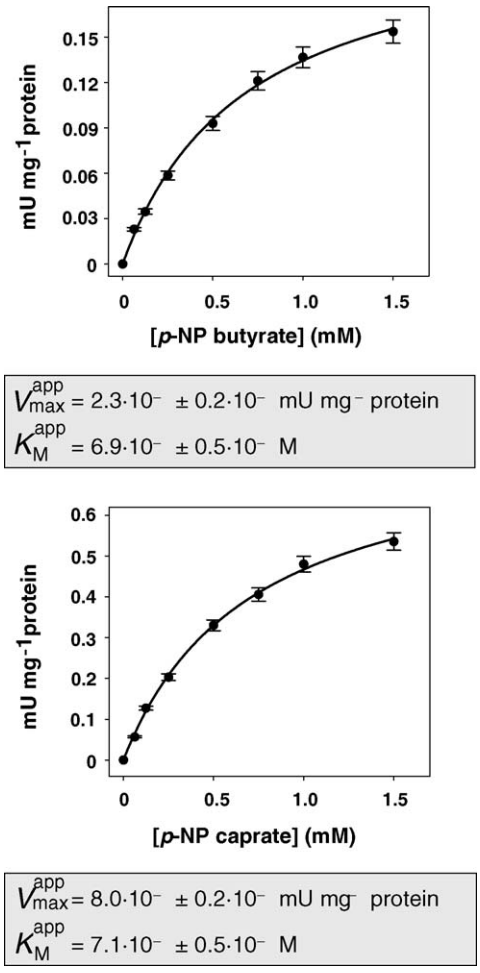

Fig. 1. Kinetic behaviour of GehA on *p*-NP butyrate and *p*-NP caprate.

When the kinetic behaviour of GehA was analysed on *p*-NP butyrate and *p*-NP caprate, the enzyme showed the typical Michaelis–Menten behaviour of carboxylesterases, with no interfacial activation, as described for carboxylesterases and a few "true" lipases [29,30]. Interestingly, although the $V_{max\ app}$ on *p*-NP caprate was nearly three-fold higher than that on *p*-NP butyrate, the $K_{M\ app}$ was almost the same, indicating that the affinity of the enzyme for both substrates was similar (Fig. 1). These data cannot be directly compared to previous results, since GehA activity on these derivatives was never assayed, with the exception of *p*-NP acetate [13]. Nevertheless, the results obtained are in agreement with the activity of GehA on a wide range of triacylglycerols (tributyrin, trilaurin, tricaprylin, trimyristin, tripalmitin, tristearin and triolein) previously reported [12–15]. Moreover, the considerable activity of GehA on $C_{2–18}$ substrates is also in agreement with the wide range of lipids found in human skin [31].

The effect of temperature and pH on the activity of GehA was determined using MUF-butyrate as a substrate. The highest activity was found at 37 °C and pH 7, displaying also high activity (more than 50%) from 20 to 50 °C, and from pH 5 to 7.5 (not shown). Furthermore, the enzyme remained active for at least 30 days when stored at 4 °C and pH 7. These optima are in agreement with those previously reported [13,15], and with the fact that GehA is a secreted enzyme acting on lipids of sebaceous glands under acid-neutral pH conditions and at 37 °C.

3.3. Inhibition of GehA

The effect of different agents on the activity of GehA was determined using *p*-NP laurate (Fig. 2). Among the cations analysed, only Ba^{2+} and Co^{2+} caused a significant inhibition of the enzyme (22.5 and 71.2% residual activity, respectively) at 1 mM, whereas Ag^{+}, Ca^{2+}, Hg^{2+}, Mg^{2+}, Ni^{2+} and Pb^{2+} strongly activated GehA (residual activity higher than 125%) at this concentration. When cations were assayed at 10 mM, Ag^{+}, Ba^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+} and Zn^{2+} caused a high inhibition (residual activity lower that 10% except for Zn^{2+}), whereas Ca^{2+}, Cu^{2+}, Mg^{2+} and Pb^{2+} strongly activated GehA (residual activity 138–271%). The strong activation produced by Ca^{2+} and the low inhibition caused by Zn^{2+} are in agreement with previous results [32,33], whereas the effect of the other cations analysed has not been described before to our knowledge, but is in agreement with the general effects of these compounds on other lipases [34].

