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Biochemical and biophysical characterization of lysozyme modified by PEGylation

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ABSTRACT

PEGylation is a strategy that has been used to improve the biochemical properties of proteins and their physical and thermal stabilities. In this study, hen egg-white lysozyme (EC 3.2.1.17; LZ) was modified with methoxypolyethylene glycol-p-nitrophenyl carbonate (mPEG-pNP, MW 5000). This PEGylation of LZ produced conjugates that retained full enzyme activity with glycol chitosan, independent of degree of enzyme modification; its biological activity with the substrate *Micrococcus lysodeikticus* was altered according to its degree of modification. The conjugate obtained with a low degree of mPEG-pNP/NH_2 modification was studied by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), demonstrating a spectral peak at m/z 19,988 Da with 77% of its original enzymatic activity. Spectroscopic studies of Fourier transform infrared (FTIR) and circular dichroism (CD) did not show any relevant differences in protein structure between the native and conjugate LZ. Studies of the effects of pH and temperature on PEGylated LZ indicated that the conjugate was active over a broad pH range, stable at 50 °C, and demonstrated resistance to proteolytic degradation.

1. Introduction

While protein and peptide drugs hold great promise as therapeutic agents, many are degraded by proteolytic enzymes, can be rapidly cleared by the kidneys, generate neutralizing antibodies, or have short circulating half-lives. The covalent attachment of polyethyleneglycol (PEG) chains onto peptides and proteins is a process known as PEGylation, and this technique has attracted growing interest as it offers the possibility of enhancing the therapeutic and biotechnological potential of strategic proteins (Abuchowski et al., 1977; Harris and Veronese, 2003). When PEG is properly linked to a polypeptide it modifies many of its features, although the main biological functions (such as enzymatic activity or receptor recognition) can often be maintained.

The PEG conjugation often leads to a modified protein with improved solubility and temperature stability, enhanced resistance to enzymatic degradation, increased serum half-life, decreased renal clearance, and lower immunogenicity, thus enhancing its biological effectiveness (Harris et al., 2001; Veronese, 2001; Colonna et al., 2008; Veronese and Mero, 2008). The PEGylated forms of adenosine deaminase and asparaginase (Soares et al., 2002; Zhang et al., 2004; Veronese and Pasut, 2005) were approved for human use by the U.S. Food and Drug Administration, and other PEG-modified proteins are being developed for possible therapeutic use, including PEG epidermal growth factor, PEGylated single-chain Fv proteins (Lee et al., 1999; Daly et al., 2005), and PEGylated drugs for the treatment of hepatitis C, acromegaly, rheumatoid arthritis and various cancers (Veronese and Pasut, 2005). Covalent attachment of PEG strands to proteins, however, is occasionally accompanied by a substantial loss of functional biological activity that may be due to the presence of the PEG moiety on or near the protein's active or regulatory site (Lee et al., 2001).

PEG size and the degree of modification have increasingly been explored with the aim of preserving protein activity. However, the loss of functional biological activity is a particularly severe problem for proteins acting on macromolecular substrates as LZ and most receptor-binding proteins, such as, cytokines and chemokines (Caliceti et al., 1990; Chiu et al., 1993; Zalipsky, 1995; Nodake and Yamasaki, 2000).

Numerous approaches have been explored to deal with the problem of biological activity losses as a result of PEGylation: site-specific PEGylation, sometimes in combination with site-specific mutagenesis (Goodson and Katre, 1990; Kinstler et al., 1996; Gaertner and Offord, 1996; Sato et al., 1996; Cazalis et al., 2004); protection of receptor-binding site during conjugation (Caliceti et al., 1993, 1994); and reversible PEGylation (Peleg-Shulman et al., 2004; Zalipsky et al., 2007). Each of these approaches has its merits and potential effectiveness in specific situations, although all of them have nontrivial technical obstacles.

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Lysozyme (LZ) is an important natural bactericidal enzyme (EC 3.2.1.17) that is widely distributed in nature that helps protect against microbial infections. LZ lyses the cell wall of various Gram-positive bacteria by splitting β (1–4) linkages between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan in bacterial cell walls and is widely used in food preservation protocols, pharmacology, and has various therapeutic applications (Mine et al., 2004; Johnson and Larson, 2005). LZ is a small protein (14.4 kDa, similar in size to various cytokines and chemokines) but it has lytic activity directed toward a very large substrate – the bacterial cell wall peptidoglycan. LZ therefore represents a suitable model for other small proteins whose biological activity involves enzymatic interactions with large substrates (Nodake and Yamasaki, 2000; Zalipsky et al., 2007).

