Journal of Biochemistry and Molecular Biology, Vol. 34, No. 5, September 2001, pp. 457-462

JOURNAL OF
Biochemistry and Molecular Biology

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Comparative Kinetic Studies of Two Staphylococcal Lipases Using the Monomolecular Film Technique

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Received 8 May 2001, Accepted 31 July 2001

Using the monomolecular film technique, we compared the interfacial properties of *Staphylococcus simulans* lipase (SSL) and *Staphylococcus aureus* lipase (SAL). These two enzymes act specifically on glycerides without any detectable phospholipase activity when using various phospholipids. Our results show that the maximum rate of racemic dicaprin (*rac*-dicaprin) hydrolysis was displayed at pH 8.5, or 6.5 with *Staphylococcus simulans* lipase or *Staphylococcus aureus* lipase, respectively. The two enzymes interact strongly with egg-phosphatidyl choline (egg-PC) monomolecular films, evidenced by a critical surface pressure value of around 23 mN · m^{-1}. In contrast to pancreatic lipases, β-lactoglobulin, a tensioactive protein, failed to inhibit *Staphylococcus simulans* lipase and *Staphylococcus aureus* lipase. A kinetic study on the surface pressure dependency, stereoselectivity, and regioselectivity of *Staphylococcus simulans* lipase and *Staphylococcus aureus* lipase was performed using optically pure stereoisomers of diglycerides (1,2-*sn*-dicaprin and 2,3-*sn*-dicaprin) and a prochiral isomer (1,3-*sn*-dicaprin) that were spread as monomolecular films at the air-water interface. Both staphylococcal lipases acted preferentially on distal carboxylic ester groups of the diglyceride isomer (1,3-*sn*-dicaprin). Furthermore, *Staphylococcus simulans* lipase was found to be markedly stereoselective for the *sn*-3 position of the 2,3-*sn*-dicaprin isomer.

Keywords: Critical pressure, Monomolecular films, Staphylococcal lipases, Stereoselectivity, Regioselectivity

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Introduction

Lipases (glycerol ester hydrolases EC 3.1.1.3) are found in all living species of the animal kingdom, as well as in plants and microorganisms such as yeast, bacteria, and fungi. Under physiological conditions, mammalian lipases hydrolyse the ester bonds that are found in acylglycerides. Lipases have been reported to have broad substrate specificity. In addition to natural glycerides, some lipases can hydrolyse synthetic lipids and/or phospholipids (Jaeger *et al.*, 1994). Maximum lipase catalytic activity is usually expressed in the presence of a lipid-water interface. The physico-chemical properties of the interface play an important role in lipolysis (Egloff *et al.*, 1995).

Some staphylococcal lipases have been purified and biochemically characterized. These enzymes can hydrolyse tri-, di-, and monoglycerides of various chain-lengths, as well as polyoxyethylene sorbitan fatty acyl esters (tweens) (Tysky *et al.*, 1983; Rollof *et al.*, 1987). The catalytic activity of some of these lipases appeared to be stimulated by calcium ions, while chelators, such as EDTA, were reported to act as inhibitors (Muraoka *et al.*, 1982; Rosenstein and Götz, 2000). The lipase from *Staphylococcus hyicus* is unique among the staphylococcal and bacterial lipases since it has a high phospholipase activity, as well as lipase activity (Rosenstein and Götz, 2000). Lipase genes of *S. aureus* strains PS54 (Lee and Iandolo, 1986) and NCTC8530 (Nikoleit *et al.*, 1995), *S. epidermis* strain 9 (Farrell *et al.*, 1993), and *S. hyicus* (Götz *et al.*, 1985) have been identified. It appears that the mature forms of these lipases are very homologous, and share a 50 to 77% amino acid sequence identity. Despite the high similarity of these enzymes, important differences in biochemical characterization, pH profile, substrate specificity, and chain length selectivity have been described (Rosenstein and Götz, 2000).

