

# GENERAL DISCUSSION

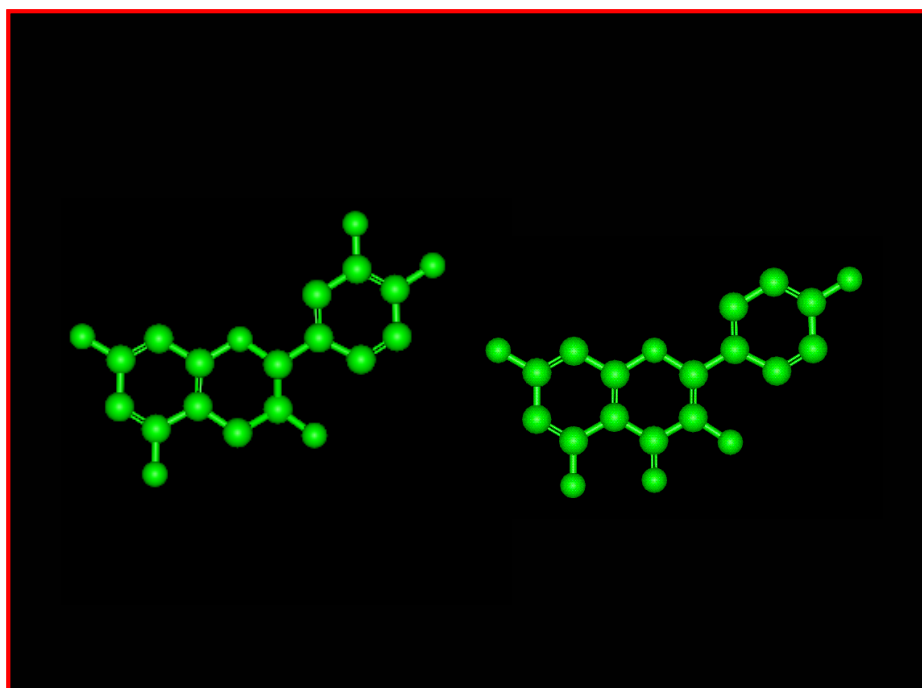


Figure D.1 Three-dimensional structure of (±)-catechin (left) and kaempferol (right).



This discussion is focused on some topics concerning related results obtained from different chapters, since the rest of aspects have been discussed at their corresponding chapters. Specifically, we will go more deeply into the similarities and differences found among *Bacillus*-related lipases (Chapters 1, 2 and 3), and into the similarities and differences found among different lipases with respect to their inhibition and activation by natural substances and other compounds (Chapters 1, 3, 4 and 5).

## 1 LIPASES FROM *Bacillus* AND RELATED GENERA

Lipases from Bacillales have attracted a great interest due to their biotechnological potential, which has led to the isolation of several lipolytic enzymes from *B. subtilis* and other species from the genus *Bacillus*, *Geobacillus* or *Paenibacillus* (Gupta *et al.*, 2004; General Introduction 3.3.5). However, knowledge about the structure, physicochemical properties, regulation and physiological role of these enzymes is still low, although they may be involved in providing carbon sources, in regulation of membrane composition, in pathogenicity, or in detoxification of biocides (Schmid & Verger, 1998; Khalameyzer *et al.*, 1999).

Interestingly, a related lipolytic system has been found among the *Bacillus*-related strains analyzed in this work. Zymogram analysis revealed the production by *B. megaterium* CECT 370, *Bacillus* sp. BP-6 and *Bacillus* sp. CR-179 of one or more cell-bound carboxylesterases (CEs) with similar properties (MW, pI and activity on MUF-butyrate) among them, and being also similar to the cell-bound CEs from family VII of bacterial lipases such as PnbA from *B. subtilis* (Zock *et al.*, 1994), EstA from *Paenibacillus* sp. BP-23 (Prim *et al.*, 2000) and EstA1 from *Bacillus* sp. BP-7 (Prim *et al.*, 2001). Moreover, high similarities have been found with respect to the secreted lipolytic enzymes of these strains. *B. megaterium* CECT 370, *Bacillus* sp. BP-6,

