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Biochemical and biopharmaceutical properties of PEGylated uricase

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ABSTRACT

PEGylation is a successful strategy for improving the biochemical and biopharmaceutical properties of proteins and peptides through the covalent attachment of polyethylene glycol chains. In this work, purified recombinant uricase from *Candida* sp. (UC-r) was modified by PEGylation with metoxypolyethilenoglycol-p-nitrophenyl-carbonate (mPEG-pNP) and metoxypolyethyleneglycol-4,6-dichloro-s-triazine (mPEG-CN). The UC-r-mPEG-pNP and UC-r-mPEG-CN conjugates retained 87% and 75% enzyme activity respectively. The K_M values obtained 2.7×10^{-5} M (mPEG-pNP) or 3.0×10^{-5} M (mPEG-CN) for the conjugates as compared to 5.4×10^{-5} M for the native UC-r, suggesting enhancement in the substrate affinity of the enzyme attached. The effects of pH and temperature on PEGylated UC-r indicated that the conjugates were more active at close physiological pH and were stable up to 70 °C. Spectroscopic study performed by circular dichroism at 20 °C and 50 °C did not show any relevant difference in protein structure between native and PEGylated UC-r. In rabbit and Balb/c mice, the native UC-r elicited an intense immune response being highly immunogenic. On the other hand, the PEGylated UC-r when injected chronically in mice did not induce any detectable antibody response. This indicates sufficient reduction of the immunogenicity this enzyme by mPEG-pNP or mPEG-CN conjugation, making it suitable for a possible therapeutical use.

1. Introduction

Uricase (EC 1.7.3.3, UC) is an enzyme belonging to the class of the oxireductases and catalyses the oxidation of uric acid, producing allantoin, acting in the purine degradation pathway.

$$\text{uric acid} + 2H_2O + O_2 \xrightarrow{\text{Uricase}} \text{allantoin} + H_2O_2 + CO_2.$$

This enzyme is commonly used in laboratory analysis for the detection of uric acid (Huang et al., 2004; Arora et al., 2007). UC is a tetramer with a molecular weight around 145–150 kDa, found in many living organisms such as bacteria, fungi, plants and animals (Mahler, 1970; Brogard et al., 1972, 1978; Schiavon et al., 2000) but, due to a nonsense codon inserted into this gene, this enzyme is produced as a truncated, 10 aminoacids long, inactive fragment in humans and apes (Bomalaski et al., 2002). Due to the absence of UC in humans, the plasma concentration of uric acid is rather high (around 300 μM) (Colloc'h et al., 2006) and an abnormal raise of this metabolite can promote renal failure and contribute to the development of diseases known as gout. Hyperuricemia is also a serious complication, very common in patients with neoplasic diseases, and may occur during chemotherapy. The severity of hyperuricemia is due to the wide spectrum of pathological consequences in kidneys, brains, subcutaneous tissues and articulations. Furthermore, an abrupt raise of uric acid blood levels can be lethal (Masera et al., 1982; Cammalleri and Malaguarnera, 2007). The treatment of these metabolic disorders has been performed with drugs that induce forced diuresis, urinary alcalinization, as well as a decrease in uric acid synthesis by inhibiting xanthine-oxidase. However, these treatments can sometimes be inefficient, leading to serious clinical complications (Schiavon et al., 2000; Cannella and Mikuls, 2005).

An interesting alternative for the treatment of gout and hyperuricemia has been the administration of UC, since this enzyme reduces uric acid plasma levels (London and Hudson, 1957; Altman et al., 1959; Masera et al., 1982) being also effective for the prevention and treatment of hyperuricemia caused by tumoral lysis and organ transplants (Bomalaski and Clark, 2004; Sherman et al., 2008). The *Aspergillus flavus* native enzyme is available for clinical use in France and Italy (Uricozyme®) (Bomalaski et al., 2002) and its recombinant form, Rasburicase®, is effective to prevent acute tumoral lysis syndrome (Cannella and Mikuls, 2005). How-

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ever, since humans do not express uricase, the enzyme would be expected to be seen by the immune system as a foreign protein. Indeed, UC from microorganisms and animals, when administered in patients, is highly antigenic, and the chronical treatment with this enzyme frequently results in allergic reactions and anaphylactic shock (Bomalaski et al., 2002). This represents a serious limiting factor for the use of UC as a therapeutic agent. For this reason, the development of a methodology to modify UC, which would diminish its immunogenicity while preserving biological activity, making it more suitable for therapeutic purposes has been investigated.

Recent studies (Colonna et al., 2008; Song et al., 2008; Veronese and Mero, 2008; Zappe et al., 2008; Morille et al., 2009; Zhou et al., 2009) showed that PEGylation is a well succeeded methodology, used to widen the therapeutic and biotechnological uses of proteins, through the coupling of polyethylene glycol (PEG), an inert and non-toxic polymer to the target biomolecule. PEGylation can modify important physico-chemical properties of proteins and improve solubility and thermal stability, increase plasmatic half-life while lowering renal clearance as well as reduce immunogenicity. PEGylated forms of adenosine deaminase (Adagen®) and asparaginase (Oncaspar®) and two forms of α-interferon (Pegasys®, PEG-Intron®) were approved by FDA (U.S. Food and Drug Administration) for human use and other PEG-proteins are being developed for therapeutical purposes, including growth factor and PEGylated drugs for the treatment of hepatitis C, acromegaly, reumathoid arthritis and cancer (Veronese and Pasut, 2005; Pasut et al., 2008).

