



Histaminase PEGylation: Preparation and characterization of a new bioconjugate for therapeutic application

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Abstract

Copper amine oxidase catalyses the oxidative deamination of primary amino groups of several biogenic amines, one of which is histamine, the principal chemical mediator of the first phase of allergic reactions. Looking forward to a possible future therapeutic application of this enzyme in the field of histamine-mediated afflictions, we developed a simple method for the purification of a histaminase from grass pea shoots, a source particularly enriched with the enzyme. Furthermore, in order to improve its therapeutic potential, in particular to reduce the high impurity due to its heterologous source, we conjugated the protein with poly(ethylene glycol) and tested the molecular, immunogenic and pharmacokinetic properties of the native and modified forms. The PEGylated enzyme showed molecular and enzymatic properties similar to those of the unmodified one, but the PEGylation extended the permanence of the injected drug in the body and eliminated its high immunogenic behaviour. The considerable ease of native histaminase production as well as the improved properties after PEGylation, make this engineered plant enzyme a suitable drug candidate for alternative treatment of histamine-mediated afflictions.

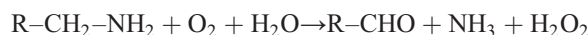
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1. Introduction

Biogenic amines are organic bases widely distributed among living organisms: animals, plants, bacteria and viruses [1]. They include a number of compounds involved in a variety of important biological processes such as mono-, di- and poly-amines, histamine, agmatine and catecholamines. The oxidation of biogenic amines to the corresponding imines is catalysed by two main classes of enzymes, which are grouped based on the chemical nature of their cofactors: the copper-containing amine oxidases, which possess high specificity for primary amines [2], and the flavin-containing amine oxidases, which catalyse primary, secondary or tertiary amines [3]. In both cases, molecular oxygen is the electron acceptor while hydrogen peroxide is formed as reaction product. Diamine oxidases (DAOs), originally referred as

histaminases [4], are homodimeric copper amine oxidases (Cu-AOs) (E.C.1.4.3.6) where each 70–95 kDa subunit contains a single copper ion and in most cases 2,4,5-trihydroxyphenylalanine quinone (TPQ) [5]. They catalyse the oxidative deamination of several primary biogenic amines according to the general reaction:



DAOs are ubiquitous enzymes widely distributed in mammals where they preferentially oxidize putrescine, cadaverine, agmatine and histamine [6]. The high DAO levels observed in the mucosal layer of intestine suggest a specific protective function against either the endogenous histamine [7,8] or histamine-related food poisoning [9], which produce a number of gastrointestinal, cutaneous, hemodynamic and neurological symptoms. DAOs have also been found to modulate cardiac anaphylactic response in guinea pig [10] and exert a significant cardio-protection against post-ischemic reperfusion damage in rats [11]. For such reasons, DAOs present good perspectives for therapeutic applications, in myocardial ischemia treatment.

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Recently, an International Patent Application (n. PCT/EP01/13770) proposing a histaminase-based drug of vegetable origin for the treatment cardiac anaphylaxis, allergic asthma, allergic and septic shock and in general in all histamine-mediated affections, such as allergic rhinitis and conjunctivitis, urticaria and atopic dermatitis has been described. As compared to their mammalian counterparts, plant DAOs display: (i) high turnover rates of catalysis and high rates of reduced enzyme re-oxidation; (ii) high binding affinity for histamine [12]; (iii) and high stability. Also, plant DAOs are present in several types of legume seedlings at high concentrations (up to 4% of total protein content) and can be easily purified by means of a few low-cost chromatographic steps. However plant proteins, due to their phylogenetic distance from mammalian sources, generally exhibit great difference in primary sequence and consequently high immunogenicity.

In order to overcome this problem and increase the *in vivo* residence time, grass pea DAOs was modified with polyethylene glycol (PEG), a polymer that in virtue of its high hydration yields derivatives with decreased immunogenic/antigenic character and prolonged half life as recently demonstrated in a number of therapeutically useful proteins [13].

This paper reports an easy purification method of grass pea DAO, the characterization of the physicochemical, enzymatic, immunogenic and pharmacokinetic properties of the native and PEG modified enzyme. It may be regarded as a contribution to the development of a new protein drug for the treatment of histamine-mediated afflictions.