*Bacillus* sp. BP-7 and *Bacillus* sp. CR-179 produced a secreted CE belonging to subfamily I.4 of bacterial lipases (Arpigny & Jaeger, 1999) and showing 99–100% identity to *B. subtilis* LipB (BSLipB). *B. subtilis* possesses another secreted CE belonging to subfamily I.4, the enzyme LipA (BSLipA), which is 66.6% identical to a lipase fragment obtained from *Bacillus* sp. CR-179. This lower identity with respect to that found for BSLipB-like proteins could explain the fact that CR-179 *lipA* fragment was amplified using consensus primers for the central region of *B. subtilis lipA*-related genes, but the whole ORF could not be obtained using specific primers for *B. subtilis lipA*. Moreover, these results suggest that a lipase similar to BSLipA, but not identical enough to be amplified by using specific primers, could also exist in *B. megaterium* CECT 370, *Bacillus* sp. BP-6 and *Bacillus* sp. BP-7. Nevertheless, the possibility that these strains do not possess such a lipase also exists, since *B. megaterium* CECT 370 and *Bacillus* sp. BP-6 display an additional secreted lipolytic enzyme of 56–57 kDa not yet characterized. Furthermore, the analysis of other lipolytic strains and species from *Bacillus* and related genera has led to the isolation of additional lipases belonging to subfamily I.4 and being 70–80% similar to BSLipA such as those from the mesophilic bacteria *B. pumilus* (Moeller *et al.*, 1991; Kim *et al.*, 2002) and *B. licheniformis* (Nthangeni *et al.*, 2001). Therefore, it seems that *B. subtilis* and other related *Bacillus* strains or species possess a common lipolytic system constituted by at least one cell-bound CE, and at least two secreted CEs, one of them highly similar to BSLipB, and another showing a moderate similarity to BSLipA. On the contrary, the existence of a secreted lipase of 56–57 kDa seems not to be a general trait, considering the present knowledge on the lipases from all these microorganisms.

The different structure, biochemical properties and regulation of these lipases suggest that they perform different physiological functions. On the one hand, it has been suggested that PnbA from *B. subtilis* and other *Bacillus*-related cell-bound CEs are involved in pathogenicity and in detoxification of biocides (Zock *et al.*, 1994; Schmid & Verger, 1998; Khalameyzer *et al.*, 1999). Moreover, as it has been mentioned before (Chapter 3), these enzymes could play a role in the turnover of cell-membrane lipids and lipid-anchored proteins since they are highly inhibited by free fatty acids, a phenomenon which could be related to avoiding a high continuous activity of these enzymes on the cell membrane lipids surrounding them. In fact, it has been described

that changes in cell membrane composition contribute to the adaptation of some microorganisms to external conditions such as variations in pH, osmolarity, etc (Tannaes *et al.*, 2001), although phospholipases have been considered as the main responsible enzymes for these changes (Schmidt & Verger, 1998). Nevertheless, cell-bound CEs could also participate in this role, perhaps in collaboration with other enzymes such as *B. subtilis* phospholipase (Kennedy & Lennarz, 1979).

On the other hand, the biological role of secreted CEs from *Bacillus* spp. is more difficult to assess. If we analyze BSLipA and BSLipB, they show some similarities but also several differences. Both enzymes have a similar structure since they share a conserved core structure. On the contrary, almost all their different residues are located at the protein surface, although they do not affect the active site of these enzymes (Eggert *et al.*, 2001). These lipases show other common molecular and biochemical features such as a similar size, pI and stability (pH and temperature). Both enzymes show substrate preference towards short–medium triacylglycerols and *p*-nitrophenyl esters. However, BSLipA displays higher activity on long-chain lipids (including triolein), whereas BSLipB is less active on these substrates, and does not hydrolyze triolein at all (Kennedy & Lennarz, 1979; Dartois *et al.*, 1992; Lesuisse *et al.*, 1993; Eggert *et al.*, 2000; Chapter 1).