In our laboratory, the lipase of *Staphylococcus simulans* was recently produced, purified, and some catalytic properties were determined using classical emulsified systems. Using

tripropionin (TC_3), a partially water soluble synthetic short chain triglyceride, as a substrate, SSL as well as SAL (Simons *et al.*, 1996) did not present the well-known interfacial activation phenomenon.

Jaeger *et al.* (1994) established that microbial lipases, such as those used in this study, may be involved in pathologic processes related to lipid hydrolysis. The monolayer technique can be used as a membrane model to better characterize the interaction of staphylococcal lipases with various lipidic films.

In this study, we proposed to use the monomolecular film technique (Dervichian, 1971; Zografi *et al.*, 1971) in order to establish the penetration power, stereoselectivity, and regioselectivity of two staphylococcal lipases (*Staphylococcus simulans* lipase and *Staphylococcus aureus* lipase).

Materials and Methods

Lipases *Staphylococcus simulans* lipase (SSL) was purified to homogeneity from a culture medium after ammonium sulfate precipitation, Sephacryl S-200 gel filtration, cation exchange chromatography (carboxymethyl Sephadex), and Superose-12 filtration using fast pressure liquid chromatography (FPLC). Pure SSL was eluted as a tetrameric protein (160 kDa), corresponding to the association of the four lipase molecules (unpublished work). His-tagged mature *Staphylococcus aureus* lipase NCTC8530 (SAL), a generous gift from Prof. Maarten Egmond (Utrecht, The Netherlands), was prepared according to Simons *et al.* (1996). The presence of a short N-terminal extension that contained six successive histidine residues did not alter the kinetic properties of this enzyme, compared to the wild-type form (Simons *et al.*, 1996).

Lipids The 1,2-*sn*- and 1,3-*sn*-dicaprin were from Sigma. The 2,3-*sn*-dicaprin was prepared from tricaprin (Sigma Chemical Co., St. Louis, USA) by stereospecific enzymatic hydrolysis of the *sn*-1 ester bond as described previously (Rogalska *et al.*, 1995). Egg-phosphatidyl choline (egg-PC), 1,2-dilaurylphosphatidyl ethanolamine (diC_{12}-PE), 1,2-dilaurylphosphatidyl serine (diC_{12}-PS), 1,2-dilaurylphosphatidyl glycerol (diC_{12}-PG), and *rac*-dicaprin were from Fluka. As expected, the surface pressure-molecular area curves of 1,2-*sn*- and 2,3-*sn*-dicaprin are superimposable (data not shown). They are characteristic of the liquid expanded state, and show no sign of discontinuity in the full range of surface pressure. The collapse pressure of 1,2-*sn*- and 2,3-*sn*-dicaprin is 40 $mN \cdot m^{-1}$, and that of 1,3-*sn*-dicaprin is 32 $mN \cdot m^{-1}$ (Rogalska *et al.*, 1995).

Proteins β-lactoglobulin and bovine serum albumin (BSA) were from Sigma (Sigma Chemical Co., St. Louis, USA).

Protein concentration The protein concentration was determined as described by Bradford (1976), or by spectrophotometry at 280 nm using the following absorption coefficients ($E^{1\%}_{1\,cm}$): β-lactoglobulin, 9.6; BSA, 6.7.

Measurement of the lipase penetration into the egg-PC monolayer The surface pressure increase that was due to the penetration of lipase into the egg-PC/water interface was measured in a cylindrical trough that was drilled in a Teflon block (surface area 7 cm^2, the total volume was 5 ml of 10 mM Tris-HCl, pH 8, 150 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA).

The aqueous subphase was continuously stirred at 250 rpm with a magnetic rod. Measurements of penetration were estimated as described previously (Piéroni *et al.*, 1990).

Monomolecular film techniques Measurements were performed with KSV-2000 Baro-stat equipment (KSV-Helsinki). The principle of the method was described previously by Verger and de Haas (1973). It involves the use of a "zero-order" trough with two compartments-a reaction compartment and a reservoir compartment, which were connected to each other by a small surface channel.

