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Spectroscopic studies on poly(ethylene glycol)–lysozyme interactions

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Abstract

In the present paper, different spectroscopic methods were applied to evaluate conformational changes of hen egg-white lysozyme (HEWL) in various solvents and in the presence of poly(ethylene glycol) (PEG). In citrate (0.007 M, pH = 6), or in Tris (0.1 M, pH = 7.4), no conformational change of the protein was measured across the range of concentrations tested. In addition, HEWL in ultra-pure water revealed no irreversible conformational change and no activity loss, at least at low concentrations (≤0.2 mg/ml). Whereas PEG can induce a reorganization of water molecules, no change of the secondary and tertiary protein conformations was observed in the presence of PEG. In addition, in the presence of PEG of various molecular weights, no change of enzymatic activity of the HEWL was observed across the range of concentrations tested.

Keywords: Hen egg-white lysozyme; PEG; FTIR; Circular dichroism; Fluorescence spectroscopy; Enzymatic activity

1. Introduction

To better control drug release and to achieve targeting to specific tissues, sustained delivery systems can be formulated (Bazile et al., 1992; Stolnik et al., 1995). Biocompatibility and bioresorbability of these systems can be achieved by using polymers, such as poly(α-hydroxy acid)s (Mauduit and Vert, 1993). The double emulsion water-in-oil-in-water method, followed by solvent extraction and freeze-drying steps, is one of the most common techniques to encapsulate a wide range of drugs, proteins for instance, at high level concentrations in polymer matrices (Pistel et al., 1999). The primary emulsion step of this manufacturing process involves aqueous and organic phases. While proteins maintain their native structure in pure organic solvents, they often lose their stability and denature in organic–water mixtures (Griebenow and Klibanov, 1996; Sah, 1999). This is partly due to the adsorption of the proteins at interfaces created during the formulation process (Wang and Wu, 1998). Adsorption leads to irreversible conformational changes of the protein, unfolding, precipitation, and formation of aggregates that are almost always coupled with a biological activity loss (Morlock et al., 1997). Furthermore, the penetration of proteins in the polymer matrix, and the consequent formation of a mixed protein/polymer layer, leads to an uncompleted protein release in vitro (Boury et al., 1995; Pean et al., 1999; van de Weert et al., 2000).

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Different strategies frequently used to minimize protein adsorption were recently reviewed (van de Weert et al., 2000). Surface active compounds that compete with proteins at interfaces minimize protein adsorption. Among these compounds, one finds poly(ethylene glycol) (PEG), a polymer which presents various potential applications in the formulation area (Harris, 1992). Several studies reported a potential increase of the protein stability in the presence of PEG (Uchida et al., 1998; Castellanos et al., 2002). Our group showed that competition between PEG400 and Nerve Growth Factor (NGF) molecules at water/oil interfaces can lead to a decrease of protein denaturation by contact with the organic phase during the emulsification step, and the penetration of NGF in the interfacial film can be limited (Pean et al., 1999).

Furthermore, it is known that PEG presents an important excluded volume (Baht and Timasheff, 1992). Due to the correlated steric exclusion and to the osmotic stress, PEG can provide the driving force that generates phase separation (Lee and Lee, 1981; Arakawa and Timasheff, 1985). When added to a multicomponent system, PEG acts on the other components by increasing their effective concentrations. The effects of such crowding are, in particular, self-association, protein precipitation, or crystallization (Vergara et al., 2002). For these reasons, PEG is also used to form aqueous two-phase systems to separate and concentrate proteins (Haire et al., 1984; Lotwin and De Bernardez Clark, 1999), or to precipitate or crystallize proteins (McPherson, 1985; Moreno et al., 2000). According to some authors, PEG also interacts and weakly binds to the hydrophobic regions of unfolded and partially folded proteins (Lotwin and De Bernardez Clark, 1999; Nerli et al., 2001). For others, PEG is an inert polymer which does not interact with proteins (Harris, 1992; Pancera et al., 2002).

Our objective was to characterize conformational changes of NGF in the presence of PEG of various molecular weights by using different spectroscopic methods: infrared spectroscopy, circular dichroism (CD), and fluorescence spectroscopy. Nevertheless, for such systematic studies, hen egg-white lysozyme (HEWL) was preferred to NGF. Indeed, HEWL is a well-known characterized protein. Furthermore, the physicochemical properties of the model protein, HEWL, approach those of NGF. The active part of NGF is a homodimer with an apparent molecular weight ($\bar{M}_w$) for the monomer of 13,000 Da; HEWL is a monomeric globular protein with a $\bar{M}_w = 14,300$ Da. Both proteins are basic at neutral pH. The influence of PEG upon HEWL conformation was assessed in various buffers and at protein concentrations close to those used in the formulation area (Pean et al., 1999; Aubert-Pouessel et al., 2002; Castellanos et al., 2002).

