0273-2289/94/4402—0213$04.40

Contribution to the Study of the Alteration of Lipase Activity of *Candida rugosa* by Ions and Buffers

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Received April 27, 1993; Accepted August 5, 1993

ABSTRACT

A semipurified C. *rugosa* lipase (LS) has been prepared from commercial lipase (LC) using an economical procedure. The presence of sugars and glycopeptides has been detected in LS and LC. Pure lipase only has covalently bonded sugars. The hydrolysis of olive oil catalyzed by LS and commercial lipase (LC) is sensitive to the presence of cations Na(I), Mg(II), Ca(II), and Ba(II) and to the nature of buffer. Highest enzyme activity is obtained with 0.1*M* Tris/HCl buffers and the combination of NaCl 0.11*M* and $CaCl_2$ 0.11*M*. Fluorescence spectroscopy analysis of LC, LS, and both pure isoenzymes lipases A and B, was used to analyze the interaction of the lipase with these effectors. Inorganic cations Na or Ca do not interact with pure enzyme LA but do interact with LC and LS and do so slightly with LB. The organic cations (morfolinium or *tris*) interact with pure lipases. We postulate that the increase in the lipase activity produced by Na(I) or Ca(II) is related with interfacial phenomena, but the increase might be more specific in the hydrolysis of olive oil in the presence of Tris-HCl or morfoline-HCl buffer, owing to enzyme–buffer interaction.

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Index Entries: Lipase *C. rugosa;* influence of cations or nature of buffer on lipase activity; fluorescence spectroscopy.

Nomenclature: LC, commercial lipase; LS, semipurified lipase; LP, purified lipase; LA, lipase A; LB, lipase B.

INTRODUCTION

Biotechnological transformations catalyzed by enzymes have been extensively investigated from a synthetic organic chemistry point of view. In this way, lipases (EC 3.1.1.3) in general, and lipase from *Candida rugosa* in particular, have been used in the hydrolysis (*1,2*) and synthesis (*3,4*) of many organic compounds. Nevertheless, even in the simplest reaction, hydrolysis of olive oil, the fundamental aspects of the reaction mechanism, are not well understood. It is well known that the hydrolysis reaction takes place in the oil–water interface (*5,6*). Hence, the instantaneous rate and direction of the reaction depend on the concentrations of reactants within the surface phase. Using this finding, several kinetic models have been described in literature for the enzymatic activity (*5,7–9*), where a conformational change of a hydrosoluble protein is necessary—as a previous step—for the fixation of the protein to the interface. This conformational change has been observed in phospholipase A_2 and in some fungi lipases (*10,11*).

In this model, the observed increases in the enzymatic activity of the lipase from *Candida rugosa* induced by the presence of Na(I) and Ca(II) could be related to interfacial phenomena (*4,12,13*) but the inactivation produced by other cations such as Co(II), Mg(II), Hg(II), Cu(II), and Fe(III) is not understood (*1*). In the same way, the influence of organic solvents and fatty acids or alcohols has been analyzed from the point of view of an interaction of the organic molecules with the oil–water interface (*14–16*). As we know, the influence of the nature of the buffer on the enzymatic activity has not been analyzed.

Nevertheless, many of these experiments have been carried out with impure enzymes. The purification of *Candida rugosa* lipase type VII yields two isoenzymes, lipase A and B, with different kinetic parameters (*17,18*). Also, sugar molecules weakly bound to the protein play an important role in hydrolytic and synthetic activities of lipase from *Candida rugosa* (*4*). Finally, alternation of the enantioselectivity produced by chemical modification of the protein (*19*) and the nature of the active site (*20,21*), have been reported. All these new structural evidences let us revise our understanding of the interaction of lipase with ions, organic molecules, and so on.

In the present paper we analyze the interaction of several cations and buffers with commercial *Candida rugosa* lipase and a semipurified version of the enzyme in order to review the observed enzymatic activity of commercial lipase (LC). We show that alterations must be related with the direct interaction effector-pure lipase, effector-sugar or effector-glycopeptides.

MATERIALS AND METHODS

Lipase (EC 3.1.1.3) from *C. rugosa* (formerly *C. cylindracea*) (Type VII) and bovine serum albumin were obtained from Sigma Chemical Co. (St Louis, MO) (LC). Olive oil emulsion (50:50, v/v) was supplied by Merck Chemical (Darmstadt, Germany). All other chemicals used were of the purest grade available.

Enzyme Preparations

Four different enzyme preparations have been used in this work. Commercial lipase (LC), semipurified lipase (LS) was obtained by dialysis of the crude LC according to the procedure described by Sánchez-Montero et al. using a cutoff membrane of 20,000 (*4*). Purified lipases (LA and LB). Both isoenzymes were prepared according to the procedure described by Rua et al. (*17,18*).

Protein Measures

The amount of protein was determined by the colorimetric Hartree method (*22*). According to the electrophoretic analysis (data not shown), there are many glycopeptides (mol wt < 60,000) in commercial sample LC and some in LS (20,000 < mol wt < 60,000) but only one band is observed in LA and LB samples (*18*).

Enzyme Assays

Lipase activity was monitored in a Crisson pH-stat (Micrott 2022, Microbur. 3031 and Microstirrer 2038). In a typical experiment, 7 mL of 0.1*M* buffer solution (Tris-HCl, KH_2PO_4-NaOH, NA_2HPO_4-NaH_2PO_4 or Morfoline-HCl) and 1 mL of lipase solution (8 mg/mL) in the appropriate buffer solution was placed in the 37°C thermostated cuvet of the pH-stat. The mixture was vigorously shaken for 10 min at 37°C. Then 2 mL of olive oil (0.1137 mg/mL in 0.1*M* appropriate buffer solution) was added.

The hydrolysis of olive oil was carried out in the presence of the different buffers and ionic strength. Experiments were performed in triplicate and the maximum deviations from the mean were less than 5%. One unit of lipase activity is defined as the amount of enzyme needed to produce 1 μmol of oleic acid/h at 37°C.

Determination of Tryptophan

Spectroscopic determination of tryptophan in lipase from *C. rugosa* was measured by the Edelhoch methods (*23*).

The ratio of the numbers of tryptofan and tyrosine residues in a protein may be determined from the absorbances at 288 nm in neutral 6*M* guanidium chloride using the formula:

$$\text{Trp} / \text{Tyr} = A_{288^*1280} - A_{280^*385} / A_{280^*4812} - A_{288^*5690}$$

To a 0.8 mL of guanidium chloride 6*M* solution were added 200 μL of protein solution. The absorbance was measured in an IV-visible spectrophotometer Shimadzu 2100.