The present work examined the modification of LZ by PEGylation with methoxypolyethyleneglycol-p-nitrophenyl carbonate (mPEG-pNP), investigating important properties of the resulting conjugates such as their degree of modification, time course reaction, enzyme activity, stability and resistance to proteolysis, as well as structural aspects as revealed by MALDI-TOF, FTIR and circular dichroism.

2. Materials and methods

2.1. Materials

Hen egg-white lysozyme (E.C 3.2.1.17), methoxypolyethylene glycol-p-nitrophenyl carbonate 5000 Da (mPEG-pNP 5000), and glycol chitosan were purchased from Sigma (St. Louis, MO). Gelcolde® Blue Stain Reagent from Pierce (Rockford, USA); Sephadex G-50 from Amersham Pharmacia Biotech AB (Uppsala, Sweden). *Micrococcus lysodeikticus* was obtained from ICN Pharmaceuticals (Costa Mesa, CA, USA); Proteomix® (trypsin and chymotrypsin) from Biobrás (Montes Claros, MG, Brazil); Protex 6L® (bacterial protease) and fungal protease from Genencor International, Inc. (New York, USA). All other reagents used were of analytical grade.

2.2. Enzyme assays

2.2.1. Lytic activity with M. lysodeikticus

The enzymatic activity of lysozyme and its conjugate was determined by measuring turbidity changes in *M. lysodeikticus* bacterial cell suspensions (0.5 mg/ml) in 50 mM of pH 7.0 phosphate buffer, as previously reported by Shugar (1952). Absorbance of the suspension was measured at 450 nm, and a decrease in absorbance of 0.001 was defined as 1 unit of lysozyme activity. The protein concentration was determined by the Bradford method (Bradford, 1976) at 595 nm, using a standard curve for lysozyme.

2.2.2. Enzyme activity with glycol chitosan

The enzyme activities of lysozyme and the modified lysozyme were also assayed using the substrate glycol chitosan, following the method of Imoto and Yagishita (1971). A mixture of 1 ml of 0.05% (w/v) glycol chitosan in 100 mM of pH 5.5 acetate buffer and 100 μl of lysozyme or modified lysozyme solution (2 mg/ml) was incubated at 40 °C for 30 min. After reacting, 2 ml of the color reagent (0.5 g of potassium ferricyanide in 1 l of 0.5 M sodium carbonate) was added and the mixture was immediately heated to boiling and held at a boil for 15 min (the control solutions contained no enzyme). After cooling, absorbance was read at 420 nm.

2.2.3. Electrophoresis (SDS-PAGE) of the modified lysozyme

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and staining with Gelcolde® Blue Stain Reagent was used to analyze PEGylated lysozyme.

Table 1
Different molar ratio mPEG-pNP 5000/NH_2.

	Lysozyme (mg)				
	10	10	10	10	10
mPEG-pNP5000/LZ (m/m)[a]	2	1	0.5	0.25	–
mPEG-pNP 5000/NH_2[b]	0.86	0.43	0.21	0.11	–

[a] Mass ratio mg/mg.
[b] Molar ratio mPEG-pNP 5000/NH_2. Each molecule of lysozyme own 7 amine group (6 ε-lysine and 1 N-terminal).

2.2.4. Time course reaction of PEGylation of lysozyme

The LZ PEGylation was followed for different time periods. A volume of 500 μl of a 4 mg/ml LZ solution and 200 μl of 10 mg/ml of mPEG-pNP (molar ratio: 0.43) solution in pH 7.5 Hepes buffer were placed in a reaction tube and the reaction was allowed to proceed at 30 °C. Aliquots (20 μg) were withdrawn after 1, 3, 5, 7, 10, 20, 60 and 120 min and added to 30 μl of sample buffer and immediately boiled to halt the reaction. The samples were then analyzed on 10% SDS-PAGE gel.

2.2.5. Chemical modification of lysozyme with different molar ratio of mPEG-pNP 5000

Different molar ratios of mPEG-pNP/NH_2 were used to examine their effects on the degree of enzyme modification. A 10 mg/ml solution of lysozyme was prepared in 100 mM pH 8 phosphate buffer and stoichiometric amounts of mPEG-pNP 5000 were added to obtain different molar ratios of mPEG-pNP/NH_2 (Table 1). The mixtures were incubated at 30 °C for 2 h. Aliquots (20 μg) of each sample were taken and electrophoresed on 10% SDS-PAGE gels to determine their degree of LZ modification. The lytic activity of each sample (with different molar ratios of mPEG-pNP/NH_2) was measured using *M. lysodeikticus* and glycol chitosan substrates.

