Specific Anion Effects on Enzymatic Activity in Nonaqueous Media

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The present work shows that salt anions affect the activity of *Pseudomonas cepacia* lipase both in aqueous and in nonaqueous media (NAM) according to a Hofmeister series. The biocatalytic assay in water was the hydrolysis of *p*-nitrophenyl acetate, whereas the esterification between 1-hexyl-β-D-galactopyranoside and palmitic acid was followed in an organic solvent. The solid lipase preparations to be used in NAM were obtained through lyophilization in the presence of concentrated solutions of Hofmeister salts (Na_2SO_4, NaH_2PO_4/Na_2HPO_4, NaCl, NaBr, NaI, NaSCN). Salts affect enzyme activity in organic media through two mechanisms: (1) enzyme protection during lyophilization; (2) enzyme activation during the reaction. At least in our case, the latter seems to be more important than the former. The decrease of the activation energy caused by the stabilization of the transition state due to "kosmotropic" anions might be the driving force of enzyme activation. According to the most recent findings, dispersion forces may be responsible of specific anion enzyme activation/deactivation in NAM.

1. Introduction

The observation that enzymes can work in nonaqueous media[1] (organic solvents, supercritical fluids, solvent-free media, and ionic liquids) has opened up the possibilities of new synthetic routes so creating a new area of biotechnology. In these media, in the presence of low amounts of water the equilibrium of hydrolysis reactions can be reversed so that hydrolytic enzymes can catalyze esterification and transesterification reactions (alcoholysis, acidolysis, interesterifications).[2] Enzyme activity in nonaqueous media (NAM) is usually low in comparison with that in conventional water media.[3,4] For this reason big efforts have been performed in order to decrease the activity gap between aqueous and nonaqueous media. Lyophilization of enzymes dissolved in concentrated salt solutions produces a very active preparation (up to 10^4 times more than salt-free preparation) for reactions in NAM.[5] The activity of subtilisin Carlsberg was correlated to the "so-called" salt kosmotropicity through the Jones−Dole *B* coefficient.[6] The same coefficient was used by Zhao to correlate the enzyme activity of aqueous solutions of hydrophilic ionic liquids.[7] In fact, the *B* coefficient measures how much a specific salt increases or decreases the bulk viscosity η of dilute water electrolyte solutions with concentration c according to the Jones−Dole equation:[8]

$$\eta/\eta_0 = 1 + Ac^{1/2} + Bc \qquad (1)$$

where η_0 is the viscosity of pure water and A is a constant related to the long-range interactions. The term $Ac^{1/2}$ is predominant in very dilute solutions. The coefficient B is related to the interaction between the ions and the solvent and is interpreted as a measure of the structure-forming and structure-breaking capacity of an electrolyte in solution. Equation 1 is valid up to a few tenth molar.[9] Thus, although the correlation with enzyme activity in NAM follows the order of kosmotropicity established by the *B* Jones−Dole coefficient, some other parameters (i.e., surface tension increment, lyotropic number, polarizability)[10] might have been used for the same purpose. Indeed, all them are related to the ubiquitous Hofmeister or specific ion effects in aqueous media.[10] These range over a whole gamut of phenomena starting with the original specific ion dependence of protein precipitation experiments of Hofmeister,[10,11] now including the Born energy of ions,[12] activity coefficients of electrolytes,[12] salt dependence of interfacial tensions,[13] specific ion dependence of pH measurements in buffers,[14] colloidal interactions, enzymatic activity in aqueous media,[15−17] and growth rates of microorganisms.[18]

One explanation for Hofmeister effects associates the phenomena with changes in bulk water structure induced by ions (ionic hydration).[19−22] Then ion specificity is associated with electrolyte-induced changes in water structure that depend on the capacity of ions to form (kosmotropic), or to break (chaotropic) hydrogen bonds. Such correlations are adduced from an intuition that derives from, and makes sense in, a standard theory that admits only electrostatic and hydration forces between ions and water. In more recent approaches,[23,24] it has been shown that standard theories are deficient in that they omit nonelectrostatic, ion-specific electrodynamic fluctuation (called for brevity dispersion or NES) forces. When these forces are included consistently, specific ion effects emerge even for the continuum solvent approximation. When, for water, the continuum solvent approximation is relaxed, electrostatic, hydration, and dispersion forces all conspire together to give a more complicated picture that is still being elucidated theoretically. The attribution of Hofmeister effects exclusively to bulk, or to surface, effects disappears. Electrostatic and dispersion forces work together to induce local water structure around ions (kosmotropy, chaotropy), and the interactions, long- and short-range, between such "dressed" ions are reflected in bulk

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properties like activities.[25] Similarly, at an interface, whatever the ion-specific water structuring effects are that show up in activities or viscosities, surface-induced water structure changes due to the electrostatic and dispersion interactions of "dressed" ions also occur. The dispersion forces and their interplay with water have to be treated consistently at the same level as the forces due to the classical electrostatic potential.[24] In any event ions are specifically adsorbed at interfaces, the strength of the adsorption potentials depending on the ion excess dynamic polarizabilities and the dielectric properties of the interface.[23] The effect is stronger for anions since they are more polarizable than cations.