Fluorescence Assays

All fluorescence measurements were made at 37°C at 280 nm excitation and 330 nm emission, in a Perkin Elmer (Norwalk, CT) 3700 fluorescence spectrometer. The lipase solution was performed in 950 μL the 0.1*M* appropriate buffer, pH = 7.0 containing different concentrations of NaCl and/or $CaCl_2$. Then 50 μL of the lipase solution (0.5 mg/mL) were added. The concentration of enzyme in cuvet was 0.025 mg/mL, in all experiments.

Influence of the Nature of Buffer

The buffers used were Tris-HCl, KH_2PO_4-NaOH, Na_2HOP_4-NaH_2PO_4, or Morfoline-HCl 0.1*M*, pH = 7.0. The ionic strength of the medium was increased by addition of NaCl.

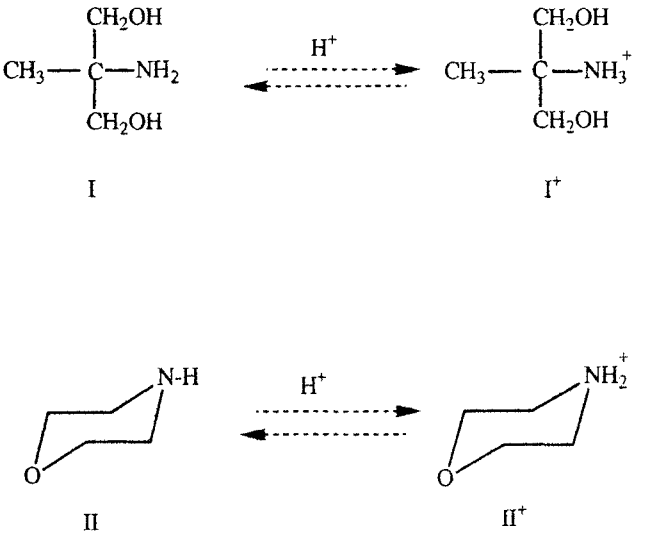
Influence of the Cations

The cations used were Ba(II), Ca(II), and CA(II)/Na(I). These assays are obtained with Tris-HCl buffer pH = 7.0.

RESULTS AND DISCUSSION

The hydrolysis of olive oil by commercial and semipurified lipases was carried out in presence and absence of NaCl. The hydrolysis experiments were performed using 0.8 mg of protein/mL (*see ref. 24*).

The optimum pH was 7.0 and the pH profile was the same in both enzymatic preparations LC and LS. This value is the same as that described in the hydrolysis of tributyrin in Sánchez-Montero et al. using commercial lipase (*4,25*) and by Rua (*17*) using pure lipase A and B. The presence of NaCl (0.154*M*) does not alter the optimum pH (as has been described elsewhere (*3,12,13*)), but increases the enzymatic activity from 213 U/mg to 749 U/mg. According to the traditional explanation of this finding, the presence of Na(I) favors the formation of hydrophilic fatty acid sodium salts that are removed from the interface to the water, letting new triglyceride molecules interact with the enzyme in the interface. This increases the "turnover" number enhancing the amount of triglyceride molecules hydrolyzed per unit of time, but this cannot be related to an effector-enzyme interaction. The presence of Na(I) also stabilizes the droplets of the microemulsion, favoring the interfacial phenomena. Nevertheless an other explanation could be analyzed according to the fluorescence spectra (*see* the Fluorescence Spectra section).



Scheme 1

In Fig. 1, we show the influence of the nature of buffer (pH = 7.0) at different ionic strengths—in lipase activity of commercial (LC) (Fig. 1A) and semipurified (LS) (Fig. 1B) lipases. The greatest lipase activity was obtained with Tris-HCl buffer. Both phosphate buffers (obtained with different initial compounds KH_2PO_4-NaOH or Na_2HOP_4-NaH_2PO_4) show similar profiles for the enzymatic activity of LC. The morfoline-HCl buffer curve lies between Tris-HCl and phosphate buffers curves, in LC. For both LC and LS, the enzymatic activity in Tris-HCl buffer is greater than in the phosphate buffer. Therefore we can conclude that the nature of buffer plays a role in the hydrolysis of olive oil. In the literature only the use of phosphate buffer pH = 7.0 (*4,19,26*), pH = 8.0 (*13*), and pH = 7.7 (*7*) is described and no one has studied other buffers that, in fact, enhance the activity. From the results showed in Fig. 1 we can deduce a strong influence of the nature of the buffer ions on enzymatic activity. A possible explanation would be that the protein is negatively charged in the reaction conditions (isoelectric points of lipase A and lipase B are 5.0 and 5.8, respectively) (*17*). Thus the interaction with the buffer cations would be different according to the nature of this cation. The organic cations from Tris and morfoline buffer (more lipidic) (Scheme 1) at the same concentration would interact better with the protein than Na(I) and Ca(II) (more hydrophilic) because hydrophobic and electrostatic interactions would occur in the first case and only electrostatic ones in the case of Na (I), Ca(II), and so on.

It is well known that the presence of Na(I) or Na (I) and Ca(II) in the medium increases the enzymatic activity of both native and immobilized lipase from *Candida rugosa* on agarose and alumina (*13,27,28*). The results obtained in our work are shown in Fig. 2. All the experiments were carried out in 0.1*M* Tris-HCl, pH = 7.0 to avoid precipitation of divalent cations with phosphate anions.

