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Role of Methoxypolyethylene Glycol on the Hydration, Activity, Conformation and Dynamic Properties of a Lipase in a Dry Film

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Abstract

A combined approach based on the use of ATR-FT/IR and steady-state fluorescence spectroscopy allowed to shed light on the effects of the additive methoxypolyethylene glycol (MePEG) on the hydration, conformation and dynamic properties of lipase from *Burkholderia cepacia* dehydrated to form a film. Spectroscopic data show that the additive has little effect on the structure of the protein; however, H/D exchange kinetic and fluorescence anisotropy suggest a more flexible enzyme molecule when in the presence of MePEG. By infrared spectroscopy, we estimated that, after conditioning the films at water activity of 1, the water content in the lipase dehydrated with MePEG is 5.4- and 4.7-fold higher than in the absence of the additive and the additive alone, respectively. Additionally, our infrared data suggest that MePEG acts by hindering intermolecular protein–protein interactions and contributing to increase the accessibility and flexibility of the lipase in the dehydrated solid film. These factors also explain the enhancement of the enzyme catalytic activity (i.e., up to 3.7-fold in neat organic solvent) when in the presence of MePEG. The method and results presented might better address the use of additives for the preparation of enzymes employed in non-aqueous media or of proteins used in a dry form in different fields of biotechnology.

Keywords

tertiary structure; secondary structure; protein dehydration; infrared spectroscopy; fluorescence anisotropy; hydrogen bonds

Introduction

The effects of dehydration and the role of additives (e.g., sugars, polysaccharides, peptides, etc.) on proteins after the drying process is a topic of major interest in different fields of protein applications.

Often, (freeze)-drying is a procedure adopted to prepare protein samples in a form less prone to degradation (e.g., reducing the protease action and/or slowing the unfolding processes), a subject particularly important in the pharmaceutical field due to the increasing number of peptides and proteins used as drugs. Furthermore, enzymes are prepared in a dehydrated form to carry out biotransformations in non-aqueous media. This is an established methodology employed also at industrial level due to its numerous advantages, including the possibility to

transform substrates unstable or poorly soluble in water, to prevent many side-reactions that are water dependent, and to favor the synthesis of ester and amide bonds (mainly by means of lipases and proteases) over hydrolysis (Carrea and Riva, 2000; Gupta and Roy, 2004; Klibanov, 2001). To optimize their activity in organic solvents, enzymes usually are used after lyophilization (in the presence of additives or salts) or immobilized (by adsorption or deposition) on different kinds of organic or inorganic supports (Lee and Dordick, 2002; Ru et al., 1999; Secundo and Carrea, 2003). In this latter case, the enzyme can be considered as a dry film layered on a support. Because of this, the study herein presented was carried out with the enzyme as a dry film. However, it has to be emphasized that the occurrence of protein dry films concerns also other biotechnological applications, including the integration of enzymes or even photosynthetic protein molecular complexes in solid state electronic devices (e.g., biosensors or detectors and photovoltaic cells) (Das et al., 2004) and the production of biodegradable film as products for packaging and capsules (Rakotonirainy and Padua, 2001).

Polyethylene glycol (PEG) and its methoxy derivative (MePEG) are additives largely employed in the pharmaceutical industry (e.g., for the formulation of protein-based drug) and in the biotechnological field (e.g., to increase the activity of several enzymes in non-aqueous media) (Dai and Klibanov, 1999; Secundo et al., 1999a, 2001, 2006). Several infrared spectroscopy studies have shown that MePEG is useful to preserve the native conformation of proteins during the freeze-drying process (Prestrelski et al., 1993a,b; Vecchio et al., 1999). However, most of the studies were concerned with the secondary structure of the protein before and after lyophilization and little is known on protein hydration and flexibility. A better understanding of the role of additives to control these latter parameters is extremely important for more efficient protein applications ranging from biotransformations and biosensors to vaccines and drug delivery.