Specific ion effects on enzymatic activity in aqueous medium have been known for more than 40 years.[26,27] Recently, we gave some evidence that the direct interaction of anions with the enzyme surface and active site, rather than a water structure change, may be responsible of enzyme activation/deactivation.[16,17] Some very recent studies seem to agree with this interpretation.[28] Ions affect only the first hydration shell; they neither enhance nor weaken the hydrogen-bond network. Thus, bulk water structure is not likely to be a relevant factor.[29−31] In summary, Hofmeister effects with proteins are likely to find at least partial explanation in interactions of the ions with the macromolecule surface and its first hydration shell.[32]

In the present work we report a comparison of the catalytic behavior of *Pseudomonas cepacia* lipase both in water and in a nonaqueous medium. In the latter case, even in the presence of very low amounts of water a Hofmeister effect seems to emerge in ordering the enzyme activity of salt−enzyme lyophilized preparations.

2. Materials and Methods

2.1. Chemicals. Lipase AH, a commercial preparation of *P. cepacia* lipase (E.C. 3.1.1.3), was purchased from Amano Enzymes (Nagoya, Japan) Sodium phosphate monobasic anhydrous (98%), sodium phosphate dibasic anhydrous (>99%), sodium sulfate (99%), sodium thiocyanate (98%), and stearyl alcohol (99%) were from Sigma-Aldrich (Milan, Italy). Sodium bromide (>99%), sodium iodide anhydrous (>99%), palmitic acid (98%), and magnesium chloride hexahydrate were from Acros Organics (Geel, Belgium). Sodium chloride (>99.5%), acetonitrile (HPLC grade), and molecular sieves (3Å) were from Merck (Darmstadt, Germany). 2-Methyl-2-butanol (99%) and 1-butanol (99%) were from Fluka (Milan, Italy). 1-Hexyl-β-D-galactopyranoside (99%) and 6-palmitoyl-1-hexyl-β-D-galactopyranoside (99%) were synthesized and purified in the research laboratory of Dr. Vladimir Mastihuba (Institute of Chemistry, Slovak Academy of Science) and characterized by Dr. Maria Mastihubová (Institute of Chemistry, Slovak Academy of Science).

2.2. Preparation of Salt Solutions in Phosphate Buffer (20 mM, pH 7). A weighed amount of the salts (Na_2SO_4, NaH_2PO_4, Na_2HPO_4, NaCl, NaBr, NaI, NaSCN), previously dried overnight at 110 °C, was dissolved in 10−15 mL of 20 mM sodium phosphate buffer pH = 7.00. The pH was set to 7.00 by adding a suitable volume of 20 mM Na_2HPO_4 solution and 20 mM sodium phosphate buffer (pH 7.00) to the final volume (25 mL). The pH measurements of the buffer and buffer/salt solutions were performed by using a pH-526 WTW pH meter, equipped with a SenTix pH glass electrode. All readings were made at 298 K. All the solutions were performed by using distilled water purified through a Millipore system (Simplicity 185) having a conductivity <0.054 μS cm^{-1}.

2.3. Enzymatic Activity Measurements in Aqueous Medium. The lipase-catalyzed hydrolysis of *p*-nitrophenylacetate (*p*-NPA) to *p*-nitrophenol was used to determine the enzymatic activity.[17] A typical experiment was performed by using an amount of 200 μL of a 50 mM *p*-NPA solution in 2-propanol that was mixed with 1.75 mL of buffer solution (20 mM) with or without salt. The reaction was started by adding 200 μL of 0.5 mg/mL lipase AH solution.