2. Materials and methods

2.1. Chemicals

Putrescine, cadaverine, spermidine, agmatine, histamine, lysine, ornithine, aminoantipyrine, 3,5-dichloro-2-hydroxybenzenesulphonic acid, horseradish peroxidase, were purchased by Sigma (St. Louis, MO). Diethylaminoethyl cellulose (DE52) was from Whatman Bioscience (Cambridge, UK). HiTrap SP Sepharose was from Amersham Pharmacia Biotech (Piscataway, NJ). All other chemicals were of analytical purity grade obtained from Fluka (Buchs, Switzerland). Linear 10 kDa PEG activated as *p*-nitrophenyl ester (10 kDa PEG-*p*NO₂) and linear 5 kDa PEG functionalised with the amino acid norleucine (Nle) and activated as hydroxyl succinimidyl ester (5 kDa PEG-OSu) were synthesised as previously described [14]. Branched 20 kDa PEG (PEG₂-OSu) was furnished by Nektar Therapeutics (Huntsville, AL).

Male BALB/c mice weighing 22 ± 2 g fed *ad libitum* used for the *in vivo* studies were from the Department of Pharmaceutical Sciences, University of Padua. The animal experiments were performed in accordance with Italian law (DL n. 116/92) and European guidelines (EEC n. 86/609).

2.2. Enzyme assay and protein determination

Amine oxidase activity was determined spectrophotometrically by following the formation of a pink adduct ($\epsilon_{515\text{ nm}} = 2.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$), as a result of the oxidation by H₂O (by product of the CuO reaction) and subsequent condensation of aminoantipyrine

(0.1 mM) and 3,5-dichloro-2-hydroxybenzenesulphonic acid (1 mM) catalysed by the ancillary enzyme horseradish peroxidase (80 µg/ml) [15]. The measurements were performed at 25 °C in 0.1 M potassium phosphate buffer, pH 7.0 with putrescine (2 mM) as a substrate [16]. Enzyme activities were expressed in units (1 U is the amount of enzyme that catalyses the oxidation of 1 µmol substrate per min). Protein content was determined according to the Bradford procedure [17] with BSA as standard. SDS/PAGE was performed according to the method of Laemmli [18].

2.3. Enzyme purification and characterization

Commercial seeds of *Lathyrus sativus* (grass pea) were soaked in aerated tap water for 12 h and grown in moistened vermiculite for 7 days in the dark at 23 °C. Freshly collected shoots of etiolated grass pea seedlings (500 g) were homogenized at room temperature in a Waring blender with 4 volumes of 50 mM KH₂PO₄/1 µM Cu (II), strained through nylon mesh cloth and the solid residue, mainly constituted by cell walls, washed four times with 3 volumes of the same buffer and the enzyme was finally eluted from the solid residue with 1 volume of 50 mM KH₂PO₄, 25% saturated ammonium sulphate. The filtrate, previously clarified by centrifugation, was adjusted to pH 7.0 and precipitated by the addition of ammonium sulphate to 70% saturation. The precipitate, collected by centrifugation, was suspended in 15 mM potassium phosphate buffer, pH 7.0, and after overnight dialysis loaded onto a DE52-cellulose column (5 × 30 cm²) previously equilibrated with 15 mM potassium phosphate buffer, pH 7.0. The fractions with $A_{280} > 0.1$, containing more than 80% of the total loaded amine oxidase activity, were collected, the pH was adjusted to 5.5 and applied onto a HiTrap SP Sepharose column equilibrated with 50 mM potassium phosphate at the same pH. The column was washed with the buffer until the A_{280} decreased to 0, followed by one volume of 50 mM potassium phosphate buffer pH 5.5, 0.1 M NaCl, while the amine oxidase was finally eluted by increasing the NaCl concentration to 0.2 M. The enzyme-containing fractions were pooled, dialyzed and lyophilised to yield 120 mg of enzyme whose homogeneity was verified by SDS electrophoresis, gel filtration and reverse phase HPLC. The specific activity was 60 U/mg. The total content of neutral sugars of the purified DAO was determined by the phenol-sulphuric acid method according to a method reported in the literature [19]. Enzyme substrate specificity was determined at 25 °C in 0.1 M potassium phosphate buffer, pH 7.0, towards putrescine, cadaverine, spermidine, agmatine, histamine, benzyl lysine and ornithine as substrates. The K_m and V_{max} values were calculated from Lineweaver–Burk plots at pH 7.0 in 0.1 M phosphate buffer with putrescine as a substrate. Reported activity values are the mean of three independent experiments, each performed in duplicate. *P* values have been calculated with Student's *t*-test, comparing K_m and V_{max} values exhibited by PEG₂ 20-DAO with respect to native DAO. **P*-value ≤ 0.05.