2.2.6. Preparation and purification of modified lysozyme

To evaluate the enzymatic activity and stability of the modified LZ, conjugate with lower degree of modification was prepared and purified by adding 2.5 mg of mPEG-pNP at a lower molar ratio of 0.11–1 ml of LZ solution (10 mg/ml) in 100 mM pH 8.0 phosphate buffer. The reaction was carried out at 30 °C for 2 h and incubated at 8 °C for 16 h. Excess mPEG-pNP and any unreactive enzyme were removed by size exclusion chromatography using Sephadex G-50. The column was pre-equilibrated with 100 mM pH 7.0 phosphate buffer and eluted with same buffer. Fractions showing enzyme activity were pooled and used as source of the PEGylated enzyme. The samples were assayed for enzyme activity against the substrate *M. lysodeikticus* as described above, and molecular mass modifications were examined using SDS-PAGE and MALDI-TOF. This conjugate was also submitted to studies of FTIR, circular dichroism, evaluation of enzymatic activity, stability at different pHs and resistance to proteolysis.

2.2.7. MALDI-TOF mass spectrometry

Both the native and conjugate LZ was analyzed in a MALDI-TOF mass spectrometer (Ettan-MALDI-TOF Pro 2.01 Amersham Biosciences). The samples were prepared using sinapinic acid as a matrix dissolved in acetonitrile/H_2O_2 [50% (v/v)]. A 100 μM solution of native or conjugate LZ was mixed with sinapinic acid 1:50 (v/v) and 0.5 μl of the mixture was spotted on the target surface. Typically, 200 laser shots were averaged. MALDI-TOF operating conditions were set as follows: the operating mode was linear, polarity positive, with an acceleration voltage of 20,000 V. Cytochrome C and bovine albumin were used as molecular mass standards for external calibration.

2.2.8. Fourier transform infrared spectroscopy (FTIR) analysis

Aliquots (1 ml) the native LZ (10 mg/ml) and LZ-mPEG-pNP (3.0 mg/ml) solution in 10 mM potassium phosphate, pH 7.4 buffer containing 2% (w/v) mannitol were frozen at −70 °C and lyophilized an FTS Systems freeze drier at chamber pressure 0.1 mbar. At 1 mg of lyophilized powder (native LZ or conjugate LZ) was weighed and mixed with a 100-fold excess weight of KBr. Infrared spectra were obtained with a Bomem MB100 FTIR spectrometer equipped with a DTGS detector. The spectra were recorded between 400 and 4000 cm^{-1} and for each sample, a total of 64 scans were collected at a resolution of 4 cm^{-1}. The spectra obtained were analyzed in the amide I band region (1700–1600 cm^{-1}).

2.2.9. Circular dichroism (CD) analysis

Spectroscopic characterization was carried out using 1 μM solutions of native LZ or conjugate LZ in 20 mM phosphate buffer pH 7.0, in a JASCO J-810 spectropolarimeter with a 0.1 cm path length quartz cell, at 20 °C. The CD spectra were recorded in the 189–260 nm range with a data pitch of 0.1 nm. Spectrums were obtained by averaging five individual scans. The spectra of buffer blanks were measured before the samples, and were subtracted from the sample CD spectra. CD spectra were analyzed in terms of α-helix content in the range 200–240 nm using the K2D2 software package that is available online (http://www.ogic.ca/projects/k2d2).

2.2.10. Evaluation of enzymatic activities and the stability of native and modified lysozyme at different pHs

Enzymatic activity was determined using buffers that varied from pH 4 to 12. Suspensions of *M. lysodeikticus* (0.5 mg/ml) were prepared in a series of buffers (pH 4–5, 50 mM acetate; pH 6–8, 50 mM Hepes and pH 9–12, 50 mM Tris–HCl) at 25 °C and enzymatic activity was immediately measured by reading the absorbance at 450 nm as described above. The assays were conducted in three independent experiments.

Enzyme stability was determined after incubation for 1 h in the same buffers (pH 4–5, 50 mM acetate; pH 6–8, 50 mM Hepes and pH 9–12, 50 mM Tris–HCl) at 50 °C. At the end of this 1 h period, enzymatic activity was determined against the substrate *M. lysodeikticus* as described above. The assays were conducted in three independent experiments.

2.2.11. Resistance to proteolysis

The resistance of native LZ and its conjugates to proteolysis was examined using Proteomix® (trypsin and chymotrypsin), Protex 6L® (bacterial protease), and fungal protease. 20 IU of each protease was added to 100 μl of native LZ (600 IU) or 100 μl LZ-mPEG-pNP (465 IU) in a total volume of 1 ml of 100 mM pH 7.0 phosphate buffer. The reaction mixture was incubated at 37 °C and aliquots were withdrawn at 0, 15, 30, 60, 120 min for immediate determination of enzymatic activity. The assays were conducted in three independent experiments.

