

CHAPTER 3

Activation and inhibition of *Candida rugosa* and *Bacillus*-related lipases by saturated fatty acids, evaluated by a new colorimetric microassay



Figure C3.1 Colorimetric microassay.

1 INTRODUCTION AND OBJECTIVES

Lipases play an important role in pathologies such as obesity and infective diseases produced by *P. acnes*, *H. pylori* and other lipolytic microorganisms (General Introduction 3.4). Therefore, research on new lipase inhibitors for the therapy of these diseases has generated a great interest (General Introduction 4).

Moreover, inhibition studies on lipases could contribute to better understand the mechanism of action of these enzymes, which could help in the designing of new inhibitory compounds of either pharmacological or biotechnological interest, and in the designing of novel substrate specificities and new selective lipases for catalyzing specific reactions useful for biotechnological applications (Simons *et al.*, 1999; General Introduction 4).

Therefore, there is a high interest in analyzing the activity of lipases and lipase inhibitors. However, lipase activity and lipase inhibition is difficult to determine since lipases are water soluble enzymes, whereas lipase substrates and many lipase inhibitors are water insoluble or sparingly soluble in water. For these reasons, a large number of methods for measuring the activity of lipases, or their inhibition, have been reported. These methods differ on the process used for substrate solubilization, on the activity marker employed or on the detection system. Most of them are not suitable for non-purified samples or for large number of assays because they are expensive or time-consuming. Thus, efforts to automation and miniaturization of such assays are desirable, and chromogenic and fluorimetric assays are by far the simplest, the most reliable and the easiest for large-scale experiments (Beisson *et al.*, 2000; Wahler & Reymond, 2001; General Introduction 2.3).

On the other hand, lipases from Bacillales have attracted a great interest due to their biotechnological potential (Jaeger *et al.*, 1999; General Introduction 3.3.5). However, knowledge about the enzymatic properties, the regulation and the physiological role of these enzymes is still low. For example, the effect of saturated fatty acids on *Bacillus*-related lipases is yet unknown despite of being the natural products of lipolytic activity. Moreover, fatty acids are well-known competitive inhibitors of the hydrolysis and synthesis reactions catalyzed by many lipases (Markweg-Hanke *et al.*, 1995; Hari Krishna & Karanth, 2001), are involved in the regulation of the gene expression of some lipases and virulence factors (Ruzin & Novick, 2000; Gupta *et al.*, 2004), and can inhibit the growth of several microorganisms such as *H. pylori* (Sun *et al.*, 2003), *P. acnes* (Higaki, 2003) and other lipolytic bacteria (General Introduction 3.1.1 and 4.2).

Therefore, this chapter is focused on the development of a fast and sensitive colorimetric microassay for the evaluation of lipase activity and inhibition, and on the evaluation of the effect of saturated fatty acids on *Bacillus*-related lipases. The exact aims of this work were:

- 3. To develop and validate a fast, simple, and reliable colorimetric microassay suitable for high-throughput evaluation of lipolytic activity and lipase inhibition.**

- 4. To analyze the effect of saturated fatty acids on *Candida rugosa* lipase and five *Bacillus*-related lipolytic enzymes: *Paenibacillus* sp. BP-23 EstA, *Bacillus* sp. BP-7 EstA1, *Bacillus subtilis* LipA, *Bacillus megaterium* LipA and *Bacillus* sp. BP-6 LipA, using the previously developed colorimetric microassay.**

◆ **NOTE:** The work presented in this chapter was mainly performed at the Università degli studi di Roma “*La Sapienza*”, in collaboration with Dr. S. Falcochio and Dr. E. Xoxi, under the supervision of Dr. L. Saso.

2 MATERIALS AND METHODS

Unless otherwise stated, the materials and methods used were those previously described in the General Materials and Methods section.

2.1 LIPASES USED

Candida rugosa lipase (CRL) was obtained from Sigma (cat. No. L-1754), and was prepared in 50 mM phosphate buffer (pH 7). CRL was also prepared in 50 mM Tris–HCl buffer (pH 7.4) when the assays were performed using this buffer.

The (*Paeni*)*Bacillus* lipases used were: *Paenibacillus* sp. BP-23 EstA (BP23EstA; Prim *et al.*, 2000), *Bacillus* sp. BP-7 EstA1 (BP7EstA1; Prim *et al.*, 2001), *B. subtilis* LipA (BSLipA; Dartois *et al.*, 1992; Sánchez *et al.*, 2002), *B. megaterium* LipA (BMLipA; this work, Chapter 1) and *Bacillus* sp. BP-6 LipA (BP6LipA; this work, Chapter 1). Crude cell extracts obtained from the corresponding *E. coli* recombinant clones (General Materials and Methods, Table M.1), and prepared in 50 mM phosphate buffer (pH 7) as described (General Materials and Methods 4.1.1), were used as enzyme source.

2.2 EVALUATION OF LIPASE ACTIVITY AND LIPASE INHIBITION BY A NEW COLORIMETRIC MICROASSAY

A previously described colorimetric assay (Prim *et al.*, 2000) was adapted and simplified to obtain the fast, simple, and more sensitive colorimetric microassay described in the General Materials and Methods 5.3.2 and summarized in Figure C3.2.