2.4. PEG conjugation

Linear 5 kDa Nle-OSu, linear PEG 10 kDa activated as *p*NO₂ phenyl formate (PEG-*p*NO₂) or branched 20 kDa PEG were added at room temperature in small portions and under rapid

stirring to diamine oxidase (0.5 mg/ml) dissolved in 0.1 M phosphate buffer, pH 8.5. Amounts of activated polymer of 50-, 175- or 350-fold molar excess over protein were used in different experiments. The conjugation reaction was carried out for 4 h, while the pH was maintained with 0.05 M NaOH. The reaction was stopped by adding molar excess of glycine and adjusting the pH to 6.0 with HCl 0.1 M. The obtained conjugates, namely 5 kDa PEG–Nle–DAO (PEG 5-DAO), 10 kDa PEG–DAO (PEG 10-DAO) and 20 kDa PEG₂–DAO (PEG₂ 20-DAO), were purified from the unreacted polymer and the side reaction products by gel filtration chromatography using a HiLoad 26/60 Superdex 200 column on a FPLC system eluted with 0.1 M phosphate buffer, 0.3 M NaCl, pH 7.0. Analytical gel filtration chromatography was performed in a HPLC system with a Phenomenex Biosep Sec S3000 column, eluted with 10 mM phosphate buffer, 0.3 M NaCl, pH 7.0.

The number of PEG-bound chains was calculated indirectly by the determination of free amino groups according to the trinitrobenzene sulphonic acid-based colorimetric methods [20,21] and by the PEG content in the conjugates according to the colorimetric iodine test [22].

2.5. Immunological evaluation

Forty BALB/c mice were divided into four groups of 10 animals each and immunized on days 0, 7, 14, 21, 28 and 35 with native amino oxidase (Group 1), PEG 5-DAO (Group 2), PEG 10-DAO (Group 3) or PEG₂ 20-DAO (Group 4). The animals were immunized by injection of 200 μ l (100 μ l subcutaneously and 100 μ l intraperitoneally) of 100 μ g/ml of amino oxidase or equimolar solutions of the conjugates prepared by dissolving 100 μ g of protein equivalent in 0.5 ml of 20 mM phosphate buffer, 0.15 M NaCl, pH 7.2 and 0.5 ml of Freund's adjuvant. Complete adjuvant was used for the first injection and incomplete adjuvant for boosting. Blood samples (50 μ l) were taken by retro-orbital bleeding once a week for 8 weeks and processed according to a validated procedure reported in the literature [23]. Samples taken at the same time from animals in the same group were centrifuged for 2 min at 3500 rpm and serum aliquots of 20 μ l were pooled. The serum was serially diluted in 20 mM phosphate buffer, 0.15 M NaCl, pH 7.2 and the anti DAO IgM and IgG were determined by ELISA. A solution of native DAO (10 μ g/ml in 0.1 M NaHCO₃, pH 9.5) was used for microplate coating (100 μ l), which was then saturated using 200 μ l of a 100 μ g/ml solution of BSA. The anti DAO IgG and IgM content in the serum was separately determined using alkaline phosphatase-conjugated goat anti IgG or anti IgM mouse antibodies. The immunoresponse was determined from the O.D. at 405 nm of the serially diluted serum samples after addition of 100 μ l of 1 mg/ml of *p*-nitrophenol phosphate (SIGMA¹⁰⁴) in 1 M diethylamine, 5 mM MgCl₂, pH 9.8. The antibody level was expressed as the greatest dilution yielding the smallest reproducible response (O.D. 0.3).

2.6. Structural investigation

2.6.1. CD spectroscopy

Circular dichroism (CD) spectra of native and PEG₂ 20-DAO were registered with a Jasco J-810 spectropolarimeter (Jasco Ltd.,

Japan) between 250 and 190 nm (far UV) and between 350 and 250 nm (near UV). The CD analysis were carried out using native protein and PEG conjugate solutions in 20 mM phosphate buffer, pH 7.2. The native and PEG₂ 20-DAO concentrations were 0.1 mg/ml and 0.6 mg/ml (expressed as protein content) for the far and near UV analysis, respectively. The CD spectra were normalised to $\Delta\epsilon$ units using the molar extinction coefficient at 280 nm = 300,000 M⁻¹ cm⁻¹.