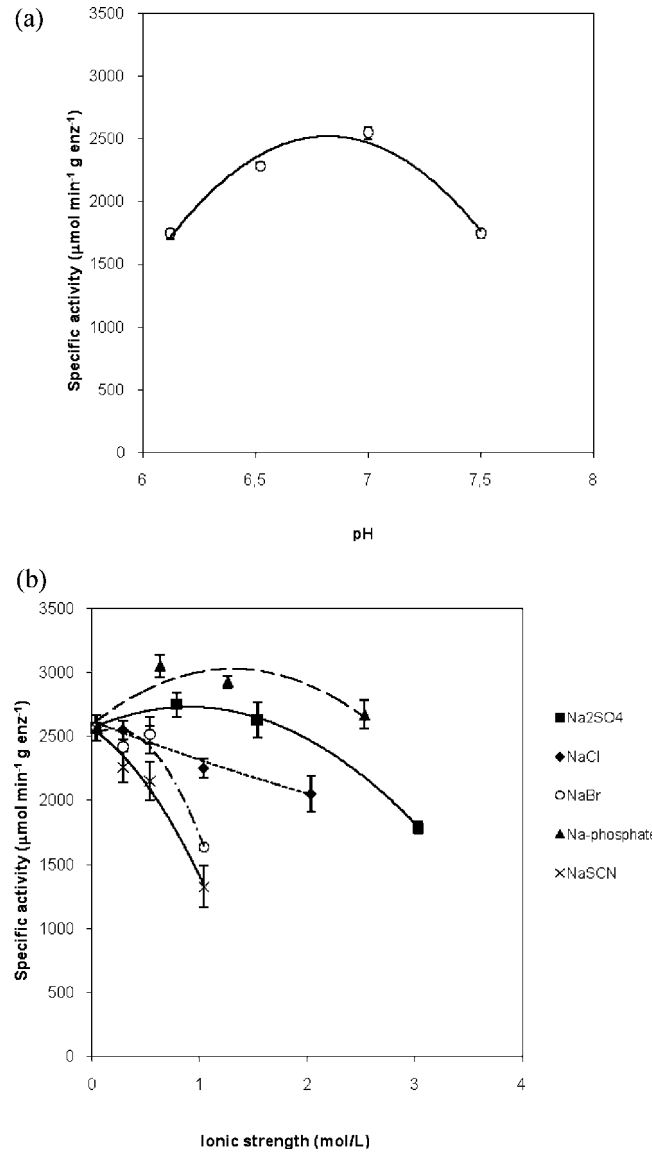


Figure 1. Behavior of *P. cepacia* lipase hydrolytic activity toward pH changes (a) and ionic strength obtained with different salts (b).

Spectrophotometric measurements were carried out after a determination of calibration lines of *p*-nitrophenol absorbance versus concentration, at all pH values, and for all salt types and concentrations. Each calibration was performed by reading the absorbance at 400 nm, using a Cary 50 UV−vis spectrophotometer, of several *p*-nitrophenol (*p*-NP−OH) standards dissolved in the buffer solution at the given sodium salt concentration. Spontaneous hydrolysis of *p*-NPA was experimentally quantified and enzymatic activity data were corrected by taking into account this phenomenon. One unit of hydrolytic activity is defined as the amount of enzyme that releases 1 μmol of *p*-NP−OH min^{-1}. All measurements were repeated from 3 to 5 times. Standard deviations, as error bars, are reported in Figure 1.

2.4. Lyophilization of Lipase Salt Solutions. A sample of 50 mg of lipase AH was dissolved in a 2 mL of buffer or buffer/salt solution. Enzyme solutions were shaken inside a 5 mL

capped glass vial (80 oscillations min^{-1}) at 30 °C, frozen at −80 °C, and then lyophilized in a Freezone4.5 (Labconco) freeze-drier at a condenser temperature of −40 °C or colder and a pressure of 133×10^{-3} mbar or lower. Lyophilized lipase samples were used for the esterification reaction after pre-equilibration at the desired water activity.

2.5. Equilibration of Substrates and Enzyme Preparations at Different Water Activities (a_w). Open vials containing the substrates solution (1-hexyl-β-D-galactopyranoside and palmitic acid) in 2-methyl-2-butanol and the enzymatic preparations were pre-equilibrated at 298 K for several days inside closed vessels containing saturated salt solutions. Five different salts were chosen in order to cover the water activity range (0−1), namely, LiCl ($a_w = 0.113$), $MgCl_2$ ($a_w = 0.328$), NaBr ($a_w = 0.576$), NaCl ($a_w = 0.753$), and K_2SO_4 ($a_w = 0.973$).