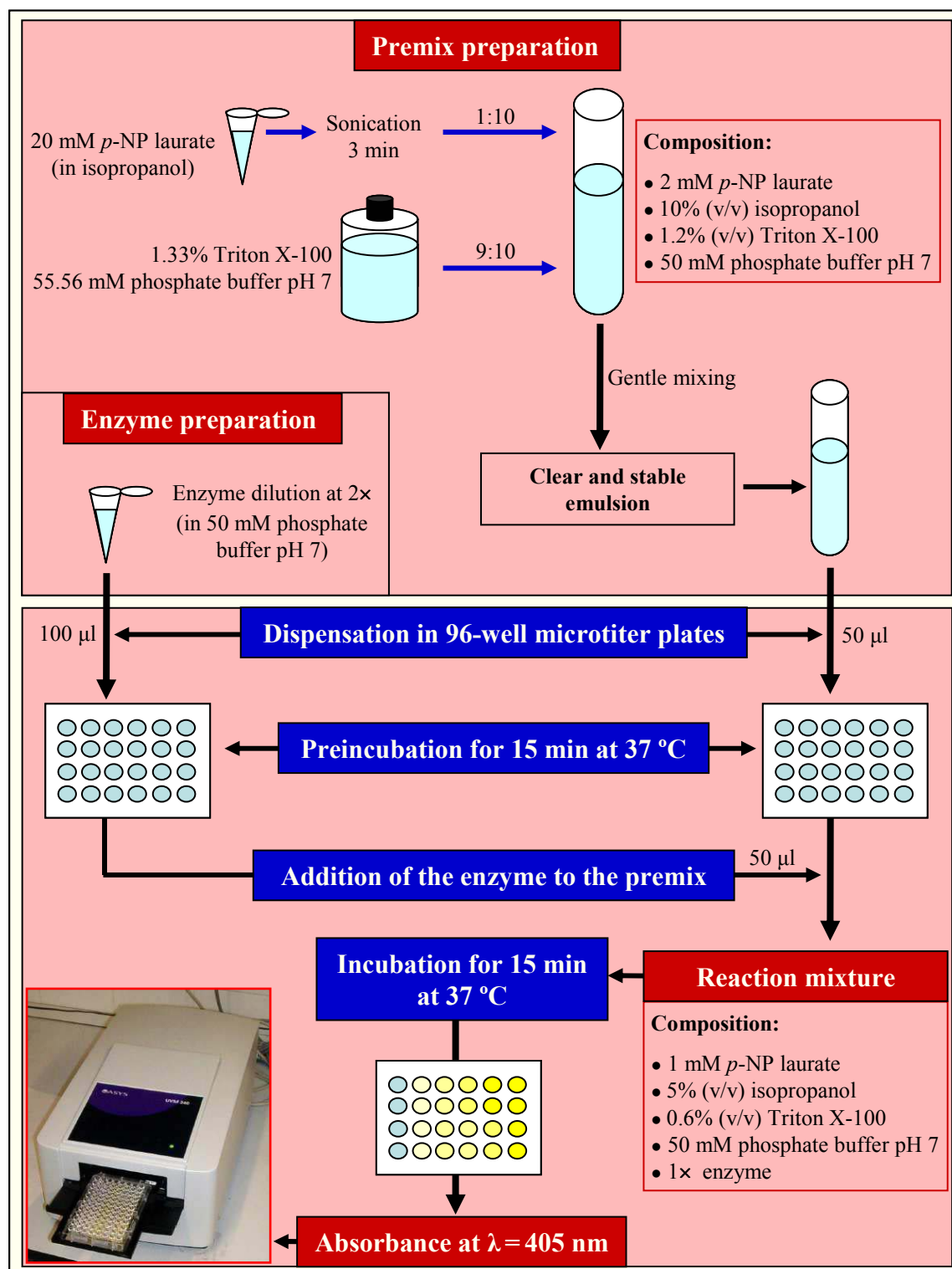


Figure C3.2 Scheme of the new colorimetric microassay developed in this chapter.

In the scheme, the colorimetric microassay is performed with *p*-NP laurate 1 mM (final concentration), although other concentrations or *p*-NP-derivatives can be used preparing the corresponding calibration curves, provided that good emulsions are achieved in the premix. Moreover, *p*-NP-derivative stocks can be prepared at higher concentrations than that shown in

the scheme (20 mM) to reduce the amount of isopropanol present in the final reaction mix. Phosphate buffer can be replaced by other buffers as far as the corresponding standards are prepared, since *p*-NP absorbance changes at different pH-values (Iacazio *et al.*, 2000). However, these buffers can not be used if they contain agents producing precipitates, excessive turbidity or interfering with the substrate, or if they are too acid or too basic because they would produce a high autohydrolysis of the *p*-NP-derivatives and high absorbance changes. Other preincubation and incubation conditions (time and temperature) can be used to adapt the assay to the optimum conditions of the lipolytic enzymes analyzed.

The method is also suitable for inhibition–activation assays since the only additional step is to prepare a properly concentrated solution of the compound under assay and to add it to the premix (prepared at a higher concentration to take into account the additional dilution produced by the tested agent). Since the solvent used can affect to the enzymatic reaction, it should be also added to the premix not containing the tested agent (100% activity).

The substrate used in all activity and activation–inhibition assays was *p*-nitrophenyl laurate (*p*-NPL), whereas the preincubations and incubations of the microtiter plates were performed for 15 min at 37 °C.

The colorimetric microassay was used to analyze the lipolytic activity of CRL and the previously described (*Paeni*)*Bacillus* lipases. The highest protein concentrations within the linear range of activity *vs.* protein–concentration curves were chosen to estimate the enzymatic activity with respect to the *p*-NP calibration curve, and were used for further activity and activation/inhibition assays. CRL kinetics were calculated from activity *vs.* substrate–concentration curves as described before (General Materials and Methods 5.6.5). Activity assays were performed at least in duplicate, being each replicate the result of an independent assay performed in duplicate.

