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PREPARATION OF LONG-ACTING SUPEROXIDE DISMUTASE USING HIGH MOLECULAR WEIGHT POLYETHYLENE GLYCOL (41,000–72,000 DALTONS)

RALPH SOMACK, MARK G.P. SAIFER and L. DAVID WILLIAMS

DDI Pharmaceuticals, Inc. 518 Logue Avenue, Mountain View, CA 94043 USA

Superoxide dismutase (SOD) has demonstrated therapeutic potential for treating a variety of conditions including radiation injury, oxygen toxicity, reperfusion injury, and inflammation, especially arthritis. However, the native enzyme's short half-life in plasma (6 minutes in mice, 25 minutes in man) limits the enzyme's effectiveness in many applications, or requires infusion of large doses. High doses of SOD derived from either natural or rDNA sources may increase the potential for immunologic sensitization. One effective use of native SOD is intra-articular administration for treatment of arthritis, where injection of SOD into joints retards elimination (15 hour terminal half-life), allowing the effective use of lower doses.

To overcome the limitations resulting from rapid clearance, various researchers have increased the persistence of SOD by cross-linking SOD or by attaching polymeric substances, including dextrans, albumin, Ficoll, polyvinyl alcohol or polyethylene glycol (PEG). PEG is relatively safe; however, the amount of modification by PEG, is the MW range 1,900–5,000 daltons, which is necessary to optimally increase serum persistence and reduce immunogenicity, results in the loss of much of the enzymatic activity.

In this report we describe the preparation of SOD adducts containing 1 to 4 strands of high MW PEG (41,000–72,000 daltons). The MW range of these adducts, measured by steric exclusion HPLC based on protein standards, is 200,000 to over 1,000,000 daltons. The number of PEG strands attached per SOD dimer (32,000 daltons) was measured by HPLC. Because of the low degree of protein modification required to produce very high MW products, these PEG-SODs retain 90%–100% of the SOD activity of the native enzyme. Additionally, these very large adducts demonstrate longer persistence and lower immunogenicity and antigenicity compared to the more highly modified PEG-SODs containing low MW PEG (i.e., 7-16 strands of 5,000 dalton methoxy-PEG).

KEY WORDS: Long-acting, SOD. PEG, polyethylene glycol, polyethylene oxide, PEG-SOD.

INTRODUCTION

Superoxide dismutase (SOD) and other proteins have been modified with a variety of polymeric substances in attempts to increase persistence and reduce potential immunogenicity.[1] Polyethylene glycol (PEG) or methoxy-PEG, typically in the 1900–5000 daltons MW range, most often have been used because of high solubility and polarity as well as low toxicity and immunogenicity. A terminal hydroxyl group on each PEG is coupled to protein nucleophilic groups (predominantly of lysine residues), typically by activation with such agents as cyanuric chloride[2] or phenylchloroformates[3] or by conversion of the hydroxyl group to a carboxyl group followed by coupling via active esters.[4] Although the protein's antigenicity is often reduced and serum persistence extended, the high degree of modification with low MW PEG required to achieve these effects leads to substantial losses of enzyme activity.[2-5]

In this report we describe the synthesis and properties of PEG-SOD adducts containing fewer than 4 strands of 41,000–72,000 MW PEG. Because of the relatively low degree of modification employing a high MW polymer, these adducts retain more

enzymatic activity, in addition to being substantially larger than the more highly derivatized PEG-SODs described by others. Some of the pharmacologic and immunologic properties of these novel PEG-SOD adducts are described in a separate report in these Proceedings.