2. Materials and methods

2.1. Materials

HEWL (prod. no. L-6876; 95%; 3× crystallized, dialyzed, and lyophilized) and *Micrococcus lysodeikticus* (fraction V 96–99% albumin) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France), and used without any further purification. PEG400 was supplied by Cooper (Melun, France). PEG2000 and PEG5000 were purchased from Servilab (Le Mans, France). Citrate (AR grade) was supplied by Prolabo (Paris, France). Ultra-pure water was obtained from a Millipore® system (Milli-Q Plus 185, Molsheim, France). NaCl (for analysis, ACS, ISO) was supplied by Merck (Nogent-sur-Marne, France). Tris(hydroxymethyl)aminomethane (Tris) was obtained from Acros Organics (Noisy-le-Grand, France).

2.2. Preparation of protein solutions

HEWL was dissolved either in water (pH = 6), citrate (0.007 M, pH = 6) or Tris (0.1 M, pH = 7.4) buffer, and solutions were gently stirred during 15 min. Citrate and Tris buffers were previously chosen as solvents by our group in protein encapsulation studies or in vitro protein release studies (Pean et al., 1999; Aubert-Pouessel et al., 2002). Protein solutions were added to appropriate quantities of PEG to give final HEWL/PEG ratios of 5:1 and 1:10 (w/w); the mixtures were stirred for 15 additional minutes. These HEWL/PEG ratios were also tested in studies previously reported by our group (Pean et al., 1999; Aubert-Pouessel et al., 2002).

2.3. Fourier transform infrared spectroscopy (FTIR) analysis

Spectra of HEWL (10 mg/ml) in distilled water, in absence or presence of PEG400, PEG2000, or PEG5000 (2 or 100 mg/ml), were recorded (Vector 22 Spectrometer, Brücker) between 400 and 4000 cm^{-1} at 4 cm^{-1} intervals using an attenuated total reflection (ATR) cell. All spectra were analyzed in the amide I band region (1720–1580 cm^{-1}). No correction of the spectra was made for the solvent. Due to the intense O–H bending mode around 1650 cm^{-1}, the presence of water molecules makes it difficult to investigate the secondary structure of proteins. In numerous papers (Prestrelski et al., 1991; Costantino et al., 1995; Hadden et al., 1995; Pouliot et al., 2002), this problem was overcome by subtracting the water spectrum. Nevertheless, difficulties arise when the contribution of water must be subtracted. Indeed, the scaling factor applied to the water spectrum prior to subtraction is rarely objective, and personal uncertainty can therefore result. In a previous paper, Robert et al. (2002) proved the relevance of Principal Component Analysis (PCA), a multivariate statistical treatment, to study the secondary structure of proteins in aqueous solution. The proposed data treatment makes it possible to extract relevant information on the conformation of proteins without performing a spectral subtraction of the water spectrum. Moreover, using the PCA, one can study spectra without deleting wavelengths and handle large data tables without making any preliminary assumption concerning the data. For these reasons, PCA was used in the present study to analyze FTIR spectra of HEWL.

PCA describes the data by assessing a small number of variables which are linear combinations of the original variables. The synthetic variables, called principal components, are uncorrelated and highlight the main variations observed in the data. The computation of principal components is based on the diagonalization of the variance–covariance matrix:

$$\boldsymbol{V} = \boldsymbol{X}'\boldsymbol{X}$$

where $\boldsymbol{X}$ is the matrix of the original data and $\boldsymbol{X}'$ the transpose of $\boldsymbol{X}$.

Diagonalization realizes the decomposition of $\boldsymbol{V}$ into eigenvectors $\boldsymbol{L}$ and eigenvalues $\boldsymbol{S}$. The eigenvectors are used for assessing the principal component scores $\boldsymbol{C}$ from the original data:

$$\boldsymbol{C} = \boldsymbol{X}\boldsymbol{L}$$

The eigenvalues indicate the percentage of total variance described by the components which are subsequently ranged according to this percentage. The power of the analysis largely lies in the graphical display obtained. Similarity maps of the samples can be plotted from the scores of two given principal components. Spectral patterns explaining similarities between samples were obtained using the eigenvectors (Robert et al., 1996).

2.4. CD analysis

The circular dichroic spectra were taken in the 185–260 nm range with a CD6 Dichrograph Instrument (Jobin-Yvon, Longjumeau, France) at 25 °C. Cuvettes of 0.1 cm path length were used. Sample concentrations were precisely determined after filtration on 0.2 μm filters (Anotop filters, Alltech, France) from the optical density at 281 nm, corrected for turbidity, using the value $A^{0.1\%}_{1\,cm} = 2.47$ (Fasman, 1976). Concentrations were typically 0.2 ± 0.03 mg/ml. All protein spectra in absence or presence of PEG were corrected for the solvent.