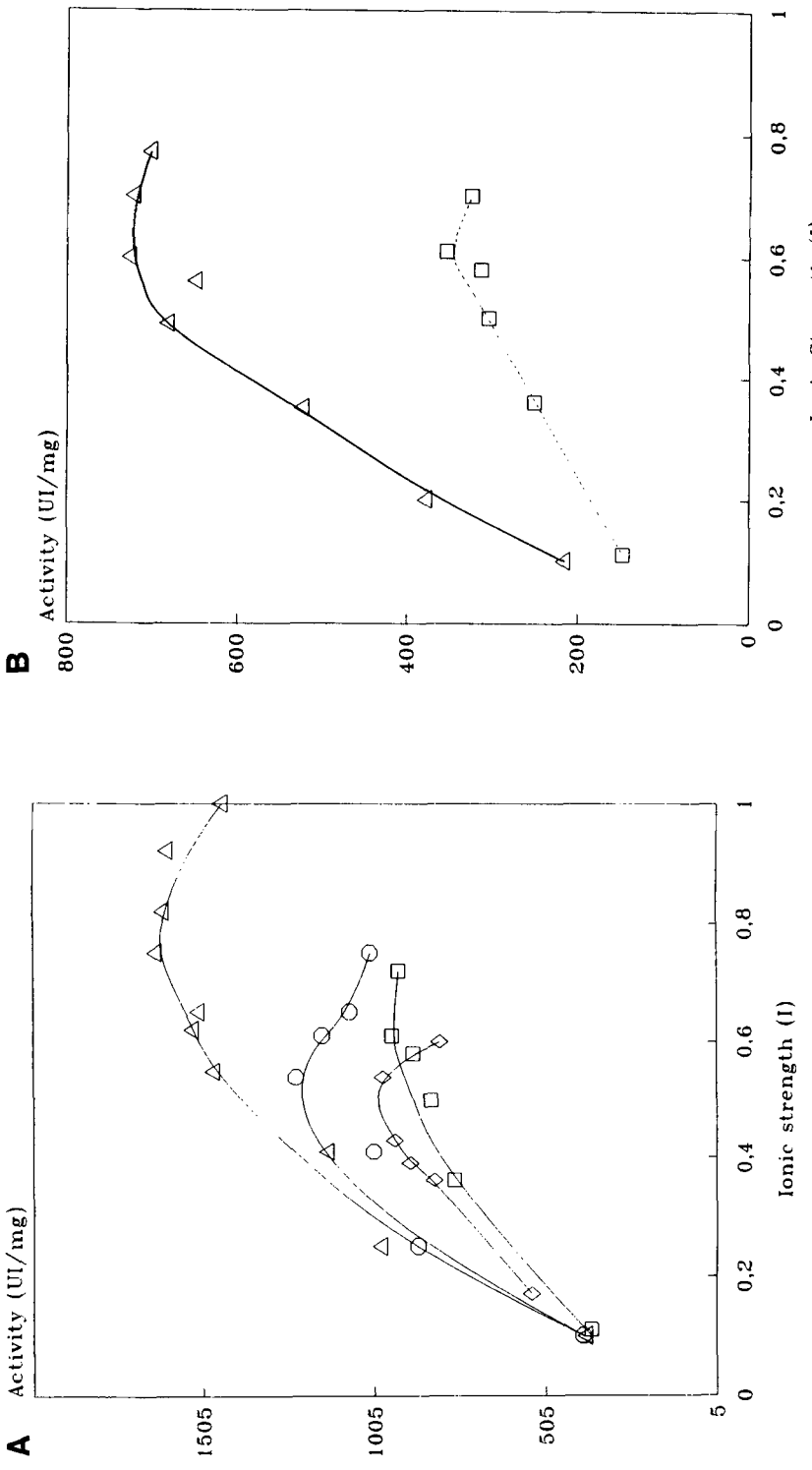


Fig. 1. Influence of the nature of the buffer 0.1*M*, on the lipase activity at 37°C and pH = 7.0 at different ionic strength. This parameter was increased by addition of NaCl; (A) Commercial lipase: 1. ◇ KH_2PO_4/NaOH; 2. ○ Morfioline/HCl; 3. Δ Tris/HCl; 4. □ Na_2HOP_4/NaH_2PO_4. (B) Semipurified lipase 1. Δ Tris-HCl; 2. □ Na_2HOP_4/NaH_2PO_4.