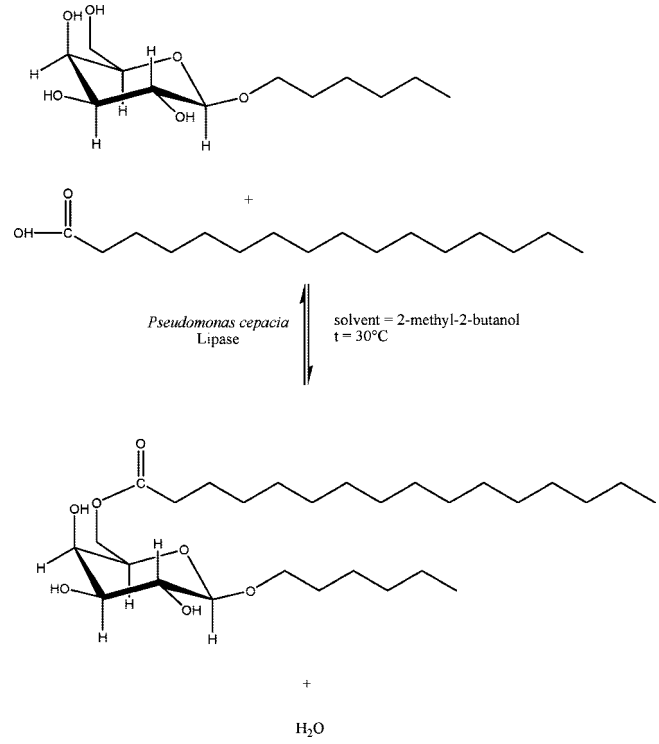
2.6. Enzymatic Activity Measurements in Nonaqueous Media. The enzymatic activity of salt−lipase lyophilized preparations suspended in the substrates solution in 2-methyl-2-butanol was determined by following the esterification of 1-hexyl-β-D-galactopyranoside with palmitic acid to form 6-palmitoyl-1-hexyl-β-D-galactopyranoside. 1-Hexyl-β-D-galactopyranoside (50 mg) and palmitic acid (244 mg) were dissolved in 2-methyl-2-butanol (2.6 mL) and pre-equilibrated at the chosen water activity. The reactions were started by the addition of the lyophilized lipase samples (all containing 50 mg of the original lipase AH powder and pre-equilibrated at the chosen water activity) to the pre-equilibrated substrates solutions. The reactions in dry media ($a_w = 0$) were performed by adding to the substrates solution 300 mg of 3Å molecular sieves. Reaction samples (25 μL) were withdrawn, mixed with 50 μL of internal standard (stearyl alcohol in 1-butanol), diluted with 1 mL of 1-butanol, and analyzed by high-performance liquid chromatography (HPLC). One unit of esterification activity is defined as the amount of enzyme that esterifies 1 μmol of 1-hexyl-β-D-galactopyranoside h^{-1}.

2.7. HPLC Analysis. HPLC analysis was performed using a Merck-Hitachi HPLC system, equipped with a D-7000 computer interface, an L-7200 autosampler, an L-7100 pump, an L-7350 column oven, a Lichrosphere 100 RP-8 end-capped 5 μm column from Merck (Darmstadt, Germany), and a Sedex 75 evaporative light-scattering detector (ELSD) (Sedere, France). Analyses were carried out at 35 °C, at a constant flow of 1 mL min^{-1} with the following solvent gradient. An isocratic elution—90% acetonitrile and 10% formic acid in water (0.5%)—was run for 5 min, a linear gradient to 100% acetonitrile was achieved in 3 min; pure acetonitrile was run for 2 min, then the initial solvent mixture was achieved in 2 min. Retention times were 2.84 min for 1-hexyl-β-D-galactopyranoside, 5.29 min for palmitic acid, 6.20 min for 6-palmitoyl-1-hexyl-β-D-galactopyranoside, and 8.49 min for stearyl alcohol (the internal standard). The disappearance of 1-hexyl-β-D-galactopyranoside and the formation of 6-palmitoyl-1-hexyl-β-D-galactopyranoside were determined according to calibration curves obtained with the internal standard method.

3. Results

3.1. Hydrolytic Activity of *P. cepacia* Lipase in Water Solution. The hydrolytic activity of *P. cepacia* lipase at various pH values was determined through the standard reaction of the *p*-nitrophenyl acetate hydrolysis.[16,17] Figure 1a shows that the highest activity is reached at about pH 7. Once the optimal pH was determined, the effect of some anions spanning the Hofmeister series, namely, SO_4^{2-}, $HPO_4^{2-}/H_2PO_4^-$, Cl^-, Br^-, SCN^-, at different ionic strengths on the hydrolytic activity of *P. cepacia* lipase was investigated. Salt addition to 20 mM buffer solutions to reach high salt concentrations modifies the pH of the initial buffer solution, and the ΔpH follows a Hofmeister series.[14] When the enzymatic activity is determined in such systems, the pH and the salt effects overlap and interfere with each other.[16,17] In order to focus only on the salt effect, all the salt solutions were adjusted to the final pH of 7 by adding a suitable volume of 20 mM Na_2HPO_4 solution.

SCHEME 1: Esterification Reaction of 1-Hexyl-β-D-galactopyranoside and Palmitic Acid Catalyzed by *P. cepacia* Lipase



The effects of the salt type and ionic strength on enzymatic activity are shown in Figure 1b. Most curves display a maximum, the position of which depends on the salt type. If we consider a 1 M ionic strength, the enzymatic activity decreases, following a (Hofmeister) series: $HPO_4^{2-}/H_2PO_4^- > SO_4^{2-} > Cl^- > Br^- > SCN^-$.

This result is similar to what was obtained previously for *Candida rugosa* lipase.[17] In that case the overall effect on enzymatic activity was a result of simultaneous phenomena associated with the salts, i.e., both the salt-induced pH variation and the direct ion interaction with the enzyme surface.