In the present study ATR-FT/IR and fluorescence data obtained on a dry film were used to shed light on the modality by which MePEG (MW 5,000 Da) can improve the activity of the lipase from *Burkholderia cepacia* in organic solvent. These results highlight some peculiarities of MePEG that could be of general interest not only for biocatalysis carried out in non-aqueous systems (e.g., ionic liquids, organic solvents, solvent-free reactions) but also for the functionality of proteins in different fields of biomacromolecules, especially if used in a dry form.

Materials and Methods

Materials

Dry films of lipase from *Burkholderia cepacia* (lipase BC) used for activity and spectroscopic measurements were prepared from LP “Amano” S (IUB number: 3.1.1.3), which was kindly donated from Amano Enzyme, Inc. (Nagoya, Japan). However, in all cases before use LP “Amano” S was dissolved in potassium phosphate 5 mM potassium phosphate buffer, pH 7 and centrifuged by Nanosep 10 K Omega (from Pall Life Science, Ann Arbor, MI). This step was repeated twice and aimed to remove any stabilizer present with the commercial sample. Methoxypolyethylene glycol (MW 5000) was purchased from Sigma. All the other reagents were of analytical grade and purchased from Aldrich.

Activity Measurements

The transesterification between 1-hexanol and vinyl butyrate to give 1-hexyl butyrate and acetaldehyde was used as model reaction. In a typical experiment 1 mL of dodecane containing 0.19 M 1-hexanol and 0.79 M vinyl butyrate was added to 75 µg of lipase dehydrated under vacuum from 0.2 mL solution in 0.05 M potassium phosphate buffer, pH 7, containing or not containing 3 mg of MePEG (throughout the article we name these samples lipase BC and lipase

BC + MePEG, respectively). Before mixing, dodecane, vinyl ester, enzyme and substrate were separately adjusted to the desired water activity (a_w) in sealed containers for at least 48 h, at 25°C, with the vapor phase of saturated salt solutions of known water activity a_w (LiBr 0.06, LiCl 0.11, MgCl₂ 0.33, MgNO₃ 0.53, NaCl 0.75, KCl 0.84, K₂SO₄ 0.96) for at least 48 h (Valivety et al., 1993). The reaction mixture was shaken at 150 rpm and 25°C. At scheduled times, aliquots were withdrawn from the reaction mixtures and the conversion determined by GLC [HP-1 Crosslinked Methyl Silicone Gum, 25 m, 0.32 mm ID, Agilent Technologies (Milan, Italy); conditions: oven temperature from 35°C (initial time 5 min) to 160°C with a heating rate of 10°C/min, H₂ as carrier gas]. Control experiments showed that the non-enzymatic reaction was negligible under all conditions used.

ATR-FT/IR Measurements

ATR-FT/IR spectra were measured by using an FT/IR-610 Jasco instrument equipped with a DTGS detector. The system was managed by Jasco software and spectra were elaborated by Spectra Analysis. Spectra were recorded as the average of 32 scans at 2 cm⁻¹ resolution. During measurements the cell compartment was flushed with dry nitrogen. The ATR-FT/IR cell was endowed with an internal reflection element (ZnSe plate) whose aperture angle was 45°, allowing 17 internal reflections. Analyses were carried out with samples prepared as film on the ZnSe plate. To this end, 0.5 mg of lipase in 0.15 mL of 0.05 M potassium phosphate buffer, pH 7, were mixed with 0.15 mL of water or 0.15 mL of MePEG solution in water (20 mg/mL) and dispersed on the ZnSe plate. Water was evaporated in about 18 h at room temperature to obtain the lipase as a solid film. A film of only MePEG was analogously prepared using 0.15 mL of a MePEG solution (20 mg/mL) mixed with 0.15 mL of 0.05 M potassium phosphate buffer, pH 7. In all cases the solid film was equilibrated to the desired a_w for at least 48 h, at 25°C, by connecting (via a tube) the cell to a sealed vessel containing water (for $a_w = 1$) or a saturated salt solution of known a_w . The criterion for equilibrium was the absence of variations between two consecutive infrared spectra recorded after a time interval of 24 h. All spectra were subtracted of the blank (spectrum of the empty cell equilibrated at the same a_w value of the protein sample), smoothed and baseline adjusted.