The influence of the amino acid-modifying agents NAI (*N*-acetylimidazole, affecting tyrosine), PHMB (*p*-hydroxymercuribenzoic acid, affecting cysteine) and PMSF (phenylmethylsulfonyl fluoride, affecting serine), and the effect of EDTA, urea and SDS were also tested (Fig. 2). NAI and PHMB caused a significant reduction of GehA activity at 10 mM, suggesting that cysteine and tyrosine are involved in the proper folding and/or activity of the enzyme, whereas the serine-inhibitor PMSF was inactive on GehA, as previously reported [13]. The lack of inhibition by PMSF could seem surprising since serine is one of the catalytic amino acids of the enzyme, and could be related to a lack of ability of PMSF to fit into the active site of GehA. In fact, not all lipases are inhibited by this compound [34]. GehA activity was also inhibited by EDTA, in agreement with previous results [32], and slightly inhibited by

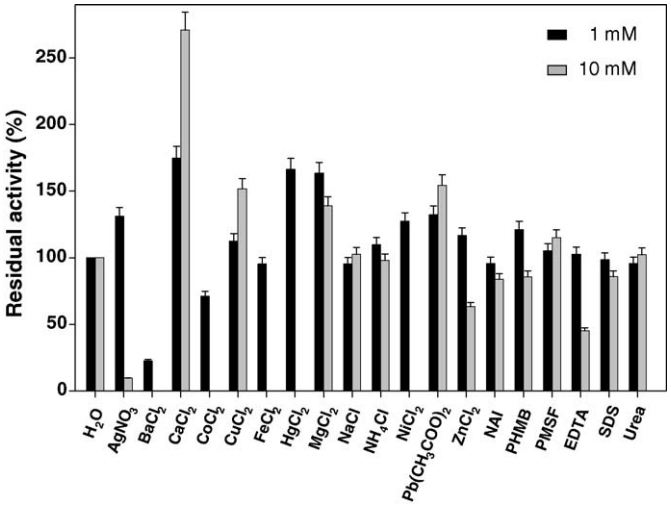

Fig. 2. Effect of several agents on GehA, assayed at 1 mM (black) and 10 mM (grey).

Table 2
Effect of natural substances on GehA lipase

Substance	S_{max} (M)	*P. acnes* GehA	
		IC_{16} (M)	IC_{50} (M)
Saponins			
β-Aescin	1.5×10^{-4}	$>S_{max}$	–
Digitonin	4.0×10^{-4}	$\sim S_{max}$	–
Glycyrrhizic acid	2.0×10^{-3}	4.0×10^{-4}	$>S_{max}$
Quillaja saponin	1.4×10^{-3}	$>S_{max}$	–
Flavonoids			
(±)-Catechin	3.0×10^{-2}	2.3×10^{-4}	3.9×10^{-4}
Kaempferol	1.4×10^{-2}	1.4×10^{-4}	2.3×10^{-4}
Alkaloids			
Rescinnamine	8.0×10^{-4}	$>S_{max}$	–
Reserpine	4.5×10^{-4}	$>S_{max}$	–

S_{max}: highest concentration at which each substance was tested; IC: concentration of inhibitor yielding a lipase inhibition of 16% (IC_{16}) or 50% (IC_{50}). The assays were performed by colorimetric microassay (37 °C and pH 7), using 1 mM *p*-NP laurate as substrate.

SDS. On the contrary, this lipase was resistant to denaturation by urea, a component of skin and sweat, confirming the adaptation of GehA to the conditions found in the sebaceous glands and the skin.