BSLipA and BSLipB-like proteins differ also in other biochemical properties, probably due to the differences existing on the surface residues of these proteins. For example, they have a different optimum pH (10 and 7–8, respectively) (Lesuisse *et al.*, 1993; Eggert *et al.*, 2000; Chapter 1), and a different response to saturated fatty acids (SFAs): BSLipA was moderately activated by low concentrations of SFAs and was inhibited at higher concentrations, whereas BSLipB-like proteins were highly activated by SFAs and were only slightly inhibited by high capric acid concentrations (Chapter 3). Moreover, secretion of BSLipA at pH 5 produces a permanent inactivation of the enzyme, probably due to protein misfolding, whereas inactivation of BSLipB when this enzyme is secreted at pH 5 can be reverted by incubation of the enzyme at pH 11 (Eggert *et al.*, 2001).

In addition, the regulation of the gene expression of *BslipA* and *BslipB* is also different, although further studies are necessary to obtain a deeper knowledge of their

regulatory system. BSLipA transcription is constitutive and independent from glucose, tributyrin, *n*-hexadecane, NaCl or ethanol, but is repressed by amino acids. On the contrary, LipB transcription only takes place in rich media, being independent from glucose, NaCl, ethanol or amino acids, but being activated by tributyrin and *n*-hexadecane (Eggert *et al.*, 2001 and 2003). Furthermore, BSLipA contains a Tat signal sequence and BSLipB contains a Sec signal sequence, which indicates that they are secreted by different pathways (the Tat system and the XCP–Sec system, respectively), although this has not been definitively confirmed (Tjalsma *et al.*, 2000; Jaeger & Eggert, 2002).

The differences mentioned for BSLipA and BSLipB suggest that these lipases perform different physiological functions (Eggert *et al.*, 2003), but also suggest that *BSlipA* gene could have duplicated and evolved to obtain a protein (BSLipB) with the same core structure but different surface residues than BSLipA. BSLipB protein would be adapted to be highly active under situations in which LipA is poorly active such as acid conditions or rich environments with a high amount of lipids containing C<sub>10</sub>–C<sub>14</sub>-SFAs, which are less specific substrates for these lipases (Eggert *et al.*, 2000, 2001 and 2003; Chapter 1). Furthermore, the high identity found among LipB-like genes suggest that this “more adapted” and ubiquitous enzyme plays a very important role in the physiology of *Bacillus* strains, which together with the fact that LipB expression is enhanced by lipid compounds such as tributyrin and *n*-hexadecane (Eggert *et al.*, 2001), reinforce the theory of a higher adaptation to lipid-rich environments of BSLipB carboxylesterase and of the promoter regions that regulate the expression of the gene encoding this enzyme. Thus, it seems that the secreted lipolytic system of *B. subtilis* and related strains would have evolved to ensure a high use of carbon sources in nutrient-rich environments with strong microbial competition, which could be related to the successful adaptation of *Bacillus* strains to many different habitats. However, further studies are necessary to determine the whole regulation and the physiological role of these lipolytic enzymes.

**General conclusion:** *B. subtilis* and other strains of the genus *Bacillus* and related genera share a quite conserved lipolytic system constituted by one or more cell-bound carboxylesterases and at least two secreted lipolytic enzymes, which would be involved in the regulation the membrane composition, in detoxification of biocides and in providing carbon sources, playing thus an important role in the adaptation of these microorganisms to nutrient-rich environments.

## 2 INHIBITION AND ACTIVATION OF LIPASES

Some considerations arise from the results obtained in the experiments performed to evaluate the effect of several substances on lipase activity. On the one hand, inhibition assays by saturated fatty acids and natural substances on the model lipase from *C. rugosa* (CRL) (Chapters 3 and 4) have revealed that significant differences in the inhibition (or activation) produced by some compounds can be found when the assays are performed using different methods or using the same method but different reaction mixture compositions.