The enzyme solution was injected into the subphase of the reaction compartment only when the lipid film covered both of them. A mobile barrier, automatically driven by the baro-stat, moved back and forth over the reservoir to keep the surface pressure (π) constant, thus compensating for the substrate molecules that were removed from the film by enzyme hydrolysis. The surface pressure was measured on the reservoir compartment with a Wilhelmy plate (perimeter 3.94 cm) that was attached to an electromicrobalance, which was connected in turn to a microprocessor that was programmed to regulate the mobile-barrier movement. Two 2-cm magnetic stirrers stirred the reaction compartment at 250 rpm. The reactions were performed at room temperature (25°C). The surface of the reaction compartment was 108.58 cm^2. Its volume was 130 ml. The reservoir compartment was 148 mm wide and 249 mm long.

Before each experiment, the Teflon trough that was used for forming the monomolecular film was cleaned with water, then gently brushed in the presence of distilled ethanol, washed again with tap water, and finally rinsed with double distilled water.

The aqueous subphase was composed of 10 mM Tris-HCl buffer, pH 8.5, 150 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA; or 10 mM phosphate buffer, pH 6.5, 150 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA.

The buffers were prepared with double-distilled water and filtered through a 0.45-μm Millipore filter. Any residual surface-active impurities were removed before each assay by a sweeping and suction of the surface.

The kinetic data were analyzed as described previously (Verger and de Haas, 1973; Ransac *et al.* 1990). Activities are expressed as the number of moles of substrate hydrolyzed by unit time and unit surface of the reaction compartment of the "zero order" trough for an arbitrary lipase concentration of 1 M.

Measurement of the pH dependency The variations with pH in the SSL and SAL activities were measured using *rac*-dicaprin monomolecular films that were maintained at a constant surface pressure of 35 $mN \cdot m^{-1}$. Assays were carried out at room temperature in a "zero-order" trough as described previously. The aqueous subphase was composed of various buffers that contained 150 mM NaCl and 2 1mM $CaCl_2$: 10 mM glycine (pH 3-4), 10 mM sodium acetate (pH 5-6), 10 mM phosphate (pH 7), 10 mM Tris-HCl (pH 8-9), or 10 mM borate (pH 9-10).

Lipase activity determination The lipase activity was measured titrimetrically at pH 8.5 and 37°C with pH-stat (Metrohm,

Switzerland) using the following tests: SSL, 0.25 ml of tributyrin in 30 ml of 2.5 mM Tris-HCl, pH 8.5, 3 mM $CaCl_2$; SAL, 0.25 ml of tributyrin in 30 ml of 2.5 mM phosphate, pH 6.5, 3 mM $CaCl_2$. One unit corresponds to 1μmole of fatty acid released per min.

Results and Discussion

Lack of phospholipase activity For both SSL and SAL, no phospholipase activity could be detected when using various phospholipids (egg-PC, diC_{12}-PE, diC_{12}-PS and diC_{12}-PG) spread as monomolecular films and maintained at various surface pressures ranging from 5 to 30 $mN \cdot m^{-1}$ (data not shown). This confirmed that SSL (unpublished work) and SAL (Simons *et al.*, 1996) are specific in the hydrolysis of glycerides only.

Interactions of SSL and SAL with egg-PC monolayers To measure the critical surface pressure of SSL and SAL, we injected a lipase sample under a monomolecular film of egg-PC at an initial surface pressure (π_i) that ranged from 2 to 30 $mN \cdot m^{-1}$. The value of the maximal surface pressure increase ($\Delta\pi_{max}$) reached equilibrium around 50 min after the injection of the lipase into the stirred aqueous subphase. It was determined and plotted as a function of the π_i. With both lipases, $\Delta\pi_{max}$ decreased linearly with increasing π_i (Fig. 1). The critical surface pressure (π_c) for each lipase was estimated by a linear extrapolation to zero surface pressure increase of the experimental points. An average comparable π_c value of 23 $mN \cdot m^{-1}$ was obtained with both SSL and SAL (Fig. 1). This result suggests that staphylococcal lipases are able to interact efficiently with egg-PC monolayers.