Spectra of lipase BC and lipase BC + MePEG obtained from the samples conditioned at the various a_w values were normalized. To this end lipase BC spectra were divided by a factor calculated from the ratio of the amide I band area (1,600–1,700 cm⁻¹ of lipase BC over that of lipase BC + MePEG, of samples both equilibrated at a_w 0.06. (The same ratio was used to normalize the spectra obtained at higher a_w .) Spectra of lipase BC + MePEG and MePEG at all a_w value were normalized for the area of the C–H stretching band, in the 2,665–3,000 cm⁻¹ region. The contribution of the C–H stretching due to the protein was negligible (less than 10%) compared to that of MePEG.

Second-derivative spectra were obtained with the derivative function of Spectra Analysis (software Jasco) using a 13-point Savitsky–Golay function to remove white noise. The second-derivative spectra for lipase BC and lipase BC + MePEG were overlaid for comparison.

The hydrogen/deuterium (H/D) exchange kinetic of a film of lipase BC or lipase BC + MePEG was carried out following the decrease of the amide II band at 1,549 cm⁻¹ as a function of time. After equilibration at a_w 0.06 (LiBr solution in H₂O) for 48 h the lipase film was brought to a_w of 0.53 by connecting the ATR-FT/IR cell to a sealed vessel containing a saturated salt solution of MgNO₃ in D₂O (Kou and Schmidt, 1999). The time of connection to the vessel containing D₂O vapors was taken as the start of exchange. A series of IR spectra (400–4,000 cm⁻¹ region) were recorded starting 30–40 s after initiating the H/D exchange. A resolution of 2 cm⁻¹ was used. The number of scans used for recording a spectrum was adjusted to the speed of the exchange (8 scan measurements in the first 30 min and 16 scans toward the end).

For the quantitative analysis of the exchange kinetic the ratio of the amide II and amide I absorbance (A), corrected with the absorbance of the baseline, at time t was defined as:

$$\omega(t) = \frac{A_{\text{amide II}}(t) - A_{\text{base}}(t)}{A_{\text{amide I}}(t) - A_{\text{base}}(t)}$$

$A_{\text{amide I}}$ and $A_{\text{amide II}}$ were measured at 1,650 and 1,540 cm^{-1} , respectively. A_{base} was measured at 1,880 cm^{-1} (flat line).

Data of the decay of ω as a function of time (t) were fitted to a two-exponential model:

$$\Delta\omega = ae_1^{(-k_1)t} + be_2^{(-k_2)t} + c$$

where k_1 and k_2 are the apparent exchange rate constants for the fast and slow exchanging amide protons, a and b are the amplitude of the decay of ω due to the fast and slow exchanging amide protons, respectively. c is the contribution to ω whose magnitude depends on the fraction of the non exchanging amide protons. The fraction (X) of each kind of exchanging protons was determined as $X_f = a/(a + b + c)$, $X_s = b/(a + b + c)$, $X_u = c/(a + b + c)$. It has to be noted that the various fractions obtained are not absolute values because proteins signals in the region 1,500–1,600 cm^{-1} could also be due to chemical groups different from N–H (e.g., C = O of carboxyl groups) whose intensity is little influenced by the H/D exchange. However, this did not hinder the comparison of different formulations of the same enzyme because they are all analogously affected by the same signals different from N–H.

Fluorescence Measurements

Intrinsic fluorescence emission spectra were measured by using a Jasco spectrofluorimeter with steady-state excitation. Fluorescence was excited at 295 nm to minimize the contribution of tyrosine. A film of lipase BC or lipase BC + MePEG was obtained on a window of quartz ($1 \times 3 \times 0.1$ cm). To this end, 1 mg of lipase dissolved in 0.15 mL of 50 mM potassium phosphate buffer, pH 7, was mixed with 0.15 mL of water or 0.15 mL of MePEG solution in water (20 mg/mL) and loaded on the quartz window. After water evaporation the dry film was equilibrated at the desired a_w value and used for the fluorescence measurements. The quartz window was placed in a mobile sample holder and positioned such that the incoming excitation beam encounters the dry film directly and is reflected towards the emission detector. Although solid samples exhibited a high level of scattering at the excitation wavelength, there was no scattering contribution beyond 310 nm.