The technology of PEGylation has been used to improve the therapeutical properties of UC. The enzyme from different sources was PEGylated: UC from *Candida utilis* (Nishimura et al., 1979; Chen et al., 1981; Davis et al., 1981; Nishimura et al., 1981; Caliceti et al., 1999, 2001), *Arthrobacter protoformiae* (Chua et al., 1988), *Bacillus fastidiosus* (Schiavon et al., 2000) and from mammals (Chen et al., 1981; Sherman et al., 2008). Among these enzymes, the one from *C. utilis* presents suitable biochemical properties for PEGylation such as higher affinity for uric acid and high catalytic rate at physiological pH (Pasut et al., 2008).

Up to now, there are few studies on PEGylation of the recombinant UC from *Candida* sp. expressed in *Escherichia coli* (UC-r) (Bomalaski et al., 2002; Bomalaski and Clark, 2004; Chohan and Becker, 2009). Thus, aiming to verify the main effects of PEGylation on this recombinant UC, in this work, we modified the enzyme with mPEG-pNP and mPEG-CN. The effects of PEGylation on the catalytic activity and stability of the enzyme were investigated, as well as structural aspects and the immunological behavior of the native and modified enzyme.

2. Materials and methods

2.1. Materials

Recombinant uricase from *Candida* sp. expressed in *E. coli*, uric acid, trinitrobenzenesulfonic acid (TNBS), monomethoxypolyethyleneglycol-p-nitrophenyl carbonate (mPEG-pNP) and 2-O-methoxypolyethyleneglycol-4,6-dichloro-s-triazine (mPEG-CN) 5000 Da, peroxidase conjugated-goat anti-mouse IgG and peroxidase conjugated-goat anti-rabbit IgG were purchased from Sigma Chemical Company. Sephadex G-100 from Amersham Pharmacia Biotec., xanthine-agarose resin from Sigma Chemical Company. Prestained protein markers were from Fermentas. All other reagents used in this study were of analytical grade. Male BALB/c mice, weighing 18 ± 2 g and a male rabbit obtained from IPEN (Instituto de Pesquisas Energéticas Nucleares, São Paulo, Brazil) were used for *in vivo* experiments.

2.2. Methods

2.2.1. Enzyme assay and protein measurement

The enzymatic activity of UC-r was evaluated with a Genesys spectrophotometer, at 25 °C as described by Mahler (1970), based on the absorbance decrease at 293 nm of a solution (1 mL) containing 48 μM of uric acid in 20 mM sodium borate buffer pH 8.5 using $\varepsilon = 12.6\ mM^{-1}\ cm^{-1}$. The specific activity was defined as the amount of enzyme that catalyses 1 μmol of uric acid per min per mg of protein. The protein concentration was determined using the Bradford method (Bradford, 1976) at 595 nm, using bovine serum albumin as standard.

2.2.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis under denaturing conditions was performed according to Laemmli (1970). Gels were stained using two visualization methods: Coomassie blue detection for protein and iodine solution staining for PEG species according to a modified procedure of Kurfurst (1992).

2.2.3. Purification of uricase

Commercial recombinant uricase from *Candida* sp. expressed in *E. coli* (UC-r) in a highly purified state was prepared by affinity chromatography with xanthine-agarose. The sample (10 mg) was applied in a column equilibrated with 50 mM sodium borate buffer pH 8.5 at a flow rate of 0.25 mL min^{-1}. The unbound fraction was removed with the same buffer. The enzyme was then specifically eluted with 1.2 mM uric acid in 20 mM sodium borate buffer pH 8.5. Fractions containing enzyme activity were pooled and used as source of pure enzyme. The activity and protein concentration were measured as above and purity was confirmed by SDS-PAGE.

2.2.4. Preparation of PEG-uricase conjugates

Purified recombinant uricase (1 mg mL^{-1}) was allowed to react with mPEG-pNP or mPEG-CN in sodium borate buffer (100 mM, pH 8.0) at 30 °C for 2 h and at 8 °C for 16 h. The PEGylation reactions were carried with 13.0 mg mPEG-pNP (a 3-fold molar excess with respect to lysines present in UC) or 8.6 mg mPEG-CN (a 2-fold molar excess with respect to lysines present in UC) in a final volume of 1 mL. The conjugation reactions were quenched by addition of lysine excess. PEG-UC-r conjugates were applied on Sephadex G-100. The column was pre-equilibrated with 100 mM sodium borate buffer pH 8.0 and eluted with the same buffer. Fractions containing enzyme activity were pooled and used as source of PEGylated enzyme. The samples were assayed for enzyme activity as above and molecular weight modifications by SDS-PAGE. The degree of modification of amino groups in the UC-r molecule with mPEG-pNP or mPEG-CN was determined by measuring the amount of free amino groups in the molecule with trinitrobenzenesulfonic acid (TNBS) as described by Habeeb (1966).

2.2.5. Kinetic studies

The K_M of native and modified UC-r was estimated by the double reciprocal plot method. Using different concentrations of uric acid (0.01–0.144 mM), the enzyme activity was assayed as described above and the K_M was calculated by the Lineweaver–Burk plotting.