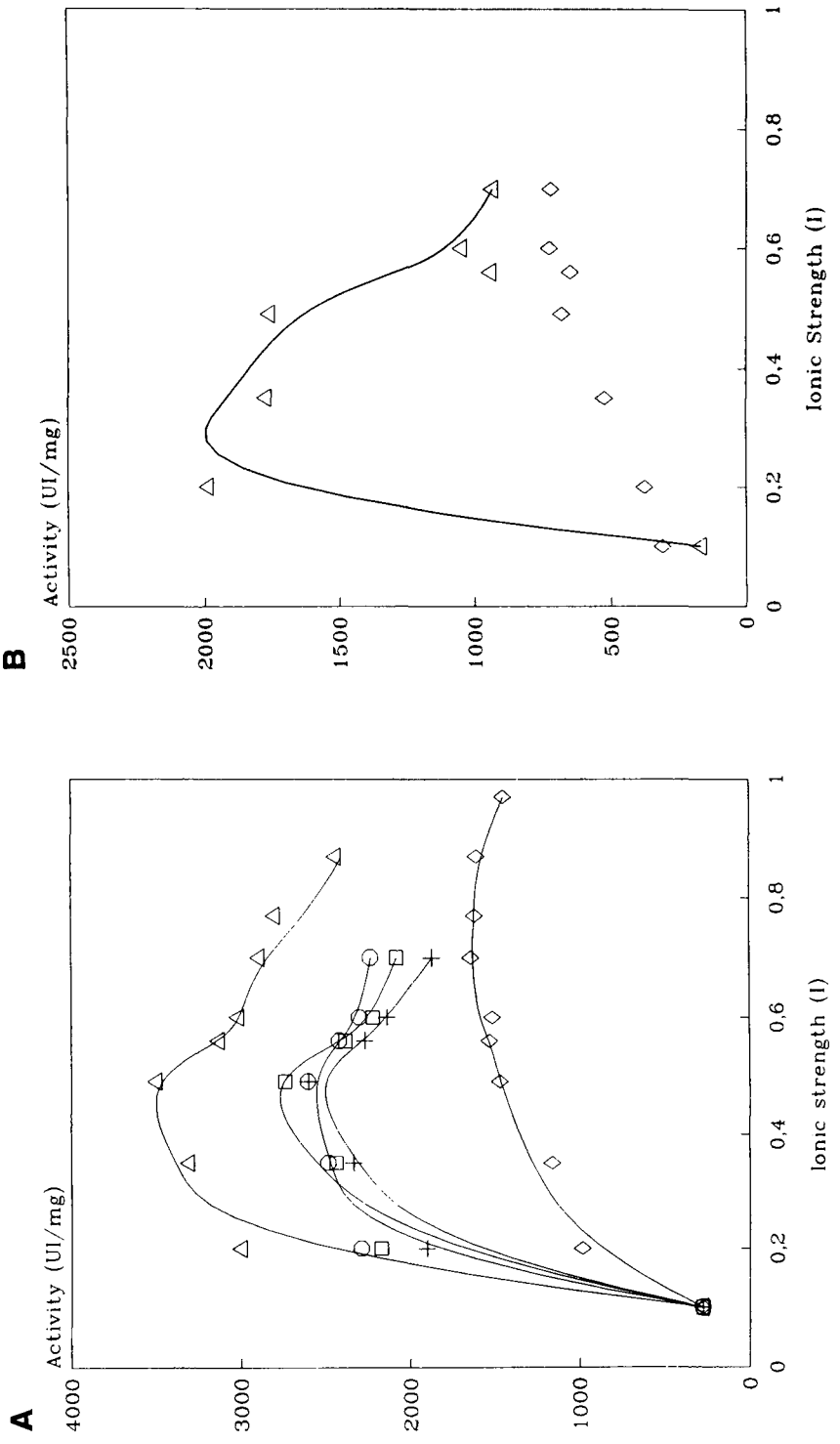


Fig. 2. Influence of the nature of cations and ionic strength in the enzymatic activity at 37°C and pH = 7.0 (The different cations are added in increasing concentrations): (A) Commercial lipase 1. ◇ NaCl; 2. □ $MgCl_2$; 3. + $CaCl_2$; 4. ○ $BaCl_2$; 5. Δ NaCl/$CaCl_2$. (B) Semipurified lipase 1. ◇ NaCl; 2. Δ NaCl/$CaCl_2$.

We observed—in both cases LC and LS—that the presence of divalent cations improves the lipase activity vs monovalent Na(I). No differences are observed when Mg(II), Ca(II), or Ba(II) are used with LC. So this effect is related to the charge of the cation and not to the volume or to the electronic field of the ion. A reasonable explanation of this finding, working at the same ionic strength, could be that each divalent cation can remove two fatty acid molecules from the interface, and each Na(I) only one but other effects, such as the stabilization of the interface, cannot be rejected. This difference would increase the substitution rate of free fatty acid molecules produced in the hydrolysis, by new triglyceride molecules, increasing the turnover number and thus, the specific enzymatic activity. Owing to the dilute conditions of our experimental model, the influence of the different solubility of fatty acid salts was not observed.

When both Na(I) and Ca(II) (NaCl 0.11*M* and $CaCl_2$ 0.11*M*) are added, the enzymatic activity of LC and LS increases (Fig. 2). This finding could be related to the stabilizing effect on micellas produced by Na(I) and to the removing of two fatty acid molecules from the interface to water by Ca(II). We have confirmed this possibility: The presence of both cations—0.2*M* NaCl and 0.003*M* $CaCl_2$—increases k_{cat} but not K_m (*27*). Otero et al. (*13*) also reported that the presence of Ca(II) increases the specific hydrolysis rate of monoglyceride to glycerol and fatty acid. Therefore, we could conclude that the effect of cations is not related to an enzyme–effector interaction but to an interfacial phenomenon. This affirmation will be discussed below.

The observed diminution of the enzymatic activity at high ionic strength must be related to the alteration of the water–oil interface and of the interaction "enzyme-interface." This effect has been observed with all lipases studied in the paper.

FLUORESCENCE SPECTRA

Fluorescence spectroscopy is a very sensitive and selective method to analyze the interaction of the protein with several effectors. Thus, the fluorescence spectra of commercial lipase (LC) and semipurified lipase (LS) have been recorded in the experimental conditions indicated above. These spectra were compared with those of pure A and B lipases obtained in the same experimental conditions. All the spectra were recorded with (Enz) = 0.025 mg/mL, in order to work in the linear region intensity/(Enz). Taking into account the percentage of protein in the different samples, four initial solutions were prepared:

1. LC (3% protein) = 16.5 mg sampl/mL = 0.495 mg Enz/mL
2. LS (13.3% protein) = 3.815 mg sampl/mL = 0.509 mg Enz/mL
3. LA (100% protein) = 0.500 mg Enz/mL
4. LB (100% protein) = 0.500 mg Enz/mL