MATERIALS AND METHODS

Materials

Bovine Cu/Zn SOD derived from liver was a non-pyrogenic, sterile pharmaceutical grade product with a purity greater than 99% (DDI Pharmaceuticals, Inc., Mtn. View, California). Monomethoxy-PEG 5000 (Methoxy PEG 5K) was from Sigma Chemical Company (St. Louis, MO), polyethylene glycol 35,000 (PEG 35K) from Fluka Chemical Corp. (Ronkonkoma, NY) and polyethylene oxide (Polyox) 100,000 (PEG 100K) from Union Carbide Corp., Specialty Chemicals Division (Danbury, CT). The apparent molecular weights of these compounds based on steric exclusion HPLC calibrated with commercial PEG standards were 4000–5000, 41,000 and 50,000 daltons, respectively. Unless otherwise noted, all molecular weights reported in this paper were obtained from steric exclusion HPLC analyses using PEG (polyethylene oxide or polyethylene glycol) calibration standards (960–847,000 daltons) obtained from Toyo Soda Manufacturing Company, Ltd. (Tokyo), Polysciences Inc. (Warrington, PA) or Polymer Laboratories, Ltd. (Church Stretton, UK). The corresponding molecular weights derived from protein-standardized HPLC range from 4 to 8 times larger. Succinic anhydride, N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) were from Sigma. QAE Sepharose-Fast Flow was from Pharmacia (Uppsala, Sweden) and DEAE Biogel A from Bio-Rad (Richmond, CA).

Methods

Agarose gel electrophoresis, SOD activity measurements and protein determinations Thin film electrophoresis was performed on Universal Agarose Film (Ciba-Corning) in Tris-glycine buffer, pH 8.5. One microliter samples were electrophoresed at 250 V for 20 minutes and stained for protein with Coomassie Blue or for SOD activity with NBT-riboflavin.[6] SOD activity was determined by the cytochrome-*c* reduction assay of McCord and Fridovich.[7] Protein was determined by biuret analysis or by HPLC, using bovine SOD standards

Molecular weight and PEG conjugation PEG-SOD MW and extent of conjugation (number of strands per SOD) were determined by aqueous steric exclusion HPLC using a Waters Ultrahydrogel 250 and 500 column pair (7.8 mm × 30 cm, each) in series, monitored by UV (214 nm) and refractive index (RI) detection. The running buffer was 0.1 M sodium phosphate buffer containing 0.1 M sodium chloride (pH 6.8). The injection volume was 10-20 mcl and the flow rate was 0.8 ml per minute. PEG showed no appreciable absorbance at 214 nm. The number of PEG strands per SOD dimer (32,000 daltons with protein standardization, or 8,000–10,000 daltons with PEG standardization) was estimated in two ways: first, by dividing the PEG-SOD peak MW (less the SOD standard peak MW) by the PEG peak MW; and second, by dividing the molar PEG content of the PEG-SOD peak (RI integral minus RI contribution of SOD, divided by the molar RI of PEG) by the molar SOD content

of the PEG-SOD peak (UV integral divided by molar UV absorbance of SOD). The molar RI and UV absorbances were determined in each assay, using appropriate PEG and SOD standards. The degree of PEG modification could not be accurately determined by titration of protein amino groups because of the low degree of derivatization (fewer than 5 strands).

Preparation of PEG succinate active esters Methoxy-PEG 5K and PEG 35K were used without further purification. PEG 100K from Union Carbide (50,000 measured by HPLC) was either used directly or fractionated by tangential flow ultrafiltration with 300,000 nmwco membranes, to obtain higher MW PEG precursors. PEG was succinylated by reaction at 70 C using a 20 to 150-fold molar excess of succinic anhydride in ethyl acetate, containing 2.5% pyridine by volume.[8] With dihydroxy-PEG, monosuccinylation was achieved by controlling the amount of reagent excess and other reaction conditions. The succinylated PEG intermediates were activated for 2 hours at 40 C in ethyl acetate using 10-fold molar excess NHS and 9-fold molar excess DCC, crystallized, washed with petroleum ether, dried under vacuum and stored refrigerated.