For the spectra of lipase BC in 50 mM potassium phosphate buffer pH 7, the concentration was 0.02 mg/mL.

Vertical (I_{vv}) and horizontal (I_{vh}) component of fluorescence intensity excited by vertical polarized light were recorded for determination of fluorescence anisotropy (r). The different sensitivity of the registering system for vertical and horizontal components was taken into account:

$$r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}}$$

where G is the ratio of the vertical and horizontal components of fluorescence intensity excited by horizontally polarized light ($G = I_{hv}/I_{hh}$) (Turoverov et al., 2002).

Results and Discussion

Activity Measurements

In order to evaluate the effect of hydration on lipase BC and lipase BC + MePEG activity, the transesterification of vinyl butyrate with 1-hexanol in dodecane as reaction medium was used as model reaction (Fig. 1). The initial rates of transesterification (formation of 1-hexyl butyrate) and hydrolysis (formation of butyric acid from vinyl butyrate) reactions, which occurred simultaneously, are shown in Figure 2a and b. It can be noted that at all a_w values lipase BC dehydrated in the presence of MePEG shows higher transesterification and hydrolytic activity than lipase BC dehydrated in the absence of the additive (e.g., at a_w 0.53, 2.8- and 3.7-fold respectively). These results agree with previous reports in which it was showed that MePEG has an activating effect on the activity of enzymes in organic solvents (Dai and Klivanov, 1999; Secundo et al., 2001, 2006).

It can be observed that the increase of a_w causes an enhancement of both transesterification (v_t) and hydrolytic rate (v_i) (Secundo et al., 1999a). This is due to the double role of water as substrate and as plasticizer that makes the enzyme more flexible and active (Zaks and Klivanov, 1988). When a_w passed from 0.06 to 0.84 the ratio v_t/v_i decreased from 0.65 to 0.12 in the case of lipase BC and from 0.72 to 0.21 in the case of lipase BC + MePEG. Above a_w 0.84 the transesterification reaction rate decreases for both lipase preparations because of the greater competition between the synthetic and the hydrolytic reaction (Fig. 1). Indeed, it is known that the presence of water is a drawback if the enzyme has to be applied in the field of organic synthesis, because it reduces the yield of the synthesis and increases the consumption of the acylating agent (e.g., vinyl ester). From this point of view the use of MePEG appears very interesting because at low a_w values it allows both a faster transesterification rate and a higher v_t/v_i ratio, that is, better conditions for synthetic reactions.

Infrared Spectroscopy Measurements

It is known that the catalytic activity of an enzyme in nonaqueous media depends on the water bound to the enzyme (Bell et al., 1995; Secundo et al., 2007; Yang et al., 2004; Zaks and Klivanov, 1988). Infrared spectroscopy has been employed to determine the number of water moles for mole of protein in dry film at different humidity values and the mechanisms by which the water/protein binding occurs (Liltoft and Maréchal, 2005; Rupley and Careri, 1991). On the other hand Bridelli et al. (2002) by means of three experimental techniques such as water sorption isotherms, thermally stimulated depolarization currents, and Fourier transform infrared spectroscopy observed that about 660 water molecules bind the enzyme when it is statically hydrated at a_w 0.89. In our study the purpose was to determine by ATR-FT/IR the effects of MePEG on the hydration of the enzyme.

Liquid water has several bands in the range 400–4,000 cm^{-1} (Fig. 3a). Particularly useful for quantification of water in proteins is the band at 670 cm^{-1} , which is due to librations (restricted rotations; i.e., rocking motions) of the whole H_2O molecule. This band does not interfere with the protein signal and its intensity is little affected by the hydrogen-bond configuration of the water molecule. To quantify this band in the infrared spectra of MePEG, lipase BC and lipase BC + MePEG conditioned at different a_w values (Fig. 3c and d) we assumed that the area between the maximum at 670 cm^{-1} and the region at wave numbers around 800 cm^{-1} gives the value of half its integrated intensity (Grdadolnik and Maréchal, 2001). However, in order to have another signal in the infrared spectrum suitable to monitor the hydration of the sample besides the libration band, we also monitored the variation of intensity at 3,500 cm^{-1} , which