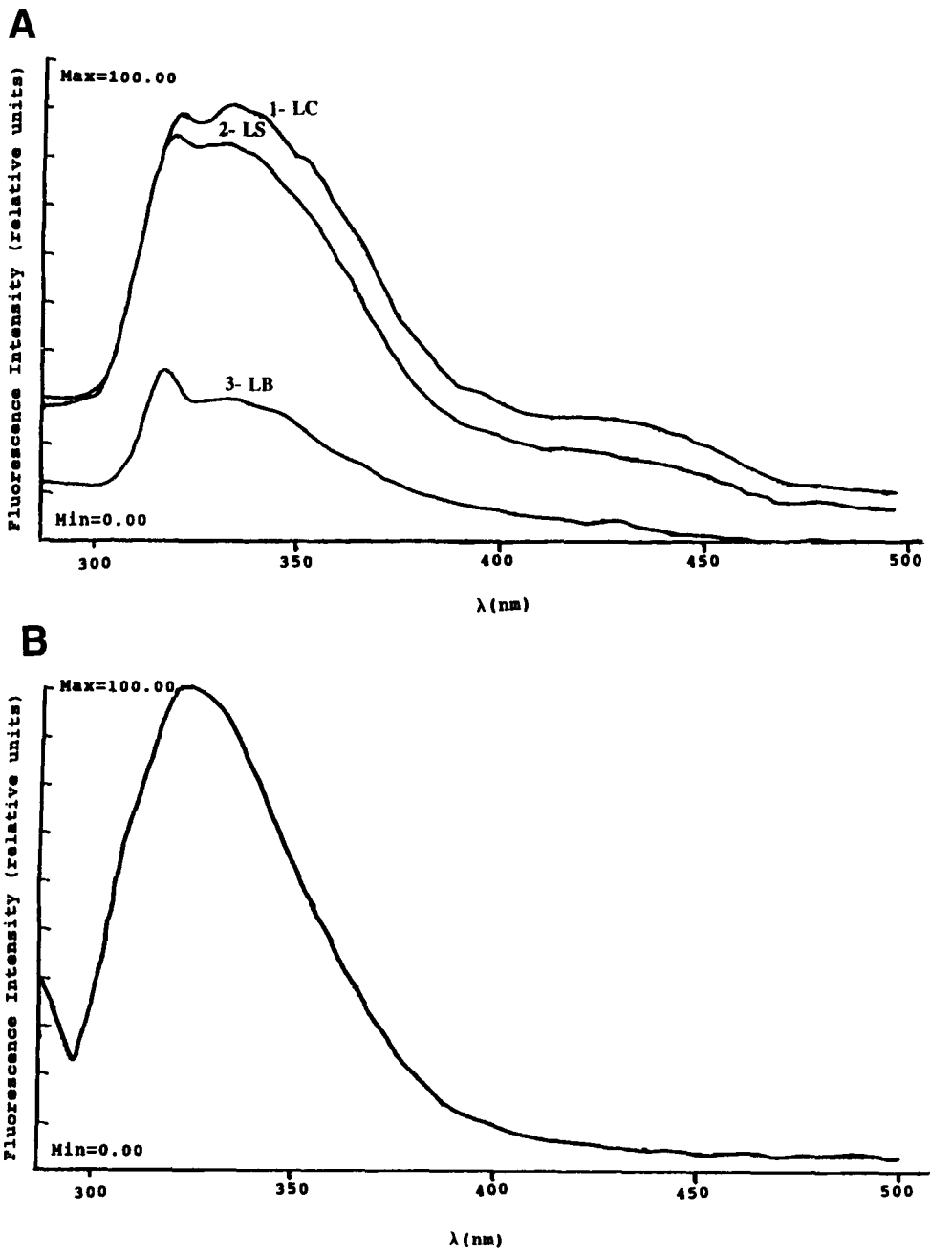


Fig. 3. Emission fluorescence spectrum of lipase of *Candida rugosa* at pH = 7.0 (Tris/HCl buffer), 37°C and λ_{Ex} = 280 nm. (A) Lipase samples: 1. commercial lipase (F = 0.2); 2. semipurified lipase (F = 0.2), and 3. pure lipase B (F = 0.2). (B) Lipase sample: pure lipase A (F = 0.5).

One hundred and fifty microliters were taken of these solutions and introduced in the fluorescence cuvet (3 mL total volume).

In Fig. 3A the emission fluorescence spectra of LC, LS, and LB are compared at the same experimental conditions (Tris-HCl buffer 0.1*M*) and the same scale factor F = 0.2. The fluorescence spectra of LA was

recorded at F = 0.5 in order to increase the spectrum. The main difference among these three spectra is the emission intensity: Those for LC and LS are larger than those for pure LB. This is directly related to the different number of Trp detected for the three samples. The number of Trp was determined by the Edelhoch method (*23*). Owing to the experimental error an interval was obtained: LC = 29 ± 3; LS = 24 ± 3 and LA = LB = 7 ± 2. The reported data in the literature are 5 Trp (*21*) and 6 Trp (*29*).

On the other hand, the emission spectrum of LA (F = 0.5), shown in Fig. 3B, is quite different to that of LB (F = 0.2) (Fig. 3A). In particular, the emission band of Tyr at 311 nm is not observed in LA. According to the results reported (*17*), the number of Tyr is slightly different in LA (14 Tyr) and LB (17 Tyr) (20 Tyr for global lipase (*20*)) or 5 Trp (*21*) or 6 Trp (*29*), but this small difference is not sufficient to explain the discrepancies observed between the two spectra. This could be explained assuming that all Tyr of LA are quickly quenched by energy transfer to Trp. This quenching is less intense for LB, so the Tyr band at 311 nm could be observed. The obvious conclusion is that the two isoenzymes have slightly different structures, which is in accordance with the differences observed in their enzymatic activities (*13,18*). Besides, their emission spectra differ. This leads us to believe that the structure of these isoenzymes is slightly different, as has been reported by Otero et al. (*13*). These authors reported different enzymatic activity for LA and LB. LA has a maximum at 320–325 nm and LB has a broader band. This behavior may be a result of the existence of two kinds of Tyr in LB—one buried in the enzyme structure (γ = 320–330 nm) and another exposed to the aqueous phase (γ = 350 nm).

In Fig. 4 we show the influence of the nature of the buffer on the fluorescence spectra of LC (Fig. 4A) and LS (Fig. 4B). We found that the Trp fluorescence depends on the nature of the buffer. In order to determine whether this alteration in fluorescence spectra is a result of a conformational change of the pure enzyme or glycopeptides with a molecular weight greater than 20,000 kDa and present in the commercial preparation (*17,18*), we recorded the fluorescence spectra of pure LA (Fig. 4C) and LB (Fig. 4D) in the same condition. We can observe that the fluorescence spectra of pure lipases change with the nature of the buffer. Thus we could conclude that the alteration in the enzymatic activity (LC or LS) observed when the nature of the buffer changes (Fig. 1) could be related to alteration in the conformation glycopeptides, but no of pure LA and LB or to alterations in the interaction commercial enzyme-interface produced by the presence of different cations and/or anions in the interface. On the other hand, we can observe that the LA spectrum (Fig. 4C) is less sensitive to the nature of the buffer than that of LB (Fig. 4D). The maximum of the fluorescence spectra of LA does not change with the nature of the buffer. Only a slight hyperchromic effect (Tris-HCl buffer) and a small hypochromic effect (Morfoline-HCl buffer) is observed versus the case of phosphate buffer. Therefore, we conclude that the buffer ions produce a bouring (Tris-HCl