2.6.2. Fluorescence quenching

Solutions of native DAO or PEG₂ 20-DAO (0.05 mg/ml, expressed in protein content) in 0.5 M phosphate buffer, 0.1 M NaCl, pH 7.7 were prepared and the fluorescence spectra were recorded between 305 and 450 nm (excitation wavelength 280 nm) before and after addition of quenching agents: KI, CsCl or acrylamide. The quenchers were added to the protein samples to reach the final concentrations of 0.02, 0.04, 0.06, 0.08 and 0.1 M. Tryptophan and *N*-acetyltryptophanamide were used as reference. The fluorescence values were plotted according to the Stern–Volmer equation:

$$F_0/F = 1 + K_{sv}[Q]$$

where F_0 represents the fluorescence of the protein without quencher, F , the fluorescence in the presence of quencher, K_{sv} , the Stern–Volmer equation (M⁻¹ super⁻¹) and $[Q]$, the molar concentration of the quenching agent [24].

2.7. Pharmacokinetic studies

About 200 μ l of 0.7 mg/ml solutions of native DAO or equimolar concentration (based on the protein content) of PEG₂ 20-DAO in 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.2, were intravenously injected into BALB/c mice. Four mice for each time point were used. Blood samples (50 μ l) were collected at different times by retro-orbital bleeding and centrifuged at 3500 rpm for 3 min. The plasma was collected and diluted 1:10 with 0.02 M phosphate buffer, 0.15 M NaCl, pH 7.2. The enzyme content in the diluted plasma was evaluated spectrophotometrically by enzyme activity assay according to the procedure reported above. Similarly, blood samples obtained from non-treated mice were centrifuged and the enzyme activity in the diluted plasma was assessed. The obtained basal enzyme activity was used to normalize the experimental data obtained from animals treated with native and PEG modified DAO. The enzyme contents in the plasma samples were calculated based on a standard titration curve.

3. Results and discussion

3.1. DAO purification and characterization

DAO purification was performed from shoots of 7-day-old etiolated seedlings by a procedure similar to that described for the purification of maize polyamine oxidase [25]. The procedure takes advantage of the fact that at acidic pH values and low ionic strengths, more than 90% of amine oxidase activity present in the

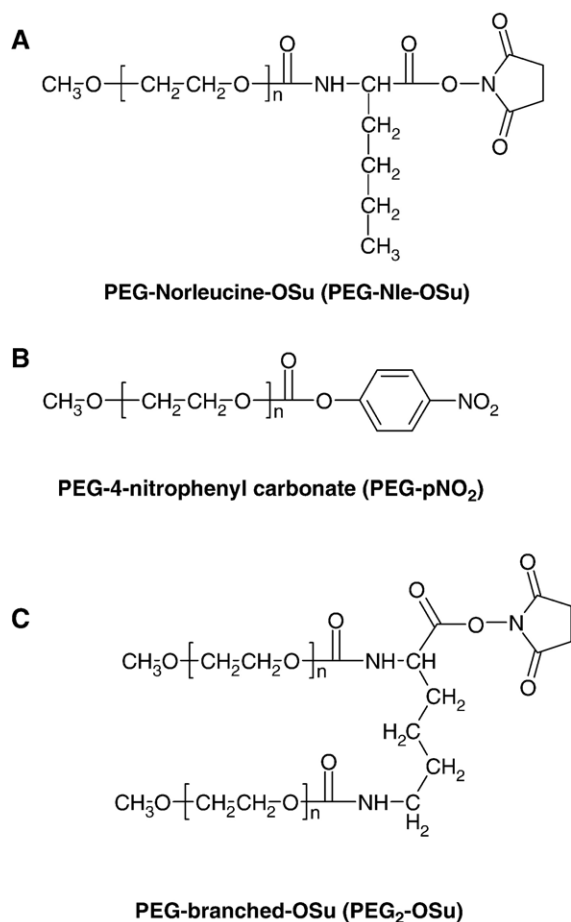


Fig. 1. PEGs structures: (A) linear 5 kDa monomethoxy–poly(ethylene glycol)–norleucine–hydroxy succinimidyl ester (PEG–Nle–OSu); (B) linear 10 kDa monomethoxy–poly(ethylene glycol)–*p*-nitrophenyl carbonate (PEG–pNO₂); (C) branched 20 kDa (2 × 10 kDa) monomethoxy–poly(ethylene glycol)–Lys–hydroxy succinimidyl ester (PEG₂–OSu).