Furthermore, interesting results were found when the effect of the same substances was analyzed under the same conditions but on different lipases. Compounds such as glycyrrhizic acid (GA) and EDTA produced a similar inhibition degree on all the enzymes analyzed, whereas PMSF, and many saponins, alkaloids, cations, and other agents were only active on some lipases (Chapters 1 and 5). On the contrary, some substances displayed an opposite behaviour when they were assayed on different lipases. For example, saturated fatty acids inhibited CRL and *Bacillus*-related cell-bound CEs, but strongly activated BSLipA and BSLipB-like CEs (Chapter 3). A similar behaviour was found for (±)-catechin and kaempferol, which strongly inhibited CRL and *P. acnes* GehA, but producing a strong activation on *H. pylori* EstV; and for other agents analyzed (SDS, some cations, etc) (Chapters 1 and 5).

These differences and similarities are related to the structure, physicochemical properties and mechanism of action of the compounds tested, and with the structural and biochemical features of the lipases analyzed. On the one hand, the differences in structure and physicochemical features among the compounds analyzed can affect to the interaction between the inhibitor and the active site or other regions of the enzyme, as well as to the interactions between these compounds and the substrate itself, the micelle, or the solvent (Patkar & Björkling, 1994; Gupta *et al.*, 2004; General Introduction 4.1; Chapter 4).

In this sense, EDTA, a compound producing a similar inhibition degree on all lipases assayed, seems to behave in a non-specific way, probably by chelating calcium or other divalent cations that could act as scavengers of the free fatty released by lipases, which are known inhibitors of these enzymes (Patkar & Björkling, 1994; Gupta *et al.*, 2004). Also SDS, urea,  $\text{Hg}^{2+}$  and other agents, which are active on most of the lipases assayed, seem to be non-specific compounds that inhibit lipase activity by a direct effect on the enzyme (conformational changes, denaturation, destabilization of the enzyme by binding to thiol and other groups, etc), or by disturbing the access of the enzyme to the interface and/or the substrate (Patkar & Björkling, 1994; Gupta *et al.*, 2004; Chapter 1 and 5).

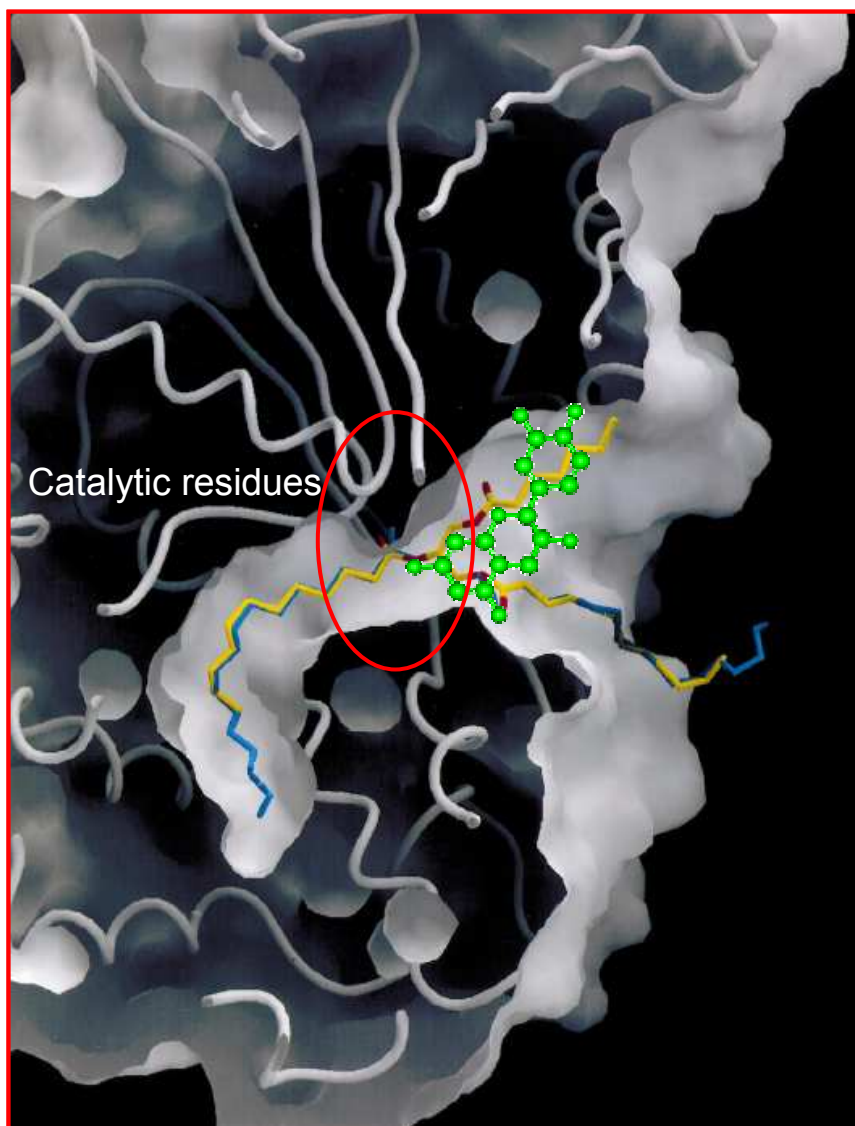
On the contrary, saturated fatty acids are considered specific reversible inhibitors that compete with the substrate for the active site of the enzyme (Markweg-Hanke *et al.*, 1995; Hari Krishna & Karanth, 2001). However, these compounds could also interact with other regions of the enzyme producing the activation found in *Bacillus*-secreted lipases (Chapter 3). PMSF is also a specific (irreversible) inhibitor (Gupta *et al.*, 2004) active on all lipases assayed with the exception of GehA, probably due to a lack of ability of PMSF to fit into the active site of this enzyme.