3.2. Esterification Activity of *P. cepacia* Lipase in Organic Solvent. Enzymatic activity of lipase from *P. cepacia* in organic solvent was determined following the esterification between 1-hexyl-β-D-galactopyranoside and palmitic acid to form 6-palmitoyl-1-hexyl-β-D-galactopyranoside (Scheme 1). The solvent (2-methyl-2-butanol) was chosen on the basis of its ability to simultaneously dissolve the two substrates.

Water activity (a_w) is a fundamental parameter that strongly affects enzymatic activity in NAM.[33] Different a_w were imposed following the standard procedure of pre-equilibration of enzyme and substrates solvent solution in a closed atmosphere at constant temperature (25 °C) given by a saturated salt solution.[33] The enzymatic esterification shown in Scheme 1 was carried out by suspending a weighed amount of enzyme powder on the reagents solution in 2-methyl-2-butanol. The amount of product versus reaction time, determined through HPLC-ELSD analysis, is shown in Figure 2. The highest yield was reached for the dry

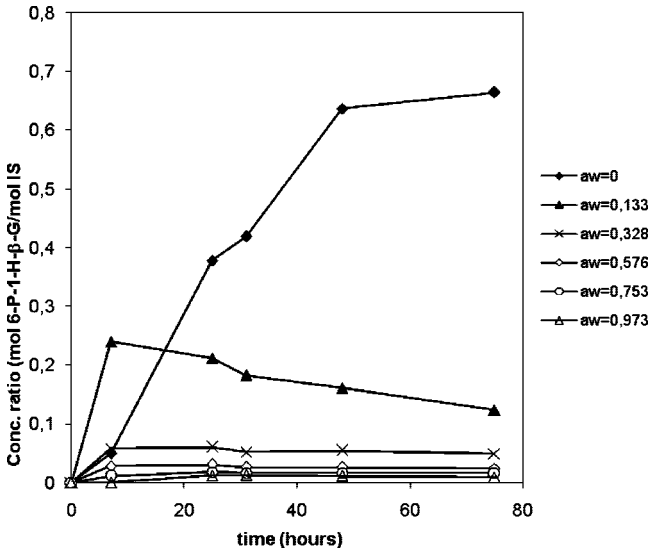


Figure 2. Effect of water activity on the production of 6-palmitoyl-1-hexyl-β-D-galactopyranoside obtained through the enzymatic esterification between 1-hexyl-β-D-galactopyranoside and palmitic acid.

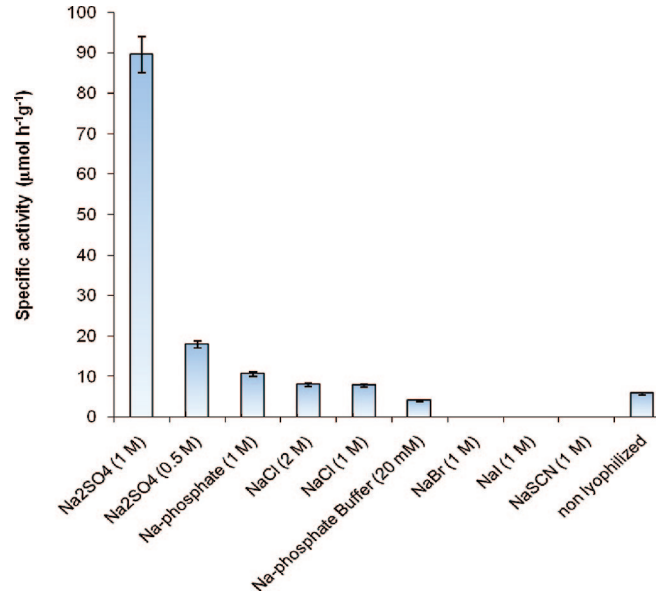


Figure 3. Effect of salt type on the esterification activity of lyophilized *P. cepacia* lipase in organic solvent.

medium ($a_w = 0$) obtained by running the reaction in the presence of molecular sieves. A water activity of 0.133 gave a higher yield in the first few hours of the reaction. Afterward, the equilibrium turned back toward reagents. Higher values of water activity gave very low yields that decreased with increasing a_w.

In the light of these results, molecular sieves were used in the subsequent parts of the work.