3. Results and discussion

3.1. Effect of reaction time on the degree of lysozyme modification

In order to couple PEG to a protein molecule it is necessary to prepare a PEG derivative with a functional group at one or both termini. The functional group must be chosen based on the type of reactive group(s) available on the receptor molecule. For LZ, lysine is the typical receptor amino acid and PEG has to be activated with functional groups suitable for undergoing reactions with lysine (Roberts et al., 2002). PEG activated with the functional group p-nitrophenyl carbonate was used in the present study.

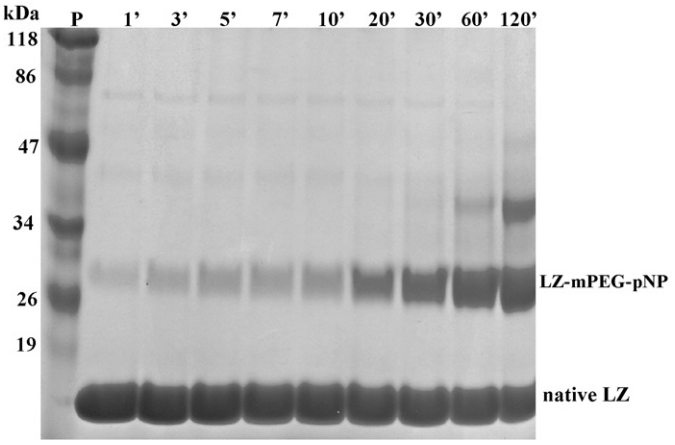

Fig. 1. Electrophoresis in polyacrylamide SDS-PAGE 10%. Time course reaction of LZ with mPEG-pNP 5000. P: standard of molecular mass. 1, 3, 5, 7, 10, 20, 30, 60 and 120 min, indicate the time interval in minutes of aliquots collected and submitted to electrophoresis.

As can be observed by Fig. 1 (reaction time course, as describe in Section 2.2.4) the reactive group p-nitrophenyl carbonate reacted relatively slowly with LZ even at high molar ratios (0.43), in contrast to other fast-reacting mPEGs such as succinimidyl succinate (Veronese and Pasut, 2005; Freitas and Abrahão-Neto, in press). This slow-reactive property of the p-nitrophenyl carbonate group permits a more precise control of the degree of modification of the LZ, and the reaction can be stopped whenever necessary to limit the degree of enzyme modification. Fig. 1 demonstrates that up to a reaction time of 30 min there is a band corresponding to a LZ-mPEG-pNP conjugate (although another band of higher molecular weight can be detected after 60 min). Thus, a homogeneous conjugate of LZ-mPEG-pNP 5000 can be obtained by adjusting the molar ratio and reaction time. The reaction can be terminated by the addition of molar excesses of lysine, which also makes it easier to separate the modified enzyme from the excess amounts of the polymer and any unreacted lysozyme.

3.2. Modification, characterization and evaluation of the enzymatic activity of the PEGylated lysozyme

The specific effects of PEGylation on the physical–chemical and biological properties of proteins are strictly determined by protein and polymer properties and by the PEGylation strategy adopted. The average size of the attached PEG moiety, as well as the total number of available attachment sites on the protein contribute to the size and net total molecular weight of the conjugated protein (Harris et al., 2001; Caliceti and Veronese, 2003). A small protein such as LZ generally has few attachment sites.

SDS-PAGE analysis of the modification of LZ using different molar ratios of mPEG-pNP (Table 1) is shown in Fig. 2. More bands are found in modified LZ using mPEG-pNP 5000/NH_2 at molar ratios of 0.21, 0.43 and 0.86 (lanes 3, 4 and 5) as compared to the use of a molar ratio of 0.11 (lane 2) (which resulted predominantly in a single band corresponding to the modified enzyme). This molar ratio (0.11) was found to be the most appropriate for our experiments because it represented the minimum concentration of PEG necessary to produce the more active LZ-mPEG-pNP conjugate (as can be seen in Figs. 2 and 3).

Nodake and Yamasaki (2000) and Lee and Park (2003) described seven residues to which the mPEG-derivative could covalently attach on the lysozyme molecule (1 α-amino group of N-terminal, and 6 ε-amino groups of the lysine residues K1, K13, K33, K96, K97 and K116), and the reactivity of these residues was determined to