is in the region where the O–H stretching falls. The choice of measuring the intensity at this wave number instead of at the maximum ($3,350\text{ cm}^{-1}$ in liquid water) is for minimizing the contribution of the stretching of the N–H groups of the protein (maximum at around $3,300\text{ cm}^{-1}$) and of the O–H groups involved in hydrogen bonds; in fact, as hydrogen bonding weakens, the covalent O–H bonds strengthen causing them to vibrate at higher frequencies (e.g., higher than $3,350\text{ cm}^{-1}$) (Barth, 2000). The water sorption isotherms, obtained by plotting the area of the band at 670 cm^{-1} or of the intensity at $3,500\text{ cm}^{-1}$ as a function of a_w , allow to compare the water adsorbed to lipase BC, lipase BC + MePEG and MePEG (Fig. 4). It can be noted that as a_w increases, lipase BC + MePEG becomes increasingly more hydrated than lipase BC and, at a_w 1, the hydration of lipase BC + MePEG is 5.4- and 4.7-fold higher than for lipase BC and MePEG, respectively. (These ratios of hydration were obtained by calculating the arithmetic mean of the two ratios obtained using the intensities at $3,500\text{ cm}^{-1}$ and the areas of the band at 670 cm^{-1} —Fig. 4). On the contrary, at lower a_w values MePEG has less effect on the enzyme hydration and at a_w 0.06 lipase BC and lipase BC + MePEG have the same water content, which is markedly higher than that of MePEG, being the water signals 3- and 10-fold higher at 670 cm^{-1} and $3,500\text{ cm}^{-1}$, respectively (insets of Fig. 3c). Then, the higher hydrophilicity of lipase BC + MePEG does not depend on the hydrophilic properties of the additive. However, MePEG favors the adsorption of water to the protein water binding sites (e.g., protein polar groups) and the formation of (multi) layers of water on the protein molecule via the condensation process (binding of water molecules to weak binding sites) (Bridelli et al., 2002; Liltorp and Maréchal, 2005). This latter event would be responsible for the steep increase of water retained with the protein at a_w above 0.84. MePEG could favor protein hydration by dispersing the enzyme and increasing the number of binding sites available to water molecules. This hypothesis also agrees with the higher catalytic activity in organic solvent of lipase BC + MePEG compared to lipase BC. In fact, it is known that dispersion of the biocatalyst (e.g., on solid supports) leads to an increase of activity in organic solvents thanks to the decrease of diffusional limitations (Rees and Halling, 2000). It is worth pointing out that a similar role of the additive was evidenced in a previous study in which lipase BC was lyophilized in the presence of sugars able to reduce intermolecular hydrogen-bonded β -sheets, even though by the method of Bedell et al. (1998) we did not observe substrate diffusional limitations in lipase BC lyophilized alone (Secundo and Carrea, 2005).

Monitoring the H/D exchange kinetic for lipase BC and lipase BC + MePEG we investigated the question if MePEG has an effect on the lipase that is not directly related to water adsorption. Evaluating the rate of H/D exchange it is possible to gain information on the overall flexibility of the enzyme molecule. However, in the present study, the rate of exchange also depends on the accessibility of D_2O to the enzyme molecules in the dry film. We found that the H/D exchange kinetic (Fig. 5) of a lipase sample brought from a_w 0.06 to 0.53 is faster when in the presence of the additive, indeed both the kinetic constants that regulate the exchange of the fast and the slow exchanging hydrogens are higher in the case of lipase BC + MePEG (Table I). It is important to note that when all the exchangeable hydrogens were exchanged (e.g., after 1,440 min) the amide II/amide I ratio (i.e., the fraction of non-exchangeable hydrogens X_u) is higher for lipase BC in the absence of MePEG (Fig. 5). These results show that the number of enzyme molecules reached by water decrease when in the absence of MePEG and suggest that the additive acts decreasing the fraction of strongly packed enzyme molecules. As the fraction of non-exchangeable hydrogens (X_u) decreases, both the fractions of the fast and slow exchangeable hydrogens (X_f and X_s) of lipase BC + MePEG increase. The rate of exchange of the slow (k_2) and fast (k_1) exchangeable hydrogens is faster in the lipase BC + MePEG preparation than in lipase BC. This faster exchange rate seems to imply, beyond a faster diffusion of water molecules to the fast-exchangeable hydrogens in lipase BC + MePEG than in lipase BC, a higher enzyme flexibility of the former at low a_w values (e.g., 0.06) too.