CD spectra were analyzed in terms of α-helix content by using the Dichroprot software, a package freely available online (http://dicroprot-pbil.ibcp.fr), which was developed by Deléage (Deléage and Geourjon, 1993, IBCP, Lyon). The Self-Consistent Method (SCM) for estimating the secondary structure content, in particular the α-helices content, was used. In this method, originally developed by Sreerama and Woody (1993), the spectrum of the protein to be analyzed is included in the basis set and an initial guess is made for the unknown structure as a first approximation. The resulting matrix equation is solved using the singular value decomposition algorithm and the initial guess is replaced by the solution. The process is repeated until self-consistency is attained. The best features of the variable selection and the locally linearized methods are incorporated into this procedure. In the Dichroprot software, the prediction for secondary structure content is calculated from four reference databases. The resulting

α-helix percentages will be given in the results as SCM_1, SCM_2, SCM_3, and SCM_4.

2.5. Fluorescence spectroscopy

Fluorescence emission spectra were obtained on a FluoroMax Spectrometer (Jobin-Yvon, Longjumeau, France) at 25 °C. For selective excitation of tryptophan, the excitation wavelength was set to 295 nm, and emission measured between 300 and 400 nm. The excitation and emission slit widths were set at 2 nm. All HEWL spectra were obtained for a protein concentration of 0.05 mg/ml and corrected for the corresponding solvent or PEG solution.

2.6. Enzymatic activity

The enzymatic activity of HEWL was determined by measuring the turbidity change in a *M. lysodeikticus* bacterial cell suspension.

The reported method by McKenzie and White (1986) was modified. HEWL was first dissolved either in pure water or Tris buffer (0.1 M) for 20 min. Thereafter, 100 μl of the protein solution was incubated in 2.9 ml of 0.015% (w/v) *M. lysodeikticus* suspension in Tris buffer (0.1 M) solution (37 °C, 4 h).

The absorbance of the suspension was measured at 450 nm (Uvikon 922 Spectrophotometer, Bio-Tek Kontron Instruments, Saint Quentin Yvelines, France). The comparison with a suspension without protein gives the lytic activity. The increase in absorbance was a second order polynomial function of HEWL mass in the concentration range studied (Aubert-Pouessel et al., 2002).

3. Results

Fig. 1a shows the similarity map obtained from infrared spectra of HEWL and HEWL/PEG mixtures in ultra-pure water. Similarity maps provide graphical comparisons of the samples by taking into account the main information extracted from the original data. Two close points observed on the similarity map characterize two similar spectra (Robert et al., 1996). The map shows a discrimination of the samples as a function of PEG concentration according to the principal component 1, but no obvious cluster due to PEG molecular weight is depicted. The principal component 2 discriminates spectra according to the presence of HEWL.

Fig. 1b shows the spectral patterns corresponding to the similarity map. The spectral pattern of the first principal component reveals an opposition between a trough at 1665 cm^{-1} and a peak at 1610 cm^{-1}, indicating a shift of the water absorption band. The spectral pattern associated with the second principal component is characterized by a negative peak near 1650 cm^{-1}. The 1650 cm^{-1} absorption band is assigned to the amide I band of the protein in α-helical conformation. In addition, the spectral pattern characteristic of the HEWL conformation revealed very small shoulders at about 1630 and 1670 cm^{-1}. These shoulders characterized the presence of small amounts of β-sheet structures as previously observed by Malzert et al. (2002).

Fig. 2a shows the CD spectra obtained for HEWL in pure distilled water, citrate and Tris buffers. No obvious difference can be observed between spectra. To more precisely compare the spectra, we used the Dichroprot software developed by Deléage (Deléage and Geourjon, 1993), and in particular the SCM. The percentage of α-helix content in each solvent assessed by using the four reference databases available is given Table 1. From the comparison of the mean SCM values in each solvent, i.e. from a statistical analysis of the variance (Fisher's test), percentages do not significantly differ between the solvents.