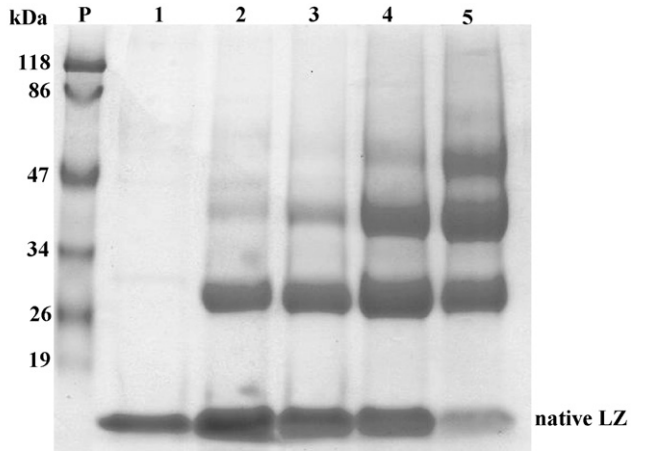

Fig. 2. Electrophoresis in polyacrylamide SDS-PAGE 10%. Lysozyme modified in different molar ratio mPEG-pNP 5000/NH_2. P: standard of molecular mass; lane 1: native LZ; lane 2: mPEG-pNP 5000/NH_2 = 0.11; lane 3: mPEG-pNP 5000/NH_2 = 0.21; lane 4: mPEG-pNP 5000/NH_2 = 0.43; lane 5: mPEG-pNP 5000/NH_2 = 0.86.

be K33 > K97 > K116 (Veerapandian et al., 1985; Yamada et al., 1986; Lee and Park, 2003; Masuda et al., 2005). Thus, the band obtained with the 0.11 molar ratio of mPEG-pNP suggests that PEGylation occurred mainly at the more reactive lysine site (K33) as observed by Nodake and Yamasaki (2000). When higher concentrations of polymer were used they apparently linked to the additional lysines (K97, K116), resulting in the production of a heterogeneous mixture of conjugated compounds with different molecular masses (Harris and Chess, 2003; Veronese and Pasut, 2005; Balan et al., 2007) as could observed by band profiles in Fig. 2, lanes 4 and 5.

Enzymatic activity is another critical property of conjugates with different degrees of mPEG-pNP attachment. The enzymatic activities of the different conjugates were determined using the substrates *M. lysodeikticus* and glycol chitosan (Fig. 3). In general, the enzyme gradually lost activity with increasing modification, probably due to the attachment of mPEG to free lysine on the protein surface that produced steric hindrance and prevented the formation of the enzyme–substrate complex. This is a particularly severe problem for proteins acting on macromolecular substrates (Caliceti et al., 1990; Chiu et al., 1993; Zalipsky, 1995; Nodake and Yamasaki, 2000). This behavior was observed in the activity profile of PEGylated LZ with the substrate *M. lysodeikticus* (Fig. 3). Using high mPEG-pNP molar ratios (0.86), for example, retained only 8% of their original enzymatic activity, while using a molar ratio of 0.11 retained 77% of its activity (Fig. 3).

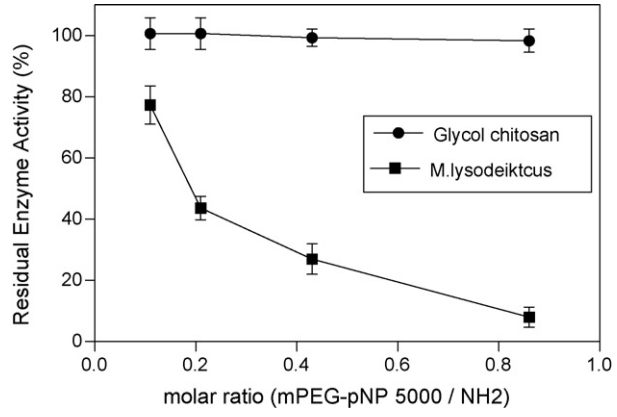

Fig. 3. Residual enzyme activity (%) of LZ-mPEG-pNP 5000 in different molar ratio (mPEG-pNP 5000/NH_2) of 0.11, 0.21, 0.43, 0.86 toward substrate *M. lysodeikticus* and glycol chitosan. (The results are means ± S.D., $n = 3$).

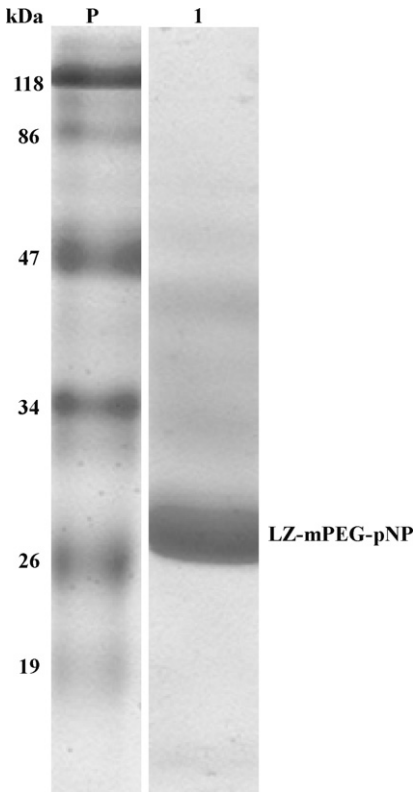

Fig. 4. Electrophoresis in polyacrylamide SDS-PAGE 10% of product of the purification of LZ-mPEG-pNP 5000. P: molecular mass standard; lane 1: conjugate LZ-mPEG-pNP.