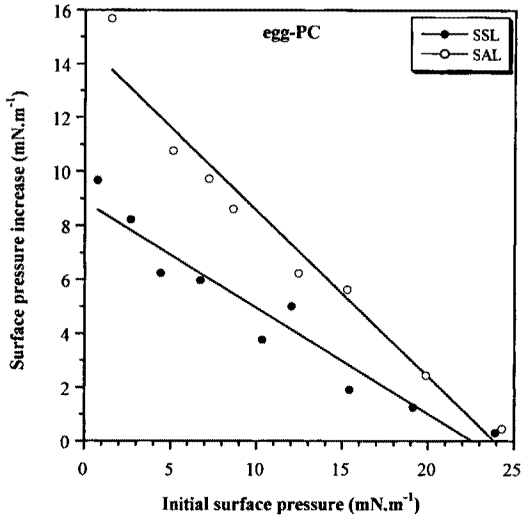

Fig. 1. Interactions of SSL and SAL with egg-PC monolayers. Maximal increase in surface pressure reached at equilibrium 50 min after injection of SSL or SAL under egg-PC monomolecular films spread at various initial surface pressures. Assays were carried out in a cylindrical Teflon trough (volume, 5 ml; Surface, 7 cm^2). Final enzyme concentration, 30 nM. Buffer: 10 mM Tris-HCl, pH 8, 150 mM NaCl, 21 mM $CaCl_2$, and 1 mM EDTA.

pH dependency of the catalytic activity of SSL and SAL We tested the pH influence of the aqueous subphase on the SSL and SAL activities (Fig. 2), using *rac*-dicaprin monomolecular films that were maintained at a constant surface pressure of 35 $mN \cdot m^{-1}$.

Fig. 2 shows clearly that the maximal SSL activity is reached at pH 8.5, while SAL presents a pH optimum of 6.5. Similar results were obtained with the same lipases using a tributyrin emulsion as substrate with the pH-stat method (data not shown).

Comparable results were also obtained by Simons *et al.* (1996) using SAL (pH optimum of 6.5) and SHL (pH optimum of 8.5). These findings confirm that staphylococcal lipases present different pH profiles.

Effect of some proteins on the hydrolysis rate of dicaprin by SSL and SAL Monolayers of dicaprin, maintained at a high and constant surface pressure (35 $mN \cdot m^{-1}$), adapt well to the study of the inhibition of lipases by proteins. Amphiphilic proteins, like β-lactoglobulin or bovine serum albumin (BSA), interact with and penetrate into the dicaprin monolayer that is maintained at 35 $mN \cdot m^{-1}$ without affecting the surface pressure (Gargouri *et al.*, 1985). Among the proteins tested by Gargouri *et al.* (1985), β-lactoglobulin is the

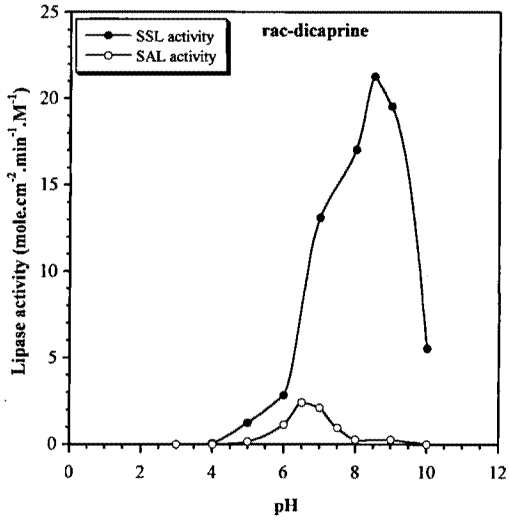

Fig. 2. pH dependency of the catalytic activity of SSL and SAL. Variations with pH in SSL and SAL activities using *rac*-dicaprin monomolecular films. The *rac*-dicaprin monolayer was spread and maintained at a constant surface pressure of 35 $mN \cdot m^{-1}$. Assays were carried out at room temperature in a "zero-order" trough (volume, 130 ml; surface, 108.58 cm^2). The final enzyme concentration was 0.11 nM. The aqueous subphase was composed of various buffers that contained 150 mM NaCl and 21 mM $CaCl_2$: 10 mM glycine (pH 3-4), 10 mM sodium acetate (pH 5-6), 10 mM phosphate (pH 7), 10 mM Tris-HCl (pH 8-9), or 10 mM borate (pH 9-10). Activities are expressed as the number of moles of substrate hydrolyzed by unit time and unit surface of the reaction compartment of the "zero order" trough for an arbitrary lipase concentration of 1 M.