After activity assays, the new colorimetric microassay was used to evaluate the effect of three saturated fatty acids (SFAs) – capric acid (C_{10:0}), lauric acid (C_{12:0}) and myristic acid (C_{14:0}) – on lipase activity, as previously described (General Materials and Methods 5.7). Briefly, the SFAs under assay were dissolved in isopropanol by sonication for 3 min and then added to the respective reaction mixtures without enzyme, at the corresponding concentrations. After a preincubation of the resulting mixtures, the enzyme solutions (also preincubated) were added to obtain the final 100- μ l reaction mixtures, which were then incubated before measuring their absorbance at $\lambda = 405$ nm ($A_{405 \text{ nm}}$). These reaction mixtures contained the following final concentrations: fatty acid (from 0 to their maximum concentration in well-emulsified reaction mixtures), 1

mM *p*-NPL, 5% isopropanol, 0.6% Triton X-100, 50 mM phosphate buffer (pH 7) and enzyme (10^{-2} mg ml⁻¹ CRL and $0.8\text{--}8.5\cdot 10^{-2}$ mg ml⁻¹ *Bacillus*-related lipases). CRL assays were also performed in a reaction mixture containing, instead of phosphate buffer, 50 mM Tris–HCl buffer (pH 7.4) plus 3.5 mM NaCl and 1.5 mM CaCl₂.

These activation–inhibition assays were performed at least in triplicate, being each replicate the result of independent assays performed in duplicate. Lipase inhibition or activation, and the concentrations yielding a lipase inhibition of 16% (IC₁₆) and 50% (IC₅₀), were calculated by regression analysis with Rsquare coefficients higher than 0.99, as previously described (General Materials and Methods 5.7). The intra- and the inter-assay coefficients of variation were calculated based on the analysis of CRL inhibition by 15 mM capric acid.

2.3 EVALUATION OF LIPASE ACTIVITY BY HPLC

Lipase activity assays by HPLC were performed as described (General Materials and Methods 5.5) with the following modifications: 200 µl reaction mixtures containing 0.46 mM β-naphtyl laurate (β-NL), 5% acetone, 0.6% Triton X-100, 50 mM phosphate buffer (pH 7) and $0\text{--}5\cdot 10^{-2}$ mg ml⁻¹ CRL were incubated for 15 minutes at 37 °C under gentle mixing. Then, β-naphtol was extracted with 200 µl of ethyl acetate and 75 µl of the organic phase were withdrawn, evaporated at room temperature under a nitrogen stream and redissolved in 150 µl methanol. Aliquots of 50 µl were then analyzed at room temperature by HPLC and the enzymatic activity was determined as previously described (General Materials and Methods 5.5). The assays were performed at least in duplicate, being each replicate the result of an independent assay performed in duplicate.

3 RESULTS

3.1 EVALUATION OF LIPASE ACTIVITY BY A NEW COLORIMETRIC MICROASSAY

A new colorimetric microassay for lipase activity determination was developed, which features a clear and stable substrate emulsion. Under the assay conditions, the $A_{405\text{ nm}}$ of the *p*-nitrophenol calibration curve was linear up to 1.8, corresponding to a *p*-NP concentration of 0.5 mM. Activity vs. protein-concentration curves were determined for CRL (Figure C3.3A) and *Bacillus*-related lipases (not shown). The highest protein concentrations within the linear range of these curves were: $1\text{--}1.5 \cdot 10^{-2}$ mg ml⁻¹ (CRL) and $0.8\text{--}8.5 \cdot 10^{-2}$ mg ml⁻¹ (*Bacillus*-related lipases). The enzymatic activities calculated for each lipase at these protein concentrations were: 2198.3 ± 102.1 , 2889.3 ± 175.1 , 316.9 ± 30.3 , 509.9 ± 47.5 , 240.5 ± 13.3 and $239.5.3 \pm 15.0$ mU mg⁻¹ protein for CRL, BP23EstA, BP7EstA1, BSLipA, BMLipA and BP6LipA, respectively.

These results were in agreement with the activity obtained for CRL using the HPLC assay (1891.98 ± 34.0 ; Table C3.1), and with the results previously obtained for BP23EstA (2900 mU mg⁻¹ protein; Prim *et al.*, 2000). On the contrary, the results obtained for the other *Bacillus*-related lipases were 1.3–7-fold higher than those obtained by the previous colorimetric assay: 250 mU mg⁻¹ BP7EstA1 (Prim *et al.*, 2001), 60 mU mg⁻¹ BSLipA (Sánchez, 2004), 41 mU mg⁻¹ BMLipA (activity at 37 °C; Chapter 1, data not shown) and 36 mU mg⁻¹ BP6LipA (activity at 37 °C; Chapter 1, data not shown). Activity vs. *p*-NPL-concentration plots were also obtained, and all enzymes displayed a Michaelis-Menten behaviour (CRL: Figure C3.3C; (*Paeni*)*Bacillus* lipases: not shown).

3.2 COMPARISON OF CRL ACTIVITY AND INHIBITION OBTAINED BY COLORIMETRIC MICROASSAY AND BY HPLC

As is shown in Figure C3.3, the activity results obtained by colorimetric microassay were compared to those obtained by HPLC.