PEG-SOD coupling reactions The NHS-activated PEG was added to solutions of SOD at 1–8 mg/ml protein in 0.1 M borate-phosphate buffer, pH 8.0 and incubated

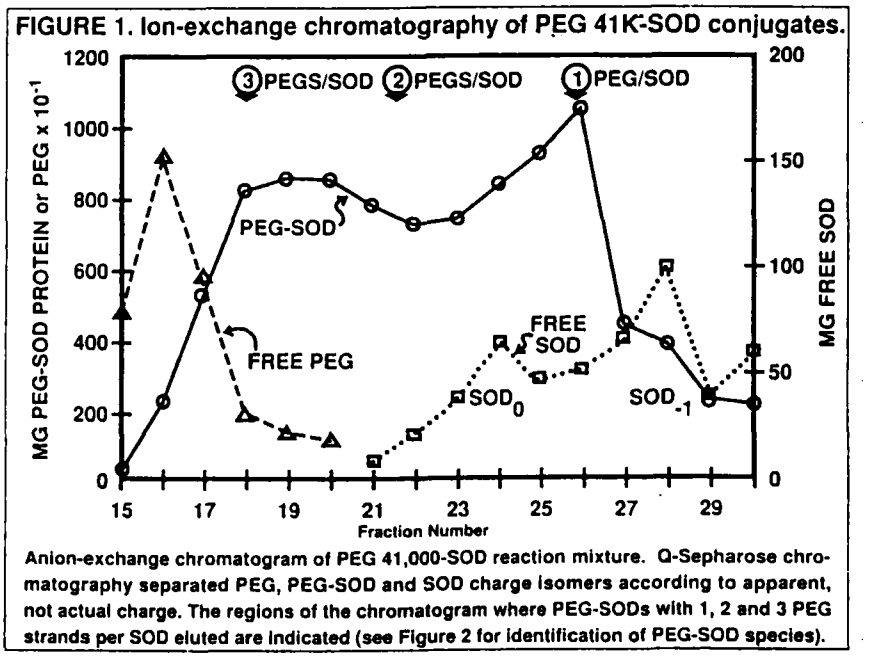

FIGURE 1. Ion-exchange chromatography of PEG 41K-SOD conjugates. A reaction mixture containing an excess of NHS-activated PEG 41K-succinate and 10 grams of bovine Cu, Zn SOD was dialyzed against the starting column buffer and chromatographed on QAE-Sephadex-FF using a salt gradient, as described in Methods. The regions of the chromatogram where PEG-SODs with 1, 2 and 3 PEGs per SOD eluted are indicated (see Figure 2 for HPLC identification of PEG-SOD species). The quantities of protein, free PEG and free SOD in each fraction are shown.

for two hours at 25C. The pH was adjusted to 6–6.5 by 10-fold dilution in 0.02 M NaH_2PO_4 or by addition of 1N acetic acid.

Purification of PEG-SOD conjugates prepared with high MW PEG The pH-adjusted reaction solutions were dialyzed to remove buffer salts and were equilibrated with 2 mM sodium phosphate buffer, pH 7.5. The sample was thenapplied to QAE or DEAE columns equilibrated with the same buffer. PEG-SOD is eluted from the column by applying a salt gradient ending with 0.1 M sodium chloride in 2 mM phosphate buffer. Fractions were adjusted to pH 6.5 with acetic acid, pooled and dialyzed against 0.9% sodium chloride.