The influence of PEG onto HEWL conformational change was analyzed by CD. The investigated HEWL/PEG ratios were kept identical to those used in FTIR. Fig. 2b displays the CD spectra recorded for HEWL in the presence of PEG having different molecular weights. The results derived from Dichroprot

Table 1
Assessment of the percentage of HEWL α-helix content in different solvents, by each of the four reference databases available in the Self-Consistent Method (SCM)

	HEWL water (pH = 6, I = 0 M)	HEWL citrate (pH = 6, I = 0.007 M)	HEWL Tris (pH = 7.4, I = 0.1 M)
SCM_1	35	54	51
SCM_2	56	51	49
SCM_3	54	48	45
SCM_4	59	54	51

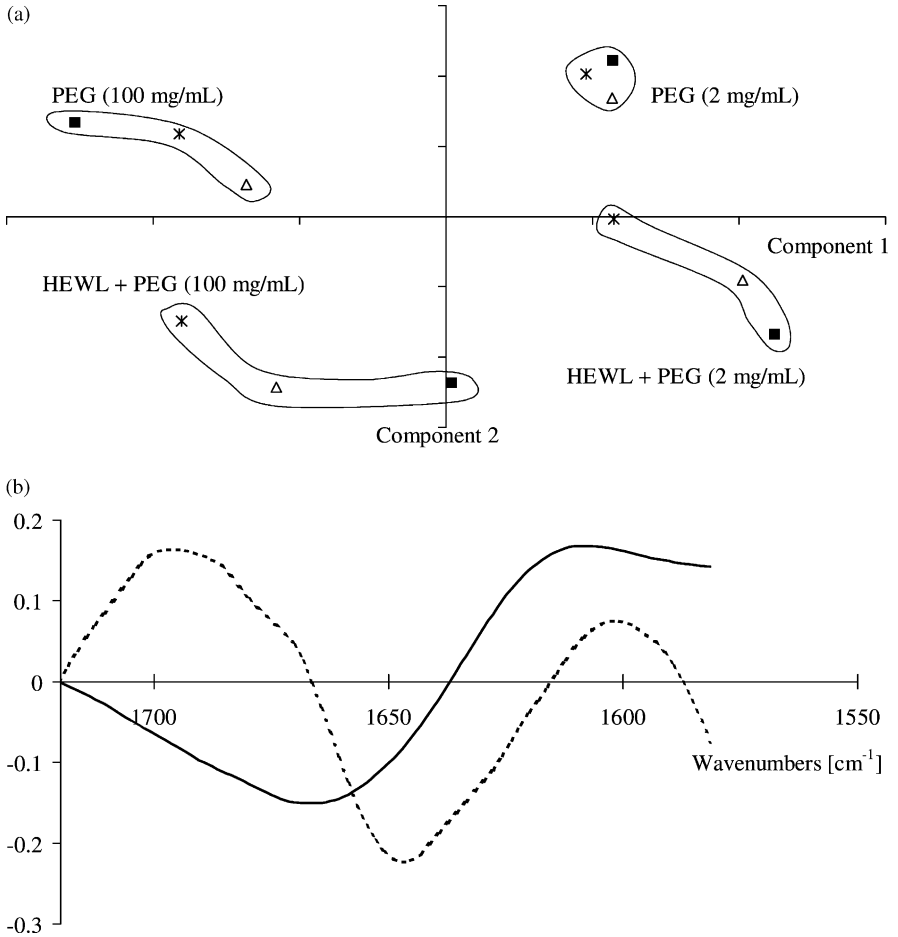

Fig. 1. (a) Similarity map defined by principal components 1 and 2 for the FTIR spectra obtained in the presence of PEG (2 and 100 mg/ml), and in the absence or presence of HEWL (10 mg/ml) in water. From that four different cases are taken into account: low/high PEG concentration; with/without HEWL. In all cases, designated by circles, PEGs of different molecular weights were used: 400 (■); 2000 (✳); 5000 (△). (b) Spectral pattern for eigenvectors 1 (—) and 2 (- - -) of the principal component analysis of the FTIR spectra obtained with or without HEWL (10 mg/ml), and with PEG400, PEG2000, and PEG5000 (2 and 100 mg/ml) in water.

analysis are given in Table 2. From the comparison of the mean SCM values in each solvent, i.e. from a statistical analysis of the variance (Fisher's test), no significant difference was observed between the percentages of α-helices of HEWL in the presence of PEGs in ultra-pure water. Studies were also carried out in citrate and in Tris buffers (results not shown), and in both cases, from a statistical point of view, no

Table 2
Assessment of the percentage of HEWL α-helix content in different PEG aqueous solutions, by each of the four reference databases available in the Self-Consistent Method (SCM)

	PEG400−	PEG400+	PEG2000−	PEG2000+	PEG5000−	PEG5000+
SCM_1	38	51	50	51	52	49
SCM_2	53	52	53	53	52	48
SCM_3	50	45	45	49	45	47
SCM_4	49	51	50	51	52	49