2.2.6. Effect of pH on the enzymatic activity and stability of native and modified uricase

The activity of UC-r was assayed at different pHs in the range from 6.0 to 10.0. The native or modified enzyme was added to 40 μL of 1.2 mM uric acid in 1 mL of different buffers (pH 6.0–8.5 100 mM Hepes and pH 9.0–10.0 100 mM sodium borate). The enzymatic activity at 37 °C was immediately measured by absorbance decrease at 293 nm. The pH at which the samples showed the high-

est activity was considered to represent 100% activity. The assays were conducted in three independent experiments.

The stability was determined in buffers (6.5 and 7.5 Hepes 100 mM; 8.5 sodium borate 100 mM) at 37 °C, 50 °C and 70 °C for 2 h. At the end of this time, the enzymatic activity of native and modified uricase was determined as above. The pH at which the samples showed the highest activity was considered to represent 100%. The assays were conducted in three independent experiments.

2.2.7. Circular dichroism analysis (CD)

The spectroscopic characterization was carried out using solutions of 1 μM of native UC-r or polymer conjugated UC-r in phosphate buffer 20 mM using a JASCO J-810 spectropolarimeter in a 0.1 cm path length quartz cell at 20 °C, 50 °C and 70 °C. The CD spectra were recorded in the range of 185–260 nm with a data pitch of 0.1 nm. Each spectrum in different temperatures was obtained by averaging five scans of the spectral region. 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 by using the K2D2 software, a package freely available online (http://www.ogic.ca/projects/k2d2).

2.2.8. Preparation of rabbit anti-uricase antibody

Anti-uricase antibody was prepared in rabbit by using purified native UC-r as a soluble antigen. Purified UC-r (100 μg) in 500 μL of PBS/Al(OH)$_3$ (50/50) was subcutaneously injected into male rabbit on day 0. The rabbit was subcutaneously boosted on days 7, 14, 21 and 28 with 100 μg of enzyme dissolved in 500 μL of PBS. The blood was collected from the marginal vein on day 35 and centrifuged. The antiserum was stored at −70 °C until antibody titration by ELISA.

2.2.9. Antibodies titration by enzyme-linked immunosorbent assay (ELISA)

Ninety six wells microplates were incubated overnight at 4 °C with 100 μL/well of coating solution (50 mM sodium bicarbonate buffer, pH 9.6) containing 1 μg of purified native or PEGylated antigen. The wells were washed three times with 200 μL of saline phosphate buffer (PBS), 5% Tween pH 7.2 (PBS-T) and then incubated for 1 h at 37 °C with 200 μL of skim milk (3%) in PBS-T. The wells, washed as described above, were incubated for 1 h at 37 °C with 100 μL of serum serially diluted in PBS-T and, after further washing, for 1 h at 37 °C with 100 μL peroxidase conjugated-goat anti-mouse IgG or conjugate-goat anti-rabbit IgG, properly diluted in skim milk in PBS-T. The wells were washed as above and 100 μL of O-phenylenediamine 25 mg mL^{-1} plus 5 μL H_2O_2 (30%) were added. The enzymatic reaction was stopped after 20 min by addition of 50 μL/well of 200 mM citric acid and the absorbance at 450 nm was determined.

2.2.10. Antibody neutralization assay

To determine if the immune response resulted in anti-uricase neutralizing antibodies, the rabbit anti-serum obtained was serially diluted in saline phosphate buffer (PBS) and incubated with native UC-r, UC-r-mPEG-pNP or UC-r-mPEG-CN for 2 h at 37 °C. After this time, the enzyme activity was determined as described above. The assays were conducted in triplicate.

2.2.11. Immunogenicity evaluations

Fifteen male BALB/c mice were divided in three groups of 5 animals and treated on days 0, 7, 14, 21 with 5 μg of native UC-r (group 1), 5 μg of UC-r-mPEG-pNP (group 2) and 5 μg of UC-r-mPEG-CN (group 3), simulating a chronical treatment (without adjuvants) with the native enzyme or its derivatives. The solutions were prepared by dissolution of the enzyme in 100 μL of PBS. The solutions were intraperitonially injected, and blood samples were taken by retro-orbital bleeding at scheduled times. The blood samples were centrifuged and the antibodies were titrated by ELISA as described in Section 2.2.9, using the corresponding antigen for coating. The assays were conducted in triplicate for each group.

2.3. Statistical analysis

The statistical analysis was performed using the Graph Pad software Prism 4.0 (San Diego, CA, USA). Data were expressed as mean ± standard deviation (S.D.). Comparison of mean values was performed using one-way analysis of variance (ANOVA). A statistically significant difference was considered when $P < 0.05$.

3. Results and discussion

3.1. Modification and characterization of PEGylated uricase

The commercial uricase (UC-r) was purified by affinity chromatography with xanthine-agarose. The yield of purified enzyme was of 75% and the specific activity was of 190 UI/mg, with a 4-fold purification factor. According to the SDS-PAGE (Fig. 1, lane 2) the enzyme was highly purified and the molecular weight of the UC-r subunit was of about 35 kDa, a value similar to the one described in literature (Koyama et al., 1996).