Since the cell wall material is considerably larger than LZ, contact between the enzyme and its substrate most likely occurs on a relatively flat surface rather than in a deep groove, and PEG covalently attached to lysozyme could prevent the formation of the enzyme–substrate complex and inhibits its activity. This result demonstrated that it is possible to obtain a conjugate with 77% of its original enzyme activity after permanent PEGylation (using a molar ratio of 0.11). Similar result was obtained in the work of Nodake and Yamasaki (2000) that the enzyme activity of modified LZ was 75%. Noteworthy, Fig. 3 shows that the different degrees of LZ modification did not have any effect on its catalytic function with the smaller and more simple glycol chitosan substrate because the catalytic function of lysozyme in hydrolyzing β-1,4-N-acetylglucosamine linkages was maintained after modification with mPEG-pNP, showing that the active site retained its functional conformation.

In addition, preliminary experiments were conducted to verify the stability of the reaction PEGylation with PEG (mPEG-succinimidyl succinate or mPEG-p-phenyl carbonate) and LZ under similar conditions for measuring enzyme activity with the substrate glycol chitosan (pH 5.5–6.5, temperature 50 °C). The samples were analyzed by SDS-PAGE and the protein profile of them indicated that the PEGylation reaction was stable and did not occur to de-PEGylation of LZ (results not shown).

3.3. Purification and structural analysis of PEGylated lysozyme

The conjugate obtained with mPEG-pNP 5000/NH_2 at a molar ratio of 0.11 was purified by size exclusion chromatography using Sephadex G-50 (as described in Section 2.2.6) with yield of 30%. The molecular mass of this conjugate was investigated using both SDS-PAGE (Fig. 4) and MALDI-TOF. The analysis of LZ-mPEG-pNP

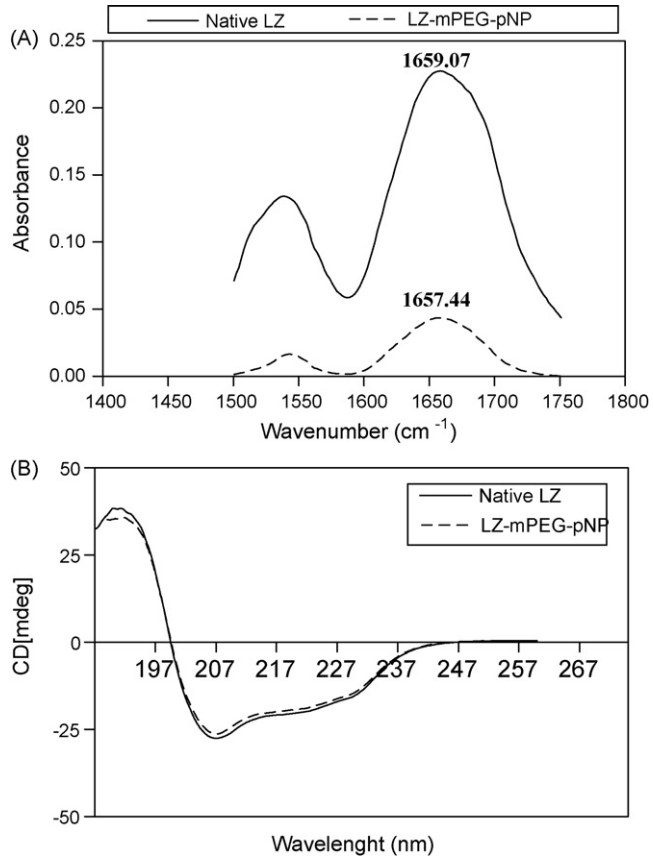

Fig. 5. Fourier transform infrared (A) and circular dichroism (B) spectra of native LZ (—) and modified LZ-mPEG-pNP (- - -).