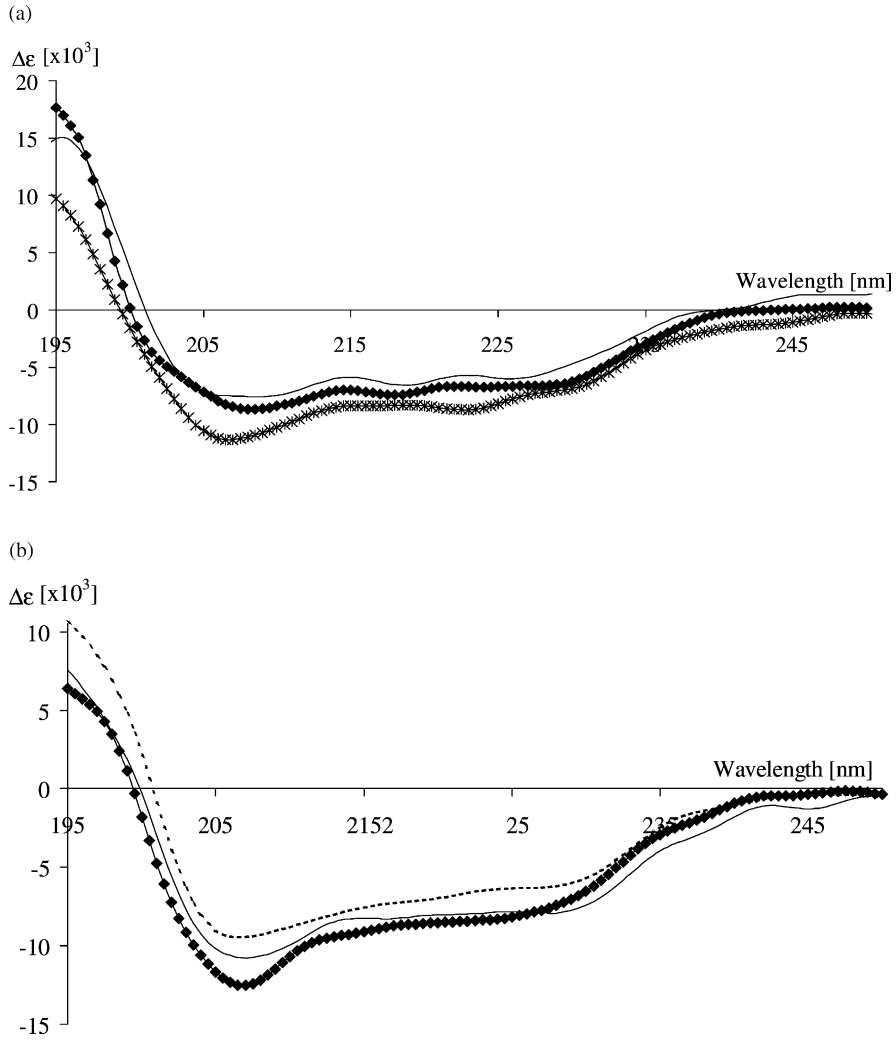

Fig. 2. (a) Dichroic spectra obtained for HEWL (0.3 mg/ml) in water (–✳–), citrate (—) or Tris (–◆–) buffer. (b) Dichroic spectra obtained for HEWL (0.3 mg/ml) in water in the presence of PEG400 (3 mg/ml) (—), PEG2000 (3 mg/ml) (- - -), and PEG5000 (0.06 mg/ml) (–◆–).

significant difference was observed between percentages of α-helices of HEWL in the presence of PEGs.

Fig. 3a and b show the fluorescence emission spectra of HEWL. In the different solvents tested, in absence or in presence of PEG of different molecular weights and concentrations, a maximum of fluorescence emission at 341 nm was observed. This indicates that no obvious conformational change of HEWL took place in the various experimental conditions tested here.

The influence of both the solvent and PEG on the enzymatic activity of HEWL was also evaluated. Fig. 4 displays the HEWL enzymatic activity curves previously obtained (Malzert et al., 2002) when both HEWL and its substrate were dissolved in Tris. In addition, the results acquired in the case of HEWL dissolved in water were also reported. No obvious difference was observed between both the curves.