Following purification, the enzyme was modified with mPEG-pNP and mPEG-CN both of 5 kDa, and displayed an interesting property, retaining 87% of the original activity after modification with mPEG-pNP and 75% when modified with mPEG-CN (Table 1). In previous works, where yeast uricase was modified with 5 kDa mPEG, with different degree of modification, residual activities of 15% and 45% were observed (Nishimura et al., 1979, 1981). Other

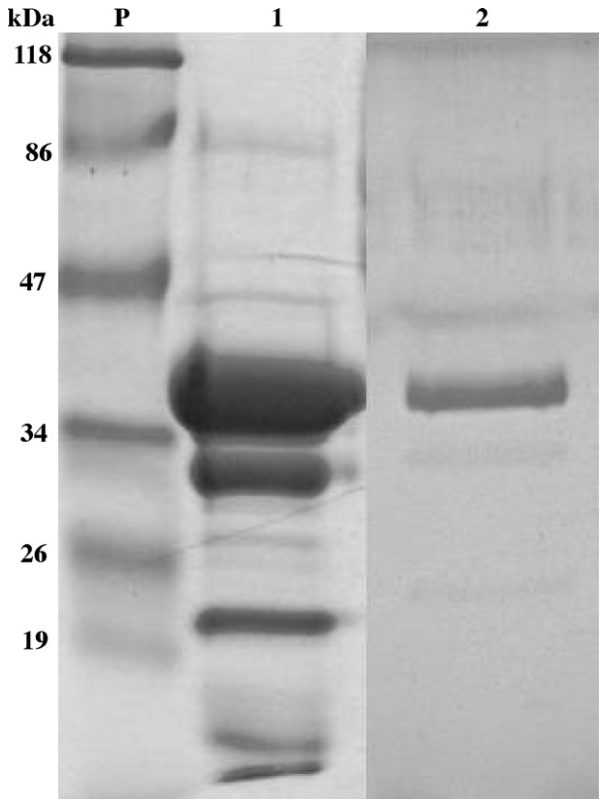

Fig. 1. Polyacrylamide gel electrophoresis SDS-PAGE (10%) of commercial recombinant uricase before and after purification. P: Molecular mass standard; lane 1: commercial recombimant uricase (UC-r); lane 2: purified UC-r obtained after affinity chromatography.

Table 1
mPEG/NH_2 molar ratio used in conjugate preparation and main properties of the modified enzyme.

Sample	Molar ratio mPEG/NH_2[a]	Extent of enzyme modification[b] (%)	Residual enzymatic activity (%)	K_M (M)[c]
Native UC-r			100	5.4×10^{-5}
UC-r-mPEG-pNP	13:1	20	87	2.7×10^{-5}
UC-r-mPEG-CN	8.6:1	20	75	3.0×10^{-5}

[a] Molar ratio mPEG/NH_2. Each molecule of recombinant uricase own 121 amino groups (120 ε-lysine and 1 N-terminal).
[b] The extent of modification was calculated on the basis of free amino groups by the trinitrobenzene sulfonic colorimetric assay.
[c] Michaelis–Menten Kinetic parameters were obtained from Lineweaver–Burk plots.

PEGylation studies were also performed using recombinant uricase expressed in *E. coli*, with succinimidyl succinimide activated 5 kDa or 20 kDa PEGs (Bomalaski et al., 2002), or with linear (5 kDa) and branched (10 kDa) PEG containing a terminal nor-leucine or lysine (Caliceti et al., 1999, 2001). In the study of Bomalaski et al. (2002) the enzyme modified with 20 kDa mPEG showed 86% of the original activity while the 5 kDa mPEG modified uricase displayed 50% residual activity.

The major factor responsible for the difference in enzymatic activity between modified enzymes is the extension of the modification obtained in each conjugate (Caliceti and Veronese, 2003). Previous studies verified that the degree of modification of *C. utilis* and porcine UC is intimately related with the molecular structure of the enzyme that directs access to the lysines and n-terminal amino groups. Furthermore, when the accessible lysine residues are close, there is a hindrance that occurs between the coupled mPEG molecule and other mPEGs that would react with neighboring lysines (Caliceti et al., 2001; Sherman et al., 2008). Thus, although the primary structure of each subunit of *C. utilis* UC-r being composed of a free amino-terminal and 30 lysines (Koyama et al., 1996) that, in theory, would be available for PEGylation, only 10–12 residues of each subunit would be accessible on the surface of the enzyme tetramer (Caliceti et al., 2001). In the present study, even using an excessive molar proportion between mPEG and enzyme, we obtained 20% modification (about 6 residues) of UC-r, according to the TNBS quantification of free amino groups (Table 1). This result suggests that the structure of UC-r promoted a steric hindrance between PEG molecules on the surface of the enzyme.

The molecular mass of the obtained conjugate is another important property related to the degree of modification of the enzyme. Thus, the conjugates were investigated by SDS-PAGE. Fig. 2A and B shows the migration profile of native UC-r and its conjugates. The gel indicates that both conjugates share similar profiles (Fig. 2A), showing that the molecular mass of the enzyme PEGylated with both the polymers, was of 50 kDa (predominant form) and 65 kDa, approximately. Furthermore, since the iodine solution gel staining procedure (Fig. 2B) is specific for PEG, the profile indicates the binding of the polymer to the protein and the molecular mass of each conjugate (Bailon and Berthold, 1998). Thus, the migration profile suggests that either 3 or 6 PEG molecules bound to each enzyme subunit, forming conjugates of approximately 200 kDa and 260 kDa respectively.