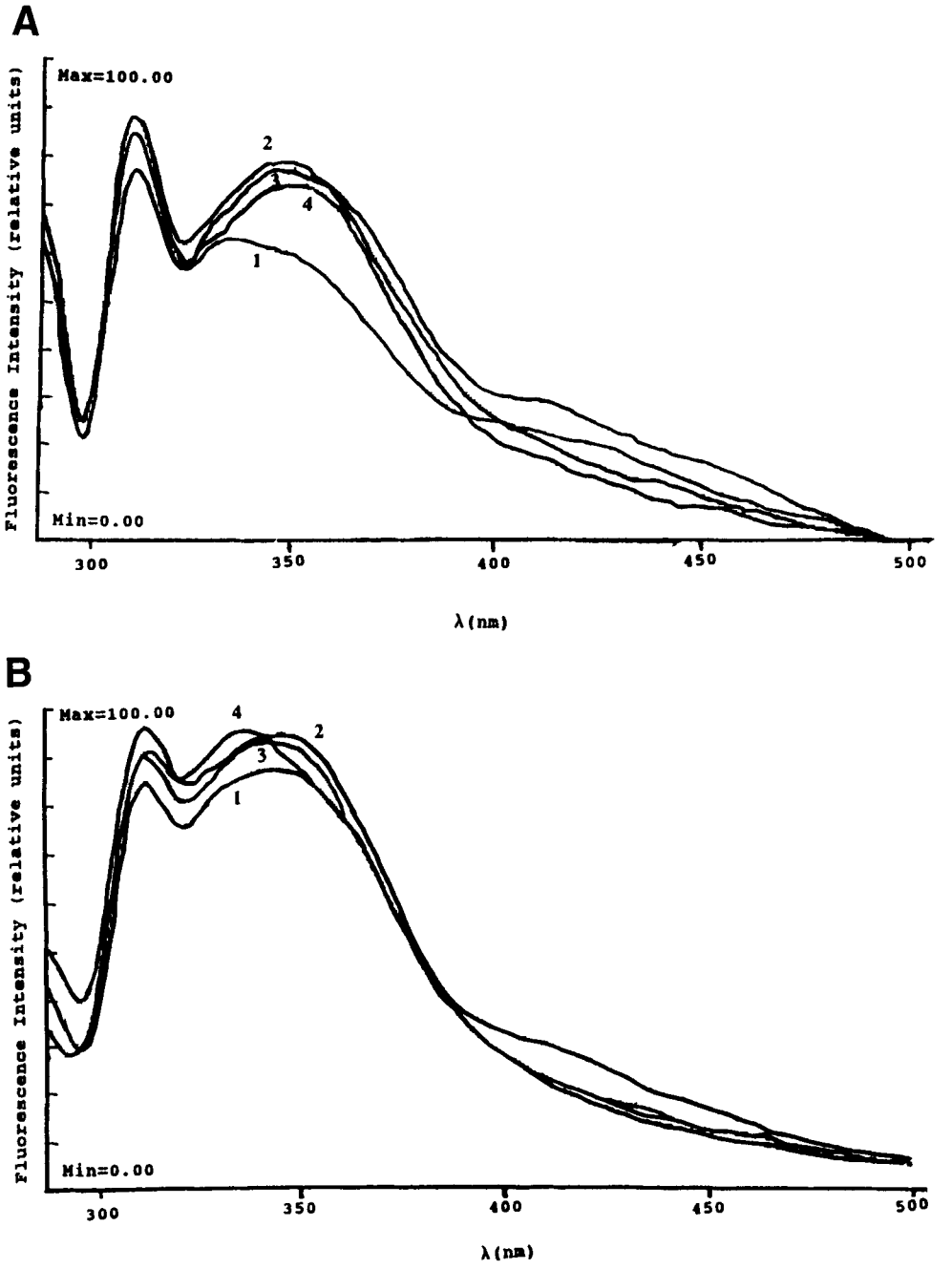


Fig. 4. Influence of buffers nature in emission fluorescence spectra of different lipase of *Candida rugosa* samples at pH = 7.0, 37°C, λ_{Ex} = 280 nm and 0.1*M* NaCl. (A) Commercial lipase: 1. Morfoline/HCl; 2. Na_2HPO_4/NaH_2PO_4; 3. Tris/HCl; and 4. KH_2PO_4/NaOH. (B) Semipurified lipase: 1. Morfoline/HCl; 2. Na_2HPO_4/NaH_2PO_4; 3. Tris/HCl; and 4. KH_2PO_4/NaOH.

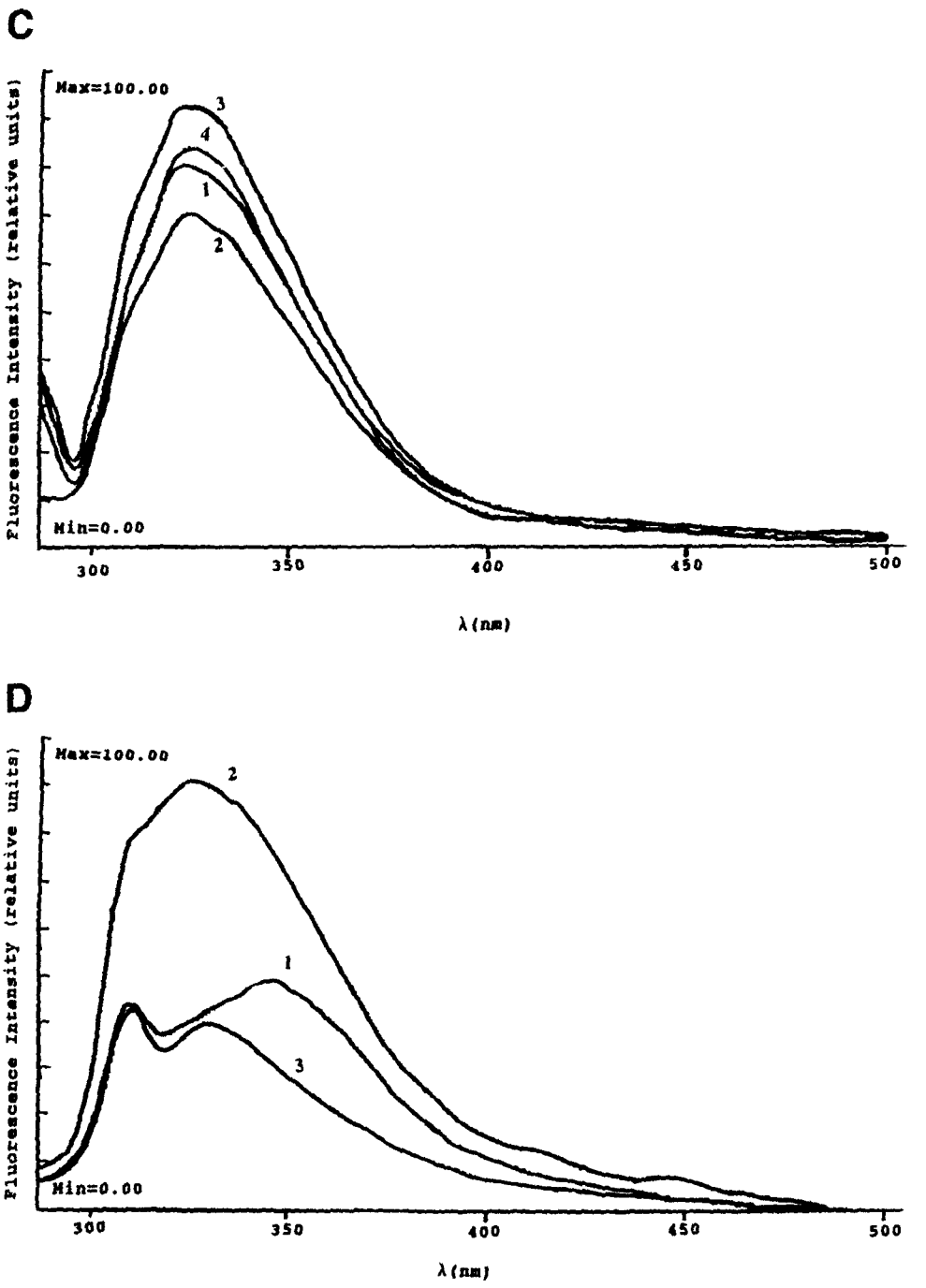


Fig. 4. (*cont'd*) (C) pure lipase A: 1. Morfoline/HCl; 2. Na_2HPO_4/NaH_2PO_4; 3. Tris/HCl and 4. $KH_2PO_4/NaOH$, and (D) pure lipase B: 1. Morfoline/HCl; 2. Na_2HPO_4/NaH_2PO_4; 3. Tris/HCl.