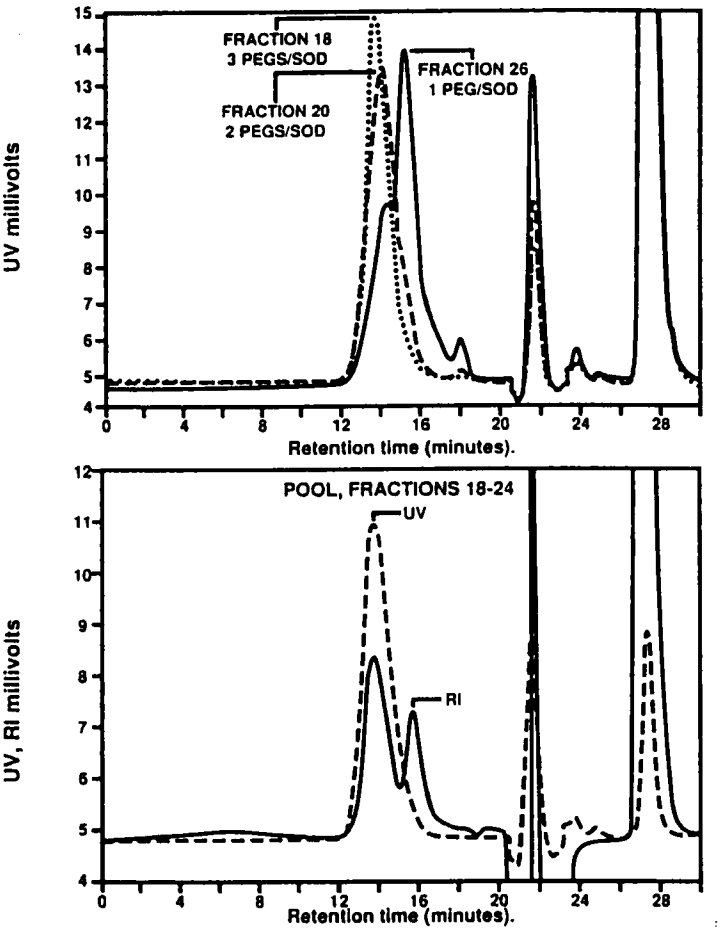

FIGURE 2. HPLC analysis of ion-exchange column fractions and product pool containing PEG 41K-SOD conjugates. Fractions from the chromatogram in Figure 1 were analyzed by aqueous steric exclusion HPLC, monitored by UV and refractive index detection (see Methods). The number of PEG strands per SOD was determined from UV retention times using PEG MW standards to measure conjugate molecular weights. The chromatograms of fractions containing predominately 1, 2 or 3 strands of PEG per SOD, as well as the product pool are shown.

Results

Purification of PEG-SOD Adducts Prepared With High MW PEG by Ion-exchange Chromatography Reaction mixtures containing SOD conjugates prepared with high MW PEG were purified by conventional ion-exchange chromatography to remove unreacted PEG and native SOD as demonstrated for a PEG 41K-SOD conjugate in Figure 1. Most of the free PEG elutes in the wash fractions with a minor fraction eluting early in the gradient. PEG-SOD elutes with increasing ionic strength during the gradient with more highly conjugated species eluting first. Unconjugated SOD elutes according to its negative charge (SOD_0 followed by SOD_{-1} with its single extra negative charge). PEG-SOD with a single PEG attached to one subunit elutes between SOD_0 and SOD_{-1}. In the example shown in Figure 1, products containing 1 to an average of 3.5 PEG strands were identified in individual column fractions by HPLC (Figure 2). Fractions 18–24 were pooled. The HPLC UV and RI elution profiles of the pool are shown in Figure 2 and the properties of the pooled product are compared to bovine Cu, Zn SOD in Table I and Figures 3 and 4. The number of PEG strands attached per SOD molecule calculated from the HPLC data is 3.3., based on molecular weight ratio and 3.1, based on molar ratio.

Extent of SOD Derivatization and Retention of Enzyme Activity PEG-SOD conjugates containing 1 to about 4 strands of PEG 41K to 72K per SOD molecule typically retained 90–100% of native SOD activity (Table II). Activity decreases when more than 4 PEG strands are attached. More extensively modified conjugates prepared by the same methods using methoxy-PEG 5K clearly show progressive loss of activity as a function of PEG conjugation when up to about 16 strands were attached per SOD molecule (Table II).

DISCUSSION

The PEG-SOD conjugates described in the present report behave as nearly neutral species demonstrating primarily endosmotic cathodic mobility when electrophoresed

TABLE I
Properties of a purified PEG 41 K-SOD conjugate compared to native bovine Cu, Zn, SOD.

Property	PEG 41 K-SOD product pool	Native, bovine Cu, Zn SOD
UV Maximum λ	259 nm	259 nm
Visible Maximum λ	680 nm	680 nm
SOD Activity (Cyt-*c*)	4197 U/mg	4578 U/mg
A280/mg/ml	0.180	0.171
A260/mg/ml	0.296	0.294
MW, HPLC (PEG Stds)[a]	145,000	8,000
MW, HPLC (Protein Stds)[b]	1,000,000	32,000
PEG Strands per SOD[c]	3.3	
PEG Strands per SOD[d]	3.1	

[a] Molecular weight determined from hydrodynamic radii by size-exclusion HPLC using PEG standards.
[b] Molecular weight determined from hydrodynamic radii by size-exclusion HPLC using protein standards.
[c] Based on HPLC MR ratio (145,000–8,000)/41,000.
[d] Based on HPLC mole ratio (moles PEG per moles SOD).