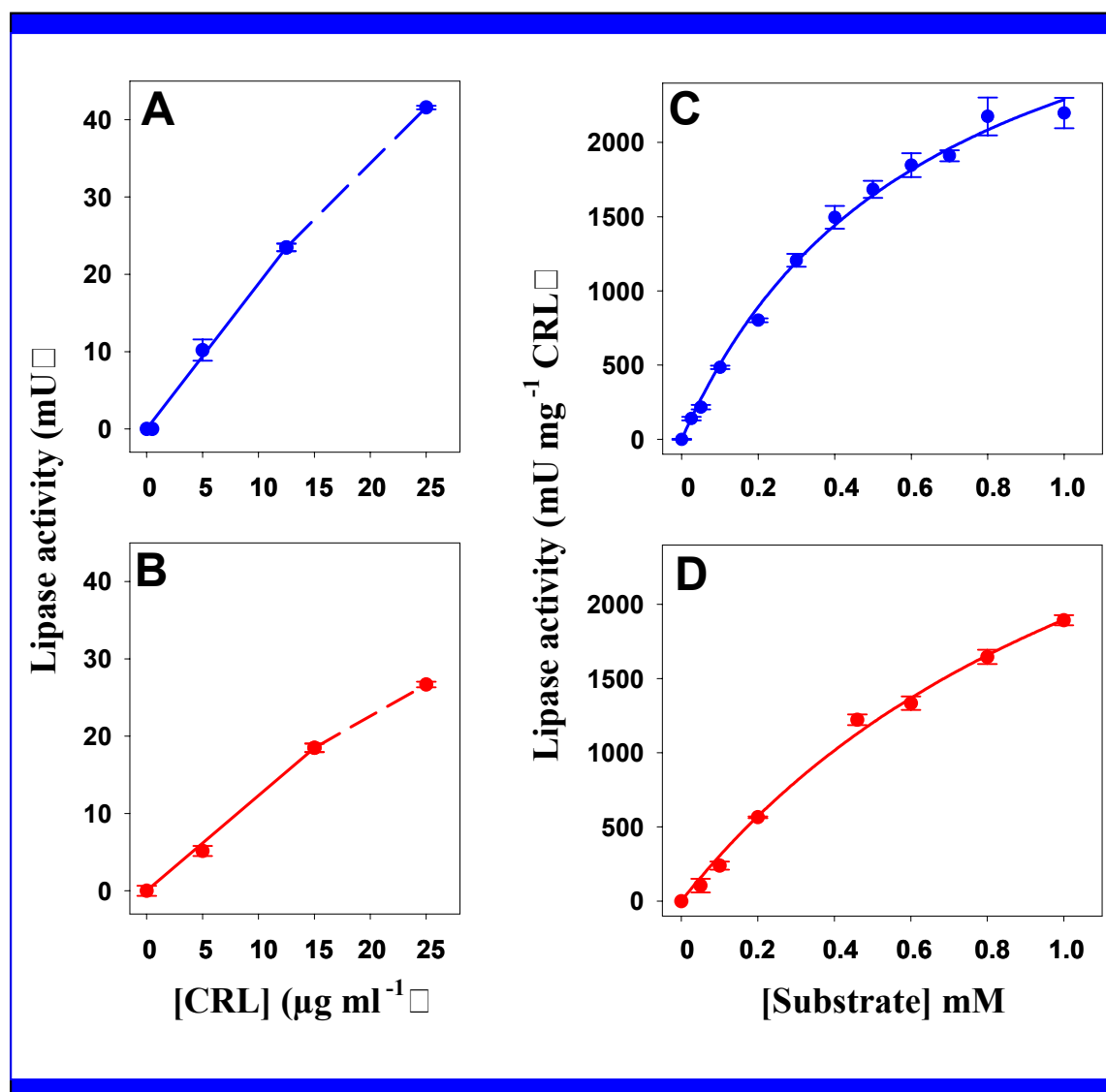


Figure C3.3 CRL activity evaluated by the colorimetric microassay and by HPLC.

CRL activity was evaluated by colorimetric microassay (A and C; in blue) and by HPLC (B and D; in red). The continuous line shows the linear range of the activity vs. CRL-concentration curves (A and B). CRL activity vs. *p*-NPL-concentration plots are also displayed (C and D).

The colorimetric microassay and the HPLC method were equivalent when CRL activity was assayed. In fact, activity vs. CRL–concentration curves had the same linear range for the colorimetric microassay (Figure C3.3A) as for the HPLC assay (Figure C3.3B). In both cases, the enzymatic activity estimated was also similar (Table C3.1). Moreover, Activity vs. substrate–concentration plots showed an equivalent kinetic behaviour and similar kinetic parameters when measured either through the colorimetric microassay or by the HPLC method (Table C3.1; Figure C3.3C–D).

Table C3.1 CRL activity evaluated by colorimetric microassay and by HPLC.

Parameter	Colorimetric microassay	HPLC
CRL concentration within the linear range of activity (μg)	Up to 12–15	Up to 12–15
Activity (mU mg^{-1} CRL at 1 mM substrate)	2198.3 ± 102.1	1892.0 ± 34.0
$V_{\text{max}}^{\text{app}}$ (mU mg^{-1} CRL)	3767.8 ± 225.7	4501.3 ± 635.4
K_M^{app} (M)	$6.5 \cdot 10^{-4} \pm 0.7 \cdot 10^{-4}$	$1.3 \cdot 10^{-3} \pm 0.3 \cdot 10^{-3}$
$k_{\text{cat}}^{\text{app}}$ (s^{-1})	3.8 ± 0.2	4.5 ± 0.6
$k_{\text{cat}}^{\text{app}}/K_M^{\text{app}}$ ($\text{s}^{-1} \text{M}^{-1}$)	$5.8 \cdot 10^3 \pm 0.4 \cdot 10^3$	$3.4 \cdot 10^3 \pm 0.5 \cdot 10^3$

Reaction conditions: 1 mM *p*-NPL (colorimetric microassay) or β -NL (HPLC), 5% isopropanol, 0.6% Triton X-100, 50 mM phosphate buffer (pH 7) and $10^{-2} \text{ mg ml}^{-1}$ CRL. An average molecular weight of 60 kDa was considered for CRL (Cygler & Schrag, 1999). The \pm refers to the standard deviations obtained from regression curves with Rsquare coefficients higher than 0.99.

In agreement with the results obtained previously by HPLC (Grippa *et al.*, 1999; Table C3.2C), when the effect of saturated fatty acids on CRL was analyzed using the colorimetric microassay (Table C3.2A; Figure C3.4), a similar low inhibitory effect was observed for the three fatty acids assayed. Small differences in the inhibition obtained were found between the two methods, but these differences drastically decreased, mainly at low fatty acid concentrations, when the colorimetric microassay was performed using reaction mixture conditions similar to those of the Grippa *et al.* (1999)

HPLC method (Table C3.2B). The intra- and inter-assay coefficients of variation of the colorimetric microassay with respect to inhibition experiments were about 3% and 4% respectively.