In the presence of PEG (HEWL/PEG ratios of 5:1 and 1:10 (w/w)), the experimental data were

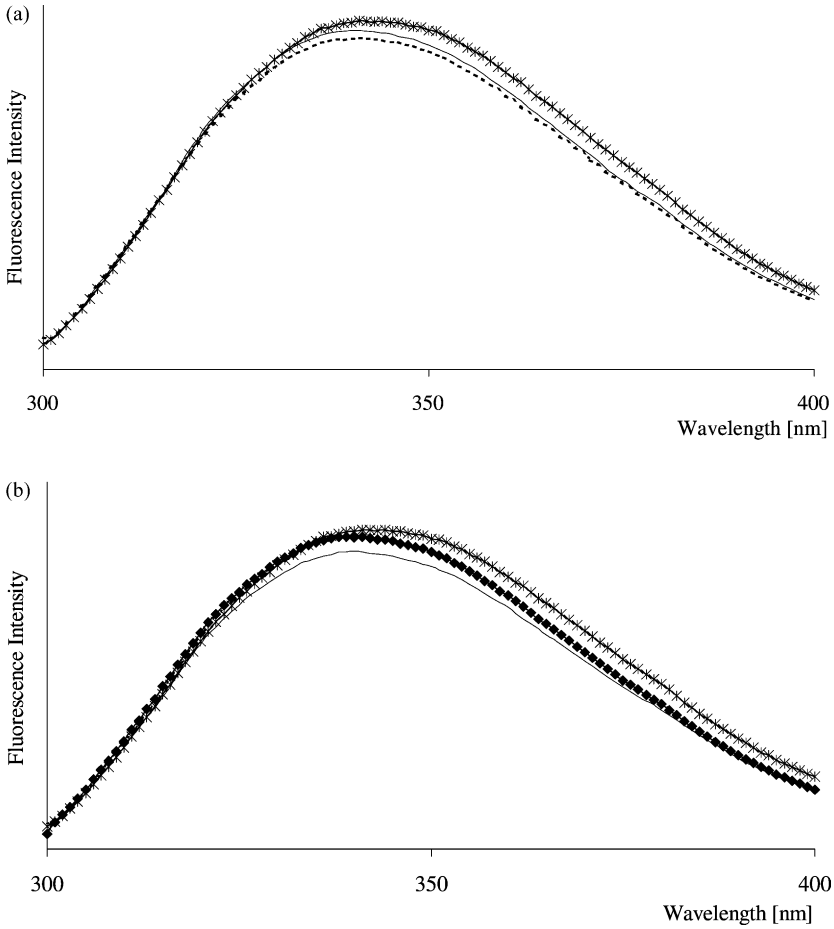

Fig. 3. (a) Fluorescence spectra obtained for HEWL (0.05 mg/ml) in water (–✳–), Tris (—) or citrate (- - -) buffer. (b) Fluorescence spectra obtained for HEWL (0.05 mg/ml) in water (–✳–), and in presence of PEG2000 (0.01 mg/ml) (- - -), PEG5000 (0.01 mg/ml) (–◆–), and PEG5000 (0.5 mg/ml) (—).

comprised within the reproducibility margin of error and support the idea that the presence of PEG does not induce any HEWL enzymatic activity change.

4. Discussion

4.1. Influence of the solvent

Far-UV CD measurements indicated that no change in the secondary structure of HEWL took place, the solvent being ultra-pure water, citrate or Tris buffer.

Regarding the tertiary conformation of the protein, fluorescence spectroscopy is applied to probe the environment of tryptophan residues. Among the six tryptophan residues of HEWL, one is buried inside the protein molecule. The others, and in particular two residues which are found in the protein split, are more solvent accessible (Fig. 5a and b). While this conformation would make the protein sensitive to the solvent environment, no change of HEWL conformation is shown in the various solvent conditions tested.

Previous studies using FTIR measurements showed the presence of some intermolecular β-sheets when HEWL was in distilled water (Malzert et al., 2002).

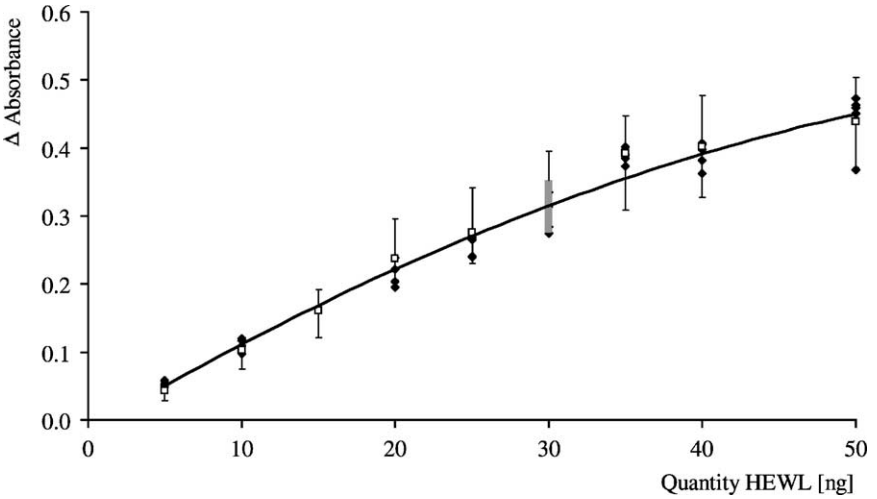

Fig. 4. Calibration curves of the enzymatic activity of HEWL when both HEWL and its substrate (*Micrococcus lysodeikticus*) are in Tris (0.1 M, pH = 7.4) (□), or when HEWL is first in water and the substrate in Tris (0.1 M, pH = 7.4) (◆). Determination of the enzymatic activity of HEWL for a quantity of 30 ng (corresponding to a total concentration in the bulk of 10 ng/ml) in the presence of PEG400, PEG2000, and PEG5000 (2 or 100 ng/ml) (▮).