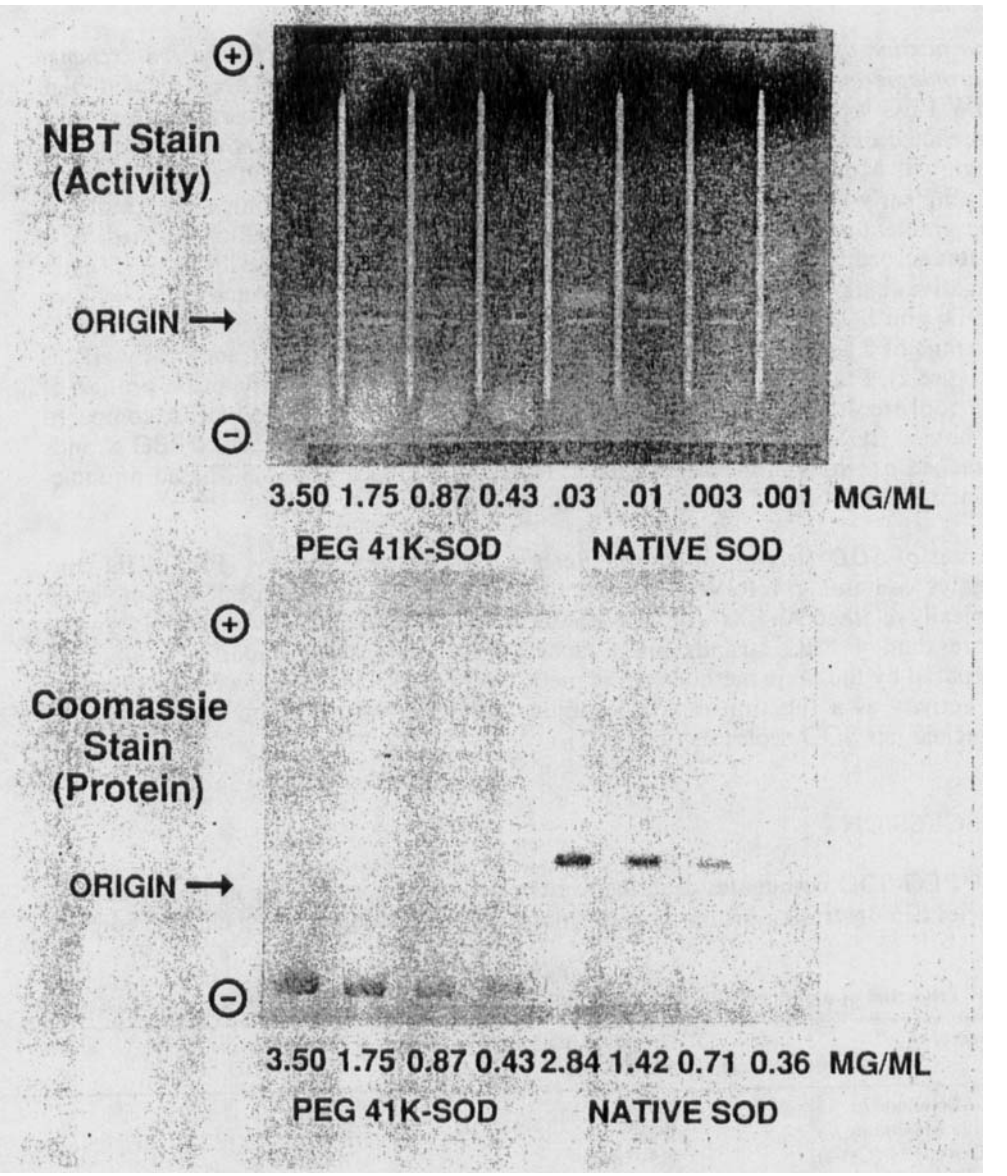

FIGURE 3. Agarose gel electrophoresis of purified PEG 41K-SOD conjugate. The PEG 41K-SOD purified by ion-exchange chromatography (fractions 18–24 of Figure 1) was electrophoresed on Agarose and stained for SOD activity (A, NBT stain) or protein (B, Coomassie stain) as described in Methods. PEG-SOD moves cathodally due to electroendosmotic flow.