Table C3.2 CRL inhibition by SFAs evaluated by several methods.

Fatty acid	S _{max} (mM)	A		B		C*	
		IC ₁₆ (mM)	IC ₅₀ (mM)	IC ₁₆ (mM)	IC ₅₀ (mM)	IC ₁₆ (mM)	IC ₅₀ (mM)
Capric acid (C _{10:0})	25	4.2	19.4	4.7	14.3	4.5	11
Lauric acid (C _{12:0})	20	4.1	14.7	1.3	14.5	1.4	10
Myristic acid (C _{14:0})	15	4	~S _{max}	1.1	13.6	2.6	10

A: colorimetric microassay. Reaction mixture: 1 mM *p*-NPL, 5% isopropanol, 0.6% Triton X-100, 50 mM phosphate buffer (pH 7) and 10⁻² mg ml⁻¹ CRL.

B: colorimetric microassay. Reaction mixture: 1 mM *p*-NPL, 5% isopropanol, 0.6% Triton X-100, 3.5 mM NaCl, 1.5 mM CaCl₂, 50 mM Tris-HCl buffer (pH 7.4) and 10⁻² mg ml⁻¹ CRL.

C*: Grippa *et al.* (1999) HPLC values. Reaction mixture: 0.46 mM β-NL, 5% acetone, 1 mM sodium taurocholate, 3.5 mM NaCl, 1.5 mM CaCl₂, 50 mM Tris-HCl buffer (pH 7.4) and 10⁻² mg ml⁻¹ CRL.

S_{max}: Highest final concentration of each fatty acid achieved in well-emulsified reaction mixtures.

3.3 EFFECT OF SATURATED FATTY ACIDS (C₁₀–C₁₄) ON *BACILLUS*-RELATED LIPASES

Table C3.3 and Figure C3.4 show the effect of capric, lauric and myristic acids on (*Paeni*)*Bacillus* lipolytic enzymes, evaluated using the colorimetric microassay. The cell-bound carboxylesterases *Paenibacillus* sp BP-23 EstA and *Bacillus* sp. BP-7 EstA1 displayed almost the same profile as CRL, showing a similar inhibition for all fatty acids assayed. Myristic acid produced the lowest inhibition, mainly at high concentrations. Among the lipases tested, BP7EstA1 revealed the strongest inhibition by fatty acids: IC₅₀ = 3.8–8.5 mM; 11% relative activity at 20 mM capric acid (Table C3.3 and Figure C3.4).

The secreted carboxylesterases *B. megaterium* LipA and *Bacillus* sp. BP-6 LipA showed a completely different behaviour, being highly activated by saturated fatty acids. Myristic acid, the strongest activator compound, showed relative activities above 225–240% from 2.5 to 15 mM, with a maximum of 285% at 5 mM. Lauric acid displayed a high activation plateau (relative activity 220–230%) at 2.5–10 mM, whereas the activation decreased at higher concentrations (125–130% relative activity at 20 mM). Capric acid caused the lowest activation of these enzymes (155–160% relative activity at 2.5–5 mM), with the activation decreasing even to 90% (10% inhibition) at 25 mM fatty acid concentration (Figure C3.4).

B. subtilis LipA reported an intermediate behaviour between cell-bound and secreted carboxylesterases. All saturated fatty acids produced a moderate activation (140–150% relative activity) at 2.5–5 mM. The activation decreased at higher concentrations to almost disappear at 15 mM. Above 15 mM an increasing inhibition (nearly 50% inhibition at 25 mM capric acid) was found (Table C3.3 and Figure C3.4).

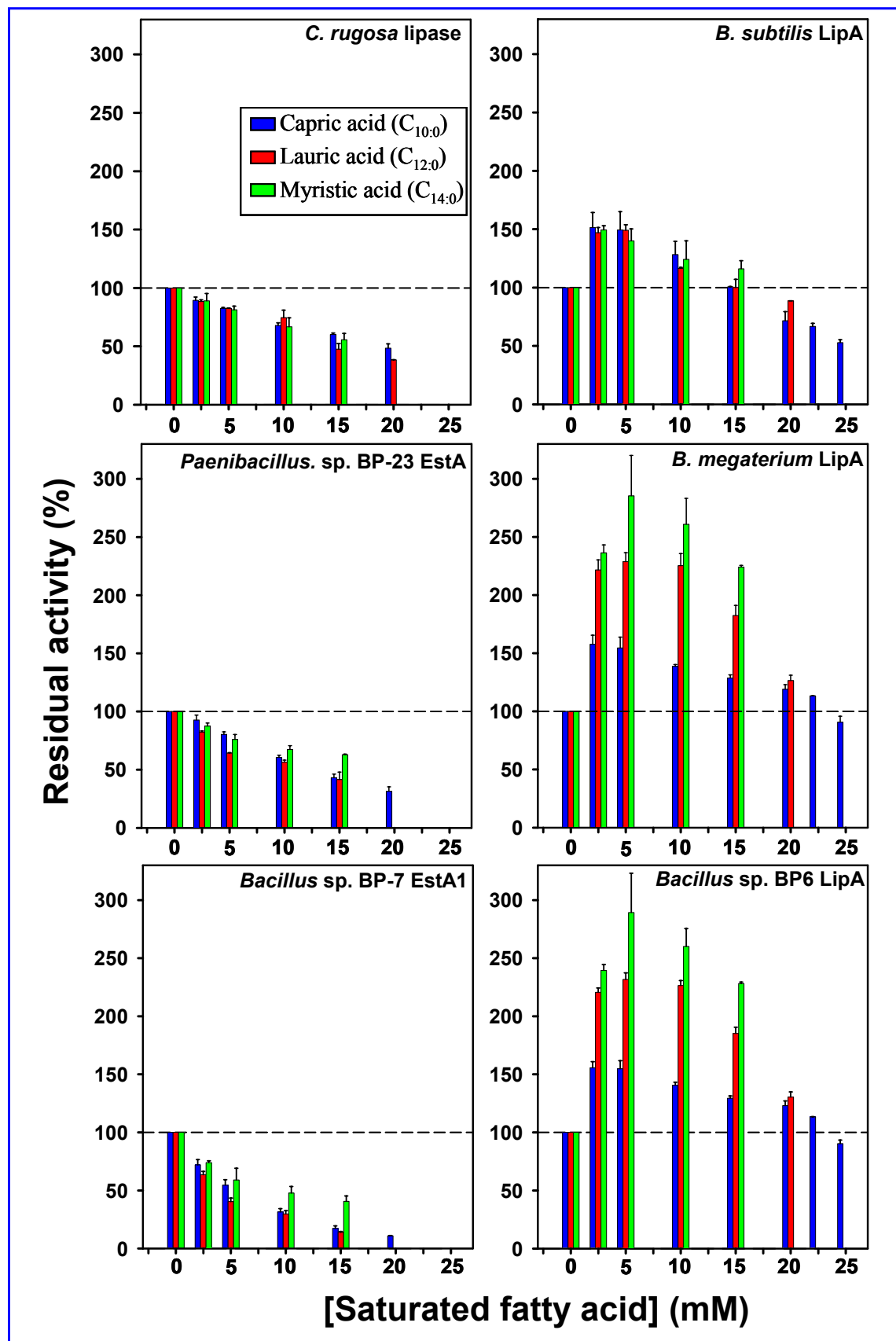
Table C3.3 Inhibition of *Bacillus*-related lipases by SFAs.