Moreover, preliminary dynamic light scattering results revealed the existence of HEWL aggregates when the protein was dissolved in water (results not shown). These aggregates had a hydrodynamic radius of 73 nm. In contrast, no aggregates were found when the protein was dissolved in 0.1 M NaCl.

Previous HEWL lytic assay showed that, in the studied conditions, the enzymatic activity of the protein was maximal when both the enzyme and its substrate were in Tris buffer, but were canceled when both were in pure water, i.e. in these conditions, no turbidity variation was measured whatever the protein concentration (Malzert et al., 2002). Absorbance values determined for the blank samples (without HEWL) were identical when *M. lysodeikticus* was dissolved in pure water or in Tris buffer (0.1 M) (Malzert et al., 2002). In the present study, no difference was observed when HEWL was first dissolved either in pure water or Tris buffer (0.1 M) for 20 min while *M. lysodeikticus* was in Tris (0.1 M). Thus, the HEWL activity loss would be reversible, and could be attributed to a conformational change of the catalytic site of HEWL in pure water, i.e. the region that concerns only two amino acid residues, Glu35 and Asp52 (Vocadlo et al., 2001). However, from CD and fluorescence spectroscopy measurements, we were not able to determine any change of the secondary and tertiary conformations of HEWL molecules.

4.2. Influence of PEG

In the present FTIR study, the similarity map corresponding to principal components 1 and 2 described the main variations in the spectral data. The main spectral variations were due to the presence of PEG and HEWL. The presence of PEG modified the water structure, shifting the infrared absorption band of water at about 1640 cm^{-1} to higher wavenumbers, whatever the molecular weight (PEG400, PEG2000, or PEG5000). It is known that two to three water molecules are linked to each PEG monomer through hydrogen bonds (Antonsen and Hoffman, 1992; Ide et al., 1999). The polymer disturbs the structure of water, giving rise to a more complex network made of water molecules and PEG segments (McPherson, 1985). No change in the secondary structure of HEWL was observed taking the first two principal components, or higher principal components, into account. While principal component 1 described the changes in the structure of water, principal component 2 characterized the presence of HEWL. Principal component 2 revealed that the HEWL was mainly in α-helical structure. PCA unfortunately failed in studying the