most potent inhibitory protein. In the presence of 0 .1μM of β-lactoglobulin in the subphase, no activity was detected with pancreatic lipases and *Rhizopus delemar* lipase (RDL) on a dicaprin monomolecular film.

In the present study, it was observed that the rate of hydrolysis by SSL and SAL of dicaprin monomolecular films, maintained at a constant surface pressure of 35 mN · m^{-1}, was unaffected by the presence of β-lactoglobulin or BSA injected into the aqueous subphase at a final concentration of 1 μM (data not shown).

Using radiolabeled lipases and proteins, Gargouri *et al.* (1986) have shown that lipase inhibition was correlated with a lack of lipase binding to mixed protein-dicaprin films, or to a desorption of lipase from the interface when inhibitory proteins were added later. Since a large proportion of the lipid film remained potentially accessible to the enzyme in the presence of the inhibitory protein, it was concluded that the observed decrease in lipase binding to the interface was due to a variation of the physiochemical properties of the lipid-water interface following binding of the inhibitory protein (Gargouri *et al.*, 1986).

A positive correlation was established between the penetration power (as defined by the initial rate of surface pressure increase, $(\Delta\pi/\Delta t)_{t=0}$, measured with a dicaprin film spread at 20 mN · m^{-1}) of various proteins and their capacity to inhibit several lipases (Gargouri *et al.*, 1985). The interfacial inhibition by proteins could be related to the respective penetration rates of the lipases and inhibitory proteins. We conclude that SSL and SAL, which are able to bind to mixed b-lactoglobulin-dicaprin film, may possess a penetration power higher than that of pancreatic lipases and some microbial lipases like RDL.

Using emulsified substrates, Simons *et al.* (1996) have shown that SAL was able to hydrolyse essentially short chain triacylglycerols (tributyrin). No significant activity was detected using long chain triacylglycerols (triolein). This last catalytic property was used by Ferrato *et al.* (1997) as a criterion to distinguish lipases from esterases. These authors defined lipases as a family of enzymes that are capable of hydrolyzing emulsified long chain triacylglycerols (triolein) independently in the presence or absence of the interfacial activation phenomenon. Accordingly, it may be inferred that SAL, which cannot hydrolyse long chain triacylglycerols, cannot be classified as a true lipase. In this study, using the monomolecular film technique, we attempted to show that SAL possesses a penetration power which allows its binding and the expression of its catalytic activity on dicaprin monolayer films that is maintained at a high surface pressure (35 mN · m^{-1}). Thus, when lipases were specific towards short and/or medium chain triacylglycerols, like SAL, the hydrolysis of monomolecular films of dicaprin could be used as a criterion to distinguish lipases from esterases.

As described by Fojan *et al.* (2000), esterases show an expected decrease in non-polar residue composition with increasing solvent accessibility, as commonly observed in water-soluble proteins. In contrast, lipases display an enhanced content of non-polar residues around 50 to 80% solvent accessibility. This increased hydrophobic patch that is located in this solvent accessible area of the proteins may facilitate the lipase attachment to the hydrophobic substrate aggregate (Sabuquillo *et al.*, 1998; Van Kampen *et al.*, 1999; Fojan *et al.*, 2000).