Fatty acid	S_{\max} (mM)	BP23EstA		BP7EstA		BSLipA		BMLipA		BP6LipA	
		IC ₁₆	IC ₅₀	IC ₁₆	IC ₅₀	IC ₁₆	IC ₅₀	IC ₁₆	IC ₅₀	IC ₁₆	IC ₅₀
		(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)
Capric acid (C _{10:0})	25	4.3	12.8	1.3	5.6	18.9	~ S_{\max}	>> S_{\max}	-	>> S_{\max}	-
Lauric acid (C _{12:0})	20	2.0	12.6	0.9	3.8	>> S_{\max}	-	>> S_{\max}	-	>> S_{\max}	-
Myristic acid (C _{14:0})	15	3.0	~ S_{\max}	1.2	8.5	>> S_{\max}	-	>> S_{\max}	-	>> S_{\max}	-

S_{\max} : Highest final concentrations of fatty acid achieved in well-emulsified reaction mixtures.

Next page: Figure C3.4 Effect of saturated fatty acids on CRL and *Bacillus*-related lipases.

Lipase activity in absence of fatty acid (100% activity) is indicated by dotted lines. Higher and lower activities mean activation and inhibition by fatty acids, respectively.



4 DISCUSSION

4.1 DEVELOPMENT OF A NEW COLORIMETRIC MICROASSAY

A previously described colorimetric assay for the evaluation of lipase activity and inhibition (Prim *et al.*, 2000) was adapted and simplified in order to obtain a fast, simple, accurate and more sensitive colorimetric microassay with a low cost and suitable for high-throughput analysis of a large number of samples using either purified or non-purified lipolytic enzymes.

Candida rugosa lipase (CRL), one of the best-known lipases, widely used in biotechnology, and being commercially available (Benjamin & Pandey, 1998; General Introduction 2.4), was used as a model lipase to develop and to validate the assay. Inhibition of *C. rugosa* enzymes is also interesting because, although *C. rugosa* is considered as a GRAS microorganism, some strains refractory to antifungal therapy are relevant in veterinary mycology and in emerging pathologies affecting immunocompromised patients (Colombo *et al.*, 2003).

Methods of measuring lipase activity using *p*-NP-derivatives should be used with care since the carbonyl function of these esters is electronically activated, which makes them liable to undergo non-enzymatic hydrolysis under drastic pH conditions (Beisson *et al.*, 2000; Wahler & Reymond, 2001). However, this disadvantage no longer applies when medium or long acyl-chain *p*-NP-derivatives and inert micellar detergents are used (Beisson *et al.*, 2000). For this reason, we chose Triton X-100 as non-ionic detergent, and *p*-NPL as substrate, which is stable and shows an intermediate behaviour between short and long low-soluble *p*-NP-esters. In fact, *p*-NPL micellized with Triton X-100 is a good physicochemical system widely used for lipolytic assays because the surface of the micelles is reproducible and easy to handle, and due to *p*-NPL spontaneous hydrolysis is negligible during 8 h (Redondo *et al.*, 1995).

Obtaining a clear and stable substrate emulsion made it possible to perform the assays using the same initial reaction mixture for all samples and without any mixture-

clearing centrifugation step. This allowed us to use multichannel pipettes and microtiter plates to automate and miniaturize the assay, which leads to a reduction of the handmade error, the costs of the assay and the amount of enzyme used, a limiting factor in experiments performed with lipases difficult to obtain.

Activity experiments on CRL and *Bacillus*-related lipases validated the new colorimetric method developed in this work. CRL activity and the linear range of CRL activity *vs.* enzyme–concentration plots appeared similar when evaluated by both the colorimetric microassay and by HPLC (Table C3.1; Figure C3.3). The activity found was nearly 10 times higher than that reported by Grippa *et al.* (1999) using a different reaction mixture in the HPLC method, and it was more similar to that reported by the manufacturer (11733.3 mU mg⁻¹ CRL, on olive oil and under different assay conditions). Therefore, using higher amounts of a better-emulsified substrate (nearly 2 times more concentrated) allows an increased detection of activity by both assays. Activity *vs.* substrate–concentration plots were also performed. Very similar Michaelis-Menten plots and kinetic parameters were observed for CRL using both methods (Table C3.1; Figure C3.3C–D). The Michaelis-Menten behaviour and the K_M^{app} obtained by the colorimetric microassay were also similar to those reported by Redondo *et al.* (1995) in CRL activity assays using *p*-NPL micellized with Triton X-100.