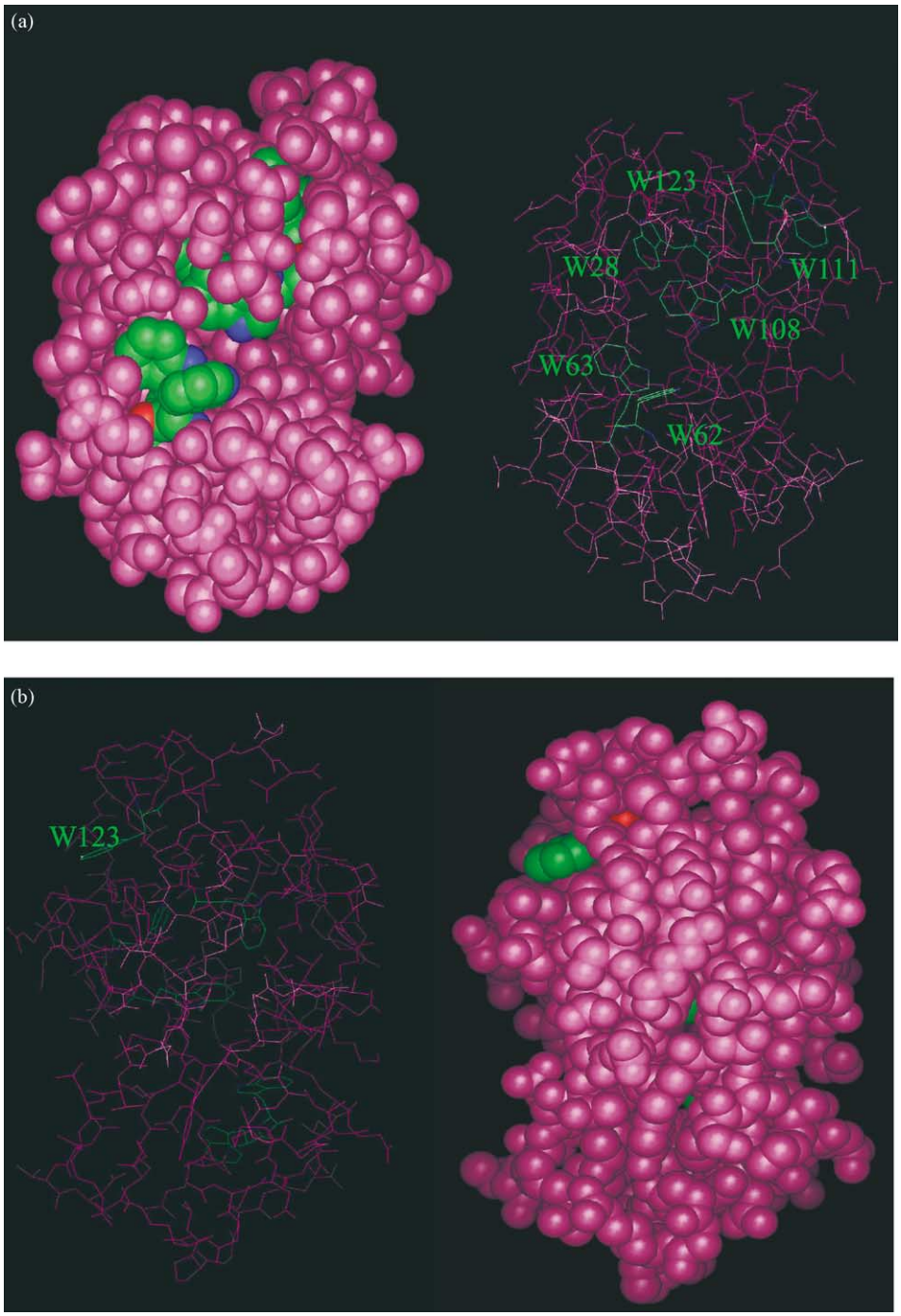

Fig. 5. Localization of tryptophan (W) residues from 193l (Protein Data Bank) HEWL molecular modeling images. (a) HEWL split overview, (b) HEWL back view.

influence of PEG on the conformational changes of HEWL in the bulk. The main changes in infrared spectra concerned the water structure and the presence of protein. Hypothetical changes in the secondary structure of HEWL were too small to be depicted using PCA. Indeed, the first two principal components took more than 95% of the total inertia into account. Castellanos et al. (2002) investigated by FTIR the conformation of γ-chymotrypsin after encapsulation in poly(D,L-lactic-co-glycolic) acid microspheres. According to the authors, PEG would be able to prevent encapsulation-induced structural changes of the protein.

From far-UV CD measurements, a statistical analysis of the α-helix content indicates that the presence of PEG does not induce any change of the HEWL secondary structures. Similar results were reported in the literature for another HEWL/PEG ratio (Lee and Lee, 1981).

From the fluorescence spectroscopy results, no major change of the environment of aromatic residues of the protein in the presence of PEG was depicted. Other authors reported that the conformational stability of bovine serum albumin was unaffected by the addition of PEG10,000 and PEG35,000 (Jiang and Schwendeman, 2001).

The addition of different concentrations and various molecular weights of PEG did not disturb the enzymatic activity of the protein. This result supports all the spectroscopic data about the absence of conformational change of the protein in the presence of PEG. It was also reported that no significant change in HEWL enzymatic activity was observed in the presence of PEG400 (Kang et al., 2002). Nevertheless, other authors reported that in comparison to the enzymatic activity measured in pure Tris buffer (pH = 7.5), in the presence of PEG400 (20 wt.%) or PEG4000 (20 wt.%), the activity of glucose-6-phosphate dehydrogenase increased by 20% (Pancera et al., 2002). These results indicate that the influence of PEG on protein conformation and enzymatic activity could be protein type dependent.

5. Conclusion

In accordance with the previous FTIR measurements, preliminary dynamic light scattering results revealed the presence of HEWL aggregates in ultra-pure water. Nevertheless, the results obtained on HEWL stability by various spectroscopic methods and enzymatic activity assays indicated that no irreversible conformational change of HEWL in ultra-pure water occurs, at least at low HEWL concentrations ($\leq$0.2 mg/ml).

Whereas PEG can provoke a reorganization of water molecules, its presence in an HEWL solution induces no change of the protein secondary and tertiary conformations in conditions relevant to the formulation processes (Pean et al., 1999; Aubert-Pouessel et al., 2002), as shown by CD and fluorescence spectroscopy studies, respectively. The enzymatic activity of the protein is also maintained and remains unmodified in the presence of PEG.

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