The effect of natural substances is more difficult to explain and would require further experiments. The degree of CRL inhibition produced by saponins seems to be related to larger and more branched carbohydrate side chains (Chapter 4). Detergents such as saponins are considered unspecific reversible lipase inhibitors that possibly interfere with the enzyme, the substrate or the interface (Patkar & Björkling, 1994), which could explain the general inhibitory effect of GA on all lipases assayed. On the



contrary, the higher inhibition produced on CRL by saponins with larger and branched chains could be due to a specific effect of these compounds on CRL, probably related to the deep and narrow tunnel containing the active site of this enzyme, since this rule do not applies in the other lipases analyzed. However, we can not discard a specific effect of GA on the active site of the enzymes analyzed, since this saponin, being the smallest, could more easily interact with the active site or close regions of different enzymes, whereas larger saponins such as QS (not active on GehA and EstV) are more difficult to accommodate into the active site or other regions of different lipases.

On the contrary, the flavonoids analyzed are small molecules able to enter the tunnel of CRL. Moreover, these compounds could interact specifically with the hydrophobic and hydrophilic residues of CRL tunnel (Pleiss *et al.*, 1998; Cygler & Schrag, 1997 and 1999), since they are aromatic molecules with hydrophilic groups (mainly hydroxyl groups). More precisely, inhibition by flavonoids on CRL seems to be related the number and disposition of their hydroxyl groups, mainly with the presence of a hydroxyl group at position 7 (ring A) (Chapter 4). Figure D.2 shows a hypothetical model for the interaction between ( $\pm$ )-catechin and the active site and tunnel of CRL. We can see how this compound would be able to compete with the substrate for the active site of CRL, and the importance of the hydroxyl group at position 7. This group, whose disposition would be similar to that of the ester bond of a triacylglycerol, could interact with the catalytic amino acids of this lipase, favouring even more the inhibition produced by this compound. Similar considerations can be made for kaempferol, whereas 3-hydroxyflavone and 5-hydroxyflavone, two flavonoids with a similar structure but without the hydroxyl group mentioned, are less active. This specific inhibition could be also the responsible for the inhibition produced by ( $\pm$ )-catechin and kaempferol on GehA (Chapter 5), although fitting of flavonoids into the active site of this enzyme, as well as a lower inhibition by 3-hydroxyflavone and 5-hydroxyflavone on this lipase, should be confirmed. On the contrary, the mechanism of action responsible for the activation produced by flavonoids on EstV (Chapter 5) remains unknown, although it could be related to an increased stabilization of this lipase produced by an interaction between these substances and some surface regions of the enzyme.