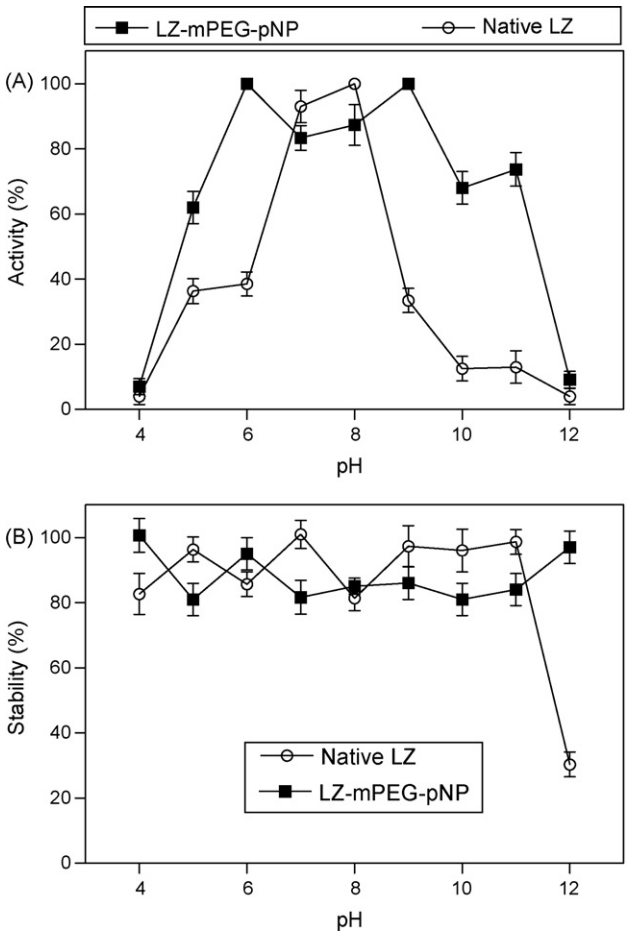

Fig. 6. Evaluation of the enzymatic activity (A) and stability (B) of native LZ (○) and LZ-mPEG-pNP (■). The activity was determined in different buffers varying the pH from 4 to 12 (4–5, 50 mM acetate; 6–8, Hepes 50 mM and 9–12 Tris–HCl 50 mM) at 25 °C. The stability was determined after incubation by 1 h in the same buffers at 50 °C. At the end of this time the enzymatic activity was determined against substrate *M. lysodeikticus* as described in methods. (The results are means ± S.D., $n = 3$.)

by MALDI-TOF showed a spectral peak at *m/z* 19,988 Da, while native LZ showed a spectral peak at *m/z* 14,117 Da (results not shown). All of the SDS-PAGE analyses until the present moment, on the other hand, have shown a band near 30 kDa for LZ-mPEG-pNP. This discrepancy may be explained by the overestimation of the molecular mass of the conjugate by SDS-PAGE analysis. This phenomenon is related to the fact that the amount of SDS bound by LZ decreases when its amino groups are modified, leading to a decrease in its mobility on SDS-PAGE which could be enhanced by the greater hydrodynamic volume of the conjugate PEG-LZ (Swank and Munkers, 1971). As a result, SDS-PAGE analysis gives higher than expected MWs when compared with MW marker proteins (Bailon and Berthold, 1998; Caliceti and Veronese, 2003). MALDI-TOF results indicate that the conjugate obtained most likely represents a single mPEG-pNP 5000 strand attached to the more reactive lysine residue (K33) of LZ.

Spectroscopic studies were performed to investigate if the conjugation of mPEG to LZ induced conformational changes in structure of the protein. Secondary structure predictions were estimated using the FTIR amide I band (1700–1600 cm^{-1}) and CD spectra of both the native and conjugate LZ. The native LZ is a predominantly α-helical protein, (Pribic et al., 1993; Meyer et al., 2004) as are presented in Fig. 5A. The main peak obtained for native enzyme was 1659.07 cm^{-1} and a very close value was obtained for the conjugate LZ-mPEG-pNP 1657.44 cm^{-1}, demonstrated that no significant differences can be seen between them.

In addition to the analysis of FTIR were performed analyses of CD that are more accurate to determine the content of α-helix in protein. The CD spectra of the native and modified LZ at 20 °C are shown in Fig. 5B and underline that the secondary structure of the LZ was not affected by mPEG conjugation. Similar results were obtained by Malzert et al. (2003). Additionally, the data was analyzed with K2D2 software, as described in Section 2.2.9, that calculated the percentage of structural elements of the native and modified LZ. The results obtained for both enzymes were 84.3% of α-helix, confirming that the secondary structure of the LZ was maintained after PEGylation.

3.4. Enzyme activity and stability of LZ-mPEG-pNP

The activity and stability of both the native LZ and LZ-mPEG-pNP are summarized in Fig. 6(A) and (B) respectively, for the entire pH range considered in this study. The modified LZ remained active within a wide pH range (Fig. 6A) and actually increased its pH-activity profile (mainly between 6.0 and 9.0). This conjugate LZ-mPEG-pNP also remained stable at 50 °C and at a high pH (pH = 12), indicating that its stability had increased in comparison to the unmodified enzyme (Fig. 6B). The development of stable formulations of LZ could considerably extend the uses of this enzyme (mostly for use in food preservation) as PEGylation of LZ would protect it against denaturation (most likely due to increased hydrophobic interactions) upon exposure to extreme temperature and pH (Johnson and Larson, 2005).