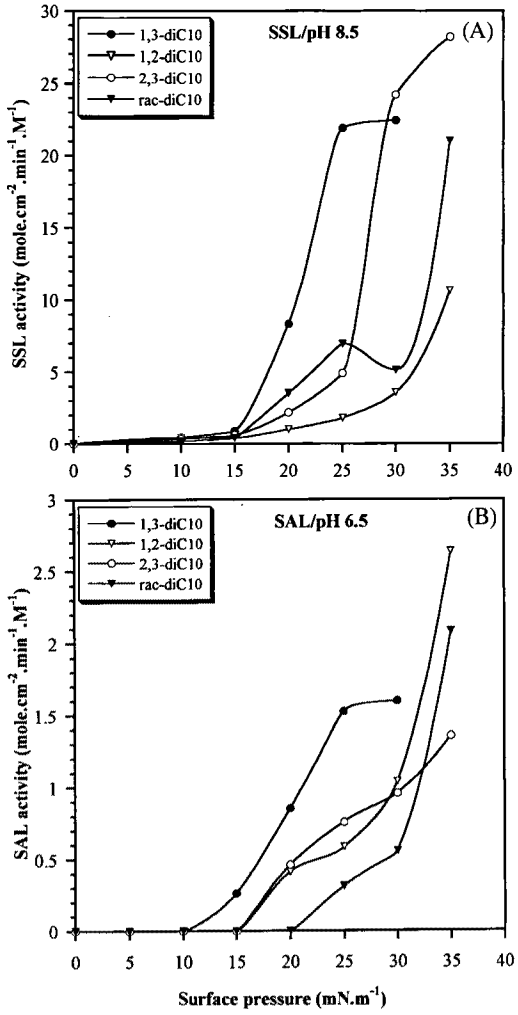

Fig. 3. Variations with surface pressure in SSL (A) and SAL (B) activities using pure dicaprin (diC10) isomers. Assays were carried out in a "zero-order" trough (volume, 130 ml; surface, 108.58 cm^2). The final enzyme concentration was 0.11 nM. SSL buffer: 10 mM Tris-HCl, pH 8.5, 150 mM NaCl, 21 mM $CaCl_2$ and 1 mM EDTA. SAL buffer: 10 mM phosphate, pH 6.5, 150 mM NaCl, 21 mM $CaCl_2$ and 1 mM EDTA. Activities are expressed as the number of moles of substrate hydrolyzed by unit time and unit surface of the reaction compartment of the "zero order" trough for an arbitrary lipase concentration of 1 M.

Variations with surface pressure in SSL and SAL activity using various dicaprin isomers Fig. 3 shows that the catalytic activity of SSL and SAL increased continuously with increasing surface pressure. This is similar to the behavior of

Table 1. Stereoselectivity and regioselectivity of SSL and SAL. The vicinity index (V.I.) and the stereoselectivity index (S.I.) of lipases were calculated using the following definitions: V.I. = $[A_{1,3}\text{-}1/2(A_{2,3}+A_{1,2})]/[A_{1,3}+1/2(A_{2,3}+A_{1,2})]$ and S.I. = $(A_{2,3}-A_{1,2})/(A_{2,3}+A_{1,2})$ at two surface pressure values (15 and 23 $mN \cdot m^{-1}$), where $A_{1,3}$, $A_{1,2}$ and $A_{2,3}$ correspond to lipase activities measured with 1,3-*sn*-dicaprin, 1,2-*sn*-dicaprin, and 2,3-*sn*-dicaprin, respectively. n.m., not measurable under the experimental conditions.

	Surface pressure ($mN \cdot m^{-1}$)	V.I.	S.I.
SSL	15	0.288	0.180
	23	0.760	0.479
SAL	15	1	n.m.
	23	0.395	0.060

other lipases from group B (Rogalska *et al.*, 1995). Under all of these circumstances, SSL more efficiently hydrolyses dicaprin monomolecular films than SAL. Using 2,3-*sn*-dicaprin monolayers as a substrate, the maximal ratio of 20 was found between the catalytic activities of SSL and SAL, measured at their respective pH optima. It is worth noting that a ratio of about 60 was reached using emulsified tributyrin as a substrate (see Materials and Methods). With this latter substrate, the specific activities of 1000 and 15 U/mg were obtained with SSL and SAL, respectively (data not shown).