The fact that MePEG is able to reduce protein–protein intermolecular interactions in the dry lipase sample was also evinced analyzing the components of the amide I band, which gives definite information about the secondary structure of the protein. It is worth pointing out that the bands of MePEG do not interfere with the amide I band ($1,600\text{--}1,700\text{ cm}^{-1}$) of the protein (Fig. 3b–d), allowing the comparison of the secondary structure of lipase BC dried in the presence or in the absence of the additive. Both lipase BC and lipase BC + MePEG show in the second derivative spectra (Fig. 6) components at around $1,639$, $1,656$ and $1,664\text{ cm}^{-1}$ that can be assigned to the secondary structures of β -sheets, α -helix and β -turns, respectively (Secundo and Carrea, 2005). The high similarity of the spectra suggests that there are no significant effects of MePEG on the enzyme secondary structure. Nevertheless, in the case of lipase BC there are two components at around $1,619$ and $1,685\text{ cm}^{-1}$ that might be assigned to β -sheets involved in intermolecular interactions, typically observed when in the presence of protein aggregates (Griebenow and Klibanov, 1995; Halverson et al., 1991; Mizutani et al., 2003; Secundo and Carrea, 2005). The absence of these components in the spectrum of lipase BC + MePEG suggests that MePEG breaks the protein intermolecular interactions, and decreases the packing density of enzyme molecules.

Fluorescence Measurements

Fluorescence spectra of both lipase BC and lipase BC + MePEG as film equilibrated at a_w 0.06 showed a λ_{max} of emission at 335 nm (Fig. 7). This value is very similar to that found for lipase BC in water (334 nm) indicating that the lipase BC undergoes only minor modification of the tertiary structure upon dehydration, independently of the presence of MePEG. This latter result agrees with our previous report in which lipase BC co-lyophilized with MePEG and dissolved in 1,4-dioxane showed a fluorescence spectrum similar to that in water (Secundo et al., 1999b).

To assess if the faster H/D exchange rate observed with ATR-FT/IR was due to an higher enzyme flexibility and not only to the faster diffusion of water in the dry film thanks to the presence of MePEG, we investigated the fluorescence anisotropy (r) of the film obtained in the absence and in the presence of MePEG. It is known that the value of r decreases with the increase in the mobility of the fluorophore. We found r equal to 0.1 for lipase BC and to 0.06 for lipase BC + MePEG, suggesting that the Trp residues of lipase BC are more mobile when the enzyme is dried and conditioned to a_w 0.06 in the presence of the additive. The higher mobility of Trp residues is also consistent with a more flexible enzyme, which appears to be the consequence of a lower degree of compactness rather than a higher water content associated with the enzyme molecule.

Conclusions

In the present study we have shown that MePEG is able to increase the activity of a lipase in organic solvent. This effect can be ascribed to the ability of MePEG to act not only as lyoprotectant (e.g., preserving the native secondary and tertiary structure of proteins), but also avoiding the “packing” of protein molecules. A better dispersion of the enzyme and lower intermolecular protein–protein interactions would favor the access of water to the enzyme (and of the substrate to the catalytic active site) increasing its hydration. This latter event makes the enzyme more flexible and able to adopt a more catalytically active conformation.