Aiming a therapeutical application of the conjugate, beside molecular mass, another parameter that has to be investigated is its functionality, which can be evaluated through K_M. Since the intention is to catalyze the oxidation of plasmatic uric acid, it would be desirable that the conjugates present high affinity for the substrate. In the present work, the K_M for the native UC-r was of 5.4×10^{-5} M, similar to the K_M described for uricase from other sources such as *B. fastidiosus* and *A. protoformiae* that have a K_M of 5.0×10^{-5} M, and *A. flavus* with a K_M of 6.1×10^{-5} M or *C. utilis* $K_M = 2.0 \times 10^{-5}$ M (Schiavon et al., 2000; Bomalaski et al., 2002).

Unexpected K_M results were obtained for the UC-r-mPEG-pNP ($K_M = 2.7 \times 10^{-5}$ M) and UC-r-mPEG-CN ($K_M = 3.0 \times 10^{-5}$ M) conjugates (Table 1), indicating that the PEGylation reaction enhanced the affinity of the enzyme for its substrate. Probably, this lower K_M value is related to the higher diffusibility of substrate, elicited by the amphipathic character of the polymer that facilitates the binding of the enzyme to the substrate which is poorly soluble. These results about the K_M of modified UC-r are of physiological and clinical relevance since the lowering of the K_M in the conjugates is an interesting aspect for their use as a therapeutic agent. A previous work also verified a lowering of the K_M of uricase after its immobilization (Arora et al., 2007) and, a similar fact also occurred with trypsin modified by PEGylation (Treetharnmathurot et al., 2008).

3.2. Effect of pH on the enzymatic activity and stability of native and modified uricase

Our results (Fig. 3) show that the native UC-r optimal pH is between 9.0 and 9.5. Interestingly, this optimal pH is dislocated towards the alkaline side, a common property from UC of different

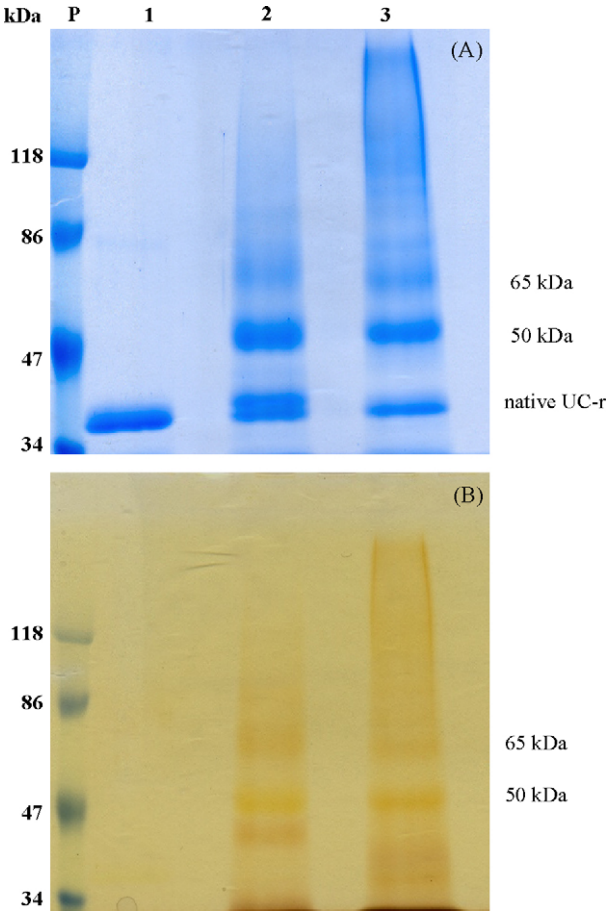

Fig. 2. Polyacrylamide gel electrophoresis SDS-PAGE (7.5%) of uricase after PEGylation, using two visualization methods: (A) Coomassie blue detection for protein. (B) Iodine staining for PEG. P: molecular mass standard; lane 1: native UC-r; lane 2: UC-r-mPEG-pNP; lane 3: UC-r-mPEG-CN.

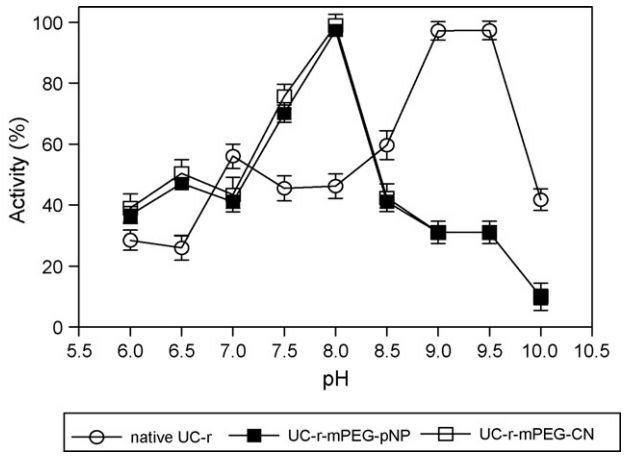

Fig. 3. Effect of pH on enzymatic activity (%) of native UC-r (○) and UC-r-mPEG-pNP (■), UC-r-mPEG-CN (□) conjugates. The activity was determined in different buffers varying the pH from 6.0 to 10.0 (6.0–8.5 Hepes 100 mM and 9.0–10.0 sodium borate 100 mM) at 37 °C. The results are mean ± S.D. ($n = 3$).

sources. Studies of subcellular localization indicate that uricase is an enzyme located in the peroxysomes (Usuda et al., 1988; Volkl et al., 1988) which are intracellular organelles that have high pH, therefore all the UCs are characterized by an alkaline optimal pH for catalytic activity (Bomalaski et al., 2002). Another feature observed in this study was that the native enzyme lost considerable enzymatic activity in pHs below 6.0 or above 10.0 (data not shown).