on agarose gels at pH 8.5 (Figure 3). This would appear to be an unexpected result since neutralization of positively charged lysine residues by attachment of PEG to the anionic SOD should increase the net negative charge, yielding products more anionic than SOD. However, similar electrophoretic behavior has been described for other

TABLE II
Properties of PEG-SOD prepared with PEG 5k and PEG 41k–72k.

Peak average MW Series I	Series 2	PEG MW	Strands PEG/SOD	SOC Activity (% Native SOD)
19K		5K	2.2	95
44K		5K	7.2	81
	47K	5K	7.8	85
54K		5K	9.2	68
	59K	5K	10.2	61
60K		5K	10.4	42
	63K	5K	11.0	49
64K		5K	11.2	43
	76K	5K	13.6	37
88K		5K	16.0	35
58K, 90K		41K	1.7	95
100K		41K	2.2	93
120K		41K	2.7	91
*145K		41K	3.3	92
166K		41K	3.8	92
178K	50K	3.4	90	
*211K		50K	4.0	99
223K	50K	4.3	84	
282K	50K	5.5	66	
*220K		72K	2.9	98

The molar PEG-to-SOD coupling input ratios for the PEG 5K adducts ranged from 10:1 to 400:1 and for the PEG 41K to PEG 72K adducts, 4:1 to 16:1. Conjugates identified by an asterisk (*) were purified by ion-exchange chromatography (see Methods).

PEG-protein conjugates and has been explained on the basis of charge-shielding by the hydrophilic polymer, rendering the conjugates transparent to the electric field.[9] The dominating influence of the charge-shielding effect of PEG conjugation which is apparent upon electrophoresis is also observed during ion-exchange chromatography (Figures 1 and 2) where the more highly modified, and therefore "more negative" products elute earlier from anion exchangers, as though they were less negatively charged. For example, PEG-SOD with a single PEG on one subunit elutes as though it has less than one extra negative charge. Shielding of charge increases with increasing PEG conjugation. The shielding of the net negative charge of the protein by PEG might influence its biological properties.

Previous workers have attached PEG or methoxy-PEG of low MW, typically about 5000 daltons, to SOD and other proteins, to obtain adducts demonstrating increased serum persistence and reduced immunogenicity;[1] however, the extent of modification with low MG PEG required to achieve these results often leads to substantial loss of enzyme activity. For example, Boccu *et al.*[5] showed that PEG-SODs with 7–18 methoxy-PEG 5K strands demonstrated serum half-lives of about 25 hours in mice and retained between 50 and 60% of native SOD activity. Attaching only 3 strands preserved about 90% of SOD activity but reduced the serum half-life to about 10 hours. Yabuki and Iwashita[10] replaced methoxy-PEG 5K with bifunctional PEG 5K to prepare higher MW SOD copolymers in which both ends of the PEG are attached to different SOD molecules. One group[11] has claimed that using, 1.1′ carbonyldiimidazole as the PEG activating agent produced SOD derivatives with up to 19 amino