extract remains associated with cell walls, from which it is released by increasing ionic strength. Furthermore, the high specific DAO activity found in the cell wall extract allowed to obtain homogeneous enzyme by two chromatographic step only with an overall recovery of over 50%. The enzyme was chromatographically and electrophoretically homogeneous, showing a single band in SDS–PAGE corresponding to a molecular mass of 72 kDa. The high recovery yield, high purity degree and the easy of purification suggest this method as suitable for large-scale preparation of this therapeutically promising enzyme.

Table 1
Degree of NH₂ conjugation, residual enzymatic activity of PEG–DAO conjugates obtained with PEGs of different mol wt. and shape at increasing ratio of PEG over protein

Final conjugate	Ratio between polymer chains and protein	Degree of NH ₂ modification (%)	Residual enzymatic activity (%)
PEG ₂ 20-DAO	50	8	95
PEG ₂ 20-DAO	175	31	100
PEG ₂ 20-DAO	350	54	110
PEG 10-DAO	350	51	105
PEG 5-DAO	350	52	100

The molecular and enzymatic properties of the purified DAO were similar to those shown by the grass pea enzyme isolated and characterized by Šebela and co-workers [26] and to other plants DAOs [27]. In particular, the pure enzyme was pink, the extinction coefficient at 500 nm was $3.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, the carbohydrate content was 13% and the specific activity was of 60 U/mg. Moreover, it exhibited broad substrate specificity since it was found to oxidise rapidly putrescine, cadaverine, spermidine, agmatine, and histamine. Monoamines were also oxidised, but at a much lower rate, while negligible conversion of the amino acids lysine and ornithine was observed. These results confirm the limited specificity of action typical of this class of enzymes.

3.2. DAO PEGylation

In order to attain an enzyme derivative with physicochemical, immunological and pharmacokinetic properties suitable for therapeutic application, DAO was modified using four activated PEGs with different molecular weight and shape: 5 kDa PEG–Nle–OSu, 10 kDa PEG–pNO₂ and 20 kDa PEG₂–OSu (Fig. 1). The modification reaction was carried out using various polymer/protein molar ratios. The preparation conditions and the main molecular properties of the derivatives are described in Table 1. Gel filtration analysis of the reaction mixture revealed that a conjugate devoid of unmodified protein was obtained at 350:1 polymer/protein molar ratio as reported in Fig. 2 for the branched PEG 20 kDa. Under these conditions about half of the amino groups were modified. This degree of modification could not be increased irrespective of the PEG molecular weight or the value of PEG/protein ratio, suggesting a limited availability of the amino groups at the protein surface. This hindered PEGylation may be ascribed to the presence of sugars on the protein surface and/or to the dimeric structure of DAO that prevents, for steric reasons, the access of the polymer to all of the protein amino residues.

3.3. DAO and PEG-DAO structural characterization

PEGylation did not decrease the DAO activity, but on the contrary a significant (P value ≤ 0.05) even if slight increase

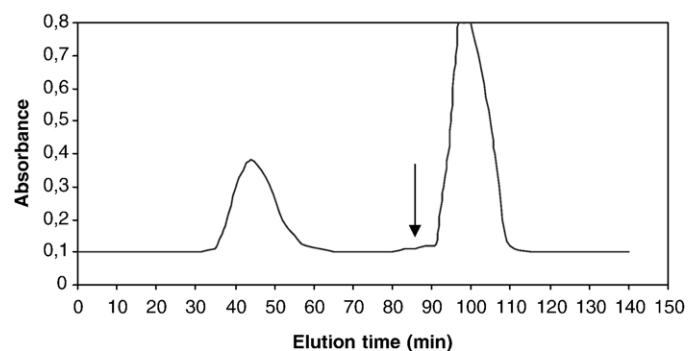


Fig. 2. HPLC analysis of the DAO PEGylation mixture obtained at a 350:1 PEG/protein molar ratio with PEG₂–OSu, 20 kDa. A Hiload 26/60 Sephadex 200 preparative grade column was used. The eluent was NaH₂PO₄ 0.1 M, NaCl 0.3 M pH=7. The elution peak at 43 min corresponds to the conjugated enzyme, the peak at 100 min corresponds to the leaving group hydroxysuccinimide. The unmodified DAO, if present, should have been eluted at 84 min (see arrow in figure).