With the modified UC-r, we observed an optimal pH shift from 9.0–9.5 to 8.0 (Fig. 3). This lowering of the optimal pH for the modified UC-r is noteworthy, being an important feature of the obtained conjugates and suggests that the complexation of the enzyme with the used polymers caused chemical alterations on the protein that modified the activity of the enzyme derivatives. In previous work with different enzyme, we observed that following PEGylation, there is a modification of the optimal pH of the enzyme, bringing it closer to the isoelectric point of the protein (Soares et al., 2002). This could indicate that, due to its amphipathic properties, PEG would facilitate charge distribution around the active site.

At close to physiological pH, we observed that the conjugates showed higher activity than the native enzyme, with a residual activity of around 70% (70 ± 5%) while native UC-r had about 40% (40 ± 6%) (Fig. 3). When in physiological solution (NaCl 0.9%; pH 7.4), the modified UC-r also presented higher activity than its native counterpart (data not shown). This observation is of great relevance because it indicates an important feature of the modified enzyme that favors its therapeutic use. The catalytic activity of uricase from other sources at physiological pH was investigated and the authors observed that the *C. utilis* enzyme is the one with higher activity, when compared with the enzymes from *A. flavus*, *A. protoformiae* and hog liver (Bomalaski et al., 2002). Thus, among the available UCs, the native or recombinant *C. utilis* enzymes are an interesting option for modification and consequently, for therapeutic applications.

In the present work, we investigated the effects of incubation at different pHs and temperature on stability of the native and modified UC-r. Fig. 4A and B shows that the native uricase and its conjugates remained 100% stable for 2 h at 37 °C, independently of the pH. When raising the temperature to 50 °C and 70 °C, the conjugated forms showed a higher stability, mostly close to physiological pH. We observed that the UC-r-mPEG-pNP and UC-r-mPEG-CN conjugates have high stability at pH 7.5 and 37 °C, 50 °C and 70 °C showed significant difference ($P < 0.0001$) between them and native UC-r. This is an interesting property of these conjugates, since besides having higher activity than the native enzyme at physiological pH (Fig. 3), they are also considerably stable. A possible mechanism to explain the stabilization of the enzyme might be the protection of the active site by the PEG molecules that increase the structural rigidity of the protein. Without the protecting effect, the structure in the active site of the native enzyme is more susceptible (Soares et al., 2002), mostly at pHs below 8.5.

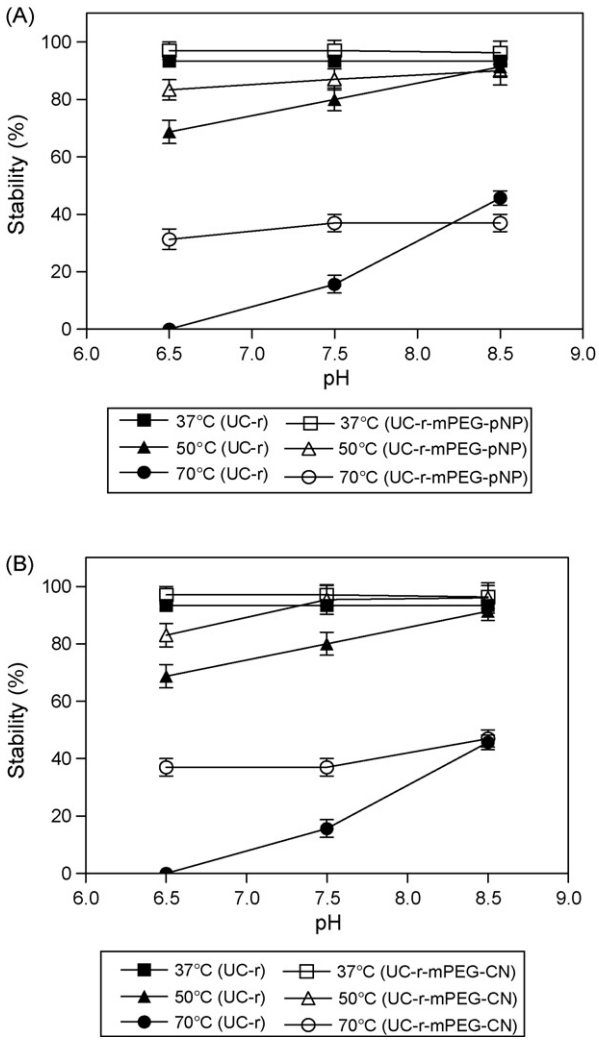

Fig. 4. Stability (%) of native and modified UC-r. (A) Stability of UC-r-mPEG-pNP conjugate and (B) UC-r-mPEG-CN conjugate. The stability was determined after incubation for 2 h at pH 6.5, 7.5 or 8.5 at different temperatures 37 °C, 50 °C and 70 °C. At the end of this time the enzymatic activity was determined as described in Section 2. The results are mean ± S.D. ($n = 3$).