The effect of salts on enzymatic activity in organic solvent was studied by lyophilizing a lipase aqueous solution having a 1 M salt concentration, set to pH 7 before lyophilization (the optimal value obtained for the enzymatic activity in water solution). This was made according to the "pH memory effect", that is, the lyophilized enzyme retains the ionization state imposed by the pH of the original aqueous solution when suspended in NAM.[34] The enzymatic activities measured for the preparations obtained in the presence of the different salts are reported in Figure 3 in comparison with the nonlyophilized enzyme along with that lyophilized from a 20 mM phosphate buffer (pH 7) solution. The activity of the enzyme samples lyophilized from concentrated salt solutions follows a Hofmeister series: $SO_4^{2-} > H_2PO_4^-/HPO_4^{2-} > Cl^- > Br^- = I^- = SCN^-$. The commonly observed (Hofmeister) neutral effect of chloride was observed also in this case, whereas the preparations obtained from bromide, iodide, and thiocyanate solutions gave almost zero activity. The high activity measured for the enzyme lyophilized from a sodium sulfate solution, and also the effect of sodium sulfate concentration, are remarkable. Conversely, but not surprising, almost no difference in activity was measured for lipases lyophilized from 1 and 2 M NaCl solutions. The results obtained for *P. cepacia* lipase parallel what found for subtilisin Carlsberg.[6]

4. Discussion

According to what usually observed, the enzymatic activity in organic solvent was lower than in aqueous media. Here we got about a 25 000-fold less active biocatalyst in NAM (0.1 μmol min^{-1} g^{-1}) compared to that in water (2500 μmol min^{-1} g^{-1}). Nonetheless, the lipase is similarly affected by the presence of the salts of the Hofmeister series both in aqueous and in nonaqueous media. In the latter case the small amount of water occurring both in the medium and in the enzyme formulation seems to play a fundamental role. NMR studies on lyophilized subtilisin Carlsberg showed that two populations of bound water exist.[35] Tightly bound water consists of water molecules that do not exchange with other water molecules in the enzyme formulation and in the bulk solvent. The second population (loosely bound water) exchanges freely with the bulk solvent and has chemical and motional properties indistinguishable via NMR from those of free water. So it has been hypothesized that the loosely bound water induces an increase in enzyme activity by increasing enzyme flexibility and active site polarity.[35] This point is usually addressed by studying the effect of water activity. Results in Figure 2 show also that the highest product yield was obtained in the presence of molecular sieves ($a_w = 0$). A possible explanation of the observed trend is that the water present in the system—both in the solvent and that loosely bound to the salt−enzyme preparation—as a result of a_w pre-equilibration is not fundamental or even harmful for enzyme catalysis. Indeed, already at initial $a_w = 0.133$ the equilibrium reaction is shifted back toward the reagents after a few hours. On the other hand, at zero water activity and in the presence of molecular sieves, the water is continuously removed by the equilibrium thus favoring the product formation.

On the other hand, tightly bound water is necessary to maintain the catalytically active conformation of the enzyme. When lyophilized, an enzyme is deprived of the fundamental water; thus, using suitable salts as lyoprotectants may help to protect the protein against destabilizing influences during lyophilization and promote hydration of the protein in the final lyophilized formulation. Although it was not quantified here, it has been reported that the percentage of water retained during lyophilization is an important parameter. Its amount parallels the Hofmeister series, being high for kosmotropic salts and low for the chaotropics.[6] In the case of *P. cepacia* lipase we have compared the activities of the nonlyophilized enzyme and of the lyophilized enzyme, in the presence of only 20 mM phosphate buffer and in the presence of 1 M salts. As shown in Figure 3, the activity of the nonlyophilized enzyme was similar to that of the lyophilized enzyme in the presence of only buffer. This means that the lyophilization process per se does not inactivate the lipase. On the other hand the lyophilization in

TABLE 1: Jones−Dole Viscosity Coefficient (*B*), Lyotropic Number (*N*), Surface Tension Increment (σ), and Effective Anion Polarizability in Solution (α) for Some Anions of the Hofmeister Series

anion	B^a	N^b	σ (μN m^2 mol^{-1})^b	α (Å^3)^b
SO_4^{2-}	0.208	2.0	2.74	6.32
HPO_4^{2-}	0.382	8.2	2.33	
$H_2PO_4^-$	0.340			
Cl^-	−0.007	10.0	1.55	3.42
Br^-	−0.032	11.3	1.31	4.85
I^-	−0.068	12.5	1.02	7.40
SCN^-	0.022	13.25	0.54	6.47

[a] From ref 6. [b] From ref 10.

the presence of salts produce a biocatalyst whose activity follows a Hofmeister series. At least in our case, salt enzyme activation seems to be more important than enzyme protection during lyophilization. In that case the pools of water at the enzyme surface should have a very high ionic strength. In this microenvironment we might expect the reoccurrence of the specific ion effects as in the case of bulk water media,[16,17] that is, in the light of the recent advances in the understanding of the Hofmeister effect, the direct interaction of anions with the enzyme surface through nonelectrostatic (dispersion) forces.[28]

4.1. Physicochemical Parameters Related to NES Forces. As reported above, several parameters have been used for correlation with Hofmeister effects.[36] Table 1 reports the values of Jones−Dole *B* coefficient,[6] lyotropic number (*N*), surface tension increment (σ), and polarizability (α) for the anions used in this work.