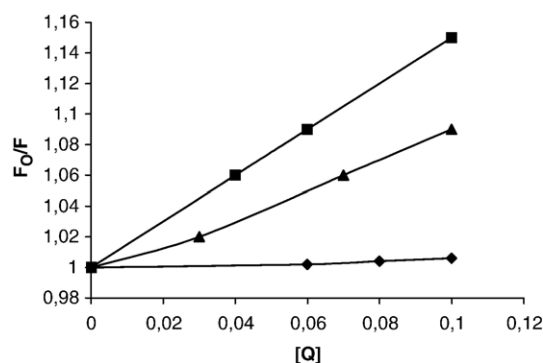


Fig. 3. Fluorescence quenching of: unmodified DAO (◆), PEG₂ 20-DAO (▲) and tryptophan amide (■), by the positively charged cesium chloride. The excitation was at 280 nm and the fluorescence recorded between 305 and 450 nm.

was observed at high PEG conjugation values (Table 1). This behaviour, already observed with other enzymes [28,29], may be explained as the result of subtle conformational variations in the surroundings of the active site which make it more favourable for catalysis. Evidence for this phenomenon could not be found by far UV and near UV circular dichroism measurements (data not shown) while structural variations came from fluorescence quenching studies showing that the addition of the positively charged cesium chloride decrease the fluorescence of the conjugated enzyme more efficiently than of the native DAO (Fig. 3), suggesting the exposure of attracting negative charges close to the protein fluorophores in the PEGylated specie. The fluorescence of both native and PEG conjugated protein was unaltered upon addition of the neutral quenching agent acrylamide, or of the negatively charged quenching agent potassium iodine. The increased accessibility of the positive quencher to the protein fluorophores in the PEGylated form, is in agreement with the higher accessibility of the positively charged substrates to the PEGylated enzyme, as revealed by its lower K_m as compared to the native enzyme (P value ≤ 0.05) (Table 2). In general, conformational alterations may be detrimental for the therapeutic properties of proteins since they may be responsible of increased immunogenicity. However, this is not the case of PEGylated products where the highly hydrated polymer masks the antigenic sites.

3.4. Immunogenic properties of the PEGylated DAO

In order to gain information on the immunological properties of either native or modified DAO, the ability to elicit anti-DAO

Table 2
Enzymological parameters of native DAO and PEG₂ 20-DAO

	K_m (1×10^{-4} M)	V_{max} (UE/mg protein)	pH optimum
Native DAO	3.0	60	7.00
PEG ₂ 20-DAO	2.7*	67*	7.00

The polymer/protein molar ratio was 350:1. A standard deviation of $\pm 3\%$ was estimated for both K_m and V_{max} values. P values have been calculated comparing K_m and V_{max} values exhibited by PEG₂ 20-DAO with respect to native DAO.

* P -value ≤ 0.05 .

IgG and IgM immunoresponse after mouse boosting with native DAO, PEG 5-DAO, PEG 10-DAO and PEG₂ 20-DAO was evaluated. This comparative study was carried out with the conjugates obtained by extensive modification which present similar amino group derivatisation. These derivatives were supposed to present the best immunological properties since the high polymer mass on the protein surface could mask efficiently the protein structure. IgG and IgM were selected as immunogenic biomarkers because their generation is directly correlated to the protein immunogenicity. IgMs are the first immunoglobulins to be generated by an individual exposure to an antigen and the IgM production time course indicates the potential immunotoxicity of the antigens. IgGs represent the long-term protection from antigens and are involved in various mechanisms of protein clearance. Finally, since the present study was aimed at evaluating the effect of the polymer conjugation on the immunogenic properties of DAO, only the anti protein IgG and IgM levels were assessed. Nevertheless, it should be noted that PEGylation may be responsible for the generation of new antigenic and immunogenic sites due to the conformational microalterations [23].