3.3. Circular dichroism analysis

Spectroscopic studies were performed to investigate if the conjugation of mPEG to UC-r induced conformational changes in the protein. The CD spectra of the native and modified enzymes at different temperatures are shown in Fig. 5. When we compare the spectra obtained at 20 °C and 50 °C, no difference was observed between the spectra of the native and modified enzymes (Fig. 5A and B). Similar results were obtained with modified uricase from other sources, indicating that the secondary structure of the protein was not affected by the conjugation with mPEG (Caliceti et al., 2001). With another protein, hen egg white lysozyme (HEWL), no differences were observed between the CD spectra of the native and PEGylated protein (Malzert et al., 2003). On the other hand, at 70 °C (Fig. 5C) we observed that native UC-r underwent denat-

Table 2
Assessment of the percentage of native UC-r, UC-r-mPEG-pNP, UC-r-mPEG-CN α-helix content at different temperatures.

Sample	α-Helix content		
	20 °C (%)	50 °C (%)	70 °C (%)
Native UC-r	36	32	15
UC-r-mPEG-pNP	35	35	26
UC-r-mPEG-CN	40	40	40

uration, as indicated by the secondary structure alterations, while the modified forms of the enzyme showed higher thermal stability, mainly UC-r-mPEG-CN that did not showed any detectable structural modification up to 70 °C.

In order to compare the CD spectra, the data were analyzed with the K2D2 software that calculated the percentage of structural elements of the native and modified enzyme at each temperature (Table 2). Based on the content of α-helix, we noticed that the native UC-r suffered structural modifications with the temperature increase, presenting a loss of 58% of α-helix at 70 °C, indicating the denaturation of the enzyme. With the UC-r-mPEG-pNP conjugate, we could also observe a slight decrease of α-helix (26%) at 70 °C, however, PEGylation induced a partial resistance to thermal denaturation of the enzyme. The UC-r-mPEG-CN did not present any measurable decrease in α-helix content, suggesting that this conjugation provided higher stabilizing forces.

3.4. Immunological properties of native and PEGlated uricase

In a first experiment, we immunized a rabbit with purified native UC-r, aiming to produce antibodies that would be used to test the immunoreactivity of the conjugated enzymes. The fungal enzyme was highly immunogenic, with the antiserum showing a 1:512,000 titer. These antibodies were then used to probe the PEGylated enzymes and, even at high IgG concentrations, the catalytic activity was almost fully preserved, even with the native enzyme (Fig. 6A), although we observed antibody binding to the native and modified enzyme (Fig. 6B). These data suggest that the active site is poorly accessible. According to the 3D structure of uricases of other sources, the catalytic domain is deeply buried in a circular shape structure, and this would indeed difficult the access of antibodies (Colloc'h et al., 1997). Attempts to crystallize the native protein and solve its structure were performed but, in all the assayed conditions, the enzyme precipitated. Thus, the hypothesis of the active

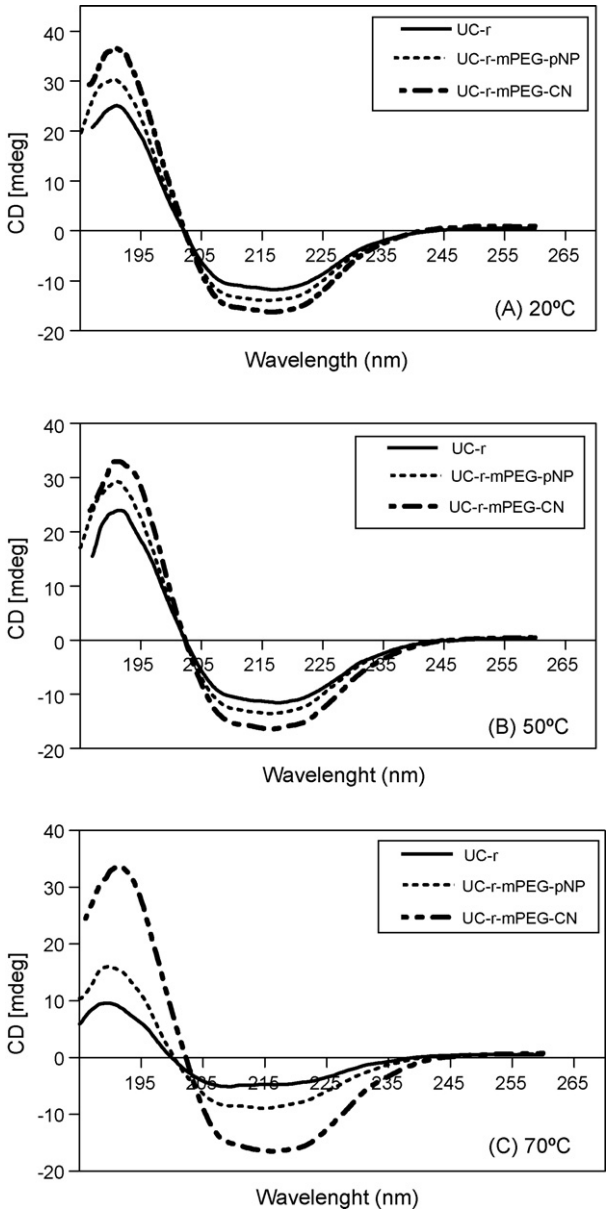

Fig. 5. Circular dichroism spectra of native UC-r (—) and modified UC-r-mPEG-pNP (···), UC-r-mPEG-CN (▬) at different temperatures (A) 20 °C, (B) 50 °C and (C) 70 °C.