Furthermore, the activities determined for the *Bacillus*-related lipases were 6–13 times higher than those obtained using the previous colorimetric method at the same temperature (Prim *et al.*, 2001; Sánchez, 2004; Chapter 1). Only BP23EstA, a 5–10-fold more active enzyme, showed almost no differences (Prim *et al.*, 2000). As shown above for the HPLC method, using higher amounts of a better-emulsified substrate (2 times more) made the new assay more sensitive, and explains the increase in activity detected for the lipases with a lower activity. Activity *vs.* *p*-NPL–concentration assays with these lipases confirmed also the typical Michaelis-Menten behaviour previously obtained for these enzymes using fluorogenic substrates (Prim *et al.*, 2000; Prim *et al.*, 2001, Sánchez *et al.*, 2002, Chapter 1).

The activity experiments reported above contributed to the validation of the new colorimetric method. The low intra- and inter-assay variations obtained reinforced also the reliability of the method. Moreover, the method confirmed the relatively low

inhibitory effect of saturated fatty acids on CRL (Table C3.2A) previously detected by HPLC (Grippa *et al.*, 1999; see Table C3.2C), confirming also the reliability of the system with regard to the inhibition assays. Small differences of inhibition were found, but they were less significant when the colorimetric method was performed using a reaction mixture (Table C3.2B) more similar to that of Grippa *et al.* (1999) (Table C3.2C). The changes in the substrate used: 1 mM *p*-NPL (Table C3.2A–B), 0.46 mM β -NL (Table C3.2C) may explain the differences found in CRL inhibition. Both substrates are structurally related and yield similar activities for CRL at the same concentration. Therefore, the lower inhibition (higher IC₅₀) of CRL achieved in the colorimetric microassays at high fatty acid concentrations (Table C3.2A–B) is probably due to the higher amount of substrate used, as fatty acids are known competitive inhibitors of lipases (Markweg-Hanke *et al.*, 1995; Hari Krishna & Karanth, 2001). An increased ionic strength (Table C3.2B–C) seems to be also an important factor because it can affect protein folding, the interaction between charged substances, and the emulsion properties by reducing the critical micellar concentration (CMC) of detergents (Edwards *et al.*, 1994). Furthermore, the use of Ca²⁺ (Table C3.2B–C) can reinforce the inhibition of a lipase, as is described for *P. acnes* GehA (Weaber *et al.*, 1971). Additionally, changes in the detergent used: Triton X-100 (Table C3.2A–B), sodium taurocholate (Table C3.2C) were important to achieve good emulsions at higher substrate concentrations. Small pH variations: 7 (Table C3.2A)–7.4 (Table C3.2B–C) showed a low effect on CRL as they were in the optimum pH range of the enzyme (6.5–7.5; Cygler & Schrag, 1999), but should be considered when analyzing other lipases or inhibitors. Therefore, the composition of the reaction mixture should be taken into account when lipase inhibition determined by different methods is compared.

4.2 EFFECT OF SFAs ON *Bacillus*-RELATED LIPASES

Knowledge about the regulation and biochemical properties of lipases from Bacillales is gaining a high interest due to the biotechnological potential of these enzymes (Jaeger *et al.*, 1999; General Introduction 3.3.5). Moreover, the physiological

role of these lipases is still unclear, although they may be involved in providing carbon sources, in regulation of the membrane composition, in pathogenicity, or in detoxification of biocides (Schmid & Verger, 1998; Khalameyzer *et al.*, 1999). For these reasons, the new colorimetric microassay was used to analyze the effect of saturated fatty acids (SFAs) on several *Bacillus*-related lipases, since these compounds are well-known competitive inhibitors of lipases (Markweg-Hanke *et al.*, 1995; Hari Krishna & Karanth, 2001), and are involved in regulation of gene expression and microbial growth (Ruzin & Novick, 2000; Gupta *et al.*, 2004). The enzymes analyzed were *Paenibacillus* sp. BP-23 EstA (BP23EstA; Prim *et al.*, 2000) and *Bacillus* sp. BP-7 EstA1 (BP7EstA1; Prim *et al.*, 2001), two cell-bound carboxylesterases (CEs) highly similar to *B. subtilis* PnbA (Zock *et al.*, 1994) and belonging to family VII of bacterial lipases, and three secreted lipolytic enzymes from subfamily I.4 of bacterial lipases: *B. subtilis* LipA (BSLipA, Dartois *et al.*, 1992; Sánchez *et al.*, 2004), *B. megaterium* LipA (BMLipA; Chapter 1) and *Bacillus* sp. BP-6 LipA (BP6LipA, Chapter 1), the latter ones highly similar to *B. subtilis* LipB (BSLipB; Eggert *et al.*, 2000).

Strong differences with respect to the effect of SFAs on these lipases were found (Table C3.3, Figure C3.4), which indicates 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.

On the one hand, BP23EstA and BP7EstA1 displayed a moderate–high inhibition by SFAs similar to that found on CRL (Table C3.2A, Figure C3.4). The inhibition observed could represent a negative feedback regulatory system controlled by product as suggested by the higher inhibition found by shorter fatty acids, which are closer to the enzyme optimal substrate. Such a regulation suggests that these *Bacillus*-related cell-bound CEs could also be involved in the turnover of cell-membrane lipids and lipid-anchored proteins. In this case, the enzyme product would avoid a high continuous activity on the cell membrane lipids surrounding these enzymes, which are active even at low substrate concentrations since do not require interfacial activation to display activity. However, further studies are necessary to know if the inhibition found is competitive or/and specific, and to solve the regulation and physiological role of these lipolytic enzymes.