Fig. 4A and B show that native DAO is highly immunogenic as its administration elicited high anti DAO IgM and IgG immuno-response since the second immunization. Maximum value was reached after 4 immunizations, after that the IgM levels

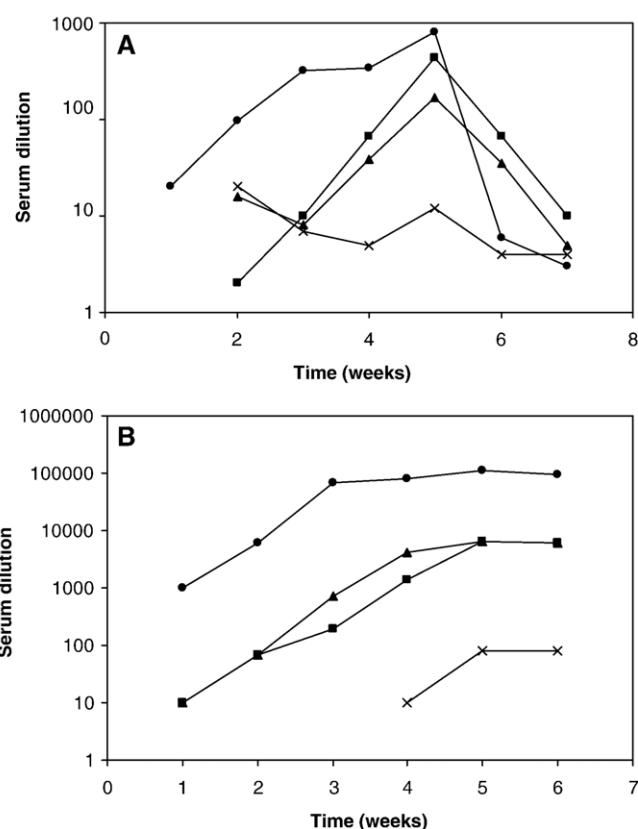


Fig. 4. (A) Anti-DAO IgM immunoresponse after immunization with: DAO (●), PEG 5-DAO (■), PEG 10-DAO (▲) and PEG₂ 20-DAO (×). (B) Anti-DAO IgG immunoresponse after immunization with: DAO (●), PEG 5-DAO (■), PEG 10-DAO (▲) and PEG₂ 20-DAO (×). Each point represents the mean value obtained pooling the blood from with 10 animals.

slowly decreased according to the typical time-limited IgM profiles. The 5 kDa and 10 kDa PEG derivatives elicited a slower immunoresponse and the maximal IgM concentration reached after the 5th immunization was about 50% and 80% lower, respectively, as compared with the native protein. The best result was obtained with PEG₂ 20-DAO, that elicited only negligible immunoresponse.

The anti-DAO IgG profiles confirm the high immunogenic properties of this protein. The native protein induced maximal anti-DAO IgG concentration after 3 immunizations. PEG 5-DAO and PEG 10-DAO induced a slower immunoresponse and the antibody generation was about 95% lower than that obtained with the native protein. Also in this case the immunogenic character of PEG₂ 20-DAO was negligible.

These results demonstrate that the reduction of immunogenic character of DAO is strictly related to the structural properties of PEG used in conjugation. Although high degree of modification was obtained with 5 kDa and 10 kDa PEGs, the derivatives maintained partially their immunogenic potential, demonstrating that linear PEG does not mask efficiently the protein structure. On the contrary, the use of the branched high molecular weight PEG₂ altered profoundly the immunogenic properties of this protein. This is probably due to a combination of parameters: higher mass of polymer on the protein surface and “umbrella like” shape of PEG, which results in high masking effect. This result may open the way to the use of this conjugate when, for therapy, repeated administrations should be needed.

3.5. Pharmacokinetic properties of native and PEG₂ 20-DAO

The pharmacokinetic behaviour of native and of the most promising PEGylated DAO was evaluated by determining the enzymatic activity time course in serum after single intravenous administration to mice.

Fig. 5 shows that PEG₂ 20-DAO exhibits a significant increase in circulating life with respect to the native enzyme, as the half-life was about 10 min for the native enzyme and 3 h for the conjugate.