**Figure D.2 Hypothetical interaction between (±)-catechin and CRL.**

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The three-dimensional structure of the active site region of CRL, shown in a cutaway view, was obtained by Cygler & Schrag (1999) using CRL crystallized in the presence of two hexadecanesulfonate molecules (in blue). From this structure, they made the superposition of a triacylglycerol molecule (in yellow) on the two inhibitor molecules observed in the crystal structure. This structure and model was used to hypothesize the interaction between (±)-catechin and the active site of CRL. We can see how (±)-catechin structure would fit with that of the hexadecanesulfonate molecules and with that of the model triacylglycerol, suggesting that (±)-catechin could be a specific inhibitor competing for the active site of the enzyme. Moreover, the hydroxyl group at position 7 of (±)-catechin seems to be important since its disposition would be similar to that of the ester bond of the triacylglycerol, suggesting that it could interact with the catalytic amino acids of the enzyme. However, further assays are necessary to confirm this hypothesis.

With respect to inhibition of CRL by alkaloids, their effect could be related to their hydrophobicity and to some structural features such as the type and length of lateral groups (mainly for rescinnamine and reserpine; Chapter 4). However, the different results obtained for rescinnamine and reserpine on EstV, and mainly on GehA, seem to indicate that the mechanisms proposed for these substances would be only specific for CRL.

On the other hand, it is interesting to point out that the differences in activation and inhibition found among the lipases assayed were higher among lipases from different families than for lipases belonging to the same or related microorganisms. For example, when the effect of saturated fatty acids was analyzed, *Bacillus*-related cell-bound CEs from family VII of bacterial lipases displayed a similar behaviour than the fungal lipase CRL, but a very different behaviour than *Bacillus* secreted CEs from subfamily I.4 (Chapter 3). Moreover, the effect of saponins, flavonoids and alkaloids on GehA, a bacterial lipase from subfamily I.7, was more similar to the effect of these substances on CRL than to that produced by them on EstV, a bacterial lipase from family V (Chapters 4 and 5). Therefore, these differences are related to the biochemical and structural features of the enzymes analyzed, and indicate that the results obtained from inhibition–activation assays cannot be extrapolated directly from one lipase to another, mainly when they do not belong to the same lipase family.

Nevertheless, taking into account that several substances active on CRL were also active on *Bacillus*-related lipases, GehA and EstV (Chapters 3, 4 and 5), we can consider that screening of lipase inhibitors using model lipases is a useful tool to select compounds with a potential activity on other lipases more difficult to obtain or analyze by large-scale experiments. In particular, model lipases are very useful in the detection of those lipase inhibitors such as GA, which have a similar general effect in a wide range of lipases (Chapters 4 and 5), although the activity of each inhibitor on other lipases must be always confirmed. Moreover, inhibition assays on model lipases represent a useful tool to determine the mechanism of action of these compounds, as mentioned before (e.g. see Figure D.2 for a hypothesis about the interaction between CRL and ( $\pm$ )-catechin), which could help in the designing of improved inhibitors, or in improving the catalytic properties of lipases with biotechnological interest (Simons *et al.*, 1999).

In fact, using the model lipase of *C. rugosa* has allowed us to find out several natural substances that inhibit as well GehA, a lipase difficult to obtain, and EstV, a lipolytic enzyme previously unknown. Most of these inhibitors (GA, (±)-catechin and kaempferol for GehA, and β-aescin and GA for EstV), have a high potential for the treatment of acne and *H. pylori*-related ulcers since they are strong lipase inhibitors with other beneficial properties and with low toxicity.

**General conclusion:** Model lipases are useful for the screening of lipase inhibitors, although the results obtained should be considered with care since lipase inhibition depends on the method and reaction conditions used, on the structure and physicochemical features of the compounds tested and on the structural and biochemical properties of the lipases analyzed.