On the other hand, BSLipA showed a different response to SFAs than cell-bound CEs, but also than BSLipB-like carboxylesterases (BMLipA and BP6LipA). BSLipA showed a moderate activation at low SFAs concentrations, and a moderate inhibition at medium–high concentrations, whereas BSLipB-like CEs were moderately (capric acid) and highly (myristic and lauric acids) activated, and only slightly inhibited at high concentrations of capric acid.

To our knowledge, direct activation by fatty acids has not been described before for bacterial lipases. These compounds act usually as inhibitors, and only reactivation of pancreatic lipase–colipase complexes inhibited by phospholipid–bile salts mixed micelles (Larsson *et al.*, 1986), and interfacial activation without an interface of fungal lipases in the presence of long acyl-CoAs below their CMC (Bano *et al.*, 2003) have been reported before. The possibility of an artifact was discarded, as the assay conditions were the same as those used for CRL and cell-bound esterases inhibition detection.

Therefore, BSLipA and, mainly, BSLipB-like CEs seem to be adapted to produce a high activity in the presence of C₁₀–C₁₄-SFAs containing lipids, which are lower specific substrates since these enzymes display preference for C₄–C₁₀-triacylglycerols (Eggert *et al.*, 2000; Chapter 1). It is known that fatty acids can bind to lipoprotein lipase in three different ways: at the active site, at specific sites, and cooperatively by hydrophobic interaction (Edwards *et al.*, 1994). For this reason, we hypothesize that the different activation of BSLipA and BSLipB-like CEs achieved at low SFA-concentrations occurs by specific or highly cooperative binding of SFAs to the protein surface, where are located almost all the different residues among these proteins (Eggert *et al.*, 2001). The inhibition found could be due to a competition with the substrate for the active site because it appears at high fatty acid concentrations. Therefore, the activation–inhibition of BSLipA and BSLipB-like CEs produced by SFAs may be the result of the competition between these two different bindings.

The different regulation by SFAs found here for BSLipA and BSLipB-like CEs, as well as the differences in structure, biochemical properties and gene regulation reported for BSLipA and BSLipB by other authors (Eggert *et al.*, 2000, 2001 and 2003)

seems to indicate a different physiological role of these enzymes, as will be explained in more detail in the General Discussion.

4.3 FUTURE PERSPECTIVES

The simple, sensitive and miniaturized colorimetric microassay developed in this work constitutes a powerful tool for high-throughput analysis of lipase activity and inhibition including enzyme characterization assays, detection of lipase-containing fractions during purification processes, screening of the lipolytic activity of novel microbial isolates, screening of new synthetic or natural lipase inhibitors, etc. In fact, this method has been used for a rapid confirmation of the inhibition results obtained in Chapter 4, and during the purification and/or characterization of lipolytic enzymes from *P. acnes* and *H. pylori* described in Chapter 5.

With respect to *Bacillus*-related lipases, further assays are necessary to obtain a deeper knowledge about their biochemical and genetic regulation and to establish the physiological role of these enzymes. In particular, it would be very interesting to find out the exact mechanism of activation by fatty acids found in BSLipA and BSLipB-like proteins (crystallographic analyses, effect of other fatty acids, etc), as well as to find out if activation by fatty exists also in other lipases. Moreover, activation of these lipases is a promising approach that could be very useful to improve the application of these enzymes in biotechnological processes.

5 CONCLUSIONS

- I. A previously described colorimetric assay for the evaluation of lipase activity and inhibition has been adapted, simplified and miniaturized in order to obtain a fast, simple, accurate and more sensitive colorimetric microassay with a low cost and suitable for high-throughput analysis of purified or non-purified lipolytic enzymes.
- II. Comparison of *Candida rugosa* lipase activity and inhibition with previous HPLC results validated the new method and confirmed the inhibition produced by saturated fatty acids on this enzyme. Comparison of *Bacillus*-related lipases activity with results previously obtained by the original colorimetric method and by fluorimetric assays contributed also to validate the method.
- III. The assays performed to validate the new method revealed the importance of the reaction mixture composition when lipase activity or inhibition determined by different methods is compared.
- IV. The colorimetric microassay has been used to evaluate the effect of saturated fatty acids on *Bacillus*-related lipases. These assays have demonstrated 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.
- V. The saturated fatty acids analyzed produced a moderate inhibition of the cell-bound carboxylesterases *Paenibacillus* sp. BP-23 EstA and *Bacillus* sp. BP-7 EstA1, suggesting a negative feedback regulation by product, and a potential role of these enzymes in cell membrane turnover.
- VI. *B. subtilis* LipA was moderately activated by low concentrations of saturated fatty acids and was inhibited at higher concentrations, whereas *B. megaterium* LipA and *Bacillus* sp. BP-6 LipA were highly activated by myristic and lauric acids, and were only slightly inhibited by high capric acid concentrations.

- VII. Direct activation by fatty acids on bacterial lipases has been described here for the first time.
- VIII. The activation–inhibition profile found for *B. subtilis* LipA, *B. megaterium* LipA and *Bacillus* sp. BP-6 LipA could be the result of a competition between the activation produced by a specific or highly cooperative binding of saturated fatty acids to the protein surface, and the inhibition produced by these compounds due to competing with the substrate for the active site of the enzyme.
- IX. The different activation–inhibition profile found for *B. subtilis* LipA and *B. subtilis* LipB-like enzymes could be related to previously reported differences in the structure, activity and regulation of these proteins, and seems to indicate a different physiological role for these enzymes.

6 PUBLICATIONS

The work corresponding to this chapter has been published in the following scientific article (see Annex I for the full text):

- **Ruiz, C., Falcocchio, S., Xoxi, E., Pastor, F.I.J., Díaz, P. and Saso, L.** (2004) Activation and Inhibition of *Candida rugosa* and *Bacillus*-related lipases by Saturated Fatty Acids, Evaluated by a New Colorimetric Microassay. *Biochim. Biophys. Acta* 1672: 184–191.