Besides the already defined Jones−Dole *B* coefficient a brief description of the other parameters follows. The lyotropic number, *N*, is an empirical evaluation of lyotropic activity based upon several different phenomena, including salting-out, swelling and gelation of lyophilic colloids, viscosity of salt solutions, rates of reactions, and flocculation of lyophobic colloids.[36]

The parameter σ indicates the surface tension change of water after addition of the salt:[10]

$$\sigma = \frac{\partial \Delta\gamma}{\partial c} \tag{2}$$

$\Delta\gamma$ being the surface tension change of the salt solution with respect to pure water.

All these parameters, previously used to describe specific ion effects, seem to correlate with the enzymatic activity both in water and in organic solvent and must be due to a common phenomenon. Indeed, Kunz et al. have shown that *B*, *N*, and σ correlate with an intrinsic property of the ion: the polarizability α.[10] Thus, among the parameters reported in Table 1 used to relate the specific anion effects with enzyme activity in organic media, polarizability, or better, the excess polarizability of the ion in water $\alpha_i^*(0)$, seems to be the most appropriate property since it is directly related to dispersion NES forces. The NES potential (U_{NES}), felt by an ion at a distance *x* from an interface, is given by the equation

$$U_{NES}(x) \approx \frac{(n_{water}{}^2 - n_{substrate}{}^2)\alpha_i^*(0)\hbar\omega_i}{8x^3} \tag{3}$$

where $n_{substrate}$ is the refractive index of the substrate, n_{water} is that of water, and $\hbar\omega_i$ is the electron affinity (or ionization potential) for the ion. These forces include, in the special limiting case of dilute media, the dipole−dipole (Keesom), dipole−induced dipole (Debye), and dispersion (London) forces.[23]

As already observed by Kunz et al.,[10] and also in our case, the correlation of both enzymatic activity and the other parameters of Table 1 with polarizability is not perfect. That is the case of sulfate anion that, although known as one of the most kosmotropic anions ($B = 0.208$), has a high value of polarizability ($\alpha = 6.32$) as a chaotropic anion. But sulfate being the only divalent ion considered, the correlation would be restored if the polarizability per unit of charge (α/z) is considered. On the other hand, for SCN^-, known as one of the most chaotropic anions, enzyme activity gives a better correlation with polarizability rather than the Jones−Dole *B* coefficient (Table 1).

4.2. Hypothetical Mechanism of the Salt Enzyme Activation/Deactivation. Lipases act through a ping−pong Bi−Bi mechanism occurring in two steps each one involving the binding of a substrate and the release of a product.[37] As found for the whole family of serine proteases a highly polar tetrahedral intermediate (an oxyanion) is formed in the transition state.[37] Three amino acids (the catalytic triad) are involved in the catalytic action, and other two (the oxyanion hole) contribute to the stabilization of the transition state through formation of hydrogen bonds.[17] The catalytic triad of *P. cepacia* lipase is constituted by Ser87, His286, and Asp264, and the oxyanion hole is constituted by Leu17 and Gln88.[38] The resolved structure reveals a highly open conformation with solvent-accessible active site.[39] A deep pocket is found in the center of the large active site cleft. The nucleophile Ser87 is located in the middle of the pocket. The pocket and the active site cleft around it are rich in hydrophobic residues.

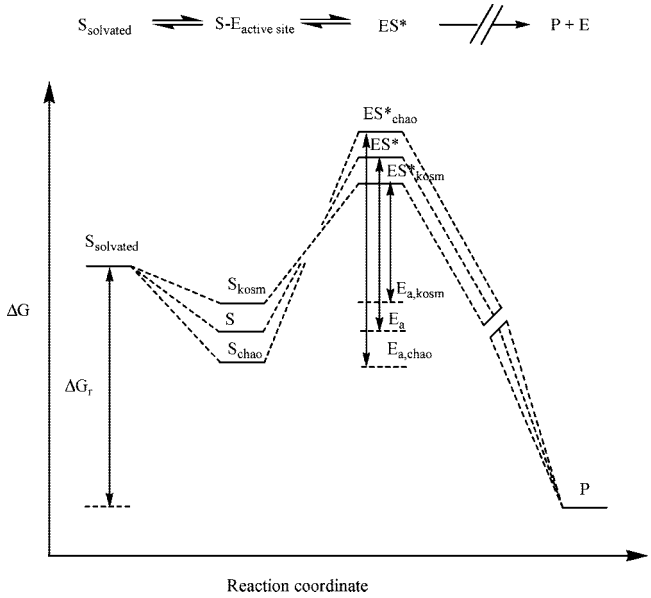
Michels et al. used a Kirkwood analysis to evaluate the electrostatic Gibbs energy of activation, ΔG_E, for an enzymatic reaction (S → P) that proceeds through the transition state.[40] This is a generalization of Born's original treatment of charge solvation for systems containing arbitrary distribution of charge centers.