The importance of GehA in acne development and the effectiveness of Kampo formulations [5] for the treatment of acne led us to analyze the effect of several natural substances that could help in the therapy or prevention of *P. acnes*-related diseases. The results obtained by colorimetric activity microassay [22] with respect to the effect on *P. acnes* GehA of several saponins (β-aescin, digitonin, glycyrrhizic acid (GA), and *Quillaja* saponin (QS)), flavonoids ((±)-catechin and kaempferol), and alkaloids (rescinnamine and reserpine) are shown in Table 2 and Fig. 3. GehA was strongly inhibited by the flavonoids (±)-catechin and kaempferol ($IC_{50} = 2.3–3.9 \times 10^{-4}$ M), whereas GA produced a lower inhibition ($IC_{16} = 4.0 \times 10^{-4}$ M) of this enzyme. Digitonin produced a similar inhibition than GA at low concentrations (14.2% inhibition at 4.0×10^{-4} M and 16% inhibition at 4.1×10^{-4} M, respectively); however, their inhibition at higher concentrations could not be compared due to the low solubility of digitonin. On the contrary, the other substances analysed produced almost no effects on this lipase (Table 2; Fig. 3).

The inhibition produced by (±)-catechin, kaempferol, glycyrrhizic acid (GA) and digitonin could explain the high anti-GehA and anti-acne properties of kampo compositions containing *Glycyrrhizae radix*, a plant rich in GA and flavonoids [5]. Up to present, it was thought that the beneficial effects of GA and flavonoids was the result of their anti-inflammatory properties, and due to their inhibition of *P. acnes* growth, which in turn would lead to a reduction in lipase activity [5]. However, the results obtained in this work indicate that their therapeutic effects are also tightly related to their inhibitory effects on *P. acnes* lipase, as in the case of those produced by the strong GehA inhibitors (±)-catechin and kaempferol. Therefore, the wide anti-acne effects of these substances, combined with their low toxicity [35,36], make them very suitable candidates for the

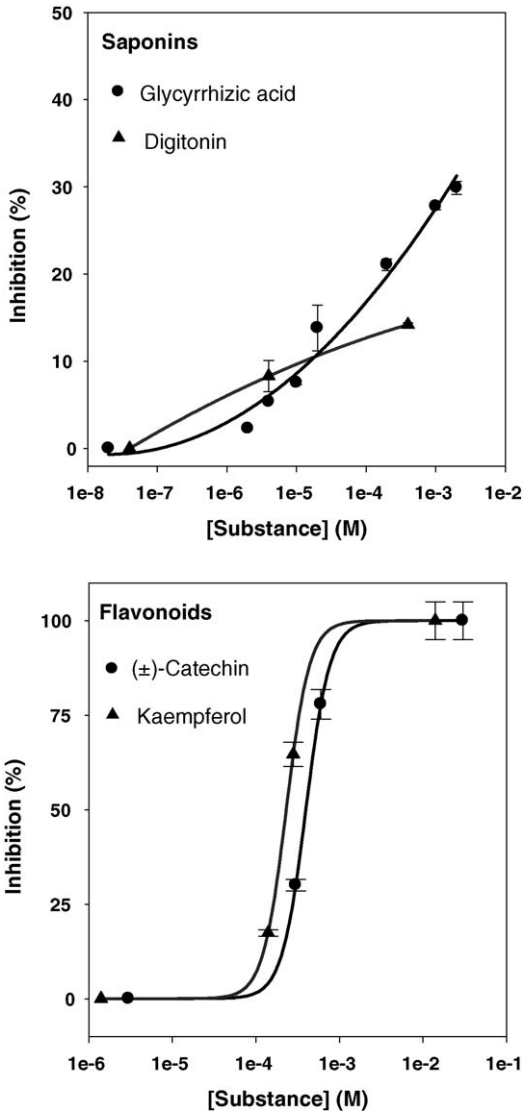

Fig. 3. Inhibition (and activation) of GehA by selected natural substances.

treatment of acne and other *P. acnes*-related diseases. Moreover, these compounds are poorly absorbed [35,37], which would reduce their side effects when administrated as topical agents. Future perspectives include further in vivo experiments to confirm the pharmacological potential of the mentioned substances and to consider them as useful anti-acne therapeutical agents.

4. Conclusions

P. acnes GehA, a lipase considered a major etiological agent in the pathogenesis of acne [5], has been cloned and characterized in more detail, which has shown that this enzyme is very adapted to the skin conditions. The effect of several natural substances on GehA has been evaluated revealing that glycyrrhizic acid, (±)-catechin and kaempferol are promising candidates for the treatment of acne due to their strong inhibitory activity on GehA, as well as to their other anti-acne effects and their low toxicity.

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