buffer) or an exposure to the polar medium (H_2O of the interface) of Trp but not an alteration in the dielectric constant of the microenvironment surrounding the Trp. On the contrary, strong changes are observed in the fluorescence spectra of LB depending on the nature of the buffer. Taking phosphate buffer as a reference, Tris-HCl buffer produces a strong hypochromic effect and a slight batochromic effect that must be related to the exposure of Trp to a more polar medium. On the other hand, the Morfoline-HCl buffer produces, as does the Tris-HCl buffer, not only a strong hypochromic but also a bathochromic effect as a consequence of the exposure of Trp to a very polar medium.

The spectra of LC, LS, and LB in the presence of Na(I), Ca(II), and Na(I) + Ca(II) vary (Fig. 5). If we compare the influence of Na(I) and Ca(II) in the fluorescence spectra of LA (Fig. 5C) and LB (Fig. 5D) with those of LC (Fig. 5A) and LS (Fig. 5B) we can see that there is no influence of cations on the fluorescence spectrum of LA (Fig. 5C), but in LB only at large concentrations of both Na and Ca cations (Fig. 5D). Therefore, we can conclude that, at the ratio of (Enz)/(cation) analyzed by us, there are no detectable conformational changes of LB or LA produced by Na(I) or Ca(II). This finding could be explained by the fact that both isoenzymes are negatively charged at pH = 7.0 (isoelectric point 5.0 for LA and 5.8 for LB (*17*)) with Na(I) as contraion and the variation of ionic strength is too small to produce electrostatic field alteration. This spectra supports the hypothesis indicated above that Na(I) and Ca(II) interacts with the olive oil–water interface and not directly with the pure LA and slightly with LB.

In contrast, LC (Fig. 5A) and LS (Fig. 5B) show greater alteration of the emission in the fluorescence spectra than LA and LB. Owing to the fact that the fluorescence spectra were recorded at the same protein amount according to the purity data, we could conclude that these qualitative variations (Fig. 5) should be related to the alteration of small glycopeptides (mol wt < 60,000) that have Trp according to the Trp determination (29 ± 3 in LC and 7 ± 2 in pure La and LB). These small molecules will be located in the microenvironment of the lipase and will strongly interact with the medium. Evidently, these changes should be attributed to the conformational changes of glycopeptides (with Trp Fig. 5) that are placed outside the protein. These glycopeptides interact with the interface and with the water and the ions, owing to the presence of sugar that makes them very hydrophylic.

Therefore, the different experimental conditions used in literature: e.g., only Na(I) (*4*), 0.01*M* $CaCl_2$ + 0.4*M* NaCl (*28*), 0.00375*M* $CaCl_2$ + 0.137*M* NaCl (*30*), or 0.005*M* $CaCl_2$ + 0.1*M* NaCl (*31*) and the conditions shown in Fig. 1, must be homogenized in order to have the reference conditions because the presence of ions alters the turnover number or the specific enzymatic activity (μmol olive oil/mg Enz·min). Nevertheless, this alteration is not related to a conformational change of the pure enzyme, because