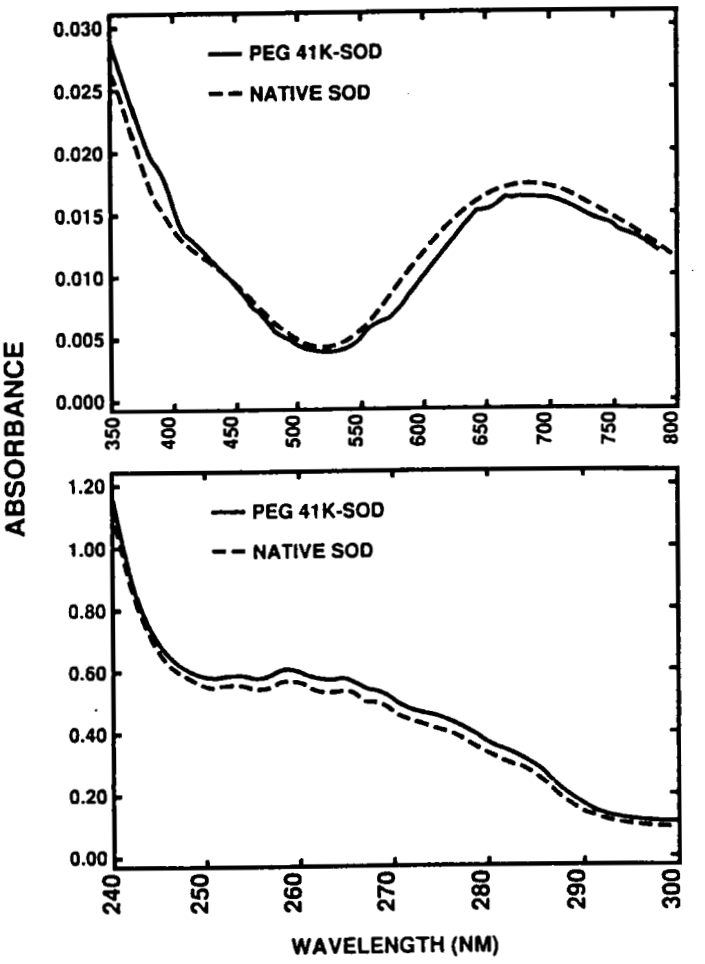

FIGURE 4 UV and visible spectra of purified PEG 41K-SOD conjugate. The PEG 41K-SOD purified by ion-exchange chromatography (Figure 1) was diluted in Sorenson's glycine buffer (pH 8.5) to 2 mg per ml protein for the UV and visible spectra. The spectra are characteristic of the SOD used in the synthesis of PEG-SOD.

groups modified, yet still retaining 95% of native SOD activity. Although more active PEG-L-asparaginase conjugates could be prepared when fewer protein groups were modified with a chlorotriazine analogue substituted with 2 strands of methoxy-PEG 5K,[12] a SOD substituted with 7–12 residues of this compound demonstrated only 50% of native SOD activity.[13] The potential immunogenticity of the triazine ring would also argue against introducing such agents into pharmaceutical preparations.

The loss of activity associated with extensive substitution may be explained by the nature of residues near the protein's active site. PEG coupling methods described in the literature involve modifying protein nucleophilic groups, principally lysine residues. Many of SOD's lysine residues are situated within the active site region where they are postulated to form a positive electrostatic gradient which guides the substrate, superoxide radical anion (O_2^-), into the active site.[14] The importance of this

electrostatic potential to enzyme activity can be appreciated from the fact that the positive domain is highly conserved in SOD molecules from diverse species.[15] Therefore, it is not surprising that appreciable neutralization of charge in this region, brought about by PEG-conjugation, reduces enzyme activity. This has been analogously demonstrated using 1-H-tetrazole and acetic anhydride to nearly completely modify lysine residues, resulting in 50% inactivation.[16]

Our results indicate that PEG-SOD conjugates exhibiting at least 90% of native enzyme activity can be made by coupling up to about 4 strands of either PEG 5K or PEG 41K–72K (Table II). The PEG 5K conjugates we synthesized with 7 to 16 PEG strands per SOD retained about 83% to 35% of the native enzyme activity, respectively. The inactivation of SOD by increasing PEG modification has been reported by others.[2–5]

Using fewer strands of high MW PEG to prepare conjugates results in less modification of lysine residues and consequently less inactivation, producing more active products of larger size. For example, the largest PEG 5K-SOD conjugate which can be produced using monofunctional PEG has a molecular weight of about 100,000 and retained less than half the native SOD activity, whereas the products containing 2–4 strands of PEG 41K–72K (Table II) exhibited at least 90% activity and molecular weights in the 100,000–300,000 range (based on PEG standardization). In a separate report published in these Proceedings, we show that conjugates described here, prepared with fewer strands of higher MW PEG, also demonstrate superior biological properties related to potential therapeutic applications, including longer circulating half-lives and less antigenicity at each degree of substitution.

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