$$\Delta G_E = -N_A\left(\frac{\mu^2}{r^3}\right)\left[\frac{\varepsilon - 1}{2\varepsilon + 1}\right] \tag{4}$$

Equation 4 gives the Gibbs energy upon transfer of a mole of dipoles of moment μ from vacuum ($\varepsilon = 1$) into a spherical cavity of radius *r*, inside an isotropic continuum of dielectric constant ε. According to eq 4 the ΔG_E depends on μ variations. The presence of different anions in contact with the enzyme molecule should affect the energetic levels of the transition state through dipole moment variations via NES forces (eq 3). On the basis of previous considerations, it is reasonable to suggest that the tightly bound water kept by the lyophilized salt−enzyme preparation acts in a synergic way together with the kosmotropic/chaotropic anions through two mechanisms that favor/disfavor enzyme catalysis. The different salts of the Hofmeister series interact differently with hydrogen bonds that are needed to stabilize the oxyanion in the transition state. Kosmotropic anions are expected to increase the transition state polarity thus decreasing the activation energy, whereas chaotropic anions should act in the opposite way. Moreover they may distort the active site three-dimensional enzyme structure. These effects should lead to the destabilization of the transition state resulting in the lipase inactivation.

Another mechanism acting jointly to the previous one, and so coresponsible of the observed trend of specific anion effects on enzyme activity in NAM, involves the binding energy between the enzyme and the substrate. The substrate is desolvated during its transfer from the bulk media to the enzymatic active site. The more energetically favorable the desolvation

SCHEME 2: Qualitative Energetic Diagram of the Steps Involved in the Enzyme Action in NAM: Substrate and Transition State Stabilization/Destabilization Due to Specific Anion Effects[a]



[a] $S_{solvated}$ is the energy level on the substrate in the solvent (2-methyl-2-butanol); S is the energy level of the desolvated substrate bound at the active site; ES* is the energy of the transition state; E_a is the activation energy; P is the energy level of products; the subscripts kosm and chao in the previous symbols (i.e., S_{kosm} and S_{chao}) refer to the same parameters in the presence of kosmotropic and chaotropic anions, respectively.

the greater the net binding energy becomes. Many enzymes (i.e., lipases) are hydrophobic at the active site, so they prefer working with hydrophobic substrates because there is a large energetic gain during their transfer from the bulk solvent to the active site. In the case of kosmotropic anions (i.e., sulfate and phosphate) a polar environment constituted by the salt-bound water at the active site level may destabilize a strongly hydrophobic substrate, as palmitic acid, during the substrate binding step. This should result in a decrease of the activation energy barrier. Chaotropic anions (i.e., Br^-, I^-, SCN^-) may act in the opposite way. Scheme 2 displays the qualitative energetic diagram of the two mechanisms here invoked to explain the specific anion effects on enzyme activity in NAM. The solvated substrate binds at the enzyme binding site losing solvating molecules: once the transition state is reached, the products formation follows. In this scheme it is assumed that the energy levels of the solvated substrate S and of the product P do not depend on the added electrolyte. In fact the transfer of an ion from pure water to a water/organic solvent gives rise to the "solvent medium effect". This is a measure of the change in the total solvation Gibbs energy of a solute when it is transferred from a reference solvent to another, or to a solvents mixture.[41] Nevertheless, the dielectric constant of 2-methyl-2-butanol (5.8) being lower than that of water (78.5), this assumption makes sense since the solubility of the salt in the organic solvent is very low, and in any case, the formation of soluble ion pairs rather than dissociated ions is likely to occur.[42] Hence most salt is present as a suspended solid thus affecting the energy levels of both solvated reagents and products only at a low extent.

5. Conclusions

The catalytic activity of a lipase lyophilized from concentrated salt solutions follows a Hofmeister series as found in water media.[16,17] This fact has two important implications. Since the water content in NAM is very low, it is hard to believe that the enzyme activation/inactivation is due to the different salt-induced bulk water structures. Even in this case it is more likely that the Hofmeister effect on enzyme activity in NAM is due to the direct interactions of ions with the enzyme surface, without water structure mediation. This interaction has to be linked to developments in understanding ionic interactions in water. The new developments involve previously missing dispersion forces that do capture some essentials of specific ion effects.[23] There is obviously more to it, and more investigations are required, but it agrees with the most recent findings in the field.[30,31,43] Finally, polarizability—an intrinsic property of ions—rather than the Jones−Dole B coefficient[6,7]—a parameter related to a bulk property of solutions (viscosity)—can be better used to describe the correlation with the enzymatic activity in NAM.

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