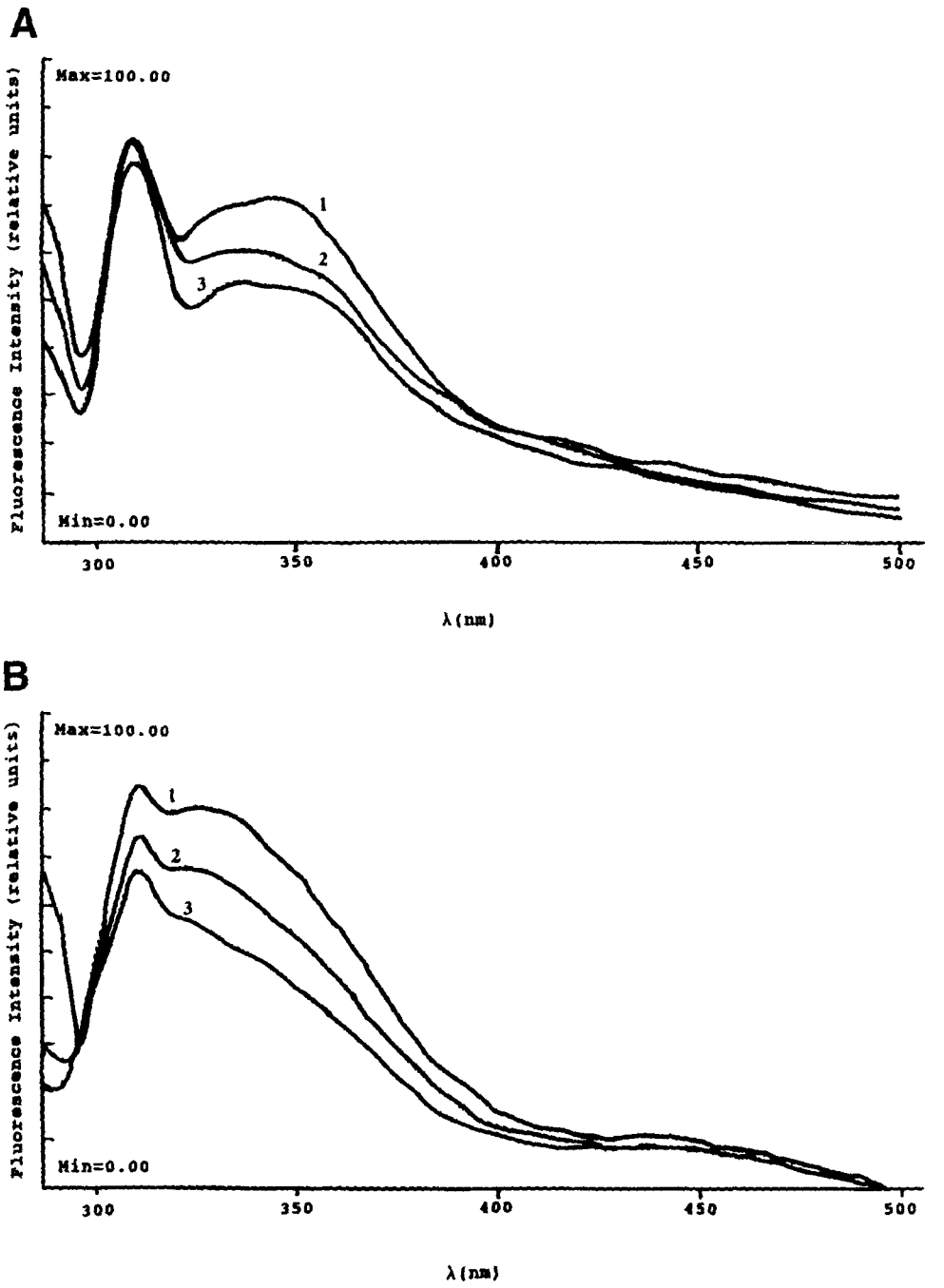


Fig. 5. Influence of cations (Na^+ and Ca^{2+}) concentration in emission fluorescence spectra of different lipase of *Candida rugosa* samples at pH = 7 (Tris/HCl 0.1*M* buffer), 37°C and λ_{Ex} = 280 nm: (A) Commercial lipase: 1. [NaCl] = 0.445*M*; (2) ($CaCl_2$) = 0.178*M* and 3. (NaCl) = ($CaCl_2$) = 0.11*M*. (B) semipurified lipase 1. (NaCl) = ($CaCl_2$) = 0.00*M*; 2. (NaCl) = 0.10*M* and ($CaCl_2$) = 0.005*M* and 3. (NaCl) = 0.01*M* and ($CaCl_2$) = 0.4*M*.

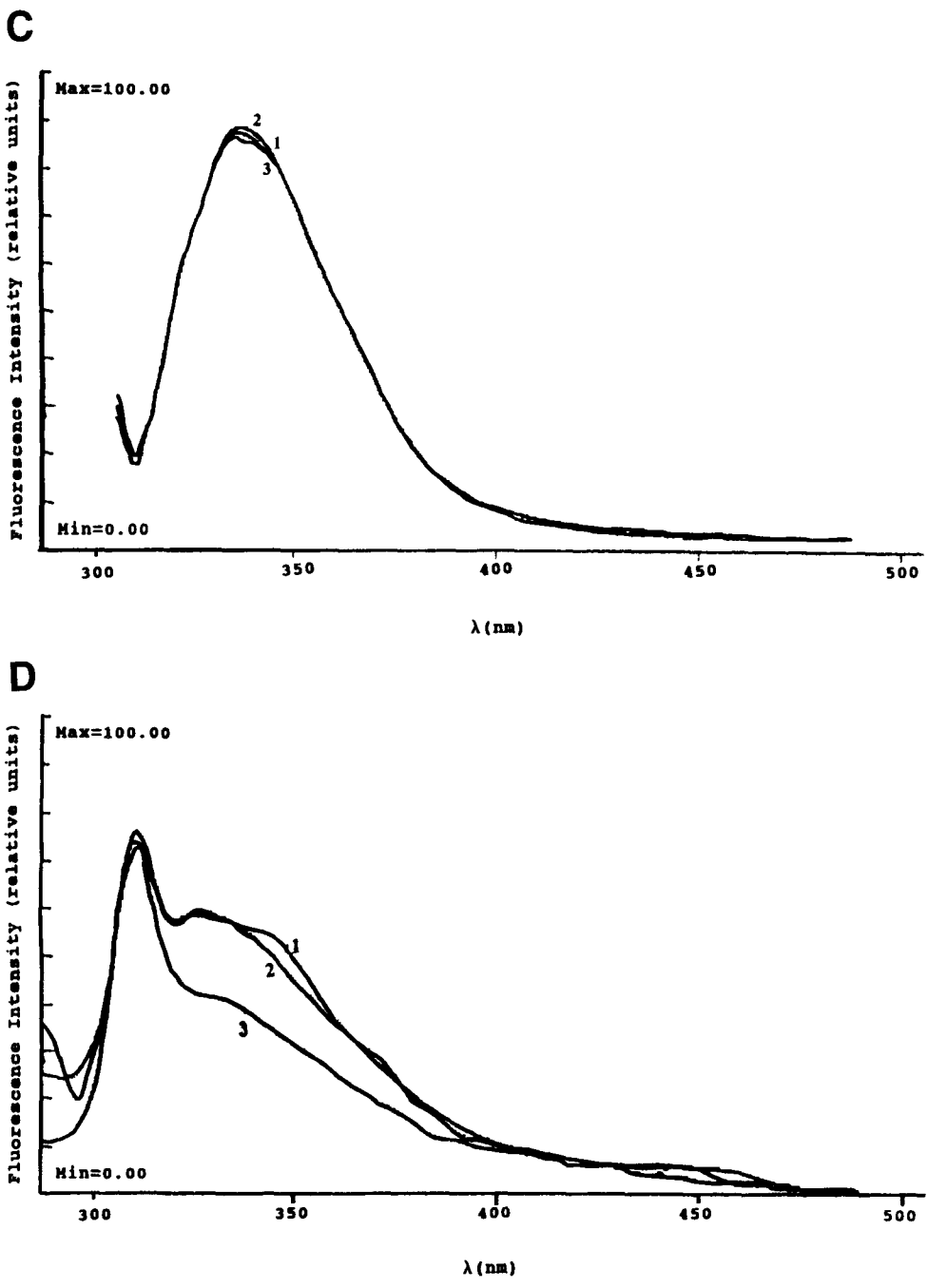


Fig. 5. (*cont'd*) (C) pure lipase A: 1. (NaCl) = 0.445*M*; 2. ($CaCl_2$) = 0.178*M* and 3. (NaCl) = 0.11*M* and ($CaCl_2$) = 0.11*M*. (D) pure lipase B 1. (NaCl) = ($CaCl_2$) = 0.00*M*; 2. (NaCl) = 0.1*M* and ($CaCl_2$) = 0.005*M* and 3. (NaCl) = 0.4*M* and ($CaCl_2$) = 0.01*M*.

LB and LA spectra are almost insensitive to the presence of the ions in a large interval. Thus, we can conclude that the increase of the enzymatic activity—observed in LC and LS—because of the increase in ionic strength, is related to a small alteration of peptides that impurify LC and LS that could favour the enzymatic activity or the enzyme-interface interaction and is also related to the interfacial interaction of the fatty acid molecule with the cation. This fact is not related to the interaction of the pure protein with the cation.

ACKNOWLEDGMENT

This work has been supported by the Ministerio de Educación y Ciencia of Spain (Grant PB-090-010-02).

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