An interesting issue related to the role of MePEG as dispersant is how it acts as support for proteins. The results presented in this paper suggest that when in aqueous solution MePEG forms a sort of macromolecular net in which the protein molecules are trapped and unable to form protein–protein intermolecular interactions. After dehydration the resulting non-covalent protein/MePEG complexes are stable enough to be soluble (or at least homogeneously dispersed to form clear solutions) even in some organic solvents (Secundo et al., 1999b). Using this

approach the notable result is that the protein itself can be made soluble in organic solvent. The model of a protein surrounded by a macromolecular net of MePEG also agrees with the results found in our previous study (Secundo et al. 2002) where we showed that the catalytic activity in organic solvents of lipase BC increased as a function of the MePEG/enzyme molar ratio, and reached a plateau above a molar ratio of 750 (molecular mass of MePEG used was 5,000 Da). Such a high ratio (observed also in the case of lipase B from *Candida antarctica* and subtilisin) indicates that the enzyme is fully activated when is completely surrounded or “enveloped” by molecules of the additive, supporting the model of a macromolecular net.

We have also shown how the hydration properties of a single kind of macromolecule (e.g., proteins) can change dramatically when mixed with other kind of macromolecules (e.g., MePEG). This appears particularly interesting especially in the prospective to generate new materials based on different proteins/polymers combinations.

Finally, we have shown that by means of simple, fast and convenient techniques (ATR-FT/IR and fluorescence) it is possible to obtain a deep knowledge of protein/additive systems, facilitating the preparation of new and more efficient protein formulations, including that of protein-based drugs whose administration could be improved. Furthermore, in the field of biocatalysis carried out in nonaqueous media, the results herein reported together with the discovery of new hydrolases (and, more in general, new enzymes) with improved catalytic properties is a step toward the development of efficient non-aqueous biocatalytic systems suitable for a large variety of industrial applications.

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Figure 1. Mechanism of catalysis of lipase BC (Enz) with vinyl butyrate as acyl donor and 1-hexanol or water as nucleophile.

Figure 2. Initial transesterification (a) and hydrolytic (b) activity of lipase BC (white squares) and lipase BC + MePEG (black squares) in dodecane as a function of a_w .

Figure 3.

ATR-FT/IR spectra of liquid water (a) and of MePEG (b), lipase BC (c), lipase BC + MPEG (d) obtained from a_w 0.06 to a_w 1. Arrows point to the direction of increasing a_w . Insets in c compare regions of the spectra of lipase BC (thin line), MePEG (thin dashed line) and lipase BC + PEG (thick dashed line) at a_w 0.06. (Spectra were truncated at 640 cm^{-1} because of a low transmittance at lower wave numbers.)

Figure 4. Magnitude of the area of the band at 670 cm^{-1} and of the intensity at $3,500\text{ cm}^{-1}$ as a function of a_w , measured for lipase BC (crosses), lipase BC + MPEG (squares) and MePEG (triangles) in the spectra of Figure 3b–d.

Figure 5.

H/D exchange kinetic of lipase BC (squares) and lipase BC + MePEG (crosses). The value of ω of the non-deuterated protein (ω for $t = 0$) was determined from spectra of the samples equilibrated at a_w 0.06 and resulted of 0.68 and 0.64 for lipase BC and lipase BC + MePEG, respectively. As expected the presence of MePEG did not significantly affect ω . The value of ω for the fully deuterated protein was obtained from the samples incubated in the presence of the vapor phase of D_2O for 24 h (no significant variation was observed after 48 h) and resulted 0.508 and 0.405 for lipase BC and lipase BC + MePEG, respectively.

Figure 6. Second derivative ATR-FT/IR spectra of lipase BC (thin line) and lipase BC + MePEG (thick line) at a_w 0.06.

Figure 7. Fluorescence spectra of lipase BC (a) and N-acetyl-L-tryptophan ethyl ester (d) in buffer and of a dry film of lipase BC (b) or lipase BC + MePEG (c). Note that there is not scattering contribution above 310 nm.

Table I
Kinetic parameters of the amide H/D exchange measurements of a dry film of Lipase BC and lipase BC + MePEG.

Enzyme film	X_f	k_1	X_s	k_2	X_u
Lipase BC	0.150	0.0073	0.100	0.0010	0.750
Lipase BC + PEG	0.195	0.0129	0.152	0.0013	0.653

X_f , X_s , X_u are the fractions of the fast, slow and non-exchanging amide protons (see Materials and Methods Section for their determination). k_1 and k_2 are the apparent exchange rate constants for the fast and slow amide protons, respectively.