In both cases, the experimental data were found to fit a bi-exponential behaviour indicating that either the native or the modified enzyme undergo distribution into a peripheral com-

Table 3

Pharmacokinetic parameters obtained by intravenous injection into BALB/c mice of native DAO and PEG₂ 20-DAO

	Native DAO	PEG ₂ 20-DAO
$t_{1/2\alpha}$ (min)	11	207
$t_{1/2\beta}$ (min)	55	1141
Cl (ml min ⁻¹)	28×10^{-3}	2×10^{-3}
V_{ss} (ml)	1.58	2.54
AUC (μm ml ⁻¹ min ⁻¹)	4966	66 794

partment. The pharmacokinetic parameters calculated by the elaboration of the experimental data highlighted the differences between the two enzyme forms. The α and β phase half-lives ($t_{1/2\alpha}$ and $t_{1/2\beta}$, respectively) reported in Table 3 show that PEGylation induces a dramatic prolongation of both distribution and elimination phases. The prolonged permanence in the blood stream of the PEGylated DAO is also demonstrated by the reduced clearance (Cl), which, in the case of the PEG₂ 20-DAO, is about ten times smaller as compared to the native protein.

The pharmacokinetic values obtained with the PEGylated DAO are in line with the results of many other proteins reported in the literature [30]. Indeed, it is known that the attachment of PEG chains on the protein surface increases the protein hydrodynamic volume reducing the kidney ultrafiltration and prevents the degradation or the clearance of the protein from the bloodstream by immunological processes or cell uptake. Only the distribution volume (V_{ss}) was found in countertendency as compared to the typical effects of PEGylation on the pharmacokinetic of proteins. Usually, PEGylation decreases the protein distribution into the peripheral compartment thus promoting their permanence in the bloodstream. On the contrary, the PEGylated DAO displays higher distribution volume (V_{ss}) as compared to the native protein indicating that it undergoes higher disposition into the peripheral compartment. Probably, the rapid elimination of the native enzyme prevents its extensive tissue distribution, while the PEGylated form can slowly extravasate. The prolonged permanence in the bloodstream and high biodistribution reflect its higher systemic bioavailability (AUC) as compared to the native protein.

However, it is worth noting that, as observed in the immunogenic investigations, the pharmacokinetic properties of the conjugates depend strictly on the polymer mass on the protein surface: higher polymer mass correlated to higher exposure in the circulation. While in many proteins extensive conjugation is accompanied by reduction or loss of biological activity, in the case of DAO the attachment of several high mass PEG chains was possible because it did not provoke a decrease in enzymatic activity.

4. Conclusions

Plant Cu-amino oxidases are enzymes possessing a variety of biological roles, which are potentially useful for many therapeutic applications. Actually, the common high immunogenic character of the proteins extracted from a plant source, the poor pharmacokinetic properties of native DAOs, together with the

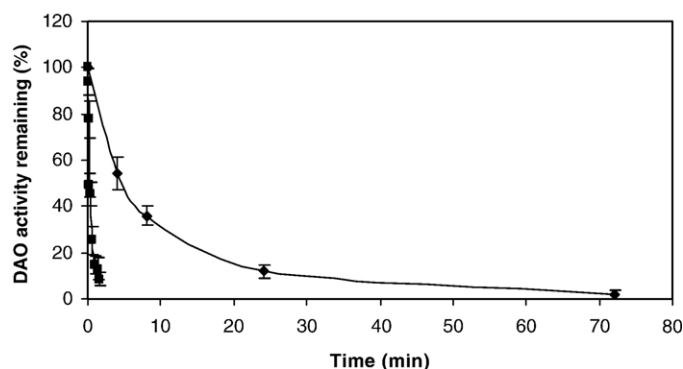


Fig. 5. Blood residence time of PEG₂ 20-DAO administrated to mice (●), as compared to that of the native enzyme (■). The standard deviation values ($\pm$ S.D.) were calculated based on four determinations.

difficulty to purify efficiently these enzymes from homologous sources, limited their prospective use in medicine. A solution was found in devising a procedure to obtain a high amount of enzyme at low cost and with high purity from pea seeds. Furthermore, PEGylation with high mass branched PEG was demonstrated useful in overcoming the high immunogenic character of this plant enzyme. The residence time in blood were also greatly increased and the enzyme activity and substrate specificity was maintained.

In conclusion we can say that, thanks to the properties of this enzyme, in particular after conjugation with high mass PEG, new treatments for histamine-related disorders can be envisaged.

Acknowledgments

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