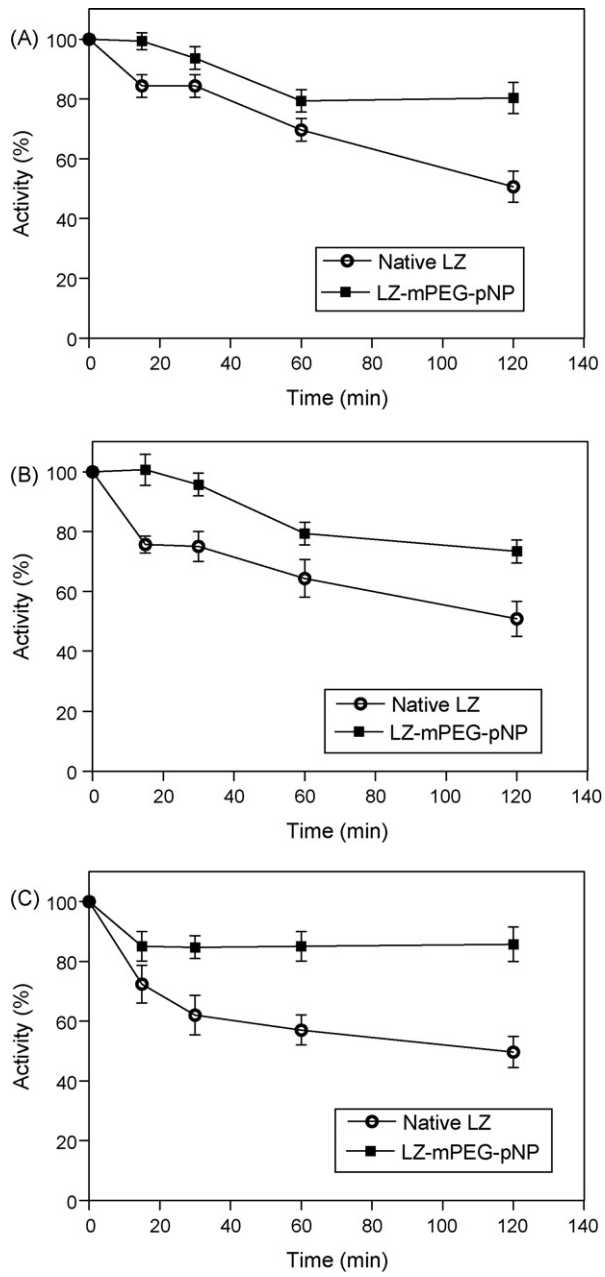

Fig. 7. Resistance to proteolysis of native lysozyme (○) and LZ-mPEG-pNP (■), digestion as assessed by enzyme activity upon incubation with Proteomix® (A), Protex 6L® (B) and fungal protease (C). (The results are means ± S.D., $n = 3$).

3.5. Resistance of modified lysozyme to proteolysis

Previous studies demonstrated the resistance of native LZ to proteolytic degradation by papain, trypsin, chymotrypsin (Mine et al., 2004) and other proteases (Polverino De Laureto et al., 1995). This proteolysis resistance is an interesting property of this enzyme and has contributed to the analysis of the structure and dynamics of other proteins (Polverino De Laureto et al., 2002). In the present study, the resistance of native and modified LZ to proteolytic degradation was examined using different proteases and the results are presented in Fig. 7. The use of excess protease (20 UI of proteases: trypsin + chymotrypsin [A], bacterial protease [B] or fungal protease [C]) resulted in a 50% decrease in enzyme activity within 2 h for native LZ, while the enzymatic activity of modified LZ remained at around 80% under the same conditions. This proteolytic resistance can be related to mPEG linkages that resulted in increased molecular mass and greater steric hindrance (Veronese, 2001; Roberts et al., 2002; Lee and Park, 2003). Other enzymes modified by PEGylation, including ribonuclease, catalase, trypsin, and L-asparaginase (Monfardini et al., 1995; Soares et al., 2002) have also been observed to become more resistant to proteolytic degradation.

4. Conclusions

In spite of the existence of other approaches to resolve the problem of the loss of biological activity resulting from PEGylation (particularly for proteins that act on macromolecular substrates), the current study successfully utilized the most common strategy of PEGylation with mPEG-pNP 5000 Da and obtained a conjugate (m/z 19,988 Da, attachment of one mPEG-pNP 5000) with 77% its of original enzyme activity against the substrate *M. lysodeikticus*. Activity and spectroscopic studies (CD, FTIR) indicated that no relevant differences in protein structure were detectable after PEGylation. The conjugate was active over a much broader pH range, was stable at 50 °C and showed high resistance to the proteolytic degradation.

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