SSL and SAL have the same surface pressure threshold (10 $mN \cdot m^{-1}$) below which enzymatic activity is undetectable. It has been established that a low surface pressure corresponds to a high surface free energy. Also, proteins can unfold under such conditions (Mac Ritchie, 1978; Graham and Phillips, 1979). Pancreatic, gastric, and some microbial lipases have been shown to be irreversibly denatured at a low surface pressure (Gargouri *et al.*, 1995; Rogalska *et al.*, 1995).

As established previously (Rogalska *et al.*, 1995), various isomers of dicaprin can be used to quantify the stereospecificity and the regioselectivity of lipases. In this study, we measured, as a function of surface pressure, the hydrolysis rates of various isomers of dicaprin monomolecular films by SSL (Fig. 3A) and SAL (Fig. 3B). Both lipases clearly distinguish between all three of the isomeric forms of dicaprin within the whole surface pressure range that was investigated. Comparable results were obtained previously using some bacterial lipases, like *Pseudomonas fluorescens* lipase and *Pseudomonas glumae* lipase (Rogalska *et al.*, 1995).

To describe quantitatively the lipase preference for the distal versus adjacent ester groups (Table 1) of diglyceride isomers, we calculated the vicinity index (V.I.), defined by Rogalska *et al.* (1995), as follows:

$$V.I. = [A_{1,3}\text{-}1/2(A_{2,3}+A_{1,2})]/[A_{1,3}+1/2(A_{2,3}+A_{1,2})]$$

where $A_{1,3}$, $A_{1,2}$ and $A_{2,3}$ are lipase activities measured with 1,3-*sn*-dicaprin, 1,2-*sn*-dicaprin, and 2,3-*sn*-dicaprin, respectively (Table 1). The V.I. value was calculated at two arbitrary chosen values of the surface pressure 15 and 23 $mN \cdot m^{-1}$, respectively (Rogalska *et al.*, 1995).

According to the V.I. values that were obtained in the present study, it may be concluded that SSL and SAL prefer distal ester groups of the diglyceride isomers (1,3-*sn*-dicaprin). At a low surface pressure (15 $mN \cdot m^{-1}$), SAL presents an absolute preference for distal ester groups (Table 1).

Comparison of the catalytic activities, using 1,2-*sn*- and 2,3-*sn*- dicaprin at two surface pressure values (15 and 23 $mN \cdot m^{-1}$), gave an estimation of the lipase stereopreferences. The stereoselectivity index (S.I.) was calculated using the following definition (Rogalska *et al.*, 1995):

$$S.I. = (A_{2,3}\ A_{1,2})/(A_{2,3} + A_{1,2})$$

where $A_{2,3}$ and $A_{1,2}$ are lipase activities with 2,3-*sn*- and 1,2-*sn*-dicaprin, respectively (Table 1).

SAL had no detectable activity at 15 $mN \cdot m^{-1}$ (Fig. 3B). Thus, its S.I. cannot be calculated. A similar behavior was reported by Rogalska *et al.* (1995) using *Candida antarctica* B lipase.

As judged by the S.I., SSL is markedly stereoselective for the *sn*-3 position of the 2,3-*sn*-enantiomer of dicaprin as measured both at 15 and 23 $mN \cdot m^{-1}$ (Table 1).

In conclusion, our results show that despite the high similarity of the amino acid sequence of SAL (Simons *et al.*, 1996) and SSL (unpublished work), important differences were observed in the pH profile, the specific activity, and the stereoselectivity.

Acknowledgments We are grateful to Dr H. Mejdoub (ENIS) for fruitful discussions during the preparation of this work. Our thanks also go to Dr H. Chahinian, Dr F. Carriere, and Dr K. Abousalham for discussions and kind help, and I. DOUCHET for technical assistance (UPR 9025 du CNRS, Marseilles, France). We thank Mr. A. Hajji (ENIS) for his help in correcting the English. This work is part of a doctoral thesis by Adel Sayari. This work received financial support from the DGRST (99/UR/09-19) and DGRST-CNRS Project (00/R0904).

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