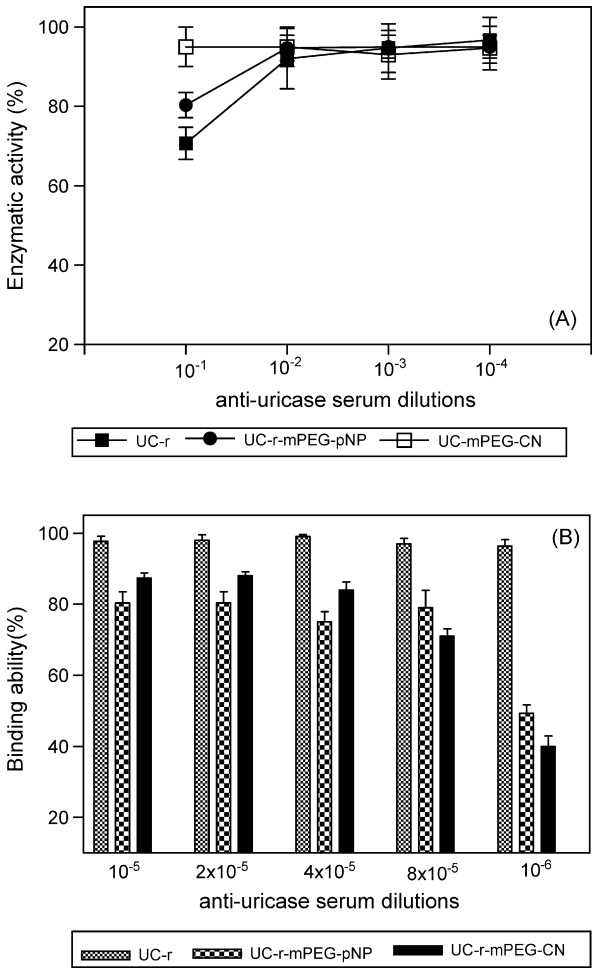

Fig. 6. (A) residual enzymatic activity (%) and (B) evaluation of antibody binding ability (%) of native UC-r and UC-r-mPEG-pNP, UC-r-mPEG-CN conjugates after incubation with anti-native uricase rabbit serum. The results are mean ± S.D. ($n = 3$).

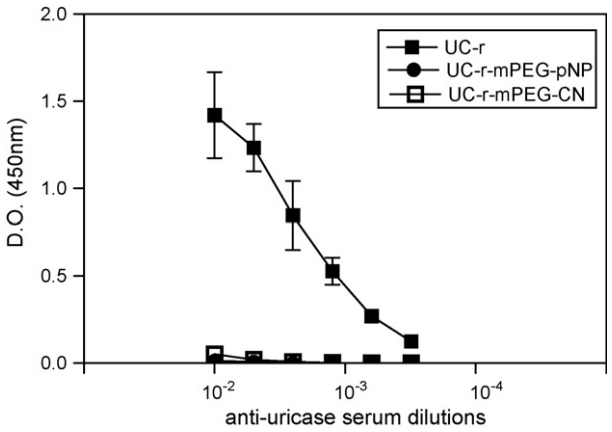

Fig. 7. Immunogenicity of native and modified UC-r in mice. The results are mean ± S.D. obtained with five animals/group.

site being surrounded by mPEG binding moieties has yet to be proven. In a second experiment, we treated mice with the native and modified enzyme as described in Section 2, simulating a chronical exposure to uricase (without adjuvants), as would occur if the protein was used as therapeutic agent, with a protein concentration similar to the one described by Sherman et al. (2008) for phase 2 clinical trials. After three weeks of treatment, blood samples were collected and we investigated the presence of anti-uricase antibodies in the sera. While the group treated with native uricase showed high IgG titers (>1:50,000), the PEGylated proteins did not induce any detectable antibody response (Fig. 7) and statistical analysis between native and conjugate UC-r confirmed this significant difference ($P < 0.0001$). This suggests sufficient reduction of the immunogenicity of this non-human protein, a basic requirement for its use as a therapeutic agent (Sherman et al., 2008).

4. Conclusions

The results shown in this work indicate that the purified recombinant uricase from *Candida* sp. (UC-r) modified by PEGylation with mPEG-pNP and mPEG-CN retained 87% and 75% of the enzyme activity respectively. The K_M values obtained were of 2.7×10^{-5} M or 3.0×10^{-5} M for the conjugates as compared to 5.4×10^{-5} M for the native UC-r, suggesting enhancement in the affinity of the modified enzyme. The pH and temperature stability assays on PEGylated UC-r indicated that the conjugates were more active at close to physiological pH and were stable up to 70 °C. Spectroscopic studies performed by circular dichroism at 20 °C and 50 °C did not show any relevant difference in protein structure among native and PEGylated UC-r. While the native UC-r elicited an intense immune response in rabbit and Balb/c mice, the PEGylated UC-r when injected in mice did not induce any detectable antibody response. The modifications resulting from the reaction of activated PEGs with UC-r improved its physico-chemical, biological and immunological properties, leading to significant advance in the transformation of a fungal enzyme in a useful therapeutic drug.

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